Glyceollin: A Phytoalexin in Leaf Blight of *Costus speciosus*

S. Kumar, R. S. Shukla, K. P. Singh, J. D. Paxton, and A. Husain

Senior, second, and third authors, scientists; and fifth author, director, Central Institute of Medicinal and Aromatic Plants, Post Bag No. 1, Ram Sagar Misra Nagar, Lucknow-226016, India; and fourth author, associate professor, College of Agriculture, Department of Plant Pathology, University of Illinois, Urbana-Champaign 61801, USA.

We thank R. S. Thakur, Division of Medicinal Chemistry, Central Institute of Medicinal and Aromatic Plants, Lucknow, and K. Rinehart, Department of Chemistry, University of Illinois at Urbana-Champaign for their interest and suggestions during these investigations.

Accepted for publication 20 June 1984.

ABSTRACT

Kumar, S., Shukla, R. S., Singh, K. P., Paxton, J. D., and Husain, A. 1984. Glyceollin: A phytoalexin in leaf blight of *Costus speciosus*. Phytopathology 74:1349-1352.

Leaves of *Costus speciosus* were inoculated with a leaf blight-causing strain of *Drechslera rostrata* and a nonpathogenic strain of *Drechslera longirostrata* to investigate the reactions of this plant. *D. longirostrata* caused accumulation of phytoalexins during the interaction. In contrast, the pathogenic *D. rostrata* did not cause accumulation of phytoalexins. Glyceollin II and III were the major phytoalexins accumulated in this plant. *D. rostrata* produced phytotoxic metabolites.

Costus speciosus (Koen) Sm. (Zingerberaceae) is an important medicinal plant. Its rhizomes bear appreciable amounts of diosgenin, a steroidal sapogenin used for the synthesis of cortisone, sex hormones, and oral contraceptives (3). The plant grows wild throughout India, and attempts have been made to cultivate it as a source of diosgenin.

Recently, a leaf blight disease caused by *Drechslera* sp. severely damaged an experimental plantation. The disease symptoms first appear as small, necrotic spots which enlarge rapidly and turn dark brown with distinct chlorotic margins (Fig. 1); eventually it causes defoliation and death. Isolations from the infected leaves yielded two *Drechslera* spp. (21). These were identified by the Commonwealth Mycological Institute (CMI), England, as *Drechslera rostrata* (CMI 244749) and *D. longirostrata* (CMI 244750). Pathogenicity tests on healthy potted plants showed that only one of the isolates of *D. rostrata* was virulent and caused symptoms within 24 hr.

Several workers have suggested that the accumulation of phytoalexins is involved in disease resistance of plants (8,11,13,18,19). Phytoalexins restrict the growth of nonpathogens and play an important role in host-pathogen interactions (4,5). Cruickshank et al (2) described phytoalexins as antimicrobial substances that play a "key role" in defense mechanisms of plants. An antifungal role for phytoalexin accumulation in soybeans (*Glycine max*) was reported by Klarman and Sanford (14) and Keen et al (12).

The present investigation was designed to study the relation of phytoalexin production to disease development and growth of the pathogen. Special emphasis was given to studying the accumulation of phytoalexins in response to nonpathogenic *D. longirostrata* on *C. speciosus*. The possible involvement of toxic metabolites produced by pathogenic and nonpathogenic strains also was investigated.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

MATERIALS AND METHODS

Cultures of *D. longirostrata* and *D. rostrata* were maintained on PDA. Fifteen-day-old leaves of *C. speciosus* were cleaned thoroughly and floated on sterilized distilled water in 20-cm-diameter petri dishes. The upper surfaces of the leaves were inoculated with a spore suspension from a 12-day-old culture of *D. longirostrata*. The diffusates were collected by the facilitated diffusion technique (9) after 48 and 60 hr of incubation. The leaves were shaken intermittently with 40% aqueous ethyl alcohol for 8 hr and kept for 24 hr at 10 C. The supernatant solution was centrifuged and reduced to half of its original volume in vacuo at 40 C. The procedure was repeated to collect the phytoalexin diffusates from leaves treated with the pathogenic *D. rostrata*. This solution was extracted with diethyl ether and the aqueous phase was discarded. The ether extract was chromatographed (TLC-Silica gel G, 0.25 mm thick) with chloroform, acetone, and ammonium hydroxide (50:50:1, v/v) as the developing solvent. Glyceollin was detected as a fluorescence-quenching spot at R_f 0.50. Inhibitory spots were eluted from the first chromatogram and TLC was repeated on these compounds with chloroform:acetone:ammonium hydroxide (40:60:1, v/v) as the developing solvent for glyceollins (R_f 0.70). The mixture of glyceollins was separated by HPLC in hexane:isopropanol (97:3, v/v) on a silica column (Waters Associates, Milford, MA 01757) and analyzed by proton resonance spectroscopy.

The pathogenic and nonpathogenic strains, *D. rostrata* and *D. longirostrata* (respectively), were separately inoculated into Fries' modified liquid medium No. 3 (20) and incubated for 12 days at 27 ± 1 C. The medium was then filtered through Whatman No. 1 filter paper. The culture filtrates at pH 4.2 or uninoculated medium adjusted to pH 4.2 were spotted separately on an attached, pierced leaf surface of *C. speciosus* and incubated for 24 hr at a relative humidity of 86 ± 5% and 30 ± 4 C (Fig. 2).

The antifungal activity in both diffusates was tested by the inhibition of spore germination (1). A spore suspension of *Cladosporium cucumerinum* was prepared from 8-day-old cultures. These were suspended in sterilized distilled water and washed thrice by centrifugation, then used to test for inhibitory activity.

Fig. 1. Leaf of *Costus speciosus* showing characteristic symptoms of leaf blight disease caused by *Drechslera rostrata*.

RESULTS

The diffusate was found to possess antifungal properties. Spore germination of *C. cucumerinum* was 2% in 48-hr diffusates compared to that of the control (98%) as given in Table 1.

Glyceollin in the 48-hr diffusate extract on TLC plates was noted by a characteristic fluorescence-quenching band (7). The band was eluted in diethyl ether and identified by UV, HPLC, and PMR (16) as glyceollin II and III when compared with known glyceollins supplied by R. Lyne (Shell Research Laboratories, Sittingbourne, England). The mixture appears to contain glyceollin II as a major constituent and a minor amount of glyceollin III. The assignments for the protons of glyceollin II are shown in Table 2.

TABLE 1. Percentage of spore germination of *Cladosporium cucumerinum* in leaf extracts from *Costus speciosus*

Treatments	Spore germination (%)		
		P-48[a]	P-60[b]
Sterilized distilled water	97		
Control	98		
Inoculated with:			
D. longirostrata		2	63
D. rostrata		89	96

[a] P-48 denotes phytoalexin diffusates collected in 40% ethanol 48 hr after inoculation.

[b] P-60 denotes phytoalexin diffusates collected in 40% ethanol 60 hr after inoculation.

TABLE 2. NMR spectrum of glyceollin II from *Costus speciosus* in deuterochloroform

Proton	Glyceollin II[a]	J (Hz)
H-1	7.15 s	
H-4	6.21 s	
H-6(a)	4.04 d	12
H-6(b)	4.13 d	12
H-7	7.21 d	8
H-8	6.43 q	8.2
H-10	6.25 a	2
H-11(a)	5.25 s	
H-12	6.41	10
H-13	5.65 d	10
Me-15	1.37 s	
Me-16	1.40	

[a] Letters in this column: s = singlet, d = doublet, and q = quartet.

The additional signals on PMR for C-12 (2 H), C-13 (1 H), and C-15 (2 H) are indicative of the presence of glyceollin III (16).

Culture filtrates of the pathogenic strain, applied to leaves, caused spots after 24 hr due to the toxicity of the compounds produced by the pathogenic strain. The culture filtrate of the nonpathogen did not cause any spots around the pierced point on the leaf surface, as shown in Fig. 2.

DISCUSSION

Glyceollin I, II, III, and several other isoflavanoids accumulate in soybean leaves inoculated with nonpathogenic *Pseudomonas* sp. (7,10,13). HPLC analysis indicates the presence of glyceollin II and III as the major constituents of the phytoalexins accumulated by *C. speciosus* in response to *D. longirostrata*. Glyceollin has not been reported in plant species outside *Glycine* spp. and is a member of a group of related isoflavanoid-derived phytoalexins found throughout the Leguminosae.

The diffusates, in terms of inhibition of spore germination of *C. cucumerinum*, suggest that phytoalexin accumulation 48 hr after inoculation by *D. longirostrata* is twice that accumulated by 60 hr. This suggests some degradation of glyceollin by the plant or pathogen. Such responses were observed by Frank and Paxton (4) in the accumulation of phytoalexins and by Oku and Ouchi (19) in the death of hypersensitive cells. Mansfield et al (17) also reported that the concentration of phytoalexin varies at different periods of incubation.

D. longirostrata (nonpathogenic) inoculated leaves accumulated significant quantities of glyceollin II and III, but glyceollin could not be detected in response to the pathogenic strain, *D. rostrata*. Therefore, glyceollin meets the usual criteria needed to define phytoalexins (12,15,16,19), and this is the first report of the same phytoalexins from taxonomically unrelated plants. It has been suggested by Ingham and Harborne (6) that phytoalexins can be used to demonstrate taxonomic relationships. Therefore, it is surprising to find that *Costus*, in a different plant family, produces glyceollin isomers found previously only in *Glycine* spp. of the Leguminosae (N. T. Keen, *personal communication*).

The conspicuous chlorotic spots due to leaf tissue damage induced by culture filtrates of the pathogenic strain, show its toxic

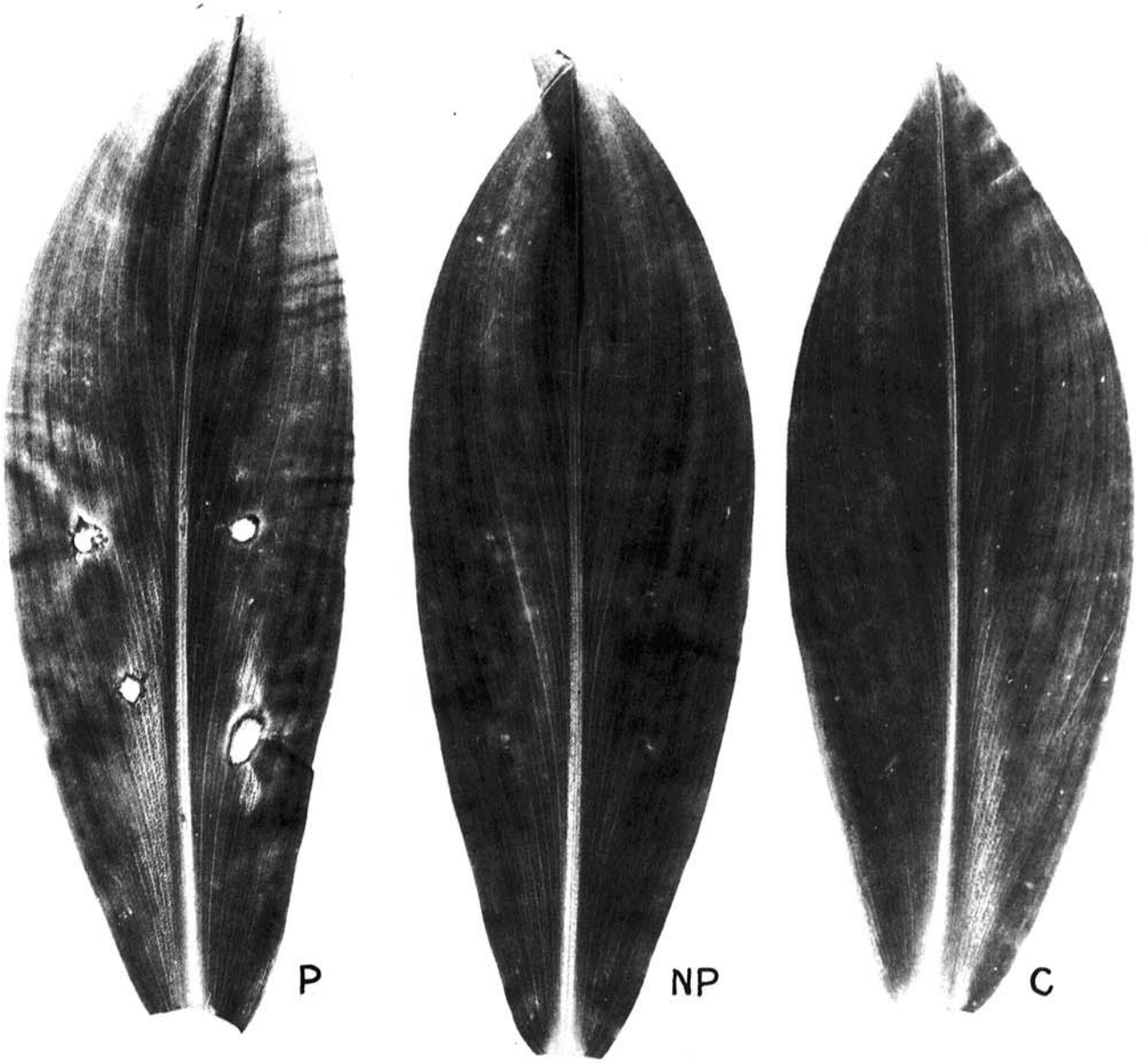

Fig. 2. Effect of toxic metabolite(s) produced by *Drechslera longirostrata* and *D. rostrata* on pierced leaves of *Costus speciosus*. Control medium (C) and culture filtrates from pathogenic (P) and nonpathogenic (NP) isolates applied to leaves.

activity. The nonpathogenic *D. longirostrata*, which does not colonize the plant, cannot block the defense mechanism. And elicited accumulation of glyceollin may have prevented its successful entrance into plant tissues. We feel that the phytoalexin accumulation provoked by the nonpathogen has no relation to the production of toxic metabolite(s). These separate phenomena operate at different levels of pathogenesis.

LITERATURE CITED

1. American Phytopathological Society, Committee on Standardization of Fungicidal Tests. 1947. Test tube dilution technique for use with the slide germination method of evaluating protectant fungicides. Phytopathology 37:354-356.
2. Cruickshank, I. A. M., Biggs, D. R., and Perrin, D. R. 1971. Phytoalexins as determinants of disease reaction in plants. J. Indian Bot. Soc. 50A:1-11.
3. Dasgupta, B., and Pandey, W. B. 1970. A new Indian source of diosgenin (*Costus speciosus*). Experientia 26:475-476.
4. Frank, J. A., and Paxton, J. D. 1970. Time sequence of phytoalexin production in Harosoy and Harosoy 63 soybeans. Phytopathology 60:315-318.
5. Heuvel, J. van den, and Grootveld, D. 1978. Phytoalexin production in French bean leaves infected by *Botrytis cinerea*. Neth. J. Plant Pathol. 84:37-46.
6. Ingham, J. L., and Harborne, J. B. 1976. Phytoalexin induction as a new dynamic approach to the study of systematic relationships among higher plants. Nature 260:241-243.
7. Ingham, J. L., Keen, N. T., Mulheirn, L. J., and Lyne, R. L. 1981. Inducibly formed isoflavonoids from leaves of soybeans. Phytochemistry 20:795-798.
8. Keen, N. T. 1971. Hydrophaseollin production by soybean resistant and susceptible to *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 1:265-275.
9. Keen, N. T. 1978. Phytoalexins: Efficient extraction from leaves by facilitated diffusion technique. Phytopathology 68:1237-1239.
10. Keen, N. T., and Kennedy, B. W. 1974. Hydroxyphaseollin and related isoflavonoids in the hypersensitive resistant response of soybeans against *Pseudomonas glycinea*. Physiol. Plant Pathol. 4:173-185.
11. Keen, N. T., and Littlefield, L. J. 1978. Association of phytoalexins with resistance in flax to *Melampsora lini*. Proc. Am. Phytopathol. Soc. 4:101-102.
12. Keen, N. T., Sims, J. J., Erwin, D. C., Rice, E., and Partridge, J. E. 1971. 6-a hydroxyphaseollin: An antifungal chemical induced in soybean hypocotyls by *Phytophthora megasperma* var. *sojae*. Phytopathology 61:1084-1089.
13. Klarman, W. L. 1968. The importance of phytoalexin in determining resistance of soybeans to three isolates of *Phytophthora*. Neth. J. Plant Pathol. 74:171-175.
14. Klarman, W. L., and Sanford, J. B. 1968. Isolation and purification of an antifungal principle from infected soybeans. Life Sci. 7:1095-1103.
15. Lyne, R. L., and Mulheirn, L. J. 1978. Minor pterocarpinoids of soybean. Tetrahedron Lett. 34:3127-3128.
16. Lyne, R. L., Mulheirn, L. J., and Leworthy, D. P. 1976. New pterocarpinoid phytoalexins of soybeans. J. Chem. Soc., Chem. Commun. 497-498.
17. Mansfield, J. W., Hargreeves, J. A., and Boyle, F. C. 1974. Phytoalexin production by live cells in broad bean leaves infected with *Botrytis cinerea*. Nature (Lond.) 252:316-317.
18. Obi, I. U., Hooker, A. L., and Lim, S. M. 1980. Phytoalexin production in corn resistant to three species of *Helminthosporium* fungi. Phytopathol. Z. 9:228-236.
19. Oku, M., Shiraishi, T., and Ouchi, S. 1975. The role of phytoalexin as the inhibitor of infection establishment in plant disease. Naturwissenschaften 10:486.
20. Pringle, R. B., and Scheffer, R. P. 1963. Purification of the selective toxin of *Periconia circinata*. Phytopathology 53:785-787.
21. Shukla, R. S., Kumar, S., Singh, K. P., and Husain, A. 1983. A new leaf spot disease of *Costus speciosus* caused by *Drechslera rostrata* in India. Indian Phytopathol. 36:571-573.