Further Studies on the Relationship between Glyceollin Accumulation and the Resistance of Soybean Leaves to *Pseudomonas syringae* pv. *glycinea*

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ABSTRACT

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Several isolates of *Pseudomonas syringae* pv. *glycinea* (Psg) previously classified as new races or not race typed were inoculated into the standard differential soybean cultivars. Based on the observed visible reaction types, the only clearly defined races in the collection were the previously described races 1, 4, 5, and 6. Certain of the other isolates fell into these existing races, but some gave ambiguous intermediate reactions or otherwise failed to justify the status of unique races. Plants of soybean cultivars Hardee and Peking gave unique reactions with Psg races 1, 4, 5, and 6 and accordingly are useful supplements to the standard differentials. The production of glyceollin in several newly tested soybean-Psg race combinations was consistently associated only with reactions visually classified as hypersensitive resistant (HR). Bacterial multiplication in inoculated leaves of several cultivars was negatively correlated with glyceollin levels formed in the leaves ($r = -0.94$). Significantly, some cultivar-race reactions typified by a visible HR resulted in considerably lower levels of glyceollin accumulation and higher bacterial multiplication than other hypersensitive interactions. Unlike certain other plants, inoculated soybean leaves maintained under high humidity exhibited visible HR, glyceollin production, and inhibition of bacterial populations similar to the same leaves incubated at ambient humidity. Mixed inocula of incompatible and compatible races of Psg gave a visible HR and glyceollin accumulation similar to the incompatible race alone and partial, but not complete, restriction of bacterial populations to the level of the incompatible race inoculated alone. The results, therefore, indicate that the incompatible race is physiologically dominant and that the soybean HR and glyceollin accumulation are associated with restricted bacterial multiplication.

Additional key words: *Glycine max*, hypersensitive reaction, pathogen races, race-specific resistance.

Several races of *Pseudomonas syringae* pv. *glycinea* (Psg) have been defined on the basis of the reactions of seven differential soybean cultivars (2,9,23). A single dominant resistance gene has been demonstrated in the soybean cultivar Harosoy by crossing (14), but the inheritance of resistance to other races has not been determined. In conjunction with the recent demonstration of single avirulence genes in two races of Psg by the molecular cloning experiments of Staskawicz et al (21, and *unpublished*), however, the Psg-soybean interaction appears to behave as a typical gene-for-gene system. Previous research suggested but did not prove that the restriction of bacterial populations in soybean leaves undergoing a hypersensitive resistant reaction (HR) is due at least in part to accumulation of the phytoalexin, glyceollin (14). Since bacterial populations had not been monitored in several cultivar-race combinations, we studied phytoalexin production and bacterial multiplication when different bacterial races or mixtures were inoculated into the leaves of various differential soybean cultivars.

MATERIALS AND METHODS

Seed of the seven standard differential soybean cultivars (2) was increased by M. Holliday, E. I. DuPont deNemours, Experiment Station, Wilmington, DE. Seeds of cultivars Hardee and Peking were supplied by C. Napoli, International Plant Research Institute, San Carlos, CA. Plants were grown from seed in 10-cm-diameter pots containing UC mix (50% fine sandy loam and 50% peat moss) overlaid with vermiculite, in growth chambers at 21–22 C as previously described (1,13) or on the greenhouse bench.

Sources of the isolates of Psg are listed in Table 1. During this investigation we assessed the frozen methylcellulose method of Suslow and Schroth (22) for long-term maintenance of Psg stock cultures. The method appeared to be very satisfactory, since all cultures of Psg that were tested survived for more than 2 yr at −20 C in 1% methylcellulose and gave the expected reactions in soybean leaves after recovery. Cells of Psg for plant inoculation were grown to log phase in the semisynthetic medium previously described (1). Mutants resistant to kasugamycin (*kas*) naladixic acid (*nal*), or rifampicin (*rif*), were obtained previously (13) and gave the same reactions as the wild types when tested in soybean plants. Primary leaves of 11- to 14-day-old plants (primary leaves just fully expanded) were inoculated with water suspensions of bacteria adjusted to 0.1 absorbance unit at 500 nm ($\sim 8 \times 10^7$ cells per milliliter). Suspensions were introduced into the leaves with a hand sprayer or a Hagborg device (13).

Bacterial populations (colony-forming units [cfu]) in soybean leaves were determined as previously described (13). Standard dilution plating was onto agar medium alone or supplemented with either kasugamycin, 70 $\mu g \cdot ml^{-1}$; naladixic acid, 400 $\mu g \cdot ml^{-1}$; or rifampicin, 100 $\mu g \cdot ml^{-1}$. Glyceollin was extracted from soybean leaves and quantitated by the previously described method (12), except that the phytoalexin was extracted from concentrated diffusates with chloroform instead of ethyl acetate. Continuous water-soaking of leaves after inoculation was achieved by placing inoculated plants in a mist chamber.

RESULTS

Plant reactions to isolates of *P. syringae* pv. *glycinea*. Several isolates from various geographical locations were tested for reaction type on the seven standard soybean cultivars. The nonpathogenic control, *P. syringae* pv. *pisi* induced hypersensitive reactions on all the differentials; the saprophytes *P. fluorescens* and *Escherichia coli* gave no reaction, as expected. Isolates of Psg previously described as races 1, 2, 3, 4, 5, 6, and 7 were supplied by B. W. Kennedy (2); races 1, 4, 5, and 6 produced the expected plant reactions shown in Table 1. On the other hand, isolate R7 produced intermediate host reactions and isolates R2 and R3 were indistinguishable from R4; accordingly, we do not consider R2, R3,

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and R7 sufficiently unique to warrant independent race status (Table 1). Isolate PgB3, previously described as incompatible on Lindarin and therefore defined as race 8 (23) also produced compatible reactions on all cultivars including Lindarin (Table 1). To further test this isolate, glyceollin was extracted and bacterial populations were estimated from leaves at 48 hr after inoculation. Glyceollin levels were uniformly low (0–32 $\mu g \cdot g^{-1}$ fr wt of leaves) and bacterial populations high ($1.8–4.0 \times 10^{10}$ $cfu \cdot g^{-1}$ fr wt of leaves) in the seven differential cultivars, thus confirming the compatible nature of the interactions. Therefore, isolate PgB3 is now best classified as race 4.

Two isolates previously classified as race 10 (F118 and F120 [9]) gave reactions which were indistinguishable from race 5, and accordingly we now reclassify these as race 5 (Table 1). Two isolates from Hungary supplied by T. Érsek (1e and 2e [10]) also proved to be race 5, while two New Zealand isolates (4180 and 3482) from Robin Mitchell by way of J. V. Leary gave reactions similar, but not identical, to those of race 4. Because of the apparent intermediate reactions of some cultivars to these isolates, no race designation is made. Ferreira (6) reported that several Brazilian isolates of Psg gave unique reaction types on the standard soybean differentials and he classified them as new races 8–17. In our tests, however, these isolates all behaved as race 4 in repeated tests (Table 1).

During the course of this work, it was observed that the soybean cultivars Hardee and Peking were useful supplemental differentials for distinguishing Psg races 1, 4, 5, and 6 (Table 2). Since the reactions of cultivars Lindarin and Merit are the same as cultivar Harosoy to races 1, 4, 5, and 6 (Table 1), they have been replaced by cultivars Hardee and Peking in studies involving these races (21).

Phytoalexin production in various cultivar-race interactions. Reactions of the standard soybean differential cultivars to the well-defined races 1, 4, 5, and 6 of Psg are given in Table 2 together with their accumulation of glyceollin following inoculation. Many of these combinations had not been previously examined for phytoalexin production, but the absolute correlation of high glyceollin production with incompatible, but not compatible, reactions was observed as in past work. Noteworthy, however, was the observation that certain of the hypersensitive reactions (such as all race 6-incompatible reactions and races 1 and 5 on certain, but not all, differentials) resulted in substantially lower phytoalexin production than others. Since lower phytoalexin production has been associated with substantially greater pathogen development in certain incompatible reactions of two other gene-for-gene plant-parasite interactions (16,18), we further examined glyceollin production and bacterial populations in various interactions of soybean and Psg.

Comparison of the reciprocal combinations of race 1 and race 5 on cultivars Harosoy and Acme showed that glyceollin did not accumulate significantly in either compatible combination (Table 2, Fig. 1) and bacterial multiplication was substantial (Fig. 1). Accumulation of glyceollin was considerable in both incompatible interactions, but was less rapid in the Acme-race 5 interaction; significantly, bacterial populations were also greater in this combination than in the Harosoy-race 1 interaction. Investigation of the incompatible Harosoy-race 6 interaction (Fig. 1) confirmed previous data showing that less glyceollin accumulated than in the Harosoy-race 1 combination. The bacterial counts further disclosed that the multiplication of race 6 was approximately 10-fold more rapid than that of race 1 in cultivar Harosoy.

TABLE 1. Reaction of seven soybean cultivars to various isolates of *Pseudomonas syringae* pv. *glycinea*, *Pseudomonas syringae* pv. *pisi*, *Pseudomonas fluorescens*, and *Escherichia coli*[a]

Bacterial isolate[b]	Race designation	Disease reaction[c] of cultivars: Acme	Chippewa	Flambeau	Harosoy	Lindarin	Merit	Norchief
P. syringae pv. *glycinea*								
R1	1	C	I	C	I	I	I	I
2159	1	C	I	C	I	I	I	I
R2	4	C	C	C	C	C	C	C
R3	4	C	C(int)	C	C	C	C	C
R4	4	C	C	C	C	C	C	C
A-29-2	4	C	C	C	C	C	C	C
J3-20-4A	4	C	C	C	C	C	C	C
Brazilian	4	C	C	C	C	C	C	C
PgB3	4	C	C	C	C	C	C	C
R5	5	I	I	I	C	C	C	I
J3-17-2	5	I	I	I	C	C	C	I
F118	5	I	I(int)	I	C	C	C	I
F120	5	I	I	I	C	C	C	I
1e	5	I(int)	I	I	C	C	C	I
2e	5	I	I	I	C	C	C	I(int)
R6	6	I	I	C	I	I	I	C
R7	?	C(int)	I(int)	I(int)	I(int)	I(int)	I(int)	I(int)
4180	?	C	C(int)	C(int)	C(int)	C(int)	C	C
3482	?	C	I(int)	C	C(int)	C	C	C
pv. *pisi*	–	I	I	I	I	I	I	I
P. fluorescens	–	–	–	–	–	–	–	–
E. coli	–	–	–	–	–	–	–	–

[a] Primary soybean leaves that had just fully expanded were infiltrated with about 10^8 bacterial cells per milliliter by using the Hagborg device. Leaves were examined daily for 6 days. Results represent four repeated experiments replicated three times.

[b] Isolates R(race) 1, 2, 3, 4, 5, 6, and 7 were obtained in 1972 from B. W. Kennedy (2); R1, R4, R5, and R6 are considered as the type isolates of the respective races. Isolates 2159, A-29-2, J3-20-4A, and J3-17-2 were obtained from W. F. Fett, USDA, Philadelphia, PA; strains PgB3, 4180, and 3482 were obtained from J. V. Leary, University of California, Riverside; F118 and F120 were from S. Gnanamanickam, Agriculture Canada, London, Ontario; 1e and 2e were obtained from Tibor Érsek, Research Institute for Plant Protection, Budapest, Hungary. Brazilian isolates supplied as races 8, 9, 10, 11, 12, 13, 16 and 17 were obtained from L. P. Ferreira, Universidado de São Paulo, Brazil. Since all of the latter isolates were compatible on all 7 cultivars, they are listed as a single entry under race 4. The *P. syringae* pv. *pisi* and *P. fluorescens* isolates were from previous work (13). *E. coli* strains DH-1, HB101, and RR-1 all gave similar results.

[c] C = compatible combination, typified by water-soaking at 2–4 days followed by necrosis. C(int) = intermediate compatible reaction with water-soaking following necrosis. I = incompatible, hypersensitive resistant reaction; necrosis observed within 24 hr after inoculation with no subsequent water-soaking. I(int) = intermediate incompatible reaction; no necrosis after 24 hr but necrosis within 72 hr; no water-soaking observed. – = no reaction observed with the exception of occasional mild chlorosis at the inoculation site.

When inoculated leaves were maintained in a water-soaked condition following inoculation, the visible hypersensitive symptoms, phytoalexin production, and restricted bacterial populations were similar to those in the same leaves incubated under the usual conditions (Table 2). The occurrence of compatible symptoms on leaves maintained water-soaked was difficult to determine visually, but bacterial populations were similar to those in leaves that were not water-soaked. Accordingly, both compatible and hypersensitive reactions appeared to occur normally when inoculated soybean leaves were maintained water-soaked.

Comparison of three soybean cultivars and three bacterial races resulted in a linear inverse relationship between glyceollin accumulation and bacterial populations (Fig. 2). Regression analysis disclosed a highly significant inverse relationship between glyceollin accumulation and bacterial populations with a correlation coefficient of −0.94. These data also demonstrate that certain cultivar-race interactions considered to be incompatible resulted in substantial bacterial multiplication and less glyceollin accumulation than others. All compatible combinations, however, resulted in glyceollin accumulation of less than 50 $\mu g \cdot g^{-1}$ fresh weight leaf tissue at 48 hr after inoculation, concomitant with high bacterial multiplication.

Inoculation of soybean leaves with mixtures of compatible and incompatible bacterial races. Mixed inoculum of race 1 and race 2 cells gave the same visible hypersensitive reaction and glyceollin accumulation in Harosoy leaves as race 1 cells alone (Table 3), confirming previous work (15). Bacterial populations in the leaves inoculated with mixtures of cells were approximately 10-fold higher than race 1 cells alone, but about 10-fold lower than race 2 cells alone. To investigate further, the experiment was repeated with the antibiotic-resistant strains race 5 *rif* and race 1 *nal*. Again, leaves inoculated with the mixed inoculum gave a visible HR and glyceollin accumulation levels similar to race 1 *nal* only (Table 3),

TABLE 2. Glyceollin accumulation and leaf reactions of different soybean cultivars 48 hr after inoculation with various races of *Pseudomonas syringae* pv. *glycinea* (Psg)

		Ambient humidity		Mist chamber	
Soybean cultivar	Psg race[a]	Glyceollin[b] ($\mu g \cdot g^{-1}$ leaves)	Plant reaction[c]	Glyceollin[b] ($\mu g \cdot g^{-1}$ leaves)[b]	Plant reaction[c]
Acme	1	14	C	35	C
	4	2	C		
	5	395	I	366	I
	6	145	I	179	I
Chippewa	1	270	I		
	4	22	C		
	5	102	I		
	6	41	I		
Flambeau	1	11	C	76	C
	4	29	C		
	5	405	I	394	I
	6	21	C	216	C
Harosoy	1	642	I	527	I
	4	20	C		
	5	14	C	26	C
	6	140	I	200	I
Lindarin	1	177	I		
	4	0	C		
	5	18	C		
	6	50	I		
Merit	1	369	I		
	4	11	C		
	5	9	C		
	6	126	I		
Norchief	1	368	I		
	4	0	C		
	5	64	I		
	6	7	C		
Hardee	1		I		
	4		C		
	5		C		
	6		C		
Peking	1		C		
	4		C		
	5		C		
	6		I		

[a] Plants were inoculated with the type races 1, 4, 5, and 6 at about 8×10^7 cells per milliliter. Results represented means of three repeated experiments.
[b] Glyceollin levels were determined at 48 hr after inoculation; a fresh weight basis was used for calculations.
[c] Plant reaction: C = compatible and I = incompatible.

TABLE 3. Visible reaction, glyceollin accumulation, and bacterial populations in leaves of soybean cultivar Harosoy after infiltration with incompatible or compatible races of *Pseudomonas syringae* pv. *glycinea* or mixed incompatible and compatible races

Inoculum[a]	Visible reaction[b]	Glyceollin[c] ($\mu g \cdot g^{-1}$ leaves)	Bacteria population[c] (CFU·g^{-1} leaves)
Race 2	C	35	$6.5 \pm 1.3 \times 10^{10}$
Race 1	I	425	$7.5 \pm 1.4 \times 10^{8}$
Race 1 + Race 2	I	393	$6.4 \pm 2.5 \times 10^{9}$
Race 5 *rif*	C	9	$2.9 \pm 2.0 \times 10^{10}$
Race 1 *nal*	I	470	$4.2 \pm 2.2 \times 10^{8}$
Race 5 *rif* + Race 1 *nal*	I	451	$3.4 \pm 2.5 \times 10^{9}$
Race 5 *rif*	-	-	$3.1 \pm 2.3 \times 10^{9}$
Race 1 *nal*	-	-	$3.2 \pm 1.8 \times 10^{8}$

[a] Inoculum adjusted to about 8×10^7 cells per milliliter; mixed inoculum about 4×10^7 cells of each race per milliliter. Race 5 *rif* = rifampicin resistant mutant, and Race 1 *nal* = naladixic acid resistant mutant.
[b] Visible hypersensitive reaction (I) or compatible reaction (C). The hypersensitive reaction was observed at 24 hr and the compatible reaction at 72 hr after inoculation.
[c] Glyceollin was extracted and bacteria populations were determined at 48 hr after inoculation. Results represent mean and standard deviation of three different experiments.

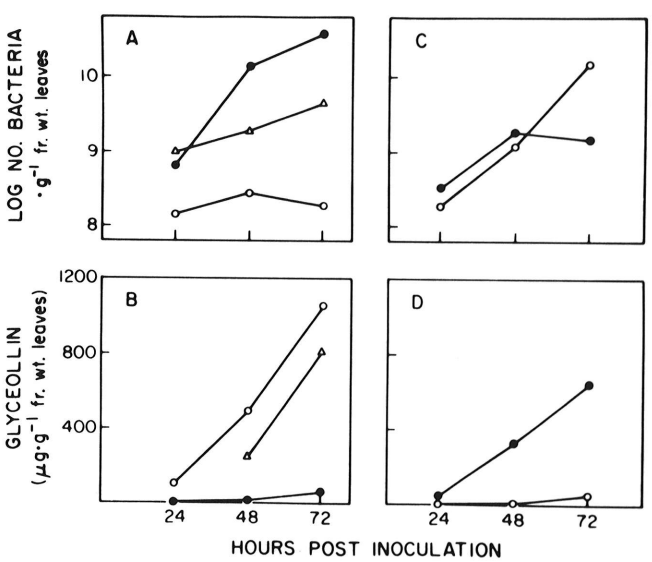

Fig. 1. Populations of *Pseudomonas syringae* pv. *glycinea* and glyceollin accumulation at various times after infiltration of soybean leaves with race 1 (o), race 5 (•), or race 6 (Δ). **A** = populations and **B** = glyceollin accumulation in cultivar Harosoy; **C** = populations and **D** = glyceollin accumulation in cultivar Acme. The data are typical of several experiments that were performed.

and the total bacterial population was again intermediate between leaves inoculated only with compatible race 5 *rif* or incompatible race 1 *nal*. However, plating on the respective antibiotic media demonstrated that race 1 *nal* cells in the mixed inoculum multiplied similarly to the same cells inoculated alone; race 5 *rif* cells in the mixed inoculum multiplied at a higher rate, but attained cell numbers only about 0.1 of that for the same cells inoculated alone (Table 3). This suggests that some but not all of the race 5 *rif* cells were influenced by a defense reaction elicited by the race 1 cells. On the other hand, the absence of stimulation of race 1 *nal* populations in the presence of race 5 *rif* cells indicates that the latter did not "induce" the compatibility or susceptibility of soybean leaves to the normally incompatible race 1 cells.

DISCUSSION

We could not confirm several descriptions of new races of Psg. Only races 1, 4, 5, and 6 appeared to constitute well-defined classifications for the isolates we tested. For instance, original races 2, 3, and 7 (2) no longer reproduced the original host reactions. The same was true for isolate PgB3, originally described as race 8 (23), for isolates F118 and F120 described as race 10 (9) and for all the Brazilian isolates described as new races 8–17 (6). Isolates previously classified as race 9 (8) were subsequently shown to be *P. syringae* pv. *phaseolicola* (19), but the basis for the altered plant reactions of the other isolates is not known. It is possible that factors such as leaf age or growth conditions may have influenced the reactions, but we noted no significant change in reaction type when young primary, older primary, or trifoliate soybean leaves were inoculated. One important factor may be the different environmental conditions in which plants were maintained both before and after inoculation by various investigators. Finally, it is possible that mutation or other variation may have led to changes in bacterial phenotype; however, isolates R1, R4, R5, and R6, which are the type isolates for the respective races, have not produced detectable changes in pathogenicity or race phenotype in over 10 yr of storage in our laboratory.

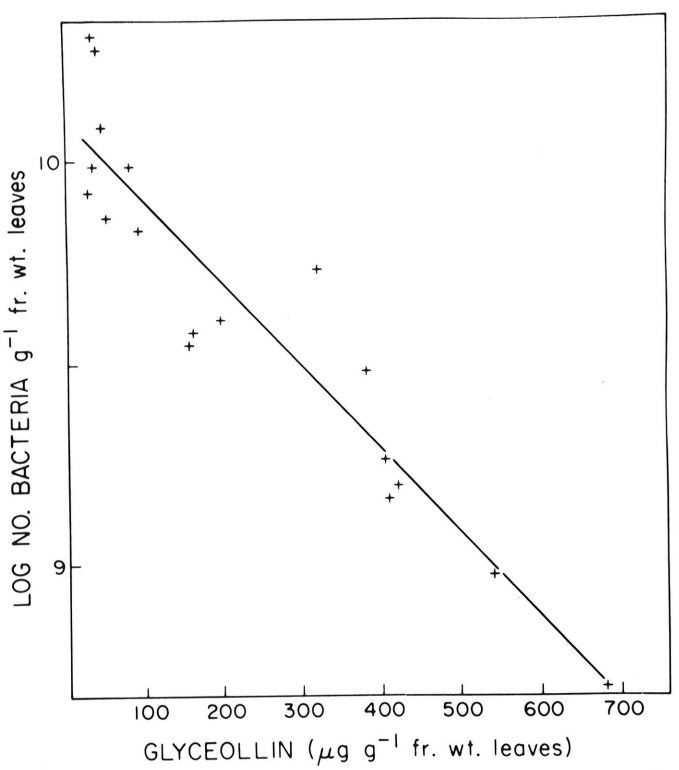

Fig. 2. Bacterial populations and glyceollin accumulation in soybean leaves of cultivars Harosoy, Flambeau, and Acme inoculated with races 1, 5, or 6 and maintained at ambient humidity or in a mist chamber. Phytoalexin data are plotted from those in Table 2. Bacterial populations were determined in the same experiment at 48 hr after inoculation. The line is a best-fit regression line, and the data gave a correlation coefficient of −0.94.

The results do not conclusively prove the involvement of glyceollin with resistance to Psg, but they confirm and extend previous reports (12,15) that restriction of bacterial populations in soybean cultivars is correlated with the rate of phytoalexin accumulation. For example, all cultivar-race combinations in which glyceollin accumulation at 48 hr was $\leqslant 100\ \mu g \cdot g^{-1}$ fr wt of leaves were invariably typified by high bacterial multiplication and the appearance of classic water-soaking symptoms. Significantly, several cultivar-race combinations considered to be incompatible based on visible plant responses in fact supported bacterial populations that were intermediate between a fully compatible reaction and the most incompatible reaction that was studied, that of Harosoy and race 1. The Harosoy-race 6 interaction was previously noted to result in markedly lower glyceollin accumulation than that of Harosoy and race 1 (1,12). We have now observed that the Harosoy-race 6 interaction is also typified by considerably more bacterial multiplication than in Harosoy leaves inoculated with race 1 (Fig. 1). In several interactions, some of which supported intermediate bacterial populations, a close correlation between glyceollin levels and bacterial populations was observed (Fig. 2). This offers additional support for the possibility that the phytoalexin glyceollin is causally involved in restricting bacterial multiplication in incompatible reactions. It is also noteworthy that the soybean-Psg system fits the pattern observed in two other gene-for-gene host-parasite systems (the systems involving flax-*Melampsora lini* and oat-*Puccinia coronata* f. sp. *avenae*) in which intermediate phytoalexin production has been related to intermediately resistant reactions while high phytoalexin production occurs only in highly resistant reactions (16,18).

Érsek and Hevesi (5) reported that race 2 led to an intermediate hypersensitive reaction and moderate glyceollin production in two soybean cultivars, but multiplied as a fully compatible race. These observations were employed to question the possible role of glyceollin accumulation in the expression of resistance to incompatible Psg races. Inspection of Fig. 1b in the paper by Érsek and Hevesi (5), however, shows that glyceollin accumulation did not exceed $100\ \mu g \cdot g^{-1}$ fr wt of tissue until well after 48 hr. As shown in our Fig. 2, all race-cultivar interactions leading to glyceollin accumulation of $<100\ \mu g \cdot g^{-1}$ fr wt of leaves at 48 hr are typified by rapid bacterial multiplication. Thus, we see no incongruity between the data of Érsek and Hevesi (5) and our own.

The incompatible reactions of tobacco (17), bean (11), and pepper (20) leaves to incompatible bacteria are blocked by maintaining the intercellular spaces water-soaked after inoculation. The failure of resistance expression in these cases presumably results from prevention of bacterial attachment to the surfaces of plant cells. In soybean leaves, however, water-soaking had little or no influence on the development of the visible HR, phytoalexin production, or restriction of bacterial populations (Table 2, Fig. 2). The reason for this difference is not clear, but it is noteworthy that ultrastructural studies have failed to disclose the occurrence of bacterial cell attachment specifically associated with incompatible soybean-Psg cell interactions (7). Érsek et al (4) however, reported the selective attachment of incompatible Psg cells to soybean cells shortly after inoculation. Thus, recognitional events occurring between incompatible bacteria and soybean cells may involve transient, but not long-duration, bacterial attachment as in certain other plants (14).

The mixture of incompatible and compatible Psg cells prior to inoculation of soybean leaves invariably resulted in visible HR, glyceollin accumulation, and restriction of bacterial populations similar to that induced in leaves inoculated only with the incompatible race (Table 3). Thus, as observed in most gene-for-gene host-parasite systems (3), the incompatible trait in Psg is physiologically dominant to compatibility. This conclusion has recently been fully confirmed by the molecular cloning of an avirulence gene from race 6 (21) and the demonstration that it conferred all race 6 incompatibilities when transferred to other Psg races. Therefore, the data strongly suggest that incompatibility is the specifically determined trait in the soybean-Psg system and that compatibility results from the passive failure of host cells to detect the bacteria and initiate a hypersensitive defense response (14).

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