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Phytochemistry 62 (2003) 865–874
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Lactofen induces isoflavone accumulation and glyceollin elicitation competency in soybean

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Received 29 July 2002; received in revised form 23 September 2002

Abstract

Lactofen, the active ingredient of the soybean disease resistance-inducing herbicide, Cobra, induces large accumulations of isoflavone conjugates and aglycones in soybean tissues. The predominant isoflavones induced in cotyledon tissues are daidzein (and its conjugates) and formononetin and glycitein aglycones. The latter two isoflavones are usually present only at very low levels in soybean seedling tissues. In leaves, the predominant lactofen-induced isoflavones are daidzein and formononetin aglycones and the malonyl-glucosyl conjugate of genistein. Isoflavone induction also occurs in cells distal to the point of treatment, but is only weakly systemic. Lactofen also induces elicitation competency, the capacity of soybean cells to accumulate the pterocarpan phytoalexin glyceollin in response to glucan elicitors from the cell wall of the pathogen *Phytophthora sojae*. Comparison of the activity of a series of diphenyl ether herbicides demonstrated that while all diphenyl ethers tested induced some degree of elicitation competency, only certain ones induced isoflavone accumulation in the absence of glucan elicitor. As a group the diphenyl ethers are thought to inhibit protoporhyrinogen oxidase, eventually leading to singlet oxygen generation. Another singlet oxygen generator, rose bengal, also induced elicitation competency, but little isoflavone accumulation. It is hypothesized that diphenyl ether-induced activated oxygen species mimic some aspects of hypersensitive cell death, which leads to elicitation competency in infected tissues.

Keywords: *Glycine max*; Papilionoideae; Soybean; Phenylpropanoid; Isoflavone; Genistein; Daidzein; Diphenyl ether; Herbicide

1. Introduction

Among the first metabolic profiling procedures developed was an HPLC-UV profiling protocol that was designed to be readily applicable to a wide range of plants and microbes (Graham, 1991a). This protocol required very small tissue samples and allowed the simultaneous analysis of well over a hundred UV-absorbing metabolites. Thus, "snap shots" of aromatic metabolic pathways could be taken with time in very discrete cell populations, leading to a detailed and dynamic picture of the shunting of metabolites in what Barz and co-workers termed the metabolic grid (Barz and Hoesel, 1979). Coupled with the many advantages of cotyledon tissues as a system for the analysis of cellular response and cell-to-cell communication, this metabolic profiling protocol was used as a critical tool in unraveling the multiplicity and some of the cellular aspects of deployment of soybean defense responses to the pathogen *Phytophthora sojae* and its cell wall glucan elicitors (WGE).

A nearly universal and early resistance response to incompatible pathogens is the hypersensitive response (HR) that leads to the oxidative death of host cells in immediate contact with the pathogen. In soybean, metabolic profiling studies with infected tissues or tissues treated with WGE clearly defined several phenylpropanoid defense responses that occur in a zone of "proximal" cells immediately surrounding the HR. These include the rapid deposition of phenolic polymers in the walls of proximal cells (Graham and Graham, 1991a), the hydrolytic release of the isoflavones genistein and daidzein from pre-formed conjugates and the subsequent accumulation of the phytoalexin glyceollin (Graham et al., 1990). Genistein is toxic to several classes of fungal and oomycetic pathogens (Rivera-Vargas

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doi:10.1016/S0031-9422(02)00709-4

et al., 1993), while daidzein is the first committed precursor for the synthesis of glyceollin (Ebel, 1986). Thus, proximal cells deploy multiple and complementary defense responses. In cells distal to (up to several hundred cells distant from) the infection court, there is a net accumulation of conjugates of daidzein and genistein (Graham and Graham, 1991b) which has been shown to play at least a partial role in increasing the defense potential of these cells (Park et al., 2002).

All of the above responses involve distinct branches of phenylpropanoid metabolism. The most relevant branch pathways for soybean defense are shown schematically and in abbreviated form in Fig. 1. Some of the key defense-related end products are shown in bold italics. Based on many years of metabolic profiling work with soybean, we have developed a working model towards grouping the enzymes of these pathways into regulatory units (indicated by numbers in Fig. 1). We call these hypothetical regulatory groupings of enzymes "metabolic cassettes". Certain combinations of cassettes are required to generate certain products. For example, to synthesize glyceollin one could envision up-regulation of enzymes of cassettes 1, 3, 5, 6, 9 and 10, if synthesis was *de novo* from phenylalanine, or alternatively just 8, 9 and 10, if synthesis proceeded from pre-formed daidzein conjugates. We are currently developing molecular probes for the analysis of the expression of the mRNAs of representatives of each of these groups to allow us to confirm and/or further refine their regulation as suggested by metabolic profiling. This type of organization of metabolic enzymes into coordinately regulated transcriptional groups was pioneered by Klaus Hahlbrock and coworkers (Hahlbrock, 1981).

The metabolic and functional multiplicity of the proximal defense responses suggested that complex regulatory processes might coordinate their deployment. Indeed it was found that the capacity for the various proximal defense responses to WGE is activated in proximal cells by a complex network of signaling events associated with hypersensitively dying or wounded cells (Graham and Graham, 1994, 1999). This induced state of responsiveness of proximal cells was called elicitation competency. Interestingly, treatment of non-activated cells with WGE leads to default expression of the distal cell response, the net accumulation of isoflavone conjugates. In this response, the glucan elicitor is the primary signal for the accumulation of daidzein conjugates, while light is the primary signal for the accumulation of genistein conjugates (Graham and Graham, 1996). Thus, the deployment of the proximal and distal cell responses is in fine balance, and the nature of, cellular aspects of, and signaling events involved in the regulation of the associated phenylpropanoid responses has been particularly well characterized in soybean.

The herbicide Cobra has been shown to protect soybean plants against several diseases in the field. These include white mold (caused by *Sclerotinia sclerotiorum*, Dann et al., 1999), sudden death syndrome (Sanogo et al., 2000), and the soybean cyst nematode (Levene et al.,

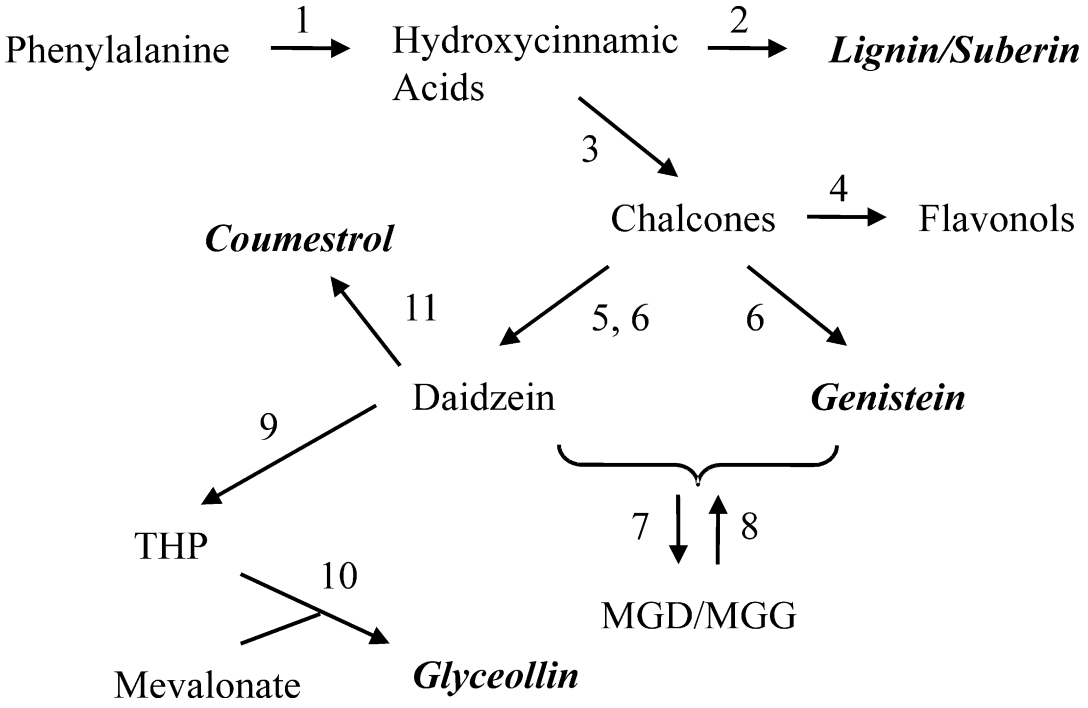

Fig. 1. Potential metabolic cassettes involved in soybean defense. In this simplified working diagram of phenylpropanoid defense pathways in soybean, the end products implicated in defense are shown in bold italics. The numbers refer to groups of enzymes that are hypothesized to be under coordinate regulation. The isoflavone 7-*O*-glucosyl-6″-*O*-malonates of daidzein and genistein are designated as MGD and MGG respectively.

1998). While little is known about the protective mode of action of Cobra, analysis of treated field plants suggests that there is a net increase in the pterocarpan phytoalexin, glyceollin, in treated tissues (Dann et al., 1999). The active ingredient of Cobra is the diphenyl ether compound, lactofen. As with other members of the diphenyl ether class of herbicides, lactofen's herbicidal activity is thought to be mediated through the inhibition of protoporphyrinogen IX oxidase (Protox), leading to the build-up of protoporphyrinogen, which is subsequently oxidized to protoporphyrin at the plasma membrane (Ahrens, 1994). Light activation of protoporphyrin, in turn, generates activated oxygen species (AOS) including singlet oxygen.

Due to our extensive work on the biochemistry of soybean defense responses, we sought to further characterize the effects of lactofen on these various responses. Here we report that lactofen appears to have two complementary activities. The first of these is a dramatic induction of a net accumulation of isoflavone conjugates and aglycones. The second activity is the activation of elicitation competency; that is, lactofen activates the capacity of soybean cells to accumulate glyceollin in response to the well characterized glucan elicitor (WGE) from the pathogen *P. sojae*.

2. Results and discussion

2.1. Characterization of the activity of lactofen

Due to a number of factors, including their relatively simple and uniform cellular architecture and the ease of thin-sectioning, soybean cotyledons have served as a very useful model system for investigation of isoflavone responses and the cellular processes involved in their regulation. Of particular use has been the minimal wound snapped cotyledon assay (Graham and Graham, 1996). An HPLC metabolic profile showing the response of such cotyledon cells to 200 μM lactofen is shown in Fig. 2B. Profiles are purposely plotted with major peaks off scale to show the resolution and finer detail of response. An array of isoflavone aglycones and conjugates are induced by lactofen. These include dramatic increases in aglycones of the isoflavones glycitein and formononetin, which are normally only at very low levels in soybean tissues (Graham, 1991b). Although not as obvious from the HPLC as plotted, higher levels of daidzein aglycone and its conjugates daidzin and MGD also accumulate. These daidzein-related responses are shown quantitatively in later figures. Finally some accumulation of genistein aglycone and coumestrol are

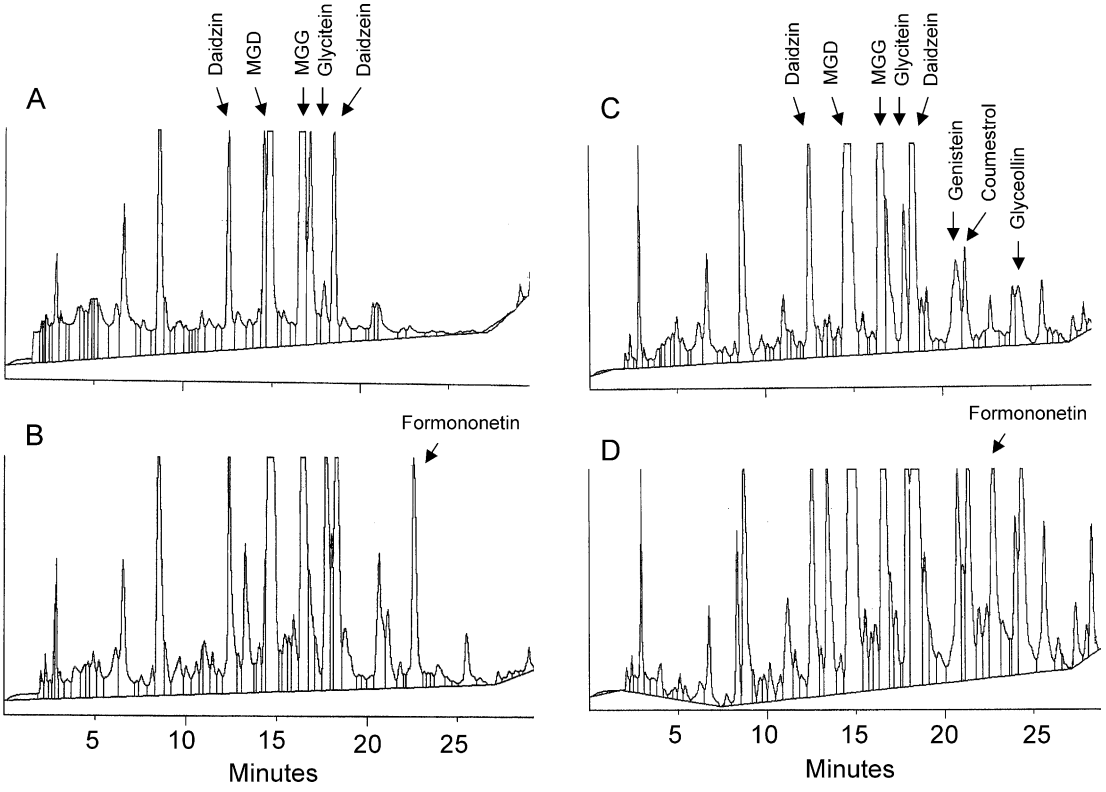

Fig. 2. HPLC profiles of the effects of lactofen and WGE treatment on soybean cotyledon tissues. Cotyledons were treated with water (A), 200 μM lactofen (B), 30 μg/ml WGE (C) or WGE and lactofen (D). Tissues were harvested at 48 h for HPLC. The major metabolites in Fig. 1 are identified. Daidzin is the simple 7-*O*-glucoside of daidzein.

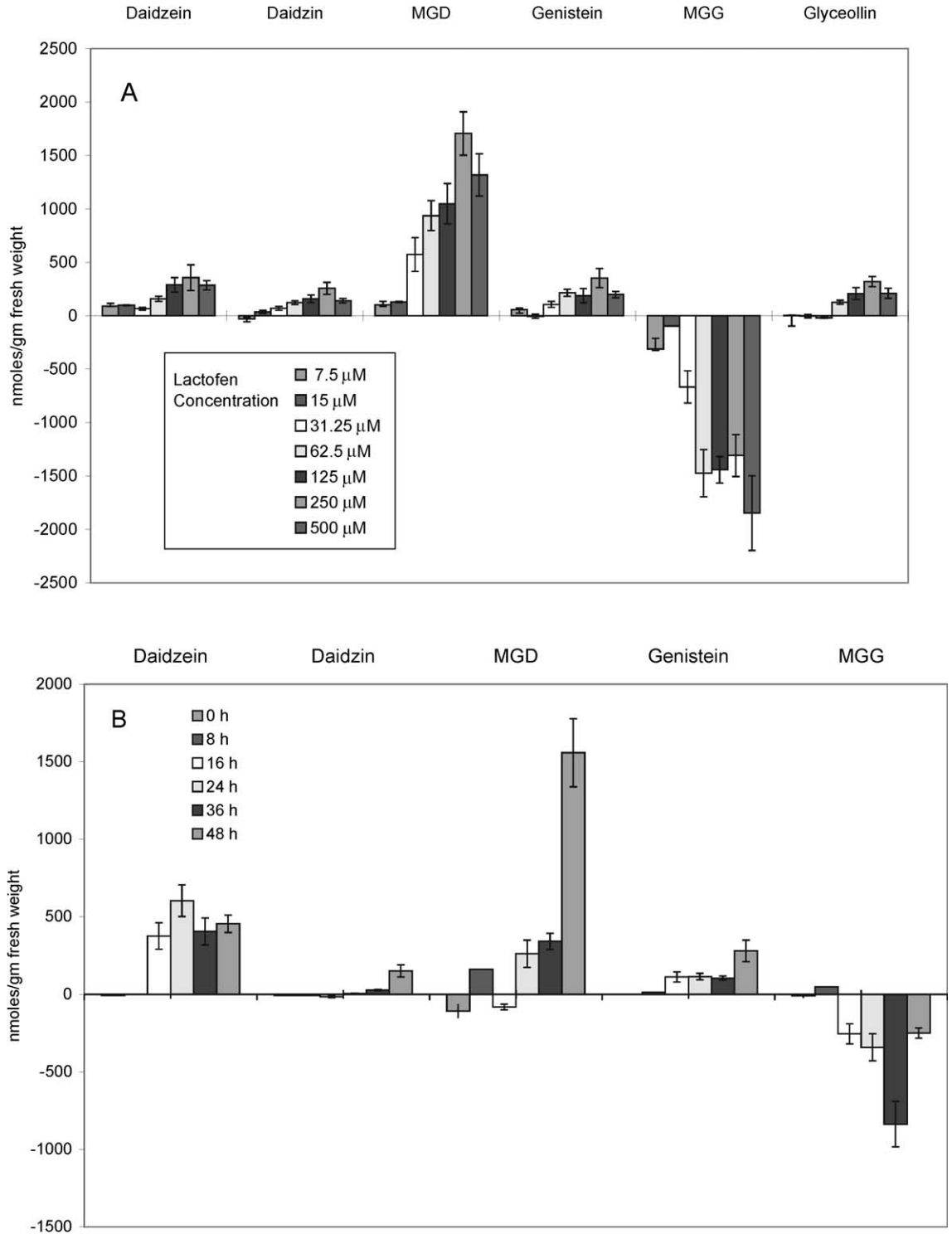

Fig. 3. Effects of lactofen on isoflavone accumulations in soybean cotyledons. Defense-related isoflavones were quantified by HPLC profiling of tissues harvested 48 h after treatment with various levels of lactofen (A). Alternatively a time course of response to 200 μM lactofen was examined in proximal (B) or distal (C) cotyledon cells. Isoflavone levels in water controls were subtracted from the plotted values, which are the average of two experiments ± standard error.

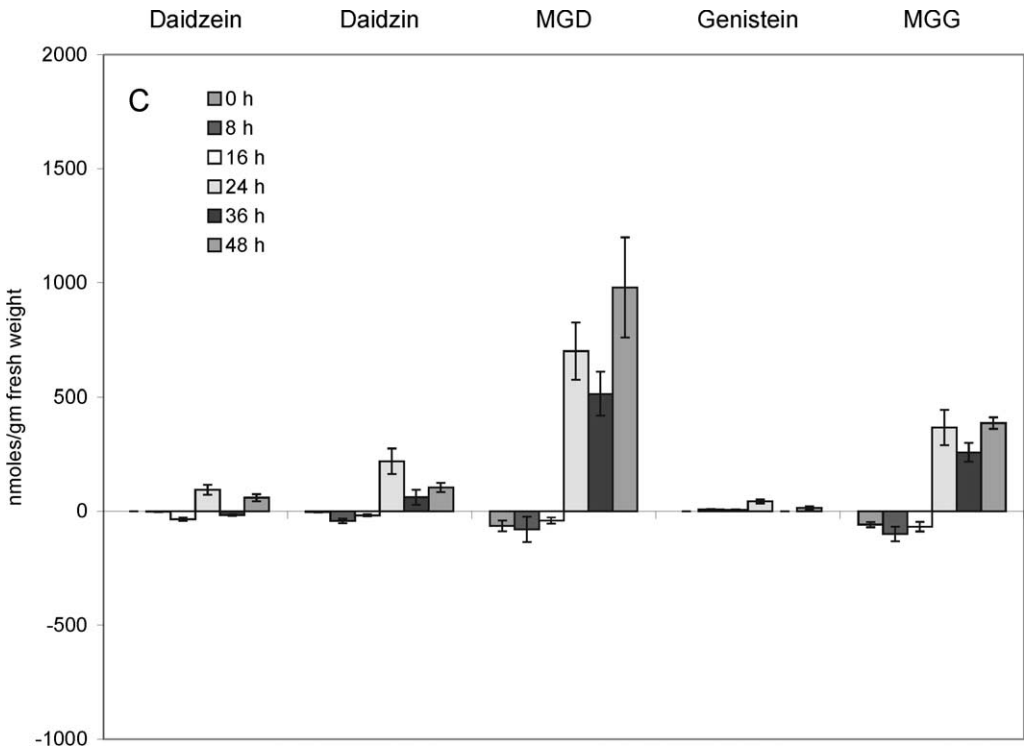

Fig. 3. (*continued*).

also apparent. For comparison, the induction of isoflavones and their conjugates by the well characterized glucan elicitor (WGE) is shown in Fig. 2C. Again, though not obvious from the profile as plotted, there are large net accumulations of daidzein and genistein conjugates, MGD and MGG. There are also some accumulations of glycitein, genistein, coumestrol and glyceollin, but little formononetin. Simultaneous treatment with both lactofen and WGE (Fig. 2D) leads to an extremely complex profile. In fact, in over 15 years of experience with metabolic profiling of soybean tissues this is the most complex pattern we have ever observed. It serves well to show the resolving power of the protocol. In addition to the variety of isoflavones induced by lactofen (in the presence or absence of the glucan), lactofen induces much higher overall levels of isoflavone aglycones compared to other treatments we have investigated. Under normal physiological conditions, and even in response to the biotic WGE (Graham and Graham, 1991b), the isoflavone aglycones in soybean seedling tissues are rapidly conjugated and do not normally accumulate to such high levels as the free aglycones. An exception to this is at the root tip, where free daidzein and genistein can reach appreciable levels (Graham, 1991b).

Dose–response curves for the well characterized defense-related isoflavones in proximal cells following treatment with various levels of lactofen are shown in Fig. 3A. The rate of application of lactofen in the field is approximately 1.5 mM. Plotted in Fig. 3A are responses to serial 1:2 dilutions of lactofen from 500 to 7.5 μM. Optimal accumulation of the malonyl-glucosyl conjugate of the 5-deoxyisoflavone daidzein (MGD) is seen at 250 μM, 1/6th the field rate, and some induction is still apparent at 31 μM, which is nearly 1/50th the field rate. Rates higher than 500 μM lead to lowered response (data not shown). In contrast to MGD, levels of the malonyl-glucosyl conjugate of genistein (MGG) actually decrease in this tissue in response to lactofen treatment. Lactofen also induces low levels of glyceollin. A time course of induction for the defense-related isoflavones is shown in Fig. 3B. Accumulations of free daidzein are apparent within 16 h of treatment, with accumulation of MGD not beginning until 24 h. Lactofen also causes isoflavone accumulations in distal cells (Fig. 3C). However, in these cells both MGD and MGG accumulate with little accumulation of free aglycones. Although not shown, the range of isoflavones induced by lactofen in distal cells is much simpler, with daidzein and genistein greatly predominating and negligible amounts of glycitein or formononetin. Thus, somewhat different responses are seen in proximal and distal cells.

In soybean leaves, the predominant constitutive phenylpropanoid metabolites are glycosides of the flavonols kaempferol and quercetin (Graham, 1991b). Although MGG is present in leaves, it is a comparatively minor component. Daidzein aglycone is sometimes also present, but at even lower levels (Graham, 1991b). Fig. 4

shows HPLC profiles of control and lactofen-treated trifoliate soybean leaves from greenhouse grown plants. Leaves from field grown plants showed similar responses. As shown in this typical experiment, lactofen induces the accumulation of MGG (3.6-fold over controls) and daidzein and formononetin aglycones (7.8- and 48-fold over controls, respectively). The flavonol glycosides were unaffected. Thus, the pattern of induction in leaves is somewhat different from that in cotyledons. In leaves, daidzein accumulates nearly exclusively as the aglycone and MGG (which decreases in cotyledons) also accumulates. Moreover, while formononetin accumulates to high levels in leaves, glycitein does not. Analysis of untreated leaves further up the stem from treated leaves, or leaves which emerged after treatment, from greenhouse and field plants (data not shown) showed that the levels of isoflavones accumulating in response to lactofen in these tissues are very low.

Because the analysis of field plants showed higher levels of glyceollin in treated plants (Dann et al., 1999), we wished to determine what effects lactofen might have on the elicitation of this phytoalexin. As noted above (in both Figs. 2B and 3A), when applied alone, lactofen is only a weak elicitor of glyceollin. To assess its possible effects on elicitation by the biotic WGE elicitor, we treated snapped cotyledon tissues simultaneously with lactofen and WGE. In this minimal wound assay, WGE normally induces both MGD and MGG, but little glyceollin (see, for example, the HPLC profile in Fig. 2C) and thus effects on glyceollin elicitation competency can be clearly followed. As shown in Fig. 5 (and in the corresponding HPLC profile in Fig. 2D), under these conditions lactofen greatly enhances glyceollin elicitation competency in response to WGE. Its effects on MGD accumulation are slightly less strong in the presence of WGE, probably reflecting the already strong induction of MGD by this elicitor. While WGE induces the formation of MGG, lactofen suppress the accumulation of this metabolite as it does when applied alone.

2.2. *Activities of metabolites of lactofen, other diphenyl ether herbicides and of the singlet oxygen generator rose bengal*

Fig. 6 shows the activities of a series of diphenyl ether herbicides for their induction of accumulation of MGD

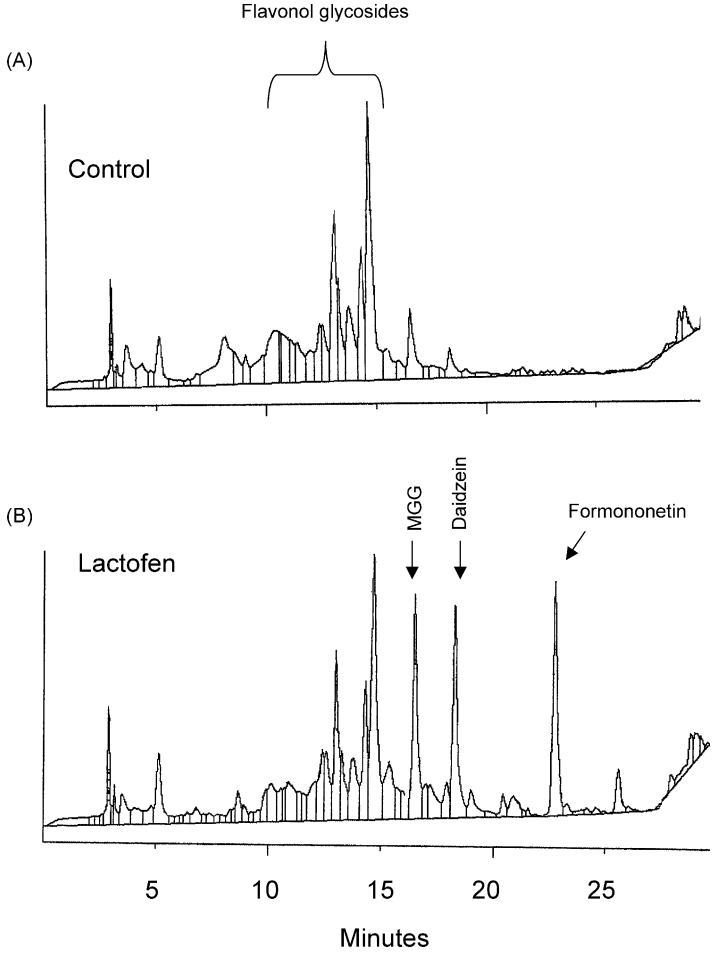

Fig. 4. HPLC profiles of the effects of lactofen on soybean trifoliate leaves. Leaves were treated with either formulation control (A) or 120 μM lactofen (B) and harvested at 48 h for HPLC.

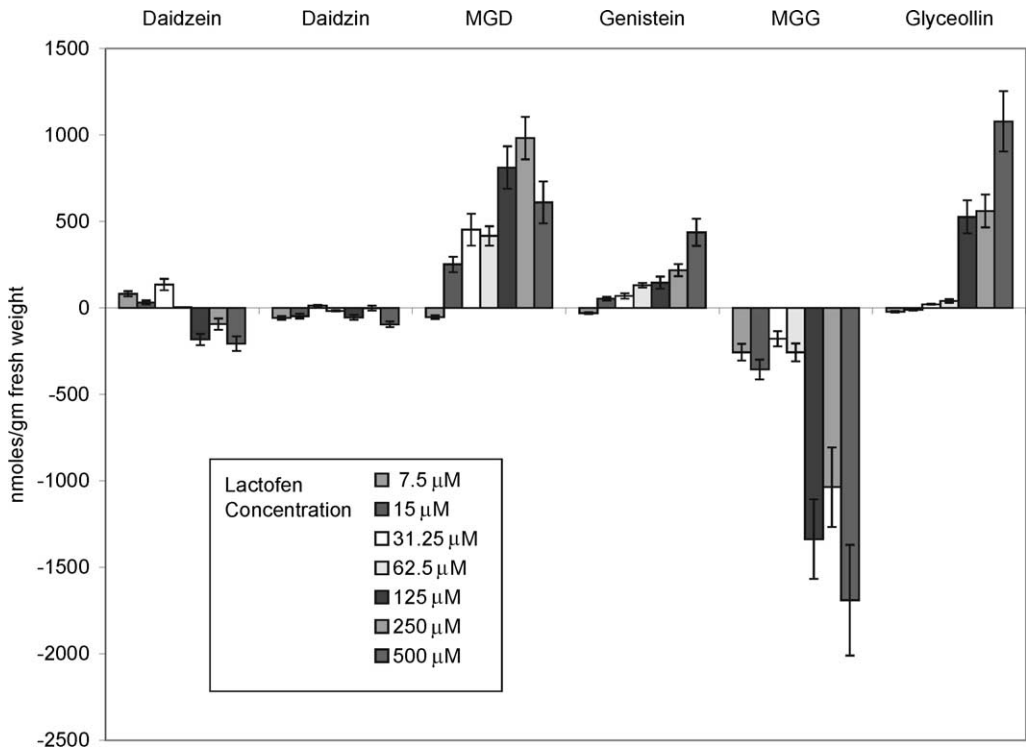

Fig. 5. Effects of various concentrations of lactofen on defense-related isoflavones and glyceollin elicitation in the presence of 30 μg/ml WGE. Tissues were harvested at 48 h for HPLC. Levels of the metabolites in WGE-treated controls were subtracted from the plotted values, which are the average of two experiments ± standard error.

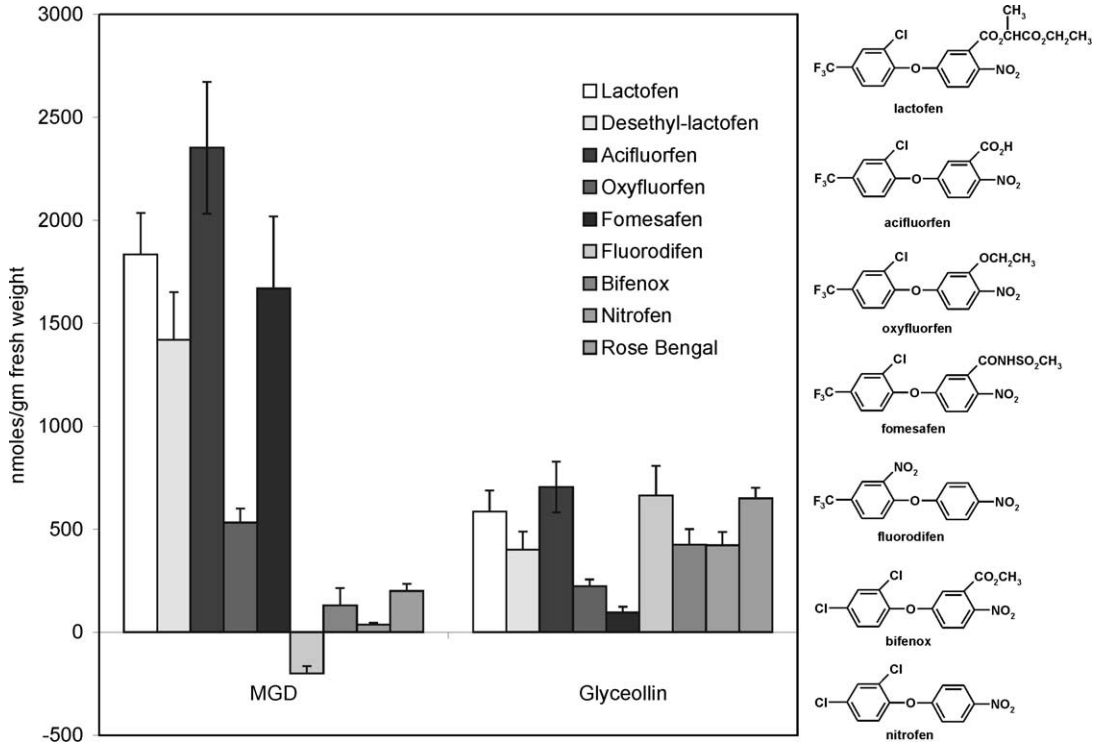

Fig. 6. Effects of various diphenyl ether compounds and of rose bengal on isoflavone accumulation (in the absence of WGE) and glyceollin elicitation (in the presence of 30 μg/ml WGE). All compounds were tested at 166 μM, except rose bengal, which was tested at 12.5 μM. Metabolite levels in the appropriate water or WGE-treated controls were subtracted from the values plotted, which are the average of two experiments ± standard error.

and for their activation of glyceollin elicitation competency in the presence of WGE. Although dose response experiments were done with each of these compounds, for simplicity we show their comparative activity at 166 uM. Among these compounds are two plant metabolites of lactofen, desethyl-lactofen (structure not shown) and acifluorfen. Both of these compounds are rapidly formed *in planta* from lactofen (Ahrens, 1994) and retain the diphenyl ether configuration. As shown in Fig. 6, they induce MGD to levels comparable to that of lactofen. Of the other diphenyl ether compounds, however, only fomesafen causes a comparable induction of MGD. While several of the diphenyl ether compounds activate glyceollin elicitation competency, the activity profile is clearly different from that for MGD. Fluorodifen, bifenox and nitrofen, which do not induce MGD, induce glyceollin elicitation competency. On the other hand, fomesafen, which is a potent inducer of MGD, is comparatively poor in activating elicitation competency. Only lactofen, desethyl-lactofen and acifluorfen are strongly active for both responses. These data suggest a possible superimposition of several structure–function relationships among these compounds. The multiple positive effects of lactofen and its metabolites on defense expression may help explain its efficacy in the field.

Although our experiments did not assess this activity in soybean, all of the diphenyl ether chemicals listed in Fig. 6 are known Protox inhibitors. The fact that all of the compounds induced some degree of elicitation competency led us to hypothesize that their activation of the accumulation of activated oxygen species might mimic some aspects of hypersensitive cell death, the endogenous event associated with activation of elicitation competency. In particular, the damage caused by this class of herbicides has been linked to singlet oxygen generation. Rose bengal, a photosensitizer and singlet oxygen generator (Bilski et al., 1998) is often used in comparative studies on diphenyl ether mode of action (see, e.g. Matsumoto et al., 1999). As shown in Fig. 6, at 12.5 uM rose bengal is as potent as the most active diphenyl ethers in activating glyceollin elicitation competency, while having comparatively little effect on MGD accumulation in the absence of WGE. Rose bengal activates elicitation competency at levels as low as 3 uM (data not shown). At 100 uM, response to the glucan is enhanced by over 1500 nmol glyceollin/gm tissue. As expected from its mode of action, rose bengal is inactive in the dark. Likewise, the herbicidal activity of the diphenyl ether compounds requires light and a series of experiments with lactofen and fluorodifen show that these compounds are inactive in inducing elicitation competency in the dark (data not shown).

The diphenyl ether compounds most active as isoflavone inducers (lactofen, desethyl-lactofen, acifluorfen and fomesafen) all posses an ionic group, or the easy potential for the formation of such a group (e.g. esters and amides). This class of diphenyl ethers has been shown to possess peroxisome proliferator activity in mammals (Gonzalez et al., 1998). In other work, we have demonstrated that other peroxisome proliferators, estrogens and fungal steroids also possess isoflavone inducing activity in soybean (Graham, 2000). While these compounds all share activity as nuclear receptor ligands, there are several other possible modes of action common to them. We are currently performing in depth experiments to determine the targets of these isoflavone-inducing chemicals.

3. Conclusions

As a class, the diphenyl ether herbicides tested all activated some degree of glyceollin elicitation competency in soybean. It seems likely that this may be due to the oxidative damage done by these herbicides, which as previously pointed out by Dann et al. (1999) may mimic some aspects of hypersensitive cell death, which in turn has been associated with generation of the elicitation competent state in infected tissues (Graham and Graham, 1999, 2000). There are a number of observations that support or are consistent with this notion. First of all, the activities of the diphenyl ethers as herbicides and as activators of elicitation competency both require light. Secondly, rose bengal, a photoactivated singlet oxygen generator, shows activity closely parallel to the diphenyl ethers active in inducing competency. Some degree of mimicry of HR by Protox inhibitors is also consistent with the observation that mutations in the *Les22* gene in corn lead to an HR lesion mimic or cell death phenotype. *Les22* encodes uroporphyrinogen decarboxylase (Hu et al., 1998). Its inactivation by mutation leads to the accumulation of the photoexcitable uroporphyrin. Conversely, the activity of diphenyl ethers as inducers of isoflavone accumulation may require the presence of (or likely generation of) an ionic group, a structural requirement for their activity as peroxisome proliferators. Both of these possible structure–function relationships, however, are tentative and will require more in depth work.

It is interesting that among the chemicals tested only lactofen and its metabolites possess both strong isoflavone-inducing and competency-activating activities. These two phenomena are highly complementary to the potential defense of treated tissues. First, these chemicals induce the accumulation of daidzein, the precursor for glyceollin and secondly they activate the capacity of soybean cells to utilize this precursor for the production of glyceollin in response to pathogen elicitor. Finally, they induce the formation of genistein conjugates in leaves and in distal cells in cotyledons, an event that could raise the defense potential of these tissues (Park et

al., 2002). Perhaps these multiple modes of action work together to at least partially account for the disease resistance-inducing activity of lactofen in the field. Indeed, Dann et al. (1999) found that both lactofen and acifluorfen were active in the field against Sclerotinia. We are currently examining lactofen-protected soybeans in greenhouse studies using both metabolic profiling and defense gene expression analysis to further test this hypothesis.

4. Experimental

4.1. Chemicals and preparations

The intact wall glucan elicitor (WGE) was prepared from the cell walls of race 1 of *Phytophthora sojae* (Kauf. and Gerde.) according to Ayers et al. (1976) and as described previously (Graham and Graham, 1991b). Before use, the unfractionated and insoluble wall glucan preparation was sonicated and then autoclaved for 3 h in deionized double distilled water (Ayers et al., 1976). The composition of this complex carbohydrate elicitor has been described (Ayers et al., 1976; Graham and Graham, 1991b). The concentrations reported here are based on weight per volume of the elicitor preparation.

Lactofen and desethyl-lactofen were obtained from Valent Technologies. All other diphenyl ether herbicides were obtained from Chem Service (West Chester, PA, USA). Rose bengal was obtained from Sigma Chemical Company St. Louis, MO, USA.

4.2. Growth and treatment of soybean seedlings

Soybean (*Glycine max* L. [Merr.]) cv. Williams was obtained from Dr. A. E. Dorrance (Department of Plant Pathology, The Ohio State University and the Ohio Agricultural Research and Development Center, Wooster, OH). Seedlings were grown as described previously (Graham et al., 1990) with slight modifications. Seedlings were grown in vermiculite at 26 °C with 500 $\mu E\ m^{-2}\ s^{-1}$ of light and a 14-h photoperiod. The flats were immediately watered very thoroughly for germination. After 3 days, the plants were watered every other day from the top. Plants were not fertilized.

For cotyledon assays, cotyledons from 6- to 11-day-old seedlings, unless otherwise noted, were harvested in small batches and used immediately. The minimal-wound snapped cotyledon assay was performed as described previously (Graham and Graham, 1996). Briefly, cotyledons were removed from seedlings by gently twisting them off the plant. They were then snapped in two at a point about 1/3 the distance away from the point of attachment of the petiole and placed petiole-side down into 0.55% water agar. The appropriate treatment was applied in 15 μl to the freshly exposed cells. Ten replicate cotyledons were used for each treatment and pooled for HPLC analysis. Soybean trifoliate leaves from 20 day old seedlings were treated by spraying with or without lactofen in 25% acetone and 0.4% Tween 20. Ten replicate leaves were pooled for analysis.

4.3. HPLC analysis

Treated cotyledons were usually harvested after 48 h of incubation unless otherwise noted in the figures. Also unless otherwise noted, only the uppermost 0.5 mm section (proximal cells) of cells was analyzed (Graham and Graham, 1991b, 1996). In some cases the next 0.5 mm section (distal cells) was also analyzed. The 10 sections per treatment were pooled together and either extracted and analyzed immediately for soluble metabolites by HPLC or stored intact at −20 °C for later extraction. Extraction was performed directly in 1.5 ml microfuge tubes in 80% ethanol (400 μl per 50 mg tissue) using a miniature polypropylene pestle (Kontes Glass Co., Vineland, NJ). Tubes were centrifuged at 18,000×*g* for 5 min before HPLC of the supernatant. HPLC was performed as described previously (Graham, 1991a). Injections of 20 μl were made onto an analytical C18 reverse phase column (LiChrosorb RP-18 10 μ, 250 mm×4.6 mm, Alltech Associates, Deerfield, Illinois), which was eluted at 1.5 ml/min with a linear gradient of 0–55% acetonitrile in water (27 min), followed by a 2 min wash with 100% acetonitrile and a 2 min wash with water. Standards were either purchased (coumestrol, daidzein, daidzin, genistein) or purified and authenticated by UV spectra and NMR and/or mass spectral analysis (MGD, MGG, glycitein, formononetin, coumestrol, glyceollin). Individual peaks were quantified by periodically running standards and determining the appropriate integration response factors. Glyceollin concentrations reported are the total of the peaks for all glyceollin isomers. Each HPLC analysis takes 30 min and large numbers of samples are analyzed for any given experiment. For this reason, rather than analyzing individual replicates, we take the approach of pooling 10 replicate cotyledon subsamples (Graham and Graham, 1991b, 1996). Performed in this way, the HPLC analysis itself has very low variability (see Graham, 1991a or statistics). Statistical analyses are then performed as the standard error between experiments, where somewhat greater sources of variation are seen (Graham, 1991a).

Acknowledgements

We would like to particularly thank Mr. Ted Bean of Valent Technologies for helpful discussions and suggestions throughout the work. We would also like to acknowledge initial aspects of the characterization of

the effects of rose bengal done by Ryan Poling in our laboratory. Funding for this project was provided by gift monies from Valent Technologies and by an Industry Matching Grant from the Competitive Research Enhancement Grant Program (CREGP) at the Ohio Agricultural Research and Development Center (OARDC), the Ohio State University. This latter grant involved equal matching funds from OARDC and Valent Technologies. Additional salaries and research support were also provided by State and Federal Funds appropriated to the OARDC.

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