Effects of Naphthenic Acids on Rooting of In Vitro Grown *Sequoia sempervirens*

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Abstract

The study describes the effect of naphthenates and their fractions on rooting of in vitro grown *Sequoia sempervirens* (Lamb. ex D. Don) Endl. shoots. Natural naphthenic acids have been isolated by alkaline extraction from the middle gas fraction of the crude oil. *Sequoia sempervirens* shoots (1 cm in length) were grown on Murashige and Skoog (1962) (MS) medium supplemented with either total naphthenate preparation, naphthenate fractions obtained by extraction on different pH (pH 2, pH 4, pH 7 and pH 9), or indole-3-butyric acid (IBA) in different concentrations (twenty treatments tested). The rooting testing was based on number and total length of roots formed after four weeks of in vitro growth. Similarly to total length of roots, the highest number of roots per explant (≈7) was achieved in medium containing 50 µM of the naphthenate fraction extracted at pH 2 and in medium containing 50 µM of the fraction extracted at pH 9. That is triple higher than in the control and significantly better than in the best IBA-treatment (50 µM IBA), where five roots per explants in average were formed. Similar to the results obtained for some agricultural and tree forest species, our results with *Sequoia sempervirens* confirm the possibility of rooting stimulation by naphthenates.

INTRODUCTION

Naphthenic acids represent a complex mixture of cycloalkyl and alkylcarboxylic acids that are found in raw oil, and could contain more than 3000 compounds (Qian and Robbins, 2001; Clemente and Fedorak, 2005). These compounds exhibited a certain biological activity with respect to uptake of various ions (Kevrešan et al., 2005a), as well as an activity similar to auxin (Ćirin-Novta et al., 2002). Naphthenic acids from this oil fraction stimulate rooting of sunflower cuttings (Kevrešan et al., 2003a), poplar hardwood cuttings (Kevrešan et al., 2003b) and softwood cuttings of *Thuja occidentalis* L. (Kevrešan et al., 2006). Naphthenate treatment influenced the rooting of black locust, *Robinia pseudoacacia* shoots in vitro (Kevrešan et al., 2005b) and caused biochemical changes in softwood cuttings of *Robinia pseudoacacia* (Kevrešan et al., 2007).

Sequoia sempervirens (Lamb. ex D. Don) Endl. (called redwood or coast redwood) is a vulnerable species native to the central and northern California coast (IUCN, 2007). It has highly decorative value as introduced species in Europe. However, its limited distribution and the shortage of seeds, as well as its low germination capacity emphasize the necessity of development of vegetative means of propagation, especially micropropagation (Iliev and Trifonov, 1996).

The aim of this work was to investigate the effects of naphthenates, in total preparation and by examined fractions, on rooting of in vitro grown *Sequoia sempervirens* shoots.

MATERIAL AND METHODS

Isolation, Characterization and Fractionation of Naphthenic Acids

Total naphthenic acids preparation was isolated by alkaline extraction from the middle gas fraction (distillation interval 168-290°C, obtained from NIS Oil Refinery, Novi Sad) of the crude oil "Velebit" (oil-gas field "Velebit", southeastern part of Pannonian Basin, Autonomous Province of Vojvodina, Republic of Serbia) and characterized by physico-chemical methods, as described earlier by Ćirin-Novta et al. (2002). Preparation of total naphthenic acids was fractionated based on acid ionization constants. The naphthenic acids were dissolved in a 5% solution of NaOH at pH 11 and the pH was subsequently decreased with H_2SO_4 and at different pH (pH 2, pH 4, pH 7 and pH 9); undissolved naphthenic acids were obtained by extraction with petroleum ether. In all experiments the sodium salt of naphthenic acids (sodium naphthenates) were used.

Characterization of total preparation of naphthenic acids showed the presence of five classes of carboxylic acids with different content in total acid mixture (% mass): aliphatic $C_nH_{2n}O_2$ (2%), monocyclic $C_nH_{2n-2}O_2$ (21%), bicyclic $C_nH_{2n-4}O_2$ (42%), tricyclic $C_nH_{2n-6}O_2$ (28%) and tetracyclic $C_nH_{2n-8}O_2$ (6%). The average molecular mass of naphthenic acids was determined to be 262, and this value was used to prepare solutions for rooting experiments.

Rooting Experiments

In vitro-grown *Sequoia sempervirens* (Lamb. ex D. Don) Endl. plants were selected for rooting experiments. Shoots (1 cm in length) were grown on Murashige and Skoog (1962) (MS) medium supplemented with 1 mg L^{-1} vitamins, 30 g L^{-1} sucrose and 7 g L^{-1} agar (Sigma). The pH was adjusted to 5.7 before autoclaving. The plants were grown at 24°C during a 16 h light photoperiod with a light intensity of 40 mmol m^{-2} s^{-1} photosynthetic active radiation (PAR) provided by cool white fluorescent tubes. The shoots were gained by micropropagation from nodal segments consisting of a piece of stem about 15 mm in length were transferred to fresh medium and incubated as mentioned above. Subcultures of the plants were performed every four weeks.

Culture medium was supplemented with either total naphthenates preparation, naphthenate fractions obtained by extraction on different pH (pH 2, pH 4, pH 7 and pH 9), or indole-3-butyric acid (IBA). Twenty treatments were tested. The abbreviations for the treatments consist from three parts. The first part describe whether the active substance is mixture of naphthenic acids or IBA, the second (only for naphthenates) the pH of extraction or "tot" for total naphthenates preparation, and the third that refers to the concentration. Following treatments were examined: na-tot-10, na-tot-50, na-tot-100 treatments with total naphthenic acids at concentrations 10, 50 and 100 μM, respectively; na-pH2-10, na-pH2-50, na-pH2-100 treatments with fraction of total naphthenic acids obtained at pH 2 at concentrations 10, 50 and 100 μM, respectively; na-pH4-10, na-pH4-50, na-pH4-100 treatments with fraction of total naphthenic acids obtained at pH 4 at concentrations 10, 50 and 100 μM, respectively; na-pH7-10, na-pH7-50, na-pH7-100 treatments with fraction of total naphthenic acids obtained at pH 2 at concentrations 10, 50 and 100 μM, respectively; na-pH9-10, na-pH9-50, na-pH9-100 treatments with fraction of total naphthenic acids obtained at pH 9 at concentrations 10, 50 and 100 μM, respectively; IBA-10, IBA-50, IBA-100, IBA-1g treatments with indole-3-butiric acid at concentrations 10, 50, 100 μM and 4.92 mM, respectively. In Control treatment MS-medium without any of examined substances was used. Shoots were placed in jars (one per jar) and six rooted shoots per one treatment were measured. The experiment was repeated twice. The rooting assessment was based on number (RN) and total length (RL [cm]) of roots formed after four weeks of in vitro growth. Number of roots was transformed by square transformation ($\sqrt{X}$). Statistical analysis was based on the average value for six jars of the treatment within repetition of the experiment and included ANOVA and LSD-test. Statistical program package STATISTICA 7.1 was used (StatSoft Inc., 2006).

RESULTS AND DISCUSSION

Characterization of total preparation of naphthenic acids showed the presence of five classes of carboxylic acids with different content in total acid mixture (%mass): aliphatic $C_nH_{2n}O_2$ (2%), monocyclic $C_nH_{2n-2}O_2$ (21%), bicyclic $C_nH_{2n-4}O_2$ (42%), tricyclic $C_nH_{2n-6}O_2$ (28%) and tetracyclic $C_nH_{2n-8}O_2$ (6%).

Almost all examined fractions, had significant stimulative effect on rooting of *Sequoia sempervirens* shoots in vitro (Table 1). The results of analysis of variance indicated very significant differences among treatments for bought examined traits (number of roots (RN) and total length of roots (RL)). The highest number of roots in all cases was obtained in treatments where the concentration of active substances had been 50 μM. The highest number of roots per explants (≈7, triple more than in the control), and total length of roots (over 10 cm, almost triple more than in the control) was achieved in medium supplemented 50 μM of the fraction extracted at pH 2 (na-pH2-50) and medium supplemented with 50 μM of the fraction extracted at pH 9 (na-pH9-50) (Table 1). Also, after bought treatments the examined traits were significantly higher than after the best IBA treatment (IBA-50). Stimulative effect of 50 μM for total napthenates preparation was observed by Kevrešan et al. (2003a) and Kevrešan et al. (2005b). Rooting stimulation of examined fractions of total naphtenates preparation (that could be considered as a mixture of numerous compounds, according to Qian and Robbins (2001) and Clemente and Fedorak (2005) suggests the presence of one or more active substances in every examined fraction of the total naphtenates preparation.

Treatments with the highest concentrations (100 μM) of total preparation of napthenates and examined fractions usually gave the least stimulative effect on rooting of in vitro *Sequoia sempervirens* plants. Kevrešan et al. (2003a) observed inhibitory effects of high concentrations of Na-naphthenates on rooting of sunflower green cuttings. Also, the inhibitory and toxic effect of higher concentrations of naphthenic acids is a well known ecological problem (Clemente and Fedorak, 2005).

Several researchers suggested potential of naphthenic acids to influence metabolic processes in plants, especially in the course of the formation and activity of roots system. Severson (1972) concluded that potassium-naphthenates stimulated the glucose uptake by root tips of bean plants, while Kevrešan et al. (2005a) showed that low concentrations of Na-naphthenates influence the uptake of some metal ions by soybean plants. Loh and Severson (1974) found that one-day treatment with potassium naphthenates had a stimulant effect on indolacetic acid oxidase activity, one of the key enzymes associated with the process of initiation and activation of root primordia. Ćirin-Novta et al. (2002) reported auxin-like effects of naphthenates, while Kevrešan et al. (2007) found their simulative rooting potential in *Robinia pseudoacacia* softwood cuttings, at the base of biochemical indicators of root initiation (activity of IAA-oxidases, peroxidases and amylases and content of glucose).

Considering the effect on the number of formed roots and total length of roots on in vitro grown *Sequoia sempervirens*, our work confirm the results of previous works on rooting stimulation by naphthenates. More formed roots means higher chances for survival during acclimatization phase and further in the field conditions. Napthenates are relatively easy to obtain, and could be a cheap alternative to widely used auxins like IBA or NAA. It could be interesting for further praxis of micropropagation of *Sequoia sempervirens*, and should be tested in the propagation of this vulnerable species by softwood cuttings.

CONCLUSION

Our results confirm the rooting stimulation potential of naphthenates for *Sequoia sempervirens*, like it was reported previously for some agricultural and forest tree species. The best results we obtained with na-pH2-50 and na-pH9-50 treatments that appeared to be twice or triple better then the control treatment, and gave significantly better results than the best examined IBA treatment (IBA-50). These results suggest high potential for the use of napthenates in rooting of *Sequoia sempervirens*.

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Tables

Table 1. Number of roots and total length of roots per microshoot of *Sequoia sempervirens* (Lamb. ex D. Don) Endl. four weeks after the examined treatment.

Number of roots (RN)			Total length of roots (RL [cm])		
Treatments*	LSD-test		Treatments	LSD-test	
na-pH2-50	7,64	a	na-pH2-50	12,08	a
na-pH9-50	6,78	ab	na-pH9-50	10,25	b
na-pH2-10	5,78	bc	na-pH2-10	9,58	b
IBA-50	5,18	c	na-tot-50	8,00	c
na-pH7-50	5,13	c	na-pH4-50	7,92	c
na-pH7-10	4,73	cd	na-pH7-50	7,25	cd
na-pH2-100	4,64	cde	IBA-50	7,17	cde
na-pH4-50	4,60	cdef	na-pH4-10	7,00	cdef
na-pH9-10	3,84	defg	na-pH2-100	6,75	cdefg
IBA-100	3,66	defg	na-pH4-100	6,08	defgh
na-tot-50	3,59	defg	na-pH7-10	5,83	efgh
na-pH4-10	3,59	defg	na-tot-10	5,75	fgh
na-pH7-100	3,50	efg	na-tot-100	5,75	fgh
na-pH9-100	3,46	fg	IBA-100	5,50	gh
na-tot-10	3,12	gh	na-pH7-100	5,00	hi
IBA-10	2,93	ghi	IBA-10	5,00	hi
na-pH4-100	2,25	hij	IBA-1g	5,00	hi
na-tot-100	2,08	ijk	na-pH9-100	4,92	hi
Control	2,01	jk	na-pH9-10	4,08	i
IBA-1g	1,36	k	Control	3,83	i

* Labels of treatments: na-tot-10, na-tot-50, na-tot-100 treatments with total naphthenates preparation at concentrations 10, 50 and 100 μM, respectively; na-pH2-10, na-pH2-50, na-pH2-100 treatments with fraction of total naphthenats obtained at pH 2 at concentrations 10, 50 and 100 μM, respectively; na-pH4-10, na-pH4-50, na-pH4-100 treatments with fraction of total naphthenats obtained at pH 4 at concentrations 10, 50 and 100 μM, respectively; na-pH7-10, na-pH7-50, na-pH7-100 treatments with fraction of total naphthenats obtained at pH 2 at concentrations 10, 50 and 100 μM, respectively; na-pH9-10, na-pH9-50, na-pH9-100 treatments with fraction of total naphthenats obtained at pH 9 at concentrations 10, 50 and 100 μM, respectively; IBA-10, IBA-50, IBA-100, IBA-1g treatments with indole-3-butiric acid at concentrations 10, 50, 100 μM and 4.92 mM respectively; Control- treatment without examined active substances.