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Isolation of a phytoalexin, *trans-p*-coumaryl aldehyde, from *Cucurbita maxima*, Cucurbitaceae

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Abstract

An induced antifungal compound, *trans-p*-coumaryl aldehyde, was isolated from fruit tissue of *Cucurbita maxima* which had been elicited with pectinase. Its chemical structure was identified by NMR and MS. This is the first report of a phytoalexin from a member of the Cucurbitaceae. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Lignification, formation of a physical barrier and phytoalexin production, synthesis of a chemical barrier, have been established as general mechanisms of disease resistance in plants (Vance, Kirk, & Sherwood, 1980; Nicholson & Hammerschmidt, 1992; Boudet, Lapierre, & Grima-Pettenati, 1995; Kuc, 1995). Studies on lignification in cucurbits are numerous and serve as the basis for much of our understanding of this defense mechanism (Grand & Rossignol, 1982; Hammerschmidt, Bonnen, Bergstorm, & Baker, 1985; Dean & Kuc, 1987; Robertsen & Svalheim, 1990). Although many researchers have examined the composition of polymeric material late in the disease interaction (Grand & Rossignol, 1982; Hammerschmidt et al., 1985; Robertsen & Svalheim, 1990), little work has focused on biochemical analysis early in the interaction. Previous researchers (Hammerschmidt & Kuc, 1982) postulated that the lignin precursor, coniferyl alcohol, may function as a phytoalexin in cucumber. Although synthetic coniferyl alcohol was highly fungitoxic, detectable quantities were not present in planta (Hammerschmidt & Kuc, 1982). Subsequent analysis of the lignin-like material deposited in disease interactions indicates that it is primarily derived from *p*-coumaryl alcohol, not coniferyl alcohol (Grand & Rossignol, 1982; Hammerschmidt et al., 1985; Robertsen & Svalheim, 1990).

To date, phytoalexins in their native form have not been identified in the Cucurbitaceae. However, recent research suggests that glycosylated phenolics may play a role in defense (Daayf, Bel-Rhlid, & Bélanger, 1997; Fawe, Abou-Zaid, Menzies, & Bélanger, 1998). Two antifungal phenolics, *p*-coumaric acid methyl ester (Daayf et al., 1997) and rhamnetin (Fawe et al., 1998) have been isolated from acid-hydrolyzed methanol extracts of cucumber leaves challenged with powdery mildew. However, when extracts were assayed prior to acid hydrolysis there was no evidence of induced antifungal compounds (Fawe et al., 1998).

We report here that elicitation of tissue from the fruit of green acorn squash, *Cucurbita maxima* Duchesne, with pectinase induces the synthesis of lignin-like material as well as an antifungal compound,

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trans-p-coumaryl aldehyde. This compound was isolated from tissues in its native and active form.

2. Results and discussion

Previous investigators reported that the defense response in cucurbits may be triggered by fungal infection by (Grand & Rossignol, 1982; Hammerschmidt et al., 1985) or by pectin degrading enzymes (Robertsen, 1987, 1990). In our studies we found that commercial pectinase from *Aspergillus niger* strongly elicited the formation of lignin-like material in the cut surfaces of squash fruit tissue (Stange & McDonald, in press). The amount of lignin-like material in tissue, as measured by selective staining with Schiff's reagent, increased rapidly for 12 h after elicitation and remained constant thereafter (Stange & McDonald, in press). Microscopic examination of tissues 18 h after elicitation revealed that the outer three to seven cell-layers turned bright red when treated with phloroglucinol–HCl (PG–HCl), indicating the presence of lignin-like material (Robertsen & Svalheim, 1990). In the process of analyzing the tissues for lignin, we noticed that EtOH extracts of elicited tissues turned pinkish to red when PG–HCl was added. This suggested that EtOH–soluble aldehyde- or allyl-compounds (Feigl & Anger, 1966) as well as lignin-like material, were induced. To our knowledge such compound(s) have not been previously described in cucurbits, so we focused our efforts on determining the biological significance and identity of these induced compound(s).

Ethanol extracts of elicited and control squash were concentrated and separated by TLC. When visualized with PG–HCl, no reacting compounds were present in extracts from the control tissue. However, in extracts from elicited tissue, a single cherry-red spot was revealed. Extracts of control and elicited tissues were separated by column chromatography on silica gel and the fractions bioassayed using *Cladosporium cucumerinum* (Keen, Sims, Erwin, Rice, & Partridge, 1971). These experiments revealed the presence of one induced antifungal compound. The zone of antifungal activity corresponded to the compound forming a red product where the plate had been sprayed with PG–HCl. There was no evidence of any antifungal activity in extracts of control samples.

Extracts from 1.0 kg of elicited squash tissue were purified by extensive column chromatography on silica gel and yielded 3 mg of a white crystalline compound identified as *trans-p*-coumaryl aldehyde. Antifungal activity in TLC bioassays was apparent where 4 μg or more of the compound was applied. By comparing the sizes of the zones of inhibition made by known amounts of the compound, we estimate its concentration in responding tissue to be 10 μg/g fr. wt. The presence of *p*-coumaryl aldehyde was also confirmed in extracts of elicited-cured fruit tissues from buttemut squash, pumpkin, cucumber and bitter melon by spraying chromatograms of extracts with PG–HCl. Similar levels of a related compound, coniferyl aldehyde, were reported to accumulate in flax inoculated with an incompatible race of *Melampsora lini* (Keen & Littlefield, 1979). *p*-Coumaryl aldehyde has been isolated from *Alpinia galanga* (Barik, Kundu, & Dey, 1987) and the female inflorescence of *Sarcophyte sanguinea* (Naidoo, Drewes, Van Staden, & Hutchings, 1992) but to our knowledge this is the first report of it as an induced antifungal compound.

The role of low molecular weight phenolic compounds in resistance in the Curcurbitaceae has only recently become apparent (Daayf et al., 1997; Fawe et al., 1998). This is the first report of the isolation of an induced antifungal compound in its native form from this family. The concentration of a related compound, *p*-coumaric acid methyl ester (presumably in its glycosylated form) has been shown to increase in leaves of cucumbers made resistant to powdery mildew by spraying them with extracts of *Reynoutria sachalinensis* (Naidoo et al., 1992). We presume on the basis of its similarity to known lignin precursors that *p*-coumaryl aldehyde is also related to the synthesis of the lignin-like material deposited in the disease resistance responses described in the cucurbits (Grand & Rossignol, 1982; Hammerschmidt et al., 1985; Robertsen & Svalheim, 1990). Whether it is simply an intermediate in the production of *p*-coumaryl alcohol or if it is directly incorporated into a polymer via a pathway analogous to lignification, or by some other mechanism, remains to be determined.

3. Experimental

3.1. Plant material

Mature fruit, weighing 0.5–1.0 kg, of green acorn squash, *C. maxima*, were purchased from local markets. Only recently harvested fruit, as evidenced by a living 5 green stem, showing no signs of injury or decay were used.

3.2. TLC bioassay

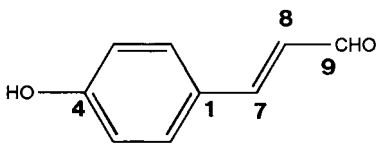
Antifungal activity of extracts and chromatographic fractions was determined by TLC bioassay. The volumes of extracts from control and elicited-cured tissues spotted were adjusted to compare them on an equivalent fr. wt. basis. Silica gel TLC plates were spotted and developed in hexane–EtOAc (1:1). Air dried plates were first sprayed with a dense conidial suspension of *C. cucumerinum* and then with half-

strength potato-dextrose agar (Keen et al., 1971). Plates were incubated in the dark for 2 days in water saturated atmosphere at 20°. Antifungal activity was indicated by a white spot on a background of brown–black fungal growth.

3.3. Induction and isolation of phytoalexin

Fruit were scrubbed with a soft brush in soapy water, rinsed with tap water and then rinsed twice with ethanol. Surface-sterilized fruit were then sliced 4–5 mm thick, rinsed for one min in tap water and placed in a single layer on moist Kraft paper. Cut surfaces were sprayed to wetness with an aq. soln containing 3.6 U *A. niger* pectinase ml^{-1} and 15 mg $CaCl_2$ ml^{-1}. Slices were incubated in the dark at 25° and 90% relative humidity for 17–21 h. The outer surfaces of the slices were removed with a potato peeler. Tissue (1.0 kg) was homogenized in 1.8 l EtOH with 30 g $NaHCO_3$. Liquid was removed by filtration and the residue washed with 0.6 l EtOH. Combined ethanolic extracts were concentrated under reduced pressure at 37°. This solution was successively partitioned against hexane and EtOAc. The components in the EtOAc fraction were separated by column chromatography on silica gel, first in hexane, C_6H_6, CH_2Cl_2 and CH_2Cl_2–Et_2O, second in hexane with increasing percentage EtOAc and finally in hexane with increasing percentage Et_2O.

Compound **1** eluted in CH_2Cl_2–Et_2O (3:1), hexane–EtOAc (3:1) and hexane–Et_2O (3:2). Because of concerns about the stability of the compound being isolated, extracts and column fractions were not reduced to dryness until completion of the purification procedure. Column fractions were reduced to 2 ml under vacuum and assayed for biological activity using the TLC-bioassay described previously. Control extracts were prepared from freshly sliced squash fruit tissue using the method describe above.



Purification yielded 3 mg of white crystalline compound. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.62 (1H, dd, J = 15.9, 7.7, H8), 6.90 (2H, d, J = 8.5, H3 and H5), 7.42 (1H, d, J = 15.9, H-1), 7.50 (2H, d, J = 8.5, H2 and H6) 9.67 (1H, d, J = 7.7, H-9). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 116.12 (C3 and C5), 126.56 (C-8), 126.98 (C1), 130.60 (C2 and C6), 152.72 (C-7), 158.46 (C4), 193.82 (C-9). EIMS M^+ 148(94), 147(100), 131(24), 120(34), 119(32), 107(21), 94(22), 91(42), 65(18). Spectral data correlates well with literature: MS (Barik et al., 1987; Naidoo et al., 1992) and NMR data in acetone-d_6 (Naidoo et al., 1992) and DWSO-d_6 (Barik et al., 1987). Samples were stored under N_2 at 5°.

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