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Full Length Research Paper

In vitro clonal propagation of *Mucuna pruriens* var. *utilis* and its evaluation of genetic stability through RAPD markers

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The *Mucuna pruriens* var. *utilis* is an important legume cover crop. Almost all the parts of the plant are reported to contain L-3,4-dihydroxy phenylalanine (L-Dopa). Here we report a rapid and reliable method for high fidelity micro-propagation. Auxiliary bud explants from 14-day-old seedlings were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations of cytokinins. During the first culture on 3.5 µ*M* 6-benzylamino purine (BAP) maximum of 6.70 ± 1.15 shoots with an average shoot-length of 1.07 ± 0.21 cm were produced. The number of shoots increased up to 16.33 ± 0.58 recording average length of 1.16 ± 0.29 cm, when the intact shoots were subjected to re-culturing on the same hormonal medium. The shoots exhibited adequate elongation of 4.00 cm on 2.89 µ*M* gibberellic acid (GA_3). The elongated shoots produced a maximum of 16.67 ± 2.89 roots on half-strength MS liquid medium supplemented with 16.20 µ*M* α-naphthalene acetic acid (NAA). The plantlets were acclimatized by transferring them first to peat moss: compost (1:1) mixture followed by sand: soil (1:1) mixture, recording 95% survival. The genetic fidelity of the regenerated shoots was confirmed using randomly amplified polymorphic DNA (RAPD) analysis employing 15 operon primers. This system provides high fidelity micro-propagation system for efficient and rapid micro-propagation of this important green manure cover crop with medicinal properties.

Key words: *Mucuna pruriens* var. *utilis*; auxiliary bud, multiple shoots, rapid micro-propagation, fidelity, RAPD.

INTRODUCTION

The genus *Mucuna* belongs to the family Fabaceae (Leguminoceae) and includes about 150 species of annual and perennial legumes of pantropical distribution. Many species of the genus offer an excellent source as cover crop and green manure, in addition to their traditional use as feed and food (Janardhanan and Lakshmanan, 1985; Mohan and Janardhanan, 1993; Capo-chichi et al., 2003). Almost all the species are reported to contain L-3,4-dihydroxy phenylalanine (L-Dopa), a non-protein amino acid that acts as precursor for the neurotransmitter dopamine, used in the treatment of Parkinson's disease (Manyam, 1995). In addition, *Mucuna* is also traditionally used in various other applications like, dye (Standley and Steyermark, 1946), treatment of pain and numbness of joints, and irregular menstruation (Ding et al., 1991).

India is one of the natural centers of origin of the *Mucuna* in the world (Eilittä et al., 2002). Rich genetic diversity coupled with wide-ranging traditional knowledge on various usage practices offers a great scope for biotechnological improvement of the *Mucuna* species of India for diverse applications. However, these efforts have been seriously constrained due to absence of well-characterized germplasm for augmenting the need of gene pool in the genetic improvement programs. Hence, there is a need to develop *in vitro* germplasm of the wild and cultivated species of *Mucuna* in India. There is also a need to standardize high fidelity, rapid and reliable protocol for micro-propagation of *Mucuna pruriens* var. *utilis*, one of the commercially important varieties of the *M. pruriens*. To the best of our knowledge, this is the first report on standardization of micro-propagation protocol

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Abbreviations: BAP, 6-benzylamino purine; GA3, gibberellic acid; NAA, α-naphthalene acetic acid.

from *M. pruriens* var. *utilis* and use of DNA marker for testing genetic fidelity of micro-propagated plants in any *Mucuna* species. The protocols presented in this paper could also go a long way for the future genetic improvement programs in this important medicinal plant.

MATERIALS AND METHODS

Seed material and sterilization

Mature seeds collected from well-dried pods of six-month-old *M. pruriens* var. *utilis* plant, grown at departmental garden of Biotechnology Department, Sir M Visvesvaraya Institute of Technology (Sir MVIT) was used as seed source. The seeds were initially washed with detergent Extran® MA 02 (Merck) for 10 min followed by wash under running tap water for 30 min. The seeds were then surface sterilized in a mixture of 0.1% mercuric chloride + 0.05% cetrimide + 0.05% bavistin for 5 min and rinsed four times with sterile distilled water.

Media and culture conditions

Analytical grade chemicals obtained from Himedia laboratories and hormones and vitamins from Sigma-Aldrich chemicals were used for preparing the stock solutions and subsequent media preparation. Murashige and Skoog's (1962) salt with 3% (w/v) sucrose was used as basal medium excepting seed germination medium, which completely lacked sucrose. After adding the growth regulators, the pH of the medium was adjusted to 5.7 ± 0.1 followed by gelling with 0.8% of agar in case of solid medium. The media was autoclaved at 121°C and 1.06 kg/cm^2 pressure for 20 min. All the cultures were incubated in a growth chamber maintained at a temperature of 25 ± 2°C, relative humidity, 70-80% and photoperiod of 16:8 h duration under photon flux density of 50 µE mol m^{-2}s^{-2} provided by day light fluorescent tubes.

Explant preparation

The surface sterilized seeds were germinated on MS basal medium without sucrose and the seedlings were allowed to grow for 12-14 day (d) until the auxiliary buds become prominent (Figure 1a). The explants were then prepared as per the technique described by Jayanand et al. (2003). Auxiliary buds measuring 0.8 - 1.0 cm lengths were aseptically inoculated onto MS medium supplemented with various shoot induction hormones.

Multiple shoot induction

For initial multiple shoot induction, the explants were cultured on Murashige and Skoog's medium supplemented with BAP (0.00-22.20 µ*M*), kinetin (Kn) (0.464 -23.2 µ*M*) and combinations of BAP (4.44 µ*M*) + Kn (0.93 - 4.65 µ*M*), BAP (4.44 µ*M*) + NAA (1.08 - 5.40 µ*M*) and BAP (4.44 µ*M*) + 3-Indolebutyric acid (IBA) (1.14 - 5.70 µ*M*). The induced shoots were allowed to grow for 30 d. At the end of 30 d period, the explants producing maximum number of multiple shoots were re-cultured on MS + BAP (0.44 - 4.44 µ*M*) for 30 more d for production of more number of shoots.

Elongation and rooting

Small micro shoots grown on subculture medium were transferred to MS media supplemented with GA_3 (2.89 -14.43 µ*M*) for elongation. The elongated shoots of 3-4 cm length were subsequently transferred to half strength liquid MS medium supplemented with NAA (0.00 - 16.2 µ*M*) for rooting. At this stage, the shoots were placed on the arc-shaped filter-paper wad of two centimeter width, with a centrally drilled hole (2 mm dia) and dipped in liquid medium to facilitate easy rooting of shoots.

In vitro and *ex vitro* hardening of plantlets

After 20 d, plantlets of 3 - 4 cm height with well-developed roots were carefully removed and washed thoroughly under running tap water for 2-3 min to remove the traces of medium. Plantlets were then transferred to plastic pots (10 cm) containing autoclaved peat moss and compost mixture (1:1) and covered with polythene bags to maintain humidity. After initial hardening in growth chamber for two-weeks, the plantlets were transferred to pots (15 cm) containing sand: soil (1:1) mixture for 30 d before finally transferring to field.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by Tukey's HSD test with 5% level of significance.

DNA isolation and RAPD analysis

For RAPD analysis, leaf samples collected from mother plant and ten randomly selected tissue culture clones of *M. pruriens* var. *utilis* were used. DNA from young leaves was extracted using modified Doyle and Doyle (1990) method. To the extraction buffer containing cetyltrimethylammonium bromide (CTAB), 0.5% charcoal along with 0.2% β-mercaptoethanol were added to avoid polyphenol oxidation. After propanol precipitation, DNA was re-suspended in 0.5 cm^3 of 1 X Tris-EDTA buffer (pH 8.0) and quantified spectrophotometrically by taking the absorbance at 260 nm. Purity of DNA was determined by digesting the isolated DNA with four restriction enzymes viz., *Eco*R I, *Hind* III, *Bam*H I and *Alu* I. RAPD assay was carried out in 0.025 cm^3 reaction mixture containing 0.2 m*M* dNTP's, 10 m*M* Tris-HCl, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1%Triton X-100, 1.0 U Taq DNA polymerase, 15 pmol primers (*Operon Technologies*, Alameda, USA) and 50 ng of genomic DNA. Amplification was performed in a thermal cycler (MJ Research, USA) as follows: After the initial cycle of 2 min at 94°C, 2 min at 36°C and 2 min at 72°C, 38 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C were performed. The last cycle was followed by 7 min extension at 72°C. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. Amplified products were resolved in 1.4% agarose gel (1 X TAE) followed by ethidium bromide staining.

RESULTS AND DISCUSSION

Seed germination

In vitro inoculated seeds germinated within 3 - 4 d on MS basal medium without sucrose, recording 100% germination. Roots appeared after 3 - 4 d and the cotyldenory leaves emerged after sixth day of culturing. All the shoots attained a height of 5-6 cm within 14 d of culturing, exhibiting synchronized seed germination pattern. Auxiliary bud explants obtained from these seedlings were inoculated onto MS medium supplemented with different hormonal combinations for the induction of multiple shoots.

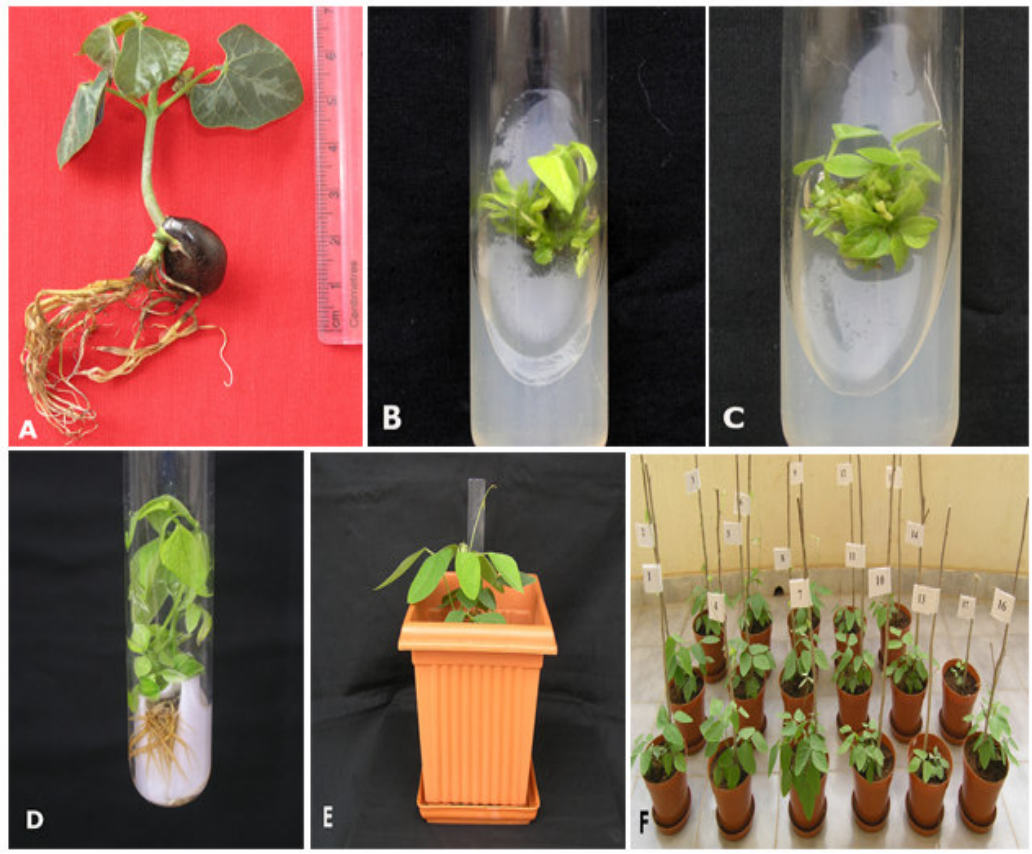

Figure 1. Rapid micropropagation of *Mucuna pruriens* var. *utilis* using axillary bud explants. **a.** 14 d old seedling of *Mucuna pruriens* var. *utilis* germinated *in vitro*. **b.** *In vitro* multiplication of shoots from axillary buds on MS + BAP (3.55 µ*M*) after 30 d of culture. **c.** Multiplication in the number of shoots, after subculturing the shoots on the same medium. **d.** Adequate rooting on half-strength liquid MS medium augmented with NAA (16.20 µ*M*). **e & f.** Acclimatization of *in vitro* regenerated plantlets in pots.

Induction of multiple shoots

Multiple shoots were observed at the end of second week, after transferring the auxiliary buds. Auxiliary buds cultured on hormone-free MS medium resulted in formation of single shoot. More than one shoot was regenerated on MS medium supplemented with growth regulators singly as well as in combinations. The mean number of shoots produced per explant on different combinations of hormones is shown in Table 1. Of the various hormones tested, BAP alone was more effective over Kn and other combinations of hormones. Superiority of BAP over Kn for multiple shoot formation was also demonstrated in *Eclipta alba* (Franca et al., 1995), *Sapium sebiferum* (Siril and Dhar, 1997), and *Pterocarpus marsupium* (Suresh and Ajay, 2004).

Percentage response for shoot formation from the explants differed in different media supplemented with various concentrations of plant growth regulators. A maximum of 85.5% (data not shown) of explants cultured on MS medium containing 3.55 µ*M* BAP formed shoots after 30 days of culture. The same medium also resulted in highest number of 6.70 ± 1.15 shoots per auxiliary bud (Figure 1b), with an average shoot length of 1.07 ± 0.21 cm. MS + Kn (23.20 µ*M*) induced 5.33 ± 0.58 shoots per auxiliary bud with an average shoot length of 1.20 ± 0.00 cm. Steady increase in number of shoots was noticed up to 3.55 µ*M* in case of BAP and 23.20 µ*M* in case of Kn, respectively. Cytokinin concentrations beyond these adversely affected the shoot development, as the regenerated shoots became stunted and dense. The stunted nature of shoot formation corresponding to increased concentration of BAP in the medium was also reported in *Orthosiphon* (Lai-Keng and Leng, 2004) and *Eupatorium* (Martin, 2004). However, sub-culturing of shoots obtained on BAP (3.55 µ*M*) on to the same hormonal medium for 30 d promoted shoot multiplication by nearly 2.5 times producing 16.33 ± 0.58 shoots (Figure 1c), with average shoot length of 1.16 ± 0.29 cm (Table 2). Stimulatory effect of increased passage of subculturing on shoot bud induction is also reported in *E. alba* (Neeti and Kothari, 2005).

Table 1. Effect of various concentrations and combinations of growth regulators on shoot induction from auxiliary bud.

S/N	Growth regulators (µ*M*)	No. of shoots/explant[y]	Shoot length (cm)[y]
1	Control[x]	1.00 ± 0.00 e[z]	1.27 ± 0.26
2	BAP 0.89	5.33 ± 0.58 abc	1.63 ± 0.03
3	BAP 1.78	5.67 ± 1.53 ab	0.67 ± 0.29
4	BAP 2.66	6.00 ± 0.00 ab	1.43 ± 0.40
5	BAP 3.55	6.70 ± 1.15 a	1.07 ± 0.21
6	BAP 4.44	5.67 ± 1.15 ab	0.93 ± 0.11
7	BAP 13.32	4.00 ± 0.00 bcd	0.63 ± 0.05
8	Kn 2.32	2.33 ± 0.58 de	1.50 ± 0.00
9	Kn 4.64	4.00 ± 0.00 bcd	2.83 ± 1.04
10	Kn 9.28	4.33 ± 0.58 bcd	1.83 ± 0.58
11	Kn 23.20	5.33 ± 0.58 abc	1.20 ± 0.00
12	BAP 4.44 + Kn 2.79	3.33 ± 0.58 cd	2.83 ± 0.29
13	BAP 4.44 + Kn 4.65	4.33 ± 0.58 bcd	2.67 ± 0.29
14	BAP 4.44 + NAA 3.24	4.00 ± 1.00 bcd	1.00 ± 0.00
15	BAP 4.44 + NAA 5.40	4.33 ± 0.58 bcd	1.00 ± 0.00
16	BAP 4.44 + IBA 4.56	4.67 ± 0.58 abc	1.67 ± 0.28
17	BAP 4.44 + IBA 5.70	3.33 ± 0.58 cd	1.50 ± 0.00

[x]Control = Hormone free MS medium.
[Y]Values are mean ± standard deviation of three independent experiments, each treatment considered after 20 replicates.
[z]Means followed by same letters are not significantly different at the 5% significance level, as determined by Tukey's HSD test.

Table 2. Effect of different concentrations of BAP on sub-culturing of multiple shoots.

S/N	BAP (µ*M*)	No. of shoots/explant[y]	Shoot length (cm)[y]
1.	BAP 0.44	13.33 ± 0.58 c[z]	1.67 ± 0.58
2.	BAP 0.89	15.33 ± 0.58 ab	1.00 ± 0.00
3.	BAP 1.78	14.00 ± 1.00 bc	1.00 ± 0.00
4.	BAP 2.67	14.33 ± 0.58 bc	1.17 ± 0.29
5.	BAP 3.55	16.33 ± 0.58 a	1.16 ± 0.29
6.	BAP 4.44	15.67 ± 0.58 ab	1.33 ± 1.04

[Y]Values are mean ± standard deviation of three independent experiments, each treatment considered after 20 replicates.
[z]Means followed by same letters are not significantly different at the 5% significance level, as determined by Tukey's HSD test.

Among the combinations of hormones tried, even the best hormonal combinations such as MS + BAP (4.44 µ*M*) + Kn (4.65 µ*M*), MS + BAP (4.44 µ*M*) + NAA (5.40 µ*M*), as well as MS + BAP (4.44 µ*M*) + IBA (4.56 µ*M*) induced only 4-5 shoots per axillary buds. This shows that, in *M. pruriens* var. *utilis*, combination of hormones do not produce synchronized effect towards large-scale bud-break leading to multiple shoot formation as seen in other *M. pruriens* varieties. Chattopadhyay et al. (1995) and Faisal et al. (2006a, b), have earlier reported maximum multiple shoot induction on NAA + 2-isopentyl adenine (2ip) and NAA+BAP in *M. pruriens* var. *pruriens* and another variety of *Mucuna* (not specified), respectively. However, the latter hormonal combination produced only 4 - 5 shoots and induced significant amount of basal callus from explants in the present study. These differential responses, revealed by different *Mucuna pruriens* varieties for the type and concentrations of the hormones used indicate possible existence of genotype specific optimum responses within large group of *M. pruriens* varieties. Similar results have also been reported in *Morus* cultivars (Tewary et al., 1996). It is established fact that, in the cultured tissues, the requirement for exogenous hormone depends on the endogenous level of the plant tissue, which varies with organ, plant genotype, and the phase of the growth

Table 3. Effect of different concentrations of NAA on root induction.

S/no.	NAA (µ*M*)	Response (%)	No. of roots/explant[y]	Root length (cm)[y]
1.	Control[x]	00.00	0.00 ± 0.00 c[z]	0.00
2.	NAA 2.70	60.00	3.67 ± 0.58 bc	4.61 ± 1.14
3.	NAA 5.40	100.00	5.00 ± 1.00 b	2.62 ± 0.74
4.	NAA 10.8	100.00	6.67 ± 1.15 b	2.27 ± 0.84
5.	NAA 16.2	100.00	16.67± 2.89 a	2.78 ± 0.51

[x]Control = Hormone free MS medium.
[Y]Values are mean ± standard deviation of three independent experiments, each treatment considered after 20 replicates.
[z]Means followed by same letters are not significantly different at the 5% significance level, as determined by Tukey's HSD test.

(Suresh and Ajay, 2004). Thus, the result establishes the need for independent standardization and examination of micro-propagation protocols in each of the varieties of *M. pruriens*, after careful consideration of variety identity, in this mopho-agronomically diverse species (Padmesh et al., 2006) of the genus *Mucuna*.

Elongation and rooting

Elongation of shoots was found to be best on GA_3 containing medium over other media supplemented with different hormones. Transferring the micro shoots to MS + GA_3 (2.89 - 14.43 µ*M*) produced enhanced elongation (data not shown), with GA_3 (2.89 µ*M*) producing maximum elongation of 4.0 cm. The positive effect of GA_3 on shoot elongation is well-established fact in plant tissue culture as reported by Jayanand et al. (2003). The elongated shoots were rooted on half strength liquid MS medium supplemented with NAA (0.00 -16.2 µ*M*). Results of rooting experiments employing different concentrations of NAA are shown in Table 3. Hundred percent cultures exhibited rooting on NAA (16.20 µ*M*), producing maximum of 16.67 ± 2.89 roots per shoot with average root length of 2.78 ± 0.51 cm (Figure 1d) in 20 d of culture. NAA as a key hormone in inducing rhizogenesis is also reported in several established micro-propagation protocols like *M. pruriens* var. *pruriens* (Chattopadhyay et al., 1995), *Pisonia alba* (Jagadish Chandra et al., 1999), *Hyptis suaveolens* (Britto et al., 2001), *Jatropha curcas* (Rajore et al., 2002). In addition, for suppressing basal callus formation during rooting, an innovative method of supporting the shoot on arc-shaped filter paper wad with centrally drilled hole was employed during the present study. This method not only reduced the basal callus formation but also facilitated easy separation of shoots from the media during hardening process.

Hardening and transplantation

Plantlets directly transferred from rooting medium to sand: soil mixture (1:1) exhibited low rate of survival (60 - 70%). To subvert this, plantlets were first transferred to autoclaved mixture of peat moss and compost mixture (1:1) and maintained in growth chamber for 14 d. The plantlets were then transferred to sand: soil (1:1) mixture and allowed to harden for 30 d (Figure 1e, f) before finally transferring to field. This resulted in 95% survival of the plants. All the survived plants were successfully transplanted to the soil.

RAPD analysis

Modified Doyle and Doyle (1990) method employed during the present study yielded high purity DNA, suitable for PCR amplification. Addition of 0.5% activated charcoal along with 0.2% β-mercaptoethanol to the extraction buffer, tried out first time for *Mucuna* in this study, fully reduced the otherwise high polyphenol oxidation. Charcoal in the extraction buffer greatly reduces polyphenol oxidation by preventing the irreversible interaction of DNA with polyphenolics (Bi et al., 1996; Krizman et al., 2006). Restriction digestion of isolated DNA with *Eco*R I, *Hind* III, *Bam*H I and *Alu* I showed characteristic smear on 0.8% agarose gel indicating high purity of the isolated DNA for molecular biology applications.

RAPD analysis following Williams et al. (1990) was employed in order to assess the genetic integrity of tissue culture clones. RAPD pattern of 10 randomly selected micropropagules obtained from auxiliary bud explants were compared with mother plant. Of the fifteen primers (Table 4) screened, 14 primers yielded clear, reproducible bands. The number of bands for each primer varied from 03 in OPA-06 to 09 in OPB-01. Each primer produced amplification products in the size range 0.2 kb in OPB-04 to 2.0 kb in OPA-01. The 14 tested primers yielded totally 925 scorable bands (number of propagules X number of screened markers) with an average of six bands per primer. All the tested primers produced monomorphic pattern across all the shoots, confirming the genetic uniformity of the micro-propagated plant material.

Table 4. Number of amplification products generated with the use of RAPD primers in the analysis of genetic fidelity of *in vitro* propagated *Mucuna pruriens* var. *utilis* plants.

S/N	Primer	Sequence (5'-3')	Number of generated bands
1	OPA-01	CAGGCCCTTC	07
2	OPA-02	TGCCGAGCTG	05
3	OPA-03	AGTCAGCCAC	05
4	OPA-04	AATCGGGCTG	06
5	OPA-05	AGGGGTCTTG	08
6	OPA-06	GGTCCCTGAC	03
7	OPA-07	GAAACGGGTG	06
8	OPA-08	GTGACGTAGG	05
9	OPA-09	GGGTAACGCC	05
10	OPA-10	GTGATCGCAG	08
11	OPB-01	GTTTCGCTCC	09
12	OPB-02	TGATCCCTGG	08
13	OPB-04	GGACTGGAGT	06
14	OPB-05	TGCGCCCTTC	04

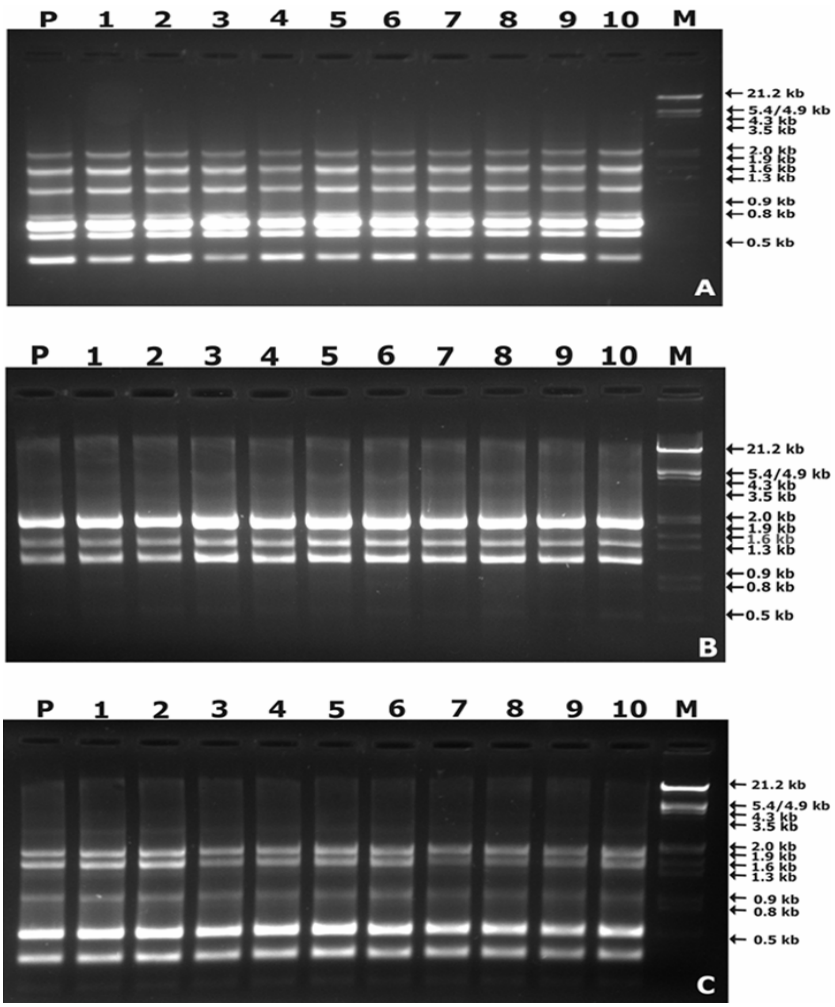

Figure 2. Agarose gel electrophoresis of RAPD products of *M. pruriens* var. *utilis* mother lines and regenerates generated with primer OPA-01 (A), OPA-06 (B) and OPA-07 (C). Lane 1(P), mother plant; lanes 2 - 11, regenerates; lane 12(M), marker (λ/*Eco*R I + *Hind* III molecular weight marker indicated in Kb).

As an example, the pattern obtained for primers OPA-01 and OPA-06 and OPA-07 are shown in Figure 2a-c.

The genetic integrity of micro-propagated plants can be determined with the use of various techniques. In this study, RAPD technique was employed, as it has the advantage of simplicity, is quickly performed and is of relatively low cost. It requires very little plant material and quick DNA extraction protocols are suitable (Rafalski et al., 1993). The limitation of this technique lies in the possible comigration of different amplification products and questionable reproducibility (Rieseberg, 1996). The results presented in this paper demonstrate that the RAPD technique proved to be effective in generating reproducible results useful in assessment of genetic fidelity of micro-propagated propagules in *Mucuna* cultivars. Various authors have found RAPD technique useful in examining genetic fidelity of tissue culture-clones. With the use of RAPD markers, clonal fidelity of micro-propagated plants has been determined in *Pinus thunbergii* (Goto et al., 1998), *Lilium* (Varshney et al., 2001) and *Tylophora indica* (Jayanthi and Mandal, 2001). Anna and Ewa (2004) have confirmed the suitability of RAPD technique for determining the genetic fidelity in two micro-propagated *Drosera* species, *D. anglica* and *D. binata*.

In conclusion, a simple, efficient and high fidelity protocol for mass propagation of *M. pruriens* var. *utilis* from auxiliary bud explants has been established. Using this protocol it is possible to produce viable, uniform and healthy plants with maximum survival rate for the proposed *in vitro* germplasm. The protocol should also provide an efficient means for large-scale cultivation and *in vitro* manipulation of *M. pruriens*, an important green manure cover-crop with medicinal properties.

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