



EFFECTIVITY OF STRAINS *Azotobacter* sp. AND *Bacillus* SP. FOR A CONTROL THE FUNGI SPECIES ASSOCIATED WITH VEGETABLE

Efectividad de cepas de *Azotobacter* sp. y *Bacillus* sp. para el control de especies fúngicas asociadas a hortalizas

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ABSTRACT. The genus *Azotobacter* and *Bacillus* have a potential to atmospheric nitrogen fixation, mineral elements solubilization and producing a group of stimulator of vegetal growth substances. In addition, *Bacillus* is recognizing by its antagonistic activity. The reasons justify its selection with actives principles of biofertilizer products. The presence of fungus diseases in vegetable cultures is a problem for Cuban agriculture. The objective of the present work was to evaluate the antagonistic activity of *Azotobacter* and *Bacillus* strains against fungus that caused diseases in vegetables cultures. For those were employed *Fusarium chlamyosporum*, *Corynespora cassiicola* and *Cladosporium oxysporum*. All strains belong at the INIFAT collections. For the development of work, “Dual Culture Bring” Method was used with permitting the selections those to possess previous activity and descript, in turn, principle affectations caused to fungi structures. Results emit that inside of two genus exist strains that inhibit the micelial growth. Among strains of *Azotobacter*, five result promissory against *Cladosporium oxysporum*, two respond to *Fusarium chlamyosporum* and only one was effective against *Corynespora cassiicola*. The activity shows for *Bacillus* genus is major. In this case, two strains show activity against *Corynespora cassiicola*, six against *Cladosporium oxysporum* and eight against *Fusarium chlamyosporum*. Highlight the finished that exists strains of *Azotobacter*sp capable of inhibit more than one fungi species, which results very new for being quoted few times the genus activity against vegetable pathogens.

RESUMEN. Los géneros *Azotobacter* y *Bacillus* tienen la potencialidad de fijar nitrógeno atmosférico, solubilizar elementos minerales y producir un grupo de sustancias estimuladoras del crecimiento vegetal. *Bacillus* se reconoce, además, por su actividad antagonista. Estas razones justifican su selección como principios activos de productos biofertilizantes. La presencia de enfermedades causadas por hongos en los cultivos hortícolas, constituye un problema en la agricultura cubana. El objetivo del presente trabajo fue evaluar la actividad antagonista de cepas de los géneros *Azotobacter* y *Bacillus* contra hongos que causan enfermedades a cultivos hortícolas. Para ello se emplearon las especies *Fusarium chlamyosporum*, *Corynespora cassiicola* y *Cladosporium oxysporum*. Todas las cepas pertenecen a las colecciones del INIFAT. Para desarrollar este trabajo se utilizó el Método de “Enfrentamiento de Cultivos Duales”, que permitió seleccionar aquellas que poseen dicha actividad y describir, a la vez, las principales afectaciones que provocan a las estructuras fúngicas. Los resultados arrojaron que dentro de los dos géneros hay cepas que logran inhibir el crecimiento micelial. Dentro de las cepas de *Azotobacter* cinco resultaron promisorias contra *Cladosporium oxysporum*, dos responden frente a *Fusarium chlamyosporum* y una sola resultó efectiva contra *Corynespora cassiicola*. La actividad mostrada por el género *Bacillus* fue mayor. En este caso, dos cepas muestran efectividad contra *Corynespora cassiicola*; seis contra *Cladosporium oxysporum* y ocho contra *Fusarium chlamyosporum*. Se comprobó que existen cepas de *Azotobacter* capaces de inhibir a más de una especie fúngica, lo que resulta novedoso por encontrarse poco citada la actividad del género contra patógenos de hortalizas.

Key words: antagonistic bacterias, biological control, fungus diseases

Palabras clave: bacterias antagonistas, control biológico, enfermedades fúngicas

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INTRODUCTION

Plant growth promoting rhizobacteria (PGPRs) are a heterogeneous group of microorganisms that inhabit the rhizosphere, on the root surface or in

association with them and exert beneficial effects on plants through direct mechanisms and indirect (1).

Direct mechanisms include those that influence the metabolism of crops, through the incorporation and mobilization of nutrients essential for their growth and development. These include the biological fixation of atmospheric nitrogen, the solubilization of nutrients and the production of physiologically active substances. On the other hand, the indirect mechanisms provide protection to plants against the attack of phytopathogenic organisms. These include antibiotic production, induction of systemic resistance, and competition for nutrients or ecological niche (2, 3).

Azotobacter and *Bacillus* are recognized as plant growth promoting bacteria. The microorganisms belonging to the genus *Azotobacter* are associative bacteria of free life. Among its potentialities are the biological fixation of atmospheric nitrogen and the production of phytohormones. *Bacillus* is noted for its great diversity, its resistance to adverse environmental conditions (4, 5) and the production of a wide range of compounds with fungicidal or fungistatic activity (6).

Diseases caused by phytopathogenic fungi cause significant yield losses under different production systems. In Cuba, epizootics are common, because climatic conditions are favorable for the reproduction of multiple causative agents of diseases. The three fungal genera used in this study are included among those that cause severe affections and two of them are considered to be emerging under protected cultivation, due to yield losses that have originated on tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annum* L.) in tropical regions (7).

The objective of this work was to evaluate the antagonistic activity of *Azotobacter* and *Bacillus* strains against plant pathogenic fungi from horticultural crops.

MATERIALS AND METHODS

The research was carried out in the Laboratory of Biofertilizers, belonging to the Agrobiotechnology Group, the Institute of Fundamental Investigations in Tropical Agriculture "Alejandro de Humboldt" (INIFAT) and in the laboratory of production of biological means of the National Institute of Plant Health (INISAV).

For the study bacterial strains conserved in the INIFAT Beneficial Bacteria Collection were used. Phytopathogenic fungi come from the Fungi Collection of the entity itself (recognized

as the 853 of the World Federation of Crop Collections, WFCC according to its acronym in English).

The antagonistic activity was evaluated against the fungi *Cladosporium oxysporum* (strain 2133); *Corynespora cassiicola* and *Fusarium chlamydosporum* (2022). The method of "Clash of Dual Cultures" (8) was used on Petri plates 90 mm in diameter. The method consisted in sowing a streak of the bacteria of interest 15 mm from the edge of the plate and at the other end, a pre-culture of the phytopathogenic fungi evaluated in the form of a disc of 7 mm diameter. After performing both plantings the plates were incubated at 28 °C for seven days for *C. oxysporum* and *F. chlamydosporum* and 15 days for *C. cassiicola*. The pre-culture of the fungi was carried out for a period of seven days under similar incubation conditions.

For the fungi *C. oxysporum* and *C. cassiicola* were used solid medium of Potato-Dextrose-Agar (PDA) and for *Fusarium chlamydosporum* the medium Agar-Oat^A. The trials counted on so many treatments (strains) were evaluated and with ten replicates for each treatment. The evaluations were performed at the end of the test in a qualitative way, by describing the effects caused to the fungal colonies. The percentage of mycelial inhibition was also calculated with the use of the following formula.

$$\text{Mycelial inhibition (\%)} = \frac{dc - dt}{dc} \times 100$$

where: dc = control diameter and dt = treatment diameter

Statistical processing of data was performed with STATGRAPH Plus version 5.0 (9) program, According to the Kolmogorov-Smirnov, Cochran C, Hartley and Bartlett tests, normality and homogeneity were verified. When there were significant differences among treatments, the means were compared according to Duncan's test at 5 % significance.

RESULTS AND DISCUSSION

C. oxysporum CKr. It is a fungus that has not been widely used for the development of in vitro works, as it was not urgent to find biocontrol agents, since it is not a potential hazard for crops of agricultural interest. However, the fact of being considered in recent years as an emerging disease on tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annum* L.) crops in crop

^A Centro Nacional de Biopreparados. *Catálogo de Medios de Cultivo* [en línea]. BIOCEN, 2013, 44 p. [Consultado: 23 de enero de 2015]. Disponible en: <<http://www.biocen.cu>>.

systems protected, promotes the search for biological alternatives for its control.

The presence of the genus *Fusarium* has been demonstrated in grasses and Fabaceae (10, 11), in numerous vegetables, among which tomatoes and peppers, as well as in fruit trees (7, 12); Also attacks ornamental plant species (13). Representatives of the genus *Fusarium* cause severe diseases in all these crops, since they are associated with symptoms such as vascular wilt and belongs to the damping-off fungi complex (7). y pertenece al complejo de hongos causantes del damping-off (7).

Likewise, *C. cassiicola* (Berk & Curt) Wei, affects large areas of tomato under protected cultivation in the world, as it induces the appearance of "Tomato Leaf Blight". In Cuba, in recent years it has become an emerging disease for this production system, due to the favorable conditions of humidity and temperature that prevail in it.

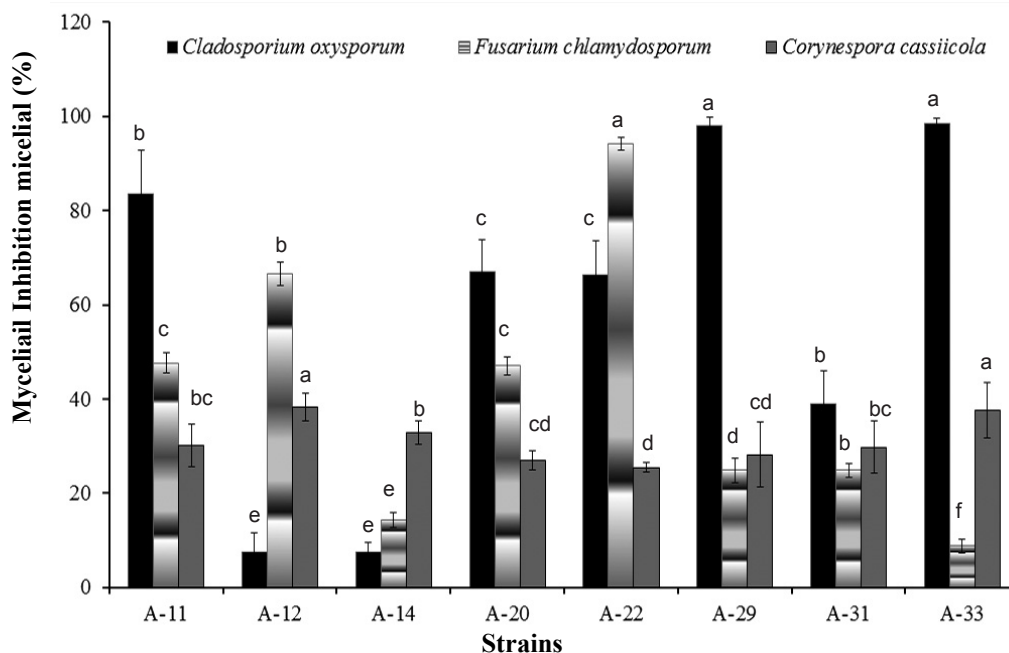
The antagonist activity of the eight strains of *Azotobacter* evaluated against *C. oxysporum* (2133), showed that only two of them (A-12 and A-14) did not show any type of activity against the pathogen. These allowed the contact with the fungus, which also manifested an abundant process of conidium genesis and did not provoke inhibition of its growth. In some replicates both microorganisms were mixed without any damage to fungal structures being observed. Another showed a medium activity (A-31) as it deformed the mycelium towards the edges and caused rupture of hyphae, but did not limit conidium genesis or severely inhibit fungal growth.

Three of the remaining five strains (A-11, A-20 and A-22) did not allow contact with the fungus, partially inhibited the conidium genesis process and caused mycelial rupture in some areas. However, the most notable were A-29 and A-33, because they completely repressed the conidium genesis, allowed a poor extension of the mycelium, from the disk that served as inoculum and produced swelling of the fungal colony, attached to Strong color changes in the culture medium and in the colony itself.

These manifestations are related to the production of metabolites with antifungal characteristics. Swelling of fungal colonies and changes in coloration demonstrate the presence of compounds with antibiotic characteristics or the production of hydrolytic and chitin lytic enzymes that minimize the growth potential of fungi by influencing their reproductive capacity (14).

The inhibition behavior of the mycelial growth caused by the different strains is shown in Figure 1, where it is observed that the trend corresponds to the damage caused to the pathogen. A-29 and A-33 strains stand out that do not show significant differences between them and reach values higher than 98 % of growth decrease. These differ significantly from all other microorganisms used in the study.

With regard to *F. chlamydosporum*, two strains showed no activity (A-14 and A-29) and did allow contact with the fungus without causing damage to the mycelial fungal structure or impeding the development of the conidium genesis process; In addition, both strains achieved percent inhibition of less than 25 % (Figure 1).



Equal letters do not differ significantly from each other for $p \leq 0.05$ according to Duncan's Multiple Rank Test. For *C. oxysporum* Se X: 1,79058; CV (%): 60,17; for *F. chlamydosporum* Se X: 0,612145; CV (%): 66,46; for *C. cassiicola* Se X: 1,38064; CV (%): 19,61

Figure 1. Effect of the *Azotobacter* sp strains on the mycelial inhibition of three phytopathogenic fungi

Three of the microorganisms deformed the mycelial structure, due to the rupture of numerous hyphae at the edges that became straight and rolled back but did not limit sporulation. The remaining (A-12, A-22 and A-33) caused a limitation of the conidium genesis process and showed signs of substance production by the accumulation on the colony edges of a bright dark yellow substance obscuring the days; however, among these three strains there are significant differences in the percentage of mycelial inhibition reached, with emphasis on strain A-22.

The differences achieved with respect to this indicator can be attributed to the production of substances of different nature and with a variable degree of efficiency over the pathogen. The influences of different types of compounds produced by bacteria and the mechanisms of action that may be involved in the results have been described (15). According to these authors, phytohormones, siderophores, peptides and certain enzymes may interfere with the growth of fungi, which prevents the development of diseases in plants.

Against *C. cassiicola* all strains of *Azotobacter* evaluated, showed some level of effectiveness. A-11 deformed the mycelial structure and prevented the formation of new hyphae. A-22 strain showed similar behavior and signs of substance production which resulted in the autolysis of hyphae within the colonies evidenced by the formation of transparent areas to the underside. A-12, A-20, A-29, A-31 and A-33 caused similar damage and limited sporulation. Equal manifestations showed A-14 strain (Figure 1). However, only strains A-12 and A-33 succeed in inhibiting the mycelial growth of the pathogen over 35 % and maintain significant differences with the rest of the strains studied.

The behavior of *Azotobacter* strains evaluated on *C. cassiicola* showed lower growth inhibition values than those against *C. oxysporum* and *F. chlamydosporum*, which may be related to the habit and growth rate of this fungus.

It should be noted that this is the first time that this group of microorganisms has been used to study its antagonistic activity against vegetable pathogens. Regarding the genus *Azotobacter*, few studies were found in the consulted literature about the production of metabolites with enzymatic or antifungal characteristics used for the control of harmful organisms. However, the strain role of this genus, isolated in association with cereals, with the potential to produce a fungistatic, ether-soluble and thermosetting substance, which reduced the growth and fungi incidence of the genera

Fusarium, *Alternaria* and *Helminthosporium* In wheat (*Triticum aestivum*) and millet (*Sorghum bicolor*) (10).

It is also known about the production of cysts, which are considered structures of resistance, by most of the strains belonging to this genus. They are wrapped in matrices of exopolysaccharides of the alginate type and polyhydroxybutyrates. Both types of substances are carbon sources and involved in processes, such as the protection of the enzyme nitrogenase in the presence of oxygen and its adaptation to stress conditions; Participate in biochemical processes that allow the integrity of cell membranes, as well as in the formation of biofilms. Polyhydroxybutyrates are also given the property of repressing the sporulation of some fungal genera, so they may be involved in this response (16). For *Azotobacter*, the production of polyhydroxyphenols and phenolates has been recently recognized, preventing membrane synthesis processes in fungi of the genus *Fusarium* (17).

In the case of the genus *Bacillus*, 11 strains were evaluated, of which two had no activity against *C. oxysporum*, three showed mean values and six were effective. The absence of activity against the pathogen was manifested in all replicates of these two microorganisms (B-3 and B-75). In these cases the growths were mixed and both managed to develop one on top of the other, without causing damage to the fungal structure. B-4, B-39 and B-61 show as main behavior that after beginning the hyphal extension, many spores jump and move away from the growth, their germination gives rise to the formation of new colonies. In this respect it was noticed the death or what is equal, the non-germination of the spores closest to the bacterial streak and the total structure deformation of the same ones, due to the rupture of large zones of the mycelium, that at the same time caused The change in coloration to white from the colonies to the back, indicating that the process of conidium genesis was partially interrupted.

Six strains showed effectiveness (B-8, B-12, B-15, B-28, B-31 and B-51) were able to decrease mycelial growth due to the rupture of numerous hyphae. In addition, they showed production signs of compounds with fungistatic activity that changed the colony color of the dark olive green to fluorescent shades of yellow or brown. They created swelling in the colonies and totally prevented the release of the first conidia.

As for the mycelial inhibition, caused by these strains, differences are observed (Figure 2). Six of them resulted in mycelial inhibition in excess of 80 %, which can be considered a high value, a sample approximately 60 % and three shed values

below 40 %. Only inhibition caused by strain B-3 can be considered negligible.

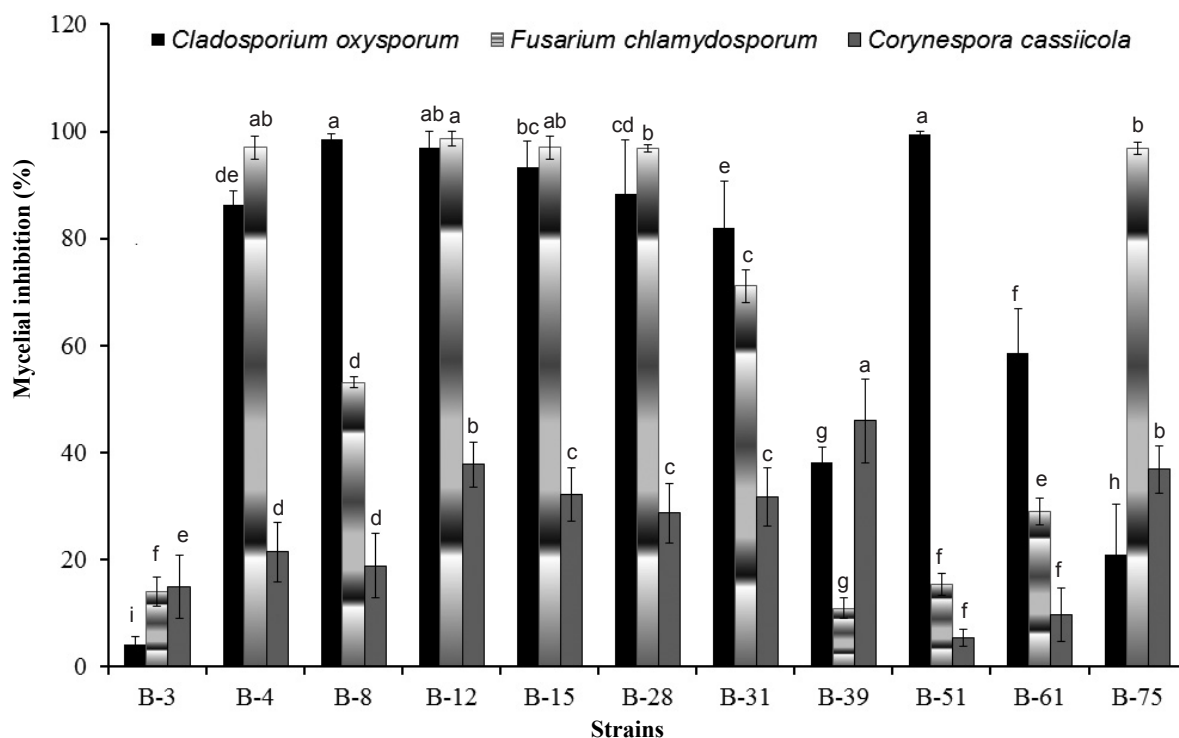
The bacteria included in the genus *Bacillus* show a greater diversity in the responses to *F. chlamyosporum*. B-3 and B-39 show coexistence with the fungus, since the growths were mixed with each other, without causing any damage to the fungal structures. B-51 and B-61 deformed the edges of the colonies into straight shapes, without causing more severe damage to the structures of the fungus. B-8 and B-31 deform the edges into inward-facing shapes, where hyphae are lysed, cause many of them to rupture inward, but only partially limit conidium genesis. On the other hand, B-4, B-12, B-15, B-28 and B-75 inhibit, severely, mycelial growth of the pathogen (Figure 2), without significant differences between them. In addition, they cause the accumulation of dark yellow substances bright around the colony and prevented the development of the conidium genesis process.

Among the strains of *Bacillus* there were several tendencies towards *C. cassiicola*. B-3, B-51, B-61, B-4 and B-31 showed low levels of inhibition because they only deformed the borders of the colonies to straight forms and induced the rupture of some hyphae, which did not cause substantial damage to the mycelium. B-15, B-28 and B-8 show signs of production of compounds with antifungal activity, which was manifested by

the appearance of autolysis zones on the back of the colonies; In addition, changes in coloration of the hyphae were evidenced to an olive greenish tone that turns blackish with the days or the accumulation of a pink substance in the edges or around the disc that served as inoculum for the assembly of the method. B-12, B-39 and B-75 were promising because in addition to the described manifestations, they were able to inhibit, to a greater extent, the fungal growth and to limit the process of conidium genesis.

It was confirmed that the formation of autolysis zones on the back of *Curvularia lunata* colonies when they were confronted with several strains belonging to the Bacilli class (18). These authors report that this effect is caused by substances of the antibiotic type that can be produced by these. In this case there may also be strains with the ability to produce certain types of substances with a high concentration or stability to cause damage to the fungal species studied.

The results suggest a certain specificity of the metabolites involved according to the phytopathogenic fungus to be controlled. Against *C. oxysporum* showed high levels of effectiveness of A-29 and A-33 strains, the latter was also highlighted against *C. cassiicola* accompanied by strain A-12. For the control of *F. chlamyosporum* only the A-22 strain stands out.



Equal letters do not differ significantly from each other for $p \leq 0,05$ according to Duncan's Multiple Rank Test. For *C. oxysporum*. EsX: 1.87437: C H (%): 47.41; For *F. chlamyosporum* EsX: 0.640883: CV (%): 59.26; For *C. cassiicola* EsX: 1.27966: CV (%): 48.08

Figure 2. Effect of Bacillus strains on mycelial inhibition of three phytopathogenic fungi

In the case of the genus *Bacillus*, this criterion is valid, although it could be related to this activity, a greater number of different nature compounds, due to the production of antibiotics such as iturin, subtilin and others recognized for the genus (18), Interference signals (17) or substances that induce systemic resistance in plants (19). However, B-8 and B-51 strains stand out against *C. oxysporum*, B-12 against *F. chlamydosporum* and B-39 against *C. cassiicola*.

CONCLUSIONS

- ◆ Strains of the two bacterial genera possess antagonistic activity against one of the fungal species used in the study.
- ◆ Strains A-29 and A-33 show high levels of effectiveness against *C. oxysporum*; the strains A-33 and A-12 stand out against *C. cassiicola*; while A-22 was efficient against *F. chlamydosporum*.
- ◆ In the case of the genus *Bacillus*, the B-8 and B-51 strains stand out against *C. oxysporum*, B-12 against *F. chlamydosporum* and B-39 against *C. cassiicola*.

RECOMMENDATION

To continue the studies for the quantification of the activity by *in vitro* and *in vivo* methods, linked to the obtaining of a product with multiple effects, which can be used as active principle of products with biofertilizer effect.

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Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial