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## Gestion durable des maladies du bois de la vigne au vignoble : Délivrer des agents de biocontrôle et les molécules bénéfiques associées

Thèse dirigée par Mme. **Florence FONTAINE, Université de Reims Champagne-Ardenne**

Et par M. **Josep ARMENGOL, Université Polytechnique de Valence**

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ABIES DOCTORAL SCHOOL

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7th DECEMBER 2022

## Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules

Thesis directed by Ms. **Florence FONTAINE, University of Reims Champagne-Ardenne**

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"*If we knew what it was we were doing,*

*it would not be called research, would it*?"

Albert Einstein

"*Success is 1% inspiration, 98% perspiration,*

*and 2% attention to detail*."

Phill Dunphy

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## Abstract

Grapevine (*Vitis vinifera* L.) plants are exposed to a wide variety of pathogens, such as bacteria, viruses, phytoplasmas, fungi and nematodes. Since nursery stages, grapevine material can be infected with innumerous pathogens, that can cause unsuspected significant diseases. Nowadays, grapevine fungal trunk diseases (GTDs) are amongst the main constraints for the productivity of this crop. Once infected, plant productivity is decreased, leading to a plant slow or apoplectic death that causes important economic losses and limits vineyard sustainability. With recent environmental conscious practices, chemical treatments for GTDs have been hardly reduced, leaving viticulturists with few effective treatments. Investigation of biocontrol agents (BCAs) capable to forestall or at least to minimize the impact of GTDs, while being a sustainable treatment, is viewed as a research priority. One potential BCA was deeply characterized, and together with a biological commercial product, both BCAs were tested against several GTD pathogens, in greenhouse under controlled conditions, and during the grapevine propagation process.

Results from the full genomic analysis of *Bacillus subtilis* PTA-271 (as BCA with a potential) show a functional swarming motility system, strong survival capacities and a set of genes encoding for bioactive substances known to stimulate plant growth or defenses, influence beneficial microbiota, and counteract pathogen aggressiveness. When tested against *Neofusicoccum parvum* Bt67 (thereafter *Np*-Bt67) in greenhouse cuttings, *B. subtilis* PTA-271 (*Bs* PTA-271) and *T. atroviride* SC1 (*Ta* SC1) proved that the cultivar contributes to their beneficial effects against *Np*-Bt67. The simultaneous application of both BCAs was further proved to be even more effective to protect Tempranillo cuttings. Moreover, the transcriptomic analysis from the same samples showed extensively the plant physiology changes induced by the pathogen but also by each BCA, *Bs* PTA-271 on Chardonnay and *Ta* SC1 on Tempranillo, to protect grapevine from *Np*-Bt67 infection. Thus, Chardonnay cuttings infected with *Np*-Bt67 showed overexpressed genes implicated on abscisic acid (ABA) biosynthesis and signaling pathways. In Tempranillo, the infection with *Np*-Bt67 leads to more substantial changes in gene expression, related mostly with amino acid import, chloroplast and photosystem related processes, plant responses to biotic stimulus, and biosynthesis of secondary metabolites. Protection induced by *Bs* PTA-271 in Chardonnay targets genes related to ABA biosynthesis, phenylpropanoid pathways and secondary metabolites, and cell

wall structure/organization in relationship with carbohydrate metabolism that requires much more consideration. Protection with *Ta* SC1 in Tempranilllo requires a larger number of changes related to transporters, cell wall integrity and extension, cell division and pathogen induced cell death, multidirectional active proteins, and microbiome interactions.

During the grapevine nursery process, the results demonstrated a significant reduction on the percentage of infected plants with Botryosphaeria dieback and Black-foot pathogens in the material treated with *Ta* SC1 and *Bs* PTA-271 respectively. The simultaneous treatments with both BCAs presented a reduction on infected plants with both Botryosphaeria dieback and Black foot pathogens. When testing the effect of *Bs* PTA-271 and *Ta* SC1 in grapevine rhizosphere microbiome of two different soil infected with Black foot pathogens, results show that the inoculation of BCAs seems to improve the rhizosphere microbiome networks and sanitation status, however, the beneficial effect of BCAs can be soil-dependent. Moreover, as observed in the other experiments, the combination of both BCAs improves their beneficial effect in the rhizosphere microbiome.

Overall, this study brought new insights on the use of one or more BCAs against several GTD pathogens, from nursery to adult grapevines. Moreover, highlighted both BCAs mode of action in grapevine protection. Thus, these findings provide, not only a better understanding of BCAs, grapevine, and pathogens interactions, but are also a strong contribution for the future development of sustainable GTDs management strategies.

**Keywords:** *Bacillus subtilis* PTA-271, Biocontrol agents, Defense mechanisms, Grapevine trunk diseases, Microbiome, Nursery process, Plant protection, Sustainable disease management, *Trichoderma atroviride* SC1, *Vitis vinifera* L..

## Résumé

La vigne (*Vitis vinifera* L.) est exposée à une grande variété d'agents phytopathogènes, tels que les bactéries, les virus, les phytoplasmes, les champignons et les nématodes. Dès la pépinière, la vigne peut être infectée par de nombreux pathogènes qui pourront provoquer des maladies très dommageables ultérieurement. Aujourd'hui, les maladies fongiques du bois de la vigne (MDB) comptent parmi les principales responsables de la baisse de productivité de cette culture. Une fois infectée, le dépérissement de la vigne s'enclenche et la productivité de la plante diminue, entraînant une mort lente ou rapide (apoplectique) de la plante avec des pertes économiques importantes et un risque majeur pour la durabilité du vignoble. La récente prise en compte de l'environnement dans le développement des pratiques de lutte a conduit à réduire fortement les traitements chimiques contre les MDB, laissant les viticulteurs avec peu de traitements efficaces. La priorité est alors donnée au développement d'alternatives durables, via la recherche de microorganismes bénéfiques (MOB) auxiliaires de la vigne, pour développer des produits de biocontrôle (BCA) capables de prévenir ou de minimiser l'impact des MDB. Un MOB doté d'un potentiel prometteur contre les MDB, associé à un produit biologique déjà commercialisé contre les MDB, ont été testés contre plusieurs pathogènes responsables des MDB, en serre sous conditions contrôlées et en pépinière lors du processus de multiplication de la vigne. Ces travaux ont été menés chez les 2 cépages, Chardonnay et Tempranillo.

Une analyse génomique complète a d'abord été réalisée sur le MOB *Bacillus subtilis* PTA-271, afin de connaître l'étendue de son potentiel prometteur contre les MDB. Les résultats obtenus ont montré un système fonctionnel de mobilité, une forte capacité de survie, et un ensemble de gènes codant pour des molécules bioactives capables de stimuler la croissance des plantes et leurs défenses, mais aussi d'influencer leur microbiote bénéfique et de contrer l'agressivité d'agents phytopathogènes. Lors de tests complémentaires sur boutures en serre contre *Neofusicoccum parvum* Bt67 (*Np*-Bt67), *Bacillus subtilis* PTA-271 (*Bs* PTA-271) et *Trichoderma atroviride* SC1 (*Ta* SC1) ont montré que le cépage influençait leurs effets bénéfiques contre *Np*-Bt67. L'application simultanée des deux BCA s'est avérée plus efficace pour protéger les boutures de Tempranillo. L'analyse transcriptomique des mêmes échantillons a par ailleurs montré des réponses physiologiques de la vigne, induites par la pathogène, mais aussi par chaque MOB protecteur contre *Np*-Bt67, *Bs* PTA-271 pour le Chardonnay et *Ta* SC1 pour Tempranillo. Ainsi, les boutures de Chardonnay infectées par *Np*-Bt67 montrent une surexpression des gènes impliqués dans la synthèse et les voies de signalisation de l'acide

abscissique (ABA). Chez Tempranillo, l'infection par *Np*-Bt67 entraîne des changements plus substantiels d'expression de gènes, liés principalement à l'importation d'acides aminés, aux activités chloroplastiques et des photosystèmes, aux réponses des plantes aux stress biotiques, et à la synthèse de métabolites secondaires. La protection induite par *Bs* PTA-271 chez Chardonnay cible des gènes liés à la synthèse de l'ABA, à la voie des phénylpropanoïdes et des métabolites secondaires, et à la structure et l'organisation de la paroi cellulaire en lien avec le métabolisme des glucides qui mérite désormais plus d'attention. La protection induite par *Ta* SC1 aux boutures de Tempranilllo implique un plus grand nombre de changements qui sollicitent à la fois des transporteurs, l'intégrité et l'extension de la paroi cellulaire, la division cellulaire versus la mort cellulaire provoquée par les agents pathogènes, des protéines actives aux effets multidirectionnels, et des interactions avec le microbiome.

Lors des essais en pépinière, les résultats obtenus ont montré une réduction significative du pourcentage de plants infectés par les pathogènes responsables du dépérissement à Botryosphaeria et de la maladie du pied noir, lors des traitements avec *Ta* SC1 et *Bs* PTA-271 respectivement. Les traitements simultanés avec les deux MOB ont montré une réduction des plants infectés par l'ensemble des pathogènes, qu'ils soient responsables du dépérissement à Botryosphaeria ou de la maladie du pied noir. En testant l'effet de *Bs* PTA-271 et *Ta* SC1 dans le microbiome de la rhizosphère de la vigne de deux sols différents infectés par les pathogènes du pied noir, les résultats montrent que l'inoculation de BCAs semble améliorer les réseaux de microbiome de la rhizosphère et l'état d'assainissement, cependant, l'effet bénéfique des BCAs peut être dépendant du sol. De plus, comme observé dans les autres expériences, la combinaison des deux BCAs améliore leur effet bénéfique dans le microbiome de la rhizosphère.Dans l'ensemble, cette étude a apporté de nouvelles connaissances sur l'utilisation d'un ou plusieurs MOB contre plusieurs pathogènes responsables des MDB, de la pépinière à la vigne adulte. De plus, elle a mis en évidence le mode d'action des deux MOB dans la protection de la vigne. Ainsi, ces résultats permettent non seulement de mieux comprendre les interactions entre les MOB, la vigne et les pathogènes, mais ils sont également une contribution non négligeable pour le développement futur de stratégies de gestion durable des MDB.

**Mots-clés:** Agents de biocontrôle, *Bacillus subtilis* PTA-271, Gestion durable des maladies, Maladies du bois de la vigne, Mécanismes de défenses, Microbiome, Pépinière, Protection de la plante, *Trichoderma atroviride* SC1, *Vitis vinifera* L..

## Resumen

Las plantas de vid (*Vitis vinifera* L.) están expuestas a una gran variedad de patógenos, como bacterias, virus, fitoplasmas, hongos y nematodos. Desde las etapas de vivero, las plantas de vid pueden estar infectadas con innumerables patógenos, que pueden causar enfermedades importantes. En la actualidad, las enfermedades fúngicas de la madera de la vid (GTDs) se encuentran entre los principales factores que limitan la productividad de este cultivo. Una vez las vides están infectadas, la productividad de la planta disminuye, provocando una muerte lenta o apoplética, que causa importantes pérdidas económicas y limita la sostenibilidad del viñedo. Con la creciente concienciación ambiental, los fungicidas de síntesis química para el control de las GTDs se han reducido, dejando a los viticultores con pocos tratamientos efectivos disponibles. La investigación de agentes de control biológico (BCAs) capaces de prevenir, o al menos minimizar, el impacto de las GTDs, se considera una prioridad de investigación. En esta Tesis Doctoral se caracterizó en profundidad un agente de biocontrol potencial y, junto con un producto comercial biológico ya registrado, ambos BCAs fueron probados contra varios patógenos agentes causales de GTDs, en invernadero bajo condiciones controladas, y también durante el proceso de propagación de la vid en vivero.

Los resultados del análisis genómico completo de *Bacillus subtilis* PTA-271 muestran un sistema funcional de motilidad de enjambre, una fuerte capacidad de supervivencia y un conjunto de genes que codifican sustancias bioactivas conocidas por estimular el crecimiento o las defensas de las plantas, influir en la microbiota beneficiosa y contrarrestar la agresividad de los patógenos. Cuando *B. subtilis* PTA-271 se probó contra *Neofusicoccum parvum* BT67 en plantas injertadas de invernadero, *B. subtilis* PTA-271 y *Trichoderma atroviride* SC1 demostraron que el cultivar contribuye a los efectos beneficiosos de *Bs* PTA-271 y *Ta* SC1 contra *Np*-Bt67. La aplicación simultánea de ambos BCAs demostró ser beneficiosa contra este patógeno en vides del cultivar Tempranillo. El análisis transcriptómico de las mismas muestras mostró ampliamente los cambios en la fisiología de la planta inducidos tanto por *Bs* PTA-271 como por *Ta* SC1 para proteger la vid ante la infección por *Np*-Bt67. En Chardonnay, las plantas infectadas con *Np*-Bt67 presentan genes sobreexpresados que están implicados en las vías de señalización del acido absicico (ABA). En Tempranillo, la infección con *Np*-Bt67 provoca cambios de expresión en más de 200 genes, relacionados sobre todo con la importación de aminoácidos, procesos relacionados con el cloroplasto y el fotosistema, respuestas de la planta a estímulos bióticos y biosíntesis de metabolitos secundarios. La protección de *Bs* PTA-

271 en Chardonnay implica a genes relacionados con la biosíntesis de ABA, las vías de los fenilpropanoides, los metabolitos secundarios, y la estructura y organización de la pared celular. La protección de *Ta* SC1 en las plantas de Tempranilllo implica un mayor número de cambios, que abarcan tanto el metabolismo primario como el secundario, relacionados con cambios en las señales hormonales, como con el aicdo absicico (ABA). Durante el proceso de producción de la vid en vivero, los resultados demostraron una reducción significativa del porcentaje de plantas infectadas con los patógenos asociados a las enfermedades de decaimiento por Botryosphaeria y Pie negro en el material de vivero tratado con *Ta* SC1 y *Bs* PTA-271 respectivamente. Los tratamientos simultáneos con ambos BCAs presentaron una reducción en el porcentaje de plantas infectadas con ambos tipos de patógenos. Al probar el efecto de *Bs* PTA-271 y *Ta* SC1 en el microbioma de la rizosfera de la vid de dos suelos diferentes infectados con patógenos del pie negro, los resultados muestran que la inoculación de los BCAs parece mejorar las redes del microbioma de la rizosfera y el estado de saneamiento, sin embargo, el efecto beneficioso de los BCAs puede ser dependiente del suelo. Además, como se observó en otros experimentos, la combinación de ambos BCAs mejora su efecto beneficioso en el microbioma de la rizosfera.

En general, este estudio aportó nuevos conocimientos sobre el uso de uno o más BCAs contra varios patógenos asociados a las GTDs, tanto en el vivero como en vides adultas (viñedo). Además, se destacó el modo de acción de ambos BCAs en la protección de la vid. Por lo tanto, estos hallazgos proporcionan, no sólo una mejor comprensión de las interacciones entre los BCAs, la vid y los patógenos, sino que también son una fuerte contribución a una estrategia de gestión sostenible de las GTDs.

**Palabras clave:** Agentes de biocontrol, *Bacillus subtilis*, Enfermedades de la madera de la vid, Gestión sostenible de enfermedades, Mecanismos de defensa de la planta, Microbioma de la vid, Proceso de vivero de la vid, Protección contra patógenos, *Trichoderma*.

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**Abbreviation list**

**ABA:** abscisic acid

**BCAs:** biocontrol agents

**BF:** black foot

***Bs* PTA-271:** *Bacillus subtilis PTA-271*

**BOT:** Botryosphaeria dieback

**CWDEs:** Cell wall degrading enzymes

**DAMPs:** damage-associated molecular patterns

**DNA:** Deoxiribonucleic acid

**dpi:** days post-inoculation

**EPS:** exopolysaccharides

**ET:** ethylene

**GTDs:** grapevine trunk diseases

**hpi:** hours post inoculation

**IAA:** Indole-3-acetic acid

**ISR:** induced systemic resistance

**JA:** jasmonic acid

**LPS:** lipopolysaccharides

**MAMPs:** microbial associated molecular patterns

**NO:** nitric oxide

***Np*-Bt67:** *Neofusicoccum parvum* BT67

**NRP:** non-ribosomally synthesized peptides

**PA:** polyamines

**PK:** polyketides

**RNA:** Ribosomal ribonucleic acid

**RNA-seq:** RNA sequencing

**RP:** ribosomally synthesized antimicrobial peptides

**RiPP:** post-translationally modified RP

**ROS:** reactive oxygen species

**SA:** salicylic acid

***Ta* SC1:** *Trichoderma atroviride* SC1

**ViC:** inorganic volatile compound

**VOC:** organic volatile compound

*Chapter I*

# Introduction

### 1.1. *Vitis vinifera* – One of the oldest and most important crops worldwide

The European grapevine *Vitis vinifera L.*, belonging to the Vitaceae family genus *Vitis*, is the wild ancestor of most cultivated vines diversified into countless varieties of significant economic interest. It is from this vine that almost all the wines in the world are produced. Around 6,000 cultivated varieties exist throughout the world, with just over 200 in France. Subspecies are also described for *Vitis vinifera*, among which *V. vinifera subsp. sylvestris* (more simply referred to as *V. sylvestris*) which includes populations returned to the wild, and *V. vinifera subsp. vinifera* (more simply referred to as *V. vinifera*) which includes cultivated varieties resulting from the domestication of wild species (Levadoux et al. 1956). To differentiate *V. sylvestris* from *V. vinifera* subspecies, phenotypic attributes are considered, such as: flower sex (dioecious male or female for wild populations, versus hermaphroditic for cultivated grapevines) and seed morphology (spherical with a small beak for sylvestris, and pyriform with a well-developed beak for domesticated cultivars) (De Andrés et al. 2012; Imazio et al. 2013; Lacou et al. 2011; Terral et al. 2010; Zohary et al. 1995).

Domestication of *V. vinifera subsp. vinifera* selected morphological characteristics that favors the plant cultivation and exploitation. Propagation of *vinifera* varieties gave preferences to the largest, juiciest, and tastiest berries, to gradually select identical grape varieties. In Western Europe, grape varieties can also result from selections and crosses between local wild plants and plants already selected. Therefore, from *V. vinifera* subsp. *vinifera*, the various grape varieties make up the vast range of vine varieties cultivated worldwide, with more than 6,000 "cultivars" or “cépages” (a botanical term for varieties of plant species obtained by crossing and cultivated). Genetic diversity is thus generally preserved within a cultivar genome of *V. vinifera*, except when mutations may accumulate over time to generate distinguishable clones and significantly increase the genomic variability (Franks et al. 2002; Riaz et al. 2002; Zhou et al. 2019). The most concentrated areas with the presence of *V. vinifera* are Central and Southern Europe, Western and Middle East of Asia, China, Mediterranean coast of Africa, South Africa, North America, South America, Australia, and New Zealand, however, nowadays, *V. vinifera* is also present in America, Africa, Asia, and Oceania.

Considering the genotype impact of the variety, the cultivar or even the clone, it is important to consider genomic traits when choosing for the plant characteristics. Additionally, cultivated grapevines are mainly grafted propagated (> 80%), so their genetic diversity could be further increased both by the scion (aerial part of the grafted plant, variety, or cultivar) and the rootstock (underground part of the grafted plant). For instance, some rootstocks are

described to promote the plant protection against pathogens and abiotic stress (drought, waterlogging, adaptation to different soils, nutrients intake, etc.), and to influence the scion vigor and grape composition (Chacón-Vozmediano et al. 2021; Gindro et al. 2003; Larignon et Dubos, 1997; Marchi 2001; Monteiro et al. 2013; Yıldırım et al. 2018), but there is no specific cultivar or clone resistant to grapevine trunk diseases (GTDs) (Bertsch et al. 2013).

The use of specific training systems or management techniques is also described to influence plant features (Leao et al. 2019; Reynolds et al. 2009; Zahavi et al. 2001). Depending on the training system, it is possible to facilitate mechanical maintenance (e.g. cut to reduce the density of perennial woods), avoid competition between grapevines, control the light exposure, floor and canopy management, etc., to ultimately result in healthier plants, with better quality bunches and higher yields (Reynolds et al. 2009).

**1.2. Focus on Biotic factors inducing plant diseases, as a Challenge to productivity.**

Diseases are one of the most important factors decreasing plant health, and consequently, the quantity and quality of plant resources/productivity (Lucas 2009; Flood 2010). Plant diseases are caused by microorganisms (viruses, bacteria, fungi, mycoplasmas, viroids), that can be carried by other biotic factors such as animals (birds, insects, mammals, nematodes, parasitic competitor plants), and can be facilitated by abiotic factors (i.e., climate changes and soil physico-chemical parameters) or pollutants (Abdulkhair and Alghuthaymi 2016).

Several biotrophic pathogens (pathogens that establish a long-term feeding relationship with the living cells of their hosts, rather than killing the host cells as part of the infection process) threaten grapevine every year. The main phytosanitary threat in all regions remains the mildews (Bois et al. 2017). Powdery mildew is caused by *Erysiphe necator*, seen as ash-grey to white powdery growth on green tissue of the vine (Gadoury et al. 2012), and downy mildew caused by *Plasmopara viticola*, can be seen as yellow leaf lesions that look oily, with a cottony growth (mycelium), on the lower leaf surface (Gessler et al. 2011).

Full necrotrophic pathogens (pathogens that kill host plant cells and use the contents to support their own growth) are also threatening for vineyard productivity. Indeed, the grey mold caused by *Botrytis cinerea* is another important grapevine disease leading to heavy yield losses, and for which pathogen pressure can changes yearly. This disease occurs when the grapes turn soft and brown, showing a fluffy grey mold on the grapes (Emanuel et al. 2000). Black rot caused by *Guignardia bidwellii*, also concerns the viticulture community, showing brown spots on the leaves and shoots, that dry and die (Pezet and Jermini 1989).

Finally, focus is made on some important pathogens described as hemibiotroph (initially invade live cells prior to transitioning to a necrotrophic lifestyle to obtain nutrients from killing the host cells) that increasingly threaten grapevine and other woody plants productivity. Indeed, more and more species of woody plants exhibit an inevitably decline called dieback (degeneration of plant tissues following a succession of multiple negative events), which leads to the death of the plant in the long term (low dieback phenotype) or in the short term (apoplectic dieback phenotype) (Anderson et al. 2004; Ciesla et al. 1994; Manion 1999). Due to undetermined period of latency between the asymptomatic to symptomatic states, early detection and management remain of these diseases, presents a challenge in nurseries, mother fields and vineyards, and only few preventives, but no effective and sustainable curative methods, are available (Mondello et al. 2018a,b). This situation worries the fruit and vine growers all around the world, triggering numerous investigations to better understand their causes, the ways to avoid and to treat them. Although per year, 0.5 to 1.5% of the woody plants die due to different factors such as fungi, fire, grazing and climate change, the main mortality factor remains undetermined (Bettenfeld et al. 2020; Boyd et al. 2013). Among them, grapevine trunk diseases (GTDs) are one of the most important groups of plant diseases for grape growers in all wine regions worldwide. These diseases are caused by several fungi that colonize and live in the plant perennial woods for undetermined period of latency (asymptomatic state) before triggering symptoms (symptomatic state) causing necrotic wood lesions that lead to the occurrence of typical leaf symptoms with discolorations and finally to a completely dead branch (Bertsch et al. 2013; Mondello et al. 2018). GTDs for which new pathogens and vectors are described and discovered yearly, and for which there is uncertainty about the role of the annual pathogen load on both the disease symptom expression and the speed of its expression. Focus will also be made on the grapevine plant and immune defenses.

### 1.3. Plant defenses to Biotic stresses

Main plant defenses are already described (Figure 1.) when the plant metabolism reacts against the pathogen and related associated motifs (MAMPs and PAMPs recognized by the plant receptors PRRs), damages (DAMPs recognized by the plant receptors PRRs), or against the effectors (such as phytotoxins) secreted by the pathogen inside the plant cell. Early (minutes to hours) to late defenses (days or longer) are described but are not necessarily specific from the virulence to the activation of local and distal defense signaling (Li et al. 2020). **Plant pattern recognition receptors (PRRs)** perceive a wide range of elicitors, including pathogen-derived cell wall/membrane components

(**the SYSTEMIC dimension of plant defenses**, e.g., peptidoglycan, chitin), pathogen associated proteins (**PAMPs** Pathogen-Associated Molecular Patterns, e.g., flagellin, effectors), and host-derived danger associated molecular patterns (**DAMPs** Damage Associated Molecular Patterns; e.g., cuticle) (Ziv et al. 2018). In few minutes (3 min) **reactive oxygen species** (ROS, such as $H_2O_2$) are generated in the apoplast as secondary signalling messenger that activates **ions fluxes** such as $Ca^{2+}$ influx (Li et al. 2020 for review). Some minutes later (5 min) a complex series of **phosphorylation-dependent signalling cascade** is activated, including mitogen-activated protein kinases (MAPK) and calcium-dependent protein kinase (CPDK) as synergistic, pro-immune activators (Li et al. 2020). MAPK and CDPK acts as defenses executor proteins that lead to nuclear-based **transcriptional reprograming** (changes in microfilament remodelling are detected within 5–15 min), before activating by phosphorylation (in 0-12 hours post infection, hpi) the stress-responsive **transcription factors** (TF, eg. AP2/ERF, bHLH, bZIP, MYB, NAC, WRKY, etc.). These **TFs then transcriptionally activate** (6-24 hpi) **sustained and robust defense processes on a large-scale and in the long-term**: (**a**) induction of defense hormone accumulation and signaling, (**b**) cytoskeleton/organelle remodeling, (**c**) regulation of the secretome and cell wall/apoplast composition, and (**d**) cellular motion (e.g., stomatal closure) as described by Li et al. (2020). When pathogens possess multiple elicitors (e.g., PAMPs, effectors), various synergistic signaling cascades are stimulated. In such conditions, it is difficult to reason by measuring each early (ca. minutes to hours) events, but rather by evaluating changes over the duration of the interaction, which can last days or longer. Thus, the activation of immunity is not a sequential series of events, but rather, represents a complex network of processes, each of which can be activated or attenuated multiple times during the host-pathogen interaction.

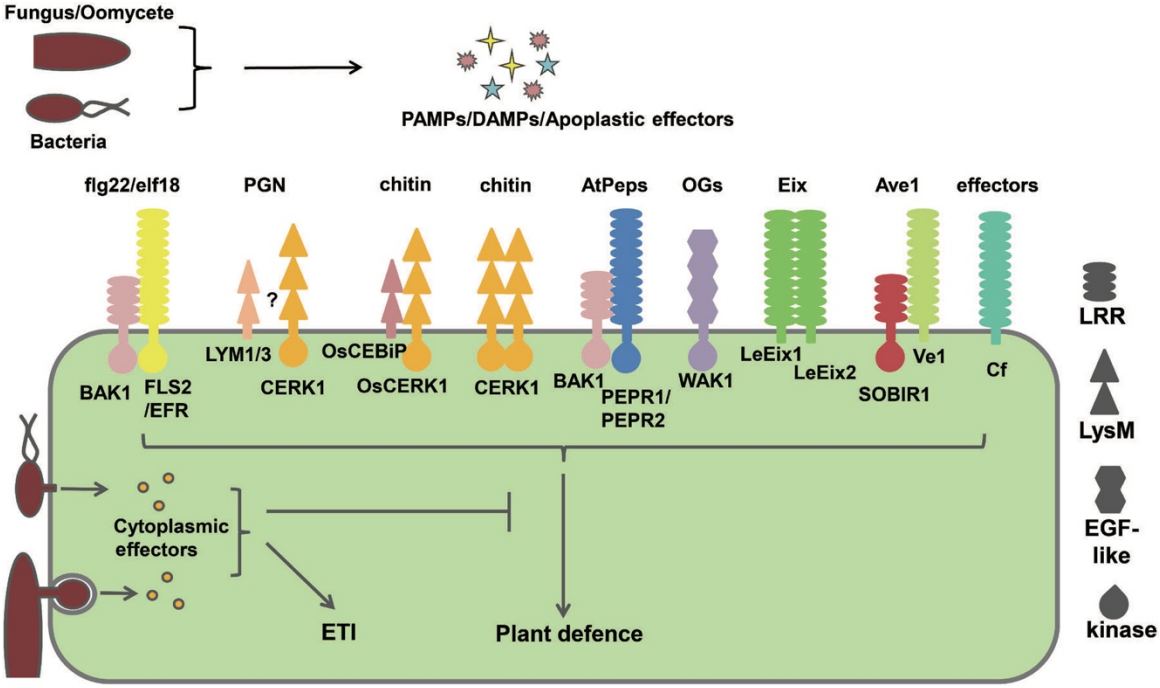

**Figure 1.** Representative PRRs and their ligands. During bacterial, fungal and oomycete pathogen infection, many danger signals such as pathogen-derived PAMPs and effectors, and plant- derived DAMPs are recognized by plasma membrane-localized RLK and RLP complexes. Pathogens can secrete effectors to suppress plant defense response. However, many of the effectors are recognized by NLRs to activate effector-triggered immunity (ETI). The LRR-RLKs FLS2 and EFR recognize bacterial flg22 and elf18, respectively. BAK1 is a co-receptor and forms a hetero-dimer with FLS2 or EFR upon ligand stimulation. Bacterial peptidoglycan (PGN) is recognized by LysM RLPs LYM1 and LYM3. LysM RLK CERK1 is also required in PGN recognition and probably forms a complex with LYM1 and LYM3 although the direct interaction has not been demonstrated. Rice and Arabidopsis have homologous yet distinct chitin receptors. The Arabidopsis CERK1 directly binds chitin and forms a homo-dimer. In rice, LysM RLP OsCEBiP is the chitin receptor and interacts with OsCERK1 for full signaling. The fungal ethylene-inducing xylanase (Eix) is recognized by tomato LRR-RLPs LeEix1 and LeEix2 complex. Tomato Ve1 recognizes the apoplastic effector Ave1 and interacts with homologs of Arabidopsis SOBIR1 which is a positive cell death regulator. In addition, several pathogen-derived effectors, e.g., Avr2, Avr4, Avr9 and Avr4E, are recognized by LRR RLPs Cf-2, Cf-4, Cf-9 and Cf-4E. Oligogalacturonides (OGs) are a classical DAMP and recognized by WAK1. Besides, LRR-RLKs PEPR1 and PEPR2 are receptors for another group of DAMPs, AtPeps, and are shown to interact with BAK1. **Note 1:** PRRs = RLKs (= receptor-like kinases) + RLPs (= receptor-like proteins). **Note 2:** PRRs share certain features: they are single-transmembrane receptor-like kinases or receptor-like proteins, containing a leucine-rich repeat (LRR), LysM, EGF-like, or lectin domain for ligand binding within the apoplast (Boutrot and Zipfel, 2017) (Adapted from Wu and Zhou et al. 2013).

In parallel to the activation of **local immune signaling**, plants also employ long-distance signaling as a mechanism to prime defense activation in advance of pathogen proliferation. This strategy enables to halt pathogen spread via the mobilization of a core, evolutionarily conserved, class of highly specific signaling molecules. Once mobilized, these signals activate defense responses in distal uninfected cells and tissues, which reduces secondary pathogen invasion, proliferation, and disease. Consequently, noninfected cells are primed to enter a pro-immune status. This process, referred to as systemic acquired resistance (SAR) (Durrant and Dong 2004; Shine et al. 2019), provides protection against a broad range of pathogens, including bacteria, fungi, and viruses.

Following pathogen perception, a broad spectrum of **distal immune signaling** is activated (the **systemic dimension** of plant defenses). The first, the electrical wave due to ions fluxes stimulates membrane potential without transporting molecules to distal cells. Based on the robustness and speed of the Ca2+ signal, it is reasonable to hypothesize that the **Ca2+ wave** represents the first phase of long-distance signaling in response to biotic stress perception. The second class of immune signal that has been described is broadly classified as **messenger/signal molecules**, including **hormones** (e.g., SA, JA/ET), **RNA, proteins, and peptides**. These signal molecules, which transmits by themselves, are long-distance messengers transmitting signals with high specificity, robustness, and durability, at the expense of speed. Moreover, each of these characteristics determines their biological function to induce and maintain the second phase of distal immunity, when massive pathogen inhibitory molecules are synthesized (Li et al. 2020). To facilitate the activation and **propagation of distal signals**, plants use not only symplastic and apoplastic pathways, but also vascular systems (e.g., phloem). **Phytohormones are long-range mobile signalling molecules**, which play an essential role in stress signalling. Salicylic acid (SA, phenylpropanoid path) is useful to protect the plant against biotrophic pathogens, while Jasmonic acid (JA, lipoxygenase path) / ethylene (ET) are described as useful against necrotrophic pathogens and pests. Due to crosstalk between induced TF, other phytohormones (e.g., abscisic acid, ABA, etc.) can also interfere with plant defenses against pathogens. Once mobilized, **signal molecules activate defense responses in distal uninfected cells and tissues**, to reduce secondary pathogen invasion, proliferation, and disease. Consequently, noninfected cells also show a pro-immune status called **systemic acquired resistance (SAR**) (Durrant and Dong 2004; Shine et al. 2019) (Figure 2.).

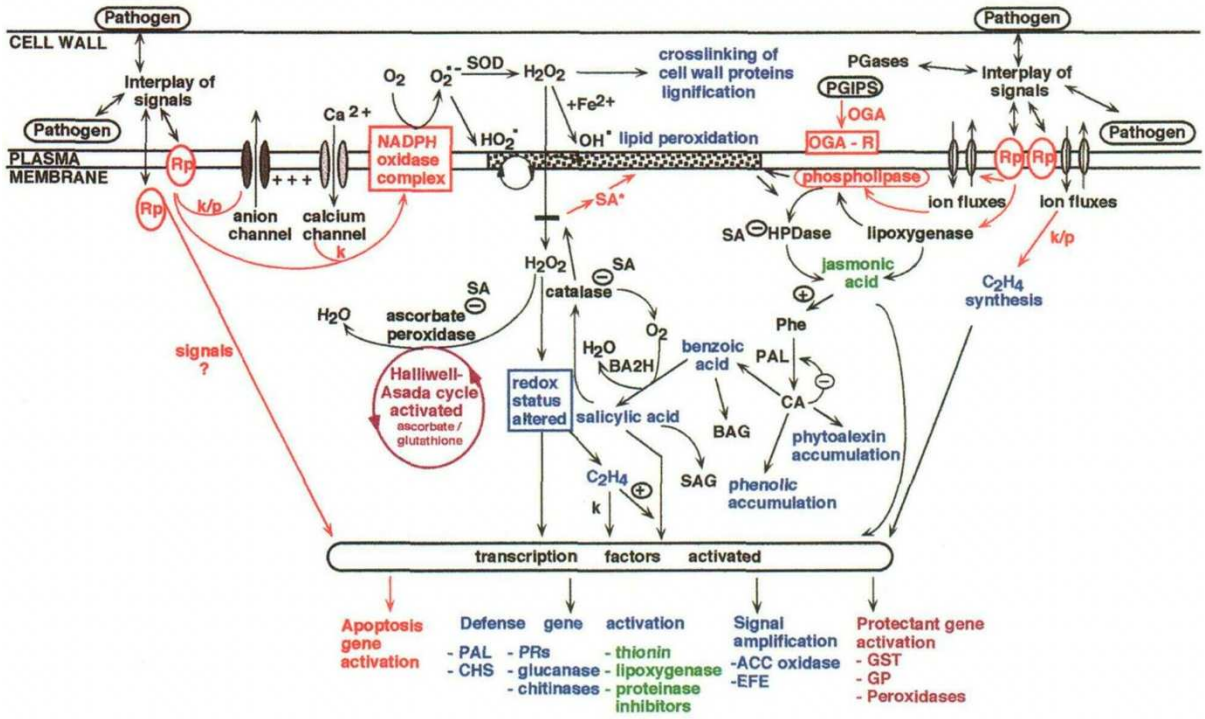


**Figure 2.** Complexity of Signaling Events Controlling Activation of Defense Responses. Plant receptor proteins (Rp) intercept pathogen-derived or interaction-dependent signals. These signals include the direct or indirect products of Avr genes, physical contact, and general components of each organism, such as chitin, enzymes, and plant cell wall fragments. Plant receptor proteins may or may not be the products of R genes. The immediate downstream signaling events are not known but involve kinases, phosphatases, G proteins, and ion fluxes. Several distinct and rapidly activated outcomes are recognized, including the production of ROS, direct induction of defense gene transcription, or possibly apoptosis genes, JA biosynthesis, and/or ethylene biosynthesis. Amplification of the initial defense response occurs through the generation of additional signal molecules, other ROS, lipid peroxides and BASA. This, in turns, induce other defense-related genes and modify defense proteins and enzymes. Concomitant alterations to cellular redox status and/or cellular damage will activate preformed cell protection mechanisms and induce genes encoding various cell protectants. Defense-related stress may also induce cell death. Cross-talk between the various induced pathways will coordinate the responses. (+) indicates positive and (-) indicates negative interactions. Components and arrows indicated in red are only postulated to be present in plant cells, whereas those in blue indicate known plant defense responses; green indicates plant defense responses also activated by JA, and purple indicates plant protection mechanisms. ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; BAG, benzole acid gluco-side;BA2H,benzoleacid

2hydroxylase;CA,cinnamicacid;CHS,chalconesynthase;EFE,ethylene-formingenzyme;HO2 ,hydroperoxylradical; HPDase, hydroxyperoxidedehydrase; GP, glutathione peroxidase; GST, glutathione S-transferase; k, kinase; O2, superoxide anion; OH , hydroxyl radical; OGA and OGA-R, oligalacturonide fragments and receptor; p, phosphatase; PAL, phenylalanine ammonia-lyase; PGases, polygalac- turonases; PGIPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA and SAG, salicylic acid and salicylic acid glucoside; SA*, SA radical; and SOD, superoxide dismutase (Adapted from Hammon-Kosack and Jones 1996).

To date, plants are described to activate different defense mechanisms depending on pathogen lifestyle (Garcia-Brugger et al., 2006). Jasmonic acid- (JA) and ethylene- (ET) dependent defense responses of plants appear to be useful to combat necrotrophic pathogens, whereas the salicylic acid (SA) dependent defense responses are useful against biotrophic pathogens. But what do we know about GTD such as Botryosphaeriaceae described as hemibiotrophic?

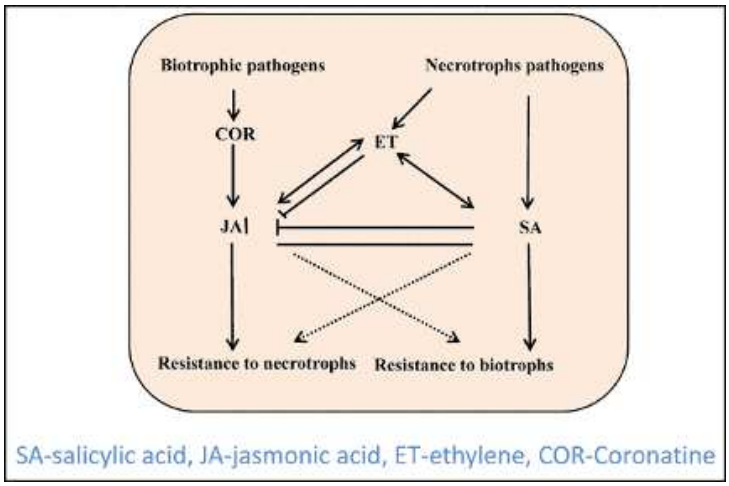


**Figure 3.** Type of defense pathways induced by biotrophic and necrotrophic pathogens. Adapted from Yang et al. 2015.

During their coevolution with pathogens, plants have developed a **set of defense reactions** based on **two levels of immunity according to the zig-zag model** theorized by Jones and Dang (2006): PTI (PAMP-Triggered Immunity) and ETI (Effector-Triggered Immunity) (Figure 4.).

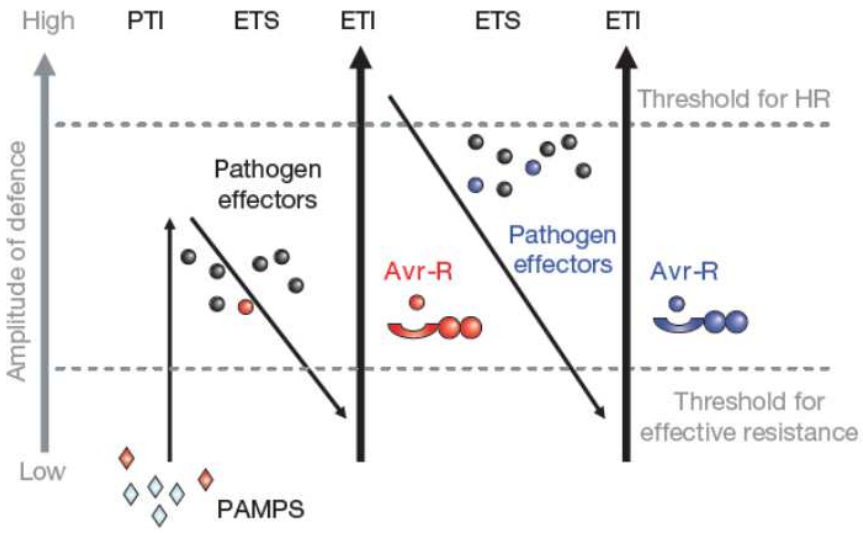


**Figure 4.** This model of plant immunity consists of four phases. The first phase consists of the recognition by the plant of highly conserved molecules of the pathogens called Pathogen-Associated Molecular Patters (PAMPs). This phase is referred to as PTI PAMP. Some adaptive pathogens are able to bypass the PTl by solving a class of molecules used as drivers. This model is called ETS (Efector-Triggered Susceptibility). The resistant plants have resistance proteins (R) that recognize a given detector and trigger ETI (Elector Triggered Immunity). The pathogen can suppress the ETI by various means. The coevolution between health and its pathogenic agent leads to an "arms". The coevolution between health and its pathogenic component leads to an "arms race" that can last for several cycles of bites on the evolutionary scale. The amplitude of the defense reactions associated to the phases of PTI and ETI differ. Indicated on the graph with two thresholds for the development of a resistance and effective resistance against a pathogen. Adapted from Jones and Dang (2006).

**The establishment of a PTI-type immune response** requires the recognition of the pathogen molecules (PAMPs or DAMPs) by the plant receptors (PRR). **PAMPs/MAMPs** (e.g., flagellin, lipopolysaccharides LPS, elongation factor EF-Tu, chitin, glucans, etc.) **and endogenous DAMPs** (e.g., galacturonic acid, oligogalacturonides) **elicit PTI** (first level of plant immunity) as indicated above: (1) PAMPs/MAMPs are recognized by the host PRR receptors belonging to the class of Receptor-Like Proteins (RLPs) or Receptor-Like Kinases (RLKs) (Monaghan and Zipfel 2012; Naito et al. 2008; Zipfel and Robatzek 2010), as well as endogenous DAMPS, once the plant cells wall are attacked by the pathogen CWDEs (Seong and Matzinger 2004). It seems that the specificity of PAMP/ PRR recognition is a result of the coevolution of the plant and its pathogen. (2) signaling pathways activating PTI (ROS, ion fluxes

and Ca signaling, MAPK/CDPK, activating TF and TF-dependent expression of defense genes. (3) hormonal signaling. (4) activation of TF responsive to hormone and expression of the related TF-dependent defence genes. (5) **PTI leads to the secretion of several types of molecules designed to limit or stop the development of the pathogen** (Wise et al. 2007). The class of molecules most produced under infection conditions is the **Pathogenesis-Related (PR) proteins** (Van Loon et al. 2006). This class includes 17 families based on sequence homologies and antimicrobial properties. Among the PR proteins are chitinases that degrade the fungal wall, protease inhibitors that inhibit the activity of fungal CWDEs, etc. Some of them are specific to a pathogen, while others have a broader spectrum of action. PR1 of infected tobacco has strong antimicrobial activity (Van Loon and Van Kammen 1970) and is commonly used as a marker gene for PTI reactions. PR1 antimicrobial properties are related to its ability to physically interact with ergosterols in the plasma membrane of the pathogen's plasma membrane and cause damage (Gamir et al. 2017). **Secondary metabolites** are also involved in defense reactions: phytoalexins (the most abundant antimicrobial compound), terpenes (Avenacin A-1 from oats), alkaloids (tomatine from tomatoes, camalexin from *A. thaliana*) or phenols (pisatine from peas) (Bednarek 2012). **The final means of combating the invasion of a pathogen is the focused secretion of callose**, a glucan polymer secreted by the plant at infection sites to form a physical barrier (Luna et al. 2010). Callose deposits are also frequently used as a marker for the induction of PTI or its suppression by suitable pathogens.

**ETS or effector-mediated repression of defense responses** is the second phase of the zigzag model, in response to apoplastic or cytoplasmic effectors. Effectors are known to manipulate the structure or functions of the host cell, and **some of them are recognized by the plant.** Resistant plants were able to resist infection by an adapted pathogen through a highly specific gene-for-gene interaction (Flor 1971; Thakur and sohal 2013; Xu et al. 2020). According to this model, **effectors of the pathogen are recognized by resistance proteins (R) of the resistant plant.** The recognition of this effector leads to a second level of immunity called effector-triggered Immunity (ETI). **The recognized effector is then qualified as an avirulence protein**, and its **recognition leads to a whole set of defense reactions**, including most often a **localized cell death called hypersensitive response (HR)** and the **stopping of the colonization of the plant by the pathogen**. Unlike PTI, which is common to several plants in the same phylogenetic group, **ETI is genotype specific.** Effector recognition is most often mediated by two types of resistance proteins: membrane receptors of the RLP or RLK type, and cytoplasmic proteins Nucleotide-binding/Leucine Rich repeat (NLR). This leads to signaling pathways and the activation of ETI-specific defense reactions (activation of resistance proteins

and signaling pathways leading to ETI). In contrast, when either the Avr gene in the pathogen or the R gene in the host is absent or mutated, recognition does not occur and the plant is said to be susceptible, resulting in disease (De Witt 1995; Flor 1971).

In natura, generally, the first infecting pathogen immunizes the plant against further infection by homologous pathogens, even though the plant may not carry a gene that determines crop-specific resistance. If the plant's promptness to resist subsequent attacks by pathogens spreads to the entire plant, the response is called systemic acquired resistance (SAR) to distinguish from the local one (LAR, with strong HR). The development of SAR is often associated with various cellular defense responses as described in Figure 3 (Ryals et al. 1996).

**Publication 1- REVIEW: Key levers to consider to sustainably manage grapevine trunk diseases from nursery to vineyards**

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# *REVIEW: Key levers to consider to sustainably manage grapevine trunk diseases from nursery to vineyards*

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**Abstract:**

Grapevine trunk diseases (GTDs), described as early as the end of the 19th century, are currently considered as one of the most relevant challenges for the viticulture worldwide. This group of grapevine diseases are caused by several fungal pathogens that develop and colonize the wood of the perennial organs causing wood necrosis and discoloration, vascular infections, and white decays. This review relates (1) the nature of GTD pathogens and their economic impact, (2) their main hosts and worldwide distribution, (3) the key events events conditioning their spread, (3) current knowledges on grapevine protection and defenses when facing one the most aggressive pathogen leading to GTDs, and its (4) dependency to a healthy microbiome; enabling to point out (5) microorganisms with biocontrol potential against GTDs, then to (6) give advices for a durable sustainable management of GTDs from nursery to vineyard.

**Keywords:** Grapevine trunk diseases, Botryosphaeria Dieback, Biocontrol agents, Trichoderma, Bacillus, Sustainable management.

## 1. Fungal trunk diseases: a challenge for grapevine productivity

During their entire lifespan, grapevine (*Vitis vinifera* L.) plants are exposed to a wide variety of pathogens, such as bacteria, viruses, phytoplasmas, fungi and nematodes (Armijo et al. 2016). Nowadays, grapevine fungal trunk diseases (GTDs) are amongst the main constraints for the productivity of this crop. GTD pathogens can

infect and severely damage grapevines from nurseries to mature plants in vineyards (Figure 1). Once infected, plant productivity is decreased, leading to a plant slow or apoplectic death that causes important economic losses and limits vineyard sustainability (Bertsch et al. 2013; Fontaine et al. 2016; Mondello et al. 2018).

In nurseries, it has been proven that the plant material can be infected with several GTD pathogens from the scion or rootstock mother blocks (Berbegal et al. 2020; Berlanas et al. 2020; Gramaje and Armengol 2011; Gramaje and Di Marco 2015), which can act as a primary source of infection (Edwards and Pascoe 2004; Edwards et al. 2001; Gramaje and Di Marco 2015; Pascoe and Cottral 2000; Waite et al. 2018). Additionally, most nursery fields allow mother vines to sprawl on the ground, which in combination with flood irrigation, can favor new fungal infections (Halleen and Fourie 2016; Hunter et al. 2004; Stamp 2003). In many cases, the presence of GTDs in the scion and rootstock mother plants is not directly related to visible external symptoms, emphasizing the high potential risk of mother vines as an inoculum source for GTD pathogens all along the vegetative propagation process (Aroca et al. 2010; Fourie and Halleen 2004; Hrycan et al. 2020).

GTD pathogens can be detected at various stages of the nursery propagation process. One of the earliest is during the hydration (soaking in water) mainly prior to cold storage, either by the water itself or by field-acquired microorganisms dispersing into the soaking water from one single contaminated plant (Gramaje and Armengol 2011; Waite and Morton 2007). Additionally, several wounds are produced in the nursery process, during disbudding, grafting, or upon root transfer in the field. These large numbers of cuts and wounds make the nursery propagation material very susceptible to infections, providing many entry points for GTD pathogens, both during the nursery process and in nursery fields (Berbegal et al. 2020; Lade et al. 2022; Waite et al. 2018).

Surveys conducted in the main grape growing countries worldwide highlighted that nursery propagation material used for new plantations is mainly infected with fungi involved in **Petri disease** (caused by *Phaeomoniella chlamydospora* and several *Cadophora* and *Phaeoacremonium* species) and **Black foot** (caused by species from *Cylindrocarpon*-like asexual morphs belonging to the genera *Campylocarpon*, *Cylindrodendrum*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon* and *Thelonectria*) (Akgül et al. 2022; Berlanas et al. 2020; Edwards et al. 2007; Gramaje and DiMarco 2015; Guerin-Dubrana et al. 2019). Petri disease and



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black foot are the two most common GTDs observed in young vineyards (<5 years old) (Gramaje and Armengol 2011). Foliar symptoms associated with both diseases (overall stunting, delayed budbreak, shortened internodes, chlorotic foliage with necrotic margins, and wilting of leaves or entire shoots) can not only overlap but resemble symptomatology associated with abiotic disorders making these diseases hard to diagnose (Gramaje and Armengol 2011). However, Petri disease can be recognized by the presence of a dark-colored sap due to the presence of phenolic compounds in the xylem vessels of trunk, while black foot can be recognized by black, sunken, necrotic lesions on roots and reddish-brown discoloration in the base of the trunk of infected vines (Gramaje and Armengol 2011). Based on these phenotypes, many authors have attributed a drastic increase in grapevine mortality to Petri disease and black foot in new plantings over the last few years (Agustí-Brisach and Armengol 2013; Agustí-Brisach et al. 2016; Aroca et al. 2010; Carlucci et al. 2017). The annual financial cost of the replacement of dead cv Tempranillo plants due to both diseases is estimated to be 7.16 million €/year in La Rioja (northern Spain) (Martínez-Diz et al. 2019).

Indeed, in the nursery fields, many GTDs-infected plants from the propagation process can develop internal symptoms, invisible externally, but allowing pathogens to spread to other plants. New infections can also occur in these nursery fields, as well as in vineyards, yet adding load and pressure from the fungal pathogens by their air-dispersed spores still present in the environment (Fig. 1), such as the ascospores or conidia of many GTD pathogens, which are released from perithecia or pycnidia embedded in the bark and/or on the surface of dead grapevine wood (Eskalen and Gubler 2001; Gramaje et al. 2018; Rooney-Latham et al. 2005; Trese et al. 1980; Urbez-Torres et al. 2010). Additionally, as non-host specific, many of these pathogens can be found in a wide range of woody perennial crops as new sources of inoculum when they are close to vineyards (Carter 1991; Gramaje et al. 2016; Hrycan et al. 2020; Petzoldt et al. 1983). Favourable environmental conditions for the release of ascospores and conidia are rainy events or high relative humidity, with temperatures above freezing that favour spore germination (Urbez-Torres et al. 2010; Van Niekerk et al. 2010). Consecutive dissemination of spores is ensured by rain droplets, wind, and arthropods until they reach the cuts or pruning wounds, their major entry points, to germinate and start colonizing new xylem vessels and pith parenchyma cells (Gramaje et al. 2018; Mostert et al. 2006; Moyo et al. 2014) (Fig.1). Depending on the pathogen,

wounds can remain susceptible to infection for up to 2 to 4 months (Rolshausen et al. 2010; Van Niekerk et al. 2010). As the wood of diseased vines can be simultaneously infected by various fungal pathogens typically associated with different GTDs, internal and external symptoms can overlap, making them hard to identify. Additionally, GTD pathogens possess an undetermined latency period (asymptomatic status) that can last up to 10 years, leading to a late diagnosis when only considering the visual external plant phenotype, and subsequently making them hard to treat.

In vineyards, the most relevant GTDs affecting young and mature vines are the following:

**Eutypa dieback** is caused by fungi belonging to the Diatrypaceae family, the most virulent and common of which is *Eutypa lata* (Kuntzmann et al. 2010). Foliar symptoms of Eutypa dieback include stunted shoots with chlorotic leaves that are often cupped and with necrotic margins. The foliar symptomatology is reproduced with purified toxic metabolites secreted by *E. lata* in woody plants (Mahoney et al. 2005; Molyneux et al. 2002; Tey-Rulh et al. 1991). Foliar symptoms can appear 3 to 8 years after infection (Tey-Rulh et al. 1991) and can vary from year to year (Sosnowski et al. 2007). Bunches on stunted shoots ripen unevenly, are small, and in severe cases, the berries shrivel and die. Woody symptoms of Eutypa dieback include cordon dieback, with loss of spurs and internal, necrotic, wedge-shaped staining in the cross-section of cordons and trunks. External cankers appear as the dieback progresses, characterized by flattened areas of the wood with no bark, leading to eventual vine death (Gramaje et al. 2018).

**Botryosphaeria dieback** (BD) is caused by Botryosphaeriaceae fungi from the genera *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria* and *Spencermartinsia* (Mondello et al. 2018). BD often presents as lack of spring growth from affected spurs with shoot dieback, bud, and xylem necrosis (Urbez-Torres 2011). Pycnidia develop from dead or cankered wood. The main wood symptom of BD is wedge-shaped perennial cankers, or circular to non-uniform central staining of the wood observed in cross-sections of affected wood (Mondello et al. 2018; Urbez-Torres 2011). Visible external symptoms can appear in the field only 1 or 2 years after infection (Urbez-Torres et al. 2006) and are mainly observed in mature vineyards (over 8-year-old).

**Esca and Grapevine Leaf Stripe Diseases** (GLSD) are due to a broad range of taxonomically unrelated fungal trunk pathogens associated with esca diseased vines



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(Mondello et al. 2018). However, the function of these fungi and how they interact with the primary fungi responsible for disease symptoms is still unclear. The main hypothesis is that young vines infected with pioneer fungi such as *P. chlamydospora*, *Cadophora* spp. or *Phaeoacremonium spp.* can later develop esca symptoms following a further colonization by several basidiomycetous species from the genera *Inocutis*, *Inonotus*, *Fomitiporella*, *Fomitiporia*, *Phellinus* or *Stereum* (Cloete et al. 2015). Two forms are also known to affect grapevines: chronic (mild) and acute (apoplectic). In the chronic or mild form, called GLSD, the most characteristic foliar symptom is the 'tiger-stripe' pattern, in which leaves display multiple banding discolorations surrounding dry, light, or red-brown necrotic tissue on the leaf blade, often bordered by narrow red or yellow blotches (Surico 2009). The acute or apoplectic form, called "esca proper", is characterized by a sudden wilting of the entire plant or of one arm or several shoots. Leaf symptoms include scorching, dropping, and shrivelling. The drying of grape clusters is also frequently observed (Mugnai et al. 1999). Foliar symptoms of both forms of esca appear in late spring or summer and can vary from year to year. Cross-sections of esca affected trunks reveal a variety of internal woody symptoms, such as black spots in the xylem, eventually surrounded by pink to brown discoloration of the wood, brown to black vascular streaking, or dry wood with a silver appearance. In oldest vines, the wood may develop a white to soft yellow rot (Fischer 2002).

**Phomopsis dieback** (PD) can be caused by species of the genera *Diaporthe* (Baumgartner et al. 2013; Dissanayake et al. 2015; Urbez-Torres et al. 2013). Among them, the most frequent species isolated of this genus in Europe are *D. eres* and *D. ampelina* (syn. *Phomopsis viticola*) (Guarnaccia et al. 2018). Both species are shown to be pathogenic on grapevine (Guarnaccia et al. 2018). The most characteristic symptoms attributed to PD include perennial cankers in the framework of the vine and lack of budbreak from infected spurs (Urbez-Torres et al. 2013).

**Cytospora canker** is caused by *Cytospora* species, known to be destructive to over 85 woody plants (Adams et al. 2005, 2006; Sinclair et al. 1987). They colonize the periderm and underlying sapwood of Angiosperms, causing brown/black discolouration of the wood and loss of hydraulic conductivity within the xylem. However, it was not long ago that this genus has been associated with diseased grapevines. Fotouhifar et al. (2010) reported two *Cytospora* species, isolated from diseased or dead grapevine wood in Iran, although the pathogenicity of these two species was not tested. Species like *Cytospora chrysosperma* (Adams et al. 2006)



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were isolated from symptomless grapevine wood and was thus classified as endophytes by Gonzalez & Tello (2011). Therefore, over a long time, *Cytospora* species were not considered as grapevine pathogens, and were not associated with GTDs. However, recent studies have characterized pathogenic *Cytospora* isolates such as *C. chrysosperma* from wood cankers of declining grapevines as pathogens that cause symptoms like those of other GTDs (Arzanlou and Narmani, 2015). New *Cytospora* species were further characterized as pathogenic to grapevine in America (*C. viticola* and *C. vinacea*) and Turkey (*C. viticola*) as indicated by Lawrence et al. (2017) and Oksal et al. (2020). More studies are now required to better understand the interactions between cytospora canker and that of other GTD pathogens. The epidemiology of cytospora canker has been deeply studied in other woody plants, and this information might help to guide future research on the spread of this disease in vineyards. As *Cytospora* species are primarily wound pathogens in tree crops (Biggs, 1989), they may infect vines through the pruning wounds generated on grapevines every season.

## 2. Are there any cultivar traits of tolerance or susceptibility of *Vitis vinifera* against GTDs?

**2.1. *V. vinifera* subsp. *vinifera*** (more simply referred to as *V. vinifera*) results from a human domestication to select morphological characteristics that favor grapevine cultivation and exploitation. Propagation of *V. vinifera* varieties gave preferences to the largest, juiciest, and tastiest berries, to gradually select identical grape varieties (a population for a variety). Grape varieties can also result from selections and crosses between local wild plants and plants already selected. Therefore, from *V. vinifera* subsp. *vinifera*, the various grape varieties make up the vast range of vine varieties cultivated worldwide, with more than 6,000 "cultivars" or “cépages” (a botanical term for varieties of plant species obtained by crossing and cultivated). Genetic diversity is thus generally preserved within a cultivar genome of *V. vinifera*, except when mutations may accumulate over time to generate distinguishable clones and significantly increase the genomic variability (Franks et al. 2002; Riaz et al. 2002; Zhou et al. 2019).

Nowadays, *V. vinifera* is present worlwide, the most concentrated areas being: Central and Southern Europe, Western and Middle East of Asia, China,



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Mediterranean coast of Africa, South Africa, North America, South America, Australia, and New Zealand (Figure 2A). Current patterns of genetic variation in *V. vinifera* grapes are associated with geographic and climatic variation (Magris et al. 2021). Distribution of GTDs around the world (Figure 2B) also depends on geographic and climatic variations (Kenfaoui et al. 2022; Songy et al. 2019).

Considering that both (i) genotype diversity within grapevine varieties, cultivars or even clones, and (ii) GTD distribution and pressure, depend on the geographical area, it could be important to deeply consider the *V. vinifera* genomic traits to better combat GTD pathogens worldwide, for instance by investigating a genomic comparison of vines from distinct geographical/climatic areas. Additionally, as cultivated grapevines are mainly grafted propagated (> 80%), their genetic diversity could be further increased, by both the scion (aerial part of the grafted plant, variety) and the rootstock (underground part of the grafted plant) (Ollat et al. 2014; Songy et al. 2019).

The susceptibility of cultivars to GTDs has been studied (Bertsch et al. 2013; Borgo et al. 2016; Fussler et al. 2008; Martínez-Diz et al. 2019; Murolo and Romanazzi 2014; Songy et al. 2019; Sosnowski et al. 2007; Travadon et al. 2013). Some cultivars could be considered susceptible to some GTDs such as Chardonnay and Merlot due to the higher proportion of symptomatic vines observed under specific climatic conditions (Andreini et al. 2009; Bruez et al. 2013; Christen et al. 2007; Murolo and Romanazzi, 2014). Grapevines with small xylem vessels appear less prone to express leaf symptoms due to limited xylem cavitation (induced by drought), and thus tend to be more resistant to some GTDs, such as Petri disease and Eutypa dieback (Pouzoulet et al. 2014; Ramsing et al. 2021). Rootstocks play an important role in grapevine production for their capacity to promote plant protection against pathogens and abiotic stress (drought, waterlogging, adaptation to different soils, nutrients intake, etc.), and to influence the scion vigor and grape composition (Chacón-Vozmediano et al. 2021; Gindro et al. 2003; Larignon and Dubos, 1997; Marchi 2001; Marín et al. 2021; Monteiro et al. 2013; Songy et al. 2019; Yıldırım et al. 2018). In a study of wood necrosis in rootstock mother vines, Liminana et al. (2009) determined that rootstock 1103 Paulsen was the least susceptible to GTDs (33% mean percent necrotic area) and 101-14 MGT was the most susceptible (71% mean percent necrotic area). Murolo

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and Romanazzi (2014) also noted that vines grafted to the drought tolerant 1103 Paulsen rootstock had lower incidence of esca symptoms than those grafted to SO4.

**2.2. *V. vinifera* subsp. *sylvestris*** (more simply referred to as *V. sylvestris*) is another subspecies of *V. vinifera* that includes populations returned to the wild. A great genetic diversity was also observed within *V. sylvestris* varieties, depending on the soil, the environment, and the local climate (Regner et al. 1998). For GTDs, Guan et al. (2016) reported that populations of *V. sylvestris* provide interesting genetic resources for breeding new strategies with enhanced resistance to BD. Comparing *V. vinifera* and *V. sylvestris* susceptibility responses to Botryosphaeriacaea infection, revealing a concept in which the phenylpropanoid metabolism of resistant genotypes is rapidly and specifically channeled to bioactive stilbenes (Khattab et al. 2021). Resistant lines would thus accumulate specific bioactive stilbenes such as viniferine trimers, to contrast with susceptible genotypes accumulating significant higher proportion of glycosylated stilbenes (Khattab et al. 2021).

## 3. Events that occur between the time spores of GTD pathogens encounter the plant and the appearance of symptoms: focus on Botryosphaeriaceae and their virulence factors.

In the Botryosphaeriaceae, asexual reproduction is the most common mode of reproduction (Baskarathevan et al. 2012a; Phillips et al. 2002; Van Niekerk et al. 2006). As indicated in the part 1 and in Figure 3, ascospores of GTD pathogens infect grapevines through pruning wounds, grafting wounds or natural wounds, and colonise the woody tissues. These wounds are entry points over a long period of time and natural inoculation depends on environmental conditions (time of year, climatic conditions).

Botryosphaeriaceae can remain latent as endophytes until the host plant is exposed to stress (Dakin et al. 2010; Hrycan et al. 2020; Slippers and Wingfield 2007). As heterotrophs, they use cell wall degrading enzymes (CWDEs) to take nutrients from the host showing no signs of disease (Giovannoni et al. 2020). Among CWDEs classified as carbohydrate active enzymes (CAZy) are those attacking lignin (ligninases such as laccases, polyphenol-oxidases, manganese peroxidases), cellulose (such as glucanases, cellobiohydrolases, glucosidases), hemicellulose



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(hemicellulases such as xylanases, xylosidases, arabinofuranosidases), and pectin (pectinases such as polygalacturonases, pectate lyases).

Several endophytic fungi are ubiquitous and can therefore colonise different hosts according to two modes of transmission: (1) vertical transmission (hypha penetration into a seed, pollen grain or propagule of the host plant) characterised by the colonization of a new progeny host of the primary host. The endophytic fungus remains genetically identical (propagation by asexual reproduction). (2) Horizontal transmission is characterised by the colonization of a new host that is usually unrelated to the primary host. It proceeds from the dissemination of spores by a dispersal vector. After germination, the hypha penetrates the new host either through the stomata or by direct penetration through the epidermis (Rodriguez et al. 2009). Most species with endophytic stage, such as *Botryosphaeriaceae*, present a horizontal mode of transmission (Rodriguez et al. 2009; Slippers and Wingfield 2007).

Fungal endophytes are described to interfere with plant ecology, fitness, and evolution (Rodriguez et al. 2009; Slippers and Wingfield 2007). Depending on the environmental conditions and the physiological state of the host, the lifestyle of the fungus can change from endophyte to necrophyte parasite. This type of pathogenic fungus is called hemibiotroph (Rajarammohan 2021). It is pathogenic in its necrotrophic state in the case of the hemibiotrophic *Botryosphaeriaceae,* with an intracellular growth that distinguish them from the true necrotrophic pathogens, considered as “extracellular hemibiotrophs” (Rajarammohan 2021). During its endophytic lifestyle, the hemibiotroph can positively impact its host (Kogel et al. 2006). During its necrotrophic phase, the hemibiotroph has a negative impact on its host in the short or long term through an excess of deleterious nutrients on the host (such as phytotoxins and other virulence and pathogenicity factors).

Depending on the biotope and environmental conditions, fungal species could adopt several modes of life during its existence (Kogel et al. 2006). This is particularly the case of endophytic Botryosphaeriaceae species, such as *Neofusicoccum parvum* which is considered as pathogenic once turning necrotroph (Figure 4).

Switching its lifestyle (“bang-mixed” to “bang-bang” strategies) would allow the pathogen to increase its aggressiveness to complete its life cycle and thus ensure its population survival when it senses a decrease in the size of the plant resource pool depending on biotic and abiotic pressures (Andanson et al. 2010). The "bang-mixed"

strategy operates when the host resources are considered inexhaustible, and only limited by the maximum number of energy channels (suction ways) that the pathogen can develop. In this case, the infection strategy is first characterised by a latent phase entirely dedicated to intra-host growth, followed by an infectious period, where part of the energy is allocated to spore production and the other part to the maintenance of intra-host multiplication structures, whose density remains constant. For the "bang-bang" strategy (aggressive infection), the pathogen senses a finite resource pool, thus a fixed size since the beginning of the infection. In this case, the infection strategy is first characterised by a latent phase dedicated to intra-host growth, followed by an infectious period switching energy exclusively towards the production of spores (avoidance of any extinction risk), thus leading to a decrease in the intra-host multiplication structures.

Plant genotype can impact the size of the resource pool depending on the metabolic changes induced upon environmental changes (Cordovez et al. 2019). Pathogen aggressiveness can thus adapt to the plant susceptibility/responses to its environment. Severity of disease symptoms were reported to vary with grapevine cultivars, inoculated tissues, and abiotic conditions (Chacon et al. 2020; Leal et al. 2021b; Ramirez-Suero et al. 2014; Spagnolo et al. 2014; Songy et al. 2019; Urbez-Torres 2011).

Pathogen genotype can parallely acquire new traits to ensure its survival and fitness (lifespan, way of reproduction, dispersal, spore germination, etc.) upon abiotic and biotic changes. We can thus speculate that hemibiotroph could constitute a transition towards necrotrophy, which would represent their evolutionary achievement (Horbach et al. 2011). In a changing environment with selection pressures, only one part of the fungal population succeeds to reproduce, thanks to acquired traits (e.g., tolerance to temperature changes, resistance to xenobiotic, etc.). Research is still in progress to better understand pathogen adaptation and aggressiveness to environmental factors both *in planta* and *in vitro*, focusing on fungal morphology, physiology and behaviour leading to survival and adaptability. Analyses need to be strengthened to better understand the weight of genetic polymorphism in the adaptation and aggressiveness of pathogens in a changing environment, to ensure its survival (Trotel-Aziz et al. 2022).

Among pathogenic factors are CWDEs, phenolic phytotoxins (toxins), growth regulators (hormones), effector proteins, and fungal viruses (Peng et al. 2021).



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Incompatible interactions would thus enable the plant metabolism to react against the pathogen and related associated motifs (PAMPs, Pathogen-Associated Molecular Patterns, e.g., effector proteins) thanks to external plant receptors PRRs (Plant pattern Recognition Receptors) that should be able to elicit a first level of plant immunity (PTI, PAMP triggered immunity). Research on these extracellular aspects remains to be deeply explored for GTD pathogens. To date BD symptoms are described to result mainly from the synergistic actions of CWDEs and phytotoxic metabolites produced and secreted by these fungi inside the plant cells (Abou-Mansour et al. 2015; Masi et al. 2018; Salvatore et al. 2021; Stempien et al. 2017; Trotel-Aziz et al. 2019, 2022). BD fungi are indeed only detected in wood (entry through CWDEs), but never in symptomatic leaves (Larignon et al. 1997), whereas phytotoxins are suspected to be involved in the production of foliar symptoms once transported from the infection site to the leaves via the xylem (Mugnai et al. 1999).

In general, effectors secreted by pathogens (eg. secondary metabolites (SM) such as phytotoxins) can suppress plant triggered immunity (such as PTI) responses and facilitate plant colonization, by targeting plant susceptibility (S) proteins, resulting in an effector-triggered susceptibility (ETS) (Li et al. 2020a; Peng et al. 2021; Van Schie and Takken 2014). Literature also describes a second layer of defenses, involving plant resistance (R) proteins (eg. nucleotide-binding leucine-rich repeat proteins, NLRs) able to recognize and detoxify the pathogen-produced effectors, resulting in effector-triggered immunity (ETI) (Jones and Dangl 2006; Kourelis and Van der Hoorn 2018; Peng et al. 2021). But to evade such effector recognition, pathogens can undergo loss or mutation of the corresponding effector genes (Jones and Dangl 2006). Pathogens and plants are thus evolving in a perpetual arm race due to relatively fast evolution of effectors, as well as of the R and S proteins (Jones and Dangl 2006; Peng et al. 2021; Rouxel and Balesdent 2017; Rouxel et al. 2011). Effectors increasing virulence are called virulence factors, while effectors recognized by R proteins are called avirulence factors (Avr proteins) (Peng et al. 2021; Stotz et al. 2014).

Focusing now on *Botryosphaeriaceae* species, several phytotoxic SM are produced and have already been identified (Table 1), but their specific roles in the infection process still remain to be clarified (Abou-Mansour et al. 2015; Andolfi et al. 2012; Djoukeng et al. 2009; Evidente et al. 2010; Larignon et al. 1997; Masi et al. 2018; Reveglia et al. 2019, 2020; Salvatore et al 2021; Trotel-Aziz et al. 2019, 2022). In general, phytopathogenic toxins are mostly low-molecular-weight SM that can produce



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specific symptoms such as wilting, growth inhibition, chlorosis, necrosis, and leaf spotting (Yin et al. 2016). At very low concentrations, phytopathogenic toxins are described to act on cell membrane, mitochondria, chloroplasts, and to inhibit the synthesis of proteins and nucleic acids in the host plant, resulting in physiological disorders, cell death, and even death of the plant itself (Peng et al. 2021; Shang et al. 2016; Soyer et al. 2015; Zeilinger et al. 2016).

According to Li et al. (2020), it is reasonable to assume that hemibiotrophic pathogens such as *Botryosphaeriaceae* have already acquired the potential to inhibit the plant's defense responses during their endophytic phase, thus before confronting any plant immune system. To do that, appressorium are useful structures enabling pathogen temporal advance (effector accumulation) to bypass plant defenses, by secreting these effectors simultaneously with the plant's perception of the pathogen. Especially since no R proteins were yet identified in grapevine to stop BD. Maybe it can be explained by the high level of polymorphism in genes involved in phytotoxin synthesis, such as (-)-terremutin and (*R*)-mellein biosynthesis among *N. parvum* isolates (Trotel-Aziz et al. 2022). This high level of genetic variations within a species, could be a consequence of the evolution and adaptation of *N. parvum* to its biotic environment (Garcia et al. 2021). It is important to remind that *N. parvum* is described as a highly aggressive plant pathogenic fungus of the *Botryosphaeriaceae* family (Larignon et al. 2015; Urbez-Torres 2011). Additionally, Trotel-Aziz et al. (2019, 2022) highlighted that, once inside the plants, phytotoxins succeed to weaken grapevine immunity to promote BD symptoms. Altogether, these recent results strongly suggest that (1) the *N. parvum* phytotoxins are effectors that trigger ETS in grapevine, and that (2) grapevine alone would not succeed to combat BD, pointing that beneficial microorganisms could be helpful to compensate such grapevine weakness.

Genetic differences also exist among the isolates of *N. parvum* that are described as a highly aggressive plant pathogenic fungus of the *Botryosphaeriaceae* family (Larignon et al. 2015; Urbez-Torres 2011), especially regarding genes involved in phytotoxin biosynthesis (Trotel-Aziz et al. 2022).

## 4. Grapevine protection and defenses when facing pathogens leading to GTDs: focus on *N. parvum*.



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When exposed to GTDs, grapevine symptoms (as indicated in part 1; i.e. delayed budburst, stunted development, leaf chlorosis, stem necrosis, canker, dead branch to full plant death) can vary depending on the pathogen genotype.

Focusing on three *N. parvum* isolates (Np-Bt67, NpB and NpB-UV9) differing in their aggressiveness and SM profiles, Np-Bt67 was characterized by its ability to produce large amount of (-)-terremutin without (*R*)-mellein and is the most pathogenic on Chardonnay grapevine (Trotel-Aziz et al. 2022). It causes strong BD symptoms (dead branch) within 10 days on grapevine cuttings (Leal et al. 2021b; Trotel-Aziz et al. 2019). NpB produced mid-amounts of both (*R*)-mellein and (-)-terremutin, while NpB-UV9 produced large amount of (*R*)-mellein without (-)-terremutin, and both were less pathogenic isolates on grapevine (stem necrosis without dead branch) than Np-Bt67 (Trotel-Aziz et al. 2022). Comparing the effects of fungal isolates and purified (-)-terremutin on cuttings, authors suggested that (-)-terremutin and (*R*)-mellein would act more as a virulence factors (i.e. increasing quantitatively the disease) than as pathogenicity factors (i.e. required to trigger the disease) upon infection (Chooi et al. 2015; Reveglia et al. 2020; Trotel-Aziz et al. 2022).

Despite they differe in their aggressiveness, the wild-type isolates Np-Bt67 and NpB displayed similar trends in plant defenses induction: an up-regulation of salicylic acid (SA) responsive genes (*PR2*, *PR5* and *PR10*), while the expression of jasmonic acid and ethylene (JA/ET) responsive genes was either unaffected or moderately up-regulated (Trotel-Aziz et al. 2022). Yang et al. (2015) reported that the plant SA-dependent defenses are not helpful to combat necrotrophic fungi and that SA-signalling could antagonize the expression of the useful JA-responsive defenses, resulting in facilitation of disease development. Among upregulated genes during cuttings infection with pathogens, are also those encoding for enzymes involved in the biosynthesis of antimicrobial phytoalexins (*PAL*, *STS*) and in detoxification process (*GST1*) (Trotel-Aziz et al. 2022). *GST1* is also strongly upregulated in plantlets treated with (-)-terremutin, therefore suspected to ensure detoxification of this phytotoxin (Trotel-Aziz et al. 2022). In contrast, phytotoxins supply to plantlets repressed the expression of *CHI* (chalcone isomerase involved in flavonoid synthesis), while they did not impact the expression of *PAL* and *STS* known to produce phytoalexins potentially metabolizable by *Botryosphaeriaceae* (Stempien et al. 2017). Trotel-Aziz et al. (2022) further suggested that (-)-terremutin could inhibit the expression of flavonoid pathway to favour the stilbene production (since they share a common precursor), resulting in



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enhancing pathogen fitness. Additionnally, these auhors demonstrated that (-)-terremutin weakened the expression of the useful host JA-dependent-defenses, confirming therefore that (-)-terremutin production by *N. parvum* would support the pathogen own development in infected grapevine tissues by interfering with host defenses. Finally, these authors suggested that *GST1, PR2*, *PR3*, *PR4*, *PR5* and *PR10* are useful defenses against *N. parvum*, being the most up-regulated in response to the most aggressive Np-Bt67 isolate, while not at all upregulated in plantlets treated with (-)-terremutin (except *GST1* as explained above). They also hypothesized that the repression of *PR1* could be beneficial for plant protection against *N. parvum*, since *PR1* was only down-regulated in response to the less aggressive isolate NpB, while it was up-regulated by both phytotoxins. Regarding the responsiveness of these genes to hormones, *PR1*, *PR5* and *PR10* are described to be regulated by SA (Caarls et al. 2015; Dufour et al. 2013; Naznin et al. 2014), *PR3* and *PR4* by JA/ET, and *PR2* by various phytohormones such as SA, JA/ET, and abscisic acid (ABA) (Liu et al. 2011).

Comparing cultivars with distinct susceptibility to GTDs (Chacon et al. 2020; Fontaine et al. 2016b; Reveglia et al. 2021; Travadon et al. 2013), an early detection of defense compounds (such as the PR2 and PR5 proteins) was observed in tolerant genotypes (Claverie et al. 2020; Spagnolo et al. 2014). A greater susceptibility to BD has also been reported for Tempranillo compared to Chardonnay (Cobos al. 2019; Leal et al. 2021b; Luque et al. 2009). Leal et al. (2021b) indicated a greater constitutive expression of some targeted defense genes in Tempranillo compared to Chardonnay (eg. SA-responsive genes), even upon challenge with Np-Bt67, possibly explaining Tempranillo's highest susceptibility to BD. Authors conclude that grapevine susceptibility to BD is cultivar-dependent and suggest that a possible high basal content of SA in Tempranillo could explain its highest susceptibility to *N. parvum*. Indeed, JA is described as useful to combat necrotrophs, but many studies have reported that endogenously accumulated SA antagonises JA-dependent defences, thus prioritising SA-dependent resistance over JA-dependent defence (Pieterse et al. 2012; Van der Does et al. 2013). Supporting this hypothesis, Leal et al. (2021b) highlighted a common feature for both cultivars: SA-dependent defences were strongly decreased in plants protected by an additional supply of beneficial microorganisms acting as biocontrol agents (BCA; see Part 6 for more details about BCA), to contrast with symptomatic plants.

Lacking self-defence means, the plant can ultimately opt for an avoidance strategy, by inducing a dead branch to confine the BD fungi. But this strategy may emerge late once the plant is infected by a cryptic pathogen that can modulate its host cellular environment, for instance by producing oxalic acid that may suppress the initiation of the host cell death in the early stages of infection (Rajarammohan 2021; Williams et al. 2011). Fortunately, following pathogen infection and CWDE activities, plants can recognize neoproduced DAMPs (Damage Associated Molecular Patterns) as signalling molecules able to activate death signaling and turn resilient (Hou et al. 2019). As for a systemic acquired resistance (SAR) triggered by a tolerant/resistant plant upon a pathogen attack (Durrant and Dong 2004; Shine et al. 2019), DAMPs can mediate an immune priming in the distal cells and tissues, to prevent pathogen progression. DAMPs are perceived by plasma membrane localized receptors, whose activation leads to the initiation of signaling cascades (e.g., MAPK, $Ca^{2+}$) and transcriptional reprograming (Hou et al. 2019). DAMPs can be peptides, ATP, proteins released from damaged cells, and degraded cell wall polysaccharides that function as PTI amplifiers (Hou et al. 2019). Among peptide signaling molecules are systemin and pep1 able to trigger JA biosynthesis, and related systemic defenses against a fungal pathogen. Among ATP is the extracellular adenosine 5-triphosphate (eATP) that can also modulate the expression of genes involved in SA and JA signalling once eATP was perceived by the lectin receptor kinase DORN1 (DOes not Respond to Nucleotides) that induces the PTI-like signalling processes. The co-factor $NAD^+$ is also a host-derived-elicitor of immune signalling able to activate PTI-like responses, especially those specific to SA. Among proteins from damaged plant cell is HMGB3. Among degraded polysaccharides from damaged plant cell are oligogalacturonides (OGs), cellooligomers, xyloglucan and oligosaccharides, methanol. Functioning as PTI amplifier, DAMPs signaling is essential to compensate MAMP (Microbial-Associated Molecular Patterns) signaling when MAMP-triggered defenses are compromised by a malfunctioning/disappearance of natural endophytic beneficial microorganisms (see Part 6 for more details about BCA and related MAMPs).

Altogether, little information exists to establish a list of potential markers of grapevine cultivar resistance or tolerance to GTD pathogens, especially considering that the pathogen bypasses the plant's useful defences, leaving it defenceless since the plant alone seems to have no useful means of self-defence (Figure 5). Currently, Leal et al. (2021b) clearly established that SA-dependent defenses are strongly



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decreased in plants protected by BCAs and stated that complementary approaches are in progress to highlight any useful changes induced by BCAs in cultivars to protect them against GTDs, considering both repressed and induced responses.

## 5. Does GTD pathology may ultimately depend on a fine tune regulation of grapevine microbiome?

Considering that grapevine alone has no useful means of self-defense, whereas it can be strongly protected when treated with beneficial microorganisms, we can wonder about the importance of its microbiome and microbiota in their protective effects against GTDs. Indeed, besides fungi, the endophytic bacterial and viral microbiome of grapevine and their roles in GTDs are largely unexplored (Bettenfeld et al. 2021; Nerva et al. 2022).

Few studies have compared the composition of the microorganism community in asymptomatic and symptomatic vines. Fotios et al. (2021) provided evidence that GTD symptomatic plants support a specific wood microbiome, showing cultivar and biogeography-dependent patterns, possibly useful to discriminate healthy from diseased vines. They also pointed out the strong interactions between the bacterial and fungal wood microbiome in asymptomatic vines, which still need to be investigated deeply to find out new bacterial BCAs. Nerva et al. (2019) characterised the microbiome from grapevines showing or not esca symptoms and found more than 100 distinct fungal isolates from 20 different families, in symptomatic plants compared to asymptomatic plants. They also reported 38 new mycoviruses, some capable of infecting grapevine trunk pathogens. Del Frari et al. (2019) also investigated the mycobiome of grapevines wood in a vineyard with history of esca. They identified 289 taxa, including five genera reported for the first time in association with grapevine wood symptoms of esca (Debaryomyces, Trematosphaeria, Biatriospora, Lopadostoma, and Malassezia). Interestingly, the mycobiome qualitative and quantitative composition of the woody tissue in proximity to symptomatic leaves, as well as in symptomatic canes, was highly similar to that in the woods of plants not exhibiting yet leaf symptomatology. This observation supports the current understanding that leaf symptoms are not directly linked with the sole presence of pathogenic fungal communities in the woods. In good accordance, *Botryosphaeriaceae* are described as non-pathogenic



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endophytes before switching pathogenic (once necrotroph). Altogether, these recent results strongly suggest that endophytic microorganisms (beneficial bacteria and beneficial fungi) and/or virus are key partners to consider conditioning the grapevine health status once infected by a fungal pathogen responsible of GTDs.

Focusing on the bacterial genera from asymptomatic and symptomatic Greek grapevines, Fotios et al. (2021) identified a strong enrichment of *Bacillus* (and widely of members of the family *Bacillaceae*) and *Streptomyces* in the former asymptomatic. This enrichment was particularly prominent in the cv Xinomavro and Agiorgitiko, while in Vidiano both *Bacillus* and *Corynebacterium* were ubiquitous. Bruez et al. (2015, 2020) also identified *Bacillus* spp. as major components of the wood bacterial community in vines, but they did not observe any significant differentiation in their abundance between symptomatic and asymptomatic plants. The prevalence of *Bacillus* and *Streptomyces* genera in asymptomatic plants (Fotios et al. 2021), coexisting with the GTD pathogens *Phaeoacremonium, Phaeomoniella* and *Seimatosporium*, suggests the capacity of these bacterial genera to act as BCAs in several plants including grapevines (Alfonzo et al. 2009, Viaene et al. 2016). Indeed Rezgui et al. (2016) and Haidar et al. (2016) reported a high suppressive activity of native endophytic *Bacillus* strains isolated from vines against several GTD-related pathogens, like *P. chlamydospora, Lasidiodiplodia pseudotheobromae, N. parvum* and *Schizophyllum commune*. Endophytic *Streptomyces* were also isolated from asymptomatic grapevines, then shown to prevent the development of *P. chlamydospora* and *P. minimum in planta* and *in vitro* (Alvarez-Perez et al. 2017). Similarly, Trotel Aziz et al. (2019) and Leal et al. (2021b) showed that a native *B. subtilis* strain PTA-271, isolated from grapevine rhizosphere, was able to attenuate BD through the different direct and indirect key levers indicated in part 6, and the origin of these mechanisms being also deeply investigated by a genomic analysis of this BCA (Leal et al. 2021a).

Focusing on the fungal genera from asymptomatic compared to symptomatic plants, the wood mycobiome of healthy vineyards is dominated by endophytes, some of which hold potential for biological control of wood pathogens amongst the genera *Trichoderma, Aureobasidium, Acremonium, Cladosporium, Alternaria* and *Epicoccum*, (Dissanayake et al. 2018; González and Tello 2011; Pancher et al. 2012). Mycoviruses from grapevine microbiome, like the ones from the genus betapartitivirus or betachrysovirus detected by Nerva et al. (2019), are also suspected to influence the

effect of beneficial microorganisms like *Trichoderma*. Indeed, Chun et al. (2018) reported that *T. harzianum* isolates infected with betapartitivirus displayed an increased antagonistic activity compared to the virus-free isolates. Ongoing high-throughput isolation efforts continue to explore in depth the rich beneficial endophytic bacterial/fungal/viral community of asymptomatic plants (healthy or resilient ones) versus symptomatic ones, considering that: (i) grapevine may support diverse communities from roots to berries (Bokulich et al. 2014; Deyett et al. 2020; Fotios et al 2021; Zarraonaindia 2016) and (ii) grapevine genotype may also influence its microbiome composition (Berlanas et al. 2019; Bettenfeld et al. 2021; Marasco et al. 2018).

To date, the broadly knowledges accumulated when considering a "one pathogen – one disease" hypothesis has allowed us to identify new plant diseases and disease-causing organisms, as well as to develop effective control strategies. However, this hypothesis is not sufficient to explain the disease process, as emerging evidence have indicated that complex pathogenic communities (or pathobiome) contribute to disease development (Mannaa and seo 2021). Living in close association with diverse communities of microbes and virus, the plant should be considered as a 'holobiont' viewed as a complex system in continuous interaction with its resident microbes and surrounding environment (Gordon et al. 2013). Microorganisms with their functional genes represent the plant microbiome (or phytobiome) whose qualitative and quantitative composition may differ among each plant, as well as over the different stages of growth or depending on the organs and tissues of a same plant. To date, BCAs such as *Trichoderma* and *Bacillus* are described to (1) assist plants in nutrient uptake, (2) support plant growth at different stages starting from seed germination, and (3) promote plant resistance to biotic and abiotic stress. Pathogenic microbes are also part of the phytobiome (Baltrus 2017), and obviously some microbial genera are beneficial members at certain stages of a plant species (supporting growth) and turn pathogenic at another stage or in other plant species (i.e., some *Rhizoctonia* members are beneficial to some plants, while others are devastating pathogens). Therefore, the plant holobiont can be summed up as a continuous fight/equilibrium between the allies of pathogenic microbes (or pathobiome) (Bass et al. 2019; Mannaa and Seo 2021) and the allies of beneficial microbes (or symbiome). Healthy plants are closely associated with a stable and diverse community of beneficial microorganisms (symbiome) that serve the important functions of the host. Recent advancements in multi-omics

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(metagenomics, meta-transcriptomics, meta-proteomics and metabolomic) have especially pointed out the occurrence of diseases with multi-species of causal agents and mixed infections, referred as “disease complex” (i.e. esca complex). Therefore, the “one pathogen - one disease” hypothesis based on the fundamental Koch’s postulates is not sufficient to describe the disease process in a realistic way, particularly when complex communities of organisms are involved (Broberg et al. 2018). Thus, even when considering a single pathogen, other plant endophytic microorganisms are likely to modulate the pathogenic effects and should thus be considered as part of the disease process (Bettenfeld et al. 2020; Brader et al. 2017; Stopnisek et al. 2016).

## 6. Microorganisms with biocontrol potential against GTDs: Focus on *Bacillus* spp. and *Trichoderma spp.*

Biocontrol microorganisms commonly found in nature include bacteria, fungi, virus, yeasts, and protozoans, controlling plant diseases either directly or indirectly (Köhl et al. 2019). Focus is now made on Bacillus *spp.* and *Trichoderma* spp. as the two main microorganisms-based BCAs used in viticulture (Harman 2006; Mondello et al. 2018; Muckherjee et al. 2012; Waghunde et al. 2017), and the most widely investigated against GTDs (Berbegal et al. 2020; Di Marco et al. 2002, 2004; Halleen et al. 2010; John et al. 2008; Leal et al. 2021b; Martínez-Diz et al. 2021a,b; Schmidt et al. 2012; Trotel-Aziz et al. 2019).

### 6.1. *Bacillus subtilis* assets

A broad range of beneficial molecules are produced or encoded by the genome of *Bacillus* spp. (Table 3), both to induce or elicit plant defenses (with MAMPs, such as phytohormones precursors, lipopolysaccharides, siderophores, etc.) and to directly compete, antagonize, or alter plant pathogens or their aggressiveness (Kloepper et al. 2004; Leal et al. 2021a; Ongena and Jacques 2008). Among *Bacillus* spp., *B. subtilis* is one of the most frequently tested against GTDs (Mondello et al. 2018), and *B. subtilis* PTA-271 has shown promising results in reducing infections caused by the aggressive

strain *N. parvum* Bt67 (Trotel-Aziz et al. 2019). *Bacillus* spp. are also known for the stable biofilm they can form, and for their attachment and persistence on the plant roots; being considered as highly protective against any stress (Chen et al. 2013).

Assets of *Bacillus* species (summarised in Figure 6) and especially those of *B. subtilis* PTA-271 for sustainable biocontrol strategy against GTDs were deeply described by Leal et al. (2021a). This starts with the *B. subtilis* motility and adhesion capacities, allowing it to colonize plant roots. Motility is due to the flagellum, enabling to move towards a vital nutrient source by chemotaxis thanks to genes involved in both the flagella maintenance (*flh* genes) and chemotaxis (*che* genes) (Henrischen 1972; Leal et al. 2021a).

The direct pathogen attack by *Bacillus spp* can then be achieved by several types of molecules also encoded by the *B. subtilis* genome (Leal et al. 2021a). *B. subtilis* can control fungal phytopathogens through the production of the exoenzymes CAZymes (carbohydrate activity enzymes) such as CWDEs, able to degrade the fungal cell wall with proteases and chitinases (Liu et al. 2011). Additionally, volatile organic compounds (VOCs) like 3-hydroxy 2-butatone and acetoin, and volatile inorganic compounds (VICs) like nitric oxide (NO), can inhibit fungal spore germination and hyphal growth (Chen et al. 2008). Other antimicrobial molecules, such as bacillaene, subtilosin and bacilysin can contribute to *Bacillus* direct confrontation with fungal pathogens. The lytic enzymes chitosanases and proteases are also pointed to produce the lipopeptides (LP) surfactin and fengycin described as powerful fungicides against pathogen survival. Finally, bacterial siderophores, like bacillibactin, can also directly alter pathogen fitness and aggressiveness, by depriving pathogen growth of iron while providing it for plant growth.

*Bacillus spp*. can also stimulate the plant defenses and induce the plant Systemic Resistance (ISR) (Devendra et al. 2007; Leal et al. 2021b; Magnin-Robert et al. 2007; Pieterse et al. 2002; Trotel-Aziz et al. 2008, 2009; Van Loon 2000) by the mean of several types of eliciting molecules (MAMPs) encoded by the *B. subtilis* genome (Leal et al. 2021a). Cell surface components of *Bacillus*, such as lipopolysaccharides (LPS) and Flagella, can trigger the plant defense-associated responses leading to ISR (Choudhary et al. 2007; Erbs and Newmann 2003; Gómez-Gómez 2004; Meziane et al. 2005). Hormonal changes can also be induced by *B. subtilis* that can synthetize precursors of SA, ABA, and GA, involved in stress

regulation (Blake et al. 2021; Leal et al. 2021a; Pacifico et al. 2019; Zhang et al. 2010;). Microorganisms also produce several transcription factors (TF) able to regulate plant defenses pathways (Liu et al. 2011), but also polyamines described as plant cell protectors upon water deficit (Ebeed et al. 2017; Leal et al. 2021a), temperature changes (Tian et al. 2012) and salinity (Leal et al. 2021a). The Lipopeptides (LP) surfactin and fengycin encoded by the genome of *Bacillus spp* (*sfr* and *fen* genes) are also described as ISR elicitors (Heloir et al. 2019; Leal et al. 2021a; Ongena and Jacques 2008), as well as the DAMPs monomers formed by *Bacillus* CWDEs from pathogen Exopolysaccharides and LPS (i.e., chitosan, glucans, etc.) (Heloir et al. 2019; Leal et al. 2021a; Pieterse et al. 2014).

*Bacillus spp.* can also increase the plant root growth, and consequently plant productivity (Hashem et al. 2019). Indeed, according to Leal et al. (2021a), *B. subtilis* can improve nutrient availability, modulate the homeostasis of plant growth hormones, and improve plant tolerance to abiotic stresses. Regarding nutrient availability, *B. subtilis* can improve the intake of several nutrients promoting the plant growth, such as nitrogen, phosphorus, and iron (Hayat et al. 2010; Leal et al. 2021a). Plants are unable to directly use atmospheric nitrogen but rely on the help of microbial symbionts. *B. subtilis* is known to fix atmospheric nitrogen as well as to promote nodulation by other bacteria (Blake et al. 2021; Elkoca et al. 2007). Microorganisms can also induce the uptake of urea, a very good nitrogen source (Glick 2012; Zaidi et al. 2010), and release organic acids such as gluconic acid able to increase the phosphate bioavailability for plants (Glick 2012; Van Schie et al. 1987). The siderophores produced by *B. subtilis*, like bacillibactin, have an affinity for iron, transporting it near the plant roots, and increasing its intake (Powel et al. 1982). Regarding hormones, *B. subtilis* strains can produce tryptophan, the main precursor of auxin (IAA), described to promote the plant lateral root development, resulting in increased intake of nutrients by the root system (Glick 2012). The production of polyamines precursors by *Bacillus* can improve the plant growth too, since these molecules are described to induce cell division, to promote the regeneration of plant tissues and cell cultures (Chen et al. 2019), and to delay senescence. *B. subtilis* can also produce hydroxy-2-butanone (acetoin), 2,3-butanediol and cytokinin (Arkhipova et al. 2005), but also abscisic acid (ABA) described as one of the major plant hormones involved in abiotic stress regulation (Blake et al. 2021). Woo et al. (2020) recently showed that inoculation with *B. subtilis* GOT9 increases the tolerance of *Arabidopsis thaliana* and *Brassica campestris* to drought

and salt stress by modulating the plant gene expression, including an up-regulation of the genes encoding for ABA biosynthesis. Volatile compounds (VICs and VOCs) have also been described to stimulate plant growth (Ortíz-Castro et al. 2009).

Finally, *Bacillus* protect itself and associated community (allies) against biotic stress (Leal et al. 2021a). *B. subtilis* is a surprising species that is largely adapted to grow in a variety of environments in the biosphere. Like all members of the genus *Bacillus, B. subtilis* can form highly resistant dormant endospores (Ricca et al. 2004; Sonenshein et al. 2002), providing better survival chances and longer shelf lives in commercial products used for biocontrol (Wu et al. 2015). Fortunately, to date, many studies described that BCAs applied to plants correlate with a reduction of the target pathogen and an enrichment of known beneficial plant bacteria, such as *Pseudomonas* (Tao et al. 2020), *Bacillus* (Yin et al. 2013), *Verrucomicrobia* (Eltlbany et al. 2019; Schmidt et al. 2014), and *Acidobacteria* (Wang et al. 2018).

### 6.1. *Trichoderma spp.* assets

As summarised in Figure 6, *Trichoderma spp.* have been described to directly antagonize GTD pathogens by competition for nutrients or space, by parasitism using CWDEs to enter its host, and by antibiosis (Sood et al. 2020). Effects of *Trichoderma* spp. on plants have been described, such as the biostimulation of the plant growth, but also the induction of local and systemic defense responses (Sood et al. 2020). Finally, genes encoded by the genome of *Trichoderma spp* have also highlighted self protection systems and mechanisms involved in microbiome protection against biotic and abiotic stress.

The direct pathogen attack by *Trichoderma spp.* can be ensured by several kind of molecules, and especially CWDEs. Once a phytopathogenic fungus is detected, *Trichoderma* first moves towards the other fungus to parasite it (Chet et al. 1981). Transporters take first an active part to this mycoparasitism, such as the tripeptide transporter ThPTR2 and the ABC transporter Taabc2 dependent on ATP, but also genes encoding for *Trichoderma* adherence such as *Qid74* (Srivastava et al. 2014). *Trichoderma* then produces several CWDEs to degrade the cell walls of these other fungi. CWDE activities release oligomers from pathogen cell wall (as DAMPs), such as the oligalacturonides (OGs) considered as elicitors able to induce the production of endochitinases by *Trichoderma*, enzymes continuing to attack the pathogen cell wall

(Srivastava et al. 2014). Among CWDEs are N-acetyl-β-D-glucosaminidase (exc1, exc2), endochitinase (ech42), chitinase (chit42, chit33), protease (Prb1), adenalyte-cyclase (tac1), xylanase (xyl) and cellulase, serine protease (tvsp1), β-glucanase (bgn 13.1) such as the β-1,3- and β-1,6-glucanase (tvbgn2, tvbgn3), β-1,3-glucosidase (tag83), and endo-poly-galacturonase (ThPG1) (For review check Sharma et al. 2011). The chitinases and endochitinases encoded by *Tga1*, *ech42*, *Th-Chit* are also described to produce antifungal metabolites, such as antibiotics with activity against phytopathogens (Cardoza et al. 2006; Reithner et al. 2005; Saiprasad et al. 2009; Woo et al. 1999). *Trichoderma* also contains genes encoding for β-Tubulins, described as cell structural components able to interact with benzimidazole fungicides to reinforce *Trichoderma*'s biocontrol process without inducing pathogen resistance (Buensanteai et al. 2010; Li and Yang 2007). Production of VOCs is also ensured by the *trichodiene synthase* (*tri5*) encoding for trichodermin, and by *Thctf1* encoding for 6-pentyl-2H-pyran-2-one (6pp) (Gallo et al. 2004; Lazazzara et al. 2021; Rubio et al. 2009; Sharma et al. 2011; Tijerino et al. 2011). All these antibiotic aggressive molecules can be stored in the appressoria formed by *Trichoderma* when in touch with the target fungus it aims to kill (Schirmböck et al. 1994).

*Trichoderma spp*. can stimulate plant defenses by the mean of several kind of molecules. Among these *Trichoderma*'s MAMPs recognized as elicitors by the plant to trigger ISR are proteins such as VOCs (Lazazzara et al. 2021), but also: the ET-inducing xylase (xynz/Eix), ThPG1, hydrophobin-like SSCP orthologues and swollenin TasSwo with local and systemic impacts, and cellulases triggering the SA and ET dependent defenses in the host plant (for review, see Hermosa et al. 2012). DAMPs resulting from the degradation of the fungal pathogen's chitin, such as OG, can also trigger the plant ISR, as well as the phytohormone like compounds such as IAA-like compounds produced by *Trichoderma* species (Hermosa et al. 2012). Indeed, *Trichoderma* secretion of IAA near the plant roots stimulates ET biosynthesis via the *in-planta* ACC synthase. In turn, ET triggers an increase in ABA biosynthesis; ABA being described as an essential signal for plant resistance to pathogens by modulating JA biosynthesis (Adie et al. 2007). Stepanova et al. (2007) further indicated that ET and IAA in the roots can reciprocally regulate each other's biosynthesis. In contrast, some enzymes of *Trichoderma* can metabolise the plant precursor of ET, namely ACC, thus decreasing its availability for ET biosynthesis *in planta* (Viterbo et al. 2010; Jaroszuk-Ściseł et al. 2019). Consequently, reductions in ET *in planta* may

successfully promote plant growth through gibberellin signaling (Jaroszuk-Ściseł et al. 2019; Viterbo et al. 2010). Regarding secondary metabolites, they can act as MAMPs at a low concentration, while at high doses they have an antimicrobial power (Hermosa et al. 2012). Among them are: 6-pentyl-α-pyrone, harzianolide and harzianopyridone, described both to activate plant defenses and to regulate the plant growth, suggesting that the defense mechanisms of plants and their developmental responses to *Trichoderma* share common components. Another secondary metabolite is alamethicin, a peptaibol described to elicit JA and ET dependent defenses (Vinale et al. 2008; Viterbo et al. 2007).

*Trichoderma* can also increase the plant root growth, and consequently plant productivity (Sood et al. 2020) by impacting the plant hormonal balance (as indicated in the previous paragraph), but also by increasing the plant nutrient uptake and nitrogen use, and helping to solubilize soil nutrients (Pozo et al. 2002). How these processes occur at the molecular level is still unknown, but it is known that there are differences in the responses of plants depending on their species.

Finally, *Trichoderma* protects itself against biotic stresses. Indeed, *Trichoderma* species have genes that encode for molecules responsible for tolerance to biotic stresses, such as putative Kelch-repeat protein (Thkel1) that regulates glucosidase activity and enhance tolerance to salt and osmotic stress (Hermosa et al. 2011). *Hsp70* gene leads to an increase of fungal resistance to heat, salt, osmotic stress, and oxidation (ManteroBarrientos et al. 2008), and *TvGST* gene is related to cadmium tolerance (Dixit et al. 2011; Sharma et al. 2011).

## 7. How to develop a durable sustainable management of GTDs? From nursery to vineyard.

The control of GTDs is a major challenge for vine growers, nurserymen, and scientists, mostly because of their complexity compared to other grapevine diseases (Gramaje and Armengol 2011). One of the most problematic aspects of GTD pathogens is their indeterminate latency (endophytic asymptomatic status) and subsequent internal lesions development leading to silent extensive damages of wood before the first external symptoms appear (Calzarano and Di Marco 2007; Mondello et al. 2018). This leaves winegrowers with few options to reduce the impact of GTDs in vineyards when the symptoms turn visibles. In nurseries too, infected asymptomatic cuttings can be

very dangerous when used to establish new plantations. Thus, a lack of GTD control would lead to an unsuspected spread of infected plants, first in the nursery and then in the vineyard (Aroca et al. 2010; Gramaje and Armengol 2011; Gramaje and Di Marco 2015).

Therefore, securing the future of management against GTDs in both nursery and vineyard using biocontrol, but also disease-resistant varieties and cultural practices is a prerequisite (Barzman et al. 2015; Collinge et al. 2019; Raymaekers et al. 2020). The Europe definition of biocontrol involves 4 categories that can be used for preventive or curative practices, among which microorganisms BCAs, semiochemicals (bio-based active molecules with a signal value between living organisms), natural substances (chemical coumpound from animal, plant or mineral origin, found in nature) and invertebrate BCAs (IBMA website). Currently, few curative methods exist, among which are curettage (Cholet et al. 2021; Mondello et al. 2018; Pacetti et al. 2021) and low copper-based fungicides (Battiston et al. 2021; Mondello et al. 2022; Reis et al. 2021). Hot-water treatments are also used successfully in nurseries to disinfest plant material since the 19th century (Baker 1962; Birchfield and Van Pelt 1958).

Cultural practices, as well as the use of microorganisms BCAs are among the best candidates for the sustainable preventive control of GTDs, while the use of disease-resistant varieties still requires further study to discriminate the grapevine R-genes that target key early-stage effectors (Elena and Luque 2016; Urbez-Torres et al. 2010).

Some products of biological control have already been registered against GTD pathogens (Mondello et al. 2018), and increasing research is currently devoted to the effect of BCAs in nursery, with *Trichoderma species*. Among them, *T. atroviride* SC1 was shown to strongly reduce the infections caused by some GTD pathogens in several cultivars, among which Tempranillo, both in nurseries and established vineyards at the registered dose rate of 2 g/L, equivalent to the density of $2 \times 10^{10}$ conidia/L recommended by the commercial product (Berbegal et al. 2020; Leal et al. *submitted*; Martínez-Diz et al. 2021a; Pertot et al. 2017;). Other BCAs (e.g. *B. subtilis*, *Fusarium lateritium, Erwinia herbicola, Cladosporium herbarum, Aureobasidium pullulans, Pythium oligandrum* and *Rhodotorula rubra*) and natural molecules (e.g., chitosan and cysteine) have also been reported to be effective against GTDs pathogens *in vitro* or in nurseries, alone or in combination with fungicides (Bertsch et

al. 2013; Leal et al. 2021b; Trotel-Aziz et al. 2019). In vineyards, *Trichoderma*-based registered products against GTDs are applied in pruning wounds (Berbegal et al. 2020; Blundell et al. 2022; Martinez-Diz et al. 2021; Mounier et al. 2016; Urbez-Torrez et al. 2020). Pruning wounds application aims to prevent infections caused by the annual contamination with spores of fungal pathogens (Di Marco et al. 2002, 2004; Halleen et al. 2010; John et al. 2005; Kotze et al. 2011; Mutawila et al. 2011, 2015; Sosnowski et al. 2004; Pitt et al. 2012). *Trichoderma*-based commercialized products are called Vintec® which contains *T. atroviride* SC1, or Remedier® that contains *Trichoderma asperellum* and *Trichoderma gamsii* (Di Marco et al. 2022). Among the commercialized BCA strains are Serenade® which contains *B. subtilis*. Research is also in progress considering the use of combined BCAs in nursery (Leal et al. *submitted*).

Before being considered for formulation then commercialisation, numerous beneficial microorganisms are assayed for their protective effect *in planta* under controlled conditions (Di Marco et al. 2002, 2004; John et al. 2005; Haidar et al. 2016; Leal et al. 2021b; Trotel-Aziz et al. 2019; Yacoub et al. 2016). Such plant model in controlled conditions enable to better understand the origin of a BCA efficiency against GTDs, by deciphering grapevine-BCA-pathogen interactions (Reis et al. 2019). The BCAs modes of actions can include (1) a direct antagonism against the pathogen or its virulence/pathogenicity factors, and/or (2) the triggering of grapevine ISR by the mean of MAMPs. For instance, *Bs* PTA-271 has been shown to induce ISR by priming the expression of the plant JA/ET-dependent defenses in Chardonnay rootlings infected with *N. parvum* (Trotel-Aziz et al. 2019) as it did with a true necrotroph such as *Botrytis cinerea* (Trotel-Aziz et al. 2008). Against *N. parvum*, *Bs* PTA-271 was also shown to be antagonistic by delaying the pathogen mycelial growth, and finally was shown to detoxify the virulence factors (*R*)-mellein and (-)-terremutin produced by *N. parvum* (Trotel-Aziz et al. 2019). *Bs* PTA-271 fungistatic effect might be explained by the release of various antifungal compounds, including surfactins or other lipopeptides which production was shown to depend on temperature (Leal et al. 2021a; Ongena and Jacques 2008; Pinto et al. 2018; Trotel-Aziz et al. 2019). Such a fungistatic effect gives the plant more time to defend itself and reduces the biosynthesis and secretion of the pathogen's virulence factors. Indeed, once inside the plant phytotoxins were shown to strongly lower the expression of most of the plant induced defenses (Trotel-Aziz et al. 2019), and (-)-terremutin was especially shown to repress the host useful

JA/ET-dependent defenses against Np-Bt67 (Trotel-Aziz et al. 2022). Fortunately, *Bs* PTA-271 can detoxify both (-)-terremutin and (*R*)-mellein (Trotel-Aziz et al. 2019). Authors suggested that this detoxifying capacity of PTA-271 was another asset to counter pathogen aggressiveness, preventing the phytotoxin level from reaching a too high level that will contribute to reprogram the useful immunity of the grapevine, thus favoring the bypassing of the host defenses by the pathogen and thus promoting the disease.

However, cultivars were also shown to condition BCAs efficiency towards BD in controlled conditions. When comparing cultivars with distinct susceptibility to GTDs (Cobos al. 2019; Leal et al. 2021b; Luque et al. 2009), Leal et al. (2021b) suggested that the high basal expression of SA-dependent defenses in Tempranillo may both explain this cultivar highest susceptibility to Np-Bt67 and the ineffectiveness of *Bs* PTA-271 alone to protect Tempranillo against Np-Bt67. Indeed, it has already been reported that early activation of SA signaling could antagonize the expression of the JA/ET-dependent defenses (Pieterse et al. 2012; Van der Does et al. 2013), although the latter are known to be useful for the plant (as basal or as induced) to fight a pathogen once entered in its necrotrophic phase (Yang et al. 2015). Combining BCAs with complementary ways of protection would thus enable to develop strongest biocontrol strategies and covering a wider range of pathogens and cultivars, especially susceptible ones. To improve the efficiency of single BCA, combinations with other BCAs or with naturel products are currently underway.

Indeed, literature reports that the combination of two or more BCAs can improve the management of plant diseases (El-Tarabily 2006; Guetsky et al. 2001; Leal et al. 2021b; Magnin-Robert et al. 2013; Weller 1988; Yobo et al. 2011), probably due to additive or synergistic effects of combined mechanisms in a complex changing environment (Meyer and Roberts 2002). Since the BCA *T. atroviride* is one of the most widely used commercialized biopesticides to control GTDs (Gramaje et al. 2018; Mondello et al. 2018), assays combining *T. atroviride* with *B. subtilis* PTA-271 were carried out by Leal et al. (2021b) in greenhouse conditions. These authors reported for the first time the biocontrol potential of the combination of Bs PTA-271 and Ta SC1 against Np-Bt67 in Tempranillo (91% protection), greater than Ta SC1 alone (80% protection), despite Bs PTA-271 and Ta SC1 can antagonize each other. This beneficial BCAs collaboration against Np-Bt67 confirms the interest of using both BCAs in combination to optimize both the plant ISR and the BCA direct antagonism



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against Np-Bt67 in some cultivars such as Tempranillo. It is also believed that the combination of several BCAs would ensure a more successful transfer of biocontrol efficacy from the greenhouse to the vineyard conditions.

Combining BCAs with natural products are currently underway, such as Bs PTA -271 and/or Ta SC1 with the molecules listed in Figure 6 (namely: elicitors of grapevine ISR against GTDs and/or direct effectors of the GTD pathogens). As mentioned earlier, such practices will (1) enable to seriously reduce the amount of chemicals used in the field, (2) restore the beneficial biodiversity damaged by years of unreasonable chemical control, (3) make the BCA products more effective in vineyards, and (4) improve the quality of the transfer of BCA results from the greenhouse to the vineyards, with the strongest guarantee of their reproducibility *in-situ*.

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## Tables

**Table 1.** Biosynthetic Gene Clusters (BGCs) involved in Secondary Metabolism (SM) of the *Neofusicoccum parvum* isolate Np-UCR-NP2-v3 showing significant similarities (>30%, protein level) with SM gene clusters from other fungi. The genome sequence of the reference isolate Np-UCR-NP2-v3 was analyzed with AntiSMASH fungal version, with defaults parameters (Blin et al. 2019). **Adapted from Trotel-Aziz et al. (2022).**

| Gene Type | Most similar known cluster | Similarity with known BGCs |
|---|---|---|
| NRPS | dimethylcoprogen | 100% |
| | hexadehydroastechrome / terezine-D / astechrome | 37% |
| NRPS,T1PKS | pyranonigrin E | 100% |
| | ilicicolin H | 50% |
| T1PKS | azanigerone A | 33% |
| | terremutin/terreic acid | 44% |
| | melanin | 100% |
| | (-)-mellein | 100% |
| | alternapyrone | 60% |
| | patulin | 33% |
| terpene | dimethylcoprogen | 100% |

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## Figures Caption

**Figure 1**. GTD infections during grapevine life cycle from (A) Nursery grapevine propagation process to (B) Nursery fields and mature grapevines in vineyards.

**Figure 2.** A. Area under vines in 2018 (OIV), B: Global distribution of grapevine fungal trunk diseases (Kenfaoui et al. 2022).

**Figure 3.** Life cycle of Botryosphaeriaceae in relation to the development cycle of the vine. Adapted from Larignon et al. (2001).

**Figure 4.** Possible lifestyles of hemibiotrophic fungi pre- and post- endophytism (yellow and red squares), under environmental, physiological, and genetic control. Adapted from Kusari and Spiteller (2012).

**Figure 5.** Suspected mode of colonization of hemibiotrophic fungal pathogens such as *Neofusicoccum parvum* in grapevine. **(A)** Endophytic phase of hemibiotrophic fungus forms an appressorium that develops hyphae (haustorium) enabling to store phytotoxins, but also to deliver cell wall degrading enzymes (CWDEs, to obtain nutrients from the living tissue) and OA (oxalic acid, or equivalent molecule, to prevent host cell death). **(B)** Necrotrophic phase of hemibiotrophic fungus starts with the formation of secondary thinner intracellular hyphae able to deliver effectors such as phytotoxins into the host, to prevent the PTI (PAMPs Triggered Immunity) by suppressing the useful host defenses, resulting in the host death rapidly. Hyphal penetration is facilitated by the dissolution of the host cell wall by an increased secretion of CWDEs. Efforts to identify and characterize determinant early-stage effectors, or to confirm the determinant value of those previously cited, still need to be deeply investigated to search for effectors potentially recognized by the host surveillance system (R-genes), and thus pave the way for gene-for-gene resistance and self-combat against hemibiotrophic pathogens. Adapted from Pradhan et al. (2021).

**Figure 6**. *Trichoderma* spp. and *Bacillus subtilis* biocontrol mechanisms and molecules.

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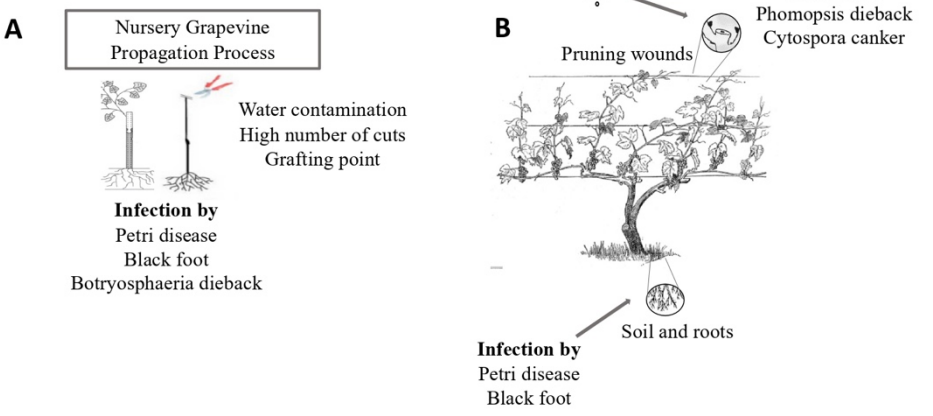
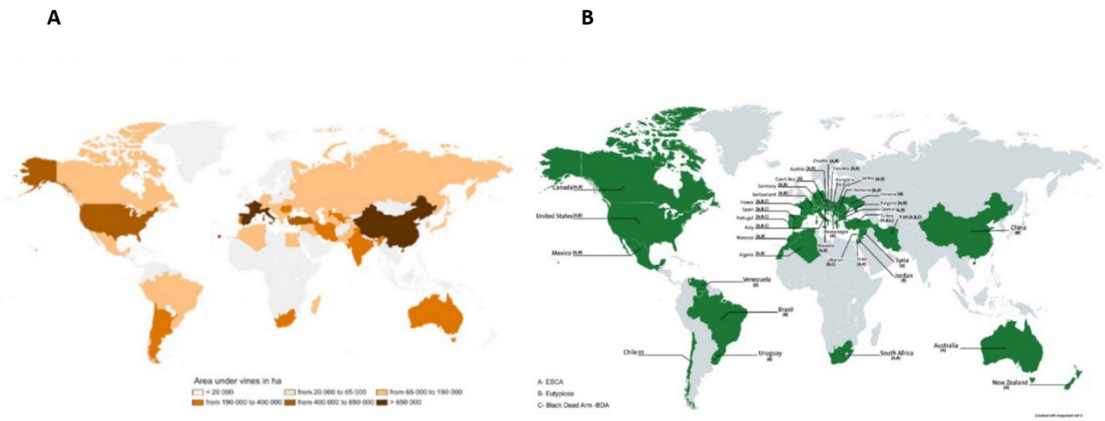
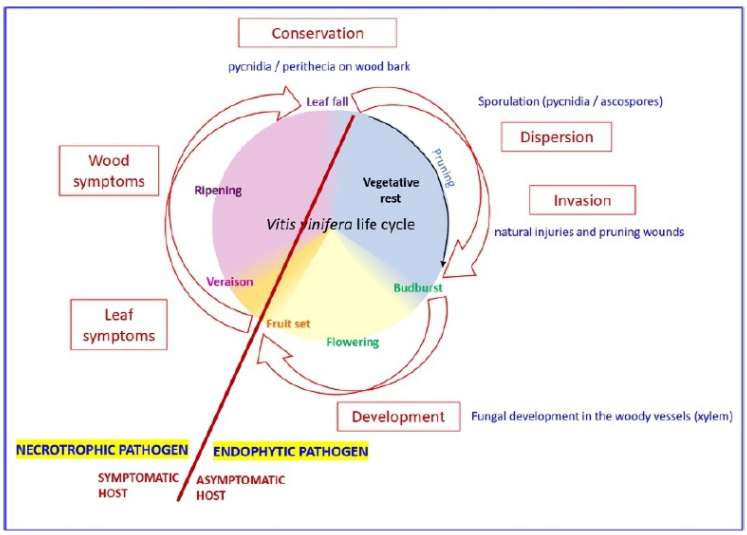

A
Nursery Grapevine
Propagation Process
Water contamination
High number of cuts
Grafting point
Infection by
Petri disease
Black foot
Botryosphaeria dieback
Vineyard
B
Spores
Infection by
Eutypa dieback
Botryosphaeria dieback
Esca/GLSD
Phomopsis dieback
Cytospora canker
Pruning wounds
Soil and roots
Infection by
Petri disease
Black foot

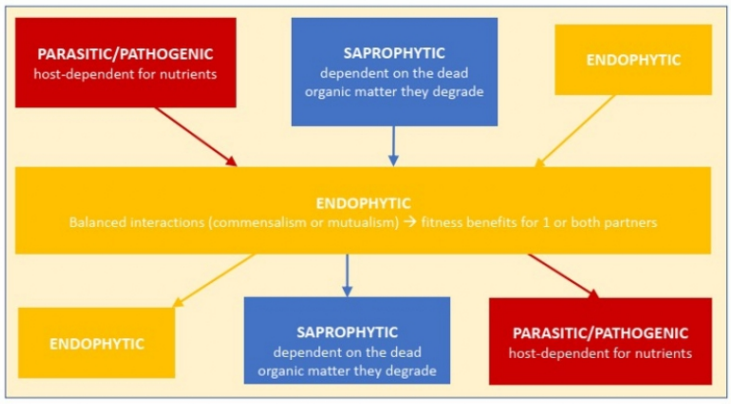



A
B
Area under vines in ha
A: ESCA
B- Eutypiose
C- Black Dead Arm -BDA



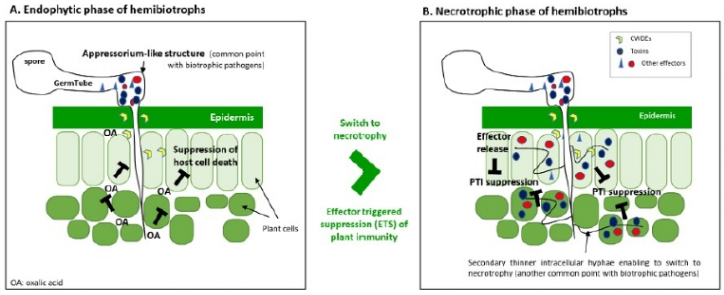

Life cycle of Botryosphaeriaceae in relation to the development cycle of the vine. Adapted from Larignon et al. (2001).

160x113mm (144 x 144 DPI)




Possible lifestyles of hemibiotrophic fungi pre- and post- endophytism (yellow and red squares), under environmental, physiological, and genetic control. Adapted from Kusari and Spiteller (2012).

132x74mm (144 x 144 DPI)




Suspected mode of colonization of hemibiotrophic fungal pathogens such as Neofusicoccum parvum in grapevine. (A) Endophytic phase of hemibiotrophic fungus forms an appressorium that develops hyphae (haustorium) enabling to store phytotoxins, but also to deliver cell wall degrading enzymes (CWDEs, to obtain nutrients from the living tissue) and OA (oxalic acid, or equivalent molecule, to prevent host cell death). (B) Necrotrophic phase of hemibiotrophic fungus starts with the formation of secondary thinner intracellular hyphae able to deliver effectors such as phytotoxins into the host, to prevent the PTI (PAMPs Triggered Immunity) by suppressing the useful host defenses, resulting in the host death rapidly. Hyphal penetration is facilitated by the dissolution of the host cell wall by an increased secretion of CWDEs. Efforts to identify and characterize determinant early-stage effectors, or to confirm the determinant value of those previously cited, still need to be deeply investigated to search for effectors potentially recognized by the host surveillance system (R-genes), and thus pave the way for gene-for-gene resistance and self-combat against hemibiotrophic pathogens. Adapted from Pradhan et al. (2021).

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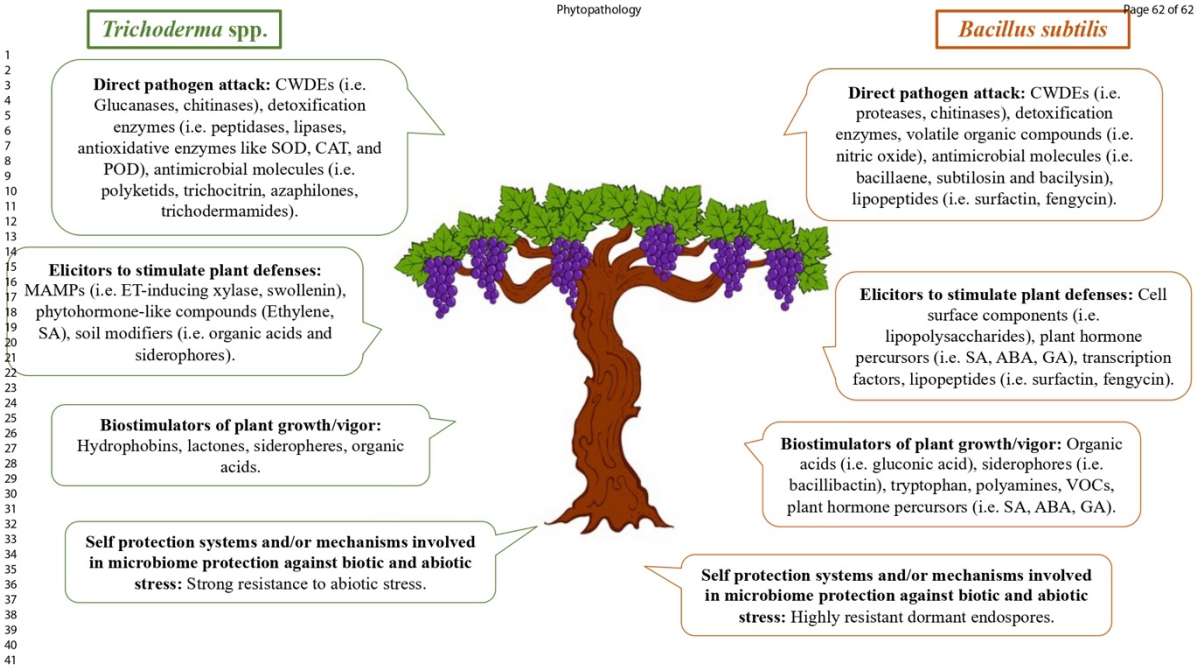
*Trichoderma* spp.
**Direct pathogen attack:** CWDEs (i.e. Glucanases, chitinases), detoxification enzymes (i.e. peptidases, lipases, antioxidative enzymes like SOD, CAT, and POD), antimicrobial molecules (i.e. polyketids, trichocitrin, azaphilones, trichodermamides).
**Elicitors to stimulate plant defenses:** MAMPs (i.e. ET-inducing xylase, swollenin), phytohormone-like compounds (Ethylene, SA), soil modifiers (i.e. organic acids and siderophores).
**Biostimulators of plant growth/vigor:** Hydrophobins, lactones, siderophores, organic acids.
**Self protection systems and/or mechanisms involved in microbiome protection against biotic and abiotic stress:** Strong resistance to abiotic stress.
*Bacillus subtilis*
**Direct pathogen attack:** CWDEs (i.e. proteases, chitinases), detoxification enzymes, volatile organic compounds (i.e. nitric oxide), antimicrobial molecules (i.e. bacillaene, subtilosin and bacilysin), lipopeptides (i.e. surfactin, fengycin).
**Elicitors to stimulate plant defenses:** Cell surface components (i.e. lipopolysaccharides), plant hormone percursors (i.e. SA, ABA, GA), transcription factors, lipopeptides (i.e. surfactin, fengycin).
**Biostimulators of plant growth/vigor:** Organic acids (i.e. gluconic acid), siderophores (i.e. bacillibactin), tryptophan, polyamines, VOCs, plant hormone percursors (i.e. SA, ABA, GA).
**Self protection systems and/or mechanisms involved in microbiome protection against biotic and abiotic stress:** Highly resistant dormant endospores.

### 1.4. Objectives

The biggest concerns to GTDs integrated management approaches, are the improvement of the phytosanitary quality of the nursery produced grapevines, and the prevention of infections through pruning wounds in the vineyard from the moment of planting. Biocontrol agents (BCAs) *Bacillus subtilis* and *Trichoderma* spp. have been shown to be promising strategies to be applied in vineyard as well as in nurseries, against GTDs. A better knowledge of the BCAs mode of action, as well as GTD pathogens aggressive molecules, and grapevine's own defensive capacity against GTD pathogens, is a key component to better understand the antagonistic effects or immunostimulatory effects of BCAs or their natural molecules. This project proposes to integrate 2 BCAs in nursery treatments to optimize the integrated control of GTDs. The efficiency of *T. atroviride* SC1 and *B. subtilis* PTA-271 was also be investigated in greenhouse, as well as their effect in grapevine rhizosphere microbiome.

We aim to (Figure 5):

1) Characterize in detail the genome of *B. subtilis* PTA-271 in order to explore its biotechnological potential for biocontrol of GTD pathogens (Chapter II). This study will also allow to identify potential beneficial molecules encoded by the genome of *T. atroviride* SC1 and *B. subtilis* PTA-271 (Chapter II).

2) Explore the protective potential (in terms of efficacy and modes of action) of the two BCAs, alone or in combination, against a very aggressive pathogen involved in GTDs and artificially inoculated to two grapevines with different sensitivities to this pathogen, under controlled conditions (Chapter III).

3) Screening for plant defense markers against GTD-causing pathogens (Chapter IV).

4) Understand the colonization and biocontrol potential of the 2 BCAs, *T. atroviride* SC1 and *B. subtilis* PTA-271, against different pathogens responsible for GTDs in nurseries (under natural conditions) (Chapter V).

5) Explore the potential of the 2 BCAs to modulate the microbiome of grapevine plants in contact with GTD pathogens in two different soil types. (Chapter VI).

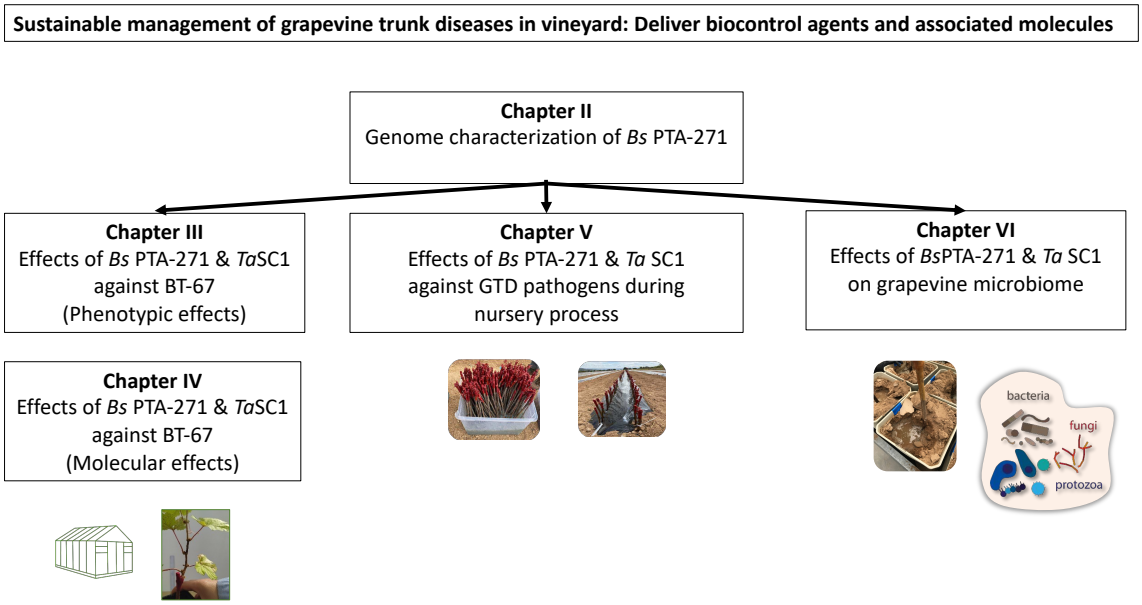


**Figure 5.** Aims of each chapter of the thesis entitled "Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules".

## *Chapter I* (French version)

Introduction

**Généralités sur la vigne**

La vigne européenne *Vitis vinifera L.*, du genre *Vitis* et de la famille des Vitaceae, est l'une des cultures les plus anciennes et les plus importantes au monde. Ancêtre sauvage de la plupart des vignes cultivées, diversifiées en d'innombrables variétés d'intérêt économique majeur, c'est à partir de cette vigne que sont produits presque tous les vins du monde. Environ 6 000 variétés cultivées existent dans le monde, dont un peu plus de 200 en France.

La domestication de *V. vinifera subsp. vinifera* a permis de sélectionner des caractéristiques morphologiques qui favorisent la culture et l'exploitation de cette plante. La multiplication des variétés de *vinifera* a privilégié les baies les plus grosses, les plus juteuses et les plus savoureuses, permettant de sélectionner progressivement des cépages génétiquement reproductibles. En Europe occidentale, les cépages peuvent également résulter de sélections et de croisements entre des plantes sauvages locales et des plantes déjà sélectionnées. Ainsi, à partir de *V. vinifera* subsp. *vinifera*, les différents cépages créés constituent la vaste gamme des variétés de vigne cultivées dans le monde, avec plus de 6 000 "cultivars" ou "cépages" (terme botanique désignant les variétés d'espèces végétales obtenues par croisement et cultivées). Les zones les plus concentrées en *V. vinifera* sont l'Europe centrale et méridionale, l'Asie occidentale et le Moyen-Orient, la Chine, la côte méditerranéenne de l'Afrique, l'Afrique du Sud, l'Amérique du Nord, l'Amérique du Sud, l'Australie et la Nouvelle-Zélande, bien que *V. vinifera* soit également présente en Amérique, en Afrique, en Asie et en Océanie.

**La vigne est exposée à de multiples pathogènes, dont ceux responsables des maladies du bois**

Tout au long de leur vie, les plants de vigne sont exposés à une grande variété d'agents phytopathogènes, tels que des bactéries, des virus, des phytoplasmes, des champignons et des nématodes (Armijo et al. 2016). Aujourd'hui, les maladies fongiques du bois de la vigne (MDB) sont parmi les principales contraintes pour la productivité de cette culture. Les agents pathogènes responsables des MDB peuvent infecter et endommager gravement les vignes, de la pépinière aux vignobles. En effet, dès lors qu'un sarment est infecté, la productivité de la plante est diminuée, conduisant à une mort lente (forme chronique) ou rapide (apoplectique) de la plante avec des pertes économiques importantes et une remise en question de la durabilité des vignobles (Bertsch et al. 2013 ; Fontaine et al. 2016 ; Mondello et al. 2018).

Parmi les différentes MDB, nous nous sommes plus particulièrement intéressés au dépérissement aux Botryosphaeria (BOT). Il est causé par des champignons appartenant à la famille des Botryosphaeriaceae appartenant aux genres *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria* et *Spencermartinsia* (Mondello et al. 2018). Le BOT se reconnaît souvent au dépérissement des pousses avec une nécrose des bourgeons (Úrbez-Torres 2011). Dans le bois, le principal symptôme est la présence de chancres, avec une coloration centrale circulaire (Mondello et al. 2018 ; Úrbez-Torres 2011). Des pycnides se développent également à partir du bois mort ou chancré. Toutefois, des symptômes tardifs peuvent n'apparaître au vignoble qu'1 ou 2 ans après la première infection (Urbez-Torres et al. 2006). Ils sont principalement observés dans les vignobles adultes (plus de 8 ans). La maladie du pied noir ( nommée aussi Black-Foot, BF) est une autre MDB fréquemment observée dans les jeunes vignobles (<5 ans) (Gramaje et Armengol 2011). Elle est causée par des espèces fongiques asexuées apparentées aux *Cylindrocarpon*, de genres *Campylocarpon*, *Cylindrodendrum*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon* et *Thelonectria*) (Akgül et al. 2022 ; Berlanas et al. 2020 ; Edwards et al. 2007 ; Gramaje et DiMarco 2015 ; Guerin-Dubrana et al. 2019). La maladie du pied noir est caractérisée par des nécroses noires et profondes au niveau des racines, associées à une décoloration brun-rouge à la base du cep des vignes infectées (Gramaje et Armengol 2011). Sur la base de ces phénotypes, de nombreux auteurs ont attribué aux MDB l'augmentation drastique de la mortalité des vignes au cours des dernières années (Agustí-Brisach et Armengol 2013 ; Agustí-Brisach et al. 2016 ; Aroca et al. 2010 ; Carlucci et al. 2017). Il en résulte un coût financier annuel conséquent pour remplacer les ceps morts à cause des MDB, partout en Europe (Mondello et al. 2018 ; Martínez-Diz et al. 2019).

**Implication du microbiome de la vigne dans l'expression des maladies du bois**

A ce jour, peu d'études ont comparé les communautés microbiennes de vignes asymptomatiques et symptomatiques. Fotios et al. (2021) semblent toutefois montrer que les plantes symptomatiques abritent un microbiome spécifique au bois, dépendant du cépage et du terroir. Ces données semblent donc prometteuses pour distinguer les vignes saines des vignes malades. Fotios et al. (2021) ont également souligné de fortes interactions entre les communautés bactériennes et fongiques du bois de vignes asymptomatiques. L'étude du mycobiome du bois de vigne, dans un vignoble avec antécédants d'esca, a permis à Del Frari et al. (2019) d'identifier 289 taxons, dont cinq genres sont signalés pour la première fois pour

être associés aux symptômes des MDB dans le bois de la vigne (les genres *Debaryomyces, Trematosphaeria, Biatriospora, Lopadostoma* et *Malassezia*). Il est intéressant de noter que la composition qualitative et quantitative du mycobiome du bois à proximité des feuilles symptomatiques, ainsi que dans les rameaux symptomatiques, est très similaire à celle du bois des plantes asymptomatiques. Cette observation soutient l'hypothèse selon laquelle les symptômes foliaires en réponse aux MDB ne seraient pas uniquement liés à la seule présence des pathogènes responsables de ces maladies. En bon accord, les Botryosphaeriaceae sont d'abord décrits comme des champignons endophytes non pathogènes avant de devenir pathogènes (une fois nécrotrophes). Globalement, ces résultats récents suggèrent fortement que les microorganismes endophytes (bactéries et champignons bénéfiques) et/ou les virus sont des partenaires clés à prendre en compte pour conditionner l'état de santé de la vigne dès lors qu'elle est infectée par un champignon phytopathogène responsable des MDB. Nerva et al. (2019) ont effectivement signalé 38 nouveaux mycovirus, dont certains sont capables d'infecter les agents pathogènes responsables des MDB de la vigne.

**Des micro-organsimes bactériens bénéfiques contre les MDB ?**

La comparaison des microbiomes de vignes grecques asymptomatiques et symptomatiques a permis à Fotios et al. (2021) d'identifier un fort enrichissement en *Bacillus* (et largement en membres de la famille Bacillaceae) et *Streptomyces* dans les plants asymptomatiques. Cet enrichissement est particulièrement important dans les cépages Xinomavro et Agiorgitiko, mais aussi dans le cépage Vidiano où les genres *Bacillus* et *Corynebacterium* sont omniprésents. Bruez et al. (2015, 2020) ont également désigné les *Bacillus* spp. comme genre bactérien majeur de la communauté bactérienne du bois de la vigne, sans pour autant observer de différences significatives en terme d'abondance entre les plantes symptomatiques et asymptomatiques. La prévalence des genres *Bacillus* et *Streptomyces* dans les plantes asymptomatiques (Fotios et al. 2021), coexistant avec des pathogènes des MDB, souligne toutefois la capacité de ces genres bactériens à agir en tant que BCA chez la vigne (Alfonzo et al. 2009, Viaene et al. 2016). Dans ce sens, Rezgui et al. (2016) et Haidar et al. (2016) ont rapporté une forte activité inhibitrice de souches endophytes indigènes de *Bacillus* contre plusieurs pathogènes liés aux MDB. De même, Trotel-Aziz et al. (2019) et Leal et al. (2021b) ont montré qu'une souche indigène de *B. subtilis* PTA-271 (*Bs* PTA-271), isolée de la rhizosphère de la vigne, était capable d'atténuer le BOT par différents leviers clés directs et indirects (Cf. Chapitre 6). L'origine des mécanismes protecteurs susceptibles d'être mis en jeu

par ce BCA sera abordée plus en détail ultérieurement, tant par une analyse génomique de *Bs* PTA-271 (Leal et al. 2021a), que par une analyse physiologique et transcriptomique des mécanismes induits *in-planta* (Trotel-Aziz et al. 2019 ; Leal et al. 2021b ; Leal et al. 2022 - revue soumise à Phytopathology).

**Des micro-organsimes fongiques bénéfiques contre les MDB ?**

La comparaison des mycobiomes de plantes asymptomatiques et symptomatiques montre que les bois de vignes symptomatiques sont dominés par des endophytes, dont certains ont un potentiel en tant qu'agents de lutte biologique contre les pathogènes responsables des MDB. Parmi ces BCA potentiels, d'origine fongique, sont les genres *Trichoderma, Aureobasidium, Acremonium, Cladosporium, Alternaria* et *Epicoccum* (Dissanayake et al. 2018 ; González et Tello 2011 ; Pancher et al. 2012). Les mycovirus du mycobiome de la vigne, comme ceux du genre betapartitivirus ou betachrysovirus décrits par Nerva et al. (2019), sont notamment soupçonnés influencer les effets des BCA du genre *Trichoderma*. Chun et al. (2018) ont aussi signalé que les isolats de *T. harzianum* infectés par le betapartitivirus présentaient une activité antagoniste accrue par rapport aux isolats sans virus. Les travaux de recherche actuels dévolus au séquençage haut débit exploratoires de la richesse des communautés bactériennes/fongiques/virales endophytiques bénéfiques de plantes asymptomatiques (saines ou résilientes) par rapport aux plantes symptomatiques, montrent désormais que : (i) la vigne peut soutenir diverses communautés microbiennes des racines aux baies (Bokulich et al. 2014 ; Deyett et al. 2020 ; Fotios et al 2021 ; Zarraonaindia 2016) et (ii) le génotype de la vigne peut également influencer la composition et l'efficacité de son microbiome ou de ses BCA (Berlanas et al. 2019 ; Bettenfeld et al. 2021 ; Marasco et al. 2018 ; Leal et al. 2021b).

**Deux micro-organismes bénéfiques au potentiel prometteur contre les MDB : *Bacillus spp*. et *Trichoderma spp*. ?**

Les espèces des genres *Bacillus spp.* et *Trichoderma spp.* constituent deux catégories de BCA largement utilisés en viticulture (Harman 2006 ; Mondello et al. 2018 ; Muckherjee et al. 2012 ; Waghunde et al. 2017), et plus particulièrement dans le contexte des MDB (Berbegal et al. 2020 ; Di Marco et al. 2002, 2004 ; Halleen et al. 2010 ; John et al. 2008 ; Leal et al. 2021b ; Martínez-Diz et al. 2021a,b ; Schmidt et al. 2012 ; Trotel-Aziz et al. 2019).

Les atouts des espèces de *Bacillus* et notamment de *B. subtilis* PTA-271 pour développer une stratégie de biocontrôle durable contre les MDB sont décrits par Leal et al. (2021a) et rappelés dans la revue de Leal et al. (2020, soumise à Phytopathology). Parmi ceux-ci : 1) sa mobilité et sa capacité d'adhésion à une surface, lui permettent de coloniser rapidement les racines des plantes. 2) L'attaque directe des agents pathogènes par diverses molécules bioactives telles que les CAZymes, les composés organiques volatils et d'autres molécules antimicrobiennes. 3) La stimulation des défenses des plantes susceptibles d'induire la résistance systémique de la plante (ISR) (Devendra et al. 2007 ; Leal et al. 2021b ; Magnin-Robert et al. 2007 ; Pieterse et al. 2002 ; Trotel-Aziz et al. 2008, 2019 ; Van Loon 2000 ; Leal et al. 2021b) au moyen de plusieurs types de molécules élicitrices (Leal et al. 2021a). 4) La stimulation de la vigueur de la plante au moyen de diverses molécules parmi lesquelles les hormones microbiennes, susceptibles de favoriser le développement des racines latérales de la plante (Glick 2012).

Les atouts des espèces de *Trichoderma* et notamment de *T. atroviride* SC1 pour développer une stratégie de biocontrôle durable contre les MDB sont décrits dans la revue de Leal et al. (2022, soumise à Phytopathology). Parmi ceux-ci : 1) L'attaque directe des agents pathogènes, par compétition pour les nutriments ou l'espace, par parasitisme en utilisant des CAZymes contre la paroi cellulaire du champignon phytopathogène, et par antibiose à l'aide de nombreuses autres molécules antifongiques (Sood et al. 2020 ; Leal et al. 2022 – revue soumise à Phytopathology). 2) Les effets de *Trichoderma spp.* sur les plantes, comme la biostimulation de la croissance des plantes, mais aussi l'induction de réponses de défense locales et systémiques (Sood et al. 2020 ; Leal et al. 2022 – revue soumise à Phytopathology). 3) Enfin, les gènes codés par le génome des *Trichoderma spp.* ont également mis en évidence des systèmes d'autoprotection et des mécanismes impliqués dans la protection du microbiome contre les stress biotiques et abiotiques.

**La lutte contre les maladies du bois, de la pépinière au vignoble**

Le contrôle des MDB est un défi majeur pour les viticulteurs, les pépiniéristes et les scientifiques, principalement en raison de leur complexité par rapport aux autres maladies de la vigne (Gramaje et Armengol 2011). L'un des aspects les plus problématiques des agents pathogènes responsables des MDB est leur latence indéterminée (phase endophyte asymptomatique) associée au développement de lésions internes conduisant à des

dommages « silencieux » dans le bois avant-même l'apparition des premiers symptômes externes (Calzarano et Di Marco 2007 ; Mondello et al. 2018). Cela laisse donc peu d'options aux viticulteurs pour réduire l'impact des MDB dans les vignobles dès lors que les symptômes deviennent visibles. Dans les pépinières, les jeunes plants asymptomatiques infectées peuvent être très dangereux lorsqu'ils sont utilisés pour établir de nouvelles plantations. Un manque de moyens de lutte contre les MDB apparaît donc clairement, et risque de conduire à une propagation insoupçonnée de plants de vigne infectés, d'abord en pépinière puis au vignoble (Aroca et al. 2010 ; Gramaje et Armengol 2011 ; Gramaje et Di Marco 2015). Par conséquent, il devient urgent de contrôler durablement les pathogènes responsables des MDB, de la pépinière au vignoble, en mettant à profit tant : le biocontrôle, que les variétés de vigne plus ou moins résistantes aux pathogènes responsables des MDB, et des pratiques culturales plus adaptées à la lutte contre ces maladies (Barzman et al. 2015 ; Collinge et al. 2019 ; Raymaekers et al. 2020).

Les pratiques culturales associées à l'utilisation de BCA apparaissent ici comme les meilleurs candidats à court terme pour contrôler durablement les MDB, en préventif comme en curatif. Quant à l'utilisation de variétés de vigne résistantes aux MDB, cette technique nécessite encore des études complémentaires, notamment pour discriminer précocément les gènes de la vigne codant pour des protéines de résistance (protéines R) ciblant en particulier les effecteurs clés sécrétés par les pathogènes responsables des MDB dès lors qu'ils entrent en phase nécrotrophe (Elena et Luque 2016 ; Úrbez-Torres et al. 2010).

**Une gestion durable retenue**

Actuellement, les approches de gestion intégrée des MDB relèvent principalement de l'amélioration de la qualité phytosanitaire des plants de vignes produits en pépinière et de la prévention des contaminations par les plaies de taille au vignoble. Toutefois, des agents de biocontrôle (BCA) existent, tels que *Bacillus subtilis* PTA-271 et *Trichoderma atroviride* SC1, et s'avèrent être des candidats prometteurs pour lutter efficacement contre le développement des MDB de la pépinière au vignoble. Optimiser la lutte contre les MDB nécessite aussi de mieux connaître les champignons responsables de ces MDB (mode d'action et molécules agressives inclues) ainsi que la capacité défensive de la vigne, seule ou associée aux effets antagonistes et/ou immunostimulateurs des BCAs ou de leurs molécules naturelles.

Ce projet de thèse propose donc d'intégrer 2 BCAs (*Bacillus subtilis* PTA-271 et *Trichoderma atroviride* SC1) dans : A/ des traitements en pépinière pour optimiser la lutte intégrée des MDB. B/ D'étudier parallèlement l'efficacité des 2 BCAs (1) *in-planta* en serre en conditions contôlées sur boutures artificiellement infectées, et (2) *in-vitro* directement contre l'agent pathogène. 3/ D'appréhender les effets des 2 BCAs sur le microbiote (au point de greffe) et le microbiome (de la rhizosphère) de la vigne.

**Les objectifs de la thèse sont donc de (Figure 5):**

1) Caractériser en détail le génome de *B. subtilis* PTA-271 pour explorer son potentiel biotechnologique en matière de biocontrôle contre les pathogènes responsables des MDB (Chapitre II). Cette étude va permettre aussi d'identifier des molécules bénéfiques potentielles codées par le génome de *T. atroviride* SC1® et de *B. subtilis* PTA-271 (Chapitre II).

2) Explorer le potentiel protecteur (en termes d'efficacités et de modes d'actions) des 2 BCAs, seuls ou en traitements combinés, contre un pathogène très agressif impliqué dans les MDB et inoculé artificiellement à 2 cépages de sensibilités différentes à ce pathogène, en conditions contrôlées (Chapitre III).

3) Cribler des marqueurs de défense des plantes contre les pathogènes responsables des MDB (Chapitre IV).

4) Comprendre le potentiel de colonisation et de biocontrôle des 2 BCA, *T. atroviride* SC1 et *B. subtilis* PTA-271, contre différents agents pathogènes responsables des MDB en pépinière (en conditions naturelles) (Chapitre V).

5) Explorer le potentiel des 2 BCAs à moduler le microbiome de plants de vigne en contact avec les agents pathogènes de MDB dans deux types de sol différents. (Chapitre VI).

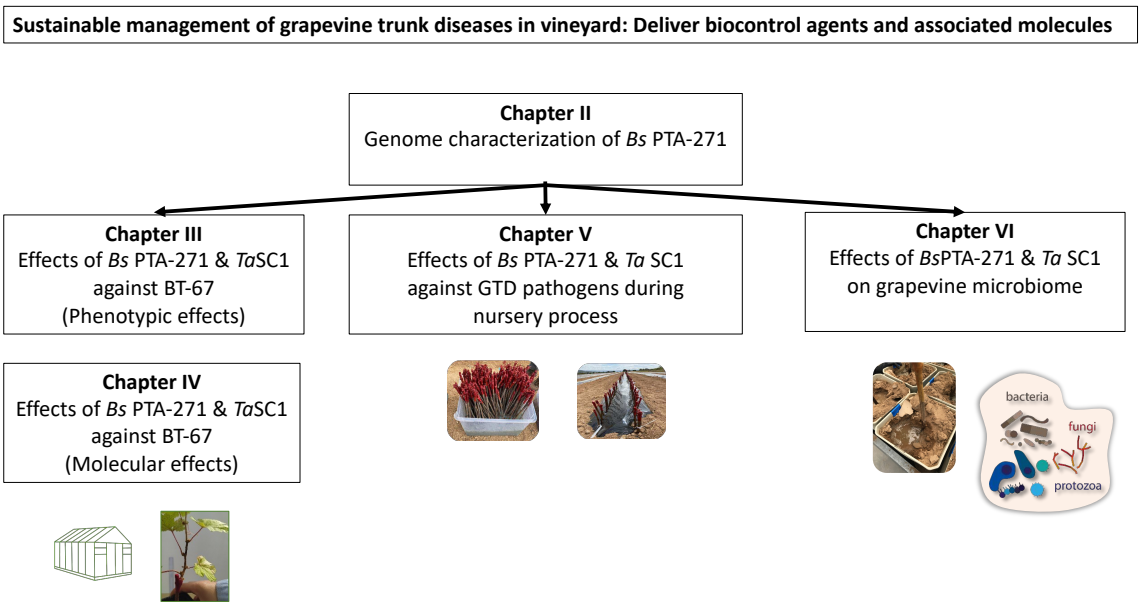


**Figure 5.** Objectifs de chaque chapitre de la thèse intitulée "Gestion durable des maladies du bois de la vigne dans le vignoble : Délivrer des agents de biocontrôle et des molécules associées".

# Results and Discussion

***Chapter II***

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# Genome characterization of *Bacillus subtilis* PTA-271

## 2.1. Contexte

*Bacillus subtilis* est une bactérie Gram-positive, considérée comme un microorganisme prometteur pour la protection des plantes, capable de survivre dans le sol pendant de longues périodes malgré des conditions environnementales difficiles (Nicholson et al. 2000). Les avantages des *Bacillus spp* sont bien connus dans les secteurs de l'industrie, de l'agriculture et de la viticulture (Borriss 2011). *Bacillus spp* sont notamment décrits pour induire la résistance systémique (ISR) des plantes lors d'attaques de pathogènes, promouvoir leur croissance, ou encore avoir un effet direct sur les pathogènes des plantes ou leurs molécules agressives (Magnin-Robert et al. 2007 ; Trotel- Aziz et al. 2019 ; Trotel-Aziz et al. 2008 ; Wang et al. 2018).

Dans les plantes, l'ISR est décrite pour être induite par la voie de l'acide jasmonique (JA) et de l'éthylène (ET), ou par celle de l'acide salicylique (SA) (Pacifico et al. 2019 ; Pieterse et al. 2009 ; Pieterse et al. 2014 ; Van Loon et al. 1998). Certains micro-organismes bénéfiques sont également décrits pour moduler l'équilibre hormonal des plantes, soit par interaction avec leurs voies métaboliques, soit en produisant les mêmes hormones que la plante ou leurs précurseurs (ET, SA, auxines, gibbérellines, cytokinines, polyamines...) (Pacifico et al. 2019). De nombreux éliciteurs bactériens de l'ISR ont aussi été identifiés dans les plantes, parmi lesquels les exopolysaccharides (EPS), les lipopolysaccharides (LPS), les sidérophores tels que la pyoverdine régulée par le fer, le fer, les flagelles, les biosurfactants, la N-acyl-L-homosérine lactone, la benzylamine N-alkylée et les composés volatils (Kloepper et al. 2004 ; Pacifico et al. 2019 ; Van Loon et al. 1998 ; Zamioudis et al. 2015). Certains d'entre eux ont déjà été identifiés dans les espèces du genre *Bacillus spp.* tel que *B. subtilis* (Akram et al. 2015 ; Audrain et al. 2015 ; Kloepper et al. 2004 ; Pacifico et al. 2019). Les changements hormonaux dans les plantes peuvent également impacter leur croissance et leur développement, comme la réduction d'ET qui favorise la croissance des plantes (Glick 2014 ; Pacifico et al. 2019 ; Xie et al. 2014). Le microbiote est d'ailleurs décrit pour agir sur la croissance et le développement des plantes en modulant la disponibilité en nutriments, par ses activités de minéralisation et chélation, mais aussi pour produire des composés volatils décrits pour leur activité de biocontrôle (Sharifi et Ryu 2018 ; Tyagi et al. 2018). Les effets bénéfiques des *Bacillus* spp. passent également par leur auto-préservation et la protection du microbiote de la plante, de façon directe ou indirecte, en conditions de stress abiotiques et biotiques (Huang et al. 2014 ; Pacifico et al. 2019). Les BCA peuvent effectivement (se) protéger grâce à des transporteurs d'extrusion, des enzymes de détoxication, des enzymes d'extinction et des enzymes

homologues à celles des pathogènes, contribuant ainsi indirectement à la protection des plantes (Pacifico et al. 2019). Enfin, *B. subtilis* est décrit pour produire une large gamme de molécules antimicrobiennes, de chélateurs et d'enzymes lytiques qui limitent la capacité d'adaptation et l'agressivité des agents pathogènes (Caulier et al. 2019). D'après la littérature, ces molécules bénéfiques sont des peptides antimicrobiens synthétisés au niveau ribosomique (RP), des peptides non synthétisés au niveau des ribosomes (NRP), des polykétides (PK), ainsi que d'autres composés volatils antimicrobiens (ViC et COV, pour inorganiques et organiques, respectivement) et des métabolites secondaires dérivés de la voie des terpénoïdes. Les spécificités propres à chaque souche de *B. subtilis* peuvent donc impacter tant les conditions biochimiques de leur milieu de vie (y compris *in-planta*) que les interactions entre les différentes espèces en présence, et moduler par conséquent les rapports de force et/ou d'équilibre entre les communautés microbiennes complexes et leurs hôtes.

Concernant la souche *B. subtilis* PTA-271, son effet protecteur a été montré sur vigne contre *Neofusicoccum parvum* Np-Bt67 et *Botrytis cinerea* (Magnin-Robert et al. 2007, 2013 ; Trotel-Aziz et al. 2008 ; Trotel-Aziz et al. 2019), les agents responsables du dépérissement aux Botryosphaeriacées et de la pourriture grise respectivement. La capacité des *B. subtilis* à sporuler pour résister aux changements climatiques (Nicholson et al. 2000), et les propriétés bénéfiques décrites ci-dessus pour *B. subtilis* PTA-271, renforcent notre intérêt pour cette souche de BCA capable de lutter contre une large gamme de pathogènes dont ceux associés aux MDB. Les MDB comptant parmi les maladies de la vigne les plus dommageables au plan économique, mais pour lesquelles il manque actuellement des stratégies de lutte efficace pour enrayer leur essor en France, en Europe, mais aussi à travers le Monde (Mondello et al. 2018 ; Trotel-Aziz et al. 2019).

Apparaissant comme un BCA au potentiel prometteur, et dans l'objectif de connaître toute l'étendue du potentiel de *B. subtilis* PTA-271 pour lutter efficacement contre les pathogènes impliqués dans les MDB, ce travail de thèse a débuté par l'analyse du génome complet de *B. subtilis* PTA-271 en comparaison à d'autres souches de *Bacillus* déjà connues (Figures 5 et 6).

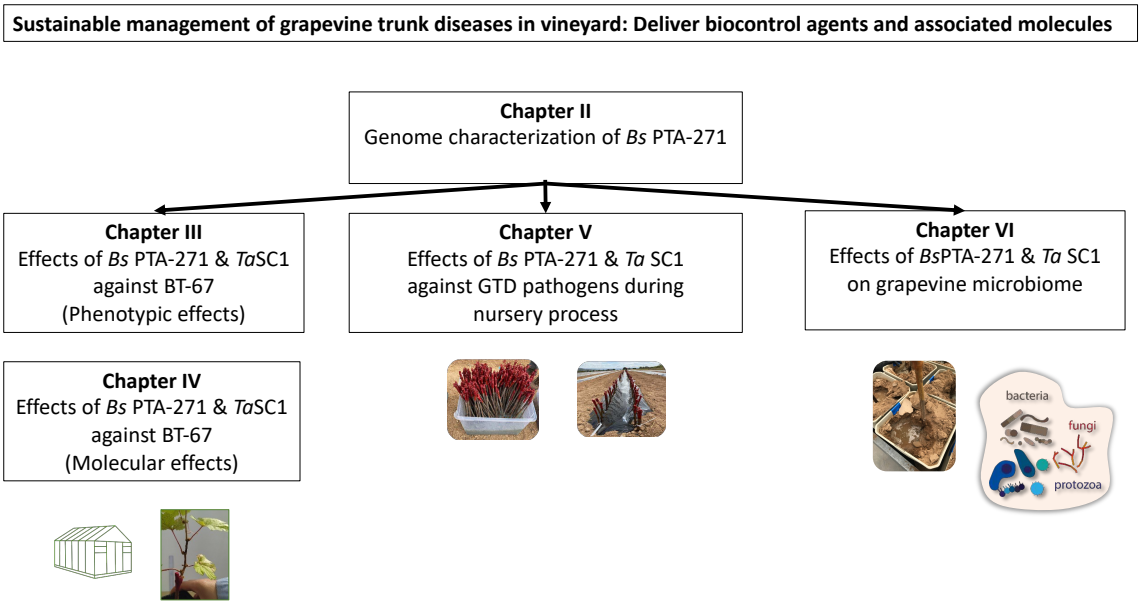


**Figure 5.** Aims of each chapter of the thesis entitled "Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules".

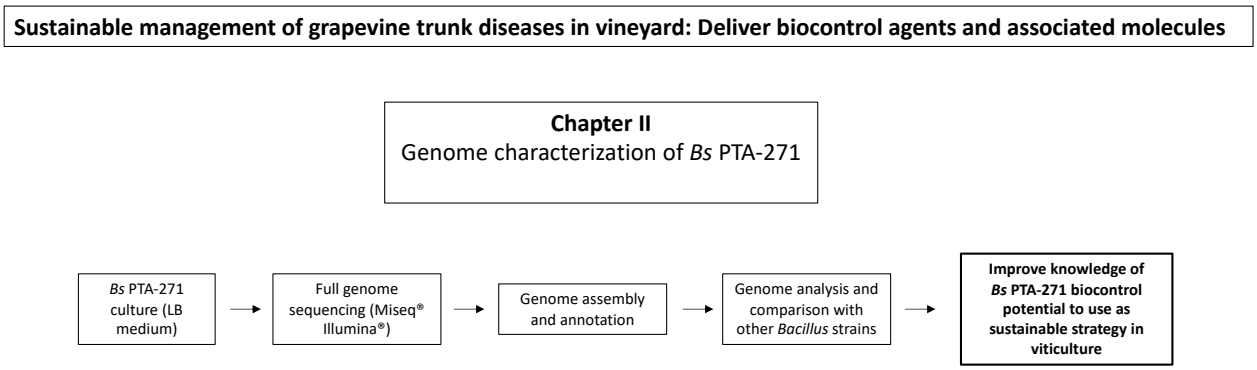


**Figure 6.** Aims and methodology of Chapter II entitled "Genome characterization of *Bacillus subtilis* PTA-271".

**Publication 2- Genome sequence analysis of the beneficial *Bacillus subtilis* PTA-271 isolated from a *Vitis vinifera* (cv. Chardonnay) rhizospheric soil: assets for sustainable biocontrol**

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RESEARCH ARTICLE Open Access

# Genome sequence analysis of the beneficial *Bacillus subtilis* PTA-271 isolated from a *Vitis vinifera* (cv. Chardonnay) rhizospheric soil: assets for sustainable biocontrol

Catarina Leal[1], Florence Fontaine[1], Aziz Aziz[1], Conceiçao Egas[2], Christophe Clément[1] and Patricia Trotel-Aziz[1*]

**Abstract**

**Background:** *Bacillus subtilis* strains have been widely studied for their numerous benefits in agriculture, including viticulture. Providing several assets, *B. subtilis* spp. are described as promising plant-protectors against many pathogens and as influencers to adaptations in a changing environment. This study reports the draft genome sequence of the beneficial *Bacillus subtilis* PTA-271, isolated from the rhizospheric soil of healthy *Vitis vinifera* cv. Chardonnay at Champagne Region in France, attempting to draw outlines of its full biocontrol capacity.

**Results:** The PTA-271 genome has a size of 4,001,755 bp, with 43.78% of G + C content and 3945 protein coding genes. The draft genome of PTA-271 putatively highlights a functional swarming motility system hypothesizing a colonizing capacity and a strong interacting capacity, strong survival capacities and a set of genes encoding for bioactive substances. Predicted bioactive compounds are known to: stimulate plant growth or defenses such as hormones and elicitors, influence beneficial microbiota, and counteract pathogen aggressiveness such as effectors and many kinds of detoxifying enzymes.

**Conclusions:** Plurality of the putatively encoded biomolecules by *Bacillus subtilis* PTA-271 genome suggests environmentally robust biocontrol potential of PTA-271, protecting plants against a broad spectrum of pathogens.

**Keywords:** Genome draft, Beneficial bacterium, *Bacillus subtilis* PTA-271, Grapevine trunk diseases, Grey mold, Wide protective spectrum, Sustainable biocontrol

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## Background

*Bacillus subtilis* is a Gram-positive endospore-forming bacterium from *Bacillus* genera considered as a promising plant beneficial organism that can survive in the soil for extended time periods under harsh environmental conditions [1]. Benefits of species from the *Bacillus* group are well described in many sectors of industry, agriculture and viticulture [2]. Focusing on the *B. subtilis* species, it has been described to provide plants with a broad range of benefits that include induced systemic resistance (ISR) upon pathogen attacks, growth promotion, or the direct control of plant pathogens [3–6].

Primed defenses during ISR are regulated either by jasmonic acid (JA) and ethylene (ET) signaling or by salicylic acid (SA) signaling [7–10]. Beneficial microorganisms may modulate the plant hormonal balance by either altering hormone synthesis or by producing similar hormones or their precursors (ET, SA, auxins, gibberellins, cytokinins, polyamines…) [8]. Numerous bacterial elicitors of ISR are also reported in several plant species, such as exopolysaccharides (EPS), lipopolysaccharides (LPS), siderophores such as the iron-regulated pyoverdin, iron, flagella, biosurfactants, N-acyl-L-homoserine lactone, N-alkylated benzylamine and volatile compounds [8, 9, 11, 12]. Some of these have already been identified in species of *B. subtilis* or *Bacillus* genera [8, 11, 13, 14]. Changes in the phytohormonal-balance also impact plant growth and development, since the reduction of ET may promote plant growth [8, 15, 16]. Microbiota support plant growth and development by modulating nutrient availability through mineralization and chelation, as well as through the production of volatile compounds that support biocontrol [17, 18]. Efficient beneficial effects of *Bacillus spp.* also assume direct and indirect bacterium and microbiota preservation, upon abiotic and biotic stressful conditions [8, 19]. When biocontrol agents protect themselves through extrusion transporters, detoxifying enzymes, quenching enzymes and pathogen homologous enzymes, they also contribute indirectly to plant protection [8]. Finally, *B. subtilis* produces an extensive range of antimicrobial molecules, chelators and lytic enzymes that limit pathogen fitness and aggressiveness [20]. According to literature, these beneficial molecules include ribosomally synthesized antimicrobial peptides (RP, including the post-translationally modified peptides RiPP), non-ribosomally synthesized peptides (NRP), polyketides (PK), as well as other uncommon antimicrobial volatile compounds (the inorganic and organic ViCs and VOCs, respectively) and terpenoid secondary metabolites as listed in Table 1. Individual strain specificities may thus impact both biochemical conditions and species ratios, and in turn interactions among complex microbial communities and their hosts.

Focusing on *B. subtilis* PTA-271, its protective effect has been published in grapevine against *Neofusicoccum parvum* and *Botrytis cinerea* [3–5], the causal agents of Botryosphaeria dieback and grey mold respectively. The ability of *B. subtilis* species to sporulate in order to resist climate changes and common disinfectants [1], combined with the fact that *B. subtilis* PTA-271 is a non-pathogenic species, make this microorganism suitable to control a wide spectrum of pathogens among which the most economically significant grapevine trunk disease (GTD) pathogens currently lack of efficient control strategies [3, 22]. In this study, we report the draft genome sequence of the *B. subtilis* strain PTA-271, analyze and compare with other known *Bacillus* strains sequences, to expand our knowledge of *B. subtilis* PTA-271 benefits, as well as design efficient and sustainable biocontrol strategies for viticulture.

## Methods

### *B. subtilis* PTA-271 GENERAL INFORMATION AND FEATURES

*B. subtilis* PTA-271 was isolated in 2001 (Table 2) from the rhizospheric soil of healthy Chardonnay grapevines (*V. vinifera* L., cv Chardonnay) from a vineyard located in Champagne (Marne, France). Rhizospheric samples were directly suspended in a sterile 0.85% NaCl solution (1 g of soil: 10 ml of NaCl) and bacterial isolates were obtained by serial dilutions of the soil samples ($10^7$, $10^3$, $10^2$ cfu/g soil) in triplicate onto LB-agar (Luria–Bertani-agar), King's B-agar and glycerol–arginine-agar plates by incubating at 30 °C for 24–72 h. All different colonies were then re-isolated on LB-agar, cultured in LB at 30 °C for 24 h and screened for their protective role against *Botrytis cinerea* by using grapevine plantlet leaf assays pretreated with bacterium [4]. Selected biocontrol microorganisms were then identified, calculated to establish the density formula and stored in a sterile 25% glycerol solution at − 80 °C for complementary purposes. The classification and general features of *B. subtilis* PTA-271 are in Table 2. The taxonomic information for this strain was already described by Trotel-Aziz et al. (2008) [4] and remains unaltered to this date.

### *B. subtilis* PTA-271 GENOMIC SEQUENCING INFORMATION

#### *Genome project history*

*B. subtilis* PTA-271 was selected for sequencing due to its efficient capacity to protect grapevine against several pathogens with distinct lifestyles such as *Botrytis cinerea* and *Neofusicoccum parvum* [3–5]. This beneficial microorganism can not only modulate grapevine defenses, but also antagonize the growth of pathogens and detoxify aggressive molecules. These beneficial bacteria provide protection against a broad spectrum of pathogens, due to its genetic traits of physical and chemical tolerance (endospore forming, withstand large pH and salinity range, Table 2). Altogether, there are advantages to sequence

**Table 1** *Bacillus subtilis* known antimicrobial molecules, chelators and lytic enzymes [6, 20, 21]

| | | |
|---|---|---|
| **RP** | **Bacteriocins** | such as the lantibiotics: lanthionine, nysin and subtilin, the pediocin-like peptides, the thuricin-like peptides and other linear peptides |
| | **Quorum quenching enzymes** | such as lactonase, decarboxylase, acylase and deaminase), |
| | **Cell wall degrading enzymes (CWDE)** | such as cellulases, proteases, chitinases, glucanases, etc… |
| | **Detoxifying-enzymes** | such as transferases and oxygenases |
| **NRP** | **Thiotemplate NRPs-lipopeptides** | such as fengycin, surfactin, iturin, bacillomycin |
| | **Thiotemplate NRPS-siderophores to compete pathogen nutrition** | such as the catecholic siderophores: itoic acid and bacillibactin |
| | **Non-thiotemplate NRPs** | such as the di- and tri- peptides rhizocticins, bacylisin, chlorotetain and the cyclic polypeptides bacitracin and mycobacillin |
| **PK** | **Acetogenins, ansamycins, enedyines, macrolides, polyenes, polyethers and tetracyclines** (such as bacillaene, difficidin and macrolactin that selectively inhibit protein synthesis) | |
| **VICs** | **Carbonated, hydrogenated, sulfur or nitrogen-containing compounds** (such as $H_2$, HCN, $H_2S$, $NH_3$). | |
| **VOCs** | **Fatty acids derivatives** (70% of the VOC) | such as the benzenoids 1,3-butadiene or 2,3-butanediol |
| | **Sulfur-containing VOCs** (VSCs) | such as dimethyl disulfide DMDS, dimethyl trisulfide DMTS, S-methyl thioacetate or S-methyl butanethioate |
| | **Nitrogen-containing VOCs** | such as azoles, pyrazines, pyridines, pyridazines, and pyrimidines |
| **Other II$^{ary}$ metabolites** | **Terpenoids** | such as isoprene and monoterpene a-terpineol |

*RP* Ribosomally synthesized antimicrobial peptides (including the post-translationally modified peptides RiPP), *NRP* Non-ribosomally synthesized peptides, *PK* Polyketides, *VIC* Inorganic antimicrobials volatile compounds, *VOC* Organic antimicrobials volatile compounds

the *B. subtilis* PTA-271 genome to better understand its key beneficial levers and develop better sustainable biocontrol strategies regardless of field conditions or soil parameters (pH, salinity, etc.).

The whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JACERQ000000000. The version described in this paper is version JACERQ000000000 and all related information is represented in Table 3.

#### *Genomic DNA preparation*

Genomic DNA of *B. subtilis* PTA-271 was extracted using the Wizard® Genomic DNA Purification kit (Promega), from the pellet of a 1 mL-overnight culture incubated at 28 °C in LB medium. DNA integrity was confirmed on a 0.65% agarose gel electrophoresis in TAE buffer. DNA concentration and quality were read from 1 µL of DNA with the NanoDrop-ONE spectrophotometer (Ozyme).

#### *Library preparation and genome sequencing*

DNA library for bacterial genome sequencing was prepared from 0.5 nanograms of high-quality genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, USA) and sequenced using paired-end (PE) 2 × 300 bp on the MiSeq® Illumina® platform at Genoinseq (Cantanhede, Portugal). All the procedures were performed according to standard manufacturer protocols.

#### *Genome assembly and annotation*

Sequenced reads were demultiplexed automatically by the Illumina® Miseq® sequencer using the CASAVA package (Illumina, San Diego, USA) and quality-filtered with Trimmomatic version 0.30 [24]. High-quality adapter-free reads were assembled with SPAdes version 3.9.0 [25] and contigs with size < 500 bp or coverage lower 10x were removed from the assembly. Assembly metrics were calculated with Quast version 4.6.1 [26].

**Table 2** Classification and features of *Bacillus subtilis* PTA-271 according to MIGS recommendations [23]

| MIGS ID | Property | Term | Evidence code |
|---|---|---|---|
| | Classification | Domain *Bacteria* | TAS |
| | | Phylum *Firmicutes* | TAS |
| | | Class *Bacilli* | TAS |
| | | Order *Bacillate* | TAS |
| | | Family *Bacilaceae* | TAS |
| | | Genus *Bacillus* | TAS |
| | | Sepecies *Bacillus subtilis* | TAS |
| | | Strain: PTA-271 | |
| | Gram strain | Gram-positive | IDA |
| | Cell shape | Rod-shaped | IDA |
| | Motility | Motile | NAS |
| | Sporulation | spore-forming | NAS |
| | Temperature range | Unreported | |
| | Optimum temperature | 37°C | NAS |
| | pH range, optimum | 4-9.5, 8 | NAS |
| | Carbon source | Organic carbon compounds | NAS |
| MIGS-6 | Habitat | Soil, grapevine | IDA |
| MIGS-6.3 | Salinity | 0-50 g/l; salt tolerant | NAS |
| MIGS-22 | Oxygen requirement | Aerobic | NAS |
| MIGS-15 | Biotic relationship | Free-living | IDA |
| MIGS-14 | Pathogenicity | Non-pathogenic | NAS |
| MIGS-4 | Geographic location | Champagne region, Marne, France | IDA |
| MIGS-5 | Sample collection | 2001 | IDA |
| MIGS-4.1 | Latitude | 49° 15' 15'' N | |
| MIGS-4.2 | Longitude | 4° 09' 28'' E | |
| MIGS-44 | Altitude | 105-206 m | |

[a]Evidence codes (from the Gene ontology project [58] – *IDA* Inferred from Direct assay, *TAS* Traceable Author Statement (i.e., a direct report exists in the literature), *NAS* Non-traceable Author Statement (i.e., not directly observed from the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence)

Contigs were checked for contamination and completeness using CheckM 1.0.9 [27]. Coding gene predictions were made with Prodigal version 2.6 [28], rRNA and tRNA genes were detected using Barrnap version 0.8 and CRISPR regions were detected by Minced version 0.2.0. Coding gene annotation was performed with Prokka version 1.12 [29] using the following repositories: SwissProt (The UniProt Consortium, 2017), HAMAP [30], TIGRFAMs [31] and Pfam [32]. Coding genes were also annotated for Pathway using KEGG [33], for peptidases using MEROPS [34] and for carbohydrate-active enzymes with dbCaN [35].

## Results and discussion

### *B. subtilis* PTA-271 GENOME PROPERTIES AND COMPARISON WITH OTHER BACILLUS STRAINS

The general features of *B. subtilis* PTA-271 are in Table 4 and Fig. 1, performed using Artemis version 16.0.0. The draft genome sequence of *B. subtilis* PTA-271 presented an estimated genome size of 4,001,755 bp divided in 20 contigs. The G + C content of this sequence was 1,751,999 bp, representing about 43.78% of the whole genome. Genome analysis showed that *B. subtilis* PTA-271 contained 4038 genes, among which 3945 (97.69%) were protein coding genes. This genome draft

**Table 3** *Bacillus subtilis* PTA-271 genomic sequencing information

| MIGS ID | Property | Term |
|---|---|---|
| MIGS 31 | Finishing quality | High-quality draft |
| MIGS28 | Libraries used | Illumina paired-end library (2×300 bp insert size) |
| MIGS29 | Sequencing platforms | Illumina MiSeq |
| MIGS31.2 | Fold coverage | 300 X |
| MIGS30 | Assemblers | SPAdes |
| MIGS32 | Gene calling method | Prodigal v.2.6 |
| | Locus tag prefix | H0Z15 |
| | WGS accession :<br>SUBID<br>BioProjet<br>BioSample | <br>SUB7775359<br>PRJNA646528<br>SAMN15546529 |
| | DDBJ/ENA/GenBank accession | JACERQ000000000 |
| MIGS13 | Source Material Identifier | *Bacillus subtilis* PTA-271 |
| | Project relevance | Biocontrol, Grapevine, GTD |

predicts 92 RNA genes among which 11 rRNA genes were identified and no CRISPR repeats. From 4,001,755 bp of the genome size, 3,550,299 bp correspond to coding genes representing 88.73% of the whole genome. From this, 3440 genes had function prediction, 3183 were assigned to the COG categories described in Table 5, and 3517 genes had Pfam domain descriptions.

## *B. subtilis* PTA-271 ASSETS FOR PLANT SUSTAINABLE BIOCONTROL

*Bacillus* species offer a broad range of benefits to plants: (1) plant growth promotion, (2) induced systemic plant defenses and protection against pathogens, and (3) prevention of pathogen fitness or aggressiveness, by producing many compounds able to interact with the host plants, the pathogens or their tripartite intricate communication. Considering this, the genome analysis of *B. subtilis* PTA-271 tried to highlight some useful characteristics directly or indirectly beneficial for a sustainable plant protection against a broad spectrum of pathogens.

### *Motility and adhesion: assets for plant root colonization*

Motility of a bacterium is due to the flagellum, enabling it to move towards a vital nutrient source (chemotaxis). In this sense, *B. subtilis* PTA-271 contains genes (Supplementary Table S1) putatively encoding for flagella maintenance (*flh* genes) and chemotaxis (*che* genes). Once reaching a comfortable area, adhesion is due to bacterium pili,

**Table 4** Genome statistics

| Attribute | Value | % of Total[a] |
|---|---|---|
| Genome size (bp) | 4,001,755 | 100 |
| DNA coding (bp) | 3,550,899 | 88.73 |
| DNA G + C (bp) | 1,751,999 | 43.78 |
| DNA scaffolds | 20 | - |
| Total genes | 4,038 | 100 |
| Protein coding genes | 3,945 | 97.69 |
| RNA genes | 92 | 2.27 |
| Genes with function prediction | 3,440 | 85.19 |
| Genes assigned to COGs | 3,183 | 78.82 |
| Genes with Pfam domains | 3,517 | 87.10 |
| CRISPR repeats | 0 | 0 |

[a] The total is based on either the size of genome in base pairs or the total number of genes in the predicted genome

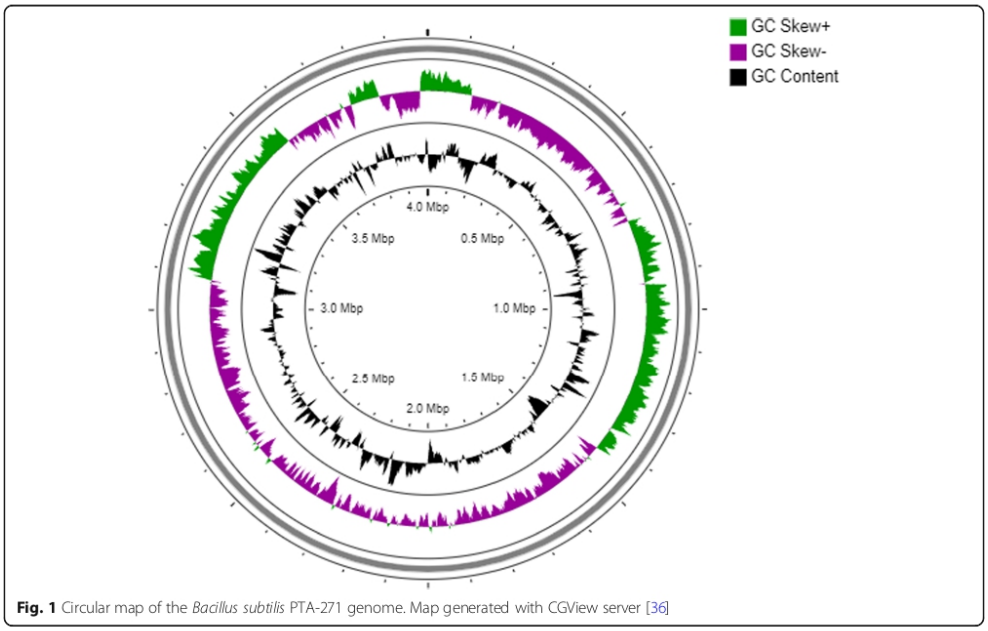


**Fig. 1** Circular map of the *Bacillus subtilis* PTA-271 genome. Map generated with CGView server [36]

allowing the initiation of biofilm formation where both chemotaxis and gene exchanges among microorganisms of microbiota can be amplified.

*B. subtilis* spp. are also described for their strong swarming motility [37]. The gene *swrC* putatively encoding for swarming motility protein is predicted in the genome of *B. subtilis* PTA-271 (Supplementary Table S1). Swarming motility requires the production of functional flagella, pili and surfactant to reduce surface tension.

Motilities and adhesion are considered advantageous characters for a successful host colonization and *B. subtilis* spp. are already described to grow in biofilm mode involved in root colonization [38]. To this end, the transcription factors (TF) Spo0A and AbrB were described as positive and negative regulators of biofilm formation, respectively [39]. Genes putatively encoding for these 2 TFs are also predicted in the genome of *B. subtilis* PTA-271 as *Spo0A* and *AbrB* (Supplementary Tables S1 and S2).

Beneficial microorganisms that successfully colonize the plant, particularly by the root system, would be advantageous, both for plant growth promotion and for plant biocontrol [40, 41].

#### *Biofertilizing and morphogenic effects: assets for plant vigor*

Plant nutrition depends on soil retention capacity of minerals and nutrient availabilities, thus both on chelating process, mineralization by decomposers and minerals bioavailability towards the plant consumer. Upon nitrogen starvation, some bacteria are described to upregulate the *ure* gene cluster, since urea is an easy nitrogen source. Such *ure* genes are predicted in *B. subtilis* PTA-271 genome (*ureA*, *ureB*, *ureC*). This cluster of genes is known to be controlled by the global nitrogen-regulatory protein *TnrA*, also predicted in *B. subtilis* PTA-271 genome (Supplementary Table S2). Regarding other nutrient access due to phosphate-solubilizing bacteria (PSB) [6, 42, 43], genes encoding for proteins involved in the production of gluconic acid and precursor of citric acid are also predicted in the genome of *B. subtilis* PTA-271 (S19-40_03830, S19-40_03828). Organic acids may lower the soil pH to solubilize phosphate and thus increase its availability to the plant [42]. Bacterial secondary metabolites (PyrroloQuinoline Quinone, PQQ) are also known to control gluconic acid production [44], and *B. subtilis* PTA-271 has 3 genes predicted to be related to PQQ production *pqqL*, *pqqF and pqqC* [45]. Additionally, *B. subtilis* PTA-271 contains the phytase gene *phy*, described in the other *Bacillus spp.* to encode for phosphatases able to hydrolyze organic complex in order to liberate phosphate and make it available for plants [46]. Iron is another very important nutrient for plant growth and development. *B. subtilis* PTA-271 possesses the *fur* gene (Supplementary Table S2) described in the literature to encode for a regulatory protein coordinating the homeostasis of iron uptake depending on its

**Table 5** Number of genes associated with general COG functional categories

| Code | Value | %age[a] | Description |
|---|---|---|---|
| J | 173 | 4.38 | Translation, ribosomal structure and biogenesis |
| A | 0 | 0 | RNA processing and modification |
| K | 313 | 7.93 | Transcription |
| L | 118 | 2.99 | Replication, recombination and repair |
| B | 1 | 0.02 | Chromatin structure and dynamics |
| D | 39 | 0.98 | Cell cycle control, cell division, chromosome partitioning |
| V | 59 | 1.49 | Defense mechanisms |
| T | 171 | 4.33 | Signal transduction mechanisms |
| M | 210 | 5.32 | Cell wall/membrane/envelope biogenesis |
| N | 62 | 1.57 | Cell motility |
| U | 49 | 1.24 | Intracellular trafficking, secretion, and vesicular transport |
| O | 109 | 2.76 | Posttranslational modification, protein turnover, chaperones |
| C | 183 | 4.63 | Energy production and conversion |
| G | 313 | 7.93 | Carbohydrate transport and metabolism |
| E | 328 | 8.31 | Amino acid transport and metabolism |
| F | 86 | 2.17 | Nucleotide transport and metabolism |
| H | 134 | 3.39 | Coenzyme transport and metabolism |
| I | 118 | 2.99 | Lipid transport and metabolism |
| P | 184 | 4.66 | Inorganic ion transport and metabolism |
| Q | 91 | 2.30 | Secondary metabolites biosynthesis, transport and catabolism |
| R | 443 | 11.22 | General function prediction only |
| S | 360 | 9.12 | Function unknown |
| W | 0 | 0 | Extracellular structures |
| Y | 0 | 0 | Nuclear structure |
| Z | 1 | 0.02 | Cytoskeleton |
| - | 762 | 19.31 | Not in COGs |

[a] The total is based on the total number of protein-coding genes in the genome

availability in the soil [47]. Regarding soils containing abundant ferric form ($Fe^{3+}$) which is poorly available to plants, the literature described bacteria producing siderophores with high specificity and affinity for iron, capable of binding, extracting and transporting iron near the plant roots [48]. *B. subtilis* PTA-271 genome also predicted the production of such siderophores, namely the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin encoded by 5 genes (*dhbA* to *dhbF*). Surfactants produced by beneficial bacteria also contribute to increase the availability of hydrophobic nutrients. In this sense, *B. subtilis* PTA-271 is suspected to produce surfactin (with *srfAA* to *srfAD*), a powerful biosurfactant due to its amphiphilic nature that strongly anchor with lipid layers, interfering with the structure of biological membranes [49].

Plant root morphology is also described to impact nutrient uptake and thus plant growth due to the stimulation of lateral root formation and root hair formation [50, 51]. Plant hormones are key elements for root morphology changes. Some beneficial bacteria are also described to produce them [51]. Regarding *B. subtilis* PTA-271 genome, it predicts the *trp* group, described in literature to produce tryptophan as the main precursor of the auxin IAA (indole-3-acetic acid) [42]. The genome of *B. subtilis* PTA-271 also predicts genes such as *yvdD* (Supplementary Table S2), linked in the literature to cytokinin synthesis which is known as a plant growth regulator (cell division, organogenesis) in combination with IAA. Gibberellins (GA) produced by some bacteria also affect the plant growth and survival [51]. Regarding the *B. subtilis* PTA-271 genome, it predicts *ispD* and *GerC3_*

*HepT*, described in the literature to be respectively linked to 2-C-methyl-D-*erythritol* 4-phosphate (MEP) and geranylgeranyl diphosphate (GGPP) production, two successive precursors of GA and abscisic acid (ABA) synthesis in plants [52].

Genes described to encode for other plant growth regulators, namely polyamines (PAs), are also predicted in the genome of *B. subtilis* PTA-271. Among them: *speA*, *speB*, *speG* and *speE* are respectively described in literature to encode for putative ADC (arginine decarboxylase), agmatinase (leading to putrescine), then spermidine- and spermine- synthases. Additionally, genes encoding for putative S-adenosyl-methionine (SAM) decarboxylase (*speH*) and SAM-methyltransferase (S19-40_00450) are predicted in *B. subtilis* PTA-271 genome, and these proteins are mentioned to complete PA synthesis from putrescine [53]. PAs are known to promote flowering and to play important roles in inducing cell division, promoting regeneration of plant tissues and cell cultures [54], as delaying senescence [55].

Volatile compounds (VOCs) produced by some beneficial rhizospheric bacteria have also been identified as elicitors promoting plant growth. Regarding *B. subtilis* PTA-271, its predicted genes encode putatively for (1) acetoin (*acuA*, *acuC*…) and (2) 2,3-butanediol (*butA* and *butC*) [20, 56]. VOCs are especially reported to interact with plant hormones [57–59].

### *Host induced defenses and microbiota preservation: assets for plant protection*

**PLANT INDUCED DEFENSES upon biotic stress** Host primed defenses during ISR are regulated by hormones, depending on either JA and ET signaling or SA signaling [7, 8, 10, 60]. Beneficial microorganisms may modulate the plant hormonal balance or directly elicit the plant defenses. Regarding the genome of *B. subtilis* PTA-271, the *metK* gene is predicted to encode for SAM synthase that would appear ISR-useful for plants which possess the complementary ET metabolic machinery [53, 55]. SA is another hormone for which several genes encoding its metabolic pathways (from synthesis to hydrolysis) are predicted in *B. subtilis* PTA-271 genome, among which *pchA* putatively encoding for the salicylate biosynthesis isochorismate synthase.

Many elicitors also induce host immunity, coming from microorganisms (MAMPs, microbial associated molecular patterns) but also from the plant host (DAMPs, damage-associated molecular patterns). MAMPs can act from the external surface of a beneficial microorganism (flagellin) or result from a secretion outside or inside the host (surfactin, fengycin, VOCs, etc.) [58, 61–63]. Flagellin proteins are putatively encoded by the *hag* gene predicted in *B. subtilis* PTA-271 (Supplementary Table S1). The lipopeptides surfactin and fengycin are other elicitors of plant ISR putatively encoded by some genes predicted in the genome of *B. subtilis* PTA-271 (*srf* and *fen* genes, respectively). VOCs produced by rhizospheric bacteria, as the 3-hydroxy 2-butanone and acetoin which are putatively encoded by *B. subtilis* PTA-271 genome, are also well known to induce ISR [58]. Among VICs, the ubiquitous nitric oxide (NO) is another signal molecule [64]. Different genes related to NO metabolic pathways are predicted in *B. subtilis* PTA-271 genome, among which the gene *nos* putatively encoding for a NO synthase oxygenase. Exopolysaccharides (EPS) and lipopolysaccharides (LPS) are other elicitors reported in several *Bacillus* genera [9–14, 60]. Regarding the genome of *B. subtilis* PTA-271, it predicts several genes putatively encoding for EPS (S19-40_00800, S19-40_00870, S19-40_00999, S19-40_01009, S19-40_01427) and LPS (*lptB*, *lapA*, *lapB*), additionally to the other elicitors predicted to be encoded by *B. subtilis* PTA-271 genome (siderophores, flagella, N-acyl-L-homoserine lactone, etc.).

DAMPs are alternative elicitors produced by lytic enzymes (chitosan, glucans, etc.) of microorganisms (either beneficial or pathogenic) or plants [62]. Genes encoding for lytic enzymes are predicted in *B. subtilis* PTA-271 genome, such as those encoding for putative chitosanase and ß-glucanase (Supplementary Table S3). Many other genes are also predicted to encode for lytic enzymes in the *B. subtilis* PTA-271 spore cortex (Supplementary Table S4) for which the roles remain unclear.

**PLANT INDUCED DEFENSES upon abiotic stress** Some previously cited hormones are also useful for plant defense against abiotic stress, such as ABA and GA [8], of which precursors are predicted to be encoded by genes identified in the genome of *B. subtilis* PTA-271 (*GerC3_HepT*, *ispD*). From GGPP, the kaurene pathway may lead to GA, while the phytoene path may lead to ABA [52], and in the genome of *B. subtilis* PTA-271, *yisP* (a *crtb* KEGG gene) encodes for a putative 15-cis-phytoene/all-trans-phytoene synthase. ET is another useful hormone for plant defense against abiotic stress [8], and *B. subtilis* PTA-271 genome has genes identified to putatively produce SAM (*metK*). Altogether these data predict that *B. subtilis* PTA-271 genome may putatively encode for key precursors of phytohormones that may influence actively ABA and ET contents in plants. In plants, ABA, GA and ET signaling pathways interfere altogether through different transcription factors (TF) or small proteins (GiD, DELLA, EIN, etc.) that physically interact [65, 66]. In the genome of *B. subtilis* PTA-271, many genes are predicted to encode for sigma factors and many TF (Supplementary Table S2). It is noteworthy to understand that useful TF upon abiotic stress could also be useful upon biotic stress. The set of genes under common regulatory controls (operons) are also listed in the Supplementary Table S2.

PAs such as those predicted to be encoded by the genome of *B. subtilis* PTA-271 are also described to protect

plant cells upon water deficit [67], temperature changes [68] and salinity [69].

**MICROBIOTA quality and preservation** As energy and carbon sources, plant root exudates (sugars, organic acids, amino acids, lipophilic compounds, etc.) would enable the selective recruitment of biosurfactant producers [70, 71]. In return, these beneficial bacteria can facilitate the bioavailability of root exudates and biofilm formation, thus the colonization of host-plants by beneficial bacteria [49, 70, 72], maybe such as *B. subtilis* PTA-271 which is suspected to produce surfactin. SA was also shown to mediate changes in the composition of root exudates, then in the qualitative microorganism recruitment by plants [19]. Regarding the *B. subtilis* PTA-271 genome, some genes are also predicted to produce SA (*pchA*), highlighting another key lever that putatively influence the composition of plant microbiome.

Beneficial microbial interactions can additionally depend on bacterial auto-inducers (AI) that are low-molecular weight signal molecules activating the interactive competences of a bacterium in a quorum-sensing (QS) dependent manner [73]. Among AI, the furanosyl-borate-diester (AI-2) is described as universal for interspecies communication both in Gram-positive and Gram-negative bacteria [74]. Regarding *B. subtilis* PTA-271 genome, the predicted *luxS* gene putatively encodes for AI-2 production, while the predicted *EntF* and *AM373* putatively encode oligopeptides or auto-inducing peptide (AIP) precursors. AIP is another class of AI consisting of 5–34 amino acids residues and produced by Gram-positive bacteria for their intercellular communication [75].

When interacting with the environment, a microorganism must also remain metabolically active to exert beneficial effects. Upon biotic interactions, *Bacillus* species are exposed to host defenses that include reactive oxygen species (ROS) [76]. Regarding the system of sensing, protection and regulation of ROS in the genome of *B. subtilis* PTA-271, genes are predicted to putatively encode for resistance to hydroperoxide (*ohrA*, *ohrB*, *ohrR*). Upon abiotic stress, beneficial bacteria must survive dehydration, wounding, cold, heat or salinity that in turn lead to regulation of the water status. For this end, bacterial species can control their intracellular solute pools [77, 78]. Regarding the genome of *B. subtilis* PTA-271, genes predicted to encode for potassium uptake proteins (*KtrA*, *KtrB*) putatively enable survival in high salinity environments. Interestingly, the genome of *B. subtilis* PTA-271 also predicts genes to detoxify or resist compounds accumulating in the environment [79, 80], such as arsenite (*arsR*), organic pesticides or nitroaromatic compounds (*sugE, qacC, mhqR, mhqA*) among others (Supplementary Tables S2 and S5).

Upon extreme environmental conditions, some beneficial bacteria can sporulate, turning on endospore form [1, 81]. Regarding the genome of *B. subtilis* PTA-271, several genes are predicted to be involved in the sporulation process (Supplementary Table S4): *spo* (sporulation control), *ger* (germination control), *cot* (endospore external layer) and *cw* (spore cortex lytic enzymes), putatively enabling it to survive long lasting periods while preserving all beneficial strengths for plant profits.

***Direct confrontation with pathogens or aggressive molecules*** Upon direct confrontation, *Bacillus* species also need to protect themselves against pathogen defenses. In addition to ROS protection, diverse transporters mediate antibiotic extrusion, whether specific to a substance or a group of substances. Regarding the genome of *B. subtilis* PTA-271, the specific transporters predicted would putatively confer it resistance towards: tetracyclin (*tetA*, *tetR*, *tetD*), fosfomycin (*fosB*), erythromycin (*msrA*, *msrB*), bacillibactin (*ymfD*), bacitracin (*BceA*, *BceB*, *BcrC*), bleomycin (*ble*) and riboflavin (*ribZ*, *rfnT*) for example. Among the non-specific transporters (or multidrug transporters) predicted in the genome of *B. subtilis* PTA-271 are: *mepA*, *ebrA* and *ebrB*; *ykkD* and *ykkC*; *bmrA* and *bmr3*; *emrY*, among others.

*Bacillus* species can additionally directly detoxify some pathogen aggressive molecules targeting plants, such as phytotoxins, by the mean of antitoxins or detoxifying enzymes such as transferases and CYP450s [82, 83]. In the genome of *B. subtilis* PTA-271, the main transferases predicted are glutathione-S-transferases GST, malonyltransferases MT, glucosyl-transferases GT and many others, while the main CYP450s predicted are monooxygenases and dioxygenases (Supplementary Table S5). Quenching enzymes constitute another lever for beneficial bacteria to directly target pathogen aggressive molecules, by preventing their QS-dependent production [8, 84]. Indeed, *Bacillus* species share *aiiA* gene encoding for *N*-acetyl homoserine lactonase able to hydrolyze the lactone ring of the AHLs (Acyl-homoserine lactones) involved in the QS production of some pathogen virulent factors. The genome analysis of *B. subtilis* PTA-271 predicts such genes putatively encoding for quenching enzymes such as lactonases, β-lactamases, deaminases, deacetylases and other (de)acylases (Supplementary Table S6).

Polyketide synthases (PKS) are another type of transferases, namely acetyltransferases, described to produce plant beneficial molecules as microbicide for phytopathogens: the polyketides (PK) [85, 86]. Regarding the genome of *B. subtilis* PTA-271, 15 genes are predicted to encode for putative PKS, many others for acetyltransferases or for enzymes sharing similar part of the PKS functions (Supplementary Table S7). According to antiSMASH 5.1.0, *B. subtilis* PTA-271 genome predicts 11 secondary metabolites gene clusters, among which: 1 PKS cluster and 1 hybrid PKS-NRPS cluster (Supplementary Table S8).

An extensive range of pathogen direct effectors are additionally produced by *Bacillus* spp., such as the RP (ribosomally synthesized peptides) and NRP (non-ribosomally synthesized peptides) antimicrobial molecules [20, 87]. Some of them are predicted as encoded by the genome of *B. subtilis* PTA-271, such as: Baillaene (*pksD)*, subtilosin *(sboA, albG, albE, albD, albB, albA)* and bacilysin (*bacE, bacF, bacG*) (Supplementary Table S3). Lipopeptides are other NRP antimicrobial molecules [49, 88], which encoding genes are predicted in the genome of *B. subtilis* PTA-271 to putatively produce the powerful antifungal substances fengycin and surfactin (Supplementary Table S3). Besides antibiotics and surfactants, bacterial siderophores can also directly alter pathogen fitness and aggressiveness, by depriving pathogen growth of iron while providing it for plant growth [89]. Regarding the genome of *B. subtilis* PTA-271, predicted genes putatively encode for the siderophore Bacillibactin (Supplementary Table S3). Lytic enzymes (CWDE) are other important feature of *Bacillus* spp. that can both alter pathogen survival and produce MAMPs [90]. Regarding the genome of *B. subtilis* PTA-271, several genes are predicted to encode for putative CWDE: 1 chitosanase (*csn*), 1 β-glucanase (*bglS*), 1 β-glucanase / cellulase (*eglS*) and about 80 proteases (Supplementary Table S3).

Besides these NRP and RP antimicrobial molecules, the genome of *B. subtilis* PTA-271 also predicts the genes *hcnC*, *acu* and *but*, putatively encoding for the volatile antimicrobial compounds: VIC (hydrogen cyanide, HCN) and VOC (acetoin and 2,3-butanediol), respectively [8, 20, 56].

According to COG categories, 2.30% of *B. subtilis* PTA-271 genome is predicted to be devoted to the production of secondary metabolites, considered as one of the most important features in biocontrol activities. AntiSMASH 5.1.0 predicts 11 secondary metabolites gene clusters in *B. subtilis* PTA-271 genome, among which 3 NRPS clusters and 2 RiPPs clusters (Supplementary Table S8).

## *B. subtilis* PTA-271 GENOME COMPARISON WITH OTHER GENOMES

To understand the magnitude of the differences between *B. subtilis* PTA-271 and other *Bacillus* strains, the PTA-

**Table 6** Comparative NCBI genome analysis of *Bacillus subtilis* PTA-271 with strains showing ≥99% of *16 s* similarity

| | Strain | Gb accession number | Isolation source | Country | Genome size (bp) | G+C content (%) | Protein-coding sequences | tRNA coding genes | rRNA |
|---|---|---|---|---|---|---|---|---|---|
| | *Bacillus subtilis* subsp. subtilis strain PTA-271 | - | rhizosphere, roots, leaves and stems (Grapevine) | France | 4.002 | 43.8 | 3945 | 81 | 11 |
| Type-strains | *Bacillus subtilis* 9407 | PISO01000016 | apple tree | China | 4.19 | 43.4 | 4012 | 79 | 9 |
| | *Bacillus subtilis* subsp. subtilis NCIB 3610 | CP034484 | not available/ unknown | not available/ unknown | 4.3 | 43.34 | 4390 | 86 | 30 |
| | *Bacillus subtilis* subsp. subtilis 168 | CP019662 | soil | Germany | 4.22 | 43.5 | 4284 | 86 | 30 |
| | *Bacillus amyloliquefaciens* subsp. plantarum FZB42 | CP000560 | soil (sugar beet) | Germany | 3.92 | 46.5 | 3687 | 89 | 31 |
| | *Bacillus velezensis* KCTC 13012 | LHCC01000001 | river velez | spain | 4.04 | 46.3 | 3806 | 80 | 9 |
| Non type-strains | *Bacillus subtilis* subsp. subtilis QB5413 | CP017313 | not available/ unknown | France | 4.22 | 42.7 | 5099 | 84 | 30 |
| | *Bacillus subtilis* SRCM104005 | CP035164 | food | South Korea | 4.14 | 43.8 | 4106 | 86 | 30 |
| | *Bacillus subtilis* QB61 | CP029461 | soil | China | 4.12 | 43.7 | 4086 | 85 | 28 |
| | *Bacillus subtilis* subsp. subtilis QB5412 | CP017312 | not available/ unknown | France | 4.22 | 43.5 | 4252 | 86 | 30 |
| | *Bacillus subtilis* SR1 | CP021985 | soil | India | 4.09 | 44.2 | 3911 | 83 | 30 |
| | *Bacillus subtilis* SG6 | CP009796 | luffa | China | 4.08 | 43.8 | 4030 | 84 | 21 |
| | *Bacillus subtilis* SRCM103773 | CP035397 | food | South Korea | 4.05 | 43.6 | 4113 | 86 | 30 |
| | *Bacillus subtilis* PR10 | CP040528 | Tobacco rhizosphere soil | China | 4.07 | 43.8 | 3978 | 86 | 30 |
| | *Bacillus subtilis* GQJK2 | CP020367 | rhizosphere | China | 4.07 | 43.8 | 3976 | 86 | 30 |
| | *Bacillus subtilis* subsp. subtilis BS155 | CP029052 | marine sediment | China | 4.33 | 43.5 | 2718 | 86 | 30 |
| | *Bacillus subtilis* SRCM103835 | CP035400 | food | South Korea | 4.14 | 43.8 | 4108 | 86 | 30 |
| | *Bacillus subtilis* SRCM103837 | CP035401 | food | South Korea | 4.14 | 43.8 | 4113 | 86 | 30 |
| | *Bacillus subtilis* SRCM103641 | CP035390 | food | South Korea | 4.11 | 43.7 | 4136 | 86 | 30 |
| | *Bacillus subtilis* H19 | CP039935 | seawater | China | 4.06 | 43.9 | 4001 | 86 | 30 |
| | *Bacillus subtilis* BS38 | CP017314 | soybean paste | South Korea | 4.01 | 43.6 | 3876 | 86 | 30 |
| | *Bacillus subtilis* subsp. spizizenii SW83 | CP030925 | Turbinaria ornata | India | 3.98 | 43.8 | 3936 | 83 | 8 |
| | *Bacillus subtilis* subsp. subtilisGFR-12 | CP032852 | Fermented soybean paste | South Korea | 4.2 | 43.3 | 4117 | 87 | 30 |
| | *Bacillus subtilis* subsp. subtilis 2KL1 | CP032872 | Fermented soybean paste | South Korea | 4.2 | 43.3 | 4110 | 93 | 33 |
| | *Bacillus subtilis* SRCM103886 | CP035162 | food | South Korea | 4.21 | 43.3 | 4121 | 87 | 30 |
| | *Bacillus subtilis* subsp. subtilis 2RL2-3 | CP032857 | Fermented soybean paste | South Korea | 4.19 | 43.4 | 4083 | 87 | 33 |
| | *Bacillus subtilis* SRCM103576 | CP035402 | food | South Korea | 4.01 | 43.5 | 3986 | 86 | 30 |
| | *Bacillus subtilis* subsp. subtilis N4-2 | CP032867 | Natto | Japan | 4.12 | 43.5 | 4154 | 87 | 30 |
| | *Bacillus* sp. M4U3P1 | CP041372 | Suaeda salsa | China | 4.12 | 43.5 | 4068 | 87 | 30 |
| | *Bacillus subtilis* subsp. subtilis N3-1 | CP032865 | Natto | Japan | 4.12 | 43.5 | 4069 | 87 | 30 |

271 genome has been compared to the complete genomes of 5 type-strains (*B. subtilis* NCIB 3610, *B. subtilis* 168, *B. subtilis* 9407, *B. amyloliquefaciens subsp. plantarum* strain FZB42, and *B. velezensis* KTCT 13012) [91] and 32 non-type strains, represented in Table 6. Among non-type strains showing ≥99% of the *16S* ribosomal gene similarity with PTA-271 are 31 distinct strains of *B. subtilis* and 1 *Bacillus velezensis*. For this genomic comparison, was used the GGDC 2.1 web server [92], the DSMZ phylogenomics pipeline to estimate DNA-DNA hybridization (DDH) [92], and the JSpecies WS web server to estimate the Average Nucleotide Identity (ANI) through pairwise comparisons [93]. The DDH value was estimated using the recommended formula (formula two) for draft genomes, at the GGDC website [94]. The ANI values were calculated using Ezbiocloud [95]. The whole data analysis enabled to obtain the intergenomic distances between genomes and their probability of belonging to the same species or subspecies. The general comparison of genomes is reported in Table 6, while the intergenomic distances (DDH estimate and ANI) are shown in Table 7.

Among the type strain genomes, the closest strain to *B. subtilis* PTA-271 was *B. subtilis* 9407, with a 0.0104 distance, a DDH estimate of 91.60%, and an ANIm of 99.02%. As expected, the most distant strain was *B. velezensis* KTCT 13012, with a 0.2268 distance, a DDH estimate of 19.40% and a 0% probability of being the same species, corroborated with an ANIm percentage of 77.02%. Concerning the non-type strain genomes, the closer strains to PTA-271 were *B. subtilis* QB5413, *B. subtilis* SRCM 104005, and *B. subtilis* QB61 with distances of 0.0112, 0.0119 and 0.0119 respectively, and DDH estimates of 90.90, 90.20 and 90.20% respectively. The most distant strain was *B. velezensis* strain ATR2, with a distance of 0.2144 and a DDH estimate of 20.50% corroborated with an ANIm percentage of 77.1%. The most distant *B. subtilis* strain to PTA-271 was *B. subtilis*

**Table 7** Comparative genome distances analysis with other strains, using DNA-DNA hybridization and average nucleotide identities

| | Strains | Gb accession number | DNA-DNA hybridization (DDH method) | | | | | Average nucleotide identities (ANIm) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Distance | DDH estimate | Probability that DDH >70% (same species) | Probability that DDH >79% (same subspecies) | Difference in % G+C | ANIm (%) | Aligned (bp) |
| Type-strains | *Bacillus subtilis* 9407 | PISO01000016 | 0.0104 | 91.60% | 96.29% | 67.69% | 0.56 | 99.02 | 1,456,961 |
| | *Bacillus subtilis* subsp. subtilis NCIB 3610 | CP034484 | 0.0121 | 90.00% | 95.80% | 65.28% | 0.27 | 98.88 | 2,509,972 |
| | *Bacillus subtilis* subsp. subtilis 168 | CP019662 | 0.0122 | 90.00% | 95.78% | 65.19% | 0.27 | 98.87 | 2,962,217 |
| | *Bacillus amyloliquefaciens* subsp. plantarum FZB42 | CP000560 | 0.2144 | 20.50% | 0.00% | 0.00% | 2.69 | 77.1 | 1,854,058 |
| | *Bacillus velezensis* KCTC 13012 | LHCC01000001 | 0.2268 | 19.40% | 0.00% | 0.00% | 2.84 | 77.02 | 437,224 |
| Non type-strains | *Bacillus subtilis* subsp. Subtilis QB5413 | CP017313 | 0.0112 | 90.90% | 96.08% | 66.63% | 1.05 | 98.76 | 2,529,290 |
| | *Bacillus subtilis* SRCM104005 | CP035164 | 0.0119 | 90.20% | 95.87% | 65.63% | 0.00 | 98.84 | 2,941,570 |
| | *Bacillus subtilis* QB61 | CP029461 | 0.0119 | 90.20% | 95.85% | 65.52% | 0.04 | 98.82 | 3,023,981 |
| | *Bacillus subtilis* subsp. subtilis QB5412 | CP017312 | 0.0122 | 89.90% | 95.78% | 65.17% | 0.27 | 98.85 | 2,985,990 |
| | *Bacillus subtilis* SR1 | CP021985 | 0.0124 | 89.70% | 95.70% | 64.82% | 0.24 | 98.86 | 2,881,899 |
| | *Bacillus subtilis* SG6 | CP009796 | 0.0124 | 89.70% | 95.70% | 64.83% | 0.05 | 98.81 | 2,968,883 |
| | *Bacillus subtilis* SRCM103773 | CP035397 | 0.0126 | 89.50% | 95.63% | 64.50% | 0.16 | 98.81 | 2,633,309 |
| | *Bacillus subtilis* PR10 | CP040528 | 0.0129 | 89.30% | 95.56% | 64.15% | 0.01 | 98.77 | 2,648,038 |
| | *Bacillus subtilis* GQJK2 | CP020367 | 0.0129 | 89.30% | 95.55% | 64.14% | 0.01 | 98.79 | 2,856,573 |
| | *Bacillus subtilis* subsp. subtilis BS155 | CP029052 | 0.0131 | 89.10% | 95.49% | 63.83% | 0.29 | 98.69 | 2,805,710 |
| | *Bacillus subtilis* SRCM103835 | CP035400 | 0.0132 | 89.00% | 95.44% | 63.61% | 0.02 | 98.75 | 2,640,533 |
| | *Bacillus subtilis* SRCM103837 | CP035401 | 0.0132 | 89.00% | 95.44% | 63.61% | 0.02 | 98.76 | 2,637,514 |
| | *Bacillus subtilis* SRCM103641 | CP035390 | 0.0137 | 88.60% | 95.29% | 62.96% | 0.07 | 98.67 | 2,561,457 |
| | *Bacillus subtilis* H19 | CP039935 | 0.0144 | 88.00% | 95.05% | 61.95% | 0.11 | 98.62 | 2,750,901 |
| | *Bacillus subtilis* BS38 | CP017314 | 0.0149 | 87.40% | 94.84% | 61.09% | 0.19 | 98.52 | 2,702,006 |
| | *Bacillus subtilis* subsp. spizizenii SW83 | CP030925 | 0.0152 | 87.20% | 94.73% | 60.64% | 0.02 | 98.56 | 2,801,320 |
| | *Bacillus subtilis* subsp. subtilisGFR-12 | CP032852 | 0.0153 | 87.10% | 94.69% | 60.50% | 0.47 | 98.49 | 2,649,573 |
| | *Bacillus subtilis* subsp. subtilis 2KL1 | CP032872 | 0.0153 | 87.10% | 94.69% | 60.47% | 0.43 | 98.48 | 2,636,663 |
| | *Bacillus subtilis* SRCM103886 | CP035162 | 0.0153 | 87.10% | 94.68% | 60.45% | 0.48 | 98.49 | 2,651,166 |
| | *Bacillus subtilis* subsp. subtilis 2RL2-3 | CP032857 | 0.0153 | 87.10% | 94.68% | 60.44% | 0.43 | 98.50 | 2,597,779 |
| | *Bacillus subtilis* SRCM103576 | CP035402 | 0.0155 | 87.00% | 94.64% | 60.28% | 0.29 | 98.51 | 2,578,601 |
| | *Bacillus subtilis* subsp. subtilis N4-2 | CP032867 | 0.0155 | 86.90% | 94.62% | 60.21% | 0.31 | 98.52 | 2,684,907 |
| | *Bacillus* sp. M4U3P1 | CP041372 | 0.0155 | 86.90% | 94.62% | 60.21% | 0.32 | 98.51 | 2,595,536 |
| | *Bacillus subtilis* subsp. subtilis N3-1 | CP032865 | 0.0155 | 86.90% | 94.62% | 60.20% | 0.31 | 98.54 | 2,616,955 |
| | *Bacillus subtilis* subsp. subtilis N2-2 | CP032863 | 0.0155 | 86.90% | 94.62% | 60.20% | 0.31 | 98.48 | 2,667,845 |
| | *Bacillus subtilis* subsp. subtilis N1-1 | CP032861 | 0.0155 | 86.90% | 94.62% | 60.19% | 0.29 | 98.56 | 2,473,375 |
| Non type-strains | *Bacillus subtilis* ATCC 21228 | CP020023 | 0.0156 | 86.90% | 94.60% | 60.12% | 0.33 | 98.53 | 2,668,973 |
| | *Bacillus subtilis* subsp. subtilis MH-1 | CP032853 | 0.0158 | 86.60% | 98.80% | 77.62% | 0.51 | 98.44 | 2,611,384 |
| | *Bacillus subtilis* SX01705 | CP022287 | 0.0175 | 85.10% | 93.79% | 57.14% | 0.08 | 98.31 | 2,686,386 |
| | *Bacillus subtilis* subsp. subtilis RO-NN-1 | CP002906 | 0.0203 | 82.60% | 92.41% | 52.68% | 0.09 | 98.04 | 2,857,714 |
| | *Bacillus velezensis* strain ATR2 | NZ_CP018133 | 0.2144 | 20.50% | 0% | 0% | 2.69 | 77.1 | 1,854,058 |

subsp. *subtilis* RO-NN-1 with a distance of 0.203 and a DDH of 82.60%.

## Conclusion

With a genome size of 4,001,755 bp containing 97.69% of protein encoding genes, the draft genome of *B. subtilis* PTA-271 highlights all the qualities of a promising plant beneficial microorganism. The most relevant predicted genes encode for: (1) a functional swarming motility system highlighting advantageous colonizing capacity of host and a strong interacting capacity within plant microbiota; (2) a strong survival capacity, due to sporulation but also to complex detoxifying systems, auto-inducing metabolic paths and recruiting capacities for adding microbiota values; and (3) the delivery of many bioactive substances (hormones, elicitors, effectors and quenchers, siderophores and lytic enzymes, etc.), facilitating the stimulation of plant growth or defenses, or else, disturbing pathogen fitness or aggressiveness. Interestingly, the putative capacity of *B. subtilis* PTA-271 to produce a wide range of phytohormone analogous (SA, ET precursor, ABA etc.), as well as diverse direct effectors and lytic enzymes against plant pathogens, highlight a significant potential for biocontrol strategies. Altogether, the plurality of the biomolecules putatively encoded by the genome of *B. subtilis* PTA-271 PTA-271 are putative strengths to impact both biochemical conditions, species ratios and their interactions, predicting an ability to combat a broad spectrum of plant pathogens such as grapevine trunk disease [3].

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-021-00372-3.

**Additional file 1: Table S1.** *Bacillus subtilis* PTA-271 encoding genes for motility, adhesion and plant root colonizing capacity.

**Additional file 2: Table S2.** *Bacillus subtilis* PTA-271 encoding genes for some Transcriptional regulators and Operons.

**Additional file 3: Table S3.** *Bacillus subtilis* PTA-271 encoding genes for antimicrobial molecules, other effectors and lytic enzymes.

**Additional file 4: Table S4.** *Bacillus subtilis* PTA-271 encoding genes for sporulation.

**Additional file 5: Table S5.** *Bacillus subtilis* PTA-271 encoding genes for some CYP450 and for Transferases.

**Additional file 6: Table S6.** *Bacillus subtilis* PTA-271 encoding genes for lactonases, β-lactamases, deaminases, deacetylases.

**Additional file 7: Table S7.** *Bacillus subtilis* PTA-271 encoding genes for PKS and other acetyltransferases.

**Additional file 8: Table S8.** Anti-SMASH 5.1.0 prediction of gene clusters responsible for secondary metabolite production in *Bacillus subtilis* PTA-271.

### Abbreviations

ABA: abscisic acid; DAMPs: damage-associated molecular patterns; EPS: exopolysaccharides; ET: ethylene; GTD: grapevine trunk diseases; ISR: induced systemic resistance; JA: jasmonic acid; LPS: lipopolysaccharides; MAMPs: microbial associated molecular patterns; NO: nitric oxide; NRP: non-ribosomally synthesized peptides; PA: polyamines; PK: polyketides; RP: ribosomally synthesized antimicrobial peptides; RiPP: post-translationally modified RP; ROS: reactive oxygen species; SA: salicylic acid; ViC: inorganic volatile compound; VOC: organic volatile compound

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### Authors' contributions

All authors contributed to writing and revising the manuscript. The author(s) read and approved the final manuscript.

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### Availability of data and materials

The whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JACERQ000000000. The version described in this paper is version JACERQ000000000 and all related information is represented in Table 3.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All authors approved the final version and consent for publication.

### Competing interests

The authors declare that they have no competing interests.

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***Chapter III***

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# Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* BT-67 (Phenotypic effects)

### 3.1. Contexte

Les changements environnementaux mondiaux (modifications climatiques, pollutions, etc.) favorisent l'incidence des maladies des plantes en augmentant la pression des agents pathogènes et/ou leur agressivité, ou en rendant les plantes plus sensibles à ces derniers (O'Brien 2017 ; Velásquez et al. 2018). Les MDB font partie des maladies les plus dommageables pour la vigne dans le monde entier et préoccupent tous les pays viticoles (Mondello et al. 2018). Attaquant les parties pérennes de la vigne pour conduire à la mort inexorable du cep à court ou à long terme, les agents pathogènes responsables des MDB sont très préjudiciables pour la durabilité de l'industrie viticole (Hofstetter et al. 2012). Les MDB affectent aussi bien les jeunes vignes dès la pépinière, que les vignes adultes implantées au vignoble (Gramaje et Armengol 2011), occasionnant à la fois une baisse de la productivité et de la longévité du vignoble, avec des pertes économiques massives (O'Brien 2017).

Le dépérissement dû aux Botryosphaeriacées (BOT) est l'une des MDB les plus importantes (se manifestant en pépinière et au vignoble), impliquant plus de 25 espèces de Botryosphaeriaceae, y compris *Neofusicoccum parvum* défini comme BOT très agressif (Billones-Baaijens et Savocchia 2019 ; Larach et al. 2020 ; Reis et al. 2019 ; Úrbez-Torres 2011). Lorsque les symptômes sont visibles, les vignes symptomatiques ont un phénotype de dépérissement chronique (forme lente de développement de la maladie) ou sévère (forme dite apoplectique). Ce dépérissement est caractérisé par un faible taux de débourrement des bourgeons, un faible développement végétatif, un chancre externe au niveau du tronc associé à des lésions nécrotiques longitudinales internes pouvant conduire à un bras mort (Billones-Baaijens et Savocchia 2019 ; Larach et al. 2020 ; Larignon et al. 2001, 2009 ; Larignon 2004). La sensibilité de la vigne aux pathogènes BOT est également décrite pour différer selon les cépages (Chacon et al. 2020 ; Claverie et al. 2020 ; Fontaine et al. 2016a ; Travadon et al. 2013 ; Leal et al. 2021b).

En raison de la durée de latence indéterminée avant que les pathogènes responsables des MDB n'induisent des symptômes (état asymptomatique), la détection précoce et la gestion des pathogènes responsables de ces MDB restent actuellement un défi, tant en pépinière qu'au vignoble. Des méthodes de lutte préventives sont toutefois disponibles, comparativement aux méthodes curatives qui sont quasi-inexistantes (mis à part le curetage, Cholet et al. 2020). En effet, peu de produits chimiques sont appliqués sur les plaies de taille dans les vignobles pour empêcher la contamination par les champignons pathogènes

responsables des MDB (Sosnowski et al. 2004 ; Gramaje et Armengol 2011 ; Gramaje et al. 2018). De plus, ces traitements ne peuvent pas éradiquer les pathogènes lorsqu'ils sont établis dans le cep au vignoble (Calzarano et al. 2004 ; Gramaje et Armengol 2011). En alternative ou en complément aux méthodes de lutte pré-citées appliquées au vignoble, l'utilisation des agents de biocontrôle (BCA) apparaît prometteuse (Mondello et al. 2018). A ce jour, le potentiel de plusieurs BCA a déjà été évalué, à la fois *in vitro* et *in planta* contre les pathogènes du BOT (Compant et Mathieu 2017 ; Compant et al. 2013 ; Di Marco et al. 2004 ; Hunt et al. 2001 ; John et al. 2004 ; Trotel-Aziz et al. 2019 ; Leal et al. 2021b). Parmi ces derniers, nous pouvons citer des bactéries appartenant aux genres *Pseudomonas*, *Bacillus* et *Enterobacter*, et des champignons appartenant aux genres *Fusarium* et *Trichoderma* (Mondello et al. 2018). Certains d'entre eux, comme *Trichoderma atroviride*, *Trichoderma harzianum*, *Bacillus subtilis* et *Bacillus amyloliquefaciens*, sont mêmes déjà commercialisés contre certains agents pathogènes des MDB, ou contre d'autres pathogènes hémibiotrophes et nécrotrophes, notamment contre *Botrytis cinerea*, *Fusarium oxysporum*, ou encore bien d'autres (Alfiky et Weisskopf 2021 ; Elad 2000 ; Kuzmanovska et al. 2018 ; Schmidt et Panstruga 2011 ; Thambugala et al. 2020).

À ce jour, les produits de biocontrôle à base de *Trichoderma* sont les plus utilisés en viticulture (Harman 2006 ; Muckherjee et al. 2012 ; Waghunde et al. 2016), notamment pour lutter contre les MDB (Berbegal et al. 2020 ; Di Marco et al. 2002, 2004 ; Halleen et al. 2010 ; John et al. 2008 ; Martínez-Diz et al. 2021a,b). Les espèces du genre *Trichoderma* sont déjà décrits pour leur antagonisme direct contre les pathogènes responsables des MDB, par compétition pour les nutriments et l'espace, par mycoparasitisme, par production d'enzymes de dégradation de la paroi cellulaire (CWDEs) des champignons phytopathogènes, et par antibiose ou sécrétion de molécules à effet antifongique direct (Harman 2006 ; Pieterse et al. 2014 ; Van Wees et al. 2008 ; Vinale et al. 2008 ; Waghunde et al. 2016). Les *Trichoderma* spp. sont également décrits pour leur capacité à stimuler la croissance et les défenses des plantes (Harman 2006 ; Pieterse et al. 2014 ; Van Wees et al. 2008 ; Vinale et al. 2008 ; Waghunde et al. 2016). Parmi les espèces de *Trichoderma* utilisées en biocontrôle, la souche *Ta* SC1 réduit fortement les infections causées par certains pathogènes des MDB, en pépinière et au vignoble, à la dose prescrite de 2 g/L, équivalente à la densité de $2 \times 10^{10}$ conidies/L recommandée par le produit commercial (Berbegal et al. 2020 ; Martínez-Diz et al. 2021a ; Pertot et al. 2016).

Les souches bactériennes appartenant au genre *Bacillus* font aussi partie des micro-organismes largement étudiés comme BCA, et leurs effets protecteurs directs et indirects sur les plantes ont été rapportés contre des agents pathogènes aux modes de vie différents (Magnin-Robert et al. 2007, 2013 ; Nguyen et al. 2020 ; Trotel-Aziz et al. 2008), y compris les agents pathogènes hémibiotrophes des MDB (Halleen et al. 2010 ; Kotze et al. 2019 ; Rezgui et al. 2016 ; Schmidt et al. 2001 ; Trotel-Aziz et al. 2019 ; Leal et al. 2021b). Le génome des espèces de *Bacillus* spp. code notamment pour une large gamme de molécules bénéfiques, capables non seulement d'induire les défenses des plantes (via des précurseurs de phytohormones, des lipopolysaccharides, des sidérophores, etc.), mais aussi de concurrencer, inhiber ou modifier directement la croissance des pathogènes ou leur capacité à produire des molécules agressives (Kloepper et al. 2004 ; Leal et al. 2021 ; Ongena et Jacques 2008). Parmi les espèces du genre *Bacillus*, *B. subtilis* est l'un des plus fréquemment testé contre les MDB (Mondello et al. 2018). La souche *B. subtilis* PTA-271 a ainsi montré des résultats prometteurs dans la réduction des infections causées par la souche pathogène très agressive *N. parvum* Np-Bt67 (Trotel-Aziz et al. 2019 ; Leal et al 2021b). La littérature indique également que la combinaison de deux ou plusieurs BCA peut encore renforcer le contrôle des maladies des plantes (El-Tarabily 2006 ; Guetsky et al. 2002 ; Magnin-Robert et al. 2013 ; Weller 1988 ; Yobo et al. 2011 ; Leal et al. 2021b), potentiellement en raison d'effets agonistes, sinon synergiques, résultants de mécanismes protecteurs complémentaires ou combinés dans un environnement complexe (Meyer et Roberts 2002).

Les interactions microbiennes bénéfiques conférées par le BCA à la plante peuvent conduire à la résistance systémique induite (ISR) de l'hôte, lui assurant potentiellement une protection contre un plus large spectre d'agents pathogènes (Alström 1991 ; De Vleesschauwer et Höfte 2009 ; Van Peer et al. 1991 ; Wei et al. 1991). L'ISR est associée à une pré-activation précoce (état de veille), suivie de réponses de défense fortes et rapides dès l'infection de la plante par un pathogène ; on parle alors de potentialisation ou de priming (Conrath et al. 2001, 2015 ; Pieterse et al. 2014). Parmi les réponses de défense induites par un BCA, celles dépendantes du jasmonate (JA) et de l'éthylène (ET) sont décrites pour être activées et utiles contre les pathogènes nécrotrophes (Nie et al. 2017 ; Niu et al. 2011 ; Pieterse et al. 2001, 2014 ; Verhagen et al. 2004 ; Van der Ent et al. 2009). L'ISR peut également être activée par la voie de l'acide salicylique (SA), notamment pour induire des défenses utiles pour lutter contre les pathogènes biotrophes (Niu et al. 2011 ; Yang et al. 2015). En résumé, la diversité des modes de protection des BCA peut dépendre à la fois du BCA et du pathogène, mais aussi de la plante

et même du cépage (Mutawila et al. 2011 ; Nguyen et al. 2020 ; Pacifico et al. 2019 ; Stempien et al. 2020).

Dans cette étude, les effets combinés d'un BCA potentiel (*Bs* PTA-271) et d'un produit commercial à base du BCA *Trichoderma atroviride* (*Ta* SC1) ont été évalués sur la protection des cépages Chardonnay et Tempranillo, connus pour exprimer des sensibilités distinctes aux MDB (Figures 5 et 7). Le pathogène sélectionné est Np-Bt67, décrit pour être un membre très agressif de la famille des BOT. Chaque BCA individuel ayant déjà montré un potentiel bénéfique pour l'un de ces 2 cépages (Berbegal et al. 2020 ; Martínez- Diz et al. 2021a ; Trotel-Aziz et al. 2019), leur effet bénéfique conjoint restait à appréhender sur chaque cépage, en conditions similaires et contrôlées, comparé aux effets individuels de chaque BCA sous conditions identiques. Pour comparer les activités des deux BCA, les densités d'application ont été déterminées sur la base de la densité optimisée pour *Bs* PTA-271 sur boutures de Chardonnay. Le mode d'action de chaque BCA (*Bs* PTA- 271, *Ta* SC1) pour conduire à la protection de la vigne contre Np-Bt67 a ensuite été étudié plus en détails, en termes de bénéfices indirects et directs. Les effets de chaque BCA, seuls ou combinés, ont donc été étudiés à la fois sur les défenses de la vigne *in-planta* et sur la croissance réciproque du pathogène et des BCA *in-vitro*.

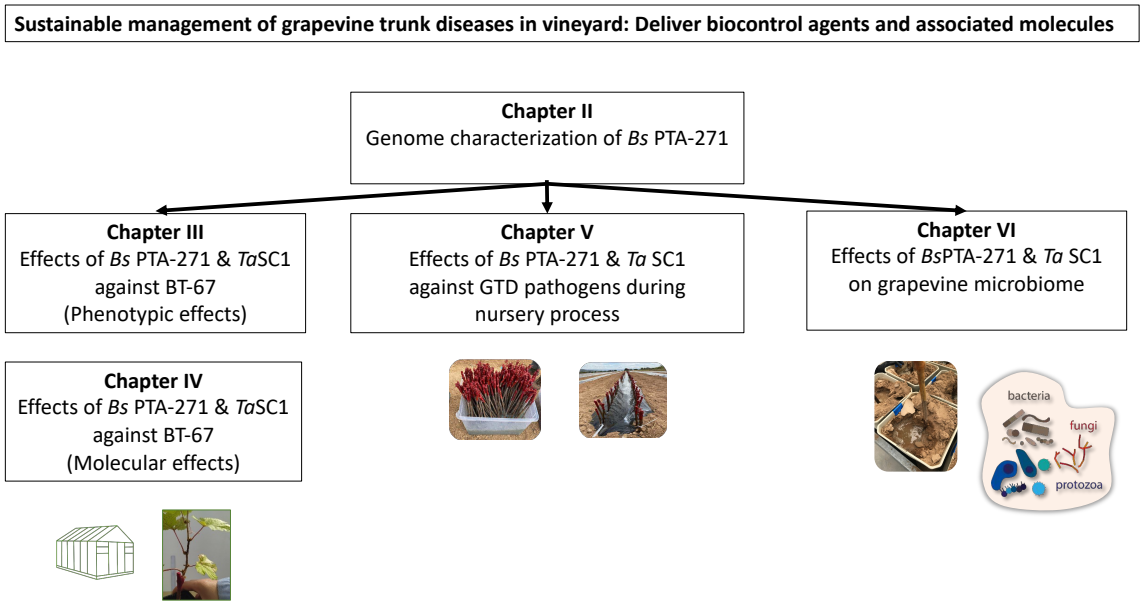


**Figure 5.** Aims of each chapter of the thesis entitled "Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules".

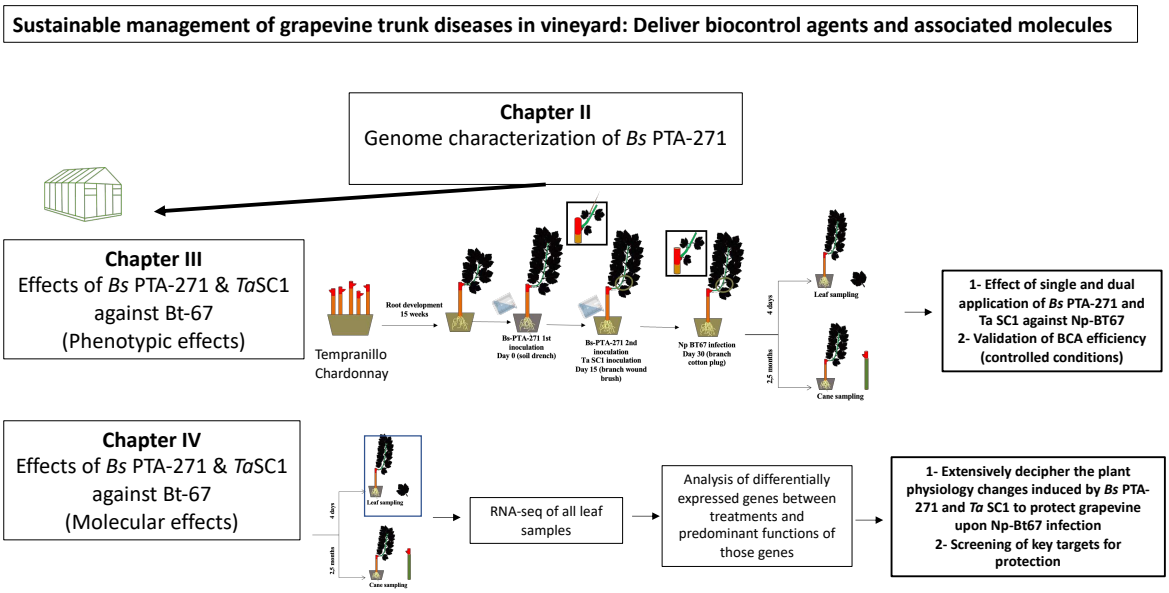


**Figure 7.** Aims chapter II and IV entitled "Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* BT-67 (Phenotypic effects)" and "Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* BT-67 (Molecular effects)", respectively.

**Publication 3- Cultivar Contributes to the Beneficial Effects of *Bacillus subtilis PTA-271* and *Trichoderma atroviride* SC1 to Protect Grapevine Against *Neofusicoccum parvum***

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# Cultivar Contributes to the Beneficial Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to Protect Grapevine Against *Neofusicoccum parvum*

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Grapevine trunk diseases (GTDs) are a big threat for global viticulture. Without effective chemicals, biocontrol strategies are developed as alternatives to better cope with environmental concerns. A combination of biological control agents (BCAs) could even improve sustainable disease management through complementary ways of protection. In this study, we evaluated the combination of *Bacillus subtilis* (*Bs*) PTA-271 and *Trichoderma atroviride* (*Ta*) SC1 for the protection of Chardonnay and Tempranillo rootlings against *Neofusicoccum parvum* Bt67, an aggressive pathogen associated to Botryosphaeria dieback (BD). Indirect benefits offered by each BCA and their combination were then characterized *in planta*, as well as their direct benefits *in vitro*. Results provide evidence that (1) the cultivar contributes to the beneficial effects of *Bs* PTA-271 and *Ta* SC1 against *N. parvum*, and that (2) the *in vitro* BCA mutual antagonism switches to the strongest fungistatic effect toward *Np*-Bt67 in a three-way confrontation test. We also report for the first time the beneficial potential of a combination of BCA against *Np*-Bt67 especially in Tempranillo. Our findings highlight a common feature for both cultivars: salicylic acid (SA)-dependent defenses were strongly decreased in plants protected by the BCA, in contrast with symptomatic ones. We thus suggest that (1) the high basal expression of SA-dependent defenses in Tempranillo explains its highest susceptibility to *N. parvum*, and that (2) the cultivar-specific responses to the beneficial *Bs* PTA-271 and *Ta* SC1 remain to be further investigated.

**Keywords: Chardonnay, Tempranillo, biocontrol, *Neofusicoccum parvum*, plant immunity, synergistic effect**

## INTRODUCTION

Global environmental changes promote the incidence of plant diseases by increasing the pathogen pressure or make the plants more susceptible to them (O'Brien, 2017; Velásquez et al., 2018). Grapevine trunk diseases (GTDs) are among the most important groups of grapevine diseases all over the world, creating a big concern in all wine-producing countries (Mondello et al., 2018).

Attacking the plant perennial part and leading inevitably to the short- or long-term death of vines, the pathogens responsible of GTDs are described as very injurious for the sustainability of the winemaking industry (Hofstetter et al., 2012). As main restrictors for viticulture, GTDs can lead to high economic losses, less table grape for consumers, and social and environmental disturbances (O'Brien, 2017). Both young and mature vines are affected by GTDs, even as nursery staged plants, reducing both productivity and longevity of the vineyard, thereby causing massive economic losses (Gramaje and Armengol, 2011).

Botryosphaeria dieback (BD) is one of the most significant GTDs, triggerable by more than 25 distinct species of Botryosphaeriaceae including the aggressive *Neofusicoccum parvum* (Úrbez-Torres, 2011; Billones-Baaijens and Savocchia, 2019; Reis et al., 2019; Larach et al., 2020). Symptomatic plants develop a low or apoplectic dieback phenotype, including a low budburst rate, a poor vegetative development, external canker, and internal longitudinal necrotic lesions that can lead to a full dead branch (Larignon et al., 2001, 2009; Larignon, 2004; Billones-Baaijens and Savocchia, 2019; Larach et al., 2020). Susceptibility to BD pathogens also differs between cultivars (Travadon et al., 2013; Fontaine et al., 2016a; Chacon et al., 2020; Claverie et al., 2020).

Due to the undetermined period of latency within the vines (asymptomatic state), early detection and management of GTDs remain presently a challenge in both nursery and vineyard, and only few preventives, but no curative methods, are available. Indeed, few chemicals are applied on pruning wounds in vineyards to prevent dissemination of the conidia of fungal pathogens (Sosnowski et al., 2004), preferring cultural practices in vineyard (Mondello et al., 2018) and sanitation methods (Gramaje and Armengol, 2011; Gramaje et al., 2018). However, these kinds of treatments cannot eradicate the pathogens once established in a vineyard (Calzarano et al., 2004; Gramaje and Armengol, 2011). An interesting alternative and complement to the previously cited GTDs control methods in vineyard is the use of biological control agents (BCAs) as reported by Mondello et al. (2018). For instance, several microorganisms have already been evaluated, both *in vitro* and *in planta* against BD pathogens (Hunt et al., 2001; Di Marco et al., 2004; John et al., 2004; Compant et al., 2013; Compant and Mathieu, 2017; Trotel-Aziz et al., 2019). Among them there are bacterial BCAs such as *Pseudomonas*, *Bacillus*, and *Enterobacter* species, and fungal BCAs such as several *Fusarium* and *Trichoderma* species (Mondello et al., 2018). Some of them, such as *Trichoderma atroviride*, *Trichoderma harzianum*, *Bacillus subtilis*, and *Bacillus amyloliquefaciens*, are already commercialized against some GTDs pathogens, or against other hemibiotrophic and necrotrophic pathogens including *Botrytis cinerea*, *Fusarium oxysporum*, or many others (Elad, 2000; Schmidt and Panstruga, 2011; Kuzmanovska et al., 2018; Thambugala et al., 2020; Alfiky and Weisskopf, 2021).

To date, *Trichoderma* species are the most used fungal-based BCA in viticulture (Harman, 2006; Muckherjee et al., 2012; Waghunde et al., 2016) and have been also widely investigated as BCA against GTDs (Di Marco et al., 2002, 2004; John et al., 2008; Halleen et al., 2010: Berbegal et al., 2020; Martínez-Diz et al., 2021a,b). *Trichoderma* spp. are described to directly antagonize GTD pathogen aggressiveness by competition for nutrients and space, mycoparasitism, cell-wall degrading enzymes, and antibiosis (Harman, 2006; Van Wees et al., 2008; Vinale et al., 2008; Pieterse et al., 2014; Waghunde et al., 2016). *Trichoderma* spp. have also been described as plant growth and defense stimulators (Harman, 2006; Van Wees et al., 2008; Vinale et al., 2008; Pieterse et al., 2014; Waghunde et al., 2016). Among *Trichoderma* spp., *T. atroviride* SC1 was shown to strongly reduce the infections caused by some GTD pathogens in nurseries and established vineyards at the registered dose rate of 2 g/L, equivalent to the density of $2 \times 10^{10}$ conidia/L recommended by the commercial product (Pertot et al., 2016; Berbegal et al., 2020; Martínez-Diz et al., 2021a). *Bacillus* strains are another group of microorganisms extensively studied as BCA and reported to directly and indirectly protect plants against pathogens with different lifestyles (Magnin-Robert et al., 2007; Trotel-Aziz et al., 2008; Nguyen et al., 2020), including the GTD hemibiotrophic pathogens (Schmidt et al., 2001; Halleen et al., 2010; Rezgui et al., 2016; Kotze et al., 2019; Trotel-Aziz et al., 2019). A broad range of beneficial molecules are produced or encoded by the genome of *Bacillus* spp., both to induce or elicit plant defenses (as with phytohormones precursors, lipopolysaccharides, siderophores, etc.) and to directly compete, antagonize, or alter plant pathogens or their aggressiveness (Kloepper et al., 2004; Ongena and Jacques, 2008; Leal et al., 2021). Among *Bacillus* spp., *B. subtilis* is one of the most frequently tested against GTDs (Mondello et al., 2018), and *B. subtilis* PTA-271 has shown promising results in reducing infections caused by the aggressive strain *N. parvum* Bt67 (Trotel-Aziz et al., 2019). Literature also reports that the combination of two or more BCAs can improve the management of plant diseases (Weller, 1988; Guetsky et al., 2002; El-Tarabily, 2006; Yobo et al., 2011; Magnin-Robert et al., 2013), probably due to additive or synergistic effects of combined mechanisms in a complex changing environment (Meyer and Roberts, 2002).

Beneficial microbial interactions conferred by BCA lead to induced systemic resistance (ISR) in the plant, giving it greater protection to pathogens in spatially separated parts of the plant (Alström, 1991; Van Peer et al., 1991; Wei et al., 1991; De Vleesschauwer and Höfte, 2009). ISR is associated with an early, strong, and rapid activation of plant defenses upon pathogen infection, a phenomenon known as the priming state (Conrath et al., 2001, 2015; Pieterse et al., 2014). Among the BCA-induced defense responses, the most relevant are jasmonate (JA)- and ethylene (ET)-dependent ones, described as useful defenses against necrotrophs (Pieterse et al., 2001, 2014; Verhagen et al., 2004; Van der Ent et al., 2009; Niu et al., 2011; Nie et al., 2017). However, Niu et al. (2011) also reported that some BCAs may mediate ISR in a salicylic acid (SA)-dependent manner. In brief, the diversity of BCA ways of protection may depend on both the BCA and the pathogen, but also on the plant and even the cultivar (Mutawila et al., 2011; Pacifico et al., 2019; Nguyen et al., 2020; Stempien et al., 2020).

In this study, we evaluated the effect of combining a potential BCA, *B. subtilis* PTA-271 (thereafter *Bs* PTA-271), and a BCA-commercial product containing *T. atroviride* SC1 (thereafter *Ta* SC1), on the protection of two distinct grapevine

cultivars, Chardonnay and Tempranillo, potentially showing distinct susceptibilities to GTDs. The pathogen selected was *N. parvum* Bt67 (thereafter *Np*-Bt67), described as a very aggressive pathogen associated to BD. As each BCA has already been recognized as beneficial to at least one cultivar (Trotel-Aziz et al., 2019; Berbegal et al., 2020; Martínez-Diz et al., 2021a), their beneficial effect was additionally investigated on the other cultivar, as single BCA and in dual combination of two BCAs. To compare the two BCAs, densities were aligned to the density optimized for *Bs* PTA-271 with Chardonnay rootlings. After looking for the protective capacity of BCAs *in planta*, their ways of action leading to protection were further explored. Thus, the indirect and direct benefits offered by each BCA and their combination were investigated, focusing on both grapevine immunity and the direct beneficial or detrimental physical interplays among the microorganisms *in vitro* (*Bs* PTA-271, *Ta* SC1, and *Np*-Bt67).

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Three-node-long cuttings of grapevine *Vitis vinifera* L. cv. Tempranillo (clone RJ-26) and cv Chardonnay (clone 7535) were provided by Viveros Villanueva nursery (Navarra, Spain) and Pommery's vineyards in Reims (France), respectively. Tempranillo cuttings were surface-sterilized for 6 h in a 0.05% cryptonol (8-hydroxyquinoline sulfate) solution, waxed and stored at 4°C in a cold chamber for 3 weeks, and then rehydrated with 0.05% cryptonol solution overnight. Chardonnay cuttings were directly surface-sterilized with 0.05% cryptonol solution overnight. Cuttings of both cv were then rooted as described by Lebon et al. (2005), using an indole-3-butyric acid (1 g/L) solution before being placed by 15 in 350-ml pots containing the soil Sorexto (horticultural soil M4600, Grenoble, France) in a culture chamber (24/20°C day/night, 55–65% relative humidity day/night, and 16-h photoperiod at 400 μmol/$m^2$/s). They were watered three times a week. Only rootlings that have developed roots (30% rooting rate in 15 weeks) were kept for further experiments and transferred to individual 200-ml pots with the same culture conditions.

### Biocontrol Agent's Growth and Plant Treatments

#### *Bacillus subtilis* PTA-271

*Bacillus subtilis* PTA-271 (GenBank Nucleotide EMBL Accession No. AM293677 for 16S rRNA and DDBJ/ENA/GenBank Accession No. JACERQ000000000 for the whole genome) was isolated in 2001 from the rhizosphere of healthy Chardonnay grapevines (*V. vinifera* L. cv. Chardonnay) from a vineyard located in Champagne (Marne, France) (Trotel-Aziz et al., 2008; Leal et al., 2021). Bacterial growth started by adding 100 μl of glycerol stock suspension to sterile Luria Bertani (LB) medium and incubating at 28°C with agitation (100 rpm). Experiments were performed when the bacterial culture is at the exponential growth phase. After centrifugation (5,000 *g*, 10 min), the pellet was washed once with a sterile 10 mM $MgSO_4$ medium and resuspended in a same $MgSO_4$ medium. Bacterial density was measured by spectrophotometry at 450 and 650 nm, and the mean density was adjusted with a sterile $MgSO_4$ medium before treatment according to Trotel-Aziz et al. (2019). The bacterial suspension was applied twice by drenching the soil at the root level of rootlings at a final density of $10^8$ CFU/g soil. Inoculations were carried out when rootlings were 16 weeks old (considered as day 0) and 2 weeks later (day 15) as indicated in **Figure 1**. Control rootlings were similarly drenched twice with $MgSO_4$ solution (**Figure 1**).

#### *Trichoderma atroviride* SC1

*Trichoderma atroviride* SC1 (Vintec®, Belchim Crop Protection, Bi-PA; $10^{10}$ conidia per gram of formulated product) was suspended in water at $10^8$ CFU/ml to compare the effect of each BCA at an equal density. In order to also take advantage of an eliciting effect, the *Ta* SC1 fungal suspension was applied once with a paintbrush to the second node of the previously wounded lignifying stem (5 mm diameter and 1 mm deep; made just before *Ta* SC1 apply) of 18-week-old rootlings (**Figure 1**). The inoculation site was immediately covered with parafilm (day 15).

### Pathogen Strain and Growth

*Neofusicoccum parvum* strain Bt67 isolated from Portuguese vineyards (Estremadura region) is inscribed in the HIA collection (Lisbon University, Portugal). This fungus was maintained on potato dextrose agar (PDA, Sigma, Saint-Quentin-Fallavier, France) plates and stored at 4°C (Trotel-Aziz et al., 2019). The resulting mycelium was plated on PDA medium and incubated in the dark at 22°C for 7 days before inoculation.

### Pathogen Inoculation to Plants, Quantification of Disease Symptoms, and Re-isolation of the Pathogen

Half of the 20-week-old rootlings that were treated with *Bs* PTA-271 (day 0 and day 15) and/or *Ta* SC1 (day 15) were further infected with the pathogen at the wounding area with a 3-mm-diameter mycelial plug from a 7-day-old culture of *Np*-Bt67 strain (day 30), thus at distinct time points (days 0–30) as summarized in **Figure 1**. The inoculation site was then covered with moist hydrophilic cotton before sealing with parafilm. The experiment was composed of five repetitions for each modality (treatment), with 8–10 replicate plants per treatment (**Figure 1**). To confirm that lesions were due to pathogen infection and not to the injury, the control plants were also injured and inoculated with sterile 3-mm PDA plugs (**Figure 1**). Rootlings, namely, "control," are those that are neither treated with BCA nor infected with the pathogen. After inoculation, vines were kept in the same culture chamber and BD symptoms were assessed at 2.5 months post-inoculation (mpi) (**Figure 1**). Disease symptoms were evaluated as described by Trotel-Aziz et al. (2019) by quantifying the percentage of the full dead shoots from inoculated rootlings (**Figure 2A**, "Full dieback") and by measuring both the external canker area and the internal necrosis length of the other lignified shoots (**Figures 2B,C**, respectively). To check the success of the infection and the lack of contaminations, re-isolation of pathogen

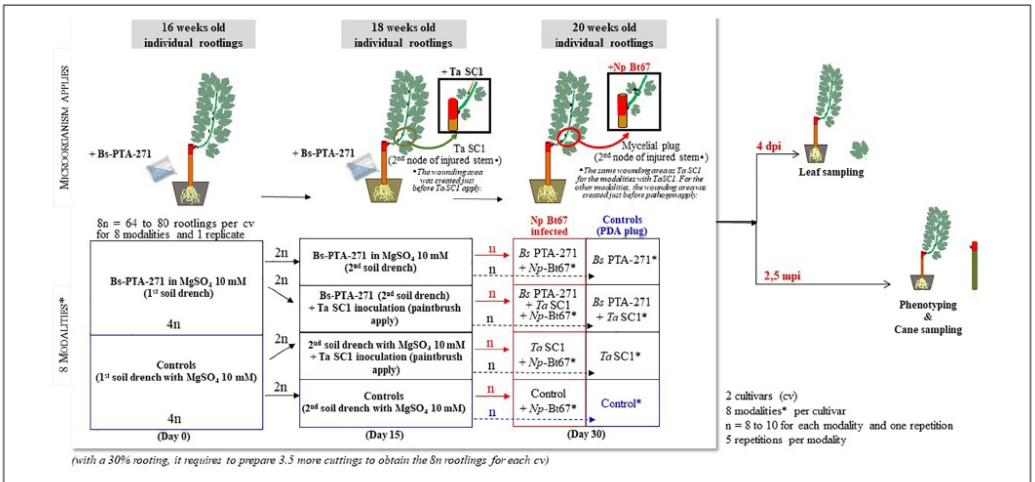


**FIGURE 1 |** Diagram showing the *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 inoculations, *Neofusicoccum parvum* strain Np-Bt67 infection, and sample collection process. *Modalities: Control, Bs PTA-271, Ta SC1, Bs PTA-271 + Ta SC1, Control + Np Bt67, Bs PTA-271 + Np Bt67, Ta SC1 + Np Bt67, Bs PTA-271 + Ta SC1 + Np Bt67.

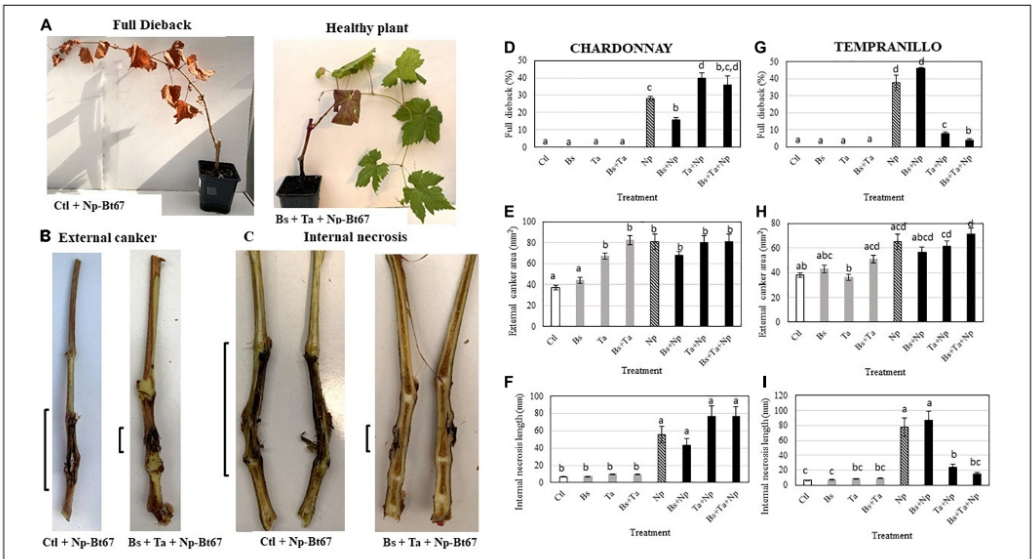


**FIGURE 2 |** The beneficial combination of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 attenuates the Botryosphaeriaceae dieback symptoms induced in Chardonnay **(D–F)** and Tempranillo **(G–I)** rootlings by the *Neofusicoccum parvum* strain Np-Bt67. One-month pretreated grapevine rootlings with PTA-271, *Ta* SC1, and both BCA (Bs, Ta, and Bs + Ta) and non-treat plants (Ctl) were inoculated with pathogen mycelium (Np). Non-infected plants were inoculated with sterile medium without pathogen (Control). Compared to healthy asymptomatic rootlings (**A**, right), the infected symptomatic rootlings showed the typical Botryosphaeria dieback symptoms: full dieback percentage (**A** left, **E**), stem external canker **(B,E,H)** and stem internal necrosis **(C,F,I)** that were photographed **(A–C)** and quantified **(D–I)** at 2.5 months post-inoculation. Data are means ± standard deviation (SD) for at least three independent experiments with 10 biological replicates per treatment. Vertical bars with different letters are significantly different (multiple comparison procedures with Tukey's test, $p < 0.05$).

was performed as described by Pinto et al. (2018), by quickly passing the infected stems onto the flame, then removing the top of the necrotic zone with a scalpel, before plating seven small pieces of tissue per plant onto PDA plates. Plates were then incubated at 28°C for at least 7 days. For every repetition of the experiments, re-isolations were performed with seven of the infected rootlings per infected combination (i.e., Np, Bs + Np, Ta + Np, and Bs + Ta + Np) and each replicate, and one negative control rootling per non-infected combination (i.e., Control, Bs, Ta, and Bs + Ta).

## Direct Confrontation Tests With Biocontrol Agents and Pathogen

Antagonism among microorganisms was checked *in vitro*, using *B. subtilis* PTA-271, *T. atroviride* SC1, and *N. parvum Np*-Bt67, in dual or three-way confrontation tests on PDA plates (9 mm diameter). *Bs* PTA-271 grown in LB medium and *Ta* SC1 resuspended in sterilized water were both used at $10^8$ CFU/ml (5 µl drop), while a 7-day-old mycelium plug (3 mm) was used for the pathogen. Three types of direct confrontation were performed: (A) the pathogen/BCA combinations were plated at the same time, but at distinct areas (i.e., 5 cm away from each other); (B) one or two isolates (*Bs* PTA-271, *Ta* SC1, or *Np*-Bt67) were plated 48 h before the other(s), and at distinct areas; and (C) isolates were plated simultaneously and at the center of the plate. Controls containing one single isolate (*Bs* PTA-271, *Ta* SC1, or *Np*-Bt67) were also made, either on the side of the plate or at the center. All plates were incubated in the dark at 28°C for at least 11 days and photographed daily. Antagonistic effect was characterized by an inhibition zone around the BCA and/or the pathogen. Since the first kind of confrontation (A) added no more information compared to the others (B and C), it was not shown in this study. The experiment was conducted twice with three replicate plates per treatment, and the area occupied by each microorganism was measured daily using ImageJ software (Rueden et al., 2017), based on a reference distance common to all images.

## RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis

From the *in planta* assays with rootlings, leaf samples were collected 4 days post-inoculation of pathogen (dpi), ground in liquid nitrogen, and then stored at −80°C. RNA was extracted from powdered 40 leaves of 8 rootlings per replicate of each modality. Total RNA was extracted from 50 mg of leaf powder with Plant RNA Purification Reagent according to manufacturer instructions (Invitrogen, Pontoise, France), and DNase treated as described by the manufacturer's instructions (RQ1 RNase-Free DNase, Promega). RNA quality was checked by agarose gel electrophoresis, and total RNA concentration was measured at 260 nm for each sample using the NanoDrop One spectrophotometer (Ozyme) and adjusted to 100 ng $\mu l^{-1}$. First-strand cDNA was synthesized from 150 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, United States). Polymerase chain reaction (PCR) conditions were the ones described by Gruau et al. (2015). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed with Absolute Blue qPCR SYBR Green ROX Mix according to manufacturer instructions (Thermo Fisher Scientific, Inc., Waltham, MA, United States), in a BioRad C1000 thermocycler using the Bio-Rad manager software CFX96 Real-Time PCR (BioRad, Hercules, CA, United States). A set of six defense-related genes, selected for their responsiveness to pathogen or priming state induced by beneficial microorganisms (Trotel-Aziz et al., 2019), was tracked by qRT-PCR using specific primers (**Table 1**). Quantitative RT-PCR reactions were carried out in duplicate in 96-well plates in a 15-µl final volume containing Absolute Blue SYBR Green ROX mix including Taq polymerase ThermoPrime, dNTPs, buffer, and $MgCl_2$ (Thermo Fisher Scientific, Inc., Waltham, MA, United States), 280 nM forward and reverse primers, and 10-fold diluted cDNA according to the manufacturer's protocol. Cycling parameters were 15 min of Taq polymerase activation at 95°C, followed by 40 two-step cycles composed of 10 s of denaturation at 95°C and 45 s of annealing and elongation at 60°C. Melting curve assays were performed from 65 to 95°C at 0.5°C $s^{-1}$, and melting peaks were visualized to check amplification specificity. EF1 and 60SRP genes were used as references and experiments were repeated five times. Relative gene expression was determined with the formula fold induction: 2(−11Ct), where 11Ct = [Ct TG (US) − Ct RG (US)] − [Ct TG (RS) − Ct RG (RS)], where Ct is cycle threshold, Ct value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is target gene, RG is reference gene, the US is an unknown sample, and RS is reference sample. Integration of the formula was performed by the CFX Manager 3.1 software (Bio-Rad). Although the genes analyzed were considered significantly up- or downregulated when changes in their expression were >2-fold or <0.5-fold, respectively, we still performed a statistical analysis. Control samples for the rootlings model are cDNA from leaves of rootlings untreated with BCA and inoculated with sterile PDA plugs (1× expression level).

## Statistical Analysis

Data of canker area and length of external and internal necrosis of the stems were obtained by the analysis of photos using ImageJ software (Rueden et al., 2017). Statistical analyses were carried out using all the vines of three replicates among five for each modality with RStudio software (Horton and Kleinman, 2015). For modality significance, mean values were compared by Tukey's test ($p < 0.05$). Results of confrontation tests are from one representative repetition out of two showing the same trends. Statistical analyses were carried out using the SigmaStat 3.5 software. For treatment effect, mean values were compared by Tukey's test ($p < 0.05$). Results of gene expression by qRT-PCR analysis correspond to means ± SEM deviation from three representative repetitions out of five showing the same trends. Statistical analyses were carried out using the XLSTAT 2021.1.1 5 software (Addinsoft, Paris, France). For treatment effect, mean values were analyzed using one-way analysis of variance (ANOVA). When differences in the means

**TABLE 1 |** Primer sequences used for qRT-PCR analysis of defense-related genes (Trotel-Aziz et al., 2019).

| Gene | Name | Accession number[1] | Forward primer (5′-3′) | Reverse primer (5′-3′) | Annealing temperature (°C) | Amplicon size (bp) | Efficiency of primers pairs (%) |
|---|---|---|---|---|---|---|---|
| *60RSP* | 60S ribosomal protein L18 | XM_002270599[1] | ATCTACCTCAAGCTCCTAGTC | CAATCTTGTCCTCCTTTCCT | 60 | 166 | 100.0 |
| *EF1* | Elongation factor 1-alpha | XM_002284888[1] | AACCAAAATATCCGGAGTAA AAGA | GAACTGGGTGCTTGATAGGC | 60 | 164 | 100.0 |
| *LOX9* | Lipoxygenase | NM_001281249[1] | CCCTTCTTGGCATCTCCCTTA | TGTTGTGTCCAGGGTCCATTC | 60 | 101 | 90.0 |
| *PR1* | Pathogenesis-related protein 1 | XM_002273752[1] | GGAGTCCATTAGCACTCCTTTG | CATAATTCTGGGCGTAGGCAG | 60 | 168 | 90.0 |
| *PR2* | Class I beta-1,3-glucanase | NM_0012809671 | TCAATGGCTGCAATGGTGC | CGGTCGATGTTGCGAGATTTA | 60 | 155 | 97.2 |
| *GST1* | Glutathione-*S*-transferase | NM_001281248[1] | TGCATGGAGGAGGAGTTCGT | CAAGGCTATATCCCCATTTTCTTC | 60 | 98 | 90.0 |
| *PAL* | Phenylalanine ammonia lyase | XM_003635637[1] | TCCTCCCGGAAAACAGCTG | TCCTCCAAATGCCTCAAATCA | 60 | 101 | 92.9 |
| *STS* | Stilbene synthase | NM_001281117[1] | AGGAAGCAGCATTGAAGGCTC | TGCACCAGGCATTTCTACACC | 60 | 101 | 94.3 |

[1]*NCBI accession number.*

were significant, Fisher's LSD *post hoc* test ($\alpha = 0.1$) was applied to determine which treatments were significantly different from others.

## RESULTS

### Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 on Two Cultivars Infected With *Neofusicoccum parvum* Bt67

In control Chardonnay infected with the pathogen, the results of infection showed a rate of 28 ± 1.24% for dead shoot (full dieback), of 80.6 ± 7.35 $mm^2$ for external canker size, and of 55.4 ± 9.44 mm for internal necrosis length (see *Np*-Bt67 in **Figures 2D–F**), while in Tempranillo, they reached 37.5 ± 4.56%, 65.2 ± 6.23 $mm^2$, and of 77.8 ± 11.95 mm, respectively (see *Np*-Bt67 in **Figures 2G–I**).

In BCA-treated Chardonnay rootlings then infected with the pathogen, the results of the biocontrol assays showed that *Bs*-PTA-271-pretreated plants presented a significant lower number of plants with full dieback (by approximately 45%) than the infected control (**Figure 2D**). Infected plants pretreated with *Ta* SC1 did not reduce the full dieback development compared to infected control plants, while infected plants pretreated with both BCAs showed a great variability in the development of full dieback symptoms. Similarly, the external canker area (**Figure 2E**) and the internal necrosis length of infected Chardonnay (**Figure 2F**) were solely slightly reduced in *Bs*-PTA-271-pretreated rootlings (by 16 and 22%, respectively), but insignificantly (**Figure 2E**). In contrast, necrosis length was increased in *Ta* SC1 and both BCAs pretreated plants compared to infected control, although non-significant (**Figure 2F**).

In BCA-treated Tempranillo rootlings then infected with the pathogen, the results of the biocontrol assays showed that *Ta*-SC1- and combined-BCA-pretreated plants showed a significant lower number of full dieback (by approximately 80 and 91%, respectively) and length of stem internal necrosis (by approximately 70 and 81%, respectively) than the infected control (**Figures 2G,I**, respectively). In contrast, infected plants pretreated with *Bs* PTA-271 showed that neither full dieback development nor internal stem necrosis reduced, compared to infected control plants. Looking at the external canker area (**Figure 2H**), none of the treatments with *Bs*-PTA- 271-, *Ta*- SC1-, and combined-BCA-pretreated plants, consecutively infected, induced any significant difference with the infected control. Therefore, external canker may not appear as a relevant indicator for Np dieback with this experimental model, for both Tempranillo and Chardonnay.

Re-isolations of the pathogen confirmed that (1) there was no background infection elsewhere than in the artificially infected rootlings, satisfying thus the Koch's postulates, and that (2) the pathogen was still alive in both dead and living stems of plants, as well as in infected plants pretreated or not with BCA. From all infected plants, the pathogen was successfully isolated with a percentage of success >90% that indicated no fungicidal effect from BCA toward *Np*-Bt67.

### Effects of Biological Control Agents on the Basal Defense of Chardonnay and Tempranillo

The ability of *Bs* PTA-271 or *Ta* SC1 or both BCAs to enhance grapevine immunity was addressed in leaves of control rootlings. Six selected defense genes were targeted by qRT-PCR: the lipoxygenase *LOX9* involved in oxylipin synthesis and described as dependent to JA/ET (Hamiduzzaman et al., 2005; Naznin et al., 2014); *PR1* described to be regulated by SA(Dufour et al., 2013; Naznin et al., 2014; Caarls et al., 2015); the β-1,3-glucanase *PR2* described to be regulated by various phytohormones such as SA, JA, and ET (Liu et al., 2010); the glutathione-*S*-transferase *GST1* putatively involved in the detoxification process; the

phenylalanine ammonia-lyase *PAL* catalyzing the first step in the phenylpropanoid pathway; and the stilbene synthase *STS* involved in the synthesis of phytoalexins. Since BCAs were not detected in leaves (not shown) where defenses were induced, the induction of plant defense by BCAs is systemic.

Data showed some differences in the level of expression of the basal defense genes between the greenhouse cultivars (**Figure 3**), despite the fact that rootlings all grew in the same chamber of the greenhouse with similar culture conditions. The cultivar Chardonnay exhibited a weak constitutive expression of targeted defense genes (**Figure 3A**) compared to Tempranillo (**Figure 3B**). In Chardonnay (**Figure 4** and **Supplementary Figure 1A**), the application of *Bs* PTA-271 at root level induced a 2.8-fold increase of *PR1* and *PR2* expression in leaves, while the application of *Ta* SC1 at stem level did not induce any consistent changes in the expression of these same defense genes in leaves. Interestingly, the application of both BCAs induced the expression of the greatest number of targeted genes in the leaves: a 2.6-fold expression of *LOX9* and a 6.6- or 6.8-fold expression of *PR2* and *STS*, respectively. *Bs* PTA-271, alone or together with *Ta* SC1, may thus act as a priming stimulus for Chardonnay cultivar.

Tempranillo (**Figure 3B**) showed a high basal expression of the targeted gene responsive to SA (i.e., *PR1*) compared to Chardonnay (**Figure 3A**). Interestingly, while the application of *Bs* PTA-271 (**Figure 5** and **Supplementary Figure 1B**) did not induce any consistent changes of defense gene expression, that of *Ta* SC1 induced the expression of the greatest number of studied genes: by a factor of 2.4 for *LOX9*, 13.0 for *PR2*, 12.0 for *GST1*, 4.3 for *PAL*, and 6.2 for *STS*. To contrast with Chardonnay, no relevant number of targeted genes were overexpressed with the application of both BCAs (2.7-fold for *PR2*, 9.6-fold for *GST1*, 3.6-fold for *PAL*, and 5.3-fold for *STS*) compared to *Ta* SC1 alone. *Ta* SC1, alone or together with *Bs* PTA-271, may thus act as a priming stimulus for Tempranillo.

## Effects of Biological Control Agents on the Induced Defense of Chardonnay and Tempranillo Upon Pathogen Challenge

In leaves of Chardonnay rootlings infected with Np-Bt67, data from qRT-PCR showed that except for *PR1* (0.8-fold expression), the expression of all targeted defense genes was consistently upregulated from 2.9- to 9.8-fold (4.3 for *LOX9*, 4.7 for *PR2*, 2.9 for *GST1*, 3.7 for *PAL*, and 9.8 for *STS*) (**Figure 4** and **Supplementary Figure 1A**). As indicated before, in the absence of pathogen infection, *Bs* PTA-271 only induced a weak expression of *PR2* and *PR1* (2.8-fold increase) but was not significant according to ANOVA analysis (**Figure 4** and **Supplementary Figure 1A**), suggesting that *Bs* PTA-271 may act as a priming stimulus in Chardonnay. However,

**A**

| CHARDONNAY | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rootlings | | | | Rootlings challenged with pathogen | | | |
| 4 dpi | Control | Bs | Ta | Bs + Ta | Ctl + Np | Bs + Np | Ta + Np | Bs + Ta + Np |
| *LOX9* | 1,00 a | 0,68 a | 1,11 a | 2,62 a,b,c | 4,34 b,c,d,e | 1,18 a,b | 1,80 a,b,c,d | 7,39 c,d,e,f |
| *PR1* | 1,00 b | 2,85 b | 0,51 a,b | 2,65 a,b | 0,81 a,b | 0,13 a | 1,20 a,b | 3,42 b |
| *PR2* | 1,00 a | 2,86 a,b | 1,06 a | 6,64 a,b | 4,74 a,b,c | 3,94 a,b | 6,55 b,c,d | 13,04 c,d,e |
| *GST1* | 1,00 a,b | 1,55 a | 0,24 a | 0,69 a | 2,98 a,b,c | 0,91 a | 1,32 a | 3,12 a,b,c |
| *PAL* | 1,00 a,b,c,d | 0,95 a | 0,90 a,b | 1,50 a,b,c | 3,70 c,d,e | 1,91 a,b,c,d,e | 3,35 b,c,d,e | 14,30 e,f |
| *STS* | 1,00 a | 1,42 a,b | 1,35 a,b | 6,89 a,b,c | 9,86 d,e,f,g | 4,13 a,b,c,d | 11,17 d,e,f,g | 27,86 e,f,g,h |

**B**

| TEMPRANILLO | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rootlings | | | | Rootlings challenged with pathogen | | | |
| 4 dpi | Control | Bs | Ta | Bs + Ta | Ctl + Np | Bs + Np | Ta + Np | Bs + Ta + Np |
| *LOX9* | 8,20 e,f,g,h | 7,34 d,e,f,g,h | 19,97 h,i | 15,11 g,h,i | 43,97 i | 13,97 f,g,h,i | 13,26 h,i | 5,14 c,d,e,f,g |
| *PR1* | 41,09 c | 38,51 c | 37,23 c | 58,55 c | 82,17 c | 144,52 c | 88,57 c | 59,31 c |
| *PR2* | 5,68 b,c,d | 4,99 a,b,c | 74,02 e,f | 15,57 c,d,e,f | 179,29 f | 67,72 f | 31,78 d,e,f | 6,51 b,c,d |
| *GTS1* | 1,83 a,b,c,d | 1,57 a,b,c | 22,05 d,e | 17,68 c,d,e | 33,24 b,c,d,e | 11,64 c,d,e | 20,56 e | 4,70 a,b,c,d |
| *PAL* | 8,53 e,f,g | 10,28 e,f | 36,69 f,g | 30,69 f,g | 112,01 g | 58,70 g | 32,43 g | 5,74 d,e |
| *STS* | 7,71 c,d,e,f | 9,98 b,c,d,e,f | 47,94 g,h | 40,94 f,g,h | 167,18 h | 43,24 g,h | 102,82 h | 7,15 b,c,d,e |

≤ 1 — 7 — ≥ 27.86

**FIGURE 3 |** *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 attenuates induced differential expression of defense-related genes in leaves of Chardonnay **(A)** and Tempranillo **(B)** rootlings before and after pathogen challenge. Twenty-week-old rootlings untreated or pretreated with PTA-271 or SC1 or both were further infected with sterile PDA plugs (Control, Bs, Ta, and Bs + Ta, respectively) or with mycelium plugs of Np-Bt67 (Ctl + Np, Bs + Np, Ta + Np, and Bs + Ta + Np, respectively). Transcript levels of defense-related genes monitored by qRT-PCR in plant leaves after 4 days of inoculation. Uninfected control of Chardonnay was considered as a reference sample (1× expression level) for both cultivars, and heatmaps represent changes in the transcript expression levels as indicated by the color shading. Data are the means from three representative replicates among five showing the same trends. Different letters indicate statistically significant differences between the treatments (ANOVA, Fisher's LSD *post hoc* test, $\alpha = 0.1$). Legends for genes are LOX9, lipoxygenase 9; PR1, pathogenesis-related protein 1; PR2, class I β-1,3-glucanase; GST1, glutathione-*S*-transferase 1; PAL, phenylalanine ammonia lyase; STS, stilbene synthase.

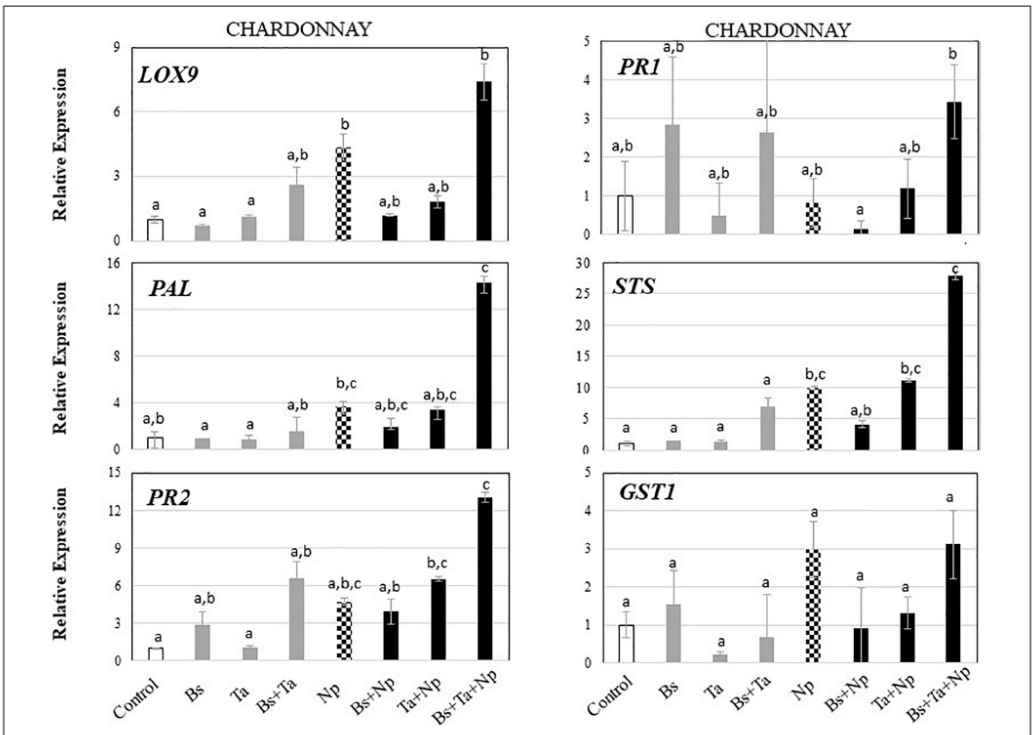


**FIGURE 4 |** *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 attenuates induced differential expression of defense-related genes in leaves of Chardonnay rootlings before and after pathogen challenge. Twenty-week-old rootlings untreated or pretreated with PTA-271 or SC1 or both were further infected with sterile PDA plugs (Control, Bs, Ta, and Bs + Ta, respectively) or with mycelium plugs of Np (Ctl + Np, Bs + Np, Ta + Np, and Bs + Ta + Np, respectively). Transcript levels of defense-related genes monitored by qRT-PCR in plant leaves after 4 days of inoculation. Uninfected control was considered as the reference sample (1× expression level). Data are the means from three representative replicates among five showing the same trends. Different letters indicate statistically significant differences between the treatments (ANOVA, Fisher's LSD *post hoc* test, $\alpha = 0.1$). Legends for genes are as in **Figure 2**.

upon pathogen challenge, no post-priming was observed in *Bs*-PTA-271-pretreated plants since it did not induce any stronger activation of the targeted plant immune defenses compared to Chardonnay-infected control at 4 dpi (**Figure 3**). In contrast, *Ta* SC1 showed no sign of priming stimulus in control Chardonnay, but it induced similar expression of *PR2* (6.5-fold increase) and *STS* (11.1-fold increase) than infected control (4.7 and 9.8, respectively). Interestingly, the application of both BCAs enabled to reach the highest level of Chardonnay defense gene expression upon pathogen challenge (3.1–27.8) compared to infected control (0.8–9.8), a priming effect shown as consistent according to the discriminating capacity of the qRT-PCR technique, but still not yet significant according to the ANOVA analysis. Anyway, such a synergy at 4 dpi can result from a priming stimulus by *Bs* PTA-271, followed by a post-primed phase upon pathogen inoculation with a more rapid and strong activation of immune defenses due to interactions among each actor (*Bs* PTA-271, *Ta* SC1, *Np*-Bt67, and Chardonnay).

In leaves of Tempranillo infected with *Np*-Bt67, the expression of all targeted defense genes was consistently upregulated from 2.0- to 31.5-fold, and significantly for *LOX9*, *PR2*, *PAL*, and *STS* (**Figure 5** and **Supplementary Figure 1B**). Compared to infected Chardonnay (**Figure 3A**), expression of the basal defense genes was significantly stronger in infected Tempranillo (**Figure 3B**), highlighting a higher basal defense level in Tempranillo toward *Np*-Bt67 than in Chardonnay at 4 dpi. The ability of each BCA or both to enhance Tempranillo immunity was also addressed (**Figure 5** and **Supplementary Figure 1B**). As reported above, in the absence of pathogen infection, *Ta* SC1 alone induced a consistent expression of almost all the targeted defenses (2.44–13.03 for *LOX9*, *PR2*, *GST1*, *PAL*, and *STS*), suggesting that *Ta* SC1 may act as a priming stimulus for Tempranillo cultivar, but in a lesser extent when combined with *Bs* PTA-271 (1.84–9.66). However, upon pathogen challenge, no post-priming was observed in *Ta* SC1-pretreated rootlings since it did not induce any stronger expression of the targeted immune defenses

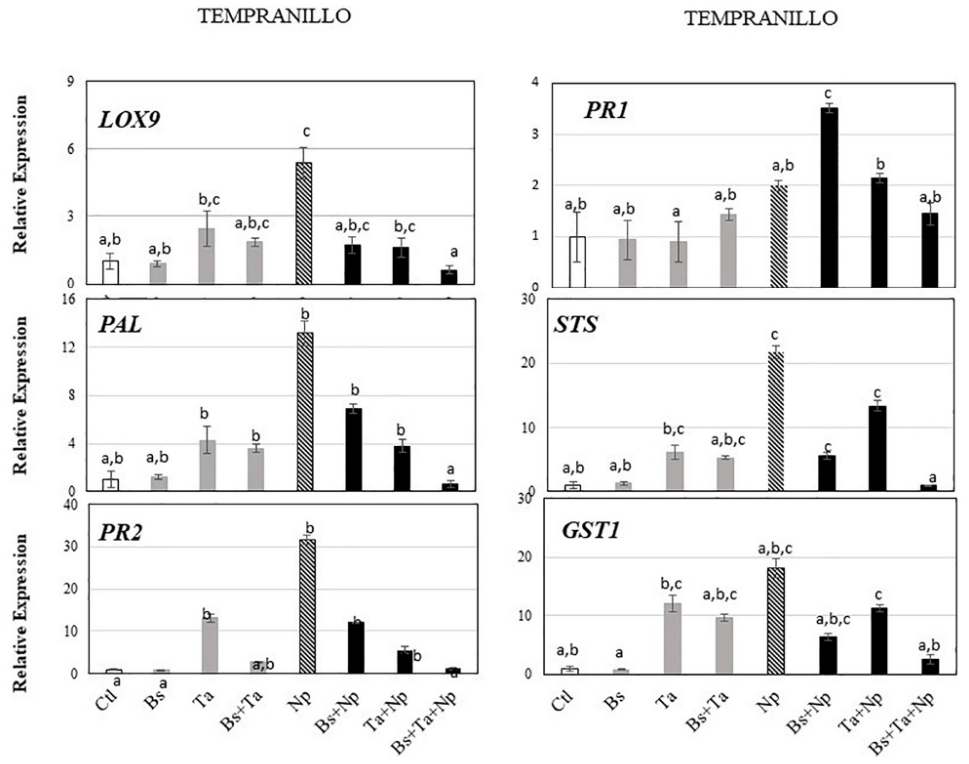


**FIGURE 5 |** *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 attenuates induced differential expression of defense-related genes in leaves of Tempranillo rootlings before and after pathogen challenge. Twenty-week-old rootlings untreated or pretreated with PTA-271 or SC1 or both were further infected with sterile PDA plugs (Control, Bs, Ta, and Bs + Ta, respectively) or with mycelium plugs of *Np*-Bt67 (Ctl + Np, Bs + Np, Ta + Np, and Bs + Ta + Np, respectively). Transcript levels of defense-related genes monitored by qRT-PCR in plant leaves after 4 days of inoculation. Uninfected control was considered as the reference sample (1× expression level). Data are the means from three representative replicates among five showing the same trends. Different letters indicate statistically significant differences between the treatments (ANOVA, Fisher's LSD *post hoc* test, $\alpha = 0.1$). Legends for genes are as in **Figure 2**.

than in the Tempranillo-infected control at 4 dpi, but lower (**Figure 5** and **Supplementary Figure 1B**). Regarding *Bs* PTA-271 effect (i.e., Bs + Np), while it showed no sign of priming stimulus in control Tempranillo, it induced the expression of almost all targeted defenses, but similarly to *Ta* SC1, thus in a lower extent than the infected control (thus, no more priming in that condition with *Bs* PTA-271). Additionally, the pretreatment with both BCAs induced a lower expression level of Tempranillo defense genes upon pathogen challenge (0.63–2.57) than in infected control (2.0–31.56). However, such apparent non-expression of Tempranillo defenses at 4 dpi did not presume any useful induced defenses at other key times or among other defenses that were not targeted in this study.

Taking the uninfected control of Chardonnay as the reference sample for both cultivars, we can compare the immunity between Tempranillo and Chardonnay upon pathogen challenge (**Figures 3A,B**, respectively). As observed in control condition (i.e., high basal expression of *PR1*), infected Tempranillo showed once again a high expression of this targeted gene presumably responsive to SA, compared to Chardonnay. This suggests that Tempranillo would use SA-dependent defense pathways toward *N. parvum*, even when overexpressing *PR2*, *GST1*, *PAL*, and *STS*, unlike Chardonnay. These data thus highlight the possible role of SA signaling in Tempranillo, especially when infected and pretreated with *Bs* PTA-271 (i.e., *PR1*). Curiously, the JA/ET-responsive gene *LOX9* was also highly upregulated in infected Tempranillo, while *LOX9* was severely downregulated in infected Tempranillo pretreated with single or both BCAs, as well as *PR2*, *PAL*, and *STS*. Opposite trends were observed in Chardonnay: (i) no prominent role of SA signaling in infected Chardonnay, especially when pretreated with *Bs* PTA-271; and (ii) *LOX9* was not so highly upregulated in infected Chardonnay (i.e., Ctl + Np),

but *LOX9* was upregulated in infected Chardonnay pretreated with both BCAs, and *PR2*, *PAL*, and *STS* with *Ta* SC1 or both BCAs. These data could suggest the prominent role of JA/ET signaling in Chardonnay, especially when infected and pretreated with BCA. Interestingly, *PR2*, *PAL*, and *STS* are common defenses induced by each BCA against *Np*-Bt67 for the two cultivars, prossibly through two distinct signaling pathways.

## Direct Beneficial or Detrimental Interplays Between *Bacillus subtilis* PTA-271, *Trichoderma atroviride* SC1, and the Pathogen *Neofusicoccum parvum*-Bt67

Regarding the *in vitro* tests with *Np*-Bt67 (**Figure 6**), results showed that *Bs* PTA-271 and *Ta* SC1 antagonize *Np*-Bt67 when plated 48 h before the pathogen. As shown in **Figures 6A,B**, the growth of *Np*-Bt67 was consistently reduced by *Ta* SC1 or *Bs* PTA-271 in dual confrontation compared to the control. However, while the growth of *Np*-Bt67 was completely repressed from day 3 by *Ta* SC1 (**Figure 6A**), it was half-repressed by *Bs* PTA-271 (**Figure 6B**), enabling the pathogen to grow consistently less than the control over the same time period. Thus, *Ta* SC1 antagonistic effect was stronger than that of *Bs* PTA-271, although only a fungistatic effect was observed between them (since when transplanted, the pathogen grows back).

In three-way confrontations (**Figures 6C,D**), the antagonistic effect of *Bs* PTA-271 against *Np*-Bt67 was still reinforced in the presence of *Ta* SC1, even applied 48 h later (**Figure 6C**). Such benefit was yet reinforced when the two BCAs were both applied 48 h before the pathogen, in which the growth of *Np*-Bt67 was close to 5 $mm^2$ (**Figure 6D**). However, it should be noted that *Ta* SC1 did not grow as fast when applied 48 h after *Bs* PTA-271, since *Ta* SC1 slopes are not parallel but weaker in **Figure 6C** than in **Figure 6D**.

To check the *Ta* SC1 capacity to keep its antagonistic effect when applied simultaneously with *Bs* PTA-271, dual confrontations were made between the two BCAs applied in the same area, with or without pathogen (**Figure 7**). As shown in **Figure 7A**, the growth of *Ta* SC1 was slowed down with *Bs* PTA-271, leading to a smaller *Ta* SC1 area than for the *Ta* SC1 control over the same time period. Interestingly, this detrimental effect of *Bs* PTA-271 on *Ta* SC1 disappeared in the three-way confrontation with the pathogen *Np*-Bt67 (**Figure 7B**), being all applied simultaneously at the same area. Thus, *Ta* SC1 and/or *Bs* PTA-271 may keep their strong antagonistic activity when facing a common adversary.

## DISCUSSION

In search of an effective protective BCA combination against *N. parvum*, we investigated a potential BCA and a BCA-commercial product, each already described as protectors against GTDs on distinct cultivars: *B. subtilis* PTA-271 with Chardonnay (Trotel-Aziz et al., 2019) and *T. atroviride* SC1 with different cultivars (Pertot et al., 2016; Berbegal et al., 2020; Martínez-Diz et al., 2021a). This study assessed the combined impact of these two BCAs on two cultivars artificially infected or not with one pathogen. *N. parvum* Bt67 was used, as a very

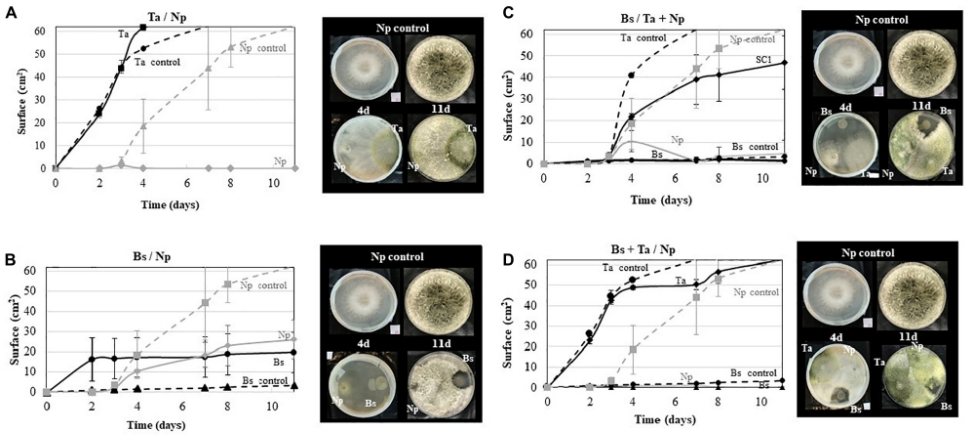


**FIGURE 6 |** Antagonistic activity of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against the *Neofusicoccum parvum* strain Bt67. **(A)** *T. atroviride* SC1 (Ta) was applied 48 h before *N. parvum* (Np) in the opposite sides of PDA plates. **(B)** *B. subtilis* PTA-271 (Bs) was applied 48 h before *N. parvum* (Np-Bt67) in the opposite sides of PDA plates. **(C)** *B. subtilis* PTA-271 (Bs) was applied 48 h before *N. parvum* (Np) and *T. atroviride* (Ta) at distinct areas of the PDA plates. **(D)** *B. subtilis* PTA-271 (Bs) and *T. atroviride* SC1 (Ta) were applied 48 h before *N. parvum* (*Np*) at distinct areas of the PDA plates. All plates were incubated at 28°C. Pictures of each plate condition were taken from day 1 to day 11 after the first inoculation. Photos on top are the control of *Np*-Bt67 and photos at the bottom indicate the confrontation assay at day 4 (left) and day 11 (right).

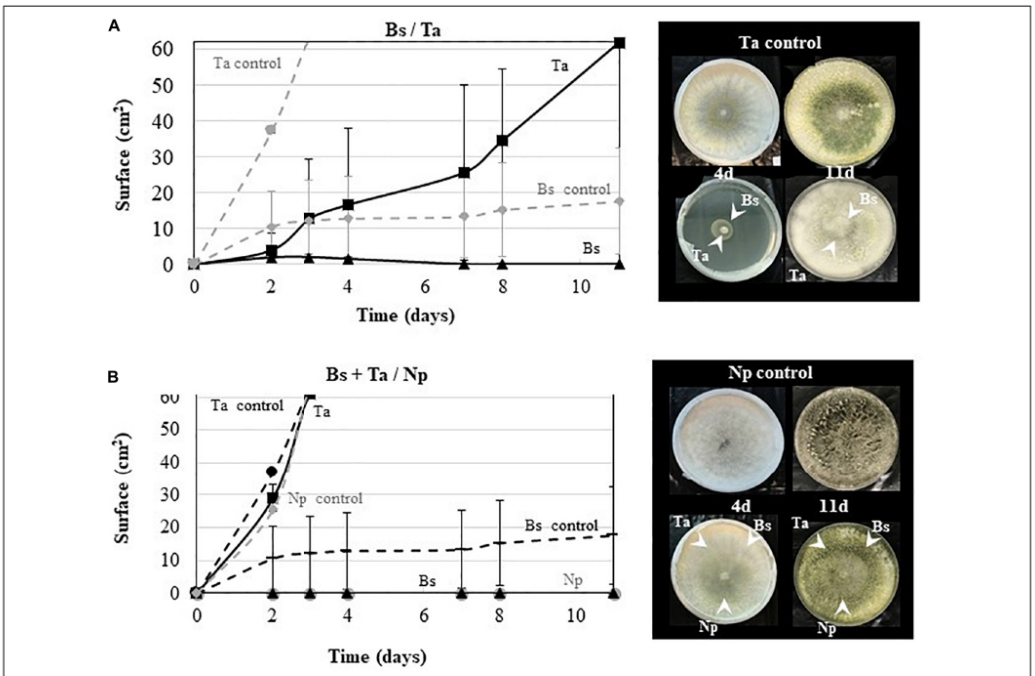


**FIGURE 7 |** Antagonistic activity of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* Bt67. **(A)** *B. subtilis* PTA-271 (Bs) and *T. atroviride* SC1 (Ta) were applied simultaneously in the center of the PDA plates. **(B)** *B. subtilis* PTA-271 (Bs), *T. atroviride* SC1 (Ta), and *N. parvum* (Np) were applied simultaneously in the center of the PDA plates. All plates were incubated at 28°C. Pictures of each plate were taken from day 1 to day 11 after the first inoculation. Photos on top are SC1 control **(A)** and *Np*-Bt67 control **(B)**, and pictures at the bottom indicate the confrontation assay at day 4 (left) and day 11 (right).

aggressive pathogen associated to BD. Our investigation focused on the capacity of single and combined BCAs to counteract BD symptoms on both cultivars. To compare the two BCAs, densities were aligned to the density optimized for *Bs* PTA-271. After looking for the protective capacity of BCA *in planta*, their modes of action leading to protection were further explored. Thus, we investigated whether these BCAs could affect pathogen growth *in vitro* and cultivar immunity upon infection *in planta*.

*Neofusicoccum parvum*-Bt67 caused BD symptoms on the rootlings of the two grapevine cultivars, as shoot full dieback, canker external necrosis, and shoot internal necrosis. Interestingly, the full dieback symptoms were more severe on Tempranillo than on Chardonnay (i.e., 37.5 and 28%, respectively, **Figures 2D,G**), suggesting a greater susceptibility to BD for Tempranillo than Chardonnay, as already reported by Luque et al. (2009) and Cobos et al. (2019). Although there is a lack of comparative data between cultivars, the distinct susceptibility of some cultivars to GTDs has already been reported (Travadon et al., 2013; Fontaine et al., 2016b; Chacon et al., 2020; Reveglia et al., 2021), even within a same cultivar from one region to another or depending on the vintage (Mimiague and Le Gall, 1994). However, in Chardonnay and Tempranillo rootlings pretreated with one or both BCAs before inoculation of the pathogen, BD symptoms were significantly reduced with *Bs* PTA-271 or *Ta* SC1, respectively. Grapevine effective protection against *Np*-Bt67 has already been reported with *Bs* PTA-271 on Chardonnay rootlings (Trotel-Aziz et al., 2019), and with *Ta* SC1 on Tempranillo in nursery and vineyards conditions (Berbegal et al., 2020; Blundell et al., 2021). Additionally, in our experimental conditions, Chardonnay seems to favor the beneficial effect of *Bs* PTA-271, while Tempranillo favors that of *Ta* SC1 beneficial effect, highlighting the relationship between cultivar response and BCA effect. Manter et al. (2010) suggested that the differences between cultivars may result from minor changes in the composition of their endophyte community, with *Trichoderma* species being among the most common endophytic fungal isolates from Tempranillo (González and Tello, 2011). Therefore, Tempranillo could be subjected to *Trichoderma*'s influence. Similarly, the efficiency of *Bs* PTA-271 toward Chardonnay may be explained by its origin of sampling from an established Chardonnay vineyard, screened from healthy vines (Trotel-Aziz et al., 2008). In combination and according to our experimental conditions, despite cited as compatible strains (Kumar, 2013), *Ta* SC1 + *Bs*

PTA-271 are less protective against *Np*-Bt67 in Chardonnay than *Bs* PTA-271 alone. The authors reported that *Trichoderma* spp. can interfere the plant signaling networks and secrete an arsenal of degrading enzymes (i.e., proteases) and secondary metabolites (Tiwari and Verma, 2019; Alfiky and Weisskopf, 2021), suggesting that *Ta* SC1 may alter both *Bs* PTA-271 integrity and beneficial effects in Chardonnay. However, the application of both BCAs enabled to reach the highest level of Chardonnay defense gene expression upon pathogen challenge (see **Figure 3A**), and the highest protection in Tempranillo cultivar (see **Figure 2G**), highlighting that *Ta* SC1 on its own would not interfere with the beneficial effects of *Bs* PTA-271.

Beneficial effects of combined BCAs have yet been reported in different pathosystems (Yobo et al., 2011; Magnin-Robert et al., 2013), and our study reports for the first time the biocontrol potential of the combination of *Bs* PTA-271 and *Ta* SC1 against *Np*-Bt67 in Tempranillo. Our results showed that *Ta* SC1 efficiently protects Tempranillo, and this protection is still observed in rootlings pretreated with both BCAs (see **Figure 2G**). Thus, a significant benefit is observed when using both BCAs in Tempranillo, despite the fact that they could antagonize each other. In this respect, Leal et al. (2021) reported that the genome of *Bs* PTA-271 encodes for the synthesis of bacillaene, a polyketide already described to antagonize *Trichoderma* spp. (Caulier et al., 2019). Therefore, the positive contribution of *Bs* PTA-271 and *Ta* SC1 to Tempranillo protection against *Np*-Bt67 suggests a fine-tuned orchestrated cooperation of BCAs when facing adversity, as supported by the **Figure 7** results and highlighted by Alfiky and Weisskopf (2021). However, the application of BCAs to rootlings was spatially separated.

Empowered with aggressive molecules, *Bacillus* spp. and *Trichoderma* spp. can possibly exert direct beneficial or detrimental interplays within the host's microbiome, and especially on the other BCA and pathogens such as GTDs fungi (Di Marco et al., 2002; Kloepper et al., 2004; Ongena and Jacques, 2008; Haidar et al., 2016; Trotel-Aziz et al., 2019; Yacoub et al., 2020; Úrbez-Torres et al., 2020; Blundell et al., 2021). *In vitro* dual confrontation tests confirmed the antagonistic activity of *Bs* PTA-271 or *Ta* SC1 toward *Np*-Bt67 since each of them significantly inhibits the mycelium growth of *Np*-Bt67 (see **Figures 6A,B**). This also prompts us the idea that the not-yet-convincing protection assay of *Bs* PTA-271 and *Ta* SC1 in infected Chardonnay would not result from a detrimental effect of *Ta* SC1 on some putative direct effects of *Bs* PTA-271. Indeed, the strong direct antagonistic activity of *Bs* PTA-271 and *Ta* SC1 toward *Np*-Bt67 also operates when the two BCAs were applied both 48 h before the pathogen (see **Figure 6D**). This antagonistic benefit is in accordance with the significant synergy of the protection in Tempranillo by both BCAs (see **Figure 2G**), and it confirms the benefit of using them in combination in Tempranillo to optimize the direct fight against *Np*-Bt67. A similar outcome was reported by Alexander and Phin (2014) using an effective combination of *Bacillus* spp. and *Trichoderma* spp. against *Ganoderma* spp. Such direct effects of BCA against pathogens are important life traits to protect grapevine from BD, still deprived of effective curative treatments nowadays (Mondello et al., 2018). In nursery too, healthy mother plants require a control of their sanitary status (Pertot et al., 2016), eventually provided by an early inoculation of such beneficial BCA with a strong antagonistic activity toward pathogens.

Direct beneficial or detrimental interplays between BCAs also condition their capacity to live together in symbiosis, even *in planta* as part of the holobiont (Bettenfeld et al., 2020). Dual confrontation was thus also performed between *Bs* PTA-271 and *Ta* SC1. As expected, *Bs* PTA-271 antagonized *Ta* SC1 (see **Figure 7A**; Caulier et al., 2019; Leal et al., 2021). However, this detrimental effect of *Bs* PTA-271 on *Ta* SC1 disappears in a three-way confrontation with *Np*-Bt67, when they were all applied simultaneously at the same area (see **Figure 7B**). These data confirm that *Ta* SC1 and *Bs* PTA-271 can positively interact to better confine *Np*-Bt67 and can lead to a direct positive contribution of this combination to the protection of Tempranillo against *Np*-Bt67. However, such a direct positive contribution of combined BCAs did not operate on the infected Chardonnay rootlings. Our experimental conditions could have altered the ability of *Ta* SC1 to exert its direct fungistatic effect (applied once at $10^8$ CFU/ml with a paintbrush over a 5-$mm^2$ area). This also strongly suggests that Chardonnay itself alters the fine-tuned orchestrated cooperation of BCAs, probably targeting the indirect *Ta* SC1 beneficial effect since BCAs are spatially separated. This prompts us to pursue our investigations further in order to decipher the indirect interactions driving to a beneficial outcome in grapevine control of *Np*-Bt67.

According to our previous works, a focus on grapevine systemic immunity was made by targeting six selected defense genes in leaves: the lipoxygenase *LOX9* involved in oxylipin synthesis and described as dependent to JA/ET; *PR1* described to be regulated by SA; the β-1,3-glucanase *PR2* described to be regulated by various phytohormones such as SA, JA, and ET; the glutathione-*S*-transferase *GST1* putatively involved in the detoxification process; the phenylalanine ammonia-lyase *PAL* catalyzing the first step in the phenylpropanoid pathway; and the stilbene synthase *STS* involved in the synthesis of phytoalexins (Trotel-Aziz et al., 2019). Interestingly, basal immunity results in a weak constitutive expression of the targeted defense genes in Chardonnay compared to Tempranillo (see **Figure 3**), despite the fact that literature described Chardonnay as less susceptible to BD than Tempranillo (Luque et al., 2009). Maybe our six targeted genes are not sufficiently exhaustive to presume at this preliminary stage of the susceptible versus tolerant status of both cultivars. However, looking at the specific red and white cultivar responses, the same studied genes are of interest: those specific to white grape cultivars include transcription factors from the ET pathway and lipid metabolism (e.g., lipoxygenase), while those specific to red grape cultivars are linked to the secondary metabolism in connection with the pathway of phenylpropanoids (e.g., PAL and its derivatives) and are expressed more strongly in the red cultivars, to distinguish them from the white ones (Lambert et al., 2012; Massonnet et al., 2017). Upon abiotic or biotic stress, other authors pointed out the highest synthesis of resveratrol in the most tolerant grapevine (Corso et al., 2015, Lakkis et al., 2019), but a gain of protection due to the BCA presence in susceptible cultivar (Lakkis et al., 2019). Considering that susceptible plants rather benefit from the help of BCA to

induce their immunity, unlike resistant plants that already have high basal immunity, we examined the immunity induced by both cultivars studied.

In Tempranillo (see **Figures 3B**, **5** and **Supplementary Figure 1B**) under our experimental conditions, application of *Bs*-PTA-271 did not induce significant changes in plant defense responses compared to control, whether infected or not. Since Tempranillo basal immunity strongly upregulates *PR1* as a marker of SA-dependent defenses, we can speculate that high basal SA content might contribute to prevent the beneficial effect of *Bs* PTA-271 on Tempranillo immunity. Our previous study showed that *Bs* PTA-271 primed the expression of the plant JA/ET-dependent defenses in Chardonnay rootlings (Trotel-Aziz et al., 2019), whereas it is reported that early activation of SA signaling could antagonize the expression of these JA/ET-dependent defenses (Pieterse et al., 2012; Van der Does et al., 2013). In this sense, *Bs* PTA-271 did not provide protection in Tempranillo against *Np*-Bt67. These results show therefore that cultivars differing in their basal immunity can condition the success of a BCA protection toward a pathogen. In contrast, *Ta* SC1 alone or together with *Bs* PTA-271 acts as a priming stimulus for Tempranillo, but no post-priming was observed with *Ta* SC1 alone and with *Ta* SC1 + *Bs* PTA-271 upon pathogen challenge. Looking at the defenses induced by these two protective modalities (*Ta* SC1 and *Ta* SC1 + *Bs* PTA-271) against *Np*-Bt67: the SA-dependent defenses (i.e., *PR1*, *PAL*, and *STS*) were rather strongly decreased in protected plants (i.e., asymptomatic despite infected), while they were the highest in symptomatic plants (*Bs* PTA-271). Since Botryosphaeriaceae are known to specifically metabolize grapevine phytoalexins (Stempien et al., 2017), which benefits pathogen fitness, we could suggest that the SA stimulation of the phenylpropanoid pathway and derivatives would wrongly serve the plant. In the case of Tempranillo exposed to Botryosphaeriaceae, the high constitutive expression of SA-dependent defense genes could thus appear as a disadvantage, confirming that Tempranillo would be less tolerant than Chardonnay to BD, as already reported by Luque et al. (2009) and Cobos et al. (2019). Fortunately, in the Tempranillo pretreated with both BCAs, the expressions of genes *PR1*, *PAL*, and *STS* were repressed, and in the Tempranillo pretreated with *Ta* SC1 alone, the expression of the genes *PAL* and *STS* were repressed. Therefore, the beneficial effect of *Ta* SC1 on Tempranillo to control *Np*-Bt67 could result from a repressive effect on detrimental SA-dependent defenses. Complementary approaches are in progress to screen the induced key levers able to trigger ISR in the whole plant. Additionally, the *Ta* SC1 beneficial effect on Tempranillo could also result from a direct antagonism toward *Np*-Bt67 since it is applied in the same area as *Ta* SC1.

In non-infected Chardonnay rootlings (see **Figures 3A**, **4** and **Supplementary Figure 1A**), *Ta* SC1 shoot application did not induce any significant changes of the selected targeted responses of plant defenses in leaves, while *Bs* PTA-271 root application upregulated the *PR1* and *PR2* gene expression and the combined application induced the expression of almost all targeted genes at a higher level than *Bs* PTA-271 alone. *Bs* PTA-271, alone or together with *Ta* SC1, may thus act as a priming stimulus in Chardonnay. However, upon pathogen challenge (4 dpi, designed as a relevant sampling time point for such experiment), *Bs* PTA-271 did not prime any of the targeted defenses, probably due to low to medium pathogen pressure compared to that reported in Trotel-Aziz et al. (2019). However, *Bs* PTA-271 beneficial effect on Chardonnay is supported by the phenotype of the *Bs*-PTA-271 treated rootlings, showing a significant protection for Chardonnay against *Np*-Bt67 at 2.5 mpi. This contrasts with the detected non-benefit provided by the combined application of both BCAs at 2.5 mpi, despite the fact that defenses were primed at 4 dpi, possibly due to a very high level of reactive oxygen species (ROS) that could amplify the plant defenses. It is interesting to note that *GST1* (involved in the ROS detoxification process) is the only useful targeted gene that was weakly expressed in non-protected Chardonnay pre-treated with both BCAs and further infected with *N. parvum* (see **Figure 3A**). This may suggest that when a pathway with high induced defenses is not combined with sufficient ROS detoxification, a plant could potentially trigger many symptoms. In our experimental conditions, Chardonnay therefore favors *Bs* PTA-271's beneficial effect, probably thanks to the different key levers including the induced grapevine immunity. Present at the root level, these results and those reported in Trotel-Aziz et al. (2019) suggest that systemic induced immunity conferred by *Bs* PTA-271 drove the plant ISR against *Np*-Bt67. Amazingly, the application of both BCAs enabled to reach the highest level of Chardonnay defense gene expression upon pathogen challenge (see **Figure 3A**), despite no protection at 2.5 mpi (see **Figure 2D**). Since *Ta* SC1 contributes to actively reducing the SA-dependent defenses in Tempranillo, one can hypothesize that *Ta* SC1 would also promote the *Bs* PTA-271 way of triggering immunity in Chardonnay. The authors also indicated that *Trichoderma* spp. may produce enzymes (i.e., ACC deaminase) able to shunt ET synthesis (Alfiky and Weisskopf, 2021), but in Tempranillo, the application of both BCAs enabled reaching the highest protection (see **Figure 2G**). Thus, it would be surprising that *Ta* SC1 on its own would succeed to interfere with the beneficial effects of *Bs* PTA-271 in Chardonnay. This opens the discussion of how the cultivar interacts with BCAs and the pathogen to condition the beneficial or detrimental outcome against *Np*-Bt67. Complementary approaches are already in progress to screen which are the induced key levers useful to control ISR in the whole plant.

## CONCLUSION

Altogether, our results provide evidence that grapevine susceptibility to BD is cultivar-dependent, as well as the BCA beneficial effects. *Bs* PTA-271 was confirmed as an effective protector for Chardonnay against *Np*-Bt67, and *Ta* SC1 was shown for the first time as a good protector for Tempranillo. This study also reports for the first time the biocontrol potential of the combination of *Bs* PTA-271 and *Ta* SC1 against *Np*-Bt67 in Tempranillo. This is a promising result for an improved efficiency of sustainable biological control in a proven context of lack of effective chemicals to manage GTDs.

Endowed with aggressive molecules, *Bs* PTA-271 and *Ta* SC1 can antagonize each other, but *Bs* PTA-271 inhibits *Np*-Bt67 development with a greater efficiency in a three-way confrontation. This beneficial BCA collaboration against *Np*-Bt67 still operates in Tempranillo and confirms the interest of using both BCAs in combination to optimize the direct fight against *Np*-Bt67. These results are of great interest for effective curative treatments to obtain healthy mother plants in the nursery and to control BD in vineyard. However, the direct beneficial effect of combined BCAs did not operate to protect Chardonnay, suggesting that Chardonnay itself probably alters the fine-tuned orchestrated cooperation of BCAs that drives such direct beneficial effect.

Plant systemic immunity was also affected by each BCA. Our findings suggest a common feature for the two cultivars: the defenses that are greatly diminished in BCA-protected plants appear to be those that are responsive to SA, in contrast to symptomatic plants. For Tempranillo, the high basal expression of SA-dependent defenses may thus explain the highest susceptibility to BD and also the ineffectiveness of *Bs* PTA-271 in our experimental conditions. Complementary approaches are underway to further investigate the responses of each cultivar to both *Bs* PTA-271 and *Ta* SC1 under controlled conditions and upon pathogen challenge.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

CL, PT-A, and FF designed and planned the research. CL performed most of the experiments, analyzed the data, and wrote the manuscript with input and discussion from PT-A, FF, JA, and DG. JA and DG validated the planned research and provided expertise for all stages of this work. J-FG ensured the quality of the mother cuttings and greenhouse conditions. NR assured the quality of the qRT-PCR analyses and data. All authors contributed to writing and revising the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.726132/full#supplementary-material

**Supplementary Figure 1 |** *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 attenuates induce differential expression of defense-related genes in leaves of Chardonnay **(A)** and Tempranillo **(B)** rootlings before and after pathogen challenge. Twenty weeks old rootlings untreated or pretreated with PTA-271 or SC1 or both were further infected with sterile PDA plugs (Control, Bs, Ta, and Bs + Ta, respectively) or with mycelium plugs of *Np*-Bt67 (Ctl + Np, Bs + Np, Ta + Np, and Bs + Ta + Np, respectively). Transcript levels of defense-related genes monitored by qRT-PCR in plant leaf after 4 days of inoculation. Uninfected control of each cultivar was considered as a reference sample (1 × expression level) for the same cultivar, and heatmaps represent changes in the transcript expression levels as indicated by the color shading. Data are the means from three representative replicates among five showing the same trends. Legends for genes are as in **Figure 2**.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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***Chapter IV***

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# Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* BT-67 (Molecular effects)

### 4.1. Contexte

L'étude précédente (Chapitre III) a permis d'évaluer le potentiel protecteur de *B. subtilis* PTA-271 (*Bs* PTA-271) et *T. atroviride* SC1 (*Ta* SC1) sur les cépages Chardonnay et Tempranillo, contre *N. parvum* Bt67 (Np-Bt67) (Leal et al. 2021b). Il a alors été montré que l'effet bénéfique de chaque BCA était dépendant du cépage. *Bs* PTA-271 a notamment été confirmé comme un BCA protecteur efficace sur Chardonnay contre Np- Bt67, et *Ta* SC1 a été confirmé dans son efficacité sur Tempranillo contre ce même pathogène. Cette étude a également révélé, pour la première fois, le potentiel bénéfique d'une combinaison de BCA contre Np-Bt67 en particulier sur Tempranillo (gain de protection de 10% par rapport à *Ta* SC1 seul). Par ailleurs, malgré une protection cépage-dépendante pour chaque BCA, une caractéristique commune aux 2 cépages protégés a été révélée : les défenses dépendantes de la voie du SA sont significativement réprimées au niveau systémique. Ce levier d'action commun à la protection de cépages distincts contre un même pathogène, en valorisant 2 BCA distincts, suggère une induction de défenses végétales spécifiques contre un pathogène cible qui méritent d'être analysées plus en détail. En effet, les changements physiologiques communs induits par chaque BCA dans la plante pourraient s'avérer utiles non seulement pour détecter de façon précoce un dépérissement à BOT asymptomatique, mais aussi pour proposer des solutions de lutte efficaces et durables, pour répondre aux besoins actuels des professionnels.

Au cours de la dernière décennie, la méthode de séquençage de l'ARN (RNA-seq) a permis de décrypter bon nombre de réponses des plantes ligneuses aux pathogènes et aux BCA. Les changements révélés dans l'expression des gènes de la vigne soumise à ces interactions multipartites portent sur : la biosynthèse de la paroi végétale, la signalisation hormonale et le métabolisme secondaire. Ces derniers pourraient donc être considérés comme des acteurs importants des interactions multipartites plante/pathogène/BCA (Xu et al. 2013, Camps et al. 2010, Yacoub et al. 2020).

La présente étude a donc aussi permis de réaliser une analyse transcriptomique pour mieux décrypter les changements physiologiques de la plante induits en réponse à *Bs* PTA-271 et *Ta* SC1 pour protéger la vigne contre une infection à Np-Bt67 (Figures 5 et 7). Ces analyses transcriptomiques sont complémentaires aux travaux de Leal et al. (2021b) présentés dans le chapitre III ; et ont donc été réalisées avec les mêmes pools d'échantillons de feuilles. L'accent a ainsi été mis sur les réponses moléculaires de la plante à son infection par Np-Bt67, en utilisant des boutures de vigne cv Chardonnay et Tempranillo préalablement inoculées avec

le(s) BCA *Bs* PTA-271 et/ou *Ta* SC1, en comparaison à leurs témoins (sans BCA et/ou sans pathogènes). A notre connaissance, il s'agit de la première étude transcriptomique sur les changements induits par deux BCA distincts à deux cépages distincts, infectés artificiellement par *Np*-Bt67.

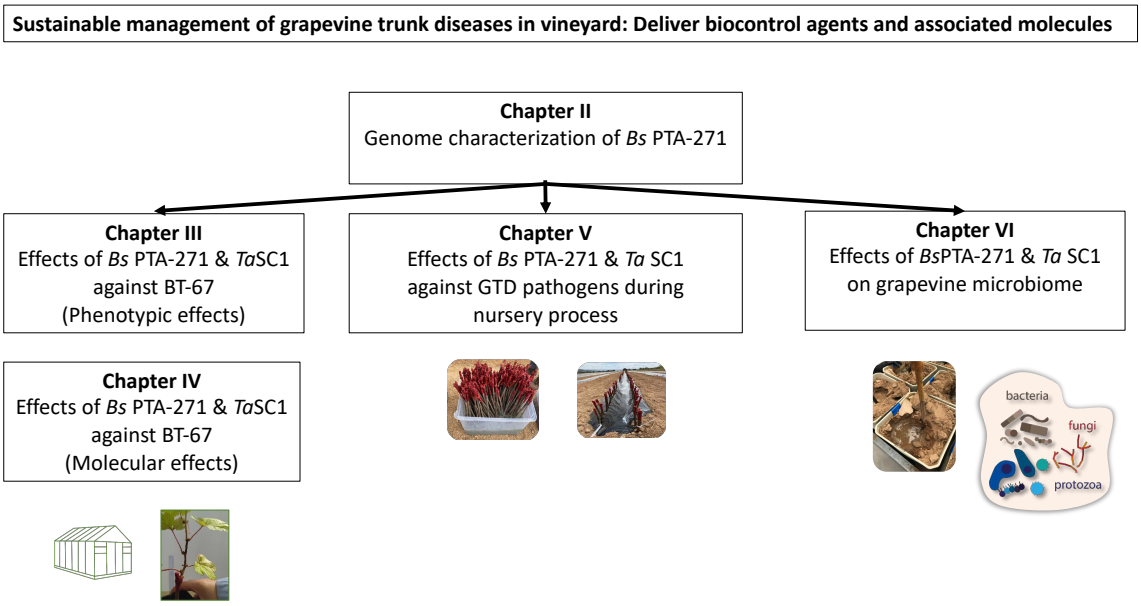


**Figure 5.** Aims of each chapter of the thesis entitled "Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules".

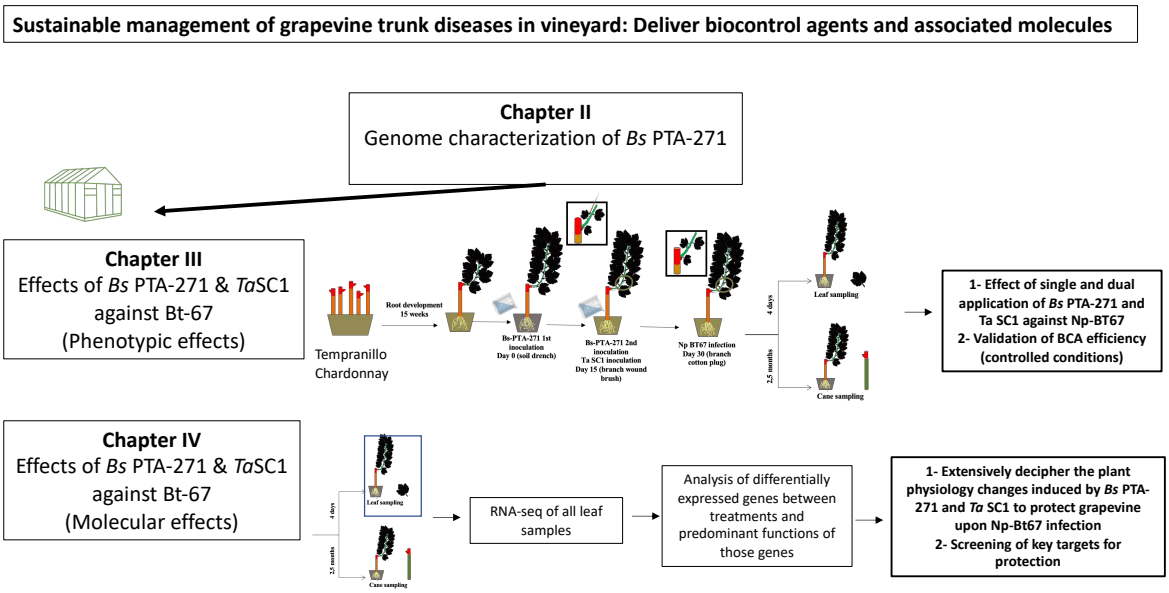


**Figure 7.** Aims chapter II and IV entitled "Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* BT-67 (Phenotypic effects)" and "Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against *Neofusicoccum parvum* BT-67 (Molecular effects)", respectively.

**Publication 4 – RNA-seq analysis of *Vitis vinifera* Chardonnay and Tempranillo during its beneficial interaction with *Trichoderma atroviride* SC1 and *Bacillus subtilis* PTA-271 against *Neofusicoccum parvum***

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***Manuscript in preparation for Frontiers in Microbiology***

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**RNA-seq analysis sequencing of *Vitis vinifera* Chardonnay and Tempranillo during its beneficial interaction with *Trichoderma atroviride* SC1 and *Bacillus subtilis* PTA-271 against *Neofusicoccum parvum***

These transcriptomic analyses are complementary to the work of Leal et al. (2021, doi: 10.3389/fmicb.2021.726132), trying now to decipher the main levers triggered by *Trichoderma atroviride* SC1 and *Bacillus subtilis* PTA-271 to protect grapevine artificially inoculated with *Neofusicoccum parvum* Np-Bt67 under controlled conditions.

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**Abstract**

Grapevine trunk diseases (GTDs) represent one of the most concerning types of grapevine diseases, worrying viticulturists in all wine-producing countries. Since the ban of sodium arsenite in the 2000s, the treatments available for GTDs, are mainly preventive and mitigative, with a lack of effective curative treatments. To contrast, the biological control based on the use of BCAs, such as *Trichoderma* spp. and *Bacillus* spp., has already shown potential and is still the focus of several studies. In the present study, we intend to extensively decipher the plant physiology changes induced by both *Bs* PTA-271 and *Ta* SC1 to protect grapevine upon Np-Bt67 infection, by using transcriptomic analysis.

Our results provide new insight on the molecular changes associated with either (1) the infection by Np-Bt67 in Chardonnay and Tempranillo, or (2) their BCA-protection against this pathogen. Chardonnay plants infected with *Np*-Bt67 over-expressed genes involved in ABA

production or signaling, while the changes in infected Tempranillo plants concern amino acid import, chloroplast and photosystem related processes, plant responses to biotic stimulus, and biosynthesis of secondary metabolites. Protection with *Bs* PTA-271 in Chardonnay targets genes related to ABA biosynthesis, phenylpropanoid pathways and secondary metabolites, and cell wall structure/organization. Protection with *Ta* SC1 in Tempranillo include changes related to transporters, cell wall integrity and extension, cell division and pathogen induced cell death, multidirectional active proteins, and microbiome interactions.

**Keywords:** RNA-Seq, grapevine, biocontrol, Botryosphaeria dieback, plant immunity

## 1. Introduction

Grapevine trunk diseases (GTDs) represent one of the most concerning types of grapevine diseases, worrying viticulturists in all wine-producing countries (Mondello et al. 2018). Indeed, GTDs lead to important economic losses and vineyard sustainability disturbances, restricting both the productivity and the longevity of grapevines (Hofstetter et al. 2012; De la Fuente et al. 2016; O'Brien 2017; Mondello et al. 2018). By affecting the plant perennial part, GTDs lead to an unavoidable short- or long-term death of vines, reminding the very aggressive nature of the pathogens responsible for GTDs and their strong detrimental effect for the sustainability of the winemaking industry (Hofstetter et al. 2012). Both young and mature vines are not spared, as well as nursery staged plants, adding yet to the massive economic losses of the grapevine industry (Gramaje and Armengol 2011). Their undetermined period of latency as endophyte (vines in asymptomatic state) before switching pathogenic (symptomatic plants with chronic and dieback symptoms) makes GTD pathologies difficult to detect (Leal et al. submitted to Phytopathology, 2022). Therefore, an early detection of useful markers and an early management of GTDs remain presently a challenge in both nursery and vineyard (Gramaje and Armengol 2011; Gramaje et al. 2018; Leal et al. 2021b).

Among all, Botryosphaeria dieback (BD) is a common disease of vineyards and nursery plants (Leal et al. submitted to PMS, 2022), and thus represents one of the main GTDs. BD is prompted by several Botryosphaeriaceae species, including the very aggressive *Neofusicoccum parvum* (Úrbez-Torres 2011; Billones-Baaijens and Savocchia 2019; Reis et al. 2019; Larach et al. 2020; Trotel-Aziz et al. 2022). *N. parvum* has a hemibiotrophic lifestyle

(Yang et al. 2015; Leal et al. 2022 review submitted to Phytopathology), with an initial heterotrophic endophytic phase allowing fungal hyphae to colonize the plant tissue from any plant wounds (e.g., pruning wounds) (Dakin et al. 2010; Hrycan et al. 2020; Slippers and Wingfield 2007). Botryosphaeriaceae can remain latent as endophytes until the host plant is exposed to stress (Dakin et al. 2010; Hrycan et al. 2020; Slippers and Wingfield 2007). As heterotrophs, they use cell wall degrading enzymes to take nutrients from the host showing no signs of disease (Giovannoni et al. 2020). During this endophytic lifestyle, the hemibiotroph can positively impact its host (Kogel et al. 2006). This stage is followed by a necrotrophic phase during which the hemibiotroph has a negative impact on its host in the short or long term due to an excess of deleterious nutrients secreted inside the host (namely, virulence and pathogenicity factors). For instance, toxins are secreted by the pathogens inside the host cells that could suppress the plant immune defenses and promote disease symptoms (Leal et al. review submitted to Phytopathology in 2022) possibly actively. Depending on the biotope and environmental conditions, fungal species could reversibly adopt several modes of life during their existence (Kogel et al. 2006; Leal et al. review submitted to Phytopathology, 2022). Once pathogenic, the Botryosphaeriaceae infection causes chronic foliar discoloration and necrosis, but more often triggers a slow or apoplectic dieback that alters the permanent structures of the vines through partial necrosis (localized death of the distal buds, or shoots, or wood canker) or the full trunk necrosis (Larignon et al. 2001; Úrbez-Torres 2011). Such reversible pathogen lifestyle, triggering more or less damaging and/or reversible symptoms for the host makes BD pathology particularly difficult to manage. The development of specific markers for an early detection of this disease, focusing either on pathogen detection or on induced plant defenses, could thus be useful indicators or levers to better manage biocontrol strategies to combat BD.

Since the ban of sodium arsenite in the 2000s, the treatments available for GTDs, both in nursery and vineyard, are mainly preventive and mitigative, with a lack of effective curative treatments that eliminate GTD pathogens from grapevines. Few chemicals are applied on pruning wounds in vineyards to prevent colonization by fungal pathogens (Sosnowski et al. 2004), favoring cultural practices in vineyard (Mondello et al. 2018) and sanitation methods (Gramaje and Armengol 2011; Gramaje et al. 2018). However, these treatments cannot eradicate the pathogens once established in a vineyard (Calzarano et al. 2004; Gramaje and Armengol 2011). To contrast, the biological control based on the use of beneficial microorganisms (BCA) has already shown potential and is still the focus of several studies (Del Frari et al. 2019; Landum et al. 2016; Pertot et al. 2016; Trotel-Aziz et al. 2019; Leal et al.

2021b). Beneficial endophytes are defined as microorganisms that colonize healthy internal plant tissues without causing any apparent disease symptoms, while possibly inducing their host tolerance to environmental stresses and pathogens (Billar de Almeida et al. 2020; Oono et al. 2015). Fungi like those belonging to the *Trichoderma* genus and bacteria including *Bacillus* species, have already demonstrated such efficiency as biological control agents both in grapevine nurseries and vineyards.

Currently, *Trichoderma* species have been extensively investigated for their use against GTDs (Di Marco et al. 2002, 2004; John et al. 2008; Halleen et al. 2010; Berbegal et al. 2020; Martínez-Diz et al. 2021a, b; Leal et al. 2021b) and are the most widely used fungal-based BCAs commercialized in viticulture (Harman 2006; Muckherjee et al. 2012; Waghunde et al. 2016). It is reported that *Trichoderma* spp. (1) can directly antagonize GTD pathogen aggressiveness through competition for nutrients and space, mycoparasitism, cell-wall degrading enzymes, and antibiosis, and (2) can also act as plant growth and defense stimulators (Harman 2006; Van Wees et al. 2008; Vinale et al. 2008; Pieterse et al 2014; Waghunde et al. 2016; Leal et al. review submitted to Phytopathology, 2022). A second group of beneficial microorganisms, namely *Bacillus* strains, has also been extensively studied as BCAs and reported to protect plants directly and indirectly against pathogens with different lifestyles (Magnin-Robert et al. 2007, 2013; Trotel-Aziz et al. 2008; Nguyen et al. 2020), including the GTD hemibiotrophic pathogens (Schmidt et al. 2001; Halleen et al. 2010; Rezgui et al. 2016; Kotze et al 2019; Trotel-Aziz et al. 2019; Leal et al. 2021b). As for *Trichoderma* species, the genome of *Bacillus* spp. encodes for a broad range of molecules that can both induce or elicit plant defenses (i.e. phytohormones precursors, lipopolysaccharides, siderophores) and compete with, antagonize, or directly modify plant pathogens or their aggressiveness (Kloepper et al. 2004; Ongena and Jacques 2008; Sood et al. 2020; Leal et al. 2021a; Leal et al. review submitted to Phytopathology, 2022).

In a previous study, Leal et al. (2021b) evaluated the effect of *B. subtilis* PTA-271 (*Bs* PTA-271) and *T. atroviride* SC1 (*Ta* SC1), on the protection of the two distinct grapevine cultivars Chardonnay and Tempranillo, against *N. parvum* Bt67 (Np-Bt67). The beneficial effect of each BCA has been proved to be cultivar dependent. *Bs* PTA-271 was confirmed as an effective protector for Chardonnay against Np-Bt67, and *Ta* SC1 was shown effective for Tempranillo plants. Authors further showed that even if the effect of each BCA differed depending on the cultivar, there was a common feature to protected cultivars: the SA-dependent defenses were significantly repressed at a systemic level. Although this study confirmed the beneficial effects of *Bs* PTA-271 and *Ta* SC1 in protecting grapevine against Np-

Bt67, and their key action lever, there is still a need to deeply decipher the plant physiological changes induced by each BCA to achieve an effective protection against this pathogen, whatever the cultivar. Indeed, the most precise information will be useful to propose the most effective biocontrol solutions adapted to professional needs.

This recent decade, the RNA sequencing (RNA-seq) method starts such a deciphering of the woody plant responses to phytopathogens and BCAs. The revealing changes in the expression of genes involved in cell wall biosynthesis, but also hormone signaling and secondary metabolism, could therefore be considered important players in the multi-party plant/pathogen/BCA interactions (Xu et al. 2013, Camps et al. 2010, Yacoub et al. 2020).

In the present study, we intend to extensively decipher the plant physiology changes induced by both *Bs* PTA-271 and *Ta* SC1 to protect grapevine upon Np-Bt67 infection, by using transcriptomic analysis. These transcriptomic analyses are complementary to the work of Leal et al. (2021b) and have been performed with the same pool of leaf samples described as statistically robust. The focus was therefore made on the molecular responses of plant to Np-Bt67 using grapevine cuttings cv Chardonnay and Tempranillo, whether or not they have been previously inoculated with *Bs* PTA-271 and/or *Ta* SC1. To our knowledge, this is the first transcriptomic study on the induced changes by two distinct BCAs to two different grapevine cultivars artificially infected with Np-Bt67.

## 2. Material and methods

### 2.1 Biological material and Experimental design

These transcriptomic analyses are complementary to the work of Leal et al. (2021b) using the same samples as those from the experiments conducted and described in Leal et al. (2021b). Therefore, three-node-long cuttings of grapevine cv. Tempranillo and cv. Chardonnay were rooted in a culture chamber (24/20°C day/night, 55–65% relative humidity day/night, with a 16-h photoperiod at 400 µmol/m2/s) then maintained in individual 200-ml pots under the same conditions. The *B. subtilis* PTA-271 (*Bs* PTA-271) inoculations were carried out on 16 weeks old rootlings (considered as day 0) and 2 weeks later (day 15), by drenching the soil at the root level for a final density of $10^8$ CFU/g soil. The inoculation with *T. atroviride* SC1 (*Ta* SC1) was performed once with a paintbrush at the second node of previously wounded lignified stems (5 mm diameter and 1 mm deep) of 18-week-old rootlings, at a final density of $10^8$ CFU/ml (day 15) as described by Leal et al. (2021b). Half of the 20-week-old rootlings that were treated with *Bs* PTA-*271* (day 0 and day 15) and/or *Ta* SC1 (day 15) were further infected

with Np-Bt67 at the wounding area with a 3-mm-diameter mycelial plug from a 7-day-old culture (day 30) as described by Leal et al. (2021b). The experiment was composed of five repetitions for each treatment, with 8–10 rootlings per repetition for each treatment.

**2.2 Sample collection**

Leaf samples from the rootlings (40 leaves of 8 rootlings per replicate of each treatment) were collected 4 days post-inoculation of pathogen (dpi), ground in liquid nitrogen, and then stored at −80°C for RNA extraction. Three replicates per treatment were then selected for the RNA extraction and RNA-seq analysis.

**2.3 RNA extraction and DNAse treatment**

Total RNA was extracted from 50 mg of leaf powder with Plant RNA Purification Reagent according to manufacturer instructions (Invitrogen, Pontoise, France), and DNase treated as described by the manufacturer's instructions (RQ1 RNase-Free DNase, Promega). RNA quality was assessed with an Experion standard sensitivity RNA chip (Bio-Rad, Hercules, CA), and total RNA concentration was measured at 260 nm for each sample using the NanoDrop One spectrophotometer (Ozyme) and adjusted to 100 ng$\mu l^{-1}$.

**2.4 RNA-seq bioinformatic analysis: sample pairwise comparisons based on *V. vinifera* genes**

RNA-seq analysis from the leaf samples were performed by Genewiz® (Azenta Life Sciences, Leipzig, Germany). RNA samples were subjected to poly(A)+ selection, cDNA synthesis, library preparation and deep sequencing using Illumina NovaSeq (2x150 bp reads), leading to 1,328,791,424 reads that were compiled in fastq format.

The estimation of the reads was performed by FastQC (Andrews et al. 2010). Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36 (Bolger et al. 2014). The trimmed reads were mapped to the Vino_Marie reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences.

Unique gene hit counts were calculated using feature Counts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted.

Using DESeq2, a comparison of gene expression between the defined groups of samples was performed. The Wald test was used to generate p-values and log2 fold changes. Genes with

an adjusted p-value < 0.05 and absolute log2 fold change > 1 were called as differentially expressed genes for each comparison.

Venn diagrams were designed using a webtool from Bioinformatics & Evolutionary Genomics (https://bioinformatics.psb.ugent.be/webtools/Venn/). A Gene Ontology (GO) analysis was performed using the GOslim software. The significant enrichment of GO terms based on hypergeometric distribution followed by FDR <0.05 correction was used for comparison between the experimental set and reference set of grapevine species using ShinyGO v0.61 (http://bioinformatics.sdstate.edu/go/) (Ge et al. 2020).

## 3. Results

### 3.1 Brief reminder of published phenotypic results prior to RNA-seq analysis

Leal et al. (2021b) have shown that grapevine susceptibility to BD is cultivar-dependent, as well as BCA beneficial effects. They confirmed *Bs* PTA-271 as an effective protector for Chardonnay against Np-Bt67, and *Ta* SC1 as a good protector for Tempranillo. Authors additionally highlighted a common feature for both protected cultivars: SA-dependent defenses were strongly decreased by the BCA and suggested that the high basal expression of SA-dependent defenses in Tempranillo explains its highest susceptibility to Np-Bt67. They finally reported the beneficial potential of BCA combination against Np-Bt67 in Tempranillo, but not in Chardonnay, and suggested that Chardonnay itself could interfere the fine-tuned cooperation of BCAs that remains to be further investigated.

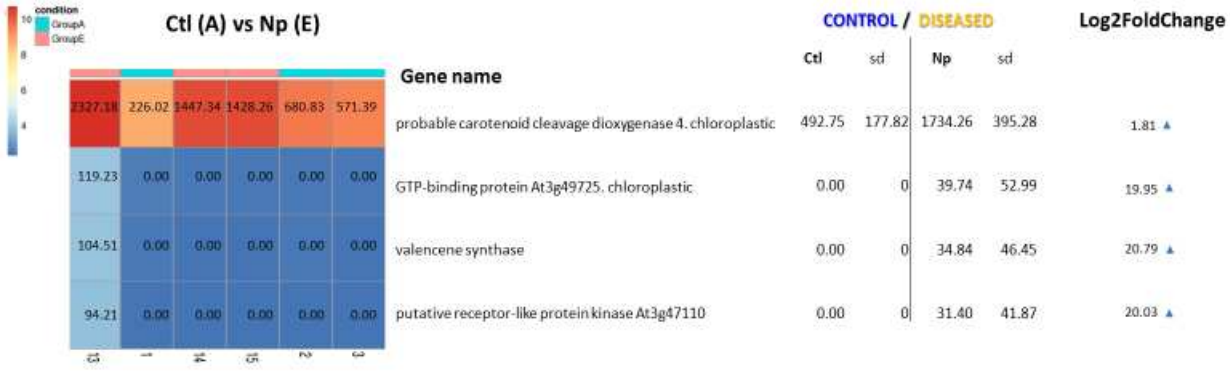
### 3.2 RNA-seq analysis on Chardonnay samples

#### 3.2.1 Differentially Expressed Genes (DEGs) in the leaves of plants infected with Np-Bt67

Compared to control plants (Ctl), plants infected with Np-Bt67 (Np) have four genes significantly overexpressed at 4 dpi (**Fig. 1A**), that encode for: a carotenoid cleavage dioxygenase, a GTP-binding protein, a valence synthase, and a putative receptor-like protein kinase. Similar trends are observed when comparing diseased (Np) with protected plants by *Bs* PTA-271 (NpBs) (**Fig. 1Ba**). However, the expression level of Valencene synthase and GTP-binding protein are indifferently overexpressed in the leaves of diseased plants infected with Np-Bt67 (Np) and unprotected with combined BCA (NpBsTa) (**Fig. 1Bb**), suggesting that these two genes would not be useful key markers associated to Chardonnay disease expression. In contrast, the two genes encoding for a carotenoid cleavage dioxygenase and a putative

receptor-like protein kinase could be useful markers associated to Chardonnay disease expression of BD. They also could be consider as key targets to better protect Chardonnay against Np-Bt67 since they are overexpressed in the leaves of diseased plants, but not in the leaves of plants protected by *Bs* PTA-271.

A




| Gene name | CONTROL / Ctl | sd | DISEASED / Np | sd | Log2FoldChange |
|---|---|---|---|---|---|
| probable carotenoid cleavage dioxygenase 4, chloroplastic | 492.75 | 177.82 | 1734.26 | 395.28 | 1.81 ▲ |
| GTP-binding protein At3g49725, chloroplastic | 0.00 | 0 | 39.74 | 52.99 | 19.95 ▲ |
| valencene synthase | 0.00 | 0 | 34.84 | 46.45 | 20.79 ▲ |
| putative receptor-like protein kinase At3g47110 | 0.00 | 0 | 31.40 | 41.87 | 20.03 ▲ |

B

**a** DISEASED / Protected

| | Np | NpBs |
|---|---|---|
| Valence synthase | 38.00 | 0.00 |
| GTP-binding protein AT3g49725 chloroplastic | 43.00 | 0.00 |

**b** DISEASED / DISEASED

| | Np | NpBsTa |
|---|---|---|
| Valence synthase | 34.95 | 0.00 |
| GTP-binding protein AT3g49725 chloroplastic | 39.88 | 0.00 |

**Figure 1.** Differentially expressed genes (DEGs) in Chardonnay leaves at 4 dpi between: **(A)** control plants (Ctl) and diseased plants once infected with *N. parvum* Bt67 (Np); **(Ba)** diseased plants (Np) and plants protected with *Bacillus subtilis* PTA-271 (NpBs); **(Bb)** diseased plants due to unprotective treatment with combined BCA upon pathogen challenge (NpBsTa) and diseased plants due to pathogen infection (Np). sd: standard deviation.

### 3.2.2 DEGs in leaves of plants protected with *Bs* PTA-271

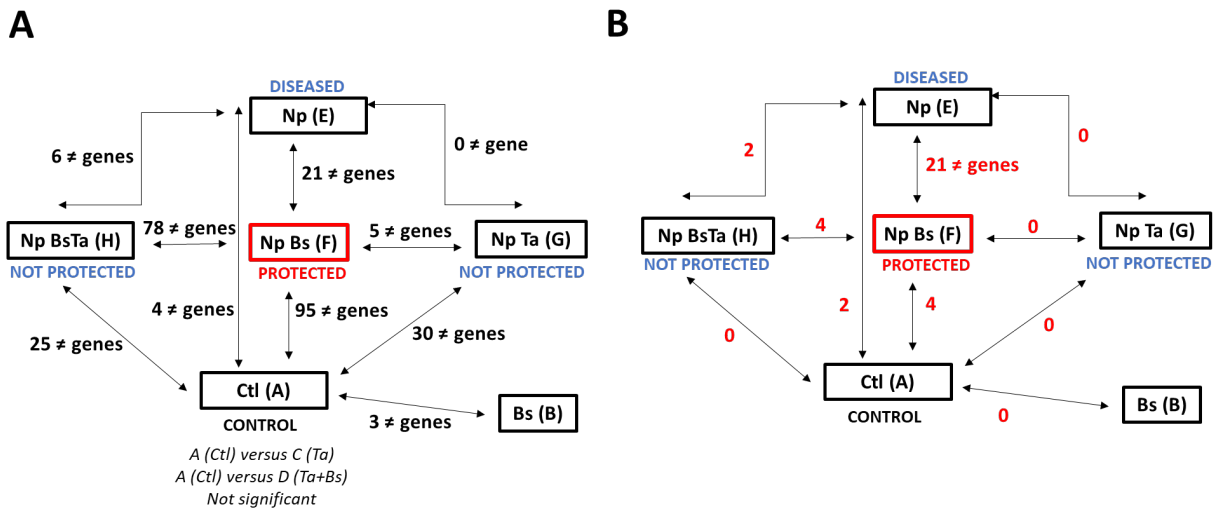
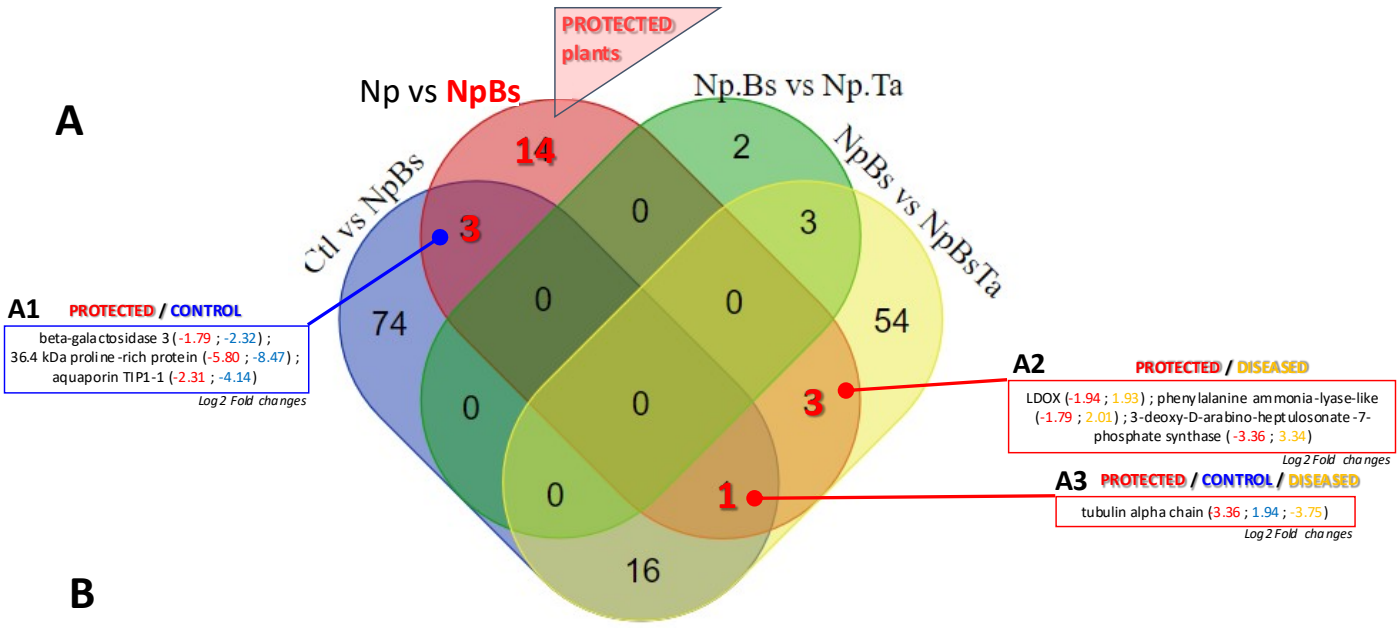



**Figure 2.** DEGs in Chardonnay leaves at 4 dpi: **(A)** between all treatments, and **(B)** common to the 21 DEGs between Np vs NpBs. Treatments are: twenty-week-old rootlings untreated or pretreated with *Bs* PTA-271 or *Ta* SC1 or both, before being infected for 4 days with sterile PDA plugs (Ctl, Bs, Ta, and Ta+Bs, respectively) or with mycelium plugs of Np-Bt67 (Np, Np Bs, Np Ta, and Np BsTa, respectively).

The comparison between all treatments shows 21 DEGs between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Bs* PTA-271 (Np Bs) (**Fig.**



**2A**). The expression level of some of these 21 genes is also modulated in the leaves of unprotected plants (**Fig. 2B**, Np BsTa vs Np, and Np BsTa vs Np Bs), making it necessary to observe in depth their expression level (**Fig. 3**).

**Figure 3.** Common DEGs in Chardonnay leaves at 4 dpi between: control (Ctl), protected (NpBs) and unprotected treatments (Np, NpTa, NpBsTa) of Chardonnay cultivars. Legend is as in Fig. 2.

As indicated in **Fig. 3**, the 21 DEGs (red) in Chardonnay leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Bs* PTA-271 (Np Bs): the tubulin alpha chain is overexpressed at similar levels both in the leaves of uninfected Control plants (**Fig. 3BB1**) and in the leaves of diseased plants (**Fig. 3BB2**). This suggests that this gene would not be a useful key marker associated to Chardonnay disease expression. For the genes encoding for the beta-galactosidase 3, the 36.4 kDa proline-rich protein and the aquaporin TIP1-1, similar expression levels are observed in protected plants with *Bs* PTA-271 (NpBs) and in uninfected Control (Ctl) plants (**Fig. 3BB1**, from 9.15 to 3237.13) as diseased plants (Np, **Fig. 4**, from 288.40 to 569.08). This suggests also that these 3 genes would not be useful key markers associated to Chardonnay disease expression of BD. Genes encoding for LDOX, PAL and a phosphate synthase show opposite trends of expression only in protected plants with *Bs* PTA-271 (NpBs) compared to unprotected diseased plants (**Fig. 3B2, A2 and B2**), suggesting that they could be key targets to consider to better protect Chardonnay against Np-Bt67.

A focus is carried out on the last 14 DEGs of **Fig. 3A**, which are the genes differing in their expression level (**Fig. 4**, highlighted red) between diseased (Np) and protected plants with *Bs* PTA-271 (NpBs). The **Fig. 4** shows all the information for the 21 DEGs observed in Chardonnay leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Bs* PTA-271 (Np Bs).

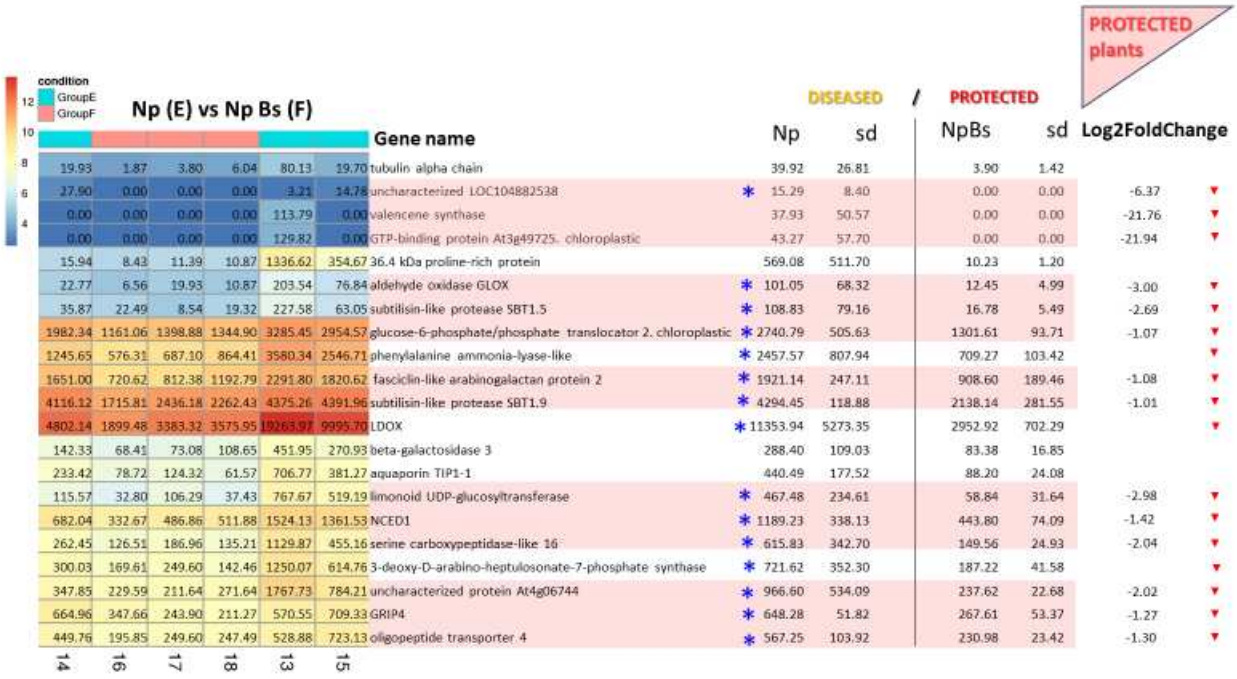



**Figure 4.** Expression levels of the differently expressed genes (DEGs) in Chardonnay leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Bs* PTA-271 (Np Bs). Highlighted red, are the 14 DEGs not yet analyzed in the Figure 3. Genes marked with an asterisk are suspected to be of interest to understand the mode of action of *Bs* PTA-271 to protect Chardonnay grapevines against Np-Bt67. sd: standard deviation.

Among the 21 DEGs identified in Chardonnay leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Bs* PTA-271 (Np Bs), 15 genes are related to the mode of action of *Bs* PTA-271 against Np-Bt67 (**Fig. 4**, blue asterisk).

As shown in **Fig. 4**, all the 15 DEGs correlated with *Bs* PTA-271 protective effect on Chardonnay plants infected with Np-Bt67 are repressed in protected plants (NpBs) compared to diseased plants (Np). These genes of interest are: an uncharacterized LOC104882538, an aldehyde oxidase GLOX, a subtilisin-like protease SBT1.5, a glucose-6-phosphate/phosphate translocator 2 chloroplastic, a phenylalanine ammonia-lyase like, a fasciclin-like arabinogalactan protein 2, a subtilisin like protease SBT1.9, a LDOX, a limonoid UDP-glucosyltransferase, NCED1, a serine carboxypeptidase-like 16, a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, an uncharacterized protein At4g06744, GRIP4, and an oligopeptide transporter 4.

### 3.2.3 Predominant Functions (GO) of *V. vinifera* DEGs in leaves of Chardonnay plants infected with Np-Bt67 and protected with *Bs* PTA-271

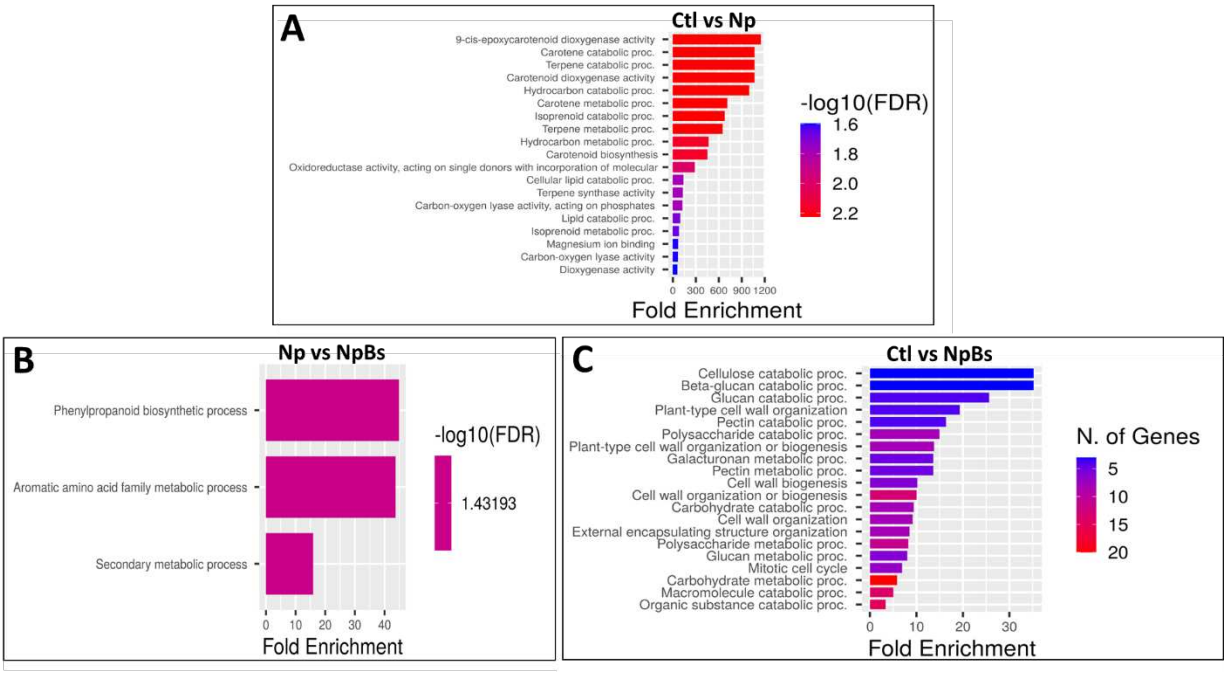



**Figure 5.** Predominant functions of DEGs in Chardonnay leaves at 4 dpi between: **(A)** control and infected plants (Ctl vs Np), **(B)** infected and protected plants with *Bacillus subtilis* PTA-271 (Np vs NpBs), and **(C)** control and protected plants with *Bacillus subtilis* PTA-271 (Ctl vs NpBs). FDR: False discovery rate.

As shown in **Fig. 5A**, plants infected with Np-Bt67 (Np) have DEGs compared to control plants (Ctl), that may induce changes in dioxygenase activities and oxidoreductase activities, in relationship with pigment production (carotene and carotenoid biosynthesis), hormonal balance (terpene/ isoprenoid pathway and cellular lipidic catabolic processes) and hydrocarbon metabolic processes (among which carbon-oxygen lyase activity, that may act on phosphatases).

In **Fig. 5B**, infected plants protected with *Bs* PTA-271 (NpBs) compared to infected plants (Np) show DEGs associated to changes in the phenylpropanoid biosynthetic process, aromatic amino acid family metabolic process, and secondary metabolic process.

Finally, DEGs in infected plants protected with *Bs* PTA-271 (NpBs) compared to control plants (Ctl), highlight changes in mitotic cell cycle and catabolic processes of organic substances / macromolecules. This impacts thus the cell wall structure organization and

biogenesis, through changes in galacturonan, pectin, cellulose, polysaccharide, glucan and carbohydrate metabolic processes **(Fig. 5C)**.

### 3.3 RNA-seq analysis on Tempranillo samples

#### 3.3.1 Major DEGs in the leaves of plants infected with Np-Bt67

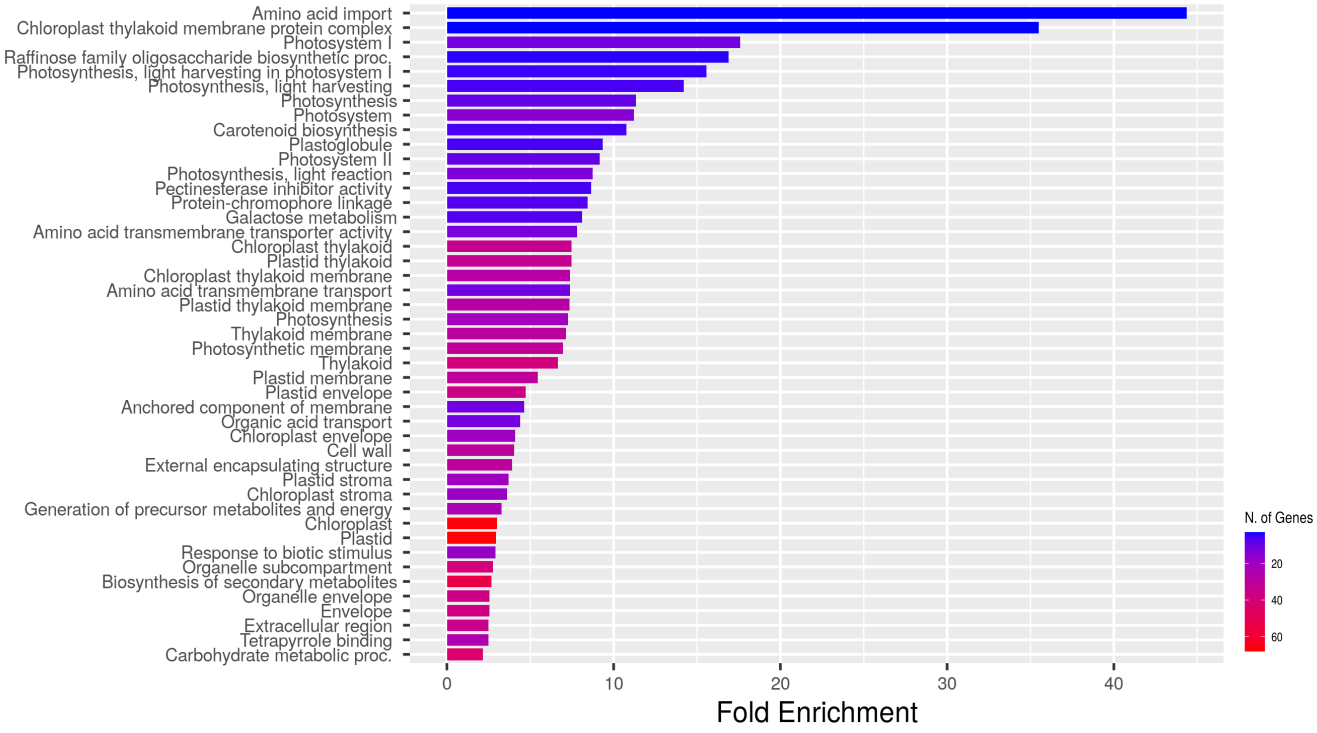



**Figure 6.** Predominant functions of DEGs in Tempranillo leaves at 4 dpi between control plants and Np-Bt67 infected plants.

Compared to control plants, the leaves of plants infected with Np-Bt67 showed 567 differentially expressed genes (DEGs) at 4 dpi. **Figure 6** only reports the predominant function of these genes which are related to amino acid import, chloroplast, and photosystem related processes, as well as the plant responses to biotic stimulus, and biosynthesis of secondary metabolites.

### 3.3.2 DEGs in leaves of plants protected with *Ta* SC1

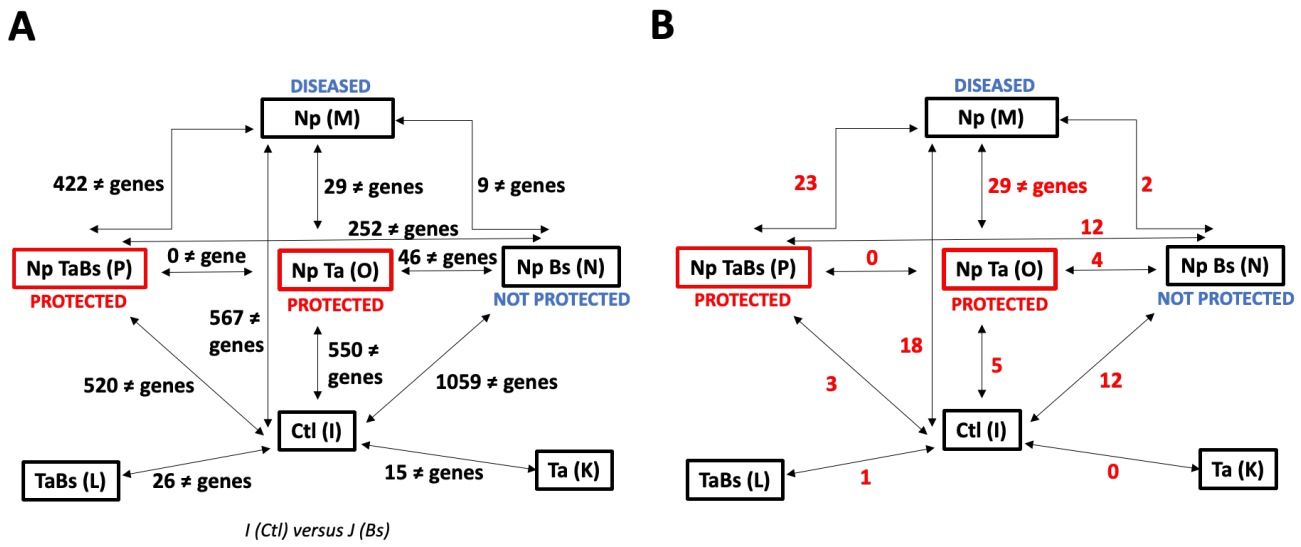
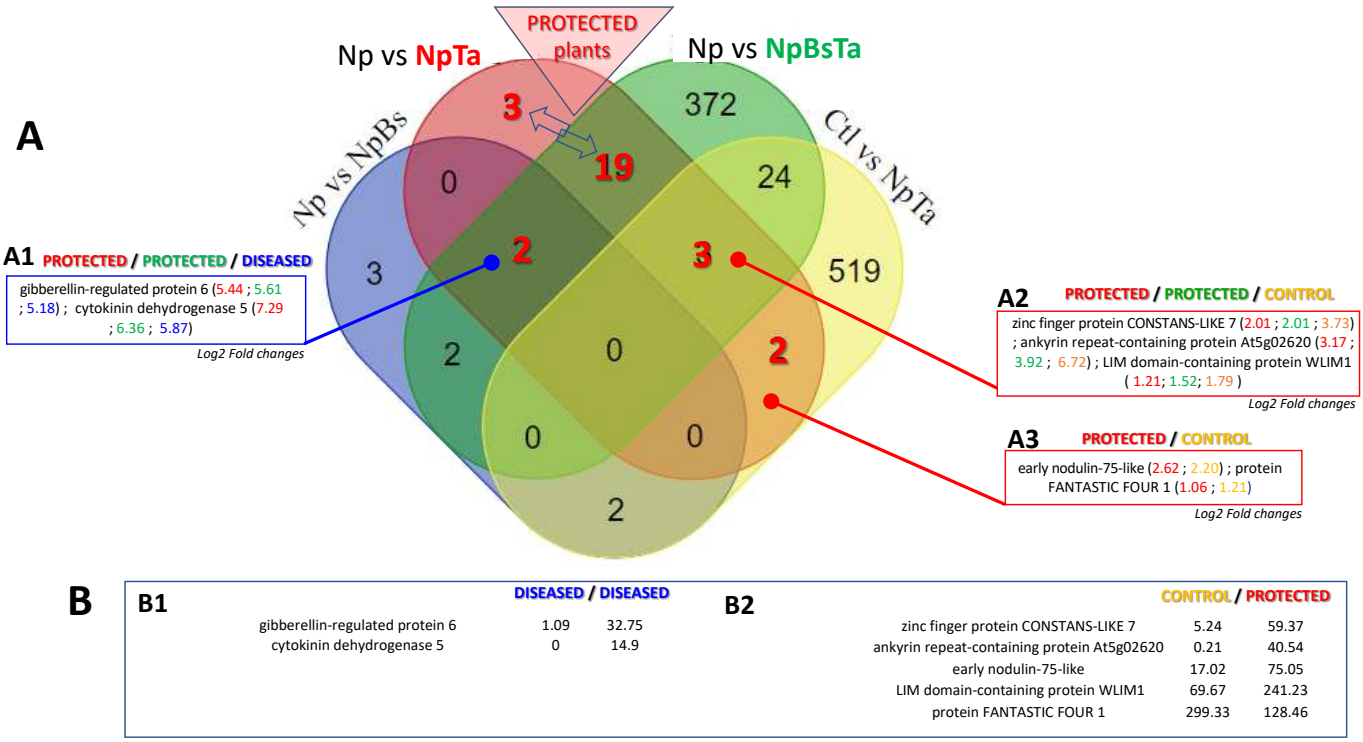



**Figure 7.** Differently expressed genes (DEGs) in Tempranillo leaves at 4 dpi: **(A)** between all treatments, and **(B) common to the 29** DEGs between Np vs NpTa (B). Treatments are: twenty weeks old rootlings untreated or pretreated with *Bs* PTA-271 or *Ta* SC1 or both, before being infected for 4 days with sterile PDA plugs (Ctl, Bs, Ta, and Ta+Bs, respectively) or with mycelium plugs of Np-Bt67 (Np, Np Bs, Np Ta, and Np BsTa, respectively).

The comparison of all treatments (**Fig. 7A**) shows 29 DEGs between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Ta* SC1 (Np Ta). The expression level of some of these 29 genes is also modulated in the leaves of unprotected plants (**Fig. 7B**, Np TaBs vs Np Bs, and Np Ta vs Np Bs), hence the need for in-depth observation of their expression level (**Fig. 8**).




**B**

**B1**

| | DISEASED | DISEASED |
|---|---|---|
| gibberellin-regulated protein 6 | 1.09 | 32.75 |
| cytokinin dehydrogenase 5 | 0 | 14.9 |

**B2**

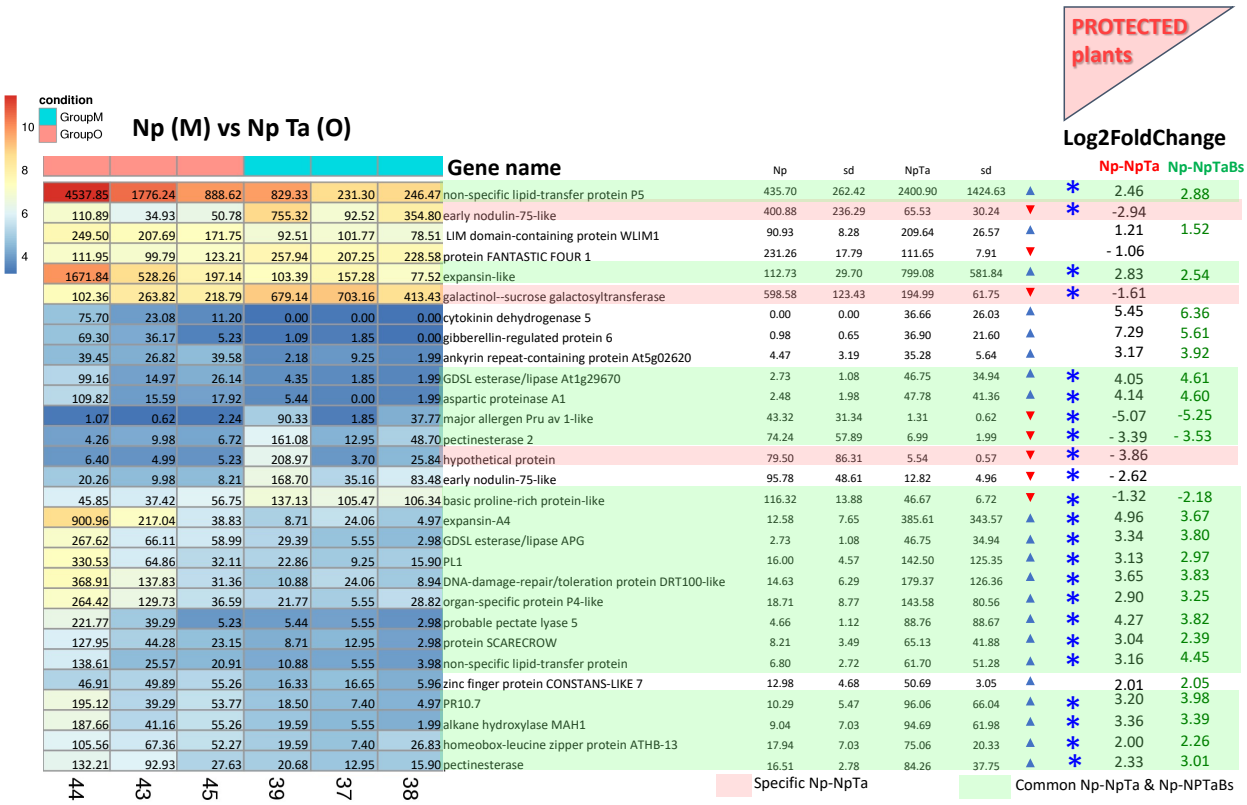
| | CONTROL | PROTECTED |
|---|---|---|
| zinc finger protein CONSTANS-LIKE 7 | 5.24 | 59.37 |
| ankyrin repeat-containing protein At5g02620 | 0.21 | 40.54 |
| early nodulin-75-like | 17.02 | 75.05 |
| LIM domain-containing protein WLIM1 | 69.67 | 241.23 |
| protein FANTASTIC FOUR 1 | 299.33 | 128.46 |

**Figure 8.** Common DEGs in Tempranillo leaves at 4 dpi between: control (Ctl), protected (NpTa and NpBsTa) and unprotected treatments (Np and NpBs) of Tempranillo cultivars. Legend is as in Fig. 7.

As represented in **Fig. 8**, among the 29 DEGs (red) in Tempranillo leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants protected with *Ta* SC1 (NpTa): cytokinin dehydrogenase 5 and gibberellin-regulated protein 6 are expressed at similar levels (**Fig. 8 AA1**) in protected and unprotected plants. This suggests that these two genes would not be a useful key marker associated to Tempranillo disease expression of BD, nor be key targets to consider to better protect Tempranillo against Np-Bt67. Similarly, patterns are observed for genes encoding zing finger protein CONSTANS-LIKE 7, ankyrin repeat-containing protein At5g02620, LIM domain-containing protein WLIM1 and the protein FANTASTIC FOUR 1 (Fig. 8 **BB2)**. This suggests that these 4 genes would not be useful key markers associated to Tempranillo disease expression of BD. In contrast, gene encoding for the early-nodulin-75-like is less expressed in control (Ctl, 17.02 in **Fig. 8 BB2**) and protected plants with *Ta* SC1 (NpTa, 75.05 in **Fig. 8 BB2**) than in diseased plants (Np, 400.88 in **Fig. 9**).

The last 23 DEGs (**Fig. 8A)** are the genes differing in their expression level between diseased (Np) and protected plants with *Ta* SC1 (NpTa) or with both BCA (NpTaBs) (**Fig. 9**). The

**Fig. 9** shows all the information for the 23 DEGs observed in Tempranillo leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Ta* SC1 (Np Ta).




| | | | | | | Gene name | Np | sd | NpTa | sd | | | Np-NpTa | Np-NpTaBs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4537.85 | 1776.24 | 888.62 | 829.33 | 231.30 | 246.47 | non-specific lipid-transfer protein P5 | 435.70 | 262.42 | 2400.90 | 1424.63 | ▲ | * | 2.46 | 2.88 |
| 110.89 | 34.93 | 50.78 | 755.32 | 92.52 | 354.80 | early nodulin-75-like | 400.88 | 236.29 | 65.53 | 30.24 | ▼ | * | -2.94 | |
| 249.50 | 207.69 | 171.75 | 92.51 | 101.77 | 78.51 | LIM domain-containing protein WLIM1 | 90.93 | 8.28 | 209.64 | 26.57 | ▲ | | 1.21 | 1.52 |
| 111.95 | 99.79 | 123.21 | 257.94 | 207.25 | 228.58 | protein FANTASTIC FOUR 1 | 231.26 | 17.79 | 111.65 | 7.91 | ▼ | | - 1.06 | |
| 1671.84 | 528.26 | 197.14 | 103.39 | 157.28 | 77.52 | expansin-like | 112.73 | 29.70 | 799.08 | 581.84 | ▲ | * | 2.83 | 2.54 |
| 102.36 | 263.82 | 218.79 | 679.14 | 703.16 | 413.43 | galactinol--sucrose galactosyltransferase | 598.58 | 123.43 | 194.99 | 61.75 | ▼ | * | -1.61 | |
| 75.70 | 23.08 | 11.20 | 0.00 | 0.00 | 0.00 | cytokinin dehydrogenase 5 | 0.00 | 0.00 | 36.66 | 26.03 | ▲ | | 5.45 | 6.36 |
| 69.30 | 36.17 | 5.23 | 1.09 | 1.85 | 0.00 | gibberellin-regulated protein 6 | 0.98 | 0.65 | 36.90 | 21.60 | ▲ | | 7.29 | 5.61 |
| 39.45 | 26.82 | 39.58 | 2.18 | 9.25 | 1.99 | ankyrin repeat-containing protein At5g02620 | 4.47 | 3.19 | 35.28 | 5.64 | ▲ | | 3.17 | 3.92 |
| 99.16 | 14.97 | 26.14 | 4.35 | 1.85 | 1.99 | GDSL esterase/lipase At1g29670 | 2.73 | 1.08 | 46.75 | 34.94 | ▲ | * | 4.05 | 4.61 |
| 109.82 | 15.59 | 17.92 | 5.44 | 0.00 | 1.99 | aspartic proteinase A1 | 2.48 | 1.98 | 47.78 | 41.36 | ▲ | * | 4.14 | 4.60 |
| 1.07 | 0.62 | 2.24 | 90.33 | 1.85 | 37.77 | major allergen Pru av 1-like | 43.32 | 31.34 | 1.31 | 0.62 | ▼ | * | -5.07 | -5.25 |
| 4.26 | 9.98 | 6.72 | 161.08 | 12.95 | 48.70 | pectinesterase 2 | 74.24 | 57.89 | 6.99 | 1.99 | ▼ | * | - 3.39 | - 3.53 |
| 6.40 | 4.99 | 5.23 | 208.97 | 3.70 | 25.84 | hypothetical protein | 79.50 | 86.31 | 5.54 | 0.57 | ▼ | * | - 3.86 | |
| 20.26 | 9.98 | 8.21 | 168.70 | 35.16 | 83.48 | early nodulin-75-like | 95.78 | 48.61 | 12.82 | 4.96 | ▼ | * | - 2.62 | |
| 45.85 | 37.42 | 56.75 | 137.13 | 105.47 | 106.34 | basic proline-rich protein-like | 116.32 | 13.88 | 46.67 | 6.72 | ▼ | * | -1.32 | -2.18 |
| 900.96 | 217.04 | 38.83 | 8.71 | 24.06 | 4.97 | expansin-A4 | 12.58 | 7.65 | 385.61 | 343.57 | ▲ | * | 4.96 | 3.67 |
| 267.62 | 66.11 | 58.99 | 29.39 | 5.55 | 2.98 | GDSL esterase/lipase APG | 2.73 | 1.08 | 46.75 | 34.94 | ▲ | * | 3.34 | 3.80 |
| 330.53 | 64.86 | 32.11 | 22.86 | 9.25 | 15.90 | PL1 | 16.00 | 4.57 | 142.50 | 125.35 | ▲ | * | 3.13 | 2.97 |
| 368.91 | 137.83 | 31.36 | 10.88 | 24.06 | 8.94 | DNA-damage-repair/toleration protein DRT100-like | 14.63 | 6.29 | 179.37 | 126.36 | ▲ | * | 3.65 | 3.83 |
| 264.42 | 129.73 | 36.59 | 21.77 | 5.55 | 28.82 | organ-specific protein P4-like | 18.71 | 8.77 | 143.58 | 80.56 | ▲ | * | 2.90 | 3.25 |
| 221.77 | 39.29 | 5.23 | 5.44 | 5.55 | 2.98 | probable pectate lyase 5 | 4.66 | 1.12 | 88.76 | 88.67 | ▲ | * | 4.27 | 3.82 |
| 127.95 | 44.28 | 23.15 | 8.71 | 12.95 | 2.98 | protein SCARECROW | 8.21 | 3.49 | 65.13 | 41.88 | ▲ | * | 3.04 | 2.39 |
| 138.61 | 25.57 | 20.91 | 10.88 | 5.55 | 3.98 | non-specific lipid-transfer protein | 6.80 | 2.72 | 61.70 | 51.28 | ▲ | * | 3.16 | 4.45 |
| 46.91 | 49.89 | 55.26 | 16.33 | 16.65 | 5.96 | zinc finger protein CONSTANS-LIKE 7 | 12.98 | 4.68 | 50.69 | 3.05 | ▲ | | 2.01 | 2.05 |
| 195.12 | 39.29 | 53.77 | 18.50 | 7.40 | 4.97 | PR10.7 | 10.29 | 5.47 | 96.06 | 66.04 | ▲ | * | 3.20 | 3.98 |
| 187.66 | 41.16 | 55.26 | 19.59 | 5.55 | 1.99 | alkane hydroxylase MAH1 | 9.04 | 7.03 | 94.69 | 61.98 | ▲ | * | 3.36 | 3.39 |
| 105.56 | 67.36 | 52.27 | 19.59 | 7.40 | 26.83 | homeobox-leucine zipper protein ATHB-13 | 17.94 | 7.03 | 75.06 | 20.33 | ▲ | * | 2.00 | 2.26 |
| 132.21 | 92.93 | 27.63 | 20.68 | 12.95 | 15.90 | pectinesterase | 16.51 | 2.78 | 84.26 | 37.75 | ▲ | * | 2.33 | 3.01 |
| 44 | 43 | 45 | 39 | 37 | 38 | | | | | | | | | |

**Figure 9.** Expression level of the differently expressed genes (DEGs) in Tempranillo leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Ta* SC1 (Np Ta). Genes marked with an asterisk are suspected to be of interest to understand the mode of action of *Ta* SC1 to protect Tempranillo grapevines against Np-Bt67. The last column shows the differential expression of the genes of interest in response to the two protective treatments, using either *Ta* SC1 (Np-NpTa) or combined BCA (Np-NpTaBs).

Among the 29 DEGs identified in Tempranillo leaves at 4 dpi between diseased infected plants with Np-Bt67 (Np) and infected plants that are protected with *Ta* SC1 (Np Ta), 23 genes are considered involved in the mode of action of *Ta* SC1 against Np-Bt67 (**Fig. 9**, blue asterisk). Indeed, the analysis performed from **Fig. 7 to Fig. 8** allows to exclude the 6 irrelevant genes not marked with an asterisk (namely: LIM domain-containing protein WLIM1, the protein FANTASTIC FOUR 1, cytokinin dehydrogenase 5, gibberellin-regulated protein 6, ankyrin repeat-containing protein At5g02620, and zing finger protein CONSTANS-LIKE 7).

As shown in **Fig. 9**, all the 23 DEGs correlated with *Ta* SC1 protective effect on Tempranillo plants infected with Np-Bt67 are either repressed or over-induced in protected plants (NpTa and Np-NpTaBs) compared to diseased plants (Np). There are 7 genes in which the expression is repressed in protected plants: 2 genes encoding for early-nodulin-75-like proteins, a galactinol-sucrose glucosyltransferase, a major allergen Pru av1-like, a pectin esterase 2, a basic proline-rich protein-like, and another gene encoding for a hypothetical protein. There are 16 genes whose expression is over-expressed in protected plants, encoding for: non-specific lipid-transfer protein P5, another non-specific lipid-transfer protein, expansin-like, expansin-A4, GDSL esterase/lipase At1g29670, GDSL esterase / lipase APG, aspartic proteinase A1, PL1, DNA-damage-repair / toleration protein DRT100-like, organ-specific protein P4-like, probable pectate lyase 5, protein SCARECROW, PR10.7, alkane hydroxylase MAH1, homeobox-leucine zipper protein ATHB-13, and pectin esterase.

### 3.3.3 Predominant Functions (GO) of *V. vinifera* DEGs in leaves of Tempranillo plants infected with *Np-Bt67* and protected with *Ta* SC1

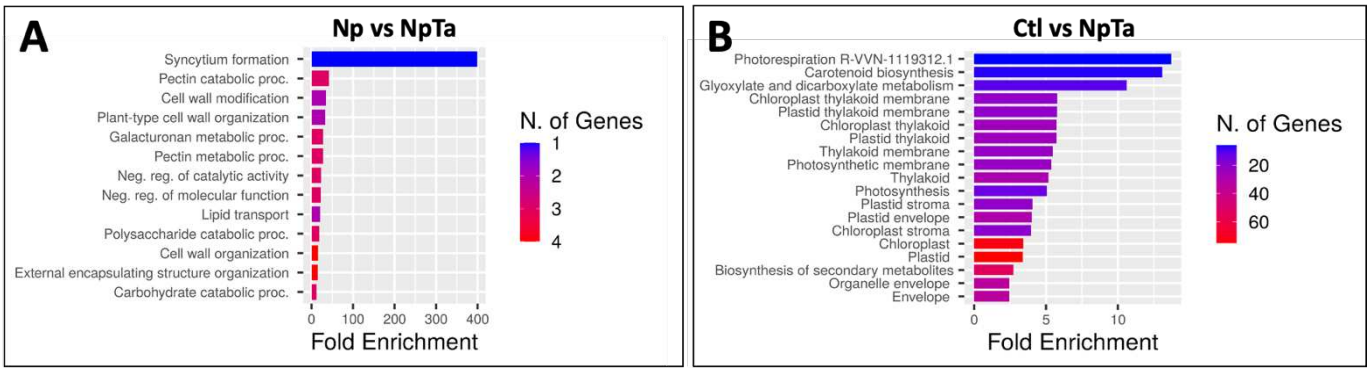



**Figure 10.** Predominant functions of DEGs in Tempranillo leaves at 4 dpi between: **(A)** infected and protected plants with *Trichoderma atroviride Ta* SC1 (Np vs NpTa), and **(B)** control and protected plants with *Trichoderma atroviride Ta* SC1 (Ctl vs NpTa).

As shown in **Fig. 10A**, the DEGs in infected plants protected with *Ta* SC1 (NpTa) compared to infected plants (Np), are related to cell and cell wall formations (syncytium formation, pectin catabolic process, cell wall modification, plant-type cell wall organization, galacturonan and pectin metabolic process), protein quenching and their expression silencing, but also lipid transport, and carbohydrate/polysaccharide catabolism.

When comparing infected plants protected with *Ta* SC1 (NpTa) to control plants (Ctl), changes concern chloroplast related processes such as photosynthesis and photorespiration, and in biosynthesis of secondary metabolites **(Fig. 10B)**.

## 4. Discussion

In Leal et al. (2021b), we evaluated the effect of *Bs* PTA-271 and *Ta* SC1 on the protection of two distinct grapevine cultivars, Chardonnay and Tempranillo, against Np-Bt67 symptoms. We provided evidence that grapevine susceptibility to BD is cultivar-dependent, as well as the BCA beneficial effects. Indeed, *Bs* PTA-271 was effective on the protection of Chardonnay against Np-Bt67, while *Ta* SC1 alone or combined with *Bs* PTA-271 was effective to protect Tempranillo against Np-Bt67 (80 and 90%, respectively). Authors additionally highlighted a common metabolic feature for both protected cultivars and suggested that plant metabolism conditions both pathogen infection and the beneficial effects of BCA.

In the present study, the same leaf samples as those used by Leal et al. (2021b) were deeply analyzed by RNA-seq, to further study the plant molecular changes associated with both (1) the infection by Np-Bt67, and (2) the protection induced by *Bs* PTA-271 and/or *Ta* SC1.

In **Chardonnay plants infected with Np-Bt67**, **2** up-regulated genes encoding for a **carotenoid cleavage dioxygenase** and a putative receptor-like protein kinase are associated to Chardonnay disease expression. In good accordance, the predominant functions (GO terms) impacted in the leaves of Np-Bt67 infected Chardonnay plants involved dioxygenase and oxidoreductase activities, in relationship with pigment production (carotene and carotenoid biosynthesis), hormonal balance (terpene / isoprenoid pathway and cellular lipidic catabolic processes) and hydrocarbon metabolic processes (among which carbon-oxygen lyase activity, acting on phosphatases). Indeed, carotenoids in plants are responsible for the biosynthesis of some phytohormones such as abscisic acid (ABA) and the strigolactones through the isoprenoid pathway (Walter and Strack 2011). The oxidative cleavage of carotenoids is catalyzed by enzymes from the dioxygenase family (CCD, carotenoid cleavage dioxygenase), that later initiate the first step of ABA biosynthesis pathway (Zhang et al. 2015). The ABA phytohormone is not only important to mediate plant adaptation to abiotic stress, but it is

also described as playing roles in plant immunity (Cao et al. 2011). The second category of upregulated genes upon Np-Bt67 infection in Chardonnay, involved a putative receptor-like protein kinase. **Receptor-like kinases (RLKs)** play critical roles in the regulation of plant developmental processes, signaling networks and disease resistance (Ye et al. 2017). As membrane-localized receptors, RLKs perceive extracellular stimuli through an extracellular ligand-binding domain, then transduce the signal by phosphorylating substrates through a cytoplasmic kinase domain (Jose et al. 2020). Interestingly, among the GO terms impacting leaves of Chardonnay plants infected with Np-Bt67 are hydrocarbon metabolic processes that involve a carbon-oxygen lyase activity acting on **phosphatases**. Indeed, ABA signaling pathways is initiated by a physical inactivation of phosphatases by ABA, allowing further ABA signal transduction through auto-phosphorylation processes (Umezawa et al. 2009). **In summary, ABA signaling appears to be involved in disease expression of BD on Chardonnay plants** since the two upregulated genes associated with Chardonnay disease expression lead to both ABA production and ABA signaling. Similar results were observed with the red cultivar Cabernet Sauvignon trunk when exposed to esca-associated fungal pathogens (Romeo-Olivan et al. 2022). In this study we further propose that the two genes encoding for a carotenoid cleavage dioxygenase and a putative receptor-like protein kinase could represent useful dieback markers of Chardonnay leaves exposed to *Np*-Bt67. They could also be targets to consider to better protect Chardonnay plants against Np-Bt67 since these genes are both repressed in the leaves of plants protected by *Bs* PTA-271.

**In infected Chardonnay plants protected with *Bs* PTA-271**, GO terms analyses indicate that the *Bs* PTA-271 protection involves changes in the phenylpropanoid biosynthetic process, aromatic amino acid family metabolic process, and secondary metabolic process. Compared to infected diseased plants, only **15** genes are downregulated in protected plants, encoding for : an uncharacterized LOC104882538, an aldehyde oxidase GLOX, a subtilisin-like protease SBT1.5, a glucose-6-phosphate/phosphate translocator 2 chloroplastic, a phenylalanine ammonia-lyase like, a fasciclin-like arabinogalactan protein 2, a subtilisin like protease SBT1.9, a LDOX, a limonoid UDP-glucosyltransferase, NCED1, a serine carboxypeptidase-like 16, a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, an uncharacterized protein At4g06744, GRIP4, and an oligopeptide transporter 4. Since the downregulation of these genes by a BCA is correlated with *Vitis vinifera* protection against *Np*-Bt67, we suggest that these genes could be additional targets to consider to better protect Chardonnay against Np-Bt67. Some of them encode for a key enzyme involved in **ABA biosynthesis** (He et al. 2018;

Yao et al. 2022), namely *NCED1* (*9-cis-epoxycarotenoid dioxygenase*), whose repression directly prevents the ABA production, previously suggested to favour disease expression. Another gene also potentially related to ABA signaling is the s*ubtilin-like protease SBT1.5*, a serine protease (or subtilase) described to contribute to the regulation of cell wall structure, plant immune responses, and cell-to-cell signaling during symbiosis by suppressing host defense (Schaller et al. 2017; Xu et al. 2019). Proteases are also described to recycle the receptors required to ABA signaling (Irigoyen et al. 2014), receptors themselves stabilized by ABA (Irigoyen et al.,2014). Thus, decreasing their expression level would seriously interfere with ABA signaling. Genes linked to the **phenylpropanoid pathways and derivatives** are: the *phenylalanine ammonia-lyase-like* (*PAL*) that encodes for an enzyme involved in the biosynthesis of many phenolic compounds such as flavonoids and stilbenes (Urban et al. 2018); The *3-deoxy-D-arabino-heptulosonate-7-phosphate synthase* (*DHAP*) is the first enzyme in a series of metabolic reactions that are responsible for the biosynthesis of amino acids, like phenylalanine (substrate of PAL) (Ganson et al. 1986); *Serine carboxypeptidase-like 16* is involved in the synthesis of flavonoids, facilitating the transacylation reaction of a large variety of phenolics, acids, saponins, and other compounds (Ahmad et al. 2020); *Leucoanthocyanidin dioxygenase* (*LDOX*) is involved in the flavonoid pathway, catalyzing hydroxylation to protect plants against abiotic stress (Wang et al. 2021). Thus, a decrease in the expression level of these genes could possibly prevent the production of flavonoids, in favor of stilbenes that are described as useful secondary metabolites with antimicrobial properties to consider against Np-Bt67 (El Kattab et al. 2021). **Secondary metabolites** include terpenes, phenolic compounds and alkaloids, which activities can be modified by glycosylation, acetylation, hydroxylation, redox changes, etc. Therefore, the contribution of *Bs* PTA-271 to repress the expression of enzymes involved in such processes could contribute to activate useful plant molecules once treated with *Bs* PTA-271, unless their repression aims to inactivate damaging molecules produced by the pathogen Np-Bt67. Genes encoding for such enzymes are: *Glyoxal oxidases* described as genes encoding for the extracellular $H_2O_2$-generating enzymes GLOX (Daou and Faulds 2017). The ubiquist *oligopeptide transporter 4* (*OPT4*) known to transport tetra- and penta-peptides, and peptide-like substrates such as glutathione (Pike et al. 2009); Glycosyltransferases, like *limonoid UDP-glucosyltransferase*, involved in hormone homeostasis, defense responses such as detoxification processes, but also biosynthesis and modification of secondary metabolites (Huang and Hou 2009). *Fasciclin-like arabinogalactan proteins* (*FLAs*) are a subclass of arabinogalactan proteins (AGPs), which are described as plant cell wall hydroxyproline-rich glycoproteins (HRGPs) post-translationally

modified by glycosylation (Showalter and Basu 2016). To date, HRGPs are designated as important to strengthen plant cell walls and contribute to plant defense reactions (Deepak et al. 2010). But the decrease in the expression level of *FLA* genes in Chardonnay plants treated with *Bs* PTA-271 suggests that a less rigid cell wall structure could favor the beneficial effects of BCA against Np-Bt67. The GO terms analyses, when comparing the infected plants protected with *Bs* PTA-271 to control plants, also point out that the beneficial effect of this BCA requires changes in macromolecules and carbohydrate metabolic processes, impacting on the biogenesis and organization of the **cell wall structure**. Looking at genes involved in carbohydrate metabolism, the *glucose-6-phosphate/phosphate translocator 2* is described to regulate and stabilize photosynthetic electron transport and carbon metabolism, and to accumulate in the plant leaves that collect sugars (Li et al. 2019). Therefore, the decrease in the expression level of this gene in infected plant protected with *Bs* PTA-271 could contribute to prevent sugar accumulation that would be more favorable to pathogen development or aggressiveness (Leal et al. 2022, review submitted to Phytopathology). **In summary, *Bs* PTA-271 beneficial effect on Chardonnay plants against BD** correlates with the downregulation of genes involved in ABA biosynthesis and phenylpropanoid pathway, especially flavonoid derivatives, as well as a downregulation of genes encoding for post-translational modifications of secondary metabolites whatever their origin. Changes in the biogenesis and organization of the plant cell wall structure also appear to assert *Bs* PTA-271 beneficial effect against Np-Bt67, in relationship with carbohydrate metabolism that require much more consideration.

**Tempranillo plants infected with Np-Bt67** present much more molecular changes in their leaves (567 differentially expressed genes) than in Chardonnay plants (2 upregulated genes). GO term analyses of Tempranillo data indicate predominant transcriptomic changes for **amino acid import**, **chloroplast and photosystem related processes**, plant **responses to biotic stimulus**, and biosynthesis of **secondary metabolites**. In particular, the upregulation of ***early-nodulin-75-like*** in diseased plants, compared to control and protected plants, suggests that protein transporters are critical for disease expression. Indeed, protein transporters can redirect or control the flow of nutrients, amino acids, hormones, and many other solutes such as carbohydrates, within the plant and/or towards the pathogen to reinforce its fitness or aggressiveness due to phytotoxin production (Trotel-Aziz et al. 2019, Leal et al. Review to Phytopathology 2022). Therefore, ***early-nodulin-75-like*** represents a useful early-marker associated with Np-Bt67 disease expression in Tempranillo plants. **Altogether**, despite much more consistent changes in the infected Tempranillo cultivars compared to the Chardonnay

plants, our whole data on the two infected cultivars are in agreement with several recent studies indicating that grapevine infection with a GTD pathogen induces a profound reprogramming of defense gene expression in the plant trunk and leaves to better serve pathogen expansion and/or aggressiveness (Pierron et al., 2016; Massonnet et al., 2017; Gonçalves et al., 2019; Trotel-Aziz et al., 2019; Labois et al., 2020).

**In infected Tempranillo protected with *Ta* SC1**, GO terms analyses indicate that *Ta* SC1 protection also correlates with changes in **cell wall formation** (syncytium formation, pectin catabolic process, cell wall modification, plant-type cell wall organization, galacturonan and pectin metabolic process), **protein quenching and expression silencing**, **lipid transport**, and **carbohydrate/polysaccharide catabolism**. GO terms analyses comparing infected plants protected with *Ta* SC1 and control plants, additionally highlighted that *Ta* SC1 beneficial effect requires changes in chloroplast related processes such as **photosynthesis and photorespiration**, and biosynthesis of **secondary metabolites**. RNA-seq analysis reveals **7 DOWNREGULATED GENES**, among which: 2 genes encoding for early-nodulin-75-like proteins, a galactinol-sucrose glucosyltransferase, a major allergen Pru av1-like, a pectin esterase 2, a basic proline-rich protein-like, and another gene encoding for a hypothetical protein. **Silencing** of transporters such as *early-nodulin-75-like proteins* is described to be linked to a suppression of plant defense signals to allow a better colonization by beneficial microorganisms such as BCAs (Tripathi et al. 2019). **Transporters** can also redirect or control the flow of many nutrients (e.g. amino acids, hormones and many other solutes) to favor pathogen fitness and aggressiveness, instead of that of the host. Silencing the *galactinol-sucrose galactosyltransferase* involved in the production of galactinol and raffinose, could also enable to prevent their use as **nutrients** for pathogens (Meyer et al. 2018). The encoding allergen *Pru av1-like* is described as a lipid transfer protein (Scheurer et al. 2001) whose silencing by *Ta* SC1 is suggested to decrease Tempranillo **susceptibility to pathogen** infection. *Proline rich proteins* (*PRPs*) are other indispensable plant proteins that support several developmental processes from germination to plant death. Downregulating *PRP* genes is described as helpful to suppress **pathogen induced cell death** and to enhance the plant resistance to diseases (Yeom et al. 2012; Gujjar et al. 2019). *Pectin esterase* genes encode for proteins that catalyze the hydrolysis of pectin into pectate and methanol. In plants, they are described to play important roles in cell wall metabolism during fruit ripening, cell wall extension during pollen germination and can vary in plant response to pathogens (Liu et al. 2018). The silencing of *pectin esterase* genes by *Ta* SC1 is suggested to preserve **cell wall integrity** and avoid the use

of cell wall components (e.g., lipid, carbohydrates, etc.) as nutrients able to favor pathogen fitness or aggressiveness. RNA-seq analysis also reveals 16 upregulated genes **in infected Tempranillo protected with *Ta* SC1**, among which: two non-specific lipid-transfer protein (nsLTPs such as P5), two expansin-like (as A4), two GDSL esterase/lipase (as At1g29670 and APG), the aspartic proteinase A1, PL1, DNA-damage-repair / toleration protein DRT100-like, organ-specific protein P4-like, probable pectate lyase 5, protein SCARECROW, PR10.7, alkane hydroxylase MAH1, homeobox-leucine zipper protein ATHB-13, and pectin esterase. Most of these genes encode for substances that confer resistance to biotic and abiotic stresses, with antifungal and antimicrobial potential. For instance, gene encoding for non-specific Lipid Transfer Proteins (*nsLTPs*) are small basic proteins able to (1) bind and transport a variety of hydrophobic molecules, and to (2) work in synergy with other antimicrobial peptides such as defensins and thionines, thus taking part to a greater a **network of immunologically active proteins** (Missaoui et al. 2022). Genes encoding for GDSL esterase/lipases (GELPs) are also overexpressed by *Ta* SC1 (*GDSL esterase/lipase At1g29670* and *GDSL esterase/lipase APG*). GELP proteins represent a variety of lipolytic enzymes that hydrolyze diverse lipidic substrates including thioesters, aryl esters, and phospholipids. They play an important role in vegetative and reproductive development, plant metabolism, and especially plant-environment interactions, like **microbiome interactions** (Chen et al. 2022). Other proteins encoded by *expansin like* genes can be produced by *Trichoderma species* to rapidly induce the **extension of plant cell walls** (Brotman et al. 2008; Marowa et al. 2016); Bacteriocins, such as putidacin L1 (encoded by *PL1*) are **proteic toxins**, that, when over-expressed *in planta*, provide an effective resistance against microbial pathogens (Rooney et al. 2019, 2020); **PR-10 proteins** are suggested as small non-specific binders to different kind of ligands such as a plant hormone, but also to several ligands creating then another molecule in its conformation, in order to promote plant development or defense systems (Lebel et al. 2010). Aspartic proteinase A1 functions are still unclear, however, their involvement in **protein turn-over** could be useful **to control hormonal signaling pathways** and improve defenses against phytopathogens (Simões and Faro 2004; Cheung et al. 2020). Similarly, the *homeobox-leucine zipper protein ATHB-13* encodes for a **transcription factor** (TF) whose function in development is largely unknown, despite the fact that TFs are known as proteins controlling molecular regulations (Ribone et al. 2015). **Relationship with hormones** needs further investigation since proteins encoded by *scarecrow* genes are involved in the complex regulation of hormone biosynthesis, that largely contribute to plant defense against pathogens, herbivores, and weeds (Wang et al. 2019, Yang et al. 2021; Sanchez et al. 2019). **Cytochrome P450s** (CYPs) are

other **versatile enzymes involved in multiple processes** of plant growth and development, thus playing an essential role in stress response. CYPs can protect plants from stresses through the biosynthesis and regulation of hormones, fatty acids, sterols, cell wall components, biopolymers, and several other defense compounds (terpenoids, alkaloids, flavonoids, furanocoumarins, glucosinolates, allelochemicals) (Pandian et al. 2020; Samuels et al. 2008). For instance, *Alkane hydroxylase MAH1* encodes for a CYP450 involved in the formation of secondary alcohols and ketones in stem **cuticular wax**, to better protect the plant tissues from environmental stresses. Tempranillo plants protected with *Ta* SC1 can also induced-changes in **cell wall composition** (i.e. cellulose, hemicellulose, pectin and lignin) by over-expressing a gene encoding for P4-like organ-specific protein (Wan et al. 2021). Finally, *Ta* SC1 upregulates both the DRT100-like proteins suspected to have significant roles in **DNA-damage-repair/toleration** (Fujimori et al. 2014), and gene encoding for the probable pectate lyase 5 proteins (*Pectate lyase gene*) mediating the maintenance of normal **cell division through auxin signaling** pathway (Sun et al. 2018; Leng et al. 2017). **In summary, *Ta* SC1 beneficial effect on Tempranillo plants against BD** correlates with: (1) the **silencing of many transporters** suggested to prevent the use of nutrients by pathogens as reported by Esparza-Reynoso et al. (2021); (2) the **preservation of cell wall integrity and extension** suggested (2a) to prevent the use of cell wall components as nutrients to favor pathogen fitness or aggressiveness, but also (2b) to better protect the plant tissues from environmental stresses (hormonal production of cuticular wax, changes in cell wall composition); (3) the **preservation of cell division** (possibly through auxin signaling) suggested to suppress pathogen induced cell death ; (4) **a network of immunologically active proteins**, among which nsLTPs, proteic toxins, proteinases, but also different TF or assimilated (PR-10 proteins), whose relationship needs further investigations with hormone signalling pathways and DNA-damage-repair/toleration as reported by Moran-Diez et al. (2021); (5) **microbiome interactions.** Compared to *Bs* PTA-271 beneficial effect on Chardonnay plants against BD, **much more changes are thus induced** by the beneficial effect of *Ta* SC1 in the leaves of infected Tempranillo, **probably because of the endophytic location of *Ta* SC1, forced thus to exploit the same "ecological niche" as the pathogen *N. parvum* Bt67.** They are thus **suspected to compete for the same space and nutrients**, which induce a highest number of systemic molecular changes inside Tempranillo leaves.

## 5. Conclusion

This study reports the transcriptomic analyses of artificially infected grapevine cultivars differentially protected by distinct BCA, simultaneously. Compared to recent publications investigating too BCA effects against GTD pathogens, our results provide new insight on the molecular changes associated with either (1) the infection by Np-Bt67 in Chardonnay and Tempranillo, or (2) their BCA-protection against this pathogen.

Considering the infection side, Chardonnay plants infected with *Np*-Bt67 over-expressed genes involved in ABA production or signaling, while the changes in infected Tempranillo plants concern amino acid import, chloroplast and photosystem related processes, plant responses to biotic stimulus, and biosynthesis of secondary metabolites.

Our study also provides deep insights on the protection induced by *Bs* PTA-271 in Chardonnay, and *Ta* SC1 in Tempranillo. Protection with *Bs* PTA-271 in Chardonnay targets genes related to ABA biosynthesis, phenylpropanoid pathways and secondary metabolites, and cell wall structure/organization in relationship with carbohydrate metabolism that requires much more consideration. Protection with *Ta* SC1 in Tempranillo requires a larger number of changes, probably because of *Ta* SC1 location in the same "ecological niche" as the pathogen Np-Bt67, thus forcing them to compete in order to ensure each actor survival. In brief, *Ta* SC1 beneficial effect on Tempranillo plants against BD correlates with many changes related to transporters, cell wall integrity and extension, cell division and pathogen induced cell death, multidirectional active proteins, and microbiome interactions.

Comparative studies with other cultivars and pathogens, might help to better decipher *Ta* SC1 beneficial effect and to better distinguish the effects of BCA from those of the plant resistance/susceptibility to infections. Further work remains now to be done on the microbiome/microbiota quality and dynamic evolution in relationship with each scenario involving our key players (1 BCA, 1 pathogen, 1 cultivar). Finally, as GTD infection are still very recurrent in vineyards, and can be undetected for years, the insight on the molecular changes in infections by GTD pathogens, as well as the changes with the application of BCAs, will become increasingly important.

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## *Chapter V*

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Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against GTD pathogens during grapevine propagation process

### 5.1. Contexte

Les infections latentes par les pathogènes responsables des MDB sont particulièrement dangereuses au moment du procédé de propagation en pépinière (Gramaje et al. 2018). Ce futur matériel de plantation asymptomatique est potentiellement déjà infecté par les agents pathogènes responsables des MDB, et peut aussi s'infecter lors des différentes étapes de leur production en pépinière (hydratation, stockage au froid, greffage, formation de cals, etc.), puis ainsi conserver et transmettre les pathogènes aux vignobles nouvellement plantés (Gramaje et al. 2018). Le matériel de vigne en pépinière est effectivement très sensible aux infections par les champignons responsables des MDB, en raison des nombreuses coupures et blessures faites tout au long du procédé de pépinière (Waite et al. 2018). Si ces infections ne sont pas contrôlées à un stade précoce de leur développement, une propagation insoupçonnée des plantes infectées peut alors se produire, d'abord dans la pépinière puis ensuite au vignoble (Aroca et al. 2010 ; Gramaje et al. 2011, 2015, 2018 ; Waite et al. 2018). Ainsi, l'amélioration de la qualité phytosanitaire des vignes produites en pépinière apparaît comme une étape des plus importante à prendre en compte pour gérer durablement les MDB, et à considérer en plus de la protection préventive ultérieure des plaies de taille contre les infections au vignoble dès lors que la plantation est réalisée (Berbegal et al. 2020).

Dans ce chapitre et en complément à ce qui a été étudié précédemment *in planta* et en serre (chapitre III et IV), les 2 BCA sont appliqués en pépinière à une densité optimisée spécifiquement pour ce modèle pépinière, soit : $10^6$ cfu/ml pour *Bs* PTA-271 et 2g/L pour *Ta* SC1 (Figure 5 et 8). Les plants utilisés sont des greffés-soudés (Tempranillo pour le greffon, et Richter 110 pour le porte-greffe). Les applications de BCA ont été effectuées à trois moments clés du procédé de pépinière : à l'étape d'hydratation des sarments (bain d'1 jour à température ambiante, juste avant le greffage), pendant la période d'incubation de 20 jours à 24°C qui favorise la consolidation de chaque greffé-soudé (ou étape de la « prise de greffe » sur de la sciure de bois imbibée de BCA), et au moment de la plantation en champs de pépinière (par trempage des racines pendant 1h dans la suspension ad'hoc de BCA juste avant la mise en sol des greffés-soudés). Ce modèle pépinière présente ainsi l'avantage de tester chaque BCA et leur mélange en conditions naturelles, donc contre des pathogènes se développant naturellement. Pour accueillir ces 3 modalités et leur témoin sans BCA, 2 champs distants de 10km ont été divisés en blocs complets randomisés de quatre répétitions par

traitement (4 x 50 plantes = 200 plantes au total) pendant 2 années consécutives, dans la Province de Valencia en Espagne.

Lors de la réalisation d'expériences au vignoble avec des BCA, les méthodes les plus courantes pour étudier la qualité de l'application des BCA sont les réisolations en boîtes de Pétri. Bien que ces méthodes soient relativement faciles, qu'elles donnent des résultats visuellement rapides et qu'elles soient moins coûteuses que les techniques moléculaires, elles ne peuvent pas garantir que la souche ré-isolée correspond à la souche de BCA inoculée, et elles dépendent donc pour cela des techniques moléculaires. La qRT-PCR sera ici mise à profit, non seulement pour détecter la présence des BCA *in-planta* (garantissant le succès des inoculations réalisées), mais également pour les quantifier. Cette technique présente aussi l'avantage de pouvoir distinguer chaque souche inoculée (en comparaison aux souches natives présentes en très faible densité), par surexpression, y compris dans des échantillons complexes tels que des échantillons de racines ou de sol (Sanzani et al. 2014 ; Schena et al. 2004).

Ainsi, les objectifs de ce travail sont (Figure 5 et 8): (i) d'évaluer l'efficacité biocontrôle de *Trichoderma atroviride SC1* et *Bacillus subtilis PTA-271*, seuls ou combinés, contre des pathogènes naturels associés au BOT et au Black-Foot, lors du procédé de propagation de la vigne en pépinière. L'efficacité des BCA est appréhendée en considérant : (a) le nombre de plantes germées ; (b) le nombre de plantes vivantes commercialisables, vivantes non commercialisables, et mortes (à la fin de la saison de croissance) ; (c) la présence et la fréquence (incidence et sévérité) des pathogènes ré-isolés et identifiés à partir des sarments de vigne (analyse ITS) : avant leur entrée en pépinière (greffon et porte-greffe) et en sortie de pépinière (racine et tige basale du porte-greffe au point de greffe). Le 2$^{ème}$ objectif de ce chapitre est (ii) d'évaluer la qualité des inoculations en BCA réalisées lors de ce même procédé en pépinière, par quantification des BCA par qRT-PCR.

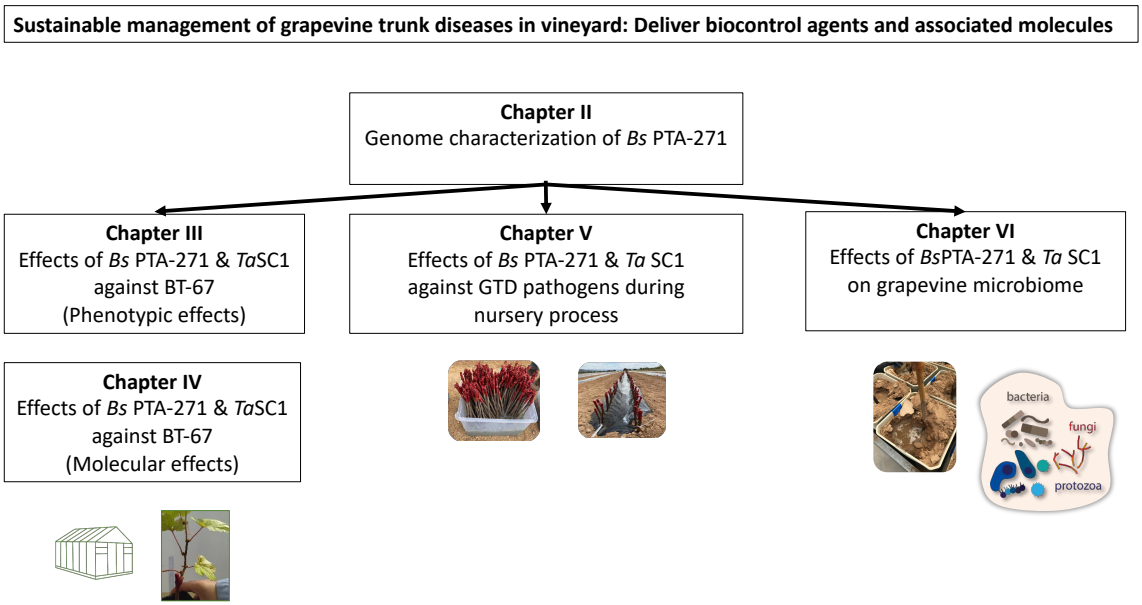


**Figure 5.** Aims of each chapter of the thesis entitled "Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules".

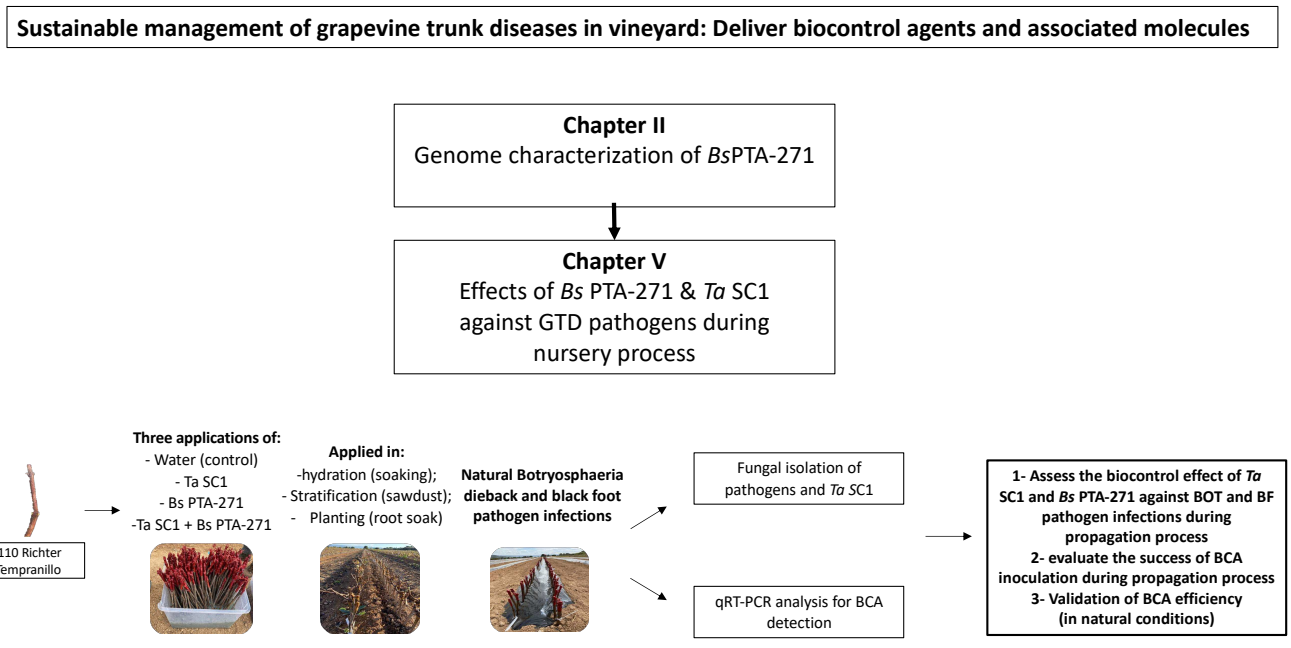


**Figure 8.** Aims chapter V entitled "Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against GTD pathogens during grapevine propagation process".

**Publication 5- Evaluation of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to control Botryosphaeria dieback and black-foot pathogens in grapevine propagation material**

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# Evaluation of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to control Botryosphaeria dieback and black-foot pathogens in grapevine propagation material

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## Abstract

**BACKGROUND**: Grapevine trunk diseases (GTDs) are a complex group of diseases, that lead to major economic losses in all wine producing countries. The investigation of biocontrol agents (BCAs) capable to forestall or at least minimize the development of GTDs has, recently, become a priority. Nursery experiments were set up to (i) assess the biocontrol effect of *Trichoderma atroviride SC1* and *Bacillus subtilis PTA-271*, alone and in simultaneous application, against Botryosphaeria dieback (BOT) and black foot (BF) associated pathogens during the grapevine propagation process, and (ii) evaluate the success of the BCA inoculation during the grapevine propagation process, using qRT-PCR techniques.

**RESULTS:** The results demonstrated a significant reduction on the percentage of infected plants and the percentage of fungal isolation from wood fragments of BOT and BF pathogens in nursery material treated with *Ta* SC1 and *Bs* PTA-271 respectively. In one of the experiments, the simultaneous treatments with *Bs* PTA-271 and *Ta* SC1 presented a reduction on both percentage, infected plants and fungal isolation, from wood fragments of BOT and BF pathogens.

**CONCLUSION:** These beneficial biological treatments may be a relevant component of an integrated approach, using complementary management strategies to limit infection by GTDs pathogens, but further research is still needed to cover as many changing conditions as possible in order to finally erase the variability of such beneficial results in field conditions.

**Keywords:** Botryosphaeria dieback, black foot, biocontrol agents, grapevine trunk diseases, grapevine propagation process, pathogen control.

## 1. Introduction

Grapevine trunk diseases (GTDs), despite having been extensively studied since the early 20th century, are still considered one of the most relevant challenges for viticulture, leading to important economic losses all around the world[1,2,3]. Botryosphaeria (BOT) and Eutypa diebacks, and Esca, Petri, and black foot (BF) diseases, are the predominant GTDs currently found in vineyards[1,2,4].

The control of GTDs is a major challenge for grape growers, nurserymen, and scientists, mostly because of their complexity compared to other grapevine diseases. It is well known that more than one GTD can be expressed within the same plant, and one of the most problematic aspects of the fungal pathogens associated with GTDs is their indeterminate latency (endophytic status)[5]. By the time the first external symptoms appear, the grapevine wood may already be extensively damaged[3,6], leaving viticulturists with few options to reduce the impact of GTDs in vineyard[2]. Latent infections are also dangerous during nursery propagation processes, as asymptomatic planting material infected by GTDs pathogens during the various steps of nursery plant production (hydration, cold storage, grafting, callus formation, etc.) can preserve and then transmit the infections to newly planted vineyards[2]. The nursery grapevine material is very susceptible to GTDs infections due to the several cuts and wounds made during the nursery steps[7], and there may be an unsuspected spread of infected plants if these infections are not controlled at an early stage, first in the nursery and then in the vineyard[2,7,8,9,10]. In this sense, the most important aspects to be covered for the sustainable management of GTDs are the improvement of the phytosanitary quality of the vines produced in nursery, and the subsequent prevention of pruning wound infections in the vineyard from the time of planting[11].

Investigation of biocontrol agents (BCAs) capable to forestall or at least to minimize the impact of GTDs is viewed as a research priority. In the past 10 years, many efforts have been made to develop new microbial antagonists, including fungi, bacteria and oomycetes[11,12,13,14,15,16,17,18,19,20]. *Trichoderma* spp. currently represent one of the most studied fungal-based BCAs used in agriculture. *Trichoderma* have a broad range of benefits for plants[21,22]. They can parasitize and suppress other fungi (mycoparasitism)[23,24], and produce several secondary metabolites, such as antibiotics[21,23] that help to control fungal pathogens.



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Besides pathogen control, *Trichoderma* can increase both plant and root growth by enhancing nutrient and nitrogen uptake and inducing plant systemic defenses[21,24]. In grapevine, *T. atroviride* strains USPP-T1, USPP-T2, I-1237 and SC1 have shown good performances in the protection of grapevine against GTDs, as well as other *Trichoderma* spp. like the *T. harzianum*, *T. asperellum* and *T. gamsii* strains[11,19,20,25,26].

*Bacillus subtilis* strains have also been widely tested to be used as BCAs against fungal pathogens[18,20,27,28,29]. They are well known for their ability to enhance the systemic defenses of the plant against subsequent biotic stresses and promote plant growth by mobilizing nutrients, increasing their availability to the plant[20,30,31]. *Bacillus subtilis* strains can also directly suppress pathogens due to the production of several secondary metabolites such as antimicrobial molecules, siderophores, lytic enzymes, and lipopeptides[20,30,31,32]. *B. subtilis* PTA-271[20], *B. subtilis* F62[33] and *B. subtilis* BBG127 and BBG131[34], have been studied and shown potential to protect grapevines against GTDs.

When performing field experiments with BCAs, the most common methods to study the quality of BCA application, are the re-isolations in petri dishes. Although these methods are relatively easy, show visually fast results, and can be cheaper than molecular techniques, they cannot ensure that the re-isolated strain corresponds to the inoculated one, and rely on molecular techniques to do so. Moreover, with these methodologies, it is not possible to accurately quantify BCAs. By using molecular techniques, especially qRT-PCR, it is possible to, not only detect BCAs presence, but also quantify the microorganism. qRT-PCR technique also allows us to distinguish between bioaugmented inoculated strains and the low-density native ones, even in complex samples such as root or soil samples[35,36].

Thus, the objectives of this work were (i) to assess the biocontrol effect of *Trichoderma atroviride SC1* and *Bacillus subtilis PTA-271*, alone and in simultaneous application, against BOT and BF associated pathogens during the grapevine propagation process, and (ii) to evaluate the success of the BCA inoculation during the grapevine propagation process, using qRT-PCR methods.

## 2. Material and methods

### 2.1 Grapevine scion and rootstock material



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Two independent nursery trials (A and B) were performed using scions of cv. Tempranillo and cuttings of 110 Richter rootstock from a commercial nursery located in Valencia province, Spain in 2021. In each trial, prior to the grafting process, 25 scion fragments and 25 rootstock fragments were randomly selected from the plant material and analyzed for the presence of GTDs pathogens. To do so, in each material type, isolations were performed from 3 cm long sections. The sections were washed with tap water, surface-disinfested using 70% ethanol and passed in a flame. Then, ten internal wood fragments per section were placed on malt extract agar (MEA) complemented with 0.5 g $L^{-1}$ of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS) (five fragments per two Petri dishes). Plates were incubated for 10–15 days at 25 ◦C in the dark, and all evolving colonies were then transferred to potato dextrose agar (PDA). For confirmation of species identity, fungal mycelium and conidia from pure cultures grown on PDA for 15-20 days at 25 ◦C in the dark were scraped and mechanically disrupted using FastPrep-24™5G (MP Biomedicals, Santa Ana, CA, USA). Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following manufacturer's instructions. The quality and integrity of the DNA was visualized on 1% agarose gels stained with REALSAFE (Durviz S.L., Valencia, Spain). All DNA samples were stored at −20 ◦C. The identification of all isolates was performed by analysis of the internal transcribed spacer (ITS) region amplified using the fungal universal primers ITS1F and ITS4[37,38]. Further molecular identification was then conducted for specific groups of pathogens. *Cadophora* and *Phaeoacremonium* spp. were identified by sequence analysis of the *β*-tubulin gene, with the primers BTCadF and BTCadR and T1 and Bt2b respectively[39,40,41]. Identification of Botryosphaeriaceae spp. was confirmed by analysis of the translation elongation factor *1-α* gen amplified using EF1F and EF2R primers[42]. Identification of *Cylindrocarpon*-like asexual morphs was confirmed by sequencing part of the *histone H3* gene with primers CYLH3F and CYLH3R.48[43].

### 2.2 Biological control agent's preparation

#### 2.2.1 *Bacillus subtilis* PTA-271

*Bacillus subtilis* PTA-271 (GenBank Nucleotide EMBL Accession No. AM293677 for 16S rRNA and DDBJ/ENA/GenBank Accession No. JACERQ000000000 for the whole genome), therefore *Bs* PTA-271, was isolated in 2001 from the rhizosphere of healthy Chardonnay grapevines on a vineyard located in Champagne (Marne, France)[27,31]. Bacterial growth started with addition of 1 ml of glycerol stock suspension to sterile Luria Bertani (LB) medium and



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incubating at 25-28°C. Experiments were performed when the bacterial culture was at the exponential growth phase. After centrifugation (5,000 g, 10 min), the pellet was resuspended with a sterile 10 mM MgSO4 medium adjusting the density to $10^6$ cfu $mL^{-1}$.

#### 2.2.2 *Trichoderma atroviride* SC1

*Trichoderma atroviride* SC1 (Vintec®, Belchim Crop Protection, Bi-PA; $10^{10}$ conidia per gram of formulated product), therefore *Ta* SC1, was suspended in water at 2g $L^{-1}$ as indicated by the producer. The viability of the conidia in the commercial product was checked to be at a minimum of 85% before each trial, as described by Pertot et al. (2016)[13]. A serial dilution of the conidia suspension was plated on PDA (Biokar-Diagnostics, Zac de Ther, France) and the colony forming units were counted after 24-48 h incubation at room temperature.

### 2.3 Nursery experiments

Grapevine propagating material (cuttings of 110 R rootstock subsequently grafted with Tempranillo cultivar) were treated with *T. atroviride* SC1, *B. subtilis* PTA-271 or the simultaneous application of both BCAs at three stages of the grapevine propagating process as performed by Berbegal et al. (2020)[11]: (i) 1-day soak in suspension prior to grafting, (ii) 20-days application of suspension in sawdust at stratification, and (iii) 1-h soak of the basal parts of the plants in a BCA suspension before planting in the rooting field (Fig. 1). All propagation material was subjected to the three treatments, and the untreated control was treated with water at all stages. Two separate experiments were performed at different times and corresponding grafted plants were transferred to different rooting fields, using four replicates per treatment with 50 plants in each replicate. The two experiments were managed separately with plant material coming from different mother blocks (Fig. 1).

Grafted plants were transfered in two different nursery-rooting fields separated by more than 10 km (experiment A was located at Llanera de Ranes and experiment B was located at Rotglà i Corberà, both in Valencia province) in 19th of May 2021 and 28th of May 2021, respectively. Both fields were arranged in a randomized complete block design with four replicates per treatment (200 plants in total). Cultural practices were performed according to the integrated pest management (IPM) guidelines and only copper compounds and wettable sulphur were applied at label dosages to control downy and powdery mildew, respectively, when required. Plants were uprooted in November 2021 and wrapped in individual perforated plastic bags to avoid cross-contamination, but also to prevent oxygen deprivation and fermentation, without exposing the cuttings to dehydration.



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### 2.4 Fungal isolation at the end of the experiments

In each trial, 20 plants per treatment and replicate were selected randomly for fungal isolation analyses. Isolations were made from 3 cm long sections cut from three different areas: the grafting point, the basal end of the rootstock cuttings and the root system. These materials were washed, surface-sterilized and isolations were performed as described in section 2.1. Ten wood fragments per each type of area (five fragments per two Petri dishes) were analysed (30 wood fragments per plant). The 3 cm sections were washed with tap water, surface-disinfested using 70% ethanol and passed in a flame. Then, ten internal wood fragments per section were placed on malt extract agar (MEA) complemented with 0.5 g $L^{-1}$ of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS) (five fragments per two Petri dishes). Plates were incubated for 10–15 days at 25 ºC in the dark, and all evolving colonies were transferred to PDA petri dishes. All GTDs pathogens were identified using the techniques explained in section 2.1, and a selection of *Trichoderma* colonies (10%) were analyzed using PCR[44] to confirm the specific *Trichoderma* strain.

### 2.5 Quantification of biological control agents on plant material by qRT-PCR

From the 20 plants selected for fungal isolation, 10 were randomly selected for Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) analysis of *Bs* PTA-271 and *Ta* SC1. Thus, after fungal isolation, the remaining wood tissues of the 3 cm long sections from the rootstock base were grinded in liquid nitrogen and stored at -20 °C. DNA was extracted from 200 mg of powdered wood, according to the protocol from Alfonzo et al. (2012)[45]. DNA quality was checked by agarose gel electrophoresis, and total DNA concentration was measured for each sample using the NanoDrop One spectrophotometer (Ozyme) and adjusted to 50 ng/µl. Both *Ta* SC1 and *Bs* PTA-271 were tracked by qRT-PCR using specific primers. For *Ta* SC1, *endochitinase* gene (*ech42*) primers described in Savazzinni et al. (2008)[46] was used. For *Bs* PTA-271, a set of primers were designed targeting the *DNA polymerase III* (*dnaE*) gene. The *DNA polymerase III* gene sequence of *Bs* PTA-271 full genome was aligned with other *Bacillus dnaE* sequences retrieved from GenBank nucleotide database using Clustal W (1.82) Multiple Alignment Program. A set of primers was designed (5' – TGGATGAAGCGAGACAGCAG – 3', 3'- TCTTCACTCAGGACAACGCC- 5'), using NCBI primer designer tool[47]. A standard curve was prepared to calculate the efficiency of the primers. The curve was constructed using



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7 consecutive 1:5 dilutions from genomic DNA from a *Bs* PTA-271 culture in LB medium starting with 50 ng/µl. One millilitre from each bacterial concentration was subjected to DNA extraction by means of the Wizard® Genomic DNA purification kit (Promega). Average Ct values (from duplicates) and standard deviations were calculated for each dilution. The threshold cycle (Ct) value was plotted against the log concentration of the template DNA, and the slope of the obtained regression line was used for calculating the efficiency (E) with the following equation: E = [10(−1/slope)]–1*100[48]. The primers presented an efficiency of 99.8%.

QRT-PCR reactions were carried out in duplicate in 96-well plates in a 15 µl final volume containing Absolute Blue SYBR Green ROX mix including Taq polymerase ThermoPrime, dNTPs, buffer, and $MgCl_2$ (Thermo Fisher Scientific, Inc., Waltham, MA, United States), 280 nM forward and reverse primers, and 10 ng $\mu L^{-1}$ gDNA according to the manufacturer's protocol. Cycling parameters were 15 min of Taq polymerase activation at 95°C, followed by 40 two-step cycles composed of 5 s of denaturation at 95°C and 20 s of annealing and elongation at 60°C. Melting curve assays were performed from 60 to 95°C at 0.5°C $s^{-1}$, and melting peaks were visualized to check amplification specificity. Elongation factor *1-alpha* (*EF1*) gene was used as reference (5'-AACCAAAATATCCGGAGTAAAAGA-3', 3'GAACTGGGTGCTTGATAGGC-5')[18]. Relative gene expression was determined with the formula fold induction: 2(−11Ct), where 11Ct = [Ct TG (US) − Ct RG (US)] − [Ct TG (RS) − Ct RG (RS)], where Ct is cycle threshold, Ct value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is target gene, RG is reference gene, the US is an unknown sample, and RS is reference sample. Integration of the formula was performed by the CFX Manager 3.1 software (Bio-Rad).

### 2.6 Statistical analyses

The isolation of pathogens belonging to the family Botryosphaeriaceae, causal agents of BOT disease, and *Cylindrocarpon*-like asexual morphs, causal agents of BF disease, from the grafted plants was expressed as the mean percentage of infected plants and the mean percentage of positive fungal isolation from wood fragments per each group of pathogens. Statistical analyses were carried out using JASP 0.16.1 (JASP team 2022). For treatment effect, mean values were analysed using Kruskal-Wallis test. When differences in the means were significant, Dunn post hoc test ($\alpha = 0.05$) was applied to determine which treatments were significantly different from others.



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## 3. Results

### 3.1 Pre-existing latent infection at the nursery entrance

In the experiment A, it was not possible to detect any infection by GTDs pathogens on the 25 scions of cv Tempranillo and the 25 cuttings of 110 Richter rootstocks collected prior to the grafting process. In the experiment B, only two rootstock cuttings showed infection by *Neofusicoccum vitifusiforme,* proving that the initial infection level was very low (data not shown).

### 3.2 *Ta* SC1 commercial viability, recovery from trial plants and compatibility with *Bs* PTA-271

In both trials, the percentage recovery of this strain was close to 100% (90-98%) on treated plants and wood fragments colonized by *Ta* SC1, and it was not recovered from untreated plants (data not shown). Moreover, the presence of *Bs* PTA-271 did not alter wood colonization by *Ta* SC1, since they were both > 90% (data not shown).

### 3.3 Impact of BCA treatments on fungal pathogen recovery at the end of the nursery process

At the end of both experiments, fungal pathogens associated with BOT (Botryosphaeriaceae spp.) and BF (*Cylindrocarpon*-like asexual morphs) were recovered from the grafted plants. Pathogens associated with Petri disease were recovered only in very few plants, thus, they were considered insufficient for statistical analysis. Pathogens associated with BF were preferably found on the roots, while those associated with BOT were preferably found on the grafting point. Therefore, the statistical comparison of treated and untreated plants was performed grouping the results of the different pathogens associated with BOT and BF diseases and their preferred areas for infection.

### 3.4 Impact of BCA treatments on the percentage of infected plants and percentage of fungal isolation from wood fragments at the end of the propagation process



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### 3.4.1 Experiment A

At the end of the propagation process Botryosphaeriaceae isolated fungi were *N. parvum, Lasiodiplodia citricola, Diplodia seriata, N. luteum* and *L. pseudotheobromae*, and isolated *Cylindrocarpon*-like asexual morphs were *Ilyonectria liriodendri, Dactylonectria novozelandica* and *D. torresensis*. Data from the different pathogens were grouped according to the two main GTDs considered in figures 2 and 3.

The percentage of plants infected with BOT (Fig. 2) and BF (Fig. 3) associated fungi in untreated plants was 33.75% (BOT) and 18.75% (BF), in plants treated with *Ta* SC1 it was 13.75% and 11.25%, in plants treated with *Bs* PTA-271 it was 37.5% and 7.5%, and in plants treated simultaneously with both BCAs 7.5% and 2.5%, respectively. Regarding the percentage of fungal isolation from wood fragments, untreated plants presented 13.75% (BOT) and 4.25% (BF), plants treated with *Ta* SC1 8.5% and 2.75%, plants treated with *Bs* PTA-271 20.25% and 0%, and plants treated simultaneously with both BCAs 3.75% and 0.75%, respectively (Figs. 2, Fig.3). The analyses of variance revealed a significant reduction of the percentage of infected plants and the isolation from wood fragments of BOT associated pathogens in plants treated with *Ta* SC1 and plants treated simultaneously with both BCAs (Fig. 2). Plants treated simultaneously with *Bs* PTA-271 and *Ta* SC1 presented the lowest percentage of BOT infected plants and isolation from wood fragments, although this difference was not statistically different from *Ta* SC1 alone. Plants treated with *Bs* PTA-271 showed no significant differences when compared to untreated plants. Regarding fungi associated with BF, plants treated simultaneously with both BCAs was the only treatment that showed significantly lower percentage of infected plants and isolation from wood fragments, than untreated plants (Fig. 3). However, the values were not significantly different from the treatments with one BCA alone.

### 3.4.2 Experiment B

At the end of the propagation process, Botryosphaeriaceae isolated fungi were *N. parvum, L. pseudotheobromae, D. seriata* and *N. algeriense*, and isolated *Cylindrocarpon*-like asexual morphs were *I. liriodendri, D. novozelandica* and *D. torresensis*. As for the experiment A, data from the different pathogens were grouped according to the two main GTDs considered in figures 4 and 5.



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The percentage of plants infected with BOT (Fig 4.) and BF (Fig. 5) associated pathogens in untreated plants was 37.5% (BOT) and 10% (BF), in plants treated with *Ta* SC1 7.5% and 9.25%, in plants treated with *Bs* PTA-271 33.75% and 8.75%, and in plants treated plants treated simultaneously with both BCAs 10% and 6.25%, respectively. Regarding the percentage of fungal isolation from wood fragments, untreated plants presented 17.5% (BOT) and 2% (BF), plants treated with *Ta* SC1 2.25% and 2.5%, plants treated with *Bs* PTA-271 16.5% and 1.75%, and plants treated plants treated simultaneously with both BCAs 3% and 2%, respectively (Fig. 4 and Fig. 5). The analyses of variance revealed significant reduction of the percentage of infected plants and the fungal isolations from wood fragments of BOT associated fungi in plants treated with *Ta* SC1 and plants treated simultaneously with both BCAs. Plants treated with *Ta* SC1 presented the lowest percentage of BOT infected plants and isolation from wood fragments. Plants treated with *Bs* PTA-271 showed no significant differences when compared to untreated plants (Fig. 4). Regarding fungi associated with BF, there was not a statistically significant reduction of the percentage of infected plants or the percentage of fungal isolation from wood fragments with any treatment (Fig. 5).

### 3.5 BCA detection by qRT-PCR at the end of the propagation process

Since *Bs* PTA-271 is more often recovered from roots, and *Ta* SC1 more often recovered from the rootstock and grafting point, the molecular analysis was performed with wood sections from the base of the rootstock, in order to facilitate the simultaneous detection of both BCAs. In both experiments, *Ta* SC1 and *Bs* PTA-271 were detected in every treated plant, and in untreated plants, neither *Ta* SC1 nor *Bs* PTA-271 were detected (Fig. 6 and Fig. 7).

#### 3.5.1 Experiment A

In experiment A (Figure 6), plants treated with *Bs* PTA-271 presented 2.65-fold more *Bs* PTA-271 than the untreated plants, and plants treated simultaneously with both BCAs presented 3.96-fold more *Bs* PTA-271 than the untreated plants. Plants treated only with *Ta* SC1 showed similar quantity of *Bs* PTA-271 as the control, showing no signs of crossed contamination. Regarding *Ta* SC1, plants treated with *Ta* SC1 presented 13.1-fold *Ta* SC1 than the untreated plants, and the plants treated simultaneously with both BCAs showed 12.2-fold *Ta* SC1 than the untreated plants. Plants treated with only *Bs* PTA-271 showed similar quantity of *Ta* SC1 as the control, showing no signs of crossed contamination.



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### 3.5.2 Experiment B

In experiment B (Figure 7), plants treated with *Bs* PTA-271 presented 2.5-fold more *Bs* PTA-271 than the untreated plants, and plants treated simultaneously with both BCAs presented 3.2-fold more *Bs* PTA-271 than the untreated plants. Plants treated only with *Ta* SC1 showed similar quantity of *Bs* PTA-271 as the control, showing no signs of crossed contamination. Regarding *Ta* SC1, plants treated with *Ta* SC1 presented 29.09-fold more *Ta* SC1 than the untreated plants, and the plants treated simultaneously with both BCAs showed 12.48-fold more *Ta* SC1 than the untreated plants (Fig. 7). Plants treated only with *Bs* PTA-271 showed similar quantity of *Ta* SC1 as the control, showing no signs of crossed contamination (Fig. 7).

## 4 Discussion

The aim of the current study was to investigate the biocontrol effect of *Ta* SC1 and *Bs* PTA-271, in single and simultaneous application, against BOT and BF associated pathogens, during the grapevine propagation process. Leal et al. (2021b)[20] already evaluated the biocontrol effect of *Ta* SC1 and *Bs* PTA-271, in single and simultaneous application, against *N. parvum* Bt67 in Chardonnay and Tempranillo grapevines in controlled greenhouse conditions. Although the layout of both works can be somewhat related, nursery experiments enabled us to work upon much more realistic environmental conditions, including natural infections with GTDs pathogens, and additional microorganism interactions, delivering much more truthful results.

It is well known that the grapevine grafting process leads to an increased risk of infection by GTDs pathogens due to the several cuts and wounds produced during this process, by the use of grafting machines and scissors infected with pathogen spores, and during callusing (subjected to high temperature and humidity) and field rooting phases[2,7,9]. It is also during the propagation process that grapevine acquires the first GTDs pathogens infections, that will progress in the plant, leading to the appearance of GTDs symptoms, years later[49]. For this reason, when performing experiments with nursery grapevines, it is not possible to detect GTDs symptoms, since the infections are recently acquired[9]. In fact, in our nursery experiments, prior to the grafting process, the infection levels of GTDs in the plant material were zero or almost negligible, with only one pathogen, *N. vitifusiforme* found in experiment B. However, at the end of the propagation process, the diversity of GTDs pathogens recovered from untreated



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plants increased significantly. Gramaje et al. 2022[50] studied the temporal dynamics of fungal microbiome in rootstocks during the propagation process, and concluded that during the process, the abundance of plant pathogens largely increases, and *Neofusicoccum* genus was found as a persistent taxon in different plant material, in agreement with our results. In both experiments, BOT and BF associated pathogens were prevalent, emphasizing the importance of trying to contain these early infections in the propagation process, to avoid BOT and BF symptoms development later in the vineyard, which have been reported as some of the most common GTDs worldwide[2,3,9,51].

The colonization of the BCAs *Ta* SC1 and *Bs* PTA-271, alone or simultaneously, in treated plants, was successful in both experiments, with higher quantities of the BCAs in treated plants, compared to untreated plants. This was concluded using, for the first time in nursery field experiments, the qRT-PCR methodology, applied to wood samples collected from the rootstock base. qRT-PCR has the sensitivity and accuracy necessary to detect specific bioaugmented strains of microorganisms, lacking in the traditional PCR technique, and can be used to monitor BCA population changes over a period of time, representing the future of pathogens and BCA detection[33,34].

In both experiments, plants treated with *Ta* SC1 presented significantly less percentage of infected plants and percentage of fungal isolations from wood fragments, of BOT associated pathogens, compared with untreated plants. Berbegal et al. (2019)[11], also proved this effect during the propagation process. Concerning BF associated pathogens, plants treated with *Ta* SC1 showed no significant differences in neither the percentage of infected plants nor the percentage of isolation from wood fragments, when compared to the untreated plants. In experiment B, this is most likely, due to the initial lower percentage of infected plants (untreated plants presented 10%, compared to 18% in experiment A). However, it is known that *Trichoderma* spp., as any other BCA present inconsistencies in its biocontrol effect, depending not only on the types of pathogens, but also on the environmental conditions (temperatures, relative humidity, surface wetness, gases, and air movement), plant cultivar metabolism, and other present microorganisms. In fact, several studies have shown the lack of effect of *Trichoderma* spp. against BF associated pathogens[11,19,52,53].

Focusing now on *Bs* PTA-271 effect, several authors have previously pointed out a positive effect of *B. subtilis* to control infections by BOT associated fungi [1,33,54,55,]. More specifically, Trotel-Aziz et al. (2019)[18] and Leal et al. (2021b)[20] had already shown the beneficial effect of *Bs* PTA-271 in reducing the symptoms of one BOT associated fungus (*N. parvum*) in grapevine



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cv. Chardonnay, but could not detect the same positive effect in the cv. Tempranillo. Corroborating with the previous findings, the treatments with *Bs* PTA-271 did not reduce either the percentage of infected plants or the percentage of fungal isolation from wood fragments of BOT associated fungi.

In contrast, it is noteworthy to highlight that *Bs* PTA-271 showed promising effects against BF associated fungi. Indeed, in experiment A, plants treated with *Bs* PTA-271 presented a reduced percentage of infected plants, and percentage of isolation from wood fragments of BF associated fungi, when compared with untreated plants, although in experiment B it was not possible to detect any significant difference. These results are promising when considering that BF pathogens are particularly difficult to manage due to many parameters influencing its development, such as soil nature, but also environmental and plant stress factors such as the soil mineral deficiency, poor drainage, soil compaction, heavy crop loads on young plants, poorly prepared soil, and improper plant holes[56]. More importantly, during the grapevine propagation process and especially at the time of planting, the susceptible basal ends of most of the nursery cuttings are partly or even fully exposed, since the young callus roots easily break during the planting process, resulting in additional wounds susceptible to infection by soilborne pathogens[57]. The grapevine roots are therefore an area of intense infection where BF soil-borne pathogens establish a parasitic relationship with the plant. But to succeed to infect the root tissues, BF pathogens must compete with the beneficial microorganisms that make up the complex rhizosphere microbiome for nutrients and ecological niches[58], explaining why this disease is so unpredictable and hard to control[59]. Currently, no curative control measures are available to eradicate BF pathogens in nurseries, and the management of this disease involves improving the quality of grapevine planting material by maintaining healthy microbiome, but also good hygiene and wound protection. During the last decades, several studies have been carried out to find effective strategies to manage BF pathogens in nursery and established vineyard[19,51,60,61,62], and a treatment with *Bs* PTA-271 could be a potential effective way to reduce the infection by BF associated fungi during the grapevine propagation process.

For both BOT and BF associated pathogens, one BCA (*Ta SC1* or *Bs* PTA-27), when well selected, could be the basic and simplest way to reduce infections caused by these fungi. However, both BCAs presented inconsistencies, and only showed a beneficial effect against Botryosphaeriaceae spp. (*Ta* SC1) or *Cylindrocarpon*-like asexual morphs (*Bs* PTA-271). The simultaneous application of *Ta SC1* and *Bs* PTA-271 presented a significant decrease in the percentage of infected plants and the percentage of isolation from wood fragments, for both



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Botryosphaeriaceae spp., and *Cylindrocarpon*-like asexual morphs, when compared to untreated plants, but only observed in experiment A. Simultaneous applications of BCAs have already been indicated as a possible effective strategy to overcome inconsistent control of plant pathogens in vineyards[19,63,64], and literature has been reporting that the interaction of more than one BCA can improve specific disease management and promote plant development and therefore resistance[20,64,65,66,67,68]. However, further research is necessary to recomend the benefits of the simultaneous applications of *Ta* SC1 and *Bs* PTA-271 in grapevine nurseries.

## 5 Conclusion

The results obtained in this study showed a promising biocontrol potential of treatments with *Bs* PTA-271 and *Ta* SC1 against BOT and BF pathogen infections during the grapevine propagation process of cv. Tempranillo. Alone, *Ta* SC1 confirmed its effectiveness to reduce the percentage of infected plants with BOT associated pathogens and the percentage of isolation from wood fragments, and *Bs* PTA-271 demonstrated, for the first time, effectiveness to reduce infections caused by BF associated pathogens during the propagation process. In one of the experiments, the simultaneous application of both BCAs presented a significant decrease in the number of infected plants and the number of infected wood fragments for both groups of pathogens.

In our study, qRT-PCR techniques were used for the first time in grapevine nursery experiments, enabling accurate quantification of the inoculated BCAs, confirming the successful colonization by BCAs in treated plants, and the lack of crossed contaminations between treatments.

Since the pathogen inoculum of GTDs pathogens can be strongly affected by environmental factors, further research is still needed to cover as many changing conditions as possible in order to finally erase the variability of such beneficial results in field conditions. Nevertheless, these beneficial biological treatments, may be a relevant component of an integrated approach, using complementary management strategies to limit infection by GTDs pathogens.

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**Conflict of Interest Statement**

*Trichoderma atroviride* SC1 is commercialized by the company Belchim Crop Protection, which partially financed the research.

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**Figure Legends**

**Figure 1**. Process scheme of nursery experiments A and B, and BCA inoculations of all treatments (control, *Bs* PTA-271, *Ta* SC1, and both BCAs).

**Figure 2.** Percentages of plants infected with Botryosphaeriaceae spp., and percentage of isolations of Botryosphaeriaceae spp. from wood fragments observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs and untreated plants (control) in the nursery experiment A. Mean percentages are based on four replicates of 20 plants per treatment and 10 wood fragments per plant. Letters a and b represent significant differences (P-value <0.05) between treatments.

**Figure 3.** Percentages of plants infected with *Cylindrocarpon*-like asexual morphs, and percentage of isolations of *Cylindrocarpon*-like asexual morphs from wood fragments observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs and untreated plants (control) in the nursery experiment A. Mean percentages are based on four replicates of 20 plants per treatment and 10 wood fragments per plant. Letters a and b represent significant differences (P-value <0.05) between treatments.

**Figure 4.** Percentages of plants infected with Botryosphaeriaceae spp., and percentage of isolations of Botryosphaeriaceae spp. from wood fragments observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs and untreated plants (control) in the nursery experiment B. Mean percentages are based on four replicates of 20 plants per treatment and 10 wood fragments per plant. Letters a and b represent significant differences (P-value <0.05) between treatments.

**Figure 5.** Percentages of plants infected with *Cylindrocarpon*-like asexual morphs, and percentage of isolations of *Cylindrocarpon*-like asexual morphs from wood fragments observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs and untreated plants (control) in the nursery experiment B. Mean percentages are based on four replicates of 20 plants per treatment and 10 wood fragments per plant. Letters a and b represent significant differences (P-value <0.05) between treatments.

**Figure 6.** Relative quantification of *Bs* PTA-271 (a) and *Ta SC1* (b), in experiment A on plants treated with *Bs* PTA-271, *Ta* SC1 and simultaneously with both BCAs. The quantification of each BCA is in comparison with untreated plants (Control) that represents 1x.



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**Figure 7.** Relative quantification of *Bs* PTA-271 (a) and *Ta SC1* (b), in experiment B on plants treated with *Bs* PTA-271, *Ta* SC1 and simultaneously with both BCAs. The quantification of each BCA is in comparison with untreated plants (Control) that represents 1x.

For Peer Review

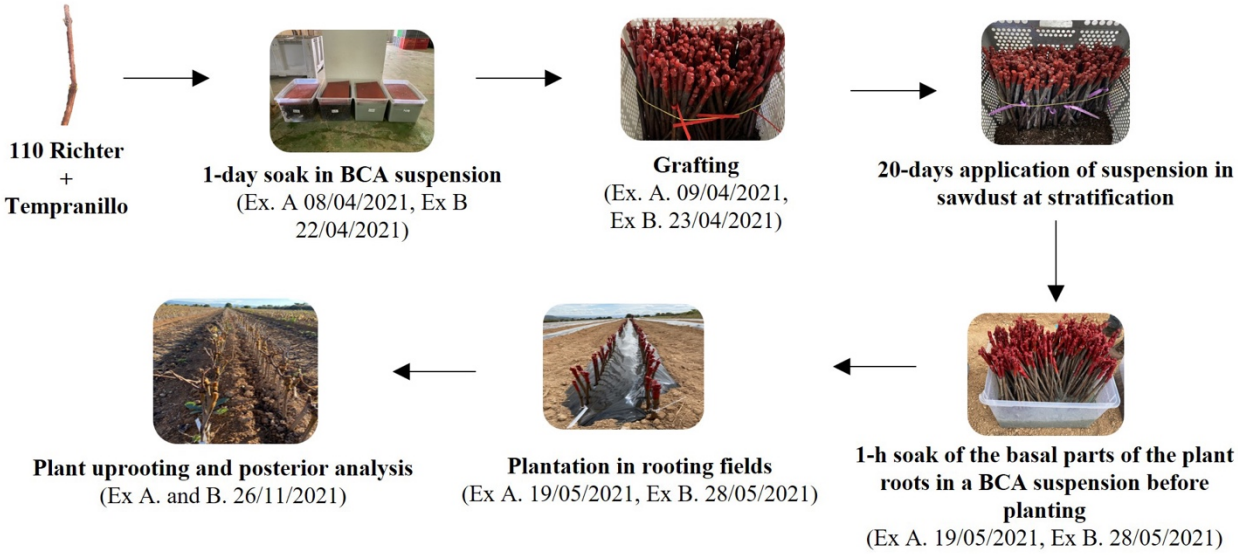
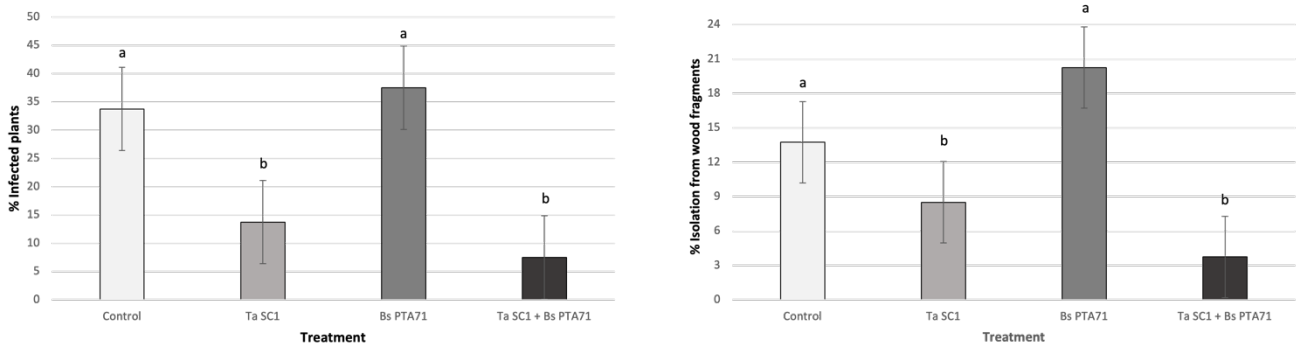



**Figure 1.**

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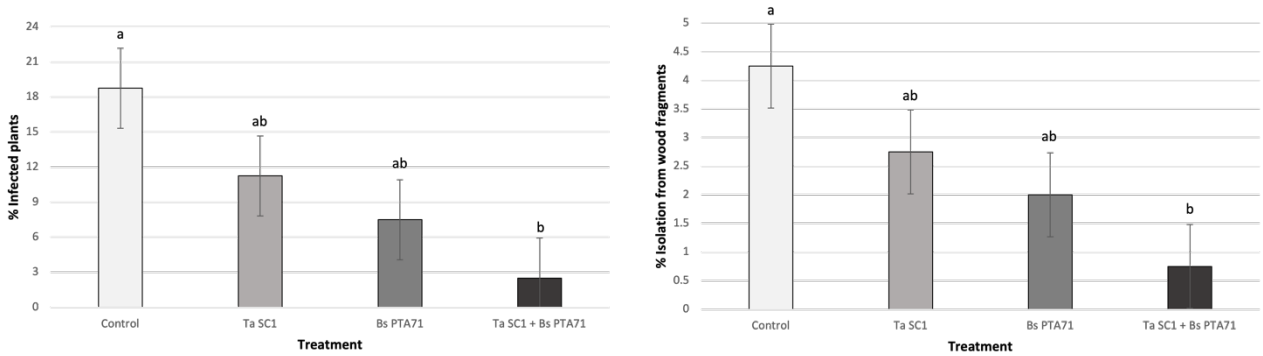


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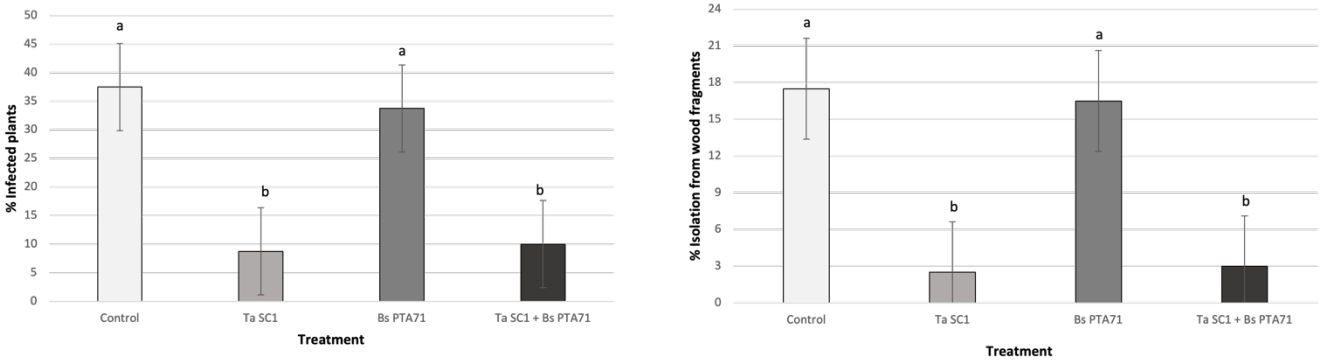



**Figure 4.**

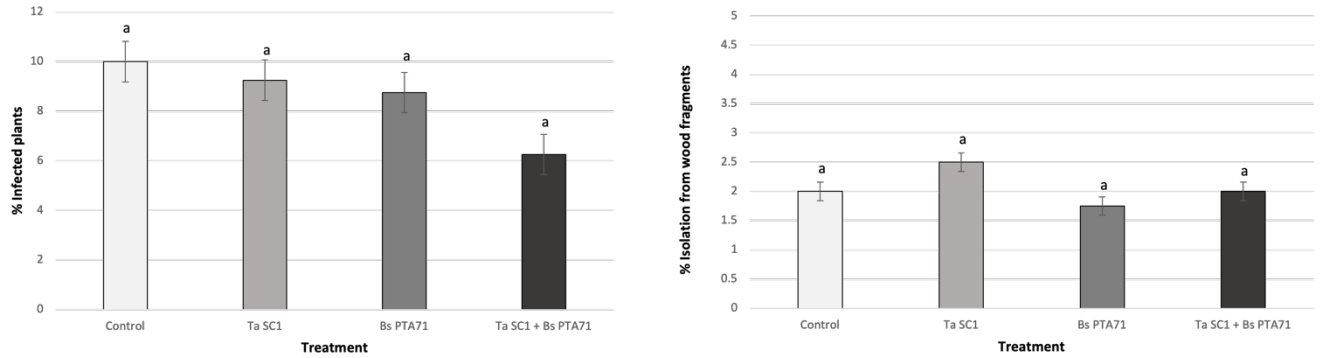
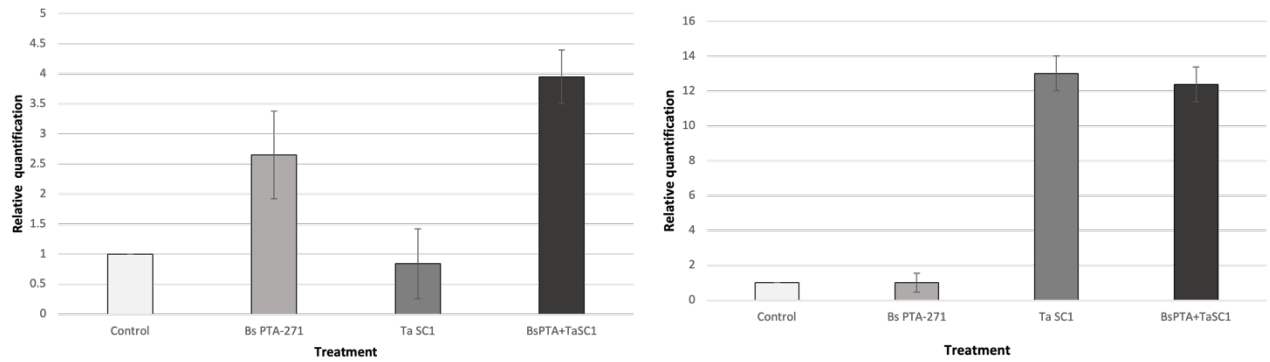



**Figure 5.**

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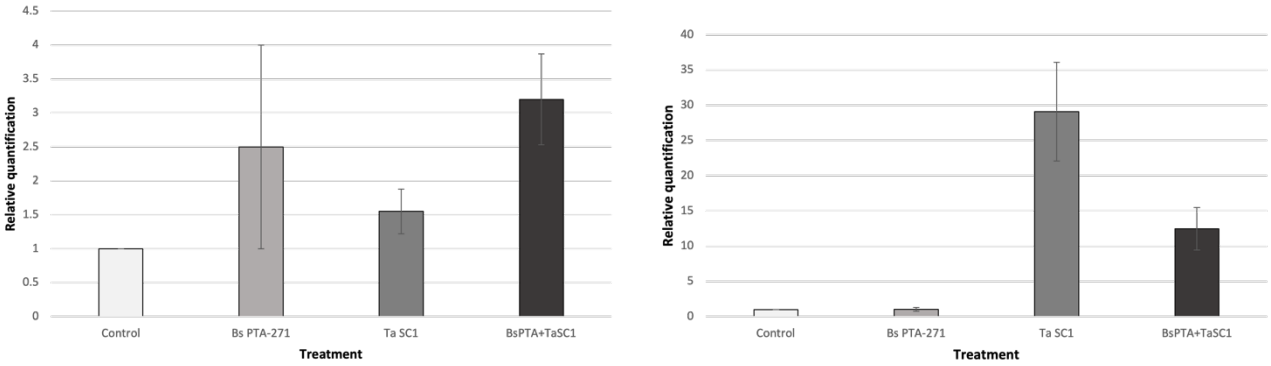
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Relative quantification
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Ta SC1
BsPTA+TaSC1
Treatment

**Figure 7.**

***Chapter VI***

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# Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 on grapevine microbiome

## 6.1. Contexte

La rhizosphère (zone étroite du sol autour des racines des plantes et influencée par les sécrétions racinaires) est l'endroit où les pathogènes du sol et les micro-organismes bénéfiques aux plantes exercent des influences sur la croissance et la santé des plantes hôtes (Raaijmakers et al. 2009 ; Liu et al. 2014). Les agents pathogènes des plantes présents dans le sol peuvent constituer une limitation majeure de la production de rendement, plus récalcitrante à la gestion et au contrôle que les agents pathogènes attaquant les parties aériennes de la plante (Bruehl 1987). Les micro-organismes qui nuisent à la croissance et à la santé des plantes sont les champignons pathogènes, les oomycètes, les bactéries et les nématodes (Raaijmakers et al. 2009 ; Li et al. 2021). Les bactéries ont besoin de blessures ou d'ouvertures naturelles pour pénétrer dans la plante et causer des infections, et la plupart des nématodes dans le sol sont libres, bien que certains puissent parasiter les racines des plantes (Siddiqui et al. 2012 ; Perri et Moens 2006). Les champignons et les oomycètes sont désignés comme les agents pathogènes du sol les plus importants. Ils sont décrits comme pénétrant dans les racines des plantes en utilisant des enzymes dégradant la paroi cellulaire et modifiant la pression mécanique de turgescence pour coloniser le cortex racinaire (Knogge 1996). Plusieurs maladies et symptômes des plantes sont attribués aux pathogènes fongiques du sol, tels que la réduction de la taille des plantes, le rabougrissement, le stress dû à la sécheresse et les carences en nutriments (Raaijmakers et al. 2009). Ces maladies sont difficiles à diagnostiquer car ces symptômes s'expriment au niveau des parties souterraines (Raaijmakers et al. 2009).

Les microbiomes associés aux plantes sont divers et complexes, et la compréhension (i) des mécanismes et des facteurs qui maintiennent des communautés microbiennes spécifiques associées aux plantes, et (ii) des facteurs qui interviennent dans l'équilibre des microbiomes, est encore limitée (Reinhold-Hurek et al. 2015). La vigne est considérée comme un excellent modèle végétal pour la recherche approfondie sur le microbiote fongique et bactérien (Gramaje et al. 2022), en utilisant les nouvelles technologies de séquençage telles que le séquençage massif de nouvelle génération (NGS). Cette technologie fournit des outils puissants pour caractériser et quantifier rapidement les communautés microbiennes avec des identifications fiables (Azevedo-Nogueira et al. 2022). Le principal avantage de ces méthodes est l'analyse du microbiome à grande échelle, pour chaque échantillon simultanément, sans qu'il soit nécessaire d'isoler les agents détectés (Azevedo-Nogueira et al. 2022). Ces

méthodologies permettent également d'identifier et de quantifier relativement la présence de chaque agent microbien (Eichmeier et al. 2018 ; Morales-Cruz et al. 2018 ; Nerva et al. 2022) et peuvent être utilisées sans détruire et mettre en danger les vignes échantillonnées. Les MDB représentent l'un des plus grands défis de la viticulture de nos jours, puisque les moyens de contrôle sont limités en termes d'efficacité. Les MDB (c'est-à-dire l'Eutypiose, le dépérissement aux Botryosphaeriacées, le complexe d'Esca, le chancre de Cytospora, le dépérissement de Phomopsis, la maladie du pied noir et la maladie de Petri) affectent principalement la partie pérenne de la vigne, du tronc aux charpentes, incluant le système racinaire (Nogueira-Azevedo et al. 2022). Les infections se produisent à travers les plaies de taille et le matériel de propagation en pépinière. À ce jour, les traitements les plus courants contre les MDB comprennent quelques produits chimiques qui sont appliqués sur les plaies de taille dans les vignobles pour empêcher la contamination par des spores (Sosnowski et al. 2004), des méthodes prophylactiques au vignoble (Mondello et al. 2018) et à des méthodes sanitaires en pépinière (Gramaje et Armengol 2011 ; Gramaje et al. 2018). Cependant, ces traitements ne peuvent pas éradiquer les pathogènes une fois qu'ils sont établis dans la plante, que ce soit au vignoble ou en pépinière (Calzarano et al. 2004 ; Gramaje et Armengol 2011).

Une alternative et un complément intéressants aux méthodes de lutte contre les MDB citées précédemment dans le vignoble est l'utilisation d'agents de lutte biologique (BCA) tels que *Trichoderma spp* et *Bacillus subtilis* (Mondello et al. 2018 ; Trotel-Aziz et al. 2019 ; Berbegal et al. 2020 ; Leal et al. 2021a,b). Les espèces de *Trichoderma* sont les BCA à base de champignons les plus utilisées en viticulture (Harman 2006 ; Muckherjee et al. 2012 ; Waghunde et al. 2016) et sont décrites comme antagonistes directs de l'agressivité des pathogènes des MDB par la compétition pour les nutriments et l'espace, le mycoparasitisme, les enzymes de dégradation de la paroi cellulaire et l'antibiose (Harman 2006 ; Van Wees et al. 2008 ; Vinale et al. 2008 ; Pieterse et al. 2014 ; Waghunde et al. 2016). Les *Trichoderma* spp. ont également été décrits comme des stimulateurs de croissance et de défense des plantes (Harman 2006 ; Van Wees et al. 2008 ; Vinale et al. 2008 ; Pieterse et al. 2014 ; Waghunde et al. 2016 ; Romeo-Olivan et al. 2022 ; Leal et al. 2021b). Les *B. subtilis* spp. sont également parmi les micro- organismes bactériens les plus fréquemment testés contre les MDB (Mondello et al. 2018), qui produisent une large gamme de molécules bénéfiques, à la fois pour induire ou susciter les défenses des plantes (comme avec les précurseurs de phytohormones, les lipopolysaccharides, les sidérophores, etc.) et pour concurrencer, antagoniser ou modifier directement les

phytopathogènes ou leur agressivité (Kloepper et al. 2004 ; Ongena et Jacques 2008 ; Leal et al. 2021).

Ces dernières années, des études se sont ainsi concentrées sur les effets de l'inoculation de plantes avec des micro-organismes bénéfiques sur la santé des plantes et/ou les agents pathogènes des plantes. Toutefois, peu d'études se sont intéressées à l'impact de l'introduction d'un nouvel BCA dans la rhizosphère, sur les communautés microbiennes indigènes du sol (Gao et al. 2012 ; Gramaje et al. 2022, Kozdrój et al. 2004). Dans cette étude, nous avons donc étudié (Figure 5 et 9) explorer le potentiel des 2 BCAs à moduler le microbiome de plants de vigne en contact avec les agents pathogènes de MDB dans deux types de sol différents. (Chapitre VI).

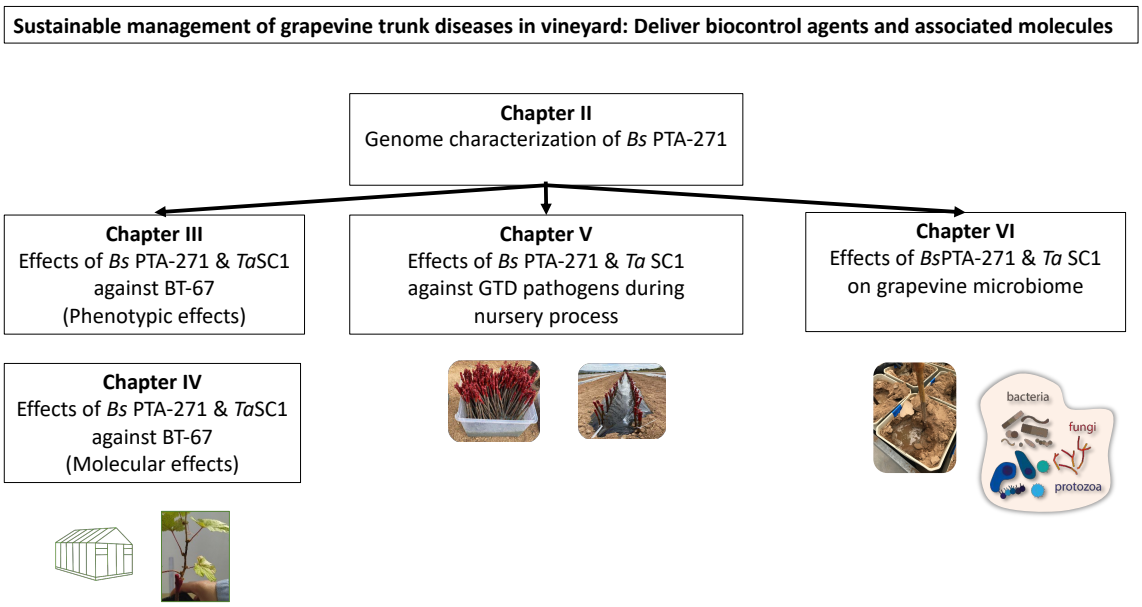


**Figure 5.** Aims of each chapter of the thesis entitled “Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules”.

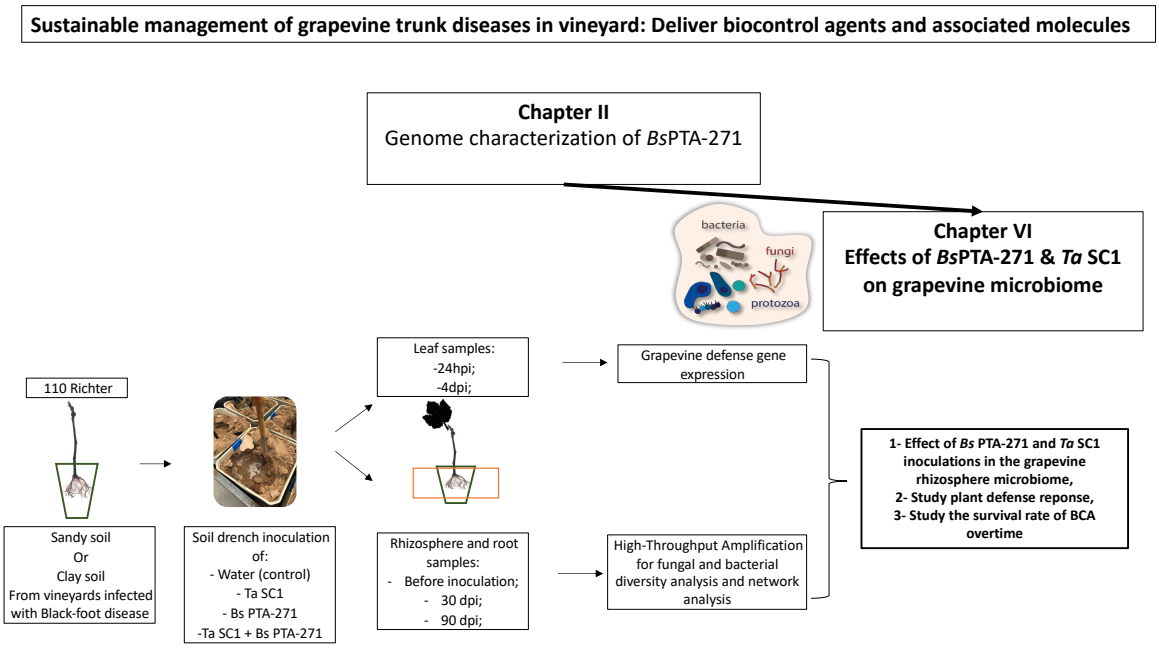


**Figure 9.** Aims chapter VI entitled "Effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 on grapevine microbiome".

**Publication 6 - Effect of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 on grapevine defenses and temporal dynamics of fungal and bacterial microbiome in grapevine rhizosphere**

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# Effect of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 on grapevine defenses and temporal dynamics of fungal and bacterial microbiome in grapevine rhizosphere

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## Abstract

The rhizosphere is where soil-borne pathogens and plant beneficial microorganisms exert influence on the growth and health of plant hosts. Soilborne plant pathogens can be a major limitation in yield production and are also strongly recalcitrant to management and control, compared to pathogens that attack the above-ground portions of the plant. Among them, are those involved in Grapevine trunk diseases (GTDs) which represent one of the biggest challenges for sustainable viticulture nowadays, as there is no current effective control to combat them. The use of biocontrol agents (BCA) like *Trichoderma* spp. and *Bacillus* spp. is being widely studied as a solution to treat GTDs.

In this study, we investigated with two distinct soils (sandy and limestone) : 1) the effect of *Bacillus subtilis* PTA-271 (*Bs* PTA-271) and *Trichoderma atroviride* SC1® (*Ta* SC1) inoculations in the grapevine rhizosphere microbiome, focusing especially on the soilborne pathogens involved in GTD such as black foot; 2) the survival capacity of *Bs* PTA-271 and *Ta* SC1 over time; and 3) the effect of *Bs* PTA-271 and *Ta* SC1 inoculations in grapevine defenses. Results suggest that: 1) the inoculation of BCAs seems to improve the rhizosphere microbiome networks and sanitation status; 2) the beneficial effect of BCAs in grapevine rhizosphere microbiome can be soil-dependent; and finaly 3) the combination of both BCAs improves their beneficial effect in the rhizosphere microbiome.

**Keywords:** Bacterial and fungal recruitment, beneficial microorganisms, black-foot disease, microbial ecology, microbiome, rhizosphere, rootstock.

## 1. Introduction

The rhizosphere (narrow zone of soil around plant roots and influenced by root secretions) is where soil-borne pathogens and plant beneficial microorganisms exert influences on the growth and health of plant hosts (Raaijmakers et al. 2009; Liu et al. 2014). Soilborne plant pathogens can be a major limitation in yield production more recalcitrant to management and control compared to pathogens attacking the above-ground parts of the plant (Bruehl 1987). Microorganisms that adversely affect plant growth and health are the pathogenic fungi, oomycetes, bacteria, and nematodes (Raaijmakers et al. 2009; Li et al. 2021). Bacteria require wounds or natural openings to penetrate the plant and cause infections, and most nematodes in soil are free-living, although some can parasitize plant roots (Siddiqui et al. 2012; Perri and Moens 2006). Fungi and oomycetes are designated as the most important soilborne pathogens. They are described to penetrate in plant roots by using cell wall-degrading enzymes and mechanical turgor pressure to colonize the root cortex (Knogge 1996). Several plant diseases and symptoms are attributed to fungal soilborne pathogens, such as a plant size reduction, stunting, drought stress and nutrient deficiencies (Raaijmakers et al. 2009). These diseases are difficult to diagnose because these symptoms mostly occur below ground (Raaijmakers et al. 2009).

Plant-associated microbiomes are diverse and complex, and there is still a limited understanding (i) of the mechanisms and factors that maintain specific plant-associated microbial communities, and (ii) of the factors that stimulate the appropriate balance microbiomes (Reinhold-Hurek et al. 2015). The grapevine is considered an excellent model plant system deeply research on fungal and bacterial microbiota (Gramaje et al. 2022), using new technologies of sequencing such as the massive next generation sequencing (NGS) that provide powerful tools to characterize and quantify microbial communities fastly with reliable identifications (Azevedo-Nogueira et al. 2022). The main benefit of such methods is the wide microbiome analysis, for each sample simultaneously, without the need to isolate the detected agents (Azevedo-Nogueira et al. 2022). These methodologies also allow to identify and relatively quantify the presence of each microbial agent (Eichmeier et al. 2018; Morales-

Cruz et al. 2018; Nerva et al. 2022) and can be performed without destroying and endangering the sampled vines.

Grapevine trunk diseases (GTDs) represent one of the biggest challenges in viticulture nowadays, as there is no current control. GTDs (i.e., Eutypa dieback, Botryosphaeria dieback, Esca complex, Cytospora canker, Phomopsis dieback, Black foot disease and Petri disease) affect mainly the perennial part of grapevines, from the trunk to shoots, and down to the root system (Nogueira-Azevedo et al. 2022). Infections happen trough pruning wounds and propagation material, with pathogen inoculum present in the soil and traveling by air. To date, the most common treatments for GTDs include few chemicals that are applied on pruning wounds in vineyards to prevent dissemination of the conidia of fungal pathogens (Sosnowski et al. 2004), associated to cultural practices in vineyard (Mondello et al. 2018) and sanitation methods in nurseries (Gramaje and Armengol 2011; Gramaje et al. 2018). However, these types of treatments cannot eradicate the pathogens once established in a vineyard or in nurseries (Calzarano et al. 2004; Gramaje and Armengol 2011).

An interesting alternative and complement to the previously cited GTD control methods in vineyard is the use of biological control agents (BCAs) such as *Trichoderma* and *Bacillus subtilis* (Mondello et al. 2018; Trotel-Aziz et al. 2019; Berbegal et al. 2020; Leal et al. 2021). *Trichoderma* species are the most used fungal-based BCA in viticulture (Harman 2006; Muckherjee et al. 2012; Waghunde et al. 2016) and are described to directly antagonize GTD pathogens aggressiveness by competition for nutrients and space, mycoparasitism, cell-wall degrading enzymes, and antibiosis (Harman 2006; Van Wees et al. 2008; Vinale et al. 2008; Pieterse et al. 2014; Waghunde et al. 2016). *Trichoderma* spp. have also been described as plant growth and defense stimulators (Harman 2006; Van Wees et al. 2008; Vinale et al. 2008; Pieterse et al. 2014; Waghunde et al. 2016; Romeo-Olivan et al. 2022; Leal et al. 2021b). *B. subtilis* spp. are also among the most frequently bacterial microorganisms tested against GTDs (Mondello et al. 2018), that produce a broad range of beneficial molecules, both to induce or elicit plant defenses (as with phytohormones precursors, lipopolysaccharides, siderophores, etc.) and to directly compete, antagonize, or alter plant pathogens or their aggressiveness (Kloepper et al. 2004; Ongena and Jacques 2008; Leal et al. 2021).

Over the years, studies have focused on the effects that inoculating plants with beneficial microorganisms has on plant health and/or plant pathogens, but few studies have looked at the impact of introducing a new biocontrol organism in rhizosphere, on the indigenous soil microbial communities (Gao et al. 2012; Gramaje et al. 2022, Kozdrój et al. 2004). In this study, we therefore investigated: 1) the effect of *B. subtilis* PTA 271 (*Bs* PTA-271) and *T. atroviride*

SC1 (*Ta* SC1) inoculations in the grapevine rhizosphere microbiome; 2) the survival capacity of *Bs* PTA-271 and *Ta* SC1 over time; and finally, 3) the effect of *Bs* PTA-271 and *Ta* SC1 inoculations in grapevine defenses.

# 2. Materials and Methods

## 2.1. Planting Material

Dormant grapevine cuttings of rootstocks 110 Richter (110 R) were obtained from commercial nursery mother fields in Larraga (Navarra, northern Spain). Two stocks of 32 cuttings were used. Each stock was collected in different mother fields separated by 800 m. Rootstock mother vines were 12-year-old and were cultivated along the ground from a self-supporting crown approximately 40 cm above the soil surface. Within each mother field, the 16 cuttings were randomly collected from 32 plants (one cutting per plant) near the crown of the mother vine. All rootstock cuttings were 40 cm long and 1.5 cm in diameter.

## 2.2. Biological control agent's preparation

### 2.2.1. *Bacillus subtilis* PTA-271

*Bacillus subtilis* PTA-271 (GenBank Nucleotide EMBL Accession No. AM293677 for 16S rRNA and DDBJ/ENA/GenBank Accession No. JACERQ000000000 for the whole genome), thereafter *Bs* PTA-271, was isolated in 2001 from the rhizosphere of healthy Chardonnay grapevines on a vineyard located in Champagne (Marne, France) (Trotel-Aziz et al. 2008). Bacterial growth started with addition of 1 ml of glycerol stock suspension to sterile Luria Bertani (LB) medium and incubating at 25-28°C. Experiments were performed when the bacterial culture was at the exponential growth phase. After centrifugation (5,000 g, 10 min), the pellet was resuspended with a sterile 10 mM MgSO4 medium adjusting the density to $10^9$ cfu $mL^{-1}$.

### 2.2.2. *Trichoderma atroviride* SC1

*Trichoderma atroviride* SC1 (Vintec®, Belchim Crop Protection, Bi-PA; 1010 conidia per gram of formulated product), therefore *Ta* SC1, was suspended in water at 2g $L^{-1}$ as indicated by the producer. The viability of the conidia in the commercial product was checked to be at a minimum of 85% before each trial, as described by Pertot et al. (2016). A serial dilution of the conidia suspension was plated on Potato Dextro Agar (Biokar-Diagnostics, Zac de Ther, France) and the colony forming units were counted after 24-48 h incubation at room temperature.

## 2.3. Experiment layout

Rootstock 110R cuttings were placed in individual pots with one of two types of soil: limestone soil (8 plants) or sandy soil (8 plants). The soils were collected from vineyards with a history of severe Black-foot disease incidence in Logroño, (La Rioja, northern Spain) (Berlanas et al. 2017). Cuttings were placed in the greenhouse for 12 months to allow plant natural infections and watered every 48h. Before BCA application, rhizosphere samples were collected from all plants (T1). Plants were then inoculated with a soil drench with *Bs* PTA-271 (2 plants per type of soil), *Ta* SC1 (2 plants per type of soil), or *Bs* PTA-271 and *Ta* SC1 (2 plants per type of soil). Control plants were treated with a water soil drench. Twenty-four hours (24 hpi) and four days after the inoculation (4 dpi), leaf samples were collected from all plants to analyze expression of plant defense genes. Rhizosphere of every plant was also collected 30 days after the inoculation (T2, 30 dpi), and 90 days after inoculation (T3, 90 dpi) for microbiome analysis.

## 2.4. RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis

Leaf samples were collected 48h post-inoculation of BCAs, ground in liquid nitrogen, and then stored at −80°C. Total RNA was extracted from 50 mg of leaf powder with Plant RNA Purification Reagent according to manufacturer instructions (Invitrogen, Pontoise, France), and DNase treated as described by the manufacturer's instructions (RQ1 RNase-Free DNase, Promega). RNA quality was checked by agarose gel electrophoresis, and total RNA concentration was measured at 260 nm for each sample using the NanoDrop One spectrophotometer (Ozyme) and adjusted to 100 ng $\mu l^{-1}$. First-strand cDNA was synthesized from 150 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc.,

Waltham, MA, United States). Polymerase chain reaction (PCR) conditions were the ones described by Gruau et al. (2015). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed with Absolute Blue qPCR SYBR Green ROX Mix according to manufacturer instructions (Thermo Fisher Scientific, Inc., Waltham, MA, United States), in a BioRad C1000 thermocycler using the Bio-Rad manager software CFX96 Real-Time PCR (BioRad, Hercules, CA, United States). A set of six defense-related genes, selected for their responsiveness to pathogen or priming state induced by beneficial microorganisms (Trotel-Aziz et al. 2019), was tracked by qRT-PCR using specific primers (Table 1). Quantitative RT-PCR reactions were carried out in duplicate in 96-well plates in a 15-µl final volume containing Absolute Blue SYBR Green ROX mix including Taq polymerase ThermoPrime, dNTPs, buffer, and MgCl2 (Thermo Fisher Scientific, Inc., Waltham, MA, United States), 280 nM forward and reverse primers, and 10-fold diluted cDNA according to the manufacturer's protocol. Cycling parameters were 15 min of Taq polymerase activation at 95°C, followed by 40 two-step cycles composed of 10 s of denaturation at 95°C and 45 s of annealing and elongation at 60°C. Melting curve assays were performed from 65 to 95°C at 0.5°C $s^{-1}$, and melting peaks were visualized to check amplification specificity. EF1-a and 60RSP genes were used as references and experiments were repeated five times. Relative gene expression was determined with the formula fold induction: 2(−11Ct), where 11Ct = [Ct TG (US) − Ct RG (US)] − [Ct TG (RS) − Ct RG (RS)], where Ct is cycle threshold, Ct value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is target gene, RG is reference gene, the US is an unknown sample, and RS is reference sample. Integration of the formula was performed by the CFX Manager 3.1 software (Bio-Rad). Although the genes analyzed were considered significantly up- or down-regulated when changes in their expression were >2-fold or <0.5-fold, respectively, we still performed a statistical analysis. Control samples are cDNA from leaves of untreated plants (1× expression level).

**Table 1.** Primer sequences used for qRT-PCR analysis of defense-related genes (Trotel-Aziz et al. 2019).

| Gene | Name | Accession number[1] | Forward primer (5′-3′) | Reverse primer (5′-3′) | Annealing temperature (°C) | Amplicon size (bp) | Efficiency of primers pairs (%) |
|---|---|---|---|---|---|---|---|
| *60RSP* | 60S ribosomal protein L18 | XM_002270599[1] | ATCTACCTCAAGCTCCTAGTC | CAATCTTGTCCTCCTTTCCT | 60 | 166 | 100.0 |
| *EF1* | Elongation factor 1-alpha | XM_002284888[1] | AACCAAAATATCCGGAGTAA AAGA | GAACTGGGTGCTTGATAGGC | 60 | 164 | 100.0 |
| *LOX9* | Lipoxygenase | NM_001281249[1] | CCCTTCTTGGCATCTCCCTTA | TGTTGTGTCCAGGGTCCATTC | 60 | 101 | 90.0 |
| *PR1* | Pathogenesis-related protein 1 | XM_002273752[1] | GGAGTCCATTAGCACTCCTTTG | CATAATTCTGGGCGTAGGCAG | 60 | 168 | 90.0 |
| *PR2* | Class I beta-1,3-glucanase | NM_0012809671 | TCAATGGCTGCAATGGTGC | CGGTCGATGTTGCGAGATTTA | 60 | 155 | 97.2 |
| *GST1* | Glutathione-*S*-transferase | NM_001281248[1] | TGCATGGAGGAGGAGTTCGT | CAAGGCTATATCCCCATTTTCTTC | 60 | 98 | 90.0 |
| *PAL* | Phenylalanine ammonia lyase | XM_003635637[1] | TCCTCCCGGAAAACAGCTG | TCCTCCAAATGCCTCAAATCA | 60 | 101 | 92.9 |
| *STS* | Stilbene synthase | NM_001281117[1] | AGGAAGCAGCATTGAAGGCTC | TGCACCAGGCATTTCTACACC | 60 | 101 | 94.3 |

[1]*NCBI accession number.*

## 2.5. DNA Extraction, Sequencing and Data Analysis of the High-Throughput sequencing Assay

Genomic rhizosphere DNA was extracted from 0.5 g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the kit protocol. DNA yields were quantified using the Invitrogen Qubit 4 Fluorometer with Qubit dsDNA HS (High Sensitivity) Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the extracts were adjusted to 10-15 ng/µL.

For fungal library preparation, the complete fungal ITS2 region was amplified using the primers ITS86F (Turenne et al. 1999) and ITS4 (White et al. 1990). For bacterial library preparation, the 16s rRNA was amplified. Primers were modified to include the Illumina sequencing primers. PCR reactions were carried out in a final volume of 25 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 12.5 µL of Supreme NZYTaq 2× Green Master Mix (NZYTech), and ultrapure water up to 25 µL. PCR amplifications consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 49 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. Libraries were purified using the

Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, Norcross, GE, USA), following the instructions provided by the manufacturer. The purified libraries were pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA, USA). Samples were sequenced in the MiSeq platform (Illumina, San Diego, CA, USA) at the AllGenetics and Biology SL (Galicia, Spain), using a paired-end 2 × 300 bp (PE 300) sequencing and the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA). Negative controls during library preparation and DNA extraction, and a positive control containing DNA of a grapevine rhizosphere sample (Berlanas et al. 2019) were included.

## 2.6. Fungal and bacterial Diversity, Taxonomy Distribution and Statistical Analysis

Alpha-diversity was calculated by analyzing the Chao1 richness and Shannon diversity in Phyloseq package. Differences in fungal and bacterial alpha-diversity among soils and BCA treatments were inferred by multiple mean comparisons using Tukey's honestly significant difference range test ($p \leq 0.05$). PERMANOVA was used to infer which OTUs significantly differed in relative abundance among experimental factors after Bonferroni corrections. The relationship in OTUs composition among samples was investigated by calculating Bray Curtis metrics and visualized in PCoA plots. Good's coverage values and rarefaction curves were also calculated. All diversity analyses were made using MicrobiomeAnalyst (Chong et al. 2020). Persistent and transient microbiota were inferred using TIME (Baksi et al. 2018). Persistent fungal and bacterial microbiota was defined as those taxa observed in 20% or more of the sampling times but with at least 90% of those observations being consecutive (Caporaso et al. 2011). Transient fungal and bacterial microbiota was defined as those taxa observed in at least 60% of the samples, but with at most 75% of those observations being consecutive (stages of sample development) (Caporaso et al. 2011).

The identification of fungal and bacterial taxa that differed in relative abundance among sampling times was performed by computing the Linear Discriminant Analysis Effect Size (LEfSe) algorithm in MicrobiomeAnalyst. The Linear Discriminant Analysis (LDA) threshold score was set up at 1.0 and Wilcoxon p-value at 0.05. Correlation networks were computed with the SparCC algorithm to identify potential interactions between genera that could represent parasitic, commensal, mutualistic or competitive relationships, using

MicrobiomeAnalyst. *p*-value threshold was set up at 0.05 with 120 permutations, and the correlation threshold at 0.6.

Heatmaps were employed to visualize the abundances of fungi and bacteria at each sampling time using MicrobiomeAnalyst, with Euclidean as distance measure and Ward as a clustering algorithm.

# 3. Results

## 3.1. Microorganisms Abundance

### 3.1.1. Fungi

Regarding the rhizosphere fungal microbiome of plants inoculated with *Bs* PTA-271, *Ta* SC1, both BCAs, and control plants, differences were found according to the applied treatment, and the time of sampling (Figure 1).

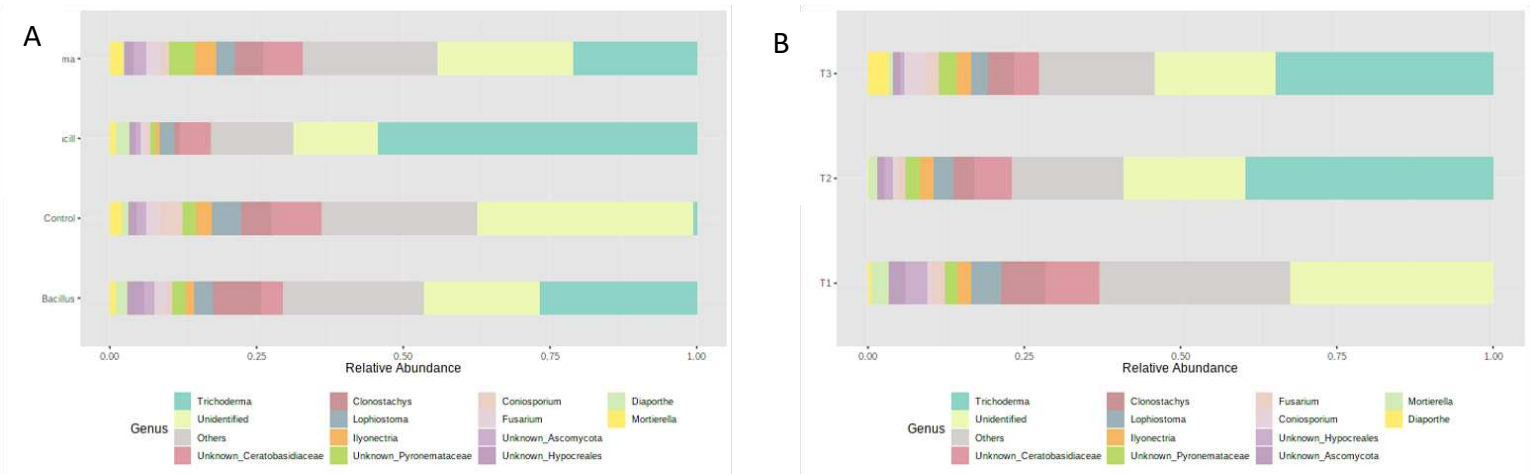


**Figure 1.** Fungal relative abundance comparison between (A) applied treatments (Control, *Bs* PTA-271, *Ta* SC1, *Bs* PTA-271 + *Ta* SC1), and (B) sampling times (T1, T2, T3).

As indicated by the Figure 1A, the main changes concerns *Trichoderma* spp., for which control plants presented a significant lower abundance, when compared to other treatments. However, plants inoculated with *Bs* PTA-271 alone had a considerable abundance of

*Trichoderma* spp., similar to the treatment with *Ta* SC1 alone. The treatment with both BCAs showed a significantly higher abundance of *Trichoderma* spp. than the treatments with only one BCA.

Concerning sampling time (Figure 1B), *Trichoderma* spp. was undetected at T1 (before inoculation), while its abundance increased significantly at both T2 and T3. However, the abundance of *Trichoderma* spp. decreased at T3, since samples presented less *Trichoderma* spp. abundance compared to T2 samples (Figure 1B).

### 3.1.2. Bacteria

The bacterial microbiome of the rhizosphere presented more microorganisms, when compared to the fungal microbiome (Figure 2). Unlike *Trichoderma* spp., *Bacillus* spp. is not the most abundant groups of bacteria. Globally, *Rudobacte*r spp. was the most abundant bacterial group in the rhizosphere (Figure 2A). Looking at *Bacillus* spp., control plants presented a significant abundance of *Bacillus* spp., and there were no significant differences on *Bacillus* spp. abundance, according to the treatments (Figures 2A) and the sampling times (Figure 2C). However, *Bacillus* spp. was more abundant in sandy soil, compared with limestone soil samples (Figure 2B).

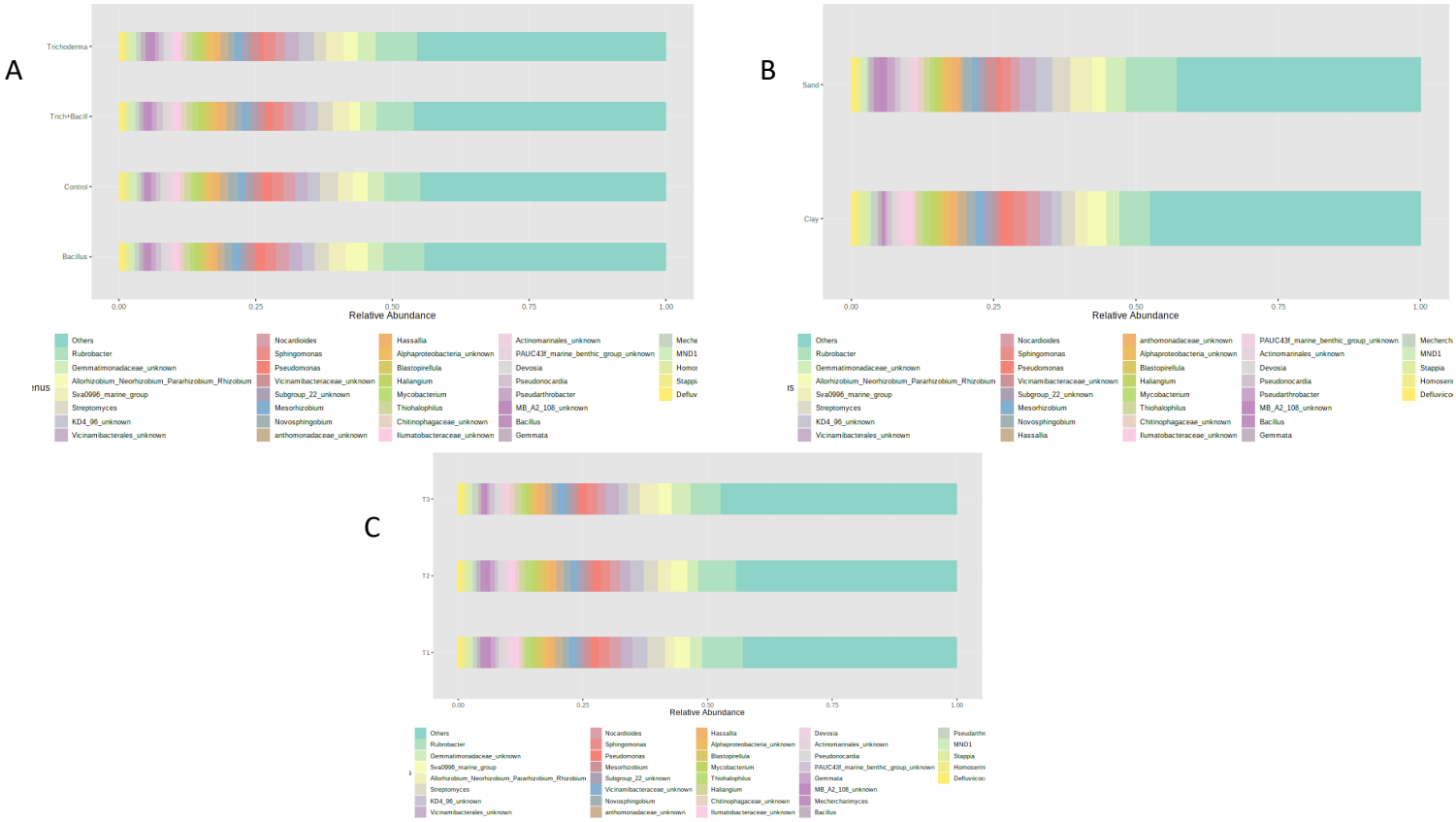


**Figure 2.** Bacterial relative abundance comparison between applied treatments (Control, *Bs* PTA-271, *Ta* SC1, *Bs* PTA-271 + *Ta* SC1) (A), type of soil (B) (sandy and limestone), and sampling times (T1, T2, T3) (C).

## 3.2. Alpha diversity

### 3.2.1. Fungi

On sandy soil there were significant differences on the diversity between the 2 compartments, roots and rhizosphere. In case of rhizosphere, there were significant differences between treatments and sampling time, therefore we focused our further analysis on rhizosphere only (Table 2).

**Table 2.** Statistical analysis of fungal alpha diversity in sandy soil samples, using Chao1 index and Shannon index. p: p-value, F: F statistics.

| Sandy soil | | | |
|---|---|---|---|
| Dataset | Factor | Indexes | |
| | | Chao1 | Shannon |
| Sandy soil | Treatment | p: 0.97885; F: 0.06377 | p: 0.013127; F: 3.7815 |
| | Compartement | p: 1.0881e-36; F: 21.601 | p: 3.8454e-07; F: 5.5378 |
| | Sampling | p: 0.7041; F: 0.35216 | p: 0.5345; F: 0.63066 |
| | Treatment_Sampling | P: 0.99942; F: 0.14286 | p: 0.063168; F: 1.8196 |
| Sandy soil/Rhizosphere | Treatment | p: 0.68457; F: 0.49948 | p: 0.00012458; F: 8.6653 |
| | Sampling | p: 0.15655; F: 1.933 | p: 0.025993; F: 3.9626 |
| | Treatment_Sampling | p: 0.86101; F: 0.54219 | p: 1.4285e-08; F: 11.115 |
| Sandy soil/Root | Treatment | p: 0.13665; F: 1.9425 | p: 0.68432; F: 0.49985 |
| | Sampling | p: 0.47012; F: 0.76758 | p: 0.010596; F: 5.0393 |
| | Treatment_Sampling | p: 0.20794; F: 1.4164 | p: 0.29356; F: 1.247 |

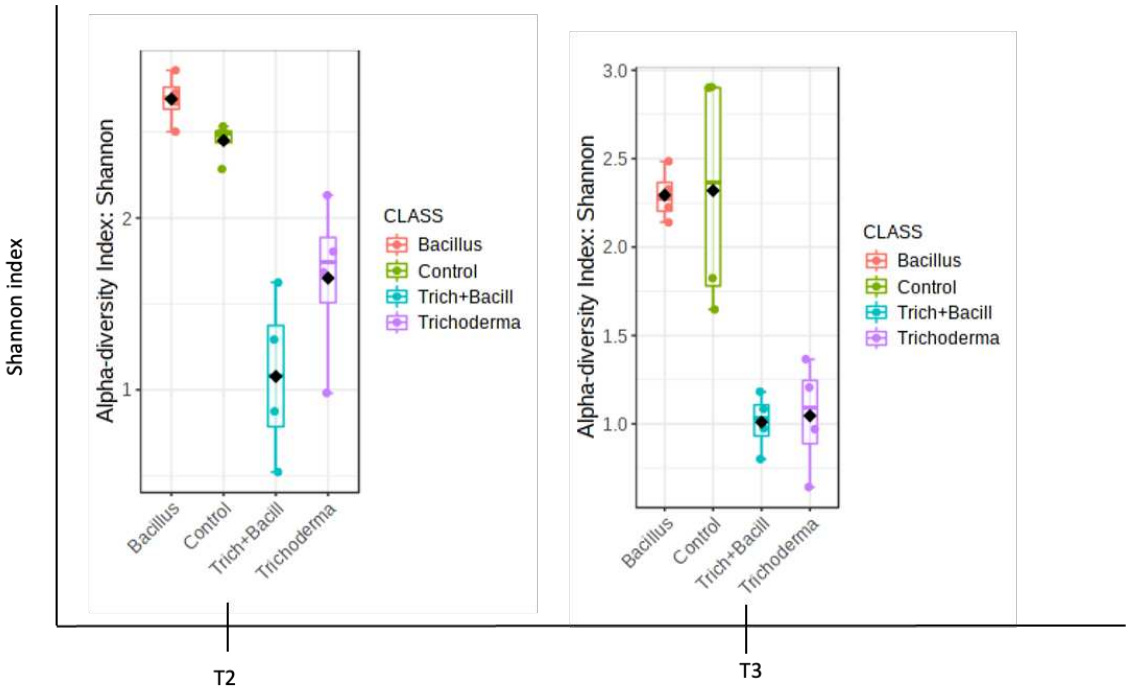


**Figure 3.** Differences in fungal alpha-diversity in sandy soil samples, between treatment applied (Control, *Bs* PTA-271 (*Bacillus*), *Ta* SC1 (*Trichoderma*), and *Trich + Bacill* (*Bs* PTA-271 + *Ta* SC1)), in sampling time T2 and T3, using Shannon index.

On sandy soil, at the sampling times T2 and T3, the treatments with *Ta* SC1 and both BCAs, presented significant lower fungal diversity than non-inoculated treatments (Figure 3).

On limestone soil there were significant differences on diversity between compartments (roots and rhizosphere). In case of rhizosphere, there were significant differences between treatments and sampling time, therefore we focused further analysis on the rhizosphere only (Table 3).

**Table 3.** Statistical analysis of fungal alpha diversity in limestone soil samples, using Chao1 index and Shannon index. p: *p*-value, F: F statistics.

| Limestone soil | | | |
|---|---|---|---|
| Dataset | Factor | Indexes | |
| | | Chao1 | Shannon |
| Limestone soil | Treatment | p: 0.92065; F: 0.16355 | p: 0.0011323; F: 5.7928 |
| | Compartement | p: 1.7327e-37; F: 22.989 | p: 4.4596e-08; F: 6.2158 |
| | Sampling | p: 0.6081; F: 0.50009 | p: 0.022458; F: 3.9554 |
| | Treatment_Sampling | p: 0.99576; F: 0.21976 | p: 0.0017627; F: 3.0531 |
| Limestone soil/Rhizosphere | Treatment | p: 0.33803; F: 1.154 | p: 7.1432e-06; F: 12.008 |
| | Sampling | p: 7.4878e-05; F: 11.82 | p: 0.018984; F: 4.3348 |
| | Treatment_Sampling | p: 0.017008; F: 2.5468 | p: 2.4087e-08; F: 10.673 |
| Limestone soil/Root | Treatment | p: 0.19994; F: 1.6131 | p: 0.31083; F: 1.2281 |
| | Sampling | p: 0.55562; F: 0.5954 | p: 0.33492; F: 1.1209 |
| | Treatment_Sampling | p: 0.16228; F: 1.5336 | p: 0.59129; F: 0.85294 |

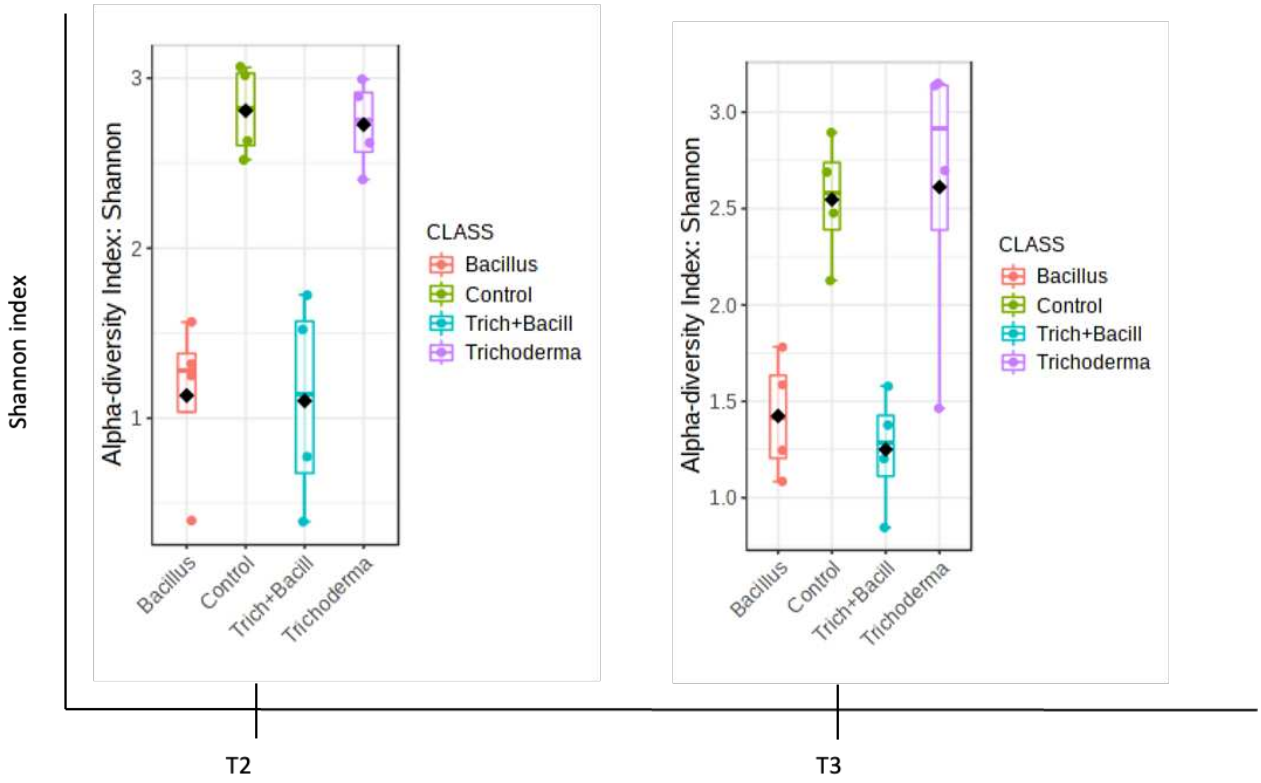


**Figure 4.** Differences in fungal alpha-diversity of limestone soil samples, between treatment applied (Control, *Bs* PTA-271 (*Bacillus*), *Ta* SC1 (*Trichoderma*), and *Trich + Bacill* (*Bs* PTA-271 + *Ta* SC1)), in sampling times T2 and T3, using Shannon index.

On limestone soil, at the sampling times T2 and T3, plants inoculated with *Bs* PTA-271 and both BCAs, presented significantly lower diversity than plants inoculated with *Ta* SC1 alone, and control plants (Figure 4.).

### 3.2.2. Bacteria

On sandy soil there were significant differences on diversity between compartments (roots and rhizosphere). In case of rhizosphere, there were significant differences between sampling times, therefore we focused further analysis on rhizosphere only (Table 4.).

**Table 4.** Statistical analysis of bacterial alpha diversity in sandy soil samples, using Chao1 index and Shannon index. p: p-value, F: F statistics.

| Sandy soil | | | |
|---|---|---|---|
| Dataset | Factor | Indexes | |
| | | Chao1 | Shannon |
| Sandy soil | Treatment | p: 0.93156; F: 0.1467 | p: 0.66954; F: 0.52005 |
| | Sampling | p: 0.26353; F: 1.3529 | p: 0.34303; F: 1.0823 |
| | Compartement | p: 1.8697e-26; F: 21.636 | p: 1.1661e-24; F: 16.964 |
| | Treatment_Sampling | p: 0.98753; F: 0.28295 | p: 0.97168; F: 0.34782 |
| Sandy soil/Rhizosphere | Treatment | p: 0.34341; F: 1.14 | p: 0.4937; F: 0.81274 |
| | Sampling | p: 0.0039894; F: 6.2613 | p: 0.0073146; F: 5.4967 |
| | Treatment_Sampling | p: 0.059909; F: 1.986 | p: 0.24222; F: 1.3425 |
| Sandy soil/Root | Treatment | p: 0.79329; F: 0.34446 | p: 0.15545; F: 1.831 |
| | Sampling | p: 0.00024413; F: 10.064 | p: 0.07751; F: 2.7083 |
| | Treatment_Sampling | p: 0.075435; F: 1.8829 | p: 0.35822; F: 1.1442 |

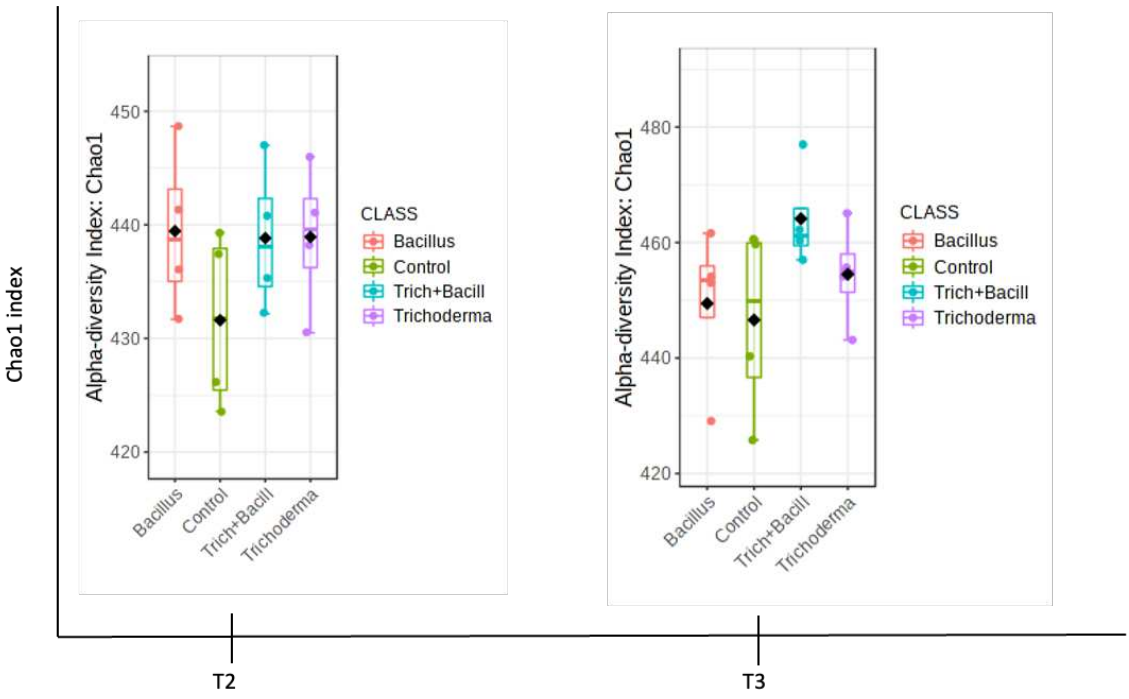


**Figure 5.** Differences in bacterial alpha-diversity of sandy soil samples, between treatment applied (Control, *Bs* PTA-271 (*Bacillus*), *Ta* SC1 (*Trichoderma*), and *Trich + Bacill* (*Bs* PTA-271 + *Ta* SC1)), in sampling times T2 and T3, using Chao1 index.

On sandy soil, at the sampling times T2 and T3, plants inoculated with any BCA presented higher bacterial diversity than control plants. Moreover, at T3, plants inoculated with both BCAs presented higher diversity than other treated plants (Figure 5).

On limestone soil there were significant differences on diversity between compartments (roots and rhizosphere). In case of rhizosphere, there were significant differences between sampling times, therefore we focused further analysis on rhizosphere only (Table 5).

**Table 5.** Statistical analysis of bacterial alpha diversity in limestone soil samples, using Chao1 index and Shannon index. p: *p*-value, F: F statistics.

| Limestone soil | | | |
|---|---|---|---|
| Dataset | Factor | Indexes | |
| | | Chao1 | Shannon |
| Limestone soil | Treatment | p: 0.99973; F: 0.0033513 | p: 0.84411; F: 0.27386 |
| | Sampling | p: 0.58565; F: 0.53813 | p: 0.22722; F: 1.5057 |
| | Compartement | p: 3.8175e-29; F: 25.174 | p: 8.9455e-22; F: 16.694 |
| | Treatment_Sampling | p: 0.99863; F: 0.17128 | p: 0.92856; F: 0.44911 |
| Limestone soil/Rhizosphere | Treatment | p: 0.7099; F: 0.4625 | p: 0.26051; F: 1.3829 |
| | Sampling | p: 0.061759; F: 2.9641 | p: 0.014797; F: 4.6336 |
| | Treatment_Sampling | p: 0.13058; F: 1.6342 | p: 0.07506; F: 1.8852 |
| Limestone soil/Root | Treatment | p: 0.89202; F: 0.20554 | p: 0.41773; F: 0.96504 |
| | Sampling | p: 0.03358; F: 3.6632 | p: 0.00012811; F: 11.01 |
| | Treatment_Sampling | p: 0.45059; F: 1.0185 | p: 0.003698; F: 3.242 |

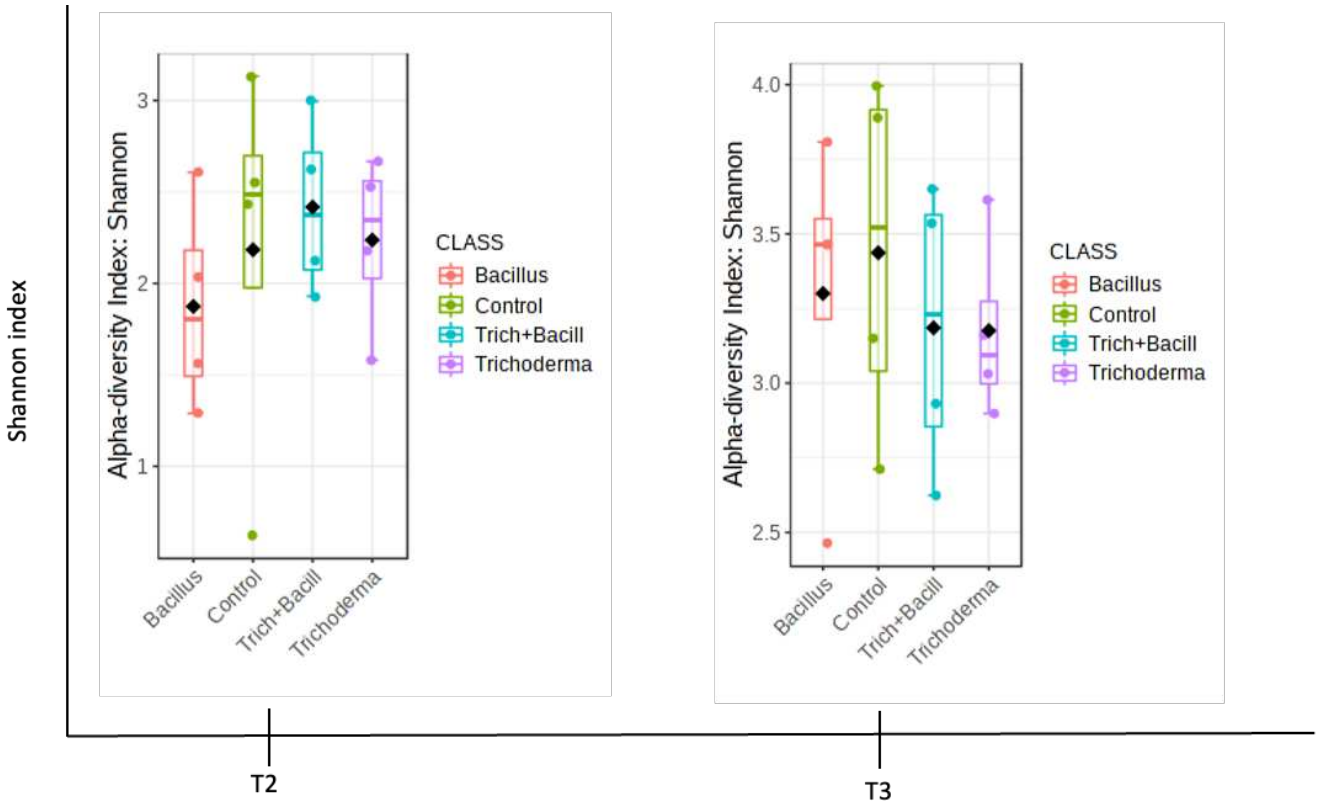


**Figure 6.** Differences in bacterial alpha-diversity of limestone soil samples, between treatment applied (Control, *Bs* PTA-271 (*Bacillus*), *Ta* SC1 (*Trichoderma*), and *Trich + Bacill* (*Bs* PTA-271 + *Ta* SC1)), in sampling times T2 and T3, using Shannon index.

On limestone soil, few differences were observed in bacterial diversity between sampling times according to the treatment. However, at T3, plants inoculated with *Ta* SC1 alone and both BCAs seem to present lower diversity when compared to control plants, and plants treated with *Bs* PTA-271 alone (Figure 6).

## 3.3. Microbial relationships in rhizosphere

### 3.3.1 Fungi

In sandy soil, the control plants reduced all positive relationships over time, as well as plants inoculated with *Bs* PTA-271 alone. In plants inoculated with *Ta* SC1, this reduction was not so prominent. In limestone soil, control plants presented an increase of positive relationships very accentuated at 30 dpi, however at 90 dpi both positive and negative relationships were reduced. Plants treated with *Bs* PTA-271 presented a very significant increase of positive relationships and a significant decrease of negative relationships overtime. Plants treated with *Ta* SC1 presented an increase of positive relationships at 30 dpi which then reduce at 90 dpi. Negative relationships reduced initially, were then stabilized 90 dpi. Plants treated with both BCAs had the most significant increase in positive relationships and reduction in negative relationships that improved 90 dpi (Table 6).

In sandy soil, the plants rhizosphere was mainly colonized by fungi from the genus *Clonostachys* (Out 85) and Ceratobasidiaceae (Otu 536). The genus *Fusarium* (Out 165) and Ascomycota (Otu 638) were also presented in higher quantities. Over time, control plants rhizosphere was mainly colonized by fungi from the family Ceratobasidiaceae and the genus *Fusarium*. At 30 dpi, plants inoculated with *Bs* PTA-271 did not present any dominant genus of fungi, however, at 90 dpi, the rhizosphere of these plants were mainly colonized by *Clonostachys*. In plants inoculated with *Ta* SC1 and both BCAs, *Trichoderma* (Out 490) was the main genus colonizing rhizosphere both at 30 and 90 dpi (Figure 7).

In limestone soil, the plants rhizosphere was mainly colonized by fungi from the family Ceratobasidiaceae (out 536), and the genera *Mortierella* (Out 287), *Lophiostoma* (Otu 253), and *Clonostachys* (Out 85). Control plants did not present any dominant genus at 30 dpi, while at 90 dpi they were mainly colonized by *Coniosporium* (Otu 91). Plants inoculated with *Bs* PTA-271 and both BCAs became colonized mainly with *Trichoderma* at 30 dpi, that is maintained at 90 dpi. Plants inoculated only with *Ta* SC1 did not show any dominant genus at 30 dpi, and at 90 dpi, plants were mainly colonized with *Coniosporium* (Figure 8).

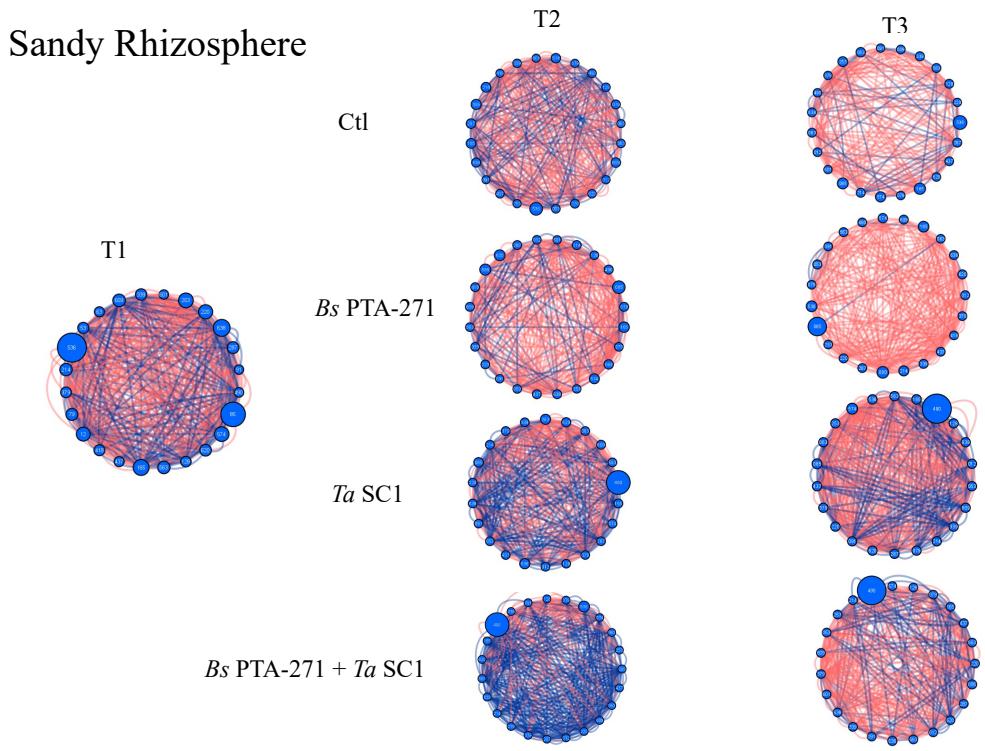


**Figure 7.** Co-occurrence networks from fungal rhizosphere structural communities in sandy rhizosphere samples, inoculated with *Bs* PTA-271, *Ta* SC1, *Bs* PTA-271 + *Ta* SC1, and control plants (CTL), on sampling times 1, 2 and 3 (T1, T2, T3). Numbers correspond to the OTU numbers, and abundance of each module is sorted by size. Red lines represent negative correlations, and blue lines represent positive correlations.

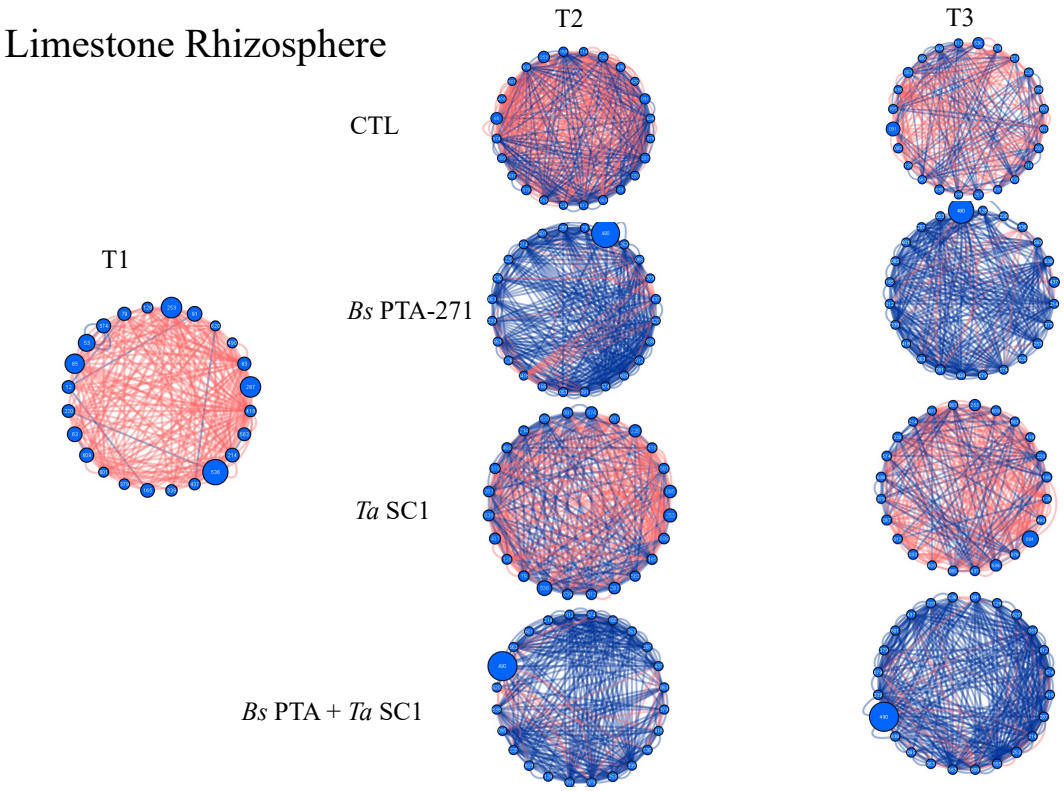


**Figure 8.** Co-occurrence networks from fungal rhizosphere structural communities in limestone rhizosphere samples, inoculated with *Bs* PTA-271, *Ta* SC1, *Bs* PTA-271 + *Ta* SC1, and control plants (CTL), on sampling times 1, 2 and 3 (T1, T2, T3). Numbers correspond to the OTU numbers, and abundance of each module is sorted by size. Red lines represent negative correlations, and blue lines represent positive correlations.

**Table 6.** Number of positive (blue) and negative (red) fungal correlations between sandy and limestone soil samples, between treatments (Control, *Bs* PTA-271, *Ta* SC1, and *Bs* PTA-271 + *Ta* SC1), in sampling times 1, 2 and 3 (T1, T2, T3).

| | Sandy soil | | | | Limestone soil | | | |
|---|---|---|---|---|---|---|---|---|
| | **Ctl** | ***Bs* PTA-271** | ***Ta* SC1** | ***Bs* PTA-271 + *Ta SC1*** | **Ctl** | ***Bs* PTA-271** | ***Ta* SC1** | ***Bs* PTA-271 + *Ta SC1*** |
| **T1** | | | 183/259 | | | | 11/207 | |
| **T2** | 124/205 | 73/245 | 184/164 | 286/104 | 181/268 | 315/40 | 138/178 | 271/27 |
| **T3** | 41/160 | 8/235 | 163/226 | 105/210 | 126/136 | 260/26 | 107/178 | 309/39 |

### 3.3.2. Bacteria

In sandy soil, plants tended to increase positive relationships over time. However, plants inoculated with *Bs* PTA-271 and both BCAs decreased positive relationships and increased negative ones at 90 dpi.

In limestone soil, plants decreased positive relationships at 30 dpi, and increased them again at 90 dpi while negative relationships are increased at 30 dpi and maintained at 90 dpi. Plants inoculated with *Bs* PTA-271 decreased positive relationships and increased negative relationships over time. Plants inoculated with *Ta* SC1 maintained positive relationships at 30 dpi, but highly decreased them at 90 dpi. For plants treated with both BCAs, positive relationships strongly decreased at 30 dpi, but highly increase at 90 dpi (Table 7).

In both, sandy and limestone soils, plants rhizosphere was mainly colonized by bacteria from the genus *Rubrobacter* (Otu 660) that was maintained over time. No treatment caused any changes in the bacterial microbiome on any type of soil (Figures 9 and 10).

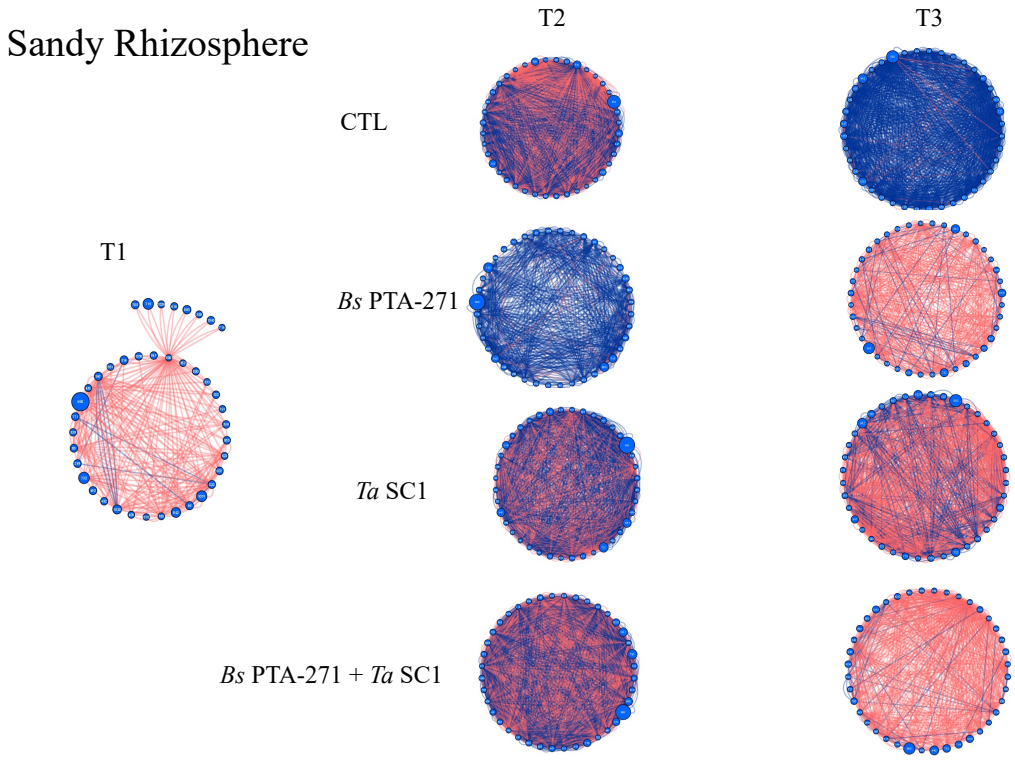


**Figure 9.** Co-occurrence networks from bacterial rhizosphere structural communities in sandy rhizosphere samples, inoculated with *Bs* PTA-271, *Ta* SC1, *Bs* PTA-271 + *Ta* SC1, and control plants (CTL), on sampling times 1, 2 and 3 (T1, T2, T3). Numbers correspond to the OTU numbers, and abundance of each module is sorted by size. Red lines represent negative correlations, and blue lines represent positive correlations.

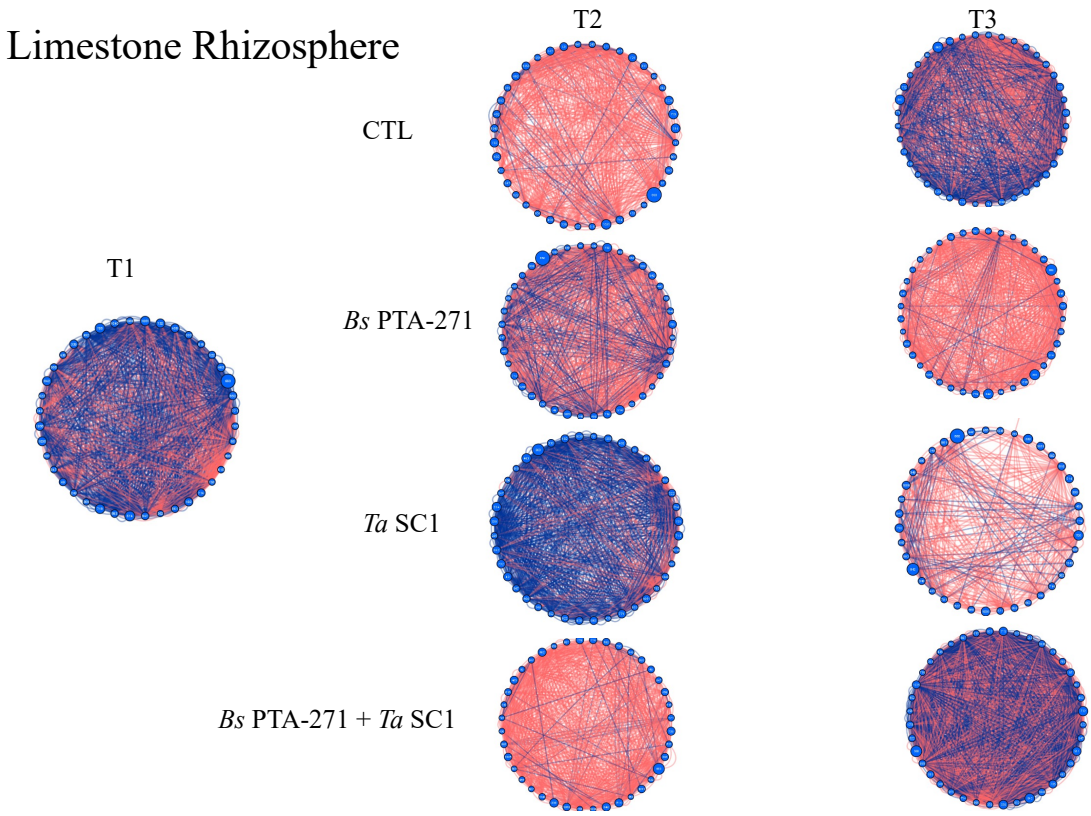


**Figure 10.** Co-occurrence networks from bacterial rhizosphere structural communities in limestone rhizosphere samples, inoculated with *Bs* PTA-271, *Ta* SC1, *Bs* PTA-271 + *Ta* SC1, and control plants (CTL), on sampling times 1, 2 and 3 (T1, T2, T3). Numbers correspond to the OTU numbers, and abundance of each module is sorted by size. Red lines represent negative correlations, and blue lines represent positive correlations.

**Table 7.** Number of positive (blue) and negative (red) bacterial correlations between sandy and limestone soil samples, between treatments (Control, *Bs* PTA-271, *Ta* SC1, and *Bs* PTA-271 + *Ta* SC1), in sampling times 1, 2 and 3.

| | Sandy soil | | | | Limestone soil | | | |
|---|---|---|---|---|---|---|---|---|
| | **Ctl** | ***Bs* PTA-271** | ***Ta* SC1** | ***Bs* PTA-271 + *Ta SC1*** | **Ctl** | ***Bs* PTA-271** | ***Ta* SC1** | ***Bs* PTA-271 + *Ta SC1*** |
| **T1** | | | 9/229 | | | | 615/397 | |
| **T2** | 763/785 | 584/40 | 708/554 | 820/696 | 63/525 | 293/567 | 680/224 | 49/685 |
| **T3** | 1083/41 | 58/424 | 326/704 | 52/518 | 564/552 | 56/672 | 74/284 | 919/773 |

### 3.4. Plant defense gene expression

According to the selected plant defense markers followed, plant leaving on sandy soil appeared much more impacted by BCA inoculation than plants in limestone soil. In sandy soil, plants inoculated with *Ta* SC1 and both BCAs presented a strong repression in almost all tested genes expression at 24hpi (except *PR1*). On the opposite, plants treated with *Ta* SC1 at 4dpi had an overexpression on all tested genes (*PR1* and *PR2* in a lesser extent), while the treatment with both BCAs presented the strongest overexpression for *LOX9*, *GST1*, *PAL* and *STS*. Interestingly, while *Bs* PTA-271 did not affect significantly the expression of the plant defense genes at 24hpi, plants inoculated with *Bs* PTA-271 showed a slight repression of *PR1, PAL* and *STS* expression at 4dpi (Figure 11).

In limestone soil, except for *PR1*, plants inoculated with *Bs* PTA-271 presented an overexpression of all selected genes (*LOX9, PR2, GST1, PAL* and *STS*) at 24hpi, that intensified at 4 dpi (except for *GST1*). Plants treated with *Ta* SC1 showed similar trends than with *Bs* PTA-271 (despite less marked at 24hpi, but also intensified at 4dpi). In contrast, plants inoculated with both BCAs showed a repression of *LOX9*, *PR1*, and *GST1* expression at 24hpi, that persist only for *GST1* at 4 dpi (Figure 12). Indeed, the sole slight overexpression observed with the treatment combining BCA are for *STS* at 24hpi, then for *LOX9*, *PR2* and *PAL* at 4dpi.

| | Sandy Soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24h after inoculation | | | | 4 days after inoculation | | | |
| | Ctl | *Bs* PTA-271 | *Ta* SC1 | *Bs* PTA-271 + Ta SC1 | Ctl | *Bs* PTA-271 | *Ta* SC1 | *Bs* PTA-271 + Ta SC1 |
| *LOX9* | 1.00 | 1.29 | 0.13 | 0.16 | 1.00 | 1.03 | 53.80 | 11.71 |
| *PR1* | 1.00 | 1.71 | 0.35 | 0.94 | 1.00 | 0.91 | 11.25 | 4.35 |
| *PR2* | 1.00 | 2.27 | 0.02 | 0.01 | 1.00 | 1.45 | 17.76 | 3.32 |
| *GST1* | 1.00 | 1.33 | 0.09 | 0.07 | 1.00 | 1.47 | 48.07 | 11.86 |
| *PAL* | 1.00 | 1.52 | 0.00 | 0.00 | 1.00 | 0.84 | 264.26 | 92.17 |
| *STS* | 1.00 | 1.24 | 0.00 | 0.00 | 1.00 | 0.39 | 108.82 | 57.08 |




**Figure 11.** Induced differential expression of defense-related genes in leaves of sandy soil plants. Samples were collected 24h and 4 days after inoculation with four treatments (Control, *Bs* PTA-271, *Ta* SC1, and *Bs* PTA-271 + *Ta* SC1). Heatmaps represent changes in the transcript

expression levels as indicated by the color shading. Data are the means from three representative replicates. Different letters indicate statistically significant differences between the treatments (ANOVA, Fisher's LSD post hoc test, α = 0.1). Legends for genes are *LOX9*, lipoxygenase 9; *PR1*, pathogenesis-related protein 1; *PR2*, class I β-1,3-glucanase; *GST1*, glutathione-S-transferase 1; *PAL*, phenylalanine ammonia lyase; *STS*, stilbene synthase.

| | Limestone soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24h after inoculation | | | | 4 days after inoculation | | | |
| | Ctl | *Bs* PTA-271 | *Ta* SC1 | *Bs* PTA-271 + Ta SC1 | Ctl | *Bs* PTA-271 | *Ta* SC1 | *Bs* PTA-271 + Ta SC1 |
| *LOX9* | 1.00 | 3.03 | 1.55 | 0.22 | 1.00 | 13.35 | 15.39 | 1.88 |
| *PR1* | 1.00 | 1.13 | 1.20 | 0.50 | 1.00 | 1.22 | 1.48 | 0.93 |
| *PR2* | 1.00 | 2.48 | 2.26 | 1.11 | 1.00 | 13.26 | 43.90 | 2.51 |
| *GST1* | 1.00 | 2.69 | 2.10 | 0.62 | 1.00 | 1.21 | 1.36 | 0.12 |
| *PAL* | 1.00 | 4.80 | 2.32 | 1.52 | 1.00 | 47.97 | 67.64 | 2.64 |
| *STS* | 1.00 | 5.98 | 3.01 | 3.27 | 1.00 | 38.77 | 35.44 | 1.53 |




**Figure 12.** Induced differential expression of defense-related genes in leaves of limestone soil plants. Samples were collected 24h and 4 days after inoculation with four treatments (Control, *Bs* PTA-271, *Ta* SC1, and *Bs* PTA-271 + *Ta* SC1). Heatmaps represent changes in the transcript expression levels as indicated by the color shading. Data are the means from three representative replicates. Different letters indicate statistically significant differences between the treatments (ANOVA, Fisher's LSD post hoc test, α = 0.1). Legends for genes are *LOX9*, lipoxygenase 9; *PR1*, pathogenesis-related protein 1; *PR2*, class I β-1,3-glucanase; *GST1*, glutathione-S-transferase 1; *PAL*, phenylalanine ammonia lyase; *STS*, stilbene synthase.

## 4. Discussion

In this study, we examined the temporal dynamics of the fungal and bacterial microbiome in 110R rootstocks naturally infected with black-foot pathogens and inoculated with *Bacillus subtilis* PTA 271 (*Bs* PTA-271) and *Trichoderma atroviride* SC1 (*Ta* SC1), in two different types of soil (sandy and limestone soils). Samples were collected from rhizosphere in 3 different times (before inoculations, 30dpi, and 90dpi), using a non-destructive method.

Generally, research on grapevine microbiome is predominantly focused on the scion cultivar, the visible half-part of the vine that produces grapes. However, more than 80% of the vineyards worldwide are currently grafted onto rootstocks (Ollat et al. 2016), which have a significantly effect on grape yield and quality and contribute to confer protection against pathogens (Marín et al. 2021).

### 4.1. Microorganisms abundance

When analyzing the fungal microbiota, the rhizosphere is initially colonized mainly by Ascomycota (Cloronostachys and Lophiostoma) and Basidiomycota (Ceratobasideaceae). These fungi are commonly found in soil and dead wood tissues in all geographic locations (Jacquemyn et al. 2017; Sun et al. 2020; Hashimoto et al. 2018). Genera of pathogenic fungi related with GTDs were also detected in the studied plants, such as *Ilyonectria* (black-foot disease pathogen) (Probst et al. 2019), and *Diaporthe* (Phomopsis dieback pathogen) (Dissanayake et al. 2015). In our experiments, control plants did not have a significant abundance of *Trichoderma* spp., showing no contamination with *Ta* SC1. In contrast, when plants are inoculated with *Bs* PTA-271 they presented a considerable abundance of *Trichoderma* spp., and similarly with plants inoculated with both BCAs that contain more *Trichoderma* than plants inoculated with only *Ta* SC1. This indicate that *Bacillus* species can recruit other microorganisms towards the plant rhizosphere to modulate the microbiome activity. Sabaté and Brandán (2022) already demonstrated that soil inoculation with *Bacillus amyloliquefaciens* B14 improves the *Trichoderma* spp. soil abundance. Interestingly, we observed that *Trichoderma* spp. abundance decreases from 30dpi to 90dpi, showing that this BCA had problems to maintain after inoculation. This could happen due to the complex nature of the rhizosphere and root microbiome (Berendsen et al. 2012), or due to abiotic factors that can change the interaction between micoorganisms (Niu et al. 2020). Martinez-Diz et al. (2018) also observed a decrease in *Trichoderma* recovery in grapevine rhizosphere over time. The bacterial microbiota described in the sampled plants was much more complex and abundant than the fungal microbiota. The plant rhizosphere was mainly colonized by *Rubrobacter*, *Streptomyces*, *Rhizobium* and Vicinamibacterales, as well as *Bacillus* spp. that, although present did not present a significant abundance. These bacterial genera are commonly found in grapevine soil and rhizosphere (Darriaut et al. 2022; Mocali et al. 2020; Dries et al. 2021). Looking at *Bacillus* spp. evolution overtime, no significant differences were observed between the rhizosphere of BCA treated plants, probably because *Bacillus* spp. is not the main bacterial genera detected in these conditions (BCA treated plants). To contrast

with *Trichoderma* spp., control plants already presented a significant abundance of *Bacillus* spp., that could lead to an impossibility of detecting differences in *Bacillus* spp. evolution overtime.

### 4.2. Alpha diversity

Our results indicated that the fungal alpha diversity fluctuated very differently over time according to the soil samples. In sandy soil, treatments with *Ta* SC1 showed a lower diversity compared with the control plants and plants treated with *Bs* PTA-271 at 30dpi. The *Ta* SC1 successful inoculation leads to a generalized colonization of this microorganism in rhizosphere and soil, giving less space and nutrients for other fungi, therefore reducing the fungal diversity. Li et al. (2022) reported a decrease in fungal diversity in soil of plants inoculated with *Trichoderma atroviride* strain HB 20111. On limestone soil, plants inoculated with *Bs* PTA-271 presented a much lower fungal diversity than control plants and plants treated with *Ta* SC1. Garbeva et al. (2004) reported that the two main factors affecting the soil microbial community structure are plant type and soil type, due to the complex interactions between microorganisms, soil, and plants. Therefore, physicochemical characteristics of the soil used in this experiment could lead to secondary metabolites production by *Bs* PTA-271 that may lead to a decrease in fungal diversity. Moreover, limestone soils are denser and heavier than sandy soils, creating complications for microorganisms to colonize plant roots (Marshal 1975). However, without further analysis on the soil composition, it is not possible to confirm this theory. The bacterial alpha diversity also varied depending on the type of soil. In sandy soil, the BCAs inoculation improved bacterial diversity in grapevine rhizosphere, and at 90dpi, the treatment with both BCAs presented higher bacterial diversity than plants inoculated with only one BCA. Previous studies pointed the BCA inoculation as a factor that can increase microbiome diversity (Gadhave et al. 2018; Li et al. 2011). In limestone soil, as in the fungal diversity, plants inoculated with *Ta* SC1 show less bacterial diversity. Environmental heterogeneity, such as the soil physicochemical properties and moisture content have been identified as major factors shaping the spatial scaling of the rhizosphere microbiome in many previous studies (Costa et al., 2006; Tan et al., 2013; Schreiter et al., 2014), including grapevine (Fernández-Calviño et al., 2010; Corneo et al., 2014; Burns et al., 2015; Zarraonaindia et al., 2015; Holland et al., 2016).

### 4.3. Microbial relationships in rhizosphere

Network analysis of taxon co-occurrence patterns provides new insight into the structure of complex microbial communities, patterns that are otherwise more difficult to reveal using standard alpha/beta diversity measures widely used in microbial ecology (Barberán et al. 2012), and also used in this study. It has been suggested that complex soil microbial community networks (networks with high number of nodes, number of links, and average connectivity), rather than the simple ones, benefit plants (Jiemeng et al. 2018). Indeed, complex networks contribute to better cope with environmental changes or to suppress soilborne pathogens. For instance, tobacco plants associated with rhizosphere microbial communities exhibiting complex networks showed lower incidence of bacterial wilt disease compared to plants associated with communities displaying less connections in their networks (Yang et al. 2017). Our results show that the correlation networks of bacterial taxa are more complex than the fungal networks. The study of the fungal taxa correlations in rhizosphere revealed that, in sandy soil, positive relationships is reduced over time, although in plants inoculated with *Ta* SC1 and both BCAs the reduction on positive correlations is subtler. Sui et al. (2022) also observed a reduction in correlations in wheat rhizosphere of plants inoculated with *T. atroviride,* probably due to the direct confrontation between *Trichoderma* and other fungi, possibly pathogenic. In limestone soil, plants treated with *Bs* PTA-271 have a very significant increase of positive relationships while negative relationships significantly decrease. As mentioned before, *Bacillus* species can recruit specific microorganisms into the microbiome, improving its quality (positive correlations).

In our study, bacterial microbiome tended to stabilize overtime, increasing positive relationships and reducing negative relationships. However, unexpectedly, plants inoculated with *Bs* PTA-271 and both BCAs decreased positive relationships and increased negative ones at 90 dpi. This could be explained by the high amount of *Bs* PTA-271 present in the rhizosphere, leading to an increase of negative correlations with other bacteria. Again, plants treated with both BCAs increased positive correlations at 90dpi, showing the potential of inoculating a combination of these BCAs, already pointed by Leal et al. (2021).

### 4.4. Plant defenses study

Concurring our previous works, a study on grapevine systemic immunity was performed, aiming six selected defense genes in leaves: the lipoxygenase *LOX9* involved in oxylipin

synthesis and described as dependent to JA/ET; *PR1* described to be regulated by SA; the β-1,3-glucanase *PR2* described to be regulated by various phytohormones such as SA, JA, and ET; the glutathione-S-transferase *GST1* putatively involved in the detoxification process; the phenylalanine ammonia-lyase *PAL* catalyzing the first step in the phenylpropanoid pathway; and the stilbene synthase *STS* involved in the synthesis of phytoalexins (Leal et al. 2021; Trotel-Aziz et al. 2019). Interestingly, induced defenses were different in sandy soil plants and limestone soil plants. In sandy soil plants at 24 hpi with *Ta* SC1 and combined BCAs, we observed a strong suppression of both SA and JA/ET–dependent defense genes. These findings suggest that *Ta* SC1 must cope with the plant defense responses during its initial stages of the interaction, and thus by suppressing elements regulating the broad-spectrum of plant innate immunity, *Ta* SC1 ensure its own colonization of grapevine roots (Jacobs et al. 2011). After 4 dpi, plants inoculated with *Ta* SC1 and *Ta* SC1+ *Bs* PTA-271 showed stimulated defenses, with a significant overexpression of *PAL* and *STS* (through many possible hormonal inducing paths, such as SA, but also JA/ET, etc.), well known to be involved in the production of antimicrobial compounds such as phytoalexins. This would suggest that the plant finally succeed to detect *Ta* SC1 as a foreigner, and it could constitute an interesting pre-priming state for the plant. Plants inoculated with *Bs* PTA-271 do not induce any changes in plant defenses during the first 24hpi, while at 4dpi a slight repression of *PAL* and *STS* is observed. This could indicate that these experimental conditions did not favor *Bs* PTA-271 in its ability to stimulate its host defenses, compared to *Ta* SC1, unless *Bacillus spp.* are already detected as non-threatening beneficial microorganisms by the plants at the selected experimental time. In limestone soil plants, results were slightly different from the sandy soil plants. In this study, our results showed that *Bs* PTA-271 or *Ta* SC1 treated-plants at 24hpi, show a slight increase in the expression of all selected genes (*LOX9, PR2, GST1, PAL* and *STS*), that intensified at 4 dpi (except for *GST1*). *Ta* SC1 effect being less marked at 24hpi, but also intensified at 4dpi. In contrast, plants inoculated with both BCAs showed also a slight overexpression for *STS* at 24hpi, then for *LOX9*, *PR2* and *PAL* at 4dpi. Since limestone soils are high in nutrients (Marshal 1975), one can thus assume, that colonization of plant roots by BCAs in limestone soil is less stressful for the plant in sandy soils (Vose, 1982). Thus, contrary to sandy soil plants, all treatments stimulate the plant defenses, even weakly and lately, but as expected from a biocontrol agent that is not pathogenic to the plant (thus only leading to defense corresponding to a pre-priming state, not to a disease state). The late changes when using combined BCA doesn't exclude a direct confrontation between BCAs, until they find their balance.

## 5. Conclusion

Altogether, our results provide evidence that the success of *T. atroviride* SC1 and *B. subtilis* PTA-271 inoculations in grapevine rhizosphere microbiome is soil-dependent. Sandy soil seems to favor *Ta* SC1 implantation in the rhizospheric microbiome, consistent with its fungal nature (better adapted to acidic pH), while *Bs* PTA-271 thrives in a limestone soil. These results were corroborated with the study of plant defense gene expression.

The inoculation of *Ta* SC1 and *Bs* PTA-271 demonstrated a positive effect in rhizosphere correlation networks, improving positive correlations and reducing negative ones. In addition,, we report the beneficial effect on grapevine rhizosphere microbiome of combining the two BCAs in the same application. This is a promising result to reduce inconsistencies observed with the use of only one BCA.

Droplet digital PCR (ddPCR) analysis are being carried out to specifically quantify *Ta* SC1 and *Bs* PTA-271 to better understand the success of the inoculations. Finally, further studies are needed to associate soil physicochemical properties with BCA beneficial effect to determine the best BCA depending on several factors (cultivar, physicochemical parameters of the soil, wine region, meteorological conditions, etc.).

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# *Concluding remarks and Perspectives*

Finding sustainable management approaches for GTDs is of utmost importance, as the present treatments are either not effective to eliminate these diseases completely or rely on the use of arch chemicals that are harmful for the environment. It is also imperative to better understand the relationships between GTDs pathogens, grapevines, and BCAs that can be

added as treatments, to better treat GTDs. In this context, the overall findings of this thesis documented the importance to deeply study biological microorganisms, their modes of action, and their interactions with plants and pathogens, as they can be the answer to control GTDs, but due to their nature, they can also be unpredictable and inconsistent on their biocontrol potential.

Firstly, this work unrevealed the biocontrol potential of a new *Bacillus subtilis* strain (*Bs* PTA-271) to be used against GTDs. With a full genomic analysis, this study highlighted the genes encoding for biocontrol related processes. The most relevant predicted genes encode for: (1) a functional swarming motility system highlighting advantageous colonizing capacity of host and a strong interacting capacity within plant microbiota; (2) a strong survival capacity, due to sporulation but also to complex detoxifying systems, auto-inducing metabolic paths and recruiting capacities for adding microbiota values; and (3) the delivery of many bioactive substances (hormones, elicitors, effectors and quenchers, siderophores and lytic enzymes, etc.), facilitating the stimulation of plant growth or defenses, or else, disturbing pathogen fitness or aggressiveness. Interestingly, the putative capacity of *B. subtilis* PTA-271 to produce a wide range of phytohormone analogous (SA, ET precursor, ABA etc.), as well as diverse direct effectors and lytic enzymes against plant pathogens, highlight a significant potential for biocontrol strategies.

This study also provide evidence of the biocontrol potential of *Bs* PTA-271 and *Ta* SC1 against *N.parvum* BT67, *in vitro*, and in two grapevine cultivars (*in planta*). Our results provide evidence that grapevine susceptibility to *Np*-BT67 is cultivar-dependent, as well as the BCA beneficial effects. *Bs* PTA-271 was confirmed as an effective protector for Chardonnay against *Np*-Bt67, and *Ta* SC1 was shown for the first time as a good protector for Tempranillo. This study also reports for the first time the biocontrol potential of the combination of *Bs* PTA-271 and *Ta* SC1 against *Np*-Bt67 in Tempranillo. This is a promising result for an improved efficiency of sustainable biological control in a proven context of lack of effective chemicals to manage GTDs.

Endowed with aggressive molecules, *Bs* PTA-271 and *Ta* SC1 can antagonize each other, but *Bs* PTA-271 inhibits *Np*-Bt67 development with a greater efficiency in a three-way confrontation. This beneficial BCA collaboration against *Np*-Bt67 still operates in Tempranillo and confirms the interest of using both BCAs in combination to optimize the direct fight against *Np*-Bt67. These results are of great interest for effective curative treatments to obtain healthy mother plants in the nursery and to control BOT in vineyard. However, the direct beneficial effect of combined BCAs did not operate to protect Chardonnay, suggesting that

Chardonnay itself probably alters the fine-tuned orchestrated cooperation of BCAs that drives such direct beneficial effect. Plant systemic immunity is also affected by each BCA. Our findings suggest a common feature for the two cultivars: the defenses that are greatly diminished in BCA-protected plants appear to be those that are responsive to SA, in contrast to symptomatic plants. For Tempranillo, the high basal expression of SA-dependent defenses may thus explain the highest susceptibility to BOT and the ineffectiveness of *Bs* PTA-271 in our experimental conditions. In transcriptomic analysis from these experiments, our results provide a new insight of molecular changes associated with the infection by Np-Bt67 in Chardonnay and Tempranillo. In Chardonnay, plants infected with *Np*-Bt67 present overexpressed genes that are implicated on ABA signaling pathways. In Tempranillo, the infection with *Np*-Bt67 leads to expression changes in more than 200 genes, related mostly with amino acid import, chloroplast and photosystem related processes, plant responses to biotic stimulus, and biosynthesis of secondary metabolites. Our study also provides deep insights of the protection induced by *Bs* PTA-271 in Chardonnay, and *Ta* SC1 in Tempranillo. Protection from *Bs* PTA-271 in Chardonnay involves genes related to ABA biosynthesis, phenylpropanoid pathways, secondary metabolites and cell wall structure and organization. Protection of *Ta* SC1 in Tempranilllo plants involves a larger number of changes, that cover both primary and secondary metabolism, related to changes in hormonal signals, such as with ABA.

One of the most important approaches to GTDs integrated management is the improvement of the phytosanitary quality of the nursery produced grapevines, as this can prevent future pathogen dissipations. This study, especially the work conducted in chapter V, reveals how promising these BCAs can be to reduce pathogen infections during the grapevine propagation process, but how inconsistent these treatments can sometimes be. These results showed a promising biocontrol potential of treatments with *Bs* PTA-271 and *Ta* SC1 against Botryosphaeria dieback and black foot pathogen infections during the grapevine propagation process of cv. Tempranillo. Alone, *Ta* SC1 confirmed its effectiveness to reduce the percentage of infected plants with BOT associated pathogens and the percentage of isolation from wood fragments, and *Bs* PTA-271 demonstrated, for the first time, effectiveness to reduce infections caused by BF associated pathogens during the propagation process. In one of the experiments, the simultaneous application of both BCAs presented a significant decrease in the number of infected plants and the number of infected wood fragments for both groups of pathogens. In this study, qRT-PCR techniques were used for the first time in grapevine nursery experiments,

enabling accurate quantification of the inoculated BCAs, confirming the successful colonization by BCAs in treated plants, and the lack of crossed contaminations between treatments. Complement to the work on chapter V, the work conducted to test the effect of *Bs* PTA-271 and *Ta* SC1 in the grapevine rhizosphere microbiome, also highlights the potential of this microorganisms to be integrated in sustainable disease management in grapevines. Our results provide evidence that the success of *Ta* SC1 and *Bs* PTA-271 inoculations in grapevine rhizosphere microbiome is soil-dependent. Sandy soil seems to favor *Ta* SC1 implantation in the rhizospheric microbiome, consistent with its fungal nature (better adapted to acidic pH), while *Bs* PTA-271 thrives in a limestone soil. These results were corroborated with the study of plant defense gene expression. The inoculation of *Ta* SC1 and *Bs* PTA-271 demonstrated a positive effect in rhizosphere correlation networks, improving positive correlations and reducing negative ones. In addition, we report the beneficial effect on grapevine rhizosphere microbiome of combining the two BCAs in the same application. This is a promising result to reduce inconsistencies observed with the use of only one BCA.

Although the outcomes of this thesis provide new knowledge on the grapevine interactions with pathogens and BCAs, and the potential of using more than one BCA to treat grapevine GTDs, there are still unanswered questions that need further clarification. The first question, related to the contribution of an BCAs in a non-sterile environment, raises the question of the origin of the protection: is it BCA-dependent or is it the result of a change induced by the BCA on the microbiome of the vine? This is a crucial point to investigate further, whether it involves intermediate physiological changes in the host plant.

The living conditions of plants and microorganisms such as BCAs also deserve more attention, especially the quality of their support, which is the soil. Depending on the geographical location, the nature of the soil fluctuates depending on the nature of the bedrock on which it rests and the climatic conditions of the station where it is located. The key elements to consider having a quality soil conducive to the good physiological state of plants and microorganisms that it carries are among others: its porosity (structure conditioned by its texture or granulometry), its pH, and its organic matter content. The use of a sandy substrate and a limestone substrate are the two extremes on the texture scale. The sandy soil offers a microporosity not very favorable to a good biological activity, but on the contrary, it is favorable to the development of an acid pH and to a bad degradation of the organic matter (if however, this one was retained). The limestone soil is rich in nutrients but can be denser and

heavier than sandy soils, which complicates the colonization of plant roots by micro-organisms.

The way the vineyard is cultivated must also be considered, in particular its irrigation, which can influence the development or not of a particular pathogen likely to infect the plant, or modulate the pressure of pathogens, etc. Further research on these aspects is still needed. This would allow us to cover as many changing conditions as possible in order to better channel/erase the variability that may be observed in the protection induced by our BCAs when transferring the results obtained to field conditions. The combined use of a larger number of BCAs is also desired, in an attempt to ensure effective protection despite fluctuations in environmental factors.

Finally, the differential effects of Bs PTA-271 and Ta SC1 against Np-Bt67 in two different grapevine varieties also revealed grapevine effects on their sensitivities to pathogens, but also to BCAs, and potentially to other environmental factors not yet understood. It is therefore necessary to further open our molecular/genetic investigations to grapevines, for example by comparing the genomes and defense responses of grapevines from very different climatic regions. The idea is to discover potential genes for tolerance or resistance to GTDs in grapevines since: (i) the diversity of genotypes within grapevine varieties, grapevines or even clones, and (ii) the distribution and pressure of pathogens responsible for GTDs, are both conditioned by the geographical area.

# *Conclusions generales et Perspectives*

Les MDB font partie des maladies de la vigne les plus dommageables au plan économique, en France, mais aussi en Europe et à travers le Monde entier. Actuellement dépourvues de moyens de lutte efficaces pour les éradiquer ou freiner leur développement, la gestion des

MDB repose sur des méthodes de lutte prophylactiques à efficacité limitée et dans certains pays à une utilisation de produits chimiques nocifs pour l'environnement. Il est donc urgent de trouver et de développer des alternatives de gestion durable pour éradiquer ou enrayer l'expansion de ces MDB. Afin de mieux lutter contre elles, il est également impératif de mieux comprendre les relations entre les agents pathogènes responsables des MDB, les cultivars de vigne et les BCA susceptibles d'être ajoutés comme traitements alternatifs. Les résultats globaux de cette thèse soulignent bien ici toute l'importance d'étudier en profondeur les impacts biologiques et biochimiques des microorganismes bénéfiques (BCA), leurs modes d'action conduisant à la protection de la plante, qui peuvent aussi dépendre des interactions multipartites entre la plante, le(s) pathogène(s) et tous les microorganismes associés au microbiome/microbiote de la plante. En effet, l'ensemble des interactions multipartites entre tous ces acteurs peut moduler tant les réponses de la plante, que l'activité des BCA ou celle des pathogènes responsables des MDB. Par ailleurs, compte-tenu des contraintes environnementales imprévisibles et du contexte climatique incertain irréversible, valoriser la biodiversité par combinaison de BCA devrait rajouter au développement de stratégies de biocontrôle plus durables.

Tout d'abord, ce travail de thèse a confirmé le potentiel d'une nouvelle souche de *Bacillus subtilis* (*Bs* PTA-271) en tant qu'agent de lutte biologique (ou biocontrôle) valorisable désormais pour lutter contre les MDB. Grâce à l'analyse génomique complète de ce BCA (Leal et al. 2021a), cette étude a aussi mis en évidence des gènes codant pour de nombreuses molécules bénéfiques, dont l'efficacité reste à tester de façon plus directe pour en valoriser tout le potentiel dans des stratégies de biocontrôle. Cette étude génomique aide par ailleurs à mieux comprendre les interactions potentielles qui sous-tendent les effets bénéfiques de *Bs* PTA-271 et donc leur capacité à protéger la vigne contre les MDB. Les gènes identifiés comme pertinents codent pour : (1) une bonne capacité de colonisation de l'hôte et d'interaction avec le microbiome/microbiote de la plante ; (2) une bonne capacité de survie, en conditions contraignantes abiotiques et biotiques ; et (3) la synthèse de nombreuses molécules bioactives facilitant la stimulation de la croissance de la plante et/ou ses défenses, ou au contraire perturbant la croissance et/ou l'agressivité des pathogènes.

Cette thèse fournit également des preuves substantielles du potentiel de *Bs* PTA-271 et *Ta* SC1 à lutter efficacement contre *N. parvum* Bt67 *in vitro* et *in planta* chez deux cultivars de vigne infectés avec ce pathogène agressif. Nos résultats ont notamment mis en évidence une

sensibilité de la vigne à Np-Bt67 qui est dépendante du cépage, ainsi qu'une efficacité des BCA cépage-dépendante (Léal et al. 2021b). *Bacillus subtilis* PTA-271 a été confirmé comme un protecteur efficace sur Chardonnay contre Np-Bt67, et *Trichoderma atroviride* SC1 a été confirmé comme un bon protecteur sur Tempranillo contre Np-Bt67. Cette étude rapporte également pour la première fois l'efficacité accrue d'une combinaison d'agents de biocontrôle (*Bs* PTA-271 et *Ta* SC1) contre Np-Bt67 sur Tempranillo (gain de protection de 10% par rapport à l'effet protecteur de *Ta* SC1 seul, Léal et al. 2021b). Il s'agit d'un résultat prometteur pour garantir un meilleur transfert des résultats obtenus en conditions contrôlées vers le terrain, et garantir ainsi une meilleure efficacité de la lutte biologique durable dans un contexte environnemental complexe où les moyens de lutte pour gérer durablement les pathogènes responsables des MDB manquent cruellement.

Dotés de molécules agressives, *Bs* PTA-271 et *Ta* SC1 peuvent exercer un effet antagoniste mutuel/réciproque sur la croissance et/ou le développement de l'un sur l'autre (en confrontation directe bi-partite). Toutefois, cette thèse montre que cet effet négatif disparaît au profit d'une meilleure efficacité contre l'agent pathogène Np-Bt67 lorsque ces 2 BCA sont en confrontation tri-partite avec Np-Bt67. Cette collaboration bénéfique des BCA contre Np-Bt67 semble toujours opérer sur Tempranillo puisque la combinaison des 2 BCA offre un gain supplémentaire de protection de 10% par rapport à *Ta* SC1 seul. Ces résultats soulignent l'intérêt d'utiliser les deux BCA en combinaison pour optimiser la lutte directe contre Np-Bt67 d'une part, mais aussi pour anticiper sur les fluctuations environnementales aux champs qui pourraient transitoirement être défavorables à l'un ou l'autre des BCA. Ces résultats sont donc encourageants pour envisager des traitements curatifs efficaces et développer des plants de pépinière le plus sain possible, et si possible dotés d'une biodiversité optimisée capable de mieux contrôler les MDB au vignoble (BOT et BF notamment). La contribution du cépage à l'efficacité d'un BCA ou à leurs interactions bénéfiques reste cependant à prendre en compte. En effet, les bienfaits des BCAs combinés n'opèrent pas pour protéger Chardonnay contre Np-Bt67, suggérant que le Chardonnay lui-même altère probablement la capacité de coopération de ces 2 BCA.

Outre leur capacité antagoniste directe sur le pathogène, les BCA peuvent aussi stimuler les défenses des plantes, ou les reprogrammer au bénéfice de la protection de leur hôte contre un agent phytopathogène (Leal et al. 2021b). Ainsi, l'immunité systémique de la vigne est ici modulée par chaque BCA, et nos résultats ont montré une caractéristique commune aux deux

cultivars : leurs défenses dépendantes du SA sont fortement diminuées dans les plantes protégées par les BCA (contrairement aux plantes symptomatiques dont les défenses SA-dépendantes sont surexprimées). Nous suggérons que la forte expression de défenses basales SA-dépendantes chez Tempranillo pourrait expliquer sa plus grande sensibilité au BOT, étant donné que l'agressivité des BOT s'exprime lorsque le pathogène entre en phase nécrotrophe et que les défenses SA-dépendantes de l'hôte ne sont pas utiles pour lutter contre un pathogène entré en phase nécrotrophe (Yang et al. 2015). Au contraire la présence de SA en grande quantité pourrait même freiner/retarder l'expression de défense utiles chez un hôte, tel que Tempranillo, ce qui pourrait alors peut-être expliquer l'inefficacité de *Bs* PTA-271 dans ces conditions expérimentales et avec ce cépage précis.

L'analyse transcriptomique réalisées sur ces mêmes expériences a apporté un nouvel éclairage à nos résultats (Chapitre IV, Leal et al. à soumettre à Frontiers in Microbiology). En effet les changements moléculaires associés à l'infection par Np-Bt67 chez Chardonnay montrent une surexpression des gènes impliqués dans la biosynthèse et la voies de signalisation de l'ABA. En bon accord, la protection conférée par *Bs* PTA-271 au cépage Chardonnay contre Np-Bt67 souligne une répression des gènes liés à la biosynthèse de l'ABA, mais aussi une répression de la voie des phénylpropanoïdes, notamment des dérivés flavonoïdes, ainsi qu'une régulation à la baisse des gènes codant pour les modifications post-traductionnelles des métabolites secondaires quelle que soit leur origine. Des changements dans la biogenèse et l'organisation de la structure de la paroi cellulaire de la plante semblent également contribuer à l'effet bénéfique de *Bs* PTA-271 contre Np-Bt67, en relation avec le métabolisme des glucides qui mérite désormais qu'on y prête plus attention. Chez Tempranillo, l'infection par Np-Bt67 entraîne des changements beaucoup plus profonds, liés principalement au transport d'acides aminés, aux activités chloroplastiques et des photosystèmes, aux réponses des plantes aux contraintes biotiques, ainsi qu'à la synthèse de métabolites secondaires. Ces résultats tendent donc à montrer que l'infection de la vigne par un pathogène responsable des MDB induit une profonde reprogrammation de l'expression des gènes de défense dans la plante et jusque dans ses feuilles, probablement pour mieux servir l'expansion et/ou l'agressivité d'un pathogène en pleine demande d'extension. Quant à la protection conférée par *Ta* SC1 aux boutures de Tempranillo, elle-aussi nécessite un plus grand nombre de changements, possiblement en raison de la localisation de *Ta* SC1 qui occupe la même " niche écologique " que l'agent pathogène Np-Bt67 auquel il est confronté. Cet espace commun à partager oblige probablement les deux protagonistes microbiens (*Ta* SC1 et Np-Bt67) à entrer en compétition

pour l'espace et les nutriments afin d'assurer leur propre survie. En bref, l'effet bénéfique de *Ta* SC1 sur les boutures de Tempranillo contre Np-Bt67 est corrélé à de nombreux changements liés aux transporteurs, à l'intégrité et à l'extension de la paroi cellulaire, à la division cellulaire versus la mort cellulaire induite par le pathogène, à l'expression de protéines actives multidirectionnelles et aux interactions intra-microbiome. Par opposition directe aux effets de Np-Bt67 sur Tempranillo, *Ta* SC1 met entre autres sous silence divers transporteurs décrits pour faciliter la colonisation par les micro-organismes, mais aussi pour moduler les flux de nombreux nutriments (comme par exemple les acides aminés, les hormones et de nombreux autres solutés tels que des glucides plus ou moins complexes) qui seraient susceptibles de favoriser l'expansion du pathogène et/ou son agressivité au détriment de la bonne santé de l'hôte.

A défaut de disposer d'un large panel de solutions de biocontrôle d'ores-et-déjà commercialisées, la gestion intégrée des MDB peut déjà contribuer à améliorer la qualité sanitaire des plants de vigne produits en pépinière, et prévenir ainsi la dissipation des pathogènes. Le chapitre IV de cette thèse est donc dévolu aux effets des BCA contre les pathogènes responsables des MDB et de leur propagation lors de la production de jeunes plants en pépinière (Leal et al., 2022 soumis à PMS). Nos résultats ont donc confirmé le potentiel biocontrôle prometteur des BCA *Bs* PTA-271 et *Ta* SC1 contre les pathogènes responsables du dépérissement à Botryosphaeria (BOT) et de la maladie du pied noir (BF), y compris en pépinière pour le cv. Tempranillo. En pépinière, *Ta* SC1 a donc confirmé son efficacité contre les pathogènes associés au BOT, et *Bs* PTA-271 a été montré pour la première fois efficace pour réduire les symptômes causés par les pathogènes responsables du BF. Une expérience (parmi les 2 réalisées) a par ailleurs montré que l'application simultanée des deux BCA a diminué significativement l'incidence et la sévérité des symptômes causés par ces deux groupes de pathogènes (BOT et BF). Cette même étude valorise également les techniques de qRT-PCR, appliquées pour la première fois à des expériences réalisées en pépinière de vigne, pour quantifier précisément les BCA inoculés, et confirmer ainsi leur capacité à coloniser avec succès les plantes traitées, les localiser, et confirmer par la même occasion l'absence de contaminations croisées entre les traitements (Leal et al. 2022 soumis à PMS). En complément du travail sur le chapitre V, le travail effectué pour tester l'effet de Bs PTA-271 et Ta SC1 dans le microbiome de la rhizosphère de la vigne, souligne également le potentiel de ces microorganismes à être intégrés dans la gestion durable des maladies de la vigne. Nos résultats montrent que le succès des inoculations de Ta SC1 et Bs PTA-271 dans le microbiome

de la rhizosphère de la vigne dépend du sol. Les sols sableux semblent favoriser l'implantation de Ta SC1 dans le microbiome rhizosphérique, ce qui est cohérent avec sa nature fongique (mieux adaptée au pH acide), alors que Bs PTA-271 se développe dans un sol calcaire. Ces résultats ont été corroborés par l'étude de l'expression des gènes de défense des plantes. L'inoculation de Ta SC1 et Bs PTA-271 a démontré un effet positif dans les réseaux de corrélation de la rhizosphère, améliorant les corrélations positives et réduisant les négatives. En outre, nous rapportons l'effet bénéfique sur le microbiome de la rhizosphère de la vigne de la combinaison des deux BCAs dans la même application. Il s'agit d'un résultat prometteur pour réduire les incohérences observées avec l'utilisation d'un seul BCA

Bien que les résultats de cette thèse apportent de nouvelles connaissances sur les interactions de la vigne avec les pathogènes responsables des MDB, et sur le potentiel de 2 BCA pour lutter efficacement contre les MDB de la vigne, des questions sans réponse méritent encore d'être clarifiées. La première question, liée à l'apport d'un BCA dans un environnement non stérile pose question quant à l'origine de la protection : est-elle réellement BCA-dépendante ? ou est-elle le fruit d'un changement induit par le BCA sur le microbiome ou le microbiote de la vigne ? Il s'agit là d'un point crucial à approfondir, qu'il passe ou non par des modifications physiologiques intermédiaires de la plante hôte.

Les conditions de vie des plantes et des microorganismes tels que les BCA méritent aussi plus d'attention, en particulier la qualité de leur support qui est le sol. Selon la situation géographique, la nature du sol fluctue eu égard à la nature de la roche mère sur lequel il repose et les conditions climatiques de la station où il se situe. Les éléments clefs à considérer en premier lieu pour avoir un sol de qualité propice au bon état physiologique des plantes et des microorganismes qu'il porte sont entre autres : sa porosité (structure conditionnée par sa texture ou granulométrie), son pH, et sa teneur en matière organique. L'utilisation d'un substrat sableux et d'un substrat argileux sont les deux extrêmes sur l'échelle de la texture. Le sol sableux offre effectivement une macroporosité peu propice à une bonne activité biologique, mais au contraire propice au développement de pH acide et à une mauvaise dégradation de la matière organique (si toutefois celle-ci était retenue). Le sol argileux est quant à lui Le sol argileux est riche en nutriments mais peut être plus dense et plus lourd que les sols sableux, ce qui complique la colonisation des racines des plantes par les micro-organismes.

La conduite de la culture de la vigne reste aussi à prendre en compte, en particulier son irrigation, qui peut influencer le développement ou non de tel ou tel pathogène susceptible d'infecter la plante, ou de moduler la pression en pathogènes, etc. Des recherches complémentaires sur ces aspects sont encore nécessaires. Cela permettrait de couvrir autant de conditions changeantes que possible afin de mieux canaliser/gommer la variabilité qui pourra être observée dans la protection induite par nos BCA lors du transfert des résultats obtenus vers les conditions de terrain. L'utilisation combinée d'un plus grand nombre de BCA est également souhaitée, pour tenter de garantir une protection efficace malgré les fluctuations des facteurs environnementaux.

Enfin, les effets différentiels de *Bs* PTA-271 et de *Ta* SC1 contre le Np-Bt67 chez deux cépages différents de vigne a aussi mis en évidence des effets cépages sur leurs sensibilités aux pathogènes, mais aussi aux BCA, et potentiellement à d'autres facteurs environnementaux non encore appréhendés. Il convient donc d'ouvrir encore nos prospections, d'ordre moléculaires/génétiques à des cépages offrant des réponses de défense beaucoup plus contrastées à Np-Bt67 en situation de contraintes abiotiques, par exemple en comparant les génomes et les réponses de défense de deux cépages issus de 2 régions climatiques très différentes. L'idée est de découvrir de potentiels gènes de tolérance ou de résistance aux MDB chez la vigne puisque : (i) la diversité des génotypes au sein des variétés de vigne, des cépages ou même des clones, et (ii) la distribution et la pression des pathogènes responsables des MDB, sont tous les deux conditionnés à la notion de zone géographique.

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## *Appendix*

## Support to chapter II

**Table s1.** *Bacillus subtilis* PTA-271 encoding genes for motility, adhesion and plant root colonizing capacity.

| Locus tag ID | Gene | Function | | |
|---|---|---|---|---|
| Motility (swarming), biofilm and root colonization | | | | |
| S19-40_00277 | *ylxH* | Flagellum site-determining protein | | |
| S19-40_00278 | *flhF* | Flagellar biosynthesis protein | | |
| S19-40_00279 | *flhA* | Flagellar biosynthesis protein | | |
| S19-40_00280 | *flhB* | Flagellar biosynthetic protein | | |
| S19-40_00283 | *fliP* | Flagellar biosynthetic protein | | |
| S19-40_00286 | *fliN* | Flagellar motor switch protein | | |
| S19-40_00287 | *fliM* | Flagellar motor switch protein | | |
| S19-40_00289 | *flgG* | Flagellar basal-body rod protein | | |
| S19-40_00293 | *fliJ* | Flagellar protein | | |
| S19-40_00296 | *fliG* | Flagellar motor switch protein | | |
| S19-40_00297 | *fliF* | Flagellar M-ring protein | | |
| S19-40_00298 | *fliE* | Flagellar hook-basal body complex protein | | |
| S19-40_00299 | *flgC* | Flagellar basal-body rod protein | | |
| S19-40_00300 | *flgB* | Flagellar basal body rod protein | | |
| S19-40_00290 | *flgD* | Basal-body rod modification protein | | |
| S19-40_00310 | *flhB* | Flagellar biosynthetic protein | | |
| S19-40_00890 | *flgK* | Flagellar hook-associated protein 1 | | |
| S19-40_00891 | *flaB3* | Flagellar filament 31 kDa core protein | | |
| S19-40_00893 | *fliW* | Flagellar assembly factor | | |
| S19-40_00895 | *hag* | Flagellin | | |
| S19-40_00897 | *fliD* | B-type flagellar hook-associated protein 2 | | |
| S19-40_00898 | *fliS* | Flagellar protein | | |
| S19-40_00557 | *motB* | Motility protein B | | |
| S19-40_02745 | *cheR* | Chemotaxis protein methyltransferase | | |
| S19-40_00115 | *cheY* | Chemotaxis protein | | |
| S19-40_00272 | *cheD* | Chemoreceptor glutamine deamidase | | |
| S19-40_00273 | *cheC* | CheY-P phosphatase | | |
| S19-40_00274 | *cheW* | Chemotaxis protein | | |
| S19-40_00275 | *cheA* | Chemotaxis protein | | |
| S19-40_00276 | *cheB* | Chemotaxis response regulator protein-glutamate methylesterase | | |
| S19-40_00285 | *cheY* | Chemotaxis protein | | |
| S19-40_00519 | *cheV* | Chemotaxis protein | | |
| S19-40_02745 | *cheR* | Chemotaxis protein methyltransferase | | |
| S19-40_03165 | *swrC* | Swarming motility protein | | |
| S19-40_00882 | *spo0A* | Response regulator receiver domain protein | | |

**Table s2.** *Bacillus subtilis* PTA-271 encoding genes for some Transcriptional regulators and Operons.

| | Gene | Function |
|---|---|---|
| *Transcriptional regulators* | | |
| S19-40_00006 | purR | LacI family transcriptional regulator, purine nucleotide synthesis repressor |
| S19-40_00012 | immR | HTH-type transcriptional regulator ImmR |
| S19-40_00023 | gltC | HTH-type transcriptional regulator GltC |
| S19-40_00025 | sinR | XRE family transcriptional regulator, master regulator for biofilm formation |
| S19-40_00049 | slyA | Transcriptional regulator SlyA |
| S19-40_00060 | gltC | HTH-type transcriptional regulator GltC |
| S19-40_00064 | yofA | HTH-type transcriptional regulator YofA |
| S19-40_00173 | glnR | HTH-type transcriptional regulator GlnR |
| S19-40_00212 | betI | HTH-type transcriptional regulator BetI |
| S19-40_00255 | infB | Translation initiation factor IF-2 |
| S19-40_00268 | tsf | Elongation factor Ts |
| S19-40_00374 | dksA | RNA polymerase-binding transcription factor DksA |
| S19-40_00374 | yocK | General stress protein 16O |
| S19-40_00405 | mraZ | Transcriptional regulator MraZ |
| S19-40_00506 | argP | HTH-type transcriptional regulator ArgP |
| S19-40_00516 | ykuD | Putative L,D-transpeptidase YkuD |
| S19-40_00529 | splA | Transcriptional regulator protein (SplA) |
| S19-40_00549 | ytcD | putative HTH-type transcriptional regulator YtcD |
| S19-40_00558 | mhqR | HTH-type transcriptional regulator MhqR |
| S19-40_00593 | mhqR | HTH-type transcriptional regulator MhqR |
| S19-40_00596 | tnrA | HTH-type transcriptional regulator TnrA |
| S19-40_00599 | splA | Transcriptional regulator protein (SplA) |
| S19-40_00614 | ohrR | Organic hydroperoxide resistance transcriptional regulator |
| S19-40_00667 | yusO | putative HTH-type transcriptional regulator YusO |
| S19-40_00690 | crp | CRP/FNR family transcriptional regulator, cyclic AMP receptor protein |
| S19-40_00758 | ywnA | Putative HTH-type transcriptional regulator YwnA |
| S19-40_00761 | zntR | Zn(II)-responsive transcriptional regulator |
| S19-40_00768 | hipB | Transcriptional regulator, y4mF family |
| S19-40_00779 | - | putative HTH-type transcriptional regulator/GBAA_1941/BAS1801 |
| S19-40_00794 | ulaR | HTH-type transcriptional regulator UlaR |
| S19-40_00809 | gltR | HTH-type transcriptional regulator GltR |
| S19-40_00814 | lrpC | HTH-type transcriptional regulator LrpC |
| S19-40_00823 | hdfR | HTH-type transcriptional regulator HdfR |

| | | |
|---|---|---|
| S19-40_00834 | scrR | LacI family transcriptional regulator, sucrose operon repressor |
| S19-40_00841 | ywtF | Putative transcriptional regulator YwtF |
| S19-40_00865 | lytR | Transcriptional regulator LytR |
| S19-40_00879 | yvhJ | Putative transcriptional regulator YvhJ |
| S19-40_00882 | degU | Transcriptional regulatory protein DegU |
| S19-40_00882 | *spo0A* | Response regulator receiver domain protein |
| S19-40_00912 | betI | HTH-type transcriptional regulator BetI |
| S19-40_00924 | zitR | Transcriptional regulator ZitR |
| S19-40_00927 | adcR | Transcriptional regulator AdcR |
| S19-40_00929 | nanR | Transcriptional regulator NanR |
| S19-40_00962 | phoB | Phosphate regulon transcriptional regulatory protein PhoB |
| S19-40_00987 | yvdT | putative HTH-type transcriptional regulator YvdT |
| S19-40_00997 | sinR | HTH-type transcriptional regulator SinR |
| S19-40_01016 | lutR | HTH-type transcriptional regulator LutR |
| S19-40_01017 | lacR | HTH-type transcriptional regulator LacR |
| S19-40_01028 | desR | Transcriptional regulatory protein DesR |
| S19-40_01035 | hdfR | HTH-type transcriptional regulator HdfR |
| S19-40_01050 | opcR | HTH-type transcriptional regulator, osmoprotectant uptake regulator |
| S19-40_01060 | opcR | HTH-type transcriptional regulator, osmoprotectant uptake regulator |
| S19-40_01067 | yodB | HTH-type transcriptional regulator YodB |
| S19-40_01068 | rghR | HTH-type transcriptional repressor RghR |
| S19-40_01069 | rghR | HTH-type transcriptional repressor RghR |
| S19-40_01078 | tetR | Mycofactocin system transcriptional regulator |
| S19-40_01084 | csoR | Copper-sensing transcriptional repressor CsoR |
| S19-40_01118 | walR | Transcriptional regulatory protein WalR |
| S19-40_01132 | liaR | Transcriptional regulatory protein LiaR |
| S19-40_01138 | ethR | HTH-type transcriptional regulator EthR |
| S19-40_01140 | cssR | Transcriptional regulatory protein CssR |
| S19-40_01148 | gltR | HTH-type transcriptional regulator GltR |
| S19-40_01152 | yusO | putative HTH-type transcriptional regulator YusO |
| S19-40_01185 | yurK | putative HTH-type transcriptional regulator YurK |
| S19-40_01273 | comA | Transcriptional regulatory protein ComA |
| S19-40_01288 | dcuR | Transcriptional regulatory protein DcuR |

| | | |
|---|---|---|
| S19-40_01325 | ulaR | HTH-type transcriptional regulator UlaR |
| S19-40_01340 | opcR | HTH-type transcriptional repressor OpcR |
| S19-40_01355 | - | Transcriptional regulator, Acidobacterial, PadR-family |
| S19-40_01359 | tetR | Tetracycline repressor protein class B from transposon Tn10 |
| S19-40_01367 | csoR | Copper-sensing transcriptional repressor CsoR |
| S19-40_01369 | tipA | HTH-type transcriptional activator TipA |
| S19-40_01375 | gltR | HTH-type transcriptional regulator GltR |
| S19-40_01379 | gltR | HTH-type transcriptional regulator GltR |
| S19-40_01398 | cmpR | HTH-type transcriptional activator CmpR |
| S19-40_01412 | adhR | HTH-type transcriptional regulator AdhR |
| S19-40_01421 | dgaR | Transcriptional regulatory protein DagR |
| S19-40_01424 | - | Sugar-specific transcriptional regulator TrmB |
| S19-40_01430 | bm3R1 | HTH-type transcriptional repressor Bm3R1 |
| S19-40_01468 | cymR | HTH-type transcriptional regulator CymR |
| S19-40_01482 | comN | Post-transcriptional regulator ComN |
| S19-40_01499 | yebC | putative transcriptional regulatory protein YebC |
| S19-40_01559 | yusO | putative HTH-type transcriptional regulator YusO |
| S19-40_01574 | betI | HTH-type transcriptional regulator BetI |
| S19-40_01606 | infC | Translation initiation factor IF-3 |
| S19-40_01618 | nrdR | Transcriptional repressor NrdR |
| S19-40_01621 | ytcD | putative HTH-type transcriptional regulator YtcD |
| S19-40_01629 | phoP | Alkaline phosphatase synthesis transcriptional regulatory protein PhoP |
| S19-40_01660 | cmpR | HTH-type transcriptional activator CmpR |
| S19-40_01683 | yttP | putative HTH-type transcriptional regulator YttP |
| S19-40_01722 | ytzE | putative HTH-type transcriptional regulator YtzE |
| S19-40_01733 | yesS | HTH-type transcriptional regulator YesS |
| S19-40_01744 | lacR | HTH-type transcriptional regulator LacR |
| S19-40_01764 | ytrA | HTH-type transcriptional repressor YtrA |
| S19-40_01802 | dksA | RNA polymerase-binding transcription factor DksA |
| S19-40_01845 | gmuR | HTH-type transcriptional regulator GmuR |
| S19-40_01860 | rspR | HTH-type transcriptional repressor RspR |
| S19-40_01863 | slyA | Transcriptional regulator SlyA |
| S19-40_01866 | tcaR | HTH-type transcriptional regulator TcaR |

| | | |
|---|---|---|
| S19-40_01872 | betI | HTH-type transcriptional regulator BetI |
| S19-40_01894 | cmtR | HTH-type transcriptional regulator CmtR |
| S19-40_01896 | norG | HTH-type transcriptional regulator NorG |
| S19-40_01900 | aseR | HTH-type transcriptional repressor AseR |
| S19-40_01901 | - | putative HTH-type transcriptional regulator |
| S19-40_01904 | yybR | putative HTH-type transcriptional regulator YybR |
| S19-40_01906 | yybR | putative HTH-type transcriptional regulator YybR |
| S19-40_01909 | gabR | HTH-type transcriptional regulatory protein GabR |
| S19-40_01921 | rhaS | HTH-type transcriptional activator RhaS |
| S19-40_01923 | carD | RNA polymerase-binding transcription factor CarD |
| S19-40_01927 | - | Transcriptional regulator, y4mF family |
| S19-40_01969 | dcuR | Transcriptional regulatory protein DcuR |
| S19-40_01990 | lrpC | HTH-type transcriptional regulator LrpC |
| S19-40_01999 | mtlR | Transcriptional regulator MtlR |
| S19-40_02005 | kipR | HTH-type transcriptional regulator KipR |
| S19-40_02028 | gabR | HTH-type transcriptional regulatory protein GabR |
| S19-40_02044 | srrA | Transcriptional regulatory protein SrrA |
| S19-40_02058 | benM | HTH-type transcriptional regulator BenM |
| S19-40_02064 | gabR | HTH-type transcriptional regulatory protein GabR |
| S19-40_02082 | hpr | HTH-type transcriptional regulator Hpr |
| S19-40_02087 | yciB | Putative L,D-transpeptidase YciB |
| S19-40_02114 | mhqR | HTH-type transcriptional regulator MhqR |
| S19-40_02148 | natR | Transcriptional regulatory protein NatR |
| S19-40_02154 | yxaF | putative HTH-type transcriptional regulator YxaF |
| S19-40_02167 | walR | Transcriptional regulatory protein WalR |
| S19-40_02172 | lutR | HTH-type transcriptional regulator LutR |
| S19-40_02207 | - | Transcriptional regulator SlyA |
| S19-40_02257 | ybbH | putative HTH-type transcriptional regulator YbbH |
| S19-40_02291 | xre | HTH-type transcriptional regulator Xre |
| S19-40_02305 | exuR | putative HTH-type transcriptional repressor ExuR |
| S19-40_02344 | manR | Transcriptional regulator ManR |
| S19-40_02402 | spxA | Transcriptional regulator, Spx/MgsR family |
| S19-40_02449 | - | Transcriptional regulator, Acidobacterial, PadR-family |

| | | |
|---|---|---|
| S19-40_02468 | norG | HTH-type transcriptional regulator NorG |
| S19-40_02472 | degA | HTH-type transcriptional regulator DegA |
| S19-40_02497 | sgrR | HTH-type transcriptional regulator SgrR |
| S19-40_02539 | bm3R1 | HTH-type transcriptional repressor Bm3R1 |
| S19-40_02555 | hpr | HTH-type transcriptional regulator Hpr |
| S19-40_02602 | gabR | HTH-type transcriptional regulatory protein GabR |
| S19-40_02607 | cysL | HTH-type transcriptional regulator CysL |
| S19-40_02612 | nsrR | HTH-type transcriptional repressor NsrR |
| S19-40_02617 | liaR | Transcriptional regulatory protein LiaR |
| S19-40_02643 | ytrA | HTH-type transcriptional repressor YtrA |
| S19-40_02786 | srrA | Transcriptional regulatory protein SrrA |
| S19-40_02834 | xre | HTH-type transcriptional regulator Xre |
| S19-40_02872 | ykuD | Putative L,D-transpeptidase YkuD |
| S19-40_02880 | - | Zn(II)-responsive transcriptional regulator |
| S19-40_02904 | argR | Arginine repressor |
| S19-40_02924 | efp | Elongation factor P |
| S19-40_02931 | mntR | Transcriptional regulator MntR |
| S19-40_02940 | sinR | HTH-type transcriptional regulator SinR |
| S19-40_02959 | spxA | Transcriptional regulator, Spx/MgsR family |
| S19-40_02992 | zur | Fur family transcriptional regulator, zinc uptake regulator |
| S19-40_03061 | marR | MarR family transcriptional regulator, multiple antibiotic resistance protein MarR |
| S19-40_03147 | lrpC | HTH-type transcriptional regulator LrpC |
| S19-40_03152 | trpR | TrpR family transcriptional regulator, trp operon repressor |
| S19-40_03164 | kstR2 | HTH-type transcriptional repressor KstR2 |
| S19-40_03183 | - | putative transcriptional regulatory protein |
| S19-40_03199 | yesS | HTH-type transcriptional regulator YesS |
| S19-40_03219 | mprA | Transcriptional repressor MprA |
| S19-40_03221 | IF5B | translation initiation factor 5B |
| S19-40_03237 | yfmP | HTH-type transcriptional regulator YfmP |
| S19-40_03254 | citT | Transcriptional regulatory protein CitT |
| S19-40_03272 | treR | HTH-type transcriptional regulator TreR |
| S19-40_03308 | glvR | HTH-type transcriptional regulator GlvR |
| S19-40_03316 | degU | Transcriptional regulatory protein DegU |

| | | |
|---|---|---|
| S19-40_03325 | yusO | putative HTH-type transcriptional regulator YusO |
| S19-40_03379 | - | Transcriptional regulator PadR-like family protein |
| S19-40_03402 | liaR | Transcriptional regulatory protein LiaR |
| S19-40_03450 | mgrA | HTH-type transcriptional regulator MgrA |
| S19-40_03463 | cynR | HTH-type transcriptional regulator CynR |
| S19-40_03472 | slrA | Transcriptional regulator SlrA |
| S19-40_03472 | slrA | Transcriptional regulator SlrA |
| S19-40_03494 | - | Transcriptional regulator, Acidobacterial, PadR-family |
| S19-40_03530 | cysL | HTH-type transcriptional regulator CysL |
| S19-40_03537 | iscR | HTH-type transcriptional regulator IscR |
| S19-40_03547 | btr | HTH-type transcriptional activator Btr |
| S19-40_03554 | czrA | HTH-type transcriptional repressor CzrA |
| S19-40_03562 | desR | Transcriptional regulatory protein DesR |
| S19-40_03567 | dksA | RNA polymerase-binding transcription factor DksA |
| S19-40_03599 | yodB | HTH-type transcriptional regulator YodB |
| S19-40_03634 | yusO | putative HTH-type transcriptional regulator YusO |
| S19-40_03677 | kdgR | HTH-type transcriptional regulator KdgR |
| S19-40_03703 | tcrX | putative transcriptional regulatory protein TcrX |
| S19-40_03711 | walR | Transcriptional regulatory protein WalR |
| S19-40_03729 | yybR | putative HTH-type transcriptional regulator YybR |
| S19-40_03734 | mta | HTH-type transcriptional activator mta |
| S19-40_03739 | gltC | HTH-type transcriptional regulator GltC |
| S19-40_03743 | slyA | Transcriptional regulator SlyA |
| S19-40_03753 | - | putative HTH-type transcriptional regulator |
| S19-40_03760 | purR | HTH-type transcriptional repressor PurR |
| S19-40_03790 | yydK | putative HTH-type transcriptional regulator YydK |
| S19-40_03799 | gntR | putative D-xylose utilization operon transcriptional repressor |
| S19-40_03804 | nicR | MarR family transcriptional regulator, lower aerobic nicotinate degradation pathwa |
| S19-40_03806 | yxaF | putative HTH-type transcriptional regulator YxaF |
| S19-40_03815 | dhaS | HTH-type dhaKLM operon transcriptional activator DhaS |
| S19-40_03885 | infA | Translation initiation factor IF-1 |
| S19-40_03911 | tuf | Elongation factor Tu |
| S19-40_03912 | fusA | Elongation factor G |

| | | |
|---|---|---|
| S19-40_03940 | ctsR | Transcriptional regulator CtsR |
| S19-40_03944 | - | Transcriptional regulator, y4mF family |
| ***Operons*** | | |
| S19-40_00023 | cysL | LysR family transcriptional regulator, transcriptional activator of the cysJI operon |
| S19-40_00049 | HpaR | homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_00060 | gltC | LysR family transcriptional regulator, transcription activator of glutamate synthase |
| S19-40_00271 | fliA | RNA polymerase sigma factor for flagellar operon FliA |
| S19-40_00371 | pyrR | pyrimidine operon attenuation protein / uracil phosphoribosyltransferase |
| S19-40_00482 | fruR2, fruR | DeoR family transcriptional regulator, fructose operon transcriptional repressor |
| S19-40_00506 | cysL | LysR family transcriptional regulator, transcriptional activator of the cysJI operon |
| S19-40_00529 | splA | transcriptional regulator of the spore photoproduct lyase operon |
| S19-40_00533 | glcT | PtsGHI operon antiterminator |
| S19-40_00558 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_00593 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_00599 | splA | transcriptional regulator of the spore photoproduct lyase operon |
| S19-40_00614 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_00779 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_00823 | hcaR | Hca operon transcriptional activator HcaR |
| S19-40_00834 | scrR | LacI family transcriptional regulator, sucrose operon repressor |
| S19-40_00884 | comFA | ComF operon protein 1 |
| S19-40_00885 | comFB | ComF operon protein 2 |
| S19-40_00887 | YvyF | YvyF: flagellar operon protein |
| S19-40_00912 | mexL | TetR/AcrR family transcriptional regulator, mexJK operon transcriptional repressor |
| S19-40_00927 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_01035 | gltC | LysR family transcriptional regulator, transcription activator of glutamate synthase |
| S19-40_01037 | araR | GntR family transcriptional regulator, arabinose operon transcriptional repressor |
| S19-40_01078 | acrR | TetR/AcrR family transcriptional regulator, multidrug resistance operon repressor |
| S19-40_01106 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_01116 | fliA | RNA polymerase sigma factor for flagellar operon FliA |
| S19-40_01138 | nemR | TetR/AcrR family transcriptional regulator, transcriptional repressor for nem opero |
| S19-40_01152 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_01185 | frlR | GntR family transcriptional regulator, frlABCD operon transcriptional regulator |
| S19-40_01325 | srlR | Glucitol operon repressor |

| | | |
|---|---|---|
| S19-40_01365 | pspE | phageshock_pspE: phage shock operon rhodanese PspE |
| S19-40_01421 | gfrR | sigma-54 dependent transcriptional regulator, gfr operon transcriptional activator |
| S19-40_01430 | fatR, bscR | TetR/AcrR family transcriptional regulator, repressor of fatR-cypB operon |
| S19-40_01559 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_01660 | ytlI | LysR family transcriptional regulator, regulator of the ytmI operon |
| S19-40_01683 | fatR, bscR | TetR/AcrR family transcriptional regulator, repressor of fatR-cypB operon |
| S19-40_01695 | cytR | LacI family transcriptional regulator, repressor for deo operon, udp, cdd, tsx, nupC, |
| S19-40_01733 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_01744 | cytR | LacI family transcriptional regulator, repressor for deo operon, udp, cdd, tsx, nupC, |
| S19-40_01764 | - | trehalos_R_Bsub: trehalose operon repressor |
| S19-40_01771 | caiE | Carnitine operon protein CaiE |
| S19-40_01845 | - | trehalos_R_Bsub: trehalose operon repressor |
| S19-40_01860 | rspR | GntR family transcriptional regulator, rspAB operon transcriptional repressor |
| S19-40_01901 | mexL | TetR/AcrR family transcriptional regulator, mexJK operon transcriptional repressor |
| S19-40_01911 | fatR, bscR | TetR/AcrR family transcriptional regulator, repressor of fatR-cypB operon |
| S19-40_01921 | rhaS | AraC family transcriptional regulator, L-rhamnose operon regulatory protein RhaS |
| S19-40_01984 | bcsB | cellulose synthase operon protein B |
| S19-40_01999 | mtlR | mannitol operon transcriptional antiterminator |
| S19-40_02105 | gltC | LysR family transcriptional regulator, transcription activator of glutamate synthase |
| S19-40_02114 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_02154 | lmrA, yxaF | TetR/AcrR family transcriptional regulator, lmrAB and yxaGH operons repressor |
| S19-40_02186 | - | trehalos_R_Bsub: trehalose operon repressor |
| S19-40_02202 | rhaS | AraC family transcriptional regulator, L-rhamnose operon regulatory protein RhaS |
| S19-40_02233 | arsR | Arsenical resistance operon repressor |
| S19-40_02262 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_02263 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_02344 | manR | activator of the mannose operon, transcriptional antiterminator |
| S19-40_02472 | cytR | LacI family transcriptional regulator, repressor for deo operon, udp, cdd, tsx, nupC, |
| S19-40_02473 | araC | Arabinose operon regulatory protein |
| S19-40_02500 | cytR | LacI family transcriptional regulator, repressor for deo operon, udp, cdd, tsx, nupC, |
| S19-40_02539 | fatR, bscR | TetR/AcrR family transcriptional regulator, repressor of fatR-cypB operon |
| S19-40_02594 | merR1 | Mercuric resistance operon regulatory protein |
| S19-40_02623 | glpP | Glycerol uptake operon antiterminator regulatory protein |

| | | |
|---|---|---|
| S19-40_02649 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_02717 | birA | BirA family transcriptional regulator, biotin operon repressor / biotin---[acetyl-CoA- |
| S19-40_02933 | pspE | phageshock_pspE: phage shock operon rhodanese PspE |
| S19-40_02946 | comGG | ComG operon protein 7 |
| S19-40_02950 | comGC | ComG operon protein 3 |
| S19-40_02952 | comGA | ComG operon protein 1 |
| S19-40_03041 | comEC | ComE operon protein 3 |
| S19-40_03042 | comEB | ComE operon protein 2 |
| S19-40_03043 | comEA | ComE operon protein 1 |
| S19-40_03075 | nemR | TetR/AcrR family transcriptional regulator, transcriptional repressor for nem opero |
| S19-40_03104 | gutR | LuxR family transcriptional regulator, glucitol operon activator |
| S19-40_03152 | trpR | TrpR family transcriptional regulator, trp operon repressor |
| S19-40_03199 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_03219 | emrR, mprA | MarR family transcriptional regulator, negative regulator of the multidrug operon e |
| S19-40_03237 | yfmP | MerR family transcriptional regulator, repressor of the yfmOP operon |
| S19-40_03272 | - | trehalos_R_Bsub: trehalose operon repressor |
| S19-40_03300 | acoR | Acetoin dehydrogenase operon transcriptional activator AcoR |
| S19-40_03308 | glvR | RpiR family transcriptional regulator, glv operon transcriptional regulator |
| S19-40_03325 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_03357 | perR | Peroxide operon regulator |
| S19-40_03384 | licT, bglG | beta-glucoside operon transcriptional antiterminator |
| S19-40_03432 | licR | putative licABCH operon regulator |
| S19-40_03450 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_03453 | sacY | Levansucrase and sucrase synthesis operon antiterminator |
| S19-40_03463 | gltC | LysR family transcriptional regulator, transcription activator of glutamate synthase |
| S19-40_03473 | acrR, smeT | TetR/AcrR family transcriptional regulator, acrAB operon repressor |
| S19-40_03488 | sacY | Levansucrase and sucrase synthesis operon antiterminator |
| S19-40_03530 | cysL | LysR family transcriptional regulator, transcriptional activator of the cysJI operon |
| S19-40_03547 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_03634 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| | | sigma-54 dependent transcriptional regulator, acetoin dehydrogenase operon tra |
| S19-40_03642 | acoR | AcoR |
| S19-40_03677 | kdgR | LacI family transcriptional regulator, kdg operon repressor |

| | | |
|---|---|---|
| S19-40_03739 | gltC | LysR family transcriptional regulator, transcription activator of glutamate synthase |
| S19-40_03760 | scrR | LacI family transcriptional regulator, sucrose operon repressor |
| S19-40_03790 | - | trehalos_R_Bsub: trehalose operon repressor |
| S19-40_03799 | gntR | putative D-xylose utilization operon transcriptional repressor |
| S19-40_03804 | HpaR | HpaR: homoprotocatechuate degradation operon regulator, HpaR |
| S19-40_03806 | lmrA, yxaF | TetR/AcrR family transcriptional regulator, lmrAB and yxaGH operons repressor |
| S19-40_03815 | dhaS | HTH-type dhaKLM operon transcriptional activator DhaS |
| S19-40_03823 | srlR | Glucitol operon repressor |
| S19-40_03839 | btr | AraC family transcriptional regulator, transcriptional activator for feuABC-ybbA ope |
| S19-40_03856 | lsrR | lsr operon transcriptional repressor |
| S19-40_03865 | hutP | Hut operon positive regulatory protein |
| S19-40_03979 | purR | Pur operon repressor |

**Table s3.** *Bacillus subtilis* PTA-271 encoding genes for antimicrobial molecules, other effectors and lytic enzymes.

**Table s4.** *Bacillus subtilis* PTA-271 encoding genes for sporulation.

| **Locus tag ID** | **Gene** | **Function** |
|---|---|---|
| ***Bacteriocin/Bacteriocin-like-peptides*** | | |
| S19-40_00081 | menE | AMP-binding enzyme |
| S19-40_00234 | albE | Antilisterial bacteriocin subtilosin biosynthesis protein AlbE |
| S19-40_00679 | albG | Antilisterial bacteriocin subtilosin biosynthesis protein AlbG |
| S19-40_00681 | albE | Antilisterial bacteriocin subtilosin biosynthesis protein AlbE |
| S19-40_00682 | alBOT | Antilisterial bacteriocin subtilosin biosynthesis protein AlBOT |
| S19-40_00683 | btuD | Lantibiotic protection ABC transporter, ATP-binding subunit |
| S19-40_00684 | albB | Antilisterial bacteriocin subtilosin biosynthesis protein AlbB |
| S19-40_00685 | albA | Antilisterial bacteriocin subtilosin biosynthesis protein AlbA |
| S19-40_00690 | crp | CRP/FNR family transcriptional regulator, cyclic AMP receptor protein |
| S19-40_00726 | ywlC | Threonylcarbamoyl-AMP synthase |
| S19-40_00946 | hisI | Phosphoribosyl-AMP cyclohydrolase |

| | | |
|---|---|---|
| S19-40_00964 | btuD | Putative bacteriocin export ABC transporter, lactococcin 972 group |
| S19-40_01244 | dhbE | 2,3-dihydroxybenzoate-AMP ligase |
| S19-40_01246 | - | AMP-binding enzyme |
| S19-40_01575 | - | AMP-binding enzyme |
| S19-40_01689 | - | AMP-binding enzyme |
| S19-40_01755 | bceB | Bacitracin export permease protein BceB |
| S19-40_01756 | bceA | Bacitracin export ATP-binding protein BceA |
| S19-40_01915 | - | Bacteriocin-protection, YdeI or OmpD-Associated |
| S19-40_01966 | nukF, mcdF, sboF | lantibiotic transport system ATP-binding protein |
| S19-40_01998 | - | AMP-binding enzyme |
| S19-40_01998 | - | AMP-binding enzyme |
| S19-40_02046 | btuD | Putative bacteriocin export ABC transporter, lactococcin 972 group |
| S19-40_02071 | licA | lichenysin synthetase A |
| S19-40_02165 | btuD | lantibiotic protection ABC transporter, ATP-binding subunit |
| S19-40_02198 | - | Trypsin |
| S19-40_02227 | skfF | Putative bacteriocin-SkfA transport system permease protein SkfF |
| S19-40_02641 | btuD | Lantibiotic protection ABC transporter, ATP-binding subunit |
| S19-40_03432 | licR | lichenan operon transcriptional antiterminator |
| S19-40_03433 | licB | Lichenan-specific phosphotransferase enzyme IIB component |
| S19-40_03434 | licC | Lichenan permease IIC component |
| S19-40_03435 | licA | Lichenan-specific phosphotransferase enzyme IIA component |
| S19-40_03456 | licC | Lichenan permease IIC component |
| S19-40_03706 | - | Trypsin-like peptidase domain protein |
| S19-40_03836 | btuD | Putative bacteriocin export ABC transporter, lactococcin 972 group |
| ***Other effectors*** | | |
| S19-40_00073 | fenC | fengycin family lipopeptide synthetase A |
| S19-40_00074 | fenD | fengycin family lipopeptide synthetase B |
| S19-40_00075 | - | fengycin family lipopeptide synthetase C |
| S19-40_00076 | fenA | fengycin family lipopeptide synthetase D |
| S19-40_00077 | fenB | fengycin family lipopeptide synthetase E |
| S19-40_00208 | pksD | bacillaene synthase trans-acting acyltransferase |
| S19-40_00567 | bacF | Transaminase BacF |
| S19-40_00679 | albG | Antilisterial bacteriocin subtilosin biosynthesis protein AlbG |

| | | |
|---|---|---|
| S19-40_00681 | albE | Antilisterial bacteriocin subtilosin biosynthesis protein AlbE |
| S19-40_00682 | alBOT | Antilisterial bacteriocin subtilosin biosynthesis protein AlBOT |
| S19-40_00684 | albB | Antilisterial bacteriocin subtilosin biosynthesis protein AlbB |
| S19-40_00684 | albB | Antilisterial bacteriocin subtilosin biosynthesis protein AlbB |
| S19-40_00685 | albA | Antilisterial bacteriocin subtilosin biosynthesis protein AlbA |
| S19-40_00686 | sboA | Subtilosin-A |
| S19-40_02068 | srfAD | Surfactin synthase thioesterase subunit |
| S19-40_02069 | srfAC | Surfactin synthase subunit 3 |
| S19-40_02070 | srfAB | Surfactin synthase subunit 2 |
| S19-40_02071 | srfAA | Surfactin synthase subunit 1 |
| S19-40_03525 | bacE | Putative bacilysin exporter BacE |
| S19-40_03526 | bacF | Transaminase BacF |
| S19-40_03527 | bacG | NADPH-dependent reductase BacG |
| ***Siderophores*** | | |
| S19-40_01242 | dhbA | 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase |
| S19-40_01243 | dhbC | Isochorismate synthase DhbC |
| S19-40_01244 | dhbE | 2,3-dihydroxybenzoate-AMP ligase |
| S19-40_01245 | dhbB | Isochorismatase |
| S19-40_01246 | dhbF | Dimodular nonribosomal peptide synthase |
| ***Lytic enzymes*** | | |
| S19-40_00094 | - | Cellulase (glycosyl hydrolase family 5) |
| S19-40_00541 | sleB | Spore cortex-lytic enzyme |
| S19-40_00651 | sleB | spore cortex-lytic enzyme |
| S19-40_01400 | csn | Chitosanase |
| S19-40_02204 | sleB | spore cortex-lytic enzyme |
| S19-40_02296 | sleB | spore cortex-lytic enzyme |
| S19-40_02768 | sleB | Spore cortex-lytic enzyme |
| S19-40_03055 | sleB | spore cortex-lytic enzyme |
| S19-40_03077 | sleB | spore cortex-lytic enzyme |
| S19-40_03385 | bglS | Beta-glucanase |
| ***Proteases*** | | |
| S19-40_00020 | ydeA | putative protease YdeA |
| S19-40_00164 | - | CAAX protease self-immunity |

| | | |
|---|---|---|
| S19-40_00193 | aprX | Serine protease AprX |
| S19-40_00233 | albF | Putative zinc protease AlbF |
| S19-40_00239 | clpP | ATP-dependent Clp protease proteolytic subunit |
| S19-40_00247 | - | putative zinc protease |
| S19-40_00262 | rasP | Regulator of sigma-W protease RasP |
| S19-40_00302 | clpY | ATP-dependent protease ATPase subunit ClpY |
| S19-40_00303 | clpQ | ATP-dependent protease subunit ClpQ |
| S19-40_00414 | lon | Lon protease |
| S19-40_00475 | - | Thermophilic metalloprotease (M29) |
| S19-40_00555 | clpE | ATP-dependent Clp protease ATP-binding subunit ClpE |
| S19-40_00578 | htpX | Protease HtpX |
| S19-40_00609 | isp | Major intracellular serine protease |
| S19-40_00640 | htrA | Serine protease Do-like HtrA |
| S19-40_00669 | rip2 | Putative zinc metalloprotease Rip2 |
| S19-40_00680 | albF | Putative zinc protease AlbF |
| S19-40_00908 | ctpB | Carboxy-terminal processing protease CtpB |
| S19-40_00980 | clpP | ATP-dependent Clp protease proteolytic subunit |
| S19-40_01141 | htrB | Serine protease Do-like HtrB |
| S19-40_01229 | paiB | Protease synthase and sporulation protein PAI 2 |
| S19-40_01403 | lasA | LasA protease |
| S19-40_01404 | lasA | LasA protease |
| S19-40_01415 | yraA | Putative cysteine protease YraA |
| S19-40_01448 | ydcP | putative protease YdcP |
| S19-40_01449 | ydcP | putative protease YdcP |
| S19-40_01512 | - | Cysteine protease Prp |
| S19-40_01538 | lon1 | Lon protease 1 |
| S19-40_01539 | lon2 | Lon protease 2 |
| S19-40_01540 | clpX | ATP-dependent Clp protease ATP-binding subunit ClpX |
| S19-40_01953 | gluP | Rhomboid protease GluP |
| S19-40_02198 | mpr | Extracellular metalloprotease |
| S19-40_02280 | - | Putative phage serine protease XkdF |
| S19-40_02300 | ftsH | ATP-dependent zinc metalloprotease FtsH |
| S19-40_02446 | nprB | Neutral protease B |

| | | |
|---|---|---|
| S19-40_02479 | wprA | Cell wall-associated protease |
| S19-40_02527 | htpX | Protease HtpX |
| S19-40_02732 | bepA | Beta-barrel assembly-enhancing protease |
| S19-40_02769 | prsW | Protease PrsW |
| S19-40_02776 | - | CAAX protease self-immunity |
| S19-40_02969 | gluP | Rhomboid protease GluP |
| S19-40_03037 | gpr | Germination protease |
| S19-40_03091 | tsaD | Glycoprotease family protein |
| S19-40_03093 | tsaD | Glycoprotease family protein |
| S19-40_03100 | - | CAAX protease self-immunity |
| S19-40_03218 | yccA | modulator of FtsH protease |
| S19-40_03275 | pfpL | Intracellular protease, PfpI family |
| S19-40_03413 | htpX | Protease HtpX |
| S19-40_03455 | epr | Minor extracellular protease Epr |
| S19-40_03486 | vpr | Minor extracellular protease vpr |
| S19-40_03605 | ctpA | Carboxy-terminal processing protease CtpA |
| S19-40_03706 | htrA | Serine protease Do-like HtrA |
| S19-40_03756 | - | CAAX protease self-immunity |
| S19-40_03937 | clpX | ATP-dependent Clp protease ATP-binding subunit ClpX |
| S19-40_03955 | ftsH | ATP-dependent zinc metalloprotease FtsH |
| S19-40_03982 | yabG | Sporulation-specific protease YabG |

**Table s5.** *Bacillus subtilis* PTA-271 encoding genes for some CYP450 and for Transferases.

| Locus tag ID | Gene | Function |
|---|---|---|
| *Bacteriocin/Bacteriocin-like-peptides* | | |
| S19-40_00081 | menE | AMP-binding enzyme |
| S19-40_00234 | albE | Antilisterial bacteriocin subtilosin biosynthesis protein AlbE |
| S19-40_00679 | albG | Antilisterial bacteriocin subtilosin biosynthesis protein AlbG |
| S19-40_00681 | albE | Antilisterial bacteriocin subtilosin biosynthesis protein AlbE |
| S19-40_00682 | alBOT | Antilisterial bacteriocin subtilosin biosynthesis protein AlBOT |
| S19-40_00683 | btuD | Lantibiotic protection ABC transporter, ATP-binding subunit |
| S19-40_00684 | albB | Antilisterial bacteriocin subtilosin biosynthesis protein AlbB |
| S19-40_00685 | albA | Antilisterial bacteriocin subtilosin biosynthesis protein AlbA |
| S19-40_00690 | crp | CRP/FNR family transcriptional regulator, cyclic AMP receptor protein |
| S19-40_00726 | ywlC | Threonylcarbamoyl-AMP synthase |
| S19-40_00946 | hisI | Phosphoribosyl-AMP cyclohydrolase |
| S19-40_00964 | btuD | Putative bacteriocin export ABC transporter, lactococcin 972 group |
| S19-40_01244 | dhbE | 2,3-dihydroxybenzoate-AMP ligase |
| S19-40_01246 | - | AMP-binding enzyme |
| S19-40_01575 | - | AMP-binding enzyme |
| S19-40_01689 | - | AMP-binding enzyme |
| S19-40_01755 | bceB | Bacitracin export permease protein BceB |
| S19-40_01756 | bceA | Bacitracin export ATP-binding protein BceA |
| S19-40_01915 | - | Bacteriocin-protection, YdeI or OmpD-Associated |
| S19-40_01966 | nukF, mcdF, sboF | lantibiotic transport system ATP-binding protein |
| S19-40_01998 | - | AMP-binding enzyme |
| S19-40_01998 | - | AMP-binding enzyme |
| S19-40_02046 | btuD | Putative bacteriocin export ABC transporter, lactococcin 972 group |
| S19-40_02071 | licA | lichenysin synthetase A |
| S19-40_02165 | btuD | lantibiotic protection ABC transporter, ATP-binding subunit |
| S19-40_02198 | - | Trypsin |
| S19-40_02227 | skfF | Putative bacteriocin-SkfA transport system permease protein SkfF |
| S19-40_02641 | btuD | Lantibiotic protection ABC transporter, ATP-binding subunit |
| S19-40_03432 | licR | lichenan operon transcriptional antiterminator |
| S19-40_03433 | licB | Lichenan-specific phosphotransferase enzyme IIB component |
| S19-40_03434 | licC | Lichenan permease IIC component |
| S19-40_03435 | licA | Lichenan-specific phosphotransferase enzyme IIA component |
| S19-40_03456 | licC | Lichenan permease IIC component |

| | | |
|---|---|---|
| S19-40_03706 | - | Trypsin-like peptidase domain protein |
| S19-40_03836 | btuD | Putative bacteriocin export ABC transporter, lactococcin 972 group |
| ***Other effectors*** | | |
| S19-40_00073 | fenC | fengycin family lipopeptide synthetase A |
| S19-40_00074 | fenD | fengycin family lipopeptide synthetase B |
| S19-40_00075 | - | fengycin family lipopeptide synthetase C |
| S19-40_00076 | fenA | fengycin family lipopeptide synthetase D |
| S19-40_00077 | fenB | fengycin family lipopeptide synthetase E |
| S19-40_00208 | pksD | bacillaene synthase trans-acting acyltransferase |
| S19-40_00567 | bacF | Transaminase BacF |
| S19-40_00679 | albG | Antilisterial bacteriocin subtilosin biosynthesis protein AlbG |
| S19-40_00681 | albE | Antilisterial bacteriocin subtilosin biosynthesis protein AlbE |
| S19-40_00682 | alBOT | Antilisterial bacteriocin subtilosin biosynthesis protein AlBOT |
| S19-40_00684 | albB | Antilisterial bacteriocin subtilosin biosynthesis protein AlbB |
| S19-40_00684 | albB | Antilisterial bacteriocin subtilosin biosynthesis protein AlbB |
| S19-40_00685 | albA | Antilisterial bacteriocin subtilosin biosynthesis protein AlbA |
| S19-40_00686 | sboA | Subtilosin-A |
| S19-40_02068 | srfAD | Surfactin synthase thioesterase subunit |
| S19-40_02069 | srfAC | Surfactin synthase subunit 3 |
| S19-40_02070 | srfAB | Surfactin synthase subunit 2 |
| S19-40_02071 | srfAA | Surfactin synthase subunit 1 |
| S19-40_03525 | bacE | Putative bacilysin exporter BacE |
| S19-40_03526 | bacF | Transaminase BacF |
| S19-40_03527 | bacG | NADPH-dependent reductase BacG |
| ***Siderophores*** | | |
| S19-40_01242 | dhbA | 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase |
| S19-40_01243 | dhbC | Isochorismate synthase DhbC |
| S19-40_01244 | dhbE | 2,3-dihydroxybenzoate-AMP ligase |
| S19-40_01245 | dhbB | Isochorismatase |
| S19-40_01246 | dhbF | Dimodular nonribosomal peptide synthase |
| ***Lytic enzymes*** | | |
| S19-40_00094 | - | Cellulase (glycosyl hydrolase family 5) |
| S19-40_00541 | sleB | Spore cortex-lytic enzyme |

| | | |
|---|---|---|
| S19-40_00651 | sleB | spore cortex-lytic enzyme |
| S19-40_01400 | csn | Chitosanase |
| S19-40_02204 | sleB | spore cortex-lytic enzyme |
| S19-40_02296 | sleB | spore cortex-lytic enzyme |
| S19-40_02768 | sleB | Spore cortex-lytic enzyme |
| S19-40_03055 | sleB | spore cortex-lytic enzyme |
| S19-40_03077 | sleB | spore cortex-lytic enzyme |
| S19-40_03385 | bglS | Beta-glucanase |
| ***Proteases*** | | |
| S19-40_00020 | ydeA | putative protease YdeA |
| S19-40_00164 | - | CAAX protease self-immunity |
| S19-40_00193 | aprX | Serine protease AprX |
| S19-40_00233 | albF | Putative zinc protease AlbF |
| S19-40_00239 | clpP | ATP-dependent Clp protease proteolytic subunit |
| S19-40_00247 | - | putative zinc protease |
| S19-40_00262 | rasP | Regulator of sigma-W protease RasP |
| S19-40_00302 | clpY | ATP-dependent protease ATPase subunit ClpY |
| S19-40_00303 | clpQ | ATP-dependent protease subunit ClpQ |
| S19-40_00414 | lon | Lon protease |
| S19-40_00475 | - | Thermophilic metalloprotease (M29) |
| S19-40_00555 | clpE | ATP-dependent Clp protease ATP-binding subunit ClpE |
| S19-40_00578 | htpX | Protease HtpX |
| S19-40_00609 | isp | Major intracellular serine protease |
| S19-40_00640 | htrA | Serine protease Do-like HtrA |
| S19-40_00669 | rip2 | Putative zinc metalloprotease Rip2 |
| S19-40_00680 | albF | Putative zinc protease AlbF |
| S19-40_00908 | ctpB | Carboxy-terminal processing protease CtpB |
| S19-40_00980 | clpP | ATP-dependent Clp protease proteolytic subunit |
| S19-40_01141 | htrB | Serine protease Do-like HtrB |
| S19-40_01229 | paiB | Protease synthase and sporulation protein PAI 2 |
| S19-40_01403 | lasA | LasA protease |
| S19-40_01404 | lasA | LasA protease |
| S19-40_01415 | yraA | Putative cysteine protease YraA |

| | | |
|---|---|---|
| S19-40_01448 | ydcP | putative protease YdcP |
| S19-40_01449 | ydcP | putative protease YdcP |
| S19-40_01512 | - | Cysteine protease Prp |
| S19-40_01538 | lon1 | Lon protease 1 |
| S19-40_01539 | lon2 | Lon protease 2 |
| S19-40_01540 | clpX | ATP-dependent Clp protease ATP-binding subunit ClpX |
| S19-40_01953 | gluP | Rhomboid protease GluP |
| S19-40_02198 | mpr | Extracellular metalloprotease |
| S19-40_02280 | - | Putative phage serine protease XkdF |
| S19-40_02300 | ftsH | ATP-dependent zinc metalloprotease FtsH |
| S19-40_02446 | nprB | Neutral protease B |
| S19-40_02479 | wprA | Cell wall-associated protease |
| S19-40_02527 | htpX | Protease HtpX |
| S19-40_02732 | bepA | Beta-barrel assembly-enhancing protease |
| S19-40_02769 | prsW | Protease PrsW |
| S19-40_02776 | - | CAAX protease self-immunity |
| S19-40_02969 | gluP | Rhomboid protease GluP |
| S19-40_03037 | gpr | Germination protease |
| S19-40_03091 | tsaD | Glycoprotease family protein |
| S19-40_03093 | tsaD | Glycoprotease family protein |
| S19-40_03100 | - | CAAX protease self-immunity |
| S19-40_03218 | yccA | modulator of FtsH protease |
| S19-40_03275 | pfpL | Intracellular protease, PfpI family |
| S19-40_03413 | htpX | Protease HtpX |
| S19-40_03455 | epr | Minor extracellular protease Epr |
| S19-40_03486 | vpr | Minor extracellular protease vpr |
| S19-40_03605 | ctpA | Carboxy-terminal processing protease CtpA |
| S19-40_03706 | htrA | Serine protease Do-like HtrA |
| S19-40_03756 | - | CAAX protease self-immunity |
| S19-40_03937 | clpX | ATP-dependent Clp protease ATP-binding subunit ClpX |
| S19-40_03955 | ftsH | ATP-dependent zinc metalloprotease FtsH |
| S19-40_03982 | yabG | Sporulation-specific protease YabG |

**Table s6.** *Bacillus subtilis* PTA-271 encoding genes for lactonases, β-lactamases, deaminases, deacetylases.

| Locus tag ID | Gene | Function |
|---|---|---|
| *Sporolation related genes* | | |
| S19-40_00078 | *yjcA* | Sporulation protein YjcA |
| S19-40_00117 | *ynzD* | Spo0E like sporulation regulatory protein |
| S19-40_00119 | *sirA* | Sporulation inhibitor of replication protein SirA |
| S19-40_00176 | *spoVK* | Stage V sporulation protein K |
| S19-40_00177 | *cwlC* | Sporulation-specific N-acetylmuramoyl-L-alanine amidase |
| S19-40_00222 | *spoVS* | Stage V sporulation protein S |
| S19-40_00246 | *ylmC* | Sporulation protein, YlmC/YmxH family |
| S19-40_00387 | *spoIIGA* | Sporulation sigma-E factor-processing peptidase |
| S19-40_00401 | *spoVD* | Stage V sporulation protein D |
| S19-40_00416 | *ylbJ* | Sporulation integral membrane protein YlbJ |
| S19-40_00471 | *kinA* | Sporulation kinase A |
| S19-40_00495 | - | Sporulation protein cse15 |
| S19-40_00522 | *kinA* | Sporulation kinase A |
| S19-40_00539 | *stoA* | Sporulation thiol-disulfide oxidoreductase A |
| S19-40_00540 | *ykvU* | Sporulation protein YkvU |
| S19-40_00541 | *sleB* | Spore cortex-lytic enzyme |
| S19-40_00545 | *ydhD* | Putative sporulation-specific glycosylase YdhD |
| S19-40_00546 | *ykvP* | Spore protein YkvP |
| S19-40_00547 | *ykvP* | Spore protein YkvP |
| S19-40_00559 | *kinD* | Sporulation kinase D |
| S19-40_00561 | *spo0E* | stage 0 sporulation regulatory protein |
| S19-40_00573 | *kinE* | Sporulation kinase E |
| S19-40_00649 | *spoIISA* | Stage II sporulation protein SA |
| S19-40_00650 | *spoIISB* | Stage II sporulation protein SB |
| S19-40_00708 | *spo0F* | Sporulation initiation phosphotransferase F |
| S19-40_00746 | *spoIID* | stage II sporulation protein D |
| S19-40_00766 | *spoIIQ* | Stage II sporulation protein Q |
| S19-40_00780 | *spoIIID* | Stage III sporulation protein D |
| S19-40_00844 | *gerBB* | Spore germination protein B2 |
| S19-40_00845 | *gerBA* | Spore germination protein B1 |
| S19-40_00958 | *whiA* | Sporulation transcription regulator WhiA |
| S19-40_00982 | *cotR* | Putative sporulation hydrolase CotR |
| S19-40_01032 | *ydhD* | Putative sporulation-specific glycosylase YdhD |

| | | |
|---|---|---|
| S19-40_01057 | *sdpC* | Sporulation delaying protein C |
| S19-40_01058 | *sdpB* | Sporulation-delaying protein SdpB |
| S19-40_01059 | *sdpA* | Sporulation-delaying protein SdpA |
| S19-40_01133 | *gerAC* | Spore germination protein A3 |
| S19-40_01134 | *gerAB* | Spore germination protein A2 |
| S19-40_01135 | *gerAA* | Spore germination protein A1 |
| S19-40_01208 | *yunB* | Sporulation protein YunB |
| S19-40_01211 | *YhcN* | Sporulation lipoprotein, YhcN/YlaJ family |
| S19-40_01229 | *paiB* | Protease synthase and sporulation protein PAI 2 |
| S19-40_01261 | *Ytvl* | Sporulation integral membrane protein YtvI |
| S19-40_01297 | *kinE* | Sporulation kinase E |
| S19-40_01298 | *kinE* | Sporulation kinase E |
| S19-40_01483 | *spoVB* | Stage V sporulation protein B |
| S19-40_01500 | *coxA* | Sporulation cortex protein CoxA |
| S19-40_01501 | *safA* | SpoIVD-associated factor A |
| S19-40_01510 | *spo0B* | Sporulation initiation phosphotransferase B |
| S19-40_01514 | *spoIVFB* | Stage IV sporulation protein FB |
| S19-40_01515 | *spoIVFA* | Stage IV sporulation protein FA |
| S19-40_01523 | *spoIIB* | Stage II sporulation protein B |
| S19-40_01529 | *spoVID* | Stage VI sporulation protein D |
| S19-40_01557 | *gerM* | Spore germination protein GerM |
| S19-40_01560 | *gerE* | Spore germination protein GerE |
| S19-40_01642 | *ytrH* | Sporulation membrane protein YtrH |
| S19-40_01643 | *ytrI* | Sporulation membrane protein YtrI |
| S19-40_01727 | - | Sporulation protein cse60 |
| S19-40_01859 | *ydhD* | Putative sporulation-specific glycosylase YdhD |
| S19-40_01951 | *ydcC* | Sporulation protein YdcC |
| S19-40_02076 | *nucB* | Sporulation-specific extracellular nuclease |
| S19-40_02162 | *cwlJ* | Spore cortex-lytic enzyme |
| S19-40_02177 | *spo0A* | Stage 0 sporulation protein A |
| S19-40_02216 | - | Sigma-G-dependent sporulation-specific SASP protein |
| S19-40_02229 | *skfC* | Sporulation-killing factor biosynthesis protein SkfC |
| S19-40_02230 | *skfB* | Sporulation killing factor maturation protein SkfB |

| | | |
|---|---|---|
| S19-40_02231 | *skfA* | Sporulation killing factor |
| S19-40_02296 | *cwlA* | Spore cortex-lytic enzyme |
| S19-40_02371 | *yjcA* | Sporulation protein YjcA |
| S19-40_02372 | *cotV* | Spore coat protein V |
| S19-40_02373 | *cotW* | Spore coat protein W |
| S19-40_02374 | *cotX* | Spore coat protein X |
| S19-40_02375 | *cotY* | Spore coat protein Y |
| S19-40_02376 | *cotZ* | Spore coat protein Z |
| S19-40_02450 | *sdpA* | Sporulation-delaying protein SdpA |
| S19-40_02451 | *sdpB* | Sporulation-delaying protein SdpB |
| S19-40_02452 | *sdpC* | Sporulation delaying protein C |
| S19-40_02483 | *yisI* | Spo0E like sporulation regulatory protein |
| S19-40_02484 | *gerPA* | Spore germination protein gerPA/gerPF |
| S19-40_02485 | *gerPB* | Spore germination GerPB |
| S19-40_02486 | *gerPC* | Spore germination protein GerPC |
| S19-40_02487 | *gerPD* | Spore germination protein GerPD |
| S19-40_02488 | *gerPE* | Spore germination protein GerPE |
| S19-40_02489 | *gerPF* | Spore germination protein gerPA/gerPF |
| S19-40_02559 | *yhaL* | Sporulation protein YhaL |
| S19-40_02674 | *spo0M* | Sporulation-control protein spo0M |
| S19-40_02725 | *YpjB* | Sporulation protein YpjB |
| S19-40_02753 | *spoIVA* | Stage IV sporulation protein A |
| S19-40_02767 | *ypeB* | Sporulation protein YpeB |
| S19-40_02768 | *sleB* | Spore cortex-lytic enzyme |
| S19-40_02812 | *spoVAF* | stage V sporulation protein AF |
| S19-40_02813 | - | stage V sporulation protein AE |
| S19-40_02814 | - | stage V sporulation protein AE |
| S19-40_02815 | *spoVAD* | stage V sporulation protein AD |
| S19-40_02816 | *spoVAC* | stage V sporulation protein AC |
| S19-40_02817 | *spoVAB* | stage V sporulation protein AB |
| S19-40_02818 | *spoVAA* | stage V sporulation protein AA |
| S19-40_02820 | *spoIIAB* | stage II sporulation protein AB (anti-sigma F factor) |
| S19-40_02821 | *spoIIAA* | stage II sporulation protein AA (anti-sigma F factor antagonist) |

| | | |
|---|---|---|
| S19-40_02828 | *spoIIM* | Stage II sporulation protein M |
| S19-40_02901 | *spo0A* | Stage 0 sporulation protein A |
| S19-40_02902 | *spoIVB* | SpoIVB peptidase |
| S19-40_02915 | *spoIIIAH* | Stage III sporulation protein AH |
| S19-40_02918 | *spoIIIAE* | Stage III sporulation protein AE |
| S19-40_03018 | *yqfD* | Sporulation protein YqfD |
| S19-40_03019 | *yqfC* | Sporulation protein YqfC |
| S19-40_03055 | *cwlH* | Spore cortex-lytic enzyme |
| S19-40_03059 | *nucB* | Sporulation-specific extracellular nuclease |
| S19-40_03771 | *soj* | Sporulation initiation inhibitor protein Soj |
| S19-40_03960 | *spoIIE* | Stage II sporulation protein E |
| S19-40_03969 | *gerD* | Stage V sporulation protein B |
| S19-40_03970 | *spoVT* | Stage V sporulation protein T |
| S19-40_03977 | *spoVG* | Putative septation protein SpoVG |
| S19-40_03982 | *yabG* | Sporulation-specific protease YabG |

| Locus tag ID | Gene | Function |
|---|---|---|
| *P450 mono-oxigenases* | | |
| S19-40_00039 | hpaB | 4-hydroxyphenylacetate 3-monooxygenase |
| S19-40_01036 | luxA | Alkanal monooxygenase alpha chain |
| S19-40_01390 | ncd2/npd | Nitronate monooxygenase |
| S19-40_01651 | moxC | Putative monooxygenase MoxC |
| S19-40_01653 | camP | 2,5-diketocamphane 1,2-monooxygenase |
| S19-40_02002 | mhuD, hmoB | Antibiotic biosynthesis monooxygenase |
| S19-40_02030 | ycnE | Putative monooxygenase YcnE |
| S19-40_02134 | limB | Limonene 1,2-monooxygenase |
| S19-40_02347 | - | Antibiotic biosynthesis monooxygenase |
| S19-40_02506 | otcC | Anhydrotetracycline monooxygenase |
| S19-40_02544 | hmoB | Heme-degrading monooxygenase HmoB |
| S19-40_02664 | ssuD | Alkanesulfonate monooxygenase |
| S19-40_02844 | - | Antibiotic biosynthesis monooxygenase |
| S19-40_03213 | hmoA | Heme-degrading monooxygenase HmoA |
| S19-40_03220 | kmo | Kynurenine 3-monooxygenase |
| S19-40_03485 | luxB | Alkanal monooxygenase beta chain |
| S19-40_03847 | ntaA | Nitrilotriacetate monooxygenase component A |
| *dioxygenases* | | |
| S19-40_00125 | fosB | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_00563 | mtnD | Acireductone dioxygenase |
| S19-40_00645 | mhqA | Putative ring-cleaving dioxygenase MhqA |
| S19-40_00719 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_01175 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_01333 | cdoA | Cysteine dioxygenase |
| S19-40_01883 | mhqO | Putative ring-cleaving dioxygenase MhqO |
| S19-40_02093 | hcaC | 3-phenylpropionate/cinnamic acid dioxygenase ferredoxin subunit |
| S19-40_02313 | hcaD | 3-phenylpropionate/cinnamic acid dioxygenase ferredoxin--NAD(+) reductase compone |
| S19-40_02851 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_02870 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_03214 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_03313 | catE | Catechol-2,3-dioxygenase |
| S19-40_03371 | - | 2OG-Fe dioxygenase |
| S19-40_03457 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |

| | | |
|---|---|---|
| S19-40_03602 | mhqE | Putative ring-cleaving dioxygenase MhqE |
| S19-40_03717 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_03759 | - | Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily protein |
| S19-40_03807 | qdoI | Quercetin 2,3-dioxygenase |
| ***GST-GT-MT related genes*** | | |
| S19-40_00083 | gcvH | Glycine cleavage system H protein |
| S19-40_00328 | faBOT | Malonyl CoA-acyl carrier protein transacylase |
| S19-40_00397 | ftsW | putative peptidoglycan glycosyltransferase FtsW |
| S19-40_00399 | mraY | Phospho-N-acetylmuramoyl-pentapeptide-transferase |
| S19-40_00434 | ftsW | putative peptidoglycan glycosyltransferase FtsW |
| S19-40_00547 | - | Glycosyl transferases group 1 |
| S19-40_00588 | - | Glycosyl transferase family 2 |
| S19-40_00592 | ugtP | Processive diacylglycerol beta-glucosyltransferase |
| S19-40_00642 | - | putative glycosyltransferase |
| S19-40_00852 | - | glyco_rSAM_CFB: glycosyltransferase, GG-Bacteroidales peptide system |
| S19-40_00853 | - | Glycosyl transferase WecB/TagA/CpsF family protein |
| S19-40_00856 | - | Glycosyl transferase family 2 |
| S19-40_00857 | - | CDP-Glycerol:Poly(glycerophosphate) glycerophosphotransferase |
| S19-40_00861 | - | Glycosyl transferase 1 domain A |
| S19-40_00862 | - | Glycosyl transferases group 1 |
| S19-40_00863 | - | Glycosyl transferase family 2 |
| S19-40_00869 | - | Glycosyl transferase family 2 |
| S19-40_00872 | - | Glycosyl transferases group 1 |
| S19-40_00876 | - | Glycosyl transferase family 2 |
| S19-40_00877 | - | Glycosyl transferases group 1 |
| S19-40_00878 | - | Glycosyl transferase family 4 |
| S19-40_01001 | - | Glycosyl transferases group 1 |
| S19-40_01002 | - | Glycosyl transferase family 2 |
| S19-40_01003 | - | Glycosyl transferases group 1 |
| S19-40_01005 | - | Glycosyl transferase family 2 |
| S19-40_01007 | - | Glycosyl transferase family 2 |
| S19-40_01589 | - | Glyoxalase-like domain protein |
| S19-40_01806 | - | Glycosyl transferases group 1 |

| | | |
|---|---|---|
| S19-40_01809 | - | Glycosyl transferases group 1 |
| S19-40_01858 | - | UDP-glucoronosyl and UDP-glucosyl transferase |
| S19-40_01985 | - | Glycosyltransferase like family 2 |
| S19-40_01997 | - | Methyltransferase domain protein |
| S19-40_02321 | - | MGT: glycosyltransferase, MGT family |
| S19-40_02705 | - | Transglycosylase |
| S19-40_02719 | - | Glycosyl transferases group 1 |
| S19-40_02740 | - | Glycosyl transferase family, a/b domain |
| S19-40_02973 | - | Glycosyl transferases group 1 |
| S19-40_03228 | - | Glycosyl transferase family 2 |
| S19-40_03344 | - | Glycosyl transferase family 2 |
| S19-40_03391 | - | Methyltransferase domain protein |
| S19-40_03452 | - | Glycosyl transferase family 8 |
| S19-40_03483 | - | Cell cycle protein |
| S19-40_03497 | - | Glycosyl transferase family 2 |
| S19-40_03504 | - | Glycosyl transferase family 2 |
| S19-40_03586 | - | MGT: glycosyltransferase, MGT family |
| S19-40_03626 | - | Glycosyl transferase family 2 |
| S19-40_03656 | - | Monogalactosyldiacylglycerol (MGDG) synthase |
| S19-40_03859 | - | Glycosyl transferase family, a/b domain |
| ***Other transferases*** | | |
| S19-40_00001 | araP | L-arabinose transport system permease protein AraP |
| S19-40_00002 | araQ | L-arabinose transport system permease protein AraQ |
| S19-40_00053 | ydaF | Putative ribosomal N-acetyltransferase YdaF |
| S19-40_00065 | - | g_glut_trans: gamma-glutamyltransferase |
| S19-40_00083 | - | methylmalonyl-CoA carboxyltransferase 1.3S subunit |
| S19-40_00089 | gtaB | UTP--glucose-1-phosphate uridylyltransferase |
| S19-40_00090 | DPM1 | dolichol-phosphate mannosyltransferase |
| S19-40_00101 | plsY | Glycerol-3-phosphate acyltransferase |
| S19-40_00174 | pat | Putative phenylalanine aminotransferase |
| S19-40_00185 | miaA | tRNA dimethylallyltransferase |
| S19-40_00207 | - | Acyl transferase domain protein |
| S19-40_00208 | - | Acyl transferase domain protein |

| | | |
|---|---|---|
| S19-40_00219 | miaB | miaB-methiolase: tRNA-i(6)A37 thiotransferase enzyme MiaB |
| S19-40_00456 | arnB | UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase |
| S19-40_00588 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_00642 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_00842 | - | MFS transporter, SP family, arabinose:H+ symporter |
| S19-40_00856 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_00869 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_00876 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_01002 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_01005 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_01007 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_01011 | arnB | UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase |
| S19-40_01037 | araR | Arabinose metabolism transcriptional repressor |
| S19-40_01038 | araE | Arabinose-proton symporter |
| S19-40_01182 | araP | L-arabinose transport system permease protein AraP |
| S19-40_01183 | araQ | L-arabinose transport system permease protein AraQ |
| S19-40_01592 | araQ | L-arabinose transport system permease protein AraQ |
| S19-40_01593 | araP | L-arabinose transport system permease protein AraP |
| S19-40_01599 | araA | L-arabinose isomerase |
| S19-40_01735 | araQ | L-arabinose transport system permease protein AraQ |
| S19-40_01999 | mtlR | mannitol operon transcriptional antiterminator |
| S19-40_02017 | mtlD | Mannitol-1-phosphate 5-dehydrogenase |
| S19-40_02018 | mtlF | Mannitol-specific phosphotransferase enzyme IIA component |
| S19-40_02019 | mtlA | PTS system mannitol-specific EIICB component |
| S19-40_02074 | kdsD, kpsF | arabinose-5-phosphate isomerase |
| S19-40_02473 | araC | Arabinose operon regulatory protein |
| S19-40_02501 | arnB | UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase |
| S19-40_02720 | mshB | thiol_BshB1: bacillithiol biosynthesis deacetylase BshB1 |
| S19-40_02770 | ypdA | Bthiol_YpdA: putative bacillithiol system oxidoreductase, YpdA family |
| S19-40_03197 | araQ | L-arabinose transport system permease protein AraQ |
| S19-40_03210 | araQ | L-arabinose transport system permease protein AraQ |
| S19-40_03228 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_03323 | yfiT | DinB superfamily protein |

| | | |
|---|---|---|
| S19-40_03344 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_03497 | arnC | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase |
| S19-40_03506 | arnB | UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase |
| S19-40_03591 | BshB2 | thiol_BshB2: bacillithiol biosynthesis deacetylase BshB2 |
| S19-40_03695 | mshD | Mycothiol acetyltransferase |
| S19-40_03700 | rocD | Ornithine aminotransferase |
| S19-40_03723 | arnT, pmrK | 4-amino-4-deoxy-L-arabinose transferase |
| S19-40_03723 | - | Dolichyl-phosphate-mannose-protein mannosyltransferase |
| S19-40_03744 | - | Acetyltransferase |
| S19-40_03746 | wecD | dTDP-fucosamine acetyltransferase |
| S19-40_03758 | maa | Maltose O-acetyltransferase |
| S19-40_03774 | rsmG | Ribosomal RNA small subunit methyltransferase G |
| S19-40_03791 | - | phosphotransferase system, EIIB |
| S19-40_03848 | ytmI | putative N-acetyltransferase YtmI |
| S19-40_03859 | - | Glycosyl transferase family, a/b domain |
| S19-40_03911 | cysN | Sulfate adenylyltransferase subunit 1 |
| S19-40_03918 | rlmG | Ribosomal RNA large subunit methyltransferase G |
| S19-40_03927 | - | Putative TrmH family tRNA/rRNA methyltransferase |
| S19-40_03930 | cysE | Serine acetyltransferase |
| S19-40_03933 | ispD | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase |
| S19-40_03948 | - | ilvE_I: branched-chain amino acid aminotransferase |
| | | glmU: UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate |
| S19-40_03976 | glmU | N-acetyltransferase |
| S19-40_03983 | rsmA | Ribosomal RNA small subunit methyltransferase A |
| S19-40_03989 | rsmI | Ribosomal RNA small subunit methyltransferase I |
| S19-40_03991 | yfiC | tRNA1(Val) (adenine(37)-N6)-methyltransferase |
| S19-40_03998 | glyA | Serine hydroxymethyltransferase |

| Locus tag ID | Gene | Function |
|---|---|---|
| *lactone hydrolase* | | |
| S19-40_01709 | ahlD, aiiA, attM, blcC | N-acyl homoserine lactone hydrolase |
| S19-40_02014 | - | Dienelactone hydrolase family protein |
| S19-40_02855 | ahlD, aiiA, attM, blcC | N-acyl homoserine lactone hydrolase |
| S19-40_03601 | - | Dienelactone hydrolase family protein |
| β-*lactamases* | | |
| S19-40_00019 | penP | Beta-lactamase |
| S19-40_00225 | - | Beta-lactamase |
| S19-40_00240 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_00467 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_00991 | - | Beta-lactamase |
| S19-40_01661 | - | Beta-lactamase superfamily domain protein |
| S19-40_01709 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_02214 | ybxI | putative beta-lactamase YbxI |
| S19-40_02259 | - | Beta-lactamase |
| S19-40_02532 | - | Beta-lactamase superfamily domain protein |
| S19-40_02855 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_02861 | - | Beta-lactamase superfamily domain protein |
| S19-40_02961 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_03257 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_03550 | bla2, blm, ccrA, blaB | metallo-beta-lactamase class B |
| S19-40_03707 | - | Metallo-beta-lactamase superfamily protein |
| S19-40_03742 | bla2, blm, ccrA, blaB | metallo-beta-lactamase class B |
| *deaminases* | | |
| S19-40_00468 | adeC | Adenine deaminase |
| S19-40_00611 | guaD | Guanine deaminase |
| S19-40_00930 | nagB | Glucosamine-6-phosphate deaminase 1 |
| S19-40_01533 | hemC | Porphobilinogen deaminase |
| S19-40_01675 | mtaD | 5-methylthioadenosine/S-adenosylhomocysteine deaminase |
| S19-40_02187 | nagB | Glucosamine-6-phosphate deaminase 1 |
| S19-40_02802 | tadA | tRNA-specific adenosine deaminase |
| S19-40_03012 | cdd | Cytidine deaminase |

| | | |
|---|---|---|
| S19-40_03042 | tadA | tRNA-specific adenosine deaminase |
| S19-40_03150 | yerA | Putative adenine deaminase YerA |
| S19-40_03978 | yabJ | 2-iminobutanoate/2-iminopropanoate deaminase |
| S19-40_04015 | tadA | tRNA-specific adenosine deaminase |
| ***deacetylases*** | | |
| S19-40_00248 | pdaA | Peptidoglycan-N-acetylmuramic acid deacetylase PdaA |
| S19-40_00383 | argE | Acetylornithine deacetylase |
| S19-40_00501 | ykuR | N-acetyldiaminopimelate deacetylase |
| S19-40_00931 | nagA | N-acetylglucosamine-6-phosphate deacetylase |
| S19-40_01649 | - | N-acetyldiaminopimelate deacetylase |
| S19-40_01692 | - | Histone deacetylase domain protein |
| S19-40_02104 | cah | Cephalosporin-C deacetylase |
| S19-40_02121 | - | N-acetyldiaminopimelate deacetylase |
| S19-40_02333 | pdaC | Peptidoglycan-N-acetylmuramic acid deacetylase PdaC |
| S19-40_02535 | lysK | N-acetyl-lysine deacetylase |
| S19-40_02547 | - | N-acetyldiaminopimelate deacetylase |
| S19-40_02585 | pgdA | Peptidoglycan-N-acetylglucosamine deacetylase |
| S19-40_02586 | cobB | NAD-dependent protein deacetylase |
| S19-40_02720 | bshB1 | N-acetyl-alpha-D-glucosaminyl L-malate deacetylase 1 |
| S19-40_02857 | argE | acetylornithine deacetylase |
| S19-40_03287 | pdaA | Peptidoglycan-N-acetylmuramic acid deacetylase PdaA |
| S19-40_03411 | icaB | Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase |
| S19-40_03591 | bshB2 | putative N-acetyl-alpha-D-glucosaminyl L-malate deacetylase 2 |
| S19-40_03620 | argE | Acetylornithine deacetylase |
| S19-40_03852 | - | N-acetyldiaminopimelate deacetylase |
| S19-40_03867 | pdaA | Peptidoglycan-N-acetylmuramic acid deacetylase PdaA |
| ***other deacetylases*** | | |
| S19-40_02720 | - | GlcNAc-PI de-N-acetylase |
| S19-40_03287 | - | spore_pdaA: delta-lactam-biosynthetic de-N-acetylase |
| S19-40_03591 | - | GlcNAc-PI de-N-acetylase |
| S19-40_02720 | - | GlcNAc-PI de-N-acetylase |
| S19-40_03287 | - | spore_pdaA: delta-lactam-biosynthetic de-N-acetylase |
| S19-40_03591 | - | GlcNAc-PI de-N-acetylase |

| | | |
|---|---|---|
| S19-40_03824 | mmsA, iolA, ALDH6A1 | malonate-semialdehyde dehydrogenase (acetylating) / methylmalonate-semialdehyde dehydrogenase |
| ***other (de)acylases*** | | |
| S19-40_00208 | faBOT | Malonyl CoA-acyl carrier protein transacylase |
| S19-40_00210 | pksC | Polyketide biosynthesis malonyl CoA-acyl carrier protein transacylase PksC |
| S19-40_00328 | faBOT | Malonyl CoA-acyl carrier protein transacylase |
| S19-40_01475 | dtd | D-aminoacyl-tRNA deacylase |
| S19-40_02338 | ybaK | Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase YbaK |
| S19-40_02881 | DBT, bkdB | 2-oxoisovalerate dehydrogenase E2 component (dihydrolipoyl transacylase |
| S19-40_03846 | - | Penicillin acylase |

**Table s7.** *Bacillus subtilis* PTA-271 encoding genes for PKS and other acetyltransferases.

| Locus tag ID | Gene | Function |
|---|---|---|
| *Polyketide related genes* | | |
| S19-40_00196 | pksS | Polyketide biosynthesis cytochrome P450 PksS |
| S19-40_00197 | pksR | Polyketide synthase PksR |
| S19-40_00198 | pksN | Polyketide synthase PksN |
| S19-40_00199 | pksM | Polyketide synthase PksM |
| S19-40_00200 | pksL | Polyketide synthase PksL |
| S19-40_00201 | pksJ | Polyketide synthase PksJ |
| S19-40_00202 | pksI | Putative polyketide biosynthesis enoyl-CoA isomerase PksI |
| S19-40_00203 | pksH | putative polyketide biosynthesis enoyl-CoA hydratase PksH |
| S19-40_00204 | pksG | Polyketide biosynthesis 3-hydroxy-3-methylglutaryl-ACP synthase PksG |
| S19-40_00205 | pksF | Polyketide biosynthesis malonyl-ACP decarboxylase PksF |
| S19-40_00206 | acpK | Polyketide biosynthesis acyl-carrier-protein AcpK |
| S19-40_00207 | pksE | Polyketide biosynthesis protein PksE |
| S19-40_00208 | pksD | Polyketide biosynthesis acyltransferase PksD |
| S19-40_00210 | pksC | Polyketide biosynthesis malonyl CoA-acyl carrier protein transacylase PksC |
| S19-40_00211 | pksB | putative polyketide biosynthesis zinc-dependent hydrolase Pl |
| S19-40_01385 | pksS | Polyketide biosynthesis cytochrome P450 PksS |
| *Acyl transferase related genes* | | |
| S19-40_00207 | pfaD | Acyl transferase domain protein |
| S19-40_00208 | faBOT | Malonyl CoA-acyl carrier protein transacylase |
| S19-40_00210 | faBOT | Acyl transferase domain protein |
| S19-40_00210 | pksC | Polyketide biosynthesis malonyl CoA-acyl carrier protein transacylase PksC |
| S19-40_00328 | faBOT | Malonyl CoA-acyl carrier protein transacylase |
| S19-40_00328 | faBOT | Malonyl CoA-acyl carrier protein transacylase |
| S19-40_02881 | DBT, bkdB | 2-oxoisovalerate dehydrogenase E2 component (dihydrolipoyl transacylase) |
| S19-40_03846 | - | Penicillin acylase |
| *Keto related genes* | | |
| S19-40_00120 | tkt | Transketolase |
| S19-40_00205 | fabF | beta-ketoacyl-acyl-carrier-protein synthase II |
| S19-40_00220 | - | 8-amino-7-oxononanoate synthase/2-amino-3-ketobutyrate |

| | | |
|---|---|---|
| | | coenzyme A ligase |
| S19-40_00407 | apbA | Ketopantoate reductase PanE/ApbA |
| S19-40_00460 | dxs | Transketolase, pyrimidine binding domain |
| S19-40_00476 | apbA_panE: 2-dehydropantoate 2-reductase | Ketopantoate reductase PanE/ApbA |
| S19-40_00565 | mtnX | 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate phosph |
| S19-40_00566 | mtnW | 2,3-diketo-5-methylthiopentyl-1-phosphate enolase |
| S19-40_00966 | ghrB | glyoxylate/hydroxypyruvate/2-ketogluconate reductase |
| S19-40_01097 | - | Aldo/keto reductase family protein |
| S19-40_01158 | fadA | 3-ketoacyl-CoA thiolase |
| S19-40_01396 | - | Aldo/keto reductase family protein |
| S19-40_01493 | csbX | Alpha-ketoglutarate permease |
| S19-40_01547 | ilvC | Ketol-acid reductoisomerase (NADP(+)) |
| S19-40_01623 | - | Aldo/keto reductase family protein |
| S19-40_01653 | camP | 2,5-diketocamphane 1,2-monooxygenase |
| S19-40_01995 | ydaE | putative D-lyxose ketol-isomerase |
| S19-40_02000 | - | Aldo/keto reductase family protein |
| S19-40_02005 | pcaR_pcaU | beta-ketoadipate pathway transcriptional regulators, PcaR/PcaU/PobR family |
| S19-40_02073 | ulaD | 3-keto-L-gulonate-6-phosphate decarboxylase UlaD |
| S19-40_02145 | - | Aldo/keto reductase family protein |
| S19-40_02175 | xylA | alpha-ketoglutaric semialdehyde dehydrogenase |
| S19-40_02310 | dlgD | 2,3-diketo-L-gulonate reductase |
| S19-40_02418 | fabF | fabF: beta-ketoacyl-acyl-carrier-protein synthase II |
| S19-40_02521 | fadA | 3-ketoacyl-CoA thiolase |
| S19-40_02597 | - | Aldo/keto reductase family protein |
| S19-40_02838 | - | Aldo/keto reductase family protein |
| S19-40_02882 | - | Transketolase, pyrimidine binding domain |
| S19-40_02895 | fadA | 3-ketoacyl-CoA thiolase |
| S19-40_03297 | dxs | Transketolase, pyrimidine binding domain |
| S19-40_03393 | scoA | putative succinyl-CoA:3-ketoacid coenzyme A transferase subunit A |
| S19-40_03394 | scoB | putative succinyl-CoA:3-ketoacid coenzyme |

| | | |
|---|---|---|
| | | A transferase subunit B |
| | | putative succinyl-CoA:3-ketoacid coenzyme |
| S19-40_03621 | scoB | A transferase subunit B |
| S19-40_03674 | kdgT | 2-keto-3-deoxygluconate permease |
| S19-40_03678 | kduI | 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase |
| S19-40_03822 | - | Aldo/keto reductase family protein |
| S19-40_03832 | iolI | 2-keto-myo-inositol isomerase |
| ***Dehydratase related genes*** | | |
| S19-40_00109 | leuC | 3-isopropylmalate dehydratase large subunit |
| S19-40_00332 | sdhA | L-serine dehydratase, alpha chain |
| S19-40_00333 | sdhB | L-serine dehydratase, beta chain |
| S19-40_00564 | mtnB | Methylthioribulose-1-phosphate dehydratase |
| S19-40_00785 | fabZ | 3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ |
| S19-40_00942 | hisB | Imidazoleglycerol-phosphate dehydratase |
| S19-40_01000 | pglF | UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase |
| S19-40_01470 | tcdA | tRNA threonylcarbamoyladenosine dehydratase |
| S19-40_01507 | - | Prephenate dehydratase |
| S19-40_01531 | hemB | Delta-aminolevulinic acid dehydratase |
| S19-40_01543 | leuD1 | 3-isopropylmalate dehydratase small subunit 1 |
| S19-40_01544 | leuC | 3-isopropylmalate dehydratase large subunit |
| S19-40_01736 | - | NAD dependent epimerase/dehydratase family protein |
| S19-40_01805 | - | NAD dependent epimerase/dehydratase |
| S19-40_02013 | fabZ | 3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ |
| S19-40_02171 | garD | Galactarate dehydratase (L-threo-forming) |
| S19-40_02173 | gudD | Glucarate dehydratase |
| S19-40_02176 | - | putative 5-dehydro-4-deoxyglucarate dehydratase |
| S19-40_02303 | uxaA | Altronate dehydratase |
| S19-40_02308 | uxuA | Mannonate dehydratase |
| S19-40_02782 | aroD | 3-dehydroquinate dehydratase |
| S19-40_02853 | dsdA | D-serine dehydratase |
| S19-40_02891 | prpD | 2-methylcitrate dehydratase |
| S19-40_02926 | yqhS | 3-dehydroquinate dehydratase |
| S19-40_03226 | rfbG | CDP-glucose 4,6-dehydratase |

| | | |
|---|---|---|
| S19-40_03334 | - | NAD dependent epimerase/dehydratase family protein |
| S19-40_03405 | - | GDP-mannose 4,6 dehydratase |
| S19-40_03419 | nnrD | ADP-dependent (S)-NAD(P)H-hydrate dehydratase |
| S19-40_03512 | rfbB | dTDP-glucose 4,6-dehydratase |
| S19-40_03631 | pseB | UDP-N-acetylglucosamine 4,6-dehydratase (inverting) |
| S19-40_03641 | ilvA | L-threonine dehydratase biosynthetic IlvA |
| S19-40_03651 | ilvD | Dihydroxy-acid dehydratase |
| S19-40_03828 | iolE | Inososе dehydratase |
| S19-40_03831 | iolE | Inososе dehydratase |
| ***Enoyl reductase related genes*** | | |
| S19-40_00232 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_00327 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_00514 | fadH | putative 2,4-dienoyl-CoA reductase |
| S19-40_00548 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_01324 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_01390 | - | putative enoyl-[acyl-carrier-protein] reductase II |
| S19-40_01662 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_01996 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02024 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02139 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02346 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02378 | fabI | Enoyl-[acyl-carrier-protein] reductase [NADH] FabI |
| S19-40_02513 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02516 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02605 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_02899 | fadH | 2,4-dienoyl-CoA reductase (NADPH2) |
| S19-40_03349 | fabL | Enoyl-[acyl-carrier-protein] reductase [NADPH] FabL |
| S19-40_03372 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_03395 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_03523 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_03527 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_03679 | - | Enoyl-(Acyl carrier protein) reductase |
| S19-40_03816 | - | Enoyl-(Acyl carrier protein) reductase |

| *Other acetyltransferase related genes* | | |
|---|---|---|
| S19-40_00053 | ydaF | Putative ribosomal N-acetyltransferase YdaF |
| S19-40_00166 | speG | Spermidine N(1)-acetyltransferase |
| S19-40_00220 | kbl, GCAT | glycine C-acetyltransferase |
| S19-40_00408 | ylbP | putative N-acetyltransferase YlbP |
| | | Dihydrolipoyllysine-residue acetyltransferase |
| S19-40_00459 | pdhC | component of pyruvate dehydrogenase complex |
| S19-40_00502 | dapH | 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransfe |
| S19-40_00527 | - | Acetyltransferase (GNAT) family protein |
| S19-40_00621 | - | Acetyltransferase (GNAT) domain protein |
| S19-40_00725 | argA | amino-acid N-acetyltransferase |
| S19-40_00765 | ywnH | Putative phosphinothricin acetyltransferase YwnH |
| S19-40_00936 | dapH | 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransfe |
| S19-40_00960 | nat | Arylamine N-acetyltransferase |
| S19-40_01010 | epsM | Putative acetyltransferase EpsM |
| S19-40_01045 | yvbK | putative N-acetyltransferase YvbK |
| S19-40_01228 | paiA | Spermidine/spermine N(1)-acetyltransferase |
| S19-40_01347 | - | Acetyltransferase (GNAT) family protein |
| S19-40_01371 | bltD | Spermine/spermidine acetyltransferase |
| S19-40_01402 | mdpB3 | acetyltransferase/esterase |
| S19-40_01427 | oatA | O-acetyltransferase OatA |
| S19-40_01551 | ysnE | putative N-acetyltransferase YsnE |
| S19-40_01659 | ytmI | putative N-acetyltransferase YtmI |
| S19-40_01771 | cysE | Serine acetyltransferase |
| S19-40_01853 | ypeA | Acetyltransferase YpeA |
| S19-40_01870 | yycN | putative N-acetyltransferase YycN |
| S19-40_01898 | - | GNAT acetyltransferase |
| S19-40_01930 | ydaF | Putative ribosomal N-acetyltransferase YdaF |
| S19-40_01994 | ydaF | Putative ribosomal N-acetyltransferase YdaF |
| S19-40_02207 | slyA | rimI: ribosomal-protein-alanine acetyltransferase |
| S19-40_02340 | - | Acetyltransferase (GNAT) domain protein |
| S19-40_02357 | ydaF | Putative ribosomal N-acetyltransferase YdaF |
| S19-40_02365 | yjcF | putative N-acetyltransferase YjcF |

| | | |
|---|---|---|
| S19-40_02403 | yjbC | Putative acetyltransferase YjbC |
| S19-40_02431 | argA | Amino-acid acetyltransferase |
| S19-40_02432 | argJ | ArgJ: glutamate N-acetyltransferase/amino-acid acetyltransfe |
| S19-40_02456 | - | Acetyltransferase (GNAT) domain protein |
| S19-40_02457 | - | Acetyltransferase (GNAT) domain protein |
| S19-40_02521 | yhfS | Putative acetyl-CoA C-acetyltransferase YhfS |
| S19-40_02525 | wecD | dTDP-fucosamine acetyltransferase |
| S19-40_02601 | phnO | Aminoalkylphosphonate N-acetyltransferase |
| S19-40_02798 | - | rimI: ribosomal-protein-alanine acetyltransferase |
| S19-40_02843 | mshD | Mycothiol acetyltransferase |
| S19-40_02845 | - | ectoine_EctA: diaminobutyrate acetyltransferase |
| | | Dihydrolipoyllysine-residue acetyltransferase |
| S19-40_02881 | pdhC | component of pyruvate dehydrogenase complex |
| S19-40_02887 | pta | Phosphate acetyltransferase |
| S19-40_02895 | mmgA | Acetyl-CoA acetyltransferase |
| S19-40_03092 | rimI | Ribosomal-protein-alanine acetyltransferase |
| S19-40_03191 | - | Acetyltransferase (GNAT) family protein |
| S19-40_03241 | - | Acetyltransferase (GNAT) family protein |
| | | Dihydrolipoyllysine-residue acetyltransferase |
| S19-40_03298 | pdhC | component of pyruvate dehydrogenase complex |
| S19-40_03441 | patA | Peptidoglycan O-acetyltransferase |
| S19-40_03507 | wecD | dTDP-fucosamine acetyltransferase |
| S19-40_03529 | pta | Phosphate acetyltransferase |
| S19-40_03548 | rimL | rimI: ribosomal-protein-alanine acetyltransferase |
| S19-40_03619 | yodP | N-acetyltransferase YodP |
| S19-40_03625 | rimL | rimI: ribosomal-protein-alanine acetyltransferase |
| S19-40_03695 | yycN | putative N-acetyltransferase YycN |
| S19-40_03740 | yjcF | putative N-acetyltransferase YjcF |
| S19-40_03744 | - | Acetyltransferase |
| S19-40_03746 | wecD | dTDP-fucosamine acetyltransferase |
| S19-40_03758 | maa | Maltose O-acetyltransferase |
| S19-40_03848 | ytmI | putative N-acetyltransferase YtmI |
| S19-40_03930 | cysE | Serine acetyltransferase |

**Table s8.** Anti-SMASH 5.1.0 prediction of gene clusters responsible for secondary metabolite production in *Bacillus subtilis* PTA-271.

| Secondary metabolite | Enzyme | Gene clusters | Compound | Similarity |
|---|---|---|---|---|
| Betalactone-NRPS | NRPS | 1 | Fengycin | 100% |
| NRPS | NRPS | 2 | Bacillibactin | 100% |
| | | | Surfactin | 78% |
| Other | NRPS | 1 | Bacilysin | 100% |
| T3PKS | PKS | 1 | - | 0% |
| TransATPKS-NRPS-PKSlike | PKS/NRPS | 1 | Bacillaene | 100% |
| tRNA-dependent cyclodipeptide synthases | - | 1 | - | 0% |
| Terpene | - | 2 | - | 0% |
| Sactipeptide | RIPP | 2 | Subtilosin A | 100% |
| | | | Sporulation killing factor | 100% |

## Gestion durable des maladies du bois de la vigne au vignoble : Délivrer des agents de biocontrôle et les molécules bénéfiques associées

Les maladies du bois de la vigne (MDB) comptent parmi les principales maladies limitant la productivité des vignobles, causant d'importantes pertes économiques et limitant la durabilité des vignobles. La recherche d'agents de biocontrôle (BCA) efficaces pour prévenir ou minimiser l'impact des MDB est désormais considérée comme une priorité de recherche. L'objectif de cette étude est alors d'investiguer le potentiel biotechnologique de *Bacillus subtilis* PTA-271 et de *Trichoderma atroviride* SC1 contre les agents pathogènes des MDB. Les effets de ces deux BCA ont aussi été étudiés dans la plante et sur son microbiome, dans le but ultime de contribuer plus efficacement à une viticulture durable. Les résultats obtenus ont montré que les BCA testés, ainsi que les pathogènes, induisent plusieurs changements physiologiques dans la plante. Le cépage influence également les effets bénéfiques des BCA. En pépinière, le matériel végétal traité avec *Bs* PTA-271 et *Ta* SC1, a montré une réduction du pourcentage d'infection par les pathogènes responsables des MDB. Les BCA inoculés améliorent non seulement l'état sanitaire des plants de vigne, mais aussi les relations bénéfiques de leur microbiome rhizosphérique. Enfin, le traitement combinant les deux BCAs semble améliorer les effets bénéfiques observés contre les pathogènes MDB, bien que d'autres études soient nécessaires pour valider ces résultats. Les résultats de cette étude fournissent de nouvelles connaissances sur les interactions de la vigne avec les pathogènes et les BCA, et sur le potentiel lié à l'utilisation de plusieurs BCA pour lutter plus efficacement contrer les MDB de la vigne. Cette étude révèle par ailleurs des marqueurs de défense de la vigne et des molécules bénéfiques de BCA contre les pathogènes MDB, et apporte de nouvelles connaissances sur les changements physiologiques induits par les pathogènes et les BCA. Ces résultats apportent une contribution importante aux stratégies de gestion durable des MDB, de la pépinière au vignoble.

**Mots-clés :** *Vitis vinifera* L., gestion durable de la vigne, agents de biocontrôle, *Bacillus subtilis* PTA-271, *Trichoderma atroviride* SC1*,* réponses de défense de la vigne, microbiome rhizosphérique, production de jeunes plants en pépinière.

## Sustainable management of grapevine trunk diseases in vineyard: Deliver biocontrol agents and associated molecules

Grapevine trunk diseases (GTDs) are amongst the main restrictors of vineyard productivity, causing important economic losses and limiting vineyard sustainability. Investigation of sustainable biocontrol agents (BCAs) efficient to forestall or to minimize the impact of GTDs, is now viewed as a research priority. The aim of this study is then to investigate the biotechnological potential of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against GTD pathogens. The effects of these two BCAs were also studied, both in the plant, and its microbiome, with the ultimate goal to contribute more efficiently to sustainable viticulture. Results showed that BCAs, as well as pathogens, induce several plant physiological changes. Grapevine cultivar also influences BCA beneficial effects. In nursery, plant material treated with *Bs* PTA-271 and *Ta* SC1, showed a reduced percentage of infection by GTD pathogens. Inoculated BCAs not only improve the health status of grapevine, but also the beneficial relationships of their rhizosphere microbiome. Finally, the combined treatment with both BCAs seems to improve the beneficial effects observed against GTD pathogens, although more studies are required to validate these results. The outcomes of this study provide new knowledges on the grapevine interactions with pathogens and BCAs, and on the potential of using several BCAs to better control GTDs. This study also reveals grapevine defense markers and BCA beneficial molecules, and provide new knowledges on pathogen- and BCA- induced plant physiological changes. These findings provide a strong contribution to sustainable disease management strategies for GTDs, from nursery to vineyard.

**Keywords:** *Vitis vinifera* L., grapevine sustainable management, biocontrol agents, *Bacillus subtilis* PTA-271, *Trichoderma atroviride* SC1*,* grapevine defense responses, rhizosphere microbiome, grapevine propagation process in nursery.

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