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PRELIMINARY CONSERVATION EFFORT ON *RHIZOPHORA ANNAMALAYANA* KATHIR., THE ONLY ENDEMIC MANGROVE TO INDIA, THROUGH *IN VITRO* METHOD

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Abstract: An efficient protocol was established for *in vitro* clonal propagation for *Rhizophora annamalayana* Kathir. the only endemic mangrove species to India. Initially the explants were surface sterilized appropriately with 0.1% mercuric chloride for one minute, 70% ethanol for 30 seconds and 3% hydrogen peroxide for one minute. Then the explants were treated with three different antioxidants for reduction of phenol browning of the explants. Among the antioxidants, 10 mg/L ascorbic acid was found to be the best. Among the five different tissue culture media - B5, WPM, MS, SH and Y3- tested, MS medium was chosen to be the best for meristem culture. Among the growth regulators, cytokinins (benzyl adinine, kinetin and zeatin) used alone and in combination with auxins (naphthalene acetic acid, indole acetic acid, indole butyric acid), the shoot growth was better observed after 20 days when MS medium was incorporated with 3.0 mg/L of benzyl adinine and 3.0 mg/L of kinetin with coconut milk. This is the initial step of tissue culture for the recovery of the fast disappearing *Rhizophora annamalayana* Kathir. Further research is progressing on mass multiplication and field transfer.

Keywords: mangroves, *in vitro* propagation, growth regulators, *Rhizophora annamalayana*.

Introduction

Rhizophora annamalayana Kathir. is fast disappearing mangrove species and is the only species endemic to India (Fig. 1a), restricted to Pichavaram mangrove forests of Southeast coast of India. This species faces serious problem of poor flowering, fall of flower buds and extremely poor seed setting making natural regeneration of the species very difficult [KATHIRESAN, 2000]. Since the rate of species recovery by conventional method is less effective, the present work attempted micropropagation of the species through tissue culture, which is a universally accepted alternative method to save and recover the species. The initiative has been taken by the author, one who has identified the species (Fig. 1b). Such attempts in mangroves have already been made, but with little favorable effect: callus initiation from the leaf explants of *Bruguiera sexangula* (Lour.) Poir. on MS medium supplemented with amino acid, 2 µM 2,4-dichlorophenoxy acetic acid and 2 µM N- (2-chloro-4-pyridyl)-N-phenyl urea [MIMURA & al. 1997; VANDER VELDE & VANDER VELDE, 2005]. However, an attempt was made here in *Rhizophora annamalayana* Kathir. for the first time. For the flourishing tissue culture practice, standardization of several aspects is required for culture media, hormones, prevention of phenolic browning of explants and microbial contaminations. Hence, the present

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investigation was made to optimize such requirements for successful tissue culture operations.

Materials and methods

Sterilization of Glasswares

All the glasswares were washed thoroughly with detergent (Teepol) in tap water and rinsed with double distilled water twice and dried in an oven at 30 ºC. The distilled water and other accessories such as forceps, blade holder, cotton, etc., were autoclaved at 121 °C for 15-20 minutes. After autoclaving, they were kept in an oven until use. And aseptic transfer of tissue was done in laminar air-flow hood (Airtech, India) and its interior was swabbed with 95% ethanol before inoculation. The autoclaved instruments were flame sterilized thrice in 95% ethanol before using them for tissue transfer.

Sample collection and surface sterilization

Fresh shoot tip and leaf explants of *Rhizophora annamalayana* Kathir., were collected from the Pichavaram, Southeast coast of India. The explants were washed thoroughly in water immediately after collection and treated with 10 mg/L of three antioxidants - citric acid, polyvinyl pyrolidone and activated charcoal separately for prevention of phenolic browning of explants. Then the explants were washed in 0.1% mercuric chloride followed by 3.0% hydrogen peroxide and 70% ethanol. Thereafter the explants were disinfected with a detergent solution (2% Teepol, Reckitt and Colman, India) for 10 min.

Stock solution preparation

Among the five different tissue culture media (B5, WPM, MS, SH and Y3) tested, MS medium was chosen for meristem culture. For the preparation of MS basal salt medium [MURASHIGE & SKOOG, 1962], separate stock solution of macronutrients, micronutrients, iron supplements, vitamins and amino acid were prepared by dissolving required amounts of chemicals in double distilled water and were stored at 4 ± 1 °C in a refrigerator. Individual growth regulators such as benzyl adenine, kinetin and zeatin alone or in combinations with different auxins like naphthalene acetic acid, indole acetic acid, indole butyric acid were also prepared and kept at 4 ± 1 °C.

Media preparation

For preparing the MS medium, all the stock solutions were taken in appropriate proportions and the final volume was made up to required quantity by adding double distilled water. Sucrose was added at 3% (w/v) to the medium as a source of carbon. Various concentrations and combinations of growth regulators were added to the medium before adjusting the pH to 5.8 using 1.0 N NaOH or 1.0 N HCl and solidified with 0.8 % agar. The culture media with all necessary ingredients were dispensed into culture tubes, conical flasks and phyta jar and sterilized. The autoclaved media were kept in inoculation room until use.

Aseptic transfer of explants

After sterilization, all the explants were cut into small pieces (1.0 to 1.5 cm long) and were individually placed on 25 x 150 mm culture tubes (Borosil, India) containing 15 ml of MS medium supplemented with various concentrations and combinations of cytokinins and auxins along with 3.0 % sucrose and 0.8 % agar (Himedia, Mumbai, India).

Micropropagation

The MS medium was prepared and to it different antioxidants {ascorbic acid (w/v) [5-15mg/L]; polyvinyl pyrolidone (w/v) [5-15mg/L] and activated charcoal (w/v) [5-15mg/L]} were added separately, solidified with 0.8% agar (Himedia, India) and the pH was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min at 15 lb. The medium was poured in to phyta jar (25 ml/jar). After the aseptic transfer of explants, the phyta jars were incubated at 25 ± 2 °C for a 16:00 h photoperiod and observations on browning and percentage survival of explants were recorded.

Effect of media on shoot induction

Shoot-tip and leaf explants were cultured on test tube containing 15 ml of culture media of B5, WPM, MS, SH and Y3 (Annexure-1). All the cultures were incubated at 25 ± 2 °C under continuous irradiation with a white fluorescent tube (30 µmol $m^{-2}s^{-1}$) for a photoperiod of 16:00 h light per day. A total of 40 explants were used for the experiment and was repeated thrice. The culture conditions remained the same for all experiments unless and otherwise specified. Data on per cent response with number of shoot formation per explants was recorded after 25 days of culture.

Effect of cytokinin and auxin combinations on shoot induction

The leaf and shoot-tip explants were cultured on MS basal medium supplemented with 0.5 to 5.0 mg/L of individual cytokinins such as benzyl adenine/zeatin/ kinetin in combinations with 0.1–0.7 mg/L of naphthalene acetic acid or indole acetic acid in addition with coconut milk. Sub-culturing was done after two week interval. The culture conditions stated above remained the same for all experiments unless and otherwise specified. A total of 20 explants were taken for each experiment and were repeated thrice. Results were recorded on number of shoot formed per explants after 21 days of culture.

Results and discussion

The present investigation was aimed at *in vitro* shoot multiplication using shoot tip explants of *Rhizophora annamalayana* for its micropropagation as the first attempt. A major recurring problem which confronts tissue culture especially of tree species is the exudation of phenolic substances from the cut surface of the explants. As a result, the medium turns dark brown in colour due to oxidation of phenolics which becomes toxic to the explant tissue leading to its death under tissue culture conditions. Naturally phenolic compounds are abundant in plants, particularly in woody species, playing an important role in hormone balance, disease resistance and protection of injured tissue from infection [COMPTON & PREECE, 1986]. In the present study, MS medium without coconut milk showed the high phenolics in shoot explant and this problem was totally overcome when the medium was incorporated with 1000 µl/L of coconut milk and 10 mg/L of activated charcoal. However, other antioxidants such as ascorbic acid and polyvinyl pyrolidone showed the poor response and high phenolic exudation.

Prevention of microbial contamination in plant tissue culture is critical for effective micropropagation. Epiphytic and endophytic microorganisms can cause severe losses to micropropagated plants at each stage of growth [CASSELLS, 1991; DEBERGH & VANDERSCHAEGHE, 1988; LEIFERT & al. 1991]. In order to arrest the microbial growth, the time duration of surface sterilization using different chemicals such as mercuric

chloride, ethanol and hydrogen peroxide were optimized to prevent microbial contamination in the explants. The surface sterilization was optimized to arrest the growth of microbial contamination for the explants when treated with 0.1% for one minute in mercuric chloride, 30 seconds in 70% ethanol and one minute in 3% hydrogen peroxide.

The addition of growth regulators in MS medium induced microshoot formation in *Rhizophora annamalayana* from the shoot-tip explants (Fig. 1d) cultured on MS basal salts, 3.0% sucrose and 1000 µl/L of coconut milk and 0.8% agar medium supplemented with individual hormones such as benzyl adenine, zeatin and kinetin. The explants responded to bud sprouting after 7 days (Fig. 1e).The microshoot was observed from 3.0 mg/L of benzyl adenine and 3.0 mg/L of kinetin and 1000 µl of coconut milk. Shoot-tip explants responded well with high frequency (70%) of shoot induction (Fig. 1f). Leaf (Fig. 1c) explants did not show any microshoot induction in all the treatments of growth regulators.

Conclusions

In order to recover the mangrove species *Rhizophora annamalayana* Kathir, the present work was attempted through tissue culture. Microshoot formation was achieved in the species by 70% using shoot tip as explant when the MS medium incorporated with 3.0 mg/L of BA and 3.0 mg/L of kinetin with 1000 µl of coconut milk. The culture techniques were standardized for surface sterilization and prevention of phenolic browning and microbial contaminations of the explants. The results are promising for micropropagation of the rare mangrove species.

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Fig. 1. a. *Rhizophora annamalayana* from the habitat, b. a rare propagule of *Rhizophora annamalayana* Kathir., shown by the author (Prof. K. Kathiresan), c. leaf explant on MS medium, d. meristem tissue on MS medium, e. bud sprouting from meristem tissue, f. shoot bud sprouting after 20 days.