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Discovery of Natural Resistance to Roundup (Glyphosate) Herbicide in Soybean

Guo-Liang Jiang

gjiang@vsu.edu

Agricultural Research Station, Virginia State University, Petersburg 23806, VA, USA

Hicholas Hall

South Dakota State University

William Townsend

Virginia State University

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Abstract

Soybean (*Glycine max* (L.) Merr.) is a globally important crop that provides a sustainable supply of plant protein and vegetable oil. Roundup herbicide with active ingredient glyphosate, as a broad-spectrum herbicide, has been extensively used to control post-emergent weeds in agricultural production, along with commercial growth of Roundup-ready (RR) crops. Widespread overuse of Roundup herbicide has led to the evolution of natural resistance in many weed species. On the other hand, the concerns about the potential health, environmental and cross-contamination effects of genetically modified organism (GMO) events have remained. Growing non-GMO resistant cultivars could eliminate the concern related to cross-contamination and help significantly reduce seed costs. However, no report has indicated that natural resistance to Roundup existed in soybean and other major crops. Here we report the very first discovery of natural Roundup resistance in conventional soybeans. The natural or non-GMO herbicide resistance discovered in conventional soybeans could be transmitted from the Roundup-survived plants to their offspring and thus could be used by selection for genetic improvement.

Introduction

Roundup herbicide with glyphosate as the active ingredient is a broad-spectrum or non-selective herbicide[1], and it has been the most widely used post-emergent herbicide in the production of major crops soybean, corn, cotton, canola, sugar beets and alfalfa, to control the weeds since Roundup-ready (RR) varieties were grown in 1990s[2,3]. Subsequently, concerns about the potential health and environmental effects of genetically modified organism (GMO) events and the effects of cross-contamination on conventional variety integrity have been raised and lingering[4]. Development of non-GMO resistant cultivars to herbicides could eliminate the concern related to cross-contamination incurred with GMO varieties and result in significantly reduced seed costs of agricultural production. Widespread overuse of Roundup herbicide has led to the evolution of natural resistance in about 50 weed species, such as horseweed, Palmer amaranth, and ryegrass[5]. Glyphosate-resistant weeds, also described as super weeds, can survive Roundup application at a lethal dosage used previously. However, there has been no report indicating the existence of natural resistance to Roundup herbicide in soybean and other major crops. The market is still dominated by transgenic or genetically engineered resistance, although other new technologies like gene editing have been explored to develop desired traits[6].

An unpredicted incident occurred with Roundup application in the field trials of conventional soybeans inspired the author to explore natural resistance to herbicide specifically Roundup in soybeans. In that incident, Roundup herbicide was sprayed by accident in some blocks of the conventional yield trials of South Dakota State University (SDSU) soybean breeding and genetics program. It was supposed that all the plants in the affected areas would be killed because of the non-selectivity of Roundup. However, surprisingly, some plants survived the Roundup application. Subsequently, several possible reasons for the phenomenon came to the breeder's mind, such as contamination or impurity due to mechanical mixing or cross-pollination, and surviving due to missed spraying. Natural resistance might be another possibility. It would be of broad interest and great significance if natural resistance to Roundup was revealed and could be developed through artificial selection and/or plant breeding. In this article, we report the very first discovery of natural Roundup resistance in conventional soybeans.

Results and Discussion

Discovery of Natural Resistance to Roundup and Preliminary Tests

As usual, yield trials of the SDSU soybean breeding and genetics program were planted at the Agricultural Research and Extension Center, Beresford, South Dakota in May of 2011. To control weeds, post-emergent herbicides were applied in July of 2011. However, Roundup herbicide was sprayed unintentionally in some blocks of conventional breeding lines for preliminary yield evaluation. Then some plants were observed to be surviving, although most plants were killed (Fig. 1). To determine the factors leading to the Roundup resistance, 276 surviving plants were selected from the affected plots and sampled for RR tests. Of the 276 samples, each representing a single plant, 263 exhibited positive reactions to the enzyme-linked immunosorbent assays (ELISA) test, indicating that majority of the survived plants carried Roundup ready events (Table 1, Supplementary Table S1). The Roundup-ready plants resulted most likely from mechanical or biological contamination. However, negative reaction was also detected in 13 survived plants from 10 plots, implying no Roundup-ready existed in these plants.

Table 1
Reaction of Roundup-survived plants of conventional soybeans in ELISA and PCR tests for Roundup-ready events

2011 Summer/Fall_ Field				**2012 Early Spring_ Greenhouse**				**2012 Early Spring_ Greenhouse (repeated)**			
ID_Plant #	ELISA	RR1	RR2	ID_Plant #	ELISA	RR1	RR2	ID_Plant #	ELISA	RR1	RR2
C2015-1	N	N	N	T01_1	N	N	N				
				T01_2	N	N	N				
				T01_3	N	N	N				
				T01_4	N	N	N				
				T01_5	N	N	N				
				T01_6	N	N	N				
C2015-2	N	N	N	T02_1	N	N	N				
				T02_2	N	N	N				
				T02_3	N	N	N				
				T02_4	N	N	N				
				T02_5	N	N	N				
				T02_6	N	N	N				
C2018-5	N	N	N	T03_1	N	N	N	T14 (as T03)_1	N	N	N
				T03_2	N	N	N	T14 (as T03)_2	N	N	N
				T03_3	N	N	N	T14 (as T03)_3	N	N	N
				T03_4	N	N	N	T14 (as T03)_4	N	N	N
				T03_5	N	N	N	T14 (as T03)_5	N	N	N
C2050-2	N	N	N	T04_1	N	N	N	T15 (as T04/T05)_1	N	N	N
				T04_2	N	N	N	T15 (as T04/T05)_2	N	N	N
				T04_3	N	N	N	T15 (as T04/T05)_3	N	N	N

Note: N = negative, P = positive, and Hemi = heterozygous.

2011 Summer/Fall_ Field				2012 Early Spring_ Greenhouse				2012 Early Spring_ Greenhouse (repeated)			
				T04_4	N	N	N	T15 (as T04/T05)_4	N	N	N
				T04_5	N	N	N				
				T04_6	N	N	N				
				T04_7	N	N	N				
				T04_8	N	N	N				
				T04_9	N	N	N				
				T04_10	P	Hemi	N				
				T04_11	N	N	N				
C2050-3	N	N	N	T05_1	N	N	N				
				T05_2	N	N	N				
				T05_3	N	N	N				
				T05_4	N	N	N				
				T05_5	N	N	N				
				T05_6	N	N	N				
				T05_7	N	N	N				
				T05_8	N	N	N				
				T05_9	N	N	N				
				T05_10	N	N	N				
				T05_11	N	N	N				
D1015-1	N	N	N	T06_2	N	N	N	T16 (as T06/T07)_1	N	N	N
				T06_3	N	N	N	T16 (as T06/T07)_2	N	N	N
				T06_4	N	N	N	T16 (as T06/T07)_3	N	N	N
				T06_5	N	N	N	T16 (as T06/T07)_4	N	N	N
				T06_1, 6, 7	P	Hemi	N	T16 (as T06/T07)_5	N	N	N
D1015-2	N	N	N	T07_3	N	N	N	T16 (as T06/T07)_6	N	N	N

Note: N = negative, P = positive, and Hemi = heterozygous.

2011 Summer/Fall_ Field				2012 Early Spring_ Greenhouse				2012 Early Spring_ Greenhouse (repeated)			
				T07_4	N	N	N	T16 (as T06/T07)_7	N	N	N
				T07_5	N	N	N				
				T07_6	N	N	N				
				T07_7	N	N	N				
				T07_1, 2	P	Hemi	N				
D1016-5	N	N	N	T08_2	N	N	N	T17 (as T08)_1	N	N	N
				T08_4	N	N	N	T17 (as T08)_2	N	N	N
				T08_5	N	N	N	T17 (as T08)_3	N	N	N
				T08_6	N	N	N	T17 (as T08)_4	N	N	N
				T08_1, 3	P	Hemi	N	T17 (as T08)_5	N	N	N
D1025-3	N	N	N	T09_1	N	N	N	T18 (as T09)_1	N	N	N
								T18 (as T09)_2	N	N	N
								T18 (as T09)_3	N	N	N
								T18 (as T09)_4	N	N	N

Note: N = negative, P = positive, and Hemi = heterozygous.

Polymerase Chain Reaction Tests of Roundup-Ready Events

To avoid false conclusion based on the ELISA test only, all the surviving plants were resampled from the field plots where at least one ELISA-negative plant was identified and then tested by polymerase chain reaction (PCR) for RR1 and RR2 events. The results indicated that all the 13 ELISA-negative plants showed negative in both RR1 and RR2 tests (Table 1, Supplementary Table S2), confirming that these plants did not contain RR1 and/or RR2 events. The Resistance to Roundup in these plants was not attributed to genetical engineering but might result from natural variation or mutation. In addition, some survived plants exhibited a homozygous or heterozygous status to RR1 in the PCR tests (Table 1, Supplementary Table S2), implying that natural hybridization occurred in the parental plants with RR1 plants. Natural hybridization due to cross-pollination in soybean was evidenced in other studies although its occurrence is low[7].

Confirmation of the Resistance in Offsprings

Only a trait that can transmit to next generations is of significance and useful for genetic improvement. To further determine the inheritability of the Roundup resistance observed, a part of seeds harvested from the Roundup-survived plants in the field were grown in the greenhouse at SDSU in early 2012 (Fig. 2). At stage V2 [8], fully developed unifoliolate leaves were treated for all the plants by smearing/touching with a Roundup-soaked paintbrush. Then the surviving plants were sampled and subjected to tests for RR events again. Of the 108 samples in ELISA test, 34 showed positive reaction but 74 were negative (Fig. 3, Table 1, Supplementary Table S3). Excluding 2 plants not tested in the PCR tests, 72 of the 74 plants with negative ELISA results also exhibited negative reactions to both RR1 and RR2 events (Fig. 3, Table 1, Supplementary Table S4), indicating that no RR events existed in these plants, consistent with the results of their parental plants. It was further demonstrated that the resistance to Roundup in the conventional soybean plants was not derived from GOM events but was natural and could pass from the Roundup-survived plants to their offspring. Eight CP4-positive plants were heterozygous for RR1 but negative for RR2, indicating that they were derived from hybrid seeds between conventional and RR1 plants. With few exceptions, three checks exhibited the expected results consistent with their nature of reaction to Roundup treatment (Supplementary Table S4). All plants with non-GMO or natural Roundup resistance and eight RR1-heterozygous plants were individually harvested. The seeds were then sown in the greenhouse to produce next generations for further study and selection.

In the summer of 2012, the plants derived from the eight RR1-heterozygous plants were treated with Roundup as described above. Of them, 70 plants survived and then were sampled for PCR detection of RR homozygosity. As a result, 32 and 38 were homozygous and heterozygous for RR1, respectively (Supplementary Table S5). It was further verified that their parental plants were hybrids that might result from a natural cross[7]. Therefore, more attention should be paid to the effects of GMO varieties on the genetic integrity of conventional crops due to cross-pollination.

Additional Evidence of Natural Resistance to Herbicide in Soybean

Natural resistance to herbicides in conventional crops is truly rare but it deserves to be explored. In soybeans, it was observed in another incident that occurred in the soybean yield trials at Virginia State University (VSU) Randolph Research Farm. In 2018, the yield test trials including 386 genotypes were planted in five trials each with 80 entries in a randomized complete block design (RCBD) with three replications except two for a preliminary yield trial (PYT). All the genotypes including 376 breeding lines and ten check cultivars are edamame and conventional soybeans. As applied generally, the post-emergence herbicide Poast was selected to control grass weeds. In early August of 2018, the herbicide was sprayed in the field trials. However, serious damage was observed unexpectedly (Fig. 4), which was totally different from previous results because the Poast herbicide is a selective herbicide specifically for grass weed control but ineffective for broad-leaf species. Then the breeder checked the issue with the technician and was informed that the herbicide was mixed with other unknown herbicides, most likely in the sales store. One month after the herbicide spraying, the damage was scored in a 0–9 scale on September 10th and 11th. The data indicated that based on the means of three replications, the scores averaged 4.3 ± 1.7, ranging from 0–9 among the 386 lines and cultivars. An approximately normal distribution was shown in the frequency analysis (Fig. 5), and broad-sense heritability was estimated to be 71.54%. This result also supported the hypothesis that natural herbicide resistance existed in conventional soybeans, although the resistance seemed to be complicated because the herbicide that damaged conventional soybeans was not elucidated in this case.

In conclusion, it was discovered in this study, for the first time, that there were conventional soybean plants and their offspring that could survive Roundup herbicide application and did not carry Roundup-ready events. The results demonstrated that natural or non-GMO Roundup resistance existed in conventional soybeans and the resistance was inheritable or transmittable from the Roundup-survived plants to their offspring. It would be possible and feasible to control weeds using non-GMO but natural herbicide resistance. This study sheds light on development of conventional crops with resistance to herbicides, especially Roundup, using natural resistance instead of genetic engineering or GMO technology. It is worth noting that the results presented in this article were preliminary, and further study is needed to elucidate the genetic mechanisms of the trait. Large-scale germplasm screening and additional selection for natural resistance to herbicides like roundup would facilitate development of herbicide-resistant cultivars of conventional crops.

Methods

Materials and Field trials

As usual, yield trials of the SDSU soybean breeding and genetics program were planted in a replicated Randomized complete block design (RCBD) at the Agricultural Research and Extension Center, Beresford, South Dakota in May of 2011. Post-emergent herbicides were applied to control weeds. However, an unpredicted incident happened in the preliminary yield trials of conventional soybean breeding lines. In July of 2011, Roundup herbicide was sprayed unintentionally in some blocks of the preliminary yield trials 2C and 2D. Some plants were observed to survive the Roundup application although most of the plants in the affected areas were killed (Fig. 1). To determine if the survived plants came from mechanical impurity or were derived from natural crosses between conventional and Roundup-ready genotypes and if they had non-GMO or natural resistance to Roundup, the survived plants were tagged, and fresh leaves were sampled from each of the plants, pending RR tests.

Roundup-Ready Tests

First, the leaf samples were tested using enzyme-linked immunosorbent assays (ELISA) for detection of RR trait, i.e., the CP4 EPSPS protein from Agrobacterium tumefaciens strain CP4 used in development of RR soybeans[9]. The tests were accomplished in a Monsanto-authorized laboratory (Biodiagnostics). The leaf samples were labelled as plot number followed by a plant number. Second, polymerase chain reaction (PCR) tests were conducted to specifically identify RR1 (GTS 40-3-2) and RR2 (MON 89788) events[10,11]. Similarly, another set of fresh leaves were sampled from each of the plants that exhibited negative results in the ELISA tests and were tested by PCR/DNA marker screening[12] in another Monsanto-authorized laboratory (SGS). The negative RR1 and RR2 reacted plants were supposed to have non-GMO resistance to Roundup. Therefore, all the plants with negative reactions in PCR tests were individually harvested in the field after full maturity for further tests, while the plants with positive results were discarded.

To confirm the inheritability of the resistance, seeds harvested from the nine Roundup-resistant conventional plants were grown in a greenhouse at SDSU in early 2012 (Fig. 2). Seeds of each plant were planted in multiple pots, each with three seeds sown. The pots were labelled as RR-T01 through RR-T09 indicating individual Roundup-resistant plants harvested in the 2011 field trials. RR and conventional cultivars were also grown as checks, designated as RR-T10 (CK1), RR-T11 (CK2) and RR-T12 (CK3), respectively. At the growth stage V2 [8], fully developed unifoliolate leaves of all the plants were treated by smearing/touching with a Roundup-soaked

paintbrush. Then leaf samples were taken from each of the surviving plants about 8 weeks after the Roundup-treatment. The leaf samples were designated as RR-T01 through RR-T09 with an additional plant number followed to indicate individual plants sampled. ELISA and PCR tests were conducted as described above. In addition, the tests were repeated using another set of samples taken from the plants grown in the greenhouse using the seeds harvested from the same sources/lines where Roundup was not sprayed in previous year, following the same Roundup treatment and ELISA and PCR test methods as described[9–11]. The samples were designated as RR-T13 through RR-T18 followed by a plant number. RR-T13 had the same source as RR-T01 and RR-T02 but was missing in the test report. RR-T14 had the same source as RR-T03, RR-T15 as RR-T04 and RR-T05, RR-T16 as RR-T06 and RR-T07, RR-T17 as RR-T08, and RR-T18 same as RR-T09, respectively. All the surviving plants with negative reactions in PCR tests were harvested individually and the seeds were planted in the greenhouse to advance next generations. The seeds from those plants with non-negative reactions to PCR tests (i.e., heterozygous for RR1) were grown in the greenhouse in the summer/fall of 2012 and PCR tests were performed again to confirm homozygosity of the Roundup-ready events.

Declarations

Conflict

Statement: The authors declare no conflict of interest.

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Author Contribution

G-L J. raised the conception, designed the research and experiments, secured fundings, managed the project, analyzed data and wrote the manuscript. N.H. and W. T. implemented the experiments and assisted in data collection. All authors reviewed and approved the manuscript.

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Data Availability

All data generated or analyzed during this study are included in this published article and the supplementary tables.

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Figures



Figure 1

Conventional soybean plots after Roundup spraying and surviving plants tagged 2011



Figure 2

Plants derived from the Roundup-survived conventional plants in the 2011 field trials were planted in greenhouse in 2012. Left, before treatment, and right, after treatment. The necrosis was caused by the Roundup treatment.



Figure 3

Surviving conventional plants after Roundup treatment in the greenhouse 2012. The necrosis was caused by the Roundup treatment. Top, RR checks after treatment, and middle and bottom, conventional resistant plants after treatment.



Figure 4

Conventional soybean plots showing variable responses to application of Poast mixed with other unknown herbicide(s) sprayed at VSU Randolph Farm, 2018

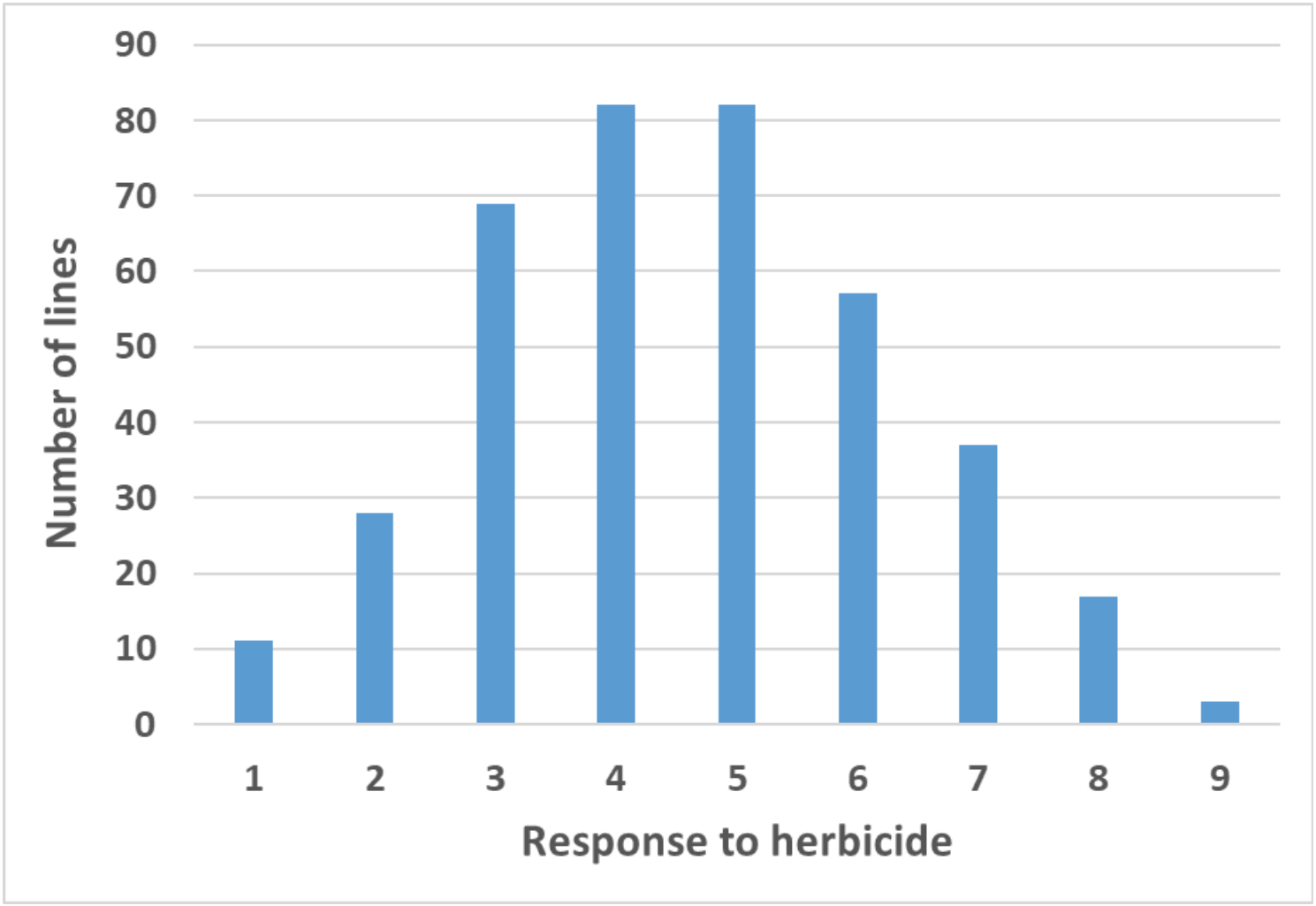


Figure 5

Frequency distribution of responses to application of Poast mixed with unknown herbicide(s) in 386 conventional soybean genotypes (2018)