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Some features of cultivating different *Chamaenerion angustifolium* (L.) Scop. forms *in vitro*

D.A. Egorova[1], O.I. Molkanova[1], Yu.N. Gorbunov[1], A.A. Gulevich[2] and E.N. Baranova[1,2,*]

[1]N.V. Tsitsin Main Botanical Garden of Russian Academy of Sciences, Botanicheskaya street 4, RU127276 Moscow, Russia
[2]All–Russian Scientific Research Institute of Agricultural Biotechnology, Timiryazevskaya street 42, RU127550 Moscow, Russia
[*]Correspondence: greenpro2007@rambler.ru

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Abstract. *Chamaenerion angustifolium* (L.) Scop. characterized by a wide range of economically useful properties. White-flowered form of *Ch. angustifolium* (L.) Scop. Is extremely rare in nature. At the same time, it is promising as a source of biologically active substances and as a highly decorative plant. The optimal way to reproduce this form is clonal micropropagation. Methods for obtaining *Ch. angustifolium in vitro* were developed, as well as the optimal selection timing of starting material for micropropagation was determined. In addition, the effect of a mineral composition of nutrient medium and plant growth regulators on the regeneration of microshoots was studied. The highest values of morphometric parameters were achieved on MS medium (Murashige & Skoog, 1962) supplemented with 0.5 mg L^{-1} BAP. The multiplication factor of the lilac–flowered form was 8.4 ± 0.2, of the white-flowered form - 9.2 ± 0.6. Comparative analysis of morphometric parameters during cultivation of *Ch. angustifolium* showed no significant difference between the lilac-flowered and white-flowered forms. The effect of antioxidants on the growth and development of regenerants has been shown. The most optimal nutrient medium for clonal micropropagation of the lilac-flowered form was MS medium (Murashige & Skoog, 1962) containing 0.5 mg L^{-1} of BAP, 50 mg L^{-1} of ascorbic acid and 50 mg L^{-1} of citric acids and for micropropagation of the white–flowered form it was the medium, containing 100 mg L^{-1} PVP.

Key words: clonal micropropagation, phenolic exudation, plant growth regulators, fireweed.

INTRODUCTION

Fireweed or *Chamaenerion angustifolium* (L.) Holub is a long-rhizome perennial. The stem of this herb reaches a height of 2 m. Leaves are alternate, narrow-lanceolate, pointed, whole-edged or with indistinct denticles. Inflorescence is a long raceme. The flowers are large, four-petal, pink or purple. The area covers a large territory of the Northern Hemisphere. In Europe, the fireweed is distributed throughout the all forest zone (Adamczak et al., 2019).

D.P. Syreishchikov (2020), in addition to the widespread, typical narrow-leaved, lilac-flowered form, noted the presence of two rare forms (modifications) of fireweed: f. *macrophyllum* Hausskn (large-leaved, characterized by oval leaves) and f. *albiflorum* Hausskn (white-flowered). In a garden a rare white coloured form is used for creating a solitaire plant, background plantings, flower beds as an alternative to delphinium and stock–rose. Traditionally *Chamaenerion angustifolium* (L.) Holub cultivar 'Album' (*Epilobium angustifolium* cv. 'Album') is cultivated (Mayer et al., 2017).

Various organs of fireweed contain a whole complex of chemical compounds: essential oil, tannins, rich elemental composition, carbohydrates (starch, sugars, pectin), flavonoids, vitamin C, amino acids and other substances. The rich chemical composition determines the broad biological activity of the plant. *Ch. angustifolium* has antioxidant, vasoconstrictor, anti-inflammatory, analgesic, antipyretic, antimicrobial and antiviral effects (Kosalec et al., 2013; Frolova et al., 2014; Shrivastava et al., 2014; Tsarev et al., 2016).

Ch. angustifolium is also used in traditional and folk medicine for a variety of diseases (for headaches, stomach ulcers, gastritis and colitis, as a hypnotic and sedative, etc.). It is a very promising medicinal plant that can be used in official medicine (Kosalec et al., 2013; Tsarev et al., 2016; Kadam et al., 2018). In recent decades, special attention has been paid to the antitumor effect of fireweed preparations (Maruška et al., 2017; Gryszczyńska et al., 2018). The white-flowered form of fireweed is especially promising as a raw material for the production of these drugs. The absence of pigments in flowers can facilitate the release of pharmaceutical substances for the drug, most of which are represented by phenolic compounds. This form can be in demand both as a valuable pharmaceutical raw material and as an exclusive variation of ornamental garden culture. The uniqueness of this form necessitates its introduction *in vitro*.

Fireweed has also a nutritional value. In Northern Europe, the herbal drink obtained by brewing fermented leaves of *Ch. angustifolium* was known for a long time, and was popular with the general population (Svanberg, 2012). In recent years, it has regained its former popularity as herbal tea (Kalle et al., 2020). Fireweed rhizomes were using as a vegetable, they are suitable for baking bread. Root sprouts and young stems were eaten like asparagus and cabbage (Adamczak et al., 2019). The leaves are suitable for preparing salads and as a seasoning for meat dishes and broths (Svanberg, 2012; Kalle et al., 2020). Rhizomes and young shoots are promising for use in food fresh and pickled. Fireweed shoots in terms of protein content are close to high-protein legumes, and the protein composition indicates its high quality (Tsarev et al., 2016).

Fireweed is one of the best honey plants among wild plants. Nectar production from 1 ha of its thickets reaches 500–600 kg, and in terms of sugar - 250–300 kg. Fireweed honey has a pleasant odor, delicate taste, greenish colour, and it is very sweet (Adamczak et al., 2019).

It can be stated that willow herb is a very promising plant with great potential for integrated use. Hence, it becomes necessary to introduce it into a wider culture. The proposed technologies of seed and vegetative propagation (by rhizomes and green cuttings) are economically unprofitable. They do not allow obtaining a large number of plants in a short period. Also *Ch. angustifolium* is characterized by interspecific hybridization, which complicates the production of genetically homogeneous material (Molkanova et al., 2018). The most promising method for obtaining a large number of fireweed planting material is clonal micropropagation. Clonal micropropagation of

plants is a tissue culture method that ensures the preservation and multiplication of a large number of cultivars of valuable decorative and medicinal plant species, widely used for commercial propagation (Rout et al., 2000). An important advantage of this method is the ability to carry out breeding work throughout the year.

MATERIALS AND METHODS

The research was carried out at the Plant Biotechnology Laboratory at the N.V. Tsitsin Main Botanical Garden of the Russian Academy of Sciences. The studied plants were provided by the Department of Cultivated Plants (Fig. 1) (Egorova et al., 2016).

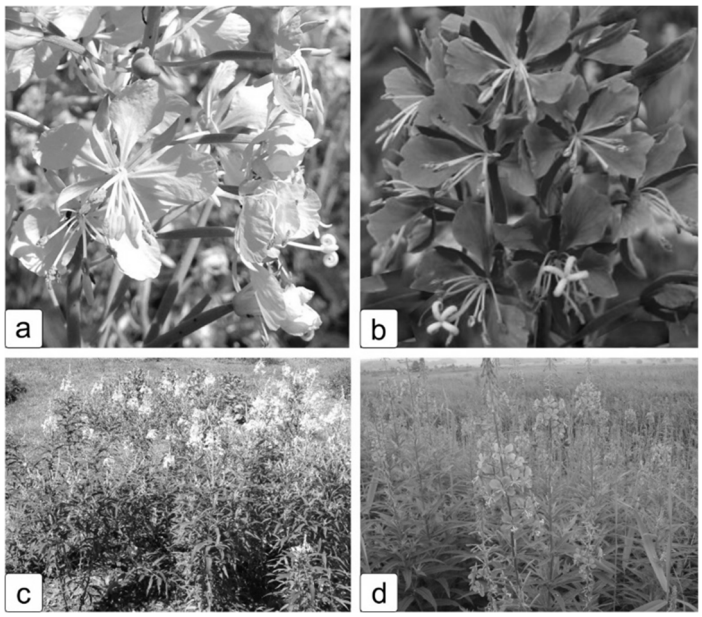

Figure 1. The source material of two varieties of purple and white fireweed (*Ch. angustifolium*).

Cultivation of plants *in vitro* was carried out according to generally accepted biotechnological methods and techniques developed in our laboratory (Egorova et al., 2016; Mitrofanova et al., 2018.; Molkanova et al., 2018). At the initiation stage, axillary meristems located on different parts of the shoot at different times of the vegetation phase were used as explants. Explants were kept in a 2–4% solution of a systemic fungicide with difenoconazole, then they were immersed in 70% ethyl alcohol for 1–2 min and transferred to 7% calcium hypochlorite solution and stirred for 5–10 min.

MS (Murashige & Skoog, 1962) and QL (Quoirin & Lepoivre, 1977) nutrient media with addition of 0.1–1.5 mg L^{-1} 6-benzylaminopurine (BAP) were used at the stage of micropropagation. Media were adjusted to a pH 5.7–5.8 with 0.1 N KOH and autoclaved at 120 °C for 20 min. Citric and ascorbic acid in a concentration of 50 and 100 mg L^{-1}, a combination of ascorbic and citric acids (in a concentration of 25 and 50 mg L^{-1}), as well as PVP (polyvinylpyrrolidone) in a concentration of 50 and 100 mg L^{-1} were added to reduce phenolic exudation. Cultures were placed in the growth chamber at 25 ± 2 °C and 16 h photoperiod under cool–white fluorescent lights. Plantlets were subcultured after

30–40 days. Multiplication factor (mean of new axillary shoots produced per microshoot) and mean length of shoots were recorded after 30 days of culture. Explants were also visually evaluated for leaf necrosis, hyperhydricity and chlorosis. The experiments were carried out in threefold replicate, 10 explants in each variant. The length of microshoots was measured and multiplication factor was calculated at the propagation stage. Results are presented as means ± standard error. Statistical analyses were done using ANOVA followed by the Tukey's Honestly Significant Difference test. Statistical significance was defined as $P \leq 0.05$ and marked with different letters in superscript: a, b, c, d. Statistical data were processed in the data analysis software packages - PAST (PAleontological STatistics).

RESULTS AND DISCUSSION

In the process of studying the effect of different explant sterilization timing on the viability of developing microshoots of various fireweed forms it was found that the highest indicator of shoot viability (70–90%) was typical for plants in the period from April to early June. When studying the effect of various exposures of calcium hypochlorite on the process of axillar meristems disinfection, it was found that exposure of 5–6 min was not enough to obtain aseptic fireweed explants. The highest yield of aseptic explants of the white-flowered form (93.3 ± 2.0%) and the lilac–flowered form of *Ch.angustifolium* (86.36 ± 9.9%) was observed at an exposure of 7 min. Increased exposure resulted in decreased contamination, but increased the number of non-viable explants (Fig. 2).

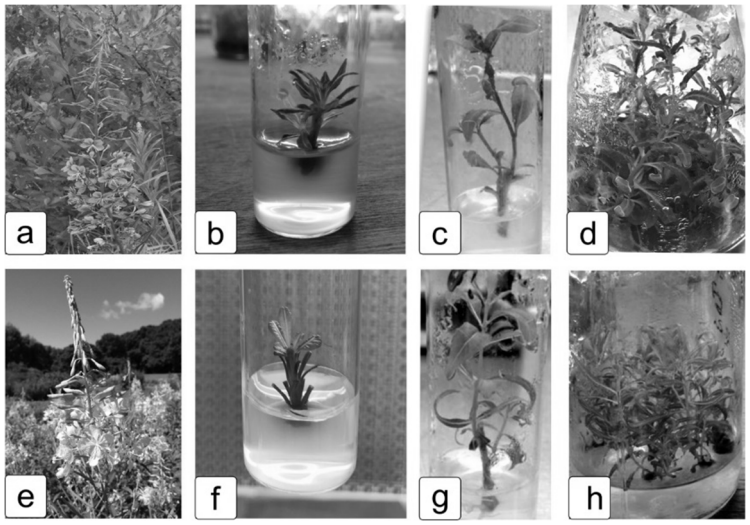

Figure 2. Micropropagation of lilac- and white-flowered forms of *Ch. angustifolium*; a – parent plant of fireweed, lilac form; b – phenolic exudation of lilac form at the initiation stage; c – the beginning growth of axillar buds of lilac form; d – the multiplication of lilac form on MS medium supplemented with 0.5 mg L^{-1} BAP, 50 mg L^{-1} ascorbic acid, and 50 mg L^{-1} citric acid; e – parent plant of white form of *Ch. angustifolium*; f – the beginning growth of axillar shoot of white form; g – the beginning growth of axillar buds of lilac form; h – the multiplication of white form on MS medium supplemented with 0.5 mg L^{-1} BAP and 100 mg L^{-1} PVP.

One of the main factors influencing the processes of morphogenesis and the intensity of micropropagation is the mineral content of nutrient medium (Dreger & Wielgus, 2015). Initially, nutrient media differing in mineral composition (MS and QL) have been compared (Table 1).

Table 1. Influence of nutrient medium on microshoots length and multiplication factor of different *Ch. angustifolium* forms

Ch.angustifolium form	Nutrient medium	Microshoot length, mm	Multiplication factor
Albiflorous form	MS	39.6 ± 5.5^{a}	4.1 ± 0.6^{a}
	QL	15.6 ± 4.3^{b}	2.4 ± 0.5^{b}
Lilac form	MS	38.6 ± 4.2^{a}	4.0 ± 0.4^{a}
	QL	14.2 ± 3.3^{b}	2.4 ± 0.5^{b}

*Values represent mean + standard error. Means followed by the same letter within the column do not differ significantly ($P \leq 0.05$) according to a Tukey's Honestly Significant Difference test.

The MS medium turned out to be the supreme better result in all respects. An active plant growth was observed and new microshoots were formed on this medium. The multiplication factor of lilac-flowered and white-flowered forms was 4.0 ± 0.4 and 4.1 ± 0.6, respectively. Plant growth was retarded on QL medium; sometimes plants were dying immediately after passage. Also, a plant chlorosis was observed 10–15 days after transplantation on this medium. It should be noted that a chlorosis was also observed in the case of long-term cultivation (more than 40 days) on MS medium.

Plant growth regulators have a significant effect on various morphophysiological processes in plants, including growth and development. The proper choice and optimal ratios of growth regulators (cytokinins and auxins) are essential for the *in vitro* enhanced growth of microshoots (Trettel et al., 2020). In the course of the study, the most optimal concentrations of exogenous cytokinin for enhanced growth induction of fireweed microshoots were identified (Fig. 3–4).

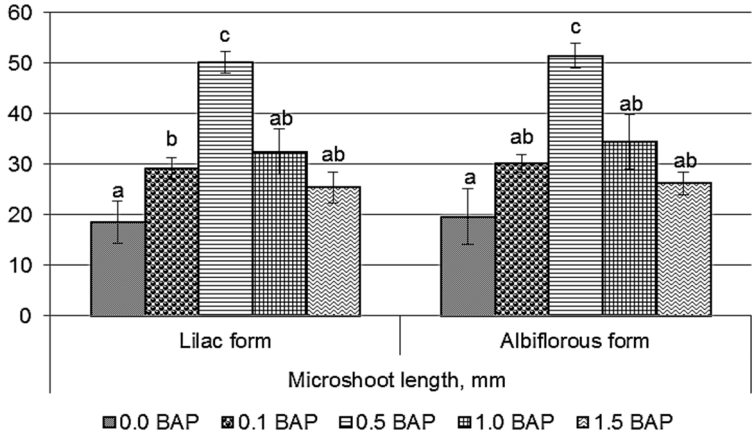

Figure 3. Influence of different BAP concentrations on *Ch. angustifolium* microshoots length.
*Values represent mean + standard error. Means followed by the same letter within the column do not differ significantly ($P \leq 0.05$) according to a Tukey's Honestly Significant Difference test.

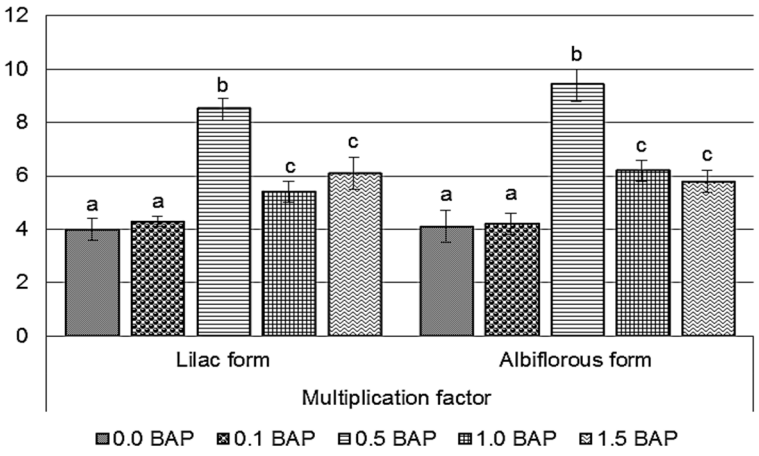

Figure 4. Influence of different BAP concentrations on *Ch. angustifolium* multiplication factor.
*Values represent mean + standard error. Means followed by the same letter within the column do not differ significantly ($P \leq 0.05$) according to a Tukey's Honestly Significant Difference test.

BAP concentrations from 0.1 to 0.5 mg L^{-1} was found to provide an increase in the multiplication factor. But a higher concentration caused tissue hydration, overgrowth of callus and a decrease in the microshoots proliferation. Under the influence of 0.5 mg L^{-1} BAP, the length of microshoots of white-flowered and lilac-flowered forms reached the maximum values: 50.2 ± 2.2 mm and 51.5 ± 2.5 mm, respectively. The multiplication factor also reached its maximum: 8.4 ± 0.2 in white–flowered form and 9.2 ± 0.6 in lilac-flowered form. Comparative analysis of morphometric parameters during micropropagation of *Ch. angustifolium* showed no significant difference between the lilac-flowered and white-flowered forms. Thus, the optimal medium for the cultivation of fireweed turned out to be a medium supplemented with 0.5 mg L^{-1} BAP, which showed the highest morphometric parameters.

Comparative analysis of the obtained data showed that nutrient media of different composition are required for two forms of *Ch. angustifolium* in order to increase their morphogenic potential (Table 2).

Table 2. The effect of antioxidants *on Ch. angustifolium* morphometric parameters

Antioxidants	Concentration of antioxidants, mg L^{-1}	Microshoot length, mm		Multiplication factor	
		Lilac form	Albiflorous form	Lilac form	Albiflorous form
–	–	8.5 ± 0.7^{a}	6.2 ± 0.6^{a}	2.1 ± 0.3^{a}	1.2 ± 0.4^{d}
Ascorbic acid	50	13.1 ± 1.1^{b}	23.9 ± 0.8^{d}	3.4 ± 0.3^{b}	4.0 ± 0.4^{b}
	100	18.0 ± 1.4^{c}	24.0 ± 1.1^{d}	4.0 ± 0.4^{b}	4.1 ± 0.4^{b}
Citric acid	50	14.1 ± 1.4^{b}	10.1 ± 1.6^{b}	3.1 ± 0.4^{b}	2.3 ± 0.3^{a}
	100	12.5 ± 0.8^{b}	12.1 ± 1.1^{b}	3.4 ± 0.3^{b}	2.5 ± 0.4^{a}
Ascorbic acid + citric acid	50	16.9 ± 1.1^{c}	23.2 ± 1.4^{d}	4.0 ± 0.3^{b}	4.0 ± 0.3^{b}
	100	23.3 ± 1.1^{d}	18.0 ± 1.1^{c}	5.2 ± 0.3^{c}	4.2 ± 0.4^{b}
PVP	50	14.6 ± 1.6^{b}	24.5 ± 1.4^{d}	3.7 ± 0.4^{b}	4.5 ± 0.3^{b}
	100	12.5 ± 1.7^{b}	28.4 ± 0.8^{e}	3.2 ± 0.3^{b}	5.5 ± 0.4^{c}

*Values represent mean + standard error. Means followed by the same letter within the column do not differ significantly ($P \leq 0.05$) according to a Tukey's Honestly Significant Difference test.

Different concentrations of various antioxidants showed to be effective in growth stimulation of microshoots. So, the maximum length (23.3 ± 1.1 mm) of the lilac-flowered form was observed on a medium containing 50 mg L^{-1} of ascorbic and citric acids, and the one of the white-flowered form on a medium containing 100 mg L^{-1} PVP (23.2 ± 1.4 mm). The smallest value of the length (6.2 ± 0.6 mm and 8.5 ± 0.7 mm) was observed in the control variant (without antioxidants), which was characterized by partial death of explants due to high phenolic exudation, as well as a slowdown in the growth and development of microshoots.

It was found that all the studied antioxidants and sorbents contributed to an increase in the multiplication factor. In the lilac-flowered form, this indicator significantly exceeded the values obtained using other concentrations and amounted to 5.2 ± 0.3 on a medium with the addition of ascorbic acid and citric acid (50 mg L^{-1} each). The highest multiplication factor of the white-flowered form of *Ch. angustifolium* was observed on culture medium containing 100 mg L^{-1} PVP (5.5 ± 0.4). In other variants, the multiplication factor varied slightly.

The induction of morphogenesis and the ways of the morphogenetic potential expression in fireweed, like other plants, depend on the genotype, initial explant, composition of the nutrient medium and cultivation conditions (Dreger et al., 2020). One of the most crucial stages in plants introduction *in vitro* is a selection of the explant isolation timing. Different explants were used for *in vitro* reproduction of fireweed. In study of Turker et al. (2008) was shown that root explants produced more shoots than other explants. In Dreger's work (2016), the best shoot regeneration was obtained from stem fragments (96%) and root explants (60%), which formed many shoots. In our study, only axillary buds at stem fragments regeneration were used, and the percentage of regeneration was 98%. Thus, propagation of fireweed plants by means of axillary buds gives a better result in comparison with other explants.

The browning of tissues of the primary explants and nutrient media due to the exudation of phenolic compounds from the cut surface often represents a significant difficulty for obtaining a sterile culture and further successful clonal micropropagation of medicinal plants. To solve this issue, antioxidants (ascorbic, citric acids) and sorbents (polyvinylpyrrolidone, activated charcoal) are added into the nutrient medium, which prevent the activation of hydrolytic enzymes and the death of explants (Nayanakantha et al., 2010). Ascorbic and citric acids detoxify reactive oxygen formations. The antioxidant impact of organic acids manifests itself by a tendency to increase the adaptive potential of plants and to optimize the processes of growth and productivity (Ulianych et al., 2020). Phenolic exudation in explants from *Ch. angustifolium* plants has also been observed in other studies. Turker et al. (2008) were able to overcome this problem only by adding 100 mg L^{-1} of ascorbic acid into the medium. However, the content of phenolic compounds depends on many factors, including the genotype. It is possible that in our study the colour mutation in the white-flowered form somewhat mitigated the intensity of the synthesis of phenolic compounds, which favourably affected the decrease in the amount of phenols neutralized by organic acids.

CONCLUSIONS

Thus, the optimal timing for isolating *Ch. angustifolium* explants is the initial stage of the growing season: from April to early June. We also report that a 7% solution of

calcium hypochlorite in 7 minutes exposure was the most effective surface sterilization procedure for the viability of explants. MS nutrient medium with the addition of 0.5 mg L^{-1} BAP is the most efficient at the propagation stage. There were no differences in a morphogenic potential between lilac-flowered and white-flowered *Ch. angustifolium* forms. Combination of ascorbic acid and citric acid (50 mg L^{-1}) was effective in reduction of phenolic exudation, but the best result was achieved for MS medium containing 100 mg L^{-1} PVP.

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