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NAPHTHENIC ACIDS – ALTERNATIVE ROOTING STIMULATORS IN BLACK LOCUST MICROSHOOTS

ABSTRACT: The study describes the rooting effect of naphthenates and their fractions on *in vitro* grown *Robinia pseudoacacia* L. shoots. Natural naphthenic acids have been isolated by alkaline extraction from middle fraction of crude oil type "Velebit" from Vojvodina, characterized and fractionated. Black locust shoot bases were immersed in ACM medium [Ahuja, 1984] without agar supplemented with either 10, 50 or 100 μM of basic naphthenate preparation, naphthenate fractions obtained by extraction at different pHs (pH 2, pH 4, pH 7 and pH 9), or indole-3-butyric acid (IBA). Treated shoots have been then grown on hormone-free medium for four weeks. Significant differences among test treatments were recorded during the third and the fourth week of *in vitro* cultivation. Final evaluation was performed on the basis of rooting percentage after four weeks of cultivation. The highest rooting percentage (>70%) was achieved after the treatment with solution containing 50 μM of IBA. However, treatment with 10 μM of naphthenate preparation achieved also positive effect on rooting (>60%). Average rooting percentage in the control treatment was just 45%. Our results with black locust confirm previous results gained with some other agricultural and forest tree species that naphthenates have the potential to stimulate rooting in shoots and cuttings.

KEYWORDS: naphthenates, micropropagation, rooting stimulators, *Robinia pseudoacacia*

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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 genotype shoots *in vitro* [Kevrešan et al., 2005b] and caused biochemical changes in softwood cuttings of *Robinia pseudoacacia* [Kevrešan et al., 2007].

The aim of this work was to determine if naphthenates could be used as alternative rooting stimulators for black locust shoots.

MATERIAL AND METHODS

Total preparations of naphthenic acids were isolated by alkaline extraction from the middle gas fraction of crude oil type "Velebit" (Autonomous Province of Vojvodina, Republic of Serbia) and characterized by physico-chemical methods, as described earlier [Ćirin Novta et al., 2002]. Preparations of total naphthenic acids were fractionated according to acid ionization constants. The naphthenic acids were dissolved in a 5% solution of NaOH at pH 11, the pH was subsequently decreased by H_2SO_4, and at different pHs (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 naphthenate) was used.

The genotype of *Robinia pseudoacacia* with fastigiata tree form was used in the experiment. The basis of 1.5–2.0 cm long shoot was immersed for one minute in solution prepared as ACM – medium without agar, supplemented with either 10, 50 or 100 μM of basic naphthenate preparation; naphthenate fractions obtained by extraction at different pHs (pH 2, pH 4, pH 7 and pH 9) or indole-3-butyric acid (IBA). Treated shoots were then grown in hormone-free ACM medium (Ahuja, 1984) for four weeks. Treatments are presented in Table 1. In control treatment shoots were immersed in hormone-free ACM medium. Five shoots were placed per jar and five jars were set per treatment. The rooting was analyzed on the basis of average number of roots per shoot (RN) and percentage of rooted shoots (RP [%]) one, two, three and four weeks after the treatment. Statistical analysis included ANOVA and LSD-test. The percentage of rooted shoots (RP) was transformed by arcsine transformation. Statistical program package STATISTICA 12 was used [StatSoft Inc., 2012].

Table 1. Test treatments applied to shoots of *Robinia pseudoacacia in vitro*

Treatment solution	Total naphthenates	Fraction of total napthenates obtained at	Indol-butiric acid	Concentration of tested active substance (μM)
na-tot-10	+			10
na-tot-50	+			50
na-tot-100	+			100
na-pH2-10		pH2		10
na-pH2-50		pH2		50
na-pH2-100		pH2		100
na-pH4-10		pH4		10
na-pH4-50		pH4		50
na-pH4-100		pH4		100
na-pH7-10		pH7		10
na-pH7-50		pH7		50
na-pH7-100		pH7		100
na-pH9-10		pH9		10
na-pH9-50		pH9		50
na-pH9-100		pH9		100
IBA-10			+	10
IBA-50			+	50
IBA-100			+	100
IBA-1g			+	4921.26
Control				

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%). The average molecular mass of naphthenic acids was determined to be 262, and this value was used to prepare solutions for rooting experiments.

In all test fractions, at least one of the concentrations had stimulative effect on rooting of black locust shoots (Figure 1). The results of analysis of variance indicated significant differences among test treatments: for number of roots per explant in the third and for the percentage of rooted shoots in the third and the fourth week (Table 2). The best results were obtained in the treatment with 50 μM IBA (IBA-50). Only after the treatment na-pH9-100 (100 μM of fraction extracted on pH 9) the percentage of rooting was not significantly different from RP on the best treatment with IBA (58% and 64%, respectively). However, almost every fraction, except fraction obtained at pH 2, achieved stimulative

effect on rooting percentage comparing to control treatment in at least one test concentration. Stimulative effect of naphthenates total preparation on rooting is in agreement with the results of Kevrešan et al. [2003a] and Kevrešan et al. [2005b]. Rooting activity of test fractions of total preparation of naphtenates, with its different values depending on concentrations, suggests the presence of numerous active substances in the total preparation of naphtenates and their presence in all test fractions. It is especially obvious for the fraction obtained at pH 9 that at concentration of 10 μM it achieved inhibited and at concentration of 100 μM stimulated rooting of black locust shoots.

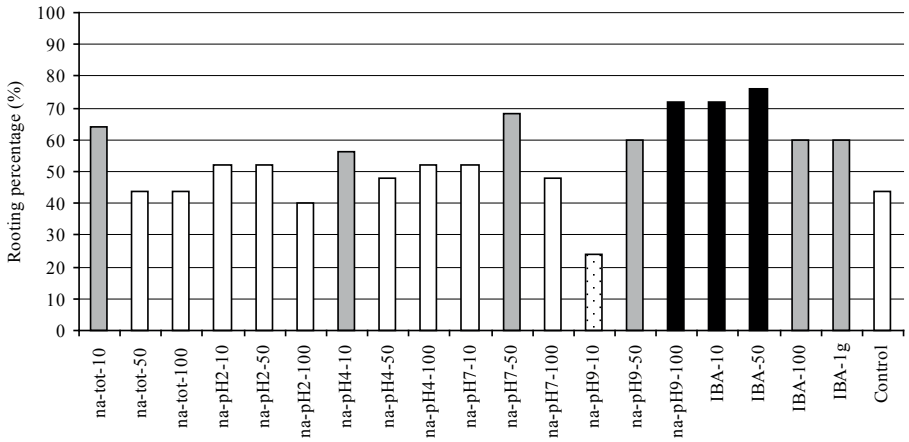

Figure 1. Rooting percentage of black locust (*Robinia pseudoacacia*) four weeks after the treatment with test solutions

Labels of treatments: explained in Tab 1.
Colors of columns: Dotted columns – treatments whose effect was significantly worse than the effect of control treatment; White columns – treatments whose effect was not significantly different from the effect of control treatment, but also significantly worse than the effect of the best IBA-treatment (IBA-50); Grey columns – treatments whose effect was significantly better than the effect of control treatment; Black columns – treatments whose effect was not significantly different from the effect of the best IBA-treatment (IBA-50)

The basis of stimulatory effect of naphthenic acids on rooting is not completely understood [Wort, 1976; Clemente and Fedorak, 2005]. 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 [1975] found that one-day treatment with potassium naphthenates had stimulative effect on the activity the indolacetic acid oxidase, one of the key enzymes in the process of initiation and activation

of root primordia. Ćirin-Novta et al. [2002] found auxinic effect of naphthenic acids, while Kevrešan et al. [2007] found their stimulative rooting potential in *Robinia pseudoacacia* softwood cuttings, on the bases of biochemical indicators of root initiation (activity of IAA-oxidases, peroxidases and amylases and content of glucose). However, the effect of naphthenats on some other important processes that influence rooting, like phenol-peroxidases activity and indolacetic acid conjugation, as well as their influence on ethylene synthesis is still poorly examined.

Treatments with 100 μM of total preparation of napthenates and test fractions (except fraction obtained at pH 9) usually failed to achieve stimulative effect on rooting of tested black locust genotype *in vitro*. Kevrešan et al. [2003a] observed inhibitory effect 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].

Table 2. Analysis of variance for test treatments applied to shoots of *Robinia pseudoacacia*

	Number of roots				Percentage of rooted shoots			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Degree of freedom								
Treatment	19	19	19	19	19	19	19	19
Error	80	80	80	80	80	80	80	80
Total	99	99	99	99	99	99	99	99
Sum of squares								
Treatment	0.572	0.591	0.677	0.454	9451.69	8008.60	10574.69	6047.62
Error	1.827	1.801	1.626	1.495	29711.85	21647.01	15932.69	9191.38
Total	2.399	2.392	2.303	1.948	39163.55	29655.61	26507.39	15239
Mean square								
Treatment	0.030	0.031	0.036	0.024	497.46	421.51	556.56	318.30
Error	0.023	0.023	0.020	0.019	371.40	270.59	199.16	114.89
F-test	1.318	1.382	1.754*	1.277	1.339	1.558	2.795**	2.770**
p-value	0.196	0.160	0.044	0.222	0.183	0.089	0.0007	0.0008

CONCLUSION

Our results confirm the possibility of rooting stimulation by naphthenic acids in black locust. The best results we obtained in *Robinia pseudoacacia* with treatment na-pH9-100. The rooting was significantly better than in the control treatment and at the level of the effect of the best IBA treatment (IBA-50). This suggests high potential for implementation of napthenates in rooting of *Robinia pseudoacacia*, which should be tested in the future.

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НАФТЕНСКЕ КИСЕЛИНЕ – АЛТЕРНАТИВНИ СТИМУЛАТОРИ ОЖИЉАВАЊА КОД МИКРОИЗБОЈАКА БАГРЕМА

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РЕЗИМЕ: Рад описује ефекат нафтената на ожиљавање микроизбојака багрема *in vitro*. Нафтенске киселине су изоловане базном екстракцијом из средње фракције сирове нафте типа „Велебит“, која је описана у ранијим радовима. Доњи део микроизбојка је урођен један минут у течни АСМ медијум [Ahuja, 1984] у који је додато 10, 50 или 100 μM основне мешавине натријум-нафтената или њених појединих фракција добијених екстракцијом на различитим pH (pH 2, pH 4, pH 7 или pH 9), односно 10, 50, 100 μM или 1g/l индол-3-бутерне киселине (IBA). Контролни третман је чинио АСМ медијум без испитиваних активних материја. Третирани микроизбојци су затим гајени на чврстој АСМ подлози без хормона. Значајне разлике међу испитиваним третманима су забележене током треће и четврте недеље узгоја у *in vitro* условима. Коначна оцена је изведена на основу процента ожиљавања након четири недеље узгоја. Највиши проценат ожиљавања је постигнут раствором са 10 μM натријум нафтената, након чега је остварен значајан позитиван ефекат на проценат ожиљавања (>60%) у односу на контролни третман (око 45%). Резултати до којих смо дошли код багрема потврђују раније резултате који су добијени код пољопривредних и шумских дрвенастих врста о могућности стимулације ожиљавања микроизбојака и резница солима нафтенских киселина.

КЉУЧНЕ РЕЧИ: нафтенати, микроизбојци, стимулатори оживљавања, *Robinia pseudoacacia*