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RESEARCH ARTICLE

MICROPROPAGATION OF *Lippia nodiflora* USING SHOOT TIP AND NODAL EXPLANTS UNDER *IN VITRO* CONDITIONS

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ABSTRACT

Lippia nodiflora (L.) (Verbenaceae) is an important medicinal plant with scanty roots. It has a number of medicinal properties used in treatment of diseases such as chronic indolent ulcers, gastrointestinal and respiratory ailments and diabetes, etc. Nodes and shoot tips were used as the explants for the initiation of multiple shoots and cultured on Murashige and Skoog (MS) medium supplemented with cytokinin namely Benzyladenine (BA) and Kinetin (KIN).The maximum number of shoots were produced in BA 3.0 mg/l. An average of 14.66 ±1.30 shoots was produced from each explant. Healthy shoots were transferred to rooting medium with half and full strength MS medium supplemented with IBA and IAA at different concentrations such as 0.1, 0.5, 1.0 and 1.5 mg/l. Half strength MS medium containing IBA of 1.0 mg/l produced the maximum number of roots (10.3 ± 4.00). Rooted plants were hardened and those grown in sand: cocopith (1:1) showed good response than others.

INTRODUCTION

Lippa nodiflora (L.) is a medicinal plant distributed in India, south and Central American countries, and tropical African territories (Terblanche and Kornelin, 1996). They are traditionally utilized as gastrointestinal and respiratory remedies (Morton 1981). Some of the other *Lippa* species have shown antimalarial (Gasquet *et al*., 1993), antiviral (Abad *et al*., 1995) activity. *L. nodiflora* possesses a number of ethanobotanical uses (Kirtikar and Basu, 1975). The plant also possesses cooling, diuretic and febrifuge properties. In unani system of medicine, the fresh plant is used to prepare a paste or poultice and applied as suppurant for boils, swollen cervical glands and chronic indolent ulcers. A bitter infusion prepared from the plant is given to children for indigestion and women after child birth. The plant is also used in the treatment of osteoarticular pains and respiratory diseases (Hooker, 1885; Kartikar, 1918; Chopra *et al*., 1956). The chemical composition of the plants includes several flavones, glycosides, alkaloids and essential oils (Basu *et al*., 1969; Nair *et al*., 1973; Francisco *et al*., Forestieri *et al.,* 1996). Nodifloridin-A and Nodifloridin-B have also been isolated from *L. nodiflora* (Singh and. Bharate, 2006). Halleridone and Hallerone compounds from *Lippia nodiflora* (Ravikanth *et al*., 2000) proved to have anticancer, anti-tumor, antimalarial, antifungal and cytotoxic activities (Nishino *et al*., 1988).

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Recent study showed antidiabetic activity of γ- sitisterol isolated from *L. nodiflora* (Balamurugan *et al*., 2011). Due to the tremendously growing world population, increasing anthropogenic activities, and rapidly eroding natural ecosystem, the natural habitat for a great number of herbs and trees are dwindling and many of the species are facing extinction. In order to cope up with this alarming situation, the recent developments in biotechnology have come as a boon to consume and mass produce these important medicinal plants (Sharma *et al*., 2010). Tissue culture is a potential tool and has opened extensive areas of research for biodiversity conservation. Plant tissue culture techniques offer a viable tool for mass multiplication and germplasm conservation of medicinal plants in order to meet the needs of pharmaceutical industry (Sahoo and Chand, 1998).

MATERIALS AND METHODS

Shoot tips and nodal segments, collected from mature plants were used as explants for establishing a protocol for mass production of *Lippia nodiflora* plantlets. The explants were washed with running tap water for 15 to 20 minutes. They were then immersed in 5% (v/v) detergent solution (Tween 20) for 3 minutes and washed with tap water for 15 minutes, to eliminate the dust particles as well as fungal and bacterial spores found on the surface of these explants. The surface sterilization was done by treating the explants with 70% ethanol for 30 seconds and rinsed with sterile double distilled

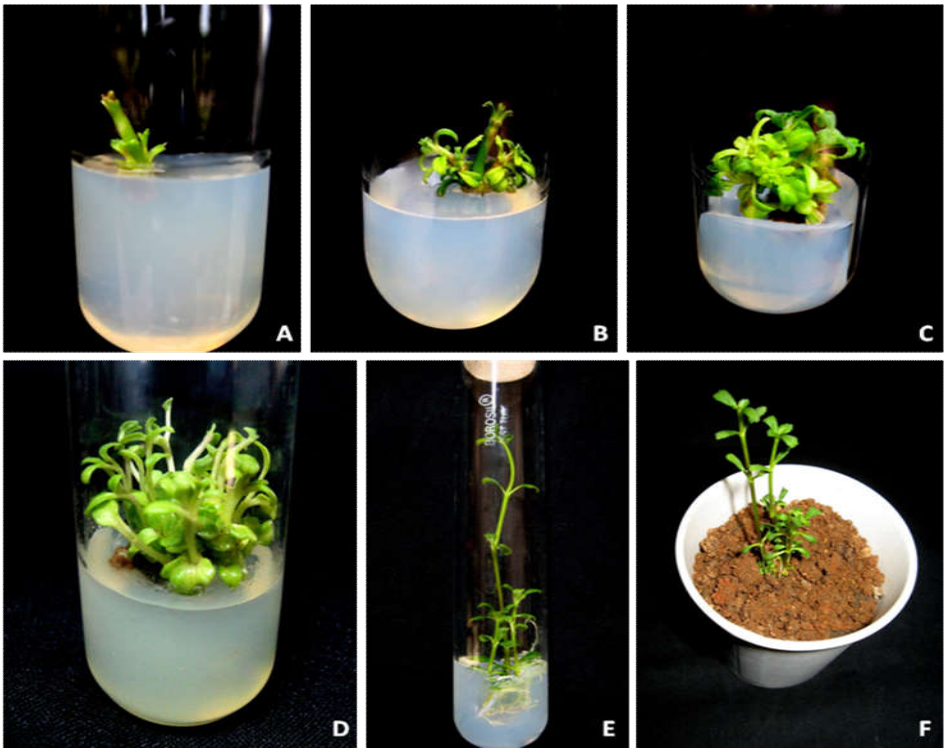

Fig. 1. ***In vitro*** **Multiplication of** ***lippia nodiflora*** **L. A - Shoot tip explant after7 days of culture on MS medium containing 3.0mg/l BAP; B - Multiple shoots formed after 2 weeks; C - multiple shoot formation after 3 weeks; D - Multiple shoots after 4 weeks; E - Shoots subcultured in MS medium containing IBA showing root formation; F - Hardening**

Table 1. Effect of cytokinin (BA and KIN) at different concentrations individually and in combination with auxins NAA and 2, 4-D on the multiple shoot formation from shoot tip explants of *Lippia nodiflora*

Plant Growth Regulator (mgL^{-1})		% of shoot formation	No. of total shoots per explants	Average length of shoot per culture
Cytokinin	Auxin			
BA	-			
1	-	90	8.44 ± 2.07	5.25 ± 1.62
2	-	80	13. 88 ± 2.03	5.42 ± 0.83
3	-	80	14.63 ± 1.30	6.32 ± 1.46
4	-	90	10.56 ± 1.24	4.91 ± 1.58
5	-	70	8.57 ± 2.23	5.27 ± 1.38
BA	NAA			
1	1	80	6.50 ± 2.14	4.39 ± 0.94
2	1	80	12.00 ± 1.93	4.14 ± 1.02
3	1	90	9.11 ± 2.20	5.11 ± 1.39
4	1	100	8.80 ± 1.23	4.36 ± 1.56
5	1	80	7.00 ± 1.22	3.68 ± 1.33
BA	2,4-D			
1	1	100	2.20 ± 0.63	2.16 ± 0.86
2	1	100	4.20 ± 1.23	2.44 ± 0.86
3	1	90	4.56 ± 2.46	3.58 ± 1.82
4	1	90	7.56 ± 2.60	2.32 ± 0.67
5	1	90	3.89 ± 1.69	1.78 ± 0.50
KIN	-			
1	-	100	2.10 ± 0.88	2.73 ± 0.82
2	-	80	2.63 ± 0.74	3.20 ± 1.25
3	-	80	3.13 ± 0.83	3.34 ± 1.49
4	-	80	2.50 ± 1.07	2.32 ± 0.32
5	-	80	1.25 ± 0.46	2.19 ± 1.17
KIN	NAA			
1	1	90	4.78 ± 1.72	5.34 ± 1.78
2	1	70	4.86 ± 1.57	3.05 ± 1.17
3	1	80	3.25 ± 1.58	3.34 ± 1.78
4	1	70	3.71± 1.50	2.28 ± 1.07
5	1	80	1.88 ± 0.64	2.01 ± 0.76
KIN	2,4-D			
1	1	100	1.70 ± 0.48	1.99 ± 0.86
2	1	80	3.25 ± 0.71	2.47 ± 0.74
3	1	100	2.38 ± 0.92	3.02 ± 1.12
4	1	90	1.78 ± 0.44	2.25 ± 1.05
5	1	80	1.80 ± 0.63	1.59 ± 0.42

Table 2. Effect of cytokinin (BA and KIN) at different concentrations individually and in combination with auxins NAA and 2, 4-D on the multiple shoot formation from nodal explants of *Lippia nodiflora*

Plant Growth Regulator (mgL^{-1})		% of shoot formation	No. of total shoots per explants Mean ±S.D	Average length of shoot per culture Mean ± S.D
Cytokinin	Auxin			
BA				
1	-	90	6.78 ± 1.92	4.75 ± 0.96
2	-	90	8.33 ± 1.73	4.74 ± 1.37
3	-	100	10.20 ± 1.55	6.01 ± 2.04
4	-	90	6.56 ± 1.13	3.99 ± 1.33
5	-	90	5.89 ± 1.45	3.93 ± 0.83
BA	NAA			
1	1	80	5.75 ± 1.04	3.93 ± 0.62
2	1	90	6.89 ± 1.76	4.08 ± 0.81
3	1	80	6.00 ± 1.93	4.66 ± 2.00
4	1	80	4.25 ± 1.04	4.23 ± 1.87
5	1	60	3.17 ± 1.17	4.00 ± 1.41
BA	2,4-D			
1	1	80	2.13± 0.83	1.83 ± 0.44
2	1	100	4.50 ± 1.18	2.82 ± 0.92
3	1	100	4.70 ± 1.49	3.73 ± 1.06
4	1	90	8.00 ± 2.00	2.70 ± 0.91
5	1	100	3.80 ± 1.48	1.66 ± 0.93
KIN				
1	-	100	2.40 ± 0.70	1.58 ± 0.39
2	-	90	2.44 ± 0.73	2.22 ± 0.86
3	-	70	3.29 ± 1.11	2.90 ± 1.40
4	-	90	1.78 ± 0.67	2.65 ± 1.10
5	-	80	1.38 ± 0.52	1.36 ± 0.62
KIN	NAA			
1	1	90	3.67 ± 1.66	4.34 ± 1.52
2	1	90	3.89 ± 1.05	3.20 ± 1.10
3	1	90	3.00 ± 1.50	3.88 ± 1.94
4	1	80	4.00 ± 1.07	2.80 ± 1.20
5	1	70	1.86 ± 0.69	2.07 ± 0.80
KIN	2,4-D			
1	1	90	1.56 ± 0.53	3.06 ± 1.04
2	1	90	2.33 ± 0.87	3.32 ± 0.88
3	1	100	1.50 ± 0.71	3.18 ± 0.96
4	1	90	1.56 ± 0.53	2.17 ± 1.00
5	1	80	2.00 ± 0.76	1.87 ± 0.27

Table 3. Effect of different concentrations of Auxins IAA and IBA on root induction

Plant growth regulators(mg/l)	Full strength MS medium		Half strength MS medium	
IAA	No. of roots per shoot Mean ±S.D	Root Length (cm) Mean ±S.D	No. of roots per shoot Mean ±S.D	Root Length (cm) Mean ±S.D
0.1	4.3 ± 1.4	1.38±0.40	5.0 ± 1.24	2.21±0.70
0.5	6.0 ± 1.94	2.27±0.68	7.2 ± 1.9	3.08±0.34
1.0	5.6 ± 1.57	3.1±0.78	6.6 ± 2.54	4.01±0.67
1.5	5.2 ± 1.68	2.09±0.49	6.1 ± 1.28	2.45±0.29
IBA				
0.1	5.2 ± 1.22	1.16±0.32	6.4 ± 1.3	1.29±0.44
0.5	6.9 ± 2.46	2.11±0.48	8.6 ± 3.8	2.39±0.32
1.0	7.9 ± 3.21	1.87±0.18	10.3± 4.0	1.81±0.49
1.5	6.4 ±1.95	1.41±0.33	7.9 ± 2.13	1.45±0.42

Table 4. Hardening of *in vitro* grown plants on different planting substrates for their acclimatization and establishment in natural conditions

Planting substrates	No. of plants transferred	No. of acclimatized plants	Acclimatization percentage
Sand+soil	25	17	68
Cocopit+soil	25	23	92
Sawdust+soil	25	13	52
sand+cocopith+sawdust	25	15	60

water for 3 to 4 times. This was followed by treatment with 0.1% mercuric chloride solution for 3 minutes and thoroughly washed with double distilled water for 3 to 4 times inside the Laminar Air Flow chamber. After surface sterilization, the nodes and shoot tips (about 0.5 cm long) were placed in MS (1962) medium supplemented with various concentrations of Cytokinins namely BA (benzyladenine) and KIN (kinetin) 1.0 – 5.0 mg/l alone or in combination i.e. BA 1-5 mg/L and NAA 1.0 mg/l or 2, 4-D and KIN 1.0 - 5.0 mg/l and NAA 1mg/l or 2, 4-D 1.0 mg/l for multiple shoot formation. Sucrose 3% (w/v) was used as the carbon source and agar 0.8% (w/v) was used for gelling. The pH of the medium was adjusted to 5.7

with NaOH before autoclaving at 121°C for 20 min. The culture was incubated at a constant temperature of 25 ± 2°C with 16 h lights (2000 lux) and 8 h darkness. After shoot initiation from nodal and shoot tip segments 2 weeks old shoots were sub cultured on MS medium supplemented with same combination for further proliferation and elongation. Elongated shoots (3-4 cm) were excised and inoculated on the rooting medium consisting of half strength and full strength MS medium. The medium was variously supplemented with auxins namely IAA and IBA at different concentrations (0.1,0.5,1.0 and 1.5mg/L). For acclimatization, the rooted shoots from one month old cultures were removed from the gelled rooting medium and washed under running water to remove traces of medium. The plantlets were transferred into paper cups with sand & soil (1:1), cocopith & soil (1:1), sawdust & soil (1:1) and sand & cocopith & sawdust (1:1:1) for hardening.

RESULTS

Multiple shoots were successfully induced from shoot tip and nodal explants of *Lippia nodiflora* cultured on MS medium. The shoot formation percentage ranged from 80-100%. In control (without addition of PGR) only 2 shoots emerged, whereas in those grown in presence of BA and KIN the number of shoots was high (Table 1&2). BA at concentration of 3.0 mg/l was able to induce up to 14.63 ± 1.30 (Fig. 1D) shoots when the shoot tips were used as explants and an average of 10.20 ± 1.55 shoots produced when nodal explants were used. In the presence of KIN only about 3 shoots were produced both in shoot tip and nodal explants (Table 1).

The combined effect of BA along with auxins such as NAA and 2, 4-D was also studied. Results show that maximum of 12.00 ± 1.93 (Table 1) shoots were produced when the medium contained 2.0 mg/l BA along with 1.0 mg/l NAA when shoot tips were used as explants, and a maximum of 6.89 ± 1.76 shoots were produced from the nodal explants when the medium contained 2.0 mg/l BA and 1.0 mg/l NAA (Table 2). However, a combination of 4.0 mg/l BA and 1.0 mg/l 2, 4-D was able to produce an average of 8 shoots per explant with reference to shoot tip and node (Table 1). Multiple shoot induction was minimal when medium contained KIN alone or in combination with auxins 2, 4-D and NAA (Table 1&2). The results clearly indicate that BA without supplementation of auxins was able to induce maximum number of shoots in both shoot tips and nodal explants. The length of shoots were measured on the 4th week and it was found that the average length of the shoots was maximum (6.32 ± 1.46) when BA was used at 3.0 mg/l in the case of shoot tip explants (Table 1). Whereas in the case of nodal explants the maximum length recorded was 6.01 ± 2.04 (Table 2). Combination of BA / KIN with NAA / 2, 4-D did not increase the shoot length. The effect of auxin on *in vitro* rooting was examined using IAA and IBA. The micro shoots were separated and cultured in full and half strength MS medium supplemented with IAA and IBA at different concentrations of 0.1, 0.5, 1.0 & 1.5 mg/l. Root induction was observed within a period of two weeks. The present study showed that maximum number of roots per shoot (10.3 ± 4.00) was observed in half strength MS basal medium with 1.0 mg/l IBA (Fig.1E). Whereas 7.2 ± 1.9 roots per shoot were noted in half strength MS medium supplemented with 0.5 mg/l IAA.

We observed that the addition of coco pith and sand (1:1) had increased the survival rate of the acclimatized plantlets. An average of 95 – 98% of the acclimatized plantlets survived after two weeks of transferring into field. After one month of transfer to field conditions the propagated plants exactly resembled the mother plants, no morphology variations were observed.

DISCUSSIONS

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 best effective PGR for multiple induction and multiplication (Shoots/Node) in *L. nodiflora* was achieved on MS medium containing 3.0 mg/l BA, as compared to KIN, at concentration of 2.0 mg/l. Similar response was noted in *Lippia alba* at 2.0 mg/l BA (Gupta *et al*., 2001) and *L. junelliana* (Julani *et al*., 1999). Kinetin produced less number of shoots when compared to that of BA, similar results were observed in *L. alba*. Kinetin has been reported as an ineffective Cytokinin for shoot proliferation in some plants (Gupta *et al*., 2001), *L. junelliana* (Julani *et al*., 1999). In *Vitex negundo* BA was found to be more effective than KIN in the induction of multiple shoots from the nodal explants (Noman *et al*., 2008), in *Dictyospermum ovalifolium* (Thoyajaksha and Rai, 2001) and in *Vitex agnus-castus* BA was found to be superior to KIN in numbers of shoots produced (Balaraju *et al.,* 2008). Some plant species can root easily without any plant growth regulators while some need the assistance of auxin for successful *in vitro* rooting of micro-shoots. On the other hand, Arikat *et al*. (2004) reported that auxin was needed for the rooting of *Salvia fruticosa*. For root induction IBA was found to be more suitable than IAA (Table 3). Half strength MS medium supplemented with IBA 1.0 mg/l produced maximum number of roots (10.3±4.0) and in full strength maximum was observed in 1.0 mg/l (7.9 ±3.21).The effective response of IBA in rooting has been reported in other medicinal plants such as *Eclipta alba* (Baskaran and Jeyabalan, 2005), *Vitex negundo* (Vadawale *et al*., 2005), *Hemidesmus indicus* (Sreekumar *et al*., 2000), *Withania somnifera* (Vadawale *et al*., 2004). In *Bauhinia cheilantha* , maximum rooting was observed in IBA (Gutierrez *et al* 2010).

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