Chapter V

MICROPROPAGATION OF *Mussaenda luteola* Delile.

MICROPROPAGATION OF *Mussaenda luteola* Delile.

Introduction:

Origin and distribution: *Mussaenda Linn,* belonging to the family Rubiaceae, is a genus of shrubs rarely herbs, distributed mainly in the tropical and sub-tropical regions of the world. In India, found in tropical Himalayas from Dehradun eastwards, Khasi hills, West Bengal, Assam to Nepal eastwards , to Deccan peninsula and Andaman island.

General Description: It is a handsome, erect and scandent shrub, sometimes a small tree commonly cultivated in gardens. The bark is grey in colour with leaves elliptic or oblong-lanceolate, conaceous or ovate. Leaves are bright to dull green, flowers in terminal cymes, tubular funnel shaped, one calyx lobe occasionally becoming coloured and folaceous. Fruits, berries which are sub-globulose or ovoid (Anonymous, 1956).

Ornamental value: About fifteen species occur in India and a few exotics are cultivated in gardens. The ornamental species of the genus are very pretty for their floral value. As one of the calyx lobes develops into a large petaloid sepal, which may be red yellow or white coloured, making the plant conspicuous and attractive. The sale of this plant ranges from 50,000 to 75,000 plants in major metropolis like Delhi and Chennai per annum. It occupies an important position among first fifteen ornamental plants. Among the many species found the important ones are *Mussaenda frondosa, Mussaenda erythrophylla, Mussaenda luteola, Mussaenda roxburghii and Mussaenda glabra* (Anonymous 1962).

Different species of Mussaenda :

***Mussaenda frondosa* Linn:** A handsome erect or scandent shrub, sometimes a small tree, found in tropical Himalayas from Dehradun eastwards, Khasi - hills, Deccan Peninsula and Andaman Island, commonly cultivated in gardens. It has grey bark, leaves elliptic-oblong or ovate; flowers in terminal cymes, tubular, funnel shaped,

yellowish green outside and orange red within, one calyx lobe occasionally becoming white and foliaceous; berries sub-globulose or ovoid (Anonymous, 1956).

***Mussaenda glabra*:** A large, scandent, often climbing shrub found in tropical Himalayas from Nepal eastwards, Bihar, West Bengal and Assam, ascending to an altitude of 1,500 m with leaves elliptic or oblong - lanceolate, conaceous, sometimes mottled; flowers in terminal cymes, yellow; berries globose. The plants are often grown in hedge. Young leaves are said to be appetizing and eaten in salad and chuttneys, leaves are chewed with betel and are used as infusion to relieve cough; a decoction of the root is also used for the same purpose. The flowers are reported to possess diuretic properties and used in asthma, recurrent fevers and dropsy.

***Mussaenda erythrophylla*:** It is an ornamental scandent – shrub grown in gardens, particularly on the hills, for its bright green leaves and yellow flowers with showy, scarlet calycine lobes. The root is said to be useful in coughs. It is also chewed as an appetizer (Anonymous, 1998).

***Mussaenda roxburghii*:** It is a large ornamental shrub found in tropical Himalayas from Nepal eastwards to north Bengal and Assam. It is grown in gardens and is suitable for hedges. The leaves are eaten as vegetables. An infusion of leaves is used for colouring baskets (Anonymous, 1998).

***Mussaenda luteola* Delile.:** A pretty shrub with dull green foliage, yellow flowers and foliaceous sepals. It is a native of Africa, commonly grown in Indian gardens. It is also suitable for hedges.

Earlier Das et al, (1993) has reported induction of somatic embryogenesis from callus cultures of *Mussaenda erythrophylla* cvs. **Queen Sirikit** and **Rosea**. Although work has been reported in other species of *Mussaenda* (Cramer and Bridgen, 1997) but there is no report on micropropagation of *Mussaenda luteola*. Therefore this species of *Mussaenda* was selected for the present studies for standardizing a protocol for micropropagation through axillary bud proliferation.

Medicinal value: Leaves as well as foliaceous calycine lobes of flowers are eaten as pot herbs. Leaves are also used as manure; wood is used for making small articles such as spoon and ladles, and also for turnery, as the wood is white, soft to moderately hard. The leaves and flowers are used in external application for ulcers. Decoction of dried shoots given to children for coughs. Roots demulcent, used for white leprosy and eye troubles. Decoction of leaves is also used against intestinal worms (Anonymous, 1994). It is used as a folk medicine for treatment of common cold, acute gastroenteritis and diarrhoea. In Fujian province of China it is used as contraceptive. The aqueous extract of this plant and its precipitate obtained by adding 95% ethyl alcohol showed significant effects in terminating pregnancy in rats. Moreover, isolation and elucidation of structure of three new saponins, named Mussaendosides have been reported (Zhao et al, 1994).

Need for micropropagation: In India, *Mussaenda* is in great demand as ornamentals because of its petaloid sepal, which may be red, yellow or white. The sale of this plant ranges from 50,000 to 75,000 plants in major metropolis like Delhi and Chennai per annum and occupies a formidable position among the first fifteen ornamental. It costs about Rs 175/- (about a feet tall) and Rs. 550/- (about a meter tall). Thus, the cost of plant might also serve as a deterrent. The saleability and commercial value of the plant can be increased by bringing down its price for more sales. It is also proved that micropropagation can bring down the cost of ornamental plants to one - eighth or one-tenth of its original cost (Bloemenveilingen, 1996).

Although *Mussaenda luteola* Delile. which is generally propagated by seeds and stem cuttings, conventional propagation is slow and is dependent upon the season. It is also inadequate for producing large number of uniform progenies. Keeping all the above factors in mind an *in-vitro* propagation of *Mussaenda luteola* using axillary bud explants was standardized with an ultimate objective of a rapid propagation of *Mussaenda.* To date there has not been any report on *in vitro* propagation of *Mussaenda luteola* except our report (Jasrai et al, 1999).

Materials and methods:

Plant Material: Shoot cuttings of *Mussaenda luteola* Delile. were collected from the Botanical Garden, M.S University of Baroda (Fig 14-a). From the cuttings, the leaves were removed and divided into single node explants.

Sterilization of explants: The explants were washed under running tap water (15 min). The washed explants were further cleaned with 0.1% (v/v) liquid detergent-Teepol and rinsed with distilled water several times. This was followed by the pre-treatment of explants with Bavistin (1%; w/v) for 2 hr on shaker (100 rpm) and then they were treated with 70% alcohol for 30 sec and thrice washed with distilled water. Finally, the explants were treated with (0.1%; w/v) $HgCl_2$ for 3 min in the LFH and rinsed with distilled water (3-4 times) to remove the traces of $HgCl_2$. The explants after inoculation in MS basal medium were incubated in 16 hr photoperiod under a light intensity of 50-60 $\mu Em^{-2}s^{-1}$, provided by white cool fluorescent lamps (Phillips, India) at $25^{\circ} \pm 2^{\circ}$ C.

Initiation of cultures: For bud-break and multiple shoot induction combinations of cytokinin-BA and auxin-IAA in MS medium were used. For elongation of shoots BA and IAA alongwith GA_3 was used. For induction of roots the *in vitro* shoots of 4-8 cm length were pulse treated with very high concentration of auxin-IBA for 10 min and transferred to ½ strength MS basal medium as reported in Jasrai et al, (1999). For acclimatization and hardening of regenerated plants procedure were adopted as described in Chapter 3 and 4.

Results and discussions:

Bud-break induction: In woody species, the frequency of *in vitro* responses generally depends upon the age and source of the plant (Rao and Venkateshwaran, 1985). However here, axillary bud sprouting and shoot elongation was obtained in all the BA and IAA concentrations tried. Both BA and IAA at very low and equal concentrations gave best result. The best response (Table-5.1) was obtained at (0.44

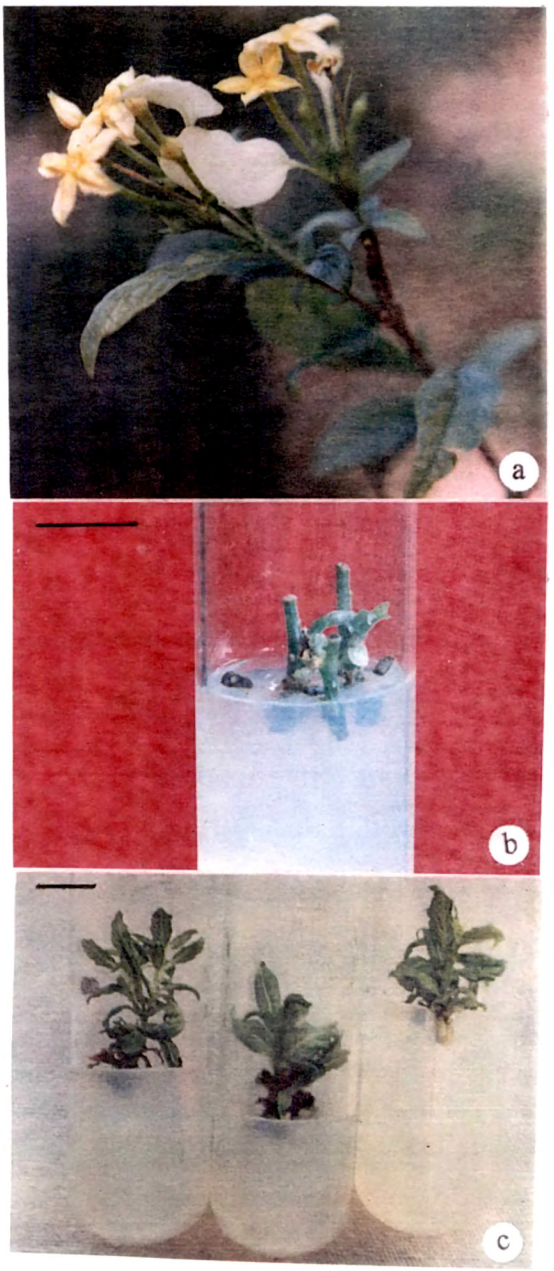
Fig 14: Establishment and growth of axillary buds of *Mussaenda luteola* Delile.

(a) A twig of the plants growing in the Botanical garden of M S University of Baroda.

(b) Bud break response of axillary buds on MS medium containing BA (0.44 μM) and IAA (0.571 μM).

(c) Growth of the axillary shoots on BA (0.44 μM) containing MS medium.

In figures, the bar = 1 cm


a
b
c

μM) BA and (0.571 μM) IAA (Fig 14-b). Increasing the concentration of both BA and IAA did not favour bud-break response. BA at higher concentration caused reduced shoot length, where as low concentration of BA incited increased shoot length (Fig 14-c). In *Mussaenda* **Dona luz** the ideal medium for proliferating shoot required high concentration of BA (10-20 μM), but if longer shoots desired, the BA levels in the medium needed to be decreased (Cramer and Bridgen, 1997).

Table-5.1: Effect of various combinations of BA and IAA in MS medium on bud-break induction in *Musaenda luteola* Delile after 10 days of incubation.

GROWTH REGULATOR (μM)		BUD BREAK RESPONSE (%) * MEAN ± S.E.	SHOOT LENGTH (cm)* MEAN ± S.E.
BA	IAA		
0.44	0.571	85 ± 3.5	0.73 ± 0.098
0.44	1.14	70 ± 14.14	0.542 ± 0.74
0.88	1.71	55 ± 3.5	1.14 ± 0.15
0.88	2.28	45 ± 3.5	0.73 ± 0.09
1.32	0.571	30 ± 7.07	1.23 ± 0.19
1.32	1.14	35 ± 3.5	0.62 ± 0.14
1.77	1.71	20 ± 0	0.75 ± 0.28
1.77	2.28	25 ± 3.5	0.28 ± 0.052

± S.E. – Standard error
*Values are average of three independent experiments

Initiation of multiple shoots: IAA (0.571 μM) concentration in combination with BA (0.88 μM to 8.87 μM) was found to be successful for multiple shoot induction. Almost all the concentration of BA tried with IAA induced multiple shoots, however the best response was achieved with (4.43 μM) of BA and (0.571 μM) IAA with respect to number of multiple shoot induced and the length of the multiple shoots. With this concentration maximum 70% explants responded for multiple shoot induction (Table-5.2) and on an average 4-7 multiples were produced after 21 days of incubation (Fig 15-a). On the contrary, the other cytokinin-KN used in combination with auxin-IAA (0.571 μM) could not give such results. Moreover, at high concentration of KN (9.29 μM) in combination with IAA completely failed to

induce multiple shoots. Infact, BA is the most effective cytokinin used for induction of multiple shoots in cultures (Nair et al, 1979).

Table-5.2: Multiple shoot induction in *Mussaenda luteola* Delile. with various concentrations of cytokinins (BA / KN) in combination with IAA in MS medium after 21 days of incubation.

GROWTH REGULATORS (μM)		NUMBER OF MULTIPLES* MEAN ± S.E.	LENGTH OF MULTIPLES (cm)* MEAN ± S.E.
BA	IAA		
0.88	0.571	2 ± 0	0.68 ± 0.14
1.10	0.571	2.6 ± 0.8	0.78 ± 0.26
2.21	0.571	4.0 ± 1.0	0.9 ± 0.25
4.43	0.571	4.4 ± 0.45	1.05 ± 0.33
8.87	0.571	3 ± 0.47	1.06 ± 0.19
KN	IAA		
0.92	0.571	2.5 ± 0.35	0.52 ± 0.12
1.16	0.571	2 ± 0	0.8 ± 0.35
2.32	0.571	3.8 ± 1.6	0.93 ± 0.11
4.64	0.571	4.5 ± 0.35	0.86 ± 0.11
9.29	0.571	--	--

± S.E. – Standard error
*Values are average of three independent experiments

	LEVENE'S TEST FOR EQUALITY OF VARIANCES		T-TEST FOR EQUALITY OF MEANS FOR NUMBER OF MULTIPLE SHOOTS		
	f	Sig.	t	df	Sig. (2 tailed)
Equal variances assumed	47.204	.000	4.080	198	.000**
Equal variances not assumed			4.080	157.721	.000**

*The data was subjected to paired t test at $p \leq 0.05$ significance
**Significant

	LEVENE'S TEST FOR EQUALITY OF VARIANCES		T-TEST FOR EQUALITY OF MEANS FOR LENGTH OF MULTIPLE SHOOTS		
	f	Sig.	t	df	Sig. (2 tailed)
Equal variances assumed	4.458	.040	1.222	52	.227 (NS)**
Equal variances not assumed			1.590	46.891	.118 (NS)**

*The data was subjected to paired t test at $p \leq 0.05$ significance
**NS implies non significant

The statistical analysis by paired t test revealed the results to be highly significant for number of shoots produced on two different cytokinins (BA / KN) with auxin-IAA. Whereas, the result were non significant for the average length of multiple shoots produced with both cytokinins BA and KN in combination with IAA.

On further subculturing of 21 days interval each, the number of multiple shoots formed after first subculture were 9-10 shoots. After each subsequent subculture the multiples produced increased by 9-10 shoots. Thus, producing maximum 32 shoots(Table-5.3) after three subcultures (Fig 15-b). BA in combination with IAA elicited a better response for multiple shoot induction than KN where maximum 50% explants responded to the multiple shoot induction and only 3 to 4 multiple shoots were produced in medium containing KN (2.32 μM) and IAA (0.571 μM). At the base (cut-end) of the shoots, callus formation was observed on subculturing to the same medium, which caused poor root formation and ultimately leading to poor survival rate of regenerated plants on transfer to the field. In *Mussaenda erythrophylla* also, the intensity of callus proliferation was greater in media containing BA in combination with IAA than in media with BA alone (Das et al, 1993). Similarly, in another shrubby ornamental-Rose, both KN and 2iP in comparison with BA were ineffective in stimulating multiple shoot formation (Hasegawa, 1979).

Table-5.3: Influence on the number of multiple shoots and their length during subculture cycles with BA (2.32 μM) and IAA (0.571 μM) in MS medium. Each subculture cycle was of 21 days duration.

NUMBER OF SUBCULTURE	NUMBER OF MULTIPLES* MEAN ± S.E.	LENGTH OF MULTIPLES (cm)* MEAN ± S.E.
1	10.5 ± 0.86	2.4 ± 0.27
2	19.8 ± 0.84	6.7 ± 0.27
3	29.6 ± 2.3	8.1 ± 0.7

± S.E. – Standard error
*Values are average of three independent experiments

Elongation of shoots: Medium containing higher concentration of IAA (2.28 μM) in combination with lower concentration of BA (0.44 μM) and 0.26 μM of GA_3 caused good elongation growth of the shoots. Gibberellins are supplemented normally for elongation of shoot buds (Schnabdrauch and Sink, 1979). However, the shoots were very weak with poor rooting response. These results were contrary to those reported for *Dianthus*, where GA_3 caused reduction in the adventitious shoot formation and promoted induction of floral buds (Sankhla et al, 1994). However, in the present studies no such induction of floral buds was observed.

Rooting: Rhizogenesis on regenerated shoots was noted in the same multiplication medium (BA-4.43 μM and IAA-0.571 μM), however they were extremely thin, spongy and lacked mechanical strength (fragile) therefore resulted in poor survival of plants during acclimatization (Fig 15-b). To overcome this problem shoots of 4 – 8 cm length were pulse treated with various concentrations of IBA (9.84 μM, 17.22 μM, 22.60 μM, 36.90 μM, 49.20 μM) for 10 min and transferred to MS basal medium. A pulse treatment with IBA (49.20 μM) for 10 min and then transferring to half strength MS basal liquid medium (Table-5.4) proved to be best treatment for root induction on such shoots. The roots with laterals were formed within 10 days of incubation (Fig 15-c). Lower concentrations than 49.20 μM of IBA failed to produce adequate and healthy roots with laterals. Earlier, this pulse treatment with high concentration of

Fig 15: Induction of multiple shoots, their growth and root induction in *Mussaenda luteola* Delile.

(a) Multiple shoots produced (4-5) on MS solid medium with BA (2.21 μM) and IAA (0.571 μM).

(b) Further multiplication of shoots after 3 subcultures (21 days each). (Note: thin spongy weak roots produced here).

(c) Isolated micro-shoots transferred to MS liquid medium after pulse-treatment with IBA (49.20 μM) for 10 min.

In figures, the bar = 1 cm


12
13
14
a
b
c

auxin and then transfer to auxin free medium has also proved to be successful in inducing roots on shoots for *Gmelina* (Kannan and Jasrai, 1996) and woody ornamental *Photinia* (Malagon, 1997). IBA is an effective auxin for pulse treatment in root induction in a wide range of woody species. A 30 min pulse treatment with IBA (54.13 μM) have been shown to stimulate rooting of *Camellia sinensis* shoots cultured *in vitro* (Agarwal et al, 1992). Earlier, a pulse treatment of 24 hr with 3.1 mM was effective for induction of rhizogenesis in *Alnus cordata* (Barghchi, 1988), whereas, Hartman et al (1990), has recommended a pulse treatment as high as 19.7 mM of IBA for 24 hr for rooting in several Cupressaceae members.

Table-5.4: Effect of different concentrations of IBA on the number of roots induced under pulse treatment regime (10 min) to the shoots and transfer to half strength MS basal liquid medium.

AUXIN IBA (μM)	NUMBER OF ROOTS * MEAN ± S.E.
9.84	2.5 ± 0.35
17.22	2.0 ± 0.15
22.60	2.33 ± 0.97
36.90	3.2 ± 0.53
49.20	7.8 ± 1.76

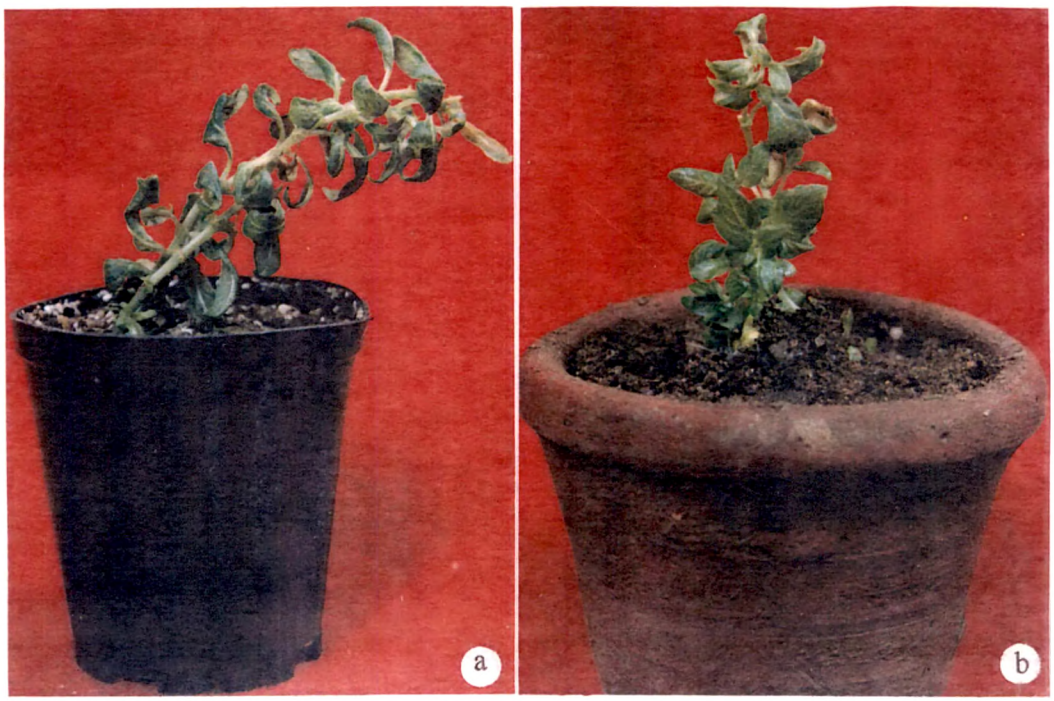
± S.E. – Standard error
*Values are average of three independent experiments

Acclimatization: Well grown plantlets of 6-7 cm length were transferred to the sterile plastic pots (2 cm diameter) containing a mixture of (1:1; v/v) perlite and vermiculite (Fig 16-a) and kept it covered with beaker for about 15 days followed by transferring them to small earthen pots (8 cm diameter) with a mixture of garden soil and compost (1:1; v/v) until their greenhouse transfer after 15 days (Fig 16-b). The plants were hardened in the same manner as discussed in Chapter 3, reported earlier for *Gmelina* (Kannan and Jasrai, 1996). The field survival response of these plants was very low being 60%.

Fig 16: Acclimatization of plantlets of *Mussaenda luteola* Delile.

(a) Plantlets in plastic pots (2 cm diameter) containing vermiculite and perlite (1:1).

(b) Well-developed, acclimatized plants in earthen pots containing garden soil (after 15 days after their field transfer).


a
b

Comparative field study of regenerated plants: Physical appearance of regenerated plants and conventionally propagated parent plant in the field revealed no significant difference in the morphological characters studied like number of leaves per shoot, size of the leaf (length and breadth), phylotaxy of leaves etc. Absence of variation in morphology of *Mussaenda luteola* Delile. regenerated plants in the present study strongly supports that plants are similar to parental lines and hence, clonally propagated. However, the low survival rate of regenerated plants may be attributed to the fact that plants undergo a shock during acclimatization from *in vitro* to *ex vitro* conditions (Smith and Hamil, 1996).

From a single nodal explant of *Mussaenda luteola* on an average four shoots with three nodes were produced on multiplication media after 21 days of incubation. These shoots were further excised into single nodal explants producing (4x3) 12 nodal explants, which produced (12x4) 48 shoots after six weeks. On further subculturing, the 48 shoots into single nodal explants resulted in (48x3) 144 explants. When transferred for multiple shoot induction (144x4) 576 shoots could be obtained in another three weeks. These shoots can be further rooted (10 days) and acclimatized (15 days) for field transfer. Thus in 3 months' cycle approximately 576 plantlets from a single nodal explant can be produced indicating high multiplication rate of the plant.

Flow chart (Fig 17) for the clonal multiplication of *Mussaenda luteola* Delile. is included in the following page:

Fig 17: Flowchart for clonal multiplication of *Mussaenda luteola* Delile.