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Induction of Callus in Catharanthus roseus (L.) G. Don and Screening for secondary metabolites in callus cells

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Abstract

Protocol for micropropagation of a known medicinal plant Vinca (Catharanthus roseus (L.) G. Don) from axenic explants had been standardized. Internodes and shoot tips were cultured on MS medium containing a varied concentration of IAA, 2-4 D, NAA, BAP and Kinetin in combination were used. The best combination of growth regulators for callus induction was at 1 mg L-1 kinetin with 0.9 mg L-1 of IAA, closely followed by 1.0 mg L-1 of kinetin with 1.0 mg L-1 of IAA for internodes as well as shoot tip. While for internodal culture 0.5 mg L-1 of 2,4-D and 1.5 mg L-1 BAP was optimum. A method of alkaloid extraction and assay of secondary metabolite in callus culture was accomplished. The HPLC assays method quantified major compounds of the secondary metabolism of callus cultures of C. roseus.

Introduction

Catharanthus roseus (L.) G. Don has been used as a folk remedy for diabetes in Natal and various other parts of S. Africa and also in India and Ceylon. The juice of the leaves is used as an application for wasp stings. Vinca is the commercial source of alkaloids used for cancer research. The roots contain various alkaloids like Ajmalicine, Reserpine, Serpentine, Vincristine, Vinblastin and Catharanthin etc. Beside the alkaloids possesses hypotensive, sedative and properties more than that of. They are antiviral, antibacterial, antiglycemic, anti-carcinogenic and many more (George and Eapen, 1999). All the plant parts are useful in deriving these alkaloids. The problem of cultivating & harvesting the plant as whole is time consuming. So, with the idea to overcome these problems work was undertaken to develop cell lines, which could yield considerable amount of these alkaloids without taking much efforts.

Catharanthus roseus produces widely used alkaloids such as the anticancer drugs vinblastine and vincristine, as well as the antihypertensive compounds ajmalicine and serpentine. Catharanthine, tabersonine, lochnericine. To study the overall secondary metabolism of C. roseus, HPLC method was proposed to quantify these compounds in a crude extract of C. roseus (Dagnino et al., 1996). The methanolic extract of callus could be used to analyze alkaloids and the extraction, concentration, purification and analysis can be done using this simplified method.

MATERIAL AND METHODS:

Plant Material:

Explants of *Catharanthus roseus* from young and healthy garden grown were collected. The explants were washed with clean running tap water and allowed to dry it. The apical meristem was cut along with some leaves with a sharp scalpel. Explants were surface sterilized in 10% sodium hypochlorite for 5 minutes. The explants were washed thrice with sterile distilled water and immediately used for the culture initiation.

Culture initiation:

Basal nutrient medium used for all the experiments consisted salts and vitamin of Murashige and Skoog with 3% (w/v) Sucrose and 0.7% (w/v) agar was purchased from Himedia Laboratories, Mumbai. Growth regulators were used individually or in combinations. pH was adjusted to 6.0 before adding agar. Culture tubes (25x150 mm) containing aliquots (15 ml) were autoclaved at 10 lbs for 30 min. The tubes were incubated alternate under 12 hr. light and dark photo regime (25-50 μ Em-2 s^{-1}; fluorescent tubes, Philips India Ltd, Mumbai).

Shoot initiation:

Experiments were conducted with nodes and shoot tips isolated from plants. They were tested with different concentrations of IAA (0.1 to 5.0 mgL^{-1}) 2,4-D (0.1 to 5.0 mgL^{-1}) BAP (0.1 to 5.0 mg L^{-1}) and kinetin (0.1 to 5.0 mgL^{-1}). Excised segments were placed on the medium supplemented with PGRs at varied concentrations and combinations. Cultures were incubated 12 hrs. light at 27±2 ^{0}C. Each treatment consisted of 20 to 40 replicates with one explant per culture tube and each experiment was repeated at least once.

Suspension culture:

Suspension cultures of Catharanthus roseus L G. Don were maintained as described before (Dagnino et al., 1996)and were harvested during the stationary phase.

Extraction:

Fifty mg of lyophilized dried biomass was taken in a test tube and 0.5 ml of water was added to it. The contents were extracted with 5 ml of dichloromethane by vortexing. The extract was centrifuged and the dichloromethane layer was collected, the procedure was repeated twice. The extraction with dichloromethane was repeated and dichloromethane extracts were pooled and dried under low pressure.

The biomass previously extracted with dichloromethane was reextracted with 5 ml of methanol by vortexing, centrifuged, and the methanol layer pooled. The extraction with methanol was repeated; the methanolic extracts were combined and dried under low pressure. The extract was redissolved in 0.5 ml of 1 M phosphoric acid and filtered through cotton. A volume of 100 µ1 of the filtered extract were injected into the HPLC system as described earlier (Dagnino et al., 1995)..

RESULT AND DISCUSSION:

The main objective of this study was to establish a rapid micropropagation system for *Catharanthus roseus* (L.) G. Don. Explants of mature plant, cultured on MS based medium turned brown in 5 days and necrosed in 15 days without exudation into the medium. Manipulation such as replacement of agar by liquid medium, change of light/dark region couldn't save the explants from turning brown.

Callus was induced in shoot tips of about 0.2 to 0.3 mm. length while these shoot were capable of producing shoots. Initially attempts were made to induce callus in the explants with 2% sucrose without growth regulators. However explants did not respond on the basal medium alone, though the explants did not turned brown but showed no sign of growth. Similar observations were made by Satheeshkumar and Seeni (2000).

The tip burst was Cytokinin dependent and occurred only in presence of Cytokinin or a combination of Cytokinin and other growth regulators. The combinations of plant growth regulators in the medium induced direct and indirect shoot regeneration. MS Complete Salt solution with 2% Sucrose was supplemented with wide range of concentration of Kinetin and IAA (0.1 to 5.0 mg L^{-1}). However there has no browning recorded in the lower concentration of 0.1 to 2.0 mg L^{-1} but the browning was recorded above 2.0 mg L^{-1} concentration. The response to kinetin and BAP concentration within the range of 1.0 to 2.0 mg L^{-1} was positive. The optimum growth of callus using alone the kinetin in MS salt solution was at 1.5 mg L^{-1} whereas the response to BAP as compared to Kinetin was low.

The response of IAA was also assessed in the concentration range of 0.5 to 5.0 mg L^{-1}. As there was browning recorded in kinetin, no such observations were made here. However in all range of concentration the explant remained green in colour without browning. The response to higher & lower concentration was insignificant. The optimum growth of callus alone with IAA was at 0.9 mg L^{-1}, closely followed was 1.0 mg L^{-1}. Effect of Auxin alone on shoot growth in grain legumes was reported by Kartha *et al*. (1981). Smith & Murashige, (1970) reported the variation of shoot tip growth from different species with the supplementation of Auxin in the medium. Lower concentrations of Auxin promoted the shoot growth whereas it failed in the higher concentration.

Cytokinin which were usually used for shoot tip cultures were BAP and Kinetin. The presence of BAP (0.5mg/1) in the medium could induce multiple shoots through the callus phase in the

present studies. BAP is most effective for meristem, shoot tip and bud cultures followed by Kinetin. (Mujib, 1995). Kartha (1978) reported multiple shoots from shoot tip cultures on medium containing BAP alone in some legumes.

Micropropagation occurs as in shoot tip cultures either direct or indirect shoot formation through callus phase. In the study direct shoot regeneration was of common occurrence. Media for nodal cultures are intrinsically the same as those suitable for shoot tip cultures. Addition of growth regulators either singly or combination to the medium induced shoot formation on MS+IBA (2 mg^{-1}) or NAA ($1mg^{-1}$) and BAP ($0.1mg^{-1}$) single shoot formation was resulted accompanied by rhizogenesis.

The methanolic extract of C. roseus callus culture was injected to evaluate the separation efficiency of the alkaloids. The extraction yield was measured for all the compounds detected.. Ajmalicine and secologanine were extracted from the callus culture in methanol whereas catharanthine, serpentine, tabersonine, vindoline and vincristine were extracted were extracted in dichloromethane. The chromatograms of crude extract of C. roseus exhibited pure compounds with different retention times as shown in chromatograms.

HPLC methods showed separation of eight C. roseus alkaloids in C. roseus crude extracts with dichloromethane and methanolic extract. Catharanthine, serpentine, tabersonine, ajmalicine, secologanine and tryptamine were successfully quantified in callus cell cultures, whereas vindoline, vincristine, vinblastine and tryptophan were detected in low qunatities. Separation of alkaloids was achieved in the crude plant extracts with a simple extraction procedure. The rapid quantification of many alkaloids was developed which will be helpful in future studies on the secondary metabolites and alkaloids of C. roseus callus culture.

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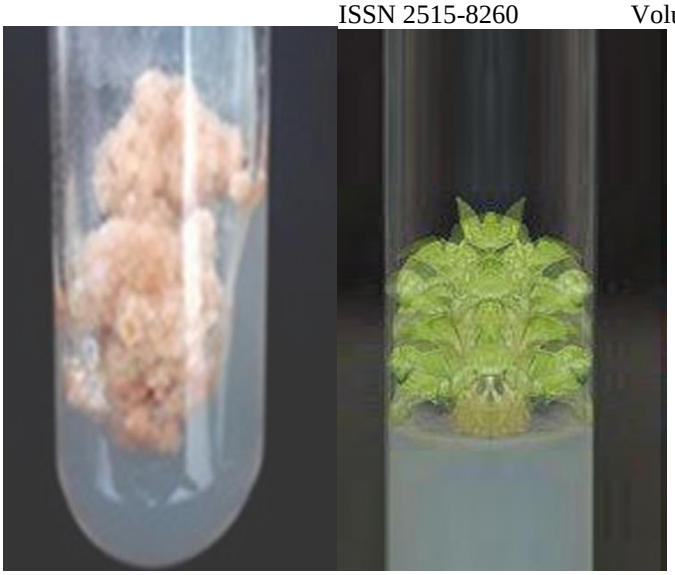
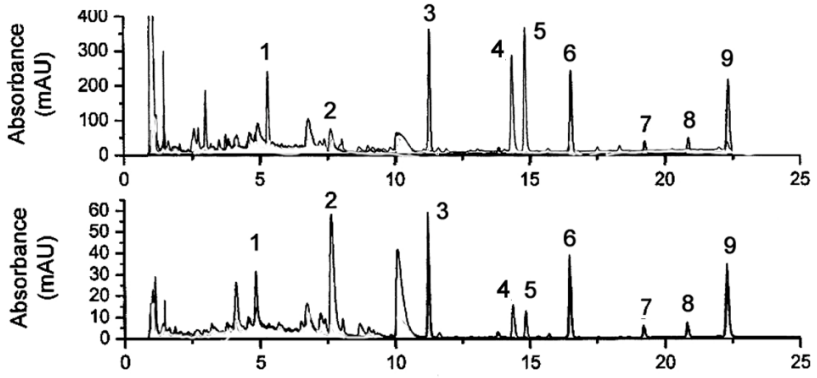


Figure 1. Development of callus and multiple shoot induction in *Catharantus roseus*



HPLC chromatograms : Methanolic extracts(Top) and Dichloromethane extract (bottom).
1.Unknown 2. Serpentine., 3. Vincristine, 4. Catharathine 5. Vinblastine 6. Unknown 7.Unknown 8. Tabersonine 9.Unknown.

Figure 2: HPLC chromatogram of the *Catharantus roseus* callus culture extract