**NEW APPROACHES FOR *IN VITRO* PROPAGATION OF *ROSA DAMASCENA* MILL.**

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**Abstract**

This research was undertaken to overcome two essential problems which arise during the process of micropropagation of *Rosa damascena* Mill. f. *Trigintipetala* - *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 GA3, 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 hydrolisate (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 optimise 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 damascenа* Mill.

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

**Introduction**

*Rosa damascena* Mill. f. *Trigintipetala*, 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 developing. *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 KA et al, 1997; Bhat MS, 1992; Pati, P.K. et al, 2006; Mirza, M. Q. B., 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 meristem, which is known as apex necrosis that causes a significant reduction of propagation rate. (Gaspar,T 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 O2. The live cells release CO2 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* – 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 – Kasanlak 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 *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 laboratory of Agrobioinstitute, Sofia.

***Culture establishment***

Axillary buds of *Rosa damascena* Mill. f. *Trigintipetal*a 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% HgCl2 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 GA3, 0.1 g l-1 Fe- EDDHA, 8 g l-1 agar

***Rooting and acclimatization***

The *in vitro* rooting of *R. damascena* Mill. micro-cuttings very often has 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 I. 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 the plantlets were transferred to plastic pots with turf soil : sand soil : perlite 2:1:1.

***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 GA3, 0.1 g l-1 Fe- EDDHA at immersion frequency 15 min flooding, 8 and 12 h stand-by 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.

**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 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 GA3 and 30 g l-1 glucose. QL medium was chosen in accordance to the best results for proliferation rate by Bhoomsiri and Masomboon, 2003. Several reports concerning shoot multiplication indicate that glucose is better for shoot proliferation, while sucrose is better for rooting (Bhattacharjee, S. K.**,** 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, 2cm long shoots were transferred to different rooting media. It was observed that during this period 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 (Fig 1). In our experiments were used 3 basal media - MS, QL and WPM which are the most common media used for rose micropopagation. The concentration of sugar and macro salt were reduced, fellow the reportthat improve the rooting in many woody and herbaceous species (Jabbarzadeh and Khosh khui, 2005). The influence of reduced sucrose and organic salt concentration on root initiation for many plants is reported by Mirza et al., 2011. 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 a highpercentage of rooted plants were observed in mediums №4 (50%), №11 (60%), № 21 (90%). The plants were cultivated in WPM (№4) and QL (№ 11 and № 21) medium with 0.4 mg/l IBA, 0.4 IAA, 20g/l 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 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 D, Nayar NK., 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 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 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 till 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. Shoot (4-5 cm long) was separated individually and transferred to rooting medium. 100 shoots were placed on medium №21(TableI) at immersion frequency 15 min flooding, 12 h stand-by periods, at 22 °C and 16/8 photoperiod. After 45 days it was enumerate that there are 85 rooted plants with 1 to 5 roots per shoot. The observation showed that leaves are green. We did not observe any chlorosis, nerosis or vitrification. (Fig 2)

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 was developed an efficient protocol to overcome two essential problems in micropropagation process of *Rosa damascena* Mill. f.*Trigintipetala* – i.e. *in vitro* rooting and top necrosis. The liquid media had better rooting response than solid media. Shoot 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 best results.

The use of bioreactors improves the efficiency of *in vitro* propagation and rooting of *R. damascena* Mill. plants. The most important factors influence on development system for propagation by bioreactor are the immersion time, homogenation of medium, 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*

**Acknowledgement**

The authors gratefully acknowledge for the cooperation and assistance of Dr. I. Ivanov from Department of Applied Microbiology, Laboratory of Applied Biotechnologies (Plovdiv), The Stephan Angeloff Institute of Microbiology, BAS and Assoc. Prof. N. Kovacheva from Institute of Roses, Essential and Medical Cultures for general helping.

**References**

Aitken-Christie J., Kozai T., Takayama S. (1995). Automation in plant tissue culture. General introduction and overview In: Aitken-Christie J, Kozai T & Smith MAL (eds) Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, The Netherland: 1–15

Akula A., Becker D., Bateson M. (2000). High-yielding repetitive somatic embryogenesis and plant recovery in a selected tea clone, ‘TRI-2025’, by temporary immersion. Plant Cell Rep, 19: 1140–1145.

Ara K.A., Hossain M.M., Quasim M.A., Ali M., Ahmed J.U. (1997). Micropropagation of rose (Rosa sp cv). Plant Tissue Cult, 7: 135– 42.

Bhat M.S. (1992). Micropropagation in rose. Indian Hortic, 37: 17– 9.

Bhattacharjee S. K. (2010). The complete book of roses, Jaipur (Raj.) India, 531pp.

Bhoomsiri Ch., Masomboon N. (2003). Multiple shoot induction and plant regeneration of *Rosa damascena* Mill. Silpakorn University International Journal, 3 (1-2): 229-239.

Canli F.A., Kazaz S. (2009). Biotechnology of roses: progress and future prospects. Süleyman Demirel Üniversitesi Orman Fakültesi Dergisi: 167-183.

De Silva M.A.N., Senarath W.T.P.S.K. (2009). Development of a Successful Protocol for in vitro Mass Propagation of *Celastrus paniculatus* Willd. A Valuable Medicinal Plant. Tropical Agricultural Research, 21(1): 21-29.

Edwin F.G., Paul D.Sh. (1984). Plant Propagation by Tissue Culture Handbook and Directory of Comerical Laboratories. Exegetics Ltd., Eversley, Basingstoke, Hants, UK, 709 pp.

Escalona M., Lorenzo J.C., Gonza´ lez B., Daquinta M., Gonza´ lez J.L., Desjardins Y., Borroto C.G. (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Rep, 18: 743–748.

Etienne H., Lartaud M., Michaux-Ferrie`re N., Carron M.P., Berthouly M., Teisson C. (1997). Improvement of somatic embryogenesis in *Hevea brasiliensis* (Müll. Arg) using the temporary immersion techniques. In Vitro Cell. Biol. 33: 81–87.

Gaspar T., Kevers C., Franck T., Bisbis B., Pillard J.P., Huault C., LeDily F., Petit-Paly G., Rindeau M., Penel C., Crèvecoeur M., Greppin H.(1995): Paradoxical results in the analysis of hyperhydric tissues considered as being under stress: questions for a debate. Bulgarian Journal of Plant Physiology, 21:80–9

Iliev I., Gajdoŝová A., Libiaková G., Mohan Jain S. (2010). Plant Micropropagation, In M.R.. Davey and P. Anthony (Editors), Plant Cell Culture: Essential Methods: 1-24.

Jabbarzadeh Z., Khosh-Khui M. (2005). Factors affecting tissue culture of Damask rose (*Rosa damascena* Mill.) Scientia Horticulturae, 105: 475-482.

Khatun M. M., Khatun H., Khanam D., Al-Amin M.D. (2010) In vitro root formation and plantlet development in Dendrobium Orchid. Bangladesh J. Agril. Res, 35(2) : 257-265.

Kim C.J.U., Jee S.O., Chung J.D. (2003). *In vitro* micropropagation of *Rosa hybrida* L. J. Plant Biotechnol., 5: 115-119.

McAlister B., Finnie J., Watt M.P., Blake way F.C. (2005). Use of temporary immersion bioreactor system (RITA) for the production of commercial *Eucalyptus* clones at Mondi Forests (SA). Plant Cell. Tissue. Organ Cult, 81: 347-358.

McCown B.H., Sellmer J.C. (1987). General media and vessels suitable for woody plant cultures. In: Bonga, J.M., Durzan, D.J. (Eds.), Tissue culture in forestry - General principles and biotechnology, Vol. 2. Martinus Nijhoff Publ., Dordrecht, Boston:. 4-6.

Mirza M. Q. B., Ishfaq A. H., Azhar H., Touqeer A., Nadeem A. A. (2011). An efficient rotocol for in vitro propagation of *Rosa gruss an teplitz* and *Rosa centifolia*. Afri. J. Biotechnol, 10 (22): 4564-4573.

Morteza S., Jafari B., Tarinejad A. (2011). *In vitro* Multiplication of Rose (*Rosa hybrida* cv. Baccara). American-Eurasian J. Agric. & Environ. Sci, 11(1): 111-116.

Murashige T., Skoog F. (1962). Revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum, 15: 473-479.

Pati P. K, Rath S. P., Sharma M., Sood A., Ahuja P. S. (2006). In vitro propagation of rose: A review. Biotechnol. Adv., 24: 94-114.

Quoirin M., Lepoivre P. (1977). Improved media for in vitro culture of *Prunus* species. Acta Hort., 78: 437-442.

Rashid A., Street H.F. (1973). The development of haploid embryoids from anther cultures of *Atropa belladonna* L. Planta, 113:263–270

Rout G. R., Mohapatra A., Mohan Jain S. (2006). Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. Biotechnology Advances, 24: 531–560.

Saffari V. R., Khalighi A., Lesani H., Bablar M., Obermaier J. F. ( 2004). Effects of different plant growth regulators and time of pruning on yield components of *Rosa damascena* Mill. Int. J. Agric. Biol., 6 (6): 1040-1042.

Thomas T. D.( 2008). The role of activated charcoal in plant tissue culture.Biotechnology Advances, 26(6): 618-631.

Van der Salm TP.M., Van del' Toorn C.J.G., Hanisch ten Cate C.B., Dubois LA.M., De Vries D.P., Dons H.J.M.(1994). Importance of the Iron Chelate for Micropropagation of *Rosa hybrida L.* 'Moneyway'. Plant Cell, Tissue Organ Culture, 37: 73-77.

Vijaya N., Satyanarayana G., Prakash J., Pierik R.L.M. (1991). Effect of culture media and Growth Regulators on in vitro Propagation of rose. Curr. Plant Sci. Biotechnol. Agric., 12: 209-214.

Wilson D., Nayar N. K. (1995). Effect of activated charcoal on in vitro rooting of cultured rose shoots. South Indian Horticulture, 43(1-2): 32-34

Ziv M. (2000). Bioreactor technology for plant micropropagation. Horticultural Review, 24: 1–30.

**Table I. 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 | ½ MS | ½ QL | WPM | WPM | ½ MS | ½ MS | ½ QL | ½ QL | ½ MS | ½ MS | ½ QL | ½ QL | ½ MS | ½ QL | ½ QL | ½ QL | ½ QL | ½ QL | ½ QL | ½ QL | ½ QL |
| Micro | 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 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 250 | 250 | 250 | 250 |
| Sucrose g/l | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| NAA mg/l | 0.5 | 0.5 | - | - | - | 2.5 | - | 2.5 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| IBA mg/l | - | - | 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 | - | - | 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 | - | - | - | - | 2.5 | - | 2.5 | - | - | - | - | - | 1 | 1 | - | - | - | - | - | - | - |
| PVP 10-1 µl/l | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 50 | 100 | - | 50 | 100 | - | - |
| Methionin mg/l | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 100 | - | - | 100 | - |
| L-Tyrosin mg/l | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 100 | - | - | 100 | - |
| Casein hydrolisate mg/l | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 250 | 250 | 250 | 250 |
| Agar g/l | - | - | 8 | - | - | 8 | - | 8 | - | 8 | - | 8 | 8 | 8 | - | - | - | - | - | - | - |
| Act. Charcоal mg/l | - | - | - | - | - | - | - | - | - | - | **-** | - | 500 | 500 | - | - | - | - | - | - | - |

**Fig1. Effect of different basal media and plant growth hormones on rooting proces**



**c**

**b**

**a**

**Fig. 2 *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 GA3, 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.**