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Disease- and Performance-Related Traits of Ethylene-Insensitive Soybean

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ABSTRACT

Ethylene controls many beneficial responses in plants but also promotes chlorosis, senescence, disease, and fruit over-ripening. The present study compared previously isolated ethylene-insensitive lines of soybean [*Glycine max* (L.) Merr.] to their isogenic, ethylene-sensitive parent with respect to disease resistance, seed yield, and other field performance traits. In laboratory tests, ethylene insensitivity reduced root colonization by soybean cyst nematode. Using healthy young plants, ethylene-insensitivity also reduced ethylene-activated leaf chlorosis and abscission. However, in the field, leaf chlorophyll and late-season senescence were not altered, suggesting that ethylene is not a main determinant of late-season senescence of soybean leaves. Field studies also revealed no changes in susceptibility to Septoria brown spot disease (caused by *Septoria glycines* Hemmi), flowering date, plant height, or seed total protein and oil concentration. Field studies did demonstrate elevated susceptibility to white mold disease [*Sclerotinia sclerotiorum* (Lib.) deBary], poor stand establishment in some but not all environments, altered plant architecture, and earlier maturity date in the ethylene-insensitive lines. Seed yield was notably undependable, being similar to the parental line in some field locations but significantly reduced in most environments. To avoid these negative impacts on overall performance, manipulation of plant ethylene responses should be targeted to specific tissues, growth stages, or growth environments.

The plant hormone ethylene controls a wide array of plant processes including fruit ripening, fading of flowers, organ shedding (abscission), gravitropism, petiole epinasty, abiotic stress responses, and responses to pathogens (Abeles et al., 1992; Bleecker and Kende, 2000). Accordingly, ethylene biology has for many decades attracted the attention of plant physiologists, plant breeders, and those who grow and market plant products. More recently a great deal has been learned about the molecular mechanisms of ethylene perception and signal transduction, especially from studies of *Arabidopsis thaliana* (L.) Heynh. (Chang, 2003; Guo and Ecker, 2004; Stepanova and Ecker, 2000). The Arabidopsis work has been driven strongly by the study of plant mutants that exhibit altered responses to ethylene. Work with tomato (*Lycopersicon esculentum* Mill.) has also been prominent, including a number of recombinant DNA-based approaches to gain economically viable control over tomato fruit ripening (Klee, 2004). Efforts to genetically control ethylene responsiveness have subsequently been pursued in a number of other crop and horticultural species.

The role of ethylene in plant responses to pathogens is complex; ethylene apparently can promote both disease susceptibility and disease resistance (Boller, 1991; Feys and Parker, 2000). The association of ethylene responses with chlorosis, senescence, and ripening (rotting) led some plant pathologists over 30 yr ago to predict that reduction of ethylene responses could reduce disease severity. Counter to this, ethylene was postulated to foster some degree of disease resistance after it was found to induce production of phytoalexins and pathogenesis-related proteins, which have predicted disease resistance activity. Tests of these ideas in whole-plant–whole-pathogen assays have provided support for both concepts. Enhanced disease tolerance (a reduction in disease damage despite similar pathogen population levels) was observed following bacterial infection in ethylene-insensitive *Arabidopsis ein2* mutants (Bent et al., 1992). This disease tolerance was not observed in *Arabidopsis etr1* mutants, possibly because of residual ethylene responsiveness in the *etr1* lines. Studies with ethylene-insensitive lines of tomato, tobacco (*Nicotiana tabacum* L.), soybean, and other species have subsequently extended this observation of tolerance to bacterial diseases and of roles that ethylene can play in promoting susceptibility to bacterial diseases (Block et al., 2005; Hirsch et al., 2002; Hoffman et al., 1999; Knoester et al., 1998; Lund et al., 1998; O'Donnell et al., 2003).

The initial study of disease resistance in ethylene insensitive plants also showed that strong *R* gene-mediated disease resistance was not observably reduced (Bent et al., 1992). Our subsequent work with ethylene-insensitive soybean demonstrated that this was not always the case. *R* gene-mediated resistance against *Pseudomonas syringae* pv. *glycinea* (Coerper) Young, Dye & Wilkie remained effective, but resistance against some races of *Phytophthora sojae* (Kaufmann and Gerdemann), mediated by *Rps1-k,* was disrupted (Hoffman et al., 1999). Further research on disease resistance that does not involve *R* genes has shown that in *Arabidopsis*, ethylene signaling is important for the activation of induced systemic resistance but not systemic acquired resistance (Thomma et al., 2001; Ton et al., 2002). Ethylene signaling contributes at least part of the partial resistance that is expressed against some but not all fungi during compatible

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Abbreviations: SCN, Soybean Cyst Nematode; SPAD, leaf chlorophyll.

interactions in *Arabidopsis* (e.g., Bohman et al., 2004; Thomma et al., 1999; van Wees et al., 2003).

Our previous work with ethylene-insensitive soybean, which documented enhanced tolerance to infection by virulent *Pseudomonas syringae* pv. *glycinea*, also documented slightly increased susceptibility to the fungal pathogens *Septoria glycines* and *Rhizoctonia solani* Kühn (Hoffman et al., 1999). Conversion of tobacco plants to ethylene insensitivity caused susceptibility to *Pythium sylvaticum* Campbell & Hendrix, which is not usually pathogenic on tobacco, as well as sensitivity to *Colletotrichum destructivum* O'Gara (Chen et al., 2003; Knoester et al., 1998). The above results and other studies have suggested that although reduction of ethylene responsiveness can be benign or even beneficial with respect to some plant diseases, it can make plants more susceptible to other pathogens, and to fungal pathogens in particular. However, reports that document the behavior of ethylene-insensitive plant lines in a field setting have been lacking. One goal of the present study was to assess the impact of altered ethylene responsiveness on plant resistance to two fungal pathogens, in a field setting.

The present work investigated interaction with fungal pathogens primarily through studies of white mold, a disease caused by the ascomycete fungus *Sclerotinia sclerotiorum* that has been intermittently prominent in central U.S. soybean cropping in many recent years. Areas infested with *S. sclerotiorum* develop severe disease in environmentally conducive years, and genetic disease resistance has been only partially effective (Grau et al., 2004). More tests were also conducted using *S. glycines*, which is the causal agent of Septoria brown spot, a foliar disease that (like ethylene) causes necrotic leaf lesions, spreading chlorosis and defoliation. The impact of Septoria brown spot on soybean seed yields is relatively minor (Grau et al., 2004), but previous laboratory-based studies revealed slightly worse brown spot symptoms in ethylene-insensitive soybeans relative to wild-type controls (Hoffman et al., 1999) and this finding merited re-examination in a field setting. Soybean cyst nematode (*Heterodera glycines* Ichinohe) is the most damaging pathogen of soybean in the USA (Wrather et al., 2001), and so studies with this pathogen were also pursued.

As noted above, ethylene affects many traits other than interaction with pathogens. Two economic traits that have received substantial attention are ripening control in climacteric fruits and senescence control for cut flowers (Giovannoni, 2004; Reid and Wu, 1992). These traits are not relevant to soybean, but altered ethylene perception could potentially alter soybean traits such as seedling emergence, plant architecture, stress responses, leaf senescence, maturity date, seed protein and oil composition, and seed yield. As an example, although *Arabidopsis* ethylene-insensitive plants grow with a morphology roughly similar to their wild-type, ethylene-responsive parent (Bleecker et al., 1988; Guzman and Ecker, 1990), close inspection reveals that ethylene-insensitive *Arabidopsis etr1* and *ein2* mutants do develop slightly larger leaves and exhibit a roughly 1 wk delay in bolting and rosette leaf senescence. We hypothesized that soybean *etr1–1* plants would also exhibit altered morphology and delays in flowering and leaf senescence, as well as maturity (first date on which seed harvest is advisable). However, in previous casual observation of the *etr1–1* soybean line (Hoffman et al., 1999), we had noted alterations in canopy morphology but not in flowering time, senescence, or maturity. The present study, conducted across multiple sites and years in a field setting, allowed more careful documentation of these traits.

Additional traits of field crops could potentially be affected by altered ethylene responsiveness and merit examination. Ethylene has been widely shown to accelerate leaf chlorosis and senescence in dicotyledonous plants (Abeles et al., 1992; Bleecker and Patterson, 1997; Mattoo and Aharoni, 1988), but a significant role for ethylene in monocarpic senescence of soybean has been questioned (Nooden, 1984), leaving open to inquiry the extent to which genetically altered ethylene responsiveness would affect late-season leaf senescence. Maturity date (first harvestable date) is another complex trait that can be affected by multiple factors. Shortening daylengths play a major role in inducing the maturity process, during which northern soybean varieties grown in conducive environments typically drop most or all leaves, the main stem and pods turn brown or gray and become very dry, and seed moisture loss (to below 15–16%) becomes the rate-limiting step determining when a field is ready to harvest (Pedersen, 2004). Protein concentration and oil concentration are two key output traits of cultivated soybean (Wilson, 2004). Seed yield is typically the most significant trait for soybean growers, and the impact of altered ethylene responsiveness on yield is not known for any field crop. Ethylene is only one of the many modulators of the above complex traits, each of which has been described extensively in the research literature.

Although field-grown plants have been studied after treatment with ethylene or inhibitors of ethylene signaling, plants with a stable genotype that reduces ethylene responsiveness have received minimal field study. In soybean, the impacts of ethylene signaling on growth and development have received little study in the field or laboratory. Hence, the broader goal of the present study was to grow ethylene-insensitive soybean lines and isogenic control lines under conditions relevant to soybean productivity, to document the impact of reduced ethylene responsiveness on an array of growth phenotypes.

MATERIALS AND METHODS

Plant Lines

The cultivar Hobbit 87 has been described (Cooper et al., 1991). The isolation and initial characterization of the *etr1–1* line (original name T119N54) and the T124N38 line has also been described (Hoffman et al., 1999); see also (Schmidt et al., 1999), but briefly, these are independent, strongly ethylene-insensitive lines derived in the M_3 generation from two separate NMU-mutagenized Hobbit 87 M_1 plants. The present study focused primarily on Hobbit 87 and the more well-

characterized Hobbit 87 *etr1–1* line (Hoffman et al., 1999), but data for T124N38 are presented for all instances in which T124N38 was included in the experiment.

Experimental Design

Field trials to measure seed yield and other plant growth traits were conducted in 1998 and 1999 in productive fields with a recent history of corn (*Zea mays* L.)–soybean rotation and cultivation under standard commercial management practices, which were continued. The same five field sites were used each year and each field received similar cultivation and herbicide both years, but a different segment of each field was utilized in the two separate years. The field site names, characteristics and husbandry were: Urbana (University South Farm, Urbana, IL, 2 km south of University of Illinois, Urbana, IL): Catlin silt loam, fall chisel plow followed by spring disk/field cultivator, metolachlor [2-choloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)acet-*o*-toluidide]/metribuzin (4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one) herbicide (pre plant); Cruse (4 km south of University of Illinois, Urbana, IL): Flanagan silt loam, fall chisel plow followed by spring disk/field cultivator, metolachlor/metribuzin herbicide (pre plant); Dekalb (13 km north of Shabbona, IL), Drummer silty clay loam, Fall plow followed by Spring mulch-finisher, dimethenamid [(*RS*)-2-chloro-N-(2,4-dimethyl-3-thienyl)-N-(2-methoxy-1-methylethyl)acetamide]/imazethapyr [(*RS*)-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid] herbicide (preemergence); Dwight (8 km northeast of Dwight, IL), Reddick silty clay loam, fall disk followed by spring disk/field cultivator, Pursuit herbicide (preemergence); Newton (9 km southeast of Greenup, IL), Cisne silt loam, no fall tillage, spring disk/soil finisher, cloransulam [3-chloro-2-(5-ethoxy-7-fluoro[1,2,4]triazolo[1,5-c]pyrimidin-2-ylsulfonamido)benzoic acid]/clomazone [2-(2-chlorophenyl) methyl-4,4-dimethyl-3-isoxazolidinone] herbicide. The Septoria field tests were conducted at Urbana (South Farm; see above) in 1998 and 1999, but on different segments of this site in the two different years, and on field plots separated from the plots used to study seed yield and other plant growth traits in the same years. The white mold field studies were conducted in 1998 and 1999 at C-FAR plots (3 km south of University of Illinois, Urbana, IL), Drummer silty clay loam, fall chisel plow followed by spring disk/field cultivator, metolachlor/metribuzin herbicide (preemergence). The white-mold study utilized the same field segment in both years, but as at all other sites, did use a different randomized block randomization in the second year.

Field tests all used a randomized block design with three blocks, with a soybean border surrounding the experimental plots. Unless specifically noted, soybeans were grown in rows 76.2 cm (30 inch) apart, planted at approximately 32.8 seeds m^{-1} (10 seeds $foot^{-1}$), with four 3.28-m (10 foot) rows $plot^{-1}$. Plots were monitored for appearance of off-type plants, which were very rare, and were removed. ANOVA tests were run with data for all plant varieties in the experiment, most of which were not relevant to the present study (25 varieties at Cruse and Newton, 35 varieties at Urbana, seven varieties at Dekalb and Dwight, 12 varieties at Urbana 1998 Septoria experiment, 24 varieties at Urbana 1999 Septoria experiment, and 25 varieties at C-FAR white mold experiment). The analysis included tests for normality and for block effects, which were not significant unless noted. From within these larger trials, sample mean and statistical significance data are presented only for the two ethylene-insensitive lines and the isogenic Hobbit 87 parent, and in the white mold and Septoria tests, for susceptible and partially resistant control lines. Tests of seed yield data were conducted by the GLM procedure in the SAS (SAS, 1989), with LSD values determined as *t* statistic groupings. Other trait data were analyzed by the MiniTab GLM procedure in MiniTab (Minitab, 2004) and then via one-way ANOVA to calculate Fisher's protected LSD values.

White Mold

Field white mold assays were conducted as described by Hoffman et al. (2002) under the experimental design described above. Briefly, plots (3.7-m row length planted at 19-cm row spacing) were grown until the majority of plants had reached R1 (flowering) stage (Pedersen, 2004), and then 1 wk later, at which point most plots had canopy closure estimated at >95% of ground surface area, plants were inoculated with dried, finely ground wheat kernels that had previously been colonized with *Sclerotinia sclerotiorum* mycelium. Inoculum was broadcast onto plants wetted by overhead mist-irrigation, targeting the upper-middle tier of the canopy in the middle two rows of the plot. In 1999, canopy closure was specifically noted for each plot, and for all lines reported in the present study, the closure was visually judged to be roughly 98% or greater at the time of the first inoculation. A second inoculation was performed 2 to 3 wk after the first inoculation. Canopy humidity was maintained between the R1 and R7 stages of plant growth by fine overhead mist irrigation. Disease severity was assessed as described by Hoffman et al. (2002), rating 30 plants per plot using a 0–3 scale (3 = severe disease), with disease severity index (DSI) = 100 × [(sum of ratings for 30 plants)/90] that normalizes to a top score of 100 if all 30 rated plants in the plot receive the maximum score of 3. The same field site was used in both 1998 and 1999. 'Asgrow 2242' and 'Syngenta S19–90' were included as susceptible and partially resistant checks, respectively (Hoffman et al., 2002).

Septoria Brown Spot

Septoria brown spot field tests were conducted as in Lee and Hartman (1996) under the experimental design described above except that 2-row plots were used rather than 4-row plots. Briefly, plants were inoculated with conidial suspensions of *Septoria glycines* twice in mid-summer (R1–R4 stage plants) by foliar misting on cool evenings roughly 2 wk apart. Successful establishment of brown spot within the experiment was confirmed in both years by comparison to the same lines grown in nearby noninoculated experiments. During 1998, disease severity was scored as number of nodes defoliated on a date approximately 1 mo after the first inoculation, stand counts (number plants/row) were recorded mid-season, and maturity date and seed yield data were collected at the end of each season. In 1999, disease severity was scored on a date when plants were at R6 to R7 stage by a 1-to-5 scale, where 1 = no or few symptoms, 2 = brown spot leaf symptoms present and approx. 20% of nodes defoliated (typically at bottom of plant), 3 = 50% of nodes defoliated, 4 = 75% defoliated, 5 = total defoliation; maturity, lodging, and height data were also collected. During 1999, R92–2335, and R92–2760 were included as susceptible and partially resistant checks, respectively.

Soybean Cyst Nematode Root Colonization Assays

Hobbit and *etr1–1* mutant seeds were surface sterilized and germinated in the dark for 3 d on 1.5% (w/v) agar. Approximately 2 cm of root radical was cut from germinated seeds and placed on 1.2% Nobel Agar plates containing Gamborg's B5 salts and vitamins (Sigma, St. Louis, MO, USA). Roots (two per plate) were allowed to grow on the plates for three more days and then 10 mature axenic SCN females (NL1-RH) were placed around each root and crushed to release eggs. Plates

were incubated for 33 d at 25°C in the dark, and the numbers of swollen females were then counted in each of five plates carrying Hobbit 87 or the *etr1–1* mutant line. The SCN population NL1-RH, which interacts with soybean differentials in the manner of Race 3, has been maintained on greenhouse-grown soybeans at USDA, ARS, Beltsville, MD, and can be obtained from the SCN Stock Center (T. Niblack, University of Illinois, Champaign-Urbana, IL). Hobbit 87 exhibits a compatible (susceptible) interaction with NL1-RH, relative to known SCN-resistant and susceptible soybean lines, as expected for this Race 3 SCN isolate. The axenic SCN used in these experiments had been maintained on similarly cultured Williams 82 roots where fresh root radicals were cyclically inoculated each month with mature females from an earlier culture plate.

Leaf Abscission and Leaf Chlorophyll Assays

For abscission assays, wild-type and mutant seeds were germinated and grown in the greenhouse in flats of Perlite (Geiger, Harleysville, PA) and watered daily without fertilizer. Greenhouse conditions in June when these experiments were completed are typically 27°C day and 18°C night with approximately 15 h of daylight. After 14 d seedlings were cut at soil level, leaf blades removed and the cut ends immersed in water. Fourteen Hobbit 87 explants and nine *etr1–1* mutant explants were put into a dark 35-L chamber where 25 μL L^{-1} ethylene in air at 25°C (a regulated flow of 5 mL L^{-1} ethylene in nitrogen mixed with a regulated flow of air) was continuously passed through at a flow rate of 2 L min^{-1}. After the indicated interval of time the explants were temporarily removed from the chamber and petioles that fell off with a gentle touch were recorded as abscised. To check for ethylene-induced chlorosis in healthy recently expanded trifoliate leaves, excised leaves were placed on moist paper towels in a dark chamber at 23°C, the air was brought to 10 μL L^{-1} ethylene by injection with a known amount of pure ethylene, and leaves were then visually checked for signs of chlorosis at the indicated number of days after start of the ethylene treatment. Leaves for this test were taken from adjacently grown wild-type and mutant plants from Urbana field-plots (described above) and from plants grown in the greenhouse (27°C day/20°C night; 16 h daylength at minimum 200 μmol m^{-2} s^{-1} maintained by automated supplemental high pressure sodium lighting) in individual pots holding 4 to 5 L of steam-sterilized 1:1:1 mix of soil:sand:vermiculite. Nondestructive measurements of relative leaf chlorophyll were made in field-grown plants from yield trials at the Urbana site (described above) with a handheld Minolta (Mahwah, NJ) SPAD-502 chlorophyll meter (Schepers et al., 1992). Readings were made on 15 plants in each plot (first year) or 20 plants in each plot (second year) at a standardized location away from the mid-vein and leaf margins on the youngest fully expanded trifoliate leaf present on the day of sampling, and the experimental unit for statistical analysis was the sample mean for the plot (rather than the SPAD reading for each individual plant). For graph presentation of SPAD data the date of full senescence for a given year was determined ±4 d as the date on which most leaves had visibly lost turgor as part of normal end-of-season senescence; all other data points for that year are placed precisely relative to this date.

Plant Architecture and Developmental Timing

The standardized VE through R8 nomenclature is used to designate soybean developmental stages (Pedersen, 2004). Flowering time data were collected from plants grown in 2-m rows (single-row plots) in 1997 and 1998 at the Urbana site (soils and cultivation noted above), and detailed notes on plant canopy shape were from the noninoculated Urbana and Cruse yield sites noted above. Lodging and maturity data were collected at all five of the above-noted yield trial sites in 1999 as well as at other sites where specifically noted, with lodging scored on a 1-to-5 scale [1 = almost all plants erect; 3 = all plants leaning moderately (45°) or 25–50% of the plants down; 5 = almost all plants down], and maturity scored as the first date on which 95% or more of pods have ripened to harvestable dryness (Pedersen, 2004). Stand count data were collected in 1998 by taking a mid-season (June/July) count, for each plot, of the number of plants in one of the middle plant rows.

Seed Yield

Seed yield was determined by harvesting all plants from the two inner rows of the four-row plot. Yield data were normalized to 13% (130 g kg^{-1}) moisture content. A 25-g sample of clean seed for each variety from each of the five 1999 yield sites was analyzed for protein and oil content by infrared reflectance (Rinne et al., 1975) at the USDA-ARS National Center for Agricultural Utilization Research at Peoria, IL, and reported as dry-weight percentage. Dry-weight percentage values were obtained with a Foss (Eden Prairie, MN) Model 1255 Infratec NIR food and feed analyzer. Two aliquots of 10 to 12 g each of whole seeds were analyzed with results automatically averaged and stored.

RESULTS

Enhanced Susceptibility to White Mold

Field-based tests were conducted to examine the impact of ethylene insensitivity on white mold resistance, as part of a larger set of contiguous plots from which other studies have been published (Hoffman et al., 2002). These plots used overhead misting after dispersal of ground, sclerotia-infested wheat kernels into the leaf canopy of flowering soybeans (first inoculum exposure at growth stage R1–R2). In both trial years, the ethylene-insensitive line Hobbit 87 *etr1–1* line was notably more susceptible to white mold than the isogenic Hobbit 87 parent (Table 1). The susceptible and partial resistant controls, Asgrow 2242 and Syngenta-S19–90, performed as expected (Table 1; Hoffman et al., 2002).

To control for possible impacts from other mutations in the *etr1–1* plant line, additional tests were performed with a second, independently isolated ethylene-insensi-

Table 1. White mold disease scores for Hobbit 87 and two ethylene-insensitive derivatives of Hobbit 87, as well as control genotypes known to be partially resistant (Syngenta S19–90) or susceptible (Asgrow 2242) to white mold. Data are from field trials performed at C-FAR test site (Urbana, IL) in 1998 and 1999.

		1998	1999
Plant line	Description	Disease†	Disease†
Asgrow 2242	susceptible check	58.6	30.4
Syngenta S19–90	resistant check	29.7*	14.1
Hobbit 87	parent of mutants	47.5	23.3
etr1–1	ethylene insensitive	72.8*	50.0*
T124N38	ethylene insensitive	58.9	34.8

* Significantly different from Hobbit 87 (LSD $P < 0.05$; LSD value for 1998 = 11.2, for 1999 = 12.7).

† Disease Severity Index (0 = no disease, 100 = 100% of plants girdled on main stem and dying).

Table 2. Septoria brown spot disease occurrence in field plots inoculated with *Septoria glycines*, evaluated in 1998 and 1999 in Urbana, IL using two different experimental designs and disease scoring systems.

Plant line†	1998 Septoria disease	1998 Stand count	1998 Maturity	1998 Yield	1999 Septoria disease	1999 Maturity
	defoliated nodes	plants m^{-1}	date	kg ha^{-1}	severity score†	date‡
Hobbit 87	10.3	27.7	19 September	2648	3.8	24 September
etr1–1	11.5	21.2*	11 September*	1817*	4.0	16 September
T124N38					4.3	15 September*
R92–2335					4.0	12 September*
R92–2760					2.3*	29 September

* Significantly different from value for Hobbit 87 (LSD $P < 0.05$).
† 1 = few or no symptoms; 5 = total defoliation.
‡ R92–2335 and R92–2760 are susceptible and partially resistant controls for Septoria brown spot sensitivity.

tive mutant of Hobbit 87 called T124N38. T124N38 was isolated after the *etr1–1* line (Hoffman et al., 1999) but by similar methods and stood out from the other few dozen candidate ethylene-insensitive lines because it exhibited particularly strong ethylene-insensitivity, similar to the *etr1–1* line (Hoffman et al., 1999). As with *etr1–1* plants, the white mold disease severity index was also consistently higher for T124N38 than for Hobbit 87 (Table 1), but in both years, the difference for T124N38 was significant only at $P = 0.10$ and not at $P = 0.05$. This statistical significance did, however, lend modest support to the finding made with the more well-characterized *etr1–1* line that ethylene-insensitivity causes soybean to be more susceptible to white mold.

Minimal Impact on Resistance to *Septoria glycines*

In a second set of experiments, the ethylene-insensitive and ethylene-sensitive Hobbit 87 lines were grown in field plots provided with *S. glycines* inoculum. Table 2 reveals that the ethylene-insensitivity trait did not significantly affect Septoria brown spot disease severity. Table 2 also shows that in 1998, stand counts were 23% lower for the ethylene-insensitive lines before *S. glycines* inoculation; hence, it was not surprising that seed yield was reduced for these lines. The difference between Hobbit 87 and *etr1–1* with respect to stand count, maturity date, and seed yield were very similar to the differences observed in noninoculated field trials (discussed below). The 1999 Septoria field study, conducted under a somewhat different experimental design and disease scoring system, also did not reveal significant differences in disease severity among Hobbit 97 and the Hobbit 87 ethylene-insensitive lines *etr1–1* or T124N38 (Table 2). These three lines had disease severity similar to the susceptible control (line R92–2335), while the partially resistant control (line R92–2760) developed significantly less Septoria brown spot disease. Subtle differences in brown spot disease development might have been detected by monitoring disease progress at earlier time points, but no significant differences in severity were detected by either of the two assessment methods reported in Table 2.

Table 3. Trifoliate leaf and leaflet characteristics of field-grown plants measured at V4-V5 growth stage among plants grown at the 1998 Urbana field site (no pathogen treatments).

Plant line	Blade length	Blade width	Petiole length
	cm		
Hobbit 87	8.07	5.45	11.52
etr1–1	8.08	5.03	9.75*

* Significantly different from value for Hobbit 87 (LSD $P < 0.05$).

Reduced Cyst Formation in Response to Soybean Cyst Nematode

The ability of a compatible soybean cyst nematode isolate to infect and develop on roots from wild-type Hobbit 87 and Hobbit 87 *etr1–1* mutant plants was examined. In two independent experiments, significantly fewer females developed on *etr1–1* roots than on wild-type control roots 1 mo after inoculation ($P = 0.006$ for ANOVA; mean ± std error of mean = 118 ± 36 and 67 ± 10 for *etr1–1* in the two respective experiments, and 182 ± 27 and 126 ± 13 for Hobbit 87). The morphology of infested roots was similar between Hobbit 87 and Hobbit 87 *etr1–1* plants.

Subtle Changes in Plant Morphology and Growth Habit

The morphology of field-grown adult ethylene-insensitive soybean plants (R1–R5; healthy mid-season plants in the early stages of flowering and pod development) was subtly different relative to ethylene-sensitive controls, with similar leaf shape but different stem–petiole architecture. Leaf blade width and length was similar between Hobbit 87 and *etr1–1* plants, but the measured petioles of Hobbit 87 were significantly longer (Table 3).

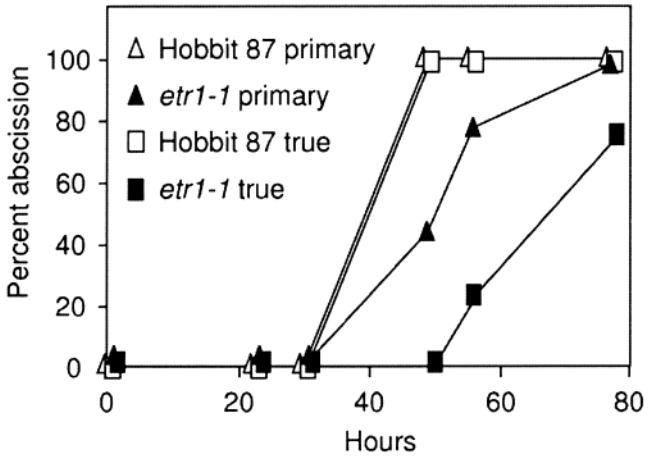

Fig. 1. Leaf abscission in response to ethylene. The percentage of petioles that had abscised in response to a gentle touch was recorded at several intervals of time for Hobbit 87 and Hobbit 87 *etr1–1* explants exposed to 25 μL L^{-1} ethylene in air at 25°C. The petioles were debladed before ethylene treatment. The abscission of petioles for the primary (unifoliate) leaves and true (trifoliate) leaves were recorded separately.

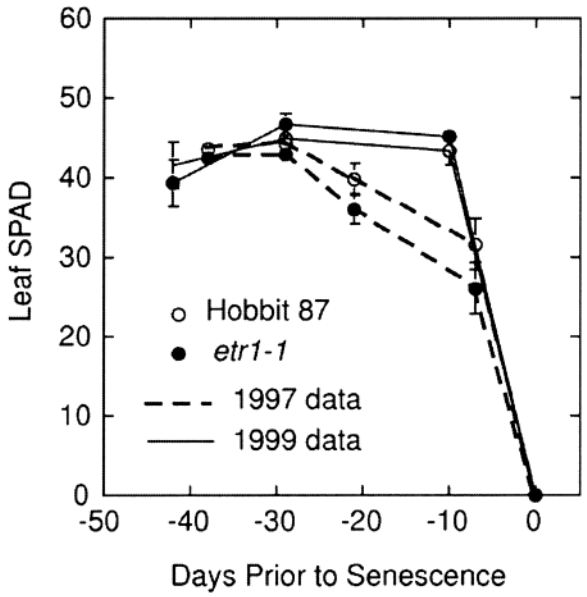

Fig. 2. Relative leaf chlorophyll content estimated with a SPAD meter. The youngest fully expanded trifoliate leaf on each of 15 plants (1997) or 20 plants (1999) per plot was sampled on each date except the last date. Data are for one experimental site in each year (three blocks per experiment), and are graphed as mean and 95% confidence interval.

Both lines were of similar height when growing in healthy stands. However, leaves in the upper canopy of Hobbit 87 were more upright, leading to a smoothly arching upper canopy along a row grown at 76-cm row spacing, while the petioles of upper leaves of *etr1–1* tended to extend more horizontally, giving the upper canopy of a row of *etr1–1* a flatter and more rectangular shape. The upper leaves of *etr1–1* plants appeared to have "flopped" and fallen sideways as they matured because the upper canopy was thin over the center of the stems. Although cause and effect were not determined, this did permit more overhead light to reach into lower tiers along the main stem of the plant, and it was also noted that at mid-height there was thicker foliage closer to the stem of *etr1–1* plants as compared with Hobbit 87. Hence, in stands of similar overall plant density, the even canopy shape of an individual Hobbit 87 plant or of a row of Hobbit 87 plants grown at 76-cm row spacing (6–10 plants per foot) was replaced in *etr1–1* with a canopy that was slightly more dense and narrow at mid-height (more leaves closer to the main stem) and broader and more uneven near the top. This canopy difference was noted at multiple sites and in multiple years and was also noted to a lesser degree for T124N38.

No significant difference in flowering date was noted between Hobbit 87 *etr1–1* plants and the parental Hobbit 87 line across 2 yr at the Urbana–South Farm location. No significant difference in plant lodging was recorded among Hobbit 87, *etr1–1*, and T124N38 at any of five field sites in 1999 (LSD $P = 0.05$; data not shown) or in casual observation of plants grown in 1997 or 1998.

Ethylene Responsiveness Less Important for Late-Season Leaf Chlorosis and Senescence, but Does Alter Maturity Date

Leaf senescence, abscission, and maturity date were investigated further. The penetrance of the *etr1–1* ethylene-insensitivity trait in mid-season plants was confirmed by exposure of healthy excised trifoliate leaves to ethylene. Leaves from *etr1–1* plants remained green for many days during exposure to 10 $\mu L\ L^{-1}$ ethylene, as would be predicted for an ethylene-insensitive plant, while leaves of the parental Hobbit 87 line became strikingly chlorotic over the first 3 d after ethylene treatment (data not shown). Leaf abscission was also studied. In healthy soybean seedlings exposed to 25 $\mu L\ L^{-1}$ ethylene, petiole abscission was delayed by roughly 1 d in the *etr1–1* plants (Fig. 1). However, the above delays in chlorosis and abscission under artificially applied ethylene did not translate into a significant delay in the normal late-season senescence of leaves on field-grown soybean plants. A SPAD meter was used to track the relative chlorophyll content of leaves starting midway through the growing season. Figure 2 shows that in both of 2 yr, no significant differences were observed among green, mid-season trifoliate leaves. Subsequent leaf senescence in the field setting (leaf yellowing, browning, and late-season insect–saprophyte damage) was quite uneven within single leaves and across leaves within a plot, making it difficult to obtain valid data with the SPAD meter later in the season. However, leaf senescence occurred over a roughly similar 10-d period for both the Hobbit 87 and *etr1–1* lines. The above results suggest that, although ethylene clearly can stimulate leaf senescence, ethylene-mediated processes are not the primary driving force in the normal late-season (monocarpic) leaf senescence of soybean varieties such as Hobbit 87.

Table 4 presents data for maturity dates (date on which pods/seeds have dried to maturity), which were recorded at field sites that did not receive pathogen inoculation. The *etr1–1* line often matured 5–8 d sooner than the isogenic Hobbit 87 parent. The same was true of the less well-characterized T124N38 ethylene-insensitive line.

Table 4. Maturity Date (first harvestable date) evaluated at five different sites, and at Urbana IL in two different years. Note that these trials did not receive any pathogen inoculation treatments.

Plant line	Urbana 1998	Urbana 1999	Cruse 1999	Newton 1999	Dwight 1999	Dekalb 1999
	date					
Hobbit 87	15 September	16 September	19 September	17 September	23 September	8 October
etr1–1	09 September*	8 September*	12 September*	15 September	24 September	3 October*
T124N38	7 September*	7 September*	9 September*	12 September*	17 September*	1 October

* Maturity date different from parental line (Hobbit 87) within same trial (LSD $P < 0.05$).

Table 5. Seed protein and oil concentrations (% of total seed weight) evaluated in 1999 at the same Urbana, Cruse, Newton, Dwight and Dekalb field sites from which data were obtained for Tables 4 and 6. Mean values across the five sites are presented; none of the values within columns are significantly different from each other (LSD $P < 0.05$). Text describes additional statistical tests.

Plant line	Protein	Oil
	%	
Hobbit 87	39.18	21.60
etr1–1	41.06	20.86
T124N38	40.06	21.28

No Change in Seed Protein and Oil Concentration

No significant differences in oil concentration among the Hobbit 87, *etr1–1*, and T124N38 plant lines were observed for seed samples harvested at five different field locations (Table 5). For protein data as well, no significant differences were observed in ANOVA studies that used 95% confidence intervals on the basis of pooled standard deviations, but the protein concentration of *etr1–1* seed was significantly greater than Hobbit 87 when Fisher LSD 95% individual confidence intervals were used (Table 5). A paired *t* test (pairing seed for Hobbit 87 and *etr1–1* at each site) yielded $P = 0.056$, narrowly rejecting any difference in protein concentration. No significant differences in protein concentration were evident in any of the tests when data for Hobbit 87 and T124N38 were compared, suggesting overall that the ethylene insensitivity trait caused no difference in seed protein concentration in this five-site field study.

Highly Variable Seed Yield in Ethylene-Insensitive Soybean Lines

In the initial generations of greenhouse and field propagation, the ethylene-insensitive mutants seemed to grow similarly to their parent lines, but after seed had been increased and multisite, multiyear trials were performed, it became apparent that the seed yield of ethylene-insensitive soybean lines is quite undependable. Table 6 shows that the ethylene-insensitive lines performed similarly to their isogenic parent at some sites, but at most sites, there was significant yield depression. Seed yields at Dwight and Dekalb in 1999 supported prior anecdotal observations that ethylene-insensitivity did not prevent performance similar to isogenic ethylene-sensitive controls in at least some environments. However, the ethylene-insensitive lines clearly did not cope well with other environments. Early season stand establishment was a major cause of yield depression at some sites. Stand count data were collected in 1998 and are presented in Table 7 (and in Table 2 for the separate Septoria experiment). Using the identical seed source at all sites, *etr1–1* plots produced depleted stands at the Cruse and especially the Urbana site but not Newton, while Hobbit 87 (and the other six nonmutagenized soybean varieties in the experiment, for which data are not shown) all established fully populated stands at all three sites. However, stand establishment was not the only thing constraining seed yield in the ethylene-insensitive soybeans. In the Newton 1998 trial, *etr1–1* stand counts were statistically indistinguishable from Hobbit 87, but *etr1–1* plants then grew less well and yielded much worse than Hobbit 87.

Table 7. Stand count data from 1998 yield trials conducted at field sites that received different husbandry treatments typical of commercial production and which did not receive any pathogen inoculation treatments.

Plant line	Stand count		
	Urbana 1998	Cruse 1998	Newton 1998
	plants m^{-1}		
Hobbit 87	26.3	32.5	33.7
etr1–1	5.5*	22.0*	30.9
T124N38	20.9*	29.9	27.2*

* Significantly different from value for Hobbit 87 within same trial (LSD P 0.05).

DISCUSSION

The goal of this study was to investigate the impacts of ethylene responsiveness on a broad array of macroscopically observable soybean traits. Ethylene-insensitive mutants offer a unique opportunity for such studies because external ethylene response activators or inhibitors do not need to be applied broadly and/or on some type of constant basis for the studies to proceed. However, plants carrying a relatively constitutive, systemic loss of ethylene responsiveness may become habituated in abnormal ways or may fail to grow in a normal fashion before onset of the stimulus of interest (such as pathogen infection). Hence, stable genetic changes, pharmacological treatments, and inducible rather than constitutive mutational studies (e.g., inducible expression of silencing constructs or dominant negative constructs) each offer particular advantages for studies of ethylene-mediated processes.

Soybean plants with reduced ethylene sensitivity exhibited a clear increase in disease susceptibility to one fungal pathogen (*Sclerotinia sclerotiorum*) but not to another (*Septoria glycines*) and exhibited decreased colonization by soybean cyst nematode. This finding is

Table 6. Seed yield data from 1998 and 1999 yield trials conducted at field sites that received different husbandry treatments typical of commercial production and which did not receive any pathogen inoculation treatments.

Line	Urbana 1998	Cruse 1998	Newton 1998	Urbana 1999	Cruse 1999	Newton 1999	Dwight 1999	Dekalb 1999
	kg ha^{-1}†							
Hobbit 87	4434	3261	3106	3766	4156	1870	3282	4143
etr1–1	1828*	2402*	990*	2461*	3262*	1480*	3067	3389*
T124N38	3358*	2239*	1817*	2401*	3726*	1587*	3363	3490

* Seed yield different from parental line (Hobbit 87) within same trial (LSD $P < 0.05$).
† Mean values of seed yield per plot for middle two rows of four-row plots, converted to kg/ha.

consistent with laboratory-based studies performed to date, most extensively with *Arabidopsis*, which have shown that blockage of ethylene responsiveness differentially affects the plant response to different pathogens. Reduction of ethylene responsiveness can be detrimental, neutral, or beneficial, depending on the pathogen [present study and Bohman et al. (2004), Chen et al. (2003), Feys and Parker (2000), Hoffman et al. (1999), Knoester et al. (1998), and Thomma et al. (1999)]. This variable impact should not be surprising given the different plant colonization and virulence mechanisms of different pathogens and the multiplicity of plant pathogenesis responses and defense responses. If ethylene response traits of soybean are to be manipulated positively or negatively with the goal of reducing disease severity, it will be necessary to target changes in plant ethylene responsiveness to infection sites or events that are common to certain pathogens but not others, and to subsequently investigate the impact of these changes with multiple pathogen species. Plant testing at multiple field locations can contribute to this by revealing susceptibility to commonly occurring pathogens that are not normally an issue with adapted soybean cultivars.

Regarding the nematode experiments, an early study by Glazer et al. (1985) demonstrated that excised tomato roots infected with the root knot nematode *Melodiogyne javanica* (Treub) Chitwood produced three- to sixfold as much ethylene as noninfected roots. Moreover, excised roots supplemented with aminocyclopropane-1-carboxylic acid or ethrel compounds that further increase ethylene, showed an increase in gall formation, whereas treatments with ethylene synthesis or perception inhibitors decreased the number of galls formed. More recently, Wubben et al. (2001) demonstrated that *Arabidopsis* mutants that overproduce ethylene were more susceptible to infection by the sugar beet cyst nematode, *Heterodera schachtii* Schmidt, and, conversely, ethylene-insensitive mutants were less susceptible. Our observation that *etr1–1* reduced or delayed soybean cyst nematode reproduction is consistent with these earlier results. Simple deployment of ethylene insensitivity, however, is constrained by its detrimental impacts on other soybean traits.

Many traits were not affected in an obvious fashion by reduced ethylene responsiveness, including overall seed protein–oil concentration, flowering date, plant height, leaf chlorophyll, and late-season leaf senescence. It should be remembered, however, that more detailed effects such as changes in specific protein or oil components were not measured. The observed impacts on plant architecture suggest the subtle and possibly intriguing ways in which a constitutive reduction in ethylene responsiveness may be manifested. The "flopping" petioles, although straight for much of their length, may represent a subtle epinastic response caused by altered feedback in ethylene-mediated pathways. The observed concentration of the mid-height leaf canopy closer to main stems may be a product of increased light incidence, or may be due to light-independent developmental alterations affected by ethylene insensitivity. These and other hypotheses remain to be investigated.

Seed yield is the most important trait for most soybean growers and it was affected negatively by reduced responsiveness to ethylene. As alluded to in the Results, the reasons for this reduction in seed yield may be multiple and complex. Reduced seedling establishment was a factor in some trials. Ethylene insensitivity may cause poor seedling establishment via a number of traits, such as poor seedling growth in wet soils, poor emergence through a crusted soil surface, or enhanced susceptibility to *Phytophthora sojae* Kaufmann & Gerdemann, *Rhizoctonia*, *Pythium*, or other damping-off pathogens. However, reduced seed yields sometimes occurred despite normal seedling establishment, suggesting that other deficiencies later in the growing season could also be responsible for reductions in seed yield. The similar seed yield of ethylene-insensitive and wild-type soybeans in some field environments suggests that instances of yield depression cannot be attributed to a general and consistently expressed defect in plant development, photosynthesis, seed formation or other core processes. The most likely causes of reduced seed yield in the ethylene-insensitive soybean lines are therefore less effective responses to pathogens, abiotic stresses, or other factors that vary substantially across sites and years.

This study and the studies of Hoffman et al. (1999) and Schmidt et al. (1999) identify areas in which genetically altered ethylene responsiveness affects soybean performance and other equally important areas that are left relatively unperturbed. Further macroscopic studies of many of these areas may be informative. These areas also may be appropriate targets for renewed physiological, cellular, and molecular studies that investigate molecular causation. Expression of ethylene insensitivity in particular tissues, at particular growth stages, or in response to specific stimuli is now possible using particular promoter sequences to drive genes that encode dominant negative ethylene receptor alleles (Wilkinson et al., 1997). The present study reinforces the suggestion that, because of the pleiotropic effects of ethylene, this targeted expression of ethylene insensitivity may be the most productive route for future research toward the development of improved plant varieties.

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