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INDUCTION OF CINNAMIC ACID 4-HYDROXYLASE IN DEVELOPING MAIZE SEEDLINGS

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Zea mays*; Gramineae; maize; germination; cinnamic acid-4-hydroxylase; enzyme induction.

Abstract—The change in the activity of cinnamic acid 4-hydroxylase in light- and dark-grown maize seedlings was studied during their early growth. The total enzyme activity in shoots of light-grown developing seedlings reached its highest activity on day five after planting, and then decreased gradually. The highest specific activity, on a fresh weight basis, of the enzyme was seen in day two. A similar pattern was observed in dark-grown seedlings. The enzyme activity was detected in roots and cotyledons where the activity also increased during early growth and then decreased. The increase in activity in the developing shoots of light-grown seedlings was completely suppressed by cycloheximide.

INTRODUCTION

Cinnamic acid 4-hydroxylase (EC 1.14.13.11), which catalyses the formation of *p*-coumaric acid from *trans*-cinnamic acid, is a key enzyme in the synthesis of phenylpropanoids. This enzyme which catalyses the second reaction of the core phenylpropanoid sequence, was first detected in microsomal membranes of pea seedlings by Russell and Conn [1, 2] and has subsequently been shown to be widely distributed in plants [3–12]. The induction of cinnamic acid 4-hydroxylase has been reported in several experimental systems, e.g. cut-injured sweet potato roots [12] and Jerusalem artichoke tubers [4]; illuminated excised buckwheat hypocotyls, gherkin cotyledons and pea seedlings [3, 5, 6], irradiated cell-suspension cultures of *Petroselinum hortense* [8, 10] and wheat leaves infected with *Botrytis cinerea* [11]. However, only a few temporal studies of the enzyme activity in relation to the growth of plants have been performed in the absence of external factors other than light. The present paper describes the induction of cinnamic acid 4-hydroxylase in developing maize seedlings and the effect of cycloheximide on that induction.

RESULTS

Changes in cinnamic acid 4-hydroxylase activity in the developing maize seedlings

All plant materials were harvested at the same time of day to ensure comparable growth conditions. The growth rate of seedlings under fluorescent light was lower than that of seedlings grown in the dark (Fig. 1).

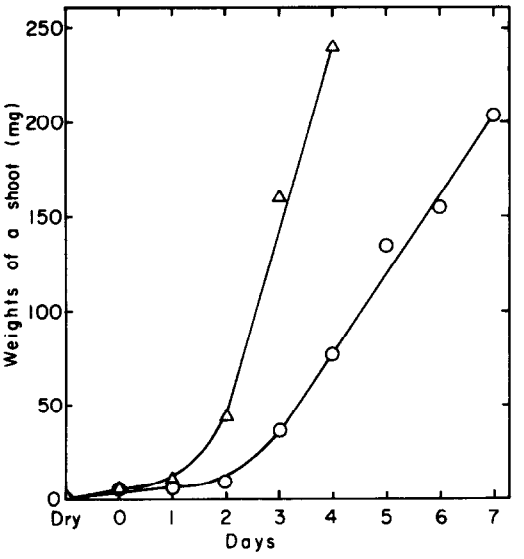

Fig. 1. Fresh weight of a shoot of developing maize seedling. (○) and (△): light- and dark-grown seedlings, respectively.

Changes in the activity of cinnamic acid 4-hydroxylase during development of the seedlings grown under light and dark conditions are shown in Figs 2 and 3, respectively. The total amount of enzyme activity in a shoot of developing light-grown seedling increased up to day five after a one-day lag period, and then decreased. The highest specific activity (activity/g fr. wt) of the enzyme occurred on day two. Phenolic acid(s) located below *p*-coumaric acid on the TLC sheet and coumarin(s) located at the origin of the TLC sheet were also detected in the extract of shoots of day two light-grown seedlings and the amounts increased up to day seven (data not shown).

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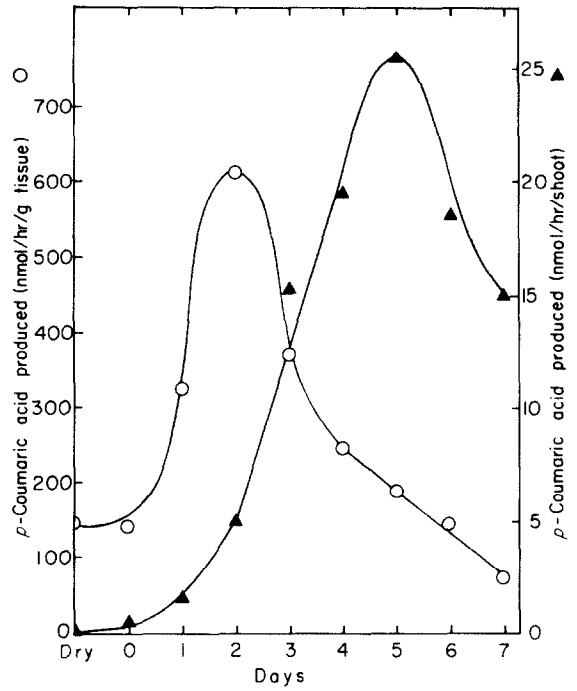

Fig. 2. Change in cinnamic acid 4-hydroxylase activity in developing light grown maize seedlings, (○) per gram tissue (▲) per shoot.

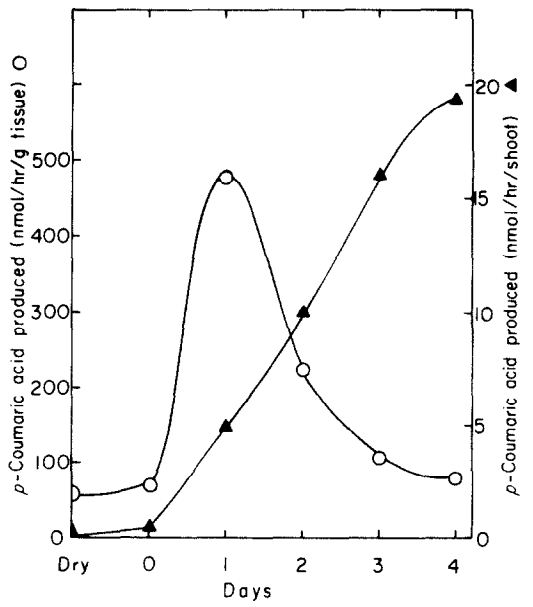

Fig. 3. Change in cinnamic acid 4-hydroxylase activity in developing dark grown maize seedlings, (○) per gram tissue (▲) per shoot.

In dark-grown seedlings, the total enzyme activity per shoot increased during the period from days one to four but the high specific activity (activity/g fr. wt) was observed on day one. Phenolic compound(s) were also detected in the extracts of shoots of day two dark-grown seedlings, but no coumarin(s) were observed. Both phenolic compound(s) and coumarin(s) were detected in the extract of day four dark-grown seedling (data not shown).

The relative amounts of activity in the roots and cotyledons of light- and dark-grown seedlings are shown in Tables 1 and 2 respectively. The enzyme activity in cotyledons and embryos of dry seeds was 34 and 60 nmol/hr/g tissue respectively (corresponding to 91 and 14 nmol/hr/100 organs). Thus, the enzyme activity in both tissues increased dramatically during germination. The activity increased in both organs during early stage of growth and paralleled those in the shoots, but then decreased more rapidly after reaching maximum levels (data not shown).

Effect of cycloheximide on the increase and subsequent decrease in cinnamic acid 4-hydroxylase activity in light-grown maize seedlings

Cycloheximide completely inhibited any increase in the activity of cinnamic acid 4-hydroxylase as well as germination when seeds were soaked and germinated in solutions containing 5 μg/ml cycloheximide (Fig. 4A). On day two, the weights of germinating embryos of 100 plants germinated in water and cycloheximide solution (5 μg/ml) were 946 mg and 324 mg, respectively. Lower concentration of cylcoheximide (1 μg/ml) did not affect either the increase in cinnamic acid 4-hydroxylase activity and or germination (data not shown). Since the specific activity of cinnamic acid 4-hydroxylase decreases after two days (Figs 2 and 4A), we examined the effect of cycloheximide on the decrease when this inhibitor was administered to two-day-old seedlings grown initially in water. As shown in Fig. 4A, cycloheximide (5 μg/ml) did not affect the decrease in specific activity (activity/g fr. wt) during the subsequent three days but completely

Table 1. Cinnamic acid 4-hydroxylase activity in organs of light grown maize seedlings

	p-Coumaric acid produced (nmol/hr)	
Organs	per g tissue	per 100 organs
Shoots	645	1650
Roots	352	1130
Cotyledons	517	3910

10 000 *g* supernatant of three-day-old seedlings were used as the enzyme source.

Table 2. Cinnamic acid 4-hydroxylase activity in different organs of dark grown maize seedlings

	p-Coumaric acid produced (nmol/hr)	
Organs	per g tissue	per 100 organs
Shoots	394	1620
Roots	232	1220
Cotyledons	334	2200

10 000 *g* supernatant of two-day old seedlings were used as the enzyme source.

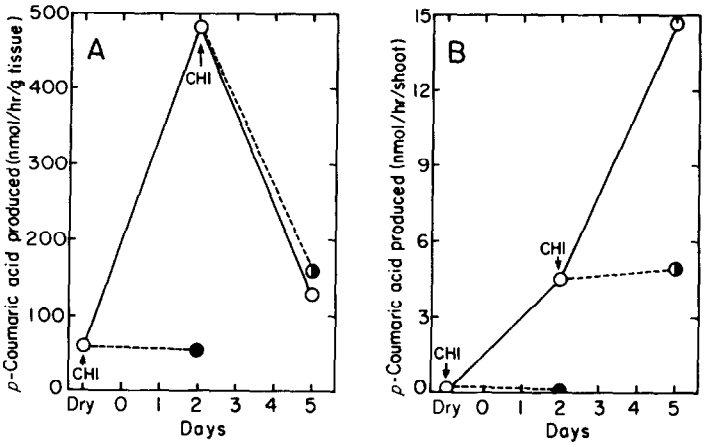

Fig. 4. Effect of cycloheximide on the increase and subsequent decrease in cinnamic acid 4-hydroxylase activity in light grown maize seedling. A and B, the enzyme activity per g fresh weight and per shoot, respectively. Dry seeds were soaked and germinated in water (○) or in cycloheximide solution (5 μg/ml, ●). In a third group of seedlings water was replaced by cycloheximide solution (5 μg/ml) on day two and the experiment continued until day five. (◑).

inhibited the increase in the total activity per shoot during that time (Fig. 4B). On day five, the weight of shoots of 100 seedlings grown continually in water and in cycloheximide solution (5 μg/ml) from day two to day five was 11.2 and 3.1 g, respectively. These data strongly suggest that the increase in cinnamic acid 4-hydroxylase activity in the developing maize seedlings over the five days involves the *de novo* synthesis of the enzyme.

DISCUSSION

The present study has shown that the total amount of cinnamic acid 4-hydroxylase per shoot in light grown developing maize seedlings increased up to day five, and then decreased rapidly. The specific activity of the enzyme, on a fresh weight basis, reached its highest levels even earlier, i.e. on day two (Fig. 2). A qualitatively similar pattern for specific activity was observed in dark grown developing shoots (Fig. 3), although the total activity per shoot had not declined after day four. In other work (data not shown) the highest specific activity of the enzyme was observed in one-day-old light grown shoots of *Sorghum* seedlings and in the apical buds of sweet clover (*Melilotus alba*) leaves.

The concomitant induction of cinnamic acid 4-hydroxylase and phenylalanine ammonia-lyase and has been observed in excised buckwheat hypocotyl [3], gherkin cotyledons [6] and suspension cultures of parsley [8–10] upon illumination. Both enzymes are also induced with similar time course patterns in sweet potato root in response to wounding [12], in cell suspension cultures of soybean prior to stationary phase [9], and in cell cultures of *Phaseolus vulgaris* during phaseollin production [7]. Cinnamic-4-hydroxylase induction has also been observed in other tissues in response to wounding [4, 5], fungal infection [11] and the administration of a number of xenobiotics such as phenobarbital, ethanol and herbicides [13, 14]. All of these treatments result in increased phenylpropanoid metabolism and suggest that the flow of carbon into specific end products is facilitated by increased activity of cinnamic acid 4-hydroxylase.

EXPERIMENTAL

Plant materials. Seeds of maize (*Zea mays*, Hybrid sweet corn NK199 obtained from Northrup King and Company, Minneapolis, MN) were soaked in aerated H_2O for 24 hr and planted in trays of moist vermiculite. They were then allowed to germinate for 7 days either under fluorescent light (1700 ft-c) employing a 16:8 hr (light:dark) photoperiod or in a dark cupboard at 26°. When seeds were germinated in the presence of cycloheximide, a solution of cycloheximide (5 μg/ml) was used for moistening the vermiculite. When cycloheximide was administered to 2-day-old seedlings, the vermiculite was washed 3 times with the cycloheximide solution and seedlings were grown for a further 3 days in trays of vermiculite soaked with cycloheximide. At intervals the seedlings were harvested, and the cotyledons, shoots and roots were separated.

Enzyme preparation. Plant tissues (*ca* 1 g fr. wt) were ground in a cold mortar with 4 vols of 50 mM Tris–HCl buffer (pH 8.0) containing 0.33 M sorbitol, 4 mM $MgCl_2$, 4 mM EDTA and 2 mM 2-mercaptoethanol. Where green tissues were used sand was added to assist grinding. The homogenate was squeezed through four layers of cheese cloth. The filtrate was centrifuged at 10 000 *g* for 10 min, and the supernatant solution was passed through a Sephadex G-25 column which had been equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 2 mM 2-mercaptoethanol to remove polyphenols and other low-M_r substances. The protein fraction eluted from the column with the same buffer (10 000 *g* supernatant solution) was further centrifuged at 100 000 *g* for 1.5 hr. The resulting pellet was resuspended with a Teflon-glass homogenizer in 1 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 2 mM 2-mercaptoethanol to yield the microsomal fraction. The 10 000 *g* supernatant solution and the microsomal fraction were both used for enzyme assay.

Assay for cinnamic acid 4-hydroxylase. The reaction mixture was composed of 50 mM Tris–HCl buffer (pH 8.0), 0.5 mM DDT, 1 mM glucose-6-P, 1 mM NADPH and 1 mM 2-mercaptoethanol containing 0.1 unit of glucose-6-P dehydrogenase and 0.25 μCi *trans*-[3C^{14}]cinnamic acid (sp. act., 4.51 μCi/μmol) in a final volume of 250 μl. The reaction was started by adding enzyme solution and incubated at 30° for 20 min.; it then was stopped by adding 35 μl of 20% TCA. After adding 0.3 μmol of *p*-coumaric acid (30 μl), the protein precipitated was removed by centrifugation.

The supernatant solution was vigorously mixed with 0.7 ml of EtOAc and the EtOAc layer was removed. The resulting aqueous layer was again extracted with 0.7 ml of EtOAc. The two fractions of EtOAc were combined and evapd under N_2. The residue was dissolved in 50 μl EtOAc and spotted on a TLC sheet 1B-F (J. T. Baker Chemical Co.) and developed with benzene–acetic acid (4:1). The positions of cinnamic acid and *p*-coumaric acid on the TLC sheet were visualized under UV light, where the respective R_f values were 0.85 and 0.60. Radioactivities corresponding to cinnamic acid, *p*-coumaric acid and other regions were counted by a liquid scintillation spectrometer (Beckman Co.). The radioactive product corresponding to *p*-coumaric acid region on the TLC sheet was identified as *p*-coumaric acid by radio GC using a gas chromatograph equipped with a FID and a column of 10% SE-52 on GCQ 60–80 mesh (15).

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