Chapter 5

Activities of nitrogen metabolism enzymes as influenced by growth regulators during formation of regenerants from protocorm-like-bodies
Nitrogen assimilation, both ammonium ($\text{NH}_4^+$) and nitrate ($\text{NO}_3^-$) ions, has an important role in the growth and differentiation. Though, nitrogen metabolism enzymes have been studied extensively in plants (Jackson et al., 1986), very little information is available in the case of orchids (Hew et al., 1993). Physiological and biochemical studies have been carried out on different aspects during in vitro organogenesis (Kavi Kishore and Mehta, 1988; Auer et al., 1992; Gopalan et al., 1992). It is well established now that energy cycles like Kreb's, glycolytic and pentose phosphate pathway are enhanced during organogenesis which may be attributed to the need for energy regeneration (Kavi Kishore, 1988).

Compounds containing nitrogen constitute only a small proportion of the total dry weight of plants but they are extremely important physiologically. Nitrogen is required for growth and nitrogen deficiency is the most common limitation on plant growth and development after water stress. Required for
production of proteins and formation of new cells, chlorophyll and cytochrome, the demand for nitrogen is closely related to the amount of growth and differentiation (Kramer and Kozlowski, 1979). Growth regulators play an important role in differentiation (Ozias-Akins and Vasil, 1985), and regulation of enzyme activity by growth regulators has been reported by various researchers (Varner and Ho, 1977; Letham et al., 1978; Moore, 1980). Developmental changes during growth are associated with changes in enzyme levels. Ashton (1976) reported that differentiation processes are controlled by the interactions between several hormones. Hence, presence of a hormone in different tissues or in the same tissue during different stages of its development may influence enzyme activity/synthesis through different mechanisms. Studies on enzymatic activities during in vitro differentiation of orchids as influenced by various growth regulators are, however, scanty (Kumaria et al., 1990). Activities of the main enzymes of nitrogen metabolism during in vitro differentiation of D. wardianum as influenced by different growth regulators are presented here.

Materials and Methods

Plbs (derived from shoot apices) and multiplied in MS liquid hormone-free medium as described in chapter VI, were used for this study. Two week old plbs were transferred to MS semi-solid medium containing 0.8% (w/v) agar, 3% (w/v) sucrose and
supplemented with IAA, IBA, NAA and BAP at a concentration range of 0-5 mg/l. Cultures were incubated at 24±2°C at 2,000 lux light intensity (16 h photoperiod) from cool, white, fluorescent light. Samples collected every 0, 15, 30 and 45 days of growth were used for assay of enzyme activities.

Enzyme Extraction

500 mg plbs were homogenized in 0.1M phosphate buffer (pH 7.5) containing 1mM disodiumethylene diaminetetraacetate, 1% polyvinylpyrrolidone, 1mM dithiothreitol and 1% bovine serum albumin, and centrifuged at 10,000 x g for 30 min. The supernatant was collected and used for measurement of nitrate reductase (NR) and nitrite reductase (NiR) activity. For glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT), Tris-HCl buffer (pH 7.6) with 5mM cysteine was used instead of phosphate buffer.

Enzyme Assay

NR activity was assayed using the method of Jaworski (1971). The 5 ml reaction mixture contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 2.5 ml of 0.2 M potassium nitrate, 0.25 ml of 5% n-propanol, 1.0 ml of distilled water and 0.25 ml of enzyme extract. Incubation was carried out at room temperature (25°C) for 30 min. Reaction was terminated by adding 1.0 ml of 1% sulphanilamide in 3 N HCl and 1.0 ml of 0.02% N- (1 naphthyl) ethylenediamine dihydrochloride. The absorbance of the solution was read at 540 nm and activity is expressed as μM
nitrite produced per min per mg protein. Standard curve was made by taking sodium nitrite at a range of concentration and making up the volume to 5 ml with distilled water. Reaction was terminated by the addition of sulphailamide followed by naphthyl ethylenediamine reagent. Absorbance was read at 540 nm.

NiR assay was done following the method of Hucklesby et al. (1972). The reaction mixture contained 0.25 ml of 0.1 M phosphate buffer (pH 7.5), 0.08 ml of 1 mM NaN02, 0.08 ml of 0.155 mM methylviologen, 0.39 ml of distilled water and 0.1 ml of enzyme extract. The reaction was started by addition of 0.1 ml of 5 mM sodium dithionite buffered in NaHCO3. After 15 min of incubation at room temperature, the reaction was stopped by vigorous agitation in order to oxidize methylviologen. Nitrites were determined by addition of 1.0 ml of 1% sulphanilamide and 1.0 ml of 0.02% N - (1- naphthyl) ethylenediamine dihydrochloride as described above. The enzyme activity is expressed as the amount of nitrite (µM) reduced per min per mg protein.

GDH activity was assayed by using the amination reaction based on glutamate formation (Thalouarn, 1988). The 2.5 ml reaction mixture contained 1.0 ml of 50 mM Tris-HCl (pH 8.0), 0.4 ml of 180 mM ammonium acetate, 0.3 ml of 16 mM α-ketoglutarate, 0.12 ml of 0.12 mM NADH and 0.1 ml of enzymatic extract. Incubation of the reaction mixture was carried out at room temperature for 30 min followed by recording of change in absorbance at 340 nm. The amount of NADH oxidised is calculated from the molar extinction coefficient. Activity is expressed as
µM NADH oxidised per min per mg protein.

GS was assayed by using the biosynthetic reaction based on glutamyl hydroxamate formation as described by Sadashivam and Manikam (1992). The 4 ml reaction mixture contained 2.0 ml of 0.2 M glutamine, 0.5 ml of 20 mM sodium arsenate, 0.3 ml of 3 mM manganese chloride, 0.5 ml of 50 mM hydroxylamine, 0.5 ml of 1 mM adenosine-di-phosphate and 0.2 ml enzyme extract. Incubation of the reaction mixture was carried out at room temperature for 30 min. Reaction was stopped by addition of 1.0 ml of ferric chloride reagent [10g trichloro-acetic acid (TCA) and 8g ferric chloride in 250 ml of 0.5 N HCl] and absorbance was read at 540 nm. Enzyme activity is expressed as µM - glutamyl hydroxamate formed per min per mg protein.

GOGAT activity was determined by following NADH oxidation (Sadasivam and Manikam, 1992). The 5 ml reaction mixture contained 1.8 ml of 50 mM Tris-HCl buffer (pH 7.6), 1.0 ml of 5 mM glutamine, 1.0 ml of 5 mM 2-oxoglutarate, 1.0 ml of 0.25 mM NADH and 0.2 ml enzyme extract. Reaction mixture was incubated at room temperature for 30 min and change in absorbance recorded at 340 nm. Activity is expressed as µM NADH oxidised per min per mg protein.

Soluble proteins were also determined in the extract (Lowry et al., 1951). 500 mg of each of the treated tissue was macerated in alcohol using chilled mortar and pestle. The volume was made up to 5 ml with 80% alcohol and centrifuged at 5,000 rpm for 20 min. The supernatant was discarded and the residue suspended in
5% TCA for 15 min after which 2 ml aliquot was taken and resuspended in 10% TCA in the ratio 1:1 (v/v) for 15 min. The mixture was centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and the residue was washed twice with distilled water and dissolved in 1.0 ml of 0.5 N NaOH and diluted to desired volume. Suitable volume of this solution was mixed with 5.0 ml freshly prepared alkaline solution (1.0 ml of 0.3% copper sulphate in 1% sodium-potassium tartarate mixed with 50 ml of 2.0% sodium carbonate solution). The reaction mixture was allowed to stand for 10 min. at room temperature. After incubation, 0.5 ml Folin-Ciocalteau reagent (equally diluted with water) was added with immediate shaking and held at room temperature for 30 min. The colour was read at 750 nm. Protein is expressed as µg per 100 mg fresh wt. of tissue using standard curve prepared by bovine serum albumin.

Besides, the tissue extract was subjected to polyacrylamide gel electrophoresis (PAGE) for localizing isozymes of soluble proteins according to the method of Ornstein (1964). Following solutions were used for electrophoresis:

Solution A : 1 N HCl
- 48 ml
Tris(hydroxymethyl) amino methane - 36.6 g
N,N,N',N'- tetramethylethlenediamine - 0.46 ml

Final volume was made up to 100 ml with double distilled water.

Solution B : Acrylamide
- 30.0 g
Methylene-bis-acrylamide - 0.8 g
Final volume was made up to 100 ml with double distilled water.

Solution C: Ammonium per sulphate - 0.14 g

Final volume was made up to 100 ml with double distilled water.

All the stock solutions except solution C (to be prepared fresh at the time of use) were kept in thoroughly washed amber coloured bottles at 4°C.

Tank buffer: Tris-glycine (25 mM, pH 8.3) was made by dissolving 0.6 g Tris (hydroxymethyl) amino methane and 2.88 glycine in 1 litre of double distilled water.

Preparation of gel

Stock solutions were brought to room temperature to avoid bubble formation in the gels. Solutions A and B were mixed gently in 1:1 ratio followed by 2 parts of solution C. The mixed gel solution was immediately loaded in gel tubes (0.5 cm x 7.0 cm) with one end sealed with parafilm. A layer of water was loaded at the top of the gel to prevent contact with atmospheric oxygen, and to straighten the meniscus. Gels were allowed to polymerise for 30 min at room temperature.

Preparation of tissue

A suitable weight of freshly chopped and chilled tissue was ground in chilled pestle and mortar in a medium containing Tris glycine buffer, 40% sucrose and 5% polyvinyl pyrrolidone. The extract was strained through four folds of muslin.
Electrophoresis

Electrophoresis was done at 4°C. Bromophenol blue (500 mg/10ml distilled water) was used as the marker dye by adding 1 or 2 drops of it to the extract. In each gel tube, suitable extract (containing 200-300 ug protein) was loaded on top of the gel. A current of 2 mA per tube was used for the first 15-20 min which was subsequently increased to 4 mA per tube. The power was turned off when the marker dye reached 5 mm above the bottom. The gels were removed from the tubes by squirting water from the syringe between the gel and the glass wall.

Staining of gels

Proteins were localized by staining the gels with amidoblight (0.1%) in acetic acid (7%) for 30 min followed by destaining in 7% acetic acid. Proteins appeared as dark blue bands against a clear background.

Results

Effects of growth regulators on the nitrogen metabolism enzyme activity were appraised.

Nitrate Reductase

The activity of NR along the time course of the experiments in control and treated plbs at different concentrations of growth regulators showed different profile plots (Fig. 14a-d). An increase in the activity on 30th day was observed in plbs treated
Fig. 14(a-d) NR activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).
Fig. 14
with BAP followed by a decline thereafter. Highest level of enzymatic activity was recorded on the 30th day in plbs treated with 1.0 mg/l BAP. In plbs treated with IBA, a steady increase in the activity on day 15 followed by a decrease was observed at higher concentrations, however, in plbs treated with 0.5 mg/l IBA a slight decrease in the activity from zero to 15 day followed by an increase till day 30 and a decline thereafter was observed. An increase in the NR activity from day zero to day 15 was also noted in plbs treated with 0.5 and 1.0 mg/l NAA which then declined till the end of the study. IAA treated plbs, at all concentrations showed a decline from zero to 15 day followed by a slight increase on the 30th day and a decline after that. Enzymatic activity in control declined on 15th day and showed a peak value on 30th day and declined later.

Nitrite Reductase

NiR profiles observed for different growth regulators understudy were more or less similar to those of NR (Fig. 15a-d). An increase in NiR activity in plbs treated with IBA and NAA was observed from zero to 15th day which then declined till the end of the study. IAA treated plbs, however, showed a decline in the NiR activity from zero to 15th day that increased in the case of 0.5 and 1.0 mg/l treated plbs on day 30 and decreased thereafter. BAP treated plbs showed an increase in activity on the 30th day which then decreased till the end of the study. The pattern of NiR activity in control plbs was similar to NR.
Fig. 15(a-d) NiR activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).
Glutamate dehydrogenase

Profiles of GDH activity were observed to be different from those of NR and NiR (Fig. 16a-d). In IBA, NAA, IAA and BAP treated plbs, a decline in the activity was observed from day zero onwards till the end of the study except for 1.0 mg/l IBA and 2.5 mg/l BAP treatments where an increase in the activity was recorded on 15th day followed by a decline. A decline in GDH activity from day zero onwards was also observed in control which continued till the end of the study.

Glutamine synthetase

Significant differences in GS activities were observed amongst different concentrations of growth regulators (Figs. 17a-d). The highest enzymatic activity was recorded in plbs treated with 2.5 mg/l BAP on the 15th day. A slight increase on the 15th day followed by a decrease till the end of the study was, however, observed in plbs treated with lower concentrations of BAP. A marked increase in GS activity on day 15 was also observed in plbs treated with 1.0 mg/l IBA. IAA treated plbs, at all concentrations, showed a decline in activity from zero day till the end of the study. A similar decline in activity was also observed in the case of control.

Glutamate synthase

Highest GOGAT activity was recorded on day 15 in plbs treated with 1.0 mg/l BAP followed closely by 1.0 mg/l IBA.
Fig. 16(a-d) GDH activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).
Fig. 16
Fig. 17(a-d)  GS activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).
Fig. 18(a-d) GOGAT activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).
Fig. 18
Increase in activity was also observed in all NAA treated plbs and in plbs treated with 0.5 mg/l IAA, on day 15. A decline in activity was observed which continued till the end of the study in plbs treated with higher concentrations of IBA and IAA. Control plbs showed an increase in the activity on 15th day, declining thereafter.

**Proteins**

The protein profile of control and treated plbs is depicted in Fig. 19a-d. An increase in soluble protein content was observed from zero day onwards which continued up to the end of the study in all the treatments. Maximum level of soluble proteins was observed in plbs treated with 1 mg/l IBA which was slightly higher than that in 2.5 mg/l BAP treated plbs. On 45th day, control had higher soluble protein levels than IAA and NAA treated plbs of the same age.

Determination of the electrophoretic profile for proteins revealed the presence of five major bands in control and all the growth regulator treatments. The bands, however, differed in their Rm, colour intensity and width (Fig. 20a-e).

**Discussion**

Relations between nitrogen metabolism and endogenous hormones are reciprocal. Not only do these hormones control certain phases of protein synthesis and degradation but two of the three main classes of hormones, the auxins and the cytokinins are themselves
Fig. 19(a-d) Soluble protein content of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).
Fig. 20(a-e) Electrophoretic profiles of protein isozymes of plbs cultured in MS (a) and medium supplemented with different growth regulators [IAA (b), IBA (c), NAA (d) and BAP (e)]. 1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations.
Fig. 21(a–c) Regeneration of multiple shoots from nodal explants
a) shoot formation from nodal bud cultured on MS + 2.5 mg/l BAP (after 8 weeks)
b) multiple shoots transferred to MS hormone-free medium (after 10 weeks)
c) well developed rooted shoots.
Table 4: Effect of different concentrations of NAA+BAP and IAA+BAP on morphogenetic responses of cultured nodal buds in MS medium*.

<table>
<thead>
<tr>
<th>Growth regulators</th>
<th>Conc. (mg/l)</th>
<th>Morphogenetic responses (%)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS medium (control)</td>
<td>-</td>
<td>40</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td>MS + BAP</td>
<td>0.5</td>
<td>30</td>
<td>3-5 ms formation</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>50</td>
<td>7-10 ms formation</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>20</td>
<td>2-4 ms formation</td>
</tr>
<tr>
<td>MS + IAA+BAP</td>
<td>0.5+0.0</td>
<td>10</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td></td>
<td>0.5+0.5</td>
<td>10</td>
<td>1-2 shoot formation</td>
</tr>
<tr>
<td></td>
<td>0.5+2.5</td>
<td>20</td>
<td>2-4 ms formation</td>
</tr>
<tr>
<td></td>
<td>0.5+5.0</td>
<td>30</td>
<td>2-4 ms formation</td>
</tr>
<tr>
<td></td>
<td>2.5+0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.5+0.5</td>
<td>10</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td></td>
<td>2.5+2.5</td>
<td>10</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td></td>
<td>2.5+5.0</td>
<td>20</td>
<td>1-2 shoot formation</td>
</tr>
<tr>
<td></td>
<td>5.0+0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5.0+0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5.0+2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5.0+5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS + NAA+BAP</td>
<td>0.5+0.0</td>
<td>20</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td></td>
<td>0.5+0.5</td>
<td>20</td>
<td>2-3 ms formation</td>
</tr>
<tr>
<td></td>
<td>0.5+2.5</td>
<td>40</td>
<td>3-5 ms formation</td>
</tr>
<tr>
<td></td>
<td>0.5+5.0</td>
<td>30</td>
<td>2-4 ms formation</td>
</tr>
<tr>
<td></td>
<td>2.5+0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.5+0.5</td>
<td>10</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td></td>
<td>2.5+2.5</td>
<td>10</td>
<td>Single shoot formation</td>
</tr>
<tr>
<td></td>
<td>2.5+5.0</td>
<td>20</td>
<td>1-2 shoot formation</td>
</tr>
<tr>
<td></td>
<td>5.0+0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5.0+0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5.0+2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5.0+5.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Data based on 10 replicates per treatment, collected after 8 weeks.

ms: Multiple shoots
-: No response
nitrogen containing compounds whose production is inevitably linked with the nitrogen metabolism of the plant ( Luckwill, 1968). In plants, the rate at which nitrate can be assimilated into simple organic forms and eventually into protein, depends largely on the activity of the NR and NiR systems in the plant and under certain conditions, these may well become limiting factors. During the course of a growth passage in tissue culture, the initially rich nutrient culture medium is progressively depleted by the tissue growth and development until one or more nutrient factors becomes limiting, and growth ceases on entry into stationary phase (Robinson et al., 1992). NR, a substrate-inducible enzyme, mediates the reduction of nitrate to nitrite. In the present study, increase in NR activity was recorded in NAA and IBA treatments on 15th day which is possibly due to progressive uptake of \( \text{NO}_3^- \) from the medium and consequent stimulation of nitrogen metabolism enzyme activities. NR activity in BAP treated plbs increased up to day 30. Also, highest NR activity was observed in 1.0 mg/l BAP treated plbs which could be due to its stimulatory effect as cytokinins are reported to be capable of inducing NR synthesis (Dwivedi et al., 1984). Stimulation of NR synthesis due to the presence of BAP in the medium has also been reported by Kende et al. (1971). Decline in the NR activity in the later stages could be due to a decrease in the \( \text{NO}_3^- \) concentration in the medium, in agreement to references that both induction and steady state level of NR activity are affected by nitrate concentration (Knypal, 1973; Chantarotwong et
Decrease might also be due to NR-inactivating factors as suggested by Shaner and Boyer (1976). An initial decline in activity from zero day to 15th day in control could be due to the readjustment to the new conditions, required by the plbs when subcultured to a fresh medium as suggested by Santos and Salema (1989).

NiR activity was always higher than that of NR. Thus, a nitrite accumulation, toxic for the plbs, is avoided. A steady availability of the substrate to act upon could be the possible reason for the higher nitrite activity which in its turn provides substrate for GDH. A decrease in the activity in the later stages of the study might be due to a parallel decrease in the NR activity.

GDH activity in control and treated plbs was found to be low in comparison to other nitrogen metabolism enzyme activities studied. No significant differences in GDH activity were observed among plbs treated with different growth regulators at different concentrations. Except for plbs treated with 1.0 mg/l IBA and 2.5 mg/l BAP concentrations which showed an increase in activity on 15th day, all other growth regulator treated plbs showed a decline in the GDH activity from day zero which continued till the end of the study suggesting thereby that ammonium assimilation might not be taking place via this pathway in the case of D. wardianum. The route of $\text{NH}_4^+$ assimilation, thus, fades away in favour of GS-GOGAT pathway, an assumption supported by high GS and GOGAT activities (Figs. 17a-d, 18a-d). Low GDH
activity can also be attributed to its low affinity for ammonium as suggested by Sprent (1979). Until recently, controversy existed regarding the metabolic role of GDH. A number of researchers assigned GDH a function in ammonium assimilation (Yamaya et al., 1986; Srivastava et al., 1987; Rhodes et al., 1989) whereas others suggested its role in catalyzing the oxidation of glutamate, thus providing carbon-skeletons to the TCA cycle (Furuhashi and Takahashi, 1982; Robinson et al., 1990). Robinson et al. (1992) attribute increased GDH activity in their study during the stationary phase of carrot cell cultures to carbon limitation and protein catabolism leading to the release of glutamate which is oxidised by GDH to 2-oxoglutarate with the concomitant release of ammonia. The primary role of GDH, thus, according to Robinson et al.(1992) is to provide carbon skeletons for TCA cycle under conditions of carbon limitation, the enzyme thereby playing an important role in linking the carbon and nitrogen metabolism.

GS activity in plbs treated with higher concentrations of IBA and BAP was more on 15th day decreasing thereafter. Also, an increase in activity was observed in plbs treated with NAA at 0.5 and 1.0 mg/l concentrations on 15th day, declining in the later stages. A much greater affinity of GS for ammonium than GDH has been reported by Sprent (1979). This additional benefit of GS, of rapid assimilation of ammonium from pools of even low concentration prevents ammonium accumulation in sufficient quantities thus checking toxicity. Hew et al. (1993) while
studying the nitrogen uptake by tropical orchids have reported a much higher activity of GS than GDH in the case of *Cymbidium*, *Dendrobium* and *Bromheadia* and have suggested that GS in orchids might be playing a major role in nitrogen assimilation. In the present study too, an increased GS-GOGAT activity indicates greater involvement of GS in the nitrogen metabolism.

GOGAT activity reaches its highest level with BAP treated plbs followed by IBA and NAA respectively. The activity depends on glutamine synthesis which acts as its substrate, thus GS activity decides the follow up activity of GOGAT. However, some diversion of glutamine towards other pathways might take place as this metabolite is essential for nucleic acid and protein synthesis. Low activities of the nitrogen metabolism enzymes in the later stages of the study could be accounted for by a depletion in the nutrients of the medium. Many tissue culture experiments have attempted to examine the relationship between exogenous plant hormones in the medium and subsequent plant growth and development (Fosket, 1980; Skivirsky *et al.*, 1982). The developmental response of explants to exogenous hormones is the result of a variety of biochemical processes including hormone uptake, transport and metabolism (Horgan, 1987). Thus, although explant growth is typically described in relation to the hormone concentration in the medium, hormone concentration does not necessarily reflect the level of active endogenous hormone in the explant (Auer *et al.*, 1992) which might account for the results with different auxins in the present study. Also,
suitability of some growth regulators over others in controlling the morphogenetic responses and their inhibitory action in some has been well documented in orchids (Liu et al., 1988). Lower activities of the enzymes in control when compared to treated plbs might be due to the absence of the growth regulators. Moreover, a higher soluble protein content could be the result of the active state of other enzymatic pathways in the present case. The present study on nitrogen metabolism enzyme activities with _D. wardianum_ in _vitro_ cultures, thus, suggest that GS plays an important role in nitrogen assimilation and also that growth regulators modify the activities of the nitrogen metabolism key enzymes.