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Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L.)

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Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L.)

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LIST OF ABBREVIATIONS

ABA	abscisic acid
AFLP analysis	Amplified Fragment Length Polymorphism
ANOVA	analysis of variance
ArMV	Arabis mosaic virus
AS	alternative solution
BAP	benzylaminopurine
BFS	bovine foetal serum
BM	basal medium
DMSO	dimethylsulfoxide
D-V	droplet-vitrification
ED	encapsulation-dehydration
EG	ethylene glycol
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FWB	fresh weight base
GA3	gibberellic acid
GFLV	Grapevine fanleaf virus
GLRaV-1	Grapevine leafroll-associated virus 1
GLRaV-3	Grapevine leafroll-associated virus 3
GVA	Grapevine virus A
IAA	indole acetic acid
IFVV	French institute of vine and wine
LMM	ligation master mix
LN	liquid nitrogen
LS	loading solution
M-AFLP	microsatellite-amplified fragment length polymorphism
MC	moisture content
NaOCl	sodium hypochlorite
pAMM	preamplification master mix
PBS	phosphate buffer saline
PFD	paraformaldehyde
PVS2	Plant vitrification solution 2
PVS3	Plant vitrification solution 3
RBDV	Rapsberry bushy dwarf virus
REGW test	Ryan-Einot-Gabriel-Welsh test
RFU	Relative fluorescent unit
sAMM	amplification master mix
SAMPL	selective amplification of microsatellite loci
RMM	restriction master mix
SE	somatic embryos
SD	mean percentages with standard error of the mean
SM	simple matching
SPCs	stabilized polyembryonic cultures
VS	vitrification solution
ZR	zeatin-riboside
-LN	non-cryopreserved
+LN	cryopreserved

ABSTRACT

Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L.)

This study aimed at establishing a cryopreservation protocol for grapevine shoot tips and at testing the efficiency of cryopreservation in eliminating selected grapevine viruses. In vitro cultures of healthy genotypes of eight Croatian autochthonous grapevine cultivars Plavac mali, Maraština, Pošip, Debit, Grk, Lasina, Plavina and Vugava and of virus-infected genotypes of Plavac mali were successfully established. Differences in survival, regrowth and growth parameters were genotype-specific. Infected cultivars were less reactive compared to healthy ones. A PVS2-based cryopreservation protocol was successfully established. Modifications in sucrose preculture conditions and use of PVS2-derived alternative vitrification solutions did not improve growth recovery. By contrast, the physiological state of the plant material played a critical role in cryopreservation. Actively growing buds sampled from single-node microcuttings displayed higher regrowth compared to buds sampled directly on in vitro plantlets. The position of buds on the stem of in vitro mother-plants affected regrowth after cryopreservation. The addition of benzylaminopurine in the shooting medium had a positive effect on regrowth after liquid nitrogen exposure, while no such positive effect was observed with zeatine riboside or proline. The cryopreservation protocol established led to approximately 50% recovery with cultivar Portan and three of the four international cultivars tested. By contrast, no or very low recovery was noted with the Croatian cultivars tested. Based on ELISA tests, the GFLV virus was eliminated from 82.4% of non-cryopreserved samples and from 77.8% of cryopreserved samples in cultivar Chardonnay and the GLRaV-3 virus was eliminated from 100% of both non-cryopreserved and cryopreserved samples in cultivar Cabernet Sauvignon. These results may be related with our immunolocalisation studies, which showed that GFLV was found in the apical dome and meristematic tissues in cultivar Pinot Noir and GLRaV-3 in sieve elements of cultivar Merlot. Genetic stability of plants regenerated from cryopreserved shoot tips was studied using AFLP markers. With the eight AFLP primer combinations employed on the 43 plants tested, no polymorphism was observed after sucrose preculture, treatment with the loading solution and half-strength PVS2. However, polymorphic fragments were observed in non-cryopreserved and cryopreserved samples treated with PVS2 solution, the number of which increased with increasing durations of exposure to PVS2 solution.

Key words: grapevine, cryopreservation, cryotherapy, genetic stability

RÉSUMÉ

Cryoconservation et cryothérapie de la vigne (*Vitis vinifera* L.)

Cette étude visait à établir un protocole de cryoconservation pour des apex de vigne et à tester l'efficacité de la cryoconservation pour éliminer des virus de la vigne sélectionnés. Des cultures in vitro de génotypes sains de huit cultivars croates autochtones de vigne, Plavac mali, Maraština, Pošip, Debit, Grk, Lasina, Plavina et Vugava et de génotypes infectés de Plavac mali ont été établies. Les différences de survie, de reprise et des paramètres de croissance observés se sont avérées spécifique des génotypes. Les cultivars infectés se sont montrés moins réactifs que les cultivars sains. Un protocole de cryoconservation basé sur droplet-vitrification avec PVS2 a été établi. Les modifications de la préculture avec le saccharose et l'utilisation de solutions alternatives de vitrification n'ont pas amélioré la reprise. A l'opposé, l'état physiologique du matériel végétal a joué un rôle essentiel pour la cryoconservation. Des bourgeons en croissance active prélevés sur des microboutures mononodales ont montré une reprise plus élevée que des bourgeons prélevés directement sur les vitroplants. La position des bourgeons sur la tige des plantes-mères in vitro a affecté la reprise après cryoconservation. L'addition de benzylaminopurine au milieu de culture des microboutures a eu un effet positif sur la reprise après immersion dans l'azote liquide, alors qu'aucun effet positif n'a été observé sur la reprise avec la zéatine riboside ou la proline. Le protocole de cryoconservation établi a permis d'obtenir environ 50% de reprise avec le cultivar Portan et avec trois des quatre cultivars internationaux utilisés. Par contre, aucune reprise ou une reprise très faible a été observée avec les cultivars croates testés. En se basant sur les tests ELISA réalisés, le virus GFLV a été éliminé de 82,4% des apex non cryoconservés et de 77,8% des apex cryoconservés chez le cultivar Chardonnay et le GLRaV-3 a été éliminé de 100% des apex non cryoconservés et cryoconservés chez le cultivar Cabernet Sauvignon. Ces résultats sont à relier à nos études d'immunolocalisation, qui ont montré que le GFLV était présent dans le dôme apical et les tissus méristématiques chez le cultivar Pinot Noir et que le GLRaV-3 était présent dans les tubes criblés chez le cultivar Merlot. La stabilité génétique des plantes régénérées à partir des apex cryoconservés a été étudiée en utilisant les marqueurs AFLP. Avec les huit combinaisons d'amorces utilisées sur les 43 plantes testées, aucun polymorphisme n'a été observé après la préculture au saccharose, le traitement avec la solution de loading et la solution de PVS2 diluée de moitié. Par contre, des fragments polymorphes ont été observés sur des explants non cryoconservés et cryoconservés traités avec la solution PVS2, dont le nombre augmentait avec l'augmentation de la durée d'exposition à la solution PVS2.

Les mots clés : la vigne, cryoconservation, cryothérapie, la stabilité génétique

SAŽETAK

Krioprezervacija i krioterapija vinove loze (*Vitis vinifera* L.)

Istraživanje je provedeno s ciljem uspostave protokola za krioprezervaciju vegetacijskih vršaka vinove loze i testiranja učinkovitosti ove metode u eliminaciji odabranih virusa na vinovoj lozi. Uspješno je uspostavljena *in vitro* kultura zdravih genotipova hrvatskih autohtonih sorata Maraština, Pošip, Debit, Grk, Lasina i Plavac mali te virusima zaraženih genotipova Plavca malog. Utvrđeno je da su razlike u preživljavanju i parametrima rasta sortno uvjetovane te da su zaraženi genotipovi lošije reagirali na uvjete *in vitro* od zdravih. Uspješno je uspostavljen krioprezervacijski protokol s krioprotektantom PVS2. Modifikacijom u predkulturi saharozom, kao ni upotrebom alternativnih krioprezervacijskih otopina temeljenih na PVS2 nije postignuto poboljšanje preživljavanja niti regeneracije nakon smrzavanja. Nasuprot tome, pokazalo se da fiziološki status biljnog materijala ima značajnu ulogu u preživljavanju nakon krioterapije. Pupovi uzeti s mikroreznica pojedinačnih nodijskih odsječaka u fazi aktivnog rasta regenerirali su bolje od pupova uzeti direktno s izboja *in vitro* biljaka. Položaj pupa na majčinskoj biljci *in vitro* također je imao utjecaj na uspješnost regeneracije nakon krioprezervacije. Dodatak benzilaminopurina u medij za kultivaciju mikroreznica pozitivno je utjecao na regeneraciju biljaka nakon izlaganja tekućem dušiku (LN), za razliku od dodatka zeatin ribozida ili prolina kod čega nije utvrđen ovakav utjecaj. S uspostavljenim krioprezervacijskim protokolom postignuto je otprilike 50% regeneracije kod sorte Portan i tri od četiri svjetski rasprostranjene sorte vinove loze uključene u istraživanje. Za razliku od njih, kod hrvatskih autohtonih sorata nije došlo do regeneracije ili je postotak regeneriranih biljaka nakon krioprezervacije bio vrlo nizak. Rezultati ELISA testa pokazali su da je GFLV virus eliminiran u 82,4% uzoraka sorte Chardonnay nakon provedenih predtretmana ali bez smrzavanja tekućim dušikom te 77,8% uzoraka kod kojih je proveden čitav protokol, uključujući i korak smrzavanja. Kod svih uzoraka Cabernet Sauvignona, kako onih koji su testirani prije, tako i onih nakon smrzavanja, došlo je do eliminacije GLRaV-3 virusa.

Ovi rezultati mogu se povezati s nalazima imunolokalizacije, kod kojih je GRLV virus pronađen i u meristemu vegetacijskog vrška sorte Pinot crni, dok je prisutnost GLRaV-3 virusa utvrđena samo u staničju floema sorte Melot. Genetička stabilnost biljaka regeneriranih nakon krioprezervacije testirana je upotrebom AFLP markera. Analiza 46 biljaka pomoću osam kombinacija AFLP početnica nije pokazala polimorfizam uzoraka uzetih nakon predkulture saharozom, izlaganja dehidritizirajućoj otopini niti polovičnoj koncentraciji PVS2. Ipak, polimorfni fragmenti utvrđeni su prije i nakon smrzavanja tekućim dušikom u uzorcima tretiranim s otopinom PVS2, a njihov broj rastao je sa dužinom izlaganja ovom krioprotektantu.

Ključne riječi: vinova loza, krioprezervacija, krioterapija, genetska stabilnost

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1 INTRODUCTION

1.1 Conservation of *Vitis vinifera* genetic resources

Wine may have an archeological record going back more than 7.5 thousand years, with the earliest suspected wine residues dating from the early to mid-fifth millenium B.C (McGovern et al., 1996). Grapevines are commonly grouped in the genus *Vitis* in the family *Vitaceae*. The genus has tipically been divided into two sections or subgenera, *Euvitis* and *Muscadinia*. *Vitis vinifera* subsp. *sativa* has been domesticated from wild populations of vine, *Vitis vinifera* subsp. *sylvestris*, where over time significant changes in the biology and physiology of the species have occurred (Jackson, 2000). The genus *Vitis* is of major agronomic importance. It consists of ~60 inter-fertile species that exist almost exclusively in the Northern Hemisphere. Among them, *Vitis vinifera* is the only species extensively used in the global wine industry. Thousands of *V. vinifera* cultivars exist, but the global market for wine production is dominated by only a few cultivars and few of them market a perception of wine. The cultivars are generally classified according to their final production: wine grapes, table grapes and raisins (This et al., 2006).

Conservation and management of genetic resources is a very important activity for valuable and endangered cultivars. The importance of grapevine as the leading fruit culture and the diversity of ecological conditions in the areas where it is grown, together with the loss of genetic diversity within this highly polymorphous species, raise the importance of *Vitis vinifera* preservation to an even higher level.

Genetic resources of grapevine are traditionally conserved *ex situ* as whole plants in field collections. The number of different varieties held in germplasm collections around the world is estimated at ~10,000, and so far, based on genetic DNA analysis, 5,000 cultivars have been identified. Nearly every winegrowing country has its own grapevine germplasm collection, owing to the quarantine restrictions and the need to maintain the material in the field as living plants (This et al., 2006). The largest grapewine collection in Vassal, (near Montpellier, in France) has 3,200 varieties represented as 7,200 clones.

Field collections of many plant species currently act as both active (for distribution) and base (for conservation) collections, and frequently no duplicate is available (Jarret and Florkowski 1990). It is indeed important to maintain field collections for evaluation, characterization, identification, and distribution purposes. Field collections may be preferred for genotypes which commonly produce variants since they are more easily identified and rogued in the field than *in vitro*. Engelmann (2012) reported that the field genebank has been the *ex situ* storage method of choice for species for which conservation as seed is problematic. According to the First Report on the State of the World's Plant Genetic Resources for Food and Agriculture (FAO, 1996), around 527,000 accessions were maintained in field genebanks at that time. The genetic resources under conservation in field collections can be readily accessed and observed, thus permitting detailed evaluation.

1.1.1 Problems and objectives

However, field conservation presents major drawbacks which limit its efficacy and threaten the safety of plant genetic resources conserved in this way. Collections remain exposed to natural disasters, attacks by pests and pathogens, and moreover, labor costs and the

requirement for technical personnel are very high. In addition, distribution and exchange from field genebanks is difficult because of the vegetative nature of the material and the greater risks of disease transfer from country to country (Engelmann, 1997a).

During the past decade, international interest in preserving the genetic resources of grapevines has risen to a higher level. In October 2001, the ECPGR (European Cooperative Programme for Plant Genetic Resources) agreed on the establishment of a Working Group on *Vitis*. The objective was to strengthen European collaboration for the purpose of improving the conservation and sustainable use of *Vitis* genetic resources in Europe. International projects, aiming at preserving genetic resources of grapevine have been launched during the last decade (GENRES 081, GRAPEGENE06, GRAPENET). Their main objective was to find out the exact extent of grape genetic resources in Europe, presented by EU partners (the grapevine collections presently hold approx. 25,583 accessions). Within the GENRES 081 project, 19 partners from 14 countries started to make an inventory aiming at establishing a European Vitis Database, by selecting identical descriptors for agronomic trait evaluation and utilising a fairly equal set of molecular markers for genetic analysis. All activities initiated in this project have been continued in the following GRAPEGENE06. Within GRAPEGENE06 a comprehensive list of *Vitis* databases within the Europeen Union has been established (Lacombe et al., 2011), aiming at supporting the implementation of European regulations on propagation of grapevine plants within the EU, and at assessing prioritiy actions, by technical and research institutes, for the conservation of grapevine genetic resources. Croatia was a member of these projects. The current GRAPENET project (supported by the European Science Fundation, entitled: East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding) enables researchers from Eastern and Western Europe having different capacities and approaches in vineyard practices. This project allows the grapevine migration route from the Caucasus and Asia to contribute to the interpretation of grapevine genetic resources in European viticulture.

1.1.2 Preservation of Croatian autochthonous cultivars

Croatian viticulture has a long and rich history (Maletić et al., 2008). In addition to contributing to national income, the importance of Croatian autochthonous cultivars is significant in contributing to the development of viticulture while preserving national identity. According to Jelaska and Briza (1967), at the end of 19th and beginning of 20th century there were more than 400 grape varieties under cultivation in Croatia, with more than 200 cultivars in Dalmatia alone (Bulić 1949). However, because of introduced pests and pathogens, modern economic pressure and introduction of world well known cultivars, a drastic germplasm erosion has occurred.

During the last decade, many efforts in the preservation of grapevine biodiversity in Croatia have been undertaken. A detailed inventory of wine-growing regions has shown that numerous autochthonous cultivars can still be found, but many of them are highly underutilized and endangered.

To preserve them from extinction, a national *ex situ* collection at the Faculty of Agriculture in Zagreb, in the experimental station “Jazbina”, has been established since 2001, consisting nowadays of more than 140 native accessions. In addition, several regional (back-up) collections have also been established to keep duplicates and enable economic evaluation in appropriate climatic conditions (Pejić and Maletić, 2010).

Along with conservation efforts, a thorough ampelographic description and genetic fingerprinting of cultivars have been carried out. The availability of new genotyping tools such as microsatellites, opened the possibility to compare the genetic profiles among local germplasm, but also among all other European cultivars. Microsatellites enabled clarification of synonyms and homonyms as well as analysis of parent-progeny relationships (Maletić et al., 1999.; 2004.; Piljac et al., 2002.; Crespan et al., 2006; Malenica et al., 2011; Žulj Mihaljević et al., 2013; Zdunić et al., 2013). The research results showed that Croatia is one of the gene centers from which distinct groups of grapevine cultivars arose (Sefc et al., 2000.; Maletić et al., 2004; Benjak et al., 2006).

In case of some neglected cultivars, a high quality potential has been observed, and their economic revitalization is in progress. Due to the increasing interest of producers and consumers in Croatia for autochthonous varieties, clonal and sanitary selection programs have been started, aimed at healthy ("virus-free") planting material production (Maletić et al., 2012).

1.1.3 Grapevine virology

However, surveys of virus diseases in different winegrowing regions of Croatia disclosed a widespread presence of viruses and a low occurrence of non-infected vines (Poljuha et al., 2004; Karoglan Kontić et al., 2009; Vončina et al., 2009a; Vončina et al., 2009b, Vončina et al., 2010 Vončina et al., 2011,). For further propagation of cultivars which are totally virus infected it will be necessary to undertake their sanitation through tissue culture and other suitable methods.

Observations from different vine-growing countries in Europe showed similar results, disclosing very high rate of virus-infected vines in some cultivars and production areas (Flak and Gangl, 1994; Kominek and Holleinova, 2003; Lázár, 2003). Grapevine is one of the woody species on which a large number of viruses have been detected (Martelli and Boudon-Padieu, 2006). Depending on the strain, grapevine cultivar and environmental conditions, virus infections can negatively influence the yield, sugar content and acidity of the must, berry color, resistance to biotic and abiotic stress, length of growing cycle, etc. On virus-infected vines characteristic symptoms can be observed, although cases exist of latent infections without much symptom expression. Thus, much attention has been paid to the sanitary status of plant material. As regards varietal improvement, the best results can be achieved if clonal and sanitary selection proceed together (Goheen, 1989; Maninni, 2000; Mannini, 2003). Despite such a large number of viruses (more than 60 documented so far (Martinelli and Gribaudo, 2009)) that can infect grapevine, only a restricted number can be considered economically important. Therefore, according to the recommendations of the International Council for the Study of Viruses and Virus Diseases of Grapevine (2003) (http://www.icvg.ch/data/recomm.pdf), during the process of plant material certification the absence of the following viruses is usually required: Arabis mosaic virus (ArMV), Grapevine fanleaf virus (GFLV), Grapevine leafroll-associated virus 1 (GLRaV-1) and Grapevine leafroll-associated virus 3 (GLRaV-3).

Intensive research activities on grapevine virology started in the 1980's, focusing towards the identification, screening and elimination of viruses from the plants. Panattoni et al. (2013) observed elimination of viruses in plants and reported that among woody plants, grapevine, apple and peach are the most frequent targets of sanitation protocols because their health status is strictly regulated.

Two methods mostly used to free vegetatively propagated plants from virus are meristem tip culture and thermotherapy. These methodologies allow quick propagation of plant material, producing healthy plants from a single individual in a short period of time, regardless of the location or season of the year (Youssef et al., 2009). After the discovery of substances capable of blocking virus nucleic acid synthesis, chemotherapy has also been included in sanitation programs (Vicente and De Fazio, 1987). Recently, Wang et al. (2003b) performed cryotherapy on grapevine shoot tips, and by exposing plant tissue to the temperature of liquid nitrogen (-196°C), they succeeded in eliminating Grapevine virus A (GVA). Besides grapevine, cryotherapy has been shown to eradicate seven unrelated groups of viruses from the shoot tips of other crops such as potato, sweet potato and fruit trees including Citrus species, berry plants as well as banana (Wang and Valkonen, 2008a).

In order to help understand the process of virus eradication, in some investigations, thorough examination of important grapevine viruses in cell structure has been performed. Goussard et al. (1991) studied elimination of Fanleaf virus (GFLV) and Grapevine leafroll-associated viruses in grapevines through somatic embryogenesis. Results showed the absence of grapevine leafroll associated viruses in regenerated embryos and plantlets, which indicated that there was no translocation of these viruses from infected tissue via proliferating callus (without vascular tissue) to embryos.

These results support the findings of Namba et al. (1979) that closterolike-virus particles are restricted to vascular tissue (phloem). It was clearly shown that GFLV particles were present in very young meristematic tissues (i.e. meristematic domes of shoot apices) of grapevine material infected with fanleaf. Kim et al. (2003) also confirmed earlier reports that symptomatic leaves infected with GFLV-3 showed long, flexuous, filamentous particles in comparison with healthy leaves. Virus-like particles observed in parenchyma cells were reported as a specific cytopathic feature of Closterovirus. A review of Grapevine Leafroll-associated Virus type 3 by Charles et al. (2006) showed that long thin flexuous particles, called “clostero-like“ have a length of above 900 nm.

The distribution of Grapevine fanleaf virus (GFLV), Grapevine leafroll-associated virus-3 (GLRaV-3) and Grapevine virus A (GVA) in embryogenic callus of grapevine was investigated by *in situ* hybridization (Gambino et al., 2010). The distribution of viruses in callus and the regeneration of healthy embryos/plantlets were related to mechanisms of virus movement in the tissues, and most probably to the characteristics of the callus and its evolution after several months of culture. GFLV, a nepovirus, has a rapid diffusion in the tissues. The replication of the two viral genomic RNAs of GFLV occurs in endoplasmic reticulum (ER)-derived membranes vesicles. The movement of the virus in the plant could explain the ability of GFLV to invade young cells and meristematic tissues. GVA and GLRaV-3 are phloematic viruses which could enter the newly formed callus possibly through the plasmodesmata and the sieve elements associated to small tracheid-like elements often observed in callus. However, their level of infection was low, probably because of their slow movement outside the phloem.

For better understanding of virus spreading in plant tissues, immunolocalisation can be used (Gallard et al., 2011). The goal of immunolocalisation, a combination of immunochemistry and morphology, is to define the cellular location of biochemically defined antigens. Immunocytochemical techniques can be applied to all types of cells. Plant cells, however, require the development and application of specific techniques to optimize their handling and

observation. The presence of the cell wall, hindering the penetration of antibodies and other natural barriers such as cuticles, are features of plants that make working with plant tissues more difficult than with animal tissues (Hawes and Satiat-Jeunemaitre, 2001).

1.2 *In vitro* research on grapevine

In vitro techniques allow the maintenance of explants in a pathogen-free environment, germplasm transfer under regulated phytosanitary control and lower conservation costs. The main advantages of these techniques are a high rate of multiplication and the possibility to eliminate viruses using the techniques described previously. These modern techniques have already been applied for multiplication, storage and collection of germplasm of more than 1000 species (George 1993).

In vitro techniques have been developed as an alternative and a supplementary measure in response to problems related to the conservation, management, sanitation and propagation of plant germplasm (Silva et al., 2012). In grapevine, these methods have been successfully used for rapid clonal multiplication and virus elimination (Skiada et al., 2009; Alizadeh et al., 2010; Diab et al., 2011). Virus eradication has been achieved by different methods of *in vitro* culture: meristem culture (Youssef, 2009), thermotherapy and meristem culture (Barlass et al., 1978.) and naturally by high vineyard temperatures (Valero et al., 2002). Recently, cryopreservation has been successful in eliminating grapevine virus GVA (Wang et al., 2003b). Efforts have also been put into the development of efficient protocols for long-term maintenance of grapevine biodiversity *in vitro*, as a safer and more cost-efficient alternative to field collections (Roubelakis-Angelakis, 2010).

A prerequisite to developing such approaches for grapevine is the development of efficient *in vitro* regeneration protocols. However, such protocols are usually highly genotype-specific and need to be adjusted to certain cultivars (Banilas and Korkas, 2007; Mederos-Molina, 2007; Péros et al., 1998). Some authors have indicated that the optimal composition of grapevine culture medium depends on the species and cultivar under consideration, so that the results obtained with one genotype on a given medium may differ from those obtained with other genotypes (Reisch, 1986; Botti et al., 1993). Other possible limitations of these techniques are the long exposure to high temperatures, which can be harmful to the plants and numerous manipulations which can induce somaclonal variation (Baránek et al., 2009).

There have been very few reports on *in vitro* propagation of Croatian native grapevine cultivars (Hartl et al., 1989; Karoglan Kontić et al., 1990; Hartl and Maleš, 2000; Hartl et al., 2005); moreover the cultivars tested were of unknown sanitary status and information about their specific culture requirements is still scant.

Regarding clonal and sanitary programs undertaken for Croatian autochthonous cultivars of economic importance, an *in vitro* laboratory has been established in 2011 in the Faculty of Agriculture, for their evaluation. The main aim is to test the special requirements of these cultivars in tissue culture conditions and their long-term conservation.

1.3 Cryopreservation

1.3.1 Cryopreservation – Introduction

Cryopreservation is the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (-196ºC). Over the past 40 years scientists developed and tested a range of cryopreservation techniques for preserving plant cells and tissues, but routine storage of plant germplasm other than seeds in liquid nitrogen (LN) is a relatively new practice (Engelmann, 2004). Many protocols are available in the literature; however they are often difficult to interpret for routine use (Towill, 2002). The procedures themselves are not difficult, but initial implementation of cryopreservation procedures can be daunting where financial and human resources are limited. Often the inital steps to define protocols and set up procedures for testing, screening, and ultimately storing the range of genotypes in each collection are beyond the scope of many plant laboratories (Reed et al., 1998).

Research on the utilization of cryopreservation for long-term conservation of plant material is a relatively new science area, in comparison with its application to animal and human materials (Whiters and Engelmann, 1998). Following the pioneering work of a few scientists during the second half of the 20th century, it is only during the last 30 years or so that plant cryopreservation research really expanded. Today, the number of plant species for which cryopreservation protocols have been developed has dramatically increased (Engelmann, 2000). Moreover, dramatic progress has been made in the past 20 years in the development of simple, efficient and broadly applicable cryopreservation techniques and there is now an increasing number of cases where cryopreservation is moving from the research laboratory to the genebank for routine application to the long-term conservation of plant germplasm (Benson, 1999; Engelmann and Takagi, 2000; Reed, 2008).

1.3.2 Cryopreservation theory

The theory of cryopreservation encompasses several interconnected disciplines ranging from physiology to cryophysics. Water status and cryoprotection are the most influential determinants of survival in combination with physiological factors. Osmotic pressure is the pressure that must be applied to a solution to prevent the flow of a solvent across the semipermeable membrane. Water flows from a weaker to a stronger solution: when equilibrated, solutions are termed isotonic. Tonicity measures the extent to which a concentrated solution causes water to move in or out of cells. In plants, cellular swelling caused by hypotonic solutions creates turgor pressure. Turgor pressure is structurally important in plants because it maintains them in an erect condition. When a plant cell is placed in a hypertonic solution the plasma membrane recedes from the wall as the vacuole loses water; this process is plasmolysis. Water has unique physio-chemical properties essential for cell function. Cryobiological manipulations influence the different states of water placing it in an important and central role in cryopreservation and storage stability (Reed, 2008).

Critical to the development of cryopreservation protocols is the control and/or avoidance of intracellular ice nucleation. It occurs when local thermal properties of a system make it energetically possible for H_2O molecules to come together to form sufficient H-bonds to enable initiation of an embryonic ice crystal. When liquid water is removed from the system to form ice, solutes become more concentrated. This lowers the temperature at which further ice is formed. One way of considering this is that the solute molecules become increasingly

concentrated and impede the ability of the remaining water molecules to interact to form crystals. Above the temperature of homogeneous ice formation, the freezing temperature of water becomes increasingly depressed to a point known as the eutectic. At this stage, the whole system solidifies and there is no further change as all available water has "frozen out". The eutectic has coordinates for temperature and concentration and it applies to two or more substances that are able to form solid solutions with one another and lower each other's freezing point to a minimum temperature (Mazur, 2004). After the initial ice nucleation event, ice crystals have the capacity to grow into complex clusters forming networks that can grow exponentially as more and more water molecules participate in complex alignments with each other. Ice nucleation affects the structural, osmotic and colligative integrity of cells causing physical ruptures and mechanical injury (Taylor et al., 2004).

To maintain the viability of hydrated cells and tissues it is essential to avoid lethal intracellular ice formation that occurs during rapid coling in LN (Sakai and Yoshida, 1967). Cells and tissues that are to be cryopreserved in LN need to be sufficiently dehydrated before being immersed in LN.

So far cryopreservation has been considered in terms of the liquid and solid (ice) phases of water. It is also possible to cryopreserve plants by the process of "vitrification", the solidification of liquids without crystallization. This comprises a "glassy state" as the system is amorphous, lacks organized structure but possesses the mechanical and physical properties of a solid (Taylor et al., 2004). Vitrification of water in biological systems is dependent on increased cell viscosity, occurring as cell solutes become concentrated. Increased viscosity inhibits the coming together of water molecules to form ice. The vitrified state is metastable, meaning that it can relatively easily revert back to a liquid and/or devitrify to form ice. Vitrified systems are particularly unstable during rewarming as changes in molecular mobility and energy may be sufficient to allow water molecules to rearrange (relax) and form ice. Vitrification can be achieved in a number of ways but all usually have the end result of increasing solute concentration to a critical viscosity.

Vitrification was proposed as a method for the cryopreservation of biological materials because it would avoid the potentially detrimental effects of extracellular and intracellular freezing (Luyet, 1937). Thus, vitrification is an effective freeze-avoidance mechanism. As glass fills space in a tissue it may prevent additional tissue collapse, solute concentration, and pH alteration during dehydration (Reed, 2008).

1.3.3 Cryopreservation techniques – introduction

According to Engelmann (2004), the cryopreservation techniques employed and the physical mechanisms upon which they are based are different in classical and new cryopreservation techniques. Classical techniques involve freeze-induced dehydration, whereas new techniques are based on vitrification.

1.3.3.1 Classical techniques

Classical techniques involve slow cooling down to a defined prefreezing temperature, followed by rapid immersion in LN. With temperature reduction during slow cooling, cells and the external medium initially supercool, followed by ice formation in the medium (Mazur, 1984). The cell membrane acts as a physical barrier and prevents the ice from seeding the cell interior and the cells remain unfrozen but supercooled. Depending upon the rate of cooling and the prefreezing temperature, different amounts of water will leave the cell before

the intracellular contents solidify. Rewarming should be as rapid as possible to avoid the phenomenon of recrystallization in which ice melts and reforms at a thermodynamically favorable, larger and more damaging crystal size (Mazur, 1984).

Classical freezing procedures include the following successive steps: pregrowth of samples, cryoprotection, slow cooling (0.5-2.0°C min^{-1}) to a determined prefreezing temperature (usually around -40°C), rapid immersion of samples in LN, storage, rapid rewarming, and recovery. Classical techniques are generally operationally complex since they require the use of sophisticated and expensive programmeble freezers. In some cases, their use can be avoided by performing the slow freezing step with a domestic or laboratory freezer (Kartha and Engelmann, 1994; Engelmann, 1997b).

1.3.3.2 New cryopreservation techniques

In vitrification-based procedures, cell dehydration is performed prior to cooling by exposure of samples to concentrated cryoprotective media and/or air desication. This is followed by rapid cooling. As a result, all factors that affect intracellular ice formation are avoided. Glass transitions (changes in the structural conformation of the glass) during cooling and rewarming have been recorded using thermal analysis (Sakai et al., 1990). Vitrification-based procedures offer practical advantages in comparison to classical freezing techniques. Like ultra-rapid freezing (above), they are more appropiate for complex organs (shoot tips, embryos) which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration. By precluding ice formation in the system, vitrification-based procedures are operationally less complex than classical ones (e.g., they do not require the use of controlled freezers) and have greater potential for broad applicability, requiring only minor modifications for different cell types (Engelmann, 1997b).

A common feature to all these new protocols is that the critical step to achieve survival is the dehydration step, and not the cooling step, as in classical protocols. Therefore, if samples to be cryopreserved are amenable to desiccation down to sufficiently low water contents (which vary depending on the procedure employed and the type and characteristics of the propagule to be cryopreserved) with no or little decrease in survival in comparison to non-dehydrated controls, no or limited further drop in survival is generally observed after cryopreservation (Engelmann, 1997b).

In recent years, new cryopreservation protocols based on vitrification of intracellular solutes have been developed, including droplet-vitrification (D-V) (Panis et al., 2005). D-V is a combination of vitrification and cooling in droplets of PVS2 plant vitrification solution (VS) on aluminium strips and presents new technological development in the area of plant cryopreservation. The main advantage of this technique is the possibility of achieving very high cooling/warming rates, due to the very small volume of VS in which the explants are placed, which increase the probability to obtain a vitrified state during cooling and to avoid recrystallization during rewarming (Panis et al., 2005). Therefore, the structural integrity of cryopreserved samples is conserved, which allows high recovery percentages and direct and rapid regrowth of cryopreserved explants.

Three standard protocols are used as the basis for most plant cryopreservation. Each has some basic steps that can help make them effective for all users. The choice of technique is related to the needs of the facility involved. Personnel, equipment, expertise, plant type, and available facilities may influence which technique is most appropiate (Table 1)

Table 1: Advantages and disadvantages of some commonly used cryopreservation techniques (From Reed 2001)

Technique	Advantages	Disadvantages
Controlled rate cooling	Stability from cracking, relatively nontoxic cryoprotectants, takes little technician time	Requires equipment, low applicability to tropical species
Vitrification	No special equipment needed, fast procedure, fast recovery	Vitrification solutions are toxic to many plants, cracking is possible, requires careful timing of solution changes
Encapsulation dehydration	No special equipment needed, nontoxic cryoprotectants, simple warming procedures, fast recovery	Requires handling each bead several times, some plants do not tolerate the high sucrose concentrations

Seven different vitrification-based procedures can be identified: (1) encapsulation-dehydration; (2) vitrification; (3) encapsulation-vitrification; (4) dehydration; (5) pregrowth; (6) pregrowth-dehydration; and (7) droplet-vitrification. In this review we focus on three main procedures: E-D, vitrification and D-V which are the most relevant to our research.

1.3.3.2.1 Encapsulation-dehydration

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose for 1-7 d, partially desiccated in the air current of a laminar airflow cabinet or with silica gel to a water content around 20% (fresh weight basis), then cooled rapidly (Dereuddre et al., 1991). Survival is high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. The encapsulation-dehydration technique was first developed with species from temperate climates including shoot tips of eucalyptus (Poissonnier et al., 1992) and grape (Plessis et al., 1991) and carot somatic embryos (Dereuddre et al., 1991).

A modified protocol has been proposed by Sakai et al. (2000) in which encapsulation and pregrowth in medium with sucrose and glycerol are performed simultaneously. This technique has been applied to apices of numerous species from temperate and tropical origin as well to cell suspensions and somatic embryos of several (Engelmann, 1997b; Engelmann and Takagi, 2000).

1.3.3.2.2 Encapsulation-vitrification

In order to treat a large number of meristems at the same time with vitrification protocols, the encapsulation-vitrification method has been developed an (Matsumoto et al., 1995). The method is a combination of E-D and vitrification, in which explants are encapsulated in alginate beads, then treated with a vitrification solution (VS) and processed as described below (steps of cryopreservation protocols).

1.3.3.2.3 Vitrification

This technique uses glycerol-based VSs designated PVS2 (Sakai et al., 1990;1991) and PVS3 (Nishizawa et al., 1993). Plant vitrification solution 2 contains 30% glycerol (w/v), 15% ethylene glycol (w/v), and 15% dimethylsulfoxide (DMSO; w/v) in basal culture medium

(without growth regulators) containing 0.4 M sucrose (pH 5.8), and PVS3 consists of 40% glycerol (w/v) and 40% sucrose (w/v) in basal medium.

1.3.3.2.4 Droplet-vitrification

D-V is a combination of vitrification and cooling in droplets of PVS2 solution on aluminium strips.

This technique is derived from the droplet-freezing technique developed by Kartha et al. (1982) for freezing cassava shoot tips, in which the shoot tips are placed in droplets of cryoprotective medium and cooled slowly in a programmable freezer. In the D-V protocol, shoot tips are loaded, treated with the PVS2/PVS3 VSs, put individually in 5-10 µl droplets of PVS2/PVS3 placed on a piece of aluminium foil, which is then immersed in LN. For warming, the aluminium foils are plunged in liquid medium containing 1.2 M sucrose and after 20 min unloading, shoot tips are retrieved and placed on recovery medium. The main interest of this technique is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotective medium in which the explants are placed.

This is a very recent technique which has been applied until now to a limited number of plant species only (Table 2). This technique has been suggested for conservation of garlic and raspberry genetic resources (Kim et al., 2007; Consello et al., 2010). The very promising results achieved call for a broader utilization of D-V.

Recently, D-V has been improved using the aluminium cryo-plate technique (Yamamoto et al., 2011) which allows to control carefully procedures for dehydration and prevention of injury by chemical toxicity or excessive osmotic stress during treatment with PVS solutions. In this technique, explants are placed on aluminium plates in individual wells and encapsulated in minute calcium alginate beads to make them adhere to the plates. They are then treated as described below for a vitrification procedure.

Table 2: List of plant species cryopreserved using the droplet-vitrification technique. Protocols developed for shoot tips, if not specified otherwise. SE: somatic embryos. (from Sakai and Engelmann 2007).

Species	Species
Allium sativum	*Solanum tuberosum*
Pelargonium ssp.	*Ipomoea batatas*
Colocasia esculenta	*Pelargonium*
*Saccharum officinarum (*SE)	*Musa* spp.
Dentranthema grandiflora	*Thymus ssp.*
Solanum spp.	*Solanum stenotomum*
Dioscorea spp.	*Fragaria x ananassa*

1.3.4 Steps of cryopreservation protocols

Cryopreservation protocols comprise several steps, for each of which optimal conditions have to be determined. Some steps are specific of the technique used, but generally there are four main steps of a protocol: osmoprotection, dehydration, cooling-rewarming and regrowth.

1.3.4.1 Osmoprotection

Encapsulation Osmoprotection corresponds to the treatment of encapsulated explants in medium with sucrose for several hours or days immediately before desiccation and cryopreservation. Beads containing the explants are placed in Erlenmeyer flasks in agitated liquid medium with between 0.50 and 1.25 M sucrose. The sucrose concentration most commonly employed is 0.75 M. Gradual increase of sucrose by 0.25 M incremental steps ensured over 80% survival for shoot tips of grape for 1 M final sucrose concentration and 70% for 1.5 M (Plessis et al., 1991), whereas direct exposure to these media led to very low recovery.

Vitrification Precultured meristems are placed in a 2 ml cryotube and osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20-30 min at 25°C. An osmotic loading treatment increases the osmolarity of the cell and minimizes osmotic damage caused by the vitrification solution.

1.3.4.2 Dehydration

Encapsulation After osmoprotection, beads are rapidly surface-dried on sterile filter paper to remove any remaining liquid medium and are submitted to physical dehydration by evaporation at room temperature. Two desiccation methods can be employed: dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel. The bead water content that ensures highest regrowth after cooling in LN is around 20%, which corresponds to the amount of unfreezable water in the samples. This value may vary depending on the species and the type of samples.

Vitrification Dehydration with a VS (PVS2 or PVS3). After removing the loading solution 1-2 ml of PVS2 is added and gently mixed. Five minutes later, PVS2 is replaced with 1-2 ml of fresh PVS2 and held at 25°C or 0°C for different periods of time. Wrapping several dissected meristems together in a small piece of tissue paper (1.5 cm x 1.5 cm) may facilitate the implementation of the osmoprotection, dehydration, cooling, warming, and dilution steps (Thinh, 1997).

1.3.4.3 Cooling-rewarming

Encapsulation After dehydration, beads are placed in 1 or 2 ml polypropylene sterile cryotubes for cryopreservation. In most cases cooling is performed by direct immersion of the cryotubes in LN. In some cases, including grape (Plessis et al., 1991; Zhao et al., 2001), potato (Fabre and Dereuddre, 1990) and Citrus (González-Arnao et al., 1998) shoot tips, controlled rate cooling using a programmable freezer followed by immersion of samples in LN was required to obtain higher regrowth after cryopreservation. For rewarming, beads are taken out of the cryotubes and placed in open Petri dishes in the laminar air flow cabinet for about 5 min or the cryotubes are stirred in a water bath for 2–3 min at 40°C or they are transferred directly onto the recovery medium.

Vitrification The meristems are suspended in 0.5 ml PVS2 in cryotubes, and the cryotubes are then directly plunged into LN (cooling rate: about 300°C min^{-1}) and held at -196°C for a minimum of 1 h. For rewarming, the cryotubes are plunged in a clean water bath at 40°C for 1 min. The surface of cryotube is disinfected with 70% ethanol. The cryotubes are opened and the content is immersed in unloading solution, containing 1.2 M sucrose for 20 min. The explants are dried and transferred to recovery medium.

1.3.4.4 Regrowth

Whatever the cryopreservation technique used, it is particularly important that cryopreserved meristems produce plants with a phenotype and genotype identical to that of untreated plants. A callus phase before shoot formation is undesirable because callusing potentially increases the frequency of genetic variants. The callus phase can be avoided in clonally propagated shoots, through the optimization of both the cryopreservation protocol and the recovery stages (Reed, 2008). Also a method for elimination or reduction of callus is the removal of auxins from the recovery medium (Chang and Reed, 2000).
Regrowth generally takes place on standard semi-solid culture medium in the dark for a short period (around 1 week) to avoid photo-oxidation phenomena. Explants are then transferred to standard conditions. With encapsulation-dehydration, in some cases it is necessary to extract the explants from the beads and to place them directly on the recovery medium to ensure their regrowth, as with microspore embryos of oilseed rape (Uragami, 1993), apices of grape (Plessis et al., 1991) and mulberry (Niino et al., 1992).

If the structural integrity of explants has been well preserved and regrowth conditions are satisfactory, they can resume growth within few days and develop shoots directly without intermediary callus formation. However, there are examples where explants remain green after rewarming, but do not develop further as observed in the case of grapevine (Miaja et al., 2000).

1.3.5 Cryopreservation of vegetatively propagated species

Several review papers have been published recently, which provide lists of species which have been successfully cryopreserved (Engelmann, 1997a, b; Cyr, 2000; Engelmann and Takagi, 2000; Sakai et al., 2002; Reed, 2008). For vegetatively propagated species, cryopreservation has a wide applicability both in terms of species coverage, since protocols have been successfully established for root and tubers, fruit trees, ornamentals, forestry and plantation crops, from temperate and tropical origin, and in terms of numbers of genotypes/varieties within a given species. With a few exceptions (e.g. potato, pear, mulberry), vitrification-based protocols have been employed. It is also interesting to note that in many cases different protocols can be employed for a given species and produce comparable results. Survival is generally high to very high and up to 100% survival could be achieved in some cases, e.g. *Allium*, yam, potato and conifers. Regeneration is rapid and direct and callusing is observed only in cases where the technique is not optimized.

Different reasons can be mentioned to explain these positive results (Engelmann, 2000). The meristematic zone of apices, from which organized growth originates, is composed of a relatively homogeneous population of small, actively dividing cells, with few small vacuoles and a high nucleo-cytoplasmic ratio. Cells of embryogenic somatic tissues display the same characteristics. These characteristics make such cells more likely to withstand desiccation than highly vacuolated and differentiated cells. No ice formation takes place in vitrification-based procedures, thus allowing direct, organized regrowth of cryopreserved explants. Other reasons for the good results obtained are linked with tissue culture protocols. Many vegetatively propagated species successfully cryopreserved until now are cultivated crops, often of great commercial importance, for which cultural practices, including *in vitro* propagation, are well established. In addition, *in vitro* material is “synchronized“ by the tissue culture and pregrowth procedures. Relatively homogeneous samples in terms of size, cellular composition, physiological state, and growth response are employed for cryopreservation,

thus increasing the chances of positive and uniform response to treatments. Finally, vitrification-based procedures allow the use of samples of relatively large size (shoot tip of 0.5-3 mm) which can regrow directly without difficulty.

Cryopreservation techniques are now operational for large-scale experimentation and routine application with an increasing number of vegetatively propagated plants. In view of the wide range of efficient and operationally simple techniques available, any vegetatively propagated species should be amenable to cryopreservation, provided that the tissue culture protocol is sufficiently operational for this species (Engelmann, 2004).

Over the past 40 years cryopreservation has been used for preserving plant cells and tissues, but routine storage of plant germplasm other than seeds in LN is a relatively new practice (Engelmann, 2004). Although the slow-cooling approach was already introduced in the 70's, for a long time cryopreservation of plant tissues was not studied on a wide scale. This was mainly due to the complexity of procedures and the high cost of cryo-freezers. With the development in the early 90's of “new” and simplified cryopreservation protocols, based on the prevention of intra- and extra-cellular ice crystals by means of vitrification of intracellular solutes and direct immersion of explants in LN, cryostorage of genetic resources has become a realistic target for many plant species (Panis and Lambardi, 2005).

Cryopreserved storage is now in place for many important collections of algae, cell cultures, and organized plant tissues, and techniques are now available for many more. Cryopreservation protocols are available for many economically important, vegetatively propagated plants including monocots (such as asparagus *Asparagus officinalis* L. and garlic *Allium sativum* L.), grasses (such as *Conadyn*, *Zoysia* and *Lolium*), sugarcane (*Saccharum officinarum* L.), potato (*Solanum* spp) lilies (*Lilium*), orchids (*Orchidaceae*), pineapple (*Ananas comosus* L. Merr.), taro (*Colocasia esculenta* L. Schott), herbaceous species (such as hop *Humulus lupulus* L.), hausa (or Chinese) potato (*Solenostemon rotundifolius* Poiret J.K. Morton), mint *(Mentha*), papaya (*Carica papaya* L.), *Pelargonium*, sealavender *(Limonium*), strawberry (*Fragaria*) and wasabi (*Eutrema wasabi* Sieb. Maxim.), as well as woody plants including azalea (*Rhododendron*), silver birch (*Betula pendula* L.), chestnut (*Castanea sativa* Miller), olive (*Olea europaea* L.), poplar (*Populus alba* L.) and many other species (Reed, 2008).

Every plant species is theoretically amenable to cryopreservation. If samples to be cryopreserved are amenable to desiccation down to sufficiently low water contents with no or little decrease in survival in comparison to non-dehydrated controls no or limited further drop in survival is generally observed after cryopreservation (Engelmann, 1997a). Nowadays, although the vitrification protocol can almost be considered as a standardized protocol, a large amount of the work is still performed in the framework of academic studies and involves only one or a few accessions per plant species (Panis and Lambardi, 2005).

1.3.6 Cryotherapy

Pathogen-free plant material is pivotal for productivity of agricultural and horticultural crops and for the value of ornamental plants (Waterworth and Hadidi, 1998). Vegetatively propagated plants, like grapevine, are especially threatened by pathogen accumulation because of this method of production.

Shoot tip or meristem culture *in vitro* has been used for many decades to eliminate viruses from vegetatively propagated plants (Faccioli and Marani, 1998). Shoot tips used for micropropagation are anatomically defined as structures that consist of the apical or lateral shoot meristem (1.0–1.5 mm in size) with three to four leaf primordia (Benson, 1999; Takada and Tasaka, 2002). The youngest meristematic cells are usually free of viruses and other obligate parasites, such as phytoplasma and bacteria. Therefore, excision and regeneration of tiny meristems might result in plants free of these pathogens. Pathogen eradication using meristem culture is challenged by the difficulty of mechanically excising very small meristems to remove the infected tissues and ensuring the survival and regeneration of the tiny meristems.

Cryotherapy is a novel application of plant cryopreservation. It eliminates plant pathogens such as viruses, phytoplasmas and bacteria by briefly treating shoot tips in LN using cryopreservation protocols. Healthy plants are regenerated from the surviving pathogen-free meristematic tissue. This method facilitates the treatment of large numbers of samples and is independent of shoot tip size (Brison et al., 1997; Wang et al., 2003b; Wang et al., 2006; Wang and Valkonen, 2008a; Ding et al., 2008). It has the potential to replace more traditional virus eradication methods such as meristem culture (Wang and Valkonen, 2008c).

In cryotherapy, infected cells are eliminated by the lethal effects of the ultra-low temperature and/or subsequent warming; no mechanical removal is necessary. The number of regenerating shoot tips might be lower after cryotherapy than after shoot tip culture, but the difference is compensated for by the easier excision of larger shoot tips, fast regrowth stage and the higher proportion of pathogen-free regenerants obtained with cryotherapy (Brison et al.,1997; Wang et al., 2003b; Wang et al., 2006; Wang and Valkonen, 2008a; Ding et al., 2008).

Cells in the meristematic zone differ from more differentiated cells by being smaller and having small vacuoles and a higher nucleo–cytoplasmic ratio (Wang et al., 2008). The meristematic cells are hence more likely to withstand dehydration than the differentiated cells whose physiology is also disturbed by infection with pathogens. Cryotherapy takes advantage of these physio-anatomical differences between meristematic and other tissues and is carried out to destroy the differentiated, infected cells by a brief cryo-treatment in LN. The localization of viruses (Wang et al., 2008; Wang and Valkonen, 2008a) and phytoplasmas (Wang and Valkonen, 2008b) in the shoot tips subjected to cryotherapy, the indispensability of the treatment of shoot tips in liquid nitrogen for obtaining a high proportion of virus-free regenerants and ultrastructural studies on the injured cells (Wang et al., 2008; Helliot et al., 2002) are consistent with the proposed model according to which cryotherapy eliminates pathogens by killing the infected differentiating cells (Helliot et al., 2002). The surviving pathogen-free meristematic cells are able to regenerate, divide and produce daughter cells that differentiate into the organs of the new shoot. A high proportion of the shoots regenerated by this method can be pathogen-free.

Recently, cryotherapy has been demonstrated to eradicate seven unrelated groups of viruses and/or two types of bacteria-like pathogens from the shoot tips of root and tuber crops such as potato (*Solanum tuberosum* L.; *Solanaceae*) and sweet potato (*Ipomea batatas* L. Lam.; *Convolvulaceae*), grapevine (*Vitis vinifera* L.; Vitaceae), fruit trees including *Citrus* spp. (*Rutaceae*) and *Prunus* (*Rosaceae*), berry plants (raspberry, *Rubus idaeus* L.; *Rosaceae*) and meristematic clumps of banana (*Musa* spp.; *Musaceae*) (Waterworth and

Hadidi, 1998; Brison et al., 1997; Helliot et al., 2002; Wang et al., 2003b; Wang et al., 2006; Wang and Valkonen 2008,a b, c, Wang et al., 2008; Ding et al., 2008). These plants represent six families and some of the world's most important crops in terms of food supply (potato, sweet potato and banana) and trade (citrus and stone fruits). More recently, Gallard et al. (2011) showed that the plants regenerated after cryotherapy tested by ELIZA were virus-free, but the immunolocalization studies detected some virus signals. This study implies a strong need for further research of this technique. The wider use of cryotherapy is possible because it relies on plant cryopreservation protocols that are available for many additional economically important, vegetatively propagated plants.

Rapsberry bushy dwarf virus (RBDV) in *Rubus* species is an example where cryotherapy was not successfull by itself. Only thermotherapy in combination with cryotherapy resulted in virus elimination (Wang et al., 2008). Cryotherapy depends on the availability of tissue culture and cryopreservation protocols for the plant of interest. Furthermore, genotypes (cultivars) of the same species might differ in their responsiveness to the cryo-treatment, which is a well-known challenge in plant cryopreservation (Benson, 2008; Engelmann, 2004) and also affects the applicability of cryotherapy.

1.3.7 Genetic stability

Cryopreservation is a complex procedure during which plant explants are exposed to very low temperatures (-196°C), potentially stressing events, including contact with cryoprotectants during pregrowth and cryoprotection, dehydration, cooling and rewarming, recovery and plant regeneration on media containing growth regulators. All such events are capable of inducing somaclonal variation in regenerated plants.

Post-cryopreservation genetic stability assessments have been performed. The effect of encapsulation–dehydration on the stability of plants after regrowth was assessed for several species. No modifications were revealed at the morphological, agronomical, chromosomal, biochemical and/or molecular levels for sugarcane (González-Arnao, 1996), apple (Hao et al., 2002), yam (Sangeeta et al., 2002; Sonali-Dixit et al., 2005), kiwi and grape (Zhai et al., 2003). Konan et al. (2007) compared plants originating from control and cryopreserved stabilized polyembryonic cultures (SPCs) of six elite oil palm clones 12 years after field transfer. If the average survival of plantlets in the nursery was higher from cryopreserved SPCs in comparison with controls and a small delay in the first flowering of cryopreserved SPCs was noted, no differences were observed afterwards in the vegetative and floral characteristics of these palms. More recently, Castillo et al. (2010) tested the genetic stability of *Rubus* germplasm and compared plants coming from explants cryopreserved for 12 years and subcultured for 7 months, plants regenerated from cryopreserved material immediately after rewarming and as control, field-grown plants propagated in the greenhouse. AFLP polymorphisms were detected in three of the four tested *Rubus* genotypes (regrown after cryopreservation) after they were subcultured for 7 months in comparison with control plants. In-field behavior of banana plants (*Musa* AA sp.) regenerated from control and cryopreserved embrygenic cell suspensions was studied (Côte et al., 2000). Observations of the morphological descriptors showed no difference between cryopreserved-derived plants and control plants.

1.3.8 Cryopreservation of grapevine

1.3.8.1 Shoot tips sampled from in vivo plants

1.3.8.1.1 Vitrification protocol

Ezawa et al. (1989), were the first to report cryopreservation of grapevine. Shoot tips, 1-2 mm in size were excised from field-grown vines of *Vitis labrusca* L. in September, November and December. The explants were placed in cryoprotectant solution containing 10% DMSO and 60g/L glucose at 20°C for 2 h, then cooled down to the prescribed temperature (prefreezing, cooling rate: 0,5°C/min) and immersed in LN (cooling rate: 500°C/min). The frozen samples were rewarmed in water at 38°C (rewarming rate: 420°C/min) before culture on solid medium. Results showed that half of cv. “Buffalo“ shoot tips and most of cv. „Delaware“ shoot tips taken in September lost their viability at -30°C (without LN immersion). By contrast, in November and December, 80% shoot tips survived even after LN exposure. The optimum prefreezing temperature before LN immersion was -30°C in “Delaware and Buffalo“. Almost all cv. “Campbell Early“ shoot tips frozen to any temperature in this experiment formed calli. Survival of “Buffalo“ shoot tips was 80% after 18 months of storage in LN.

In September shoot tips, a large part of the meristems was injured by freezing, because freezing resistance of the shoot tips was not sufficient. Therefore, the leaf primordia of cryopreserved shoot tips elongated, but no shoot formation was achieved. Regarding the effect of prefreezing temperature on survival of the November and December-collected shoot tips, especially in cv. “Buffalo“, it is assumed that intracellular freezing occurred in cells of the shoot tips prefrozen to -20°C when plunged into LN, because cells might not be dehydrated sufficiently by prefreezing. It is probably due to low freezing resistance that callus induction and leaf primordia elongation without shoot formation were observed in cv. “Cambell Early“ shoot tips frozen to -20°C,-30°C,-40°C and -196°C. The authors emphasized that winter dormant buds (meristems) have been often used for freeze-preservation studies, because they have high freezing resistance (Katano et al, 1983; Moriguchi et al., 1985.; Sakai and Nishiyama, 1978). However, the correlation between hardening conditions and physiological-structural mechanisms of freezing tolerance are not yet explained clearly.

1.3.8.2 Shoot tips sampled on in vitro plantlets

1.3.8.2.1 Encapsulation-dehydration

A comprehensive study on cryopreservation of grapevine shoot tips was made by Plessis et al. (1991) who tested encapsulation-dehydration with axillary and anticipated buds as material. Sucrose preculture was performed as follows: direct preculture for 2 days (0.1-1.5M) or progressive preculture for 9 days (every 2 days with successively 0.3, 0.5 and 0.75 M, and then every day for further increases in concentration (1, 1.25, and 1.5 M, successively). Two types of freezing were performed: rapid freezing, by direct immersion in LN and two-step freezing, where samples were progressively cooled from +20°C to -80°C before immersion in LN. These authors observed the following results: survival remained high (>72%) whatever the final sucrose concentration employed during preculture, if sucrose was added progressively. The tolerance of shoot tips to dehydration depended also on the preculture. After direct preculture with 0.75 M sucrose and 4 h dehydration (25% residual water), survival was below 17%. After 6 days of progressive preculture to the same sucrose concentration, survival after a similar dehydration remained higher than 75%. After

progressive preculture (from 0.3 to 1.5 M sucrose) and 4 h dehydration, two-step freezing yielded better results than rapid cooling. The best survival was obtained after preculture with 0.75, 1 and 1.25 M sucrose with an optimum of 24,7% with 1 M.

Plantlet recovery on Barlass and Skene (1978) regeneration medium was recorded 4 weeks after rewarming (Plessis et al., 1991). Addition of 1% of bovine foetal serum (BFS) improved recovery. Subculture of explants on MS medium led to the formation of plantlets. This fact indicates that regrowth of alginate-coated *Vitis* shoot tips required using a special medium for reactivation. Prolonged preculture on media enriched with sucrose may be an alternative for species which are sensitive to low temperatures. Low survival following cooling in LN may be related to physiological differences caused by the position of shoot-tips along the stem axis and correlative inhibition induced by apical dominance.

Afterwards, Plessis et al. (1993) performed a more thorough study of encapsulation-dehydration focusing on the effects of pretreatment, cooling and postculture. Considering their rank on the stem, there was no significant difference in survival of axillary shoot tips whatever their position. Shoot regrowth remained very close to shoot tip survival except for shoot tips of rank 2 for which less than half of surviving shoot tips developed into shoots. Several precooling temperatures were tested from +20°C to -100°C, before quenching in LN. Shoot tip survival increased significantly as precooling temperature decreased. These authors compared the effect of a 2 and 7-day preculture with, up to 1 M sucrose, followed by rapid cooling or two-step cooling. Reduction of the preculture duration did not affect shoot tip survival at any stage of the cryopreservation process. Whatever the duration of preculture, shoot tip survival after two-step cooling was higher than after rapid cooling by direct immersion in LN.

The addition of DMSO during the last step of a 7-day stepwise preculture, did not significantly alter shoot tip viability, which remained above 70% (Plessis et al., 1993). As regards postculture conditions, concentrations of sucrose and duration of postculture were studied. High survival (46-48%) was observed when shoot tips were extracted from the beads after only 1 day of postculture on 0.3 M sucrose medium and 7 days of postculture on 0.1 M sucrose medium. The best results (40-60% survival and 30% shoot recovery without callus formation) were obtained after a 2-day stepwise preculture in medium containing 1 M sucrose, two-step cooling, and direct subculture on standard culture medium.

In this comprehensive study, the importance of sucrose preculture was highlighted. Plessis et al. (1993) indicated that sucrose and other carbohydrates maintain plasma membrane integrity by water substitution on the membrane surface and ensure protein protection. Only stepwise preculture with high sucrose concentrations allowed grapevine shoot tips to survive dehydration and subsequent cooling in LN. In organised cell systems such as meristems subjected to long preculture, sucrose first induces osmotic dehydration, and may enter cells and contribute to maintenance of the integrity of plasma and inner membranes during dehydration and freezing.

Wang et al. (2000) cryopreserved *in vitro*-grown shoot tips of grapevine using encapsulation-dehydration. Shoot tips (1 mm in size) were excised from 4-week old stock cultures of rootstock LN 33 hybrid (*Vitis* L.) and cv. Superior. A comparison between the effects of dehydration with silica gel and by air drying on cryopreserved shoot tips showed that survival was dependent on water content but not on the dehydration method. The rewarming method

markedly affected survival of cryopreserved shoot tips, and rewarming at 40°C for 3 min (fast rewarming) was found best compared to slow rewarming at room temperature for 15 min. A post-culture medium composed of half-strength MS (Wang et al., 2003a) supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA was used. With these optimized parameters, 60 and 40% survival of cryopreserved shoot tips were obtained for the LN33 hybrid and cv. Superior, respectively.

Miaja et al. (2000) tested encapsulation-dehydration of axillary buds. The alginate beads were grown stepwise for one day in liquid culture media containing increasing concentrations (0.3 M; 0.6 M; 1.0 M) of sucrose and then dehydrated under a sterile air flow at room temperature for 4 h so that the content of water in the embedded buds was 30%. A two-step freezing method was used: samples were progressively cooled at the rate of 0.5°C/min from +20°C to -80°C before immersion in LN and after immersion in LN, rewarmed at +40°C in a sterile water bath and transferred to recovery medium (MS medium containing 0.6% agar, 0.1 M sucrose, 10 µM BA and 1% bovine fetal serum). Storage of buds in LN was not successful even though the fluorescein diacetate test gave positive results with all the cultivars tested. This technique gave only indications of post-thaw viability, but not of regeneration capability. The explants remained green but they did not develop further.

Zhao et al. (2001) performed encapsulation-dehydration with axillary buds of four grapevine cultivars (Cabernet franc, Chardonnay, Fengh 51 and rootstock LN 33). These authors studied various parameters including desiccation duration, preculture of mother plants (cold-hardening, long period without subculture before excision), two cooling procedures (rapid and two-step cooling) and post-culture conditions. The regrowth percentage of buds decreased with decreasing bead moisture content (from 0 to 6 h of desiccation). Corresponding to bead moisture contents of 21 and 18%, a dramatic drop was observed in regrowth percentage from 66.1% for 5 h to 34.5% for 6 h of dehydration. When mother-plants were cold-hardened and placed under a modified photoperiod for one month after a 4-month period without subculture, regrowth was obtained after both rapid and slow cooling but was much higher with slow cooling. When mother-plants were kept for 3 months without subculture, regrowth of prefrozen buds was obtained for all prefreezing temperatures tested. After cryopreservation, regrowth increased progressively from 12.5% without prefreezing to 40.9% for a prefreezing temperature of -40°C. Various results in this comprehensive study implied that the physiological state of mother plants affects cryopreservation success.

1.3.8.2.2 Vitrification protocol

Matsumoto and Sakai (2003) were the first to use vitrification as a technique for cryopreservation of *in vitro* grown grape shoot tips. They tested a two-step vitrification protocol: osmo-protected shoot tips were first dehydrated with half strength PVS2 for 30 min, followed by PVS2 for 50 min at 0°C. This procedure was applied to 10 other *Vitis* cultivars with an average recovery of 64%.

Grassi et al. (2004) reported cryopreservation of grape rootstock “Kober 5BB” using a one-step vitrification protocol where they tested different sucrose pretreatments and different exposure durations to PVS2 solution. Cryopreserved shoot tips showed the best survival (60%) after treatment with 0.4 M sucrose, 30 min incubation in the PVS2 solution and rapid rewarming at 40°C.

Recently, Ganino et al. (2012) showed on grapevine rootstock „Kober 5BB“ that pretreament alone markedly affected shoot tip survival. Plantlets regenerated from shoot tips which underwent 60 min or more exposure to PVS2 showed signs of malformation. The consequence of prolonged PVS2 treatment was convex plasmolysis. This research showed what are the possible limits of PVS2 exposure on the cell structure, but to confirm these findings, application to numerous cultivars is necessary.

Attempts to develop efficient protocols have been performed using shoot tips (Plessis et al., 1991,1993; Wang et al., 2000, 2003; Zhao et al., 2001; Matsumoto and Sakai, 2003; Ganino et al., 2012). However, if these studies showed that cryopreservation could be efficiently applied to grape, no large scale use of these protocols has been performed so far. One of the reasons can be the large number of grapevine cultivars in use and the high influence of genotype (Peros et al., 1998). Other limitations should be analysed.

1.3.8.3 Cell suspensions

Grape embryogenic cell suspensions have been cryopreserved, as the most suitable plant material for grapevine genetic transformation (Hebert, 1993). The first report of grapevine cell suspension cryopreservation was on rootstock ‘41 B’ (*Vitis vinifera* ‘Chasselas’ x *Vitis berlandieri*) made by Dussert et al. (1991). These authors tested the influence of pretreatment, cooling and rewarming conditions. They obtained 60% viability using controlled cooling (0.5-1.0°C/min). This technique was suggested as efficient for cryopreservation, but additional studies on the conversion rate of cryopreserved embryos and their development *in vitro* were required. In their following publication, Dussert et al. (1992) observed post-thaw culture of grape embrygenic cell suspensions. Optimal growth recovery was achieved with the addition 1 mg/l naphtoxyacetic acid to the culture medium.

Wang et al. (2002) tested encapsulation-dehydration for cryopreservation of grape embryogenic suspensions. These authors showed that optimal viability of cryopreserved cells was achieved when encapsulated cells were subjected to 2-4 days of preculture on 1 M sucrose then dehydrated to 20.6% water content. They obtained 42-76% viability of cryopreserved cells using the encapsulation-vitrification technique. Cell suspensions were precultured with a final sucrose concentration of 1 M and dehydrated with PVS2 at 0°C for 270 min.

Gonzalez-Benito et al. (2009) improved viability of embryogenic cell suspensions by increasing preculture duration. Cryopreserved cells reached up to 50% viability, with a 1 M sucrose preculture for 4 days.

1.3.8.4 Genetic stability research on grapevine

Grapevine varieties are the result of several processes: multiple domestication centers from local wild *Vitis sylvestris* vines (Arroyo-Garcia et al., 2006), subsequent crosses between domesticated and local wild vines, the old practice of growing seedlings from spontaneous crosses and to a lesser extent during the last century conventional breeding (Pelsy et al., 2010). However, all the vines of a single variety are descendants from a single selected vine grown from a single seedling. Selected seedlings have been multiplied by vegetative propagation and all vines of a variety should be genetically uniform (identical), provided that somatic mutations have not occurred (Pelsy, 2010). However, within the populations of varieties, clones showing phenotypical variation can be observed (Forneck, 2005; Maletić et al., 2008; Meneghetti et al., 2011).

Many changes in the phenotype and genotype of plants after exposure to stress have been recorded (Jain, 2001; Hazarika, 2006; Saker et al., 2006; Oh et al., 2007). In the case of grapevine, the stress is arising from the manipulation of grapevine clones which may possess unique characteristics as a result of purposeful selection carried out over centuries. Such changes are mostly undesirable. The stress caused by *in vitro* culture, thermotherapy and virus infection has been revealed by AFLP analysis (Barânek et al., 2009). The average polymorphism observed between *in vitro* cultivated somaclones was (2.1%) and (1.7%) for Riesling and Müller Thurgau variants, respectively, whereas somaclones after thermotherapy showed 2.1% and 1.3% polymorphism, respectively.

1.3.8.5 Cryotherapy on grapevine

Cryotherapy has been successful in eliminating grapevine virus GVA from grapevine shoot tips (Wang et al., 2003b). Two cryopreservation protocols have been performed: encapsulation-dehydration and vitrification. They were compared in their efficiency in virus eradication. Survival after cryopreservation was higher for encapsulation-dehydration (62%) in comparison with vitrification (50%). Percentage of virus eradication was equal for both protocols tested and a 97% GVA-free rate was achieved in plants regenerated from cryopreserved shoot tips of grapevine. It has been published that different cryogenic procedures may result in differences in virus elimination efficiency (Brison et al., 1997). This report clearly showed no differences in the efficiency of GVA elimination using different cryopreservation procedures. This is the only report of cryotherapy performed on grapevine.

2 HYPOTHESIS AND GOALS OF THESIS

Hypothesis

1. Maintenance of grapevine *ex situ* collections is expensive, labour intensive and accessions are often threatened by natural hazards. *In vitro* storage is a method of choice for establishing their safe duplicates, enabling also sanitation of plant material by meristem culture, thermotherapy and recently cryotherapy. So far, *in vitro* culture of a considerable number of international grapevine cultivars has been established.
   It is possible to establish successful *in vitro* cultures of healthy as well as virus-infected genotypes of Croatian autochthonous varieties.
2. The potentially safest method to maintain germplasm for long time periods without changes is storage in LN (cryopreservation). Although cryopreservation has a wide applicability for some agricultural crops, so far no efficient protocol suitable for long-term storage of a large number of grapevine cultivars has been established.
   By studying the influence of different parameters and customizing cryopreservation protocol steps, it is possible to enhance survival and regrowth of shoot tips of diverse grapevine cultivars (international as well as Croatian autochthonous ones) after LN exposure.
3. Cryotherapy of shoot tips is a novel technique used for pathogen (viruses, phytoplasmas and bacteria) eradication from different important agricultural crops. So far, successful sanitation by cryotherapy of grapevine infected only with GVA has been reported.
4. At the temperature of LN (-196°C) metabolic and most physical processes are stopped and explants do not undergo cellular division. Grapevine cultivars preserved under cryogenic storage will maintain their genetic stability. The genetic uniformity of offsprings after successful regeneration assessed by molecular markers is expected.

Goals

1. To establish *in vitro* culture of healthy genotypes of Croatian autochthonous grapevine cultivars Plavac mali, Maraština, Pošip, Debit, Grk, Lasina, Plavina and Vugava and virus-infected genotypes of Plavac mali. To test specific requirements of each cultivar and the influence of virus infection on *in vitro* growth.
2. To establish an efficient protocol for grapevine shoot tips cryopreservation by testing:
   a. different steps and parameters of the protocol (preculture conditions, vitrification procedure, choice of cryoprotectants, exposure to cryoprotectants, etc.)
   b. physiological state of plant material (source of material, position of the bud on the stem, size of explants, etc.)
3. To study the efficiency of cryotherapy on grapevine shoot tips infected with two economically important grapevine viruses: GFLV and GLRaV-3.
4. To test regenerated plants deriving from all steps of cryopreservation protocol (including controls) for their genetic uniformity using molecular markers.

3 MATERIALS AND METHODS

3.1 Plant materials

In this work, plant materials of different origins were used for different purposes. Croatian cultivars were used for *in vitro* introduction and cryopreservation experiments. Cultivar Portan was used to establish the cryopreservation protocol. A set of four international cultivars were used to test the developed cryopreservation protocol and in cryotherapy experiments.

Immunolocalisation experiments were performed with infected Croatian cultivars, using Portan as healthy control and with healthy and infected international cultivars.

Cultivar Portan was used for genetic stability experiments.

3.1.1 Croatian cultivars

Eight Croatian grapevine varieties were selected, including Plavac mali, Maraština, Pošip, Debit, Grk, Lasina, Plavina and Vugava. All of them are commercially grown in Dalmatia and are thus of great economic importance. In order to ensure production of certified plant material, virus screening and mass positive selection were conducted during three consecutive production years. Healthy genotypes showing good production characteristics were propagated and planted in the pre-base plantation “Baštica” near Zadar.

Plant material of healthy genotypes was taken from this plantation, while material of infected genotypes of cultivar Plavac mali originated from the grapevine germplasm repository of the Institute for Adriatic Crops and Carst Reclamation near Split. Comprehensive studies were performed recently on Croatian autochthonous cultivars, allowing their classification in several groups (A, B, C, D) according to their genetic distance (Zdunić et al., 2013). Cultivars were compared accordingly in our study. Hardwood cuttings were taken at the beginning of December 2010 and were further exposed to low temperature (-10°C) for 2 weeks in order to break dormancy and ensure uniform shoot grow.

3.1.2 International cultivars and Portan

Cuttings from IFVV screenhouses taken from plants of four cultivars, Chardonnay, Cabernet Sauvignon, Merlot and Pinot Noir of known viral status (infected or not infected) were introduced *in vitro* and multiplied to obtain at least 50 plants for each non-infected and infected accession.

In vitro cultures of cultivar Portan were initally established from field-grown plants in the grape germplasm collection of Institut national de la recherche agronomique in Vassal (France).

3.2 Methods

3.2.1 *In vitro* introduction of Croatian cultivars

3.2.1.1 In vitro inoculation and in vitro culture

Hardwood cuttings were cultured in a growth chamber to obtain shoot growth from winter buds. Shoots developed from these hardwood cuttings with leaves removed were first washed with tap water for 15 min, immersed in 70 or 96% ethanol for 30 s, then in 5%

sodium hypochlorite (NaOCl) for 30 min and rinsed three times for 5 min each in sterile distilled water. Shoots were then cut into 1.5 cm long nodal segments which were planted in individual test tubes (100 mm x15 mm) containing 10 ml culture medium.

For healthy genotypes, three media based on MS (Murashıge and Skoog, 1962) basal salts as the most commonly used in tissue culture of different grapevine varieties (Torregrosa et al., 2001) were compared: 1) M1: half-strength MS; 2) M2: full-strength MS; and 3) M3: full-strength MS + 4.4 µM benzylaminopurine (BAP), each containing 30 g/l sucrose and 8 g/l agar. Experiments with virus-infected Plavac mali genotypes were conducted using M1. Cultures were incubated under a 16 h light/28°C - 8 h dark/24°C dark photo/thermoperiod, with a light intensity of 40 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

3.2.1.2 Evaluation of sanitary status

Although selected genotypes had been tested for the presence of viruses during the process of mass positive selection, according to the European Union Council Directive 92/34/EEC, they were retested for the purposes of our research. Virological tests were carried out at the Department of Phytopathology of the Faculty of Agriculture, University of Zagreb. Samples of six genotypes of cv. Plavac mali, (PMC 201, PMC 008/1, PMC 010/1, PMC 064/3, PMC 225/2 and PMC 201/6) were tested for presence of Grapevine leafroll-associated virus 1 (GLRaV-1), Grapevine leafroll-associated virus 3 (GLRaV-3) and for Grapevine virus A (GVA) using enzyme-linked immunosorbent assay (ELISA). An additional test using RT-PCR was conducted for GVA. For the ELISA test, phloem tissues from mature canes, collected throughout the dormant season, were used as potential sources of antigen. They were ground in a mortar after addition of LN and diluted in extraction buffer (pH 8.2) with a 1/15 ratio. All other ELISA steps were conducted according to manufacturer's instructions (Agritest, Valenzano, Italy). For GVA, total RNA was extracted from cortical scrapings using the RNeasy plant mini kit (QIAGEN, Germany) according to manufacturer's instructions and RT-PCR was conducted as described by MacKenzie (1997).

3.2.1.3 Evaluation of survival/regrowth and statistical analysis of results

For each cultivar, three repetitions of 15 test tubes per medium were performed. Survival was evaluated 2 weeks after inoculation by counting the number of shoots, which showed any type of growth, while regrowth was defined as the development of plants with at least four leaves, 8 weeks after inoculation. Contamination, recorded 2 weeks after inoculation, was consistently below 5% (data not shown). After 8 weeks, two growth variables, shoot length (cm) and number of nodes, were recorded on grown plants for all tested cultivars. For infected genotypes of cv. Plavac mali, survival was evaluated after 4 weeks and regrowth after 12 weeks, due to their low shoot growth. Shoot length and number of nodes were recorded 12 weeks after inoculation. Survival and regrowth data are presented in % of inoculated explants.

The influence of genotype and culture medium and their interaction on survival and recovery were analyzed using logistic regression. Success of survival/recovery was coded as binary variable (1 for success and 0 for lack of survival/recovery).

Factorial ANOVA was performed for shoot length and number of nodes based on cultivar, medium and their interaction. One-way ANOVA and means comparison was performed using Duncan's multiple range test for shoot length and number of nodes between eight cultivars and three media.

A canonical discriminant analysis was performed using PROC CANDISC in the SAS software (SAS 2004), based on the following variables: shoot length, number of nodes and average length of nodes of the eight cultivars studied grown on the three different media tested and the first two canonical variables were plotted.

The Ryan-Einot-Gabriel-Welsh test (REGW) was performed using the SAS software (SAS, 2004), for comparison of non-parametric values for survival and regrowth percentages between healthy and infected genotypes of cultivar Plavac mali ($P \leq 0.05$).

3.2.2 Cryopreservation

3.2.2.1 In vitro culture of international cultivars

In vitro plantlets of international cultivars were cultured on basal medium (BM) composed of Galzy medium (Galzy et al., 1990) with 1.5 % sucrose and 0.7% agar (Sigma) at pH 5.8. They were cultured at 24 ± 2°C under a 16 h light/8 h dark photoperiod with a light intensity of approx. 40 µE $m^{-2}s^{-1}$ provided by a mixture (2:1) of cool white and Grolux fluorescent tubes. *In vitro* mother-plants were kept without subculture for 3 months to reach a length of approximately 12 cm before use for cryopreservation experiments.

3.2.2.2 In vitro culture of cultivar Portan

In vitro plantlets of cultivar Portan were cultured on basal medium (BM) composed of half-strength MS (Murashige and Skoog, 1962) mineral elements with Morel's vitamins, 3% sucrose and 0.7% agar (Sigma) at pH 5.8. They were cultured at 27 ± 2°C under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. *In vitro* mother-plants were kept without subculture for 2 months to reach a length of approximately 12 cm before use for cryopreservation experiments. They were excised and used as axillary buds (Figure 1).

3.2.2.3 Production of shoot tips for cryopreservation experiments

In vitro plantlets were cut into single node microcuttings of approx. 1.5 cm in length, which were transferred to 9 cm Petri dishes (20 microcuttings/Petri dish) and placed on ½ MS medium containing 20 g/l sucrose, 7 g/l microagar and 1 µM benzyladenine (BA). Petri dishes were placed at 27 ± 2°C under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. Shoot tips were excised from microcuttings after 2 weeks and used for cryopreservation experiments.

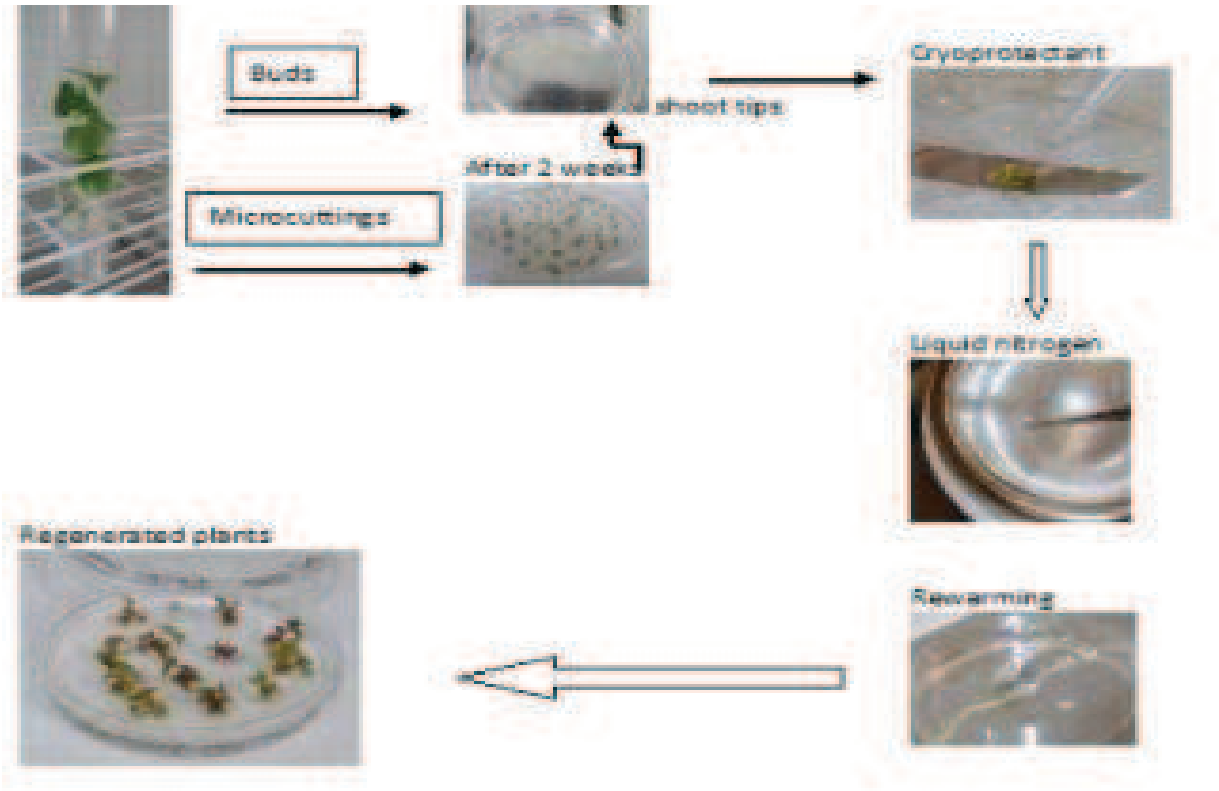


Figure 1: Schematic representation of the base cryopreservation protocol

3.2.2.4 Encapsulation-dehydration

The standard E-D protocol was the following. Excised shoot tips (1 mm long, were suspended in half-strength MS medium devoid of calcium, supplemented with 3% (w/v) Na-alginate (low viscosity, Sigma A2158), 2 M glycerol and 0.4 M sucrose, according to Wang et al. (2000). The mixture with the shoot tips was dispensed dropwise with a sterile pipette into a 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose at room temperature for 30 min, to form beads (about 4 mm in diameter, each bead containing one shoot tip. The beads, placed in 250 ml Erlenmayer flasks, were then precultured stepwise in liquid (100 ml) medium with progressively increasing sucrose concentrations over a period of 2 days (12 h for each step with 0.25, 0.5, 0.75 and 1.0 M sucrose). Beads were afterwards dehydrated with silica gel at 103 °C (10 beads above 80 g silica gel in 120 ml flasks) to 22.3% moisture content (MC, fresh weight basis), which was achieved after 4 h. Dehydrated beads were placed in cryotubes and directly immersed in LN. Encapsulated shoot tips subjected to dehydration alone or to dehydration and LN exposure were post-cultured on medium containing 1µMol benzyladenine (BAP), maintained in the dark at 26°C for 7 days and then transferred to the conditions described for mother-plants.

Two experiments were performed with ED. In the first experiment, we studied the effect of bead MC on recovery of grapevine shoot tips. After preculture as described above, encapsulated shoot tips were dehydrated to 16.2%, 20.3% and 22.3% MC (achieved after 4, 5 and 6 h, respectively), immersed, or not, in LN and transferred to recovery medium. In the second experiment, we investigated the effect of explant size on recovery. The standard ED protocol was thus applied to 1- and 2-mm long shoot tips.

3.2.2.5 Droplet-vitrification

3.2.2.5.1 Base protocol

Excised shoot tips (1 mm long) were precultured on solid ½ MS medium with 0.1 M sucrose for 24 h. Precultured shoot tips were then treated with a loading solution (LS) containing 2 M glycerol + 0.4 M sucrose in MS medium (Sakai et al., 1991) for 20 min at room temperature. Shoot tips were dehydrated with half-strength PVS2 or PVS3 at room temperature for 30 min, then with full strength PVS2 at 0°C or PVS3 at room temperature. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% dimethylsulfoxide (DMSO) and 0.4 M sucrose in MS medium (Sakai et al., 1990) and PVS3 (50% (w/v) glycerol and 50% (w/v) sucrose in basal medium (Nishizawa et al., 1993). Shoot tips were then placed on aluminium foils in 5 µl droplets of PVS2/PVS3 and immersed in LN for at least 1 h. For rewarming, the aluminium foils with the shoot tips were immersed for 20 min at room temperature in unloading solution containing 1.2 M sucrose (Sakai, 1997), then transferred to recovery medium, half-strength MS supplemented with 1 µMol BAP.

3.2.2.5.2 Effect of physiological state of explants

3.2.2.5.2.1 Shooting medium

Two experiments were performed to test the effect of growth regulators in shooting medium on growth recovery of cryopreserved shoot tips.

In the first experiment, microcuttings were placed on shooting medium with 1 µMol ZR and buds were excised after 2, 4 or 6 weeks. Control buds were excised directly from *in vitro* plantlets. This experiment was carried out in IRD Montpellier.

In the second experiment, microcuttings were cultured on shooting medium devoid of growth regulators or containing 1 µMol BAP or ZR and buds were excised after 2 weeks. This experiment was carried out in Zagreb University.

3.2.2.5.2.2 Duration of culture on medium with zeatine riboside

Single node microcuttings were placed on culture medium containing 1 µM ZR, and kept on this medium for 4 to 16 weeks with subcultures on new medium every 4 weeks. Control samples consisted of shoot tips, which were not cultured on medium with ZR. Mean explant number per treatment was 10.

The D-V technique was employed for shoot tip cryopreservation. After excision, shoot tips were placed for 24 h on a medium containing 0.3 M sucrose, treated for 20 min with a loading solution containing 1.2 M glycerol + 0.4 M sucrose, then with cold (0°C) half-strength PVS2 vitrification solution (Matsumoto and Sakai 2003) for 30 min, dehydrated with full-strength PVS2 for 25, 50 or 75 min, cooled rapidly in liquid nitrogen in PVS2 droplets placed on aluminium foils, rewarmed rapidly by immersion of foils in an unloading solution containing 1.2 M sucrose for 20 min, then transferred for recovery on ½ MS based with 1µM BAP (Wang et al., 2003a).

3.2.2.5.2.3 Source of material

Two experiments were performed in Zagreb University to test the effect of source of material on growth recovery of cryopreserved shoot tips.

In the first experiment, buds were dissected from 2 month-old *in vitro* plantlets, bearing eight-nine buds. Buds were split in three groups depending on their position on the stem and used directly for cryopreservation experiments. The first group included terminal buds (node 1), the second group buds from nodes 3 and 4 from the top and the third one buds from nodes 6 and 7 from the top.

In the second experiment, *in vitro* plantlets were cut into single node microcuttings, which were split in the three groups described above. These microcuttings were cultured for 2 weeks on shooting medium with 1 µMol BAP before excision and cryopreservation of buds. Control buds were dissected from nodes 1, 3-4, 6-7 and used directly for cryopreservation experiments.

3.2.2.5.2.4 Pretreatment with proline

Single-node explants isolated from *in vitro*-grown plantlets were cultured for 2 weeks on shooting media (half-strength MS-based medium plus 1µM BAP) containing no proline (control) or 50, 500, or 2000 µM filter-sterilized L-proline.

After 2 weeks, in a first series of explants (at least 37 per condition), the number of shoot-developing nodes was counted and individual shoots length and number of newly-developed nodes were recorded. Shoot tips excised from micro-shoots obtained on the different shooting media were subjected to a PVS2-based D-V protocol and plunged into LN for at least 1 h. Control explants (treated with loading, PVS2, and unloading solutions, but not exposed to LN) and LN-treated, rewarmed explants were grown on the same recovery medium containing 1µM BAP. Thirteen to eighteen explants were used per condition.

3.2.2.5.2.5 Sucrose preculture

Three different sucrose preculture procedures were tested to define which pretreatment was suitable for axillary buds of grapevine. The aim was to observe how sucrose preculture affects survival and regrowth of axillary buds before they are subjected to cryopreservation. Thus, two individual trials were performed.

In the first experiment, buds were subjected to three different sucrose preculture procedures: 1) shoot tips were excised and precultured on solid ½ MS medium with 0.1 M sucrose for 24 h, named “base“ protocol; 2) shoot tips were excised and precultured on solid ½ MS medium with 0.3 M sucrose for 72 h (based on Matsumoto and Sakai, 2003); 3) shoot tips were excised and precultured on solid ½ MS medium with 0.3 M sucrose for 24 h, 0.5 M sucrose for 24 h and 0.7 M sucrose for 8 h (based on Kim et al., 2009) and exposed to PVS2 for 25 and 50 min without LN exposure.

In the second experiment, buds were subjected to procedures 2) and 3) above and exposed to PVS2 for 15, 30 and 45 min without LN exposure.

3.2.2.5.3 Effect of alternative vitrification solutions

Two experiments were performed to compare the effect of PVS2 and of PVS2-derived alternative solutions, A3 (37.5% glycerol, 15% DMSO, 15% EG and 22.5% sucrose), A7 (37.5% glycerol, 10% DMSO, 10% EG and 32.5% sucrose) developed by Kim et al. (2009). In the first experiment, performed with axillary buds, the vitrification solutions were applied for 15, 30 or 45 min. In the second experiment, shoot tips sampled from microcuttings were treated with the three VSs for 50 min.

In the case of PVS3-derived solutions, the effect of PVS3 was compared with two alternative solutions, B3 (45% glycerol and 45% sucrose) and B4 (40% glycerol and 50% sucrose). Axillary buds were treated with these three VSs for 60, 120, or 180 min.

3.2.2.5.4 Cryopreservation of Croatian and international cultivars

3.2.2.5.4.1 Croatian cultivars

The base cryopreservation protocol was applied to the four Croatian cultivars studied using three different durations of PVS2 exposure (25, 50 and 75 min).

3.2.2.5.4.2 International cultivars

The base cryopreservation protocol (PVS2 treatment for 50 min) was applied to healthy and infected genotypes of the four international cultivars studied, Cabernet Sauvignon, Pinot Noir, Chardonnay and Merlot.

3.2.2.5.5 Evaluation of survival/regrowth and statistical analysis of results

Survival was evaluated 2 weeks after cryopreservation by counting the number of shoots that showed any type of growth, while regrowth was defined as the development of apices into shoots with expanded leaves 8 weeks after rewarming. Both survival and regrowth percentages were expressed relative to the total number of shoot tips treated. This manual counting was applied in every experiment where only one repetition was performed.

In all experiments which were replicated thrice, with 10-15 shoot tips per experimental condition, survival and regrowth data were presented as mean percentages with standard error of the mean (SD). Statistical differences between mean values of all parameters were assessed by analysis of variance (ANOVA) and Duncan's Multiple Range Test for mean separation. Duncan's Multiple Range Test was performed for comparing percentages between conditions ($P \leq 0.05$) using the SAS software. Only in experiments on duration of exposure to ZR shooting medium, survival and regrowth data, presented as mean percentages with standard error of the mean (SD) were subjected to arcsine transformation.

In the proline pretreatment experiment, shoot length data were subjected to one-way ANOVA, then to pair-wise comparisons (unilateral Student's t-test with uneven variances, 5% level).

3.2.2.6 Phytosanitary status of international cultivars

Cuttings of four cultivars, Chardonnay, Cabernet Sauvignon, Merlot and Pinot Noir from IFVV screenhouses taken from plants of known viral status (infected or healthy) were introduced *in vitro* and multiplied to obtain at least 50 plants for each non-infected and infected accession.

Table 3: Sanitary status of accessions subjected to cryotherapy

Genotype	Code in grapevine collection	Sanitary status
Chardonnay	E313	GFLV
Pinot Noir	E95	GFLV
Merlot	E124	GLRaV-3
Cabernet Sauvignon	E88	GLRaV-3

3.2.2.7 Cryopreservation protocol

Excised shoot tips (1 mm long) were precultured on solid ½ MS medium with 0.1 M sucrose for 24 h. Precultured shoot tips were then treated with a loading solution (LS) containing 2 M glycerol + 0.4 M sucrose in MS medium (Sakai et al., 1991) for 20 min at room temperature. Shoot tips were dehydrated with half-strength PVS2 at room temperature for 30 min, then with full strength PVS2 at 0°C for 50 min. Shoot tips were then placed on aluminium foils in 5 µl PVS2 droplets and immersed in LN for at least 1 h. For rewarming, the aluminium foils with the shoot tips were immersed for 20 min at room temperature in an unloading solution containing 1.2 M sucrose (Sakai, 1997), then transferred to recovery medium, half-strength MS supplemented with 1µMol BAP.

3.2.2.8 Analyzed samples

Even though survival was obtained with all four cultivars tested, regeneration of fully developed *in vitro* plantlets was possible only with Chardonnay and Cabernet Sauvignon. Table 4 shows the number of regenerated plants from control and cryopreserved apices and the number of samples used for ELISA testing. In total, 26 plantlets/plants of cv. Chardonnay were tested for the presence of Grapevine fanleaf virus (GFLV) and 38 plantlets/plants of cv. Cabernet Sauvignon for presence of Grapevine leafroll-associated virus 3 (GLRaV-3) after cryotherapy

Table 4: Number of plants regenerated from control and cryopreserved apices and number of samples used for ELISA testing (*from some of regenerated plants several leaves were taken for virus testing)

	Regenerated plants		Total number of samples for ELISA virus testing*	
Condition	CON 50'	FRE 50'	CON 50'	FRE 50'
Cultivar				
Chardonnay	15	7	17	9
Cabernet Sauvignon	20	18	15	16

CON50':exposure to PVS2 for 50 min; FRE 50' : exposure to PVS2 for 50 min followed by LN exposure

3.2.2.9 Serological assays

For identification of both viruses, double antibody sandwich ELISA was used and reagents provided by Agritest (Valenzano, Italy). All buffers were prepared according to manufacturer's instructions. As a source of potential antigen, small-sized *in vitro* plants (average fresh weight of ca 0.050 g) were ground into powder in a mortar using a pestle with the addition of 2 ml LN. Ground samples were diluted in an adequate volume of grapevine extraction buffer (ratio 1:15, w:v) and all other ELISA steps were carried out according to manufacturer's instructions using a working volume of 200 µl. Two h after adding the p-nitrophenylphosphate substrate (Sigma, USA) results were read at 405 nm using an EL800 spectrophotometer (BioTek, USA). According to Agritest's instructions, samples with absorbance greater than three times the average value of the negative controls were considered positive, while samples with absorbance between two and three times average value of negative control were considered questionable (potential low concentration of the virus).

3.2.2.10 Immunolocalization

3.2.2.10.1 Testing of protocol

Plant material

In vitro grape plantlets of cv. Maraština, Plavac mali, Portan and Vugava were cultured on basal medium (BM) composed of half-strength MS (Murashige and Skoog, 1962) mineral elements with Morel's vitamins (1948), 3% sucrose and 0.7% agar (Sigma) at pH 5.8. All cultivars were infected with Grapevine leafroll-associated virus 1 (GLRaV-1). The first testing of the protocol was performed with this virus. They were cultured at 24 ± 2°C under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. *In vitro* mother-plants were kept without subculture for 2 months to reach a length of approximately 12 cm before use for immunolocalisation experiments. Axillary buds were excised from *in vitro* plantlets and subjected to immunolocalisation experiments (following the first step of fixation of samples).

Preparation of samples for immunolocalisation

The goal of immunolocalisation is to define the tissular and cellular localization of biochemically defined antigens.

With the objective to study if is it possible to eliminate viruses through cryopreservation, histological studies based on immunolocalisation protocols were performed. The main goal was to determine the occurrence of virus epitope signals in shoot tips of grapevine. The first trial was performed in order to define if the immunolocalisation protocol developed in the CIRAD Montpellier laboratory also worked for grapevine as a species.

The immunolocalisation protocol was performed as follows:

a) Fixation of samples
b) Embedding in agarose and cutting
c) Saturation of non-specific sites
d) Labeling with specific antibodies using a primary antibody raised against the virus epitope and a secondary fluorescent antibody raised against the primary antibody
e) Observation of the cuts under a Laser microscope

Fixation of samples

Grapevine axillary buds, of a length of 1.5 cm, (with a portion of stem) were subjected to fixation. According to the protocol, fixation of samples should be performed after cutting with a vibratome. Problems occurred (the buds were expelled out the agarose) during cutting of samples and fixation before embbeding in agarose was necessary.

Fixation was performed as follows: axillary buds (Figure 2) were placed in a fixation solution (4% paraformaldehyde (PFD) in 0.01M PBS buffer) for 16 h (overnight). They were rinsed with PBS + glycine 0.1 M for 15 min, then twice with PBS (0.1 M) for 15 min. After fixation, samples were left in 70% ethanol overnight. Samples were embbeded in 7% agarose (Sigma; A-4018 Lot 90HO474) (Fig. 2) and prepared for cutting.

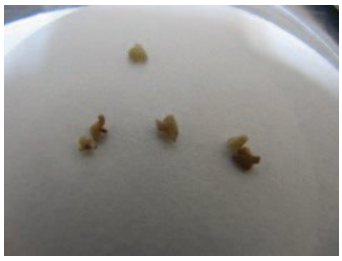


Figure 2: Buds prepared for embbeding in agarose

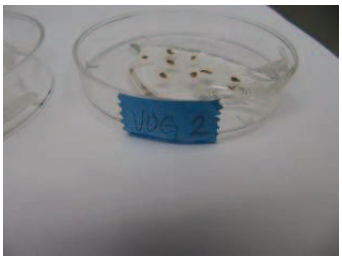


Figure 3: Samples prepared in agarose blocks

Cutting of samples was performed in CIRAD Montpellier (Histology and Plant Cell Imaging Platform) with a vibratome (MICROM HM 650 V) (Figure 4) using the following parameters: tickness: 60 µm, program: 100, frequency: 30, amplitude: 0.2 and speed: 16. Samples were selected under a binocular microscope (Figure 5) and the best ones were put in glass cups for immunolocalization.

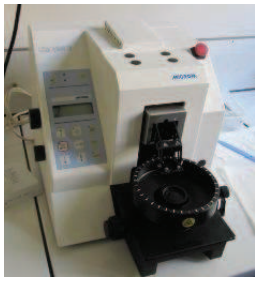


Figure 4: Vibrotome HM 650 V

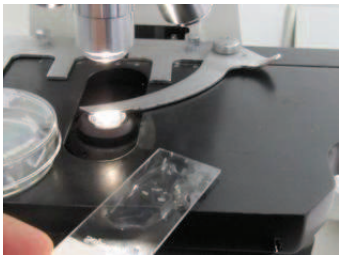


Figure 5: Selection of cuts under the microscope

Saturation of non-specific sites

To block non-specific sites, samples were saturated in PBS buffer containing a blocking reagent (blocking solution ROCHE, 5%) and left overnight at 4°C.

Labeling with specific antibodies

Monoclonal labelled anti-GLRaV-1 antibody raised from mouse (BIOREBA, AG, Reinach, Switzerland, 120415, IgG) was added. In the first assay, the primary antibody was diluted 500 fold. In the second assay, two concentrations of primary antibody were prepared and diluted to: 1) 1/1000 and 2) 1/100. After exposition overnight, a secondary antibody specific to the first antibody was added: 1) in the first assay : Alexa 488 (goat-anti mouse, Invitrogen: A11078); and 2) in the second assay Alexa 633 (anti-mouse) and diluted to 1/500.

Before observation, cuts were spread on glass slides and mounted into Mowiol solution.

Three types of samples were used for virus localization: 1) as controls, apices from virus-free cv. Portan, without any antibody applied, infected samples of cv. Plavac mali without any antibody applied; 2) samples with primary and secondary antibody; and 3) cv. Maraština samples with primary antibody, to check and optimize the dilution of the primary antibody.

Apices were cut with a vibratome in 60 µm sections, the fixation protocol was performed and the control samples were processed as described previously.

Chlorophyll autofluorescence and virus labelling with fluorochrome Alexa Fluor ^{R} secondary antibodies 488 were excited by laser, i.e. He-Ne (543nm) and Ar (488nm) respectively, and emission (at 610 nm and 525 nm, respectively) was observed under a confocal light scanning microscope (510 Meta Confocal microscope from ZEISS). The first test was performed with cultivars Pošip and Maraština, to adjust the procedure of cutting with the vibratome (thickness of cuts) and dilution of primary antibodies. For each experiment, two controls were performed: sections without antibody, in order to confirm the absence of autofluorescence at the fluorochrome wavelength, and sections with only primary antibody, in order to adjust the dilution of the primary antibody.

In further experiments, the secondary antibody (Alexa 633, anti-mouse) was added, labelled with phosphatase-alkaline and samples were observed.

In this first test, one grapevine virus was tested (GLRaV-1) with several cultivars (Maraština, Plavac mali and Pošip). Some samples were eliminated due to problems with the sectioning of shoot tips or high signal of autofluorescence. In the following experiments, additional viruses were studied.

DAPI application protocol:

If an antibody conjugated with fluorescent dye is used, the addition of DAPI is advisable to prevent fading of the dye. The DAPI application protocol was the following: slides were incubated in secondary antibody, diluted in 5 % PBS, at 37°C for 120 min or 1 h at room temperature (humid chamber). They were rinsed thrice in PBS for 10 min, counterstaining was made with DAPI (1 µg/ml in PBS) to stain DNA (15 min), washing twice with PBS, 10 min each. Mounting of sections was performed in anti-fading reagent e.g. Citifluor (in glycerol-PBS) using nail-varnish to seal the cover slips on the slides.

3.2.2.10.2 Application of immunolocalisation protocol to international and Croatian cultivars

Immunolocalisation experiments were performed with infected Croatian cultivars, using Portan as healthy control and also with healthy and infected international cultivars. The plants were cultivated as described in Sections 3.1.1 and 3.1.2. Their sanitary status was evaluated as described in Section 3.2.1.2 and 3.2.2.6.

The immunolocalisation protocol was applied to healthy and infected cultivars of cv. Chardonnay, Cabernet Sauvignon, Merlot and Pinot Noir. Chardonnay and Cabernet Sauvignon showed high and wide autoflorescence and they were thus eliminated from this part of observations. Pinot Noir contains GFLV virus and Merlot GLRaV-3 virus.

The immunolocalisation protocol developed in the first experiment was applied. Explants were cut with a vibratome in 60 µm sections and the fixation protocol was performed. The polyclonal non-labelled anti-GFLV antibody (BIOREBA, AG Reinach, Switzerland; A120415), raised from rabbit, was added and diluted 50 fold. Monoclonal non-labelled anti-GLRaV-3 (BIOREBA, AG Reinach, Switzerland; A122315), raised from rabbit and mice, was added and diluted 50 fold. The secondary antibody was applied (Alexa 546) and diluted 500 fold. In the first experiment, during which the protocol was tested, the primary antibody was labelled. Alexa 488 was chosen as secondary antibody because it conjugated to alkaline phosphatase. In the second experiment, during which numerous cultivars were used, this step was not necessary, because we defined the window for each cultivar where autoflorescence was weak or nil. Observations were made under a confocal light scanning

microscope (510 Meta Confocal microscope from ZEISS). For each experiment, healthy parts of tissues: meristem, vascular bundles and leafs were used as controls.

In immunolocalisation experiments, different Alexa-secondary antibodies labelled with different fluorochromes were applied as grapevine shoot tips displayed high autofluorescence. When testing different cultivars, we defined for each genotype a spectral window (whenever possible), with no or low autofluorescence. The fluorescence emission of secondary antibodies applied were: 1) Alexa 488 (450-550 nm wavelength); 2) Alexa 633 (600-700 nm wavelength) and 3) Alexa 546 (550-650 nm wavelength). For example, for cultivar Pinot Noir, it was observed that there was no autofluorescence around 550 and 650 nm wavelength corresponding to the emisson spectra of Alexa 546 nm. Therefore we chose a secondary antibody labelled with Alexa 546 for the second experiment on international cultivars. However, some cultivars like Chardonnay showed a high level of autofluorescence and it was not possible to observe results of virus localization in these samples.

3.2.3 Genetic stability

3.2.3.1 Samples subjected to genetic stability experiment

The cryopreservation protocol was performed (with three PVS2 durations tested: 25, 50 and 75 min) to obtain regenerated plants which were subjected to genetic stability testing. After recovery, regenerated plants were maintained in tissue culture conditions for 4 months before samples were taken for DNA isolation. Each condition contained a number of plants that regenerated after LN exposure. Plants cultivated for 2-months were considered as control samples.

Table 5: Number of plantlets regenerated after different steps of the cryopreservation protocol and subjected to genetic stability test

Step of the cryopreservation protocol		Number of regenerated plants
Sucrose preculture	preculture on ½ Ms medium with 1M sucrose	4
LS	loading solution	5
PVS2/2	dehydration with half-strength PVS2	11
CON 25'	exposure to PVS2 for 25 min	0
FRE 25'	exposure to PVS2 for 25 min followed by LN exposure	1
CON 50'	exposure to PVS2 for 50 min	6
FRE 50'	exposure to PVS2 for 50 min followed by LN exposure	8
CON 75'	exposure to PVS2 for 75 min	3
FRE 75'	exposure to PVS2 for 75 min followed by LN exposure	0
Control	*in vitro* plants	6

3.2.3.2 AFLP protocol

The protocol employed was that designed by Vos et al. (1995).

A. Digestion

DNA digestion was performed with two different restriction enzymes, EcoRI and MseI, using the following protocol:

1. On ice add 20 µl DNA at the concentration of 50 ng μl^{-1} in 20 µl RMM mix (Table 6) and mix gently, centrifuge briefly at 7000 rpm.
2. Incubate the mix in a Thermostat Plus (Eppendorf) as follows:
   - 3 h at 37°C (restriction of DNA)
   - 15 min at 70°C (denaturation of enzymes)

Table 6: Reagents and final concentration needed per sample of restriction master mix (RMM)

Reagent	Final concentration
NEB-Buffer EcoRI (10X)	1X
BSA (100X, 10 mg ml^{-1})	1X (0,1 mg ml^{-1})
EcoRI-endonuclease (100 U μl^{-1})	5 U
MseI-endonuclease (50 U μl^{-1})	5 U

B. Ligation

Before starting the ligation protocol, solutions of adaptors EcoA and MseA were prepared

1. The Ligation master mix (LMM) was vortexed and 15 µl LMM solution were added to 35 µl digested DNA, then centrifuged briefly at 7000 rpm.
2. The mix prepared was incubated in a ThermostatPlus for 3 h at 37°C.
3. After ligation, the mixture was diluted with 150 µl T10E0.1 Buffer and placed at -20°C.

Table 7: Sequences of adaptors used in AFLP analysis

Name of adaptors		Sequences of adaptors
EcoA:	EcoA1	5' CTC GTA GAC TGC GTA CC 3'
	EcoA2	5' AAT TGG TAC GCA GTC 3'
MseA:	MseA1	5' GAC GAT GAG TCC TGA G 3'
	MseA2	5' TAC TCA GGA CTC AT 3'

Table 8: Reagents and final concentration of LMM per sample

Reagent	Final concentration
EcoA adapter (5 µM)	0.33 µM
MseA adapter (50 µM)	3.33 µM
ATP (100 mM)	0.67 mM
T4-Buffer (10X)	1X
T4-ligase (5 U μl^{-1})	0.33

C. Preamplification

For preamplification, primers with one selective nucleotide were used. The sequences of these primers are presented in Table 9.

Table 9: Name and sequences of primers

Name of primers	Sequence of primers
E01	5' GAC TGC GTA CCA ATT CA 3'
M02	5' GAT GAG TCC TGA GTA AC 3'

Preamplification was performed using 20 µl pAMM (preamplification master mix) solution (Table 10) per sample.

Table 10: Reagents and final concentration of pAMM per sample

Reagens	Final concentration
PCR-Buffer (10X)	1X
$MgCl_2$ (25 mM)	3 mM
dNTP (2 mM)	0.2 mM
E01-primer (10 µM)	0.25 µM
M02- primer (10 µM)	0.25 µM
Taq-polymerase (5 U $µl^{-1}$)	0.5 U

Five µl sample DNA after restriction and ligation were added to 15 µl pAMM solution and the mix was centifuged for 30 s at 4000 rpm.

Preamplification consisted of 20 cycles, with the following sequence: denaturation of DNA for 20 s at 94°C, overlapping the primers at 56°C for 30 s and synthesis of new DNA chains at 72°C for 2 min. This was followed by final extension for 30 min at 60°C.

After preamplification, 20 µl of solution with replicated DNA fragments were diluted in 500 µl T10E0.1 (in range 1:25) buffer and stored at -20°C.

D. Selective amplification

For selective amplification, we used the eight primer combinations presented in

Table 11.

One of the primers used was marked with a fluorescent color, making it possible to detect amplified fragments with a capillary sequencer.

Selective amplification was performed in 20 µl sAMM (amplification master mix) solution per sample (Table 12).

Table 11: Combinations and sequences of primers used in selective amplification

Marker name	Sequence
Selective markers Labelled EcoRI markers: E10 (6-FAM) E32 (VIC) E35 (6-FAM) E37 (6-FAM) E40 (VIC) *VIC – green color, FAM – blue color	 5'-GAC TGC GTA CCA ATT CACA-3′ 5'-GAC TGC GTA CCA ATT CA AC-3 5'-GAC TGC GTA CCA ATT CAT A-3′ 5'-GAC TGC GTA CCA ATT CAC G-3′ 5'-GAC TGC GTA CCA ATT CAG C-3′
Unlabelled MseI markers: M15 M16 M40 M61 M62	 5′-GAT GAG TCC TGA GTA ACA-3′ 5′-GAT GAG TCC TGA GTA AAT C-3′ 5′-GAT GAG TCC TGA GTA AGC-3′ 5′-GAT GAG TCC TGA GTA ACT G-3′ 5′-GAT GAG TCC TGA GTA ACT T-3′

Table 12: Reagents and final concentration of sAMM per sample

Reagents	Final concentration
PCR-Buffer (10X)	1X
$MgCl_2$ (25 mM)	3 mM
dNTP (2 mM)	0.2 mM
Exx-primer (10 µM)	0.25 µM
Mxx-primer (10 µM)	0.25 µM
Taq-polimerase (5 U $µl^{-1}$)	0.5 U

Five µl diluted preamplified DNA were added to 15 µl sAMM solution. The mixture was centifugated 30 s at 4000 rpm.

Selective amplification was performed in 30 cycles. The temperatures and durations of some phases of the reaction were equal to those used for preamplification with the exception of the temperature of overlapping primers, which was 66°C during the first cycle, then decreased of 1°C at each following cycle down to 56°C. The following 20 cycles were performed at 56°C.

3.2.3.3 Genomic DNA isolation

Leaves of *in vitro* plants were freeze-dried using Christ Beta 1-4 freeze dryer and stored at -20°C. For isolation of genomic DNA Macherey-Nagel NucleoSpin II Plant Kit (Macherey-Nagel, Germany) was used according to manufacter's instructions.

3.2.3.4 Observations of results

The experiments were performed using an ABI3130 Genetic Analyzer (Applied Biosystems). The GeneMapper 4.0 software was used for detection of the peaks. The detailed manual scoring of peaks was undertaken to ensure the highest possible accuracy.

A peak height of 50 RFU (Relative fluorescent units) was chosen as minimum height for peak scoring. Every amplified peak was compared between all samples and a binary matrix was constructed. The NTSys v 2.10e software was used for further analysis (Rohlf, 2000).

To test the efficiency of the AFLP method, eight control samples were used. Control samples were *in vitro* plants grown on half-strength MS medium originating from several mother plants.

Genetic polymorphism is the occurrence in the same population of two or more alleles at one locus. AFLP markers are scored as a binary matrix of presence/absence of a specific amplified fragment. If all analysed samples have a certain fragment, the fragment is monomorphic. If there are some differences between samples in presence or absence of that fragment, the fragment is polymorphic.

Simple matching (SM) is a ratio of the total number of matches to the total number of occurrences or fragments. The SM coefficient was calculated according to Sokal and Michener (1958), following the formula:

$$S_{SM}^{ij} = \frac{a+d}{a+b+c+d}$$

The method is based on comparisons between co-occurring bands (indicated by ‘ones’ in common in the data matrix) and different bands (indicated by ‘one and zero’ or ‘zero and one’) between each genotype pair. Within these matrix data obtained, genetic similarity was calculated for groups (steps) of the cryopreservation protocol.

4 RESULTS

4.1 *In vitro* establishment of Croatian cultivars

Establishing an efficient *in vitro* culture protocol for Croatian native grapevine cultivars is a prerequisite for their micropropagation and virus sanitation as well as for research work on cryopreservation and cryotherapy protocol development. The objectives of this work were to evaluate the response to *in vitro* introduction of eight economically important Croatian grapevine cultivars, and to study the impact of their sanitary status, by comparing the response to *in vitro* introduction of healthy and virus-infected genotypes of one cultivar.

4.1.1 Effect of cultivar and medium on survival and regrowth

For the evaluation of survival and regrowth, the cultivars studied were divided in three groups according to their genetic characterization (Zdunić et al., 2013). In group A, high survival (84.5-100.0%) was reached on M1 for all cultivars (Table 13). Survival of cultivar Plavac mali was above 90% whatever the culture medium used. Differences in survival were observed between the other cultivars on M2 and M3 (containing BAP). On M2, survival was lower for cultivars Debit, Grk and Lasina (57.1-61.0%). On M3, survival of cultivars Grk and Plavina was only 36.4% and 64.3%, respectively. Survival of cultivars of groups B and C was highest on M3 and lowest on M1.

Table 13: Survival (%) and regrowth (%) of nodal segments of eight native Croatian grapevine cultivars from three genetic groups after *in vitro* inoculation on three different media (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 µM BAP).

Cultivar	Genetic group*	Survival (%)			Regrowth (%)		
		M1	M2	M3	M1	M2	M3
Debit	A	100.0	61.0	87.5	42.1	61.0	83.3
Grk	A	90.9	60.0	36.4	59.0	20.0	22.7
Lasina	A	93.3	57.1	100.0	83.3	19.0	100.0
Plavac mali	A	97.5	96.0	91.7	95.0	80.0	91.7
Plavina	A	84.6	89.3	64.3	46.2	39.3	46.4
Pošip	B	82.2	96.7	100.0	80.0	80.6	100.0
Vugava	B	65.6	70.0	93.3	37.5	36.6	93.3
Maraština	C	66.6	84.3	100.0	66.6	62.5	100.0

*According to Zdunić et al. (2013).

After 8 weeks, Plavac mali was the most reactive cultivar of group A, with regrowth between 80.0 and 95.0% on the three media tested (Table 13). The lowest regrowth, between 20.0% on M2 and 59% on M1, was observed for Grk, followed by Plavina, with 39.3% regrowth on M2, 46.2% on M1 and 46.4% on M3. Regrowth of cultivar Debit was high on M2 and M3 and low on M1 (42.1%), while cultivar Lasina showed very high regrowth on M1 and M3 but only 19.0% on M2. In groups B and C, cultivar Pošip showed very high regrowth on all three media tested, while regrowth of cultivars Vugava and Maraština was lower on M1 and M2 compared to M3.

When studying the effect of cultivar, medium and cultivar*medium interaction on survival and regrowth of the eight cultivars studied, logistic regression analysis showed that only the cultivar and the cultivar*medium interaction had a significant effect on survival and regrowth (Table 14).

Table 14: Effect of cultivar and medium composition on the survival and regrowth of nodal segments of eight native Croatian grapevine cultivars, as revealed by logistic regression analysis.

	Survival			Regrowth		
Effect	DF	Chi-Square	Pr > Chi-Square	DF	Chi-Square	Pr > Chi-Square
Cultivar	7	22.4272	0.0021	7	22.4272	0.0021
Medium	2	2.5546	0.2788	2	2.5546	0.2788
Cultivar*Medium	13	25.1122	0.0223	13	25.1122	0.0223

4.1.2 Effect of cultivar and medium on shoot growth

After 8 weeks in culture, the length of shoots, the number of nodes and the length of nodes produced from inoculated nodal segments were measured. Variance analysis indicated a high level of significance both for the principal factors considered (cultivar and culture medium) and their interaction (medium*cultivar) (Table 15).

Table 15: Factorial ANOVA for the effect of cultivar, medium and their interaction on shoot length, number of nodes, and average internode length from shoots produced from nodal segments of eight native Croatian grapevine cultivars studied.

Variable	Source of variance	Degrees of freedom	Sum of Squares	Mean Square	F - Ratio	P - Value
Shoot length (cm)	Medium	2	224.8	112.4	38.94	<0.0001
	Cultivar	7	123.1	17.6	6.09	<0.0001
	Medium*Cultivar	14	368.9	26.3	9.13	<0.0001
Number of nodes	Medium	2	140.0	69.8	14.52	<0.0001
	Cultivar	7	361.3	51.6	10.73	<0.0001
	Medium*Cultivar	14	547.5	39.1	8.13	<0.0001
Average internode length	Medium	2	2.01	1.01	13.73	<0.0001
	Cultivar	7	2.73	0.39	5.33	<0.0001
	Medium*Cultivar	14	4.61	0.33	4.49	<0.0001

On average for the eight cultivars studied, significant differences in shoot length were observed between the three media tested (Table 16). M2 induced the highest average shoot length, M1 gave intermediate results while M3 gave the lowest results. On M2, differences in shoot length between cultivars were not significant, except between Maraština, which displayed the highest shoot length (7.4 cm), and Pošip, which displayed the lowest shoot length (4.4 cm). On M1, shoot length was highest for Plavac mali, intermediate for Lasina and Plavina, and lower for the other five cultivars. On M3, shoot length was highest for Plavina, Grk and Pošip, and lower for the other five cultivars.

Table 16: Effect of culture medium (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 µM BAP) on shoot length produced from nodal segments of eight native Croatian grapevine cultivars studied. One way ANOVA and means comparison using Duncan's multiple range test were performed to study the effect of cultivar and medium composition

Cultivar	Genetic group	Shoot length (cm)[a]			F value	p	Average[b]
		M1	M2	M3			
Debit	A	2.8 ± 1.2 b/C	5.6 ± 2.1 a/AB	3.3 ± 1.1 b/C	11.4	0.0002	3.9 CD
Grk	A	3.4 ± 1.3 b/C	6.2 ± 1.0 a/AB	4.4 ± 2.8 ab/AB	3.2	0.063	4.1 BCD
Lasina	A	4.7 ± 0.9 b/B	5.7 ± 2.3 a/AB	3.4 ± 0.9 c/BC	13.5	<0.0001	4.2 BCD
Plavac mali	A	6.3 ± 2.1 a/A	5.9 ± 3.3 a/AB	2.3 ± 0.7 b/D	23.0	<0.0001	5.2 A
Plavina	A	4.8 ± 3.2 a/B	5.3 ± 1.8 a/AB	4.6 ± 2.6 a/A	0.2	0.791	4.9 AB
Pošip	B	2.8 ± 1.0 b/C	4.4 ± 2.0 a/B	3.9 ± 0.9 a/ABC	14.2	<0.0001	3.7 D
Vugava	B	2.6 ± 1.0 b/C	5.2 ± 1.9 a/AB	3.0 ± 1.6 b/CD	9.2	0.0004	3.5 D
Maraština	C	3.6 ± 1.9 b/BC	7.4 ± 2.9 a/A	3.1 ± 0.6 b/CD	47.8	<0.0001	4.7 ABC
	F value	17.8	3.3	5.8			
	P	<0.0001	0.0035	<0.0001			
	Average[c]	4.1b	5.8 a	3.5 c			

[a] Different lowercase letters indicate significant differences between media for the same cultivar; different uppercase letters indicate significant differences between cultivars for the same medium
[b] Average of cultivars on three media (see Table 15 for F and p values)
[c] Average of medium for eight cultivars (see Table 15 for F and p values)

Table 17: Effect of culture medium (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 µM BAP) on number of nodes produced from nodal segments of the eight native Croatian grapevine cultivars studied. One way ANOVA and means comparison using Duncan's multiple range test were performed to study the effect of cultivar and medium composition.

Cultivar	Genetic group	Number of nodes			F value	p	Average[b]
		M1	M2	M3			
Debit	A	3.2 ± 0.4 b/D	5.3 ± 2.0 a/CD	4.1 ± 1.5 ab/D	4.3	0.021	4.3 D
Grk	A	4.9 ± 1.0 b/C	10.0 ± 2.6 a/A	6.0 ± 4.4 b/ABC	5.3	0.146	5.9 ABC
Lasina	A	6.0 ± 1.5 a/BC	5.2 ± 2.8 a/CD	7.0 ± 2.1 a/A	4.7	0.0121	6.6 AB
Plavac mali	A	7.3 ± 2.3 a/AB	6.3 ± 3.1 ab/BCD	5.3 ± 2.5 b/BCD	4.2	0.0178	6.6 AB
Plavina	A	8.3 ± 4.1 a/A	7.0 ± 2.6 a/BCD	6.5 ± 3.9 a/AB	0.5	0.589	7.1 A
Pošip	B	2.9 ± 1.2 c/D	4.6 ± 2.6 b /D	5.8 ± 1.4 a/ABCD	29.4	<0.0001	4.5 D
Vugava	B	2.6 ± 1.1 c/D	7.9 ± 3.0 a/ABC	4.7 ± 2.9 b/CD	11.0	0.0001	5.0 CD
Maraština	C	4.5 ± 2.0 b/C	8.1 ± 2.9 a /AB	4.5 ± 1.3 b/CD	31.7	<0.0001	5.8 BC
	F value	25.3	4.9	5.3			
	P	<0.0001	<0.0001	<0.0001			
	Average[c]	5.0 b	6.5 a	5.5 b			

[a] Different lowercase letters indicate significant differences between media for the same cultivar; different uppercase letters indicate significant differences between cultivars for the same medium.
[b] Average of cultivars on three media (see Table 15 for F and p values)
[c] Average of medium for eight cultivars (see Table 15 for F and p values)

The average number of nodes for the eight cultivars studied was significantly higher on M2, compared to M1 and M3 (Table 17). The number of nodes produced on M1 and M2 varied broadly between cultivars, from 2.6 (Vugava) to 8.3 (Plavina) on M1 and from 4.6 (Pošip) to 10.0 (Grk) on M2. By contrast, on medium M3, the difference between cultivars was less marked, with number of nodes between 4.1 (Debit) and 7.0 (Lasina).

Group B and C cultivars and cultivar Debit (group A) produced longer internodes compared to other group A cultivars (Table 18). Internode length was also influenced by the culture medium, with M3 (containing BAP) producing shorter ones compared to M1 and M2. On M3 hyperhydricity was observed with all cultivars (data not shown).

Table 18: Effect of culture medium (M1: half-strength MS; M2: full-strength MS; M3: full-strength MS + 4.4 µM BAP) on average length (cm) of internodes of shoots produced from nodal segments of the eight native Croatian grapevine cultivars studied. One way ANOVA and means comparison using Duncan's multiple range test were performed to study the effect of cultivar and medium composition

Cultivar	Genetic group	Internode length (cm)[a]			F value	p	Average[b]
		M1	M2	M3			
Debit	A	0.8 ± 0.2 b/BC	1.0 ± 0.2 a/AB	0.8 ± 0.2 b/A	4.0	0.0255	0.9 A
Grk	A	0.6 ± 0.1 a/DC	0.6 ± 0.0 a/C	0.8 ± 0.1 a/A	1.9	0.1686	0.7 C
Lasina	A	0.8 ± 0.2 b/BC	1.1 ± 0.1 a/A	0.5 ± 0.1 c/B	24.5	<.0001	0.6 C
Plavac mali	A	0.8 ± 0.1 a/BC	0.9 ± 0.2 a/ABC	0.5 ± 0.2 b/B	21.8	<.0001	0.7 BC
Plavina	A	0.5 ± 0.1 b/D	0.7 ± 0.1 a/BC	0.7 ± 0.1 a/A	5.9	0.0076	0.7 C
Pošip	B	1.0 ± 0.3 a/AB	1.0 ± 0.4 a/AB	0.6 ± 0.1 b/A	18.3	<.0001	0.9 A
Vugava	B	1.1 ± 0.5 a/A	0.7 ± 0.3 b/C	0.7 ± 0.4 b/A	3.3	0.0452	0.8 AB
Maraština	C	0.7 ± 0.2 ab/BC	0.9 ± 0.1 a/ABC	0.7 ± 0.1 b/A	3.8	0.0271	0.8 ABC
	F value	5.5	3.8	6.4			
	P	<0.001	<0.001	<0.001			
	Average[c]	0.8 a	0.9 a	0.6 b			

[a] Different lowercase letters indicate significant differences between media for the same cultivar; different uppercase letters indicate significant differences between cultivars for the same medium.
[b] Average of cultivars on three media (see Table 15 for F and p values.)
[c] Average of medium for eight cultivars (see Table 15 for F and p values)

Based on the three parameters studied, shoot length, number of nodes and length of internodes produced by nodal explants, differences between cultivars of the three groups identified were observed (Table 16, Table 17, Table 18). Group B cultivars (Pošip and Vugava) were the least reactive to *in vitro* culture conditions, while group A cultivars (except Debit) were the most reactive. Maraština, the only representative of group C, displayed intermediate reactivity.

A canonical discriminant analysis of shoot length, number of nodes and average length of nodes of the eight cultivars studied produced on the three media tested was performed. The first two canonical variables (CAN1 and CAN2) explained 92.7% of the total variability between cultivars grown on different media. Based on CAN1 (which explained 54.5% of total variability) and CAN2 (which explained 38.2% of total variability) a scatter plot was designed (Figure 7). Shoot length was highly positively correlated (0.93) with CAN1, while CAN2 was

highly positively correlated with number of nodes (0.88) and highly negatively correlated (0.81) with average length of nodes. Based on the position on the scatter plot of centroids of the eight cultivars grown on three different media and on the correlation of the variables studied with CAN1 and CAN2, the growth differences of the eight cultivars studied on the three media tested could be explained. Based on the position of vectors (angles between vectors), a positive correlation between node number and shoot length was observed. By contrast, there was no correlation between average internode length and shoot length. There was a low negative correlation between average shoot length and the number of nodes.

4.1.3 Effect of virus infection on *in vitro* growth parameters

In order to test the influence of sanitary status on *in vitro* growth parameters, nodal segments of virus-infected genotypes of cultivar Plavac mali were introduced *in vitro*. Genotypes with single and double virus infection were selected. The virus infection status of selected genotypes, as revealed by ELISA and RT-PCR, is presented in Table 19.

No significant difference in survival was observed between the healthy control genotype (PMC201) and infected ones, with values ranging between 82.8 and 97.5% (Table 20). By contrast, significant differences in regrowth were noted between the healthy control and infected genotypes. Almost all surviving explants of the healthy genotype displayed regrowth, while regrowth of infected ones dropped to 5.5-31.4%.

Statistically significant differences in shoot length and number of nodes were observed between healthy and infected genotypes (Table 21). Shoots of the healthy genotype reached an average length of 6.3 cm, while PMC 201/6, the most reactive infected genotype, reached only 2.6 cm. Shoots of the other infected genotypes measured between 1.2 and 2.6 cm. (Figure 6). There was no significant effect of the phytosanitary status of Plavac mali genotypes on internode length.

Table 19: Virus infection status determined by ELISA and RT-PCR (GVA) testing of selected genotypes of grapevine cultivar Plavac mali. All samples were tested for the presence of GFLV, GLRaV-1, GLRaV-3 and GVA viruses

Genotype	Virus
PMC 201	none detected
PMC 008/1	GLRaV-3 + GVA
PMC 010/1	GLRaV-1 + GLRaV-3
PMC 064/3	GLRaV – 1
PMC 225/2	GLRaV – 3
PMC 201/6	GFLV

Table 20: Effect of virus infection status on survival (%) and regrowth (%) of nodal segments of six genotypes of grapevine cultivar Plavac mali

Genotype	Virus infection	Survival (%)	Regrowth (%)
PMC 201	healthy	97.5a	95.0a
PMC 201/6	GFLV	85.1a	18.5bc
PMC 010/1	GLRaV-1 + GLRaV-3	82.8a	28.5bc
PMC 064/3	GLRaV-1	84.3a	15.6bc
PMC 008/1	GLRaV-3 + GVA	88.5a	31.4b
PMC 225/2	GLRaV-3	86.1a	5.5c

In columns, values followed by the same letter are not significantly different according to Ryan's multiple range test.

Table 21: Effect of virus infection status on shoot length (cm), number of nodes and average internodes length (cm) produced from nodal segments of six genotypes of grapevine cultivar Plavac mali

Genotype	Viral status	Shoot length (cm)	Number of nodes (n)	Average internode length (cm)
PMC 201	healthy	6.3 ± 2.1a	7.3 ± 2.3a	0.85a
PMC 201/6	GFLV	2.6 ± 1.8b	3.0 ± 1.9b	0.87a
PMC 010/1	GLRaV-1 + GLRaV-3	2.4 ± 1.7b	2.6 ± 1.6bc	0.91a
PMC 064/3	GLRaV-1	1.6 ± 0.7cb	1.7 ± 0.6cd	0.97a
PMC 008/1	GLRaV-3+GVA	1.5 ± 0.7cb	1.8 ± 1.3cd	0.86a
PMC 225/2	GLRaV-3	1.2 ± 0.4c	1.2 ± 0.5d	0.99a

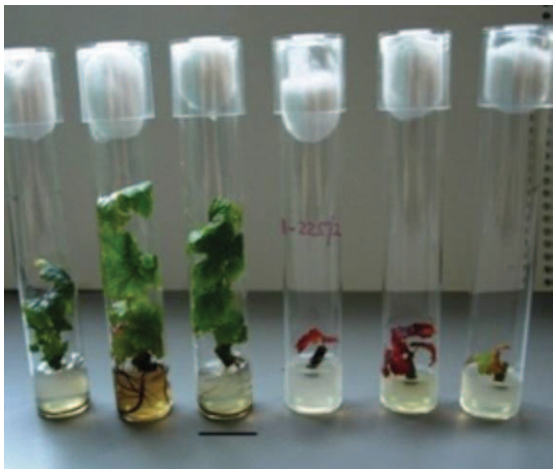


Figure 6: Shoots of healthy (three test tubes on the left) and infected genotype (three tubes on the right) of cultivar Plavac mali, 2 months after *in vitro* inoculation of nodal segments. Bar: 1 cm.

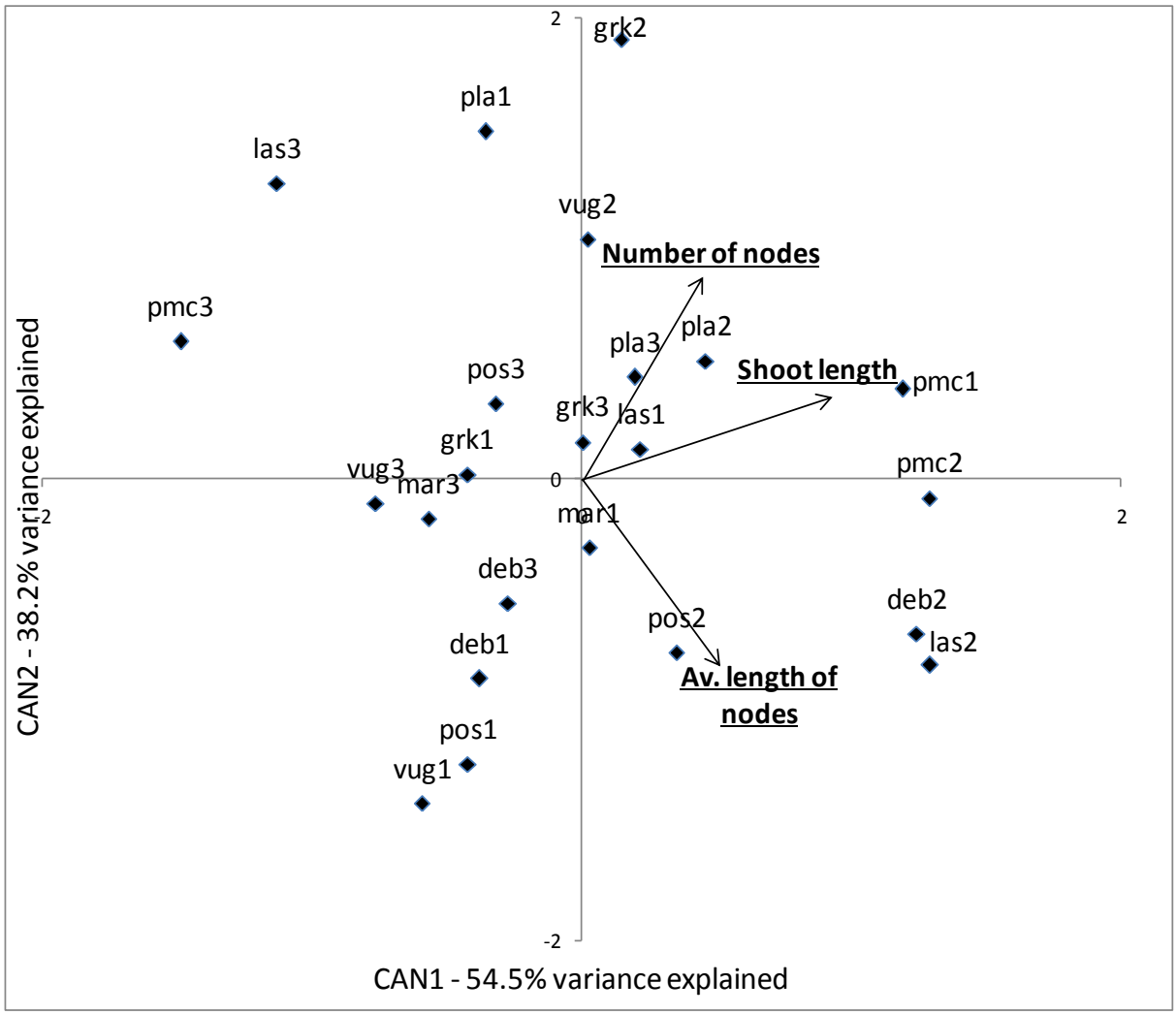


Figure 7: Position of the eight Croatian grapevine cultivars studied grown on three different media in the space defined by the first two canonical variables of the canonical discriminant analysis performed using three growth parameters (shoot length, number of nodes and average internodes length) and vector diagram of correlations between the three growth parameters studied (shoot length, number of nodes and average internode length) and the first two canonical variables. Plavac mali: pmc; Maraština: mar; Pošip: pos; Debit: deb; Grk: grk; Lasina: las; Plavina: pla; Vugava: vug. 1: M1 (half-strength MS); 2: M2 (full-strength MS); 3: M3 (full-strength MS + 4.4 µM BAP)

4.1.4 Discussion

In present study, we studied the response of eight autochthonous cultivars (Debit, Grk, Plavina, Lasina, Plavac mali, Pošip, Vugava and Maraština) essential to Croatian viticulture following their introduction *in vitro*. The results revealed that cultivar and interaction between cultivar and medium had a significant effect on survival and regrowth, whereas the effect of medium was not significant. Differences were observed between the three groups of cultivars selected according to their genetic distance (Zdunić et al., 2013). Group A cultivars showed good survival and regrowth on M1 (half strength MS medium devoid of cytokinin), while group B and C cultivars developed better on M3 (full strength MS medium with BAP). Plavac

mali yielded the best overall results while Grk had the worst performance for *in vitro* inoculation.

After 8 weeks in culture, variance analysis for shoot length and number of nodes showed a high degree of significance both for the principal factors considered (cultivar and culture medium) and their interaction (medium*cultivar). For all cultivars, the most suitable medium regarding the obtained shoot length and number of nodes appeared to be M2 (full-strength MS medium) and the least suitable M3. The canonical discriminant analysis performed showed a positive correlation between node number and shoot length, while average internodes length was not correlated with stem length. Moreover, only a low negative correlation existed between internodes length and the number of nodes. Nevertheless, according to our results some cultivars such as Grk, Plavina, Lasina and Plavac mali showed a tendency towards shorter internodes and Debit towards longer internodes. Internode elongation was influenced by the medium as well, and cultivars grown on M3 (containing BAP) usually produced shorter internodes compared with the other two media. Decrease of internode length in presence of BAP has been observed in many species (George, 1993) including *Vitis* (Goussard, 1981; 1982).

The variability observed in *in vitro* performance of the eight cultivars studied confirmed that the efficiency of *in vitro* multiplication is strongly genotype-dependent, as previously reported in *Vitis* (Galzy, 1964; Favre and Grenan, 1979; Harris and Stevenson, 1982; Chee and Pool, 1983; Galzy et al., 1990; Mayerson et al., 1994; Torregrosa and Bouquet, 1996). Some studies (Roubelakis-Angelakis and Zivanovitc, 1991) suggested that the variable efficiency of *in vitro* techniques between grapevine varieties could be linked to differences in endogenous plant regulator content and/or to different responses to exogenous growth regulators. This hypothesis is supported by our results, which showed that growth initiation for some cultivars was enhanced by the addition of BAP to the culture medium, while this was not the case for other cultivars.

Previous studies have shown that the cytokinin BAP is effective in inducing shoot development in *Vitis* (Lee and Wetzstein, 1990; Heloir et al., 1997; Alizadeh et al., 2010). At relatively low concentrations (2.5-5.0 μM), BAP enhanced bud multiplication, whereas a higher concentration (10μM) it induced hyperhydricity (Banlas and Korkas, 2007). The addition of cytokinin inhibited shoot elongation in some cultivars such as Refošk (Koruza and Jelaska, 1993). In our study, M3 (full strength MS medium supplemented with 4.4 μM BAP) enhanced survival and regrowth of cultivars, especially those of groups B and C, as well as Lasina and Plavac mali (group A). However, in most cultivars, it negatively affected growth parameters, notably by means of internode shortening and hyperhydricity.

Micropropagation of cultivars Debit, Plavac mali, Plavina, Pošip and Vugava was first performed by Hartl et al. (1989) using a MS medium supplemented with 2.85 μM indole acetic acid (IAA), 0.44 μM BAP and 3% sucrose. Hartl and Maleš (2000) reported that *in vitro* culture of cultivars Plavac mali and Vugava on MS medium with 4.4 μM BAP was suitable for inducing bud growth, but that increasing BAP concentration to 8.9 μM caused multiple shoot proliferation with compact nodal segments and abnormal leaf shape. However, these authors emphasized that growth of Plavac mali shoots was erratic and *in vitro* propagation of this cultivar was strongly limited. By contrast, in our conditions, Plavac mali was the most reactive cultivar based on survival, regrowth (regardless of medium) and shoot elongation (on medium M1 and M2); however, decrease in shoot growth and nodal segment compaction

were observed on the medium even with lower concentrations of BAP (Hartl and Maleš, 2000). The differences observed in the reactivity of the eight cultivars studied imply that, even though *in vitro* introduction was achieved with all of them, the specific requirements of each cultivar should be thoroughly tested. In accordance with our results, Péros et al. (1998) observed a high variability in the response to *in vitro* culture of 23 grapevine cultivars. Some cultivars showed excellent growth, whereas others exhibited relatively poor growth. As also observed in our study, these authors indicated that shoot length was highly positively correlated with the number of nodes.

The surveys of Croatian native cultivars performed in recent years (Poljuha et al., 2004; Zdunić et al., 2007; Karoglan Kontić et al., 2009; Vončina et al., 2009a) highlighted the serious problem of virus infestation in their populations. For cultivars showing a high percentage of vines infected with viruses having high economic impact (GFLV, GLRV-1, GLRV-3 and GVA), the initiation of a virus eradication program is urgently needed. *In vitro* techniques have been successfully used for virus eradication in *Vitis* (Staudt and Kassemeyer, 1994; Skiada et al., 2009; Youseff et al., 2009) but a prerequisite to their utilisation is the establishment of *in vitro* cultures of infected genotypes. This provided the scientific basis for our study on the influence of the most common single and double virus infections on *in vitro* introduction of infected Plavac mali genotypes. Studies by Barba et al. (1993) and Monis and Bestwick (1996) revealed that the virus titre of *in vitro* propagated grapevine tissues was higher (up to 30 times higher in some samples) than in the original actively growing vines. Correspondingly, the growth rate of infected genotypes was significantly lower than that of healthy ones (Barba et al., 1993; Monis and Bestwick, 1996). It has been reported that virus infection strongly influenced regeneration potential (Smerea et al., 2010). However, the results published on the influence of specific virus strains and virus combinations on *in vitro* growth of grapevine are not consistent. Indeed, Buciumeanu et al. (2010) observed a higher multiplication rate of GFLV-infected plants compared to healthy plants, while very poor growth of plants infected with GLRaV-1+3 was noted. By contrast, Walter (1988) and Abracheva et al. (1994) reported that GFLV caused growth reduction of *in vitro* grapevine plantlets. In our experiments comparing the *in vitro* growth of one healthy and several infected Plavac mali genotypes, all growth parameters values measured were significantly higher in the healthy genotype. Differences in growth parameters between genotypes infected with different viruses or virus combinations have been reported (Walter and Martinelli, 1997). In our conditions however, no such differences were observed between the five infected Plavac mali genotypes studied, regardless of their virus contamination status.

4.2 Cryopreservation

The availability of an efficient and reliable cryogenic protocol yielding a high recovery percentage is a prerequisite for large scale, routine application of cryopreservation (Matsumoto and Sakai, 2003).

The objective of this study was to compare the efficiency of two techniques E-D and D-V for cryopreservation of *in vitro* shoot tips of the grapevine cultivar Portan. This was the first time that D-V was tested on grapevine shoot tips. The effect of several parameters of these protocols was also studied, including sucrose pretreatment, bead MC, and size of explants for E-D and nature of the vitrification solution and duration of exposure to the vitrification solution for D-V.

4.2.1 Encapsulation-dehydration

In the first experiment, we studied the effect of bead MC on survival and recovery of grapevine shoot tips cryopreserved using the standard ED protocol. Survival and regrowth of shoot tips were high after sucrose pretreatment (Table 22). Survival and regrowth of dehydrated shoot tips decreased in line with decreasing MCs, from 90.0% (survival) and 40.0% (regrowth) at 22.3% MC to 30.0% (survival) and 10.0% (regrowth) at 16.2% MC. After cryopreservation, survival and regrowth were low, reaching similar values for three MCs tested, i.e. 20.0-32.5% for survival and 10.0% for regrowth.

In the second experiment, we studied the effect of explant size on survival and recovery of grapevine shoot tips. After sucrose pretreatment, survival and regrowth of smaller (1 mm) and larger (2 mm) shoot tips were comparable (Table 23). After dehydration, survival of larger shoot tips was higher compared to smaller explants; however, regrowth reached similar values for explants of both sizes. After cryopreservation, survival and regrowth were higher for smaller explants, reaching 45.5% (survival) and 37.1% (regrowth) for 1 mm-long shoot tips, against 24.0% (survival) and 16.0% (regrowth) for 2 mm-long shoot tips.

Table 22: Effect of bead MC (%, FWB) on survival and regrowth (%) of grapevine cv. Portan shoot tips after sucrose pretreatment, dehydration (-LN) and cryopreservation (+LN) using the standard ED protocol

Treatment	Survival (%±SD)		Regrowth (%±SD)	
	-LN	+LN	-LN	+LN
Sucrose pretreatment	100.0±0a	-	75.0±35.3a	-
Dehydration to 22.3% MC	90.0±14.1ab	20.0±0c	40.0±0ab	10.0±14.1b
Dehydration to 20.3% MC	70.8±5.9b	32.5±10.6c	29.2±5.9b	10.0±14.1b
Dehydration to 16.2% MC	30.0±14.1c	20.0±0c	10.0±14.1b	10.0±14.1b

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

Table 23: Effect of explant size on survival and regrowth (%) of grapevine cv. Portan shoot tips after sucrose pretreatment, dehydration (-LN) and cryopreservation (+LN) using the standard ED protocol.

	Survival (%±SD)				Regrowth (%±SD)			
Size of explants	1mm		2mm		1mm		2mm	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Sucrose pretreatment	100.0 ±0a	-	95.8 ±5.9a	-	75.0 ±35.4a	-	66.7 ±23.6a	-
22.3% beads MC	30.0 ±14.1b	45.5 ±5.4ab	73.3 ±18.9ab	24.0 ±1.4b	16.7 ±4.7a	37.1 ±4.2a	11.7 ±2.4a	16.0 ±0.9a

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

4.2.2 Droplet-vitrification

4.2.2.1 Establishment of base protocol

Loading and treatment of shoot tips for 30 min with half-strength PVS2 had no significant effect on their survival and regrowth (Table 24). Increasing the duration of dehydration with PVS2 decreased survival and regrowth from 60.0% for 25 min treatment to 30.0% after 75 min. After LN exposure, survival decreased from 70.0% for 25 min treatment to 30.0% after 75 min, while regrowth was 40.0% for 25 min treatment, 50.0% for 50 min and 30.0% for 75 min treatment.

In the second experiment, we compared the effect of shoot tip dehydration with PVS2 and PVS3 VSs. PVS3 was highly toxic since survival and regrowth were only 10.0% after 40 min treatment and were nil for longer durations (Table 25). No survival was achieved after LN exposure following PVS3 treatment. By contrast, with the PVS2 VS, survival and regrowth were 30.0% after 40 min treatment and low survival and regrowth (10.0%) were still noted after 120 min treatment. After cryopreservation, survival and regrowth were achieved only following a 40 min PVS2 treatment, with values reaching 40.0 % for both survival and regrowth.

Table 24: Effect of duration of dehydration with PVS2 solution on survival and regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine cv. Portan shoot tips

Treatment	Survival (%±SD)		Regrowth (%±SD)	
	-LN	+LN	-LN	+LN
Loading	100.0±0.0a	-	100.0±0.0a	-
½ PVS2 /30 min	100.0±0.0a	-	83.03±23.6ab	-
PVS2 /25 min	60.0±0.0b	70.0±14.1b	60.0±28.3bc	40.0±0.0d
PVS2 /50 min	50.0±14.1bc	50.0±14.1bc	40.0±0.0cd	50.0±14.1bcd
PVS2 /75 min	30.0±14.1c	30.0±14.1c	30.0±14.1cd	30.0±14.1cd

In columns, mean values for survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

Table 25: Effect of duration of dehydration with PVS2 and PVS3 solution on survival (%) and regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine cv. Portan shoot tips

	Survival (%±SD)				Regrowth (%±SD)			
	PVS2		PVS3		PVS2		PVS3	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Loading control	70.0 ±14.1b	-	70.0 ±14.1a	-	70.0 ±14.1a	-	70.0 ±14.1a	-
½ PVS/30 min	100.0 ±0.0a	-	60.0 ±0.0a	-	90.0 ±14.1a	-	50.0 ±14.1b	-
PVS/40 min	30.0 ±14.1cd	40.0 ±0.0c	10.0 ±14.1b	0.0b	30.0 ±14.1bc	40.0 ±0.0b	10.0 ±14.1c	0.0c
PVS/80 min	8.3 ±11.8de	0.0e	0.0b	0.0b	8.3 ±11.8cd	0.0e	0.0c	0.0c
PVS/120 min	10.0 ±14.1de	10.0 ±14.1de	0.0b	0.0b	10.0 ±14.1cd	0.0e	0.0c	0.0c

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

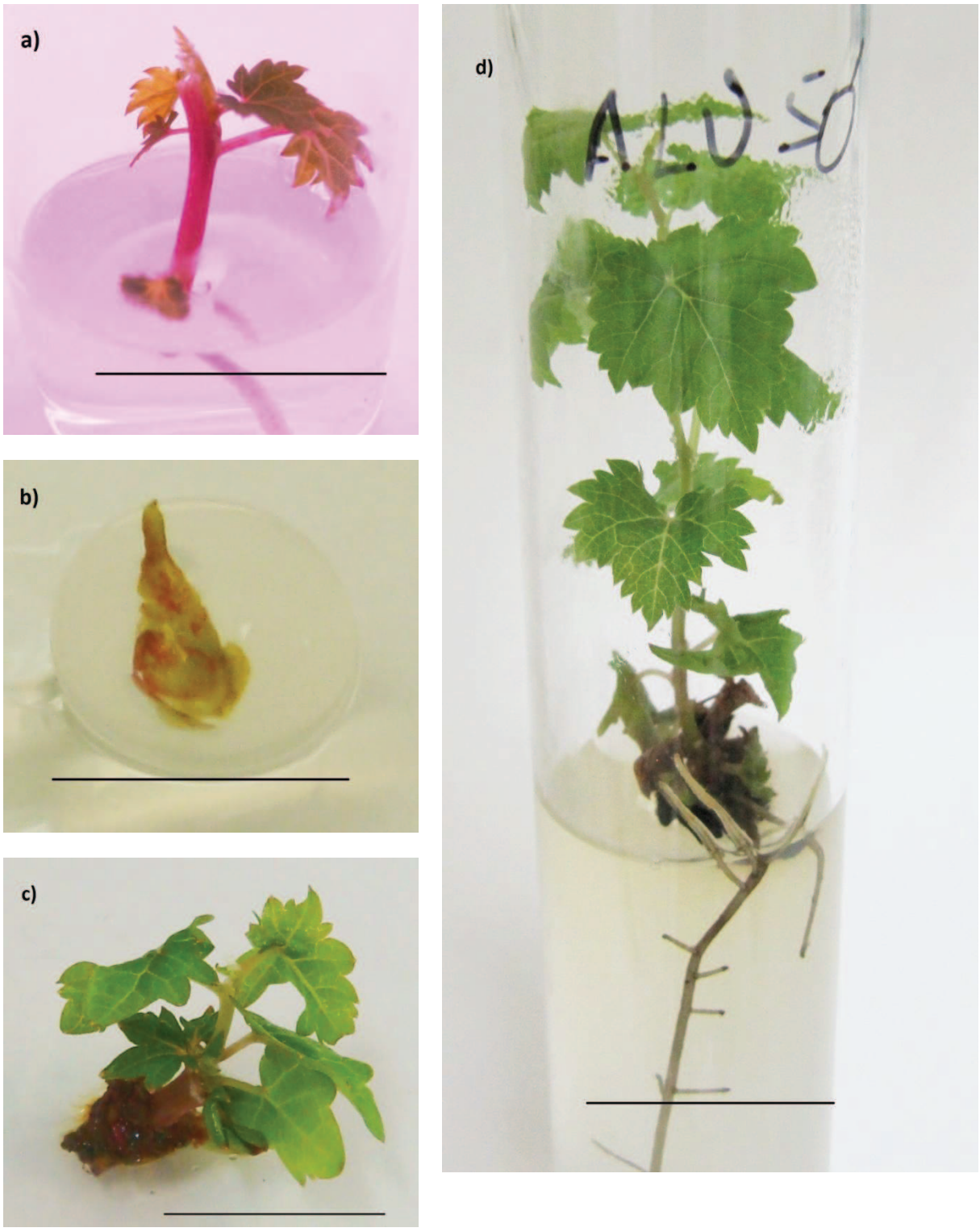


Figure 8: (a) One node microcutting before dissection of shoot tip (bar 10.0 mm).
(b) Surviving shoot tip cryopreserved by ED (dehydration to 22.3% MC) 2 weeks after rewarming (bar 4.0 mm)
(c) Recovery of shoot tip cryopreserved by DV (75 min PVS2 exposure) 6 weeks after rewarming (bar 10.0 mm).
(d) Regenerated plant 8 months after cryopreservation by DV within (50 min PVS2 exposure) (bar 10.0 mm).

4.2.2.2 Effect of physiological state of explants

4.2.2.2.1 Shooting medium

Two experiments were performed to test the effect of growth regulators in shooting medium on growth recovery of cryopreserved shoot tips. In the first experiment, microcuttings were placed on shooting medium with 1 µMol ZR and buds were excised after 2, 4 or 6 weeks. In the second experiment, microcuttings were cultured on shooting medium devoid of growth regulators or containing 1 µMol BAP or ZR and buds were excised after 2 weeks.

When buds were excised from microcuttings cultured on shooting medium with 1 µMol ZR, survival of LS and ½ PVS2 controls was very high (87.5-100.0%), whatever the microcutting culture duration tested (Table 26). With all microcutting culture durations tested, survival of non-cryopreserved explants decreased progressively with increasing PVS2 treatment durations. A similar pattern was observed with cryopreserved buds but survival values were generally lower compared to non-cryopreserved explants, especially for the shorter PVS2 treatment durations tested.

Table 26: Effect of duration of culture (weeks) of grapevine single node microcuttings on medium with 1 µMol ZR and of duration (min) of PVS2 treatment on survival (%) of non-cryopreserved (-LN) and cryopreserved (+LN) buds.

	Survival (% ±SD)			
Duration of culture on shooting medium (weeks)	0	2	4	6
Control LS 20 min	100.0 ± 0.0 a	96.9 ± 3.5 a	100.0 ± 0.0 a	97.9 ± 3.6 a
Control ½ PVS2 30 min	96.6 ± 6.6 a	98.3 ± 3.3 a	95.1 ± 6.3 ab	87.5 ± 9.6 ab
PVS2 25 min (-LN)	70.8 ± 28.4 ab	74.2 ± 19.8 b	73.0 ± 19.6 bc	71.4 ± 13.0 bc
PVS2 50 min (-LN)	51.3 ± 24.6 bc	81.6 ± 14.7 ab	50.8 ± 13.7 cde	63.2 ± 25.9 bc
PVS2 75 min (-LN)	42.2 ± 14.2 bc	41.8 ± 10.1 cd	35.0 ± 12.2 de	32.3 ± 11.9 de
PVS2 25 min (+LN)	41.6 ± 15.7 bc	44.4 ± 1.4 cd	59.1 ± 27.5 cd	58.8 ± 8.3 c
PVS2 50 min (+LN)	31.9 ± 36.9 c	53.3 ± 12.5 c	51.2 ± 10.6 cde	50.5 ± 10.7 cd
PVS2 75 min (+LN)	30.7 ± 19.3 c	33.2 ± 16.4 d	26.9 ± 16.0 e	24.4 ± 15.3 e

Figures followed by different letters are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

Regrowth of LS and ½ PVS2 controls was high whatever the microcutting culture duration employed, comprised between 61.9 and 81.5% (Table 27). Regrowth of non-cryopreserved buds decreased progressively in line with increasing PVS2 treatment durations although this decrease was more pronounced for longer (4 and 6 weeks) microcutting culture durations. After LN exposure, regrowth also decreased in line with increasing PVS2 treatment durations. Regrowth of buds cryopreserved directly after excision from *in vitro* plantlets was consistently lower, compared to buds sampled from microcuttings. The highest regrowth values, 48.1 and 40.6%, were achieved after a 25-min PVS2 treatment duration of buds sampled from 4 and 6-week old microcuttings, respectively.

Table 27: Effect of duration of culture (weeks) of grapevine single node microcuttings on medium with 1 µMol ZR and of duration (min) of PVS2 treatment on regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) buds.

	Regrowth (% ±SD)			
Duration of culture on shooting medium (weeks)	0	2	4	6
Control LS 20 min	80.3 ± 19.6 a	72.5 ± 15.4 a	81.5 ± 13.6 a	77.0 ± 29.5 a
Control ½PVS2 30 min	62.5 ± 37.8 ab	60.6 ± 9.5 ab	71.9 ± 16.0 ab	61.9 ± 29.3 ab
PVS2 25 min (-LN)	54.1 ± 21.1 abc	43.8 ± 17.6 bc	57.4 ± 17.6 bc	48.8 ± 9.6 abc
PVS2 50 min (-LN)	35.2 ± 25.9 bc	36.6 ± 15.8 c	42.6 ± 6.3 cde	44.8 ± 13.7 abc
PVS2 75 min (-LN)	36.2 ± 16.5 bc	32.1 ± 6.6 c	22.6 ± 2.1 ef	21.6 ± 2.8 c
PVS2 25 min (+LN)	25.0 ± 17.5 bc	31.8 ± 6.1 c	48.1 ± 15.6 cd	40.6 ± 12.3 bc
PVS2 50 min (+LN)	23.4 ± 25.1 c	30.4 ± 19.5 c	35.4 ± 4.5 def	32.1 ± 8.5 bc
PVS2 75 min (+LN)	22.6 ± 16.8 c	24.4 ± 12.8 c	20.1 ± 20.9 f	24.4 ± 15.4 c

Figures followed by different letters are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

When comparing shooting media devoid of growth regulators and supplemented with ZR or BAP, it appeared that the treatment had a significant effect but that the shooting medium had no significant effect on bud survival and regrowth (Table 28).

Table 28: Factorial ANOVA showing the effect of shooting medium and of treatment on survival and regrowth of grapevine buds

Variable	Source of variance	Degree of freedom	Sum of Squares	Mean Square	F - Ratio	P - Value
Survival	Shooting medium	2	0.26	0.13	1.69	0.2328
	Treatment	7	3.46	0.49	6.31	0.0050
Regrowth	Shooting medium	2	0.18	0.09	2.95	0.0984
	Treatment	7	3.68	0.52	16.48	<.0001

Survival of LS and ½ PVS2 controls was very high (90.00-100.0%) for all three shooting media tested (Table 29). Cryopreservation induced a decrease in survival for all PVS2 treatment durations tested, even though changes were statistically significant only in a few experimental conditions. In most experimental conditions, the hormonal content of the shooting medium had no effect on survival of non-cryopreserved buds. After cryopreservation, survival was significantly higher for buds sampled on shooting medium with BAP compared to ZR, for 25 and 75 min PVS2 treatments. After a 50 min PVS2 treatment, survival was similar for buds originating from ZR and BAP shooting media and significantly lower for shooting medium devoid of growth regulators.

Table 29: Effect of shooting medium containing half-strength MS without growth regulators, supplemented with benzyladenine (BAP) or zeatin-riboside (ZR) and of PVS2 treatment duration on survival (%) of control (-LN) and cryopreserved (+LN) grapevine buds

Treatment	Survival (%)		
	No GR	BAP	ZR
Control LS 20 min	90.0 a/A	90.0 a/A	100.0 a/A
Control ½ PVS2 30 min	90.0 a/A	90.0 a/A	100.0 a/A
PVS2 25 min (-LN)	-	100.0 a/A	100.0 a/A
PVS2 25 min (+LN)	-	63.6 a/AB	20.0 b/C
PVS2 50 min (-LN)	61.9 a/B	80.0 a/A	70.0 a/AB
PVS2 50 min (+LN)	21.0 b/C	66.6 a/AB	60.0 a/B
PVS2 75 min (-LN)	-	100.0 a/A	58.3 b/B
PVS2 75 min (+LN)	-	40.0 a/B	20.0 b/C

Different lowercase letters indicate significant differences between media; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$). -: not tested.

Regrowth of LS and ½ PVS2 controls was high (90.0%) for all three shooting media tested, except for ½ PVS2 controls sampled from shooting medium without growth regulators, which was 60.0% (Table 30). Regrowth of non-cryopreserved controls decreased in line with increasing PVS2 treatment durations. After cryopreservation, regrowth was highest (40.0-44.4%) after 50 min PVS2 treatment (Table 30). The presence of ZR or BAP in the shooting medium had generally no significant effect on regrowth of non-cryopreserved and cryopreserved buds. However, regrowth of control and cryopreserved buds sampled on medium without growth regulators was significantly lower compared to buds sampled on shooting medium containing BAP or ZR.

Table 30: Effect of shooting medium containing half-strength MS without growth regulators, supplemented with benzyladenine (BAP) or zeatin-riboside (ZR) and of PVS2 treatment duration on regrowth (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Treatment	Regrowth (%)		
	No GR	BAP	ZR
Control LS 20 min	90.0 a/A	90.0 a/A	90.0 a/A
Control ½ PVS2 30 min	60.0 a/B	90.0 a/A	90.0 a/A
PVS2 25 min (-LN)	-	100.0 a/A	90.0 a/A
PVS2 25 min (+LN)	-	18.8 a/B	20.0 a/B
PVS2 50 min (-LN)	9.5 b/C	30.0 a/B	40.0 a/B
PVS2 50 min (+LN)	10.5 b/C	44.4 a/B	40.0 a/B
PVS2 75 min (-LN)	-	50.0 a/B	8.3 b/B
PVS2 75 min (+LN)	-	20.0 a/B	10.0 a/B

Different lowercase letters indicate significant differences between media; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$). -: not tested

4.2.2.2.2 Duration of culture on medium with zeatine riboside

Single node microcuttings were placed on culture medium containing 1 µM ZR, and kept on this medium for 4 to 16 weeks with subcultures on new medium every 4 weeks. Control samples consisted of shoot tips, which were not cultured on medium with ZR

Recovery of cryopreserved shoot tips was achieved after up to 8 weeks of culture on ZR medium (Table 31). No regrowth was obtained after longer culture durations. The highest regrowth percentages (40-45%) were noted with shoot tips cultured on ZR medium for 4 or 8 weeks, which had been treated with PVS2 for 75 min. Regrowth of cryopreserved shoot tips was rapid and direct (Figure 9). Extended culture duration on ZR medium induced callogenesis during the development of microcuttings (Figure 10).

Table 31: Effect of culture duration on medium with ZR and of duration of exposure to PVS2 on recovery (%) of grapevine shoot tips after cryopreservation.

Culture duration on ZR medium (weeks)	Recovery (%) Duration of exposure to PVS2 (min)		
	25	50	75
0	33	6	20
4	18	20	40
8	0	30	45
12	0	0	0
16	0	0	0

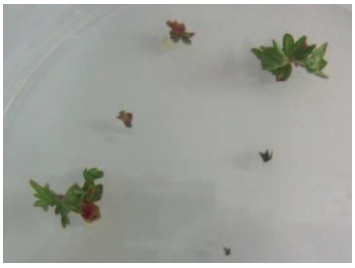


Figure 9: Regrowth of cryopreserved shoot tips treated with PVS2 for 75 min



Figure 10: Extended culture duration of shoot tips on ZR medium induced callogenesis

4.2.2.2.3 Source of material

Effect of source of material

Two experiments were performed to test the effect of source of material: 1) buds dissected from 2 month-old *in vitro* plantlets were split in three groups depending on their position on the stem and used directly for cryopreservation experiments; and 2) *in vitro* plantlets were cut into single node microcuttings, which were split in the same three groups and cultured for 2 weeks on shooting medium with 1 µMol BAP before excision and cryopreservation of buds.

Variance analysis showed that the treatment had a significant effect but that the position of buds had not a significant effect on bud survival and regrowth (Table 32). Survival of LS and ½ PVS2 controls was between 57.1-100.0% for buds of the three groups studied (Table 33). Survival of non-cryopreserved buds N° 3-4 and 6-7 remained high after the three PVS2 treatment durations tested, whereas survival of terminal buds decreased to 30.0 and 20.0% after 50 and 75 min PVS2 treatment, respectively. After cryopreservation, no survival of terminal buds was achieved after 50 and 75 min PVS2 treatment. In the case of buds N° 3-4 and 6-7, survival was comprised between 21.4 (25 min PVS2 treatment) and 50.0% (75 min PVS2 treatment).

Table 32: Factorial ANOVA showing the effect of treatment and position of buds on survival and regrowth of grapevine buds.

Variable	Source of variance	Degree of freedom	Sum of Squares	Mean Square	F - Ratio	P - Value
Survival	Position of buds	2	0.06	0.03	0.21	0.8124
	Treatment	7	4.65	0.66	4.12	0.0117
Regrowth	Position of buds	2	0.41	0.20	1.81	0.1992
	Treatment	7	2.67	0.38	3.37	0.0252

Table 33: Effect of position of bud on the stem of *in vitro* mother-plants and of PVS2 treatment duration on survival (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Treatment	Survival (%)		
	Bud N°1	Buds N° 3-4	Buds N° 6-7
Control LS 20 min	100.0 a/A	57.1 b/BCD	100.0 a/A
Control ½ PVS2 30 min	100.0 a/A	87.5 a/AB	100.0 a/A
PVS2 25 min (-LN)	100.0 a/A	100.0 a/A	50.0 b/C
PVS2 25 min (+LN)	30.0 a/B	21.4 a/D	33.3 a/BC
PVS2 50 min (-LN)	30.0 a/B	50.0 a/CD	53.3 a/C
PVS2 50 min (+LN)	0.0 b/B	46.1 a/CD	41.6 a/C
PVS2 75 min (-LN)	20.0 b/B	82.3 a/ABC	85.7 a/AB
PVS2 75 min (+LN)	0.0 b/B	46.6 a/CD	50.0 a/C

Different lowercase letters indicate significant differences between media; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$).

Regrowth of LS and ½ PVS2 controls was between 35.7-100.0% for buds of the three groups studied (

Table 34). Regrowth of non-cryopreserved apical buds decreased drastically for longer PVS2 treatment durations, reaching 10.0% after 75 min. No regrowth of cryopreserved apical buds was achieved after 50 and 75 min PVS2 treatments. As regards buds N° 3-4 and 6-7, there was no significant effect of their origin, of the duration of the PVS2 treatment and of LN exposure on regrowth. Regrowth of non-cryopreserved N° 3-4 and 6-7 buds varied between 35.7 and 41.6% and regrowth of cryopreserved buds between 21.0 and 50.0%.

Table 34: Effect of position of bud on the stem of *in vitro* mother-plants and of PVS2 treatment duration on regrowth (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Conditions	Regrowth (%)		
	Bud N°1	Buds N° 3-4	Buds N° 6-7
Control LS 20 min	100.0 a/A	50.0 b/A	100.0 a/A
Control ½ PVS2 30 min	60.0 b/B	35.7 b/A	100.0 a/A
PVS2 25 min (-LN)	50.0 a/BC	35.7 a/A	35.7 a/B
PVS2 25 min (+LN)	30.0 a/BCD	21.4 a/A	33.3 a/B
PVS2 50 min (-LN)	30.0 a/D	41.6 a/A	40.0 a/B
PVS2 50 min (+LN)	0.0 b/D	41.6 a/A	25.0 ab/B
PVS2 75 min (-LN)	10.0 b/CD	41.1 a/A	35.7 a/B
PVS2 75 min (+LN)	0.0 b/D	40.0 a/A	50.0 a/B

Different lowercase letters indicate significant differences between positions of buds; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$).

Survival and regrowth of non-cryopreserved buds sampled directly on *in vitro* plantlets or on microcuttings had similar values whatever the node from which they originated (Table 35). Explants were exposed to PVS2 for 50 min as this duration had shown the highest results in previous experiments. After cryopreservation, survival was nil or very low (9.0%) for shoot tips sampled directly on *in vitro* plantlets or on microcuttings, respectively. Survival was between 38.6-46.6% for buds N° 3-4 and 6-7 sampled directly on *in vitro* plantlets or on microcuttings. Regrowth of buds N°3-4 was higher compared to buds N°6-7 and terminal buds. After cryopreservation, no regrowth was achieved with terminal buds sampled directly on *in vitro* plantlets. Regrowth was not significantly different in the other experimental conditions. The maximal regrowth (41.6%) was achieved with buds N°3-4 sampled directly on *in vitro* plantlets.

Table 35: Effect of node number from which buds were sampled, either directly or after microcutting culture for 2 weeks, on survival and regrowth (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Node N°	Survival (%)				Regrowth (%)			
	Directly		Microcuttings		Directly		Microcuttings	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
1	30.0 b	0.0 b	30.0 b	9.0 b	30.0 b	0.0 b	20.0 b	9.0 ab
3/4	50.0 a	46.1 a	76.9 a	40.0 a	41.6 ab	41.6 a	76.9 a	20.0 ab
6/7	53.3 a	41.6 a	63.6 a	38.8 a	40.0 b	25.0 ab	27.2 b	11.1 ab

Different lowercase letters indicate significant differences between different sources of material within columns, according to Duncan's Multiple Range Test ($P < 0.05$).

4.2.2.2.4 Pretreatment with proline

Shoot developed on the different shooting media did not demonstrate notable differences in aspect (Figure 11) although leaves produced on media with the higher two proline concentrations displayed a slightly lighter green; similarly, a comparable number of shoots and nodes were produced over the range of media, although a slightly lower number of nodes were formed on the medium containing the highest proline concentration (Figure 12).

Shoots arising from the control medium and media with the lower two proline concentrations did not notably differ in their development with similar shoot length measured, whereas the highest concentration of proline inhibited shoot development (Figure 13) with a significant global difference between treatments. As no other pair-wise comparisons were significant, this could be explained by a slightly enhancing effect of proline used at 500 µM and a slightly depressive effect of the 2000 µM treatment, both not large enough to differ from the control but statistically different when compared. Thus, in our conditions, proline did not markedly alter shoot development although some toxicity could be suspected at high concentrations. Indeed, excess proline has been shown to affect negatively chlorophyll-binding proteins (Nanjo et al., 2003) which could account for the loss of coloration observed at the highest two proline concentrations. In addition, both the number of nodes formed and micro-shoot length mean value were lower (but not significantly) when explants were grown at the highest proline concentration.

After explant pretreatment with loading/PVS2 solutions with or without LN exposure, shoot tips survival and regrowth were recorded after 2 and 8 weeks (Table 36). Carry-over effects for the 2000 µM proline medium were observed since lower survival and regrowth were obtained both for LN-treated or non-treated explants excised from micro-shoots obtained on this medium. No significant differences in survival and regrowth were observed for explants subjected to pre-treatment phases without LN exposure. A slightly enhancing effect (although non-significant) on post-cryopreservation survival was observed for explants derived from shoots developed on 50 or 500µM proline, but no significant improvement of regrowth was observed for these two conditions.

Table 36: Effect of different proline shooting media (observed after 2 and 8 weeks respectively) of grapevine shoot tips subjected to a PVS2-based droplet-vitrification protocol on survival and regrowth (%) of control (-LN) and cryopreserved (+LN) grapevine buds

Shooting medium	Survival		Regrowth	
	-LN	+LN	-LN	+LN
Control	52.9	27.8	41.4	27.8
50 µM Pro	57.1	38.8	50.0	33.3
500 µM Pro	61.5	40.0	61.5	30.0
2000 µM Pro	42.8	23.0	28.5	15.4
	ns*	ns	ns	ns

*non significant (ANOVA, p=5%)

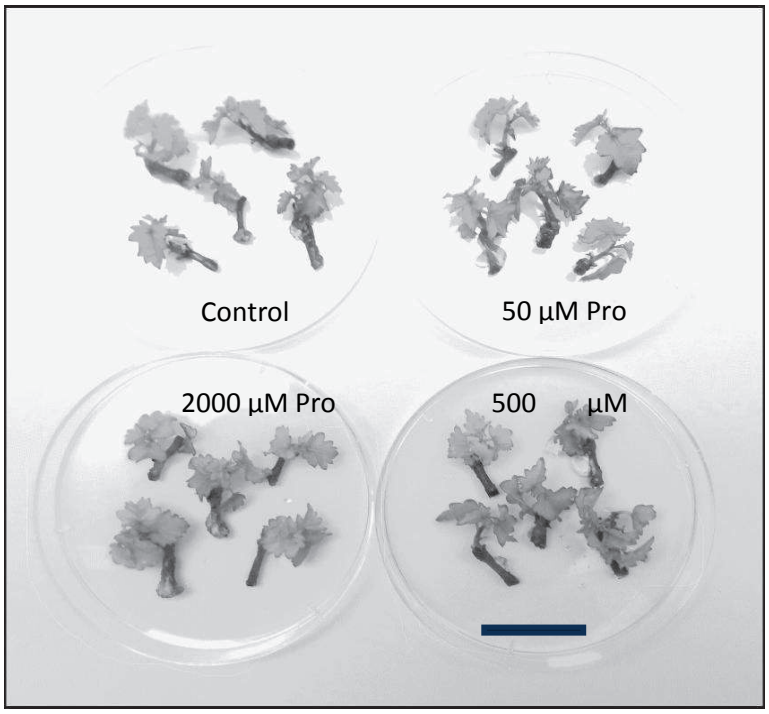


Figure 11: Representative samples of shoots developed after two weeks on different shooting media. Bar=2cm

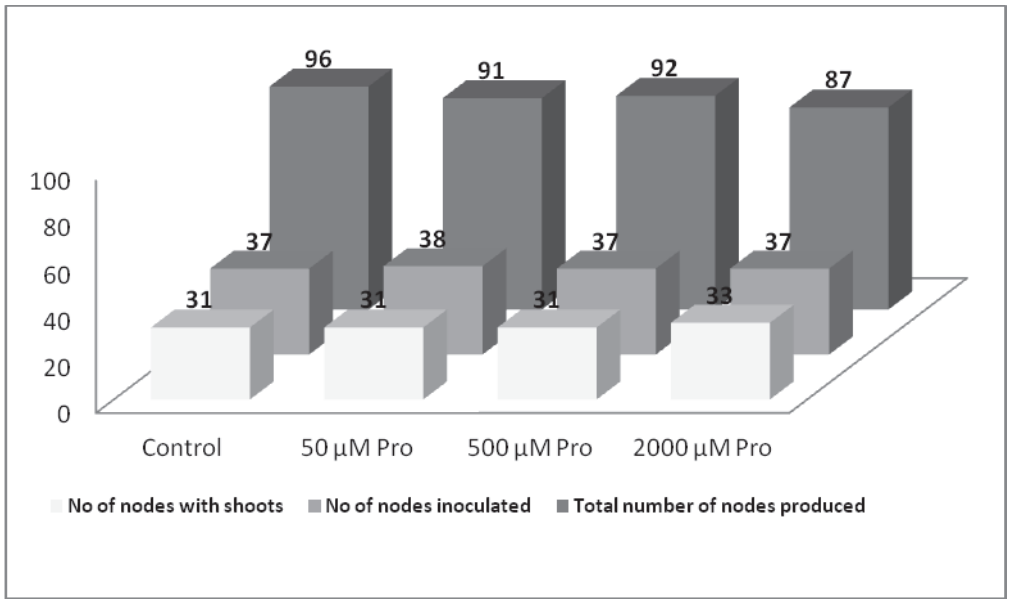


Figure 12: Number of shoots developed and total number of nodes formed two weeks after inoculation on the different shooting media.

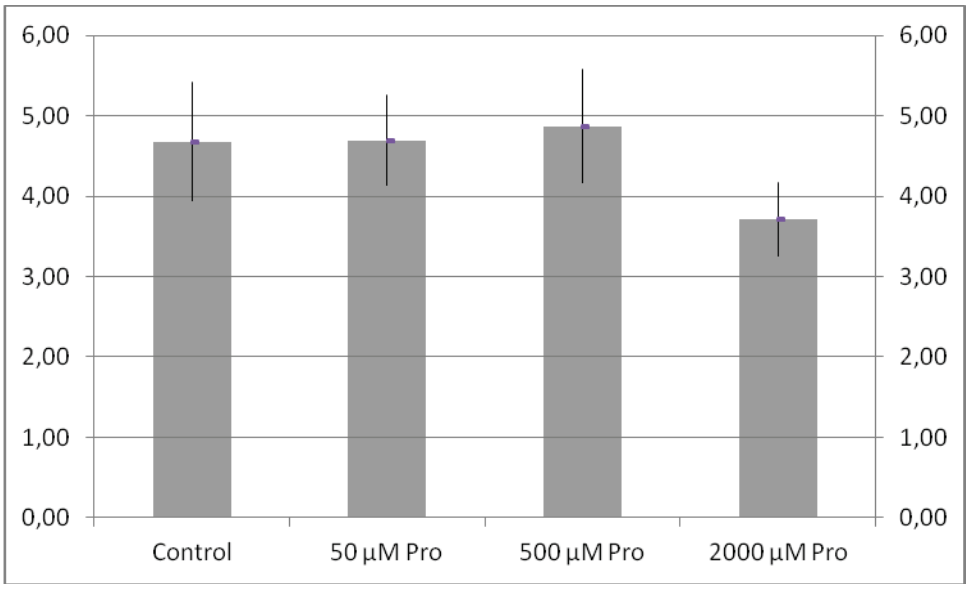


Figure 13: Mean shoot length (mm) obtained on the different shooting media with 5% confidence intervals. Means values differing by one letter are significantly different (Student's t-test, a=5%)

4.2.2.2.5 Sucrose preculture

In these experiments we compared the effect of three different sucrose preculture procedures on survival and regrowth of non-cryopreserved grapevine axillary buds and shoot tips: 1) preculture on 0.1 M sucrose medium for 24 h (base protocol); 2) preculture on 0.3 M sucrose for 3 days (Matsumoto and Sakai, 2003); and 3) preculture on 0.3 M for 24 h, 0.5 M for 24 h and 0.7 M for 8 h (Kim et al., 2009).

Only the preculture procedure had a significant effect on survival of grapevine buds (Table 37). With all three preculture procedure tested, a significant decrease in survival was observed after exposure to PVS2 solution. Survival was significantly higher following preculture according to Kim et al. (2009) compared to the base protocol and M&S procedure (Table 38). By contrast, regrowth was not significantly different after PVS2 exposure for the three preculture procedures tested (Table 39). When using Kim et al. (2009) preculture, survival and regrowth decreased in line with increasing treatment durations (Fig.14).

Table 37: One-way ANOVA showing the effect of pretreatment and condition of protocol on survival and regrowth of non-cryopreserved (-LN) grapevine buds.

Variable	Source of variance	Degree of freedom	Sum of Squares	Mean Square	F - Ratio	P - Value
Survival	Pretreatment	2	0.30	0.15	0.97	0.4061
	Condition of protocol	4	1.60	0.40	6.62	0.0072
Regrowth	Pretreatment	2	0.26	0.13	2.26	0.1469
	Condition of protocol	4	0.33	0.08	1.32	0.3275

Pretreatment – includes sucrose pretreatment or preculture with sucrose
Condition of protocol – includes each step of cryopreservation protocol (loading, half-strength PVS2 etc.)

Table 38: Effect of three sucrose pretreatment procedures (base protocol, M & S and Kim) on survival (%) of non-cryopreserved (-LN) grapevine buds

Conditions	Survival (%)			F-Value	Pr > F
	Base protocol	M & S	Kim		
Sucrose pretreatment	70.8 ab/B	100.0 a/A	89.5 a/AB	4.4	0.0156
Control LS 20 min	90.0 a/A	95.0 a/A	100.0 a/A	1.1	0.3316
Control ½ PVS2 30 min	80.0 a/A	90.4 ab/A	90.0 a/A	0.6	0.5512
PVS2 25 min	48.1 b/A	70.0 b/A	65.0 b/A	1.3	0.2800
PVS2 50 min	20.7 c/B	32.5 c/B	65.2 b/A	6.3	0.0026
F- Value	10.3	17.9	4.0		
Pr > F	<.0001	<.0001	0.0046		

Different lowercase letters indicate significant differences between conditions of the cryopreservation protocol; different uppercase letters indicate significant differences between three different sucrose pretreatment, according to Duncan's Multiple Range Test (P < 0.05).

Base protocol: sucrose preculture on 0.1 M sucrose medium for 24 h
M & S: sucrose preculture on 0.3 M sucrose for 3 days
Kim: sucrose preculture on 0.3 M for 24 h, 0.5 M for 24 h and 0.7 M for 8 h

Table 39: Effect of three sucrose pretreatment procedures (standard, Matsumoto & Sakai and Kim) on regrowth (%) of non-cryopreserved (-LN) grapevine buds

Conditions	Regrowth (%)			F-Value	Pr>F
	Base protocol	M & S	Kim		
Sucrose pretreatment	62.5 b/A	33.3 a/A	63.1 a/A	2.5	0.0873
Control LS 20 min	90.0 a/A	30.0 a/B	27.3 b/B	14.5	<.0001
Control ½ PVS2 30 min	55.0 b/A	33.0 a/A	25.0 b/A	2.0	0.1335
PVS2 25 min	48.1 b/A	35.0 a/A	35.0 b/A	0.5	0.5723
PVS2 50 min	13.8 c/A	25.0 a/A	8.6 b/A	1.5	0.2193
F- Value	9.4	0.2	4.2		
Pr > F	<.0001	0.9249	0.0033		

Different lowercase letters indicate significant differences between conditions of the cryopreservation protocol; different uppercase letters indicate significant differences between three different sucrose pretreatment, according to Duncan's Multiple Range Test (P < 0.05)

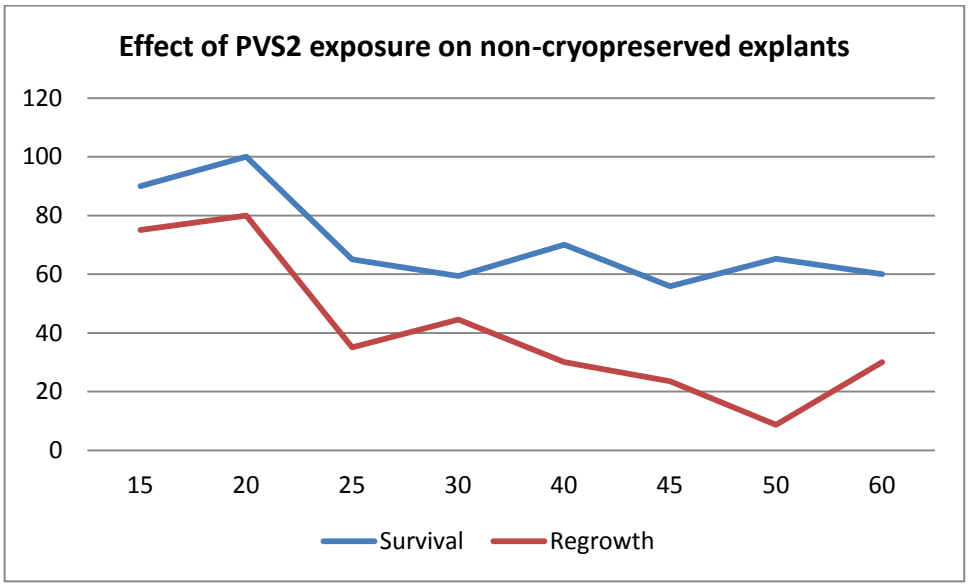


Figure 14: Effect of PVS2 exposure on non-cryopreserved explants subjected to sucrose preculture by Kim et al.(2009)

4.2.2.3 Effect of vitrification solution

4.2.2.3.1 Effect of PVS2 and PVS2-derived alternative solutions

The effect of two alternative vitrification solutions, PVS2-A3 (37.5% glycerol, 15.0% DMSO, 15.0% EG and 22.5% sucrose) and PVS2-A7 (37.5% glycerol, 10.0% DMSO, 10.0% EG and 32.5% sucrose) was compared to PVS2-A1 (30.0% glycerol, 15.0% DMSO, 15.0% EG and 13.7% sucrose), as employed in the base protocol. Experiments were performed both on axillary buds sampled directly on *in vitro* plantlets and on shoot tips sampled on microcuttings cultivated as described in Section 3.2.2.3.

A) Axillary buds

Survival of non-cryopreserved axillary buds remained relatively high after treatment with the three vitrification solutions tested (Table 40, Table 41 and 42). It decreased drastically after LN exposure with PVS2-A1 and PVS2-A7 and was nil with PVS2-A3. Regrowth of non-cryopreserved buds decreased in line with increased durations of treatment with the three vitrification solutions tested. After LN exposure, only very low regrowth was achieved with PVS2-A1 (16.7%) and PVS2-A7 (11.1%).

Table 40: Effect of treatment with VS PVS2-A1 vitrification solution on survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) axillary buds of cultivar Portan.

Treatment	Survival (%)		Regrowth (%)	
	-LN	+LN	-LN	+LN
	PVS2 – A1			
Pregrowth control	96.7±5.8ab	-	93.4±11.5a	-
Loading control	96.7±5.8ab	-	63.2±31.7b	-
PVS2 /15 min	100.0±0.0a	0.0 d	59.6±0.6b	0.0 c
PVS2 /30 min	83.3±15.3b	24.4±9.6c	76.7±15.3ab	16.7±15.3c
PVS2 /45 min	83.7±5.4b	11.3±1.4c	57.9±19.4b	0.0c

This experiment was repeated thrice containing 15 explants per conditions.

Table 41: Effect of treatment with VS-A7 vitrification solution on survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) axillary buds of cultivar Portan.

Treatment	Survival (%)		Regrowth (%)	
	-LN	+LN	-LN	+LN
	PVS2 – A7			
Pregrowth control	100.0 a	-	90.0 a	-
Loading control	90.0 a	-	30.0 b	-
PVS2 /15 min	100.0 a	0.0 c	66.6 a	0.0 b
PVS2 /30 min	88.8 a	0.0 c	22.2 b	0.0 b
PVS2 /45 min	90.0 a	44.4 b	30.0 b	11.1 b

Table 42: Effect of treatment with VS-A3 vitrification solution on survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) axillary buds of cultivar Portan.

Treatment	Survival (%)		Regrowth (%)	
	-LN	+LN	-LN	+LN
	PVS2 – A3			
Pregrowth control	100.0 a	-	100.0 a	-
Loading control	100.0 a	-	100.0 a	-
PVS2 /15 min	100.0 a	0.0 c	88.8 a	0.0 c
PVS2 /30 min	80.0 a	0.0 c	50.0 b	0.0 c
PVS2 /45 min	40.0 b	0.0 c	20.0 c	0.0 c

B) Shoot tips sampled on microcuttings

The effect of alternative solutions on survival and regrowth of shoot tips sampled from microcuttings was observed after a treatment duration of 50 min. Table 43 shows the effect of alternative solutions and treatments on survival and regrowth of non-cryopreserved (-LN) grapevine buds. Alternative solutions had a significant effect on survival, whereas the treatment had a significant effect on regrowth.

Table 43: Factorial ANOVA showing the effect of alternative solution and of treatment on survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine buds.

Variable	Source of variance	Degree of freedom	Sum of Squares	Mean Square	F - Ratio	P - Value
Survival	Alternative solution (AS)	2	6.08	0.66	4.42	0.0365
	Treatment	3	1.81	1.40	9.25	0.0019
	AS x Treatment	6	7.90	0.09	0.60	0.7234
Regrowth	Alternative solution (AS)	2	8.06	0.33	2.17	0.1568
	Treatment	3	1.87	2.32	14.86	0.0002
	AS x Treatment	6	9.93	0.06	0.44	0.8364

Alternative solution (AS) – exposure to PVS2-A1 or PVS2-A3 or PVS2-A7.
Treatment – considers each step of cryopreservation protocol.

Survival decreased after a 50 min exposure to solutions PVS2-A1 and PVS2-A3 but was not significantly different without and with cryopreservation. In case of solution PVS2-A7, survival of non-cryopreserved shoot tips remained high (80.0%). It decreased to 60.0% after LN exposure.

No regrowth was achieved of non-cryopreserved and cryopreserved shoot tips following treatment with vitrification solution PVS2-A7. In case of solutions PVS2-A1 and PVS2-A3, regrowth was observed not significantly different before LN exposure, reaching 26.5% and 20.7% for PVS2-A1 and PVS2-A3, respectively, and after LN exposure, reaching 13.3% and 12.9%, respectively.

Table 44: Effect of treatment with vitrification solution PVS2-A1, PVS2-A3 or PVS2-A7 on survival of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of cultivar Portan.

Conditions	Survival (%)		
	PVS2-A1	PVS2-A3	PVS2-A7
Loading control	100.0 a/A	90.0 a/A	100.0 a/A
Control ½ PVS2 30 min	100.0 a/A	75.0 a/B	95.0 a/A
-LN	47.0 b/B	34.5 b/B	80.0 ab/A
+LN	33.3 b/B	12.9 b/B	60.0 b/A

Different lowercase letters indicate significant differences between conditions of the cryopreservation protocol; different uppercase letters indicate significant differences between three different cryoprotective solutions, according to Duncan's Multiple Range Test (P< 0.05).

Table 45: Effect of treatment with vitrification solution PVS2-A1, PVS2-A3 or PVS2-A7 on regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of cultivar Portan.

Conditions	Regrowth (%)		
	PVS2-A1	PVS2-A3	PVS2-A7
Loading control	100.0 a/A	90.0 a/A	60.0 a/B
Control ½ PVS2 30 min	100.0 a/A	75.0 a/B	75.0 a/B
-LN	26.5 b/A	20.7 b/AB	0.0 b/B
+LN	13.3 b/A	12.9 b/A	0.0 b/A

Different lowercase letters indicate significant differences between conditions of the cryopreservation protocol; different uppercase letters indicate significant differences between three different cryoprotective solutions, according to Duncan's Multiple Range Test (P< 0.05).

4.2.2.3.2 PVS3 and PVS3-derived alternative solutions

This experiment was performed using buds sampled directly from 2-month old *in vitro* plantlets. We compared the effect of PVS3-B1 (50.0% sucrose + 50.0% glycerol) and of two PVS3-derived alternative solutions developed by Kim et al. (2009.), solution PVS3-B3 (45.0% glycerol + 45.0% sucrose) and PVS3-B4 (40.0% glycerol + 50.0% sucrose).

Survival of non-cryopreserved buds decreased progressively with increasing treatment durations (Table 46). After cryopreservation, no survival was achieved for a 60 min treatment duration and no recovery for a 180 min duration. Low survival and recovery were achieved with PVS3-B1 and PVS3-B3 after 120 min treatment.

Table 46: Effect of treatment with vitrification solution PVS3-B1, PVS2-B3 or PVS2-B4 and of treatment duration on survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) axillary buds of cultivar Portan.

Experiment	PVS3-B1		PVS3-B3		PVS3-B4	
Condition	Surv.%	Regr.%	Surv.%	Regr.%	Surv.%	Regr.%
Sucrose PC control	100.0	100.0	100.0	100.0	100.0	100.0
LS 20min	100.0	100.0	100.0	100.0	100.0	29.4
-LN 60'	70.0	30.0	80.0	47.0	93.0	47.0
+LN 60'	0.0	0.0	0.0	0.0	0.0	0.0
-LN 120'	40.0	20.0	45.0	18.0	87.0	33.0
+LN 120'	40.0	20.0	9.0	9.0	6.7	0.0
-LN 180'	30.0	20.0	73.0	54.0	47.0	27.0
+LN 180'	20.0	0.0	0.0	0.0	13.0	0.0

4.2.3 Cryopreservation on Croatian and international cultivars

Cryopreservation protocol was first tested on Croatian cultivars during the establishment of the laboratory in Zagreb. One infected genotype of Plavac mali (containing GLRaV-1 virus) and two infected genotypes of Maraština (MAR 034, containing GLRaV-3 and MAR 058, containing GLRaV-3+GVA) were subjected to droplet-vitrification protocol using three PVS2 treatment durations (25, 50 and 75 min).

The second cryopreservation experiment was performed with four Croatian cultivars, Plavac mali, Maraština, Pošip and Škrlet using the base protocol.

4.2.3.1 Cryopreservation of Croatian cultivars

Table 47: Survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) axillary buds of virus infected genotypes of Plavac mali and Maraština

Results; *during laboratory establishment		Survival (%)		Regrowth (%)	
		-LN	+LN	-LN	+LN
PMC 095/4 (GLRaV-1)	LS 20'	100.0	-	80.0	-
	PVS2half 30'	100.0	-	80.0	-
	PVS2 25'	66.0	28.0	33.0	14.0
	PVS2 50'	0.0	0.0	0.0	0.0
	PVS2 75'	0.0	0.0	0.0	0.0
MAR 034 (GLRaV-3)	LS 20'	100.0	-	80.0	-
	PVS2half 30'	70.0	-	30.0	-
	PVS2 25'	30.0	50.0	10.0	10.0
	PVS2 50'	60.0	25.0	0.0	0.0
	PVS2 75'	41.0	27.0	0.0	0.0
MAR 058 (GLRaV-3+GVA)	LS 20'	100.0	-	90.0	-
	PVS2half 30'	40.0	-	13.0	-
	PVS2 25'	21.0	10.0	14.0	0.0
	PVS2 50'	0.0	0.0	0.0	0.0
	PVS2 75'	0.0	0.0	0.0	0.0

*Experiments were performed before the establishment of the base cryopreservation protocol with cv. Portan

The base cryopreservation protocol was tested with four Croatian cultivars, Plavac mali, Maraština, Pošip and Škrlet. Relatively high survival and regrowth were obtained after treatment with the loading solution and the ½ strength PVS2 solution. However, treatment with the PVS2 solution induced a drastic decrease in survival and recovery. After cryopreservation, very low survival and recovery were obtained with only two (Plavac mali and Maraština) of the four cultivars tested.

Table 48: Effect of PVS2 treatment duration on survival and regrowth of non cryopreserved (-LN) and cryopreserved (+LN) shoot tips of four Croatian autochthonous cultivars

Genotypes	Condition	Survival (%)		Regrowth (%)	
		-LN	+LN	-LN	+LN
Plavac mali	LS 20'	40.0	-	40.0	-
	PVS2half 30'	60.0	-	60.0	-
	PVS2 25'	30.0	10.0	30.0	10.0
	PVS2 50'	0.0	0.0	0.0	0.0
	PVS2 75'	20.0	0.0	20.0	0.0
Maraština	LS 20'	80.0	-	80.0	-
	PVS2half 30'	60.0	-	60.0	-
	PVS2 25'	-	-	-	-
	PVS2 50'	44.0	22.0	33.0	11.0
	PVS2 75'	-	-	-	-
Pošip	LS 20'	100.0	-	85.0	-
	PVS2half 30'	83.0	-	83.0	-
	PVS2 25'	0.0	0.0	0.0	0.0
	PVS2 50'	0.0	0.0	0.0	0.0
	PVS2 75'	0.0	0.0	0.0	0.0
Škrlet	LS 20'	-	-	-	-
	PVS2half 30'	-	-	-	-
	PVS2 25'	-	-	-	-
	PVS2 50'	22.0	15.0	22.0	0.0
	PVS2 75'	-	-	-	-

The trials were performed on approximately 10-15 explants per experimental condition

4.2.3.2 Cryopreservation on international cultivars

Survival of non-cryopreserved and cryopreserved shoot tips of the four international cultivars tested was generally high (Table 49). However, no regrowth was achieved with control and cryopreserved shoot tips of cultivar Pinot noir. With the three other cultivars tested, intermediate (Chardonnay) to high (Merlot) regrowth was achieved before and after cryopreservation. Similar results were obtained with healthy and virus infected cultivars.

Table 49: Survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) of shoot tips sampled from healthy and infected *in vitro* plantlets of four international cultivars. PVS2 treatment duration 50 min

Treatment	Survival (%)		Regrowth (%)	
(PVS2 50')	-LN	+LN	-LN	+LN
Pinot Noir, healthy	76.6	47.7	0	0
Pinot Noir, infected	90.0	37.9	0	0
Chardonnay, healthy	77.5	61.4	25	30
Chardonnay, infected	69.4	50.9	55.6	30.7
Cabernet S., healthy	75.0	57.7	35.0	46.6
Cabernet S., infected	86.9	61.8	62.3	41.6
Merlot, healthy	90.0	75.0	90.0	70.0
Merlot, infected	85.5	67.9	79.2	61.0

Sanitary status of infected genotypes is presented in Table 50.

4.2.4 Discussion

In the present work, we first compared the efficiency of ED and DV techniques for cryopreserving shoot tips of grapevine cv. Portan. Recovery of cryopreserved samples was achieved with both techniques; however, DV, which was used for the first time with grapevine shoot tips, produced higher regrowth. Fifty % regrowth was obtained with DV following 50 min exposure of shoot tips to PVS2. No regrowth was achieved following treatment of shoot tips with PVS3 vitrification solution. By comparison, with ED, 37.1 % regrowth after LN exposure was achieved using 1 mm-long shoot tips and only 16.0 % with 2 mm-long shoot tips.

The ED technique comprises several successive steps, the conditions of which must be optimized to achieve maximal recovery after cryopreservation. Preculture of beads with progressively increasing sucrose concentration has produced improvement in recovery percentage (Plessis et al.,1993; Wang et al., 2000; 2003b). In our case, sucrose preculture reached high values in survival (95.8-100.0), as well as in regrowth percentage (66.7-75.0). Explant dehydration is one of the crucial steps in this protocol. In general, the bead MC, which ensures highest regrowth after cooling in LN is approx. 20%, which corresponds to the amount of unfreezable water in the samples (Benelli et al., 2012). This value may vary depending on the species and explant type. In the case of grapevine, Plessis et al. (1993) achieved 20% survival of cryopreserved shoot tips following dehydration to 25 % MC, Wang et al. (2000) achieved 59.3 % survival after dehydration to 16.1% MC, and Zhao et al. (2001) noted 40% survival after dehydration to 21% MC. In our work, we did not define an optimal MC for cryopreserving grape shoot tips and regrowth after cryopreservation was lower, compared with the results of Plessis et al. (1993) and of Wang et al. (2000). Indeed, we

obtained a similar, low regrowth of 10 % after LN exposure following dehydration to three MCs, 22.28%, 20.26% and 16.1%. These results may be due to the high sensitivity of cv. Portan shoot tips to the ED technique, and/or to the fact that the preculture and dehydration conditions selected were not optimal.

An important parameter of cryopreservation protocols is the size of shoot tips employed as experimental materials. Explants must be of a sufficiently small size to avoid the occurrence, in too large specimens, of gradients between external and more internal cell layers during cryoprotection, cooling and warming, which would be detrimental to survival. When cryopreserving pear shoot apices using the ED technique, Scottez (1993) showed that smaller shoot tips, of a size comprised between 1-2 mm, produced higher recovery compared to those of the two other categories tested, which had a size of 3-4 and 5-6 mm. In the case of grape, Wang et al. (2003b) compared the survival after cryopreservation of shoot tips of four different sizes (0.5, 1.0, 1.5 and 2.0 mm). The highest survival (65%) was obtained with 1.0-1.5 mm-long shoot tips, while larger or smaller shoot tips produced lower survival. We obtained comparable results in our experiments, with the smaller (1 mm long) shoot tips employed producing significantly higher regrowth, compared with larger ones (2 mm long).

In the DV technique, explants are rapidly dehydrated with highly concentrated vitrification solutions, resulting in the vitrification of internal solutes upon cooling in LN (Sakai and Engelmann, 2007). Because of their high concentration in cryoprotective agents, these vitrification solutions are often highly toxic. Optimizing the duration of exposure of samples to these solutions is therefore of paramount importance to achieve high regrowth after cryopreservation (Reed, 2008). The optimal duration of exposure to vitrification solutions is associated with the size and structure of the excised shoot tips and appears to be highly species-specific at the same temperature. Ganino et al. (2012) showed on grapevine shoot tips that even exposure to vitrification solutions, not followed by storage in LN, markedly affected shoot tip survival. After a 30 min exposure, regrowth percentage of shoot tips decreased from 94 % (control) to 57 %, and dropped to 15 % when the treatment was prolonged up to 60 min. Optimal treatment durations with PVS2, which contains the penetrating cryoprotectants DMSO and EG, are in the magnitude of 30-60 min, while longer exposures are generally required with PVS3, which contains only the non-penetrating cryoprotectants sucrose and glycerol (Sakai and Engelmann, 2007). In our experiments, the optimal PVS2 treatment duration was 50 min, which was comparable to the results obtained by Matsumoto and Sakai (2003) with grapevine shoot tips cryopreserved with the vitrification technique.

For plant materials, which are highly sensitive to PVS2, dehydration with the PVS3 VS, which does not contain penetrating cryoprotectants and is generally less toxic, often produces high regrowth after cryopreservation. This was the case with chrysanthemum shoot tips, which showed significantly higher regrowth after cryopreservation following PVS3 dehydration, compared with PVS2 dehydration (Kim et al., 2009). By contrast, shoot tips of other plant species such as garlic and sugarcane proved to be equally tolerant to dehydration with PVS2 and PVS3 (Kim et al., 2009; Barraco et al., 2011). Our experiments showed that grapevine shoot tips had an original behaviour, as they were extremely sensitive to PVS3 exposure. It can be hypothesized that grapevine shoot tips are highly sensitive to the high concentrations of sucrose and/or glycerol present in the PVS3 VS.

We studied the effect of growth regulators (type and duration of exposure) in shooting medium on grapevine shoot tip recovery after cryopreservation.

We used a shooting medium in order to produce more homogenous and vigourously growing shoots. In our conditions, preliminary experiments had shown that explants developed more homogeneously and more rapidly when growth regulators were included in the shooting medium (data not shown). When comparing shooting media supplemented with ZR or BAP at the same concentration, no significant difference was observed on regrowth of cryopreserved buds after the optimal PVS2 treatment duration, which suggested that the cytokinin type did not influence explant response. However, with that same optimal PVS2 treatment, a significant difference in regrowth was observed between media with and without cytokinin, indicating that cytokinins, even at the low concentrations employed, afforded some specific protection against LN exposure. This is in contrast with results obtained on *Anisoganthos* shoot tips by Turner et al. (2001.) who demonstrated that cytokinins had no effect on cryopreservation success when added at the preculture stage, although they positively affected post-cryopreservation regrowth when combined to gibberellic acid (GA3) in the recovery medium. On the other hand, Petijová et al. (2012.) stated that, whereas short-term pretreatment with BAP did not modify *Hypericum perforatum* shoot tip response to cryopreservation, its use prior to LN exposure in combination to abscisic acid (ABA) decreased post-cryopreservation recovery.

In our experiments, the duration of the growth phase of microcuttings on shooting medium had little influence on explant survival and regrowth after LN exposure. To the best of our knowledge, no other report on the influence of microcutting culture duration before cryopreservation has been published. When comparing buds taken directly from grapevine *in vitro* plantlets and from microcuttings, buds displayed generally lower survival and regrowth before and after LN exposure.

Our study demonstrated that microcutting culture duration on ZR affected shoot tip recovery percentage after cryopreservation. Shoot tips should be sampled on microcuttings cultured on ZR medium for 0-8 weeks. Although a stimulatory effect of preculture cannot be undoubtedly demonstrated from these experiments, it can be remarked that the highest recovery percentages obtained in this study were achieved after one or two preculture cycles on ZR-containing medium.

Grapevine is a species which displays a strong apical dominance. Axillary buds in grapevine are organized as bud complexes (with several distinct meristems) (Huglin, 1986). Buds located at different levels on a stem are at different physiological stages (George, 1993). In our experiments, shoot tips completely failed to regrow after PVS2 exposure durations longer than 50 min. Buds N° 6-7 reached the highest regrowth after cryopreservation following a 75 min exposure to PVS2. As regards buds N° 3-4 and 6-7, there was no significant effect of their original position on survival and regrowth after PVS2 exposure.

The effect of the position of buds on the stem of donor *in vitro* plantlets on their recovery after cryopreservation has been tested in carnation, grape and potato (Dereuddre et al., 1988; Plessis et al., 1993; Halmagyi et al., 2005). In the case of carnation, the lower the position of axillary buds on the stem (starting from the terminal bud) the lower their regrowth after LN exposure, with regrowth values ranging from 90% for the former to less than 10% for the latter (Derreudre et al., 1998). Similar results were obtained with potato (Halmagyi et al.,

2005). By contrast, Plessis et al. (1993) did not observe any effect of bud position on the stem on their regrowth after cryopreservation. Our results are in accordance with those of Dereuddre et al. (1988) and of Halmagyi et al. (2005) and differ from those of Plessis et al. (1993). The differences noted between our results and those of Plessis et al. (1993) may be due to the fact that experiments were performed with different varieties, and possibly with plant materials which had been multiplied for different durations since their introduction *in vitro*. Also, mother plant physiology could have been different, notably in terms of culture conditions (type of vessel and gazeous exchanges) and duration of donor plant culture.

In our experiments with proline, although a slight increase in survival could be observed, no significant beneficial effect of proline pretreatment on post-cryopreservation regrowth could be evidenced in our conditions. The 2-week period before explant excision could have allowed at least partial catabolism of exogenous proline back to glutamate (Szabados and Savouré, 2010). Exposure to exogenous proline at higher (45 mM) concentration has also been shown to modify the endogenous amino acid pool (Chen et al., 2011). We therefore cannot rule out the fact that interpretation of the results observed in our conditions could have been made difficult as a result of complex interactions. Shorter exposures to proline can then be envisaged in order to reduce proline catabolism and better evidence its effect proper. The actual rate of proline uptake by *in vitro* grapevine explants is not known, but endogenous proline content of *Arabidopsis* seedlings increased almost 100-fold within 24 h when they were treated with 45 mM proline (Chen et al, 2011) and a 6-h treatment with 20 mM proline was sufficient to effectively reduce oxidative stress in grapevine leaves excised from *in vitro* plants (Ozden et al., 2009). It is therefore likely that shorter proline treatments applied just before exposure to LN (i.e. in the 24-h period after explant excision and before explant pre-treatment with vitrification solutions) could evoke a clearer response as to the possible protective effect of proline during grapevine *in vitro* shoot tips cryopreservation.

The results obtained in this study underlined the paramount importance of the physiological state of the plant material for cryopreservation success. Actively growing buds sampled from microcuttings displayed higher regrowth compared to buds sampled directly on *in vitro* plantlets. The addition of growth regulators to the shooting medium had a positive effect on regrowth after LN exposure. We also confirmed that the position of buds on the stem of *in vitro* mother-plants affected regrowth after cryopreservation. Further experiments should include testing the results obtained with additional grapevine varieties. An histological comparison of buds displaying different responses after cryopreservation would provide a more accurate view of key factors involved in grapevine tolerance to cryopreservation.

The most commonly employed VSs are PVS2 (Sakai and Engelmann, 2007) and PVS3 (Nishizawa et al., 1993), which have been developed by the late Prof. Sakai's group. PVS2 is considered more efficient but also more toxic to sensitive materials, compared to PVS3 (Sakai et al., 2008). Recently, Kim et al. (2009) have developed a series of alternative VSs, some of which proved more efficient than the original PVS2 and PVS3. When applied to sensitive *Chrysanthemum* shoot tips, some of the PVS3-based VSs led to increased recovery compared to the original PVS3 solution. Contrarily to the observations of Kim et al. (2009), our results showed that the PVS2- and PVS3-based alternative solutions tested did not lead to an increased recovery of grapevine shoot tips, the original PVS2 consistantly providing the best results.

The base cryopreservation protocol established was applied to four Croatian and four international cultivars. In case of Croatian cultivars, very low recovery was achieved with only two of the four cultivars tested. This low success might be due to the fact that cryopreservation experiments were performed using cultures which had been introduced *in vitro* very recently and which were thus not adapted to *in vitro* culture conditions. Another possibility could be that the *in vitro* culture conditions employed were not adapted to the multiplication of these Croatian cultivars. Much higher success was achieved with three of the international cultivars (Chardonnay, Merlot and Cabernet Sauvignon). The regrowth percentage was between 30-70%, which was comparable to the results obtained by Matsumoto and Sakai (2003.) on 10 *Vitis* cultivars, which produced an average recovery of 64%. By contrast, cultivar Pinot Noir shoot tips completely failed to recover after cryopreservation. This illustrates the fact that the response to cryopreservation can be genotype-specific, and that different conditions for the same protocol or different protocols may have to be used to achieve cryopreservation of different genotypes (Reed 2011). Another important result was that the health status of the plants cryopreserved had no effect on their cryopreservation response, healthy and infected shoot tips providing similar regrowth percentages after LN exposure.

4.3 Virus eradication – cryotherapy

In vitro accessions of four cultivars: Chardonnay, Cabernet Sauvignon, Merlot and Pinot Noir were cultivated in INRA's *in vitro* laboratory. ELISA tests were used to evaluate the sanitary status of these cultivars. Chardonnay and Pinot Noir were found infected with GFLV (Grapevine fanleaf virus) virus and Merlot and Cabernet Sauvignon with GLRaV-3 (Grapevine leaf roll-associated virus-3) (Table 50). Cuttings from IFVV screenhouses taken from a plant of known viral status (infected or not infected) were introduced *in vitro* to obtain at least fifty plants for each non-infected and infected accession. They were used as stock plants for virus eradication experimentation. All infected accessions/cultivars were subjected to cryotherapy during the cryopreservation experiments.

Table 50: Sanitary status of accessions subjected to cryotherapy

Genotype	Code in grapevine collection	Sanitary status
Chardonnay	E313	GFLV
Pinot Noir	E95	GFLV
Merlot	E124	GLRaV-3
Cabernet Sauvignon	E88	GLRaV-3

ELISA tests for two grapevine viruses (GFLV and GLRaV-3) on initially infected plants regenerated after cryotherapy have been performed with chemicals from Agritest producer. The results of sanitary status are presented in Table 50 and details are explained in Table 51. Chardonnay and Cabernet Sauvignon regenerated after cryopreservation experiment and these cultivars were subjected to test of virus eradication. The efficiency of virus elimination resulted in 82.4% for non-cryopreserved samples and 77.8% for cryopreserved samples for Chardonnay cultivar containing GFLV virus. The virus elimination was completely efficient eliminating GLRaV-3 virus in cultivar Cabernet Sauvignon in non-cryopreserved and cryopreserved samples.

Table 51: Results of ELISA tests for two grapevine viruses (GFLV and GFLaV-3) performed on plantlets regenerated from control and cryopreserved buds

		Number of samples for ELISA virus testing		% of negative (healthy) samples	
Condition		-LN 50'	+LN 50'	-LN 50'	+LN 50'
Cultivar	Virus				
Chardonnay	GFLV	17	9	82.4	77.8
Cabernet Sauvignon	GLRaV-3	15	16	100	100

4.3.1. Immunolocalization

4.3.1.1 Protocol optimization

During the first trials, we faced difficulties with cutting of samples. Buds excised from *in vitro* grapevine plantlets were around 1.5 cm in length, containing one bud with a portion of stem, which was cut diagonally to ensure good embedding into agarose. The initial agarose concentration tested was 6%, which was not high enough to properly maintain the buds during cutting with the vibratome. This problem was overcome by increasing the agarose concentration to 7%. The defined procedure of bud preparation for agarose embedding following fixation is presented in

Figure 15. Buds need to be excised from 2-4 month old plantlets, i.e. at a stage where they have a well defined meristem and two leaf primordiums (otherwise tissues are too aqueous). Then, buds were subjected to fixation with paraformaldehyde and PBS buffer. After fixation, they were left in 70% ethanol for several days. Dried buds were carefully immersed in agarose and placed in the upper layer of the agarose blocks. Afterwards, pyramidal agarose blocks containing one bud, were cut and glued on the metal surface of the vibratome holder for cutting.

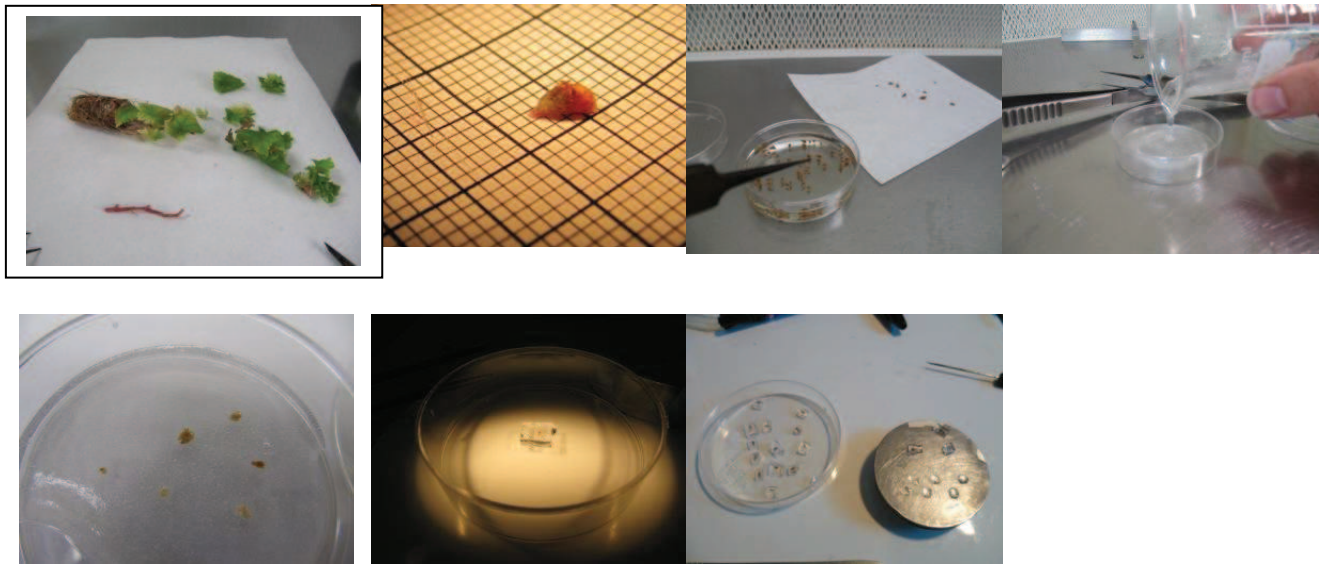


Figure 15: Grapevine bud preparation for immunolocalisation: a) Taking buds from *in vitro* plantlets; b) Isolated bud 1 mm in length; c) Fixation of buds; d) and e) Embbeding in agarose; f) and g) Glueing pyramidal blocks of agarose containing buds on the vibratome plate before cutting

Preliminary experiments showed that the optimal primary antibody concentration should be less than 100 fold, which was tested in our experiments and that a cut tickness of 60 µm was satisfactory.

Figure 16 shows a shoot tip of cv. Maraština used for testing the immunolocalization protocol. Green signal parts reveal the occurrence of virus in this sample. Nuclei were stained blue with DAPI. This picture shows that the GLRaV-1 signal was located in vascular bundles of the left leaf primordiums. There was no signal in the apical dome and in sub-apical tissues. There was a strong signal in the right side of the meristem, but it was not clear if it was, or not, part of the right leaf primordium.

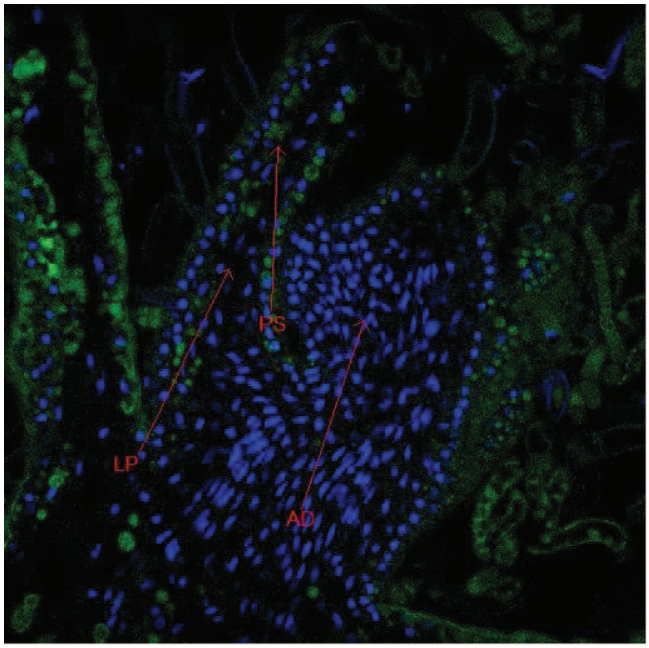


Figure 16: Immunolocalization of GLRaV-1 virus epitope inside an shoot meristem of an infected genotype of cultivar Maraština-1; (GLRaV-1 virus) primary + secondary antibody labelled with Alexa 488 (green signal), Nucleus are stained in blue by DAPI
AD- shoot meristematic dome, LP-leaf primordium, PS Provascular strands

The sample displayed in Figure 17 was used as control for infected ones. This sample did not have any antibody applied. The structure of the meristem was visible with the submeristematic dome and two leaf primordia.

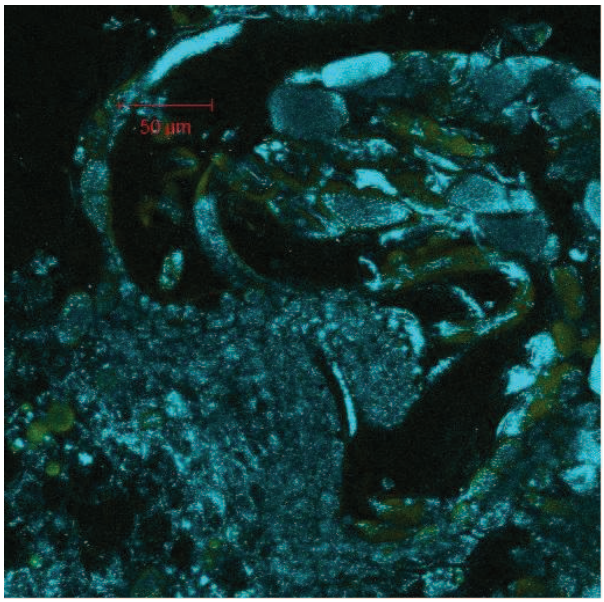


Figure 17: Control sample of infected Plavac mali cultivar (sample not treated with antibody Figure 18 displays the area below the meristematic dome. Cell walls and nucleus were stained in blue. The brown color indicated a potential virus signal, spread all over the tissues surrounding the meristem.

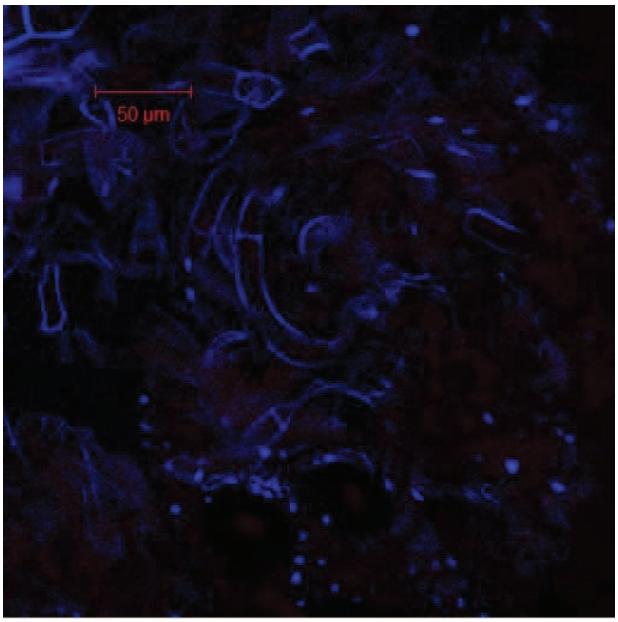


Figure 18: Immunolocalization of GLRaV-1 virus epitope inside the area below the shoot meristematic dome of infected genotype of cultivar Pošip-1, (GLRaV-1 virus) labelled with secondary antibody Alexa 633. (dark red signal) Nucleus and are stained in blue by DAPI; red staining indicates a potential virus signal

Figure 19 shows the same shoot tip of cultivar Maraština, labelled with another secondary antibody Alexa 633. DAPI stained nuclei in blue and the red staining indicated a virus signal. A virus signal was spread in vascular bundles of the leaf primordium and in tissues surrounding the shoot meristem.

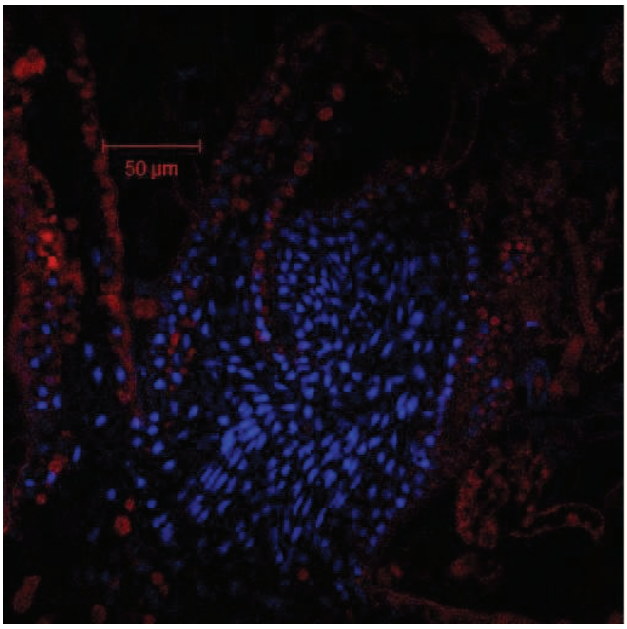


Figure 19: Immunolocalization of GLRaV-1 virus epitope inside a shoot meristem of an infected genotype of cultivar Maraština-1, (GLRaV-1 virus) primary + secondary Antibody labelled with Alexa 633

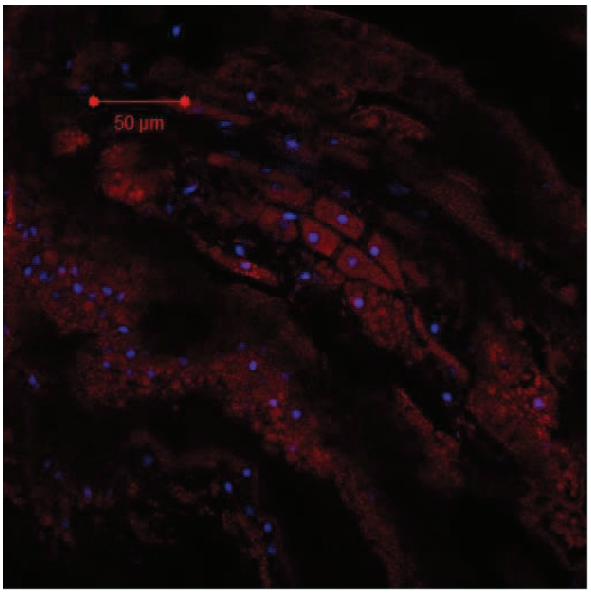


Figure 20: Immunolocalization of GLRaV-1 virus epitope inside vascular bundles of an infected genotype of cultivar Pošip-1; (GLRaV-1 virus) primary antibody diluted 100 fold, labelled with Alexa 633 (red signal). A signal of the GLRaV-1 virus was spread in cells and in sieve elements all over the tissues

In Figure 20, the dilution of primary antibody was 100 fold. The objective was to see whether the primary antibody concentration was sufficient to clearly observe the virus signal. Nuclei were visible and the signal of the GLRaV-1 virus was spread in cells and in sieve elements all over the tissues. However, to better see the signal within the structure of the cells, the primary antibody had to be more concentrated.

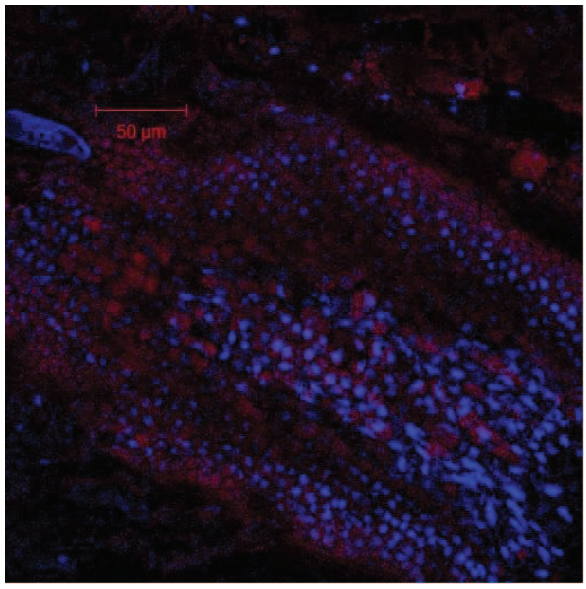


Figure 21: Immunolocalization of GLRaV-1 virus epitope inside a shoot meristem of an infected genotype of cultivar Pošip-1, (GLRaV-1 virus) primary antibody diluted 1000 fold and labelled with Alexa 633 (red signal)

In Figure 21, DAPI stained the nuclei, but the GLRaV-1 virus signal in red was not strong enough, because of the low concentration of primary antibody.

In conclusion, the optimal primary antibody concentration should be less than 100 fold so in further experiments 50 folds will be applied and a tickness of 60 µm, which approved satisfactory in preliminary experiments performed.

4.3.1.2 Application of immunolocalisation protocol on international cultivars

Immunolocalisation experiments were performed on cv. Pinot Noir (GFLV virus), Chardonnay (GFLV virus), Cabernet Sauvignon (GLRaV-3) and Merlot (GLRaV-3). Cultivars Chardonnay and Cabernet Sauvignon showed a high level of autoflorescence and therefore were not included in this study. Different types of plant tissue were observed: meristems (shoot tips), vascular bundles of stem tissue and vascular bundles of leaves.

4.3.1.2.1 Immunolocalisation study on cv. Pinot Noir

Figure 22 presents a healthy shoot tip of cv. Pinot Noir. In the upper left window, we can clearly see that there was no natural autoflorescence. This sample was used as control for infected shoot tips of the same cultivar (Pinot Noir) but also no signal linked with the epitope virus tested as the sample has been treated with Ab1 and Ab2 .

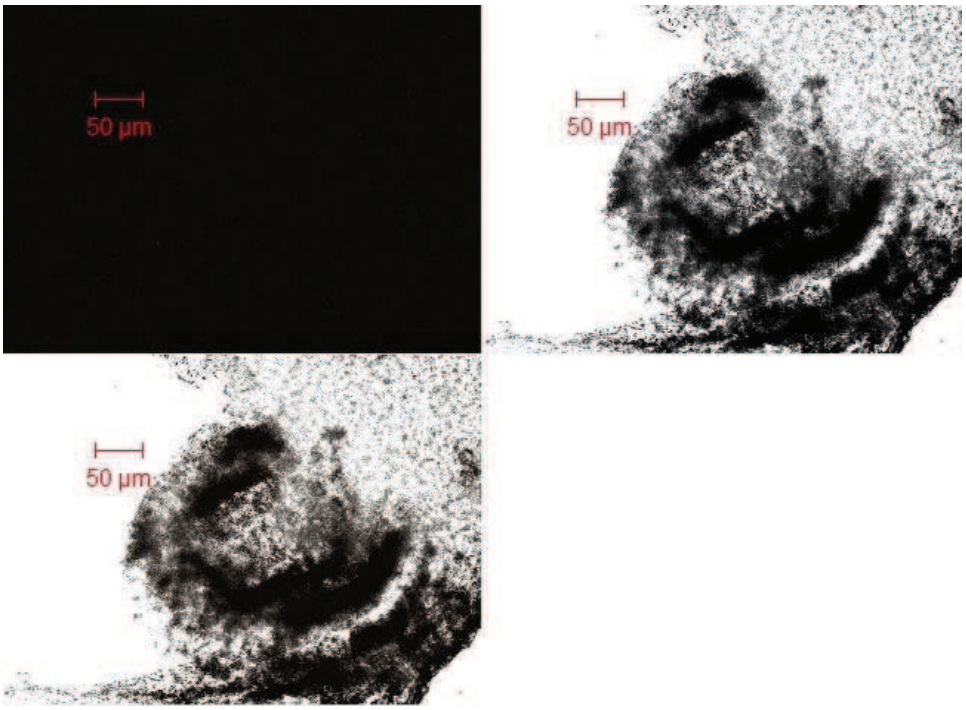


Figure 22: Immunolocalization of a shoot tip of a healthy genotype of cultivar Pinot Noir; GFLV virus primary antibody diluted 50 fold, labelled with Alexa 546

Figure 23 shows an infected shoot tip of cultivar Pinot Noir infected with GFLV virus. In the left upper window a very weak signal could be observed. This signal should correspond to the signal of virus in this meristem. This means that even if the sample has been infected, virus epitope is not detected in the shoot meristem.

Figure 24 presents the immunolocalisation observation of GFLV virus epitope of another shoot tip of an infected genotype of cv. Pinot Noir. On this picture the virus signal was clear. In the upper left window, we could see the virus signal located in the meristematic dome, in the internal tissues of the meristem and in the leaf primordia. The virus was also observed in the sub-meristematic dome.

Based on the observation of the two meristems of cv. Pinot Noir observed on Fig. 23 and 24, which derived from the same batch of material, the epitope of the virus could be located inside the meristematic dome but some shoot meristems of the same cultivar were virus epitope-free (no detection possible).

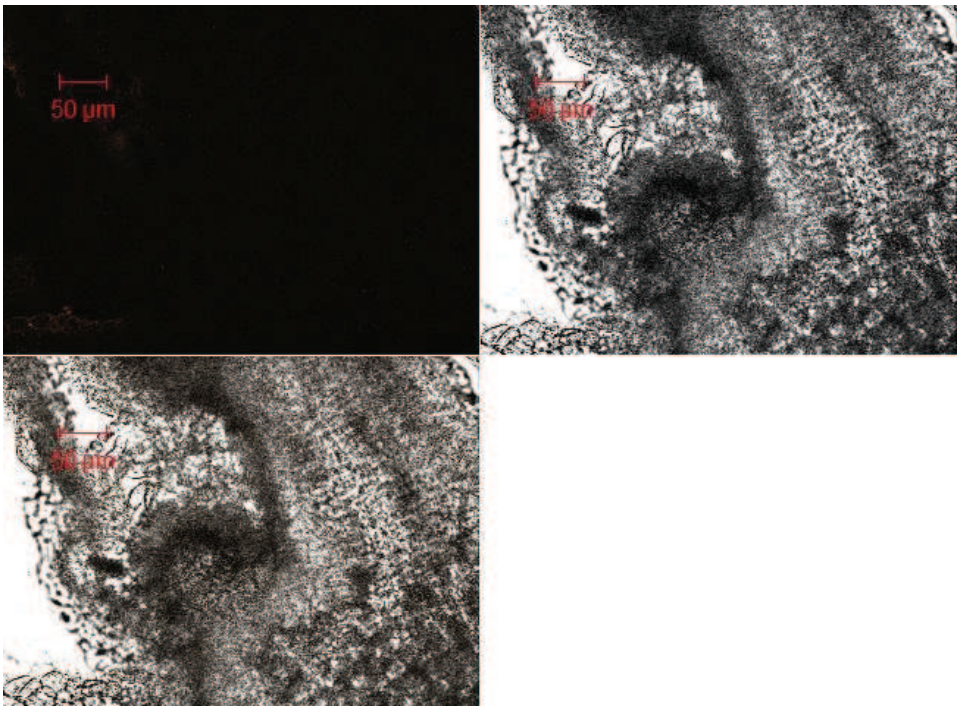


Figure 23: Immunolocalization of GFLV virus epitope inside a shoot tip of an infected genotype of cultivar Pinot Noir. Primary antibody diluted 50 fold, labelled with Alexa 546

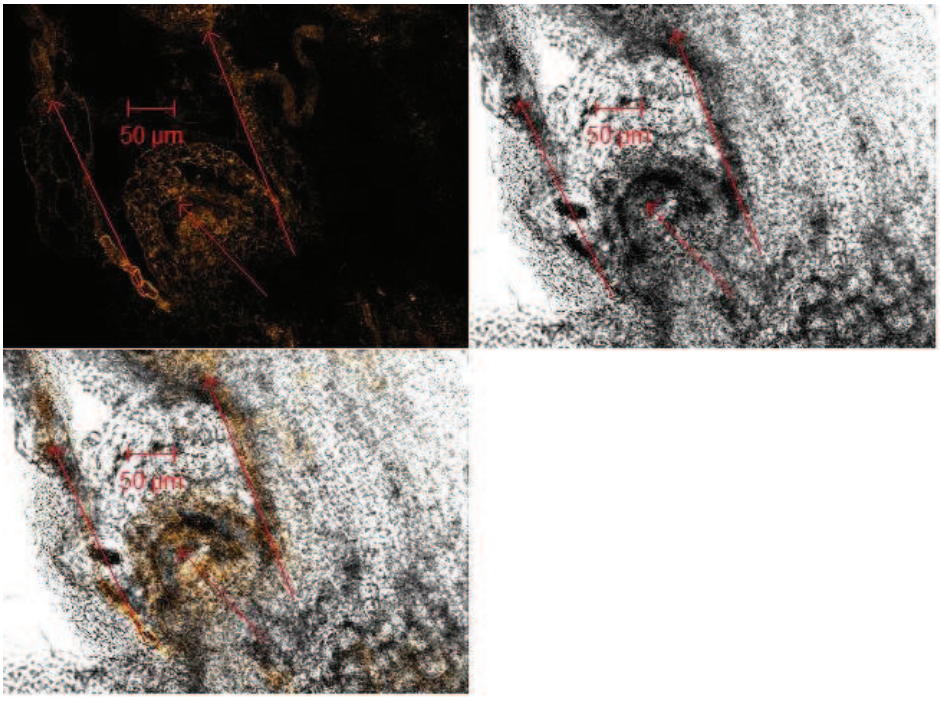


Figure 24: Immunolocalization of GFLV virus epitope inside a shoot tip of an infected genotype of cultivar Pinot Noir; GFLV virus primary antibody diluted 50 fold, labelled with Alexa 546 (orange signal is localised inside the shoot meristem dome and inside leaf primordias

Vascular bundles of cv. Pinot Noir were also observed Figure 25 shows the vascular bundles of a healthy genotype of cultivar Pinot Noir. No signal observed: in the upper left window, implying that there was no autoflorescence in this spectra and no aspecific labelling due to the secondary antibody fixation on the meristem tissues.

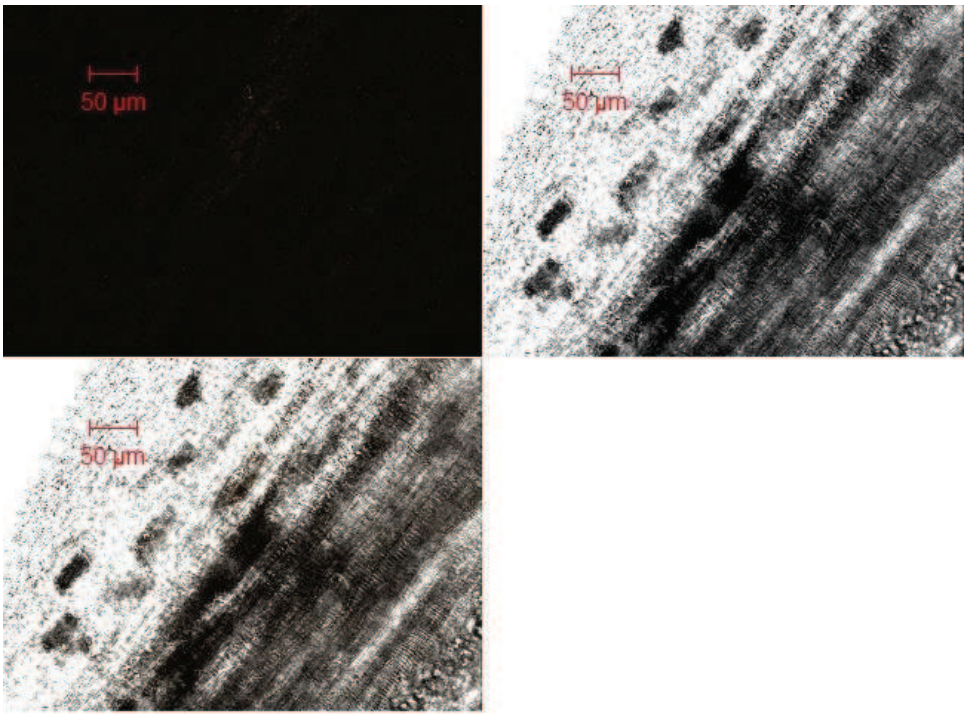


Figure 25: Immunolocalization of healthy parts of vascular bundles of cultivar Pinot Noir; primary antibody diluted 50 fold, labelled with Alexa 546

Figure 26 presents vascular bundles of an infected genotype of cultivar Pinot Noir (GFLV). In the upper left window, a strong signal was observed which corresponded to a virus signal. This signal was spread all over the vascular bundles.

Furthermore, Figure 27 presents the observation of leaf vascular bundles of a healthy genotype of Pinot Noir. There was no obvious signal in the upper left window.

Figure 28 and Figure 29 show leaf section of an infected genotype of cultivar Pinot Noir. In both Figures, the virus signal was located in tha vascular bundles inside the sieve elements of the leaf tissues. No signal is located inside the xylem vessels.

Our results revealed that a strong GFLV signal was observed in meristem and leaf tissues of infected genotype of cv. Pinot Noir. As there was no strong autoflorescence, it could be concluded that this signal was due to the presence of the GFLV virus in this cultivar.

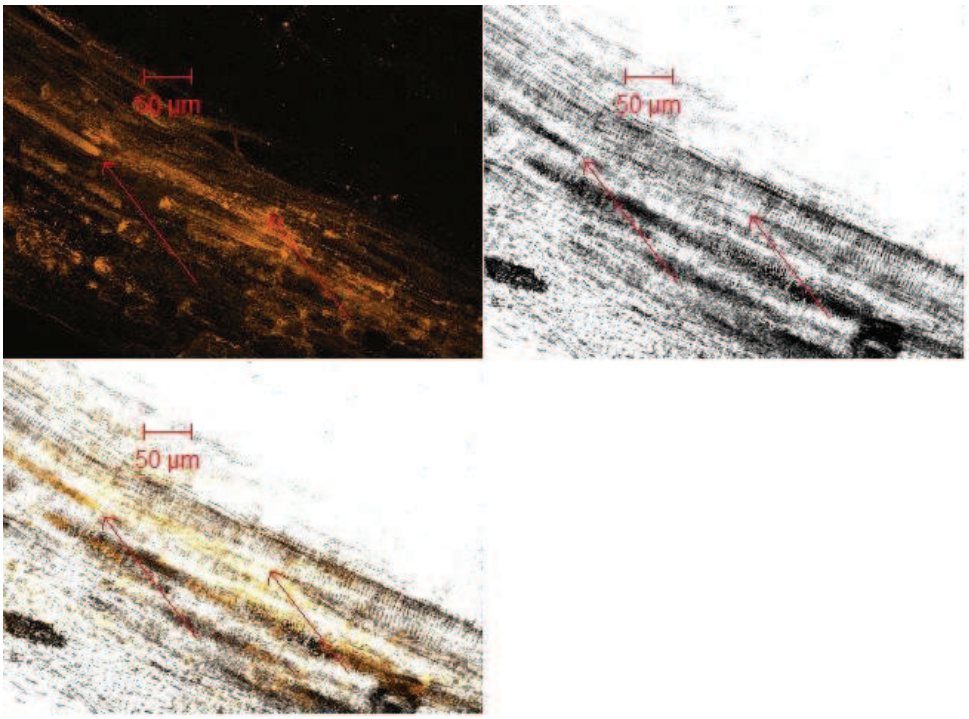


Figure 26: Immunolocalization of GFLV virus epitope inside vascular bundles of an infected genotype of cultivar Pinot Noir; GFLV virus primary antibody diluted 50 fold, labelled with Alexa 546

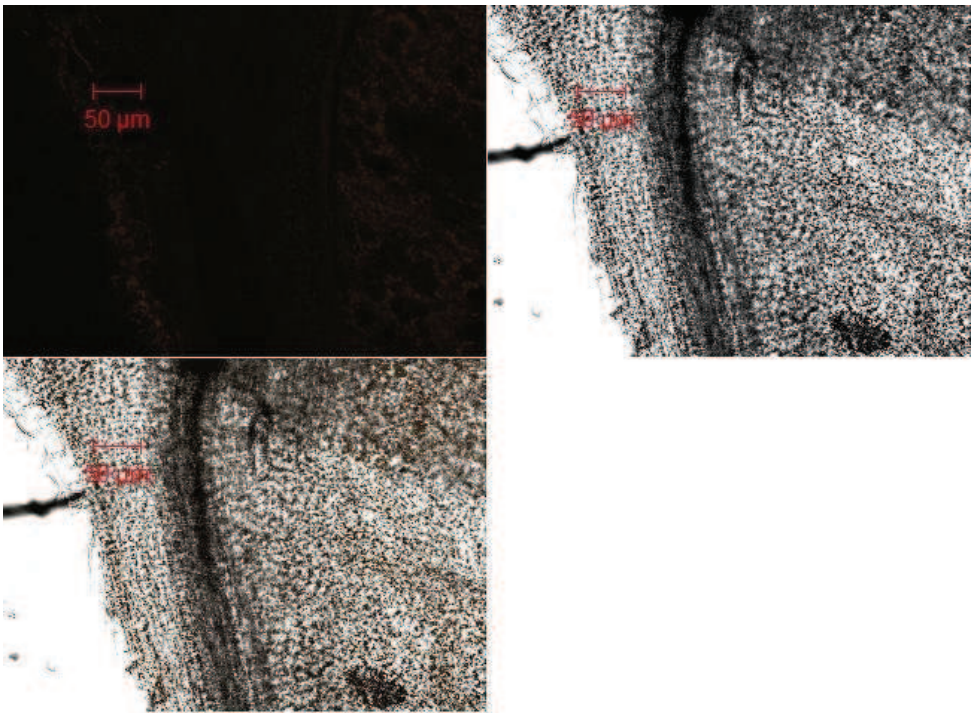


Figure 27: Immunolocalization of leaf vascular bundles of a healthy genotype of cultivar Pinot Noir; primary antibody diluted 50 fold, labelled with Alexa 544 VB: vascular bundle

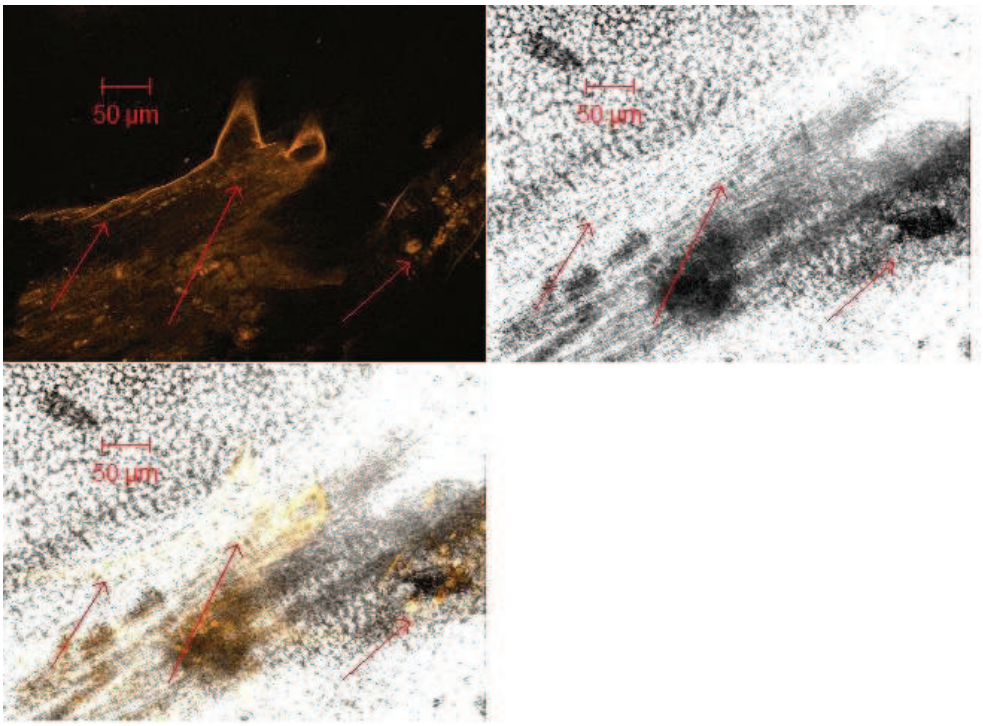


Figure 28: Immunolocalization of GFLV virus epitope inside the leaf of an infected genotype of cultivar Pinot Noir; GFLV virus primary antibody diluted 50 fold, labelled with Alexa 546 (orange signal)

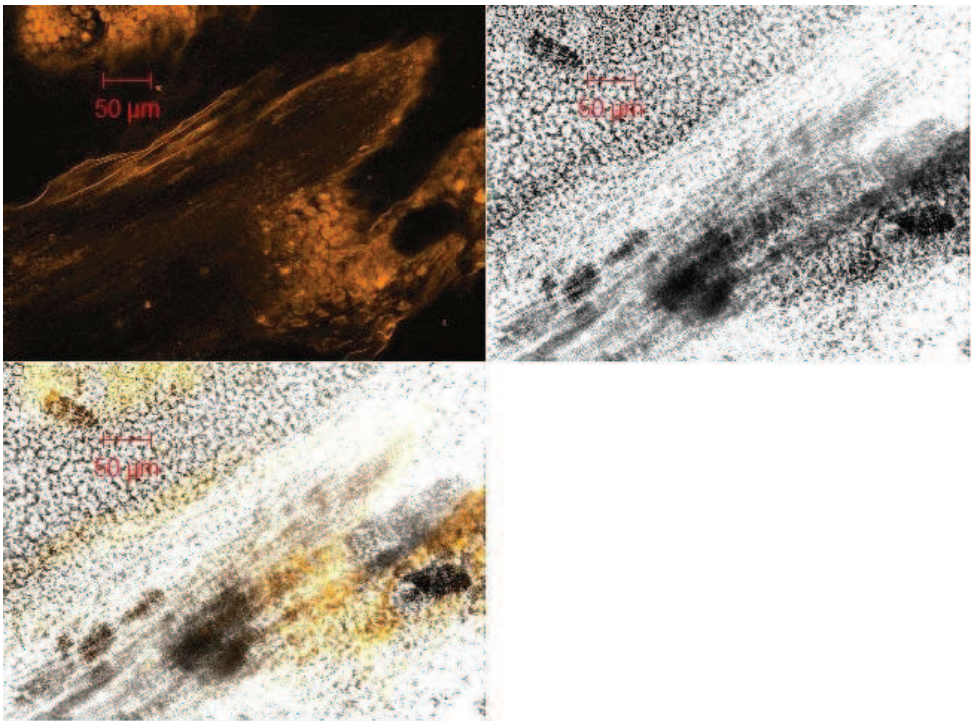


Figure 29: Immunolocalization of GFLV virus epitope inside the leaf of an infected genotype of cultivar Pinot Noir; GFLV virus primary antibody diluted 50 fold, labelled with Alexa 546

In conlusion, in cultivar Pinot Noir the virus epitope of GFLV virus was spread all over the meristematic dome and leaf primordiums of infected meristems, as well as in vascular bundles of infected leafs. Our results clearly showed that even though some meristems were infected, the virus epitope was not clearly observed. We can conclude that the virus epitope of GFLV virus is uneven in shoot meristems and consistent in vascular bundles.

4.3.1.2.2 Immunolocalization study on cv. Merlot

Additional experiments were performed with cultivar Merlot infected with GLRaV-3 virus. Observations were performed on leaf sections.

Figure 30 and Figure 31 display the leaf structure of a healthy (Figure 30) and infected (Figure 31) genotype of cv. Merlot. In Figure 30, a faint signal corresponding to back-ground was observed in the left upper window. By contrast, a strong signal was observed in leaf veins in Figure 31 indicating the presence of GLRaV-3 virus.

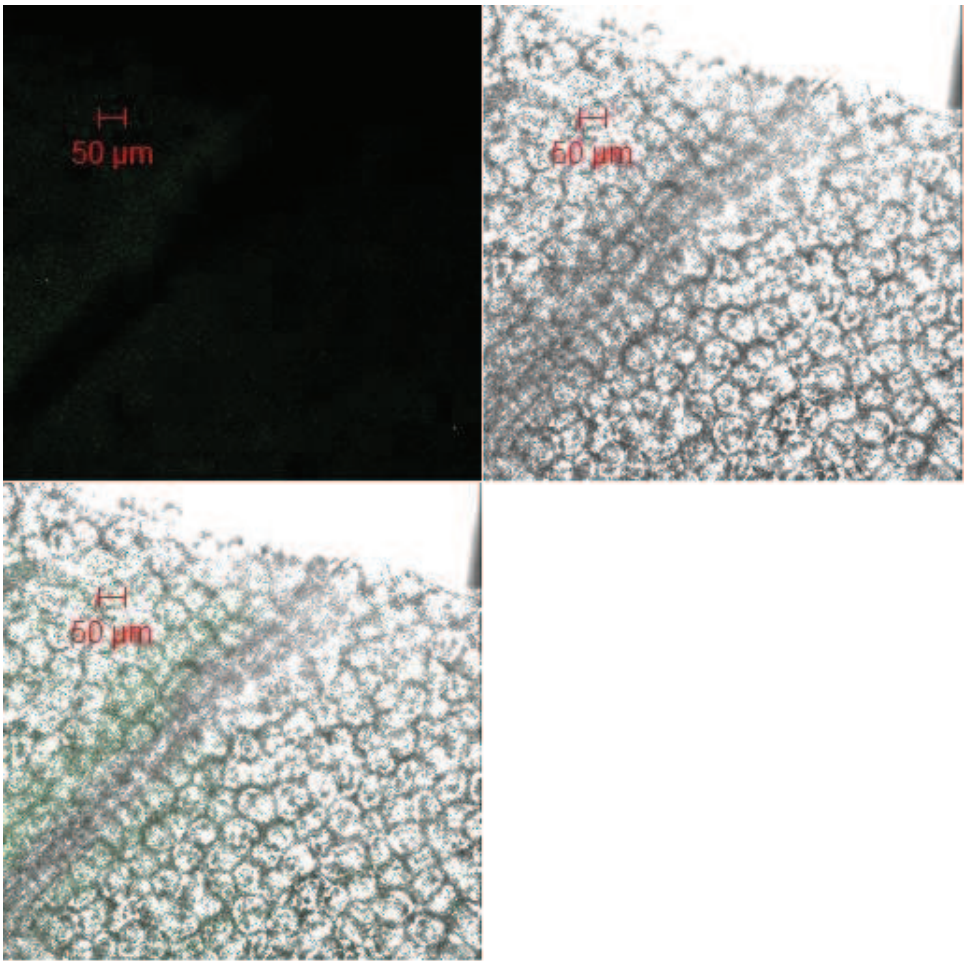


Figure 30: Immunolocalization on a leaf section of a healthy genotype of cultivar Merlot; primary antibody diluted 50 fold, labelled with Alexa 546.

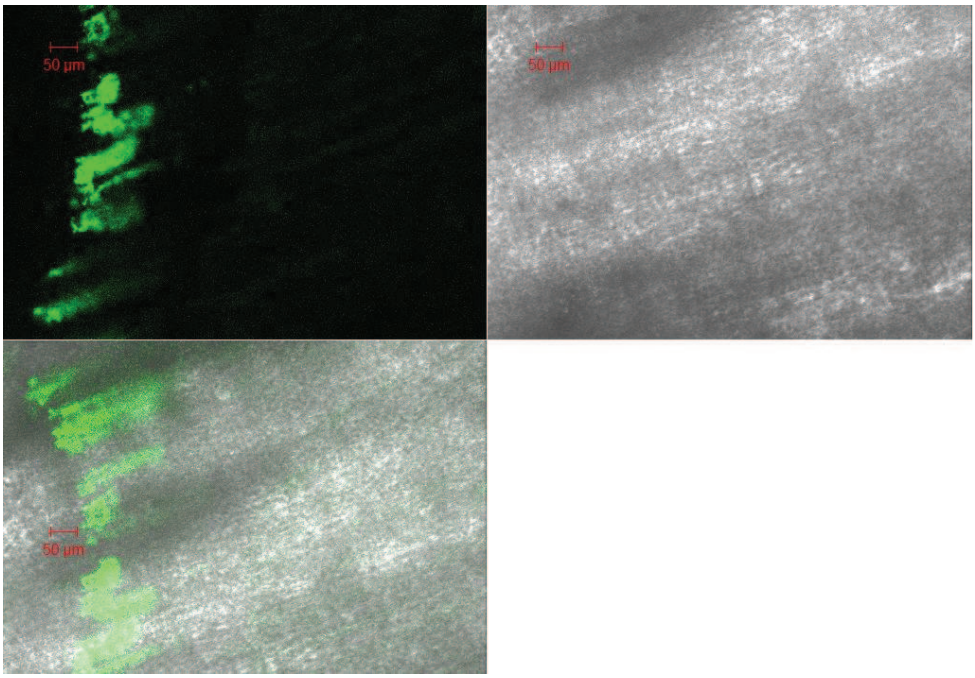


Figure 31: Immunolocalization of GLRaV-3 virus epitope inside the leaf of an infected genotype (GLRaV-3 virus) of cultivar Merlot; primary antibody diluted 50 fold, labelled with Alexa 488 (green signal).

4.3.1.3 Discussion

The immunolocalisation protocols were perfomed to detect the virus signals of two economically important grapevine viruses (GLRaV-3 and GFLV) in plant tissues of selected cultivars. In our experiments, this technique was performed on non-cryopreserved plant material. However, this technique can be useful for observation of virus presence in plant tissues after cryopreservation.

The immunolocalization experiments performed required a lot of work to improve the protocol due to the high autofluorescence in grapevines depending on the genotype and also to our limited knowledge on the primary antibodies used, which was one of the obstacles in protocol development. Finally the protocol was adapted to grapevine and tested on international cultivars.

After performed cryopreservation on international cultivars selected, regrowth of Merlot (both control plants and LN exposed ones) was very poor, so there was not enough plant material to perform any ELISA test. As reported previously, no regrowth of healthy or infected Pinot noir was obtained after cryopreservation.

The results of the cryotherapy experiments revealed that a high proportion of Chardonnay and Cabernet Sauvignon plants were virus-free after being subjected to cryopreservation. GLRaV-3 was completely eliminated, while GLRV was eliminated in about 78% of the regenerated plants.

Virus testing was also performed on control plants treated with PVS2 and the proportion of healthy plants was almost the same as with LN exposed ones for both viruses.

The difference noted in the sanitation rate between the two viruses studied could be expected. Indeed, it is well documented that nepoviruses (GFLV) and closteroviruses (GLRaV3) generally behave differently depending on the technique used to eliminate them and to regenerate virus-free plants (Valero et al., 2002). This differential response is connected with different capacities of parenchymatic (GFLV) and phloematic (GLRaV-3) viruses to invade and spread through the tissues of the host plant.

Closterolike-virus particles of GLRaV-3 are known to be restricted to vascular tissues (phloem) (Namba et al., 1979) while GFLV particles are present even in very young meristematic tissues (i.e. meristematic dome of shoot apices) of infected grapevine. For this reason leafroll associated viruses are usually well responding to sanitation by means of meristem culture, while the success of GFLV eradication is significantly lower, reaching about 40.8 % of healthy plants (Gribaudo et al., 2012).

In our immunolocalization studies, it was also observed that GLRaV-3 was restricted to the veins and leaf primordia of shoot tips of cv. Merlot. By contrast, GFLV was observed even in meristematic tissues of the apical dome of shoot tips of cultivar Pinot Noir.

Various reports on different viruses and species have shown that explant size is crucial for the success of virus eradication (Brison et al., 1997; Faccioli and Marani, 1998; Ding et al., 2008). The larger the size of the meristem cultured, the greater the number of regenerated plants; however the number of virus-free plantlets produced is inversely proportional to the size of the cultured tips (Faccioli and Marani, 1998). One of the advantages of cryotherapy, compared with meristem culture, is the possibility of using large explants. These explants contain large cells, which are more likely to be infected by the spreading virus than smaller meristematic ones. However, cryopreservation kills large cells, which cannot survive because of their higher water content, and thus increases the proportion of healthy cells in large shoot tips.

Based on our results on cryopreservation protocol optimization, 1 mm long explants were used in these experiments. Such small explants may already be free of infected cells, which could be one of the reasons for the high percentage of healthy plants obtained even without LN exposure. This is in accordance with the study of Youssef et al. (2009) who reported that meristem tips of 1 mm in length were found to be optimal for elimination of GFLV and GLRaV-1 from infected grapevines using meristem tip culture.

Another explanation for the high percentage of virus-free plants obtained from control and cryopreserved shoot tips might be the exposure of explants to cryoprotectants which penetrate deeply in shoot tips and may have a kind of “chemotherapeutic” effect. It has been shown that some chemicals such as DMSO can inhibit viral replication through blocking of virus nucleic acid synthesis (Vicente and De-Fazio, 1987) in animal (Scholtissek and Müller, 1998) and human tissues (Aguilar et al., 2002). Their use as another possible method of virus elimination has been studied (Vicente and De Fazio, 1987; Hu et al., 2012; Guţă et al., 2009), but so far it has not proved efficient enough to be used for large-scale therapy of plant viral diseases. Yet another explanation for this result might be that the osmotic shock caused by exposure of shoot tips to the highly concentrated PVS2 solution kills differentiated and virus-infected cells, without the need for LN exposure. This was already reported by Helliot et al. (2002) who obtained 2% virus-free plants for cucumber mosaic virus (CMV) and 95% virus-free plants for banana streak virus (BSV) in *Musa spp.* after 1-h cryoprotection with

PVS2 solution. In contrast, Wang et al. (2003b) did not report the occurrence of any virus-free plants before the LN exposure. Additional experiments are thus needed to confirm our preliminary observations of virus elimination without LN exposure.

4.4 Genetic stability

4.4.1 Results

To test the genetic stability of plants after cryopreservation, AFLP analysis was performed. In this experiment, cryopreserved and non-cryopreserved shoot tips (sampled after sucrose preculture, loading and treatment with half-strength PVS2 solution) were included. There was no callus formation and shoot tips developed directly into plantlets. The recovery percentage of shoot tips differed after the successive steps of the cryopreservation protocol and each condition produced a different number of regenerants. The AFLP analysis was performed using eight primer combinations on a total of 43 plants.

A total of 777 fragments were amplified. Out of eight control samples, two of them had missing data and were discarded from further analysis. Comparing the AFLP profiles of control samples 41 polymorphic peaks were detected, corresponding to 5.27% (data not shown). Polymorphism between control samples could be a result of clonal differences between mother vines used for creating *in vitro* culture. However, these polymorphisms could also be a result of a PCR error or a sequencer (ABI3130) error. As could not find the cause of the polymorphism, all 41 fragment were removed from all the samples resulting in 736 amplified fragments used for further analysis

Table 52: Number of amplified and polymorphic bands per primer combination and percentage of polymorphism for each step of the cryopreservation protocol and sucrose and loading treatment

Primer combination	Total bands amplified	Polymorphic bands					
		SUCROSE	LS	PVS2half	CON 50	FREE 50	CON 75
E10-M16	57	4	4	13	20	15	8
E40-M40	82	8	7	25	11	64	3
E40-M61	88	2	0	18	12	57	1
E37-M61	93	1	2	21	10	27	1
E40-M62	122	4	4	30	26	87	17
E32-M40	93	2	6	15	62	54	11
E35-M40	103	30	46	18	21	41	9
E37-M62	98	4	7	11	10	25	1
Total	736	55	76	151	172	370	51
		7.5%	10.3%	20.5%	23.4%	50.3%	6.9%

LS: treatment with loading solution for 20 min; Sucrose: treatment with 0.3 M sucrose for 24 h; PVS2half: treatment with half-strength PVS2 solution for 30 min; FRE25: treatment with PVS2 for 25 min followed by LN exposure; CON50: treatment with PVS2 for 50 min; FRE50: treatment with PVS2 for 50 min followed by LN exposure; CON75: treatment with PVS2 for 75 min.

All steps of the cryopreservation protocol produced different numbers of polymorphic fragments. From 736 amplified fragments, 136 were monomorphic. Looking at the

successive steps of the cryopreservation protocol (Table 52), the lowest average percentages of polymorphism were 6.9% and 7.5% for the CON75 and sucrose treatments, respectively. The highest average percentage of polymorphic fragments was achieved with treatment FRE 50 (50.3%).

When comparing plants regenerated after sucrose preculture, loading treatment and dehydration with half- strength PVS2 (sucrose, LS and PVS2half), with the six control plants cultivated on MS2/2 medium, it was observed that all plants clustered very close to each other, (coefficient of genetic similarity 0.93) except three of them (PVS2half_2, LS_4 and PVS2half_6) (Figure 33).

On Figure 34, we compared all plants from the successive steps of the cryopreservation protocol. It was noted that differences between samples increased with the number of treatments to which they were exposed. Looking at the last two clusters, it was visible that increasing the duration of exposure to PVS2 influenced the number of polymorphic fragments, even without LN exposure. Exceptions were samples CON 50_5 and CON 50_6, which were very close to control samples.

On Figure 35, we compared the six plants regenerated from non-cryopreserved shoot tips treated with PVS2 for 50 min (CON 50) with the eight plants regenerated from cryopreserved shoot tips treated with PVS2 for 50 min (FRE 50). Samples split into two clusters with a similarity coefficient of 0.66. One cluster included the untreated control, all CON 50 and three FRE 50 plants. The other cluster included the five other FRE 50 plants. This result showed that the level of polymorphic fragments increased with the number of treatments to which they were exposed.

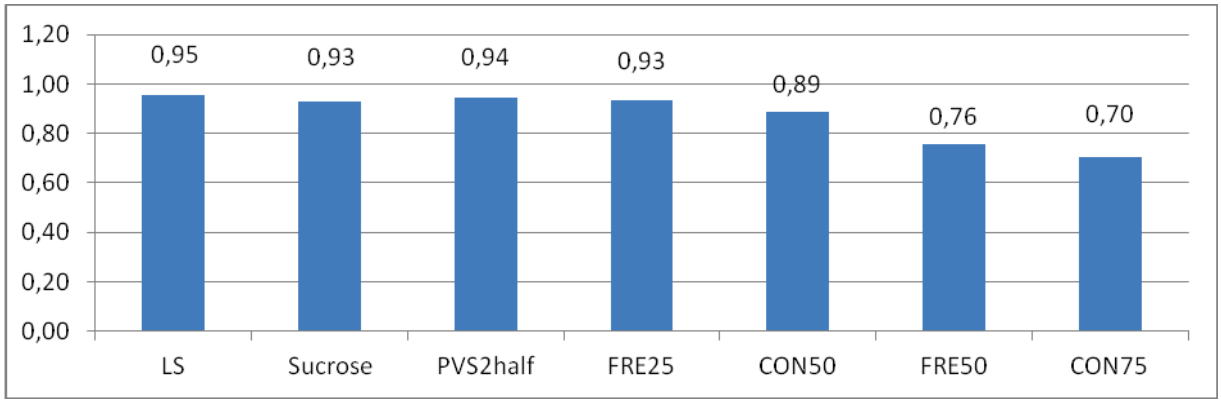
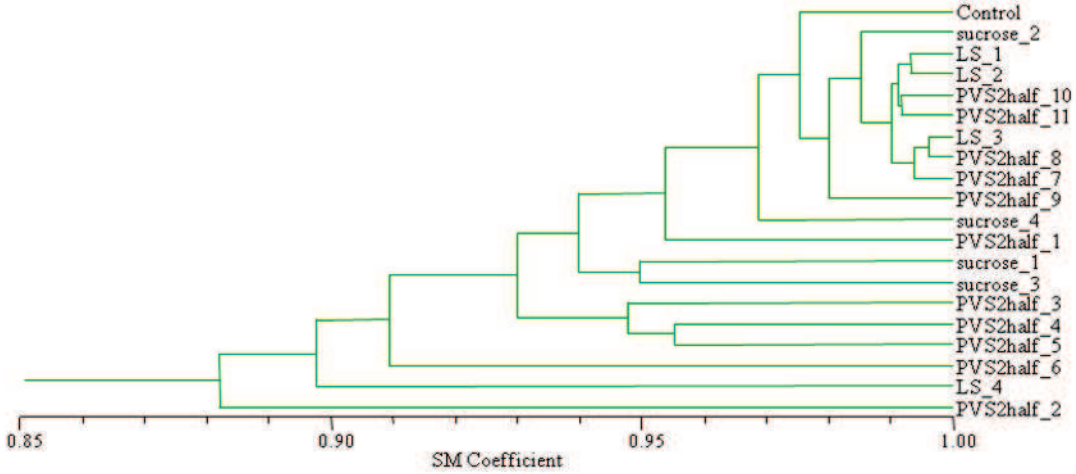


Figure 32: Genetic similarity between the seven steps of the cryopreservation protocol calculated based on their SM coefficient. LS: treatment with loading solution for 20 min; Sucrose: treatment with 0.3 M sucrose for 24 h; PVS2half: treatment with half-strength PVS2 solution for 30 min; FRE25: treatment with PVS2 for 25 min followed by LN exposure; CON50: treatment with PVS2 for 50 min; FRE50: treatment with PVS2 for 50 min followed by LN exposure; CON75: treatment with PVS2 for 75 min.

We compared the similarity, based on a simple matching coefficient, between non-treated samples and samples taken after each step of the cryopreservation protocol (Fig 32). The first three steps of the cryopreservation protocol showed a high similarity with a 0.93-0.95 average SM value. With the following treatments the average value of SM coefficient

decreased to 0.76 and 0.70 for FRE 50 and CON 75, respectively. We could thus observe a



slight decrease of the average value of the SM coefficient with the number of treatments.

Figure 33: Comparison between control conditions of cryopreservation protocol with control (*in vitro* plants) LS: treatment with loading solution for 20 min; Sucrose: treatment with 0.3 M sucrose for 24 h; PVS2half: treatment with half-strength PVS2 solution for 30 min; FRE25: treatment with PVS2 for 25 min followed by LN exposure; CON50: treatment with PVS2 for 50 min; FRE50: treatment with PVS2 for 50 min followed by LN exposure; CON75: treatment with PVS2 for 75 min.

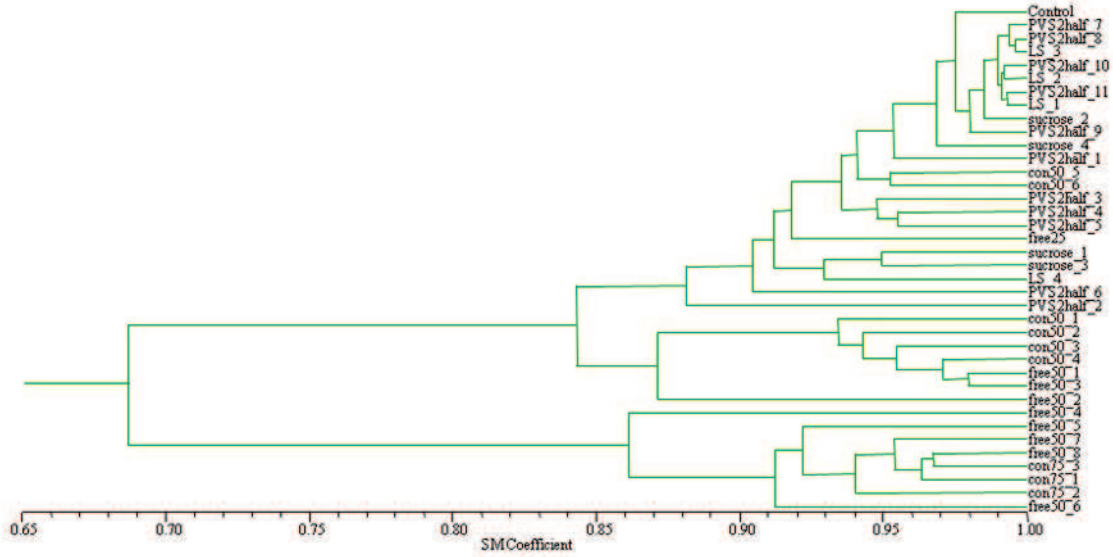


Figure 34: Comparison between all steps of cryopreservation protocol with control (*in vitro* plants)) LS: treatment with loading solution for 20 min; Sucrose: treatment with 0.3 M sucrose for 24 h; PVS2half: treatment with half-strength PVS2 solution for 30 min; FRE25: treatment with PVS2 for 25 min followed by LN exposure; CON50: treatment with PVS2 for 50 min; FRE50: treatment with PVS2 for 50 min followed by LN exposure; CON75: treatment with PVS2 for 75 min.

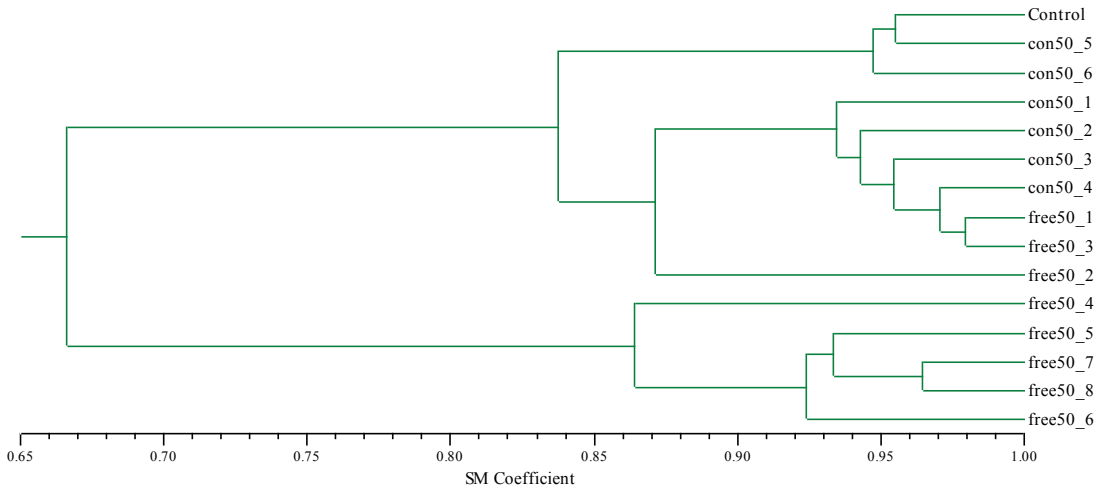


Figure 35: Comparison between plants non-cryopreserved (CON) and cryopreserved (FRE) after PVS2 for 50 min exposure with control (*in vitro* plants)) LS: treatment with loading solution for 20 min; Sucrose: treatment with 0.3 M sucrose for 24 h; PVS2half: treatment with half-strength PVS2 solution for 30 min; FRE25: treatment with PVS2 for 25 min followed by LN exposure; CON50: treatment with PVS2 for 50 min; FRE50: treatment with PVS2 for 50 min followed by LN exposure; CON75: treatment with PVS2 for 75 min.

4.4.2 Discussion

In this study, AFLP markers were employed to test the genetic stability of grapevine *in vitro* plantlets regenerated following a cryopreservation experiment. With the eight AFLP primer combinations employed on the 43 plants tested, no significant differences in AFLP profiles were observed after sucrose preculture, treatment with the loading solution and with half-strength PVS2. However, an increase in polymorphic fragments was observed in non-cryopreserved and cryopreserved samples treated with PVS2 solution, the number of polymorphic fragments increasing with longer durations of exposure to PVS2 solution.

The use of AFLP molecular markers was suggested to identify possible clones among one grape cultivar (Vokurka, 2003). Anhalt et al. (2011) used AFLP markers to classify mutations in 86 Riesling clones of *Vitis vinifera* and enhanced our understanding of the dynamics of grapevine clones analyzed by AFLP fingerprints. AFLP markers were used successfully to identify clones in combination with retrotransposon-based markers (Castro et al., 2012), to study the distribution and to evaluate the genetic variability between clones of grapevines (Meneghetti et al., 2011) and the intravarietal variability (Meneghetti et al., 2012). AFLP markers were used for discriminating all Malvasia genotypes at the molecular level, with the addition of SAMPL (selective amplification of microsatellite loci) and M-AFLP (microsatellite-amplified fragment length polymorphism) markers. Based on these studies which aimed at identifying differences between grapevine clones, AFLP markers appeared appropriate to investigate possible differences in genomic structure of plants regenerated after the successive steps of a cryopreservation protocol. However, this method has some shortcomings, of technical nature, as reported by Benjak et al. (2006). These authors showed that AFLP results can be influenced by the DNA extraction protocol. Using the same samples and three different DNA extractions, Benjak et al. (2006) obtained different results (different level of polymorphism) for every DNA extraction method tested. It is very important

to treat all samples in the same way (identical method of sample preparation, identical DNA isolation protocol, etc.) to ensure that any polymorphism detected between samples is the result of differences at the DNA level and not due to technical inconsistencies of AFLP method.

Genetic stability of cryopreserved material was tested in some earlier studies (Harding, 2004). Zhai et al. (2003) used RAPD markers on cryopreserved grapevine shoot tips. These authors did not find any difference between the DNA patterns obtained with plantlets regenerated from control and cryopreserved plantlets. Castillo et al. (2010) tested the genetic stability of cryopreserved *Rubus* multiplied for 7 months in tissue culture conditions and planted in the field. SSR and AFLP analysis was performed on material recovered directly from cryopreserved shoots subcultured for 7 months and on field-grown plants. No SSR polymorphisms were detected, between cryopreserved samples and mother-plants regardless of the subculture. AFLP polymorphisms were detected in three of the four *Rubus* genotypes after they had been subcultured for 7 months. Field-grown plants from the polymorphic shoot tips of *R. grabowskii* and Silvan displayed the same AFLP finger-prints as their corresponding mother-plants. Only 7-month cultured *in vitro* shoot tips displayed polymorphism *in vitro*, and they were no longer detected when the plants were grown *ex vitro*. This study showed that polymorphism can vary depending on the environmental conditions of the plantlets. It is also obvious that different techniques used for polymorphism detection may produce different results. Our study, which revealed differences between non-cryopreserved and cryopreserved material, is in accordance with the results of Castillo et al. (2010) who found polymorphism in plantlets derived from cryopreserved shoot tips subcultured for 7 months but contradicts the results of Zhai et al. (2003) who did not observe any difference between control and cryopreserved samples.

Many factors can induce genetic variation in cryopreserved materials, including the type of explants selected, the freezing procedure employed and regrowth pattern achieved (Aronen et al., 1999; Harding 1996; 1999; 2004). The control and optimisation of these parameters are important in order to avoid inducing genetic variation. In our experiments, shoot-tips were the explants selected for cryopreservation because they are programmed to directly develop into shoots. The technique used was D-V, which showed the best overall results in our experiments. The protocol we developed used the PVS2 solution, which is known for its efficiency but also for its toxicity (Sakai and Engelmann, 2007; Reed, 2008). One hypothesis may be that, even though, to the best of our knowledge, no such changes have been reported in the literature, exposure of grape shoot tips to PVS2 for 50 min induced the polymorphisms observed. LN exposure increased the level of polymorphism obtained. Nevertheless, several reports claimed that *in vitro* culture itself can be mutagenic. There are some reports of phenotypic or morphological changes induced by *in vitro* culture in grapevine: Grenan (1984) described the morphological changes of leaves in plants produced *in vitro*; Dami and Hughes (1995) reported that leaves from *in vitro* cultivated plants lacked normal palissade layers, contained larger mesophyll cells, had greater intercellular pore spaces and fewer chloroplasts than those originating from greenhouse-grown plants. Gribaudo et al. (2000) observed that after some years in the vineyard, plants produced by micropropagation reached a higher yield and had a different shape of leaves compared to plants multiplied in the nursery. Already from these reports, we can conclude that *in vitro* culture can cause some changes in the structure of plants, without involving any toxic chemicals.

Our results are not sufficient to conclude that the cryopreservation protocol employed induced polymorphism in regenerated plants. Using eight AFLP primer combinations and amplifying 736 fragments, only a small portion of the genome was covered. In addition, a relatively low number of plants was tested. However, our results suggest that polymorphism may increase after some steps of the cryopreservation protocol and that caution is necessary.

It is reasonable to assume that all individual regenerants do not pass the same path of regeneration and the level of mutation events can be different at the end. This could explain observed exceptions that in spite of evident trend of more polymorphism (mutations) after more treatments there are some individuals that clustered close to control ones. If so, it would be reasonable to conclude that proper test for genetic stability (in our case AFLP) should be applied as regular measure after cryopreservation and subsequent regeneration in order to select “the most stable” regenerants prior to further propagation. This should ensure, or at least increase the genetic fidelity of the original genotype.

5 GENERAL DISCUSSION

Vitis vinifera includes a large number of cultivars, which have enabled spreading viticulture worldwide. The importance of conserving grapevine genetic diversity having been recognized, international efforts have been made to collect and characterize remaining *Vitis vinifera* cultivars. Grapevine genetic resources have traditionally been conserved *ex situ* in field collections and, as in other European countries, the national collection of grapevine cultivars has also been established in Croatia with the main focus on collecting and characterizing Croatian autochthonous varieties. A survey of virus diseases in the Croatian grapevine collections disclosed the widespread presence of viruses and the low occurrence of non-infected vines. Although field collections play a preeminent role in grapevine conservation programmes, their maintenance is expensive and time consuming and they are exposed to the risk of accession loss due to natural hazards.

Over the past decades, plant cryopreservation technologies have been evolving rapidly, opening the door to the possibility of safe and cost-effective long-term storage of valuable genetic resources of many species. It has also been shown recently that cryopreservation could be effective in eradicating viruses, a process termed cryotherapy.

The main objective of our research project was to define an efficient protocol for long-term conservation of grapevine through cryopreservation. A series of parameters influencing the recovery of grapevine shoot tips after LN exposure were studied, starting with the source and physiological state of the plant material and proceeding with different steps of the cryopreservation protocol.

After establishing the protocol, the efficiency of LN exposure on virus elimination was examined. In order to understand the occurrence of the viruses studied in shoot apices, immunolocalisation techniques were employed. The plant material regenerated from cryopreserved explants was then submitted to molecular marker analysis to test the possible effects of cryopreservation on genetic stability.

5.1 Establishment of *in vitro* culture of Croatian cultivars

There are currently 5,000 grape varieties identified worldwide (This et al., 2006). In Croatia, approximately 130 different varieties have been identified, which are considered as native, 14 of which are considered to be economically important (Maletić et al., 2004).

A prerequisite to the application of different biotechnological approaches for the conservation and sanitation of grapevine cultivars is the development of an efficient *in vitro* regeneration protocol. The study performed with eight economically important Croatian autochthonous cultivars revealed that *in vitro* multiplication was strongly genotype-dependent, which was in accordance with the conclusions of previous reports (Banilas and Korkas, 2007; Mederos-Molina, 2007; Péros et al., 1998). This implies the need for protocol adjustment to certain cultivars.

Differences between the three groups of cultivars selected according to their genetic distance were observed. Plavac mali yielded the best overall results while Grk had the worst performance for *in vitro* inoculation. Half-strength MS was the most suitable medium but not all cultivars responded well to the BAP concentration chosen.

In addition to the non-uniformity in the regenerative potential of explants introduced *in vitro*, our experiments comparing *in vitro* growth of healthy and virus-infected Plavac mali genotypes highlighted the negative impact of virus infection on regeneration potential. So, together with genotypic specificity, the sanitary status of an accession has to be taken into account when introducing genotypes in tissue culture, following application of virus eradication programs.

5.2 Establishment of a cryopreservation protocol for grapevine

The E-D technique has been the most frequently used in earlier research on cryopreservation of grapevine shoot tips (Plessis et al., 1991; 1993, Wang et al., 2000; 2003a,b; Zhao et al., 2001). However, the rate of recovery with this technique remained relatively low (around 35%). Matsumoto and Sakai (2003) established a protocol for grapevine shoot tips using the vitrification technique. With the two-step vitrification protocol developed, recovery increased up to 80%. This protocol was applied to 10 cultivars of *Vitis* with an average recovery of 64%.

The vitrification technique has been improved using banana shoot tips (Panis et al., 2005) by putting explants in 5-10 µL droplets of vitrification solution and placing them on aluminium strips which are immersed in LN. The advantage of this technique, named D-V, is the possibility of achieving very high cooling/warming rates due to the very small volume of vitrification solution in which explants are placed.

In our research, we decided to compare E-D and D-V, which had, so far, never been employed for cryopreservation of grapevine shoot tips. D-V produced higher regrowth compared to E-D and was thus selected as the base technique for further experiments.

Due to its high *in vitro* reactivity, cultivar Portan was selected to establish the cryopreservation protocol. In order to avoid any possible genotypic effect, most experiments concerning the optimization of the cryopreservation protocol were performed with this cultivar. This cultivar fulfilled the main aim, which was the establishment of a cryopreservation protocol for grapevine shoot tips.

In cryopreservation experiments of grapevine, axillary buds are usually used as initial explants. Axillary buds along the stem are not equally developed. This is true for grapevine (George, 1993) as well as for other plant species and it can influence the establishment of nodal and organogenic cultures (Hung and Trueman, 2011).

As previously observed by Scottez (1993) and Wang et al. (2003), the size of buds affected shoot tip recovery after LN exposure. In order to produce more homogenous buds for cryopreservation, we added a mononodal microcutting culture step before cryopreservation, as suggested by Charoensub et al. (2003) for cassava. This was the first time microcuttings were used in cryopreservation of grapevine.

When comparing the recovery of buds of different origins after cryopreservation, those sampled on microcuttings reached higher recovery compared to those taken directly from *in vitro* plantlets, whatever the level of the node they originated from. Microcuttings originating from basal buds reached higher recovery compared to those sampled at other levels on the stem.

The effect of microcutting culture conditions was thoroughly studied. Different durations of exposure to a shooting medium containing ZR and shooting media containing BAP or ZR were tested. An earlier report on grapevine growth (Goussard, 1981) had shown that ZR induced a higher level of shoot proliferation than BAP, and that the optimal single concentration of BAP and ZR differed considerably. In our experiments, both cytokinins had a stimulatory effect on shoot tip recovery, even though BAP produced better results.

Our experiments also clearly showed that the culture duration of microcuttings on shooting medium containing ZR should not be longer than 8 weeks, to avoid negative effects on shoot tip recovery after cryopreservation. This finding was important for planning cryopreservation experiments using microcuttings as a source of shoot tips.

It had previously been observed with begonia that adding proline in the pregrowth medium (7 days before cryopreservation) significantly improved recovery after freezing (Buritt, 2008). Our experiments, which included a 2-week culture of microcuttings on proline-containing medium, did not lead to any improvement in recovery. Shorter proline treatment durations may thus be tested.

All these experiments led to improvements in the cryopreservation protocol; however, in view of the results achieved, further improvements are still needed before routine application of the cryopreservation protocol can be envisaged.

Many cryopreservation protocols include a sucrose preculture step, which often consists of a progressive increase in the sucrose concentration, in order to allow explants to withstand high final sucrose concentrations (Wang et al., 2000; 2003, Matsumoto and Sakai, 2003; Zhao et al., 2001). In our experiments, we tested three different sucrose preculture procedures including culture on medium with 0.1 M sucrose for 24 h (base protocol); 2) culture on medium with 0.3 M sucrose for 72 h (Matsumoto and Sakai, 2003); and 3) culture on medium with 0.3 M sucrose for 24 h, 0.5 M sucrose for 24 h and 0.7 M sucrose for 8 h (Kim et al., 2009). No marked differences in recovery of shoot tips after exposure to PVS2 were noted following these three preculture procedures.

Kim et al. (2009) designed alternative vitrification solutions by increasing or decreasing the concentration of the constitutive cryoprotectants of the original PVS2 and PVS3 solutions and tested their applicability using the D-V procedure. Two model plant species were used: large bulbous garlic and dehydration-sensitive chrysanthemum. Garlic shoot apices showed similar recovery whatever the vitrification solution used, whereas chrysanthemum showed high sensitivity to DMSO, EG and glycerol-based vitrification solutions.

In our experiments, exposure to PVS2 for 50 min produced the highest recovery. This duration of exposure to PVS2 and the recovery percentage were similar to those reported by Matsumoto and Sakai (2003). In an attempt to improve recovery, two PVS2- and PVS3-derived alternative solutions were applied. However, no increase in recovery was achieved with the alternative vitrification solutions employed.

After the cryopreservation protocol was established with cultivar Portan, its was tested on other cultivars. We first focused on Croatian autochthonous cultivars of interest, as the main objective of our work was to conserve this material. Unfortunately, because of the difficulties linked to the *in vitro* establishment and multiplication of the Croatian autochthonous cultivars studied, only a small number of cultures of four cultivars could be produced for

cryopreservation experiments. Low recovery was achieved with only two of these four cultivars. The main hypothesis to explain this result is that *in vitro* culture conditions for the Croatian cultivars used were not optimal and that their growth rate was still low. Another possibility could be that the conditions of the cryopreservation protocol were not adapted to the cultivars tested.

We also tested the cryopreservation protocol using infected and healthy genotypes of four international cultivars: Cabernet Sauvignon, Chardonnay, Merlot and Pinot Noir. Matsumoto and Sakai (2003) reported cryopreservation of cultivars Cabernet Sauvignon and Merlot using a two-step vitrification protocol, with recovery of 80% and 86.7%, respectively. Using an E-D protocol, Plessis et al. (1991; 1993) obtained around 30% recovery of Chardonnay shoot tips after LN exposure. In our experiments with the D-V technique, we obtained comparable recovery with Chardonnay, 70% recovery with Merlot and 46.6% recovery with Cabernet Sauvignon.

The differences observed between cultivars and the similar recovery results obtained in our experiments and in other reports with the same cultivars indicate that the response of grapevine shoot tips to cryopreservation is highly genotype-specific. Cultivars may prove more tolerant or sensitive to a particular procedure and each cultivar may also have its own limitation no matter which protocol is applied.

5.3 Cryotherapy of grapevine shoots tips

Recently, Engelmann (2012) reported new possibilities provided by cryopreservation for improving *ex situ* conservation of plant biodiversity in genebanks. One of them is cryotherapy as a novel method for virus eradication (Wang et al., 2008).

In cryotherapy, plant pathogens such as viruses, phytoplasmas and bacteria are eradicated from shoot tips by exposing them to LN. The uneven distribution of viruses and obligate vasculature-limited microbes in shoot tips allows their elimination through the destruction of infected cells by the cryotreatment and regeneration of healthy shoots from the surviving pathogen-free meristematic cells.

Being a vegetatively propagated species, grapevine is often highly virus- infected. This is the case of Croatian grapevine autochthonous cultivars located in the costal part of Croatia (Karoglan Kontić et al., 2009). GLRaV-3 is the most widespread virus in this wine region.

Cryotherapy was efficient in eradicating seven unrelated groups of viruses in different plant species including *Prunus, Citrus,* berry plants, banana, grape, potato and sweet potato, (Brison et al.,1997; Ding et al., 2008; Helliot et al., 2002; Wang et al., 2003b; Wang et al., 2006; Wang et al., 2008; Wang and Valkonen, 2008a,b,c). The only previous report of cryotherapy with grapevine mentioned the elimination of the GVA virus (Wang et al., 2003b). GVA had been mentioned as being difficult to eliminate from infected grapevine compared with closteroviruses (Gribaudo et al., 1997). In this cryotherapy study, the effect of cryopreservation on GVA elimination was associated exclusively with the freezing step (Wang et al., 2003b); however, many details were missing to conclude how elimination occurred.

In our experiments, we applied cryotherapy to healthy and infected genotypes of international cultivars. Infected genotypes contained economically important grapevine viruses: GFLV and GLRaV-3.

After cryopreservation, recovery was achieved with only two cultivars (Chardonnay and Cabernet Sauvignon), from which mature plants were regenerated and subjected to virus testing. Our results revealed that GLRaV-3 was completely eliminated from the tested material and that GFLV was eliminated in 77% of the plants tested. The possible reasons for this difference in the success of elimination of these two viruses may be related to their localization in infected tissues. Closterolike-virus particles, such as GLRaV-3, are restricted to vascular tissues (phloem) (Namba et al., 1979) whereas GFLV particles were present in very young meristematic tissues (i.e. meristematic domes of shoot apices) of grapevine material infected with fanleaf. Our immunolocalisation observations indicated that GLRaV-3 was located in leaf veins of cultivar Merlot. Cultivar Pinot Noir infected with GFLV had a virus signal in the apical dome and meristematic tissues.

Contrarily to the results of Wang et al. (2003), who showed that GVA virus elimination from grapevine shoot tips occurred following LN exposure, our results revealed that elimination of GFLV and GLRaV-3 was possible following PVS2 treatment, i.e. before LN exposure.

A possible explanation may be the relatively small size of explants used (1 mm), which could already be free of infected cells, as it is well documented on different viruses and species that explant size is crucial for the success of virus eradication (Brison et al., 1997; Faccioli and Marani, 1998; Ding et al., 2008). The other, more likely, explanation may be found in the exposure of shoot tips to the highly concentrated PVS2 vitrification solution, which would penetrate deeply in shoot tips and kill the cells containing viruses, thus having a "chemotherapeutic" effect. Apart from the work of Wang et al. (2003), who did not mention the production of virus-free plants before LN exposure, no other cryotherapy report includes virus testing of plants regenerated from treatment steps before LN exposure.

5.4 Genetic stability

During the cryopreservation process, the plants are exposed to a series of streses. Many factors are capable of inducing genetic variation in cryopreserved materials such as the type of explants selected, the freezing procedure employed and regrowth pattern achieved.

In this study, AFLP markers were employed to test the genetic stability of grapevine *in vitro* plantlets regenerated following cryopreservation. Our results clearly showed no significant differences in AFLP profiles in controls treatments such as sucrose preculture, treatment with the loading solution and with half-strength PVS2. However, an increase in polymorphic fragments was observed in non-cryopreserved and cryopreserved samples treated with PVS2 solution, the number of polymorphic fragments increasing with longer durations of exposure to PVS2 solution.

These results are not sufficient to conclude that cryopreservation induces genetic changes in regenerated plants. The genetic stability test should be repeated to determine the main reason why polymorphism occurred. There are no reports so far, which observe similar results.

However, it should be noted that there are some reports of a high level of polymorphism in grapevine. Stajner et al. (2009) performed AFLP analysis using nine primer combinations on 14 Syrah and 22 Malbec clones and obtained a total of 411 bands, of which 65% were polymorphic. These authors reported 33.82% and 63.88% of polymorphic bands in Syrah and Malbec clones, respectively. Meneghetti et al. (2012) tested 10 Malvasia genotypes by AFLP, SAMPL and M-AFLP markers and found a high level of intra genetic variability. Using 16 primer combinations, they obtained 682 bands from AFLPs and 308 of them were polymorphic. These results confirmed a high intra-variability within grapevine clones. Based on these studies, the level of polymorphism obtained in our results is in the range of that observed between clones within a grapevine cultivar. Additional studies should thus be performed with plants regenerated from cryopreserved shoot tips treated with PVS2 for 50 min. Other molecular markers like MSAP and SNP should be included in additional genetic fidelity assessments. Most importantly, plants should be followed for several years in field conditions in order to compare their morphological and important agronomic characteristics with those of mother plants.

6 CONCLUSIONS AND GENERAL PERSPECTIVES

6.1 Conclusion

1. It has been possible to successfully establish *in vitro* cultures of eight Croatian autochthonous varieties: Debit, Grk, Plavina, Lasina, Plavac mali, Pošip, Vugava, Maraština and virus-infected genotypes of Plavac mali. Although differences were observed in growth parameters between cultivars based on their genetic relationship, our objective, which was to provide a sufficient quantity of plant material for experiments, has been fulfilled.
2. We have shown that cultivar and interaction between cultivar and medium had a significant effect on survival and regrowth of nodal segments *in vitro*. Infected genotypes were less reactive, but a sufficient quantity of plant material for virus eradication could be obtained. No difference was observed between genotypes of Plavac mali infected with different virus strains.
3. An efficient cryopreservation protocol has been established for grapevine shoot tips using the D-V technique, reaching approx. 50% recovery. The results obtained highlighted the importance of adjusting different steps and parameters of the protocol.
   - The duration of exposure and the choice of the vitrification solution influenced significantly recovery after LN exposure. In comparison with PVS3 and some PVS2- and PVS3-derived alternative solutions, PVS2 led to the highest recovery percentage. Out of all treatment durations tested, ranging from 15 to 75 min, a 50 min exposure to PVS2 appeared to be most suitable for grapevine.
   - The position of the buds on the stem of *in vitro* mother-plants influenced the success of cryopreservation. Actively growing buds sampled from microcuttings displayed higher regrowth after LN exposure compared to buds sampled directly from *in vitro* plantlets. The addition of BAP to the shooting medium had a positive effect on regrowth after LN exposure; exposure to ZR shooting medium should be no longer then 8 weeks.
4. The response of grapevine to cryopreservation was highly genotype- specific. Three out of the four international cultivars tested produced high recovery, comprised between 30 and 70%. By contrast, recovery of Croatian autochthonous cultivars was very low.
5. Cryotherapy has a high potential for elimination of grapevine viruses. The cryopreservation protocol used led to 100% elimination of GLRaV-3 and to 77% elimination of GFLV. This result has been substantiated with immunolocalisation studies, which showed that GLRaV-3 was located in sieve elements of grapevine tissues while GFLV was found in the meristematic dome. In some cases virus elimination was observed already following PVS2 treatment. This may be due to the small size of explants used or to a “chemotherapeutic” effect of the PVS2 vitrification solution.
6. Genetic analyses performed with AFLP markers showed some polymorphism in plants regenerated from shoot tips following exposure to PVS2 solution. All control steps of cryopreservation protocol were close to control *in vitro* plantlets, whereas three samples deriving from cryopreserved material previously exposed to PVS2 for 50 min showed higher level of polymorphism with a similarity coefficient of 0.66.

6.2 General perspectives

In this study it was confirmed that one of the main prerequisites for the use of cryopreservation and cryotherapy for long-term maintenance and sanitation of grapevine genetic resources is the availability of a tissue culture protocol suitable to different grapevine genotypes. The rate of survival and regrowth after cryopreservation would be increased by using plant material of genotypes well adapted to *in vitro* conditions. So, further efforts will be put into the *in vitro* introduction of a broader range of cultivars, especially Croatian autochthonous ones, and into adjusting the *in vitro* culture medium in order to get a satisfactory multiplication rate for each of them. Special attention will be paid to the virus-infected genotypes because of their lower *in vitro* response.

The droplet-vitrification cryopreservation protocol established produced satisfactory recovery. The availability of a reliable cryogenic protocol producing a suitable recovery percentage with one cultivar is a basic requirement for its application to other cultivars. Our cryopreservation protocol achieved suitable results with cv. Portan, the most used in our experiments, but its broader application did not produce the expected results. Possible reasons may be the high genotype specificity of grapevine as a species or the low adaptability of the protocol for wider application. Therefore, our experiments suggest further steps to define a protocol that would be applicable to a wider range of grapevine cultivars. Testing the newly developed D-cryoplate and V-cryoplate techniques may also prove relevant. As preparation of material in the form of microcuttings improved our results, there are still possibilities for further adjustments in BAP concentration in shooting medium. Exposure to the PVS2 solution for 50 min led to 50% recovery after LN exposure. The use of additional alternative vitrification solutions designed for sensitive species may improve this recovery percentage.

Although a high sanitation rate was obtained using cryotherapy, our results were only very preliminary since they concerned only two cultivars and two viruses and since only a small number of plants were analyzed. The high proportion of virus-free plants obtained before LN exposure indicated that treatment of shoot tips with a vitrification solution may have a chemotherapeutic effect on virus infection. Further studies on the use of PVS2 and of other vitrification solutions for virus elimination without LN exposure should be performed. Experiments should be carried out using explants of different sizes, additional cultivars and virus strains, including immunolocalisation studies after the different steps of protocol. The efficiency of exposure to a vitrification solution and of cryotherapy on virus eradication should be compared with standard methods, such as meristem culture.

Genetic stability is a major concern in relation to *in vitro* storage and cryopreservation of plant material. In our experiments, some polymorphism was observed in the regenerants analyzed by AFLP molecular markers after different steps of protocol. Our results are not sufficient to conclude that cryopreservation induces genetic instability in plants (because they reflect the screening of only a small portion of the whole genomic DNA), but they certainly call for particular caution in some steps of the protocol. In order to get a better understanding of the nature and meaning of this variability, some additional molecular markers (such as MSAP and SNP) should be used in the future. Most importantly, in addition to genetic analysis, monitoring of morphological and agronomical traits of plants of a set of genotypes deriving from selected steps of the cryopreservation protocol should be performed for some years after their transfer to in vivo conditions. This study would provide a more comprehensive insight into the impact of cryopreservation on genetic stability of regenerants.

From a practical perspective, we have succeeded in establishing *in vitro* cultures of Croatian cultivars. We also have established an efficient PVS2-based cryopreservation protocol, which has proved to be efficient in eliminating two grapevine viruses. These results pave the way to the large scale application of cryopreservation for the long-term conservation and sanitation of important Croatian grapevine cultivars.

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8 ANNEXES

1- Participation in International Congres of Croatian Genetics, Krk, 2012.
2- Participation in 44th Croatian and 4th International Symposium on Agriculture, Opatija, 2009.
3- Participation in *In vitro micropropagation and conservation* course held by Suceava Gene Bank, Romania, 2009.
4- Presented poster and short publication in Proceeding of COST871 meeting, Angers, 2011.
5- Presented poster and abstract in Congres of Croatian Genetics, Krk, 2012.
6- Article *Effect of shooting medium and source of material on grapevine (Vitis vinifera L.) shoot tip recovery after cryopreservation*, Marković; 2014 CryoLetters 35, in press.
7- Presented short report in Fort Collins, on International Symposium of Plant Cryopreservation, Marković; Acta Horticulturae accepted for publication.
8- Article *In vitro introduction of healthy and virus-infected genotypes of native Croatian grapevine cultivars*, first page and abstract, Marković; submitted to Central European Journal of Biology.
9- Article *Cryopreservation of grapevine (Vitis vinifera L.) in vitro shoot tips using encapsulation-dehydration and droplet-vitrification*, Marković, published in Central European Journal of Biology (2013) 8(10),993-1000.
10- Article *Effect of proline pretreatment on grapevine shoot-tip response to a droplet-vitrification protocol*; Marković 2013. American Journal of Plant Sciences 4, in press.

ANNEX I

Hrvatskogenetičkodruštvo
CroatianGeneticSociety

Kršnjavoga 25, 10 000 Zagreb, Croatia Tel.+385 1 48 36 013 Tel/Fax. +385 1 48 36 016
http://www.genetika.hr

CERTIFICATE OF ATTENDANCE

We certify that Zvjezdana Marković has attend 3rd Congress of Croatian Genetics with international participation held in Krk, May 13-16, 2012.

Professor Jasna Franekić
President of the Scientific Committee

HRVATSKO GENETIČKO DRUŠTVO ZAGREB Kršnjavoga 25

Professor Verica Garaj-Vrhovac
President of the Organizing Committee

ANNEX II

Croatian 2009 International Symposium on Agriculture

44th Croatian and 4th International Symposium on Agriculture
16 – 20 February 2009 Opatija, Croatia

CERTIFICATE OF ATTENDANCE

We hereby confirm that

Zvjezdana Marković

has attended the

44th Croatian & 4th International Symposium on Agriculture
held in Opatija (Croatia), February 16 - 20, 2009

President of the Organising Committee

Prof.dr. Vlado Guberac

Hrvatski 2009 Međunarodni Simpozij Agronoma

ANNEX III



SEEDNet

CERTIFICATE

Zvjezdana Marković

Has successfully completed training course on

***In vitro* micropropagation and conservation**

Held by the Suceava Gene Bank

19 - 22 of October 2009

SUCEAVA GENEBANK

Director
Dr. Silvia Străjeru

ANNEX IV

Duration of culture of grapevine (*Vitis vinifera*) microcuttings on medium with zeatin riboside affects shoot tip recovery after cryopreservation

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Introduction

Cryopreservation is the only method currently available for the safe, cost-effective, long-term conservation of vegetatively plant species such as grapevine. Cryopreservation protocols have been established for shoot tips sampled from grapevine *in vitro* plantlets (Wang, 2000; Matsumoto & Sakai, 2003).
An important parameter for successful cryopreservation is the physiological state of the plant material. Zeatin riboside (ZR) is a growth regulator, which has been shown to stimulate bud proliferation (Goussard, 1987). Culture duration on ZR medium can affect the physiological state of the plant material. In this study, we investigated the effect of the duration of culture on ZR medium of grape microcuttings on shoot tip recovery after cryopreservation.

Material and methods

Plant material

The plant material employed in this study consisted of *in vitro* plantlets of grapevine (*Vitis vinifera*) cultivar Portan, (initial material courtesy of the late Dr A. Bouquet) obtained from the INRA grapevine germplasm field repository in Vassal, France.

Methods

In vitro culture

In vitro plantlets were cultured on ½ MS medium (Murashige & Skoog, 1962) containing 20 g/l sucrose and 7 g/l agar. They were kept without subculture for 2 months at 26 ±1°C under a 16 h light/8 h dark photoperiod, with a light intensity of 3000 lux until they were approximately 12 cm long. The plantlets were then dissected in microcuttings consisting of a stem fragment of 1,5 cm with one bud, which were placed on culture medium containing 1 µM ZR, and kept on this medium for 4 to 16 weeks with subcultures on new medium every 4 weeks. Control samples consisted of shoot tips, which were not cultured on medium with ZR.

Cryopreservation

The droplet-vitrification technique was employed for cryopreservation of shoot tips. After excision, shoot tips were placed for 24 h on medium containing 0.3 M sucrose, treated for 20 min with a loading solution containing 1.2 M glycerol + 0.4 M sucrose, then with half-strength PVS2 vitrification solution (Matsumoto & Sakai, 2003.) for 30 min, dehydrated with full-strength PVS2 for 25, 50 or 75 min, cooled rapidly in liquid nitrogen in PVS2 droplets placed on aluminium foils, rewarmed rapidly by immersion of foils in unloading solution containing 1.2 M sucrose for 20 min.

Results

Recovery of cryopreserved shoot tips was achieved after up to 8 weeks of culture on ZR medium (Table 1). No regrowth was obtained after longer culture durations. The highest regrowth percentages (40-45%) were noted with shoot tips cultured on ZR medium for 4 or 8 weeks, which had been treated with PVS2 for 75 min. Regrowth of cryopreserved shoot tips was rapid and direct (Fig. 1). Extended culture duration on ZR medium induced callogenesis during the development of microcuttings (Fig. 2).

Table 1. Effect of culture duration on medium with ZR and of duration of exposure to PVS2 on recovery (%) of grapevine shoot tips after cryopreservation.

Culture duration on ZR	Recovery (%)		
medium (weeks)	Duration of exposure to PVS2 (min)		
	25	50	75
0	33	6	20
4	18	20	40
8	0	30	45
12	0	0	0
16	0	0	0

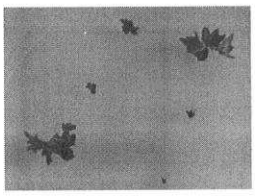

Figure 1. Regrowth of cryopreserved shoot tips treated with PVS2 for 75 min.

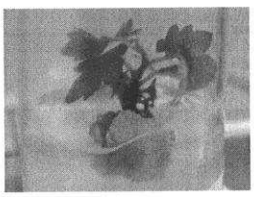

Figure 2. Extended culture duration on ZR medium induced callogenesis

Conclusion

This study demonstrated that the culture duration of microcuttings on ZR had an effect on recovery of shoot tips after cryopreservation. Shoot tips should be sampled on microcuttings cultured on ZR medium for 0-8 weeks.
Only intermediate recovery was obtained after cryopreservation in these preliminary experiments. Improved results may be achieved notably by modifying the duration of treatment with PVS2, or by using other vitrification solutions such as PVS3 or alternative solutions developed by Kim *et al.* (2009).

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ANNEX IV

Duration of culture of grapevine (*Vitis vinifera*) microcuttings on medium with zeatin riboside affects shoot tip recovery after cryopreservation

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1. Introduction

Cryopreservation is the only method currently available for the safe, cost-effective, long-term conservation of vegetatively propagated plant species such as grapevine. Cryopreservation protocols have been established for shoot tips sampled from grapevine *in vitro* plantlets (Plessis et al 1993; Wang et al 2000; Zhao et al 2001; Matsumoto and Sakai 2003).
An important parameter for successful cryopreservation is the physiological state of the plant material, and buds taken at different levels on the shoots of the stock plants could differ in this regard. It can then be advisable to preculture microcuttings to obtain axillary shoots from which terminal buds can be sampled, constituting a more homogenous explant population. A cytokinin can be used at this stage to achieve quicker and more homogenous shooting from the microcuttings. Zeatin riboside (ZR) has been shown to stimulate bud proliferation in grapevine (Goussard 1987). As culture duration on ZR medium could affect the physiological state of the plant material, we investigated the effect of this parameter on grape shoot tip recovery after cryopreservation.

2. Material and methods

2.1.Plant material

The plant material employed in this study consisted of *in vitro* plantlets of grapevine (*Vitis vinifera*) cultivar Portan, (initial material courtesy of the late Dr A. Bouquet) obtained from the INRA grapevine germplasm field repository in Vassal, France.

2.2.Methods

In vitro culture

In vitro plantlets were cultured on ½ MS medium (Murashige and Skoog 1962) containing 20 g/l sucrose and 7 g/l agar. They were kept without subculture for 2 months at 26 ±1 ℃ under a 16 h light/8 h dark photoperiod, with a light intensity of 3000 lux until they were approximately 12 cm long. The plantlets were then dissected in microcuttings consisting of stem fragments of approx. 1.5 cm with one bud, which were placed on culture medium containing 1 µM ZR, and kept on this medium for 4 to 16 weeks with subcultures on new medium every 4 weeks. Control samples consisted of shoot tips, which were not cultured on medium with ZR. Mean explants number per treatment was 10.

2.3.Cryopreservation

The droplet-vitrification technique was employed for shoot tip cryopreservation. After excision, shoot tips were placed for 24 h on a medium containing 0.3 M sucrose, treated for

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20 min with a loading solution containing 1.2 M glycerol + 0.4 M sucrose, then with cold (0°C) half-strength PVS2 vitrification solution (Matsumoto and Sakai 2003) for 30 min, dehydrated with full-strength PVS2 for 25, 50 or 75 min, cooled rapidly in liquid nitrogen in PVS2 droplets placed on aluminium foils, rewarmed rapidly by immersion of foils in an unloading solution containing 1.2 M sucrose for 20 min, then transferred for recovery on ½ MS based with 1µM BA (Wang et al. 2003).

3. Results

Recovery of cryopreserved shoot tips was achieved after up to 8 weeks of culture on ZR medium (Table 1). No regrowth was obtained after longer culture durations. The highest regrowth percentages (40-45%) were noted with shoot tips cultured on ZR medium for 4 or 8 weeks, which had been treated with PVS2 for 75 min. Regrowth of cryopreserved shoot tips was rapid and direct (Fig. 1). Extended culture duration on ZR medium induced callogenesis during the development of microcuttings (Fig. 2).

Table 1. Effect of culture duration on medium with ZR and of duration of exposure to PVS2 on recovery (%) of grapevine shoot tips after cryopreservation.

Culture medium	Recovery (%) Duration of exposure to PVS2		
	25	**50**	**75**
0	33	6	20
4	18	20	40
8	0	30	45
12	0	0	0
16	0	0	0

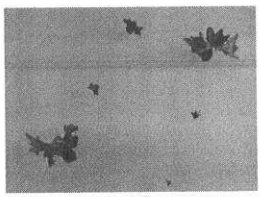

Figure 1. Regrowth of cryopreserved shoot tips treated with PVS2 for 75 min.



Figure 2. Extended culture duration of shoot tips on ZR medium induced callogenesis.

4. Discussion

This study demonstrated that microcutting culture duration on ZR affected shoot tip recovery percentage after cryopreservation. Shoot tips should be sampled on microcuttings cultured on ZR medium for 0-8 weeks. Although a stimulatory effect of preculture cannot be undoubtedly demonstrated from these experiments, it can be remarked that the highest recovery percentages obtained in this study were achieved after one or two preculture cycles on ZR-containing medium.

Only intermediate recovery was obtained after cryopreservation in these preliminary experiments. Improved results may be achieved notably by modifying PVS2 treatment duration, or by using other vitrification solutions such as PVS3 or recently developed alternative solutions (Kim et al. 2009).

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ANNEX V

In Vitro Regeneration Capability Of Healthy And Infected Genotypes Of Cv. *Plavac Mali*

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Introduction

Plavac mali is the major red wine cultivar (Vitis vinifera L.) in Croatia (Bulić, 1949). The survey of virus diseases in commercial vineyards of the coastal vine-growing region disclosed a widespread presence of viruses and a low occurrence of non-infected vines in population of this cultivar. Viruses that cause negative effects on quality and quantity of the yield are grapevine fanleaf virus (GFLV), rugose wood virus, grape leafroll associated viruses (GLRaVs) and grape fleck virus (GFKV) (Martelli 2003).
In order to start sanitation program, we tested *in vitro* requirements of healthy and infected genotypes of Plavac mali.

Materials and Methods

Plant material

The plant material of six genotypes cv. *Plavac mali* was taken at the beginning of December 2010. from the Institute for Adriatic Crops and Carst Reclamation grapevine germplasm repository Duilovo, Split.

Table 1: Results of serological test (ELISA) for six genotypes of cv. *Plavac mali*

No.	Genotype	Virus
1.	PMC 201	-
2.	PMC 008/1	GLRaV-3 + GVA
3.	PMC 010/1	GLRaV-1 + GLRaV-3
4.	PMC 064/3	GLRaV-1
5.	PMC 225/2	GLRaV-3
6.	PMC 201/6	GFLV

In vitro inoculation

Sterilized grapevine buds were cultured on ½ MS medium (M1) (Murashige & Skoog, 1962) - all genotypes, and variants M2 (full-strength MS) and M3 (MS+1mg/L BAP) - healthy genotype containing 20 g/l sucrose and 7 g/l agar. Observations of growing rate: shoot length (mm) and the number of leaves, were initiated 8 weeks after inoculation.

Results

Healthy genotype

Table 2: Influence of mediums on *in vitro* growth parameters in node culture of cv. *Plavac mali*

Genotype	N	Shoot lenght (cm)	Number of leaves
½ MS	39	6,33[a]	7,36[a]
MS	20	5,99[a]	6,3[ab]
MS+BAP	22	2,37[b]	5,36[b]

Mean values followed by the same letter are not significantly different according to Duncan's range test (P ≤ 0.05)



Picture 1. PMC 201 on M1 medium



Picture 2. PMC 201 on M3 medium

The highest growth parameters were achieved on M1 medium. Occurrence of hyperhydration, callus formation and axillary branching were observed on M3 medium, supplemented with BAP.

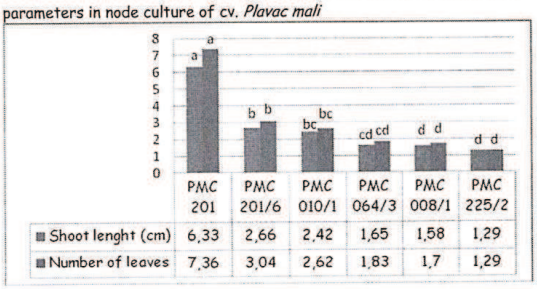
Infected genotypes

Table 3: Influence of virus infection on *in vitro* growth parameters in node culture of cv. *Plavac mali* after 8 weeks on M1 medium

Genotype	N	Shoot lenght (cm)	Number of leaves
Healthy	81	5,17[a]	6,56[a]
Infected	137	1,92[b]	2,09[b]

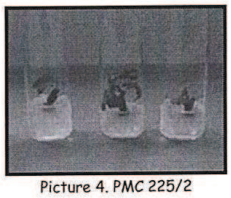
Mean values followed by the same letter are not significantly different according to Duncan's range test (P ≤ 0.05)

Table 4: Influence of infection with different viruses on *in vitro* growth parameters in node culture of cv. *Plavac mali*





Picture 3. PMC 064/3



Picture 4. PMC 225/2

Conclusion

Obtained results showed that sanitary status influenced significantly on regeneration capacity of nodal segments in culture *in vitro*. Growth parameters of healthy Plavac mali were significantly higher than those of any other infected genotype. The genotype with virus GLRaV-3 showed the lowest results in shoot length and the number of leaves. Visual observation confirmed weak growth of infected genotypes.

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IN VITRO* REGENERATION CAPABILITY OF HEALTHY AND INFECTED GENOTYPES OF cv. *PLAVAC MALI

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Plavac mali is Croatian autochthonous cultivar of great economic interest. The survey of virus diseases in commercial vineyards of the coastal vine-growing region disclosed a widespread presence of viruses and a low occurrence of non-infected vines in population of this cultivar. In order to start sanitation program, we tested *in vitro* requirements of healthy and infected genotypes of Plavac mali. Excised nodal segments of healthy genotype were inoculated on three different media: half-strenght MS (M1), full-strenght MS (M2) and full-strenght MS supplemented with 1mg/L^{-1} BAP growth regulator (M3). Growing rate, evaluated on the base of shoot length (mm) and the number of leaves, were significantly higher on M1 and M2, media without growth regulator, in comparison with M3 medium where callus formation and vitrification were observed. Five genotypes infected with specific combination of viruses (GFLV,GLRaV-1,GLRaV-3,GLRaV-1+GLRaV-3 and GLRaV-3+GVA) were grown on M1 medium. Obtained results showed that sanitary status influenced significantly on regeneration capacity of nodal segments in culture *in vitro*. Shoot length and the number of leaves of healthy Plavac mali were significantly higher than those of any other infected genotype. Difference in growing rate between genotypes infected with different viruses or virus combination where also observed. The genotype with virus GLRaV-3 showed the lowest results in shoot length and the number of leaves.

ANNEX VI

EFFECT OF SHOOTING MEDIUM AND SOURCE OF MATERIAL ON GRAPEVINE (*Vitis vinifera* L.) SHOOT TIP RECOVERY AFTER CRYOPRESERVATION

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Abstract

In this work we investigated the effect of the physiological state of grapevine buds on their regrowth after liquid nitrogen exposure. In a first set of experiments, we tested the regrowth of cryopreserved buds sampled from microcuttings cultured on shooting medium containing benzylaminopurine or zeatin riboside for various durations. Regrowth of cryopreserved buds sampled from microcuttings was higher (30%), compared to buds sampled directly from *in vitro* plantlets (23%), for all culture durations of microcuttings on shooting medium tested (2-6 weeks). Addition of cytokinin in the shooting medium improved regrowth of cryopreserved buds compared to buds sampled from microcuttings cultured on medium devoid of growth regulators; however similar results were obtained with the two cytokinins tested. In a second set of experiments, we studied the regrowth after liquid nitrogen exposure of buds sampled from different positions on the stem of *in vitro* plantlets. Buds sampled on nodes 3-4 and 6-7 (from the top of the stem) displayed higher regrowth compared to shoot tips. No significant differences were noted in regrowth after cryopreservation between buds sampled from microcuttings produced from the terminal node, or nodes 3-4 and 6-7.

Keywords: droplet-vitrification, shoot tips, growth regulators, position of buds.

INTRODUCTION

Cryopreservation (liquid nitrogen [LN], -196°C) is the only method currently available for safe and cost-effective long-term conservation of vegetatively propagated plant species such as grapevine. A range of efficient vitrification-based cryopreservation techniques has been developed over the last 25 years and applied to numerous plant species (26, 22). Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, whilst avoiding the formation of crystalline ice (4). Among the vitrification-based procedures established, encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet-vitrification are the most commonly employed for bud cryopreservation (26).

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In the case of grapevine, cryopreservation protocols have been established for shoot tips sampled from *in vitro* plantlets using encapsulation-dehydration (20, 21, 29, 31), vitrification (14, 15) and, more recently, droplet-vitrification (13).

Selecting experimental material at the optimal physiological stage is of paramount importance for successful cryopreservation (3). In the case of cold-tolerant species, mother-plants can be exposed to low temperature treatments to induce cold-acclimation processes. Shoot tips are generally excised from actively growing mother-plants, thus ensuring that they are composed of actively dividing meristematic cells, a characteristic that increases their tolerance to dehydration and LN exposure.

Buds located at different positions on a shoot are at different physiological stages (7), which may impact cryopreservation results. In the case of carnation, the lower the position of axillary buds on the stem (starting from the terminal bud) the lower their regrowth after LN exposure (2). By contrast, Plessis *et al.* (21) indicated that survival of grape shoot tips after cryopreservation was independent of their position on the stem. In order to reduce any potential heterogeneity in the physiological state of the buds employed for cryopreservation, Charoensub *et al.* (1) suggested cutting *in vitro* mother-plants into mononodal microcuttings and culturing those on fresh shooting medium for a given period of time prior to excision of homogenous and actively growing apices.

The composition of shooting medium, including notably the nature and concentration of growth regulators, can have a strong effect on the growth of microcuttings and thus on the physiological state of the buds. In the case of grapevine, benzylaminopurine (BAP) has been frequently used for multiplication (6, 10, 11, 16). However, hyperhydricity of cultures was noted when BAP was used over a threshold concentration (11). Zeatin riboside (ZR) has also been shown to stimulate bud proliferation in grapevine (8). In relation to cryopreservation, Wang *et al.* (30) investigated the effect of BAP addition to the recovery medium on regrowth of cryopreserved grapevine shoot tips. However, no study on the effect of growth regulator addition in the shooting medium has yet been performed with grapevine.

The objective of the present study was to investigate shoot regrowth from cryopreserved buds sampled from microcuttings cultured on shooting medium containing BAP or ZR at various concentrations and for different durations, and to study shoot regrowth after LN exposure of buds sampled at different levels on the stem of *in vitro* plantlets.

MATERIALS AND METHODS

Plant material

The plant material employed in this study consisted of 2-month old *in vitro* plants of grapevine (*Vitis vinifera* L.) cultivar Portan. These cultures were initially established from field-grown plants in the grape germplasm collection of Institut national de la recherche agronomique (INRA), Vassal (France).

Methods

In vitro culture

In vitro culture of mother-plants and of single node microcuttings

Grape *in vitro* plantlets were cultured on basal medium (BM) composed of half-strength MS (18) mineral elements with Morel's vitamins (17), 3% sucrose and 0.7% agar (Sigma) at pH 5.8. They were cultured at 24 ± 2 °C under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. *In vitro* mother-plants were kept without subculture for 2 months to reach a length of approximately 12 cm before use for cryopreservation experiments. These *in vitro* mother-plants were cut into single node

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microcuttings of approx. 1.5 cm in length, which were transferred to 9 cm Petri dishes (20 microcuttings/Petri dish) and placed on shooting medium consisting of ½ MS medium containing 20 g/l sucrose, 7 g/l microagar and 1 µMol ZR or BA or devoid of growth regulators. Petri dishes were placed in the environmental conditions described above. One-mm long shoot tips were excised from microcuttings after different durations and used for cryopreservation experiments.

Effect of growth regulators in shooting medium

Two experiments were performed to test the effect of growth regulators in shooting medium on growth recovery of cryopreserved shoot tips.

In the first experiment, microcuttings were placed on shooting medium with 1 µMol ZR and buds were excised after 2, 4 or 6 weeks. Control buds were excised directly from *in vitro* plantlets. This experiment was carried out in IRD Montpellier.

In the second experiment, microcuttings were cultured on shooting medium devoid of growth regulators or containing 1 µMol BAP or ZR; the buds were excised after 2 weeks. This experiment was carried out in Zagreb University.

Effect of source of material

Two experiments were performed in Zagreb University to test the effect of material source on growth recovery of cryopreserved shoot tips.

In the first experiment, buds were dissected from 2 month-old *in vitro* plantlets, bearing eight-nine buds. Buds were split in three groups depending on their position on the stem and used directly for cryopreservation experiments. The first group included terminal buds (node 1), the second group buds from nodes 3 and 4 from the top and the third one buds from nodes 6 and 7 from the top.

In the second experiment, *in vitro* plantlets were cut into single node microcuttings, which were split in the three groups described above. These microcuttings were cultured for 2 weeks on shooting medium with 1 µMol BAP before bud excision and their cryopreservation.

Control buds were dissected from nodes 1, 3-4, 6-7 and used directly for cryopreservation experiments.

Cryopreservation

Excised buds were cryopreserved using the droplet-vitrification technique developed by Markovic *et al.* (13). They were precultured on solid ½ MS medium with 0.1 M sucrose for 24 h. Precultured buds were then treated with a loading solution (LS) containing 2 M glycerol + 0.4 M sucrose in MS medium (25) for 20 min at room temperature. Buds were dehydrated with half-strength PVS2 at room temperature for 30 min, then with full strength PVS2 at 0°C for 25, 50 and 75 min, except in the second experiment on the effect of source of material, where only a 50 min PVS2 treatment was used. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% dimethylsulfoxide (DMSO) and 0.4 M sucrose in MS medium (24). Buds were then placed on aluminium foils in 5 µl droplets of PVS2 and immersed in LN for at least 1 h. For rewarming, the aluminium foils with the buds were immersed for 20 min in unloading solution containing 1.2 M sucrose (23) at room temperature, then transferred to recovery medium. Explants were post-cultured on medium containing 1µMol BAP, maintained in the dark at 26°C for 7 days and then transferred to the conditions described for stock cultures.

Assessment of survival and regrowth and statistical analyses

Survival was evaluated 2 weeks after cryopreservation by counting the number of shoots that showed any type of growth, while regrowth was defined as the development of apices into

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shoots with expanded leaves 8 weeks after rewarming. Both survival and regrowth percentages were expressed relative to the total number of shoot tips treated. Experiments studying the effect of growth regulators in shooting medium were replicated thrice, with 10-15 shoot tips per experimental condition and in all other experiments 15-20 explants per experimental condition were used.

In the first experiment the effect of growth regulators in shooting medium, survival and regrowth data, presented as mean percentages with standard error of the mean (SD) were subjected to arcsine transformation. Statistical differences between mean values of all parameters were assessed by analysis of variance (ANOVA) and Duncan's Multiple Range Test for mean separation. Duncan's Multiple Range Test was performed for comparing percentages between conditions ($P \leq 0.05$), using the SAS software (27).

RESULTS

Effect of growth regulators in shooting medium

When buds were excised from microcuttings cultured on shooting medium with 1 µMol ZR, survival of LS and ½ PVS2 controls was very high (88-100%), for all times of exposure tested (Table 1); however, survival of non-cryopreserved explants decreased progressively with increasing PVS2 treatment durations. A similar pattern was observed with cryopreserved buds but survival values were generally lower compared to non-cryopreserved explants, especially for the shorter PVS2 treatment durations tested.

Table 1. Effect of time of exposure (weeks) of grapevine single node microcuttings on medium with 1 µMol ZR and of duration (min) of PVS2 treatment on survival (%) of non-cryopreserved (-LN) and cryopreserved (+LN) buds. Data followed by different letters are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

	Survival (% ±SD)			
Time of exposure on shooting medium (weeks)	0	2	4	6
Control LS 20 min	100 a	97 ± 4 a	100 a	98 ± 4 a
Control ½ PVS2 30 min	97 ± 7 a	98± 3 a	95 ± 6 ab	88 ± 10 ab
PVS2 25 min (-LN)	71 ± 28 ab	74 ± 20 b	73± 20 bc	71 ± 13 bc
PVS2 25 min (+LN)	42 ± 16 bc	44 ± 1 cd	59 ± 28 cd	59 ± 8 c
PVS2 50 min (-LN)	51 ± 25 bc	82 ± 15 ab	51 ± 14 cde	63 ± 26 bc
PVS2 50 min (+LN)	32 ± 37 c	53 ± 13 c	51 ± 11 cde	51 ± 11 cd
PVS2 75 min (-LN)	42 ± 14 bc	42 ± 10 cd	36 ± 12 de	32 ± 12 de
PVS2 75 min (+LN)	31 ± 19 c	33 ± 16 d	27 ± 16 e	24 ± 15 e

Regrowth of LS and ½ PVS2 controls was high whatever the microcutting culture duration employed, and was between 61 and 82% (Table 2). Regrowth of non-cryopreserved buds decreased progressively in line with increased time of PVS2 treatment, although this decrease was more pronounced for longer times of exposure on shooting medium (4 and 6 weeks). After LN exposure, regrowth also decreased in line with increasing PVS2 treatment durations. Regrowth of buds cryopreserved directly after excision from *in vitro* plantlets was consistently lower, compared to buds sampled from microcuttings. The highest regrowth values, 48 and 41%, were achieved after a 25-min PVS2 treatment of buds sampled from 4 and 6-week old microcuttings, respectively.

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Table 2. Effect of time of exposure (weeks) of grapevine single node microcuttings on medium with 1 µMol ZR and of duration (min) of PVS2 treatment on regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) buds. Data followed by different letters are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

	Regrowth (% ±SD)			
Time of exposure on shooting medium (weeks)	0	2	4	6
Control LS 20 min	80 ± 20 a	73 ± 15 a	82 ± 14 a	77 ± 30 a
Control ½PVS2 30 min	63 ± 38 ab	61 ± 10 ab	72 ± 16 ab	62 ± 29 ab
PVS2 25 min (-LN)	54 ± 21 abc	44 ± 18 bc	57 ± 18 bc	49 ± 10 abc
PVS2 25 min (+LN)	25 ± 18 bc	32 ± 6 c	48 ± 16 cd	41 ± 12 bc
PVS2 50 min (-LN)	35 ± 26 bc	37 ± 16 c	43 ± 6 cde	45 ± 14 abc
PVS2 50 min (+LN)	23 ± 25 c	30 ± 20 c	35 ± 5 def	32 ± 9 bc
PVS2 75 min (-LN)	36 ± 17 bc	32 ± 7 c	23 ± 2 ef	22 ± 3 c
PVS2 75 min (+LN)	23 ± 17 c	24 ± 13 c	20 ± 21 f	24 ± 15 c

When comparing shooting media devoid of growth regulators and supplemented with ZR or BAP, it appeared, based on factorial ANOVA, that the treatment had a significant effect but that the shooting medium had no significant effect on bud survival and regrowth.

Survival of LS and ½ PVS2 controls was very high (90-100%) for all three shooting media tested (Table 3). Cryopreservation caused a decrease in survival for all times of PVS2 exposure tested, even though changes were statistically significant only in a few experimental conditions. In most experimental conditions, the hormonal content of the shooting medium had no effect on survival of non-cryopreserved buds. After cryopreservation, survival was significantly higher for buds sampled on shooting medium with BAP compared to ZR, for 25 and 75 min PVS2 treatments. After a 50 min exposure to PVS2, survival was similar for buds originating from ZR and BAP shooting media and significantly lower for shooting medium devoid of growth regulators.

Table 3. Effect of shooting medium containing half-strength MS without growth regulators, supplemented with benzyladenine (BAP) or zeatin-riboside (ZR) and of PVS2 treatment duration on survival (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Treatment	Survival (%)		
	No GR	BAP	ZR
Control LS 20 min	90 a/A	90 a/A	100 a/A
Control ½ PVS2 30 min	90 a/A	90 a/A	100 a/A
PVS2 25 min (-LN)	-	100 a/A	100 a/A
PVS2 25 min (+LN)	-	64 a/AB	20 b/C
PVS2 50 min (-LN)	62 a/B	80 a/A	70 a/AB
PVS2 50 min (+LN)	21 b/C	67 a/AB	60 a/B
PVS2 75 min (-LN)	-	100 a/A	58 b/B
PVS2 75 min (+LN)	-	40 a/B	20 b/C

Different lowercase letters indicate significant differences between media; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$). -: not tested.

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Regrowth of LS and ½ PVS2 controls was high (90%) for all three shooting media tested, except for ½ PVS2 controls sampled from shooting medium without growth regulators, which was 60% (Table 4). Regrowth of non-cryopreserved controls decreased with increasing time of PVS2 treatment. After cryopreservation, regrowth was highest (40-44%) after 50 min PVS2 treatment (Table 4). The presence of ZR or BAP in the shooting medium had no significant effect on regrowth of non-cryopreserved and cryopreserved buds. Regrowth of control and cryopreserved buds sampled on medium without growth regulators was significantly lower compared to buds sampled on shooting medium containing BAP or ZR.

Table 4. Effect of shooting medium containing half-strength MS without growth regulators, supplemented with benzyladenine (BAP) or zeatin-riboside (ZR) and of PVS2 treatment duration on regrowth (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Treatment	Regrowth (%)		
	No GR	BAP	ZR
Control LS 20 min	90 a/A	90 a/A	90 a/A
Control ½ PVS2 30 min	60 a/B	90 a/A	90 a/A
PVS2 25 min (-LN)	-	100 a/A	90 a/A
PVS2 25 min (+LN)	-	19 a/B	20 a/B
PVS2 50 min (-LN)	10 b/C	30 a/B	40 a/B
PVS2 50 min (+LN)	11 b/C	44 a/B	40 a/B
PVS2 75 min (-LN)	-	50 a/B	8 b/B
PVS2 75 min (+LN)	-	20 a/B	10 a/B

Different lowercase letters indicate significant differences between media; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$). -: not tested.

Effect of source of material

Variance analysis showed that the treatment had a significant effect but that the position of buds had not a significant effect on bud survival and regrowth ($P<0.05$). Survival of LS and ½ PVS2 controls was between 57-100% for buds of the three groups studied (Table 5).

Table 5. Effect of bud position on the stem of *in vitro* mother-plants and of PVS2 treatment duration on survival (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Treatment	Survival (%)		
	Bud N°1	Buds N° 3-4	Buds N° 6-7
Control LS 20 min	100 a/A	57 b/BCD	100 a/A
Control ½ PVS2 30 min	100.a/A	88 a/AB	100 a/A
PVS2 25 min (-LN)	100 a/A	100 a/A	50 b/C
PVS2 25 min (+LN)	30 a/B	21 a/D	33 a/BC
PVS2 50 min (-LN)	30 a/B	50 a/CD	53 a/C
PVS2 50 min (+LN)	0 b/B	46 a/CD	42 a/C
PVS2 75 min (-LN)	20 b/B	82 a/ABC	86 a/AB
PVS2 75 min (+LN)	0 b/B	47 a/CD	50 a/C

Different lowercase letters indicate significant differences between bud position; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$).

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Survival of non-cryopreserved buds N° 3-4 and 6-7 remained high after the three PVS2 treatment durations tested, whereas survival of terminal buds decreased to 30 and 20% after 50 and 75 min PVS2 treatment, respectively. After cryopreservation, no survival of terminal buds was achieved after 50 and 75 min of PVS2 exposure. In the case of buds N° 3-4 and 6-7, survival was between 21 (25 min PVS2 treatment) and 50% (75 min PVS2 treatment).
Regrowth of LS and ½ PVS2 controls was between 36-100% for buds of the three groups studied (Table 6). Regrowth of non-cryopreserved apical buds decreased drastically for longer PVS2 treatment durations, reaching 10% after 75 min. No regrowth of cryopreserved apical buds was achieved after 50 and 75 min PVS2 treatments. As regards buds N° 3-4 and 6-7, there was no significant effect of their origin, of the duration of the PVS2 treatment and of LN exposure on regrowth. Regrowth of non-cryopreserved N° 3-4 and 6-7 buds varied between 36 and 42% and regrowth of cryopreserved buds between 21 and 50%.

Table 6. Effect of position of bud on the stem of *in vitro* mother-plants and of PVS2 treatment duration on regrowth (%) of control (-LN) and cryopreserved (+LN) grapevine buds.

Conditions	Regrowth (%)		
	Bud N°1	Buds N° 3-4	Buds N° 6-7
Control LS 20 min	100 a/A	50 b/A	100 a/A
Control ½ PVS2 30 min	60 b/B	36 b/A	100 a/A
PVS2 25 min (-LN)	50 a/BC	36 a/A	36 a/B
PVS2 25 min (+LN)	30 a/BCD	21 a/A	33 a/B
PVS2 50 min (-LN)	30 a/D	42 a/A	40 a/B
PVS2 50 min (+LN)	0 b/D	42 a/A	25 ab/B
PVS2 75 min (-LN)	10 b/CD	41 a/A	36 a/B
PVS2 75 min (+LN)	0 b/D	40 a/A	50 a/B

Different lowercase letters indicate significant differences between positions of buds; different uppercase letters indicate significant differences between conditions of the cryopreservation protocol, according to Duncan's Multiple Range Test ($P < 0.05$).

Survival and regrowth of non-cryopreserved buds sampled directly from *in vitro* plantlets or from microcuttings was similar regardless of node position on the stem (Table 7). After cryopreservation, survival of shoot tips sampled directly from *in vitro* plantlets or from microcuttings was low (0 and 9%, respectively). Survival was between 39-47% for buds N° 3-4 and 6-7 sampled directly from *in vitro* plantlets or from microcuttings. Regrowth of buds N°3-4 was higher compared to buds N°6-7 and terminal buds. After cryopreservation, no regrowth was achieved with terminal buds sampled directly from *in vitro* plantlets. Regrowth was not significantly different in the other experimental conditions. The maximal regrowth (41.6%) was achieved with buds N°3-4 sampled directly from *in vitro* plantlets.

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Table 7. Effect of bud position on grapevine *in vitro* plantlets, excised either directly from *in vitro* plantlets or from single-node microcuttings cultured for 2 weeks, on survival and regrowth (%) after cryopreservation.

Node N°	Survival (%)				Regrowth (%)			
	Directly		Microcuttings		Directly		Microcuttings	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
1	30 b	0 b	30 b	9 b	30 b	0 b	20 b	9 ab
3/4	50 a	46 a	77 a	40 a	42 ab	42 a	77 a	20 ab
6/7	53 a	42 a	64 a	39 a	40 b	25 ab	27 b	11 ab

Different lowercase letters indicate significant differences between different sources of material within columns, according to Duncan's Multiple Range Test ($P < 0.05$).

DISCUSSION

In this paper, we studied the effect of growth regulators (type and duration of exposure) in shooting medium on grapevine shoot tip recovery after cryopreservation and the effect of bud position on their response to our cryopreservation protocol.

We used a shooting medium in order to produce more homogenous and vigourously growing shoots. In our conditions, preliminary experiments had shown that explants developed more homogeneously and more rapidly when growth regulators were included in the shooting medium (data not shown). When comparing shooting media supplemented with ZR or BAP at the same concentration, no significant difference was observed on regrowth of cryopreserved buds after the optimal PVS2 treatment duration, which suggested that the cytokinin type did not influence explant response. However, with that same optimal PVS2 treatment, a significant difference in regrowth was observed between media with and without cytokinin, indicating that cytokinins, even at the low concentrations provided some specific protection against LN exposure. This is in contrast with results obtained on *Anisoganthos* shoot tips by Turner *et al.* (28) who demonstrated that cytokinins had not effect on cryopreservation success when added at the preculture stage, although they positively affected post-cryopreservation regrowth when combined to gibberellic acid (GA_3) in the recovery medium. On the other hand, Petijová *et al.* (19) stated that, whereas short-term pretreatment with BAP did not modify *Hypericum perforatum* shoot tip response to cryopreservation, its use prior to LN exposure in combination to abscised acid (ABA) decreased post-cryopreservation recovery.

In our experiments, the duration of the growth phase of microcuttings on shooting medium had little influence on explant survival and regrowth after LN exposure. To the best of our knowledge, no other report on the influence of microcutting culture duration before cryopreservation has been published. When comparing buds taken directly from grapevine *in vitro* plantlets and shoot tips sampled from microcuttings, buds displayed generally lower survival and regrowth before and after LN exposure.

Grapevine is a species which displays a strong apical dominance (5). In grapevine axillary buds are organized as bud complexes comprising prompt-buds and latent buds (12). Buds located at different levels on a stem are at different physiological stages (7). In our experiments, shoot tips completely failed to regrow after PVS2 exposure longer than 50 min. Buds N° 6-7 showed the highest regrowth after cryopreservation following a 75 min exposure to PVS2. For buds N° 3-4 and 6-7, no significant effect of their original position on survival and regrowth after PVS2 exposure was observed.

The effect of the bud position on the stem of donor *in vitro* plantlets on their recovery after cryopreservation has been tested in carnation, grape and potato (2, 9, 21). In the case of carnation, the lower the position of axillary buds on the stem (starting from the terminal bud)

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the lower their regrowth after LN exposure, with regrowth ranging from 90% for the former to less than 10% for the latter (2). Similar results were obtained with potato (9). By contrast, Plessis *et al.* (21) did not observe any effect of bud position on the stem of grapevine *in vitro* plantlets on regrowth after cryopreservation. Our results are in accordance with those of Dereuddre *et al.* (2) and of Halmagyi *et al.* (9) and differ from those of Plessis *et al.* (21). The differences noted between our results and those of Plessis *et al.* (21), may be due to the fact that experiments were performed with different grapevine varieties, and possibly with plant materials which had been multiplied for different durations since their introduction *in vitro* and may thus be in a different physiological state.

In conclusion, the results obtained in this study underlined the paramount importance of the physiological state of the plant material for cryopreservation success. Actively growing buds sampled from microcuttings displayed higher regrowth compared to buds sampled directly on *in vitro* plantlets. The addition of growth regulators to the shooting medium had a positive effect on regrowth after LN exposure. We also confirmed that the position of buds on the stem of *in vitro* mother-plants affected regrowth after cryopreservation. Further experiments should include testing different grapevine varieties. An histological comparison of buds displaying different responses after cryopreservation, would provide a more accurate view of key factors involved in grapevine tolerance to cryopreservation.

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ANNEX VII

Effect of Proline Pretreatment on Grapevine Shoot-tip Response to a Droplet-Vitrification Protocol

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Keywords: *Vitis vinifera* L., cryopreservation, *in vitro* culture, stress alleviation, amino acid, medium supplementation

Abstract

Proline has been shown to accumulate in plants in response to biotic and abiotic stresses. Exogenous proline has thus been used for improving some plant cryopreservation protocols. Further enhancement of cryopreservation efficiency for *in vitro* grapevines could be expected if stresses linked to cryopreservation procedures could be reduced. We therefore studied the possible beneficial effect of proline in grapevine cryopreservation.

Single-node explants from *in vitro* grown grapevine plantlets (*Vitis vinifera* L. cv Portan) were cultured on shooting media (half-strength MS + 1µM BAP) containing no proline (control) or 50, 500, or 2000 µM filter-sterilized L-proline. Shoot tips excised from these micro-shoots were subjected to a PVS2-based droplet-vitrification procedure. Control and rewarmed explants were grown on a recovery medium containing 1µM BAP.

Shoot development on control medium and lower proline concentrations did not notably differ whereas the highest concentration of proline inhibited shoot development. Carry-over effects were observed since lower survival and regrowth were obtained both for non-frozen or LN-treated explants excised from micro-shoots obtained on the 2000 µM proline medium. No significant differences in survival and regrowth were observed for non-frozen explants subjected to pretreatment without LN exposure. A slightly enhancing effect (although non-significant) on post-cryopreservation survival was observed for explants derived from shoots developed on 50 or 500µM proline, but no significant improvement of regrowth percentage was observed for these two conditions.

Although a slight increase in survival could be observed, no significant beneficial effect of proline pretreatment on post-cryoconservation regrowth could be evidenced in our conditions. However, the 2-week period before explant excision could have allowed at least partial metabolism and catabolism of exogenous proline; the results observed could thus have been the consequence of complex interactions. Shorter proline treatments applied closer to the actual LN exposure step might produce different results and allow for clearer interpretation.

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INTRODUCTION

Proline has been shown to accumulate in plant cells and organs in response to biotic (plant-pathogen interaction) and abiotic (water limitation, redox potential modification, freezing, etc…) stresses, even though this amino acid seems to accumulate in highest concentrations in the event of a drought stress (Verslues and Sharma, 2010). These stress-mitigating properties have prompted the inclusion of exogenous proline in some plant cryopreservation protocols in order to improve explant survival and regrowth (Burritt, 2012).

Although its modes of action are not fully known, proline could likely act protectively during the dehydration steps included in all cryopreservation protocols. Indeed, dehydration is known to evoke the formation of reactive oxygen species (Smirnov, 1993) and exogenous proline has recently been shown to alleviate H_2O_2-mediated oxidative stress in grapevine leaves (Ozden *et al*, 2009).

Thus, while satisfactory efficiency of cryopreservation is documented for *in vitro* grapevines (Benelli *et al*, 2012), further improvement could be expected if stresses linked to cryopreservation procedures (such as oxidative stress) could be reduced. To our knowledge the possible beneficial effect of proline in grapevine cryopreservation has not been tested yet and we therefore considered it worthwhile to investigate its influence in a vitrification-based protocol.

MATERIAL AND METHODS

Single-node explants isolated from *in vitro*-grown grapevine plantlets (*Vitis vinifera* L. cv Portan) were cultured for 2 weeks on shooting media (half-strength MS-based medium plus 1µM BAP) containing no proline (control) or 50, 500, or 2000 µM filter-sterilized L-proline.

After 2 weeks of culture of single-node explants (at least 37 explants per experimental condition) on the different shooting media tested, the number of shoot-developing nodes was counted and individual shoot length and number of newly-developed nodes were recorded. Shoot tips excised from micro-shoots obtained on the different shooting media were subjected to a PVS2-based droplet-vitrification protocol (Marković et al., 2013) and plunged into liquid nitrogen for at least 1 h. Control explants (treated with loading, PVS2, and unloading solutions, but not exposed to LN) and LN-treated, rewarmed explants were grown on the same recovery medium containing 1µM BAP. Thirteen to eighteen explants were used per condition.

RESULTS AND DISCUSSION

Effect of proline on shoot development and growth parameters

Shoots developed on the different shooting media did not demonstrate notable differences in aspect (Fig. 1) although leaves produced on media with the higher two proline concentrations displayed a slightly lighter green; similarly, a comparable number of shoots and nodes were produced over the range of media, although a slightly lower number of nodes were formed on the medium containing the highest proline concentration (Fig. 2).

Shoots arising from the control medium and media with the lower two proline concentrations did not notably differ in their development with similar shoot length measured, whereas the highest concentration of proline inhibited shoot development (Fig. 3) with a significant global difference between treatments. As no other pair-wise comparisons were significant, this could be explained by a slightly enhancing effect of proline used at 500 µM and a slightly depressive effect of the 2000 µM treatment, both not large enough to differ from the control but statistically different when compared. Thus, in our conditions, proline did not markedly alter shoot development although some toxicity could be suspected at high

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concentrations. Indeed, excess proline has been shown to negatively affect chlorophyll-binding proteins (Nanjo *et al*, 2003), which could account for the loss of coloration observed at the highest two proline concentrations. In addition, both the number of nodes formed and micro-shoot length mean value were lower (but not significantly) when explants were grown at the highest proline concentration.

Effect of proline on post-cryopreservation survival and regrowth

After explant pretreatments with loading/PVS2 solutions with or without LN exposure, shoot tip survival and regrowth were recorded after 2 and 8 weeks (see Table). Carry-over effects for the 2000 µM proline medium were observed since lower survival and regrowth were obtained both for LN-treated or non-treated explants excised from micro-shoots obtained on this medium. No significant differences in survival and regrowth were observed for explants subjected to pretreatment phases without LN exposure. A slightly enhancing effect (although non-significant) on post-cryopreservation survival was observed for explants derived from shoots developed on 50 or 500µM proline, but no significant improvement of regrowth was observed for these two conditions.

Increasing explant number in repeat experiments would clearly improve statistical significance. However, the 2-week period before explant excision could have allowed at least partial catabolism of exogenous proline back to glutamate (Szabados and Savouré, 2010); Exposure to exogenous proline at higher (45 mM) concentration has also been shown to modify the endogenous amino acid pool (Chen et al., 2011). We therefore cannot rule out the fact that interpretation of the results observed in our conditions could have been made difficult as a result of complex interactions. Shorter exposures to proline can then be envisaged in order to reduce proline catabolism and better evidence its proper effect. The actual rate of proline uptake by *in vitro* grapevine explants is not known, but endogenous proline content of *Arabidopsis* seedlings was increased almost 100-fold within 24 h when they were treated with 45 mM proline (Chen et al., 2011) and a 6-h treatment with 20 mM proline was sufficient to effectively reduce oxidative stress in grapevine leaves excised from *in vitro* plants (Ozden et al., 2009). It is therefore likely that shorter proline treatments applied just before exposure to liquid nitrogen (*i.e.* in the 24-h period after explant excision and before explant pretreatment with vitrification solutions) could evoke a clearer response as to the possible protective effect of proline during grapevine *in vitro* shoot tip cryopreservation.

CONCLUSIONS

Over the range of concentrations tested, proline did not strongly alter shoot development. Detrimental carry-over effects of proline at 2000µM could be suspected as shoot tips regrowth after vitrification treatments and LN exposure was negatively affected. After LN exposure, and although a slight increase in survival could be observed at the lowest concentrations tested, no significant effect of proline pre-treatment on post-cryopreservation regrowth could be evidenced in our conditions. However, repeating the experiment with shorter durations of exposure to proline would likely allow clearer interpretation by limiting the effects of proline metabolism and likely interactions resulting therefrom.

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ANNEX VIII

In vitro introduction of healthy and virus-infected genotypes of native Croatian grapevine cultivars

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22 Abstract

23 We evaluated the response of eight economically important Croatian grapevine cultivars and
24 studied the impact of their sanitary status to *in vitro* introduction, by comparing the response
25 of healthy and virus-infected genotypes of one cultivar. Survival of nodal explants was
26 measured after 2 weeks and regrowth after 8 weeks of culture on three media, M1 (half-
27 strength MS), M2 (full-strength MS) or M3 (full-strength MS with 4.4 µM
28 benzylaminopurine). Logistic regression analysis showed that cultivar and cultivar*medium
29 interaction effects on survival and regrowth were significant. After 8 weeks, average shoot
30 length and node number were significantly higher on M2 compared to M1 and M3. M3
31 induced significantly shorter average internode length, compared to M1 and M2. Canonical
32 discriminant analysis of growth parameters on all three media revealed growth differences
33 between the eight cultivars studied. Survival of the healthy and of the five infected genotypes
34 of cultivar Plavac mali was 97.5 and 82.8-87.5%, respectively. Regrowth in the healthy
35 genotype reached 95.5%, but dropped to 5.5-31.4% in infected ones. The healthy genotype
36 showed significantly higher shoot length (6.3 cm) and node number (7.3) compared to
37 infected genotypes, with shoot length between 1.2-2.6 cm and node number between 1.2-3.0.
38 By contrast, internode length was not significantly different between the healthy and the
39 infected genotypes. The present work represents the first successful *in vitro* introduction for
40 three of the eight native Croatian cultivars studied.

41

42 Keywords: grapevine, Croatian autochthonous cultivars, *in vitro* inoculation, growth
43 parameters, infected genotypes

44

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VERSITA

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Cryopreservation of grapevine (*Vitis vinifera* L.) *in vitro* shoot tips

Research Article

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Abstract: In this work, we compared the efficiency of encapsulation-dehydration and droplet-vitrification techniques for cryopreserving grapevine (*Vitis vinifera* L.) cv. Portan shoot tips. Recovery of cryopreserved samples was achieved with both techniques; however, droplet-vitrification, which was used for the first time with grapevine shoot tips, produced higher regrowth. With encapsulation-dehydration, encapsulated shoot tips were precultured in liquid medium with progressively increasing sucrose concentrations over a 2-day period (12 h in medium with 0.25, 0.5, 0.75 and 1.0 M sucrose), then dehydrated to 22.28% moisture content (fresh weight). After liquid nitrogen exposure 37.1% regrowth was achieved using 1 mm-long shoot tips and only 16.0% with 2 mm-long shoot tips. With droplet-vitrification, 50% regrowth was obtained following treatment of shoot tips with a loading solution containing 2 M glycerol + 0.4 M sucrose for 20 min, dehydration with half-strength PVS2 vitrification solution (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% dimethylsulfoxide and 0.4 M sucrose in basal medium) at room temperature, then with full strength PVS2 solution at 0°C for 50 min before direct immersion in liquid nitrogen. No regrowth was achieved after cryopreservation when shoot tips were dehydrated with PVS3 vitrification solution (50% (w/v) glycerol and 50% (w/v) sucrose in basal medium).

Keywords: *Encapsulation-dehydration • Droplet-vitrification • Shoot tip size*

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1. Introduction

Grapevine is one of the economically most important fruit worldwide [1]. Its long cultivation has resulted in the development of large numbers of cultivars adapted to a wide diversity of climates. Many grapevine cultivars are now endangered and international efforts aiming at preserving grapevine biodiversity have been undertaken. Although field collections play a preeminent role in grapevine conservation programmes, maintenance of plant genetic resources only in field collections is risky, as valuable germplasm can be lost because of pests, diseases and various calamities such as adverse weather conditions [2,3]. Today, biotechnology offers a broad range of techniques, which allow optimizing plant genetic resource conservation.

Among these techniques, cryopreservation (liquid nitrogen [LN], -196°C) is a highly suitable and efficient tool for long-term storage of plant germplasm, requiring minimum space and maintenance [2]. The cryopreservation protocols developed recently do not require controlled cooling, thereby allowing cells and shoot tips to be cryopreserved by direct transfer to LN. The development of simple and reliable cryopreservation methods should allow much borader use of cryopreserved cultured cells, shoot tips and somatic embryos [4]. Among the techniques developed, encapsulation-dehydration, vitrification and droplet-vitrification are the most frequently used for cryopreservation of shoot tips.

Encapsulation-dehydration is based on the technology developed for producing artificial seeds

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[5]. Explants are encapsulated in calcium alginate beads (usually 3% w/v), osmotically dehydrated in liquid medium enriched with sucrose at concentrations between 0.75 and 1.25 M for 1 to 7 days, then physically dehydrated to moisture contents (MC) around 20% (fresh weight basis) and cooled rapidly.

Vitrification involves osmotic dehydration of shoot tips with cryoprotectants. Shoot tips are treated with a loading solution with intermediate concentration (usually 2 M glycerol + 0.4 M sucrose, [6]), then dehydrated with highly concentrated vitrification solutions (total molarity around 5-7 M), cooled and rewarmed rapidly; cryoprotectants are removed by placing shoot tips in an unloading solution (containing 0.8-1.2 M sucrose) and apices are transferred on recovery medium for regrowth. The most commonly employed vitrification solutions are the so-called Plant Vitrification Solutions PVS2 [7] and PVS3 [8] developed by the group of the late Prof. Sakai in Japan.

Droplet-vitrification is the latest technique developed [9]. In a droplet-vitrification protocol, shoot tips are first treated with a loading solution, then with a vitrification solution, placed on aluminium foils in minute droplets of vitrification solution and cooled rapidly in LN. The high recovery percentages achieved with this technique are due to the very high cooling and rewarming rates achieved because of the direct contact between samples and LN during cooling, and between samples and the unloading solution during rewarming. There is an increasing number of species to which droplet-vitrification has been successfully applied [4].

The availability of an efficient and reliable cryogenic protocol yielding a high recovery percentage is a prerequisite for large scale, routine application of cryopreservation [10].

Although cryopreservation research has been initiated over 20 years ago on grapevine, no such routinely applicable protocol is yet available. Different cryopreservation techniques have been tested with grapevine *in vitro* shoot tips. Grapevine apices sampled from *in vitro* plantlets have been cryopreserved using encapsulation-dehydration, with recovery ranging between 24 and 40% [11,12] or 40-60% [13]. Zhao *et al.* [14] cryopreserved shoot tips of three grape cultivars using the encapsulation-dehydration technique combined with a slow cooling-dehydration to -40°C. Recovery ranged between 15% and 40%. The vitrification technique has been successfully employed with *in vitro* shoot tips of four grapevine cultivars [15]. In these experiments, the average recovery ranged from 47% to 85%. This vitrification protocol was improved by incorporating a two-step dehydration procedure and successfully applied to ten *Vitis* cultivars or species, with shoot recovery ranging between 60% and 80% [10].

The objective of this study was to compare the efficiency of two techniques (encapsulation-dehydration and droplet-vitrification) for cryopreservation of *in vitro* shoot tips of the grapevine cultivar Portan. This was the first time that droplet-vitrification was tested on grapevine shoot tips. The effect of several parameters of these protocols was also studied, including sucrose pretreatment, bead MC, and size of explants for encapsulation-dehydration and nature of the vitrification solution and duration of exposure to the vitrification solution for droplet-vitrification.

2. Experimental Procedures

2.1 Plant material

The plant material employed in this study consisted of *in vitro* plants of grapevine (*Vitis vinifera* L.) cultivar Portan. These cultures were initially established from field-grown plants in the grape germplasm collection of the Institut national de la recherche agronomique (INRA), Vassal (France).

2.2 *In vitro* culture

Grape *in vitro* plantlets were cultured on basal medium (BM) composed of half-strength MS [16] mineral elements with Morel's vitamins [17], 3% sucrose and 0.7% agar (Sigma) at pH 5.8. They were cultured at 24±2°C under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE m^{-2} s^{-1} provided by cool white fluorescent tubes. *In vitro* mother-plants were kept without subculture for 2 months to reach a length of approximately 12 cm before use for cryopreservation experiments. *In vitro* plantlets were cut into single node microcuttings of approx. 1.5 cm in length (Figure 1), which were transferred to 9 cm Petri dishes (20 microcuttings/Petri dish) and placed on ½ MS medium containing 20 g/l sucrose, 7 g/l microagar and 1 µMol zeatin riboside (ZR). Petri dishes were placed in the environmental conditions described above. Shoot tips were excised from microcuttings after 2 weeks and used for cryopreservation experiments.

2.3 Cryopreservation

2.3.1 Encapsulation-dehydration (ED)

The standard ED protocol was the following. Excised shoot tips (1 mm long, Figure 1b) were suspended in half-strength MS medium devoid of calcium, supplemented with 3% (w/v) Na-alginate (low viscosity, Sigma A2158), 2 M glycerol and 0.4 M sucrose, according to Wang *et al.* [13]. The mixture with the shoot tips was dispensed dropwise with a sterile pipette into a 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose at room temperature for 30 min, to form beads (about

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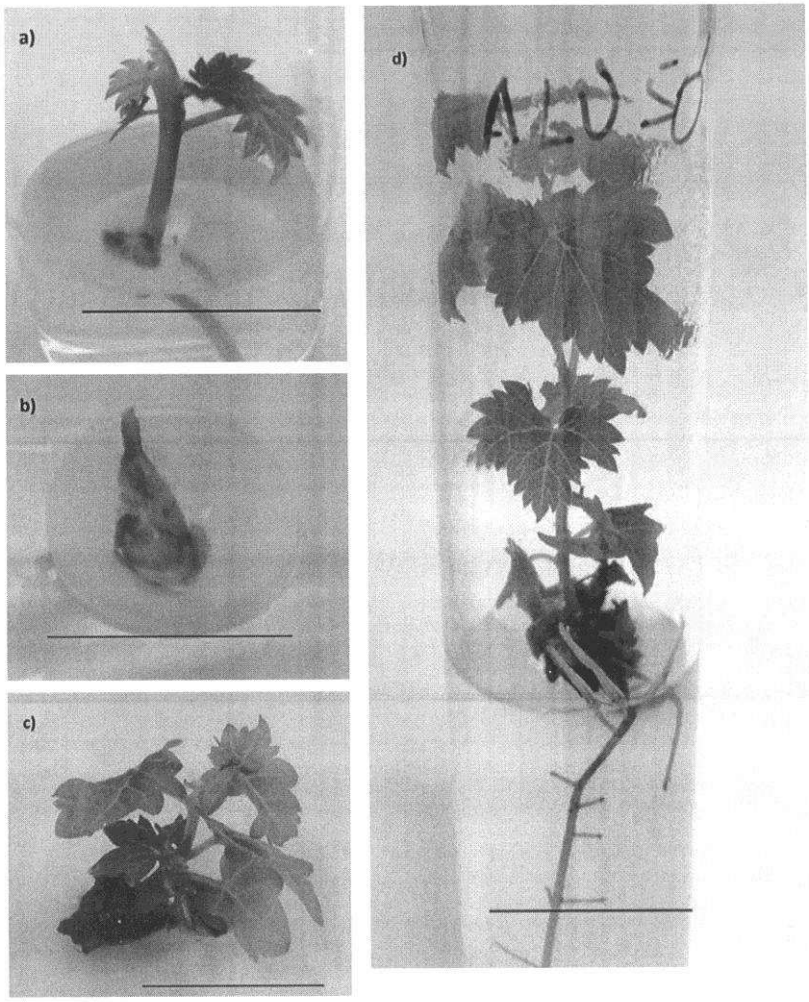

Figure 1. Successive steps of grape shoot tip cryopreservation using ED and DV. (a) One node microcutting before dissection of shoot tip (bar 10.0 mm). (b) Surviving shoot tip cryopreserved by ED (dehydration to 22.3% MC) 2 weeks after rewarming (bar 4.0 mm) (c) Recovery of shoot tip cryopreserved by DV (75 min PVS2 exposure) 6 weeks after rewarming (bar 10.0 mm). (d) Regenerated plant eight months after cryopreservation by DV within (50 min PVS2 exposure) (bar 10.0 mm).

4 mm in diameter, Figure 1c), each bead containing one shoot tip. The beads were precultured over a period of 2 days in 250 ml Erlenmayer flasks in liquid (100 ml) BM with progressively increasing (every 12 h) sucrose concentrations (0.25 M, 0.5 M, 0.75 M and 1.0 M sucrose). Beads were afterwards dehydrated with silica gel (10 beads above 80 g silica gel in 120 ml flasks) to 22.28% MC (fresh weight basis), which was achieved after 4 h. Dehydrated beads were placed in cryotubes and directly immersed in LN. Encapsulated shoot tips

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subjected to dehydration alone or to dehydration and LN exposure were post-cultured on BM containing 1 µM benzyladenine (BA), maintained in the dark at 26°C for 7 days and then transferred to the conditions described for stock cultures.

Two experiments were performed with ED. In the first experiment, we studied the effect of bead MC on recovery of grapevine shoot tips. After preculture as described above, encapsulated shoot tips were dehydrated to 16.16%, 20.26% and 22.28% MC (achieved after 4, 5 and 6 h, respectively), immersed, or not, in LN and transferred to recovery medium. In the second experiment, we investigated the effect of explant size on recovery. The standard ED protocol was thus applied to 1 and 2 mm long shoot tips.

2.3.2 Droplet-vitrification

Excised shoot tips (1 mm long, Figure 1b) were precultured on solid ½ MS medium with 0.1 M sucrose for 24 h. Precultured shoot tips were then treated with a loading solution (LS) containing 2 M glycerol + 0.4 M sucrose in MS medium [18] for 20 min at room temperature. Shoot tips were dehydrated with half-strength PVS2 or PVS3 at room temperature for 30 min, then with full strength PVS2 at 0°C or PVS3 at room temperature. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% dimethylsulfoxide (DMSO) and 0.4 M sucrose in MS medium [7] and PVS3 50% (w/v) glycerol and 50% (w/v) sucrose in basal medium [8]. Shoot tips were then placed on aluminium foils in 5 µl droplets of PVS2/PVS3 and immersed in LN for at least 1 h. For rewarming, the aluminium foils with the shoot tips were immersed for 20 min in unloading solution containing 1.2 M sucrose at room temperature [19], then transferred to recovery medium, as described for ED.

Two experiments were performed with DV. In the first experiment, we studied the effect of duration of exposure to PVS2 on recovery of grapevine shoot tips. Shoot tips were treated with PVS2 for 25, 50 and 75 min, immersed, or not, in LN, treated with unloading solution and transferred to recovery medium. In the second experiment, we compared the effect of treatment with PVS2 and PVS3 on recovery of grapevine shoot tips. Shoot tips were treated with PVS2 at 0°C for 40, 80 and 120 min or with PVS3 at room temperature for 40, 80 and 120 min immersed, or not, in LN, treated with unloading solution and transferred to recovery medium.

2.4 Assessment of survival and regrowth and statistical analyses

Survival was evaluated 2 weeks after cryopreservation by counting the number of shoots that showed any type of growth, while regrowth was defined as the development of apices into shoots with expanded leaves 8 weeks after rewarming. Both survival and regrowth percentages were expressed relative to the total number of shoot tips treated. Experiments were replicated twice, with 10 shoot tips per experimental condition. Survival and regrowth data, presented as mean percentages with standard error of the mean (SD) were subjected to arcsine transformation. Statistical differences between mean values of all parameters were assessed by analysis of variance (ANOVA) and Duncan's Multiple Range Test for mean separation.

3. Results

3.1 Encapsulation-dehydration

In the first experiment, we studied the effect of bead MC on survival and recovery of grapevine shoot tips cryopreserved using the standard ED protocol. Survival and regrowth of shoot tips were high after sucrose pretreatment (Table 1). Survival and regrowth of dehydrated shoot tips decreased in line with decreasing MCs, from 90.0% (survival) and 40.0% (regrowth) at 22.28% MC to 30.0% (survival) and 10% (regrowth) at 16.16% MC. After cryopreservation, survival and regrowth were low, reaching similar values for the three MCs tested, *i.e.* 20-22% for survival and 10.0% for regrowth.

In the second experiment, we studied the effect of explant size on survival and recovery of grapevine shoot tips. After sucrose pretreatment, survival and regrowth of smaller (1 mm) and larger (2 mm) shoot tips were comparable (Table 2). After dehydration, survival of larger shoot tips was higher compared to smaller explants; however, regrowth reached similar values for explants of both sizes. After cryopreservation, survival and regrowth were higher for smaller explants, reaching 45.5% (survival) and 37.1% (regrowth) for 1 mm-long shoot tips, against 24.0% (survival) and 16.0% (regrowth) for 2 mm-long shoot tips.

3.2 Droplet-vitrification

Loading and treatment of shoot tips for 30 min with half-strength PVS2 had no significant effect on their survival and regrowth (Table 3). Increasing the duration of dehydration with PVS2 decreased survival and regrowth from 60% for 25 min treatment to 30% after 75 min. After LN exposure, survival decreased from 70% for 25 min treatment to 30% after 75 min, while regrowth was 40% for 25 min treatment, 50% for 50 min and 30% for 75 min treatment.

In the second experiment, we compared the effect of shoot tip dehydration with PVS2 and PVS3 vitrification

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Treatment	Survival (%±SD)		Regrowth (%±SD)	
	-LN	+LN	-LN	+LN
Sucrose pretreatment	100.0±0a	-	75.0±35.3a	-
Dehydration to 22.28% MC	90.0±14.1ab	20.0±0c	40.0±0ab	10.0±14.1b
Dehydration to 20.26% MC	70.8±5.9b	32.5±10.6c	29.2±5.9b	10.0±14.1b
Dehydration to 16.16% MC	30.0±14.1c	20.0±0c	10.0±14.1b	10.0±14.1b

Table 1. Effect of bead MC (%, FWB) on survival and regrowth (%) of grapevine cv. Portan shoot tips after sucrose pretreatment, dehydration (-LN) and cryopreservation (+LN) using the standard ED protocol.

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

Size of explants	Survival (%±SD)				Regrowth (%±SD)			
	1 mm		2 mm		1 mm		2 mm	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Sucrose pretreatment	100.0±0a	-	95.8±5.9a	-	75.0±35.4a	-	66.7±23.6a	-
22,3% beads MC	30.0±14.1b	45.5±5.4ab	73.3±18.9ab	24.0±1.4b	16.7±4.7a	37.1±4.2a	11.7±2.4a	16.0±0.9a

Table 2. Effect of explant size on survival and regrowth (%) of grapevine cv. Portan shoot tips after sucrose pretreatment, dehydration (-LN) and cryopreservation (+LN) using the standard ED protocol.

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

Treatment	Survival (%±SD)		Regrowth (%±SD)	
	-LN	+LN	-LN	+LN
Loading	100.0±0a	-	100.0±0a	-
½ PVS2 /30 min	100.0±0a	-	83.03±23.6ab	-
PVS2 /25 min	60.0±0b	70.0±14.1b	60.0±28.3bc	40.0±0d
PVS2 /50 min	50.0±14.1bc	50.0±14.1bc	40.0±0cd	50.0±14.1bcd
PVS2 /75 min	30.0±14.1c	30.0±14.1c	30.0±14.1cd	30.0±14.1cd

Table 3 Effect of duration of dehydration with PVS2 solution on survival and regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine cv. Portan shoot tips.

In columns, mean values for survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

solutions. PVS3 was highly toxic since survival and regrowth were only 10% after 40 min treatment and were nil for longer durations (Table 3). No survival was achieved after LN exposure following PVS3 treatment. By contrast, with the PVS2 vitrification solution, survival and regrowth were 30% after 40 min treatment and low survival and regrowth (10.0%) were still noted after 120 min treatment. After cryopreservation, survival and regrowth were achieved only following a 40 min PVS2 treatment, with values reaching 40 % for both survival and regrowth.

4. Discussion

In the present work, we compared the efficiency of ED and DV techniques for cryopreserving shoot tips of grapevine cv. Portan. Recovery of cryopreserved samples was achieved with both techniques; however, DV, which was used for the first time with grapevine shoot tips, produced higher regrowth. Fifty percent regrowth was obtained with DV, following 50 min exposure of shoot tips to PVS2. No regrowth was achieved following treatment of shoot tips with PVS3 vitrification solution.

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	Survival (%±SD)				Regrowth (%±SD)			
	PVS2		PVS3		PVS2		PVS3	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Loading control	70.0±14.1b	-	70.0±14.1a	-	70.0±14.1a	-	70.0±14.1a	-
½ PVS/30 min	100.0±0a	-	60.0±0a	-	90.0±14.1a	-	50.0±14.1b	-
PVS/40 min	30.0±14.1cd	40.0±0c	10.0±14.1b	0.0b	30.0±14.1bc	40.0±0b	10.0±14.1c	0.0c
PVS/80 min	8.3±11.8de	0.0e	0.0b	0.0b	8.3±11.8cd	0.0e	00.0c	0.0c
PVS/120 min	10.0±14.1de	10.0±14.1de	0.0b	0.0b	10.0±14.1cd	0.0e	00.0c	0.0c

Table 4. Effect of duration of dehydration with PVS2 and PVS3 solution on survival (%) and regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine cv. Portan shoot tips.

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

By comparison, with ED, 37.1% regrowth after LN exposure was achieved using 1 mm-long shoot tips and only 16.0% with 2 mm-long shoot tips.

The ED technique comprises several successive steps, the conditions of which must be optimized to achieve maximal recovery after cryopreservation. Bead preculture with progressive sucrose concentration has been shown to improve recovery [12,13,20]. In our case, sucrose preculture allowed high survival (95.8-100.0) and regrowth (66.7-75.0) percentages. Explant dehydration is one of the crucial steps in this protocol. In general, the bead MC, which ensures highest regrowth after cooling in LN is approx. 20%, which corresponds to the amount of unfreezable water in the samples [21]. This value may vary depending on the species and explant type. In the case of grapevine, Plessis *et al.* [12] achieved 20% survival of cryopreserved shoot tips following dehydration to 25% MC, Wang *et al.* [13] achieved 59.3% survival after dehydration to 16.1% MC, and Zhao *et al.* [14] noted 40% survival after dehydration to 21% MC. In our work, we did not define an optimal MC for cryopreserving grape shoot tips and regrowth after cryopreservation was lower, compared with the results of Plessis *et al.* [12] and of Wang *et al.* [13]. Indeed, we obtained a similar, low regrowth of 10% after LN exposure following dehydration to three MCs, 22.28%, 20.26% and 16.1%. These results may be due to the high sensitivity of cv. Portan shoot tips to the ED technique, and/or to the fact that the preculture and dehydration conditions selected were not optimal.

An important parameter of cryopreservation protocols is the size of shoot tips employed as experimental materials. Explants must be of a sufficiently small size to avoid the occurrence of gradients between external and more internal cell layers during cryoprotection and cooling and warming, which would be detrimental to survival. However, at the same time, shoot tips must be of a sufficiently large size for rapid and direct regrowth to take place after cryopreservation [5]. When cryopreserving pear shoot apices using the ED technique, Scottez [22] showed that smaller shoot tips, of a size comprised between 1-2 mm, produced higher recovery compared to those of the two other categories tested, which had a size of 3-4 and 5-6 mm. In the case of grape, Wang *et al.* [20] compared the survival after cryopreservation of shoot tips of four different sizes (0.5, 1.0, 1.5 and 2.0 mm). The highest survival (65%) was obtained with 1-1.5 mm-long shoot tips, while larger or smaller shoot tips produced lower survival. We obtained comparable results in our experiments, with the smaller (1 mm long) shoot tips employed producing significantly higher regrowth, compared with larger ones (2 mm long).

In the DV technique, explants are rapidly dehydrated with highly concentrated vitrification solutions, resulting in the vitrification of internal solutes upon cooling in LN [4]. Because of their high concentration in cryoprotective agents, these vitrification solutions are often highly toxic. Optimizing the duration of exposure of samples to these solutions is therefore of paramount importance to achieve high regrowth after cryopreservation [23]. The optimal duration of exposure to vitrification solutions is associated with the size and structure of the excised shoot tips and appears to be highly species-specific at the same temperature. Ganino *et al.* [24] showed that exposure of grapevine shoot tips to vitrification solutions markedly affected shoot tip survival. Regrowth of shoot tips decreased from an initial value of 94% to 57% after a 30 min exposure and to 15% after 60 min. Optimal treatment durations with PVS2, which contains the penetrating cryoprotectants DMSO and EG, are in the magnitude of 30-60 min, while longer exposures are generally required with PVS3, which contains only the non-penetrating cryoprotectants sucrose and

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glycerol [4]. In our experiments, the optimal PVS2 treatment duration was 50 min, which was comparable to the results obtained by Matsumoto and Sakai [6] with grapevine shoot tips cryopreserved with the vitrification technique.

For plant materials, which are highly sensitive to PVS2, dehydration with the PVS3 vitrification solution, which does not contain penetrating cryoprotectants and is generally less toxic, often produces high regrowth after cryopreservation. This was the case with chrysanthemum shoot tips, which showed significantly higher regrowth after cryopreservation following PVS3 dehydration, compared with PVS2 dehydration [25]. By contrast, shoot tips of other plant species such as garlic and sugarcane proved to be equally tolerant to dehydration with PVS2 and PVS3 [25,26]. Our experiments showed that grapevine shoot tips had an original behaviour, as they were extremely sensitive to PVS3 exposure. It can be hypothesized that grapevine shoot tips are highly sensitive to the high concentrations of sucrose and/or glycerol present in the PVS3 vitrification solution.

In conclusion, we showed for the first time that the DV technique could be successfully applied for cryopreservation of grapevine shoot tips. Recovery was significantly higher with DV, in comparison with the ED technique. The current results might be improved by optimizing various steps of the DV protocol, including conditioning *in vitro* plants or microcuttings on medium enriched with growth regulators such as BAP or zeatine riboside [27], or using alternative loading and vitrification solutions [28,29]. Before its large scale application can be envisaged, the optimized protocol should be validated through its application to a range of grapevine cultivars.

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Scientific Research

Effect of Proline Pretreatment on Grapevine Shoot-Tip Response to a Droplet-Vitrification Protocol

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ABSTRACT

Proline has been shown to accumulate in plants in response to biotic and abiotic stresses. Exogenous proline has thus been used for improving some plant cryopreservation protocols. Further enhancement of cryopreservation efficiency for *in vitro* grapevines could be expected if stresses linked to cryopreservation procedures could be reduced. We therefore studied the possible beneficial effect of proline in grapevine cryopreservation. Single-node explants from *in vitro* grown grapevine plantlets (*Vitis vinifera* L. cv Portan) were cultured on shooting media (half-strength MS + 1 µM BAP) containing no proline (control) or 50, 500, or 2000 µM filter-sterilized L-proline. Shoot tips excised from these micro-shoots were subjected to a PVS2-based droplet-vitrification procedure. Control and rewarmed explants were grown on a recovery medium containing 1 µM BAP. Shoot development on control medium and lower proline concentrations did not notably differ whereas the highest concentration of proline inhibited shoot development. Carry-over effects were observed since lower survival and regrowth were obtained both for non-frozen or LN-treated explants excised from micro-shoots obtained on the 2000 µM proline medium. No significant differences in survival and regrowth were observed for non-frozen explants subjected to pretreatment without LN exposure. A slightly enhancing effect (although non-significant) on post-cryopreservation survival was observed for explants derived from shoots developed on 50 or 500 µM proline, but no significant improvement of regrowth percentage was observed for these two conditions. Although a slight increase in survival could be observed, no significant beneficial effect of proline pretreatment on post-cryoconservation regrowth could be evidenced in our conditions. However, the 2-week period before explant excision could have allowed at least partial metabolism and catabolism of exogenous proline; the results observed could thus have been the consequence of complex interactions. Shorter proline treatments applied closer to the actual LN exposure step might produce different results and allow for clearer interpretation.

Keywords: *Vitis vinifera* L.; Cryopreservation; *In Vitro* Culture; Stress Alleviation; Amino Acid; Medium Supplementation

1. Introduction

Proline has been shown to accumulate in plant cells and organs in response to biotic (plant-pathogen interaction) and abiotic (water limitation, redox potential modification, freezing, etc.) stresses, even though this amino acid seems to accumulate in highest concentrations in the event of a drought stress [1]. These stress-mitigating properties have prompted the inclusion of exogenous proline in some plant cryopreservation protocols in order to improve explant survival and regrowth [2]. Although its modes of action are not fully known, proline could likely act protectively during the dehydration steps included in all cryopreservation protocols. Indeed, dehydration is known to evoke the formation of reactive oxygen species [3] and exogenous proline has recently been shown to alleviate H_2O_2-mediated oxidative stress in grapevine leaves [4].

Thus, while satisfactory efficiency of cryopreservation is documented for *in vitro* grapevines [5], further improvement could be expected if stresses linked to cryopreservation procedures (such as oxidative stress) could be reduced. To our knowledge the possible beneficial

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effect of proline in grapevine cryopreservation has not been tested yet and we therefore considered it worthwhile to investigate its influence in a vitrification-based protocol.

2. Materials and Methods

Single-node explants isolated from *in vitro*-grown grapevine plantlets (*Vitis vinifera* L. cv Portan) were cultured for 2 weeks on shooting media (half-strength MS-based medium plus 1 μM BAP) containing no proline (control) or 50, 500, or 2000 μM filter-sterilized L-proline.

After 2 weeks of culture of single-node explants (at least 37 explants per experimental condition) on the different shooting media tested, the number of shoot-developing nodes was counted and individual shoot length and number of newly-developed nodes were recorded. Shoot tips excised from micro-shoots obtained on the different shooting media were subjected to a PVS2-based droplet-vitrification protocol [6] and plunged into liquid nitrogen (LN) for at least 1 h. Control explants (treated with loading, PVS2, and unloading solutions, but not exposed to LN) and LN-treated, rewarmed explants were grown on the same recovery medium containing 1 μM BAP. Thirteen to eighteen explants were used per condition.

3. Results and Discussion

3.1. Effect of Proline on Shoot Development and Growth Parameters

Shoots developed on the different shooting media did not demonstrate notable differences in aspect (**Figure 1**) although leaves produced on media with the higher two proline concentrations displayed a slightly lighter green; similarly, a comparable number of shoots and nodes were produced over the range of media, although a slightly lower number of nodes were formed on the medium containing the highest proline concentration (**Figure 2**).

Shoots arising from the control medium and media with the lower two proline concentrations did not notably differ in their development with similar shoot length measured, whereas the highest proline concentration inhibited shoot development (**Figure 3**) with a significant global difference between treatments. As no other pairwise comparisons were significant, this could be explained by a slightly enhancing effect of proline used at 500 μM and a slightly depressive effect of the 2000 μM treatment, both not large enough to differ from the control but statistically different when compared. Thus, in our conditions, proline did not markedly alter shoot development although some toxicity could be suspected at high concentrations. Indeed, excess proline has been shown to negatively affect chlorophyll-binding proteins [7], which could account for the loss of coloration observed at the highest two proline concentrations. In addition, both the number of nodes formed and micro-shoot length mean value were lower (but not significantly)

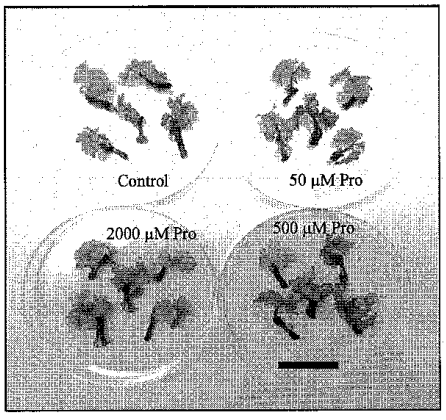

Figure 1. Representative samples of shoots developed after 2 weeks on different shooting media. Bar = 2 cm.

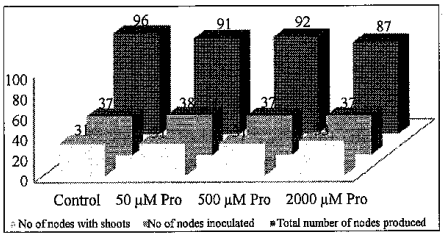

Figure 2. Number of shoots developed and total number of nodes formed 2 weeks after inoculation on the different shooting media.

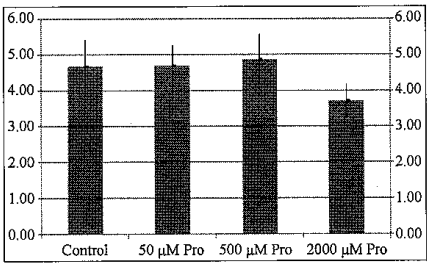

Figure 3. Mean shoot length (mm) obtained on the different shooting media with α = 5% confidence intervals. Mean values differing by one letter are significantly different (Student's t-test, α = 5%).

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Table 1. Percentage of survival and regrowth (observed after 2 and 8 weeks, respectively) of control (−LN) and cryopreserved (+LN) shoot tips excised from different shooting media and subjected to a PVS2-based droplet-vitrification protocol.

Shooting medium	Survival		Regrowth	
	−LN	+LN	−LN	+LN
0 Pro	52.9	27.8	41.4	27.8
50 µM Pro	57.1	38.8	50.0	33.3
500 µM Pro	61.5	40.0	61.5	30.0
2000 µM Pro	42.8	23.0	28.5	15.4
	ns*	ns	ns	ns

when explants were grown at the highest proline concentration.

3.2. Effect of Proline on Post-Cryopreservation Survival and Regrowth

After explant pretreatment with loading/PVS2 solutions with or without LN exposure, shoot tip survival and regrowth were recorded after 2 and 8 weeks (**Table 1**). Carry-over effects for the 2000 µM proline medium were observed since lower survival and regrowth were obtained both for LN-treated or non-treated explants excised from micro-shoots obtained on this medium. No significant differences in survival and regrowth were observed for explants subjected to pretreatment phases without LN exposure. A slightly enhancing effect (although non-significant) on post-cryopreservation survival was observed for explants derived from shoots developed on 50 or 500 µM proline, but no significant improvement of regrowth was observed for these two conditions.

Increasing explant number in repeat experiments would clearly improve statistical significance. However, the 2-week period before explant excision could have allowed at least partial catabolism of exogenous proline back to glutamate [8]. Exposure to exogenous proline at higher (45 mM) concentration has also been shown to modify the endogenous amino acid pool [9]. We therefore cannot rule out the fact that interpretation of the results observed in our conditions could have been made difficult as a result of complex interactions. Shorter exposure to proline can then be envisaged in order to reduce proline catabolism and better evidence its proper effect. The actual rate of proline uptake by *in vitro* grapevine explants is not known, but endogenous proline content of *Arabidopsis* seedlings was increased almost 100-fold within 24 h when they were treated with 45 mM proline [9] and a 6-h treatment with 20 mM proline was sufficient to effectively reduce oxidative stress in grapevine leaves excised from *in vitro* plants [4]. It is therefore likely that shorter proline treatments applied just before LNexposure (*i.e.* in the 24-h period after explant excision and before explant pretreatment with vitrification solutions) could evoke a clearer response as to the possible protective effect of proline during grapevine *in vitro* shoot tip cryopreservation.

4. Conclusion

Over the range of concentrations tested, proline did not strongly alter shoot development. Detrimental carry-over effects of proline at 2000 µM could be suspected as shoot tips regrowth after vitrification treatments and LN exposure was negatively affected. After LN exposure, and although a slight increase in survival could be observed at the lowest concentrations tested, no significant effect of proline pretreatment on post-cryopreservation regrowth could be evidenced in our conditions. However, repeating the experiment with shorter durations of exposure to proline would likely allow clearer interpretation by limiting the effects of proline metabolism and likely interactions resulting therefrom.

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