

Pretreatment with abscisic acid accompanied by sucrose improves callus survival after cryopreservation of hazel (*Corylus avellana* L.) by desiccation

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Abstract

A simple cryopreservation method (desiccation) is examined on *Corylus avellana* L. callus in current study. The effect of abscisic acid (ABA) concentration, preculture length on Murashige and Skoog (MS) medium containing ABA + 10% sucrose, and storage length in liquid nitrogen (LN) on callus survival rate were investigated. After cryo-storage, the calli were heated at 40°C followed by cold tap water for one minute, then transferred to the recovery medium. Calli survival was assessed 6–8 weeks after exiting from LN. There was a significant difference between ABA concentrations and different culture periods on medium containing mentioned hormone in survival rate but no significant difference was observed between the storage periods in LN. In the present study, the highest survival rate (45/79%) was obtained in both the treatment of 20 days preculture on medium containing 2 mg l⁻¹ ABA following 120 minutes desiccation-one day storage in LN and 150 minutes desiccation-one month storage in LN.

Key Message

Desiccation method with the help of ABA and sucrose was an effective method in the successful cryopreservation of *Corylus avellana* L. callus.

Introduction

Conservation of plant genetic resources is an important challenging issue. As these resources are lost and genetically eroded due to biotic and abiotic stresses or human errors, nevertheless cryopreservation is regarded as a reliable method of conserving plant resources wherethrough samples are stored in LN (-196°C), which allows plant materials to be safely stored for long-lasting time (Shibli et al. 2006; Ochatt et al. 2021). At this temperature, all cellular activities such as cell division, metabolism, and biochemical activities are stopped, and samples can be stored for a long time period without modification (Walters et al. 2009). Cryopreservation is a useful solution for storing undifferentiated plant cells for extended duration as a source to supplementary manipulation such as secondary metabolite production (Shibli et al. 2006).

The amount of water in the cell is the most significant factor in the germplasm's ability to be stored successfully in LN (Stanwood 1985). Forming ice crystals in plant cells is the most destructive event that causes irrecoverable damage during cryo-storage (Dumet and Benson 2000). Many methods have been developed in cryopreservation technique in order to decrease water content of the cells and among all, desiccation is the simplest one. In this procedure, samples are dehydrated using laminar air flow (without accurate control on temperature and air humidity) or vials containing silica gel (Panis et al. 2001).

The success of the cryopreservation protocols depends on the tolerance and sensitivity of the plant germplasm to the stresses resulting from the protocols used for dehydration (Reed et al. 2005). The dehydration process can lead to physical and chemical damages in most cells (Takagi 2000). Tolerance induction to desiccation of tissue is a critical stage, prior to the immersion of the material in LN, that can

be achieved by preculturing plant materials for 1–7 days on medium including high concentrations of sucrose (Engelmann et al. 2003; Suzuki et al. 2006) and also, exogenous ABA supplementation that contributes to protein synthesis and compatible solutes, which are significant in freezing tolerance (Stewart and Voetberg 1985).

ABA plays an important role in developing drought tolerance in various plant species. Adding ABA to the culture medium before cryopreservation increases the survival rate of cryopreserved specimens, so this plant hormone has been used in cryopreservation protocols of many species (Edesi et al. 2020)

The common hazel, *Corylus avellana* L. is one of the most important species of *Corylus* genus (Gürcan et al. 2010), and an economically nut tree native to Europe and Asia and commonly grows in moderate climates such as Turkey, Spain, and Italy (Torello Marinoni et al. 2018). Among all parts of this species, its fruit is the main product (Kubiak-Martens 1999) which includes wealth of proteins, oils, vitamins, phenolic compounds, and antioxidants (Gaderi et al. 2012). Furthermore, Paclitaxel as one of the most powerful anticancer medicines is found in small amounts in hazel (Hoffman 1998).

Firstly, Paclitaxel was insignificantly derived from the bark of yew tree (*Taxus baccata*) (Hoffman and shahidi 2009). Since the large quantities of bark are needed, this tree was considered as endangered wild species (Shinwari and Qaiser 2011). Accordingly, finding a new source of taxol could be a best solution for the purpose of preserving this limited population as well as minimizing the drug's production costs. Plant cell culture is a promising eco-friendly technology for producing paclitaxel in massive amounts (Gibson et al. 1993; Espinosa-Leal et al. 2018). Since hazel cell cultures are readily available, rapid-growing, and easier to culture in vitro, they are a promising alternative taxol-producing supply in comparison to taxus (Bestoso et al. 2006). Some previous studies on hazelnut callus have indicated that taxol production in this system can be improved by adding fungal elicitors to hazel cell culture medium or optimizing the culture medium of hazel (Salehi et al. 2017; Farhadi et al. 2020). Undifferentiated tissues such as callus are genetically unstable and may be susceptible to epigenetic changes that result in lack of regeneration, genetic mutations, and changes in secondary metabolite synthesis (Meijer et al. 1991). Additionally, since continuous cultures have high costs and specimens may be lost due to disease or human/technical mistakes, cryopreservation seems to be a reasonable choice for long-term and secure storage of tissue culture samples (Harding 2004). Although, cryopreservation technique has been done successfully on hazel's embryogenic axes, oil seeds, pollen using desiccation, shoot tips by vitrification and axillary buds by droplet vitrification (Gonzalez-Benito and Perez 1994; Michalak et al. 2013; Shukla et al. 2016; Nebot et al. 2018; Sgueglia et al. 2021), to date no study have focused on cryopreservation of *C. avellana* callus. Therefore, this study was designed to investigate the possibility of successful cryopreservation of *C. avellana* callus as a precious source of taxol.

Materials And Methods

Induction and maintenance of callus culture

A stable 6-year-old diploid *C. avellana* callus was used to establish the *C. avellana* callus. Principally, seed cotyledons were cultured on MS medium (Murashige and Skoog, 1962) enriched with 0.2 mg l⁻¹ 6-benzylaminopurine (BAP) and 2 mg l⁻¹ dichlorophenoxyacetic acid (2,4-D) and solidified with 8 g l⁻¹ agar agar in order to induce callus. Prior to autoclaving for 20 minutes at 121°C, the pH of all media was set to 5.8 with either KOH or HCl. All cultures were placed in dark at 25 ± 2°C until the calli appeared. Several subcultures of calli were carried out on the same medium to produce a homogeneous callus. Every 25 days, calli were periodically subcultured (Salehi et al. 2017). Materials used in the experiments including culture medium ingredients and plant growth regulators were offered by Sigma and Merck Chemical Companies.

C. avellana calli were cryopreserved by desiccation method as described by Popova et al. (2009) with some modifications.

Pretreatment and desiccation

Preparation of preculture medium

Firstly, basal medium was prepared and autoclaved for 20 mins at 121±1°C. Under the laminar airflow cabinet, ABA was sterilized through the syringe filter (0.22 µm CA) and added to cooled basal medium. The prepared culture medium was finally distributed in volumes of 50 ml inside jars.

First experiment

Callus fragments with 50±5 mg of fresh weight were separated at 24th day of subculture and incubated for 7 days on MS solidified medium without plant growth regulators in order to eliminate phenolic compounds. Hormone free MS medium was prepared and autoclaved for 20 mins at 121±1°C. 7-day-old calli were moved on MS solidified medium supplemented with 1,2 and 3 mg l⁻¹ ABA and 100 g l⁻¹ sucrose and 8 g l⁻¹ agar agar for 5, 10, and 15 days (pretreatment) to induce desiccation endurance. Then, desiccation was performed at a relatively constant temperature (27±1 °C) by transferring calli in 100 mm open petri dishes inside the laminar airflow cabinet and exposing them to sterile airflow for 0-180 minutes with 30 minutes intervals.

Second experiment

Callus fragments were cultured on hormone free MS medium for 7 days as mentioned in first experiment. Then calli were transferred on MS medium supplemented with 2 mg l⁻¹ ABA and 100 g l⁻¹ sucrose solidified with 8 g l⁻¹ agar agar for 10, 15, 20, and 25 days. after pretreatment, calli were subjected to sterile airflow for 0-300 minutes with 30 minutes intervals (temperature: 27±1 °C).

Cryopreservation

After desiccation, as control, half of the calli were transferred on MS medium including 0.2 mg l^{-1} 6-benzylaminopurine (BAP) and 2 mg l^{-1} dichlorophenoxyacetic acid (2, 4-D) and 8 g l^{-1} agar agar, sealed and kept on dim shelf at $25 \pm 1 \text{ }^{\circ}\text{C}$. The other half of the calli were transferred to cryovials and sealed (4 ml in volume, Simport company) then plunged immediately in LN for one day in first experiment and for one day and one month in second test.

Rewarming and recovery

Water bath was warmed up previously up to 40°C . Samples were pulled out from LN, and instantly rewarmed in water bath at for 1 minute followed by 1 minute rewarming in cold tap water. Then cryovials were surface-sterilized with 70% ethanol. Then calli were transferred on MS medium containing 0.2 mg l^{-1} 6-benzylaminopurine (BAP) and 2 mg l^{-1} dichlorophenoxyacetic acid (2, 4-D) and 8 g l^{-1} agar agar in order to evaluate survival rate. Cryostored calli were kept 6-8 weeks on dim shelf at $25 \pm 1 \text{ }^{\circ}\text{C}$ in darkness. Subculture was performed every two weeks in recovery period.

DATA analysis

The number of fresh callus clumps emerging from cryostored calli after 6-8 weeks culturing on recovery medium was used to determine survival and presented in percentage. Three replications were performed for each treatment; In each replication, 10-12 callus clumps were treated.

SAS software (9.4) was used for statistical analysis of data. Before analyzing the variance of the data, normality test was performed on the residuals and due to the lack of normal distribution of the residuals, first the data were converted using the formula ($y = \text{ARSIN}(\text{SQRT}(y / 100))$) and then the analysis of variance was performed using the general linear model (GLM). GLM procedure was carried out as a factorial experiment in a completely randomized design with three replications. The least significant difference (LSD) test at the level of 5% was used to compare the means of the treatments. When the interactions were significant, the mean comparison of the main effects was avoided. After analysis of variance and mean comparison, using the formula ($\text{SIN}(y)^2 \times 100$), the means were converted into the main data unit. The charts were drawn using Excel software.

Results

Experiment 1

With regard to calli without storing in LN (L0 = control), with no desiccation, in all three concentrations of ABA, calli viability were high and equivalent. However, for calli grown on MS medium containing 3 mg l^{-1} ABA, extending preculture period to 10 and 15 days suppressed calli growth (Fig. 1a). With increasing desiccation time, calli grown on medium containing 1 mg l^{-1} ABA for three different preculture times had a lower survival rate compared to calli grown on medium containing 2 and 3 mg l^{-1} ABA, so that after two hours of desiccation, they entirely lost the capability to survive (Fig. 1b-d). By increasing ABA concentration to 3 mg l^{-1} , calli could tolerate desiccation up to 150 min and the highest survival rate

(45.96%) was observed for calli precultured 10 days (A3P10L0) with 150 min desiccation (Fig. 1e). Besides, calli could survive after 180 min desiccation by increasing preculture duration on medium containing 2 mg l^{-1} ABA (Fig. 1f) and the highest survival rate (94.54%) at 120 min desiccation related to this medium was recorded for 15 days preculture (Fig. 1d).

By one day storage in LN, calli precultured on medium including 3 mg l^{-1} ABA could survive up to 150 min desiccation and also by extending preculture time to 15 days on medium consisting of 2 mg l^{-1} ABA, survival rate was observed until 180 min desiccation (Fig. 1). Nevertheless, the highest survival rate (37.99%) for frozen calli was related to 15 days of preculture on medium containing 2 mg l^{-1} ABA and 120 min desiccation and (Fig. 1d).

Experiment 2

Concerning calli without storing in LN (L0), calli with 10 days preculture on MS medium containing 2 mg l^{-1} ABA could survive up to 150 min desiccation. With increasing preculture time to 15 and 20 days, calli could tolerate desiccation up to 240 min (Fig. 2a-h). Calli could only survive for 60 min of desiccation with a 16.28% survival rate by extending preculture duration to 25 days (Fig. 2b). 25 days precultured calli showed 33.18% growth without any desiccation (Fig. 2a).

Regarding calli without storing in LN, the highest survival rate for 15 and 20 days preculture was 100% after 90 and 120 min desiccation, respectively (Fig. 2c-d). At different desiccation times, calli precultured for 20-days showed a higher survival rate than those precultured for 15-days (Fig. 2).

With regard to one day storage in LN, 10 and 15 days precultured calli with 90, 120 and 150 min desiccation could survive after cryostorage (Fig. 2c,d,e) and the highest survival rate (29%) was related to 120 min desiccation (Fig. 2d). 20-days precultured calli showed survival from 90 to 210 min desiccation and desiccation for 120 and 150 min resulted in the highest survival rates (45.79%) and (43.30%), respectively (Fig. 2d,e). 25-days precultured calli did not survive after one hour storage in LN.

For the calli stored 30 days in LN, 10 and 15 days precultured calli could survive till 150 min desiccation and the highest survival was related to 120 min desiccation (29/39%) and (25%), respectively (Fig. 2d). 20-days precultured calli could survive up to 180 min desiccation and the highest survival (45.79%) was associated with 150 min desiccation (Fig. 2e). 25-days precultured calli did not show ability to survive after 30 days of storage in LN.

Discussion

In this study, we evaluated the effect of desiccation procedure on *C. avellana* survival after cryopreservation by examining ABA concentration, length of preculture on ABA containing medium, desiccation periods and LN storage length. In both of our experiments every two-week subculture was crucial to obtain optimum growth after cryopreservation. According to our preliminary tests, without a two-week subculturing period, newly generated calli on freezed samples could not develop further and

remained tiny. This might be due to the inhibition of growth caused by phenolic compounds produced by frozen blackened calli on recovery medium.

Most plant cells comprise large amounts of water, which makes them sensitive to freezing. The sudden formation of ice during freezing process results in physical and biochemical damages to the cell. The water content inside the cells is the only important factor that affects the ability of the germplasm to be stored in LN (Stanwood 1985). Basically, cell's water amount must be declined to adequate level in order to enable cells to tolerate very cold temperature of LN (Bian et al. 2002). However, the appropriate water content for cryopreservation depends on the species and type of plant specimen and should be determined by different experiments (Popova et al. 2010).

Desiccation is one of the simplest methods for reducing cell water before immersing samples in LN. Desiccation-based cryopreservation has been found to be successful on callus of different species, such as embryogenic calli of cassava (Danso and Ford-Lloyd 2004), ginkgo biloba (Popova et al. 2009) and schisandra chinensis (Turcz.) Baill (Sun et al. 2016). In our experiments, we were also able to effectively use this approach on *C. avellana* calli. The ability to elongate the desiccation period without affecting the survival of samples is particularly advantageous for cryopreservation since the cells will lose water gradually and slowly until they reach the desired water content, besides are not damaged due to dehydration issues. This may be achieved by using different pretreatments.

Numerous researchers have examined the impact of ABA and sugars on desiccation and cryopreservation tolerances. Based on previous reports, the role of ABA as a pretreatment is to increase sucrose absorption and accumulation from the medium, hence, enhances glassy state formation in cells (Giladi et al. 1977; Wolkers and Hoekstra 2003). Besides, this hormone regulates expression of some genes which produce specific proteins and antioxidant enzymes that are responsible for cell protection and oxidative stress tolerance, respectively (Cutler et al. 2010). Burch and Wilkinson (2002) reported high recovery compare to control on cryopreservation of *Ditrichum cornubicum* (Paton) by using ABA and sucrose together, and high recovery was recorded on cryopreservation of *Brassavola nodosa* (L) Lind. (Orchidaceae) by using ABA alone as pretreatment (Mata-Rosas and Lastre-Puertos 2015).

Based on our results, in the first experiment without storing in LN, calli grown on medium containing 1 and 2 mg l⁻¹ ABA in all three preculture periods showed 100% survival without desiccation, but survival was somewhat reduced by culturing calli on medium containing 3 mg l⁻¹ ABA. Likewise, increasing the preculture period from 5 to 15 days on culture medium containing 3 mg l⁻¹ ABA has reduced survival from 98.61–75%, respectively (Fig. 1a). These findings are in line with the study of burritt, (2008) who reported 15–23% decrease in growth of adventitious shoots of *Begonia x erythrophylla* by enhancing ABA concentration from 0.5 and 1 mg l⁻¹ up to 1.76 mg l⁻¹. We observed that prolonging preculture duration up to 20 days had positive effect on calli survival after either desiccation or cryopreservation. In other words, increasing the preculturing time caused the calli to tolerate longer desiccation time, however the highest growth rate was observed within 120, 150, and 180 min of dehydration (Fig. 2d-f). 25-day preculture showed an inhibitory effect on calli growth even in case of control (Fig. 2a). Researchers

concluded that ABA causes the accumulation of sucrose and glucose in cells (Lu et al. 2009), As a consequence, pretreating samples with high concentrations of ABA, as well as extending the preculture time on media containing ABA that cause excessive sugar accumulation, may result in a decrease in growth rate.

Cryopreservation is an acceptable method for long-term storage of genetic resources because all the biological activity of cells stops at this temperature (Gonzalez-Arnan et al. 2008). Accordingly, it was hypothesized that cryopreserved materials could be preserved without alteration or modification for an extended period of time (Engelman 2011). Our findings proved that the length of the LN-storage did not affect the calli survival as no significant difference was observed in the survival of calli between one day and one month LN-storage. In this regard, our results are in agreement with some previous studies on cryopreservation of other plant species such as *Dioscorea deltoidei*, sugarcane (*Saccharum* sp.) and *Pinus radiata*, (Martinez-Montero 1998; Hargreaves et al. 2002; Mandal and Dixit-Sharma 2007). However, there are some exceptions to the effect of LN-storage period on survival rate. For example, the germination of *Hevea* pollen has decreased from 20% with one month storage to 2% after five months storage (Hamzah and Leen, 1986). Conversely, in some *Prunus mume* cultivars, the germination of pollens kept in LN for one to four years was significantly increased compared to unfrozen pollens (Withers and Engels 1990). In the study on *Anigozanthos viridis*, no reduction was observed in the survival of shoot apices after 12 months storage (Turner et al. 2001).

Conclusion

The present work demonstrated successful cryopreservation of *C. avellana* callus for the first time. Our study showed that the desiccation-based cryopreservation is easy to apply. We also examined the effects of ABA concentration, preculture duration on medium containing ABA, and desiccation length in order to achieve the highest survival rate which was obtained 45.79% in our study. However, further studies into many parameters and even other protocols are required in order to improve higher survival rates and as the samples are calli, genetic stability testing is essential.

Abbreviations

ABA: Absciscic acid

MS: Murashige and Skoog

LN: Liquid nitrogen

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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Author's contributions Shiva Mojarrad-Nanas carried out all experiment, interpreted the results and wrote the manuscript. Ali Mokhtassi-bidgoli performed data analysis. The co-author read and approved the final manuscript.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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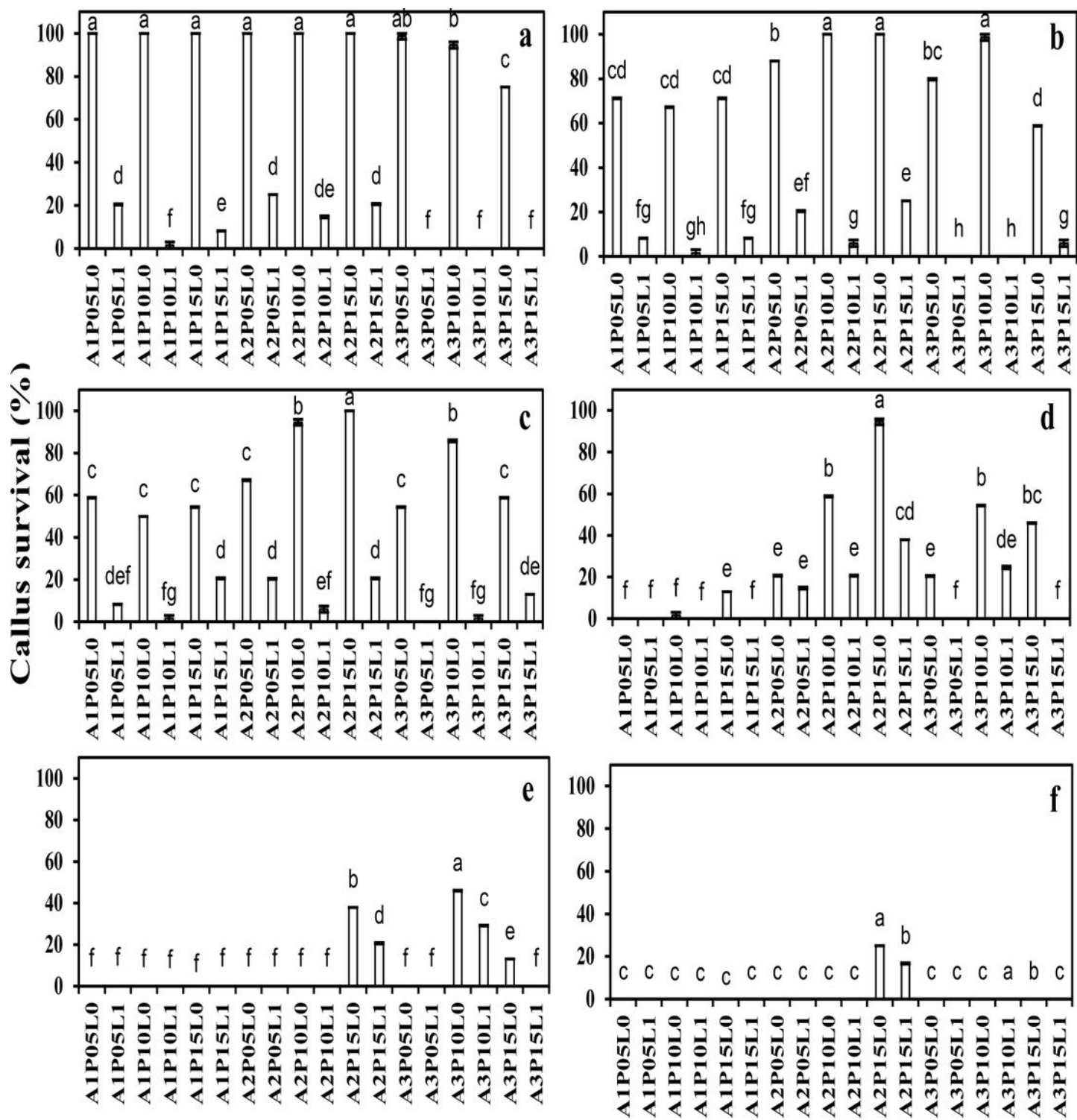
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Figures



ABA concentration, preculture time on ABA containing medium and LN storage period

Figure 1

Effects of preculture duration (P05: 5, P10: 10 and P15: 15 days) on medium containing different concentrations of ABA (A1: 1, A2: 2 and A3: 3 mg / l), drying by sterile airflow of laminar cabinet (A: 0, B: 1, C: 1.5, D: 2, E: 2.5 and F: 3 hours) and storage periods in LN (L0: 0, L1: 1 and L30: 30 days) on the

survival of the hazelnut callus. In each section of figure, the same letters on the top of the columns indicate no significant difference by ANOVA, followed by LSD test ($p \leq 0.05$)

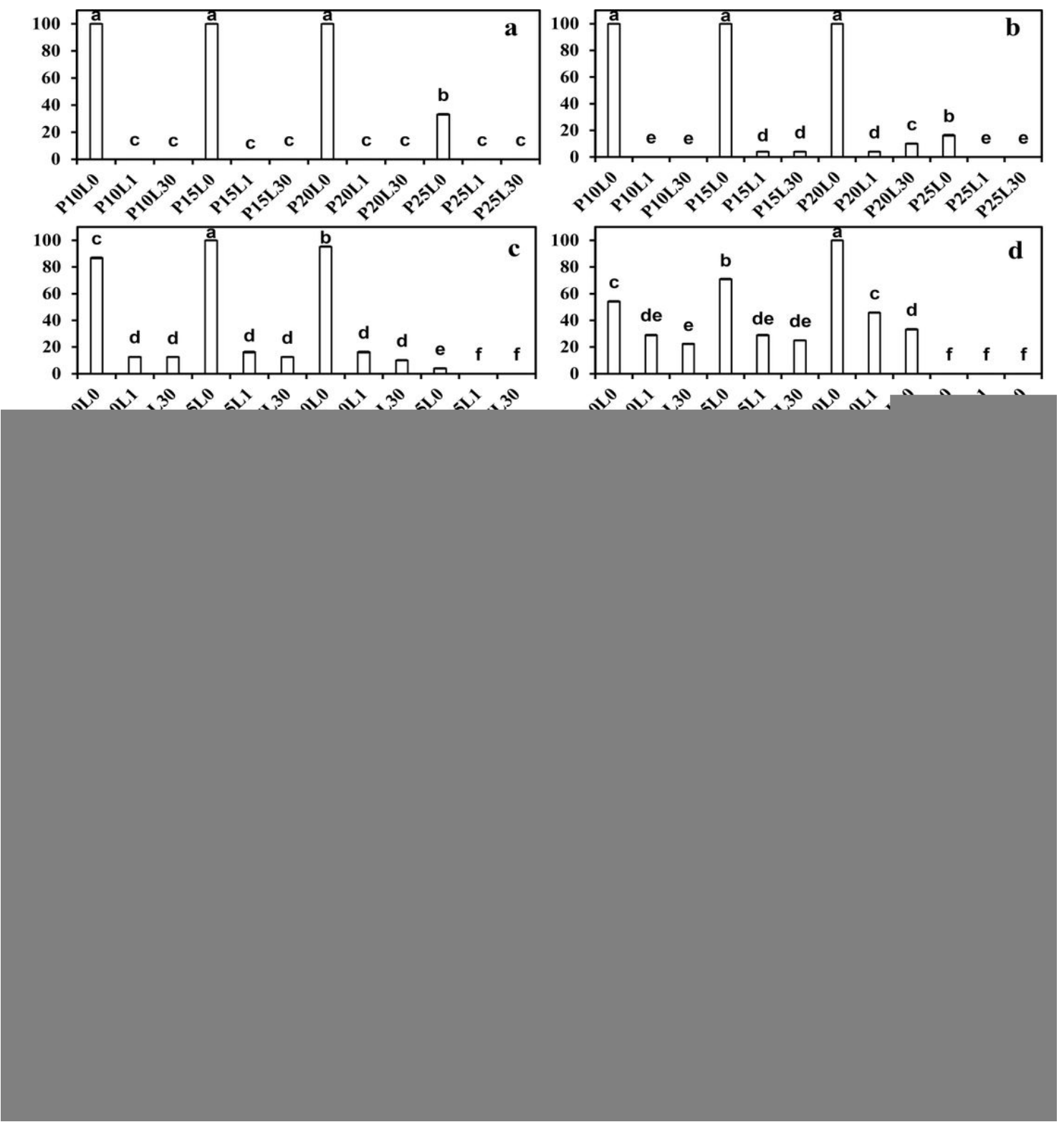


Figure 2

Effects of culture duration on preculture medium (P10: 10, P15: 15, P20: 20 and P25: 25 days) containing 2 mg l⁻¹ ABA, drying by sterile airflow of laminar cabinet (A: 0, B: 1, C: 1.5, D: 2, E: 2.5, F: 3, G: 3.5, H: 4, I:

4.5 and J: 5 hours)) and storage periods in LN (L0: 0, L1: 1 and L30: 30 days) on the survival of hazelnut callus. In each section of figure, the same letters on the top of the columns indicates no significant difference by ANOVA, followed by LSD test ($p \leq 0.05$)

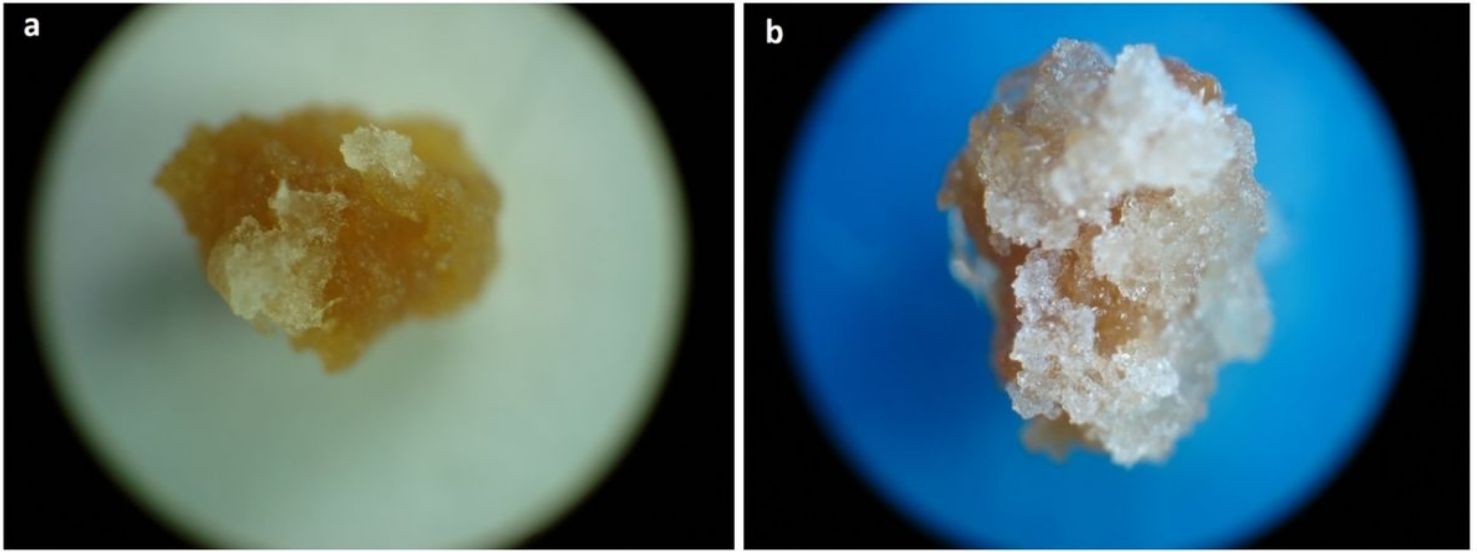


Figure 3

a Cryopreserved callus, 6 weeks after removal from LN at 100x magnification (2 mg l⁻¹ ABA, 15 days preculture, 120 mins desiccation). **b** cryopreserved callus about 6 weeks after removal from LN at 100x magnification (2 mg l⁻¹ ABA, 20 days preculture, 150 mins desiccation, one day storage in LN)

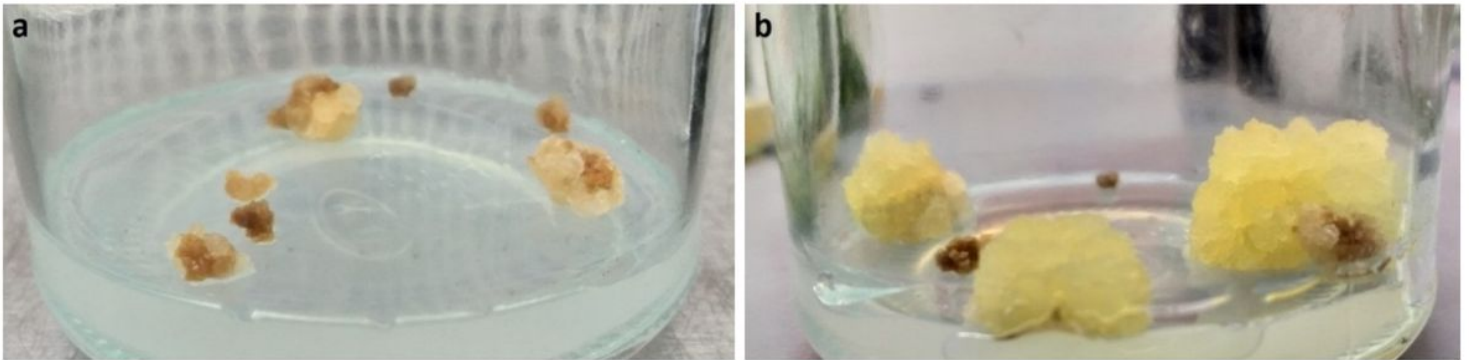


Figure 4

Calli survived after one day storage in LN followed by 20 days preculture on medium containing 2 mg l⁻¹ ABA, 150 mins desiccation **a** callus growth after 6 weeks of removing from LN **b** callus growth after 10 weeks of removing from LN