Survival and State of Garlic Explants of Two Lithuanian Cultivars after Cryopreservation

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Abstract: Cryopreservation features of Lithuanian garlic (Allium sativum L.) cultivars ‘Žiemiai’ and ‘Dangiai’ were investigated. The survival rate and state of explants, depending on the type of explant, and dehydration conditions were evaluated in the experiment. Explants isolated from cloves and bulbils were dehydrated with a plant vitrification solution 3 (PVS 3) containing 50% w/v glycerol and 50% w/v sucrose in liquid MS medium. Three different exposure durations of 1.5, 2, and 3 h in PVS3 solution were applied at 0 °C. Unloaded stem domes were planted in Petri dishes with MS medium supplemented with 1 mg L⁻¹ naphthaleneacetic acid (NAA) and 6–dimethylallylamino purine (2-iP) on 0.8% plant agar. The obtained results showed that the cryopreservation in liquid nitrogen reduced the survival rate of explants by 20–40%. The average number of surviving explants after freezing reached 64.3% for both cultivars. Cultivar ‘Žiemiai’ showed a 24% higher capability for survival than ‘Dangiai’. The total number of surviving explants of ‘Žiemiai’ reached 76.1%. Explants from bulbils were 23% more effective for cryopreservation compared to cloves. Evaluation of the effect of dehydration duration showed that the survival rate of the explants from bulbils of both cultivars was highest after treatment with PVS3 for 3 h and reached 91%. Treatment in PVS3 solution for 2 h was sufficient for survival of explants from cloves. Obtained results confirm that the efficiency of garlic cryopreservation depends on complex factors.

Keywords: Allium sativum L.; genotype; PVS3; survival; vitrification

1. Introduction

Garlic (Allium sativum L.) is a very specific and important plant in many countries of the world [1]. According to information from the Lithuanian Statistics Department, garlic production covered 1322.4 tons in our country, and the area planted with garlic was 188.91 ha in 2022 (https://osp.stat.gov.lt/zemes-ukis1 accessed on 30 March 2023). The growing area is increasing every year.

As a vegetatively propagated plant, garlic is conventionally propagated by cloves. Cold hardiness and resistance in winter are the main factors determining the geographical distribution of garlic. Scientific reports prove that garlic is very sensitive to changes in growing location and climatic conditions [2–4]. The Institute of Horticulture of the Lithuanian Research Centre for Agriculture and Forestry maintains a field collection of over 50 cultivars and populations of garlic. Studies conducted earlier confirmed higher winter hardiness of Lithuanian cultivars compared to foreign accessions [5–7]. However, winter cold damage to plants is still a major problem for garlic in our country, even for the local cultivars. In addition, the susceptibility of garlic to pests and pathogens also causes serious threats to genetic resources stored in uncontrolled field collections. In vitro techniques are being developed and used to store vegetatively propagated plants under low-temperature conditions. Cryopreservation allows for the preservation of plant material for unlimited periods without alteration [8].
A number of studies on the cryopreservation of garlic have been carried out in the past 30 years. A vitrification technique using apices sampled from cloves was reported [8,9]. Later, the differences between the use of apices sampled from bulbils and from cloves for cryopreservation were shown [10]. Maggioni et al. [1] and Keller [11] demonstrated the advantage of low-temperature preculture for *Allium* germplasm cryopreservation and its application in the activity of gene banks. Various studies reported the use of shoot tips and stem discs as explants for garlic cryopreservation [12–15]. The effects of osmotic stress and oxidative damage as critical factors during dehydration and freezing of garlic explants and the expression of genes in response to cold and osmotic stress in cryopreservation were studied [12,16–20]. The influence of the composition of different plant vitrification solutions (PVSs) on the survival of explants and regeneration frequency after cryopreservation was extensively studied [14,21–23]. Genotype-specific effects were presented in a series of garlic cryopreservation reports [9,10,13,14,17,22]. Nanoparticles and natural plant extracts can be used in the various steps of the cryo-procedure for the improvement of plant cryopreservation efficiency [24]. The aspects of cryopreservation of garlic have been studied widely in the literature. However, the success of cryopreservation depends on many factors, such as different protocols, genotypes, explant types, dormancy, etc. Therefore, for the cryopreservation of specific genotypes, a general understanding of cryopreservation is not sufficient; specific conditions are critical for the success of cryopreservation, which must be clarified each time. The results of many authors proved that it is not easy to regenerate plants after cryopreservation [10,13,16,17,21,22]. Therefore, such research does not lose its novelty and relevance.

Experiments with garlic cryopreservation have not been conducted in Lithuania. In vitro long-term storage problems and cold adaptation have been studied in our country for horticulture plants [25,26]. Cryopreservation experiments with horticulture plants are carried out in the Laboratory of Cryobiology LAMMC.

The aim of our study was to investigate the cryopreservation features of Lithuanian garlic cultivars, to evaluate the state of the explants after cryopreservation, and to determine the optimal conditions for long-term garlic preservation.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

Investigations were performed at the Institute of Horticulture LAMMC in 2018–2019. Garlic was grown in the crop rotation experimental field, where agrotechnological measurements and harvesting were carried out using conventional methods for horticulture research investigations [27].

Lithuanian garlic cultivars Žiemiai and Dangiai were used in the cryopreservation experiments. Both cultivars represent hardneck morphotypes. The bulb’s external scale of the cultivar ‘Dangiai’ is white with a purple stripe on it. The bulb has a constant number of 6–7 cloves. The cultivar Žiemiai is distinguished by the creamy white colour of the bulb’s external scales and the number of cloves per bulb, which reaches 8–12.

Cloves of garlic with an average weight of 6–7 g were planted on 18 October 2018 and bulbs and flower heads with bulbils were harvested on 21 August 2019. The plant material was then stored in a refrigerator cabinet (“Friotekno”, Italy) at 4 ± 1 °C for 9 weeks. Bulbs and cloves without an external scale were washed with tap water (3–4 min), and sterilized by rinsing with 75% ethanol and soaking in 10% sodium hypochlorite solution (7 min). Rinsing three times with sterile water was performed after both ethanol and hypochlorite treatment. Sterilized stem domes with basal parts (2 mm diameter and 3 mm length) were excised from cloves and bulbils. For the osmotic dehydration, explants were placed in 9 cm Petri dishes on a Murashige and Skoog (MS) [28] modified medium (Duchefa Biochemie, Haarlem, The Netherlands) supplemented with 13.7% sucrose and 0.8% plant agar. Petri dishes with explants were maintained at 22 ± 3 °C in the dark for 2 days. Then, stem domes of cloves and bulbils were transferred to the plastic cryotubes and incubated in a loading solution containing 18.4% w/v glycerol (Carl Roth, Germany) and
13.7% w/v sucrose in liquid MS medium for 20 min. at room temperature. Afterwards, the explants were dehydrated with plant vitrification solution 3 (PVS3) containing 50% w/v glycerol and 50% w/v sucrose in liquid MS medium for 1.5 h at 0 °C (on ice). A 1.5 h dehydration was applied in studies of the influence of genotype and explant type. For the dehydration experiment, the durations of exposure to PVS3 were 1.5, 2, and 3 h. Finally, the plant material was immersed directly in liquid nitrogen and stored for 1 h. All steps of the cryopreservation procedure were applied for the control samples except storage in liquid nitrogen (non-LN-treated) only. For rewarming, cryotubes with samples were plunged into a warm water bath (40 °C) (Heidolph instruments, Schwabach, Germany) until the cryoprotectant solution became liquid (1–2 min). Explants were rinsed two times with washing solution and placed on sterile filter paper to remove excess solution. The washing solution consisted of MS medium supplemented with 410 g·L⁻¹ sucrose. Unloaded stem domes were planted in Petri dishes with MS medium supplemented with 1 mg L⁻¹ naphthaleneacetic acid (NAA) and 6-dimethylallylamino purine (2-iP) on 0.8% plant agar, pH 5.8. Fifteen (15) explants were placed in one Petri dish. Petri dishes were placed at 25 ± 3 °C in the dark in a cooled incubator (ST2, Pol-Eko, Poland). After 7 days, Petri dishes with samples were transferred to the growth room under controlled conditions at 25 ± 3 °C, a 16/8 h day/night photoperiod, and illumination intensities of 50–150 µmol m⁻² s⁻¹.

2.2. Assessment of Cryopreservation Efficiency

Cryopreservation efficiency was determined by counting the number of surviving explants and evaluating the explant state. The survival and the state of explants and their tissues were determined visually three weeks after rewarming. Explants were characterised using a six-point rating scale: 0—dead, brown with no green; 1—etiolated, brown, pale; 2—yellowish brown colour; 3—etiolated, light-green colour; 4—not etiolated, green colour; 5—not etiolated, dark green colour, prolongation of the leaf bases (Figure 1). Survival criterion: explants with a condition of 2–5 points were considered survivors.

Figure 1. State of explants by points.

2.3. Data Analysis

Ten (10) explants of cloves and bulbils were used in every three replications (n = 3) for both genotypes and every treatment (two types of explants and three different exposure durations in PVS3). Obtained data were statistically processed by the ANOVA method according to Duncan’s multiple ranges for mean separation at a 5% significance level. The ± standard errors were submitted. Principal component analysis was used to identify grouping variables in the treatments within SPSS Statistical Package for the Social Sciences (SPSS) Software V.12 (2002) s.

3. Results and Discussion

3.1. Influence of Genotype on Survival Rate of Explants

A high influence of genotype on the efficiency of garlic cryopreservation was previously described in many scientific reports [8,10,14,21]. Morphologically different cultivars have different abilities to survive and regenerate after cryopreservation [29]. Two Lithuanian garlic cultivars were tested in our studies.
Lithuanian cultivars showed different abilities for survival after cryopreservation. The assessment of the state of explants showed that the number of dark green explants in the control (not frozen) experiment reached 92% for cultivar ‘Žiemiai’ and 82% for cultivar ‘Dangiai’. Some of the explants showed intensive prolongation of the leaf bases and rooting. This indicates that the cryoprotectants and preparational procedures did not significantly reduce the viability of the explants. ‘Žiemiai’ were a little more resistant to the treatment. The cryopreservation in liquid nitrogen reduced the survival rate of explants by 20–40%. The average number of surviving explants (state 2–5 points) of both cultivars was 64.3%. Here also, cultivar ‘Žiemiai’ showed a higher capability for survival. The total number of surviving explants of this cultivar reached 76.1%, while 39% were light-green in colour (Point 3), and 28% were yellowish-brown in colour (Point 2) (Figure 2). No significant differences were found between cultivars according to the number of explants that were in the best condition (4–5 points). The average number of non-etiolated, green-coloured explants (Point 4) reached 9.8% for both cultivars.

![Figure 2. Influence of genotype for the state of surviving explants after cryopreservation (average values for both types of explants). Scale (state of explants, points): 0—dead, brown with no green; 1—etiolated, brown, pale; 2—yellowish-brown colour; 3—etiolated, light-green colour; 4—non-etiolated, green colour; 5—non-etiolated, dark-green colour, prolongation of the leaf bases. Means followed by the same letter do not differ significantly within the column at \( p = 0.05 \) (Duncan’s multiple range test). Vertical dashes indicate the mean of standard error.](image)

The cryopreservation abilities of Lithuanian garlic cultivars were not previously studied. However, the assessment of explants’ survival after the cryo-procedure proved that a creamy white cultivar, ‘Žiemiai’, showed a higher survival rate after cryopreservation compared with the light purple ‘Dangiai’, while Chinese researchers presented results where purple garlic was more effective in survival and regeneration rates [17]. The explant’s survival rate of purple-coloured cultivars reached 65–82.6% and that of white-coloured cultivars was 7–51.2%, according to their findings. The Spanish cultivar Morado, which is covered with bold, bright-purple-coloured skin, showed that after cryopreservation, 22.5% of stem discs produced shoots [15]. Regeneration percentages ranging between 45% and 83% were presented by Zanke et al. [30] when 20 samples were subjected to cryo-freezing tests. Kim et al. [31] noted the regeneration from 72% to 95% of ten different garlic cultivars. These results confirm a high specific effect of genotype and individual response of cultivar.

3.2. Influence of Explant Type on the Survival Rate

A comparison of the effect of explant type showed that the survival rate of stem domes sampled from bulbils was significantly higher than that of stem domes sampled from cloves. For both cultivars, the average survival rate of stem domes from bulbils reached up to 79.5%, and that from cloves up to 70% in the control experiment. A similar tendency was observed with explants after cryopreservation. The average survival rate of both cultivars
was 67.8% and 45.1% for cryoprotected stem domes from bulbils and cloves, respectively. A state assessment of surviving explants showed that, significantly, the highest number (36%) was etiolated, light-green coloured (Point 3) stem domes sampled from bulbils (Figure 3). The average number of yellowish-brown coloured (Point 2) explants reached 19% and 31.1% from cloves and bulbils, respectively; the difference was also significant.

![Figure 3](image_url)

Figure 3. Influence of surviving explant type for their state after cryopreservation (average values for both cultivars). Scale (state of explants, points): 0—dead, brown no green; 1—etiolated, brown, pale; 2—yellowish-brown colour; 3—etiolated, light–green colour; 4—non-etiolated, green colour; 5—non-etiolated, dark green colour, prolongation of the leaf bases. Means followed by the same letter do not differ significantly within the column at $p = 0.05$ (Duncan’s multiple range test). Vertical dashes indicate the mean of standard error.

Our results demonstrated that the survival abilities after the cryopreservation procedure of stem domes sampled from bulbils and cloves are different. Explants from bulbils were more effective compared with cloves. Kim et al. [22] report that bulbil primordia, formed on unripe inflorescences, proved to be the most suitable material for the conservation of bolting varieties. Some authors prefer to use garlic shoot tips excised from cloves [12,13,15]. Using explants from cloves could be a good alternative for cryopreservation of garlic not forming flower stalks. The main disadvantages of explants from cloves are the limited clove number per bulb and higher contamination with infection caused by direct contact with the soil. In our experiments, an increased fungal infection level was observed with stem domes sampled from cloves compared with that from bulbils. Using garlic bulbils for cryopreservation presents the advantage of the enhancement of plant material sources. Keller [21] emphasizes bulbil size as an important factor for cryopreservation efficiency. We used explants 2 mm in diameter and 3 mm in length. Polish researchers present that the optimal size of explants is 1.5 mm in length [24]. Smaller explants may be at higher risk of meristem susceptibility during the isolation procedure. Results obtained by Kim et al. [16] showed that smaller shoot tips, measuring 1.5 or 3.0 mm in diameter, displayed higher regeneration than large ones, measuring 4.5 mm in diameter. The larger the explant, the more freeze-damaged tissue it contains, which may negatively affect the survival of meristems after freezing. In addition, the diffusion of cryoprotectants into deeper tissues during preparation depends on the size of the explant. This may affect the survival of explants after cryopreservation [32]. Makowska et al. [10] note a high survival rate (85–98%) of apices sampled from bulbils that weighed 100–420 mg more than the small ones. However, the regeneration efficiency they achieved was lower when using apices from bulbils rather than from cloves.
3.3. Influence of Dehydration Conditions on the Survival Rate of Explants

Dehydration is an important factor in garlic survival after cryopreservation [12,16]. It is proven that regeneration without the use of plant vitrification solution (PVS) is impossible [17,24]. In our experiment, the average values of survival rate for both tested cultivars and both types of explants reached 79% in the control variant and 50.5% after cryopreservation, while PVS3 was used for 1.5 h. Significant increases in the survival rate of cryoprotected explants were observed under extended PVS3 exposure duration. The highest survival rate of explants was observed when stem domes were soaked for 3 h in PVS3 solution. The total number of surviving stem domes from bulbils reached 91%, while 58.4% were light green (Point 3), and 31.4% were of a non-etiolated green (Point 4) colour (Figure 4). The survival rate of stem domes from cloves was 72%, and more than 36% of them were light green (Point 3) or non-etiolated green (Point 4). When stem domes from cloves were treated with PVS3 for 2 h, the number of high-quality explants of a light-green colour (Point 3) was significantly higher than that of the same type of explants treated with PVS3 for 3 h.

Figure 4. Influence of exposure duration PVS3 for the state of surviving explants after cryopreservation (average values for both cultivars). Scale (state of explants, points): 0—dead, brown with no green; 1—etiolated, brown, pale; 2—yellowish-brown colour; 3—etiolated, light-green colour; 4—non-etiolated, green colour; 5—non-etiolated, dark-green colour, prolongation of the leaf bases. Means followed by the same letter do not differ significantly within the column at $p = 0.05$ (Duncan’s multiple range test). Vertical dashes indicate the mean of standard error.

Thus, our results demonstrated that a 2 h exposure in PVS 3 solution was optimal for the survival of explants from cloves and a 3 h exposure for explants from bulbils, respectively. The same tendency was obtained by other researchers. Kim et al. [31] found that shoot-tip treatment with PVS3 for longer than 2 h had a positive effect on the survival and regeneration of explants after cryopreservation. Treating shoot tips with PVS3 for 2.5–3 h ensured a 92.2% survival rate and 92% regeneration after freezing, in their experiments. After testing three different genotypes, Makowska et al. [10] reported that bulbil explant survival reached 73–95% when explants were treated with PVS3 solution for 4 h. The most optimal method used for garlic in the Polish cryobank is the vitrification of shoots based on PVS3 treatment for 2 h [33,34]. Contrasting results are presented by Liu et al. [17], in which an optimal duration of treatment with PVS3 was 1.5 h compared with 2.5 and 3.5 h, respectively. Possibly, an extended duration of PVS3 treatment ensures sufficient diffusion of cryoprotectants to deeper explant layers; however, it depends on many conditions, such as explant size, explant type, etc. [32].
The scatter plot of principal coordinate analysis (PcoA) displayed diversity in garlic explant survival depending on genotype, type of explant, and treatment duration with PVS3. According to the PcoA results of the explant’s survival rate, it is possible to classify the investigated samples into several groups. The explants of both cultivars in the control experiment (ŽbC, ŽclC, DbC, and DclC) were distinguished by the highest survival dispersed in one group in the PC scatter plot area with the highest positive value (Figure 5). The stem domes from bulbils of both cultivars were treated for 2–3 h with PVS3 (ŽbII, ŽbIII, DbII, and DbIII) located in another group on the front side of the scatter plot. The rest of the samples with a lower survival rate comprised a separate group.

Figure 5. Scatter plot of garlic survival rate. Ž—cultivar Žiemiai; D—cultivar Dangiai; b—stem dome from bulbils; cl—stem dome from cloves; I—1.5 h PVS3; II—2 h PVS3; III—3 h PVS3; C—control experiment.

These experiments have provided new knowledge about the cryopreservation ability of Lithuanian garlic cultivars. The PcoA of the explant’s survival rate showed that the stem domes from bulbils of both cultivars treated with PVS3 for 2–3 h were similar in their survival ability. This confirms that the dehydration conditions and type of explant were critical factors affecting the survival rate. These factors had a higher impact on the survival of explants than did the genotype. Our results confirm also that the efficiency of garlic cryopreservation is influenced by different factors. There are some critical factors that involve all the cryopreservation steps, such as the type of plant materials, conditions of preculture, cryopreservation technique, cooling, warming, and regrowth conditions [35]. For example, different authors provide different optimal durations of exposure to PVS3 or different optimal explant types, though obviously genotype and other possible factors, even including bulb dormancy and ageing behaviour, should be considered [15]. For our study, we used garlic that was stored for 9 months at +4 °C. Preliminary studies have shown (data not presented) that it is sufficient for the cold acclimation of garlic and to complete the dormancy period. In this report, we did not provide data on the regeneration of garlic after freezing, but we believe that the data from our research clearly show the influence of genotype, explant type, and dehydration on cryopreservation. This was the purpose of our study. Additional research is necessary to improve the cryopreservation procedure for different genotypes of garlic.

4. Conclusions

The survival rate of explants after cryopreservation was influenced by different factors. The average number of surviving explants after freezing of both Lithuanian cultivars reached 64.3%. The cultivar ‘Žiemiai’ was distinguished by a higher number of surviving explants, reaching 76.1%, and showed a better capability for survival compared to ‘Dangiai’. The results confirmed that the type of explant and dehydration conditions were critical factors affecting the survival rate of garlic. The explants from bulbils were more effective for cryopreservation than the explants from cloves. Treatment in PVS3 solution for 3 h was
optimal for the survival of explants from bulbils (91%), and treatment for 2 h was optimal for the survival of explants from cloves (78.5%).

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**References**


18. Xing, X.; Liu, M.; Zhou, R.; Jiang, F.; Bai, Y.; Wie, H.; Zhang, D.; Wei, J.; Wu, Z. Ascorbic acid addition during dehydration improves garlic shoot tip cryopreservation but does not affect viral load. *Cryobiology* 2022, 107, 64–73. [CrossRef]


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