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EFFECTS OF FUNGICIDES ON ENDOPHYTIC FUNGI AND PHOTOSYNTHESIS IN SEEDLINGS OF A TROPICAL TREE, *Guarea guidonia* (*Meliaceae*)

El efecto de fungicidas sobre los hongos endófitos y la fotosíntesis en plántulas de un árbol tropical, *Guarea guidonia* (*Meliaceae*)

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ABSTRACT

Endophytes are microorganisms that live within healthy plant tissues, and include fungi and bacteria. They can be mutualists, comensals or even latent pathogens. Presence of these endosymbionts may affect host physiology, for example by consuming products of photosynthesis (endophytes are heterotrophs) or producing toxic metabolites. In this work two fungicides were used to eliminate fungal endophytes from seedlings of *Guarea guidonia*. Light-saturated photosynthesis (A_{max}) was measured in endophyte-free plants and compared with control plants. Each fungicide killed different fungal endosymbionts. *Phomopsis* was more susceptible to benomyl while *Colletotrichum* was more susceptible to propiconazole. Although suggestive, values of A_{max} were not significantly different for each treatment compared with control plants. No prediction can be made at this point about the final outcome of a given plant-endophytic fungi interaction.

Key words: endophytes, tropics, photosynthesis, fungicides, *Guarea*.

RESUMEN

Numerosos microorganismos viven asintomáticamente en las plantas, hallándose en todos los órganos y tejidos en los que han sido buscados. Estos microbios reciben el nombre de endófitos, y pueden ser bacterias y hongos. Su papel es ampliamente discutido en la actualidad, y se ha encontrado evidencia a favor de una relación mutualista entre el hospedero y el huésped, aunque en otros casos ésta no se ha podido demostrar. El efecto de los endófitos en su hospedero está siendo recientemente estudiado, y algunos casos muestran que pueden alterar algunas funciones de la planta hospedera. En este trabajo se estudió el efecto de la presencia de endófitos sobre la fotosíntesis en plántulas de *Guarea guidonia*, en las cuales se habían eliminado sus endosimbiontes naturales usando dos fungicidas comerciales. Cada fungicida tuvo efecto selectivo sobre diversos grupos de hongos y hubo diferencias en la tasa neta de fotosíntesis en ambos casos al compararlos con el grupo control. Sin embargo, los datos aquí presentados y los previamente publicados muestran que no es posible aún hacer una predicción exacta acerca del efecto de un grupo de hongos endófitos sobre la fisiología de su hospedero natural.

Palabras clave: hongos endófitos, trópico, fungicidas, fotosíntesis, *Guarea*.

INTRODUCTION

The term endophyte is used to designate microorganisms living asymptomatically inside plants (Carroll, 1988). Although plant hosts do not exhibit symptoms, there is an inherent cost of these symbioses to the plant, since endophytic microorganisms are heterotrophs. The autotrophic partner provides food and housing, but what does it receive from the heterotrophic endophytes? If nothing, then the relationship is parasitic and in this case the plant's performance should improve when endophytes are removed. Some endophytic partners are benefical to their hosts because they can increase fitness by, for example, synthesizing chemical compounds that act as defenses against herbivores (Clay, 1988; Arnold *et al.*, 2003).

The exact role and the net outcome of endophytic fungi on their hosts has not been clearly established, and is a matter of current debate. To date, very few attempts have been made to measure the effects of endophytes on plant physiology, and most of these have worked with C3 grasses in temperate climates. This can be addressed by developing experiments in which endophytic microorganisms are eliminated from hosts, and compare physiological performance with naturally infected hosts. The purpose of this work is to study the effect of naturally ocurring endophytic fungi on photosynthetic rates in seedlings of a tropical timber tree. Specifically we want to test if the saturated rate of photosynthesis (A_{max}) is lower in infected plants than in endophyte-free plants.

MATERIALS AND METHODS

Growth and treatment of plants. Seedlings of *Guarea guidonia* were collected from a secondary forest at the El Verde Research Station (EVRS), Luquillo Experimental Forest, Puerto Rico. The EVRS is 400 masl, located at 18° 19' N, 65° 49' W, with an average annual temperatute of 21-25°C, and an average annual precipitation of 3,450 mm (Brown *et al.*, 1983). Only healthy seedlings at the two leaf stage were chosen. Seedlings were individually potted in 4:1 soil:sand and grown for six months in a small clearing in the forest with *G. guidonia* trees nearby. This was done to allow plants to become naturally infected with endophytes. Plants were then moved to a shadehouse at EVRS and fertilized monthly with 17-6-10 Sierra® fertilizer. After seven months the seedlings were moved to a shadehouse at the University of Puerto Rico, Río Piedras campus. Light levels in the shadehouse were 500 μmol m^{-2} s^{-1}, roughly one third of incident light as measured late in the morning. Plants were allowed to acclimatize six month before photosynthesis measurements were taken. To remove fungi plants were sprayed with a solution of propiconazole (Tilt®, 1-{[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl] methyl}-1h-1,2,4-triazole; 3.8 mL/L water), a solution of benomyl (Benlate®, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate; 0.4 g/L), or water alone (for control plants). Plants were sprayed every four days for three weeks. Ten plants were used for each treatment.

Photosynthesis. Light saturated rate of photosynthesis (A_{max}) was measured with the Li-6400 photosynthesis system (LI-COR Inc., Lincoln, NE, USA). Three individuals were selected from each treatment. Measurements were done on the youngest fully expanded, healthy leaf (previously tagged) on each seedling. Measurement light level was set to PFD of 1500 μmol m^{-2} s^{-1}, which was provided by a LED light source. Air temperature in the leaf chamber was controlled at 26 ± 0.1 °C and relative humidity was more than 60%. The incoming air CO_2 concentration in the leaf chamber was set at 360 ppm. The leaf was placed in the leaf chamber and allowed to acclimatize to the conditions in the chamber. The readings were taken after photosynthesis and stomata had stabilized and the rates remained constant. Net photosynthesis readings were taken on two days, April 12 and April 26, 1999.

Isolation and identification of fungi. Fungi were isolated from leaves to determine extent and type of endophyte infection, and ensure that propiconazole and benomyl were effective at eliminating endophytes. The same leaves used for photosynthesis measurements were used. Four 3x5 mm pieces were cut from each leaf. Leaf pieces were surface sterilized by washing in water with Tween 20 surfactant, followed by 70% ethanol for 1 min, 2.6% sodium hypoclorite (50% Clorox®) for 3 min, and 70% ethanol for 0.5 min. After surface sterilization, leaf pieces were plated on potato dextrose agar (12 g Bacto® potato dextrose broth and 20 g agar/L, with 50 ppm each streptomycin, penicillin and tetracycline added after autoclaving), and incubated for at least two weeks at 20-22 °C. Fungi were identified by colony and spore morphology. One of the most common fungi could not be induced to sporulate, and was identified by sequencing the ITS region of nuclear ribosomal DNA using PCR primers ITS1 and ITS4 (White *et al.*, 1990). To determine statistical significance of differences in isolation frequency, number of leaf pieces with and without fungi (and with and without particular fungi) were compared with control leaves using Fisher's exact tests.

RESULTS

Photosynthesis. On both days, net photosynthesis readings were highest for benomyl-treated plants and lowest for propiconazole-treated plants (Table 1). However, differences were not significant at the 0.05 level (p = 0.615, p = 0.098, respectively). Readings on April 26 were higher than on April 12.

	April 12	April 26
Propiconizole	3.98 ± 0.34	4.90 ± 0.15
Benomyl	4.80 ± 0.43	7.43 ± 0.24
Control	4.72 ± 0.45	6.05 ± 0.18
Nested ANOVA:	$F_{2,25} = 0.50$	$F_{2,27} = 2.53$
	$p = 0.615$	$p = 0.098$

Table 1. Mean photosynthesis of *Guarea guidonia* seedlings treated with fungicides (in µmol m^{-2} s^{-1}).

Isolation of fungi. The most commonly isolated fungi from control leaves were *Phomopsis* (in 40% of leaf pieces), *Phyllosticta* (33%), and *Colletotrichum* (14%). No other fungal genera were isolated. Nine percent of leaves yielded two or more fungi, and some leaf pieces also yielded unidentified bacteria. Leaf pieces from fungicide-treated leaves yielded significantly fewer fungi than control leaves (Table 2; $p = 0.007$ for propiconazole, $p < 0.001$ for benomyl). Benomyl-treated leaves yielded significantly fewer fungi than propiconazole-treated leaves ($p = 0.002$).

	Benomyl	Propiconizole	Control
Phomopsis	5	42	40
Colletotrichum	2	0	14
Phyllosticta	7	6	33
Two fungi	2	0	9

Table 2. Frequency of endophytic fungi in fungicide-treated leaves of *Guarea guidonia* (in % of leaf pieces infected).

Phomopsis was significantly less common in benomyl-treated leaves than in control leaves ($p < 0.001$). However, frequency of Phomopsis was not significantly affected by propiconazole ($p = 0.67$). On the other hand, propiconazole significantly reduced frequency of *Colletotrichum* ($p = 0.03$) whereas benomyl did not ($p = 0.06$). Both fungicides significantly reduced frequency of *Phyllosticta* ($p = 0.01$; $p < 0.01$). When the two fungicides were compared to each other, benomyl was significantly better than propiconazole at eliminating *Phomopsis* ($p < 0.001$) but differences in *Colletotrichum* and *Phyllosticta* were not significant ($p = 1$; $p = 1$).

DISCUSSION

The data show an increase in photosynthesis of benomyl-treated plants and a decrease in propiconazole-treated plants, as compared with control plants. Nevertheless, this difference was not statistically significant, which means that we could not demonstrate that photosynthesis in *G. guidonia* seedlings was either enhanced or reduced by the presence of endophytes. Other studies in this field have shown different results. For example, a recent study in maize and banana showed a net decrease in photosynthetic efficiency due to the presence of endophytic fungi (Rodrígues *et al.*, 2000). However, *G. guidonia* is a wild plant, whereas maize and banana were domesticated long time ago. Domestication could have affected the plants' relationships with endophytes by selecting for high rates of photosynthesis under optimal conditions, and lower production of secondary metabolites. On the other hand, there are reports in which endophytic fungi enhanced photosynthetic capabilities of their grass host (Clay, 1990; Marks and Clay, 1996), but Belesky *et al.* (1987) found variable photosynthetic rates and increased stomatal resistance in infected grasses.

Grasses are the most studied group in terms of plant-endophyte interactions. The *Poaceae* has shown to be broadly infected by endophytes (White, 1987), and introduced grasses are protected by endophytic fungi against herbivory, although in natural communities the scenario seems more variable (Saikkonen *et al.*, 1999). The presence of endophytes affects plant hosts from cellular to community level, and contrasting results have been found at different levels including plant community diversity (Spyreas *et al.*, 2001). Recently, soil fertility was demonstrated to be a key factor in determining the final outcome of plant-endophyte interaction (Lehtonen *et al.*, 2005). Taken as a whole, there is not a clear scenario in which a unique status can be attributed to the plant-endosimbiont relationship. It appears as if each plant-endophytic fungi interaction should be studied separately in order to establish the nature of its relationship. Fungi isolated in this study coincide with most abundant taxa found in mature *G. guidonia* trees (Gamboa and Bayman, 2001), which supports the idea that they are real endophytes. Although some of these genera (*Phomopsis* and *Colletotrichum*) include pathogens of many plant species, no disease caused by them on *G. guidonia* has been reported in the literature, nor was any detected since the beginning of our research in 1995.

Benomyl and propiconazole are broad spectrum fungicides currently employed in agriculture, and many studies on the interaction between fungi and vascular plants (for example mycorrhizae) use benomyl as a systemic fungicide in order to get uninfected plants (Fitter and Nichols, 1988; Carey *et al.*, 1992; Merryweather and Fitter, 1996). One of the main properties of this fungicide is the almost total lack of collateral effects (see Fitter and Nichols, 1988). Propiconazole has cytokinin-like properties which could affect physiological aspects of the plants, and although we observed changes in the shape of leaves from plants treated with it, our data show that its effect on photosynthesis was negligible. Both fungicides effectively diminished the load of endophytic fungi, but we observed a differential effect of fungicides on endophytes, since *Phomopsis* was more susceptible to benomyl while *Colletotrichum* was more susceptible to propiconazole. This selective effect was previously reported in barley leaves (Riesen and Close, 1987), where propiconazole diminished populations of only certain endophytes. The meaning of this differential effect of fungicides on endophytes is unclear, and should be addressed carefully.

Carroll (1991) postulated that the boundaries between mutualism and pathogenesis are very thin, and we think that is useful and necessary to set experiments for clarifying the specific role of each species in fungal endophytic assamblages. This could be done by selectively eliminating competitors with fungicides as was done here. Since it is possible that some species are benefical while others are detrimental to the host, the net outcome of this symbiosis will be the summatory of benefits and costs for each of the endosymbionts involved (Bayman *et al.*, 2002). In *Theobroma cacao* (cacao), a neotropical rainforest tree like *Guarea guidonia*, endophyte-free plants developed more lesions than endophyte-infected plants when inoculated with a fungal pathogen (*Phytophtora* sp., Arnold *et al.*, 2003). Effects of endophytes on photosynthesis were not considered in this study. But if an endophyte simultaneously protects its host against pathogens and reduces photosynthesis, its net effect on the host will depend whether light availability or disease resistance is more critical at any given time. In conclusion, no clear generalizations can be made about the effects of endophytes on plants, since available data show a *continuum* of interactions ranging from mutualism to pathogenicity. More experiments dealing with both domesticated and wild plants are necessary, as well as studies of the net effect of endophytes on host fitness.

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