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Direct Shoots Regeneration from Nodal Meristems of *Brugmansia suaveolens* (Humb. & Bonpl. ex Willd.) Bercht. & J.Presl.

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An efficient tissue culture protocol has been developed for micropropagation of Brugmansia suaveolens. The fresh sprouts were used as explants. These were surface sterilized. The explants were responded in 15 days after inoculation on Murashige and Skoog (MS) medium supplemented with 2.0 mgl-1 Benzylaminopurine (BAP). 5-6 shoots were regenerated on this media combination. Callus was formed when auxins were added to the medium. Maximum numbers of shoots (8-9 shoots/culture) were reported on MS medium augmented with 0.5 mgl-1 each of BAP and Kinetin. The long and healthy shoots were excised and isolated from the shoot clumps and used for in vitro rooting experiments. Indole-3 acetic acid (IAA) and Indole-3 butyric acid (IBA) both are equally effective in root induction. The in vitro rooted plantlets were hardened in green house in pots which contains mixture of soil and manure..

Keyword: Micropropagation, Brugmansia Suaveolens, In Vitro, Hardening.

INTRODUCTION: *Brugmansia* is a genus of seven species of flowering plants in the family Solanaceae. *Brugmansia* species are native to tropical regions of South America but now naturalized in most parts of the world [1,2]. Their large, fragrant flowers give them their common name of Angel's Trumpets, a name sometimes used for the closely related genus *Datura*. *Brugmansia* species are woody trees or bushes, with pendulous, not erect, flowers, that have no spines on their fruits[3,4].

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Brugmansia suaveolens produce atropine alkaloids, such as hyoscyamine and scopolamine as main defense compounds. Tropane alkaloids are also typical secondary metabolites of several other solanaceous plants. These inhibit the muscarinergic acetylcholine receptor and show parasympatholytic properties. As such they are used in medicine to treat spasms, to sedate patients and for dilatation (mydriasis) of the pupil. Furthermore, tropane alkaloids affect neuronal activities and are known hallucinogens[5,6]. *B. suaveolens* has been mainly employed externally to treat rheumatic and arthritic pains, swelling, scalds, inflammations, skin rashes, haemorrhoids and wounds. Its extracts exhibit spasmolytic, antiasthmatic, anticholinergic, narcotic and anesthetic properties[7,8]. Most alkaloids are important for

the fitness and survival of the plants producing them since they help to protect against herbivores and/or infecting microorganisms[9,10].

The leaves of *B. suaveolens* are smoked as 'asthma cigarettes', steambath are prepared with the leaves for bad coughs and bronchitis, the juice is boiled and mixed with hog-lord as an external application for all types of burns, scalds, inflammations and hemorrhoids, poultices of the leaves are applied to badly healing wounds, swellings and arthritic, as an antispasmodic to control Parkinson's disease[6].

Considering its pharmaceutical importance an efficient protocol for tissue culture and plant regeneration is a pre-requisite for biotechnological processes[11] and for breeding programs[12]. The effect of jasmonic acid and aluminium on production of tropane alkaloids in hairy root cultures of *B. candida* was studied by [13]. [4] set up experiments of hairy root cultures of *B. suaveolens*.

In vitro procedures and plant regeneration are used to some degree in almost every major plant species. The success of such biotechnology requires an efficient protocol for plant regeneration from different explants[14]. However, in the literature, there are no studies that have checked the direct plant regeneration in *B. suaveolens* using mature somatic tissues without callus formation.

Materials and Methods

Plants of *B. suaveolens* were collected from Munar and Thekari regions of Tamil Nadu and Kerala and maintained in the green house to get fresh and healthy sprouts as explants. Different types of explanting materials namely, axillary/terminal shoots, shoot apices/nodal shoot segments, were harvested from selected plants. The explants were cleansed, dressed and treated with 0.1% solution of Bavistin, (a systemic fungicide) (BASF, India Ltd. Bombay). These were surface sterilized with ethanol and $HgCl_2$ (0.1%) solution under Laminar Air Flow Cabinet and inoculated aseptically onto MS basal medium[15].

The cultures were kept in growth room under controlled conditions at the temperature 26±2°C with 14h/d illumination of 30-40 µmol $m^{-2}s^{-1}$ Spectral Flux Photons (SFP) and 60-70% relative humidity (RH). The light was provided by fluorescent tubes and incandescent bulbs. Temperature was maintained by central air conditioning systems.

The cultures were inoculated on MS medium supplemented with BAP and Kinetin (0.5 mgl^{-1} to 5.0 mgl^{-1}) for shoots induction. After establishment of the cultures from the explants, the shoots were excised containing 2-3 nodes and were transferred to shoot multiplication medium. These were further multiplied by culturing of (i) clumps of shoots, (ii) single nodal shoot segments, or (iii) 2-3 nodal shoot segment, at an interval of 25-30 days, after removing the senesessed leaves. For the rooting of *in vitro* produced shoots, the shoots were isolated of appropriate size and these were rooted on half and one-fourth strengths of MS medium containing auxins (Indole-3 acetic acid and Indole-3 butyric acid) in different concentrations.

The *in vitro* rooted plantlets were washed with autoclaved distilled water to remove adhered nutrient agar and then transferred to sterilized soilrite mixture filled in glass bottles moistened with one-fourth strength of MS basal salts. Initially the hardened plantlets were kept covered with caps. After 12-15 days the caps were loosened and finally removed. Plants hardened in bottles were transferred to polybags containing sand, soilrite, organic manure and black soil in 1:1:1:1 ratio or in different ratio depending upon plant species. The hardened plantlets were finally transferred to the pots, polybags and were transferred to the field.

The cultures were regularly subcultured on fresh medium after 4-5 weeks interval. The observations were taken after every five days of inoculation. The experiments were repeated thrice with ten replicates per treatment. The rate of multiplication represents number of shoots produce per explant on a specific medium after number of days of its inoculation as mentioned in the results. The data were subjected to statistical analysis.

Results and Discussion

An efficient and reproducible plant regeneration protocol is a prerequisite for genetic transformation in any plant system. Shoot tips and nodal shoot segments were used as explanting material for multiple shoot induction. In order to ensure year-round availability of explants and to avoid microbial contamination in the cultures, shoot tips were harvested from the green house grown plants. Direct plant regeneration through multiple shoot induction from the explant was adopted, as it does not involve an intermediate callus phase, and tends to produce genetically similar plants[16]. Plant regeneration using callus system requires a longer period to establish cultures and to obtain embryogenic calli, along with the risk of somaclonal variation in the plants regenerated from callus[17,18,19]. [20]used seeds as explants of *B. suaveolens* to establish callus cultures and then regenerated shoots from these cultures.

Clonal multiplication through various explants is advantageous over conventional propagation method because a large number of plants can be produced within a short duration. The most effective medium for shoot induction and multiplication in *B. suaveolens* was achieved on MS medium containing BAP, as compared to Kinetin (**Table 1)**. This indicates that these explants contain sufficient endogenous level of auxins or capable of its *de novo* synthesis which can induce shoot formation even in a medium containing cytokinin alone[21]. At higher concentration of BAP or Kinetin the rate of shoot proliferation declined.

Table 1. Effects of cytokinin (BAP and Kinetin) on induction of shoots from the explants of *B. suaveolens* on MS medium.

Sl. No.	Kinetin (mgl^{-1})	BAP (mgl^{-1})	No. of shoots (Mean ± SD)	% Response
1	0.00	0.00	0.0 ± 0.00	0
2	0.50	-	1.2 ± 0.46	21
3	1.00	-	2.6 ± 0.38	34
4	1.50	-	3.4 ± 0.68	58
5	2.00	-	4.2 ± 0.62	69
6	2.50	-	5.8 ± 0.83	62
7	3.00	-	5.1 ± 0.23	56
8	3.50	-	4.6 ± 0.16	42
9	4.00	-	3.2 ± 0.33	26
10	5.00	-	3.0 ± 0.81	19
11	-	0.50	2.0 ± 0.16	14
12	-	1.00	3.4 ± 0.52	22
13	-	1.50	4.1 ± 0.78	38
14	-	2.00	6.6 ± 0.32	51
15	-	2.50	5.0 ± 0.92	69
16	-	3.00	4.2 ± 0.34	61
17	-	3.50	3.2 ± 0.86	53
18	-	4.00	2.8 ± 0.19	46
19	-	5.00	2.5 ± 0.51	33

The season of collection of explants is an important factor in the success of tissue culture experiments. The response of tissues in cultures is generally varying due to accumulation of phenolic compounds in the meristematic tissues[22,23]. Numbers of shoots were more in BAP containing medium as compared to Kinetin. Superiority of BAP over Kinetin was also demonstrated by several workers[24,25,26].

Shoot tips and axillary meristems proved preferred explants for *in vitro* culture due to ease of handling as compared to material from other sources. When nodal segments as well as shoot tips were cultured on regeneration medium,

swelling of the explant was observed within 4 days of culture on BAP containing medium in case of *B. suaveolens*. Single shoot growth was initially observed (**Fig. 1A & 1B**), but multiple shoot induction was subsequently observed on medium supplemented with BAP. Multiple shoots ranging from 6 to 7 were observed after 3 weeks of culture of the explants on MS medium with different concentrations of BAP (**Fig. 1C & 1D**).

To a certain extent, with increasing concentration of BAP, an increase in number of multiple shoots was observed. Higher concentrations of BAP in the medium resulted in a reduced number of shoots. BAP is involved in reprogramming and differentiation of the competent cells necessary for adventitious bud development, and has been reported to induce synthesis or accumulation of endogenous cytokinins[27]. At lower concentrations of BAP (1-2 mgl^{-1}), although lower numbers of shoots were induced, shoot growth was observed to be normal. In contrast, at higher concentrations of BAP (3-5 mgl^{-1}), although the number of shoots induced was considerably higher, stunted growth was observed.

The well developed shoots were transferred to multiplication medium containing BAP and Kinetin. The shoot multiplication initiated within a week after subculture. Shoot regeneration potentiality was 100% in all the concentrations of both the cytokinins. Though several growth regulators are available for shoot multiplication, BAP and Kinetin are widely used. Of the two cytokinins tested, BAP was more effective in shoot induction and proliferation than Kinetin. Similar to this, in several studies BAP was more effective in inducing and sprouting of a large number of shoots[28,29,30]. Several workers showed that the synergistic combination of two cytokinins was more effective for shoot differentiation[31,32]. Similarly as in the above findings, more numbers of shoots were observed in present investigation when BAP and Kinetin was used together. The maximum numbers of shoots were observed on MS medium augmented with 0.5 mgl^{-1} each of BAP and Kinetin. In an attempt to standardize the direct plant regeneration protocol for *B. suaveolens*, 7-8 shoots per culture bottle were induced on MS medium as shown in **Table 2 and Fig. 1E.**

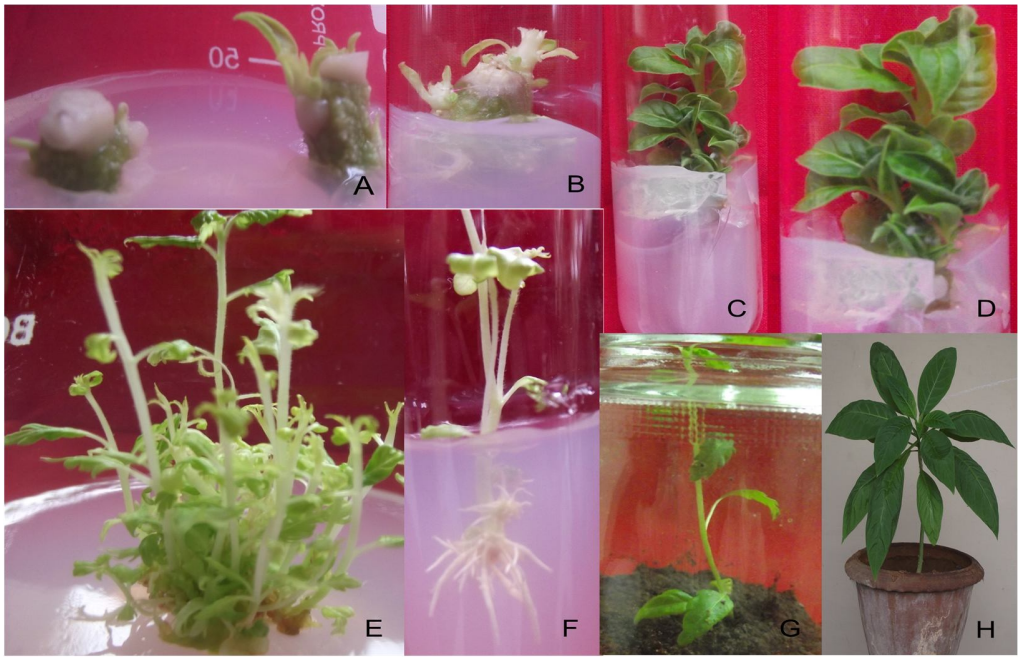
Table 2. Effects of Cytokinin (BAP and Kinetin) and their concentrations on multiplication of shoots from sub-cultured shoots on MS Medium.

Sl. No.	BAP (mgl^{-1})	Kinetin (mgl^{-1})	No. of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
1	0.00	0.00	00.00	00.00
2	0.10	-	5.2 ± 0.28	3.8 ± 0.57
3	0.50	-	5.3 ± 0.61	3.5 ± 0.23
4	1.00	-	6.2 ± 0.76	3.2 ± 0.83
5	1.50	-	6.1 ± 0.92	3.0 ± 0.30
6	2.00	-	5.6 ± 0.17	2.9 ± 0.58
7	-	0.10	4.3 ± 0.54	2.6 ± 0.8
8	-	0.50	5.1 ± 0.83	2.7 ± 0.13
9	-	1.00	5.2 ± 0.89	2.8 ± 0.67
10	-	1.50	5.2 ± 0.14	2.8 ± 0.43
11	-	2.00	4.5 ± 0.87	2.6 ± 0.19
12	0.10	0.10	6.1 ± 0.47	2.5 ± 0.92
13	0.50	0.50	7.9 ± 0.21	2.8 ± 0.73
14	1.00	1.00	7.3 ± 0.83	2.7 ± 0.68
15	1.50	1.50	6.8 ± 0.14	2.5 ± 0.31
16	2.00	2.00	5.1 ± 0.67	2.3 ± 0.29

The *in vitro* regenerated shoots were carefully cut from the base and transferred on full or half strength of MS medium supplemented with IBA and IAA for the induction of rooting. Half strength of MS medium with IBA was found to be more suitable as compared to full strength of MS medium for rooting of shoots. On half strength MS medium with IBA (2.0 mgl^{-1}), a maximum of 89% shoots were rooted with 2-3 cm in length after 4 weeks of inoculation (**Table 3 and Fig. 1F**). The superiority of IBA over other auxins was also reported by[33].

Table 3. Effects of Auxins (IBA and IAA) on *in vitro* regeneration of root from *in vitro* generated shoots on half strength MS medium.

Sl. No.	IBA (mgl^{-1})	IAA (mgl^{-1})	No. of roots (Mean ± SD)	Root length (cm) Mean ± SD)
1	0.00	0.00		
2	0.50	-	2.2 ± 0.83	1.1 ± 0.70
3	1.00	-	2.4 ± 0.14	1.3 ± 0.83
4	1.50	-	2.6 ± 0.87	1.5 ± 0.54
5	2.00	-	3.0 ± 0.30	1.7 ± 0.00
6	2.50	-	2.6 ± 0.21	1.7 ± 0.23
7	3.00	-	2.0 ± 0.69	1.4 ± 0.47
8	-	0.50	1.7 ± 0.64	1.2 ± 0.81
9	-	1.00	2.3 ± 0.83	1.3 ± 0.23
10	-	1.50	2.5 ± 0.71	1.6 ± 0.49
11	-	2.00	2.8 ± 0.82	1.7 ± 0.56
12	-	2.50	2.8 ± 0.79	1.6 ± 0.82
13	-	3.00	2.6 ± 0.32	1.5 ± 0.16



Figs. 1A & 1B. Induction of shoots from nodal meristems. 1C & 1D. Multiple shoots regeneration *in vitro*. 1E. Multiplication stage in cultures. 1F. *In vitro* root formation from the cut ends of the shoots of *B. suaveolens*. 1G & 1H. Plantlets in hardening and pot transfer stages.

After root formation, the plantlets were removed from the culture bottles carefully without destruction of the root system and after washing the roots in water; plantlets were transferred to the bottles with soilrite and nurtured with 1/4 MS liquid medium without sucrose (**Fig. 1G**). The rooted plants were hardened in green house. Gradual exposure of the plants to the open environment was necessary to avoid plant death due to desiccation. The well-established plants from bottles were transferred to earthen pots (**Fig. H**). The plants were then shifted to sunlight in field conditions. The rooted plantlets obtained from half strength of MS medium with IBA showed 95% survival rate.

Direct regeneration of plants required relatively short period (10–11 week) compared to the regeneration via callus phase (20–22 week) reported earlier by[34]. The direct plant regeneration protocol for *B. suaveolens* is highly efficient, rapid, and genotype independent. It does not involve a callus phase, and so a reduced time period is required for regeneration of complete plant, reducing the likelihood of somaclonal variation in the tissue-cultured plants. Thus, the present findings could be used for conservation and large-scale propagation of this important plant species.

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