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Cryopreservation of Hazelnut (*Corylus avellana* L.) Axillary Buds from *In Vitro* Shoots Using the Droplet Vitrification Method

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Abstract: Cryopreservation by droplet vitrification was applied to hazelnut (*Corylus avellana* L.). axillary buds of the Italian cultivated variety Tonda Gentile Romana, which were collected from *in vitro* growing shoots, immersed in ice cooled PVS2 or PVS3 for 60 or 90 min, then transferred to a droplet of vitrification solution, placed on a strip of aluminium foil, and plunged into liquid nitrogen (LN). Additionally, the effect on the recovery of the mother plant after cryopreservation was evaluated, following a cold pre-treatment at 4 °C for 3 months. The highest regrowth percentage (56.7%) was obtained after applying PVS3 for 60 min, while the application of PVS2 for the same amount of time reduced regrowth to 41.5%. Increasing the exposure to vitrification solutions to 90 min reduced regrowth to 43.3% when PVS3 was applied, and 35.6% if PVS2 was used. The cold pre-treatment on the mother plant did not significantly improve overall regrowth. The cryopreservation process did not decline the rooting ability of the recovered shoots.

Keywords: *in vitro* culture; length of dehydration; PVS2; PVS3; regrowth; rooting

Citation: Sgueglia, A.; Frattarelli, A.; Gentile, A.; Urbinati, G.; Lucioli, S.; Germanà, M.A.; Caboni, E. Cryopreservation of Hazelnut (*Corylus avellana* L.) Axillary Buds from *In Vitro* Shoots Using the Droplet Vitrification Method. *Horticulturae* **2021**, *7*, 494. https://doi.org/10.3390/horticulturae7110494

Academic Editor: Sergio Ruffo Roberto

Received: 27 September 2021
Accepted: 12 November 2021
Published: 15 November 2021

1. Introduction

The development of efficient ex situ conservation methods has an essential role in the conservation of biodiversity and avoidance of genetic erosion. Cryopreservation reduces the costs of maintenance as well as the risks of material losses caused by biotic and abiotic factors in respect to the in-field and in-greenhouse preservation [1–5]. Therefore, the studies and application of *in vitro*-based ex situ cryopreservation have been largely increased in recent years [1–3].

Several cryopreservation protocols, based on dehydration–encapsulation [6], vitrification [7], and a combination or modification of these methods [8,9], have been established for several species, including woody fruit species [10–15].

The main problems of all cryopreservation protocols are the avoidance of intracellular ice crystals (during freezing and thawing) and the improvement of tissue tolerance to cryoprotectants. Intracellular ice crystals in explant cells were avoided, during the vitrification method, by treatment with cryoprotectant solution. Sufficient dehydration with a vitrification solution is very important for a high survival rate and shoot tip recovery after liquid nitrogen (LN) [16]. PVS2 [7] and PVS3 [17] are the most used solutions.

Vitrification solutions are mostly toxic for the explants and exactly defined protocol for their application is necessary for explants regrowth [9].

Plant species differently respond to cryopreservation protocols. Thus, to develop a cryopreservation method for successful preservation, the genotype-related response must be taken into consideration [18].

The droplet vitrification (DV) technique is one of the modifications of the vitrification method. The aforementioned technique used a PVS2 solution and was developed for sweet potato shoot tips [19] and *Musa* germplasm conservation [20]. During DV, explants were frozen in a single droplet of vitrification solution, placed on small pieces of aluminium foils, which were immersed in LN. Applying this procedure, cooling, and thawing are faster than the vitrification method; thus, the lethal effects of intracellular ice crystals formation during these steps are reduced [15,20,21]. Droplet vitrification has been successfully applied to temperate fruit woody species such as apple [22,23], *Prunus* spp. [24–26], *Citrus* spp. [27], and *Vitis* spp. [28–30], and it is currently the most widely applicable cryo-protocol for cryopreserving plant germplasm within gene banks [15].

European hazelnut (*Corylus avellana* L.) is a species of the Betulaceae family and is of high interest in the world for its nuts, which are used in the confectionery and bakery industries [31]. Italy is the second largest hazelnut producer country in the world, after Turkey, and most of its production is based on native varieties [32]. Conservation of hazelnut biodiversity was performed in or ex situ [33].

The conservation of local cultivars is essential for breeders and stakeholders [33,34] and their micropropagation is a suitable tool for the production of true-to-type and disease-free plants. Micropropagation of hazelnut cultivars has been described in several studies [35–40] and it is commonly used in the USA, Chile, and Italy as a tool for propagation and conservation of genetic resources [33,41]. *In vitro* cultures also represent the base for cryopreservation and rapid clonal multiplication offering a possibility for the reintroduction of ancient cultivars.

Cryopreservation of *C. avellana* has previously been performed by using embryo axes [42–44]. However, since heterozygosity is high in hazelnut cultivars, clonal preservation, based on cryopreservation of shoot tips or axillary buds, is much more desirable than seeds or pollen preservation [45–50]. Recently, a study performed on cryopreservation of two Italian hazelnut cultivars showed the possibility of the application of an encapsulation–dehydration method on axillary buds excised from *in vitro* growing shoots [51].

The main aim of this work was to evaluate the applicability of the DV method for cryopreserving *C. avellana* germplasm. Axillary buds of the cultivated variety Tonda Gentile Romana, an old cultivar mostly diffused in Central Italy [34], were used to define how the type and the length of PVS treatment and the cold pre-treatment of the *in vitro* growing mother plant affect regrowth of explants after cryopreservation.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

In vitro propagated cultures of hazelnut (*Corylus avellana* L.), Italian cultivated variety Tonda Gentile Romana (TGR), were established from a single axillary bud, according to Gentile et al. [52]. Regenerated shoots were further multiplied *in vitro* (Figure 1A) on Molt medium, consisting of a combination of DKW (Driver and Kuniyuki medium) [53] and WPM [54] salts; DKW vitamins, according to Damiano et al. [39], were supplemented with 6-benzyladenine (BA, 1.5 mgL^{-1}), gibberellic acid (GA_3, 0.1 mgL^{-1}), and indole butyric acid (IBA, 0.01 mgL^{-1}). Sucrose 30 gL^{-1} and 5.7 gL^{-1} agar (B&V, Parma, Italy) were also supplied and pH was adjusted to 5.7. Cultures were sub-cultured every 3 weeks and maintained in standard cultural conditions at 24 ± 1 °C, under a 16 h photoperiod with light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Fluora L58 vv/77-Osram).

2.2. Explant Preparation for Cryopreservation and Loading Phase

Axillary buds were collected from *in vitro* grown shoots, at the end of subculture. Explants were transferred to 20 mL filter-sterilised loading solution, consisting of 2 M glycerol and 0.4 M sucrose dissolved in Molt liquid medium, and kept in darkness for 20 min at room temperature (Figure 1B).

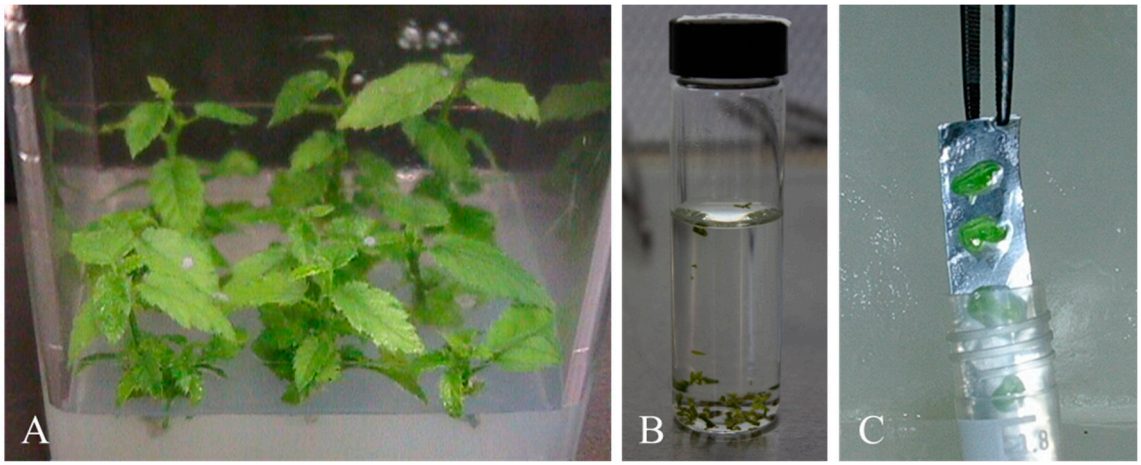

Figure 1. (**A**) shoots multiplication of hazelnut, cultivated variety Tonda Gentile Romana, (**B**) axillary buds in loading solution, (**C**) explants in droplets of vitrification solution plunged in liquid nitrogen on a strip of aluminium foil.

2.3. Explant Dehydration, Thawing and Unloading

After the loading phase, 30 explants (control) were directly transferred to the recovery solution (1.2 M sucrose dissolved in hormone free Molt liquid medium). For the others, loading solution was replaced by ice-cooled filter-sterilized PVS2 [7], consisting of 30% glycerol, 15% ethylene glycol, and 15% DMSO, dissolved in hormone free Molt liquid medium containing 0.4 M sucrose, or PVS3 [17] containing 50% (*w*/*v*) glycerol and 50% (*w*/*v*) sucrose added to hormone free Molt liquid medium. Explants were maintained immersed in the vitrification solutions for 60 or 90 min at 0 °C. Then, according to Panis et al. [20], explants in a droplet (25–30 µL) of PVS2 or PVS3 solution were placed on a strip of aluminium foil (5 mm × 20 mm) (Figure 1C) and maintained at around 0 °C during the manipulations, using a frozen cooling element. Half of the explants for each treatment were moved directly to the recovery solution while the strips with the others were plunged into LN and kept for 1 h. All the explants were maintained immersed in the recovery solution at room temperature for 15 min and then axillary buds were placed on sterile filter paper on top of a solid hormone free Molt medium containing 0.3 M sucrose. After 24 h the explants were transferred on to fresh medium without filter paper and maintained in Petri dishes in darkness for 1 week. Then, explants were transferred on Molt medium in standard cultural conditions and sub-cultured every 2 weeks.

2.4. Cold Pre-Treatment of Mother Plant

Axillary buds were collected either from *in vitro* shoots cultures maintained at 4 °C for 3 months, or from shoots maintained in standard cultural conditions, the latter used as control. Excised explants, following the steps of the protocol described above, were transferred to LS in darkness for 20 min at room temperature, then to PVS3 for 60 or 90 min, and immersed or not in LN.

2.5. Rooting Induction of Recovered Explants

Shoots originated from the axillary buds cryopreserved using PVS3 for a duration of 60 min were multiplied for five subcultures. Then, 30 of these shoots and 30 from standard cultural conditions, as a control, were induced to root, according to Sgueglia et al. [41]. Briefly, shoots were transferred to a rooting medium, containing half strength MS macro salts, full MS micro salts and organics [55], 20 gL^{-1} sucrose, 5.6 gL^{-1} agar (B&V, Parma, Italy), and 17.6 µM IBA, in standard cultural conditions. After 7 days, shoots were transferred to Jiffy-7® Peat Pellets in a greenhouse, under a polyethylene tunnel with 90% of humidity, at 10–22 °C (night-day temperature), and midday average PPF of 35 µmol $m^{-2}s^{-1}$.

Data were collected 45 days after the beginning of the root induction treatment.

2.6. Data Collection and Statistical Analysis

Regrowth was evaluated 8 weeks after transferring on to the Petri dishes with the regrowth medium, by counting the number of explants green and showing growth. Buds which resumed normal development (production of new leaves and/or new shoots) were considered to be regrowing. Each treatment and control was performed with 3 Petri dishes with 10 explants each. The rooting experiment was performed by using a total of 30 shoots in 3 vessels (10 shoots for vessel) per treatment. All the experiments followed a completely randomized design. Results are presented as mean percentages. Arcsine transformation ($Y = arcsine\sqrt{\frac{p}{100}}$), where p is the percentage and Y is the result of the transformation, was applied to percentages. Standard errors (SE) were calculated and statistical differences among means were assessed by one-way ANOVA and Tukey's test ($p \leq 0.05$).

3. Results

3.1. Effect of Type and Length of Plant Vitrification Solution Treatment

When explants were not immersed in LN, the treatment with PVS2 and PVS3, applied for 60 min, did not reduce regrowth with respect to the explants not immersed in vitrification solutions (control). The increase in the application to 90 min significantly reduced regrowth (70.0%) in the explants treated with PVS3 and more in those treated with PVS2 (60.0%) (Figure 2).

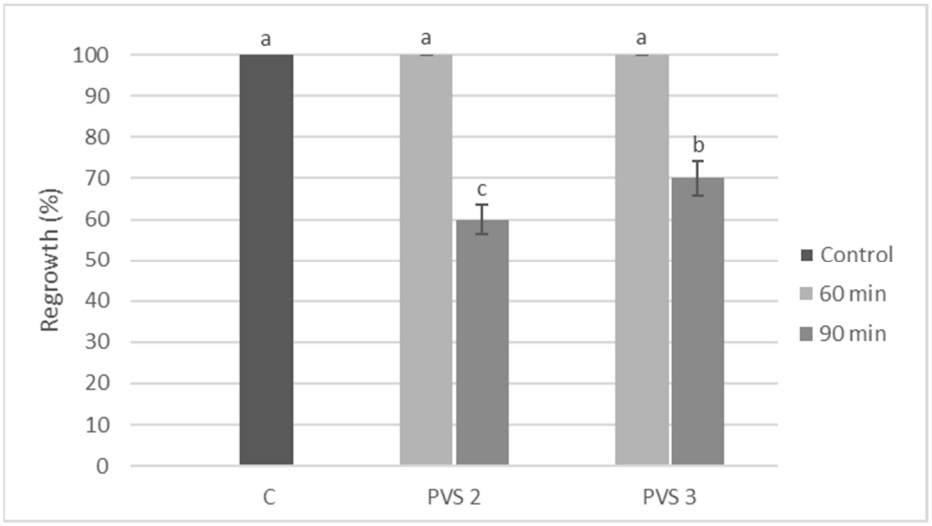

Figure 2. Effect of vitrification solutions (PVS2 or PVS3), applied for 60 or 90 min, on regrowth (%) of axillary buds of *Corylus avellana*, cultivated variety Tonda Gentile Romana, not immersed in liquid nitrogen. Control (C) indicates explants directly transferred to the recovery solution after loading phase. Error bars indicate SE. Bars marked with the same letter are not significantly different according to Tukey's test ($p \leq 0.05$).

Four weeks after transferring axillary buds immersed in LN in the regrowth medium, different types of reaction could be distinguished: completely or partially black axillary buds, indicating that there was high oxidation and necrosis of the explants (Figure 3A), or green explants (Figure 3B). Four weeks later, surviving explants already showed shoot regrowth (Figure 3C).

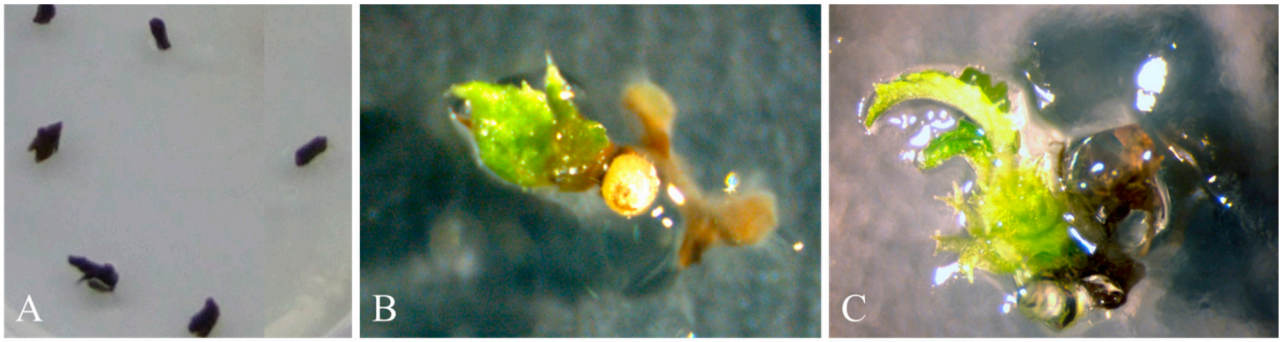

Figure 3. *Corylus avellana*, cultivated variety Tonda Gentile Romana. (**A**) Explant necrosis 4 weeks after thawing, (**B**) green explants 4 weeks after thawing, and (**C**) regrowing shoot 8 weeks after freezing.

Regrowth rates after immersion of axillary buds in LN were significantly affected either by the type of vitrification solution, or by the duration of its application (Table 1): the highest regrowth percentage (56.7%) was obtained after applying PVS3 for 60 min, while the application of PVS2 for the same duration reduced regrowth to 41.5%. Increasing duration to 90 min reduced regrowth when PVS3 and PVS2 were applied (43.3% and 35.6%, respectively).

Table 1. Effect of the type of vitrification solution (PVS2 or PVS3) and duration (60 or 90 min) on regrowth of axillary buds of *Corylus avellana*, cultivated variety Tonda Gentile Romana, 8 weeks after immersion in liquid nitrogen. Mean data are followed by Standard Error. Means followed by the same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Type of Vitrification Solution	Timing of Application (min)	Regrowth (%)
PVS2	60	41.5 ± 1.5 b
PVS2	90	35.6 ± 2.2 c
PVS3	60	56.7 ± 3.3 a
PVS3	90	43.3 ± 3.3 b

3.2. Effect of Cold Pre-Treatment of Mother Plant

Explants excised from cold treated mother plants, treated with PVS3 for 90 min and immersed in LN, showed a significant regrowth improvement in respect to explants excised from not cold treated shoots; on the other hand, reduction in regrowth was observed with 60 min of PVS3 treatment (Figure 4A). In the explants not immersed in LN, a regrowth improvement was only found with 90 min of PVS3 application, when the cold pre-treatment of mother plants was applied (Figure 4B).

3.3. Rooting of Recovered Explants

Rooting was 78% in shoots obtained from cryopreserved axillary buds and 80% in the shoots from standard cultural conditions, with a mean number of 5–6 roots, without significant differences between the responses (Table 2). No morphological differences were observed (Figure 5).

Table 2. Rooting of *Corylus avellana*, cultivated variety Tonda Gentile Romana, in cryopreserved (+LN) and in shoots grown in standard cultural conditions (SSC). Mean data are followed by Standard Error. Means on the column followed by the same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Cryopreservation	Rooting (%)	N. of Roots/Shoot
+LN	78 a	5.9 ± 0.5 a
SSC	80 a	6.1 ± 0.4 a

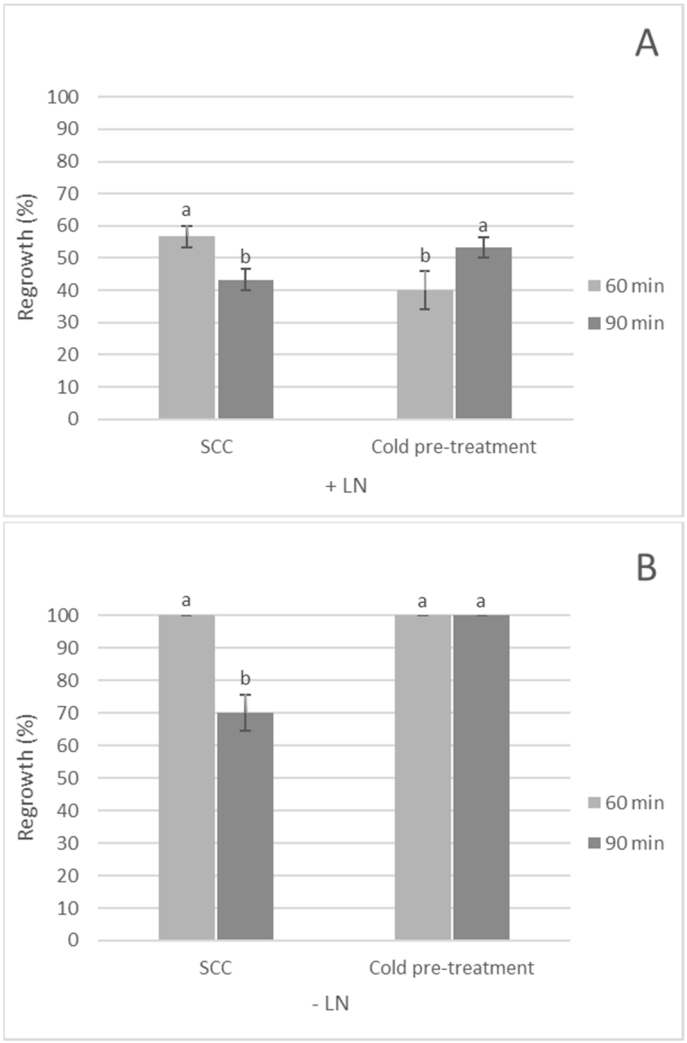

Figure 4. Effect of cold pre-treatment (4 °C) of mother plant's on regrowth (%) of axillary buds immersed in PVS3 (60 or 90 min) with (**A**) or without (**B**) liquid nitrogen (LN) immersion. SCC—standard cultural conditions. Error bars indicate SE. Bars of means marked with the same letter are not significantly different according to Tukey's test ($p \leq 0.05$).

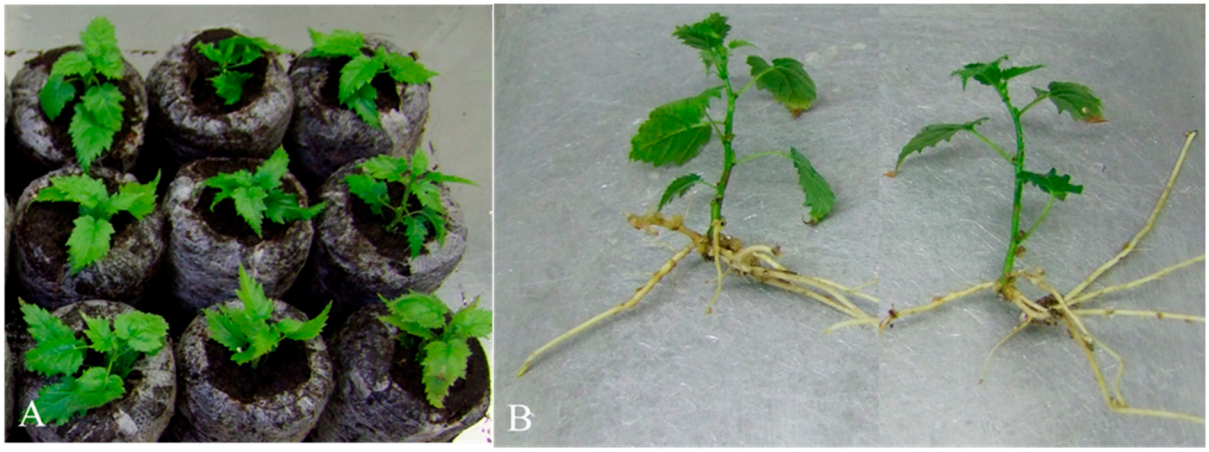

Figure 5. Rooting of *Corylus avellana*, cultivated variety Tonda Gentile Romana, after cryopreservation: (**A**) microcuttings from cryopreserved explants after rooting induction (17.6 µM IBA for 7 days) and transferring in Jiffy-7®, (**B**) rooted microcuttings originated from cryopreserved ABs (**left**) and from the control.

4. Discussion

Defining the suitable exposure duration of explants to the vitrification solutions is essential for cell dehydration, necessary to avoid the formation of intracellular ice crystals during freezing and thawing, and to prevent injury by chemical toxicity of cryoprotectants [56,57] or excessive osmotic stress [30]. In the current study, cryopreservation by DV was applied to axillary buds of *C. avellana*, the Italian cultivated variety Tonda Gentile Romana, collected from *in vitro* grown shoots. The effect on regrowth of the vitrification solution PVS2 and PVS3 and of the time of their application (60 or 90 min) was compared. Axillary buds were chosen as explants for cryopreservation by DV since this type of explant was shown to be more suitable than the shoot tips in a previous study on apple [22]. In cryopreserved axillary buds, the highest regrowth (56.7%) was obtained from the treatment with PVS3 applied for 60 min, while the application of PVS2 for the same duration reduced regrowth to 41.5%. These results are also consistent with previous findings in temperate woody species. In fact, Condello et al. [22] observed that a 60 min duration of PVS2 treatment is suitable for cryoconservation of apple cultivars and rootstocks by DV. Halmagyi et al. [58] found that 30–40 min of PVS2 application is suitable for shoot tips of other apple cultivars and Li et al. [23] found the highest shoot regrowth levels with 30–50 min duration of PVS2 exposure. On the other hand, PVS3 was successfully applied to various *Prunus* spp. and hybrids [24–26], and the best results were obtained with timings ranging from 60 to 90 min, according to the species.

The PVS2 treatment applied for 90 min induced a greater reduction in regrowth (60.0%) in the control (−LN) explants than the same duration of PVS3 application (70.0%), further suggesting that for the hazelnut axillary buds PVS3 is less toxic than PVS2. This response could be related to the presence of DMSO in the composition of PVS2, which can cause damage at cellular levels [56,59,60].

Previous studies have also shown the importance of the application of a cold hardening period to *in vitro* mother plants for the successful cryopreservation of fruit trees, including kiwi and apple [16,61–63]. In our study we present evidence that for cryopreservation of axillary buds of hazelnut, cold-treatment of *in vitro* mother plants is not critical for recovery, as previously observed in apple cryopreservation by the encapsulation–vitrification method by Paul et al. [64] and by Halmagyi et al. [58], using DV. However, axillary buds treated with PVS3 for 90 min and immersed, or not, in LN, showed higher regrowth when cold treatment was applied to the mother plant. These results, even if there was no significant overall effect on the explant regrowth, suggest that cold pre-treatment resulted in the different sensitivity of explants to cryoprotective solution, as previously observed in cryopreservation by grey poplar explant [65].

Concerning rooting of cryopreserved shoots, an increased efficiency in respect to the control was reported in *Rabdosia rubescens* after encapsulation–dehydration cryopreservation [66]. On the other hand, Kulus and Zalewska [67] and Kulus et al. [68] reported rooting inhibition after cryopreservation by encapsulation in Na-alginate in *Chrysanthemum* spp. In the present study, no influence of cryopreservation on the plant rooting efficiency was recorded, confirming results obtained in shoot tips-derived microcuttings of *Citrus* [69].

In conclusion, in this study, cryopreservation by DV was successfully applied for the first time for cryopreservation of axillary buds of *C. avellana*, the Italian cultivated variety Tonda Gentile Romana. We showed that axillary buds, excised from mother plants grown in standard cultural conditions, are suitable explants for cryopreservation in hazelnut, and we emphasised the critical role of the choice of the type of vitrification solution and the duration of its application. In addition, no influence of cryopreservation process was observed on the rooting response of the shoots obtained from survived axillary buds.

Our results highlight the potential of this method in ex situ conservation of biodiversity in this species. We show that the application of this DV protocol represents a useful option for the cryopreservation of hazelnut. In fact, cultivars of *Corylus avellana* being highly heterozygous, clonal preservation, based on axillary buds cryopreservation by DV, represents a new relevant step forward in respect to the available protocols for conservation

of seeds or embryos and an improvement of the regrowth response with regards to a previous approach based on the encapsulation–dehydration method applied to axillary buds of the cultivated variety Tonda Gentile Romana. We believe that the present DV protocol represents an efficient tool for cryopreservation and rapid clonal multiplication for the reintroduction of ancient cultivars in this species.

Author Contributions: A.S. and A.F. experiments conduction and data collection; A.S., E.C., A.G., and G.U., data elaboration and original draft writing; A.G., A.S., S.L., and E.C., review and editing; E.C., experiments planning and funding acquisition; M.A.G., supervision. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by the Italian Ministry of Agriculture (Mipaaf), National project Plant Genetic Resources/Implementation of the FAO International Treaty (RGV-FAO). Thanks are also due to University of Palermo (Italy) for the 'Mediterranean Fruit Crops' Ph.D. course, attended by Alessandra Sgueglia.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DMSO	dimethyl sulfoxide
DV	droplet vitrification
LN	liquid nitrogen
PVS2	plant vitrification solution 2
PVS3	plant vitrification solution 3

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