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# Micropropagation and assessment of genetic stability in *Celastrus paniculatus*: An endangered medicinal plant

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**Abstract:** A highly efficient protocol for *in vitro* regeneration of an indigenous, endangered medicinal plant *Celastrus paniculatus* was achieved using nodal explants. Murashige and Skoog (MS) basal medium supplemented with 0.5 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L naphthaleneacetic acid (NAA) showed maximum percentage of shoot multiplication (83.4%) with 8.2 shoots/explants. Maximum rooting of 73.3% with 4.8 roots/shoot was achieved on half-strength MS media supplemented with 0.5 mg/L indole-3-acetic acid (IAA) and the percentage of survival was 91% after acclimatization. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) marker study confirmed genetic stability for *in vitro* raised explants by showing 100% monomorphism. High multiplication rate associated with genetic stability ensure the efficacy of the present *in vitro* clonal propagation protocol of this important medicinal plant species.

**Key words:** *Celastrus paniculatus*; genetic stability; ISSR; RAPD; shoot multiplication

## Introduction

*Celastrus paniculatus* Willd. (Celastraceae) commonly known as 'Jyotishmati', "Intellect tree" or 'Bitter sweet' is an endangered medicinal tree gaining popularity in primary health care and herbal drug formulation (Rekha et al. 2005). It is a deciduous woody climber, widely used in ayurvedic system of medicine against diseases like leprosy, leucoderma, skin diseases, paralysis, depression, arthritis, asthma, cancer and fever (Sharma et al. 2001; Rekha et al. 2005). Indiscriminate over exploitation coupled with insufficient attempts for replenishment of wild stock have contributed to its threatened status requiring scientific efforts for conservation and commercial cultivation (De Silva & Senarath 2009; Lal & Singh 2010). Due to low seed viability, germination rate and lack of vegetative propagation methods, tissue culture technique is an alternate solution for conservation of those valuable and endangered medicinal plants (Rout et al. 2008; Bantawa et al. 2011; Swarna & Ravindhran 2012). Though tissue culture is recognized as one of the key areas of biotechnology for large scale propagation and conservation, but it is severely hindered due to somaclonal variations (Larkin & Scowcroft 1981). Optimization of hormonal treatment, periodic monitoring of the genetic stability is utmost important for commercial utilization of true-to-type plants of the desired genotype. Several techniques have been developed to assess the genetic purity of *in vitro* raised plants but molecular marker techniques presents a most powerful and reliable methods. Among the various molecular markers, random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analysis are the simplest and quickest method used for assessment of genetic stability with no obvious phenotypic alternations (Mohanty et al. 2011). In the present study we report an efficient protocol for production of *in vitro* raised plants of *Celastrus paniculatus* with high genetic uniformity.

Based on above facts the present investigation was carried out with the following objectives: i) standardization of protocol for large scale production of *Celastrus paniculatus*, ii) assessment of genetic stability of the *in vitro* raised plants using molecular markers.

## Material and methods

*Celastrus paniculatus* Willd. (Celastraceae) is a climbing herbs, lenticellate. Leaves obovate, elliptic or oblong, serrulate, acuminate, pubescent beneath. Flowers yellowish or greenish. Capsule yellow, globose. Seed red arilled. A nine years old *C. paniculatus* (identified by the taxonomist of Regional plant resource center (RPRC), Bhubaneswar with reference to the herbarium accession No. 3191) plant growing at medicinal garden of RPRC served as the donor mother plant for nodal explants as well as DNA stability analysis. The nodal explants were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 20 minutes followed by

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washing with distilled water at least for 3 times, then explants were trimmed and transferred to agar basal culture medium containing macro and micro nutrients as recommended by Murashige & Skoog (MS) (1962) and pH was adjusted to 8 using 0.1N NaOH and HCl. The media was solidified with 0.7% (w/v) agar and was sterilized for 15 min at 121 ℃. Optimization of phytohormone types and concentration was done by taking different hormones, i.e., 6-benzylaminopurine (BAP: 0.25–2.0 mg/L), kinetin (Kn: 0.25–2.0 mg/L) and auxins i.e., indole-3-acetic acid (IAA: 0.10–0.25 mg/L), 1-naphthalene acetic acid (NAA: 0.10–0.25 mg/L) and indol-3-butryic acid (IBA: 0.10–0.25 mg/L) alone or in combination for bud initiation followed by shoot multiplication and elongation. The culture was maintained at temperature of 25 ± 2 ℃, 16-h photoperiod having a light intensity of 3,000 lux and > 85% relative humidity. The culture was maintained by sub culturing at an interval of 30 days. The *in vitro* regenerated elongated shoots (3–4 cm) were excised aseptically and implanted on full and half strength MS medium with and without growth regulators (IAA, NAA and IBA) (0.10–1.0 mg/L) and 2% (m/v) sucrose for root induction. Rooted plantlets were thoroughly washed and planted in plastic pots containing a mixture of soil, sand and dry cow-dung manure (1:1:1, v/v/v) and kept in the greenhouse for acclimatization. All the experiments were repeated three times with a minimum of 20 replicates. All the cultures were examined periodically, and after 30 days interval the data were analyzed statistically by the Duncan's multiple range test (Harter 1960) at $P < 0.05$ for each experiment. Healthy and young leaves of *C. paniculatus* were taken both from *in vitro* plantlets and *in vivo* grown mother plant. Leaf samples were taken in every six months interval up to two years for RAPD and ISSR analysis. DNA extraction was done by following Doyle & Doyle (1990) method. The RAPD analysis was performed according to Williams et al. (1990) by using 30 decamer primers (Operon Technologies, Almeda, USA) and ISSR analysis was performed according to Zeitkiewicz et al. (1994) using 20 synthesized ISSR primers (*MS Bangalore Genei*, India). The PCR reaction was performed by following the normal steps of 5 min for complete denaturation, followed by 42 cycles having three ranges of temperature, i.e., 92 ℃ for 1 min for denaturation of template DNA, 1 min for primer annealing at appropriate annealing temperature as mentioned in Table 3, 72 ℃ for 2 min for primer extension, followed by running the samples at 72 ℃ for 7 min for complete polymerization. The PCR products obtained were analyzed in 1.5% and 2% agarose gel for RAPD and ISSR respectively. Each PCR reactions were repeated thrice. The size of the amplicons were estimated using 100 bp DNA ladder plus or DNA ladder mix (MBI Fermentas, Vilnius, Lithuania) and documented in the GelDoc (Bio-Rad, Hercules, USA).

## Results and discussion

The assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true-to-type clones (Eshraghi et al. 2005). *In vitro* germplasm conservation relies on micropropagation methods. However, phenotypic and genetic variations are reported to occur during in *vitro* regeneration processes, originating somaclonal variants (Kaeppler et al. 2000). Thus, the risks of genetic changes induced by tissue culture and the importance of assessing the genetic stability of the biological material along all phases of storage must be considered in the context of conservation (Chandrika & Rai 2009).

To our knowledge, no report was available on the comparative genetic stability of regenerants and mother plant of *C. paniculatus* by using molecular markers. In this paper a micropropagation protocol for mass production was developed and molecular markers were used to assess the genetic stability of micropropagated *C. paniculatus* plants. There are few records on the micropropagation of this valuable species through callus (Sharada et al. 2003) and through bud differentiation (Rao & Purohit 2006; Lal & Singh 2010). In this report, the effect of various concentrations of auxins (NAA, IBA and IAA) and cytokinins (BAP and Kn) were analyzed for high frequency micropropagation through direct organogenesis from intact nodal explants of *C. paniculatus*. Out of the various hormonal combinations tried, MS medium supplemented with 0.5 mg/L BAP has shown maximum percentage of bud break after 14 days of inoculation, where as MS medium fortified with 0.5 mg/L Kn bud break was noticed after 20 days of inoculation, while it was delayed at higher concentration (2.0 mg/L) in BAP and Kn. The medium devoid of growth regulators failed to induce the formation of shoot buds (data not shown). Similar results were obtained in *Crataeva nurvala* (Walia et al. 2003); *Nyctanthus arbor-tristis* (Rout et al. 2008) and *Celastrus paniculatus* (Lal & Singh 2010) on MS basal medium. Higher numbers of multiple shoot were recorded in all concentrations of BAP than the medium supplemented with the same concentration of Kn albeit with longer internodes. Moreover, combination of kinetin plus IAA or NAA did not favor the rate of shoot multiplication, whereas an inclusion of a low concentration of NAA along with BAP increases the rate of shoot multiplication. About 83.4% of cultures showed multiple shoots in the medium having 0.5 mg/L BAP and 0.10 mg/L NAA (Table 1). Maximum number of multiple shoots (8.2) was obtained in the medium containing 0.5 mg/L BAP and 0.1 mg/L NAA after 4 weeks of culture (Figs 1A, B). A higher concentration of BAP (> 2.0 mg/L) in the culture medium inhibited the growth of the shoots and stimulated callusing at the basal end where as a higher concentration of NAA suppress the rate of shoot multiplication and showed stunted growth. This result is contradictory to the result obtained by Lal & Singh (2010), as they found maximum rate of multiplication in MS medium supplemented with 1.0 mg/L BAP and 0.1mg/L NAA (*C. paniculatus*) but supported by reports given by Gopi et al. (2006), Rout et al. (2008) and Murthy et al. (2010) in *Ocimum gratissimum, Nyctanthus arbor-tristis* and *Ceropegia spiralis* respectively. The present findings suggest a high frequency of shoot production from axillary meristems by manipulating the concentration of growth regulators. This may be due to the growth regulators applied externally during *in vitro* studies might disturb the internal polarity and change the genetically programmed physiology of explants resulting in organogenesis as suggested by Lal &

Table 1. Effect of different concentrations of cytokinins and auxins on shoot multiplication from nodal explants of *Celastrus paniculatus* after 30 days of sub culture.

| MS + Plant growth regulators concentration (mg/l) | | | | | % of shoot multiplication (mean ± SE)[a] | No. of multiple shoot /explants (mean ± SE)[a] |
|---|---|---|---|---|---|---|
| BA | Kn | IAA | NAA | IBA | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.25 | 0 | 0 | 0 | 0 | 60.2 ± 1.0 d | 5.87 ± 0.3 b |
| 0.50 | 0 | 0 | 0 | 0 | 78.4 ± 1.1 f | 7.66 ± 0.6 d |
| 1.00 | 0 | 0 | 0 | 0 | 65.8 ± 0.5 d | 6.63 ± 0.5 c |
| 2.00 | 0 | 0 | 0 | 0 | 36.5 ± 0.8*b | 4.86 ± 0.3*a |
| 0 | 0.25 | 0 | 0 | 0 | 59.3 ± 1.1 c | 5.23 ± 0.7 b |
| 0 | 0.50 | 0 | 0 | 0 | 70.4 ± 1.2 e | 6.23 ± 0.5 c |
| 0 | 2.00 | 0 | 0 | 0 | 31.4 ± 1.0 a | 4.32 ± 0.5 a |
| 0.50 | 0.25 | 0 | 0 | 0 | 72.5 ± 0.8 e | 6.65 ± 0.5 c |
| 0.50 | 0 | 0.10 | 0 | 0 | 71.3 ± 0.5 e | 7.82 ± 0.4 d |
| 0.50 | 0 | 0 | 0.10 | 0 | 83.4 ± 0.8 g | 8.20 ± 0.5 e |
| 0.50 | 0 | 0 | 0.25 | 0 | 76.5 ± 1.1 f | 7.76 ± 0.4 d |
| 0.50 | 0 | 0 | 0 | 0.10 | 72.6 ± 0.9 e | 5.82 ± 0.6 b |

[a] Values are mean ± SE; 20 replicates per treatment, repeated three times; * callusing at the basal end; means having same letter within columns were not significantly different at $P < 0.05$

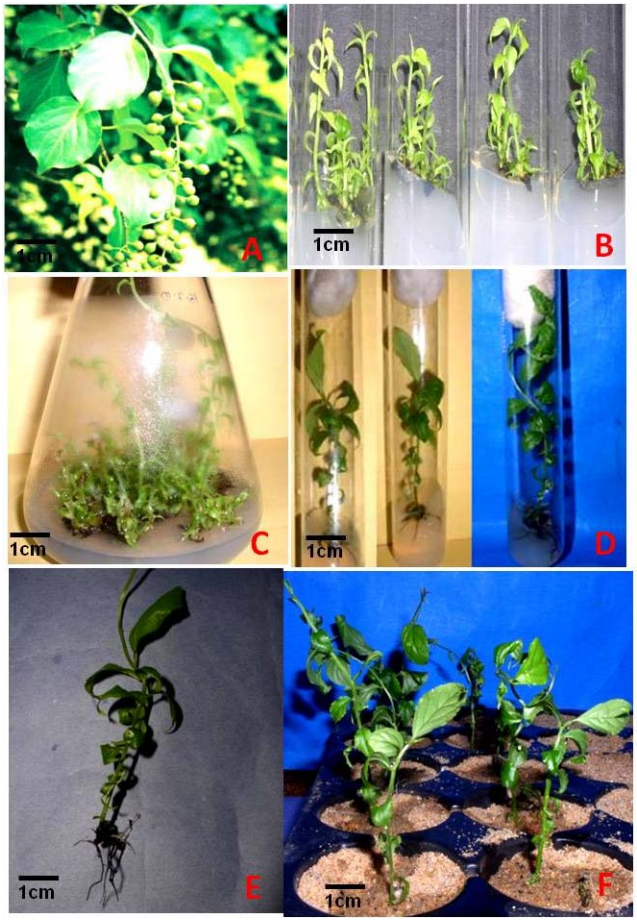


Fig. 1. *In vitro* propagation of *Celastrus paniculatus*. Source of explants (A). Development of multiple shoots from nodal explants on MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA and 3% sucrose after 2 weeks (B) and 4 weeks (C) of culture. Induction of roots from microshoots on half strength MS medium supplemented with 0.5 mg/L IBA and 2% sucrose after 2 weeks (D) and 3 weeks (E) of inoculation. *In vitro* raised rooted shoots grown in the sand: soil: cow dung in the ratio 1: 1: 1(v/v/v) after 20 days (F).

Singh 2010. The percentage of shoots forming roots and number of roots per shoot significantly varied depending on concentrations of growth regulators (Table 2). The maximum percentage of rooting (73.3%) with an average of 4.8 roots /shoot was obtained in the half MS medium containing 0.50 mg/L IAA (Figs 1C, D). Root development is very poor in the half MS medium supplemented with NAA which is contradictory to the results obtained by Lal & Singh (2010) because the donor mother plants are growing in two different regions which leads to the possible involvement of gene in modulating hormone levels (Tantikanjana et al. 2001), but supported by De Silva & Senarath (2010). Root development was, however slow at higher concentrations of auxins with callusing at the base. The rooted plantlets were transferred into plastic pots for acclimatization. About 91% of the rooted plantlets survived in the pot one month after the transfer (Fig. 1E).

There are many factors like length of culture periods, genotype and nature of explant, which could influence the stability of the tissue cultured plants (Vendrame et al. 1999; Premvaranon et al. 2011). So assessment of genetic stability of *in vitro* regenerated plantlets is highly significant for further studies. However, the reliability and efficiency of molecular markers are frequently questioned. A better analysis of genetic stability of plantlets can be made by using a combination of two types of markers which amplify different regions of the genome (Martins et al. 2004). Genetic integrity by RAPD and ISSR analysis has been reported earlier in many medicinal plant species (Mohanty et al. 2011). This is the first report on the genetic stability analysis of micropropagated *Celastrus paniculatus* using RAPD and ISSR markers. A total of 40 plantlets were analyzed taking 10 plantlets from each culture period ($2^{nd}$ sub-culture, $3^{rd}$ subculture, rooted microshoots and acclimatized plantlets). Initially, thirty RAPD and twenty ISSR primers were tested, out of which 21 RAPD and 12 ISSR primers were selected finally based on their reproducibility. Selected 21 RAPD primers gave rise to a total of 134 scorable bands with an average of 6.4 bands ranging

Table 2. Effect of different concentrations of growth regulators alone or in combinations on rooting response of *Celastrus paniculatus* after 30 days of culture.

| Half MS + Plant growth regulators concentration (mg/L) | | | % of rooting (mean ± SE) | No. of root/explants (mean ± SE) | Days to rooting |
|---|---|---|---|---|---|
| IAA | IBA | NAA | | | |
| 0.00 | 0.00 | 0.00 | 0 | 0 | 0 |
| 0.10 | 0.00 | 0.00 | 23.2 ± 0.6 a | 2.32 ± 0.3 b | 20 |
| 0.25 | 0.00 | 0.00 | 44.2 ± 0.8 d | 3.28 ± 0.5 d | 17 |
| 0.50 | 0.00 | 0.00 | 61.4 ± 1.0 f | 3.65 ± 0.6 f | 15 |
| 1.00 | 0.00 | 0.00 | 31.4 ± 1.0 b * | 1.85 ± 0.6 a* | 18 * |
| 0.00 | 0.10 | 0.00 | 29.5 ± 1.0 b | 2.82 ± 0.6 c | 19 |
| 0.00 | 0.25 | 0.00 | 53.4 ± 0.8 e | 3.50 ± 0.8 e | 16 |
| 0.00 | 0.50 | 0.00 | 73.3 ± 1.0 g | 4.82 ± 0.5 h | 15 |
| 0.00 | 1.00 | 0.00 | 38.3 ± 0.6 c * | 2.68 ± 0.3 c * | 16 * |
| 0.00 | 0.00 | 0.10 | 19.3 ± 0.8 a | 2.12 ± 0.5 a | 20 |
| 0.00 | 0.00 | 0.25 | 38.7 ± 0.6 c | 3.21 ± 0.3 d | 17 |
| 0.00 | 0.00 | 0.50 | 56.5 ± 0.6 e | 4.00 ± 0.8 g | 15 |
| 0.00 | 0.00 | 1.00 | 18.3 ± 0.6 a * | 1.68 ± 0.3 a * | 16 * |
| 0.50 | 0.10 | 0.00 | 59.2 ± 0.6 e * | 3,60 ± 0.5 f * | 16 * |
| 0.00 | 0.50 | 0.25 | 60.0 ± 0.8 e * | 3.85 ± 0.4 g * | 15 * |

[a] Values are mean ± SE; 20 replicates per treatment, repeated three times; * callusing at the basal end; means having a same letter within columns were not significantly different at $P < 0.05$.

Table 3. RAPD and ISSR banding pattern of both micropropagated and field grown mother plants.

| Primer | Sequence | Total bands | Band Range(bp) |
|---|---|---|---|
| | RAPD | | |
| OPA-01 | AATCGGGCTG | 3 | 450–1800 |
| OPA-02 | TGCCGAGCTG | 5 | 300–2000 |
| OPA-04 | AATCGGGCTG | 6 | 400–2300 |
| OPA-10 | GTGATCGCAG | 7 | 300–2100 |
| OPA-16 | AGCCAGCGAA | 5 | 600–2100 |
| OPA-18 | AGGTGACCGT | 9 | 500–2700 |
| OPC-02 | GTGAGGCGTC | 4 | 650–2000 |
| OPC-12 | GAGAGCCAAC | 7 | 350–2200 |
| OPC-20 | ACTTCGCCAC | 7 | 500–1900 |
| OPD-02 | GGACCCAACC | 5 | 300–2400 |
| OPD-05 | TGAGCGGACA | 6 | 700–2900 |
| OPD-08 | GTGTGCCCCA | 5 | 900–2700 |
| OPD-11 | AGCGCCATTG | 7 | 600–3000 |
| OPD-18 | GAGAGCCAAC | 9 | 350–2500 |
| OPD-20 | ACCCGGTCAC | 10 | 300–2900 |
| OPN-02 | ACCAGGGGCA | 4 | 500–2100 |
| OPN-06 | GAGACGCACA | 8 | 350–2300 |
| OPN-07 | CAGCCCAGAG | 7 | 400–2700 |
| OPN-08 | ACCTCAGCTC | 7 | 300–2400 |
| OPN-15 | CAGCGACTGT | 6 | 600–1400 |
| OPN-16 | AAGCGACCTG | 7 | 400–2100 |
| | ISSR | | |
| IPG-01 | $(AG)_8T$ | 7 | 300–1100 |
| IPG-02 | $(AG)_8C$ | 8 | 250–1450 |
| IPG-05 | $(AC)_8T$ | 9 | 200–1500 |
| IPG-09 | $(AC)_8G$ | 8 | 350–1800 |
| IPG-10 | $(GA)_8A$ | 7 | 300–1200 |
| IPG-11 | $(GA)_8T$ | 8 | 400–1100 |
| IPG-12 | $(GA)_8C$ | 9 | 250–1600 |
| IPG-13 | $(CT)_8A$ | 7 | 200–1000 |
| IPG-14 | $(CT)_8G$ | 10 | 200–1200 |
| IPG-16 | $TGG(AC)_7$ | 6 | 500–1800 |
| IPG-18 | $AGG(GT)_7$ | 10 | 250–1550 |
| IPG-20 | $(GACA)_4$ | 9 | 200–1100 |

from 300 to 2,900 bp. In case of ISSR analysis, 12 selected primers produced a total of 98 scorable bands ranging from < 200 to 1,800 bp with an average of 8.1 bands per primer (Table 3). The banding pattern of mi-

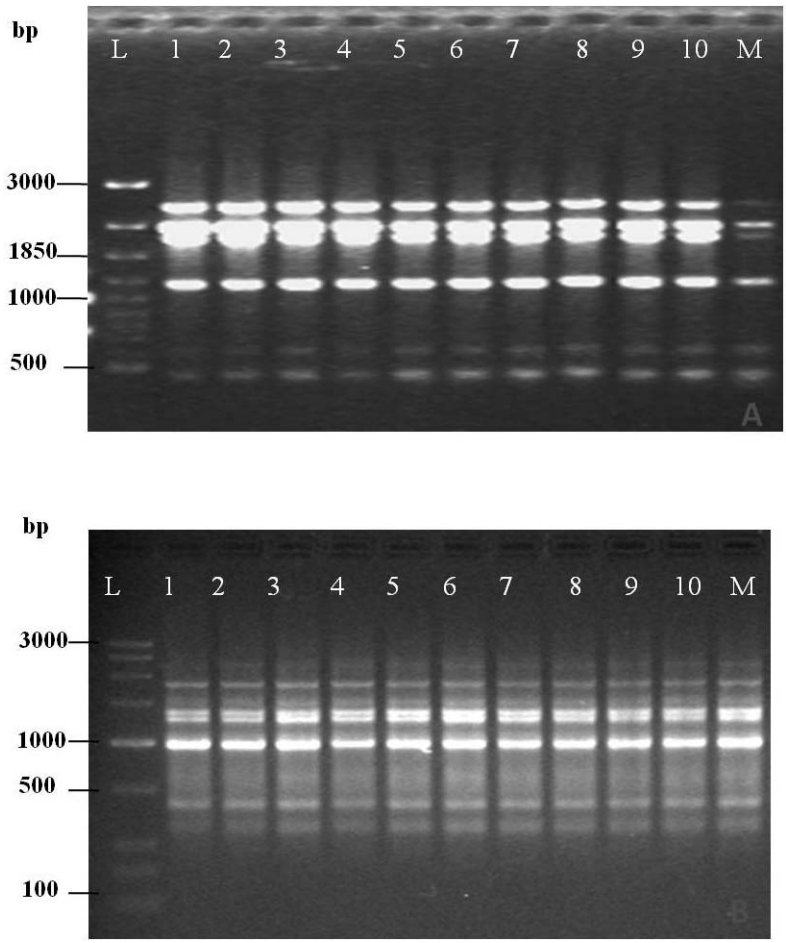


Fig. 2. Representative gel photograph showing banding pattern in micropropagated plants and field grown mother plant of *Celastrus paniculatus* revealing genetic uniformity by RAPD primer OPN-07 (A) and ISSR primer IPG-09 (B). (L – DNA ladder, lanes 1 to 10 – micropropagated plants, M – mother plant).

cropropagules along with their explant donor plant were found to be monomorphic for all the RAPD and ISSR primers tested except a few weak bands (Fig. 2) which were not consistently polymorphic in the replicates of PCR reactions.

The report concludes that the protocol described here is reproducible for large scale propagation and conservation of this valuable medicinal plant true to type. RAPD and ISSR marker system was effective in confirming the genetic fidelity of plantlets regenerated through micropropagation. Thus the procedure developed has great potential for crop improvement using biotechnological approaches like genetic transformation and production of secondary metabolites.

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