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Standardization of *in vitro* mass multiplication protocol for hybrid tea rose cv. Grand Gala

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ABSTRACT

An efficient protocol for *in vitro* multiplication of hybrid tea rose cv. Grand Gala was developed using axillary bud segments. Out of different pre-treatments of explants tried, the highest explant survival (82.16%) was obtained with carbendazim (0.2%) + diathane M-45 (0.2%) + 8-HQC (200 mg/l) for 4 h on a horizontal shaker (120 rpm). Sucrose concentration of 40 g/l in the medium seems to be optimal for *in vitro* shoot multiplication. Murashige and Skoog medium supplemented with 5.0 mg /l BAP + 0.2 mg/l NAA + 0.5 mg/l GA_3 was found most effective for culture establishment and shoot proliferation with highest number of micro-shoots (6.49 shoots / explant). Rooting of micro-shoots was induced on half-strength MS basal medium supplemented with NAA (0.5 mg/l) + IBA (0.5 mg/l). The regenerated plantlets were efficiently hardened in glass jars filled with vermiculite + agropeat (1:2) moistened with half-strength MS medium salts and covered with polypropylene lids, thereafter plants were successfully transferred to the glasshouse with good survival.

Key words: Rose, micropropagation, *in vitro* regeneration.

INTRODUCTION

Rose is a favourite plant cultivated for its beautiful flowers and is universally claimed as the "Queen of Flowers". Traditionally, hybrid-tea roses (*Rosa hybrida* L.) have been considered to be one of the most prized flowers of the world because of their high ornamental value. Their importance has grown over the years with the emergence of a global cut flower market. It is admired for their perfect blooms, exquisite colour and unique fragrance. As cut flower, it occupies top position in acreage, production and consumption. Roses are generally multiplied vegetatively by grafting and budding that are very slow and time consuming methods. Moreover, diseases and environmental hazards make the cultivar degenerate gradually.

Micropropagation procedures have facilitated mass production of good quality plantlets giving a boost to rose floriculture industry. This technique allow producing roses with higher quality under a virus indexing programme, attending in this way the market demand. Keeping this in view, the present investigation was carried out to establish an efficient and reproducible protocol for rapid and large scale propagation of 'Grand Gala' an important cut rose cultivar.

MATERIALS AND METHODS

The present study was carried out at the Central Tissue Culture Laboratory, L.B.S. Centre, IARI, New Delhi during 2010-2012. Rose cultivar Grand Gala maintained at Centre for Protected Cultivation Technology, IARI, New Delhi was used for this experiment. The bud sticks having 3 to 4 matured axillary buds were selected from the middle portion of current season flowering shoots. With secateurs they were excised during morning hours and cut into individual axillary bud segments (≥1.5 cm). The explants were washed with Teepol® (0.1%) solution for 5 min. followed by washing under running tap water for 15 min. The nodal segments were then treated with different pre-treatments such as: (i) carbendazim (0.2%) + 8-HQC (200 mg/l), (ii) carbendazim (0.2%) + diathane M-45 (0.2%) + 8-HQC (200 mg/l) along with control (distilled water) for 4 h on horizontal shaker (120 rpm). The pre-treated explants were then surface-sterilized with 0.1% mercuric chloride for 8-10 min. followed by two-three rinsings with autoclaved distilled water. The surface sterilized explants were cultured on MS medium supplemented with different concentrations of BAP (3.0, 4.0 and 5.0 mg/l), NAA (0.1 and 0.2 mg/l), and GA_3 (0.3 and 0.5 mg/l) to find out the best treatment combination for culture establishment. The surface sterilized explants were cultured on media containing M S + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l) with five different concentrations of sucrose; *i.e.*, 20, 30, 40, 50 and 60 g/l. After four weeks, individual micro-shoots (about 1 cm) were separated from the bunch and transferred onto fresh medium with the similar hormonal combination and a specific concentration of sucrose.

The sprouted shoots were then sub-cultured onto MS medium supplemented with different concentrations of BAP (3.0, 4.0 and 5.0 mg/l), NAA (0.1 and 0.2

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mg/l), and GA_3 (0.3 and 0.5 mg/l) to find out the best treatment combination for shoot proliferation. The multiplied shoots on proliferation media were separated and individual micro-shoots were transferred onto elongation media comprising basal MS medium supplemented with various concentration of GA_3 (0.5, 1.0 and 1.5 mg/l) to standardize its optimum dose for micro-shoots elongation. Elongated shoots were then transferred individually in cultured vessels containing full-and half-strength of MS medium fortified with different concentrations of auxins like NAA and IBA individually or in combination for rooting. A dose of 40 g/l of sucrose was added for culture establishment and shoot proliferation and 60 g/l was added in rooting medium. The *in vitro* rooted plantlets were removed from flasks, washed thoroughly with autoclaved distilled water to remove the sticking agar-agar to roots. The roots were then dipped in carbendazim (0.1%) for 10 sec. The plantlets were then acclimatized in glass jars filled with vermiculite + agro peat (1:2) moistened with half-strength MS medium salts (macro + micro) and covered with polypropylene lids. The plantlets were kept in culture room (15 days) before transferring to greenhouse. For culture initiation, 20-25 explants were inoculated per treatment in three replications. The cultures were maintained at 25±1°C under fluorescent white light (47 µmol m^{-2} s^{-1}) at a photoperiod of 16/8 h light and dark cycles. The data was analyzed employing completely randomized design (CRD) and percent data was subjected to Arc Sin √% transformation before ANOVA.

RESULTS AND DISCUSSION

Pre-treatment of explants with different fungicidal and bactericidal treatments had significant effect on survival of explants, microbial contamination, bud sprouting and days to bud sprouting (Fig. 1). The treatment (T_3) comprising carbendazim (0.2%) + diathane M-45 Indofil® (0.2%) + 8-hydroxy quinnoline citrate (200 mg/l) for 4 h agitation gave the highest explants survival and bud sprouting, which were significantly superior compared to the other treatments. Whereas, the minimum explant survival and bud sprouting were recorded with distilled water control (T_1). The pre-treatment of axillary bud explants with treatment (T_3) comprising carbendazim (0.2%) + diathane M-45 (0.2%) + 8-HQC (200 mg/l) for 4 h minimized microbial contamination significantly as compared to control. This pre-treatment also gave the earliest bud sprouting as compared to control. It is obvious that the fungicides used had both systemic and contact fungicides, thus gave efficient control of microbial infection. Similarly, 8-HQC was effective due to its bactericidal activities. Efficacy of these compounds has earlier been demonstrated by Machado *et al*. (10), Prasad (11), and Bharadwaj *et al*. (3) in rose.

The maximum survival of explants (86.25%) and bud sprouting (81.11%) was recorded for the treatment MS + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l) (Table 1; Fig. 3a).The minimum response was noted with hormone-free MS medium. The above treatment also gave the earliest (4.73) bud sprouting when comparing with other treatments, which was maximum delayed (7.47) in control. The efficacy of BAP in stimulating shoot proliferation has earlier been reported by Vijaya *et al*. (16), and Kumar and Prateesh (8). Earlier, Douglas *et al*. (5) and Arnold *et al*. (1) reported the efficacy of cytokinins in combination with an auxin or together with GA.

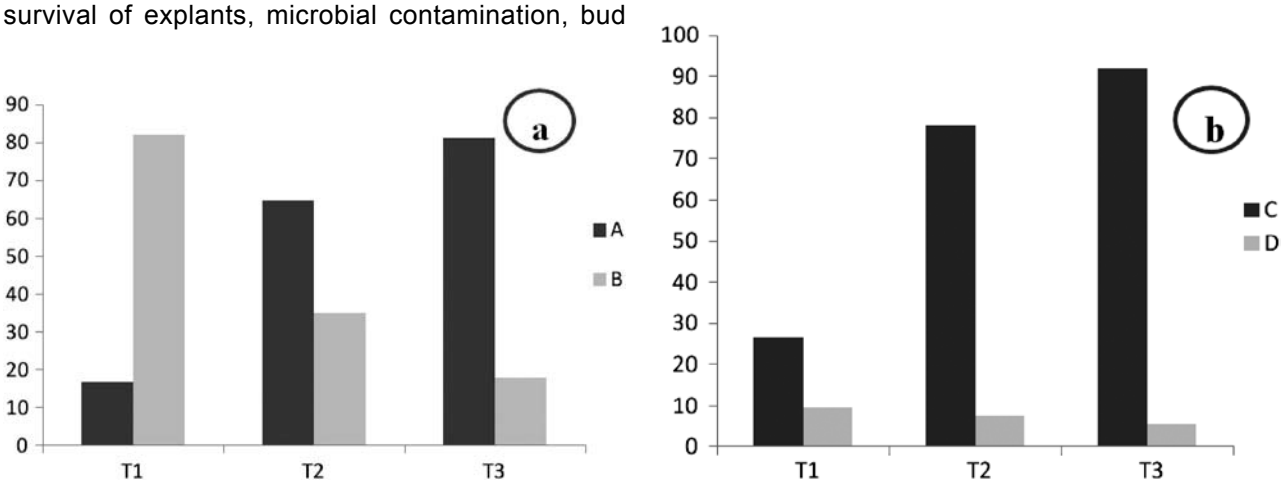

Fig. 1. Effect of different pre-treatments on *in vitro* culture initiation in hybrid tea rose cv. Grand Gala. T_1 = control (distilled water) for 3 h, T_2 = carbendazim (0.2%) + mancozeb-45 (0.2%) + 8-HQC (200 mg/l) for 3 h, T_3 = carbendazim (0.2%) + mancozeb-45 (0.2%) + 8-HQC (200 mg/l) for 4 h; A = explant survival (%), B = microbial contamination (%), C = bud sprouting (%), D = days to bud sprouting.

Table 1. Effect of growth regulators on *in vitro* culture establishment in hybrid tea rose cv. Grand Gala.

Treatment	Explant survival (%)	Bud sprouting (%)	Days to bud sprouting
MS + No hormone (control)	47.95 (43.85)	43.50 (41.28)	8.47
MS + BAP (3 mg/l) + NAA (0.1 mg/l) + GA_3 (0.3 mg/l)	66.17 (54.46)	61.85 (51.88)	6.12
MS + BAP (3 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l)	69.89 (56.75)	71.41 (57.71)	5.62
MS + BAP (3.5 mg/l) + NAA (0.1 mg/l) + GA_3 (0.3 mg/l)	77.40 (61.65)	79.18 (62.89)	5.00
MS + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l)	86.25 (68.27)	81.11 (64.27)	4.73
CD at 5%	2.72	3.65	0.76

*The values given in parentheses denote the Arc Sin √% values

The maximum number of shoots sprouted was noted with treatment MS + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l) (Table 2; Fig.3b). After first sub-culture maximum shoots per explants (5.29) was noted with the same treatment. The least number of shoots per explant (3.53) were recorded under control, *i.e.*, MS without any hormone. Shoot multiplication rate in third sub-culture revealed that maximum number of shoots per explant (6.49) were also recorded in the treatment, *i.e.*, MS + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l) compared to least (4.40) in control. Growth regulators at an optimum dose lead to good shoot proliferation and the same was observed in each sub-culture. The favourable influence of BAP and NAA in different metabolic processes (Kulaeva, 7) is known to effect plant metabolism. The better results regarding shoot proliferation in tissue culture might be due to the role of optimum dose of BAP, which enhances axillary branching and multiple shoot formation. Superiority of BAP in shoot multiplication has earlier been shown by Scotti Compos and Pais (12). It is also opined that in multiple shoots a proliferation may be due to loss of apical dominance (Bala *et al*., 2; Bressan *et al*., 4; Douglas *et al*.,5; Singh and Syamal, 14).

Table 2. Effect of growth regulators on *in vitro* shoot multiplication in hybrid tea rose cv. Grand Gala.

Treatment	No. of shoots proliferated per explant per sub-culture			
	First	Third	Fifth	Mean
MS + No hormone (control)	3.53	4.16	4.40	4.03
MS + BAP (3 mg/l) + NAA (0.1 mg/l) + GA_3 (0.3 mg/l)	4.11	4.79	5.48	4.79
MS + BAP (3 mg/l) + NAA (0.1 mg/l) + GA_3 (0.3 mg/l)	4.48	5.27	6.11	5.29
MS + BAP (3.5 mg/l) + NAA (0.1 mg/l) + GA_3 (0.3 mg/l)	5.05	5.58	6.42	5.68
MS + BAP (5.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l)	5.29	5.70	6.49	5.83
CD at 5%	0.345	NS	0.494	

Besides hormonal regime, other media components are also known to influence the *in vitro* growth response of rose cultures (Short and Roberts, 13; Skirvin *et al*., 15). Concentration of sucrose in the medium has been found to affect photosynthetic potential (Langford and Wainwright, 9) and rate of shoot multiplication. Sucrose concentration of the medium showed a distinct influence on the rate of shoot proliferation in cultures. Proliferation rate depicted by mean number of nascent shoots produced per culture in four weeks was highest in 40 g/l sucrose, intermediate in 30 and 50 g/l and lowest in 20 and 60 g/l (Fig. 2). Sucrose facilitates the induction of vascular

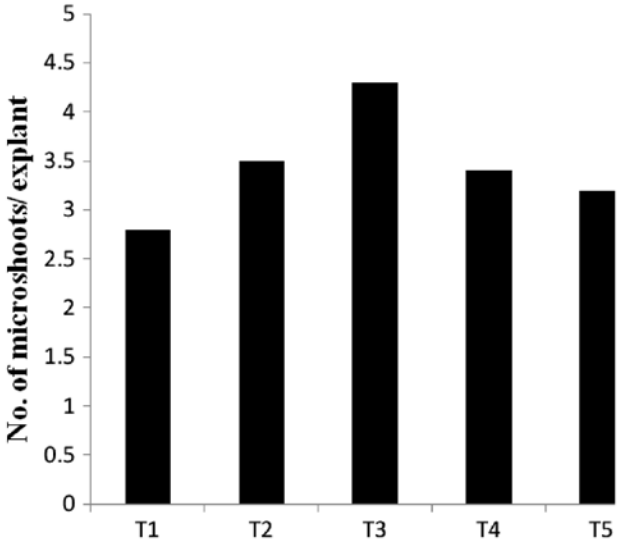

Fig. 2. Effect of different sucrose levels on shoot multiplication. T1 = 20 g/ l, T2 = 30 g/ l, T3 = 40 g/ l, T4= 50 g/ l, T5 = 60 g/ l.

tissue differentiation in cultures. Without exogenous sucrose, the formation of tracheary elements will be greatly reduced or absent. Hyndman *et al.* (6) and Langford and Wainwright (9) reported that sucrose concentration of the medium incrementally influences the photosynthetic ability of *in vitro* growing shoots up to a certain level; but higher concentrations suppress the activity. This could be the reason for inferior growth response in 60 g/l sucrose as compared to 40 g/l in the present study.

The data presented in Table 4 depicts the effect of basal medium strength and auxin on days to root initiation, rooting, number of roots per shoot, root length and root quality. The earliest root initiation (14.3 days) was noted on half-strength MS + NAA (0.5 mg/l) + IBA (0.5 mg/l) followed by half-strength MS + NAA (1.0 mg/l) + IBA (1.0 mg/l) (14.9 days) (Fig. 3e). The time taken for root initiation was most delayed in control (28.3 days). The highest rooting was observed for the treatment half-strength MS + NAA (0.5 mg/l) + IBA (0.5 mg/l). The rooting on reduced basal salt strength medium was significantly higher as compared to full-strength medium. The rooting percentage was maximum (91.67) on half-strength MS + NAA (0.5 mg/l) + IBA (0.5 mg/l) followed by half-strength MS + NAA (1.0 mg/l) + IBA (1.0 mg/l) (81.67) as compared to minimum in control (15.83). The longest root length (4.78 cm) was induced with half-strength MS+ NAA (0.5 mg/l) + IBA (0.5 mg/l). The qualitative data suggest that roots were not only few but stunted in medium devoid of auxins. When NAA was supplemented individually, roots were small stunted and thick while those cultured on medium supplemented with IBA had thin and long roots. Interestingly, roots on half-strength medium supplemented with the dual auxins were of medium length and thin. It is evident from the study that there has been synergistic effect when the two auxins were employed together. Optimum role of two auxins has been reported earlier also by Singh and Syamal (14), and Bharadwaj *et al*. (3).

Table 4. Effect of different acclimatization strategies on survival of *in vitro* raised plantlets.

Treatment	Survival (%)	Plantlet height (cm)	No. of leaves per plantlet
Plastic pot with polythene bag cover	74.26 (59.52)	6.27	3.90
Plantlet in glass jar with polypropylene lid	86.24 (68.40)	8.12	4.82
CD at 5%	4.81	1.92	0.02

The values given in parentheses denote the Arc Sin √% values

The highest plantlet survival (86.24%), the plant height (8.12 cm) with good number of leaves (4.82 per plant) were recorded for the treatment where plantlets were acclimatized in glass jars filled with vermiculite + agropeat supplemented with half-strength MS medium (macro + micro-organics) and covered with polypropylene lids as compared with, the plantlet survival in plastic pots covered with Polythene bags (Table.4) . The better results obtained in glass jars might be due to less open space but appropriate relative humidity as compared to those hardened in plastic pots covered with polythene bags. Efficacy of glass jars for *Rosa hybrida* has been earlier reported by Bala *et al*. (2); Singh and Syamal (14), and Bharadwaj *et al*. (3). The results of present investigation demonstrate that rose cultivar Grand Gala can be multiplied *in vitro* employing the above protocol.

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Table 3. Effect of basal medium strength and auxins on rooting of micro-shoots of hybrid tea rose cv. Grand Gala.

Treatment	Days to root initiation	Rooting (%)	Root length (cm)	Root quality	Plantlets growth
MS + No hormone (control)	28.3	15.83 (23.46)	1.56	St	Poor
MS + NAA (0.5 mg/l)	17.8	31.67 (34.26)	1.78	S, Th, St	Good
MS + IBA (0.5 mg/l)	18.2	25.00 (30.02)	2.54	Th, S	Good
½ MS + NAA (0.5 mg/l) + IBA (0.5 mg/l)	14.3	91.67 (73.26)	4.78	T, L	V. Good
½ MS + NAA (1.0 mg/l) + IBA (1.0 mg/l)	14.9	81.67 (64.68)	4.29	Th, L	V. Good
CD at 5%	1.28	5.10	1.16	-	-

The values given in parentheses denote the Arc Sin √% values
T = thin, Th = thick, L = long, M = medium strength, St = stunted, S = small

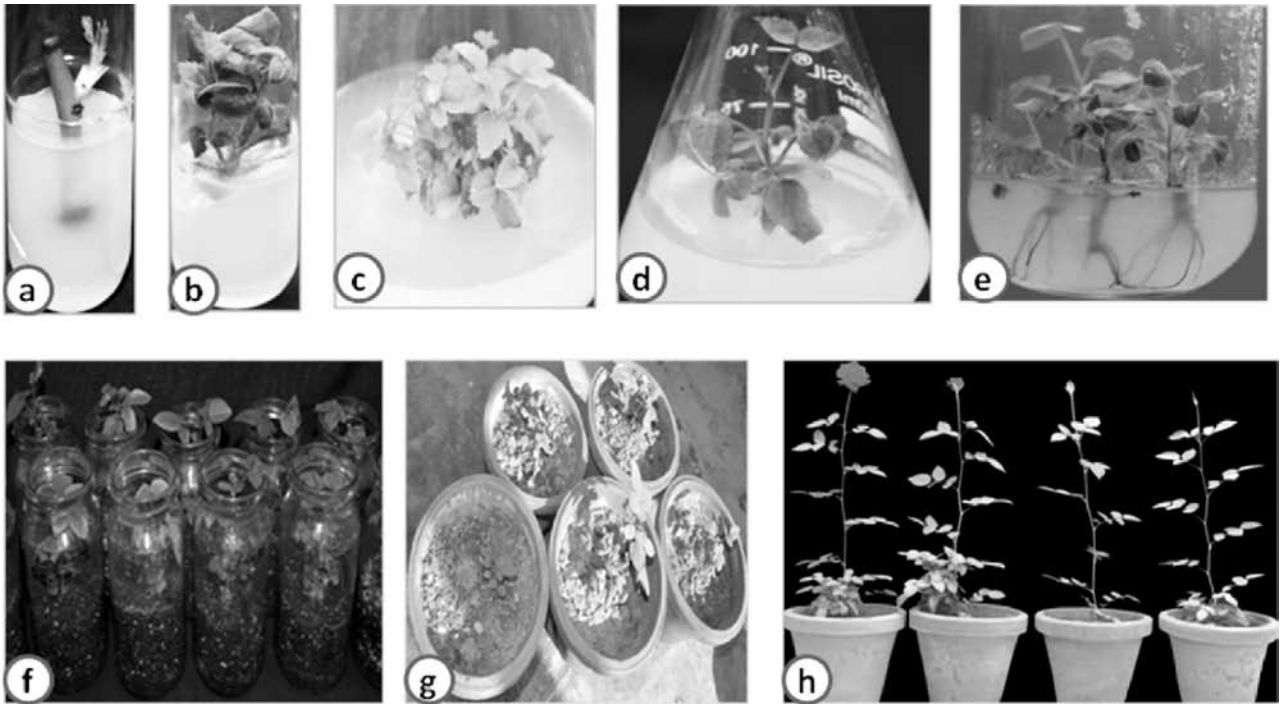

Fig. 3. *In vitro* plant regeneration in Hybrid Tea rose cv. Grand Gala. (a, b, c) Bud sprouting, Sprouting of shoots and shoot proliferation on MS + BAP (3.5 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l). (d) Elongation of shoots on MS + GA_3 (1.0 mg/l). (e) *In vitro* rooting on ½ MS + NAA (0.5 mg/l) + IBA (0.5 mg/l). (f) Gradual hardening in glass jar, (g) Acclimatized plants in soil, and (h) Acclimatized plants with flower.

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