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Peculiarities of morphogenesis of the endangered species of willow (*Salix* spp.) *in vitro*

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Conservation and reproduction of rare genotypes of *Salix* L. species, in particular the blunt-leaved willow (*Salix retusa* L.) and Jacquin's Willow (*Salix alpina* Scop.) that are listed in the Red Data Book of Ukraine in the status of rare and endangered species, is one of the urgent tasks of the present. The aim of research was to develop methods of introduction of *S. retusa* and *S. alpina* into *in vitro* culture for their mass reproduction and conservation. The plant material was cultivated on a culture medium prescribed by MS, WPM, and DKW with the addition of growth regulators according to the conventional method. Effective sterilization (over 80%) of explants of *S. retusa* and *S. alpina* was achieved by applying a stepwise method, which consisted of consistently maintaining them in solutions 0.1% $HgCl_2$ and 1.0% $AgNO_3$ for 5–6 min. Significant results in the regeneration of explants by activating the growth of available meristems *in vitro* were observed on MS with the addition of 0.25–0.5 mg/l 6-(Furfurylaminopurine, kinetin) and 2 g/l activated carbon. Our further research will serve as a base for developing microclonal propagation of *S. retusa* and *S. alpina* for their conservation and reproduction *in vitro*.

Keywords: conservation, *Salix* L., explants, microclonal propagation

INTRODUCTION

Conservation and reproduction of rare genotypes of *Salix* L. species, its natural richness, and the sources of a unique gene pool, is currently one of the urgent tasks. Such plants include the blunt-leaved willow (*Salix retusa* L.) and Jacquin's willow (*Salix alpina* Scop.), the alpine species that are listed as rare and endangered species in the Red Book of Ukraine (Red Book of Ukraine, 2010–2018; Identifier of plants, 1977; Malinovsky et al., 2002).

S. alpina Scop. (syn. *S. jacquiniana* Willd., *S. jacquinii* Host.) is a relict species with a disjunctive

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habitat in Central Europe. In Ukraine, the species is spread in the Carpathian alpine zone of the area of the Svydovets Mountain at the height of 1880 m above sea level. Alpine willow is a dioecious gametophyte, mezohygrophyte, and microtermal species that grows in damp limestone rocks in the structure of *Salicion* retuse and *Androsation* alpine union groups.

S. alpina is a low-growing shale bush, 2–15 cm tall. Young leaves are pubescent, adults are bare, only with cilia along the edge, reversed, whole, brilliant, dark green from above, up to 2 cm in length.

Bract-shaped parts are 1–3 mm long, reddish, dark on the top, hairy. The seed-bud is reddish, on a very short leg, pubescent in a young condition. The column is short; the pistil is bifurcate and reddish. Fruit box is grayish pubescent (Malynovskyi et al., 2002).

In Ukraine, specimens *of S. alpina* have a status of the disappearing species and are represented by a single isolated population occupying a small area and with a small number of individuals. The reasons for the change in the size of the species are associated with narrow ecological-coenotic amplitude, which is limited by specific environmental conditions and the influence of recreation. Plants are protected in the Carpathian Biosphere Reserve. They grow on crude limestone rocks in the alpine belt (Red Book of Ukraine, 2010–2018; Identifier of Plants, 1977; Ishchuk, 2017).

S. retusa is a Central European species with a disjunctive habitat on the eastern limits of distribution in the array of Montenegro. It grows in alpine and subalpine zones, mountain rocky slopes and rocky tops, at an altitude of 1750–2000 m above sea level. *S. retusa* is protected in the Carpathian Biosphere Reserve and Carpathian National Park (Ishchuk, 2017).

S. retusa is a creeping bush, 10–30 cm tall, with branched shoots of brown colour. The leaves are green, shiny, and reversible, with weak or strongly pronounced leaf venation, 0.8–3.5 cm long and 0.5–1.1 cm widthways (Red Book of Ukraine, 2010–2018; Identifier of Plants, 1977; Ishchuk, 2017).

Both species belong to the *Salicaceae* family. The areas of their distribution in Ukraine are the alpine belt of the Carpathians. Plants reproduce primarily from seeds and by vegetative processes (Red Book of Ukraine, 2010–2018; Malynovskyi et al., 2002).

Current situation with preservation of endangered biological diversity and their scientific reproduction role are regulated by a number of international and national regulations and standards, in particular the Convention on the Protection of Biological Diversity (Rio de Janeiro, 1992), "All-European Strategy for the Conservation of Biological and Landscape Diversity" (Pan-European Strategy, 1995), "The Concept for the Conservation of Biological and Landscape Diversity of Ukraine" (Resolution of the Cabinet of Ministers of Ukraine, 1997), as well as the laws of Ukraine "On Environmental Protection", "On the Nature Reserve Fund of Ukraine", and "On Plant World" (Laws of Ukraine, 1991; 1992; 1999).

One of the approaches to solving the problem is preservation of plant tissue culture *in vitro*. The method of culture of isolated tissues and organs *in vitro* allows obtaining healthy, genetically homogeneous plants throughout a year from a minimum amount of donor material (Butenko, 1964; Kalinin et al., 1980; Cell and Tissue Culture in Forestry, 1987).

For some genotypes of *Salicaceae* family Mirb., microclonal propagation technologies, such as the microclonal reproduction technology for individual genotypes of the family *Salicaceae* Mirb., have been tested and developed by a number of authors (Mohan, Ishii, 2003; Read, Bavougian, 2013; Bilous et al., 2017) for some genotypes of *Salix* L. (Park et al., 2008; Shi, 2014; Kandel, 2017).

However, in current scientific literature there are no studies on micropropagation of *S. alpina* and *S. retusa*.

METHODS

Objects selected in September 2017 from plant materials of *S. alpina* and *S. retusa* were 3–4-year-old donor plants. Ten to twenty pieces

of samples of live plant material were used in 4–5 repetitions. For obtaining aseptic tissue culture *in vitro*, 5–10 cm long shoots were used. Aseptic conditions were based on commonly accepted methods in biotechnology that were modified by the authors in the process of work (Butenko, 1964; Murashige, 1974; Kalinin et al., 1980; Smith, 2012). Shoot fragments 10–15 mm in length and with one lateral bud were used as explants. Sterilization of plant material consisted of soaking it in a soapy liquid and then under running tap water (15–20 min in each), rinsing with distilled water (for 1–2 min), treating with 70% alcohol (for 30–60 sec), and using a number of sterilizing substances: 0.1% $HgCl_2$ (for 5–20 min), 1.0% $AgNO_3$ (for 5–20 min), 2.5% NaClO (for 5–20 min), and 4-fold washing in sterile distilled water (for 4–5 min). The explants were introduced into the culture *in vitro* on the hormone-less culture medium prescribed by MS (Murashige, Skoog, 1962), WPM (McCown, Lloyd, 1981), DKW (Driver, Kuniyuki, 1984) and supplemented with 100 mg/l myo-Inositol, 30 g/lsucrose, and 7.0–7.3 g/l microbiological agar; the cycle of cultivation was 25–30 days.

The regenerative ability of the plant material in vitro was studied on a nutrient medium MS with the addition of cytokinin: 2-iP (6-γ,γ-Dimethylallylaminopurine), BA (6-Benzyladenine), kinetin (6-Furfurylaminopurine). In some variants of the nutrient medium, MS was supplemented with 2 g/l activated carbon. The acidity index (pH) was adjusted to the level of 5.7–5.9. MS-based media were used as control. The regenerative ability of explants *in vitro* was studied on cultivation days 35–40.

The plant material was cultivated in a light room at a temperature of 24 ± 1°C with lighting of 2000–3000 Lux with a 16-hour photoperiod and a relative humidity of 70–75%. The following study methods were applied: biotechnological (plant tissue culture *in vitro*, microclonal propagation), statistical (average, standard error, single-factor dispersion analysis). Experimental data were calculated using statistical program MS Excel.

RESULTS AND DISCUSSION

Analysis of variance revealed that concentration of different sterilization solutions and its interaction with *S. retusa* and *S. alpina* had significant difference in overcoming contamination and improving survival of shoot tip explants (Table 1).

It was established that the following solutions were inappropriate for sterilization of explants of *S. retusa* and *S. alpina* plants: 1.0% $AgNO_3$ for 5–20 min (variants 4, 5, 6), 2.5% NaClO for 5–20 min (variants 7, 8, 9), and 0.1%

Table 1. **Efficiency of sterilization of explants of *S. retusa* and *S. alpina* plants *in vitro***

Variant of experiment	Sterilization requirements	Efficiency of sterilization of explants, %
1	0.1% $HgCl_2$ duration 5–6 min	38.8 ± 4.3
2	0.1% $HgCl_2$ duration 15–16 min	72.5 ± 4.8
3	0.1% $HgCl_2$ duration 19–20 min	65.0 ± 6.5
4	1.0% $AgNO_3$ duration 5–6 min	20.0 ± 4.6
5	1.0% $AgNO_3$ duration 15–16 min	31.2 ± 5.5
6	1.0% $AgNO_3$ duration 19–20 min	32.5 ± 5.2
7	2.5% NaClO duration 5–6 min	33.8 ± 4.3
8	2.5% NaClO duration 15–16 min	50.0 ± 5.8
9	2.5% NaClO duration 19–20 min	40.0 ± 4.1
10	consequential staying in solutions for 5–6 min in each liquid: 0.1% $HgCl_2$, 1.0% $AgNO_3$ 2.5% NaClO	85.0 ± 6.5

$HgCl_2$ for 5–6 minutes (variant 1), since these procedures recorded a rather small efficiency.

According to our observations, the majority (60%) of all infections of explants were fungi (they appeared on days 3–6 of cultivation, (Fig. 1(a)), followed by bacterial inoculation – 30% (showed somewhat later, days 5–15), while the mixed type of infection reached no more than 10% (appeared on days 3–15).

Effective sterilization (over 80%) of *S. retusa* and *S. alpina* explants was achieved by applying a stepwise method, which consisted of consistently maintaining them in a number of solutions, for 5–6 min in each: 0.1% $HgCl_2$, 1.0% $AgNO_3$, and 2.5% NaClO (Fig. 1(b, c). A significant percentage of aseptic microshoot fragments (over 70%) were obtained by using 0.1% $HgCl_2$ for 15–16 min.

In order to grow an isolated culture of *in vitro* plants of the Salicaceae family, the main nutrient media of the MS, WPM and DKW have been known to be used. In our research, they were modified and tested for the suitability of obtaining regenerative explants (Fig. 2).

Experimental results of study of the regenerative capacity of plant explants showed the expediency of using the MS nutrient medium as a base at the initial stages (regeneration frequency was 93.3 ± 3.3% for *S. retusa*, and 83.3 ± 8.8 – *S. alpina*).

The influence of nutrient components on the regenerative capacity of explants was statistically significant at $\alpha = 0.05$ ($F_{cal.} > F_{cr}$; for *S. retusa*: $F_{cal.} = 19.91$, $F_{cr} = 5.14$; *S. alpina*: $F_{cal.} = 14.36$, $F_{cr} = 5.14$).

After obtaining aseptic viable plant material on basal MS medium (by using a range of antioxidants) (Fig. 1(d)), they were cut into 1.0–1.2 cm fragments and transferred to the MS nutrient medium modified (Table 2) by growth regulators: 0.25–0.5 mg/l kinetin, 0.5–1.0 mg/l BA, and 2-iP.

Significant results on the regeneration of explants by activating the growth of available meristems *in vitro* were recorded on MS with the addition of 0.25–0.5 mg/l kinetin and 2 g/l activated carbon. Activation of plant microshoots 2.5–4.0 cm in length with characteristic

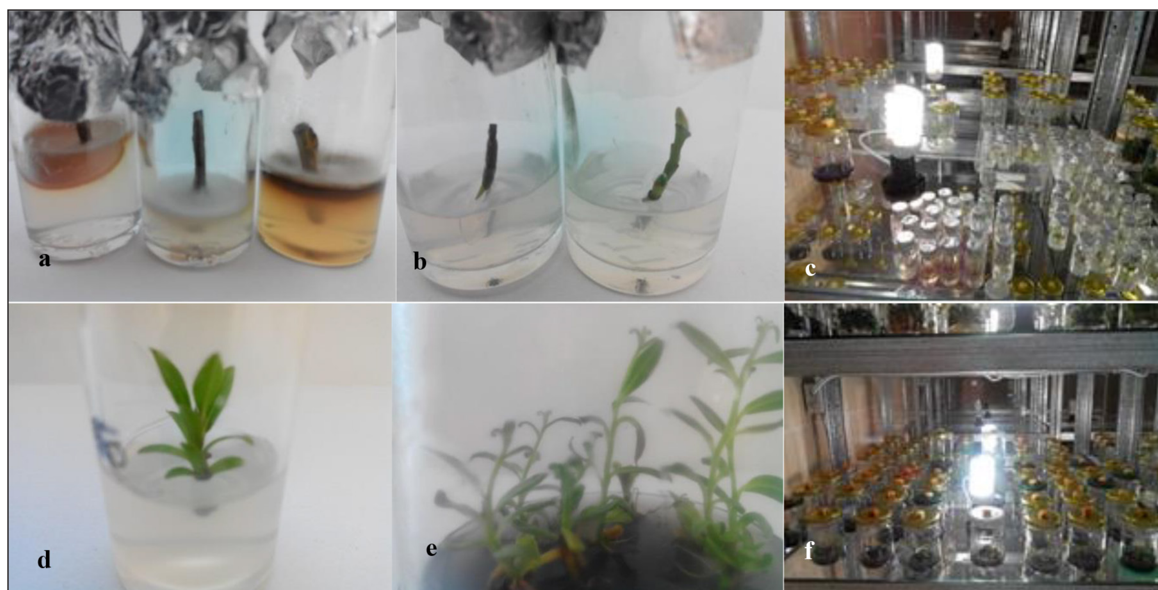

Fig. 1. Order of priorities in stages of reproduction of *S. retusa* and *S. alpina* by tissue culture method in vitro: **a** – infected plant material, on day 5 of cultivation; **b** – aseptic *S. alpina* explants, day 15 of cultivation; **c** – aseptic viable *S. retusa* explants in a cultural room under controlled conditions; **d** – proliferation of S. retusa micro segments (basal MS media) at the stage of introduction into *in vitro* culture; **e** – *S. alpina* microshoots (MS medium with 0.25 mg/l kinetine and 2.0 g/l activated charcoal); **f** – *S. retusa* plants (MS medium with 0.5 mg/l kinetine and 2.0 g/l activated charcoal) in a culture room

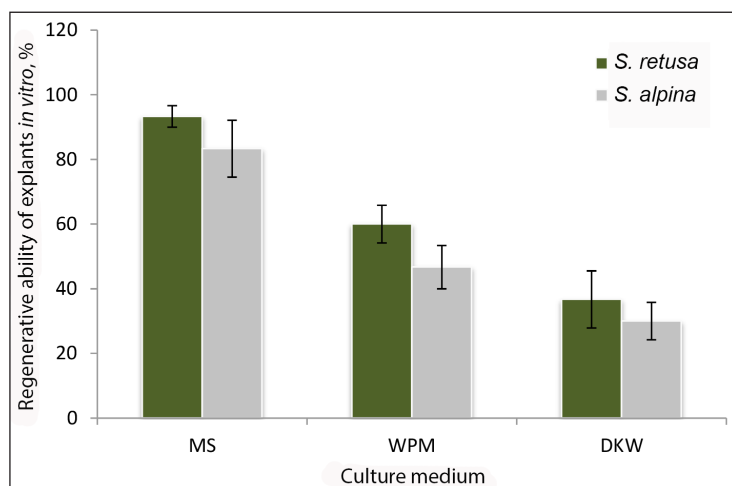

Fig. 2. Effect of different nutrient media on the regenerative ability of explants of *S. retusa* and *S. alpina* at the stage of their introduction into *in vitro* culture (days 25–30 of cultivation)

Table 2. **Characteristics of *S. retusa* and *S. alpina* plant-regeneration growth by cultivation on modified MS nutrient media (35–40 days in culture *in vitro*)**

Variant of experiment	Nutrient medium	Average value of microshoots, cm	Average number of microshoots on one explant, pcs	Colour of explants	Type of morphogenesis	Root formation, pcs
control	basal MS	1.6 ± 0.1	1.5 ± 0.3	green	Activating the growth of available meristems of explants	–
1	MS + 0.25 mg/l kin	2.0 ± 0.2	1.5 ± 0.3	–//–	Activating the growth of available meristems of explants	–
2	MS + 0.25 mg/l kin + 2 g/l activated carbon	3.0 ± 0.2	3.2 ± 0.2	–//–	Activating the growth of available meristems of explants	–
3	MS + 0.5 mg/l kin + 2 g L^{-1} activated carbon	3.6 ± 0.2	3.0 ± 0.4	–//–	Activating the growth of available meristems of explants	–
4	MS + 0.5 mg/l BA	1.7 ± 0.1	1.5 ± 0.5	light-green	Activating the growth of available meristems of explants	–
5	MS + 1.0 mg/l BA	1.2 ± 0.1	1.5 ± 0.3	–//–	Activating the growth of available meristems of explants	–
6	MS + 1.0 mg/l 2-iP	1.1 ± 0.1	1.2 ± 0.2	–//–	Activating the growth of available meristems of explants	–

pigmentation was obtained on days 35–40 of cultivation, but formation of roots on explants was not noted (Fig. 1 e, f).

Addition of a range of antioxidants – glutathione 2.0 g/l, adenine 1.0 g/l, glycine 1.0 g/l and 2 g/l of activated carbon – to MS nutrient

medium significantly improved the processes of microparticle growth, probably because of the decrease in the secondary metabolite activity. In the case of adding 0.5 mg/l BA, or 1.0 mg/l BA, or 1.0 mg/l 2-iP to MS nutrient medium, the length and the number of regenerated segments on explants were significantly lower than those of variants 2 and 3. Consequently, as a result of our research, we obtained aseptic and morphogenic active explants of *S. retusa* and *S. alpina* ready for the next stage of mass multiplication *in vitro*.

Our further research is aimed at developing biotechnology for microclonal propagation of *S. retusa* and *S. alpina* for their successful rooting *in vitro* and their adaptation to *ex vitro* conditions.

We have developed part of biotechnological procedures that can be useful in conserving rare and endangered species of willows.

CONCLUSIONS

1. Our direct plant regeneration system attempts using shoot and nodal segments were successful and could be applied in the conservation of endangered willow species.

2. Effective sterilization (over 80%) of *S. retusa* and *S. alpina* explants was achieved by applying the stepwise method, which consisted of consistently maintaining the explants in a number of solutions for 5–6 min in each: 0.1% $HgCl_2$, 1.0% $AgNO_3$, and 2.5% NaClO. In spite of high contamination of natural explants, such kind of sterilization was successfully applied.

3. At the stage of their introduction into *in vitro* culture, *S. retusa* and *S. alpina* isolated explants were cultivated on selected media. Among various tested and optimized media for the sterile morphogen culture, the basal MS medium with a range of antioxidants (glutathione 2.0 g/l, adenine 1.0 g/l, and glycine 1.0 g/l) was the most efficient one. In this case, a significant regeneration capacity was observed in *S. retusa* and *S. alpina* explants (explant regeneration frequency of over 80%).

4. Micropropagation attempts using shoot and nodal segments from stem-origin explants of *S. retusa* and *S. alpina in vitro* were successfully recovered on MS medium with 0.25–0.5 mg/l kinetin and 2 g/l activated carbon.

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NYKSTANČIOS GLUOSNIO (*SALIX* SPP.) RŪŠIES MORFOGENEZĖS YPATUMAI *IN VITRO*

Santrauka

Retų *Salix* L. rūšių genotipų, ypač uolinio karklo (*Salix retusa* L.) ir karklo *Salix alpina* Scop., esančių Ukrainos Raudonojoje knygoje, išsaugojimas ir dauginimas yra viena svarbiausių dabartinių užduočių. Šio tyrimo tikslas buvo sukurti *in vitro S. retusa* ir *S. alpina* kultūras, kad būtų galima jas išsaugoti ir dauginti. Augalinė medžiaga buvo kultivuojama pagal MS, WPM, DKW parinktoje auginimo terpėje, papildomai pridėjus augimą reguliuojančių preparatų. Efektyvus *S. retusa* ir *S. alpina* tirpalų sterilizavimas (daugiau kaip 80 %) buvo pasiektas laipsniškai palaikant tirpaluose 5–6 min.: 0,1 % $HgCl_2$, 1,0 % $AgNO_3$. Ženklinant tirpalų regeneraciją pagal *in vitro* meristemas, nustatyti reikšmingi rezultatai pridėjus 0,25–0,5 mg/l 6-furfurilaminopurino, kinetino ir 2 g/l aktyvintos anglies. Mūsų tolesni tyrimai bus skirti *S. retusa* ir *S. alpina* mikrokloniniam dauginimui, jų išsaugojimui ir reprodukcijai *in vitro*.

Raktažodžiai: išsaugojimas, *Salix* L., eksplantas, mikrokloninis dauginimas