Plant Physiol. (1992) 98, 654–659
0032-0889/92/98/0654/06/$01.00/0

Received for publication July 2, 1991
Accepted September 5, 1991

Glyphosate Suppression of an Elicited Defense Response[1]

Increased Susceptibility of *Cassia obtusifolia* to a Mycoherbicide

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ABSTRACT

The major effort in developing pathogenic fungi into potential mycoherbicides is aimed at increasing fungal virulence to weeds without affecting crop selectivity. Specific suppression of biosynthesis of a phytoalexin derived from the shikimate pathway in *Cassia obtusifolia* L. by a sublethal dose (50 micromolar) of glyphosate increased susceptibility to the mycoherbicide *Alternaria cassiae* Jurair & Khan. Glyphosate applied with conidia suppressed phytoalexin synthesis beginning at 12 hours, but not an earlier period 8 to 10 hours after inoculation. The phytoalexin synthesis elicited by fungal inoculation was also suppressed by darkness. The magnitudes of virulence of the mycoherbicide in the dark or with glyphosate in the light were both higher than after inoculation in the light with the same concentration of conidia in the absence of glyphosate. Five times less inoculum was needed to cause disease symptoms when applied with glyphosate than without. Glyphosate did not render *A. cassiae* virulent on soybean (*Glycine max*), a crop related to the host. These results suggest that a specific inhibition of a weed's elicited defense response can be a safe way to enhance virulence and improve the efficacy of the mycoherbicide.

Microbial attack induces massive biochemical changes in the host plant, including the activation of a number of "defense responses" (5, 14). These defense responses can modulate the virulence, *i.e.* the degree of pathogenicity or severity of disease caused by the pathogen. Most of these responses require transcriptional activation of genes for enzymes that can degrade the pathogen (chitinases and glucanases), or for enzymes whose products form a physiological barrier (lignin, hydroxyproline-rich glycoproteins, callose), or function as a part of a biosynthetic pathway that leads to the synthesis of "defensive" compounds (phytoalexins). The shikimate pathway produces phenolic precursors for lignin and many phytoalexins (7, 9–11, 13). The activation of several genes of the shikimate pathway requires light (6, 15, 16, 21, 30). The gene products do not appear in the dark after inoculation or after treatment with elicitors. Thus, there is no synthesis of the corresponding enzymes and their flavonoid products in the dark (16, 21). Even if there was transcription, a lack of photosynthate needed as precursors and/or as energy could limit translation in the dark.

Phytoalexins can be a major defense system of plants, and they are involved in preventing infection by many pathogens (12). The phytoalexins in legumes are mostly phenylpropane derivatives synthesized via the shikimate pathway together with the acetate-malonate pathway (10). The direct evidence for the defensive function of phytoalexins comes mainly from work on phenylpropanoid phytoalexins. Early treatment with antimetabolites that specifically inhibit phenylalanine ammonia lyase (17, 29), or 5-*enol*pyruvulshikimate-3-phosphate synthase (9, 11, 13) inhibited the accumulation of phytoalexins in beans and allowed infection by some previously noninfective strains of pathogens. A deletion of genes controlling phytoalexin degradation is correlated with a loss of pathogenicity (25), and the insertion of a gene coding for phytoalexin degradation enhances infectivity (20). Such data provide rather direct proof for the involvement of phytoalexins in plant defense.

Mycoherbicides should be formulated to be highly pathogenic and specific to weeds while not damaging the crop (26, 28). These criteria of strong virulence and great specificity seldom come together in nature (19, 24). Low virulence of mycoherbicides is commonly overcome by application of extremely high levels of inoculum (thousands of spores/cm^2) (4, 27). One approach to improve mycoherbicides and reduce the levels of inoculum are formulations that create a moist microenvironment around the spore. These formulations protect spores from drying and supply the nutrition required for the initial stages of disease establishment (18). Such emulsions can allow one spore to be infective (3), but species and pathogen selectivities can be lost (2). Another approach to the improvement of the efficacy of mycoherbicides is by transformation of the mycoherbicidal organisms with virulence genes from other fungi or bacteria (8, 19). This has risks, as the host range of the mycoherbicide may thus be genetically broadened to include crops. We presumed that the suppression of the elicited defenses of a plant by a specific antimetabolite would temporarily enhance the intensity of a disease. This would theoretically permit infection with less inoculum during the joint treatment with the antimetabolite. Later generations of spores will be less virulent due to the lack of the antimetabolite.

We describe the consequences of the inhibition of accu-

[1] This research was supported in part by a grant from the National Council for Research and Development, the Ministry of Science and Technology, Jerusalem, and the Gilbert de Botton endowed chair of plant sciences to J.G.

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mulation of a phytoalexin in the weedy legume *Cassia obtusifolia* L. infected with the mycoherbicide *Alternaria cassiae* Jurair & Khan. The phytoalexin was identified as 2-(*p*-hydroxyphenoxy)-5,7-dihydroxychromone (22). It was labeled by radioactive phenylalanine after elicitation, indicating that it was derived from the shikimate pathway (22).

MATERIALS AND METHODS

Plants and Fungi

Plants of *Cassia obtusifolia* L. and soybean (*Glycine max* [L.] Merr. cv Williams 82) were grown as previously described (3). *Cassia* plants were used at 12 to 14 d after seeding, when the first true leaf was expanded and before the development of the second leaf. Sixteen-day-old plants of soybean were used.

The source, culture, and preparation of conidia of the fungi *Alternaria cassiae* Jurair & Khan and *Alternaria crassa* (Sacc.) Rands were as previously described (3).

Inoculation of Plants

Plants or detached leaves were inoculated by either spraying a conidial suspension to runoff (23) or by application of 2-μL droplets (3) as previously described. The spray treatments contained 10^5 conidia/mL, and the droplets each contained 200 spores unless otherwise stated in figures. Glyphosate [*N*-(phosphonomethyl) glycine] (acid) was added to conidial suspensions to give a final concentration of 50 μM. Four droplets of 1 μL containing 100 μM glyphosate were placed on each leaflet when applied 6 h before inoculation to the same spot; otherwise, 50 μM was used in 2-μL droplets with conidia. In either case, there was 0.1 nmol of glyphosate in a droplet. All treatments contained 0.02% Tween-80 to prevent the clumping of conidia in water and for better spreading of the solutions on the leaves. Detached leaves were placed in Petri dishes and incubated at 24°C with 100% RH under continuous light supplied by cool white fluorescent tubes (30 $\mu E\ m^{-2}\ s^{-1}$). Petri dishes were wrapped with black cloth for darkness.

Estimation of Virulence

The amount of mycelium that developed on detached leaves of *Cassia* was quantified by a radioimmunosorbent assay developed from antibodies that were raised in our laboratory against the mycelium of *A. cassiae* (2). The dry weight of the shoots was measured when intact plants were inoculated.

Extraction and Measurement of Flavonoids and Phytoalexin

Leaves were extracted overnight at room temperature with 10 volumes of 90% methanol based on fresh weight to give 80% methanol final concentration. The methanol filtrate was evaporated *in vacuo,* the remaining water was partitioned three times against ethylacetate, and the organic phases (previously shown to contain all phytoalexin activity) were retained and dried *in vacuo* (22). Dry samples of leaf extracts were dissolved in 5 to 20 μL methanol, applied to silica gel TLC plates (Merck G254, 5 × 7.5 cm, 0.2 mm), and developed with ethylacetate:methanol (96:4, v/v). Phenolic compounds were detected by fluorescence under 366 nm UV light. TLC plates were sprayed with 1% $AlCl_3$ in absolute ethanol, and flavonoids reacting with $AlCl_3$ were detected by the specific fluorescence in 366 nm UV light. The phytoalexin was indicated by the yellow fluorescence with $AlCl_3$ at R_F 0.82, and activity was verified by bioassay (23).

The concentration of flavonoids in the above extract was measured in a Perkin-Elmer LS-5B luminescence spectrometer by the $AlCl_3$-spectrofluorimetric method detailed by Sharon and Gressel (23). The phytoalexin (R_F 0.82) was eluted from TLC plates, the solvent was dried, and the sample was redissolved in absolute methanol. The amount of phytoalexin was measured by the same procedure as for total flavonoids, and the differences between the readings before and after the addition of $AlCl_3$ were used to calculate the amount of phytoalexin in plant extracts, based on 0.56 arbitrary fluorescence units/1 nmol phytoalexin.

All experiments were performed with complete randomized designs with four replicates per treatment and were repeated at least four times.

RESULTS

Lack of Elicitation in Darkness

Light is known to be required for the elicitation and activation of genes controlling several of the flavonoid-synthesizing enzymes (16). The defense responses of *Cassia* include the synthesis of a phenylpropanoid phytoalexin. Thus, this response should be suppressed by keeping the inoculated plants in darkness. The accumulation of phenolic materials was compared in detached leaves that were kept in the light or in darkness after inoculation. A number of fluorescing compounds accumulated in inoculated plants that were kept in light but not in plants that were kept in darkness (Fig. 1, inset). Some of these phenolic materials fluoresced only after spraying with $AlCl_3$, indicating the presence of flavonoids. A very low concentration of flavonoids was found in inoculated leaves in darkness 8 h after inoculation. Flavonoids could not be detected in inoculated leaves in darkness 24 h after inoculation (Fig. 1).

Development of Infection in Darkness

The extent of fungal growth in darkness was studied by the radioimmunosorbent assay to ascertain whether the intensity of the disease in darkness is stronger due to lack of phytoalexin. Detached leaves were inoculated with different concentrations of conidia, and the development of infection was compared in the light and in darkness, 24 and 48 h after inoculation (Fig. 2). There were no significant differences in the amounts of mycelium 24 h after inoculation with any number of conidia. Twice as much mycelium developed on leaves that were kept in darkness for 48 h after inoculation than on leaves incubated in the light. This difference was only measurable when leaves were inoculated with 100 or 500 conidia per site, but not with 10 conidia per site. These results show that the inoculum threshold required for infection is not completely abolished in the dark, as 10 conidia were not

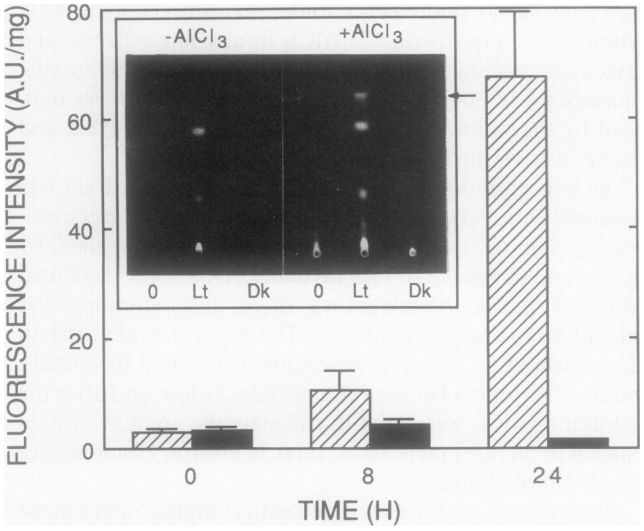

Figure 1. Lack of elicitation of defense responses by fungal infection in the dark. Detached leaves were sprayed with 10^5 conidia/mL and placed under continuous light or in darkness until sampling. Leaf discs of equal area were excised and extracted, and the relative amount of flavonoids in light (hatched) or darkness (solid) was measured. Brackets indicate standard deviations of the means. Aliquots of extracts after 24 h, equal to 5 mg leaf tissue, were applied on TLC plates, developed, and one of the plates was sprayed with $AlCl_3$ to react with flavonoids (inset). Phenolic compounds and $AlCl_3$ fluorescing flavonoids were detected under 366 nm UV light. O, uninoculated; Lt, inoculated and incubated in the light; Dk, inoculated and incubated in darkness. Arrow indicates the position of the phytoalexin.

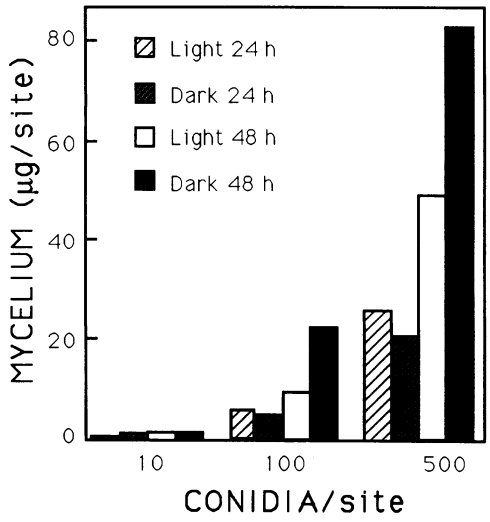

Figure 2. Growth of mycelium on inoculated leaves in light and in dark. Detached leaves were inoculated with varying numbers of conidia per droplet. At 24 and 48 h, 4 mm leaf discs were excised, and the amounts of mycelium were quantified by radioimmunosorbent assay.

enough to cause infection. Conversely, the extent of colonization was greater in darkness than in light with higher inoculum levels.

Effect of Glyphosate on Accumulation of the Phytoalexin

Glyphosate, a specific inhibitor of the enzyme 5-*enol*pyruvylshikimate 3-phosphate synthase (1), was tested as a potential inhibitor of production and accumulation of the *Cassia* phytoalexin. This pathway should be essential for the synthesis of the B ring of the phytoalexin (22). A sublethal concentration (50 μM) of glyphosate applied to plants together with the conidia decreased the amount of a few of the elicited phenolic compounds (Fig. 3). The content of $AlCl_3$ fluorescing flavonoids decreased by 57% (after subtraction of the constitutive level). Compounds with an $R_F < 0.42$ were less affected, whereas the accumulation of compounds with an $R_F > 0.42$, including the phytoalexin at R_F 0.82, were strongly suppressed in the presence of glyphosate.

The quantities of phytoalexin were measured at various concentrations of glyphosate (Fig. 4). There was no inhibition of growth of *A. cassiae* at any of these concentrations of glyphosate (data not shown), as was previously shown with other fungi (11, 13). The growth of plants was not affected by 50 μM, but with 100 μM glyphosate, newly emerging leaves were mildly bleached, *i.e.* were lacking in all pigments (data not shown). Fifteen micromolar glyphosate caused a 50%

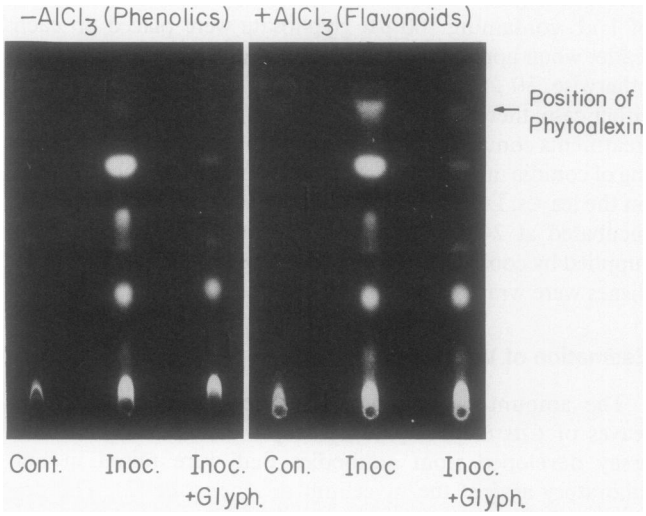

Figure 3. Glyphosate suppression of accumulation of total phenolic compounds ($-AlCl_3$) and flavonoids ($+AlCl_3$) after inoculation. Detached leaves were sprayed with 10^5 conidia/mL in water (Inoc.) or with a solution containing 50 μM glyphosate (Inoc + glyph) and compared with a mock-inoculated control (Cont). The leaves were incubated under continuous light for 24 h and the leaf discs were then excised and extracted. Aliquots equal to 5 mg leaf tissue were applied on TLC plates, the plates were developed, and total phenolic compounds ($-AlCl_3$) and flavonoids ($+AlCl_3$) including the phytoalexin were detected under 366 nm UV light. The arrow denotes the position of the phytoalexin, as verified by bioassay (23). Aliquots were taken for spectrofluorimetric measurements of the relative amount of flavonoids: Control, 4.8; inoculated, 58; inoculated + glyphosate, 28. Values are in arbitrary fluorescence units/mg leaf tissue.

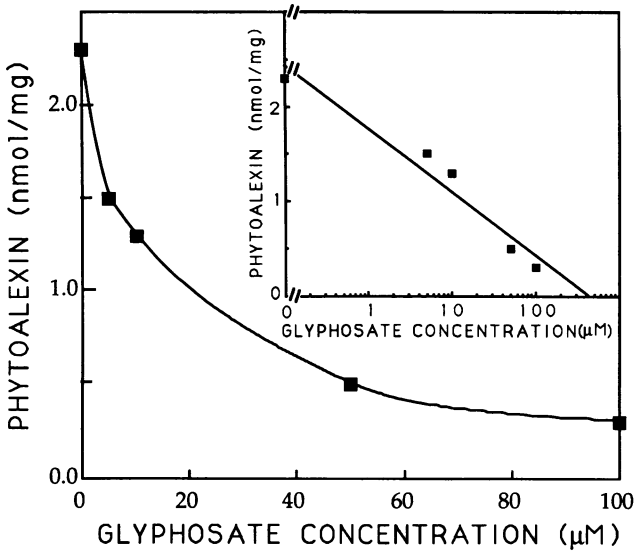

Figure 4. Concentration dependence of suppression of accumulation of *Cassia* phytoalexin by glyphosate. Detached leaves were inoculated with conidia in water or in solutions with glyphosate. Four droplets of 2 μL containing 200 conidia each were placed on each leaf. Leaf discs were excised 24 h after inoculation, and the amount of phytoalexin was measured after extraction and separation on a TLC plate and removal of the spot at R_F 0.82. The glyphosate concentration inhibiting 50% of phytoalexin accumulation was 15 μM.

inhibition of accumulation of the phytoalexin 24 h after inoculation, and 50 μM glyphosate suppressed accumulation of the phytoalexin by 80% (Fig. 4).

The inhibition of accumulation of the phytoalexin by glyphosate 24 h after inoculation corroborated our previous results (22), showing that it was synthesized *de novo* from phenylalanine. The effect of glyphosate on the early stages of synthesis and accumulation of the phytoalexin was studied to ascertain whether the initial response depends on *de novo* synthesis from the same kind of precursors. A time course experiment showed that glyphosate applied together with conidia was not effective before 12 h (Fig. 5C). Even an application of glyphosate 6 h before inoculation did not reduce the level of phytoalexin assayed 8 and 10 h after inoculation (Fig. 5D, A), whereas in the dark, there was no accumulation (Fig. 5B).

Effect of Glyphosate on Damage by Infection

The effects of glyphosate (50 μM) applied with conidia on the inoculum threshold and on damage to plants were simultaneously measured to ascertain whether the inhibition by glyphosate of the synthesis of phytoalexin enhanced the intensity of the disease. Glyphosate added to the spray gave a fivefold reduction in the concentration of conidia required to obtain the same reduction in dry weight of plants in the greenhouse (Fig. 6A). *Cassia* seedlings were killed by 10^4 conidia/mL sprayed to runoff with 50 μM glyphosate (Fig. 6B). The same concentration of conidia in water alone caused only scattered necroses.

Effect of Glyphosate on the Interaction between *A. cassiae* and Soybean

The effect of glyphosate was tested on the interaction between *A. cassiae* and soybean. Soybeans produce phenylpropanoid phytoalexins after infection by incompatible pathogens (13, 17). *A. cassiae,* which is used as a mycoherbicide to control *C. obtusifolia* in soybeans, does not infect soybean, but under certain conditions it may cause "hypersensitive" necrotic spots. This response might be changed due to inhibition by glyphosate of the synthesis of phenylpropanoid phytoalexins in beans (9, 11, 13). Thus, soybean plants were inoculated with a conidial suspension of *A. cassiae* in water or in a suspension with glyphosate (50 μM), and the infection was compared. The hypersensitive response of soybean to inoculation with *A. cassiae* was not changed by glyphosate. There was no change with glyphosate in the number nor in the size of the necrotic lesions (data not shown).

DISCUSSION

It has previously been shown that treatment of plants with glyphosate 24 h before inoculation decreased plant response to the point at which avirulent strains of pathogenic bacteria and fungi were rendered pathogenic (9, 11, 13). Our data show that glyphosate further enhanced virulence of a mycoherbicidal pathogen (Fig. 6).

The sublethal concentration (50 μM) of glyphosate that was applied with conidia decreased the content of phytoalexin (Figs. 4, 5) and rendered *Cassia* seedlings highly susceptible to the mycoherbicide *A. cassiae* (Fig. 6). Thus, the virulence of *A. cassiae* on *Cassia* was enhanced, and infection occurred with less inoculum. Treatment with glyphosate at 50 μM did

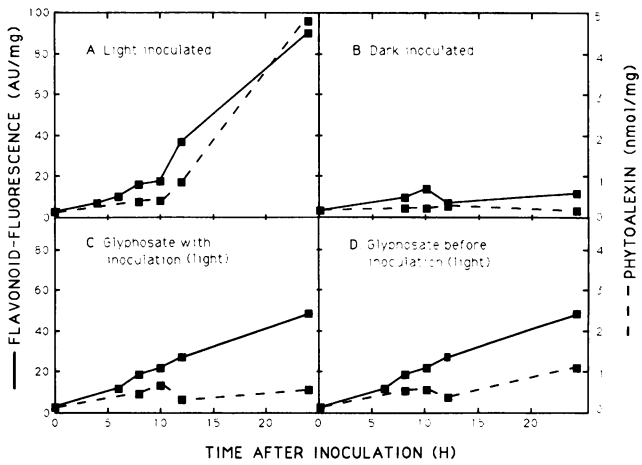

Figure 5. Suppression of early and late accumulation of total flavonoids and of the phytoalexin by glyphosate. Detached leaves were inoculated with 2-μL droplets containing 200 conidia in water or in a solution of 50 μM glyphosate. When glyphosate was applied before the conidia, the same amount of glyphosate was applied 6 h before the conidia to the same spots. The leaves of all treatments were placed in Petri dishes in an incubator in continuous light or in darkness, and the amount of total flavonoids (solid line) and phytoalexin (dashed line) was measured ($AlCl_3$ spectrofluorimetry) at various times after treatment.

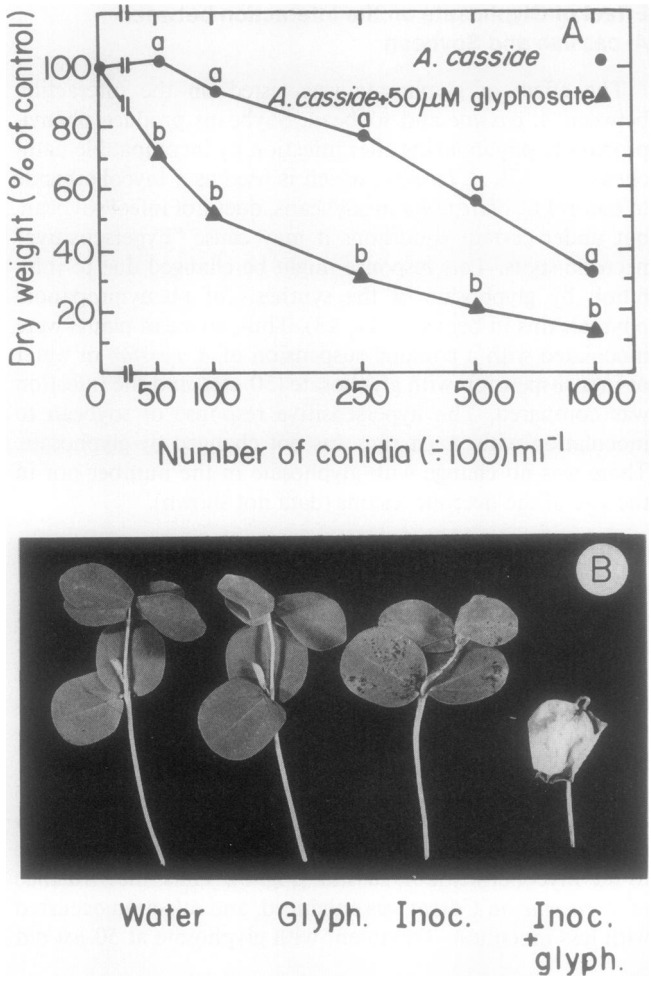

Figure 6. Enhanced damage at various levels of inoculum treatment with glyphosate. Seedlings with one first true leaf were sprayed to runoff with various concentrations of conidia in water or conidia in a solution of 50 μM glyphosate. They were placed in a dew chamber (100% RH) for 16 h, after which they were moved to the greenhouse. A, The dry weight of the shoots was recorded after 10 d. (Note semi-log plot.) B, Typical shoots 7 d after spraying to runoff with: water glyphosate (Glyph.), conidia (10^4/mL) in water (Inoc.), or the same concentration of conidia with glyphosate (Inoc. + glyph.). Mycelia in the leaf discs were measured 72 h after inoculation.

not result in pathogenicity of the fungus to soybean. This implies that this concentration of glyphosate had no significant effect on other factors that contribute to the resistance of the plant. Still, it may render soybean more susceptible to soybean-specific pathogens, as previously reported (9).

Two phases of accumulation of flavonoids as well as of phytoalexins were characterized. Only the second phase was inhibited by glyphosate (Fig. 5), indicating that the synthesis of the phytoalexin at the early stages does not require *de novo* synthesis of early precursors in the pathway. The first stage may rely on metabolites that are chemically closer to the phytoalexin, either precursors to, or conjugates with, the phytoalexin. Still, gene activation may well be required for the early stages, as in the dark there was no phytoalexin synthesis even 8 to 10 h after inoculation (Figs. 1, 5B). Conversely, there might have been gene expression, but photosynthate may have been needed to provide energy and/or precursors for the synthesis. This initial response is probably important as a first and fast local response at the immediate site of fungal penetration; in the dark, the infection was even more massive than with glyphosate in the light, indicating that the early response had some inhibitory effect on the fungus, yet it was not enough to prevent the later spread of the fungus. This phytoalexin is fungistatic (A. Sharon, J. Gressel, unpublished) and once its level decreased, the fungus could develop again.

We have shown that coapplication of a mycoherbicide with an antimetabolite that suppresses an elicited defense increased the susceptibility of the host weed and enhanced the virulence of the mycoherbicidal fungus without affecting its specificity (Figs. 3–5). Glyphosate suppressed the elicited appearance of other nonphytoalexin phenolics (Fig. 3), showing that it might inhibit the production of precursors for other defense pathways, such as lignin biosynthesis. The possibility that glyphosate will enhance the virulence of soybean pathogens cannot be ruled out. Still, the chances that a droplet of glyphosate will fall on groups of spores of a soybean pathogen are poor if mycoherbicides are applied in ultralow volume, directed sprays. Many conidia per droplet are still required to establish disease even with glyphosate. Hence, the chances of causing infection on soybean by use of glyphosate are rather improbable. Discovery of more specific antimetabolites that inhibit the synthesis of phenylpropanoid-derived as well as other type phytoalexins would further document the validity of this approach to other mycoherbicide-weed-crop situations.

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