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MULTIPLE SHOOT AND PLANT REGENERATION FROM IMMATURE LEAFLETS OF *IN VITRO* ORIGIN IN CURRYLEAF (*MURRAYA KOENIGII* SPRENG)

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SUMMARY

High frequency shoot regeneration protocol from *in vitro* raised young leaves of *Murraya koenigii* Spreng was standardized. A combination of 6.6 µM 6-benzyl amino purine (BAP) and 2.9 µM indole 3-acetic acid (IAA) has induced significantly ($p<0.05$) more number of shoots per explant (21.5). Maximum length (2.72 cm) of regenerated shoots (8 weeks after initial culture) was observed on Murashige and Skoog (MS) medium with 5.5 µM BAP and 2.9 µM IAA and on medium with 4.4 µM BAP and 2.9 µM IAA (2.69) which also gave maximum number of leaves per shoot (13.4). Maximum number of roots (recorded 6 and 8 weeks after initial culture) from cut end of induced shoots was obtained on MS medium supplemented with 26.9 or 37.6 µM α-naphthalene acetic acid (NAA). Percentage seedling establishment and whole plant fresh and dry weight were best on hardening medium containing peat, perlite and sand at 1: 1: 2 ratios.

***Key words*: Cytokinin, hardening, micropropagation, plant regeneration, tissue culture.**

INTRODUCTION

Curryleaf (*Murraya koenigii* Spreng, Rutaceae) is an important spice cum vegetable grown throughout the world. This erect growing perennial and evergreen aromatic shrub is native to *Tarai* tract of Southeast Asia and later introduced to Florida (Morten 1984). Curryleaf has been used in Ayurvedic and Yunani preparations (Joseph *et al*. 1981), rich source of vitamin A (12,600 IU), calcium (830 mg/100g) and possesses anti-bacterial and anti-fungal properties (Joseph and Peter 1985). Bark is bitter, acrid, cooling, alexeteric, anthelmintic, analgesic and used to cure piles, allay heat of body, thirst, inflammation, itching and is useful in treating blood disorders (James 1996). Though seed set is poor and results in highly heterogeneous population, seed propagation is most followed (Ranganathappa *et al*. 2001) because of high-level recalcitrance for rooting of mature stem cuttings that could be solved only with high concentration (5000 ppm) of IBA (Ranganathappa *et al*. 2002). At this juncture, tissue culture protocol for high frequency *in vitro* regeneration in curryleaf will be of much use.

Previous reports on micropropagation in curryleaf show the use of intact seedlings (Bhuyan *et al*. 1997) or internodes (Hazarika *et al*. 1995) as explants, which resulted in comparatively lesser shoot regeneration frequency. On the contrary, this paper details a high frequency regeneration protocol for curryleaf.

MATERIALS AND METHODS

Shoot tips (approx. 1 cm) collected from 6 year old plants of cultivar DWD-1 were used as explants which were rinsed thoroughly in running water and sterilized

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using 0.1% $HgCl_2$ treated for 15 min., followed by three 5 min. washes with sterile double distilled water. MS basal medium with 3 % sucrose (pH 5.8 and autoclaved at 15 lbs and 121°C for 20 min) was used for establishment of initial shoot tip cultures. About 35 ml medium was dispersed into each glass bottle (250 ml capacity) and shoot tips were inoculated after a fresh cut at the basal end. Cultures were incubated under 16 h photoperiod (50 µmol m^{-2} s^{-1} fluorescent white light) at 25±1°C. Fresh growth of leaves at shoot tip was observed after three weeks of culture (Fig. 1). Seven days after opening, individual basal leaflets with 0.5-0.7 cm length and 0.3-0.5 cm breadth were inoculated on MS medium containing different concentrations of cytokinins (BAP at 0.0, 2.2, 3.3, 4.4, 5.5, 6.6 and 7.7 µM or kinetin at 0.0, 2.3, 3.5, 4.6, 5.8, 6.9 and 8.1 µM; Sigma Aldrich) alone or with 2.9 µM IAA (Himalaya Drugs). Cytokinins were added in the medium prior to autoclaving and filter sterilized IAA was added just before dispersing into bottles. Ten leaflets were inoculated into each bottle with lower surface in contact with medium. Sub-culturing was done in every 7 days throughout the experiment and each treatment was replicated 10 times. Effect of different treatments on regeneration was quantified 8 weeks after culture in terms of number, length and number of leaves per induced shoots.

Eight weeks old shoots from regeneration media were transferred to rooting media with different levels of NAA (5.4, 16.1, 26.9, 37.6 and 53.7 µM; Himalaya Drugs). Five shoots were inoculated in each bottle, each treatment was replicated 10 times and number and length of roots induced 6 and 8 weeks after inoculation were recorded. After 8 weeks of culture, seedlings were removed from media, washed to remove agar remains and transferred to hardening media with different combinations of peat, perlite and fine sand (washed free of soil); under greenhouse conditions. For the initial week, seedlings were covered with 200 gauge polyethylene pouches to maintain the humidity. Observations on percent seedling establishment and fresh and dry (120°C for 1 hour) plant weights (using Metler digital balance) were recorded. Significant differences (CD at 5%) between treatments were calculated following Student's t-test (Rangaswamy 1995).

RESULTS AND DISCUSSION

In the experiment to study the shoot induction from immature leaflets (Table 1), all treatments except those devoid of BAP had shown initiation of shoot induction within 2 weeks of inoculation (Fig. 2) and in 4 weeks, differentiation was complete (Fig. 3). Eight weeks after culture, higher average (of all the treatments in this experiment) number of shoots per explant, length of regenerated shoots and number of leaves per shoot were observed on media containing both auxin and cytokinin (6.45, 1.33 and 6.74 respectively) compared to media

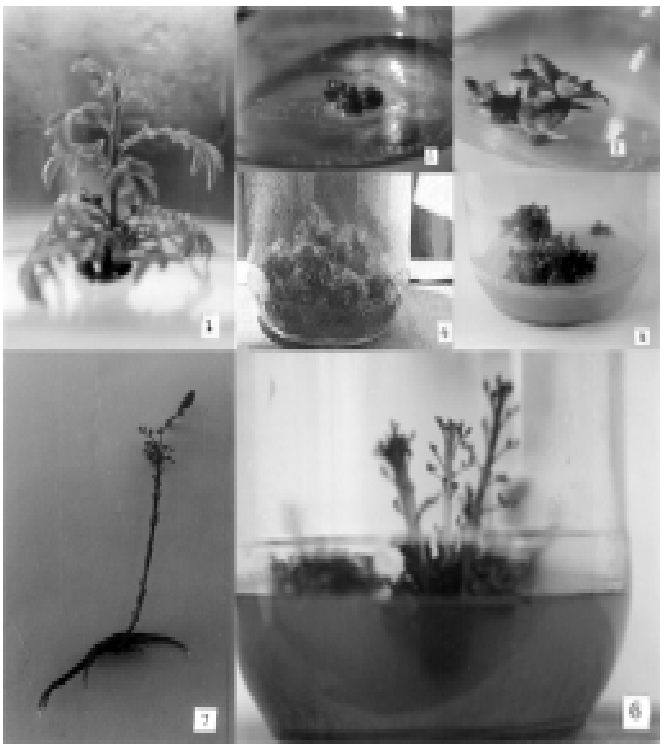

Fig. 1. *In vitro* established curryleaf shoot tip showing fresh growth of leaves after three weeks of culture on basal MS medium, Fig. 2 & 3. Initiation and completion of shoot differentiation from immature leaflet explant after two and four weeks of culture on MS medium supplemented with 7.7 µM BAP and 2.9 µM IAA Fig. 4. Rapid proliferation of shoot buds from immature leaflets of curryleaf cultured on MS medium with 6.6 µM BAP and 2.9 µM IAA Fig. 5. Comparatively lesser proliferation of shoot buds on MS medium with supra-optimal level of BAP (7.7 µM) along with 2.9 µM IAA Fig. 6. *In vitro* regenerated and elongated shoots (8 weeks old) ready to be transferred to rooting media and Fig. 7. *In vitro* regenerated complete plantlet of curryleaf after 8 weeks of culture on rooting medium containing 16.1 µM NAA

Table 1. Effect of various plant growth regulators on shoot induction from immature *in vitro* raised leaflets of curryleaf (8 weeks after culture)

MS basal with growth regulator(s) (µM)	Number of shoots induced	Mean shoot length (cm)	Leaves per induced shoot
BAP			
0.0	0.0^{k}	0.00^{l}	0.0^{m}
2.2	0.6^{jk}	1.25^{fghi}	6.7^{h}
3.3	1.4^{ijk}	1.53^{def}	6.1^{hi}
4.4	1.5^{ijk}	2.72^{a}	10.2^{d}
5.5	3.0^{gh}	2.63^{a}	11.0^{c}
6.6	5.1^{ef}	2.19^{b}	10.9^{c}
7.7	8.5^{d}	1.62^{cde}	5.8^{ij}
BAP+IAA			
0.0+2.9	0.0^{k}	0.00^{l}	0.0^{m}
2.2+2.9	1.0^{jk}	1.45^{def}	6.2^{hi}
3.3+2.9	1.6^{ijk}	1.69^{cd}	8.6^{f}
4.4+2.9	4.4^{efg}	2.69^{a}	13.4^{a}
5.5+2.9	11.2^{c}	2.72^{a}	12.0^{b}
6.6+2.9	21.5^{a}	2.23^{b}	12.3^{b}
7.7+2.9	17.4^{b}	1.39^{def}	7.4^{g}
KINETIN			
0.0	0.0^{k}	0.00^{l}	0.0^{m}
2.3	0.5^{jk}	0.28^{kl}	3.0^{l}
3.5	0.5^{jk}	0.73^{j}	5.3^{j}
4.6	0.6^{jk}	0.95^{ij}	5.4^{j}
5.8	1.8^{ijk}	1.32^{efg}	9.3^{e}
6.9	2.2^{hij}	0.33^{k}	4.1^{k}
8.1	5.3^{e}	0.90^{j}	3.9^{k}
KINETIN+IAA			
0.0+2.9	0.0^{k}	0.00^{l}	0.0^{m}
2.3+2.9	1.0^{jk}	0.40^{k}	3.1^{l}
3.5+2.9	1.6^{ijk}	0.91^{j}	4.0^{k}
4.6+2.9	3.2^{fghi}	0.99^{hij}	5.2^{j}
5.8+2.9	4.0^{efgh}	1.27^{fgh}	7.8^{g}
6.9+2.9	11.1^{c}	1.86^{c}	8.0^{fg}
8.1+2.9	12.3^{c}	1.02^{ghij}	6.3^{hi}

Replications-10, values followed by the same letter are not significantly different by student t-test at 0.05 per cent probability level

Table 2. Rooting responses with different levels of NAA on shoots of *in vitro* origin in curryleaf

MS medium	6 weeks after culture		8 weeks after culture	
with NAA (µM)	Number of roots induced	Mean length (cm) of induced roots	Number of roots induced	Mean length (cm) of induced roots
0.0	0.0[d]	0.00[d]	0.0[d]	0.00[d]
5.4	0.0[d]	0.00[d]	0.0[d]	0.00[d]
16.1	3.6[b]	1.55[ab]	4.1[c]	3.82[a]
26.9	5.4[a]	1.61[a]	8.3[a]	3.58[b]
37.6	5.6[a]	1.53[b]	8.2[a]	3.12[d]
53.7	3.2[c]	1.32[c]	5.1[b]	3.41[c]

Replications-10, values followed by the same letter are not significantly different by student t-test at 0.05 per cent probability level

Table 3. Effect of various media on establishment of 8 weeks old curryleaf seedlings of *in vitro* origin (after 6 weeks of transfer to the hardening media)

Ratio of peat, perlite and sand in the media	Seedling establishment (%)	Plant fresh weight (g)	Plant dry weight (g)
1: 1: 1	69.9[c]	21.3[de]	2.3[b]
2: 1: 1	73.3[c]	26.4[bc]	3.2[ab]
1: 2: 1	66.6[c]	23.2[cd]	2.7[ab]
1: 1: 2	96.7[a]	31.6[a]	4.1[a]
2: 2: 1	56.6[d]	18.4[e]	1.8[b]
1: 2: 2	83.3[b]	28.7[ab]	3.5[ab]

Each treatment with 15 seedlings replicated 2 times. values followed by the same letter are not significantly different by student t-test at 0.05 per cent probability level

with no auxin (2.21, 1.18 and 5.84). Increased efficiency of BAP and IAA combination over BAP alone in shoot induction was reported earlier (Phillips and Hubstenberger 1985). BAP gave higher average (of all treatments in this experiment) number of shoots per explant, length of regenerated shoots and number of leaves per shoot (5.51, 1.72, and 7.90 respectively) over kinetin (3.15, 0.78 and 4.67). Previously, the superiority of BAP over kinetin in multiple shoot induction was reported in mangosteen (Goh *et al*. 1990), carambola (Rashid and Ahmed 1992) and roseapple (Prashantha *et al*. 2003). Maximum number of shoots (21.5) was proliferated on medium with 6.6 µM BAP and 2.9 µM IAA (Fig. 4). A higher concentration of 7.7 µM BAP was supra-optimal resulting in 17.4 shoots per explant (Figure 5). Longest shoots were observed on media with 4.4 or 5.5 µM BAP alone (2.72 and 2.63 cm respectively) or in combination with IAA (2.69 and 2.72 cm respectively). Maximum number of leaves (13.4) was observed on medium with 4.4 µM BAP and 2.9 µM IAA.

In the *in vitro* rooting experiment, best response (Table 2) in terms of number (5.4) and length (1.61 cm) of induced roots was on medium supplemented with 26.9 µM NAA (Figure 6). Medium fortified with 37.6 µM NAA also gave good rooting response (5.6, 1.53 cm respectively). Eight weeks after inoculation, higher levels of NAA (26.9 and 37.6 µM) were found to induce higher

number of roots (8.3 and 8.2 respectively) but root length reduced with increase in concentration. Highest root length (3.82 cm) was obtained on medium with 16.1 µM IAA (Fig. 7) suggesting that higher level of auxin is needed for root induction only. These observations are in agreement with the previous results in pepper (Mathew 2000).

A combination of peat, perlite and sand in 1: 1: 2 ratio was best hardening medium (Table 3) in terms of seedling establishment (96.7%), fresh (31.6 g) and dry (4.1 g) plant weights. Poorest seedling establishment (56.6) in the medium with high peat and perlite (2: 2: 1) is attributed to poor drainage as evidenced by yellowing and subsequent decaying which are typical to water stagnation (Malathi 2001).

This study suggests that for efficient micropropagation of curryleaf, one week old leaflets from initially established cultures shoot tips should be cultured on MS medium with 6.6 µM BAP and 2.9 µM IAA. Regenerated shoots could be converted to whole plant using 26.9 µM NAA and best hardened on substrate containing peat, perlite and sand at 1: 1: 2 ratios.

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