Phytoalexins: Efficient Extraction from Leaves by a Facilitated Diffusion Technique

N. T. Keen

Department of Plant Pathology, University of California, Riverside, CA 92521.

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ABSTRACT

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A method has been devised for the extraction of phytoalexins from leaves challenged with pathogens. The leaves are harvested from intact plants and phytoalexins are extracted by vacuum infiltration with aqueous 40% ethanol and shaking them in the ethanol solution for several hours. Phytoalexins were readily isolated from the filtered solution. The method gave relatively efficient recovery of phytoalexins from soybean plants inoculated with *Pseudomonas glycinea* and flax plants inoculated with *Melampsora lini* with minimal quantities of interfering compounds. By using the new technique putative phytoalexins also were recovered from the leaves of pathogen-inoculated celery, sunflower, and wheat plants.

Generally, extraction of phytoalexins from leaves challenged with incompatible pathogens is complicated by the presence of foliar pigments, waxes, sterols, and other interfering compounds. Previously, to avoid this problem, drop-diffusion techniques been employed with excised leaves and water droplets (1, 7). This method is tedious, however, and is not easily applied to large amounts of tissue; the utility of the technique is diminished further by the relatively low solubility of many phytoalexins in pure water. In research with putative phytoalexins in the flax/flax-rust system (4), appraisal of these considerations led to the development of what I propose to call the "facilitated diffusion technique" for the recovery of phytoalexins from relatively large samples of freshly harvested inoculated leaves, with minimal extraction of interfering compounds. To assess further the utility of the technique, it was tested with the soybean-*Pseudomonas glycinea* host-parasite system, one in which phytoalexins are known to be associated with incompatible reactions (3).

MATERIALS AND METHODS

Soybean [*Glycine max* (L.) Merr.] plants were grown as described by Keen and Kennedy (3) except that they were maintained at a constant temperature of 21-22 C. Primary leaves of 11- to 14-day-old plants were inoculated with suspensions of various races of *Pseudomonas glycinea* Coerper or with suspensions of *P. pisi* (generally 5×10^7 cells/ml tap water) by infiltrating them into the leaves with a hand sprayer (3). Stock cultures of the *P. glycinea* races were supplied by B. W. Kennedy and single-colony isolates were stored by lyophilization in skim milk or at 4 C in saline. Flax *(Linam usitatissimum* L.) plants were grown according to Littlefield (6) and inoculated with race 1 of *Melampsora lini* (Ehrnb.) Lev. when 3 wk old.

Entire flax seedlings cut above the cotyledonary leaves or soybean leaves were harvested at intervals after inoculation and quickly weighed and placed in 250-ml Erlenmeyer flasks with approximately 15 ml/g (fresh tissue weight) of 40% aqueous ethanol and then vacuum infiltrated. The flasks containing the plant tissue immersed in the ethanol solution were stoppered and placed on a rotary shaker operating at approximately 110 cycles per min at 25 C. Shaking for 2 hr removed a majority of the extractible phytoalexins from flax leaves, but the thicker soybean leaves required 5 hr to extract near-maximal amounts of glyceollin. After the agitation by shaking, the leaves were removed by filtration and the filtrates were vacuum-concentrated to approximately one-half volume at 45 C. The concentrated solution then was extracted twice with ethyl acetate and the organic layers were pooled, dehydrated with $MgSO_4$, and taken to dryness. Following transfer of the residue to vials with peroxide-free diethyl ether and evaporation of the ether, volumes of ethyl acetate .as determined by the weight of tissue extracted (generally 0.05 ml/g fresh tissue weight) were added to the vials. Then the crude extracts were analyzed by TLC, generally on silica gel GF_{254} plates (Merck, 0.375-mm) developed with hexanes/ethyl acetate/methanol (60/40/1, v/v) for extracts from flax leaves and with chloroform/acetone/concentrated NH_4OH (50:50:1, v/v) with extracts from soybean leaves. The phytoalexins were eluted with ethanol and quantitated by UV spectrometry (3). The TLC plates also could be bioassayed directly for antifungal compounds by the TLC bioassay using *Cladosporium cucumerinum* (5).

RESULTS

As described in (4), the facilitated diffusion technique permitted the detection, isolation, and identification of

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two putative phytoalexins, coniferyl aldehyde and coniferyl alcohol, from flax leaves inoculated with incompatible races of *Melampsora lini*. Detection of the same compounds was very difficult in extracts from fresh or lyophilized inoculated leaves when they were homogenized in organic solvents. This was due not only to interference with the TLC, but also appeared to result from complexing or degradation of the two phenylpropanoid compounds during extraction.

In order to more fully test the potential of the facilitated diffusion technique for extraction of phytoalexins from other plants, it was tested with soybean leaves that had been inoculated with compatible or incompatible *P. glycinea* races or with *P. pisi*. Facilitated diffusates from soybean leaves contained negligible quantities of photosynthetic pigments when examined by TLC as compared to large quantities when leaf homogenization was used for extraction of isoflavanoids (3). Diffusates from leaves inoculated with compatible *P. glycinea* races consistently contained lower concentrations of glyceollin than did diffusates from leaves inoculated with incompatible races (Table 1). This confirms previous work using extraction by homogenizing leaves (3), extends those observations to reactions of the cultivar Flambeau, and also includes reactions of soybean leaves to races 4 and 6, not employed in the previous work.

During the course of the investigation with soybean leaves, two previously undescribed chemicals were discovered to be produced coordinately with glyceollin, coumestrol, daidzein, and sojagol observed in previous work (3). The two new compounds chromatographed at R_f 0.25 and 0.56 as 254 nm-absorbing spots on silica gel TLC plates developed with chloroform/acetone/concentrated NH_4OH (50/50/1, v/v) as compared to glyceollin (R_f 0.46). Significant quantities of the new compounds were not detected in extracts from soybean hypocotyls inoculated with incompatible races of *Phytophthora megasperma* var. *sojae*. The new chemicals were not detected in facilitated diffusates from noninoculated soybean leaves and were present in low amounts from leaves inoculated with compatible *P. glycinea* races, but higher amounts were present in diffusates from leaves inoculated with incompatible races. The two new compounds have been isolated and appear to be isoflavanoids related to the glyceollins, but do not possess detectable antifungal activity (N. T. Keen, *unpublished*).

The facilitated diffusion technique has been successfully employed to isolate phytoalexins from leaves of several wild *Glycine* spp. inoculated with *Pseudomonas pisi* (Keen et al., *unpublished*). Using the technique, I also recovered possible phytoalexins from leaves of celery and sunflower inoculated with *P. pisi* or *P. glycinea* and from wheat leaves inoculated with an incompatible race of *Puccinia graminis* f. sp. *tritici*. Although these latter chemicals have not been identified or critically tested for association with disease resistance, they encourage the thought that the facilitated diffusion technique may have general applicability to the recovery of phytoalexins from leaves.

TABLE 1. Glyceollin concentrations determined using the facilitated diffusion technique on primary soybean leaves inoculated with various races of *Pseudomonas glycinea* or *P. pisi*[a]

Leaves infiltrated with	Glyceollin (μg/g fr wt)			
	Harosoy 63	Chippewa	Acme	Flambeau
H_2O Control	31	42	30	39
P. glycinea race 1	384[b]	240[b]	37	59
P. glycinea race 2	45	...	51	83
P. glycinea race 4	65	44	165	117
P. glycinea race 5	51	55	273[b]	321[b]
P. glycinea race 6	298[b]	158[b]	150[b]	56
P. pisi	413[b]	...	...	350[b]

[a]Twelve-day-old plants were infiltrated with water or bacteria at 8×10^7 cells/ml and harvested after 52 hr.

[b]Denotes hypersensitive resistant reactions; all other reactions were compatible, involving water-soaking, necrosis, and chlorosis.

DISCUSSION

Phytoalexin research has been oriented predominantly toward plants with relatively fleshy and nonpigmented tissues such as hypocotyls, tubers, and fruit pods because of the technical difficulty of working with leaves. The facilitated diffusion technique, however, would appear to be of great utility for the isolation and quantitation of phytoalexins formed in foliar tissue in response to pathogens. Its major advantage is that it allows relatively efficient extraction of phytoalexins with little removal of pigments and other interfering leaf compounds. It would appear superior to drop diffusion methods that use excised leaves since (i) leaves may be inoculated on the plant and then harvested fresh at desired intervals for extraction; (ii) the technique may be used with relatively large amounts of leaf tissue (*viz.*, 100 g fr wt or more) for the extraction of quantities of phytoalexins suitable for chemical characterization with minimal labor; (iii) it yields photoalexins relatively free of interfering compounds but offers good recovery as compared to drop diffusion techniques or methods using homogenization–for instance, in our previous work with the soybean-*P. glycinea* system (3), maximum levels of glyceollin of about 1,000 μg/g fr wt leaves were detected by homogenization, as compared to maximum levels of about 500 μg/g using facilitated diffusion in the present work; (iv) the data in Table 1 suggest that the technique may be useful in quantitative studies of phytoalexin production in compatible and incompatible inoculated plants. However, in common with the drop-diffusion technique, errors would be expected to arise if plant varieties with differing cuticular properties or leaf thickness were compared. It is possible that factors similar to these contributed to the consistently lower recovery of glyceollins from Chippewa and Acme leaves inoculated with incompatible bacteria as compared to Harosoy (Table 1).

The facilitated diffusion technique should be of considerable utility in the search for phytoalexins from additional plant species, especially those plants that do not form large fleshy vegetative tissues or fruit pods; indeed, my preliminary research has indicated that production of phytoalexins occurs in leaves of sunflower,

celery, and wheat, all plants for which phytoalexins are not presently known.

The diffusion technique was of great use in the flax/flax-rust system (4), since even with heavy uredospore inoculation, only a relatively few host cells in leaves of incompatible genotypes come into contact with the fungus (6). The resulting dilution effect by interfering chemicals from noninfected cells makes the detection and quantitation of phytoalexins difficult if homogenization of whole leaves is utilized. In addition, there are indications that the putative flax phytoalexins coniferyl aldehyde and coniferyl alcohol (4) complex to other leaf components when whole leaf homogenates are prepared, thus recovery was low.

Data obtained using the facilitated diffusion technique confirm and extend those previously published (3) and continue to support the hypothesis that glyceollin represents a phytoalexin involved in the hypersensitive reaction of soybean leaves against incompatible races of *P. glycinea* and nonpathogenic *Pseudomonas* spp. With the exception of an unusually high value for glyceollin in Acme leaves inoculated with race 4 in the experiment shown in Table 1, all incompatible reactions consistently contained glyceollin levels about ×5 to ×10 those in compatible inoculated leaves. It was of considerable interest that race 6 resulted in less accumulation of glyceollin in the incompatible cultivars Harosoy 63 and Chippewa than did race 1. This held true in seven successive experiments and through time-course experiments from 18-116 hr after inoculation. The hypersensitive reaction of leaves inoculated with race 6 also was visibly distinct, involving a slower-developing mesophyll necrosis but with more eventual darkening and necrosis of the leaf veins occurring. The significance of this difference in plant reaction to the two incompatible races is unknown, but likely involves chemical differences in the recognition system for the two races in the plant (2).

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