Research & Reviews in Biotechnology and Biosciences
Volume-1, Issue-2, Year-2014
PP: 17-23 ISSN No: 2321-8681

Margosa tree Germplasm conservation through anther-derived organogenic callus masses

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Abstract

In several mature trees like Margosa, long- term conservation through seeds is not possible as they are highly susceptible to various pathogens and are spoiled within a few weeks during storage. Micropropagation too is difficult due to lack of any suitable juvenile explant and presence of polyphenols. Most of the explants such as leaf, stem pieces and petiole segments are recalcitrant for in vitro differentiation. Genetic improvement of such species can not be performed till a suitable regeneration protocol is established. Anthers being more juvenile as compared to other explants, respond quickly to in vitro regeneration techniques under suitable physico-chemical environment. In the margosa tree (Azadirachta indica A, Juss, Family Meliaceae) young anthers containing sporogenous tissues produced sufficient amounts of organogenic callus on various media supplemented with plant growth regulators in proper concentration and combinations. The browning of the callus could be inhibited with the help of Polyvinylpyrrolidone (PVP), or activated charcoal or L-cysteine. Whenever needed multiple shoots were differentiated by subculturing yellowish green nodular callus masses on media possessing one cytokinin (BA or Kn) and one auxin (IAA, NAA, or IBA) in combination. The regeneration potential of the callus was maintained for several years. The morphogenetic response from subcultured callus masses was in the form of caulogenesis, rhizogenesis and embryogenesis according to composition of culture media. The regenerated plants showed minimum variability in their morphological characters.

Key words: Azadirachta indica, callus, somatic embryos, subcultures

Introduction

Most of the trees are mainly conserved through seed storage practices as seed banks using proper insecticides. Some trees are mainly propagated through seeds as vegetative propagation is a very difficult and tedious process. Margosa (*Azadirachta indica*) trees are popularly known as neem in India and belongs to the family Meliaceae.It is very famous medicinal plant and used in agriculture, medicine,public health, toiletries, cosmetics, soap industry and livestock production (Solanki and Gupta, 1996). Neem is propagated through seed germination during the rainy season in North India. The morphogenetic potential of anthers for the induction of haploids and differentiation of plantlets from anther-derived callus masses during subsequent sub-culturings has been reported (Gautam *et al*.,1993). The major hurdle in performing experiments was that flowering was available only for a limited period in a year, i.e. flowering months mid May to mid June. Therefore, only limited experiments could be

performed. Whenever seeds were collected and stored during the month In July for raising *in vitro* seedlings for experimenting with juvenile explants, it was a failure as seeds could not be stored for long periods because they were highly susceptible to various pathogens. The micropropagation from *in vivo* mature explants such as stem pieces and young leaves,etc was also very difficult due to presence of many phenolic compounds which are known to be inhibitory for *in vitro* organogenesis.Therefore, we developed a method for conserving and micro propagating these trees through anther-derived callus masses. These callus masses were maintained and multiplied for several years without any decrease in their morphogenetic potential. During subsequent subculturing multiple shoots were produced. The large scale production and multiplication of callus would be beneficial for the bioreactor program used by pharmaceutical companies.

Materials and Methods

Two mm long flower buds of *Azadirachta indica* were collected from a flowering tree during April-May months growing in Delhi University Campus. At this bud length , all anthers of a bud contain sporogenous tissue or in some cases uninucleate pollen grains. The anthers were surface sterilized with saturated chlorine water for 15-20 minutes and subsequently washed three times with sterilized distilled water for 5 minutes each. Anthers were excised and inoculated under aseptic conditions on NB(Nitsch medium, 1969) and MS(Murashige and Skoog, 1962) medium containing 3% sucrose(BDH), 0.8% agar(Difco-Bacto) and various auxins such as 2,4-D, IAA, NAA as well as cytokinins like BA and Kinetin, either alone or in various combinations.. In each subculture callus masses were transferred to fresh MS media augmented with growth regulators. In the second subculture 5% coconut milk was added to enhance callus amount. In other subcultures PVP at 250-1000 mg/L was also added to reduce browning of calli. The pH of media was adjusted at 5.8 before autoclaving. All cultures were incubated under continuous cool, white light produced by Phillips fluorescent tubes of 40 watts at 25-27 0C and 45-55% relative humidity.

Results and Discussion

Anthers containing sporogenous tissue or uninucleate pollen grains produced frable greenish yellow callus masses on both NB and MS media supplemented with growth regulators. However better response in terms of callus masses and percentage were on MS medium. The superiority of MS medium for organogenesis under hormonal influences has been recorded earlier also in some trees such as *Populus* spp. (Lu *et al*.,1978) as well as *Carica papaya* (Litz,1984). The callusing was observed from several media but good amount of green nodular callus was obtained on MS+ 1.86mg/l NAA+0.21mg/l Kn, MS+1.86 NAA+ 0.22 mg/lBA and MS+1.7mg/l IA A+.22mg/lBA media within 5-6 weeks. After 13-15 weeks green nodular structures and multiple roots developed in 25% cultures on MS+1.86 mg/l NAA+ 0.21 mg/ l NAA + 0.21 mg/l Kn medium. The nodular calli were transferred to the shoot induction medium. During the first subculture, the calli grew appreciably on MS+1 mg/l BA+0.1 mg/l NAA medium. In addition to callusing, 5 % cultures also differentiated roots. Sometimes when calli were subcultured on MS+ IAA (0.5 and 1 mg/l), MS+NAA (0.5 and 1 mg/l), or MS+ 1 mg/l NAA+ 0.1mg/l BA roots developed . In some experiments , callus masses maintained on MS+ 1 mg/l BA +0.1 mg/l NAA medium differentiated nodular shoot buds as well as globular to heart shaped embryos after second subculture in either MS+0.5mg/l BA or MS+ 1

mg/l BA+ 0.1 mg/l NAA medium (Fig.1). The number of embryos per culture varied from 2-3 and 5-6% cultures showed embryo differentiation. As expected from tree tissue cultures, on prolong culturing before 4-6 weeks, calli started turning brown due to release of polyphenols in the medium, which ultimately ceased differentiation. To prevent browning callus masses were transferred to fresh medium containing PVP, or L- cysteine or activated charcoal. Of the various concentrations tried, 750 mg/l PVP proved most satisfactory in which callus masses remained healthy, green, nodular and showed vigorous growth along with embryo formation as well as multiple shoots differentiation (Figs 2& 3). These organogenetic callus masses could be maintained for several months without any loss in their morphogenetic potential, thus can be the best material for conserving germplasm of this tree for a long period. L-cysteine-HCL was used at 5-20mg/l to check browning of anthers in MS+1.86mg/l NAA+0.21mg/lKn medium. At 20 mg/l, it was most effective in yielding 41% callusing (Fig.4), but did not check browning effectively.

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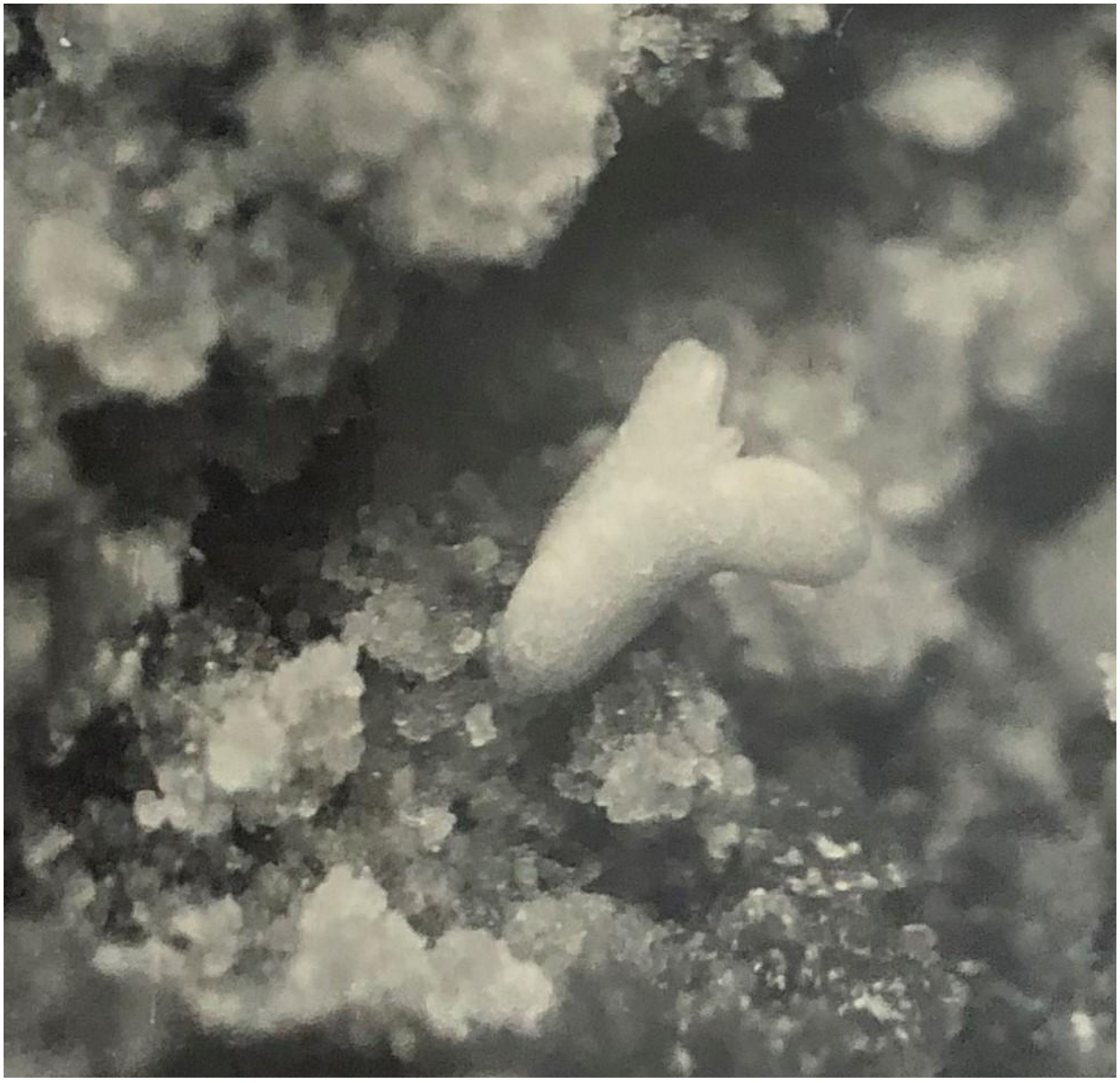

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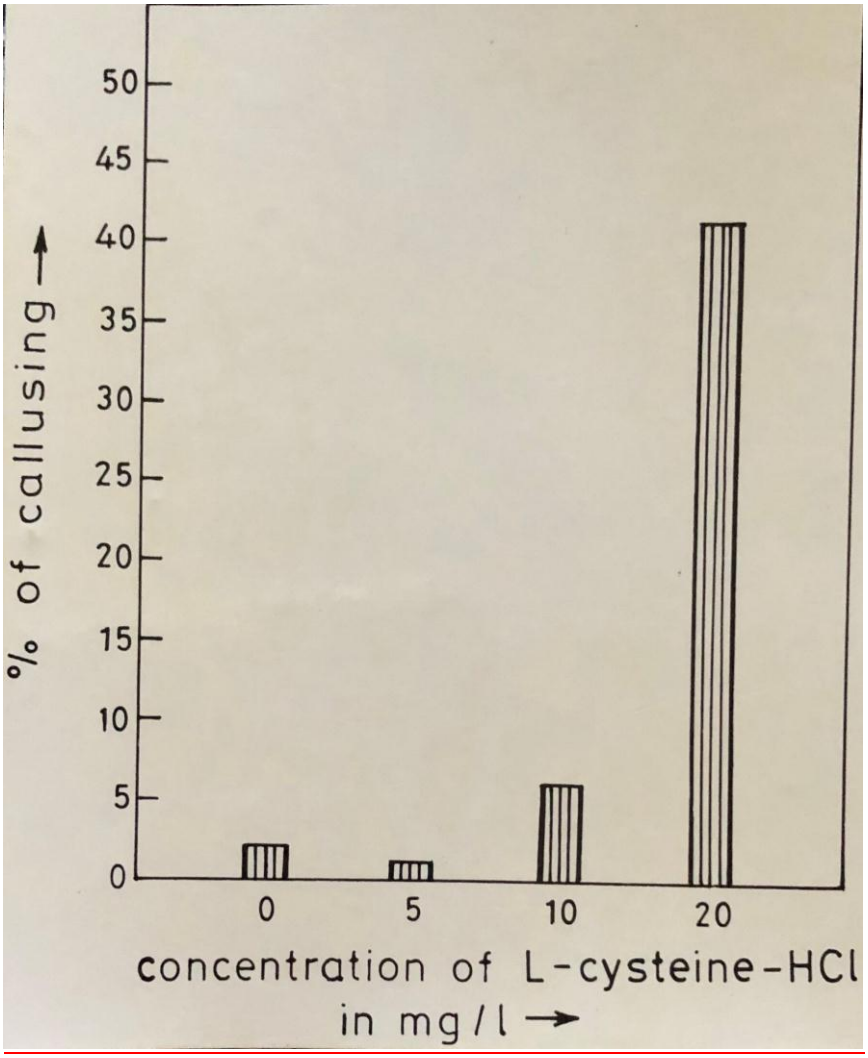

Figure 1 : Callus differentiating embryo after 63 days subculture on MS+1mg/l BA+0.1mg/l NAA X 6.

Figure 2 : Organogenic callus maintained on MS+1mg/l BA+0.1mg/l NAA+750mg/l PVP for nearly two years showing shoot differentiation. X 2.5.

Figure 3 : Multiple shoot differentiation (arrows) on medium mentioned in Fig 2 after51 days of subculture. X 3.

Figure 4 :Histogram depicting effect of L-Cysteine-HCL on anthers cultivated on MS+ 1.86mg/l NAA + 0.21mg/l Kn medium for 86 days.