# Light is Essential for Degradation of Ribulose-1,5-Bisphosphate Carboxylase-Oxygenase Large Subunit During Sudden Death Syndrome Development in Soybean

J. Ji[1], M. P. Scott[1,2], and M. K. Bhattacharyya[1,2]

[1] Interdepartmental Genetics Program and Department of Agronomy, Iowa State University, Ames, Iowa 50010, USA
[2] USDA-ARS, Corn Insects and Crop Genetics Research Unit, Ames, Iowa 50014, USA

Received: December 28, 2005; Accepted: April 3, 2006

**Abstract:** *Fusarium solani* f. sp. *glycines* (Fsg) has been reported to produce at least two phytotoxins. Cell-free *Fsg* culture filtrates containing phytotoxins have been shown to develop foliar sudden death syndrome (SDS) in soybean. We have investigated the changes in protein profiles of diseased leaves caused by cell-free *Fsg* culture filtrates prepared from *Fsg* isolates. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was conducted to investigate the protein profiles of diseased and healthy leaves. An approximately 55 kDa protein was found to be absent in diseased leaves. Matrix-assisted laser desorption-ionization time-of-flight mass spectrometric analyses and a database search revealed that the missing protein is the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit, which is involved in carbon assimilation and photorespiration. This result was confirmed by Western blot experiments. We have shown that light is essential for disappearance of the Rubisco large subunit initiated by cell-free *Fsg* culture filtrates. The disappearance of the protein is fairly rapid and occurs within 24 h, presumably due to degradation. Cell-free, *Fsg* culture-induced degradation of the Rubisco large subunit was accompanied by accumulation of reactive oxygen species under light conditions. Terminal deoxynucleotidyl transferase-mediated nick end labelling experiments suggested that programmed cell death was initiated in leaves of seedlings fed with cell-free *Fsg* culture filtrates. These results suggest that, in the presence of light, *Fsg* culture filtrates containing phytotoxins cause degradation of the Rubisco large subunit and accumulation of free radicals and, thereby, initiate programmed cell death leading to foliar SDS development in soybean.

**Key words:** *Fusarium solani* f. sp. *glycines, Fusarium virguliforme,* soybean, SDS, Rubisco, MALDI-TOF-MS, TUNEL.

***Abbreviations:***

| | |
|---|---|
| *Fsg:* | *Fusarium solani* f. sp. *glycines* |
| SDS: | sudden death syndrome |
| PAGE: | polyacrylamide gel electrophoresis |
| Rubisco: | ribulose 1,5-bisphosphate carboxylase/oxygenase |
| MALDI-TOF-MS: | matrix-assisted laser desorption-ionization time-of-flight mass spectrometry |
| TUNEL: | terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling |

Plant Biol. 8 (2006): 597–605
© Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2006-924175 · Published online July 5, 2006
ISSN 1435-8603

## Introduction

Sudden death syndrome (SDS) of soybean is caused by the fungal pathogen, *Fusarium solani* f. sp. *glycines* (*Fsg*). The fungus has recently been renamed *Fusarium virguliforme* (Aoki et al., 2003). SDS has been detected in Argentina, Brazil, and the USA (Rupe and Hartman, 1999). In the United States, the disease has been reported in more than ten states and the estimated average annual crop loss from the disease has been valued at around US$ 100 million (Wrather et al., 2001). SDS development is affected by environmental factors such as rainfall, soil fertility, and planting date. It is also affected by genotype and soybean cyst nematode infection (Rupe et al., 1991, 1993; Chang et al., 1997).

SDS is characterized by root rot, crown necrosis, premature defoliation, and flower and pod abortion (Rupe, 1989). *Fsg* is a soil-borne fungal pathogen (Roy et al., 1997). In susceptible soybean cultivars, the infected roots show crown necrosis and rotting symptoms within 2 weeks of planting. *Fsg* produces a large number of asexual spores on the surface of the severely infected soybean roots. SDS foliar symptoms, also known as leaf scorch, develop slowly. About 1 or 2 months following planting, interveinal chlorotic spots appear in infected susceptible plants. The spots develop into necrotic or chlorotic streaks and, in the late stage, premature defoliation occurs (Melgar and Roy, 1994). *Fsg* has never been isolated from aboveground portions of the soybean plant. The foliar symptoms are therefore presumably induced by *Fsg* phytotoxins (Roy et al, 1989; Rupe, 1989; Strobel, 1982). Under greenhouse conditions, leaf scorch resistance is governed by a single gene in the cultivar, Ripley, and by two genes in P9451 (Stephens et al., 1993; Ringler and Nickell, 1996). Under field conditions, SDS resistance is partial and governed by multiple genes (Chang et al., 1996, 1997; Hnetkovsky et al., 1996; Meksem et al., 1999; Njiti et al., 1996, 1997, 2002). Several quantitative trait loci for SDS resistance have been identified (Iqbal et al., 2002; Njiti et al., 2002).

In plants, programmed cell death (PCD) is initiated during development, senescence, exposure to phytotoxins and under stresses caused by high ozone concentration and pathogen attack (Buchanan et al., 2000; Greenberg, 1996; Yao et al., 2001; Pasqualini et al., 2003; Levine et al., 1996). There are several host-selective toxins that trigger PCD leading to necrosis (Wolpert et al., 2002). For example, victorin, a toxin produced by *Cochliobolus victoriae,* causes a mitochondrial oxidative burst and PCD in oat (Yao et al., 2001; Navarre and Wolpert, 1999). AAL toxin produced by *Alternaria alternata* f. sp. *lycopersici* causes PCD and the *Alternaria* stem canker disease in tomato (Lincoln et al., 2002). AM toxins I – III, produced by the *Alternaria alternata,* are host-specific. AM toxins inhibit $CO_2$ fixation and cause leaf necrosis. Chloroplasts are the primary target of AM toxins that cause leaf spot disease in apple (Miyashita et al., 2003). The wheat pathogen, *Pyrenophora tritici-repentis,* secretes at least two host-selective toxins, ToxA and ToxB, encoded by single genes. A recent study has indicated that in toxin-sensitive wheat lines a host factor(s) is involved in internalization of ToxA into the cytoplasm and chloroplasts (Manning and Ciuffetti, 2005). The toxin causes cell death in both toxin-sensitive and insensitive wheat lines if expressed through transformation procedures. The mechanism used by the toxin to cause disruption in the thylakoid structure is, however, not yet known.

Light is known to play an important role in activating phytotoxins. Cercosporin is maintained in inactive form in fungi but, after being released from the fungal hyphae, it is oxidized to acquire photodynamic activity (Daub et al., 1992). *Cercospora* toxins, Cebetin A and B inhibit the plasma membrane and chloroplast ATPases of sugar beet in a light-dependent manner (Jalal et al., 1992). The *F. solani* toxin, dihydrofusarubin, causes the degradation of tobacco leaf pigments in a light-dependent manner (Heiser et al., 1998, and references therein). In spinach, dihydrofusarubin causes the formation of reactive oxygen species (ROS), such as superoxide, by interrupting the photosynthetic electron transport chain of chloroplasts (Albrecht et al., 1998).

Tentoxin, produced by *Alternaria alternata,* inhibits photophosphorylation by binding to the chloroplast ATPase. This leads to decreased $CO_2$ fixation and an increased number of free electrons that cause ROS generation (Pinet et al., 1996). Tabtoxin, produced by *Pseudomonas syringae* pv. *tabaci,* causes chlorotic lesions in tobacco leaves in a light-dependent manner (Durbin, 1981). Rubisco carboxylation activity is inhibited and, as a result, ATP and NADPH generated from photophosphorylation are not utilized for carbohydrate assimilation and ADP and $NADP^+$ are not generated for further photophosphorylation. This leads to ROS generation through transfer of electrons from photosystem I to molecular oxygen rather than to ADP or $NAPD^+$ (Heiser et al., 1998, and references therein).

ROS produced by phytotoxins can cause peroxidative breakdown of unsaturated fatty acids. Chlorophyll is oxidized and bleached by alkoxyl radicals produced from the peroxidation of fatty acid (Heiser et al., 1998). For example, cercosporin, produced by *Cercospora* sp. *cercospori,* causes lipid peroxidation (Elstner and Osswald, 1980; Elstner et al., 1985; Heiser et al., 1998). *F. solani* naphthazarin toxins are electron acceptors for reducing oxygen during formation of ROS. These toxins enhance membrane permeability (Medentsev et al., 1988; Nemec, 1995) and cause chlorosis of citrus leaves. They disrupt chloroplasts by causing chloroplast membranes to swell and by disorganizing the granule stacks (Achor et al., 1993; Nemec, 1995).

Cell-free culture filtrates of *Fsg* have been found to cause SDS foliar symptoms routinely in 3-week-old seedlings and it was suggested that *Fsg* phytotoxins translocated from roots are responsible for development of foliar symptoms (Li et al., 1999). The cell-free *Fsg* culture filtrates can also cause cell death (Li et al., 1999). Cell-free culture filtrates isolated from non-pathogenic *F. solani* isolates did not cause leaf scorch in soybean (Li et al., 1999). Most likely, *Fsg* toxins produce leaf scorch only in soybean. Two phytotoxins have been isolated from the cell-free *Fsg* culture filtrates: monorden and a 17-kDa proteinaceous toxin. Purified fractions including the 17-kDa protein cause browning of soybean calli and necrosis in detached soybean leaves (Baker and Nemec, 1994; Jin et al., 1996). The gene encoding this proteinaceous toxin has not been isolated. The role of the toxin in leaf scorch development is also unknown. We have investigated changes in protein profiles that occur during leaf scorch development following feeding of soybean seedlings with cell-free *Fsg* culture filtrates. We have observed that the Rubisco large subunit is degraded in diseased leaves. Light is essential for the degradation of Rubisco large subunit by the cell-free *Fsg* culture filtrates. Data presented in this paper suggest that, in the presence of light, *Fsg*-specific toxin(s) causes degradation of Rubisco large subunit and accumulation of free radicals, which presumably cause PCD and leaf scorch development in soybean.

## Materials and Methods

### *Preparation of fungal culture filtrates*

*Fusarium solani* f. sp. *glycines* isolates Clinton and Scott were grown and maintained on solid Bilay medium (0.1% $KH_2PO_4$ [w/v], 0.1% $KNO_3$ [w/v], 0.05% $MgSO_4$ [w/v], 0.05% KCl [w/v], 0.02% starch [w/v], 0.02% glucose [w/v], and 0.02% sucrose [w/v]) for 12 days at 23 °C in the dark. Forty plugs were transferred into 100 ml modified Septoria medium (MSM) and incubated for 12 days without shaking at 23 °C in the dark (Song et al., 1993). Cultures were filtered through two layers of Whatman No. 1 filter paper and the pH was adjusted to 6.0 with HCl. The cultures were refiltered through a 0.45-mm stericup and then through a 0.22-mm stericup (Millipore, Inc.) and the cell-free *Fsg* culture filtrates were stored at 4 °C until use. Filtered MSM was used as the control (Li et al., 1999).

### Stem cutting assay

The soybean cultivar Williams 82 was grown in soil for 3 weeks to second trifoliate stage in a growth chamber under a 16-h photoperiod at 200 µmol photons/$m^2$/s and 25 °C, and in darkness at 16 °C. Cell-free *Fsg* culture filtrates were diluted in sterile, double distilled water. Soybean seedlings were cut below the cotyledon. Cut seedlings were placed in 50 ml Oakridge tubes containing 25 ml diluted, cell-free *Fsg* culture filtrate or diluted MSM filtrate as a control (Li et al., 1999).

## Leaf protein extraction

One g of leaf tissue was ground in liquid nitrogen. The ground tissue powder was then mixed with 5 ml chilled acetone containing 10% trichloroacetic acid (TCA) (w/v) and 0.07% β-mercaptoethanol (β-ME) (v/v) and kept at – 20 °C for 1 h. The samples were then centrifuged at 13 000 rpm for 10 min and the insoluble material was washed in ice-cold acetone containing 0.07% β-ME (v/v). The pellets were kept at – 20 °C for 20 min and then centrifuged at 13 000 rpm and 4 °C for 10 min. The washing, incubation, and centrifugation steps were repeated twice. The pellets were vacuum-dried in a Speed Vac (Savant Instruments, Inc., NY) for 3 min. One ml rehydration buffer (7 M urea, 2 M thiourea, 2 mM tributyl phosphate, 4% [w/v] CHAPS [3-{(3-cholamidopropyl) dimethylammonio}-1-propanesulfonate], 0.5% ampholytes) was added to solublize the proteins. Tributyl phosphate and ampholytes were added just before use.

## Two-dimensional electrophoresis

Electrophoresis of protein samples was conducted in a 24-cm immobilized pH gradient (IPG) gel (pH 4.0 – 7.0) at 100 V for 4 h followed by 500 V for 4 h, 1000 V for 6 h, 4000 V for 6 h, and 8000 V for 20 h in an Ettan IPGphor system (Amersham Biosciences, NJ). Subsequently, proteins were separated in the second dimension on a 12% sodium dodecyl sulfate-polyacrylamide gel at a constant current of 40 mA in an Ettan DALTsix large vertical electrophoresis system (Amersham Biosciences, NJ). The second-dimension gels were stained with modified Coomassie Blue stain (Coomassie Blue G-250 0.1% [w/v], methanol 34% [v/v], phosphoric acid 3% [v/v], ammonium sulfate 10% [w/v]), for at least 24 h. Stained gels were destained with 1% (v/v) acetic acid for at least 24 h. A cluster of protein spots, absent in protein samples prepared from diseased leaf tissues showing moderate symptoms (interveinal chlorosis and necrosis), were picked for identification.

## Protein in-gel digestion

A cluster of protein spots and a 55-kDa protein band, not present in leaf scorch diseased tissues, were identified on a two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel and single dimension (1-D) sodium dodecyl sulfate-polyacrylamide gel of healthy tissues, respectively, and isolated using a 20-gauge needle. The gel pieces were transferred into 400 µl soaking buffer (50% [v/v] acetonitrile in 25 mM ammonium bicarbonate, pH 8.0) in a 500 µl microcentrifuge tube. The gel fragments were soaked in the buffer for 15 min. The supernatant was removed and the gel pieces were washed with 400 µl soaking buffer three more times. Finally, the gel fragments were soaked in 100% acetonitrile for 5 min. The acetonitrile was then removed and gel fragments were dried for 20 to 30 min in a Speed Vac (Savant Instruments, Inc., NY). The gel fragments were rehydrated with a minimal volume of trypsin solution (10 µg of trypsin in 1 ml of 25 mM ammonium bicarbonate buffer, pH 8.0) (Promega, Inc., WI). The gel was incubated at 37 °C for 16 to 24 h. After removal of the supernatant, gel fragments were soaked in and extracted with 25 µl aliquots of 50% acetonitrile (v/v)/5% trifluoroacetic acid (v/v) for 30 to 60 min. Extracts were dried in a Speed Vac (Savant Instruments, Inc., NY) to reduce the volume to 5 to 10 µl.

## Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS)

A 1 µl digested protein sample and 1 µl of matrix (α-cyano-4-hydroxycinnamic acid [ACH], 10 mg/ml) were mixed thoroughly. One µl of this mixture was loaded onto the sample target of a Dynamo MALDI mass analyzer (Thermo Bioanalysis, Ltd, UK). After the mixture was dried, a 0.5-µl calibration standard was loaded onto the sample target spot. Voyager control software (Applied Biosystems, Inc., CA) was used to eject the sample and obtain the mass spectrum of the tryptic peptides. To identify the protein, masses of tryptic peptides determined by MALDI-TOF were compared with the calculated peptide mass fingerprints of the protein sequence databases (NCBInr.10.21.2003) with the aid of the program MS-Fit (http://prospector.ucsf.edu/ucsfhyml3.4/msfit.htm).

## *Free radical assay*

The nitro-blue tetrazolium (NBT) staining method was used to detect $O_2^-$ accumulation in leaves. Leaves were vacuum-infiltrated with 0.5 mg/ml NBT solution for 5 min three times. Then leaves were incubated in the NBT solution in darkness for 1 h at room temperature. After removal of the NBT solution, leaves were incubated at 50 °C in 80% ethanol until chlorophyll was completely bleached (Dutilleul et al., 2003). The 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining method was applied to detect $H_2O_2$ accumulation in leaves. Leaves were vacuum-infiltrated with DAB solution (1 mg/ml) for 5 min for three times. Then leaves were incubated in the DAB solution in darkness for 14 h at room temperature. After removal of the NBT solution, leaves were incubated at 50 °C in 80% ethanol until the chlorophyll was completely bleached (Dutilleul et al., 2003).

## Western blotting

Fifteen µg of total protein from each sample were separated in a 12% polyacrylamide-sodium dodecyl sulfate mini gel. Following electrophoresis, gels were electro-blotted onto a PVDF membrane (Millipore, Inc., MA). The membrane was blocked with 1% bovine serum albumin (BSA). The membrane was incubated with anti-Rubisco large subunit antibody (1 : 2000 dilution: AgriSera, Inc., Sweden) or anti-GDC-P protein antibody (1 : 1000 dilution) for 3 h. Anti-chicken IgY alkaline phosphatase conjugate (Promega, Inc., WI) and anti-rabbit IgG b + l alkaline phosphatase conjugate (Bethyl Laboratories, Inc., TX) were used as the secondary antibodies for anti-Rubisco large subunit antibody and anti-GDC-P protein antibody, respectively. The membranes were incubated in Western Blue Stabilized Substrates (Promega, Inc., WI) for detecting alkaline phosphatase activity.

## In situ *cell death detection: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) staining*

Soybean leaves showing early symptoms (chlorotic spots) were collected from cell-free *Fsg* culture filtrate-treated seedlings. Leaves collected from MSM-treated seedlings were used as the negative control. Circular leaf sections 0.6 cm in diameter were punched from the collected leaves and fixed in freshly prepared 4% paraformaldehyde and 0.25% glutaraldehyde (v/v) in 0.1 M phosphate buffered saline (PBS) (pH 7.4) over-

night at 22 °C. Leaf tissues were then washed in PBS buffer for 30 min. Leaf tissues were dehydrated in 70 to 100% ethanol for 3 h, treated with xylene and embedded in paraffin. Leaf tissues were cross-sectioned to 10 µm thickness and de-waxed. Sections were treated with proteinase K for 20 min at 23 °C. Fifty µl of TUNEL reaction solution (5 µl terminal deoxynucleotidyl transferase [TdT] and 45 µl of labelled nucleotides from an *in situ* cell death detection kit [Roche Applied Science, Inc., IN]) were added to label the free 3′-OH group of the broken end of DNA molecules in a template-independent manner. Fifty µl of TUNEL reaction solution were added to MSM-fed leaf tissues to serve as a negative control and 10 units of DNase were added to MSM-fed leaf tissues to serve as a positive control. The tissues were incubated at 37 °C in a water bath in darkness for 60 min. Then leaf tissues were washed in PBS buffer three times. The fluorescent spots were visualized with a fluorescence microscope using a green filter (515 – 565 nm wavelength). Nuclei were localized by staining the tissue preparations with 0.2 µg/ml Hoechst 33258 dye and observed under a fluorescence microscope (Carl Zeiss, Inc., Germany) using a blue filter (352 – 461 nm wavelength).

## Results

### *Cell-free* Fsg *culture filtrates cause foliar SDS symptoms*

Earlier, it was shown that symptoms of foliar SDS were induced when 3-week-old seedlings were fed with cell-free *Fsg* culture filtrates through cut stems (Li et al., 1999). This assay, termed the stem cutting assay, produces highly reproducible foliar symptoms as compared to those caused by root inoculation with *Fsg* spore suspensions. Two *Fsg* isolates, Clinton and Scott, isolated and kindly provided by X.B. Yang, produce similar disease symptoms on the susceptible soybean cultivar, Williams (Fig. **1**). The colour of culture medium containing the Clinton isolate becomes reddish after about 12 days of growth, and the production of reddish colour is associated with the development of foliar SDS. Therefore, we chose to use the Clinton isolate in this investigation.

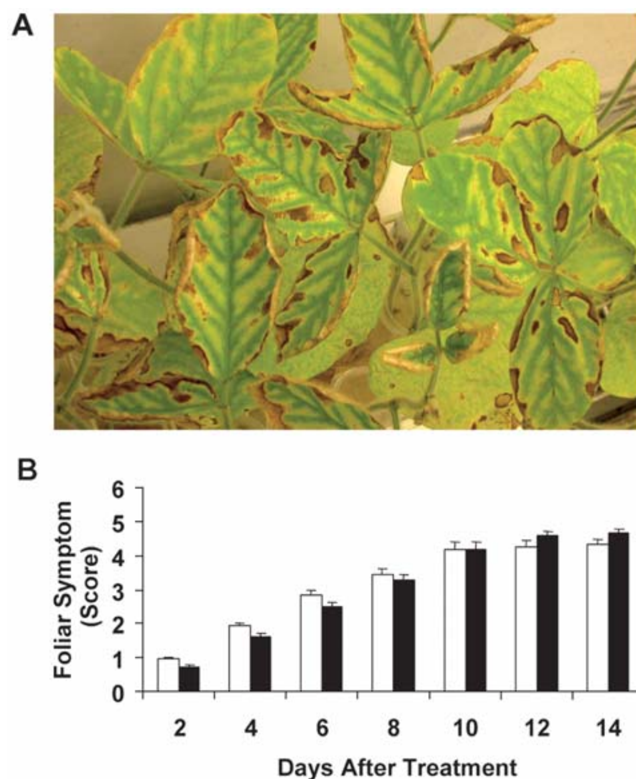


**Fig. 1** SDS foliar symptom development in soybean seedlings fed with cell-free *Fsg* culture filtrate. (**A**) Foliar symptom development 14 days after treatment of cut soybean seedlings with the cell-free *Fsg* culture filtrate prepared from the *Fsg* isolate Clinton. Culture filtrate (1 : 24 dilution) was fed through cut ends of 3-week-old seedlings. (**B**) Foliar SDS symptom development following treating of seedlings with cell-free *Fsg* culture filtrates prepared from two *Fsg* isolates: Clinton (black bar) and Scott (white bar). Foliar symptoms were evaluated as follows: 0, no symptoms; 1, chlorotic spots; 2, interveinal chlorosis and necrotic spots (> 30% of leaf area affected); 3, interveinal chlorosis (> 50% of leaf area affected); 4, interveinal necrosis (> 70% of leaf area affected); 5, severe interveinal necrosis (> 90% of leaf area affected). No symptoms were observed when MSM was fed. Similar results were obtained in three independent experiments.

### *Steady-state levels of Rubisco large subunit were decreased in diseased tissues*

The total soluble protein profiles of healthy and diseased leaves were compared using 2-D PAGE (Fig. **2**). A cluster of abundant proteins was consistently absent in diseased tissues (Fig. **2B**). MALDI-TOF MS analysis showed that the missing protein spots were Rubisco large subunit. Subsequently, results of MALDI-TOF MS analysis of a major 55-kDa protein band from a 1-D sodium dodecyl sulfate-PAGE gel that disappeared from diseased tissues also showed that the protein was Rubisco large subunit. Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) is the predominant protein in leaves. Rubisco catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PGA). Rubisco is composed of eight large (~ 56 kDa) and eight small subunits (~ 14 kDa). Western blotting of protein samples from healthy and diseased tissues using anti-Rubisco large subunit antibody confirmed that the protein was indeed the Rubisco large subunit (Fig. **2C**). It has been shown in oat that the P protein of the glycine decarboxylase complex (GDC) binds to Victorin toxin (Wolpert and Macko, 1989; Wolpert et al., 1994). We therefore investigated if steady state levels of P protein were also altered in diseased leaves by carrying out Western blot analysis. We observed that P protein of GDC was stable in diseased tissues (Fig. **2C**).

### *Light accelerates Rubisco large subunit degradation*

Light has been shown to be essential for development of toxin-induced disease symptoms (Daub and Briggs, 1983; Heiser et al., 1998, and references therein). We therefore investigated if light was essential for development of cell-free *Fsg* culture filtrate-induced leaf scorch in soybean. We observed that development of leaf scorch was not initiated when cell-free *Fsg* culture filtrate-fed soybean seedlings were incubated under dark conditions. This observation led us to investigate if absence of leaf scorch development in the dark was associated with the absence of Rubisco large subunit disappearance. We observed that, in the dark, levels of Rubisco large subunit did not change in the *Fsg* culture filtrate-fed seedlings. Exposure of the cell-free *Fsg* culture filtrate-fed seedlings to light, however, resulted in the disappearance of Rubisco large subunit and develop-

ment of leaf scorch symptoms (Fig. **3**). Rubisco large subunit completely disappeared within 24 h of exposure of the cell-free *Fsg* culture filtrate-fed seedlings to light. Rubisco is a fairly stable enzyme (Peterson et al., 1973). We, therefore, concluded that disappearance of Rubisco large subunit was a result of its degradation rather than inhibition of its synthesis.

### *Light is essential for accumulation of free radicals in* Fsg *culture filtrate-fed leaves*

The sudden loss of Rubisco in light-exposed, *Fsg* culture filtrate-fed seedlings led to failure of conversion of light energy into carbohydrates, and reducing equivalents produced in the light reaction, which were not utilized in carbon fixation. This presumably led to accumulation of free radicals, causing senescence-like cell death. We therefore hypothesized that, in absence of light, the extent of free radical accumulation in leaves of *Fsg* culture filtrate-fed seedlings should be much less than that in light-exposed leaves of *Fsg* culture filtrate-fed seedlings. We observed that exposure of cell-free *Fsg* culture filtrate-fed seedlings to light resulted in accumulation of free radicals (Fig. **4**). In the absence of light, the cell-free *Fsg* culture filtrate failed to initiate the accumulation of free radicals to the levels produced in the presence of light.

### In situ *cell death in diseased leaves*

In plants, programmed cell death (PCD), the equivalent of apoptosis in mammals, is a cell suicide process. The characteristics of PCD include cell shrinkage, chromatin condensation, DNA fragmentation, and appearance of apoptotic bodies (Greenberg, 1996). PCD has been suggested to be a mechanism for symptom development in many plant diseases (Yao et al., 2001; Lincoln et al., 2002). We therefore investigated whether PCD is observed during leaf scorch development. Endonucleolysis is the key biochemical event in apoptosis. We used TUNEL stain to detect any DNA fragmentation in nuclei. At the initial stage, PCD is characterized by fragmentation of nuclear DNA to approximately 50 kb. Labelled nucleotides were transferred to the free 3′-OH ends of oligonucleosomal DNA fragments *in situ* using terminal deoxynucleotidyl transferase (TdT) in a template-independent manner. This technique allows the labelling of broken DNA ends of only those nuclei that undergo programmed cell death. Fig. **5** shows DNA end labelling in leaf tissues from seedlings that were fed with the cell-free *Fsg* culture filtrate Fig. **5 F**), but not in the leaf tissues fed with culture medium (Fig. **5 B**).

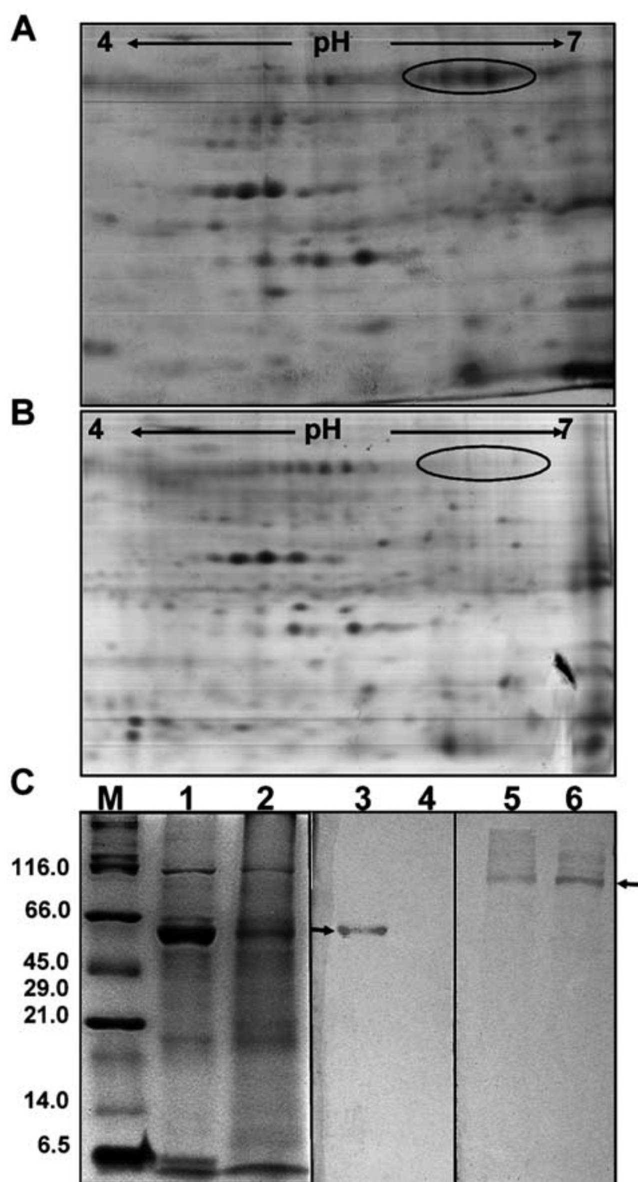


**Fig. 2** Disappearance of Rubisco large subunit in diseased leaf tissues. (**A**) Two-dimensional (2-D) sodium dodecyl sulfate-PAGE of proteins prepared from healthy leaf tissues. (**B**) 2-D sodium dodecyl sulfate-PAGE of proteins prepared from diseased leaf tissues showing moderate symptoms (interveinal chlorosis and necrosis). Gels were stained with Coomassie Blue R250. The degraded protein spots are marked with a circle. The protein spots in the circle were identified as large subunits of Rubisco by MALDI-MS analyses. (**C**) Western blot analysis of the soybean proteins isolated from diseased and healthy tissues. Lanes 1, 3, and 5, proteins prepared from control healthy leaf tissues; lanes 2, 4, and 6, proteins prepared from diseased leaf tissues (interveinal chloroisis); lanes 1 and 2, Coomassie blue-stained sodium dodecyl sulfate-PAGE gel; lanes 3 and 4, Western blot of Rubisco large subunit; lanes 5 and 6, Western blot of the GDC-P protein. In each lane, 15 µg of total protein were separated. M, protein marker in kDa. Similar results were obtained from an additional experiment.

## Discussion

Cell-free *Fsg* culture filtrate contains phytotoxins that cause foliar SDS (Li et al., 1999). We have observed that active components of cell-free *Fsg* culture filtrate causing foliar SDS bind to an anion exchange column suggesting that the active principle is negatively charged (data not shown). The 17 kDa, proteinaceous toxin of *Fsg* has been reported to also be negatively charged and was shown to cause browning of calli and necrosis of detached leaves in soybean (Jin et al., 1996).

Foliar symptoms developed following feeding of soybean seedlings with cell-free *Fsg* culture filtrates include chlorophyll loss and degradation of Rubisco, which are two hallmarks of senescence (Greenberg, 1996; Navarre and Wolpert, 1999). Rubisco degradation is associated with two important physiological processes: senescence and plant responses to environmental stresses. During senescence, Rubisco, the most abundant leaf protein, provides amino acids to reproductive

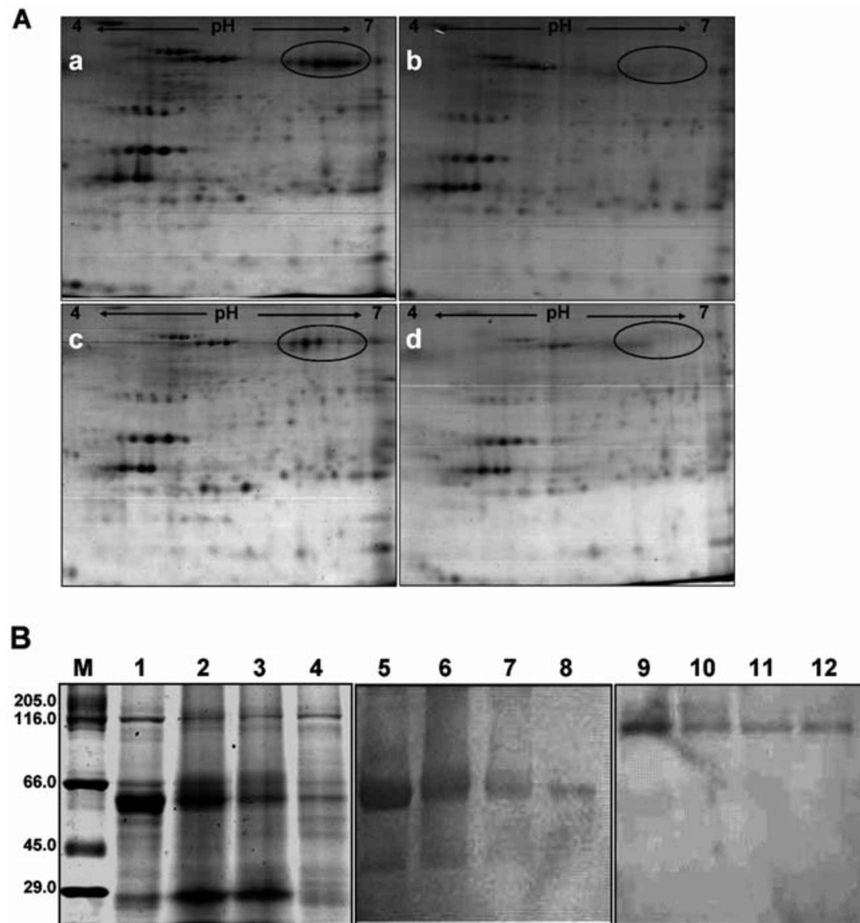


**Fig. 3** Light is required for the disappearance of Rubisco large subunit by the *Fsg* culture filtrates. (**A**) 2-D sodium dodecyl sulfate-PAGE. The seedlings were fed with either cell-free *Fsg* culture filtrates or MSM through cut ends for 12 h and then exposed to light. Following light exposure, seedlings were returned to darkness for the remaining time of a total 36-h period. **a** Leaf proteins from seedlings exposed to light for 0 h following feeding with cell-free *Fsg* culture filtrate; **b** leaf proteins from seedlings exposed to light for 1 h following feeding with cell-free *Fsg* culture filtrate for 12 h; **c** leaf proteins from seedlings exposed to light for 8 h following feeding with culture medium only for 12 h; **d** leaf proteins from seedlings exposed to light for 8 h following feeding with cell-free *Fsg* culture filtrate for 12 h. The location of Rubisco large subunit is shown by circles. (**B**) Western blot analysis of the soybean leaf proteins isolated from dark- and light-exposed *Fsg* culture filtrate-fed seedlings. Experimental design is similar to that of Fig. **3 A**. Lanes 1, 5, and 9: leaf proteins from seedlings exposed to light for 0 h following feeding with cell-free *Fsg* culture filtrate; lanes 2, 6, and 10: leaf proteins from seedlings exposed to light for 1 h following feeding with cell free *Fsg* culture filtrate for 12 h; lanes 3, 7, and 11: leaf proteins from seedlings exposed to light for 4 h following feeding with cell-free *Fsg* culture filtrate for 12 h; lanes 4, 8, and 12: leaf proteins from seedlings exposed to light for 8 h following feeding with cell-free *Fsg* culture filtrate for 12 h. Following light treatment, seedlings were returned to darkness for the remaining time of a total 36-h incubation period. Lanes 1 – 4: Coomassie blue-stained sodium dodecyl sulfate gel; lanes 5 – 8: Western blot of Rubisco large subunit; lanes 9 – 12: Western blot of the GDC-P protein; lanes 1 – 12, about 15 µg protein were separated in each lane. M, protein markers in kDa.

organs (Peoples et al., 1980; Makino et al., 1984; Ferreira and Teixeira, 1992). Environmental stresses such as low $CO_2$, oxidative stress, and toxin treatments can also induce Rubisco degradation (Ferreira and Davies, 1989; Casano and Trippi, 1992; Navarre and Wolpert, 1999). Interestingly, ROS are the common products generated during senescence and environmental stress (Desimone et al., 1996).

How the active principle of the *Fsg* culture initiates the degradation of Rubisco is yet to be uncovered. We have shown that light is essential for Rubisco degradation and accumulation of free radicals. The degradation of Rubisco presumably disrupts electron transport in chloroplasts. ATP and NADPH generated from photophosphorylation in chloroplasts are utilized by Rubisco for carbohydrate assimilation. In *Fsg* culture-fed leaves, ATP and NADPH are not utilized due to degradation of Rubisco. As a result, ADP and $NAPD^+$ are not released for further photophosphorylation, leading to transfer of electrons from photosystem I to molecular oxygen and accumulation of ROS. Free radicals, produced in the presence of light presumably result in damage to leaf tissues, including initiation of PCD. The TUNEL assay showed nuclear DNA fragmentation in diseased leaves, an important characteristic of apoptosis. Similar results were observed in response to application of other host-selective toxins. For example, victorin causes oxidative damage in light-incubated oat tissues (Navarre and Wolpert, 1999). Host-selective and chlorophyllin toxins such as AAL and T toxins also require light for inducing necrosis (Moussatos et al., 1993; Bhullar et al., 1975).

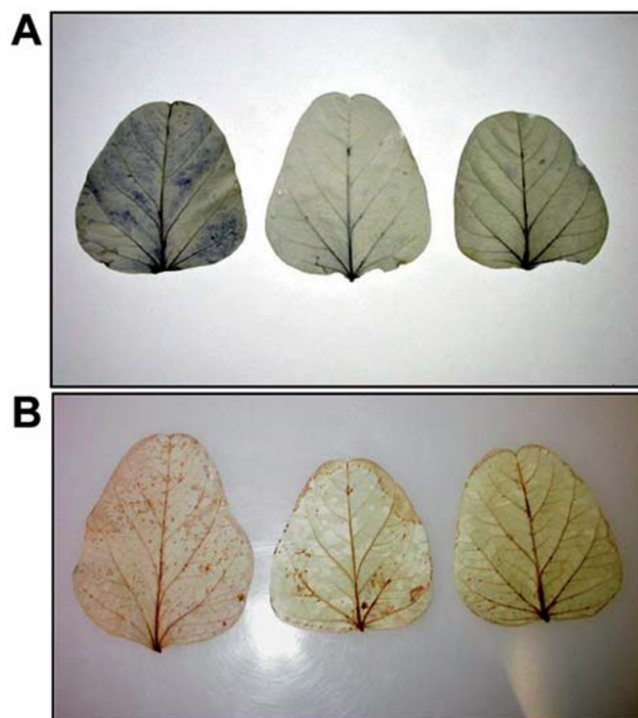


**Fig. 4** *In situ* $O_2^-$ and $H_2O_2$ accumulation in light-exposed *Fsg* culture filtrate-fed seedlings. Leaflets on the left are from cell-free *Fsg* culture filtrate-fed seedlings exposed to 1 h light. Leaflets in the middle are from cell-free *Fsg* culture filtrate-fed seedlings exposed to 0 h light. Leaflets on the right are from MSM-fed seedlings exposed to light for 1 h. The seedlings were fed with cell-free *Fsg* culture filtrates or MSM for 15 h in the dark prior to exposure to light. Following light exposure, leaves were immediately used for nitroblue tetrazolium or 3,3′-diaminobenzidine tetrahydrochloride assays. (**A**) *In situ* $O_2^-$ identification by nitroblue tetrazolium assay. The blue colour indicates the $O_2^-$ accumulation. (**B**) *In situ* $H_2O_2$ identification by 3,3′-diaminobenzidine tetrahydrochloride assay. The brown spot indicates accumulation of $H_2O_2$. Similar results were obtained from two additional experiments.

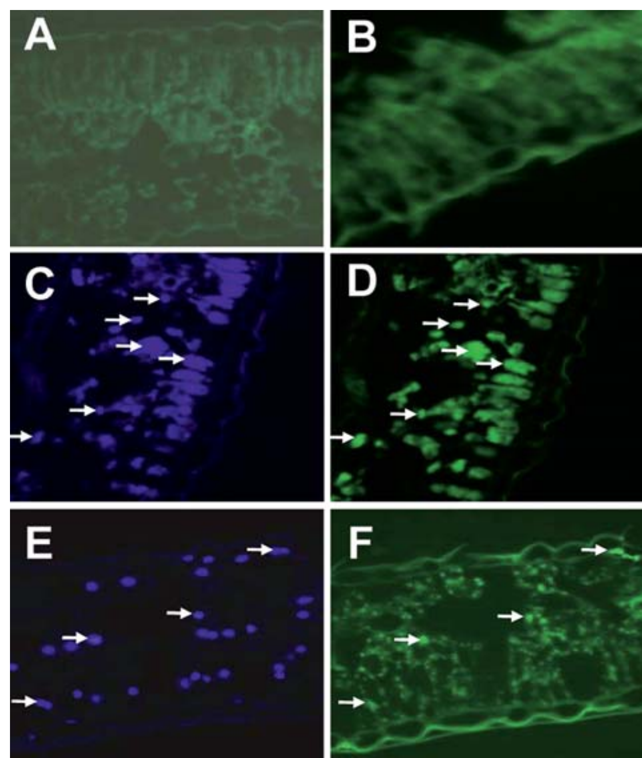


**Fig. 5** DNA fragmentation in leaf tissues in response to exposure of seedlings to cell-free *Fsg* culture filtrates. (**A**) Leaf tissues were treated with TUNEL reaction solution without terminal deoxynucleotidyl transferase (TdT). (**B – F**) Leaf tissues were treated with TUNEL reaction solution containing TdT. Broken DNA ends in **D, F** were localized by labelling with the TUNEL reaction. Green spots shown by arrows are nuclei containing fragmented DNA. Blue spots represent nuclei stained with Hoechst 33258 dye in **C, E**. Staining of nuclei with Hoechst 33258 dye was conducted following the TUNEL reaction. **A – D** show leaf tissues from seedlings fed with MSM. Tissues shown in **C, D** were treated with DNAase before the TUNEL reaction; tissues shown in **E, F** are from seedlings fed with cell-free *Fsg* culture filtrates. Note that DNAse treatment of a MSM-fed leaf in **D** resulted in nuclei with broken DNA fragments localized by TUNEL reagents (shown by arrows). Without DNAse treatment, no such nuclei were observed in an MSM-fed leaf (**B**). Whereas, in the leaf of a seedling fed with the cell-free *Fsg* culture filtrate, nuclei contain broken or fragmented DNA labelled with TUNEL reagents, shown by arrows in **F**. Locations of the TUNEL-positive nuclei in **F** are shown in **E** with arrows. Similar results were obtained in an additional experiment.

Rubisco initiates the oxidative photosynthetic carbon pathway that requires cooperative interaction between chloroplasts, peroxisomes, and mitochondria. Rubisco catalyzes the oxygenation of ribulose 1,5-bisphosphate in chloroplasts. Glycine decarboxylase complex (GDC) is the key enzyme of this photorespiration pathway in mitochondria. Along with serine hydroxymethyl transferase, GDC metabolizes glycine into serine to end the photorespiration pathway. The four proteins that constitute GDC are P protein (100 kDa), H protein (15 kDa), T protein (45 kDa), and L protein (61 kDa). Victorin binds to the P protein of GDC and also to Rubisco (Wolpert and Macko, 1989; Navarre and Wolpert, 1995; Curtis and Wolpert, 2004). Our Western blot results showed that the GDC P protein was stable in diseased tissues. It is yet to be investigated if *Fsg* toxins cause mitochondrial dysfunction, as observed in oat leaves following Victorin treatments (Wolpert et al., 2002).

There are several similarities between physiological changes that occur in oat and soybean following exposure to Victorin and *Fsg* phytotoxins, respectively. They are: (i) degradation of Rubisco large subunit; (ii) accumulation of free radicals under light conditions; (iii) initiation of programmed cell death; and (iv) necrosis in leaves. Therefore, it is most likely that both toxins cause diseases through a common mechanism. We are currently purifying the *Fsg* phytotoxin(s) in order to identify the *Fsg* toxin binding host protein(s) and will attempt to discover the mechanism by which the Rubisco degradation pathway and SDS development are initiated in soybean.

## Acknowledgements

We thank X. B. Yang and D. Oliver, ISU, for kindly providing us with *Fsg* isolates and the anti-GDC P protein antibody, respectively. We also thank S. Li, University of Illinois, for her invaluable advice, and A. Bogdanove, C. Bronson, and J. M. Peterson, Iowa State University, for reviewing the manuscript. We thank the United Soybean Board for financial support funded through D. Lightfoot, University of Southern Illinois.

## References


Achor, D. S., Nemec, S., and Baker, R. A. (1993) Effect of *Fusarium solani* naphthazarin toxins on the cytology and ultrastructure of rough lemon seedlings. Mycopathologia 123, 117 – 126.

Albrecht, A., Heiser, I., Baker, R., Nemec, S., Elstner, E. F., and Osswald, W. (1998) Effect of the *Fusarium solani* toxin dihydrofusarubin on tobacco leaves and spinach chloroplasts. Journal of Plant Physiology 153, 462 – 468.

Aoki, T., O'Donnell, K., Homma, Y., and Lattanzi, A. R. (2003) Sudden-death syndrome of soybean is caused by two morphologically and phylogenetically distinct species within the *Fusarium solani* species complex-*F. virguliforme* in North America and *F. tucumaniae* in South America. Mycologia 95, 660 – 684.

Baker, R. A. and Nemec, S. (1994) Soybean sudden death syndrome: isolation and identification of a new phytotoxin from cultures of the causal agent, *Fusarium solani* (abstract). Phytopathology 84, 1144.

Bhullar, B. S., Daly, J. M., and Rehfeld, D. W. (1975) Inhibition of dark $CO_2$ fixation and photosynthesis in leaf discs of corn susceptible to the host-specific toxin produced by *Helminthosporium maydis,* race T. Plant Physiology 56, 1 – 7.

Buchanan, B., Gruissem, W., and Jones, R. L. (2000) Biochemistry and Molecular Biology of Plants. Rockville, MD: American Society of Plant Physiologists, 1044 – 1099 p.

Casano, L. M. and Trippi, V. S. (1992) The effect of oxygen radicals on proteolysis in isolated oat chloroplasts. Plant Cell Physiology 33, 329 – 332.

Chang, S. J. C., Doubler, T. W., Kilo, V., Suttner, R. J., Klein, J. H., Schmidt, M. E., Gibson, P. T., and Lightfoot, D. A. (1996) Two additional loci underlying durable field resistance to soybean sudden death syndrome (SDS). Crop Science 36, 1624 – 1628.

Chang, S. J. C., Doubler, T. W., Kilo, V., Suttner, R. J., Schmidt, M. E., Gibson, P. T., and Lightfoot, D. A. (1997) Association of field resistance to soybean sudden death syndrome (SDS) and cyst nematode (SCN). Crop Science 37, 965 – 971.

Curtis, M. J. and Wolpert, T. J. (2004) The victorin-induced mitochondrial permeability transition precedes cell shrinkage and biochemical markers of cell death, and shrinkage occurs without loss of membrane integrity. The Plant Journal 38, 244 – 259.

Daub, M. E. and Briggs, S. P. (1983) Changes in tobacco cell membrane composition and structure caused by cercosporin. Plant Physiology 71, 763 – 766.

Daub, M. E., Leisman, G. B., Clark, R. A., and Bowden, E. F. (1992) Reductive detoxification as a mechanism of fungal resistance to singlet oxygen-generating photosensitizers. Proceedings of the National Academy of Sciences of the USA 89, 9588 – 9592.

Desimone, M., Henke, A., and Wagner, E. (1996) Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. Plant Physiology 111, 789 – 796.

Durbin, R. D. (1981) Toxins in Plant Disease. New York: Academic Press, 66 p.

Dutilleul, C., Garmier, M., Noctor, G., Mathieu, C., Chetrit, P., Foyer, C. F., and Paepe, R. D. (2003) Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. Plant Cell 15, 1212 – 1226.

Elstner, E. F. and Osswald, W. (1980) Chlorophyll photobleaching and ethane production in dichlorophenyldimethylurea-(DCMU) or paraquat-treated *Euglena gracilis* cells. Zeitschrift für Naturforschung 35, 129 – 135.

Elstner, E. F., Osswald, W., and Youngman, R. J. (1985) Basic mechanisms of pigment bleaching and loss of structural resistance in spruce *(Picea abies)* needles – advances in phytomedical diagnostics. Experientia 41, 591 – 597.

Ferreira, R. B. and Davies, D. D. (1989) Conversion of ribulose-1,5-bisphosphate carboxylase to an acidic and catalytically inactive form by extracts of osmotically stressed *Lemna minor* fronds. Planta 179, 448 – 455.

Ferreira, R. M. B. and Teixeira, A. R. N. (1992) Sulfur starvation in *Lemna* leads to degradation of ribulose-bisphosphate carboxylase/oxygenase without plant death. Journal of Biological Chemistry 267, 7253 – 7257.

Greenberg, J. T. (1996) Programmed cell death: a way of life for plants. Proceedings of the National Academy of Sciences of the USA 93, 12094 – 12097.

Heiser, I., Osswald, W., and Elstner, E. F. (1998) The formation of reactive oxygen species by fungal and bacterial phytotoxins. Plant Physiology and Biochemistry 36, 703 – 713.

Hnetkovsky, N., Chang, S. C., Doubler, T. W., Gibson, P. T., and Lightfoot, D. A. (1996) Genetic mapping of loci underlying field resistance to sudden death syndrome. Crop Science 36, 392 – 400.

Iqbal, M. J., Yaegashi, S., Njiti, V. N., Ahsan, R., Cryder, K. L., and Lightfoot, D. A. (2002) Resistance locus pyramids alter transcript abundance in soybean roots inoculated with *Fusarium solani* f. sp. *glycines*. Molecular Genetics and Genomics 268, 407 – 417.

Jalal, M. A. F., Hossain, M. B., Robeson, D. J., and van der Helm, D. (1992) *Cercospora beticola* phytotoxins: cebetins that are photoactive, magnesium ion-binding, chlorinated anthraquinone-xanthone conjugates. Journal of American Chemical Society 114, 5967 – 5971.

Jin, H., Hartman, G. L., Nickell, C. D., and Widholm, J. M. (1996) Characterization and purification of a phytotoxin produced by *Fusarium solani,* the causal agent of soybean sudden death syndrome. Phytopathology 86, 277 – 282.

Levine, A., Pennell, R. I., Alvarez, M. E., Palmer, R., and Lamb, C. (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Current Biology 6, 427 – 437.

Li, S., Hartman, G. L., and Widholm, J. M. (1999) Viability staining of soybean suspension cultured cells and a stem-cutting assay to evaluate phytotoxicity of *Fusarium solani* culture filtrates. Plant Cell Reports 18, 375 – 380.

Lincoln, J. E., Richael, C., Overduin, B., Smith, K., Bostock, R., and Gilchrist, D. (2002) Expression of the *antiapoptotic baculovirus* p35 gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. Proceedings of the National Academy of Sciences of the USA 99, 15217 – 15221.

Makino, A., Mae, T., and Ohira, K. (1984) Relation between nitrogen and ribulose-1,5-bisphosphate carboxylase in rice leaves from emergence through senescence. Plant Cell Physiology 25, 429 – 437.

Manning, V. A. and Ciuffetti, L. M. (2005) Localization of Ptr ToxA produced by *Pyrenophora tritici-repentis* reveals protein import into wheat mesophyll cells. Plant Cell 17, 3203 – 3212.

Medentsev, A. G., Baskunov, B. P., and Akimenko, V. K. (1988) Formation of naphthoquinone pigments by the fungus *Fusarium decemcellulare* and their influence on the oxidative-metabolism of the producer. Biochemistry (Moscow) 53, 353 – 363.

Meksem, K., Doubler, T. W., Chancharoenchai, K., Njiti, V. N., Chang, S. J. C., Arelli, A. P. R., Cregan, P. E., Gray, L. E., Gibson, P. T., and Lightfoot, D. A. (1999) Clustering among loci underlying soybean resistance to *Fusarium solani,* SDS and SCN in near-isogenic lines. Theoretical and Applied Genetics 99, 1131 – 1142.

Melgar, J. and Roy, K. W. (1994) Soybean sudden-death syndrome – cultivar reactions to inoculation in a controlled environment and host-range and virulence of causal agent. Plant Disease 78, 265 – 268.

Miyashita, M., Nakamori, T., Miyagawa, H., Akamatsu, M., and Ueno, T. (2003) Inhibitory activity of analogs of AM-toxin, a host-specific phytotoxin from the *Alternaria alternata* apple pathotype, on photosynthetic $O_2$ evolution in apple leaves. Bioscience, Biotechnology and Biochemistry 67, 635 – 638.

Moussatos, V., Witsenboer, H., Hille, J., and Gilchrist, D. (1993) Behavior of the disease resistance gene *Asc* in protoplasts of *Lycopersicon esculentum* Mill. Physiological and Molecular Plant Pathology 43, 255 – 263.

Navarre, D. A. and Wolpert, T. J. (1995) Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. Plant Cell 7, 463 – 471.

Navarre, D. A. and Wolpert, T. J. (1999) Victorin induction of an apoptotic/senescence-like response in oats. Plant Cell 11, 237 – 249.

Nemec, S. (1995) Stress-related compounds in xylem fluid of blight-diseased citrus containing *Fusarium solani* Naphthazarin toxins and their effects on the host. Canadian Journal of Microbiology 41, 515 – 524.

Njiti, V. N., Shenaut, M. A., Suttner, R. J., Schmidt, M. E., and Gibson, P. T. (1996) Soybean response to sudden death syndrome: inheritance influenced by cyst nematode resistance in Pyramid × Douglas progenies. Crop Science 36, 1165 – 1170.

Njiti, V. N., Suttner, R. J., Gray, L. E., Gibson, P. T., and Lightfoot, D. A. (1997) Rate-reducing resistance to *Fusarium solani* f. sp. *phaseoli* underlies field resistance to soybean sudden death syndrome. Crop Science 37, 132 – 138.

Njiti, V. N., Meksem, K., Iqbal, M. J., Johnson, J. E., Kassem, M. A., Zobrist, K. F., Kilo, V. Y., and Lightfoot, D. A. (2002) Common loci underlie field resistance to soybean sudden death syndrome in Forest, Pyramid, Essex, and Douglas. Theoretical and Applied Genetics 104, 294 – 300.

Pasqualini, S., Piccioni, C., Reale, L., Ederli, L., Della Torre, G., and Ferranti, F. (2003) Ozone-induced cell death in tobacco cultivar Bel W3 plants. The role of programmed cell death in lesion formation. Plant Physiology 133, 1122 – 1134.

Peoples, M. B., Beilharz, V. C., Waters, S. P., Simpson, R. J., and Dalling, M. J. (1980) (*Ttriticum aestivum* L.) II. Chloroplast senescence and the degradation of ribulose-1,5-bisphosphate carboxylase. Planta 149, 241 – 251.

Peterson, L. W., Kleinkopf, G. E., and Huffaker, R. C. (1973) Evidence for lack of turnover of ribulose 1,5-diphosphate carboxylase in barley leaves. Plant Physiology 51, 1042 – 1045.

Pinet, E., Cavelier, F., Verducci, J., Girault, G., Dubart, L., Haraux, F., Sigalat, C., and Andre, F. (1996) Synthesis, structure, and properties of MeSer(1)-tentoxin, a new cyclic tetrapeptide which interacts specifically with chloroplast F-1 $H^+$-ATPase differentiation of inhibitory and stimulating effects. Biochemistry 35, 12804 – 12811.

Ringler, G. A. and Nickell, C. D. (1996) Genetic resistance to *Fusarium solani* in Pioneer Brand 9451. Soybean Genetics Newsletter 23, 144 – 148.

Rupe, J. C. (1989) Frequency and pathogenicity of *Fusarium solani* recovered from soybeans with sudden-death syndrome. Plant Disease 73, 581 – 584.

Rupe, J. C., Gbur, E. E., and Marx, D. M. (1991) Cultivar responses to sudden-death syndrome of soybean. Plant Disease 75, 47 – 50.

Rupe, J. C., Sabbe, W. E., Robbins, R. T., and Gbur, E. E. (1993) Soil and plant factors associated with sudden death syndrome of soybean. Journal of Production Agriculture 6, 218 – 221.

Rupe, J. C. and Hartman, G. L. (1999) Sudden death syndrome. In Compendium of Soybean Diseases (Hartman, G. L. et al., eds.), St. Paul, MN: APS Press, pp. 37 – 39.

Roy, K. W., Hershman, D. E., Rupe, J. K., and Abney, T. S. (1997) Sudden death syndrome of soybean. Plant Disease 81, 1100 – 1111.

Roy, K. W., Lawrence, G. W., Hodges, H. H., Mclean, K. S., and Killebrew, J. F. (1989) Sudden death syndrome of soybean: *Fusarium solani* as incident and relation of *Heterodera glycines* to disease severity. Phytopathology 79, 191 – 197.

Song, H. S., Lim, S. M., and Clarke, J. M. Jr. (1993) Purification and partial characterization of a host specific pathotoxin from culture filtrate of *Septoria glycines*. Phytopathology 83, 659 – 661.

Stephens, P. A., Nickell, C. D., and Kolb, F. L. (1993) Genetic analysis of resistance to *Fusarium solani* in soybean. Crop Science 33, 929 – 930.

Strobel, G. A. (1982) Phytotoxins. Annual Review of Biochemistry 51, 309 – 333.

Wolpert, T. J., Dunkle, L. D., and Ciuffetti, L. M. (2002) Host-selective toxin and avirulence determinants: what's in a name? Annual Review of Phytopathology 40, 251 – 285.

Wolpert, T. J. and Macko, V. (1989) Specific binding of victorin to a 100-kDa protein from oats. Proceedings of the National Academy of Sciences of the USA 86, 4092 – 4096.

Wolpert, T. J., Navarre, D. A., Moore, D. L., and Macko, V. (1994) Identification of the 100-kDa victorin binding-protein from oats. Plant Cell 6, 1145 – 1155.

Wrather, J. A., Stienstra, W. C., and Koenning, S. R. (2001) Soybean disease loss estimates for the United States from 1996 to 1998. Cananadian Journal of Plant Pathology 23, 122 – 131.

Yao, N., Tada, Y., Park, P., Nakayashiki, H., Tosa, Y., and Mayama, S. (2001) Novel evidence for apoptotic cell response and differential signal in chromatin condensation and DNA cleavage in victorin-treated oats. The Plant Journal 28, 13 – 26.

M. K. Bhattacharyya

G303 Agronomy Hall
Iowa State University
Ames, Iowa 50011-1010
USA

E-mail: mbhattac@iastate.edu

Editor: C. M. J. Pieterse