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Trichoderma species form endophytic associations within *Theobroma cacao* trichomes

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ARTICLE INFO

Article history:
Received 21 April 2009
Received in revised form
26 August 2009
Accepted 10 September 2009
Available online 16 September 2009
Corresponding Editor: Paola Bonfante

Keywords:
Fungal endophyte
Theobroma cacao
Trichoderma
Trichome

ABSTRACT

Trichoderma species are usually considered soil organisms that colonize plant roots, sometimes forming a symbiotic relationship. Recent studies demonstrate that *Trichoderma* species are also capable of colonizing the above ground tissues of *Theobroma cacao* (cacao) in what has been characterized as an endophytic relationship. *Trichoderma* species can be re-isolated from surface sterilized cacao stem tissue, including the bark and xylem, the apical meristem, and to a lesser degree from leaves. SEM analysis of cacao stems colonized by strains of four *Trichoderma* species (*Trichoderma ovalisporum*-DIS 70a, *Trichoderma hamatum*-DIS 219b, *Trichoderma koningiopsis*-DIS 172ai, or *Trichoderma harzianum*-DIS 219f) showed a preference for surface colonization of glandular trichomes versus non-glandular trichomes. The *Trichoderma* strains colonized the glandular trichome tips and formed swellings resembling appresoria. Hyphae were observed emerging from the glandular trichomes on surface sterilized stems from cacao seedlings that had been inoculated with each of the four *Trichoderma* strains. Fungal hyphae were observed under the microscope emerging from the trichomes as soon as 6 h after their isolation from surface sterilized cacao seedling stems. Hyphae were also observed, in some cases, emerging from stalk cells opposite the trichome head. Repeated single trichome/hyphae isolations verified that the emerging hyphae were the *Trichoderma* strains with which the cacao seedlings had been inoculated. Strains of four *Trichoderma* species were able to enter glandular trichomes during the colonization of cacao stems where they survived surface sterilization and could be re-isolated. The penetration of cacao trichomes may provide the entry point for *Trichoderma* species into the cacao stem allowing systemic colonization of this tissue.

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Introduction

Theobroma cacao, the source of chocolate, is a native species in tropical forests of Central and South America (Wood & Lass 2001). The putative center of origin of *T. cacao* is the upper waters of the Amazon at the foot of the Andes (Cheesman 1944), currently the borders of Colombia and Ecuador. Cacao trees are vast reserves for endophytic microbial populations (Arnold & Herre 2003; Arnold *et al.* 2003; Evans *et al.* 2003; Rubini *et al.* 2005) including many species of *Trichoderma*, some of which are new species. Newly identified endophytic *Trichoderma* species include *Trichoderma ovalisporum* (Holmes *et al.* 2004), *Trichoderma martiale* (Hanada *et al.* 2008), *Trichoderma stromaticum* (Samuels *et al.* 2000), *Trichoderma theobromicola*, and *Trichoderma paucisporum* (Samuels *et al.* 2006b), *Trichoderma koningiopsis*

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E-mail address: bryan.bailey@ars.usda.gov
0953-7562/$ – see front matter Published by Elsevier Ltd on behalf of The British Mycological Society.
doi:10.1016/j.mycres.2009.09.004

(Samuels et al. 2006a), and *Trichoderma evansii* (Samuels & Ismaiel 2009).

Although *Trichoderma* species are typically considered common soil saprophytes, they are capable of more intimate associations with plant root systems in what has been characterized as an opportunistic avirulent symbiotic relationship (Harman et al. 2004). The critical characteristic of this association is the penetration of the plant's root system by *Trichoderma* and the persistent survival of the fungus within living plant tissues. Recent research results, principally with *T. cacao*, demonstrate that *Trichoderma* species can persist not only within the plant's root system but also within above ground tissues in endophytic associations (Evans et al. 2003; Bailey et al. 2006, 2008).

Many of the endophytic *Trichoderma* species isolated from cacao environments are being studied for their potential to protect cacao against diseases. Black Pod (*Phytophthora* species), Witches' Broom (*Moniliophthora perniciosa*), and Frosty Pod Rot (*Moniliophthora roreri*) are major cacao diseases that colonize above ground tissues. All three diseases occur in South and Central America although their distributions vary, as does their relative importance (Bowers et al. 2001; Wood & Lass 2001). Fungal leaf endophytes are common but for the most part exclude *Trichoderma* species (Arnold & Herre 2003; Arnold et al. 2003; Hanada et al. 2008). Some fungal leaf endophytes have been shown to protect cacao against *Phytophthora* species in a response thought to include induced resistance (Arnold et al. 2003). *Trichoderma* species have also been identified with potential for limiting yield losses due to Witches' Broom (Samuels et al. 2000), Frosty Pod (Holmes et al. 2004), and Black Pod (Tondje et al. 2007; Hanada et al. 2008).

Although *Trichoderma* species have been extensively studied for their abilities to colonize soils and roots, there is very little information on how *Trichoderma* species colonize tissues above ground. The *Trichoderma* strains we are studying were isolated from above ground tissues and have demonstrated potentials for endophytically colonizing the above ground tissues of cacao seedlings (Evans et al. 2003; Bailey et al. 2006, 2008). It is unknown how these *Trichoderma* strains penetrate cacao tissues when establishing the endophytic association. A common structure encountered by microbes on above ground plant parts is the trichome. Trichomes, like root hairs, are modified cells of the epidermis (Hülskamp 2004; Ishida et al. 2008). Trichomes come in many forms but in general terms can be characterized as glandular or non-glandular (Hülskamp 2004; Ishida et al. 2008). Glandular trichomes have the ability to produce and release complex mixtures of metabolites onto plant surfaces (Wagner 1991; Walters et al. 1991). Trichomes are typically discussed in terms of their involvement in drought tolerance, insect resistance, and disease resistance (Wagner 1991; Lai et al. 2000; Wagner et al. 2004). The objective of the research presented here was to characterize the interaction between four *Trichoderma* species and the trichomes of cacao. A complex interaction was observed where *Trichoderma* colonized cacao glandular trichomes both externally and internally, identifying a potential path for further endophytic colonization of the cacao stem and other tissues.

Methods

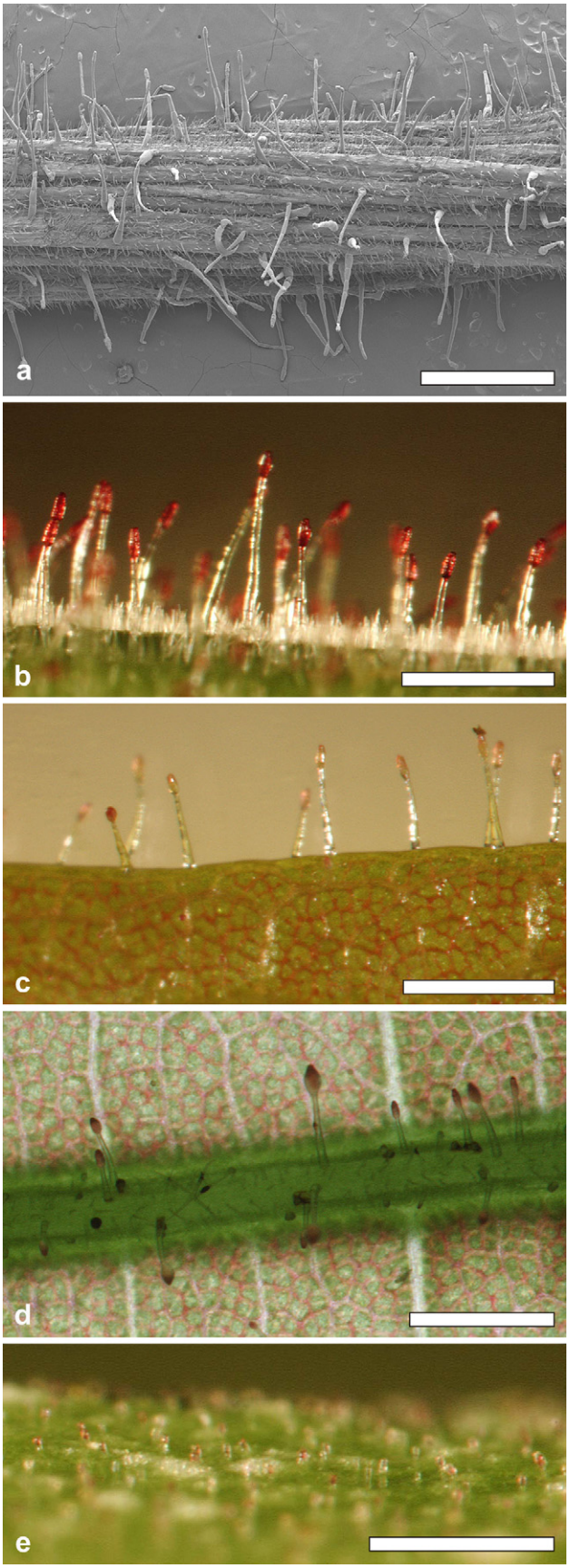
Trichoderma *inoculation*

Four *Trichoderma* strains representing four species of *Trichoderma* were chosen for study (Bailey et al. 2006): DIS 70a (*Trichoderma ovalisporum*, CBS 113299, AY376037), DIS 219b (*Trichoderma hamatum*, CBS 121697, EU248626), DIS 219f (*Trichoderma harzianum*), DIS 172ai (*Trichoderma koningiopsis*, CBS 121697, DQ284972). Strain DIS 219f (FJ463325) is held in the collection of the Systematic Mycology and Microbiology Lab, USDA, ARS, Beltsville, Maryland 20705 (contact Dr. Gary J. Samuels). All of the strains were provided by G.J. Samuels, who identified them by sequencing a 0.65 kb region of the translation-elongation factor 1-alpha (*tef1*) gene as was described by Samuels et al. (2006b); their respective GenBank numbers are given above. The method by which these fungi were originally isolated from the field was described by Evans et al. (2003). The *Trichoderma* strains were isolated from complex tropical environments where *Theobroma* species grow as a part of the natural ecosystem. DIS 70a (*T. ovalisporum*) was isolated from high tropical forest along the Pañ acocha-Río Yanayacu, Napo River, Sucumbios Province, Ecuador in 1999 (Holmes et al. 2004). The tissue was a Witches' Broom on a liana, identified by a local Quechua guide as ayahuasca (*Banisteriopsis caapi*, Malpighiaceae). DIS 219b (*T. hamatum*) and DIS 219f (*T. harzianum*) were isolated from a pod of *Theobroma gileri* found in Guadual, Lita, Esmeraldas Province, Ecuador (Evans et al. 2003). DIS 172ai (*T. koningiopsis*) was isolated from the stem of a 50–60 Y old *Theobroma grandiflorum* tree located in EMBRAPA, Belem, Para, Brazil (Samuels et al. 2006a).

The *Trichoderma* strains were grown on cornmeal agar (1.5 % Agar, Difco Laboratories, Detroit, MI, USA) amended with 20 % dextrose (CDA) and incubated at 23 °C for 5 d without light before use. Two agar plugs (0.5 cm in diameter) were added to soilless mix (2:2:1, sand:perlite:promix), in double magenta boxes (77 × 77 × 194 mm; Magenta, Chicago, IL, USA). Control magenta boxes were uninoculated. The magenta boxes contained 9 cm of sterile soilless mix. Sterile water (25 mL) was added to the soilless mix at the time of inoculation. The sealed magenta boxes were maintained in growth chambers as described below for 14 d before being planted with cacao seed.

Plant materials and microscopy

For the general observations concerning trichome types and distributions, the Sustainable Perennial Crops Laboratory has a cacao collection that includes mature trees grown from seed and therefore of variable genotype (fruit bearing trees more than 3 Y old) and young trees (less than 2 Y old and not flowering) of the verified clones SCA6, ICS1, and CCN51. The pattern of trichome formation was observed on green stems, leaf midribs, and leaf blades for the cacao clones listed above. Cacao pods from mature trees were also studied. The tissues were observed and photographed under a Nikon SMZ1500 dissecting scope (Nikon, Inc., 1300 Walt Whitman Road, Melville, NY, USA) equipped with a Nikon



Digital Camera (DXM1200) at 50–100× magnification. Fresh tissue was harvested from newly emerged orthotropic stems from SCA6, ICS1, and CCN51. The number of each type of five trichome types was counted at three positions on the midrib, 1.5 cm from the base, in the middle, and 1.5 cm from the tip on both the abaxial and adaxial surfaces. In addition, two views of the central blade area were counted on both surfaces. The stem trichomes were counted 4 cm from the apex. All of the counts were normalized to the number of trichomes per square mm. The stem and leaf blade areas were considered flat. Since the leaf midrib is approximately cylindrical, the total outer area of the midrib was determined using the midrib radius and the length of midrib and the formula $A_{MR} = 2\pi rh$. The upper surface area of the midrib was considered approximately flat and area calculated using $A_{UMR} = L \times W$. The area of the lower midrib A_{LMR} was calculated as the midrib surface area (A_{MR}) minus the area of the upper midrib (A_{UMR}).

Trichomes were harvested using a small artist paintbrush. The trichomes were rubbed off the tissues in the presence of 0.5 mL of sterile distilled water. This involved repeatedly dipping the brush in the water and rubbing the tissue surface in contact with the water. The trichomes were transferred to a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA, USA) and photographed under a Nikon Eclipse E600 compound scope (Nikon, Inc., 1300 Walt Whitman Road, Melville, NY 11747-3064, USA) equipped with a Nikon Digital Camera DXM1200 at 200× magnification.

For the *Trichoderma* colonization studies, seeds of *Theobroma cacao* variety comun (Lower Amazon Amelonado type) were used from the Almirante Cacau, Inc. farm (Itabuna, Bahia, Brazil). Seeds were surface sterilized after removal of the seed coat in 14 % sodium hypochlorite for 3 min, and then washed 3 times with sterile distilled water. Sterilized seeds were germinated on 1.5 % water agar plates under fluorescent lights for 3 d at 22 °C. The germinated seeds were planted 3-cm deep into the sterile soilless mix in double magenta boxes without *Trichoderma* (Control) or with *Trichoderma*. There were 3 boxes (replications) for Control and 3 boxes for each of the four *Trichoderma* strains. Seedlings were grown for 21 d in a controlled environment chamber (model M-2, EGC Corp., Chagrin Falls, OH, USA) with the following conditions: 12-h light/12-h dark photoperiod at 25 °C, 50 % or higher relative humidity, and 50 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). The experiment was carried out two times, once for light microscope observation and once for SEM observation, although multiple preliminary experiments were carried out prior to setting the final experimental conditions.

For isolation and plating of trichomes, seedling stems were harvested by cutting the stem 1 cm above the soil line and

Fig 1 – Trichomes associated with cacao tissue. (a) SEM photo of cacao variety comun seedling stem after fixation. (b) Young green stem on cacao clone ICS1. (c) Upper leaf surface and leaf margin on cacao clone ICS1. (d) Lower leaf surface and midrib on cacao clone ICS1. (e) Surface of young pod from cacao variety comun. The fresh tissues (panels b–e) were photographed with a dissecting scope at 50× magnification.

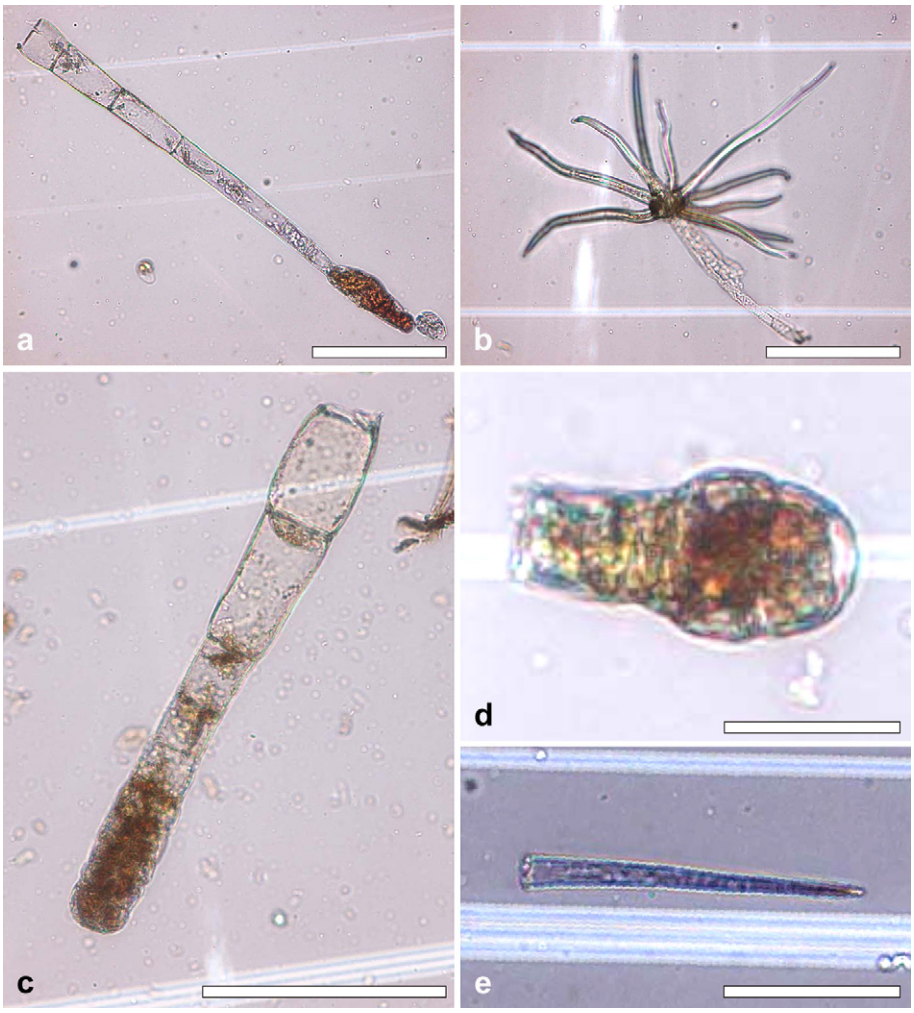


Fig 2 – Trichome types identified on cacao tissues. The trichomes were harvested and transferred to a hemacytometer and photographed using a compound microscope at 200× magnification. (a) Tall glandular trichome from young green stem of cacao clone ICS1. (b) Complex stellate non-glandular hair from young green stem of cacao clone ICS1. (c) Intermediate glandular trichome from cacao variety comun seedling stem. (d) Short glandular trichome from cacao pod. (e) Simple non-glandular hair from stem of cacao clone ICS1. Scale bars: a–c, 125 μm; d, 25 μm; e, 50 μm.

1 cm below the cotyledons resulting in an approximately 6 cm stem section. The stem sections were surface sterilized in 14 % sodium hypochlorite for 3 min followed by three washes in sterile distilled water. In some early studies stem sections were directly plated on water agar plates and observed under the dissecting microscope. Trichomes were isolated using a small artist paintbrush. The trichomes were rubbed off the stem in the presence of 0.5 mL of sterile distilled water. This involved repeatedly dipping the brush in the water and rubbing the stem surface in contact with the water. The trichome in water mixture was spread onto a water agar plate using a sterile bent glass rod. The water agar plates were incubated at 23 °C without light. As soon as 6 h and as late as 20 h after the trichomes were plated they were observed and photographed under the dissecting scope and the compound microscope. Individual trichomes with fungal hyphae emerging were transferred to CDA plates and incubated for up to two weeks as described above. The four *Trichoderma* strains being studied have distinct morphology and culture characteristics that were used to confirm the identities of the isolated cultures. Images of the respective *Trichoderma* species can be seen at Samuels *et al.* (2009). Photographs documenting colony morphology were made with a Nikon COOLPIX 990 digital camera with close ups being made under the dissecting microscope.

For SEM analysis, stem sections were harvested as described above while holding the stem in its middle with forceps. The stem was then dissected into sections (1–1.5 cm each). Sections were placed in fixative (2.5 % glutaraldehyde, 2 % formaldehyde in 0.1 M sodium cacodylate buffer, pH 6.8) in order to allow observation of the *Trichoderma* on the stem surface with minimal disturbance. The stems were then rinsed in 0.1 M sodium cacodylate buffer, pH 6.8 three times, 30 min per rinse and then post-fixed in 1 % aqueous osmium tetroxide for 2 h. The stems were rinsed in deionized water three times, at least 30 min per rinse

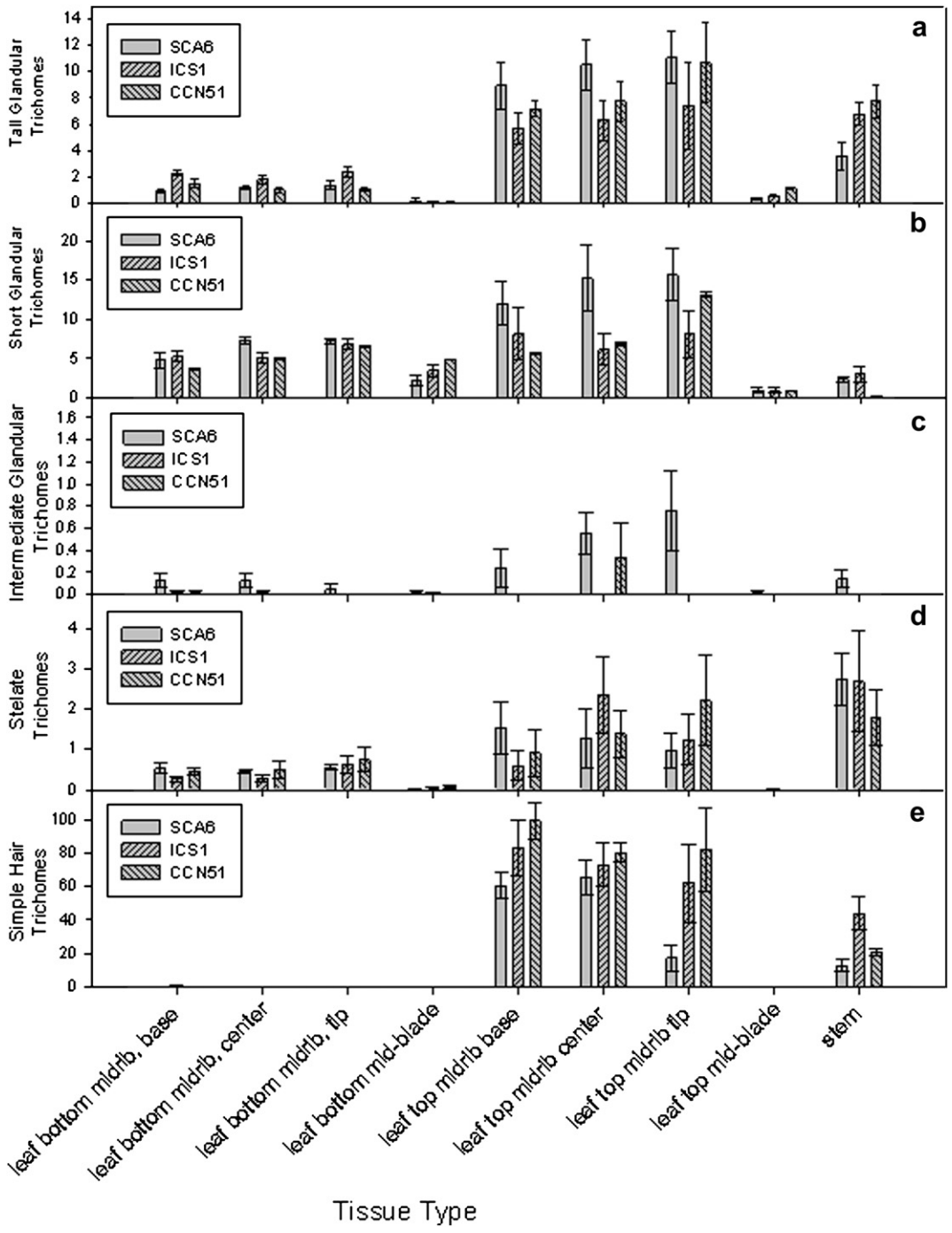


Fig 3 – Trichome densities on stems, leaf blades, and midribs of cacao clones SCA6, ICS1, and CCN51. Fresh tissue was harvested from newly emerged orthotropic stems. The number of each type of five trichome types was counted at three positions on the midrib, 1.5 cm from the base, in the middle, and 1.5 cm from the tip on both the abaxial and adaxial surfaces. In addition, two views of the central blade area were counted on both surfaces. The stem trichomes were counted 4 cm from the apex. All of the counts were normalized to the number of trichomes sq. mm^{-1}.

and dehydrated in a graded ethanol series (30-50-75-95-100 × 3), at least 30 min per exchange. Stems were critical point dried in a Tousimis Autosamdri-815 (Rockville, MD, USA) critical point dryer and each piece was then slit lengthwise with a razor blade. Each slit stem piece was mounted flat-side down onto aluminum specimen stubs using double-adhesive coated carbon tabs (Ted Pella, Inc., Redding, CA, USA) and coated with gold palladium in a Denton Desk II (Denton Vacuum, Inc., Moorestown, NJ, USA) sputter coating unit. The samples were viewed and photographed in a Hitachi S4700 field emission scanning electron microscope (Hitachi, Japan).

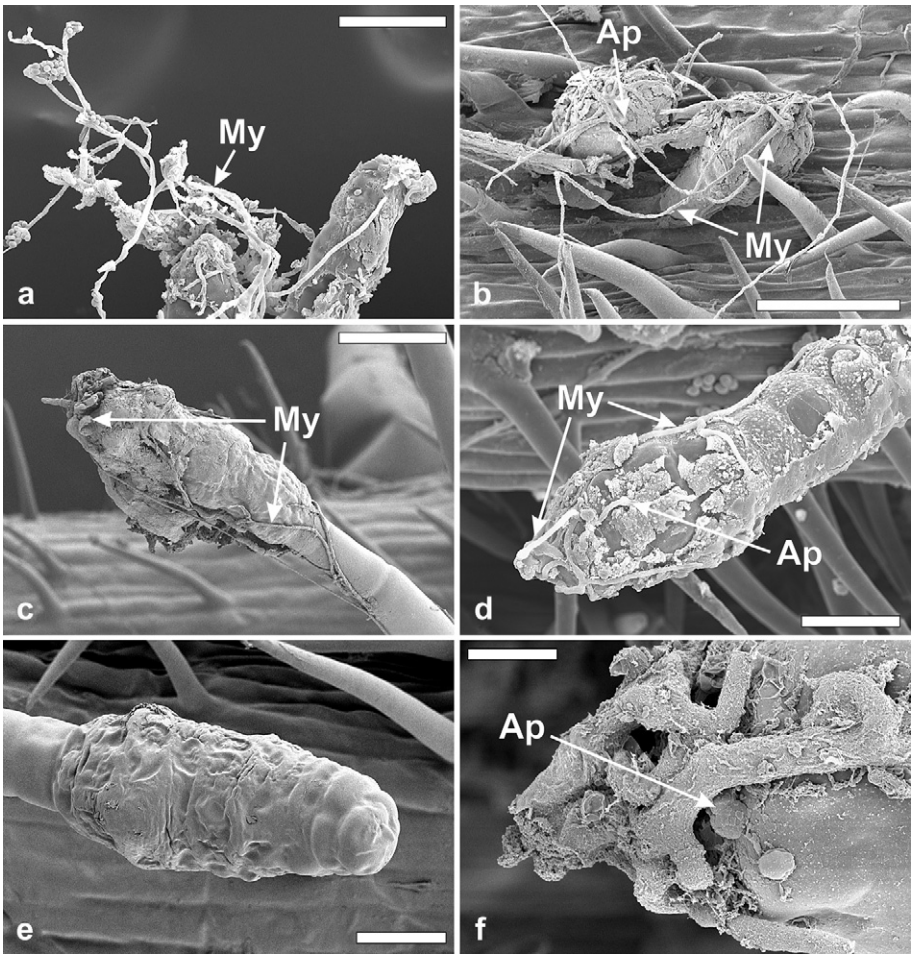


Fig 4 – Surface colonization of cacao seedling glandular trichomes. Stem sections were harvested and fixed as described in the text and photographed by SEM. (a) Tall glandular trichomes colonized by strain DIS 219b. (b) Short glandular trichomes colonized by strain DIS 219f. (c) Tall glandular trichome colonized by DIS 70a. (d) Intermediate glandular trichome colonized by strain DIS 172ai. (e) Tall glandular trichome from an uninoculated cacao seedling. (f) Close up of the trichome tip colonized by strain DIS 219f. Ap, appresorium; My, mycelium. Scale bars: a–c, 50 μm; d, e, 25 μm; f, 5 μm.

Results

There are numerous trichomes of several different shapes and sizes on the hypocotyls of cacao variety comun seedlings (Fig 1a). We were able to identify three types of glandular trichomes (tall, intermediate, and short, Fig 2a,c,d respectively), each with uniseriate stalks and multicellular heads, and two types of non-glandular trichomes (complex stellate hairs and simple hairs, Fig 2b,e respectively) on the leaves, stems, and pods of cacao (Fig 1). When compared to the tall glandular trichomes, the intermediate glandular trichomes tended to have less tapered stalks with a cylindrical secretory head (Fig 2c) and were most commonly found on the hypocotyls of young cacao seedlings. The various trichome types could be identified, at least occasionally, on stems, leaves, and pods. On leaves, trichomes were principally associated with epidermal tissues covering the veins and along the leaf margins (Fig 1c,d). While trichomes can be very dense on cacao leaves in association with leaf veins, the mature leaf blade itself is sparsely covered by trichomes of any type. Cacao pods are, on the other hand, primarily covered with short glandular trichomes (Fig 1e) with large glandular trichomes and non-glandular trichomes being present but sparse in occurrence.

The upper leaf midrib and stem had a higher density of tall glandular, stellate hairs, and simple hairs than the lower leaf midrib or leaf blade (Fig 3a,d,e). The density of short glandular trichomes was higher on the upper leaf midrib and the stem than on the lower leaf midrib or leaf blade (Fig 3b). The density of the different trichome types varied with clone and tissue type. ICS1 had more tall glandular trichomes on the bottom of the leaf midrib than CCN51 or SCA6 while ICS1 had fewer tall glandular trichomes on top of the leaf midrib than CCN51 and SCA6 (Fig 3a). SCA6 had more short glandular trichomes on the top leaf midrib than ICS1 or CCN51 (Fig 3b). CCN51 had very few short glandular Trichomes on the stem compared to the other clones (Fig 3b). Intermediate glandular trichomes were rarely observed on stems and leaves of older plants (Fig 3c). ICS1 had more simple hair trichomes on stems

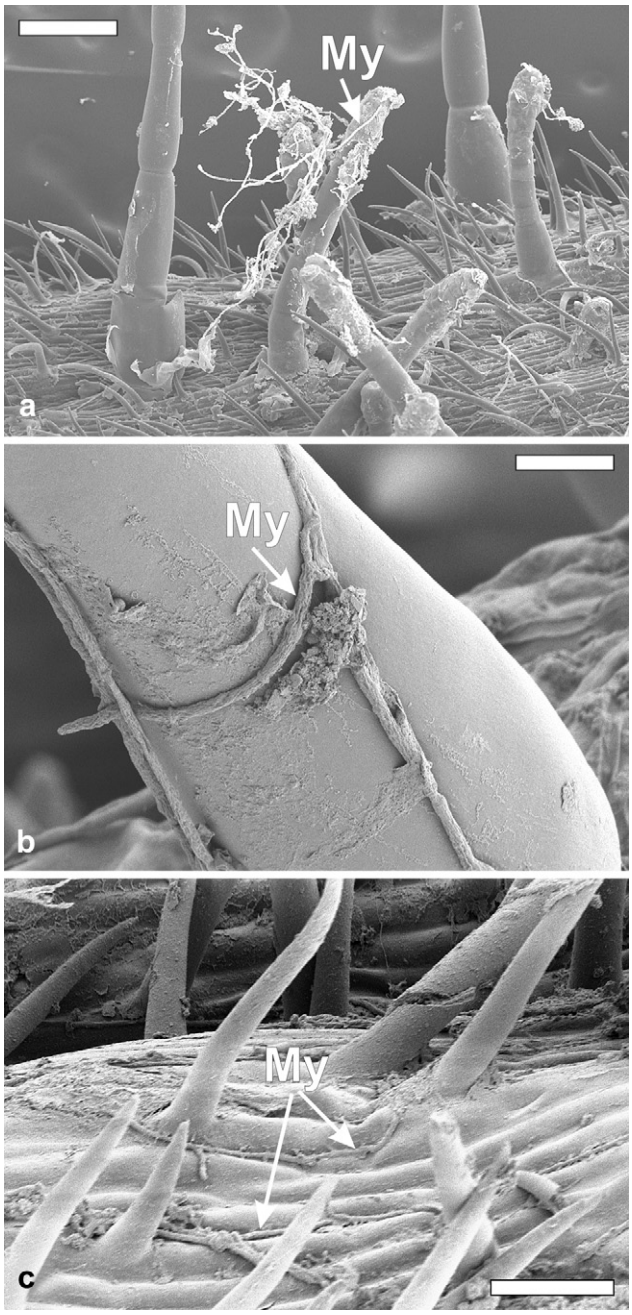


Fig 5 – Association of *Trichoderma* mycelia with the surface of cacao variety comun seedling stems. Stem sections were harvested and fixed as described in the text and photographed by SEM. (a) Strain DIS 219b growing loosely on the stem surface in association with tall glandular trichomes. (b) Mycelia of strain DIS 70a growing in close association with the stalk of a tall glandular trichome on a cacao variety comun seedling stem, (c) Mycelia of strain DIS 219f growing in close association with the stem epidermal surface. My, mycelium. Scale bars: a, 100 μm; b, 10 μm; c, 25 μm.

than SCA6 or CCN1 (Fig 3e). It was also observed that the tall glandular trichomes of the clone SCA6 had much less red pigmentation than those on the other available clones, tending to remain light green on leaves and stems (data not shown).

Colonized cacao stem tissues were examined by SEM in order to better detail the surface interactions between the *Trichoderma* strains and the trichomes in addition to other surfaces. Mycelium of all four *Trichoderma* was observed colonizing the cacao trichomes (Fig 4). Portions of the surface and associated trichomes of some hypocotyl stem segments were covered by a mass of mycelial growth in a loose association (Fig 5a). In other areas the mycelium was observed forming a close association with the stalk cells of trichomes (Fig 5b) and the stem surface (Fig 5c). *Trichoderma* mycelium was not observed in close association with the non-glandular trichomes for any of the four strains studied. On the glandular trichome head the *Trichoderma* mycelium often formed complex multilayered structures (Fig 4f). Within these structures, mycelial swellings were observed that resembled appresoria. In most cases, trichomes colonized by *Trichoderma* were also covered with a granular/powdery material (Fig 4d). This material was not always associated with *Trichoderma* colonized trichomes indicating that it was of plant origin. All three types of glandular trichomes were observed by SEM to be surface colonized by *Trichoderma*. Fig 4b,c,d represent short, tall, and intermediate glandular trichomes respectively.

When stem sections of cacao seedlings colonized by *Trichoderma* were observed within 24 h after surface sterilization, *Trichoderma* hyphae appeared to be emerging from the tips of glandular trichomes (Fig 6). This was a general observation for many different *Trichoderma* species and strains and was not limited to the specific strains of *Trichoderma* shown.

Trichomes were isolated from colonized cacao seedlings after surface sterilization to verify that they were internally colonized by *Trichoderma* species. No *Trichoderma* was isolated from the uninoculated control seedlings. All four *Trichoderma* species studied were re-isolated from glandular trichomes (Fig 7). Intermediate and tall glandular trichomes, but not short glandular trichomes, with emerging *Trichoderma* hyphae were observed and isolated. *Trichoderma* hyphae most often emerged from the trichome head but were also observed emerged from the broken cell in the trichome stalk opposite the head (Fig 7d). On occasion, mycelium emerged from the head and the stalk base of relatively intact trichomes with long multicelled stalks (Fig 8). The efficiency of isolating the inoculated *Trichoderma* strain from the trichomes with emerging hyphae was 100 % for all four strains studied. This included 10 trichomes colonized with DIS 70a, DIS 219b, and DIS 219f and 8 trichomes colonized with DIS 172ai. The colony morphology and spore production patterns were unique for the four strains studied, allowing easy verification of the fungal cultures (Fig 9). The morphology of the *Trichoderma* isolated from each trichome was always consistent with that of the strain with which the source seedling had been inoculated.

Discussion

Cacao trichome diversity

Trichomes, like root hairs, are extensions of cell of the epidermis (Wagner 1991; Ishida *et al.* 2008). The protodermal cells differentiate into trichomes by undergoing a series of

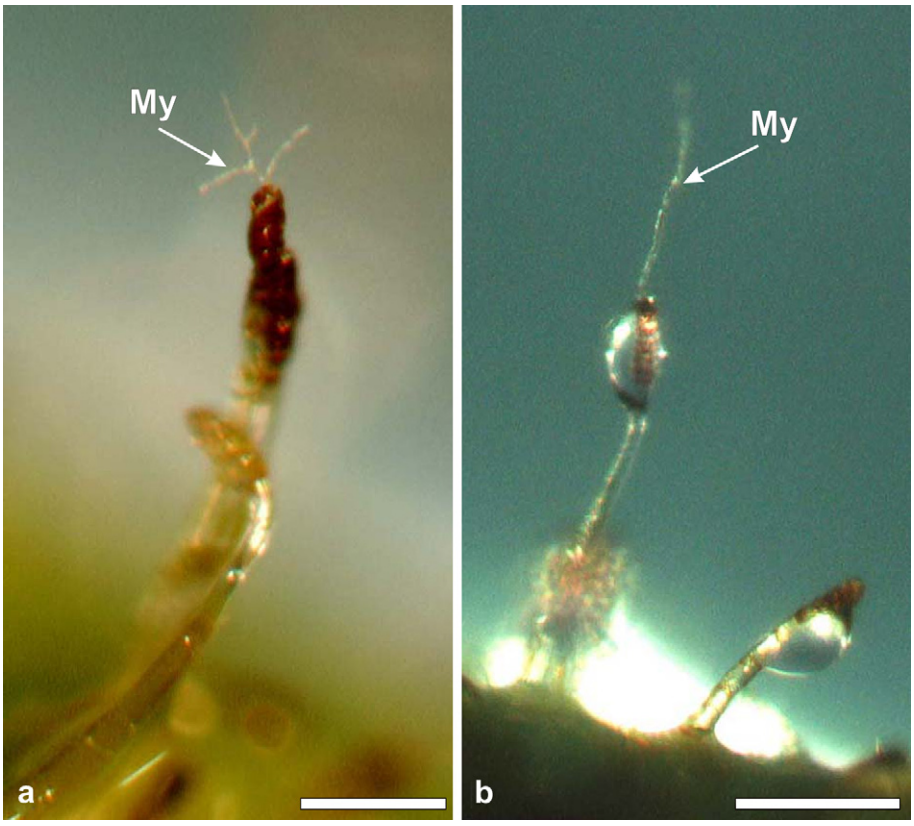


Fig 6 – *Trichoderma* mycelia emerging from tall glandular trichomes attached to surface sterilized cacao variety comun seedling stems: (a) 219f, (b) 219b. Stem sections from colonized seedlings were observed and photographed under the dissecting scope at 112.5× magnification. My, mycelium. Scale bars: a, 100 μm; b, 250 μm.

anticlinal and periclinal cell divisions (Wagner 1991). Glandular trichomes come in many different shapes and sizes. Glandular trichomes have specially developed cells in the trichome head that produce exudates with the potential to contain secondary metabolites with various activities, an important distinction from non-glandular trichomes. The list of functions attributed to glandular trichomes in plants is long and includes providing resistance to insects and diseases (Wagner *et al.* 2004). An example of these functions can be found in *Solanum berthaulltii* a wild potato species. *S. berthaulltii* possesses type A and type B glandular trichomes on its leaf surface that confer resistance to insects. In intercrosses between *S. berthaulltii* and *Solanum tuberosum*, resistance to *Phytophthora infestans* was inversely correlated with type A trichome density and with polyphenol-oxidase (PPO) activity of type A trichome glands. Information on the glandular trichomes of cacao is limited. In this study, we identified three types of glandular trichomes on cacao stems: short, intermediate, and tall, all with multicellular heads (Fig 2). The intermediate and tall glandular trichomes are more complex structurally than the short glandular trichomes. Nakayama *et al.* (1996) identified four types of trichomes on mature leaf and stem of the cacao, two tectorial and two glandular, apparently not distinguishing as unique the intermediate glandular trichomes. The density of the trichomes varied with tissue type (Fig 3). Examples of this include the higher frequency of intermediate glandular trichomes on cacao seedling hypocotyls compared to other tissues, the association of trichomes on cacao leaves with epidermal tissues covering the veins, the higher density of several trichome types on the upper leaf midrib and stems, and the prevalence of short glandular trichomes on cacao pods.

A genetic basis for diversity in trichome type, frequency, and distribution has been documented in association with pest resistance (Taylor 1956; Sorensen *et al.* 1986; Van Dam *et al.* 1999). For example Van Dam *et al.* (1999) characterized the inheritance of sticky (glandular) verses non-sticky (non-glandular) phenotypes in *Datura wrightii* and hypothesized that stress caused by insect feeding was an important factor in maintaining this trait in nature. Susilo *et al.* (2007) found that a higher density of trichomes (short glandular trichomes as described in our study) was associated with resistance of cacao to cocoa pod borer. Here we noted that the density of the most common trichome types, tall glandular trichomes, short glandular trichomes, and simple hair trichomes, varied with clone and tissue type. In addition we observed that the tall glandular trichomes of SCA6 have very little red pigmentation on stems and leaves compared to other clones (data not shown). These observations should be verified on field grown trees under varying environmental conditions.

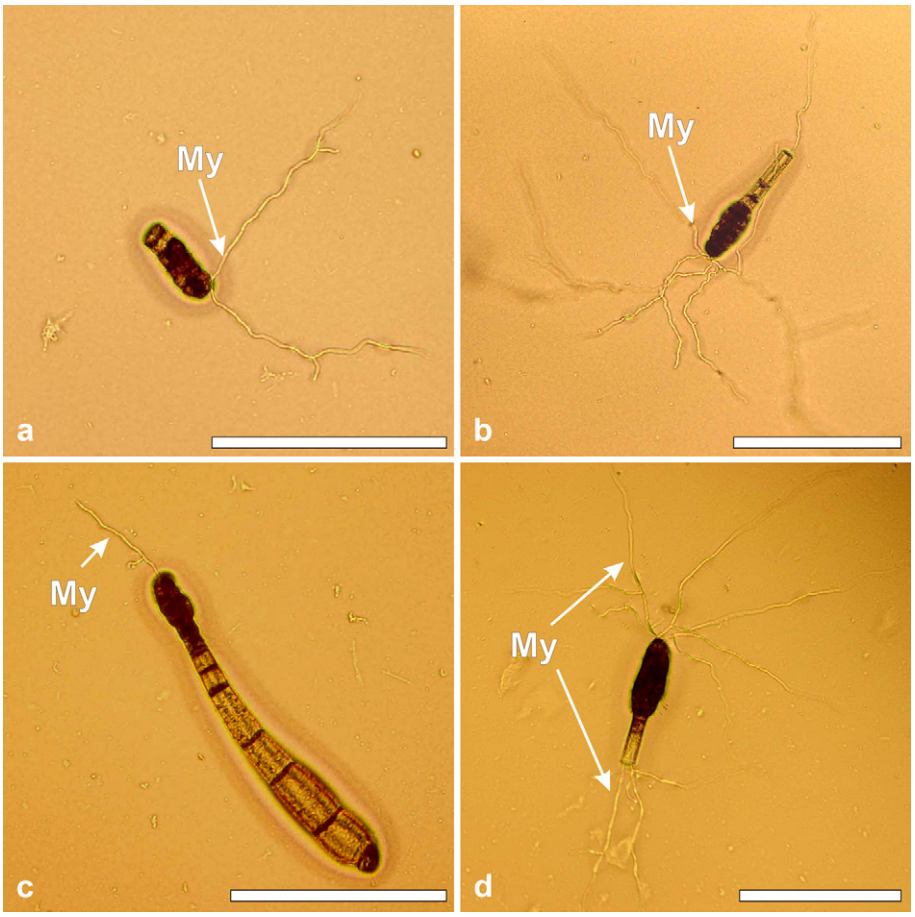


Fig 7 – *Trichoderma* mycelia emerging from trichomes from surface sterilized stems of cacao variety comun seedlings. Trichomes were isolated from colonized stem sections and plated on water agar plates. The trichomes were observed for emerging mycelia and photographed using a compound microscope at 200× magnification. (a) Intermediate glandular trichome with emerging mycelia of *Trichoderma* strain DIS 219b. (b) Tall glandular trichome head with mycelia of strain DIS 219f emerging. (c) Tall glandular trichome with mycelia of strain DIS 70a emerging. (d) Tall glandular trichome head with mycelia of strain DIS 172ai emerging from tip and broken stalk cell. My, mycelium. Scale bars: a–d, 125 μm.

The glandular function of trichomes

Glandular trichomes are encompassed within the cuticle layer, which can either rupture to release the exudates, the exudates may pass through pores, or components of the exudates may volatilize and pass through the cuticle (Sharma *et al.* 2003; Valkama *et al.* 2004). The practical consequences of these possibilities are as follows: A) if the cuticle ruptures on its own the *Trichoderma* could bypass this barrier and have direct access to the trichome cell surfaces and the spaces between cells or B) the *Trichoderma* would need to break down the cuticle to gain access to the trichome cell surfaces and to the spaces between cells. It is unclear which of these two options are employed here. The appresorium-like swelling observed on the trichome head could function during direct penetration of the cuticle. A dried granular matrix was often observed on the heads of glandular trichomes. This material likely originated from the glandular trichomes since it was observed on occasion on trichomes not colonized by *Trichoderma*. Also, the dried granular matrix was observed on all three types of glandular trichomes in association with trichome colonization.

Trichome epiphytic and endophytic colonization by Trichoderma

Trichomes themselves have and are receiving extensive study of late. Although fungal associations with trichomes have been described (Dornelo-Silva & Dianese 2004; Pereira-Carvalho *et al.* 2008), there is little information on endophytic associations between fungi and trichomes of any plant species. Pereira-Carvalho *et al.* (2008) described several new fungal genera associated with the trichomes of neotropical native plants growing in the cerrado of Brazil and suggested that trichomes need further study as an unexplored source of novel fungal diversity. However, the nature of the fungal/trichome association and the types of trichomes (glandular or non-glandular) involved in the interactions were unclear.

Trichome exudates are most often studied for their inhibitory activities against insects and microbes (Wagner 1991; Lai

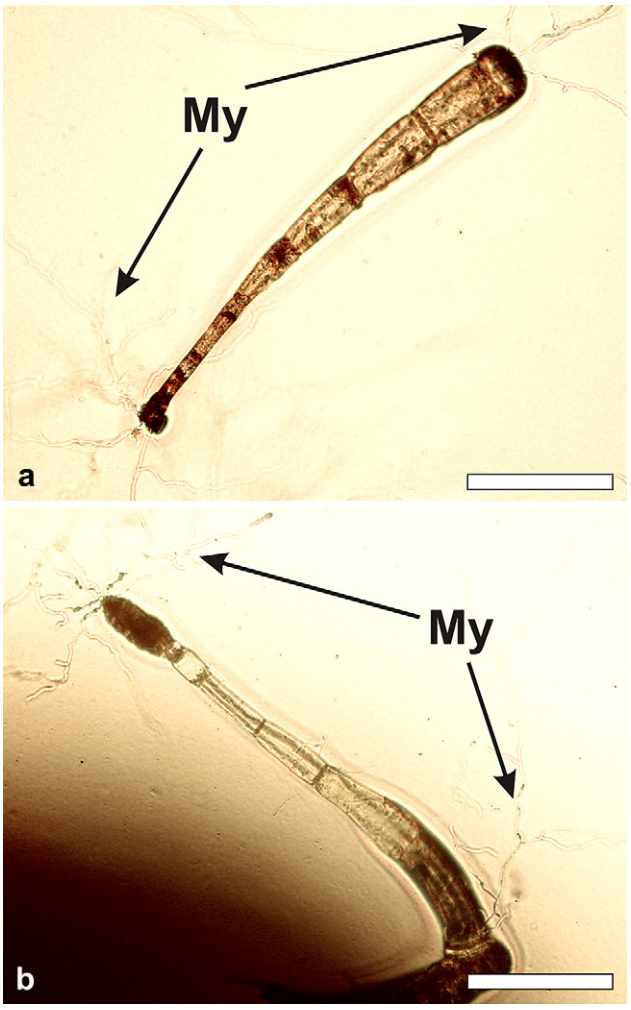


Fig 8 – *Trichoderma* mycelia emerging from the head and basal stalk cell of tall glandular trichomes from cacao variety comun seedling stems. Trichomes were isolated from colonized stem sections and plated on water agar plates. The trichomes were observed for emerging mycelia and photographed using a compound microscope at 200× magnification. (a) Strain DIS 219b, (b) Strain DIS 219f. My, mycelium. Scale bars: a, b, 125 μm.

et al. 2000; Wagner et al. 2004), but in cacao seedlings *Trichoderma* species seem to thrive on the resulting exudates to the extent that the trichomes are actively endophytically colonized. The three types of glandular trichomes were observed by SEM to be surface colonized by *Trichoderma* (Fig 4b,c,d). The association between *Trichoderma* and cacao trichomes was not *Trichoderma* species specific since four distinct *Trichoderma* species were able to colonize cacao seedling glandular trichomes. An indication of special adaptation to colonization was observed both in the development of complex multilayered mycelial structures on the trichome head (Fig 4f) and, within that structure, the formation of mycelial swellings that resemble appresoria.

Root hairs, like trichomes, also differentiate from epidermal cells (Ishida et al. 2008). Molecular genetic analyses using *Arabidopsis* mutants have demonstrated that the differentiation of trichomes and root hair/hairless cells is regulated by similar molecular mechanisms although they work in somewhat opposite fashion (Ishida et al. 2008). A close relationship with root hairs has been described for the *Trichoderma asperellum* T-203, an intensely studied biocontrol fungus, when colonizing roots of cucumber (Yedidia et al. 2000). Hyphae were observed to curl around root hairs and form swellings similar to appresoria. *Trichoderma harzianum* strain T22, characterized as a root competent strain, was shown to colonize root hairs of maize (Harman 2000). Although we were unable to see the penetration of *Trichoderma* through the trichome wall, we were able to demonstrate *Trichoderma* emerging from surface sterilized trichomes for all four species studied. The emerging *Trichoderma* could be observed as soon as 6 h after being plated on water agar. *Trichoderma* was re-isolated from intermediate and tall glandular trichomes but not short glandular trichomes. It is unclear why we could not convincingly re-isolate *Trichoderma* from the short glandular trichomes. We were able to show colonization of the short glandular trichomes by SEM analysis of colonized tissues (Fig 4b). It is possible the short glandular trichomes are not endophytically colonized but it may be more likely that they are unstable once colonized by *Trichoderma*, or, due to their small size, the *Trichoderma* is killed within the trichome during surface sterilization.

Trichomes as a pathway for endophytic colonization of the cacao stem

Trichoderma species have been shown using TEM to penetrate the root directly (Yedidia et al. 2000), although penetration has generally been restricted to the first few cells encountered. *Trichoderma* strains DIS 70a, DIS 219b, DIS 219f, and DIS 172ai have been studied in considerable detail for their endophytic association with above ground cacao tissues (Bailey et al. 2008) and their ability to alter cacao gene expression during colonization (Bailey et al. 2006). Strain DIS 70a (Holmes et al. 2004) and other *Trichoderma* strains are being further studied for their potential to control cacao diseases and ameliorate damage caused by abiotic stresses. We have been able to re-isolate the *Trichoderma* strains studied here from the xylem of cacao seedlings, demonstrating their ability to penetrate deeply within the cacao stem (Bailey et al. 2008). Although we cannot definitively say *Trichoderma* enters more deeply into the stem through glandular trichomes, there are several types of evidence that suggest this is possible. First from our observations on individual isolated glandular trichomes it is clear *Trichoderma* can move through the head into and through the stalk cells (Fig 6). In addition, we were able to isolate relatively intact tall glandular trichomes where the *Trichoderma* hyphae were observed to emerge simultaneously from both the trichome head and the basal cell of the trichome stalk (Fig 7). It should be mentioned that these observations could result from separate penetration events. Since the *Trichoderma* is closely associated with the stem epidermis and the trichome stalk in many areas, penetration could result from these associations. Once through the cuticle, the *Trichoderma* would have to either move through the cells of the trichome stalk or between the cuticle and stalk cells, ultimately penetrating the epidermis into the stem.

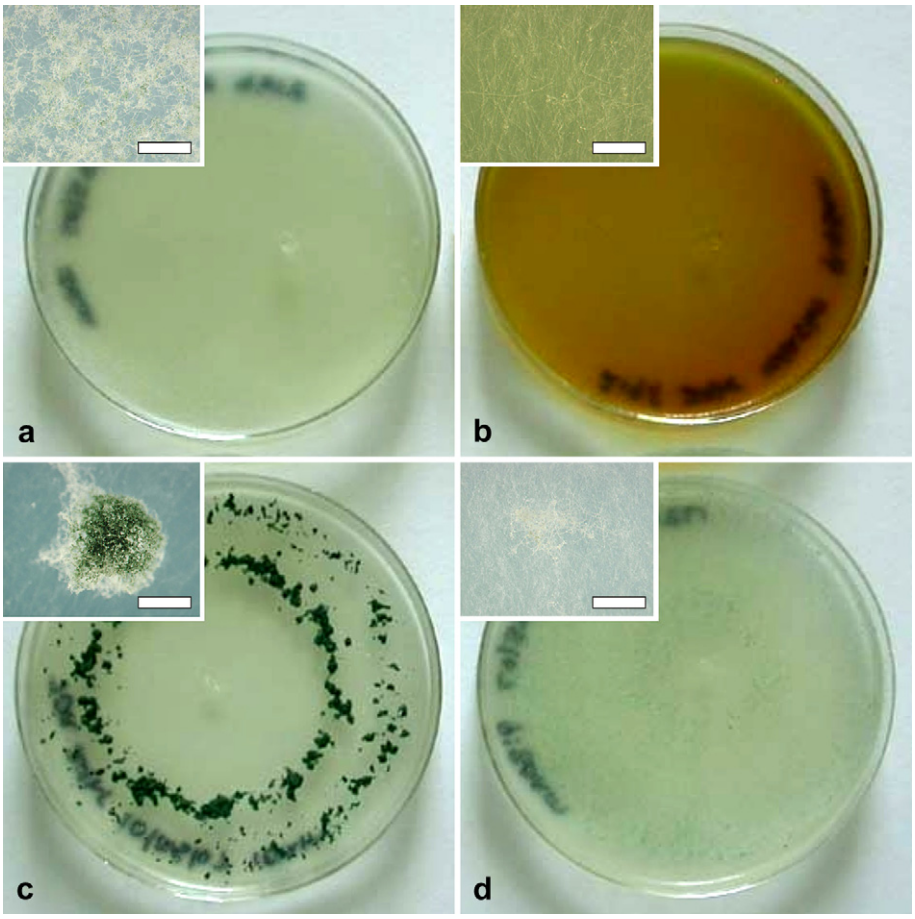


Fig 9 – Characteristic colony morphology of the four *Trichoderma* strains after re-isolation from colonized cacao variety comun seedling stem trichomes. The colonized seedling stems were surface sterilized and the trichomes were isolated as described in the text. Individual trichomes with emerging mycelia were re-isolated and plated on CDA plates. The cultures were grown for up to two weeks in the dark. Culture plates were photographed with a digital camera with the close ups being made with the dissecting microscope at 50× magnification. (a) DIS 219b, (b) DIS 219f, (c) DIS 70a, (d) DIS 172ai. Scale bars: a–d insets, 500 μm.

Conclusion

Four *Trichoderma* strains representing four different species colonized the glandular trichome tips and formed swellings resembling appresoria. The four *Trichoderma* species were able to enter glandular trichomes during the colonization of cacao stems where they survived surface sterilization and could be re-isolated. Cacao trichomes may provide the entry point for *Trichoderma* species into the cacao stem allowing systemic colonization of this tissue contributing to the establishment of this unique endophytic association.

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