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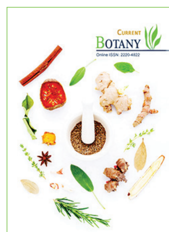

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In vitro regeneration of plantlets from leaf segments of *Pupalia lappacea* (L.) Juss. for mass propagation

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ABSTRACT

The *in vitro* study was carried out to standardize the protocol for induction of callus, proliferation of shoots, initiation of roots, and plant regeneration in *Pupalia lappacea* (L.) Juss. using the artificial nutrient culture medium. Maximum callus induction (76%) from the young leaf explants was achieved by using MS medium supplemented with 0.5 mg L^{-1} of 2, 4-D and 2.5 mg L^{-1} of NAA. The maximum shoot induction (78%) was observed on MS medium containing 2.0 mg/L of BAP and 0.8 mg/L of GA_3. The highest root initiation (70%) was obtained on MS medium with IBA (1.2 mg L^{-1}) and IAA (0.5 mg L^{-1}). The acclimation process was successfully completed in the garden soil with a better survival rate (72%).

KEYWORDS: *Pupalia lappacea*, *In vitro* studies, Growth regulators, Acclimatization

INTRODUCTION

The medicinal plant, *Pupalia lappacea* (L.) Juss. Belongs to the Amaranthaceae, which is distributed in the Kolli Hills of Namakkal District, the Eastern Ghats, Tamil Nadu, India. *P. lappacea* is a perennial erect or prostrate herbaceous plant with potential therapeutic values. Inflammatory disorders and bone fractures are treated by the application of *P. lappacea* leaf paste with edible oil in folklore medicine (Jalalpure *et al.*, 2008). The decoction is extracted from the fruits of *P. lappacea* and mixed with palm oil to treat boils and wounds. The fruit juice is also used in the treatment of fever and cough. In Africa, the fruit is one of the ingredients for the preparation of enema, which is mixed with palm oil and applied to treat leprosy and boils skin. The leaf juice is used to treat flatulence, and it is also used to cure malaria, jaundice, diarrhoea, erectile dysfunction, and paralysis (Bero *et al.*, 2009). Therefore, large-scale cultivation of species is needed to conserve the species due to the high demand for potential medicinal *P. lappacea* for the preparation of medicine. In general, various conservation techniques have been practiced worldwide for the protection of plant species; the most important one is tissue culture (Parabia *et al.*, 2007). The plant tissue culture technique has great promise for pharmaceutical industries, plant breeders, and others, besides helping in the conservation of plant species for the maintenance of natural wealth. Therefore, the present study was aimed to standardize the protocol for *in vitro* regeneration through leaf explants of *P. lappacea* for mass multiplication.

MATERIALS AND METHODS

Healthy seeds of *Pupalia lappacea* were collected from the Kolli Hills of Namakkal District. The mother plants were raised by sowing seeds in pots and maintained in the green house. Young leaves were collected from mother plants, washed twice with running tap water, and then treated with a 5% tween-20 solution and rinsed with running tap water. Further explants were treated with 5% Amphicillin and Rifampicin antibiotics for the elimination of fungal contamination. After three rinsings of the explants with distilled water, the surface sterilization of the explants was done with 0.1% $HgCl_2$ for 3 minutes and rinsed with double-distilled water 3-4 times. Murashige and Skoog (1962) medium was prepared by using 3% sucrose with 1% agar (Hi-Media, India). Before autoclaving at 121 °C for 15 min, the pH value of 5.6-5.7 was adjusted prior to the addition of agar to the MS medium. Culture bottles were maintained inside the culture chamber with a relative humidity of 60-65% at 24 ± 2 °C under a photoperiod of 16/8 hr (light/dark) with a light intensity of 2000 lux provided by white fluorescent tubes.

The leaf segments were inoculated in the MS medium supplemented with various concentrations and combinations of 2,4-D and NAA for callus initiation. For shooting, MS medium was prepared with different concentrations and combinations of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L of BAP and 0.8 mg/L of GA_3. After shoot induction, *in vitro* regenerated shoots with a length of 5-6 cm were excised and transferred onto the rooting medium

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supplemented with various concentrations and combinations of IBA, Kn, and IAA for rooting. For acclimatization, the rooted plantlets were removed from culture bottles, washed with running tap water, and transferred to plastic cups filled with hardening medium containing garden soil, sand, and vermicompost (1:1:1) and maintained in the greenhouse for the study of plantlet survivability. All the experiments were done at least two times, in triplicate. The statistical analysis was done, and mean values were compared using Duncan's multiple range test ($P<0.05$).

RESULTS AND DISCUSSION

The callus initiation was archived after 25 days of inoculation of leaf segments in the MS medium (Figure 1a). Maximum callus induction (76%) was observed in the MS medium containing 2.5 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} of NAA (Table 1 & Figure 1b). A similar effect of 2,4-D on callus formation in the leaf explants of another species, *Achyranthes aspera*, belongs to the family Amaranthaceae as observed by Sen *et al.* (2014). Further studies were conducted to find out the shoot induction capacity of the callus. It was observed that the maximum shoot induction (78%) was observed on MS medium containing 2.0 mg L^1 of BAP and 0.8 mg L^1 of GA_3. The maximum length of shoot (6.6 cm) and number of multiple shoots (8.37 shoots/callus) were obtained on the MS medium with the same combinations and concentrations of growth hormones (Table 2 & Figure 1c). The maximum effect of BAP over other cytokinins on shoot proliferation of several medicinal plants has been reported (Jebakumar & Jayabalan, 2000; Hussain & Anis, 2006; Faisal & Anis, 2003; Chakradhar & Pullaih, 2014; Magendiran *et al.*, 2022). Among the different concentrations of cytokinins, BAP gives the highest number of numerous shoots, and this hormone can help avoid plant

Table 1: Effect of growth regulators on callus induction from leaf explants of the species *Pupalia lappacea*

Growth regulators (mg/L)				Days required for callus formation after inoculation	Callus formation (%)
2,4-D	BAP	NAA	IAA	Leaf Explant	Leaf Explant
0.0	0.5	0.2	0.0	14	24.11 ± 0.52[g]
0.0	1.0	0.4	0.0	15	35.02 ± 1.21[h]
0.0	1.5	0.8	0.0	17	40.34 ± 0.82[j]
0.0	2.0	1.0	0.0	20	44.14 ± 1.21[l]
0.0	2.5	1.2	0.0	21	50.23 ± 1.63[i]
0.0	3.0	1.4	0.0	19	53.18 ± 0.82[i]
0.5	0.0	0.5	0.0	23	57.56 ± 1.24[a]
1.0	0.0	0.5	0.0	25	60.76 ± 0.82[b]
1.5	0.0	0.5	0.0	26	67.17 ± 1.41[c]
2.0	0.0	0.5	0.0	28	72.26 ± 1.21[f]
2.5	0.0	0.5	0.0	25	76.00 ± 0.82[h]
3.0	0.0	0.5	0.0	21	59.21 ± 0.67[b]
0.0	0.2	0.0	0.3	20	32.38 ± 1.63[d]
0.0	0.4	0.0	0.3	21	39.45 ± 0.63[g]
0.0	0.6	0.0	0.3	23	45.64 ± 0.82[h]
0.0	0.8	0.0	0.3	19	55.32 ± 1.12[i]
0.0	1.0	0.0	0.3	17	58.17 ± 1.42[i]
0.5	0.0	0.0	0.2	19	28.89 ± 0.21[a]
1.0	0.0	0.0	0.4	21	33.43 ± 0.82[b]
1.5	0.0	0.0	0.6	23	37.00 ± 0.42[d]
2.0	0.0	0.0	0.8	20	40.00 ± 1.34[e]
2.5	0.0	0.0	1.0	22	45.00 ± 0.12[e]

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 2: Effect of different concentrations of growth regulators on shoot initiation, shoot number and shoot length after the subculturing of leaf derived callus of the species *Pupalia lappacea*

Growth regulators (mg/L)					Culture response (%)	No. of shoots/callus	Shoot length (cm)
BAP	GA_3	Kn	IBA	NAA			
0.5	0.0	0.0	0.2	0.0	23.14 ± 0.62[a]	2.42 ± 0.32[a,b,c]	1.9 ± 0.02[a]
1.0	0.0	0.0	0.4	0.0	31.17 ± 1.03[f,g]	3.00 ± 1.63[a,b]	2.9 ± 0.82[a,b,c]
1.5	0.0	0.0	0.6	0.0	43.10 ± 0.12[h]	4.42 ± 0.62[b,c,d]	3.1 ± 1.63[a,b,c]
2.0	0.0	0.0	0.8	0.0	44.08 ± 1.63[c]	5.10 ± 1.63[d,e,f]	3.5 ± 0.82[a,b,c]
2.5	0.0	0.0	1.0	0.0	42.24 ± 0.82[d,e]	4.99 ± 1.21[c,d,e]	4.2 ± 0.62[a,b,c]
3.0	0.0	0.0	1.2	0.0	40.07 ± 1.23[c,d]	4.20 ± 0.82[b,c,d]	4.4 ± 1.21[a,b,c]
0.5	0.2	0.0	0.0	0.0	56.48 ± 1.21[h]	4.75 ± 0.16[c,d,e]	3.7 ± 1.34[a,b,c]
1.0	0.4	0.0	0.0	0.0	64.00 ± 0.42[i]	5.59 ± 1.63[e,f,g]	4.5 ± 0.82[a,b,c]
1.5	0.6	0.0	0.0	0.0	70.13 ± 0.61[j]	7.58 ± 0.41[d,e,f]	5.4 ± 1.14[b,c]
2.0	0.8	0.0	0.0	0.0	78.07 ± 1.23[i]	8.37 ± 1.43[h,i]	6.6 ± 1.24[b,c]
2.5	1.0	0.0	0.0	0.0	75.67 ± 0.16[k]	6.26 ± 1.21[i]	6.0 ± 0.12[c]
3.0	1.2	0.0	0.0	0.0	58.06 ± 1.21[h]	6.08 ± 0.82[g,h,i]	4.9 ± 0.82[a,b,c]
0.5	0.0	0.2	0.0	0.0	33.38 ± 0.82[g]	3.76 ± 0.82[f,g,h]	3.2 ± 1.61[a,b,c]
1.0	0.0	0.2	0.0	0.0	37.40 ± 0.82[d,e]	4.12 ± 1.63[d,e,f]	3.6 ± 0.42[a,b,c]
1.5	0.0	0.2	0.0	0.0	43.67 ± 1.23[h]	4.86 ± 1.63[a,b,c]	4.2 ± 0.02[a,b,c]
2.0	0.0	0.2	0.0	0.0	49.89 ± 0.82[i]	5.28 ± 0.12[a,b]	4.9 ± 0.82[a]
2.5	0.0	0.2	0.0	0.0	53.55 ± 0.62[j]	5.67 ± 0.62[a]	5.1 ± 1.63[a,b,c]
3.0	0.0	0.2	0.0	0.0	55.26 ± 1.63[h]	6.08 ± 0.31[b,c,d]	5.9 ± 0.82[a,b,c]
0.5	0.0	0.0	0.0	0.1	30.12 ± 0.82[i]	3.25 ± 1.03[c,d,e]	3.2 ± 1.63[a,b,c]
1.0	0.0	0.0	0.0	0.2	34.66 ± 0.42[j]	4.06 ± 1.21[a,b,c]	4.1 ± 1.23[a,b,c]
1.5	0.0	0.0	0.0	0.3	39.37 ± 0.14[g]	4.26 ± 0.22[a]	4.5 ± 0.82[a,b,c]
2.0	0.0	0.0	0.0	0.4	49.47 ± 1.21[e,f]	4.44 ± 0.82[a,b]	3.6 ± 0.82[a,b,c]
2.5	0.0	0.0	0.0	0.5	54.76 ± 1.63[c,d]	5.16 ± 1.23[b,c,d]	3.9 ± 1.63[a,b,c]
3.0	0.0	0.0	0.0	0.6	64.00 ± 0.82[b]	5.98 ± 0.82[d,e,f]	4.7 ± 0.32[a,b,c]

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 3: Effect of different concentrations of growth regulators on rooting percentage, root number and root length after subculturing the leaf derived callus of the species *Pupalia lappacea*

Growth regulators (mg/L)			Shoots rooted (%)	No. of roots/ shoot	Root length (cm)
IBA	Kn	IAA			
0.5	0.2	0.0	31.16 ± 0.23[b]	3.25 ± 0.19[a,b,c]	2.7 ± 0.40[a-d]
1.0	0.4	0.0	35.29 ± 1.43[d]	3.67 ± 0.41[a,b,c]	3.3 ± 0.62[ab]
1.5	0.6	0.0	38.09 ± 0.24[e]	4.08 ± 1.23[b,c,d]	3.9 ± 0.82[abc]
2.0	0.8	0.0	42.43 ± 0.41[g]	4.75 ± 0.82[a,b,c]	4.1 ± 0.31[c-f]
2.5	1.0	0.0	51.34 ± 1.21[h]	5.26 ± 0.12[c,d,e]	4.7 ± 0.35[ef]
3.0	1.2	0.0	55.10 ± 0.17[d]	4.91 ± 1.21[b,c,d]	3.9 ± 0.42[b-e]
0.3	0.0	0.5	38.11 ± 0.25[c]	3.96 ± 0.31[a,b,c]	3.6 ± 0.22[abc]
0.6	0.0	0.5	46.45 ± 1.42[e]	4.28 ± 0.24[b,c,d]	4.7 ± 1.41[abc]
0.9	0.0	0.5	65.17 ± 0.11[f,g]	6.78 ± 0.82[a]	5.8 ± 0.29[b-e]
1.2	0.0	0.5	70.18 ± 0.42[i]	8.76 ± 0.24[c,d,e]	6.7 ± 0.82[a]
1.5	0.0	0.5	63.26 ± 0.12[i]	7.14 ± 0.41[d,e]	6.0 ± 0.32[d,e,f]
1.8	0.0	0.5	60.38 ± 1.34[j]	7.11 ± 1.21[e]	5.4 ± 0.23[f]
0.0	0.5	0.3	29.16 ± 1.27[a]	2.48 ± 1.32[a,b]	1.9 ± 1.43[a,b,c]
0.0	1.0	0.3	39.45 ± 0.82[b]	3.43 ± 0.71[a,b]	2.6 ± 0.16[b-f]
0.0	1.5	0.3	43.34 ± 1.43[d]	4.20 ± 1.40[a,b,c]	3.4 ± 0.82[a,b,c]
0.0	2.0	0.3	48.14 ± 0.24[d]	4.85 ± 0.41[b,c,d]	3.8 ± 0.34[a-d]
0.0	2.5	0.3	50.28 ± 1.19[e]	5.45 ± 1.24[c,d,e]	3.5 ± 0.42[ab]
0.0	3.0	0.3	38.27 ± 0.42[e,f]	3.97 ± 0.82[a,b,c]	3.2 ± 0.21[a-d]

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 4: Effect of different composition of hardening medium on survivability rate of leaf callus derived plantlets of the species *Pupalia lappacea*

Hardening medium composition (V/V)	No. of plantlets under hardening	No. of plantlets survived	Survivability (%)
Red soil + sand (1:1)	50	24	45 ± 1.14[a]
Garden soil + sand + vermicompost (1:1:1)	50	43	72 ± 0.24[e]
Decomposed coir waste + perlite + compost (1:1:1)	50	37	68 ± 0.43[d]
Vermicompost + soil (1:1)	50	31	60 ± 1.21[c]
Red soil + sand + vermicompost (1:1:1)	50	28	54 ± 0.61[b]

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT

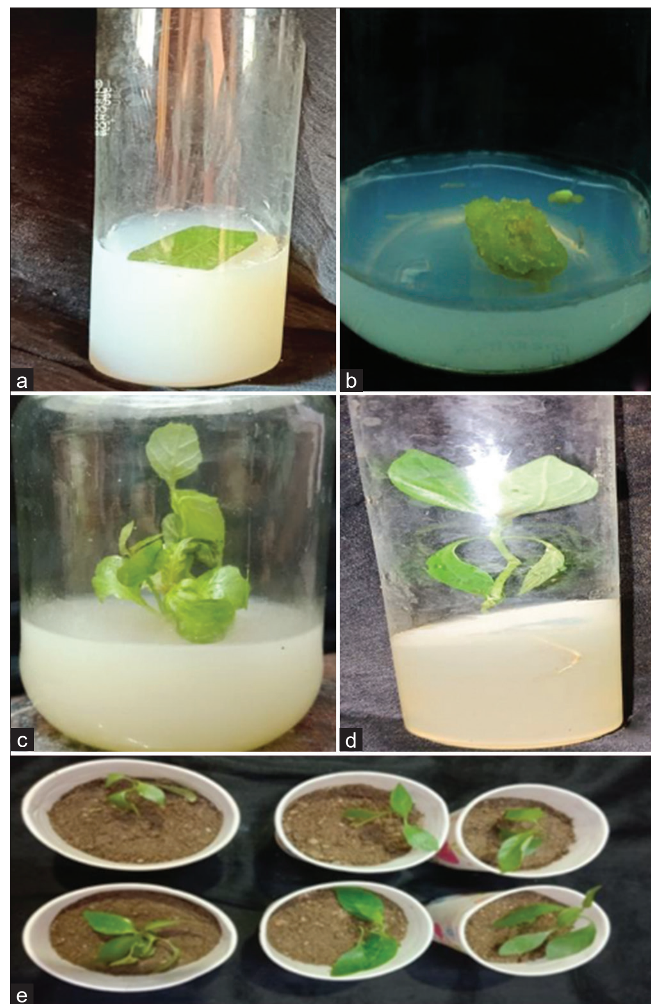

Figure 1: *In vitro* regeneration through leaf explant of *Pupalia lappacea*. a) Leaf explant inoculation, b) Callus initiation, c) Multiple shoot proliferation, d) Root initiation and e) Acclimatized plantlets

abnormalities (Kasilingam *et al.*, 2018). Ghose *et al.* (2022) also confirmed that MS media supplemented with BAP and other growth regulars like NAA showed the highest efficiency in producing multiple shoots.

Root induction is an important step in the *in vitro* regeneration of any plant species. Multiple shoots were excised from the culture bottles and inoculated on MS medium supplemented with IAA and IBA for rooting. The rooting attributes of the study species are given in Table 3. The maximum root induction (70%), root length (6.7 cm), and number of roots (8.76 roots per shoot) were observed when MS medium was fortified with IBA (1.2 mg L^{-1}) and IAA (0.5 mg L^{-1}) (Table 3 & Figure 1D). The same results have already been confirmed by many *in vitro* studies (Sreekumar et al., 2000; Ramulu *et al.*, 2002; Martin, 2002; Lemma *et al.*, 2020; Prajapati *et al.*, 2023; Sarropoulou *et al.*, 2023). The *in vitro* rooted plantlets were successfully transferred to plastic cups filled with a hardening medium containing garden soil, sand, and vermicompost (1:1:1) and maintained in the greenhouse (Figure 1e). The survivability rate of leaf-derived plantlets was higher (72%) in the green house conditions (Table 4).

CONCLUSION

The present *in vitro* regeneration study has described an efficient protocol for callus induction and successful acclimatization of *P. lappacea* from leaf explants. The plant regeneration was successful in various concentrations and combinations of growth regulators. This protocol provides great potential for large-scale production of plantlets and to meet the needs of different pharmaceutical industries.

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