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Full Research Article

In vitro Sterilization, Rooting and Acclimatization of Difficult-to-root *Bougainvillea* Cultivars

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Abstract

Bougainvillea is a widely used high value landscape plant. It is commonly propagated by hardwood cuttings but this method is tedious and time consuming. Moreover, there are certain cultivars where rooting is very low. For easy, quick, and mass multiplication of such cultivars, tissue culture technique can be put to use. Tissue culture has been proved to be useful for successful multiplication in case of a number of vegetatively propagated shrubs. Present investigation was carried out in order to standardize a protocol for *in vitro* rooting and acclimatization of two difficult-to-root *Bougainvillea* cultivars, viz., Mahatma Gandhi and Refulgens. Nodal sections with axillary buds were excised, surface-sterilized and cultured on MS medium supplemented with plant growth regulators. Agitation of explants in carbendazim (0.1%) + Metalaxyl (0.1%) +8-HQC (200 mg l^{-1}) for 3 hr followed by quick dip in ethyl alcohol (70%; v/v) for 30 sec and surface sterilization in $HgCl_2$ (0.1%) for 5 min was found to be best for eliminating microbial contamination prior to inoculation. *In vitro* grown micro-shoots were grown in MS media along with various concentrations of NAA and/or IBA in order to induce rooting. Highest *in vitro* rooting (64.99%) of micro-shoots was noted in the treatment where half-strength MS medium was supplemented with 1.0 mg l^{-1} IBA. Acclimatization was most effective in glass jar with polypropylene cap. The hardened plantlets were successfully transferred to the glasshouse after a short period of *in vitro* acclimatization.

1. Introduction

Bougainvillea belongs to family Nyctiginaceae and is native of South America. It flourishes well in all the metropolitan cities. It is one of the most important garden plants, particularly in the tropical and sub-tropical regions of the world. *Bougainvillea* is a versatile plant and rich in its varietal wealth which can be used in different ways like bush, standard shrub, climber, hedge, pot plant, bonsai and ground cover for sloppy lands and to make the garden colourful for most part of the year (Singh et al., 2011). It has earned a pride position in floriculture and land scaping due to its lush green foliage and its colourful bracts. Use of diesel, petrol, in all ways and smoke from fire crackers, leaf burning, plastic/wood burning greatly affects the ambient air quality. *Bougainvillea* is a pollution tolerant plant and can help in the mitigation of air pollution besides its ornamental value in the landscaping (Sharma et al., 2005). It is also drought tolerant, salt tolerant, and wind resistant (Kobayashi et al., 2007). *Bougainvillea* flourishes well everywhere, even in the heavily polluted areas of big cities and industrial towns and that is why it is called as 'Glory of the Garden'. Its broad leaf surface area has the capacity for absorbing toxic substances and thus it works as pollution sink.

Now-a-days nurserymen are taking keen interest in the propagation of *Bougainvillea* to produce large number of plants. *Bougainvillea* is primarily propagated by stem cuttings, but lack of competence to form adventitious roots by cuttings occurs routinely and is an obstacle for the vegetative propagation (Celine et al., 2006). The use of cuttings from stems, leaves, roots or terminal buds are considered the most commonly applied technique due to its practicability and simplicity (Okunlola and Ibironke, 2013). But, in case of certain *Bougainvillea* cultivars there is difficulty in rooting of

cuttings (Hartman and Kester, 1989). Also, the plants which are produced by air-layering are in small quantity and need more skill and labour (Ahmad et al., 2007). These issues curtail the free use of elite cultivars like Mahatma Gandhi, Refulgens, Sweat Heart, Dr. H.B. Singh, Mary Palmer, Thimma, etc. Tissue culture is a well-established method for the rapid propagation and multiplication of plants, which are otherwise difficult to multiply by conventional methods. It can be used to propagate plants of economicimportancein large quantities out of season (Malik et al., 2007). The present research was undertaken in order to overcome such problems, with an objective to provide an easy, viable and reproducible method for *in vitro* rooting of two difficult-to-root *bougainvillea* cultivars, Mahatma Gandhi and Refulgens.

2. Materials and Methods

The experiments were conducted during July, 2013 to April, 2014 at the Central Tissue Culture Laboratory, LBS Centre, IARI, New Delhi. Two difficult-to-root cultivars, *B. spectabilis* cv. Refulgens and *Bougainvillea peruviana* cv. Mahatma Gandhi (syn. Mrs. H.C. Buck) maintained at the 'International *Bougainvillea* Registration Authority' Repository' at IARI, New Delhi were selected for the study.

2.1. Explant

The middle portion of the new growing tender stems, were selected and cuttings were taken in the early morning. Small sections of 2.0–2.5 cm in length, each containing a dormant bud were made, and were put under running water for about half an hour and thereafter washed in Teepol® (0.1%) solution and thoroughly rinsed 3–4 times with tap water. Final rinsing was done using double distilled water.

2.2. Surface sterilization

The initial pre-treatments and surface sterilization of explants were merged to form five combination treatments, which were carbendazim (0.5%, 0.5%, 0.2, 0.1) along with Metalaxyl (0.5%, 0.5%, 0.2, 0.1) for varying durations of (30, 60, 120, 180) mins., all these treatments were supplemented with 200 mg l^{-1} 8-HQC for 30 minutes and treatment with ethyl alcohol (70%, v/v) for 30 sec followed by mercuric chloride (0.1%) for 5 minutes Treatment only with 0.1% $HgCl_2$ for 5 minutes was taken as control. The explants were established on MS + sucrose (40 g l^{-1}) + BAP (3 mg l^{-1}) +NAA (0.1 mg l^{-1}) +GA_3 (0.5 mg l^{-1}). Further experiments were conducted using the best pre-treatment of the above mentioned pre-treatment and surface sterilisation combination experiment.

2.3. Rooting media

MS medium supplemented with BAP (4.0 mg l^{-1}) and kinetin (0.5 mg l^{-1}) was used for shoot proliferation/multiplication. In order to optimize the most suited medium for rooting *in vitro* established micro-shoots, they were excised individually and sub-cultured onto half-strength MS medium fortified with different concentrations of NAA (0.5, 1.0, 2.0, 3.0, 4.0) and IBA (0.5, 1.0, 2.0) and NAA+IBA (2.0, 2.0) along with 60 gl^{-1} sucrose.

2.4. Acclimatization

For acclimatization of rooted plantlets (28 day-old), two types of strategies were tried, i.e. glass jars (with polypropylene caps) and plastic pot (4.5') with polythene cover. Before transferring the rooted plantlets for acclimatization, they were washed with sterile distilled water followed by dip in carbendazim (0.2%). The potting media used for cclimatization comprised of cocopeat+perlite (1:1) moistened with 1/4th strength MS basal salts devoid of organics. For initial 15 days, the plantlets were kept in culture room (white fluorescent light 57 µmol m^{-2} s^{-1} with 16/8 hr light and dark photoperiod, 65–70% RH, 26±1 °C). Thereafter the lid was loosened for the next 15 days. The polythene cover was punctured after 15 days to reduce moisture levels. After complete acclimatization, the plants were transferred to shade net house for further growth and maintenance.

The data was analyzed employing Factorial Completely Randomized Block Design (FCRD) and the percent data were subjected to angular transformation before ANOVA.

3. Results and Discussion

For *in vitro* culture establishment suitable Pre-treatment and surface sterilization technique was standardized in explant collected from field-grown plants of the two *Bougainvillea* cultivars (Table 1 and 2).

3.1. Microbial contamination

As evident, the different pre-treatments and surface sterilization treatments gave significant effect in reducing contamination. The lowest microbial contamination (31.94%) was observed in the treatment comprising 3 hr agitation in 0.1% carbendazim + 0.1% Metalaxyl+8-HQC (200 mg l^{-1}) followed by surface sterilization using 70% ethanol (30 sec) followed by agitation in 0.1% $HgCl_2$ (5 minutes), The treatment comprising carbendazim 0.2%+Metalaxyl 0.2%+8-HQC 200 mg l^{-1} for 2 hr followed 70% EtoH (30 sec) and 0.1% $HgCl_2$ (5 minutes) was as par with the previous mentioned treatment, where the microbial infection was 36.11%. In control treatment, only 0.1% $HgCl_2$ (5 minutes) without any pre-treatment was tried, the microbial contamination was found to be highest (73.61%).The minimum microbial contamination (22.22%) was observed in Mahatma Gandhi with pre-treatment for 3 hr agitation in 0.1% carbendazim+0.1% Metalaxyl+ 200 mg l^{-1}

Table 1: Standardization of the pre-treatments on *in vitro* culture establishment in *Bougainvillea* cultivars

Treatment	Microbial infection (%)		Mean	Culture establishment (%)		Mean	Bud sprouting (%)		Mean	Days to bud sprouting		Mean
	Refulgens	Mahatma Gandhi		Refulgens	Mahatma Gandhi		Refulgens	Mahatma Gandhi		Refulgens	Mahatma Gandhi	
0.1% $HgCl_2$ 5 min. (control)	77.78 (62.54)	69.44 (56.48)	73.61	20.25 (26.71)	20.45 (26.85)	20.35	13.33 (21.39)	12.22 (20.44)	12.77	11.11	9.25	10.18
Carbendazim 0.5%+ Metalaxyl0.5%+8-HQC 200 mg l^{-1} (30 min.) +70% EtoH (30 sec)	45.00 (43.13)	38.89 (38.59)	41.90	38.21 (38.17)	29.30 (32.77)	33.75	35.00 (36.28)	24.44 (29.6)	29.72	11.05	11.18	11.12
Carbendazim 0.5%+ Metalaxyl 0.5%+8-HQC 200 mg l^{-1} (60 min.)+ 70% EtoH (30 sec)	45.56 (42.48)	33.33 (35.24)	39.40	38.56 (38.41)	32.3 (34.63)	35.43	33.33 (35.24)	31.78 (34.33)	32.55	10.9	10.26	10.58
Carbendazim 0.2%+ Metalaxyl 0.2%+8-HQC 200 mg l^{-1} (120 min.)+ 70% EtoH (30 sec)	44.44 (41.78)	27.78 (31.82)	36.11	46.38 (42.94)	39.5 (38.94)	42.94	42.33 (40.57)	35.56 (36.63)	33.95	10.88	10.11	10.50
Carbendazim 0.1%+ Metalaxyl 0.1%+8-HQC 200 mg l^{-1} (180 min.)+ 70% EtoH (30 sec)	41.66 (40.16)	22.22 (28.11)	31.94	53.65 (47.06)	49.50 (44.71)	51.57	50.56 (45.34)	42.67 (40.80)	46.61	10.77	10.47	10.62
Mean	50.88	38.33		39.41	34.21		34.91	27.33		10.942	10.254	
CD (p=0.05)												
Treatment (T)			23.71			20.53			12.77			0.36
Genotype (G)			13.01			NS			29.72			NS
T×G			NS			21.25			32.55	11.11	9.25	0.50

8-HQC followed by surface sterilization with 30 sec dip in 70% EtoH followed by agitation in 0.1% $HgCl_2$ (5 minutes).

3.2. Culture establishment

Irrespective of the pre-treatment/ surface sterilization treatment tried, the two cultivars varied significantly in their response with regard to culture initiation. Amongst cultivars, Refulgens was found to give better response (50.88%) compared to Mahatma Gandhi (38.33%).

The interaction effect of treatment×genotype was found to be non-significant. Among the various treatments, highest culture establishment and bud sprout (51.57 and 46.61% resp.) were observed in the treatment where 3 hr agitation in 0.1% carbendazim+0.1% Metalaxyl+8-HQC (200 mg l^{-1}) followed by surface sterilization treatment with 70% ethanol (30 sec.) and agitation in 0.1% $HgCl_2$ (5 minutes) was given. The treatment comprising carbendazim 0.2%+Metalaxyl 0.2%+8-HQC 200 mg l^{-1} for 2 hr followed 70% EtoH (30 sec) and 0.1% $HgCl_2$ (5 minutes) was on par with the previous mentioned treatment for both culture establishment and bud sprouting (49.50 and 33.95%, respectively). Lowest culture establishment aswell as bud sprouting (20.45 and 12.77%) were recorded in the control treatment where the explants were kept in double-distilled water and 0.1% $HgCl_2$ treatment (5 minutes) was given before inoculation.

Further, for treatment×genotype interaction, significant effect was noted. The maximum culture establishment (53.65%) was observed in Refulgens with pre-treatment having 3 hr agitation in 0.1% carbendazim+0.1% Metalaxyl+200 mg l^{-1} 8-HQC followed by surface sterilization with 30 sec dip in 70% EtoH followed by agitation in 0.1% $HgCl_2$ (5 minutes), also the minimum culture establishment (20.25%) was observed in Refulgens in control treatment.

3.3. Bud sprouting

Among the various pre-treatment and surface sterilizations

Table 2: Standardization of the pre-treatments on the *in vitro* culture establishment in *Bougainvillea* cultivars

Treatment	No of micro-shoots formed		Mean	Callusing, if any		Remarks
	Refulgens	Mahatma Gandhi		Refulgens	Mahatma Gandhi	
0.1% $HgCl_2$ 5 min. (control)	1.00	1.00	1.00	+	++	Pale yellow leaves and stunted growth
Carbendazim 0.5%+Metalaxyl0.5%+8-HQC 200 mg l^{-1} (30 min.)+70% EtoH (30 sec)	1.00	1.06	1.03	+	++	-do-
Carbendazim 0.5%+Metalaxyl 0.5%+8-HQC 200 mg l^{-1} (60 min.)+70% EtoH (30 sec)	1.00	1.07	1.04	+	++	Normal shoots
Carbendazim 0.2%+Metalaxyl 0.2%+8-HQC 200 mg l^{-1} (120 min.)+70% EtoH (30 sec)	1.05	1. 02	1.03	+	++	Normal shoots
Carbendazim 0.1%+Metalaxyl 0.1% + 8-HQC 200 mg l^{-1} (180 min.)+70% EtoH (30 sec)	1.15	1.04	1.09	+	++	Greener and longer normal
Mean	1.104	1.06				
CD (p=0.05)						
Treatment (T)			NS			
Genotype (G)			0.08			
T×G			0.14			

given, the effect of the given treatments was significant. Highest (46.61%) bud sprouting was observed in carbendazim 0.1%,Metalaxyl 0.1% and 8-HQC 200 mg l^{-1} (180minutes) followed by 70% EtoH (30 sec) and 0.1% $HgCl_2$ (5 minutes). While the control treatment showed the lowest (12.77%) bud sprout. Fastest (10.18 days) bud sprouting was observed in the control treatment (Table 1), where only 0.1% $HgCl_2$ (5 minutes) dip was given, while the slowest (11.12 days) sprouting was seen in carbendazim 0.5%, Metalaxyl 0.5% and 8-HQC 200 mg l^{-1} (30 minutes) followed by 70% EtoH (30 sec) and 0.1% $HgCl_2$ (5 minutes).

The number of micro-shoots formed per explant (Table 2) after giving the pre-treatments and surface sterilization were found to be non-significant. While amongst the treatments, the treatment comprising 3 hours agitation in 0.1% carbendazim+0.1% Metalaxyl+8-HQC (200 mg l^{-1}) followed by surface sterilization treatment with 70% ethanol (30 sec) followed by agitation in 0.1% $HgCl_2$ (5 minutes) led to formation of highest number of micro-shoots per explant (1.09). The lowest (1.0) number of micro-shoots were formed in the control treatment.

The observations on callus induction showed that amongst the two cultivars, the cultures of Mahatma Gandhi induced higher callusing in comparison to Refulgens irrespective of the growth regulator treatments.

The importance of pre-treatment has been emphasized by a number of previous workers. Bacteria, which may be introduced in cultures as epiphytes and endophytes or later during culture handling, sometimes remain covert or latent and go unnoticed in the absence of specific indexing (Thomas, 2004). Plant pathogens are less likely to be introduced into plant tissue culture if explants are screened for absence of blemishes and disease symptoms or if pre-treatments (e.g. fungicides and/or antibiotics) are applied to donor plants (Sivasithamparam et al., 2002).

Surface of plant parts carry a wide range of microbial contaminants. To avoid this source of infection, the tissue/explant must be thoroughly surface-sterilized before planting it on the nutrient medium; tissues with systemic or latent fungal or bacterial infections are usually discarded in tissue culture studies (Bhojwani and Razdan, 1983).

Successful disinfestation of explantsis a re-requisite for *in vitro* culture and often involves a standard set of treatments, which vary with the type of explant and species in question (Thorpe and Patel, 1984).

The efficacy of carbendazim, metalaxyl and 8-HQC have earlier been demonstrated by (Kadam et al., 2013) in Achyranthes aspera, (Singh et al., 2013) in rose. The pre-treatment of axillary bud explants with carbendazim (0.2%)+ diathane-M-45 (0.2%)+8-HQC (200 mg l^{-1}) for 3 h was found to minimize microbial contamination in rose (Singh et al., 2013).

The *in vitro* establishedmicro-shoots were transferred to medium supplemented auxin(s) (Table 3), in order to optimize rooting. A total of nine treatments were tried. The auxin treatment.

effect irrespective of the genotype was found to be significant.

Table 3 : Effect of auxin(s) on *in vitro* rooting of *Bougainvillea* micro-shoots

Treatment	Rooting (%)			No. of days required for root initiation		
	Refulgens	Mahatama Gandhi	Mean	Refulgens	Mahatama Gandhi	Mean
NAA (0.5 mg l^{-1})	14.25 (22.14)	16.66 (24.04)	15.45	18.45	18.33	18.39
NAA (1.0 mg l^{-1})	10.56 (19.00)	16.66 (24.06)	13.61	22.55	19.66	21.10
NAA (2.0 mg l^{-1})	44.44 (41.78)	38.45 (38.29)	41.45	12.83	17.45	15.14
IBA (0.5 mg l^{-1})	24.45 (29.60)	46.66 (41.90)	35.55	24.57	21.0	22.7
IBA (1.0 mg l^{-1}	35.55 (36.57)	94.44 (76.31)	64.99	23.45	15.6	19.52
IBA (2.0 mg l^{-1})	30.45 (33.45)	49.99 (44.94)	40.22	24.45	23.1	23.77
IBA (2 mgl^{-1}) + NAA (2 mg l^{-1})	29.45 (32.83)	16.66 (24.06)	23.05	21.35	24.25	22.80
NAA (3.0 mg l^{-1})	72.22 (58.18)	15.42 (23.11)	43.82	12.75	20.35	16.55
NAA (4.0 mg l^{-1})	77.77 (61.82)	20.35 (26.78)	49.06	11.86	9.35	10.61
Mean	64.81	35.03		19.14	18.78	
CD (p=0.05)						
Treatment			8.56			7.32
Genotype			10.05			NS
T×G			13.65			14.18

Highest (64.99%) rooting was noted in the treatment where half-strength MS medium was supplemented with IBA (1.0 mg l^{-1}). Meagre rooting (13.61%) was recorded in the treatment where MS medium was supplemented with NAA (1.0 mg l^{-1}).

Among the two cultivars, Refulgens showed the highest (72.22%) rooting on NAA (3.0 mg l^{-1}), while Mahatma Gandhi responded best (94.44%) with IBA (1.0 mg l^{-1}) (Figure 1).

Amongst the cultivars, there was significant response for rhizogenesis. Refulgens showed higher rooting (64.81%) compared to Mahatma Gandhi where the rooting was low (35.03%). The interaction effect of the treatment×genotype was found to be significant. Irrespective of auxin levels and cultivars, the highest (94.44%) rooting was observed in Mahatma Gandhi in the treatment where MS medium was supplemented with IBA (1.0 mg l^{-1}). However, poorest rooting (10.56%) was observed in Refulgens in the treatment, where MS medium was supplemented with NAA (1.0 mg l^{-1}).

The treatments varied significantly for the number of days required for root initiation. Earliest (10.61 days) root initiation was observed in case of the treatment where MS medium was supplemented with 4.0 mg l^{-1} NAA, which was non-significantly followed by the treatment where MS medium was supplemented with NAA (2.0 mg l^{-1}). The treatment where MS medium was supplemented with IBA (2.0 mg l^{-1}) took the longest number of days (23.77) to induce rooting.The rooting response of the two cultivars was found to be non-significant. The interaction effect of the treatment×genotype was found to be significant. Irrespective of the auxin treatments and genotype combinations, earliest (9.35 days) rooting was observed in Mahatma Gandhi with MS medium fortified by NAA (4.0 mg l^{-1}). The rooting was most delayed (24.45 days) for Refulgens in micro-shoots with MS medium fortified with IBA (1.0 mg l^{-1}).

In *Bougainvillea glabra*, Chaturvedi (1978) induced roots in micro-shoots in medium containing 0.1 mg l^{-1} each of IBA and 2, 4, 5-T. (Sharma et al., 1981; Javed et al., 1996; Ahmad et al.,

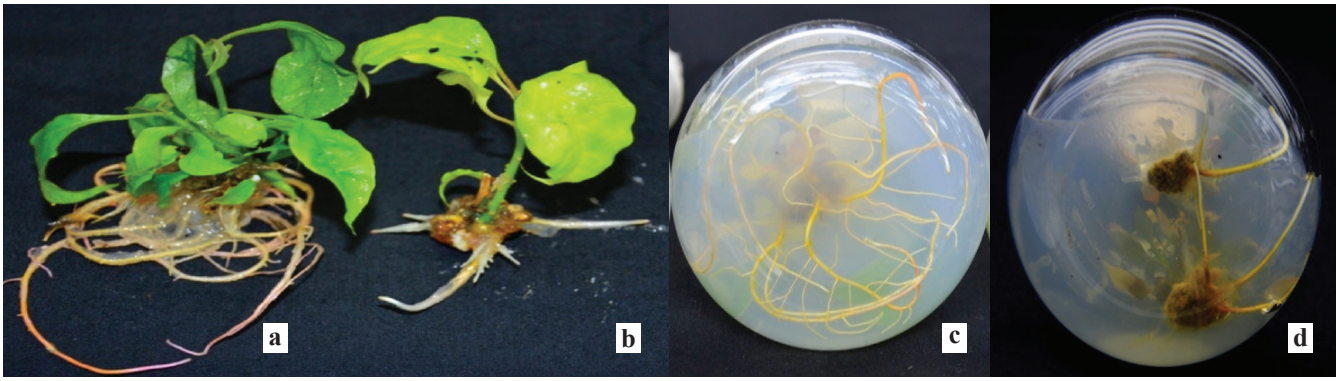

Figure 1: *In vitro* rooting in Bougainvillea cv. 'mahatma Gandhi' and 'Refulgens'. Mahatma Gandhi (a & c) half MS+1 mg l^{-1} IBA, (b & d) half MS + 1 mg l^{-1} NAA.

2007) also advocated the use of IBA for *in vitro* root-induction in case of *Bougainvillea*.

Sharma and Chaturvedi (1988) reported cent percent rooting in difficult-to-root cultivar 'Scarlet Queen Variegated' when the medium was supplemented with 5 mg l^{-1} NAA for 15 days and then transferred onto a medium (pH 4.5) containing 0.5 mg l^{-1} NAA. (Kumari et al., 2002) also obtained good rooting on medium supplemented with 1 mg l^{-1} NAA in chrysanthemum. Recently in 2012, Datta and Mandalin *Bougainvillea* cvs Los Banos Variegata and Mary Palmer Special reported rooting of individual shoots on 4 mg l^{-1} NAA. In other shrubs too, like *Clerodendrum colebrookianum* (Mao et al., 1995), *Lavandula Stoec* has (Nobre et al., 1996) and *Rotula aquatic* (Martin et al., 2003) the efficacy of use of NAA for *in vitro* has been reported. So, NAA was used as a treatment to induce rooting.

Use of combination of auxins can been observed in previous research works too. (Shah et al., 2006) observed that 2.5 mg l^{-1} NAA combined with 2.5 mg l^{-1} IBA gave 100% root induction. Datta and Mandal (2012) reported that neither NAA nor BA alone but lower BA concentration (0.5 mg l^{-1}) of in combination with all tested concentrations of NAA was effective in *Bougainvillea*.

In all the treatments put to test for root initiation, increased (60 mg l^{-1}) concentration of sucrose was taken for inducing root initiation. Increasing the concentration of the carbon source leads to root initiation, this has been reported by (Conner et al., 1993) the use of 6% sucrose to be best in asparagus. Khan (1999) also reported that sucrose concentration was positively correlated with rooting percentage, root number shoot^{-1} and root length.

Amongst the two cultivars, there was significant difference in their responses, Refulgens showed the highest (72.22%) rooting on NAA (3.0 mg l^{-1}), and in Mahatma Gandhi showed the highest (94.44%) rooting in IBA (1.0 mg l^{-1}). This may be attributed to the difference in cultivars used. It is well documented that plant growth regulators effective for one species may not be equally effective for another cultivar or species (Novak and Juvova, 1982/83; (Jang et al., 2003).

3.4. Acclimatization

For the acclimatization of the rooted plantlets, two strategies were tried (Table 4) (Figure 2), and the response of the two cultivars (survival percentage and shoot length) were found to be significant. Irrespective of the cultivars, use of glass jar with polypropylene cap was found to be better, where the

Table 4: Effect of different acclimatization strategies on the survival of *in vitro* raised *Bougainvillea* plantlets

GA_3 (mg l^{-1})	Survival (%)			Shoot length (cm)		
	Refulgens	Mahatama Gandhi	Mean	Refulgens	Mahatama Gandhi	Mean
Glass jar with PP cap	45.50 (42.42)	85.50 (67.86)	65.5	4.25	5.50	4.87
Plastic pot (4.5")	30.75 (33.65)	62.50 (52.24)	46.62	3.55	4.25	3.90
Mean	38.12	74.00		3.90	4.90	
CD (p=0.05)						
Treatment			5.99			0.32
Genotype			12.00			0.37
T×G			17.10			0.82

Figure 2: (a & c) Use of Glass jar with PP cap or polythene cover (depending on the length of the plant) with Cocopeat: perlite (1:1) medium for hardeningin Refulgens and Mahatma Gandhi resp. (b & d) FYM: sand: garden soil (2: ½ : ½) in refulgens and Mahatma Gandhi resp.

survival rate was 65.5% with mean shoot length was 4.87 cm. The genotype effect was found significant. Mahatma Gandhi plantlets showed higher survival (74.00%) with longer shoots (4.90 cm) in comparison to Refulgens plantlets. Refulgens had low survival (38.12%) with shorter mean shoot length (3.90 cm).

Acclimatization of micropropagated plantlets on a large scale is generally carried out in a polyhouse where a gradation of high-to-low humidity and low-to-high levels of irradiance are maintained (Bhojwani and Razdan, 1983). There is a lack of knowledge about the biological behavior of plantlets during acclimatization, as slow growing *in vitro* plants are extremely sensitive to environmental conditions, especially to water stress. Different aspects of water resistance, such as cuticle development and osmotic adjustment, need the accumulation of compounds such as lipids, waxes, sugars and amino acids. These compounds require large amounts of carbon and energy, which would therefore not be available for growth (Lakso et al., 1986). Numerous recommendations and procedures have been identified those contributing towards successful hardening percentages of *in vitro* rooted plantlets. Most of these techniques are based on controlling the ambiance of the developing plantlets in terms of humidity, temperature and nutrition in a pre-sterilized medium.

In the present study, *in vitro* raised plantlets were successfully transferred to the glasshouse after a short period of acclimatization. Use of glass jar with polypropylene cap was found to be better, for the hardening of the rooted plantlets. Earlier, (Singh et al., 2011) while comparing different hardening strategies, glass jar with polypropylene cap (PP) found to be most effective as far as hardening success (66.67%) was concerned in grape.

Hatzilazarou (2003) in *Bougainvillea* sp. 'Alexandra' reported that the transplanting losses during acclimatization were higher for plantlets derived from *in vitro* rooted micro-cuttings than from *ex vitro*-rooted micro-cuttings. Acclimatization in the fog system favoured the survival rate and reduced the transplanting losses. On the other hand, the transplanting losses were much higher on the greenhouse bench and especially under full sunlight.

Both the difficult-to-root cultivars (Mahatma Gandhi and Refulgens) rooted well under *in vitro* conditions.

4. Conclusion

Minimization of microbial contamination (31.94 %) by agitation of the explants in a solution of 0.1% carbendazim+0.1% Metalaxyl®+ 8-HQC (200 mg l^{-1}) for 3 hrsfollowed by surface sterilization in 70% ethanol (30 sec) and agitation in 0.1% $HgCl_2$ (5 minutes). *In vitro* rhizogenesis (94.44 and 77.77%) on half-strength MS medium supplemented with 1.0 mg l^{-1} IBA in Mahatma Gandhi and 3.0 mg l^{-1} NAA in Refulgens, respectively. Acclimatization in glass jar with PP cap filled with cocopeat+perlite (1:1).

5. References

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