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Cryopreservation Protocols for Grapevine Shoot Tips

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Abstract

Grapevine is an important plant species known worldwide, counting more than 10,000 cultivars of *Vitis vinifera* spread all over the world. There is a strong need for long-term conservation of grapevine genetic resources. With so polymorphic species, it is highly difficult to obtain relevant results considering that cryopreservation protocols were established testing only few very often specific cultivars. Regarding cryopreservation protocols, many factors are influencing the final result. Research articles processing cryopreservation protocols of grapevine are reporting a percentage of recovery, but a broader application of some protocol on a large range of *Vitis vinifera* cultivars is limited. How to design an efficient cryopreservation protocol, starting from plant material to the appropriate observation of recovery, will be discussed in this chapter.

Keywords: grapevine shoot tips, cryopreservation protocol, testing of protocol, observation of recovery

1. Introduction

Grapevine is an important plant species known worldwide. Grape is used to produce wines and it is its primary importance. There are many *Vitis vinifera* cultivars; recent research confirmed more than 10,000, but the global market for wine production is dominated by only a few cultivars [1]. Grapevine cultivars do not have equal importance regarding its quality nor potential for wine production. The cultivars are generally classified according to their final production: wine grapes, table grapes, and raisins [1]. Regarding wine cultivars territorially spread within one country, some of them are economically important, whereas some of them have just cultural importance presenting a national viticultural treasure aiming to preserve

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biodiversity of the viticultural country. Genetic structure in cultivated grapevines has been shaped mostly by human uses, in combination with a geographical effect [2].

Wine countries are giving a great importance on preservation of grapevine collections and 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 [3]. Recently in Croatia revitalization of some threatened cultivars is in progress [4, 5]. Mentioned activities are tough and demanding very often with high maintenance costs and the need for additional workers. Thus, a risk of natural hazards to which these collections are exposed is still present. Especially, important long-term storage of plant material can be for small wine countries, like Croatia, because the majority of autochthonous cultivars are economically important, but there are local varieties that present local heritage for some regions or subregions [6]. Particular variety can be highly important for some remote parts within already isolated island (e.g., cultivar Grk at Lumbarda on an island Korčula). One specific example of one cultivar is "Zlatarica from Blato," whereas amphelographic and economic characteristics observed a middle-quality grape variety, but its importance is in conservation of biodiversity of grapevine resources on island Korčula [7]. Also, the big problem can be a virus infection of some variety that has a small population because of the loss of intravarietal variability [8]. Varieties that are intended for wine production need to be conserved in grapevine collections, and there should be a means for its constant evaluation and maintenance regarding their qualitative potential [6]. Wine country like Croatia has many important native grapevine cultivars (Babić, Debit, Grk, Kraljevina, Plavac mali, Plavina, Pošip) with significant wine production. These cultivars are also scientifically explored [9-11]. So, there is a general need in Croatia to make a selection of cultivars that will be maintained in grapevine collections all over Croatia and the rest of the majority of them to be cryopreserved and stored in liquid nitrogen.

The alarming loss of plant biodiversity both in nature and within agricultural systems, for grapevine resources as a polymorphic species, has led us to look for alternatives to in situ conservation. Cryopreservation as a method for long-term conservation exists more than 60 years, and there are experimental protocols already used with a range of crops and geno types, but a broader application of this method has not been achieved in many plant species [12]. Therefore, maybe cryopreservation as a method should be revealed as a useful tool for longterm maintenance of select germplasm [13]. There are examples of gene banks with more or about 100 accessions for potato, cassava, and pear, over the world in different research laboratories [14], but researchers for many plant crops are facing this method, as a means for longterm conservation, as a hardly applicable technique. Often, the initial 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 [15]. A starting point that is also critical is choice of initial plant material for experiments. In the case of grapevine, this factor was decisive in many cryopreservation experiments [16]. Water status and cryoprotection are the most influential determinants of survival in combination with physiological factors, and that is why crop expert should choose plant tissue that they assume it should survive [13]. The published research mostly relates to *Vitis vinifera*, and the few studies applied to other species show that the protocols need to be improved [17].

In designing cryopreservation experiments, researchers often overlook one major area like culture conditions. In clonally propagated plants, the condition of the plant or the culture can greatly affect the success of a cryopreservation protocol [18].

Research articles dedicated to cryopreservation of grapevine revealed variations in response according to the genotype [19]. The main objective of those articles is to present a cryopreservation protocol with exact result of recovery of some particular genotype of interest for some viticultural area or viticultural country. In so polymorphic crop, as grapevine, it is almost impossible to conclude this method as a technique working or not and whether it is just a matter of genotype.

Some variations in response can be due to the different research laboratories and conditions within. Still in a case of one laboratory that is dedicated to cryopreservation protocols, variations in results with same genotype were observed between years, researchers, and slightly modified culture conditions (data not shown). How to overcome these problems and set up an efficient cryopreservation protocol for the genotypes of interest will be processed in this research note article.

1.1. Establishment of an efficient cryopreservation protocol

Cryopreservation of grapevine first started in 1989 by Ezawa [20], but more detailed cryopreservation protocol for grapevine was reported by Plessis in 1991 [21]. The authors reported survival >72%, but the recovery was not evaluated in the mentioned research [21]. Since then, many techniques of cryopreservation were used by different researchers. The preculture with sucrose was noted as deciding factor for recovery in many of them [21–27]. However, no article revealed how exactly recovery was achieved, and only one research article observed the physiological state of plant material [16]. It is known that the age of mother plants, the size of explants, and some other culture conditions are of paramount importance for in vitro growth of grapevine plants. Also, already in in vitro conditions, great variations between cultivars are reported [28]. Therefore, it is hard to conclude what exactly gave so various results between cultivars. In regeneration in vitro for some cultivars, high genotypic influence is revealed [29–31].

These variations in response according to the genotype are reported in recent review articles of cryopreservation of grapevine [19, 32]. Even if it is noted that any vegetatively propagated species should be amenable to cryopreservation [33], in the case of grapevine, there are examples where explants remain green after rewarming, but do not develop further [24]. The same situation has been revealed in our laboratory, whereas some important native cultivars survived cryopreservation protocol, and they remain green during the following 4 weeks, but unfortunately recovery was not achieved (data not shown).

The most suitable explant for cryopreservation is shoot tip of grapevine, and it is the most used explant in testing of recent cryopreservation protocols for grapevine [16, 17, 34–37]. However, the right time and size of suitable shoot tip are also of a great importance for cryopreservation success as a condition that is adapted to each individual laboratory. Considering that shoot tips of grapevine have the lowest possibility of somaclonal variations, it can be a good choice in testing of protocols. Regarding the cryopreservation protocols recently

established for grapevine, shoot tips were chosen as the most appropriate material for experiments. Experiments of cryopreservation protocols with shoot tips taken from field-grown plants are in a progress in our laboratory (data not shown). This prestep can avoid in vitro culture and simplify the cryopreservation protocol. Furthermore, some of the genotypes are very sensitive to culture conditions and often is difficult to resolve which factors are affecting a weak growth of in vitro plants or inability to regenerate. Unfortunately, to apply cryopreservation protocol, it is necessary to have numerous populations of in vitro material. The situation is more complicated if the success of a particular method requires some preliminary results, where a large amount of already rare plant material will be spent. This is the case of some cultivars from the isolated areas with a small amount of material. Four cultivars from place Kaštela near Split were introduced in tissue culture conditions (2011). Initial growth of plant material was satisfying, but in the period when we should already have adult plants for experiments, the plants start to yellow and browning and gradually decay. Urgent attempts of intervention with various media with higher concentration of growth regulators have been applied. More or less the same situation with same cultivars was repeated following 4 consecutive years with fewer deviations. The material was taken from a virus-infected plant. Also, international cultivars (Cabernet Franc, Cabernet Sauvignon, Grenache, Merlot) were taken from infected vines and introduced in tissue culture conditions in order to test the method of cryotherapy. Cultivars Cabernet Sauvignon and Grenache were very sensitive to culture conditions where initial culture already failed. Cabernet Franc and Merlot grew well, but regular multiplication was not successful enough to give the requested amount of plants to carry out the experiments [38]. This problem has been investigated on a cell level with "Gala" apple cryopreserved shoot tips [39]. They found cell membrane damage and alternation in mitochondria which caused a slower shoot proliferation in cryopreserved shoot tips. Even if our study does not include cryopreservation, this can be due to the virus infection that causes a weak growth in tissue culture conditions of infected cultivars tested. How to obey this problem for the cultivars of interest will be investigated in the following experiments in our laboratory.

Choosing the right method for testing a cryopreservation protocol is necessary to observe laboratory capacities and, in general, to define the final aim of the procedure. The current situation revealed a lot of tested protocols, significantly different, resulting without the broader application of this technique for grapevine. How and when will this important method in viticulture come to life depends on how it is presented in scientific and expert area. Testing of the cryopreservation protocol is a too demanding procedure if we want to only reach some good percentage of survival or to get fast results. The final aim should observe the cultivar of interest in a given condition for long-term conservation, and only then this type of experiments is reasonable. In the case of just testing, the protocol with representative data is an example of this method made on irrelevant cultivars or rootstocks that have reached high percentages of regeneration. This is a method of scientific approach that should not be performed with the aim of not giving the wrong picture of the method in general. Of course, some non-important cultivars and rootstocks should be tested because of their national importance and the need for long-term preservation that has been already mentioned above. From all experiments made in one laboratory, "model cultivar" should arise from all thoroughly tested steps from

choosing the appropriate material for experiment, defining a duration of exposure to cryoprotectant solutions to the already selected most appropriate medium for regeneration. Once the protocol is set up for cryopreservation as the "standard" one, then it could be concluded how many laboratories and which one advanced in that area with cryopreservation protocols. That assume that all culture condition requirements are suitably adjusted undoubtedly not affecting the procedure of cryopreservation protocol. When exact protocol is adopted, it can be tested in several different laboratories on the same cultivar with a comparison of obtained results.

Knowledge of growth, development, and regeneration of grapevine in tissue culture conditions is a crucial factor in achieving success. From the beginning of tissue culture on grapevine, there are many protocols in use, but there is only limited use in viticulture.

It was reported that in vitro establishment was achieved with nodal segments taken outdoor from just mature canes of 2-month old [40]. Alizadeh [40] also reported that micropropagation will inevitably be necessary for mass propagation in different horticultural crops. Chee and Pool [41] first designed a large scale of micropropagation protocol where in 8 weeks one person can deliver 2000 vines of 6 cm high and with six to nine nodes. This presents a grown plant of grapevine in vitro. This definition should be a copy in defining recovery after cryopreservation of grapevine in vitro plants. The most similar results were obtained in our in vitro laboratory, when achieving in vitro grown plants, if the protocol of micropropagation is working, is needed at least 2 to 3 months. To record the recovery or regeneration after cryopreservation, grown plants should be achieved; otherwise, it is not clear enough does regeneration has been achieved. Considering that laboratories are describing recovery differently, any other regeneration of grapevine in vitro plants can be questionable.

1.2. Preculture of explants

In the case of cryopreservation protocols for grapevine, preculture means exposing explants, which are intended for cryopreservation protocol to solutions with sucrose or some other additives. The accumulation of endogenous cryoprotectants such as sugar and sugar alcohol may increase the stability of membranes under conditions of severe dehydration [13]. It is noted that the most frequent cryopreservation protocols applied on grapevine include preculture, no matter which method has been applied [19]. The main aim is to induce tolerance of in vitro stock cultures and explants to dehydration and subsequent freezing in LN.

Protocols initially developed for grapevine, including the method of encapsulation-dehydration, allude the treatment of explants with increasing the sucrose contents in a medium [21, 34, 36, 42]. However, in order to increase the level of regeneration, researchers explore different types of preculture. In recently developed protocols with the method of droplet vitrification [16], preculture was a basal solid medium with 0.1 M sucrose concentration. In his first improvement of droplet vitrification method, Pathirana et al. [17] increase the level of regeneration with addition of 0.1 mM of salicylic acid in pretreatment. More detailed research revealed that preculture medium can be composed with more components: 0.3 M sucrose, salicylic acid, glutathione and ascorbic acid [43]. In our laboratory, research made on the influence of preculture on survival after cryopreservation revealed that explants should be cultivated in a form of microcuttings on the medium with addition of growth regulators [36]. Although the procedure of preparation of microcuttings is homogenizing the material where all axillary buds take over the role of shoot tip, addition of benzyladenine (BAP) in a medium can improve regeneration [16] after cryopreservation that was reported. At any rate for grapevine, explants themselves in a size of 1 mm, the greatest size for survival, can not survive an extremely stressful process of freezing, without the previous preparation.

In our case, a preculture with 0.1 M sucrose in a medium for 24 hours, just before procedure of cryopreservation [36], resulted as the greatest for grapevine shoot tip survival after cryopreservation and was applied in the following research [16, 37]. The importance of preculture arises from the fact that direct immersion in liquid nitrogen of one *Vitis vinifera* cultivar resulted without survival after cryopreservation [44] even if the report is quite old.

Some presteps or addition of some steps in the standard protocol of cryopreservation has been tested. Improvement of plant regeneration in cryopreserved kiwi fruits was revealed [45]. Cold acclimation of donor plantlets at 4°C for 2 weeks followed by sucrose preculture of shoot tips and supplementation of ascorbic acid (0.4 mM) in all media throughout the procedure registered 40% regeneration after cryopreservation. Cold-acclimated plantlets and ascorbic acid pretreated shoot tips exhibited severe plasmolysis and some disruption of membrane and vacuoles. This study revealed that only those cells that have been dehydrated and plasmolyzed can withstand cryopreservation without vitrification [45]. This type of experiment design is highly needed in cryopreservation of grapevine.

1.3. Role of genotypes

In a recent review article about cryopreservation of grapevine, it was noted that this method is highly genotype sensitive. Even the previously thoroughly tested protocols applied on different grapevine cultivars revealed significantly various results [25], they observed regrowth rates from 30% in Teleki 5BB (*V. berlandieri* × *V. riparia*) to 86.7% in Merlot (*V. vinifera*). Whenever more than one genotype tested in the success of cryopreservation protocol on grapevine shoot tips, a strong genotype effect was reported [17, 23, 37, 46, 47]. In this overview it was noted that significantly more *Vitis vinifera* cultivars were tested in comparison with rootstocks and hybrids. Considering the general need for conservation of grapevine genetic resources [19, 32], *Vitis vinifera* cultivars are rather a better choice. However, regarding the main aim of the method, each research laboratory is trying to find solution for the cultivars of interest where consequently so many cultivars were tested through this method.

Cryopreservation of grapevine shoot tips has been tested with different methods of cryopreservation. Firstly, Ezawa et al. [20] reported two-step cooling with three *V. labrusca* genotypes reaching 96.7% of survival, respectively. Esensee and Stushnoff [44] tested one *Vitis vinifera* cultivar without any survival by direct immersion in liquid nitrogen, as reported earlier. Neither two-step cooling nor direct immersion in liquid nitrogen was tested with grapevine shoot tips afterward. Combination of encapsulation-dehydration and two-step

cooling was made on nine Vitis vinifera cultivars [22, 46, 47], and even in different research laboratories, there was a quite stable rate of recovery reaching around 30%. With a method of encapsulation-dehydration, six Vitis vinifera cultivars were tested reaching 37-63% of recovery [23, 34, 36, 42, 48]. Encapsulation-vitrification as a method was tested with only one hybrid (V. berlandieri x V. riparia) with no specified number of survival [49]. The most used method for grapevine shoot tips was vitrification and more recently droplet vitrification. Regarding vitrification various results were obtained with different types of genotypes reaching from 30 to 86.7% of recovery [25, 27, 34, 48, 50-52]. Even if the method of droplet vitrification was designed to achieve some improvements, extremely low rate of 6% of recovery was reported [17], and high percentage (76%) of recovery was noted [53]. From the mentioned results, it is difficult to conclude which cultivar or genotype is adaptable to cryopreservation protocol in order to test the protocol. Results clearly revealed that there is still no "standard protocol" for grapevine shoot tips effective in each research laboratory. Thus, a major problem arises from the fact that different cultivars are responding differently, and in many cases to achieve high percentage of recovery, a cultivar of interest should be previously tested. From our point of view, there should be one cultivar responding very well in tissue culture conditions that should be tested in each cryopreservation laboratory. The proposed solution is the only scientific way we could observe efficiency and repeatability of cryopreservation laboratories.

2. Conclusion

A general perspective for cryopreservation of grapevine is questionable considering the long period needed for adjustment of the protocol. Also, in adjustment of the protocol to the one cultivar, we are in a large risk to waste the time and money, if tested protocol does not work on the range of cultivars. In the review article [19], it was observed that more than 25 reports were made on grapevine shoot tips; however, no wide range of application was made, even needed for grapevine genetic resources. With here listed and suggested guidelines, we could surely take the opportunity to make a common research and with joint forces come to the solution.

There is no existing a grapevine gene bank fo far, so we assume that all grapevine cryopreservation specialists have the same interest and that is the application of tested protocols to a greater number of cultivars. Consequently, some type of association testing grapevine cryopreservation protocols, at least with regard to material, variety, and tested protocols, should be united. The highly demanding protocol for grapevine that will give more or less the same results on one grapevine cultivar, chosen as a "model cultivar," through cryopreservation research laboratories around the world is surely the beginning that all trustworthy laboratories should aspire.

Hopefully, that part of our proposals will start to be implemented in the near future, and we will continue to work intensively on developing a single variety protocol and further testing of cultivars of interests that need long-term preservation.

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References

- [1] This P, Lacombe T, Thomas MR. Historical origins and genetic diversity of wine grapes. Trends in Genetics. 2006;**22**:511-519
- [2] Bacilieri R, Lacombe T, Le Cunff L, Vecchi-Staraz MD, Laucou V, Genna B, et al. Genetic structure in cultivated grapevines is linked to geography and human selection. BMC Plant Biology. 2013;13:25. DOI: 10.1186/1471-2229-13-25
- [3] Roubelakis-Angelakis KA. Grapevine Molecular Physiology and Biotechnology. New York: Springer; 2010
- [4] Maletić E, Pejić I, Karoglan Kontić J, Preiner D, Šimon S. Vitis genetic resources in Croatia preservation, evaluation and revitalization of grapevine varieties. Plant Genetic Resources and their Exploitation in the Plant Breeding for Food and Agriculture/ Hauptvogel, Pavlov; Benedikova, Daniela; Hauptvogel, Rene (ur.). Piestany: Research Institute for Plant Production Piestany, 2007;129-130
- [5] Maletić E, Pejić, I, Karoglan Kontić J, Preiner D, Šimon S. Grapevine genetic resources in Croatia – preservation, evaluation and revitalization of autochthonous varieties. Konferencija o izvornim pasminama i sortama kao dijelu prirodne i kulturne baštine - pod pokroviteljstvom Vlade Republike Hrvatske - Šibenik, 13. - 16. studenog 2007. Knjiga sažetaka. Šibenik: State Institute for Nature Protection. 2007;166-167
- [6] Maletić E, Karoglan Kontić J, Pejić I, Preiner D, Zdunić G, Bubola M, et al. Green Book Indigenous Grapevine Varieties Of Croatia. Zagreb: State Institute for Nature Protection; 2015

- [7] Karoglan Kontić J, Marković Z, Preiner D, Maletić E. Revitalizacija ugrožene sorte vinove loze Zlatarice blatske na otoku Korčuli. Book of Abstracts 2nd Conference on Native Breeds and Varieties as part of Natural and Cultural Heritage with international participation / Davorin Marković, Jasna Jeremić (ur.). Zagreb: State Institute for Nature Protection; 2010. p. 49
- [8] Žulj Mihaljević M, Anhalt UCM, Rühl E, Tomić Mugoša M, Forneck A, Zdunić G, et al. Cultivar identity, intravarietal variation, and health status 3 of native grapevine varieties in Croatia and Montenegro. American Journal of Enology and Viticulture. 2015;66(4):531-541
- [9] Preiner D, Horvat I, Maletić E. Ampelografske karakteristike klonskih kandidata sorte Plavina u pokusnom nasadu "Baštica" u 2010. Godini. Glasnik zaštite bilja. 2011a;**34**(5):74-80
- [10] Preiner D, Lešić J, Stupić D, Andabaka Ž, Edi M. Ampelografske karakteristike klonskih kandidata sorte Grk (V. vinifera L.) u pokusnom nasadu "Baštica" u 2010. Godini. Glasnik zaštite bilja. 2011b;34(6):68-79
- [11] Preiner D, Žugec I, Marković Z, Andabaka Ž, Stupić D, Maletić E. Ampelografske karakteristike klonskih kandidata sorte Pošip (V. vinifera L.) u pokusnom nasadu "Baštica" u 2010. godini. Glasnik zaštite bilja. 2012;4:64-73
- [12] Towill LE. Cryopreservation of plant germplasm. In: Towill LE, Bajaj YPS, editors. Biotechnology in Agriculture and Forestry: Cryopreservation of Plant Germplasm II. Berlin: Springer-Verlag; 2002. pp. 3-21
- [13] Reed BM. Plant Cryopreservation: A Practical Guide. New York: Springer Science and Business Media LLC; 2008. pp. 33-58
- [14] Gonzalez-Benito ME, Clavero-Ramírez I, López Aranda JM. Review. The use of cryopreservation for germplasm conservation of vegetatively propagated crops. Spanish Journal of Agricultural Research. 2004;2(3):341-351
- [15] Reed BM, Paynter CL, DeNoma J, Chang Y. Techniques for medium-and long-term storage of (*Pyrus* L.) genetic resources. Plant Gen Res Newsletter. 1998;115:1-4
- [16] Marković Z, Chatelet P, Preiner D, Sylvestre I, Kontić JK, Engelmann F. Effect of shooting medium and source of material on grapevine (*Vitis vinifera* L.) shoot tip recovery after cryopreservation. Cryo Letters. 2014;**35**:40-47
- [17] Pathirana R, McLachan A, Hedderley D, Panis B, Carimi F. Pretreatment with salicylic acid improves plant regeneration after cryopreservation of grapevine (*Vitis* spp.) by droplet vitrification. Acta Physiologiae Plantarum. 2016;**38**(1):1-11. DOI: 10.1007/ s11738-0152026-1
- [18] Reed B. Culture conditions are as important as the protocol in successful cryopreservation. Cryobiology. 2018;80:156-195. DOI: 10.1016/j.cryobiol.2017.10.065

- [19] Bi WL, Pan C, Hao XY, Cui ZH, Kher MM, Marković Z, et al. Cryopreservation of grapevine (*Vitis* spp.)—a review. In Vitro Cellular & Developmental Biology. Plant. 2017;**53**:449-460. DOI: 10.1007/s11627-017-9822-9
- [20] Ezawa T, Harada T, Yakawa T. Studies on freeze-preservation of fruit tree germplasm: III Freeze-preservation of grape shoot tips. Journal of the Faculty of Agriculture, Hokkaido University. 1989;64:51-55
- [21] Plessis P, Leddet C, Dereuddre J. Resistance to dehydration and to freezing in liquid nitrogen of alginate coated shoot tips of grapevine (*Vitis vinifera* L. cv. Chardonnay). Comptes Rendus Acad Sci Paris Ser III Sci Vie. 1991;**313**:373-380
- [22] Plessis P, Leddet C, Collas A, Dereuddre J. Cryopreservation of *Vitis vinifera* L. cv. Chardonnay shoot tips by encapsulation-dehydration: effect of pretreatment, cooling and postculture conditions. Cryo Letters. 1993;14:309-320
- [23] Wang QC, Tanne E, Arav A, Gafny R. Cryopreservation of in vitro-grown shoot tips of grapevine by encapsulation dehydration. Plant Cell, Tissue and Organ Culture. 2000;63:41-46
- [24] Miaja ML, Gribaudo I, Vallania R, Fernandez LF. Low temperature storage and cryopreservation of a *Vitis vinifera* L. germplasm collection: first results. Eucarpia symposium on Fruit Breeding and Genetics. Acta Horticulture. 2000;538
- [25] Matsumoto T, Sakai A. Cryopreservation of axillary shoot tips of in vitro-grown grape (*Vitis*) by a two-step vitrification protocol. Euphytica. 2003;**131**:299-304
- [26] Grassi S, Benelli C, Fabbri A. Cryopreservation of buds of grape rootstock "Kober 5BB" (Vitis berlandieri × Vitis riparia). 2004. VII Giornate Scientifiche SOI, Napoli
- [27] Ganino T, Silvanini A, Beghé D, Benelli C, Lambardi M, Fabbri A. Anatomy and osmotic potential of the *Vitis* rootstock shoot tips recalcitrant to cryopreservation. Biologia Plantarum. 2012;56:78-82
- [28] Marković Z, Preiner D, Bošnjak A, Safner T, Stupić D, Andabaka Ž, et al. *In vitro* introduction of healthy and virus-infected genotypes of native Croatian grapevine cultivars. Central European Journal of Biology. 2014;9:1087-1098
- [29] Banilas G, Korkas E. Rapid micro-propagation of grapevine (cv. Agiorgitiko) through lateral bud development. Journal of Science and Technology. 2007;2:31-38
- [30] Mederos-Molina S. Culture medium requirements for micropropagation of *Vitis vinifera* L. cv. Listan Blanco. Acta Horticulturae. 2007;(754):265-271
- [31] Péros JP, Torregrosa L, Berger G. Variability among *Vitis vinifera* cultivars in micropropagation organogenesis and antibiotic sensitivity. Journal of Experimental Botany. 1998;49:171-179
- [32] Bettoni JC, Costa MD, Pereira Gardin JP, Kretzschmar AA, Pathirana R. Cryotherapy:A new technique to obtain grapevine plants free of viruses. Revista Brasileira de Fruticultura. 2016;38(2):e-833. DOI: 10.1590/0100-29452016833

- [33] Engelmann F. Plant cryopreservation: Progress and prospects. In Vitro Cellular & Developmental Biology. Plant. 2004;**40**:427-433
- [34] Wang QC, Mawassi M, Li P, Gafny R, Sela I, Tanne E. Elimination of virus A (GVA) by cryopreservation of in vitro-grown shoot tips of *Vitis vinifera* L. Plant Science. 2003;165:321-327
- [35] Marković Z, Chatelet P, Peyrière A, Preiner D, Engelmann-Sylvestre I, Karoglan J, et al. Effect of proline pretreatment on grapevine shoot-tip response to a droplet-vitrification protocol. American Journal of Plant Sciences. 2013:2414-2417
- [36] Marković Z, Chatelet P, Sylvestre I, Kontić J, Engelmann F. Cryopreservation of grapevine (*Vitis vinifera* L.) in vitro shoot tips. Central European Journal of Biology. 2013;8:993-1000
- [37] Marković Z, Preiner D, Stupić DS, Andabaka Ž, Šimon S, Vončina D, et al. Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L.). Vitis. 2015;**54**:247-251
- [38] Šikuten I. In vitro multiplication of virus infected grapevine genotypes through the method of cryotherapy [Master thesis]. 2016
- [39] Wang MR, Hao XY, Zhao L, HuaCui Z, Volk GM, Wang QC. Virus infection reduces shoot proliferation of in vitro stock cultures and ability of cryopreserved shoot tips to regenerate into normal shoots in 'Gala' apple (Malus × domestica). Cryobiology. 2018; (in press. DOI: 0.1016/j.cryobiol.2018.08.002
- [40] Alizadeh M, Singh SK, Patel VB. Comparative performance of in vitro multiplication in four grape (*Vitis* spp.) rootstock genotypes. International Journal of Plant Production. 2010;4:41-50
- [41] Chee R, Pool RM. *In vitro* vegetative propagation of *Vitis:* Application of previously defined culture conditions to a selection of genotypes. *Vitis.* 1983;**22**:363-374
- [42] Bayati S, Shams-Bakhsh M, Moieni A. Elimination of grapevine virus A (GVA) by cryotherapy and electrotherapy. Journal of Agricultural Science and Technology. 2011;13:443-450
- [43] Bettoni JC. The development of a droplet-vitrification method to conserve Vitis collections in the USDAARS National Plant Germplasm System and UDESC-CAV Santa Catarina State University in Brazil. Third International Symposium on Plant Cryopreservation, Thailand, Bangkok; March 2018
- [44] Esensee V, Stushnoff C. Cryoconservation of dormant grape (Vitis sp.) buds. In: Contributed Papers (Oral and Poster) 87th Annual Meeting of the American Society for Horticultural Science 1990; 25. p.190
- [45] Mathew L, McLachlan A, Jibran R, Burritt DJ, Pathirana R. Cold, antioxidant and osmotic pre-treatments maintain the structural integrity of meristematic cells and improve plant regeneration in cryopreserved kiwifruit shoot tips. Journal of Agricultural Science and Technology. 2018;13(3):443-450

- [46] Zhao YH, Wu YJ, Engelmann F, Zhou M. Cryopreservation of axillary buds of grape (*Vitis vinifera*) in vitro plantlets. Cryo Letters. 2001;**22**:321-328
- [47] Zhai ZY, Wu YJ, Engelmann F, Chen RZ, Zhao YH. Genetic stability assessments of plantlets regenerated from cryopreserved in vitro cultured grape and kiwi shoot tips using RAPD. Cryo Letters. 2003;24:315-322
- [48] Wang QC, Li P, Batuman O, Gafny R, Mawassi M. Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured in vitro. Cryo Letters. 2003;24:293-302
- [49] Benelli C, Lambardi M, Fabbri A. Low temperature storage and cryopreservation of the grape rootstock "Kober 5BB". Acta Horticulturae. 2003;(623):249-253
- [50] Shatnawi M, Anfoka G, Shibli R, Almazra' Awi M, Shahrour W, Arebiat A. Clonal propagation and cryogenic storage of virus-free grapevine (*Vitis vinifera* L.) via meristem culture. Turkish Journal of Agriculture and Forestry. 2011;35:173-184
- [51] Hassan NA, Haggag AM. Cryopreservation of two Egyptian grape (*Vitis vinifera*) cultivars using two steps vitrification protocol. World Applied Sciences Journal. 2013;**28**:254-258
- [52] Lazo-Javalera MF, Tiznado-Hernández ME, Vargas-Arispuro I, Valenzuela-Soto E, Rocha-Granados M del C, Martínez-Montero ME, et al. Data on antioxidant activity in grapevine (*Vitis vinifera* L.) following cryopreservation by vitrification. Data in Brief. 2015;5:549-555
- [53] Bi WL. Cryopreservation of shoot tips of grapevine (*Vitis* spp.) and cryotherapy for eradication of grapevine leafroll-associated virus 3 [PhD thesis]. Yangling, China: Northwest A&F University; 2017

