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Standardization of protocol for *in vitro* multiplication of rose (*Rosa* × *hybrida*) cv. Happiness

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ABSTRACT

An efficient protocol for *in vitro* multiplication of *Rosa* × *hybrida* L. cv. Happiness was standardized using axillary bud segments. Out of different pre treatments for explants, the highest explant survival (80.25%) was obtained with T_1 pre-treatment comprising 0.2% Carbendazim + 0.2% Mancozeb-45 +150 mg/l 8-HQC for 4 hragitation on a horizontal shaker (200 rpm). Sucrose concentration of 30g/l in the medium was found to be optimum for *in vitro* shoot multiplication. Murashige and Skoog (MS) medium supplemented with 2.5 mg/l BAP + 5.0 mg/l kinetin + 0.1 mg/l NAA + 0.5 mg/l GA_3 was found most effective for culture establishment, however, MS medium comprising 2.5 mg/l BAP + 2.5 mg/l kinetin + 0.1mg/l NAA+ 0.5mg/l GA_3 along with 40 mg/l adenine sulphate was found to be better for shoot proliferation with highest number of micro shoots (7.10 shoots/explant). Rooting of micro shoots was induced on half strength MS basal medium supplemented with NAA (0.5 mg/l) and IBA (0.5 mg/l) rooting growth regulators. The regenerated plantlets were efficiently hardened in glass jars filled with coco peat + vermiculite + perlite (2:1:1) 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: Axillary bud, *In vitro*, Micropropagation, *Rosa* × *hybrida*, Regeneration

Rose is universally known as "Queen of Flowers". It belongs to the family Rosaceae. Traditionally, roses (*Rosa* × *hybrida*) have been considered to be one of the most valued flowers of the world because of their high ornamental value. The importance of beautiful flowers has grown over the years with the emergence of a global cut flower market. Roses are also recognized for economical benefits, being the best source of raw material to be used in cosmetics and perfumery industry.

Roses are generally multiplied vegetatively by cutting, budding and grafting methods. Although, multiplication by vegetative means is a predominant technique in roses, however, it does not ensure healthy and disease free plants. These methods of multiplication are very slow and time consuming. Moreover, environmental hazards make the cultivar degenerate gradually. Micropropagation procedures have facilitated mass production of good quality plantlets giving a boost to rose floriculture industry. It is one of the most important techniques of tissue culture that offers cost-effective implication in commercial floriculture (Soomro *et al*. 2003, Pati *et al*. 2006). This technique allow producing roses with high quality under a virus indexing programme, attending in this way the market demand. Several micropropagation protocols have been proposed in roses, however, cost efficient protocol capable of producing a large number of good quality micro shoots can be successful (Bressan *et al.* 1982). With this background in view, the present study was carried out to establish an efficient and reproducible protocol for rapid and large scale propagation of Happiness, an important cut rose.

MATERIALS AND METHODS

The rose cultivar Happiness maintained at Research Farm of the Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi was used for this experiment. The bud wood having 4-5 matured axillary buds were selected from the middle portion of current season flowering shoots. The bud wood was excised during morning hours with secateurs and cut into individual axillary bud segments of 1.5 cm length.

The explants were washed with Teepol at 0.1% solution for five minutes followed by washing under running tap water for 15 minutes. The nodal segments were then treated with different pre-treatments such as distilled water (Control; T_0), {Carbendazim (0.2%) + Mancozeb-45 (0.2%) + 8-Hydroxy Quinoline Citrate (8-HQC) (150

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mg/l) (T_1)}; {Carbendazim (0.2%) + 8-HQC (150 mg/l) (T_2)}; { Mancozeb-45 (0.2%) + 8-HQC (150 mg/l) (T_3)} and {Carbendazim (0.2%) + Mancozeb-45 (0.2%) (T_4)} for 4 hr agitation on horizontal shaker (200 rpm). The pre-treated explants were then surface sterilized with 0.1% mercuric chloride for three minutes followed by one washing of 70% ethanol and three rinsings with autoclaved distilled water.

The surface sterilized explants were cultured on MS medium supplemented with adenine sulphate (40 mg/l) and different concentrations of growth regulators, viz. 6-benzylaminopurine (BAP) (1.0 and 2.5 mg/l), α-naphthaleneacetic acid (NAA) (0.1 mg/l), kinetin (1.0, 2.5 and 5.0 mg/l) and gibberellic acid (GA_3) (0.5 mg/l) to find out the best treatment combination for culture establishment. The surface sterilized explants were also cultured on medium containing MS + BAP (2.5 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5mg/l) with five different concentrations of sucrose, i.e. 10, 20, 30, 40 and 50 g/l.

The sprouted shoots were then sub-cultured on MS medium supplemented with different concentrations of BAP (1.0 and 2.5 mg/l), NAA (0.1 mg/l), kinetin (1.0, 2.5 and 5.0 mg/l) and GA_3 (0.5 mg/l) to find out the best treatment combination for shoot proliferation. The multiplied shoots were separated and individual micro shoots were transferred onto elongation media comprising basal MS medium supplemented with concentrations of GA_3 (0.5 and 1.0 mg) to standardized its optimum dose for micro shoots elongation. Afterwards, elongated shoots were transferred individually in culture 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 30 g/l of sucrose was added for culture establishment and 40 g/l for shoot proliferation and elongation and 60 g/l was added in rooting medium. The *in vitro* rooted plantlets were removed from flasks, washed thoroughly with autoclaved double distilled water to remove the sticking agar-agar on roots. The rooted plantlets were dipped in Carbendazim (0.1%) for 10 seconds. The rooted plantlets were then acclimatized in glass jars and plastic pots filled with agro peat medium comprising of soilrite + cocopeat + perlite in a ratio of 1:1:1, supplemented with 1/2 strength liquid inorganic MS medium without calcium, growth regulators, sucrose and organic components. These glass jars were covered with polypropylene lids and polythene bags whereas plastic pots were covered with polythene bags. The plantlets were kept in culture room for 15 days before transferring to green house. For culture initiation, 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 hr light and dark cycles.

The data was analyzed using completely randomized design (CRD) and per cent data was subjected to Arc Sin √ % transformation before ANOVA.

RESULTS AND DISCUSSION

Explant sterilization

Pre-treatment of explants with different fungicidal and bacterial treatments has significant affect on explant survival, microbial contamination, bud sprouting and days to bud sprouting (Fig 1). Pre-treatment (T_1) comprising Carbendazim (0.2%) + Mancozeb-45 (0.2%) + 8-HQC (150 mg/l) for 4 hr agitation gave the highest explant survival and bud sprouting and was significantly superior to the other pre-treatments. Whereas, the minimum explant survival and bud sprouting were recorded with distilled water control (T_0). The pre-treatment of axillary bud explants with treatment T_1 comprising Carbendazim (0.2%) + Mancozeb-45 (0.2%) + 8-HQC (150 mg/l) for 4h reduced microbial contamination significantly as compared to control. Since the fungicides used in the treatment were both systemic and contact types, thus, they gave efficient control over microbial contamination. Similarly, 8-HQC was effective due to its bactericidal activities. Efficacy of these compounds has earlier been reported by Machado *et al.* (1991), Bharadwaj *et al.* (2006) and Kumari *et al.* (2013) in rose.

Culture establishment

The treatment T_6 comprising MS + BAP 2.5 mg/l+ kinetin 5.0 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l resulted in maximum survival of explants (89.35%), bud sprouting (8.11%) and earliest bud sprouting (3.25 days). The minimum response with respect to explant survival, bud sprouting and delayed sprouting was noted with growth

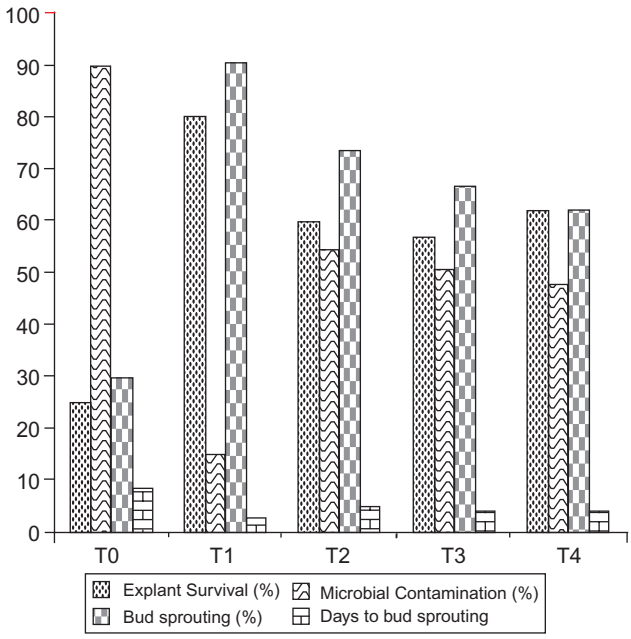

Fig 1 Effect of pre-treatments on *in vitro* culture initiation in hybrid tea rose cv. Happiness. T_0 = Control (Distilled water) for 4 hr agitation; T_1 = Carbendazim 0.2 % + Mancozeb-45 0.2% + 8 HQC 150 mg/l for 4h; T_2 = Carbendazim (0.2%) + 8-HQC (150 mg/l) for 4 hr; T_3 = Mancozeb-45 (0.2%) + 8-HQC (150 mg/l) for 4h; T_4 = Carbendazim (0.2%) + Mancozeb-45 (0.2%) for 4 hr.

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

Treatment	Explant survival (%)	Bud sprouting (%)	Days to bud sprouting
T_0 MS + Without growth regulators	42.95 (40.83)	45.15 (42.18)	8.65
T_1 + MS + BAP 1.0 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	64.14 (52.46)	64.82 (52.80)	6.25
T_2 + MS + BAP 2.5 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	67.12 (55.25)	73.24 (58.81)	5.78
T_3 + MS + BAP 1.0 mg/l + kinetin 1.0 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	75.4 (60.25)	80.18 (63.12)	5.15
T_4 + MS + BAP 2.5 mg/l + kinetin 1.0 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	82.15 (63.15)	83.11 (65.30)	4.12
T_5 + MS + BAP 2.5 mg/l + kinetin 2.5 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	86.25 (68.27)	85.35 (68.15)	3.85
T_6 + MS + BAP 2.5 mg/l + kinetin 5.0 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	89.35 (70.10)	87.45 (75.54)	3.25
CD (P=0.05)	2.68	3.35	0.82

The values given in parentheses denote the Arc Sin % values

regulator free MS medium (control) (Table 1, Fig 3a). The efficacy of BAP in stimulating shoot proliferation has earlier been reported by Bala *et al.* (2010) in *Rosa* × *Hybrida*, Kumar and Pratheesh (2004) in *Rosa indica*, Carelli and Echeverrigaray (2002) in *Rosa rugosa* and Vijaya *et al.* (1991) in rose. Arnold *et al.* (1992) and Douglas *et al.* (1989) reported the efficacy of cytokynin in combination with auxin or together with GA_3.

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

Treatment	Number of shoots proliferated/explant/subculture			
	First	Third	Fifth	Mean
T_0 MS + Without growth regulators	3.42	4.25	4.43	4.03
T_1 + MS + BAP 1.0 mg/l + NAA 0.1 mg/l + GA_3 0.5mg/l	4.15	4.7	4.97	4.61
T_2 + MS + BAP 2.5 mg/l + NAA 0.1 mg/l + GA_3 0.5mg/l	4.42	5.21	5.82	5.15
T_3 + MS + BAP 1.0 mg/l + kinetin 1.0 mg/l + NAA 0.1 mg/l + GA_3 0.5mg/l	5.25	5.6	6.33	5.73
T_4 + MS + BAP 2.5 mg/l + kinetin 1.0 mg/l + NAA 0.1 mg/l + GA_3 0.5mg/l	5.86	6.16	6.72	6.25
T_5 + MS + BAP 2.5 mg/l + kinetin 2.5 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	6.4	7.1	7.8	7.10
T_6 + MS + BAP 2.5 mg/l + kinetin 5.0 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	6.05	6.67	7.21	6.64
CD (P=0.05)	0.34	0.42	0.52	

Shoot proliferation

In vitro shoot multiplication largely depends on media containing BAP and kinetin as major plant growth regulators in combination with a low concentration of NAA or IBA (Syamal and Singh 1996, Singh and Syamal 1999, Carelli and Echeverrigaray 2002). The maximum number of shoots sprouted with treatment MS + BAP 2.5 mg/l + kinetin 2.5 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l along with adenine sulphate (40 mg/l) (Table 2, Fig 3b). After first sub culturing, maximum shoots per explant (6.40) was observed with the same treatment whereas, least number of shoots per explant (3.42) were noted in control, i.e. MS medium without any growth regulator. Shoot proliferation rate in third sub-culturing revealed that maximum number of shoots per explant (7.10) were recorded in the treatment T_5 compared to least number (4.25) in control (T_0) (Table 2). The optimum dose of growth regulators leads to good shoot proliferation and the same was observed in each sub culture. The favourable influence of BAP, kinetin and NAA in different metabolic processes (Kulaeva 1980) is known to effect plant metabolism. The better shoot proliferation in tissue culture might be due to the role of optimum dose of BAP and kinetin which enhance axillary branching and multiple shoot formation. Previous studies indicated that shoot proliferation may be due to loss of apical dominance (Bala *et al.* 2010 and Douglas *et al.* 1989).

Effect of sucrose

In addition to growth hormones, other media components are known to influence the *in vitro* growth response of rose cultures (Skirvin *et al.* 1990 and Chort and Roberts 1990). Sucrose concentration in medium showed a

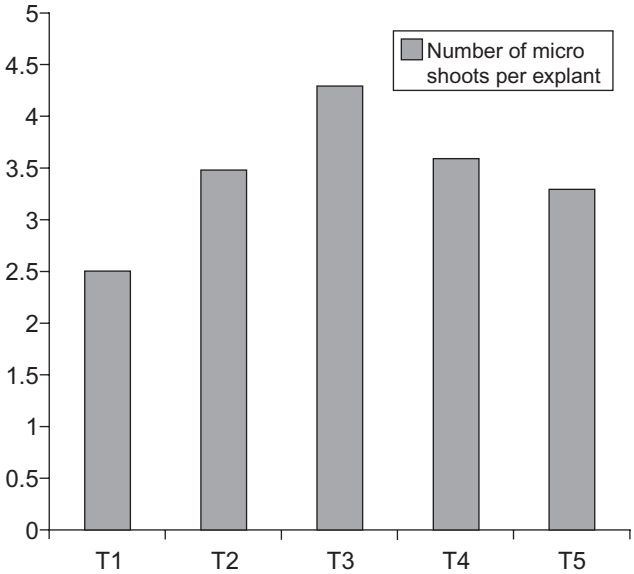

Fig 2 Effect of different sucrose levels on shoot multiplication. T_1 = 10g/l; T_2 = 20 g/l; T_3 = 30 g/l; T_4 = 40 g/l; T_5 = 50 g/l.

distinct influence on the rate of shoot proliferation in cultures. Proliferation rate depicted by mean number of micro shoots produced per culture in four weeks was highest in 30g/l sucrose, intermediate in 20 and 40 g/l and lowest in 10 g/l (Fig 2). Sucrose facilitates tissue differentiation in cultures without exogenous sucrose, the formation of tracheary elements may be greatly reduced or absent. Sucrose concentration of the medium increasingly influences the photosynthetic ability of *in vitro* growing shoots up to certain level but higher concentrations of sucrose suppress the activity (Hyndman *et al.* 1982 and Langford and Wainwright 1988). This could be the reason for inferior growth response in higher concentration, i.e. 50 g/l as compared to 30 g/l in the present investigation. However, Xing *et al.* (2010) reported browning of leaves and then inhibition of shoot growth on medium supplemented with 3% sucrose.

Rhizogenesis

Rooting of elongated plantlets was achieved on medium containing NAA in combination with IBA. The data presented in Table 3 depicts the effect of basal medium strength and auxins on days to root initiation, rooting percentage, 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) (15.5 days) (Fig 3d).

Table 3 Effect of basal medium strength and auxins on *in vitro* rooting of micro shoots of hybrid tea rose cv. Happiness

Treatment	Days to root initiation	Rooting (%)	Root length (cm)	Root quality	Plantlet growth
MS + Without growth regulators (control; T_0)	26.5	14.35 (22.46)	1.87	St	Poor
MS + NAA 0.5mg/1 (T_1)	20.2	33.27 (35.25)	2.34	S, Th	Good
MS + IBA 0.5mg/l (T_2)	18.3	25.15 (30.05)	3.59	S, Th	Good
1/2 MS + NAA 0.5 mg/l + IBA 0.5 mg/l (T_3)	14.3	90.17 (72.15)	4.71	Th, L	Very good
1/2 MS + NAA 1.0 mg/l + IBA 1.0 mg/l (T_4)	15.5	83.62 (65.72)	4.65	Th, L	Very good
CD (P=0.05)	1.23	5.32	1.21		

The values given in parentheses denote the Arc Sin √% values
St = Stunted; S= Small; L= Long; Th= Thick

However, delayed root initiation was exhibited in control i.e. 1/2 MS without auxins (26.5 days). The highest rooting was observed for the treatment half strength MS along with

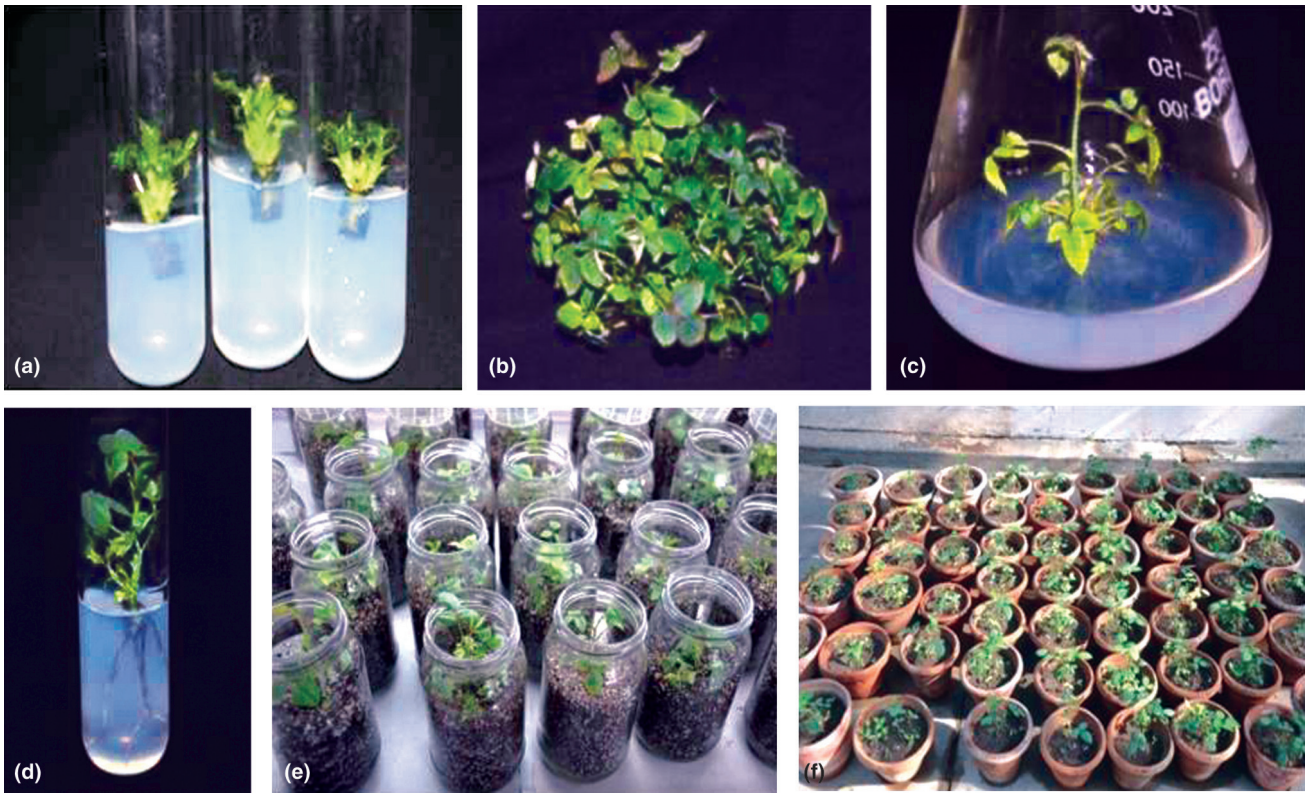

Fig 3 *In vitro* plant regeneration in Hybrid Tea rose cv. Grand Gala. (a) Explant establishment in MS medium comprising BAP 2.5 mg/l + kinetin 5.0 mg/l + NAA 0.1mg/l + GA_3 0.5 mg/l (b) Proliferation in MS medium comprising BAP 2.5 mg/l + kinetin 2.5 mg/l + NAA 0.1 mg/l + GA_3 0.5mg/l along with adenine sulphate (40 mg/l), (c) Elongation of shoots on MS medium along with GA_3 (0.5 mg) and adenine sulphate (40 mg) / lt , (d) *In vitro* rooting on MS medium containing NAA (0.5 mg/l) + IBA (0.5 mg/l), (e) Gradual hardening in glass jar, (d) Acclimatized plants in pots under glasshouse.

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

Treatment	Survival (%)	Plant height (cm)	Plant height leaves/plantlet
Plastic pots with polythene bag cover	73.12 (58.47)	6.75	3.59
Glass jar with polythene bag	79.25 (62.50)	8.15	4.6
Glass jar with plastic lid	88.25 (69.55)	8.65	5.85
CD (P=0.05)	5.82	2.15	0.03

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

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 maximum per cent rooting (90.17) was found in 1/2 MS + NAA (0.5 mg/l) + IBA (0.5 mg/l) followed by 1/2 MS + NAA (1.0 mg/l) + IBA (1.0 mg/l) (83.62), whereas minimum was in control (14.35). The longest root length (4.71 cm) was observed in half strength MS + NAA (0.5 mg/l) + IBA (0.5 mg/l). However, shortest root length was observed in control MS medium without auxins (1.87 cm).

The qualitative data revealed that root were not only few but were having stunted growth in medium devoid of auxins. The roots were small and thick in medium supplemented with NAA and IBA individually. However, roots on half strength medium supplemented with both the auxins (NAA and IBA) were thick and long. It was evident from the study that there has been synergistic effect when the two auxins were employed together and optimum role of two auxins was reported earlier also by Bala *et al.* (2010), Kumari *et al.* (2013) and Bharadwaj *et al.* (2006) in rose.

Plantlet acclimatization

The treatment where plantlets were acclimatized in glass filled with agro peat medium supplemented with half strength MS medium (macro and micro organics) and covered with polypropylene lids exhibited highest plantlet survival (88.25 %), plant height (8.65 cm) and good number of leaves (5.85/plantlet) (Table 4, Fig 3e). These results might be due to less open space and appropriate relative humidity as compared to those hardened in plastic pots covered with polythene bags. Efficacy of glass jar covered with polypropylene lid was also reported earlier by Bala *et al.* (2010) and Kumari *et al.* (2013). In vitro raised plants were acclimatized in pots under glasshouse. The results of present study demonstrate that cv. Happiness can be multiplied *in vitro* and further used for the induction of variation through *in vitro* mutagenesis in *Rosa × hybrida.*

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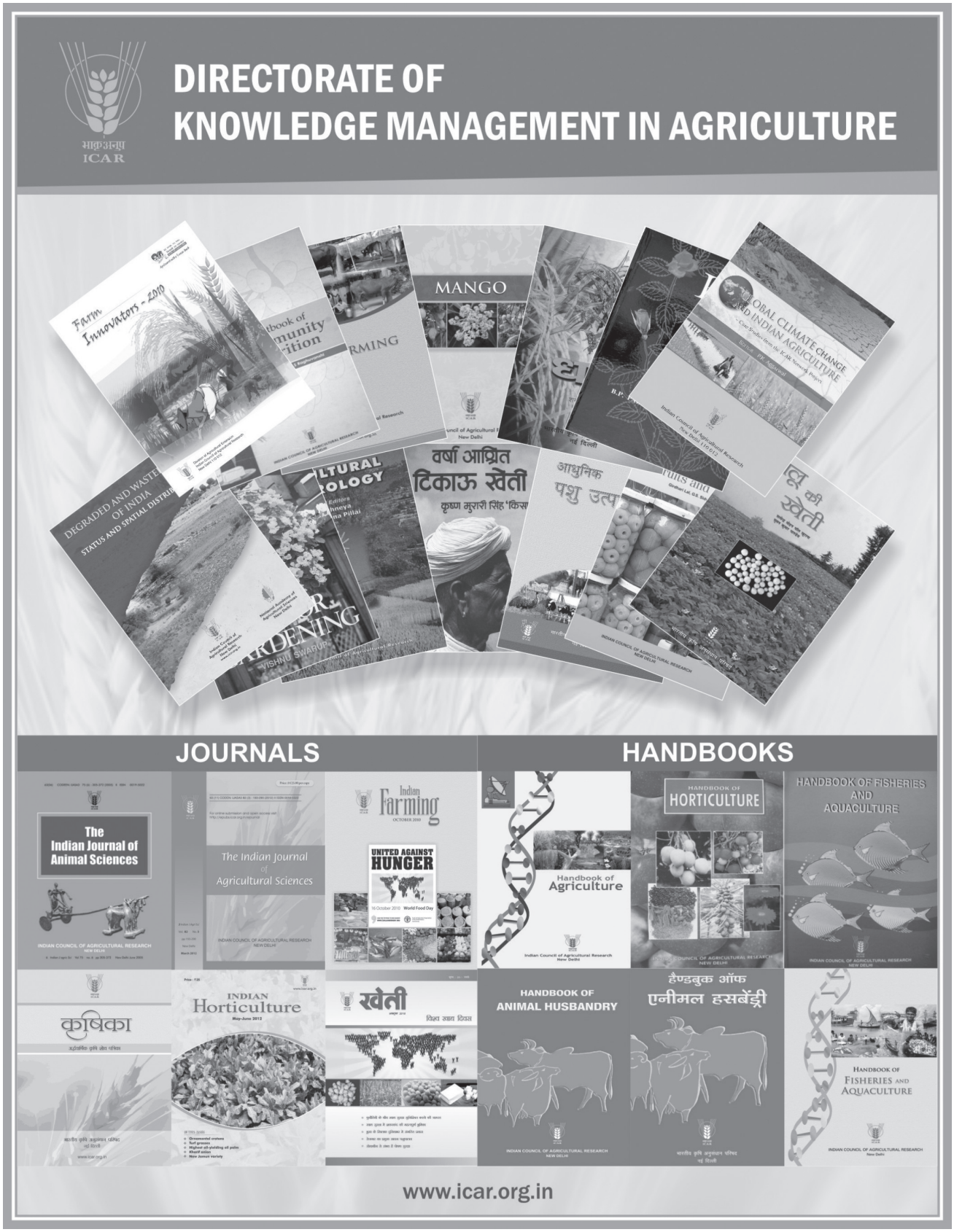
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