Cytotoxicity of Ethanol Extracts of in vivo, in vitro and Biotized Grown Plants of Vernonia divergens on EAC Cell Lines

Rajeev Kumar, Anand Prakash, Sanju Kumari, Utkarshini, Nishi Kumari, Rohit Kumar, Kanak Sinha, *Santosh Kumar

UGC Centre of Special Assistance, DRS Department of Botany, B R A Bihar University, Muzaffarpur, India.

ABSTRACT
The present study was attempted to enhance the anticancer activities in in vitro grown medicinal plant, Vernonia divergens, generated under the stress of a fungus, Piriformospora indica and to evaluate the effect of such biotization against EAC cell lines using standard MTT assay. The effect of adenine sulphate in addition to the phytohormones was assessed for micropropagation of V. divergens from shoot tip explants. The regenerated plantlets were biotized with a fungus, P. indica through soil drenching method. After acclimatization of 30 days, the ethanol extracts of these regenerants were screened for their inhibitory action against EAC mouse cell lines at different concentrations for 72 hours of treatment and compared with that of the inhibitory action of both in vivo and in vitro (non-biotized) generated plantlets. The combination of adenine sulphate and phytohormones sufficiently increased the number of shoots and roots per explants. Biotization of micropropagated plantlets not only enhanced the survival rate and growth of root, shoot and leaf of the regenerated plants but it also increased the anticancer potentiality. The level of inhibition of ethanol extract against EAC cell lines of these regenerated plants was found to be low as compared with that of the natural garden plants but the stress of biotization with this fungus helped the regenerants to regain the inhibitory action against the cell lines. Thus, biotization with the fungus, P. indica is vital not only in the growth promotion of plants but also demonstrated enhanced anticancer potentialities in micropropagated plants, grown under stress.

Key Words: Anticancer, Biotization, Micropropagation, Vernonia divergens, Piriformospora indica

INTRODUCTION
Pharmaceutically important phytochemicals which are claimed to be in practice as anticancer drugs are routinely derived from medicinal plants. The synthesis of these compounds depends on the growth rate of the plants. However, the geographical and environmental conditions as well as the genetic make-up of the plants largely contribute in the production and accumulation of these chemicals. Stereo-specificity of these compounds may be another major impediment for the chemical synthesis of these drugs in plants. At this juncture, plant tissue culture technique can be an alternative to overcome these problems for the synthesis of specific compounds. The present work was an attempt to study the anticancer activity of ethanol extracts from Vernonia divergens, a medicinal plant employing cell and tissue culture techniques. One part of the study was devoted to the regeneration of in vitro plantlets through tissue culture and its biotization with a fungus, Piriformospora indica during growth under stress. The other part deals with the study of the effect of the stress due to biotization on anticancer activity of ethanol extracts of in vivo grown garden plant as well as in vitro generated non-biotized and biotized plantlets.

In recent years tissue culture techniques has been widely exploited especially in the micropropagation of endangered medicinal plants. However, mortality rate of in vitro generated plants on transfer in soil has been the matter of concern for industries because survival of these in vitro plants as compared to in vivo plants remains questionable under adverse environmental stresses. The mutual beneficial relationship with useful microorganisms like bacteria including actinomycetes and fungi, which are otherwise absent under in vitro conditions could be one of the reasons. The colonization of bacteria into the plants improves plant performance under stress environment and also enhances yield. Studies with different crops, vegetables and medicinal plants have shown plant-microbe interactions. It is an established fact that rate of translocation of nutrients becomes high during plant-microbes interaction. Microbes around plant root system are treated as plant growth promoting rhizobacteria (PGPR). These arbuscular mycorrhizal (AM) fungi and PGPRs have increased the growth and biomass of different plants. P. indica, a root endosymbiotic fungus, having property of AM fungi, increases plant growth and overall biomass by translocating phosphorus to the host by an energy dependent process.
Vernonia divergens is an important medicinal plant which does not produce viable seeds and is propagated vegetatively. This plant, commonly known as insulin plant, is a potent sugar killer and is used as an excellent...
Table 1: Effect of Adenine sulphate (25 mg/L) in combination with BAP and NAA on multiplication of shoots from shoot tip explants of *Vernonia diverseng* after 25 days of MS medium containing 0.8% agar and 5% sucrose.

<table>
<thead>
<tr>
<th>Growth regulators</th>
<th>NAA</th>
<th>Shoot formation</th>
<th>No. of shoots [Explants^−1]</th>
<th>Height of shoots [cm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>45±1.00</td>
<td>1.32±0.10</td>
<td>1±0.01</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>62±1.00</td>
<td>5.75±0.13</td>
<td>1.00±0.03</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>70±1.50</td>
<td>7.63±0.25</td>
<td>1.25±0.01</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>76±1.75</td>
<td>10.25±0.65</td>
<td>1.95±0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>100±0.00</td>
<td>20.00±0.080</td>
<td>3.00±0.01</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>100±0.00</td>
<td>25.00±1.04</td>
<td>4.00±0.00</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>100±0.00</td>
<td>15.00±0.54</td>
<td>3.25±0.01</td>
</tr>
</tbody>
</table>

Data represent the mean of 20 cultures, ± Standard Deviation (SD), where n = 3

Explants cultured with solid MS medium containing 0.8% agar, 3% sucrose and supplemented concentrations (1.0, 2.0 and 3.0 mg/L) of 6-benzylaminopurine (BAP), (0.5, 1.0 and 1.5 mg/L) of α-naphthalene acetic acid (NAA) and (25 mg/L) of adenine sulphate (ADS). The pH of the medium was adjusted to 5.8 before addition of agar and autoclaving at 121°C. The cultures were maintained at 25±2°C. Multiplication of shoots from the shoot tips once established on MS medium supplemented with different hormones at desirable concentrations were rooted on MS medium supplemented with IBA (1.0, 2.0 and 3.0 mg/L). Thus, obtained complete plantlets were put for hardening in tray-beds in the acclimatizer room once they were biotized with the fungus, *P. indica*.

**Materials and Methods**

Plant and microbial materials: Shoot tips (2-3 cm) as explants were excised from two year old *V. diverseng* plant maintained in the SAP garden of DRS Department of Botany, B R A Bihar University, Muzaffarpur. The explants were washed with 5% (v/v) teepol solution for 10 min; surface sterilized with 0.2 % HgCl₂ for 2-3 min and rinsed 3-4 times with sterile double distilled water.

Table 2: Extent of colonization after 30 days time intervals in acclimatizer and its effect on survival of micropropagated plantlets of *V. diverseng*.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Colonization of <em>P. indica</em> (%)</th>
<th>Plant Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.0 ± 1.34</td>
<td>66.0 ± 1.28</td>
</tr>
<tr>
<td><em>P. indica</em></td>
<td>78.0 ± 0.88</td>
<td>86.0 ± 1.66</td>
</tr>
</tbody>
</table>

± = S D, n = 3.
Plant Biomass: Roots and shoots were washed in tap water and then in 0.1% HCl to remove adhering soil particles. Dry weights were recorded by drying root and shoot samples in an oven at 70°C for 2 days. Root and shoot length were also measured. Various parameters including per cent survival, per cent colonization of *P. indica*, population count of biotized roots, root-shoot length and dry weight as well as the length and number of leaves were recorded after 30 d of transplantation under humidified acclimatized room condition.

MTT assay: The cytotoxicity assay was done through quantitative determination of viable cells as prescribed by previous protocol. EAC cells (5X10³ per well) were cultured on a flat bottomed 96-well plate. After 24 hrs, 48 hrs and 72 hrs of incubation (at 37°C, 5% CO₂), 20 μl of MTT stock solution was added to each well of the assay plate, which was again incubated for four hours at 37°C. The incubation of stock solution confirmed the formation of formazan crystals by the reduction of tetrazolium salt by the mitochondria of living cells. The medium was removed and 150 μl of DMSO was added to each well to dissolve the MTT metabolic product. The plates were read in ELISA plate reader at the wavelength of 540 nm. Untreated cells are used as a control of viability (100%) and the results are expressed as % viability relative to the control.

**RESULTS**

Micropropagation of the plant was achieved through shoot tips as explants, which were excised from two year old plant of *Vernonia divergens* (Figure 1a) on MS medium supplemented with plant growth regulators at different concentrations. BAP (3mg/L) with adenine sulphate (25 mg/L) was effective for shoot multiplication but the combination with NAA (1mg/L) gave better results. Data revealed that the three good concentrations of BAP supplemented with NAA and same concentration of adenine sulphate were standardized for excellent multiplication of shoots. These concentrations were: 1mg/L BAP + 0.5 mg/L NAA, 2mg/L BAP + 1.0 mg/L

![Graph](image)

**Fig. 2:** Effect of different concentration of ethanol extracts of in vivo (■), in vitro (□) and in vitro biotized plants (▲) against EAC cell line growth inhibition (Relative Cell Cytotoxicity) in MTT assay.

**Fig. 3:** Relative Cell Cytotoxicity of ethanol extracts of in vivo ( ], in vitro ( ] and in vitro biotized plants ( ▲) against EAC cell line: The IC₅₀ values were extrapolated from the cytotoxicity curve at 50% of inhibition of EAC cell line after 72 hours of extract treatment.
NAA and 3.0 mg/L BAP +1.5mg/L NAA. Among these, 2mg/L BAP +1mg/L NAA along with adenine sulphate (25 mg/L) was responsible for rapid and heavy multiplication of shoots and the best morphogenic response was obtained (25-30 per explants) (Figure 1b; Table 1). Micro-plants were separated and sub-cultured on different concentrations of IBA (Figure 1c). Attempts to develop rooting in the regenerated shoots were tried successfully by supplementing IBA (2 mg/L) (Figure 1d). With the help of P. indica (Figure 1e), the micropropagated plantlets were biotized and after 30 days, they were examined for colonization (Figure 1f). Histological studies of the roots of V. divergens colonized by P. indica showed inter- and intra-cellular spread of hyphae and the formation of chlamydospores. The colonization was studied on the basis of presence of hyphae and pear shaped chlamydospores in the cortical cells of stained roots (Figure 1g). Survival rate after 30 days of acclimatization in soil was found to be maximum with P. indica biotization. By soil drenching method it was observed that the per cent colonization ranged from 58.0 to 78.0 within 30 days to 90 days of transplantation and per cent plant survival ranged from 86.0 to 100 in this period as compared to the control plants (non-biotized) which ranged from 66.0 to 68.0 (Figure 1h, Table 2). It established the impact of colonization since all the treated plants transferred after one month under stress of P. indica of growth were survived (Figure 1i). Significant increase in length and dry weight of root and shoot as well as number of lateral roots was observed due to colonization as compared to the un inoculated control plants. An increase in shoot length and shoot dry weight was recorded in vitro plants biotized with P. Indica after 30 d of growth in humidified acclimatized room. Leaf of V. divergens deserved special attention since it was frequently used by diabetic patients. Therefore, it was conceived to observe the effect of P. indica on the length and no. of leaves of micropropagated plants. The length of leaf was 5.6 cm as compared to 3.5 cm in control. Similarly the number of leaves was 7.00 per biotized plant as compared to 4.25 in control (Table 3). The concentrated ethanol extracts from three sources, viz., in vivo natural, garden plants, in vitro generated plantlets (non-biotized) and in vitro generated biotized plants were screened for in vitro percentage cytotoxicity at 72 hours of treatment. The profile of EAC cell growth inhibition after being treated with plant extracts of three sources mentioned above could be observed (Figure 2). It was found that all the three extracts showed slight cytotoxicity at the concentration below 125 μg/mL. At higher concentrations (125, 250, 500 and 1000 μg/mL), the in vivo plant extract was inhibitory on EAC mouse cell lines, the percent inhibitions were 65, 66, 78 and 88%, respectively whereas there was 20% less inhibition in ethanol extract of in vitro plantlets as reported previously. However, the level of inhibition in EAC cell lines was almost restored (62, 63, 76 and 85 μg/ml) in in vitro generated biotized and acclimatized plantlets (Figure 2), which were allowed to grow under stress of colonization of the fungus, P. indica. The trend was also found to be similar in the IC50 value in in vivo and in vitro biotized plants; it was 100 and 95.5 μg/ml, respectively, whereas the IC50 value was raised to 660.6 μg/ml (Figure 3) in in vitro non-biotized plant extract. The ethanol extracts significantly (p <0.05) reduced the viability of EAC cells after incubation for 72 hours in a manner that was directly related to the concentrations (0 to 1000 μg/ml) of the extracts in all the three sources.

DISCUSSION
The present work is an attempt to study the anticancer activity of secondary metabolites from selected medicinal plants employing cell and tissue culture techniques. Plant micropropagation via direct shoots regeneration allowed large scale multiplication of plantlets in vitro by preventing clonal variation as opposed to regenerated from calli alone, which often leads to somaclonal variation. Generally BAP is considered as most suitable hormone for increasing the large scale multiplication and micropropagation of various plant species. Addition of adenine sulphate resulted into highest percentage of multiple shoot regeneration. In general, micropropagated plants, exhibit high mortality rates upon their transfer to soil. Even 5% mortality causes a huge loss during commercial plants production. The humidified acclimatized room and field possess relatively lower humidity, higher light intensity and septic environment that are stressful to micropropagated plants as compared to in vitro conditions. The benefit of any micropropagation system can be fully realized only by the successful transfer of plantlets from tissue culture vessels to the ambient conditions found ex vitro. Biotization of micropropagated V. divergens with P. indica increased resistance of plants from stresses at the time of transplantation, thus protected micropropagated young plantlets from ‘transplantation shock’. Plant growth and biomass is greatly influenced by nutrients and environmental conditions. P. indica too helped in nutrient uptake by extending its hyphae in the rhizospheric region where even finest roots cannot reach. Thus, role of this probiotic fungus in nutrient uptake and growth of micropropagated V. divergens was found to be significant.

Table 3: Effect of P. indica on root, shoot and leaf of micropropagated V. divergens after 30 d of transplantation under humidified acclimatized room condition.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root Length (cm/plant)</th>
<th>Root dry wt (g/plant)</th>
<th>Shoot Length (cm/plant)</th>
<th>Shoot dry wt (g/plant)</th>
<th>Leaf Length (cm)</th>
<th>Leaf Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.4± 0.38</td>
<td>0.05± 0.05</td>
<td>4.0± 0.72</td>
<td>1.10± 0.44</td>
<td>3.5± 0.65</td>
<td>4.25± 0.46</td>
</tr>
<tr>
<td>P. indica</td>
<td>10.5± 0.88</td>
<td>0.18± 0.05</td>
<td>8.3± 1.00</td>
<td>2.30± 0.85</td>
<td>5.6± 1.25</td>
<td>7.00± 0.55</td>
</tr>
</tbody>
</table>

± = S D, n = 3
effective studies in field conditions. Biological assay such as tumor cell cytotoxicity is a sensitive method to scrutinize the anticancer principle present using EAC cell lines in vitro. 17. The significant (p< 0.05) decrease in the cancer cell viability with increasing dose and time indicates that the ethanol extracts are cytotoxic even in cultivated plant tissues, if exploited 16. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan 25. The above results confirm the production of secondary metabolites with anticancer properties 17, even during the morphological differentiation at low concentration 16, but it can be enhanced by new biotechnological interventions such as fungal mediated cultures 26. Vernonia divergens, growing in the field conditions (in vivo) acquires the biochemical pathway to produce anticancer compounds. These compounds may be altered during in vitro conditions of tissue culture but under stress of the biochemical pathway to produce anticancer compounds. Further research in this area may lead to better treatment of cancer. Phytochemical analyses of the in vivo and in vitro derived plantlets confirmed the presence of alkaloid, phenols, terpenoids, flavonoids, glycosides and steroids (Unpublished observations). Pharmacognostical studies and evaluation of total phenolic and flavonoid contents 27 of this tradionally used anti diabetic plant species is in progress.

ACKNOWLEDGEMENT
The authors acknowledge the University Grants Commission, New Delhi for granting financial assistance to carry out the present research work under the aegis of Special Assistance Program(SAP) at DRS-Phase I(F.3-13/2009) to the Department of Botany, B R A Bihar University, Muzaffarpur. Thanks are also due to Dr Anita K Verma, Department of Nanotechnology and Zoology, K M College, Delhi University, Delhi, India for carrying out experiments on EAC cell line.

REFERENCES