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INHIBITION OF RADISH GERMINATION AND ROOT GROWTH BY COUMARIN AND PHENYLPROPANOIDS

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Abstract—Thirteen natural and synthetic phenylpropanoids as well as coumarin (2×10^{-4}M) were tested for their biological activity on radish germination and subsequent root growth in light and darkness. Coumarin was the most potent inhibitor. With some exceptions, phenylpropanoids with a carboxylic group in the side chain inhibited root growth. Coumarin was formed spontaneously by photooxidation of 2-hydroxycinnamic acid. Microscopic observations of root treated with coumarin suggest that this substance inhibits the elongation of cells of the differentiating zone of the root.

Key Words—Coumarin, dormancy, light, phenylpropanoids, radish, *Raphanus sativus*, seed, root inhibition.

INTRODUCTION

It is well known that phenolic compounds such as simple phenols, phenylpropanoids, and coumarins may play an important role in allelopathy by either inhibiting germination or reducing growth of seedlings (Bewley and Black, 1985; Putnam and Tang, 1986). However, clear insight into the precise physiological perturbation caused by these substances has not been obtained because of the

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difficulty in determining the specific site of the action (Rice, 1984; Einhellig, 1986).

Recently, we have shown that some free forms of glucosylated phenylpropanoids possess antialgal activity *in vitro* (Aliotta et al., 1992a) and that in radish seeds coumarin inhibits germination in the presence of light (Aliotta et al., 1992b). These data have led us to investigate the structure–activity relationship of some synthetic and natural phenylpropanoids and coumarin upon germination and seedling growth of radish in light and in darkness. Moreover, the ultrastructure of the root of radish seedlings treated with coumarin was studied to identify the morphological aspects of growth reduction.

METHODS AND MATERIALS

Compounds **1, 2, 3, 4, 5, 6, 7, 8**, and **9** (Table 1) were purchased from Aldrich-Chemical Company (Milwaukee, Wisconsin); the remaining phenylpropanoids were synthesized.

To synthesize (*E*)-propenylbenzene **(10)** and (*Z*)-propenylbenzene **(11),** allylbenzene **(9)** (600 mg) was refluxed with ethanol saturated with KOH (3 ml). After 5 hr, the solution was neutralized with 2 N HCl and extracted with Et_2O. The organic material (450 mg) was then separated by argentation preparative TLC [petrol–Et_2O (9 : 1)] to give **10** (310 mg) and **11** (110 mg).

To synthesize 2-methoxycinnamic **(12),** 3-methoxycinnamic **(13),** and 4-methoxycinnamic **(14)** acids, a commercial sample (200 mg) of the corresponding hydroxy acid was treated with ethereal CH_2N_2 to give the permethylated derivative, which was refluxed in aqueous methanolic KOH for 3 hr to give, after acidification, the methoxy acid.

Bioassay. Seeds of *Raphanus sativus* L. Saxa, collected during 1990, were purchased from Imperatore Co., Naples. The seeds were surface-sterilized in 95% ethanol for 15 sec and germinated on 30-ml layers of Bacto-Agar gel (10 g/liter H_2O) in covered 9-cm sterilized Petri dishes. Germination conditions were 25 $\pm$ 1°C with a continuous irradiance of 25 $\mu E/m^2/sec$ or darkness. Different amounts of neutral substances **8**, **9**, **10**, and **11**, whose concentrations ranged from 5×10^{-5} to 2×10^{-4} were dissolved in acetone and adsorbed by Whatman filter paper disks (diameter 0.6 cm). Disks were inserted in the middle of Petri dishes after the solvent had evaporated. An acetone control filter disk was included in each treatment series. Concentrations corresponding to those of neutral compounds were tested for acidic compounds **(1, 2, 3, 4, 5, 6, 7, 12, 13,** and **14),** previously neutralized with $NaHCO_3$. Aliquots of these compounds were placed directly in Petri dishes with agar. Seeds were placed in Petri dishes only after uniform distribution of the substances in agar had been obtained by diffusion (three days). The actual time required varied from a few hours for

TABLE 1. INHIBITION OF PHENYLPROPANOIDS AND COUMARIN (2×10^{-4} M) ON GERMINATION AND ROOT GROWTH OF RADISH AFTER FIVE DAYS OF SOWING.

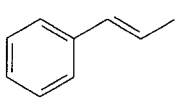
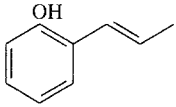
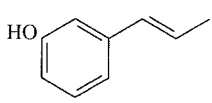
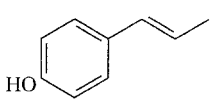
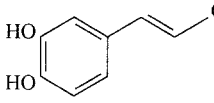
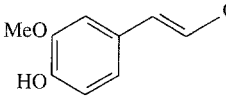
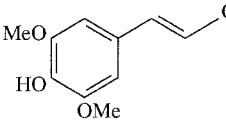
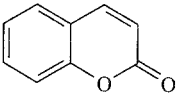
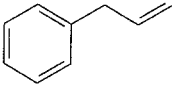
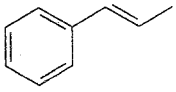
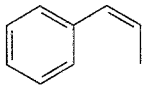
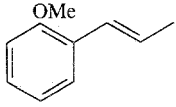
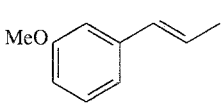
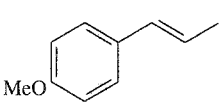
Compound	Germination (%)		Root Length (mm)	
	Light	Dark	Light	Dark
CONTROL	88 ± 3	99 ± 1	76 ± 8	97 ± 7
 cinnamic acid **1**	88 ± 5	95 ± 1	54 ± 6	41 ± 4
 2-hydroxycinnamic acid **2**	40 ± 5	99 ± 1	12 ± 8	42 ± 4
 3-hydroxycinnamic acid **3**	90 ± 4	99 ± 1	75 ± 10	96 ± 9
 4-hydroxycinnamic acid **4**	80 ± 3	99 ± 1	65 ± 9	42 ± 6
 3,4-dihydroxycinnamic acid **5**	91 ± 4	99 ± 1	54 ± 8	62 ± 8
 4-hydroxy-3-methoxycinnamic acid **6**	87 ± 6	99 ± 1	65 ± 10	64 ± 6
 4-hydroxy-3,5-dimethoxycinnamic acid **7**	88 ± 5	99 ± 1	75 ± 8	95 ± 4
 coumarin **8**	50 ± 6	90 ± 5	6 ± 3	8 ± 4
 allylbenzene **9**	85 ± 6	99 ± 1	75 ± 7	92 ± 5

TABLE 1. CONTINUED

Compound	Germination (%)		Root Length (mm)	
	Light	Dark	Light	Dark
 (*E*)-propenylbenzene **10**	89 ± 7	99 ± 1	79 ± 8	94 ± 6
 (*Z*)-propenylbenzene **11**	90 ± 2	99 ± 1	78 ± 7	95 ± 7
 2-methoxycinnamic acid **12**	89 ± 4	99 ± 1	45 ± 6	85 ± 7
 3-methoxycinnamic acid **13**	89 ± 5	99 ± 1	46 ± 5	61 ± 9
 4-methoxycinnamic acid **14**	89 ± 3	99 ± 1	45 ± 5	63 ± 8

[a]Data are expressed as percentages of germination ± SD and root length ± SD.

neutralized phenylpropanoids to three days for **8, 9, 10,** and **11**. The time of this equilibrium distribution was established by assaying the substances over time in the filter paper disk as well as in three cylinders of agar having the same volume (0.132 ml, diameter 0.6 cm) and the distances of the centers from the middle of the filter paper disks of 1.2, 2.4 and 3.6 cm, respectively (Aliotta et al., 1992b). For each substance, concentrations corresponding to those used were observed in agar cylinders after the times indicated above. Germination and root growth were followed in light and in darkness. Seeds were considered germinated when the protrusion of the radicle became evident. Effects on root elongation were determined by measuring, to the nearest millimeter, the length of the radicle of each seedling five days after placing the seeds on the agar medium. Each determination was replicated three times using five Petri dishes containing 20 seeds each. Data are expressed as mean ± standard deviation (SD).

Estimation of Coumarin Formed by 2-Hydroxycinnamic Acid. The agar containing 2-hydroxycinnamic acid was dissolved at 70°C for 3 min and extracted

for 6 hr with ethyl acetate in a Soxhlet apparatus. The extract was dried and chromatographed on preparative TLC [$CHCl_3$–AcOEt (3:1)] to separate 2-hydroxycinnamic acid from coumarin. The coumarin was identified from NMR spectral data. To determine the rate of transformation, a neutralized solution (120 ml) of 2-hydroxycinnamic acid (360 mg) was placed into 12 flasks (10 ml each). Six flasks were kept at 25°C with a continuous irradiance of 25 $\mu E/m^2/$ sec and six flasks were kept in darkness. Every day, one flask from the light and one from the dark treatments were acidified with 2 N HCl and extracted with diethyl ether. The extract was dried and chromatographed on TLC [$CHCl_3$–AcOEt (3:1)]. Coumarin and unchanged 2-hydroxycinnamic acid were identified by comparison with authentic samples.

Light and Electron Microscopy. Control and coumarin-treated root tips were excised 96 and 170 hr after sowing, respectively. The difference in time was due to a delay in germination of seeds treated with coumarin (2×10^{-4} M). In this respect, control and coumarin-treated root tips were observed 48 hr after their protrusion. Root tips were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 hr at room temperature. The root tips were then placed for 1 hr into 2% OsO_4 in 0.1 M phosphate buffer (pH 7.3) before being dehydrated in a graded series of ethanol solutions and embedded in Epon 812 resin (Luft, 1961).

Thick sections (ca. 1 μm each) were stained with 0.1% toluidine blue and observed with a Zeiss light photomicroscope.

Thin sections, obtained with a diamond knife on a Supernova microtome, were sequentially stained at room temperature with 2% uranyl acetate (aqueous) for 5 min and by lead citrate for 10 min (Reynolds, 1963). Ultrastructural studies were made using a Philips CM12 transmission electron microscope operated at 80 kV.

Some root tips, after fixation and ethanol dehydration, were also critical-point dried and finally coated with carbon and gold in a sputter-coater. These specimens were observed at 20 kV with a Cambridge 250 Mark3 scanning electron microscope.

The observations were carried out at Centro Interdipartimentale di Ricerca sulle Ultrastrutture Biologiche (Faculty of Sciences, University of Naples).

RESULTS AND DISCUSSION

As can be seen, germination and root growth of radish were slightly inhibited by light; moreover, coumarin and 2-hydroxycinnamic acid were the most potent inhibitors of both germination and subsequent root growth (Table 1). Radish seed inhibition induced by coumarin was higher in light than in darkness, which differs from what has been seen with lettuce (Berrie et al., 1968). Percent

inhibition of germination of radish seeds by coumarin in light and darkness, however, was less than its inhibition of root length. For the phenylpropanoids tested, it appears that those with a carboxylic function in the side chain and only a hydroxyl or methoxyl group in the ring were most active on root growth, except for 3-hydroxycinnamic acid. 4-Hydroxy-3, 5-dimethoxycinnamic acid and the neutral compounds were inactive. No clear pattern of root growth inhibition for light vs dark was observed. In particular, 4-hydroxycinnamic acid is more active in darkness; in contrast 2-methoxycinnamic acid is more active in light.

It must be noted that 2-hydroxycinnamic acid is closely related to coumarin. Indeed, the presence of coumarin in Petri dishes containing neutralized 2-hydroxycinnamic acid was first noted by its aroma of mown hay and then confirmed by its spectral data. This is consistent with the report of Murray et al. (1982). Figure 1 shows that 2-hydroxycinnamic acid is transformed to coumarin in the presence of light. In this respect, the inhibition observed with 2-hydroxycinnamic acid may be due to the formation of coumarin.

It must be emphasized that light, an important ecological and morphogenetic factor (Fenner, 1985), influences both the biological effects of coumarin and its formation from 2-hydroxycinnamic acid.

Root Anatomy. Figure 2 shows a comparison of longitudinal median sections of a control radish root and one chosen among the few seedlings grown in light in presence of coumarin. These roots were excised 48 hr after their germination. As can be seen, the pattern of radish root development in the

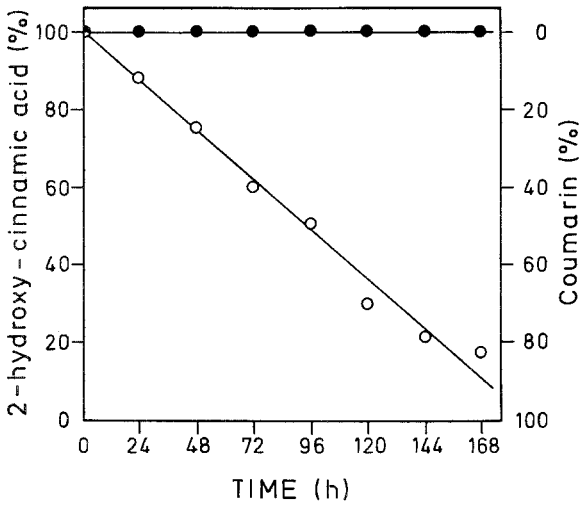


FIG. 1. Time-course of oxidation of 2-hydroxycinnamic acid to coumarin in presence of continuous light (○) and dark (●).

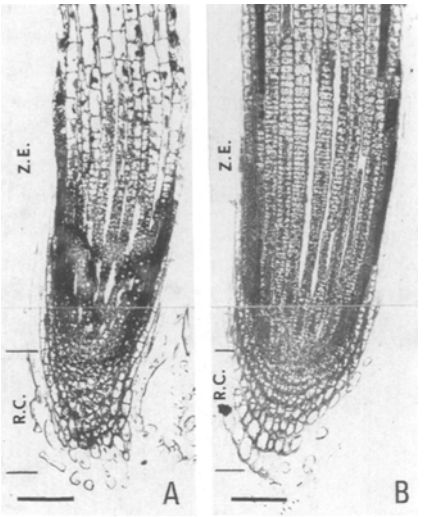


FIG. 2. Anatomy of radish root tips. (A) control; (B) treated with coumarin. Bar = 100 μm. R.C. = root cap; Z.E. = zone of elongation.

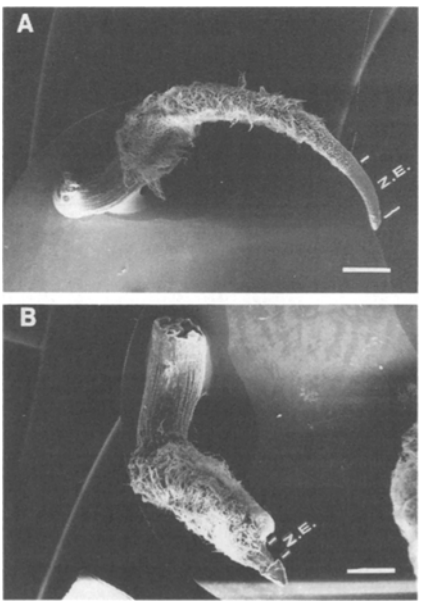


FIG. 3. Scanning electron micrographs of radish root tips. (A) control; (B) treated with coumarin. Bar = 1 mm. Z.E. = zone of elongation.

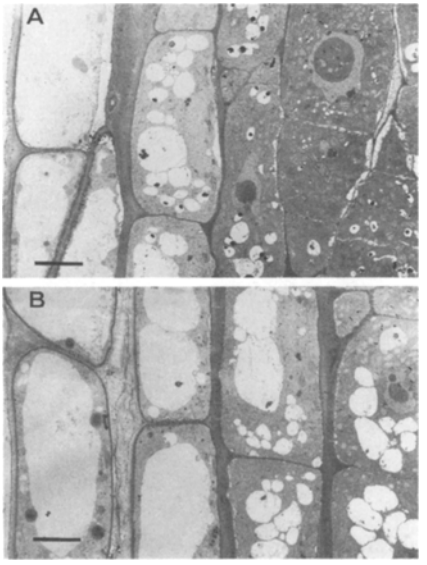


FIG. 4. Transmission electron micrographs of the meristematic zone. (A) control; (B) treated with coumarin. Bar = 0.5 μm.

presence of coumarin (Figure 2B) differs from that of the control (Figure 2A). In fact, the architecture of the control root system is elongated, unlike the coumarin-treated root system, which is more compact in growth. According to Avers and Goodwin (1956) and Jankay and Muller (1976), coumarin appears to inhibit the elongation of cells of the differentiating zone of the root. Scanning electronic micrographs of the same roots confirmed that the elongation zone of the root is shorter for the treated coumarin root (Figure 3B) than for the control (Figure 3A). Moreover, in the coumarin root system, there was an apical shift of root hair differentiation to form a tuft (Figure 3B) not observed in the control (Figure 3A).

A low-magnification view of a transverse cross-cut of the meristematic areas obtained with transmission electron micrographs shows that cells treated with coumarin (Figure 4B) are highly vacuolated compared with the control (Figure 4A). Further studies are in progress to identify the sequence of cellular events causing growth reduction.

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