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High *in Vitro* Production of Secondary Metabolites or Regeneration of *Catharanthus roseus* Plant by using Plant Tissue Culture Technique

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Abstract

Catharanthus roseus (periwinkle) is one of the most important medicinal and ornamental plants in the world. In this research, we will study about plant tissue culture of *Catharanthus roseus* and regeneration of plant to produce primary and secondary metabolites. An efficient protocol was standardized using axillary bud and shoots tip explants of *Catharanthus roseus*-an anticancer medicinal plant. In this investigation, periwinkle seeds, after sterilization were cultured on MS medium. Petiole segments of seedlings (4 day old) were su cultured to medium containing various concentrations of NAA accompanied with Kin and sub cultured t regenerate the callus and root. Callus and roots were obtained from petioles in some of treatments. The extracts of callus and roots from different treatments were analysed by spectrophotometer, TLC and HPLC with respect to the indole alkaloids producing capacity. Most of the secondary metabolites were produced in new roots and callus roots.

Keywords: Plant tissue culture • Primary metabolites • Secondary metabolites • *Catharanthus roseus*

Introduction

Catharanthus roseus (L.) belongs to the family *apocynaceae* and commonly known as sadabahar or periwinkle which originates from Madagascar. It is one of the important medicinal and ornamental plants in the world. The periwinkle is source of several commercially valuable secondary metabolites which are used by the pharmaceutical industry for the treatment of cancer and cancerous tumors. More than 400 alkaloids including vincristine, vinblastine, resperine, ajmalcine, vinceine and raubasin etc. are known in Catharanthus roseus and have their importance medical field.

In Plant tissue culture technique, plant tissues and organs are grown *in vitro* on artificial media, under aseptic and controlled environment. The technique depends mainly on the concept of tot potentiality of plant cells which refers to the ability of a single cell to express the full genome by cell division. Along with the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant. Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium. Murashige and Skoog medium (MS medium) is most extensively used for the vegetative propagation of many plant species *in vitro*. The pH of the media is also important that affects both the growth of plants and activity of plant growth regulators. It is adjusted to the value between 5.4-5.8. Both the solid and liquid medium can be used for culturing. The composition of the medium, particularly the plant hormones and the nitrogen source has profound effects on the response of the initial explant.

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Establishment of plant tissue culture

In vitro culturing of plant tissue culture involves the following steps

- Collecting and sterilization of glassware tools/vessels
- Preparation of explants
- Surface sterilization of explant
- Production of callus from explants
- Proliferation of culture
- Sub culturing of callus
- Suspension culture

Materials and Methods

Growth regulators required for plant tissue culture

Plant growth regulators (PGR's) play an essential role in determining the development pathway of plant cell and tissues in culture medium. The auxins, cytokinins and gibberellins are most commonly used plant growth regulators. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue organ cultured and the objective of the experiment Auxins and cytokinins are most widely used plant growth regulators in plant tissue culture and their amount determined the type of culture established or regenerated. The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus. Hormones such as BAP (Benzylaminopurine) and NAA (Naphthalene Acetic Acid) to stimulate growth and root development, respectively (Table 1).

Compounds	Mg/Ml
KNO_3	1,900.00
$CaCl_2$ (anhyd)	332.20
$MgSO_4$ (anhyd)	180.70
KH_2PO_4	170.00
Na_2EDTA	37.25

$FeSO_4.7H_2O$	27.80
H_3BO_3	6.20
$MnSO_4.H_2O$	16.90
$ZnSO_4.H_2O$	5.37
KI	0.83
$Na_2MO_{4\cdot2}H_2O$	0.25
Sucrose	30,000.00
i-Inositol	100.00
Thiamine.HCl	0.40
NH_4NO_3	1,650.00

Table 1. Inorganic and organic supplements.

Antibiotics required for plant tissue culture to avoid infection in plant

- Streptomycin
- Kenamycin
- Activated charcoal

Other organic supplements

- Protein
- Coconut milk
- Yeast
- Malt extract
- Orange juice and tomato juice

Water

- Demineralized or distilled water

Solidifying agents

- Agar
- Gelatin

Results and Discussion

Sterilization of media

The prepared media should be sterilized by ISI mark Autoclave (for large amounts) by 121° or Domestic pressure cookers (for small amounts). For the sterilization of glassware and metallic equipment's Hot air oven with adjustable tray is required (Figure 1).

Figure 1. For the sterilization of glassware and metallic equipments Hot air oven with Adjustable tray is required.

Explant preparation

Explants of *Catharanthus roseus* was taken from the laboratory of Madhya Pradesh Council Science and Technology Laboratory, Bhopal.

Preparation and sterilization of growing medium when not provided pre-poured. These steps will make 1 L of growth medium, which is enough to prepare about 65 growing tubes.

• MS mixture was dissolved in about 800 ml of distilled water. Stir the water continuously while adding the salt mixture. 30 g sugar was added and stirs to dissolve. PH was adjusted to 5.8 using 1M Na Oh or 1M H Cl as necessary while gently stirring. Then distilled water was added to make the total volume up to 1 litre.

• Weigh out 8 grams of agar and add it to the MS solution. Heat the solution gently while stirring until all the agar has dissolved.

• Keeping the mixture warm so that it does not solidify, divide it equally into two prewarmed containers. Each container can be used to prepare about 30 tubes as above.

• The first container had BAP added at the rate of 2.0 mg/l. The second container had the NAA hormone added at the rate of 0.1 mg/L. To do this, it is necessary to make concentrated solutions of both BAP (2.0 mg/ml) and NAA (1.0 mg/ml). Then 1 ml of the concentrated BAP was added to each 1 liter of medium that was prepared..

This study used a well clinically defined 5-year retrospective data set from HIV seropositive subjects with and without ART alongside with both their baseline and control data set, which are void of all possible confounding factors. The data consist of serum protein levels, which are mostly markers of hepatic damage and measured in a cohort of well clinically defined HIV-1 mono-infected patients with and without ART and attending University of Maiduguri Teaching Hospital, Nigeria as well as the control group. The study accesses the impact of ART, the likely pathophysiology in subjects receiving ART and those not on ART as reflected on the serum protein level as well as the potential of these serum proteins in discriminating between the study groups.

Preparation of a sterile transfer chamber and equipment

A transfer chamber was made from a clean glass aquarium turned on its side. Scrub the aquarium thoroughly with a 30% bleach solution. Rinse with sterile distilled water, turn upside down on a clean counter or paper towels and allow drying. Cut holes in a clean plastic sheet to allow arms to reach into the chamber and reinforce the cut edges with tape if necessary. Tape the clean plastic sheet over the open side of the aquarium making sure that the arm holes are located at a convenient height. Plastic sleeves could also be fitted to these holes to make it easier to prevent the entry of airborne spores into the chamber. The finished aquarium chamber was also sterilized by spraying with 10% chlorox bleach just prior to each use and drying with sterile paper towel.

Wrap the forceps, scalpels, razor blades, paper towel and gloves (rubber or surgical) in aluminium foil, seal with tape and sterilize by processing them in a pressure cooker for twenty minutes. These items were also sterilized by placing in an oven at 350°F for 15 minutes.

Alternatively the forceps and blades was sterilized by dipping in 10% bleach and then rinsing in sterile water, or dipping in alcohol and then placing in a flame. If we choose to dip in bleach and rinse in sterile water, it is best if fresh solutions are available since the water can easily be contaminated if care is not used. These liquid containers should only be opened once they are inside of the sterile chamber.

Plant preparation

Plant material must first be surface-sterilized to remove any bacteria or fungal spores present. The aim is to kill all microorganisms, but at the same time not cause any adverse damage to the plant material.

• *Catharanthus roseus* should be cut into small sections of florets about 1 cm across.

• The prepared plant material was washed in a detergent-water mixture for about 20 minutes.

• The washed plant material was transferred to the sterilizing chlorox solution. Shake the mixture for 1 minute and then leave to soak for 10-20 minutes. Carefully pour off the bleach solution using the lid to keep the plan tissue from coming out and then carefully cap the container.

Transfer of plant material to tissue culture medium

• The plant material was placed in the chlorox bleach sterilizing container, the containers of sterile water, the sterilized forceps and blades, some sterile paper towel to use as a cutting surface and enough tubes containing sterile medium into the sterile aquarium. The outside surfaces of the containers, the capped tubes and the aluminium-wrapped supplies should be briefly sprayed with 70% alcohol before moving them into the chamber.

• The gloves was sprayed with a 70% alcohol solution and hands rubbed together to spread the alcohol just prior to placing hands into the chamber.

• The container with the plant material was opened carefully and enough sterile water was poured in to half fill the container. Replace the lid and gently shake the containe to wash tissue pieces (explants) thoroughly for 2-3 minutes to remove the bleach. Pour off the water and repeat the washing process 3 more times.

• The sterilized plant material from the sterile water was removed and placed on the paper towel or sterile Petri dish. Cut the *Catharanthus roseus* into smaller pieces about 2 to 3 mm across.

• A prepared section of plant material in sterile forceps was taken and placed into the medium in the polycarbonate tube. *Catharanthus roseus* pieces should be partly submerged in the medium, flower bud facing up.

• The cap was replaced tightly on the tube (Figure 2).

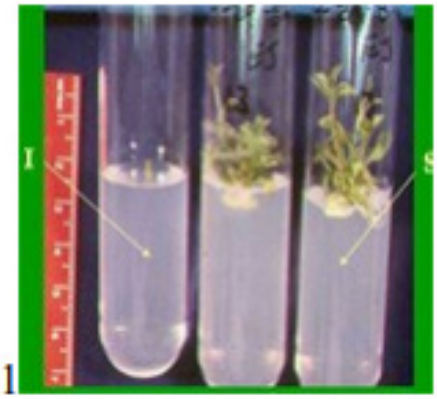

Figure 2. The plant material was placed in the chlorox bleach sterilizing container.

Growing the plants

• The tubes containing plant sections may be placed in a well-lit area not in direct sunlight. The shoots will probably grow more quickly if the explants are placed under fluorescent or grow-lights to provide at least 12 hours of light per day. The aquarium can be used as a growth chamber with the lighting about 20-25 cm (8-10") overhead. This will also help maintain a more regular and warm temperature. Ensure that the temperature does not go over 28°C. New shoots should develop within 2 weeks, and should be well advanced in 3 to 4 weeks. The tubes were checked daily and discard any signs of contamination (before discarding, first sterilize in the pressure cooker or add bleach into the tube).

• Roots can appear within 6 weeks on catharanthus. This transfer to the second, rooting medium must be conducted under the same sterile conditions as at the initiation of the culture. All necessary equipment and the aquarium should be set up as before and properly sterilized

• Working inside the sterile aquarium chamber, the cap from the culture tube was removed There will usually be several shoots that have arisen from each explant. These shoots should be carefully separated by gently removing the whole explant from the medium with sterile forceps and then separating the shoots by gently pulling them apart using two pairs of forceps. Each shoot was then placed into a tube of rooting medium and the bottom of the shoot pushed into the medium so that good contact is made. The cap was replaced and the shoots were then allowed to grow as in step 1 until roots are formed, usually within 2-3 weeks.

Potting the clones

• Once roots are well formed the plants are ready to be transferred into soil (Figure 3).

Figure 3. Roots are well formed the plants are ready to be transferred into soil.

• Each plant was carefully removed from its tube of medium and planted into a small pot containing a clean light potting mix. The agar medium was gently washed prior to planting. The plants will still need to be protected at this stage since they are not acclimated to the drier air when compared to the moist environment of the tube.

• All of the pots onto a tray was placed and cover loosely with a plastic dome or tent. The plants in an area was placed with 12-16 hours of light (either natural or artificial) but not direct sunlight.

• After a week, the cover can be gradually removed and the plants acclimated to stronger light and drier atmospheric conditions [1-8].

• We now have a collection of plants that are genetically exactly the same. We could use these plants to carry out other experiments knowing that one common source of variation in the experiment has been eliminated. Some of these tests could include looking at plant responses to low light levels, to drought, or to saline soil conditions (Figure 4).

Figure 4. The regenerative phase of C roseus, Note: 1: Sowing in MS medium; 2: Callus induction in MSCP1 medium; 3, 4: Shoots initiation in MSCP2 and MSCP3 medium separately). B Shooted plantlet. C Rooted plantlet D, E Transgenic plantlets in soil. F Flower seedlings. Scale bar=3 cm in A, 1 cm in B, F, 2 cm in C and 1.5 cm in D.

Conclusion

Various plants of *Catharanthus roseus* were observed or regenerated for the production of primary and secondary metabolites. C. roseus can be very harmful whenever devoured orally by people, and is referred to in the Louisiana State Act 159. All pieces of the plant are toxic. On utilization, manifestations comprise of gentle stomach cramps, cardiovascular difficulties, Hypotension, Systematic paralysis eventually leading to death.

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