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RESEARCH ARTICLES

# Micropropagation and clonal fidelity assessment of acclimatized plantlets of *Crotalaria longipes* Wight & Arn. using ISSR markers

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**Abstract**
An efficient protocol for micropropagation of *Crotalaria longipes* Wight & Arn., an endemic and endangered Indian legume, was developed. Nodal explants cultured on Murashige and Skoog (MS) supplemented with Thidiazuron (TDZ) 1.0 mg $L^{-1}$ and NAA 0.5 mg $L^{-1}$ showed maximum shoot induction. The multiplied shoots were subsequently sub-cultured every 3 weeks on the same medium. The separated microshoots cultured on half-strength MS basal salts supplemented with 0.5 mg $L^{-1}$ indole-3-butyric acid (IBA) showed maximum root induction. After 4 weeks of culture, the rooted shoots (> 6 cm) were planted in pots containing coco peat and garden soil (1:1) covered with plastic domes and maintained at 25 ºC for 2 weeks. After 2 weeks, the potted plants were transferred to a glasshouse, maintained at 25 °C with a relative humidity of 80%. The acclimatized plants were then successfully established in soil with an 80% survival rate. Clonal fidelity assessment of acclimatized plantlets was confirmed using inter simple sequence repeat (ISSR). All the in vitro developed plants showed monomorphic banding pattern similar to the mother plant, thus ascertaining the true-to-type nature of the in vitro raised plants.

**Keywords** Clonal fidelity · *Crotalaria longipes* · ISSR markers · Legume · Micropropagation

## Introduction

The genus *Crotalaria* (Fabaceae), is the largest genus of legumes in India with 97 species (Sanjappa 2001). *Crotalaria longipes* is a herbaceous plant endemic to Andhra Pradesh and Tamilnadu. This species is listed under the threatened category (Nayar and Sastry 1987). Jayanthi (2012) reported that the distribution of *C. longipes* is restricted only in Kolli hills, Southern Eastern Ghats of Tamil Nadu. However, our field studies in various areas of Nilgiris, the Southern Western Ghats; and Kolli and Shevaroys hills of Southern Eastern Ghats of Tamil Nadu revealed the occurrence of *Crotalaria longipes*. However, the distribution is very scarce, invariably in all the areas surveyed, which is mainly due to deforestation activities. Plant tissue culture techniques provide a suitable alternative for mass multiplication of this threatened species. A protocol for the in vitro propagation through multiple shoot induction in *Crotalaria longipes* is not currently available. Ex situ conservation of threatened plant species using plant tissue culture techniques may lead to show somaclonal variation (Ilezuk and Jacygrad 2016; Larkin and Scowcroft 1981), especially while conserving them for a longer duration under in vitro condition. Screening of in vitro developed cultures is, therefore, necessary to remove any mutated plants to maintain the genetic integrity of the germplasm. Polymerase chain reaction (PCR) based deoxyribonucleic acid (DNA) markers, especially ISSR are more preferable for confirming genetic integrity of the micropropagated plants (Mao et al. 2018; Rani and Raina 2000) because it is cost-effective, technically simple, and does not require sequence information of the template DNA (Kumar et al. 2009). The applications of ISSR markers to ascertain the genetic fidelity of the in vitro developed clones is evident from different plant systems viz., *Psidium guajava* L. (Kamle et al. 2014), *Albizia procera* (Mohammad et al.2016), *Cornus alba* L. (Ilczuk and Jacygrad 2016), *Rhododendron wattii* (Mao et al. 2018). Therefore, in the present study, ISSR markers were used to ascertain the genetic integrity of the in vitro developed seedlings of *C.longipes* and to ensure mass multiplication of genetically stable true-to-type plants of this species before being introduced in their natural habitat. The present study was initiated firstly

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to develop a suitable protocol for the rapid micropropagation of *Crotalaria longipes* and secondly to ascertain the genetic fidelity of the acclimated plants using ISSR markers.

## Material and methods

### Plant material and culture initiation

Plant material for tissue culture was obtained from the wild plants of *Crotalaria longipes* (Fig. 1a) grown in the garden premises of National Orchidarium and Experimental Garden, Botanical Survey of India, Southern Regional Centre, Yeraud, Salem District, Tamilnadu, India.

For culture initiation, young vegetative shoots measuring 30–45 cm in length were collected and thoroughly washed under running tap water for 10 min to remove any adhering surface dirt particles. The nodal segments of 2–3 cm, excised from the vegetative shoots, were rinsed in 1.0% (*v/v*) Dettol (Reckitt Benckiser, India Ltd.) for 7 min and washed thoroughly in distilled water. The nodal segments were then sterilized with 10% (*v/v*) sodium hypochlorite (4% concentrated) solution containing 1 mL of Tween 20 per 100 mL for 10 min. After three washes in sterile distilled water, the final sterilization was carried out using 0.1% (*w/v*) mercuric chloride for 3 min with subsequent washing thrice in sterile distilled water. The nodal explants were trimmed and inoculated onto MS (Murashige and Skoog

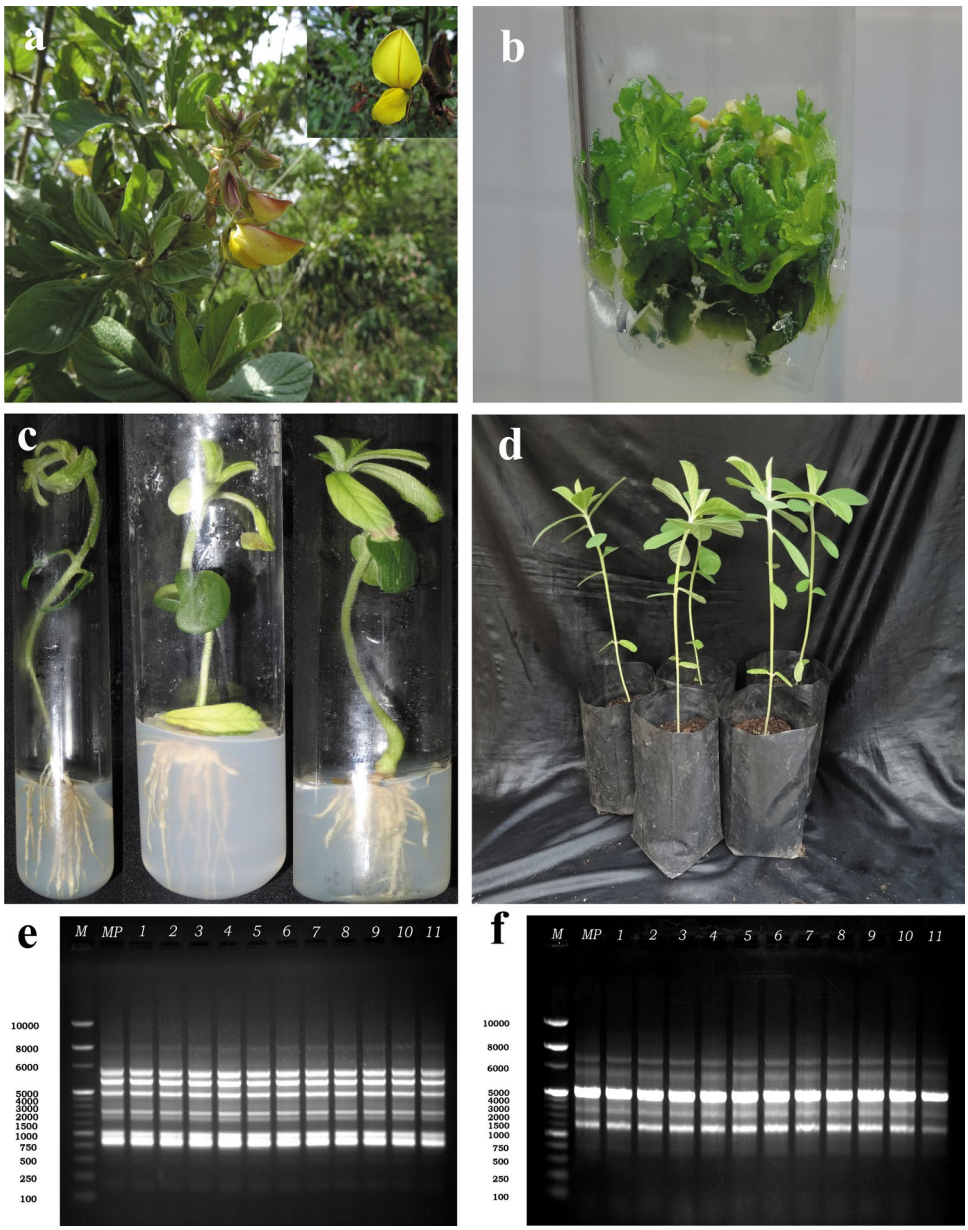


**Fig. 1** In vitro propagation *Crotalaria longipes* (**a**) *C. longipes* growing in its natural habitat with opened flower shown as insert. **b** Multiple shoot induction from nodal explants. **c** In vitro rooting after 7 wk of culture on MS medium containing 0.5 mg $L^{-1}$ IBA. **d** 8-wk-old plantlets grown in polythene bags for acclimation. **e** Genetic fidelity analysis using ISSR 834 and (**f**) ISSR 836. *Lane M* represents 1 kb ladder, *Lane MP* represents mother plant and *Lanes 1—11*represents randomly chosen acclimated plants of *C. longipes*

**Table 1** Effect of different cytokinins [thidiazuron (TDZ), 6-benzyl aminopurine (BAP), kinetin (KN)] and an auxin [(α-napthaleneacetic acid (NAA)] on shoot regeneration from nodal segments of in vitro raised *Crotalaria longipes* seedlings

| Plant Growth Regulators (mg/l) | | | | Regeneration rate (%) | No. of shoots per explant | Length of shoots (cm) |
|---|---|---|---|---|---|---|
| TDZ | BAP | KN | NAA | | | |
| 0 | 0 | 0 | 0 | 40 | $0.7 \pm 0.48^{gijkl}$ | $1.02 \pm 0.37^{fmo}$ |
| 0.5 | 0 | 0 | 0.5 | 80 | $12.1 \pm 2.51^{dh}$ | $2.64 \pm 0.30^{chjk}$ |
| 1.0 | 0 | 0 | 0.5 | 95 | $28.6 \pm 2.27^{a}$ | $2.87 \pm 0.27^{bhi}$ |
| 2.0 | 0 | 0 | 0.5 | 75 | $18.7 \pm 2.40^{b}$ | $4.17 \pm 0.58^{a}$ |
| 3.0 | 0 | 0 | 0.5 | 70 | $14.8 \pm 2.61^{ch}$ | $2.29 \pm 0.14^{dijl}$ |
| 4.0 | 0 | 0 | 0.5 | 55 | $5.5 \pm 2.06^{e}$ | $2.11 \pm 0.99^{ekl}$ |
| 5.0 | 0 | 0 | 0.5 | 50 | $2.2 \pm 0.78^{fi}$ | $1.00 \pm 0.00^{gm}$ |
| 0 | 0.5 | 0 | 0.5 | 60 | $5.5 \pm 1.58^{ah}$ | $2.26 \pm 0.25^{dij}$ |
| 0 | 1.0 | 0 | 0.5 | 85 | $7.7 \pm 2.05^{b}$ | $2.49 \pm 0.31^{bhi}$ |
| 0 | 2.0 | 0 | 0.5 | 75 | $4.4 \pm 0.96^{ch}$ | $3.31 \pm 0.35^{a}$ |
| 0 | 3.0 | 0 | 0.5 | 55 | $2.2 \pm 0.63^{dij}$ | $2.31 \pm 0.19^{chj}$ |
| 0 | 4.0 | 0 | 0.5 | 45 | $1.0 \pm 0.00^{eik}$ | $1.59 \pm 0.29^{e}$ |
| 0 | 5.0 | 0 | 0.5 | 40 | $1.0 \pm 0.00^{fil}$ | $1.00 \pm 0.00^{gk}$ |
| 0 | 0 | 0.5 | 0.5 | 55 | $3.3 \pm 1.15^{bhi}$ | $1.51 \pm 0.20^{gh}$ |
| 0 | 0 | 1.0 | 0.5 | 70 | $5.5 \pm 1.50^{a}$ | $1.72 \pm 0.17^{ag}$ |
| 0 | 0 | 2.0 | 0.5 | 65 | $3.0 \pm 0.94^{ch}$ | $1.39 \pm 0.11^{bhi}$ |
| 0 | 0 | 3.0 | 0.5 | 50 | $3.0 \pm 0.94^{di}$ | $1.20 \pm 0.14^{cijkl}$ |
| 0 | 0 | 4.0 | 0.5 | 45 | $1.0 \pm 0.00^{ej}$ | $1.09 \pm 0.11^{djmn}$ |
| 0 | 0 | 5.0 | 0.5 | 40 | $1.0 \pm 0.00^{fk}$ | $1.00 \pm 0.00^{flno}$ |

Means (±SD) within a column followed by the same letter are not significantly different at $p \leq 0.05$

1962) medium. Cytokinins viz., thidiazuran (TDZ), 6-benzylaminopurine (BAP), and kinetin (Kn) (0.5–5.0 mg $L^{-1}$) and auxin, α-naphthalene acetic acid (NAA) (0.5 mg $L^{-1}$) were used for initial shoot induction (Table 1). After three weeks, the nodal explants with initial axillary shoot induction were transferred to fresh MS medium supplemented with the different combinations of cytokinin and an auxin (Table 1) for shoot elongation. Data for regeneration frequency, shoot number, and shoot length were recorded, four weeks after continuous culture.

In all the experiments, the nutritional media contained MS basal salts, vitamins, 30 g/l sucrose, 0.8% (*w/v*) agar and supplemented plant growth regulators. The pH was adjusted to 5.8 with 1 N NaOH and 1 N HCl before autoclaving at 121 ℃ at 110 kPa for 20 min.

All macronutrients, micronutrients, vitamins, hormones, sucrose, agar and other chemicals used in the present study were obtained from Himedia Laboratories Pvt. Ltd. Mumbai, India. All the cultures were maintained at 25 ± 2 ℃ with a 16/8 h light/dark cycle. The light intensity was 40 µmol $m^{-2}$ $s^{-1}$ provided by the cool white fluorescent tubes (Philips 36 W, Philips, India).

## In vitro rooting

The regenerated shoots, 3–4 cm in length, were dissected and cultured on half-strength MS basal salts supplemented

**Table 2** Effect of different auxins [indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and α-napthaleneacetic acid (NAA)] on root induction from the microshoots of *Crotalaria longipes* after 7 weeks

| Plant growth regulators (mg/l) | | | Rooting rate (%) | No. of roots per shoot | Root length (cm) |
|---|---|---|---|---|---|
| IBA | IAA | NAA | | | |
| 0 | 0 | 0 | 60 | $0.80 \pm 0.42^{eg}$ | $1.01 \pm 0.36^{ef}$ |
| 0.5 | 0 | 0 | 75 | $12.9 \pm 0.73^{ab}$ | $4.26 \pm 0.71^{a}$ |
| 1.0 | 0 | 0 | 90 | $12.0 \pm 1.15^{a}$ | $2.83 \pm 0.39^{b}$ |
| 2.0 | 0 | 0 | 65 | $6.8 \pm 1.39^{c}$ | $2.21 \pm 0.18^{c}$ |
| 3.0 | 0 | 0 | 50 | $2.2 \pm 0.78^{d}$ | $1.38 \pm 0.18^{df}$ |
| 0 | 0.5 | 0 | 55 | $9.1 \pm 1.66^{a}$ | $3.51 \pm 0.24^{a}$ |
| 0 | 1.0 | 0 | 60 | $4.7 \pm 1.15^{b}$ | $2.40 \pm 0.41^{b}$ |
| 0 | 2.0 | 0 | 50 | $2.2 \pm 0.78^{cf}$ | $1.92 \pm 0.20^{c}$ |
| 0 | 3.0 | 0 | 45 | $1.0 \pm 0.00^{dfg}$ | $1.29 \pm 0.15^{df}$ |
| 0 | 0 | 0.5 | 60 | $4.9 \pm 0.73^{a}$ | $2.11 \pm 0.15^{a}$ |
| 0 | 0 | 1.0 | 65 | $3.1 \pm 0.99^{b}$ | $1.81 \pm 0.21^{ae}$ |
| 0 | 0 | 2.0 | 55 | $2.0 \pm 0.81^{c}$ | $1.51 \pm 0.16^{be}$ |
| 0 | 0 | 3.0 | 50 | $1.0 \pm 0.00^{dg}$ | $0.76 \pm 0.22^{df}$ |

Means (±SD) within a column followed by the same letter are not significantly different at $p \leq 0.05$

with auxins, viz., IBA or NAA or IAA, at different concentrations (0.5–3.0 mg $L^{-1}$) (Table 2) for root induction. The regenerated shoots cultured on half-strength MS basal salts without auxin served as the control treatment. Rooting percentage, number of roots per shoot, and root length were recorded after seven weeks of culture.

### Plant acclimatization to *ex vitro* condition

The rooted plantlets after 7 week were removed from the culture and were washed thoroughly in distilled water to remove any adhered agar particles. The washed plantlets were placed in transparent plastic containers filled with an autoclaved mixture of garden soil, vermicompost, and sand (1:1:2) and covered with a polythene dome. The plastic containers with plantlets were maintained in the culture room at 25 ± 2 °C with a relative humidity of 75% and 35 µmol $m^{-2}$ $s^{-1}$ light intensity emitted from cool-white fluorescent tubes (36 W, Philips India Pvt. Ltd) in a 16-h photoperiod for 4 weeks. During the period of acclimation, the relative humidity was reduced gradually to 60% after 10 days from the beginning of the hardening process. The plantlets were transferred to a glasshouse without the polythene dome and watered every alternate day, for four weeks. The acclimatized plants were then transferred to the field after 8 weeks.

### Genetic fidelity analysis of acclimated plants

The total genomic DNA was extracted from the leaf material (1 g) of both wild plants and 10-weeks-old in vitro raised *Crotalaria longipes* plantlets using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987).

### ISSR polymerase chain reaction

Ten ISSR primers (Priority life science, Coimbatore, Tamil Nadu, India) were selected for assessment of polymorphisms (Table 4). Polymerase chain reaction (PCR) was carried out using a DNA thermal cycler (Eppendorf®, Mastercycler™ nexus gradient, Hamburg,Germany) with the final reaction mixture volume of 25 µL contained 10 X PCR reaction buffer (500 mM Tris−HCl, 160 mM $(NH_4)SO_4$ pH 9.2), 1.5 µl $MgCl_2$ (2 mM), 0.5 µl dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP), 1 µL primers, 0.3 µL of DNA Taq polymerase, 1 µL of 25 ng template DNA and sterile distilled water. PCR conditions used amplification consists of an initial denaturation step at 94 °C for 5 min; followed by 35 cycles of 1 min at 94 °C for denaturation, 1 min at 50 °C for annealing, 2 min at 72 °C for extension; and a final extension at 72 °C for 10 min. After amplification, the PCR products (10 µL) per lane were compared by 1.2% (*w/v*) agarose gel electrophoresis in 1X Tris–acetic acid–EDTA (TAE) buffer containing 0.25 µg/µL, along with 1Kbp DNA ladder (Himedia Pvt. Ltd., India) as size markers. The amplified PCR products were visualized under gel documentation system (Gelstan-1312 series, Medicare, India). ISSR- PCR reactions were analyzed in a binary data scored for presence (1) and absence (0) of banding patterns for each plantlet.

### Data analysis

One-way analysis of variance (ANOVA) was used to evaluate the significance of the difference of means of data from various experiments using SPSS statistical software package (Trial version: 16). The values were presented as mean (± SD) and $P < 0.05$ is considered as significant. All the data pertaining to multiple shoot induction and root induction were taken after seven weeks of continuous culture in vitro.

## Results and discussion

### Multiple shoot induction

Shoot induction from the nodal explants of *C. longipes* was achieved on MS basal medium supplemented with various concentrations of three cytokinins (TDZ, BAP and KIN) in combination with an auxin (NAA). Regardless of the combinations of auxin and cytokinins used, the nodal explants showed axillary bud regeneration within 2 weeks after inoculation. Among the different combinations of TDZ (0.5 mg–5.0 mg $L^{-1}$, Table 1) and NAA (0.5 mg $L^{-1}$) tested, TDZ (1.0 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) showed the maximum regeneration frequency (95%) and shoot number (28.6 ± 2.15 per nodal explant) (Fig. 1b). On the other hand, maximum shoot length (4.17 ± 0.55 cm) was attained with TDZ (2.0 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$). However, there was a decline in the regeneration frequency, shoot number and length as the TDZ concentration increased. This trend was invariably true in other PGR combinations viz., BAP + NAA and KIN + NAA. The synergistic effect of TDZ and NAA in promoting shoot induction was reported in *Astragalus cariensis* leaf explants (Erisen et al. 2011; Murthy et al. 1998). Further it is reported that TDZ when combined with other plant growth regulators showed positive impact on shoot induction as compared to TDZ alone (Huetteman and Preece 1993; Lincy and Sasikumar 2010). Thus the present study is in conformity on the synergistic effect of TDZ and NAA. In a similar trend, as that of TDZ + NAA, the positive effect of BAP in combination with NAA was observed on shoot induction in *C. longipes*. The maximum regeneration frequency (85%), mean shoot number (7.7 ± 2.05 per nodal explant) was achieved with BAP (1.0 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$). However, maximum shoot length (3.31 ± 0.33 cm) was achieved with BAP (2.0 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$). The positive effect of

**Table 3** List of primers, their sequences, number of scored monomorphic bands and size of the amplified fragments generated by inter simple sequence repeat (ISSR) markers, expressed as base pairs (bps), used to assess genetic integrity of in vitro propagated clones of *C. longipes*

| S.No | Primer used | Primer sequence (5′-3′) | Annealing temperature (°C) | Number of scored bands per plant | Amplified product size (bps) 100–3000 bps |
|---|---|---|---|---|---|
| 1 | ISSR 818 | $(CA)_7G$ | 44 | 3 | 300–1200 |
| 2 | ISSR 812 | $(GA)_8A$ | 48 | 2 | 100–1400 |
| 3 | ISSR 808 | $(AG)_8C$ | 50 | 4 | 200–2000 |
| 4 | ISSR 811 | $(GA)_8C$ | 50 | 3 | 600–2700 |
| 5 | ISSR 830 | $(TG)_8G$ | 50 | 3 | 200–1700 |
| 6 | ISSR 834 | $(AG)_8YT$ | 50 | 5 | 300–1, 800 |
| 7 | ISSR 836 | $(AG)_8YA$ | 50 | 6 | 200–1, 400 |
| 8 | ISSR 840 | $(GA)_8YT$ | 50 | 5 | 300–1600 |
| 9 | ISSR 841 | $(GA)_8YC$ | 52 | 2 | 300–1000 |
| 10 | ISSR 842 | $(GA)_8YG$ | 52 | 4 | 200–1400 |
| Total number of bands | | | | 37 | |
| Average number per primer | | | | 3.7 | |

BAP in combination with NAA was reported in *Astragalus cariensis* (Erisen et al. 2010). Adlinge et al. (2014) also ascertained that BAP in combination with NAA showed a positive impact on the maximum shoot induction in *Vigna mungo* (L.) Hepper cv. Sarala. In a different set of experiment, the effect of different combinations of Kn + NAA (Table 1) on the maximum regeneration frequency, mean shoot number and mean shoot length was tested. The results showed a maximum regeneration frequency (70%), mean shoot number (5.5 ± 1.43 per nodal explants) and mean shoot length (1.72 ± 0.16 cm) with Kn (1.0 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$). Effect of kinetin on shoot induction was reported by earlier workers in other plant species viz., *Caralluma bhupenderiana* (Ugraiah et al. 2011), *Lens culinaris* (Singh and Raghuvanshi 1989; Williams and McHughen 1986), *Crotalaria laburnifolia* (Rajender et al. 2012). The present study, on the whole, revealed that the combination effect of TDZ + NAA on multiple shoot induction in nodal explants of *C. longipes* was found to be significantly higher, followed by BAP + NAA and Kn + NAA. Present study is in agreement with the earlier workers to ascertain that the low concentration of an auxin in combination with cytokinins can substantially improve the shoot induction frequency (Kaliamoorthy et al. 2008; Martin 2002, 2003; Sreekumar et al. 2000; Wotavova-Novotna et al. 2007).

## In vitro rooting

Shoots more than 3–4 cm were transferred to half strength MS basal salts supplemented with IBA, IAA and NAA (Table 2) for rooting. Root induction was observed in all the treatments including the control. Invariably in all the treatments, the emergence of root primordia at the base of the shoot was observed after two weeks of culture. However, the shoots treated with 0.5 mg $L^{-1}$ IBA resulted in the maximum rooting rate (90%), mean root number (12.9 ± 0.70 per shoot) and root length (4.26 ± 0.68 cm) (Fig. 1c). The positive effect of IBA on root induction was reported in *Vigna mungo* (Ignacimuthu et al. 1997), *Cajanus cajan* (Franklin et al. 2000a), *Pisum sativum* (Franklin et al. 2000b), *Harpagophytum procumbens* (Kaliamoorthy et al. 2008), Rhododendrons (Mao et al. 2011, 2018) *Cicer microphyllum* (Singh et al. 2019). The present study supports the results of earlier workers pertaining to the positive effect of IBA in *C. longipes.*

## Acclimatization

Around 80% of the in vitro raised plants of *C.longipes* transferred from lab to greenhouse condition survived (Fig. 1d). The successfully acclimatized plants were then transferred to soil beds developed in the garden premises and maintained. A protocol for the in vitro propagation through multiple shoot induction in *C. longipes* is not available and therefore this is the first report on in vitro regeneration in *C. longipes*. This efficient and reproducible protocol can be used for in situ and ex situ germplasm conservation.

## ISSR fingerprinting

Mass multiplication of an endemic and threatened plant through micropropagation method can be achieved. However, it may not be successful from the conservation point of view, unless genetic fidelity is maintained. During the course of in vitro propagation, especially when cultures are being stored in vitro for longer duration, the chances of genetic mutations are quite high (Rani and Raina 2000). In view of this, genetic integrity of the acclimatized plants of *C.longipes* was assessed using 10 ISSR primers (Table 3) and compared with a representative mother plant growing naturally in the garden premises of BSI, SRC, Yercaud, Salem District, Tamil Nadu, India. The results yielded a total

of 37 amplified products generated from 10 ISSR primers with 10 acclimatized plants and a mother plant. The ISSR markers each generated an average of 3.7 monomorphic bands and their lengths varied from 100 to 2700 base pairs (Table 3) (Fig. 1e, f). The results of genetic fidelity assessment using ISSR markers revealed that all the bands produced by the acclimatized clones were monomorphic and were similar to that of the mother plant growing in the wild. The effective use of ISSR markers in clonal fidelity assessment of micropropagated plants is well documented in various studies (Mao et al. 2018; Teeluck et al. 2016).

Present study has provided a simple, efficient and reproducible protocol for a high frequency shoot regeneration from the nodal explants of *Crotalaria longipes* Wight & Arn., an endemic and threatened plant of south India was developed. This protocol could be effectively used for mass multiplication of this endemic and threatened plant species for re-introduction in the wild and also to maintain a germplasm in the botanical gardens.

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