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EVALUATION OF ANTIFUNGAL ACTIVITY OF *Trichoderma asperellum* Samuels AGAINST FUNGAL PATHOGENS THAT AFFECT SOYBEAN (*Glycine max* L.) CROP

Evaluación de la actividad antifúngica de *Trichoderma asperellum* Samuels ante patógenos fúngicos que afectan al cultivo de la soya (*Glycine max* L.)

Ariel Cruz Triana[1]✉, Deyanira Rivero González[1], Benedicto Martínez Coca[2], Anayza Echevarría Hernández[1] and Aida Tania Rodríguez[1]

ABSTRACT. Among the factors that adversely affect crop yield of soybean (*Glycine max* L.) the incidence of pests and more specifically pests caused by fungi stand out. Numerous fungicides in increasing doses are used in the phytosanitary control of these agents, and in most cases the desired control is not achieved, a situation that increases production costs and significantly reduces farmer benefits. Based on these antecedents, the objective was to evaluate fungal diseases of highest incidence in soybean crop and antifungal activity of *Trichoderma asperellum* Samuels. The experiment was carried out at the Basic Scientific and Technological Unit "Los Palacios". The *in vitro* antagonism of *T. asperellum* strains by the method of dual culture in Petri dishes was assessed. Besides, the distribution and the attack index of soybean crop key diseases under field conditions when the crop was treated with the Ta. 13 strain of *T. asperellum* were evaluated. Generally, *T. asperellum* showed a high potential for the biological control of evaluated diseases, significantly reducing its distribution and infection percentage and it provoked that the number of pods per plant were higher.

Key words: biological control, *Cercospora kikuchii*, *Fusarium* sp., *Phakopsora pachyrhizi*

RESUMEN. Entre los factores que inciden negativamente en el rendimiento del cultivo de la soya (*Glycine max* L.) se destaca la incidencia de numerosas plagas y en específico las causadas por hongos. En el control fitosanitario de estos agentes se emplean numerosos fungicidas en dosis cada vez mayores, y en la mayoría de los casos no se logra el control deseado, situación que incrementa los *costos* de producción y reduce significativamente los beneficios de los productores. Sobre la base de estos antecedentes, el objetivo del trabajo fue evaluar las enfermedades fúngicas de mayor incidencia en el cultivo de la soya, así como la actividad antifúngica de *Trichoderma asperellum* Samuels. El experimento se desarrolló en la Unidad Científica Tecnológica de Base "Los Palacios". Se evaluó el antagonismo *in vitro* de cepas de *T. asperellum* por el método de cultivo dual en placas de Petri. Además se evaluó la distribución e índice de ataque de enfermedades claves del cultivo de la soya en condiciones de campo al ser tratado con la cepa de *T. asperellum* Ta. 13. De manera general *T. asperellum* mostró un elevado potencial para el control biológico de las enfermedades evaluadas, disminuyendo significativamente su distribución y porcentaje de infección, e incidió en que el número de vainas por plantas fuera superior.

Palabras clave: control biológico, *Cercospora kikuchii, Fusarium* sp., *Phakopsora pachyrhizi*

INTRODUCTION

Soybeans (*Glycine max* L.) is one of the most important oilseeds in the world (1,2). Soybean is the main crop in Argentina due to its adaptation to soils, the incorporation of technology with the use of direct sowing and the price of the international market.

[1]Unidad Científico Tecnológica de Base "Los Palacios", Km 1 ½ carretera La Francia, Los Palacios, Pinar del Río, Cuba
[2]Grupo de Fitopatología Protección de plantas. Centro Nacional de Sanidad Agropecuaria (CENSA), Carretera Jamaica Km 3,5 San José de las Lajas, Mayabeque, Cuba
✉ actriana@inca.edu.cu

The physical, chemical and biological properties of each soil are modified by the type of tillage system used. The environment generated by tillage alters the growth and functional balance of arable crops. In 2006, an essay was installed in the Ezeiza Party (Pampa Ondulada, both for the volumes marketed as seed, and for the important byproducts obtained, which are part of a long series of agroindustrial chains. , its grains contain around 35 % of proteins and possess almost all the essential amino acids. The world production of soy has exceeded 250 million tons, distributed mainly between the United States (45 %) and Brazil (26 %), and followed by Argentina, China, India, Paraguay and Canada (3).

In Cuba it is imported from Brazil, Argentina and Asian countries, which makes it necessary to allocate large resources to acquire grain, which is an important component in the intensive production of poultry and pig meat, production of milk, yogurt, oil and other food (4). Based on this premise, in the last five years there was a notable increase in the areas for the production of soybean and the technification of the crop in the country.

The edaphoclimatic conditions in Cuba are favorable for the development of soybean cultivation, reaching yields higher than 2 t ha^{-1}. However, high temperatures and relative humidity are conducive to the proliferation of numerous pests, and damage caused by fungi. Diseases manifest themselves at any stage of plant development or simply during their entire cycle; reducing the production of soybeans, affecting the physical, physiological, nutritional and commercial quality of the product, both in commercial grain and seed, causing annual losses of around 10 % of production.

Fungal soil diseases are difficult to control and seed treatment does not achieve crop protection for long periods of time (5). The repeated application of chemical fungicides against these pathogens has favored the appearance of resistant strains and imbalances in the soil microbiota, which decrease the antagonistic activity of beneficial microorganisms that are present in the soil (5). Taking these aspects into account, the demands for alternatives to control diseases become more important. In this sense, the application of *Trichoderma* spp. in different plant-pathogen interactions it has demonstrated its biological feasibility globally (6). This antagonistic fungus is a natural inhabitant of the soil that has excellent qualities for the biological control of fungal pathogens and has different mechanisms, through which it exerts its action, emphasizing among them, the microbial competition acting as colonizer of the roots and not leaving ecological niche to other phytopathogenic fungi (6). They also produce metabolite that favor health, root mass, and consequently yields.

On the basis of these premises, it is proposed that products based on *Trichoderma* spp. they constitute a new ecological alternative to the use of chemical products in agriculture.

The objective of this work is to evaluate the antifungal activity of *Trichoderma asperellum* against pathogens that affect the cultivation of soybean (*Glycine max* L.).

MATERIALS AND METHODS

Determine the pests of fungal origin of greater incidence in the soybean cultivation

The experiment was developed in the Base Scientific Science Unit “Los Palacios”, Pinar del Río, Cuba; belonging to the National Institute of Agricultural Sciences (INCA), during the rainy season of 2014, in a system of flat terraces and rice soil classified as Hydroelectric Gley Nodular Petroferric (7).

An area of 250 m^2 was planted with the Vietnamese cultivar DT-20, following the norms described in the technical guide for the production of soybeans, with the variant that no chemical fungicide was applied (8).

The present fungal diseases and their distribution were evaluated with a frequency of 15 days; for this purpose, 100 cross-diagonal plants were counted, and the percentage of plants with symptoms of diseases was determined. The determination of the causal agents of the diseases was based on the comparison between the presented symptomatology and the bibliography (8). Stereo observations were also made (Novel Model with 100x magnification) and an optical microscope (Model Novel N-800M, with an increase of 400x), with the support of morphological identification keys for pathogens (9). The formula used in the field evaluation is as follows:

$$P= (a/N)\ 100$$

a.- Number of diseased plants or organs
N.- Total plants in the sample

In vitro antagonism of *T. asperellum* isolates against the most widely distributed fungal species under field conditions

The antagonistic effect of strains *Ta*.3, *Ta*.13, *Ta*.17, and *Ta*.85 of +- against the fungus of greater distribution under field conditions was evaluated.

For this, the dual culture method was used from cultures of the fungus seeded in Potato-Dextrose-Agar medium (BIOCEN) at pH 5.5, incubated at 26 °C and dark for seven days. The isolates of *T. asperellum* used in this trial were isolated and characterized (10), and they are conserved in the Plant Mycology Laboratory of the National Center for Agricultural Health (CENSA).

Discs of 0,6 cm in diameter of the antagonist and of the pathogen, diametrically opposed, were seeded on Petri dishes of 9 cm in diameter with PDA medium. They underwent similar incubation conditions for 11 days of observation. A control of the pathogen was used, incubated under similar conditions. Five replicates were used per treatment.

The competition for substrate was evaluated. For this, the radius of the colonies was measured at 16, 24, 40, 48, 64, 72, 64, 72, 88, 96, 184, 208 and 232 hours, by using a graduated ruler (mm) and determined the degree of antagonism according to Scale described by Bell and collaborators (11):
1-Trichoderma sp. It grew completely on the culture medium and on the pathogenic organism.
2- *Trichoderma* sp. It grew completely on the culture medium and on the pathogenic organism.
3- *Trichoderma* sp. reaches 2/3 of the surface of the culture medium.
4- *Trichoderma* sp. and the pathogen have colonized approximately half the surface of the medium and none of them dominates over the other.
5- The pathogenic organism colonizes at least 2/3 of the surface of the medium.
6- The pathogenic organism grows above *Trichoderma* sp. and occupies the surface of the medium.

At 64 hours, the percentage of inhibition of radial growth of the pathogen (PICR) was determined according to the formula:

$$ICR = \frac{r_1 - r_2}{r_1} * 100$$

where:
r_1: radial growth of the control colony.
r_2: radial growth of the isolation facing the antagonist.

Effectiveness of *T. asperellum* against the fungi of greater incidence in the cultivation of soybean under field conditions

The experiment was developed in the Los Palacios Base Scientific and Technological Unit. Planting was carried out on the furrow ridge, and soybeans were used, cultivate Vietnamese DT-20, at a planting density of 160,000 plants per hectare and the ridge distance of 0,70 m, following the guidelines described in the guide technique for the cultivation of soybean (8).

The *T. asperellum Ta*.13 strain was used and the applications were carried out with a backpack, in the morning hours. Soil treatment was applied five days before sowing, in young plants it was applied at the base of the plant and the last treatment was carried out at the beginning of flowering, on the foliage of the plants. In all the variants, the dose of *T. asperellum* was 1 kg ha^{-1} at a concentration of 10^9 conidia g^{-1}.

Plots of 30 m^2 (5x6) were made in a randomized block design, with three repetitions for each treatment, with the following variants:
1- Control treatment of pathogens with application of chemical fungicides (C + Q)
2- Control treatment without chemical application (C-Q)
3- Treatment of *T. asperellum* to the soil (*Ta*. 13 + S)
4- Treatment of *T. asperellum* to the soil and stem of young plants (*Ta*.13 + S + T)
5- Treatment of *T. asperellum* to the soil, to the stem of young plants and before flowering (*Ta*.13 + S + T + F)

In the control treatment of pathogens, Celest Top products were used at a dose of 3,0 ml kg^{-1} of seed and a pre-flowering application with Amistar 250 SC at a dose of 0,3 L ha^{-1}.

The present symptoms were assessed bimonthly and 50 plants were sampled following the methodology of the English flag (12). Subsequently, the distribution of the disease and its incidence was determined.

The percentage of infection was calculated using the evaluation scales and the Townsend and Heuberger formula (13).

$$I = [\sum (a.b)/NK] \, 100$$

I.- Infection percentage.
[∑ (a.b).- Sum of the products of the number of plants (a) by their corresponding grade (b).
N.- Total number of plants observed.
K.- Greater degree of scale.

Soil disease assessment scale (14):

- No visible symptoms of the disease.
- Light discoloration, without necrotic lesions or with 10% of the hypocotyl and root tissues covered with lesions.
- Approximately 25 % of the hypocotyl and root tissues are covered with lesions, with strong discoloration, although the tissues are firm.
- Approximately 50 % of the hypocotyl and root tissues are covered with lesions that combine with softening, decay and considerable reduction of the root system.
- Approximately 75 % or more of the hypocotyl and root tissues are affected by advanced stages of decay, in combination with a severe reduction of the root system.

Depending on the area of the air organ of the affected plant, the following scale was developed:

0- Apparently healthy leaves
1- Some spots
2- Up to 10 % of the surface of an organ with symptoms.
3- Attack of 11 to 25 % of the surface of an organ with symptom.
4- Attacked from 26 to 50 % of the surface of an organ with symptoms.
5- More than 50 % of the surface of an organ with symptoms.

30 plants were selected at random for each treatment and the number of pods per plants and the mass of 100 grains was determined.

With the distribution data, percentage of infection and yield, a simple variance analysis was performed for each disease and performance variable evaluated. The statistical package STATGRAPHYCS version 5.1 (15) was used. The means were doubled by Duncan's multiple range test with a significance level of p≤0.05 (16).

RESULTS AND DISCUSSION

Evaluation of the fungal pests with the highest incidence in the cultivation of soybeans

The fungi that affected under the experimental conditions evaluated were *Fusarium* sp., *Cercospora kikuchii* (Matsumoto & Tomoyasu) MW Gardner and *Phakopsora pachyrhizi* Sydow & P Sydow and more notably *Fusarium* sp., with a distribution superior to 3 % regardless of the moment of the evaluation (Table I). It was observed that the distribution of *C. kikuchii* and *P. pachyrhizi* increased over time which corroborates that they are diseases of higher incidence in the final stages of crop development. At 52 days there was a significantly greater distribution of *C. kikuchii* with respect to *P. pachyrhizi* and at 66 days an inverse behavior was manifested. The times of greatest incidence of the diseases under study agree with those described by other authors under similar experimental conditions (17).

Table I. Distribution (%) of the main diseases present in soybeans

Momento ddg	*Fusarium* sp.	*C. kikuchii*	*P. pachyrhizi*	ESx̄
21	3,21 a	0,0 b	0 b	0,234
52	3,018 a	1,3 b	0,2 c	0,275
66	3,64 a	2,9 a	3,51 a	0,110

Different letters differ statistically according to the Duncan Multiple Range test (p≤0,05)

In vitro antagonism of *T. asperellum* isolates against the main fungal agent of soy

Competition for the substrate

Ta.13 and *Ta*.85 isolates of *T. asperellum* were located in degree 1 of the Bell Scale, compared to the evaluated pathogen, while the *Ta*.3 and *Ta.* 17 showed degree 2 of antagonism (Table II).

This shows a high antagonistic capacity of them, even when the latter do not grow completely on the pathogen. This gives them an important competitive advantage over space with respect to phytopathogenic fungi, even before showing their mycotoxin arsenal and mycoparasitic activity (18).

Table II. Antagonism of the isolates of *T. asperellum* on *Fusarium* sp., according to the Bell scale, 10 days after the culture

Hongos patógenos	Aislamientos de *T. asperellum*			
	Ta. 3	Ta. 13	Ta. 17	Ta. 85
Fusarium sp.	2*	1	2	1

* Degrees of the Bell Scale *et al*.

This is of greater importance since the *Trichoderma* strains colonize the soil quickly, because they are naturally resistant to many toxic chemical compounds, including herbicides, fungicides, pesticides and phenolic compounds, and even more so if we take into account that many of these are strong opportunistic invaders and producers of powerful antibiotics (19).

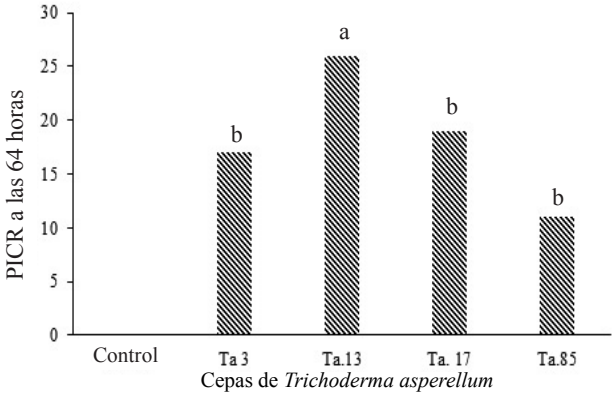
Ta.3, *Ta.*13, *Ta*.17 and *Ta*.85 isolates of *T. asperellum* show an inhibitory effect of radial growth with respect to the control, at 64 hours, which varies from 11 to 26 % with respect to the control.

Isolation *Ta*.13 was the one with the highest PICR against the pathogen (26 %), with significant differences with respect to the other strains evaluated, so from this analysis it can be concluded that the best isolation in terms of the PICR against the pathogen it was *Ta.* 13 (Figure).

The PICR values of the *Ta* 13 strain were similar to those obtained by other authors when evaluating the antibiosis of *Trichoderma* spp. isolates in front of *Botrytis cinerea* in Mora and *Rhizoctonia* sp. (18,20).

The mycoparasitic process is complex and may involve the chemotrophic growth of *Trichoderma* towards the host stimulated by molecules derived from it, such as amino acids and sugars; the recognition mediated by lectins; the formation of hooks or appressoria-like structures that contain high

concentrations of osmotically active solutes such as glycerol and that facilitate penetration; the secretion of extracellular hydrolytic enzymes; and finally the penetration and death of the host. The antibiotic capacity of the genus *Trichoderma* is mainly due to the production of antibiotics as peptaibol, trichoziannins A1 and B1. By producing these metabolites *Trichoderma* sp. they manage to inhibit the germination of the spores and the elongation of hyphae of phytopathogenic fungi. On the basis of the foregoing, it is reasonable to think that the efficacy of the strain *Ta*13, could be due to the fact that it produces high concentrations of these metabolites (18).



Different letters differ statistically according to the Duncan Multiple Range test (p≤0,05)

Figure. PICR of *Fusarium* sp. in dual culture with *T. asperellum* isolates (ESx = 0,21)

EFFECTIVENESS OF *T. ASPERELLUM* AGAINST PHYTOPATHOGENIC FUNGI *FUSARIUM* SP., *C. KIKUCHII* AND *P. PACHYRHIZI* UNDER FIELD CONDITIONS

Regardless of the disease evaluated, the distribution of the diseases in the treatments with *T. asperellum* was significantly lower than in the treatment without chemical application, except the three treatments with *T. asperellum* against the fungus *P. pachyrhizi*, where no differences were observed. This could be due to the fact that in treatment three only the application of *T. asperellum* was applied to the soil and this pathogen affects the aerial parts and in the final stages of crop development (21), so the action of *T. asperellum* was limited (Table III).

The distribution of *Fusarium* sp., in all treatments with *T. asperellum* was significantly lower compared to the control without application, so it is not justified to perform the three applications for the control of this disease.

With respect to chemical treatment, there was less distribution of the diseases *C. kikuchii* and *P. pachyrhizi* when the three applications with *T. asperellum* were made.

When evaluating the infection percentage, a tendency similar to the distribution was observed; however, to reduce the affectations of the fungi *C. kikuchii* and *P. pachyrhizi*, at least two applications with the biocontroller were necessary (Table IV).

Table III. Distribution percentage of *Fusarium* sp., *Cercospora kikuchii* and of *Phakopsora pachyrhizi* at 66 days after the germination of soybeans

Tratamiento	*Fusarium* sp.	*C. kikuchii*	*P. pachyrhizi*
1.Control químico	0,1 c	11,13 c	12,21 b
2.Control sin aplicación	20,62 a	15,46 a	17,92 a
3.T. asperellum+suelo	3,2 b	13,6 b	17,2 a
4.T. asperellum+suelo+tallo	3,8 b	13,78 b	7,86 c
5.T. asperellum+suelo+ tallo+ floración	2,8 b	10,14 d	8,01 c
ESẋ	0,049	0,032	0,028

Different letters differ statistically according to the Duncan Multiple Range test (p≤0,05)

Table IV. *Fusarium* sp., *Cercospora kikuchii* and *Phakopsora pachyrhizi* infection percentage in Soy

Tratamiento	*Fusarium* sp.	*C. kikuchii*	*P. pachyrhizi*
1.Control químico	8,33 b	18,63 d	7,21 c
2.Control sin aplicación	11,67 a	25,61 a	15,35 a
3.T. asperellum + suelo	6,27 c	27,07 a	16,6 a
4.T. asperellum + suelo + tallo	7,04 bc	13,50 bc	10,03 b
5.T. asperellum + suelo + tallo + floración	7,03 bc	11,42 cd	10,1 b
ESẋ	0,091	0,143	0,134

Different letters differ statistically according to the Duncan Multiple Range test (p≤0.05)

It should be noted that from the first application of *T. asperellum*, both the distribution and the percentage of *Fusarium* infection was significantly lower with respect to the control without application. This could be due to the fact that both share the same habitat and favor the early activation of mechanisms of action such as competition for the substrate, antibiosis and mycoparasitism of *Trichoderma* on the pathogen (5).

The treatments with the biological control had a greater number of pods with respect to the treatment without application; however, no significant differences were observed between the treatments with respect to the weight of the grains (Table V). The differences in the number of pods per plant, could be due to *Trichoderma* promotes plant growth. According to Viterbo (22) *Trichoderma* enhances seminal germination, radical growth and development, nutrient intake and use, resistance to abiotic stress, more abundant and early flowering, an increase in height and weight of plants, including an increase in the returns. These processes are mediated by the synthesis or stimulation of phytohormone production by the plant due to the interaction with some strains of *Trichoderma*, such as molecules similar to cytokinins (zeatin) and gibberellin GA3; as well as by the acidification of the surrounding environment due to the excretion of organic acids: gluconic, citric and fumaric, which allow the solubilization of phosphates, micronutrients and trace minerals (iron, manganese and magnesium) (19,23).

Table V. Performance variables in the DT.20 soy bean cultivar

Tratamientos	No. Vainas/ Planta	Masa 100 granos (g)
1- C+Q	147,05 a	16,37 ns
2- C-Q	128,6 c	16,19 ns
3- Ta.13+Suelo	139,03b	16,34 ns
4- Ta. 13+Suelo+Tallo	143,45 b	16,37 ns
5- Ta.13+Suelo+Tallo+Flor	148,20 a	16,40 ns

Letras diferentes difieren estadísticamente según prueba de Rangos Múltiple de Duncan (p≤0,05)

CONCLUSIONS

The *Ta* 13 strain of the species *Trichoderma asperellum* has biocontrol potential due to the inhibition and antagonism on *Fusarium* sp. *in vitro* and on *Fusarium* sp., *C. kikuchii* and *P. pachyrhizi* under field conditions. It also stimulates the plant growth of soy by increasing the number of pods per plant.

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