A PROJECT COMPLETION REPORT
OF

MINOR RESEARCH PROJECT
ENTITLED

"NITROGEN METABOLISM IN *Arthocnemum indicum* Moq."

: Submitted to :

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: Submitted by:

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Enclosure-I

PROJECT COMPLETION REPORT OF MINOR RESEARCH PROJECT

A brief Project Completion Report (PCR) of Minor Research Project entitled "Investigating the Antifungal Potential of *Cassia fistula* (L.)." sanctioned to Dr. Abdul Qaiyum Ansari, Associate Professor, Department of Botany, Maharashtra College of Arts, Science and Commerce, Mumbai- 400 008.

Introduction:

Mangrove ecosystem represents an important wetland ecosystem providing shelter to diverse population of plants and animals. However, with indiscriminate urbanization and reclamation of land for building houses the halophytic flora is rapidly vanishing from India. It's becoming increasingly important to save this important group of plants. These plants also grow in nitrogen deficient environment. The present study would give an insight on the enzyme machinery which enables them to efficiently utilize and assimilate the nitrogen which is available.

Salt-tolerant shrubs and trees (*Prosopis,Tamarix* and others) can be planted for conservation, stabilization, rehabilitation of degraded environments, and to reverse desertification. They may be harvested for much-needed fuel wood and timber. The replanting of mangrove forests (*Avicennia* and *Rhizophora*) on the tropical coasts represents another immediate low-cost opportunity for both environmental restoration and sustainable commercial production.

The frontiers of knowledge can be expanded by searching the untapped diversity for discovering new resources for new genes – chemicals – metabolites etc. Thus, this will add to the value of biotechnology, diversity of molecules in the organism that can be used in various aspects. It will be significantly useful to study the impact of soil salinity on nitrogen metabolism. It will be useful to understand the adaptive mechanisms of the plants growing in physiological drought such as saline habitat. Thus it will help designing the strategy to reclaim saline habitat and growing useful crops.

There is also potential to use halophytes or diversities among and within halophyte species to improve crop production in saline areas. Many of their unique features could be valuable in plant breeding programs. To give one example, *Agropyron junceum* has been used as a source of salt tolerance genes for wheat.

***Review*:**

Salinity is an important environmental stress from which natural and cultivated plants suffer. High salinity is extremely heterogeneous in space and time at any environment and many plants use windows of low salinity levels of organisation (Orcutt and Nilsen, 2000). Salt-induced soil degradation is common in arid and semi-arid areas in many parts of the world, and is particularly serious in irrigated land. Over the last few decades, it has increased steadily in large-scale irrigation schemes in a number of countries Salt-affected soils are found in at least 75 countries and occupy more than 20% of the world's irrigated area (Ghassemi et al. 1995).

In some countries, they occur on more than half of the irrigated land (Cheraghi 2004).

Soils in dry areas are particularly prone to salinization. This is because the rate of water evaporation from the soil exceeds the water input from rainfall, allowing salts to accumulate near the surface as the soil dries (Szabolcs 1989). This is likely to increase due to climate change, which will probably cause dry regions to become drier (Dore 2005). The vast tracts of saline soils in dry lands limit the amount and quality of vegetation available for livestock grazing. Accumulation of salts in these soils originates either from the weathering of parent salt bearing minerals (primary salinity) or from anthropogenic activities involving unsustainable management of land and water resources (secondary salinity).

Secondary (human-induced) salinization affects a smaller area than primary salinity (Ghassemi et al. 1995, Oldeman et al. 1991) but is a more serious problem because it mainly affects cropland.

Arable land is scarce in dry regions; yet salinity-affected land is frequently abandoned because amelioration is expensive (Dregne 1995). The problem is aggravated by the low salt tolerance of major agricultural crops (Abrol et al.1988) compared to wild salt-tolerant plants (halophytes), which are adapted to saline soils (Glenn 1995). Salt build-up is perhaps the largest productivity constraint in irrigated agriculture in the dry areas (Ghassemi et al. 1995).

In recent decades, progress has been made in understanding and avoiding practices that lead to soil salinization, and in developing new crops, exploring genetic variation within crops and developing agronomic techniques for the management of salt-affected soils so they remain in production without requiring complete restoration.

PRESENT STUDY

Halophytes are a group of plants growing in saline habitats. Extensive studies have been carried out on halophytes in the recent times and large amount of literature has been published on them. Some of the most important reviews have been written by Navalkar (1973), Poljakoff-Mayber and Gale (1975) and Sen and Rajpurohit (1982).

In Mumbai exentensive research has been carried out on halophytes like Salvadora persica *(Amonkar, 1977 and Ingle, 1984)* Sonneratia acida *and* Sonneratia apetala *(Rao, 1975)*, Acanthus ilicifolius *(Siddhanti, 1977, Mukundan, 1981 and Sanglikar, 1982)* Suaeda maritima *(Chittar, 1971, Ghanekar, 1979 and Gokhale, 1982)*, Pentatropis cynanchoides *(Lokhande, 1983) and* Aeluropus lagopoides *(Sarangdhar, 1986)*.

A survey of literature on halophytes however revealed dearth of information on nitrate metabolism of halophytes. Earlier, Beadle *et al.* (1957) studied nitrogen content in different species of *Atriplex,* while Goodman and Caldwell (1971) and Stewart *et al.* (1972) later studied nitrate metabolism in desert halophytes. Studies on nitrate reductase have been undertaken in halophytes like *Pentatropis cynanchoides* (Lokhande, 1983) *Agieceras corniculatum* and *Sessuvium portulacastrum* (Bhosale, 1978) *Aeluropus lagopoides* (Sarangdhar, 1986). These studies were however, restricted to plants growing in the natural environment of marshy land. Therefore, it was felt that nitrogen metabolism of a background halophyte would be useful in understanding the relationship of nitrogen metabolism of submerged halophyte, background halophyte and glycophytes and contribute to the existing knowledge of mineral nutrition and its effects on metabolic reactions in this plant, thus for a present study a succulent halophyte *Arthocnemum indicum Moq.* was undertaken.

Arthrocnemum indicum is a plant that is succulent halophyte (salt tolerant) which grows in salt marshes on tropical areas of the world. This plant belongs to Chenopodiaceae family. Arthrocnemum indicum is a traditional medicinal halophyte common as salt marshes (R. Ksouri *et al*., 2012). It is used in the treatment of poisonous snakebites and scorpion stings. It also plays

a prominent role in traditional oriental medicine and ancient Indian medicine (Ayurveda). *Arthocnemum indicum* Moq may be useful as a candidate in the treatment of the colon cancer in a specific manner. In fact, the high anticancer and antioxidant activities found in shoots of this halophyte could be ascribed to the high total polyphenol content (Mondher B *et al*., 2013). Therefore, it was felt that nitrogen metabolism of *Arthocnemum indicum* Moq which is one of the most salt tolerant species along with *Arthocnemum macrostachyum (*Moric.) (Vicente *et al*., 2007, 2009) should be undertaken to get more insight in stress adaptation to nitrogen assimilation by halophytes.

It is often said that the plant is the product of its external environment. In order to understand the mineral composition of the plant it is thus absolutely essential to study the chemical composition of the soil. The soil supporting *Arthocnemum indicum* Moq was analyzed for its salts and nitrogen content. The Plant of *Arthocnemum indicum Moq* was analyzed for its salts and nitrogen content.

Nitrogen metabolism in *Arthocnemum indicum* Moq have been studied. The effect of the salinity on the activity of nitrate reductase (NR), glutamine synthtetase (GS), glutamate dehydrogenase (GDH), alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT) obtained from leaves and roots have also been determined. Beside, electrophoretic studies were undertaken to determine isoenzymes of GDH and AspAT enzymes and also to determine protein profile of the plant.

CHAPTER-I

(A) SOIL ANALYSIS

The nutritional status of a plant is largely dependent on its soil environment. It is now well known that these mineral nutrients are at the centre of the most metabolic activities in plants. Plant physiologists are of the opinion that halophyte-vegetation studies might help in solving some of these problems provided they are thoroughly investigated for mechanism of salt tolerance. It has long been felt that the information obtained through such studies could furnish a clue as to how salt affected wastelands can be managed and brought under cultivation. A significant contribution in this field of study has been made by Ashby and Beadle (1957) and

Greenway (1962 a,b). These studies indicated that certain glycophytes were salt tolerant while some halophytes could thrive in non- saline habitats.

In his review of mangroves, Walsh (1974) has aptly remarked that there is a need for research, which relates species and habitat to elemental composition. On the basis of these reports, it was felt that *Arthocnemum indicum* Moq could represent a plant ideally suited for a study of salinity problem and ion regulation.

In order to correlate the mineral contents of this plant with that of the environment, soil analysis studies were undertaken. Soil supporting *Arthocnemum indicum* Moq was analyzed for its soluble salts, chlorides, sulphates, and nitrogen content. In addition to this, exchangeable sodium, potassium, calcium, and magnesium were also determined.

MATERIAL AND METHODS

Preparation of soil sample:

The soil supporting roots of *Arthocnemum indicum* Moq was collected from different places and dead plant material and litter were removed and air dried in plastic trays. The air-dried soil was gently crushed with wooden pestle and sieved through 2mm mesh. The soil thus obtained was used for analysis.

Total water-soluble salts:

The total water-soluble salts were estimated by the method of Piper (1966).

Preparation of soil extracts:

Soil extract was prepared according to the method of Piper (1966). Air-dried soil and ammonium acetate were mixed in proportion of 1: 5 and allowed to stand overnight. This was filtered through Whatman No.44. The soil was continuously leached with ammonium acetate till approximately one liter of the filtrate was collected. This solution was evaporated to 2-3 ml and then digested according to the method of Toth *et al.* (1948).

Chlorides, Sulphates:

Chlorides were estimated by Volhard's method (1956).

Sulphates were estimated by the method of Bower and Huss (1948).

Nitrogen content:

The soil sample was digested by the Kjeldahl's method and total nitrogen content was determined by the method of Kjeldahl as described by Hawk *et al.* (1947).

Na, K, Ca and Mg:

Dried soil sample was digested using nitric-perchloric acid digestion procedure (Toth *et al.*, 1948).

The digested samples were diluted to volume and analyzed for Na, K, Ca and Mg. Sodium and potassium were analyzed using flame photometer whereas calcium and magnesium were analyzed by the method of Cheng and Bray (1951).

Soil salinity:

Salinity problem, according to Chapman (1974) arises when the concentrations of sodium salts or magnesium salts are present in excess. The greater the excess, the more pronounced are the effects of salinity.

Salinity in *Arthocnemum indicum Moq* is 9.969% in terms of NaCl. The high salinity of the soil as compared to *Pentatropis* soil (9.44%, Lokhande, 1983) and soil supporting *Avicennia* (3.39%, Navalkar, 1959) may be due to more frequent inflow of sea water, since *Arthocnemum indicum Moq* grows in marshes.

RESULTS

Total soluble salts in the soil:

Total soluble salts in saline soil mostly include sulphates, nitrates, chlorides and bicarbonates of sodium, potassium, calcium and magnesium. Saline soils contain a high percentage of chlorides especially of sodium; hence, they usually show higher values for total salts than non-saline soils. The limit for the percentage of salts in saline soils has been suggested by Kearney and Schofield

(1936). According to these workers, soils containing more than 0.1% salts can be termed as saline soils. Thus, the values for salinity of soil supporting *Arthocnemum indicum* Moq (9.969%) as well as the total soluble salt content (6.65%) (Table 1.1) of the soil from the natural habitat indicate that these values are above the normal level of salinity.

Our results (Table 1.1) indicate that the soil from natural habitat of *Arthocnemum indicum* Moq is highly saline, since it contains 6.65% total soluble salts. The high soluble salt content of such soil is generally due to chloride of sodium and magnesium.

Soil sulphates:

The soil of natural habitat of *Arthocnemum indicum* Moq is comparatively poor in sulphates, which is 0.098% i.e. 2.04 *meq*/100 g air-dried soil. (Table 1.2).

The ratio of sulphates to chloride in *Arthocnemum indicum* Moq soil is below unity. Thus, in *Arthocnemum indicum* Moq soil chloride absorption is favoured than sulphate absorption.

(B) PLANT ANALYSIS

(1) Mineral content:

Preparation of plant extract:

1g of dry plant material was powdered and digested with hot concentrated HNO_3 till the contents dissolved. The extract was cooled and decolorised by the addition of minimum amount of perchloric acid (Toth *et al.,* 1948).

Na, K, Ca and Mg were analyzed as described in soil analysis.

Total nitrogen content:

From available information on desert halophytes (Beadle *et al.*, 1957 and Dwyer and Wolde-Yohannis, 1972) it is apparent that these plants are able to maintain reasonably high tissue nitrogen levels.

The values for total nitrogen in roots and leaves of *Arthocnemum indicum* Moq estimated are 5.35 and 4.04 g/100 g fresh tissue. The high total nitrogen content may be due to efficient nitrogen metabolism and it suggest that these coastal halophytes, like desert halophytes, appear to be well adapted to conditions where availability of soil nitrogen is low. Obviously, they must possess an efficient nitrogen uptake mechanism.

RESULT:

Table 1.3 depicts the mineral analysis of the soil from the natural habitat of *Arthocnemum indicum* Moq. In the soil, among the anions, chlorides and sulphates are the major constituents while among the cations, sodium is dominant ion. Calcium occupies the second position followed by magnesium and potassium.

Sodium content in the soil and the plant:

The value for exchangeable sodium in the soil supporting *Arthocnemum indicum* Moq is depicted in the Table 1.2. Sodium is the most dominant cation in this soil, mainly because seawater loads the saline soil with sodium. The sodium adsorption ratio (SAR) of the soil, which represents the interaction of total salt concentration and sodium concentration. The SAR of the soil supporting *Arthocnemum indicum* Moq was determined by the equation:

$$SAR = \frac{Na}{(Ca + Mg)^{0.5}}$$

Where Na, Ca, Mg are expressed in miliequivalents.

The soil supporting *Arthocnemum indicum* Moq is rich in calcium content indicating that they have high sodium absorption.

It will be observed that, amongst halophytes, which accumulate sodium and chloride ions, *Arthocnemum indicum* Moq has high sodium content.

Potassium content in soil and plant:

Along with sodium, potassium represents another major cation in halophytes. Potassium is known to play an important role in salt tolerance due to its penetration into cell sap and causing protoplasmic changes leading to increased water stressed plants (Singh and Tripathi, 1979). According to Kingsbury *et al.* (1984), there may be an increased potassium requirement when plants are grown under sodium salt stress.

Table 1.4 depicts potassium content in soil and plant, along with the potassium absorption ratio (PAR) and exchangeable potassium percentage (EPP) of *Arthocnemum indicum* Moq.

Table 1.1. Moisture content, organic matter content, total nitrogen content, total soluble salts, chloride content and sulphate content of soil supporting *Arthocnemum indicum Moq.*

Moisture content	11.46%
Organic matter content	1.094%
Total nitrogen content	0.2%
Total soluble salts	6.65%

Table 1.2. Chloride, sulphate, and exchangeable cations in soil supporting Arthocnemum *indicum Moq.*

	Percentage*	meq/100g air dried soil
Chlorides	9.969	265.8
Sulphates	0.098	2.04
Exchangeable sodium	6.74	291.2
Exchangeable potassium	1.96	50.26
Exchangeable calcium	3.56	178
Exchangeable magnesium	0.83	69.16

*Values expressed in g per 100 g air-dried soil.

The saline soil and its higher values for PAR and EPP coupled with the high potassium content of *Arthocnemum indicum* Moq indicate a well developed absorption mechanism in this halophyte. Cramer *et al.* 1986 also reported increased K: Na ratio under NaCl salinity due to better uptake of potassium.

Calcium content in soil and plant:

In saline soil the survival of halophytes depends on the presence of calcium (Epstein, 1961) hence, the soil, which supports the halophytes, is usually rich in calcium (Mishra, 1967). In

Table 1.2, the data reveal that the soil supporting *Arthocnemum indicum Moq* contains 3.56% exchangeable calcium (178 *meq*/ 100 g air dried soil).

Magnesium content in soil and plant:

The values for Ca/Mg in the soil supporting *Arthocnemum indicum Moq* are shown in Table 1.3. In the present investigation, Ca/Mg ratio for the soil is 1.7:1. In the light of Berger's (1969) observation, the conditions are adequate for uptake of magnesium in the soil supporting *Arthocnemum indicum Moq,* but the soil is also rich in sodium, thus availability of magnesium to the plant is hampered, to some extent. The leaves of *Arthocnemum indicum Moq* contain 40 meq/ 100 g dry tissue (Table 1.3).

Table 1.3. sodium. potassium, calcium and magnesium content of *Arthocnemum indicum Moq.*

Cation	Plant*	Soil #
Sodium	412.00	248.00
Potassium	98.40	28.80
Calcium	65.00	120.00
Magnesium	40.00	69.16

*Values expressed as meq per 100 g dry tissue
Values expressed as meq per 100 g air-dried soil.

Table 1.4. Potassium content of soil* and plant#, potassium adsorption ratio (PAR) and exchangeable potassium percentage (EPP) of habitat.

Potassium content in plant	98.40
Exchangeable potassium in soil	50.26
PAR (%)	1.89
EPP (%)	35.78

* Values expressed as meq per 100 g dry soil
# Values expressed as meq per 100 g dry tissue

CHAPTER-II

A)KINETICS OF NITROGEN ASSIMILATING ENZYMES

1. NITRATE REDUCTASE

MATERIAL AND METHODS

Healthy plants of *Arthocnemum indicum* Moq were collected from natural habitat and brought to the laboratory in a polythene bags. The plants were washed with distilled water and air-dried. These plants were used for *in vivo* assay.

Chemicals:

(1) 0.1M Tris-hydroxy methyl amino methane (Tris-HCl) buffer of pH 7.5
(2) 0.1M Potassium nitrate (KNO_3)
(3) 1% sulfanilamide dissolved in 1.5N HCl
(4) 0.02% *N*-(1-Napthyl) ethylene diamine dihydrochloride

***In vivo* assay of NR**

The *in vivo* assay of NR was carried out according to the method of Klepper *et al*. (1971). Leaves from healthy plants were cut with a pair of scissor into very small pieces. The reaction mixture consisted of 2ml of Tris-HCl buffer of pH 7.5, 2ml of 0.1N KNO_3 and chopped leaves (50mg). The leaves were weighed prior to placement in the reaction mixture. The tubes were incubated for one hour at 37˚C after which the leaves were removed and the reaction was terminated by adding 1ml of 1% sulfanilamide. This was followed by the addition of 0.02% *N*-(1-Napthyl) ethylene diamine dihydrochloride, as a result of which a pink colour developed. The absorbance was measured spectrophotometrically (Systronic PC Based UV/VIS Spectrophotometer) at 540 nm. The specific activity of the enzyme NR is expressed as ΔOD/g leaves/hour.

NR of the leaves was assayed at different pH ranging from 4 to 9. Tris-HCl buffer was used for the assay. NR of the leaves of *Arthocnemum indicum* Moq was assayed at different substrate concentration ranging between 100 mM and 500 mM KNO_3.

RESULTS

(1) Effect of H-ion concentration on NR activity:

NR exhibited optimum activity at pH 7.5 (Figure 2.1). Although the enzyme showed a two-peak response to changes in H-ion concentration, a pH of 7.5 was apparently more favourable than pH

5.5, since at latter pH the enzyme activity was less by about 10%. In view of this, in all further experiments the pH for the enzyme assay was maintained at 7.5.

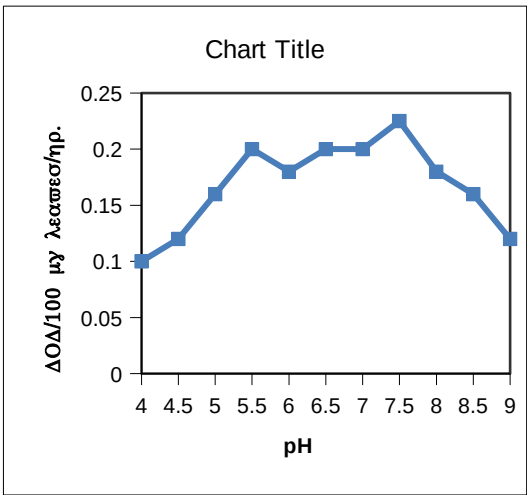


Figure 2.1. Effect of pH variation on the *in vivo* NR activity in *Arthocnemum indicum Moq.*leaves.

(2) Effect of substrate concentration on NR activity:

The effect of different concentrations of the substrates is illustrated in figure 2.2. The rate of NR activity was linear upto 200 mM KNO_3 and increased further till 200 mM KNO_3. At 300 mM KNO_3 the enzyme activity reached its maximum. The concentration of substrate was therefore maintained at 100 mM KNO_3 in all further *in vivo* assays. The rate of the reaction is calculated in terms of ΔOD/g leaves/hour.

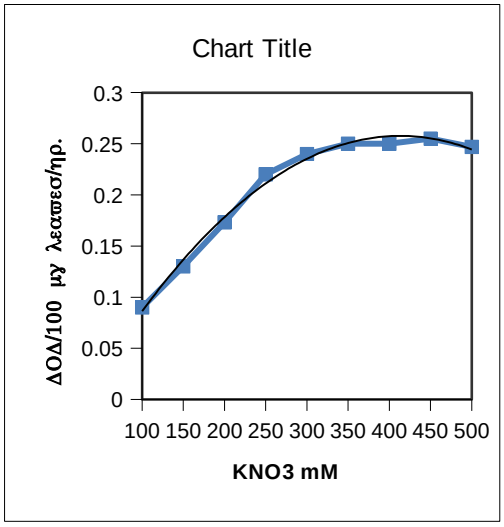


Figure 2.2. Effect of substrate variation on the *in vivo* activity of NR in *Arthocnemum indicum Moq.* leaves.

(3) Effect of enzyme concentration on NR activity:

Figure 2.3 depicts the effect of varying concentrations of enzymes on the rate of NR activity. The rate of enzyme reaction was linear upto 0.1g plant tissue. With increase in the quantity of tissue from 0.1g to 0.3g, there was gradual increase in the NR activity following a rectangular hyperbola. In further experiments, the concentration of the enzyme was maintained at 0.1g leaf material.

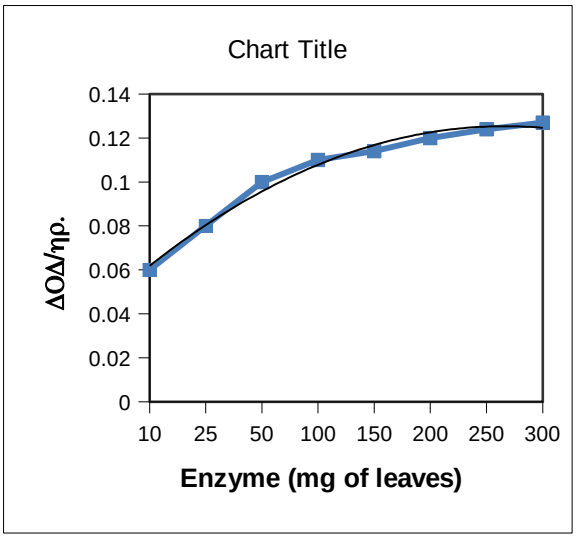


Figure 2.3. Effect of enzyme variation on the *in vivo* activity of NR in *Arthocnemum indicum Moq.* leaves.

DISCUSSION

NR has been widely studied in crop plants, but beside the work done on this enzyme by Bhosale (1978) in *Aegiceras coniculatum* and *Sessuvium portulacastrum*, Sarangdhar (1986) in *Aeluropus lagopoides*, other known reports pertain only to desert halophytes (Goodman and Caldwell, 1971; Stewart *et al.*, 1972). In spite of voluminous work done on this enzyme; several features remain to be elaborated. At this stage, it would be worthwhile to summarize some of the essential features of NR already known because of studies undertaken by a host of workers on crop plants and other glycophytes.

NADH-NR has been studied in detail by Beevers *et al.* (1964), and Maratzki *et al.* (1967). NADH generated by the glycolytic triose phosphate dehydrogenases, was reported by Klepper *et al.* (1971) to function as the reductant for nitrate reduction. Sawheny *et al*. (1978) observed that NADH generated beyond the triose phosphate dehydrogenase steps of glycolysis was also

attained by NR. Kadam *et al.* (1980), while working with *Sorghum* seedlings, had reported that NADH generated by the TCA cycle could also be used.

The induction of NR by the substrate was reported by Afridi and Hewitt (1964). Li *et al.* (1995) have shown induction of NR by glutamine. Similarly Shiraishi *et al.* (1992) studied synthesis of NR in cultured spinach cells, which is controlled by glutamine.

From the above cited reports it is evident that NR has been extensively studied in crop plants, more so, in relation to carbohydrate supply, nitrate availability, irradiance, source of energy supply etc. In the present study, NR of *Arthocnemum indicum* Moq was characterized. The *in vivo* assay reveals that the NR system in *Arthocnemum indicum* Moq has pH optima of 7.5, K_m value of 126.66 mM, V_{max} of 0.204 ΔOD/100mg leaves/hour and the temperature coefficient (Q_{10}) is 1.36 for 20-30°C and 0.76 for 30-37°C.

Table 2.1 Characteristics of Aspartate aminotransferase.

	Leaf	Root
V_{max} (2-oxoglutarate)	0.071ΔOD/0.1mg protein/10 min.	0.054ΔOD/0.1mg protein/10 min
V_{max} (Asparate)	0.072ΔOD/0.1mg protein/10 min.	0.053ΔOD/0.1mg protein/10 min
K_m(Aspartate)	4.3 mM	5 mM
K_m (2-oxoglutarate)	0.08 mM	0.09 mM
Ea(27-37˚C)	5.75 Kcal/mole	4.64 Kcal/mole
Q_{10}	1.38	1.28
pKa	5.2and 7.55	5.2 and 7.5

2. ASPARTATE AMINOTRANSFERASE (AspAT EC: 2.6.1.1)

MATERIAL AND METHODS

Enzyme Extraction:

Plants were collected from natural habitats and brought to the laboratory in polythene bags. The plants were washed with deionized water and blotted dry. One g fresh leaf material was ground vigorously in 10 ml chilled extraction buffer (0.2M Tris-HCl pH 8, 3.5 mM $MgCl_2$ and 2.5% Polyethylene glycol) using a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 20 minutes around 0˚C±2˚C temperature. The supernatant was used as the enzyme source. The enzyme activity has been represented as change in absorbance/mg protein /unit time. The protein concentration was determined by the method of Lowry *et al.* (1951)

Enzyme Assay:

Two ml of assay mixture contained:

i) Tris-HCl (pH 8.0) 0.1M, (ii) Aspartate 20 mM,

(iii) 2-oxoglutarate 0.3 mM and (iv) Enzyme source

The above prepared assay mixture was incubated for 30 minutes at 37^{o}C. Then the reaction was stopped by adding 0.1 ml aniline citrate, which converts oxaloacetate to pyruvate. After 20 minutes 2,4-Dinitrophenylhydrazine (1ml) was added which converted pyruvate to hydrazone. This hydrazone in alkaline medium was read at 525nm. The activity of AspAt was expressed as ΔOD/0.1mg protein/30 minutes.

AspAT of leaves and root of *Arthocnemum indicum* Moq were assayed at different pH ranging from 4 to 9. Tris-HCl buffer was used for the assay. The enzyme from both root and leaves were subjected to variation of substrates *viz.* aspartate concentration ranging from 5 mM to 50 mM and 2-oxoglutarate concentration ranging from 0.01 mM to 0.1 mM.

RESULTS

(1) Effect of pH variation on AspAT activity:

The activity of the enzyme AspAT extracted from the leaves and the roots of *Arthocnemum indicum* Moq as a function of change in H^+ ion concentrations is depicted in figure 2.4. The maximum activity of AspAT of leaves and the roots was seen at pH 7.5. Although the enzyme showed a two-peak response to changes in H^+ ion concentration, a pH of 7.5 was more favourable than pH 5.5, since at latter pH the enzyme activity was less by about 12% and 13% respectively. The enzyme activity was minimum at pH 4 in the leaves as well as the roots and the activity in both declined beyond pH 7.5. In view of this, in all further experiments the pH for the enzyme assay was maintained at 7.5.

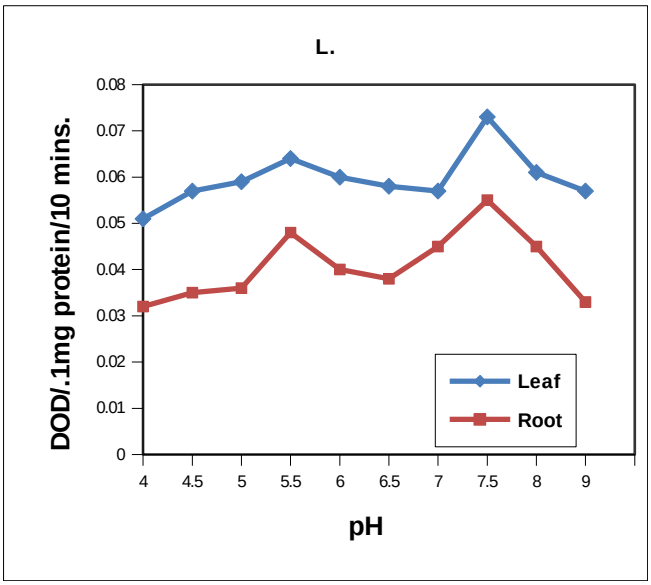


Figure 2.4. Effect of pH variation on the *in vitro* activity of AspAT in *Arthocnemum indicum Moq.*

(2) Effect of aspartate variation on AspAT activity:

Aspartate is an amino acid, which supplies an amino group in transamination reaction to a keto acid, and a new amino acid is formed. Figure 2.5 depicts data on the effect of aspartate variation on the aspartate aminotransferase activity. The transamination reaction of aspartate aminotransferase from the leaves and the roots of *Arthocnemum indicum* Moq plants showed a typical hyperbolic pattern response. The rate of AspAT activity were linear upto 20 mM aspartate concentration, further increase in aspartate concentration resulted in a steady state. The concentration of aspartate was therefore maintained at 20 mM for the assay of the enzymes extracted from the leaves and the roots of *Arthocnemum indicum* Moq in further studies. The rate of reaction was calculated in terms of ΔOD/0.1mg protein/10 min. The V_{max} and K_m of leaves and the roots of *Arthocnemum indicum* Moq are tabulated in Table 2.1.

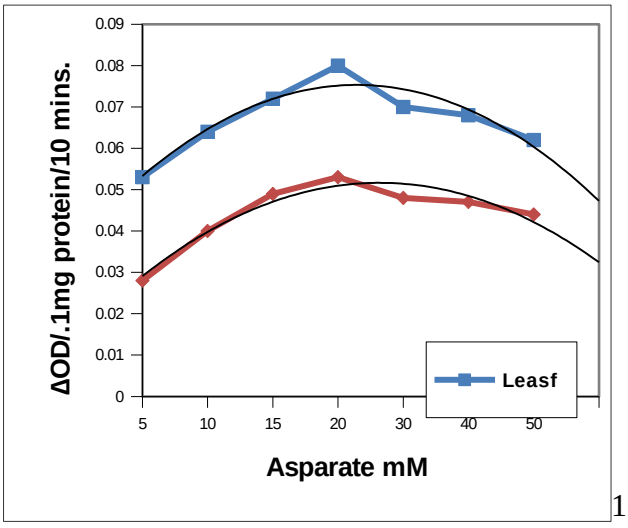


Figure 2.5. Effect of asparate variation on the *in vitro* activity of AspAT in *Arthocnemum indicum Moq.*

(3) Effect of 2-oxoglutarate variations on AspAT activity:

2-oxoglutarate is a keto-acid involved in several metabolic pathways. In the aspartate aminotransferase system, 2-oxoglutarate participates in reductive amination as a substrate and accept amino group from aspartate to form oxaloacetic acid and glutamic acid. Figure 2.6 depicts the effect of 2-oxoglutarate on the AspAT activity from the leaves and the roots. The concentration used in the assay mixtures ranged from 0.01mM to 0.08mM 2-oxoglutarate. In the leaves the rate of AspAT activity showed a linear response upto 0.04 mM 2-oxoglutarate concentration and thereafter with increase in 2-oxoglutarate concentration it showed a steady state activity even upto 0.08 mM concentration. Whereas in the roots the initial response was very much similar to leaves upto 0.04 mM 2-oxoglutarate concentrations, but at higher concentration, the activity showed a decline.

In view of the above results, the concentration of the 2-oxoglutarate was therefore maintained at 0.04 mM for *in vitro* assay. The V_{max} and K_m for the leaf enzyme as calculated from the Michaelis-Menten formula are 0.071ΔOD/0.1mg protein/10min and 0.08 mM 2-oxoglutarate concentration and for the root enzyme it is 0.054ΔOD/0.1mg protein/10min and 0.09 mM 2-oxoglutarate concentration.

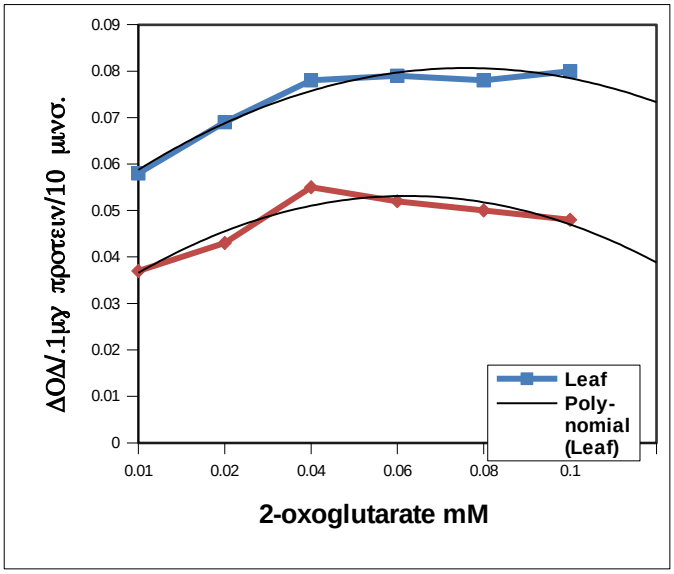


Figure 2.6. Effect of 2-oxoglutarate on the *in vitro* activity of AspAT in *Arthocnemum indicum Moq.*

3. ALANINE AMINOTRANSFERASE (AlaAT EC 2.6.1.2)

MATERIAL AND METHODS

Enzyme extraction was carried out as described for AspAT. AlaAT was estimated by the colorimetric method described by Reitman and Frankel (1957).

Enzyme Assay:

A total volume of 2 ml assay mixture contained:

Tris-HCl buffer (pH 7.0) 0.2M, Alanine 100 mM,

2-oxoglutarate 0.34 mM and Enzyme source

The assay mixture was incubated for 30 minutes at 37°C. The reaction was then stopped by 1 ml of the chromogen solution 2-4 Dinitrophenyl hydrazine. The hydrazone formed in the alkaline medium was spectrophotometrically read at 525 nm. The specific activity is calculated in terms of Δ OD / 0.1 mg protein/hour.

AlaAT of leaves and root of *Arthocnemum indicum* Moq were assayed at different pH ranging from 4 to 9. Tris-HCl buffer was used for the assay. The enzyme from both root and leaves were also subjected to variation of substrates *viz.* alanine concentration ranging from 40 mM to 200 mM and 2-oxoglutarate concentration ranging from 0.01mM to 0.1mM.

RESULTS

(1) Effect of hydrogen ion concentration on AlaAT activity:

AlaAT extracted from the leaves and the root of *Arthocnemum indicum Moq.* was subjected to variation in the H^+ concentration in the range of pH 4 to 9. AlaAT from the leaves as well as the root showed peak activity at pH 7. The enzyme is less active in acidic as well as alkaline range (Figure 2.7).

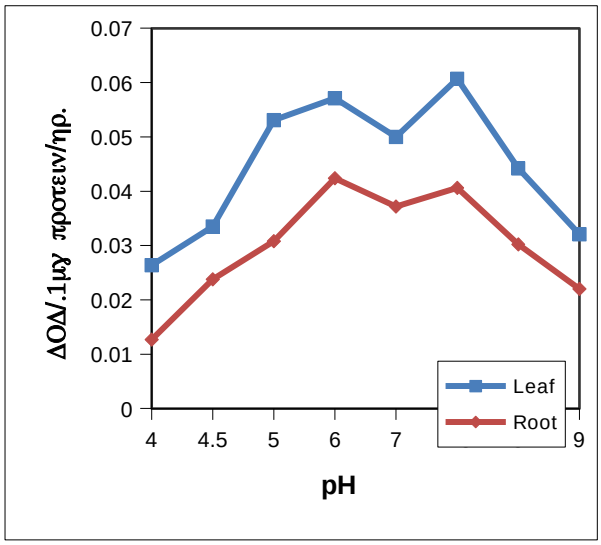


Figure 2.7. Effect of pH variation on the *in vitro* activity of AlaAT in *Arthocnemum indicum* Moq

(2) Effect of alanine variation on AlaAT activity:

Figure 2.8 represents the AlaAT activity of the enzyme extracted from the leaves and the roots of *Arthocnemum indicum* Moq when studied as a function of alanine concentration in the assay mixture.The enzyme extracted from the leaves appears to be highly reactive when compared with that from the root enzyme. The AlaAT system reaches saturation at 100 mM alanine concentration in the reaction mixture (Figure 2.8). Further increase upto 140 mM alanine concentraion resulted in a decline in the AlaAT activity. The V_{max} and K_m for the leaves calculated from the formula are 0.062ΔOD/0.1 mg protein/hour and 31.3 mM alanine respectively.

The enzyme from the root shows a linear response to the increase in the concentration of alanine in the assay mixture upto 140 mM. However, the activity falls suddenly at 200 mM alanine concentration. The V_{max} and K_m calculated from the formula are summarized in Table 2.2.

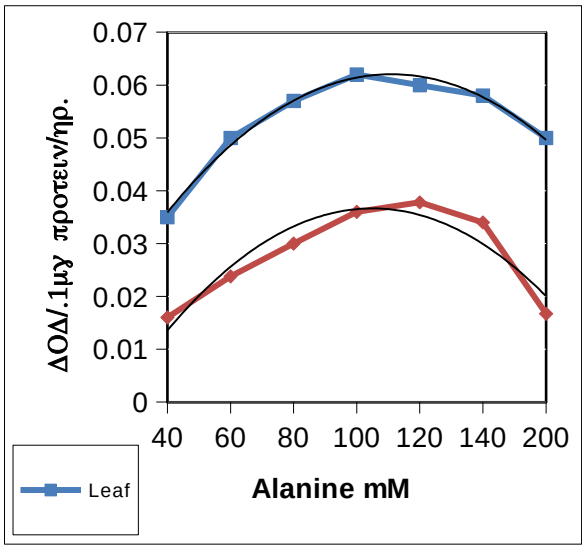


Figure 2.8. Effect of Alanine variation on the AlaAT activity in *Arthocnemum indicum Moq.*

(3) Effect of 2- oxoglutarate variation on AlaAT activity:

AlaAT utilizes alanine and 2-oxoglutarate as a substrate when the reaction is studied in the direction of the synthesis of glutamate and pyruvate. In this system, alanine donates the amino group through aldimine/ketimine linkage with the enzyme to 2-oxoglutarate. With this, the aldehyde form of the enzyme is once again regenerated, for the second sequence of reactions. In this set of experiments AlaAT activity from the leaves and the root of *Arthocnemum indicum* Moq was studied as a function of 2-oxoglutarate concentration in the assay mixture. During these studies, the other parameters for the assay were maintained at a constant level.

Figure 2.9 represents the Michaelis-Menten plot of the data on the effect of 2-oxoglutarate variations on the AlaAT activity. The activity is maximum at 0.6 mM 2-oxoglutarate concentration in the assay mixture for both leaves and the root. Beyond 0.6 mM 2-oxoglutarate concentration has inhibitory effect on the AlaAT activity. At 1 mM 2-oxoglutarate concentration the AlaAT activity drops by 43% in the leaves and 32% in the root.

The V_{max} and K_m for the enzyme extracted from the leaves and the roots are summarized in the Table 2.2.

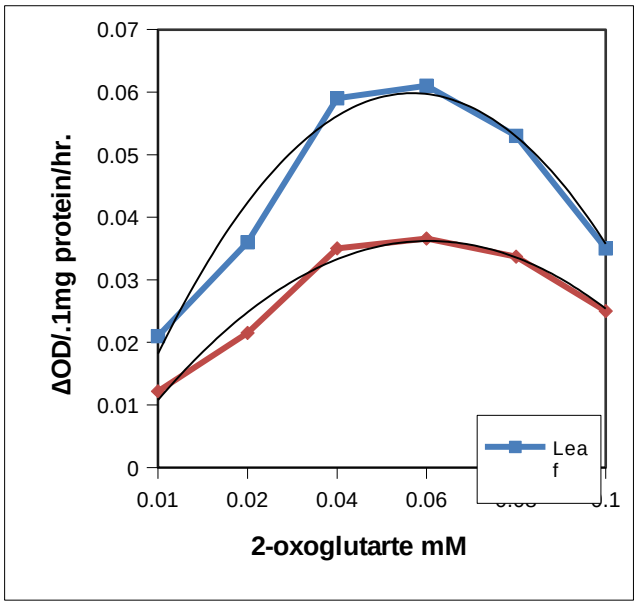


Figure 2.9.Effect of 2-oxoglutarate on the activity of AlaAT (GPT) in *Arthocnemum indicum Moq.*

Table 2.2 Characteristics of alanine aminotransferase.

	Leaf	Root
V_{max}(2-oxoglutarate)	0.061ΔOD/0.1mg protein/hour	0.036ΔOD/0.1mg protein/hour
V_{max}(Alanine)	0.062ΔOD/0.1mg protein/hour	0.0393ΔOD/0.1mg protein/hour
K_m (2-oxoglutarate)	0.09 mM	0.12 mM
K_m (Alanine)	31.3 mM	56.5 mM
Ea (27-37˚C)	7.792 Kcal/mole	9.54 Kcal/mole
Q_{10}	1.524	1.676
pKa	6.7	6.9

DISCUSSION

Higher plants are able to incorporate nitrogen of nitrate and nitrite or ammonium ions into amino acids. The metabolism of amino acids commits them to a variety of fates. Their biosynthetic pathway leads to proteins whereas their degradation leads to deamination. Amino acid catabolism is initiated by the removal of the amino group by transamination, thereby generating 2-oxoglutaric acid. The most common acceptor is the citric acid intermediate, 2-oxoglutarate, which is transaminated to glutamate. Braunstein and Kritzman (1937) were the pioneers to demonstrate the transamination reaction. Later, much information has accumulated regarding the occurrence and the properties of amino tranferases in bacteria, animal and plant tissues. These enzymes are of importance in the biosynthesis, degradation and formation of important products of amino acids.

Unlike the aminotransferases from the animal sources plant amino transferases have not been studied extensively for their characterization due to the presence of inhibiting and denaturing factors such as organic acids and phenolic compounds. With the result that a number of different aminotransferases are known to operate in a single plant species, but their substrate specificity is not well understood. In a few cases, the potential amino acid and ketoacid substrates for the different amino transferases present in a single species were examined in detail. Wilson *et al.* (1954) demonstrated in white lupin seedlings transamination reactions with 17 free protein amino acids using 2-oxoglutarate as the amino group acceptor. Subsequently, Forest and Wightman (1972) investigated potential transamination reactions of 22 free protein amino acids with 2-oxoglutarate, oxaloacetate, pyruvate or glyoxylate as amino group acceptors in the roots, cotyledons and shoots of *Phaseolus vulgaris* seedlings..

Gamborg and Keeley (1966) and Forest and Wightman (1972) observed that in plant tissues much of the aminotransferase activity is found in the cytosol. Huang *et al.* (1976) working on spinach leaves observed that out of the total AspAT activity 45% was due to chloroplastic isozyme, 26% due to cytosol isozyme, 19% due to mitochondrial isozyme and 3-10% due to peroxisomal isozyme.

Aminotransferases participate in an electron shuttle system, which transfers reducing equivalents between the cytosol and other compartments such as the chloroplasts and the peroxisomes (Haber, 1974). A role was proposed for AspAT and AlaAT in the C_4 pathway of photosynthesis (Hatch and Mau, 1973). The level of AspAT and AlaAT in C_4 plants is 20 times more than that of C_3 plants.

In the present investigation on transaminases of *Arthocnemum indicum* Moq *viz.* AspAT and AlaAT, it was observed that the enzymes from the leaves and the root were active at 37°C and both the enzymes showed two pH optimas. AspAT utilizes aspartate and 2-oxoglutarate as substrates with K_m values 0.08 mM (leaf) and 0.09 mM (root) of 2-oxoglutarate and 4.3 mM (leaf) and 5 mM (root) of aspartate. AlaAT utilizes alanine and 2-oxoglutarate as substrates with K_m values of 0.09 mM and 0.12 mM of 2-oxoglutarate; and 31.3 mM and 56.5 mM of alanine for leaf and root respectively.

4. GLUTAMINE SYNTHETASE (GS) (EC: 6.3.1.2)

MATERIAL AND METHODS

Chemicals:

(1) **Extraction buffer**-0.1M Tris-HCl containing 1mM EDTA, 1mM cystein and 0.1% v mercaptoethanol (pH 7.0)

(2) **Assay buffer**-0.1M Tris-HCl, pH 7.0

(3) $MgSO_4.7H_2O$ 0.1M (pH adjusted to 7.0 with NaOH)

(4) Hydroxylamine-0.1M (pH adjusted to 7.0 with NaOH)

(5) ATP-0.06M.

(6) Sodium glutamate- 0.6M.

(7) Ferric chloride reagent- Prepared from equal volumes of 10% $FeCl_3.6H_2O$ in 0.2 N HCl, 24% Trichloroacetic acid and 50% HCl.

GS activity was estimated by the method of Elliot (1955) with some modifications.

Enzyme Extraction:

Enzyme extraction was carried out as described for AspAT with extraction buffer as given above for GS.

Enzyme Assay:

Initial 3ml of assay mixture consisted of:

Assay buffer 0.1M, $MgSO_4.7H_2O$ 7.5 mM, Hydroxylamine 2.5 mM,

ATP 7.5 mM, Sodium glutamate 75 mM and Enzyme source

The reaction was initiated by the addition of sodium glutamate, which was replaced in the blank by buffer. After incubation at 30°C for 30 minutes, 1ml of ferric chloride reagent was added to each tube and the absorbance was read at 540 nm in Systronic PC Based UV/VIS Spectrophotometer. The protein content of the enzyme was estimated by the method of Lowry *et al.* (1951). The specific activity of the enzyme is expressed as ΔOD/mg protein/30 minutes.

GS extracted from the leaves of *Arthocnemum indicum* Moq was assayed at different pH ranging from 6 to 8.5. The buffer was prepared from Tris-HCl. Effect of variation of substrates

concentrations were also studied. ATP concentration was varied between 1.5 mM and 10.5 mM, Na-glutamate was varied between 75 mM and 375 mM and hydroxylamine concentration was varied between 0.625 mM and 5 mM in the assay mixture.

RESULTS

(1) Effect of H^+ ion variation on glutamine synthetase activity:

Glutamine synthetase (GS) enzyme extracted from the leaves of *Arthocnemum indicum* Moq was subjected to changes in H^+ ion concentration in the range of pH 6 to 8.5 (Figure 2.10). The enzyme showed maximum activity at pH 7. The enzyme was less active in lower range of pH, i.e. acidic range and as well as higher range of pH i.e. neutral and alkaline range. When the pH of reaction mixture was changed from pH 7 to pH 6, the GS activity was lowered by 85%.

In further experiments, the assay mixture consisted of buffer of pH 7. The specific activity of the GS enzyme is represented as ΔOD/mg protein/30 minutes.

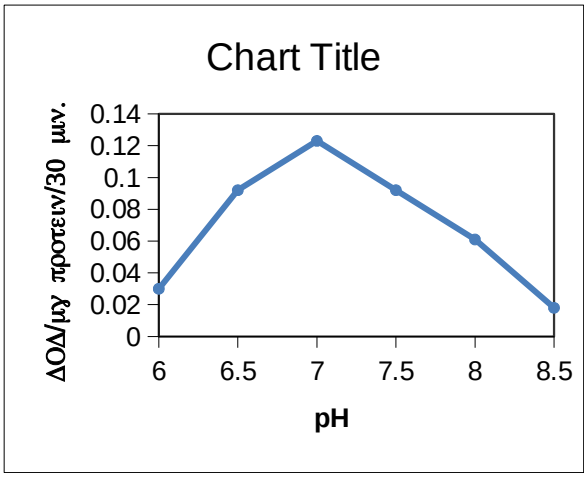


Figure 2.10. Effect of pH variation on the *in vitro* activity of glutamine synthetase in *Arthocnemum indicum Moq.* Leaves

(2) Effect of ATP variation on glutamine synthetase activity:

The GS catalyses the ATP dependent formation of glutamine from glutamate and hydroxylamine. Figure 2.11 represent the rate of activity of the GS extracted from the leaves of *Arthocnemum indicum* Moq as a function of ATP concentration in the assay medium. The enzyme activity had a linear relationship with ATP till a concentration of 7.5 mM, and then reached a steady state. The V_{max} obtained from the graph for 10.5 mM ATP concentration in the assay mixture is 0.141 ΔOD/mg protein/30 minutes with a corresponding K_m of 3.1 mM ATP.

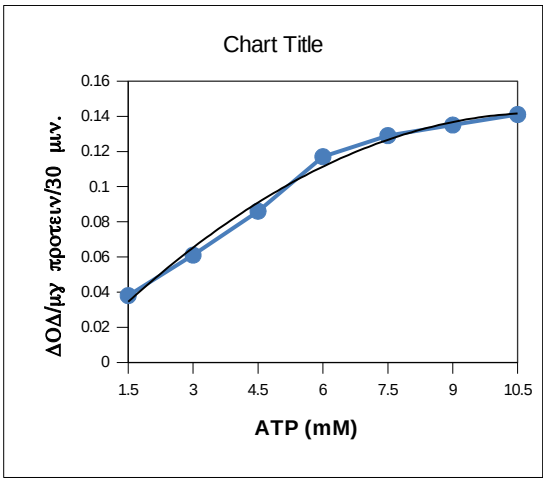


Figure 2.11. Effect of ATP variation on the *in vitro* activity of glutamine synthetase in *Arthocnemum indicum Moq.* in leaves.

(3) Effect of hydroxylamine variation on glutamine synthetase activity:

The hydroxylamine is a substrate, which provides the necessary amino group for the production of glutamine from the glutamate by GS activity in presence of ATP. The GS enzyme extracted from the leaves of *Arthocnemum indicum Moq*, was studied as a function of hydroxylamine concentration. Figure 2.12 depicts the rate of GS activity. The plot of hydroxylamine variation showed a linear relationship upto 2.5 mM hydroxylamine concentration. Further, beyond 3.75 hydroxylamine concentration showed a decrease in GS activity. The V_{max} from the formula for 3.75 mM hydroxylamine concentration in the assay mixture is 0.135 ΔOD/mg protein/30 minutes with a corresponding K_m 0.63 mM hydroxylamine (Table 2.3).

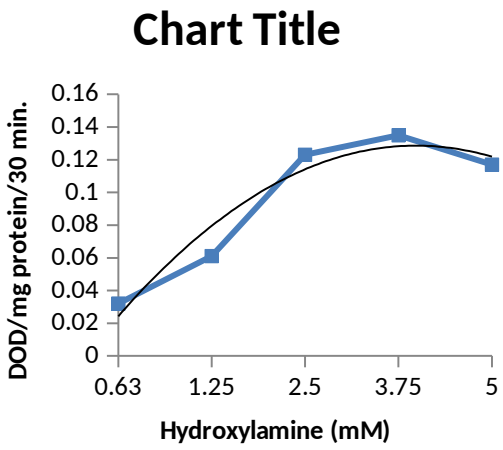


Figure 2.12. **Effect of hydroxylamine variation on the *in vitro* activity of glutamine synthetase in *Arthocnemum indicum Moq.* leaves**

(4) Effect of Na-glutamate variation on glutamine synthetase activity:

In GS system the Na glutamate accept the amino group from the hydroxylamine and is converted into glutamine in presence of energy mediator ATP. The enzyme extracted from the leaves of *Arthocnemum indicum Moq* was subjected to variation in Na-glutamate concentration. The enzyme showed a rapid increase in the activity in the range of 75 mM to 225 mM Na-glutamate concentration. Thereafter, the enzyme attained a steady state (Figure 2.13).

The V_{max} obtained from the calculation is 0.135 ΔOD/mg protein/30 minutes with a corresponding K_m of 75 mM Na-glutamate (Table 2.3).

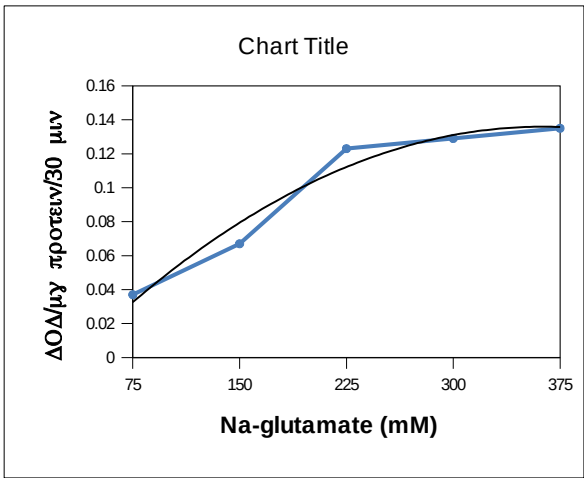


Figure 2.13. Effect of Na-glutamate variation on the *in vitro* activity of glutamine synthetase in *Arthocnemum indicum Moq*.leaves.

Table 2.3 Characteristics of Glutamine synthetase.

V_{max} (Na-glutamate)	0.135ΔOD/mg protein/30 min
V_{max} (ATP)	0.141ΔOD/mg protein/30 min
V_{max} (Hydroxylamine)	0.135ΔOD/mg protein/30 min
K_m (Na-glutamate)	75 mM
K_m (ATP)	3.1 mM
K_m (Hydroxylamine)	0.63 mM
pKa	6.9

DISCUSSION

Because of the central importance of GS in the assimilation of NH_4^+ derived from such reactions as nitrate reduction, photorespiration, protein degradation and nitrogen fixation, GS has been extensively studied in higher plants. There have been many studies with various higher plants on occurrence of GS isoform and their localization (Botella *et al.,* 1988) as well as their biochemical characteristics (Hirel and Gadal, 1980), gene structure and expressions (Lightfoot *et al.,* 1988;

Sakamoto *et al.,* 1989; McGrath and Coruzzi, 1991, Becker *et al.,* 1992) and physiological functions (Wallsgrove *et al.,* 1987; Edwards *et al*., 1990; Kamachi *et al.,* 1992 b).

In halophytes, like *Suaeda maritima* var. macrocarpa when grown in optimal saline conditions exhibits a high GS activity (Boucaud and Billard, 1979). Variations in the level of GS activity have been recorded in *S. maritima* grown in the lower marsh, drift marsh and upper marsh (Stewart and Rhodes, 1978). While plants located in the lower marsh, exhibits maximum GS activity. The enzyme acivity is at a lower level in the drift lime zone and at the lowest level in plants of the upper marsh. In various halophytes examined (*Aster tripolium, Halimone portulacoides, Honkenya peploides, Plantago maritima, Puccinella maritima, Salicornia europa, Spartina anglica, Spergularia media, Suaeda maritima, Triglochin maritima*) the levels of GS and GDH decrease in roots in response to an increase in external salinity. On the other hand, in the shoot tissues, an increase in external salinity brings about a corresponding increase in the level of GS activity. According to Stewart and Rhodes (1978) such changes possibly reflect the tendency for the shoot to play a greater role in nitrogen assimilation under saline conditions

Zink (1989) observed increase in GS activity with an increase in NO_3^- or NH_4^+ in the growth medium. NH_4^+ ions are also responsible for the regulation of GS activity by adenylation and deadenylation mechanism (Streicher *et al.,* 1974).

GS activity has been reported to decrease with plant growth (Lara *et al.,* 1983; Canovas *et al.,* 1986). Cullimore and Bennet (1988) suggested that changes in GS activity during development are because of differential expression of isoenzymes.

An increase in GS activity in salt tolerant plants and decrease in the activity in susceptible plants under saline conditions has been reported by Rakova *et al.* (1978 a) and Bottasin *et al.* (1985). According to Bottasin *et al.* (1985), salt resistance depends on the capacity of plants to withstand the inhibition of GS by NaCl, as salt adaptation in halophyte is the shift of nitrogen metabolism towards glutamine route. In accordance with the findings of Bottasin (1985), the resistance to salt is well exhibited by *Arthocnemum indicum* Moq plant as it has shown good GS activity.

5. GLUTAMATE DEHYDROGENASE (GDH) (EC: 1.4.1.2)

MATERIAL AND METHODS

Enzyme Extraction:

Enzyme extraction was carried out as described for AspAT with chilled extraction buffer (0.1 M Tris-HCl, pH 8)

GDH activity was estimated by the method described by Chou and Splittstoesser (1972).

Enzyme Assay:

3 ml of assay mixture contained:

Tris-HCl buffer (pH 8) 0.1M, 2-oxoglutarate 5 mM, NADH 0.1 mM

NH_4Cl 5 mM and Enzyme source.

The reaction was initiated by the addition of NH_4Cl. The enzyme activity was measured as the decrease in NADH absorbance at 340 nm using Systronic PC Based UV/VIS Spectrophotometer. GDH activity is expressed as ΔOD/0.1 mg protein/ 15 minutes.

GDH extracted from the leaves of *Arthocnemum indicum Moq* was assayed at different pH ranging from 6.5 to 9.The buffer was prepared from Tris-HCl. Effect of variation of substrate concentrations were also studied. 2-oxoglutarate concentration was varied between 1.25 mM and 7.5 mM, NADH was varied between 0.025 mM and 0.2 mM and NH_4Cl concentration was varied between 2.5 mM and 20 mM in the assay mixture.

RESULTS

(1) Effect of H^+ ion variation on GDH activity:

Glutamate dehydrogenase enzyme extracted from the leaves of *Arthocnemum indicum Moq* was subjected to changes in the H^+ ion concentration in the range of pH 6.5 to 9.0 (Figure 2.14).

The GDH system from the leaves seems to be more sensitive to the changes in the pH in the alkaline range. This is evident from the observation that there is more than 95% increase in the activity of the enzyme when the pH of the medium is altered from pH 7.0 to 7.5. However, at pH

between 8.0 and 9.0 the enzyme showed a decrease in the activity in comparison with activity at pH 7.5 and showed a 30% to 39% lower response. On the basis of these results, it was decided to use pH 7.5 for all further studies.

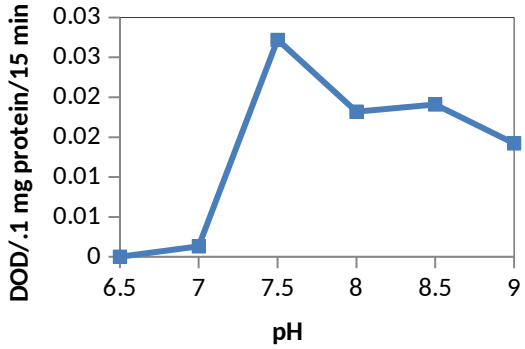


Figure 2.14. Effect of pH variation on the *in vitro* activity of GDH in *Arthocnemum indicum Moq*.leaves.

(2) Effect of NADH variation on GDH activity:

The activity of GDH was studied in the direction of synthesis of glutamate in which 2-oxoglutarate served as the acceptor of NH_4^+, provided through ammonium chloride, in the assay mixture.

Figure 2.15 depicts the rate of activity of the enzyme extracted from the leaves of *Arthocnemum indicum Moq* as a function of NADH concentration in the assay medium. The plot showed a linear relationship upto 0.05 mM NADH concentration, and then

followed a typical hyperbolic pattern with the system apparently indicating the tendency towards saturation beyond a concentration of 0.1 mM of NADH in the assay medium. The V_{max} obtained from the Michaelis-Menten formula is 0.039 ΔOD/0.1 mg protein/15 minutes with corresponding K_m of 0.037 mM NADH (Table 2.4).

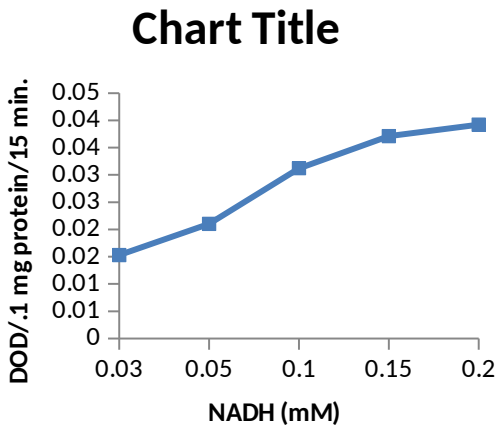


Figure 2.15. Effect of NADH variation on the *in vitro* activity of GDH in *Arthocnemum indicum Moq.*leaves.

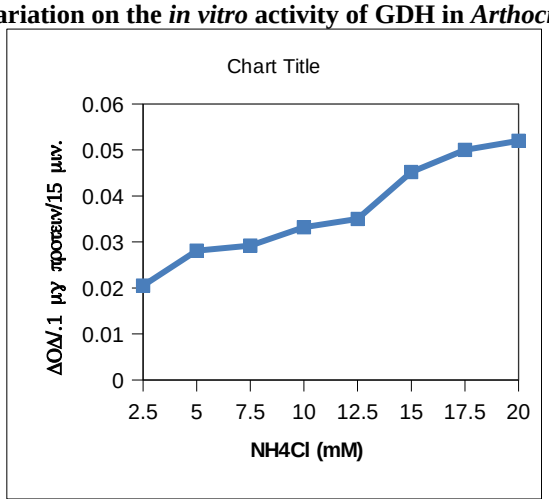


Figure 2.16. Effect of NH4Cl variation on the *in vitro* activity of GDH in *Arthocnemum indicum Moq.*leaves.

(3) Effect of NH_4Cl variation on GDH activity:

Ammonium chloride provides the amino group for the reductive amination of 2-oxoglutarate to yield glutamate. The figure 2.16 depicts the data on the effect of NH_4Cl variation of the GDH system of the leaves of *Arthocnemum indicum Moq.*

Figure 2.16 reveals that the GDH extracted from the leaves when studied as a function of NH_4Cl concentration followed a peculiar two step of activation. At lower concentration of NH_4Cl, upto 10 mM, the system apparently became saturated. The apparent K_m at this stage is 2.5 mM NH_4Cl. When the concentration of NH_4Cl was further increased to 20 mM, a second saturation became apparent with K_m 5.2 mM NH_4Cl. The maximum velocity i.e. V_{max} at these saturations are 0.035 and 0.052 ΔOD/0.1 mg protein/15 minutes (Table 2.4).

(4) Effect of 2- oxoglutarate variation on GDH activity:

2-oxoglutarate is a keto acid used in several metabolic pathways. In the GDH system, 2-oxoglutarate participates in reductive amination as a primary substrate and accepts amino group to form glutamate as the product. Figure 2.17 represents data on the effect of 2-oxoglutarate variations on the GDH systems obtained from the leaves of *Arthocnemum indicum Moq.*

The Michaelis-Menten plot gives a typical hyperbola (Figure 2.17). The GDH system showed a linear relation upto 0.5 mM 2-oxoglutarate concentration, further increments in the 2-oxoglutarate concentration showed a decline in the GDH activity. The K_m as calculated from the data is 1.2 mM 2-oxoglutarate activity with a corresponding V_{max} is 0.027 ΔOD/0.1 mg protein/15 minutes.

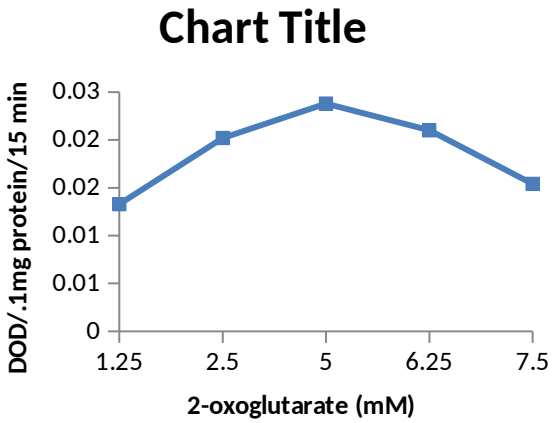


Figure 2.17. Effect of 2-oxoglutarate variation on the *in vitro* activity of GDH in *Arthocnemum indicum Moq.*leaves.

Table 2.4 Characteristics of Glutamate dehydrogenase.

V_{max} (2-oxoglutarate)	0.0273 ΔOD/0.1mg protein/ 15 min.
V_{max} (NADH)	0.0392 ΔOD/0.1mg protein/15 min.
V_{max} (NH_4Cl)	0.035 ΔOD/0.1mg protein/15 min. 0.052 ΔOD/0.1mg protein/15 min.
K_m (2-oxoglutarate)	1.2 mM
K_m (NADH)	0.037 mM
K_m (NH_4Cl)	2.5 mM and 5.2 mM
pKa	7.6

DISCUSSION

GDH has been studied extensively since glutamate and 2-oxoglutarate not only occupy pivotal positions in protein metabolism but also constitute intermediates in several pathways. GDH provides a route for incorporation of nitrogen into organic compounds and thus it establishes a link between carbohydrate and amino acid metabolism.

The reaction mechanism of plant GDH systems is proposed as the compulsory ordered binding of NADH, 2-oxoglutarate and NH_4^+ followed by the release of glutamate and NAD^+ and *vice versa* for either direction (Srivastava and Singh, 1987).

GDH systems, from bacterial, animal, fungal and plant sources that have been studied extensively are known to be regulated either by induction, repression effects or by allosteric effects. The effectors can be divided into three general classes, purine nucleoside, di and triphosphates (ADP, GDP, GTP etc.), substrates and coenzymes and certain steroid hormones and lipids (Smith *et al.,* 1975). GDH from plant sources appears to be an allosteric enzyme in which the properties of the enzyme can be modified by the availability of effectors like Ca^{+2} (Yamaya *et al.,* 1984).

Amino acids affect the activity of GDH in various tissues of plants. Glutamate increases the aminating enzyme activity in *Lemna* (Rhodes *et al.,* 1976) and the deaminating activity in *Zea* leaves (Singh and Srivastava, 1983). It has been suggested that glutamate acts as a competitive inhibitor with respect to 2-oxoglutarate and also as an allosteric modulator of the enzyme, either directly or via some metabolic products. The activity of GDH is inhibited by exogenously supplied sugars such as glucose and fructose (Sahulka and Lisa, 1980) and sucrose (Nauen and Hartmann, 1980); probably the supply of sugars results in the increased activity of GS, which in turn represses the GDH activity (Sahulka and Lisa, 1980). The supply of ammonia, nitrate and urea as nitrogen sources influences GDH activity in plants. According to Givan (1979) isozyme of GDH detoxifies the excess ammonia absorbed by the tissue, since ammonia accumulation is harmful to the metabolism of the cells. Singh and Srivastava (1982) are of the opinion that the increase in GDH activity may not be only due to the *de novo* synthesis but probably also due to allosteric regulation by ammonium as there is an *in vitro* stimulation of GDH activity. It has been

suggested that ammonia produced by proteolysis during senescence might be responsible for the *de novo* synthesis of GDH enzyme (Lauriere and Daussant, 1983).

GDH catalyses the reversible conversion of 2-oxogluarate and L-glutamate for the entry of ammonia into the organic acid cycle as well as for its release (Srivstava and Singh, 1987). The aminating role of GDH in the presence of high levels of NH_4^+ in preventing ammonia toxicity is well known (Choe and Thimann, 1977).

In 1987, Yamaya and Oaks have summarized the role of GDH in leaf metabolism. According to these workers the mitochondrial GDH functions in the direction of the biosynthesis of glutamate, and they have, therefore, proposed that one function of mitochondrial GDH is to synthesize glutamate from some of the NH_4^+ released by photorespiration. This glutamate thus represents a reserve for the use in biosynthetic reaction.

6. ELECTROPHORETIC STUDIES

MATERIAL AND METHODS

The plant material of *Arthocnemum indicum Moq.*were collected from the natural habitat and brought to the laboratory in polythene bags and used for the study of protein profile and isozymes of glutamate dehydrogenase (GDH) and aspartate aminotransferase (AspAT).

Enzyme Extraction:

Enzymes GDH and AspAT were extracted as described earlier with difference that minimum quantity of buffer was used to get concentrated extract.

Polyacryamide Gel Electrophoresis:

PAGE was carried out at low temperature (4˚C±1˚C) according to the method of Zweig and Whitaker (1967). The gels used for the separation of anionic samples in the present experiment were 7.5% running gels; stacking pH 8.3. The gels were cast in neutral glass plates (7 cm long, 8 cm wide and 0.75 mm thickness with 10 wells) of vertical electrophoresis unit (GEL TECH VERTICAL UNIT). The gel was polymerized with polymerizing catalysts like ammonium

persulphate and TEMED (accelerator of polymerization of gel). For the electrophoresis, the buffer was prepared using 6g Tris-HCl, 28.8 g glycine and volume was made to one liter. The buffer was diluted 10 times and pH was adjusted to 8.3 before use.

The enzyme extract was mixed with bromophenol blue as a front marker and loaded in a polymerized gel wells. The entire gel plate was fitted in the electrophoretic unit, it was flooded with running buffer and the anodal, and cathodal ends were connected to the power system. The electrophoretic run was carried out at a current of about 2 mA /well, at a constant voltage of 150 volts. The run was carried out for about 90 minutes until the bromophenol marker front migrated to the other end of the plate. Subsequently, the gel was loosened with a jet of water and removed from the glass plate.

Detection of Proteins:

The gels were fixed in 10% trichloro acetic acid for 20 minutes and the bands of proteins were detected by staining with 0.1% coomasie brilliant blue (R-250) in methanolic solution (Methyl alchol: water: glacial acetic acid:: 1: 1: 2) for 30 minutes. Gradual destaining of the gels was carried out by placing them in 7% acetic acid until the distinct protein bands appeared on the gels.

Detection of GDH:

Isoenzymes of GDH were detected on the gels by the method of Brewer and Singh (1970).

The staining mixture was prepared by dissolving 0.25 M L-glutamic acid, 1.5 mM NAD, 0.163 mM phenazine methosulphate (PMS) and 0.43 mM nitroblue tetrazolium (NBT) in 0.125M phosphate buffer (pH 9.0). The staining mixture was prepared shortly before use as the reactants are less stable in solution.

For detecting GDH isoenzymes, the gels were incubated in the staining mixture at 37˚C. GDH activity generates NADH, which reduces phenazine, which in turn reduces NBT. The reduced NBT is an insoluble formazan, which is coloured.

Detection of AspAT:

Isoenzyme of AspAT were detected on the gels by the method of Brewbaker *et al.* (1968) using fast violet B salt. The fast violet B salt is specific for oxaloacetic acid and forms a chromogenic compound.

The following aquous stock solution were prepared

(i) 0.2 M phosphate buffer, pH 7.5

(ii) 5 mg/10 ml pyridoxal-5-phosphate

(iii) 3 g/100 ml bovine albumin

(iv) 0.2 M aspartic acid, pH adjusted to 7.5 with KOH.

(v) 0.1 M 2-oxoglutaric acid, pH adjusted to 7.5 with KOH.

Pyridoxal-5-phosphate solution was stored frozen and other solution can be kept in the refrigerator without freezing.

The following solutions were prepared just before use.

(a) Diazonium salt solution-120 mg of fast violet B salt was dissolved in 8 ml of water.

(b) 1 g of polyvinylpyrrolidone (PVP) was mixed with 22.0 ml of solution (i), 0.8 ml of solution (ii), 1.6 ml of solution (iii), 6.8 ml of solution (iv) and 2.0 ml of solution (v).

For detecting the isoenzymes of AspAT, the gels were preincubated in solution (b) for 30 minutes. After preincubation the gels were transferred in solution (a). Pinkish red bands developed immediately indicating the zones of transaminase activity.

RESULTS AND DISCUSSION

Electrophoretic studies of proteins, GDH and AspAT enzyme of *Arthocnemum indicum Moq.*

Protein profile:

Protein profile in leaves of *Arthocnemum indicum Moq* showed ten bands whereas protein profile of the root showed nine bands. On comparison of the protein profile of leaves and root, it was observed that some bands were common, *viz.* bands P1, P6, P7, P10 and P11, whereas bands P2, P4, P5, P9 and P13 were different, which were not found in the root protein profile. Similarly,

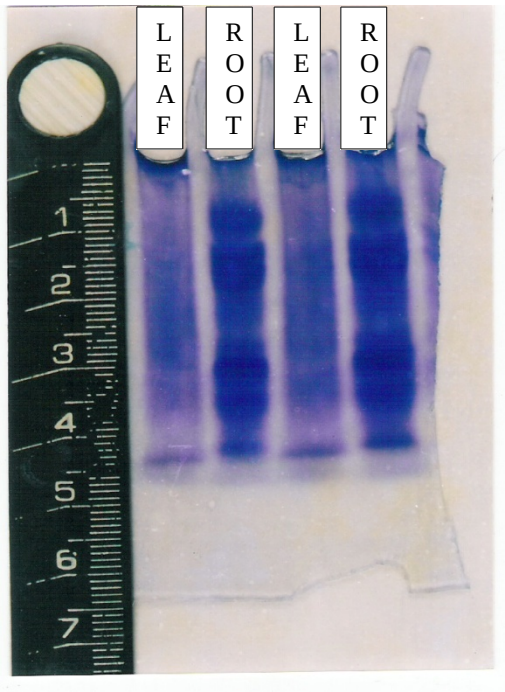
bands P3, P8, P12 and P14 of root protein profile were missing in the leaves protein profile. In all, there were P1 to P14 bands in protein profile of *Arthocnemum indicum Moq*. From the electrophoretic studies, it was observed that amount of protein in root was more; hence, root bands were more intensified on staining. (Photoplate 2.1 and Figure 2.18).

Isoenzymes:

GDH

Four bands G1, G2, G3, and G4 of Rf values of 0.05, 0.21, 0.43 and 0.67were observed representing GDH activity in the leaves of *Arthocnemum indicum* Moq. (Figure 2.19). Isoenzymic nature of GDH in higher plants is well established (Thurman *et al.*, 1965; Yue, 1969). Number of GDH isoenzymes varied amongst different plants studied and upto 7 isozymes have been reported in *Pisum sativum* (Hartman, 1973), *Medicago sativa* (Hartman *et al.*, 1973), *Arabidopsis thaliana* (Cammaerts and Jacob, 1985) and *Vitis vinifera* (Loulakakis and Angelakis, 1990). Ratajczak *et al.* (1986) observed 8 isozymes of GDH in lupin root nodules. According to Srivastava and Singh (1987), isozymic number of GDH enzyme varies with plant species as well as with nutritional and environmental conditions.

Kanamori *et al*. (1972) detected new isozymes of GDH on the zymogram of PAGE due to ammonia treatment. Similar observation of synthesis of new isozymes of GDH under high levels of ammonia in the cellular environment was made by several workers (Ratajczak *et al.*, 1977, Givan, 1979). According to Loulakakis and Angelakis (1991), ammonia induces expression of isozyme. Plants growing under high salt concentration are known to accumulate high concentration of ammonia (Strogonov, 1964), which may lead to synthesis of new isozyme of GDH. Isozymic studies of GDH in leguminous plants have suggested that the GDH isozymic pattern is the result of an adaptation of the cell to nitrogen metabolism (Mazurowa *et al.*, 1980, Avhad 2012) suggesting a physiological role of GDH isoenzymes in the regulation of nitrogen metabolism. However, in the present study three isozymes of GDH were detected in the leaves. This may be in response to halophytic nature of the plant.



Photoplate 2.1. Photograph depicting spectrum of proteins extracted from the leaves and roots of *Arthocnemum indicum Moq.*

AspAT

Electrophoretic studies of the AspAT enzyme gave a 2-band pattern in both leaves and roots of *Arthocnemum indicum Moq.* Two bands *viz.* A1 and A4 of Rf values of 0.395 and 0.54 were observed representing AspAT activity in leaves of *Arthocnemum indicum Moq* and two bands (A2 and A3), representing AspAT activity, were also observed in the root extracts of *Arthocnemum indicum Moq* of Rf values of 0.437 and 0.52 (Figure 2.20).

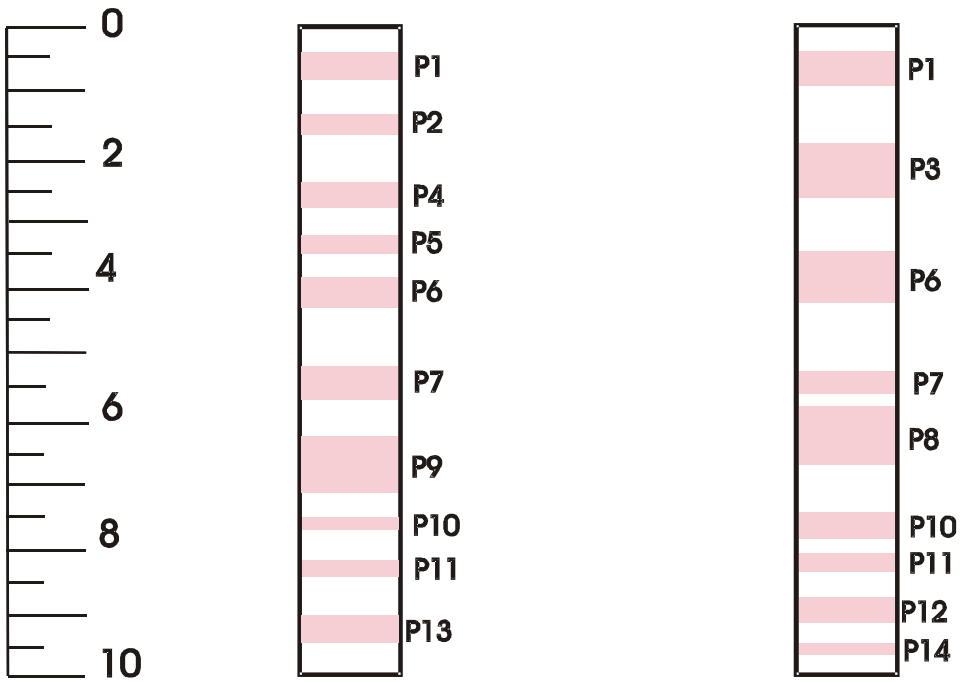


Figure 2.18. Zymogram depicting proteins extracted from the leaves and roots of *Arthocnemum indicum Moq.*

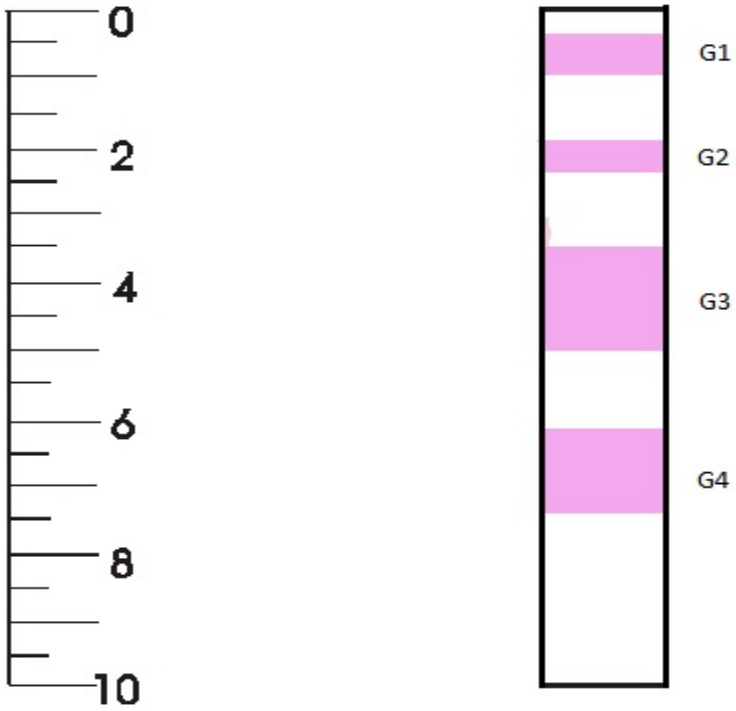


Figure 2.19 Zymogram depicting isoenzymes of Glutamate dehydrogenase (GDH) from the leaves of *Arthocnemum indicum Moq.*

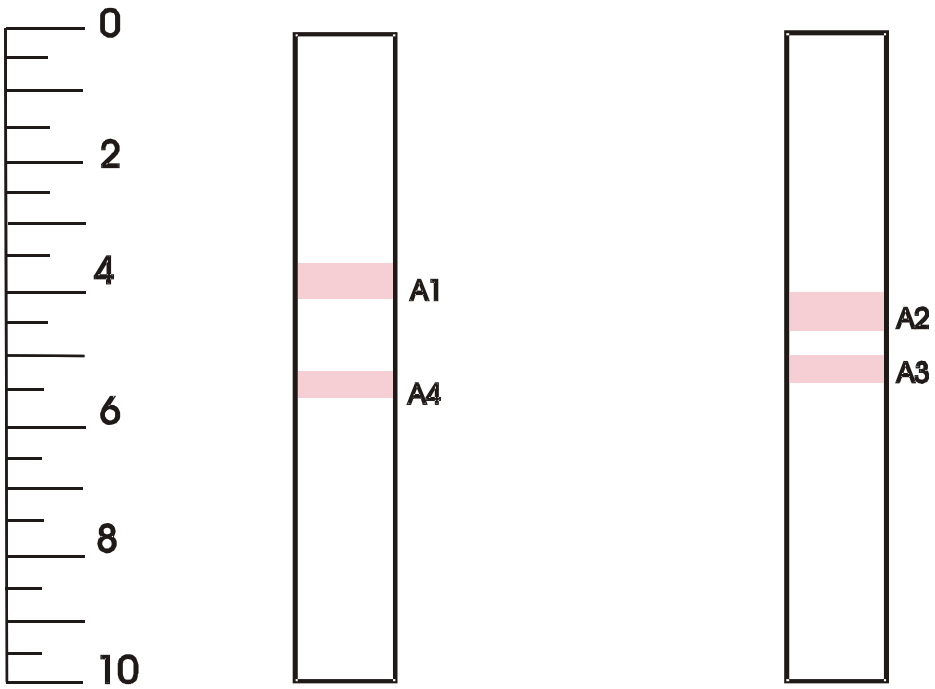


Figure 2.20 Zymogram depicting isoenzymes of Aspartate Aminotransferase (AspAT) from the leaves and roots of *Arthocnemum indicum Moq.*

CONCLUSION

1 *Arthocnemum indicum Moq.* is an erect dwarf shrub, with much branched hairy stem. It bears small thin, light green leaves. The plant belongs to family Convolvulaceae.

2 *Arthocnemum indicum Moq.* flourishes during the month of October to May in saline soil.

3 The total soluble salt present in soil supporting *Arthocnemum indicum Moq* is 6.65% which is above the normal level of salinity.

4 From the exchangeable sodium percentage (ESP) and sodium adsorption ratio values of soil supporting *Arthocnemum indicum Moq*, it can be concluded that it has an efficient sodium absorption and accumulation mechanism which is essential for maintaining osmotic potential of the cells and the tissues.

5 The soil supporting *Arthocnemum indicum Moq* contain 3.56% exchangeable calcium and 0.83% exchangeable magnesium. The high calcium content of the soil can be ascribed to the efficient sodium absorption mechanism.

6 The plant has low magnesium content because high sodium content of the soil interferes with uptake of magnesium.

7 The high total nitrogen is due to efficient nitrogen metabolism and these halophytes appear to be well adapted to condition where availability of soil nitrogen is low.

8 The *in vivo* studies of NR activity of *Arthocnemum indicum Moq* leaves revealed that leaf NR shows optimum activity at pH 7.5 with a pKa value of 6.7. The enzyme has a V_{max} of 0.204ΔOD/mg protein/hour with a corresponding K_m of 126.66 mM KNO_3.

9 *Arthocnemum indicum Moq* plant showed an efficient transaminase system both aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) were active in the leaves and root. The leaf and root AspAT showed two pH optimas, one at 5.5 and the other at 7.5. The enzyme utilizes 2-oxoglutarate and aspartate as substrates with K_m values of 0.08 mM (leaf) and 0.09 mM (root) of 2-oxoglutarate and 4.3 mM (leaf) and 5 mM (root) of aspartate.

10 Similar to AspAT, AlaAT also showed two pH optimas of 6 and 7.5.. AlaAT utilizes 2-oxoglutarate and alanine as substrates. The enzyme has a V_{max} of 0.061ΔOD/0.1mg protein/hour and 0.036ΔOD/0.1mg protein/hour for 2-oxoglutarate with corresponding K_m values of 0.09 mM and 0.12 mM for the leaf and the root enzymes respectively. The V_{max} values for aspartate are 0.062ΔOD/0.1mg protein/hour and 0.039ΔOD/0.1mg protein/hour with corresponding K_m values of 31.3 mM and 56.5 mM for the leaf and the root enzymes respectively.

11 The nitrogen assimilating enzymes *viz.* glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were active only in the leaves of *Arthocnemum indicum Moq.* Glutamine synthetase (GS) extracted from the leaves of *Arthocnemum indicum Moq* showed optimum activity at pH 7. The enzyme utilizes sodium glutamate, ATP and hydroxylamine as substrates with K_m values of 75 mM of Na-glutamate, 3.1 mM of ATP and 0.63 mM of hydroxylamine.

12 Glutamate dehydrogenase (GDH) showed optimum activity at pH 7.5. GDH utilizes 2-oxoglutarate, NADH and NH_4Cl as substrates with 1.2 mM of 2-oxoglutarate, 0.037 mM of NADH; and 2.5 mM and 5.2 mM of NH_4Cl.

13 The soluble protein when separated by electrophoresis revealed 10 bands for the leaf extracts and 9 bands for the root extracts of which 5 bands had similar Rf values.

14 The GDH enzyme extracted from the leaves of *Arthocnemum indicum Moq,* when separated by electrophoresis showed presence of 3 isoenzymes of Rf values of 0.062, 0416 and 0.656.

15 Electrophoretic studies of aspartate aminotransferase (AspAT) enzyme revealed 2 isoenzymes both in the leaf (Rf values 0.395 and 0.54) and the root (Rf values 0.437 and 0.52) of *Arthocnemum indicum Moq*.

16 From the present investigation on the analysis of soil and plant of *Arthocnemum indicum Moq* and the enzymes of nitrogen metabolism in *Arthocnemum indicum Moq* plant, it can be concluded that even though the plants of *Arthocnemum indicum Moq* grow in soil which is poor in nitrogen content, it has a high nitrogen content in the plant which is due to an efficient enzyme machinery responsible for assimilating the nitrate and ammonia available to the plant.

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