Use of *Trichoderma* fungi in spray solutions to reduce *Moniliophthora roreri* infection of *Theobroma cacao* fruits in Northeastern Costa Rica

John Seng[1], Geovanny Herrera[2], Christopher S. Vaughan[3] & Michael B. McCoy[3]

1. Grinnell College, Grinnell, IA, USA; sengjohn@grinnell.edu
2. Milwaukee Public Museum, Milwaukee, WI 53233-1478, USA; wesgeova@yahoo.com
3. Associated Colleges of the Midwest, San Pedro de Montes de Oca, Costa Rica; cvaughan@acm.edu, mmccoycolton@yahoo.com

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Abstract: Cacao (*Theobroma cacao*) is an important cash crop in tropical climates such as that of Latin America. Over the past several decades, the infection of cultivated cacao by *Moniliophthora roreri*, known commonly as "monilia", has significantly hindered cacao production in Latin America. Studies have proposed the use of *Trichoderma* sp. fungi in biocontrol treatments to prevent and reduce monilia infection, yet tests of *Trichoderma*-containing spray treatments on cacao agroforests have produced mixed results. Researchers and agricultural workers have suggested that addition of soil, fly ash, or other carbon sources to a *Trichoderma* spray may improve its efficacy in fighting monilia. To test these suggestions, we designed a series of spray mixtures including *Trichoderma* cultures, soil, and all necessary controls. We applied the spray mixtures to 80 cacao trees (20 trees for each of four resistant-selected clones to monilia) at the FINMAC organic cacao plantation in Pueblo Nuevo de Guacimo, Limón Province, in northeastern Costa Rica in March-April 2013. Five treatments were applied (control, water, water plus sterilized soil, water plus *Trichoderma*, and water plus sterilized soil plus *Trichoderma*). Each treatment was applied to four trees of each clone. We monitored the incidence of monilia infection under each spray treatment over the course of 35d. We found that spraying entire cacao trees two times with a mixture containing *Trichoderma* and sterilized soil significantly reduced the incidence of monilia infection by 11% ($p \leq 0.05$) in only 35d, as compared to the control. This reduction in loss of cacao pods translates into an increase of plantation mean productivity of 1 500kg dried beans/ha by 198kg/ha up to 1 698kg/ha or by a total increase over the whole 110ha plantation by 21 780kg. We propose that using such an antifungal spray over the whole course of a crop cycle (120 days) would decrease infection incidence even more. Application of this fungal control measure has the potential of revitalizing the production of cacao in the region. Rev. Biol. Trop. 62 (3): 899-907. Epub 2014 September 01.

Key words: *Theobroma cacao*, biological control, cacao, Costa Rica, monilia, organic fungicide, *Trichoderma*.

Cultivation of the cacao tree, *Theobroma cacao* (L.), is very important to the economies and ecosystems of Latin American countries such as Costa Rica. Cacao seeds, the primary ingredient in chocolate, are a valuable cash crop which historically has comprised a large amount of Latin American agricultural exports (Bamba & Reed, 2004). In addition, cacao agroforest systems are thought to provide habitat and migration corridors between forest fragments for such diverse species as sloths (Vaughan, Ramirez, Herrera, & Guries, 2007; Ramirez, Vaughan, Herrera, & Guries, 2011), howler monkeys (Muñoz, Estrada, Naranjo, & Ochoa, 2006), numerous birds (Reitsma, Parrish, & McLarney, 2001), foraging ants (Roth, Perfecto, & Rathcke, 1994), terrestrial amphibians and reptiles (Whitfield et al., 2007) and dung beetles (Harvey, González, & Somarriba, 2006). Yet the Latin American cacao industry today is struggling with persistent crop disease.

The infection of cacao fruits throughout Central and South America by the fungus *Moniliophthora roreri*, known commonly as "monilia", has decimated much of Latin American cacao production over the past four decades (Hoopen, Rees, Aisa, Stirrup, & Krauss, 2003; Aime & Phillips-Mora, 2005; Phillips-Mora & Wilkinson, 2007). Monilia infection is extremely concerning to chocolate producers. Throughout Latin America, monilia infection claims some 30 000MT of potential cacao production annually (Bowers, Bailey, Hebbar, Sanogo, & Lumsden, 2001). Additionally, loss of this agroforestry crop as an economic option has aided the recent expansion of banana and pineapple monocultures, very destructive ecologically, as well as, socially.

Recent studies demonstrated the potential of the fungus, *Trichoderma* sp., which thrives in the soil of cacao agrosystems and similar environments (Mpika, Kebe, & N'Guessan, 2011), as a natural antagonist to *M. roreri* (Stefanova, Leiva, Larrinaga, & Coronado, 1999; Benítez, Rincón, Limón, & Codón, 2004, Nalimova, 2007). Some plantations, including FINMAC, have proposed a passive management strategy where diseased pods are cut from trees and buried under the leaf litter near the topsoil under the assumption that the soil population of *Trichoderma* will kill off the monilia spores as described in Krauss & Soberanis (2002). Yet the current rate of loss attributed to monilia at FINMAC (25-30% pod loss/ yr) indicates that simply burying the pods and using semi-resistant on-farm-produced clones is not enough to combat infection effectively.

Previous research also suggested the possibility of spraying cacao pods, branches, and/ or trunks with *Trichoderma* cultures as a preventative management strategy to control the spread of monilia. However, *Trichoderma* normally thrives in the soil, meaning that the fruits and branches of a cacao tree do not provide ideal growth conditions or nutrients. To compensate for this environmental mismatch in agricultural applications, some studies have proposed and tested (Krauss & Soberanis, 2002) the addition of nutrient sources when applying a *Trichoderma* spray.

The objectives of this study were to test the temporal impact of *Trichoderma* fungal spray: a) on reduction of monilia incidence and degree of infection, b) with and without addition of sterilized soil as a fungal nutrient or adherent, and c) on four farm-produced clonal cacao varieties.

MATERIALS AND METHODS

Study site: The study was conducted on the organic cacao (*Theobroma cacao*) plantation at FINMAC, in Pueblo Nuevo de Villa Franca de Guacimo, Limón Province, Costa Rica (10°20" N - 83°20" W). The FINMAC plantation sits at 40m above sea level (Wieme, 2011) and is located within the Premontane Wet Forest Life Zone (Holdridge, 1964). Annual rainfall averages 6000mm and mean annual temperature is 26ºC (Holdridge, 1964; Vaughan et al., 2007; Wieme, 2011; Cornwell, 2012). Canals throughout the plantation provide irrigation and drainage. Additional plant species intermixed with the cacao, including *Eucalyptus deglupta*, *Cocos nucifera*, *Leucaena leucocephala* and *Musa acuminata*, ensure that cacao trees rarely receive more than 3hrs of direct sunlight per day. The cacao (*Theobroma cacao*) agroforest within the plantation is at times home to parrots, macaws, two-and-three-toed sloths, ants, termites and wasps.

Study system: *Theobroma cacao* at FINMAC is planted with 3m spacing between all trees. Six distinct clones, referred to here as clones A, B, C, D, E and F, are cultivated at the plantation. The land was converted from livestock pasture to cacao plantation circa 1982, and has been organically managed since 1997 (Cornwell, 2012). The FINMAC farm is situated on mostly flat to slightly rolling ground on moist soil with moderate-to-high amounts of leaf litter. Cornwell (2012) worked at FINMAC to characterize and quantify soil quality and composition. She explained that soil moisture is maintained by subterranean drainage tubes, soil is fertilized with composted coffee husks, and organic herbicides have been applied intermittently. During the Cornwell (2012) study, the soil on the organic cacao plantation had a mean pH of 5.8. Mean cation exchange capacity in the agroforest soil is 22.6cmol$^+$/L (Cornwell, 2012). For more detailed information regarding soil composition and quality, see Cornwell (2012).

Test subject selection: Twenty trees each of clones B, C, D and E were selected within a 100 x 260m block at FINMAC. Clones A and F were not included since their fruits had recently been harvested at the time of study. Trees were chosen by walking inwards from the beginning of each clone-row (from the main farm road), marking every other tree, and skipping those which did not have at least four healthy fruits longer than 15cm. For clones B and C, 20 trees meeting the guidelines were available within single clone-rows. For clones D and E, 10 trees were selected from each of two clone-rows because fewer trees meeting the guidelines were available within each row. Marked trees were encircled with bright orange twine wrapped around adjacent trees to the selected tree. Plantation workers were asked not to cut or touch fruits in marked trees.

Treatments: Five treatments were randomly assigned in equal frequency to trees from each clone using the random integer generator in the "R" base package (R Development Core Team, 2012) so that each treatment was applied to four trees per clone. In cases where clones were split between two rows, treatments were assigned at equal frequencies between the rows. Eighteen liters of each treatment were prepared 24hrs prior to application at room temperature in 18-l plastic screw-top tanks as follows: (1) nothing applied; (2) water with 700g soil (3) water (4) water with 10g Trich-Aid (a *Trichoderma* inoculum powder); and, (5) water, 10g Trich-Aid, and 700g soil. Treatments 2, 3, 4 and 5 contained 50ml of Limonoil, an agricultural treatment adhesion solution (www.grupocolono.com).

Soil was obtained from a nearby cow pasture, finely sifted, and sterilized in the plantation cacao-drying oven at 250ºC for 25min. Water used was non-chlorinated, obtained from a spigot on the plantation. Trich-Aid is a powdered *Trichoderma* inoculum, manufactured by Bio-Tech (Guapiles, Limón, Costa Rica). Treatments were applied twice throughout the study period: 22 March and 9 April 2013, using a Carpi 18-l manually-operated backpack sprayer. Sprays were delivered at a rate of 1l•min^{-1} for 60 ±20s per tree, wetting the entire trunk, branches, leaves and fruits from the ground up to 2.5m.

Monitoring: Fruits in each experimental tree were observed for monilia infection at twelve time points between 23 March and 26 April 2013: March 23 and 25, and April 4, 8, 10, 12, 15, 17, 19, 22, 24 and 26. At each time point the number of fruits greater than 15cm in length was first counted. The number of fruits longer than 15cm with monilia infection was then recorded using a four-stage infection scale (Fig. 1): (0) no infection, (1) infection entering, indicated by irregular discoloration and/or irregular bump, (2) infection consuming fruit, indicated by irregular brown to black patch, and (3) production of fine, thickly layered cream colored spores. Infected fruits were marked with brightly colored flagging tape. Fruits with stage 3 monilia infections were cut from their trees and thoroughly buried under a layer of leaf litter, and were notated as grade three infected fruits in all subsequent observations. Fruits with any degree of monilia infection at the first time point were excluded from analysis.

Mean incidence of monilia infection was calculated using the cumulative percentage of fruits which became infected throughout the course of the study. Mean degree of monilia infection was calculated by taking the arithmetic mean of degrees of monilia infection, zero to three, throughout time points, so as to provide a hypothetical mean degree of infection for any time point. Differences between mean incidence and mean degree of infection between treatments, clones and time points were estimated by parametric 2-way ANOVA using R software (R Development Core Team, 2012). *A posteriori* comparisons between means were

Fig. 1. Guide to monilia infection grades. Top to bottom: characteristic examples of infections of grades one, two, and three.

performed using Tukey "honestly significant difference" (HSD) tests with the "agricolae" package for R (De Mendiburu, 2013). Graphs were generated with the base R software package (R Development Core Team, 2012).

RESULTS

Impact of spray treatments: Treatment five, "*Trichoderma* plus soil" significantly reduced mean incidence of monilia infection by 11% and mean infection degree by 0.121 ($p \leq 0.05$) (Table 1) as compared to the control (treatment one).

Differences between clones: When results are split by clone, treatment five continued to reduce mean incidence (Fig. 2A) and mean degree (Fig. 2B) of monilia infection in clones C, D and E, though the change is only statistically significant in the cases of clone C, for infection incidence, and clone E, for infection degree. In clone B, treatment four significantly reduced mean incidence and degree of monilia infection.

Infections over time: Examination of mean incidence of monilia infection over time, grouped by treatment (Fig. 3A) revealed that fruits under treatment five were significantly less infected than the control (treatment one) at every time point after 12 March 2013 and

TABLE 1
Monilia infection under each treatment

Treatment[1]	Mean incidence[2,4]	Mean degree[3,4]
1	16.74[a]	0.206[a]
2	13.62[ab]	0.178[ab]
3	12.66[ab]	0.158[ab]
4	9.15[ab]	0.144[bc]
5	5.78[b]	0.085[d]

1. **1**=hard control (nothing applied), **2**=water, soil, and Limonoil, **3**=water and Limonoil, **4**=water, limonoil and *Trichoderma*, **5**=water, soil, Limonoil and *Trichoderma*.
2. percent of fruits observed displaying any degree of monilia infection.
3. on the 0-3 scale of infection, as described in methods.
4. means with the same letter(s) are not significantly different (Tukey HSD test, $p \leq 0.05$).

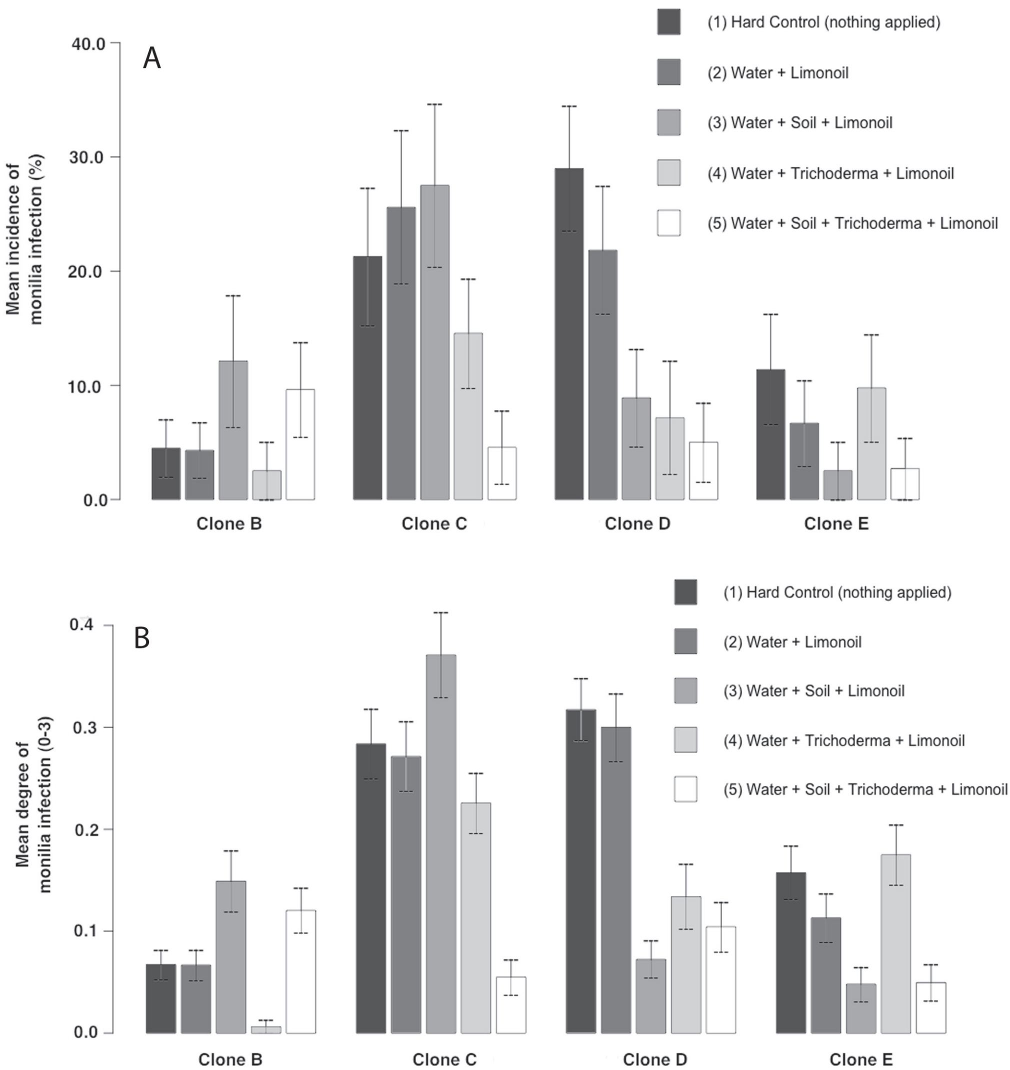

Fig. 2A. Mean incidence of monilia infection, grouped by clone and treatment. Vertical lines are ±1 Tukey 95% confidence intervals. FINMAC plantation, Pueblo Nuevo de Guácimo, Costa Rica. March-April 2013.
Fig. 2B. Mean degree of monilia infection, grouped by clone and treatment. Vertical lines are ±1 Tukey 95% confidence intervals. FINMAC plantation, Pueblo Nuevo de Guácimo, Costa Rica. March-April 2013.

fruits under treatment four were significantly less infected than the control (treatment one) at every time point after 19 April 2013.

Differences between clones over time: Grouping mean incidence of monilia infection by clone over time (Fig. 3B) revealed that fruits of clones C and D were generally significantly more likely to become infected by monilia than those of clones B and E.

DISCUSSION

Trichoderma sprays reduced monilia infection so greatly that its use to combat monilia pod rot in cacao within the study area

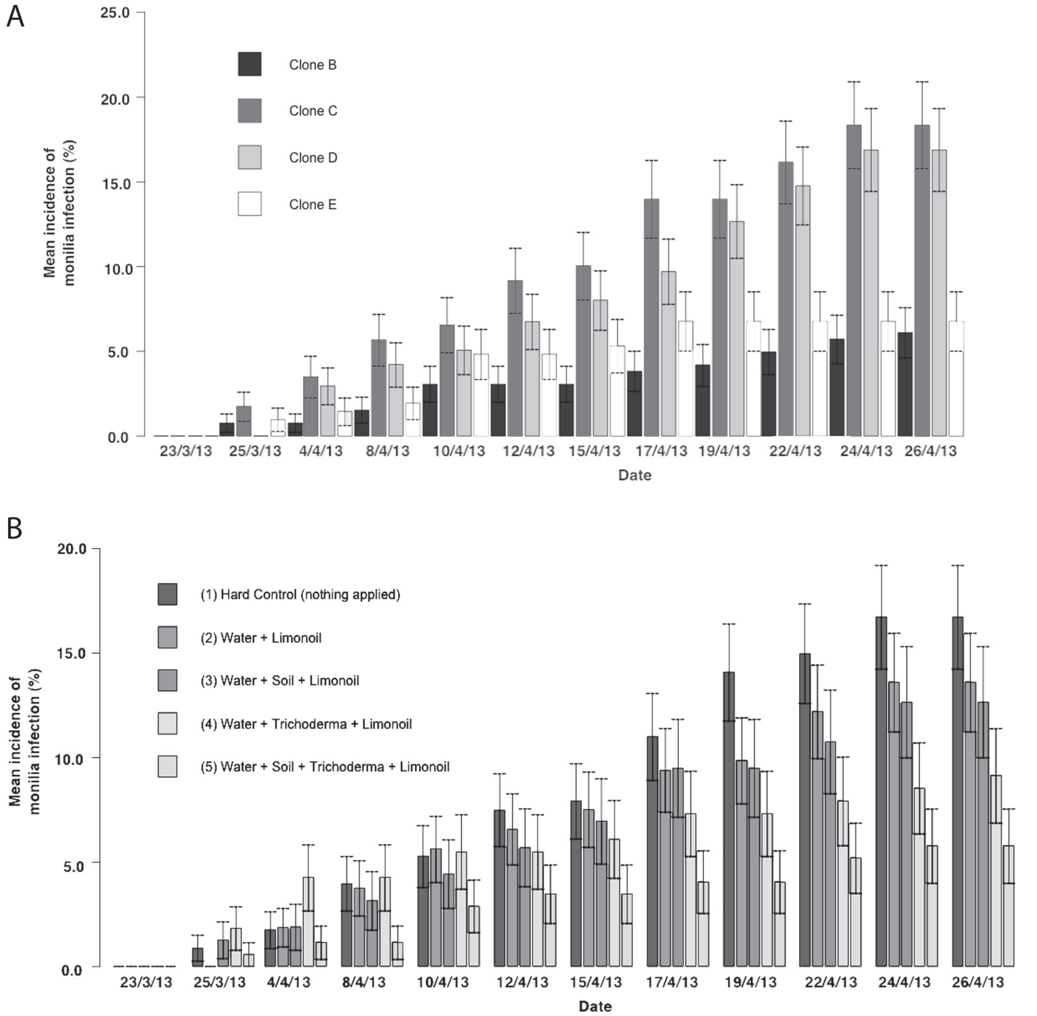

Fig. 3A. Mean incidence of monilia infection at each time point, grouping treatments. Verticle lines are ±1 Tukey 95% confidence intervals. FINMAC plantation, Pueblo Nuevo de Guácimo, Costa Rica. March-April 2013.
Fig. 3B. Mean incidence of monilia infection at each time point, grouping clones. Verticle lines are ±1 Tukey 95% confidence intervals. FINMAC plantation, Pueblo Nuevo de Guácimo, Costa Rica. March-April 2013.

is supported. Krauss & Soberanis (2002) found a 5.2% reduction in mean incidence of monilia infection in eastern Peru with numerous periodical *Trichoderma* sp. sprays over a 4-mo period. Our reduction in monilia mean incidence of infection by twice (11%) that amount was surprising, since our study was only 35d in length with only two *Trichoderma* applications.

Regarding viability of soil as an additive, differences between treatments four and five provided a preliminary justification for the addition of soil to *Trichoderma* sprays as a natural aid to antifungal activity. In this study we propose that the soil added to some treatment sprays provided a nutrient source for *Trichoderma,* aiding in survival and thus improving its ability to fight monilia infection. However, there are also a number of alternate possibilities which remain to be tested. For instance, the soil may simply help the fungi stick to the tree and fruit surfaces. It is also possible that additional soil microbes, which were not killed in the heat-sterilization process, may have had an effect on monilia infection.

In addition to soil, it would be useful to test other nutrient sources, such as fly ash as an organic carbon source. Use of ash in sprays would certainly be cheap but may be difficult to prepare and deliver using a backpack sprayer. Many classic studies of *Trichoderma* sp. have grown cultures in dedicated fungal media such as potato dextrose broth (Hermosa et al., 2000) or malt extract broth (Castle et al., 1998). A high-volume solution of *Trichoderma* culture in pre-sterilized potato dextrose or malt extract broth could easily be inoculated, incubated to grow the fungus to a high concentration, and stored indefinitely. The concentrated stock could then be diluted and efficiently delivered as needed via manual or motorized backpack sprayers.

Given the variation in treatment response between clones, we propose that the genetic variation between different clones has the potential to affect their response to spray treatments. Clone B exhibited an unusually strong response to treatment four and a relative lack of response to treatment five in the reduction of monilia infection incidence and degree. The unique response of Clone B to treatment as compared to other clones suggests that genetic variation in cacao can affect its response to *Trichoderma*-based antifungal spray treatments.

Furthermore, clones B and E tended to experience significantly lower incidences of monilia infection without *Trichoderma* application, implying that genetic variation in cacao also can influence its resistance to monilia infection. However, genetically resistant clones may not have desirable cacao bean production or quality. Studies involving more clones should be performed in order to learn more about the variable response of cacao to monilia and *Trichoderma* throughout various clones. Furthermore, molecular and genetic work incorporating sequence comparisons between tested clones could be used to identify common genes associated with monilia resistance and *Trichoderma* response, thus aiding in the process of clone breeding and selection. Differences between clones in treatment response should be used to inform selection of clones in order to maximize crop yield.

Regarding management implications, though the addition of sterilized soil to a *Trichoderma* spray per treatment five did appear to improve the antifungal activity of this treatment, it was not efficient enough for use as a larger scale treatment, simply because the soil tends to clog the backpack sprayer. Furthermore, heating and sifting enough soil to spray an entire plantation would take a lot of time. One solution to the backpack sprayer problem might be to use motorized backpack sprayers with better filters. Yet the more viable solution would be to develop an organic nutrient additive which is more easily prepared and delivered, such as the broths proposed above.

The 11% reduction in monilia incidence, in only 35d, by treatment five as observed through the course of the study period is in itself economically intriguing. Given such an improvement, the plantation mean productivity of 1 500kg dried beans/ha (Cornwell, 2012) would rise by nearly 200kg/ha to 1 698kg/ha. Treating the entire 110ha plantation with *Trichoderma* and soil would thus increase total mean productivity of dried beans by 21 780kg.

It is important to note that current monilia losses of cacao pods of 25-30% or more per year are common on FINMAC farm. However, even more interesting is to remember this study was only 35d in length, where infection incidence decreased at a positive linear rate (Fig. 3A, 3B). Would infection incidence decrease even more over a 4-mo growing period with periodical *Trichoderma* applications? Could the normal 30% loss be eliminated over this time period? If so, the economic, ecological and social benefits of *Trichoderma* control of monilia, promoting expanded cacao production, would be nothing short of phenomenal.

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RESUMEN

Uso de aspersión fungicida de *Trichoderma* para reducir infección de frutos de *Theobroma cacao* por *Moniliophthora roreri* en el noreste de Costa Rica. El cacao (*Theobroma cacao*) es un cultivo comercial importante en los climas tropicales como los de América Latina. A lo largo de las últimas décadas la infección de cacao cultivado con *Moniliophthora roreri,* conocida comúnmente como "monilia", ha dificultado la producción del cacao en América Latina de manera significativa. Algunos estudios han propuesto el uso del hongo *Trichoderma* sp. en tratamientos de control biológico para prevenir y reducir la infección por monilia. No obstante, pruebas realizadas con tratamientos por aspersión que contenían *Trichoderma* en cultivos de cacao agroforestales produjeron resultados diversos. Investigadores y trabajadores agrícolas han sugerido que la adición de tierra, cenizas volantes u otras fuentes de carbón a la aspersión de *Trichoderma* podría mejorar su eficacia en la lucha contra la monilia. Para probar la validez de estas sugerencias, diseñamos una serie de mezclas para la aspersión que incluían cultivos de *Trichoderma*, tierra y todos los testigos necesarios. Aplicamos aspersiones a 80 árboles de cacao (20 árboles para cada uno de cuatro clones seleccionados anteriormente por su resistencia a la monilia) en la finca de cacao orgánico FINMAC en Pueblo Nuevo de Guácimo, provincia de Limón, noreste de Costa Rica durante marzo y abril de 2013. Se aplicaron cinco tratamientos (testigo, agua, agua con tierra esterilizada, agua con *Trichoderma*, y agua con tierra esterilizada y *Trichoderma*). Se aplicó cada tratamiento a cuatro árboles de cada clon. Medimos la tasa de incidencia de infección por monilia bajo cada tratamiento por aspersión durante 35d. La aplicación de dos aspersiones a los árboles completos con una mezcla de *Trichoderma* y tierra esterilizada redujo la tasa de incidencia de infección por monilia en 11% ($p \leq 0.05$) en solo 35d, en comparación con el tratamiento testigo. Esta reducción en la pérdida de frutos de cacao representa un aumento de 198kg/ha de semillas secas sobre la producción media de 1 500kg/ha, o un aumento total de 21 780kg en toda la plantación de 110ha. Proponemos que el uso de tal aspersión fungicida a lo largo de todo el ciclo de cultivo (120d) produciría una disminución aún mayor de la tasa de incidencia de infección. La aplicación de este método de control fungicida tiene el potencial de revitalizar la producción de cacao en la región.

Palabras clave: *Theobroma cacao*, cacao, control biológico, Costa Rica, fungicida orgánico, monilia, *Trichoderma*.

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