RESEARCH PAPER:

Micropropagation of *Talinum cuneifolium* (Vahl.) Willd. through petiole culture

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SUMMARY

A simple and reliable protocol was developed through leaf petiole explant of *Talinum cuneifolium* for multiple shoot regeneration. This leafy vegetable tuberous shrub has been successfully *in vitro* propagated on MS (Murashige and Skoog) nutrient medium. The effect of auxins (IAA, NAA, IBA) and cytokinins (BA, Kn) were examined singly and also in different combinations and concentrations. IBA at 0.5 mgl^{-1} proved better results by achieving light brown coloured compact calli with roots. Shoot regeneration was succeeded by alone and both combinations of auxins and cytokinins. High frequency (56.04 ± 0.05) of shoot regeneration was achieved using BA at 2 mgl^{-1} alone and maximum mean length (2.72 ± 0.05) of the shoots were established in the medium fortified with the combination of BA 1 mgl^{-1} + Kn 2 mgl^{-1}. Elongated shoots were excised and sub cultured for rooting on half strength MS medium fortified with 1 mgl^{-1} IBA. High frequency (85.70 ± 0.07) regeneration of roots with maximum mean number (12.46 ± 0.02) and maximum mean length (4.60 ± 0.01) were established. The *in vitro* regenerated plantlets were successfully acclimatized in paper cups containing vermiculite, then transferred to lab and green house. Hardened plants were transplanted in to sand and soil (1:1) and supplied ¼ strength MS medium for highest (85 to 90%) survival rate. An attempt was also made to determine the extent of clonal purity of the *in vitro* regenerated plants at the biochemical level by employing peroxidase isozyme as marker, in order to get an insight into the impact of somaclonal variations in the course of their regeneration.

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Talinum cuneifolium (Vahl.) Willd., / *Talinum portulacifolium* (Forsk.) Asch. Ex Schw. – an erect shrub with subterranean tuber belongs to the family Portulacaceae. The leaves of *Talinum cuneifolium* commonly known as Ceylone bachalli, are eaten as a cooked vegetable or as raw in salad, alone or with young stem parts. It is cultivated in Africa (like spinach), and is used as a green leafy vegetable due to its rich vitamin A and mineral content (Anonymous, 2004). The leaves can also be stored dry for later use. The plant is a palatable fodder for cattle and goats. It is also an important medicinal plant in the local systems of medicine. Indian system of medicine (ISM) refers that the leaves and roots are medicinally important parts. The supplementation of the leaves of this plant is reported to be a better diet for strengthening the body. 5 to 10 leaves are eaten daily in the morning to control blood sugar level in the diabetic patients (Savithramma, 2003). The powdered leaves are used in treatment of diabetic, mouth ulcer and aphrodisiac. Roots are used for cough, gastritis, diarrhoea and pulmonary tuberculosis (Madhavachetty *et al.,* 2008). In Ethiopia the leaves are applied medicinally against eye diseases and the root against cough and gonorrhoea (Saradvathi, 2009).

This valuable plant has markedly depleted to satisfy the local food and medicinal needs. The growth of plant is very slow and takes long time. One of the constraints associated with the conventional propagation was very short span of seed viability and low survival rate by stem cuttings in *Talinum cuneifolium* which restricts its mass propagation via conventional methods. No alternative mode of multiplication was available to propagate and to conserve genetic stock of this plant. *In vitro* multiple shoot regeneration may give higher rate of propagation within very short time and space.

Isoenzymes can be considered to be the direct expression of the gene function of cells

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during differentiation and their variations were often associated with somaclonal variations. A detailed analysis of their changing pattern during development may lead to some understanding of the basic mechanism of cellular differentiation to obtain efficient plant regeneration *in vitro*. Peroxidases and esterases were widely distributed among higher plants. The application of isozymes as markers in morphological and regeneration studies has been reported by several workers (Bhatt *et al.,* 1992; Feuser *et al.,* 2003) to detect clonal fidelity. The current problem facing the regenerating system in plant tissue culture was the occurrence of uncontrollable somaclonal variations that were undesirable in any clonal propagation and conservation programmes. Propagation by all methods of indirect organogenesis carries a risk that the regenerated plants will differ genetically from each other and from the mother plant (George, 1993). There have been no reports of a regeneration system for *Talinum cuneifolium*. Therefore, the present study was undertaken with an aim of establishing an efficient protocol for *in vitro* plant regeneration from leaf petiole explant.

MATERIALS AND METHODS

Leaves of *Talinum cuneifolium* (Vahl.) Willd. were collected from S.V. University Botanical gardens, Tirupati, A.P. and cut the petiole as explant from leaf . These explants were initially washed under running tap water with Teepol solution (5% v/v) for 15 min. followed by 4 to 5 washings with distilled water. Disinfestations of these explants was then made under laminar air flow chamber by keeping them in 70% alcohol for 60 sec. followed by rinsing for 3 times in sterile distilled water. Finally the explants were immersed in 0.1% $HgCl_2$ (mercuric chloride) for 3 min. The surface sterilization was followed by 5 to 6 rinses in sterile distilled water. The surface sterilized explants were cultured on MS basal medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.8% (w/v) agar. Explants were implanted in different combinations and concentrations of growth regulators (BA, IAA, IBA, NAA and 2,4-D) singly as well as in combinations for shoot proliferation. The pH of the medium was adjusted to 5.8 by using 0.1N HCl (hyderocloric acid) or 0.1N NaOH (sodium hydroxide) solutions before autoclaving. All cultures were incubated in a culture room at 25 ± 2^0C with a relative humidity of 50 to 60% and 16 h photoperiod at a photon flux density of 15-20 µE m^2/s^{-1} from white cool fluorescent tubes. For each treatment, 12 replicates were used and each experiment was repeated at least thrice. The cultures were examined periodically.

MS medium has been designated for tissue culture of shrubs. Each plant requires different quantities of inorganic and organic nutrients for its morphogenic response, so no single medium will give satisfactory results with all tissues used. Selection of an appropriate nutrient medium was also essential for the success of all experimental systems in plant tissue culture. In order to formulate a suitable medium for a new system, it was best to start with a well known basal medium such as MS medium (Bhojwani and Razdan, 1983). Accordingly MS medium was initially used in the present study. Medium composition greatly affected the callus and shoot regeneration of *Talinum cuneifolium.*

Non-SDS-PAGE (Poly Acrylamide Gel Electrophoresis) of peroxidase isozyme was carried out to test the clonal purity as per the method of Van Eldic *et al.* (1980).

RESULTS AND DISCUSSION

Auxins and cytokinins play a significant role in *in vitro* culture of plants. The auxin metabolism plays a critical role in the regeneration of plants from cell culture because removal of auxin from growth medium induces morphogenesis in callus culture (Ammirato, 1977). Callus developed on the above mentioned optimal medium was used for further studies to evaluate the effect of BA and Kn at various concentrations on indirect shoot regeneration. In general high concentrations of cytokinin and low concentration of auxin promote the induction of shoot organogenesis. There were also numerous cases, where the observed type of morphogenesis was opposite that which was expected of the phytohormone balance to which the explants were exposed (Thorpe, 1980).

Callus initiation:

In general juvenile explants were more responsive in proliferation of callus than explants derived from mature trees (Thorpe *et al.,*1991). Maximum callus was produced on MS medium supplemented with NAA and BAP from leaf explants of *Solanum aviculare* (Kittipongpatana *et al.,* 1998). Light yellow coloured nodular compact calli were initiated from the leaf petiole explant on MS medium supplemented with 0.5 mgl^{-1} NAA (Table 1 and Plate 1A). Light cream coloured nodular calli were produced when MS medium was supplemented with 0.5 mgl^{-1} IAA (Table 1 and Plate 1B). The callus was white in colour with roots emerging from the sides of the callus in leaf explants on MS medium supplemented with IBA as reported in *Spilanthes paniculata* (Chandra *et al.,* 2008). Light brown coloured compact friable calli with roots were produced from the leaf petiole explant on MS medium supplemented with 0.5 mgl^{-1} IBA (Table 1 and Plate 1C).

Table 1 : Effect of different plant growth regulators on callus induction from leaf petiole explant of *Talinum cuneifolium*

Plant growth regulators (mgl^{-1})			Nature of response and morphogenic ability
NAA	IAA	IBA	
0.5			Light yellow coloured nodular compact calli
	0.5		Light cream coloured nodular friable calli
		0.5	Light brown coloured compact calli with roots

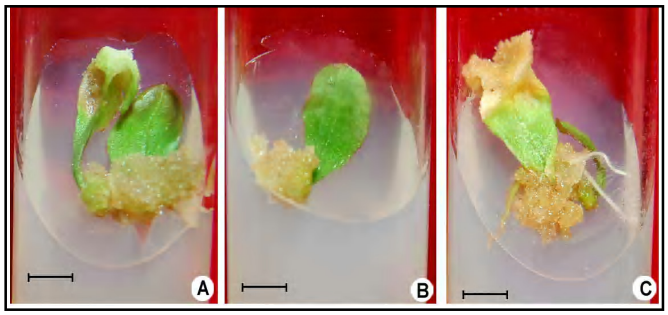

Plate 1 (a), (b), (c) : Induction of callus from petiole culture on MS medium containing A) 0.5 mg l-1 NAA (1cm Bar = 6.09 mm), B) 0.5 mg l-1 IAA (1cm Bar = 6.09 mm) and C) 0.5 mg l-1 IBA (1cm Bar = 5.95 mm)

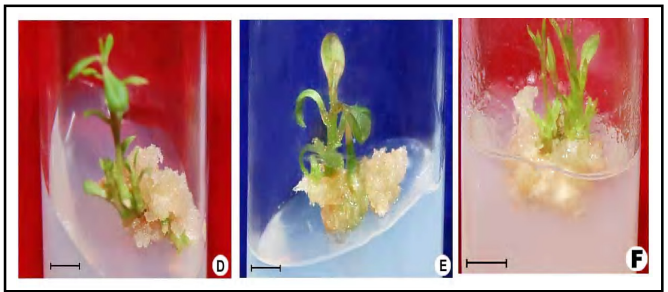

Plate 1 (d), (e), (f) : Multiple shoot regeneration from petiole culture on MS medium containing D) 2 mg l-1 BA (1 cm Bar = 3.96 mm), E) 1 mg l-1 BA + 2 mg l-1 Kn (1 cm Bar = 7.81 mm) and F) 2 mg l-1 BA + 0.1 mg l-1 IAA (1 cm Br = 8.06 mm)

Shoot multiplication:

Shoot buds were initiated from petiole and leaf explants when cultured on MS medium supplemented with BA or Kn and also combination with NAA reported *Spilanthes paniculata* (Chandra *et al.,* 2008). Regeneration of shoot buds from leaf explants were reported in *Albizzia amara* (Ramamurthy and Savithramma, 2003). Leaf petiole showed the highest number of shoots per responsive explant on MS medium with 2 mgl^{-1} BA in primary subculture. BA is effective in single and combination with Kn and IAA. Highest number of shoots were developed from leaf explant of *Plumbago* (Das and Rout, 2002). It was found that BA alone performed better than in combinations with 4 multiple shoots obtained in *Sterculia* (Anitha and Pullaiah, 1999). 2 mgl^{-1} BA alone showed remarkable results by production of high frequency (56.04 ± 0.05) of shoot regeneration (Table 2 and Plate 1D) and also high mean number (3.03 ± 0.08) of shoots were established. 1 mgl^{-1} BA + 2 mgl^{-1}Kn achieved high frequency (42.4 ± 0.21) of shoots regenerated (Table 2 and Plate 1E). Addition of BAP along with NAA or IAA, was essential for the induction of multiple shoots as reported in *Withania somnifera* (Kannan *et al.,* 2005). 2 mgl^{-1} BA in combination of 0.1 mgl^{-1} IAA produced moderate mean length (2.42 ± 0.04) of the shoots (Table 2 and Plate 1F).

Table 2 : Effect of different plant growth regulators on indirect shoot regeneration from the callus derived from leaf petiole explant of *Talinum cuneifolium*

Plant growth regulators (mgl^{-1})			Frequency of shoot regeneration	Mean no. of shoots/ explant	Mean length of the shoot (cm)
BA	Kn	IAA			
2			56.04±0.05^{h}	3.03±0.08^{c}	2.03±0.06^{d}
1	2		42.4±0.21^{b}	2.25±0.05^{b}	2.72±0.05^{f}
2		0.1	37.05±0.05^{a}	1.30±0.04^{a}	2.42±0.04^{e}

('±' indicates the standard error). Mean values having the same letter in each column don't differ significantly at P≤0.05 (Duncans Test).

Root initiation:

Rooting of *in vitro* shoots in low salt medium has been reported in *Philodendron* sp. (Maene and Debergh, 1985). Rooting was succeeded by the single and combination with different concentrations of NAA, IBA and IAA. NAA to be the most effective auxin for initiation and growth of roots reported in *Mussaenda erythrophylla* (Thakur and Kumar, 2007). 0.1 mgl^{-1} NAA in combination with 0.5 mgl^{-1} IAA produced low frequency (44.71 ± 0.01) of root regeneration, low mean number of roots (2.83 ± 0.09) and also low mean length (1.02 ± 0.02) of the roots (Table 3 and Plate 2A and B) were achieved. Maximum number of elongated roots were developed on MS medium containing IBA as observed in *Oroxylum indicum* (Gokhale and Bansal, 2009). 1 mgl^{-1} IBA showed good frequency (85.70 ± 0.07) of root regeneration, higher mean number (12.46 ± 0.02) of roots and higher mean root length (4.60 ± 0.01) were achieved. (Table 3 and Plate 2C and D)

Table 3 : Effect of different auxins on the root induction of *in vitro* raised shoots of *Talinium cuneifolium* on half strength MS medium

Plant growth regulators (mgl^{-1})			Frequency of root regeneration	Mean no. of roots/ explant	Mean length of the root (cm)
NAA	IBA	IAA			
	0.5		78.81±0.09^{k}	8.64±0.01^{j}	3.50±0.04^{h}
	1.0		85.70±0.07^{l}	12.46±0.02^{k}	4.60±0.01^{i}
		0.5	46.70±0.02^{b}	2.18±0.01^{a}	1.28±0.03^{b}
		1.0	54.36±0.04^{e}	3.36±0.02^{c}	1.50±0.03^{c}
0.5			58.21±0.05^{g}	4.62±0.08^{f}	2.15±0.02^{d}
1.0			67.41±0.03^{i}	7.90±0.05^{i}	3.26±0.02^{g}
0.5	0.1		74.64±0.02^{j}	5.31±0.01^{g}	3.03±0.10^{f}
1.0	0.1		63.92±0.01^{h}	7.60±0.03^{h}	3.30±0.03^{g}
	0.5	0.1	52.363±0.02^{d}	4.40±0.01^{e}	1.60±0.04^{c}
	1.0	0.1	55.05±0.03^{f}	5.32±0.06^{g}	2.55±0.06^{c}
0.1		0.5	44.71±0.01^{a}	2.83±0.09^{b}	1.02±0.02^{a}
0.1		1.0	52.23±0.02^{c}	3.60±0.04^{d}	1.18±0.01^{b}

Values represented above are the means of 12 replicates ('±' indicates the standard error). Observations after 4 weeks of culture. Mean values having the same letter in each column don't differ significantly at P≤0.05 (Duncans Test).

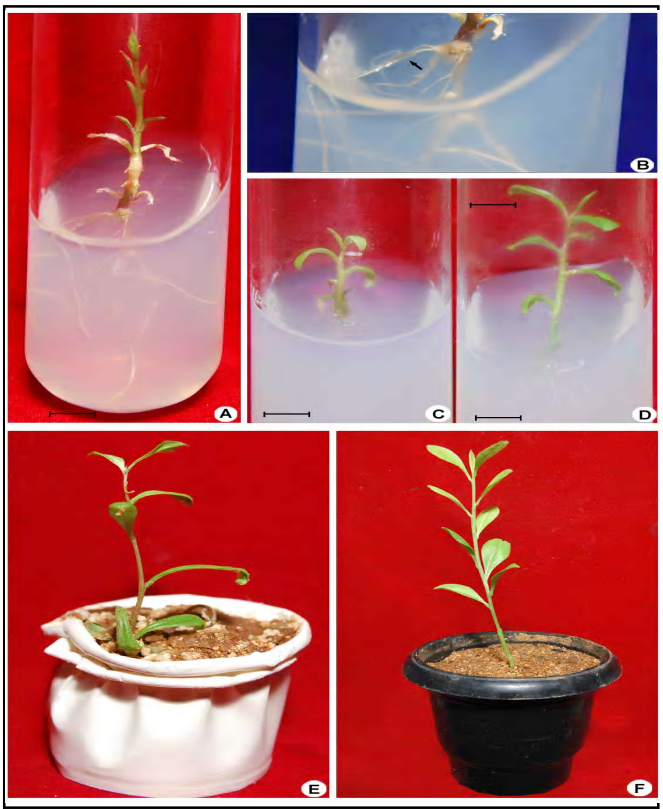

Plate 2 : Induction of tubers from the root of *Talinum cuneifolium* on half strength MS medium supplemented with A) 0.5 mg l^{-1} BA and 100 g sucrose (1 cm Bar = 5.10 mm); B) Magnified view of transparent spindle shaped mini tuber in the roots of *Talinum cuneifolium* after 30 days (1cm Bar= 2.80 mm); C and D) Elongation and rooting of plantlets on half strength MS medium supplemented with 0.5 mg l^{-1} IBA (1cm Bar = 5.10 mm); Transplanted plantlets: E) in a paper cup after 2 weeks and F) in a plastic pot after 1 month

Hardening and acclimatization:

Acclimatization of regenerated plants to the external environment was very important and depends on different plant and environmental factors as suggested by (Donelly and Tindall, 1993). Acclimatization was the final, but necessary step in all micro-propagation schemes during which plants have to adopt to the new environmental conditions such as lower relative humidity, higher light intensity fluctuating temperature and constant disease stress (Preece and Suttler, 1991). Accordingly rooted plants were gradually acclimatized with an increase in temperature from 25 to 28^{0}C and decrease in relative humidity from 80 to 50% for a period of 15 to 20 days. The paper cups containing *in vitro* derived plantlets were kept in the culture room temperature and cups were covered with polythene bags to maintain high humidity and kept in mist chamber covered with coir mat. Survival of *in vitro* plants after planting was largely dependent on the components of the potting media (Plate 2E).

Rate of rooting and establishment in soil were comparable to the *Spilanthes acmella* Murr. (Saritha *et al.,* 2002) and *Philodendron* sp. (Maene and Debergh, 1985). In the present study, garden soil and sand (1:1) was found suitable for highest survival of plants. These plants were irrigated with ¼ strength MS salts and exposed gradually to external environment. The plants propagated *in vitro* were well established and able to sustain easily on their own after transfer to the field condition (Plate 2F).

Clonal uniformity:

The observations obtained with peroxidase isoenzyme pattern in the current investigation substantiated the uniformity of the multiple shoots derived from leaf explant which was most desirable in any micropropagation system. Bhaskaran *et al.* (1987) suggested that the biochemical traits such as isozymes provides an alternative tool to study the extent of somaclonal variation. These findings were also supporting the view that biochemical traits such as isozyme provides an evidence to study the extent of somaclonal variations in a manner analogous to their use in elucidating genetic variation in natural population (Bhaskaran *et al.,* 1987).

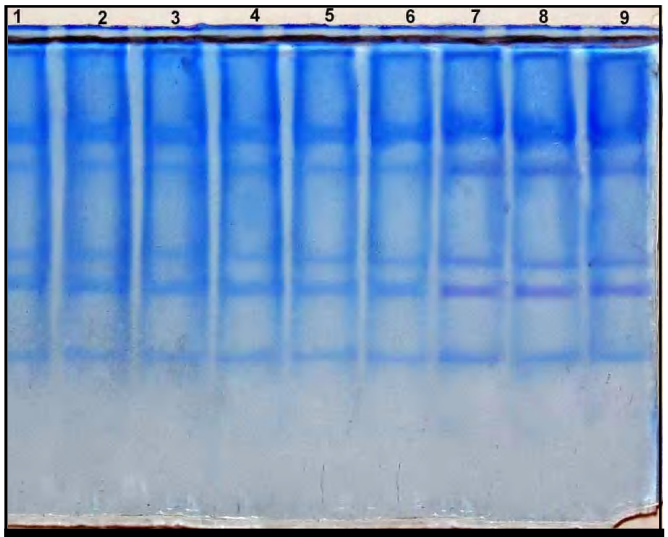

Plate 3 : Banding pattern of peroxidase isozyme in leaf tissue of *Talinum cuneifolium*.1, 2, 3 Field grown; 4, 5, 6 Direct regenerated shoots and 7, 8, 9 Indirect regenerated shoots

Results showed no variation in the banding pattern of peroxidase isozyme between the parent plants and the plants regenerated through direct organogenesis (Plate-3). Sudha (1996) used isozymes as markers to the screen clonal purity in tissue cultured medicinal plants. This observation substantiated the uniformity of the clonal plants. All the isozyme bands in plants regenerated directly from mature explants were identical in their intensity of staining with those of the parent plant. The intensity of bands of plants regenerated from leaves were nearly same as those of parent plant. Of the six bands observed in isoenzyme profile, three bands stained darker and the remaining were stained faintly. But the plants regenerated through callus showed variations in intensity of staining with that of parent plants (Plate-3). The efficient micropropogation technique described here may be highly useful for raising desirable genotypes of *T. cuneifolium* for commercial cultivation.

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