Relationship of Safynol and Dehydrosafynol Accumulation to Phytophthora Resistance in Safflower

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ABSTRACT

Six-week-old safflower plants of the breeding line Biggs and the variety Nebraska-10 were wound-inoculated in the first internode with *Phytophthora drechsleri* (virulent to Nebraska-10, avirulent to Biggs) and *P. megasperma* var. *sojae* (avirulent to both). Plants were held at 30 C with 2,200 ft-c of continuous light. Two antifungal polyacetylenes, safynol (*trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol), and dehydrosafynol (*trans*-11-tridecene-3,5,7,9-tetrayne-1,2-diol), were extracted from infected stems and quantitated.

When inoculated with *P. drechsleri*, Biggs stems (resistant) contained 956 μg (12 hr) and 1,472 μg (24 hr) safynol, and 47 μg (12 hr) and 297 μg (24 hr) dehydrosafynol/100 g fresh wt. Nebraska-10 (susceptible), inoculated with *P. drechsleri*, contained 943 μg (12 hr) and 1,590 μg (24 hr) safynol, and 27 μg (12 hr) and 200 μg (24 hr) dehydrosafynol/100 g fresh wt.

When inoculated with *P. megasperma* var. *sojae*, the cultivars had similar safynol contents. Biggs and Nebraska-10 stems, inoculated with *P. megasperma* var. *sojae*, contained, respectively, 128 μg (12 hr) and 382 μg (24 hr), and 57 μg (12 hr) and 228 μg (24 hr) dehydrosafynol/100 g fresh wt. The rate of accumulation of dehydrosafynol was statistically correlated with the high disease resistance of Biggs.

Nebraska-10 plants, 4 days after inoculation with *P. megasperma* var. *sojae*, contained 2,100 μg safynol and 876 μg dehydrosafynol/100 g fresh wt, and were resistant to *P. drechsleri* for 2 to 4 days. Safynol and dehydrosafynol were, respectively, 1.5 and 13.5 times more toxic to *P. megasperma* var. *sojae* than to *P. drechsleri*.

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Safynol (*trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol) and dehydrosafynol (*trans*-11-tridecene-3,5,7,9-tetrayne-1, 2-diol), antifungal polyacetylene compounds, have been implicated as disease resistance factors in stem rot of safflower (*Carthamus tinctorius* L.) incited by *Phytophthora drechsleri* Tucker (1, 2, 3, 12, 13, 14). These polyacetylenes accumulate in infected, resistant safflower tissues to levels sufficient to account for cessation of lesion development 48 hr after inoculation with *P. drechsleri* (1, 2). Under greenhouse conditions, there is no significant difference between the levels of safynol in first internodes of Biggs (resistant) and Nebraska-10 (susceptible) safflowers during the first 24 hr after inoculation with *P. drechsleri* (2). However, during the next 72-hr period, the safynol concentration increases in Biggs and decreases in Nebraska-10 (N-10).

Klisiewicz & Johnson (10) observed lesion restriction 16-24 hr after hypocotyls of resistant Biggs safflower were inoculated with zoospores of *P. drechsleri*. However, in susceptible N-10, the fungus invaded the hypocotyl tissues without signs of the resistant reaction observed for Biggs. We observed similar disease reactions for these cultivars when they were inoculated with mycelium of *P. drechsleri*. In preliminary tests, the total diethyl ether-soluble compounds, which include safynol and dehydrosafynol, were extracted from Biggs and N-10 18 hr after wound-inoculation with *P. drechsleri* (2,200 ft-c, 30 C). The extract from Biggs was 4 times more toxic to the linear growth of *P. drechsleri* in lima bean broth than the extract from N-10. Extracts from healthy stems were not fungitoxic (12). These observations indicated that the resistance mechanism in Biggs is activated very early after infection.

We report here the levels of both safynol and dehydrosafynol in Biggs and N-10 held under controlled conditions during the first 24 hr after inoculation with both a virulent and an avirulent fungus.

MATERIALS AND METHODS.—Safflower plants of the cultivars Biggs (17) and Nebraska-10 (N-10) were grown in steamed soil held in porous 20-cm clay pots in the greenhouse (16). *Phytophthora drechsleri*, isolate 201, and *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildeb., a New Jersey isolate, were grown on lima bean agar supplemented with 0.5% glucose in petri dishes held at 27 C and 24 C, respectively.

For the polyacetylene determinations, 6-week-old plants were transferred from the greenhouse to a controlled environment room at 4:00 PM and held at 19 C in the dark until 8:00 AM the next day. Between 8:00 AM and 10:00 AM, the plants were wounded in the first internode with pin pricks in four vertical rows, four/row and 3 mm apart, spaced an equal distance around the stem. The wounded areas were covered with strips of inoculum (lima bean agar plate cultures) held in place with a strip of aluminum foil lined with plastic film. Wounded and inoculated, and unwounded, control plants were held at 30 C with continuous 2,200 ft-c (ca. 23700 lux) of fluorescent and incandescent light. Twelve and 24 hr after inoculation, whole cross sections (ca. 2 cm

long), cut ca. 5 mm from the upper and lower pin pricks, and similar stem sections from uninoculated, unwounded plants were immediately frozen with liquid N_2, lyophilized, and extracted with methanol. Safynol and dehydrosafynol were isolated by thin-layer chromatography and quantitated as described previously (1, 12).

The effect of inoculation of N-10 plants with *P. megasperma* var. *sojae* (avirulent to safflower) on subsequent infection by *P. drechsleri* was determined. The epidermis on one side of the first internode was scraped lightly with a knife blade. The wounded area was covered with a strip of inoculum (lima bean agar plate cultures) which was held in place with plastic film and aluminum foil. Controls were wounded, but not inoculated. After holding the plants in the greenhouse for 4 days after inoculation, the original inoculum was removed and replaced with an 8-mm disc of inoculum of the virulent fungus, *P. drechsleri*. The plants were then held in the greenhouse for 7 days. Safynol and dehydrosafynol concentrations in wound-inoculated tissue from a representative group of plants were determined at the time of inoculation with *P. drechsleri*.

The toxicities of safynol and dehydrosafynol to the linear mycelial growth of *P. megasperma* var. *sojae* were determined by a previously reported method (1, 12). Toxicity tests with *P. drechsleri* were conducted simultaneously as a check.

RESULTS.—*Safynol and dehydrosafynol content of stems inoculated with P. drechsleri*.—The content of both safynol and dehydrosafynol increased in both cultivars in the first 12 hr after inoculation. During the second 12 hr after inoculation, dehydrosafynol continued to accumulate from averages of 47 μg to 297 μg/100 g fresh wt for Biggs and from 27μg to 200 μg/100 g fresh wt for N-10. An analysis of variance of this data showed that the dehydrosafynol contents for 12 and 24 hr after inoculation were significantly different (.05 level) for each cultivar (Table 1). Although the safynol contents for the second 12 hr after inoculation appeared to increase ca. 50% for each cultivar, these differences were not significant. The average dehydrosafynol content 24 hr after inoculation in Biggs (297 μg/100 g fresh wt) was significantly greater than the corresponding dehydrosafynol content for N-10 (200 μg/100 g fresh wt). The highest contents of Biggs and N-10 in any experiment 24 hr after inoculation were 388 μg and 207 μg dehydrosafynol/100 g fresh stems, respectively. Comparisons of the weight of healthy and infected stems indicated that there was no apparent loss of fresh or dry weight due to infection for the 12- or 24-hr light periods.

Safynol and dehydrosafynol content of stems inoculated with P. megasperma var. sojae.—The content of safynol increased rapidly during the first 12 hr after inoculation, but there was no striking increase during the second 12 hr after inoculation for the two cultivars (Table 1). An analysis of variance of the data showed that there was no significant difference between the contents of safynol for the two periods, or between cultivars.

The dehydrosafynol contents increased strikingly and reached average levels of 382 μg and 228 μg/100 g fresh stems for Biggs and N-10, respectively, 24 hr after inoculation (Table 1). An analysis of variance of these levels of dehydrosafynol at 24 hr as well as

TABLE 1. Concentration of safynol[a] and dehydrosafynol[b] in Biggs and Nebraska-10 safflower stems after wound-inoculation with *Phytophthora drechsleri* and *P. megasperma* var. *sojae*

Light duration[c]	Biggs[d]		Nebraska-10[d]	
	Safynol[e]	Dehydrosafynol[e]	Safynol[e]	Dehydrosafynol[e]
hr	*μg/100 g*	*μg/100 g*	*μg/100 g*	*μg/100 g*
Noninoculated				
0	165	<.2	80	<.2
Inoculated with:				
Phytophthora drechsleri				
12	956 a	47 c	943 a	27 c
24	1,472 a	297 a	1,590 a	200 b
Phytophthora megasperma var. *sojae*				
12	1,020 ab	128 c	880 b	57 d
24	1,258 a	382 a	1,052 ab	228 b

[a] *Trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol.

[b] *Trans*-11-tridecene-3,5,7,9-tetrayne-1,2-diol.

[c] Stems were wounded with 16 pin pricks, inoculated, and held at 30 C in continuous light (2,200 ft-c). Stem sections were harvested at the end of the light period.

[d] Biggs is resistant to *P. drechsleri*; Nebraska-10 (N-10) is susceptible. Both cultivars are resistant to *P. megasperma* var. *sojae*.

[e] Average of three replications of 18 plants each. Means with the same letter are not different at the .05 level of significance. The data for the two fungal species, based on infected stems (fresh weight), were statistically analyzed separately, and the letters should not be compared for species differences.

those for 12 hr after inoculation (128 μg and 57 μg/100 g fresh stems from Biggs and N-10, respectively) showed significant differences between the cultivars for each time period. For a single experiment, the highest levels of dehydrosafynol obtained from inoculation with *P. megasperma* var. *sojae* for 24 hr were 414 μg and 243 μg/100 g fresh stems from Biggs and N-10, respectively. There was no apparent loss of weight of inoculated stem sections when compared to controls.

Cross-protection of susceptible safflower.—Both safynol and dehydrosafynol accumulated in N-10 stems which were wound-inoculated with *P. megasperma* var. *sojae*. The infected stems of plants held in the greenhouse 4 days after inoculation with this fungus contained 2,100 μg safynol and 876 μg dehydrosafynol/100 g fresh wt. Similar stem sections from healthy controls contained 80 μg safynol and less than 0.2 μg dehydrosafynol. Controls, which were wounded but not inoculated with *P. megasperma* var. *sojae*, were susceptible to *P. drechsleri* when held in the greenhouse. Lesions developed within 24 hr and expanded rapidly thereafter. Production of lesions by *P. drechsleri* in stems previously inoculated with *P. megasperma* var. *sojae* was delayed for 2 to 4 days in ca. 90% of the plants. Lesions did not develop in ca. 10% of the plants 7 days after inoculation.

Inhibition of mycelial growth of P. megasperma var. sojae with dehydrosafynol.—In three tests, the median effective doses (ED_{50}) of safynol and dehydrosafynol required to inhibit the mycelial growth of *P. megasperma* var. *sojae* were 7 ± 0.5 μg/ml and 0.15 ± 0.05 μg/ml, respectively, for measurements made 24 hr after the start of the tests. The ED_{50} for *P. drechsleri* was found to be 11.5 ± 0.5 μg safynol/ml and 2 ± 0.5 μg dehydrosafynol/ml.

DISCUSSION.—Preliminary tests for this study showed that antifungal substances in infected tissue accumulate faster in Biggs than in N-10 after inoculation with *P. drechsleri*. However, it is experimentally difficult to determine the capacity of Biggs and N-10 to accumulate antifungal substances in response to a highly virulent pathogen such as *P. drechsleri*, because host colonization is quite different for the two safflowers as early as 12-24 hr after inoculation (10). The problem of host colonization differences as related to disease resistance studies has been experimentally and theoretically defined by Bell (4). The differences in accumulation of antifungal compounds between resistant and susceptible varieties of cotton inoculated with live conidia of *Verticillium albo-atrum* (virulent) were even greater when the cultivars were treated with 10^{-3} M $CuCl_2$ or dead conidia. Apparently, these greater differences obtained with $CuCl_2$ or dead conidia were due to the deletion of differing rates of host colonization caused by inoculation with live conidia (4, 5). Biggs and N-10 safflowers give similar hypersensitive reactions to wound or nonwound inoculations with *P. megasperma* var. *sojae*, and this suggests that the number of cells involved is nearly the same for the two cultivars. In this study, the use of the avirulent *P. megasperma* var. *sojae* minimized the differences in host colonization, and allowed us to measure more accurately the different rates of accumulation of safynol and dehydrosafynol in Biggs and N-10.

Reactions of safflower cultivars resistant and susceptible to *P. drechsleri* are similar to the reactions of soybean cultivars resistant and susceptible to *P. megasperma* var. *sojae* in that the reaction type is related to rate of accumulation of an antifungal compound. Keen (9) reported that the soybean cultivars Harosoy 63 and D60-9647 differed in their rates of accumulation of the antifungal hydroxyphaseollin when they were inoculated with race 2 of *P. megasperma* var. *sojae* (virulent to D60-9647, but avirulent to Harosoy 63). With race 1, avirulent to both of these cultivars, there was no significant difference in accumulation. Biggs safflower, however, accumulates about 2 times as much dehydrosafynol on a whole stem basis as N-10 safflower when both cultivars are inoculated with *P. megasperma* var. *sojae*, avirulent to both. The resistance of Biggs to all known races of *P. drechsleri* (15) is conditioned by a single recessive factor pair (16), and differs genetically from the dominate soybean resistance.

Although dehydrosafynol, a highly fungitoxic compound, is a major antifungal factor in safflower extracts (1), it does not account for all of the antifungal activity. Bohlmann et al. (6) and Bohlmann & Zdero (7) have identified 13 acetylenic compounds, including safynol from safflower. Some of these compounds, and others not identified (8), may also accumulate in response to infection and contribute to the total fungitoxicity of ether extracts from infected safflower stems.

The significance of the relationship of safynol and dehydrosafynol levels to the disease reaction of Biggs and N-10 in continuous light is further verified by analogy to the levels of these compounds as related to the disease reaction of Biggs under intermittent high and low light (14). The relative concentrations of safynol and dehydrosafynol in infected Biggs and N-10 with continuous 2,200 ft-c (Table 1) are similar to those for moderately resistant Biggs (16 hr of 1,300 ft-c/24 hr) and moderately susceptible Biggs (8 hr of 1,300 ft-c/24 hr). These results suggest that there is a threshold of fungitoxicity (ED_{100}) which must be reached before the resistant reaction type is expressed (4). Biggs is genetically capable of reaching this threshold for *P. drechsleri*, providing that sufficient light is available. Nebraska-10 is not genetically capable of reaching this threshold even under high light, but can acquire a state of permunity to *P. drechsleri* if preinoculated with *P. megasperma* var. *sojae*.

The sensitivities of pathogens to antifungal compounds produced by plants have been correlated with virulence (11). The sensitivity of *P. drechsleri* may be close to the acquired threshold of fungitoxicity in N-10, and this may play a role in the susceptible disease reaction of this variety. *Phytophthora megasperma* var. *sojae* is about 13.5 times and 1.5 times more sensitive than *P. drechsleri*

to dehydrosafynol and safynol, respectively. The extreme sensitivity of *P. megasperma* var. *sojae* to these compounds could account for the resistant reaction to this fungus of both safflower cultivars.

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