**In vitro studies on Egyptian Catharanthus roseus (L.).**

**II. Effect of Biotic and Abiotic Stress on Indole Alkaloids Production**

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**Abstract:** Catharanthus roseus is still the only source for the powerful antitumor drugs vinblastine and vincristine. Calli Subculture had been done on MS-medium containing 1 mg/l kin. Effect of mannitol as abiotic stress at the concentrations 0, 2000, 4000 or 8000 ppm or Aspergillus niger as biotic stress at the concentrations 0, 0.05, 0.15 and 0.25 % on calli growth parameters, achievement and production of vinblastine and vincristine was investigated. Supplementation of MS-medium with 8000 ppm mannitol or 0.25 % of Aspergillus niger resulted the highest value of total alkaloids, vinblastine and vincristine production. The best results of calli growth parameters as well as enhancement the biosynthesis of indole alkaloids were recorded with leaf, stem and root calli cultures, respectively.

**Key words:** Catharanthus roseus, calli cultures, biotic and abiotic stress, vinblastine and vincristine

**INTRODUCTION**

Plant cells are considered to be excellent producers of a broad variety of chemical compounds. Many of these compounds are of high economic value such as various drugs, flavors, dyes, fragrances and insecticides. These compounds usually play a role in the interaction of the plant with its environment, e.g. as toxins to defend the plant against micro-organisms or various predators, as messengers, attractants, repellents or as camouflage. Madagaskar periwinkle (Catharanthus roseus (L.) G. Don) is one of the most extensively investigated medicinal plants and has been studied extensively for its antitumorous property. It has been reported that more than 100 phytochemicals can be produced in Catharanthus roseus, of which vincristine and vinblastine are the most important indole alkaloids. These two alkaloids have been used as therapeutic agents to treat a number of cancers. However, the yield of these compounds is notably very low. A comprehensive multidisciplinary approach has been integrated in order to improve the alkaloid contents. In this respect, various factors which influence in vitro biosynthesis of alkaloids have also been described. Singh mentioned that, elicitors can be grouped into three categories: (a) biotic elicitors, such as bacterial and fungal cell walls or glycoproteins; (b) abiotic elicitors, such as UV irradiation, salts and various non-constitutive compounds; and (c) endogenous elicitors, which are normally signal compounds produced by cells in plant. As well as the effect of different amounts of fungal elicitors on the alkaloid productivity has been tested. On the other hand, the effect of osmotic stress on achievement and production of secondary metabolites in plant cell cultures had been reported.

**MATERIALS AND METHODS**

**Plant Materials:** Seeds of Egyptian Catharanthus roseus (L.) Don. were kindly obtained from Institute of Horticulture Research, Agricultural Research Centre, Giza, Egypt. Seeds were surface sterilized under aseptic conditions of laminar flow hood, using 70 % EtOH for 30 Sec, and then transferred to a solution of 50 % Clorox (containing 5.25 % NaOCl) for 15 min. Then they were aseptically germinated on basal MS medium for 4 weeks and used as plant materials.

**Callus Production:** Three aseptically segments of leaf, stem, and root were excised from C. roseus sterilized plantlets and placed in 200 ml jars containing 40 ml of MS solid medium containing 1 mg/l each of 2,4-D and Kin (The best medium for callus production according to). Sub-culturing had been done every 4 weeks on MS-medium containing 1 mg/l kin.

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Elicitation of C. roseus Alkaloids: A biotic stress (mannitol) or biotic stress (Aspergillus niger) were used for more optimization and enhancement of calli growth parameters and vicristine, vinblastine production in different types of calli cultures.

Abiotic Stress: Mannitol was added to the MS-culture medium at the different concentrations of 0.0, 2000, 4000 and 8000 ppm.

Biotic Stress: Elicitor Preparation: The fungus Aspergillus niger was obtained from The Department of Plant Pathology of the National Research Centre. Aspergillus niger was grown in malt extract (20g/l) in shake flask (1000 ml) with 200 ml medium on a rotary shaker (120 rpm) at room temperature. After 7 days the cell suspension was autoclaved, and filtrated (on Whatman no. 1) filter paper. The mycelium was washed several times with sterilized distilled water and suspended in 100 ml water. This mixture was homogenized, autoclaved again and measured through the (P.C.V.) and used without purification. In this experiment, the following concentrations (0, 0.05, 0.15 and 0.25 %) of suspended Aspergillus niger, were added to the culture media.

Determination of Total Indole Alkaloids: Preparation of in vivo and in vitro derived tissue samples, and determination of total indole alkaloids were carried out according to the method described by Arvind et al. The obtained total alkaloids of these different calli cultures and in vivo derived samples were subjected to HPLC analysis using the following conditions:

Instrument:

- HPLC (waters).
- 600 E delivery system (pump).

Detector:

- 486 UV Detector (Waters associates).

Column:

- Nova Pak C₁₈ (Waters)3.9 x 150 mm

The results were integrated by Millennium 32 chromatography.

The standard curves were calculated at wave lengths 254 nm and 280 nm for vicristine and vinblastine, respectively.

The percentage of total alkaloids as well as vinblastine (VB) and vicristine (VC) in different calli culture and in vivo samples were determined and calculated using standard curves.

Statistical Analysis: All experiments were designed in a completely randomized design and obtained data were statistically analyzed using standers error (SE) according to the method described by Snedecor and Cochran.[24]

RESULTS AND DISCUSSION

Results:

Calli Growth Parameters:

Effect of Mannitol as Abiotic Stress: Data tabulated in Table (1) shows the effect of MS-medium containing 1 mg/l Kin and supplemented with mannitol at the different concentrations of 0, 2000, 4000 and 8000 ppm on enhancement of C. roseus calli growth parameters i.e., fresh, dry weights (g/jar) and dry matter content (%). The highest values of calli fresh weight 2.25, 1.87 and 1.73 (g/jar) were recorded with leaf, stem and root calli cultures, respectively (Fig.1). On other hand, the highest values of dry weights 0.22, 0.16 and 0.14 were recorded with leaf, stem and root calli cultures, respectively. However, the highest percentage of leaf, stem and root dry matter content 9.77, 8.66 and 8.27 were recorded with Ms-medium free mannitol. The best results of the different calli growth parameters were observed with MS medium free mannitol as a compared with other concentrations. The gradually increasing of mannitol concentration resulted reduction in calli fresh and dry weights, however, increased the dry matter content as compared with MS medium free mannitol. Leaf explants showed the best results of calli growth parameters as compared with stem and root explants, respectively.

Effect of Aspergillus niger as Biotic Stress: As shown in Table (2) fungi extract of Aspergillus niger at the concentration of 0, 0.05, 0.15 and 0.25 % was added to the MS-culture medium containing 1 mg/l Kin. Data in Table (2) revealed that, calli growth parameters, fresh and dry weights as well as the percentage of dry matter content were affected by increasing the level of Aspergillus niger concentration as biotic stress. The highest value of calli fresh weight 2.95, 2.55 and 2.38 (g/jar) were recorded with leaf, stem and root, respectively. Similarly, the best results of calli dry weight 0.25, 0.22 and 0.20 (g/jar) were resulted also from leaf, stem and root, respectively. However the best results of dry matter content (%) 9.77 was record with leaf calli cultures derived from MS medium free Aspergillus niger. The best results of calli growth parameters were observed with the MS-medium supplemented with 0.15 % of Aspergillus niger.
**Table 1:** Effect of MS-medium containing 1 mg/l Kn and supplemented with mannitol as abiotic stress at the concentrations of 0, 2000, 4000 and 8000 ppm on leaf, stem and root calli fresh, dry weights (g/jar) and dry matter content (%) of C. roseus. The initial weight of callus inoculum was ~ 250 mg/jars and incubated under light condition 16/8 h. for 4 weeks.

<table>
<thead>
<tr>
<th>Mannitol (ppm)</th>
<th>0</th>
<th>2000</th>
<th>4000</th>
<th>8000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Explanats</strong></td>
<td>F.W</td>
<td>D.W</td>
<td>D.W</td>
<td>DMC</td>
</tr>
<tr>
<td>Leaf</td>
<td>2.25±0.13</td>
<td>0.22±0.016</td>
<td>9.77</td>
<td>1.93±0.11</td>
</tr>
<tr>
<td>Stem</td>
<td>1.87±0.16</td>
<td>0.16±0.015</td>
<td>8.66</td>
<td>1.54±0.12</td>
</tr>
<tr>
<td>Root</td>
<td>1.73±0.19</td>
<td>0.14±0.062</td>
<td>8.27</td>
<td>1.45±0.09</td>
</tr>
</tbody>
</table>

*Each value is the average of 5 replicates ± SE; F.W = Fresh weight (g/jar); D.W = Dry weight (g/jar); D.M.C = Dry matter content (%); SE= Standard Error.

**Effect of Aspergillus niger as Biotic Stress:** The highest percentages of total alkaloids (%), VB and VC as relative to VB and VC of intact plant 0.83, 0.48 and 0.28 were resulted from leaf derived calli cultures. The descending order of total alkaloids, VB and VC production were recorded with leaf, stem and root calli cultures, respectively (Fig. 2).

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**Fig. 1:** Calli production from leaf, stem and root explants of Egyptian C. roseus (L.) cultured on MS-medium supplemented with 1 mg/l Kn and incubated under light condition 16/8 h. for 4 weeks.

Leaf calli cultures showed the best results of cell growth parameters as compared with stem and root calli cultures, respectively.

**Vinblastine and Vicristine Production:**

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**Effect of Mannitol as Abiotic Stress:** The highest values of total alkaloids (%), percentage of VB and VC as relative to VB and VC of intact plant 0.53, 0.42 and 0.24 were recorded with leaf derived calli cultures from MS medium which supplemented with 8000 ppm of mannitol as compared with other mannitol concentrations. Total alkaloids, VB and VC accumulation were gradually increased by increasing the level of mannitol concentrations. The descending order of total alkaloids, VB and VC production was recorded with leaf, stem and root calli cultures, respectively (Fig. 2).

**Effect of Aspergillus niger as Biotic Stress:** The highest percentages of total alkaloids (%), VB and VC as relative to VB and VC of intact plant 0.83, 0.48 and 0.28 were resulted from leaf derived calli cultures. The descending order of total alkaloids (%), VB and VC production were recorded with leaf, stem and root calli cultures, respectively. Supplementation of MS medium with of A. niger at the concentration of 0.25 % resulted in the highest values of total alkaloids (%), VB and VC as relative to VB and VC of intact plant 0.83, 0.48 and 0.28.
Fig. 2: Effect of MS-medium containing 1 mg/l Kin and supplemented with different concentrations of mannitol as abiotic stress on enhancement of total alkaloids, vinblastine and vircristine production in leaf, stem and root calli cultures as relative to C. roseus intact plant.

Fig. 3: Effect of MS-medium containing 1 mg/l Kin and supplemented with different concentrations of Aspergillus niger as biotic stress on enhancement of total alkaloids, vinblastine and vircristine production in leaf, stem and root calli cultures as relative to C. roseus intact plant.

0.25 % gave the highest values of total alkaloids, VB and VC production as compared with other concentrations (Fig. 3).

Discussion: The role of growth hormones in regulation of C. roseus indole alkaloids has been extensively studied\(^ {14,23}\). In agreement of our obtained results, Garnier et al.\(^ {10}\) and Yahia et al.\(^ {31}\) reported that that exogenously applied cytokinins to untransformed C. roseus callus or cell suspension cultures increased the content of ajmalicine and serpentine. Moreover, Arvy et al.\(^ {2}\) reported that auxins negatively influence
alkaloid biosynthesis at all levels and subculturing cells on an auxin-free medium, results in increased Tdc and Str mRNA levels, while addition of auxins rapidly decreases the Tdc mRNA level. 2,4-D strongly inhibits alkaloid production essentially during the growth phase. Furthermore, Decendit et al. reported that cytokinins are very important growth regulators which regulate many aspects of plant growth and differentiation. Also, in 1993 they reported that addition of zeatin to an auxin-free C. roseus cell cultures resulted in an increase in alkaloid accumulation and enhanced the activity of G10H and the bioconversion of secologanin to ajmalicine in C. roseus cultures. Concerning the effect of abiotic stress on enhancement of indole alkaloid production from C. roseus calli cultures, in contrast of our obtained results, Pasquali et al. reported that, although that salicylic acid (SA) has been shown to be an important compound in the defense system of plants and addition of SA to C. roseus, either seedlings or cell cultures did not affect the yield of alkaloids. A weak inducing effect on Str and Tdc steady-state mRNA levels was observed in C. roseus after addition of 0.1 mM SA. However in close of our obtained results, Smith et al. reported that increasing of sucrose concentrations in cultured cells of C. roseus, from 4 to 10 % (w/v) stimulated the alkaloid content. On other hand DiCosmo and Towers, reported that addition of 200 mM sorbitol resulted in a 63 % increase in catharanthine content.

Concerning the mechanism of elicitor action at physiological and molecular levels. The mechanism of elicitation in plants is based on elicitor–receptor interaction after which a rapid array of biochemical responses occur. The biosynthesis of TIAs can be stimulated by addition of exogenous elicitors such as fungal preparations. Many studies reveal that fungal elicitors profoundly affect regulation of indole alkaloid biosynthesis. In agreement of our obtained results Moreno et al. measured, activities of some enzymes involved in secondary metabolism in C. roseus before and after fungal elicitation and found that TDC activity is highly induced by elicitation.

Combination of abiotic and biotic elicitors added to C. roseus cell suspension cultures resulted in improvement of TIAs production. Ajmalicine and catharanthine are induced by addition of tetramethyl ammonium bromide and Aspergillus niger homogenate.

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Abbreviations:

- MS: Murashige and skoog medium.
- 2,4-D: 2,4- dichlorophenoxyacetic acid.
- Kin: Kinetin, 6-furfuryliminopurine.
- (VB): Vinblastine.
- (VC): Vicristine.

REFERENCES


