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In vitro propagation of white oil-bearing rose (*Rosa alba* L.)

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Abstract. *The influence of major factors such as explant sterilization, plant growth regulators in the multiplication and rooting media and the genotype on the in vitro multiplication of White Oil-Bearing Rose (Rosa alba L.) was studied. Explants used in the experiment were 1.0 to 1.5 cm long nodal segments from specially cultivated mother plants. The combination of two disinfectants in the following order: 0.2% HgCl2 solution for 3 min followed by treatment with 0.5% NaClO solution for 20 min and 0.25% NaClO for 30 min was determined as the most suitable sterilization scheme. The best results of multiplication were obtained in basic MS medium with added BAP in concentration of 0.5 to 3.0 mg/L. The analysis of the variance reveals that the genotype, media variants and interactions between them have a statistically significant influence on the variation of the total number of induced adventive shoots. The highest percentage of successfully rooted young plants (97, 5% on average for both genotypes) was obtained in ex vitro conditions by direct rooting in a soil mixture.*

Keywords: white oil-bearing rose, *in vitro*, propagation, variance analysis

Abbreviations: MS- Murashige and Skoog medium, IBA- Indole -3-butyric acid, BAP - 6-Benzylaminopurine, TDZ – Thidiazuron, CPPU [N-(2-chloro-4-pyridyl)-N-phenylurea]

Introduction

The oil-bearing rose is the most emblematic economic crop for Bulgaria. Number one is the world renowned Kazanlak oil-bearing rose (*Rosa Damascena* Mill.f. trigintipetala Dieck). In recent years a number of studies of the Institute of Roses in the town of Kazanlak have drawn attention to the second most important oil-bearing rose, namely the White Oil-Bearing Rose (Nedkov et al., 2009); Dobreva and Kovacheva, 2010). The unobtrusive and characteristic essential oil is widely used both in perfumery in the form of a blend and in the food industry (Dobreva and Gerdzhikova, 2014). The plants of this species are resistant to unfavourable weather conditions, diseases and pests and could be grown successfully in organic farming (Kovacheva, 2005). Unfortunately, the production of propagating material from a population of white oil-bearing rose, as well as the share of currently occupied areas, are minimized. This requires searching for opportunities to meet market needs quickly; with particular attention being paid to research related to the production of quality certified propagating material.

Clonal micropropagation in in vitro conditions is used as an alternative to traditional methods of production of propagation material for a number of economically important species and leads to the production of a larger number and better adapted plants with a better developed root system and higher quality end product (Roberts and Schum, 2003).

Thanks to the intensive research of a large number of authors summarized in the surveys of Pati et al. (2006) on the main factors on which clonal micropropagation depends - type of explants, genotype, medium, in recent years the method of clonal micropropagation has begun to replace ever more successfully the classic vegetative propagation of the rose (Ginova et al., 2012). Despite the increasing popularity of the clonal micropropagation method, there are currently no data published in the world literature related to in vitro propagation studies of the white oil-bearing rose.

The objective of the present study is to investigate the influence of major factors such as explant decontamination, the hormonal composition of the multiplication and rooting medium, and the genotype on the *in vitro* multiplication process in the white oil-bearing rose.

Material and methods

Plant material and decontamination

The plant material is obtained from elite experimental plots of the Institute of Roses and Essential Oil Crops in the town of Kazanlak, situated in the Valley of the Roses, South Central Bulgaria. As a result of a thorough selection assessment, five clones of a population of white oil-bearing rose have been isolated and subjected to steam distillation and gas chromatographic analysis to determine the quantity and parameters of the obtained essential oil. As a result, two clones of the population (R2-1 and R2-2) have been identified, which have proven economic qualities and are used as the object of this study. The experiments were carried out in a laboratory for in vitro propagation of the Industrial plants company, Kazanlak and include three main stages: introduction in culture, multiplication and rooting of the obtained plants. Nodal segments of length (5-8cm) derived from the secondary growth of the donor plants in the period end of June – beginning of July, after flowering, have been used as initial material. After sterilization, the plant material is cut into segments (1.0-1.5cm) long with one to two axillary buds and placed individually in culture medium in closed glass

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containers. Three variants of decontamination have been studied, all of which have been pretreated with 70% ethyl alcohol for 1 min on a magnetic stirrer for surface decontamination.

· The first variant involves sterilization in a 0.2% $HgCl_2$ solution for 8 min.

· The second variant involves serial sterilization in a 0.5% NaClO solution for 20 min and 0.25% NaClO for 30 min on a magnetic stirrer. In both variants, flushing in sterile water is done three times for 5 minutes, the latter being in the laminar immediately prior to placing in the culture medium.

· The third variant of sterilization takes place in two stages. First stage includes treatment with 0.2% solution of $HgCl_2$ for 3 min and subsequent flushing in sterile distilled water for 2 min. Second stage - decontamination in 0.5% NaClO solution for 20 min. This is followed by flushing in distilled water for 2-3 min. The sterilization process continues in 0.25% NaClO solution for 30 min and flushing.

A total of 40 pcs. of explants from each clone have been set for each sterilization variant. Explants are placed one per test tube and cultured in a phytostatic room at a temperature of 21°C, a luminance of 2000 lux. cool white, light/dark period - 12/10 for 20 days.

Proliferation and multiplication

The culture medium for induction and proliferation is MS Murashige and Skoog (1962) with no added hormones. The medium is solidified with 6 g/l agar, and the pH is set at 5.8 before sterilization, which takes place at 121°C at 1.1 atm for 20 min. The decontaminated live explants, 20 days after introducing into the culture, are transferred to multiplication media. A total of 8 variants of the MS basic medium have been used with addition of BAP (6-Benzylaminopurine) at concentrations of 0.5; 3 and 5 mg/L and in combination of 0.5 mg/l BAP with four other growth regulators of the cytokinin group: 1.5 mg/L kinetin, 1.5 mg/L zeatin, 1.5 mg/L TDZ (Thidiazuron) and 0.5 mg/L CPPU (N-(2-chloro-4-pyridyl)-N-phenylurea) (Table 1). In one of the variants (8) citric acid and vitamin C are added to the MS basic medium with 0.5 mg/L BAP as antioxidants at concentrations of 50 mg/L. The experiment was performed by complete randomized block design in two replications. Each replication consists of 5 glass vessels, each vessel containing 4 explants. Explants are transferred to fresh medium every 4 weeks.

Root formation and acclimatization

Two methods of root formation have been studied. First method is in vitro rooting and was applied in four variants: MS medium without hormones and with addition of three different auxins at the same concentration listed in Table 3. The process of root formation has been monitored for a period of 4 weeks. Second method applied is ex vitro rooting of shoots direct in soil mixture 1:1 /perlite : peat/, without passing through root formation medium. Prior to placing in the soil mixture, in vitro produced shoots are immersed in a solution of IBA - 0.1 mg/l for 1 minute with gentle shaking of the vessel where treated. The experiment was performed on a complete randomized block design in two replications. Each repetition consists of 10 glass vessels with culture medium/pots with fertilizer soil mixture, each vessel containing 4 explants. After rooting, the young plants are transferred into a closed greenhouse under conditions of high humidity 80-90% and temperature 30-32˚C, for 20 days. During hardening temperature and humidity gradually decrease and the greenhouse is opened.

Statistical analyses

The experiments were conducted by completely randomized block design in two replications. The data have been processed by analysis of variance ANOVA. Differences between the culture media variants have been tested by Duncan Multiple Range Test. Statistical analyses of the results obtained were made by the software package Statistica 8.0 (StatSoft, Inc. 2002).

Results and discussion

Sterilization of primary explants

Sterilization of the primary explants is an important factor on which the further success of in vitro cultivation depends. The results presented on Figure 1 related to the decontamination effect show that the highest percentage of pathogen-free and vital explants in both genotypes studied - 75% in clone R2-1 and 90% in clone R2-2 are obtained by using the third variant of sterilisation. In treatment with 0.2% $HgCl_2$ solution for 5 min (first variant) a large percentage of explants - 75% in clone R2-1 and 95% in R2-2 do not tolerate the sterilization process and die. The second variant of decontamination was found to be ineffective, and most of the explants were infected - 85% in clone R2-1 and 80% in clone R2-2. The results obtained confirm the importance of this stage of introducing explants into culture. In literature there is controversial information on the

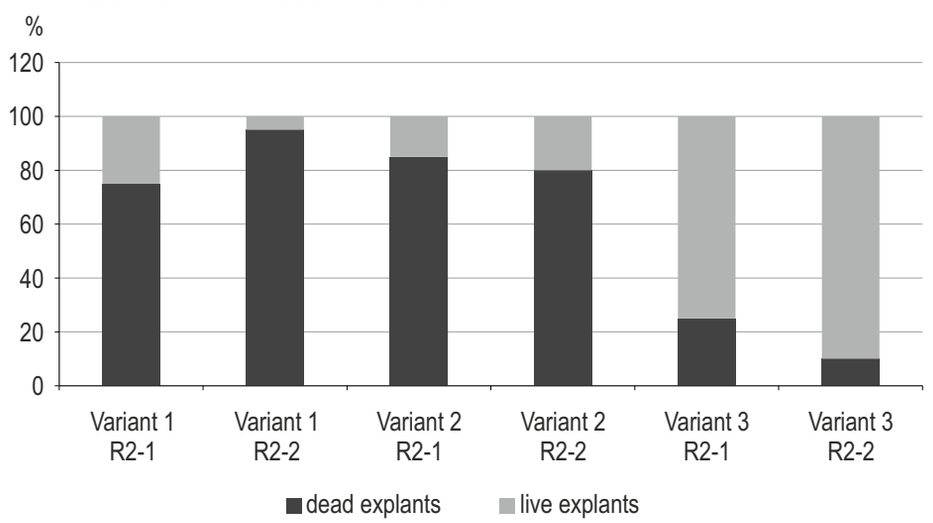

Figure 1. Influence of sterilization variants on explants viability (%) in two clones (R2-1, R2-2) of *Rosa alba* L.

effectiveness of different disinfectants in different types of roses (Khosh-Khui and Sink, 1982a, 1982b; Rout et al., 1989, 1990; Skirvin et al., 1990; Khosravi et al., 2007; Kermani et al., 2010). Our results do not confirm the efficacy of any of the most commonly used disinfectants $HgCl_2$ and NaClO in single use. Mercury dichloride ($HgCl_2$) proved to be too aggressive to explants and they died, while NaClO is inefficient in decontamination of plant material from pathogens. The maximum number of live pure explants in our experiment was obtained by combining the two disinfectants most commonly used by other authors so far by adhering to a certain sequence regarding concentration and time for reaction, which has not been reported to date for decontamination of explants of different rose species.

a – Proliferation of nodal segments in the induction medium

b – Transfer of explants in multiplication medium

c – Shoots multiplication

d- in vitro rooting

e – ex vitro rooting

Figure 2. *In vitro* propagation of *Rosa alba* L. – a, b, c, d, e

Induction and multiplication

Decontaminated and vital explants began to turn green and nodal segments swell. A noticeable prolongation of the nodal segments on the induction medium was observed within 10 days (Figure 2a). About 4 weeks after transfer of the multiplication medium (Figure 2b) nodal explants containing lateral buds formed adventive shoots (Figure 2c) in all of the culture media variants used in both genotypes. Variation in the degree of multiplication expressed by the percentage of the reacting explants, the total number of adventive shoots obtained and the number of shoots per explant was observed (Table 1).

The analysis of the variance presented in (Table 1) shows that the genotype, multiplication medium variants and the interaction among them have a statistically significant influence on the variation in explant multiplication expressed by the total number of induced adventive shoots. Percentages of total sums of squares accounted for genotype, medium and G×M interaction were used to indicate the variation attributed to each component. The variation in the total number of induced advents shoots is mostly due to medium composition (68.0%), followed by genotype-medium interaction (21.5 %).

The results presented in Table 2 show that the greatest number of explants responded by multiplication, the greatest total number of adventive shoots and the greatest number of adventive shoots per explant in both genotypes studied were obtained in medium variants 1, 2 and 3, which contain only one growth regulator BAP (benzylaminopurine) in concentration from 0.5 to 3.0 mg/L. The total number of adventive shoots obtained on average from the two genotypes did not differ significantly at the two lowest concentrations of BAP – 66 pcs. of variants 1 and 64 pcs. of variant 2, which is also confirmed by the Duncan Multiple Range Test, for reliability of differences. When combining a low BAP concentration with other cytokinins - kinetin, zeatin, Thidiazuron and CPPU (variants 4, 5, 6 and 7), the multiplication rate is lower - from 31.5 pcs. in variant 5 (combination between 0.5 $mg.L^{-1}$ BAP and 1.5 $mg.L^{-1}$ zeatin) to 45.5 pcs. in variant 6 (combination between 0.5 $mg.L^{-1}$ BAP and 1.5 $mg.L^{-1}$ TDZ). It is noteworthy that the R2 1 genotype reacts better than the R1 1 genotype to media variants containing a combination of BAP and TDZ (variant 6) and BAP and CPPU (variant 7). The total number of shoots obtained in R2 1 on variant 6 being close to those obtained on the best medium variants containing only BAP (variants 1 and 2), the average number of adventive shoots per explant being even higher, but at the expense of the lower rate of reacting explants. The lowest is the multiplication rate (21.8 total adventive shoots) in variant 8, which differs from variant 1 only by the added antioxidants. The highest number of explants multiplying more than 3 adventive shoots per explant (Figure 2c) were recorded in media 1 and 2, with a larger number of explants multiplying 4 and 5 shoots being recorded in medium 1 compared to medium 2. In media 6, 7 and 8 the number of explants that formed only one shoot prevails, and in medium 5 - the number of explants with two shoots.

Table 1. Analysis of variance for the effect of studied factors on the total number of multiplies adventive shoots

Source of variation	df	SS	MS	F	Strength of influence, %
Genotype	1	820.12	820.12***	69.063	8.53
Medium	7	6538.88	934.13***	78.663	68.0
Interaction	7	2066.87	295.27***	24.865	21.5
Error	16	190.00	11.87		

*** Significant at p< 0.001

Table 2. Effect of various types and concentrations of growth regulators on the multiplication process

Variants of the multiplication medium	Explants with reaction, %		Total number of induced shoots		Average number of shoots per explant, pcs.	
	Genotype R2-1	Genotype R2-2	Genotype R2-1	Genotype R2-2	Genotype R2-1	Genotype R2-2
1. MS+ 0.5 BAP, mg/L	100	95	67 [a]	65 [a]	3.35	3.4
2. MS+ 3.0 BAP, mg/L	85	90	68 [a]	60 [a]	4.0	3.3
3. MS+ 5.0 BAP, mg/L	100	95	45 [b]	47 [b]	2.25	2.5
4. MS -0.5 BAP+1,5 kinetin, mg/L	85	85	46 [b]	40 [b]	2.7	2.4
5. MS+ 0.5 BAP+1.5 zeatin, mg/L	80	80	38 [bc]	25 [c]	2.37	1.6
6. MS+ 0.5 BAP+1.5 TDZ, mg/L	75	80	62 [a]	29 [c]	4.4	1.7
7. MS+ 0.5 BAP+0.5 CPPU, mg/L	50	55	53 [ab]	17 [d]	5.3	1.6
8. MS mod+ 0.5 BAP+OX	55	60	14 [d]	29 [c]	1.3	2.4

Note: a, b, c, d values differ significantly at p < 0.05

Both genotypes differ to some extent in their ability to multiply under in vitro conditions, genotype R2-1 showing a slightly better response in 5 of the studied culture media (Table 2). In genotype R2-2, a higher total number of induced shoots and an average number of shoots from a single explant were obtained only in medium 3 and medium 8. Genotype R2-1 is superior to genotype R2-2 in the variants of medium 5, 6 and 7. Different reactions of both clones of Rosa alba to some of the tested media variants are a manifestation of the occurring interaction between the two factors, which is also confirmed by the variance analysis performed (Table 1).

Shoot multiplication of explants is a key phase for the success of clonal micropropagation (Pati et al., 2006). The results from that experiment confirm the significance of BAP as exogenously added cytokinin to the medium for the best course of in vitro multiplication of nodal explants in various plant species, incl. different types of roses (Vijaya et al., 1991; Pati et al., 2006; Khosravi et al., 2007). It is known that the positive effect of BAP on rose multiplication is related to its ability to reduce apical dominance and to induce the development of axillary buds (Kapchina et al., 2000). According to Roberts and Schum (2003) applying BAP in concentration of 0.45 mg/l (2mM) is suitable for multiplying most rose species and varieties, which is confirmed by the current study on the white rose. Our results are in line with the finding by a number of authors that by increasing the BAP concentration above a certain level reduction of multiplication ability in oil-bearing rose occurs. (Hameed et al., 2006; Mitrofanova et al., 2016).

Combining BAP at a low concentration of 0.5 mg/l with other types of cytokinins (kinetin, zeatin, thidiazuron and CPPU) proved to be inefficient for multiplication of the white oil-bearing rose.

The genotypic differences found in our study on the ability of the white roses to multiply confirm the importance of genotype as a major factor in which the success of in vitro clonal micropropagation depends and has so far been reported by many authors in other types of roses (Bressan et al., 1982; Horn, 1992; Dubois et al., 2000).

Root formation

According to a number of authors one of the main problems of in vitro clonal micropropagation of different plant species, incl. the rose is the rooting of the regenerated plants (Pati et al., 2006; Ginova et al., 2012). In our study, we investigate the influence of the rooting conditions - *in vitro* and ex vivo and different growth regulators from the group of auxins - IBA, IAA and NAA at low concentration of 0.1 mg/l (for *in vitro* rooting) on the efficiency of the process for the white oil-bearing rose. The analysis of the variance presented in (Table 3) shows that a statistically significant influence on root formation in our

Table 3. Analysis of variance for the effect of the studied factors on rooting of regenerated plants

Source of variation	df	SS	MS	F	Strength of influence, %
Genotype (G)	1	9.80	9.80[ns]	2.130	1.96
Medium (M)	4	371.20	92.80**	20.174	74.5
Interaction (GxM)	4	71.20	17.80*	3.870	14.29
Error	10	46.00	4.60		

** Significant at p<0.01, * Significant at p<0.05, ns – not significant

experiment has the growth regulators in medium/conditions for rooting and the interaction between growth regulators/conditions and the genotype. The influence of the genotype is statistically unproven. The variation of total number of plantlets with roots is mostly due to medium composition/conditions (74.5%), followed by the genotype medium interaction (14.29%). Our results are consistent with the studies by Kim et al. (2003), which also found that rooting depends largely on the nutrient medium and the growth regulators.

The results presented in Table 4 show that under *in vitro* conditions the rooting process (Figure 2d) proceeds best when IBA is added to the medium and the largest percentage of rooted plants is obtained – 85% (average of the two genotypes). The lowest is the percentage of rooted plants using NAA - 67.5%. *In vitro* rooting produces relatively good results on a medium without added growth regulators as well - 72.5% rooted plants. The largest percentage of successfully rooted plantlets was obtained by ex vitro direct rooting (Figure 2e) in a peat-soil mixture – 97.5% on average for both genotypes. All rooted plants are vital and have well-developed root system of the relevant length and mass.

Table 4. Effect of growth regulators and conditions on the rooting of *in vitro* regenerated plants

Rooting conditions/ growth regulators	Placed shoots, pcs.	Rooted explants, pcs.		Efficiency of rooting, %	
		R2-1	R2-2	R2-1	R2-2
in vitro					
1.MS- no hormones	40	26	32	65	80
2.MS+0.1mg/L IBA	40	32	36	80	90
3. MS+0.1mg/L IAA	40	28	31	70	77.5
4. MS+0.1 mg/LNAA	40	29	25	72.5	62.5
ex vitro					
5. Peat-soil mixture	40	40	38	100	95
Total for genotype	200	155	166	77.5	83

The two genotypes do not differ significantly in their rooting capability, but R2-2 genotype surpassing in a statistically unproven way genotype R2-1. In it 83% of all plantlets set in all media and under all conditions have been successfully rooted, whereas in R2-1 – 77. 5 %.

In the literature there is controversial information on the efficiency of various types and concentrations of auxins in rooting different species and genotypes of roses (Kirichenko et al., 1991; Chu et al., 1993; Kornova and Michailova, 1994; Pati et al., 2006). These contradictions can be explained by the genotypic differences in the levels of endogenous auxins and the differences in the residual accumulation of auxins from the previous explant cultivation. Our results for white oil-bearing rose indicate that when the rooting process takes place in vitro conditions, the best results are obtained when using 3-indole butyric acid (IBA) at low concentrations and are consistent with the studies by (Saffari et al., 2004; Ginova and Kondakova, 2013; Mitrofanova et al., 2016) for *R. damascenae* and Akhtar et al. (2015) for *R. centifolia*.

The relatively high percentage of rooted plants obtained in in vitro conditions without addition of growth regulators has been reported by other authors for other types of roses as well (Douglas et al., 1989). It may be due to the high endogenous content of auxins in the regenerated plants, and according to Mitrofanova et al. (2016) can be explained by the so-called "hormonal autonomy" of plant cells under in vitro conditions.

Our results show that both clones of white oil-bearing rose rooted excellently directly in a 1:1 peat-soil mixture (perlite:peat) without the need to place them in a root-formation medium in in vitro conditions. This allows shortening the multiplication period and saves significant resources, which is essential for the industrial production of propagation material.

Because of these obvious advantages, many rooting studies have been carried out so far in ex vitro conditions, which prove the high efficacy of this method in other types of roses, too (Pittet and Moncousin, 1982; Pati et al., 2006).

Conclusion

This study explored key stages related to the clonal in vitro propagation of white oil-bearing rose. Nodal segments 1-1.5 cm long were used as primary ng explants with one to two axillary buds from two pre-selected clones, distinguished by the best economic qualities. The most suitable sterilization scheme determined was the combination of two disinfectants in the following order: 0.2% $HgCl_2$ solution for 3 min followed by treatment with 0.5% NaClO solution for 20 min and 0.25% NaClO for 30 min. The variance analysis conducted shows that the genotype, variants of culture medium for multiplication and the interaction between them have statistically significant effect on the variation of the total number of induced adventive shoots. The largest number of explants responds by multiplication, the highest total number of adventive shoots and the highest number of adventive shoots per explant in both genotypes studied is obtained in medium variants 1, 2 and 3 containing just one growth regulator - BAP (benzylaminopurine) at a concentration of 0.5 to 3.0 mg/L. In variants 1 and 2 the largest number of explants multiplying more than 3 adventive shoots has been obtained. The root-formation proceeds best under ex vitro conditions in both clones. The largest percentage of successfully rooted plantlets (97.5% on average for both genotypes) were obtained by direct rooting in a peat-soil mixture. All rooted plants are vital and have well-developed root system of the relevant length and mass. This allows shortening the propagation period and saves significant resources, which is essential for the industrial production of propagation materials in white oil-bearing rose.

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The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods,Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

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Todorov N and Mitev J, 1995. Effect of level of feeding during dry period, and body condition score on reproductive performance in dairy cows,IXth International Conference on Production Diseases in Farm Animals, September 11–14, Berlin, Germany.

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