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ORIGINAL PAPER

Plant characterization of Roundup Ready 2 Yield® soybean, MON 89788, for use in ecological risk assessment

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Abstract During the development of a genetically modified (GM) crop product, extensive phenotypic and agronomic data are collected to characterize the plant in comparison to a conventional control with a similar genetic background. The data are evaluated for potential differences resulting from the genetic modification process or the GM trait, and the differences—if any—are subsequently considered in the context of contributing to the pest potential of the GM crop. Ultimately, these study results and those of other studies are used in an ecological risk assessment of the GM crop. In the studies reported here, seed germination, vegetative and reproductive growth, and pollen morphology of Roundup Ready 2 Yield® soybean, MON 89788, were compared to those of A3244, a conventional control soybean variety with the same genetic background. Any statistically significant differences were considered in the context of the genetic variation known to occur in soybean and were evaluated as indicators of an effect of the genetic modification process and assessed for impact on plant pest (weed) characteristics and adverse ecological impact (ecological risk). The results of these studies revealed no effects attributable to the genetic modification process or to the GM trait in the plant that would result in increased pest potential or adverse ecological impact of MON 89788 compared with A3244. These results and the associated risk assessments obtained from diverse geographic and environmental conditions in the United States and Argentina can be used by regulators in other countries to inform various assessments of ecological risk.

Keywords Soybean · Ecological risk assessment · Glyphosate tolerance

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Introduction

Prior to commercializing a genetically modified (GM) crop product, a variety of assessments must be performed to assess the safety of the product (Prado et al. 2014). In the United States the overall assessment considers factors "including but not limited to: Plant

pest risk characteristics, disease and pest susceptibilities, expression of the gene product, new enzymes, or changes to plant metabolism, weediness of the regulated article, impact on the weediness of any other plant with which it can interbreed, agricultural or cultivation practices, effects of the regulated article on nontarget organisms, indirect plant pest effects on other agricultural products, transfer of genetic information to organisms with which it cannot interbreed, and any other information which the Administrator believes to be relevant to a determination" (CFR 2008 §340.6). The ecological risk assessment (ERA) examines whether the GM crop has the potential to become a plant pest or to have other adverse ecological impacts (e.g., adverse effects on nontarget organisms).

A generally accepted approach to generate data for an ERA is based on comparative assessment of plant characteristics (e.g., phenotypic, agronomic, and ecological characteristics) between the GM plant and an appropriate non-GM conventional control variety (Conner et al. 2003; Hill and Sendashonga 2003; EFSA 2004; Roberts et al. 2013; Prado et al. 2014). The results of the assessments are evaluated for potential harmful effects as a result of the genetic modification process or the trait, are used in ecological risk assessment, and may be used for other evaluations (e.g., to confirm product efficacy evaluations). The requirement for an ERA as a component part of the product assessment and authorization process is specified by the United States Department of Agriculture–Animal and Plant Health Inspection Service (CFR 2008), the Canadian Food Inspection Agency (CFIA 2012), the European Union (EFSA 2004), and other regulatory agencies throughout the world.

Testing and development of a GM crop from initial transformation through commercialization takes many years; a survey of biotechnology companies by Phillips McDougall (2011) indicated that across crops, the average length from discovery of a trait to first commercial sale was more than 13 years. Initially a technology provider may develop hundreds to thousands of unique insertion events (Heck et al. 2005; Phillips McDougall 2011). Throughout the development cycle, these events are evaluated and only those that meet specified criteria (e.g., molecular, protein, expression, phenotypic and agronomic performance) are advanced towards potential commercialization (Mumm and Walters 2001; Prado et al. 2014). Those that do not meet these criteria are eliminated from advancement. Thus, a GM crop that is being developed for commercialization and regulator approval has already passed through many evaluations prior to reaching the stage where much of the ERA data is generated.

The ERA for pest potential and adverse ecological impact of a GM crop is based on an approach similar to that used in ecotoxicological studies (Hill and Sendashonga 2003; Raybould 2007; Nickson 2008); in the context of GM crop plants, the focus is on differences detected in the plant that might cause ecological harm, rather than on all possible changes (Raybould 2007). If one or more differences are observed between the GM crop and the control, the differences are examined to determine whether they have the potential to cause ecological harm (Horak et al. 2007; Prado et al. 2014). The overall assessment approach and individual study design is in accordance with the concept of familiarity as originally developed by the OECD (1993). Familiarity can be defined as "knowledge gained through experience over time… [that] considers the nature of the crop that was modified, the characteristics of the trait that was introduced, the likely receiving environment for the GM crop, and the likely interactions between these" (Nickson 2008). Using familiarity as a base, judgments are made as to which types of characteristics should be measured and what types of changes could possibly cause harm if they were to occur (Wolt et al. 2010). Examples of this kind of risk assessment have previously been reported for Roundup Ready® Flex cotton (Horak et al. 2007) and for drought-tolerant corn (Sammons et al. 2014).

The results of well-designed plant characterization studies conducted in the field, greenhouse, or laboratory and subsequent ecological risk assessments are used by risk assessors and regulators to evaluate whether unconfined release (e.g., cultivation) or limited release (as may occur in countries that import grain) is acceptable in their country (Roberts et al. 2013). Since the studies are conducted in diverse geographies representing a broad range of environmental conditions and agricultural ecosystems, and given the similarity of the endpoints being assessed, these results and the associated risk assessments could be "transportable" to other countries (Roberts et al. 2013), i.e., although the studies may not have been conducted in a specific country, the risk assessors in that country can use the data and assessments from other countries to inform their own assessments of potential ecological hazards.

In this report, we summarize studies performed to support the ERA for potential weediness in cropping systems and for specific potential adverse ecological effects (invasiveness of natural plant ecosystems) of Roundup Ready 2 Yield® soybean (*Glycine max* (L.) Merrill), 'MON 89788'. MON 89788 is a second-generation soybean product developed by Monsanto Company to provide tolerance to Roundup® agricultural herbicides. MON 89788 contains the 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* sp. strain CP4 (*cp4 epsps*). Expression of the gene product (CP4 EPSPS protein) renders the plant tolerant to glyphosate, which is the active ingredient in Roundup agricultural herbicides.

MON 89788 uses the CP4 EPSPS protein, which was also produced in the first-generation Roundup Ready® soybean (40-3-2), and in Roundup Ready® corn, Roundup Ready® alfalfa, Roundup Ready® cotton, Roundup Ready® canola, Roundup Ready® sugarbeets, and Roundup Ready® Flex cotton (CERA 2011). Roundup Ready soybean 40-3-2 was the first soybean product containing a biotechnology trait commercialized in the United States. The relevant existing information and experience gained from the cultivation of these Roundup Ready crops can be used to inform risk assessments for MON 89788 conducted by global regulatory agencies and to identify if additional information is needed to assess for specific hazards of interest. To date there have been no credible reports of potential weediness (of the GM crop) or adverse ecological impact (invasiveness to native plant communities or harm to nontarget organisms) of Roundup Ready crops (e.g., CERA 2011). Furthermore, the information and experience gained from the commercial production of these crops were used in the problem formulation (planning phase) for the ERA of MON 89788 soybean. Based upon the concept of familiarity (experience with these crops, with soybean, with the Roundup Ready trait, and with the potential areas of cultivation), it was hypothesized that there would be no increased weediness potential or potential adverse ecological effects (i.e., invasiveness into native plant communities) from the cultivation of MON 89788.

This report includes plant characterization data on germination characteristics, vegetative and reproductive growth characteristics, and pollen morphology of MON 89788 compared to a conventional control. These results are particularly useful in evaluating weediness and invasiveness of MON 89788. Additional studies on the ecological interactions of MON 89788 are reported in Horak et al. (submitted). The endpoints chosen to inform this risk assessment for potential weediness and potential adverse ecological effects were based on the experience of plant breeders, weed scientists, risk assessors, and other agricultural experts familiar with soybean, and those familiar with ecological risk assessment of GM crops.

Materials and methods

MON 89788, control, and reference materials

MON 89788 seed, plants, or pollen were used in these studies (Supplementary Table S1). The control material was seed, plants, or pollen of non-glyphosate-tolerant soybean, A3244. A3244 has a genetic background similar to MON 89788 with the exception of the glyphosate tolerance trait; it does not contain the inserted DNA present in MON 89788. The reference materials were seed, plants, or pollen of commercially available soybean varieties that were selected to represent a diversity of the commercially cultivated soybean and varied by study (Supplementary Table S1). The references provided a range of background values common to commercial soybean for the characteristics assessed. Details on all study sites are given in Supplementary Table S2.

Germination

MON 89788, the control, and reference seed were produced in 2005 in each of three US locations (Supplementary Table S2): Jackson County, AR (site code AR), Clinton County, IL (site code IL4) and Fayette County, OH (site code OH2). Seed testing was conducted at BioDiagnostics Inc. (River Falls, WI) with methods consistent with those established by the Association of Official Seed Analysts (AOSA 2000, 2002). Four replicate rolled towels containing 100 seed each of MON 89788, control, or reference seed produced at each location were placed into each of six germination chambers. Towels were arranged in each chamber in a split-plot design, with seed production location as the whole plot and seed material as the subplot. The chambers were maintained at a target alternating temperature of 20/30 °C (AOSA-

Table 1 Plant characteristics evaluated in studies on MON 89788, A3244, and commercial reference varieties in 2004–2006

Study	Characteristic	Evaluation timing[a]	Evaluation description
Germination	Normal germinated seed (optimal temperature only)	5 and 8 days after planting	Seedlings that exhibited normal developmental characteristics and possessed both a root and a shoot
Germination	Abnormal germinated seed (optimal temperature only)	8 days after planting	Seedlings that could not be classified as normal germinated (e.g., insufficient root and shoot development, lacked a shoot, shoot with deep cracks or lesions, or exhibited mechanical damage)
Germination	Germinated seed (additional non-optimal temperatures only)	5, 8, and 13 days after planting	Any seedling with a radicle of 2 mm or more
Germination	Viable hard seed	8 days after planting (optimal temp.) 13 days after planting (additional temp.)	Seed that did not imbibe water and remained hard to the touch
Germination	Dead seed	5 and 8 days after planting (optimal temp.) 5, 8, and 13 days after planting (additional temp.)	Seeds that had visibly deteriorated and had become soft to the touch
Germination	Viable firm swollen seed	8 days after planting (optimal temp.) 13 days after planting (additional temp.)	Seeds that had visibly swollen (imbibed water) and were firm to the touch but lacked any evidence of growth
Phenotypic Study 1, 2, 3	Early stand count	V2–V4	Number of emerged plants in two inner rows of each plot
Phenotypic Study 1, 2, 3	Growth stage monitoring	Recurring, recorded every 2–3 weeks from approx. V2 until R8	Average growth stage, using guidelines outlined in Pedersen (2004)
Phenotypic Study 1, 2, 3	Seedling vigor	V2–V4	Rated on a 1–9 scale, where 1–3 = excellent, 4–6 = average, and 7–9 = poor vigor
Phenotypic Study 1, 2, 3	Days to 50 % flowering	R1–R2	Days from planting until approx. 50 % of the plants in each plot were flowering
Phenotypic Study 1, 2, 3	Flower color	R1–R2	Color of flowers: purple, white, or mixed
Phenotypic Study 2, 3	Pubescence	R8	Phenotypic 2: Type of pubescence: hairy, hairless, or mixed Phenotypic 3: Color of pubescence: gray, tawny, light tawny, or mixed
Phenotypic Study 1, 2, 3	Plant height	R8	Distance from the soil surface to the uppermost node on the main stem of five representative plants per plot
Phenotypic Study 1, 2, 3	Lodging	R8	Rated on a 0–9 scale, where 0 = completely up and 9 = completely down
Phenotypic Study 1, 2, 3	Pod shattering	R8	Rated on a 0–9 scale, where 0 = no shattering and 9 = completely shattered
Phenotypic Study 1, 2, 3	Final stand count	R8	Number of plants in two inner rows of each plot
Phenotypic Study 1, 2	Seed moisture	R8	Percent moisture content of harvested seed
Phenotypic Study 1, 2, 3	100-seed weight[b]	R8	Mass (g) of 100 harvested seed

Table 1 continued

Study	Characteristic	Evaluation timing[a]	Evaluation description
Phenotypic Study 1, 2, 3	Yield	R8	Mass (Mg) of harvested seed produced per hectare, adjusted to 13 % moisture
Pollen	Pollen viability	Single day during flowering	Percent viable (red–purple) grains after staining
Pollen	Pollen diameter and morphology	Single day during flowering	Diameter measurement along two perpendicular axes, and qualitative assessment of morphology

[a] Plant developmental stages are as described in Pedersen (2004)

[b] Referred to in Phenotypic Studies 1 and 3 as seed test weight

recommended optimal alternating temperature) or at 10, 20, 30, 10/20, or 10/30 °C (non-AOSA, additional target temperatures). Alternating temperatures were maintained for 16 h at the lower temperature and 8 h at the higher temperature. Growth chamber temperatures were monitored with Watchdog 110 Data Loggers (Watchdog Data Loggers, Spectrum Technologies, Inc., Plainfield, IL).

Seed in all towels were evaluated 5 and 8 days after planting; germinated and dead seeds were removed on each evaluation day. Seed from the optimal-temperature tests were categorized as normal germinated, abnormal germinated, firm swollen, hard, or dead according to AOSA definitions (Table 1). For seed tested under the non-optimal temperatures, normal and abnormal germinated seed were classified together as "germinated"; all other categories were as defined for the AOSA temperature regime. In the optimal treatment, hard or firm swollen seeds present on day 8 were subjected to a tetrazolium test to assess viability (AOSA 2000). Seed in the non-optimal-temperature treatments were also evaluated 13 days after planting and any remaining hard or firm swollen seed were subjected to a tetrazolium test to assess viability.

Growth and development observations

Phenotypic Study 1

Trials were established at each of 17 US sites during spring 2005 in a randomized complete block design with six entries (MON 89788, control, four references appropriate to the site) and three replications per site (Supplementary Table S2). Agronomic practices (e.g., tillage, fertilizer, pesticides) used to prepare and maintain each study site were characteristic of those used in each respective geographic region, and plot size, seeding depth, seeding rate, and planting date varied by location. Herbicides containing glyphosate were not used in this study to avoid injury to the conventional control and reference plants and to ensure all plots were managed uniformly. Phenotypic characteristics and growth stage were evaluated throughout the life of the plant, from emergence through harvest (Table 1). The minimum and maximum mean reference values (from across the sites) were determined to represent the range of values for the phenotypic characteristics that are typical for commercially available soybean.

Phenotypic Study 2

Trials were established at each of five US sites during spring 2006 (Supplementary Table S2). Other study characteristics and methods were as described for Phenotypic Study 1.

Phenotypic Study 3

Trials were established at each of five Argentina sites during late 2004 or early 2005 (Supplementary Table S2). Other study characteristics and methods were as described for Phenotypic Study 1.

Pollen morphology and viability

Pollen samples of the MON 89788, control, and reference materials were collected from a site in Lincoln County, MO from Phenotypic Study 1 (site MO2; Supplementary Table S2). Three whole flowers were collected from each of five plants per plot on a single day

for a total of 15 flowers per plot. Pollen was extracted from each of the three flowers per plant and placed into a microcentrifuge tube. To assess pollen viability, approximately 0.1 ml of Alexander's stain (Alexander 1980) was added to each tube, and tubes were thoroughly mixed via vortex, heated in a water bath at approximately 55 °C for approximately 10 min, and then placed in cold storage (approximately 4 °C) until viewing.

Pollen in stain solution was transferred to microscope slides, viewed under a light microscope, and visualized and assessed with the aid of a digital color camera. Pollen viability was evaluated for each sample by counting viable (red to purple) and dead (blue to green, sometimes collapsed or otherwise misshaped) pollen grains. Pollen grain diameter was evaluated for ten representative viable pollen grains collected from one plant per plot. Micrographs (200×) of the ten selected pollen grains were imported into Image-Pro Plus v4.5.1.27 (Media Cybernetics, Inc.) software for diameter measurement along the *x*-axis and the *y*-axis (i.e., perpendicular to the *x*-axis). General morphology of pollen was observed for one (randomly selected) of the three micrographs for each material evaluated for pollen grain diameter.

Statistical analysis

Germination study

Germination data were analyzed according to a split-plot design using SAS software (SAS Institute 2002–2003) to compare the MON 89788 to the control for each temperature regime. The whole-plot treatment was the seed production site, arranged in a randomized complete block design. The subplot was the seed material, arranged in a completely randomized design. Statistical significance was set at $p \leq 0.05$. The data were combined across the three production sites and MON 89788 was compared to the control for the measured characteristics. MON 89788 was not statistically compared to the reference materials. The minimum and maximum mean values were determined from among the commercially available soybean reference varieties.

Phenotypic studies

For each study, the data for MON 89788 and the control were analyzed (where appropriate; e.g., flower color was not analyzed) according to a randomized complete block design using SAS software (SAS Institute 2002–2003). Statistical significance was set at $p \leq 0.05$. For each analyzed characteristic, MON 89788 was compared to the control, combined across all sites. There were no statistical comparisons between MON 89788 and the references. The minimum and maximum mean values across sites for the reference varieties were calculated.

Pollen study

Pollen data were analyzed according to a randomized complete block design using SAS software (SAS Institute 2002–2003). Weighted LSMeans (arcsine transformation) of the percentage viable pollen and mean pollen grain diameter for MON 89788 were compared to the control at the $p \leq 0.05$ significance level. For pollen viability, $n = 3$, with 5 plants (subsamples) per replicate; for pollen diameter, $n = 3$, with 20 diameter measurements per replicate per material. No statistical comparisons were made between MON 89788 and references. Minimum and maximum mean values were calculated for the references.

Data interpretation for ecological risk assessment

Figure 1 outlines the stepwise process used to determine whether a statistically significant difference between MON 89788 and A3244 for any of the characteristics assessed was potentially adverse in terms of pest potential or ecological impact. A "No" answer at any step indicated that the difference did not contribute to a biological or ecological concern for MON 89788, and subsequent steps were not considered. In the initial steps, data from MON 89788 and A3244 were compared at a single location (pollen study) or across locations (germination study and growth and development studies) (Fig. 1, Steps 1 and 2). If a statistically significant difference between MON 89788 and the control was detected, then the MON 89788 mean value was compared with the range of means obtained for the reference materials grown in that study (Fig. 1, Step 3). If the MON 89788 mean value for an assessed characteristic was outside the range of the means of the reference materials, the MON 89788 mean characteristic value was considered in the context of published literature values for

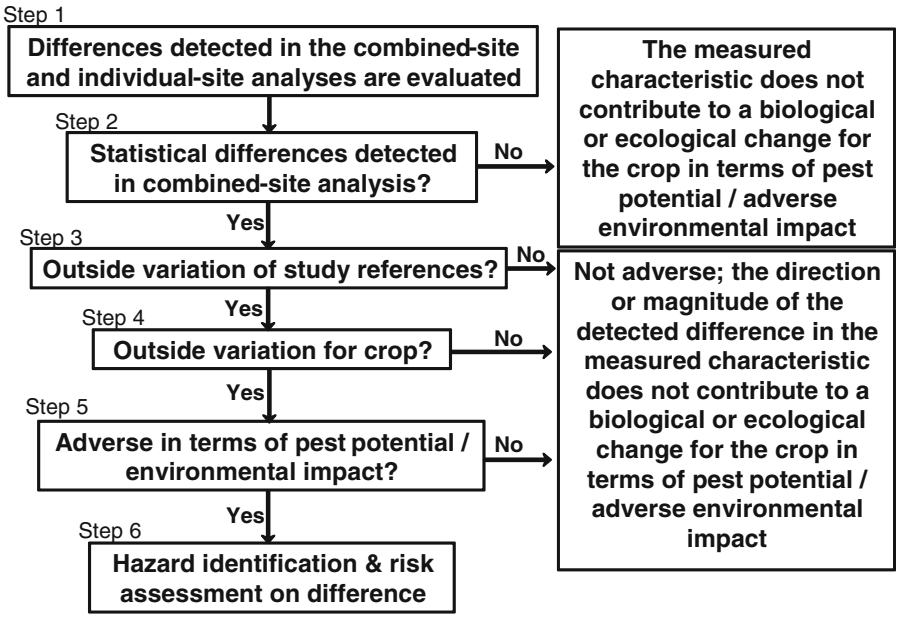

Fig. 1 A stepwise model for assessing the results of comparative phenotypic characterization experiments (adapted from Horak et al. 2007)

the characteristic for commercial varieties of the crop. If the MON 89788 mean value for a particular characteristic was outside the published characteristic values for commercial soybean varieties (Fig. 1, Step 4), the characteristic would be assessed for the magnitude of the change and for whether or not it was adverse in terms of pest (weed) potential or other ecological impact. If an adverse effect (hazard) was identified, then a risk assessment of the difference would be performed. In such a situation, the risk assessor would then consider potential contributions of the difference to elevated pest potential of the crop, the interaction of the crop with other organisms, the impact of any statistical differences detected on other measured characteristics, and the potential for and effects of transfer of a characteristic to wild soybean or other sexually compatible species.

Results and discussion

Germination study

A total of 25 comparisons were made between MON 89788 and the A3244 control in the combined-site analysis. No differences were detected between MON 89788 and the control for percent germinated, viable hard, dead, or viable firm swollen seed in the 10, 20, 30, 10/20, and 10/30 °C temperature regimes (Table 2). In addition, no differences were detected between MON 89788 and the control for percent normal germinated, abnormal germinated, viable hard, or viable firm swollen seed in the AOSA temperature regime (20/30 °C). One difference was detected between MON 89788 and the control under the AOSA temperature regime, where percent dead seed was lower for MON 89788 compared to the control (5.7 vs. 10.1 %). The mean value of percent dead seed for MON 89788 was, however, within the range of values observed for the reference varieties. Furthermore, this difference in percent dead seed was not detected in any of the five additional temperature regimes. Therefore, the difference detected in percent dead seed of MON 89788 is unlikely to be associated with the genetic modification and is unlikely to be biologically meaningful in terms of increased weed potential (Fig. 1, Step 3). No viable hard (dormant) seed were observed for MON 89788 or the control from any seed production site in any temperature regime. The results failed to reject the null hypothesis, i.e., they support a conclusion of no consistent differences in dormancy and germination characteristics.

Table 2 Comparison of MON 89788 to the control across seed production sites for germination characteristics

Temperature regime	Germination category[a]	Mean %		Reference range[b]	
		MON 89788	Control	Min.	Max.
10 °C	Germinated	94.1	94.5	49.3	99.3
	Viable hard	0.0	0.0	0.0	0.3
	Dead	5.7	5.2	0.8	49.5
	Viable firm swollen	0.3	0.3	0.0	1.3
20 °C	Germinated	92.3	90.6	44.5	99.3
	Viable hard	0.0	0.0	0.0	0.3
	Dead	7.8	9.3	0.8	55.5
	Viable firm swollen	0.0	0.1	0.0	0.0
30 °C	Germinated	94.4	93.9	57.0	98.5
	Viable hard	0.0	0.0	0.0	0.0
	Dead	5.6	6.1	1.5	43.0
	Viable firm swollen	0.0	0.0	0.0	0.0
10/20 °C	Germinated	94.5	94.2	46.3	99.0
	Viable hard	0.0	0.0	0.0	0.0
	Dead	5.4	5.8	1.0	53.3
	Viable firm swollen	0.1	0.0	0.0	0.3
10/30 °C	Germinated	94.1	93.9	50.3	99.5
	Viable hard	0.0	0.0	0.0	0.0
	Dead	5.9	6.1	0.5	49.5
	Viable firm swollen	0.0	0.0	0.0	0.3
20/30 °C (AOSA)	Normal germinated	78.4	73.2	12.5	94.5
	Abnormal germinated	15.9	16.5	4.5	36.5
	Viable hard	0.0	0.0	0.0	0.0
	Dead	5.7*	10.1	0.8	55.8
	Viable firm swollen	0.0	0.0	0.0	0.3

* Indicates a statistically significant difference between MON 89788 and the control at $p \leq 0.05$

[a] Germinated seed in the AOSA temperature regime were categorized as either normal germinated or abnormal germinated seed, whereas in the other temperature regimes these categories were combined and termed "germinated"

[b] Minimum and maximum mean values from the 12 commercial reference varieties

Growth and development observations

Phenotypic Study 1—US

In 114 growth stage observations at individual sites, MON 89788 was within the same growth stage range as the control 113 times (data not shown). The single exception was during the third observation at a single site in Iowa (site code IA2), where MON 89788 was evaluated as more mature than the control (R3 vs. R2, respectively). However, the growth stage of MON 89788 was within the range of growth stages observed for the commercial references at the site, and MON 89788 was at the same growth stage as the control during subsequent observations at that site.

In the combined-site analyses, no differences were detected between MON 89788 and the control for early stand count, seedling vigor, days to 50 % flowering, lodging, pod shattering, final stand count, seed moisture, seed test weight, or yield (Table 3). Flower color was also the same between MON 89788 and the control at all sites. The only significant difference was reduced plant height for MON 89788 compared to the control (77.7 vs. 82.0 cm; Table 3). Although plant height for MON 89788 was reduced compared to the control, the mean value observed for MON 89788 was within the range of mean values observed in the commercial references (Table 3). Furthermore, the magnitude of the difference in plant height was small (approximately 5 %), and a small decrease in plant height would not be likely to contribute to increased weed potential. Therefore, the difference detected in plant height across sites is unlikely to be biologically meaningful in terms of increased weed potential (Fig. 1, Step 3).

Table 3 Phenotypic Study 1: phenotypic characteristics of MON 89788, the control, and the references across 17 US sites

Phenotypic characteristics (units)	MON 89788	Control	Reference range[a]	
			Min.	Max.
Early stand count (# plants/2 rows)	291	299	193	360
Seedling vigor	2.5	2.4	1.7	5.0
Days to 50 % flowering	44	45	33	50
Flower color[b]	Purple	Purple	–	–
Plant height (cm)	77.7*	82.0	48.8	108.2
Lodging	0.5	0.6	0.0	5.2
Pod shattering[c]	0.0	0.0	0.0	0.2
Final stand count (# plants/2 rows)	266	270	178	297
Seed moisture (%)	11.5	11.7	8.8	15.1
Seed test weight (g/100 seed)	15.0	15.2	13.5	17.4
Yield (Mg/ha)	3.3	3.4	1.1	4.4

– Indicates that no reference range is available for categorical data

* Indicates a statistically significant difference between MON 89788 and the control at $p \leq 0.05$

[a] Reference range = minimum and maximum mean values observed among the references

[b] Flower color was either purple or white and was not statistically analyzed

[c] Not statistically analyzed due to lack of variation

Phenotypic Study 2—US

In all 39 growth stage observations across the individual sites (five sites × 7 to 10 observation/site), MON 89788 was at the same growth stage as the control (data not shown). In the combined-site analyses, no differences were detected between MON 89788 and the control for any of the 13 phenotypic characteristics assessed (Table 4).

Phenotypic Study 3—Argentina

In all 35 growth stage observations, MON 89788 had the same growth stage range as A3244 (data not shown). In the combined-site analysis, no differences were detected between MON 89788 and A3244 for seedling vigor, days to 50 % flowering, flower color, plant height, plant pubescence color, lodging, pod shattering, seed test weight, or yield (Table 5). Early stand count was greater for MON 89788 than for A3244 (199.9 vs. 177.7 plants per two rows; Table 5). Final stand count was also greater for MON 89788 than for A3244 (186.4 vs. 170.0 plants per two rows; Table 5). The magnitude of the differences in early and final stand count was approximately 12 and 10 %, respectively; however, yield was not significantly affected (Table 5). In addition, this difference was not observed in the other studies and the combined-site mean for early and final stand counts observed for MON 89788 was within the respective range of mean values observed for each characteristic in the commercial references (Table 5). Therefore, the differences detected in the combined-site analysis are unlikely to be biologically meaningful in terms of increased weed potential (Fig. 1, Step 3).

When considered across all three phenotypic studies conducted in different years (2004, 2005, and 2006) or geographic areas (US and Argentina), the differences observed in the cross-site analysis [plant height in Phenotypic Study 1 (US); early and final stand count in Phenotypic Study 3 (Argentina)] were not observed across studies. The lack of a consistent trend across years and geographies for these characteristics provides additional evidence that these differences are not associated with the genetic modification process and were within the range of what is familiar for soybean. Since for the characteristics measured, MON 89788 was not meaningfully different from conventional soybean, it is also no more likely to be a weed than commercial soybean (see "*Assessment of potential for weediness and invasiveness*" below).

Table 4 Phenotypic Study 2: characteristics of MON 89788, the control, and the references across five US sites

Phenotypic characteristic (units)	MON 89788	Control	Reference range[a]	
			Min.	Max.
Early stand (plants/m)	19.7	19.7	14.1	24.9
Seedling vigor (1–9 scale)	4.7	5.0	1.7	8.0
Days to 50 % flowering	50.5	51.3	45.0	61.0
Flower color[b,c]	Purple	Purple	–	–
Plant height (cm)	90.2	91.7	77.5	116.3
Plant pubescence[b]	Hairy	Hairy	–	–
Lodging (0–9 scale)	0.6	0.7	0.0	4.2
Pod shattering (0–9 scale)	0.2	0.1	0.0	1.1
Final stand (plants/m)	17.7	17.4	11.5	24.0
Seed moisture (%)	13.9	13.9	11.2	17.0
100-seed weight (g)	16.3	16.6	14.0	22.7
Yield (mg/ha)	3.7	3.6	2.2	4.9

No statistically significant differences were observed between MON 89788 and the control at $p \leq 0.05$

– Indicates that no reference range is available for categorical data

[a] Reference range = minimum and maximum mean values observed among the references

[b] Not statistically analyzed due to categorical nature of characteristic; no variability observed

[c] Flower color data were collected at four of the five sites. Color was either purple or white and was not statistically analyzed

Table 5 Phenotypic Study 3: phenotypic characteristics of MON 89788, the control, and the references across five sites in Argentina

Phenotypic characteristics (units)	MON 89788[a]	Control[a]	Reference range[b]	
			Min.	Max.
Early stand count (# plants/2 rows)	199.9*	177.7	68.3	245.0
Seedling vigor[c]	3.5	3.7	3.0	4.7
Days to 50 % flowering	39.9	40.3	33.7	46.3
Flower color[c]	Purple	Purple	–	–
Plant height (cm)	57.1	58.9	48.1	71.4
Plant pubescence color[c]	Gray	Gray	–	–
Lodging[c]	0.4	0.5	0.0	2.0
Pod shattering[c]	0.0	0.0	0.0	0.0
Final stand count (# plants/2 rows)	186.4*	170.0	90.0	216.0
Seed test weight (g/100 seed)	17.5	17.9	15.6	22.5
Yield (Mg/ha)	2.7	2.7	1.8	3.4

– Indicates that no reference range is available for categorical data

* Indicates a statistically significant difference between MON 89788 and the control at $p \leq 0.05$

[a] Least square means are provided for early stand count, days to 50 % flowering, plant height, final stand count, seed test weight, and yield. Means are provided for all other numeric characteristics

[b] Reference range = minimum and maximum mean values observed among the references

[c] Seedling vigor, flower color, pubescence color, lodging, and shattering had low or no variability, which precluded statistical analysis

Pollen morphology

No statistically significant differences were detected at $p \leq 0.05$ between MON 89788 and the control for average pollen grain diameter (23.7 vs. 23.1 μm, respectively; reference range 21.6–23.4) or percent viable pollen (82.0 vs. 75.3 %, respectively; reference range 56.4–80.1). No visual differences between MON 89788 and the control were observed in general pollen morphology (data not shown). The lack of differences between pollen collected from MON 89788 compared to the conventional control for the assessed characteristics supports a conclusion of no biological or ecological differences for MON 89788 compared to conventional soybean.

Application of data to ecological risk assessment of MON 89788

When assessing the results of plant characterization studies, risk assessors evaluate data from all characteristics for effects that could be associated with the genetic modification process or the trait and whether the effects could pose a hazard (Prado et al. 2014). In addition, assessors may also focus on subsets of characteristics that would allow them to draw conclusions about specific potential ecological risks such as increased weediness of the GM crop.

Assessment of germination, growth, and reproductive characteristics

In the studies described here, numerous germination, growth, development, and reproductive characteristics were evaluated to compare Roundup Ready 2 Yield soybean, MON 89788, to an appropriate control, A3244. The selected characteristics were included in the evaluations because they are well understood and known to plant breeders, weed scientists, regulators, and other agricultural experts familiar with soybean, familiar with the Roundup Ready trait, and/or familiar with ecological risk assessment of GM crops. The results of all measurements of these characteristics were considered in the context of the potential to cause harm.

This assessment tested the hypothesis that MON 89788 was unchanged in all the characteristics measured relative to what is familiar for soybeans. A weight-of-evidence approach was used to assess whether the differences were unfamiliar for soybeans and potentially indicative of an effect resulting from the genetic modification process. Out of the characteristics evaluated, few differences were detected between MON 89788 and the control, and each was subsequently assessed. These differences were not consistent across trials. If there would have been a consistent difference, this would have been further considered in the overall risk assessment for potential changes in plant weediness, pest potential, or adverse ecological impact.

Assessment of potential for weediness and invasiveness

As discussed above, a subset of the characteristics was also evaluated to assess for specific risks of increased weediness and invasiveness, and has been used to assess the potential for adverse ecological impact (Horak et al. submitted). Weed scientists have developed lists of characteristics that are observed in many common weeds such as seed dormancy, ability to compete interspecifically, adaptations for short- and long-distance seed dispersal, continuous seed production for as long as the growing season permits, high seed output in favorable environments, and some seed output in a range of environments (Baker 1974; Radosevich et al. 1997). These lists are useful to identify potential characteristics for GM crop assessment. However, assessors must also consider other factors to inform decision making. First, during GM crop product development, technology providers focus development on events that meet molecular, protein, composition, agronomic, and environmental safety criteria and eliminate those that do not meet advancement criteria (Mumm and Walters 2001; Heck et al. 2005; Phillips McDougall 2011; Prado et al. 2014). Second, the characteristics of the conventional crop need to be considered (OECD 1993); in many highly domesticated crop plants the characteristics (and associated genes) associated with weediness are reduced due to breeding for desirable characteristics (Warwick and Stewart 2005). For example, corn, soybean, and cotton breeders select against ear, pod, or boll drop, shattering, lodging, and dispersal mechanisms that with other phenotypic changes could be associated with weediness). Finally, depending upon the species, several of the identified "weedy" characteristics from these lists would likely need to be

combined for a highly domesticated plant to become weedy (e.g., corn and soybean likely would need to acquire seed dormancy, seed dispersal, and enhanced competitive mechanisms to become a significant weed in cultivated fields or native plant communities).

In this assessment, seed dormancy (hard-seededness), plant lodging, and seed pod shattering were further assessed to determine if there were changes in these characteristics that would be indicative of increased weediness potential. These were chosen based on evaluation of relevant risk hypotheses that in order for soybean to revert to a more weedy or wild state, seed dormancy would need to be present so that the seed could survive over winter or several seasons. In addition, plant lodging and seed pod shattering could potentially be associated with aspects of seed dispersal: the mature seeds would need to be dispersed to favorable niches for the plant to function as a weed of agronomic settings or native plant communities and not harvested at the end of the growing season. Although not evaluated in this assessment, additional characteristics would also likely be needed for the plant to be able to function as a weed or wild plant, such as the ability to compete with native vegetation. Since the characteristics listed above were unchanged in MON 89788, the results support a conclusion that on the basis of phenotypic and agronomic characteristics, there is no reason to believe that MON 89788 is any more weedy or invasive than the A3244 conventional control. The only potential advantage MON 89788 would have over A3244 in a non-agricultural setting is that (as expected) it could not be controlled by Roundup. As noted above, however, cultivated soybean does not have weedy characteristics and competes poorly with other species, so the need for control is minimal and could be addressed, if needed, by mechanical control or use of other herbicides (e.g., OECD 2000).

Based on the concept of familiarity, it was hypothesized that there would be no increased weediness potential or potential adverse ecological effects from the cultivation of Roundup Ready 2 Yield soybean, MON 89788. The first-generation Roundup Ready trait has been grown on tens of millions of hectares with no substantiated claims of adverse ecological effects of the plant in terms of weediness, invasiveness, or adverse ecological impacts (CERA 2011). Those observations combined with the results of the studies reported herein corroborate the conclusions for MON 89788 of a lack of weediness potential or an adverse ecological impact.

The data generated in these studies can be (and has been) used to inform environmental risk assessments in other world areas. The Argentina and US field experiments were conducted under diverse geographic and environmental conditions and yet yielded similar results and conclusions regarding potential hazards. For the assessment of specific risks, in this case increased weediness, the data generated are transportable to other countries to inform risk assessments for those regions.

In summary, the results presented here support the conclusion that Roundup Ready 2 Yield soybean, MON 89788, is no more likely to pose an increased plant pest risk or to have greater weed potential or adverse ecological impact than conventional soybean.

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