Scientific Paper

# *Effect of the inoculation with* Bradyrhizobium *sp. and* Trichoderma harzianum *in triticale (X.* Triticosecale Wittmack*), under drought stress conditions*

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**ABSTRACT:** At the Pastures and Forages Research Station Sancti Spíritus, a field trial was conducted under drought stress conditions, to evaluate the effect of the inoculation with a strain of *Bradyrhizobium* sp. and the fungus *Trichoderma harzianum*, in triticale (*X. Triticosecale Wittmack*). The experimental design was completely randomized, with four replications and seven treatments. A control fertilized with $NH_4NO_3$ (150 kg N/ha) was used, as a well as an absolute control and five inoculated treatments. The variables aerial dry weight, root dry weight, stem length, grain yield, weight of a thousand seeds and number of grains per ear were evaluated. The simple application of *Bradyrhizobium* or in combination with *Trichoderma*, in general, exerted a positive effect on the plants, and was higher than the application of chemical fertilizer. It was also observed, that the development of the aerial part and the grain yield were not related to the application of mineral nitrogen, but to the inoculation. It is recommended to conduct similar experiments on different soil types in the Sancti Spíritus province, under drought stress conditions.

Key words: microorganisms, yield, *Rhizobium*

## INTRODUCTION

Soil microorganisms contribute with a wide range of essential services to the sustainability of the dynamics of all ecosystems. They act as the main driving agents of the nutrient cycle; regulate the dynamics of the organic matter of the soil, carbon sequestration and emission of greenhouse gases; modify the soil physical structure and the water regime; and improve the efficiency in the nutrient uptake by the plants (Singh *et al*., 2011).

It is known that the inoculation of cereals with plant growth promoting microorganisms leads to plant and yield growth (Andrews *et al*., 2003). There are antecedents of the positive effect of rhizobia and other rhizobacteria in cereals belonging to the *Triticum* genus (Bécquer *et al*., 2012a; Bécquer *et al*., 2012b; Bécquer *et al*., 2012c).

On the other hand, it is known that the hydric stress limits crop growth and productivity, especially in arid and semiarid areas (Yang *et al*., 2008; Farooq *et al*., 2009). Drought is considered one of the highest natural disasters of the world, the most frequent and persistent, the one with the highest negative effect on agricultural production, and also the cause of real adverse impacts on the environment (World Meteorological Organization, 1994).

There are diverse ways to fight drought, and among the most novel ones is the use of microbial inoculants (Tikhonovich and Provorov, 2011). It is known that the positive effect of soil microorganisms is not only related to their influence on growth, because they also constitute a relevant factor in the tolerance of plants to the abiotic stress, such as the one caused by drought. Among those rhizobacteria, rhizobia occupy an important place (Uchiumi *et al*., 2004; Stiens *et al*., 2006).

Another microorganism that influences positively plant development is the filamentous fungus *Trichoderma* sp. The direct mechanisms related to its protective effect include competition, antibiosis and mycoparasitism (Howell, 1998). More recent studies indicate the induction of defense mechanisms in the plants by *Trichoderma*, as well as its plant growth promoting activity (Saber *et al*., 2009; Shaban and El-Bramawy, 2011). There are antecedents of the positive effect of *Trichoderma harzianum* in wheat, when combined with rhizobia (Bécquer *et al*., 2015).

For such reason, the objective of this study was to evaluate the effect of the inoculation of *Bradyrhizobium* and *Trichoderma* in triticale (*X. Triticosecale Wittmack*), variety INCA TT-77 at different moments, and under agricultural drought stress conditions.

## MATERIALS AND METHODS

**Location.** The trial was conducted from January, 2014, to April, 2014, in an experimental plot belonging to the Pastures and Forages Research Station Sancti Spíritus, located at 21º 53' 00" North latitude and 79º 21' 25" West longitude, and an altitude of 40 m.a.s.l.

### Provenance of the strains and their identification

***Rhizobium* strain**. The strain JJ6, belonging to the genus *Bradyrhizobium* sp. (Bécquer *et al*., 2002) was used, which is a microsymbiont of *Centrosema virginianum* (naturalized legume from Sancti Spíritus, Cuba).

***Trichoderma* strain.** The product TRICHOSAVE 34 (of LABIOFAM S.A.) was used, composed by a substratum of rice hulls and broken rice inoculated with sporulated mycelia of *T. harzianum* A-34.

**Plant material.** The cereal triticale, from the National Institute of Agricultural Sciences (INCA for its initials in Spanish) –Mayabeque, Cuba–, was evaluated. This cereal has antecedents of high yields in the Sancti Spíritus province when inoculated with rhizobia (Bécquer *et al*., 2012b), and constitutes an alternative for animal feeding in Cuba.

### Preparation of the inoculants

***Bradyrhizobium***. The strains grew on solid yeast-mannitol medium (Vincent, 1970), and they were re-suspended in liquid yeast-mannitol medium until achieving a cell concentration of $10^6$-$10^8$ CFU/mL. For the inoculation of the plants, the inoculant was diluted in proportion 1:10 in saline solution at 0,9 %.

***Trichoderma***. Following the technical recommendation of the manufacturer, tap water was added to the above-mentioned product at a rate of 35 g/L, and it was filtered with gauze before inoculating the plants.

### Inoculation of the plants

**With *Bradyrhizobium***. The inoculation was performed upon the seed germination (six days after sowing); for such purpose a graduated burette was used, whose content was poured on the newly-germinated plants, and, by regulating the jet, each plant received approximately 8-10 mL of the liquid inoculant. The re-inoculation was carried out 15 days after sowing, in an equal way, with a bacterial inoculant of the same titer.

**With *Trichoderma***. The inoculation was made upon the seed germination (six days after sowing), with a dose equivalent to 250 L/ha of solution, through the same procedure as the one used for *Bradyrhizobium*. The re-inoculation of the treatments was made 15 days after sowing, with an inoculant of the same titer.

**Fractionated inoculation**. Fifteen days after the initial microorganism inoculant was applied, according to the treatment, the fractionated inoculation was performed

**Soil preparation, sowing, irrigation**. Conventional cultivation activities were carried out: plowing, harrowing, crossing, re-crossing, harrowing and furrowing. The sowing of the experiment was made in the second ten-day period of January, with spaced drilling and a sowing dose of 60 kg/ha. The sowing frame was 50 cm between furrows. The seed was manually harvested 105 days after sowing, in the last ten days of April. Each plot measured 2 m x 4 m.

Irrigation was applied three times, by sprinkling: immediately after sowing, at the beginning of tillering and when the flag leaf was visible. Although the norm established by the Institute of Fundamental Research on Tropical Agriculture (INIFAT, 2003) is 350 $m^3$/ha, in this trial only 30 % of it was applied, in order to favor only the survival of rhizospheric microorganisms.

**Climate variables in the area.** The temperature, rainfall and relative humidity data were provided by the Meteorological Station Sancti Spíritus, belonging to the Provincial Meteorological Center.

**Rainfall**. Rainfall behaved irregularly. In November, 2013, was similar to the historical value (54,9 mm), while in December (7,0 mm), January (64,3 mm) and March (45,9 mm) it was lower. In these last two months important phenological stages of the experimental crop occurred (from germination to grain filling), which coincided with the lower occurrence of rainfall in that period. The historical averages were surpassed only in February (67,7 mm) and April (102,6 mm).

**Temperature and relative humidity**. The period was characterized by the predominance of high temperature and relative humidity (table 1).

Table 1. Temperature and humidity data in the experimental area. Year 2014.

| Month | Mean temperature (ºC) | Average relative humidity (%) |
|---|---|---|
| January | 22,2 | 76,2 |
| February | 23,9 | 77,6 |
| March | 24,1 | 71,0 |
| April | 25,3 | 71,7 |

**Determination of the status of agricultural drought.** The status of agricultural drought was determined through the Index of Aridity or Index of Agricultural Drought (IE), with the formula proposed by Solano and Vázquez (1999), to test whether the trial was conducted under hydric stress conditions:

IE = RET / PET

Where:

RET (or E): estimated real evapotranspiration, depending on the humidity status of the soil.

PET (or Eo): estimated potential evapotranspiration, depending on the atmospheric conditions.

If RET = PET, the supply of water to the soil is adequate. When RET < PET, there is water insufficiency (in this case, IE can be insufficient, critical or very critical).

The month of January ended with a critical to insufficient IE which varied in February and March to regular (Centro Meteorológico Provincial Sancti Spíritus. 2014).

**Basic agrochemical composition of the experimental soil**. The soil of the experimental area corresponds to the Brown type with carbonate differentiation, from brown to slightly dark brown clay, with slight reaction to HCl; it shows some gravel in horizon A1, good surface and internal drainage, and is moderately erodible (Hernández *et al*., 1999). It is also characterized by a macronutrient content low in phosphorus and potassium (2,63 mg/100 g of $P_2O_5$; 6,0 mg/100 g of $K_2O$), as well as 1,51 % of organic matter and pH 5,9. Due to the poor nutrient content in the soil, deep fertilization was carried out (N: 9, P: 13, K: 17) at 21 days in all the treatments, with doses of 80 kg N/ha.

**Experimental design and statistical analysis**. A randomized block experimental design was used, with seven treatments and three replications: *Trichoderma* + *Bradyrhizobium* sp. at 15 days (Trich. + JJ6 15 d.), *Bradyrhizobium* sp. + *Trichoderma* at 15 days (JJ6 + Trich.15d.), *Bradyrhizobium* sp. + *Trichoderma* at the moment of sowing (JJ6 + Trich.), *Trichoderma* at the moment of sowing (Trich.), *Bradyrhizobium* sp. at the moment of sowing (JJ6), absolute control (AC) and fertilized control (FC), which consisted in a 300 kg/ha application of nitrogen ($NH_4NO_3$).

The data were statistically processed, according to the variance analysis (ANOVA) (StatGraphics Plus, v. 5.1, 1994-2001, Statistical Graphics Corporation). The differences among means were determined by Fisher's LSD (Least Significant Difference) test ($p < 0,05$).

**Measured variables:**

- Aerial dry weight (ADW, g/m$^2$).
- Root dry weight (RDW, g).
- Stem length (SL, cm).
- Grain yield (GY, kg/ha, extrapolated).
- Number of grains per ear (No. G/ear)
- Weight of a thousand seeds (W 1 000 seeds, g).

The data of the variable with digit count (number of grains per ear) were transformed by $\sqrt{x}$ (Lerch, 1977).

## RESULTS AND DISCUSSION

The positive effect of the combined inoculation of beneficial microorganisms on triticale was proven, under agricultural drought conditions, in a season with complex characteristics that damaged the good plant growth.

With regards to the variable aerial dry weight, it was observed that the treatment in which the *Bradyrhizobium* and *Trichoderma* strain were combined at the moment of sowing (table 2) showed statistically higher values than the other treatments (613,67 g/m$^2$), and even than the fertilized control, which indicates that both microorganisms acted synergically on this productive indicator. The other treatments, except *Trichoderma* (412,67 g/m$^2$) and the fertilized control (440,67 g/m$^2$), had common superscripts with the absolute control.

Considering the drought stress to which the crop was subject, it is deduced that *Bradyrhizobium*, belonging to the group of rhizobacteria with properties of induction of systemic tolerance to environmental stress, produced cytokinins which counteracted the negative effect of the abscisic acid in the leaves, response of the plant to this type of stress (Yang *et al*., 2008).

Ahmad *et al*. (2008) also found that 80 % of the di-nitrogen fixing bacteria produce indole-acetic acid. This growth substance leads to the increase of total phenols, calcium content and the activity of the polyphenol oxidase enzyme, which protects the plant against pathogens and improves its growth through the elimination of oxygen reactive species, which are formed in the plant from a hydric stress (Chowdhury, 2003; Yang *et al*., 2008). In this sense, Vanderlinde *et al*. (2010) stated that rhizobia are likely to produce antioxidants (catalase), exopolysaccharides and other substances to be able to survive in extreme environments, especially under drought conditions. In this regard, it is known that *Rhizobium sullae* strains, isolated in the semiarid region of Tunisia and moderately tolerant to drought, significantly increased the dry aerial biomass of plants inoculated under this type of environmental stress (Fitouri *et al*., 2012).

The strain JJ6 which was used in this experiment was previously selected due to its positive effect on *Triticum secale* (Bécquer *et al*., 2006) and on *Zea mays* (Bécquer *et al*., 2008).

The simple application of *Trichoderma* also exerted a positive effect on the aerial dry weight, although the values of this treatment were lower than the one reached with the combination of the fungus with *Bradyrhizobium*. Harman *et al*. (2004) stated that the production of organic acids by *Trichoderma* favors the solubilization of phosphates, micronutrients and mineral cations, such as iron, manganese and magnesium.

Regarding the root dry weight (table 2), treatments JJ6 + Trich. (11,0 g) and JJ6 (10,2 g) were statistically equal to the fertilized control, while JJ6 + Trich. exceeded the absolute control, *Trichoderma*, JJ6 + Trich. 15d. and Trich + JJ6 15d. The simple application of *Trichoderma* produced the lowest value (7,47 g) with equal superscripts to the absolute control.

It is evident that the combination of the *Bradyrhizobium* strain with the *Trichoderma* strain did not generate antagonism between these two microorganisms, because the treatment that combined them, as well as the simple application of *Bradyrhizobium* sp., exerted a positive effect on root development. It is possible that *Trichoderma*, by using its cellulolytic degradation mechanisms on the host roots (Haram *et al*., 1996), allowed the entrance of the bacteria that were jointly inoculated. With regards to this topic, Yanni *et al*. (2001), Perrine *et al*. (2004) and Saritha Kumari *et al*. (2009) sustain that the growth promoting molecules, such as indole acetic acid, gibberellins and cytockinins produced by the rhizobia present in the rhizosphere or in the plant tissues, stimulate the higher root development and increase the nutrient absorption capacity in benefit of the plant.

In the stem length (table 2) statistical superiority of JJ6 (59,00 cm) was found with regards to the absolute control, Trich + JJ6 15d., JJ6 +Trich. 15d. and *Trichoderma*; although the first did not differ from JJ6 + Trich. (55,16 cm) or from the fertilized control. In this variable, just like in the previous one, the simple

Table 2. Performance of triticale with the different combinations of microorganisms.

| Treatment | Variable | | |
|---|---|---|---|
| | ADW ($g/m^2$) | RDW (g) | SL (cm) |
| Trich. + JJ6 15d. | 287,33$^{c}$ | 8,60$^{b}$ | 49,42$^{b}$ |
| JJ6 + Trich. 15d. | 222,63$^{c}$ | 9,07$^{b}$ | 48,75$^{b}$ |
| JJ6 + Trich. | 613,67$^{a}$ | 11,0$^{a}$ | 55,16$^{ab}$ |
| Trich. | 412,67$^{b}$ | 7,47$^{c}$ | 41,50$^{c}$ |
| JJ6 | 284,60$^{c}$ | 10,2$^{ab}$ | 59,00$^{a}$ |
| AC | 250,43$^{c}$ | 5,07$^{c}$ | 39,37$^{c}$ |
| FC | 440,67$^{b}$ | 9,07$^{ab}$ | 53,77$^{ab}$ |
| SE ± | 22,13 | 1,066 | 2,500 |

a, b, c: values with different superscripts differ at $p < 0,05$.

inoculation of *Bradyrhizobium*, as well as the combined inoculation of the bacteria with *Trichoderma* at the moment of sowing, had a higher effect than that of the absolute control, the other fractionated treatments that were applied, and the simple inoculation of *Trichoderma*.

In general, it was proven that the three studied variables increased their values when subject to the treatments of simple application of *Bradyrhizobium* and its combination with *Trichoderma*, for which the production of phytohormones by the rhizobium had incidence on the root system, and also through this system, it could influence positively the variables of the aerial part development, such as dry weight and stem length. Yanni *et al*. (2001) and Rosenblueth and Martínez-Romero (2006) considered that the rhizobia and other microorganisms can penetrate the roots of the non-leguminous species through the cracks, or through the lateral root emergence sites, and become established in the xylem and the intercellular spaces of the plants; for which the action range of the metabolites emitted by the bacteria can reach far from the root system.

From all the variables that were evaluated in this experiment, the grain yield, the weight of a thousand seeds and the number of grains per ear showed the final result of all the processes that occur in the plant, and in general, in the soil-microorganism-plant system.

Regarding the grain yield (table 3), treatments JJ6 + Trich. (815,3 kg/ha) and JJ6 (815,3 kg/ha) were statistically higher than the others, and even than the fertilized control.

Saleem *et al*. (2007) and Van Loon (2007) reported that many rhizobacteria contain the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which splits the precursor of ACC, ethylene, into α-ketobutyrate and ammonia, and reduces the ethylene levels in the plants subject to stress. This allows the root system grow without the inhibition caused by such compound, which propitiates a higher absorption of nutrients by the plant and a higher grain production.

The inoculation of the crop did not propitiate a high grain yield (between 380,0 and 815,0 kg/ha), but it was interesting that the treatment with application of 300 kg N/ha did not exceed JJ6 + Trich. or JJ6. This indicates that they exerted a positive effect on the plant in spite of the drought stress, and that they constitute a better choice than the application of chemical fertilizers under these conditions. On the other hand, the yield of the fertilized control was lower than the one obtained by Baset Mia and Shamsuddin (2010) and Bécquer *et al*. (2012b) under more favorable climate conditions.

In the weight of a thousand seeds (table 3), JJ6 + Trich. (31,33 g) showed common superscripts with the fertilized control and it was higher than the other treatments, with the exception of JJ6. In this variable, the simple treatment with *Bradyrhizobium* was significantly lower than the fertilized control, but higher than the absolute control and one of the fractionated inoculation treatments. This proved that the grain quality was also influenced by the action of these biofertilization variants, through the nutrient absorption mechanisms and the stimulating action on the root, by the plant growth promoting rhizobacteria (Saritha Kumari *et al*., 2009); as well as through the presence of rhizospheric bacteria in different parts of the plant, with their subsequent positive effect on it (Yanni *et al*., 2001; Rosenblueth and Martínez-Romero, 2006).

In the number of grains per ear (table 3) there were three treatments which did not differ from the fertilized control: JJ6 + Trich. (7,07 g), *Trichoderma* (6,72 g) and JJ6 (6,91 g), and that turned out to be statistically higher than the absolute control, Trich + JJ6 15d. and JJ6 + Trich. 15d. Like in the aerial dry weight, the positive effect of *Trichoderma* on this variable was observed.

Avis *et al*. (2008) and Shoresh *et al*. (2010) reported the biofertilizer properties of *Trichoderma*, based on the increase of the absorption of minerals and their solubilization, as well as on the production of plant growth stimulating substances. These last authors state that the above-mentioned capacity of *Trichoderma* and other biocontrolling microorganisms is a consequence of their abilities to reprogram the genetic expression of plants, probably through the activation of a limited number of metabolic pathways. Mogle and Mane (2010) reported that in tomato seeds treated with a mixture of *Trichoderma* and *Rhizobium* the incidence of pathogen fungi was reduced and germination increased. The *Trichoderma* strain which was inoculated in this trial was previously used in *Vigna luteola*, and its combination with a *Bradyrhizobium* sp. strain also showed a positive effect (Bécquer *et al*., 2004).

The fact that the simple application of *Trichoderma* had a positive effect on the aerial dry weight and the number of grains per ear confirms to a certain extent the plant growth stimulating properties of this fungus, which could be used in agricultural practice, previously

Table 3. Performance of triticale with the different combinations of microorganisms.

| Treatment | Variable | | | |
|---|---|---|---|---|
| | GY, kg/ha (extrapolated data) | Weight of a thousand seeds (g) | No. G/ear | |
| | | | Transformed data √x | Original data |
| Trich + JJ6 15d. | 380,0[c] | 21,50[d] | 5,83[b] | 34 |
| JJ6 + Trich. 15d. | 404,0[c] | 23,33[cd] | 5,88[b] | 35 |
| JJ6 + Trich. | 815,3[a] | 31,33[ab] | 7,07[a] | 50 |
| Trich. | 427,0[c] | 25,67[c] | 6,72[a] | 45 |
| JJ6 | 815,3[a] | 30,33[b] | 6,91[a] | 48 |
| AC | 376,3[c] | 24,50[cd] | 4,81[c] | 23 |
| FT | 558,0[b] | 34,00[a] | 6,5[ab] | 42 |
| SE± | 3,38843 | 1,03126 | 0,28566 | |

a, b, c, d: values with different superscripts differ at $p < 0,05$.

tested in future experiments with other crops and in different edaphoclimatic environments.

It is concluded that the simple application of *Bradyrhizobium*, or in combination with *Trichoderma*, exerted a better effect on the plants than the application of chemical fertilizer. It was also observed that the development of the aerial part and the grain yield were not linked to the application of mineral nitrogen, but to the inoculation.

It is recommended to conduct similar experiments on different soil types under drought stress conditions, in the Sancti Spíritus province.

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Received: January 21, 2015
Accepted: October 20, 2015