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NEW APPROACHES FOR *IN VITRO* PROPAGATION OF OIL-BEARING ROSE

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Abstract

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This research was undertaken to overcome two essential problems, which arise during the process of micropropagation of *Rosa damascena* **Mill**. **f**. *trigintipetala* Dieck. - *in vitro* rooting and top necrosis. These problems decrease application of *in vitro* technologies for mass production of oil – bearing rose plants.

The nodal segments from young, active growing shoots were cultured on MS medium with 0.5 mg l^{-1} BA for shoot induction. Then they are transferred on proliferation medium including QL, 0.5 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 0.1 g l^{-1} Fe-EDDHA. In this study was tested 3 media (MS, QL, WPM) with different combination of hormones for induction of roots. The combination of ½ QL macro salt, QL micro salt and vitamins, 20 g l^{-1} sucrose, 0.4 mg l^{-1} IBA, 0.4 mg l^{-1} IAA, 250 mg l^{-1} meso – inositol and 250 mg l^{-1} casein hydrolizate (liquid medium) was the most suitable treatment for rooting. The shoots, which developed roots, are 90%. It was used a temporary immersion bioreactor system RITA® for optimize the micropropagation system. It was very effective for overcome the problem with necrosis because factors, which provoke it, were eliminated.

In this article, we report for first time potential capacity of bioreactor system "RITA" for propagation of recalcitrant species as *Rosa damascena* Mill.

Key words: *Rosa damascena* Mill. f. *trigintipetala* Dieck., *in vitro* rooting, top necrosis, bioreactors

Abbreviations: MS - Murashige & Skoog Medium, QL- Quirin and Lepoivre Medium, BA - Benzyl Adenine, NAA - 1-Naphthaleneacetic acid, IAA - Indole-3-acetic acid, GA3 - Gibberellic acid, IBA - Indole-3-butyric acid, WPM – Woody Plant Medium, PVP –Polyvinylpyrrolidone, CH – Casein hydrolizate, AC – Active Charcoal

Introduction

Rosa damascena Mill. f. *trigintipetala* Dieck., named Kazanlak oil – bearing rose is the most important aromatic plant in Bulgaria of considerable economic importance to the country.

The Bulgarian rose oil is prized worldwide for its high quality and its application in parfume industry.

Traditionally, *Rosa damascena* is propagated vegetatively. However, the negative side of the application of this method is that it is very slow, time consuming and labor - intensive.The choice of reliable method for propagation plays a significant role in the processes of maintenance and preserving the oil-rose quality.

Recently, plant biotechnology methods have been rapidly developping. *In vitro* propagation of rose has played an important role in the rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants. So far, there are many reports for *in vitro* propagation of *Rosa* sp. (Ara et al., 1997; Bhat, 1992; Pati et al., 2006; Mirza et al., 2011; Canli and Kazaz, 2009; Salekjalali et al., 2011) but it is important to mention that *R. damascena* Mill. belong to group of recalcitrant species.

The micropropagation of oil-bearing rose is accompanied by many difficulties. The reason is that this rose species is very rich of polyphenols and after cutting the ends of explants, they exude dark colored compounds in medium (Iliev et al., 2010). In confined space of jars, plastic boxes or test tubes there is accumulation of ethylene and other gases due to the lack of controlled gas exchange. These factors can cause browning of tissues, dysfunction or death of primary

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meristem, which is known as apex necrosis that causes a significant reduction of propagation rate (Gaspar et al., 1995).

In order to optimise the tissue culture systems for propagation a temporary immersion bioreactor system RITA® was used. The advantages of this system are mechanical agitation and homogenation of medium; air supply with a certain concentration of O_2. The live cells release CO_2 which is led away the bioreactor along with the inert air and excessive oxygen.

Some researchers found that the immersion system RITA® increases multiplication rate of shoots in pineapple, tea, eucalyptus (Escalona et al., 1999; Akula et al., 2000; McAlister et al., 2005) and root development of rubber tree, eucalyptus (Etienne et al., 1997; McAlister et al., 2005). It is reported that plants produced through RITA® system have better acclimation rate. (Aitken-Christie et al., 1995) Other advantages are the reduced consumables and labor costs (McAlister et al., 2005) etc.

In this article we present an efficient protocol to overcome two essential problems in micro propagation process of *Rosa damascena* Mill. f. *trigintipetala* Dieck. – i.e. *in vitro* rooting and top necrosis.

Material and Methods

Plant material

The plant material was collected from The Institute of Roses, Essential and Medical Cultures – Kazanlak in spring from freshly sprouted to one year old, active growing, health branches. The nodal explants were chosen from the top and middle part of the branches. Before including *in vitro* conditions the branches are stored in a wet paper at 4°C. Twenty plants were raised for each experimental rooting medium.

The experiments were carried out in the laboratory of Agrobioinstitute, Sofia.

Culture establishment

Axillary buds of *Rosa damascena* Mill. f. *trigintipetala* Dieck. separated from young, active growing shoots of rose shrub were used for including under *in vitro* conditions. The explants were surface sterilized by dipping in 70% ethanol for 30 s, then incubated in 0.1% $HgCl_2$ with 3 drops of Tween 20 for 3 min, followed by rinse three times with sterile distilled water. Afterwards, nodal segments 1-1.5 cm long were placed on shoot induction medium - full strength Murashige and Skoog, 0.5 mg l^{-1} BA, 30 g sugar, 8 g l^{-1} agar. The pH was adjusted to 5.7-5.8 before the adding of agar. The culture media was autoclaved at 121°C. Cultures were incubated at 21-22°C under a 16/8 photoperiod at white fluorescent light having 2500 lux intensity. After 3 weeks new shoots were transferred to proliferation medium including Quoirin and Lepoivre medium, 30 g l^{-1} glucose, 0.5 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 0.1 g l^{-1} Fe- EDDHA, 8 g l^{-1} agar

Rooting and acclimatization

Very often, the *in vitro* rooting from micro-cuttings of *R. damascena* Mill. creates problems. In order to establish an efficient protocol for rooting of micro-cuttings, a profound testing of different media was performed as it is described in Table 1. For media we used MS (Murashige and Skoog, 1962), QL (Quoirin and Lepoivre, 1977), WPM (Mc Cown and Sellmer, 1982); plant growth regulators - 0.5–2.5 mg l^{-1} NAA, 0.4 mg l^{-1} IBA, 0.4–1 mg l^{-1} IAA, 1-2.5 mg l^{-1} 2.4 D; amino acids –100 mg l^{-1} L - Methionin, 100 mg l^{-1} L-Tyrosine; 250 mg l^{-1} casein hydrolisate; 500 mg l^{-1} active charcoal; with and without presence of gel agent.

After rooting stage the plantlets were transferred to seedling growing trays with turf soil: sand soil: perlite 2:1:1 in acclimatized room with controlled conditions 25°C and 16/8 h photoperiod. The top of the trays were covered with transparent plastic. This can provide a favorable environment for the plant's growth. After 15 days, the plants were discovered.

Bioreactors

At first shoots about 2 cm in length were transferred into each bioreactor (RITA) containing a working volume of 200 ml QL medium supplemented with 30 g l^{-1} glucose, 0.5 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 0.1 g l^{-1} Fe EDDHA at immersion frequency 15 min flooding, 8 and 12 h standby periods, at 22°C and 16/8 photoperiod. After 30 days, the plants were sub cultured on rooting medium consisting ½ QL macro salt, QL micro salt and vitamins, 20 g l^{-1} sucrose, 0.4 mg l^{-1} IBA, 0.4 – 1 mg l^{-1} IAA, 250 mg l^{-1} meso-inositol and 250 mg l^{-1} casein hydrolisate. After rooting, the plantlets were transferred to plastic pots with soil mixture of turf, sand and perlite in ratio 2:1:1 respectively.

Statistical Analysis

The experiment with testing of different rooting media had twenty replications for each treatment and there was one shoot per culture bottle. The experiment with bioreactors had five replications with twenty plants for each treatment. Percentage of shoots formed roots, the mean number of roots, the mean length of roots and percentage of acclimatized plants were recorded. Data were evaluated by ANOVA test.

Results and Discussion

In vitro proliferation and multiplication are largely based on medium formulations containing cytokinins and auxin (Kim et al., 2003). Vijaya et al. (1991) reported that BA was

Table 1
Testing of various hormonal balances in medium for rose root induction

Medium	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Macro salts	½MS	½QL	WPM	WPM	½MS	½MS	½QL	½QL	½MS	½MS	½QL	½QL	½MS	½QL	½QL	½QL	½QL	½QL	½QL	½QL	½QL
Micro salts	MS	QL	WPM	WPM	MS	MS	QL	QL	MS	MS	QL	QL	MS	QL	QL	QL	QL	QL	QL	QL	QL
Vitamins	MS	QL	WPM	WPM	MS	MS	QL	QL	MS	MS	QL	QL	MS	QL	QL	QL	QL	QL	QL	QL	QL
Meso-inositol mg l^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	250	250	250	250
Sucrose g l^{-1}	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
NAA mg l^{-1}	0.5	0.5	-	-	-	2.5	-	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-
IBA mg l^{-1}	-	-	0.4	0.4	-	-	-	-	0.4	0.4	0.4	0.4	-	-	0.4	0.4	0.4	0.4	0.4	0.4	0.4
IAA mg l^{-1}	-	-	0.4	0.4	-	-	-	-	0.4	0.4	0.4	0.4	1	1	0.4	0.4	0.4	0.4	0.4	0.4	0.4
2.4 D mg l^{-1}	-	-	-	-	2.5	-	2.5	-	-	-	-	-	1	1	-	-	-	-	-	-	-
PVP 10-1 µl l^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	100	-	50	100	-	-
L-Methionin mg l^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	-	-	100	-
L-Tyrosin mg l^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	-	-	100	-
Casein hydrolisate mg l^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	250	250	250	250
Agar g l^{-1}	-	-	8	-	-	8	-	8	-	8	-	8	8	8	-	-	-	-	-	-	-
Act. Charcoal mg l^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	500	500	-	-	-	-	-	-	-

the most effective growth regulator for stimulation of shoot proliferation. Three weeks after leading, the healthy plants were transferred on proliferation medium consisting full QL medium supplemented with 0.5 mg l^{-1} BA, 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 and 30 g l^{-1} glucose. Bhoomsiri and Masomboon (2003), chose QL medium in accordance to the best results for proliferation rate. Several reports concerning shoot multiplication indicate that glucose is better for shoot proliferation, while sucrose is better for rooting (Bhattacharjee, 2010). Many reports comment the effectiveness of 100 mg l^{-1} FeEDDHA added to medium for micropropagation consisting in the appearance of larger leaves and more chlorophyll content.

The influence of FeEDDHA was observed on *R. hybrida* L. where the rose shoots elongated with increasing FeEDDHA concentration in the medium and the chlorophyll content of shoots was higher than shoots cultivated on QL medium with FeEDTA (van der Salm et al., 1994). Rashid and Street (1973) described that FeEEDHA is more effective than ferric citrate. In our study, the shoots cultivated on this medium had from 1:3 to 1:4 proliferation rates. After some subculture periods, 2 cm long axillaries and adventitious shoots were transferred to different rooting media. It was observed that during this period a chlorosis followed by a necrosis of leaves and apex caused the essential problems. To solve the problem, different media and combinations of growth regulators were examined. In our experiments 3 basal media - MS, QL and WPM were used which are the most common media for rose micropropagation. The concentration of sugar and macro salt were reduced, fellow the report that improve the rooting in many woody and herbaceous species (Jabbarzadeh and Khosh Khui, 2005). Mirza et al. (2011) report the influence of reduced sucrose and organic salt concentration on root initiation for many plants. The auxin, 3-indole butyric acid (IBA) is used with great success for rooting in plant tissue culture (Saffari et al., 2011). During our experiment (Table 2) a high percentage of rooted plants were observed in media №4 (50%), №11 (60%), № 21 (90%). In these media, we observed best results for number of formed roots №4 (2.50) №11 (2.25) № 21 (2.61). The plants were cultivated in WPM (№4) and QL (№ 11 and № 21) medium with 0.4 mg l^{-1} IBA, 0.4 mg l^{-1} IAA, 20 g l^{-1} sucrose and without agar. Best root response was observed in the case of IBA and IAA auxins combination. This result is conformity with reported ones by Khatuni (2010) and Silva and Senarath (2009). Effective use of 2.4-D has also been reported for *in vitro* rooting of other plant species (Edwin and Paul, 1984). One week after the transfer on a proliferation medium, in some of the shoots (more or less 50%) was observed browning of the tissues. The necrosis started from the top of plants. These plants died before inducting of roots. Plants cultivated on QL medium had

Table 2
Effect of different basal media and plant growth hormones on rooting process

n	MS +0.5 NAA	QL +0.5 NAA	WPM+0.4 IBA +0.4 IAA +agar	WPM+0.4 IBA +0.4 IAA	MS+ 2.5 2.4D	MS+ 2.5 NAA+agar	QL+ 2.5 2.4D	QL+ 2.5 NAA+ agar	MS+0.4 IBA+ 0.4 IAA	QL+ 0.4 IBA+ 0.4 IAA
A	35	20	20	50	25	0	30	30	0	60
B	1.57±0.79	1 ±0.00	1.50±0.58	2.50±0.85	1.80±0.84	-	1.83±0.41	1.67±0.52	-	2.00±0.63	2.25±0.75
C	1.34±0.99	0.90±0.14	0.95±0.42	1.46±0.49	1.34±0.64	-	1.62±0.33	1.43±0.34	-	1.70±0.49	1.69±0.52

n	QL+ 0.4 IBA+ 0.4 IAA+ agar	MS+ INAA+ I 2.4D +agar+AC	QL+ INAA+ I 2.4D +agar+AC	QL+ 0.4 IBA+ 0.4 IAA+50 PVP	QL+0.4 IBA+ 0.4 IAA+ 100PVP	QL+ 0.4 IBA+ 0.4 IAA+ amino acids	QL+ 0.4 IBA+ 0.4 IAA+ 100 PVP+ CH	QL+0.4 IBA+ 0.4 IAA+ amino acids + CH	MS+ 0.4 IBA+ 0.4 IAA+ agar	QL+0.4 IBA+ 0.4 IAA+ CH
A	10	20	40	10	0	15	10	10	35	90
B	1.0±0	1.75±0.50	2.13±0.64	1.50±0.71	-	2.00±0.00	1.00±0.00	1.00±0.00	1.86±0.69	2.61±0.98
C	0.75±0.21	1.08±0.36	1.65±0.41	0.80±0.14	-	1.60±0.17	0.85±0.21	0.75±0.07	1.73±0.53	1.97±0.72

A – percentage of rooting plants (%); B – mean of numbers of roots; C – mean of length (cm) of roots.
The results were recorded after 4 weeks. Statistically significant at $P < 0.05$

the best rooting response. It can be explained with the composition of QL medium where the chlorine ions, to which genus Rosa is sensitive, are almost eliminated. In order to try to eliminate the necrosis we used PVP and active charcoal (medium № 13, 14, 15, 16, 18, 19). Usually the active charcoal is used to absorb phenolic compounds, toxic elements that evolve from plant. It is used with combination of auxins for stimulating the induction of roots of micropropagated shoots (Wilson and Nayar, 1995 and Thomas, 2008). The addition of PVP helps in oxidizing polyphenols leached into the medium, and promotes higher rate of organogenesis (Rout et al., 2006). However, the results in our study showed that the addition of active charcoal and PVP did not stop the development of necrosis. The root response was low (0-40%). Plants cultivated on medium № 16 died before an induction of roots.

The low percentage of rooting plants (0-30%) in other media can be explained with the large number of necrotic plants. In media with low percentage of developed roots, the mean number of roots is from 1 to 2 roots and 0.75 to 1.73 mean lengths (cm).

After rooting stage, 108 rooted plants are transferred to soil under indoor conditions. The survival percentage of the plants was 51%.

In order to overcome the problems of shoots' necrosis, we used temporary immersion system RITA® bioreactor. In 200 ml QL medium were placed 2 cm-long shoots for proliferation. It was chosen two modes of operation - immersion frequency 15 min flooding, 8 and 12 h stand-by periods, at 22°C and 16/8 photoperiod. After 4 week, the shoots are growing until 4-5 cm-long. The shoots cultivated at 12h stand - by period were more intensive green, at 8h stand-by period they are smaller and vitrified. The propagation rate was the same as in the solid medium (from 1:3 to 1:4) but the biomass is bigger and there was no necrosis of shoots. Axillaries and adventitious shoots (4-5 cm long) were separated individually and transferred to rooting medium. 100 shoots were placed on medium №21 (Table 1) at immersion frequency 15 min flooding, 12 h stand-by periods, at 22°C and 16/8 photoperiod. After 4 weeks, it was enumerate that there are 85 rooted plants with an average 3.07 roots and 2.65 lengths (cm) per shoot. The observation showed that leaves were green. We did not observe any chlorosis, necrosis or vitrification. (Figure 1) The rooted plants are transferred to soil under indoor conditions. The survival percentage of the plants was 82%.

The cultivation in liquid media using a temporary immersion system with different frequencies of immersion was described to increase plant quality and multiplication rates of banana, coffee, and rubber (Ziv, 1999). The advantages of temporary immersion bioreactors are due to fact that aerated liquid cultures in bioreactors provide a better contact between the plant biomass and the medium. Gas exchange has not restriction and its composition in both the medium and the gaseous atmosphere, can be strictly controlled. Another advantage is the ability to influence plant biomass in relation to the medium volume. The control of gas exchange prevents from accumulation of phenol and other toxic compounds. The contact between cultivated plants and medium influence on the development of biomass, such plants are more adaptive in ex – vitro conditions and more successfully overcame the acclimatization.

Conclusion

In this study an efficient protocol was developed in order to overcome two essential problems in micropropagation process of *Rosa damascena* Mill. f. *trigintipetala* Dieck. – i.e. *in vitro* rooting and top necrosis. The liquid media had better rooting response than solid media and shoots cultivated on ½ QL macro salt, QL micro salt and vitamins, 20 g l^{-1} sucrose, 0.4 mg l^{-1} IBA, 0.4 mg l^{-1} IAA, 250 mg l^{-1} meso-inositol and 250 mg l^{-1} casein hydrolisate gave the best results.

The use of bioreactors improves the efficiency of in *vitro* propagation and rooting of *R. damascena* Mill. plants. The

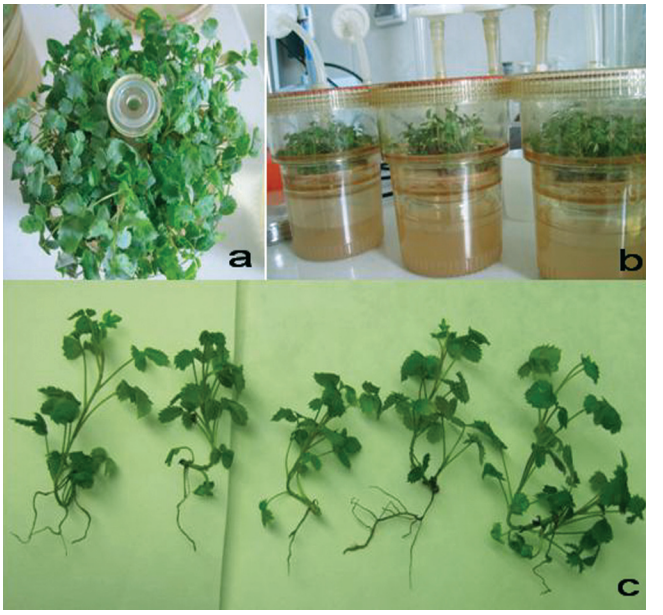

Fig. 1. *In vitro* propagation of *Rosa damascena* Mill. in bioreactor:
a) multiple shoots developed on QL medium, 30 g l^{-1} glucose, 0.5 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 0.1 g l^{-1} Fe-EDDHA;
b) RITA bioreactors;
c) rooting of *in vitro* shoots on ½ QL macro salt, QL micro salt and vitamins, 20 g l^{-1} sucrose, 0.4 mg l^{-1} IBA, 0.4 mg l^{-1} IAA, 250 mg l^{-1} meso – inositol and 250 mg l^{-1} casein hydrolisate.

most important factors influencing the efficiency of the system for propagation by bioreactors are the immersion time, homogenation of medium and air exchanged. They all allow overcoming the top necrosis.

This protocol could be used for the commercial *in vitro* propagation of *Rosa damascena* Mill. f. *trigintipetala* Dieck..

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