

## Activities of hydrolytic enzymes in callus cultures of tobacco during organogenesis

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**Abstract.** Starch, total sugars, reducing sugars and protein contents and the specific activities of hydrolytic enzymes such as amylase, Phosphorylase, soluble acid invertase, wall-bound acid invertase, sucrose synthetase, acid and alkaline phosphatases and ribonuclease were determined in root forming, shoot forming and non-organ-forming callus cultures of tobacco. Organ-forming cultures not only showed higher amounts of the above metabolites but also higher enzyme activities compared to non-organ-forming cultures. The activities of these enzymes in relation to organogenesis is discussed.

**Keywords.** Tobacco callus; hydrolytic enzymes; organogenesis.

### 1. Introduction

Factors regulating organ formation in cultured cells of higher plants is a subject of intense study (Thorpe and Meier 1974). Many investigations have been made about the physiological changes taking place during organogenesis in callus cultures (Kavi Kishor 1989; Kavi Kishor and Mehta 1988). While Dougall (1962) and Syono (1965) implicated protein synthesis in shoot formation, Lee and Skoog (1965) hypothesized that aromatic compounds increased bud formation by regulating the endogenous auxin/cytokinin level.

In this paper, changes in the activities of some of the hydrolytic enzymes in organ and non-organ-forming callus cultures of tobacco is measured. Furthermore, the levels of certain metabolites such as total sugars, reducing sugars, starch and soluble proteins during organogenesis is reported.

### 2. Materials and methods

Initiation of callus of tobacco, its maintenance, media used for callus proliferation, shoot and root differentiation has been reported earlier (Kavi Kishor and Mehta 1989). Five replicate flasks were harvested every 5rd day and analysed for metabolites and enzyme assays. The experiments were terminated by day 15, when shoots and roots appeared. The callus tissues from different flasks were pooled, weighed (1 g) and homogenized in a pre-chilled mortar using neutral glass powder, 0.1% polyvinylpyrrolidone and 0.05 M phosphate buffer pH 7.0. The tissue homogenate was centrifuged for 20 min at 50,000 g in a refrigerated centrifuge. All the steps

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in the preparation of the enzyme extract were carried out at 0–4°C and all assays were performed on the same batch of tissue. The supernatant was dialyzed for 2–3 h at 5°C in 0.05 M phosphate buffer (pH 7.0). Average values obtained in two separate experiments are shown in the figures.

Table 1 lists the methods used for estimating the metabolites and enzymes. Slight modifications in pH, substrate, enzyme and co-enzyme concentrations were made as necessary in the methods referred. This is essential to obtain the optimum activity of the enzymes.

**Table 1.** Metabolite and enzyme assay methods.

Metabolite assayed	Reference to assay method	Activity expressed* (one unit)
Total sugars	Yemm and Willis (1954)	mg of glucose/100 mg of dry tissue
Reducing sugars	Nelson (1944), Somogyi (1952)	mg of glucose/100 mg of dry tissue
Total starch	Hassid and Neufeld (1964)	mg of glucose/100 mg of dry tissue
Soluble proteins	Lowry <i>et al</i> (1951)	mg of BSA/g fresh weight of tissue
Amylase	Bernfeld (1955)	As 33 µg of maltose liberated/min <sup>-1</sup>
Phosphorylase	Turner and Turner (1960)	As 1 µg of phosphate liberated/min <sup>-1</sup>
Sucrose synthetase	Pressey (1969)	As 1 µmol of fructose liberated/min <sup>-1</sup>
Soluble acid invertase	Bacon (1955)	As 1 mg of reducing sugars liberated/h <sup>-1</sup>
Wall-bound acid invertase	Klis and Hak (1972)	As 1 nmol of sucrose hydrolyzed/mg <sup>-1</sup> dry weight of cell wall/h <sup>-1</sup>
Acid phosphatase	Zink and Veliky (1979)	As 1 mM of <i>p</i> -nitrophenol released/min <sup>-1</sup>
Alkaline phosphatase	Zink and Veliky (1979)	As 1 mM of <i>p</i> -nitrophenol released/min <sup>-1</sup>
Ribonuclease	Tuve and Arfinsen (1960)	As change in 0.01 OD/min <sup>-1</sup>

\*Specific activity of enzymes was expressed as units per mg of protein min<sup>-1</sup>.

### 3. Results and discussion

Shoot differentiation was noticed in 75% of the callus cultures of tobacco in MS medium supplemented with 0.3 mg/l indole-3-acetic acid (IAA) and 5% sucrose. Callus cultures undergoing differentiation were slightly compact and green in colour. Emergence of the shoots from callus began around day 12. This process was not completely synchronous, and shoot initiation continued for several days in the cultures. However, in most of the cultures, shoots were differentiated in 15 days. Differentiation of roots was observed on Murashige and Skoog's (MS) medium (1962) containing 2 mg/l IAA and 5% sucrose, in 13–15 days with 70% frequency. Callus growing on callus proliferating medium (non-organ-forming callus) served as control. This callus was granular, highly friable and lush green in colour in contrast to organ-forming (OF) callus. There was a rapid accumulation of total and reducing sugars in shoot forming (SF), root forming (RF) and non-organ-forming (NOF) callus tissues during the initial 5 days and then there was a gradual depletion till the termination of culture period (table 2). However, starch accumulated till day 9 in OF as well as NOF tissues. The accumulation of sugars and starch was higher in OF (both SF and RF) tissues compared to NOF callus. Total soluble protein levels were also higher in OF callus than in NOF callus (table 2).

**Table 2-** Content of certain metabolites in tobacco callus during growth and organogenesis.

Metabolite	Tissue	Days in culture					
		0	3	6	9	12	13
Total sugars mg/100 mg Dry weight	NOF	3.3	18.4	15.3	12.0	10.4	8.6
	SF	3.3	27.9 <sup>a</sup>	22.1 <sup>a</sup>	14.0 <sup>a</sup>	10.6	9.3
	RF	3.3	32.0 <sup>a</sup>	26.8 <sup>a</sup>	20.3 <sup>a</sup>	14.9 <sup>a</sup>	11.5 <sup>a</sup>
Reducing sugars mg/100 mg Dry weight	NOF	2.9	15.0	13.0	8.7	8.6	5.0
	SF	2.9	19.2 <sup>a</sup>	15.3 <sup>a</sup>	10.2 <sup>a</sup>	8.5	7.4 <sup>a</sup>
	RF	2.9	19.9 <sup>a</sup>	20.0 <sup>a</sup>	15.7 <sup>a</sup>	12.4 <sup>a</sup>	9.2 <sup>a</sup>
Total starch mg/g Fresh weight	NOF	1.9	2.4	2.6	2.7	2.3	2.0
	SF	1.9	3.1 <sup>a</sup>	4.1 <sup>a</sup>	5.9 <sup>a</sup>	5.5 <sup>a</sup>	4.7 <sup>a</sup>
	RF	1.9	3.3 <sup>a</sup>	4.4 <sup>a</sup>	5.8 <sup>a</sup>	5.7 <sup>a</sup>	5.0 <sup>a</sup>
Total protein mg/g Fresh weight	NOF	0.3	1.2	2.1	3.2	3.4	3.7
	SF	0.3	2.3 <sup>a</sup>	3.8 <sup>a</sup>	4.6 <sup>a</sup>	5.1 <sup>a</sup>	5.5 <sup>a</sup>
	RF	0.3	1.9 <sup>a</sup>	3.7 <sup>a</sup>	4.3 <sup>a</sup>	4.9 <sup>a</sup>	5.4 <sup>a</sup>

Average values from 6 replicates and two independent experiments.

<sup>a</sup>Significant  $P = 0.1$

The difference in the specific activity of enzyme amylase in SF, RF and NOF callus tissues was not significant (figure 1A). There was an increase in the activity of phosphorylase in the SF and RF tissues leading to a peak of activity on day 9. The activity of the same enzyme in the corresponding NOF cultures was however, less considerably (figure 1B). Soluble acid invertase increased till day 6 in OF cultures and declined thereafter. On the other hand, in NOF cultures, the activity remained more or less constant throughout the culture period (figure 2A). Peak activity of wall bound invertase in all the cultures was registered on day 3. Nevertheless, the activity was considerably higher in both SF and RF tissues than in the NOF tissues (figure 2B). Activity of sucrose synthetase was also higher in SF and RF cultures especially till day 9 (figure 2C). The activity of acid phosphatase although lowest at day 3, rose sharply thereafter and peaked on day 9 in OF culture's (figure 3A). Alkaline phosphatase activity likewise slightly declined and peaked by day 15 in both SF and RF cultures (figure 3B). The activities of acid and alkaline phosphatases were several folds lower in NOF cultures throughout. Ribonuclease activity was significantly higher in SF and RF tissues right from day 0 until day 15 compared to NOF tissues (figure 4).

Accumulation of starch has been found in other organogenetic processes such as initiation of cotton embryos (Jensen 1963), differentiation of shoots in tobacco callus (Kavi Kishor and Mehta 1989) and shoot forming rice callus (Kavi Kishor P B, unpublished). Based on the above observations, including the present one, the physiological significance of starch, total and reducing sugar accumulation during differentiation seems to reflect the high energy requirement of the organogenetic processes. Also accumulated starch and sugars play an important role as osmotic agents (Kavi Kishor and Mehta 1989).

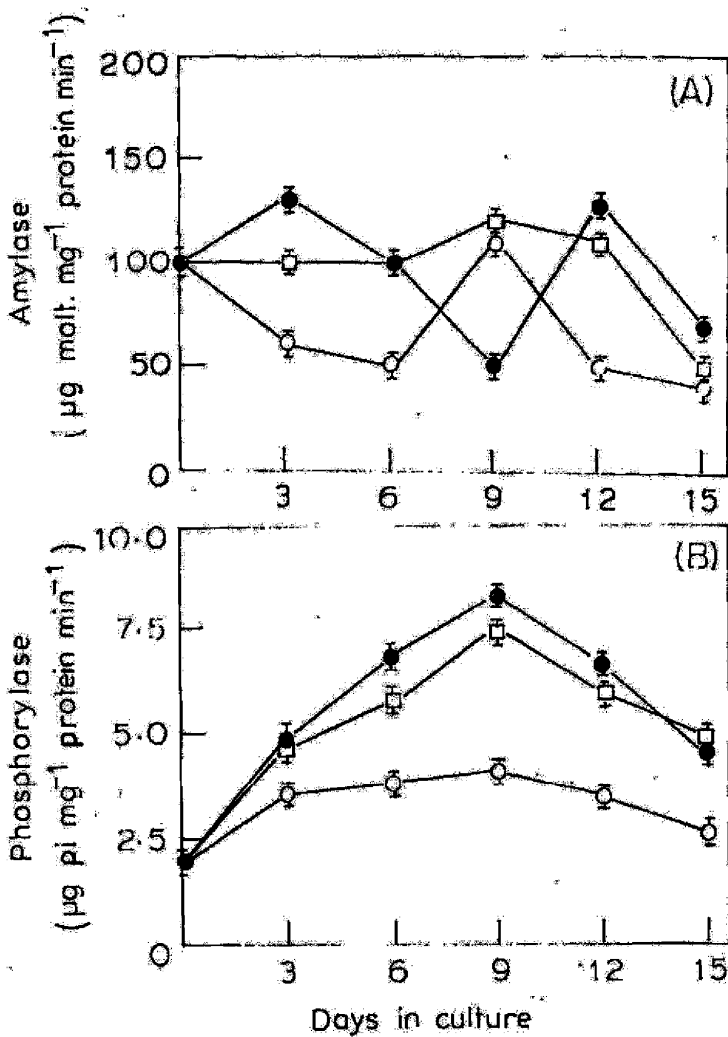


Figure 1. Specific activity of amylase (A) and Phosphorylase (B) in NOF (-O-),SF (-●-) and RF (-□-)tobacco callus.

Saka and Maeda (1975, 1974) examined the activities of some hydrolytic enzymes during shoot formation in rice callus. After 10 days in culture, the amylase activity in shoot forming regions was higher than in the non-differentiating callus parts in rice. However, there was no significant increase in the activity of amylase in OF cultures in the present study. But, the activity of Phosphorylase (considered to be important in starch degradation) was significantly higher in SF and RF than NOF tissues. Enhanced Phosphorylase activity may be important in the degradation of accumulated starch in the OF cultures. Increased activities of sucrose synthetase, soluble and wall bound acid invertases likewise may suggest increased degradation of sucrose in OF cultures to meet the higher demands at the site of organ initiation. As shown by Ross *et al* (1975) a very high and uninterrupted supply of free sugars

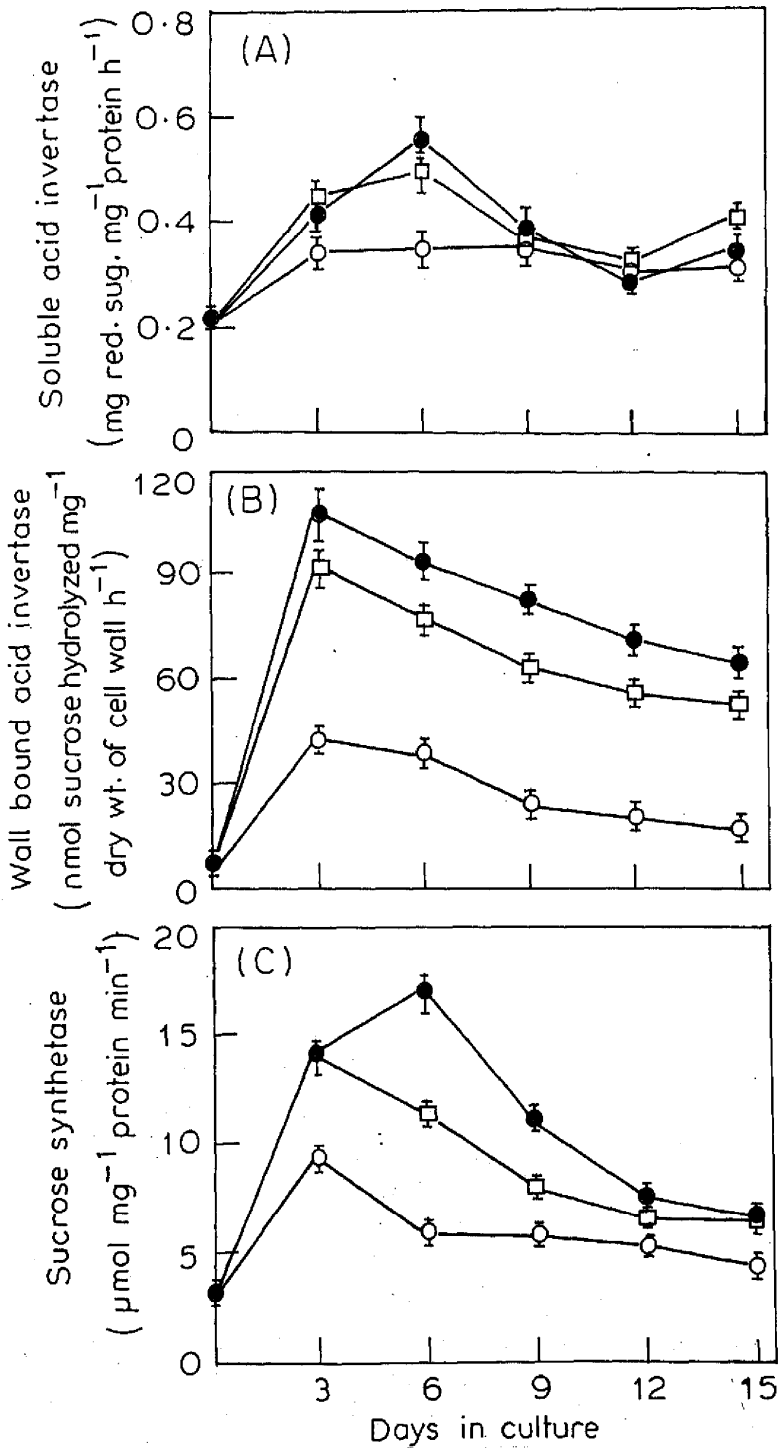
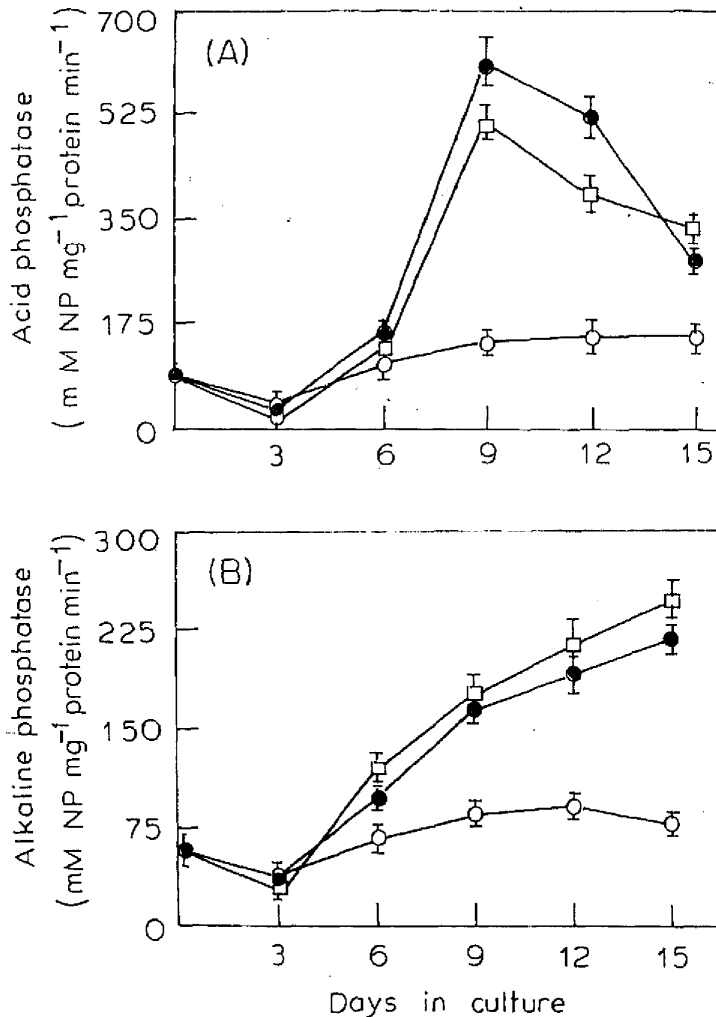


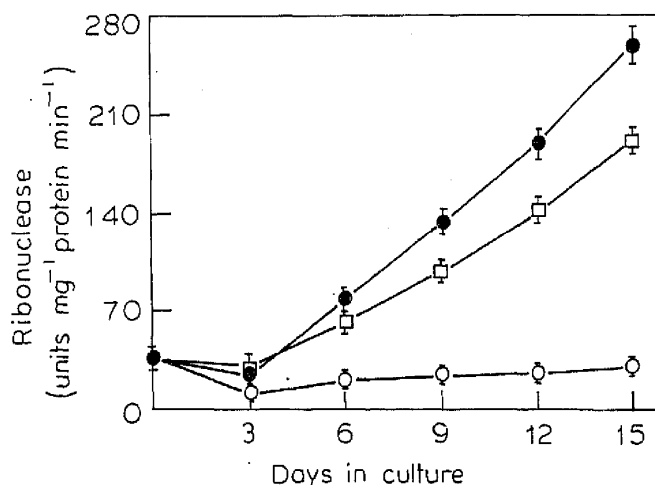
Figure 2A. Specific activity of soluble acid invertase (A), wall bound acid invertase (B) and sucrose synthetase (C) in NOF (O-O), SF (●-●) and RF (□-□) tobacco callus.



**Figure 3.** Specific activity of acid phosphatase (A) and alkaline phosphatase (B) in NOF (-O-), SF (-●-) and RF (-□-) tobacco callus.

may be essential at the site of organ initiation and therefore it is likely that the degradation products of starch and accumulated free sugars may fulfill this role.

Decreased activities of the phosphatases during the initial culture period (day 3) may be because of high inorganic phosphate levels in the medium. Pronounced increase in the activity of phosphatases in shoot and root differentiating tissues suggests that these enzymes might play a role in biochemical degradation of plasmodesmata. Such a degradation may facilitate the penetration of roots in the callus. Examining the role of acid phosphatases during the initiation and formation of adventitious roots in *Impatiens*, Malik and Kumari (1977) attributed such a role to the enzyme. It was speculated that glycosidases might cleavage wall linkages and facilitate growth. Increased activity of these hydrolytic enzymes indicated that degradation of different compounds preceded in the OF tissues and this was



**Figure 4.** Specific activity of ribonuclease in NOF (-O-), SF (-●-) and RF (-□-) tobacco callus

concurrent with the high synthetic activity that occurs during organogenesis (Kavi Kishor and Mehta 1988; Brown and Thorpe 1980).

Activities of most of the enzymes examined were found to be indicative of the pattern of organogenesis in callus cultures of tobacco.

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