# ARTIGOS

# Symbiotic and endophytic fungi as biocontrols against cocoa (*Theobroma cacao* L.) phytopathogens

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## ABSTRACT

Villamizar-Gallardo, R.A.; Ortíz-Rodriguez, O.O.; Escobar, J.W. Symbiotic and endophytic fungi as biocontrols against cocoa (*Theobroma cacao* L.) phytopathogens. ***Summa Phytopathologica,*** v.43, n.2, p.87-93, 2017.

Cocoa *(Theobroma cacao* L.) is a tropical tree, seriously affected by fungal diseases. To control several pathogens, biological methods are prescribed since they are friendly to the environment and easy to use. The main objective of this study was to assess the biocontrol effect of two native strains, *Trichoderma viride* and *Botryosphaeria quercum*, on phytopathogens such as *Phytophthora palmivora* and *Moniliophtora roreri*, causal agents of black pod and frosty pod rot diseases, respectively. In addition, biocontrolers were faced on potential mycotoxigenic fungi such as *Aspergills flavus* and *Fusarium solani, which* are very common on cocoa. The Bio-Control Index (BCI) was calculated to determine the *in vitro* biocontrol effect against the four phytopathogens. Results indicated that the best biocontrol agent of phytopathogens was *B. quercum*, showing BCI of 82.3%, 80.7%, 63.3% and 59.7% for each tested phytopathogen, respectively. Competition for substrate was the dominant biocontrol strategy. As to the origin of strains, those coming from the Department Norte de Santander and Santander showed the highest average inhibition percentage. This study provides an initial screening to the endophytic and antagonistic potential of fungi, specifically those capable of colonizing cocoa pods and soils. Thus, these strains can be used as an efficient biological control alternative against several known phytopathogens of cocoa in the field.

**Keywords**: Cocoa, pathogens, fungi, biocontrol

## RESUMO

Villamizar-Gallardo, R.A.; Ortíz-Rodriguez, O.O.; Escobar, J.W. Fungos simbióticos e endofíticos como biocontroles de fitopatógenos de cacau (*Theobroma cacao* L.)**.** ***Summa Phytopathologica,*** v.43, n.2, p.87-93, 2017.

Cacau (*Theobroma cacao* L.) é uma árvore tropical, bastante atacada por doenças fúngicas. Para o controle de doenças, métodos biológicos são prescritos uma vez que são amigáveis ao meio ambiente e fácil de usar. O principal objetivo do estudo foi avaliar o efeito de biocontrole das estirpes nativas, *Trichoderma viride* e *Botryosphaeria quercum*, em fitopatgenos: tais como *Phytophthora palmivora* e *Moniliophtora roreri*, agentes causais de doenças podridão parda e monilíase, respectivamente. Além disso, os agentes de biocontroles foram avaliados no controle de fungos micotoxigênico: como *Aspergills flavus* e *Fusarium solani*, que são muito comuns em cacau. O Índice de Bio-Controle (BCI) foi calculada para determinar o efeito *in vitro* de biocontrole contra quatro fitopatógenos. Os resultados indicaram que o melhor agente de controle biológico de fitopatogenos era *B. quercum*, que mostra 82,3% de BCI e 80,7%, 63,3% e 59,7% para cada um dos fitopatógenos testados, respectivamente. A competição por substrato foi a estratégia utilizada no controle biológico. As estirpes, provenientes do Departamento de Santander e Santander Norte apresentaram a maior inibição percentual média. Este estudo é um inicial para sobre o potencial endófito e antagonística de fungos, capazes de colonizar as especificamente bagas de cacau e solo. Assim, estas estirpes podem ser indicadas uma alternativa eficiente contra fitopatógenos de cacau importantes.

**Palavras-chave**: Cacau, patógenos,cogumelos, biocontrole

Cocoa *(Theobroma cacao* L.) is a tropical American tree flowering all year long and bearing a fruit known as pod. Colombia is the ninth producer in the world and the third one in Latin America, after Brazil and Ecuador. Estimated projections indicate that by 2021 Colombia might produce 285,600 Mg cocoa, as cocoa tree plantations are expected to reach over 230,000 ha (11); however, 50% to 90% cocoa production is affected by fungal diseases (2,4). The most prevailing infections are frosty pod rot (caused by the fungus *Moniliophthora roreri*) and black pod (the etiological agent of which is *Phytophthora* sp.), both causing cocoa crop losses of up to 100% (15). In addition, there are secondary infections caused by phytopathogens like *Aspergillus* and *Fusarium* that attack opportunistically as cocoa deteriorates, thus accelerating pod degradation and leaving traces of mycotoxins (3).

To reduce the effects of fungal phytopathogens, cocoa growers apply good cropping practices, sometimes combined with the use of fungicides. The first method is easy to apply but labor demanding and is only economically viable if the cocoa price in the market is high (8). In the case of fungicides, they certainly protect the plant

from pathogen attacks but also have deleterious effects on the fly *Forcipomyia* sp., which pollinates the cocoa flower, thus altering the ecological equilibrium of the plantation.

With the aim of mitigating the environmental effects of chemically synthesized fungicides, several biological control strategies have been studied (21). Biocontrol microorganisms such as *Trichoderma* have been commonly used since they are cosmopolitan and their isolation process is relatively simple (6). In the case of cocoa crops, *Trichoderma* has been demonstrated as capable of establishing both symbiotic and endophytic relationships with the plant, thus promoting cacao growth and protecting it against diseases (9)**.** Endophytic fungi have been investigated since they are broadly recognized for their capacity to penetrate and colonize the host, where they promote the synthesis of biological compounds that favor the plant growth (13). However, biocontrol agents have limitation in applications due to their outstanding pathogen specificity (10). This makes necessary their isolation from the ecosystem to where they are going to be applied, with the purpose of increasing their effectiveness.

For such reason, the main objective of the present study was to investigate biocontrol agents such as *Trichoderma viride* and *Botryosphaeria quercum* (antagonistic and endophytic fungi, respectively). Native strains obtained from soil and cocoa pods in three departments of Colombia were isolated with the aim of proving to *in vitro* level their biocontrol effect on cocoa phytopathogens coming from the same environment. Finally, the use of the biocontrol fungus *B. quercum* as phytopathogen in cocoa crop was reported and the Bio-Control Index (BCI) was calculated to determine the *in vitro* biocontrol effect against four phytopathogens.

## MATERIALS AND METHODS

### Sample collection

Soils were obtained through simple random sampling from 20 farms showing the highest cocoa production records according to the departments of Santander, Norte de Santander (N. de S.) and Antioquia, which belong to the Cocoa Growers Federation – FEDECACAO. Samples were taken from the plant's rhizosphere at 20 to 30 cm depth, covering 1 ha cultivated land, until 1 kg final sample was obtained for each department.

Healthy and diseased *T. cacao* L. pods were sampled from the above-mentioned farms. Samples were taken from the different cocoa materials available in the plantations (clones and hybrids). Diseased pods were those exhibiting symptoms such as deformations, black stains, oily spots, yellow halos, chocolate color stains with well-defined margins and cream color powder. The pods were packed in plastic paper, labeled and transported inside boxes to the laboratory for processing.

### Fungal isolates

Antagonistic fungi from farms of the departments Norte de Santander (N. de S.), Santander and Antioquia were sieved and 10 g of the obtained material were dissolved in 90 mL sterile distilled water. This solution was 10-fold diluted up to $10^{-3}$, then plated on agar PDA (OXOID) and incubated at 25+/-2°C (Memmert incubator) during eight days. Pure culture was prepared from heterogeneous growth until axenic cultures from each department were obtained and morphologically and molecularly characterized.

### Endophytic fungi

Healthy pods were disinfected with 5% sodium hypochlorite and sterile distilled water in order to remove the bacterial pool that is usually present on the fruit's external surface. Then, the upper cortex was peeled out so that 2mm pieces were chosen to be inoculated on potato dextrose agar (PDA) (OXOID) modified with cocoa pod cortex extract and chloramphenicol. Pure culture was prepared from heterogeneous growth until axenic cultures from each department were obtained and morphologically and molecularly characterized.

### Phytopathogenic fungi

Both primary and secondary phytopathogens were obtained from cocoa pods exhibiting the previously described typical symptoms. Spores contained in the upper and inner pod cortex were directly plated on PDA modified with cocoa pod cortex extract and chloramphenicol. Pure culture was prepared from heterogeneous growth until axenic cultures were obtained and morphologically and molecularly characterized.

### Characterization

Morphological characterization was performed by taking into account aspects such as texture, edge, and mycelium color. Reproductive structures (spores), type of hyphae and presence of septa were observed by means of staining with lactophenol blue. The photographic record was obtained in a Nikon Eclipse 80 i phase contrast optical microscope (100 X magnification). DNA was isolated by using an ultraclean microbial DNA isolation kit (Mo-Bio Laboratories, USA) and prepared according to the manufacturer's specifications. The microorganisms were lysed by using bead-based homogenizer. The released DNA was then bound to a silica spin filter and washed. DNA was recovered with DNA-free Tris buffer. Isolated DNA was amplified by employing two molecular markers corresponding to the ITS region and the β-tubulin gene (see Table 1). The amplified DNA was then sequenced in Macrogen (Korea) and analyzed according to BLAST database.

Biocontrol assay was carried out by using the dual culture method described and modified by Smitha et al. (19). Three *Trichoderma* and three *Botryosphaeria* strains, one from each department, were screened to prove their biocontrol effect against primary and secondary cocoa phytopathogens. Petri dishes containing PDA were inoculated with a 5mm agar disc with phytopathogens grown for 5 days. Another agar disc with pathogens of the same age and same diameter containing the biocontrol agent was placed at a distance of 3 cm from the

**Table 1.** ITS region and *β*-tubulin genes employed as primers for the characterization of phytopathogenic fungi isolated from cocoa crop soils.

| Primer | Molecular Marker | Sequence |
|---|---|---|
| B-tub | Bt-Lev | GTG AAC TCC ATC TCG TCC ATA |
| | Bt - T2M | CCA CTG GGC TAA GGG TCA TT |
| | PN3 | CCG TTG CTG AAC CAG CGG AGG GAT |
| IT S | | C |
| | PN16 | TCC CTT TCA ACA ATT TCA CG |

phytopathogen, assuring the same growth space for both fungi. The media were incubated at 25°C for 12 days, during which growth was daily monitored. The process was photographically recorded with an Unnikon 2500 digital camera equipped with a fixed support setting a distance of 18 cm in all cases and then analyzed with ImageJ free access software. No significant growth changes were observed after day 6, when the Bio-Control Index (BCI=A/B*100.) was calculated, where A corresponds to the area of the biocontrol agent, while B is the total area covered by the biocontrol agent+ phytopathogen, adapted from Szekeres et al. (20). A *T. harzianum* strain provided by the culture collection of Universidad de Pamplona (Colombia) was used as control.

**Assessment of the analyzed variables**

Data obtained in the present study were analyzed for the biocontrol effect of *T. viride* and *B. quercum* native strains on plant pathogens such as *P. palmovira, M. roreri, Aspergillus flavus* and *Fusarium solani*. The biological agent was tested with the following categories: *B. quercum B. and T. viride,* then with the region, corresponding to three categories: Antioquia, Norte de Santander and Santander; and finally with the associated agents such as *Moniliopthora, Phytophthora, Aspergillus and Fusarium.* The response variable was the Bio-Control Index (BCI). Therefore, for each biological agent, five replicates were considered and these values were averaged based on the region and the agents. Results were analyzed using the program Statgraphics and analysis of variance (ANOVA), comparing tests between the levels of each factor and evaluating assumptions.

## RESULTS AND DISCUSSION

**Biocontrol agent isolates**

Strains that had white and cottony mycelium subsequently becoming olive green, as well as granular texture and irregular margins, were obtained from soil samples at the departments of Santander, Norte de Santander and Antioquia. According to morphological characterization, mycelium bore ramified hyaline conidiophores in the form of a tree, exhibiting septate hyaline hyphae, bottle-shaped phialides joining the conidiophores, and round or ovate conidia (Figure 1A-1B). Molecular assays indicated that the isolated strains from all sampled departments corresponded to the species *Trichoderma viride*, coinciding with previous studies conducted by Guigón-López et al. (6) and Vargas et al. (22).

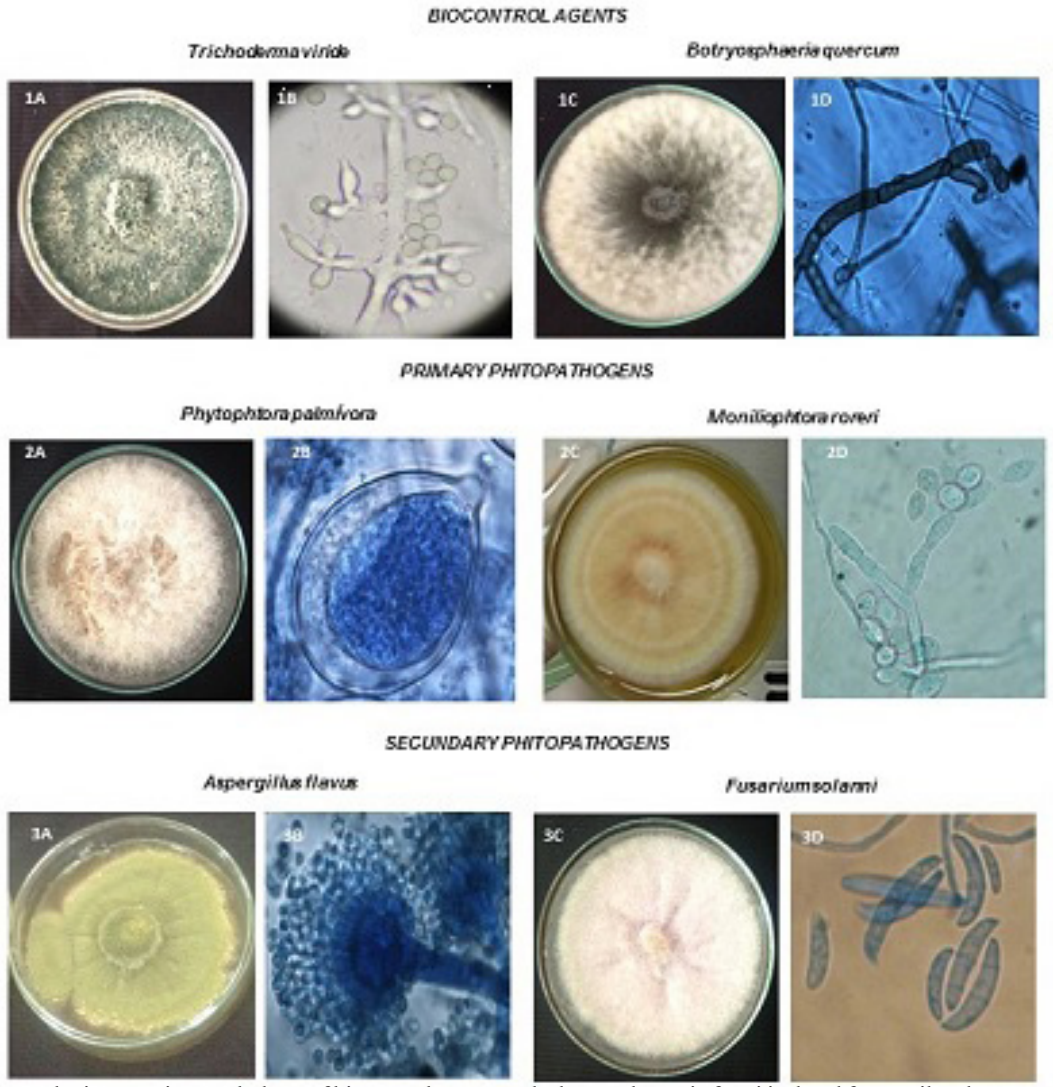


**Figure 1.** Macro and microscopic morphology of biocontrol agents and phytopathogenic fungi isolated from soil and cocoa pod samples cultured in PDA. Microscopic images were obtained from samples stained with lactophenol blue (100**X).**

Isolates obtained from healthy pods from each evaluated department developed into colonies with white filamentous mycelium that turned to gray cottony mycelium of irregular margins after six days. Under the microscope, it exhibited thick septate hyphae with humps and large ovate spores (Figure 1C-1D). Based on molecular analyses, the isolated strains from all sampled departments corresponded to the endophytic species *B. quercum* (26).

**Phytopathogenic fungi**

Primary phytopathogens such as *Phytophthora* and *Moniliophthora* and secondary phytopathogens like *Aspergillus* and *Fusarium* were among the fungi most commonly found in diseased pods. The first two are the etiological agents of black pod and frosty pod rot, respectively, while the last two are very important in terms of public health due to their mycotoxigenic potential (3, 25). *Phytophthora* presented a plushy texture, uniform sporulation, gray-cream color and papillate sporangia containing zoospores (Figure 2A-2B). According to macro and micro characteristics, the isolated strain corresponded to the species *P. palmivora* (8). *Moniliophthora* exhibited fluted texture and central and terminal rings colored beige with light and dark brown center. Microscopically, it showed globose, ovoid or ellipsoid spores and no septate hyaline hyphae (Figure 2C-2D), corresponding to the species *M. roreri*.

Macro and micro characteristics of secondary phytopathogens are also shown in Figure 1 (3A-3F), coinciding with previous reports by Pazouki & Panda (17). Molecular characterization allowed determining that the isolated strains corresponded to the species *A. flavus and F. solani,* respectively, reported by Mounjouenpou et al. (14).

**Biocontrol assay**

*T. viride* strains isolated from the departments of Santander, Norte de Santander and Antioquia, hereinafter called T.v.S., T.v.NS. and T.v.A, had biocontrol effect on the growth of the isolated phytopathogenic fungi. Macroscopically, *T. viride* clearly gains space to rapidly grow, adapt and develop, especially when faced with the primary phytopathogens. Tthe secondary phytopathogens had the strongest inhibitory effect against *Fusarium solani*, followed by *A. flavus* (Figure 2A). At the microscopic level, the antagonistic fungus recognized the phytopathogen, penetrating, wrapping, strangling and consuming it. This process is illustrated in Figure 2B, coinciding with the study performed by Bailey et al. (1).

Strains of the endophytic fungus *B. quercum* isolated from the departments of Santander, Norte de Santander and Antioquia, hereinafter called B.q.S., B.q.NS. and B.q.A, also had biocontrol effect on the growth of the isolated phytopathogen. As shown in Figure 3, the fungus develops rapidly, gaining space to grow and surrounding the phytopathogen. Similarly to *T. viride*, this phenomenon is more outstanding when the biocontrol agent is faced with *M. roreri* and *P. palmivora* (Figure 3-A). At the microscopic level, we could observe how the endophytic biocontrol agent encounters the phytopathogen and wraps it with mycelium, forming a hook that allows it to strangle the hypha, thinning and finally destroying it (Figure 3-B).

The biocontrol effect of each *T. viride* and *B. quercum* strains vs the different phytopathogens was calculated and measured as "BioControl Index - BCI". Results are shown in Table 2.

According to ANOVA, $p > 0.05$ was determined with three significant factors without any interactions, see Table 3.

Then, we analyzed which categories within each factor were significantly different between regions and the etiologic agents were evaluated. Considering the region, only Norte de Santander and Santander had significant differences. The region of Antioquia showed no significant difference, compared to the other regions (Figure 4).

For the etiological agent, there were significant differences between *A. flavus-F.solani* and *M. roreri-P. palmivora*, as shown in Figure 5.

Based on the obtained results, the best biocontrol agent of both primary and secondary phytopathogens was *B. quercum,* with BCI values of 82.3%, 80.7%, 63.3% and 59.7% against *P. palmivora, M. roreri, A. flavus and F. solani,* respectively. *T. viride* strains displayed a slightly lower biocontrol index, compared to *B. quercum*, reaching values of 78.7%, 74.1%, 51.2% and 58.6% for each tested phytopathogen.

As to the origin of strains, isolates from the department of Norte de

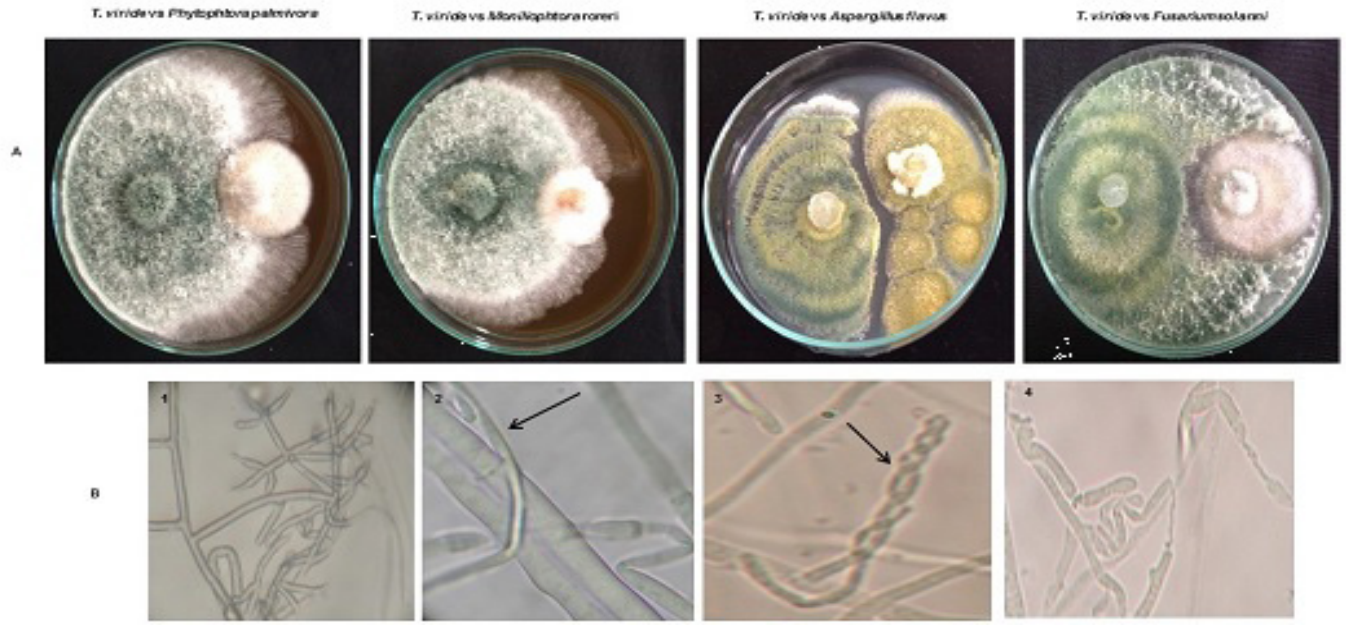


**Figure 2.** A) Biocontrol mechanism of *Trichoderma viride* vs. phytopathogens. B) Damage caused by the antagonist to the structures of *Fusarium solani.* 1) Mycelium of the antagonistic fungus 2) Wrapping 3) Strangling 4) Hyphallysis

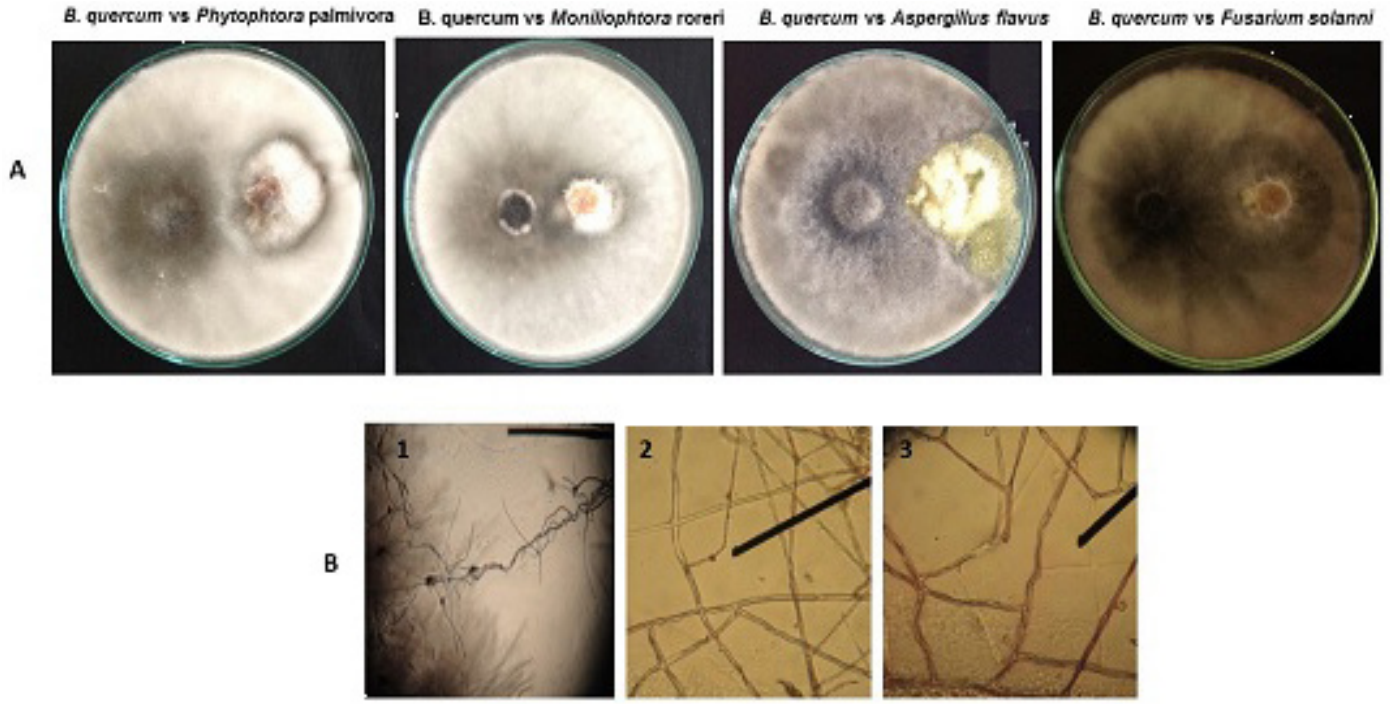


**Figure 3.** A) Inhibition test conducted within the endophytic fungus *B. quercum* faced against primary and secondary phytopathogens. B) Damage caused by the endophytic fungi to the structures of *Fusarium* sp. 1) Mycelia of endophytic vs. phytopathogenic fungi 2) Hook formation 3) Wrapping and hyphallysis.

**Table 2.** BioControl Index (BCI) for the biocontrol agents vs cocoa phytopathogens.

| | **Department** | ***M. roreri*** | ***P. palmivora*** | ***A. flavus*** | ***F. solani*** |
|---|---|---|---|---|---|
| ***B. quercum*** (BCI) | Antioquia | 68.76 | 69.98 | 63.25 | 59.03 |
| | Norte de Santander | 78.92 | 82.29 | 56.94 | 59.72 |
| | Santander | 80.67 | 78.23 | 56.08 | 52.95 |
| ***T. viride*** (BCI) | Antioquia | 58.11 | 59.75 | 46.82 | 54.03 |
| | Norte de Santander | 74.10 | 78.67 | 51.25 | 53.49 |
| | Santander | 52.00 | 52.25 | 43.51 | 49.63 |

**Table 3.** Analysis of variance for BCI; all F-ratios are based on the residual mean square error.

| **Source** | **Sum of squares** | **Df** | **Mean Square** | **F-Ratio** | **P-Value** |
|---|---|---|---|---|---|
| **Main effects** | | | | | |
| A:Biological Agent | 695.634 | 1 | 695.634 | 23.96 | 0.0027 |
| B:Region | 357.287 | 2 | 178.644 | 6.15 | 0.0352 |
| C:Agents | 1407.69 | 3 | 469.229 | 16.16 | 0.0028 |
| **Interactions** | | | | | |
| AB | 161.625 | 2 | 80.8127 | 2.78 | 0.1395 |
| AC | 78.9035 | 3 | 26.3012 | 0.91 | 0.4916 |
| BC | 265.13 | 6 | 44.1884 | 1.52 | 0.3114 |
| Residual | 174.168 | 6 | 29.028 | | |
| Total (corrected) | 3140.44 | 23 | | | |

Santander showed the highest BCI. This supports our results in which biocontrol agents had high specificity to the phytopathogen found in the same environment and ecosystem. In fact, isolated plant pathogens came from the department of Norte de Santander and the best BCIs were reflected in *B. quercum* and *T. viride* coming from the same region.

Since *B. quercum* is an endophytic fungus present in healthy pods and *T. viride* is a symbiotic fungus common in soils, we faced one to the other to verify if both fungi could be used synergistically as biocontrol agents in cocoa crops and thus enhance their action against pathogens. Both fungi developed at the same growth rate on PDA, and

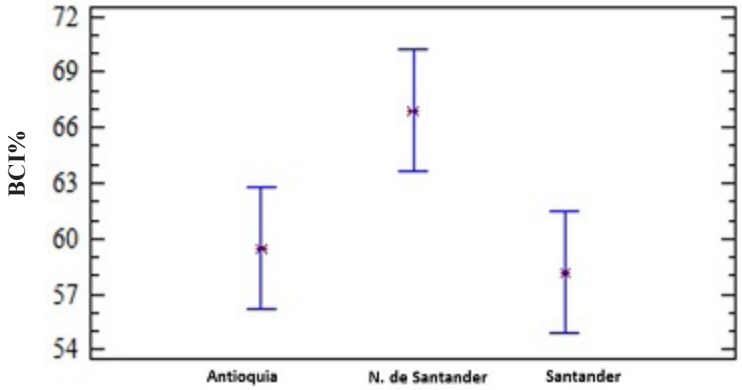


**Figure 4.** Average comparison of BCI according to the evaluated region.

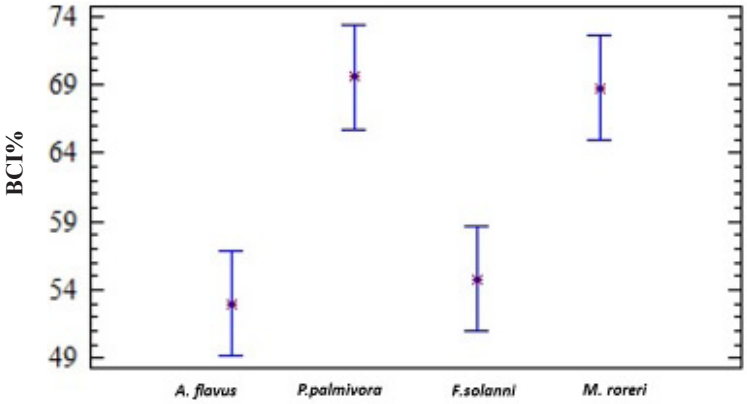


**Figure 5.** Average comparison of BCI according to the evaluated phytopathogen agent.

no competition for substrate or antibiosis was present, indicating they can coexist in the same medium without interfering with their role.

As a tropical country, Colombia offers ideal conditions for cocoa cultivation. Nevertheless, low distribution of resistant biological materials, among other reasons, determines cocoa yield to be very low. In this study, all analyzed biological materials (hybrids and clones) contained spores of *M. roreri* and *P. palmivora*. In addition, there was high prevalence of secondary pathogens like *A. flavus* and *F. solani*. Their presence in cocoa represents a high risk in terms of public health due to their mycotoxigenic potential, since they are capable of producing aflatoxins and ochratoxin type A which are carcinogenic (3).

To control these phytopathogens, this study presents two biocontrol agents isolated from cocoa crops, including fruit and soil samples, which are capable of efficiently inhibiting the growth of primary and secondary phytopathogens. One of them, characterized as *T. viride*, was regularly found in all analyzed soil samples. This corroborates the data reported by Villalobos et al. (24), who detected soil as a complex habitat that provides adequate nutrient levels and biotic and abiotic factors as protection strategies to isolate *Trichoderma* species (23).

The genus *Trichoderma* includes a vast variety of strains acting as biological control by producing metabolites (i.e., cellulases, glucanases, lipases, proteases and chitinases) with antifungal activity, competing for space and nutrients, developing mycoparasitism and promoting plant growth (5). These results were confirmed in the current study in which the confrontation of *T. viride* vs. *M. roreri*, *P. palmivora* and *F. solani,* revealed competition for substrate as the dominant biocontrol strategy. Contrastingly, the biocontrol effect of the antagonist on *A. flavus* proved to be an antibiosis process featured by the clear emergence of inhibition areas and change in the mycelial color**.**

On the other hand, in this study we characterized endophytic fungi called *B. quercum.* These fungi are closely associated with platanus crops, which are commonly used in Colombia to shade cocoa plantation, and therefore are horizontally transmitted, i.e., through the environment (7, 16). Inhibition tests revealed that the most common mechanism employed by *B. quercum* against most phytopathogens is competition for substrate, coinciding with previous reports by Mejía et al. (12), who compared the inhibitory action of different endophytic morphospecies on primary phytopathogens of cocoa. These fungi are known to produce secondary metabolites (alkaloids) plus phenolic compounds and ligninolytic enzymes that reduce the growth of phytopathogens**.**

Analyzing the behavior of phytopathogens, most of the experimental units testing the genera *P. palmivora* and *M. roreri* classified them as highly inhibited strains. This is the most remarkable result, considering that these fungi are the main etiological agents responsible for cocoa crop losses. With respect to geographical origins, strains from Norte de Santander and Santander reached the highest average inhibition percentages. *T. harzianum,* a well-known fungus used in biocontrol (18), was obtained from the culture collection of Universidad de Pamplona and was used as control. Results allowed observing that this fungus displayed the lowest inhibition effect against all tested phytopathogens, thus proving that the biological control agents must be isolated from the ecosystem to where they are going to be applied in order to enhance their effectiveness.

Finally, the outcome of this study will be used to develop guidelines for the biological management of important diseases affecting cocoa crop, which will help farmers preserve and apply biocontrol agents in plants and assess the disease control level.

*IN VITRO* evaluation of the biocontrol effect of two native symbiotic and endophytic strains associated with cocoa tree against different phytopathogens was successfully carried out based on their

biocontrol index values.

Comparing both types of fungi, the inhibition percentage of *Botryosphaeria quercum,* in contrast to all phytopathogens, was higher than the values obtained for *Trichoderma viride* and the control.

Results showed a relatively superior biocontrol performance of the endophytic fungus over the symbiotic type. However, these two biocontrol agents may be suggested as a biological protocol to be used in a synergic strategy to reduce infection caused by the usual phytopathogens to cocoa trees in the field.

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