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Mycorrhizal and Rhizobial Colonization of Genetically Modified and Conventional Soybeans▽

Jeff R. Powell,[1]* Robert H. Gulden,[2] Miranda M. Hart,[3] Rachel G. Campbell,[2] David J. Levy-Booth,[4] Kari E. Dunfield,[4] K. Peter Pauls,[2] Clarence J. Swanton,[2] Jack T. Trevors,[3] and John N. Klironomos[1]

Department of Integrative Biology,[1] Department of Plant Agriculture,[2] Department of Environmental Biology,[3] and Department of Land Resource Science,[4] University of Guelph, Guelph, Ontario N1G 2W1, Canada

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We grew plants of nine soybean varieties, six of which were genetically modified to express transgenic *cp4-epsps*, in the presence of *Bradyrhizobium japonicum* and arbuscular mycorrhizal fungi. Mycorrhizal colonization and nodule abundance and mass differed among soybean varieties; however, in no case was variation significantly associated with the genetic modification.

Herbicide-tolerant crops are among the most successful commercialized genetically modified (GM) crops; for example, herbicide-tolerant varieties were planted in 89%, 65%, and 36% of U.S. soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and corn (*Zea mays*) acreage, respectively, in 2006 (13). Most GM herbicide-tolerant varieties have been engineered to express a bacterial variant of a gene (*cp4-epsps*) encoding 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS); the broad-spectrum herbicide glyphosate inhibits wild-type EPSPS activity but not that of the *cp4-epsps* variant, facilitating in-crop chemical weed control (4). EPSPS catalyzes an intermediate step in the shikimic acid pathway responsible for production of aromatic amino acids used in the synthesis of phenolic compounds (8). Plant phenolics mediate interactions with other organisms, including microorganisms and herbivores (2, 18). Levels of isoflavones in GM and conventional soybean seeds have been compared, with few differences observed (10, 11, 14, 20). However, we do not know how expression of *cp4-epsps*, in addition to that of wild-type *epsps*, affects levels of plant phenolics in the vegetative structures of modified plants. If present, such unintended phenotypes in GM crops may have consequences for nontarget organisms and ecosystems.

Many commercialized GM crop species can benefit from associations with mutualistic microorganisms. Soybeans, in particular, form root symbioses with two distinct groups of mutualistic microorganisms: *Bradyrhizobium japonicum*, which forms nodules and fixes atmospheric N_2 (16), and arbuscular mycorrhizal (AM) fungi, which can enhance nutrient uptake and disease tolerance (19). Plant phenolic compounds have a variety of functions associated with the formation of rhizobial and mycorrhizal symbioses, including signaling during the recognition, infection, and establishment stages (1, 18). If the synthesis of these phenolic compounds is altered in GM soybeans, or if additional unintended phenotypes are associated with expression of *cp4-epsps* in GM soybeans, then rhizobial and/or mycorrhizal symbioses may be altered, with potential consequences for crop productivity and nutrient cycling.

Here, we present the results of a greenhouse experiment, using multiple GM and conventional soybean varieties, which addresses whether unintended phenotypes associated with variant *cp4-epsps* expression affect soybean root symbioses. Four GM varieties (2602R, 2702R, AG1901, and DKB06-52) were provided by First Line Seeds (Guelph, Ontario, Canada). Two GM varieties (OAC Raptor and OAC Rockwood) and all conventional varieties (OAC Bayfield, OAC Clinton, and OAC Oxford) were provided by Istvan Rajcan (Department of Plant Agriculture, University of Guelph). OAC Raptor and OAC Rockwood are sister lines derived from crosses between a herbicide-resistant variety and a conventional variety (OAC Bayfield) followed by four backcrosses with OAC Bayfield; including these three varieties allows for the comparison of genotypes differing primarily in the presence or absence of transgenic *cp4-epsps*.

Soybeans were grown in 1-gallon pots containing an autoclaved, 60:40 mixture of peat moss (Premier Pro Moss; Premier Tech, Rivière-du-Loup, Quebec, Canada) and playground sand (Hillview; Nu-Gro IP Inc., Brantford, Ontario, Canada). We inoculated pots with roots (15 g wet weight) of corn plants grown in a mixture of Turface (Profile Products LLC, Buffalo Grove, IL) and field soil (Elora Research Station, Ontario, Canada); corn roots were heavily colonized (confirmed microscopically) with a mixed community of AM fungi. Pots were also inoculated with 0.5 g of a peat-based *B. japonicum* 532C inoculum (Hi-Stick+; MicroBio, Saskatoon, SK, Canada), buried 5 cm below the soil surface, when seedlings were first transplanted. Soybean seeds were surface sterilized with 0.5% NaOCl for 5 min, germinated on moist filter paper, and, after 9 days, transplanted to the soil-filled pots (one seedling per pot). Seedling establishment was evaluated 7 days later; when necessary, dead seedlings were replaced with the remaining germinated seedlings. After 18 days, all pots were fertilized with 200 ml low-P fertilizer [0.4 mM KH_2PO_4, 2.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2.0 mM K_2SO_4, 1.0 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM FeIII EDTA, 50 μM KCl, 25 μM H_3BO_3, 2.0 μM $ZnSO_4 \cdot 7H_2O$, 2.0 μM $MnSO_4 \cdot H_2O$, 0.5 μM $CuSO_4 \cdot 5H_2O$, 0.5

* Corresponding author. Mailing address: Department of Integrative Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Phone: (519) 824-4120, ext. 56718. Fax: (519) 767-1656. E-mail: jpowell@uoguelph.ca.

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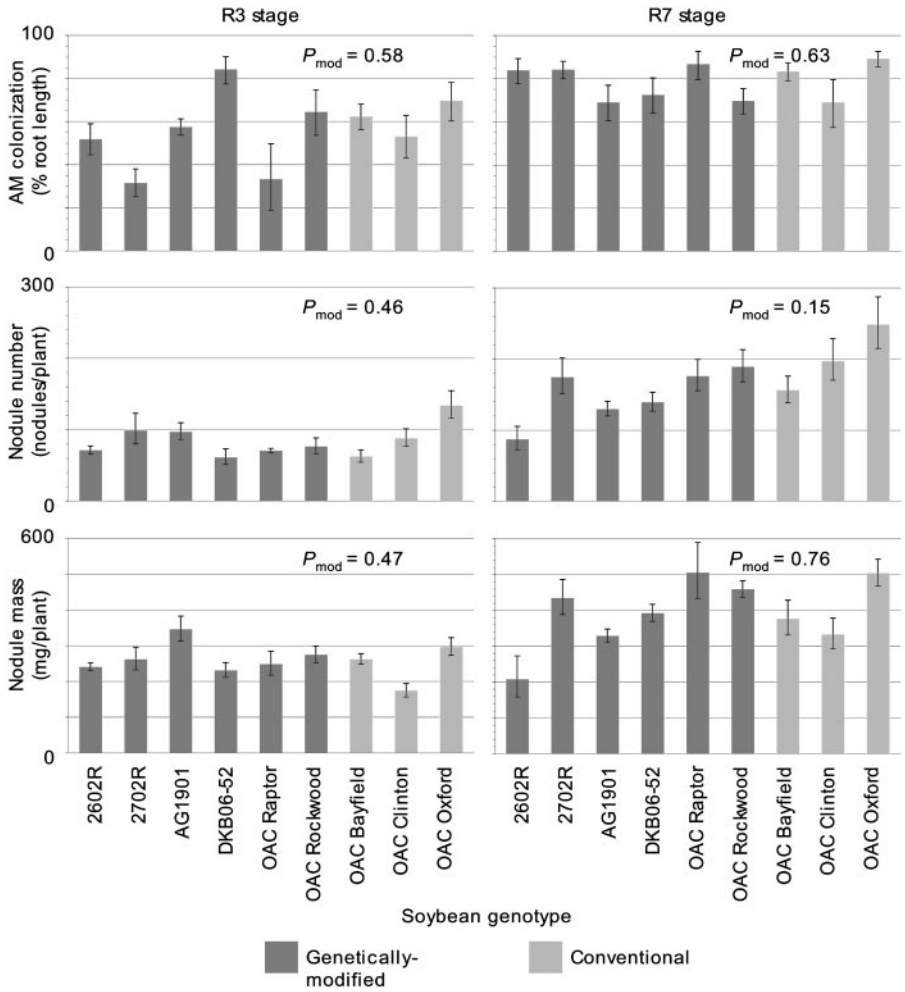

FIG. 1. AM (top panels) and rhizobial (middle and bottom panels) responses to genetically modified and conventional soybean varieties at the R3 (early pod formation; left) and R7 (physiological maturity; right) growth stages. AM colonization was measured as the percentage of root segments colonized by AM fungal hyphal structures. Rhizobial colonization was measured as the number and mass of nodules per root system. Soybean variety was a significant source of variation ($P < 0.05$) for all graphs except the top right graph. Genetic modification was not a significant source of variation in any case (indicated by P_{mod} values [i.e., P values for the variable of modification]). Bars and lines represent backtransformed means and standard errors, respectively.

μM $Na_2MoO_4 \cdot 2H_2O$]. We assumed that any effect of nitrate addition on the rhizobial symbiosis (7) was independent of the genetic modification.

We harvested soybean plants at either the R3 (initiation of pod formation; 3 to 5 replicates per variety) or R7 (physiological maturity; 7 to 10 replicates per variety) stage (5). Harvesting at these stages allowed us to estimate symbiont responses at the beginning and end of soybean pod development as well as assess differences among soybean varieties in early soybean growth rate (R3) and yield (R7). Shoots were clipped at the soil surface, and soil was removed from roots with gentle washing. Nodules were counted and removed from roots, dried to constant mass (60°C), and weighed. Five small subsamples of roots, selected systematically from different locations in the root system, were pooled, fixed in 70% ethanol, stained with Chlorazol black E (3), and examined with a compound microscope at ×200 magnification using the gridline-intersection method, which estimates the percentage of root length colonized by AM fungal hyphal structures (12). Total AM fungal colonization measures the prevalence of all AM fungal hyphal structures in roots, including arbuscules and vesicles, indicating overall hyphal growth. Arbuscular colonization specifically measures the prevalence of arbuscules, which are the AM fungal structures presumably associated with nutrient uptake by the host plant (19), potentially indicating the physiological status of the mycorrhizal association. AM responses were arcsine[square root(p)] (for which p is the proportion of root segments colonized) transformed, and rhizobial responses were $\log_{10}$ transformed to realize normality (Shapiro-Wilks test) and homogeneity (Levene's test) of error distributions. We then analyzed the responses with one-way analysis of variance (general linear model; soybean genotype as a single factor with nine levels) and, where appropriate, performed multiple comparisons with Tukey's honestly significant difference test. We tested for effects associated with the genetic modification with two-sample t tests, using the estimated mean value for each variety, after transformation, as the experimental units; there-

fore, the two samples represented GM ($n = 6$) and conventional ($n = 3$) varieties, respectively. All statistical analyses were conducted using R version 1.16 software (17).

Soybean variety had a significant effect on mycorrhizal colonization of soybean roots at the R3 stage ($P = 0.002$); however, the response was not associated with the genetic modification (Fig. 1). In addition, colonization of the OAC Raptor and OAC Rockwood varieties was not significantly different from that of the conventional parental line, OAC Bayfield (Tukey $P_{\text{adjusted}} > 0.05$ at both growth stages). All varieties had similar amounts of mycorrhizal colonization at the R7 stage ($P = 0.11$; Fig. 1). Colonization of soybean roots by arbuscules was not significantly different among varieties ($P = 0.14$).

Both nodule number ($P < 0.001$) and mass ($P < 0.001$) were significantly affected by soybean variety; however, responses with respect to nodule number or mass were not associated with the genetic modification (Fig. 1). In addition, nodule number or mass for the OAC Raptor and OAC Rockwood varieties did not differ significantly from that for the conventional parental line, OAC Bayfield (Tukey $P_{\text{adjusted}} > 0.05$ for each variable at both growth stages). Mass of nodules has been observed to correlate with acetylene reduction and N_2 fixation in other studies (9, 21), suggesting that rhizobial functioning is also unlikely to be affected by *cp4-epsps* expression.

Although we observed differences among soybean varieties in their associations with root symbionts, we found no evidence attributing these differences to *cp4-epsps* expression in GM soybeans. To our knowledge, our study was the first to address whether genetic modification represents a significant source of variation for nodulation and/or mycorrhizal fungal colonization of soybean or other crops that express *cp4-epsps* (alfalfa, corn, and cotton form AM associations; alfalfa also forms rhizobial associations; canola [*Brassica napus*] forms neither association type). Symbiont responses for these other crops may be similar to the responses observed here. However, this speculation should be confirmed experimentally, since the genomic position of transgenic *epsps* resulting from a specific transformation event may affect the types of pleiotropies that occur, or effects on synthesis of phenolic compounds may be species specific. In addition, other genetic modifications also target production of phenolic compounds in crops, including those targeting lignin production in alfalfa, sorghum (*Sorghum bicolor*), and tobacco (*Nicotiana tabacum*) (6, 15, 22). However, we cannot extrapolate our data to genetic modifications that target other biochemical mechanisms in plants. Finally, our experiment was conducted under a single set of environmental conditions and provides little insight into the community composition of the symbiotic microorganisms studied. We propose that our experimental strategy could be employed in future studies to evaluate effects of genetic modifications on root symbioses under different environmental conditions and that future studies could incorporate DNA fingerprinting techniques to estimate effects on the community structure of symbiotic microorganisms.

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