Study of culture conditions for improved micropropagation of hybrid rose

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ABSTRACT: An efficient protocol was developed for micropropagation of hybrid roses by manipulating growth regulators, photoperiods, gelling agent and subculture period. Multiple shoots were achieved from nodal explants of *Rosa hybrida* cvs. Cri Cri, Pariser Charme and First Red on the Murashige and Skoog (MS) medium supplemented with 1.5–2.0 mg/l BA (6- benzylaminopurine), 50 mg/l Ads (adenine sulfate) with 3% (w/v) sucrose. Inclusion of indole-3-acetic acid (IAA; 0.1–0.25 mg/l) into the cytokinin-rich medium promoted high frequency of shoot multiplication. The induction of multiple shoots was also affected by photoperiod and subculture period. Higher multiplication was achieved under 16 h photoperiod in all tested cultivars. The rate of multiplication was low when photoperiod both increased or decreased. The frequency of shoot multiplication was best up to the 6^{th} to 7^{th} subculture and thereafter it declined. Rooting was readily achieved upon transferring the microshoots onto half-strength MS medium supplemented with 0.25 mg/l IBA (indole-3-butyric acid) and 2% (w/v) sucrose. The percentage of rooting was less on MS medium containing NAA (1-naphthalene acetic acid) or IAA as compared with IBA. More than 60% of rooted plantlets were established in the greenhouse. The *in vitro* raised plantlets were grown normally and flowered within one month after their transfer to open field.

Keywords: growth regulators; *in vitro*; rose; shoot multiplication

Rose is one of the most popular flowering ornamentals in the world. Presently, it is a favorite ornamental for landscapes, as well as the most important commercial cut flower. Cut and pot roses are cultivated worldwide over an estimated area of 16,000 ha in glasshouses and 3,000 ha in the open field (Brichet 2003). Rose is generally propagated by vegetative methods like cutting, layering, budding and grafting. Seeds are used for propagation of species and also for production of rootstocks (Horn 1992). Some of the commercial cultivars are difficult to propagate conventionally. Although propagation by vegetative ways is a predominant technique in roses, yet it does not ensure healthy and disease-free plants. Moreover, dependence on season and slow multiplication rates are some of other major limiting factors in conventional propagation (Pati et al. 2006). *In vitro* culture technique is an alternative method for plant propagation; every year, millions of plants are required to be planted. Micropropagation of roses was reported by various researchers using cultures of axillary buds and apical meristems (Skirvin et al. 1990; Yan et al. 1996; Rout et al. 1999; Pati et al. 2006); similarly micropropagation of various rose cultivars was reported by various authors who described using axillary bud and apical meristem culture (Hasegawa 1979; Barve et al. 1984; Rout et al. 1990; Chu et al. 1993; Voyiatzi 1995). There are a few studies on the effect of growth regulators and culture environment on shoot multiplication of commercial rose cultivars. The objective of the present study was to establish an efficient protocol on micropropagation by manipulating growth regulators and culture condition.

MATERIALS AND METHODS

Explant source and culture. Internodal segments (8–10 cm long) of *Rosa hybrida* cvs Cri Cri, Pariser Charme and First Red were collected from rose garden of the Regional Plant Resource Centre, Bhubaneswar. They were recut into 3–4 cm long segments and washed thoroughly with 2% (v/v) Teepol (Qualigen, Mumbai, India) for about 15 min with constant shaking, and rinsed with running tap water. Then, segment surface was sterilized with 0.1% (w/v) mercuric chloride solution for 30 min with constant

shaking, followed by rinsing with sterile distilled water thrice. The internodal segments were further cut into 0.5–1.0 cm long with single node and used as explant source.

The nodal explants were cultured on semisolid Murashige and Skoog (1962) (MS) basal medium supplemented with different concentrations of 6-benzylaminopurine (BA) (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l), kinetin (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l), indole-3-acetic acid (IAA) or 1-napthalene acetic acid (NAA) (0, 0.1, 0.25 and 0.5 mg/l) alone or in combination. Agar-agar (6.5 g/l) was added to the medium as gelling agent after adjusting the pH to 5.7–5.8 using 0.1N NaOH or 0.1N HCl before autoclaving. 20 ml of the molten medium was dispersed into the culture tube (25 × 15 mm) plugged with non-absorbent cotton wrapped in one layer of cheesecloth and autoclaved at 121°C or 1.06 kg/cm^2 for 15 min. The explants were inoculated aseptically. The cultures were maintained at 25 ± 2°C under 16 h photoperiod. Subsequently, the cultures were maintained by regular subculture at four week intervals on fresh medium with the same composition.

In another experiment, the cultures were incubated under 0, 8, 12, 16, 20 and 24 h photoperiod from cool, white fluorescent lamps (Philips, India) with 3.0 klx. The cultures were maintained in the fresh medium (MS + 2.0 mg/l BA + 50 mg/l Ads + 0.25 mg/l IAA + 3% sucrose) at four week intervals.

In a separate experiment, two gelling agents (agar-agar and gelrite) were used separately along with the multiplication medium (MS + 2.0 mg/l BA+ 50 mg/l Ads + 0.25 mg/l IAA + 3% sucrose) for induction and proliferation of multiple shoots. The cultures were incubated at 25 ± 2°C under 16 h photoperiod.

For induction of root, the microshoots were excised from a 12-week-old culture (derived from three subculture periods) and transferred to semisolid half-strength MS medium supplemented with 2% (w/v) sucrose and different concentrations of indole-3-butyric acid (IBA), IAA and NAA (0.1, 0.25 and 0.5 mg/l) alone or in combination. One excised shoot was placed in each tube (25 × 150 mm) having 15 ml of the culture media. All the cultures were incubated at 25±2°C under 16 h photoperiod with 3.0 klx intensity. Rooted micropropagules were thoroughly washed to remove the adhering gel and planted in earthen pots containing 100% sand for 2 weeks in the greenhouse. After developing good root systems, they were subsequently transferred to 6" earthen pots containing sterile mixture of sand, soil and cow-dung manure in the ratio of 1:1:1 (v/v). The pots were kept in the greenhouse for acclimatization.

Observation of cultures and presentation of the results. Number of cultures per treatment varied from experiment to experiment; each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots per culture, mean percentage of rooting and number of roots per shoot were statistically analyzed by the Post-Hoc Multiple Comparison test (Marascuilo, McSweeney 1977). Among the treat-

Table 1. Effect of subculture on shoot multiplication of *Rosa hybrida* cvs. Cri Cri, First Red and Pariser Charme cultured on MS medium supplemented with 2.0 mg/l BA, 50 mg/l Ads and 0.25 mg/l IAA. The subculture was made in four week intervals

No. of subculture	Name of cultivar (avg No. of multiple shoots/culture ± S.E)*		
	Cri Cri	First Red	Pariser Charme
1	2.32 ± 0.9	2.44 ± 0.8	3.87 ± 0.8
2	4.62 ± 1.1	4.86 ± 0.7	5.64 ± 0.9
3	4.78 ± 1.0	4.92 ± 1.0	5.26 ± 0.8
4	5.12 ± 0.8	5.11 ± 0.8	6.66 ± 1.0
5	5.24 ± 0.6	5.34 ± 0.6	6.72 ± 1.3
6	5.85 ± 0.8	5.82 ± 1.0	7.34 ± 1.2
7	5.32 ± 1.2	6.42 ± 0.8	7.58 ± 1.1
8	5.08 ± 1.0	6.26 ± 0.6	7.23 ± 1.2
9	4.45 ± 0.8	5.72 ± 0.9	6.76 ± 0.8
10	4.11 ± 0.7	5.14 ± 1.0	6.14 ± 0.9
11	3.52 ± 0.8	4.24 ± 0.7	5.96 ± 1.0
12	3.24 ± 0.6	3.86 ± 0.8	5.42 ± 0.9

*20 cultures per subculture; repeated thrice

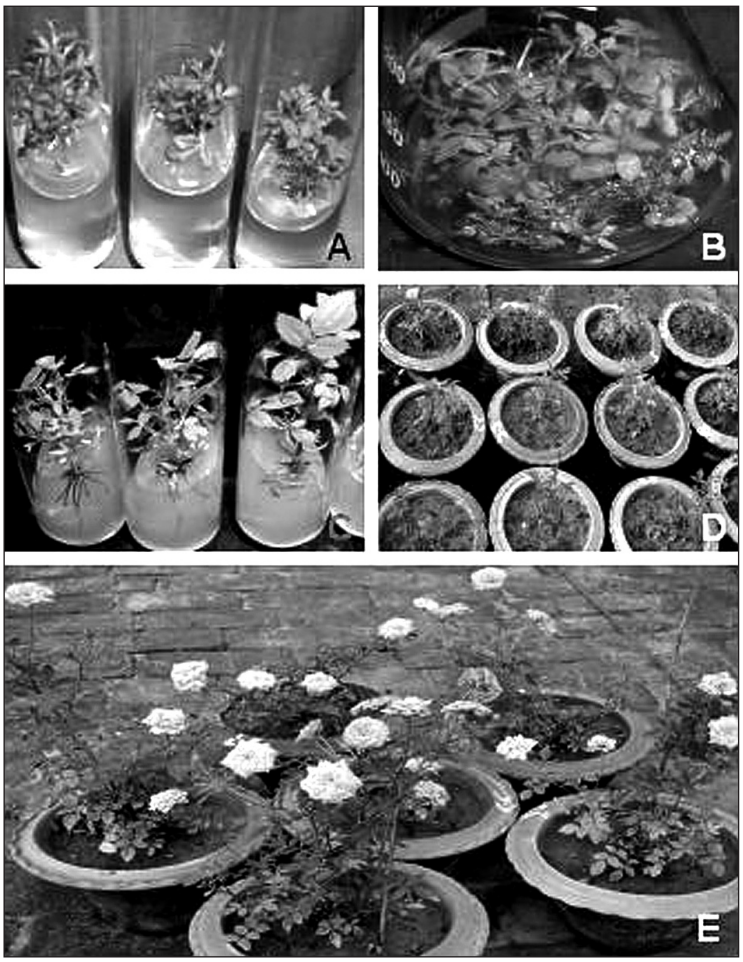

Fig. 1. A – Axillary shoot development from nodal explants of Cri Cri on MS medium supplemented with 0.5 mg/l BA+ 25 mg/l Ads + 0.01 mg/l IAA after 2 weeks of culture (Bar = 10 mm). B – Development of multiple shoots on MS medium supplemented with 2.0 mg/l BA + 50 mg/l + Ads + 0.25 mg/l IAA after 4 weeks of subculture (Bar = 25 mm). C – Induction of roots from excised shoots on ½ MS supplemented with 0.25 mg/l IBA + 20 g/l sucrose (Bar = 50 mm). D and E – *In vitro* raised plantlets established in soil bearing flowering (Bars = 50 mm for Fig. D and 1 cm for Fig. E)

ments, the average figures followed by the same letters were not significantly different at $P < 0.05$ level.

RESULT AND DISCUSSION

Shoot bud initiation and multiplication

Among the three cytokinins used, the medium containing BA plus Ads showed a higher rate of bud break subsequently developed into multiple shoots as compared to Kn or Kn + Ads. About 85–90%-bud break was achieved within 7–8 days of inoculation. Feeble callusing was observed at the base of explants in all the three cultivars tested. Zieslin and Halevy (1976) reported that cytokinins enhance the percentage of bud break in rose but this was accompanied by a higher rate of flower bud atrophy. Rout et al. (1990) reported a higher percentage of bud break in hor-

Table 2. Effect of different photoperiods on shoot multiplication of three rose cultivars on MS medium supplemented with 2.0 mg/l BA, 50 mg/l Ads and 0.25 mg/l IAA after 4 weeks of subculture

Photoperiod (hours)	Name of cultivar (avg No. of multiple shoots/culture ± S.E)*		
	Cri Cri	First Red	Pariser Charme
0	0	0	0
8	2.54 ± 0.8 a	3.82 ± 0.8 a	3.62 ± 0.8 a
12	4.33 ± 1.1 d	4.34 ± 1.0 b	4.56 ± 0.9 b
16	4.72 ± 1.2 d	4.92 ± 1.2 c	5.78 ± 1.1 c
20	4.06 ± 1.0 c	4.32 ± 0.9 b	5.56 ± 1.0 c
24	3.86 ± 0.8 b	4.10 ± 0.8 b	4.92 ± 0.8 b

*25 cultures per treatment; repeated thrice

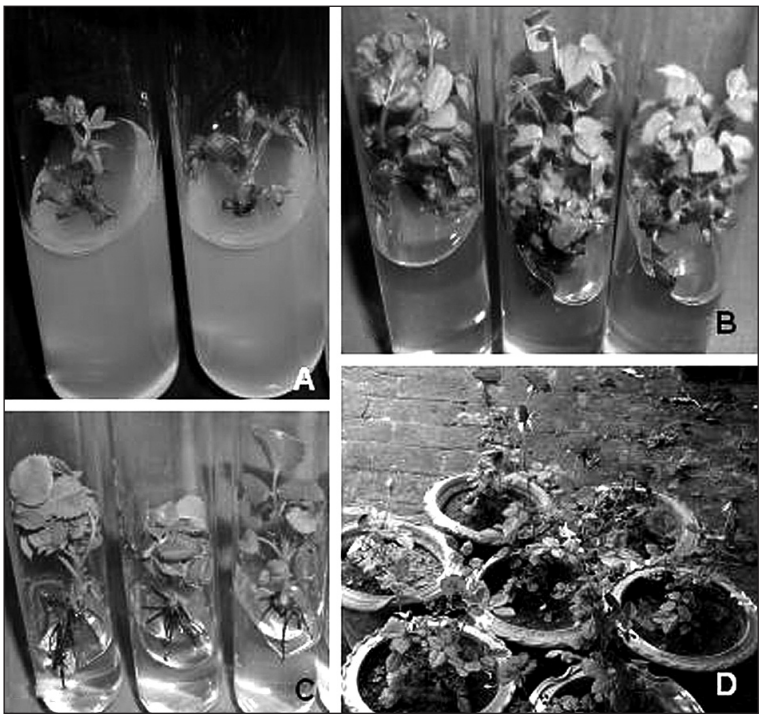

Fig. 2. A – Axillary shoot development from nodal explants of First Red on MS medium supplemented with 0.5 mg/l BA+ 25 mg/l Ads + 0.01 mg/l IAA after 2 weeks of culture. B – Development of multiple shoots on MS medium supplemented with 2.0 mg/l BA + 50 mg/l + Ads + 0.25 mg/l IAA after 4 weeks of subculture. C – Induction of roots from excised shoots on ½ MS supplemented with 0.25 mg/l IBA + 20 g/l sucrose after 3 weeks of culture (Bar = 50 mm). D – Rooted plantlets established in soil beaning flowers

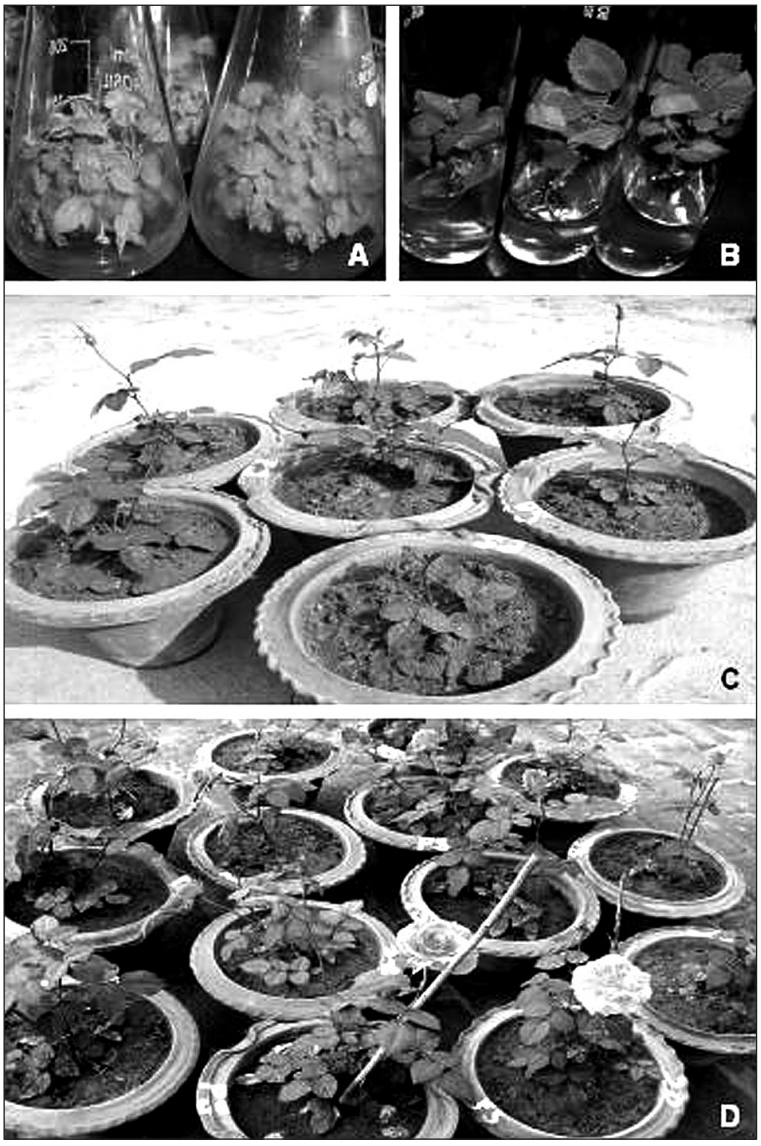

Fig. 3. A – Developmets of multiple shoots from nodal explants of Pariser Chame on MS medium supplemented with 2.0 mg/l BA + 50 mg/l Ads + 0.25 mg/l IAA after 4 weeks of subculture. B – Induction of roots from excised shoots on ½ MS supplemented with 0.25 mg/l IBA + 20 g/l sucrose (Bar = 10 mm). C – Rooted plantlets established in soil after three weeks since transfer. D – *In vitro* raised plantlets bearing flowers after one month since transfer

Table 3. Effect of auxins on root induction from microshoots of *Rosa hybrida* after 2 weeks of culture in MS + 2% sucrose

Growth regulators (mg/l)			Percentage of shoots rooted (mean ± S.E)*	Avg No. of roots/shoot (mean ± S.E)*
IAA	IBA	NAA		
Cv. First Red				
0	0	0	0	0
0.10	0	0	0	0
0.25	0	0	24.0 ± 0.5	2.12 ± 0.4
0.50	0	0	32.7 ± 0.6 +	3.14 ± 0.6 +
0	0.10	0	42.0 ± 0.7	3.11 ± 0.5
0	0.25	0	62.8 ± 1.2	5.23 ± 0.6
0	0.50	0	54.0 ± 0.7 +	4.48 ± 0.7 +
0	0	0.10	35.3 ± 0.6	3.82 ± 0.6
0	0	0.25	40.7 ± 0.6	3.68 ± 0.5
0	0	0.50	41.3 ± 0.5 +	3.0 ± 0.7 +
0.10	0.25	0	52.8 ± 0.7	4.23 ± 0.6
0.25	0.10	0	50.6 ± 0.8 +	4.06 ± 0.3 +
0.10	0	0.50	46.2 ± 0.5 +	3.27 ± 0.5 +
Cv. Cri Cri				
0	0	0	0	0
0.10	0	0	0	0
0.25	0	0	40.0 ± 0.8	2.76 ± 0.3
0.50	0	0	30.7 ± 0.7 +	3.14 ± 0.4 +
0	0.10	0	50.7 ± 0.6	4.82 ± 0.5
0	0.25	0	58.6 ± 0.7	4.76 ± 0.2
0	0.50	0	42.6 ± 0.6 +	2.53 ± 0.5 +
0	0	0.10	32.7 ± 0.8	1.32 ± 0.3
0	0	0.25	52.0 ± 1.0	2.66 ± 0.4
0	0	0.50	32.0 ± 0.4 +	2.15 ± 0.6 +
0.10	0.25	0	56.2 ± 0.6	4.72 ± 0.4
0.25	0.10	0	52.6 ± 0.7+	4.42 ± 0.3 +
0.10	0	0.50	40.2 ± 0.5 +	3.12 ± 0.5 +
Cv. Pariser Charme				
0	0	0	0	0
0.10	0	0	42.7 ± 0.7	2.82 ± 0.6
0.25	0	0	56.7 ± 0.6	3.56 ± 0.5
0.50	0	0	47.3 ± 0.4 +	3.42 ± 0.3 +
0	0.10	0	64.6 ± 0.5	4.34 ± 0.6
0	0.25	0	62.0 ± 0.6	4.22 ± 0.4
0	0.50	0	54.3 ± 0.7	3.14 ± 0.8
0	0	0.10	39.3 ± 0.6	2.94 ± 0.5
0	0	0.25	48.0 ± 0.7	2.86 ± 0.7
0	0	0.50	54.6 ± 0.8 +	3.86 ± 0.6 +
0.10	0.25	0	50.2 ± 0.6	4.10 ± 0.4
0.25	0.10	0	52.4 ± 0.7 +	3.86 ± 0.4 +
0.10	0	0.50	50.6 ± 0.6 +	3.34 ± 0.6 +

*50 microshoots per treatment; repeated thrice, + callusing at the basal cut end

mone free medium within 10–12 days but the rate of growth was found slow. Medium supplemented with BA or BA + Ads, however, induced early bud break within 6–8 days of culture. The inclusion of IAA (0.1–0.25 mg/l) in the BA + Ads containing medium enhanced the rate of shoot multiplication. The rate of multiplication varied from cultivar to cultivar. The maximum rate of shoot multiplication was achieved on medium containing 1.5–2.0 mg/l BA, 50 mg/l Ads and 0.25 mg/l IAA (Figs. 1A,B, 2A,B, 3A). The shoot multiplication rate also varied in different species and was specific to the culture medium (Davies 1980; Khosh-Khui, Sink 1982a,b; Rout et al. 1990; Pati et al. 2006). The rates of multiplication were 5.72, 4.84 and 4.56 in Pariser Charme, First Red and Cri Cri, respectively, after four weeks of subculture (data not shown). The rate of shoot multiplication depended on the period of subculture. A high frequency of shoot multiplication was obtained during the 6th to 7th subculture and it declined in subsequent subculture (Table 1).

The result showed that the frequency of shoot multiplication was higher under 16 h photoperiod as compared to continuous light (Table 2). Similar effects of photoperiod on shoot multiplication were reported in other crops (Schlegel, Schneider-Maessen 1981; Samantaray et al. 1995). The effect of light on *in vitro* morphogenesis was also reported Seibert and Kadkade (1980). Baraldi et al. (1988) also emphasized the interaction of light intensity and plant hormone triggering *in vitro* shoot differentiation in *Prunus* species.

Gelling agents also have a great impact on shoot multiplication and elongation. Between two gelling agents used, agar-agar favored the highest rate of multiple shoots production (8–10 per shoots per explant) as compared to gelrite (5–6 shoots per explant). Shoot length was observed to be very low in agar (~ 3–4 cm) as compared to greater shoot length (~ 6–8 cm) in gelrite after 4 weeks of culture.

Induction of rooting from microshoots

After successful multiplication, the microshoots were excised and transferred to different rooting media for induction of roots. The rate of rooting varied in different varieties and types of media. The result showed that the development of roots from microshoots was obtained on half-strength MS medium supplemented with IBA or NAA or IAA with 2% sucrose. It was also observed that among the three auxins tested, IBA (0.25 mg/l) favored a good response as compared to IAA or NAA (Table 3). Maximum percentage of rooting was 58.6%, 62.8% and 64.6% in Cri Cri, First Red and Pariser Charme, respectively, on the medium containing 0.25 mg/l IBA within 21 days of culture (Figs. 1C, 2C, 3B). Little friable callus was formed at the cut end of the microshoot when it was transferred to medium containing higher concentration of auxins (0.5 mg/l). Skirvin and Chu (1979) reported that the rooting was attained from microshoots of rose cultivars on solid medium without growth regulators. Many researchers reported that roots were induced from excised mature microshoots on MS medium supplemented with low concentration of auxins (IAA, IBA, NAA and 2,4-D in the range of 0.1–0.5 mg/l with the reduction of the sucrose level 2–2.5%) (Hasegawa 1979, 1980). Davies (1980) achieved 100% rooting in several rose cultivars by placing them on MS medium supplemented with 40 g/l of sucrose lacking growth regulators. Khosh-Khui and Sink (1982c) reported that half-strength MS medium supplemented with NAA (0.54μM) was adequate to induce rooting in cv. Bridal Veil of hybrid rose. Douglas et al. (1989) achieved very high percentage of rooting in cv. Queen Elizabeth in ¼ MS medium without any growth regulators. Rout et al. (1990) achieved the root initiation from seven rose cultivars within 8–10 days on ½ MS medium supplemented with 0.1–0.25 mg/l of NAA or 2.4-D alone or in combination.

Acclimatization and field establishment

After successful rooting, the rooted plantlets were transferred to pots containing sand for further development of root. Within two weeks, about 10 to 15 roots were developed per microshoot. After two weeks, the rooted plantlets were transferred to 6" arthen pots containing sand: soil: cow-dung in the ratio 1:1:1 and were kept in greenhouse for acclimatization. Watering was made at two-day intervals. About 60% of the *in vitro* raised plantlets survived and flowering occurred within one month of transfer (Figs. 1D,E, 2D, 3C,D).

In conclusion, micropropagation of three commercial rose varieties was established by manipulating the culture condition and growth regulators. This study will help for conservation and mass propagation of hybrid roses for horticulture as well as pharmaceutical industries.

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Studium kultivačních podmínek zlepšujících rozmnožování hybridních růží *in vitro*

ABSTRAKT: Pomocí manipulace s růstovými regulátory, délkou fotoperiody, zpevňující složkou média a dobou pasážování byla vypracována efektivní metodika pro rozmnožování hybridních růží *in vitro*. Proliferace výhonků bylo

dosaženo u nodálních explantátů hybridních růží odrůd Cri Cri, Pariser Charme a First Red na kultivačním médiu Murashige a Skoog (MS) s dodáním 1,5–2,0 mg/l BA (6-benzylaminopurinu), 50 mg/l Ads (adenin sulfátu) s 3% sacharózou. Doplnění kyseliny β-indolyloctové (IAA; 0,1–0,25 mg/l) do média bohatého na cytokinin podporovalo vyšší četnost proliferace výhonků. Tato četnost byla dále ovlivňována délkou fotoperiody a dobou pasážování. U všech odrůd došlo k nejvyšší intenzitě rozmnožování při délce fotoperiody 16 hodin. Při prodlužování nebo zkracování této fotoperiody se intenzita rozmnožování snižovala. K nejvyšší intenzitě proliferace výhonů docházelo až po 6. nebo 7. pasážování, později opět docházelo k jejímu poklesu. K zakořeňování docházelo snadno po přenesení mikrovýhonků na médium MS s poloviční koncentrací soli, doplněném o 0,25 mg/l IBA (kyselina indolylmáselná) a 2% sacharózu. Procento zakořeňování bylo nižší, pokud médium MS obsahovalo místo IBA NAA (kyselina α-naftyloctová) nebo IAA. Ve skleníku se podařilo dopěstovat více než 60 % zakořeněných rostlinek. Sazenice, které byly vypěstovány v podmínkách *in vitro,* rostly normálně a kvetly během jednoho měsíce po přesazení do venkovních podmínek.

Klíčová slova: regulátory růstu; *in vitro*; růže; mikrorozmnožování

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