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BIOCHEMICAL CHANGES DURING SHOOT DIFFERENTIATION IN CALLUS CULTURES OF *CHLOROPHYTUM BORIVILIANUM* SANT. ET FERNAND

ROHTAS SINGH, HANS R. DHINGRA AND S. C. GOYAL*

Plant Tissue Culture Laboratory, Department of Botany and Plant Physiology, CCS Haryana, Agricultural University, Hisar-125004, India

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SUMMARY

Biochemical changes leading to shoot regeneration during *in vitro* culture of callus derived from bud pedicel of *Chlorophytum borivilianum* on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/l BAP + 1.0 mg/l NAA were investigated. Starch content and reducing sugars were high in the control callus, which further increased significantly in shoot differentiating cultures. Contents of total soluble sugars, free amino acids, total soluble proteins and total phenols were lower in the former and increased in the shoot differentiating cultures. The activities of enzymes like α-amylase, acid-protease, acid-phosphatase and peroxidase increased up to appearance of green patches (8-12 day) and reached a peak on 16th day of inoculation that coincided with the appearance of shoots. Conversely, the acid invertase activity decreased till the appearance of shoots.

***Key words*: Biochemical changes, callus, *Chlorophytum borivilianum*, shoot differentiation.**

INTRODUCTION

Differentiation, i.e. the process of growth and morpho-physiological specialization of cells from unorganized mass of callus cells, is a prerequisite for application of biotechnology for the crop improvement. Knowledge on the control of differentiation has hardly grown since the demonstration that differentiation of organized structures in tissue culture is under the influence of growth regulators like cytokinins and auxins along with other components of the culture medium (Smith and Krikorian 1991). Little is known about the intervening biochemical events occurring in the cultured cells undergoing organogenesis (plant regeneration). Therefore, elucidation of biochemical changes accompanying differentiation, will be helpful to decipher the underlying mechanism. Reports on biochemical changes associated with root differentiation in callus tissues and hypocotyl segments are available (Kumar and Maherchandani 1988, Abe *et al.* 1996, Kalra and Bhatla 1996,1998, Sujatha *et al.* 2000, Dominic and Joseph 2004, Jeyaseelan and Rao 2005). The present study was undertaken to determine the changes in the levels of key metabolites and the associated enzymes during shoot differentiation from callus cultures of *C. borivilianum*.

MATERIALS AND METHODS

Young bud explants with pedicel were excised from the inflorescence taken from field grown plants of *C. borivilianum*. These were wetted in 1.0% teepol solution followed by surface sterilization with 0.2% mercuric chloride (2 min) and with 70% ethanol for 1 min and finally washed with sterile distilled water (4-5 times). These were then implanted on MS (Murashige and Skoog, 1962) basal medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA. The callus obtained from explants was transferred (after 4-weeks) to MS + BAP

* Corresponding author, E-mail: goyal_sc@yahoo.co.in

(2.0mg/l) medium. Visible shoot formation was observed after 12-16 days of inoculation. Sampling was done prior to inoculation (0 day) and after 4th, 8th, 12th and 16th day after inoculation on shoot differentiation medium and some key metabolites and activity of enzymes were studied.

Extraction and determination of metabolites: These were extracted by the modified method of Barnett and Naylor (1996). One hundred mg of callus was homogenized in 80% ethanol (v/v) and centrifuged for 10 min at 10,000 rpm. The homogenate was refluxed thrice for 15 min on a water bath at 60°C. The supernatants were pooled together and final volume was made to 5 ml with ethanol and used for estimation of total soluble sugars (Yemm and Willis 1954), reducing sugars (Honda *et al*. 1982), free amino acids (Yemm and Cocking 1955) and total phenols (Amorim *et al.* 1977). The pellet was hydrolysed with 4 ml of chilled 0.2 N $HClO_4$ at 4°C for 24 h. The hydrolysate was centrifuged at 5000 rpm for 15 min and the supernatant so obtained was used for starch estimation by the method of Hassid and Neufeld (1964). To the leftover pellet, 2 ml of 1N NaOH was added and left for overnight at room temperature. After centrifugation at 5000 rpm for 15 min the supernatant was collected and the pellet was rehydrolyzed with 1N NaOH and centrifuged as above. The two supernatants were pooled together and final volume was made to 5 ml and used for estimation of total soluble proteins by Bradford (1976) method.

Extraction and determination of activity of enzymes: The crude enzyme extract was prepared by homogenizing one gram of fresh callus tissue in pre-chilled pestle and mortar using 0.1 M Tris-HCl buffer (pH 7.5) containing 0.25 mM EDTA, 2.5 mM Cysteine-HCl and 0.1% PVP and the homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant thus collected was used for estimating the activity of various enzymes viz. α-amylase (Shuster and Gifford 1962), acid-invertase (Summer 1935), acid-protease (Beevers 1968), acid-phosphatase (Jones 1969) and acid peroxidase (Seevers *et al*. 1971). Activity of the enzyme peroxidase was expressed in terms of rate of increase of absorbance per min per mg protein. One unit of enzyme was arbitrarily defined as 1.0 change in O.D. per min per mg protein. Enzymatic protein content in the extract was determined as per the method of Bradford (1976).

RESULTS

Callus induction was evident within 15 days of inoculation of the bud pedicel on MS + BAP (1.0 mg/l) + NAA (1.0 mg/l) and callusing was evident in 92% cultures with good callus growth in subsequent 4-weeks. The 4-week old green hard calli inoculated on MS + BAP (2.0 mg/l) showed formation of green patches (between 8-12 days) and shoots formation between 12-16 days. The harvesting of callus tissue was done on 4, 8, 12 and 16 day after inoculation, in order to study the process of shoot differentiation (data not shown).

Cellular metabolites: Starch content decreased continuously untill the appearance of green patches on 12th day and then followed an increase with the appearance of shoots on 16th day, while in non-shoot forming callus (control), starch content was higher in the beginning (Fig. 1A). Total soluble sugars were less in control callus (0 day) and a steady increase was observed in the differentiating calli up to 12th day which declined again with shoot formation i.e. on 16th day (Fig.1A). Reducing sugars were significantly high on the day of inoculation (0 day) which declined in the differentiating calli till the appearance of shoots on 16th day (Fig. 1A).

Similarly, calli had high free amino-acid content on the day of inoculation on the shoot differentiation medium which further increased up to stage of formation of green patches and decreased at the time of shoots appearance (Fig. 1B). On the other hand, the total soluble proteins in shoot forming callus remained high throughout the period of study as compared to control. Similarly, the total phenolic content was also higher during shoots differentiation. Shoot differentiating calli had more than twice the phenolic content just prior to shoot appearance (on 12th day) as compared to control callus. However, shoot differentiation was accompanied by a drop in the phenolic content (Fig. 1B).

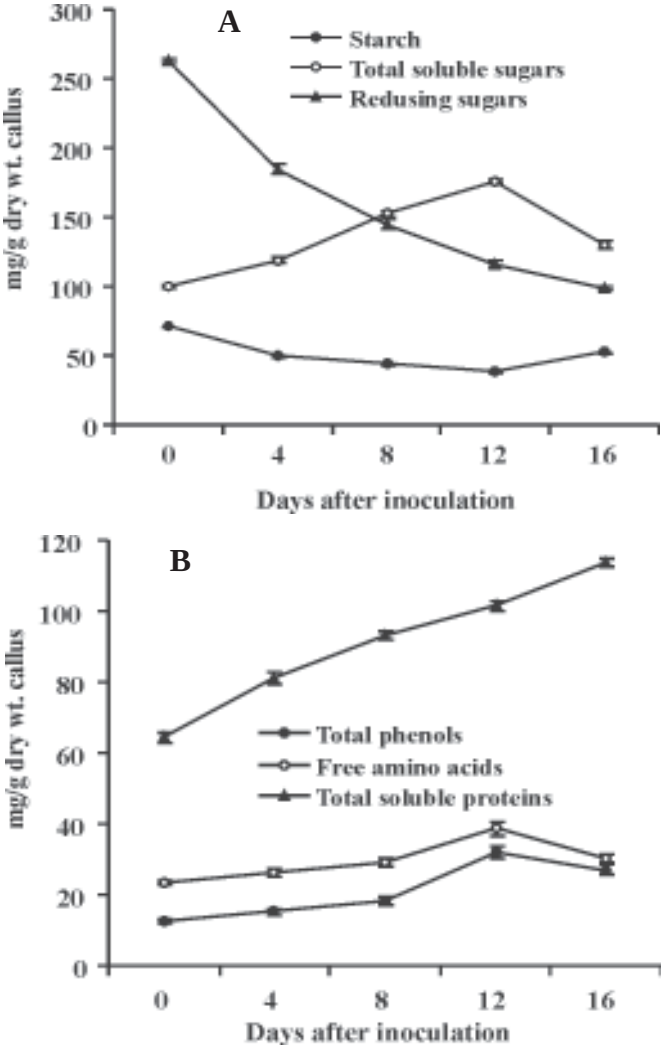

Fig. 1. Changes in the levels of starch, total soluble sugars and reducing sugars, A; total phenols, free amino acids and total soluble proteins, B; in *C. borivilianum* callus prior to inoculation on shoot forming medium (0 day) and after 4 , 8 , 12 and 16 day after inoculation (vertical lines indicate ± SE)

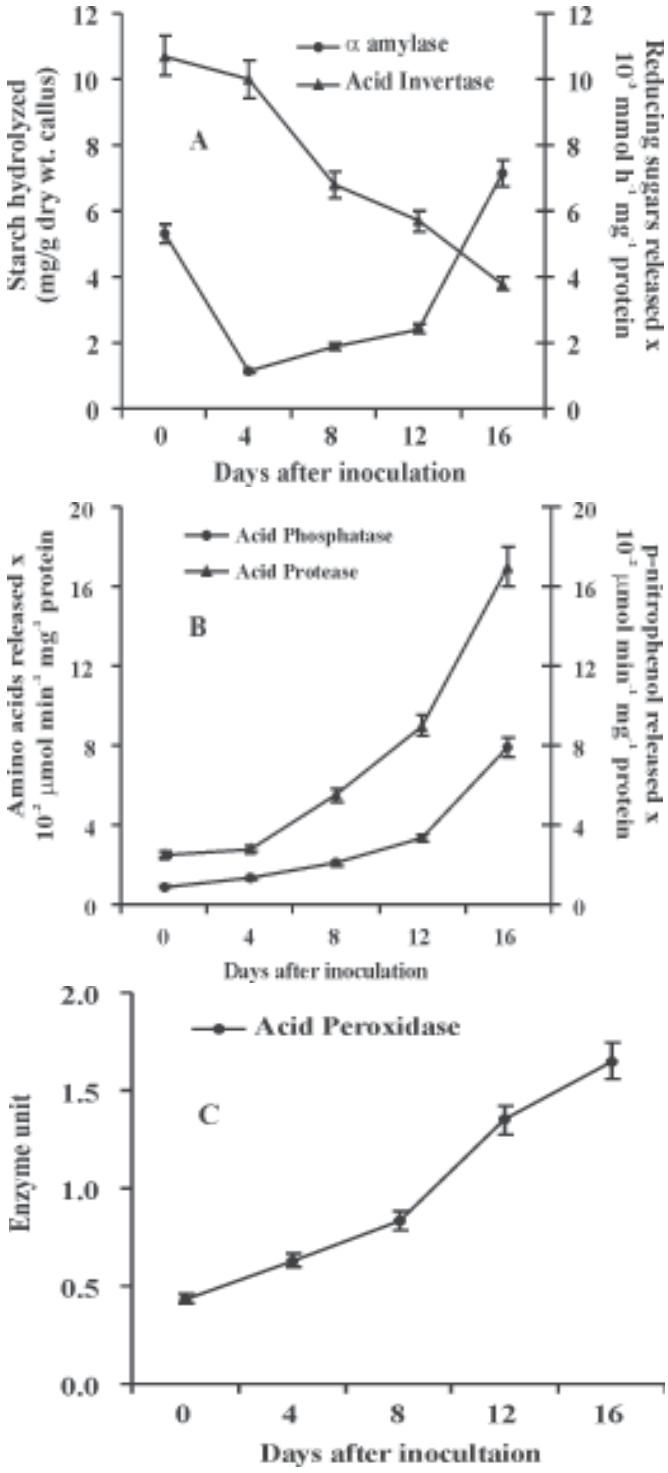

Fig. 2. Changes in the levels of α-amylase and acid invertase, A; acid phosphatase and acid protease, B; acid peroxidase, C; enzymes in *C. borivilianum* callus prior to inoculation on shoot forming medium (0 day) and after 4 , 8 , 12 and 16 day after inoculation (vertical lines indicate ± SE)

Enzymes activity: The activity of α-amylase declined sharply (more than 5 times) in shoot differentiating calli on 4th day than that of control callus which was followed by a steady increase up to the stage of formation of green patches on 12th day. Peak activity was observed on 16th day which corresponded to shoot appearance and the activity was about 7 times higher than that on 4th day (Fig. 2A). Correlation studies indicated a positive correlation of total soluble carbohydrates and negative correlation of starch with α-amylase activity. The acid-invertase activity was more in callus prior to inoculation

for differentiation and showed a continuous decrease in the activity in shoot differentiating calli (Fig. 2A) and showed a positive correlation with the reducing sugar content. Acid-protease activity was found to be higher under shoot forming conditions. Peak activity was observed on 16th day and was about 6 times higher than that in the undifferentiating callus (Fig. 2B). Total proteins as well as free-amino acids were positively correlated with protease activity. The activities of both acid-phosphatase and acid-peroxidase were more in shoot differentiating calli than that of control callus. The peak activities of these enzymes were recorded on 16th day, associated with rapid differentiation (Fig. 2 B & C). Correlation between acid-phosphatase-protein and peroxidase-phenols were highly significant.

DISCUSSION

Tissue/callus cultures have been extensively used for investigating factors regulating organogenesis (Brown and Thorpe 1986). In the present study, attempts were made to correlate levels of metabolites and enzymatic data to shoot differentiation in callus cultures of *C. borivilianum*. Differentiation is a high energy requiring process as it involves high rates of cell division as compared to steady callus maintenance and starch is a food reserve stored by the cells to meet high-energy requirements (Yadav *et al.* 1995). Present study also revealed a decline in the starch content in differentiating calli . This was coroborrated with higher α-amylase activity thereby suggesting that energy is being made available through utilization of starch reserves (Jain *et al.* 1990, Abe *et al.* 1996). Positive correlation between α-amylase activity and total soluble carbohydrates and its negative correlation with starch content appear to be making energy rich carbohydrates available to sustain higher metabolic status during differentiation (Yadav *et al.*1995). Since acid invertase activity is concerned with the hydrolysis of sucrose leading to release of reducing sugars like glucose and fructose which serve as respiratory substrates. Acid-invertase activity has been shown to have a direct correlation with differentiation (Copping and Street 1972). Gradual decline in reducing sugar content during shoot differentiation in callus could be associated with the utilization of sugars for differentiation process (Chatrath *et al.* 1996).

In calli during shoot differentiation, total proteins and free-amino acids accumulated gradually from 0 to 16th day indicating high rates of protein as well as amino acid synthesis during all the stages of organogenesis. In the course of callus cultures, accumulation of protein/free-amino acids has been observed during shoots differentiation (Yadav *et al.* 1995, Chatrath *et al.* 1996, Sujatha *et al.* 2000). A definite role of protein synthesis in organogenic processes is well established. Higher acid protease activity is correlated with high rate of degradation of pre-existing storage and other proteins, and free amino acid pool thus formed is used for the synthesis of new proteins and enzymes required during differentiation of somatic embryos/shoots from callus cultures (Dave and Batra 1995, Yadav *et al.* 1995).

Carbohydrate metabolism of plants is indirectly controlled by availability of inorganic phosphate, which in turn is regulated by the activity of acid phosphatase. In the present study, sharp increase in acid-phosphatase during shoot differentiation has a positive correlation with increased protein content and decreased sugar and starch content. Accumulation of proteins was reflective of changes in acid-phosphatase in shoots forming regions (Patel and Thorpe 1984) and maximum acid-phosphatase activity has been observed during shoots formation in cultured hypocotyls of *Jatropha integerrima* (Sujatha *et al.* 2000). Phenols are reported to participate in the formation of cross-linking of cell wall constituents via oxidative polymerization of hydroxylated cinnamyl alcohols, a process catalyzed by peroxidase (Mader and Fussel 1982). So, a positive correlation was evident between total phenolic content and activities of peroxidase. Increase in peroxidase activity has been correlated with the formation of adventitious shoot and somatic embryogenesis (Kochba *et al.* 1977, Sujatha *et al.* 2000). Peroxidase isozymes are known to be tissue specific and can be employed as markers of developmental stages. Kalra and Bhatla (1996) reported IAA induced appearance of new peroxidase isozyme (no. 4) at the time of initiation of roots in the sunflower callus which persisted till proliferation stage. A similar expression/suppression of some other specific peroxidase isozymes may be controlling shoot differentiation in *C. borivilianum* also.

Starch and reducing sugar content decreased until the shoot differentiation thereby indicating that the process is energy demanding. On the other hand, protein content remained high even after the shoot differentiation while free amino acids and phenolic compounds increased upto shoot differentiation and declined thereafter. Increased pool of free amino acids may be utilized in the synthesis of new proteins and enzymes required for differentiation process.

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