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Efficient Micropropagation of English Shrub Rose "Heritage" Under *in Vitro* Conditions

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ABSTRACT

In vitro propagation of rose has played a very important role in rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants. Micropropagation using shoot tip segments from 2 years old plants of cv. Heritage of rose using different combinations of TDZ, Kinetin and NAA and rooting using full, ½ and ¼ strength of MS macro, micro elements and vitamins was investigated. The results showed that the highest shoot proliferation was obtained on MS medium containing 0.05 mg L^{-1} TDZ, 0.2 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA. A reduction in shoot regeneration was observed, when either NAA was removed or concentration of TDZ was increased in the medium. The results also showed that the MS medium containing half concentration of macro, micro salts and vitamins was the most suitable medium for rooting of the *in vitro* regenerated shoots. The *in vitro* plant regeneration protocol for of cv. "Heritage" is considered as an important step for successful implementation of biotechnological techniques for rose improvement in Turkey.

Key Words: *In vitro;* Shoot tips; Propagation; Rooting; Rose; TDZ; Kinetin; NAA; Multiplication rate

INTRODUCTION

Rose is one of the most important commercial flower crop used in the floriculture and cut flower industry of Turkey. Besides, important cultivars of rose propagated on commercial scale, 23 wild rose cultivars are also grown and found in the Isparta province of Turkey. They are popularly used in perfumery, cosmetic industry and medicinal purposes in many regions of the world. Commercial propagation of roses is usually done by cuttings, although they can also be propagated by budding and grafting (Horn, 1992), which is difficult un-desirable and tedious process. Since so many cultivars of rose are propagated all over the world, it is very difficult to maintain their record. Moreover, most cultivars are cultivated without awareness of their genetic background. Similarly, dependence on season and slow multiplication rates are some of the other major limiting factors in conventional propagation (Pati *et al*., 2006). This makes scientific propagation work even more difficult.

In the last few years, *in vitro* propagation has revolutionized commercial nursery business (Pierik, 1991). The first shoot organogenesis from callus tissue was reported by Hill (1967) in a climbing hybrid tea rose "The Doctor". The earliest references of rose micropropagation were those of Jacob *et al*. (1969; 1970a b) and Elliott (1970) in *Rosa hybrida* cv. Superstar and *R. multiflora*, respectively. It was followed by *in vitro* micropropagation of *R. hybrida* by Skirvin and Chu (1979) and Hasegawa (1979), who used axillary buds for proliferation. Since these pioneering efforts, several studies and a number of papers have been published on the commercial propagation of hybrid rose (Davies, 1980; Bressan *et al*., 1982; Curir *et al*., 1986; Valles & Boxus, 1987; Horn *et al*., 1988; Horn, 1992; Carelli & Echeverrigaray, 2002). Significant features of *in vitro* propagation procedure are its enormous multiplicative capacity in a relatively short span of time, production of healthy and disease free plants, and its ability to generate propagules around the year (Dhawan & Bhojwani, 1986).

High heterozygosity, in the *R. hybrida* has resulted in contradictory results and low multiplication rates on important rose cultivars. The results are so contradictory, that one has to consider several times to consider using a method successfully adopted in one cultivar to another cultivar.

The most important technique in micropropagation is meristem proliferation, where in shoot meristems or nodal segments harboring an axillary bud are cultured to regenerate multiple shoots without any intervening callus phase. Multiplication of a variety through shoot tip could result in development of plants immune to fungal and viral diseases and maintain genetically pure, healthy and vigorous stock plants. Therefore, this study aimed to develop *in vitro* techniques for multiplication of economically important rose cv. "Heritage" immune to fungal and viral diseases.

MATERIALS AND METHODS

Shoot tip explants of pink colored English rose "Heritage" were collected from two years-old plants propagated through hard wood cuttings during April 2005 and maintained under the botanical garden of a commercial floriculture nursery at Ankara Turkey. Half of the selected shoots were covered holed transparent with polythene bags for four weeks to allow growth and development of new shoot tips independent of external environmental effects.

Thereafter, soft wood shoot cuttings (bearing shoot tips) from respective covered and uncovered plants were excised and brought to the laboratory for surface sterilization and subsequent use in tissue culture studies. After removing leaves, the shoots (having length of 1.8 - 2.5 cm & diameter of 3.00 to 3.5 mm) were surface sterilized with 100% (v/v) commercial bleach (Axion- Turkey), containing 5 - 6% NaOCl with one drop of Tween 20 per 100 mL for 5, 7.5 and 10 min followed by 3 X 3 min rinses each in sterile distilled water. Bleached cut ends of the sterilized shoot tip explants were removed before culturing on 0.7% agar (Sigma Type A) solidified MS (Murashige & Skoog, 1962) medium supplemented with different concentrations of TDZ, Kinetin and NAA (Table I) and 3% sucrose in Magenta GA7® vessels.

After two months of culture, *in vitro* developing shoots were rooted by culturing on 0.7% agar solidified MS medium containing full, ½ and ¼ strength of MS macro, micro mineral elements, vitamins and 3.0% sucrose.

Unless otherwise specified, the pH of all culture media was adjusted to 5.7 before autoclaving at 121°C, under pressure of 1.2 kg cm^{-2} for 20 min. All cultures were maintained at 24 ± 2°C under 16/8 h (light/dark) photoperiod provided by Sylvania day light-white fluorescent tubes with intensity of 42 µmol photons $m^{-2}s^{-1}$.

The rooted plantlets were transferred to wooden containers (50 x 90 cm) containing soil mix having compost, sand and clay (1: 1: 1), (autoclaved at 135°C for 20 min) and watered. During the fist week of transfer, the plantlets were covered with transparent polythene bags to maintain high humidity and then watered as and when required. The survival rate was examined one month after transfer.

A complete randomized design (CRD) was used both for micropropagation and rooting studies. Four explants were inoculated per Magenta vessel (containing 25 mL of culture medium) in each treatment with four replications and were repeated twice. Data taken in percentage were subjected to arcsin transformation before analysis and converted back in to percentage form for presentation in tables (Snedecor & Cochran, 1968). The data were analyzed using SPSS Version 12.00. Significant differences were assessed using Duncan's multiple range test ($P < 0.05$).

RESULTS

Micropropagation. Study with the nodal stem segments collected from un-covered plants showed high degree (100%) of fungal and bacterial contamination with any treatment time. Whereas, the explants taken from the developing shoots under polythene cover were easy to sterilize using 100% commercial bleach for 7.5 min. However, sterilization for 10 min was damaging and sterilization for 7.5 min resulted in bleaching of chlorophyll at the cut ends of the explants, which were removed before culturing, to give way to fresh conducting tissues. Surface sterilization for 10 min with the commercial bleach was damaging and 5 min was ineffective to sterilize the plants.

The nodal segments of *cv.* "Heritage" cultured on MS medium containing different concentrations of TDZ, Kinetin and NAA (Table I) showed variation in the frequency of adventitious shoot regeneration and number of shoots per explant. The results showed the highest frequency (100%) of shoot multiplication and number of shoots per explant (6.00) on MS medium containing 0.05 mg L^{-1} TDZ, 0.2 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA. It reduced to 3.33 shoots per explant and 75.00% shoot regeneration on MS medium containing 0.05 mg L^{-1} TDZ, 0.2 mg L^{-1} kinetin without NAA and to 2.5 shoots per explant with 75% shoot regeneration on MS medium containing 0.1 mg L^{-1} TDZ, 0.2 mg L^{-1} Kinetin without NAA.

Rooting. The resulting shoots cultured on MS medium containing ¼, ½ and full strength of MS macro, micro elements and vitamins showed variable response to rooting after four weeks of culture (Table II). The highest number (5.63) and frequency of rooting (50%) was obtained on MS medium containing ½ strength of MS macro, micro salts and vitamins. However, full or ¼ strength of MS macro, micro salts and vitamins were inhibitory and resulted in sporadic and low rooting of 25.00 and 16.67%, respectively. The rooted plants were not difficult to acclimatize at 24 ± 1°C and relative humidity of 80% during initial stages of development gradually reduced to 40% after 4 weeks of culture and was transferred to the greenhouse for flowering.

DISCUSSION

A successful micropropagation protocol proceeds through a series of stages, each with a specific set of requirements. These are (i) initiation of aseptic cultures, (ii) shoot multiplication, (iii) rooting of micro shoots and (iv) hardening and field transfer of tissue culture raised plants (Pati *et al*., 2006).

For initiation of aseptic cultures, a thorough knowledge of the physiological status and the susceptibility of the plant species to different pathological contaminants are required. A review of literature showed that surface sterilization of rose is problematic and different approaches have been adopted to achieve sterilization. Salehi and Khosh-Khui (1997) used sterile solution of different antibiotics (gentamycin, ampicillin, tetracycline ornamoxicillin) at different concentrations and duration for disinfection from internal contaminants. They noticed that use of an antibiotic solution before surface sterilization was un-successful. However, dipping in 100 mg L^{-1} solution of gentamycin or ampicillin after surface sterilization resulted in the highest percentage of disinfected explants. However, our experience with different explants in our laboratory shows that treatment with biocides or antibiotics is not effective especially when the percentage of endogenic

Table I. Effects of different plant growth regulators on shoot regeneration from shoot tip explant of cv. Heritage of rose under in vitro conditions

TDZ (mg/l)	Kinetin (mg/l)	NAA (mg/l)	Number of shoots per explant	Frequency (%) of shoot regeneration
0.05	0	0	1.17[1] bc[2]	25.00 c
0.05	0	0.1	1.83 bc	25.00 c
0.05	0.2	0	3.33 b	75.00 b
0.05	0.2	0.1	6.00 a	100.00 a
0.1	0	0	0.83 c	50.00 c
0.1	0.	0.1	1.67 bc	75.00 b
0.1	0.2	0	2.50 bc	75.00 b
0.1	0.2	0.1	0.72 c	75.00 b

[1] Each value is the mean of 4 replicates each repeated twice with 4 explants.
[2]Values with in a column followed by different letters are significantly different ($p<0.01$) using Duncan's Multiple Range Test.

Table II. Effects of ¼, ½ and full strength MS médium on rooting of cv. Heritage of rose

Strength of MS medium	Number of roots per explant	Frequency (%) of rooting
¼	0.67[1] c[2]	16.67 b
½	5.63 a	50.00 a
full	1.91 b	25.00 b

[1] Each value is the mean of 4 replicates each with 4 explants and was repeated twice.
[2]Values with in a column followed by different letters are significantly different ($p<0.01$) using Duncan's Multiple Range Test.

contaminants is too high and preferred in this study. Rout *et al*. (1989a,b; 1990), initially treated explants with 70% (v/v) ethanol for 20 - 30 s followed by treatment with 0.1% $HgCl_2$ for 5 - 7 min and rinsing in sterile distilled water. However, due to high toxic nature of $HgCl_2$, its use is highly undesirable and is not recommended procedure.

The results of our study showed that explants taken from un-covered shoots were difficult to sterilize under any duration of time. However, 7.5 min treatment of explants taken from shoots that grew under polythene bags for four weeks, with 100% commercial bleach (5 - 6% NaOCl) was very effective. Sterilization for ten minutes was damaging with distinguishable bleaching of explants; and sterilization for 5 min was not enough to sterilize the explants completely. These results are in line with Khosh-Khui and Sink (1982a b), Skirvin and Chu (1979) and Hasegawa (1979), who sterilized the shoot tips using sodium hypochlorite (5.25%) and "Tween 20" or Triton X (0.1%) for 5 - 10 min followed by washing in sterile distilled water.

It is very evident that, TDZ at a lower concentration in combination with Kinetin and NAA had supporting effect on the shoot regeneration. Any, increase in the concentration of TDZ with or with out Kinetin in the shoot induction medium was inhibitory and had negative effects on shoot regeneration. The results are in line with many researchers, who observed variable effects of cytokinins and auxins on shoot regeneration of different varieties of rose. Alekhno and Vysotskii (1986) obtained efficient shoot regeneration of rose on MS medium containing 0.5 - 1 mg L^{-1} BA. Bini *et al*. (1983) used BA and Zeatin for multiplication of *Rosa indica.* Similarly, Singh and Syamal (1999) used BAP, NAA and GA_3 to obtain more than 5 shoots per explant.

In most of the earlier reports, varying concentrations of different auxins have been used for root induction (Hasegawa, 1979; Khosh-Khui & Sink, 1982c; Collet, 1985; Kirichenko *et al*., 1991; Chu *et al*., 1993; Arnold *et al*., 1995). However, (Murashige, 1979) reports relatively low salt concentrations in the medium to enhance rooting of micro shoots. It was observed that rooting in "cv. Heritage" of rose was highly dependent on the concentration of MS macro, micro elements and vitamins in the medium with best rooting on ½ strength of MS salts and vitamins. In line with our results, Skirvin and Chu (1979) induced rooting of micro shoots on growth regulators free solidified medium. Similarly, Hyndman *et al.* (1982) demonstrated that a decrease in KNO_3 and NH4NO_3 concentration was the decisive factor for improving the rooting percentage. They succeeded in enhancing root number and length of *in vitro* grown shoots of *R. hybrida* cv. "Improved Blaze" by lowering total nitrogen concentration of MS salts in the culture medium keeping other salt concentrations constant. The results of our experiment are further supported by the findings of Douglas *et al.* (1989), who used full strength of MS medium or dilution of MS medium to one fourth strength containing saccharose to obtain rooting of rose. Badzian *et al*. (1991) also had very similar results, who reported use of MS medium with major elements reduced to one quarter to one third strength for root induction.

The successful acclimatization of micropropagated plants and their subsequent transfer to the field is a crucial step for commercial exploitation of *in vitro* technology. The acclimatization of micropropagated roses is reported to be a difficult procedure because of rapid desiccation of plantlets, when exposed to external conditions. No problem was observed in acclimatization of *in vitro* regenerated plantlets in the environmental chamber set to 80% humidity during early stages of development reduced gradually to 40% in the later stages of adaptation in organic matter rich soil mix contained in pots that affectively helped to maintain the soil humidity and prevented desiccation.

We conclude that present work opens an opportunity to *in vitro* propagation of cv. "Heritage" using shoot tip explants. However, the concentration of TDZ, Kinetin and NAA must be carefully chosen in order to avoid inhibitory effects of TDZ at higher concentrations.

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