Available online at www.sciencedirect.com

SciVerse ScienceDirect

http://www.elsevier.com/locate/biombioe

High frequency direct organogenesis and evaluation of genetic stability for *in vitro* regenerated *Pongamia pinnata*, a valuable biodiesel plant

*Vigya Kesari, Aadi Moolam Ramesh, Latha Rangan**

Department of Biotechnology, Indian Institute of Technology Guwahati, Assam 781 039, India

ARTICLE INFO

Article history:
Received 7 June 2010
Received in revised form
27 March 2012
Accepted 29 March 2012
Available online 14 May 2012

Keywords:
Biodiesel
Epicotyl
Genetic fidelity
Markers
Micropropagation
Nodal explants

ABSTRACT

In the present study regeneration efficiency and genetic clonality are the two major aspects for *in vitro* propagation of candidate plus tree of *Pongamia pinnata*, a versatile biodiesel legume. Woody Plant Medium (WP) and Murashige and Skoog's medium (MS) supplemented with different concentrations and combinations of plant growth regulators were screened for high frequency regeneration using nodal segment culture and axenically grown seedlings of elite genotype of *P. pinnata*. Percentage response from field-grown mature nodal segments of *P. pinnata* were highly dependant on the season, with greater than 68% of culture developing adventitious shoots during spring. Woody Plant Medium supplemented with benzyladenine (5.0 mg L^{-1}) and kinetin (0.5 mg L^{-1}) gave the greatest response to initiation and multiplication. The multiplication rate of 11 shoots per explants with an average shoot length of 3.0 cm was observed. Multiplied shoots started to produce roots in the multiplication medium itself containing BA and NAA but subsequent establishment was poor. The rooting response was enhanced in half-strength MS media with indole-3-butyric acid (0.5 mg L^{-1}). Rooted plants were hardened successfully in glass house with 70% survivability. RAPD and ISSR markers were employed to determine the genetic fidelity of *in vitro* raised plantlets.

1. Introduction

Pongamia pinnata (L.) Pierre (synonym: *Derris indica*) is a nitrogen-fixing, wild, perennial, leguminous tree native to humid and subtropical environments occurring widely along the coasts and riverbanks of India and Myanmar. The tree is hardy enough to survive a wide range of climatic conditions. It is recommended as a shade tree for pastures and a windbreak for tea. Generally tall and branchy, this tree is of great importance in protecting the soil from water erosion. Its multi-purpose benefits as a provider of green manure, stress resistance, medicine, and its role in agroforestry make it a potential candidate for large-scale plantations on marginal lands [1]. For last few years *P. pinnata* has received considerable attention as biodiesel crops for its seed yield (10 kg–160 kg per tree) and non-edible oil content (28 %–39 %) with high percentage of polyunsaturated fatty acids. Thus *P. pinnata* is a strong candidate to contribute significant amounts of fuel feedstocks in the future [2,3]. Because of its above-mentioned environmental, medicinal and industrial

Abbreviations: BA, benzyladenine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; ISSR, inter simple sequence repeat; Kn, Kinetin; MS, Murashige and Skoog basal medium; NAA, naphthalene acetic acid; PMSF, phenylmethanesulfonylfluoride; RAPD, random amplified polymorphic DNA; WP, Woody Plant medium.

* *Corresponding author*. Tel.: +91 361 2582214; fax: +91 361 2582249.
E-mail addresses: latha_rangan@yahoo.com, lrangan@iitg.ernet.in (L. Rangan).

0961-9534/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biombioe.2012.03.029

characteristics, Government of India has drawn ambitious programmes for its large-scale cultivation.

The species is generally propagated either by seeds or by root suckers, but the seed germination rate is very slow, and progenies are of highly heterozygous. The major drawback of this tree species is that they exhibit a very long flowering cycle of 4 years–7 years depending on the environmental conditions. There are few reports on vegetative propagation of *P. pinnata*. Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production, and conservation of elite and rare germplasm. Approximately 20% of land in India is considered largely wasteland where little or no agriculture is practised. *In vitro* propagation in *P. pinnata* will not only speed up mass multiplication and generation of superior planting material of true-to-type plants but can also aid in complementing the ongoing activities of conventional propagation to raise its cultivation in the wasteland or unproductive land. To meet the future demands for biodiesel, establishing extensive plantations comprising high-yielding elite lines through *in vitro* technique will offer the best possibility towards obtaining strands of homogeneous clones. Thus immense potential lies for improvement of productivity by selection of appropriate elite planting materials of *P. pinnata*. Such studies will help in future with regard to genetic engineering and improvement of the biodiesel crops for the desired traits. There are reports on *in vitro* shoot regeneration in *P. pinnata* [4,5] but the difficulty has been to establish good regenerating material in a suitable medium. Even attempts at using axillary meristem have faced problems at the multiplication stage. The inherent problem associated with commercially valuable tree species *P. pinnata* is to establish good axenic cultures that are genetically identical to the donor plant. It is important to first establish the suitability of a particular micropropagation protocol developed for a specific clone with respect to the production of genetically identical and stable plants before it is released for commercial purpose.

The incidences of somaclonal variation amongst subclones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues or organs can seriously limit the broader utility of micropropagation systems [6,7]. Several techniques have been developed to assess the genetic purity of tissue culture-raised plants such as phenotypic traits, physiological supervisions, cytological studies, isozymes [8], field assessment and molecular studies [9]. Somaclonal variation mostly occurs as response to the stress imposed on the plant in culture conditions and is manifested in the form of DNA methylations, chromosome rearrangements, and point mutations [10]. Molecular techniques are at present powerful and valuable tools used in the analysis of genetic fidelity of *in vitro* propagated plants. Several DNA markers have been successfully employed to assess the genomic stability in regenerated plants including those with no obvious phenotypic alternations [11]. Among the markers, inter simple sequence repeat (ISSR) [12] and random amplified polymorphic DNA (RAPD) [13] have been mostly favoured and well established in many tree species.

There has been no information on the comparative standardization of Woody Plant (WP) media [14] and Murashige and Skoog's (MS) media [15] for high efficiency *in vitro* propagation of elite genotypes of *P. pinnata*. However, the vegetative propagation and *in vitro* regeneration through stem cuttings and axenically grown cotyledonary meristem in MS media has been reported in *P. pinnata* [4,5,16,17]. Also no studies were reported so far on genomic stability or variation of *P. pinnata* micropropagated plants. In current study, we report an efficient *in vitro* propagation of elite genotype of *P. pinnata* with the following objectives: (1) to mass multiply using different explant sources and, (2) to establish diseased-free materials for further studies. We investigated the effect of WP media [14] traditionally used in the multiplication of tree species, and MS media [15] for multiplying *P. pinnata*. A comparison of initiation and multiplication of different explants in WP and MS media with different hormone combinations were also attempted in this study. In addition, we performed RAPD and ISSR analysis of the micropropagated plants for genetic stability assessment.

2. Materials and methods

2.1. Plant material

Seeds and nodal segments of elite mature plant (CPT, candidate plus tree) of *P. pinnata*, (NGPP 46, North Guwahati *P. pinnata*) were harvested from Sila Forest Range, North Guwahati, Assam (latitude 26°14′6″N and longitude 91°41′28″E). In our previous study for selection of CPTs, 10 locations (each representing one population) with 5 random trees (total of 50 trees named NGPP 1 to NGPP 50) from each location were scored for various morphological and reproductive characters using a combined analysis program of CROPSTAT [3]. Individuals performing above average for 75% of characters were tagged as the CPT in the first-stage analysis. In the second-stage analysis, based on pod-seed traits and oil content, the best individual CPT, NGPP 46, was identified [3]. Tendril nodal explants of new actively growing shoots were harvested from the mature elite tree NGPP 46 during three different seasons of the year 2008–2009 *viz.*, spring (February–May), rainy (June–October) and winter (November–January) whereas seeds were harvested during the month of October–December, 2008 when they are in the middle to late immature stage (green seeds). Seeds and nodal segments of NGPP 46 used as the starting material were collected from the study site at 8 am–10 am in the morning and transported to the laboratory in the sealed polythene bags and immediately used for micropropagation experiment. In the laboratory, all the extraneous leaves were removed from the fresh tendril nodal explants and cut into pieces (3 cm–4 cm), each having 1–2 axillary buds. The seeds and tendril nodal explants (3 cm–4 cm) were washed under running tap water and subsequently treated with the volume fraction of 2% Tween 20 for 5 min and rinsed with water until all traces of detergent were removed. Establishment of the *in vitro* cultures in *P. pinnata* posed considerable problems with contamination in primary cultures, which reappeared even after repeated subculturing. The problem was overcome by treating the explants with 1 g L^{-1} of bavistin, a systemic fungicide (BASF, India) for 2 h and then with the volume fraction of 70% ethanol for 2 min before surface-sterilizing with 1 g L^{-1} of mercuric chloride for 7 min,