INTRODUCTION

The genus *Mucuna* belongs to the family Fabaceae (Leguminosae) and includes about 150 species of annual and perennial legumes with pan-tropical distribution. *Mucuna pruriens* L. is a well-known medicinal plant, yet, study on its pharmacological properties and corresponding compounds still continues. Importance of the genus *Mucuna* as a medicinal plant is mainly due to presence of L-Dopa. L-Dopa (3,4 dihydroxy–L–phenylalanine) is a neurotransmitter precursor used for symptomatic relief of Parkinson’s disease. Further, it acts as a precursor for several neurologically important catecholamines such as the neurotransmitter dopamine and the important hormones, adrenaline and noradrenalin (Riley, 1997).

Reproducible regeneration of shoots from various explants is desirable in plant tissue culture for crop improvement (Christopher et al, 1991). Differentiation of structures in tissue culture is controlled by growth regulators, along with other components of the culture medium (Narender et al, 2011). Analysis of activities of various enzymes provides a reasonable and promising approach to understanding the biochemical basis of developmental pathways (Singh et al, 2009). Therefore, there is a need to study structural and biochemical aspects underlying initiation of organized development *in vitro* (Sujatha et al, 2000). The present study was aimed at investigating the biochemical changes that occur during regeneration of shoots (plantlets) in explants of two accessions of *Mucuna pruriens*, viz., Accession 1 (*M. pruriens* bearing a black seed-coat) and Accession 2 (*M. pruriens* bearing a white seed-coat). This was done by monitoring the efficiency of enzymes involved in nitrogen utilization, and changes in the level of some hydrolytic enzymes.

MATERIAL AND METHODS

**Plant material and preparation of explants**

Seeds of both the accessions of *Mucuna pruriens* were procured from University of Agricultural Sciences, Bengaluru. The seeds were surface-sterilized with 1% mercuric chloride for 5 min, followed by washing in sterile distilled water 5-6 times to remove traces of the surface-

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Biochemical changes during plantlet regeneration in two accessions of *Mucuna pruriens*

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ABSTRACT

The genus *Mucuna* is an important medicinal herb and is extensively used in traditional Indian systems of medicine for various ailments. *In vitro* culture technique provides an alternative to plant propagation and germplasm conservation. Our aim was to study the biochemical changes occurring during regeneration of shoots (plantlets) from explants of two accessions of *Mucuna pruriens*, by monitoring the efficiency of nitrogen utilization and changes in levels of some hydrolytic enzymes. A rapid micropropagation system was developed using Murashige and Skoog’s (MS) medium supplemented with BAP and IAA combined. In both the accessions, 3.0mg l\(^{-1}\) 6-BAP, in combination with 0.2mg l\(^{-1}\) IAA, induced shoot buds and shoot elongation; however for multiple-shoot induction, a slightly higher concentration of cytokinin, i.e., 3.5mg l\(^{-1}\) 6-BAP, in combination with 0.2mg l\(^{-1}\) IAA, was required. Results of the present study confirm an active growth of explants revealed by nitrate assimilation enzymes and hydrolytic enzymes. It is concluded that medium composition, growth regulator combination and culture incubation conditions are all vital in both the accessions of *Mucuna pruriens* for induction of *in vitro* plant regeneration.

Key words: *Mucuna*, *in vitro*, biochemical changes, regeneration, enzymes
sterilant. These were then germinated in vitro on basal MS (Murashige and Skoog, 1962) medium. Plants grown thus were used as the explant source. Explants were trimmed aseptically (1.5 to 2.0 cm) and inoculated onto MS medium.

**Media and culture conditions**

MS medium composed of MS salts and vitamins supplemented with sucrose (30 g l⁻¹), solidified with 0.8% (w/v) agar and pH adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were maintained at 24 ± 2°C under 16 h light/8 h dark photoperiod using light provided by cool, white, fluorescent lamps (25 µmol m⁻² s⁻¹) in a growth chamber.

**Shoot induction, multiplication and rooting**

MS basal medium supplemented with various concentrations (0.5, 1.0 to 5.0 mg l⁻¹) of different cytokinins, viz., 6-BAP (benzyl amino purine), kinetin and 2-ip [6-(γ, γ-dimethylallylamino purine)] either singly, or in combination, with 0.2 mg l⁻¹ indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA) or no plant growth regulator to evaluate morphogenic potential of the nodal explants. All the cultures were subcultured to fresh medium of the same composition every 28 days (4 weeks). Percentage response of explants producing shoots, number of shoots produced per explants and shoot-length were recorded at weekly intervals.

Rooting of shoots was done on half-strength MS medium supplemented with different concentrations of IAA and NAA (each singly at 0.5 mg l⁻¹ to 4 mg l⁻¹) or in combination with 0.1% activated charcoal.

**Enzyme extraction and assay**

Enzyme extraction and assays were performed as described below, with slight modifications when necessary, for the present investigation. For Nitrate/ Ammonia assimilating enzymes, extraction for nitrate reductase was carried out as per Altaf Ahmad and Abdin (1999), and enzyme activity was assayed as per Campbell and Smarrelli (1978). Extraction and assay for glutamine synthetase were done as per Philippe Lenee and Yves Chupeau (1989). The same extraction procedure was adopted for glutamate dehydrogenase. Optimum conditions for enzyme activity were maintained, namely, pH, temperature, substrate and cofactor concentrations. Acid and alkaline phosphatase enzyme extraction and assay were carried as per Angosto et al. (1988). For invertase, the method of Yolanada Cuadrado et al. (2001) was used for extraction, and the activity was determined using the method of Miller and Ranwala (1994). Extraction and assay of α-amylase was carried out as per Sadasivam and Manickam (2008). Peroxidase extraction was done as per Lorenza M. Bellani et al. (2002) and its activity was assayed as per Oskar Sanchez et al. (1989).

**Statistical analysis**

All the experiments were conducted in three replicates. Data were subjected to statistical analysis using Microsoft Excel (MS Office, 2003) and are presented as Mean ± SE.

**RESULTS AND DISCUSSION**

**Shoot induction and rooting in nodal explants**

Organogenesis was observed in nodal segments cultured on MS medium supplemented with each of the concentrations of BAP/kinetin/2-ip (alone, or in combination) with 0.2 mg l⁻¹ IAA/NAA in both the accessions of *Mucuna*. Morphogenic response observed was better with the aminopurine class of cytokinins (BAP and 2-ip) than with the furfuryl amine class of cytokinins (kinetin), with BAP showing a better response among the former. Therefore, for further studies, only BAP was used as the cytokinin of choice. Optimum growth of shoot occurred on medium containing 3 mg l⁻¹ BAP in combination with 0.2 mg l⁻¹ IAA (Fig. 1a and 1b) in both the accessions.
emergence) was also observed in MS medium fortified with NAA/IAA at 0.5mg l⁻¹. Morphological changes occurring in explants during the course of their proliferation on a suitable medium were monitored by determining some biochemical changes, viz., nitrate/ammonia utilizing enzymes during shoot regeneration from nodal/leaf explants, and changes in hydrolytic enzymes during organogenesis.

**Changes in nitrate reductase (NR) activity:**

Nitrate reductase (NR) is one of the key enzymes involved in the first step of nitrate assimilation in plants (Altaf Ahmed & Abdin, 1999). Table 3 shows the pattern of changes in nitrate reductase activity in both the accessions monitored from the day of inoculation up to the 30th day, at 5-day intervals.

In regenerating nodal explants of Accession 1, the activity peaked on Day 20. Thereafter, it remained the same until Day 30. Whereas, in Accession 2, two peaks of activity were observed on the 10th and 25th day (Table 3).

**Changes in GS and GDH activity:**

Glutamine synthetase (GS) and Glutamine dehydrogenase (GDH) are the other key enzymes involved in nitrate and ammonia assimilation in plants. In Accession 1, GS activity was found to be higher between the 10th and 25th day (Table 3).
20th day, and decreased thereafter. Accession 2 had higher GS activity between the 5th and 20th day, and decreased thereafter (Table 3).

In Accession 1, it was observed that activity of both the isoforms of GDH (NAD+ and NADH isoforms) from Day 0 and Day 5 remained the same; but, there was an increase in activity on Day 10, and it peaked on Day 15, decreasing thereafter. But, the activity was greater in the NADH isofarm on Day 10 compared to the NAD isoform (Table 3). In Accession 2, there was a gradual increase in the activity of both the isoforms of glutamate dehydrogenase (NAD+ and NADH isoforms) up to Day 10 and Day 15 in NADH and NAD isoforms, respectively, and decreased thereafter (Table 3).

**Table 2. Effect of different auxins (in half-strength MS medium supplemented with 0.1% activated charcoal) on root induction in two accessions of Mucuna pruriens**

<table>
<thead>
<tr>
<th>Auxin (mg l⁻¹)</th>
<th>Rooted shoots (%)</th>
<th>Mean no. of roots/shoot</th>
<th>Mean root-length (cm)</th>
<th>Plant survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Accession 1</td>
<td>Accession 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No auxin</td>
<td>07.2 ± 0.95</td>
<td>07.2 ± 0.95</td>
<td>1.4 ± 0.07</td>
<td>0.8 ± 0.02</td>
</tr>
<tr>
<td>IAA 0.2</td>
<td>34.2 ± 1.28</td>
<td>32.4 ± 1.28</td>
<td>5.6 ± 0.12</td>
<td>3.5 ± 0.12</td>
</tr>
<tr>
<td>0.3</td>
<td>48.4 ± 1.14</td>
<td>42.4 ± 1.12</td>
<td>7.6 ± 0.07</td>
<td>6.7 ± 0.06</td>
</tr>
<tr>
<td>0.4</td>
<td>76.5 ± 2.12</td>
<td>72.5 ± 2.02</td>
<td>9.6 ± 0.10</td>
<td>8.6 ± 0.01</td>
</tr>
<tr>
<td>0.5</td>
<td>94.8 ± 2.96</td>
<td>92.8 ± 2.86</td>
<td>11.9 ± 0.11</td>
<td>10.9 ± 0.1</td>
</tr>
<tr>
<td>1.0</td>
<td>72.2 ± 2.08</td>
<td>68.2 ± 1.67</td>
<td>5.2 ± 0.06</td>
<td>4.8 ± 0.05</td>
</tr>
<tr>
<td>NAA 0.2</td>
<td>63.5 ± 4.72</td>
<td>61.5 ± 3.62</td>
<td>6.9 ± 0.08</td>
<td>5.6 ± 0.04</td>
</tr>
<tr>
<td>0.3</td>
<td>78.6 ± 3.26</td>
<td>72.4 ± 2.36</td>
<td>7.7 ± 0.08</td>
<td>7.4 ± 0.07</td>
</tr>
<tr>
<td>0.4</td>
<td>89.2 ± 2.33</td>
<td>85.2 ± 2.13</td>
<td>9.9 ± 0.09</td>
<td>9.4 ± 0.08</td>
</tr>
<tr>
<td>0.5</td>
<td>96.2 ± 6.63</td>
<td>94.2 ± 5.52</td>
<td>12.4 ± 0.24</td>
<td>12.2 ± 0.23</td>
</tr>
<tr>
<td>1.0</td>
<td>46.3 ± 2.88</td>
<td>43.4 ± 2.68</td>
<td>5.6 ± 0.06</td>
<td>5.2 ± 0.05</td>
</tr>
</tbody>
</table>

(Data represent Mean ± S.E.)

Invertases exist in at least two isoforms, such as the soluble (extracellular) and wall-bound form; and, acid and alkali isoforms. In the present study, both acid (pH 5) and alkali (pH 7.5) isoforms were studied in organ-forming and non-organ-forming regenerating shoot cultures.

**Wall-bound invertase**

Activity of the wall-bound invertases in Accession 1 is presented in Table 3. Acid invertase peaked on Day 15. In the case of alkaline invertase, there was no increase in activity at all; rather, there was a gradual decrease in its activity from Day 0 to Day 30.

The activity of wall-bound invertase of Accession 2 peaked on Day 5, and, gradually decreased from Day 10 to Day 30 (Table 3); whereas alkaline invertase showed a little increase in activity on Day 5, and decreased thereafter.

**Extracellular invertase**

Activity of acid isoforms in Accession 1 peaked on Day 10, and gradually decreased thereon. Alkaline isoforms also showed maximum activity on Day 10 (Table 3). The activity in Accession 2 showed a gradual increase from Day 0 to Day 10 and remained constant up to Day 15, decreasing thereafter; whereas, the activity of alkaline isoforms peaked on Day 10.

**α-amylose**

Activity of α-amylose remained the same on Day 0 and Day 5 in both the accessions, and gradually increased from Day 10 to Day 30 (Table 3).

**Peroxidase**

Peroxidase activity in Accession 1 peaked on Day 25, and decreased thereafter; whereas, in Accession 2, peak activity was observed on Day 20.

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Nitrate uptake system in a plant must be versatile and robust, because, plants need to transport adequate amount of nitrate to satisfy the demand in the face of external nitrate concentration that can vary by five orders of magnitude (Crawford, 1995). Nitrate supplement in the medium must be converted into NH₄⁺ in plants before the nitrogen can enter amino acids and other nitrogen compounds. Nitrate reductase has been studied intensively because its activity often controls protein synthesis rate in plants absorbing NO₃⁻ as a major nitrogen-source (Srivastava, 1980; Naik et al., 1982). Genes for this have been cloned from several plants and mutants, and transgenic lines are available too (Lam et al., 1996; Lochab et al., 2007).

Results of the present investigation clearly suggest that there is a synergy that operates among enzymes for nitrate and ammonia assimilation when nitrate concentrations in the medium are high (~30mM in MS medium). This induces production of nitrate reductase, and subsequently GS, as nitrate is converted into ammonia.

Activity of nitrate reductase persists continuously even after cultures enter the stationary phase; whereas, production of GS is directly or indirectly dependent on NR activity. From the results above, it can be concluded that decrease in GS activity when cultures enter the stationary phase may be attributed to decrease in the activity of nitrite reductase; also, this could be due to exhaustion of sucrose in the medium. Results of the present investigation are supported (in other plant species) by Philippe Lenee & Yves Chupeau (1989) and Suzuki et al (1987).

In the present study, lower levels of phosphatases during the initial culture-period may be because of the high

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Accession</th>
<th>Days of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrate reductase(µmole NO₃⁻ g⁻¹ min⁻¹)</strong></td>
<td>Accession 1</td>
<td>0  5  10  15  20  25  30</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>25.24 25.24 25.24 49.4 73.95 73.95 73.95</td>
</tr>
<tr>
<td><strong>Glutamine synthetase (GS)(n moles of γ-glutamate formed min⁻¹ g⁻¹ protein)</strong></td>
<td>Accession 1</td>
<td>100 100 240 260 120 150 100</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>200 230 230 230 230 140 130</td>
</tr>
<tr>
<td><strong>Glutamate dehydrogenase (NADH-GDH)(µmole NADH g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>100 100 160 260 100 100 100</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>100 100 120 180 60 100 100</td>
</tr>
<tr>
<td><strong>Glutamate dehydrogenase (NAD⁻-GDH)(µmole NAD⁻ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>508 508 434 579 650 675 711</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>394 394 431 468 662 712</td>
</tr>
<tr>
<td><strong>Acid phosphatase(n moles of PNP released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>508 508 434 579 650 675 711</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>394 394 431 468 662 712</td>
</tr>
<tr>
<td><strong>Alkaline phosphatase(n moles of PNP released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>468 468 394 434 468 662 712</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>418 418 431 418 468 529 712</td>
</tr>
<tr>
<td><strong>Wall-bound invertase(µg of reducing sugar released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>3.05 3.05 8.76 12.8 0.876 0.292 0.292</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>2.3 2.3 2.00 1.75 0.876 0.292 0.292</td>
</tr>
<tr>
<td><strong>Extracellular invertase(µg of reducing sugar released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>3.00 3.504 4.080 3.504 0.379 0.292 0.292</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>2.00 2.300 1.750 0.876 0.292 0.292 0.292</td>
</tr>
<tr>
<td><strong>Wall-bound invertase(µg of reducing sugar released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>1.460 1.460 2.920 1.168 0.584 0.000 0.000</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>2.000 2.300 1.750 0.876 0.292 0.000 0.000</td>
</tr>
<tr>
<td><strong>Extracellular invertase(µg of reducing sugar released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>2.336 2.336 3.050 0.876 0.292 0.000 0.000</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>2.62 2.62 3.504 3.050 0.292 0.000 0.000</td>
</tr>
<tr>
<td><strong>α-Amylase(µg maltose released min⁻¹ g⁻¹ F.W.)</strong></td>
<td>Accession 1</td>
<td>30 30 32 36 38 42 46</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>28 28 30 34 36 38 40</td>
</tr>
<tr>
<td><strong>Peroxidase(Units ml⁻¹ min⁻¹)</strong></td>
<td>Accession 1</td>
<td>400 480 720 360 600 840 780</td>
</tr>
<tr>
<td></td>
<td>Accession 2</td>
<td>300 360 400 400 600 450 660</td>
</tr>
</tbody>
</table>

Mean±SE of 3 observations is not indicated due to the large amount of data.
inorganic-phosphate levels in the medium. Pronounced increases in the activity of phosphatases before manifestation of a visible morphogenic event (observed to happen prominently on Day 20), i.e., on Day 15 in both the accessions, suggests that these enzymes may have a role in biochemical degradation of plasmodesmata. Such degradation may facilitate penetration of roots/elongation of shoots (Naidu & Kavi Kishor, 1995; Kumar, 1998). Together with this, peroxidase activity was found to peak on Day 20 in Accession 2, and Day 25 in Accession 1, with gradual and progressive increase witnessed from Day 0. Peroxidases are a large group of enzymes involved in a number of biological processes such as lignification (Lagrimini et al., 1997a), cross-linking of cell wall proteins (Bradley et al., 1992) and auxin catabolism (Lagrimini et al., 1997b). Perhaps, an increase in enzyme-level indicates a role of these enzymes in tissue proliferation and differentiation. On examining involvement of acid phosphatases in initiation and formation of adventitious roots in Impatiens sps., Malik and Kumari (1977) attributed these roles to the enzymes. It was speculated that glycosidases may cleave wall-linkages and facilitate growth.

Increased activity of hydrolytic enzymes seen in the present investigation indicates that different compounds degrade in tissues, and this is concurrent with a high synthetic activity occurring during organogenesis. Results of the present investigation are supported by previous findings of activity occurring during organogenesis. Results of the degrade in tissues, and this is concurrent with a high synthetic activity of the present investigation indicates that different compounds may cleave wall-linkages and facilitate growth.

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CONCLUSION
With a view to confirm whether nutrients or, growth regulators added to the medium and conditions like of source light and temperature at which cultures were incubated, affected growth of plants in vitro, biochemical studies were undertaken. In higher plants, nitrate-assimilating and hydrolytic enzymes are regulated by light, hormones, sugars, and, carbon and nitrogen metabolites. Results of the present study confirm active growth of explants revealed by the activity of nitrate assimilating enzymes and hydrolytic enzymes (excepting GDH, the activity of which decreased, as, it is a stress induced enzyme). Thus, it may be concluded that medium composition, growth regulator combination and culture incubation conditions are cited for optimal growth in vitro in both the accessions of Mucuna pruriens for plant regeneration.
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