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Regular Article

Identification of a novel trait associated with phytotoxicity of an insecticide etofenprox in soybean

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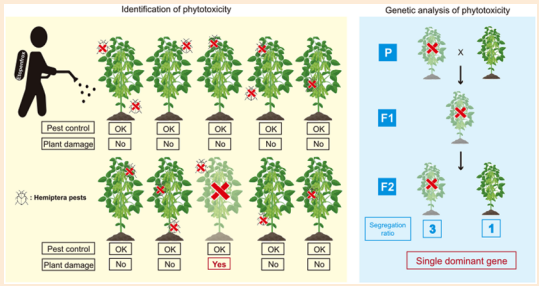
Synthetic insecticides are widely used to control pests in various crop fields. Especially in soybean [*Glycine max* (L.) Merr.] fields, the insecticide etofenprox, which is a pyrethroid derivative, has been used to manage hemiptera pests. To date, soybean phytotoxicity response has not been reported to etofenprox derivatives, two Korean cultivars, Danbaek and Kwangan, were first identified to show leaf shape shrinkage damage after etofenprox application. We confirmed that the causal substance for phytotoxicity is etofenprox and that it had dosage effects. Through genetic analysis using three F_2 populations, sensitivity to etofenprox is confirmed to be managed by a single dominant gene, and that gene is the same in Danbaek and Kwangan. Although further genetic research is required to identify the gene responsible for sensitivity to etofenprox, the results of this study will help to elucidate the interaction between plants and chemicals when breeding new cultivars or developing pesticides.



Keywords: soybean, insecticide, phytotoxicity, etofenprox, genetics.

Introduction

Soybeans [*Glycine max* (L.) Merr.] are a major seed crop worldwide, with a wide range of practical uses as protein and oil sources for livestock and humans.[1)] Due to global warming, the major pests in soybean fields have shifted from foliage-feeding coleopteran and lepidopteran pests to sap-sucking hemipteran pests during the last few decades.[2)] Various stink bugs and soybean aphid species are the major hemipteran pests of soybean.[3)] Hemipteran pests damage on soybean plants by sucking the plant juice. These insects have mouthparts for piercing and sucking, the most characteristics of hemipteran insects which are highly adapted to extract liquid contents from plant tissues.

Synthetic pyrethroid insecticides have been used to manage insects since a few decades ago. In 2002, the usage of synthetic pyrethroid insecticides had grown to represent 18% of the dollar value of the world insecticide market.[4)] The insecticidal mode of action of pyrethroids relies on the ability to bind and disrupt voltage-gated sodium channels in insect's nerve system.[5)]

Phytotoxicity is defined as a detrimental effect on various physiological processes, including seed germination, seedling growth, and water uptake.[6)] The symptoms of phytotoxicity are varied, including leaf speckling, leaf margin necrosis (browning) or chlorosis (yellowing), brown or yellow leaf spots, leaf cupping or twisting, plant stunting, and plant death.[7)] Several pesticides have been reported to result in phytotoxicities, such as visible injuries (chlorosis, leaf necrosis, vein discoloration, and terminal bud death), reduced vegetative growth, and disruption of reproductive organ development, leading to critical losses in crop yield.[8)]

In various crop species, the interactions between plant physiology and pesticides have been reported.[9)] In lettuce, methyl

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parathion and permethrin had significant effects on photosynthesis and stomatal conductance rates.[10] Haile *et al.* reported that the photosynthetic rates of lettuce were significantly reduced by the application of methomyl, endosulfan, acephate, and surfactant at the seedling stage.[11] In soybean, photosynthetic rates were not affected by insecticides such as chlorpyrifos, permethrin, carbaryl, and spinosad at either the seedling or reproductive stage.[12] However, a significant reduction (24% with malathion and 20% with carbaryl) in net photosynthesis was observed after the first application.[13] The physiological responses of plants to pesticides have been reported to be connected to photosynthesis or oxidative stress.[14] Nanomolecular particles from pesticides may disturb photosystem II and the biosynthesis of chlorophyll or chloroplast. Reactive oxygen species (ROS) induced by pesticides can cause oxidative stress in cellular components.[14]

Etofenprox has been widely used in soybean fields to manage pests. Although several physiological studies of interactions between plants and pesticides have been reported, genetic and genomic research has not been conducted to date. In this study, we introduce a novel trait, sensitivity to phytotoxicity of etofenprox in soybean. Among the soybean germplasm, breeding lines, and elite varieties, we found that only a limited number of resources showed leaf shrinkage due to the application of etofenprox. To elucidate this physiological response, two experimental approaches were conducted: (1) confirmation of the causal substance of phytotoxicity in soybean and (2) genetic analysis for sensitivity to phytotoxicity of etofenprox was conducted.

Materials and methods

1. Plant materials

To elucidate the response to etofenprox, Danbaek, Kwangan, Daepung, Samnam and Tohoku 69 were employed (Table 1). Danbaek, Kwangan and Tohoku 69 showing sensitivity to etofenprox were developed as high-protein cultivars and have been used as breeding materials by the Rural Development Administration (RDA).[15] Daepung[16] and Samnam[15] were developed as ingredients for soy sauce and tofu stance in 2002 and 1991, respectively, by the RDA. Daepung and Samnam show insensitivity to etofenprox.

To conduct genetic analysis (segregation analysis and complementation test), we developed three F_2 populations derived from crosses between Danbaek (Sensitivity parents)×Samnam (Insensitivity parents), Kwangan (Sensitivity parents)×Samnam and Danbaek×Kwangan in the Dankook University Experimental Field.

2. Etofenprox phytotoxicity in soybean

2.1. The inducing substance responsible for phytotoxicity

To determine the substance responsible for causing phytotoxicity, 10 plants of Danbaek, Kwangan, Tohoku 69, Daepung and Samnam were grown using a mixture of horticultural soil and nursery-bed soil at the a ratio of 3 : 1 in 50 deep-cells seed trays (55×27×12 cm, wide×length×height). All plants were grown in same method in this study. Three different treatments were applied, using the active-ingredient contents of etofenprox 20% emulsifiable concentrate (EC). The first treatment was an etofenprox 20% EC 1000× dilution consisting of etofenprox of 99% purity, acetone and surfactants. The second treatment was a mixture of acetone and surfactants 1000× dilution with no etofenprox added. The third treatment was distilled water as a negative control. The treatments were conducted with three biological replications. The foliar spray was used four times for two weeks from the V_1 (first trifoliate leaf fully expanded) stages. A 10 mL spraying volume was applied to each plant in each application. The phenotype was evaluated (Sensitivity/Insensitivity) a week after the final application.

2.2. Dosage-dependent phytotoxicity in soybean

To confirm dosages effect of phytotoxicity, 10 plants of Samnam (Insensitivity) and Danbaek (Sensitivity) were planted. Four treatments (negative control, 500×, 1000× and 2000×) were applied using a foliar spray method; a 1000× dilution of etofenprox 20% EC is the general concentration in insecticide application. From the V_1 stage, the treatments were applied four times for two weeks with 10 mL of spraying volume per plant in each application. All leaves were harvested one week after the final application and dried for 48 hr at 40°C for dry weight. ANOVA (analysis of variance) and LSD (least significant difference) tests were carried out using R software.[17]

3. Genetic analysis

3.1. Segregation analysis

Ninety-nine F_2 populations of Danbaek×Samnam and 125 F_2 populations of Kwangan×Samnam were used for segregation analysis. Foliar spraying was performed four times in two weeks at the V_1 stage using an etofenprox 20% EC 1000× dilution. A 10 mL spraying volume was applied to one plant in each application. One week after the final application, the phenotype was evaluated by sensitivity or insensitivity. A goodness-of-fit test for the segregation ratio was carried out using R software.[17]

3.2. Complementation test

Of the F_2 populations, 105 F_2 population derived from a crossing between Danbaek and Kwangan were used for complementation

Table 1. Responses to etofenprox 20% EC active ingredient in Samnam, Daepung, Danbaek, Tohoku 69 and Kwangan

Treatment	Samnam	Daepung	Danbaek	Tohoku 69	Kwangan
Negative control	×	×	×	×	×
Acetone+surfactant	×	×	×	×	×
Acetone+surfactant+etofenprox 99%	×	×	○	○	○

○: Response, ×: No response.

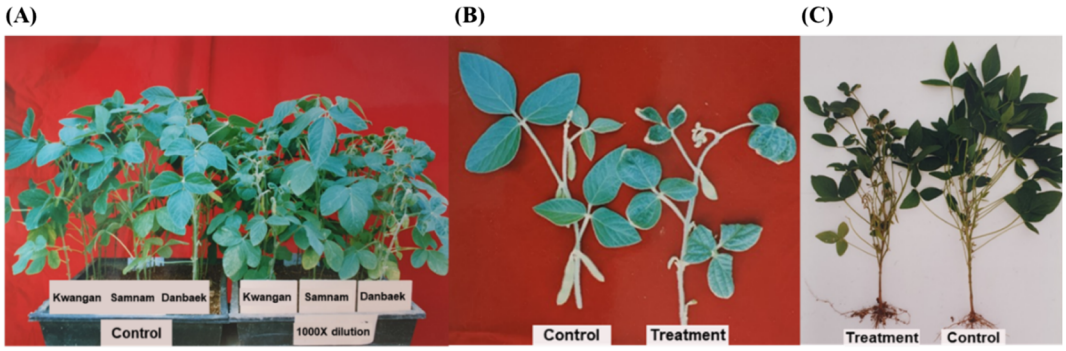

Fig. 1. Morphological responses of phytotoxicity against etofenprox 20% EC in Kwangan, Samnam and Danbaek. A: Kwangwan and Danbaek showed leaf shrinkage after treatment of etofenprox 20% EC 1000× dilution, while Samnam showed no response. B: Upper trifoliate leaves of Danbaek before/after treatment with an etofenprox 20% EC 1000× dilution. C: Whole plant of Danbaek before/after treatment with an etofenprox 20% EC 1000× dilution.

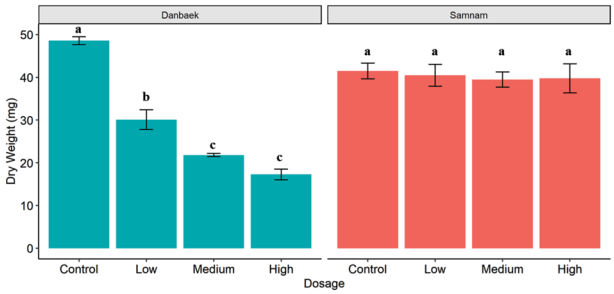

Fig. 2. Dosage effect of an etofenprox 20% EC 1000× dilution in Danbaek and Samnam. X and Y axes indicate dosage and dry weight, respectively. Control: distilled water; low: etofenprox 20% EC 2000× dilution; medium: etofenprox 20% EC 1000× dilution; high: etofenprox 20% EC 500× dilution.

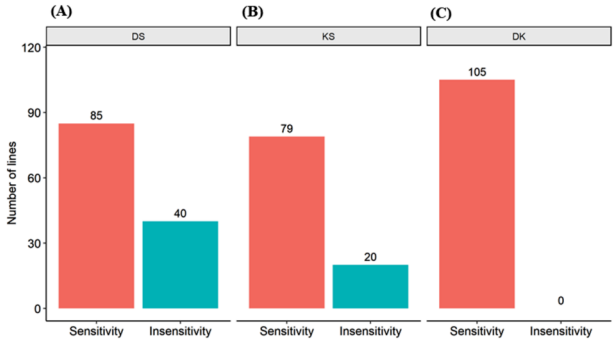

Fig. 3. Segregation analysis and complementation test for phytotoxicity caused by etofenprox using three F_2 populations. A: Segregation analysis for the Danbaek×Samnam (DS) F_2 population. B: Segregation analysis for the Kwangan×Samnam (KS) F_2 population. C: Complementation test for the Danbaek×Kwangan (DK) F_2 population.

test. Ten plants per line were grown as mentioned above. Foliar spraying was performed using an etofenprox 20% EC 1000× dilution four times in two weeks at the V_1 stage. A 10 mL spraying volume was applied to one plant in each application. One week after the final application, the phenotype was evaluated for sensitivity or insensitivity.

Results

1. Soybean response to etofenprox

1.1. The inducing substance responsible for phytotoxicity

The treatment with an active ingredient of etofenprox 20% EC was applied to confirm whether etofenprox was the causal substance of phytotoxicity in soybean. The plants treated with acetone and surfactant without etofenprox and those treated with distilled water (control) did not show any symptoms in any of the varieties (Danbaek, Kwangan, Daepung, Samnam, or Tohoku 69) (Table 1, Fig. 1A). Leaf shrinkage, the phytotoxicity symptom in soybean, was observed with the treatment including etofenprox in three varieties, Danbaek, Tohoku 69, and Kwangan. Morphological differences were clearly shown from the second trifoliate leaves to the bottom of the plants (Fig. 1B and C). As a result, we concluded that etofenprox was the key substance responsible for phytotoxicity among the components of etofenprox 20% EC (Table 1).

1.2. Dosage-dependent phytotoxicity in soybean

The dry weight of all leaves from Danbaek (Sensitivity) and Samnam (Insensitivity), except for the top trifoliate leaves that had not been treated sufficiently, was measured after 48 hr. The mean dry weights of Samnam were 41.5, 40.5, 39.5, and 39.7 mg for each dosage (control, low, medium, high) (Fig. 2). No significant difference was detected among treatments in Samnam (Fig. 2). However, the dry weight of Danbaek significantly decreased as the concentration of etofenprox increased (48.58, 30.08, 21.81, and 17.26 mg) ($p<0.001$). This result showed that the dosage of etofenprox had an effect on phytotoxicity in Danbaek.

2. Genetic analysis

2.1. Segregation analysis

To investigate the genetics underlying sensitivity to phytotoxicity of etofenprox in soybean, an etofenprox 20% EC 1000× dilution was applied to two F_2 populations. Among the 125 individuals in the Danbaek×Samnam F_2 population, 85 individuals showed sensitivity to etofenprox, and 40 individuals showed insensitivity (Fig. 3A). Among the 99 progenies of the Kwangan×Samnam F_2 population, 79 individuals showed sen-

Table 2. Segregation ratio for phytotoxicity to etofenprox in two F_2 populations

Cross combination	Observed		Expected ratio	χ^2	p
	Response	No response			
DS[a)]	85	40	3:1	3.2667	0.071
KS[b)]	79	20	3:1	1.2155	0.2702

[a)] Danbaek×Samnam (DS) F_2 population. [b)] Kwangan×Samnam (KS) F_2 population.

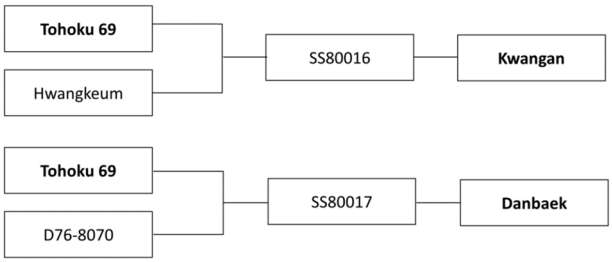

Fig. 4. Pedigree diagram of Kwangan and Danbaek. Tohoku 69 was the common ancestor of two varieties.

sitivity to etofenprox, and 20 individuals showed insensitivity (Fig. 3B). The segregation ratios in two F_2 populations were fitted to 3:1 ($p>0.05$) (Table 2). This result indicates that sensitivity to phytotoxicity of etofenprox is controlled by a single dominant gene (Table 2).

2.2. Complementation test

To examine whether the gene regulating sensitivity to phytotoxicity of etofenprox is the same gene in two different varieties, Danbaek and Kwangan, the Danbaek×Kwangan F_2 population was used for complementation test. All 105 individuals showed a shrinkage response to etofenprox (Fig. 3C), indicating that the gene causing sensitivity to phytotoxicity of etofenprox in Danbaek and Kwangan was the same, and this gene might be inherited from the common ancestor, Tohoku 69, which was also sensitive to etofenprox (Fig. 4).

Discussion

Many crops are sensitive to agrochemicals despite the fact that they are not the target of the chemical treatments. The effects of pesticides in non-target host plants, such as reduction of pollen performance in tomato,[18)] impairment of reproductive development in potato,[19)] malfunction in enzyme activities and photosynthesis in cucumber,[20)] retained germination and growth in soybean,[21)] disturbance of physiological and morphological parameters in maize,[22)] and damaged growth and production of photosynthetic pigments in tomato,[23)] have been reported. Excessive use of pesticides can disturb various processes in plants, like respiration, photosynthesis, cell growth, molecular composition, and biosynthesis.[23)] Pesticide-induced stress has been found to produce oxidative stress, contributing to toxicity in the form of ROS, such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) at the cellular level.[24)] However, phytotoxicity had not been reported in soybean, which may have affected the breeding program performed under pesticide application.

Although Tohoku 69, the common ancestor of Danbaek and Kwangan, had been widely used as a parental line in breeding programs for high protein, soybean phytotoxicity had not been reported. Phytotoxicity from insecticide is rarely observed as a morphological response in plant species because insecticides are designed to target not plants but insects, as etofenprox targets the nerve system of insects. Due to the lack of previous research on plant responses to insecticides, we conducted two experiments to elucidate the plant-insecticide interaction: segregation and complementation analysis. Segregation analysis is the statistical methodology used to determine the mode of inheritance of a phenotype, especially with a view to elucidating single gene effects.[25)] The object of complementation test is to determine whether two mutations associated with a specific phenotype represent the same gene (alleles) or are variations of two different genes.[26)] Through segregation and complementation analysis, it was confirmed that sensitivity to etofenprox in soybean was managed by a single dominant gene. In conclusion, this phenomenon was not a temporary response to an environmental factor but by genetic factor that may have been inherited stably from a common ancestor.

Danbaek is an elite cultivar developed by the RDA for high protein content, up to ~50%.[27)] Although high protein has been a major trait of interest in Korean breeding programs for the last few decades, Danbaek has rarely been used as a parental line.[15)] Because phytotoxicity from etofenprox had not been reported in soybean and leaf shrinkage, the symptom of phytotoxicity, is not easy to distinguish from damage induced by viral diseases, progenies might have had negative selective pressure on soybean fields with etofenprox application. Sensitivity to phytotoxicity of etofenprox is one of the most important traits that should be considered in a breeding program to avoid false negatives in screening procedures.

This study reported a novel phenomenon in soybean, phytotoxicity caused by etofenprox, which is a widely used insecticide. To identify the candidate gene associated with sensitivity to phytotoxicity of etofenprox, further genetic analysis is required using high-throughput genotyping system, genetic mapping tools, and recombinant inbred lines (RILs). Once the single dominant gene responsible for sensitivity to phytotoxicity of etofenprox is characterized, it can be valuable genetic tool as a selective marker in a molecular breeding system for soybean. The results of this study provide valuable information for breeding programs using soybean cultivars that show phytotoxicity.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

Conceptualization, K.-H.K. and S.K.; methodology, T.L., J.H., and J.-M.K.; software, J.-M.K.; validation, J.J. and J.-M.K.; investigation, J.J. and K.-H.K.; data curation, J.H. and S.K.; writing—original draft preparation, J.-M.K. and J.H.; writing—review and editing, J.H., J.L., and S.K.; supervision, S.K.

All authors discussed the results and agreed on the outcome of this manuscript. All authors have read and agreed to the published version of the manuscript.

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