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Joseph Byrum

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ANALYSIS OF THE OMEGA-3 FATTY ACID DESATURASE IN SOYBEAN [*Glycine max* (L.) Merr.] GENOTYPES A5 AND A23; RESISTANCE TO SCLEROTINIA STEM ROT IN SOYBEAN [*Glycine max* (L.) Merr.] IS INDUCED BY 2,6-DICHLORO-ISONICOTINIC ACID

By

Joseph Byrum

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ABSTRACT

ANALYSIS OF THE OMEGA-3 FATTY ACID DESATURASE IN SOYBEAN [*Glycine max* (L.) Merr.] GENOTYPES A5 AND A23; RESISTANCE TO SCLEROTINIA STEM ROT IN SOYBEAN [*Glycine max* (L.) Merr.] IS INDUCED BY 2,6-DICHLORO-ISONICOTINIC ACID

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Soybean genotypes A5 and A23 have reduced α-linolenate ($18:3^{\omega\ 3,6,9}$) content in their seed oil when compared with wild type soybeans. A gene controlling linolenic acid in A5 is designated *fan*(A5) and is independent of the linolenic acid gene *fan2*(A23). DNA gel-blot hybridizations using the microsomal ω-3 linoleate desaturase cDNA indicate a missing DNA fragment in the A5 when compared to A23 and lines wildtype for linolenic acid. It is likely, therefore, that the phenotype of the A5 mutant is a result of a deletion of a microsomal ω-3 linoleate desaturase gene.

Soybean frequently suffer heavy losses from white mold under conditions that are favorable for disease development. Systemic acquired resistance (SAR) is a mechanism that is characterized by the development of a disease-resistant state in plants. It was our goal to exploit this mechanism via the use of a novel synthetic immunization compound 2,6-dichloro-isonicotinic acid INA. We report here that the application of 2,6-dichloro-isonicotinic acid (INA) to soybeans results in protection against white mold.

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CHAPTER 1. ANALYSIS OF THE OMEGA-3 FATTY ACID DESATURASE IN SOYBEAN GENOTYPES A5 AND A23

BACKGROUND

The fatty acid composition of an oil determines its physical and chemical properties and thus it uses. Plants are attractive experimental objects for genetic studies of lipid metabolism for several reasons. First, because plant oils are of major economic importance and because the oil composition is less than optimal in many cases, there is substantial interest in exploring the degree to which genetic methods can be used to modify the oil composition of seeds. Second, in many cases, relatively large alterations can be made in the fatty acid composition of the storage triacylglycerol without exerting any obvious deleterious effects on the growth and development of the organism. The major edible oils have rather similar fatty acid compositions, in which 18 carbon fatty acids with one to three double bonds predominate. Thus, for many edible uses the various major oil sources are interchangeable and their consumption and competitive status in the market-place is largely controlled by price. However, the specific fatty acid composition of an edible plant oil may sometimes give it special status. Concerns about the possible contributions of saturated fats to heart disease have also put a slight premium on those vegetable oils with the lowest levels of these constituents. For example, the entry of canola oil into the edible oils market, although dependent on economic factors, has been

buoyed by publicity concerning its low saturated fatty acid content.

Soybean produces seed oil in which the fatty acid composition is not ideally suited for the intended use. Linolenic acid has been identified as the unstable component of soybean oil responsible for undesirable odors and flavors. Therefore, there has been an ongoing interest in reducing linolenic content below 3%. One of the most striking examples of the utility of the mutagenic approach is the isolation of two ethyl-methanesulfonate-induced mutants of soybean. When the two mutations were combined to make the double mutant, a line almost completely deficient in 18:3 in seed lipids was obtained. There was apparently no deleterious effect on the viability, oil content, or germination of the seeds. The simplest interpretation of these results is that there are two genes encoding two desaturases in the seeds and the products of these genes have been inactivated by the mutations. Since there was little or no effect on leaf fatty acid composition, it seems likely that there are additional desaturase genes which are leaf specific. Biochemical and genetic studies of these mutants are important to our understanding of the 18:2 desaturation step because the enzyme is an integral membrane protein that has been difficult to solubilize and, therefore, to investigate by traditional enzymological methods. In the absence of a purified enzyme, genetic techniques can be an alternative means to study the relevant genetic locus. However, it seems likely that, because of the inherent limitations of mutation

breeding, many other desirable changes in seed oil fatty acid composition may require the directed application of genetic engineering methods. Presently, specific information about the biochemistry and regulation of lipid metabolism has made it possible to predict the result of the introduction of one or a few genes that might usefully alter seed lipid synthesis. This would allow scientists to design new products that can provide expanded or new markets for excess agricultural output.

INTRODUCTION

Soybean produces seed oil in which the fatty acid composition affects the nutritional value as well as its physical and chemical characteristics (Neff et al. 1992). The polyunsaturated fatty acid α-linolenate($18:3^{\omega 3,6,9}$) is synthesized by plants, but not by most other higher eukaryotes. This fatty acid is an essential component of human nutrition because in mammals it acts as a precursor to membrane lipids and families of signaling molecules (Smith and Borgeat, 1985). It is also commercially important because it has been identified as the unstable component of soybean oil responsible for undesirable odors and flavors (Liu et al., 1992). For these reasons, variation in the linolenic acid content has been a target for genetic selection.

The application of conventional breeding methods, coupled in some cases with mutagenesis, has resulted in the production of new lines with desirable alterations in the fatty acid composition of the seed oil (Hammond and Fehr, 1983). Most of the genetic alterations in seed fatty acid composition appear be the result of mutations disrupt normal fatty acid metabolism and leads to an accumulation of intermediate fatty acid products in seed storage lipids (Ohlrogge et al., 1991). It is unclear, however, what structural genes have

been mutated in existing public soybean germplasm.

The biosynthesis of linolenic acid is the result of a desaturation reaction catalyzed by a membrane associated omega-3 desaturase which introduces the third double bond into the 18-carbon fatty acid. In leaf tissue, there are two distinct pathways for polyunsaturated fatty acid biosynthesis, one located in the microsomes and the other located in the plastid membranes. In *Arabidopsis thaliana*, the plastid ω-3 fatty acid desaturations are controlled by the *FAD 7* locus (Browse and Somerville, 1991). The major enzyme responsible for the synthesis of seed linolenic acid is the microsomal ω-3 linoleate desaturase. Its activity is controlled in *Arabidopsis* by the *FAD 3* locus (Lemieux et al., 1990) which may be equivalent to one or both of the *FAN* loci in soybean.

The lowest published content of α-linolenate($18:3^{\omega 3,6,9}$) in soybean was identified at Iowa State University from the cross of two EMS induced mutant lines, A5 and A23 (Fehr et al., 1992). A5 has a major gene controlling reduced linolenic acid, at a locus independent of *fan2*(A23). The locus in A5 is designated *fan*(A5). When the two mutants were crossed, segregates with $<25g\ kg^{-1}$ linolenic acid in seed lipids were obtained which were designated A16 and A17. The simplest interpretation of these results is that there are at least two genes encoding the microsomal ω-3 linoleate desaturase in the seeds and that the products of these genes have been inactivated by the mutations. The objective of this project was to describe the molecular nature of the ω-3

fatty acid desaturase in soybean genotypes A5 and A23.

MATERIALS AND METHODS

Genetic Material. The genotypes utilized for the study included A5, A23, A16, and A17. A23 has a major gene controlling reduced linolenic acid content, at a locus independent of *fan*(A5). The locus in A23 is designated *fan2*(A23). A16 and A17 are considered to have the genotype: *fan*(A5)*fan*(A5)*fan2*(A23)*fan2*(A23). A population of 70 $F_{4:5}$ derived individuals segregating for *fan*(A5) and *fan2*(A23) was constructed from a cross between Pioneer variety 9231 which has wildtype linolenate levels x double mutant A89-269077. Seed and fatty acid composition of each line in the population was kindly provided by Pioneer Hi-Bred International.

DNA Gel-Blot Analysis. Total genomic DNA from young soybean leaves was isolated as previously described by Anderson et al. (1992). Restriction digests, electrophoresis on agarose gels, Southern blots, hybridizations, and autoradiography was according to Berantzky and Tanksley (1986) with minor modifications. Ten micrograms of parental DNA was digested with EcoRI, Hind III, Taq I, EcoRV and Dra I. DNA from the parental genotypes was analyzed by gel-blot hybridization to the cDNA coding for the soybean microsomal ω-3 linoleate desaturase (Yadav et al., 1993) to identify polymorphisms. When polymorphisms were identified, DNA from each segregant in the $F_{4:5}$ population was digested with EcoRI and Dra I. The

populations were then analyzed by gel-blot hybridization with the microsomal ω-3 linoleate desaturase cDNA. Analysis of variance was used to test for significant association between the segregation of the cDNA and the linolenate content of the lines. Data were analyzed using the general linear model procedures of the statistical analysis system (SAS).

PCR Analysis. Eight random 30-mer oligonucleotides were designed so that four primers were in the forward orientation and four primers were in the reverse orientation based on the cDNA sequence of the microsomal ω-3 linoleate desaturase. All possible primer combinations were amplified. Amplification was carried out using standard reaction conditions in a Perkin Elmer Cetus DNA Thermal Cycler 480 programmed for 94°C/4 min and then 30 cycles of 94°C/1 min, 60°C/1 min, 72°C/3 min.

RNA Gel-Blot Analysis. Soybean embryo tissue was harvested and frozen in liquid nitrogen from the following genotypes: A5, A23, A16, and A17 at 10 DAF and 30 DAF (DAF = Days After Flowering). Total RNA was isolated as described by Ausubel et al. (1991). Yield was determined by spectrophotomeric A_{260}. After electrophoresis of 20μg of total RNA per lane in a 1.2% formaldehyde agarose gel, RNA was transferred to Hybond-N^+ membrane (Amersham) according to the manufacturers specifications. Nucleic acid hybridizations were as described previously (Yadav et al., 1993). To ensure that each lane of the RNA gel blots contained similar amounts of

undegraded RNA, filters that had been hybridized to the microsomal ω-3 linoleate desaturase probe were subsequently stripped and rehybridized with a ^{32}P labeled soybean actin gene (Psac 3). The amount of hybridization was determined by measuring the amount of bound radiolabled probe using a Molecular Dynamics (Sunnyvale, CA) PhoshorImager.

RESULTS AND DISCUSION

A5 Mutant. Previous studies have shown the low linolenic acid phenotype of *fan*(A5) to behave like a quantitative character (Fehr et al., 1992). The complexity of the biochemical interactions and the genetics of this mutant suggested that the mutation may not have directly affected a structural gene for a desaturase, but the regulation of expression of desaturase activity (Ohlrogge et al., 1991). Our study indicates a deletion of the structural gene encoding the microsomal ω-3 linoleate desaturase has occurred in this mutant. DNA gel-blot hybridizations using the microsomal ω-3 linoleate desaturase cDNA indicates a missing DNA fragment in the A5, A16, and A17 mutants when compared to A23 and lines wildtype for linolenic acid (Figure 1). The 70 $F_{4:5}$ individuals segregating for the *fan*(A5) and *fan2*(A23) loci were scored for the presence or absence of the fragment. The absence of the fragment was significantly ($P \leq 0.0001$) associated with reduced linolenic acid and accounted for 67% of the variation for linolenic acid in the population. The mean of the lines without the DNA fragment was 43 g kg^{-1} and the mean of the lines with the DNA fragment was 77g kg^{-1}. PCR was utilized to further investigate the deletion in *fan*(A5). All possible pairwise oligonucleotide combinations generated one DNA fragment in genotypes A5, A16, and A17 (Figure 2). In contrast, the wildtype and A23 mutant generated two DNA fragments. The reduction of linolenic acid

of the *fan*(A5) mutant, is thus, the result of a single codominant nuclear mutation at the microsomal ω-3 linoleate desaturase that controls the level of linolenic acid in seeds. However, the PI line from which A5 was derived via EMS treatment was not available for this study. We cannot completely rule-out the possibility that the deletion was present prior to the EMS treatment.

A microsomal ω-3 linoleate desaturase gene mapped to linkage group B2 in the USDA-ARS *G.max /G.soja* population (Byrum et al., 1995). This is consistent with the placement of *fan*(A5) at the same location in another population (Brummer et al., 1995). This further suggests that the low linolenic acid phenotype obtained with the *fan*(A5) locus is the result of a mutation in the microsomal ω-3 linoleate desaturase gene.

Figure 1. DNA gel-blot analysis of the soybean genotypes using the cDNA encoding the microsomal ω-3 desaturase. Lanes 1 - 6 were digested with Dra I. Lanes 7 - 12 were digested with Hind III. Lanes 1 - 6, 7 - 12 represent the double mutant parent, wildtype parent, A5, A23, A16 and A17, respectively.

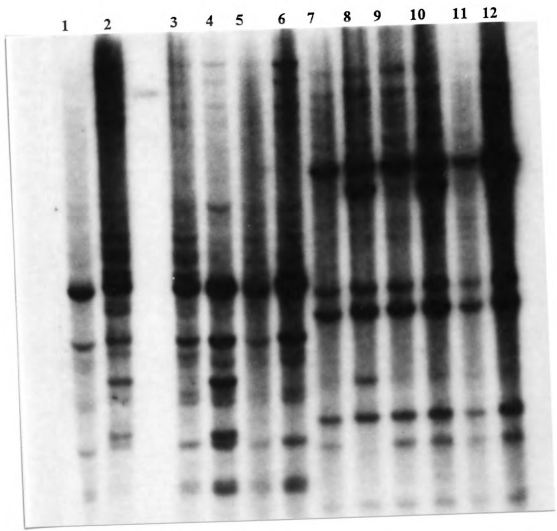
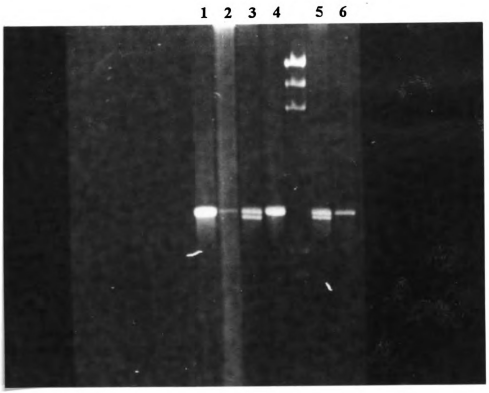


Figure 2. PCR analysis of the parental genotypes using 30-mer oligonucleotides flanking the 5' and 3' end of the cDNA encoding the microsomal ω-3 desaturase. Lanes 1 - 6 represent A17, A16, A23, A5, wildtype parent, and double mutant parent.



A23 Mutant. No polymorphisms were identified with the microsomal ω-3 desaturase cDNA to map *fan2*(A23) in the $F_{4:5}$ derived population segregating for alleles of the *fan*(A5) and *fan2*(A23) loci. Therefore, the locus from which *fan2*(A23) is derived was not definitively determined.

Northern analysis indicated no differential changes in steady state contents of the RNA levels from the microsomal ω-3 desaturase in A5, A16 and A17 when compared with A23 and wildtype lines. Since A5, A16 and A17 have a deleted microsomal ω-3 desaturase locus this would indicate the existance of more than one gene. This observation is consistent with studies by (Rennie and Tanner 1989) which reported the *fan*(A5) mutant phenotype to be temperature sensitive. The temperature effect is independent of *fan*(A5) and, thus, the result of another desaturase locus possibly but not necessarily *fan2*(A23).

CHAPTER 2. RESISTANCE TO SCLEROTINIA STEM ROT IN SOYBEAN IS INDUCED BY 2,6-DICHLORO-ISONICOTINIC ACID

BACKGROUND

An important aspect of successful agriculture is the control of plant diseases that reduce productivity, quality, and profitability. The use of intensive farming and mass monoculture resulted in the extensive use of chemical pesticides to control plant pests and diseases. The use of chemical pesticides, however, will become increasingly restricted due to concerns for the environment and health. The need for extensive toxicological and field testing prior to release of new chemicals and development of pest or pathogen resistance to some current pesticides may also contribute to a reduction in pesticide utilization. Environmentalists campaign for reduction in the use of pesticides and fertilizers that can find their way into air, water, and soil. A segment of the public clamors for food that contains no pesticide residues, yet wants an abundant supply of inexpensive, high-quality food. Extremists believe that farmers should not use any synthetic chemicals. However, sincere these proponents may be, such paradigms do not completely reflect contemporary reality. This is not to say that agriculture cannot change. The changes of the 20th century have been revolutionary in scope. The changes of the 21st century will be even more profound. While it is clear that changes are needed, is also is clear that we cannot abruptly shift into a lower gear that would cause starvation and economic collapse. Change must be based on sound research

and economic considerations. We are faced with the challenge of finding more effective, safer, and economical ways to protect plants against pests to help feed an ever increasing world population. Utilization of plants own defense mechanisms by application of technologies to induce resistance may contribute toward a solution to this problem (Metraux et al. 1991). Plant immunization can be a natural, safe, effective, persistent and durable alternative to pesticides in controlling plant diseases. Chemical inducers may provide better means of application possibilities for induction of resistance, providing that they are easily accessible and not harmful. Plant immunization may find its niche in sustainable agriculture as one of a variety of practices that promote plant health and reduce plant disease.

It is a lesser known fact that plants can also be immunized against disease-causing pathogens or feeding pests (Kuc, 1982). This phenomenon is the result of the development of systemic acquired resistance (SAR) which is characterized as an increase in resistance to subsequent pathogen attack in inoculated and uninoculated parts of the plant. SAR in plants was reviewed almost 60 years ago by (Chester, 1933). SAR to pathogens usually develops after the appearance of a necrotic lesion around the inoculation site. This localized cell suicide is called the hypersensitive response (HR). While the HR effectively traps pathogens in and around lesions, it makes the whole plant more resistant to a wide range of disease-causing microorganisms (Madamanchi and

Kuc, 1991; White and Antoniw, 1991). These local resistance responses develop more rapidly than SAR and involve cell wall and cuticle strengthening, synthesis to toxins, antifeedants, and the production of defense-related proteins, which include the pathogenesis-related (PR) proteins (Lamb and Dixon, 1990; Madamanchi and Kuc, 1991; White and Antoniw, 1991; Ross 1966; Kuc 1982; Ward et al., 1991; Paxton and Chamberlain, 1967; Svoboda and Paxton, 1972). Local resistance may be partially mediated through relatively immobile endogenous elicitor which include oligogalacturonide fragments of the plant cell wall (Lamb and Dixon, 1990). The combined effects of local and systemic resistance mechanisms protect the area around the HR lesion best against a subsequent pathogen attack (Ross, 1961). For, example, SAR induced by necrotizing viruses develops in uninfected tissues two to three days after inoculation, lasts for several weeks, and may provide protection against pathogenic bacteria, fungi, and other viruses.

Pathogen invasion triggers a complex pattern of responses in the host plant that result in either the disease state or in the development of disease resistance (Lamb et al., 1989). The choice between disease and resistance represents the end-product of a complex dialog between host and pathogen (Dixon and Lamb, 1990). A diverse array of pathogen virulence functions and plant defense functions have been described. More recently, dissection of the complex interplay between plant and pathogen has become possible through the

combined application of genetic and biochemical techniques (Daniels et al., 1988). In many cases, disease resistance depends on early recognition of the pathogen by the plant followed by elaboration of a rapid defense response (Klement, 1982). It has been postulated that for certain plant/pathogen interactions, specific recognition is mediated by pairs of "complementary" genes: a pathogen-encoded avirulence (avr) gene and a host-encoded disease resistance gene (Keen, 1990). Only interactions which involve both genes lead to pathogen recognition by the host, induction of an active defense response, and disease resistance. In several instances, race/cultivar specificities can now be explained by interactions between defined avirulence gene/resistance gene pairs.

Pathogen-induced HR and SAR are associated with the local and systemic appearance of at least five families of proteins referred to as PR proteins. Tissue localization, timing of appearance and known functions of some PR proteins suggest their involvement in SAR. However, definitive proof that the systemic induction of PR proteins causes SAR is still lacking. Plants rely on a long-distance signal molecules that at low concentrations, can activate resistance mechanisms in cells not directly invaded by the pathogen. The existence of these signals was first hypothesized by Ross (1966) and demonstrated in grafting experiments (Guedes et al., 1980). A signal in SAR should be synthesized by the plant, increase systemically following attack by a

pathogen, move throughout the plant, induce defense-related proteins and phytochemicals, and enhance resistance to pathogens.

INTRODUCTION

Sclerotinia stem rot or white mold caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is becoming an increasingly important soybean disease in Michigan. *S. sclerotiorum* may cause extensive plant mortality during pod development resulting in significant yield reduction. Soybean cultivars vary in their reaction to *S. sclerotiorum* (Grau et al., 1984; Chun et al., 1987; Buzzell et al., 1993). Cultivars range from moderately tolerant to extremely susceptible within maturity groups O through II. However, less tolerance is reported within maturity group III and later maturity groups (Nelson et al., 1991). It is unclear whether tolerance is due to avoidance mechanisms such as plant architecture, maturity, or lodging characteristics. A major unresolved issue is whether or not physiological resistance accounts for observed differences in cultivar reactions to *S. sclerotiorum*.

Disease control measures have traditionally exploited genetic resistance to pathogenic organisms present in the environment. However, plants also possess an inducible resistance mechanism called systemic acquired resistance (SAR) which is a nonspecific "immunity" to infection. Once induced, the resistance is effective against a broad range of pathogens (Ryals et al., 1992) and can last from a week to months after induction. SAR to disease has been documented for more than 100 years (Chester, 1933) and has been extensively

studied in tobacco and cucumber (Ross 1966; Kuc 1982; Ward et al., 1991).

Evidence for induced resistance in leguminous plants was demonstrated when common bean plants became resistant to virulent races of *Colletotrichum lindemuthianum* after preinfection of hypocotyls with spores of avirulent races of the same species (Elliston et al., 1971; Elliston et al., 1976; Elliston et al., 1976; Elliston et al., 1977; Raje et al., 1969; Sutton, 1979). A bean cultivar normally susceptible to all races of *C. lindemthianum* could also be made anthracnose-resistance with a preinfection of the cucumber pathogen, *Colletotrichum lagenarium*. This result shows that the presence of a strain-specific resistance gene is apparently not required for SAR to develop. Elliston et al., (1976a) tested a number of different species and strains of *Colletotrichum* in the same type of experiment. These researchers demonstrated that two species failed to give localized protection; while, two others and a race of *C. lagenarium* gave inconsistent protection. Conversely, *C. trifolii* and two other races of *C. lagenarium* gave localized protection. If the process underlying induction of systemic resistance is similiar in bean and curcurbits, then it is not likely to be confined in its effectiveness to species of *Colletorichum* as pathogens. More recently, Kutzner et. al., (1993) described SAR in bean against TNV using bean rust as inducer.

Induced resistance in cultivars lacking resistance genes was examined under microscopy of epidermal strips by Ellistion et al., (1976b). Protection

caused by *C. lagenarium* became evident 84 to 96 hours after inoculation with *C. lindemuthianum*. When the primary mycelium of the *C. lindemuthianum* ceased growing, the contents of the invaded plant cells became granular and brown. Cloud and Deverall (1987) repeated the earlier experiments of Sutton (1979) confirming the effectiveness of droplet inoculation on the first leaves in greatly diminishing symptom development on the second leaves when these were inoculated a week later.

Several lines of evidence indicate that SAR induces putative defense compounds in soybean. Soybean seedlings that were initially inoculated with *Phytophthora cactorum* were protected from subsequent infections of *P. megasperma*. This was associated with the accumulation of phytoalexins (Paxton and Chamberlain, 1967; Svoboda and Paxton, 1972). More recent work has shown that resistance induced against *P. megasperma* results in the increased levels of pterocarpanoid phytoalexins, β-1,3-endoglucanase, isoflavones, daidzein, and genistein (Hahn et al., 1985; Bonhoff and Grisebach, 1988; Yoshikawa et al., 1990; Morris et al., 1991; Graham and Graham, 1991). These defense-related proteins and phytochemicals induced during SAR enhance resistance to pathogens.

In addition to biotic inducers, certain chemicals with no direct antibiotic effect can also induce resistance in plants. These include natural products such as salicylic acid (SA) (White, 1979) and synthetic immunomodulators such as

2,6-dichloro-isonicotinic acid (INA) (Metraux et al. 1991). Experiments with INA in the common bean system showed that the application to the first leaves not only protected the second leaves against *C. lindemuthianum*, but also against *Pseudomonas syringae* pv. phaseolicola, the cause of halo blight, and *Uromyces appendiculatus*, the cause of beans rust disease (Dann, 1991). Additionally, INA treatments were equally or more effective as *C. lindemuthianum* in inducing systemic resistance against the same foliar pathogens, that is, the anthracnose and rust fungi (Dann and Deverall, 1995).

The discovery of new chemical compounds that stimulate natural disease resistance mechanisms will provide innovative strategies for crop protection. Pretreatments of crop plants to improve resistance against pathogens is an attractive disease control practice, especially when protection covers resistance against a wide range of pathogens (Kuc, 1982; Metraux et al., 1991; Ryals et al., 1992). Such an approach would complement conventional chemical control and assist crop management strategies. Progress toward implementing these strategies has been limited. Considering the eventual breakdown of genetic disease resistance coupled with the finite sources of genetic tolerance against *S. sclerotiorum*, an alternative means of control would be desirable in soybean. The objective of this study was to determine if the application of 2,6-dichloro-isonicotinic acid (INA) to soybean would induce resistance to *S. sclerotiorum.*

MATERIALS AND METHODS

General Experimental Procedure. A two-year study was conducted at East Lansing, MI on a Capac loam (fine -loamy, mixed, mesic Aeric Ochraqualfs) and at Zilwaukee, MI on a Sloan silt loam (fine-loamy, mixed, mesic Fluvaquentic Haplaquolicorganic). Four soybean cultivars were chosen for this study that varied in disease reaction to *S. sclerotiorum*: NK 1990, Elgin 87, Corsoy 79, and Williams 82 (Table 1). A combination secondary tillage tool with cultivator shovels, cutting blades, spike tooth harrow, and rolling baskets was used to prepare the seedbed at both locations. Soybean cultivars were solid-seed planted at 385,000 seeds ha^{-1} on May 18, 1993 and May 15, 1994 at both locations. The experiment was maintained weed-free with a postemergence application of 0.78 kg ai ha^{-1} bentazon with 0.21 kg ai ha^{-1} acifluorfen plus crop oil concentrate at 2.9 L ha^{-1}, followed by hand weeding twice.

Table 1. Cultivars utilized in tests. Disease susceptibility ratings are based on grower observations and can be influenced by any number of cultural practices and environmental conditions.

SOYBEAN CULTIVARS		
CULTIVAR	**MATURITY**	**DISEASE SUSCEPTIBILITY**
NK 1990	I	Highly Tolerant
Corsoy 79	II	Moderately Tolerant
Elgin 87	II	Moderately Susceptible
Williams 82	III	Highly Susceptible

Field Disease Pressure. Rainfall was supplemented with overhead irrigation to total 4 cm of water per wk at East Lansing during flowering in 1993 and 1994. The test at Zilwaukee was managed under rainfed conditions in 1993 and 1994 due to the relatively high water table at this location. Historically, the Zilwaukee research site had a high level of natural *S. sclerotiorum* infestation and was planted to susceptible crops in previous years. To ensure an adequate level of inoculum in soil at East Lansing, sclerotia were collected from cull pile screenings at a dry bean processing plant in the fall of 1990 - (present) and lightly incorporated into soil with a cultivator.

Mycelial Growth on INA-Amended PDA. Wild-type isolates were used to determine if INA had any direct antibiotic effect on *S. sclerotiorum*. Isolates were maintained on potato-dextrose agar (PDA) at 4 C and cultured on PDA at 22 to 25 C. Four PDA (Difco) solutions were prepared at INA concentrations of O, 130 mM, 260 mM, and 520 mM, autoclaved at 121 C for 20 min, then poured into petri dishes and allowed to cool. Mycelium were grown by transferring agar disks (3 mm diameter) cut from the actively growing colony margins of individual isolates and placed in the center of each PDA plate with the mycelium in contact with the surface of the medium. Four replicates of each INA concentration were placed under fluorescent lights. Colony diameters were determined after 3 days by taking the mean of two perpendicular measurements.

General Chemical Procedure. INA (2,6-dichloro-isonicotinic acid) (CIBA-GEIGY AG, Basel, Switzerland) was formulated as a wettable powder with 25% active ingredient. It was applied to 1.5 m by 6.1m plots with a hand-held, CO_2-pressurized sprayer equipped with a four-nozzle boom with 8004 type flat fan nozzles delivering a volume of 106 L ha^{-1} at 207 kPa in 1993 and 1994. The suspensions were prepared with sterile tap water. Crop injury was assessed 14 and 28 d after treatment both years, using a 0 to 100% scale, with 0 = no injury and 100% = plant death.

1993 Field Study. In 1993, treatments of INA at the East Lansing and Zilwaukee sites were arranged in a randomized complete block design with two and five blocks, respectively. INA was applied at 78 mM, 130 mM, or 182 mM on July 5 and then the same rate applied again on July 15 and July 25. The East Lansing site was harvested on November 13, 1993.

1994 Field Study. The experimental design in 1994 was a randomized complete block design. This experiment included higher INA concentrations, an earlier initial INA application, and a varying number of applications during the infection period. Treatments at the East Lansing and Zilwaukee sites were arranged in a randomized complete block design with four replications at each location. INA was applied at 182 mM, 234 mM, and 339 mM either once on June 25, three times on June 25, July 4, and July 15 or four times which included a July 28 application. The experiment was harvested on November

11, 1994 at both locations.

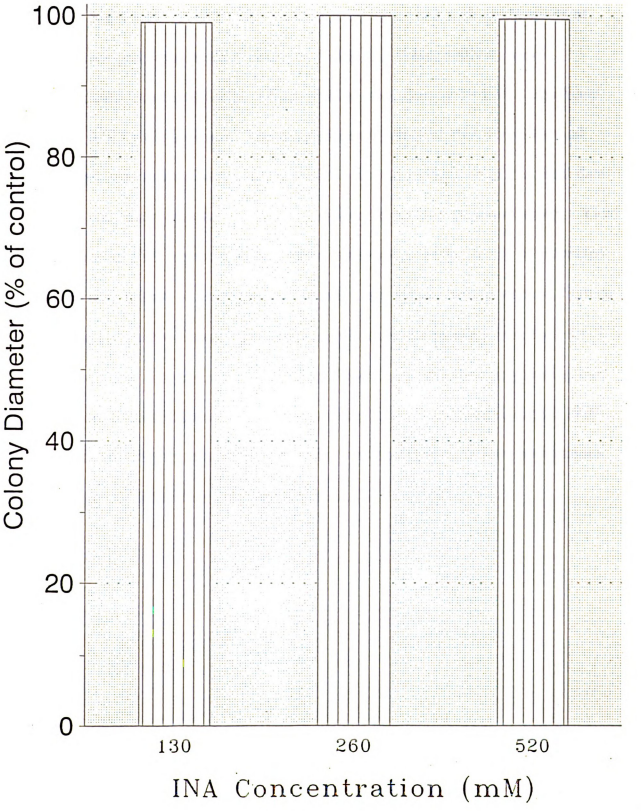
Disease Ratings. Disease was evaluated on September 9, 1993 and September 30, 1994 at the East Lansing Site and on September 11, 1993 and September 28, 1994 at the Zilwaukee site. Individual plants were rated for *S. sclerotiorum* on a 0-3 scale, where 0 = no symptoms, 1 = lesion(s) only on lateral stems, 2 = lesion(s) on main stems, and 3 = diseased plant dead (Sherwood and Hagedorn, 1958). A score of 0 would be given to a plot where no plants rated have disease symptoms and 100 to a plot where all plants rated were dead from the disease. Fifty plants in 1993 and thirty plants in 1994 were scored at random from the center three rows of each plot. A disease severity index (DSI) was calculated by the method of Sherwood and Hagedorn (1958).

Statistical Protocol. Data were analyzed using the analysis of variance and general linear model procedures of the statistical analysis system (SAS). Data from each year was analyzed separately due to the different experimental designs.

RESULTS and DISCUSSION

INA did not affect maturity, height or lodging of any cultivar in 1993 and 1994 (Data not presented). Treatments with INA were not accompanied by any crop injury at the highest concentrations in 1993 and 1994. Evaluations of fungal isolates indicate that the effect of INA is unlikely to result from a direct antibiotic effect. There was no difference in colony diameters at any concentration three days after plating (Figure 3).

Figure 3. Colony diameters of *S. sclerotiorum* grown on INA-amended PDA for 3 days, expressed as percentage of unamended control (8 cm).


100
80
60
40
20
0
Colony Diameter (% of control)
130
260
520
INA Concentration (mM)

Significant ($P \leq 0.01$) yield increases were seen with applications of INA averaged over the four cultivars (Table 2). When cultivars were individually evaluated for the effect of INA, only Corsoy 79 had a significant ($P \leq 0.01$) yield increase. The nonsignificant effect of INA for individual cultivars was at least partly because of the limited replication of the yield data. Yield was taken only for the two replications of plots in Ingham County. Yield response in INA treated soybeans were presumed to be the result of increased protection against *S. sclerotiorum*. Ideally, a producer would want to "induce resistance" before flowering. The ascospores are the primary inoculum for *S. sclerotiorum*, first colonizing senescing flower parts and then progressing into the stem tissues (Tourneau, 1979; Lumsden, 1979; Abawi and Grogan, 1979). Significant disease reduction ($P \leq 0.01$) was observed with INA applications averaged over the four cultivars (Table 3).

Table 2. Yield results from two blocks at the East Lansing site in 1993.

* Significantly different from control at LSD P ≤ 0.01.

Cultivar	INA Rates (mM)			
	0	78	130	182
	----------(kg ha^{-1})----------			
NK 1990	4048	4442	4396	4434
Corsoy 79	3287	3954	4101*	4037
Elgin 87	3550	3839	4063	3521
Williams 82	2783	3237	3247	3121
Mean	3417	3868*	3952*	3778

Table 3. DSI ratings of cultivars in 1993 at East Lansing and Zilwaukee. Fifty plants were scored at random from the center three rows of each plot. Reaction of cultivars to *S. sclerotiorum* was rated on a 0-3 scale, where 0 = no symptoms, 1 = lesion(s) only on lateral stems, 2 = lesion(s) on main stems, and 3 = diseased plant dead (Nelson et al. 1991). A disease severity index (DSI) was calculated by the method of Sherwood and Hagedorn (1958). * Significantly different from control at LSD P ≤ 0.01.

Cultivar	INA Rates (mM)			
	0	78	130	182
	----------(DSI)----------			
NK 1990	0.3	0.3	0.3	0.5
Corsoy 79	1.8	0.9	0.6	1.1
Elgin 87	14.5	8.7	3.6*	2.7*
Williams 82	37.9	20.9*	16.6*	17.7*
Mean	13.6	20.9*	5.3*	5.5*

In 1994 soybean response to INA was influenced by application timing, but not by application rate. The initial application on June 25 was applied approximently six to ten days in advance of flowering. One application INA on June 25 was not sufficient to provide significant protection against *S. sclerotiorum* in any cultivar regardless of INA application rate. A significant yield increases was seen with applications of INA for Williams 82 (Table 4). Significant disease reduction ($P \leq 0.01$) was also observed for these INA applications in Williams 82 (Table 5).

Table 4. Yield results from the 1994 tests at East Lansing and Zilwaukee sites. Rates (182 mM, 234 mM, and 339 mM) are combined within each application of each cultivar since there was not a significant rate effect. n = 8 for the control (0) and n = 24 for applications 1, 3, and 4. * Significantly different from control at LSD $P \leq 0.01$.

Cultivar	Number of INA Applications			
	0	1	3	4
	----------(kg ha^{-1})----------			
NK 1990	3662	3650	3571	3486
Corsoy 79	2888	2876	2877	2757
Elgin 87	2762	2900	2956	3062
Williams 82	1922	2057	2325	2493*
Mean	2809	2871	2932	2950

Table 5. DSI ratings of cultivars in 1994 at East Lansing and Zilwaukee. Thirty plants were scored at random from the center three rows of each plot. Reaction of cultivars to *S. sclerotiorum* was rated on a 0-3 scale, where 0 = no symptoms, 1 = lesion(s) only on lateral stems, 2 = lesion(s) on main stems, and 3 = diseased plant dead (Nelson et al. 1991). A disease severity index (DSI) was calculated by the method of Sherwood and Hagedorn (1958). Rates (182 mM, 234 mM, and 339 mM) are combined within each application of each cultivar since there was not a significant rate effect. n = 8 for the control (0) and n = 24 for applications 1, 3, and 4. * Significantly different from control at LSD P ≤ 0.01.

Cultivar	Number of INA Applications			
	0	1	3	4
	----------(DSI)----------			
NK 1990	13.8	14.5	9.1	9.5
Corsoy 79	26.5	33.8	30.5	31.0
Elgin 87	50.0	54.3	50.7	45.3
Williams 82	65.0	58.5	51.4	49.9*
Mean	38.8	40.3	35.4	33.9

The contrasting yield results between 1993 and 1994 could be the result of: (i) the effect of INA was not sufficient enough to overcome the increase disease pressure observed in 1994 versus 1993 or (ii) the environmental conditions were not conducive for allowing the INA effective entry and distribution into the plant. The INA derivative utilized in this study was in the free acid form. One factor that would enhance the effectiveness in the field would be the use of an appropriate surfactant for soybean.

S. sclerotiorum is an important fungal pathogen of many plant species and is responsible for substantial losses in crop yields in North America each year (Purdy, 1979). Disease symptoms on individual plants are often severe under suitable environmental conditions. The destructive nature of these symptoms is facilitated by its extensive host range and the production of persistent sclerotia that produce apothecia. Cultural practices aimed at minimizing *S. sclerotiorum* infection have had a marginal impact depending on the environmental conditions. Additionally, the ability to identify and manipulate heritable genetic resistance have been slow. The advent of synthetic immunomodulators such as 2,6-dichloro-isonicotinic acid (INA) which activate inducible defenses could result in a broad spectrum of protection not limited to *S. sclerotiorum* in soybean. Evidence from a related signal molecule (SA) indicates that the exogenous application of SA stimulates resistance to a variety of lesion-inducing viral, bacterial, or fungal pathogens (Mills and Wood, 1984;

Penazio et al., 1987; Ye et al., 1989; Rasmussen et al., 1991). Confirmation of broad spectrum control of disease in cucumber and arabidopsis with INA was shown by Metraux et al. (1991) and Ward et al. (1991). In our tests, 2,6-dichloro-isonicotinic acid (INA) protected the most susceptible soybean cultivars from *S. sclerotiorum* under field conditions, suggesting that INA functions indirectly by increasing the plants resistance.

CONCLUSION

Two lines of evidence suggest that the fungicidal response observed in INA immunization results not from direct effects on the pathogen but from a reaction of the plant to the chemical. First, neither INA nor its metabolites have direct antibiotic activity (Metraux et al., 1991). Second, INA has been shown to cause dramatic changes in plant gene expression that are similar to the changes observed during pathogen-induced immunization (Ward et al., 1991). Thus, INA may induce resistance by mimicking some aspects of pathogen attack, possibly accelerating the normal responses of the plant to further infection; therefore, the biological responses of soybean to chemical immunization should be investigated. Plants should be treated with varying concentrations of INA and, at increasing intervals after treatment, should be inoculated with a pathogen such as *Peronospora parasitica* that causes downy mildew. Trypan blue staining can be used to visualize the plant-fungus interaction because it penetrates fungal cells easily and preferentially stains plant cells that have suffered membrane damage (Keogh et al., 1980). Additionally, in tobacco, INA and other immunizing agents induce the dramatic accumulation of mRNA's that encode a variety of extracellular proteins (Ward et al., 1991). Using PAGE examination, proteins present in the intercellular wash fluid after INA treatment should be compared with soybeans plants that

had been treated with a water control. This would lead up to experiments defining the onset of SAR at the molecular level which can be achieved by the isolation and characterization of proteins followed by cDNA identification with oligonucleotides, cross-hybridization of known cDNA clones to previously unknown related genes and differential screening of cDNA's expressed in tissue rendered systemically resistant by INA treatment. The identification of cDNA's would allow one to implement time-course studies of resistance development.

A significant issue is arising from observations of hyperinducibility of defense genes and whether the hyperinducing signal combinations can induce resistance to pathogens. Many genes are induced by several apparently unrelated signals, such as osmotic stress and pathogen invasion, bringing into question the relationship between inductive signals and gene function (Mason et al., 1992; Xu et al., 1994). There is evidence that there are hyperinduced states of plant defense genes that result from particular signal combinations. Signal combinations may synergistically hyperinduce other plant defense genes and that such synergistic signals may be more specifically related to gene function than any single inductive signal, for example, using combined treatments of SA and INA.

Another aspect of the induced resistance state is understanding the nature and transmission of the systemic signal or signals. Elicitors of diverse chemical nature, including carbohydrates, lipids, oligosaccharides and proteins and have

been shown to trigger plant defense responses at nanomolar concentrations (Ebel and Cosio, 1994). The effects of elicitors on ion fluxes across the plasma membrane have been considered to be part of elicitor-specific signal transduction leading to the induction of plant defense responses in several plant-pathogen interactions (Farmer et al., 1989; Dietrich et al., 1990; Felix et al., 1991; Mathieu et al., 1991; Renelt et al., 1993). Thus, nonchemical signals can not be excluded at this time and would be of great interest for further investigation.

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