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Efficacy of the biocontrol agent *Trichoderma harzianum* ITEM 3636 against peanut smut, an emergent disease caused by *Thecaphora frezii*

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Abstract In this work, a bioformulation containing *Trichoderma harzianum* strain ITEM 3636, an effective biocontrol agent against the peanut pathogen *Fusarium solani*, was evaluated for control of peanut smut, an emergent disease caused by *Thecaphora frezii.* The performance of the bioformulation was evaluated during seasons 2014/2015 and 2015/2016 in experimental fields with history of peanut smut. Inoculation with *T. harzianum* ITEM 3636 significantly reduced the severity of peanut smut during both seasons by 17% and 25%, respectively. This is the first report where a consistent decrease of peanut smut symptoms is achieved in field experiments using a potential biological control agent. The identity of the causal agent of peanut smut was confirmed by sequencing the D1/D2 DNA region. *T. harzianum* ITEM 3636 caused significant increases in grain weight/plant in both years. Peanut smut and brown root rot are diseases that cause severe economic losses. Both causal agents may be present in the soil and, depending on environmental factors, cause disease. The *T. harzianun* ITEM 3636 bioformulation has high potential for controlling both diseases. Thus, the application of a single bioformulation could protect the health of peanut plants against two high impact pathogens.

Keywords *Trichoderma harzianum* strain ITEM 3636 · *Thecaphora frezii* · Peanut seeds inoculation · Peanut smut management · Plant growth promotion

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Peanut (*Arachis hypogaea* L.) is one of South America's originated oleaginous crops with high importance worldwide. Approximately 600,000 tons of peanut and peanut-derived products are exported to 88 countries, with annual incomes of nearly 800 million dollars. During the last years, peanut production has not only been increasing in yield but also in the quality of the harvested product since consumers tend to require high-quality products. Therefore, research and dissemination of technologies constitute essential elements for growing peanuts (Andrés et al. 2016).

Among the diseases affecting peanut plants, Marinelli et al. (2010) reported the presence of peanut smut in samples from the northern-central region of Córdoba, Argentina. The causal fungus, *Thecaphora frezii*, had been identified by Carranza and Lindquist in Carranza and Lindquist (1962) from infected wild peanut (*Arachis* sp.) from Brazil. The production losses

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caused by this disease are highly significant, with reductions of nearly 60% (Marraro Acuña and Murgio 2009). Cultivation practices such as crop rotation and application of fungicides are not sufficient to control peanut smut (Marraro Acuña and Murgio 2009; Oddino et al. 2010). In this context, and in addition to the risk of its spread to other regions, the study of *T. frezii* and the development of management strategies to control peanut smut become highly relevant.

Biological control agents (BCA) represent a sustainable alternative compatible with other strategies for the management and control of plant diseases. Several BCA described as belonging to the species *Trichoderma harzianum* have been proven to be effective in suppressing diseases in different plants and under different conditions (Abeysinghe 2007; Perazzolli et al. 2011; Vitale et al. 2012). Taxonomic studies have shown that the morphologically defined species *T. harzianum* is a species complex (Druzhinina et al. 2010). Chaverri et al. (2015) revised the taxonomy of the *T. harzianum* species complex and evaluated widespread commercial biocontrol products (reported to contain *T. harzianum)*. They found that none of the biocontrol strains were identified as *T. harzianum sensu stricto*, and suggested a more reliable secondary barcode, nuc-translation elongation factor 1α (TEF1), to identify species in this complex.

In previous works, we demonstrated that a formulation containing *T. harzianum* strain ITEM 3636 has biocontrol capacity against the brown root rot of peanut caused by *Fusarium solani*, under greenhouse and field conditions (Rojo et al. 2007). *F. solani* and *T. frezii* can co-exist on peanut and soil and the incidence of one or other disease will depend on climate conditions. Based on this background, and with the perspective of application against other peanut pathogen, the objective of this study was to investigate the effectiveness of this bioformulation to control peanut smut caused by *T. frezii*. As the bioformulation containing *T. harzianum* ITEM 3636 exerted positive effects on plants with an increase in plant growth (biofertilization) and, possibly, with a stimulation of plant-defense mechanisms, we hypothesized that this bioformulation could control an emergent disease such as peanut smut.

Peanut (*Arachis hypogaea* L.) cultivar Granoleico was used in this study. This Argentinian cultivar was released in the year 2002 by the breeding place "El Carmen", General Cabrera, Córdoba, Argentina, and developed by a cross between Tegua and I-JS-95-1 and its subsequent genealogical selection.

T. harzianum ITEM 3636, originally isolated from peanut cropped soil, was deposited at the Istitute Tossine e Micotossine da Parassiti Vegetali, Italy. The isolate was kept in 15% glycerol and frozen at −80 °C. The inocula of *T. harzianum* ITEM 3636 were obtained from 7-day-old cultures on Petri dishes containing malt extract agar (MEA) at 28 °C. The suspensions of conidia were harvested by covering each of 10 plates with 10 ml of sterile distilled water and scraping the surfaces of the cultures with a sterile glass spatula. The inoculum density was adjusted to 5×10^6 conidia/ml by adding sterile distilled water.

T. harzianum ITEM 3636 was tested for its tolerance to Options Advance (NOVA) composed, in g per 100 ml, by: carboxyn (5,6-dihydro-2-methyl-1,4-oxatin-3-carboxylate) 20 g, methyl thiophanate [methyl 4,4′-(o-phenylene) bis (3-thiophanate)] 10 g and metalaxyl [methyl (N-methoxy acetyl)-N-(2,6 xilil)-DL-alaninate] 1,33 g. The methodology used to test the tolerance was described in Rojo *et al*. (2007). Briefly, peanut seeds treated or untreated with the fungicide, at 400 ml/100 kg, were soaked with a mixture of carboxymethyl cellulose (CMC) (2%) and a *Trichoderma* conidial suspension (10^6 conidia/ml), each using a relation of 1 ml per 10 g of seeds. Then, the seeds were air dried in a laminar flow hood. To determine the number of spores present on the seed coat after inoculation, a seed sample was shaken in sterile distilled water and an aliquot of the resulting suspension was plated onto Dichloranchloramphenicol-Rose Bengal agar (DRBC). The count of *Trichoderma* colonies was determined after 5 d incubation at 25 °C under a 12 h light/dark cycle.

Field assays, from November 2014 to April 2015 and from November 2015 to April 2016, were conducted in commercial fields located in the same peanut-producing area (32°45′36.33″ S 63°51′21.35″ W; 32°87′84.23″ S 63°91′52.15″ W) of General Cabrera, province of Córdoba, Argentina. The fields had a long history of peanut smut incidence. Rainfall from seeding to harvest was approximately 640 mm during the 2014/2015 season and 510 mm during the 2015/2016 season. The minimal and maximum temperature values from planting to R5 stage were 6.4 and 39.6 °C for the 2014/2015 season and 8.5 and 37.7 °C for the 2015/2016 season. *T. harzianum* ITEM 3636 was applied by pelleting seeds previously coated with the Options Advance (NOVA)

chemical fungicide and 2% CMC. The treatments were: (1) inoculation: each kg of seeds was pelleted with 100 ml of 2% CMC and 100 ml of the suspension of *T. harzianum* ITEM 3636, and (2) control: each kg of seeds was treated with 100 ml of 2% CMC and 100 ml of sterile distilled water. The sowing was performed during November. Randomized complete block designs, with four replicates, were used. The plots consisted of four rows, each of 10 m long, with an area of 28 m^2 (2.8 m × 10 m). The spacing between rows was 0.7 m, with 0.08 m between plants. The seed rate was of 120 kg/ha, with a depth of 5 cm. Pesticides were applied at recommended doses to avoid the detrimental effects of weeds and pests on plant growth and crop yield. Plant emergence was evaluated 15 days post-seeding to ensure a minimum number of plants per treatment. At 75 days post-seeding, 10 plants per plot were randomly removed to assess total fresh weight, shoot dry weight, root dry weight, shoot length and root length. At harvest (120 days), plants (15–20) from 1 m^2 of each plot were collected to determine weight of grains and the number of grains with confectionery quality (greater than 7.5 mm sieve size).

The disease intensity was assessed at harvest on all of the plants obtained from 1 m^2 of plots. The smut caused by *T. frezii* was quantified through incidence (percentage of diseased pods) and severity of the disease through a 0–4 scale (Marinelli et al. 2008; see Fig. 1). From this classification, the index of disease severity (IDS) was calculated using the following formula:

$$\mathrm{IDS} = [(n \times 0) + (n \times 1) + \cdots + (n \times 4)]\, N^{-1}$$

Where n is the number of pods corresponding to each classification of the disease (0–4) and N is the total number observed.

Fig. 1 Peanut grains showing different values from the scale used to quantify peanut smut. Severity of the disease using a 0–4 scale where: 0 = healthy pod, 1 = normal pod, one seed with a small sorus, 2 = normal or deformed pod, half of one seed affected, 3 = malformed pod and a whole seed affected, and 4 = malformed pod and two seeds affected

Statistical analyzes were performed using InfoStat Professional *V 2009* (National University of Córdoba, Argentina) and Sigma Stat *V 2.03* for Windows (SPSS Inc.). All of the data were evaluated by one-way analysis of variance (ANOVA). In all cases, the residuals were tested for normality using the Holm Sidak test. Data from grain weight/plant from the 2014/2015 field assay and from disease incidence during the 2015/2016 field assay were analyzed for significance after square root transformations.

Scientific efforts for several decades have been focused on developing alternatives to chemical pesticides for managing plant diseases. A long worldwide effort has been concentrated on BCA (Tjamos et al. 2010). Our primary goal is to contribute to a safe, sustainable, and optimal peanut production. Considering this, we evaluated the effectiveness of a bioformulation developed for peanut brown root rot against the emergent disease known as peanut smut. First, experiments were conducted to evaluate the possibility of combining *T. harzianum* ITEM 3636 with a fungicide commonly used on peanut seeds thus inferring their compatibility to develop an advantageous integrated management of soilborne plant pathogens. Seeds treated with the fungicide resulted in an effective inoculum (CFU/seed), not different from that obtained from untreated seeds. The recovery of inoculum from seeds was 2.2×10^4 and 2.3×10^4 CFU/seed, without and with the fungicide, respectively. In addition, the identity of our strain was confirmed using sequence analysis of the 1α (TEF1) and calmodulin (CAL) genes. The primers EF1-728F/EF1-986R and CAL-228F/CAL-737R were used for the amplification of the 1α (TEF1) and CAL sequences, respectively (Carbone and Kohn 1999). The PCR products were sent to Macrogen (Korea) for sequencing. The DNA sequences were deposited in the GenBank (accession numbers KY595072 and KY595073).

Next, the performance of the bioformulation was evaluated during seasons 2014/2015 and 2015/2016 in experimental fields with history of peanut smut. Due to the large number of reports on the promotion of germination caused by *Trichoderma* spp. in several crops, we decided to evaluate the number of emerged inoculated plants. For this, the number of emerged or germinated plants in the different treatments was assessed 15 days after planting. The average values obtained from both harvests were not significantly different. The mean

increase in seedling emergence caused by inoculation with *T. harzianum* ITEM 3636 was 26.7% as compared to control plants ($P \leq 0.05$).

The following growth parameters were evaluated 75 days after planting: plant fresh weight, shoot dry weight, root dry weight, shoot length and root length. The plants that emerged from seeds inoculated with *T. harzianum* ITEM 3636 during the first year showed an increase in root dry weight and shoot length, as compared to control plants. However, differences were not statistically significant ($P \leq 0.05$). Notably, inoculation of *T. harzianum* ITEM 3636 significantly enhanced shoot length of peanut plants during the second year of experimentation. On the other hand, the obtained values of plant fresh weight, shoot and root dry weight and root length from both treatments were not significantly different after 75 days of experimentation in both fields ($P \leq 0.05$). The best criterion for treatment efficacy in a study of biocontrol under field conditions is to evaluate the crop yield at harvest. Therefore, we proceeded to determine the parameters of crop yield at harvest (120 days after planting). Regarding the inoculation with *T. harzianum* ITEM 3636, significant increases in grain weight/plant were observed, as compared to the controls, in both years. The maximum increase was recorded in the first year of experimentation, where the recorded increase was of 12.9 g per plant (29%) on average (Table 1). On the other hand, plants that emerged from seeds inoculated with *T. harzianum* ITEM 3636 also showed increases in the number of confectionery quality grains, as compared to the controls, the recorded increase during the first year was of 12 confectionery quality grains per plant, or 24% and of 0.57 confectionery quality grains per plant (3.5%) during the second year (Table 1). However, differences with control plants were only significant during the 2014/2015 season. Increases in quality of grains are very important for their effective marketing. On the whole, there were year-to-year variations in the evaluated parameters.

The contribution of *T. harzianum* for the biocontrol of plant diseases was amply demonstrated (Montealegre et al. 2010; Srivastava et al. 2010; Yang et al. 2011). *T. harzianum* ITEM 3636 was observed to be effective in decreasing the mean severity index of brown root rot in peanut plants, increasing the frequency of healthy plants, and boosting yield. The potential of using this strain to control the disease and the plant growth-promoting effect was confirmed in three experiments carried out in fields artificially and naturally infested with *F. solani* (Rojo et al. 2007). In this work, the bioformulation containing *T. harzianum* ITEM 3636 was effective in controlling *T. frezii* during both years in naturally contaminated fields. The incidence of peanut smut was recorded at 120 days after planting. We recorded mean values of 20.51% and 13.65% of affected pods in control treatments during the first and second year, respectively. Inoculation with *T. harzianum* ITEM 3636 consistently reduced the incidence of peanut smut by 3% during both seasons (Fig. 2). The severity of peanut smut was also recorded at 120 days after planting. Based on the above-mentioned scale we show the different levels of severity observed in our assays in Fig. 1. The severity of the disease in the fields was shown to be below the scale of 1 for the control treatments from both years. Inoculation with *T. harzianum* ITEM 3636 reduced the severity of peanut smut by 17% (severity = 0.54) as compared to the control

Table 1 Yield parameters in the field assays

Treatment	Harvest 2015		Harvest 2016	
	Grain weight/plant (g)	Number of confectionery quality grains/plant	Grain weight/plant (g)	Number of confectionery quality grains/plant
Control	43,85 ± (22,7) b	49,24 ± (27,2) b	28,04 ± (4,2) b	32,28 ± (4,9) a
T. harzianum ITEM 3636	56,74 ± (26,9) a	61,17 ± (28,9) a	29,58 ± (4,8) a	33.42 ± (4,2) a

Each value represents the mean from 4 plots

The standard deviations are shown between parentheses

Different letters in each column indicate statistically significant differences ($P \leq 0.05$)

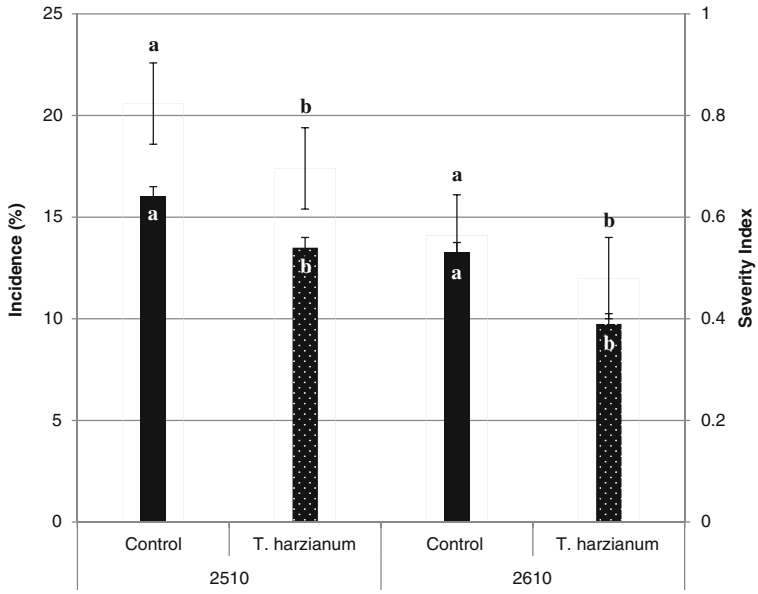

Fig. 2 Effect of seed treatment with a bioformulation containing *T. harzianum* ITEM 3636 on incidence (□) and severity (■) of peanut smut in naturally infested plots. Data were recorded in field assays during two years (2015 and 2016). Different letters on each parameter from each year indicate statistically significant differences ($P \leq 0.05$)

(severity = 0.65) during the first year and 25% (severity = 0.39) as compared to the control (severity = 0.52) during the second year (Fig. 2). This is the first report where a decrease of peanut smut's symptoms is repeatedly achieved in the field using a biological control agent.

As shown, the crop yield was higher during the first year of experimentation. The comparison between the two harvest seasons showed significant differences ($P \leq 0.05$) in grain weight/plant (g) and number of confectionery quality grains/plant (data not shown). This difference could be explained by differences in the amount of rainfall and the temperature during the two cultivation periods evaluated. The rainfall values registered from seeding to harvest were 640 mm and 510 mm for the 2014/15 and 2015/16 harvest season, respectively. Thus, during 2014/15, the peanut growth area had the best rain conditions of the last twenty years. Soil temperature is also important in determining peanut yield as the pod growth occurs in the ground. Plant dry weight depends, among other factors, on air and/or soil temperature. Pod yield is usually correlated positively with total dry matter accumulation and, therefore, any effect of temperature on total dry matter accumulation will affect pod yield (Prasad et al. 2009). The temperature for peanut growth was optimal during the 2014/15 harvest season. The highest temperatures exceeded by far the historical average, and a similar event took place with the minimal temperatures (the lowest in the last years) registered. Consequently, there was an important and beneficial daily thermal amplitude.

In addition, we confirmed the identity of peanut smut's causal agent, *T. frezii*, by amplification and sequencing of the D1/D2 region from gDNA samples using the primers D1/D2-NL1 and D1/D2-NL4 (O'Donnell 1993), which allow amplification of a fragment of about 650 bp, corresponding to a partial region of the large ribosomal subunit (28S rDNA; data not shown). The obtained sequences were deposited in the GenBank (NCBI) under the accession numbers KX944749 and KX944750.

To conclude, *T. harzianum* ITEM 3636 showed ability to control peanut smut by *T. frezii* and has the potential to exert beneficial effects on peanut plants, increasing the crop yield. Further assays are needed to thoroughly evaluate the performance of strain ITEM 3636 under different soil and climate conditions, to assess its biocontrol capacity against different peanut pathogens and to determine the impact of the formulation containing *T. harzianum* ITEM 3636 on members of the soil microbiota. In Argentina, brown root rot and peanut smut are diseases that cause severe economic losses. Both causal agents, *F. solani* and *T. frezii*, may be present in the soil and, depending on environmental factors, cause disease. *T. harzianun* ITEM 3636 is a microbial agent with high potential for controlling both diseases. Thus, the application of a single bioformulation could

protect the health of peanut plants against two high impact pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal subjects This article does not contain any studies with human or animal subjects.

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