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ROSA DAMASCENA MILL. - AN OVERVIEW FOR EVALUATION OF PROPAGATION METHODS

A. GINOVA*, I. TSVETKOV and V. KONDAKOVA
AgroBioInstitute, 8, Dragan Tsankov blvd., BG - 1164 Sofia, Bulgaria

Abstract

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Rosa damascena Mill. f.*Trigintipetala*, named Kazanlak oil – bearing rose is an emblematic plant species for Bulgaria with important economical sense. Its importance is determined by the extremely high quality of rose oil, which is obtained after distillation of rose flowers. The methods of propagation play a general role in maintenance and improving the quality of oil rose. Traditionally, the oil-bearing rose is vegetative propagated. In this review are considered the advantages and disadvantages of classical methods of propagation (by grafting, layering, cutting) and *in vitro* methods for propagation. Special attention is directed to the problems that accompany the use of these methods. The layering and grafting are not preferred for use, because their more time consuming and low multiplication rate. The cutting is the preferable way for oil rose propagation in Bulgaria. This method has high generative rate, but obtained plants are not always healthy. *In vitro* techniques are used for rapid multiplication with high rate, production of healthy and disease-free plants, but they are costly.

Key words: *Rosa damscena* Mill., classical methods of propagation, *in vitro* micro propagation, bioreactors
Abbreviations: MS - Murashige & Skoog Medium, QL- Quirin and Lepoivre Medium, BAP - 6-Benzylaminopurine, BA - Benzyl Adenine, NAA - 1-Naphthaleneacetic acid, IAA - Indole-3-acetic acid, GA_3 - Gibberellic acid, IBA - Indole-3-butyric acid, PGRs - plant growth regulators, Kin - Kinetin, TDZ - Thidiazuron, WPM – Woody Plant Medium, PVP –Polyvinylpyrrolidone, PVPP -Polyvinylpolypyrrolidone

Introduction

Rosa damascena Mill. is a beautiful aromatic flower with immense horticultural importance, signification from economical and research point of view. The *Rosa* genus belongs to the family *Rosaceae* and has over 130 species (Cairns, 2001).

Roses are grouped into classifications based on the botanical characteristics: hybrid teas, grand floras, polyanthas, floribundas, miniatures, climbing, shrub, old roses, but only a few species are scented (Gudin, 2000). These include *Rosa damascena* Mill., *R. gallica* Linn., *R. centifolia* Linn., *R. bourboniana* Desportes., *R. chinensis* Jacq., *R. moschata* Herrm. and *R. alba* Linn.

The initially rose species used for oil production are: *Rosa damascena* Mill, *Rosa gallica* Linn., *Rosa centifolia* Linn. and *Rosa moschata* Herm. (Tucker and Maciarello, 1988). Hurst (1941) classified the today Damask roses into two types according to their flowering habit: summer Damask that blooms only in early summer, and autumn Damask that blooms during the autumn. Traditionally, highly scented summer Damask roses are cultivated in commercial rose gardens. There are several main prospects for production of Damask rose (*R.. damascena*) as rose water, attar of rose and other essential oils in the perfume industry (Lavid et al., 2002). It has application in pharmacology such as anti-HIV, antibacterial, antioxidant, hypnotic, antidi-

E- mail: a.ginova@gmail.com

abetic activities, and relaxant effect on acheal chains. (Boskabady et al., 2011). Rose petals are used for preparation of jams, jellies, conserves, liqueurs. Distillation waste can be used for livestock feed and composting. In other countries, Damask rose is also used as an ornamental plant in parks and gardens.

The world main producers of rose oil are Bulgaria, Turkey and Iran and to a less extent France, India, China and Northern Africa. As *Rosa damascena* was originally introduced from the Middle East into Western Europe. In Bulgaria, the cultivation of oil – bearing rose was initiated in the 16th century after the expansion of the Ottoman Empire (Topalov, 1978). Kashan, Shiraz, Fars, Meshed and Azerbaijan are the major cultivation areas in Iran; Isparta, Burdur, Denizli, Afyonkarahisar in Turkey and the Kazanlak valley in Bulgaria. The soil and climatic properties of the Kazanlak region such as temperature, humidity, cloudiness, and precipitation in the flowering season - May and June have important contribution in obtaining rose oils with high quality in accordance with the world standards, so, the planting of rose was sustained there. During the 19th century the industrial cultivation of *R. damascena* (called also 'Kazanlashka roza': the rose of town of Kazanlak, Bulgaria) spreads throughout the completely Sub-Balkan valley from the town of Sliven to the town of Klisura. Oil rose plantations were also established on the south slopes of the mountain Sredna Gora – Strelcha, Zelenikovo and the north slopes of the Rhodopa Mountain – Bratsigovo, Peshtera. The maximum planted area has been reached in 1917 (8951 ha) and after that it gradually decreased. (Kovatcheva et al., 2010). These days the spreading of oil – bearing rose is expanded further to the west of Klisura to Mirkovo village (Sofia regions).

In Turkey, Isparta province (latitude 37^{o}45' N, longitude 30^{o}33'E, altitude 997 m) is accepted as the valley of the oil rose. The climatic conditions of Isparta proved to be favorable for the cultivation of the rose. Eighty percent of oil rose production of Turkey is from Isparta and 20% both from Burdur, Afyonkarahisar and Denizli districts. Cuttings, by budding and grafting which is a difficult and long process with unsatisfactory multiplication rate, usually do the commercial propagation of roses. Some rose genotypes have problems with rooting. Bhattacharjee, 2010 claimed that there is a correlation between plant propagation, soil and climate conditions.

In this context, we define *Rosa damascena* as recalcitrant species and looking for alternative methods for propagation with high multiplication rate and ensuring the genetic stability.

The aim of this review is to do a comparative evaluation of all methods for propagation and to determine their positive and negative sides.

Classical methods for propagation

Traditionally, *Rosa damascena* have been propagated vegetative; since the time of Theophrastus, one has been advised against growing roses from seeds, cuttings, grafting, layering or budding (Widrlechner, 1981). Several methods for propagation are used. These methods are very slowly and time-consuming and usually associated with various problems such as limitation of stock plant and prolonged production time (Skirvin et al., 1990). Some methods are suitable for some roses and not for others.

In the past in Bulgaria have used layering and grafting as a means of propagation but they were not effective enough. In nowadays only way used for the propagation of oil rose is through green cuttings.

Initially for the vegetative propagation methods head been used as "kesme" (Topalov et al., 1967; Topalov, 1978) and including branches from old plantation in trenches 40 cm deep and 35 cm wide, which after a certain time to develop shoots (aboveground organs), is launching a new rose bushes. In this method, except branches from rose bushes are used and completely uprooted trees, which are laid horizontally at the base of the trenches. Ordered rose bushes are covered with 10cm soil and 5-6 cm animal manure. During the springtime from the buds of the laid bushes are growing shoots which develop an own root system. The propagation rate is 1:8 to 1:10 (Figure 1).

The other method known as "Chinese layer" was developed from V. Topalov (1978). In this case, the roses from the row are bending and place in pre-digged trench in row spacing, close to the row. They are covered with soil and animal manure. In the autumn roses are removed, separated by native bush and planted in a

permanent place. The propagation rate reaches 1:10 to 1:15 (Figure 2).

In order to accelerate reproduction and increase the breeding rate of mother crops in the 70s year of the last century has been established suitable rootstock for the production of grafted material from oil-bearing rose.

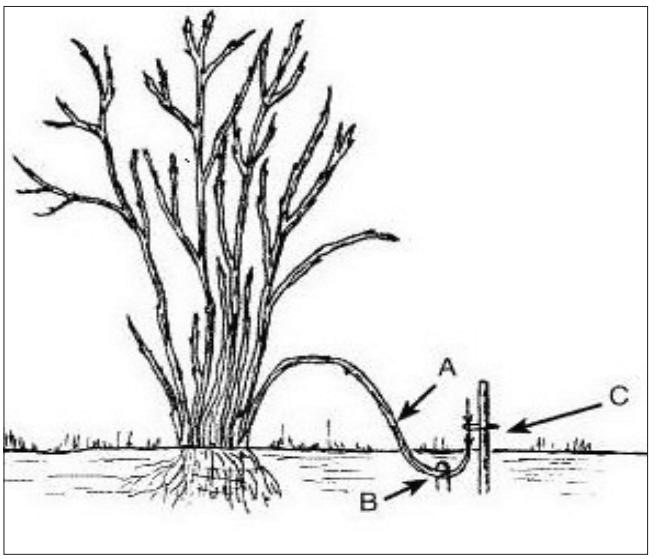

Fig. 1. Vegetative propagation by layers:
A. Pull branch down for simple layer;
B. Make wound or cut at bend;
C. Stake tip to hold upright
(http://extension.missouri.edu/p/G6970)

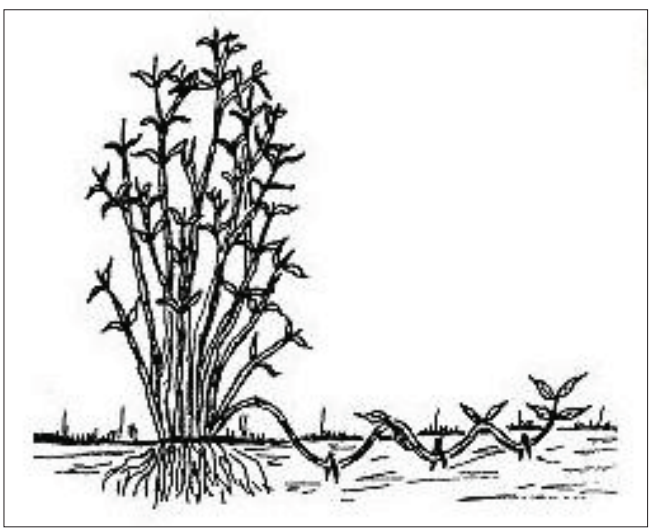

Fig. 2. Vegetative propagation by "chinese layers"
(http://extension.missouri.edu/p/G6970)

It was found that the most suitable rootstock is *Rosa canina* Brogs, which has good affinity and compatibility with *Rosa damascena* Mill. f. *trigintipetala* (Zlatev et al., 2001).

Since 1986 in Bulgarian Institute of Roses, Essential and Medical Cultures, Kazanlak, was implemented new technology for production of seedlings by rooting of green cuttings in cultivation facilities (Zlatev et al., 2001). By this method, parts of healthy leaved shoots placed under favorable conditions to form roots and above ground organs. In this method, each rooted cutting is developing new plant. The best root cuttings are obtained from strongly and moderately growing shoots. This method has high generative rate. It is the only way of propagation in Bulgaria, yet.

Layers and cuttings realize the propagation of rose in Turkey. Rose twigs or cuttings from old gardens cut at the soil level, they are placed into ditches horizontally, and their ends are overlapping in the trenches, which are then covered with soil containing manure. The cuttings necessary for planting are procured from old rose fields, not younger than six years. It is less labour consuming than other methods in which seedlings are used (Baydar H, 2006).

Primarily cuttings and suckers achieve damask rose propagation, but micropropagation is a developing method in Iran (Nikbakht et al., 2004). The cuttings have not to be younger than six years. Cuttings were taken from the base of rose shrubs and carefully inspected. Dry and diseased parts were removed. An older method for procuring cuttings was to uproot the entire rose shrub and use the roots as well. Later, this method was abandoned as inefficient, because the old rose garden died, the roots were not reliable planting material, and the procedure was too labour intensive. The woody 30-100 cm long cuttings were placed into ditches horizontally and their ends are overlapping (Haghighi et al., 2008).

The successful application of classical vegetative propagation methods requires knowledge of the strengths and weaknesses of all above description methods. Using of this methods will be well to know that they require more time, they are labor intensive and more of the case performance is not satisfactory. Propagation by layers requires rather large area for

a lay-bed, and weed control among the layers is a problem. The advantages of using green cuttings for propagation is greater efficiency (1:50 to 1:60) and material is genetically identical (Kovatcheva et al., 2009). The weakness of this method is the root system, which is not always well developed. Another negative side is the possibilities for transmission of bacterial and viral diseases that can cause a problem in future plantings.

In vitro micro propagation

In vitro methods have also been employed for achieving faster rates of multiplication, (Khosh-Khui and Sink, 1982; Ishioka and Tanimoto, 1990; Kornova and Michailova, 1994). The tissue culture method is a widely applicable for study, selection and propagation of plants. The potential of the cells to grow and develop into multicellular organisms is known as cell totipotency (Wetzstein and He, 2000). It can be employed for large-scale propagation of disease free clones and gene pool conservation.

Clonal stability of the micropropagated plants is essential for in vitro germplasm conservation. Skirvin and Janick (1976) were among the first who directed attention to the fact that clonal variation in genotypes is very important for improvement in horticultural species. Subsequently, Thorpe and Harry (1997) evidence that *in vitro* culture techniques have played on important role in the breeding programme, production and improvement of plant quality. Various types of changes were reported in cell cultures at phenotypes, karyotypic, physiological, biochemical and molecular level. Larkin and Scowcroft (1981) reviewed extensively and reported the phenotypic variation among plants regenerated after a passage through tissue and cell culture.

Recently, the propagation of *R. Damascena* by *in vitro* techniques is used mainly in Iran and India. According to Debergh and Read (1991) and Altman (2000), the micropropagation process can be divided in five different stages: selection of the mother plant, initiation of culture, multiplication, rooting and adaptation. Often, clear relations of the physiological status of the original tissue, and the reactions of explants taken thereof can be observed. In addition, the reaction of explants correlates with the growth conditions of the mother plant used to obtain explants for cultivation. Plants used for apply of tissue culture methods must be healthy, genetic identity and actively growing.

The correct choice of explants material can have an important effect on the success of tissue culture. For micropropagation of rose, the most commonly used explants is a nodal stem segment with size - 9.0–10.0-mm long; 3.0–4.0-mm diameter (Figure 3).

First report for efficient and rapid propagation of *Rosa damascena* Mill., using nodal explants from natural grown plants was reported by Bhoomsiri and Masomboon (2003). After sterilization, nodal explants were transferred on MS (Murashige and Skoog, 1962) and QL (Quirin and Lepoivre, 1977) medium with different concentration of BA and NAA. The highest number of shoot per explant was obtained on QL medium supplemented with 4.0 $mg.l^{-1}$ BA and 0.1 $mg.l^{-1}$ NAA (8.27 shoots per explants).

In Bulgaria, first report for using of apical and adventitious buds was described by Kornova et al. (2001). It was tested 11 different media on the base of MS with

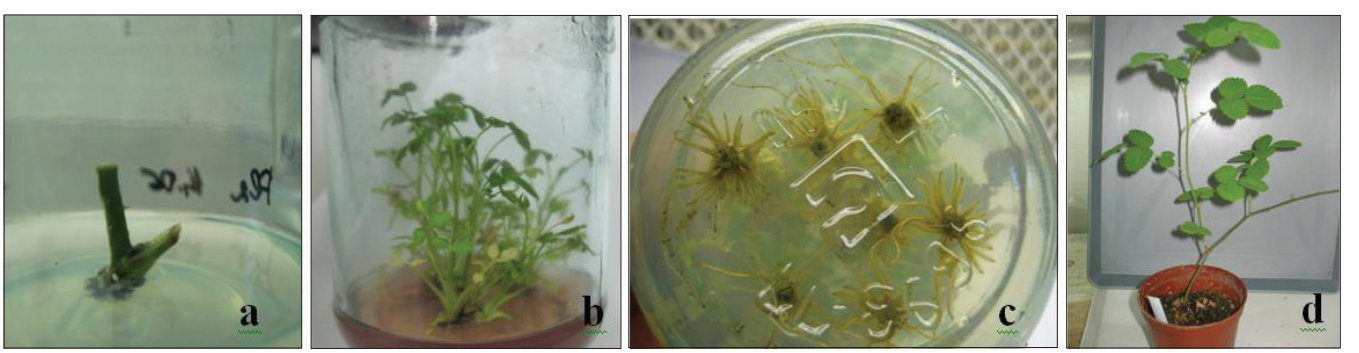

Fig. 3. *In vitro* propagation of *R. Damascena*
(a. induction of plant; b. multiplication; c. rooting; d. adaptation)

various concentration of BAP (0.5-1.5 $mg.l^{-1}$) and participation of auxins NAA and IAA (0.1 $mg.l^{-1}$). The most appropriate medium for micropropagation was MS containing BAP 0.5 – 1 $mg.l^{-1}$ with or not 0.1 $mg.l^{-1}$ IAA with optimal rate of multiplication 2.2 -2.6.

A liquid culture system using nodal segments was used for shoot proliferation and root induction in *Rosa damasena* and *Rosa burboniana* (Pati et al., 2005). For efficient and large-scale induction of roots in micro shoots, a rooting vessel was designed and developed to facilitate the micro propagation protocol. Their work highlights the significance of osmotic potential in relation to enhanced growth and development in liquid cultures, vis-à-vis agar-gelled cultivars, especially in relation to root induction during micro propagation.

In research of Nikbakht et al. (2005) was investigated the regeneration of two Iranian cultivars of Damask rose (*Rosa damascena* Mill.), "Azaran" and "Ghamsar" under *in vitro* conditions. The shoot single node segments included lateral buds were taken from bushes. Results of the study showed that among 12 different media, a liquid modified MS medium (with eliminated Cl- and reduced NH4 + ions) caused the best growth of newly proliferated shoots and no aging occurred. The choice of suitable medium with optimal multiplication effect were made between tested 32 different combination including growth regulators in different concentration- BA (0, 1, 2 and 3 $mg.l^{-1}$), GA_3 (0, 0.1, 0.25 and 0.5 $mg.l^{-1}$) and NAA (0 and 1 $mg.l^{-1}$).

Best results as proliferation, multiplication rate, appearance and leaf color were registred on medium with BA (1-2 $mg.l^{-1}$), GA_3 (0.1 $mg.l^{-1}$) and NAA (0-0.1 $mg.l^{-1}$) for cv."Azaran" and with the same concentrations of BA and GA_3, but without NAA for cv."Ghamsar". In the same time, Jabbarzadeh and Khosh-Khui (2005) reported that the combination of BA at concentrations of 2.5–3 $mg.l^{-1}$ with a low rate of IBA was the most suitable treatment for *in vitro* multiplication of Damask rose. (3.75-4.00 shoot number per single-node explants). The explants tolerated a higher concentration of plant growth regulators (PGRs) without showing any abnormality in morphology. Shoot orientation did not influence on shoot multiplication rate. Mamaghani et al. (2010) announced the effects of culture medium and combination of various plant growth regulators on shoot proliferation of three elite Iranian *R. damascena* Mill accessions - M6 (Kashan), G1 (East Azerbyjan) and G2 (West Azerbyjan). First, the effect of culture media, MS and WPM (Lloyd and McCown, 1980) containing 1.5, 2.5, 5 $mg.l^{-1}$ BAP on shoot multiplication (shoot number per explants) and shoot length were studied. Then, the effect of BAP (5 $mg.l^{-1}$) combination with 0.1 and 0.1 $mg.l^{-1}$ as well as IAA (0.1 and 0.5 $mg.l^{-1}$) and IBA (0.1 and 0.5 $mg.l^{-1}$) was studied. Finally, three levels of BAP (2, 2.5 and 5 $mg.l^{-1}$) and Kin (2, 2.5 and 5 $mg.l^{-1}$) alone and their combination by adding TDZ (0.05 $mg.l^{-1}$) and IBA (0.1 $mg.l^{-1}$) on shoot proliferation and shoot length in three accession were examined. The explants had higher shoot multiplication, shoot length and better green leaves on MS than WPM medium. The highest shoot proliferation (5.9) was obtained at a combination of 5 $mg.l^{-1}$ BAP and 0.1 $mg.l^{-1}$ TDZ. Maximum shoot length was observed in the medium with 0.5 $mg.l^{-1}$ IAA and 5 $mg.l^{-1}$ BAP and 0.01 $mg.l^{-1}$ TDZ. Shoot multiplication and shoot length were varied with genotypes and BAP as well as Kin concentration. M6 accession needed 2 $mg.l^{-1}$ BAP and 2$mg.l^{-1}$ Kin for maximum shoot multiplication and shoot length, G1 and G2 needed 2.5 $mg.l^{-1}$ BAP and 2.5 $mg.l^{-1}$ Kin. Anther culture of *Rosa* has been successfully done (Tulaeezadch and Khosh-khui, 1981). The MS media with 2.0 $g.1^{-1}$ IAA and 0.4 $mg.l^{-1}$ kinetin was the best for anther culture of *R .damascena.*

Some explants placed on culture medium exude dark colored compounds into the culture medium (phenols, pigments) that are released from the cut ends of the explants. This can cause browning of tissue and the medium, which is often connected with poor culture establishment and reduced regeneration ability. The basal media enriched with, such as activated charcoal (1-2%), various antioxidants - ascorbic acid or citric acid (50 - 100 $mg.l^{-1}$), or polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP), or the ethylene inhibitor, silver nitrate are often employed to nullify this effect. Frequent subculture, incubation in shaking liquid culture, reduced culture temperature or the use of etiolated explants, are also methods that have been used to deal with this problem (Iliev et al, 2010). The addition of PVP helps in oxidizing polyphenols leached in the medium, and promotes high rate of organogen-

esis (Rout et al., 2006). In order to decrease leaves and shoot tip necrosis additional vitamin complex, containing biotin (1 mg.l^{-1}), calcium pantothenate (0.5 mg.l^{-1}), riboflavine (0.5 mg.l^{-1}), and folic acid (0.5 mg.l^{-1}) were used (Mamaghani et al., 2010).

Roberts and Schum (2003) indicated some type of roses require more calcium salts as calcium deficiency may lead to shoot tip necrosis, hyperhydration of stem and leaf tissues. They showed that adding calcium in the multiplication medium significantly increased shoot multiplication rates and stem length. Application of calcium pantothenate complex may enhance shoot quality and root regeneration. Calcium regulated cellular process such as cell division and elongation (Ghorbanli and Babalar, 2003). Arnold et al. (1995) have suggested that calcium and potassium have a role in auxin absorption.

Vitrification is physiological disorder, which can be a serious problem in plant micropropagation. Vitrified microplants lose their ability to propagate and/or present difficulties of ex vitro acclimatization. As cause of vitrification has been suggested high concentration of cytokinins, high concentration of potassium and ammonium, calcium deficiency (Mohamed –Yassen et al., 1992).

The main problem in Damask rose *in vitro* propagation, however, is the rooting step. The auxin, 3-indole butyric acid (IBA), is usually used with great success for rooting in plant tissue culture, but the other common auxins such as 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) have been used for root induction with less percentage of success (Saffari et al., 2004). Rooting is improved in many woody and herbaceous species by lowering the concentration of sucrose from 2 or 3 to 0.5 to 1% in rooting medium (Jabbarzadeh and Khosh khui, 2005). The effect of reduced sucrose and organic salt concentration may be important for various plants root initiation (Mirza et al., 2011). Another problem which previous researcher were faced with was short lifelong of scions in rooting medium because of the appearance of brownish ends of cuttings and dying off after few days. However, we overcame this by applying activated charcoal 0.1% in MS medium to absorb phenolic compounds that evolve from woody plant part in MS medium, and considered as toxic elements for explant. Also, in parallel, it seems that darkness through applying charcoal MS medium was suitable for rooting of damask scions as well. Mirza et al. (2011) also reported highest percentage of root formation 89% on MS medium supplemented with 0.5 mg.l^{-1} IBA which is similar with present study results. A lower rooting ability was also recorded in old world spp. (*Rosa canina* and *Rosa damascena*) when compared with *Rosa hybrida* (Pati et al., 2006). Kirichenko et al. (1991) reported that rooting of micro shoots of the essential oil bearing roses was difficult when compared with the ornamental varieties. Current studies indicate that, there are genes responsible for increased number of bud initials and shoot proliferation. Moreover, the possible involvement of the gene in modulating hormone levels has also been reported (Tantikanjana et al., 2001). In *R. damascena* and R. *bourboniana* rooting was initiated as a two-step procedure where IBA (2 mg.l^{-1}) was used in MS medium and in the second step the shoots were transferred to PGR free medium containing half strength of MS (Pati et al 2005). Bhoomsiri and Masomboon (2003) reported that rooting was obtained only on MS medium supplemented with 0.5 mg.l^{-1} NAA (87.5 % of the explants exhibited root development with 2.71 roots per explant). The experimental medium of Kornova et al. (2001) with a base on modified MS and 0.1 mg.l^{-1} NAA was created good conditions for rooting. Strongly influenced by the coverage of rooting medium with a liquid film of hormone free medium. Jabbarzadeh and Khosh-Khui (2005) announced for problems in rooting too. Application of different media (MS, 1/2 MS, 1/3 MS and 1/4 MS) with different concentrations of auxins did not produce satisfactory results. Similarly, explants failed to produce roots in quick-dip method using sterilized 0–2000 mg.l^{-1} auxin solutions. Other treatments such as using various concentrations of ABA with various concentrations of IAA, IBA and NAA also applying different concentrations of sucrose and agar did not result in rooting. At last, among the treatments the successful treatment for rooting of shoots was using 2.5 mg.l^{-1} 2,4-D for 2 weeks in MS medium and then transferring the explants to MS medium without any PGRs. When shoots were kept in the rooting medium for more than 2 weeks, root-tips became brown in color and plant-

lets died after a few days. The problem was solved by transferring the plantlets from rooting medium to root elongation medium (without any PGRs). It may be concluded that auxins are necessary just for root initiation, but not for subsequent root development of Damask rose. The report by Nikbakht et al. (2005) for *in vitro* rooting include quick deep treatment of micro shoots' bottom in 2000 ppm IBA solution and then rooting in liquid half strength of the same MS medium showed the best result compared with 1000 ppm.

Most rooting media involve a modification of the MS high mineral salt medium with or without growth regulators (Khosh-khui and Sink, 1982). The most common auxins used for rose root induction are NAA (naphthaleneacetic acid 0.03-0.1 $mg.l^{-1}$), IAA (Indole-3 -acetic acid 0-1 $mg.l^{-1}$) and IBA (3.0 $mg.l^{-1}$ IBA indole-3-butyric acid). All are effective in rooting of rose *in vitro* (Khosh-Khui and Sink, 1982). Another factor that affects rooting of rose is the salt concentration of the nutrient medium. Many roses rooted well in diluted medium; half or quarter strength MS salt concentrations often promote rooting (Hasegawa, 1980 Environmental factors also affect the ability of roses to root. According to Khosh-Khui and Sink (1982) rose shoots grown at low light intensity (1.0 Klux) gave a higher rooting percentage (84%) than those grown under higher light intensities (3.0 Klux). Skirvin et al. (1990) reported that red light can have positive effect on rooting of miniature roses (*R. chinensis*) (Skirvin and Chu, 1984). They also reported their miniature roses proliferated better under cool white fluorescent light than under warm white fluorescent.

In the report of Mamaghani et al. (2010) was utilized modified MS medium contained 1/2, 1/3 and 1/4 concentrations of macronutrients, sucrose as well as full concentration of micronutrient supplemented with different concentrations of NAA (0.1, 0.2, 0.5 and 1 $mg.l^{-1}$). The results indicated that root regeneration of shoots under *in vitro* condition were difficult. 25% of shoots in G1 (East Azerbyjan) accession were rooted on modified 1/3 MS medium supplemented with 0.1 $mg.l^{-1}$ NAA. On the other hand, root regeneration of G2 (West Azerbyjan) accession was found on modified 1/2 MS medium by adding 0.2 $mg.l^{-1}$ NAA . Using TDZ with high concentration of BAP decreased adventitious root formation on micro shoots. BAP can be accumulated in plant tissue at high concentration in the form of conjugates, inactive glucosides, as well as physiologically active ribosides and ribotides (Podwyszynska, 2003). Horan et al. (1995) reported that the establishment of rooted plantlets was dependent on the age of the micro shoots. Huettman and Preece (1993) suggested that rooting of micro shoots were difficult because of carry over effect from cytokinin. Podwyszynska (2003) formulate the hypothesis that the problem with root formation of some rose genotypes may result from inadequate endogenous level of auxin and other growth regulators, phenolics or enzymes. Low rooting attributed to mature explants might have some inhibitory materials (Podwyszynska, 2003). The result indicates there were different responses among accessions for rooting. Hasegawa (1980) suggested that there is a difference in rooting requirements and responses to culture condition, as well as in rooting capacity.

The last and most important step in micropropagation is the establishment of plantlets to *ex vitro* conditions (Rahman et al., 1992; Rout et al., 1999). Once plantlets are well rooted, they must be acclimatized in normal greenhouse environment. The compost containing a high amount of organic and inorganic nutrients could increase the nutrient availability for the plants. Therefore, plants grown in sand, soil, organic matters and vermiculite mixture develop better than in other medium tested. Roses can be successfully grown on a wide range of soils, but they do best on well-drained soils, with a soil pH of 6.0 to 6.5 (Jabbarzadeh and Khosh khui, 2005).

The presence of organic matter and vermiculite tend to keep more moisture in the potting medium.This is important for plant survival during the first week after the transfer to outdoor conditions. The potted plants are covered with perforated plastic bag that can provide a favorable environment for the damask growth. It could be concluded that the composition of sand, soil, organic materials, vermiculite (2:2:1:1) was the suitable medium for acclimatization of damask rose plantlets.

Pati et al. (2005) indicate that the period of incubation of micro shoots in rooting vessel also influenced on the survival percentage of *R. damascena* plantlets under greenhouse conditions. Significantly higher sur-

vival percentage (96.66%) was observed after 6 weeks of incubation compared to the lowest (3.3%) after 1 week, suggesting a clear correlation of incubation period during root induction upon survival of plantlets during hardening or acclimatization.

During *in vitro* process, plantlets grow under very special conditions in relatively air-tight cultivation vessels, e.g., air humidity is higher and irradiance lower than in conventional culture. The use of closed vessels in order to prevent microbial contamination decreases air turbulence, which increases leaf boundary layers and limits the inflow of CO_2 and outflow of gaseous plant products from the vessels. The cultivation media are often supplemented by saccharine as carbon and energy sources. This addition decreases considerably the water potential of the medium and increases the risk of bacterial and fungal contamination.

Other application of biotechnological tools is regeneration from protoplasts culture. This system allows applying protoplast fusion technology for facilitating gene transfer between incompatible rose species holds great potential. Application of this technique allows one to bypass sexual incompatibilities thus facilitating widening of the gene pool available for rose improvement. Pati et al. (2004) reported for successful protocol for protoplast isolation from microspores of *Rosa damascena* Mill.

This technology is more complicate, requires knowledge in several research disciplines and mainly applying for genetic study.

Plant regeneration

Indirect organogenesis

First report for indirect shoot regeneration from rose callus using *Rosa damascena* was reported by Ishioka and Tanimoto (1990) who tried to examine the plant regeneration from rose callus. In this investigation was showed that adventitious buds could be successfully induced from Bulgarian rose callus tissues. Adventitious bud formation could be successfully attained, depending on the kinds of mineral salts used in the medium, auxin and cytokinin used. When callus tissues were cultured on the medium without ammonium nitrate and contained indoleacetic acid and benzyladenine, buds were formed on the callus. The number of buds was significantly increased by the simultaneous addition of calcium ionophore. When the cultures were transferred to the medium without cytokinin, roots were formed in the basal part of the buds. For induction of adventitious bud was tested various concentrations of auxins - NAA (0.02 – 0.2 mg.l^{-1}), IAA (0.02 – 0.2 mg.l^{-1}) and cytokinins – BA (0.2 – 2 mg.l^{-1}), K (0.2 – 2 mg.l^{-1}). The best results for bud formation were observed on the MS medium containing 0.2 mg.l^{-1} IAA and 2 mg.l^{-1} BA (2.6 buds per culture). The cultures with buds were transferred to the rooting medium containing 1 mg.l^{-1} IAA.

Direct organogenesis

The first report on direct shoot regeneration in scented rose was describe by Pati et al. (2004) using direct induction of shoot buds from leaf explants of in vitro-raised shoots of *R. damascena* var. Jwala.(Institute of Himalayan Bioresource Technology, Palampur) without the intervening callus phase. Petioles from fully developed young leaves, obtained from 4 wk pruned old shoots, were found to be ideal for regeneration of shoots. Initially the explants were cultured in an induction medium ½ MS including 1.5 mg.l^{-1} TDZ, 0.05 mg.l^{-1} α-NAA and 2.5 mg.l^{-1} $AgNO_3$ and subsequently transferred to the regeneration medium MS with 0.5 mg.l^{-1} BA and 0.01 mg.l^{-1} NAA for 21 days. In vitro rooting of micro-shoots was accomplish within 2 wk on ½ MS liquid medium containing 2 mg.l^{-1} IBA for 1 wk in the dark and later transferred to hormone – free medium and kept in the light. Plantlets were transferred to soil.

Somatic embryogenesis

In roses, somatic embryo genesis has been obtained from a variety of explants such as calli derived from leaf tissue, immature leaf and stem segments, immature seeds, petioles and roots and anther filaments (Bhattacharjee, 2010). It has been reported for induction of somatic embryogenesis from *Rosa damascena* in Bulgaria (Borisova, A. and Goturanov. G, unpublished data).

The use of *in vitro* technology for propagation of vegetative plants is an effective alternative to traditional methods. The plants thus obtained were free of pathogens, genetically identical but their price is higher. *In vitro* method is expensive and therefore its use should be very well grounded and motivated.

Bioreactors

Automation of tissue culture will depend on the use of liquid cultures in bioreactors; allow fast proliferation, mechanized cutting, separation, and automated dispensing (Sakamoto et al., 1995). These techniques were used for some plants, which involve minimal hand manipulation and thus reduce *in vitro* plant production costs. Eide et al. (2003) reported two liquid culture systems for plant propagation i.e. temporary immersion systems and permanent submersion of the plant cells/ tissue that requires oxygen supply through rotary shakers or bioreactors. Temporary immersion system, e.g. RITA bioreactor, seems to be better than the permanent submersion system for shoot proliferation (Figure 4).

Other approach of plant cells and tissue culture techniques is production of secondary metabolites under controlled and reproducible conditions, independent of geographic and climatic conditions. Studies on the production of plant metabolites by callus and cell suspension cultures have been carried out on an increasing scale since the end of the 1950's. The prospect of using such culturing techniques is for obtaining secondary metabolites, such as active compounds for pharmaceuticals and cosmetics, hormones, enzymes, proteins, antigens, food additives and natural pesticides from the harvest of the cultured cells or tissues.

As for the clonal propagation of a plant in large scale, regeneration from cells belonging to explants of plants is potentially very useful. (Yesil-Celiktas et al, 2010) Liquid media have been used for plant cells, somatic embryos, and organ cultures in both agitated flasks and various types of bioreactors. Scale up of plant regeneration in a liquid medium is easier than on a solid medium (Okamoto et al. 1996). Although the use of bioreactors has been directed mainly for cell suspension cultures and secondary metabolite production, research directed at improving bioreactors for somatic embryogenesis has been reported for several plant species as well. Utilization of bioreactors for clonal propagation has been attracting interest recently due to scale-up and automation advantages (Gurel, 2009). Pavlov et al (2005) investigate the low molecular metabolites (volatiles and polar compounds) produced by *Rosa damscena* Mill. 1803 cell suspension cultured in biore-

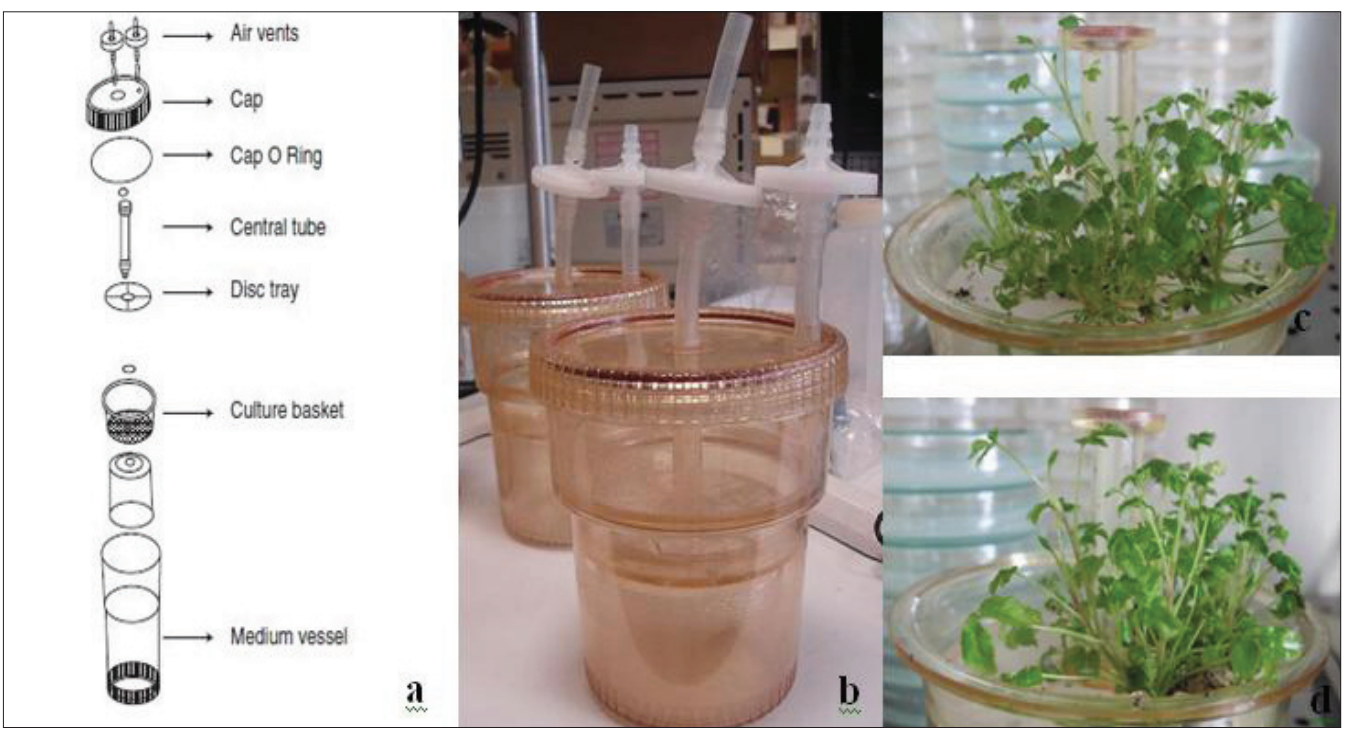

Fig. 4. Temporary immersion bioreactor system (RITA®): Components and assembly of the RITA Bioreactors (Kintzios, 2010); b. RITA® c, d. multiplication of roses in bioreactor

actor and in flasks. It was found that the main groups of volatiles were hydrocarbons and free acid and their esters. The main components of polar fraction were free acids, especially amino acids and oxidized acids.

Bioreactors have also been used for the cultivation of hairy roots mainly as a system for secondary metabolite production (Ziv, 2000). Furthermore, innovative processes have been proposed for producing secondary metabolites selectively by enzymatic reactions.

Conclusion

Rosa damascena have great importance to the Bulgaria economy. Based on our review of the methods used for generation of this culture, we can summarize their application until now having mind positive and negative side of each multiplication method.

The expansion of the areas and increase the yield of rose flowers is a priority for manufacturers and scientists dedicated knowledge and experience of it.

Choosing an appropriate method for breeding should be subordinated to the goal that we set ourselves and know that there are no perfect methods. Best result would be obtained if combining the use of different methods and the ability to use the best of them.

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