Regeneration from leaf explants of steppe cherry \((Prunus fruticosa\) Pall)

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Abstract. The article represents data on morphogenesis from leaf explants of three steppe cherry genotypes, as well as the degree of somaclonal variability at in vitro and ex vitro stages, and in the field. It was revealed that a content of 6-benzylaminopurine, 4.43 µM, in combination with auxin, 0.5–0.6 µM, stimulates in the light the direct organogenesis in the tissues of the leaf base. This reaction was observed from 16.7 to 75.0% of explants, depending on the genotype. An equal 6-benzylaminopurine - auxin ratio (1:1) led to the callus along with microshoots. Depending on the genotype, up to 30.0% of explants had such a mixed type of organogenesis. The mitotic index value in the apical leaflets differed depending on the day time. At the stage of micropropagation itself, an increase of the mitotic index was observed from 10 to 16 hours; at the stage ex vitro, no significant differences in the mitosis frequency were revealed within this time interval. No significant differences were found between the level of the mitotic index for plants obtained directly from leaves and those from buds. The mitosis passed without disturbances. No phenotypic changes in plant habit, shape and color of leaves were found.

1. Introduction

Steppe cherry in the \(Prunus\) L. genus is the main genetic source of resistance to unfavorable environmental factors. This representative of cherry is the only native species that grows in the climatic conditions of Western Siberia and the Urals with sharp seasonal fluctuations in temperature and humidity. Altai breeders, from 1933 to the present, using methods of analytical selection and remote hybridization, have created more than 17 varieties based on genome of this cherry. Currently, as a result of global climate change, increased phytopathogenic impact of dangerous diseases and pests, cherry crops require significant renewal. It is necessary to develop and accelerate propagation of adaptive varieties of a new generation, followed by the planting of mother nurseries.

The Laboratory of biotechnology and cytology of the Federal Altai Scientific Center for Agrobiotechnology (briefly NIISS) has developed a technology for microclonal propagation of promising cherry varieties by using of meristematic tissues of vegetative buds [1]. To increase the multiplication factor within the direct path of organogenesis, expand the possibilities of the breeding process, carry out genetic transformation and create transgenic forms of plants, it is of interest to use as an explant also a leaf tissue, which is capable to regenerate [2–4]. It is important to know how the plants obtained in this way will be identical to the original genotypes. Somaclonal variability is one of the main factors, which causes a loss of varietal identity after clonal micropropagation; at the same
time, it can be valuable for expanding genetic diversity and obtaining new genotypes in the breeding process [5].

The study of the leaf explants morphogenesis for perspective genotypes of steppe cherry and the estimation of the somaclonal variability degree in in vitro stage and in the resulting plant regenerants were the main aim of this investigation.

2. Material and methods

The material was the elite hybrids of steppe cherry ‘3-11-20’; ‘972-7-16’ and the ‘Pamyati Lewandowski’ variety, bred in NIISS, cultivated in vitro.

Young leaves were placed on Murashige-Skoog (MS) medium [6] with growth regulators (GR): cytokinin 6-benzylaminopurine (6-BAP) and cytokinin-like thidiazuron (TDZ) at concentrations from 4.43 to 22.73 µM; as well as auxins - β-indolyl-3-butyric acid (IBA), α-naphthylacetic acid (NAA), indoleacetic acid (IAA) at concentrations from 0.5 to 16.7 µM. Transverse incisions were made on the leaf blades. For further normal growth of shoots, the leaf was cut into fragments with a group of microshoots and transferred to nutrient media that are optimal for micropropagation of cherries [1]. Adventive microshoots were planted on a micropropagation medium. Shoots reaching 10-15 mm were moved to a rooting media. Rhizogenesis of microshoots was carried out using the ½ MS medium with 2.0 µM IBA. The rooted plants were adapted to ex vitro conditions and planted in the field.

The regeneration processes were carried out in a 16/8 hour light/dark photoperiod at 24 ± 1 °C and an illumination intensity of 2000–3000 lux, and in darkness. The data have been analyzed after 4-6 weeks. The regenerative ability of cultured tissues was assessed by the number of explants that formed morphogenic structures (buds, roots, leaves, shoots) or proliferating callus. The color and consistency of the callus were also taken into account.

The level of somaclonal variability was determined by studying the mitotic index (MI) in the young leaves meristem in regenerated plants at in vitro and ex vitro stages and by the presence of phenotypic changes in plants in laboratory and field conditions. The mitotic index in the apical leaf tissues of plants obtained from apical buds, in comparison with those in plants obtained from leaf tissue, was calculated in per thousand (%/oo) according to the common method. To calculate the number of dividing cells, paper prints of micrographs, made using a “Mikmed 6” microscope and a “TS-500” digital camera (LOMO) at x40 magnification (objective), were done. The ratio of the total number of mitoses to the total number of meristematic cells in one photograph, multiplied by 1000 was counted. The percentage of cells with chromosomal abnormalities during mitosis was also calculated [7]. To study the cellular activity, the squashed preparations stained with acetic hematoxylin were prepared [8]. Changes in the phenotype (the presence of dwarfs, plants with leaf shape and leaf color alterations, close internodes, albino or chimeric tissue patches) were assessed in at least 30 plants in ex vitro and in the field.

All the data were analyzed with the Microsoft Office Excel 2007 application package.

3. Results

Mainly the direct path of organogenesis was observed on media with a low BAP content of 4.43 µM in combination with auxins at a concentration of 0.5-0.6 µM (IBA or NAA or IAA) and using a cytokinin: auxin ratio of 8 : 1 (media no. 2, 3, 4) in leaf plates cultured in the light. It was in the form of meristematic cones, buds, leaves, and shoots. This reaction was observed from 16.7 to 75.0% of explants, depending on the genotype (figure 1). In other explants, only callus growth or explant death was observed. For hybrid ‘972-7-16’, the optimal variant turned out to be medium no. 4 with the NAA addition, on which about 75.0% of explants begin start of direct organogenesis. The maximum amount of hemogenesis for the ‘Pamyati Lewandowski’ variety was equal to 40%, and exclusively on the medium, where 0.6 µM IAA was added as auxin.
Figure 1. Genotype and treatment effects on organogenesis in steppe cherry with media compositions: 1) 4.43 BAP + 5.0 IBA; 2) 4.43 BAP + 0.6 IBA; 3) 4.43 BAP + 0.5 IBA; 4) 4.43 BAP + 0.6 NAA; 5) 4.43 BAP + 5.6 NAA; 6) 4.43 BAP + 5.6 IAA; 7) 4.55 TDZ + 0.6 IBA.

With a tenfold increase in the auxin concentration (5.0 - 5.6 µM, media no. 1, 5, 6) in combination with BAP 4.43 µM at the base of the leaf and along the edge of the leaf blade, for from 10.0 to 80.0% of explants of all genotypes, both hemogenesis and callus growth had been observed. In ‘Pamyati Lewandowsky’ variety, only 10.0% of leaf explants, along with callus, microshoots (from 1 to 8 pcs / explant) formed, and 40.0% of explants had only callus. As the exception, the ‘3-11-20’ was, in which with this GR ratio the direct organogenesis was observed in 16.7% of explants without formation of callus on the leaf blade. Genotype the ‘972-7-16’ is again demonstrated a high ability to multiply at a ratio of 4.43 BAP with 5.6 NAA (medium no. 5). Together with callus, a hemogenesis (buds, leaves, shoots) was observed on leaf blades – 80.0% of proliferating explants. Replacement of NAA by IAA (medium no. 6) led to a more than twofold decrease in the ability of the explants of this hybrid for organogenesis and for increasing a callus growth. In other genotypes, only callus developed on these media.

A further increase of BAP (8.86-13.29 µM) in the nutrient medium with the addition of IBA (10.0-15.0) or NAA by 2–3 times stimulated the callus mass growth from the base of the leaf and throughout the leaf blade. The structure of the callus was heterogeneous; there were dense and loose areas. Callus color varied from light green to brown. The addition of NAA in medium instead of IBA enhanced callus growth. Organogenesis was not observed in most explants on media with such a GR ratio. Only in individual explants of the ‘Pamyati Lewandowski’ (up to 10.0%) on these media in the light showed a hemogenesis in the form of leaves and microshoots rise (figure 2a).

Figure 2. (a) Direct organogenesis from the ‘Pamyati Lewandowski’ on the medium BAP 13.29µM and IBA 15.0 µM after 6 weeks of leaf culture; (b) Adventive shoots, emerging buds of the ‘3-11-20’ on the medium with TDZ 4.55µM and IBA 0.6 µM after 4 weeks of development.
Recently, thidiazuron (TDZ) has been used in in vitro studies. This GR, which is similar in properties to cytokinins, has a more potent activity compared to BAP, being a promoter of shoot proliferation from leaf explants of strawberry, sweet cherry, and apple [2, 9–12]. In our studies, the replacement of BAP by TDZ at concentration of 4.55 µM and in combination with a 0.6 µM of IBA (medium no. 7) led to the callus growth on the leaf blade of almost half of all explants. Direct organogenesis (33.3%) was observed in explants of the ‘3-11-20’ (figure 2b) and in ones of the ‘Pamyati Lewandowski’ (10.0%). In other cases a callus developed. Significant callus growth and indirect organogenesis on blueberry leaves, using TDZ 0.5 mg/l, were noted by R. Cappelletti and other co-authors [2]. At the same time, when scientists were working with leaf explants of the ‘Royal Gala’ apple tree, using TDZ 5.0-20.0 µM, 100% of shoot regeneration was noted [12]. In our experiment, an increase in the TDZ concentration up to 22.73 µM together with NAA 2.68 µM stimulated the growth of callus mass – to 100% of explants for the ‘Pamyati Lewandowski’ and the ‘972-7-16’. For the ‘3-11-20’ hybrid it was only up to 36.4% of explants. Growth started from the base of the leaf, spreading along the central vein.

After 30 days of cultivation, the callus completely covered the leaf. In its structure, it was heterogeneous, had a dense and bumpy, sometimes loose, consistency. Callus color varied from white to light green. Explants with dense green callus, which have bud emerging, were transferred to a medium with a more than 4-fold lower concentration of TDZ and replacement of NAA with IBA. After 4 weeks of cultivation with this GR ratio, hemogenesis only in the explants of the hybrid ‘3-11-20’ – 28.2 % was observed. Upon subsequent transfer of leaf fragments with microshoots to an optimized nutrient medium with a twofold GR content decrease, a full-fledged shoots developed for all genotypes.

The study of somaclonal variability in terms of cellular activity in the ‘3-11-20’ was carried out in vitro and ex vitro. It was revealed that in vitro the greatest number of mitoses falls to the period from 10 to 12 hours (22–37‰). At the ex vitro stage in the time period from 10 to 16 hours, no significant difference in the number of cell divisions was found – 40–48‰ (figure 3).

![Figure 3. Mitotic activity in vitro and ex vitro in leaf explants of the ‘3-11-20’ cherry within different day time periods.](image)

Chromosome aberrations usually affect significant parts of chromosomes; their morphology is changing. Its phenomenon can be detected in the form of chromosomal or chromatid "bridges" [7]. In our investigation only in a few cases (0.1–0.2% of mitosis) of "forward runs" during chromosome movement to the poles were noted, which cannot be considered a serious violation.

In the experiments carried out, no significant difference was noted in the level of mitotic activity of cherry meristem in plants obtained both from the explants of vegetative buds and from the explants of
young leaves. The mitotic index at *ex vitro* stage in the ‘3-20-11’ averaged 40.8 and 36.3\%o, respectively (*t* <0.05) – table 1.

**Table 1.** Mitotic index in the ‘3-11-20’ regenerated plants at the *ex vitro* adaptation stage, 2020.

| Explant type          | Repetition number | Total of meristematic cells, pcs. | MI, %  | Total of dividing cells, % | Student’s *t*-test |
|-----------------------|-------------------|-----------------------------------|--------|-----------------------------|-------------------|
| Meristem of vegetative bud | I                 | 1360                              | 45     | 4.5±0.6                     | –                 |
|                       | II                | 1752                              | 30     | 3.0±0.4                     | –                 |
|                       | III               | 1105                              | 51     | 5.1±0.6                     | –                 |
|                       | Average1:         |                                   |        | 40.8                        | 4.1±0.3           |
| Meristem of leaf tissue | I                 | 1294                              | 23     | 2.3±0.4                     | –                 |
|                       | II                | 729                               | 49     | 4.9±0.8                     | –                 |
|                       | III               | 1242                              | 46     | 4.6±0.6                     | –                 |
|                       | Average2:         |                                   |        | 36.3                        | 3.6±0.3           |

No reliable external manifestation of somaclonal variability in phenotypic traits was found in plants undergoing *ex vitro* as in laboratory conditions and in the field (more than 250 plants) during 2 years of observation. No dwarfs, albinos, variegated and deformed plants were found (figure 4).

**Figure 4.** Regenerated cherry plants: the ‘3-11-20’ *ex vitro* in the laboratory (a - bud; b - leaf); the ‘Memori of Lewandowski’ in field conditions (c - bud; d - leaf).

4. Discussion
It is known that the plant genotype is one of the main factors that determine the ability of somatic tissues to morphogenesis [2, 9, 12]. This is confirmed by our research for steppe cherry. The highest percentage of direct organogenesis was noted for the ‘972-7-16’ on no.4 and no.5 media with a significant difference in the auxin content. It indicates a high regenerating potential of somatic leaf tissues of this genotype.

The role of RG in the morphogenesis induction in somatic tissues of peach, raspberry, strawberry and apple is indicated by the works of many researchers [3, 5, 11]. According to S.A. Muratova et al. [4] in *Rubus* leaf explants morphogenesis is induced by cytokinin (2.0–5.0 mg/l) and auxins (0.5–1.0 mg/l) contents. For steppe cherry leaf explants, the type of cytokinin and the ratio of cytokinin is auxin
concentrations used became important factors in triggering of organogenesis processes. In most cases, organogenesis was observed for the endogenous RG ratio of 1 : 1 or 8 : 1 on medium with BAP addition. It was noted that an increase of cytokinin concentration in relation to one of auxin is stimulating the callus growth. In some experiments, RG BAP is the most effective factor for shoot regeneration from leaves of sweet cherry and sour cherry [9]. The use of TDZ to stimulate organogenesis in leaf explants of steppe cherry is also possible, but to stimulate the direct type of regeneration, it is necessary to conduct additional studies and establish its optimal balance with auxins.

5. Conclusion
It was found that young leaf tissue in studied steppe cherry genotypes, cultivated in vitro, has a high capacity for callus formation and morphogenesis. In varying degrees, depending on the genotype, it is possible to induce the direct organogenesis in *P. fruticosa* leaf explants when in addition to BAP (4.43 µM), one of the auxins (0.5–5.6 µM) is included in the medium. In this case, it is necessary to cultivate the explants in the light. This is important when valuable genotypes, and especially steppe cherry varieties, are multiplied without stimulating of genetic and phenotypic somaclonal variations.

In our studies, the absence of cellular and phenotypic somaclonal variability in cherry plants obtained in vitro by direct organogenesis under the optimized cultivation conditions confirms that along with tissues of vegetative buds, the use of leaf tissues is also promising as explants.

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