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Antimicrobial Photodynamic treatment as pest management. A study of the effects of photoactivated TPPS and Chlorophyllin on the fungus pathogen *Botrytis cinerea,* and on two plant models: Grapevine (*Vitis vinifera*) and Potato (*Solanum tuberosum*)

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“I say to the young, be happy that you were born in Italy because of the beauty of the human capital, both masculine and feminine, of this country... No other country has such human capital.”

-- Rita Levi-Montalcini

“Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid.”

--Albert Einstein

"One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away.

--Stephen Hawking

Acknowledgments

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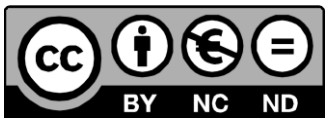
Droits d’auteurs



DEDICATION

A mio nonno, la mia stella polare. . .

A mia madre e mia sorella e alle persone che ci hanno creduto prima che ci credessi io. . .

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APDT : Antimicrobial Photodynamic Treatment

***B. cinerea* :** *Botrytis cinera*

Cat : Catalase

CFU : Colony-forming unit

Chl : Chlorophyllin (E140)

ESEM : Environmental Scanning Electronic Microscopy

GPX : Guaiacol peroxidase activity

H_2O_2 : Hydrogen peroxide

MCF : minimum fungicidal concentration

MDA : Malondialdehyde

MS : Murashige and Skoog

PDA : Potato Dextrose Agar

PDB : Potato Dextrose Broth

PDT : Photodynamic Therapy

PS : Photosensitizer(s)

ROS : Reactive Oxygen Species

SOD : Superoxide dismutase

TPPS : 5,10,15,20-(tetra-4-sulfonatophenyl) porphyrin tetra-ammonium

I. Introduction

Agriculture has always been essential for the survival of the human species, not only for food but also for dressing (cotton and flax fibers) and heating (straw). Furthermore, the farming and agricultural industry still employ a large part of global human population, estimated at 49 % of the world human active population (approximately 1.3 billion people) in 2010 (FAO, 2010; Federico, 2008). As the human population is expected to reach at least 10 billion people around 2100, it will trigger a multitude of social and economic consequences including of course, agriculture activities as reported in Figure 1 (Gerland *et al* 2014; United Nations 2019).

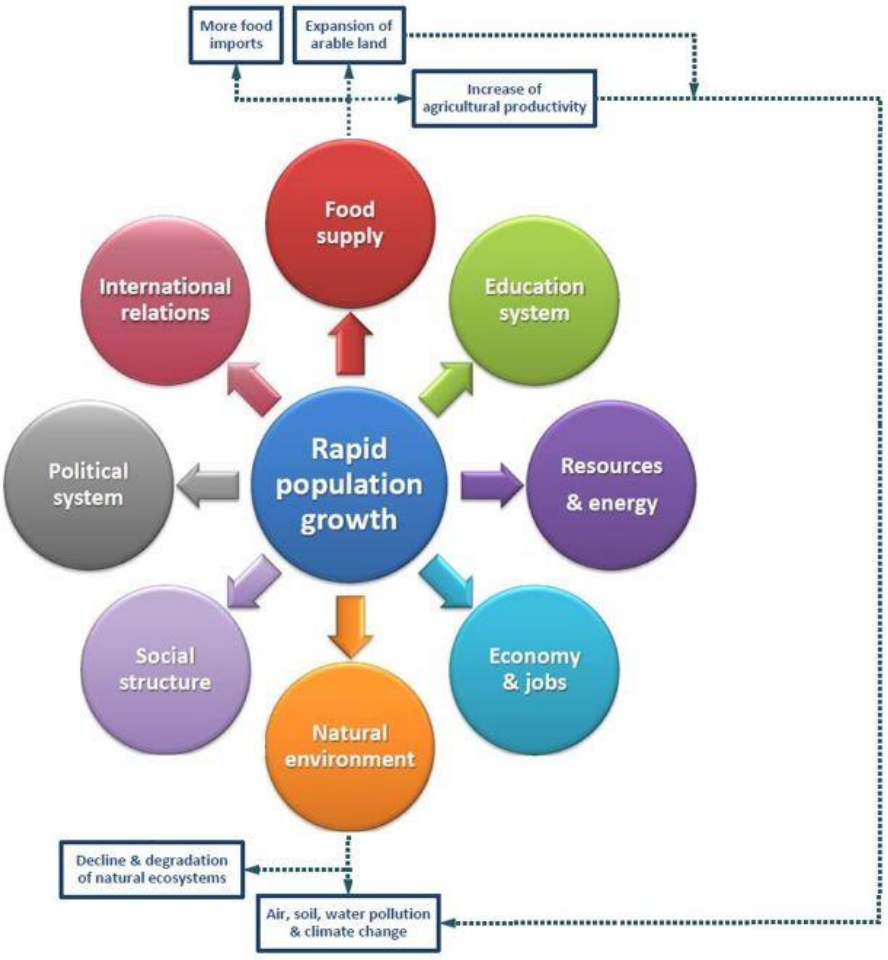

Figure 1: Effects of the rapid population growth.

Sourced and modified from Heilig 2014.

In 2009, the Food and Agriculture Organization of the United Nations (FAO) estimated that the food production must increase by 70 percent between now and 2050, in order to sustain the new population. This is predicted to cost € 75 billion per developed country, on top of the € 130 billion already spent

on culture every year (FAO, 2009). Striving for an improved crop production and for fighting against weeds and pathogens, farmers must use more and more chemical substances called pesticides.

I.1. Eternal struggle between undesirable organisms and crops: the birth of chemical agriculture

Since the beginning of agricultural practises, humans tried to develop the most effective and least time-consuming methods to cultivate their crops and defend them against phytopathogens and weeds (Savary *et al* 2019). The first method used by Sumerians (~4500 years ago) to eradicate the plant's competitors and phytopathogens, was the use of elemental sulfur and sulfides (George and Shukla 2011; Abubakar *et al* 2020). Approximately 3200 years ago, the use of mercury and arsenic ('para-pesticides') was introduced by the Chinese and maintained until the creation of the synthetic pesticides (~1950s). In 1882, the Bordeaux mixture, a mixture of copper (II) sulfate and calcium hydroxide, was developed and largely used in French wine regions to treat various grapevine diseases caused by fungi (Fleurat-Lessard *et al* 2011; George and Shukla 2011; Abubakar *et al* 2020). In the middle of the 20th century, the official development of pesticides occurred. Due to an increase in human demographic and massive rural exodus linked to industrial expansion, both the development of pesticides and farm mechanisation were the solutions to help farmers increase crop yield with less labour (Aktar *et al* 2009). The term "pesticides" refers herbicides, insecticides, bactericides and fungicides, as shown in the figure below (Figure 2).

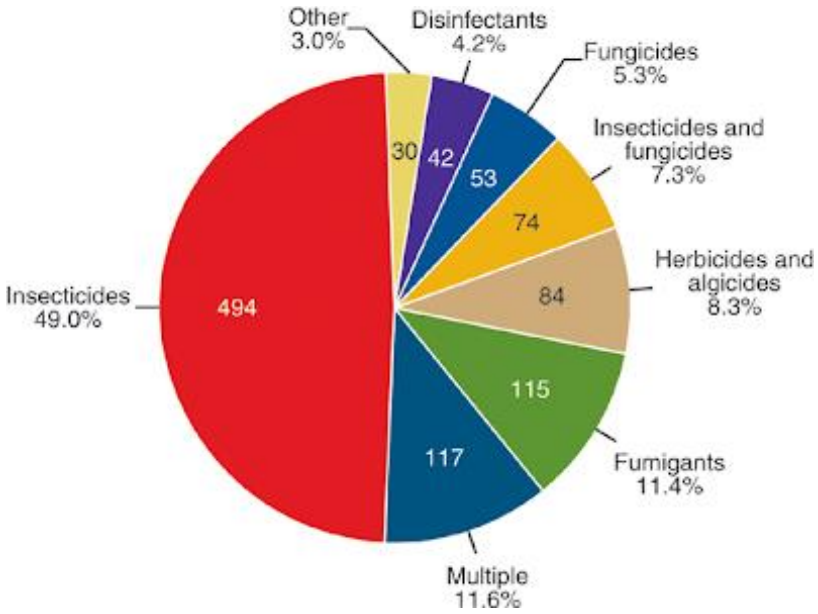

Figure 2: Classification of pesticides.

Sourced from Donaldson et al 2008.

The first modern pesticide, dichlorodiphenyl-trichloroethane (DDT), was synthesised by Paul Muller in 1939 (George and Shukla 2011; Abubakar *et al* 2020). This marked the beginning of the huge utilisation of chemical pesticides around the world, which peaked after the Second World War, when modern synthetic organic chemistry started to grow. This chemical area of research greatly contributed to the increasing quantity and quality of the harvest and to the accomplishment of the green revolution (Smith *et al* 2008).

To increase quality and quantity of crop production, farmers used huge amounts of pesticides that led to harmful effects and shown to be very dangerous for the environment and human health (Dallaire *et al* 2012; Henry *et al* 2012; Tago *et al* 2014; Donley 2019). Moreover, these substances can accumulate in the natural habitats (air, water and soil). Consequently, humans (farmers and rural population) are exposed to pesticides to the detriment of their health. The World Health Organization (WHO) reported 3 million pesticide poisoning cases per year, resulting in over 200,000 deaths in developing countries. In particular, these harmful substances can accumulate in the human body and target specific systems including the epithelial, gastrointestinal, neurological, respiratory, reproductive and endocrinal ones. These compounds could also promote cancers. Finally, the efficacy of pesticides has a limited durability and their extensive use has induced the development of multi-resistant pathogens. Taking into account these multiple dangers, it becomes urgent to largely reduce the use of pesticides (George and Shukla 2011; Mahmood *et al* 2016; Nicopoulou-Stamati *et al* 2016).

Currently, the status of the problem caused by pesticides can be determined by analysing the methods of their usage in four of the largest agricultural producers in the world: United States of America (USA), European Union (EU), Brazil and China. Generally, governments have different rules and regulatory agencies that govern the banning and/or elimination of these harmful substances. However, this analysis can provide a glimpse into the global effectiveness of the pesticide regulatory laws (Donley 2019). Among the top four largest agricultural producers, USA still uses approximately 100 pesticides in quantities of millions of kilos per year that are prohibited in the other three producers (Figure 3).

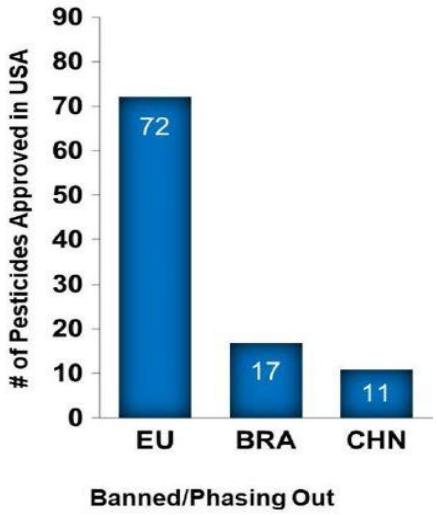

Figure 3: Number of pesticides banned in Europe, Brazil and China.

The numbers represent the amount of pesticides that are approved for use in the USA but are banned or being phased out in the Europe (EU) (72 pesticides), Brazil (BRA) (17 pesticides) and China (CHN) (11 pesticides). Sourced and modified from Donley 2019.

The gap between EU and the other large producers is very impressive. Furthermore, in the EU, there are 446 legal pesticide ingredients commercially available for use according to the European Commission (European Commission, 2019; Nagi *et al.* 2020). The main aim of the European commission is to reduce the risks and impact of pesticide use on human health and the environment. Keeping this aim in mind, in 2007, the French government introduced the "plan Ecophyto" with the objective of reducing the sale and use of pesticides by 50% over the next 10 years. Two years after, on the 21st of October 2009, the European parliament and council introduced a directive 2009/128/EC with the same objective. In 2015, it became apparent to the French government that this task was unrealistic and not achieved until 2018 (Figure 4).

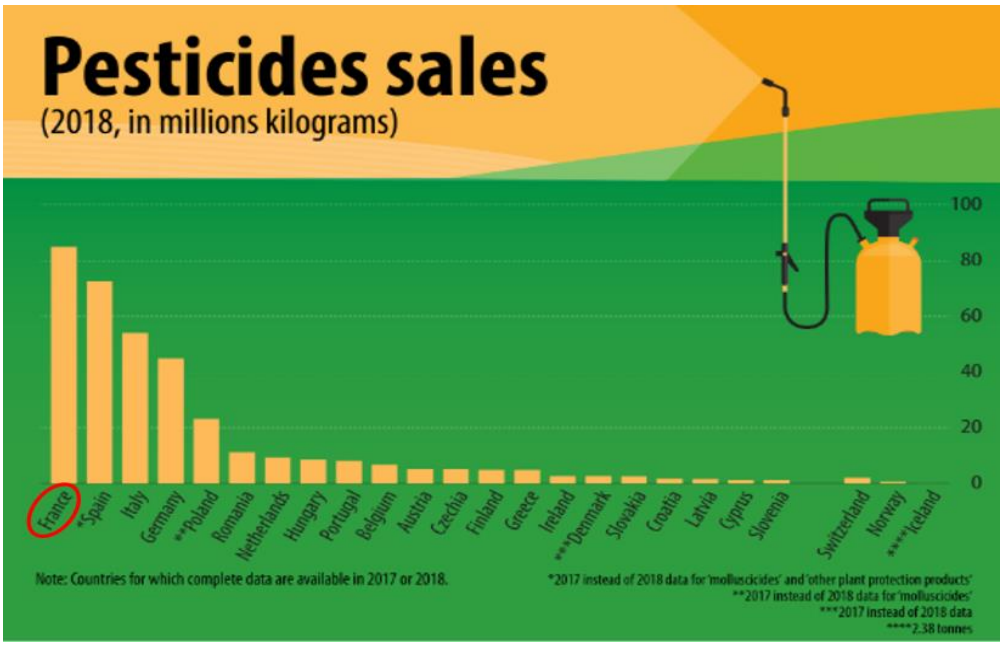

Figure 4: The sales of pesticides in European countries.

Red circle showing the France as the leader in pesticide sales in the EU. Sourced and modified from Agri-environmental indicator - consumption of pesticides, Eurostat 2018.

Therefore, the French government decided to update the plan and name it as “plan Ecophyto II.” In this new plan, decrease in the use, the risks and the impacts of agrochemical products was encouraged. This plan also highlights that the existing alternative solutions are not sufficiently set up.

I.1.1. Fungicides - the biggest sell-out pesticide category in the EU

In Europe, three other countries Spain, Italy and Germany are also great crop producers (Figure 4). Taking together, they represent over two thirds of the total EU pesticide sales (in weight), both in 2011 and 2018. When taking a closer look at the pesticide markets in the EU, the group 'Fungicides and bactericides' is the most important and sold group of pesticides (Figure 5).

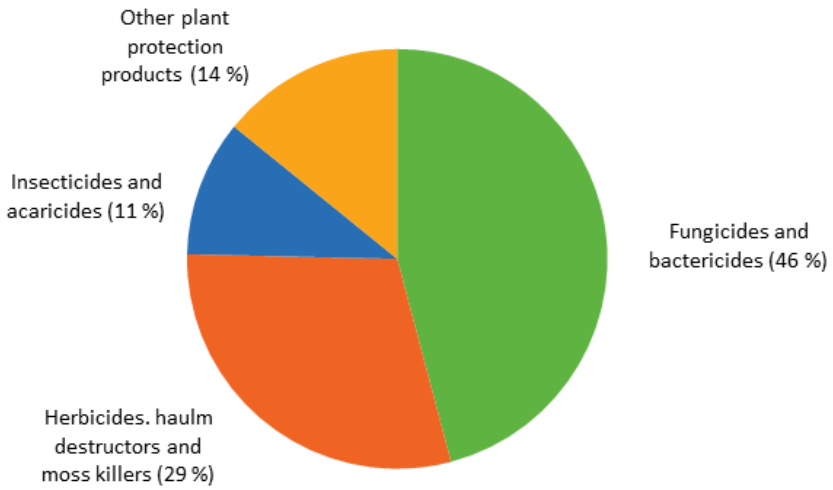

Figure 5: Percentage of total mass in kilograms of different pesticides in 2016 in the EU.

Sourced from Eurostat 2016.

The use of chemical fungicides must decrease for multiple reasons: human health, ecological and environmental major concerns (George and Shukla 2011; Mahmood *et al* 2016; Nicopoulou-Stamati *et al* 2016). In this context, it was envisaged to develop the antimicrobial photodynamic treatment (APDT) as an additive option in agriculture.

Since the PEIRENE laboratory has a long-term expertise in photodynamic treatment based on the use of a photosensitizer, it was decided to gain insight into this challenge on the agriculture side. The choice was made to work with the fungal phytopathogen, *B. cinerea* and two of its numerous hosts: grapevine, one of its major targets and potato, a minor target.

I.2. *Botrytis cinerea*

A list of the top 10 most important and dangerous plant pathogenic fungi in the scientific and economic fields was established by the community of fungal pathologists associated with the journal Molecular Plant Pathology (Table 1).

Rank	Fungal pathogen
1	*Magnaporthe oryzae*
2	*Botrytis cinerea*
3	*Puccinia* spp.
4	*Fusarium graminearum*
5	*Fusarium oxysporum*
6	*Blumeria graminis*
7	*Mycosphaerella graminicola*
8	*Colletotrichum* spp.
9	*Ustilago maydis*
10	*Melampsora lini*

Table 1: List of the major fungi impacting crop yield.

Sourced and modified from Dean et al 2012.

Magnaporthe oryzae is the first of the top ten because its target is the rice that is the most cultivated crop in the world (Khush 2005; Fernandez and Orth 2018). *B. cinerea* is ranked in second place because it induces the grey mold or rot that affects plants and their fruits. Furthermore, this fungus infects more than 200 crops, including wild plants (such as the *Rosacee* family) and cultivated plants (such as *Vitaceae*, *Solanacea* and *Fabaceae*) (Keller *et al* 2003; Williamson *et al* 2007; Elad *et al* 2016).

I.2.1. Taxonomy of *Botrytis cinerea*

I.2.1.1. The genus *Botrytis*

The *Botrytis* genus was described for the first time in 1729 by Antonio Micheli in "*Nova Plantarum Genera*." The name is derived from Greek 'botrus' means bunch of grapes. More than two centuries later, Hennebert was accredited for completing the description of the genus (*B. fuckeliana /B. cinerea*: teleomorph and anamorph, sexual and asexual reproductive forms, respectively) (Hennebert 1973). The genus Botrytis includes at least 20 species and one hybrid called *B. alli* (*B. byssoidea* x *B. aclada*) that differ by morphology and their large host range.

In 2005, Staats *et al*. proposed an approach based on genealogy. The genus *Botrytis* can then be divided into two clades: the first one includes the 4 botrytis species that attach to the dicots and the second one consists of 18 other botrytis, 3 of which attack dicot plants and 15 that attach to monocot plants. Phylogenetic analysis of the plant hosts and the botrytis species suggest that the pathogens evolve by successive infections from host to host obtaining new pathogenic determinants (Table 2; Staats *et al* 2005; Romanazzi and Feliziani 2014).

Species	Common Disease Name	Typical Host/Tissue Specificity	Host-Plant Species
B. cinerea Pers. / *B. fuckeliana* (de Bary) Whetzel	Gray mould	Fallen leaves, fruits, and flowers	>235 plant species
B. fabae Sardiña	Chocolate spot	Leaves of bean	*Vicia spp. L., Pisum spp. L., Lens spp. L., Phaseolus spp. L.*
B. calthae Hennebert		Stem of marsh-marigold	*Caltha palustris*
B. ranunculi Hennebert		Buttercup	*Ranunculus spp. L.*
B. ficariarum Hennebert		Buttercup	*Ficaria verna*
B. pelargonii Roed		Leaves of geranium	*Pelargonium spp. L.*
B. paeoniae Oud.	Peony blight	Stems of cultivated peonies	*Paeonia spp. L.*
B. hyacinthi Westerd. and Beyma	Hyacinth fire	Leaves of hyacinth	*Hyacinthus spp. L.*
B. tulipae Lind	Tulip fire	Leaves, stems, and flowers of cultivated tulips	*Tulipa spp. L.*
B. elliptica (Berk.) Cooke	Lily fire	Leaves, stems, and flowers of cultivated lilies	*Lilium spp. L.*
B. squamosa Walker	Onion leaf blight	Leaves of onion	*Allium cepa*
B. aclada (Fresen.) Yohalem	Gray-mould neck rot	Bulbs of onion, garlic, and leek	*Allium spp. L.*
B. allii[a] (Munn) Yohalem	Gray-mould neck rot	Bulbs of onion, garlic, and leek	*Allium spp. L*
B. byssoidea Walker/ *B. allii* (Sawada) Yamamoto	Mycelial neck rot	Bulbs of onion, garlic, and leek	*Allium spp. L.*
B. globosa Raabe	Neck rot	Wild garlic	*Allium ursinum.*
B. porri Buchw.		Bulbs of garlic, leek	*Allium spp. L.*
B. sphaerosperma Buchw.	Blight	Three-cornered Leek (White-flowered Onion)	*Allium triquetrum*
B. narcissicola Kleb. Ex Westerd. and Beyma	Smoulder mould	Bulbs of narcissus	*Narcissus spp. L.*
B. polyblastis Dowson	Narcissus fire	Leaves of narcissus	*Narcissus spp. L.*
B. galanthina (Berk. and Br.) Sacc.	Blight	Snowdrop	*Galanthus spp. L.*
B. convoluta Whetzel and Drayton	*Botrytis* rhizome rot	Rhizomes of cultivated iris	*Iris spp. L.*
B. croci Cooke and Massee	Crocus blight	Leaves of cultivated crocus	*Crocus spp. L.*
B. gladiolorum Timm. / *B. draytonii* (Budd. and Wakef.) Seaver	Gladiolus blight	Stems of cultivated gladiolus	*Gladiolus spp. L.*

[a] Hybrid species according to Yohalem, Nielsen, and Nicolaisen 2003.

Table 2: Species described by Hennebert 1973.

The additional headings represent the common disease names and typical host-plant tissue and species. Sourced and modified from Staats et al 2005.

I.2.1.2. *Botrytis cinerea* species

The name of *Botrytis cinerea (B. cinerea)* was given by Elias Magnus Fries in 1952 in "Fries's *Systema Mycologicum*" (Egerton 2012). This ascomycete belongs to the Leotiomycete class and to the Sclerotiniacee family (Beever and Weeds 2004). In 1866, Bary established a genetic relationship between *B. cinerea*, the asexual organism and *Botryotinia fuckeliana*, the sexual organism. Groves and Dayton (1939) observed, for the first time, the *in vitro* formation of an apothecium (a spore-

bearing structure) by *B. cinerea*. This confirmed the systematic link between the *B. cinerea* and *Botryotinia fuckeliana*. The name *B. cinerea*, the asexual form of teleomorph *Botryotinia fuckeliana*, is mainly used among the mycologists and phytopathologists even though the scientific names of fungi are mainly given by their sexual form (Table 3).

B. cinerea classification	
Super kingdom	**Eukaryota**
Kingdom	Fungi
Subkingdom	Dikarya
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Leotiomycetes
Order	Helotiales
Family	Sclerotiniaceae
Genus	Botrytis

Table 3: B. cinerea classification.

Due to its capacity to infect a large range of hosts and because even one single strain can cause infections in many plants and organs *in vitro*, *B. cinerea* was considered for a long time to not be specialized, which is the opposite for the other species of the genus. *B. cinerea* is divided into two sympatric sibling species; *transposa*, and *vacuma* (Diolez *et al* 1995; Giraud *et al* 1999; Martinez *et al* 2003). Transposa possesses two transposable elements named *Boty* and *Flipper*. Vacuma does not contain either of the transposable elements.

Transposable elements are divided into two classes: class I and class II (Kidwell and Lisch 2001). *Boty* belongs to class I that consists of elements that transpose by reverse transcription of an intermediate RNA. The first transposable element *Boty* is characterized by a long terminal repeat present in multiple copies in different regions of the genome (Diolez *et al* 1995; Giraud *et al* 1999). *Flipper*, the second transposable element, is mobile and inserts itself into nitrate reductase during spontaneous mutant selection (Levis *et al* 1997).

Both sibling species, *transposa* and *vacuma*, display different abilities to infect different hosts. For example, *vacuma* attaches to green peas when *transposa* is prevalent on bramble (Diolez *et al* 1995). This nomenclature is still widely used in the scientific community (Munoz *et al* 2002; Ma and Michailides 2005; Samuel *et al* 2012). Nevertheless, another classification exists that distinguishes

these two sibling species of *B. cinerea*. Instead of naming the groups *transposa* and *vacuma*, the first group (Group 1) contains only *vacuma* strains whereas, the second group (Group 2) contains *vacuma* and *transposa* strains (Fournier *et al* 2002; Fournier *et al* 2005; Fournier and Giraud 2008). Recently, genome sequencing provided more data about *B. cinerea* (Amselem *et al* 2011; Hahn *et al* 2014; van Kan *et al* 2017).

I.2.2. Life cycle of *Botrytis cinerea*

During the life cycle of *B. cinerea*, the fungus produces mycelium, macroconidia, microconidia, sclerotia and fruiting bodies called apothecia (Figure 6). Mycelium, macroconidia and sclerotia are part of the asexual reproduction whereas the rest are involved in the sexual reproduction (Razak and van Kan 2014).

The mycelium is comprised of an ensemble of grey or olive coloured branched filaments called hyphae, the diameter of which varies greatly depending on the growth conditions. Generally, when the mycelium is mature at the fruiting stage, it is able to produce conidia (asexual spores) on many different substrates. Conidia are approximately 10 μm in length and 5 μm in width that are dispersed by wind, rainfall, or insects (Barnett and Hunter 1998; Holz *et al* 2004; Schumacher and Tudzynski 2012). Conidia are produced by conidiophores that looks as arbuscule (Figure 6) (Holz *et al* 2004). Mycelium and conidia are able to induce the host infection when environmental conditions are favourable.

However, in unfavourable conditions, the fungus is able to create sclerotia (Jarvis 1980). The structure of sclerotia consists of mycelial branches fused together to produce a globular mass, that at the beginning is hyaline, then it turns to a brown colour due to deposition of melanine pigments in the external protection layer. This external coat keeps the fungus protected for a long period from the UV radiation and to the other external hazardous factors (Williamson *et al* 2007).

When climatic conditions are favourable, the sclerotium generates conidia or mycelium. However, it is also possible that sclerotium can induce the formation of the apothecium containing asci with ascospores (not frequently observed in nature) (Coley-Smith and Cooke 1971; Faretra *et al* 1988; Beever and Weeds 2004). The ascospores are the product of meiosis and their role is to act as a source of inoculum for disease development.

Last, but not least, are the microconidia, that also aid the fungus in propagating when environmental conditions are unfavourable. They are generated from the macroconidia germ tubes or from the mature hyphae cells (Fukumori *et al* 2004; Holz *et al* 2007; Schumacher and Tudzynski 2012; Romanazzi and Feliziani 2014). In sexual reproduction, microconidia can act as the male gamete and

they may fertilise sclerotia, that likely serves as the female gamete (Faretra *et al* 1988; Williamson *et al* 2007; Veloso and van Kan, 2018). However, sexual reproduction is a very rare event and thus the mechanism has been poorly described.

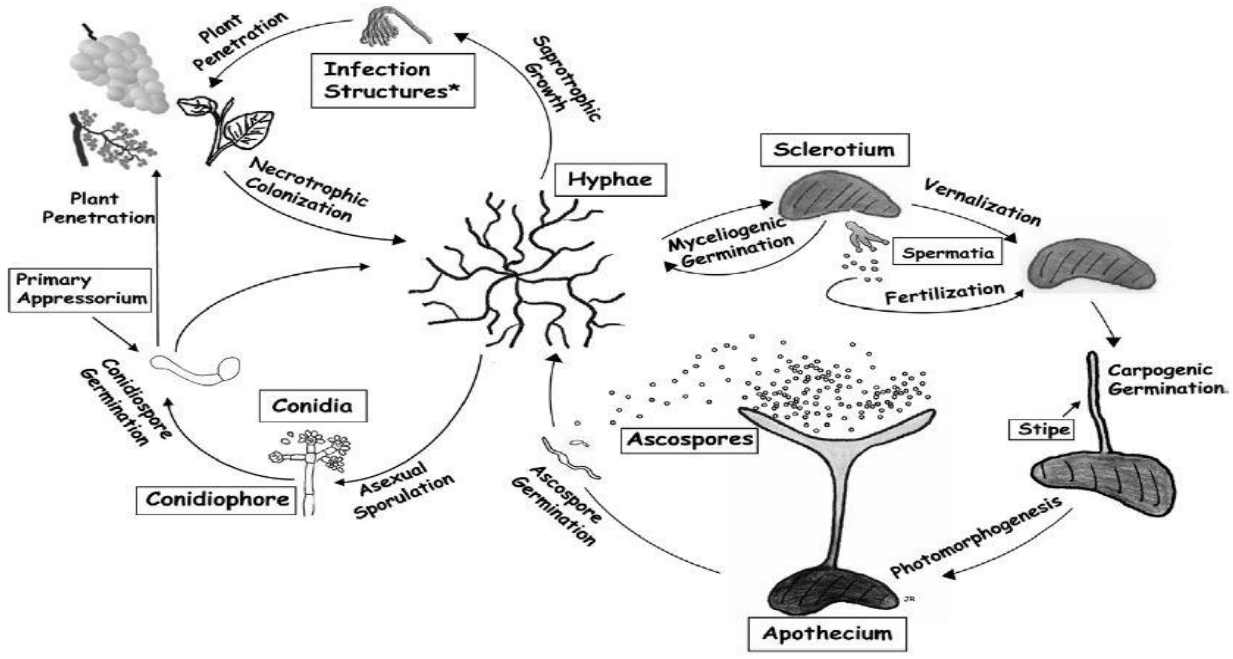

Figure 6: Life cycle of B. cinerea.

Sourced from Romanazzi and Feliziani 2014.

I.2.3. The infection process of *Botrytis cinerea*

The infection can occur when three factors are pooled concomitantly: the pathogen, the host and environment (Francl 2001). *B. cinerea* can survive in the vegetable debris in the form of mycelium, conidia and sclerotia that are the only form able to survive for a long time in the ground (Klaus 2007). When the environmental conditions become favourable, conidia are produced, dispersed and they land on leaves where they can germinate in the presence of water (Muvunyi 2012). Moreover, the sclerotia that are attached to nearby leaves on the ground, can also produce mycelium that will lead to an infection. The conidia and mycelium need to have an exogenous source of energy for their developments. For the development of the appressoria, specialized cells used for infecting the host plant, an exogenous source of energy is also needed (Figure 6) (Kosuge and Hewitt 1964; Yoder and Whalen 1975; Li *et al* 2004).

The germination of the conidia is more favoured in a nutrient medium than in water (Clark and Lorbeer 1977). In water, the germinate filament stops growing immediately after the appearance of

the conidia's germ tube. However, this is not the case when conidia are in the presence of nutrients like glucose, fructose, galactose, because in these conditions, even the old conidia can also germinate (Kosuge and Hewitt 1964, Clark and Lorbeer 1977; Shiraishi *et al* 1970a, Edwards and Seddon 2001). Another important factor favouring the infection of *B. cinerea*, is that the germ tube of conidia and mycelium are capable of penetrating the plant's surface, due to the secretion of hydrolytic enzymes, that cause cuticle lysis, or due to a pre-existing lesion (injury) in the plant host. The pathogen is then able to kill epidermal and underlying dermal cells (Jarvis 1989; Muvunyi 2012). *B. cinerea* starts the infection on the quiescent leaves and is then able to expand up to the aerial parts of the plant and the fruits. The primary infection is defined by collapsing brown-coloured tissue and defined margins. The infection progresses and the fungus becomes able to overcome the host's defence. In particular, the hyphae grow in a protrusive way and, as a secondary infection, induces the destruction of the plant tissue. The hypersensitive response of the plants to the invasion of the pathogen induces the death of the plant cells near the fungus infection. The problem with this kind of response is that it favours the development of *B. cinerea* because it is a necrotrophic fungus (Govrin and Levine 2000). Upon the death of the plant's cells, numerous compounds are secreted by the fungus: proteins, toxins, oxalic acid and reactive oxygen species (ROS). It has been proved that ROS are produced by both the fungus and the plant (Temme and Tudzynski 2009). In particular, the generation of H_2O_2 (one kind of ROS) was observed in and around the cell wall of the penetrated host. Even if the plant is able to induce oxidative stress, the fungus is immune to it. When the lesion induced from the fungus causes tissue maceration, sporulation is also induced (Van Kan *et al* 2006).

The last factor to discuss that influences the fungus' infection is climatic and microclimatic conditions (Latorre *et al* 2015). In particular, humidity and temperature play an important role in the plant's infection and for the development of the disease. It was shown that at a temperature range between 15 and 25 °C that germination can occur (Sirry 1957; Shiraishi *et al* 1970a; Shiraishi *et al* 1970b; Davidson and Krysinska-Kaczmarek 2007; Carisse 2016). Moreover, *B. cinerea* has a very good development under high humidity (above 90 %) (Yunis *et al* 1990; Nair and Allen 1993; Williamson *et al* 1995; Carisse 2016).

I.2.3.1 The bright side of the *Botrytis cinerea* infection: noble mold

B. cinerea is also the fungal agent of the noble mold that is beneficial in sweet wine production. The "disease" development is controlled and even promoted by some specific climatic conditions: alternating cycles of cold humid nights and dry sunny days. Curiously, it is not due to a specific population of *B. cinerea*. So far, according to the literature, there is no genetic link between strains

causing noble or grey mold. Consequently, winegrowers have to focus on controlling the environmental growth factors to optimize this noble rot growth. The noble rot takes place at the end of the growing season (Maygar 2011). The development of noble rot starts when the fungus penetrates the ripe berries without any alteration of the fruit skin. Subsequently, the fungus decomposes the fruit skin that becomes porous with high water evaporation. This leads to an enzymatic maceration and an important increase in sugar concentration in the berries (the *pourri rôti* stage). This increasing sugar content stops the fungal development resulting in the berries of noble mold having the desirable aroma of the resulting sweet wine (Negri *et al* 2017).

The production of these particular liquorous wines started in Hungary or Slovakia (Tokaj or Tokaji region), Germany (Rhine region) and France (Sauternes region) and grew widespread throughout the world (Australia, Northern of Italy, USA and South Africa). Examples of these wines are white wines, such as Tokaji Aszu, Sauternes and Monbazillac and red wines like Amarone (Maygar 2011; Tosi *et al* 2012; Fournier *et al* 2013).

I.2.3.2. The dark side of *Botrytis cinerea* infection: grey mold

B. cinerea represents a dramatic health problem for a large panel of plant species causing grey mold. Moreover, as saprophyte it can survive on the seeds or on plant matter in the soil for long periods (Bardin *et al* 2018). Furthermore, *B. cinerea* is also dangerous because it infects crops pre- and post-harvest. The fungal infection can even start before the harvest and can stay in a quiescent status for a long time, until the environmental conditions and changes in the host's physiology become favourable for fungal development and growth (Droby and Lichter 2007; Feliziani and Romanazzi 2014; Hua *et al* 2018)

This pathogen is able to infect a lot of vegetables and small fruit crops such as tomato, apple, grape, potato and many others (Figure 7) (Droby and Lichter 2007; Feliziani and Romanazzi 2014). The infection starts on the soft parts of the fruit tissue with the appearance of a dark spot. Depending on the light quantity, sporification can then develop from the site of infection.

Figure 7: Grey mold induced by B. cinerea on crops and flowers.

*The disease symptoms on vegetative organs (grapevine and potato leaves) or rose petals (**top panel**) or on fruits **(bottom panel)** grape, strawberry and tomatoes.*

The infection can occur before harvest and can survive at a low storage temperature (0–5°C). A recent study from the FAO estimated that, with respect to the total amounts of fruits and vegetables produced worldwide, between 15 and 50% are lost at the postharvest stage, mainly because of *B. cinerea*. Moreover, it is also very complicated to estimate precisely the real cost of the loss due to this pathogen's infection. Fungicides and biocontrol treatments that have been designed to fight against *B. cinerea*, cost more than €1 billion/annum (Dean *et al* 2012; Hahn 2014; Romanazzi and Feliziani 2014). Thus, it is clear that *B. cinerea* triggers a large expensive problem that needs a long-lasting solution. In this manuscript, two plant targets of *B. cinerea* are studied: grapevine (*Vitis vinifera*) and potato (*Solanum tuberosum*). The former is more susceptible to *B. cinerea* infection whereas this is not the case for the latter.

I.3. *Botrytis cinerea* targets: grapevine and potato.

I.3.1. Grapevine plantlets

Grapevine is one of the most cultivated fruit species in the world with a large economic impact and the main target of *B. cinerea* during its vegetative and fruit developments. Around the world, grapevine is mostly used for the production of wine but also for the production of juice, as well as fresh and dried fruit (Torregrosa *et al* 2015; Figure 8).

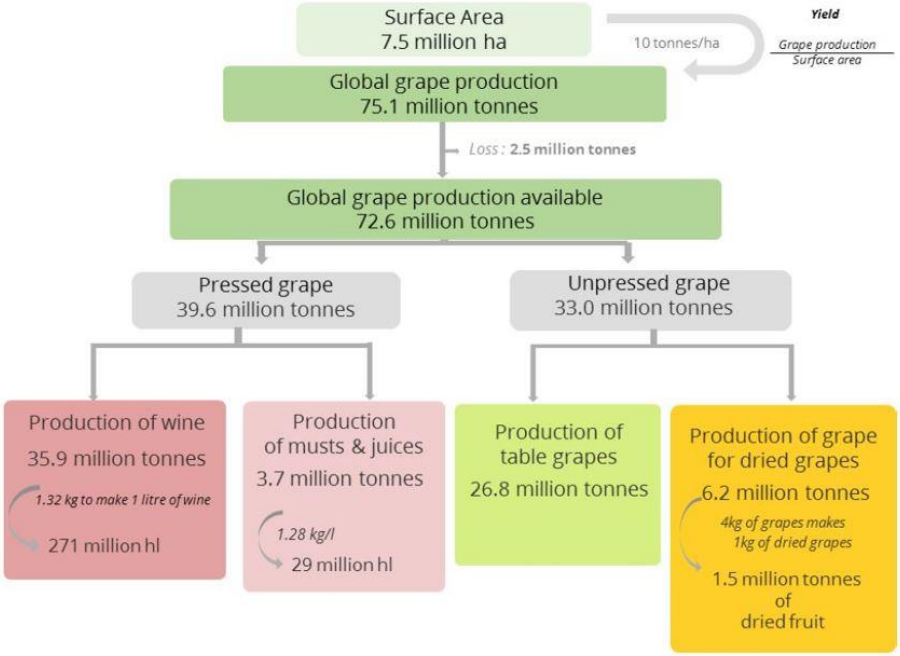

Figure 8: Schematic presentation of the global viticulture situation in 2014.

Sourced from FAO-OIV FOCUS 2016.

According to the International Organization of Wine and Vine (OIV), France is the top wine exporting country in the world, producing 17 % of the total wine produced worldwide (OIV 2019; LARVF 2017). Nevertheless, the total area planted with wine grapes in France represents only the third worldwide place after Spain and China, just before Italy (OIV 2019; Table 4).

thousand ha	2014	2015	2016	2017	2018
Spain	975	974	975	968	969
China	813	847	858	865	875
France	789	785	786	788	793
Italy	690	685	693	699	705
Turkey	502	497	468	448	448
USA	450	446	439	434	439

Table 4: Top 6 countries with the largest area under vine used for wine production.

Sourced from OIV 2019.

The wine industry is very important for the French economy and of course for some regions such as Bordeaux (Nouvelle Aquitaine), Champagne or Bourgogne that produce the most famous vintages in the world. Fifteen percent of French agriculture incomes were wine generated and this industry still ensure around 600,000 jobs. In 2019, the sale of French wine and spirits amounted to €14 billion (De la Hamaide 2020).

I.3.1.1. *Botrytis cinerea*: a deleterious agronomic and economic impact on grapevine

The grapevine losses are mainly due to *B. cinerea* (Keller 2015). It is responsible for decreasing the production yield and the overall quality of wine (colour, taste and scent) costing at least € 2 billion worldwide (Ribéreau-Gayon *et al* 1998; Pszczokowski *et al* 2001; Elmer and Michailides 2004; Steel *et al* 2013).

B. cinerea induces a reduction of amino acids concentration and a change of the typical aroma of wine by degrading the compounds that gave the characteristic bouquet of wine as a signature (La Guerche *et al* 2006). Furthermore, this pathogen excretes enzymes such as polyphenol oxidase and lacases that oxidise the phenolic compounds (present in grapes) to quinone-type compounds. This process can eventually lead to the formation of polymers that are noticed when red wine is discoloured and when white wines turn brown (Pezet *et al* 2004; Ribéreau-Gayon *et al* 1998; Pszczolkowski *et al* 2001).

I.3.1.2. Vulnerable stages of grapevine to *Botrytis cinerea*

When the grapevine is in flowering or veraison (stage where the grapes increase in size and start to accumulate sugars and aroma compounds), *B. cinerea* infection can better occur, probably due to the low level of resveratrol produced during the flowering stage (Keller *et al* 2015). Moreover, the infection could happen when pollen grains are abundant even when anthers are senescent (Chou and Preece 1968; Bulit and Dubos 1982; Pearson and Goheen 1998). After infection during the flowering stage, *B. cinerea* remains latent until the period of the veraison (Deytieux-Belleau *et al* 2009). During veraison, grapes are more sensitive to the infection because of the berry cuticle and the cell wall modifications (Deloire 2010; Keller 2015). More specifically, there is an increase in waxy deposits and large changes in the morphology of the wax surface of the berry. Furthermore, the cutin content decreases significantly (by almost two thirds) in the grapes, rendering the berries more prone to fungal infection (Deloire 2010).

I.3.1.3. Different susceptibilities of grapevine varieties to *Botrytis cinerea*

The grapevine varieties present different susceptibilities to *B. cinerea*. This makes it an essential management indicator, according to the Integrated Pest Management (Table 5) (Galet 1988; Dry and Gregory 1988; Marois *et al* 1992; Kogan 1998; Dubos 2002; Fermaud *et al* 2011).

Cultivar	a	b	c	d	e	f	g	h
Grenache Noir	4	3	-	-	4	-	3	4
Cabernet Franc	3	-	-	-	-	-	4	1
Petit Verdot	0-1	-	-	-	-	-	1	1
Cabernet Sauvignon	2	-	0	1	1	0	1	1
Mourvèdre	-	-	-	-	-	-	1	-
Merlot	3	-	-	-	-	-	3	3
Syrah	2	-	1	3	3	-	-	2
Cot	3	-	-	-	-	-	3	3
Roussanne	4	-	-	-	-	-	-	4
Chardonnay	4	-	2	2	3	-	3	3
Pinot Noir	3	4	2	3	4	-	-	3
Gewürztraminer	4	-	-	-	-	-	1	4
Sauvignon Blanc	4	-	4	3	4	-	1	4

a = Dubos (2002), b = Dry and Gregory (1990), c = Orffer (1979), d = Jackson and Schuster (1987), e = Robinson (1986), f = Marois et al. (1992), g = Galet (1988), h = ACTA (1980); 0 = highly resistant, 1 = resistant, 2 = intermediate, 3 = susceptible, 4 = highly susceptible.

Table 5: Susceptibility to B. cinerea of 13 grapevine varieties.

Sourced and modified from Pañitrur De la Fuente 2017.

Among the multitude of grapevine varieties (around 6000 across the world and those listed in Table 5), three widely grown grapevine varieties: Sauvignon, Chardonnay and Merlot were chosen and investigated (clones grown *in vitro*) in our study. The Chardonnay and Sauvignon backgrounds are highly susceptible to the infection of *B. cinerea* whereas the Merlot variety is more resistant (Galet 1988; Dry and Gregory 1990; Marois *et al* 1992; Kogan 1998; Dubos 2002; Fermaud *et al* 2011). Furthermore, these grapevine varieties are listed in the top 10 most cultivated and famous grapevines for wine production in the world (Anderson 2013).

I.3.2. Potato plant and culture

The other plant considered in this work is the potato plant (*Solanum tuberosum)*. This plant species was chosen because it is one of the most important food crops in the world. Furthermore, it is a member of Solanaceae family as tomato (*Solanum esculentum*), already studied in the PEREINE laboratory (Guillaumot *et al* 2016). Nevertheless, in contrast to tomato plants, potato plants are not the favourite *B. cinerea* host.

Potato constitutes, grown in more than 100 countries that have a large range of climate conditions (Hawkes, 1994). The total world potato production was estimated around 388,191,000 tonnes in 2017 (Figure 9) (FAOSTAT, 2019). The potato originated from Peru where some wild relatives still exist and represent a pool of genetic and morphological diversity (Spooner *et al* 2005).

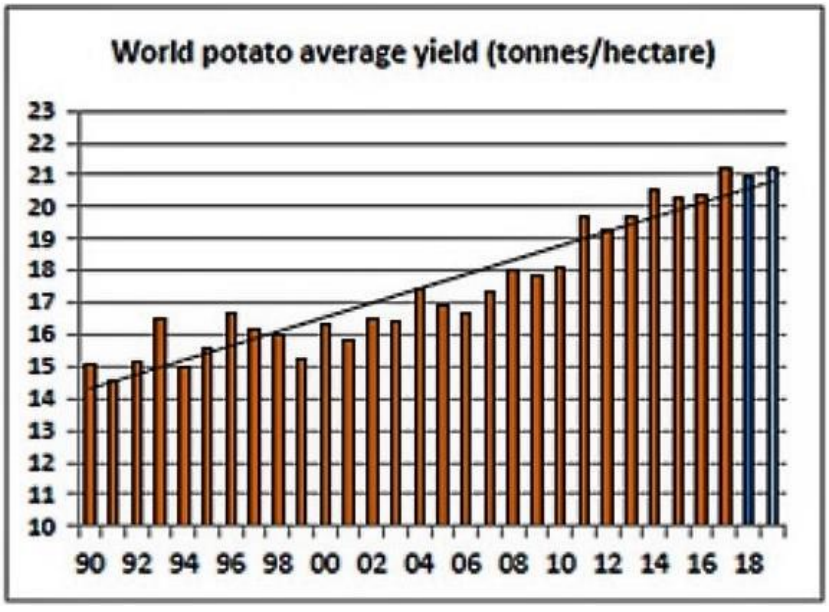

Figure 9: The average world production of potato over the last 30 years.

Estimations were done for 2018 and 2019. Sourced from Potato Pro 2020.

I.3.2.1. Potato a staple food

The ever-increasing difference between cereal demand due to population growth and the reduction in wheat crop yield, has caused basic food shortages in many places around the globe. Thus, the potato tuber has become one of the main foods to the global population. It is highly nutritious and when it is fresh, the tubers are made up to 20 % of dry matter and 80 % water. Within this dry matter, there is fibre, protein, a small amount of fatty acids and 75 % starch. On top of these food types, the dry matter also contains minerals (magnesium, phosphorus and potassium) and vitamins (C, B1, B3 and B6) (Prokop and Albert 2008; Camire *et al* 2009).

To guarantee a good yield of potato production, it is important to consider the factors that ensure the quality of this perennial plant. Other than plant infection by pests (see Table 6 and next section), the most important factor for determining a healthy potato growth is the climatic environment (Monteith, 2000). In particular, the temperature is reportedly the primary factor that drives the phenotypical and morphological development (Hodges 1991). Another main factor that influences the phenological development of the potato is the temperature of the crop from the sunlight (Ewing and Struik 1992). Additional factors such as the lack of water and the day-length can slow down the development in some plants.

I.3.2.2. Potato pathogens

The following table (Table 6) reports the principal pathogens in potatoes.

Pathogen	Disease/ Commonname	Distribution
(a) fungi		
Alternaria solani	Early blight	Worldwide
Erysiphe cichoracearum	Powdery mildew	L. America, Europe, Near-East
Fusarium spp.	Fusarium dry rot	Worldwide
Macrophomina phaseolina	Charcoal rot	Worldwide where T >28 °C
Phoma fo eata	Gangrene	N. America, Europe, Asia, Oceania
Phytophthora infestans	Late blight	Worldwide
Rhizoctonia solani	Black scurf/ stem canker	Worldwide
Spongospora subterranea	Powdery scab	Worldwide
Synchytrium endobioticum	Wart	Africa, Asia, America, Europe
Verticillium spp.	Verticillium wilt	Worldwide
(b) bacteria		
Erwinia caroto ora ssp. atroseptica	Black leg, soft rot	Worldwide
Pseudomonas solanacearum	Bacterial wilt, brown rot	Asia, Africa, S. America (probably worldwide)
Cla ibacter michiganense ar. sepedonicum	Ring rot	Worldwide
Streptomyces scabies	Common scab	Worldwide
(c) viruses		
PLRV Potato leafroll virus		Worldwide
PVY° Potato virus Y°		Worldwide
PVNⁿ Potato virus Yⁿ		Europe, USSR
PVA Potato virus A		Worldwide
PVX Potato virus X		Worldwide
PVM Potato virus M		E. Europe
(d) nematodes		
Globodera rostochiensis	Golden nematode	Worldwide
Globodera pallida	White potato cyst nematode	N.W. Europe, S. America
Meloidogyne chitwoodi	Root knot nematode	Worldwide?
Meloidogyne incognita	idem	Europe, America, Africa, Asia
Meloidogyna hapla	idem	Europe, America
Meloidogyna ja anica	idem	Africa, Asia, S. America
Nacobbus aberrans	False root-knot nematode	America, India, USSR, N.W. Europe
Pratylenchus penetrans	Lesion nematode	N. America, Europe
Pratylenchus spp.	Lesion nematode	Worldwide

Table 6: Principal pathogenic agents that cause losses in the potato.

Sourced from Jeger et al 1996.

In this list, among fungi, *B. cinerea* does not appear because grey mold is considered as a minor disease for potato crop (Kirk and Merlington, 2012). However, fungi that belong to the same division of *B. cinerea* appear in the table above. Therefore, it was supposed that the experiments conducted on *B. cinerea* could be reproduced on other ascomycota fungi in the future. Furthermore, the fungi that attack essential crops like rice and the potato are of interest for the application of this treatment. Keeping this idea in mind, four different varieties of potato are taken into consideration due to their fungal susceptibility in this PhD manuscript: Bintje, Grenadine, Hinga, and Laurette. Even though no specific data linking these potato varieties and *B. cinerea* was found, the choice of these four varieties was driven mainly by their commercial and/or historical interest in Europe. Nevertheless, the Bintje variety is highly susceptible against a large range of pests (bacteria, fungus and virus). Specifically, this variety shows a low resistance to fungal diseases, such as early blight caused by *Alternaria solani*

(Ascomycete). However, potato varieties show different susceptibilities to different kinds of pathogens. Grenadine and Laurette are very sensitive to downy mildew induced by different oomycetes (European Cultivated Potato Database; Garrigues Freres SAS Rodez (Aveyron, France), Grocep Lauriere (Haute-Vienne, France)). Lastly, the Hinga variety is highly susceptible to the potato virus M.

I.4. Disease management

B. cinerea is characterized by its genetic variability that makes the control of the disease very difficult. Moreover, since it is a saprophyte it can survive on the seeds or on plant matter in the soil for long periods (Bardin *et al*. 2018). Many treatments have been used to fight off and control this fungus. However, they are currently becoming inefficient due to the development of resistances. Some of the most important methods will be described in this section.

I.4.1. Plant prophylaxis to fight *Botrytis cinerea*

Many chemical and biological treatments are still used to fight off and control *B. cinerea*. However, they are becoming inefficient due to the development of resistance. Thus, the search for new treatments, respecting the environmental laws, is currently underway. The measures used in order to protect plants before the development of fungal infections are called prophylactic treatments (Maloy 2005). Despite preventative treatments (control of temperature and humidity), fungal infection can still occur but in a lesser extent.

Concerning *B. cinerea*, the greenhouse prophylactic treatment is based on the elimination of quiescent leaves and infected organs. The fungus, being a necrotic plant pathogen, is thus unable to grow and develop a secondary infection (Köhl *et al* 1999; Dik and Wubben 2004).

Other important methods to reduce the *B. cinerea* infection in greenhouses are the control of the number of plants per square metre and the removal of excess leaves. This can therefore allow optimal air circulation and thus, diminish humidity (Daugaard *et al* 2003; Decognet *et al* 2009). It is also known that fertilization plays an important role limiting the fungal development (Volpin and Elad 1991; Elad and Volpin 1993; Daugaard *et al* 2003). Although this kind of treatment can be useful in agriculture, it is not enough to eliminate the diseases linked to *B. cinerea*. Therefore, other treatments are summarized below.

I.4.2. Chemical treatments

Chemical treatments encompass the use of fungicides to fight against fungal plant pathogens. To this day, it remains an indispensable tool to ensure plant protection in order to have sufficient food supply for the ever-growing population. Moreover, the overuse of fungicides has led to the development of multi resistance in the fungus, thus rendering these fungicides ineffective (Wang *et al* 1986; Leroux *et al* 2002, Kretschmer et al 2009). A typical example of resistance to fungicides was observed with dicarboximides (iprodione, vinclozolin) (Pommer and Lorenz 1982). Fungicides are grouped based on their mode of action: multi-site, oligo-site and uni-site (Figure 10). The action of the former is directly against multiple enzymatic systems (glycolysis enzymes, Krebs cycle enzymes and electron transport chain). Thanks to their large spectrum of action, these class of pesticides, including dithiocarbamates (mancozebe, manebe), phthalimides (captan, folpel) and chloro-nitriles (chlorothalonil), induce a lower risk of developing resistance. The oligo-site and uni-site fungicides are more specific and block one fungal metabolic pathway (Brent and Hollomon 1998; Leroux and Gardan 2003). In particular, they can act on the biosynthesis of the most important living molecules like carbohydrates, lipids, proteins and nucleic acids.

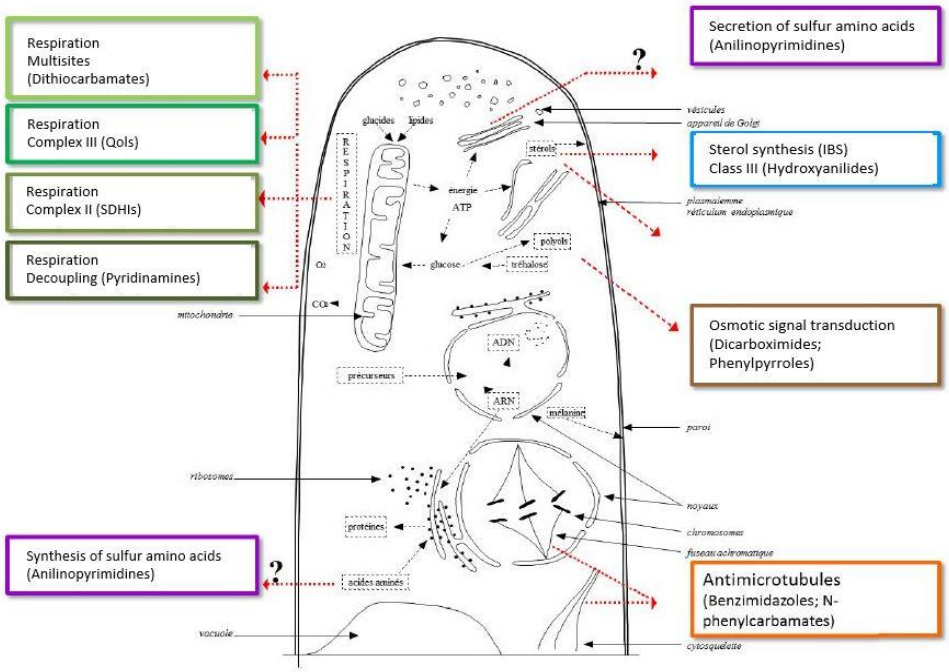

Figure 10: Action modes of some of the main organic fungicides.

Sourced and modified from Leroux and Gardan 2003 and Lepoivre 2007.

Resistant strains are adapted to new environments that have regular treatments by fungicides, thus improving the biological characteristics of this strain (Milgroom *et al* 1989). This type of resistance is called acquired resistance and it is different to natural resistance that is already inborn in the fungus before the application of the fungicide (Leroux *et al* 1999; Leroux *et al* 2013a; Wiesch *et al* 2011). Natural resistance is generated by the continuous mutations of sensitive strains.

Resistance is induced by the appearance of mutations in the fungus population treated by fungicides. DNA mutations could change the gene structure and create a new allele in the treated population (Mc Donald and Linde 2002). The number of resistant strains increases and by opposition, decreases the efficacy of fungicides. This acquired resistance is often determined by a mutation affecting the structure of the target protein of fungicides (Uesugi *et al* 1993; Leroux *et al* 2002; Wood and Hollomon 2003).

B. cinerea characteristics (sexual and asexual reproduction, large host range, etc.) make it very resistant to fungicides. For this reason, large doses of fungicides are necessary to treat the infection and this causes environmental pollution (Brent and Hollomon 1998).

I.4.3. Biological pest control

To bypass the utilization of fungicides, another treatment to control the fungus' infection is needed. In this context, a biological pest control that involves an active human management role is used and is a fundamental part of the integrated pest management (IPM) program (Barzman *et al*. 2015).

This method is based, mostly, on the use of a microbial agent that can be in a competitive relationship with the harmful pathogen. Often the microbial agent used is an organism, such as a parasite, or a pathogen itself, which acts as a natural antagonist of the undesired pathogen. However, the biological pest control can show side-effects in biodiversity, particularly if the consequences of introducing another species in a predefined system is not evaluated. In order to determine the best biological pest protection, specific requisites are necessary. Firstly, knowledge of the pathogen's biology and of the microorganism used for the treatment is required. Secondly, the experiment must be repeatable in laboratory and finally, validation in real conditions must be performed. Moreover, such as for other treatments discussed in the next sections, the final objective of this kind of treatment is to reduce the presence of the pathogen or, at the very least, minimize its activity (Cook et Baker 1984). Other biological treatments include the use of mineral agents and plant oils to fight against *B. cinerea*.

I.4.3.1. Microbial agents

The biological pest control, with the use of micro-organisms antagonists against *B. cinerea* such as other filamentous fungi, yeast and bacteria, has been intensively studied (Newhook 1951; Wood 1951; Van Lenteren 2000; Paulitz and Belanger 2001; Mari *et al* 2003; Elmer and Reglinski 2006; Droby *et al* 2009). In 1951, Newhook and Wood inoculated quiescent lettuce leaves with *B. cinerea* antagonists, *Fusarium spp.* and *Penicillium claviforme* and as a result, *B. cinerea* was not able to attach to the leaves. This strategy was later confirmed by Newhook in 1957 using tomatoes grown in a greenhouse and in order to inhibit the *B. cinerea* infection, a spore suspension of *Cladosporium herbarum* and *Penicillium sp* was used. Many other examples of micro-organisms used as a biological control against *B. cinerea* are reported in the literature (Bhatt and Vaughan 1962; Blakeman 1972; Blakeman and Sztejnberg 1974; Nelson and Powelson 1988; Elad *et al* 1995; De Meyer and Hofte 1997; Latorre *et al* 1997; Köhl *et al* 1998; Sean *et al* 1999; Nicot *et al* 2003; Buck and Jeffers 2004; Bardin *et al* 2008; Calvo-Garrido *et al* 2019; Sarven *et al* 2020).

Microorganisms can also be competitors of *B. cinerea* for essential nutrients like nitrogen, carbon, macro- and micro-elements in the soil. The lack of these nutritious elements can interfere with *B. cinerea*'s capability to produce hydrolytic enzymes necessary for infecting the plant host. They can also change the pH of the soil that must be acidic for *B. cinerea* development (Blakeman and Fokkema 1982; Paul *et al* 1997; Filonow 1998; Kapat *et al* 1998; Duffy *et al* 2003; Manteau *et al* 2003; Elad and Stewart 2004; Schoonbeek *et al* 2007). In particular, this competition leads to the reduction of spore germination or mycelium growth, which is essential for the pathogen's growth and development (Blakeman and Fokkema 1982, Blakeman 1993).

Antibiosis one of the latest method developed against *B. cinerea*, involves the antagonist organism producing toxic secondary metabolites for the target pathogenic agent. These metabolites can inhibit the germination, sporulation and the mycelial growth of the pathogenic fungus (Montesinos *et al* 2009). Finally, hyper parasitism is another strategy that can also be used against *B. cinerea* (Whipps and Gerlagh 1992; Yu and Sutton 1997; Fravel 2005).

I.4.4. Mineral and organic compounds

These composites can act as natural fungicidal agents to control pathogenic agents in plants (Tripathi and Dubey 2004). An example is chitosan and its derivatives, which show plant protection proprieties against some phytopathogens (Bautista-Banos *et al* 2006). In the case of *B. cinerea*, chitosan (Figure 11) was used as a protection on post-harvest fruits (El-Ghaouth *et al* 1997, Choi *et al* 2002, Oliveira Junior *et al* 2012).

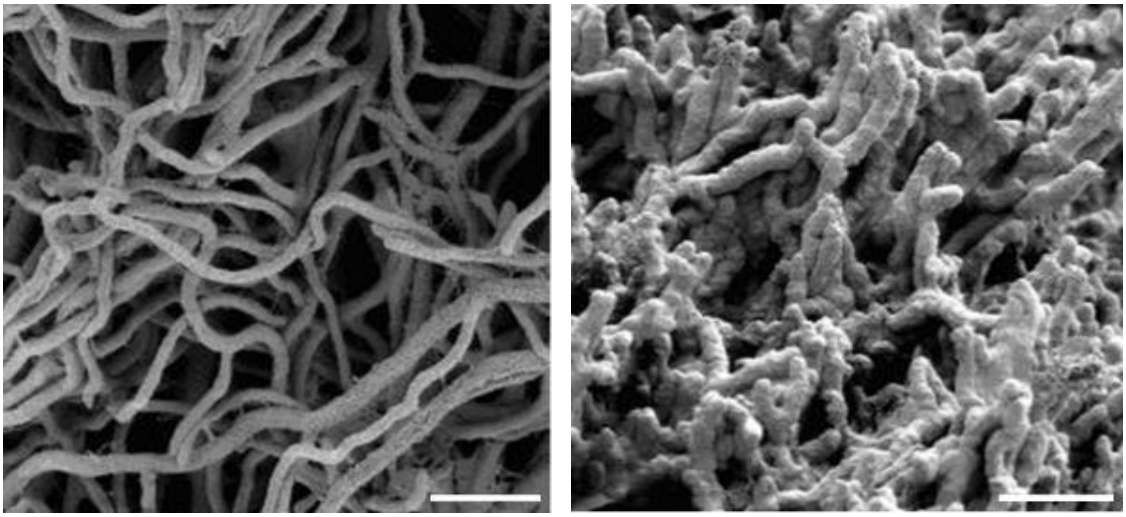

Figure 11: Effect of chitosan on mycelium of B. cinerea.

Scanning electron micrographs of B. cinerea mycelium after 5 days of cultivation at 25°C. (left): control mycelium (right): mycelium treated with chitosan. Scale bars represent 20 µm. Sourced and modified from Oliveira Junior et al. 2012.

Nigro *et al* (2006) showed the potential of nineteen salts on the control of *B. cinerea* development on post-harvest raisins. Calcium chloride, potassium carbonate, sodium bicarbonate and sodium carbonate were able to reduce the fungus incidence *in vivo* (Nigro *et al* 2006). In another interesting study (Yildirim and Mericli Yapici 2007), food additives such as potassium sorbate, sodium benzoate, methylparaben, propylparaben and sorbic acid, showed strong inhibition on the mycelial growth and conidia germination in *B. cinerea*.

I.4.5. Plant essential oils

Another method associated with biological pest control, in particular at post-harvest, involves the use of plant essential oils. These oils are extracts from plants and they show efficacy against microbial pathogens. The active ingredients are commonly aromatic substances such as flavonoids, phenols, alkaloids, sterols (Burt *et al* 2004). It has been demonstrated that the plant essential oils inhibit the mycelium growth of fungi (Manohar *et al* 2001; Marin *et al* 2004; Velluti *et al* 2004; Burgiel and Smaglowski 2008; Hadizadeh *et al* 2009). Burgiel and Smaglowski (2008) described complete growth inhibition of *B. cinerea* on media with a 0.5 % addition of tea tree oil. Banani *et al* (2018) displayed the efficacy of thyme essential oils against *B. cinerea* on apple fruit. Hou *et al* (2020) reported the capacity of phenolic acids extracted from rice straw, to induce a strong inhibitory effect

on *B. cinerea*. In conclusion, because of their reduced environmental toxicity, plant oils can be considered a good alternative to fungicides. However, while the probability to develop resistance in pathogens is reduced, it can still occur and this is a disadvantage of using plant oils against fungal pathogens (Daferera *et al* 2003).

I.5. Looking for an efficient alternative treatment for *Botrytis cinerea*: photodynamic therapy

I.5.1. Photodynamic therapy in agriculture

Photodynamic therapy (PDT) is a treatment that involves a photosensitizer (PS), light and oxygen. The PS, if activated by light, will interact with oxygen and induce the formation of reactive oxygen species (ROS), toxic for the living cells (Møller *et al* 2007; Donnelly *et al* 2008; Benov 2015). PDT has been used in medicine: oncology, ophthalmology and dermatology (Dolmans *et al* 2003; Konopka and Goslinski 2007, Robertson *et al* 2009; Babilas and Szeimies 2010; Darlenski and Fluhr 2012; Dąbrowski and Arnaut, 2015; Habermeyer and Guilard 2018).

PDT has been successfully used to inactivate microorganisms such as bacteria, yeast and fungi and has thus been named antimicrobial photodynamic therapy (APDT or PDI Photodynamic Treatment Inhibitor). Since the 1990s, resistant Gram-negative bacteria could efficiently be killed by cationic charged PSs (Hamblin and Abrahamse 2020). Since then, it has been envisaged to be used against multidrug resistant microorganisms because this therapy does not lead to the development of resistance (Maisch 2009; Huang *et al* 2010). For this reason, APDT can be considered an alternative strategy for fighting plant pathogens. Furthermore, APDT applications have already been investigated from the field of medicine to the agricultural industry, including food and water decontamination (Jori and Brown 2004; Luksiene 2005; Alves *et al* 2010; Kashef *et al* 2017; Glueck *et al* 2019; Almeida *et al* 2020).

I.5.2. Photosensitizers

In order to breakdown APDT and understand the potential of the PS, the mechanism of activation is described herein. This phenomenon was first described and explained by Jablonski and has thus explained by the Jablonski diagram (Figure 12) (Dabrowski *et al* 2016). Upon irradiation, the PS absorbs a photon and is excited from the ground state (S0) to an excited singlet state (S1). This state is unstable, so the PS can undergo two different pathways. On one hand, it is possible that it returns to its stable ground state, converting the energy into heat or light. The latter known as fluorescence.

On the other hand, the PS can undergo an intersystem crossing that brings the PS to an excited triplet state (T1). In this state, the PS can induce phosphorescence, or it can react with other molecules inducing the formation of ROS. The triplet can form ROS *via* two possible reactions mechanisms: type I and type II (Foote 1991; Baptista *et al* 2017, Hamblin and Abrahamse 2020). The type I reaction involves the PS in the excited triplet state giving an electron or a proton to biological substrates. This reaction generates radical anion or cation species (superoxide anion radicals, hydroxyl radicals and hydrogen peroxide). The type II reaction mechanism involves the reaction between the excited triplet state of the PS and molecular oxygen, thus forming cytotoxic singlet oxygen ($^1O_2$).

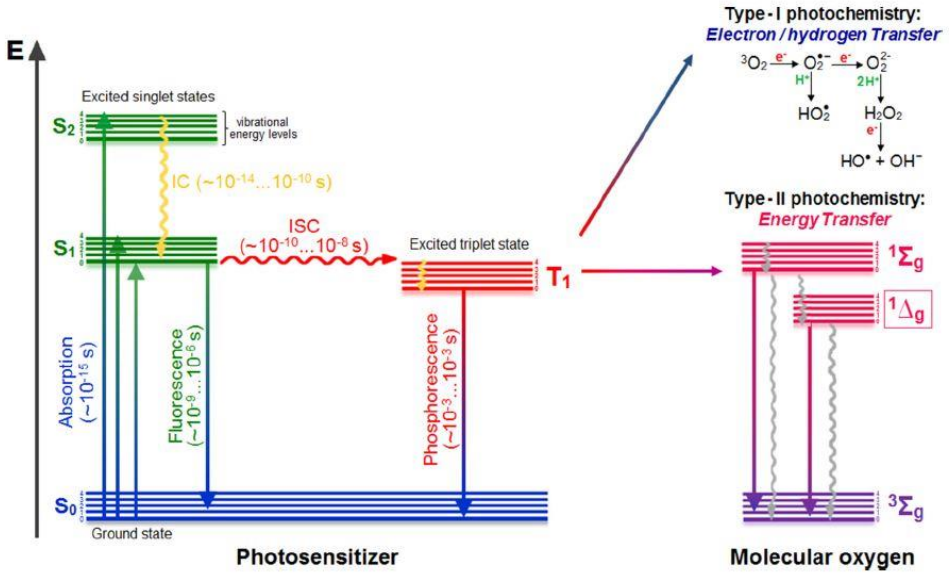

Figure 12: Photodynamic reaction pathway of PS with light and oxygen according to Jablonski. *Sourced from Dabrowski et al 2016.*

I.5.2.1. Classification of the different PS

PS can be natural and synthetic compounds. These natural PS are extracted and purified from living system (plants, fungi). Lots of applications have already been described (Azizullah *et al* 2014; Song *et al* 2014; de Menezes *et al* 2014a/b; Fracarolli *et al* 2016; Yoshida *et al* 2017; Spaeth *et al* 2018; Lukseviciute and Luksiene 2020). The second type of PS concerns molecules that can be synthesized by chemical reactions and also successfully tested on various domains (Hidalgo *et al* 2015; Nazir *et al* 2015; Abrahamse and Hamblin 2016; Song *et al* 2017; Muehler *et al* 2017; Hamblin and Abrahamse 2020).

More specifically, PS can be divided into porphyrinoid and non-porphyrin molecules (Issawi *et al* 2018). The difference between these two categories is that the porphyrinoid compounds consist of a tetrapyrrolic

backbone whereas the other groups are represented by a variety of other structures. In this manuscript, only the porphyrinoid PS are taken into consideration: porphyrin and chlorin (Figure 15 and 18).

I.5.2.2. Tetrapyrrolic photosensitizers: porphyrins and chlorins

In this present manuscript, the biological applications of porphyrins and chlorins are developed.

I.5.2.3. Porphyrins

Porphyrin means “purple” in Greek, and these PS are among the most studied and well-known photosensitizers (Milgrom 1997). They are intensely coloured compounds. The principal characteristic of porphyrins is their particular structure that consists of 4 pyrrole moieties bound together by methine bridges (Figure 13).

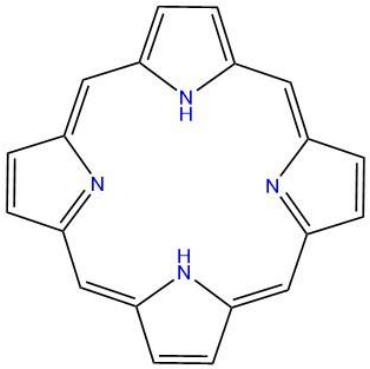

Figure 13: Basic chemical structure of porphyrin.

These macrocycles are capable of binding metals in the center of the ring, *via* the nitrogen atoms. When the porphyrin is metalated, the photophysical, photochemical and photobiological proprieties change. Porphyrins are characterized by a specific UV-Vis spectrum (from 400 nm to 800 nm). All porphyrins show a spectrum where a large intense band, the Soret band, is detected around 400 nm. In the free base porphyrin, the Soret band is accompanied by four smaller band called Q-bands whereas the metalated derivatives only have two Q bands (Valicsek and Horvath 2013) (Figures 14).

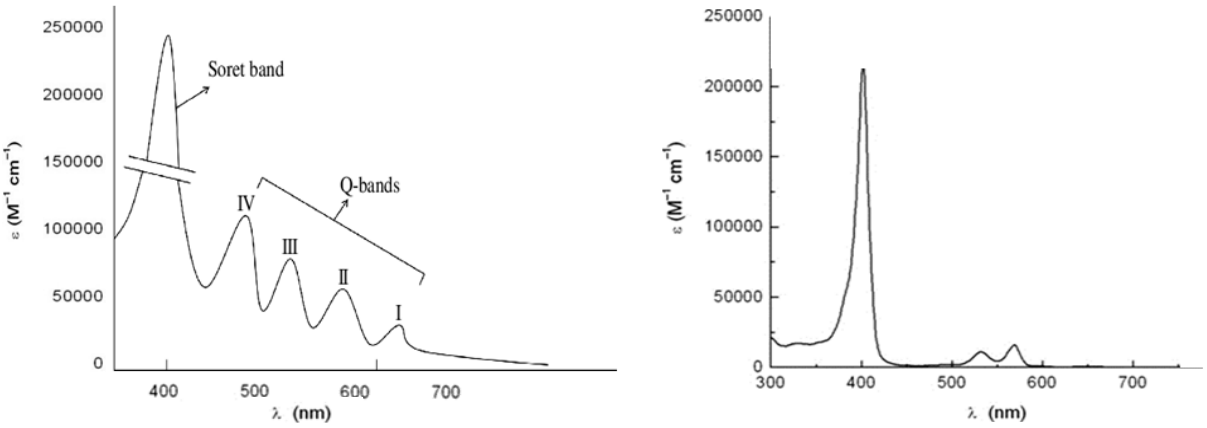

Figure 14: UV Vis absorption spectrum of free base and metalated porphyrin.

On the left: free-bases porphyrin, on the right: metalated porphyrin. Sources and modified from Josefsen and Boyle 2008 and Zakavi et al 2012.

The porphyrin taken into consideration in this manuscript is a free base 5,10,15,20-(tetra-4-sulfonatophenyl) porphyrin tetra-ammonium (TPPS) (Figure 15).

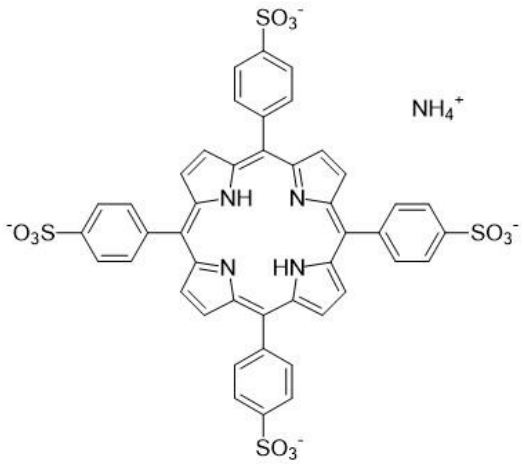

Figure 15: Chemical structure of free base 5,10,15,20-(tetra-4-sulfonatophenyl) porphyrin tetra-ammonium (TPPS).

NH_4^+ is the counter ion.

I.5.2.4. Chlorins

Chlorophyll derivatives from plants and bacteria are known to be good PSs (Alisson and Maghissi 2013). These derivatives are namely, chlorins and bacteriochlorins, and they display different

photochemical, photophysical and photobiological properties. The chlorin macrocycle is the skeleton of chlorophyll *a* and *b* in plant photosynthesis whereas bacteriochlorins provide the basis for the structure of bacteriochlorophyll *a* in bacteria. These molecules are almost identical to porphyrins in chemical structure. However, they differ as chlorin loses one double bond in one of the pyrrole moieties and the bacteriochlorin loses two double bonds (see Figure 16; Yoon *et al* 2013).

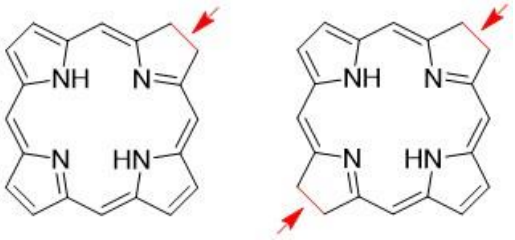

Figure 16: Basic chemical structure of chlorin and bacteriochlorin.
The arrows in red display where the double bond has been lost.

The loss of these double bonds causes a red-shift of absorption of the Q-bands (Figure 17).

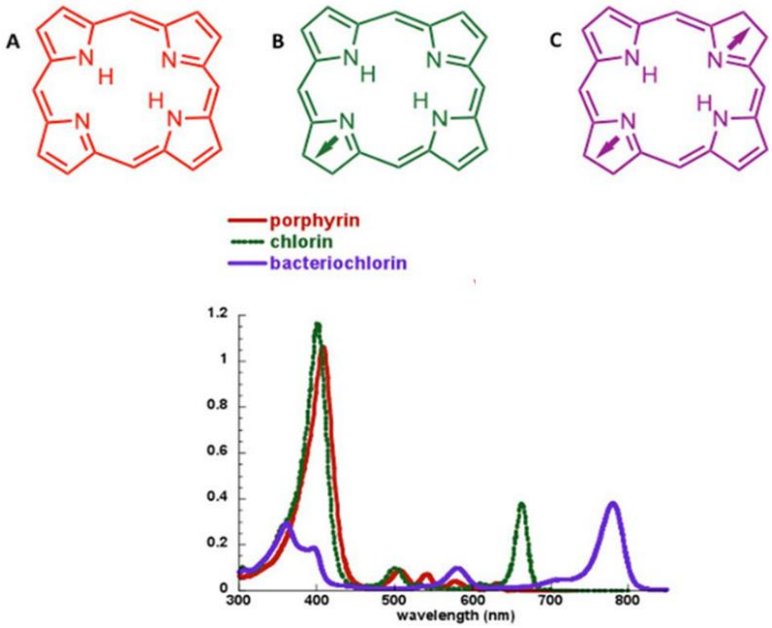

Figure 17: Difference between the UV vis spectra of porphyrin, chlorin and bacteriochlorin.
Sourced from Abrahamse and Hamblin, 2016.

The reason why bacteriochlorin is not commonly used is due to its tendency to undergo oxidation rapidly. The chlorin taken into consideration here is chlorophyllin (see structure in Figure 18). This PS is an approved food additive (E140 and E141) and it behaves like a typical PS; after light-

activation, it produces ROS that are harmful for pathogens, bacteria, viruses and fungi (Luksiene et al., 2004; Luksiene, 2005; Luksiene *et al* 2010; Luksiene and Brovko, 2013; Luksiene, 2014; Glueck et al 2019; Luksiene and Buchovec, 2019; Lukseviciute and Luksiene 2020).

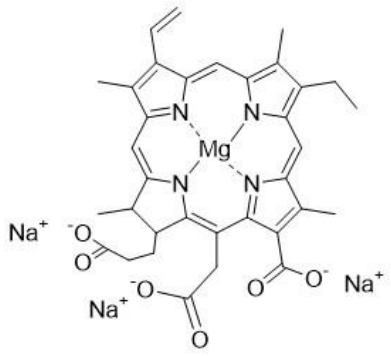

Figure 18: Chemical structure of chlorophyllin (E140).

Na^+ is the counter-ion

I.5.2.5. Natural porphyrins and tetrapyrrolic pathway

Tetrapyrrolic pathways play an important role in biological systems. Products of these pathways are naturally occurring heme and chlorophyll. These two structures consist of a porphyrin and a porphyrin-type molecule (chlorin) in the active centre, respectively (Battersby *et al* 1980, Senge *et al* 2015). Heme and chlorophyll also differ by the metal that they have in the centre. Chlorophyll has a Mg^{2+} metal, whereas heme has a Fe^{2+} (Figure 19).

The tetrapyrrolic pathway, that allows for the formation of heme and chlorophylls, is highly regulated. This pathway and a part of this regulation is discussed in the publication below (Ambrosini *et al*. 2019 “How protoporphyrinogen IX oxidase inhibitors and transgenesis contribute to elucidate plant tetrapyrrole pathway”).

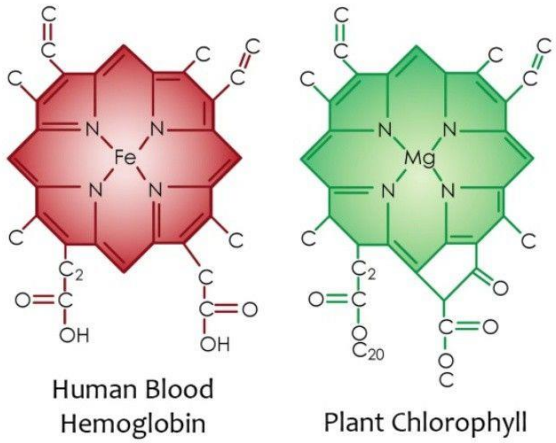

Figure 19: Chemical structure of Hemoglobin and chlorophyll.

I.5.2.6. PUBLICATION 1: How protoporphyrinogen IX oxidase inhibitors and transgenesis contribute to elucidate plant tetrapyrrole pathway

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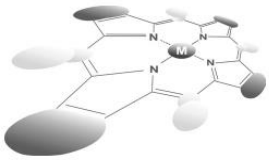

How protoporphyrinogen IX oxidase inhibitors and transgenesis contribute to elucidate plant tetrapyrrole pathway

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ABSTRACT: Several families of herbicides, especially diphenyl ether (DPE) and pyrimidinedione, target the plant tetrapyrrole biosynthesis pathways and in particular one key enzyme, protoporphyrinogen IX oxidase (PPO). When plants are treated with DPE or pyrimidinedione, an accumulation of protoporphyrin IX, the first photosensitizer of this pathway, is observed in cytosol where it becomes very deleterious under light. Indeed these herbicides trigger plant death in two distinct ways: (i) inhibition of chlorophylls and heme syntheses and (ii) a huge accumulation of protoporphyrin IX in cytosol. Recently, a strategy based on plant transgenesis that induces deregulation of the tetrapyrrole pathway by up- or down-regulation of genes encoding enzymes, such as glutamyl-*t*RNA reductase, porphobilinogen deaminase and PPO, has been developed. Against all expectations, only transgenic crops overexpressing PPO showed resistance to DPE and pyrimidinedione. This herbicide resistance of transgenic crops leads to the hypothesis that the overall consumption of herbicides will be reduced as previously reported for glyphosate-resistant transgenic crops. In this review, after a rapid presentation of plant tetrapyrrole biosynthesis, we show how only PPO enzyme can be the target of DPE and how transgenic crops can be further resistant not only to herbicide but also to abiotic stress such as drought or chilling. Keeping in mind that this approach is mostly prohibited in Europe, we attempt to discuss it to interest the scientific community, from plant physiologists to chemists, who work on the interface of photosensitizer optimization and agriculture.

KEYWORDS: diphenyl ether, herbicides, photosensitizers, plant transgenesis, protoporphyrinogen oxidase, pyrimidinedione, tetrapyrroles.

INTRODUCTION

After the Second World War, crop yields and agricultural dependencies were considerably raised, becoming largely dependent on pesticides, and making the agriculture and crop business very efficient and profitable. However, this was also very destructive for wildlife and, unfortunately, detrimental to human health. Three main classes of pesticides are largely produced by agrochemical industries: fungicides, herbicides and bactericides. These molecules are normally very specific and directed against one specific living group. For example, adventices (weeds) are eliminated by glyphosate or oxyfluorfen [1, 2]. Nevertheless, time exposures and treatments make these molecules hazardous for other groups such as humans and wildlife [3]. Thus, around the world, agricultural practices based on excessive pesticide consumption have become an increasing and major problem for both human and wildlife health. Therefore, environmental protection must be rapidly taken into account and resolved.

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In agriculture, two situations must be considered: the pre-harvest when plants are cultivated and the post-harvest when fruits and vegetables are harvested. The first is directly linked to open field farming and very dependent on pesticide inputs such as herbicides and antimicrobial molecules, for crop yield performance. The latter situation concerns vegetable and fruit conservation or preservation for the food industry. To gain insight into the exciting challenge in optimized agriculture and food industry, Antimicrobial PhotoDynamic Treatment (APDT) has been recently envisaged for the improvement of agriculture with respect to wildlife and environmental health and safety. APDT in agronomy could be a large-scale strategy for both pre- and post-harvests. Independent of the situation, APDT depends on a complex molecule excited by light called a photosensitizer (PS). The aim of this approach is to kill microorganisms or weeds that are largely responsible for food rot or decrease in crop yields, without depletions of crop growth. Recent studies based on exogenous photosensitizer treatment on various species growing *in vitro* or in field against bacteria or fungi were performed with encouraging results [4–14]. The recent studies about potential direct APDT applications on plants and/or microorganisms have been collected and largely illustrated in Issawi *et al.* [15]. Otherwise, to our knowledge, only a few research teams have implemented the APDT approach in fruit decontamination and conservation [16–19]. They tested a natural PS from chlorin group, chlorophyllin (a food additive known as E140) or curcumin on fruits such as strawberries, showing a delay of fruit rot [20–24]. This strategy was also applied to vegetables such as lettuce, tomato and spinach [19]. All the data relative to food preservation have been well reviewed and thus will not be emphasized in this review [16, 23, 25–29].

In our opinion, an additional side of plant photodynamic treatment that could be described as indirect photodynamic treatment which is based on plant transgenesis tools is generating a lot of interest but has not yet been compiled. This alternative, imagined in 2000s, only concerns herbicide resistance [30]. Until now, 60% of pesticides used worldwide correspond to herbicide molecules that are directed against undesired plants [31]. The difficulty is that plants called adventices (or weeds) have a very rapid growth rate and a huge seed production, making them very efficient and mostly dominant in crop fields.

Since the two last decades, manipulations of tetrapyrrole biosynthesis genes have been carried out to kill plants by forcing them to accumulate photosensitizers (PS) such as protoporphyrin IX, the first natural PS. Surprisingly, these transgenic plants showed herbicide resistance and all the tested herbicides recognized the same enzyme target, the protoporphyrinogen IX oxidase called Protox or PPO (Fig. 1). Indeed, at least 6 classes of herbicides are PPO inhibitors. They recognize and inhibit its catalytic site [32, 33]. In this review, only two classes: diphenyl ether (DPE) and pyrimididione, which are largely used in agriculture all over the world, will be investigated [15, 32–37]. It was thought that the strategy implying foreign PPO expression by plant transgenesis was a good way to diminish herbicide demand as previously shown with glyphosate and transgenic plants overexpressing foreign 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) that is not recognized and inhibited by glyphosate [38]. Nevertheless, in a European context, where genetically modified plants are not welcome, this type of approach should probably be given up. Regardless, transgenic plants and gene manipulations have given rise to a better understanding of tetrapyrrole pathway regulation. Furthermore, the study of these plants has shown interactions between tetrapyrrole pathway regulation and response to abiotic stress such as chilling or drought [39, 40]. Therefore, after describing the plant tetrapyrrole biosynthesis, we will focus on the effects of overexpression of PPO and other key enzymes in crops, in order to better understand the mechanism that could explain herbicide resistance in PPO transgenic plants.

Plant tetrapyrrole biosynthesis

The tetrapyrrole biosynthetic pathway has been described in mammals, plants and microorganisms [15, 41–43]. The tetrapyrrole core is a complex macrocycle that can be linked to a long linear carbon chain, as in chlorophylls, to fix them in thylids or surrounded by proteins to form hemoglobin or Cytochrome P450. Tetrapyrroles are able to bind a metal in its core, magnesium for chlorophylls or iron for hemoglobin, with metalation being under the control of chelatases. Tetrapyrroles, metallated or not, play a crucial role in sunlight collection (chlorophylls *a* and *b*), nitrogen fixation, oxygen transport, and ROS scavenging (heme) [43–46].

The first common precursor is the 5-aminolevulenic acid (5-ALA), which may come from succinyl-CoA glycine *via* the Shemin pathway or glutamate *via* the C5 pathway. The C5 pathway is characteristic of plants and of most bacteria, while the Shemin pathway is characteristic of humans, some bacteria and yeasts. After the formation of 5-ALA, a series of chain reactions leads to the formation of uroporphyrinogen III. Subsequently the uroporphyrinogen III can lead to the formation of siroheme (synthesized in bacteria, yeast and plants), cobalamin B12 (synthesized in bacteria and archaea), cofactor F430 (which only exists in methanogenic bacteria) and coproporphyrinogen III through the coproporphyrinyl oxidase [47–49]. Following formation of coproporphyringen III, protoporphyrin IX (PPIX) is produced. PPIX is the main key precursor of heme and chlorophylls (Fig. 1). Thus, its synthesis has to be highly regulated. Furthermore, PPIX must be confined in the plastidial compartment to avoid its potential

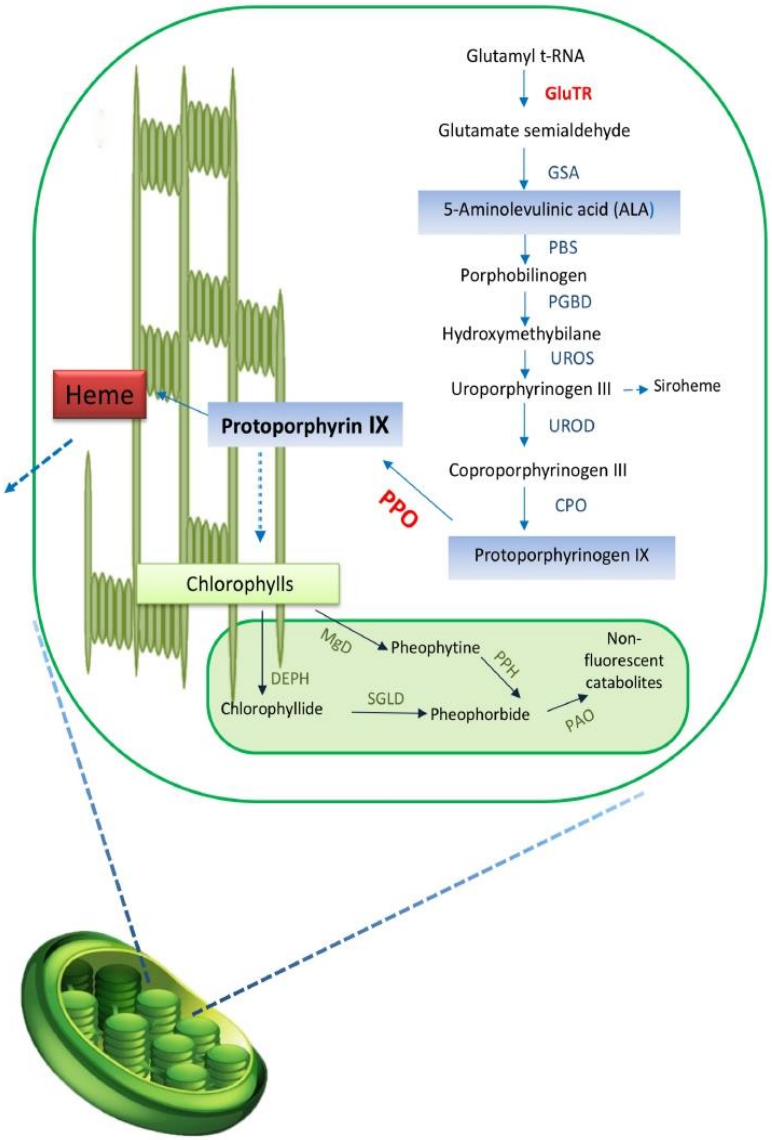

Fig. 1. Plant tetrapyrrole biosynthesis pathway and chlorophyll turnover in chloroplast. The two key enzymes of tetrapyrrole pathway: GluTR: glutamine *t*-RNA reductase and PPO: protoporphyrinogen IX oxidase are shown in red. Protoporphyrin IX is located in thylakoids of chloroplasts and leads to heme and chlorophylls. Heme is partially translocated into cytosol (blue dashed arrow). GSA: Glutamate semialdehyde aminomutase; PBS: porphybilinogen synthase; UROS: uroporphyrinogen synthase; UROD: uroporphyrinogen decarboxylase: CPO: coproporphyrinogen oxidase. Black arrows in green box indicate chlorophyll turnover pathway with the major derivatives that are also photosensitizers and enzymes. MgD: Mg-dechelatase; DEPH: dephytylase, PPH: pheophytinase, PAO: pheophorbide oxygenase; SGLD: stay-green like Mg-dechelatase

photo-activation which could be fatal to plants. Two other compounds, uroporphyrinogen III and coproporphyrinogen III, could also have the capacity to become powerful PSs [15]. Due to the vital importance of this pathway for plant life, it is thoroughly regulated at transcriptional and post-transcriptional levels. The rate-limiting step is certainly the synthesis of 5-ALA, particularly the activity of glutamyl *t*RNA reductase (GluTR) that is encoded by a small genetic family called Hem A [15, 42]. Moreover, glutamine *t*-RNA reductase (GluTR) is the second key enzyme of the pathway that is regulated by feedback by regulator protein (Flu) but it has never been identified as a potential herbicide or drug targets [42]. In plants, enzymes, especially UROS, UROD, CPO and PPO, must always be expressed in large amount even under normal conditions, in order to

supply metabolites of tetrapyrrole in case of stress [41]. In addition, at the heme/chlorophyll branching point, protoporphyrinogen oxidase and magnesium chelatase are subjected to redox regulation by thioredoxin. Moreover, in the Mg branch, the synthesis of 5-ALA is under negative control through protochlorophyllide [41, 42, 50–52].

Overexpression of PPO in crops

In the beginning of the 2000s, genes encoding PPO were cloned from bacteria, humans and plants and used to generate transgenic rice, maize and tomatoes [53–58]. In plants and algae, two classes of PPO were characterized, namely PPO-1 and PPO-2, from chloroplasts and mitochondria respectively [55]. Both plant isoenzymes PPO-1 and PPO-2 are coded by Hem Y, but the two isoenzymes of tobacco or spinach show a weak amino acid sequence identity (around 27% for tobacco PPO-1 and 2) [59]. Both PPOs are bound to the internal membranes of chloroplasts or mitochondria; PPO-1 enzymes are linked by their Nt sequence at the consensus domain (Gly-X-Gly-XX-Gly) to thylakoids. Until now, no cytoplasmic PPO has been identified [60]. The hypothesis underlying overexpression of gene encoding PPO was that the plants were not able to live anymore due to a huge accumulation of PPIX. Surprisingly, transgenic crops like Arabidopsis and rice, overexpressing human, bacterial or plastidial plant PPO genes, could survive, showing a significant amount of protoporphyrin IX responsible for bronzing necrosis or photobleaching, and an increase in superoxide dismutase and peroxidases activities that mitigate photodynamic stress [53–56, 58]. Tomato plants over-expressing plastidial *Arabidopsis thaliana* PPO under the control of 35S promoter showed significantly higher enzyme activity and a resistance to the DPE herbicide acifluorfen [55]. To a larger extent, the transgenic crops overexpressing plant plastidial, bacterial or human PPO showed resistance to DPE, pyrimidinediones such as butafenacil, and even to abiotic stress, for instance, drought and chilling. Phung *et al.* studied the response of transgenic rice expressing bacterial PPO and showed a resistance to drought stress conditions [38].

Overexpression of other key enzymes in crops

Additionally, gene encoding bacterial ferrochelatase overexpression leads to acifluorfen resistance in rice plants due to heme overexpression which plays a crucial role in signaling and ROS scavenging [61]. Furthermore, overexpression of *AtHEMA*1 encoding Glutamine *t*RNA reductase (GluTR) leads to protochlorophyllide accumulation in Arabidopsis and much more in tobacco plants [62]. *RUG1* gene encoding porphobilinogen deaminase (PGBD) was characterized and overexpressed in *Arabidopsis thaliana* showing leaf necrotic spots and supernumerary shoot meristems [63].

The chlorophyll catabolic pathway has not yet been manipulated to overproduce endogenous PS like pheophorbide, probably due to the tiny regulation of chlorophyll inside cells. However, several harmful PS like pheophytine and pheophorbide can be found within that turnover pathway [64].

Suppression of key gene expression involved in tetrapyrrole pathway

Antisense RNA was used to knock down the genes encoding key enzymes of the tetrapyrrole metabolic pathway. Mock and Grimm reduced the level of UROD leading to the accumulation of the harmful PS uroporphyrin III and subsequently growth inhibition in tobacco under intense light (Fig. 1) [65]. Intriguingly, RNA antisense against UROD and CPO in tobacco induced, beyond the accumulation of uroporphyrin III and coproporphyrin III, necrotic lesions similar to the hypersensitive reaction after pathogen attack, with the high level of pathogenesis-related proteins and salicylic acid conferring resistance against tobacco mosaic virus [66].

Moreover, PPO antisense expression leading to systemic acquired resistance was also reported in *Arabidopsis thaliana* [67]. Ayliffe and co-workers developed an activation tagging system based on a transposable element to enable UROS gene silencing in barley. They showed that the reduced UROS enzyme activity led to the accumulation of the substrate hydroxymethylbilane, which spontaneously cyclizes to form uroporphyrin I, resulting in necrotic lesions and involved in retrograde signaling [68].

Unexpectedly, antisense against the CHL-H subunit of magnesium chelatase (one of the three subunits of Mg chelatase) in transgenic barley did not cause a photosensitive reaction but instead caused a reduced level of heme and chlorophyll due to negative feedback in the early steps of the tetrapyrrole synthesis [69].

How do PPO transgenic plants resist DPE?

Indeed, deregulations of the plant tetrapyrrole pathway, especially based on PPO overexpression, lead to resistance to several families of herbicides, particularly DPE (oxyfluorfen, acifluorfen) and pyrimidinedione (butafenacil). Photodynamic herbicides and PPIX target the same site of the PPO protein [55, 70, 71]. The inhibition of PPO by DPE (acifluorfen) or pyrimidinedione (butafenacil) is a competitive inhibition that inhibits PPO activity (Fig. 2a). Moreover in tobacco, at position 392 of PPO, a Phe or Tyr in mitochondrial PPO (PPO-2), seems to be essential for the orientation of the herbicide molecule into the pocket [71]. The first ring (A) of proto IX is stacked by Phe^{392}, the second ring (B) is stacked between Leu^{356} and Leu^{372}, and the propionate carboxylate of the third ring (C) is bound to Arg^{98} while the fourth ring (D) is

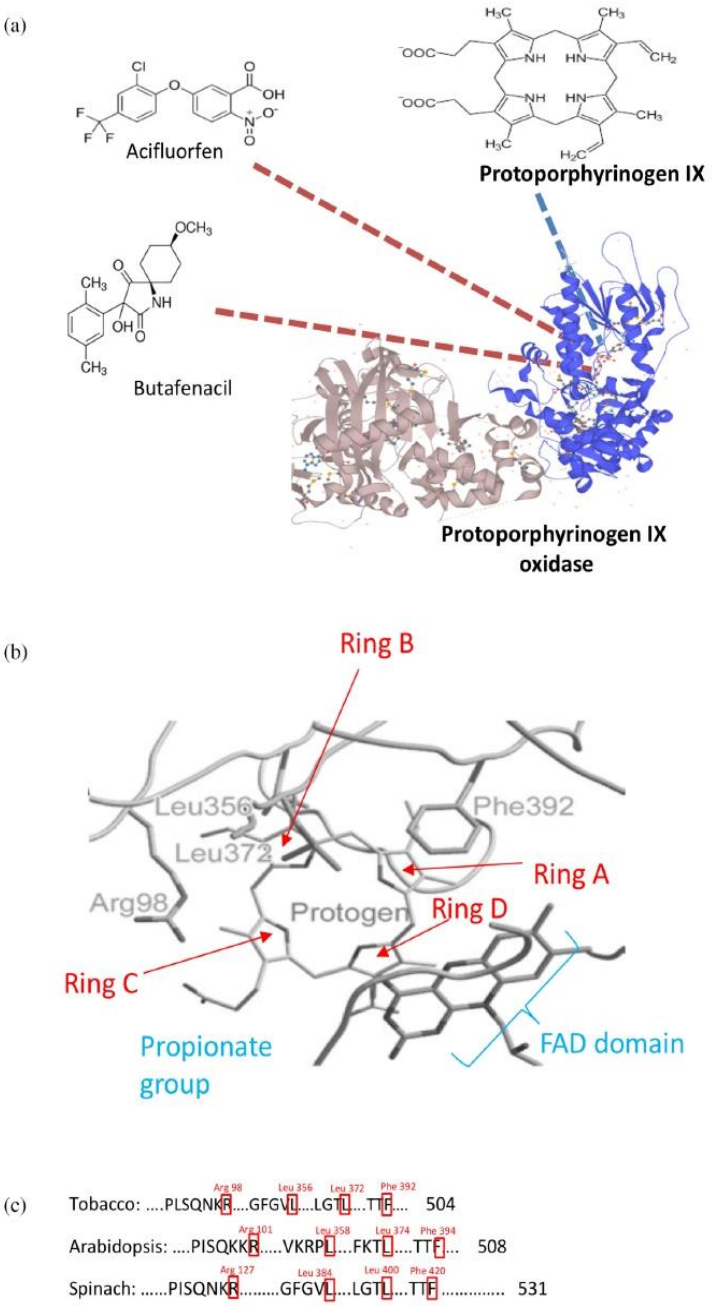

Fig. 2. Herbicides and Protoporphyrinogen IX competition **on Ct of mitochondrial PPO** (a) Tridimensional structure of mitochondrial PPO from tobacco (*Nicotiana tabacum*) is from UniProt bank (access number O24164). Herbicides are represented by acifluorfen as diphenyl ether and butafenacil as pyrimidinedione. The blue dashed arrow indicates a normal interaction between protoporphyrinogen IX substrate and the pocket of PPO with the essential position of Phe^{392} and the red dashed lines the inhibition by photo-herbicides on the same site of PPO. (b) Ring A and ring B of protoporphyrinogen IX is stacked by Phe^{392} and between Leu^{356} and Leu^{372}, respectively. The propionate carboxylate of the ring C is bound to Arg^{98} and the fourth ring (D) is located near the Flavin Adenine dinucleotide Domain (FAD domain), modified from Heinemann *et al.*, 2017 [73]. (c) Multiple amino-acid sequence alignments of plant PPO-2: *Nicotiana tabacum* (access number O24164), *Arabidopsis thaliana* (access number Q8S9J1) and *Spinacia oleracea* (access number Q94IG7). Red boxes indicate amino acids involved in substrate/herbicides recognition

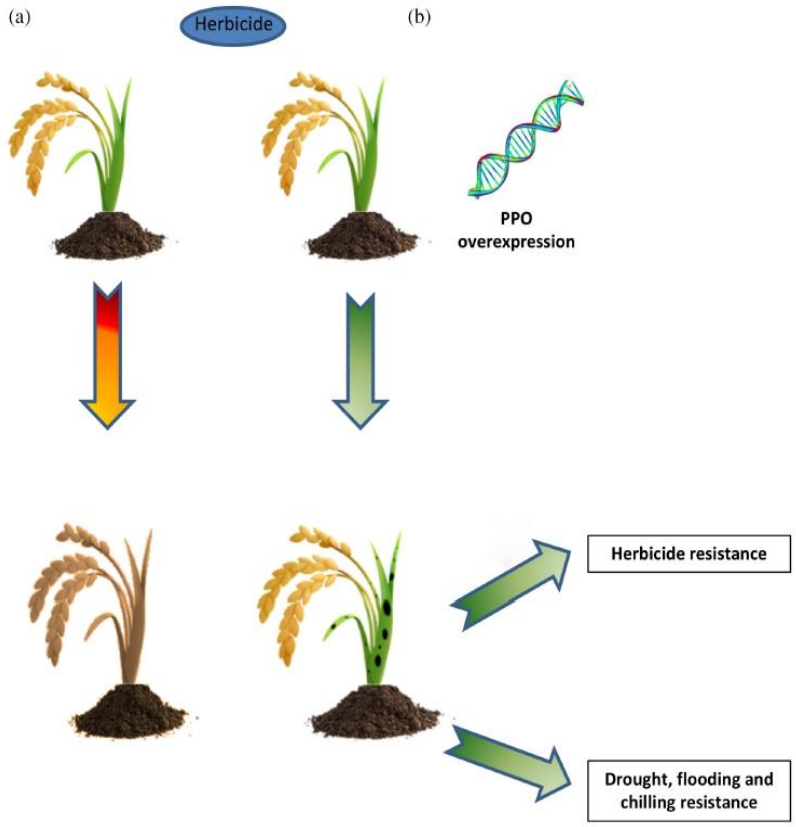

Fig. 3. Photo-herbicide, crop and PPO transgenesis strategy. (a) crop sprayed by an herbicide such as DPE or pyrimidinedione is dying. (b) Transgenic crop overexpressing PPO treated or not by DPE or pyrimidinedione, well grows showing some leaf necrosis represented by black spots. Moreover, this plant becomes multi-resistant to abiotic stresses such as drought, chilling and flooding

located near the FAD domain (Fig. 2b) [71–73]. In other species, such as Arabidopsis and spinach, these functional amino acids seem to be shifted (Fig. 2c). When DPE interacts with PPO, extra production of protoporphyrinogen IX is delocalized from the chloroplast to the cytosol and transformed into PPIX. Protein transporters from chloroplasts to cytoplasm and putative cytoplasmic PPO are still unknown.

Cytoplasmic PPIX becomes photo-activated and consequently leads to a high ROS production that triggers plasma membrane oxidation and death [33, 68]. When genes encoding PPO are overexpressed in plants, PPIX remains in chloroplasts where it is less deleterious. Indeed, PPIX triggers ROS production but as it occurs in chloroplasts that are double membrane organelles, plant cells are self-protected as supposed by Li and Nicholls [55]. When genes encoding PPO were overexpressed in plants, a decline of porphyrin intermediates (Fe-protoporphyrin IX, Mg-protoporphyrin IX, protochlorophyllide, Mg-protoporphyrin methyl ester) was observed while the level of heme and chlorophyll remained unchanged. Indeed, these metabolites act as signal molecules in the complex network controlling stress-responsive gene induction. Moreover, the unbound heme serves as a ROS scavenger through its incorporation in apoproteins such as catalase and peroxidase. Furthermore, chlorophyll plays a pivotal role in maintaining photosynthesis under stress [38, 39, 43]. Finally, when transgenic plants overexpressing PPO are treated with a photo-herbicide, they become resistant to the herbicide (Fig. 3).

CONCLUSION

Until recently, all the strategies performed to bypass or decrease herbicide use based on plant tetrapyrrole deregulation by transgenesis were essentially foreseen to increase crop yield, as was achieved for glyphosate resistance of transgenic crops [74]. Indeed, it was shown that transgenic crops resistant to glyphosate led to a true decrease in herbicide consumption the first time they were cultivated in fields. Unfortunately, with time, weeds became super-resistant to glyphosate, and farmers are now forced to use higher amounts of glyphosate, even more than before transgenesis. Thus, this type of approach

in agriculture must be considered as very dangerous for plant, wildlife, water contamination and human health as shown with glyphosate transgenic plants largely cultivated around the world [40]. Fortunately, researchers developing PPO-overexpressing transgenic crops showed a surprising result, resistance to drought, which is also of interest with the idea to generate plants able to be resistant to the Earth's global warming. Moreover, their work helped increase understanding of the high regulation of this key pathway to respond to abiotic stress [38, 39]. Therefore, to gain insight into the reduction of herbicide consumption, a direct photodynamic treatment must be developed using a very low concentration of very efficient PS, which will be rapidly photodegraded and will kill weeds without altering crop growth and development, as previously described by our team [9, 12, 15] and those of Almeida [14] and Braga [6–8, 10–11].

Acknowledgments

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ABBREVIATIONS

APDT: antimicrobial photodynamic treatment; DPE: Diphenyl ether; PPO: protoporphyrinogen oxidase, PPIX: protoporphyrin IX; PS: photosensitizer; ROS: reactive oxygen species.

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I.6. PhD objectives

Since the 1960's, the use of huge amounts of pesticides to increase crop yield caused environmental pollution, loss of fauna and flora biodiversity, and the development of multi-resistant plant pathogens. Now, agriculture practises must become environmentally friendly especially in Europe and moreover in France. Thus, new strategies to improve agriculture, without side effects for the environment and human health, need to be developed. One of the major problems in agriculture remains the struggle against pathogens, especially fungi. Various strategies to fight against fungi in particularly *B. cinerea* have been described in the introduction of this manuscript. In this PhD work, in order to combat against this fungus, a new approach called antimicrobial photodynamic treatment (APDT) is investigated. This treatment is based on the activation of a photosensitizer (PS) under sunlight.(Figure 20).

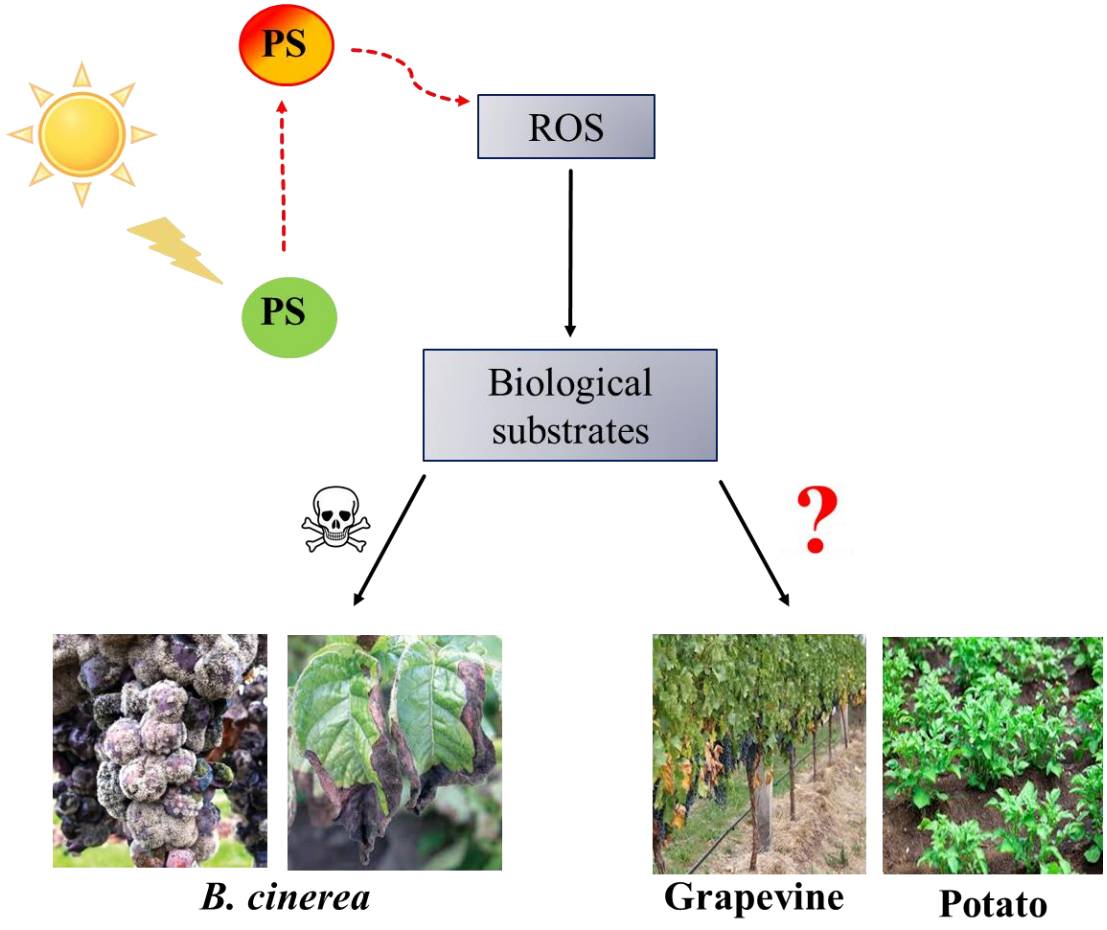

Figure 20: Scheme of APDT mechanism in the context of this manuscript

In the third chapter, the response of *B. cinerea* to exogenous TPPS and Chl treatments was investigated under a 16-hour photoperiod and dark conditions. TPPS was chosen because encouraging results were already obtained on Arabidopsis and tomato plantlets (Guillaumot *et al* 2016; Issawi *et al* 2018). More specifically, the anionic porphyrin tetra-4-sulfonatophenylporphyrin tetra-ammonium(TPPS) did not provoke any harmful effect on both plantlets, even at concentrations as high as 50 µM. However, cationic tetra(N-methylpyridyl) porphyrin, tested at 3.5 µM, inflicted harmful effects on both 14-day-old Arabidopsis and tomato plantlets.

Furthermore, TPPS is a synthetic molecule and we hypothesised that Chl, a natural water-soluble compound, could be a better candidate for future agricultural applications.

In chapter IV, the effect of TPPS was investigated on *in vitro* clonal grapevine plantlets (*Vitis vinifera*). Moreover, the experiments were conducted on three different varieties: Sauvignon, Chardonnay and Merlot because of their different susceptibilities to *B. cinerea.* The overall goal was to reproduce a "pathosystem prototype" *in vitro* by using a micromolar concentration of TPPS. This pathosystem was developed using detached grapevine leaves in contact with *B. cinerea* pre-treated or not with TPPS. The results were reported at the end of chapter IV in the manuscript (submitted paper in Scientific Reports "Photodynamic inactivation of *Botrytis cinerea* by an anionic porphyrin: an alternative pest management of grapevine).

However, in chapter V, the response of potato plantlets (S*olanum tuberosum*) to an exogenous supply of TPPS and Chl was measured. In this case, four different varieties were taken into consideration: Bintje, Hinga, Grenadine and Laurette. The fact that different varieties of well-known plants were studied represents a milestone for this type of strategy. Furthermore, a pathosystem for potato was not developed in this work as it will be more interesting to investigate the effect of the APDT against a more common potato pest such as *Alternaria solani.*

II. Material and methods

II.1. Photosensitizers

Two photosensitizers are used in this work. The first PS corresponds to a free base porphyrin 5,10,15,20-(tetra-4-sulfonatophenyl) porphyrin tetra-ammonium (named TPPS) and the second one, a chlorin: chlorophyllin (named Chl) (Figure 15 and 18, respectively).

Both PS are perfectly water-soluble and thus, prepared in sterile and pure water to a final concentration of 1 mM. TPPS can be stored for two weeks and Chl that is unstable in solution, not more than few days. Both stock solutions are kept under dark conditions at 21 °C. TPPS was purchased from the company PorphyChem (Dijon, France) and Chl from the company Carl Roth (Karlsruhe, Germany).

II.2. Fungal material and culture

II.2.1. *Botrytis cinerea* strain

The strain of *B. cinerea* (UBOCC-A-117017) was collected and isolated from infected tomato plants. This was provided by Dr Weill from the UBO Culture Collection, UBOCC-EDSIAB, Plouzané, France.

II.2.2. *Botrytis cinerea* mycelium growth curve on agar medium

The *B. cinerea* culture medium was potato dextrose agar (PDA purchased from Difco). After autoclaving (120ºC for 20 min), PDA was supplemented with or without TPPS in the concentration range of 0.5–3.5 µM or with 25 and 50 µM Chl, respectively.

To maintain the fungus growth or to follow PS effect on mycelium growth, a plug of 0.6 cm diameter of two-week old *B. cinerea* was placed in the middle of Petri dish containing PDA with or without PS at the desired concentration. Measurements of diameter of the circular mycelium growth were performed every day to draw the growth curves. For both PS, the growth was monitored under 16h photoperiod and dark conditions.

II.2.3. *Botrytis cinerea* spore isolation and germination

The culture medium for maintaining and germinating the spores was Potato Dextrose Broth (PDB), autoclaved at 120 ºC for 20 min.

II.2.3.1. Spore extraction

A plate containing at least two-week old *B. cinerea* was harvested by gently scraping the gel surface of the culture in the presence of 10–15 ml of 0.05 % solution of Tween 80. Sterile mini glass beads (2 mm diameter) were added to the suspension that contained mycelium and spores. The suspension was vortexed for 10 seconds, 3 times. Subsequently, the suspension was filtered on a 40 µm nylon filter to remove the mycelium. Spore numeration was performed under Malassez cell and the enriched spore suspension was diluted in order to reach a concentration of 10^3–10^5 spores.ml^{-1}.

II.2.3.2. Photodynamic treatment of TPPS on spore germination

To investigate TPPS potential inhibitory effect, the protocol described in Alasmari *et al* 2017 was followed and slightly modified. The experiment was conducted *in vitro* under a 4-hour illumination (photon flux density of at least 100 µmol.m^{-2}.s^{-1}) with 3.5, 12.5 and 50 µM TPPS.

The extracted spores were suspended in a phosphate buffer (pH 7) in a small Petri dish (60 mm diameter), at a concentration of at least 10^4 spores.ml^{-1}, in a 4 ml final volume. Aliquots of 2 ml of spores suspension were mixed with the same volume of TPPS solution (1:1 *v/v*). The Petri dishes were left under light for at least 4 hours under low stirring (80 rpm). In order to determine the viable spore reduction after 4-hour light exposure, 100 µl of each sample was transferred on PDA and incubated for 4–5 days at 22ºC under dark. The following calculation was used for determining the percentage reduction by counting the colony forming units (CFU):

% Reduction = (A-B)/A*100

Where:

A: the mean of spore reduction without PS after 4 hours of illumination

B: the mean of spore reduction with PS after 4 hours of illumination

II.3. Plants material: varieties and cultures

II.3.1. Grapevine clone culture

Chardonnay (clone 7535) was provided by Pr. Clément (Université de Reims, Champagne-Ardenne, France). Sauvignon (clone 379) and Merlot (clone 373) were provided by the Institut Français de la Vigne et du Vin (Bordeaux, France). Intermodal explants of grapevine were dissected and placed in glass tubes or jars containing half Chée and Pool medium and 2 % (w/v) sucrose, Sobigel solidified medium (pH 5.9) for 1 to 3 months. PS were added to the medium after autoclaving. The cultures were then exposed to an Osram spectrum white lamp (photon flux density of 120 µmol.m^{-2}.s^{-1}) for

16 hours and the temperature was maintained at 24 °C. Chée and Pool medium was purchased from Duchefa Biochemistry (Haarlem, Holland). Roots and aerial parts were rapidly dissected, frozen into liquid nitrogen and stored at -20 °C until use.

II.3.2. Infection of two- month-old grapevine leaves with *Botrytis cinerea* mycelium

Two-month-old grapevine detached leaves and four-day-old mycelium plug (0.6 cm diameter) were used to perform the "*In vitro* infection" experiment. This type of experiment was only performed for grapevine leaves. Mycelium plugs were firstly incubated in 12.5 or 50 µM TPPS and gently stirred for 8 hours at 22 °C under dark conditions. After incubation, fungus discs were placed on the upper leaf epidermis of the grapevine from Chardonnay, Merlot and Sauvignon varieties. The co-cultures were monitored daily and photographed under a Leica stereomicroscope.

II.3.3. Potato clone culture

Bintje, Hinga, Grenadine and Laurette clones were provided by Grocep, Lauriere (Haute-Vienne, France). Intermodal explants of 1-month-old clonal plantlets were dissected and placed in glass tubes or jars containing Murashige and Skoog (MS) basal medium plus MS vitamins, 2 % (w/v) sucrose and 100 $mg.L^{-1}$ myo-inositol solidified medium (pH 5.8). Photosensitizers were added to the medium after autoclaving. MS medium including vitamins was purchased from Duchefa Biochemistry (Haarlem, Holland). The cultures were then exposed to white light (photon flux density of 120 $\mu mol.m^{-2}.s^{-1}$) under a 16-hour photoperiod at 22 °C for 14 days or 1 month. Roots and aerial parts were quickly dissected before liquid nitrogen freezing and stored at -20 °C until use.

II.4. Microscopic analysis of mycelium or germinating spores

II.4.1. Confocal microscopy analysis

A LSM510META Zeiss confocal microscope (Carl Zeiss France, Marly-le-Roi, France) was used to perform the microscopic analysis on the *B. cinerea* mycelium and spores.

The samples used for the mycelium were a culture of 4 days old *B. cinerea* grown under dark on PDA containing 3.5 µM TPPS or 50 µM Chl. The spores were incubated in buffer phosphate or in PDB medium with a concentration of 10 µM TPPS or Chl at 22 °C. After incubation, the suspension was centrifuged for 5 minutes at 10,000 rpm. Subsequently, the pellet was resuspended in a new buffer phosphate or PDB medium without photosensitizers. The data was acquired using the spectral

acquisition mode for TPPS and Chl localisation (excitation wavelength was 405 nm and the emission was detected at 640 nm) and under the channel mode for examination of mycelium and spores.

II.4.2. Environmental Scanning Electronic Microscopy on mycelium fungus and infected leaves

This experiment was performed to visualise TPPS effect on mycelium structure and/or pathosystem: isolated leaves, *B. cinerea* and TPPS. Environmental Scanning Electronic Microscopy (ESEM) was conducted on *B. cinerea* mycelium. The fungus grew on plates containing, or not, TPPS for 4 days under 16 h of photoperiod or under dark. These were then examined under Environmental Scanning Electronic Microscope (ESEM Quanta 450, Felmi-ZFE, Graz, Austria). The width of hyphae was measured from the pictures obtained from ESEM. Furthermore, ESEM analysis was conducted on the two- month-old grapevine leaves infected with the 4-day-old *B. cinerea* mycelium pre-treated or not with TPPS.

II.5. Determination of enzymatic activities linked to ROS production

II.5.1. Extraction buffer composition

A) For *Botrytis cinerea*

After grinding in liquid nitrogen, the fine powder of mycelium that contains total proteins was dissolved in 1 mL of extraction buffer containing 50 mM phosphate buffer pH 7.8, 1 mM 1,4-dithiothreitol (DTT), 1 mM ethylene diamine tetra acetic acid, 1% (w/v) polyvinylpyrrolidone (PVP) and 10% (v/v) glycerol. This solution was centrifuged at 13,000 rpm for 20 minutes at 4°C. The protein concentration of the supernatant was determined according to Bradford (1976).

B) For plantlets

The protocol for the plant protein extraction was the same with the exception of extraction buffer that contains polyvinylpolypyrrolidone (PVPP) instead of PVP. PVPP inhibits interference between plant phenolic compounds and Bradford solution (Bio-Rad, France).

II.5.2. Superoxide Dismutase

The superoxide dismutase (SOD) activity protocol was performed for fungus and plants, in the absence of bathocuproine sulfonate (Guillaumot *et al* 2016). The standard curve was set so that the increase in absorbance at 560 nm was between 0.02 and 0.03 absorbance units per minute and was

monitored for 5 min. One unit of SOD was defined as the amount of enzyme required for a 50 % inhibition of NBT reduction. Five µL of the enzyme extract were used and the SOD specific enzyme activity was determined according to the protein concentration of each sample.

II.5.3. Catalase

The measurement of catalase (Cat) was performed for fungus and plants, according to the protocol described by Guillaumot *et al* 2016. Rapidly, 20 µg of total soluble proteins were transferred to a 50 mM phosphate potassium buffer (pH 7.0). To start the reaction, H_2O_2 was added to a final concentration of 20 mM and the final volume was 1 mL. The measurement of absorbance at 240 nm was monitored for 3 minutes and the H_2O_2 disappearance was calculated using the slope of the linear portion of the resulting curve using a molar extinction coefficient of 43.6 $M^{-1}.cm^{-1}$.

II.5.4. Guaiacol Peroxidase

As this enzyme activity is only described for plants, it was conducted on *in vitro* plants roots or aerial parts, according to the protocol described by Guillaumot *et al* 2016. The guaiacol peroxidase activity (GPX) was monitored at 436 nm. The increase in the absorbance is due to the formation of tetraguaiacol. For each sample, 10 µg of total soluble proteins were transferred to 50 mM phosphate potassium buffer (pH 7) and supplemented with 0.25% guaiacol (5% stock solution in 95% ethanol w/v). To start the reaction, H_2O_2 was added to a final concentration of 2.5 mM and the absorbance was monitored for 2 minutes. Molar extinction coefficient used for the calculation is of 25.5 $mM^{-1}.cm^{-1}$.

II.6. Hydrogen peroxide quantification

The measurement of hydrogen peroxide (H_2O_2) was always performed on fresh samples according to Guillaumot *et al* (2016). Fresh mycelium or plants (roots or aerial parts) are grounded in liquid nitrogen. 1 mL of extraction buffer was then added to the samples and the homogenates were centrifuged at 13,000 rpm at 4 °C for 20 min. As H_2O_2 reacts with an acidic solution of titanium (III) sulfate ($Ti_2(SO_4)_3$) to form pertitanic acid ($TiO(H_2O_2)^{2+}$), 335 µL of 0.1% titanium III sulfate (v/v) in a solution of 20% (w/v) H_2SO_4, was added to the mixture. The absorbance was then read at 415 nm. H_2O_2 amounts were expressed as $nM.g^{-1}$ protein.

II.7. Lipid peroxidation and MDA assay

This test was based on the determination of malondialdehyde (MDA) content. Approximately, 150 mg of fresh or frozen fungus or plants (roots or aerial parts), were grounded in liquid nitrogen. 1.5 mL of 20% (w/v) TCA was added to the powder. The mixture was centrifuged for 20 min at 13,000 rpm at 4 °C. The following procedure was performed in haemolysis tubes. For each sample, 0.5 ml was added to either 0.5 ml 20% TCA (−TBA) or 20% of TCA supplemented with 0.5% thiobarbituric acid (+TBA). These samples were transferred into a water bath for 30 minutes at 95 °C. Afterwards, the samples moved on ice to cool. Optical densities were determined at three wavelengths: 440, 532 and 600 nm, respectively. The peak at 400 nm is due to the possible presence of porphyrin in the extract and subsequent interference with absorbance readings (Guillaumot *et al* 2016)

1) [(Abs 532+TBA−Abs 600+TBA) − (Abs 532−TBA−Abs600−TBA)] =A.

2) {[(Abs 440+TBA − Abs 600+TBA) − (Abs 440−TBA − Abs 600−TBA)] 0.0571} = B.

3) MDA equivalents ($nmol.ml^{-1}$) = $[(A - B)/157,000].10^6$

II.8. Proline content determination

250 mg of the mycelium were grounded at 4°C in 1.5 mL of 5% (w/v) 5-sulfosalicylic acid (SSA). After centrifugation at 13,000 rpm for 15 min at 4°C, 0.2 ml of the supernatant was mixed with 0.8 ml of 1% (w/v) ninhydrin in 60% (v/v) acetic acid. The samples were incubated at 100 °C for 20 min and were allowed to cool to room temperature before the addition of 1 mL toluene. After a 4 h incubation at room temperature, the upper organic phase was isolated, collected and the absorbance was read at 520 nm. A standard curve was established with proline and the content was expressed in $\mu mol\ g^{-1}$ fresh weigh.

II.9. Total thiol Assay

After liquid nitrogen grinding, 1 ml of 0.2 N HCl was added to 100 mg of mycelium powder or plants (roots or aerial parts), the mixture was vigorously vortexed and kept on ice. A centrifugation at 13,000 rpm at 4 °C for 20 minutes was then performed. Afterward 500 µL of the extract solution were neutralized with 400 µL NaOH (0.2 M) and 50 µL NaH_2PO_4 (0.2 M). 700 µL of 0.12 M NaH_2PO_4, 6 mM EDTA and 0.1 mL of 6 mM Dithiobis 2 nitro benzoic acid (DTNB) were added to 200 µL extract. For standards, the extract was replaced by 0, 5, 10, 25 and 50 $\mu g.mL^{-1}$ glutathione (total volume 1 mL). The absorbance at 412 nm was read 5 minutes after the addition of standard or extract.

II.10. Cellular leakage of *Botrytis cinerea*

The protocol was modified from Lunde and Kubo, 2000. Cellular leakage is determined by quantifying the intracellular component's release to the medium from washed cells by a simple spectrophotometry method. Four plugs of 4-day-old mycelium were transferred into 40 ml of PDB solution for at least one week to grow. 1.5 g of fresh fungus were then weighed, washed 3 times with PBS solution and incubated with a fresh solution of 20 mL PDB solution, containing two different concentrations of TPPS (1.5 and 3.5 µM). The new tubes were incubated in agitation at least for one hour under dark conditions. Subsequently, the fungus in solution was removed by centrifugation (10 min, 10,000 rpm) and absorbance of supernatant was read at 260 and 280 nm.

II.11. Modification of pH medium by *Botrytis cinerea* extracellular secretion

Aqueous stock solutions of 1 mM TPPS were prepared in two different pH ranges: 5.5 and 10.8. From these two stock solutions at different pH, several dilutions were made: 1.5, 3.5, 10, 12.5 and 50 µM TPPS. These dilutions were made four times: into two groups of dilutions at pH 5.5 and the other two at pH 10.8. From here, 4 plugs of 0.6 cm 4-day-old or 15-day-old *B. cinerea* were placed into each 50 ml tube (water control and 1.5–50 µM TPPS) and were incubated for at least 1 hour under dark conditions and low agitation (90 rpm) (Figure 21).

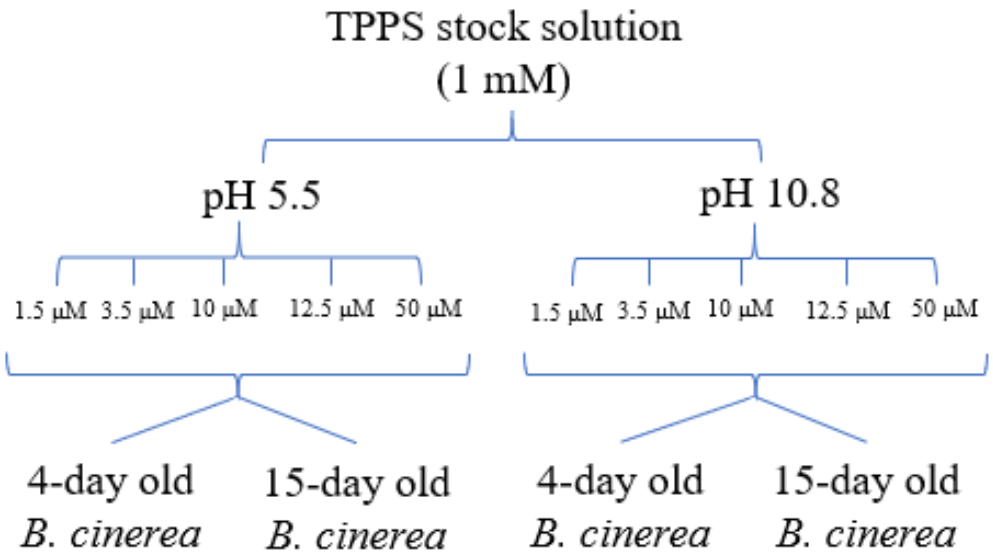

Figure 21: Diagram of solutions made for investigation of extracellular secretion starting from the stock solution of TPPS (1mM in water).

The pH of the different mixtures (control, 1.5 and 50 µM TPPS) was measured before and after adding the fungus plugs. After incubation, the fungus plugs were removed prior to UV-Vis spectrum determination.

II.12. Statistical Analysis

All biological experiments were performed at least three times independently. Results were expressed as a mean ± SD (Standard Deviation). The data were analysed by t-student test and one-way ANOVA using the PAST free software.

Chapter III. Photoactivation and inhibitory effect of TPPS and Chl on *Botrytis cinerea*

In this chapter, the effects of TPPS and Chl on *B. cinerea* were studied. Both PS were tested on the growth of *B. cinerea* myceliun under a 16 h photoperiod and dark conditions. Moreover, the TPPS efficacy was also investigated on spore germination. This is considered as a sine qua non step to develop our strategy.

III.1. Results

III.1.1. TPPS and Chl effect on *Botrytis cinerea* mycelium growth

The growth curve of *B. cinerea* was constructed using three different concentrations of TPPS (0.5, 1.5 and 3.5 µM) under a 16 h photoperiod and dark conditions.

A low concentration of photoactivated TPPS (0.5 µM) induced a delay in the mycelium growth, whereas 1.5 µM of photoactivated TPPS completely inhibited the growth of the *B. cinerea* mycelium (Figure 23). Therefore, this concentration represents the minimum fungicidal concentration (MFC). Furthermore, under dark conditions, the growth of the treated mycelium and control were identical. This thus suggests that TPPS, under dark, did not interfere with the growth and was not cyto- or genotoxic for fungal cells. Overall, TPPS, tested at 1.5 µM, seems to be a very efficient fungicide. According to our previous works on Arabidopsis and tomato plantlets that are insensitive to 50 µM TPPS, this was very encouraging to defend the PDT strategy against fungus without alteration of plant growth (Guillaumot *et al* 2016; Issawi *et al* 2018). Thus, it was decided to continue to work with TPPS to validate our approach even if it is not a natural PS.

The same test procedure was carried out for a natural PS, Chl, at 25 and 50 µM. Surprisingly, in presence of 25 µM Chl, the growth of the fungus slowed down and after the four day culture, it restarted to grow suggesting a degradation of photoactivated Chl in the medium (Figures 23-24). Furthermore, Chl tested at 50 µM was able to inhibit the fungus growth (Figures 22-23). Even if this was a very high concentration compared to the MCF of TPPS, it was considered that the photoactivated Chl was very promising. This is because of its ability to inhibit fungus growth and the fact that it is a naturally occurring PS. Moreover, it was observed that under dark conditions, the fungal growth was very slightly slowed down in both 25 and 50 µM Chl (Figure 24)

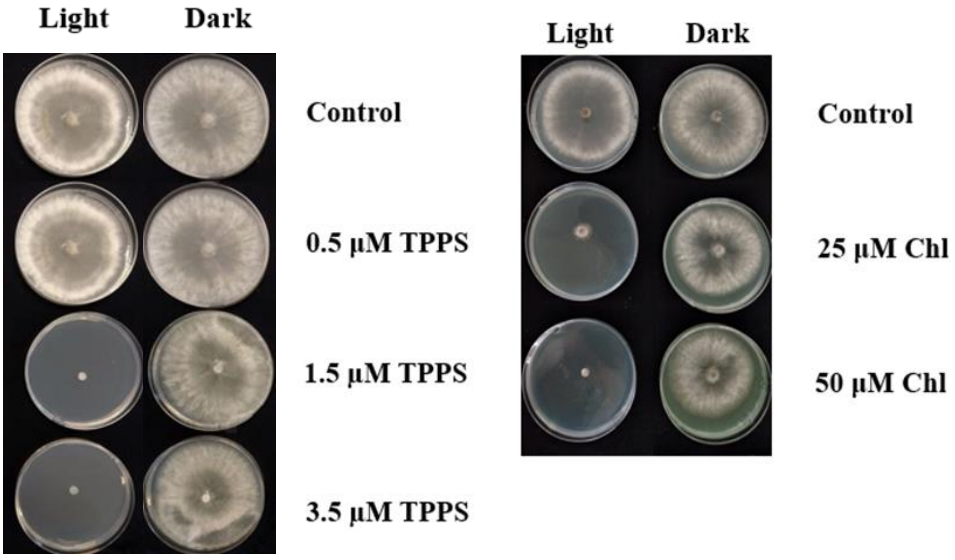

Figure 22: Mycelium growth monitoring under dark and light conditions in presence of TPPS or Chl.

Plugs isolated from a 14-day-old culture were placed in the centre of the Petri dish containing or not the PS. Petri dishes were placed under a 16h photoperiod (light) or under dark conditions. Different concentrations of TPPS (0.5, 1.5 and 3.5 µM) (left) and Chl (25 and 50 µM) (right) were tested. Petri dishes were photographed after 4 day-culture.

In order to construct the growth curve, the diameter of the fungus was measured daily, starting from 0.6 cm. As shown by the growth curve of the control under light and even under dark conditions (Figure 22), the fourth day corresponded to the end of the exponential growth phase. Therefore, all further investigations were conducted with 4-day-old *B. cinerea* mycelium.

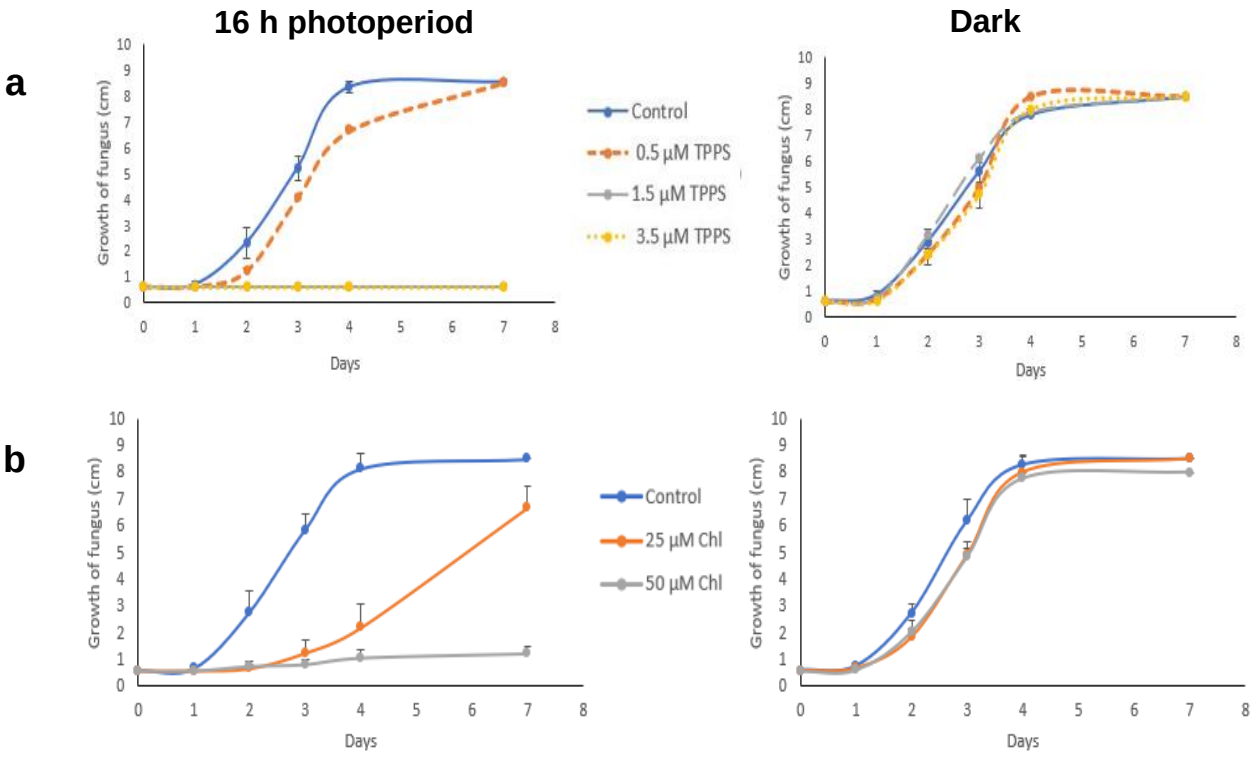

Figure 23: B. cinerea growth curve.

a): *Growth curve of B. cinerea under a 16 h photoperiod (left) and dark conditions (right) using TPPS at different concentrations.* ***b):*** *Growth curve of B. cinerea under a 16 h photoperiod and dark conditions using Chl at different concentrations. Results are the mean of three independent experiments ± sd.*

III.1.2. Localisation of TPPS and Chl in *Botrytis cinerea* mycelium and germinating spores

III.1.2.1. In mycelium

The two PS had effects on the growth of *B. cinerea*. TPPS induced a dramatic effect on the mycelium's growth curve whereas Chl slows down the growth. Therefore, it was decided to visualise the PS in the *B. cinerea* mycelium and spores by confocal microscopy. In previous work on tobacco plant cells (TBY-2 cells), where TPPS was the most efficient cell killer, it was mainly found in plant cell walls (Issawi *et al* 2019). From these findings, a similar localisation of TPPS was expected in the fungal cell wall even if the cell wall composition is completely different to that of the plant cell wall. The samples used for the mycelium were a culture of *B. cinerea* cultivated on PDA containing 3.5 µM TPPS or 50 µM Chl. Surprisingly, TPPS and Chl were found in the cytoplasm of four-day-old mycelium cells without any specific cytoplasmic localisation (Figure 24).

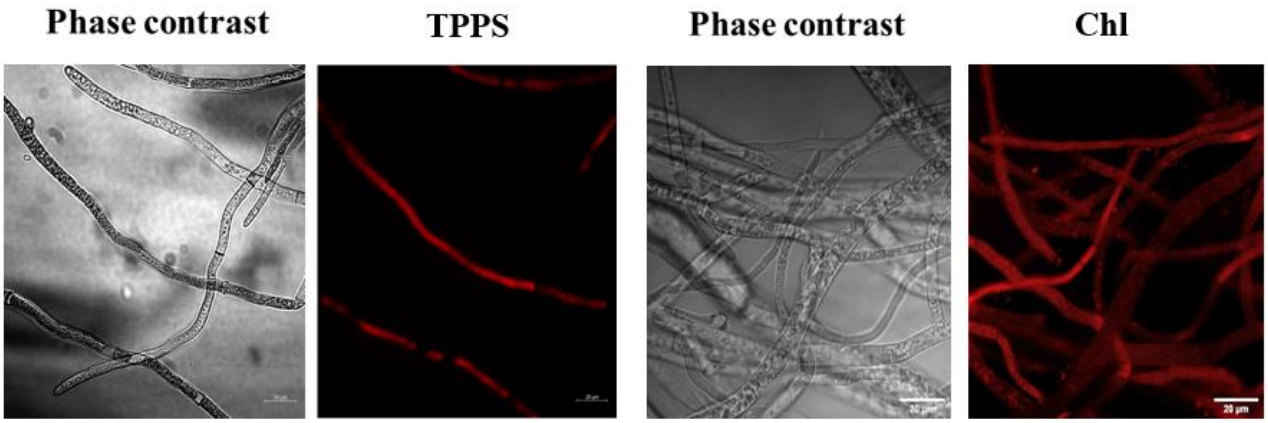

Figure 24: Cellular localisation of both PS in B. cinerea mycelium by confocal microscopy.

Left pictures: localisation of TPPS with its corresponding picture in contrast phase and right panel: Chl localisation with its corresponding picture in contrast phase. Analysis was performed on B. cinerea mycelium cultivated under dark for 4 days on PDA containing 3.5 µM TPPS or 50 µM Chl. For both TPPS and Chl detection, spectral mode was used (cf mat and meth.).

Scale bar is 20 µm.

III.1.2.2. In spores

According to literature, after 4-6 h of inoculation, the spores were swelling and after approximately 10 hours, the germ tube appeared (Figure 25; Gull and Trinci, 1971).).

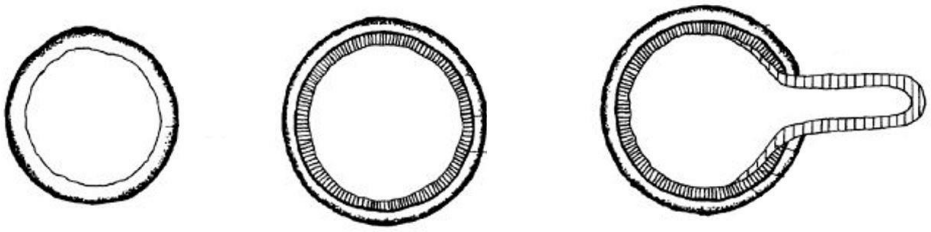

Figure 25: Scheme of spore germination.

(Left): *Dormant spore; (**Middle):** beginning of germination including swelling (conidia wall increasing thickness from 263 to 339 nm); **(Right):** Emergence of germ tube. Sourced and modified from Gull and Trinci, 1971.*

The isolated and counted spores (~10^3–10^5 spores.ml^{-1}) were incubated under dark conditions in 10 µM of both PS. The incubation with PS was performed for at least 16 hours (cf mat and meth).

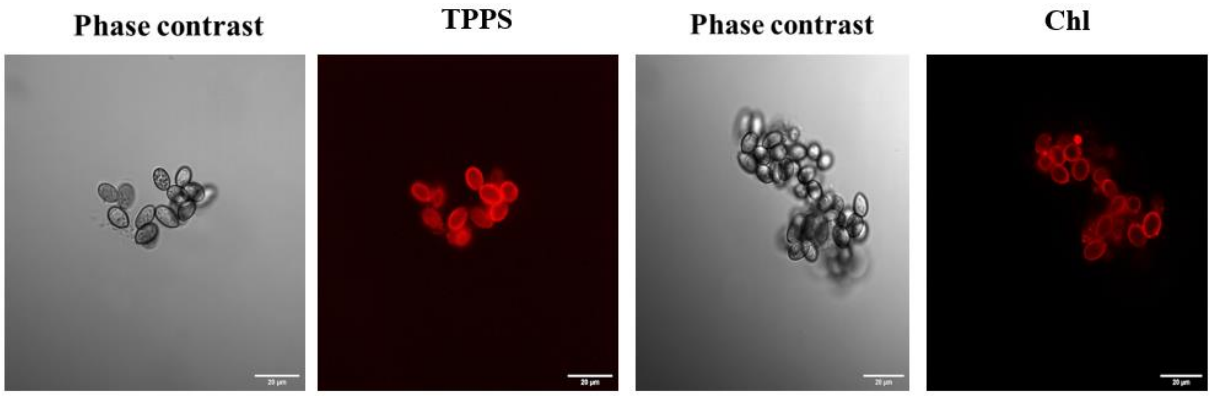

Figure 26: Localisation of TPPS and Chl in non-germinated spores of B. cinerea by confocal microscopy.

Dormant spores were incubated in the presence of 10 µM for both PS for 16 h under dark conditions. Left pictures: localisation of TPPS with its corresponding picture in contrast phase and right pictures: Chl localisation with its corresponding picture in contrast phase. Detection was performed as described in mat and meth. Scale bar is 20 µm.

TPPS was located in cell wall and inside cell whereas Chl was mainly localized in the spore cell wall (Figure 26). It was hypothesised that the charge of the PS : negative for TPPS and Chl at physiological pH, determines the localisation of the PS in the fungal cells.

Ph

Spore labelling was followed up by the study of germinating spores in PDB and confirmed dormant spore labelling for both PS (Figure 27). This is particularly true for Chl that is not localised inside the cell wall of young hyphae generating by germinating spores (Figure 27).

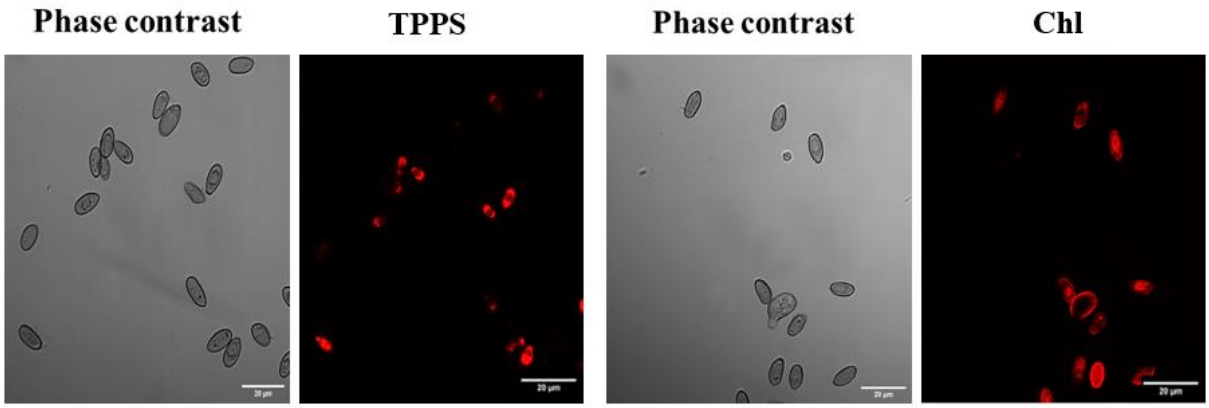

Figure 27: Localisation of both PS in germinated spores of B. cinerea by confocal microscopy. *Germinated spores were incubated in PDB containing 10 µM TPPS or Chl for 16 hours under dark conditions. Left pictures: localisation of TPP with its corresponding picture in contrast phase and right panel: Chl localisation with its corresponding picture in contrast phase. Spectral mode detection was used for PS localisation (cf mat and meth.). Scale bar is 20 µm.*

For the Chl experiments on *B. cinerea*, only growth curves as well as localisations in mycelium and spores were performed because this study started less than one year ago in 2019 and because it was not possible to work in the laboratory during lockdown. Nevertheless, according to our preliminary results on *B. cinerea*, Chl seems very promising.

III.1.3. The ability of photoactivated TPPS on *Botrytis cinerea*

III.1.3.1. Phenotypic mycelium analysis under Environmental Scanning Electron Microscopy

Environmental Scanning Electron Microscopy (ESEM) analysis was very useful to investigate the potential mycelium phenotype modifications that could not be observed under light microscope (Figure 28).

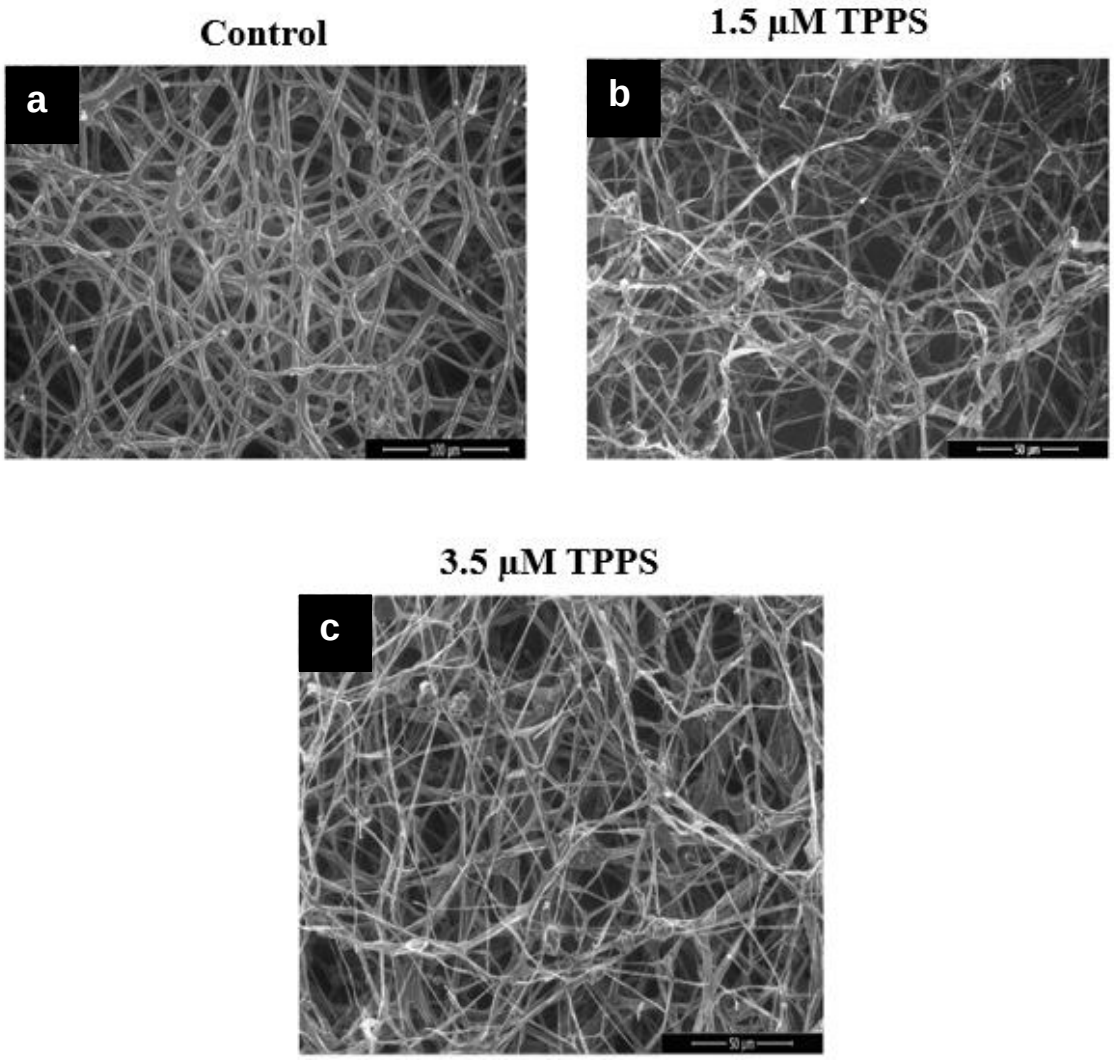

Figure 28: Four-day-old mycelium phenotype grown in presence of photoactivated TPPS. *Analyses were performed using ESEM.* ***a)*** *Untreated mycelium (control);* ***b)*** *mycelium treated with 1.5 µM TPPS;* ***c)*** *mycelium treated with TPPS tested at 3.5 µM. Scale bar (µm) is reported on each picture.*

The mycelium treated with TPPS, under a 16 h photoperiod, is stressed thus producing spores and characterised by different hyphae widths (**Table 7**). In presence of TPPS, the hyphae shows a reduced

width. For the mycelium treated with 1.5 µM TPPS, there is a 71 % reduction in width whereas the mycelium treated with 3.5 µM TPPS displays a 69 % reduction. Furthermore, the hyphae appear less organized in terms of structure and produce spores (see submitted paper).

Control	1.5 µM TPPS	3.5 µM TPPS
4.7 ± 0.9 µm	1.36 ± 0.4 µm	1.45 ± 0.2 µm

Table 7: Widths of B. cinerea hyphae in control and TPPS conditions.

Results are the average widths from three independent experiments ± sd.

III.1.3.2 PDT with TPPS on spores germination

Preliminary germination test: development of the system

Three increasing concentrations of TPPS: 3.5, 12.5 and 50 µM TPPS; were taken in consideration for evaluating the capacity of TPPS to inhibit the germination of spores. As seen previously, the MFC is 1.5 µM TPPS for mycelium. However, this concentration is not reported as it does not exhibit any show effect on the spore germination. The extracted spores were then suspended in phosphate buffer and left for 4 hours under light conditions (cf mat and meth for further details).

After 4 hours of incubation with TPPS (or without in the control), 100 µl samples from each condition were transferred and streaked on PDA-Petri dishes. The spore colony appearance and their number (CFU) were determined and evaluated related to control. Only 7 % colony reduction was determined for sample treated by 3.5 µM TPPS, a significant reduction was observed for the two other TPPS treated samples: 12.5 % reduction for 12.5 µM TPPS and 50 % for 50 µM TPPS, respectively suggesting a strong TPPS stress interfering with germination (Figure 29). This latest result is of interest for the proposed APDT strategy because of the inhibitory effect of TPPS on *B. cinerea* spore germination.

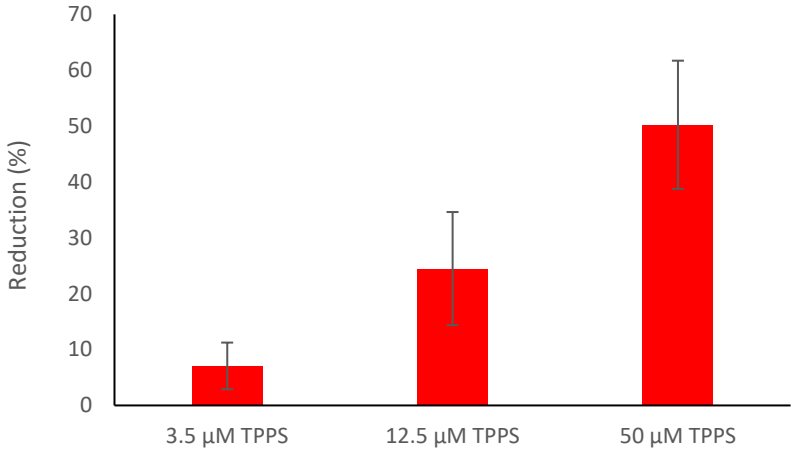

Figure 29: Effect of TPPS on spore viability.

CFU were determined as described in mat and meth. The number of spore colonies was determined for 3.5 ,12.5 and 50 µM TPPS under illumination. Results are the mean of three independent experiment ± sd.

III.1.4. ROS production and mycelium response at biochemical level conducted on the mycelium of *Botrytis cinerea* under light conditions

The first test carried out was the determination of the proline content. Proline can act as a singlet oxygen quencher as well as a free radical scavenger (Guillaumot *et al* 2016). The proline content was assayed as an indicator of general stress response in living cells and as a marker of oxidative stress too. As expected, in response to stress induced by photoactivated TPPS, the proline content increased (Figure 30a).

To gain insight of the fungal response to photodynamic treatment in presence to TPPS, total thiol assay was performed. Even after a 0.5 µM TPPS treatment, a dramatic total thiol content decrease was noticed, suggesting at a biochemical level, a strong stress not observed at phenotypical level (Figures 30a and b).

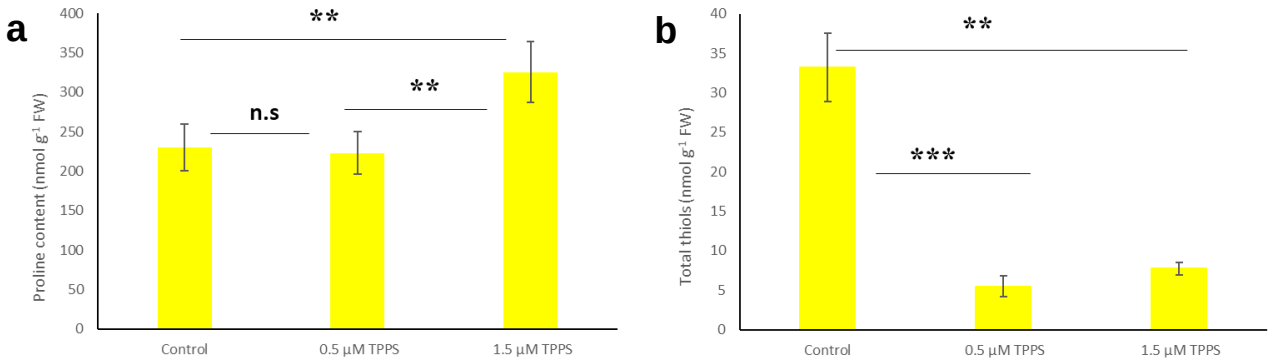

Figure 30: Proline and total thiol contents of TPPS treated mycelium grown under a 16 h photoperiod.

a) *Proline content and* ***b)*** *Total thiol content of four-day-old mycelium. Results are the mean of three independent experiments ± sd (**: p<0.01; ***: p<0.001; ns: not significant).*

As both assays (proline and thiol contents) demonstrated a strong stress undergone by TPPS treatment on *B. cinerea* mycelium, it was decided to deeply explore the response induced by photodynamic stress generated by photoactivated TPPS.

MDA assay was conducted to investigate lipid peroxidation due to TPPS photoactivation. The decline in cellular function was greatly linked to lipid peroxidation that significantly increased after treatment with 1.5 µM TPPS. This could also explain why mycelium growth alteration was not observed for 0.5 µM TPPS treated mycelium whereas thiol content decreased (Figures 30b and 31b).

As photoactivation of TPPS implied ROS production and especially H_2O_2 production, the production of this specific ROS species was quantified in the fungus' mycelium. The choice to measure H_2O_2 production is due to its stability, compared to other ROS, and its ability to diffuse into cells. The increasing amount of H_2O_2 in treated *B. cinerea* indicates that TPPS undergoes type 1 reaction to form H_2O_2 (Figure 31a).

Another side of the fungal ROS defence arsenal against oxidative burst concerns the enzymes such as catalase (Cat) and superoxide dismutase (SOD). A Cat activity was detected in 0.5 µM TPPS treated mycelium but not in that of 1.5 µM TPPS (data not shown). SOD, enzymes that catalyse the dismutation of the superoxide radical anion to produce molecular oxygen (O_2) and H_2O_2, SOD enzyme activity never changed either in the control or in TPPS-treated mycelium (Figure 31c). Thus, it appears that there was no production of the superoxide radical anion.

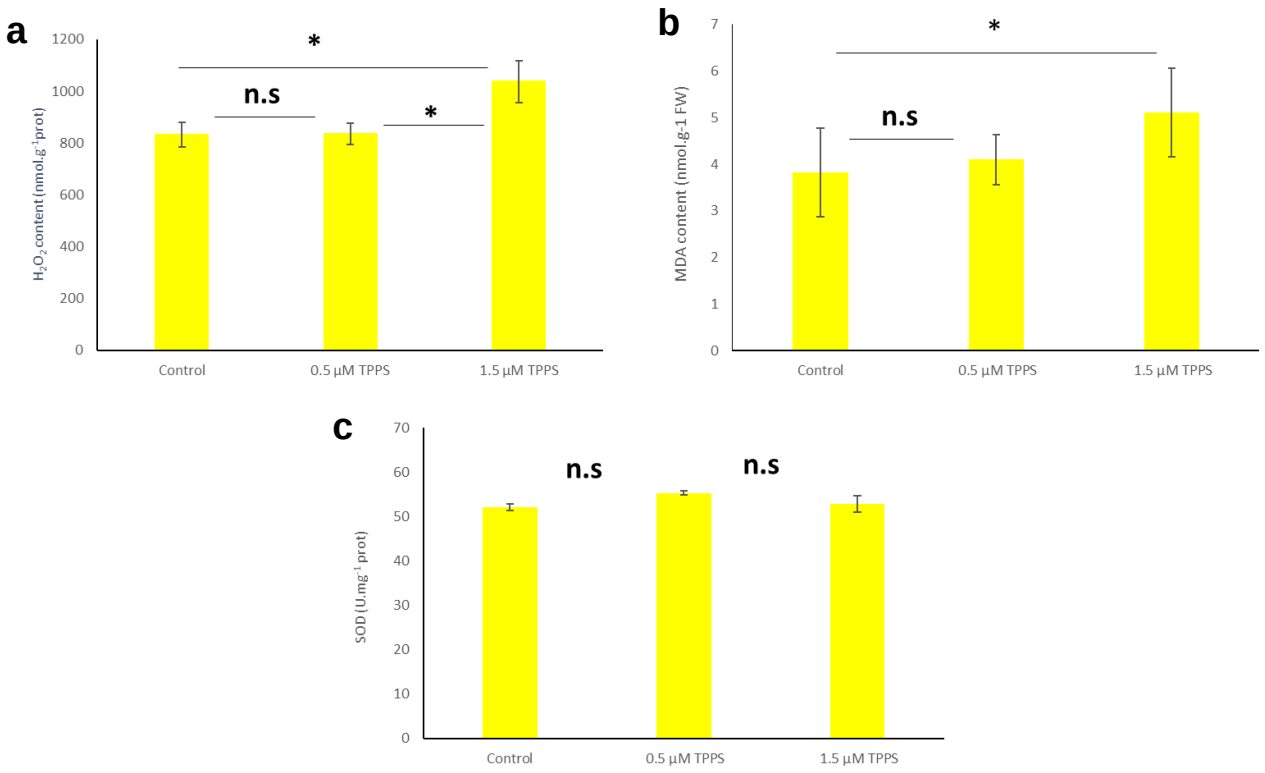

Figure 31: Biochemical assays in four-day-old TPPS treated mycelium.

a): *H_2O_2 assay of four-day-old mycelium of B. cinerea;* ***b):*** *MDA assay of four-day-old mycelium of B. cinerea;* ***c):*** *SOD enzymatic activity of four-day-old mycelium of B. cinerea. All assays were conducted in light conditions (16-hour photoperiod). Results are the mean of three independent experiments ± sd (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: not significant).*

III.2. TPPS effect on *Botrytis cinerea* mycelium under dark conditions

TPPS did not seem to influence mycelium growth under dark conditions. Indeed, the treated fungus in the presence of TPPS (0.5–3.5 µM) grew as the control (Figures 21 and 22). However, some unexpected results were obtained on TPPS treated mycelium under dark conditions that were developed in this section.

III.2.1. Mycelium phenotype analysis under dark conditions

The 4-day-old fungus was observed under ESEM and the fungus structure appeared modified even from low TPPS concentrations (Figure 32, table 8).

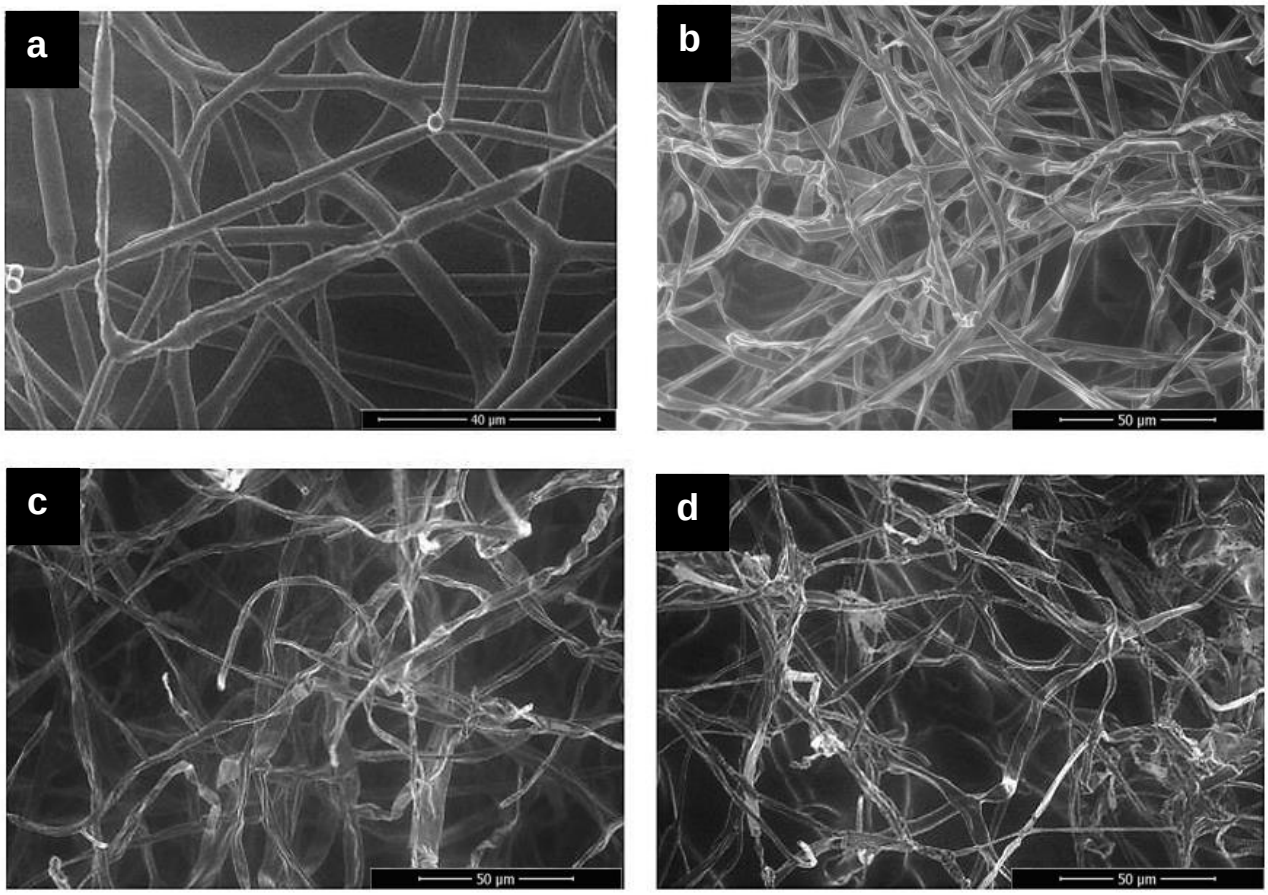

Figure 32: Four-day-old mycelium phenotype grown in presence of TPPS under dark conditions. *Analyses were performed using ESEM.* ***a)*** *Control;* ***b)*** *1.5 µM TPPS;* ***c)*** *3.5 µM TPPS;* ***d)*** *10 µM TPPS. Scale Bar is reported on each picture.*

In contrast to light conditions, the hyphae width increased under dark conditions (Table 8). Surprisingly, no photo-activated TPPS induced a stress that caused changes in the structure of the fungus hyphae. The effect did not seem to depend on the concentration of TPPS, but rather on its presence. Further experiments are required to confirm the hypothesis that TPPS can alter the form of the *B. cinerea* hyphae.

Control	1.5 µM TPPS	3.5 µM TPPS	10 µM TPPS
4.4 ± 0.8 µm	6.03 ± 1.3 µm	5.9 ± 1.12 µm	6.0 ± 1.6 µm

Table 8: Effect of TPPS on widths of B. cinerea hyphae under dark conditions.

Three increasing TPPS concentrations were tested on 4-day-old B. cinerea. Measurements were conducted via ESEM analysis (figure 33). Results represent the average widths and the mean of 3 independent experiments ± sd.

III.2.2. The fungus mycelium grown in dark – stress or not?

In order to evaluate the stress that caused these structural variations, biochemical assays were conducted. To monitor the general response of the fungus to abiotic stress, proline and total thiol contents of 4-day-old *B. cinerea* mycelium under dark conditions were measured (Figure 33).

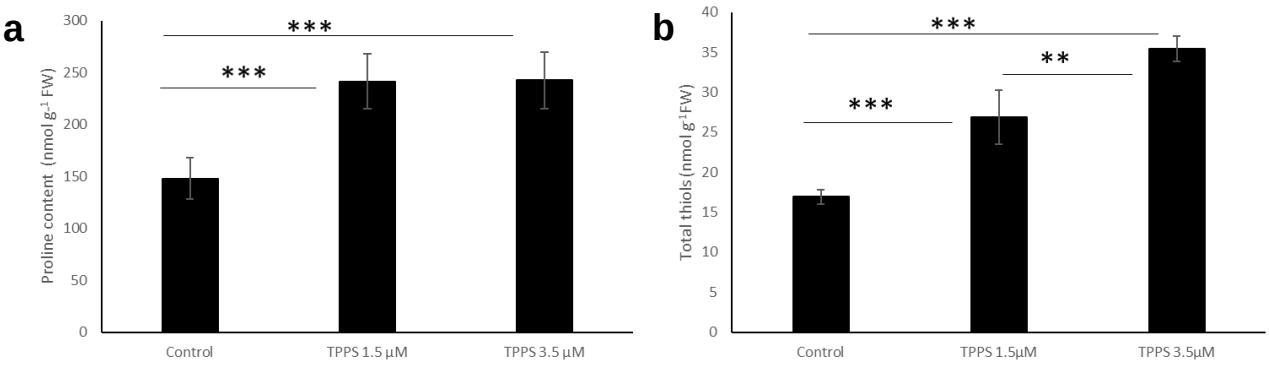

Figure 33: Proline and thiol contents in TPPS treated mycelium under darkness.

a) *Proline content and* ***b)*** *Total thiol content in 4-day-old mycelium. Results are the mean of three independent experiments ± sd (**: p<0.01; ***: p<0.001).*

III.2.2.1. Cellular leakage of *Botrytis cinerea*

To further investigate this stress, it was decided to measure the cellular leakage. The hypothesis from this experiment was that the fungus, after a 1-hour incubation with 1.5 and 3.5 µM TPPS, became stressed. Thus, releases nucleotides mainly detected at Abs 260 nm and proteins at Abs 280 nm into the medium (Figure 34). The fungus secreted in medium both nucleotides and proteins suggesting a leak from cells in response to TPPS presence.

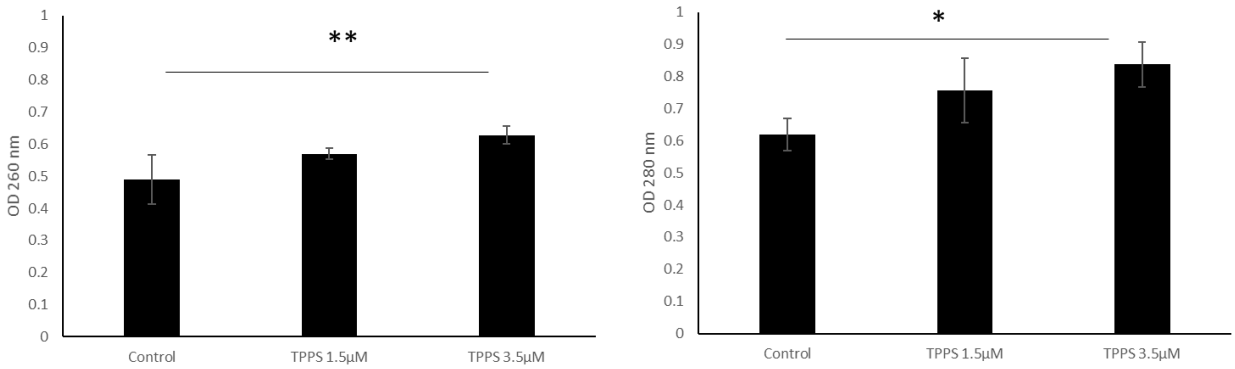

Figure 34: Cellular leakage assays.

Assays were performed as described in mat and meth. Results are the mean of three independent experiments ± sd (: p<0.05; **: p<0.01).*

III.2.3. TPPS and *Botrytis cinerea* – an investigation of different pH levels in dark conditions

Accidentally, it was observed that when the mycelium plugs were in contact with a diluted TPPS solution, the solution changed colour from brown to green suggesting an acidification of the medium. From previous work, it was known that the colour of the TPPS solution depends on pH and becomes green (protonated form) at acidic pH (Leroy-Lhez *et al* 2019; Issawi *et al* 2019).

After cultivating *B. cinerea* for 4 days and at least 15 days under dark, 4 plugs of 0.6 cm were collected from the Petri dishes and discarded into diluted TPPS solutions. More specifically, these solutions were prepared by diluting the acidic (pH 5.5) and basic (pH 10.8) TPPS stock solution (1 mM)

Behaviour of *Botrytis cinerea* mycelium in acidic pH

Four plugs of 4-day or 15-day-old *B. cinerea* were added into the diluted acidic solutions of TPPS. The solutions changed from dark brown to two different colours: lime green (4-day-old) and purple (15-day-old) (Figure 36). These colour changes were linked to the acidification of the medium which led to the protonation of TPPS for 4-day-old mycelium (Figure 36). However, an unexpected result was obtained for the 15-day-old mycelium where the fungus was able to slightly increase the pH of the solution (Figure 35-36, Table 9).

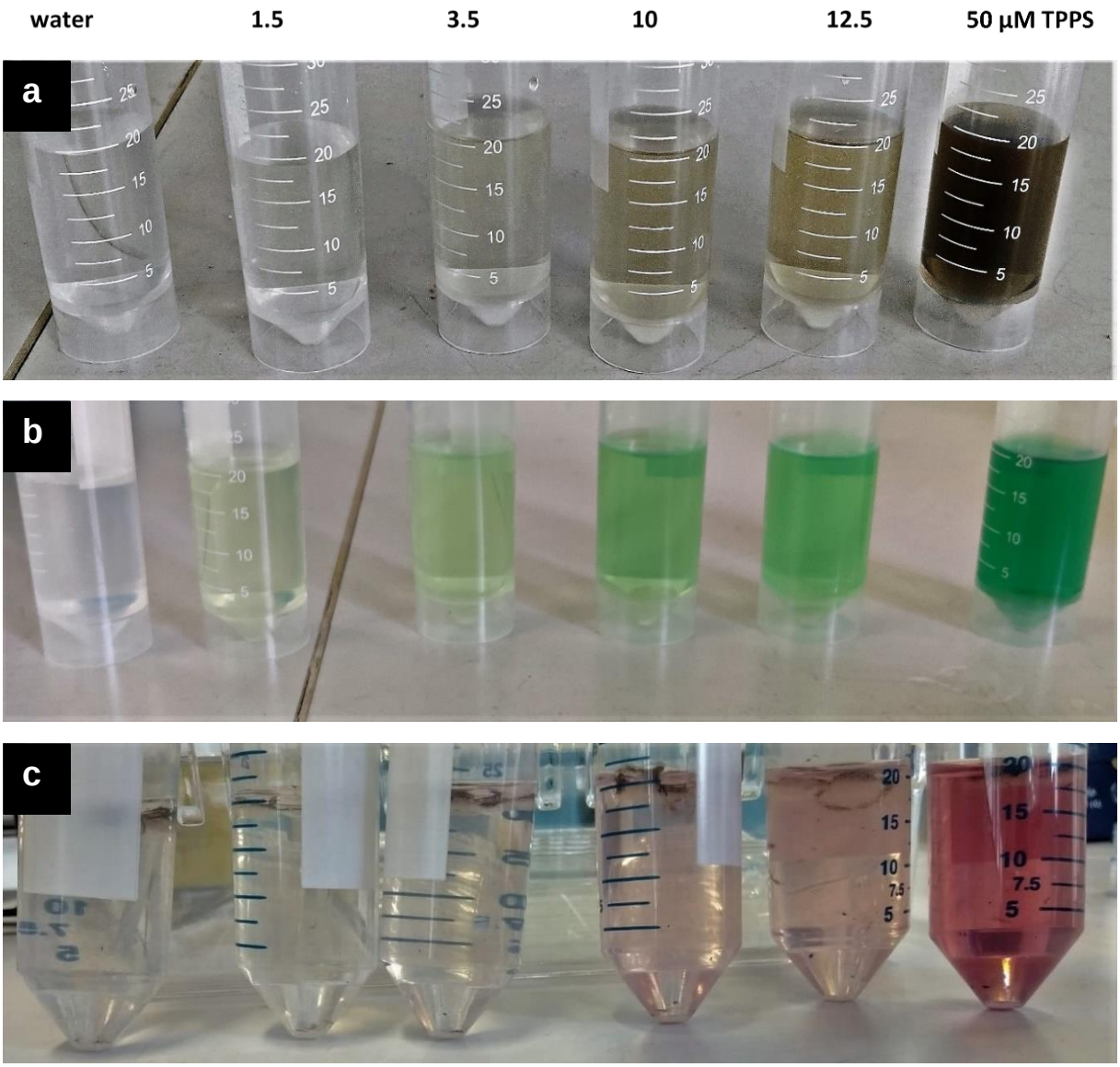

Figure 35: TPPS behaviour in acidic media with young or old mycelium.

a) *Tubes containing serial concentrations of TPPS stock solution (1 mM, pH 5.5) without fungus plugs;* ***b)*** *4 plugs of 4 day-old mycelium were added to the different TPPS concentrations and incubated at least 1 h under dark conditions;* ***c)*** *4 plugs of 15-day-old mycelium were added to the different TPPS concentrations and incubated at least 1 h under dark conditions.*

Mycelium		pH before fungus addition	pH after fungus addition
4-day-old *B. cinerea*	Control water	7.9 ± 0.33	4.45 ± 0.051
	1.5 µM TPPS	7.83 ± 0.002	4.46 ± 0.005
	50 µM TPPS	7.14 ± 0.04	4.39 ± 0.08
15-day-old *B. cinerea*	Control water	7.9 ± 0.02	7.9 ± 0.72
	1.5 µM TPPS	7.83 ± 0.06	8 ± 0.5
	50 µM TPPS	7.14 ± 0.09	7.4 ± 0.25

Table 9: pH variations of medium after mycelium incubation under dark conditions.

pH was measured before and after fungus addition in medium containing two TPPS concentrations (1.5 and 50 µM).

According to Leroy-Lhez *et al* 2019, the UV-vis spectra were monitored for both the lowest and highest concentrations of TPPS. Prior to measuring the UV-Vis spectra, the fungi plugs were removed (Figure 37).

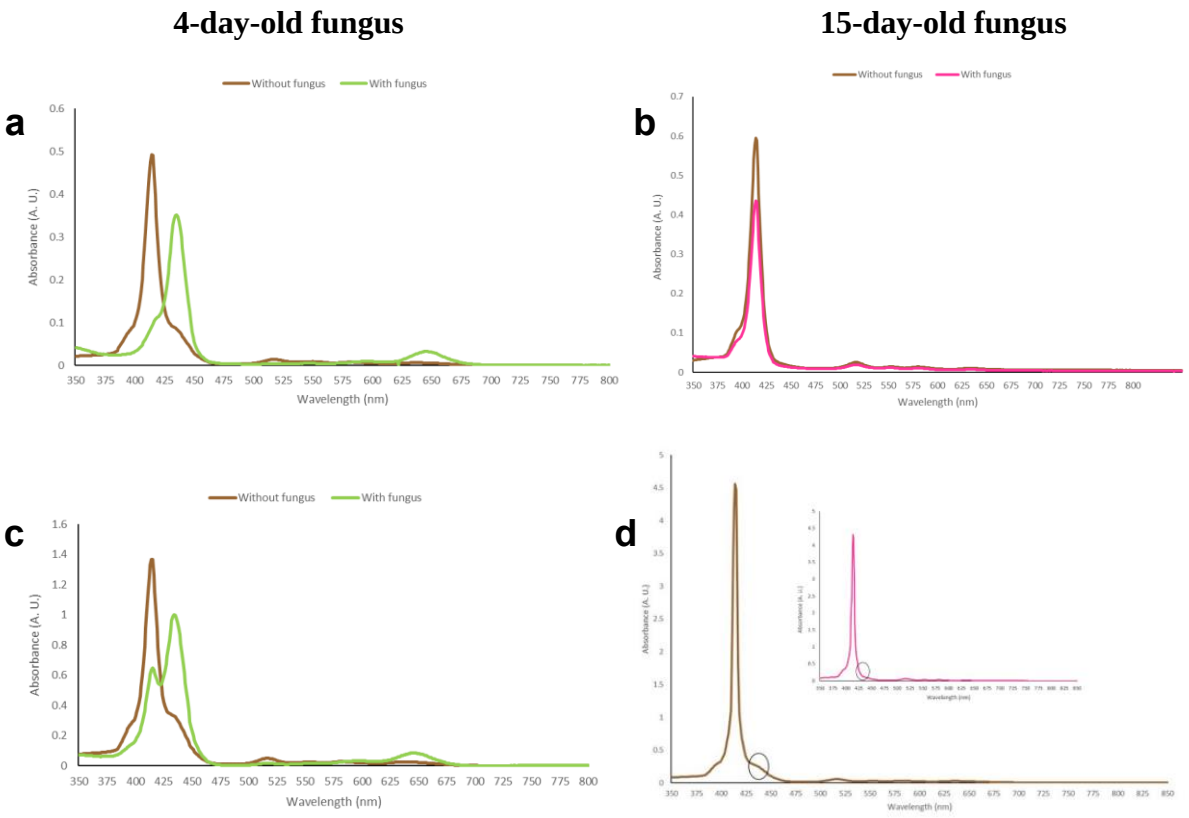

Figure 36: UV-Vis spectra of 1.5 and 50 µM TPPS solutions with and without 4-day-old or 15-day-old B. cinerea mycelium.

In the 4-day-old fungus, there are overlapped UV-Vis spectra of 1.5 ***a)*** *and 50* ***c)*** *µM TPPS in acidic pH solution incubated without (**brown**) and with (**green**) 4-day-old fungus for at least an hour. In the 15-day-old fungus, there are overlapped UV-Vis spectra of 1.5* ***b)*** *and 50* ***d)*** *µM TPPS in acidic pH solution incubated without (**brown**) and with (**purple**) 15-day-old fungus for at least an hour.*

In the case of the 4-day-old fungus, a shift of the Soret band of the TPPS that suggests its protonation was observed for both concentrations (1.5 and 50 µM) (Figure 37). However, this was not the case in the 15-day-old *B. cinerea* where a slight protonation was observed in the 50 µM TPPS acidic solution (Figure 37, table 9).

In conclusion, an acidification of the medium was observed by 4-day-old *B. cinerea* but not in the case of 15-day-old fungus. This specific result of the 15-day-old fungus has never been reported in the literature and need to be further explored in the future.

Behaviour of *Botrytis cinerea* mycelium in basic pH

The pH of the porphyrin stock solution was adjusted by adding a drop of 1 M NaOH solution to achieve a pH of 10.8. From here, the dilutions shown in Figure 37, were made. Four plugs of 4-day-old or 15-day-old *B. cinerea* were added into the dilutions of the basic solution of TPPS (pH 10.8). These solutions changed colour from purple to lime green when 4-day-old mycelium plugs were added to the solution (Figure 37b). Nevertheless, with 15-day-old mycelium, the colour of the solution stayed purple as the control without mycelium (Figure 37a and c, Table 10).

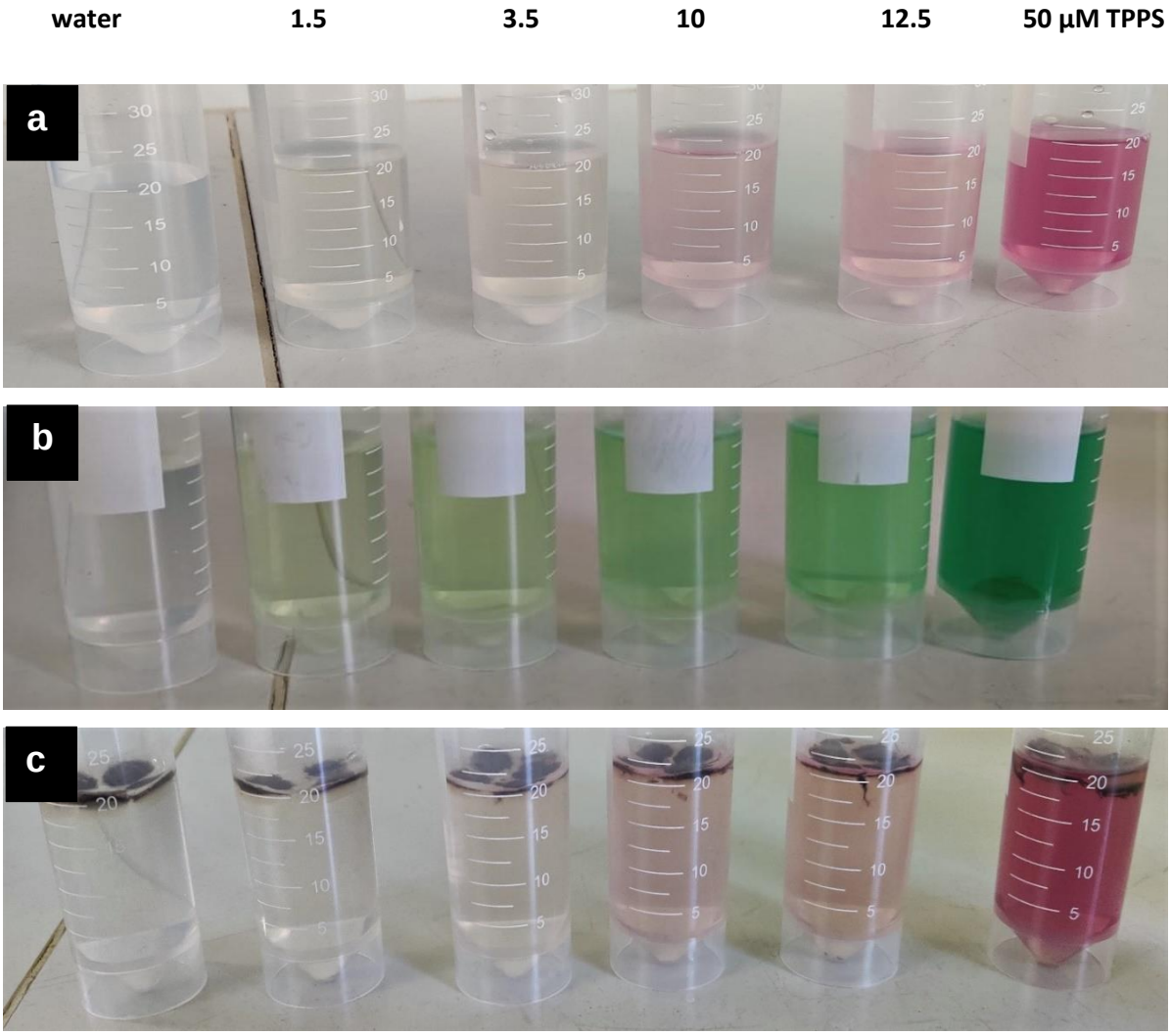

Figure 37: TPPS behaviour in basic media with young or old mycelium.

a) *Tubes containing serial concentrations of TPPS stock solution (1 mM,* pH 10.8); ***b)*** *4 plugs of 4 day-old mycelium were added to the different TPPS concentrations and incubated at least 1 h under dark conditions;* ***c)*** *4 plugs of 15-day-old mycelium were added to the different TPPS concentrations and incubated at least 1 h under dark conditions.*

Mycelium		pH before fungus addition	pH after fungus addition
4-day-old *B. cinerea*	Control water	7.9 ± 0.03	4.49 ± 0.18
	1.5 µM TPPS	8.11 ± 0.02	3.76 ± 0.05
	50 µM TPPS	7.69 ± 0.05	3.03 ± 0.013
15-day-old *B. cinerea*	Control water	7.9 ± 0.25	6.41 ± 0.03
	1.5 µM TPPS	8.11 ± 0.045	6.29 ± 0.16
	50 µM TPPS	7.69 ± 0.0722	6.30 ± 0.045

Table 10: pH variations of medium after mycelium incubation under dark and basic conditions.

pH was measured before and after fungus addition in medium containing two TPPS concentrations (1.5 and 50 µM). Four plugs from 4-day or 15-day-old B. cinerea grown under dark conditions were added to the solution for 1 hour.

Also, in this case, the UV-vis spectra were measured from the lowest to the highest concentration of TPPS. The highest concentration had to be diluted by a factor of 10 and prior to measuring the UV-Vis spectra, the fungi plugs were removed (Figure **39**).

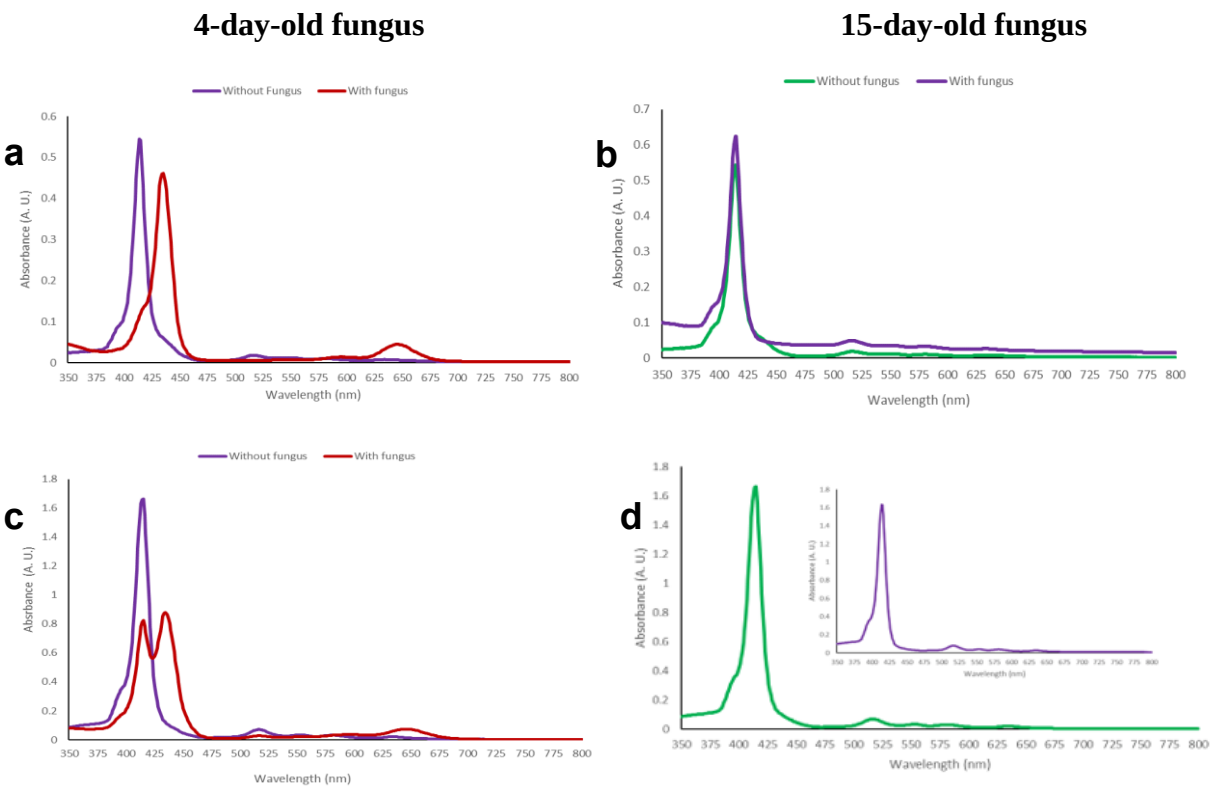

Figure 38: UV-Vis spectra of 1.5 and 50 µM TPPS solutions with and without 4-day-old or 15-day-old B. cinerea mycelium.

In the 4-day-old fungus, there are overlapped UV-Vis spectra of 1.5 ***a)*** *and* ***c)*** *50* ***(Bottom)*** *µM TPPS in basic pH solution incubated without (**purple**) and with (**red**) 4-day-old fungus for at least an hour. In the 15-day-old fungus, there are overlapped UV-Vis spectra of 1.5* ***b)*** *and* ***d)*** *50* ***(Bottom)*** *µM TPPS in basic pH solution incubated without (**green**) and with (**purple**) 15-day-old fungus for at least an hour.*

As previously noted in the acidic conditions, the 4-day-old fungus acidified the solution in an almost extraordinary fashion. In this case, the young fungus could reduce the pH from 8 to less than 4. However, the 15-day-old fungus marginally changed the pH and the solution stayed neutral.

III.3. Discussion

As discussed in the introduction, there are many different biological and chemical methods used to fight against *B. cinerea*. None of them are 100 % efficient and some generate fungus multi-resistance. Moreover, all these measures are a burden for the agronomical economy costing more than €1 billion ever year (Dean *et al* 2012; Hahn 2014; Romanazzi and Feliziani 2014). Therefore, in the fight against *B.cinerea*, a new more cost-effective treatment is needed. In this context, APDT is thought to be a new and valuable strategy that should not induce multi-resistance of fungus.

APDT and studies on other fungi

APDT against fungi has already been reported in literature. For instance, a porphyrin and potassium iodide adjuvant co-encapsulated in micelles, methylene blue psoralen and riboflavin were able to efficiently inactivate fungi like *Candida albicans* (Sousa *et al* 2019; Castro *et al* 2020). Before this, an aluminium phtalocyanine chloride nano-emulsion was investigated for its effectiveness against *Candida albicans* and *Candida tropicalis* (Rodrigues *et al* 2019). In another study concerning methylene blue by itself, it was able to kill the *Colletotrichum abscissum* fungus and proved to be harmless against plant organs; in addition, this treatment did not induce any secondary resistance (Gonzales *et al* 2017). Also, *Colletrichum acuratum* and *Aspergillus nidulans* were inactivated photodynamically by coumarins, furocoumarins and phenothiazinium (De Menezes *et al* a, b; 2014; Fracarolli *et al* 2016).

TPPS as a good candidate to fight against *Botrytis cinerea*

As seen above and until now, very few studies including APDT and *B. cinerea* have been performed (Imada *et al* 2014). As a first and key step, an efficient PS able to kill fungus, without altering plant development, must be identified. According to our results on the *B. cinerea* strain, this PS is the anionic porphyrin TPPS that inhibits mycelium growth with a MFC of 1.5 µM after photoactivation. Several points are in favour of further large use of TPPS: 1) TPPS does not show visible effect on the mycelium growth under dark conditions; 2) TPPS does not aggregate in solution and can easily permeate through cell walls and membranes (Leroy-Lhez *et al* 2019; Issawi *et al* 2019); 3) it is efficient at a very low concentration against fungus (see submitted paper); 4) it does not alter some species of plant growth *in vitro* even at high

concentrations (Guillaumot et al 2016; Issawi, 2018, cf chap IV and V of this work); 5) it is able to reduce the sporulation.

The only inherent defect of TPPS remains its chemical origin which explains the interest towards a natural PS: chlorophyllin that is also presented in this work. However, the concentration that inhibits the fungus growth is 50 µM which is far more than the MCF of TPPS. It was hypothesized that it could be due to the rapid degradation of Chl in the fungus growth medium. This hypothesis was considered because experiments determining the photostability of Chl in water and in PDB were conducted (data not shown). After 24 hours, Chl is almost completely photodegraded.

Effect of light on *Botrytis cinerea* growth

APDT is based on the use of UV-visible light. Therefore, it is of importance to take into account the general effect of light on *B. cinerea* growth. Fungi can sense light and use it to prepare themselves against stressful conditions in its environment (Schumacher 2017; Dias *et al* 2019). Previous studies showed that *B. cinerea* has a large absorption spectrum, spanning from the near-UV to the far IR region (Kumagai and Oda 1969; Tan and Epton 1973; Tan and Epton 1974; Tan 1975; Suzuki *et al* 1977; Suzuki and Oda 1979). Asexual and sexual reproduction are also regulated by light and the presence of light favours conidia formation whereas the absence (dark conditions), favours sclerotia development. However, rather surprisingly, a larger number of conidia is formed in cultures that are cultivated in light-dark cycles, than in constant light. This phenomenon shows that also the dark has an importance for the conidiation. However, the dark-pigmented sclerotia do not seem to induce a switch between the two types of reproduction whereas sclerotia is able to generate mycelium and conidia. The latter can produce microconidia and when these conidia have the female gametes, they encounter the male gametes of sclerotia. Upon illumination, these two gametes undergo sexual reproduction to form apothecia (Faretra *et al* 1988).

The effect described herein is generated after exposure to daylight or white light, but it is interesting to also understand the power that a specific wavelength can have on the pathogen and in particular, on the conidia development (Tan 1974; Tan and Epton 1974). Specifically, UV light alone allows conidia development, and this is not the case under blue light irradiation. Under this type of light, the formation of sclerotia does not occur (Suzuki *et al* 1977; Suzuki and Oda 1979). However, if *B. cinerea* is exposed to far-red light after being exposed to blue

light, the conidia can start to develop once again (Figure 40). Whereas, if blue light illumination is followed by red light exposure, conidiation does not occur (Tan 1974,1975). Despite this, green light can prevent mycelial growth and conidial germination (Zhu *et al* 2013).

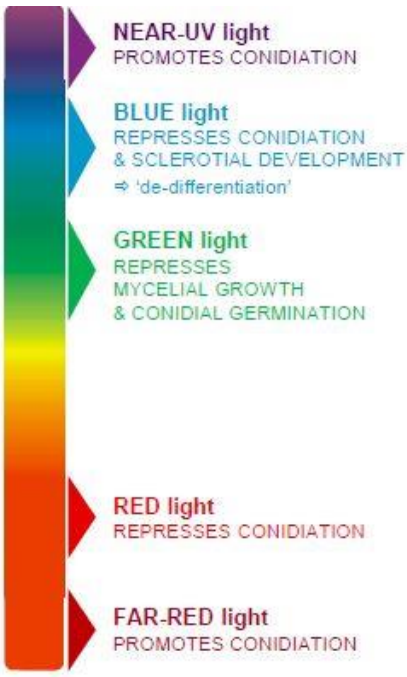

Figure 39: Different light regions and their effects in life of B. cinerea.

Sourced from Schumacher 2017.

Therefore, it is clear that the fungal infection and life cycle can be controlled by the modulation of light. From this fact alone, the idea was to utilise the therapy called Antimicrobial Photodynamic Therapy (APDT) against *B. cinerea*. This therapy incorporates the use of a molecule called a photosensitiser (PS) and light which results in the photosensitiser being excited and can react with oxygen to form reactive oxygen species (ROS; for more details see introduction; Wainwright *et al* 2017).

Effect of TPPS under dark conditions

Surprisingly, TPPS did not delay mycelium growth under dark conditions. However, the structure of the TPPS treated fungus was altered and larger than the control under ESEM analysis. The width of the control was 4.4 µm and 6 µm for the treated. Overall, it appeared that the effect does not depend on the concentration of TPPS, but rather on the presence of this

porphyrin. Under dark conditions, TPPS becomes protonated in its inner core because of medium acidification by mycelium cells. Moreover, the presence of the porphyrin induces an increased production of proline and total thiols and an increase in nucleotide and proteins secretion into the medium (called cell leakage). All of this data confirm that the fungus is under stress. It has been reported that fungal pathogens have the capability to modulate their environmental pH in order to escape the immune responses of their host and in an effort to destroy their host (Manteau *et al* 2004). In particular, at pH 5.5, multiple organic molecules such as oxalic acid and polysaccharides, or enzymes such as polygalacturonase and laccases are secreted by the fungus and can change the extracellular pH medium (Manteau *et al* 2003; Vylkova, 2017). *B. cinerea*, in particular, uses a defence strategy based on the acidification of its environment with the production of organic acids, such as oxalic acid or citric acid (Rolland *et al* 2009). This can explain why the anionic porphyrin TPPS was protonated and showed a different structure, highlighted by its UV-Visible spectrum. From the colour changes, pH measurements and UV-vis spectroscopy, it appears that the 15-day-old fungus can somehow deprotonate the porphyrin in acidic solution. To our knowledge, this is the first reported scenario where a fungus can deprotonate the porphyrin in acidic media. *B. cinerea* changes its environment from an acidic pH to basic one.

Chlorophyllin – a natural PS with lots of promises for the future

According to previous results presented above, the study of Chl on *B. cinerea* must be carefully continued. Furthermore, as shown by confocal microscopy analysis, Chl is only localised and retained in the spore cell wall and not found in hyphae. It was hypothesised that this could be one of the reasons why Chl (25 µM) only limits mycelium growth under illumination. Thus, Chl is a very promising and interesting PS that will be able to limit the fungal infection. To clarify the real power of Chl further investigations are planned.

III.4. Conclusion

So far, these promising results, in particular under light conditions, have been obtained *in vitro*. Also, the experiments discussed in this chapter should be done in a greenhouse and in fields to determine the real potential and efficacy of PS against *B. cinerea*. On one hand, it was impressive that at a low µM concentration of TPPS, was so effective against our strain of *B. cinerea*. These results highlight the potential of the use of TPPS against *B. cinerea* in agronomic

practices. On the other hand, it was so interesting the capability of the natural PS to limit the growth of the strain. From here, it should be envisaged to use the other kinds of PS to fight against this pathogen. To conclude, it would also be interesting to further investigate the potential of TPPS under dark conditions. The results obtained give hope to be able to find the right balance between PS and light to moderate the aggressiveness caused by *B. cinerea* on plants.

Chapter IV: Photoactivated TPPS effect on grapevine varieties: Chardonnay, Merlot and Sauvignon

In chapter III, the potential of photoactivated TPPS against the mycelium and spores of *B. cinerea* was demonstrated, suggesting that TPPS was a very good candidate for a potential APDT application. An important preliminary result was the effect of a low micromolar TPPS concentration (MFC = 1.5 µM) on mycelium growth. This was an excellent start for a future APDT in agriculture. To continue towards this goal, it was of interest to demonstrate that TPPS does not induce harmful effects on plant species other than Arabidopsis (*Arabidospis thaliana* var. Wassilewskija) and tomato (*Solanum Lycopersicum* var. Bali) (Guillaumot *et al* 2016; Issawi *et al* 2018).

Thus, we decided to study a woody and perennial plant species: grapevine (*Vitis vinifera*) that represents one of the major targets of *B. cinerea*. In this chapter, we worked on three widely grown grapevine varieties: Sauvignon, Chardonnay and Merlot (clones grown *in vitro*, cf mat and meth). As previously mentioned in the introduction (section I.3.1.3.), Chardonnay and Sauvignon backgrounds could be considered highly susceptible to the infection of *B. cinerea* which is contrary to the Merlot variety (Galet 1988; Dry and Gregory 1990; Marois *et al* 1992; Kogan 1998; Dubos 2002; Fermaud *et al* 2011). Furthermore, one of the aims of this work was to focus on the notion of variety and their response to the same TPPS treatment.

Therefore, this chapter is divided into three main parts: the first one targets the basic study of the three clonal varieties at the phenotypical and biochemical level, the second part concerns how they react to the presence of TPPS under a 16 h photoperiod and the third part, the development of the desired pathosystem (detached grapevine leaves/*B. cinerea* mycelium treated or not by TPPS). This last part is in the form of the submitted paper “Photodynamic inactivation of *Botrytis cinerea* by an anionic porphyrin: an alternative pest management of grapevine”. At the time that this thesis was written, this paper was currently under revision in Scientific Reports.

Finally, it is important to note that in the beginning of this PhD work, the Chardonnay clone was the only available in the laboratory and more assays were conducted on it and presented in the last part of this chapter as supplementary results.

IV.1. Chardonnay, Merlot and Sauvignon phenotypical analysis

As a first step of the grapevine work, the growth and development of the three varieties were studied under normal conditions without TPPS. Intermodal dissections of two-month-old clonal plantlets were performed and each internode (approximately 5 per plantlet), without leaf, was deposited on new medium in tubes or jars as described in mat and meth.

No obvious differences in terms of growth or phenotype were noticed (Figure 40). A nice growth was clearly observed after one month for the three varieties with well-developed root and aerial parts (Figure 40). For each variety, plantlets produced around two sets of well-expanded pairs of leaves. However, some plantlets generated from the same "parent" or clonal plantlets were stressed with a shorter stem and fewer leaves, suggesting somaclonal variations and/or external factor incidence. Moreover, some internodes never generated plantlets. Even if *in vitro* culture with clonal explants represents the highest control conditions (light, temperature, humidity, culture medium), explant cultures still show variations (seasonal for example) that could not be explained. It was considered that after 3 months, development of plantlets even in jars was no longer optimal due to physical and medium limits. For instance, leaves were twisted and appeared less green suggesting an early senescence process especially for Merlot and Chardonnay clones (Figure 40).

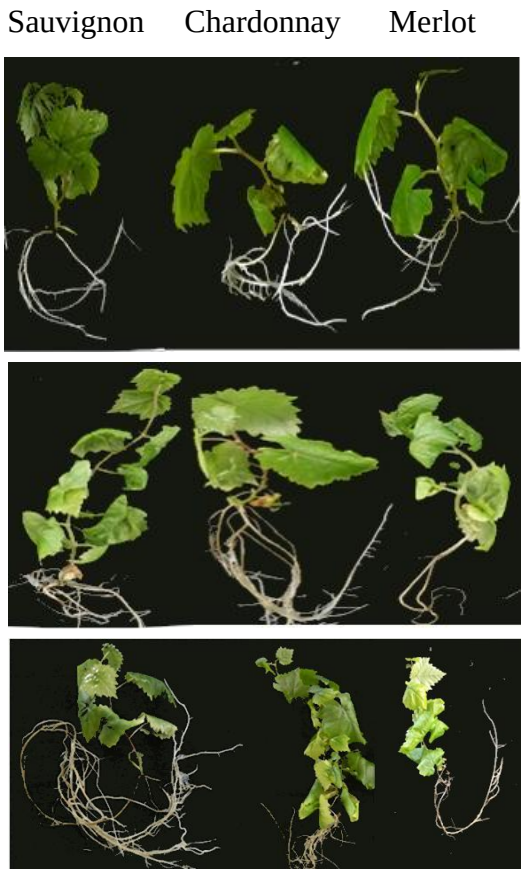

Figure 40: Growth and development of the three studied grapevine varieties: Sauvignon, Chardonnay and Merlot.

Dissected internodes were cultivated in jars for 1 month (top), 2 months (middle) and 3 months (bottom).

IV.1.1. Total thiol content in the three grapevine varieties

Even if grapevines did not seem to be affected at the phenotypical level by *in vitro* culture, this result had to be confirmed at the biochemical level. It is known in literature that measuring the thiol content measurement is a good indicator of stress (Ulrich and Jakob, 2019). Thus, the total thiol contents were determined in isolated roots (R) and aerial parts (A.P) of each variety aged 1-3 months.

The one-month-old plantlets from each variety produced high and very similar amounts of total thiols in both roots and aerial parts (around 100 nmol g^{-1} FW) (Figure 41a). After one-month culture, a low reduction of total thiol content was measured in the roots whereas it significantly doubled in the aerial parts of the Merlot and Sauvignon varieties (up to 200 nmol g^{-1} FW) (Figure 41b and c). The exception came from Chardonnay's aerial parts where the thiol content remained relatively constant around 100 nmol g^{-1} FW after 2- and 3-month-old cultures (Figure 41b and c). To conclude, the aerial

parts seemed to be more affected than the roots, probably due to the culture conditions in tubes or jars. However, Sauvignon and Merlot plantlets were more sensitive to the *in vitro* culture conditions than Chardonnay clones. This result was of importance for the rest of the study (comparison of the response of three varieties to APDT) and because it showed how relevant it was to work with different backgrounds.

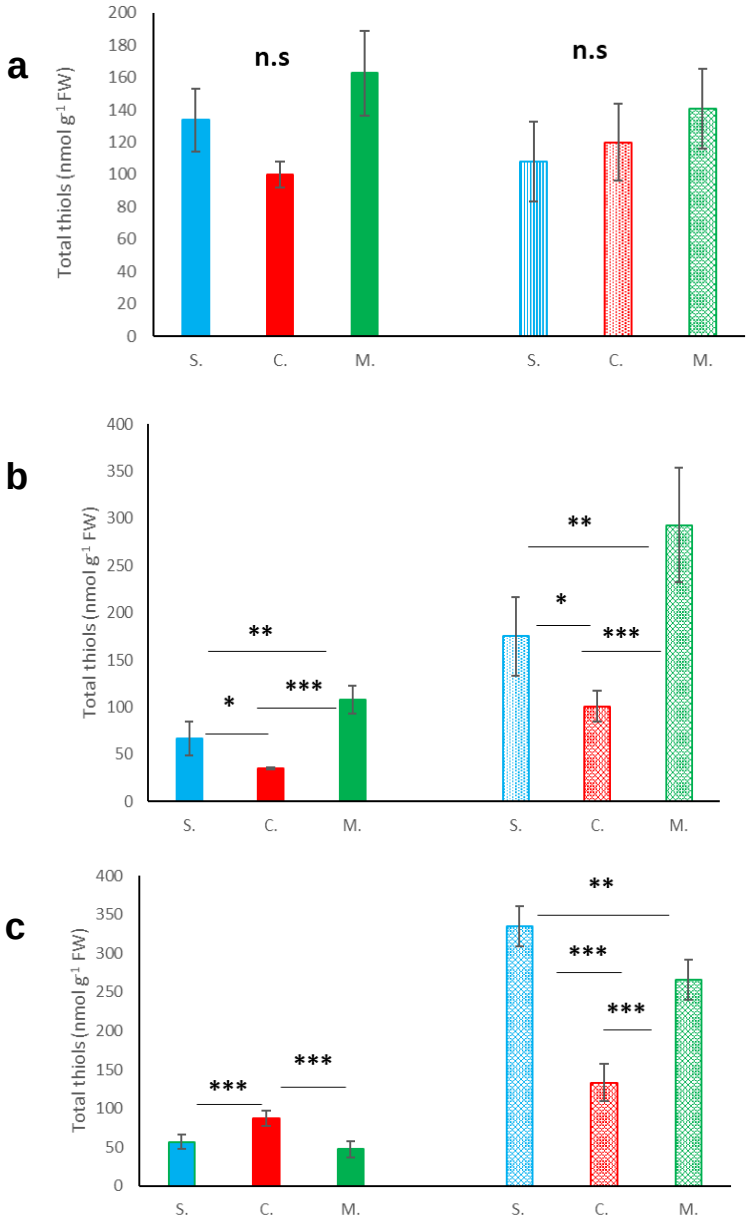

Figure 41: Total thiols assay on the three varieties of grapevine.

The filled boxes represent the roots and the dotted boxes to the aerial parts of each variety. S: Sauvignon, C: chardonnay and M: Merlot. The experiment was conducted on 1- (a), 2- (b), and 3-month (c) old plantlets culture. Results are the mean of five independent experiments ± sd (: p<0.05; **: p<0.01; ***: p<0.001; ns: not significant).*

IV.1.2. TPPS photodynamic treatment on grapevine varieties

As already mentioned, all pre-tests with photoactivated TPPS tested at various concentrations were monitored on Chardonnay plantlets and the results are given in supplementary data at the end of this chapter. As Chardonnay explants supported the presence of 12.5 µM TPPS under a 16 h photoperiod, it was decided that 12.5 µM TPPS will be the working concentration for all studies on grapevine for the three backgrounds.

IV.1.3. TPPS effect on grapevine phenotype

For the three grapevine varieties, photoactivated TPPS tested at 12.5 µM induced a slowdown of their growth and development with shorter roots and stem length for one-month-old plantlets (Figure 42).

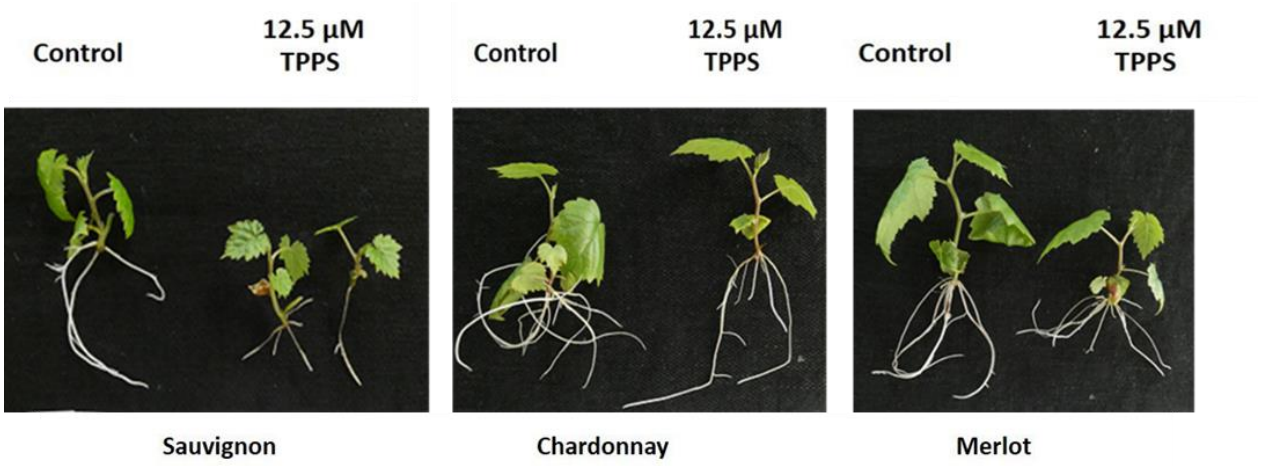

Figure 42: One-month-old grapevine plantlets grown in the presence of 12.5 µM TPPS.

Plantlets were grown in vitro under a 16 h photoperiod. The name of each variety is reported on the pictures. For the Sauvignon clone, two treated plantlets were photographed.

Differences blurred after one-month culture. The two-month-old plantlets phenotype from each variety treated or not with TPPS looked very similar suggesting that TPPS no longer had any ulterior effect (Figure 43).

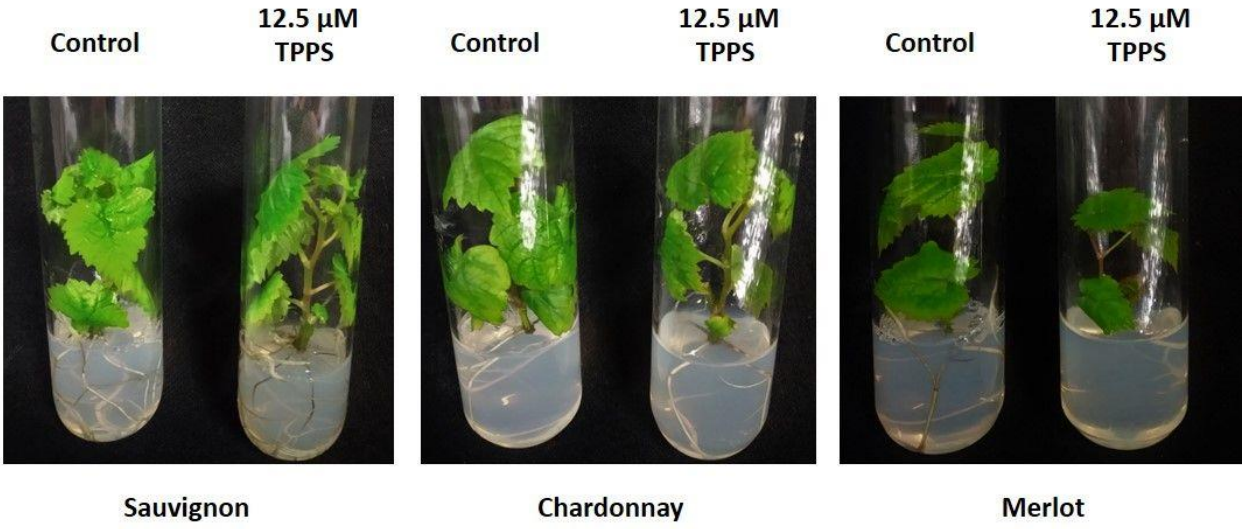

Figure 43: Two-month-old grapevine plantlets grown in the presence of 12.5 µM TPPS or not.
Sourced from Ambrosini et al. 2020.

The 3-month-old plantlets grown *in vitro* confirmed what had been phenotypically observed for the 2-month-old culture (pictures not shown). To confirm this normal phenotype in the presence of TPPS, the total thiols content and GPX activity were determined on 2- and 3-month-old plantlets.
These assays were not performed on 1-month-old plantlets because of their limited growth that led to less starting material, especially for TPPS-treated Sauvignon roots.

IV.1.4. Photoactivated TPPS effect on the two- and three-month-old plantlets

IV.1.4.1. Total thiols assays

Monitoring the thiol production in one-month-old plantlets in the presence of photoactivated TPPS was very informative for two reasons: thiols are involved in the general stress response and they constitute a pool of antioxidant molecules that act as a primary defence against biotic and abiotic stress. Thus, upon TPPS illumination in the 2- and 3-month culture, the total thiols contents were determined in organs (roots and aerial parts) isolated from plantlets of the three backgrounds (Figures 44- and 5).
As a first result, no significant difference in thiol content was determined in aerial parts for the three varieties whether after two- or three-month culture suggesting a limited stress (Figure 44). The situation was quite different for roots that were directly in contact with photoactivated TPPS. After a 2-month culture, Sauvignon- and Merlot-treated roots showed a significant increase in thiol content whereas the same treatment did not seem to disturb the Chardonnay root apparatus (Figure 44).

According to the conclusions inferred from Figure 42, Sauvignon and Merlot varieties seemed to be more sensitive to tissue culture when treated with TPPS. Thus, it was not surprising that both background responses were stronger than the one determined for Chardonnay roots after 2 month-culture. However, the Merlot response, at the root level, seemed to be boosted compared to that of the Sauvignon (Figure 44).

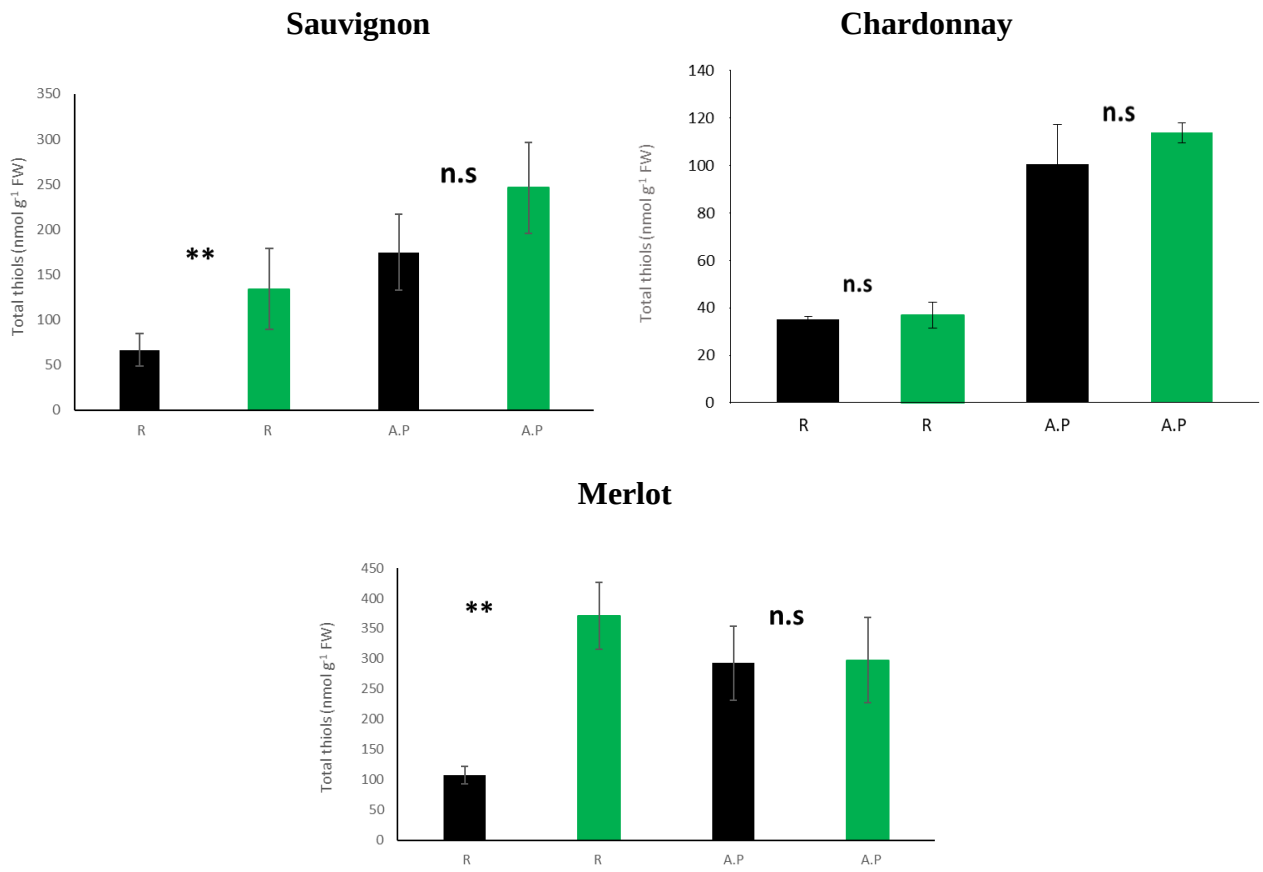

Figure 44: Total thiol assay of 2-month-old grapevine plantlets upon TPPS illumination.

*The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. Results are the mean of five independent experiments ± sd (**: $p<0.01$; ns: not significant).*

Surprisingly after a 3-month culture, Sauvignon roots treated or not with TPPS were similar in terms of thiol content suggesting that TPPS no longer had an effect. Additionally, the same situation was observed for Merlot 3-month-old treated roots with a significant decrease in thiol content compared to the huge amount in 2-month-old treated roots: from around 350 to 100 nmol g^{-1} FW (Figures 44 and 45).

To conclude, an unexpected increase in thiol contents was measured for Chardonnay treated roots (Figure 45).

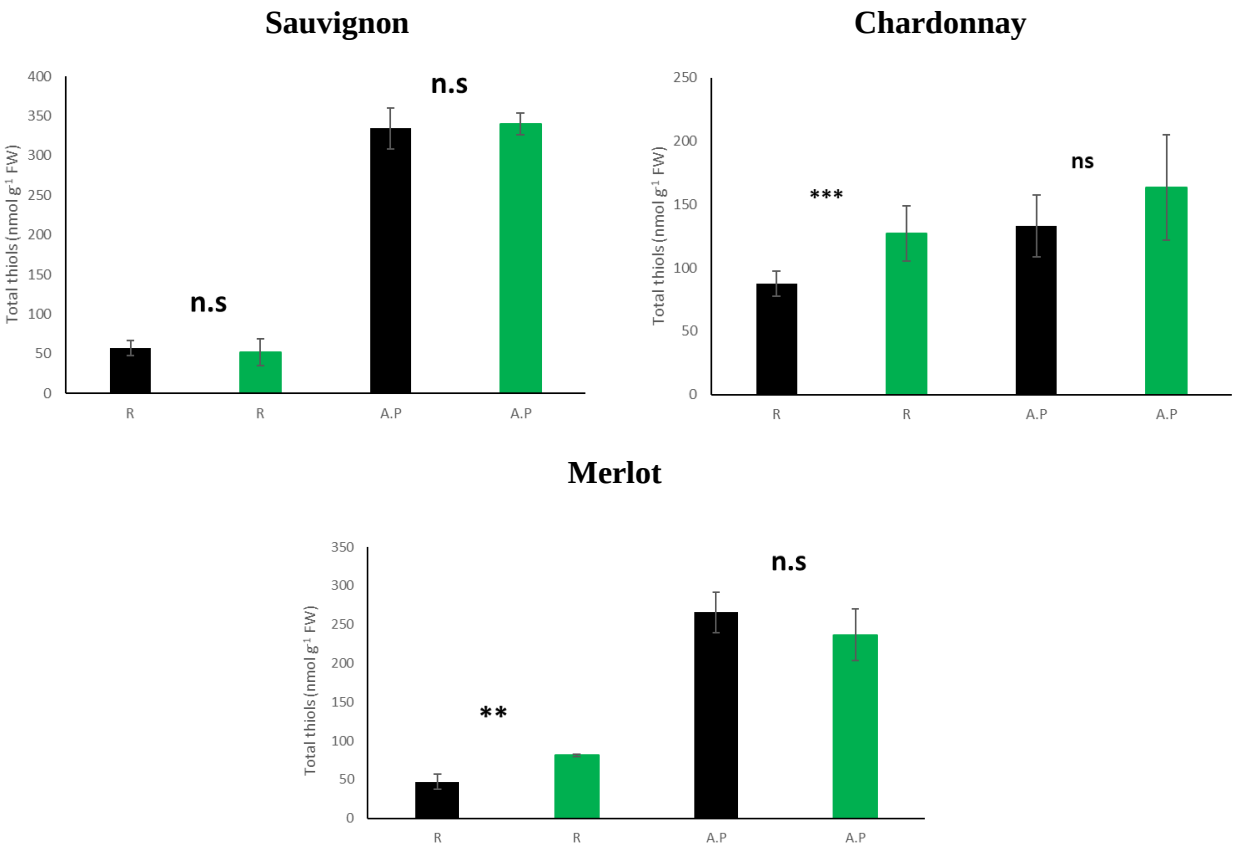

Figure 45: Total thiol assay of 3-month-old grapevine plantlets upon TPPS illumination.

*The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. Results are the mean of five independent experiments ± sd (**: p<0.01; ***:p<0.001; ns: not significant).*

IV.1.4.2. Guaiacol peroxidase activity in 2 and 3-month-old grapevines

Guaiacol peroxidase (GPX) could be involved in the scavenging of H_2O_2 produced by the photo-activation of TPPS. Thus, it was decided to measure its activity as a good indicator of oxidative burst triggered by photoactivation of TPPS. GPX enzymatic assays were carried out on the two- and three-month-old plantlets (Figures 46 and 47).

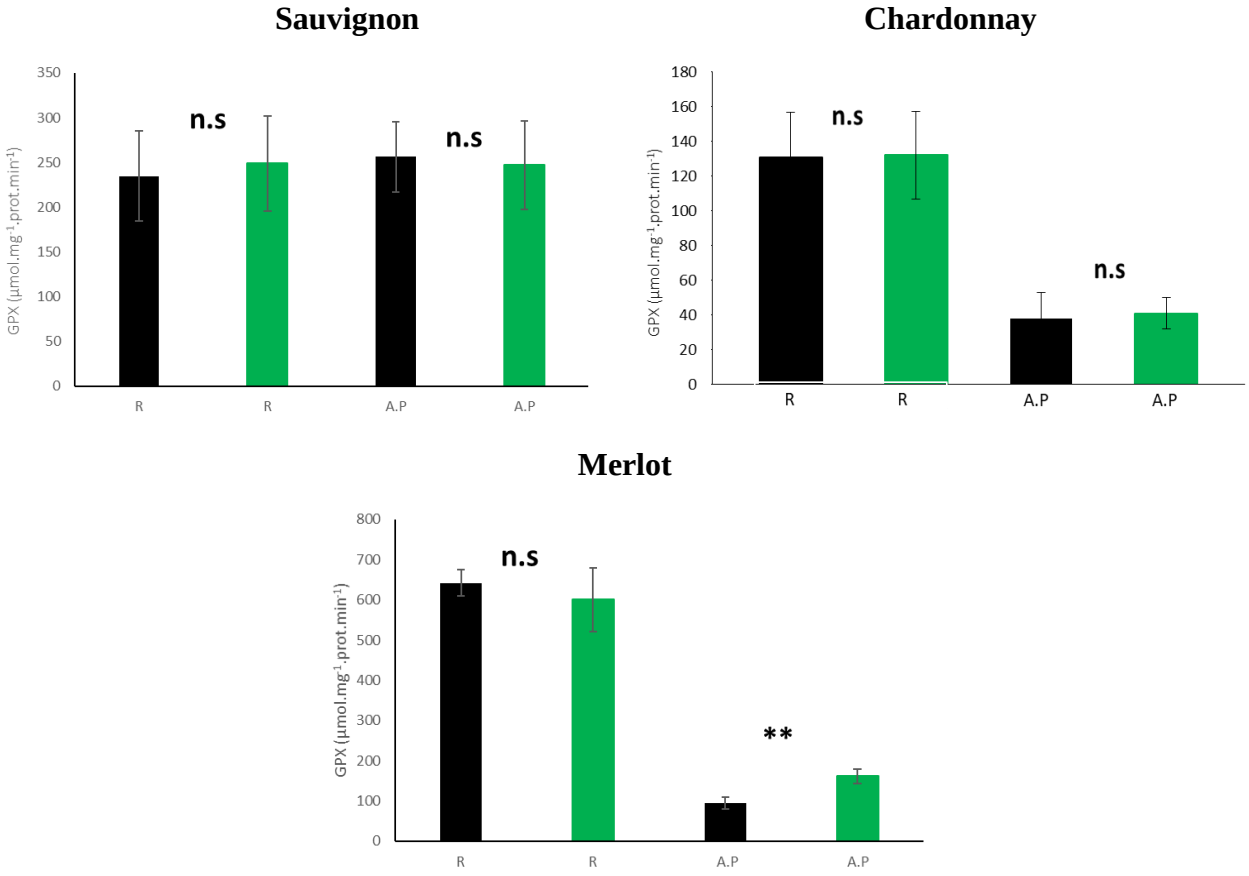

Figure 46: GPX enzymatic activity in 2-month-old isolated grapevine organs upon TPPS illumination.

*The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. Results are the mean of five independent ± sd (**: p<0.01; ns: not significant).*

In the control roots, the GPX activity was very different for the three varieties (Figure 46). While it was approximately 200 µmol.mg^{-1}.prot.min^{-1} or less in Sauvignon and Chardonnay roots, respectively, it increased until 600 µmol.mg^{-1}.prot.min^{-1} in Merlot roots (Figure 46). Moreover, while GPX remained very similar in the root and aerial parts of Sauvignon, it greatly decreased in Chardonnay and Merlot aerial parts (Figure 46). The GPX activity became less obviously marked on

3-month-old organs from the three varieties (Figure 47). It was thought that monitoring GPX in organs of the three backgrounds could contribute to their characterization.

The oxidative burst induced by photoactivated TPPS reflected a stable GPX activity, which remained undetectable after 2-month culture in Sauvignon, Chardonnay roots and aerial parts as well as in the Merlot roots (Figure 46). This activity only increased in Merlot's aerial parts although the thiol content remained constant (Figure 44 and 45). This increase in GPX activity was also observed in the 3-month-old Sauvignon and Merlot varieties but remained constant in Chardonnay aerial part (Figure 47).

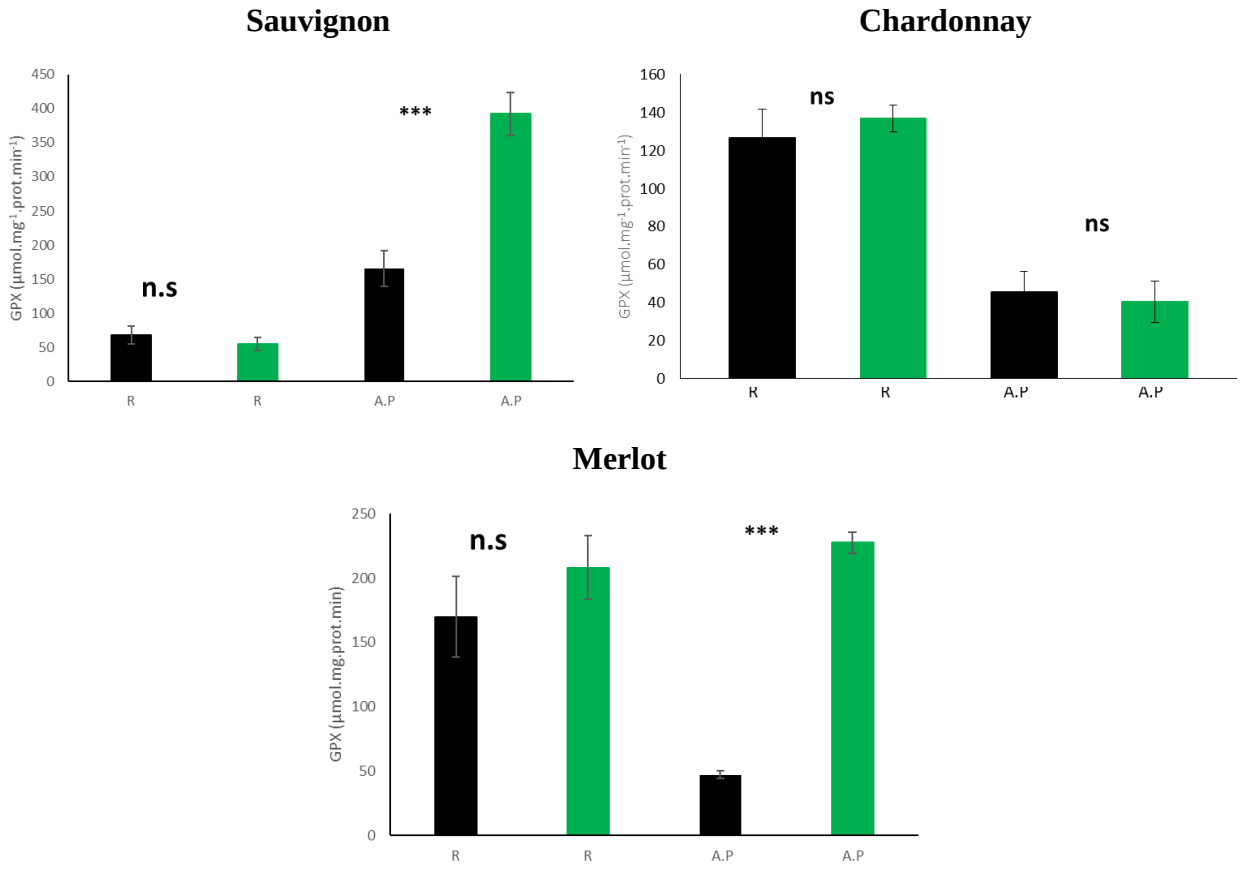

Figure 47: GPX enzymatic activity in 3-month-old isolated grapevine organs upon TPPS illumination.

*The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. Results are the mean of five independent ± sd (**: p<0.01; ***: p<0.001; ns: not significant).*

Combining all the data presented above confirmed, that under a 12.5 µM TPPS photo-treatment, Chardonnay, Sauvignon and (possibly in a lesser manner) Merlot variety were not really stressed as

shown by phenotype and biochemical assays. Thus, towards the goal of APDT, a preliminary experiment was developed using detached leaves, *B. cinerea* mycelium plugs and TPPS. The results were reported in the submitted paper in the next section.

IV.2. PUBLICATION 2: "Photodynamic inactivation of *Botrytis cinerea* by an anionic porphyrin: an alternative pest management of grapevine"

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Photodynamic inactivation of *Botrytis cinerea* by an anionic porphyrin: an alternative pest management of grapevine

Veronica Ambrosini, Mohammad Issawi, Vincent Sol & Catherine Riou✉

Botrytis cinerea **is a necrotic plant fungus that causes gray mold disease in over 200 crops, including grapevine. Due to its genetic plasticity, this fungus presents strong resistance to many fungicides. Thus, new strategies against *B. cinerea* are urgently needed. In this context, antimicrobial photodynamic treatment (APDT) was considered. APDT involves the use of a photosensitizer that generates reactive oxygen species upon illumination with white light. Tetra-4-sulfonatophenyl porphyrin tetra-ammonium (TPPS) was tested on *B. cinerea* using light. 1.5 µM TPPS completely inhibited mycelial growth. TPPS (12.5 µM) was tested on three grapevine clones from Chardonnay, Merlot and Sauvignon, grown in vitro for 2 months. Treated root apparatus of the three backgrounds increased thiol production as a molecular protection against photoactivated TPPS, leading to a normal phenotype as compared with control plantlets. Finally, 2-month-old grapevine leaves were infected with 4-day-old mycelium of *B. cinerea* pre-incubated or not with TPPS. The pre-treated mycelium was unable to infect the detached leaves of any of the three grapevine varieties after 72 h growth when subjected to a 16 h photoperiod, contrary to untreated mycelium. These results suggest a strong potential of photo-treatment against *B. cinerea* mycelium for future agricultural practices in vineyard or other cultures.**

The great challenge of agriculture is to produce sufficient food for the ever-growing world population. Since the 60 s, to access this performance, agri-business practices that include excessive uses of pesticides and fertilizers are becoming the main cause of soil, water and air pollution, as well as loss of fauna and flora biodiversity[1,2]. Moreover, this industrial agriculture triggers major public health problems such as infertility, cancers and child malformations[3,4]. Aware of the need to reduce all these dramatic environmental damages, the European Union introduced the directive 2009/128/EC to reduce the use of pesticides.

To fight off plant competitors and pathogens, new approaches are necessary for safe practices in agriculture such as genetically modified plants, genetic improvements, as well as organic and integrated agriculture[5,6]. The photodynamic treatment is a general and new concept with a large spectrum of applications for animal and plant cells, plant and animal pathogens, as well as microorganisms[7–18]. Thus, photodynamic treatment could represent an innovative and powerful strategy to fight off plant competitors and pathogens in future agricultural practices[17]. One of the key actors of APDT is a molecule called a photosensitizer (PS). When irradiated with light, this molecule produces reactive oxygen species that are toxic for cells[19]. By contrast, most PSs present low levels of cytotoxicity or genotoxicity in the dark[9,20]. Furthermore, biological applications are best conducted with water-soluble PSs which are ideally prone to quick photodegradation, thus avoiding a buildup of toxicity. PSs are classified in many groups such as porphyrins, chlorins, coumarins, furocoumarins, phthalocyanines and phenothiaziniums. Porphyrins and chlorins such as chlorophyllin, have been shown to be very effective against bacteria on kiwi leaves tested in vitro[21,22]. Coumarins, furocoumarin and phenothiaziniums were shown to be active against the plant-infecting fungi *Colletrichum acuratum* and *Aspergillus nidulans*[23–25]. Finally, when tested on *Citrus sinensis* petals and leaves, methylene blue was able to kill the *Colletotrichum abscissum* fungus and proved to be harmless to plant organs; in addition, this treatment did not induce any secondary resistance[26].

Previous research works explored the in vitro phenotypical and molecular responses of Arabidopsis and tomato plantlets to the photodynamic stress induced by an exogenous supply of PS[27,28]. The cationic tetra (N-methylpyridyl) porphyrin, either free base or zinc-complexed, tested at 3.5 µM, inflicted harmful effects

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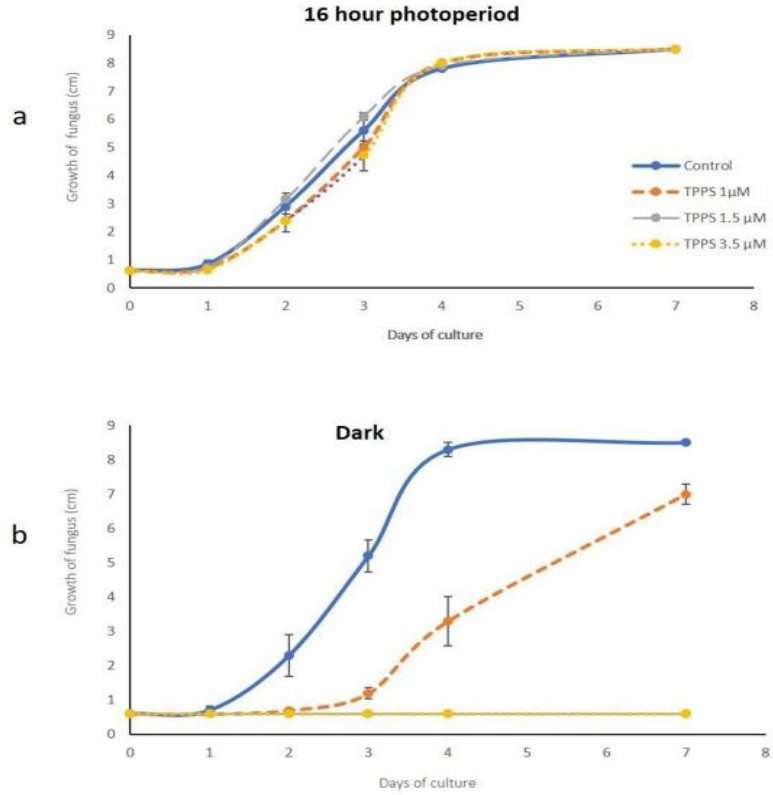

Figure 1. Growth curves of *B. cinerea* mycelium (**a**) in the dark conditions and (**b**) under 16 h photoperiod. Growth curve of *B. cinerea* was performed as follows: a plug of 0.6 cm diameter was placed in the middle of plates containing PDA medium supplemented with or without TPPS. Three TPPS concentrations: 1, 1.5 and 3.5 µM were tested in the dark and under light. Results are the mean of three independent experiments ± sd.

on both 14-day-old Arabidopsis and tomato plantlets. Nevertheless, while Arabidopsis plantlets were killed, tomato plantlets could be rescued after a 14 day-treatment[28]. Surprisingly, the anionic porphyrin tetra-4-sulfonatophenylporphyrin tetra-ammonium (TPPS) did not provoke any harmful effect on both plantlets even at concentrations as high as 50 µM[27,28]. With the aim to develop APDT for agriculture applications, TPPS could be a good candidate because of its low toxicity for plants. Moreover, TPPS remains negatively charged in many chemical environments even under acidic pH and does not aggregate in solution, allowing it to easily permeate cells through cell walls and membranes[29,30]. Therefore, we hypothesized that TPPS could be a good PS candidate to kill the plant pathogen *B. cinerea* and has potential to be a safe option for grapevine (*Vitis vinifera* L.) explants.

B. cinerea is frequently responsible for drastic reductions in crop yields at harvest and for reducing wine quality[31,32]. This fungus displays very strong resistance to many fungicides, due to its genetic plasticity which confers its diversity in morphology, mycelial growth, sporulation and virulence[33–37]. For the Integrated Pest management, the grapevine's susceptibility to *B. cinerea* can be considered an essential management indicator[38–43]. According to the classification proposed by Dubos[42] and Fermaud et al.[43], Chardonnay and Sauvignon are highly susceptible to *B. cinerea* infection whereas the Merlot variety is more resistant. Furthermore, these three varieties are listed in the top 10 most cultivated and famous grapevines for wine production in the world[44].

Therefore, as a first and necessary step, TPPS was tested separately on the three grapevine backgrounds and on *B. cinerea* mycelium with the aim of killing the plant pathogen without affecting plantlet growth and development. As a second and final step, grapevine detached leaves infected with *B. cinerea* mycelium were tested with and without TPPS pre-treatment.

Results

Mycelium growth. The effect of three increasing concentrations of TPPS was monitored on *B. cinerea* mycelium growth under dark and light conditions as described in the material and methods section. As the four curves obtained with or without TPPS in the dark coincided within the standard deviation, we confirmed that, by itself, TPPS did not exhibit any cyto- and/or genotoxic activity against the fungus (Fig. 1a). Under light and at a low TPPS concentration (1.5 µM), mycelium growth was completely inhibited (Fig. 1b). Furthermore, 1 µM TPPS significantly slowed down mycelium growth under light; however, after a 7 day-culture, the colony reached a size similar to that of the control (Fig. 1b). Therefore, 1.5 µM of photoactivated TPPS has been chosen as the minimum fungicidal concentration (MFC). As shown by the growth curves in the dark and in light conditions,

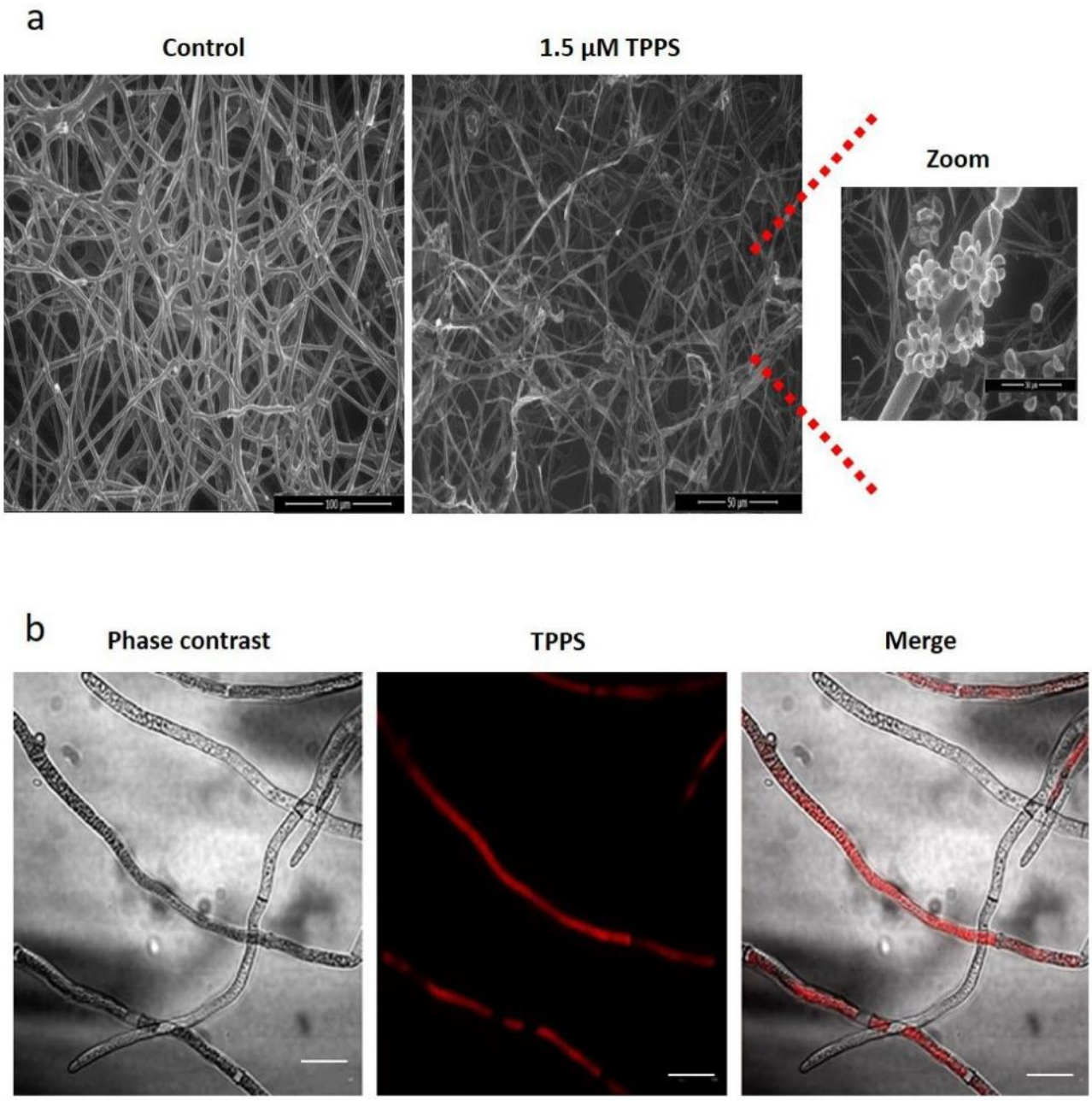

Figure 2. Microscopic observation of *B. cinerea* and TPPS localization in mycelial cells. (**a**) *B. cinerea* visualized under light using environmental scanning electronic microscope. The left picture corresponds to the control hyphae and the middle picture to the irradiated hyphae treated with 1.5 µM TPPS. Treated hyphae shows spore formation (right image). (**b**) TPPS localization in *B. cinerea* hyphae by confocal microscopy. *B. cinerea* was cultivated for 3 days in presence of 3.5 µM TPPS in the dark. Sample was excited at 405 nm and TPPS detection was performed under spectral acquisition with a peak of emission around 640 nm. Scale bar: 20 µm.

the fourth day of culture corresponded to the end of the exponential growth phase (Fig. 1). Thus, all further experiments were conducted with 4-day old mycelium.

Effect of photoactivated TPPS on hyphae morphology. As the mycelium growth was affected by photoactivated TPPS, it was decided to carefully look at the hyphae structure, using ESEM. As expected, photoactivated TPPS induced important phenotypic changes of the hyphae, compared with the control that showed very regular hyphae with a well-organized structure (Fig. 2a). In presence of 1.5 µM TPPS, hyphae were notably less organized, exhibited irregular shapes and produced some spores (Fig. 2a). Moreover, the TPPS-treated hyphae presented a reduced width (1.36 µm) compared with the control (4.7 µm) (data not shown).

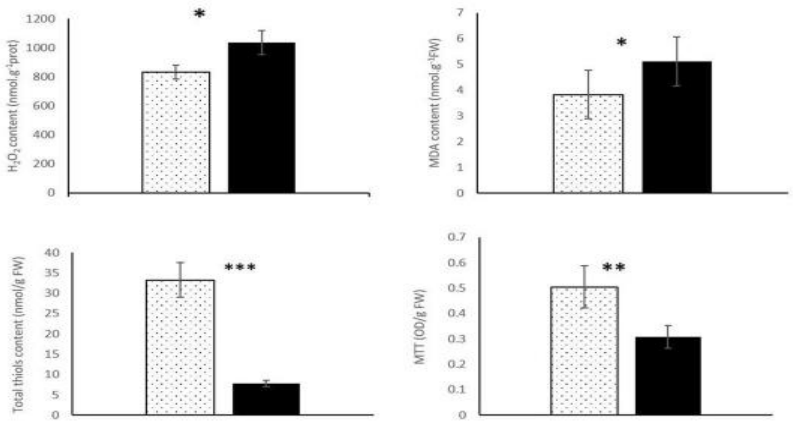

Figure 3. Biochemical activities measured in *B. cinerea* mycelium under a 16 h photoperiod white light for 4 days. Hydrogen peroxide (H_2O_2) production, MDA, total thiol contents and MTT assay were performed with control mycelium (untreated, dotted box) and mycelium treated with 1.5 µM TPPS (black box). Results are the mean of three independent experiments. Statistical significance is determined by a one way ANOVA test (*$P < 0.05$, ***$P < 0.001$).

TPPS localization inside mycelial cells. According to the phenotypical effects observed in TPPS-treated mycelium under light, it was of interest to localize TPPS inside the cells. TPPS was found inside several cells of 4-day-old mycelium (Fig. 2b). This intracellular localization could explain the very strong inhibitory effect of photoactivated TPPS on mycelial growth. As TPPS was located inside cells, this showed that it could cross the cell wall and accumulate in the cell cytoplasm.

Biochemical activities of TPPS-treated *B. cinerea* mycelium. To gain insight into the effect of photoactivated TPPS on the mycelium, biochemical assays linked to oxidative stress and cell metabolism activity, were conducted. As expected, increases in both H_2O_2 and MDA contents were observed in treated mycelium compared to the control, demonstrating at the molecular level that the fungus was stressed (Fig. 3). Metabolic activity, linked to mitochondrial respiration, was monitored with the MTT assay. Indeed, under light, formazan production decreased in the treated hyphae compared with the control, thus explaining the fungal growth inhibition (Fig. 3). While MDA indicated an increase in lipid peroxidation in the presence of photoactivated TPPS, the thiol content provided additional evidence that the fungus was not dead but only deeply stressed (Fig. 3).

TPPS and grapevine plantlets grown in vitro. Internodal explants from each variety: Merlot, Sauvignon and Chardonnay, were placed on 12.5 µM of TPPS for two months in growth chamber as described in the material and methods section. Each culture was examined during a period of 3-months and the explant subculture was performed from 2-month-old plantlets. There was no phenotypical difference between the control and the treated plantlets after a 1-month culture (Fig. 4). To confirm the absence of the phenotypical effect of photoactivated TPPS, we measured the thiol content in aerial and root organs of treated and control plantlets from the three varieties (Table 1). Roots from the three varieties, that were in contact with photoactivated TPPS, showed a significant increase in the total thiol content compared to the control (Table 1). At the aerial level, while no difference in thiol content was observed in Sauvignon and Merlot, there was a significant difference in the total thiol content measured in Chardonnay (Table 1). This could suggest that the Chardonnay variety is more sensitive to photoactivated TPPS than the two other backgrounds although no visible outcome could be seen at the phenotypic level (Fig. 4).

TPPS effect on *B. cinerea* infected leaves. The final interest of this work was to put together grapevine, *B. cinerea* and TPPS with the expectation to kill the pathogen without disturbing plants. As a preliminary assay, before the development of a complete plant pathosystem, our antifungal photodynamic treatment was tested on detached grapevine leaves from two-month-old plantlets. Experiments were conducted as described in the material and methods section, in the growth chamber. The infection was monitored for 72 h. Before 48 h, no change was observed in leaves from the three clones (data not shown). After 48 h, the untreated *B. cinerea* started to invade the leaf surface of the three varieties. The growth of the mycelium, pre-treated with 12.5 µM of TPPS for 8 h, was not completely inhibited (Fig. 5). However, after a 50 µM TPPS pre-treatment, the growth of mycelium was totally inhibited on the leaf surface suggesting that the strategy described in this study worked efficiently.

ESEM analysis was conducted on infected leaves. In uninfected leaves, the structure was well-defined and organized (Fig. 6). However, in the infected leaves when *B. cinerea* was not pre-treated with TPPS, the fungus completely invaded the leaves, making the structure unrecognizable (Fig. 6). By contrast, when *B. cinerea* was pre-treated with 50 µM of TPPS, ESEM pictures showed a leaf structure almost identical to that of the control

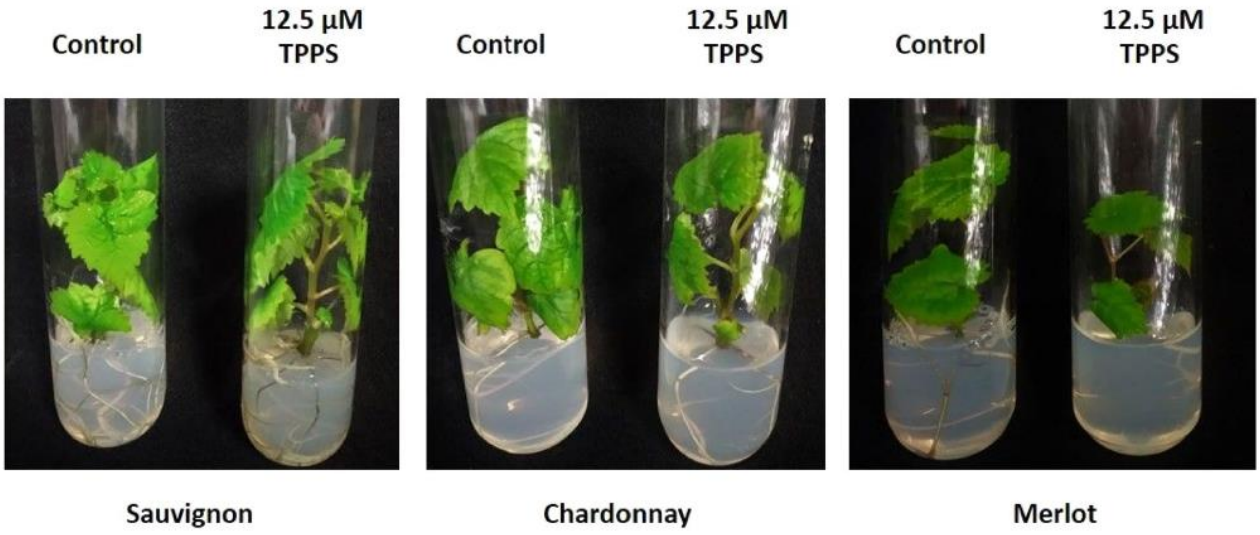

Figure 4. Grapevine varieties growing on control medium and on medium supplemented with 12.5 µM TPPS. Pictures correspond to plantlets from each variety, cultured during one month in glass tubes containing control medium (left) or medium with 12.5 µM TPPS (right).

	Total thiol content (nmol/g FW)			
	Roots		Aerial organs	
	Control	TPPS	Control	TPPS
Sauvignon	66.63 ± 17.96	134.12 ± 44.44**	174.8 ± 42.13	246 ± 55.46
Chardonnay	44.47 ± 5.8	77.5 ± 14.8**	69.35 ± 11.18	106.7 ± 13.4*
Merlot	107.7 ± 14.56	361.38 ± 55.45**	292.77 ± 60.9	297.7 ± 70.3

Table 1. Total thiol content of 2-month old grapevine plantlets. Roots and aerial organs from each variety: Sauvignon, Chardonnay and Merlot, were separately frozen and analyzed. TPPS was tested at 12.5 µM. *FW* fresh weight. Results are the mean of three independent experiments ± sd. Statistical significance is determined by a one way ANOVA test (*$P < 0.05$, **$P < 0.01$). Statistical analysis was always performed against the control. When no indication, not significant.

confirming the macroscopic phenotype (Fig. 6). It was noticed for the Sauvignon variety, that spores were present in the pre-treated *B. cinerea* suggesting that Sauvignon was more susceptible than the other two varieties.

For further investigation, biochemical assays were performed. H_2O_2 quantification, linked to oxidative stress, was conducted for the three leaf conditions and the three varieties after infection or not with TPPS. After a 72 h treatment, a basal content of H_2O_2 was detected in the three leaf varieties, very similar for Chardonnay and Sauvignon leaves and slightly lower for Merlot (Table 2). When the leaves were infected by a 4-day-old *B. cinerea* mycelium, the H_2O_2 content significantly increased for the three varieties especially for Sauvignon (more than 3.5-fold higher than the control leaf) explaining its strong susceptibility to *B. cinerea* (Table 2). An increase in H_2O_2 production in all leaf types in contact with *B. cinerea* was expected. Concerning the leaves infected by the fungus pre-treated with 50 µM TPPS for 8 h, no significant difference was observed between Chardonnay and Merlot leaves compared to leaves infected by the fungus. No signs of infection were observed after 72 h culture (Fig. 5, Table 2). *B. cinerea*, pre-treated with TPPS, was no longer able to induce a high production of H_2O_2 in Sauvignon leaves (Table 2). The H_2O_2 content nearly reached the basal level detected in the control Sauvignon leaves (Table 2). As a response to this H_2O_2 production detected in the leaves induced by *B. cinerea*, and to gain insight into the leaf's proper response, we measured the total thiol content in leaves that were subjected to the three different treatments. For Chardonnay and Merlot detached leaves, no significant difference was observed between the control, infected leaves and infected leaves pre-treated with TPPS (Table 2). A significant increase in thiol content was only observed for Sauvignon leaves between the control and the infected leaves. This result suggested that Sauvignon leaves were able to fight against the fungus infection with a thiol induced response (Table 2). Nevertheless, we also confirm that our pretreatment with TPPS inhibited *B. cinerea* growth on leaves for each variety. These results are promising for the development of APDT treatments in agriculture (Figs. 5, 6).

Discussion

Botrytis cinerea is a very serious problem in a large variety of plants. It is a necrophytic fungus that induces ROS production that contributes to plant cell destruction during its infection[45]. This fungus is able to infect leaves, stems, flowers and fruit, causing severe damages and commercial losses in agriculture. In vineyards, the fungus

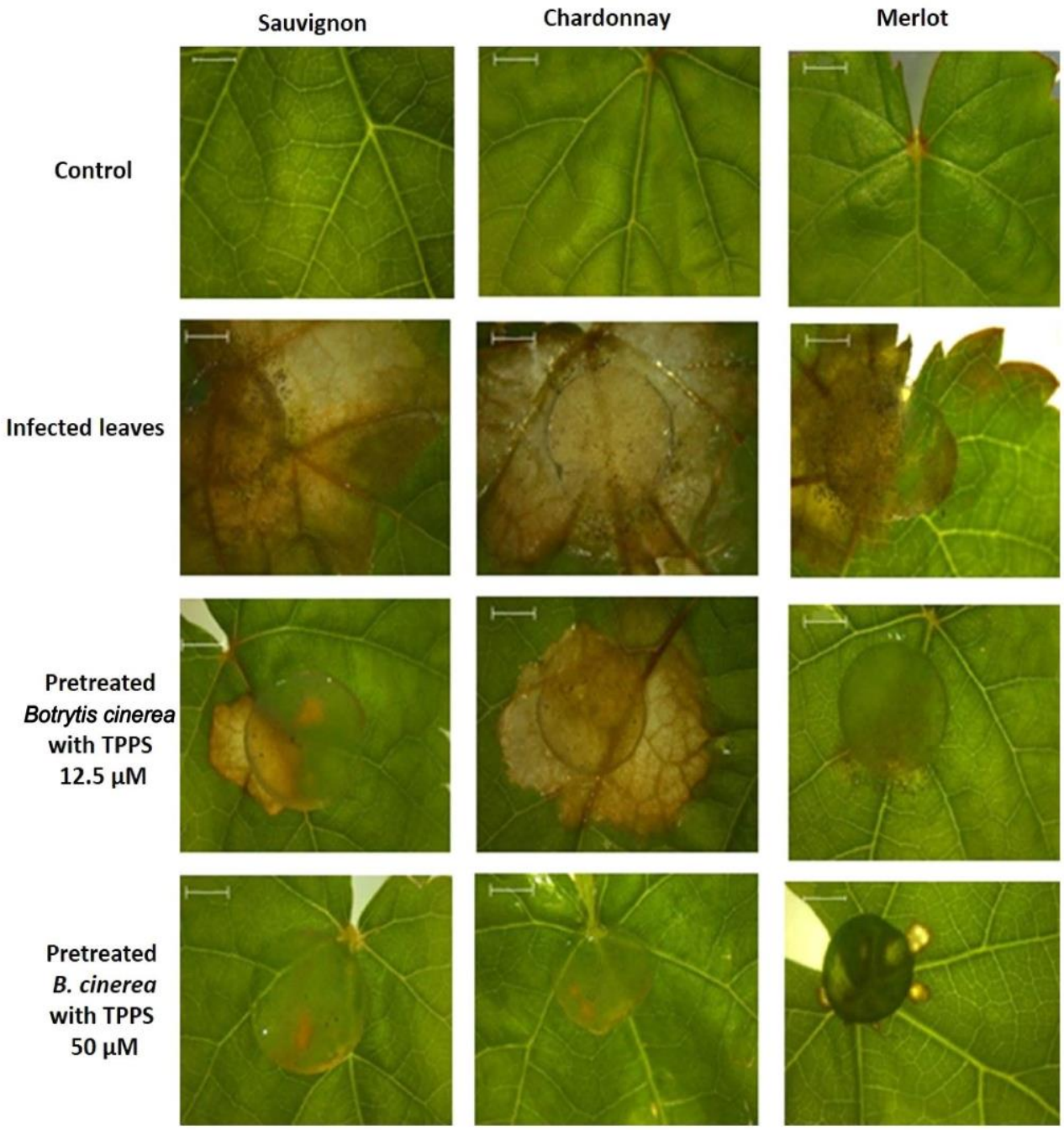

Figure 5. TPPS pre-treatment of *B. cinerea* leads to inhibition of mycelial growth on grapevine leaves. Pictures were taken after a 72 h infection of *B. cinerea*. Detached leaves from the three varieties, without any contact with *B. cinerea* (upper panel). On the three other panels, 4-day-old *B. cinerea* mycelium plugs were placed on detached leaves. Before contact with these leaves, the plugs were pre-incubated or not with 12.5 or 50 µM TPPS for 8 h under dark conditions. Subsequent to pretreatment with 12.5 µM TPPS, mycelium growth was reduced, but the infection was not inhibited. The lower panel corresponds to mycelium pretreatment with 50 µM TPPS: *B. cinerea* was no longer able to invade the leaf surface. The circular plugs correspond to the 6 mm mycelium disc placed on the leaf surface at the beginning of the experiments. Scale bar: 2 mm.

induces several deleterious effects on both quality and quantity of vine production. Despite all the damage it can cause, under specific weather conditions, its growth on grapes induces noble rot that gives rise to sweet wine. However, the fight against this pathogen remains a daily struggle especially for fruit production in summer or fall. For more than 50 years, the use of specific fungicides has largely been envisaged and in that time, *B. cinerea* found coping strategies. As a consequence, fungicide treatments gradually became inefficient, even the famous $CuSO_4$ solution also known as 'Bordeaux mixture' lost its effectiveness against fungal pathogens[46,47]. Moreover, the copper divalent ion, also toxic for plants, contributes to soil pollution[48,49].

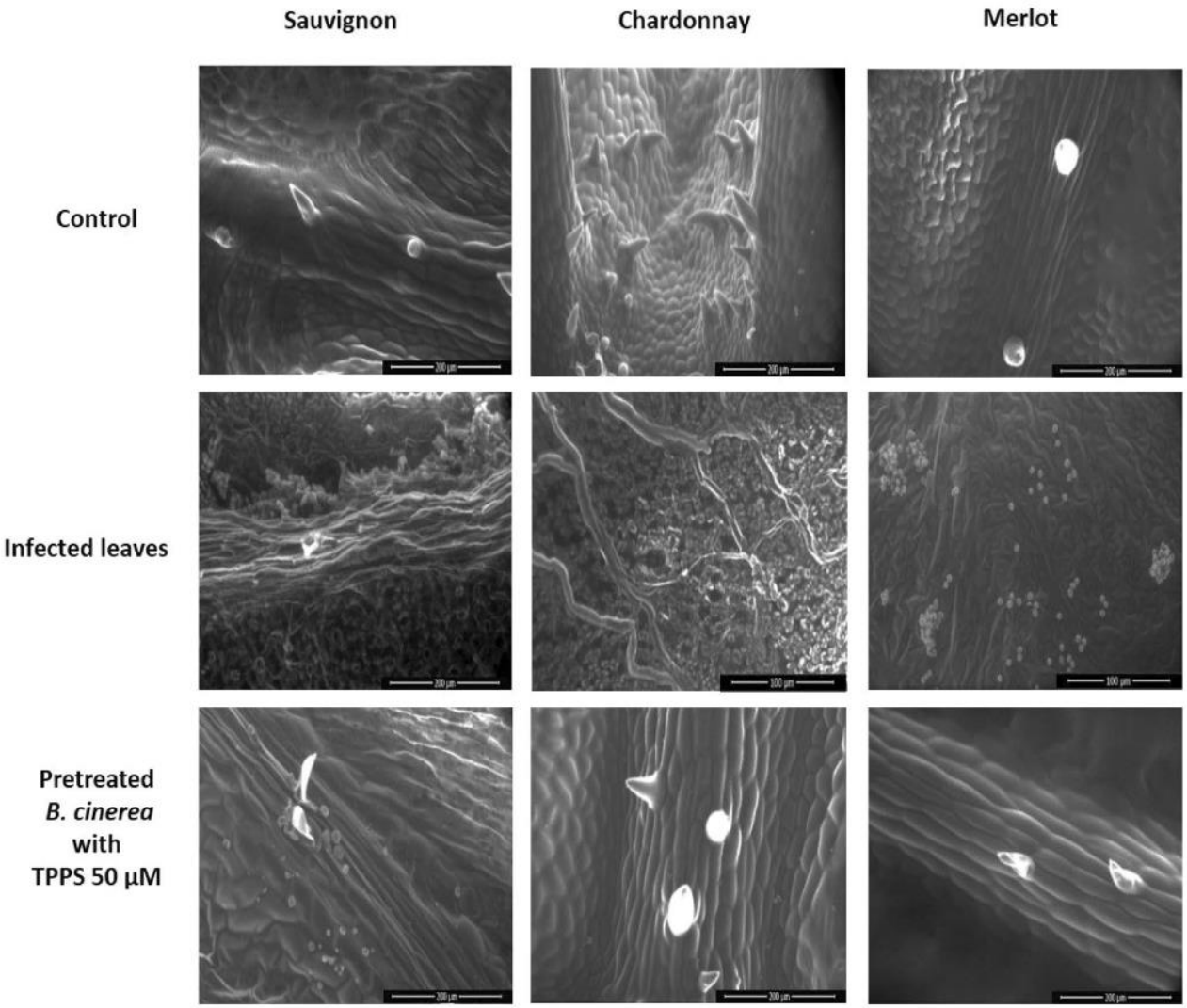

Figure 6. Scanning microscopy observations of leaves infected or not by *B. cinerea* pretreated or not with TPPS. After treatment with TPPS, the fungus was unable to infect the Chardonnay and Merlot leaves. For the Sauvignon variety, spores have been observed on leaf surface, even after TPPS pretreatment.

	Hydrogen peroxide content (nM g^{-1}prot)			Total thiol assay (nmol/g FW)		
	Control (C)	Infected leaves (IL)	Pretreated *B. cinerea* (P*Bc*)	Control (C)	Infected leaves (IL)	Pretreated *B. cinerea* (P*Bc*)
Sauvignon	255 ± 61	904 ± 99 C = ***	338 ± 88 IL = ***	337 ± 44 IL = *	434 ± 34	340 ± 41 IL = *
Chardonnay	204.6 ± 59	473 ± 86 C = ***	408 ± 57 C = ***	206.6 ± 41	291.6 ± 58.3	351.3 ± 59.5
Merlot	156 ± 35	396 ± 26 C = **	293 ± 68 C = *	282 ± 25	323.4 ± 58	259.5 ± 9

Table 2. Biochemical activity assays in detached grapevine leaves infected or not by *B. cinerea*. Detached leaves from Sauvignon, Chardonnay and Merlot varieties were tested and analyzed. *C* control (healthy leaf), *IL* leaves infected with *B. cinerea*, *PBc* leaves infected with *B. cinerea* pre-incubated with 50 µM TPPS for 8 h, *FW* fresh weight. Results are the mean of three independent experiments. Statistical significance is determined by a one way ANOVA test (*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$). Statistical analysis was performed for each assay and was represented as follows: C = *** in IL column meant C compared to IL with a $P < 0.001$. No indication means not significant.

Therefore, new strategies against fungi are urgently needed. Wang et al.[50] used naturally occurring eugenol (EC50 value of 235 µM for *B. cinerea*). This was found to mainly affect fungal mycelium growth rather than the germination of spores as shown in previous reports on fungicides, such as carbendazim and *N*-phenyl carbamates[51,52].

In a similar way, Fleurat-Lessard et al.[53] discovered a strategy whereby they investigated the potential of $FeSO_4$ and found that the sulfate anion determines the inhibition of mycelium growth in pathogenic fungi that is similar to *B. cinerea* at high concentrations (range of 0.5–20 mM). They also tested different iron salts and found that those with bromide, chloride and sulfate anions showed the best antifungal activity. In particular, the addition of an ammonium counterion to the sulfate moiety contributed to the inhibition of mycelium growth in the pathogenic fungus *Eutypa lata,* an ascomycete like *B. cinerea.*

Therefore, taking these results from previous works into account[27,28,30,53], we decided to test TPPS, a molecule that presents four external sulfonate groups linked by a tetrapyrrole ring. Indeed, TPPS with an ammonium counterion could be an excellent antifungal candidate. Moreover, it was demonstrated that TPPS remains negatively charged in a large array of chemical environments, even under acidic pH and does not aggregate in media, allowing it to diffuse through cell walls and membranes more easily[29,30]. Therefore, this PS was thought to be an excellent candidate for APDT.

Under white light, TPPS at a very low concentration (MFC= 1.5 µM) induced a severe inhibition of *B. cinerea* mycelium growth which led to death. We did not succeed to rescue the mycelium after this treatment. To our knowledge, there is little to no information available on the fungistatic or fungicidal effect of light-activated photosensitizers on *B. cinerea*[54]. The first step was to investigate whether the anionic porphyrin was able to induce any changes in the mycelium structure. In the previous study, it was shown that the structure of the *B. cinerea* hyphae changed after treatment with antibiotics, eugenol, $FeSO_4$ and tea tree oil[50,53,55,56]. Moreover, it has been shown that stressed mycelium often produces spores and could show altered cell elongation[57]. According to our data, TPPS also induced a structural change on the fungus. More specifically, the structure of the PS-treated fungus was thinner than the structure of the control. The treated mycelium produced spores implying that the fungus was under stress. Therefore, it was of interest to localize TPPS inside the cells. In tobacco plant cells, TPPS was also tested at 3.5 µM and it was the most effective porphyrin PS to induce cell death under a short light period of 5 h and it was proven to mainly localize in the cell wall[30,58]. Thus, a similar localization of TPPS was expected in the fungal cells. The multi-layer fungal cell wall is enriched in neutral sugars and proteins and poor in chitin and uronic acids, suggesting a neutral global charge of the cell wall[59]. Nevertheless, the *B. cinerea* cell wall composition and it's global charge remain controversial. It was hypothesized that this neutrality could allow TPPS to cross the fungal cell wall which is completely different from the tobacco cell wall[30,59,60]. Further analyses, such as the H_2O_2 content, MDA, MTT, total thiols and microscopy analysis, confirmed fungus stress to the point of dying. Our findings proved that TPPS, due to its characteristics, could be a valid alternative to classic fungicides[29].

From our previous studies, 50 µM TPPS was our reference for plantlets grown in vitro from seeds: Arabidopsis and tomato[27,28]. In this new study, we changed our plant model: grapevines that are obtained from clones, to establish a pathosystem with *B. cinerea*. Comparing with the other plant species, the grapevine varieties did not grow optimally at root apparatus level in the presence of 50 µM TPPS. Thus, the concentration was reduced to 12.5 µM TPPS for all the chosen varieties: Chardonnay, Merlot and Sauvignon. The choice of these three varieties was due to their different susceptibility to *B. cinerea*[42,43]. Furthermore, 12.5 µM TPPS did not induce any phenotypical nor biochemical modification of the three grapevine plantlets. In addition, this TPPS concentration was approximately ten times higher than the minimal concentration inhibiting the mycelium growth (1.5 µM); thus, the strategy presented in this article could work against pathogens without altering plant growth and development.

The final aim was to demonstrate TPPS efficiency against *B. cinerea* in 2-month-old infected grapevine leaves to validate the hypothesis of mimicking a pathosystem. The in vitro cultures and artificial system demonstrated the potential of our strategy. ESEM images from infected leaves, of the three grapevine varieties, after 72 h treatment confirmed that these leaves infected with *B. cinerea* pre-treated with 50 µM TPPS were similar to that of the control. For the Sauvignon variety, the presence of spores on infected leaves confirmed that it is more sensitive to the fungus infection than the other two varieties[42,43]. This is also confirmed by the measure of the total thiol content, considered as a primary and strong defense to infection[61].

In conclusion, TPPS was able to kill the pathogen *B. cinerea* without harming the grapevine leaves in vitro. Moreover, this molecule does not produce any biochemical nor phenotypical changes on the plantlets grown in vitro. These preliminary experiments carried out are indeed promising and, in the future, experiments could be done in a greenhouse and in fields to determine the real potential and efficacy of TPPS against plant pathogens. The results and findings presented herein are also very encouraging because the photodynamic treatment has been developed using a low concentration of PS. Therefore, we show that APDT can be used for the struggle against phytopathogens in the agronomic practices as the PS is effective against plant pathogens and exhibits non-toxic side effects toward plants.

Material and methods

Photosensitizer. 5,10,15,20-(tetra-4-sulfonatophenyl) porphyrin tetra-ammonium (TPPS) was purchased from PorphyChem (Dijon, France). The stock solution (1 mM) was prepared in distilled water and kept in the dark at room temperature for 2 weeks.

***Botrytis cinerea* culture.** The *B. cinerea* strain (UBOCC-A-117017) used in this study was isolated from infected tomatoes and provided by Dr Weill (Université de Bretagne Occidentale, Brest, France). The culture was maintained on potato dextrose agar (PDA). The growth curve of *B. cinerea* was performed as follows: a

plug (0.6 cm diameter) of 2-week old *B. cinerea* mycelium was placed in the middle of plates containing PDA medium supplemented with or without TPPS in the concentration range 0.5–3.5 µM. TPPS was added to the PDA medium just after autoclaving (120 °C, 20 min). Plates were incubated at 22 °C, either in the dark or subjected to a photoperiod of 16 h (Osram large spectrum white lamp: photon flux density of 120 µmol m^{-2} s^{-1}). Daily measurements of the diameter of the fungus were performed and reported to draw the growth curves.

Grapevine clone culture. Chardonnay (clone 7535) was provided by Pr. Clément (Université de Reims, Champagne-Ardenne, France). Sauvignon (clone 379) and Merlot (clone 373) were provided by the Institut Français de la Vigne et du Vin (Bordeaux, France). Intermodal explants of grapevine were dissected and placed in glass tubes or jars containing half Chée and Pool medium, and 2% (w/v) sucrose-solidified medium (pH 5.9) for 2 months. TPPS was added to the medium after autoclaving. Chée and Pool medium was purchased from Duchefa Biochemistry (Haarlem, Holland). The cultures were then exposed to white light (Osram large spectrum white lamp: photon flux density of 120 µmol m^{-2} s^{-1}) for 16 h and the temperature was maintained at 24 °C.

Infection of young leaves with *B. cinerea*. Two-month-old grapevine leaves and four-day-old mycelium plug (0.6 cm diameter) were used to perform the experiment. Mycelium discs were firstly incubated in 12.5 or 50 µM TPPS in the dark and gently stirred for 8 h at 22 °C. Fungus discs were then placed on the upper leaf epidermis of the grapevine (Chardonnay, Merlot and Sauvignon) and were left for at least 72 h under white light (Osram large spectrum white lamp: photon flux density of 120 µmol m^{-2} s^{-1}). The co-cultures were monitored daily and photographed using a Leica stereomicroscope.

MTT assay. A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was carried out on the fungus mycelium, that was treated with TPPS or not, and had grown for 4 days under photoperiod. The MTT assay was performed to quantify the mitochondrial activity of mycelial cells. The fungal samples were frozen in liquid nitrogen and were grounded to a powder. 1 mL of 0.1% (m/v) MTT solution was added to 150 mg of powder. Samples were left in the dark and stirred for 3 h at room temperature. The suspension was centrifuged at 4000×*g* for 10 min. Supernatant was discarded and 1 mL of isopropanol was added to the pellet. Samples were vortexed and centrifuged at 4000×*g* for 10 min. Absorbance was read at 590 nm.

Determination of malondialdehyde content. Approximately 150 mg of fresh or frozen fungal material was grounded in liquid nitrogen. 1.5 mL of 20% (w/v) TCA was added into the powder. The mixture was centrifuged at 13,000×*g* at 4 °C for 20 min. The supernatants were analyzed for their malondialdehyde (MDA) content as described by Issawi et al.[28].

Hydrogen peroxide quantification. The measurement of Hydrogen peroxide (H_2O_2) was performed according to Guillaumot et al. 2016[27]. 1 mL of extraction buffer (50 mM, pH 7.8) was added to fresh samples such as mycelium, plantlets or in vitro detached leaves. The composition of the extraction buffer added to the mycelium samples was 1 mM EDTA, 1% (w/v) PVP, 10% (v/v) glycerol and 1 mM DTT. For the plantlet and the leaf samples, the extraction buffer was almost identical, however PVPP was used in the buffer, instead of PVP. All samples had been frozen in liquid nitrogen, prior to being grounded into a powder. Homogenates were centrifuged at 13,000×*g* at 4 °C for 20 min. 335 µL of 0.1% titanium III sulfate (v/v) was dissolved in a solution of 20% (w/v) H_2SO_4 and this solution was added to supernatants. Absorbances were read at 415 nm and H_2O_2 levels were expressed as nM g^{-1} protein. Protein concentration was determined by Bradford assay using BSA as standard (Bradford, 1976)[62].

Total thiol assay. Approximately 100 mg of fine powder has been obtained from the samples (fungus, plantlets or in vitro detached leaves). After grinding in liquid nitrogen, 1 mL of 0.2 N HCl was added to the powder. A centrifugation at 13,000×*g* for 20 min was performed. Afterward, 500 µL of the supernatant was neutralized with 400 µL NaOH (0.2 M) and 50 µL NaH_2PO_4 (0.2 M). 700 µL of 0.12 M NaH_2PO_4, 6 mM EDTA and 0.1 mL of 6 mM dithiobis-2-nitro-benzoic acid (DTNB) was added to 200 µL of extract. A standard calibration curve was prepared by replacing the extract with 0, 5, 10, 25 and 50 µg/mL glutathione solutions (total volume 1 mL). Absorbance at 412 nm was read 5 min after the addition of glutathione or extract.

Environmental scanning electronic microscopy. Environmental Scanning Electronic Microscopy (ESEM) was performed on *B. cinerea* mycelium and in vitro detached leaves after fungus infection. Mycelium grew on plates supplemented or not with TPPS for 4 days under 16 h photoperiods and was examined under Environmental Scanning Electronic Microscope (ESEM Quanta 450, Felmi-ZFE, Graz, Austria). Sizes of hyphae or branching filaments that constitute the mycelium of the fungus, were measured from ESEM pictures.

Two-month-old healthy grapevine leaves and leaves infected with *B. cinerea* pre-treated or not with TPPS were examined under ESEM.

Confocal microscopy analysis. Mycelium was cultivated for 4 days on media containing 3.5 µM TPPS in the dark. Data acquisition with a LSM510META Zeiss confocal microscope (Carl Zeiss France, Marly-le-Roi, France) was performed under the spectral acquisition mode for TPPS localization inside the mycelium (excitation at 405 nm, emission detected at 640 nm) and under the channel mode for examination of the mycelium.

Statistical analysis. All biological experiments were performed at least three times independently. Results were expressed as a mean ± SD (Standard Deviation). The data were analyzed by one-way ANOVA using the PAST free software.

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Author contributions

V.A. carried out the experiments and wrote the manuscript. M.I. and V.S. contributed to the writing and the substantial re-editing of this paper. C.R. prepared the plant samples and was a major contributor to the writing of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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IV.3. Supplementary results

IV.3.1. Additional result from the last part of the submitted paper

To complete results exposed in figure 5 and Table 2 from the submitted paper, the GPX activity was measured in the detached leaf, detached leaf + *B. cinerea* and detached leaf + *B. cinerea* pre-treated with 50 µM TPPS from the three varieties (Figure 48).

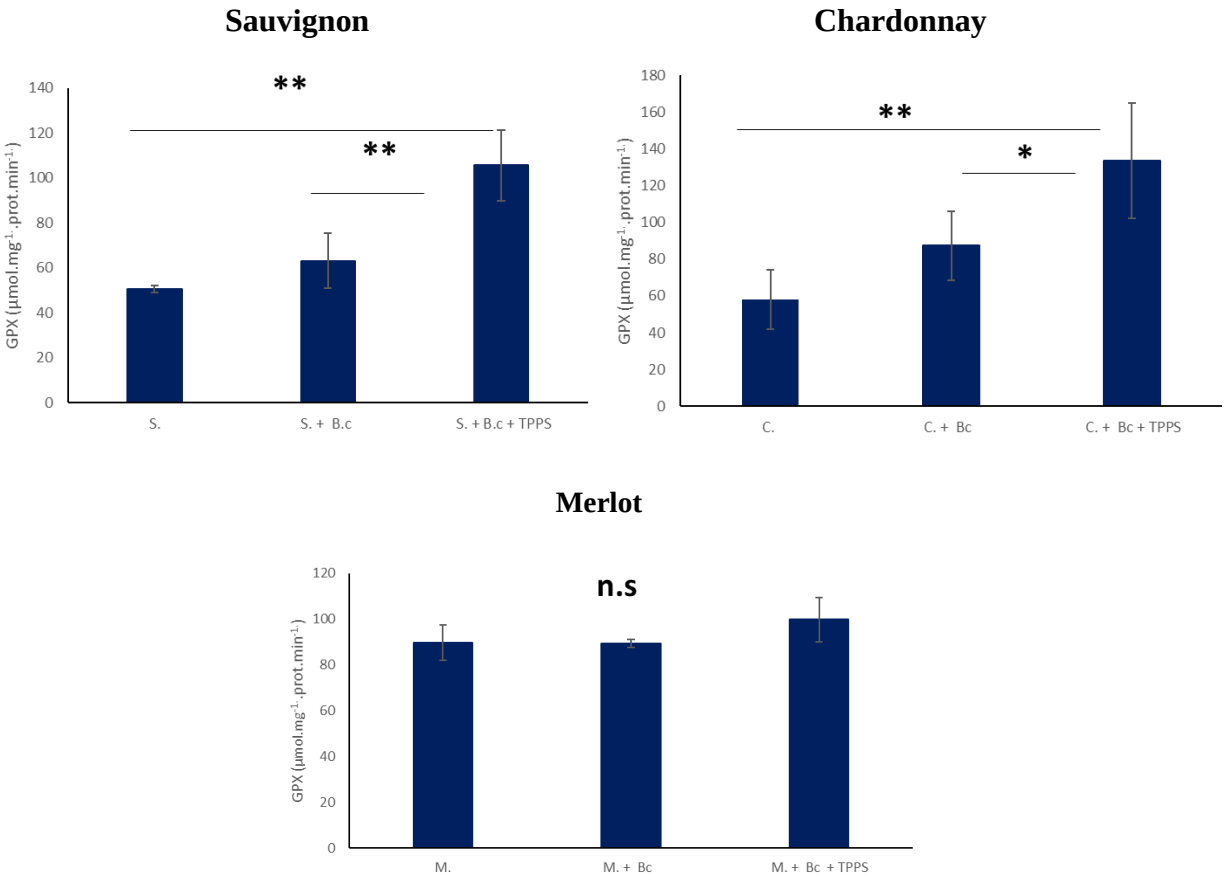

Figure 48: GPX activity in our experimental pathosystem.

Results are the mean of five independent experiments ± sd (: p<0.05; **: p<0.01; ns: not significant).*

According to the H_2O_2 contents in table 2 of the submitted paper, an increase in GPX activity was expected in Chardonnay and Sauvignon grapevine varieties especially in the leaves infected with *B. cinerea*, pre-treated or not (Table 2 of submitted paper, Figure 48). Unexpectedly, no GPX increase was observed for the Merlot infected leaf nor the pre-treated with TPPS, which may explain why this background is less sensitive to the attack of *B. cinerea* (Figure 48, Figure 5 of submitted paper).

More investigations can be envisaged because the GPX enzyme is not the only enzyme capable of scavenging H_2O_2. It was possible that other enzymes, such as catalase or ascorbate peroxidase, are involved. At least, we need to remark here that enzymatic activities in grapevine isolated organs were very difficult to determine with robustness due to important heterogeneity of the plantlet growth under both control and TPPS treatments. This was particularly true for the Chardonnay variety that was the most heterogeneous. Thus, the enzymatic tests were repeated more than 10 times independently for Chardonnay. Fortunately, this was not the case for Sauvignon and Merlot varieties, but, usually, 5 independent tests were performed anyway.

IV.3.2. Miscellaneous results from Chardonnay plantlets

It is important to state that various concentrations of TPPS were tested on the grapevine varieties. The first concentration tested on Chardonnay was 50 µM, which almost inhibited the grapevine growth (data not shown). 25 µM TPPS was then tested on the Chardonnay variety. This concentration did not largely alter the phenotypical growth and development of this variety whereas it did for the Merlot variety (Figure 49). Thus, 25 µM TPPS was not tested on Sauvignon.

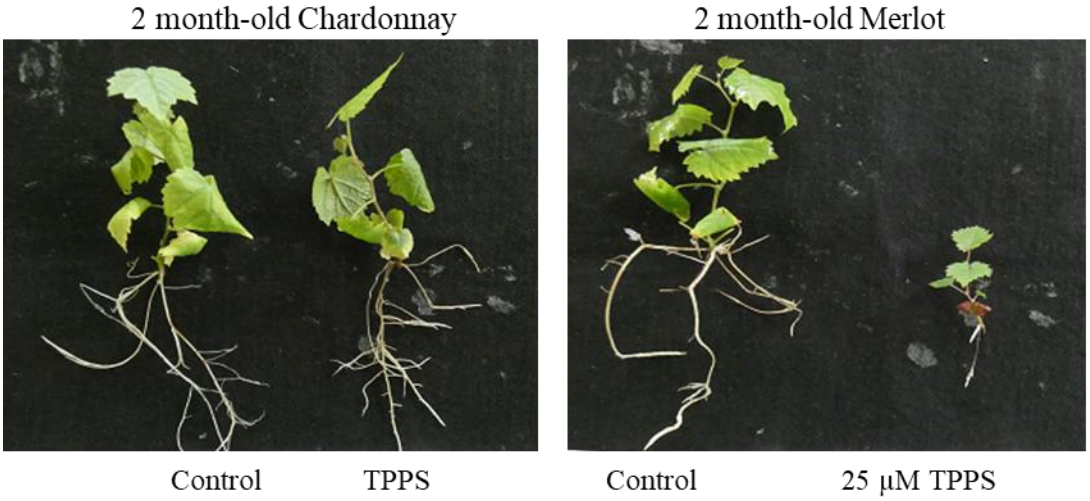

Figure 49: Chardonnay and Merlot phenotype on 25 µM TPPS.

At this point, it was decided to reduce the concentration to 12.5 µM TPPS. As already discussed, this concentration was better for the three variety. It is important to note that more experiments were carried out on Chardonnay because it has been in the laboratory for a longer time (~3 years) while the other two varieties (Sauvignon and Merlot) have been studied more recently.

For Chardonnay, the roots and aerial part measurements were conducted in the control and the plant treated with 12.5 µM TPPS (Figure 50).

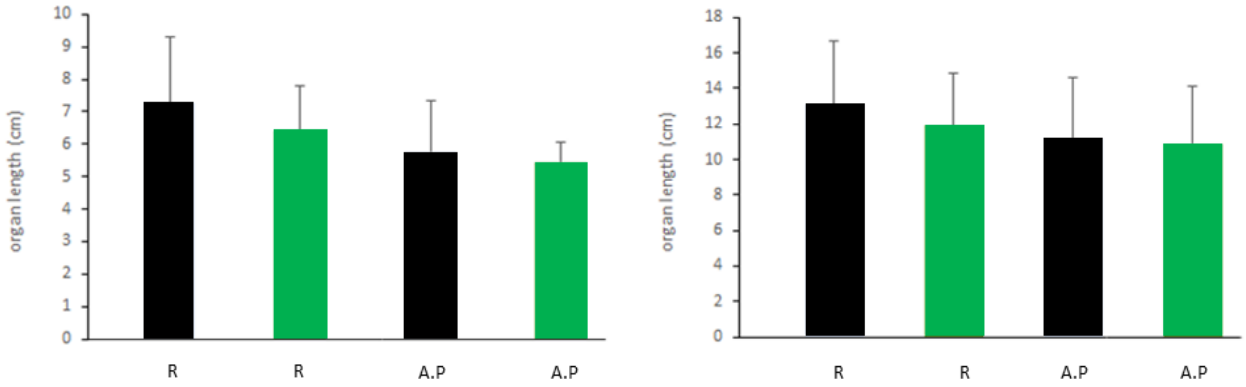

Figure 50: Chardonnay plantlets with measure of organ length.

Two- and three-month-old culture Chardonnay variety with measure of organ length; The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. Results are the mean of ten independent experiments ± sd.

Measurements confirmed no real phenotypic effect of photoactivated TPPS tested at 12.5 µM on the growth of the plant. However, in order to confirm this at a molecular level, biochemical tests were conducted.

IV.3.2.1. Biochemical tests on two- and three-month-old Chardonnay plantlets

The following biochemical assays were performed: H_2O_2, superoxide dismutase (SOD) and catalase (Cat) on two- and three-month-old Chardonnay (roots and aerial parts separately) tested on 12.5 µM TPPS under 16h photoperiod.

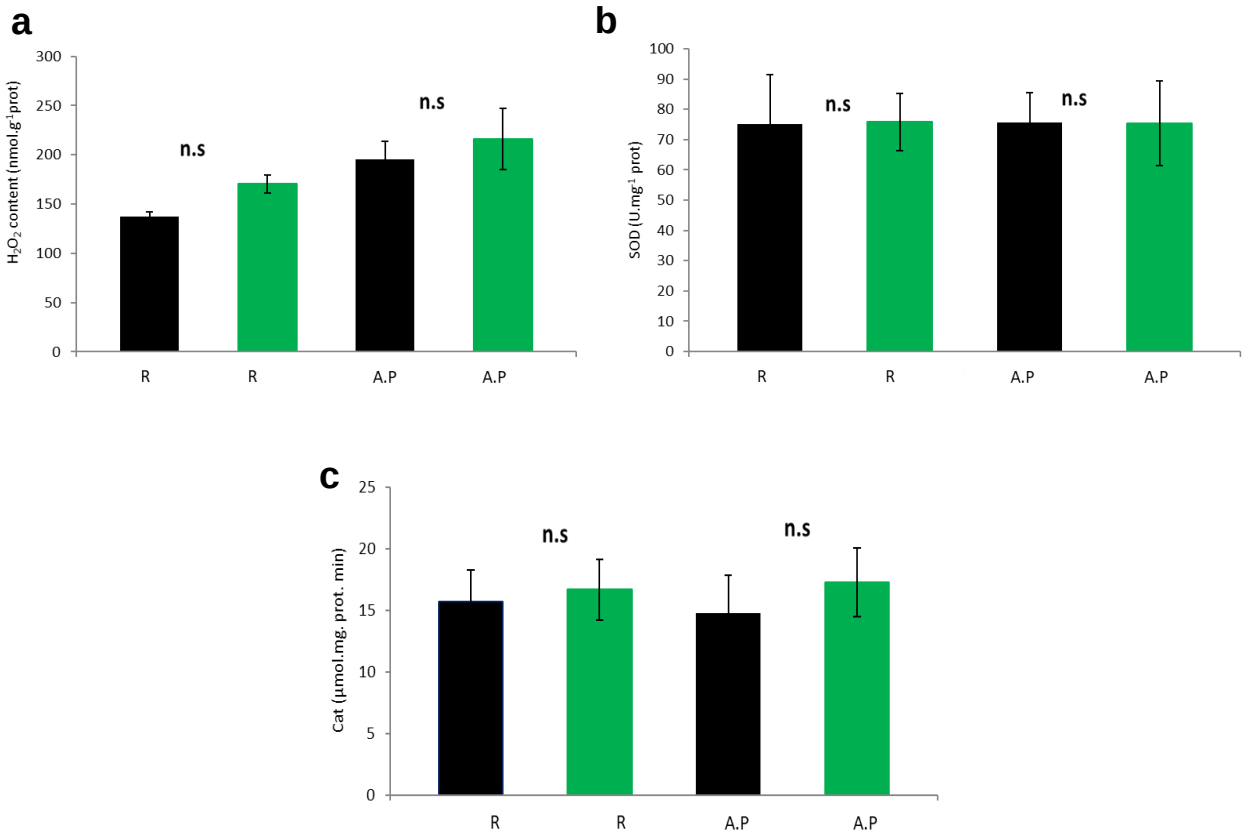

Figure 51: Biochemical assays on 2-month-old Chardonnay plantlets.

The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. ***a)*** *H_2O_2 assay;* ***b)*** *SOD activity and* ***c)*** *Catalase (Cat) activity. Results are the mean of five independent experiments ± sd (ns: not significant).*

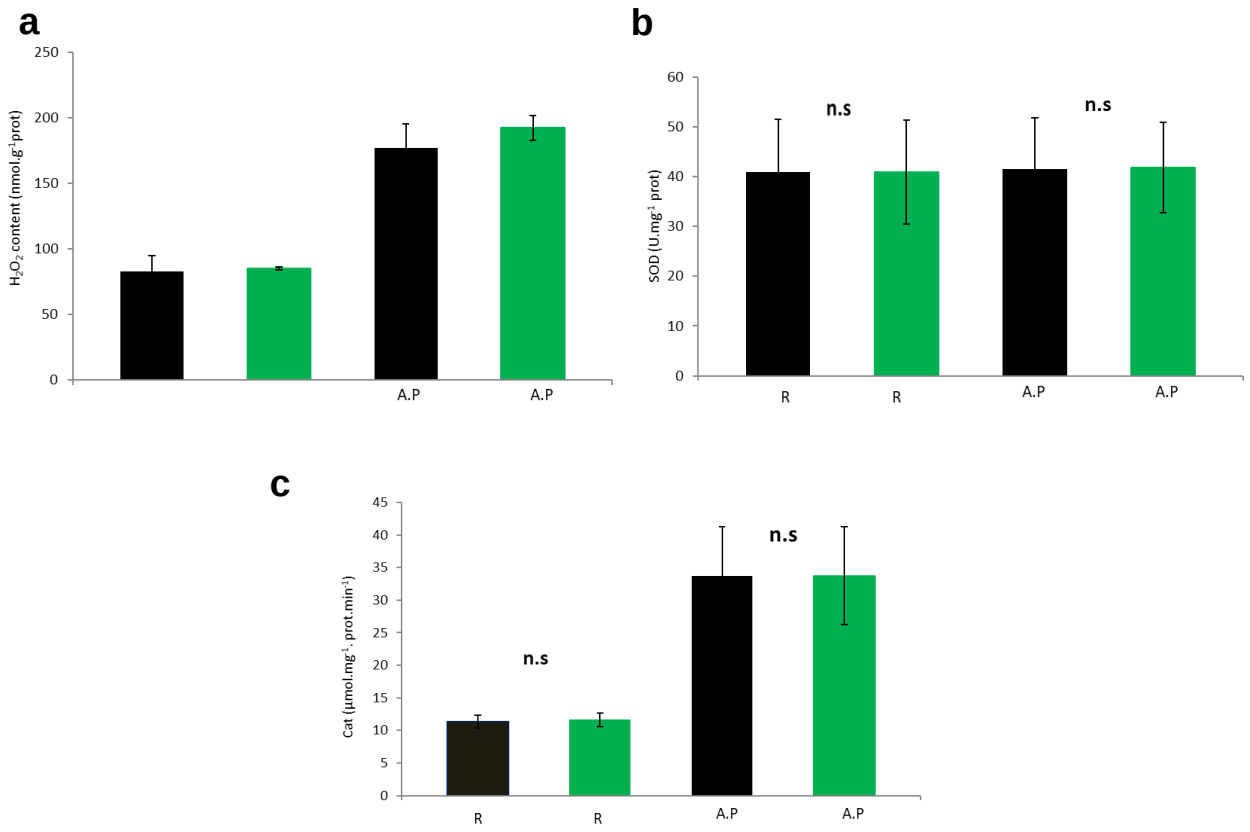

Figure 52: Biochemical assays on 3-month-old Chardonnay plantlets.

The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots and aerial parts isolated from TPPS treated plantlets. ***a)*** *H_2O_2 assay;* ***b)*** *SOD activity and* ***c)*** *Catalase (Cat) activity. Results are the mean of five independent experiments ± sd (ns: not significant).*

From biochemical assays performed on 2 and 3-month-old plantlets, no significant difference was pointed out between control or TPPS treated conditions (Figures 51 and 52). This strongly suggested that the anionic porphyrin under illumination did not affect the Chardonnay growth and development at phenotypical and biochemical levels (Figures 42, 43, 51 and 52). As it was also shown for Sauvignon and Merlot, it was clear that the photoactivated TPPS is rather safe for grapevine varieties and in a greater extent for several plant species.

IV.4. Discussion

Grapevine (*Vitis vinifera*) is a fruit extensively grown worldwide and is of great economic significance throughout the world (Laucou *et al* 2018). The French economy, concerning vineyards, is world renown particularly for the exportation of wine. 17% of the total wine available worldwide is produced in France (OIV 2016; LARVF 2017). The French wine and spirits sector is essential to preserve and further develop, with respect to the environment. Clearly, one way to achieve this is to manage the diseases that affect the grapevine plants with safer practices. Furthermore, pathogens (virus, bacteria and especially fungi) that lead to these diseases can reduce the overall yield of grapevine production (Armijo *et al* 2016). Among fungi, *B. cinerea* is one of the most dangerous for grapevine. As already discussed, this fungus is able to infect grapevine by direct penetration *via* pores, or injury present in the plant or through conidia that can remain latent until berry maturity (Viret *et al* 2004).

The new strategy to fight against *B. cinerea*, proposed in this manuscript, is APDT based on the use of an anionic porphyrin (TPPS) and sunlight. It has been shown and discussed in chapter III, the power of photoactivated TPPS against *B. cinerea*. Furthermore, in this chapter, the harmlessness of TPPS has been demonstrated on three grapevine varieties: Sauvignon, Chardonnay and Merlot. The choice of these varieties was made because of their differing sensitivities to *B. cinerea*. However, before combining the pathogen and the plants to form a pathosystem, it was important to test the plantlet's responses to TPPS under a 16 h photoperiod. Thus, Sauvignon, Chardonnay and Merlot plantlets, growing in the presence of 12.5 µM TPPS for 3 months, were phenotypically very similar to the controls that grew without TPPS. One of the hypotheses to explain the lack of phenotypic alterations in the grapevine varieties, was linked to TPPS photodegradation. Guillaumot *et al* (2016) reported that only 8 % of TPPS remained in the medium after 15 days under light. Thus, in 2-month and 3-month-old grapevine varieties, TPPS was supposed to be completely photodegraded explaining why the phenotype was similar to untreated plantlets.

However, the TPPS concentration used for grapevine (12.5 µM) was lower than that used in experiments conducted on Arabidopsis and tomato plantlets, respectively (50 µM TPPS) (Guillaumot *et al* 2016; Issawi *et al* 2018). The reason can be associated with the fact that the model species were grown from seeds whereas the grapevine varieties were grown from clonal explants. Furthermore, the *in vitro* culture of clonal explants grown under controlled conditions

still show variations that we could not explain. However, the results obtained on the grapevine varieties were very encouraging because of the MFC of *B. cinerea* (1.5 µM).

Therefore, the next step was to prove TPPS efficiency against *B. cinerea* in two-month-old infected grapevine leaves in the hopes of mimicking a pathosystem. Three different biochemical tests: H_2O_2, total thiols and GPX, were conducted on the two-month-old and three-month-old leaves infected with *B. cinerea* mycelium. The first two tests were already discussed in the submitted publication. However, the GPX activity in Sauvignon and Chardonnay increased upon addition of the *B. cinerea* mycelium treated with TPPS whereas H_2O_2 content decreased in these conditions. More investigations are required to diagnose the reason for this result.

Finally, in the near future, the overall goal to confirm our preliminary data will be to conduct spraying solutions on vegetative and reproductive developments of grapevine infected by *B. cinerea* in greenhouse.

IV.5. Conclusion

To conclude, TPPS did not cause any drastic biochemical nor phenotypical changes in the grapevine varieties grown *in vitro*. Thus, it was decided to mimick a pathosystem with this family of plants. Studying different varieties with different susceptibilities to the pathogen *B. cinerea* allowed us to better understand the effect our treatment can have on large varieties of grapevine. Moreover, the results obtained on the plants show the principle concepts of APDT: selectively kill the pathogen and not harmuff the plant host.

Chapter V. TPPS and Chl effects on potato plantlets

This chapter constitutes a continuation of the work on the PS and their effects on new plant species to gain insight into future APDT applications. With that idea in mind, potato plant species and some of its varieties were investigated. As already mentioned, one of the aims of this PhD work relative to the plant side was to focus on the notion of variety and their response to the same PS treatment.
Moreover, the susceptibility to a pathogen is rather linked to the notion of varieties rather than species. In this chapter, the four varieties investigated were: Bintje and Laurette (both commercial varieties), Grenadine and Hinga. As already mentioned, potato is a minor target of *B. cinerea*, However, as discussed in the third chapter, APDT works against *B. cinerea*. Therefore, it will be envisaged to use this treatment also against other Ascomycota fungi, like *Alternaria solani*, that is the principal pathogens of potatoes (Table 6, page 20).
In this last chapter, the growth and development of potato plantlets from the four varieties will be analysed under normal *in vitro* multiplication medium and in medium that contains photoactivated TPPS or Chl. As concluded, the effects of Chl was very promising in inhibiting *B. cinerea* mycelium growth (Chapter III), and thus this PS will be studied as a preliminary plant step on the four potato varieties. We must confess that a similar study is currently being conducted for grapevine, but we estimated that it was to preliminary to be presented in chapter IV.

V.1. Bintje, Grenadine, Laurette and Hinga phenotypical analysis

At the beginning of this PhD work, only the Bintje variety was available in the laboratory. One and a half years ago, our potato clone library grew with Laurette, Hinga and Grenadine varieties that were a gift from Grocep. These potato plantlets grew quite rapidly from internode dissections and cultures (see mat and meth). Thus, the results concerning the phenotype study and biochemical assays were performed on 14-day and 1-month-old cultures from the four varieties either with TPPS or Chl.
The first step of this study was to monitor the growth and development of the 14-day-old and 1-month-old four potato plantlets varieties without PS. The plantlets seemed to support the *in vitro* multiplication well and they looked healthy (Figure 53). No growth or development alterations were observed either after a 14 day or a 1-month culture for the four varieties suggesting an absence of stress (Figure 53). However, the Hinga variety grew slower than the three other backgrounds with very short internodes.

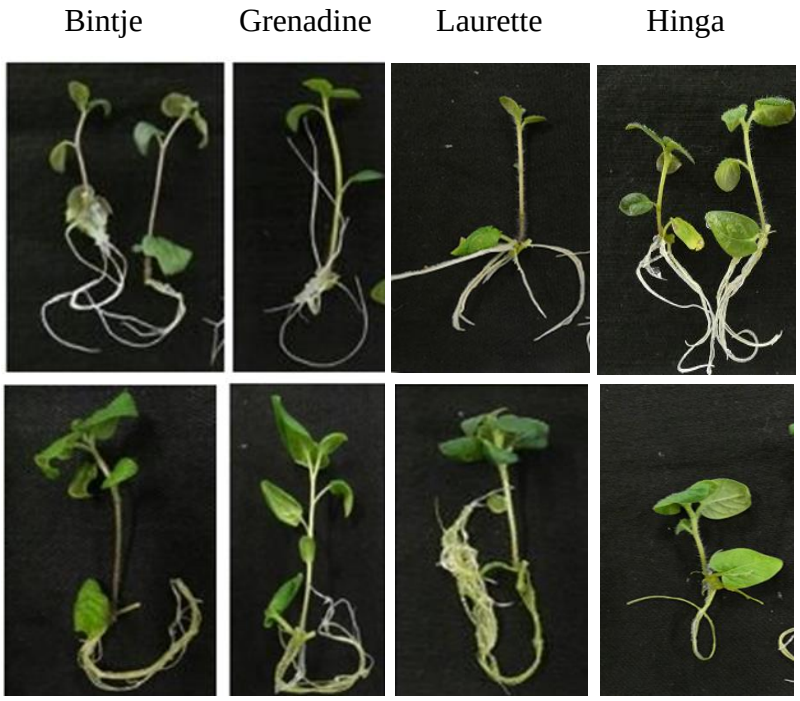

Figure 53: Phenotype of potato plantlets generated in vitro.

14-day-old plantlets grown in vitro conditions and generated from internode explant. 2 plantlets per variety were photographed except for Grenadine, (Bottom panel): 1- month-old Bintje, Grenadine, Laurette and Hinga (from left to right) control grown in in vitro conditions.

As the phenotypical evidence was not sufficient to investigate whether plantlets were stressed or not, a total thiols assay on the roots (R) and aerial parts (A.P) of each variety was conducted (Figure 54).

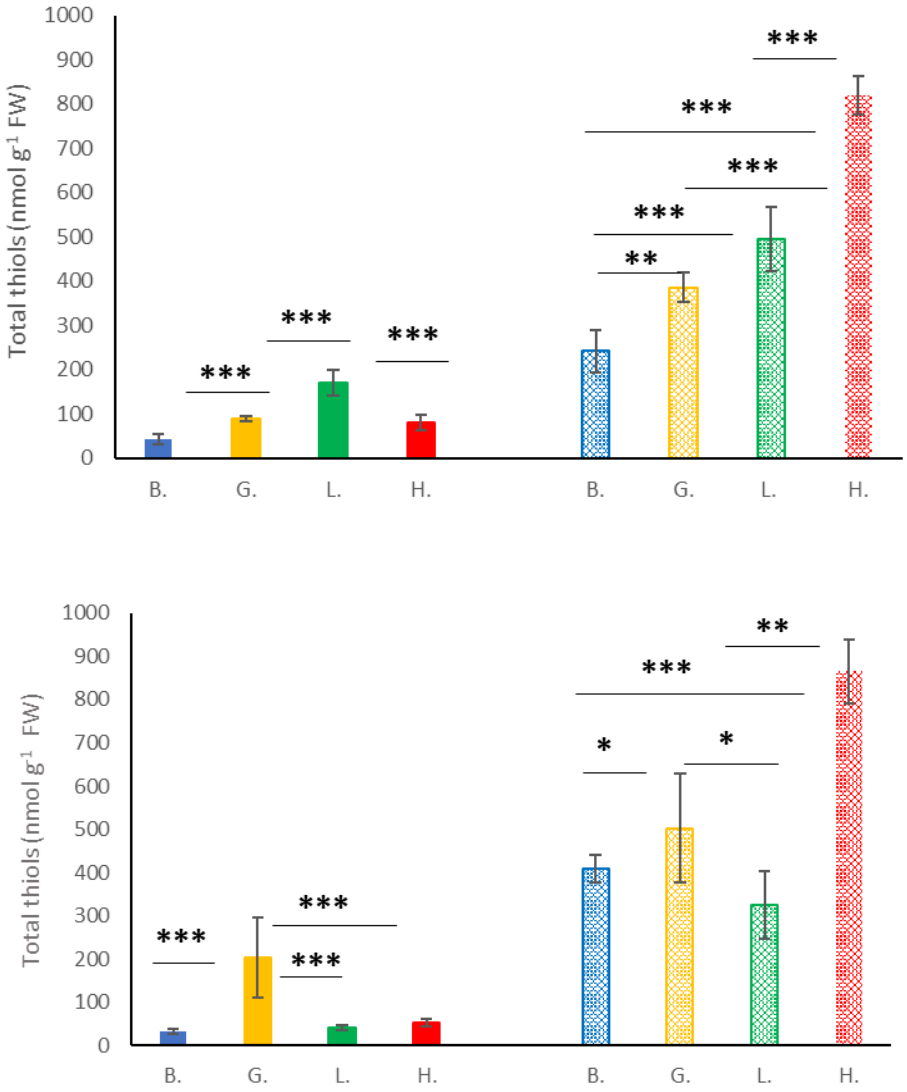

Figure 54: Total thiol assay of the roots and aerial parts of 14-day-old and 1-month-old culture potato variety of Bintje, Grenadine, Laurette and Hinga.

B., G., L. and H. represent the four potato varieties, respectively. The filled boxes on the left represent the roots and the dotted boxes on the right represent the aerial parts. Results are the mean of three independent experiments ± sd (: p<0.05; **: p<0.01; ***: p<0.001).*

The result that arose from Figure 54, was that the four varieties could be characterized by their own total thiol contents in regard to both their roots or aerial parts. For example, Bintje roots showed the lowest thiol content in roots compared to the three other backgrounds (< 50 nmol g^{-1} FW; Figure 54). In the roots of the three other varieties taken after 14-day or 1-month culture, thiol contents remained basically constant (approximately 100 nmol g^{-1} FW) with the exception of Grenadine roots after 1-month that doubled (Figure 54).

However, in the aerial parts of Bintje and Grenadine, the thiols slightly increased between the 14-day to 1-month culture. For Laurette aerial parts, the thiol content decreased between the 14-day and 1-month culture whereas for Hinga, it remained very high (up to 800 nmol mg^{-1} FW). This suggests a situation of stress due to the culture for Hinga (Figures 53-54). According to this assay and phenotype, care was taken concerning the PS effects on Hinga plantlets that were already disturbed by the culture itself.

V.2. TPPS effect on phenotypical development of potato plantlets

As seen under a 16 h photoperiod, 12.5 µM TPPS, the same concentration used for grapevine clones, strongly slowed down Bintje growth (Figure 55). Therefore, this concentration was decreased to 10 µM which appeared to be more suitable for the Bintje growth even if the plants still presented a significant delay in global growth (Figure 55).

Figure 55: 14-day-old Bintje variety treated with (Left): 12.5 µM TPPS and (Right): 10 µM TPPS.

In each picture, the control was on the left and the treated plant, on the right side.

The same situation was also noticed in the Hinga and Laurette varieties. The growth delay was still visible but less pronounced after 1-month culture for the four tested varieties (Figure 56). Whereas, at a concentration of 10 µM TPPS, the potato varieties were still quite sensitive to photoactivated TPPS, it was decided to keep this limit concentration for further investigations on the four potato varieties.

Figure 56: 14 day-old or 1-month-old Laurette and Hinga varieties treated with 10 µM TPPS.

The photos shown constitute. Upper panel: 14-day culture and lower panel: 1-month on the right. In each picture, the control was on the left and the treated plant, on the right side.

V.2.1. Total thiols content assay

To investigate whether there was stress present in the three varieties, due to the addition of TPPS in the artificial growth, the thiol contents were measured (Figure 57).

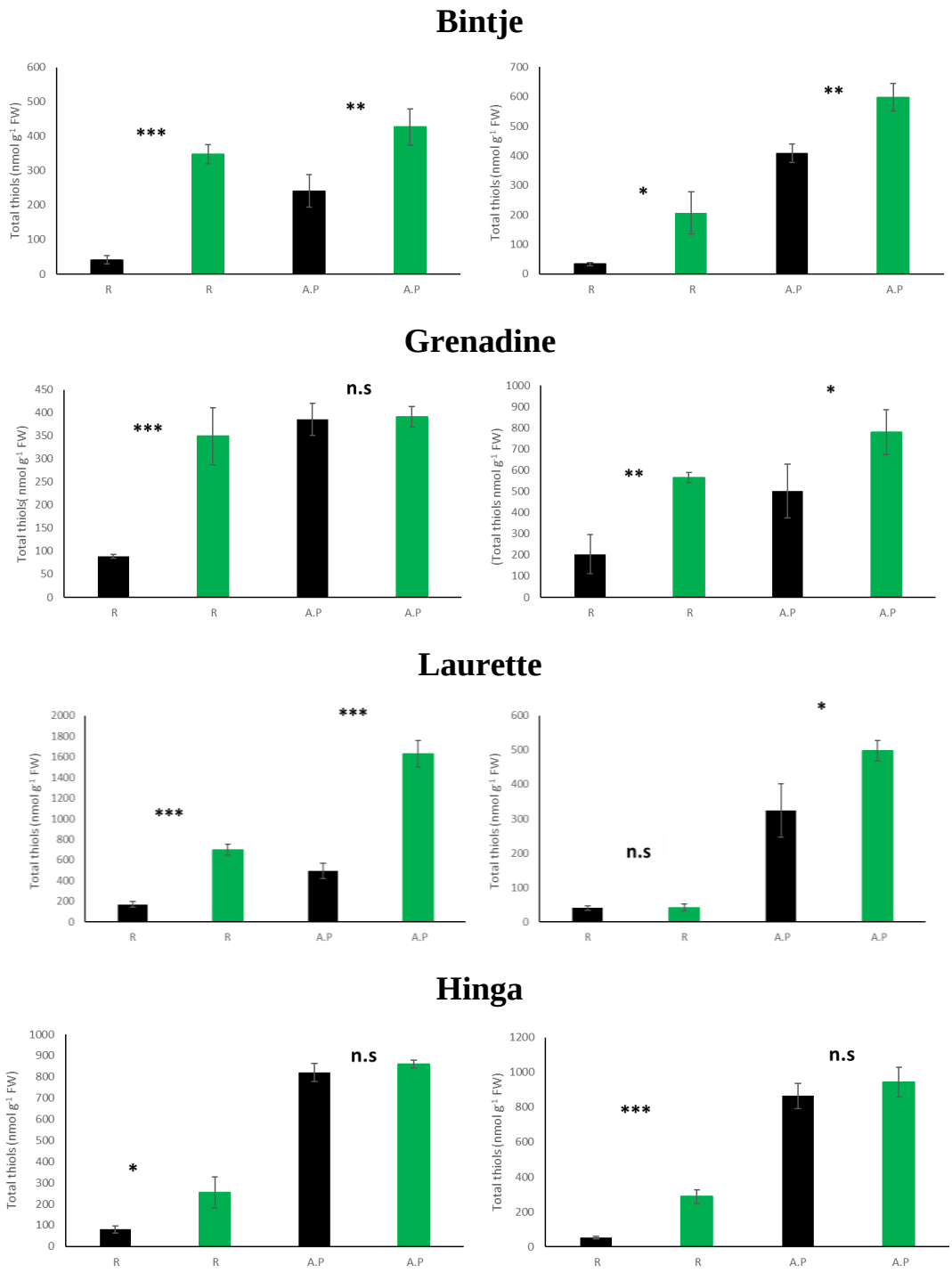

Figure 57: Total thiol content in roots and aerial parts of 14-day-old and 1-month-old potato varieties treated with 10 µM TPPS.

The graph on the left represents the 14-day-old potato plants and the graph on the right represents 1-month-old potato plants. The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots (R) and aerial parts (A.P) isolated from TPPS treated plantlets. Results are the mean of three independent results ± sd (: p<0.05, **: p<0.01, ***: p<0.001; ns: not significant).*

In the 14-day-old potato varieties, there was a significant difference in the production of thiols due to TPPS photoactivation in the roots and aerial parts of every potato variety, except the aerial parts of Grenadine and Hinga (Figure 57). For both varieties, the thiol content was already high in their control aerial parts, suggesting that it could no longer increase after a 10 µM TPPS treatment. After a 1-month culture, there was a difference in the total thiol content in the roots and aerial parts of almost every potato variety studied. However, two exceptions were pointed out with Laurette roots and Hinga aerial parts where no significant difference between the control and treated parts with 10 µM TPPS was detected.

V.2.2. GPX and Cat enzymatic activities as H_2O_2 scavengers

GPX and catalase activities were measured as scavengers of H_2O_2 that is generated by TPPS photoactivation.

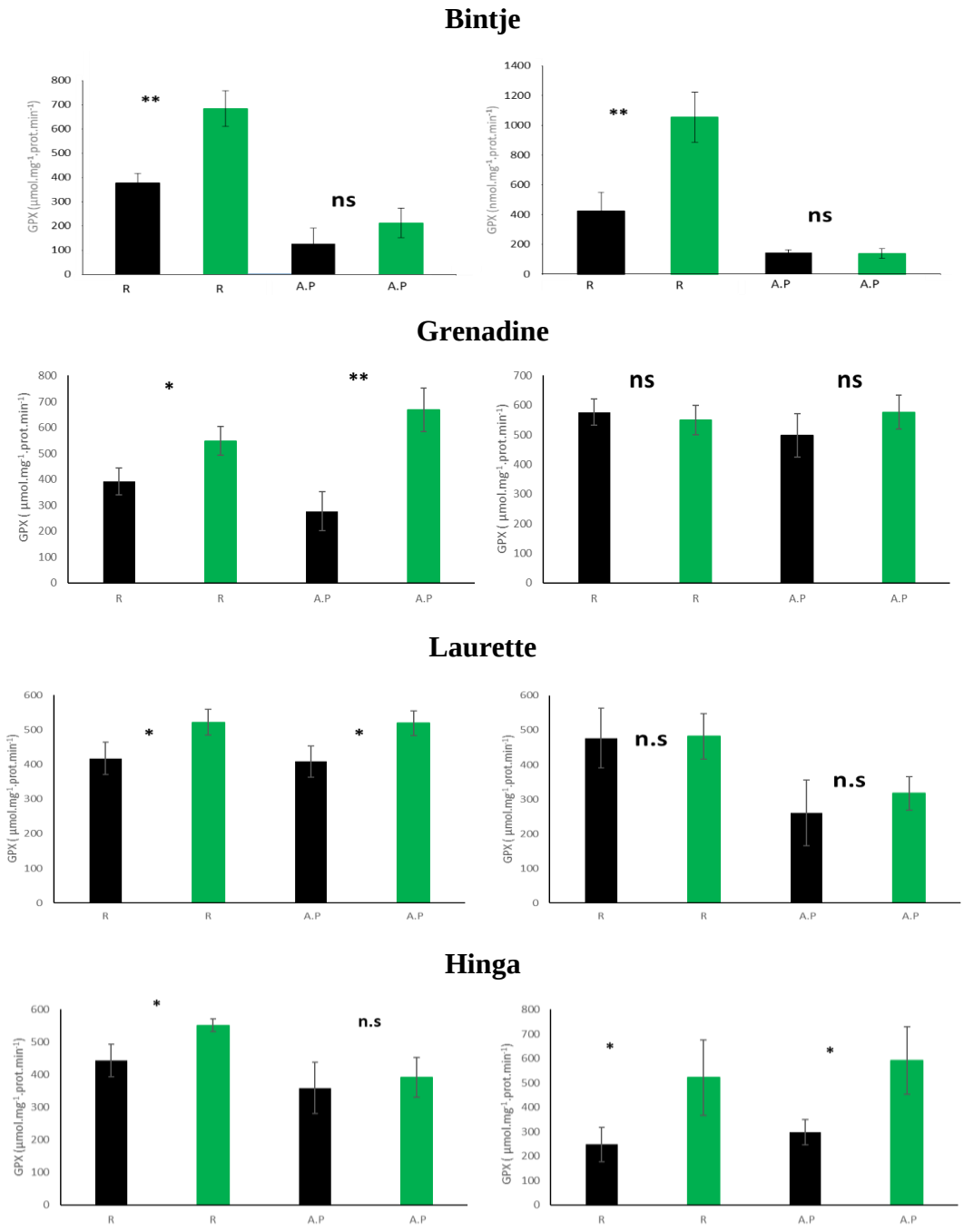

Figure 58: GPX assay of roots and aerial parts of 14-day-old and 1-month-old potato varieties treated with 10 µM TPPS.

The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots (R) and aerial parts (A.P) isolated from TPPS treated plantlets. Results are the mean of three independent results ± sd (: p<0.05, **: p<0.01, ns: not significant).*

In the 14-day-old potato varieties, there was a statistically significant difference between all organs in the four varieties, with exception to the aerial parts of both Bintje and Hinga. After a 1-month culture, no difference in GPX activity was noticed except three cases: roots of Bintje and both the roots and aerial parts of Hinga (Figure 58).

For the catalase activity, Bintje showed the most various profile after 14-day or 1-month culture with an increase in Cat activity either in treated roots or aerial parts, suggesting this enzyme could help the plantlets to resist to photoactivated TPPS (Figure 59). For the other treated plantlets, the catalase activity was more constant with the exception of 14-day-old Grenadine and Hinga aerial parts that showed a significant increase from 10 (control) to approximately 30 $\mu mol.mg^{-1}$ $prot.min^{-1}$ (treated).

From these enzymatic assays (GPX and Cat) and thiol content determination, plantlets defended themselves to oxidative burst generated by photoactivated TPPS that came over the basic stress generated by tissue culture.

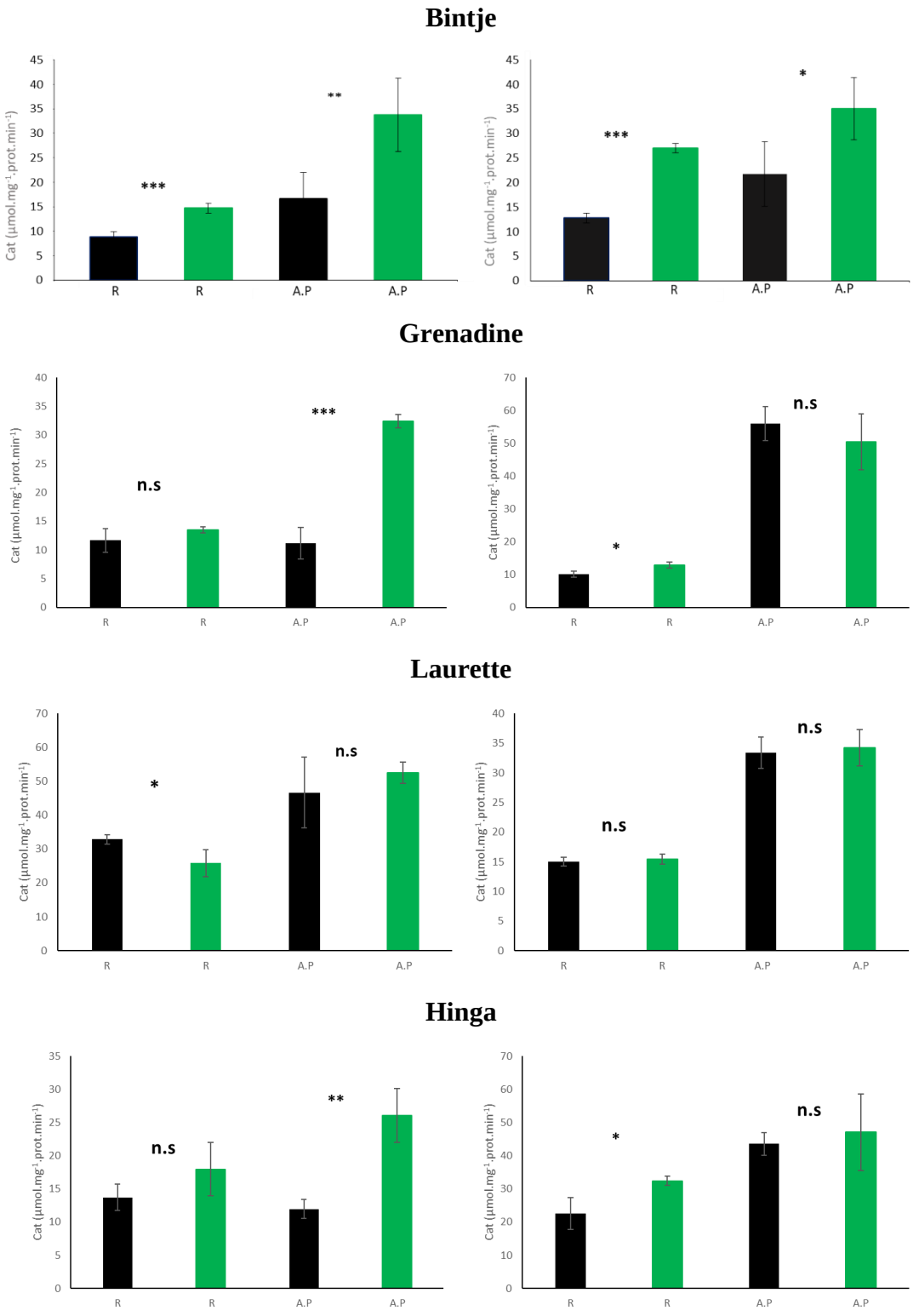

Figure 59: Cat assay of roots and aerial parts of 14-day-old and 1-month-old potato varieties treated with 10 µM TPPS.

The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots (R) and aerial parts (A.P) isolated from TPPS treated plantlets. Results are the mean of three independent results ± sd (: p<0.05; **: p<0.01; ns : not significant).*

V.3 Chlorophyllin induced phenotype

As TPPS did not seem to be the ideal PS for potato plantlets generated from the four chosen varieties, Chlorophyllin (Chl) was investigated as a natural PS from the chlorin category. In this section, the same experiments were performed as were performed for TPPS, as shown above. Surprisingly, even at concentrations as high as 50 µM Chl, there were no harmful phenotypic effects on the 14-day-old and 1-month-old Bintje potato variety (Figure 60).

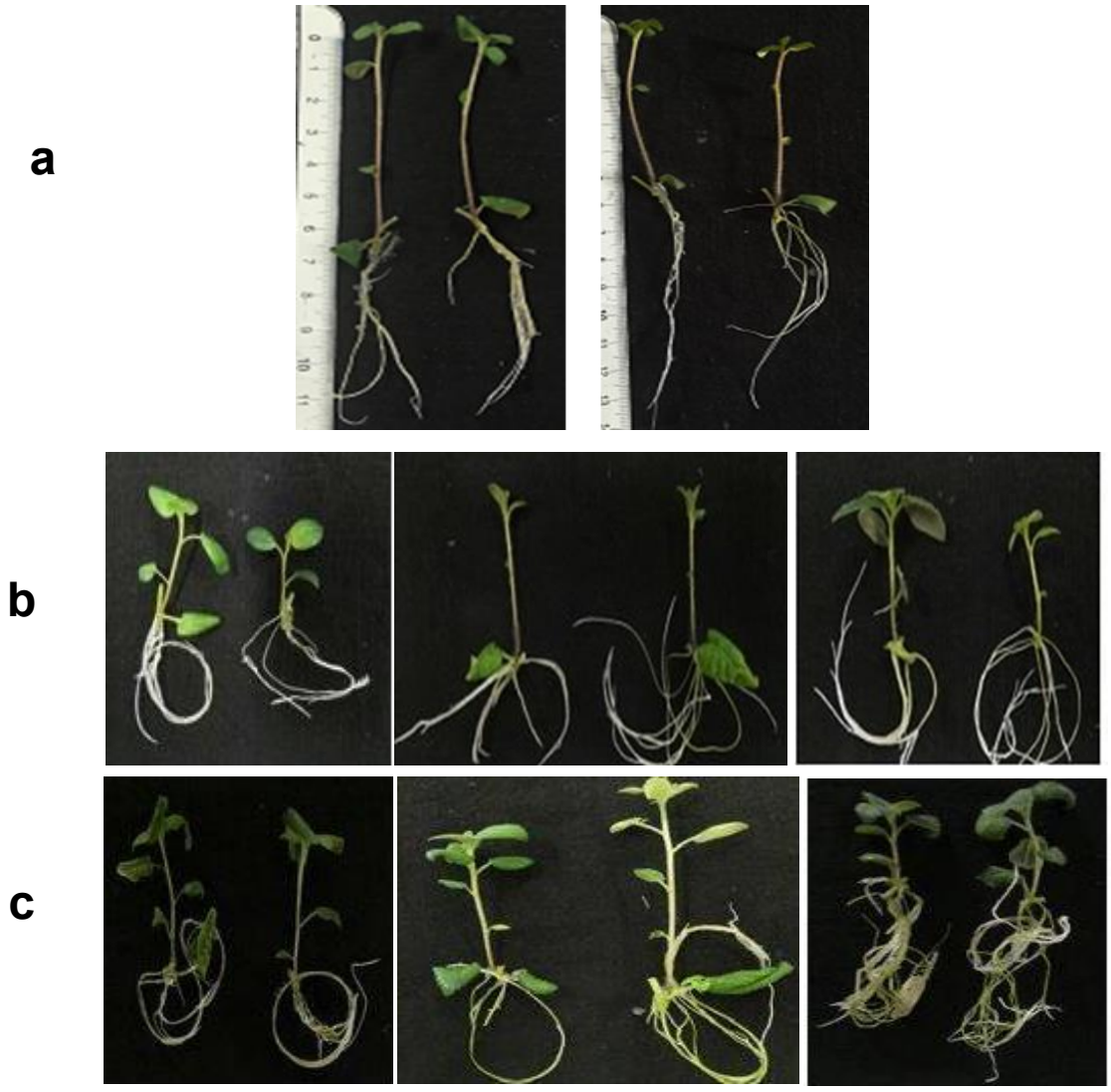

Figure 60: Chlorophyllin effect on the four potato clones.

Chl was tested at 50µM. (a) Bintje variety (left) 14-day-old control and treated with Chl, (right) 1-month-old control and treated plantlets (b) First panel corresponded to 14-day-old plantlets from left to right: Grenadine, Laurette and Hinga. (c) Second panel corresponded to 1-month-old plantlets from left to right: Grenadine, Laurette and Hinga. For each picture, the control was on the left and the treated plant on the right side.

V.3.1. Total thiol contents in Chl treated plantlets

To confirm that Chl had no harmful effects on the potato varieties, a total thiols assay was conducted on 14-day-old and 1-month-old plantlets.

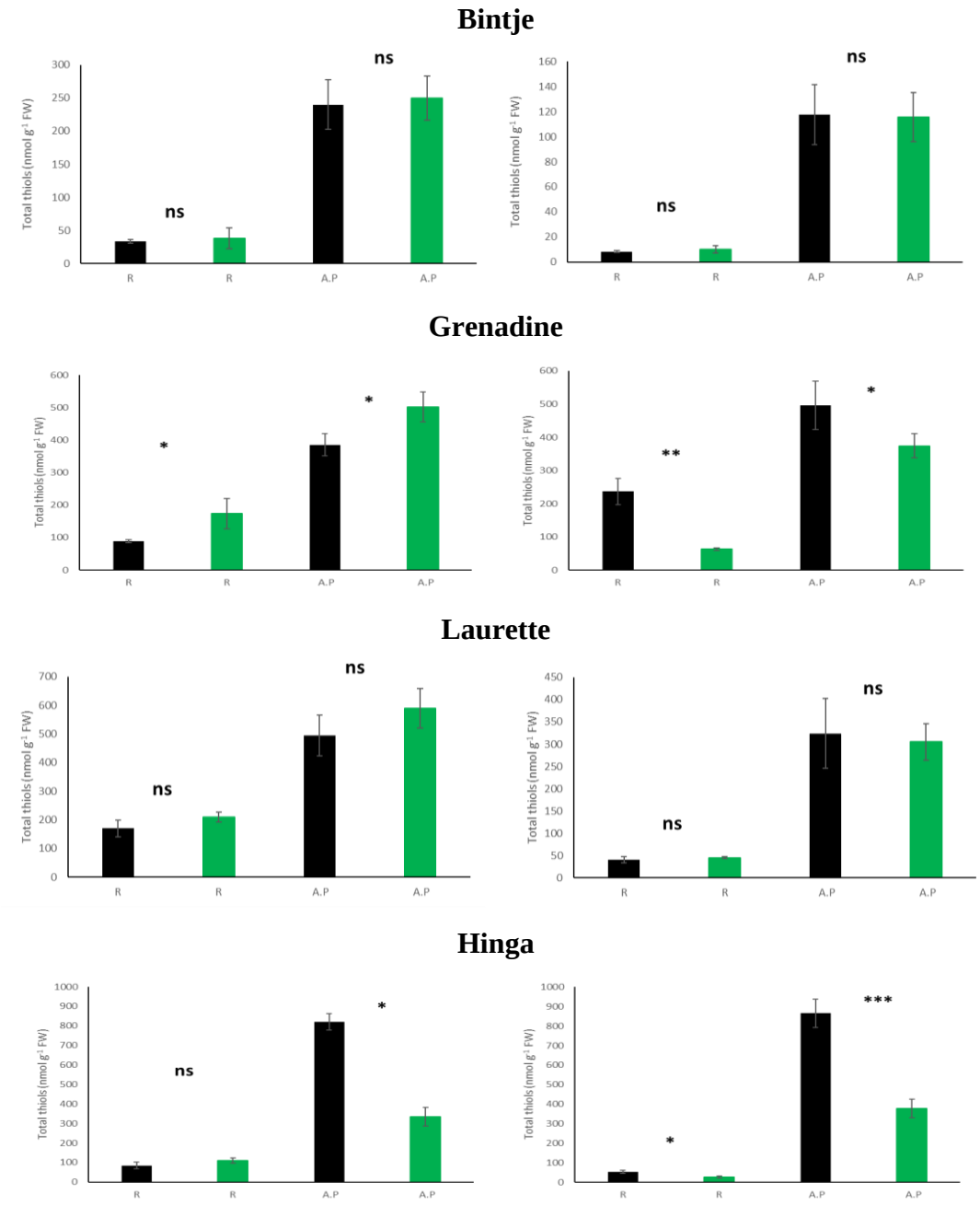

Figure 61: Total thiol content in roots and aerial parts of 14-day-old and 1-month-old potato varieties treated with 50 µM Chl.

*The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots (R) and aerial parts (A.P) isolated from Chl treated plantlets. Results are the mean of three independent results ± sd (ns: not significant; *: p<0.05, **: p<0.01, ***: p<0.001)*

The total thiol contents measured in 14-day-old or 1-month-old Bintje and Laurette varieties did not show any significant differences either in the aerial parts nor the roots nor between the control and treated thus, suggesting no Chl-induced stress (Figure 61). By contrast, the 14-day-old Grenadine aerial parts and roots treated with photoactivated Chl produced a larger amount of thiols than the control, suggesting a potential abiotic stress for the variety. Surprisingly, after 1-month, Grenadine-treated organs showed less thiol contents than the control ones, also noticed for Hinga aerial organs suggesting a beneficial effect induced by Chl.

V.3.2. GPX and Cat activities in response to Chl

To continue the investigation at the biochemical level, GPX and Cat activity assays were monitored. Interestingly, the GPX activity in each variety was always significantly higher in the control organs than in the treated samples (Figure 62).

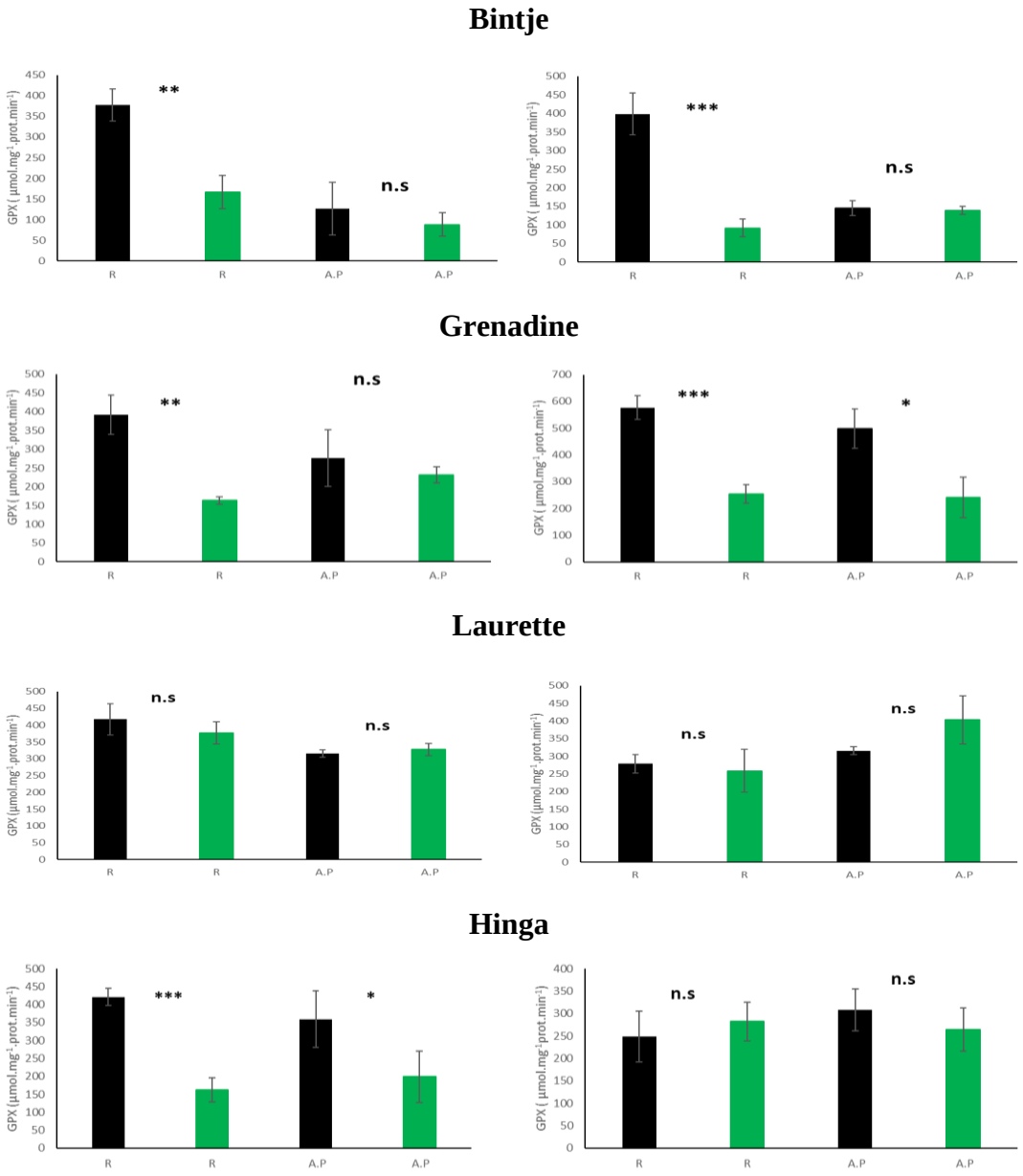

Figure 62: GPX assay of the roots and aerial parts of 14-day-old and 1-month-old potato varieties treated with 50 µM Chl.

The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots (R) and aerial parts (A.P) isolated from Chl treated plantlets. Results are the mean of three independent results ± sd (: p<0.05, **: p<0.01, ns: not significant).*

Grenadine and Hinga showed the most variable GPX and Cat activities from 14-day or 1-month culture (Figures 62-63).

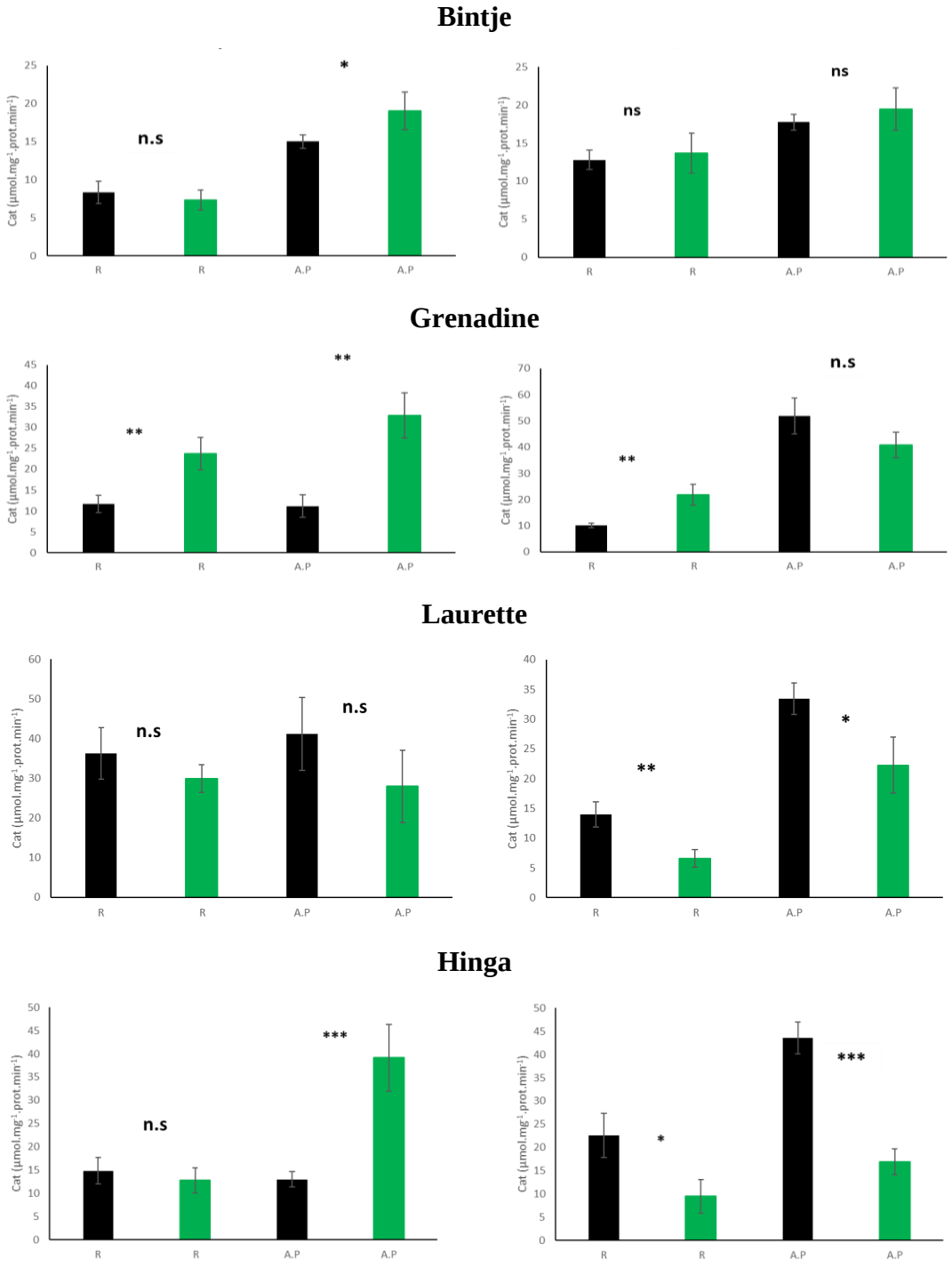

Figure 63: Catalase assay from roots and aerial parts of 14-day-old and 1-month-old potato varieties treated with 50 µM Chl.

The black boxes correspond to control roots (R) and aerial parts (A.P) and green boxes to the roots (R) and aerial parts (A.P) isolated from Chl treated plantlets. Results are the mean of three independent results ± sd (: p<0.05, **: p<0.01, ns : not significant).*

V.4. Discussion

The potato plant species is a member of Solanaceae family as is the tomato plant used in the previous study by Guillaumot et al 2016. The reasons why this work on the potato was conducted are derived from the interest to know whether plants from the same family: potato and tomato (Solanaceae) respond in the same way to an exogenous supply of PS. Furthermore, potato plants are the fourth most important food crop in the world and numerous potato diseases, in particular caused by fungi, can cause significant losses in its production (Fiers et al 2012). The idea to develop a pathosystem including potato/fungus with an efficient PS against fungi would be very interesting and useful.

Firstly, we clearly demonstrated that TPPS even tested at low concentration (10 µM) was not suitable for a normal growth and development of the four potato varieties tested in this work. This result was also very similar to grapevine varieties: therefore, the suitable concentration for all grapevine varieties was 12.5 µM TPPS under illumination. Therefore, at this point, we were able to state that different plant models support different concentrations of TPPS. We hypothesized that this wide range of responses was due to the starting plant material.

The tomato plant and Arabidopsis models were generated from seeds (Guillaumot *et al* 2016; Issawi *et al* 2018). However, potatoes and grapevine varieties come from clonal explants that, by definition, are genetically very similar or close together. Grapevine or potato internodes constituted of fragment of stem, leaf and bud, are a sum of differentiated tissues that could loss a part of their adaptation ability to TPPS presence. By contrast, seedlings are able to keep the full capacity to response to TPPS even at high concentration. Nevertheless, we can assume that TPPS could be a good candidate because we can bet that grapevine or potato in greenhouse or field could, without any problem, support photoactivated TPPS at higher concentration. Secondly, the fungus pathogen study in this thesis work is *B. cinerea* which does not commonly induce a disease or mold in potato (Kirk and Merlington, 2012). However, fungi that belong to the same division of *B. cinerea* (Ascomycota) are common and dangerous potato pests. Therefore, it was considered that the experiments conducted on *B. cinerea* could be enlarged to other Ascomycota fungi such as *Alternaria solani* in the future.

As just discussed above, TPPS did not seem to be the good PS for potato plants, making an APDT application difficult to develop. Nevertheless, and in contrary to TPPS, it was shown that 50 µM Chl did not interfere with growth and development of potato varieties.

Photoactivation of Chl led to ROS production that did not disturb plants meaning that they could defend themselves from Chl induced oxidative burst.

Moreover, 50 µM Chl under a 16 h photoperiod inhibited *B. cinerea* mycelium growth (Chapter III). Thus, the hypothesis was that Chl could be the good candidate for APDT. It will be interesting to test the potential of the Chl against a pathosystem such as: potato / *Alternaria solani*. Chl remains an intriguing compound largely used as a food additive E140 (magnesium metaled form) and E141 (copper metaled form). Moreover, it is harmful for pathogens like fungi (Luksiene et al., 2004; Luksiene, 2005; Luksiene *et al* 2010; Luksiene and Brovko, 2013; Luksiene, 2014; Glueck et al 2019; Luksiene and Buchovec, 2019; Lukseviciute and Luksiene 2020).

Zhang *et al* (2019) demonstrated that Cu-Chl can be a radical quencher that can protect plants against ROS. The only difference between the two Chl is that the Mg trapped in the tetrapyrrolic core of E140 led to less stability. This could explain why no effect in potato was observed after Chl (E140) treatment under 16 h photoperiod because it was rapidly degraded in the culture medium. Moreover, still from Zhang observations on tomato plants and Chl antioxidant activity, it could also explain the beneficial effect on the growth of Hinga and Grenadine varieties.

V.5. Conclusion

Our work on potato clones is thought as a preliminary study to fix a new pathosystem: potato /*Alternaria solani*. For the moment, we have not started to work on Alternaria however, this work on potato was very informative. TPPS was indicated as a “harmful” PS for the four tested varieties of potato, grown *in vitro*: Bintje, Grenadine, Laurette and Hinga. Thus, it decided to open our field of investigations to a natural water-soluble PS: Chl that induced an inhibitory effect on *B. cinerea* mycelium growth. Surprisingly, whereas Chl did not alter potato growth, it can even stimulate it.

In summary, we are confident that future APDT applications will depend on the use of a natural PS and Chl belongs to this category. Bearing this in mind, we are confident that Chl presents lot of advantages that could largely contribute to its development in future.

General conclusion and perspectives

In the PEIRENE Laboratory, we are researching a new strategy called antimicrobial photodynamic treatment based (APDT) on improving agriculture. Our hypothesis is that this approach will not present deleterious effects on the environment nor human health.

One of the major problems in agriculture remains the struggle against pathogens, especially fungi. Based on previous work and with the intention of developing this approach on new living systems, the potential of a different PS was investigated. Moreover, an exogenous supply of PS was supplied on two new plant species that are targets of *B. cinerea*: grapevine and potato. The main PS chosen in this thesis is TPPS and it was chosen because encouraging results were already obtained on Arabidopsis and tomato plantlets (Guillaumot *et al* 2016; Issawi *et al* 2018). It was confirmed that TPPS remains a good candidate, inhibiting *B. cinerea* mycelium growth without drastically altering plantlet growth. Nevertheless, because TPPS is a synthetic molecule, we tested a natural PS: a chlorin called Chlorohyllin (Chl). Here, a surprising result was obtained, Chl, like TPPS, was able to inhibit mycelium growth without disturbing potato plantlet growth. It was even noted that Chl was able to stimulate plant growth. This work was completed for the potato plant and presented in this manuscript. A similar study is also on the road for grapevine varieties and seems to give similar results (not shown). Therefore, according to our study on the potato plant and four of its varieties: Bintje, Laurette, Grenadine and Hinga and the *B. cinerea* response to photoactivated Chl, we claim that this PS is a better candidate than TPPS, full of promises for future agricultural applications.

All results presented in this manuscript constitute the beginning, or the first step of the APDT story. Some specific, numerous and complementary experiments must be carried out to validate the possibility to use APDT in agronomic practices. However, this strategy is very difficult to develop or to coordinate because three factors that interact together must be considered. Two factors are dependent on the living: the pathogen and its plant host and the third factor: the PS, that must “kill” the pathogen without interfering with the plants whilst being eco-friendly.

More experiments should be also monitored on *B. cinerea*. It was the first time, with the exception of Imada *et al* 2014, that the potential of a PS was studied on this fungus. This is the reason why basic, fundamental tests were conducted on the fungus after the PS treatment. Therefore, to complete this analysis at a fundamental level, it would be very interesting to monitor the *B. cinerea* response to PS treatment by studying the other kind of ROS scavengers

such as tocopherol, which should also be monitored for plants. This tocopherol assay is on the road for both *B. cinerea* and plantlets by LC-MS/MS analysis at the "Service Commun de Recherche et d'Analyse de Biomolécules de Limoges."

Nevertheless, our attention will be focused on the photoactivation phenomenon but also on the dark effect that was observed in Chapter III. In the dark, it was shown in this study that the mycelium plugs secreted some products than were able to change the pH of the medium. In the literature, it has been shown that *B. cinerea*, in particular, uses a defence strategy based on the acidification of its environment with the production of organic acids, such as oxalic acid or citric acid (Manteau *et al* 2003; Vylkova, 2017). However, in our case, acidification was not always observed for the 4- and 14-day-old fungus. It would be very interesting to understand the kind of compounds secreted from the fungus and the mechanism of this secretion.

Moreover, it will be important to determine the cell wall composition of this pathogen to understand fully how drugs can interact with the pathogen. Moreover, the Chl work on *B. cinerea* is far from finished. It would be interesting to observe whether Chl has an effect on the spore germination. Additionally, it would be beneficial to observe, *via* ESEM, the possible changes in the fungal structure that is caused by this PS under light and under dark. Furthermore, as seen previously, a minor delay in the mycelium growth was observed when treated with Chl under dark conditions.

Finally from the *B. cinerea* study and its responses to PS treatment that we described in this PhD work, we thought it will be of great interest and very informative to compare the fungus response to PS treatments under light or dark conditions at a global molecular level by a proteomic approach. This will give us a general picture of the response and may be help to discover new pathways.

Until now, all experiments on plants were performed in the laboratory with very young plantlets grown *in vitro* from clones. This type of study shows its limits in terms of plant physiology, growth and development, PS treatment (supply in the medium instead of spraying) and finally, in terms of inoculation and treatment. We are aware that the experiment showed in the submitted paper was very artificial and only tried to mimic the reality. Indeed, the next step relative to plant work and their response to PS needs to be monitored in a greenhouse. The plants and their response to PS treatment must be studied at the vegetative and reproductive levels. It will be achieved after PS treatment alone, after pathogen inoculation and after pathogen inoculation plus PS spraying. For the PS spraying, PS concentration and formulation must be considered.

From the plant side and especially potato species, its pathosystem has to be developed. *B. cinerea* is not interesting for this species and therefore, it will be of importance to look at a new fungus pathogen such as *Alternaria solani* and to further study its response to chlorophyllin.

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Etude des effets de TPPS et Chlorophylline sur Botrytis cinerea et sur deux plantes modèles : vigne et pomme de terre

Un des problèmes majeurs de l'agriculture reste la lutte contre les agents pathogènes, en particulier les champignons. Depuis les années 60, l'utilisation de quantités phénoménales de pesticides, pour garantir les rendements des cultures, a provoqué une pollution désastreuse de l'environnement, une perte de la biodiversité et le développement de pathogènes multi-résistants. Les pratiques agricoles doivent donc devenir plus respectueuses de l'environnement tout en restant optimales. Le traitement photodynamique antimicrobien (APDT) est un traitement alternatif envisagé dans la lutte contre les microorganismes pathogènes, sans effet sur les plantes de culture et sur l'environnement voir la santé humaine. Dans l'intention de développer cette approche, deux photosensibilisateurs (PS) : une porphyrine anionique (TPPS) et une chlorine (la Chlorophylline) ont été testés sur un champignon pathogène (Botrytis cinerea) et deux de ses cibles : la vigne (Vitis vinifera) et la pomme de terre (Solanum tuberosum). Nous avons montré que TPPS même à très faible concentration inhibait la croissance du mycélium de B. cinerea sans pour autant altérer le développement des jeunes plants de vigne cultivés in vitro. Des résultats très similaires ont aussi été obtenus avec la Chlorophylline (Chl), molécule naturelle. TPPS et Chl semblent donc de très bons candidats pour une approche APDT. Cependant, dans le but d'une approche éco-responsable, nous privilégions plutôt Chl molécule naturelle contre TPPS, molécule de synthèse. En conclusion, les résultats obtenus tout au long de ces travaux de thèse sont réellement encourageants et nous permettent de défendre l'APDT comme stratégie agricole d'avenir et surtout éco-friendly.

Mots-clés : *APDT, photosensibilisateurs,Botrytis cinerea, TPPS, Chlorophyllin, vigne, pomme de terre*

Study of the effects of photoactivated TPPS and Chlorophyllin on *Botrytis cinerea*, and on two plant models: grapevine and potato

Nowadays, one of the major problems in agriculture remains the struggle against pathogens, especially fungi. In the last 60 years, the use of large amounts of pesticides to increase crop yield caused environmental pollution, loss of biodiversity, and the development of multi-resistant plant pathogens. Now, more than ever, agriculture practises must become environmentally friendly and thus, new strategies to improve agriculture, without side effects for the environment and human health, need to be developed. Antimicrobial photodynamic treatment (APDT) has emerged as an alternative treatment that can be envisaged in agronomic practices to fight against microorganisms, without harming plants.

To develop this approach on complex living systems such as grapevine and potato, hosts of the fungus pathogen Botrytis cinerea and two photosensitizers were taken into consideration: an anionic porphyrin (TPPS) and a natural chlorin Chlorophyllin (Chl). More specifically, an extensive work was conducted using photoactivated TPPS. TPPS was very effectively inhibit Botrytis cinerea development and it did not cause any phenotypical or biochemical alterations of the grapevine varieties (Merlot, Chardonnay and Sauvignon). In the potato varieties, while there were alterations in its growth and development, the plants were able to defend themselves.

Furthermore, in order to find a new eco-friendly solution that is more suitable for a large range of plants we tested a natural PS: the chlorophyllin (Chl). Surprisingly, Chl was able to inhibit Botrytis cinerea mycelium growth without disturbing neither potato nor grapevine plantlet growth. In conclusion, these preliminary studies based on the use of Chl suggest that the development of a safe but efficient APDT approach in agriculture may no longer be a dream

Keywords : *APDT, photosensitizers, Botrytis cinerea, TPPS, Chlorophyllin, grapevine, potato.*