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Inoculation of soybean (*Glycine max.* (L.) Merr.) with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions

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Abstract

In short-season soybean production areas, low soil temperature is the major factor limiting plant growth and yield. The decreases in soybean yield at low temperatures are mainly due to nitrogen limitation. Genistein, the most effective plant-to-bacterium signal in the soybean (*Glycine max* (L.) Merr.) nitrogen fixation symbiosis, was used to pretreat *Bradyrhizobium japonicum*. We have previously reported that this increased soybean nodulation and nitrogen fixation in growth chamber studies. Two field experiments were conducted on two adjacent sites in 1994 to determine whether the incubation of *B. japonicum* with genistein, prior to application as an inoculant, or genistein, without *B. japonicum*, applied onto seeds in the furrow at the time of planting, increased soybean grain yield and protein yield in short season areas. The results of these experiments indicated that genistein-preincubated bradyrhizobia increased the grain yield and protein yield of AC Bravor, the later maturing of the two cultivars tested. Genistein without *B. japonicum*, applied onto seeds in the furrow at the time of planting also increased both grain and protein yield by stimulation of native soil *B. japonicum*. Interactions existed between genistein application and soybean cultivars, and indicated that the cultivar with the greatest yield potential responded more to genistein addition.

Abbreviations: DAP-days after planting; RZT-root zone temperature.

Introduction

Soybean (*Glycine max.* (L.) Merr.) is a subtropical legume which requires temperatures in the range of 25 to 30 °C for optimal growth and dry matter accumulation. In areas with relatively short growing seasons, temperature is considered the major factor limiting soybean growth and yield. When the temperature drops below 25 °C, total dry matter and grain yield are decreased (Summerfield and Wien, 1982). The decreases in soybean yield at low temperatures are mainly due to nitrogen rather than to carbon limitation (Thomas and Sprent, 1984). Current soybean production in eastern Canada is at the northernmost North American limit of the crop. In most areas of Canada early vegetative growth of soybean occurs under suboptimal temperatures. Under short season Canadian conditions, soybean plants, in association with *Bradyrhizobium japonicum*, can fix 100 to 200 kg ha^{-1} yr^{-1} of nitrogen from the atmosphere (Smith and Hume, 1987). Soybean requires 25 to 30 °C root zone temperatures (RZTs) for optimal symbiotic nitrogen fixation. However, the average RZT during the first month of the growth season in Canadian soybean production areas is often below 15 °C, and may be lower than 20 °C until July (Lynch and Smith, 1993a). Low RZTs strongly and negatively affect all stages of nodulation and nodule function (Jones and Tisdale, 1921; Hardy et al., 1968; Roughley and Date,

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1986; Lynch and Smith, 1994). However, the early infection processes are the most sensitive, with an event occurring during the first 12 h being particularly sensitive to temperature (Lynch and Smith, 1993b; Zhang and Smith, 1994). Symbiotic nitrogen fixation is a complex process involving physiological and biochemical aspects of both symbiotic partners. An early step in the nodulation process is the selective attachment to and penetration of the plant root by the bacterium. Initial recognition between *B. japonicum* and soybean involves exchange of molecular signals. Legume roots secrete phenolic compounds which act as signals to bradyrhizobia (Peters and Verma, 1990). In exudates and extracts from legume roots, flavones and isoflavones, have been identified as the inducing molecules for bradyrhizobial chemotaxis and for expression of *Bradyrhizobium* nodulation genes, e.g. genistein and daidzein in soybean (Peters and Verma, 1990).

In an attempt to overcome nitrogen limitation during early soybean development, by improving nodulation and nitrogen fixation, Zhang and Smith (1995) used genistein-preincubated *B. japonicum* inocula and found that this substantially increased nodulation and accelerated the onset of nitrogen fixation by soybean at low RZTs under controlled environment (Zhang and Smith, 1995) and field conditions (Zhang and Smith, in prep.). However, to date, there have been no investigations of whether activation of *B. japonicum* included in inocula, or already present in the soil, with genistein increases soybean seed protein and seed dry matter yield under field conditions. Therefore, in this study, we tested two hypotheses: 1) preincubation of *B. japonicum* with genistein, prior to use as inocula for soybean seeds planted in early cool spring soybean production areas increases soybean protein and grain yield under field conditions, 2) genistein directly applied into the plant rhizosphere at the time of seeding increases soybean protein and grain yield under field conditions. Different *B. japonicum* strains were preincubated with genistein and applied to different soybean cultivars to determine whether interactions existed between genistein application and *B. japonicum* strains or soybean cultivars.

Materials and methods

Site preparation and field layout

The two experiments included in this study were located at the Emile A. Lods Research Centre, McGill University, Macdonald Campus. The experiments were carried out on two adjacent sites, both on a Chicot light sandy loam soil. At one site, to prevent possible competition from native *Bradyrhizobium* or interference from other soil microflora that might obscure genistein preincubation effects, the soil was sterilized by the application of methyl bromide (50 g m^{-2}) under a plastic canopy for 72 h (sterilized site). Three days elapsed between removal of the fumigation canopy and planting (Lynch and Smith, 1993b). At the other site the soil was unsterilized. The first experiment included three factors, genistein application, *B. japonicum* strains, and soybean cultivars. The experimental design was a 2×2×2 factorial organized in a randomized complete block split-plot with four replications. The main-plot units consisted of genistein application treatments (0 and 20 μM; Zhang and Smith, 1995), while the combinations of two soybean cultivars, Maple Glen and AC Bravor, and inoculation treatments (two strains of *B. japonicum*, 532C; Hume and Shelp, 1990, and USDA 110) formed the subplot units. Two factors were included in the second experiment, genistein application, and soybean cultivars. This experimental design was also arranged as a randomized complete block split-plot design with four replications. Two levels of genistein concentration, 20 and 0 μM, were arranged as mainplot units, and soybean cultivars, Maple Glen and AC Bravor, were the sub-plot units. At the unsterilized site, each sub-plot (2×3 m) consisted of four rows of plants with 40 cm between rows. The space between plots was 80 cm and between replications 1 m. At the sterilized site, the size of each sub-plot was 1.6×2 m and consisted of three rows of plants, also with 40 cm between rows. The space between plots was also 80 cm and between replications, 2 m. In the previous year, 1993, this experimental field had been planted with oat and barley, while in 1992 green manure alfalfa was grown in this experimental area. The available soil nitrogen, indicated by the average nitrogen accumulation in the non-fixing soybean plants, was 167 kg ha^{-1}. Potassium and phosphate were provided by a spring application of 340 kg ha^{-1} of 5-20-20 (N, P_2O_5, K_2O). The 17 kg ha^{-1} of nitrogen were applied as "popup" nitro-

gen, conventionally applied in this area to help support plant growth before the onset of nitrogen fixation.

Inoculum preparation

For the first experiment, the inoculum was produced by culturing, separately, *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) for 3 d in 2 L flasks shaken at 125 rpm at room temperature (20-23 °C). For production of *B. japonicum* preincubated with genistein (4', 5, 7-Trihydroxyisoflavone, purity of 98%, Sigma, Mississauga, Ontario, Canada), 100 mL of a cell suspension from a 3-day old (log phase, 2×10^9 cells mL^{-1}) subculture were aseptically added to 500 mL of sterile genistein solution (24 μM, which made the final genistein concentration equal to 20 μM) in a 2 L Erlenmeyer flask and incubated at 30 °C without shaking for 48 h (Halverson and Stacey, 1984). Following incubation, the cell suspensions were pelleted by centrifugation at 7000 *g* for 10 min, washed once with distilled water, and resuspended in distilled water to an A_{620} of 0.08 (approximately 10^8 cells mL^{-1}) (Bhuvaneswari et al., 1980).

Planting method

Seeds of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with sterile distilled water (Bhuvaneswari et al., 1980). These cultivars were selected as they have been developed for production under the short season, cool conditions of eastern Canada and have performed well there. The seeds were planted by hand on May 11 (about one week before the normal planting date) and 18 (approximately the middle of the normal planting period) at the unsterilized and sterilized sites, respectively. The delay in planting at the sterilized site was due to the extra time required for the methyl bromide fumigation. Twenty mL of washed inoculum (for Exp. 1), or 20 mL of either genistein solution (20 μM) or distilled water (for Exp. 2) per one metre of row were applied evenly by syringe directly onto the seed along the furrow. Alcohol sterilization of the implements was used to prevent cross contamination throughout planting and all subsequent data collection procedures. Following emergence, seedlings were thinned to achieve a stand of 500,000 plants ha^{-1} (20 plants m^{-1} of row, with an average inter-plant distance of 5 cm within the row).

Data collection

Daily average air temperature, average soil temperatures at a depth of 5 cm and precipitation were recorded at the Macdonald Campus weather station, McGill University, Ste. Anne de Bellevue, Quebec, Canada, only 500 m from the experimental field. Plant samples were taken on August 12, at which time plants were at the reproductive stage 6 (R6) (Fehr et al., 1971), for investigation of growth variables such as, leaf number, leaf area, pod number and seed number. Leaf number and area per plant were determined using a Delta-T area meter (Delta-T Devices Ltd., Cambridge, UK). Pod number and seed number per plant were counted by hand. End of season grain yield was determined from a one meter row of plants taken from the middle row of each plot. Plants were harvested by hand at harvest maturity, then shelled by a plot combine (Wintersteiger, Salt Lake City, UT), oven-dried at 70 °C for at least 48 h, and weighed. Grain dry matter yield was calculated based on a 0% moisture content. Six more plants, also from the middle row, were hand-harvested, and oven-dried at 70 °C, after which the seeds were manually separated from shoots. Total shoot weight and harvest index were determined from these plants, which were enclosed within wire mesh following flowering to facilitate the collection of senescent leaves. The dried seeds from each plot were ground using a Moulinex coffee mill (Moulinex Appliances Inc., Virginia Beach, VA). The nitrogen concentration of seeds was then determined by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden). The protein concentration was calculated by multiplying nitrogen concentration by 6.25.

Statistical analysis

Results were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance (Steel and Torrie, 1980).

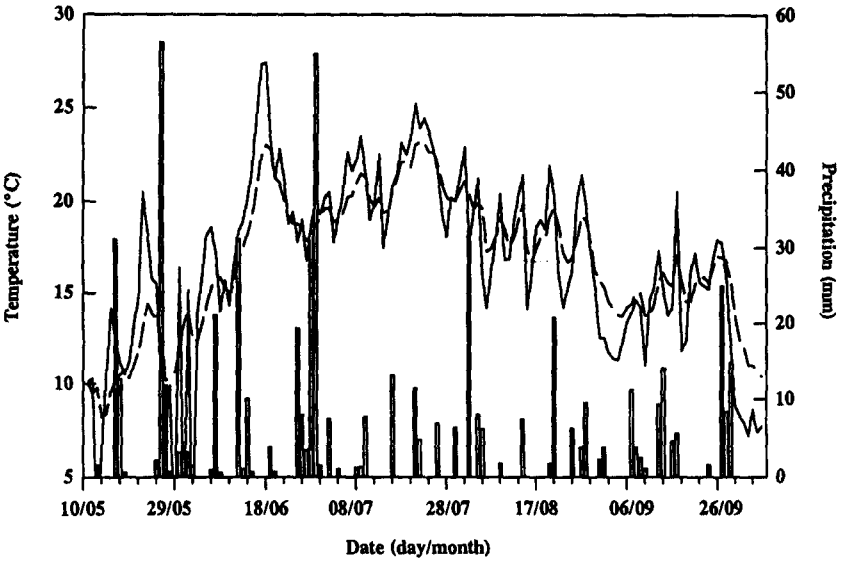

Figure 1. The average daily temperatures of air (solid line) and soil at a depth of 5 cm (dashed line) and precipitation (bar) during the soybean growing season in 1994 (Ste. Anne de Bellevue, Quebec, Canada).

Table 1. Effects of genistein application, *B. japonicum* strains, and soybean cultivars for soybean leaf number and area, grain yield components, and final protein and grain yield at the unsterilized site (data from Exp. 1)

Genistein	*B. japonicum*	Cultivar	Leaf (plant^{-1})		Number (plant^{-1})		100 seeds	Yield (t ha^{-1})		
			Number	Area (cm^2)	Pod	Seed	weight (g)	Grain protein	Total protein	Grain
20 μM	USDA110	Ac Bravor	22.27	1021.20	24.70	61.28	18.67	1.70	2.07	4.33
		Maple Glen	17.33	669.85	24.60	61.10	18.69	1.34	1.75	3.43
	532C	AC Bravor	28.80	798.65	27.03	64.08	19.62	1.67	2.12	4.05
		Maple Glen	19.03	715.23	20.53	50.40	18.74	1.59	2.07	3.91
0 μM	USDA110	AC Bravor	17.97	861.53	19.65	41.60	18.95	1.30	1.56	3.45
		Maple Glen	19.38	730.33	25.65	59.60	19.11	1.37	1.74	3.69
	532C	AC Bravor	21.60	715.93	23.15	51.20	19.34	1.48	1.81	3.90
		Maple Glen	18.53	743.78	24.28	61.85	19.01	1.52	1.89	4.01
$LSD_{0.05a}$			5.32	106.18	5.20	10.22	1.11	0.16	0.20	0.37
$LSD_{0.05a}$			6.69	100.26	7.34	9.58	1.68	0.17	0.23	0.30
Genistein			NS	**	NS	***	NS	**	**	***
B. japonicum			NS	**	***	***	***	**	**	***
Cultivar			**	***	NS	NS	NS	NS	NS	NS
Genistein*	*B. japonicum*		NS	NS	NS	NS	NS	**	**	**
Genistein*	cultivar		*	*	***	***	NS	**	**	***
Genistein*	*B. japonicum**	cultivar	NS	NS	NS	NS	NS	NS	NS	**

Means of leaf number and area, nodule number and weight, pod and seed number represent four plants from each subplot unit, at crop maturity. Means of 100 seed weight and grain dry matter yield calculated from the one meter row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparison of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicated no significant difference of significant differences at the 0,1, 0.05, and 0.01 levels, respectively.

Table 2. Effects of and genistein application and soybean cultivars on soybean leaf number and area, grain yield components, and final protein and grain yield at the sterilized site (data from Exp. 1)

Genistein	Cultivar	Leaf (plant^{-1})		Number (plant^{-1})		100 seeds	Yield (t ha^{-1})		
		Number	Area (cm^2)	Pod	Seed	weight (g)	Grain protein	Total protein	Grain
20 μM	AC Bravor	34.56	874.31	27.75	65.44	16.12	1.58	2.07	4.21
	Maple Glen	33.57	852.36	22.08	58.67	17.41	1.54	1.96	4.09
0 μM	AC Bravor	27.87	676.12	22.50	54.14	15.69	1.39	1.77	3.64
	Maple Glen	37.94	848.06	26.25	60.19	16.51	1.49	1.96	3.88
$LSD_{0.05a}$		9.31	125.00	3.71	10.66	0.54	0.17	0.19	0.45
$LSD_{0.05b}$		10.04	131.41	4.95	10.83	1.05	0.17	0.20	0.43
Genistein		NS	NS	NS	*	NS	*	*	**
Cultivar		NS	NS	NS	*	***	NS	NS	NS
Genistein*cultivar		NS	**	***	*	NS	**	**	**

Means of leaf number and area, nodule number and weight, pod and seed number represent four plants from each subplot unit at crop maturity. Means of 100 seed weight and grain dry matter yield calculated from the one meter row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparison of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Results

Air and soil temperature, precipitation and plant development

Average daily temperatures for both air and soil (at a depth of 5 cm) were below 15 °C until early June, and remained well below 20 °C until mid-July, about two months after planting (Fig. 1). Both low air and soil temperatures slowed the rate of seedling emergence, particularly for the earlier-seeded plants at the unsterilized site (Zhang and Smith, unpubl. data), such that for the May 11 planting at the unsterilized site, seeds germinated on May 25, i.e. at 14 days after planting (DAP), while seeds planted on May 18 at the sterilized site germinated at 9 DAP. Plants in both the unsterilized and sterilized sites flowered in mid-July and reached physiological maturity in early September. Precipitation during the planting period was 47 mm, while the total precipitation during the soybean growing season (May to September) was 572 mm (Fig. 1), which is sufficient for soybean production.

Experiment 1

The nodule number of uninoculated plants in Experiment 2 indicated that the native soil population of *B. japonicum* in unsterilized soil was low, with uninoculated plants forming few nodules; at physiological maturity there were fewer than three nodules per plant, although the nodules were very large, on average almost 5 times larger than those on plants in Experiment 1. However, at the sterilized site, fumigation with methyl bromide was not completely effective and the un-inoculated plants in Experiment 2 formed nearly as many nodules as the inoculated plants in Experiment 1. Therefore, main effects, two way interaction comparisons between either genistein application and *B. japonicum* strains, or genistein application and soybean cultivars and three way interaction comparisons for genistein application, *B. japonicum* strains, and cultivars were tested on the unsterilized site. At the sterilized site, only main effects and two way interactions between genistein application and soybean cultivars were tested.

Most growth variables, such as plant height, nodule number per plant, time of crop maturity, harvest index, and seed moisture content at harvest maturity, were not affected by genistein application at both the unsterilized and sterilized sites (data not shown). The leaf area of AC Bravor receiving USDA110 was increased by 20 μM genistein addition at the unsterilized site (Table 1). At the sterilized site, the leaf area of AC Bravor was increased more by 20 μM genistein application than the leaf area of Maple Glen (Table 2).

The number of seeds formed on AC Bravor plants receiving genistein-preincubated *B. japonicum* (Exp. 1) increased by 34.2 and 20.9% compared to those

receiving *B. japonicum* only at the unsterilized and sterilized sites, respectively (Tables 1 and 2). This increase in seed number was due to the higher pod numbers of AC Bravor plants receiving genistein-preincubated *B. japonicum* than of those receiving *B. japonicum* only. Maple Glen plants receiving either genistein-preincubated *B. japonicum* or *B. japonicum* only had a similar seed number at both the unsterilized and sterilized sites (Tables 1 and 2). Since seed number of AC Bravor increased, the total grain yield was increased by genistein application at both the unsterilized and sterilized sites. At the unsterilized site, grain yield of AC Bravor plants receiving preincubated-*B. japonicum* USDA 110 was 25.5% higher than by those receiving *B. japonicum* USDA110 only, whereas grain yield of AC Bravor plants receiving either preincubated-*B. japonicum* 532C or *B. japonicum* 532C only was not different between these treatments (Table 1). At the sterilized site, the final grain yield of AC Bravor treated with genistein increased due to the increase in seed number, and was 15.7% higher than that of plants without genistein treatment (Table 2). Grain protein yield and total plant protein yield of AC Bravor were increased by genistein application at both the unsterilized and sterilized sites (Tables 1 and 2). For the two *B. japonicum* strains, USDA110 and 532C, the grain protein yield of AC Bravor with genistein treatment was 21.6 and 13.7% higher than in plants without genistein treatment at both the unsterilized and sterilized sites, respectively. There was no difference in protein yield between Maple Glen receiving either preincubated *B. japonicum* or normal *B. japonicum* at either the unsterilized or sterilized site. Two way interactions between genistein application and *B. japonicum* also existed at the unsterilized site; the grain protein yield of plants inoculated with genistein-preincubated *B. japonicum* USDA110 increased by 13.4% compared to *B. japonicum* USDA110 without genistein, whereas preincubation of *B. japonicum* 532C with genistein did not increase grain protein yield (Table 1). The effects of genistein application on total plant protein yield followed the same pattern as grain protein yield at both the unsterilized and sterilized sites (Tables 1 and 2); however, total plant protein yield of plants receiving genistein-preincubated *B. japonicum* 532C was 13.5% higher than that of plants receiving *B. japonicum* 532C without genistein at the unsterilized site (Table 1).

Experiment 2

Genistein, directly applied onto seeds in the furrow at the time of planting, increased soybean growth variables and yield compared to control plants at both the unsterilized and sterilized sites (Tables 3 and 4). Generally speaking, the effects of genistein application directly onto soil, without preincubated *B. japonicum*, on soybean growth variables, yield components, and final grain and protein yield in Experiment 2 followed the same pattern as was observed in Experiment 1.

Discussion

Genistein is one of the plant-to-bacteria signals important in establishment of the soybean-*Bradyrhizobium* symbiosis (Kosslak et al., 1987). Preincubation of *B. japonicum* inocula with genistein increased nodule number and hastened the onset of N_2 fixation at suboptimal RZTs under controlled environment conditions (Zhang and Smith, 1995). These increases could lead to an increase in nitrogen fixation ability and a reduction in the nitrogen limitation of soybean growth in short season areas. Therefore, the genistein-pretreated *B. japonicum* increased the final grain yield at both the unsterilized and sterilized sites (Tables 1 and 2). For instance, at the unsterilized site the yield of AC Bravor receiving *B. japonicum* USDA110 was increased by 25.5% (Table 1).

Genistein application not only increased plant dry matter accumulation, but also increased total protein and grain protein yield for AC Bravor (Table 1). Zhang and Smith (1994) reported that low RZTs delay all of the steps in the infection of soybean roots by bradyrhizobia. For example, the period between inoculation and root hair curling was 1 and 2 days longer, respectively, for plants grown at 17.5 and 15 °C RZT than at 25 °C RZT. Presumably, the preincubation of *B. japonicum* with genistein at 30 °C prior to soybean inoculation activated the bradyrhizobial *nod* genes. Since added genistein activated bradyrhizobial *nod* genes and soybean inoculation, nodulation events and nitrogen fixation started 2 to 5 days earlier at the suboptimal RZTs (Zhang and Smith, 1995). Sprent (1979) postulated that an increase of 10% in the period of nodule activity of a grain legume, particularly between the onset of nitrogen fixation and the attainment of maximum fixation, could double the seasonal level of nitrogen fixed. In a controlled environment experiment, the total fixed nitrogen of plants receiving 20 μM

Table 3. Effects of genistein applied directly onto seed in furrow at the time of planting, and soybean cultivar on soybean leaf number and area, grain yield components, and final protein and grain yield at the unsterilized site (data from Exp. 2)

Genistein	Cultivar	Leaf (plant^{-1})		Number (plant^{-1})		100 seeds	Yield (t ha^{-1})		
		Number	Area (cm^2)	Pod	Seed	weight (g)	Grain protein	Total protein	Grain
20 μM	AC Bravor	27.48	881.60	21.53	47.15	15.74	0.83	1.16	2.76
	Maple Glen	19.28	606.50	16.35	37.73	16.00	0.86	1.12	2.72
0 μM	AC Bravor	22.03	722.98	15.73	28.53	15.33	0.78	0.92	2.24
	Maple Glen	16.65	500.58	15.45	35.05	16.18	0.73	0.94	2.41
$LSD_{0.05a}$		3.67	149.91	2.75	8.37	7.64	0.15	0.18	0.31
$LSD_{0.05b}$		5.72	157.61	2.72	10.05	7.36	0.17	0.19	0.34
Genistein		NS	*	**	*	NS	NS	**	**
Cultivar		***	***	**	NS	*	NS	NS	NS
Genistein*cultivar		NS	NS	**	**	NS	NS	NS	**

Means of leaf number and area, nodule number and weight, pod and seed number represent four plants from each subplot unit at crop maturity. Means of 100 seed weight and grain dry matter yield calculated from the one meter row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparison of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Table 4. Effects of genistein applied directly onto seed in furrow at the time of planting, and soybean cultivars on soybean leaf number and area, grain yield components, and final protein and grain yield at the sterilized site (data from Exp. 2)

Genistein	Cultivar	Leaf (plant^{-1})		Number (plant^{-1})		100 seeds	Yield (t ha^{-1})		
		Number	Area (cm^2)	Pod	Seed	weight (g)	Grain protein	Total protein	Grain
20 μM	AC Bravor	34.13	971.75	26.13	62.00	16.58	1.77	2.13	4.53
	Maple Glen	39.38	978.63	29.00	74.00	17.31	1.77	2.23	4.68
0 μM	AC Bravor	38.63	679.67	20.17	48.00	15.30	1.43	1.80	3.94
	Maple Glen	39.50	849.50	24.88	59.25	16.15	1.32	1.66	3.55
$LSD_{0.05a}$		14.27	57.99	4.54	11.34	1.88	0.24	0.28	0.41
$LSD_{0.05b}$		15.20	58.29	5.60	13.43	3.42	0.25	0.31	0.58
Genistein		NS	***	NS	*	NS	**	**	**
Cultivar		NS	***	**	**	NS	NS	NS	NS
Genistein*cultivar		NS	***	NS	NS	NS	NS	**	**

Means of leaf number and area, nodule number and weight, pod and seed number represent four plants from each subplot unit, at crop maturity. Means of 100 seed weight and grain dry matter yield calculated from the one meter row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparison of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

genistein-pretreated *B. japonicum* increased by 49.5 and 43.7% compared to non-genistein pretreated *B. japonicum* at 17.5 and 15 °C, respectively (Zhang and Smith, 1995). Also, an increase of 40% in total fixed nitrogen was obtained from a field experiment under the short soybean growing season conditions typical in Canada (Zhang and Smith, in prep.). In the study reported here, the increased grain protein yield and total protein yield at both the unsterilized and sterilized sites agrees with the findings discussed above.

In Experiment 2, genistein applied directly onto seeds in the furrow at the time of planting also increased yield components and final grain and protein yield for both cultivars, AC Bravor and Maple Glen, at the sterilized site, and for AC Bravor only at the unsterilized site (Tables 3 and 4). As the plants

were not deliberately inoculated with *B. japonicum* in this experiment, the observed increases would seem to have two possible explanations. First, since genistein has been isolated and identified as a major inducer of *nod* genes in *B. japonicum* (Kosslak et al., 1987), genistein could have induced *nod* gene expression in the native soil *B. japonicum*, resulting in increased soybean nodulation and nitrogen fixation (Zhang and Smith, in prep.). Second, increased protein yield and grain yield could be due to the growth regulator effects reported for similar compounds. Flavonoids have been reported to function as modulators of polar auxin transport (Jacobs and Rubery, 1988). However, given the small amounts of genistein added and the previously measured effects on soybean nodulation, the former of these two possibilities seems most probable. Some additional mechanisms could also have effects, although they seem unlikely to be major in this case. A recent study indicated that low molecular weight phenolic compounds not only play important roles in the plant-(*Brady*)*Rhizobium* symbiosis, but also stimulate the early events of vesicular arbuscular mycorrhizal establishment (Elias and Safir, 1987). Lane et al. (1987) reported that an isoflavone and its derivatives appear to be involved in resistance to both insects and fungi.

The proportional increases in average values of plant growth variables, yield components and final grain and protein yield were generally larger in Experiment 2 than in Experiment 1. Two possible conditions could have led to this observation. First, native bradyrhizobia proliferated and developed under low soil temperature conditions (below 15 °C) until early June (when nodules were visible). At the time when nitrogen fixation was first detected genistein addition to the soil increased nodule number by 60 and 27% at the unsterilized (June 11) and sterilized (June 17) sites, respectively. At physiological maturity (August 11), soil applied genistein increased nodule number by 15 and 12%, respectively, at the sterilized and unsterilized sites. For plants inoculated with *B. japonicum*, the onset of nitrogen fixation started 3 d earlier and at this time nodule numbers were higher with genistein addition at the unsterilized site. Nodule numbers were not different between genistein levels at the times of either onset of nitrogen fixation at the sterilized site, or at physiological maturity at both sites. Second, soils were not inoculated with *B. japonicum* in Experiment 2, and so would have had lower *B. japonicum* populations than the inoculated soils of Experiment 1. This resulted in lower nodulation and rates of N_2 fixation (Zhang and Smith, in prep.), therefore, plants in the uninoculated soils would have had a greater nitrogen deficiency stress. These plants would benefit more from a treatment that made the symbiosis more effective.

The cultivar AC Bravor tended to be more responsive to genistein application at both sites for Experiment 1 and at the unsterilized site for Experiment 2. At the sterilized site of Experiment 2 the increases in growth variables, yield components, and final grain and protein yield of Maple Glen were similar to those measured for AC Bravor (Tables 1, 2, 3, and 4). AC Bravor is a relatively late-maturing cultivar in its production region and has a higher potential yield than Maple Glen, under optimal environmental conditions (Conseil Des Productions Végétales du Québec recommendations). Thus, nitrogen limitation was more for AC Bravor growth and development. Since the effect of preincubation of *B. japonicum* with genistein on soybean nodulation and nitrogen fixation was probably greater pronounced under plant nitrogen stress conditions (Zhang and Smith, in prep.), the increase in grain yield and protein yield by AC Bravor due to genistein application should be greater than that of Maple Glen.

In summary, this is the first field experiment showing that genistein-preincubated *B. japonicum* increased soybean grain and protein yield. Interactions existed between genistein application and soybean cultivars, suggesting that genistein application to higher yield potential cultivars was more effective. Genistein directly applied into the rhizosphere also improved plant grain and protein yield, probably by stimulation of native soil *B. japonicum*. Overall, from this study it is clear that genistein-preincubated *B. japonicum*, or genistein directly applied onto seed in the furrow at the time of planting can increase soybean grain yield, grain protein yield, and total protein yield.

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