INVITRO BIOCHEMICAL STUDIES ON CERTAIN MULBERRY VERITIES OF M₅, V1, S36 AND ANANTHA

Varaprasad P.[1] and A. Vijaya Bhaskara rao[2]

[1]*Department of Sericulture, Sri Krishnadeveraya University, Anantapur-515003, A.P. India*
[2]*Department of Ecology and Environmental Sciences, Pondicherry Central University, Pondicherry*

Abstract

Some plant roots, especially cluster roots, exude carboxylates that perform acid phosphatase activity, helping to mobilize phosphorus nutrient-deficient soils. α-amylase is an enzyme that catalyses the breakdown of starch into sugars. Amylase is present in human saliva, where it begins the chemical process of digestion. Foods that contain much starch but little sugar, such as rice and potato, taste slightly sweet as they are chewed because amylase turns some of their starch into sugar in the mouth. Both α-amylase and β-amylase are present in seeds; β-amylase is present in an inactive form prior to germination, whereas α-amylase and proteases appear once germination has begun. Cereal grain amylase is key to the production of malt.

I. INTRODUCTION

Biochemical studies involves the structure, composition, and chemical reactions of substances in living systems and also study the topics as how living things obtain energy from food, the chemical basis of heredity, and what fundamental changes occur in disease. Differentiation is specific to cell type, region and organ. Therefore, identification of biochemical markers during sequential stages of organogenesis could be used to identify early events of differentiation, embryogenic potential or serve as an indication of loss of regeneration capacity with culture age. Keeping in view these facts, we have investigated the extent of biochemical and molecular variation in 4 varieties of mulberry plants, *Morus Spp* regenerated from 3- to 24-week-old auxiliary buds callus cultures. The calli were derived from cultured basal segments of auxiliary buds of a single field grown donor plants.

Carbohydrates yield energy and the carbohydrates are of special importance in plant metabolism. The principal function in organisms is production and storage of this vital commodity. Furthermore, a variety of other organic compounds are synthesized from carbohydrates. A reducing sugar is any sugar that either has an aldehyde group or is capable of forming one in solution through isomerism Carbohydrate metabolism gained importance in the physiology of a plant wherein it releases the energy locked up in the mono saccharides by breaking them down to simple carbon dioxide and water. Glutamate dehydrogenase (GDH) is an enzyme, present in most microbes and the mitochondria of eukaryotes, as are some of the other enzymes required for urea synthesis, that converts glutamate to α-ketoglutarate, and vice versa. Nitrate reductases are molybdoenzymes that reduce nitrate (NO^3) to nitrite (NO^{+2}). Nitrate molecule binds to the active site with the Mo ion in the +6 oxidation state. Electron transfer to the active site occurs only in the proton-electron transfer stage, where the Mo^{+5} species play an important role in catalysis. Acid phosphatase is a phosphatase a type of enzyme, used to free attached phosphate groups from other molecules during digestion is basically a phosphomonoesterase. It is stored in lysosomes functions when these fuse with endosomes which are acidified while they function; therefore, it has an acid pH optimum.

II. MATERIALS AND METHODS

The following biochemical studies were carried out in the leaf / explant tissue of the regenerates of four mulberry varieties for the characterization of the active growth patterns and to identify somaclonal variations in regenerations.

Metabolite estimations:

Total and reducing sugars

Total and reducing sugars were extracted from leaf tissue of *Morus Spp.* regenerants as described by Borkowska and Szezerba (1991) with slight modifications that are essential. Approximately 1 gm of leaf tissue of was homogenized into 4 ml of 10 mM sodium acetate buffer (pH 4.6) containing 0.02 mM PVP, 0.78 mM Na_2EDTA and 0.1 mM $MgCI_2$. The homogenate was centrifuged at 20,000 Xg for 15 minutes at 4°C, supernatants were recovered and were used for the estimation of total and reducing sugars. Total sugars were estimated spectrophotometrically as described by Yemm and Willis (1954) using anthrone reagent. Reducing sugars were determined according to Nelson (1944) and Somogyi (1952). Results were extrapolated from a glucose standard curve and expressed as mg of glucose g^{-1} fresh weight.

Starch

Starch content was extracted and estimated in leaf regenerates of Mulberry varieties as described by McCready *et al.*, (1950). Approximately 1 gm of leaf tissue was homogenized with 4 ml of 80% boiling ethanol (80°C) and homogenized. The homogenate was centrifuged at 5,000 Xg for 15 minutes; residues were recovered and used for the estimation of starch. Starch content was estimated spectrophotometrically as described by Yemm and Willis (1954) using anthrone reagent. Results were extrapolated from a glucose standard curve; amount of starch was calculated by multiplying the equivalent by 0.90 and expressed as mg of starch g^{-1} fresh weight.

Phosphatases

1. Acid phosphatase

Preparation of acid phosphatase enzyme extraction and assay were carried out according to Angosto *et al.*, (1988). 2 gm of leaf tissue of Mulberry Cultivars homogenized in a mortar for 5 minutes at 4°C in 4 - 5 ml of 125 mM sodium acetate buffer (pH 5.0) containing 1% (w/v) PVP and 12 mM 3-mercaptoethanol. The homogenates were centrifuged at 15,000 X g at 4°C for 30 minutes. Supernatants were recovered, subjected for dialysis and used as enzyme source. The reaction mixture was containing enzymes extract (50^1), 30 mM p-nitro phenol phosphate (10ml) and 125 mM sodium acetate buffer (pH 5.0) 350 ml. The mixture was incubated at room temperature for 7 min, and the reaction was stopped by the addition of 500 mM NaOH (500 ml). The resulting quantity of p-nitrophenol was determined by absorbance at 410 nm. One unit of phosphatase activity was defined as p.mol p-nitro-phenyl phosphate hydrolyzed min^{-1} as compared to control without substrate.

2. Alkaline phosphatase

Preparation of alkaline phosphatase enzyme extraction and assay were carried out according to Angosto *et al.*, (1988). 2 gm of leaf/explant tissue of *Morus Spp.* was homogenized in a mortar for 5 minutes at 4°C in 4-5 ml of 25 mM Tris-HCI buffer (pH 7.5), 30mM p-nitrophenyl phosphate and enzyme extract. The reaction mixture was incubated for 5 min at room temperature. The reaction was started by the addition of substrate and stopped with the addition 500 mM NaOH (0.5 ml). One unit of the enzyme activity was defined as the amount of enzyme catalyzing the release of 1 p.mol p-nitrophenol min^{-1} compared to a control without substrate.

Amylase

Amylase activity was measured in the roots and leaves of control and stressed plants according to the method of Sridhar and Ou, (1972). The fresh plant material was extracted into 10.0 ml of cold 1M acetic acid-sodium acetate buffer (pH 6.0) by macerating in a mortar with pestle. The homogenate was filtered through a muslin cloth and the filtrate was transferred in to centrifuge tubes and centrifuged at 5000 rpm in a refrigerated high speed centrifuge for 20 min at 0°C. The pellet was discarded and the supernatant was taken. 5.0 ml of enzyme extract was taken into a conical flask and 10.0 ml of 1mM acetic acid-sodium acetate buffer (pH 6.0) and 5.0 ml of 1% starch were added to it. The contents were mixed and incubated at 37°C for 24 h. After incubation 1.0 ml of reaction mixture was taken and reducing sugars released were estimated by Nelson's method (1994) as described earlier. Amylase activity was expressed in µg of reducing sugars released per mg protein.

Invertases

1. Soluble invertase

Enzyme extraction and partial purification: Lyophilized regenerates were ground to a powder and homogenized in extraction buffer containing 50 mM HEPES-NaOH, pH 7.5,5 mM ~-mercaptoethanol, 10 mM Na_2SO_J, I mM EDTA, I mM $MgCI_2$, 1 mM benzamidine and 100 flM PMSF. The homogenate was filtered through four layers ofcheesecloth and centrifuged for 30 min at 17,OOOg to remove insoluble material.Invertase assay was conducted with the dialyzed 61 to 80% (NH4)2S0. The invertase assay was a modification of the two step protocol of Goldstein and Lampen (1975). The protein fraction (20 to 50 Ill) was incubated for 30 min at 37°C with 100 mM NaOAc, pH 4.7 and 100 mM sucrose or)00 mM HEPES-NaOH, pH 8.0 and 100 mM sucrose in a 100 III total volume for acid or alkaline invertase activity assays, respectively. Reaction was stopped by addition of an equal volume of 0.5 M potassium phosphate, pH 7.0 and boiled for 3 min. Glucose concentration was determined by addition of I ml of a solution containing 14 U glucose oxidase, I U peroxidase, 24 11M o-dianisidine and 38% glycerol and incubation for 30 min at 30°C. Reaction was stopped by addition of 1.5 ml 6N HCI, and the absorbance at 540 nm was measured. Soluble invertase activity was expressed as µmol glucose h^{-1}mg protein^{-1}.

2. Cell bound invertase

Frozen regenerates samples were thoroughly homogenized in 10 cm3 ice-cold acetate buffer (100 mol m "3; pH 50) and made up to 25 cm3. After standing in ice for 30 min with occasional stirring, 15 cm3 of homogenate were centrifuged at 2500 *g* for 20 min (MSE Chilspin II; 4 °C). Acid invertase activity and the concentrations of hexose, sucrose and protein in the supernatant fractions were determined as described in Morris and Arthur (1984). Tests for the presence of low molecular weight invertase inhibitors and for possible binding of soluble invertases to insoluble tissue residues during homogenization were negative (Morris and Arthur, 1984). Furthermore, no significant hydrolysis of sucrose to hexose occurred during extraction and assay. Cell bound invertase activity was expressed as µmol glucose h^{-1}mg protein^{-1}.

Total Proteins

Total proteins were extracted from leaves of Mulberry Cultivation regenerates as described by Kavi Kishore (1989). Approximately 1 gm of leaf tissue was homogenized into 4 ml of 0.05 M phosphate buffer (ph 7.0), containing 30 mM p-mercaptoethanol, 20 mM EDTA and 0.05 g of PVP. The homogenate was centrifuged at 20,000 Xg for 15 minutes, supernatants were recovered and used for the estimation of total proteins concentrations were measured according to Bradford (1976), using bovine serum albumin (BSA) as a standard. 5 ml of the Bradford reagent and 100 µl of the protein extract were

mixed and the reaction mixture was incubated at room temperature for 20 min. The absorbance values were measured at 595 nm.

Nitrogen Metabolism
Nitrate reductase activity

Extraction of nitrate reductase was carried out as described by Altaf Ahmad and Abdin (1999) with slight and appropriate modifications essential for optimal extraction. 1 gm of Mulberry varieties leaf / explants tissue from *in vitro* raised plantlets was homogenized in 25 ml of extraction buffer containing 25mM potassium phosphate (pH 7.5), mM cysteine-HCI, 5 mM EDTA and 0.3% PVP. Extract was filtered through muslin cloth and centrifuged at 10000 x g for 15 minutes at 4°C. The crude extract was immediately assayed for the activity.

Nitrate reductase activity was assayed as described by Campbell and Smarrelli (1978). Final volume 2.0 ml, of reaction mixture, containing 0.5 ml 100 |1M potassium phosphate buffer (pH 7.5), 10 mM KNO_3, 0.5 ml pyridine nucleotides (either 50 |1M NADH, or NADPH or equimolar concentrations of each pyridine nucleotides) and 0.5 ml enzyme extract was incubated at 33°C for 30 minutes. After the completion of incubation period, reaction was stopped by the addition of zinc acetate. The mixture was centrifuged at 7000 rpm for 15 minutes; supernatant was retained for the produced nitrite estimation. To the supernatant 2 ml of colour developing agent i.e., 1% sulphanilamide in 1.5 N HCI and 0.2% NADH (N-napthylene diamine hydrochloride) were mixed in equal volume. After 10 minutes nitrite produced was estimated colorimetrically at 540 nm as described by Hageman and Huckles (1971). Amount of nitrite produced was calculated from the standard curve that was prepared by using potassium nitrite solution. Enzyme activity was expressed in terms of μ mol of nitrite released per gm fresh weight of tissue per unit time or specific activity was defined as one unit of enzyme as that amount which catalyzed the reduction of 1 n M NO_3" $mg^{"1}$ protein/ min".

Glutamate dehydrogenase (GDH)

Enzyme extraction was carried out as described by Philippe Lenee and Yves Chupeau (1989), with slight modifications as necessary for the present investigation. Assay for glutamate dehydrogease (NAD* / NADH specific) activity was carried out as described in Malik and Singh (1980) with slight modifications as necessary for the assay. For GDH activity assay reaction mixture final volume was maintained as 3.0 ml and the reaction mixture was prepared by adding the following constituents to glass cuvettes i.e., 0.2 ml of 0.2 M α-ketoglutarate, 2.3 ml of 0.1 M Tris-HCI (pH 8.0), 0.1 ml 5 mM NADH, 0.2 ml of crude enzyme extract and equilibrated for 5 minutes at 30°C. Then the cuvette was transferred to the cuvette holder of spectrophotometer set at 340 nm. Reaction was initiated by the addition of 0.2 ml of 1 M ammonium chloride. Change in absorbance was recorded at intervals of 30 seconds up to 3-5 minutes. Aminative, deaminative GDH activities were defined as n moles of cofactor oxidized or reduced ($NADH/NAD^+$) $min^{"1}$ $gm^{"1}$/fresh weight of the tissue respectively. Blanks were used for the enzymatic determinations and the rate of appearance or disappearance of the cofactor was measured in absence of α-keto glutarate and ammonium by Lizbeth Castro-concha *et* al., (1990).

Statistical analysis

Data was recorded throughout the study and was subjected for statistical analysis. Mean values and respective standard errors were calculated. The data was represented in terms of frequency of shoot and root formation viz., % of shoot forming explants, average number of shoots per each explant, percentage of rooting per shoot. Similarly in all the other studies (evaluation of suitable medium, callus induction and biochemical studies) data analysis was carried out computationally as mean ± SE. All the data of obtained for each parameter was analyzed for their significance, according to the method of Duncan's Mutltiple Range test (Duncan, 1955). The significance was calculated at 5% level ($P<0.05$).

III. RESULTS AND DISCUSSION

Carbohydrate metabolism

Carbohydrates yield energy, and the carbohydrates are all special importance in plant metabolism. The principle function in organism is production and storage of this vital commodity. Furthermore a variety of other organic compounds are synthesized from carbohydrates. Carbohydrates gained importance in the physiology of plants where in it releases the energy locked up in the monosaccharide by breaking them down to simple CO_2 and H_2O.

Total sugars

The levels of total sugars in regenerating auxiliary explants of M_5 mulberry variety were presented in figure 7.1. Total sugars were increased from day '0' to the day '15' with higher levels observed on the same day and thereafter a gradual decline in the amounts were recorded till day '30'. A similar trend was also found in the mulberry variety V_1 (Fig 7.2), S_{36} (Fig 7.3), and Anantha (Fig. 7.4).
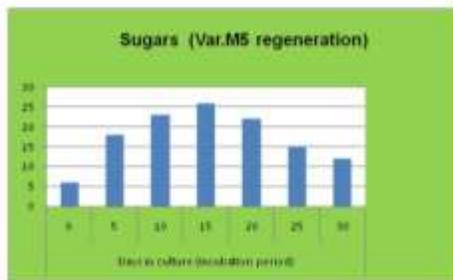
Fig. 7.1 Levels of total sugars (mg glucose/gm. f.w) during Morus Spp. M5 regeneration

Fig 7.2 Levels of total sugar (mg glucose /gm. f.w) during Morus Spp. V₁ regeneration
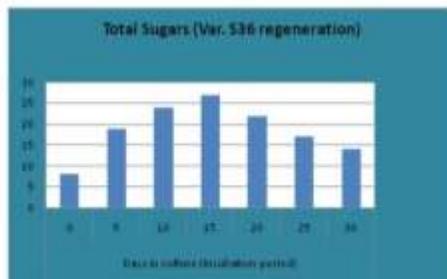
Fig. 7.3 Levels of total glucose (mg glucose/gm. f.w) during Morus Spp. S36 regeneration
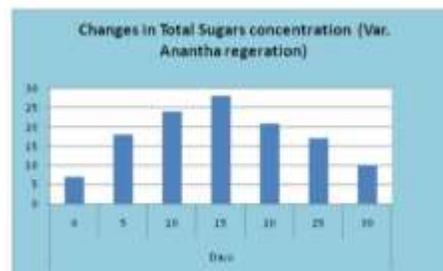
Fig. 7.4 Levels of total sugars (mg total glucose /gm. f.w) during Morus Spp. Anantha regeneration
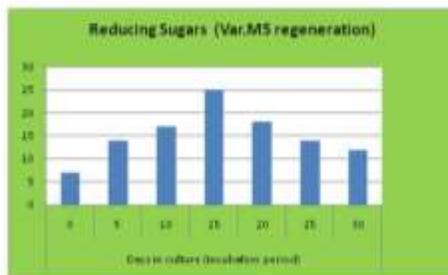
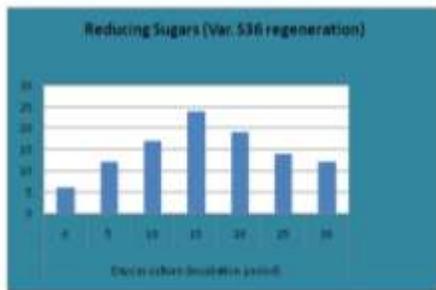
Fig. 7.5 Levels of reducing sugars (mg glucose /gm. f.w) during Morus Spp. M5 regeneration

Fig. 7.6 Levels of reducing sugar (mg glucose/gm. f.w) during Morus Spp. V1 regeneration

Reducing sugars

The levels of reducing sugars in regenerating auxiliary explants of M_5 mulberry variety were presented in figure 7.5. In regenerating shoots of Morus Spp, the reducing sugar levels were increased steeply from day '0' day '15', with higher amounts recorded on day '15', and thereafter a gradual decline was observed till day '30'. A similar trend was also found in the mulberry variety V_1 (Fig 7.6), S_{36} (Fig 7.7), and Anantha (Fig. 7.8).

Starch

The levels of reducing sugars in regenerating auxiliary explants of M_5 mulberry variety were presented in table 7.9 and figure 7.9. Starch accumulation was observed in regenerating shoots between day 'O' today '15' and thereafter a slight decline was observed till day '30' (Figure 26-33), however, the difference is not so significant between day '10' today '20'. A similar trend was also found in the mulberry variety V_1 (Fig 7.10), S_{36} (Fig 7.11), and Anantha (Fig. 7.12).

Fig. 7.7 Levels of reducing sugars (mg glucose/gm. f.w) during Morus Spp. S36 regeneration
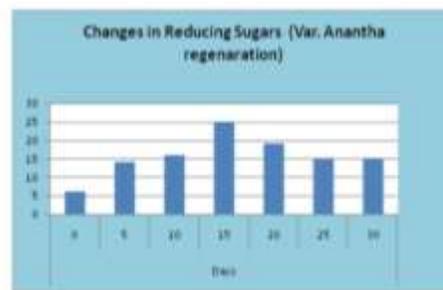
Fig. 7.8 Levels of reducing sugars (mg reducing sugars /gm. f.w) during Morus Spp. Anantha regeneration

. 7.9 Levels of starch sugars (mg starch /gm. f.w) during Morus

Fig. 7.10 Levels of starch (mg starch/gm. f.w) during Morus Spp. V₁ regeneration
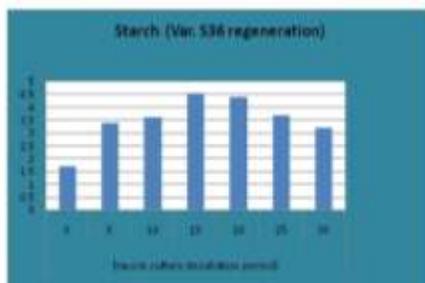
Fig. 7.11 Levels of starch (mg starch /gm. f.w) during Morus Spp. S36 regeneration

Fig. 7.12 Levels of starch (mg starch /gm. f.w) during Morus Spp. Anantha Regeneration

Hydrolytic enzymes

Acid and alkaline phosphatases

The levels of alkaline phosphatases in regenerating auxiliary explants of M_5 mulberry variety were presented in figure 7.13. In the generating shoot cultures changes in the levels of acid and alkaline phosphatase enzymes were monitored from '0' today '30'. The activity of acid phosphatase in regenerating shoots of M_5 was found to be increased gradually from day '0' to the day '30', whereas the alkaline phosphatase activity was increased from day '0' till day '20' and thereafter gradual decrease was recorded till the day '30'. A similar trend was also found in the mulberry variety V_1 (Fig 7.14), S_{36} (Fig 7.15), and Anantha (Fig. 7.16).
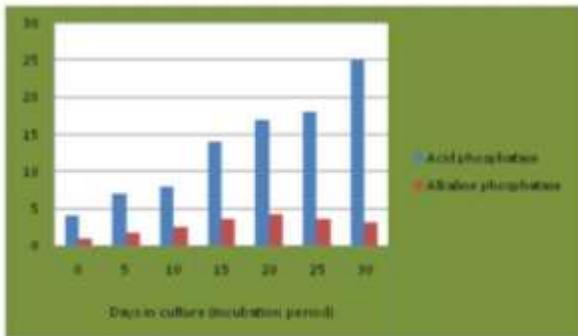
Fig. 7.13 Levels of hydrolytic enzymes of Phospohatases during Morus Spp. M5 Regeneration
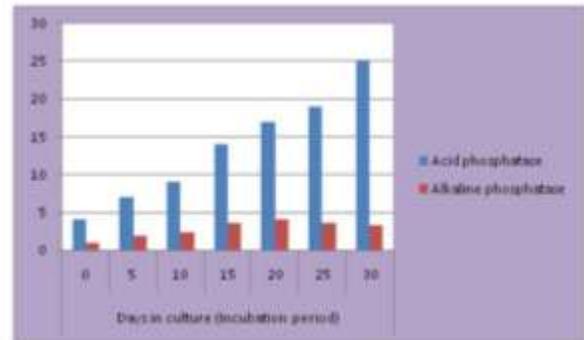
Fig. 7.14 Levels of hydrolytic enzymes of Phospohatases during Morus Spp. V1 Regeneration
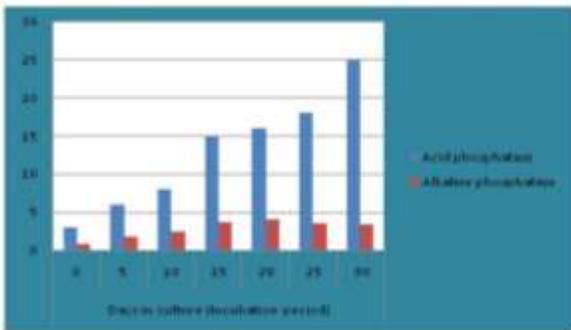
Fig. 7.15 Levels of hydrolytic enzymes of Phospohatases during Morus Spp.S36 Regeneration
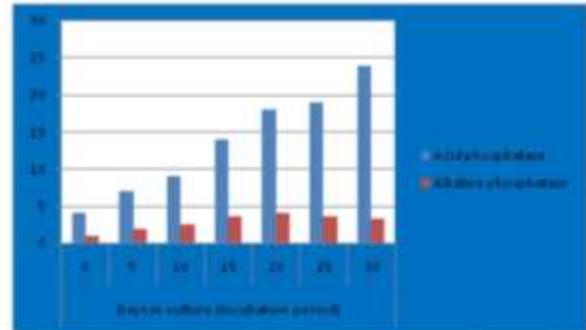
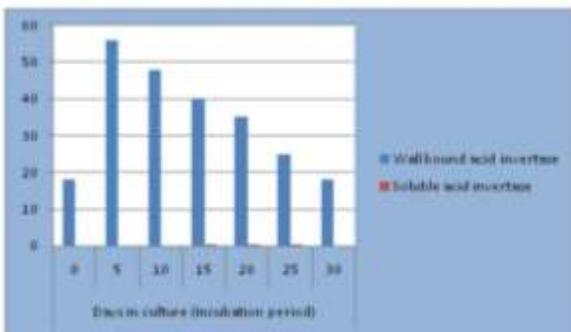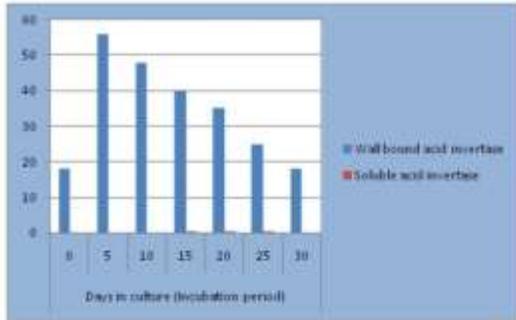Fig. 7.16 Levels of hydrolytic enzymes of Phospohatases during Morus Spp. Anantha regeneration

Fig. 7.17 Levels of hydrolytic enzymes of Invertases during Morus Spp. M5 Regeneration

Fig. 7.18 Levels of hydrolytic enzymes of Invertases during Morus Spp. V1 Regeneration

Invertases

The levels of wall bound and soluble acid invertases in regenerating auxiliary explants of M_5 mulberry variety were presented in figure 7.17. The activities of wall bound acid invertases in M_5 mulberry variety in regenerating shoots, was observed to be increased from day 5, it was a steep increase from day '0' today '5', and thereafter it was observed to be a gradual decline in the activity. The soluble acid invertase activity in regenerating shoots was observed to be increased from day '0' and peaked on day '15'. Invertase, β-h- fructosidase is a saccharide cleavaging hydrolase, which occurs in yeast fungi and higher plants. As the invertases exist in atleast two isoforms such as soluble and wall-bound and further into acid and alkali isoforms, only acid phosphatases were studied in regenerating shoots because of their importance in cellular energy mediation, metabolic reactions. A similar trend was also found in the mulberry variety V_1 (Fig 7.18), S_{36} (Fig 7.19), and Anantha (Fig. 7.20).

Fig. 7.19 Levels of hydrolytic enzymes of Invertases, during Morus Spp.S_{36} Regeneration
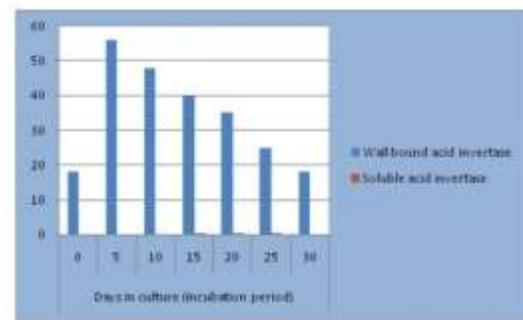

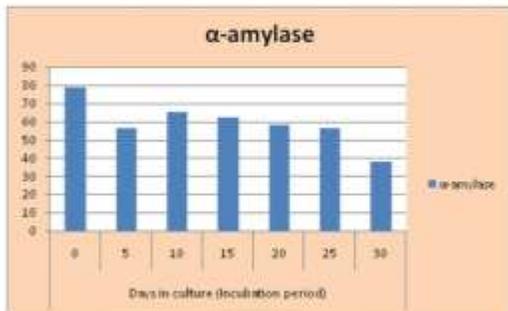Fig. 7.20 Levels of hydrolytic enzymes of Invertases during Morus Spp. Anantha Regeneration

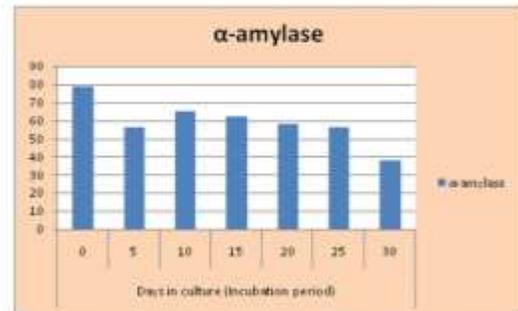Fig. 7.21 Levels of hydrolytic enzymes of α-amylase during Morus Spp. M5 Regeneration

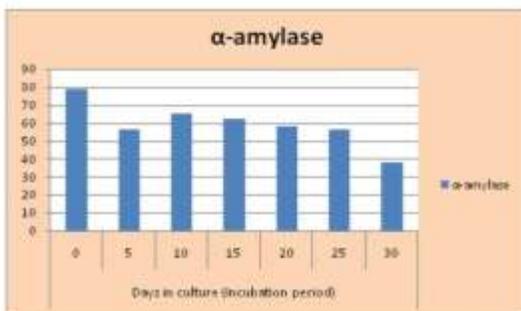Fig. 7.22 Levels of hydrolytic enzymes of α-amylase during Morus Spp. V1 Regeneration

Fig. 7.23 Levels of hydrolytic enzymes of α-amylase during Morus Spp.S_{36} Regeneration
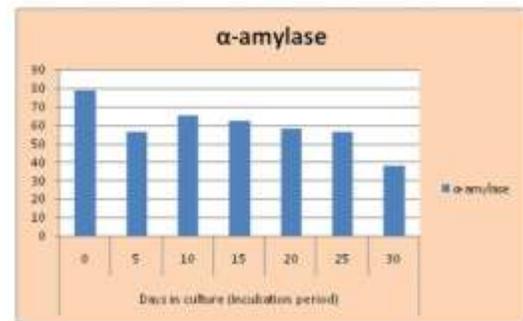

Fig. 7.24 Levels of hydrolytic enzymes of α-amylase during Morus Spp. Anantha Regeneration

α-Amylase

The levels of α-amylase in regenerating auxiliary explants of M_5 mulberry variety were presented in figure 7.21. α -Amylase activity in regenerating shoots was observed to be increased from day '0' to the day '10', followed by this a gradual decreased till day '30'. A similar trend was also found in the mulberry variety V_1 (Fig 7.22), S_{36} (Fig 7.23), and Anantha (Fig. 7.24).

Nitrogen metabolism
Total proteins

The levels of total protein**s** in regenerating auxiliary explants of M_5 mulberry variety were presented in figure 7.25. Total protein content in regenerating shoots was gradually increased with consistent progress from day '0' today '30', with highest amount recorded on day '30'. A similar trend was also found in the mulberry variety V_1 (Fig 7.26), S_{36} (Fig 7.27), and Anantha (Fig. 7.28).
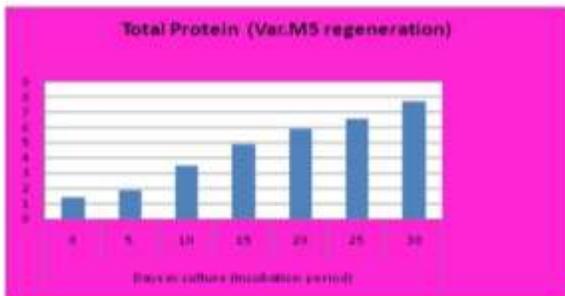

Fig 7.25 Levels of total protein (mg protein/gm. f.w) during Morus Spp. M5 regeneration
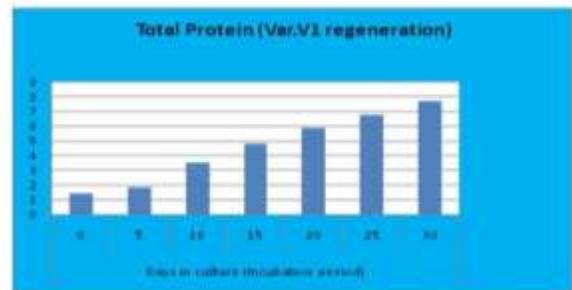

Fig. 7.26 Levels of total protein (mg Protein/gm. f.w) during Morus Spp. V1 regeneration
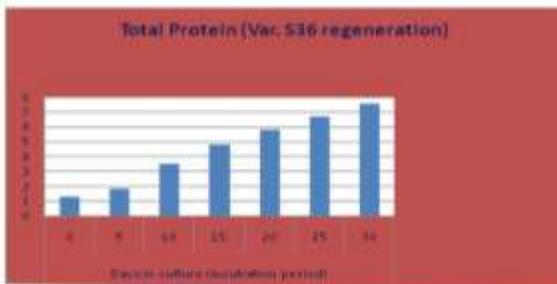

Fig. 7.27 Levels of total protein (mg total protein /gm. f.w) during Morus Spp. S36 regeneration
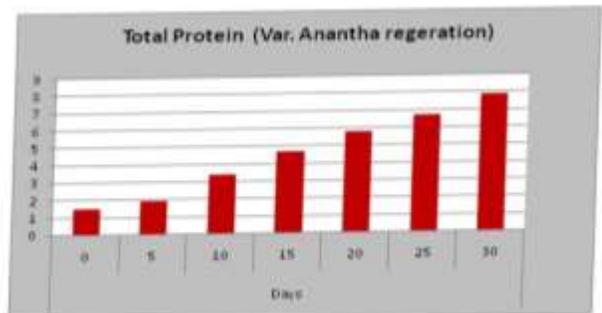

Fig. 7.28 Levels of total protein (mg total protein /gm. F.W) during Morus Spp. Anantha regeneration
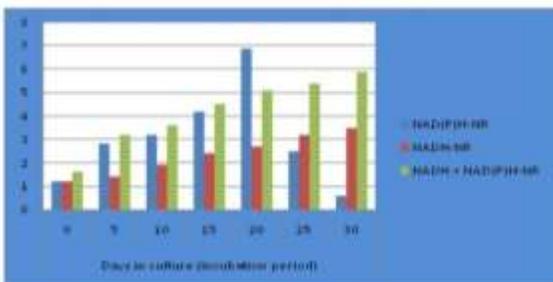

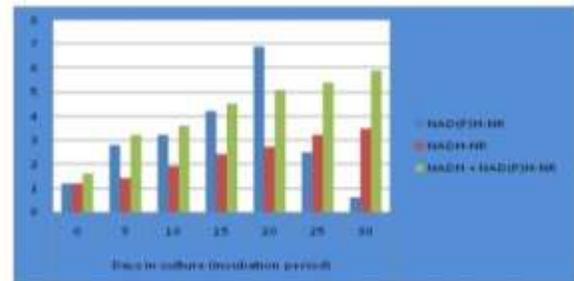Fig. 7.29 Levels of NR, and certain hydrolytic enzymes during Morus Spp. M5 regeneration

Fig. 730 Levels of NR during Morus Spp. VI regeneration Nitrate reductases Table. 7.2 Levels of GDH during Morus Spp. M5 regeneration

Activities of Nitrogen metabolism and ammonia assimilation enzymes.

Activity of Nitrate Reductase

Nitrate reductase activity in regenerating auxiliary explants of M5 was presented in the figure 7.29. In regenerating auxiliary explants, the NAD (P) H: NR activity initially on '0' day was observed to be lower than NR activity in presence of either NADH or both NADH-NAD (P) H, raised up to day '5' and further progressed till day '15' then peaked on day '20', thereafter a gradual decrease was observed till the day '30', shown lowest activity. Whereas, it was observed that from the day '0' to the day 30' a gradual increase in the enzyme activity with constant progress, in case of NADH-NR. A similar trend was also found in the mulberry variety V_1 (Fig 7.30), S_{36} (Fig 7.31), and Anantha (Fig. 7.32).
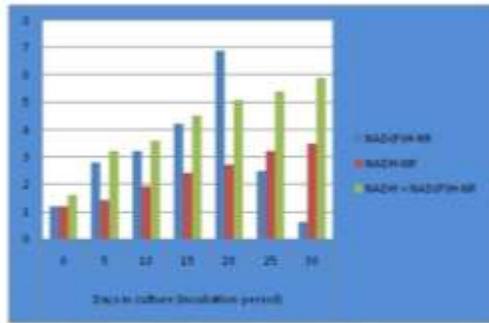
Fig. 7.31 Levels of NR during Morus Spp.S_{36} regeneration Nitrate reductases

Fig. 7.32 Levels of NR during Morus Spp. Anantha regeneration Nitrate Reductases

Fig. 7.33 Levels of GDH during Morus Spp. M5 regeneration (Glutamate dehydrogenases)

Fig. 7.34 Levels of GDH during Morus Spp. V1 regeneration Glutamate dehydrogenases
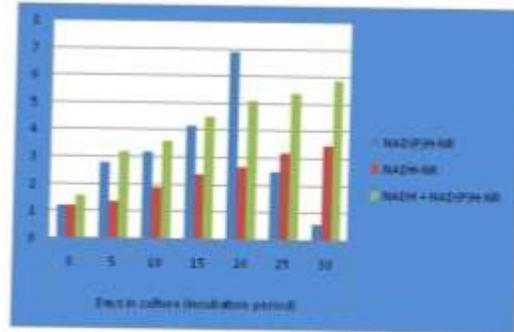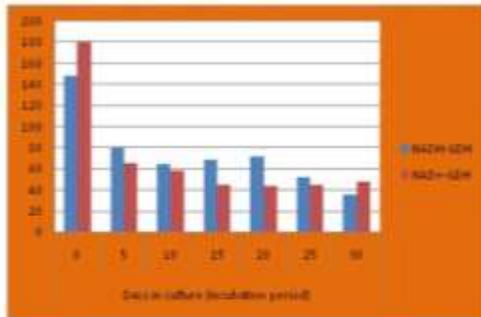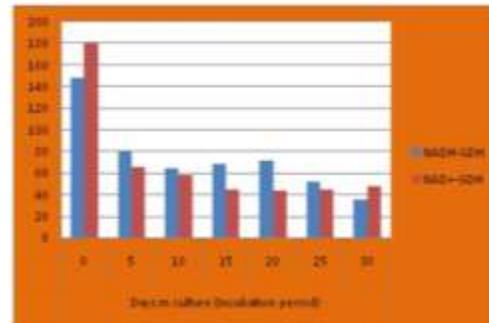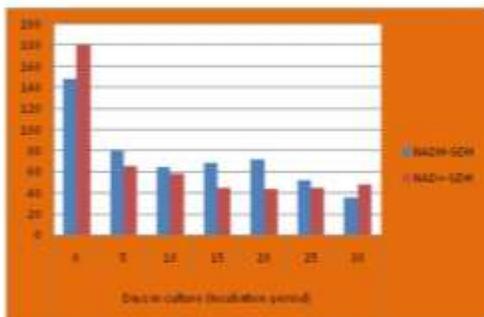

Fig. 7.35 Levels of GDH during Morus Spp.S_{36} regeneration Glutamate dehydrogenases
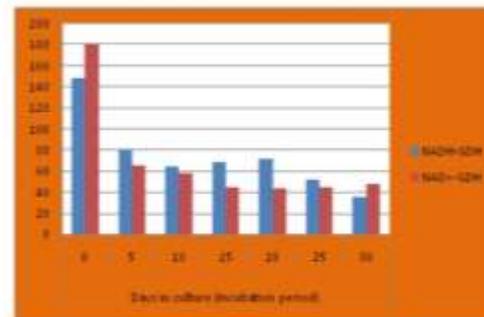

Fig. 7.36 Levels of GDH during Morus Spp. Anantha regeneration

Glutamate dehydrogenase (GDH) activity

Glutamate dehydrogenase (GDH) activity in regenerating auxiliary explants of M5 was presented in the figure 7.33. In Morus Spp. it was observed that a gradual fall in both the isoforms of glutamate dehydrogenase (both NAD* and NADH isoforms) activities from day '0' today '30'. In case of NAD$^+$–GDH (oxidative enzyme) the rate of decrease in the activity was less pronounced than the NADH-GDH. The extent of decrease in case of NAD$^+$ isoform was observed to be a gradual decline till day '30'. Whereas in case of the NADH-GDH (isoform) a sudden fall in the activity could be seen from day '0' today '5', followed by a steep decrease till day '20', thereafter it appeared to be a slight raise till day '30'. A similar trend was also found in the mulberry variety V_1 (Fig 7.34), S_{36} (Fig 7.35), and Anantha (Fig. 7.36).

During the present study it was observed that, shoot regeneration in petiole, auxillary bud, and shoot tips explants of Morus Spp. was highest percent when reared on MS medium supplemented with 3mgl^{-1} 6-BAP in combination with 1 mgl^{-1} NAA, though the organogenesis was also observed in other plant growth regulator combinations. These morphological changes in the explants during their proliferation on suitable medium were monitored by determining certain biochemical changes *viz.,* changes in metabolite concentrations, hydrolytic enzymes, nitrate/ammonia utilizing enzymes.

Carbohydrate metabolism

Similarly changes in the metabolites and hydrolytic enzymes of carbohydrate metabolism were investigated in the present study. The changes in the metabolites such as starch, reducing sugars and total sugar content indicate that the accumulation of these metabolites till day '15' seems to reflect the high energy requirement of the organogenic processes, also this accumulation of starch and sugars play an important role as osmotic agents (Kavi Kishore and Mehta, 1989; Naidu and Kavi Kishore, 1995), and further decrease in the levels is of much significance with associated visible manifestation of organogenesis.

The regenerating shoot cultures have been extensively used for investigating factors regulating organogenesis (Brown and Thorpe, 1986; Sujatha *et ai,* 2000). During the present study changes in certain hydrolytic enzymes and certain metabolites were monitored in regenerating shoot cultures to confirm tissue active proliferation. Amylases were widely occurring group of hydrolases, which cleave the α-1,4, glycosidic bonds in oligosaccharides and polysaccharides like starch, glycogen, and dextreme. α- amylase is an endoamylase, while β-amylase and ϒ-amylases are exoamylases, while α-amylases and ϒ-amylases occur in both in plants and animals. B-amylases found only in seeds. Increased activity of these hydrolytic enzymes such as amylases and acid and alkaline phosphatase enzymes during the present investigation indicated that degradation of different compounds proceeded in the regenerating tissues and this was concurrent with the high synthetic activity that occurs during organogenesis. The results of the present investigation are supported by the previous findings reported by (Brown and Thorpe 1980), (Kavi Kishore and Mehta, 1988), (Naidu and Kavi Kishore, 1995), (Kumar M 1998) in other plant species reported elsewhere.

Changes in the activities of soluble acid and wall bound invertase activities indicate that the significant role played by soluble invertases and importance of these enzymes during organogenesis. In our study both the activities of soluble acid and wall bound invertase increased up to 15 day and 25th day correspondingly. This indicates that the peak activities of the enzyme exhibits most rapid cell expansion in the regerants. Further, the rapid decline of hexose at 15th day indicates the rapid utilization of hexose. The observations reported here demonstrate a close association between cell expansion growth of regenarants. The results are coincided by Morris and Arthur (1985).

Nitrate Metabolism

The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy total demand for nitrogen in the face of external nitrate concentration that can

vary by five orders of magnitude (Nigel M Crawford, 1995). Nitrate supplemented in the medium must be converted to NH_4^+ in the plants before the nitrogen enters amino acids and other nitrogen compounds.

Nitrate reduction occurs in two distinct reactions catalyzed by different enzymes, one of which is nitrate reductase (NR) which occurs in cytosol and converts nitrate into nitrite the first reaction step, and in the second step NO_2^- is reduced to NO_4^+. Nitrate reductase has been studied intensively, because its activity often controls the rate of protein synthesis in plants absorbing NO_3^- as the major nitrogen source (Srivastava, 1980; Naik *et al*., 1982). Apparently, NR is continuously synthesized and degraded (Somers *et al*., 1983), and abundant levels of NO_3^- in the cytosol clearly increases the activity of NR, largely because of faster synthesis of the enzyme, which is a case of enzyme induction (Salisburry and Ross, 1986).

The observed results clearly suggest that there are at least 2 isoforms for NR enzymes that could use both NADH and NAD (P)H in Morus spp. as it was also suggested by many others earlier (George Sorger *et al*., 1986; Altaf Ahmed and Abdin, 1999) and the results of the present study are in accordance with the results reported earlier in mustard seedlings by Altaf and Abdin (1999), as it was explained that, whenever the nitrate concentration is more than 20mM, it is reduced to nitrite by NAD(P)H : NR as this isoform dominantly active than the other isoforms at nitrate concentrations above 20mM and can utilize either NAD(P)H alone or the combination of NADH : NAD(P)H as the coenzymes, whereas the other isoform can utilize NADH efficiently than NAD(P)H, optimally functions at low nitrate concentration (<10 mM). Hence, it could be concluded here that at 30 mM concentrations of nitrate and until day '15' to the day '20' the nitrate reduction was carried out by NAD (P) H: NR and thereafter with the decreased levels (low levels) of nitrate the isoform activity was gradually declined and NADH: NR was observed to be active at low levels of medium nitrogen. Thus, it could be concluded that the two NRs appear to be induced in sequence simultaneously during growth and active multiplication of explants into shoots/ plantlets.

However, initial activity of NADH: NR on the day '0' was observed to be slightly greater than that of NAD (P) H: NR activity. Likewise the NR activity in presence of both NADH and NAD (P) H at equimolar concentrations was observed to be greater than NADH: NR throughout the period of study and it was lower than NAD (P) H: NR activity till day '20'. However, the enzyme activity was observed to be progressed with gradual raise until the day '30' from the day '0'.

Glutamate dehydrogenase is one of the enzymes involved in the nitrogen metabolism and involved in ammonia assimilation. The NO_3^- / NO_4^+ ratio in the culture medium is a determinant factor in the explant/ tissue differentiation process (Robert *et al*., 1987; Walker and Sato, 1981). Both NADH and NADPH can serve as glutamate dehydrogenase substrates for the reductive amination reaction. Activity with NADH and NADPH was in the ratio of 8: 1 like in other systems (Daguin and Letouze, 1986). GDH isoform activities were decreased from day '0' today '30' consistently, this suggests that there was no vitrification in the explants, and the reduced nitrogen may be utilized for the amino acid biosynthesis by the other enzymes of nitrogen assimilation like GS and GOGAT, but not by GDH, the increased activity of which is a sign of verification. Thus it could be concluded that the tissue/explants are actively proliferating on MSB_5 medium with 6-BAP (3 mg l^{-1}) and NAA (1 mgl^{-1}) combination.

IV. CONCLUSION

Since the building up and breaking down of protoplasm of regenerants is concerned with certain metabolites and certain enzyme activities, some biochemical studies were carried to know the plantlet regeneration and growth pattern of Mulberry varieties such as M5, S36, V1 and Anantha, during their multiplication and regeneration into shoots and plantlets. The changes in the metabolites such as starch, reducing sugars and total sugar content indicate that the accumulation of these metabolites till day '15'

seems to reflect the high energy requirement of the organogenic processes, also this accumulation of starch and sugars play an important role as osmotic agents and further decrease in the levels is of much significance with associated visible manifestation of organogenesis.

V. ACKNOWLEDGEMENTS

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