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RESEARCH ARTICLE

Direct adventitious shoot bud formation on hypocotyls explants in *Millettia pinnata* (L.) Panigrahi- a biodiesel producing medicinal tree species

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Abstract A reproducible protocol developed for in vitro regeneration of *Milletia pinnata* using hypocotyl segments. Multiple shoots were induced from hypocotyl explants through direct adventitious shoot bud regeneration. The proximal end of hypocotyls was responsive for shoot bud induction. Silver nitrate and adenine sulphate had a positive effect on shoot bud induction and elongation. The maximum response and number of shoot bud produced in media supplemented with 8.88 μM BAP with 108.6 μM adenine sulphate and 11.84 μM silver nitrate. Elongated shoots were harvested and successful rooting of microshoots achieved on MS media supplemented with 9.84 μM IBA, with 81.1 % rooting. Remaining shoot buds sub-cultured for further multiplication and elongation. Each subculture produced eight to nine elongated microshoots up to four subcultures. The rooted microshoots were successfully hardened and transferred to field.

Keywords In vitro · Hypocotyl · Direct adventitious bud · Silver nitrate · Adenine sulphate · *Milletia pinnata*

Introduction

Milletia pinnata (syn *Pongamia pinnata*, locally known as Karanja), a biodiesel producing leguminous tree, possesses multiple uses. Among various options for biodiesel production from tree- borne oilseeds, it is the most promising candidate. It produces more quantity of biodiesel per kg of seed as compared to Jatropha (Patil et al. 2014). Traditionally, all parts of plant are used by native people for curing various ailments, including leprosy, gynecological problem, dental problem, piles and skin disease (see Yadav et al. 2011 and references therein). Pongamol and karanjin isolated from fruit showed antihyperglycemic activity (Tamrakar et al. 2008, 2011). Flavanoids and endophytes isolated from pongamia have shown anticarcinogenic activity (Minakawa et al. 2010; Verekar et al. 2014).

Lack of elite planting material is a major bottleneck in large scale plantation of *M. pinnata*. The outsourcing nature of species creates a high level of heterogeneity in population. Hence, a wide range of molecular diversity and variability for seed morphology, oil content, seed biochemicals, karanjin content exists within species (Jaisankar et al. 2014; Rao et al. 2011; Kaushik et al. 2007; Sharma et al. 2011, 2014; Pavithra et al. 2014). Further, propagation of *M. pinnata* through seed possesses several problems. The seed loses its viability soon and higher moisture content and flesh nature of seed further invites fungal attack during storage. Physical dormancy (hard seed coat) is also a major problem, which reduces the nursery germination percent (Edwards and Naithani 1999). Hence, an effective clonal multiplication is required for large-scale propagation of improved genotype. Biotechnological interventions can open path for development of genotypes with enhanced oil quality and medicinal property (Belide et al. 2010). But, the primary requirement of effective biotechnological manipulation is reproducible in vitro regeneration protocol. *Although* success has been reported for whole plant regeneration in vitro in *M. pinnata* (Belide et al. 2010; Sujatha et al. 2008; Shrivastava and Kant 2010); no report on direct adventitious bud formation from hypocotyl explants in *M. pinnata* is available to the best of our knowledge. Direct pathways reduces chances of variation in vitro (soma clonal variation)

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(Lee and Rao 1986), facilitates the production of large numbers of platelets within shorter time. It is also a preferred pathway for genetic transformation by *Agrobacterium* (Kantia and Kothari 2002).

In this paper we have shown that hypocotyl explants from axenically grown seedlings can produce direct shoot buds. The present protocol may ensure mass production of promising/elite planting stock on a large scale and open the avenues for biotechnological interventions for further improvement of this multipurpose tree species.

Materials and methods

Explant preparation and inoculation

Mature seeds of karanja (*M. Pinnata* L.) were collected from ripe fruits and washed under running tap water for 30 min followed by rinsing with 5 % aqueous solution of Tween-20 (SRL, India) for 10 min and kept in 1 % Bavistin (carnbandazim powder, BASF, India), a broad spectrum fungicide, for 15 min and rinsed 5–6 times with distilled water. The de-coated seeds were surface sterilized for 5 min with a 0.1 % $HgCl_2$ aqueous solution (SRL, India) and finally rinsed 5–6 times with autoclaved distilled water. The mature embryos were carefully excised from surface-sterilized seeds and germinated in a culture bottle containing full strength MS basal medium (Murashige and Skoog 1962) and 6 g/l agar (Bacteriological grade, Hi-media, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 120 °C and 104 kPa for 15 min. All the cultures were incubated in a culture room at a temperature of 25±2° C with relative humidity at 55±5 % and were exposed to 16 h light and 8 h dark photoperiod provided by 40 W cool white fluorescent tubes kept 50 cm above bench surface. Ten days-old mature embryo derived axenic seedlings served as the source of explants. The hypocotyl explants were excised from axenic seedling for direct shoot bud induction.

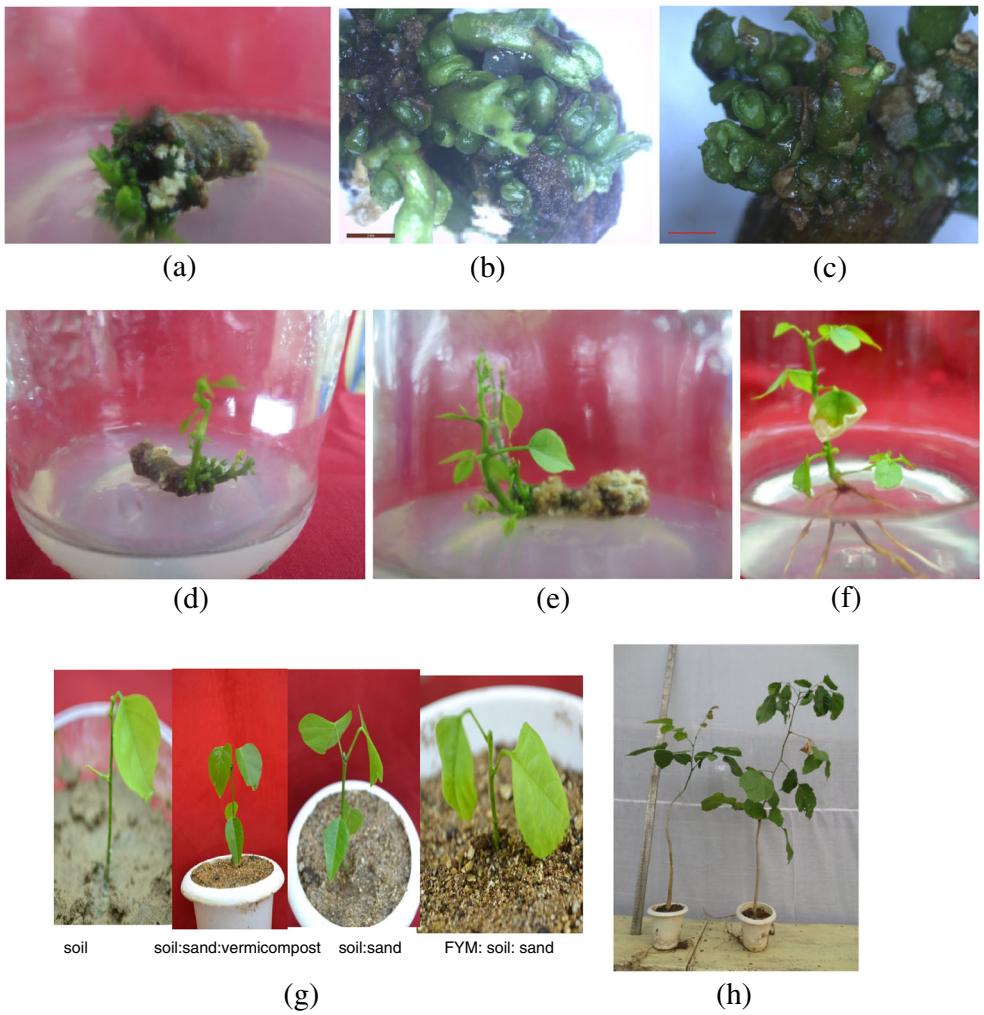

Fig. 1 In vitro regeneration of *M. pinnata* through direct adventitious shoot bud induction on hypocotyl explants **a**) shoot bud induction at proximal end of hypocotyl **b**) close view of shoot buds sprouting directly from hypocotyls in MS+8.88 μM BAP +108.6 μM Ads +11.84 μM $AgNO_3$ (bar=2 mm) **c**) close view of shoot buds elongation in MS+8.88 μM BAP +108.6 μM Ads +11.84 μM $AgNO_3$ (bar= 2 mm) **d**) shoot bud induction in MS+ 9.3 μM Kin **e**) shoot bud elongation in MS+8.88 μM BAP +108.6 μM Ads +11.84 μM $AgNO_3$ **f**) rooting of microshoots **g**) hardening of plantlets **h**) in vitro raised plants ready for plantation

Table 1 Effect of cytokinins on response and number on hypocotyl explants for shoot bud induction response, number of shoot bud per explant, number of elongated shoot and shoot length (cm) in *Millettia pinnata* after 6 weeks of culture

Growth regulators (μM)		Percent Response	Average number of shoot bud per explant	Average number of elongate shoot per explant	Average length of shoot
BAP	Kinetin				
0	0	10.68±0.33c	4.67±0.67bc	1.03±0.03c	1.37±0.22c
4.44	–	51.55±1.77b	5.67±0.33b	2.37±0.19b	1.73±0.12c
8.88	–	72.20±1.10a	7.50±0.32a	4.43±0.18a	1.80±0.06bc
11.1	–	67.68±1.76a	5.97±0.58b	4.03±0.29a	1.53±0.20c
–	4.65	50.33±3.28b	3.53±0.26 cd	1.03±0.03c	2.30±0.15a
–	9.3	50.00±1.92b	3.27±0.12d	1.30±0.10c	2.27±0.09ba
–	11.63	47.78±2.22b	4.33±0.33 cd	1.30±0.15c	2.27±0.12ba

Standardization of establishment and shoot proliferation medium

Isolated hypocotyl segments were cultured on full strength MS media supplemented without any cytokinin (control) or various concentrations of BAP (4.44, 8.88 and 11.1 μM) or Kin (4.65 μM, 9.3 μM and 11.63 μM) for shoot bud induction. Effect of silver nitrate ($AgNO_3$) and adenine sulphate (Ads) was also investigated for establishment and shoot elongation. Hypocotyl explants were cultured on MS media supplemented with 4.44 μM and 11.1 μM BAP alone or in combination with 11.84 μM $AgNO_3$ and/or 108.6 μM Ads. Shoots obtained from each harvest were cut and cultured on MS medium containing 8.88 μM BAP in combination with 108.6 μM Ads and 11.84 μM $AgNO_3$ for further multiplication and elongation. All cultures were maintained under similar conditions as described earlier for seed germination.

Rooting and acclimatization of plantlets

Shoots 1.5–2.0 cm in length were excised and transferred to half-strength MS medium supplemented with various concentrations of IBA (2.46 μM, 4.92 μM, 7.38 μM and 9.84 μM), NAA (2.69 μM, 5.37 μM, 8.06 μM and 10.74 μM) and IAA (2.85 μM, 5.71 μM, 8.56 μM and 11.42 μM). The rooted plants were removed from the culture tubes, washed free of agar with sterile distilled water and transferred to plastic pots with garden soil, soil:sand:vermicompost (1:1:1), Cocopeat, sand: soil (1:1), and FYM:soil:sand (1:1:1) for hardening. The plantlets were maintained at 70 % relative humidity by initially covering with transparent polythene. The plants were kept in 28 °C under a 12-h photoperiod for acclimatization. The plants were fertilized with 1/4th MS macronutrients twice during the course of acclimatization at an interval of 4–5 week. At the end of 6 weeks, surviving plants were noted.

Statistical analysis

For shoot induction data on per cent explants responses, average number of shoot bud per explants, average number of elongated shoots and average shoot length were recorded. For the root induction, data on response in rooting, days taken for root initiation, number of roots and average length of root

Table 2 Effect of silver nitrate and adenine sulphate on hypocotyl explants for shoot bud induction response, number of shoot bud per explant, number of elongated shoot and shoot length (cm) in *Millettia pinnata* after 6 weeks of culture

Growth regulator (μM)	Additives (μM)		Percent Response	Average number of shoot bud per explant	Average number of elongate shoot per explant	Average length of shoot
BAP	Silver nitrate	Adenine sulphate				
8.88	–	–	67.66±1.76bc	5.97±0.58d	4.03±0.29ef	1.80±0.06 cd
11.1	–	–	72.20±1.10b	7.50±0.32bc	4.43±0.18ed	1.53±0.20d
8.88		108.6	51.11±1.11d	5.73±0.91d	3.20±0.15 g	2.30±0.15c
11.1		108.6	63.33±1.92c	7.30±0.40bc	3.60±0.06 fg	2.17±0.03 cd
8.88	11.84		66.67±1.93bc	9.37±0.09bc	5.07±0.32c	3.37±0.22a
11.1	11.84		52.22±2.93d	8.50±0.06c	4.97±0.12de	3.07±0.09a
8.88	11.84	108.6	80.00±1.92a	16.00±1.16a	8.53±0.18a	3.27±0.12a
11.1	11.84	108.6	71.11±1.11b	11.33±0.88b	7.70±0.10b	3.17±0.03a

Table 3 Effect of auxins on micro-shoot derived from hypocotyls of *Millettia pinnata* for per cent root induction, days taken for rooting, number of root and length of root (cm) after 6 weeks of culture

Auxins (μM)			Rooting Percentage	Day of initiation	Number of root	Length of root (cm)
IBA	NAA	IAA				
0	0	0	–	–	–	–
2.45	–	–	31.1±1.10ef	15.66±0.33bcd	1.03±0.07 h	0.80±0.06 g
4.90	–	–	48.9±1.10c	14.66±0.33de	1.63±0.09f	1.26±0.09f
7.35	–	–	58.9±1.10b	13.33±0.67e	2.66±0.09b	4.16±0.12b
9.84	–	–	81.1±1.10a	9.33±0.33f	4.20±0.06a	7.06±0.15a
–	2.70	–	27.8±1.10 fg	14.66±0.33de	1.66±0.09ef	3.46±0.09c
–	5.40	–	25.57±1.13gh	15.00±0.57d	1.90±0.12e	2.43±0.12de
–	8.10	–	23.33±1.93 h	16.66±0.33bc	2.30±0.06c	2.73±0.12d
–	10.80	–	22.20±1.10 h	17.00±0.57a	1.80±0.06ef	1.40±0.06f
–	–	2.85	27.80±1.10 fg	15.33±0.33 cd	1.33±0.09 g	2.26±0.12e
–	–	5.70	31.10±1.10ef	15.00±0.57d	1.66±0.03ef	3.60±0.10c
–	–	8.55	28.90±1.10 fg	14.33±0.67de	1.36±0.03 g	0.93±0.09 g
–	–	11.40	33.33±1.93d	14.66±0.33de	1.16±0.07gh	1.26±0.12f

recorded. Surviving explants in hardening was recorded. Each treatment consisted of thirty replications and was repeated thrice. For hardening 10 plantlets per treatment were considered. Data were analyzed with ANOVA for a completely randomized design (CRD). Duncan's new multiple range test (DMRT) (Gomez and Gomez 1984) was used to separate the means to determine significant effects.

Results and discussion

The hypocotyls were inoculated on MS Media supplemented with various growth regulators. The response of explants to the growth regulators supplements was significantly different. Distal end of hypocotyls swelled and started callusing without showing any sign of regeneration, whereas the proximal end responded to all the treatments except the control for shoot bud formation. Similar finding has been reported for hypocotyls response to in vitro culture in *Morus multicaulis* (Xi-Ling et al. 2011) and cucumber (Mohiuddin et al. 1997). The basipetal movement of auxin along stems has been well documented (Kramer et al. 2008; Raven et al. 2005). Differential response of explants region may be due to gradient in endogenous growth hormone concentration. Role of the position of explants and the gradient of response has been reported in some other plants also (García-Luis et al. 1999; Goh et al. 1995; Moreira-Dias et al. 2001).

Shoot bud appeared in most of the treatment within 2 weeks of culture (Fig. 1a), however, there was significant variation in percent response and number of shoot bud and number of elongated shoot bud. More response to BAP as compared to Kinetin was evident. The maximum response and number of shoot bud produced by the proximal end of hypocotyls explants in media supplemented with 8.88 μM BAP followed by 11.1 μM BAP (Table 1). The number of elongated shoots was also produced maximum in media supplemented with 8.88 μM BAP. Although the longest average length observed in media supplemented with 9.3 μM Kin, the number of elongated shoots were very few in number (Fig. 1d). The positive effect of BAP in shoot multiplication has been demonstrated by most of the earlier workers in *Milletia* on various explants (Belide et al. 2010; Shrivastava and Kant 2010; Kesari et al. 2012; Sugla et al. 2007).

Direct adventitious shoot bud started to appear at the proximal end of hypocotyls from the second week of culture. Although some elongated shoot bud appeared, but these shoots showed signs of leaf senescence. Hence, in the next effort, media were supplemented with ethylene antagonist silver nitrate and growth supplement adenine sulphate (Table 2).

Adenine sulphate had no marked influence on the percent response of explants, number of shoot bud, number of elongated shoots and average shoot length. $AgNO_3$ when added into the media showed remarkably increased response of explants. Maximum response, multiplication and elongation of the shoot was observed in media supplemented with both silver nitrate and adenine sulphate, however, addition of

Table 4 Effect of different growing media on hardening of in vitro grown *Millettia pinnata* plantlets after 6 weeks

Media composition	Survival percentage
Soil	33.33
Soil:Sand:Vermicompost (1:1:1)	83.33
Coco Peat	20.00
Soil:Sand (1:1)	36.67
FYM: Soil : Sand (1:1:1)	36.67

$AgNO_3$ in the media increased the number of shoot bud and average shoot length in all media tested. Although, Ads had no major effect on evoking shoot bud or length of shoot, however when applied with $AgNO_3$, there was a significant increase in the number of shoot bud as well as in the number of elongated shoots and average length of shoots. Percent response, number of shoot buds per explants, number of shoot larger than 1.5 cm and average length of shoot was highest in the media supplemented with 8.88 μM BAP in combination with 108.6 μM Ads and 11.84 μM $AgNO_3$ (Fig. 1b, c, e). The positive effect of silver ion on shoot bud multiplication and elongation has been shown by various studies earlier (Brar et al. 1999; Hyde and Phillips 1996; Pestana et al. 1999; Giridhar et al. 2003; Sridevi and Giridhar 2014; Sgamma et al. 2015; San et al. 2015).

Elongated shoots were harvested for root induction and remaining hypocotyls along with small shoot buds were again placed on media supplemented with 8.88 μM BAP in combination with 108.6 μM Ads and 11.84 μM $AgNO_3$. The miniature shoot bud further elongated and at the node of the harvested shoots more shoot buds were produced. Up to the fourth subculture, eight- nine elongated shoots were harvested for rooting from each sub -culture.

In each sub-culture, elongated shoots were harvested and cultured in media supplemented with various concentrations of IBA, IAA or NAA in the full strength MS media. Maximum response for rooting was evoked by 9.84 μM IBA and it took minimum days in root induction. Maximum number of roots and maximum length of root also observed in MS media supplemented with 9.84 μM IBA (Table 3, Fig. 1f). The significance of IBA for the development of adventitious root has already been shown in karanja by many earlier workers (Manoj et al. 2009; Sugla et al. 2007; Kesari et al. 2009 & 2012; Shrivastava and Kant 2010).

In order to ensure higher ex vitro survival rate, rooted plantlets of *M. pinnata* were transferred from the aseptic culture environment *(*in-vitro) to various hardening media. After 6 weeks, observations were recorded on survival of plants and the results are presented in Table 4. The highest percentage of survival was obtained by using soil:sand:vermicompost (1:1:1) (Fig. 1g) and the minimum survival was observed in cocopeat. Minimum percent survival in cocopeat may be attributed to its high water holding capacity, which might have clogged the fragile root system of micropropagated plants. Similar result was reported in tomato (Devi et al. 2008).

Immediate transfer of plants from low light intensity and high humidity to controlled temperature and normal atmospheric humidity conditions caused the death of regenerates. A gradual transfer procedure was found imperative. Pots were covered with polyethylene bags to maintain high humidity and were kept in growth chamber 25±2 °C for 4 weeks. The covers were gradually removed upon appearance of new leaves. Hardened plants ready for transplantation were obtained within 2 months (Fig. 1h).

Conclusions

In conclusion, present study demonstrated an efficient and reproducible protocol for the first time for direct regeneration from hypocotyls explants. This protocol also demonstrated the significance of adenine sulphate and silver nitrate (ethylene regulator) in regeneration of *Milletia pinnata* from hypocotyl explants. This protocol will be extremely helpful for biotechnological interventions for improvement of this multipurpose tree species.

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