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Standardization of an efficient protocol for *in vitro* mass multiplication of Hybrid Tea rose cv. Raktima

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ABSTRACT

An efficient protocol for *in vitro* micropropagation of hybrid tea rose cv. Raktima was standardised using axillary bud explant. Before inoculation, explants were treated with different concentrations of fungicides and bactericides. Out of different pre-treatments tried, the highest explant survival (73.83%), bud sprouting (69.28%) with minimum microbial contamination (17.16%) were obtained with carbendazim (0.2%) + dithane M-45 (0.2%) + 8-HQC (200 mg/l) for 3 h treatment on a horizontal shaker (120 rpm). The effect of different combinations of BAP (2.5, 3.0 and 3.5 mg/l), NAA (0.1 and 0.2 mg/l), and GA_3 (0.3 and 0.5 mg/l) on culture establishment and shoot proliferation was studied. Murashige and Skoog (1962) medium supplemented with BAP (3.0 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l) was found most effective for culture establishment with highest explant survival (94.83%) and bud sprouting (91.90%) and on shoot proliferation with highest number of micro-shoots per explant (9.50). Better rooting on micro-shoots was induced on half-strength MS basal medium supplemented with NAA (0.5 mg/l) + IBA (0.5 mg/l) with highest rooting (94.13%). The regenerated plantlets were hardened in glass jars filled with vermiculite + agropeat (1:2) moistened with one quarter-strength MS salts (macro and micro) and covered with polypropyline lids resulting in maximum survival (91.86%). After hardening, plants were successfully transferred to the glasshouse with good survival.

Key words: HT rose, micropropagation, *in vitro* regeneration, Raktima.

INTRODUCTION

Rose (*Rosa* × *hybrida* L.) is one of the most popular flowering ornamentals in the world and is universally claimed as the "Queen of flowers". Presently, it is a favourite ornamental for its potentiality marketing as cut flowers and potted plants in many countries of the world. As cut flower, it ranks first in acreage, production and consumption. Roses are generally multiplied vegetatively by grafting and budding that are slow and also time consuming methods. Moreover, diseases and environmental hazards make the cultivar degenerate gradually. 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*., 12). *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 axillary buds and apical meristems (Rout *et al*., 14; Pati *et al*., 12). Micropropagation techniques allow producing roses with higher quality, under a virus-indexing programme, attending in this way the market demand. Keeping this in view, the present study was carried out to establish an efficient and reproducible protocol for rapid and large scale propagation of rose.

MATERIALS AND METHODS

The present study was carried out at the Central Tissue Culture Laboratory, LBS Centre, IARI, New Delhi. Hybrid Tea rose cultivar Raktima (Pink Parfait × Sugandha) developed by IARI, New Delhi was used. The bud-sticks having 3 to 4 matured axillary buds were selected from the middle portion of current season flowering shoots. With secateur they were excised during morning hours and cut in to individual nodal segment (≥ 1.5 cm) (Fig.1). Well prepared 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: (T_1) Control- distilled water, (T_2) carbendazim (0.2%) + 8-HQC (200 mg/l), (T_3) carbendazim (0.2%) + diathane M-45 Indofil® (0.2%) + 8-HQC (200 mg/l) for 3 h on horizontal shaker (120 rpm). The pre-treated explants were then surface-sterilized with 0.1% mercuric chloride for 5 min. followed by two-three rinsings with autoclaved distilled water. The surface-sterilized explants were cultured on MS medium supplemented with different

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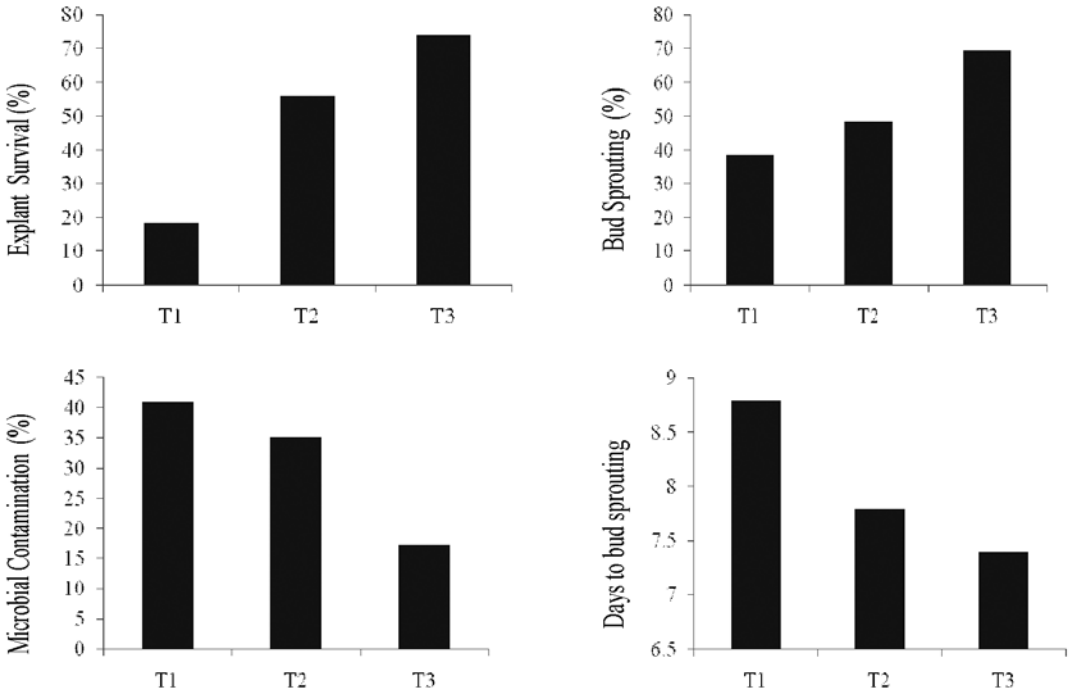

Fig. 1. Effect of different pre-treatments on explant survival, bud sprouting, microbial contamination and days to bud sprouting in rose cv. Raktima. T1 = control (distilled water) - 3 h; T2 = Carbendazim (0.2%) + 8-HQC (200 mg/l) - 3 h; T3 = Carbendazim (0.2%) + Dithane M-45 (0.2%) + 8-HQC (200 mg/l) - 3 h.

concentrations of BAP (2.5, 3.0 and 3.5 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 sprouted shoots were then sub-cultured onto MS medium supplemented with different concentrations of BAP (2.5, 3.0 and 3.5 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 shoot proliferation. The multiplied shoots on proliferation media were separated and individual micro-shoots were transferred onto elongation medium comprising basal MS medium with various concentrations 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 MS medium fortified with different concentrations of auxins like NAA and IBA individually or in combination for rooting. A constant dose of sucrose, *i.e.*, 40 g/l was used in all the combinations. 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 carbebdazim (0.1%) for few 10 sec. The plantlets were then acclimatized using two different strategies like: (i) plastic pots (4") filled with vermiculite + agropeat (1:2) moistened with one quarter-strength of MS medium salts (macro + micro) and covered with polythene bags, and (ii) glass jars filled above potting mixture and covered with polypropylene lids. The plantlets were kept in culture room (15 days) before transferring to greenhouse. For cultured 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 the percent data was subjected to Arc Sin √% transformation before ANOVA.

RESULTS AND DISCUSSION

As evident from the data presented in Fig. 1, pre-treatment of explants with different fungicidal and bactericidal treatments had significant effect on explant survival, bud sprouting, and microbial contamination. The treatment comprising carbendazim® (0.2%) + diathane M-45 Indofil® (0.2%) + 8-hydroxy quinnoline citrate (200 mg/l) for 3 h agitation gave the highest explant survival (73.83%) and bud sprouting (69.28%), which were significantly superior compared to the other treatments. Whereas, the minimum explant survival (18.50%) and bud sprouting (38.53%) were recorded with distilled water control. The pre-treatment of axillary bud explants with bavistin (0.2%)

+ diathane-M-45 (0.2%) + 8-HQC (200 mg/l) for 3 h minimized microbial contamination (17.16%) as compared to control (41.00%). This pre-treatment also gave the earliest bud sprouting (7.40 days) as compared to control (8.79 days). It is obvious that the fungicides used had both systemic and contact types, 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 Prasad (13), Bharadwaj *et al.* (3), Bala *et al.* (2) in rose, and Kadam *et al.* (5) in tuberose.

The maximum explant survival (94.83%) and bud sprouting (91.90%) were recorded for the treatment MS + BAP (3.0 mg /l) + NAA (0.1 mg /l) + GA_3 (0.5 mg/l) (Table 1). The minimum survival of explant (48.53%) and bud sprouting (42.90%) was noted on hormone-free MS medium. The above treatment also gave the earliest (6.43 days) bud sprouting when compared with other treatments, which was maximum delayed (12.10 days) in control. The efficacy of BAP in stimulating shoot proliferation has earlier been reported by Kumar and Pratheesh (8). Earlier, Douglas *et al*. (4) and Arnold *et al.* (1) reported the efficacy of cytokinins in combination with an auxin or GA_3.

The maximum number of shoot sprouted on initial explant (2.50 per explant) was noted with treatment MS + BAP (3.0 mg /l) + NAA (0.1 mg /l) + GA_3 (0.5 mg /l). After first sub-cultured maximum

Table 1. Effect of MS medium supplemented with different growth regulators on *in vitro* culture establishment and shoot multiplication in hybrid tea rose cv. Raktima.

Treatment (mg/l)	BAP	NAA	GA_3	Explant survival (%)	Bud sprouting (%)	Days to bud sprouting	No. of shoots proliferated / explant			
							Initial culture	First sub-culture	Second sub-culture	Third sub-culture
T_1 (control)	-	-	-	48.53 (44.14)*	42.90 (41.41)*	12.10	1.00	2.96	5.30	6.33
T_2	2.5	0.1	0.3	85.67 (67.70)	82.33 (65.24)	7.83	1.60	3.86	5.20	6.10
T_3	2.5	0.1	0.5	85.23 (66.58)	84.53 (66.90)	6.83	1.93	5.67	5.10	5.20
T_4	3.0	0.1	0.3	89.60 (71.19)	86.93 (68.89)	6.57	2.23	5.93	6.11	8.56
T_5	3.0	0.1	0.5	94.83 (76.82)	91.90 (73.54)	6.43	2.50	6.80	7.13	9.50
T_6	3.5	0.1	0.3	89.10 (70.72)	85.87 (67.99)	6.83	2.60	6.47	7.06	9.16
T_7	3.5	0.1	0.5	86.90 (68.78)	83.27 (65.90)	6.93	2.63	5.36	6.83	8.13
T_8	2.5	0.2	0.3	85.27 (67.37)	81.67 (64.70)	7.57	2.47	4.06	6.13	7.46
T_8	2.5	0.2	0.5	78.23 (62.17)	75.90 (60.65)	7.73	2.10	4.60	5.83	7.30
T_9	3.0	0.2	0.3	74.13 (59.41)	71.73 (57.92)	8.10	2.23	5.40	5.26	6.83
T_{10}	3.0	0.2	0.5	72.53 (58.37)	69.87 (56.87)	8.60	2.37	5.10	5.67	6.30
T_{11}	3.5	0.2	0.3	76.57 (61.00)	72.93 (58.71)	7.57	2.43	4.83	5.06	6.63
T_{12}	3.5	0.2	0.5	71.50 (57.73)	68.30 (55.83)	7.93	2.30	3.83	4.26	6.03
CD at 5%				6.39	6.16	0.62	0.41	0.94	0.64	0.68

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

shoots per explant (6.80) were recorded was noted with the same treatment. The least number of shoots per explant (2.96) were recorded under control, *i.e.*, MS without any hormone. Shoot multiplication rate estimated revealed that maximum number of shoots per explant (9.50) were also recorded in the treatment, *i.e.*, MS + BAP (3.0 mg /l) + NAA (0.1 mg /l) + GA_3 (0.5 mg /l) compared to least (6.03) in control. Growth regulators at an optimum dose lead to good shoot proliferation and the same is observed in each subculture (Table 1). The favourable influence of BAP in different metabolic processes (Kulaeva, 6) and NAA 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 (15). It is also opined that in multiple shoot proliferation may be due to loss of apical dominance (Douglas *et al*., 4; Singh and Syamal, 16; Verma *et al*., 18). It is evident from the data that treatment of MS + GA_3 (0.5 mg/l) had a marked influence on the shoot length (2.77 cm) after 20 days of transfer on elongation medium, which was drastically low in control (1.47) (Table 2). After 40 days of transfer, highest shoot length (3.53 cm) was registered in the above treatment. Gibberellins are known for inducing stem elongation in a number of crops. The application of gibberellic acid into shoot elongation medium resulted in rapid growth which might be due to the increased activity of the endogenous auxin in the presence of GA_3. Gibberellic acid is involved in several important biochemical and morphogenetic responses, which include the promotion of elongation in axial organs, such as stems and flower pedicels, along with the stimulation of root growth (Srivastava, 17). The present findings lend support from the previous work done by Prasad (12), Kumar *et al.* (7), and Latado *et al.* (9).

The data presented in Table 3, 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 (10.33 days) was noted on ½ MS + NAA (0.5 mg /l) + IBA (0.5 mg /l) followed by ½ MS + NAA (1.0 mg /l) + IBA (1.0 mg /l) (12.30 days). The time taken for root initiation was most delayed in control (28.46 days). The highest (94.13%) rooting was observed for the treatment ½ MS + NAA (0.5 mg /l) + IBA (0.5 mg /l). The rooting on reduced basal salt strength was significantly higher as compared to full-strength medium. The maximum number of roots per shoot (6.77) was induced on ½ MS + NAA (0.5 mg/l) + IBA (0.5 mg /l) as compared to minimum in control (1.97). The longest roots (5.70 cm) were induced with ½ 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 (16), and Bharadwaj *et al.* (3).

The highest plantlet survival (91.86%), the plant height (9.95 cm) with good number of branches (1.83 per plant) and leaves (5.32 per plant) were recorded for the treatment where plantlets were acclimatized in glass jars filled with vermiculite + agro-peat supplemented with one quarter-strength MS medium and covered with polypropylene lids, the plantlet survival in plastic plastic pots covered with polythene bags (Table 4) (Fig. 6). 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 been reported by Singh and Syamal (16), Bharadwaj *et al.* (3), Mandal and Datta (10), and Nahid *et al.* (11)

Table 2. Effect of MS medium supplemented with different concentrations of gibberellic acid on micro-shoot length and inter-nodal elongation in hybrid tea rose cv. Raktima.

Treatment	GA_3 (mg/l)	Shoot length (cm)		Internodal length (cm)
		After 20 days	After 40 days	After 40 days
T_1	Devoid of hormones (control)	1.47	2.43	0.80
T_2	0.5	2.77	3.53	1.27
T_3	1.0	1.80	3.20	1.17
T_4	1.5	1.60	2.83	1.13
CD at 5%		0.93	0.65	0.02

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

Treatment	Days to root initiation	Rooting (%)	No. of roots per shoot	Root length (cm)
MS devoid of hormones (control)	28.46	16.00 (23.56)*	1.97	2.20
MS + NAA (0.5 mg /l)	17.80	30.27 (33.38)	4.53	2.67
MS + NAA (1.0 mg /l)	15.63	24.27 (29.53)	4.40	2.93
MS + IBA (0.5 mg /l)	17.86	55.97 (48.46)	3.90	3.43
MS + IBA (1.0 mg /l)	18.46	51.47 (45.86)	3.50	3.27
½ MS devoid of hormones	24.03	28.86 (38.25)	2.93	3.10
½ MS + NAA (0.5 mg /l)	12.50	86.20 (68.29)	5.37	3.30
½ MS + NAA (1.0 mg /l)	12.63	84.09 (66.55)	5.27	3.77
½ MS + IBA (0.5 mg /l)	13.36	58.63 (50.00)	4.77	3.73
½ MS + IBA (1.0 mg /l)	14.60	52.87 (46.67)	4.60	3.97
½ MS + NAA (0.5 mg /l) + IBA (0.5 mg /l)	10.33	94.13 (75.09)	6.77	5.70
½ MS + NAA (1.0 mg /l) + IBA (1.0 mg /l)	12.30	88.37 (70.12)	5.50	4.60
CD at 5%	2.20	3.32	0.46	0.30

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

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

Treatment	Survival (%)	Plantlet height (cm)	No. of branches per plantlet	No. of leaves per plantlet
Plastic pots with polythene bags	86.80 (68.70)*	9.41	1.53	4.55
Plantlets in glass jars with polypropyline lids	91.86 (73.36)	9.95	1.83	5.32
CD at 5%	8.10	2.17	0.02	0.13

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

in various ornamental crops. The result of present investigation demonstrates that rose cultivar Raktima can be multiplied *in vitro* employing the above protocol (Fig. 2).

REFERENCES

1. Arnold, N.P., Binns, M.R., Barthakur, N.N. and Cloutier, D.C. 1992. A study on the effect of growth regulators and time of plantlet harvest on the *in vitro* multiplication rate of hardy and hybrid tea roses. *J. Hort. Sci.* **67**: 727-35.

2. Bala, M., Singh, K.P. and Prasad, K.V. 2010. Standardization of *in vitro* mass multiplication protocol for hybrid tea rose cv. Pusa Mohit. *Indian J. Hort.* **67**: 225-29.

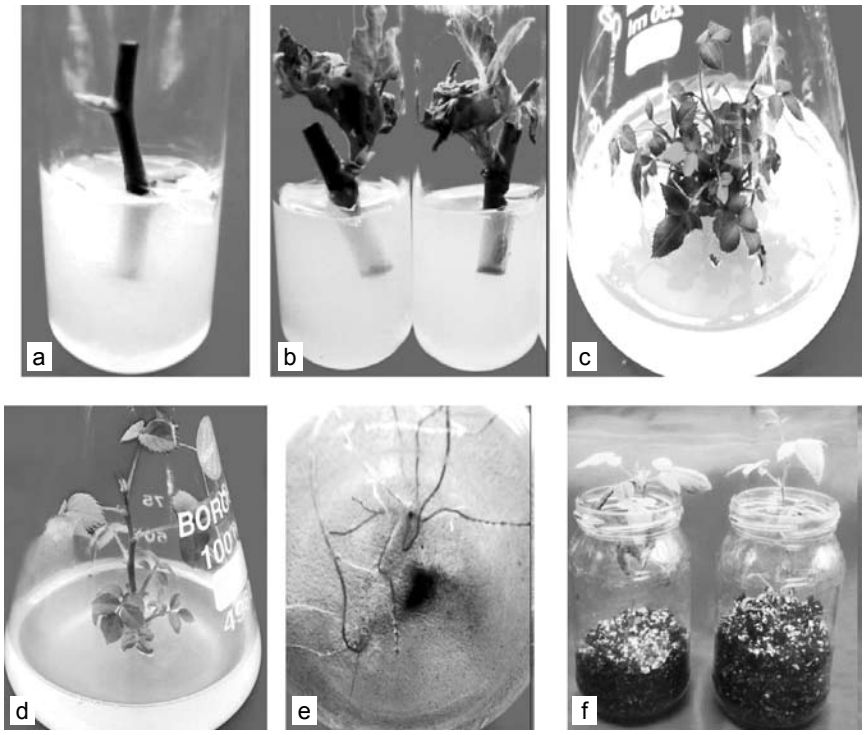

Fig. 2. *In vitro* propagation in rose cv. Raktima. (a,b,c) Culture initiation and shoot proliferation on medium MS + BAP (3.0 mg/ l) + NAA (0.1 mg/ l) + GA_3 (0.5 mg/ l); (d) Shoot elongation on MS + GA_3 (0.5 mg/l); Rhizogenesis on ½ MS + NAA (0.5 mg /l) + IBA (0.5 mg /l); (e) Rhizogenesis on ½ MS + NAA (0.5 mg /l) + IBA (0.5 mg /l); and (f) Acclimatization of *in vitro* raised plantlets in glass jars.

3. Bharadwaj, R., Singh, S.K., Pal, S. and Kumar, S. 2006. An improved protocol for micropropagtion of miniature rose (*Rosa chinensis* Jacq. var. *minima*) cultivars. *J. Orn. Hort.* **9**: 238-42.

4. Douglas, G.C., Rutledge, C.B., Casey, A.D. and Richardson, D.H.S. 1989. Micropropagation of floribunda, ground cover and miniature roses. *Plant Cell Tissue Organ Cult.* **19**: 55-64.

5. Kadam, G.B., Singh, K.P., Singh, A.K. and Jyothi, R. 2010. *In vitro* regeneration of tuberose through petals and immature flower buds. *Indian J. Hort*. **67**: 76-80.

6. Kulaeva,O.N. 1980. Cytokinins action on enzyme activity in plants. **In**: *Plant Growth Substances.*, F. Skoog (Ed.), *Proc. 10th International Conference on Plant Growth Substances*. Madison, Wisconsin, July 1979. Springer-Verlag, New York, pp. 119-28.

7. Kumar, S., Kanwar, J.K. and Sharma, D.R. 2004. *In vitro* regeneration of *Gerbera jamesonii* Bolus from leaf and petiole explants. *J. Plant Biochem. Biotech.* **13**: 73-75.

8. Kumar, M.A and Pratheesh, P.T. 2004. *In vitro* shoot multiplication in *Rosa indica* L. *Plant Cell Biotech. Mol. Biol*. **5**: 73-76.

9. Latado R.R., Adames A.H. and Neto, A.T. 2004. *In vitro* mutation of chrysanthemum (*Dendranthema grandiflora* Tzvelev) with ethyl methane sulphonate (EMS) in immature floral pedicels. *Plant Cell Tiss. Org. Cult.* **77**: 103-6, doi:10.1023/B:TICU.0000016481. 18358.55.

10. Mandal, A.K.A. and Datta, S.K. 2005. Direct somatic embryogenesis and plant regeneration from ray florets of chrysanthemum. *Biol. Plant*. **49**: 29-33, http://dx.doi.org/10.1007/s10535-005-0033-6.

11. Nahid, J.S., Saha, S. and Hottori, K. 2007. High frequency shoot regeneration from petal explants of *Chrysanthemum morifolium* Ramat. *in vitro*. *Pakistan J. Biol. Sci*., http://dx.doi.org/10.3923/pjbs.2007.3356.3361.

12. Pati, P.K., Rath, S.P., Sharma, M., Sood, A. and Ahuja, P.S. 2006. *In vitro* propagation of rose: A review. *Biotech. Adv*. **24**: 94-114.

13. Prasad, K.V. 1995. Studies on *in vitro* regeneration of roses. Ph.D. thesis submitted to Indian Agricultural Research institute, New Delhi.

14. Rout, G.R., Samantaray, S., Mottley, J. and Das, P. 1999. Biotechnology of the rose: A review of recent progress. *Scientia Hort*. **81**: 201-28.

15. Scotti Compos, P. and Pias, M.S. 1990. Mass propagation of the dwarf rose cultivar Rosamini. *Scientia Hort.* **43**: 321-30.

16. Singh, S.K. and Syamal, M.M. 2001. A short preculture soak in thidiazuron or forchlorfenuron improves Axillary shoot proliferation in rose micropropagation. *Scientia Hort.* **91**: 169-77.

17. Srivastava, L.M. 2005. *Plant Growth and Development*: *Hormones and Environment*. Academic Press, An imprint of Elsevier, San Diego, USA, pp. 172-88.

18. Verma, A.K., Prasad, K.V., Janakiram, T. and Kumar, S. 2012. Standardization of protocol for pre-treatment, surface sterilization, regeneration, elongation and acclimatization of *Chrysanthemum morifolium* Ramat. *Int. J. Hort.* **2**: 7-12.

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