Influence of cultivation factors on morphogenesis in vitro
Salix acutifolia Willd

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Abstract. Salix contains salicylates in their leaves, bark, and buds. That is why there is a necessity in elaborating microcloning propagation technologies of same genotypes which are high productive in salicin in order to create industrial plantations. The study examined the effect of 25 different combinations of BAP and NAA concentrations on the growth and development of S. acutifolia. The best results were observed on MS supplemented with 1.0 mg/l BAP and 0.01 or 0.1 mg/l NAA, and rhizogenesis on MS medium supplemented with 0.1 and 0.2 mg/l NAA. In order to study the effect of the mineral composition of basic media on the morphogenesis of S. acutifolia in an in vitro culture, DKW, GD, MS, and WPM were tested. Kn, BAP, and 2iP were compared to study the effect of cytokinins on the morphogenetic ability of S. acutifolia explants. The influence of the mineral composition of the medium on the rhizogenesis of S. acutifolia explants in vitro was evaluated on four studied media DKW, GD, WPM, and MS. Each medium contained 3% sucrose, 0.6% agar-agar, and 0.2 mg/l NAA. As a control, MS medium supplemented with 0.2 mg/l NAA was selected.

1. Introduction

In recent decades, interest in herbal medicines has been increasing, due to an increase in allergic reactions to taking medications with an artificially created structure. In connection with the increasing demand for drugs of biological origin, there is a need to create industrial plantations of highly productive plants. Most species of willow (Salicaceae, Salix) contain salicyclic alcohol derivatives and salicylates in the leaves, bark or buds. Medicines obtained from Salix plants contain salicin (2-hydroxymethyl-phenyl-β-D-glucopyranoside) and its derivatives: frailin, salicortin, 2’-o-acetylsalicortin, tremulacin, salireposide, etc. [1]. Salicylates have been used for hundreds of years as painkillers, anti-inflammatory and antipyretic drugs. Interest in natural willow preparations as an alternative to aspirin, a synthetic analogue, is increasing because willow salicylates do not have the side effect (irritation and stomach damage) that is characteristic of aspirin [2]. But most of the willow sources currently available for medicines contain less than 1% of the active ingredients. Therefore, research, selection and cultivation of willow species with a high content of salicylates is of great interest [3].

In this regard, there is a need to breed willow in order to obtain salicylates, by creating intensively planted industrial plantations with a short rotation of 1 year, when using highly productive (salicylate yield) clone varieties that are resistant to adverse environmental factors, diseases and pests.
In comparison with other species of woody plants, there are few published studies on microclonal propagation of plants of the genus Salix. Bergman studied the effect of BAP on the micropropagation of Salix clones, as well as the technology of microclonal propagation for some species of alpine willows (S. caprea L.), including hybrids S. caprea × S. viminalis L. (= S. × smithiana) [4]. A number of authors indicate the difficulties that arise during microchering and rooting of Salix plants, which, in their opinion, is associated with the influence of the genotype [5,6]. However, highly effective methods of micropropagation have been developed for some willow species and clones [7]. A hybrid between Salix Fragilis (female) x Salix Lispoclados (male) was established by ovary culture. The hybrid seedlings were grown on Schenk and Hildebrandt (SH) semi-solid medium containing 0.2 mg/l 6-benzyladenine (BAP). Shoot multiplication from nodal cuttings of in vitro hybrid seedlings was achieved on semi-solid WPM medium with 0.2 mg/l BAP [8,9].

The aim of this study was to develop the stages of microclonal propagation of S. acutifolia as a source of valuable biologically active substances. The objective of the work was to assess the influence of factors of the mineral and hormonal composition of nutrient media at the stages of proliferation and rooting of Salix shoots.

2. Materials and methods

Willow plant material selected from trees of model populations (Nizhny Novgorod region, Kirov region, Mari El republic) was used as an object of research. The experiments were carried out using apical and axillary buds in vitro. In vitro culture introduced material from annual shoots of field plants. Subsequent analysis of the accumulation of biologically active substances was carried out on the obtained regenerant plants. Plant material was collected at the beginning of February with a willow model population of the willow, previously selected for salicin content (Russia - Nizhny Novgorod Region, Kirov Region, Mari El Republic). The content of total salicin in the extract from the cortex of the selected genotype was 7.4 ± 0.2%. Buds from the annual shoots of model trees were chosen as primary explants. The introduction of S. acutifolia into in vitro culture was performed by sterilizing young axillary and apical buds. Sliced cuttings 15-20 cm long, in which the leaves were previously removed, were placed in plastic bags. The number of buds on each handle varied from 8 to 14. The bags were carefully sealed to prevent moisture loss. Prior to the experiment, the packages were stored in the dark at a temperature of +4 °C. The selected shoots were pre-washed with a soap solution with a brush and rinsed with distilled water. Cuttings were cut with a scalpel into small segments (5-10 mm) with buds, then they were cleaned of external renal scales. After that, the cuttings were soaked in an aqueous solution of “Fairy” detergent and placed on a magnetic stirrer for 15-20 minutes. At the next stage, the plant segments were washed under running water for 30-40 minutes.

All further manipulations with the samples were carried out in a laminar box. Next, these segments were placed for 30-40 seconds in 70% ethanol, and then transferred to a sterilizing solution of Na hypochlorite, in 10% commercial Domestos bleach, for 10 minutes. After that, the explants were washed three times in sterile distilled water, the ends where the cells were killed were removed with a sterile scalpel and placed on MS nutrient medium (Murashige and Skoog's) [10]. The concentration of sucrose in the medium was 30 g/l, agar-agar 6 g/l.

The study examined the effect of 25 different combinations of concentrations of BAP (0.0; 0.1; 0.5; 1.0; 2.0 mg/l) and naphthylacetic acid (NAA 0.0; 0.01; 0.05; 0.1; 0.2 mg/l) for the growth and development of explants of S. acutifolia (MS medium, sucrose 30 g/l, agar-agar 6 g/l). Cultivation was carried out at 21 °C, illumination 1800 Lux, photoperiod 16/8. 10 explants per option were laid, three repetitions. The study showed that the most intensive formation of new shoots in the studied willow genotype occurred on MS medium with the addition of 1.0 mg/l BAP in combination with NAA at a concentration of 0.01 and 0.1 mg/l, and the root formation process on MS medium supplemented with 0.1 and 0.2 mg/l NAA in concentration [11]. The stimulating effect of low concentrations of BAP (5*10^{-7} M, 10^{-6} M) on elongation of shoots from axillary buds was shown by Bergman [4], but the authors did not observe the formation of adventitious buds. Agrawal [7], when hybrid willow (S. fragilis × S.
lispoclados) was cultivated on a medium supplemented with 0.2 mg/l BAP, obtained a multiplication factor of 5–8 after the third subculture.

In order to study the effect of the mineral composition of basic media on the morphogenesis of S. acutifolia in an in vitro culture, MS [8], DKW [12], GD [13] and WPM [14] were tested. In the course of the study, microcranes with a length of 1.2–1.5 cm, ten explants were planted in culture vessels with four test media. Each medium was supplemented with 1.0 mg/l BAP, 0.1 mg/l NAA and contained 30 g/l sucrose, agar-agar at a concentration of 6 g/l. Three repetition. Cultivation was carried out at 21 °C, illumination 1800 Lux, photoperiod 16/8. Statistical analysis was performed by analysis of variance.

To study the effect of cytokinins on the morphogenetic ability of S. acutifolia explants, a comparison of hemogenesis was performed on media supplemented with Kn, BAP, and 2iP. As the main used MS medium containing 0.1 mg/l NAA, 30 g/l sucrose and 6 g/l agar-agar. Segments of shoots with a length of 12-15 mm were placed in culture vessels of 21 explants per variant, three repetition.

The influence of the mineral composition of the medium on the rhizogenesis of explants of S. acutifolia in vitro was evaluated on four studied media DKW, GD, WPM, and MS. 10 explants were planted in culture vessels. Each medium contained 30 g/l sucrose, 6 g/l agar-agar, and 0.2 mg/l NAA. As a control, MS medium supplemented with 0.2 mg/l NAA was selected. Three repetition. Cultivation was carried out at 21 ° C, illumination of 1800 Lux, photoperiod 16/8.

3. Results

Preliminary studies have shown that when using a 0.5% Na hypochlorite solution (Domestos) as a sterilizing agent within 10 minutes, the maximum number of sterile morphogenic explants of S. acutifolia is reached at 94.44%.

During an experiment to study the effect of mineral salts on the in vitro morphogenesis of S. acutifolia explants, it was noted that the studied culture medium variants have a significant difference in their regenerative activity on hemogenesis.

The largest number of formed shoots and buds per explant (2.9 and 2.8) was observed on MS and DKW media, respectively, while at the same time, on WPM, this indicator was only 2 shoots per explant. Thus, the formation of new buds was 43% more efficient on MS than on WPM, and 39% and 19% on DKW and GD, respectively.

The experiment showed that the studied media significantly differ from each other in terms of their influence on the growth of shoots of S. acutifolia in an in vitro culture. When evaluating the explant growth parameters, the best results were observed on MS medium; the shoot length in this variant was 46% longer than on GD medium. The DKW and WPM environments on this indicator practically did not differ (less than WPM by 26.8% and 24.7%, respectively). On the GD medium, short shoots were formed.

On days 14-16 of cultivation, thickenings formed in the form of overgrown tissues on the basal part of the explants, on which adventitious buds subsequently formed.

In the course of the study, a significant difference was revealed between the studied variants of culture media supplemented with Kn, BAP, 2iP cytokinins in terms of the intensity of S. acutifolia hemogenesis in vitro culture.

The largest number of newly formed buds (on average 4.2 buds/explant) was recorded on a medium with Kn, which in turn was 50% more effective than control. At the same time, the newly formed shoots were more formed than on the other two studied media. On a medium with 2iP, new buds formed 10% less than in control (1.9 buds/explant).

At the same time, Sant S. Bhojwani in his article [15] notes that in an in vitro hybrid culture, Salix BAP induces three morphological processes, namely, basal part callusing, root formation suppression and shoot proliferation. Kn causes explant callus (in the concentration range of 0.1-1.0 mg/l), but only high concentrations of the hormone inhibit rhizogenesis and induce hemogenesis.

After 8 weeks of cultivation on DKW, GD, WPM, MS media, the rhizogenesis intensity was evaluated by the following criteria: average number of rooted shoots (table 1), average number of roots per explant (table 2), average length of one root (table 3).
The influence of the medium begins to affect the average value of rooted plants from the eighth week of cultivation. At 2, 3 and 5 weeks of cultivation, the actual value of the Fisher criterion, calculated by the method of variance analysis, varies from 0.37 to 2.3 with a tabular value of 4.2, which indicates the absence of the influence of the environment on rooting at a 5% significance level. At the eighth week of the experiment, the influence of the medium on the percentage of rooted plants is significant at a 1% significance level (F fact. = 8.5 with F tab. 0.01 = 7.85).

The best result of rooting of explants of *S. acutifolia* was obtained on MS medium - 96.7%, somewhat less than rooted plants on WPM medium - 92.6%, while on DKW and GD environments, root shoots were 20% less (table 1).

| Time, weeks | DKW | GD  | MS  | WPM |
|-------------|-----|-----|-----|-----|
| 2           | 34.8| 32.8| 26.5| 57.4|
| 3           | 45.2| 45.0| 41.0| 54.1|
| 5           | 67.2| 49.7| 55.0| 75.6|
| 8           | 65.3| 76.7| 96.7| 92.6|

When assessing the average number of roots formed on one explant, it was also noted that the influence of the medium begins to affect from the eighth week of cultivation. At 2, 3 and 5 weeks of cultivation, the actual value of the Fisher criterion calculated by the analysis of variance varies from 0.92 to 2.9 with a tabular value of 4.2, which indicates that there is no influence of the environmental factor on the average number of roots per explant at a 5% level significance. However, at the eighth week of the experiment, the influence of the medium is significant at a 1% significance level (F fact. = 6.1, F tab. 0.01 = 7.85). Explants on DKW medium formed roots by 43.5% less than on MS medium (2.3 pcs/explant - control), while on GD medium the average number of roots per explant was 1.6, which is also less than in the control by 30.4% (table 2). The best results were obtained on WPM - 2.9 pcs/explant, which is more than on MS, by 26%.

| Time, weeks | DKW | GD  | MS  | WPM |
|-------------|-----|-----|-----|-----|
| 2           | 0.7 | 0.7 | 0.3 | 1.1 |
| 3           | 0.9 | 1.1 | 0.5 | 1.1 |
| 5           | 1.4 | 1.3 | 1.9 | 2.4 |
| 8           | 1.3 | 1.6 | 2.3 | 2.9 |

After three weeks of cultivation, the average root length on the test media did not differ significantly. After 8 weeks of cultivation, the minimum average root length was observed on GD medium (12.1 pcs/explant), which is 30.5% less than in the control. Unsatisfactory results were also obtained on DKW medium (12.4 pcs/explant), the average root length was 28.7% less than on MS. The maximum average root length in the experiment was 18.7 mm in the WPM medium, exceeding the control by 7.5% (table 3).

| Time, weeks | DKW | GD  | MS  | WPM |
|-------------|-----|-----|-----|-----|
| 2           | 4.7 | 5.3 | 7.3 | 5.3 |
| 3           | 8.2 | 7.1 | 10.1| 12.1|
| 5           | 11.5| 10.5| 15.8| 18.2|
| 8           | 12.4| 12.1| 17.4| 18.7|
As a result of the study, a significant difference was found between the DKW, GD, MS, and WPM media in the average root length.

After the plants of S. acutifolia formed roots in vitro, they were removed from the culture vessels and adapted to the soil substrate. Peat tablets “Jiffy-7” with a diameter of 37 mm were used as a substrate. A week later, roots began to appear from the substrate, and after another two weeks the resulting plants were planted in pots with soil mix. Further growing was carried out in open ground conditions.

4. Conclusion
Studies have shown that the studied environments differ significantly from each other in terms of the characteristics studied. When assessing the parameters of growth and development of shoots, the best results were achieved on MS. In work Skálová et al was shown that for cultivation of green young shoot apexes of S. alba and S. lapponum the best results were achieved on OK medium, and for the ovule culture of S. caprea and S. viminalis the best result was on CP medium [16]. The effect of phytohormones on the breaking of dormancy of axillary buds in Salix pseudolasiogyne and their subsequent proliferation from nodal explants were examined by Paek et al. Axillary shoots from developed in all the WPM media, a higher percentage bud break occurred on BAP supplemented media [17].

In the course of the study, a significant difference was revealed in the studied variants of culture media supplemented with K, BAP, 2iP in terms of the intensity of S. acutifolia hemogenesis in an in vitro culture. Research has shown that using K to multiply S. acutifolia shoots is more effective than BAP or 2iP. Similar results were obtained by culturing Salix nigra on WPM supplemented with one of 0.1 mg/l thidiazuron (TDZ), 0.5 mg/l or 1.0 mg/l BAP [15].

Based on the obtained results of the set of studied parameters of rooting in vitro of S. acutifolia explants, a significant difference was established between the influence of mineral salts of the cultivation medium on the processes of rhizogenesis, starting from the eighth week of cultivation. It was experimentally established that the results obtained on the WPM environment are usually 10-20% better than the results achieved on other studied environments.

References
[1] Wagner H and Bladt S 2001 Plant drug analysis (Springer, Berlin, Germany)
[2] Chrubasik S, Eisenberg E and Balan E 2000 Treatment of low back pain exacerbations with willow bark extract: a randomized double-blind study American Journal of Medicine 109 9-14
[3] Julkunen-Tiitto R and Meier B 1992 Variation in growth and secondary phenolics among field cultivated clones of Salix myrsinifolia Planta Medica 258 77-80
[4] Bergman L, Arnold S and Eriksson T 1985 Effects of N6-benzyladenine on shoots of five willow clones (Salix ssp.) cultured in vitro Plant Cell Tissue and Organ Culture 4 135-44
[5] Liesebach M and Naujoks G 2004 Approaches on vegetative propagation of difficult-to-root Salix caprea Plant Cell, Tissue and Organ Culture 79 239-47
[6] Neuner H and Beiderbeck R 1993 In vitro propagation of Salix caprea L. by single node explants Silvae Genet 42 308-10
[7] Agrawal D and Gebhardt K 1994 Rapid micropropagation of hybrid willow (Salix) established by ovary culture Plant Physiology 143 763-5
[8] Agrawal D C and Gebhardt K 1994 Rapid micropropagation of hybrid willow (Salix) established by ovary culture Journal of Plant Physiology 143(6) 763-5
[9] Amo-Marco J B and Lledo M D 1996 In vitro propagation of Salix taraconensis pau ex font quer, an endemic and threatened plant In Vitro Cellular & Developmental Biology. Plant. 32(1) 42-6
[10] Murashige T and Skoog A 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures Physiologia Plantarum 15 473-97
[11] Sergeev R and Shurgin A 2009 Reproduction in vitro of willow genotypes with a high content of
biologically active substances for plantation cultivation on salicin

[12] Driver J A and Kuniyuki A H 1984 In vitro propagation of paradox walnut rootstock Hortscience 19 507-9

[13] Gresshoff P M and Doy C H 1972 Development and differentiation of haploid Lycopersicon esculentum (tomato) Planta 107 161-70

[14] Lloyd G and McCown B H 1980 Commercially feasible micropropagation of mountain laurel, Kalmia latifolia by use of shoot tip culture Proceedings of the International Plant Propagators Society 30 421-7

[15] Bhojwani S S 1980 Micropropagation method for a hybrid willow (Salix matsudana x alba NZ-1002) New Zealand Journal of Botany 18 209-14

[16] Skálová D, Navrátilová B, Richterová L, Knit M, Sochor M and Vašut R 2012 Biotechnological methods of in vitro propagation in willows (Salix spp.) Central European Journal of Biology 7 931-40

[17] Park S Y, Kim Y W, Moon H K, Murthy H N, Choi Y H and Cho H M 2008 Micropropagation of Salix pseudolasiogyne from nodalexplants Plant Cell Tiss Organ Cult 93 341-6

[18] Lyyra S, Lima A and Merkle S A 2006 In vitro regeneration of salix nigra from adventitious shoots Tree Physiol 26(7) 969-75