

Full Length Research Paper

Somatic embryogenesis in *Mucuna pruriens*

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Accepted 1 September, 2009

This study reports the induction of somatic embryos in *Mucuna pruriens*. Different explants cultured on MS medium supplemented with 11.31 μ M 2,4-D produced golden yellow embryogenic callus that induced synchronized embryo development on MS basal liquid medium. Organization of pre-embryonic mass was noticed 15 d after sub culturing the callus, which progressively developed to globular, heart, torpedo and cotyledonary shaped embryos. Attempts to germinate them did not succeed as the cotyledonary embryos turned characteristic red and subsequently brown before finally turning black and unresponsive. The reaction was consistent across different media constituents, hormonal concentrations and pH ranges. Supplementation of anti-oxidants was also ineffective. Polyacrylamide gel electrophoretic analysis of the brown and non-brown embryos revealed quantitative differences in protein contents between the two. More detailed study is necessary to establish the precise cause for embryo browning and ways of its regulation.

Key words: *Mucuna*, somatic embryogenesis, embryo-browning, antioxidants, L-Dopa.

INTRODUCTION

The genus *Mucuna* belongs to the family Fabaceae (Leguminosae) and includes about 150 species of annual and perennial legumes of pantropical distribution. *Mucuna pruriens* is an important member of this genus and has been increasingly investigated for its potential as cover crop, green manure, feed and food crop (Capo chichi et. al., 2003). It grows luxuriantly in a wide variety of agro-climatic conditions producing high biomass (up to 10-12 t Dm ha⁻¹) and seed yield (2000 kg/ ha⁻¹/year); while its many varieties perform well as green manure/cover crop (GMCC). In spite of these, *Mucuna* is poorly adopted by the farmers because it lacks - or is perceived to lack uses as food and feed. Presence of anti-nutritional factors, especially Levodopa (L-3, 4 dihydroxy phenylalanine) in the seed is the major bottleneck for its consumption. L-Dopa has an important medicinal use in the treatment of Parkinson's disease but produces dan-

gerous side effects such as nausea, anorexia when consumed as food (Reynolds, 1989; Szabo and Tebett, 2000). Thus, the content of L-Dopa has to be either reduced or remedied, if *Mucuna's* potentials for food and feed applications are to be fully realized.

Biotechnological methods, especially genetic transformation for the traits, that can be turned "on" or "off" at specific stages of plant development and seed set would be of greater utility in working for the above goal (Temple and Huyck, 2000). However, the latent application of such technologies, to the goal of reducing L-Dopa in a tissue specific manner would require substantial research efforts in a sustained manner. Well before such strategies are considered, there is an immediate need to develop efficient regeneration protocols for large scale generation and screening of transformants. As on this date, the availability of useful data from any such efforts is very limited. To the best of our knowledge, this is the first report on induction of somatic embryos in any *Mucuna* species.

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Abbreviations: NAA, α -Naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxy acetic acid; BAP, benzyl amino purine; BA, N6-benzyladenine; L-Dopa, 3,4-dihydroxy phenylalanine; PVP, polyvinyl pyrrolidone; IZE, immature zygotic embryos; PEM, proembryonic mass; MS, Murashige and Skoog (1962) medium

MATERIALS AND METHODS

Plant material

Mature seeds collected from well-dried pods of six-month-old *M. pruriens* plant grown at the garden of Department of Biotechnology,

Sir M Visvesvaraya Institute of Technology (Sir MVIT), Bangalore were used as seed source. Seeds were initially washed with detergent Extran[®] MA 02 (Merck) for 10 min followed by wash under running tap water for 30 min. Surface sterilization was carried out by treatment with 0.1% mercuric chloride + 0.05% cetrime + 0.05% bavistin mixture for 5 min. Washed seeds were germinated on MS medium (Murashige and Skoog, 1962) containing 0.3% charcoal (w/v) and 0.8% agar (w/v), devoid of sucrose.

Explant preparation

Leaf and stem explants of 1.5-2.0 cm² size respectively, excised from 8-d old *in vitro* grown seedlings were used as explant source. For culture of immature zygotic embryos (IZE), 10 d old newly set pods collected from field grown plants were dissected at septal region to recover the embryos and 5 - 6 mm length IZE were inoculated on to callus induction medium.

Media preparation

Analytical grade chemicals obtained from Himedia laboratories, hormones and vitamins from Sigma-Aldrich chemicals were used for preparing the stock solutions and media preparation. Murashige and Skoog's salts, with 3% (w/v) sucrose were used as the basal medium. 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. Charcoal, whenever added, was added before adjusting the pH. The media was autoclaved at 121°C and 1.06 kg/cm² pressure for 20 min.

Preparation of anti-oxidants

All the antioxidants, except charcoal were filtered, sterilized and added to the pre-autoclaved media. Charcoal was added before autoclaving. Except for charcoal, in all the above cases, pH of the media was adjusted to 5.7 ± 0.1 before addition of antioxidants.

Callus induction

For callus induction leaf, stem and immature zygotic embryos were cultured on MS medium supplemented with 3% sucrose (w/v) and 0.8% agar (w/v) and different concentrations of 2,4-D (2.26 - 45.24 µM), NAA (2.69 - 5.37 µM) and combination of 2,4-D (2.26 - 22.62 µM) + BAP (2.2 - 11.05 µM) and 2,4-D (2.26-22.62 µM) + NAA (0.5-13.43 µM). Cultures were incubated, unless otherwise specified in a growth chamber maintained at a temperature of 25 ± 2°C, relative humidity, 70 - 80% and photoperiod of 16 h light, 8 h dark, under photon flux density of 50 µE mol m⁻²s⁻² provided by day-light fluorescent lamps.

Somatic embryo induction

Callus initiated from all the three explants on 2,4-D (11.31 µM) were subjected to embryogenesis by subculturing onto different reduced concentrations of 2,4-D both in solid as well as liquid cultures. The above media was also supplemented with glutamine (1 mg/l) and growth adjuvants such as casein hydrolysate (2 g/l), yeast extract (1 g/l), malt extract (2 g/l) and coconut milk (5% v/v). Liquid cultures were maintained in Orbitec LJE temperature controlled shakers at 120 rpm shaking under conditions specified for the callus induction.

Somatic embryo germination

For embryo germination, light green cotyledonary stage embryos formed in 25 - 30 d old suspension culture were transferred onto MS solid medium, both half and full strength, supplemented with or without different cytokinins. Thidiazuron (1.13-2.27 µM), BAP (0.22-2.22 µM), kinetin (1.16-2.32 µM) and zeatin (4.56-9.12 µM) individually as well as in combinations with NAA (2.69 µM) were used in germination medium and the results were compared with that in MS basal medium used as control. For controlling the browning of embryos, antioxidants, namely charcoal (0.25 - 1.0%), PVP (5 - 25 mg/l) and ascorbate (0.0- 0.1 mg/l) were supplemented in the embryo germination medium. To determine the effect of light, total illumination produced by 4 tube lights each producing photon flux density 15 µE mol m⁻²s⁻² measured at 40 cm distance using Quantum sensor (Apogee Instruments-UT) in a closed chamber (Orbitec LJE) was considered as 100% and intensities produced by 1, 2 and 3 tubes were considered as ¼, ½ and ¾ respectively. Green embryos were also incubated at temperatures ranging from 25 - 30°C and pH 5.5 - 6.0 for determining the effect of temperature and pH on embryo germination.

SDS -PAGE

One gram of embryonic tissue was homogenized in a chilled mortar and pestle with the extraction buffer containing 0.1 M Tris-HCl (pH 7.2), 0.5% polyvinyl pyrrolidone (PVP), 5% sucrose and 10 mM 2-mercaptoethanol (Siva and Krishnamurthy, 2005). The samples were centrifuged at 15,000 rpm for 10 min and the fresh supernatant was used for separation of proteins on vertical polyacrylamide gels (5% stacking and 8% separating). The gels were run in tris-glycine buffer (pH 6.8) at 20 mA for about 1½ h at room temperature and stained for 30 min using coomassie brilliant blue and visualized under white light after de-staining. The experiments were repeated thrice.

Statistical analysis

The cultures were examined at regular intervals and all the experiments were repeated at least thrice. Data were analyzed using 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.

RESULTS

Seed germination

In vitro inoculated seeds germinated within 3 - 4 d on MS basal medium without sucrose. Presence of seed coat inhibited rate and percentage of germination by nearly 40% due to oxidation of phenolic compounds (Deshpande et al., 1982; Ravindran and Ravindran, 1988; Singh, 1993). Its removal favoured germination to 90%. All the shoots attained a height of 6 - 7 cm within 8 d, exhibiting synchronized seed germination pattern.

Callus induction

Time duration for initiation of callusing differed in different

explants. While the leaf and stem explants exhibited initiation on around 7 d, IZE showed callus formation only after 10 d. Nevertheless, callus from all the three explants exhibited similar proliferation in subsequent subcultures. Best proliferation was seen on two hormonal concentrations; MS + 2,4-D (13.31 μM) and MS + NAA (5.37 μM) + 2,4-D (4.53 μM) (Figure 1G; Table 1). However, NAA added medium stimulated rhizogenesis from callus in subsequent subcultures.

Somatic embryogenesis

Callus transferred onto MS basal medium showed best embryo maturation. Explant factor appear to play key role in this process. Among the three explants used, immature zygotic embryos produced maximum number of embryonic structures. However, embryos from leaf origin exhibited better developmental synchronization with maximum number of embryos entering cotyledonary stage at a time (Table 2). Of the two culture conditions, rapid embryogenesis was observed on liquid cultures compared to solid culture. Anatomical progression leading to proembryonic mass (PEM) initiated on around 3rd d and distinct PEM were formed around 7th d after transferring the callus to MS liquid medium without hormone (Figure 1A-E). Consolidation of pre-embryonic mass into globular structures was visible around 12 - 15th d. From this point, complete progression from globular to cotyledonary embryos completed in 10 - 15 d (Figure 1H - K). Similar progression took nearly two and half months on solid culture. Even the separation and structural organization was poor on solid culture.

Somatic embryo germination

Between 25-30th d on maturation medium, embryos turned green and appeared progressing for germination. But, after 30th d all the embryos developed characteristic reddish brown shade within 4 - 5 d and progressively turned dark (Figure 1L). After this, the embryonic cells continued to exhibit active cytoplasmic streaming for nearly 120 d and all efforts to revive them failed. The browning was found to be irreversible even on transfer to varied strength of media constituents, hormonal concentrations and pH ranges. Efforts such as early subculturing to germination medium, supplementation of growth adjuncts, incremental addition of fresh media in small quantity directly to the maturation medium all failed to thwart this transformation.

Effect of anti-oxidants

In an effort to understand the role of oxidative stress in the above process, we supplemented germination

medium with three anti-oxidants; PVP, charcoal and ascorbate. PVP reduced browning but triggered active cell division leading to complete breakdown of structural organization of the embryos, where as ascorbate failed to reduce embryo browning. Greening was completely lost in charcoal supplemented medium and the extent of browning was not recordable due to difficulty in differentiating colors.

Effect of light and temperature

Embryo browning was found to be sensitive to physical conditions. Green embryos incubated in complete darkness turned colorless. Embryos incubated at 25 and 50% light intensities showed maturation and incomplete organogenesis up to rooting while 75% light and control turned brown. Browning also increased proportionally with increase in the temperature (25 - 30°C) indicating temperature sensitive nature of this process.

SDS -PAGE

The protein bands observed in the extracts of brown embryos were comparatively pale and less intense than those observed in non-brown embryonic suspension (Figure 1F). No variation was seen in terms of the number of bands.

DISCUSSION

Genetic improvement through transgenic technology is impeded due to non availability of efficient regeneration system in many grain legumes (Chandra and Pental, 2003). Embryogenic system offers an ideal tool for *in vitro* production and selection of transgenic plants (Finer and McMullen, 1991; Christou, 1997). Such methods are available only for few grain legumes such as *Glycine max* (Finer and Nagasawa, 1988), *Vigna aconitifolia* (Kumar et al., 1988), *Vigna unguiculata* (Kulothungan et al., 1995), *Cajanus cajan* (Anbazhagan and Ganapathi, 1999) Chickpea (Kiran et al., 2005) and Horsegram (Varisai et al., 2004). In majority of these cases, preference for 2,4-D for embryogenic callus induction is well established (Griga et al., 1987; Mohamed et al., 2004; Anbazhagan and Ganapathi, 1999). Immature zygotic embryos (IZE), young cotyledons or vegetative shoot apices have been the most responsive explants (Hartweck et al., 1988). However, the superiority of leaf for embryogenic development in *Mucuna* may be attributed to explant-specific developmental response owing to unique biochemical constitution of this plant. Initial browning/blackening of explants due to leaching and subsequent oxidation of polyphenols might be responsible for delayed response in IZE explants.

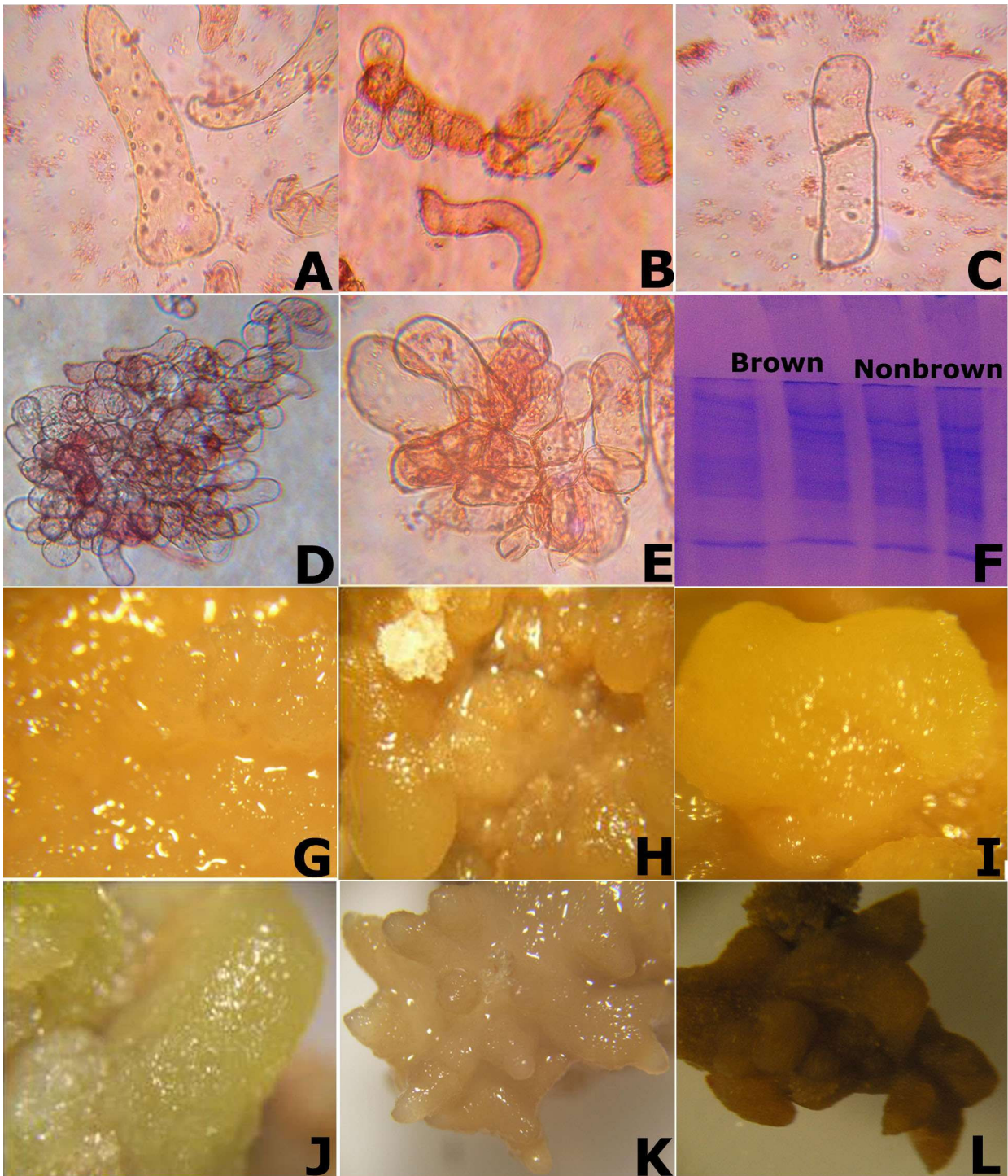


Figure 1. Stages of somatic embryogenesis in *M. pruriens*. (A to E) Cells exhibiting anatomical progression leading to formation of proembryonic mass (PEM) on transfer to MS basal liquid medium. (F) SDS- PAGE protein profile for the brown and non-brown embryo extracts. (G) Golden yellow embryogenic callus induced from leaf explants cultured on 2,4-D (11.31 μM). (H) Formation of globular embryos. (I) Development of heart shaped embryos. (J) Torpedo shaped embryo. (K) Matured cotyledonary shaped embryos. (L) Rooting from matured embryo. (L) Embryo-browning observed on germination medium supplemented with BAP (0.22 μM).

Species of the genus *Mucuna* are characterized by the presence of non-essential amino acid, 3,4-dihydroxy

phenylalanine (L-Dopa), in different parts of the plant. Reported even in other grain legumes such as *Vicia*,

Table 1. Somatic embryo induction on ms medium in different growth hormones in different explants of *M. pruriens*.

S/N	Growth regulator (μM)	No. of embryogenesis induced ^y			Number of embryos attained cotyledonary stage (%)		
		Leaf	Stem	IZE	Leaf	Stem	IZE
1	MS Basal	54.75 \pm 0.56 ^a	37.25 \pm 1.92 ^a	75.5 \pm 0.64 ^a	62.96	49.66	29.98
2	2,4-D, 1.13	40 \pm 0.81 ^c	27.25 \pm 0.83 ^d	62.5 \pm 1.8 ^{ab}	56.53	46.67	22.27
3	2,4-D, 2.2	36 \pm 0.93 ^d	20.5 \pm 1.12 ^e	54.5 \pm 0.64 ^{bc}	45.39	49.02	11.47
4	NAA 1.34	40.5 \pm 0.84 ^{cd}	30.25 \pm 1.09 ^c	70 \pm 0.81 ^{cd}	60.69	59.50	21.04
5	NAA 2.69	45.25 \pm 0.96 ^{bc}	31 \pm 0.71 ^c	65.75 \pm 0.76 ^{de}	61.45	48.37	17.00
6	2,4-D 1.13 + NAA 1.34	49.75 \pm 0.96 ^{cd}	34 \pm 0.71 ^b	75.5 \pm 0.15 ^e	35.11	12.25	29.09
7	2,4-D 2.2 + NAA 2.69	38.75 \pm 1.11 ^b	24 \pm 0.71 ^e	65.25 \pm 0.69 ^f	68.95	43.29	36.70

^yValues are mean \pm standard deviation of three independent experiments, each treatment considered after 20 replicates. Means followed by same letters are not significantly different at the 5% significance level, as determined by Tukey's HSD test.

Table 2. Response of leaf segments of *mucuna pruriens* on ms media with different growth regulators.

S/N	Growth regulator (μM)	Callus induction (%)	Type of response
1	MS basal	-	-
2	2,4-D 2.26	54.71	Embryogenic
3	2,4-D 4.53	78.39	Embryogenic
4	2,4-D 11.31	100	Embryogenic
5	NAA 2.69	68.33	Rhizogenesis
6	NAA 5.37	69.92	Rhizogenesis
7	2,4-D 2.26 + NAA 2.69	100	Embryogenic
8	2,4-D 4.53 + NAA 5.37	100	Embryogenic
9	2,4-D 2.26 + BAP 2.2	35.68	Nonembryogenic
10	2,4-D 11.31 + BAP 4.42	45.27	Nonembryogenic
11	2,4-D 2.26 + BAP 2.2	51.73	Nonembryogenic
12	2,4-D 4.53 + BAP 4.42	54.92	Nonembryogenic
13	BAP 2.2 + NAA 2.69	36.02	Nonembryogenic
14	BAP 4.42 + NAA 5.37	48.73	Nonembryogenic

Baptista and *Lupinus* (Daxenbichler et al., 1971), in *Mucuna* L-Dopa accumulation is predominant in seed (3 - 6%), while other parts such as stem, leaf and root also exhibits its presence at lower levels (Szabo and Tebett, 2000). L-Dopa is synthesized from tyrosine precursor in one step conversion process catalyzed by the enzyme tyrosine hydroxylase (Griffith and Conn, 1973) and in turn acts as precursor for the black pigment melanin (Brain, 1976). The potential cytotoxicity of the melanogenic intermediate DOPA has long been recognized and exploited as a targeting concept in experimental melanoma therapy (Urabe et al., 1994).

Considerable evidences are available on the endogenous accumulation of L-Dopa in cells of *M. pruriens* grown *in vitro* (Brain, 1976; Huizing et al., 1985). Several hormonal as well as physical factors are known to influence this process. While 2,4-D inhibits its synthesis (Brain, 1976), BA is noted to exert positive influence (Huizing et al., 1985). Light is known to promote *in vitro*

biosynthesis of L-Dopa. Due to high degree of instability, once formed, L-Dopa gets converted to its metabolite O-quinones especially dopamine and melanin (Brain, 1979; Wichers et al., 1983). This phenomenon, recognizable by browning preceded by characteristic red shade has been observed both in callus and suspension cultures of *M. pruriens*. It has been observed that pH range 5.5 - 6.5 is optimal for this process (Wichers et al., 1985). These close similarities with melanogenesis process reported from earlier studies not only explains the possible involvement of same in the embryo browning process, but also its anti-physiological effects in terms of cytotoxic activities or gene function inhibitor as revealed by electrophoretic studies. However, the factor that triggers this process in a tissue specific manner in cotyledonary embryos remains unclear. Possibly, release of phenoloxidase in cytosol due to vacuolar damage caused by the reactive oxygen species generated by the photosynthetic activities of the germinating embryos is responsible for

this oxidation. Photosynthesis is one of the pathways in which large number of free radicals gets produced (Kelvin, 1959; Marshal et al., 2002). Reduction in browning at lower light intensities also point towards involvement of light dependent process in embryo-browning. Failure of anti-oxidants to prevent this might be due to delayed supplementation of reagents in the reaction mixture.

Thus, it can be concluded that a comprehensive study is necessary to establish the role of L-Dopa, its metabolites and changes in enzymatic activities associated with oxidative stress on *in vitro* development process in *Mucuna*. This will help gain insight into molecular mechanism of embryo-browning and also possible ways by which the process could be regulated to establish efficient embryogenic system in *M. pruriens*.

ACKNOWLEDGEMENTS

Authors acknowledge Principal, Sir MVIT and Head, Department of Biotechnology, Sir MVIT for the facilities and Sri Krishnadevaraya Educational Trust (Sri KET), Bangalore for the financial support.

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