Biological control of *Heterodera glycines* by spore-forming plant growth-promoting rhizobacteria (PGPR) on soybean

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Abstract

*Heterodera glycines*, the soybean cyst nematode, is the most economically important plant-parasitic nematode on soybean production in the U.S. The objectives of this study were to evaluate the potential of plant growth-promoting rhizobacteria (PGPR) strains for mortality of *H. glycines* J2 in vitro and for reducing nematode population density on soybean in greenhouse, microplot, and field trials. The major group causing mortality to *H. glycines* in vitro was the genus *Bacillus* that consisted of 92.6% of the total 663 PGPR strains evaluated. The subsequent greenhouse, microplot, and field trials indicated that *Bacillus velezensis* strain Bve2 consistently reduced *H. glycines* cyst population density at 60 DAP. *Bacillus mojavensis* strain Bmo3 suppressed *H. glycines* cyst and total *H. glycines* population density under greenhouse conditions. *Bacillus safensis* strain Bsa27 and Mixture 1 (Bve2 + Bal13) reduced *H. glycines* cyst population density at 60 DAP in the field trials. *Bacillus subtilis* subsp. *subtilis* strains Bsssu2 and Bsssu3, and *B. velezensis* strain Bve12 increased early soybean growth including plant height and plant biomass in the greenhouse trials. *Bacillus altitudinis* strain Bal13 increased early plant growth on soybean in the greenhouse and microplot trials. Mixture 2 (Abamectin + Bve2 + Bal13) increased early plant growth in the microplot trials at 60 DAP, and also enhanced soybean yield at harvest in the field trials. These results demonstrated that individual PGPR strains and mixtures can reduce *H. glycines* population density in the greenhouse, microplot, and field conditions, and increased yield of soybean.

Introduction

*Heterodera glycines* Ichinohe, the soybean cyst nematode, was first reported in the United States in North Carolina in 1954 [1]. Now *H. glycines* has been found in every soybean-producing state in the U.S. except New York and West Virginia, due to their small soybean acreage and limited soybean production [2]. In the United States, *H. glycines* was the most important...
disease in soybean production, followed by Phytophthora root and stem rot and seedling diseases over the past 10 years [3]. Soybean yield losses caused by H. glycines were estimated to be 25% to 38% of total yield losses in 28 U.S. states, which is more than any other disease from 2006 to 2009 [4].

The removal of chemical nematicides such as Aldicarb (Temik) (Bayer CropScience, Raleigh, NC) has driven the investigation for alternative strategies for integrated pest management of plant-parasitic nematodes. Biological control agents previously assessed for the management of H. glycines were nematophagous fungi, endoparasitic fungi, female and egg-parasitic fungi, fungi producing antibiotic substances, vesicular-arbuscular mycorrhizal (VAM) fungi, Pasteuria spp., chitinolytic bacteria, and plant-growth-regulatory bacteria [5]. Monacrosporium drechsleri, an example of nematophagous fungi, has been found to attack J2 of H. glycines [6]. Hirsutella rhossiliensis and H. minnesotensis, are two endoparasitic fungi found to parasitize vermiform stages of H. glycines [7], and both were found highly effective against H. glycines through paratisizing J2 in the soil when applied at planting or two weeks prior to planting in the greenhouse [6]. The fungal genera Exophiala, Fusarium, Gliocladium, Neocosmospora, Paecilomyces, Phoma, Stagonospora, and Pochonia were commonly recovered from females and cysts of H. glycines [5]. Isolates from those fungi could be female and/or egg-parasitic fungi. Some fungi were found to produce antibiotic substance which inhibits eggs hatch or juvenile mobility. For example, an isolate of the fungus Chaetomium globosum, was found to produce a low molecular weight compound, flavipin, which inhibited in vitro egg hatch and juvenile mobility of Meloidogyne incognita and hatch of H. glycines [8]. VAM fungi were also reported to decrease numbers of H. glycines. Tylka et al. [9] found that numbers of H. glycines in roots and soil were decreased by VAM fungi by as much as 73% at the highest H. glycines inoculum level through 49 days after planting in the greenhouse experiments.

Bacteria are another large group that offered potential in reducing H. glycines population density. Pasteuria spp. was first reported to attack H. elachista in Japan in 1987 [10] and was later found to attack H. glycines in North America in 1994 [11]. Four chitinolytic bacterial strains were found to reduce numbers of H. glycines through the interaction with the chitin substrate mixed in the soil in the greenhouse [12]. Thirty-six of 201 rhizobacteria strains were also found to reduce numbers of soybean cysts, eggs, and J2 in the initial greenhouse tests [13]. Among 20 strains that suppressed (≥ 50%) H. glycines in the initial greenhouse screening test, four were Pseudomonas spp., two Bacillus spp. (B. cereus and B. pumilus), three Paenibacillus spp., and one Streptomyces spp. [13]. Plant-growth-regulatory bacteria especially plant-growth promoting rhizobacteria (PGPR) were found to have potential for the control of H. glycines. Kloepper et al. [14] found that B. megaterium, B. pumilus, and Bacillus spp. were antagonistic to H. glycines and M. incognita. Sharma [15] evaluated the efficiency of toxins from pure cultures of B. sphaericus (Bs 2362), B. thuringiensis var. israelensis (Bti-H-14), and B. thuringiensis var. kurstaki (Btk-HD-1) against H. glycines in a greenhouse pot experiment. However, none of the toxins significantly reduced the final nematode population density in relation to the untreated control. Sharma and Gomes [16] evaluated the effect of those toxins again on oviposition and J2 hatching of H. glycines race 3 in the greenhouse and found the number of hatched J2 treated with Bs 2362 was significantly less than the control in one experiment.

Among these antagonists, rhizobacteria, especially Bacillus PGPR, can promote plant growth and elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts [17], and also elicit nematicidal activity or induced systemic resistance to plant-parasitic nematodes. Many of these species produce endospores which help the bacteria survive in a wide range of environmental conditions and have long-shelf life giving them an advantage as a commercial product. Some Bacillus strains have been developed into commercial products for plant disease and plant-parasitic nematode management, such as BioNem-
WP/BioSafe (B. firmus) (AgroGreen, Israel) [18], BioYield (combination of B. amyloliquefaciens strain IN937a and B. subtilis strain GB03) (Gustafson LLC, USA) [17, 19], Nemix (Bacillus spp.) (AgriLife/Chr Hansen, Brazil) [20], VOTIVO (B. firmus GB-126) (Bayer CropScience, Germany) [21], and Pathway Consortia (mixture of B. subtilis, B. licheniformis, B. megaterium, B. coagulans, Pseudomonas fluorescens, Streptomyces spp., and Trichoderma spp.) (Pathway Holdings, USA) [22].

More research on beneficial PGPR strains as biocontrol agents for plant-parasitic nematodes management is needed. The overall objective of this project was to evaluate PGPR strains for biological control potential of H. glycines on soybean. The specific objectives were to assess the potential of PGPR strains for H. glycines J2 mortality percentage in vitro using high throughput screening and select strains to further test for H. glycines population density reduction and enhanced plant growth in the greenhouse, microplot, and field production systems.

Materials and methods

PGPR strains

A total of 663 PGPR strains were included in an in vitro study. These strains were originally isolated, identified, and maintained by J. W. Kloepper at Auburn University, Auburn, AL. Among these strains, 92.6% were Bacillus spp. including 208 strains of B. simplex, 70 strains of B. toyonensis, 53 strains of B. aryabhattai, 51 strains of B. cereus, 44 strains of B. mycoides, 41 strains of B. velezensis, 35 strains of B. safensis, 21 strains of B. altitudinis, 21 strains of B. weihenstephanensis, 15 strains of B. subtilis subsp. inaquosorum, 13 strains of B. methylotrophicus, six strains of B. pumilus, five strains of B. psychrosacccharolyticus, four strains of each B. mojavensis, B. subtilis subsp. subtilis, and B. thuringiensis, three strains of B. siamensis and B. tequilensis, and 13 strains of other Bacillus spp. The remaining 8.4% of the strains, ten were Sporosarcina globispora, nine were Paenibacillus amylolyticus, four were Paenibacillus lautus, three were unknown species, and 23 were from multiple other genera. The PGPR strains, stored in 30% glycerol at -80°C, were transferred to tryptic soy agar (TSA) (VWR, Radnor, PA) plates, and incubated at 35°C for 24 hours. Vegetative cells of each strain were suspended in 5 ml of sterile distilled water in glass tubes. The concentration of bacterial vegetative cell suspensions was adjusted to 1 × 10^7 CFU/ml.

Nematode inoculum

The H. glycines used as inoculum in vitro, in the greenhouse and microplot experiments were from a culture maintained in the greenhouse since 2000. Eggs for the experiments were extracted from a 60-day-old soybean ("Asgrow 5935", Monsanto, St. Louis, MO) stock culture maintained in 500 cm³ polystyrene pots. Soil was gently washed from the soybean roots and cysts and females were dislodged from the roots [23]. Water with the cyst and female suspension was poured through nested 850-μm-pore and 250-μm-pore sieves to separate trash from cysts and females [23]. Cysts and females were ground with a mortar and pestle to release the eggs. Eggs were washed with water and collected on a 25-μm-pore sieve and the suspension was centrifuged at 240 g for 1 minute using the sucrose centrifugation-flotation method [24]. For in vitro tests, H. glycines eggs were placed in a modified Baermann funnel [25] on a Slide Warmer (Model 77) (Marshall Scientific, Brentwood, NH) and incubated at 31°C for 5 to 7 days to obtain the J2 [26]. The J2 were collected on a 25-μm-pore sieve, transferred to 1.5 ml micro centrifuge tubes, centrifuged at 5,000 g for 1 minute, rinsed with sterile distilled water, and centrifuged at 5,000 g for 1 minute. The J2 suspensions were adjusted to 30 to 40 J2 per 10 μl of water [26, 27]. For greenhouse and microplot trials, eggs were enumerated at × 40
magnification with an inverted TS100 Nikon microscope and standardized to 2,000 eggs per cone-tainer for tests in the greenhouse or 50,000 eggs per pot for tests in the microplot [27].

Tests in vitro

In vitro tests were conducted to assess mortality percentage of *H. glycines* J2 by PGPR strains. The PGPR vegetative cell suspensions and *H. glycines* J2 inocula were prepared as described previously. Ten μl of nematode suspension containing 30 to 40 *H. glycines* J2 were added in each well of a 100 μl 96-well plate. Ninety μl of each PGPR vegetative cell suspension was transferred into each test well of the 96-well plate. Clothianidin plus *B. firmus* I-1582 (Poncho/Votivo) (Bayer CropScience, Raleigh, NC) at a 0.7 μl / well (0.424 mg ai/seed), 100 million international unit (MIU)/well of *Pasteuria nishizawai*e (Clariva) (Syngenta Greensboro, NC), and 1 granule/well of Aldicarb (Temik 15G) (Bayer CropScience, Raleigh, NC) were used as industry standards, and sterile distilled water was the untreated control. Each plate was sealed with parafilm (VWR, Radnor, PA) and incubated at room temperature for 48 hours. Numbers of live *H. glycines* J2 were enumerated and recorded at experiment initiation and 48 hours after exposure to the treatments. Viability of *H. glycines* J2 was determined using the sodium technique developed by Xiang and Lawrence [27] for high throughput screening of biological or chemical agents on plant-parasitic nematodes. Mortality percentage of *H. glycines* J2 were calculated as the following equation: [(live J2 prior to exposure – live J2 at 48 hours) / live J2 prior to exposure] × 100. Each bacterial treatment had four replications and the experiment was repeated.

Plant material

The soybean (*Glycine max*) variety “Asgrow 5935” (Monsanto, St. Louis, MO) as reported by Monsanto to be susceptible to *H. glycines* was used for all the experiments.

Trials in the greenhouse

Seventy two PGPR strains from the in vitro screenings with high J2 mortality were selected for initial evaluation in the greenhouse for their efficacy to reduce nematode population density and promote soybean plant growth. Confidential agreements were signed during this research study and only ten PGPR strains were available for further testing. These included *B. altitudinis* strains Ball11 and Ball13, *B. mojavensis* strain Bmo3, *B. safensis* strains Bsa26 and Bsa27, *B. subtilis* subsp. *subtilis* strains Bsssu2 and Bsssu3, *B. velezensis* strains Bve12 and Bve2, and *Fictibacillus solisalsi* strain Fso1. All the tests were conducted at the Plant Science Research Center (PSRC) located at Auburn University, Auburn, AL. Experiments were performed in 150 cm³ plastic cone-tainers (Stuewe & Sons Inc., Tangent, Oregon) filled with a soil:sand mix (60:40 v/v). The soil was a kalmia loamy sand (80% sand, 10% silt, and 10% clay) collected from Plant Breeding Unit (PBU) located at E.V. Smith Research Center of Auburn University near Talladega, AL. Soil was steam pasteurized at 180°C for 60 minutes to 120 minutes and cooled for 24 hours. Steam pasteurizing process was repeated prior to use. Two soybean seeds were planted 2.5 cm deep in each cone-tainer. One ml of bacterial cell suspension (1×10⁷ CFU/ml) was inoculated on each seed at planting. For the nematicide controls, soybean seeds were treated with each compound following industrial recommendations: 0.13 mg a.i./seed of Clothianidin plus *B. firmus* I-1582 (Poncho/Votivo), or 0.15 mg a.i./seed of Abamectin (Avicta) (Syngenta, Greensboro, NC), or 10,000 million international unit (MIU)/ml of *Pasteuria nishizawai*e (Clariva) (Syngenta Greensboro, NC) prior to planting. All seeds were treated with a Gustafson table-top seed treater (Bayer CropScience, Research Triangle Park, NC), mixed for 3 min in the 454-gm stainless steel bucket and allow to airdry before packaging [28]. One ml of tap
water added to the seeds was used as the untreated control. One ml containing 2,000 H. glycines eggs was pipetted into each cone-tainer at planting. Experiments were arranged in a randomized complete block design (RCBD). Each treatment had five replications and the entire experiment was repeated twice. Soybean seedlings were thinned to one per cone-tainer after emergence. Plants were watered as needed. Supplemental light of 1000 watts halide bulbs producing 110,000 lumens was supplied to maintain the day length of 14 hours per day. Greenhouse temperature was ranged from 21˚C to 35˚C. Experiments were terminated at 60 DAP. Plant and nematode measurements were recorded. Plant measurements included Plant height (PH) and Biomass including shoot and root fresh weights (SFW/RFW). Heterodera glycines cyst and vermiform stage numbers were recorded. The H. glycines cysts were extracted from the soybean roots as described previously in inoculum preparation. Water suspension containing 150 cm$^3$ of soil from cone-tainers was poured through nested 75-μm and 25-μm-pore sieve to extract vermiform stages (juveniles and males). Vermiform stages were collected on the 75-μm-pore sieve and centrifuged using sucrose centrifugation-flotation method [24].

Trials in the microplot
The performance of five strains and two strain mixtures were evaluated for nematode population density, early growth promotion, and yield enhancement of soybean in the microplots. The strains included a strain of B. altitudinis (Bal13), a strain of B. safensis (Bsa27), a strain of B. subtilis subsp. subtilis (Bsssu2), two strains of B. velezensis (Bve12 and Bve2), and two mixtures Mixture 1 (Bve2 + Bal13) and Mixture 2 (seeds treated with Abamectin + Bve2 + Bal13). Mixtures were formed from the best performing strains based on greenhouse studies. The experiments were conducted at the PSRC. Experiments were established in 26.5 liter pots filled with a Kalmia loamy sand (80% sand, 10% silt, and 10% clay) collected from PBU. Nematodes were extracted from the non pasteurized soil as previously described and H. glycines population density was below the detection level of the extraction method descripted previously. Experiments were arranged in a RCBD with 6 replications for each treatment and the experiment was repeated twice. Ten soybean seeds were hand-planted at 2.5 cm in depth in a linear pattern to simulate a linear row foot in the field [27]. One ml bacterial suspension (1 × 10$^7$ CFU/ml) was applied to each seed at planting. Five ml containing 50,000 H. glycines eggs were pipetted randomly in each pot at planting. Soybean seeds treated with Clothianidin plus B. firmus I-1582, Abamectin, and P. nishizawai as previously described were used as standards. The untreated control received 1 ml of tap water per seed. Each microplot received 30 ml per minute of water by an automatic drip irrigation system adjusted throughout the season to run for 15–45 minutes twice a day, for a total of 450–1350 ml of water per microplot per day. At 60 DAP, one representative soybean plant was dug from each microplot for PH and Biomass (SFW + RFW) measurements and nematode extraction as previously described. Cysts were extracted from the roots. Vermiform stages were extracted from 100 cm$^3$ of soil surrounding the roots. Total nematode numbers including cysts and vermiforms were recorded. At plant maturity, approximately 160 DAP, soybeans were harvested and yield was recorded as grams of soybean seed per plot.

Trials in the field
The same strains and mixtures assessed in the microplot trials were evaluated in field trials for their effect on early-season nematode population density, plant growth promotion, and yield enhancement in soybean. The experiments were established in the research stations of E.V. Smith in a Wickham fine sandy loam soil (70% sand, 16% silt, and 18% clay), Tallassee, AL and Tennessee Valley Research and Extension Center (TVREC) in a Decatur silt loam soil.
Both were artificially infested fields with soybean cysts added every year since 2011. The experiments were arranged in a RCBD with 5 replications for each treatment. The field trials were arranged in two-row plots that were 7 m long with 0.9 m row spacing. Blocks were separated by a 6 m alley. One hundred and seventy five soybean seeds were planted in each row with an Almaco plot planter (Almaco, Iowa). The PGPR treatments were applied as in-furrow spray standardized to $1 \times 10^7$ CFU/seed and applied at 32.5 liter per hectare at planting. Seeds treated with Clothianidin plus *B. firmus* I-1582, Abamectin, and *P. nishizawae* as previously described were included as industry standard controls. Tap water applied in-furrow was used as untreated control. At 60 DAP, four random soybean plants were removed from each plot. The same plant growth parameters evaluated in the microplots were evaluated in the field. *Heterodera glycines* population density was determined by extracting soybean cysts and females from the roots, and vermiform stages from the soil as described previously. Soybeans were harvested mechanically with a Almaco plot harvester (Almaco, Iowa) at plant maturity approximately 160 DAP and yield recorded and adjusted to 13% moisture content.

### Statistical analysis

Data collected from *in vitro*, greenhouse, microplot, and field trials were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using the PROC GLIMMIX procedure. Dependent variables included J2 mortality, plant height (PH), biomass (Bio), cyst, vermiform stage (VS), total SCN, and yield. Fixed effects were PGPR strains or nematicides treatments and the random effects included replication, repeat in time, and location. Student panels were generated to determine the normality of the residuals. A log-normal distribution transformation was required for the PH, Bio, cyst, VS, total SCN, and yield data to satisfy the normal assumptions. LS-means were compared between the treatments, chemical standards Clothianidin plus *B. firmus* I-1582, Abamectin, *P. nishizawae* and the untreated control by Dunnett’s method at significant level of $P \leq 0.05$ or $P \leq 0.10$. The LS-means are presented in the tables with adjusted *P* values for statistical differences.

### Results

#### Test *in vitro*

The mortality percentage of *H. glycines* J2 ranged from 0.0% to 99.9% with the PGPR strains tested (663) with an average of 16.0%. Data presented were results of 52 strains LS-means greater than 50% mortality of *H. glycines* J2 (Table 1). The PROC GLIMMIX analysis indicated the numerator and denominator df are 666 and 1875, respectively with an F value of 8.01, and $P < 0.0001$. Of those 52 strains, 24 were *B. simplex*, five were *B. altitudinis*, five were *B. toyoensis*, three were *B. aryabhattachi*, three were *B. safensis*, two were *B. mycoides*, two were *B. subtilis* subsp. *subtilis*, and the remaining were *B. lentus*, *B. methylotrophicus*, *B. mojavensis*, *B. pumilus*, *B. weihenstephanensis*, *Fictibacillus solisali*, *Paenibacillus taichungensis*, and *P. xylanexedens*. Among all the PGPR strains tested, 6.8% caused significantly greater level of mortality percentage than the biological standard Clothianidin plus *B. firmus* I-1582 ($P \leq 0.05$); 7.9% caused significantly greater level of mortality percentage than the level caused by Aldicarb ($P \leq 0.05$); 5.6% caused statistically similar mortality percentage to the level caused by Aldicarb ($P \leq 0.05$); and 13.2% caused significantly greater mortality percentage than the level caused by untreated control ($P \leq 0.05$) (Table 1). Among all the strains, 92.6% were *Bacillus* spp. strains, which was the major genera with greater mortality percentage than any other single genera.
Table 1. PGPR strains effect on *Heterodera glycines* J2 with LS-means more than 50% mortality\(^a\).

| Code  | Scientific name            | *Heterodera glycines* | Dunnett’s *P vs* (\(P < 0.05\)) |
|-------|----------------------------|------------------------|----------------------------------|
|       | J2 mortality (%)\(^b\)   | Clothianidin + *B. firmus* | *P. nishizawae* | Aldicarb | Water |
|       |                            |                        |                                 |           |       |
| Bal9  | Bacillus altitudinis       | 51.7                   | 0.1099                           | 0.0206    | <0.001 | <0.001 |
| Bal11 | Bacillus altitudinis       | 64.0                   | 0.0236                           | 0.0045    | 0.1725 | <0.001 |
| Bal12 | Bacillus altitudinis       | 54.7                   | 0.0408                           | 0.0059    | 0.0002 | <0.001 |
| Bal13 | Bacillus altitudinis       | 81.2                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bal20 | Bacillus altitudinis       | 55.1                   | 0.0353                           | 0.0050    | 0.0003 | <0.001 |
| Bar15 | Bacillus aryabhattai       | 90.5                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bar16 | Bacillus aryabhattai       | 64.9                   | 0.0180                           | 0.0033    | 0.2079 | <0.001 |
| Bar21 | Bacillus aryabhattai       | 57.5                   | 0.0136                           | 0.0016    | 0.0011 | <0.001 |
| Ble1  | Bacillus lentus            | 74.2                   | <0.001                           | <0.001    | 0.4208 | <0.001 |
| Bmo3  | Bacillus mojavensis        | 54.5                   | 0.2720                           | 0.0907    | 0.0117 | 0.0010 |
| Bmt10 | Bacillus methylotrophicus  | 51.4                   | 0.4749                           | 0.1896    | 0.0039 | 0.0033 |
| Bmy19 | Bacillus mycoides          | 66.9                   | 0.0092                           | 0.0015    | 0.3115 | <0.001 |
| Bmy32 | Bacillus mycoides          | 77.7                   | 0.0001                           | <0.001    | 0.9947 | <0.001 |
| Bpu6  | Bacillus pumilus           | 78.4                   | <0.001                           | <0.001    | 0.9982 | <0.001 |
| Bsa25 | Bacillus safensis          | 62.5                   | 0.0378                           | 0.0079    | 0.1200 | <0.001 |
| Bsa26 | Bacillus safensis          | 74.1                   | 0.0006                           | <0.001    | 0.8614 | <0.001 |
| Bsa27 | Bacillus safensis          | 79.2                   | <0.001                           | <0.001    | 0.9997 | <0.001 |
| Bsp2  | Bacillus simplex           | 60.2                   | 0.0444                           | 0.0004    | 0.0038 | <0.001 |
| Bsp3  | Bacillus simplex           | 62.0                   | 0.0437                           | 0.0095    | 0.1061 | <0.001 |
| Bsp4  | Bacillus simplex           | 93.9                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsp8  | Bacillus simplex           | 55.9                   | 0.2035                           | 0.0626    | 0.0186 | 0.0005 |
| Bsp26 | Bacillus simplex           | 64.5                   | 0.0201                           | 0.0038    | 0.1927 | <0.001 |
| Bsp53 | Bacillus simplex           | 81.9                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsp68 | Bacillus simplex           | 87.1                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsp90 | Bacillus simplex           | 52.2                   | 0.0340                           | 0.0038    | <0.001 | <0.001 |
| Bsp113| Bacillus simplex           | 63.3                   | 0.0010                           | <0.001    | 0.0144 | <0.001 |
| Bsp123| Bacillus simplex           | 74.2                   | 0.0005                           | <0.001    | 0.8715 | <0.001 |
| Bsp129| Bacillus simplex           | 99.9                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsp130| Bacillus simplex           | 61.6                   | 0.0490                           | 0.0109    | 0.0960 | <0.001 |
| Bsp133| Bacillus simplex           | 73.7                   | 0.0007                           | <0.001    | 0.8329 | <0.001 |
| Bsp139| Bacillus simplex           | 67.6                   | 0.0072                           | 0.0011    | 0.3548 | <0.001 |
| Bsp141| Bacillus simplex           | 99.9                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsp146| Bacillus simplex           | 70.9                   | 0.0021                           | 0.0003    | 0.6075 | <0.001 |
| Bsp149| Bacillus simplex           | 64.7                   | 0.0189                           | 0.0035    | 0.2013 | <0.001 |
| Bsp153| Bacillus simplex           | 89.7                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsp159| Bacillus simplex           | 56.8                   | 0.1650                           | 0.0480    | 0.0251 | 0.0004 |
| Bsp165| Bacillus simplex           | 71.4                   | <0.001                           | <0.001    | 0.2188 | <0.001 |
| Bsp168| Bacillus simplex           | 69.1                   | 0.0042                           | 0.0006    | 0.4596 | <0.001 |
| Bsp171| Bacillus simplex           | 67.3                   | 0.0079                           | 0.0013    | 0.3390 | <0.001 |
| Bsp188| Bacillus simplex           | 73.0                   | 0.0009                           | 0.0001    | 0.7829 | <0.001 |
| Bsp196| Bacillus simplex           | 95.1                   | <0.001                           | <0.001    | 1.0000 | <0.001 |
| Bsssu2| Bacillus subtilis subsp. subtilis | 74.8              | 0.0004                           | <0.001    | 0.9084 | <0.001 |
| Bsssu3| Bacillus subtilis subsp. subtilis | 74.2              | 0.0005                           | <0.001    | 0.8715 | <0.001 |
| Bto10 | Bacillus toyonensis        | 64.7                   | 0.0005                           | <0.001    | 0.0250 | <0.001 |
| Bto11 | Bacillus toyonensis        | 62.7                   | 0.0013                           | 0.0001    | 0.0114 | <0.001 |

(Continued)
Greenhouse trial

The PROC GLIMMIX analysis for the greenhouse trials indicated the numerator and denominator df are 13 and 117, respectively with an F value of 2.34, and P = 0.0083. Strains *B. mojavensis* Bmo3 and *B. velezensis* Bve2 suppressed *H. glycines* cyst population density at 60 DAP at levels statistically equivalent to Abamectin (P < 0.05) (Table 2). All ten PGPR strains significantly increased the soybean plant height compared to the standard Clothianidin plus *B. firmus* I-1582 at 60 DAP (P < 0.05) (Table 3).

Microplot trial

Five *Bacillus* PGPR strains and two mixtures were evaluated in the microplot for early plant growth promotion, reduction of *H. glycines* population density, and yield enhancement. The PROC GLIMMIX analysis for the microplot trials indicated the numerator and denominator df are 10 and 90, respectively with an F value of 2.60, and P = 0.0080. Results indicated that the *B. velezensis* strain Bve2 significantly reduced *H. glycines* cyst numbers compared to the control.
Biological control with PGPR

Table 2. Effect of ten PGPR strains on *Heterodera glycines* cyst numbers and total nematode population density in greenhouse trials at 60 DAP.

| Treatment | Scientific Name | Cyst | 60 DAP | Dunnett’s *P* vs. (P ≤ 0.10) | Abamectin | Water | Total H. glycines | Clothianidin + B. firmus | P. nishizawai | Abamectin | Water |
|-----------|----------------|------|--------|-------------------------------|-----------|-------|------------------|------------------------|---------------|-----------|-------|
| Bal11     | B. altitudinis  | 2458 | 0.9599 | 1.0000                         | 0.0400    | 1.000 | 2897             | 0.9781                 | 1.0000        | 0.10     | 1.000 |
| Bal13     | B. altitudinis  | 2154 | 0.9860 | 1.0000                         | 0.0556    | 1.000 | 3817             | 0.9781                 | 1.0000        | 0.10     | 1.000 |
| Bmo3      | B. mojavensis   | 1665 | 1.0000 | 0.9931                         | 0.2698    | 0.9678| 2319             | 1.000                  | 1.0000        | 0.10     | 1.000 |
| Bsa26     | B. safensis     | 2934 | 0.6536 | 0.9993                         | 0.0092    | 1.000 | 3781             | 0.9840                 | 0.9960        | 0.10     | 1.000 |
| Bsa27     | B. safensis     | 2754 | 0.9449 | 1.0000                         | 0.0353    | 1.000 | 3132             | 1.000                  | 1.0000        | 0.10     | 1.000 |
| Bssu2     | B. subtilis subsp. subtilis | 2140 | 0.9940 | 1.0000                         | 0.0759    | 1.000 | 2474             | 1.000                  | 1.0000        | 0.10     | 1.000 |
| Bssu3     | B. subtilis subsp. subtilis | 2064 | 0.8248 | 1.0000                         | 0.0184    | 1.000 | 2780             | 0.9966                 | 0.9959        | 0.10     | 1.000 |
| Bve2      | B. velezensis   | 1583 | 1.0000 | 0.9780                         | 0.3331    | 0.9282| 1822             | 0.9966                 | 0.9859        | 0.10     | 0.5600 |
| Bve12     | B. velezensis   | 3527 | 0.3012 | 0.9062                         | 0.0018    | 0.9644| 4197             | 0.7629                 | 0.8500        | 0.10     | 0.0047 |
| Fso1      | Fictibacillus solisalsi | 1733 | 0.9991 | 1.0000                         | 0.0944    | 1.000 | 2326             | 1.000                  | 1.0000        | 0.10     | 1.0200 |
| Control   | Active ingredient |      |        |                               |           |       |                  |                        |               |           |       |
| Poncho/ | Clothianidin     | 1745 | ...    | 0.9832                         | 0.3554    | 0.9424| 2386             | ...                    | 1.0000        | 0.1875   | 0.9999 |
| Votivo    | + B. firmus I-1582 |      |        |                               |           |       |                  |                        |               |           |       |
| Clariva   | *Pasteuria nishizawai* | 2245 | 0.9832 | ...                            | 0.0594    | 1.000 | 2562             | 1.000                  | ...           | 0.1446   | 1.0000 |
| Avicta    | Abamectin        | 1116 | 0.3715 | 0.0620                         | ...       | 0.0352| 1789             | 0.1963                 | 0.1513        | ...      | 0.0562 |
| Untreated | Water            | 2304 | 0.9343 | 1.0000                         | 0.0327    | ...   | 3274             | 0.9999                 | 1.0000        | 0.10     | ...    |

Greenhouse trials were performed in plastic cone-tainers with mixed pasteurized soil and sand (60:40 v/v) for 45 days. Data collected were repeated twice and analyzed in SAS 9.4 using PROC GLIMMIX procedure at significant level of α ≤ 0.10. Adjusted *P* values less than 0.10 indicated a significant effect. Adjusted *P* values were obtained by analyzing data according to Dunnett’s method.

*The LS-means and adjusted *P* values are presented in the tables.

*Cyst = soybean cysts and white females at 60 DAP.*

*Active ingredients for the nematicides Poncho/Votivo are Clothianidin plus B. firmus I-1582, Clariva is Pasteuria nishizawai, Avicta is Abamectin, and untreated control is water.*

*Total *H. glycines* = total numbers of soybean cysts, white females, and juveniles at 60 DAP.*

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Biological standard *P. nishizawai* at 60 DAP (P ≤ 0.10) (Table 4). *Bacillus altitudinis* strain Bal13 and Mixture 2 significantly increased plant height compared to all the industrial standards (P ≤ 0.10) (Table 5). *Bacillus altitudinis* strain Bal13, *B. safensis* strain Bsa27, and Mixture 2 significantly increased plant biomass (SFW + RFW) compared to the untreated control at 60 DAP (P ≤ 0.10) (Table 5). Number of *H. glycines* veriform stage (data not show) at 60 DAP and soybean yield (Table 5) at harvest were similar among all the PGPR strains and the industrial standards.
The PROC GLIMMIX analysis for the field trials indicated the numerator and denominator df are 10 and 71, respectively with an F value of 2.19, and $P = 0.0280$. Strains *B. safensis* Bsa27, *B. velezensis* Bve2, and Mixture 1 significantly reduced *H. glycines* cyst numbers compared to untreated control at 60 DAP ($P \leq 0.10$) (Table 6). Strain Mixture 2 (Fig 3) significantly increased soybean yield compared to the untreated control at 160 DAP ($P \leq 0.10$) (Table 6). Plant height, biomass, *H. glycines* verminform stages, and total *H. glycines* were similar among all the PGPR strains and industrial standards (data not show).

### Table 3. Effect of ten PGPR strains on soybean plant height and plant biomass in greenhouse trials at 60 DAP.

| Treatment | Scientific Name | PH$^b$ | 60 DAP | Bio$^d$ | 60 DAP |
|-----------|----------------|-------|---------|---------|---------|
|           |                |       | Clothianidin $P. nishizawae$ Abamectin Water | Clothianidin $P. nishizawae$ Abamectin Water |
| Bal11     | *B. altitudinis* | 35.3  | 0.0164 1.0000 0.9971 1.0000 | 4.9 0.1865 0.9910 0.9971 0.9845 |
| Bal13     | *B. altitudinis* | 35.1  | 0.0154 1.0000 0.9962 1.0000 | 5.4 0.0116 1.0000 1.0000 1.0000 |
| Bmo3      | *B. mohavensis* | 40.8  | 0.0002 0.6444 0.3767 0.9827 | 4.6 0.1871 0.9909 0.9970 0.9842 |
| Bsa26     | *B. safensis*   | 38.9  | 0.0014 0.9352 0.6983 1.0000 | 4.8 0.0566 1.0000 1.0000 1.0000 |
| Bsa27     | *B. safensis*   | 35.4  | 0.0109 1.0000 0.9870 1.0000 | 4.8 0.0766 1.0000 1.0000 1.0000 |
| Bssu2     | *B. subtilis*   | 41.4  | 0.0002 0.4976 0.2746 0.9227 | 5.4 0.0319 1.0000 1.0000 1.0000 |
| Bssu3     | *B. subtilis*   | 34.9  | 0.0255 1.0000 0.9997 0.9999 | 5.2 0.0399 1.0000 1.0000 1.0000 |
| Bve2      | *B. velezensis* | 34.7  | 0.0279 1.0000 0.9998 0.9998 | 5.3 0.0771 1.0000 1.0000 1.0000 |
| Bve12     | *B. velezensis* | 37.9  | 0.0020 0.9654 0.7667 1.0000 | 6.1 0.0028 0.9972 0.9921 0.9986 |
| Fso1      | *Fictibacillus* | 35.0  | 0.0187 1.0000 0.9984 1.0000 | 4.3 0.1577 0.9963 0.9990 0.9930 |

**Control**

| Active ingredient$^a$ | Poncho/Votivo | Clariva | Avicta | Untreated control |
|------------------------|---------------|---------|--------|------------------|
|                        | Clothianidin  | Pasteuria | Abamectin | Water |
|                        | + *B. firmus* | *nishizawae* |          |       |
|                        | Poncho/Votivo | 34.2    | 0.0477 1.0000 0.9990  | 5.0 0.0319 1.0000 1.0000 1.0000 |
| Clariva                |               |         | 1.0000 0.9546 5.2 0.0517 1.0000 | ...
| Avicta                 |               |         |         |         |
| Untreated control      |               |         |         |         |

Greenhouse trials were performed in plastic cone-tainers with mixed pasteurized soil and sand (60:40 v/v) for 60 days. Data collected were repeated twice and analyzed in SAS 9.4 using PROC GLIMMIX procedure at significant level of 0.05. Adjusted P values less than 0.05 indicated a significant effect. Adjusted P values were obtained by analyzing data according to Dunnett's method.

$^a$The LS-means are presented in the tables with adjusted P values for statistical differences.

$^b$PH = plant height (cm) at 60 DAP.

$^c$Active ingredients for the nematicides Poncho/Votivo are Clothianidin plus *B. firmus* I-1582, Clariva is *Pasteuria nishizawae*, Avicta is Abamectin, and untreated control is water.

$^d$Bio = soybean plant biomass including shoot fresh weight (g) and root fresh weight (g) at 60 DAP.

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Discussion

In vitro screening of the 663 PGPR strains indicated that 13 *Bacillus* species including *B. altitudinis*, *B. aryabhattai*, *B. lentus*, *B. methylotrophicus*, *B. mojavensis*, *B. mycoides*, *B. pumilus*, *B. safensis*, *B. simplex*, *B. subtilis* subsp. *subtilis*, *B. toyonensis*, *B. velezensis*, *B. weihenstephanensis*, and species of *Fictibacillus* and *Paenibacillus* caused greater than 50% mortality percentage of *H. glycines* J2 in vitro. Strains of *B. altitudinis*, *B. aryabhattai*, *B. lentus*, *B. methylotrophicus*, *B. mojavensis*, *B. mycoides*, *B. safensis*, *B. simplex*, *B. toyonensis*, *B. velezensis*, and strains of *Fictibacillus* were first documented in this study for antagonistic activity against *H. glycines*. Previously, some bacterial species have been documented to be antagonistic to *H. glycines*. *Bacillus megaterium* [14], *B. pumilus* [13, 14], *B. sphaericus* [15, 16], *B. cereus*
[13], *Paenibacillus* spp. [13] were reported for their nematicidal activity on reduction of *H. glycines* population density in greenhouse trials. None of these studies included high throughput *in vitro* screening of biological agents to *H. glycines*. Our study is the first documentation of high throughput *in vitro* screening of biological control agents on efficacy to *H. glycines*.

*Bacillus velezensis* strain Bve2 consistently reduced *H. glycines* cyst numbers at 60 DAP in the greenhouse, microplot, and field trials. *Bacillus mojavensis* strain Bmo3 suppressed *H. glycines* cyst and total *H. glycines* population density under greenhouse conditions. *Bacillus safensis* strain Bsa27 and Mixture 1 (Bve2 + Bal13) reduced *H. glycines* cyst numbers at 60 DAP in

![Fig 2. Soybean roots treated with strain *B. altitudinis* Bal13 (Right) and untreated control (Left) at 60 DAP.](https://doi.org/10.1371/journal.pone.0181201.g002)
the field trials. Individual strains of Bmo3 and Bve2 and Mixture 2 (Abamectin + Bve2 + Bal13) were previously found to reduce M. incognita eggs/g root on cotton plants in the greenhouse, microplot, and field studies [29]. This study expanded the documented nematicidal activity of the strains Bmo3 and Bve2 on H. glycines. Some studies have documented individual or mixtures of PGPR strains and/or nematicides or other agents on reduction of plant-parasitic nematode population density. Burkett-Cadena et al. [19] reported that the combination of B. amyloliquefaciens (sym. B. velezensis) strain GB99 and B. subtilis strain GB03 (BioYield, Gustafson LLC, USA) significantly reduced Meloidogyne spp. eggs per gram root, juvenile nematodes per cm³ of soil, and galls per plant on tomato. Castillo et al. [25] found that individuals strains of B. firmus GB-126 (Votivo, Bayer CropScience, Germany) and Paecilomyces lilacinus 251 (PL 251, Biological Control Products, South African), or the combination of B. firmus GB-126 and P. lilacinus reduced Rotylenchulus reniformis population density in the greenhouse, microplot, and field trials. Our results are in agreement with their studies that individual PGPR strains and mixtures have biological control potential on plant-parasitic nematodes.

### Table 4. Effect of five PGPR strains and two mixtures of PGPR strains on *Heterodera glycines* population density on soybean in microplot trials at 60 DAP.

| Treatment     | Scientific Name       | Cystb | 60 DAP Dunnett’s P vs. (P < 0.10) | Total H. glycinesa | 60 DAP Dunnett’s P vs. (P < 0.10) | Cystb |
|---------------|-----------------------|-------|-----------------------------------|-------------------|-----------------------------------|-------|
|               |                       |       | Clothianidin + B. firmusc | P. nishizawaed | Abamectin | Water | Clothianidin + B. firmusc | P. nishizawaed | Abamectin | Water |
| Bal13         | B. altitudinis        | 1123  | 0.0449   0.6546 0.0611 0.1065 1224 0.0791 0.8444 0.1114 0.2987 |       |       |
| Bsa27         | B. safensis           | 472   | 0.9998   0.3982 0.9866 0.9833 609 1.0000 0.7686 1.0000 0.9995 |       |       |
| Bssu2         | B. subtilis subsp. subtilis | 774 | 0.7977   1.0000 0.8814 0.9752 984 0.3261 1.0000 0.4340 0.8383 |       |       |
| Bve12         | B. velezensis         | 439   | 0.9899   0.1373 0.9678 0.8624 448 0.9960 0.1455 0.9793 0.7078 |       |       |
| Bve2          | B. velezensis         | 384   | 0.9042   0.0627 0.8277 0.6375 425 0.9875 0.1131 0.9543 0.6243 |       |       |
| Mixture 1a    |                       | 465   | 0.9996   0.3750 0.9977 0.9776 471 0.9998 0.3643 0.9980 0.9041 |       |       |
| Mixture 2a    |                       | 930   | 0.4621   0.9997 0.4589 0.6263 968 0.5817 1.0000 0.6898 0.9537 |       |       |
| Control Active | ingredientc         |       | Poncho/ Votivo Clothianidin | 563 | ... | 0.5400 | 1.0000 | 0.9999 | 584 | ... | 0.4944 | 1.0000 | 0.9914 |
| Clariva       | Pasteuria nishizawae  | 832   | 0.5400   ... 0.6467 0.7878 931 0.4944 ... 0.6216 0.9537 |       |       |
| Avicta        | Abamectin            | 587   | 1.0000   0.6467 ... 1.0000 | 620 | 1.0000 | 0.6216 | ... | 0.9989 |       |       |
| Untreated control | Water                | 632   | 0.9999   0.8361 1.0000 ... 736 | 0.9914 | 0.9539 | 0.9989 | ... |       |       |

Microplot trials were performed in 26.5 liter pot. Data collected were repeated and analyzed in SAS 9.4 using PROC GLIMMIX procedure at significant level of α < 0.10. Adjusted P values less than 0.10 indicated a significant effect. Adjusted P values were obtained by analyzing data according to Dunnett’s method.

a The LS-means are presented in the tables with adjusted P values for statistical differences.

b Cyst = cysts and white females from 100 cm³ of soil at 60 DAP.

c Active ingredients for the nematicides Poncho/Votivo are Clothianidin plus B. firmus I-1582, Clariva is Pasteuria nishizawae, Avicta is Abamectin, and untreated control is water.

d Total H. glycines = total numbers of soybean cysts, white females, and vermiform stages per 100 cm³ of soil at 60 DAP.

e Mixture 1 = strain Bve2 + strain Bal13; Mixture 2 = Abamectin + strain Bve2 + strain Bal13.

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Table 5. Effect of five PGPR strains and two mixtures of PGPR strains on early plant growth at 60 DAP and yield on soybean at 160 DAP in the microplota.

| Treatment | Scientific Name | PH | 60 DAP | 160 DAP |
|-----------|----------------|----|--------|---------|
|           |                |    | Dunnett's P vs. (P < 0.10) | Dunnett's P vs. (P < 0.10) |
|           |                |    | Clothianidin + B. firmus | Clothianidin + B. firmus | Clothianidin + B. firmus |
|           |                |    | P. nishizawae | Abamectin | Water | P. nishizawae | Abamectin | Water | P. nishizawae | Abamectin | Water |
| Bal13     | B. altitudinis  | 43.8 | 0.0476 | 0.0689 | 0.0938 | 0.0389 | 95.7 | 0.1523 | 0.1388 | 0.4995 | 0.0184 | 192.2 | 1.0000 | 0.9999 | 0.9748 | 0.9974 |
| Bsa27     | B. satellitas  | 41.7 | 0.1396 | 0.1903 | 0.2450 | 0.1176 | 94.7 | 0.1308 | 0.1189 | 0.4508 | 0.0150 | 175.3 | 0.9998 | 1.0000 | 0.7707 | 1.0000 |
| Bsssu2    | B. subtilis subsp. subtilis | 36.1 | 0.9462 | 0.9842 | 0.9965 | 0.9120 | 73.6 | 0.7390 | 0.7027 | 0.9986 | 0.1484 | 193.2 | 0.9996 | 0.9708 | 1.0000 | 0.8941 |
| Bve12     | B. velezensis  | 38.4 | 0.4498 | 0.5745 | 0.6872 | 0.3893 | 66.0 | 0.9773 | 0.9672 | 1.0000 | 0.3954 | 203.0 | 0.9997 | 0.9758 | 0.9999 | 0.9356 |
| Bve2      | B. velezensis  | 36.7 | 0.7664 | 0.8727 | 0.9388 | 0.7015 | 76.7 | 0.7185 | 0.6819 | 0.9979 | 0.1394 | 219.1 | 0.9782 | 0.8365 | 1.0000 | 0.6875 |
| Mixture 1 |                 | 36.8 | 0.3309 | 0.4216 | 0.5098 | 0.2884 | 74.4 | 0.7053 | 0.6742 | 0.9898 | 0.1880 | 156.3 | 0.8853 | 0.9900 | 0.3279 | 0.9944 |
| Mixture 2 |                 | 43.8 | 0.0478 | 0.0691 | 0.0940 | 0.0300 | 88.5 | 0.4328 | 0.4048 | 0.8835 | 0.0812 | 185.4 | 1.0000 | 0.9993 | 0.9925 | 0.9885 |

Microplot trials were performed in 26.5 liter pot. Data collected were repeated and analyzed in SAS 9.4 using PROC GLIMMIX procedure at significant level of $\alpha \leq 0.10$. Adjusted $P$ values less than 0.10 indicated a significant effect. Adjusted $P$ values were analyzed according to Dunnett's method.

aThe LS-means are presented in the tables with adjusted $P$ values for statistical differences.

bPH = plant height (cm) at 60 DAP.

cActive ingredients for the nematicides Poncho/Votivo are Clothianidin plus B. firmus I-1582, Clariva is Pasteuria nishizawae, Avicta is Abamectin, and untreated control is water.

dBio = plant biomass including shoot fresh weight and root fresh weight (g) at 60 DAP.

ey = soybean yield (g) obtained at 160 DAP and adjusted to 13% moisture content per pot.

fMixture 1 = strain Bve2 + strain Bal13; Mixture 2 = Abamectin + strain Bve2 + strain Bal13.

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Table 6. Effects of five PGPR strains and two mixtures of PGPR strains on early soybean plant growth and yield in the field trials.

| Treatment | Scientific Name | Bio | 60 DAP | Cyst | Yield |
|-----------|-----------------|-----|--------|------|-------|
|           |                 |     | Clothianidin | Abamectin | Water | Clothianidin | Abamectin | Water | Clothianidin | Abamectin | Water |
| Bal13     | B. altitudinis   | 70.1| 0.9933 | 1.0000 | 0.9977 | 0.3704 | 41.420 | 0.3705 | 0.997 | 0.4113 | 0.9980 |
| Bsa27     | B. safensis      | 64.9| 0.9970 | 1.0000 | 0.9572 | 1.0000 | 1.0000 | 0.9740 | 0.9972 | 0.0297 | 42.733 | 0.9543 | 0.9998 | 0.9708 | 0.5994 |
| Bssu2     | B. subtilis subsp. subtilis | 66.8| 0.9999 | 1.0000 | 0.9915 | 1.0000 | 1.0000 | 0.7477 | 0.5509 | 43.935 | 1.0000 | 0.7683 | 1.0000 | 0.1419 |
| Bve12     | B. velezensis    | 84.3| 0.9783 | 0.6804 | 0.9992 | 0.7811 | 163 | 0.3678 | 0.9160 | 0.7477 | 0.5509 | 43.935 | 1.0000 | 0.7683 | 1.0000 | 0.1419 |
| Bve2      | B. velezensis    | 78.2| 0.9883 | 0.7810 | 1.0000 | 0.9553 | 118 | 0.9968 | 1.0000 | 1.0000 | 0.0448 | 43.969 | 1.0000 | 0.8815 | 1.0000 | 0.2077 |
| Mixture 1 |                 | 71.8| 1.0000 | 1.0000 | 0.9979 | 1.0000 | 163 | 0.3678 | 0.9160 | 0.7477 | 0.5509 | 43.935 | 1.0000 | 0.7683 | 1.0000 | 0.1419 |
| Mixture 2 |                 | 77.5| 1.0000 | 0.9700 | 1.0000 | 0.9667 | 169 | 0.5460 | 0.9500 | 0.8465 | 0.8607 | 44.667 | 0.9999 | 0.4036 | 0.9996 | 0.0422 |
| Control   | Active ingredient | Poncho/Votivo | Clothianidin + B. firmus | 74.0 | 0.9966 | 1.0000 | 1.0000 | 95 | ... | 0.9816 | 1.0000 | 0.0071 | 44.056 | 0.7082 | 1.0000 | 0.1179 |
|           |                 | Clariva | Pasteuria nishizawae | 64.3 | 0.9955 | 0.9373 | 1.0000 | 125 | 0.9816 | ... | 1.0000 | 0.0700 | 42.899 | 0.7082 | ... | 0.7547 | 0.8979 |
|           |                 | Avicta | Abamectin | 75.1 | 0.9993 | 0.9961 | 151 | 0.9993 | 1.0000 | 0.0330 | 43.963 | 1.0000 | 0.7547 | ... | 0.1360 |
| Untreated control | Water | 68.7 | 1.0000 | 1.0000 | 0.9667 | 222 | 0.0071 | 0.0700 | 0.0330 | 40.555 | 0.1179 | 0.8979 | 0.1360 | ... |

Field trials were performed in E.V Smith and Tennessee Valley Research and Extension Center in 2015. Data collected were repeated and analyzed in SAS 9.4 using PROC GLIMMIX procedure at significant level of 0.10. Adjusted P values less than 0.10 indicated a significant effect. Adjusted P values were obtained by analyzing data according to Dunnett’s method.

*The LS-means are presented in the tables with adjusted P values for statistical differences.

B. firmus I-1582

Active ingredients for the nematicides Poncho/Votivo are Clothianidin plus B. firmus I-1582, Clariva is Pasteuria nishizawae, Avicta is Abamectin, and untreated control is water.

Cyst = soybean cysts and white females in 100 cm³ of soil at 60 DAP.

Yield = soybean yield (kg/ha) obtained at 160 DAP and adjusted to 13% moisture content.

Mixture 1 = strain Bve2 + strain Bal13; Mixture 2 = Abamectin + strain Bve2 + strain Bal13.

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Bacillus subtilis subsp. subtilis strains Bsssu2 and Bsssu3, and B. velezensis strain Bve12 increased early soybean growth including plant height and plant biomass in the greenhouse trials. Bacillus altitudinis strain Bal13 increased early plant growth on soybean in the greenhouse and microplot trials. Mixture 2 (Abamectin + Bve2 + Bal13) increased early plant growth in the microplot trials at 60 DAP, and also enhanced soybean yield at harvest in the field trials. Some studies have reported that individual or mixtures of PGPR strains can promote plant growth and increase yield on multiple plant hosts. Raupach and Kloepper [30] found seven PGPR seed treatments including single-strain treatments and mixtures of B. pumilus strain INR7, Curtobacterium flaccumfaciens strain ME1, and B. subtilis strain GB03 significantly promoted plant growth on cucumber in the field studies when methyl bromide was absent. The individual B. subtilis strain GB03 and mixture of B. pumilus strain INR7 plus C. flaccumfaciens strain ME1 promoted growth significantly on cucumber [30]. Liu et al. [31] found individual PGPR strains Bsa27 (AP7) and Bpu6 (AP18) promoted plant growth on Chinese cabbage and one strain mixture containing PGPR strains Bve2 (AP136) (B. velezensis), Bmo3 (AP209) (B. mojavensis), Lma1 (AP282) (Lysinibacillus macroides), Bve15 (AP305) (B. velezensis), Bsa27 (AP7) (B. safensis), Bpu6 (AP18) (B. pumilus), and Bve40 (AP218) (B. velezensis) increased shoot and root dry weights in the greenhouse test. They found that those individual strains and mixtures increased marketable yield of Chinese cabbage in the field [31]. Our study is in an agreement with previous research that individual or mixtures of PGPR strains can promote plant growth under greenhouse or field conditions and that some PGPR strains can reduce plant-parasitic nematode population density.

Conclusions

Overall, this study indicated that B. velezensis strain Bve2, B. mojavensis strain Bmo3, and Mixture 1 (Bve2 + Bal13) have the potential to manage H. glycines on soybean. These two strains also have been found to reduce the population density of Meloidogyne incognita [29]. Bacillus altitudinis strain Bal13 and Mixture 2 (Abamectin +Bve2 + Bal13) have the ability to enhance soybean yield under field conditions. In the future, the formulation of these effective PGPR strains and mixtures should be further evaluated for the integrated management of H. glycines on soybean.
Supporting information

S1 Table. PGPR isolates effect on *Heterodera glycines* J2 mortality as compared to the industry standard biologicals Poncho/Votivo, Clariva, and chemical Temik as well as an untreated control. *In vitro* tests were performed in 96-well plates. Data collected were analyzed in SAS 9.4 using PROC GLIMMIX procedure at significant level of $\alpha \leq 0.05$. $P$ value less than 0.05 indicate a significant effect. Adjusted $P$ values were obtained according to Dunnett’s method. The LS-means are presented in the tables with adjusted $P$ values to determine statistical differences.

S2 Table. Effect of PGPR strains on soybean plant growth and *Heterodera glycines* population density in the greenhouse trials at 60 DAP.

S3 Table. Effect of five PGPR strains and two mixtures on plant growth and *Heterodera glycines* population density on soybean in microplot trials at 60 DAP.

S4 Table. Effects of five PGPR strains and two mixtures of PGPR strains on soybean plant growth and *Heterodera glycines* population density in the field trials.

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