Chapter III

MICROPROPAGATION OF *Bougainvillea spectabilis* Willd. var Splendens

MICROPROGATION OF *Bougainvillea spectablis* Willd. var Splendens

Introduction:

Origin and distribution: The genus *Bougainvillea* named after De Bougainvillea (1729-1811)-a French navigator, belongs to the family-Nyctaginaceae, which is known for herbs and woody vines from warm regions. The plants belonging to this group have simple leaves, flowers without petals but often with showy bracts, imitating the flower (Anonymous, 1986). A native of South America, *Bougainvillea* is a woody shrub often climber, with very gaudy large bracts, is found throughout the tropics and subtropics. The four species: *B. butiana, B. glabra, B. peruviana* and *B. spectabilis* have been commonly grown in India and other parts of the world (Pal and Krishnamurthi, 1967).

General description: Leaves alternate, petiolated and entire; flowers small and inconspicuous, tubular, the margin 5-6 lobed; stamen 7-8 on unequal capillary filaments; ovary stipitate (Bailey, 1950). The small and inconspicuous flowers are associated with large and showy magenta-purple or red bracts that constitute the decorative value of the plants.

Ornamental value: The true flowers of *Bougainvillea* are relatively insignificant. It is the numerous striking colourful petals like bracts, which afford the glowing curtain for which these plants are famed. *Bougainvillea* can be grown as shrubs, climbers, hedges and lately as pot plants. In California, Florida and other southern regions of America, *Bougainvillea* is plentifully used as porch-covers, where they make a most brilliant show. They are valuable for summer bedding also. All the varieties make very desirable subjects for covering verandas, arches and pergolas. Planting at the base of trees, where they rapidly increase in a mass of most beautiful and highly colored flower-bracts. One of the most effective purpose to which these plants can be put is that of hedge or fence plants in tropical and sub-tropical countries. They can stand drought exceedingly well

and may be pruned with impunity (Bailey, 1950). The variable species of *Bougainvillea* are *B. brasiliensis, B. bracteata, B. peruviana* and *B. glabra.*

Different species of *Bougainvillea*:

***Bougainvillea peruviana*:** It is an immense and strong growing climber rising by means of numerous stout spines, has brick red bracts more showy than other species. Variables are also known as *B. brasiliensis, B. bracteata.*

***Bougainvillea Formosa*:** It is a semi-scandent, free flowering with purplish mauve colour and well adapted for decoration under cool treatment of warm greenhouses and conservatories, it is possibly a form of *B. glabra.*

***Bougainvillea spectabilis*:** It is a tall and stout climber, with large and thick leaves. Flowers in large panicles; bracts larger, deep rose colour but varying to purple and greenish. It is an immense and strong climber, rising by means of numerous stout hooked spines. This species of *B. spectablis* is said to be more difficult to start from cutting than the other type, at least without bottom heat (Bailey, 1950).

Vegetative propagation: The conventional propagation is by cutting and air layering where as in some of the species bedding is recommended. The conventional method of vegetative propagation by stem cuttings is slow and also dependent upon the season. Therefore it is inadequate for producing large number of propagules. Thus to meet the high demand of mass multiplication of this plant *in vitro* culture techniques offer an alternative for rapid clonal propagation. Keeping all these factors in mind *in vitro* propagation of *B. spectablis* using axillary meristem from mature shrubs was undertaken. The objective of this study is to establish an efficient method of clonal multiplication of *Bougainvillea spectablis.*

Material and methods:

Plant material: Healthy and mature plants of *B. spectabilis* Willd. var. **Splendens** growing in the M.S. University-Baroda Campus were selected as the

primary source of explants (Fig 1-a). Initially some mature branches were cut and discarded to allow the juvenile shoots to appear and grow at the cut ends. The juvenile shoots with six to eight nodes were collected and their cut ends were immediately dipped in water. For the initiation of the cultures the nodal explants bearing 2-3 nodes were excised after the removal of leaves and thorns from the juvenile shoots.

Sterilization of explants: The nodal explants were first washed under running tap-water to remove dust particles and then treated with mild detergent-Teepol (1%; v/v) for 5 min and again keeping under running tap-water (20 min). This was followed by pre-treatment of explants with a solution containing Bavistin (0.5%; w/v) and chloramphenicol (0.25%) treatment on shaker (1-2 hr). Lastly, pre-treated explants were sterilzed with $HgCl_2$ (0.1%; w/v) (5 min) and then washed repeatedly (5 times) with sterile distilled water. Single node explants were cut and immediately inoculated on agar based semi-solid MS medium (Fig 2-a).

Initiation of cultures: For bud break, elongation of shoots and multiple shoot induction combinations of cytokinin-BA with auxin – IAA in MS medium were used. For the induction of roots, the *in-vitro* shoots of 4-8 cm length were pulse-treated with different concentrations of IBA at different time intervals (1.5 – 5 min). All the shoots treated for rooting were kept in dark for 24-48 hr and then transferred to light for the further growth and elongation of the roots. For acclimatization and hardening of regenerated plants, procedures were adopted as described in Chapter 2.

Comparison between *in vitro* and *in vivo* raised plants: The regenerated plants after transferring to the field were compared with *in vivo* raised parental stock for their morphological characters like number of leaves on the shoots, length and breadth of leaves, arrangement of thorns and flower colour.

Results and discussions:

The *in vitro* propagation of many ornamental plants has been successfully accomplished (Holdgate, 1995). Considerable work has also been done on cereals also (Yamada, 1995). But similar advances have not been reported for the propagation of economically important woody plants. This is because of somewhat variable response of species and

Fig 1: (a) *Bougainvillea spectabilis* Willd. var **Splendens** plant growing in M S University of Baroda-campus.


a

cultivars to the *in vitro* environment; much research is still needed to define the cultural conditions required by woody ornamental species. Over the years clonal multiplication has been achieved (Mackay, 1996) in the evergreen small tree *Arbutus xalapensis* H.B.K. (Woodward et al, 1996), *Gassya eliptica* **Dougl** an ornamental evergreen shrub. Axillary shoot proliferation of *Lonicera* species has also been reported by (Karhu, 1997).

A preliminary report on the propagation of a woody ornamental viz *Bougainvillea glabra* **Magnifica** by tissue culture has been published (Chaturvedi et al, 1978; Sharma et al, 1981), but there are no other reports on micrpropagation of other species and cultivars of *Bougainvillea.* Micropropagation provides a useful method for the mass propagation of many plants, including woody ornamentals (Pennell, 1987).

The present research work aimed at the generation of micropropagation protocol for *Bougainvillea spectabilis* Willd. var. **Splendens** so as to produce plantlets true-to-type to the parental stock. This work also emphasizes on the various factors such as the problem of contamination, seasonal variation and different stages in the micropropagation process. Initially, a severe problem of both fungal and bacterial contamination was observed in the cultures, leading to the senescence of the explants and loss of cultures. This problem was taken care by pre-treatment of explants with the anti-fungal-Bavistin (0.5%; w/v) and anti-bacterial-chloramphenicol (0.25%; w/v) for 1-2 hr on shaker before surface sterilization. This process profoundly controlled contamination in the cultures.

With respect to the seasonal variation in bud sprouting, the best period for maximum sprouting was observed from the month of June to September (Table-3.1). This may be due to the high atmospheric levels of humidity during monsoons, as temperature and humidity play a vital role in bud proliferation (Sharma et al, 1996). It was observed that explants collected during flowering period (February-May), showed callus formation all over the cut surface of the explants. During the initiation, bud break took almost double time (20 days) in the cultures. This may be attributed to the physiological stage of the explant, which influences the type and extent of morphogenesis (Hughes, 1984) like in *Iris,* shoots could be produced from the youngest tissue near the bud from stem segments (Weiler et al, 1977). In fact, in woody species, embryo and seedling tissues have a high capacity of regeneration than other mature tissues and organs (Cheng, 1975).

Table-3.1: Seasonal influence on bud sprouting of axillary nodal explants of *Bougainvillea spectabilis* Willd. on MS medium containing BA (6.65 μM) and IAA (2.85 μM).

MONTHS	BUD SPROUTING (%)* MEAN ± S.E.
January	10 ± 1.2
February	33 ± 4
March	45 ± 4
April	45 ± 1.9
May	50 ± 2.7
June	65 ± 3.5
July	80 ± 3
August	80 ± 3.7
September	72 ± 2.2
October	45 ± 4
November	31 ± 3
December	35 ± 3.5

± S.E. represents Standard error

*Values are the percentage means of three independent experiments

Axillary Bud Sprouting: It was evident from the observations that MS medium with BA (6.65 μM) and IAA (2.85 μM) was the suitable composition for axillary bud sprouting and shoot formation (Table-3.2). At the lowest concentration of BA the response was the minimum and as the concentration increased the bud sprouting response showed an increasing trend. The response was maximum (80%) with 1.0 cm length of the shoots after 10 days of incubation on a medium BA (6.65 μM) with (2.85 μM) of IAA(Fig 2-b). Unlike BA, KN at all the concentrations used, was found to be largely ineffective for inducing good response from the axillary meristems. Similar responses were reported for *Bougainvillea glabra* (Sharma et al, 1981). In general, KN has been reported to be less effective than BA, particularly in woody plants (Murashige, 1974). Earlier Chaturvedi and Mitra, (1975) reported shoot-bud induction in callus tissue of *Citrus grandis* by BA, where as KN totally failed to replicate the results. In certain other woody species like different genotypes of mulberry BA has been employed to induce shoot differentiation (Jain et al, 1990). In *Lavandula latifolia* best results for bud sprouting in nodal explants were achieved on MS medium with 5 μM BA (Sanchez et al, 1996). However, contrastingly KN was

found to be suitable for micropropagation protocol using axillary explants of *Isoplexis canariensis* (Arrebola et al, 1997).

Table-3.2: Influence of cytokinins (BA and KN) with IAA on bud sprouting from axillary explants and growth of shoots in *Bougainvillea spectabilis* Willd. var. Splendens after 10 days.

GROWTH REGULATORS (μM)		BUD SPROUTING (%)* MEAN ± S.E.	SHOOT LENGTH (cm)* MEAN ± S.E.
BA	IAA		
-	-	-	-
1.10	2.85	15.5 ± 2.3	0.3 ± 0.02
1.10	5.70	33 ± 2.7	0.03 ± 0.02
2.21	2.85	38.3 ± 2.7	0.75 ± 0.13
2.21	5.70	50 ± 0	0.67 ± 0.09
4.43	2.85	46.6 ± 5.4	0.58 ± 0.01
4.43	5.70	63.3 ± 2.7	0.56 ± 0.01
6.65	2.85	73.3 ± 2.7	1.03 ± 0.02
6.65	5.70	-	-
KN	IAA		
1.16	2.85	3.33 ± 2.7	0.03 ± 0.02
1.16	5.70	6.66 ± 5.4	0.05 ± 0.04
2.32	2.85	6.66 ± 5.4	0.05 ± 0.04
2.32	5.70	30.00 ± 4.7	0.27 ± 0.03
4.64	2.85	26.6 ± 5.4	0.40 ± 0.05
4.64	5.70	46.6 ± 2.7	0.44 ± 0.09
6.96	2.85	30.0 ± 9.4	0.43 ± 0.04
6.96	5.70	-	-

± S.E. - Standard error
*Values are the mean percentage of three independent experiments.

	Sum Of Squares	Df	Mean Square	f	Sig.
Between groups	4.151	7	0.593	5.392	.000**
Within groups	9.020	82	0.110		
Total	13.171	89			

*The data was subjected ANOVA at $p \leq 0.05$ significance
**Significant

	Sum Of Squares	Df	Mean Square	f	Sig.
Between groups	0.491	6	8.182	1.045	0.412(NS)
Within groups	2.975	38	7.829		
Total	3.466	44			

*The data was subjected to ANOVA at $p \leq 0.05$ significance
NS-Non-Significant

In *Bougainvillea spectabilis*, the presence of auxin IAA with BA did not make any significant increase in the elongation of shoots; the presence of BA (8.87 μM) alone was very effective for growth and elongation of the shoots (Fig 2-c). In *Morus alba* (Sharma et al, 1990) and *Citrus sp* (Iriondo et al, 1995) BA was significantly superior to other cytokinins, both in terms of shoot length and number of nodes.

There was a significant difference between the bud-break response among the groups BA with IAA and KN with IAA. According to results of ANOVA the calculated f value was highly significant at $p \leq 0.05$ for each concentration, among the groups of BA and IAA. The average length of shoots showed significant difference among all the concentrations of BA with IAA tried. Similarly the f value was highly non-significant among the groups of KN and IAA. The average length of shoots of each concentration of KN and IAA did not show any significant difference.

Induction of multiple shoots: *In vitro* nodal explants were used for multiple shoot induction for bulking. For multiple shoot formation from *in vitro* nodal explants, BA gave better results than the other cytokinins (KN and AS) alone or in combination (Table-3.3). The superiority of BA over other cytokinins for multiple shoot formation in woody species has also been observed by other investigators (Monette, 1986; Brand and Lineberger, 1986; Purohit and Dave, 1996; Jain and Nessler, 1996). On the contrary, 2 iP was found to be the most effective for shoot proliferation in *Gardenia* (Economou and Spanoudaki, 1985) and KN favoured multiple shoot formation in *Dalbergia sissoo* Roxb. (Chauhan et al, 1996).

The results in the present studies revealed that apart from the ineffectiveness of the three individual cytokinins (BA, KN and AS) on multiple shoot induction of *in vitro* explants in *B. spectabilis*, the addition of all the three cytokinins was also ineffective. Thus nullifying the synergistic effect of different cytokinins to induce multiple shoot formation. Though the synergistic affect of the two cytokinins BA and KN showed best results for multiple shoot formation in other woody species of fruit-*Annona squamosa* (Nair et al, 1984) and in *Dampriera diversiplia* and *Prostanthera rotundifolia* (Williams et al, 1984).

Fig 2: Establishment and bud growth in *Bougainvillea*

(a) Single node explant established in the MS medium.

(b) Axillary bud growing on MS medium containing BA (6.65μM) and IAA (2.85μM) after 10 days.

(c) Elongation and growth of shoots on MS medium supplemented with BA (8.87 μM).

In figures, the bar = 1 cm


a
b
c

Table-3.3: Multiple shoot induction response of *in vitro* nodal explants on MS basal medium containing cytokinins (BA /KN /AS) alone and in combinations after 21 days in *Bougainvillea spectabilis* Willd. var. Splendens

CYTOKININS (μM)			NUMBER OF MULTIPLE SHOOTS MEAN ± S.E.*	SHOOT LENGTH (cm) MEAN ± S.E.*	NUMBER OF NODES / SHOOT MEAN ± S.E.*
BA	KN	AS			
8.87	-	-	2 ± 0	0.65 ± 0.1	2.2 ± 0.5
13.31	-	-	-	-	-
-	9.29	-	1 ± 0.7	0.74 ± 0.1	1.8 ± 0.1
-	13.93	-	-	-	-
-	-	7.96	1 ± 0.7	1.25 ± 0.2	2.4 ± 0.2
-	-	11.94	-	-	-
8.87	9.29	7.96	1 ± 0.7	2 ± 0.7	0.9 ± 0.2
13.31	13.93	11.94	-	-	-

± S.E. - Standard error

*Values are the mean percentage of three independent experiments

The highest percent (80%) (Table-3.4) of multiple shoots induction with maximum 5 to 6 shoots (Fig 3-a) were produced on MS medium containing BA (31.07 μM) with IAA (2.85 μM). The data showed that addition of low levels of auxin with high level of cytokinin (BA) significantly increased the multiple shoot formation. These results were completely contradictory to the results obtained with *Bougainvillea glabra*, where maximum number of multiple shoots were produced on medium containing high level of auxin-IAA with low level of cytokinin-KN (Sharma et al, 1981; Chaturvedi et al, 1981). On the contrary, in *Halesia carolina*, addition of auxin (NAA) together with BA caused reduction in the number of shoots formed with BA alone (Brand and Lineberger, 1986). However, there are other reports of significant effect of low level of auxin with high level of cytokinin on the shoot formation. In kiwi fruit, the highest number of shoots were formed with BA (9.29 μM) and (0.26 μM) of IBA (Monette, 1986). Similarly all combinations of BA and NAA tested induced axillary shoot proliferation in *Euphorbia antisyphilitica* explants (Jokobek et al, 1986). In *Sterculia urens* Roxb. also combination of cytokinins (BA and KN) failed to improve shoot multiplication and caused undesirable callus formation (Purohit et al, 1996).

Further growth of multiple shoots (Fig 3-b and c) was achieved on MS medium with BA (8.87 μM).

Table-3.4: Multiple shoot induction response and growth of shoots in *in vitro* nodal explants of *Bougainvillea spectabilis* with varying concentrations of BA in combination with IAA.

GROWTH REGULATOR (μM)		NUMBER OF MULTIPLE SHOOTS* MEAN ± S.E.	NUMBER OF NODES* MEAN ± S.E.	SHOOT LENGTH (cm)* MEAN ± S.E.
BA	IAA	**	**	**
6.6	2.85	2.1 ± 0.2^{a}	3.6 ± 0.3^{c}	2.4 ± 0.2^{f}
8.8	2.85	2.6 ± 0.2^{ab}	2.7 ± 0.2^{a}	2.1 ± 0.2^{cd}
11.1	2.85	2.6 ± 0.1^{ab}	2.9 ± 0.2^{ab}	1.6 ± 0.1^{a}
22.2	2.85	2.7 ± 0.2^{b}	3.1 ± 0.2^{b}	2.1 ± 0.2^{cd}
31.07	2.85	3.1 ± 0.2^{cb}	2.7 ± 0.2^{a}	1.9 ± 0.1^{c}
39.29	2.85	3 ± 0.12^{c}	3.5 ± 0.2^{bc}	2.3 ± 0.2^{e}

± S.E - Standard error
*Values are the mean percentage of three independent experiments
**Means within a column followed by the same superscript letter are not significantly different ($p \leq 0.05$; Duncan's Multiple Range test)

Root Induction: In the present study, the rooting of the *in vitro* grown shoots of *B. spectabilis* was induced by pulse treatment with IBA for different time intervals in both solid (Fig 4-a) and liquid medium (Fig 4-b). Similar method of root induction on shoots by pulse treatment with auxin has been reported earlier. (Kannan and Jasrai, 1996; Purohit and Dave, 1996).

In *B. glabra*, among all auxins tried for rooting, the two auxins NAA and IAA were found to be ineffective whereas a combined effect of auxins (IBA and 2, 4, 5-T) induced (100%) rooting (Chaturvedi et al, 1978). However, a good amount of callus formation preceded the root formation. This kind of rooting is undesirable for reduced mortality and better establishment of regenerated plants in the field. In *Verbena tenera* more than 90% of shoots were rooted in the medium with IBA (Hosoki et al, 1994). In *Lachenalia,* maximum root induction on micro-shoots was achieved by treatment with the K-salt of IBA (Ault, 1995).

Fig 3: Induction and growth of multiple shoots in *Bougainvillea*

(a) Multiple shoot induced on MS medium supplemented with BA (31.07 μM) and IAA (2.85 μM).

(b) Growth of multiple shoots on MS medium with BA (8.87 μM) after 3 weeks.

(c) Further growth of multiple shoots after 4 weeks.

In figures, the bar = 1 cm


a
b
c

The lower cut end of the well-grown (6-9 cm) shoots were dipped in IBA solution for pulse treatment (2 min). The IBA treated shoots were transferred to the basal medium. Initially, they were kept in dark for 48 hrs and later on transferred to the normal light conditions. Such pulse treated shoots of *B. spectabilis,* with different concentrations of IBA (Table-3.5) showed maximum response for root formation (90%) with 17 roots (Fig 4-b) and 1.8 cm maximum root length at (1.476 mM) of IBA. The MS basal liquid medium responded better than the solid medium (Fig 4-c), as long, stout primary and many lateral roots were produced in the liquid medium. Those roots which were above the level of medium, showed formation of numerous root hairs.

Table-3.5: Effect of various concentrations of IBA for 2 min pulse treatment on rooting of *in vitro* developed shoots of *B. spectabilis* Willd. var: Splendens on MS basal liquid media

AUXIN IBA (mM)	ROOTING (%)* MEAN ± S.E.	NUMBER OF ROOTS / SHOOT* MEAN ± S.E.	ROOT LENGTH (cm)* MEAN ± S.E.
0.246	-	-	-
0.492	44.2 ± 2.4	2.3 ± 0.4	0.5 ± 0.4
0.738	76.1 ± 3.8	4.4 ± 0.3	1 ± 0.8
0.984	79.3 ± 5.1	5.3 ± 0.5	0.9 ± 0.6
1.476	84.9 ± 0.6	6.9 ± 1.0	1.2 ± 0.08

± S.E. - Standard error
*Values are the mean percentage of three independent experiments

The exposure of the regenerated shoots to pulse treatment with IBA (0.738 mM) for (2 min) and then incubation in the liquid medium gave maximum results for root induction on shoots without any callus formation (Table-3.6). Further growth of these roots was achieved in the same hormone free liquid medium. However, prolonged auxin treatment and increased concentration of IBA resulted in the callus formation and poor stunted growth of roots (Fig 4-d).

Fig 4: Root induction on micro-shoots of *Bougainvillea* by pulse treatment with IBA for 2 min.

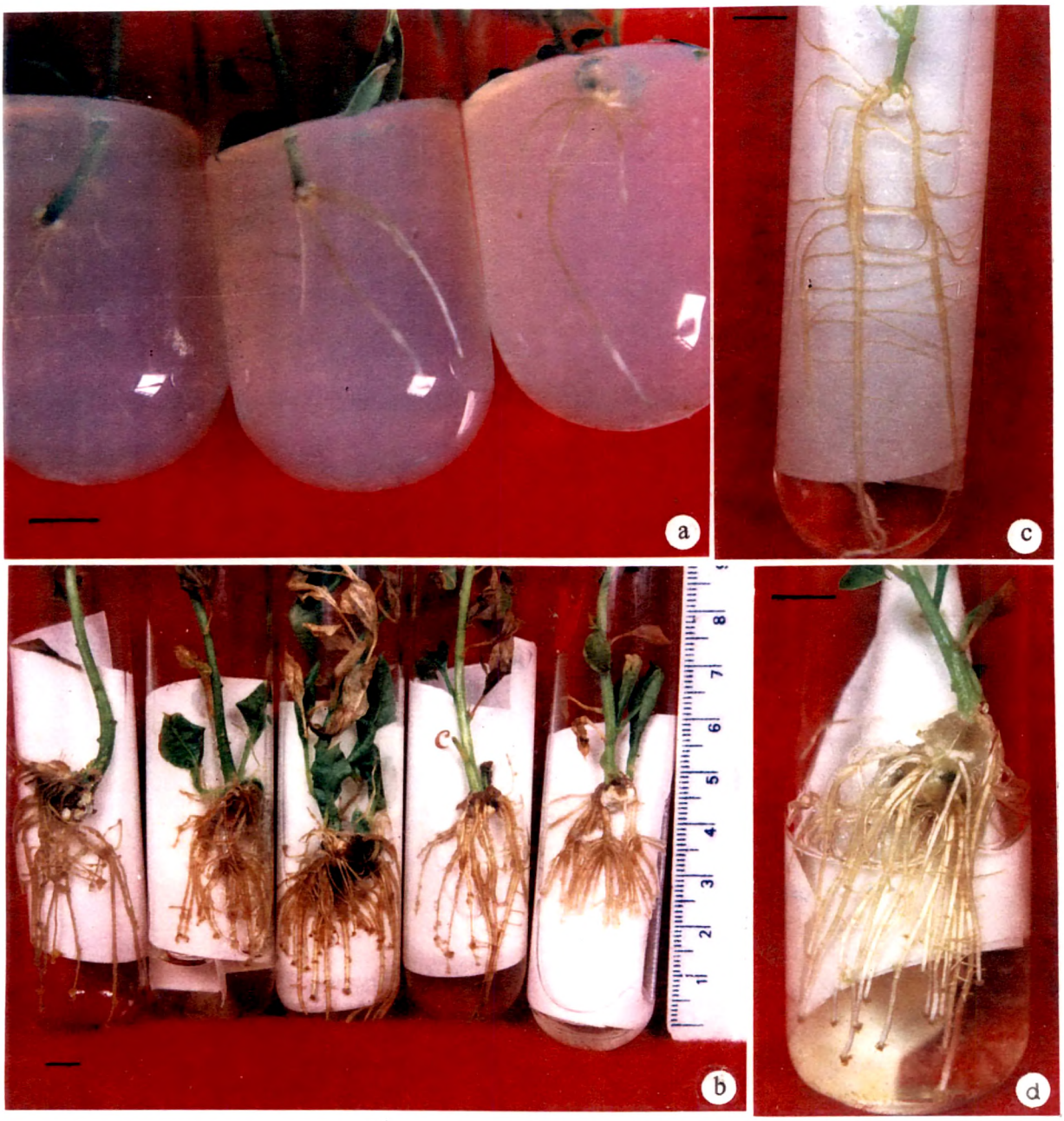
(a) Poor root formation on solid medium after pulse treatment of shoots with IBA.

(b) Influence of different levels of IBA on root formation on shoots by pulse treatment (left to right - 0.492 mM; 0.738 mM; 0. 984 mM; 1.476 mM).

(c) Well-developed roots with laterals on shoots treated with IBA (0.738 μM) for two min and transferred to MS liquid medium.

(d) Root induction with callus formation at the base with IBA (1.476 mM) treated shoot on MS liquid medium.

In figures, the bar = 1 cm


a
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d

Table-3.6: Response of rooting in *Bougainvillea* of *in vitro* shoots with respect to different time intervals of pulse treatment of IBA (0.738 mM) in both solid and liquid medium.

EXPOSURE TIME OF SHOOTS TO IBA (min)	TYPE OF MEDIUM	ROOT INDUCTION (%)* MEAN ± S.E.	NUMBER OF ROOTS* MEAN ± S.E.	ROOT LENGTH (cm)* MEAN ± S.E.
1.5	Solid	77.7 ± 11.9	1.1 ± 0.18	0.3 ± 0.04
	Liquid	66.6 ± 27.2	1.8 ± 0.4	0.7 ± 0.09
2	Solid	77.6 ± 18.2	1.3 ± 0.4	0.5 ± 0.1
	Liquid	100 ± 0	3 ± 0.3	1.4 ± 0.1
3.5	Solid	16.6 ± 13.6	0.2 ± 0.1	1 ± 0.2
	Liquid	73.3 ± 21.7	1 ± 0.3	0.35 ± 0.2
5	Solid	-	-	-
	Liquid	50 ± 23.5	0.75 ± 0.4	0.4 ± 0.1

± S.E. - Standard error
*Values are the mean percentage of three independent experiments

In *Lonicera*-a popular shrubby and climbing woody ornamental, the IBA pulse treatment resulted in similar root formation frequency as of the other auxin treatments, but with fewer and longer primary roots and more abundant lateral roots (Karhu, 1997). Moreover, basal callus was absent in the IBA-pulsed and control micro cuttings. Similarly, Williams et al (1984) *in vitro* proliferated shoots of *Dampiera diversifolia* produced callus at the base of the shoot and formed thick, stunted roots at high levels of IBA (Williams et al, 1984) and Jain et al (1996) in *Camptotheca acuminata*. The highest frequency of *in vitro* root formation at low concentration of IBA (10 to 50 μM) was found in *Cercis canadensis* (Distabanjong et al, 1997). More satisfactory results were obtained by dipping the shoots of Kiwi fruit for only a few seconds in IBA and placing them directly in the soil mix (Monette, 1986). In Dessert milkweed (Lee and Thomas, 1985) a 48 hr IBA pulse treatment (pre-culturing) of cultures followed by a 4 week subculture on hormone-free medium greatly enhanced the rooting response as well as the number of roots per culture. From our own laboratory, Kannan and Jasrai (1996) also reported similar results with *ex vitro* root induction of *Gmelina*.

In our results with *Bougainvillea spectablis,* liquid medium proved to be better for rooting. For another ornamental plant-*Chrysanthemum* also liquid medium proved to

Fig 5: Acclimatization of regenerated plantlets of *Bougainvillea spectabilis* Willd. var **Splendens.**

(a) Plantlets in plastic net pots in a tray covered with polythene sheet.

(b) Plantlets in the earthen pots with garden soil, compost and sand (2:3:1) covered with polythene sheet.

(c) Acclimatized plantlets before their field transfer.


a
b
c

be better for the growth of roots (Jaacov et al, 1972). In *Sophora toronairo* use of liquid medium led to better plantlet formation. Complete dark period during initiation phase (48 hr) was found to promote rooting frequency in *B. spectablis.* Roy et al (1988) noted that 15 days dark period was essential for maximum root induction in *Mitragyna pavifolia.* Apple micro-cuttings also showed increased rooting in darkness (Welander, 1983). Reasons for the enhanced rooting of stem cuttings under reduced irradiance or in darkness are not clear; however it has been shown that levels of certain natural growth inhibitors are lower in plant tissues grown in dark than that grown in light (Eliasson, 1971; Tillberg, 1974). The reduced levels of inhibitors may promote rooting under low light or dark condition. Absence of interferring effect of light on the root promoting action of endogenous auxin may also be responsible for increased root induction in dark (Hartmann and Kester, 1986).

Acclimatization of the plantlets: The rooted plantlets were transferred to plastic net-pots (2 cm diameter) lined with filter paper inside. The plants were acclimatized in such pots containing a mixture of sterile perlite and vermiculite (2:5; v/v). The pots (10 to 12) were kept in a tray [100 x 40 cm] lined at the base with three layers of filter paper soaked with sterile distilled water and covered with a polythene sheet (Fig 5-a). The plants were irrigated with (3 ml) half strength MS basal medium initially. Subsequently the plants were irrigated at every alternate day. After 15 days, these plants were directly transferred to small earthen pots (8 cm diameter) individually or collectively (Fig 5-b) in broader but shallow earthen pots containing a mixture of garden soil, compost and sand (2:3:1; v/v). The individual pots transferred to a tray and others in shallow pots were covered by polythene sheet, in which 5 to 6 holes size (4 mm) were made so as to expose them to the culture room conditions. Slowly and gradually the number of these holes were increased. The pots were irrigated with distilled water, whenever required. After two weeks these plantlets which attained a growth of 8-10 cm length with 8-10 leaves, were transferred to the botanical garden and kept (3 – 4 days) under the shade of a tree (Fig 5-c). These plants were then transplanted in the bigger earthen pots (12 cm diameter). Regular growth of these regenerated plants was recorded every month till one year (Fig 6-a and b). The plants attained a height of 40-50 in six months and after 9 months of their transfer to

Fig 6: Growth of regenerated plants of *Bougainvillea spectabilis* Willd. var **Splendens** in the field.

(a) Regenerated plants after one month of their growth.

(b) Growth of regenerated plants after 6 months.

(c) Regenerated plants flowering after 9 months.

(d) Closer view of flowers on the *in vitro* raised plants.


a


b


c


d

the field, plants showed flowering (Fig 6-c). The colour of the flower resembled the flowers of the parental stock (Fig 6-d).

Comparative field study of regenerated plants: The morphological characters of both regenerated and parental plants were studied such as number of leaves on the shoots, length and breadth of leaves (Table-3.7). The morphology of leaves and their arrangement, size and arrangement of thorns was found to be very similar as that of the parental plants. Statistically the characters for both number and size (length and breadth) of regenerated and parental stocks were significantly similar. This indicates that the *in vitro* raised plants were true-to-type to the parental plant and clonally propagated.

Table-3.7: Comparison of regenerated plantlets transferred to field with parent plant for morphological characters in *Bougainvillea spectabilis* Willd. var Splendens

TYPE OF PLANTS	NUMBER OF LEAVES* MEAN ± S.E.	LEAF LENGTH (cm)* MEAN ± S.E.	LEAF BREADTH (cm)* MEAN ± S.E.
Parental	43.9 ± 3.8^{a} * *	6.1 ± 0.4^{c} * *	4.1 ± 0.4^{d} * *
Regenerated	46.4 ± 2.6^{a}	5.8 ± 0.2^{c}	3.8 ± 0.05^{d}

± S.E. - Standard error
*Values are the mean percentage of three independent experiments
**Means within the column followed by the same superscript letter are not significantly different ($p < 0.05$, Fisher-Behranes test)

On an average of four multiple shoots were produced after 21 days of incubation (Fig 7) which subsequently elongated in a week in the elongation medium with four nodes each resulting in 4-5 cm shoot length. These *in vitro* grown shoots were again excised and incubated on MS medium for further multiplication into single nodal axillary explants. Thus, it was estimated that 1024 plantlets can be produced within 4 month cycle.

Thus flow chart (Fig 7) for the clonal multiplication of *Bougainvillea spectabilis* Willd. var **Splendens** is included in the following page:

Fig 7: Flowchart for clonal multiplication of *Bougainvillea spectabilis* Willd. var Splendens