



## Short Communication

# Coinoculation of soybean plants with *Bradyrhizobium japonicum* and *Trichoderma harzianum*: Coexistence of both microbes and relief of nitrate inhibition of nodulation

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## ABSTRACT

Coinoculation of plants with mixtures of beneficial microbes sometimes produces synergistic effects. In this study, the effect of soybean coinoculation with the N<sub>2</sub>-fixing *Bradyrhizobium japonicum* E109 and the biocontrol fungus *Trichoderma harzianum* Th5cc was analyzed. Nodulation by E109 was not hampered by Th5cc, which antagonized five out of seven soybean pathogens tested. Furthermore, Th5cc relieved nitrate-inhibition of nodulation, enabling the formation of nodules containing infected cells with bacteroids in the presence of the otherwise inhibitory 10 mM KNO<sub>3</sub>. Th5cc released micromolar amounts of auxin, and addition of 11 μM indoleacetic acid to soybean plants inoculated with E109 in the absence of Th5cc also induced nodulation in the presence of 10 mM KNO<sub>3</sub>. Thus, Th5cc may release auxins into the soybean rhizosphere, which hormones might participate in overcoming the nitrate-inhibition of nodulation. Our results suggest that soybean plants coinoculated with these microorganisms might benefit from biocontrol while contributing to soil-nitrogen preservation.

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## 1. Introduction

Food security demands sustainable-food provision for an increasing population, while expansion of cropping lands is becoming constrained. For instance, in Argentina, the third largest soybean producer in the world, the soybean cropped surface expanded at a rate of 825,000 hectares per year between 1998 and 2012 [1] but after this accelerated growth the surface remained constant in around 20 million hectares [2,3], indicating that the soybean agricultural frontier was reached in this country. Therefore, any current increase in production

necessitates a greater productivity per hectare, leading to higher nutrients extraction by plants possibly harming soil fertility and stability. Among the technologies available to mitigate pressure on soil health is the inoculation of pulse crops with symbiotic and plant-growth-promoting microbes (PGPMs), which are intended to diminish the use of fertilizers and pesticides. Soybean plants are regularly inoculated with N<sub>2</sub>-fixing *Bradyrhizobium* spp., but the use of other PGPMs on this crop is only beginning. It was reported that coinoculation of soybean with *Bradyrhizobium* sp. and the arbuscular mycorrhizal fungus *Glomus mosseae* lead to higher plant biomass if the soil is poor in N and P [4]. However, the production of arbuscular mycorrhizal fungi at commercial scale for use in extensive crops still awaits full development. Strains of *Bacillus* spp. were also used for coinoculation with *B. japonicum*, yielding improved nodulation with respect to plants inoculated with *B. japonicum* alone [5,6]. In addition, increases in soybean nodulation, biomass production, and crop yield were observed after coinoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense* [7] or *Streptomyces griseoflavus* [8]. However, there are no reports on coinoculation of soybean with *B. japonicum* and biocontrol fungi.

**Abbreviations:** AG, arabinose-gluconate medium; CFU, colony-forming units; CR, Congo Red; DAI, days after inoculation; IAA, indoleacetic acid; LPCB, lactophenol cotton blue; MFS, modified Fåhræus solution; PDA, potato-dextrose agar; PGPM, plant-growth promoting microbe; YM, yeast-extract mannitol medium; YMA, YM with 1.5 % (w/v) agar.

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Species of the filamentous fungus *Trichoderma* spp. are well-characterized biocontrol agents for several crop plants [9]. This PGPM possesses the advantage that it is easy to cultivate under laboratory conditions and fermentation methods for its scale production have been proposed [10–12]. In particular, *T. harzianum* inhibited 56.3 % of growth of the soybean pathogen *Sclerotinia sclerotiorum* in dual culture tests and contained the disease caused by this pathogen in plants, although effects in enhancing soybean production were not consistently observed [13,14]. Moreover, *T. harzianum* induced resistance to *Fusarium oxysporum* in soybean seedlings [15]. In addition to its biocontrol properties, *T. harzianum* elaborates plant-growth regulators [16]. However, these works did not investigate whether *T. harzianum* may be compatible with *Bradyrhizobium* spp., the main symbiont of soybean. Therefore, in this work we aimed at testing whether *B. japonicum* and *T. harzianum* could coexist in soybean rhizospheres, and if so, whether that coexistence was beneficial.

## 2. Materials and methods

### 2.1. Strains and culture conditions

*B. japonicum* E109, recommended for soybean inoculants in Argentina [17], was grown in yeast-extract-mannitol (YM) [18] or arabinose-gluconate (AG) [19] at 28 °C. When grown in liquid medium, the cultures were agitated by rotary shaking at 180 rpm and the biomass was estimated by optical density at 500 nm. For growth in solid medium, YM was supplemented with 1.5 % (w/v) agar (YMA) and 3.6 µM Congo Red (CR). *T. harzianum* Th5cc, isolated from wheat phyllosphere [20], was grown in potato-dextrose agar (PDA) or *Trichoderma*-selective medium [21], in both cases with 1.5 % (w/v) agar at 28 °C in the dark. Occasionally, this fungus was grown in YM, or in YMA with CR, as indicated.

### 2.2. Biocontrol assays

To test *T. harzianum* Th5cc biocontrol against a known soybean pathogen, two 5-mm-diameter discs were placed, one with the pathogen and the other with *T. harzianum* Th5cc, facing each other in a PDA plate. Then, both fungi were grown for 7 days at 28 °C and the growth assessed by the test-fungal-growth inhibition effected by *T. harzianum* Th5cc on a semiquantitative scale previously described by Bell et al. [22]. This scale classifies the protective capacity according to the following scores. 1: indicates a complete overgrowth of the biocontrol fungus over the pathogen fungus, 2: a growth of the biocontrol fungus over at least two-thirds of the medium surface, 3: a colonization of one-half of the surface by each of the two fungi with neither one dominating the other, 4: a colonization of at least two-thirds of the surface by the pathogen, 5: a complete overgrowth of the pathogen over the biocontrol fungus. Scores ≤ 2 indicate significant antagonism of *T. harzianum* on the pathogen. The pathogens tested were: *Alternaria* spp. D18, *Cercospora kikuchii* D33, *Phomopsis longicolla* DP38, *P. longicolla* DP41, *Rhizoctonia* spp. R24, *Rhizoctonia* spp. RM, and *Sclerotinia sclerotiorum* L50, all of them obtained from the Rizobacter collection.

### 2.3. Plant experiments

Soybean Don Mario 4800 seeds were surface-sterilized and germinated as described [23]. To evaluate nodulation, sets of 10 soybean plants were cultivated in sterile perlite/sand (2:1) and watered with sterile modified Fåhræus solution (MFS) as described [24], with each set being inoculated as follows: 1) with *B. japonicum* E109 grown to the exponential phase in AG broth, 2) with *T. harzianum* Th5cc grown in PDA, 3) with an admixture of

both microorganisms, 4) with sterile MFS. *B. japonicum* was diluted in the MFS directly from AG broths at the desired cell concentrations assessed by counting in a Neubauer chamber. To inoculate *T. harzianum*, conidia were harvested by flooding the PDA cultures with sterile distilled water and then rubbing the culture surface with a sterile glass rod followed by counting in a Neubauer chamber. Plants were cultivated for the indicated days after inoculation (DAI) in a plant-growth chamber at 30 °C/20 °C day/night temperature with a photophase of 16 h. During the cultivation, the plants were watered twice per week with sterile MFS either with or without KNO<sub>3</sub> at the indicated concentrations. All treatments were conducted with ten plants per condition, and each entire experiment was repeated three times.

### 2.4. Auxin determinations

*T. harzianum* Th5cc hyphae were transferred to liquid YM and agitated at 180 rpm for 7 days. Then, the cultures were centrifuged at 13,000 × g for 3 min and the supernatant was used for auxin determination with Salkowski's reagent [25]. Quantification was performed at 535 nm by comparison with a calibration curve constructed with purified indoleacetic acid (IAA, Sigma Chemical Co.) as a standard.

### 2.5. Microscopy

For hyphae observations, mycelium growing in YMA-CR plates either in contact with *B. japonicum* colonies or not, was examined under a Nikon Eclipse E200 microscope at the indicated magnifications. For hyphae staining inside roots, root segments from plants inoculated with *T. harzianum* Th5cc were washed, embedded in 3% (w/v) KOH for 20 min to soften the tissues, and stained with lactophenol cotton blue (LPCB). Then, the roots were observed under a Nikon Eclipse E200 microscope at 40 × magnification in search for endophytic hyphae. For nodules observations, nodules were excised from plants, transversally cut into halves, and fixed in 2% (v/v) glutaraldehyde. Fixed nodules were then dehydrated, infiltrated with epoxy resin, and sectioned. For optical microscopy, glutaraldehyde-fixed 2-µm-thick nodule sections were stained with a saturated solution of toluidine blue and analyzed under a Nikon Eclipse E200 microscope (Melville, NY). For transmission electron microscopy, 70-nm ultrathin sections were stained with uranyl acetate as described [23] and viewed with a JEM 1200 EX (JEOL, Japan Electron Optics Laboratory Co., Ltd.). The electron micrographs were obtained with an ES500W Erlangshen charge-coupled camera device (Gatan Inc., Pleasanton, CA).

## 3. Results and discussion

### 3.1. Absence of interference of *B. japonicum* and *T. harzianum* with one another

We grew *T. harzianum* Th5cc in YMA with CR [18], a medium commonly used for *B. japonicum* cultivation. To check if either of these microbes was antagonistic to the other, four equidistant *B. japonicum* E109 macrocolonies were cultured in a YMA-CR plate for 7 days, and then a 5-mm-diameter *T. harzianum* Th5cc plug was laid in the center of the plate amidst the *B. japonicum* macrocolonies. Four days later the edge of the fungal colony reached the *B. japonicum* macrocolonies, and by 10 days the fungal colony had passed over the bacteria to colonize the entire plate surface (Fig. S1a). Fungal growth rate and conidia formation were not affected by the presence of *B. japonicum* E109 (Fig. S1b). Moreover, there was no evidence of growth inhibition or mycelia damage in the *Bradyrhizobium-Trichoderma* contact zone (Fig. S1c). To verify a

normal rhizobial growth after contact with the fungus, the bacterial macrocolonies were scraped from under the mycelia and streaked in fresh YMA-CR supplemented with 100 mg.l<sup>-1</sup> cycloheximide as a fungicide. Thereafter *B. japonicum* E109 grew normally, indicating that the biocontrol fungus did not antagonize rhizobial replication.

To observe the effects of coinoculation on nodulation and plant growth, sets of 10 soybean plants received the following inoculations: 1) *B. japonicum* E109, 2) *T. harzianum* Th5cc, 3) an admixture of both microorganisms, 4) sterile MFS. Twenty-one DAI, only those plants that had received *B. japonicum* E109 became nodulated. Those plants inoculated *B. japonicum* E109 alone developed 16 ± 4 nodules per plant, while those inoculated with the admixture manifested 12 ± 3 nodules per plant (average ± SD), indicating that *T. harzianum* Th5cc had not precluded nodulation. The nodules from the coinoculated plants had the same ultrastructure as those inoculated with only *B. japonicum* E109 (Fig. 1). In addition, washes from *T. harzianum* Th5cc-infested roots cultivated in *Trichoderma*-selective medium [21] developed  $(0.97 \pm 0.24) \times 10^5$  *T. harzianum* colony-forming units (CFU). g<sup>-1</sup> root fresh weight, whereas washes from roots inoculated with the admixture produced  $(1.24 \pm 0.62) \times 10^5$  *T. harzianum* CFU. g<sup>-1</sup> root fresh weight, indicating that *T. harzianum* Th5cc had colonized the soybean rhizosphere, staying at root surfaces, and *B. japonicum* had not interfered.

To ascertain whether *T. harzianum* Th5cc may be endophytic in soybean roots, LPCB-stained root segments from plants inoculated with *T. harzianum* Th5cc were observed under the microscope. No hyphae with typical conidial-morphologic structure were observed below the root epidermis, indicating that Th5cc underwent no endophytic phase in soybeans under the conditions studied.

### 3.2. Antagonism of soybean pathogenic fungi by *T. harzianum*

To test the capability of *T. harzianum* Th5cc as biocontrol agent, dual cultures with known soybean pathogens were performed. By

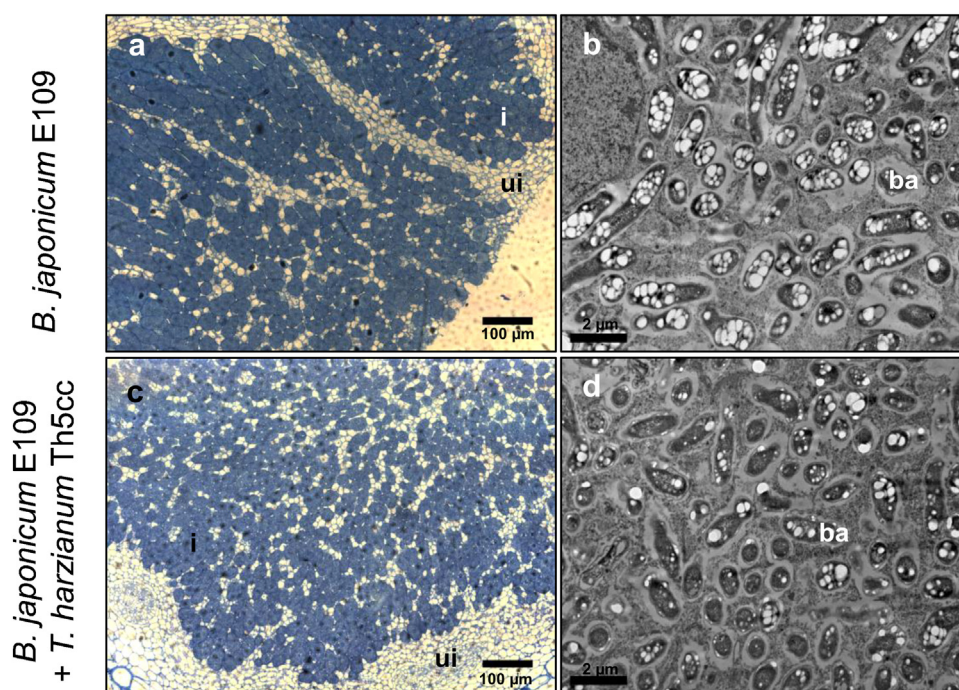
using the Bell's semiquantitative scale [22] it was observed that *T. harzianum* Th5cc inhibited *Cercospora kikuchii* D33 (score 1), *Alternaria* sp. D18, *Phomopsis longicolla* DP38, and *Rhizoctonia* sp. RM and R24 (score 2); whereas the control of *Sclerotinia sclerotiorum* L50 and *P. longicolla* DP41 was weaker (score 3; Fig. S2). According to this semiquantitative scale, antagonism cannot be considered in those interactions with score ≥ 3 [22]. Therefore, *T. harzianum* Th5cc was antagonist against 5 of the 7 pathogen strains tested.

### 3.3. Noninhibition by *T. harzianum* of soybean nodulation by *B. japonicum* in the presence of inhibitory nitrate concentrations

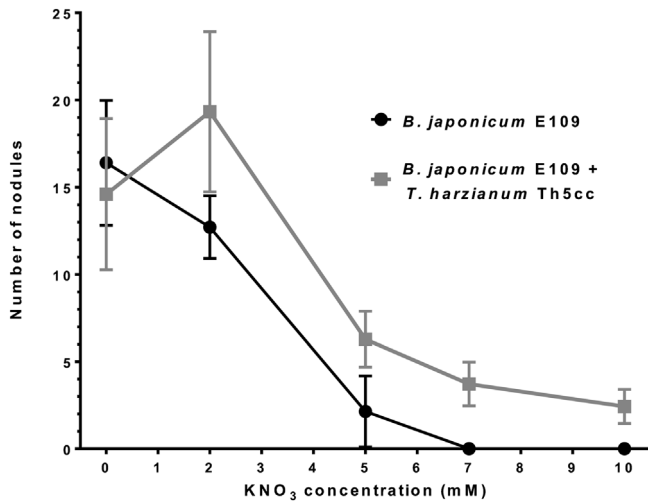
Soybean nodulation is hampered if combined nitrogen sources, and especially nitrate, are available [26]. Furthermore, plants inoculated with *T. harzianum* alone and cultivated without a nitrogen source may be nitrogen-limited, thus masking the fungus's effects on plant growth. Hence, in new experiments we evaluated the response of sets of soybean plants inoculated with or without combinations of *B. japonicum* and *T. harzianum* as described above and watered with MFS containing increasing KNO<sub>3</sub> concentrations. In the absence of *T. harzianum* Th5cc, nodulation steadily decreased until reaching an inhibition above 5 mM KNO<sub>3</sub>. By contrast, in coinoculated plants, the fungus had a positive effect on nodulation at all KNO<sub>3</sub> concentrations tested (Fig. 2). As expected, neither uninoculated controls nor plants inoculated with *T. harzianum* Th5cc alone contained nodules.

The nodules produced in coinoculated plants in the presence of 10 mM KNO<sub>3</sub> were red inside and contained infected cells and normal bacteroids (Fig. 3a–c). Upon comparing these results to the ultrastructure of nodules produced by *B. japonicum* E109 in the absence of KNO<sub>3</sub> (Fig. 1a, b), we concluded that the addition of KNO<sub>3</sub> in the presence of *T. harzianum* Th5cc did not substantially affect nodule development.

Previous results had raised the question as to whether the nodules formed in the presence of KNO<sub>3</sub> contributed to plant



**Fig. 1.** Structure of the soybean nodules produced by *Bradyrhizobium japonicum* E109 alone (a, b) or by the admixture of *B. japonicum* E109 and *Trichoderma harzianum* Th5cc (c, d). Histological cuts (a, c) and ultrastructure (b, d) of nodules formed during 21 DAI in perlite/sand pots watered with MFS. Uninfected cells (ui) are present contiguous to infected cells (i), which contain bacteroids (ba).

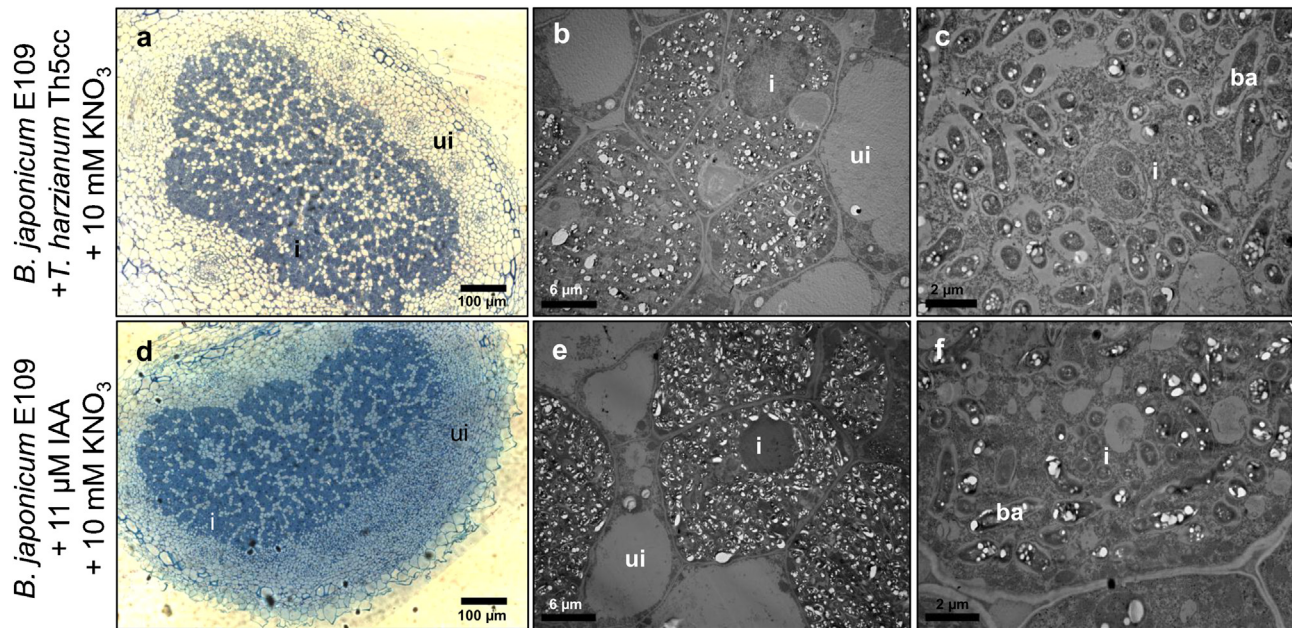


**Fig. 2.** Nodulation of soybeans inoculated with *Bradyrhizobium japonicum* E109 in the exponential growth phase at  $1.10^6$  rhizobia.  $\text{ml}^{-1}$  and *Trichoderma harzianum* Th5cc at  $1.10^7$  conidia.  $\text{ml}^{-1}$ . Nodules produced at 21 DAI (ordinate) by *B. japonicum* E109 alone (black circles) or *B. japonicum* E109 along with *T. harzianum* Th5cc (gray squares) at increasing  $\text{KNO}_3$  concentrations (abscissa). Plants inoculated without *B. japonicum* E109 did not nodulate. Values are averages  $\pm$  standard deviations (error bars).

growth. In response, we compared shoot and root dry weights produced by soybean plants inoculated with *B. japonicum* with or without *T. harzianum* and watered with N-free MFS, or MFS supplemented with 10 mM  $\text{KNO}_3$  during 40 DAI to promote a sufficient dry-mass formation. As shown in Table 1, in the condition without  $\text{KNO}_3$  all plants inoculated with *B. japonicum* E109 nodulated either with or without *T. harzianum* Th5cc, with no significant differences in nodules number between both inoculation treatments. By contrast, in the condition with 10 mM  $\text{KNO}_3$ , only the plants inoculated with the admixture contained nodules. Although shoot and root dry weights were significantly higher in plants grown with 10 mM  $\text{KNO}_3$ , there were no differences within each N-condition among the different inoculation treatments (Table 1), in agreement with previous observations on *T. harzianum* effects on soybean growth [13].

### 3.4. Possible induction of nodulation by auxins in the presence of $\text{KNO}_3$

Auxin transport and accumulation may be related to the response of nodulation to combined nitrogen [27–29]. Since *T. harzianum* may produce auxins [16] we were interested in determining whether the Th5cc strain may produce this plant growth regulator. To this end, we suspended this strain in YM medium, and quantified the auxins released to the culture supernatant. In this way, we detected the presence of ca. 11  $\mu\text{M}$  extracellular auxins in these supernatants, using IAA as standard. To assess whether auxins might be



**Fig. 3.** Structure of the soybean nodules produced at 21 DAI in perlite/sand pots watered with MFS supplemented with 10 mM  $\text{KNO}_3$  throughout the entire experiment. Histological cuts (a,d) or ultrastructure (b, c, e, f) of nodules obtained with *Bradyrhizobium japonicum* E109 coinoculated with *Trichoderma harzianum* (a–c), or *B. japonicum* E109 with 11  $\mu\text{M}$  indoleacetic acid (d–f). Uninfected cells (ui) are present next to bacteroid-containing (ba) infected cells (i).

**Table 1**

Nodulation and dry weight of soybean plants inoculated with *B. japonicum* E109, *T. harzianum* Th5cc or both, in the presence or absence of combined N.

Inoculation	Nodules per plant $\pm$ SD <sup>§</sup>		Shoot dry weight $\pm$ SD <sup>§</sup> (g.plant <sup>-1</sup> )		Root dry weight $\pm$ SD <sup>§</sup> (g.plant <sup>-1</sup> )	
	without $\text{KNO}_3$	with $\text{KNO}_3^*$	without $\text{KNO}_3$	with $\text{KNO}_3^*$	without $\text{KNO}_3$	with $\text{KNO}_3^*$
<b>E109</b>	16 $\pm$ 4 A	0	0.76 $\pm$ 0.14 A	1.56 $\pm$ 0.24 B	0.47 $\pm$ 0.06 A	0.76 $\pm$ 0.10 B
<b>Th5cc</b>	0	0	0.67 $\pm$ 0.07 A	1.32 $\pm$ 0.29 B	0.43 $\pm$ 0.09 A	0.82 $\pm$ 0.14 B
<b>E109 + Th5cc</b>	15 $\pm$ 4 A	3 $\pm$ 1 B	0.73 $\pm$ 0.13 A	1.46 $\pm$ 0.15 B	0.49 $\pm$ 0.06 A	0.88 $\pm$ 0.10 B
<b>Control</b>	0	0	0.69 $\pm$ 0.14 A	1.21 $\pm$ 0.21 B	0.41 $\pm$ 0.06 A	0.68 $\pm$ 0.08 B

<sup>§</sup> SD: Standard deviation. Values followed by different letters within each trait were statistically different with  $p < 0.005$  according to ANOVA followed by Tukey test. Differences among values followed by the same letter within each trait were non significant.

\*  $\text{KNO}_3$  concentration: 10 mM during the whole assay.

responsible for the results observed in Fig. 3a–c and to investigate this issue, soybean plants were inoculated with *B. japonicum* E109 suspended in MFS containing 10 mM KNO<sub>3</sub> and 11 μM IAA. At twenty-one DAI all plants thus inoculated and watered with MFS supplemented with 10 mM KNO<sub>3</sub> formed nodules (Fig. 3d–f). These nodules were histologically and ultrastructurally indistinguishable from the ones obtained from plants inoculated in parallel with *B. japonicum* E109 and *T. harzianum* Th5cc in the presence of 10 mM KNO<sub>3</sub> without IAA (Fig. 3a–c).

The correlation observed between auxin production by *T. harzianum* Th5cc, nodulation by coinoculation in the presence of KNO<sub>3</sub>, or *via* exogenous IAA application suggested that a possible release of auxins by *T. harzianum* Th5cc in the soybean rhizosphere might have induced nodulation by *B. japonicum* E109 in the presence of high KNO<sub>3</sub> concentrations. In contrast, the putative auxin production by *T. harzianum* Th5cc in the soybean rhizosphere had no effect on plant-biomass production under the conditions employed here.

#### 4. Conclusion

Although this work was performed with only one strain from each microorganism, the symbiotic and biocontrol properties observed here were in agreement with those reported previously with other strains [13–15,23]. However, *T. harzianum* Th5cc enabled the production of functional nodules in soybean by *B. japonicum* in the presence of inhibitory nitrate concentrations, which is a synergistic outcome that cannot be predicted from the performance of each microorganism alone. This property might be related to the production of auxins and their release by the fungus. Furthermore, nodulation in the presence of nitrate might be advantageous in preserving soil fertility because those plants coinoculated with both microorganisms might obtain part of their nutrient nitrogen from the atmosphere even in soils relatively rich in nitrogen. In addition, *T. harzianum* Th5cc demonstrated to be antagonist to five soybean pathogens, whereby this coinoculation method may allow benefits from the *T. harzianum*–biocontrol activity. Nevertheless, other strains should be analyzed to optimize the tripartite bacterium–fungus–plant relationship in order to obtain better responses for plant biomass production and crop yield.

#### CRedit authorship contribution statement

**Esteban Tomás Iturralde:** Investigation, Visualization, Formal analysis, Validation, Writing - review & editing. **Marina Celeste Stocco:** Investigation, Writing - review & editing. **Andrés Faura:** Investigation, Resources, Writing - review & editing. **Cecilia Inés Mónaco:** Supervision, Resources, Writing - review & editing. **Cristina Cordo:** Supervision, Resources, Writing - review & editing. **Julieta Pérez-Giménez:** Supervision, Conceptualization, Methodology, Validation, Project administration, Writing - review & editing. **Aníbal Roberto Lodeiro:** Conceptualization, Formal analysis, Validation, Resources, Project administration, Funding acquisition, Writing - original draft, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00461>.

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