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Establishment of *in vitro* regeneration from petiole explants and assessment of clonal fidelity by ISSR markers in *Luffa acutangula* L.Roxb

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ABSTRACT

An improved *in vitro* organogenesis was achieved from petiole explants of *Luffa acutangula* L. Roxb, a medicinally and economically important cucurbitaceous member. Morphogenetic response of petiole explants excised from *in vitro* grown plants has been studied with different concentrations of auxins and cytokinins. Higher percent of callus induction was occurred on Murashige and Skoog (MS) medium supplemented with 2mg/L BAP and 0.2mg/L NAA from the cut ends of petiole explants after 4 weeks of culture. Multiple shoots (8.28±0.85 shoots per explant) were induced after 2-3 weeks from the green compact callus on MS medium with 30g/L sucrose, 2.0mg/L BAP along with 0.2mg/L IAA. The elongation of proliferated shoots was achieved on same medium supplemented additionally with 0.3 mg/L GA_3. The elongated shoots (1cm) were rooted on MS medium supplemented with 0.5 mg/L IBA. Rooted plants were acclimatized in the greenhouse and subsequently established in soil with 85% survival rate. Clonal fidelity of *in vitro* raised plantlets was determined using ISSR marker technique. The results showed the similar banding pattern with different ISSR primers ranging from 250bp to 1000bp and indicated the absence of polymorphism in donar mother plant and as well in *in vitro* regenerated plants.

1. INTRODUCTION

The success of *in vitro* plant regeneration technique in recent years has been standardized and extended to almost every major agronomic, vegetable, medicinal and fiber crop systems and this could be the appropriate approach for maintaining unique source without any genetic change. The plants belonging to cucurbitaceae provide a major portion of vegetable and they need to be investigated further in developing elite transgenic lines for maximum utilization [1]. *Luffa acutangula* L. Roxb popularly known as ridge gourd and also called angled gourd, belongs to family cucurbitaceae with vital medicinal importance as rich in β-carotenes, flavonoids, saponins and triterpenes. The entire plant is extensively useful as traditional remedy for anemia, jaundice and in spleen enlargements [2]. It consists of a gelatinous compound called luffein, a ribosomal inactivating protein possessing antiviral properties [3]. Many comprehensive therapeutic advantages of this plant showed analgesic and antioxidant properties [4].Significant hypoglycemic [5] and hepatoprotective [6] activities of this plant extracts has been studied extensively. In the earlier attempts, successful reports for *in vitro* regeneration were available from different explants of *Luffa acutangula* L. Roxb like shoot tip explants [7], cotyledonary nodes, nodal explants [8], internodes [9] and cotyledons [10]. A promising *In vitro* flowering condition has been employed within this system as a source of viable aseptic anthers for haploid culture [11]. The evaluation of genetic stability of *in vitro* raised plantlets with molecular markers is consent with clonal identification and analyzing the variations at genetic levels. ISSR (Inter simple sequence repeats) marker system offers a highly discriminative tool in studying genetic fidelity among *in vitro* cultured plants. Similar consecutive studies with ISSR have been done in cultivar identification in strawberry [12] and also in clonal identification of *woodfordia fruticosa* [13], *Citrullus colocynthis* [14]. ISSR markers were used in studying genetic variability in two morphological variants in flower colour with in species of *Hyptis suaveolens* [15]. In the present investigation, attempts were made to standardize a reproducible protocol for *in vitro* clonal propagation of petiole explants by organogenesis and assessment of their clonal fidelity using ISSR markers in *Luffa acutangula* L.Roxb.

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2. MATERIALS AND METHODS

2.1. Explant material

Seeds of *Luffa acutangula* L. Roxb were collected from Warangal Seed Company, Telangana. They were washed with running tap water and were decoated .The decoated seeds were surface sterilized with 0.1% (W/V) mercuric chloride for 3-4 minutes, then rinsed with sterile distilled water for 2-3 times to remove traces of sterilant. The seeds were demoistened with sterile filter paper and were cultured onto Murashige and Skoog (MS) [16] medium without hormones. For all experiments, the P^H of medium was adjusted to 5.6-5.8 and solidified with 0.8 % (W/V) agar. The seeds germinated within a week of culture and subsequently developed into complete plantlets within 3-4 weeks and further experiments were conducted using petiole explants obtained from these well developed plants.

2.2 Callus initiation and shoot formation

Explants were cultured on MS medium fortified with various concentrations and combinations of different plant growth regulators for multiple shoot initiation via intervening callus phase. Different concentrations of 6-Benzyl amino purine (BAP) (0.5 mg/L – 3 mg/L), Thiadiazuron (TDZ) (0.5 mg/L – 3 mg/L) and 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.5 mg/L – 3 mg/L) alone and in combination with α-Naphthalene acetic acid (NAA) (0.1 mg/L – 0.4 mg/L) were used. Multiple micro shoot buds were initiated from well developed callus after culturing on MS medium supplemented with various concentrations of BAP and IAA within 3-4 weeks with a subculture of 25 days interval onto the same medium.

2.3 Shoot elongation and Rooting

Varied concentrations of Gibberellic acid (GA_3) (0.1 mg/L - 0.5 mg/L) with MS basal medium were used for proper elongation of regenerated shoots. The fully elongated single shoots were excised and transferred to rooting medium containing Indole-3-butyric acid (IBA) at different levels (0.5 mg/L – 3 mg/L).Fully developed plantlets with complete roots were acclimatized and shifted to greenhouse.

2.4 PCR conditions

Total genomic DNA was extracted following a modified cetyl trimethyl ammonium bromide (CTAB) DNA extraction procedure [17] from the leaf tissues of both *in vitro* raised and *in vivo* mother plants. Different ISSR primers were tested on the basis of their suitability for detailed study. PCR was performed PCR was performed using 25 µL of reaction mixture containing 1 µL (0.5 unit per reaction) of Taq DNA polymerase , 2 µL of PCR buffer ,1 µL of 2.5 mM dNTPs, 1 µL of each ISSR primer(10 pmols), 1 µL of extracted DNA(0.5ng), and 19µL of milliqwater.PCR conditions carried out for amplification consists of an initial denaturation step at 94°C for 3 min, 40 cycles of 1 min at 94°C, 40 sec at 45°C, and 2.3 min at 72°C, followed by final extension of 7 min at 72°C, with a soak temperature of 10°C.

3. RESULTS AND DISCUSSION

3.1 Callusing

A successful regeneration protocol of ridge gourd was obtained via callus induction from the cut ends of petiole segments in MS medium supplemented with auxin alone or in combination with cytokinin. In this study maximum percentage of callus induction was occurred after 4 weeks of culture and morphological features of callus were found to be different on different medium. The effect of auxins NAA and IAA which can induce callus efficiently on MS medium with BAP showed high frequency of multiple shoot regeneration from Balsam cotyledonary explants [18].However in the present investigation green callusing was observed with BAP in combination with NAA and where as the response in TDZ+NAA (43.96±4.38) and 2,4-D (43.84±4.02) alone significantly induced brown, granular to yellowish, compact callus respectively. Green compact callus was formed at a high frequency rate of 81.68±2.16 in MS medium containing 2.0 mg/L BAP with 0.2 mg/L NAA and because of their multiplication capability, these calli were used for induction of organogenesis{Fig.1 a,b}(Table 1).

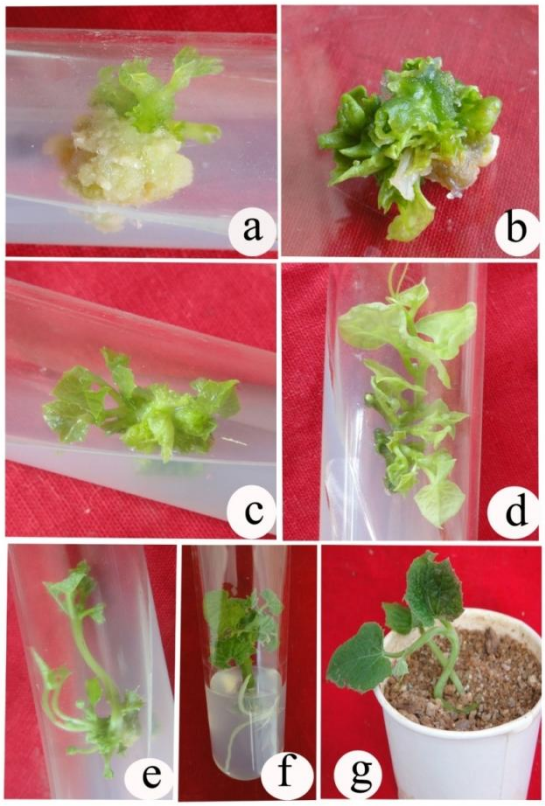

Fig.1: *In vitro* regeneration from petiole explants of *Luffa acutangula* L.Roxb. a, b. Initiation of callus derived adventitious shoots. c, d. Proliferation of shoots on MS medium containing 2mg/L BAP & 0.2mg/L IAA. e.Elongation of shoots on proliferation medium containing GA_3 (0.3mg/L). f. Rooting induction in elongated shoots on 0.5mg/L IBA. g. Hardened plant in greenhouse

Similar consecutive studies produced organogenic callus in *Momordica dioica* from different combinations of BAP with NAA and their ratios were intended to play a major role in

determining the *in vitro* response from different explants in most cucurbits [19] and further increase in NAA concentrations has showed declining effect on callus proliferation [20].At higher levels, the effect of BAP (42.92±5.32) and TDZ (28.45±6.37) was found to show low callogenic response which induced brown, granular callus with different texture and color. whereas callus from 2,4-D is found to be nonorganogenic with a low frequency rate (43.84±4.02). However the explants failed to show callogenic response on hormonal free MS medium that really explains the importance of varied levels of plant growth regulators that are supplemented to basal medium.

Table 1: Effect of various concentrations of Hormones on callus induction from petiole explants of *Luffa acutangula* L.Roxb**.**

Hormones (mg/L)	% Response	Morphological feature of callus
2,4-D		
0.5	27.68±5.21	Yellowish- brown granular
1.0	36.68±6.23	Yellowish, compact
2.0	41.44±5.58	Brown, Granular
3.0	43.84±4.02	Brown, Granular
BAP + NAA		
0.5+ 0.2	42.92±5.32	Yellowish, Nodular
1.0+ 0.2	63.28±6.19	Green, compact
2.0+ 0.2	81.68±2.16	Green, compact
3.0+ 0.2	42.92±5.32	Brown, Granular
TDZ + NAA		
0.5 + 0.2	54.08±7.09	Yellowish - brown compact
1.0 + 0.2	43.96±4.38	Yellowish brown Nodular
2.0 + 0.2	39.76±5.80	Brown, Granular
3.0 + 0.2	28.45±6.37	Brown, Granular

Values are given as Mean ± standard error (SE) represents the % of response of 25 replicates per treatment and the data was recorded after 4-weeks of culture.

3.2 Multiple shoots initiation

The combination of cytokinins with auxins play a key role in promoting the induction of multiple shoots and among the various hormonal supplements used, the best shootbud differentiating response was observed from green compact callus on MS medium with 2mg/L BAP in combination with 0.2 mg/L IAA. In this, an average of 8.28±0.85 shoots with a frequency percent of 83.2±7.90 per explants was observed. Phytohormone TDZ 1mg/L with 0.2mg/L IAA showed response in initiating 4.48±0.48 shoots per explant with 54.8±7.20 frequency whereas increased concentrations of both the hormones gave a diminishing effect on shoot multiplication{Fig.1c,d}(Table 2). Manye *et al.* studied that, the type of callus used for further proliferation of shoots and appropriate proportionate quantity of phytohormones play a key role in establishing the *in vitro* cultures by differentiating the callus and initiating the multiple shoots in *Momordica charantia* L [21]. The Individual hormonal levels of BAP and TDZ at 1mg/L alone has exhibited shooting response at an average of 2.44±0.63 and 3.36±0.60 shoots respectively and at lower frequency rate when compared to their combination with IAA. Comparatively the same findings were achieved on low shooting response when single hormonal medium is used for rapid *in vitro* studies [22].

Table 2: Induction of Multiple shoot buds on various hormonal concentrations of BAP and TDZ alone and in combination with IAA from petiole explants of *Luffa acutangula* L.Roxb.

Hormones (mg/L)	% callus induced shoots	No. of Shoots/Explant (Mean ± S.E)
0.5	31.4±8.01	2.32±0.61
1.0	38.8±8.47	2.44 ±0.63
2.0	31.8±8.34	1.92±0.52
3.0	29.4± 5.48	1.36 ±0.37
BAP + IAA		
0.5 + 0.2	18.4±6.87	2.12±0.38
1.0 + 0.2	32.2±7.54	2.96±0.66
2.0 + 0.2	83.2±7.90	8.28±0.85
3.0 + 0.2	26.2± 6.34	1.68±0.45
TDZ		
0.5	15.4±4.33	1.84±0.42
1.0	20.8±6.87	3.36±0.60
2.0	28.8±7.79	2.76±0.76
3.0	19.2±7.53	2.12±0.38
TDZ + IAA		
0.5 + 0.2	12.5±3.12	2.72 ±0.80
1.0 + 0.2	54.8±7.20	4.48±0.48
2.0 + 0.2	27.4±6.54	2.52 ±0.53
3.0 + 0.2	16.2±3.85	1.88±0.45

Values are given as Mean ± standard error (SE) represents the No.of Shoots/Explant of 25 replicates per treatment and the data was recorded after 3-4weeks of culture.

3.3 Shoot elongation and Rooting

In this study, satisfactory results were obtained with BAP and found to be more effective when compared to TDZ. The proliferated shoots that were transferred to same regeneration medium containing GA_3 (0.3mg/L) showed shoot elongation of 3.12±0.50 cm with 85% response {Fig.1e}(Table 3) and these results support the findings in *Cucumis sativus* and *Cucurbita pepo* by selvaraj *et al*. and Ananthakrishnan *et al* [23,24] respectively. Best rooting response of these elongated shoots were observed after 2 weeks of culture when transferred to MS medium supplemented with 0.5mg/L IBA with an average number of 11 roots per explants {Fig.1f}. In contrast, other auxins like NAA and IAA have been found to be effective in inducing roots in other plant systems like *Momordica charantia* [25] and however here in case of *Luffa acutangula*, there is poor rooting response in terms of inducing low number, weak and roots were found to be slender with NAA and IAA (data not shown).The rooted plants were gently washed and transferred to plastic container containing sterile soil and vermiculite mixture in 1:1 ratio and maintained in greenhouse for 2 weeks of hardening with a survival rate of 85% {Fig.1g}. The morphological features of hardened plantlets were found to be quite similar to that of parental lines.

Table 3: Effect of GA_3 on elongation of proliferated shoots from petiole explants of *Luffa acutangula* L. Roxb cultured on MS medium containing BAP (2.0 mg/L)+(0.2 mg/L) IAA.

GA3 (mg/L)	% response	Shoot Length (cm)
0.1	43	1.36±0.26
0.2	73	2.28±0.74
0.3	85	3.12±0.50

Each treatment consisted of 25 replicates and repeated thrice.

3.4 Clonal fidelity

Clonal fidelity is one of the essential consideration to determine the level of genetic similarity between regenerants and

their mother plant that are raised by *in vitro* tissue culture methods [26] and where there could be a chance of inducing somaclonal variation in micropropagated plants [27]. Molecular markers are the precise tools that are used rapidly to determine and reveal genetic differences from *in vitro* raised plants and non tissue culture derived controls. Here in this report, the assessment of genetic stability between *in vitro* regenerated plants in comparison to a single donor mother plant was carried out using ISSR fingerprinting and this could be reliable in determining variability at DNA level in plants and some of similar findings has been established in other plants [28] and also because of their simplicity and cost effectiveness this method has been adopted for the present study.

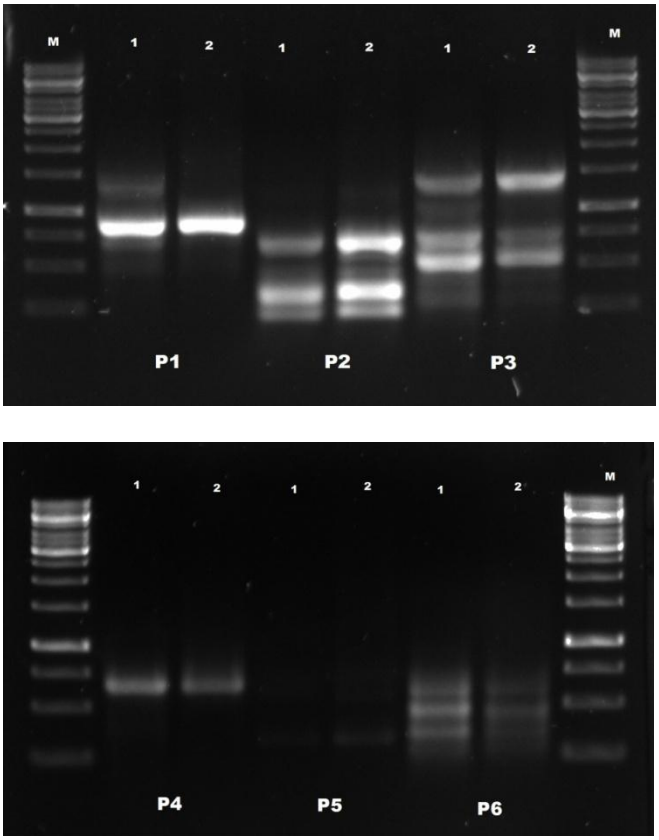

Fig. 2: Assessment of clonal fidelity in *in vitro* regenerants by ISSR markers M-Marker, 1-Mother donar plant, 2-*In vitro* regenerated plant,P1,P2,P3,P4,P5,P6-Primers used for amplification

Out of 7 ISSR primers screened, 6 primers developed distinct and scorable bands with a size ranging from 250 bp to 1000 bp {Fig. 2a,b} (Table 4). All the bands displayed monomorphic patterns. Thus ISSR marker system has revealed the genetic stability in *in vitro* regenerants with almost indicating complete uniformity. The present results are similar with various reports that have been summarized on rapid regeneration protocols from different explant sources and absence of genetic variability amongst *in vitro* raised and donar plant systems like *Asparagus officinalis* [29], *Chlorophytum arundinaceum* Baker [30] and *Momordica charantia* L [31].

Table 4: List of ISSR primers used for analyzing genetic stability in micropropagated plants of *Luffa acutangula* L.Roxb.

ISSR marker No.	Primer Sequence (5′–3′)	Size Range (bp)
P1	AGAGAGAGAGAGAGAGG	750-900bp
P2	GAGAGAGAGAGAGAGAC	250-750bp
P3	TCTCTCTCTCTCTCTCG	500-1500bp
P4	AGAGAGAGAGAGAGAGYT	500-750bp
P5	TCTCTCTCTCTCTCTCC	250-500bp
P6	AGAGAGAGAGAGAGAGG	250-1000bp
P7	GAGAGAGAGAGAGAGAT	No amplification

4. CONCLUSION

The present investigation has demonstrated a simple regeneration protocol for conservation and multiplication through callus derived shoot organogenesis from petiole segments of *Luffa acutangula* L. Roxb and this can be applied for transgenic improvement in this species. The use of BAP and TDZ in combination with IAA or NAA favoured plant regeneration and callus induction respectively and this is the first report confirming the clonal fidelity of regenerated plants with ISSR markers.

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