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Callus induction with 6-BAP and IBA as a way to preserve *Prunus ledebouriana* (Rosaceae), and endemic plant of Altai and Tarbagatai, East Kazakhstan

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Abstract. *Orazov A, Myrzagaliyeva A, Mukhitdinov N, Tustubayeva S. 2022. Callus induction with 6-BAP and IBA as a way to preserve* Prunus ledebouriana *(Rosaceae), and endemic plant of Altai and Tarbagatai, East Kazakhstan. Biodiversitas 23: 3178-3184.* This article presents the results of the study of in vitro conditions for the cultivation and induction of callus tissues of a rare plant species *Prunus ledebouriana* (Schlecht.) Y.Y.Yao (Ledebour's almond), endemic plant to East Kazakhstan. For the first time, protocol and optimal composition of Murashige & Skoog (MS) were developed for obtaining calendemiclus tissues. The main indicator of successful introduction was the frequency of callus tissue formation *in vitro*. Two types of explants were used: the whole embryos and embryos with a removed root part. The removal of the root part inhibited the organogenesis of the embryonic root and the first shoot, which allowed the accumulation of nutrients for the growth of the callus mass and increased the frequency by 7.79±0.46%. Various exogenous phytohormones such as Kinetin, 6-BAP, GA, IBA were added to the MS culture medium. A decrease in Kinetin concentrations of 0.04 mg/L resulted in lower intensity, but a higher frequency of formation (72.00±5.66%) and mass gain. The average concentration up to 0.5 mg/L of exogenous phytoregulators, such as GA and 6-BAP, resulted in frequency from 65.38±5.44% to 72.00±5.66%, the concentration of IBA up to 1 mg/L increased the frequency from 46.00±2.83 to 72.00±5.66%. The protocol for obtaining callus from an embryonic explant and the composition of the optimal nutrient medium for the induction of callusogenesis made it possible to obtain *P. ledebouriana* meristem tissues for further use in the development of callus cryopreservation technology.

Keywords: *Prunus ledebouriana*, endemic plant species, rare species, callusogenesis, in vitro

Abbreviations: 6-BAP: 6-benzylaminopurine, GA: gibberellic acid, IBA: indolylbutyric acid, NAA: Naphthaleneacetic acid, IAA: Indole-3-acetic acid

INTRODUCTION

Prunus ledebouriana (Schlecht.). syn. Y.Y.Yao *Amygdalus ledebouriana* Schltdl. (Rosaceae Juss.) is the only nut-bearing plant that forms bush thickets; a representative of the large genus *Prunus* L. This genus includes about 254 species. The studied species belongs to the tribe *Amygdaleae*, a special group of rare dwarf wild almonds of the sections *Chamaeamygdalus* Spach (Eisenman 2015). *P. ledebouriana* grows only on the shrub belts of the mountain ranges of the Altai and Tarbagatai mountain systems of East Kazakhstan (Komarov 1941; Stepanova 1962; Browicz 1989). The distribution area is limited to these mountain ranges. The shrub form of *P. ledebouriana* slightly differs from the closely related *Prunus tenella* Batsch syn. *Amygdalus nana* L. (Orazov et al. 2020). Features characterizing *P. ledebouriana* are the presence of a large shrub with wide leaves and 7-9 mm calyx tube; generally, the bush is high-up to 2 m. The base of seed is aslant-elongated. Flowering is usually observed from April to May, fruiting-from June to July. *P. ledebouriana* reproduces mainly vegetatively, in addition to sexual reproduction (Pavlov 1961). The endemism factor explains the presence of a narrow ecological niche in the geographical isolation of the Altai and Tarbagatai mountain ranges. This species also has a larger habitus (Sokolov 1980). Growth was recorded on the southern mountain slopes and foothills at an altitude of 700 to 1500 meters above sea level (Figure 1 A, B). There are also populations recorded in the territories of the Kokpektinsky and Ulansky districts of East Kazakhstan (Orazov et al. 2020).

The shape of the fruit is a dry drupe, felt-fluffy, not juicy, with a monoembryonic structure that requires a long period of stratification for a period of three to four months (Svistulenko et al. 2018). One of the limiting factors of sexual reproduction is the presence of the fruit weevil Conotrachelus nenuphar (Herbst), which is pest that harms this plant (Kolomiec and Chapalda 2020) in natural conditions on the territory of the Katon-Karagay district, Narymsky ridge of Altai (iNaturalist: 178304079), near Mount Saryshoky (Figure 1 C, D).

The main methods of protecting *P. ledebouriana* are the introduction of natural populations into specially protected natural areas of the Katon-Karagai and Tarbagatai National Parks (Orazov et al. 2021) and the introduction by scientific organizations such as the “Altai Botanical Garden” (1970), “Institute of Botany and Phytointroduction” (1960), “Mangyshlak Experimental Botanical Garden”

(2000). Also, inclusion of the species in the list the Red Book of the Republic of Kazakhstan (Baitulin 2014) and woody plants of Central Asia (Istvud et al. 2009) and giving special status to EN B1ab (iii)) a vulnerable endemic plant species.

One of the newest and most relevant methods for conserving the biodiversity of rare plant species is ex-situ, the creation of various seed collections, etc. (Khromov and Cherednichenko 2022). Biotechnological methods are also being developed to obtain callus tissues for further micropropagation or cryopreservation (Romadanova et al. 2015; Efferth 2019). Despite this, now the process of somatic embryogenesis and the induction of callus tissue, as well as other issues of *P. ledebouriana* cultivation, are poorly understood. One of the first works on organogenesis and callusogenesis of representatives of the genus *Amygdalus* was carried out in India, Albania. The leaves and embryos of the plant were studied as the optimal explant. For the introduction into *in vitro* culture, a 0.7% gel-like agar medium Murashige & Skoog (MS) with 3-6% of sucrose was used (Spahiu et al. 2015).

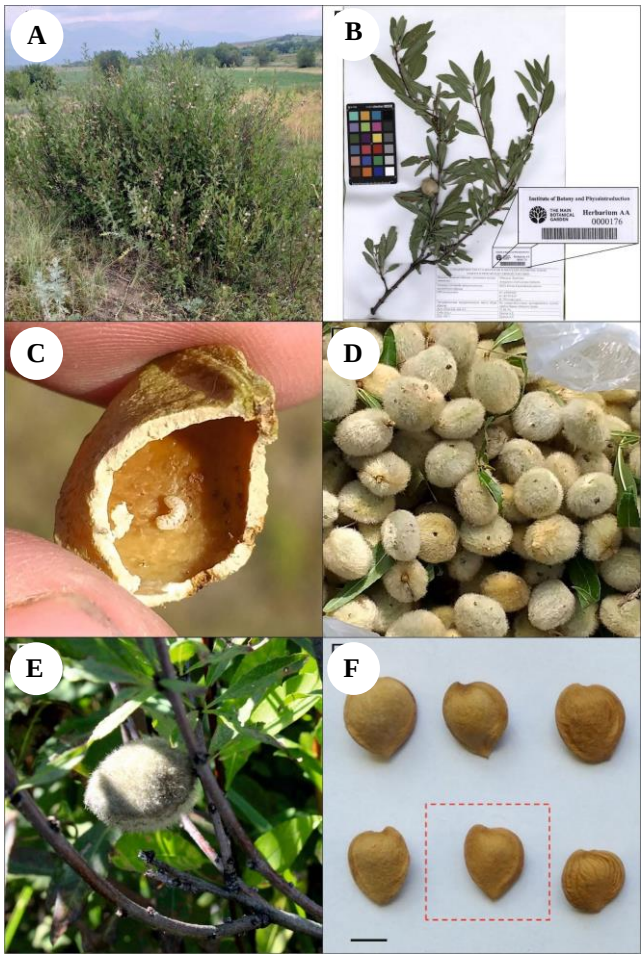

Figure 1. A. *P. ledebouriana* (Schlecht.). syn. Y.Y.Yao growing in the Katon-Karagai region (East Kazakhstan). B. Herbarium material of the Institute of Botany and Phytointroductions (Almaty, Kazakhstan) (AA: AA 0000176). C: Opened stone fruit infested with fruit weevil larvae (*Conotrachelus nenuphar* (Herbst). D: Almond fruit with oily corky growths from the larva of this insect species (photo in the field by Orazov A.E.). E: Fruits of *P. ledebouriana*. F: Stone without pericarp

In 2011, scientists from Dicle and Batman Universities (Turkey) developed methods for *in vitro* rooting of *Amygdalus communis* L. (Namli et al. 2011). Apical shoots of *Amygdalus communis* L. were cultured on Murashige and Skoog (MS) medium containing 1.0 mg^{-1} BA and dipped shoots 10, 15, 30, and 35 minutes at 1.0 g/L of IBA.

In 2017, in Kazakhstan, there were the first works on the application of micropropagation methods to *P. ledebouriana* by scientists from the Kazakh Research Institute of Forestry and Agroforestry (Kolov and Korotyaev 2017; Chebotko 2020) and S. Amanzholov East Kazakhstan University (Myrzagalieva 2017). In these works, the first protocols for introduction into *in vitro* culture had been developed. In all studies, the well-known MS nutrient medium (Isıkalan et al. 2011) was used with the addition of various exogenous phytohormones that affect the induction of callusogenesis on various explants.

The main goal of this research work is to develop a protocol and the optimal composition of the nutrient medium for obtaining highly stable callus tissue of *P. ledebouriana*. Based on the results of this scientific work, the authors received a patent for a utility model for a specific composition of the medium used for intensive division of the meristem tissue from the *P. ledebouriana* embryonic explant, which promotes callus formation (Patent 2020). Two types of explants were used – the whole embryo and an embryo with a removed root part. The developed protocol for obtaining callus tissues can be used to develop a technology for cryopreservation of *P. ledebouriana* tissues.

This article was prepared based on the results of the research work carried out as part of fundamental scientific research on the priorities of science development for 2015-2017 on the topic "2808/GF4 Development of biotechnological methods for the conservation of endemic and medicinal plants under *in vitro* conditions" (supervisor A.B. Myrzagaliyeva).

MATERIAL AND METHODS

Taking explants

Embryos of 1.5-3 mm in size isolated from mature fruits of *P. ledebouriana*, which have a high concentration of meristematic tissues for effective callus formation, were objectives for introduction into in vitro culture (Figure 1 E, F). Plant samples for in vitro cultivation were collected in specially protected areas-Katon-Karagai (49°05'46.6"N, 84°29'10.3"E) and Tarbagatai (47°09'05.7"N, 82°01' 18.9"E) national parks.

Laboratory equipment and reagents

In this study, the gel-like MS medium (Murashige and Skoog 1962) was used for the induction of callus tissue with the full composition of elements of different ratio such as macrosalts (NH_4NO_3-*ammonium nitrate* (CHEMELEMENTS, Ukraine), KNO_3-*potassium nitrate* (EMSURE, USA), $CaCl_2*2H_2O$-*calcium chloride, dihydrate* (EMSURE, USA), $MgSO_4*7H_2O$-*magnesium sulfate, heptahydrate* (JSC "Karpov Chemical Plant,

Russia"), KH_2PO_4-*potassium phosphate monobasic* (Xinxiang Huaxing Chemical Co., Ltd., China) and microsalts ($MnSO_4*5H_2O$-*manganese sulfate pentahydrate* (Newareaktiv, Russia), $ZnSO_4$-*zinc sulfate* (ETIMADEN, Turkey), H_3BO_3-*boric acid* (ETIMADEN, Turkey), KI-*potassium iodide* (Newareaktiv, Russia), $Na_2MoO_4*2H_2O$-*sodium molybdate dihydrate* (Newareaktiv, Russia), $CuSO_4$-*copper sulphate* (Newareaktiv, Russia), $CoCl_2$-*cobalt chloride* (BioReagent, SigmaAldrich, USA), source of iron ($FeSO_4$-*iron sulphate* (PANREAC, Spain), $C_{10}H_{14}N_2Na_2O_8*2H_2O$-*Trilon B* (Sigma-Aldrich GmbH, USA) vitamins ($C_{12}H_{17}N_4OS$-*thiamine* (SigmaAldrich, USA), $C_8H_{11}NO_3$-*pyridoxine* (SigmaAldrich, USA), $C_6H_5NO_2$-*nicotinic acid* (MERCK, Germany), $C_6H_{12}O_6$-*mesoinositol* (SigmaAldrich, USA), growth regulators ($C_{12}H_{11}N_5$-*6-benzylaminopurine* (SigmaAldrich, USA), $C_{10}H_9NO_2$-*indolylbutyric acid* (BioReagent, SigmaAldrich, USA), $C_{19}H_{22}O_6$-*gibberellic acid* (BioReagent, SigmaAldrich, USA), $C_{10}H_9N_5O$-*kinetin* (SigmaAldrich, USA), $C_{12}H_{10}O_2$-*1-Naphthaleneacetic acid* or α-*Naphthaleneacetic acid* (SigmaAldrich, USA), $C_{10}H_9NO_2$-*Indole-3-acetic acid* (SigmaAldrich, USA), carbon source was $C_{12}H_{22}O_{11}$-*sucrose* (Helicon, Russia). The gel-forming agent was a micro-bacteriological $(C_{12}H_{18}O_9)_n$ – *agar-agar* (Fluka, Spain). The hydrogen index (pH) varied around 5.9 (slightly acidic solution).

The main equipment for research is the experiment: Steam sterilizer (autoclave) VK-75-01 (Tyumen-Mediko, Russia); Water distiller DE-10M (Plant Elektromedoborudovaniye, Russia); Electromagnetic stirrer (Ohaus, USA); pH meter HI 98100 (Hanna Instruments, Romania); Laminar box BAV-"Laminar-S" (Lamsystems, Russia); Tool kit for preparation (Ningbo Kelsun, China); Thermostat TS-1/20 SPU (Smolensk SKTB SPU, Russia); Illuminated shelving (Novosibirsk, Russia).

Laboratory glass test tubes with a diameter of 30 and a height of 40 and chemical glassware. Test tubes Ø 10 mm, measuring cylinders V 25-1000 mL, measuring beakers V 50-1000 mL, Petri dishes Ø 40-90 mm, scalpel, tweezers, parchment paper and cotton.

All experiments used 0.7% MS agar gel medium with 3-6% of sucrose. Exogenous phytohormones were added before autoclaving in various variations according to various literature sources, and their concentrations were modified according to Table 1.

To prepare the medium and sterilize the explants, pure chemical reagents (chemically pure) were used. The reagents were dissolved in a separate small proportion of distilled water (dH_2O) and made up to the volume of 1 L. The pH of the nutrient medium was adjusted with an HCl solution (titration) in the range of 5.5-5.9 before autoclaving. Autoclaving of the nutrient medium was carried out at 121°C, at 2 atmospheres for one hour. Seed sterilization and isolation of the *P. ledebouriana* embryo was carried out according to generally accepted methods with various modifications and changes.

The explants' inoculation

Seeds (drupes) were cleaned from the fruit shell (pericarp) and washed 3 times in a weak soapy solution for 15 minutes, and then washed with distilled water. Then treated in 70% ethanol (C_2H_6O) and washed three times with sterile distilled water. The isolation of the embryo was carried out under special sterile conditions under a laminar flow hood. The drupe was opened and then wetted with sterile distilled water. Then the embryos were separated from the endosperm using tweezers and a scalpel. One of the cotyledons was moved away exposing the embryo. As explanations, a whole embryo and an embryo with a removed root part were used (Figure 2). Then the explants were placed in a test tube on hormone-free (control) MS medium and MS medium with hormones and various composition options and modifications.

Conditions of culture

For further incubation and callusogenesis, test tubes were placed at a temperature of 20-25°C in a light culture room on shelving with backlight at illumination from 2000 to 3000 lux (Philips) chiaroscuro photoperiod 17/7 hours. During the cultivation of explants, the formation of two types of calluses and the germination of the embryo on a certain day of cultivation were observed. After the callus reached a significant size, a morphogenic assessment of the callus tissue was carried out. It was determined by external properties, friability, density, color, etc.

Table 1. Variations of the nutrient medium for callusogenesis from the embryo of *P. ledebouriana* (mg/L)

Exogenous phytohormones	MS-01 (88*) (mg/L)	MS-02 (100*) (mg/L)	MS-03 (102*) (mg/L)	MS-04 (100*) (mg/L)	MS-05 (102*) (mg/L)	MS-06 (102*) (mg/L)	MS-07 (100*) (mg/L)	MS-08 (94*) (mg/L)	MS-09 (106*) (mg/L)	MS-10 (96*) (mg/L)	Control (88*) (mg/L)
Kinetin	2.50	0.10	-	-	-	0.04	-	-	-	-	-
6-BAP	-	-	1.00	1.00	0.25	0.50	-	-	-	-	-
IBA	-	-	-	0.10	0.50	1.00	-	-	-	-	-
GA	-	-	-	-	0.25	0.50	0.10	-	-	-	-
NAA	5.00	-	-	-	-	-	-	5.00	-	-	-
IAA	-	-	-	-	-	-	-	-	1.00	0.50	-

Note: *number of samples to be used

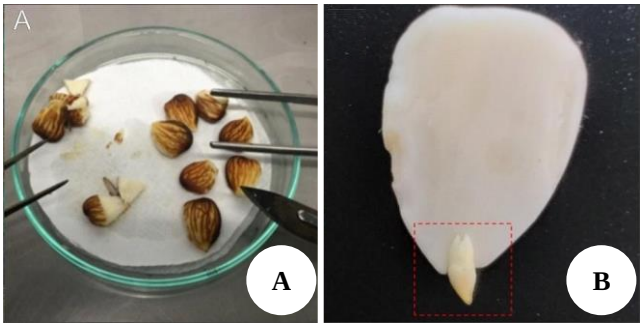

Figure 2. A. Endosperm of *P. ledebouriana* without endocarp during embryo dissection. B. Endosperm with embryo

Statistical analysis

For the statistical processing of the obtained results, the frequency of induction of callus formation under *in vitro* conditions was used. The frequency of callus formation induction was determined as the ratio of the number of callus-producing explants to the total number of planted explants for each type of explant separately. Average values were calculated with standard deviation (SD). The obtained empirical data were processed in Microsoft Excel (2019) with the construction of histograms (for each type of explant, depending on the composition of the nutrient medium). For statistical processing, the frequency of formation of callus tissue was used. The morphological structure of the callus was described according to the method of Timofeev and Rumyantseva (Timofeeva and Rumyantseva 2012). The results of statistical analysis (number of calluses and callus formation time) were described by the boxplot method.

RESULTS AND DISCUSSION

For the effective conservation of the genetic and biological diversity of endemic plant species is the use of a complex and problematic method of obtaining resistant callus tissues ready for long-term differentiation or cryo-preservation (Ngezahayo and Liu 2014). To implement these tasks, many experiments were carried out under in vitro conditions: Hasan et al. 2010; Isikalan et al. 2010; Dejampour et al. 2011; Eisenman 2015; Hervani et al. 2018; Efferth 2019, and others. In this study, materials of various types of explants were used and the optimal composition of the nutrient medium was selected to obtain stable callus from *P. ledebouriana* embryos.

Culture initiation and types of explants

The first signs of tissue structural changes in *P. ledebouriana* explants under in vitro conditions were recorded on the fifth day of cultivation on various media and types of plant explants in a permanent light culture room. Viability was observed in most explants, callus tissue was formed, direct vegetation (development of cotyledon, shoot and primary root) without the formation of callus mass (Figure 3A). On explants of the first type, vegetative leaf growth and the formation of an embryonic root with an increase in callus tissue on the neck were recorded (Figure 3B, 3C). For the type of the first explant, the percentage indicator of the conditional formation of organogenesis is 41.23% n callus formation is 35.37% of the total number of explants. The structure of the callus is loose, more homogeneous and not morphogenic (not abundant), crumbling, light brown. Buds gemmogenesis was not observed. The frequency of callus formation on this type of explant is shown in Table 2.

On explants of the second type, the process of direct vegetation was not observed. There was no root development, no cotyledon development, and the tissues were etiolated (Figure 4A). The structure of the callus tissue was more heterogeneous, brown-green and brown at the base of the section (Figure 4B). The callus was mostly morphogenic with uneven shape and a small-hilly surface. It grows not deep into the medium, but to the periphery. Buds gemmogenesis was not observed (Figure 4C).

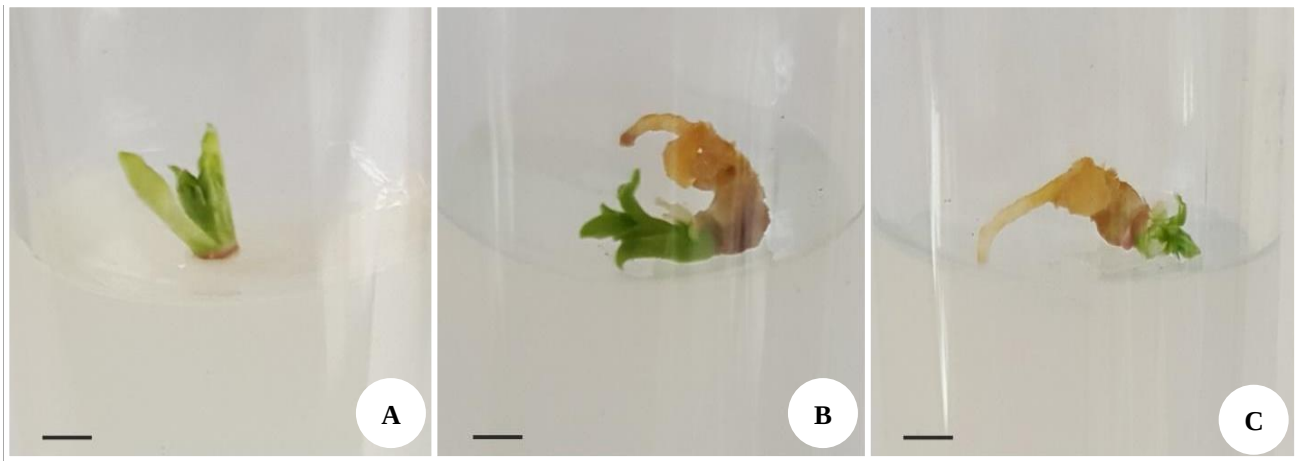

Figure 3. Direct vegetation, explant vegetation with callus formation on embryos (type 1 explants) with embryonic root and bud vegetation of *P. ledebouriana*. A. Control. B, C: MS-05: 6-BAP-0.25 mg/L, IBA-0.5 mg/L, GA-0.25 mg/L). 20th day of cultivation. Bar = 1 mm

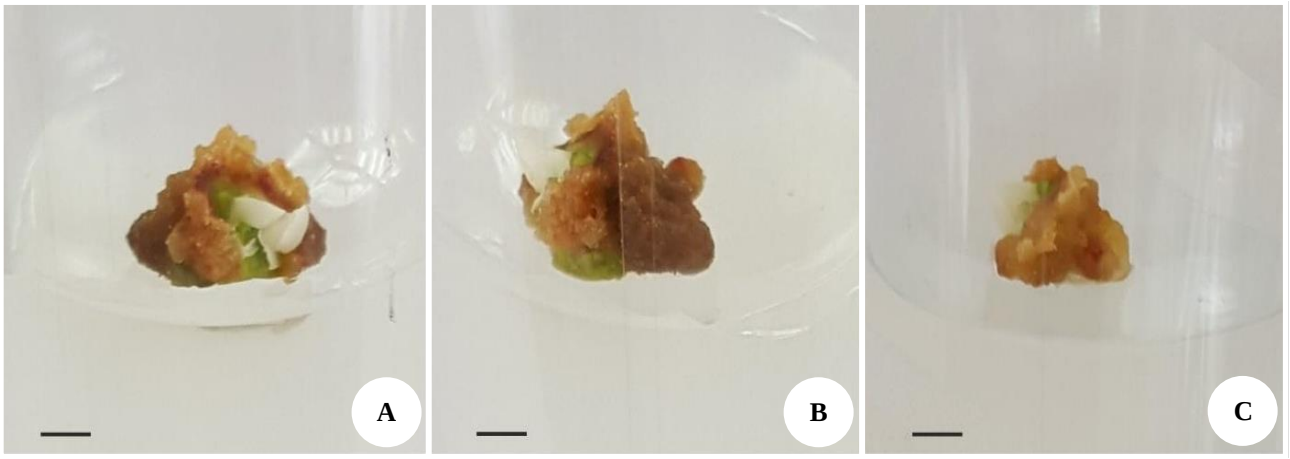

Figure 4. Callus mass on embryos with removed roots of *P. ledebouriana*. A, B, C: different angles. On MS-06: Kinetin-0.04 mg/L, 6-BAP-0.5 mg/L, IBA-1 mg/L, GA-0.5 mg/L. 20th day of cultivation. Bar = 1 mm

Compositions of the nutrient medium

The main indicator of success in the experiment was the frequency of callus formation with two reps on various media and types of explants. For the explant of first type, the average value was 38.98±6.01 %; the frequency of callus formation for two reps was 35.16±6.46 %. High rate of callus formation was recorded on the following nutrient media: MS-04 (6-BAP-1 mg/L and IBA-0.1 mg/L (type 1 explant-46.78±2.83%; type 2 explant – 50.00±8,49%)), MS-05 (6-BAP – 0.25 mg/L, IBA – 0.5 mg/L, GA – 0.25 mg/L (type 1 explant – 57.69±5,44%; type 2 explant – 56.00±5,66%)), and MS-06 (Kinetin – 0.04 mg/L, 6-BAP – 0.5 mg/L, IBA-1 mg/L, GA-0.5 mg/L (type 1 explant-72,00±5,66%; type 2 explant-65,38±5,44%)). Also, 37.35 % of explants were undeveloped (not forming callus tissue) or with necrotic tissue. The frequency of callus formation on this type of explant is shown in Table 2.

The role of exogenous phytohormones influencing the increase in the frequency of callusogenesis inductions was revealed in media with different concentrations of phytohormones: Kinetin – 2.5 mg/L, 0.1 mg/L, 0.04 mg/L (Popov 2016), 6-BAP – 0.25 mg/L, 0.5 mg/L, 1 mg/L (Isikalan 2008), IBA – 0.1 mg/L, 0.5 mg/L, 1 mg/L (Dejampour et al. 2011), GA – 0.1 mg/L, 0.25 mg/L, 0.5 mg/L (Vysotskiy 2015), BAP – 0,5 mg/L, 1 mg/L (Lestari et al. 2019). Low rate of induction frequency was recorded in media with the addition of the following phytohormones with different concentrations: NAA-5 mg/L (Hasan et al. 2010) IAA – 0.5 mg/L, 1 mg/L (Isikalan 2010); α-NAA-5 mg/L (Popov 2016). The data are presented in comparative diagram of boxplots in the figure 5.

High rate of callus formation was observed on a nutrient medium with the addition of phytohormones such as Kinetin, 6-BAP, GA, IBA. Decrease in concentrations of Kinetin up to 0.04 mg/L resulted in low intensity, but high frequency of formation (72.00±5.66%) and mass gain. An increase in the concentration of Kinetin led to a decrease in the efficiency of cell division. The frequency of callus formation varied between 22.50±3.54% and 31.25±2.95 %.

The average concentration of 0.5 mg/L of phytoregulators such as GA and 6-BAP gave a high frequency on two types of explants from 65.38±5.44% to 72.00±5.66%. Increasing the concentration of IBA to 1 mg/L increased the frequency from 46.00±2.83% to 72.00±5.66%, this effect also appeared in the work (Isikalan C. et al. 2010). These culture media showed stable callus formation at day 15 with a little tissue necrosis. Nutrient media supplemented with IAA-0.5 mg/L, IBA 1 mg/L, and NAA 5 mg/L showed a low frequency of callus formation from 26.00±8.49% to 30.00±2.83%.

Table 2. Callus formation frequency on different media and explant type

Medium	Type 1 explant (whole embryo)		Type 2 explant (embryo without root)	
	Av. (%)	SD (%)	Av. (%)	SD (%)
MS-01	31.25	2.95	22.50	3.54
MS-02	20.00	5.66	24.00	11.31
MS-03	37.04	5.24	31.25	8.84
MS-04	46.00	2.83	50.00	8.49
MS-05	57.69	5.44	56.00	5.66
MS-06	72.00	5.66	65.38	5.44
MS-07	42.00	14.14	30.00	8.49
MS-08	35.42	2.95	28.26	3.07
MS-09	26.00	8.49	16.07	7.58
MS-10	23.91	9.22	30.00	2.83
Control	37.50	3.54	33.33	5.89
Average values	38.98	6.01	35.16	6.46

Note: Av.: average value; SD: standard deviation

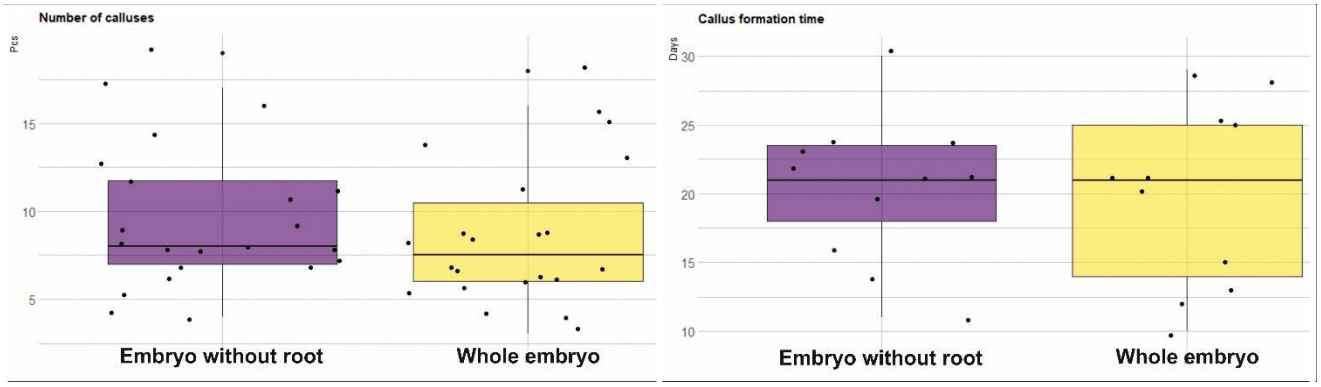

Figure 5. The diagram of boxplots (variances, standard deviation and the mean) for number of calluses and callus formation time

In conclusion, the frequency of callus formation under *in vitro* conditions for the first type of explant (whole embryo) was 38.98±6.01% and for the second type of explant (embryo with the root removed) was 46.78±6.47%. Which was 7.79±0.46% more for the second type of explant. Removal of the embryonic root from the embryo inhibited the organogenesis of the root and the first shoot, which allowed the accumulation of nutrients for the growth of the callus mass. Also, exposure to subepithelial tissue accelerated the process of delivery of exogenous phytohormones for callusogenesis.

The protocol for obtaining callus from an embryonic explant and the composition of the optimal nutrient medium for the induction of calussogenesis were successful to obtain *P. ledebouriana* calluses. The results of the research can be used to develop the technology of *P. ledebouriana* callus cryopreservation in liquid nitrogen and for further reproduction and population increase in the territory of East Kazakhstan. Further research in this area will be directed to histological analysis to study the structure of callus tissue and its organogenesis in order to obtain daughter regenerated plants.

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