Nitric Oxide Synthase-Mediated Phytoalexin Accumulation in Soybean Cotyledons in Response to the Diaporthe phaseolorum f. sp. meridionalis Elicitor

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Phytoalexin biosynthesis is part of the defense mechanism of soybean (Glycine max) plants against attack by the fungus Diaporthe phaseolorum f. sp. meridionalis (Dpm), the causal agent of stem canker disease. The treatment of soybean cotyledons with Dpm elicitor or with sodium nitroprusside (SNP), a nitric oxide (NO) donor, resulted in a high accumulation of phytoalexins. This response did not occur when SNP was replaced by ferricyanide, a structural analog of SNP devoid of the NO moiety. Phytoalexin accumulation induced by the fungal elicitor, but not by SNP, was prevented when cotyledons were pretreated with NO synthase (NOS) inhibitors. The Dpm elicitor also induced NOS activity in soybean tissues proximal to the site of inoculation. The induced NOS activity was Ca2+/ and NADPH-dependent and was sensitive to the NOS inhibitors N6-nitro-l-arginine methyl ester, aminoguanidine, and l-N6-(iminoethyl) lysine. NOS activity was not observed in SNP-elicited tissues. An antibody to brain NOS labeled a 166-kD protein in elicited and nonelicited cotyledons. Isoflavonoids (daidzein and genistein), pterocarpans (glyceollins), and flavones (apigenin and luteolin) were identified after exposure to the elicitor or SNP, although the accumulation of glyceollins and apigenin was limited in SNP-elicited compared with fungal-elicited cotyledons. NOS activity preceded the accumulation of these flavonoids in tissues treated with the Dpm elicitor. The accumulation of these metabolites was faster in SNP-elicited than in fungal-elicited cotyledons. We conclude that the response of soybean cotyledons to Dpm elicitor involves NO formation via a constitutive NOS-like enzyme that triggers the biosynthesis of antimicrobial flavonoids.

Plants respond to attack by pathogens by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spreading. Such defenses include rapid and localized cell death (hypersensitive response, HR) and the accumulation of antimicrobial compounds known as phytoalexins, which play an important role in many plant-pathogen incompatible interactions (Paxton, 1991). Glyceollins are the major pterocarpans produced in soybean (Glycine max) plants. The timing and magnitude of glyceollin accumulation differ markedly in compatible and incompatible interactions, but are consistent with their proposed role in race-specific resistance (Graham et al., 1990; Paxton, 1991; Graham, 1995 and refs. therein).

Glyceollins are pterocarpan derivatives from the phenylpropanoid pathway and they occur as a series of isomers (I–IV; Paxton, 1995). Phe ammonia-lyase (PAL), the first enzyme in this pathway, catalyzes the nonoxidative deamination of the amino acid Phe to produce cinnamate, the first structure with a phenylpropanoid skeleton in this biosynthetic pathway (Paxton, 1991). Daidzein, a dihydroxylated isoflavone, is the immediate precursor of the glyceollins (Bailey and Mansfield, 1982; Paxton, 1995). Genistein (trihydroxylated isoflavone) is another antimicrobial isoflavonoid that is accumulated in soybean tissues during incompatible reactions (Dixon et al., 1995; Dakora and Phillips, 1996).

Various substances can induce glyceollin production in soybean tissues, including fungal elicitors, oligogalacturonides, H2O2, and salicylic acid (Degousée et al., 1994; Gomez et al., 1994; Graham, 1995; Knorzer et al., 1999). Recently, nitric oxide (NO) was shown to induce the expression of genes related to phytoalexin biosynthesis in soybean and tobacco (Nicotiana tabacum) cells in culture (Delledonne et al., 1998; Durner et al., 1998). This nitrogen radical also induces phytoalexin accumulation in potato (Solanum tuberosum) tuber tissues (Noritake et al., 1996), potentiates the induction of HR in soybean cells by reactive oxygen intermediates (Delledonne et al., 1998), and induces death with the hallmarks of apoptosis in Kalanchoë daigremontiana, Taxus brevifolia (Pedroso et al., 2000a, 2000b), Arabidopsis (Clarke et al., 2000), and Citrus sinensis (Saviani et al., 2002) cells. These observations suggest the existence of an NO-mediated signaling pathway in plant defense responses to pathogens.

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In mammalian cells, the main mechanism of NO generation is through the enzyme NO synthase (NOS), which metabolizes L-Arg to L-citrulline with the formation of NO (Pollock et al., 1991). Although no NOS gene has been found, various studies have suggested the occurrence of a NOS-like enzyme in plant cells. NOS-like activity, measured by L-citrulline formation from L-Arg and/or by its sensitivity to mammalian NOS inhibitors, has been detected in several plant species (Cueto et al., 1996; Ninnemann and Maier, 1996; Delledonne et al., 1998; Durner et al., 1998; Barroso et al., 1999; Ribeiro et al., 1999). In addition, using antibodies produced against mammalian NOS isoforms, NOS-like proteins have been localized in the cytosol and nucleus of maize (Zea mays) root tips (Ribeiro et al., 1999), and in peroxisomes and chloroplasts of pea (Pisum sativum) leaves (Barroso et al., 1999). Western-blot analysis showed that the NOS immunoreactive proteins in maize and pea extracts had a molecular mass of approximately 166 and 130 kD, respectively (Barroso et al., 1999; Ribeiro et al., 1999), which is in the same molecular mass range as described for mammalian NOS (Pollock et al., 1991). The involvement of a NOS-like enzyme in plant defense responses to pathogens has also been suggested (Delledonne et al., 1998; Durner et al., 1998). Increased levels of NOS activity were observed in tobacco plants resistant to tobacco mosaic virus (Durner et al., 1998) and Ralstonia solanacearum (Huang and Knopp, 1998). Consistent with these observations, NOS inhibitors compromised the responses of Arabidopsis leaves to attack by Pseudomonas syringae (Delledonne et al., 1998).

Soybean stem-canker disease represents one of the greatest limitations to the cultivation of this crop in Brazil. Intense efforts have been made to develop soybean cultivars resistant to the fungus Diaporthe phaseolorum f. sp. meridionalis (Dpm), the causal agent of this disease. However, very little is known about the metabolic alterations that confer resistance to Dpm. One of the experimental approaches used by the Agronomical Institute of Campinas in Brazil to select for resistance to Dpm has been the observation of a red color developed in the stem of soybean plants inoculated with Dpm. Resistant cultivars develop an intense reddish color at the site of fungal inoculation, whereas in susceptible cultivars the color develops later, when the disease has already manifested itself (N.R. Braga, personal communication). This color in soybean tissues results from the accumulation of certain glyceollin precursors following exposure to various biotic and abiotic factors (Ingham et al., 1981; Zähringer et al., 1981), and its intensity is proportional to the phytoalexin content (Ayers et al., 1976b). Glyceollin precursors that have a red coloration include glycinol and the isoprenylated compounds glyceolidon I and II (Ingham et al., 1981; Zähringer et al., 1981).

Considering that phytoalexin production seems to be involved in the defense mechanism of soybean plants against attack by Dpm, and that NO may participate in plant defense responses, we have examined the involvement of an NOS-like enzyme in the activation of phenylpropanoid biosynthesis in soybean cotyledons treated with Dpm elicitor. We also compared the time course of the effects of the Dpm elicitor and an NO donor compound on the formation of phenylpropanoid intermediates in soybean cotyledons. In addition, the induction of NOS-like activity and the effect of NOS inhibitors on this protein were analyzed. Our results demonstrate the involvement of a constitutive Ca2+-dependent NOS-like enzyme in the soybean defense response to Dpm elicitor.

RESULTS

Flavonoids Elicited in Response to Dpm and Sodium Nitroprusside (SNP)

The effect of SNP, an NO donor, on phytoalexin accumulation in soybean was compared with that induced by a crude Dpm extract. A soybean cultivar resistant to Dpm (IAC-18) was treated with SNP or Dpm elicitor for different periods, using the cotyledon assay. After treatment, the diffusates were analyzed for phytoalexin content by HPLC with detection at 286 nm. As shown in Figure 1, when cotyledons were elicited with Dpm extract, the isoflavones daidzein and genistein were detected after 6 h of incubation, whereas with SNP, these metabolites accumulated earlier, being detected just 3 h after the beginning of treatment. Glyceollins, the daidzein-derived pterocarps, were detected only after a 12-h incubation with Dpm elicitor, and their production increased up to 20 h. For SNP, only daidzein and genistein were detected up to 12 h after stimulation, and glyceollins appeared only 20 h after elicitation. The Dpm extract stimulated the accumulation of a greater variety of metabolites than did SNP, the greatest difference being observed after 20 h of treatment (Fig. 1).

Figure 2 compares the time course for the production of genistein, daidzein, and glyceollins in Dpm- and SNP-elicited soybean cotyledons. In both treatments, maximal genistein production occurred after 12 h and decreased at 20 h. In contrast, the accumulation of daidzein and glyceollins showed different patterns for the two treatments. Maximal daidzein production occurred after 12 h of incubation with the Dpm elicitor. After this period, daidzein decreased and the glyceollins began to increase. The differences in the proportion of these two compounds represented the conversion of daidzein into glyceollins during prolonged incubations. Maximal production of daidzein also occurred after a 12 h of incubation with SNP, and the levels of this precursor were unchanged after 20 h. The levels of glyceollins also
remained low, indicating a limited conversion of daidzein into glyceollins.

The diffusates of Dpm- and SNP-elicited cotyledons were also analyzed for the presence of the flavonones apigenin and luteolin because a spectral analysis showed a high \( A_{350} \), a wavelength typical of these compounds. Both elicitors induced maximal accumulation of luteolin after 12 h, which then de-
creased during further incubation (Fig. 3). The response to SNP preceded that of Dpm because luteolin was already detected after 3 h of elicitation. Apigenin was induced principally by Dpm and began to appear after 12 h of elicitation, its content being higher than that detected after 20 h of treatment with SNP.

Overall, the response of soybean cotyledons to SNP was faster (flavonoid production was already detected after 3 h) and more intense (higher production) than that to Dpm. However, SNP was not as efficient as Dpm in stimulating the biosynthesis of metabolites such as glyceollins and apigenin.

NO Dose-Dependent Phytoalexin Accumulation

Phytoalexin production in Dpm-elicited cotyledons was compared with that induced by different concentrations of SNP. In these assays, cultivars susceptible (IAC-14) and resistant (IAC-18) to Dpm were used, and phytoalexin accumulation was estimated as the overall production of phenolics at 286 nm. As shown in Figure 4, phytoalexin production in soybean cotyledons induced by SNP was dose dependent and, at 10 mM, was similar to that induced by the fungal elicitor. No phytoalexin production was observed when SNP was replaced by ferricyanide, a structural analog of SNP, which is not an NO donor (data not shown). These results suggest that the NO radical generated by SNP was the signaling molecule involved in the stimulation of phytoalexin production in soybean cotyledons. There were no significant differences among the phytoalexin responses of susceptible (IAC-14) and resistant (IAC-18) cultivars after exposure to Dpm elicitor or SNP as estimated by the $A_{286}$ (Fig. 4) and by HPLC analysis (data not shown).
Effect of NOS Inhibitors on Phytoalexin Production

When soybean cotyledons (IAC-14 and IAC-18 cultivars) were pretreated with the NOS inhibitors N⁶⁷-G-nitro-L-Arg methyl ester (L-NAME) or aminoguanidine (AMG) for 5 h and were subsequently elicited with the Dpm elicitor for 20 h, lower amounts of phytoalexins were produced (50% inhibition) compared with controls not preincubated with the NOS inhibitors (Fig. 5). HPLC analysis showed that the decrease in A₂₈₆ was due mostly to a reduced content of daidzein, genistein, and glyceollins (data not shown). L-NAME is a structural analog of L-Arg that competitively inhibits inducible forms and irreversibly inhibits constitutive isoforms of NOS in animals (Baylis et al., 1995). AMG, although not a structural analog of L-Arg, is an irreversible inhibitor of both isoforms, but preferentially of the inducible form (Laszlo et al., 1995; Wolff and Lubeskie, 1995). A low uptake of the inhibitors by the tissues, the absence of the inhibitors during elicitation, and a lower affinity of the putative plant NOS enzyme for these compounds compared with that of animals could explain the incomplete inhibition of phytoalexin accumulation in Figure 5. In contrast, pretreating soybean cotyledons with the NOS inhibitors did not prevent phytoalexin formation in response to SNP elicitation (Fig. 5). These results suggested that NO produced by a NOS-like enzyme could be involved in the signaling pathway leading to phytoalexin induction in soybean tissues in response to Dpm elicitor.

Induction of NOS Activity in Elicited Cotyledons

Soybean cotyledons (cultivar IAC-18) were elicited with the fungal elicitor or with SNP for different periods, and the tissues were then prepared to determine NOS activity using the Arg-citrulline assay, as described in “Materials and Methods.” This is a more reliable method for measuring NOS activity than is NO quantification because NO can be generated in plants by routes other than NOS (see Wendehenne et al., 2001; Salgado et al., 2002). The difference between the total L-[¹⁴C]citrulline formation and that inhibited by simultaneous incubation with L-NAME and AMG was used to estimate NOS activity. The time course for NOS activity showed that cotyledon elicitation with the Dpm extract induced maximal enzyme activity after 6 h of incubation, which corresponded to 3 pmol L-[¹⁴C]citrulline h⁻¹ mg⁻¹ when 1 μM L-[¹⁴C]Arg was used (Fig. 6). In a converse manner, SNP did not evoke NOS activity in cotyledon tissues (Fig. 6).

To further characterize the NOS activity induced in soybean cotyledon tissues, the Ca²⁺ and NADPH dependence and the effect of l-N⁶-(iminoethyl) Lys (l-NIL) on L-[¹⁴C]citrulline formation after 6 h of elicitation were examined using 10 μM L-[¹⁴C]Arg. l-NIL is a potent inhibitor of animal NOS, being about 28 times more selective for inducible NOS than for the constitutive isoform (Connor et al., 1995; Moore et al., 1996). As shown in Figure 7, L-[¹⁴C]citrulline formation in control and SNP-elicited tissues was 4.5 and 4.0 pmol min⁻¹ mg⁻¹, respectively. These values did not change in the presence of EGTA or l-NIL, and they were not affected when NADPH was absent from the reaction mixture. These results indicate that there was a basal L-[¹⁴C]citrulline incorporation in these conditions that
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Figure 6. NOS activity in elicited soybean cotyledons (cultivar IAC-18). Tissues from soybean cotyledons elicited with Dpm elicitor (equivalent to 20 μg of Glc) or SNP (10 mM) for different times were assayed for NOS activity as described in “Materials and Methods” using 1 μM L-[U-14C]Arg (350 μCi μmol⁻¹). NOS activity was determined as the difference between the total L-[U-14C]citrulline production and that observed in the presence of the NOS inhibitors L-NAME and AMG, both at 3 mM. The points represent the mean ± s.e. of two experiments each done in triplicate. *P < 0.05 (by Student’s t test) compared with the control (time 0).

Figure 7. NOS activity of elicited soybean cotyledons is sensitive to L-NIL and is Ca²⁺ and NADPH dependent. Tissues from soybean cotyledons elicited with Dpm extract or SNP for 6 h were assayed for NOS activity using 10 μM L-[U-14C]Arg (70 μCi μmol⁻¹). L-[U-14C]citrulline formation was determined in complete reaction medium (Control), and in the presence of 3 mM L-NIL (+L-NIL), 2 mM EGTA (-Ca²⁺), and without NADPH (-NADPH). The bars represent the mean ± s.e. of two experiments each done in triplicate. *P < 0.05 (by Student’s t test) compared with the total L-[U-14C]citrulline production obtained in the presence of all NOS cofactors.

Figure 8. Immunoblot of proteins solubilized from soybean cotyledons probed with antibodies raised against brain NOS. Concentrated solubilized proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with rabbit anti-brain NOS antibodies. Lanes, from left to right, contain proteins from the following sources: (1) detergent extract from brain (20 μg) and 150 μg of solubilized soybean cotyledons that was not elicited (2; control) or was elicited with SNP (3; 10 mM) or Dpm (4; equivalent to 20 μg of Glc). The arrows indicate the positions of the protein standards (indicated in kilodaltons). The result shown is representative of three similar experiments.
change upon treatment with SNP or Dpm. These results suggest that the putative NOS protein involved in phytoalexin accumulation in soybean-elicited cotyledons was a constitutive enzyme, and they agree with the Ca\(^{2+}\) dependence and slight increase in NOS activity seen after elicitation with Dpm. The 166-kD protein detected in cotyledons cross-reacted very weakly with antibodies raised against mouse macrophage NOS (result not shown).

DISCUSSION

The results of this study indicate the involvement of an NOS-like enzyme in the response of soybean cotyledons to the elicitor extracted from spores of Dpm, the causal agent of stem canker disease. Phytoalexins accumulated in soybean cotyledons elicited with the Dpm extract and with the NO donor SNP. Dpm-induced phytoalexin production was inhibited when cotyledons were pretreated with mammalian NOS inhibitors, whereas these same inhibitors did not affect flavonoid accumulation when the cotyledons were elicited with SNP. Consistent with these observations, Dpm elicitation induced NOS activity in soybean tissues, but no such activity was observed in SNP-elicited tissues. In Dpm-elicited cotyledons, the induction of NOS activity preceded the activation of isoflavone (daidzein and genistein) biosynthesis. Therefore, maximal NOS activity occurred when isoflavone production was still low. In contrast, when soybean cotyledons were elicited with SNP, isoflavones accumulated earlier. As observed for isoflavones, the time course of luteolin production was faster after elicitation with SNP than after treatment with Dpm. These results agree with the hypothesis that the Dpm elicitor activated the phenylpropanoid pathway through an NOS-like enzyme. Thus, in elicitation with Dpm, phytoalexin accumulation occurred only after NOS activation, whereas the time course for the production of these metabolites was faster when NO was provided directly.

Phytoalexin production in several defense responses against attack by pathogens is regulated at the level of the enzymes PAL, chalcone synthase (CHS), and chalcone isomerase (Dixon and Paiva, 1995). The present observations that isoflavones (daidzein and genistein), pterocarps (glyceollins), and flavones (apigenin and luteolin) accumulated in Dpm-elicited cotyledons via NOS activity suggest that the NO produced endogenously in this interaction could stimulate phytoalexin biosynthesis by regulating the expression of the initial enzymes of the phenylpropanoid pathway. Delledonne et al. (1998) reported that NO\(^{-}\)-nitro-L-Arg, a structural analog of L-Arg, prevented the accumulation of PAL transcripts in soybean cell cultures inoculated with an avirulent strain of *Pseudomonas syringae*. This treatment also inhibited the transcription of CHS, the first enzyme of the phenylpropanoid pathway branch. These authors also showed that SNP induced the expression of the *PAL* and *CHS* genes in soybean cells in culture. NO donors such as S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine, and the injection of mammalian NOS in tobacco seedlings, were able to induce the expression of *PAL* genes (Durner et al., 1998).

Our results also show that although NO was effective in stimulating the initial steps of the phenylpropanoid pathway, this radical was apparently not the only signaling molecule engaged in the late conversion of daidzein into glyceollins. As shown in Figures 1 and 2, the conversion of daidzein into glyceollins was more synchronized in Dpm- than in SNP-elicited tissues, despite the high effectiveness of the NO donor in eliciting the accumulation of the precursor daidzein. In soybean, as in a number of other host species, there is good evidence that reactive oxygen species (ROS) play a major role in the initiation of the resistance against pathogens (Bolwell and Wojtaszek, 1997; Knorzer et al., 1999). For instance, hydrogen peroxide (H\(_2\)O\(_2\)) is an effective inducer of phytoalexin accumulation in soybean hypocotyls (Degoussé et al., 1994; Gomez et al., 1994) and in suspension cultures of cells (Apostol et al., 1989). Furthermore, a synergistic interaction between NO and H\(_2\)O\(_2\) has been reported for the induction of cell death in soybean cell cultures (Delledonne et al., 2001). In preliminary experiments, we observed no such synergistic interaction during phytoalexin induction in cotyledons of the soybean cultivar IAC-18. H\(_2\)O\(_2\), tested in the concentration range of 2 mM to 1 mM, was ineffective in inducing phytoalexin production. Moreover, H\(_2\)O\(_2\) dose dependently reduced the phytoalexin accumulation induced by SNP (L.V. Modolo, M.R. Braga, and I. Salgado, unpublished data). In accordance with these preliminary observations, Delledonne et al. (1998) and Durner et al. (1998) found that although NO acted synergistically with ROS to potentiate cell death, it also acted independently of ROS to induce the expression of defense-related genes, including the *PAL* gene. Previous studies have shown that the oxidative burst and the loss of cell viability are not directly linked to phytoalexin induction in soybean, cotton (*Gossypium hirsutum*), and carrot (*Daucus carota*) cells (Davis et al., 1993; Koch et al., 1998). A detailed analysis of the oxidative burst in soybean also showed that although H\(_2\)O\(_2\) plays a central role as an “orchestrator” of the HR, it was not very effective in inducing the accumulation of transcripts for PAL and CHS (Tenhaken et al., 1995). In contrast, Degoussé et al. (1994) investigated the relationship between oxidative processes and phytoalexin biosynthesis and demonstrated that H\(_2\)O\(_2\) lead to significant glyceollin elicitation in soybean hypocotyls and radicles. Together, these results and those of our preliminary experiments indicate that the uncoupling of HR from NO-mediated phytoalexin induction by Dpm elicitor in cotyledons of the soybean.
cultivar IAC-18 is not unlikely. Thus, in our system, 
$H_2O_2$ is apparently not the signal triggered by the 
Dpm elicitor that acts synergistically with NO to 
activate specific defense genes involved in glyceollin 
synthesis. On the other hand, glyceollin accumula-
tion in soybean tissues is affected by genotype, age, 
and the developmental state of specific organs, and is 
under strong regulation by several endogenous con-
ditions (Abbasi and Graham, 2001; Abbasi et al., 2001 
and refs. therein). Thus, the elucidation of the signal-
ing molecules, which act in concert with NO during 
elicitation by Dpm, requires further investigation.
The NOS-like enzyme described here showed char-
acteristics similar to most other such enzymes found 
so far in plants (Wendehenne et al., 2001) because it 
was Ca$^{2+}$ dependent, partially sensitive to inhibitors 
of different mammalian NOS isoforms, and constitu-
tively expressed. The specific activity of this putative 
enzyme was in the same range (3–4 pmol L-citrulline 
h$^{-1}$ mg$^{-1}$) as that described for maize tissues using 
the same assay technique to measure NOS activity in 
crude soluble extracts (Ribeiro et al., 1999). When a 
potent NOS inhibitor was used to characterize the 
specific NOS activity, the estimated values for soy-
bean cotyledons increased by approximately one or-
der of magnitude (to 5.8–7.8 pmol L-citrulline min$^{-1}$ 
mg$^{-1}$), reaching values similar to those found by 
Cueto et al. (1996) in soluble extracts of lupin (Lupi-
num albus) roots (2.5 pmol L-citrulline min$^{-1}$ mg$^{-1}$) at 
an equivalent L-Arg concentration. If NO is produced 
by a particular cell type and/or organelle, the mea-
sured NOS activity may be significantly higher than 
that estimated from crude soluble extracts, as re-
ported for purified peroxisomes of pea leaves (5.6 
nmol L-citrulline min$^{-1}$ mg$^{-1}$; Barroso et al., 1999). 
The NOS-immunoreactive protein detected in soy-
bean cotyledons of the cultivar IAC-18 had a molec-
ular mass of 166 kD, similar to that described in 
maize tissues (Ribeiro et al., 1999), but was more 
reactive with antibody to brain NOS. However, the 
characteristics of the soybean enzyme differed from 
those of the putative NOS protein detected in pea 
peroxisome, which has a molecular mass of 130 kD 
and is immuno-related with the murine inducible 
NOS, despite being Ca$^{2+}$ dependent and constitu-
tively expressed (Barroso et al., 1999).

Although soybean resistance to Dpm in the field 
appears to be related to phytoalexin production, in 
this study, there were no differences in the phytoto-
exin responses between the resistant and susceptible 
cultivars induced by Dpm elicitor or SNP. These 
experiments were done using detached cotyledons 
kept under controlled conditions. Factors that could 
fluence the plant response to pathogens under field 
conditions, such as high temperature and humidity 
(Classen and Ward, 1985; Graham, 1995; Smith, 
1996), were not considered here. Furthermore, plant 
resistance to a pathogen is a multifactorial phenom-
enon that includes an arsenal of defensive reactions 
(Hahn et al., 1989). In view of this and the fact that 
the mechanisms involved in the resistance of soybean 
plants to stem canker fungus are still poorly under-
stood, other responses in addition to phytoalexin 
production could be involved in the resistance of 
soybean to attack by Dpm in the field.

Whereas the antimicrobial function of phytoalexins 
is well documented, the role of flavones such as apig-
egin and luteolin in plant defense against pathogens 
remains to be established. The anti-inflammatory ac-
tivity of these molecules in mammalian cells has been 
attributed to their antioxidant property, as well as to 
a regulatory action on NO production through their 
capacity to inhibit NOS expression (Kim et al., 1999).

Thus, it seems reasonable to suppose a similar action 
for apigenin and luteolin in controlling the level of NO 
at the site of infection in soybeans. Preliminary results 
from our laboratory have shown that diffusates from 
soybean cotyledons treated with Dpm elicitor or SNP 
inhibited NO production in LPS- and/or IFN-y-
activated macrophages (L.S. Scuro and I. Salgado, un-
published data).

There is increasing evidence that NO may influ-
ence various developmental processes and that it has 
a role in plant defense responses to pathogens (Sal-
gado et al., 2002). Our data strongly suggest that 
NO-induced phytoalexin production in soybean coty-
ledons is mediated by an NOS-like enzyme and that 
the plant response to attack by Dpm is likely to 
involve this signaling pathway.

MATERIAL AND METHODS

Plant Material

Soybean (Glycine max) seeds of the cultivars IAC-14 and IAC-18, suscep-
tible and resistant to the fungus Diaporthe phaseolorum f. sp. meridionalis 
(Dpm), respectively, were provided by Dr. Nelson R. Braga (Instituto Ag-
ronômico de Campinas, Campinas, São Paulo, Brazil). The cotyledons used 
were detached from 7- to 8-d-old seedlings grown in vermiculite at room 
temperature in a greenhouse.

Preparation of the Elicitor

Elicitor from Dpm (strain 8498; kindly supplied by Dr. Margarida F. Ito, 
Instituto Agronômico de Campinas) was obtained by autoclaving (121°C, 
1.5 atm, 30 min) aqueous spore suspensions of 30- to 40-d-old cultures 
grown in potato-dextrose-agar (Merck, Darmstadt, Germany) media in the 
dark at room temperature. The autoclaved suspension was centrifuged at 
10,000g for 6 min, and the pellet was discarded. Total carbohydrates were 
quantified in the supernatant by the phenol-sulfuric procedure (Dubois et 
al., 1956) using C1C as standard.

Elicitation Assay

The production of phytoalexins was evaluated using the soybean coty-
ledon assay (Ayers et al., 1976a). A small section (l.d. = 1.0 cm) was 
removed from the adaxial surface of each cotyledon and the wounded 
surface was treated with 50 μL of test solution, as specified in the figure 
legends. The cotyledons were kept in a petri dish containing water-absorbed 
filter paper in the dark at 20°C for 20 h, unless stated otherwise. The 
cotyledons were washed with deionized water (1 mL per cotyledon), and 
phytoalexin production was estimated in the diffusates by spectrophotom-
etry at 286 nm.
Analysis of Glyceollins, Daidzein, and Genistein

Diffusates from the soybean cotyledon assay (20 cotyledons) were extracted with ethyl acetate, as described by Keen (1978). Organic fractions were evaporated to dryness and the residues were then solubilized in methanol and analyzed by HPLC in a chromatograph (DX500; Dionex, Sunnyvale, CA) fitted with a diode array detector. The samples were run on a 4.6 mm × 250 mm column (ODS C18; Zorbax, Chadds Ford, PA) with a linear gradient from 20% to 60% (v/v) acetonitrile in 0.1% (v/v) trifluoro- acetic acid (0.7 mL min⁻¹) according to Pelicice et al. (2000). Peak area versus compound concentration was plotted for various concentrations of available standards. Daidzein and genistein were identified by calibration with authentic standards (Sigma, St. Louis). Diffusates from the Williams 82 soybean cultivar from Illinois Foundation Seeds (Champaign; kindly supplied by Dr. M.G. Hahn, University of Georgia, Athens) were used as the glyceollin standard. All compounds were monitored at 286 nm.

Analysis of Apigenin and Luteolin

Aliquots of the diffusates from the soybean cotyledon assay (20 cotyle- dons) were evaporated, resuspended in methanol, and analyzed by HPLC in a chromatograph (Shimadzu, Kyoto) fitted with a UV-VIS detector. The samples were analyzed using a 4.6 mm × 250 mm column (CLS ODS C18; Shimadzu) with a mobile phase of methanol in 10% (v/v) formic acid, in a method modified from Garcia-Viguera et al. (1998). Elution was done at a flow rate of 1 mL min⁻¹ using a gradient that started with 50% (v/v) methanol and increased to 60% (v/v) at 10 min, followed by 100% (v/v) methanol for 3 min and then by holding at 50% (v/v) methanol for 4 min. Peak area versus compound concentration was plotted for various concentrations of apigenin and luteolin standards (Sigma). The runs were monitored at 336 nm and 350 nm for apigenin and luteolin, respectively.

Determination of NOS Activity

The NOS activity of cotyledon tissues treated with 50 μL of test solution was determined by the citrulline assay method modified from Rees et al. (1995). The conversion of l-[L-14C]Arg to l-[L-14C]citrulline was determined in samples with and without the NOS inhibitor l-NAME (Sigma), AMG (RBI, Natick, MA), or l-NIL (Calbiochem, La Jolla, CA). In brief, 1.0 g of tissue, together with 50 mg of polyvinylpyrrolidone, was homoge- nized in 1.0 mL of cooled extraction buffer (50 mM Tris, pH 7.4, containing 320 mM Suc, 10 μg mL⁻¹ leupeptin, 10 μg mL⁻¹ soybean trypsin inhibitor, 2 μg mL⁻¹ aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM dihio- theritol, and 10 mM reduced glutathione). The homogenate was centrifuged at 10,000g for 10 min at 4°C. Forty microliters of the supernatant was added to 100 μL of the assay buffer [40 mM HEPES, pH 7.2, containing 10 μM FAD, 10 μM FMN, 1 mM dihiotheithritol, 1.25 mM CaCl₂, 50 μM L-NAME], 2 μM l-arginine, 0.7 mL min⁻¹ tetrahydrobiopterin, 10 μg mL⁻¹ calmodulin, 1 mM β-NADPH, and 1 μM (50 nCi) or 2 μM (100 nCi) l-[L-14C]Arg; Amersham Pharmacia Biotech, Buckinghamshire, UK). After incubation for 30 min at room temperature, the reaction was stopped by adding 1 mL of Dowex-AG 50W suspended in 100 mM HEPES containing 10 mM EDTA (1:1.5, v/v). The resin was removed by centrifugation (10,000g for 10 min at 18°C). Four hundred microliters of this supernatant was added to 3 mL of scintillation liquid and was counted in a counter (LS 6000; Beckman, Fullerton, CA). Protein content was deter- mined by the Coomassie Blue-binding method (Bradford, 1976) using pro- tein reagent (Bio-Rad, Hercules, CA), and bovine serum albumin as standard.

Western Blotting

Western blotting of polypeptides solubilized from cotyledon tissues was done as described by Ribeiro et al. (1999) using a polyclonal antibody to human neuronal NOS (2 μg mL⁻¹) raised in rabbits (BD Transduction Laboratories, Lexington, KY).

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