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CHANGES IN ISOPEROXIDASES DURING SHOOT FORMATION IN TOBACCO CALLUS

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SUMMARY

Shoot formation in tobacco (*Nicotiana tabacum* L.) callus is accompanied by an increase in peroxidase activity which takes a form similar to a sigmoid curve. The "stationary" phase coincides with the period of organ formation. Characteristic changes in isoperoxidase pattern are found in the shoot-forming part of the callus. These changes are different from those in the nonshoot-forming part or in gibberellin-treated tissue, which does not form shoots.

Key words: isoperoxidases; starch gel electrophoresis; shoot formation; tobacco callus.

INTRODUCTION

Cultures of tissues in vitro usually require phytohormones for growth and differentiation. The importance of these growth substances for organized development was shown by Skoog and Miller (1). They observed that not only the concentration but the relative amounts of auxin and cytokinin influenced the pattern of differentiation, with relatively high cytokinin/auxin ratios favoring shoot formation in tobacco callus. Gibberellins also play a role in organogenesis, repressing shoot and root formation in the presence of otherwise suitable ratios of auxin and cytokinin (2-4).

The tobacco callus system has been used for several years to investigate physiological aspects of shoot formation. These studies have shown inter alia that the capacity of different strains of tobacco callus to form shoots was correlated with its scopoletin content (5); that there was an auxin-cytokinin interaction with respect to the levels of scopoletin and its glycoside scopolin (6,7); and that aromatic amino acids and other hydroxylated aromatic compounds stimulated bud formation in vitro (8). These and similar compounds are thought to affect the endogenous auxin level by interacting with peroxidases (8,9).

In this paper, we examine changes in isoperoxidase pattern during shoot formation in tobacco callus. We relate these changes to the developmental sequence of the process and, by various manipulations, attempt to separate those changes involved with growth from those associated with the organ-forming process.

MATERIALS AND METHODS

Callus was initiated from stem pith segments of tobacco (*Nicotiana tabacum* L., Wisconsin 38) in September, 1976. The method of maintenance of that callus in stock cultures and the experimental conditions for shoot production have been reported previously (10-12). Gibberellic acid (5×10^{-7} M) was incorporated into the medium before autoclaving, as earlier studies showed that it still retained its effectiveness in shoot repression (13). In some experiments the tissue was cut in half parallel to the surface in contact with the medium to give bottom and top portions which were analyzed separately. Previous studies (11,14) showed that organized development leading to shoot primordia formation occurred in the lower half of the callus.

Crude enzymic extracts were prepared by grinding 600 mg of tissue in 0.6 ml of 0.2 M phosphate buffer, pH 6.1, at 2° C. The macerate was centrifuged (25,000 × *g* for 10 min) and the supernate used as the enzyme. Guaiacol-peroxidase activity was determined as previously reported (15) and protein was assayed by the Folin-phenol method (16). Peroxidase isoenzyme patterns were determined using vertical starch gel electrophoresis (17), and the gels were developed with benzidine and o-dianisidine.

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RESULTS

As a preliminary investigation, a comparison was made of changes in total specific peroxidase activity of tobacco callus grown under shoot-forming and callus-forming conditions in the dark (Fig. 1). The kinetics of enzyme activity appeared quite different. Whereas a slow and continuous increase characterized the callus-forming tissue (*B*), a higher rate of enzyme development was measured in the earlier stages of culture in the shoot-forming tissue (*A*). This was followed by a leveling off of activity. The isoperoxidase patterns were different from each other, both on the cathodic and anodic sides (*zymograms not presented*).

Compared to the callus-forming medium, the shoot-forming medium contained a higher level of kinetin, as well as certain additives (adenine sulfate, tyrosine and increased inorganic phosphate) (10,11). Further examination was therefore restricted to culture conditions in which the above constituents were the same. A detailed comparison was made between shoot-forming ($-GA_3$) and nonshoot-forming ($+GA_3$) tissues (Fig. 2). The development in the total peroxidase activity in the absence of GA_3 was similar to that in Fig. 1*A*, with the plateau beyond 7 days in culture. The same type of curve also was observed in the presence of GA_3, with the difference, however, that the plateau occurred later in culture and with a higher total specific peroxidase activity. Some main differences characterized the isoperoxidase patterns of the tissues grown in the absence or presence of GA_3. On the anodic side, the activity of the four fast-moving bands A_{3a}, A_{3b} and A_{4a}, A_{4b} remained low in shoot-forming tissue compared to the GA_3-treated tissue in which a steady increase occurred. On the cathodic side, differences in the pattern of development of three isoenzymes were found between the two tissues. C_{1a} increased much more in intensity, whereas C_{2b} decreased more slowly, and C_4 increased more slowly in the shoot-forming tissue compared to the nonshoot-forming one.

When bottom and top portions of shoot-forming tissue were examined (Fig. 3), different patterns of development in total peroxidase activity were observed. In the bottom portion of the tissue, a distinct sigmoid-like curve with a lag phase in the first 4 days in culture and reaching a plateau after 7 days was recorded. These log and stationary phases were not found in the top part of the callus. The bottom portion was characterized by a low activity of the extreme anodic isoperoxidases, and a slower rate of decrease in the C_{2b} band, compared to the top portion. In addition, the C_3 and C_4 bands increased in intensity earlier in the bottom half of the callus. Bottom and top portions of callus-forming tissue were more alike in their isoenzyme patterns.

DISCUSSION

The developmental sequence leading to shoot formation in dark-grown tobacco callus has been described (10,11). By days 7 to 8 in culture, zones of preferential cell division activity are observed in the lower half of the callus. Within these zones meristemoids begin to appear by day 9, and these give rise to shoot primordia beginning at day 12 in

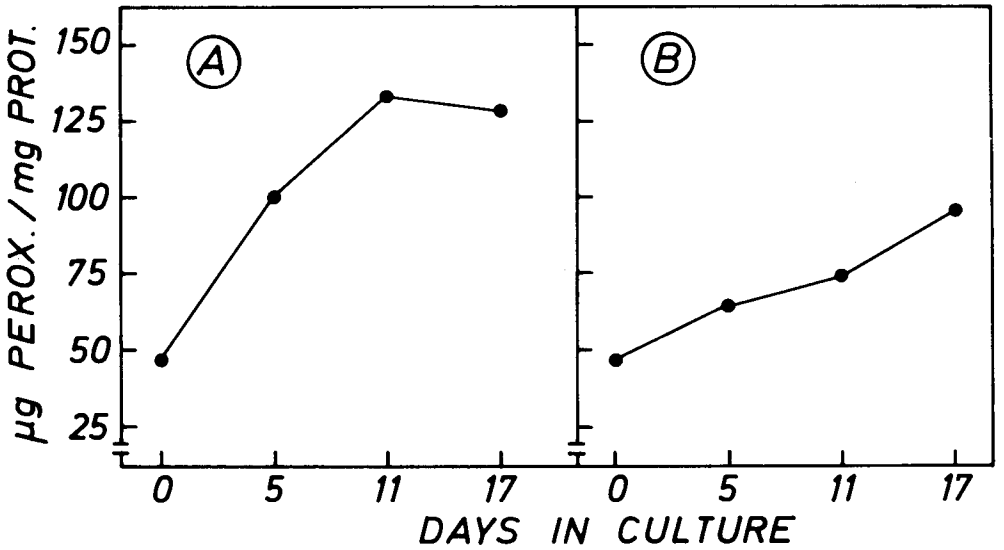

FIG. 1. Changes in total specific peroxidase activity during culture of tobacco callus grown under shoot-forming (*A*) and callus-forming (*B*) conditions in the dark.

culture. These primordia emerge from the lower half of the callus starting around day 14 and ultimately form the leafy vegetative shoots of tobacco.

Shoot formation in tobacco callus is accompanied by an increase in total specific peroxidase activity. Similar increases in peroxidase activity were observed by other workers using tobacco (18,19) and endive (20,21), and we also have observed it with epidermal explants of tobacco (22). However as Figs. 1*B*, 2 (+ GA_3), and 3 (*top*) show, increases in total specific peroxidase also are observed in nonshoot-forming tissues and the nonshoot-forming part (*top*) of shoot-forming callus. Thus this increase does not by itself characterize the differentiation process.

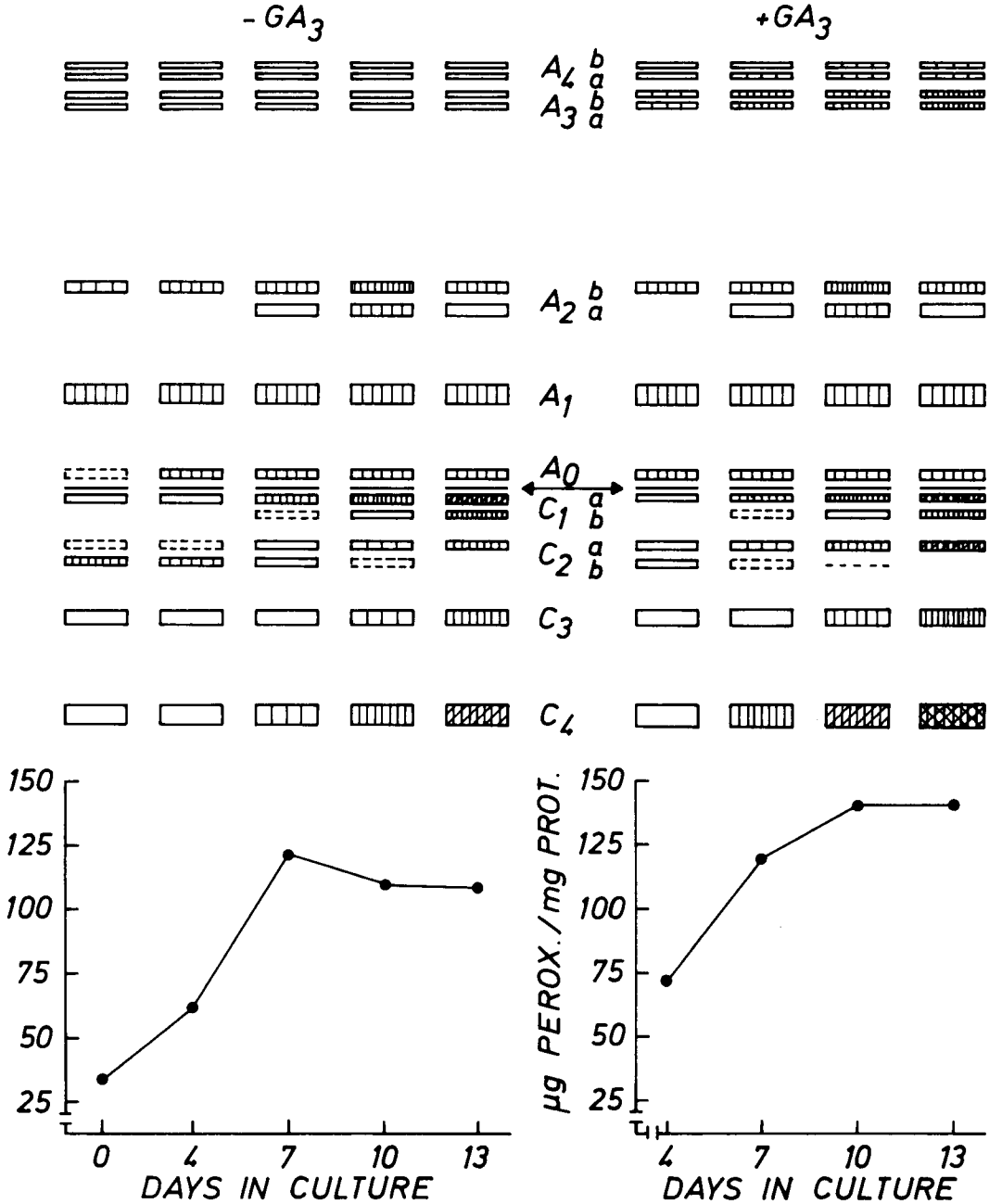

FIG. 2. Peroxidase zymogram and changes in total specific peroxidase activity during culture in the dark of shoot-forming (—GA_3) and nonshoot-forming (+GA_3) tobacco callus.

A detailed examination of shoot-forming tissues, however, indicates a definite sigmoid-like curve in peroxidase development with a plateau at the time of the initiation of organized development up to the time of primordia emergence. Such a plateau also can be found in the work of Mäder (19). The increase of peroxidase activity prior to the initiation of meristemoid formation may be indicative of the requirement for a reduction in endogenous auxin to bring about a favorable auxin-cytokinin ratio as has been suggested previously (8). During shoot differentiation in tobacco callus, a reduction in the activity of the fast-migrating anodic peroxidases was previously reported (19). We did not observe any reduction at this isoperoxidase level; but the most marked difference between shoot-forming and nonshoot-forming tissues was the very low level of activity of these most anodic bands in the former. Since the activity of these specific isoperoxidases is

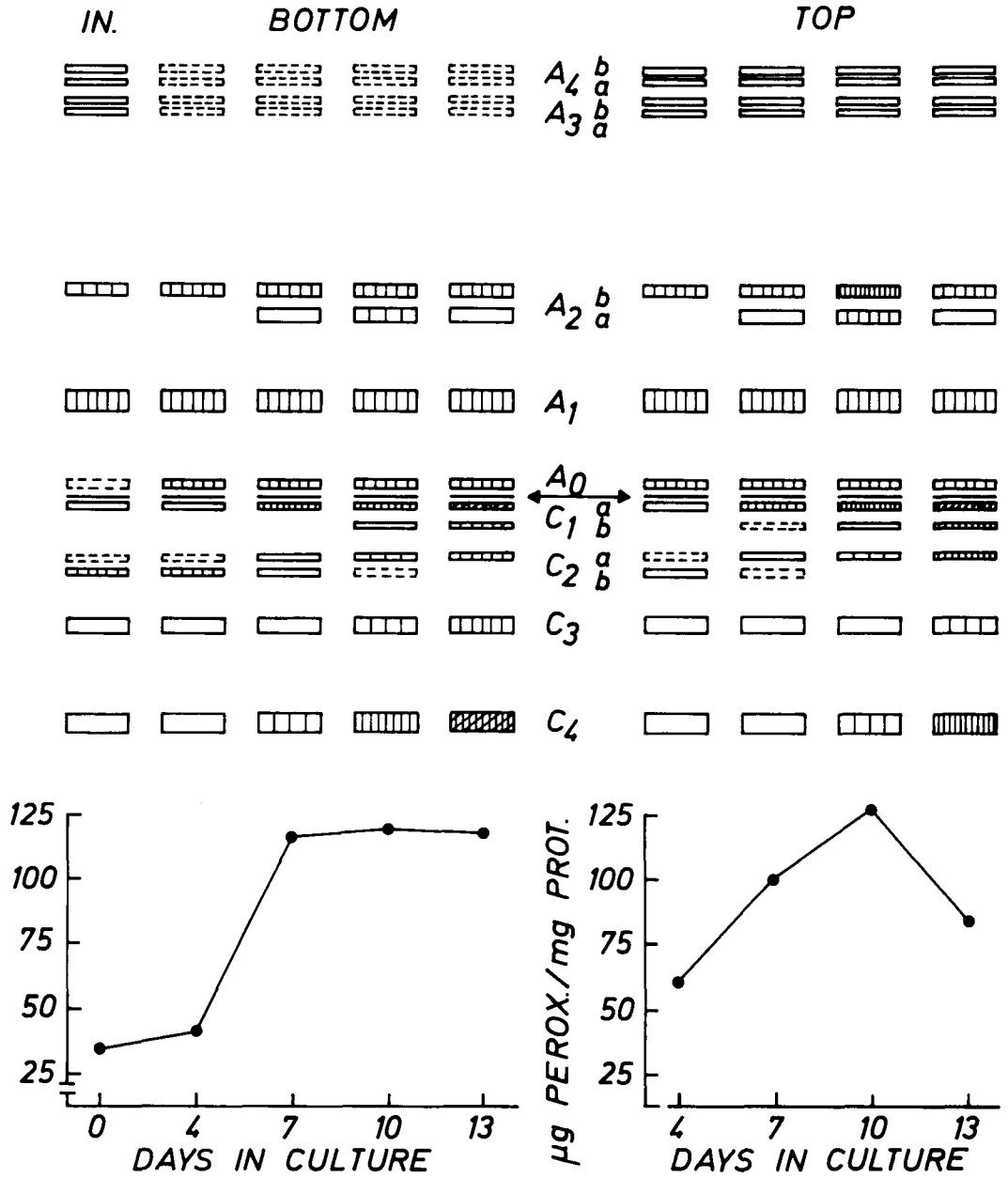

FIG. 3. Comparison of isoperoxidase patterns and total specific peroxidase activity of bottom and top portions of tobacco callus grown under shoot-forming conditions in the dark. IN - inoculum.

thought to be associated with lignification (23-25), it can be conceived that conditions leading to shoot formation maintain the tissue in a more meristematic state by reducing lignification of the cell walls. This idea is consistent with histochemical observations on shoot-forming tobacco callus (26), and on tyrosine incorporation into lignin in this tissue (27).

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