Systemic Deployment of *Trichoderma asperellum* in *Theobroma cacao* Regulates Co-occurring Dominant Fungal Endophytes Colonization

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Endophytic *Trichoderma* inoculated into roots have been known to colonize above ground tissues of cacao. In this study, we evaluated *Trichoderma asperellum* spread and impact on endophytic fungi occurring naturally in leaf, stem and root tissues after application through foliar spraying, stem infusion, and soil drenching into cacao seedling of two, four, and five months old respectively. This fungus was isolated from all plant tissues, although by stem infusion was not detected in leaf tissues, and regulated different co-occurring fungal endophytes influenced by seedling age. Dominant endophytes detected were *Fusarium* 1 and 3 in seedlings used for foliar spraying, morphospecies 1 and 3, and *Lasiodiplodia* 1 in seedlings used for stem infusion, and *Lasiodiplodia* 2, 3 and 4, and *Paecilomyces* in seedlings used for soil drenching.

In general, these dominant fungi were more numerous in tissues of the control than in those inoculated with *T. asperellum* over three weeks post inoculation, but instead four weeks post inoculation. The pattern changed in tissues following soil drenching where higher colonization of dominant fungi in treated seedlings began earlier. These data showed that *T. asperellum* can deploy systemically, through the application of foliar spraying, infusion, and soil drenching, to almost all parts of the cacao plant even in the presence of endogenous fungal endophytes and the last fungi their self can reach high colonization in the presence of *T. Asperellum*. Therefore, this presence together of introduced and co-occurrence of endophyte fungi could potentially be used to develop a method for suppressing cacao pathogens.

**Keywords:** Fungal endophytes, foliar spraying, stem infusion, soil drenching, colonization.

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Fungi derive their nutrition from a wide diversity of substrates, and many of them depend upon plants for growth and reproduction. Endophytic fungi have attracted considerable attention in the past four decades, usually, infect and live within living plant tissues without causing any manifestation of a disease. They grow within roots, stems and or leaves, sometimes emerging to sporulate at plant-tissue senescence (Samuels et al., 1979; Stone et al., 2004; Rodriguez et al., 2009). These endophytes are organized into four classes, each defined by the plant tissues it infects, and its transmission. Class 1 endophytes (C-endophytes) are members of the Clavicipitaceae, infect grasses and are seed-borne, growing from seed into leaves. Class 2-4 endophytes belong to many taxonomic groups except the Clavicipitaceae (NC-endophytes), are not borne by seed and occur in the majority of plant groups except grasses (Saikkonen et al., 2002; Arnold and Lutzoni, 2007; Rodriguez et al., 2009).

Every plant species including cacao, *Theobroma cacao* harbors endophytic fungi (Arnold et al., 2000; Herre et al., 2007). In cacao, above ground tissues are endophyte-free at emergence; they accumulate diverse endophytes.
by aerial infection through fungal spores in
the environment. A few species consistently
dominate the endophyte early on and after 2-3
weeks more uncommon species are encountered
(Arnold et al., 2003; Herre et al., 2005; Mejia et
al., 2008). The endophytic assemblage in leaves
is dominated by genera such as Colletotrichum,
Botryosphaeria, Xylaria, and Phomopsis (Arnold
et al., 2003), in branches or twigs dominant genera
are Trichoderma, Pestalotiopsis, Fusarium, and
Lasiodiplodia (Hanada et al., 2010; Rosmana
unpublished data; Rosmana et al., 2013; Rubini et
al., 2005), while in trunks the dominant endophytes
are species of Clonostachys and Trichoderma
(Crozier et al., 2006; Evans et al., 2003). Recent
evidence also shows that Class 3 endophytes
include fungi frequently associated with parasitic
or pathogenic lifestyles (Arnold and Engelbrecht,
2007). These fungal endophytes can play an
important role in host defense (Arnold et al., 2003).

Cultures of Trichoderma endophytes of cacao have been known and used in control of
diseases such as Phytophthora pod rot (Hanada
et al., 2009; Hakkar et al., 2014), witches broom
(Samuels et al., 2000), frosty pod rot (Holmes et
al., 2004), and vascular streak dieback (Rosmana
et al., 2015; Rosmana et al., 2016). Although
Trichoderma is considered effective against these
diseases, there is no evidence that the fungus spread
systemically and maintain the pace of growth
in cacao tissues in nature. The co-occurrence of
glial endophytes mentioned above is one factor
that could limit the establishment and proliferation
of endophytic Trichoderma colonization. If this
case is understood, the use of Trichoderma in the
field is not necessary a major problem because a
single inundative application has at least the
potential to colonize the entire plant. The divers
of application method should be thought with a
view to developing more effective and efficient
biological control of diseases in cacao.

The present study describes a step toward
understanding the distribution of Trichoderma
asperellum in cacao tissues and its impact on co-
occurring fungal endophytes as they can interact
each other affecting the survival and activities of
them. We applied T. asperellum on cacao seedling
firstly by inoculating through foliar spraying,
secondly by stem infusion through exposing to the
cambium, and thirdly by inoculating roots through

soil drenching. We then observed the ability of this
Trichoderma culture to colonize leaves, stems, and
roots and we enumerated its competitive success
with other seedling associated fungi. The three
application methods were done separately and not
at the same time using different seedlings; this was
for permitting to obtain more divers of endophytic
fungi

MATERIALS AND METHODS

Source and preparation of Trichoderma for
treatment

The study used Trichoderma asperellum
strain ART-4/G.J.S. 09-1559 from the collection of
the Cocoa Research Group, Faculty of Agriculture
Hasanuddin University. This fungus was cultured
on potato dextrose agar (PDA) in 9 cm diameter
Petri dishes for seven days, by which time they
had colonized the entire surface of the Petri dishes.
Cultures were then flooded with 10 mL of sterile
water, and the surface was scraped with the spatula.
The liquid containing conidia and mycelium was
then decanted into 250 mL Erlenmeyer flasks. The
flasks were shaken to separate the mycelium
from spore suspension, the contents of the flask
were filtered through sterile muslin cloth. Spore
concentrations were adjusted to approximately
106 spores/mL and these spores were used for
inoculation experiments.

Inoculation of Trichoderma asperellum into
cacao seedlings

Cacao beans of MCC 1 clone were
germinated in layers moist cloth and those that
showed the strongest germination were then
planted in poly-bags containing approximately
1.5 kg nonsterile soil. These seedlings were grown
in the greenhouse with the temperature range
of 27°C to 32°C and humidity range of 78% to
90%, to permit infection by fungal spores from
the environment. After two, four and five months
old, seedlings were inoculated with T. asperellum
through respectively foliar spraying, stem infusion,
and soil drenching.

For foliar spraying, 106 spores/mL T.
asperellum were suspended in 250 ml sterile
water and then sprayed a whole quantity of
this suspension using a hand sprayer onto the
surface of three until four young leaves in each
seedling. Before spraying, the soil surface was
covered with plastic to prevent the *Trichoderma* from entering the plants through soil. In the stem infusion experiment, the same quantity of 250 mL suspension containing 10^6 spores were filled into 1.5 L plastic bottle and by hanging and reversing of this bottle, the suspension infused into each seedling via a small hose attached to a disposable syringe with the needle placed under the bark but above the cambium. The procedure used in this inoculation technique is the same as that used in side grafting: a reverse V-cut was made in the stem by making a triangle incision on the bark begun from the top, but without the horizontal incision. This results in a pocket into which the needle of the syringe can be inserted. Once inserted, the entire site is bound with the fine cord to prevent leakage of any of the inoculum. For the soil drenching, 10^6 spores suspended in 250 mL was just flushed gradually into soil surface the stem of the seedling. Each experiment consisted of 20 seedlings inoculated with *T. asperellum* and 20 seedlings as control, which was not inoculated with *T. asperellum*; therefore, a total of 120 seedlings were used in the three experiments.

**Assessment of distribution and competitiveness of endophytic *Trichoderma asperellum***

The presence of *T. asperellum* and its competitiveness with other fungi found in leaf, stem, and root tissues was observed after the first, second, third, and fourth-week post inoculation by sampling five treated and five untreated seedlings. Leaves were cut into 1 x 0.5 cm^2^ pieces; stems and roots were cut into 1 cm sections after removing their bark. Five pieces of leaves and five sections of stem and roots respectively were sterilized in 0.5% sodium hypochlorite for three minutes, 70% ethanol for two minutes and vigorously washed several times in sterile distilled water before being placed onto PDA in Petri dishes. These Petri dishes were incubated at room temperature and examined every day for the presence of *Trichoderma* and other fungi.

The colonization of *Trichoderma* and other fungi in cacao seedling tissues was calculated using the formula of $C = a/b \times 100\%$ where $C$ was colonization percentage; $a$ was the number of pieces or sections containing the fungi and $b$ was the total number of parts in Petri dishes.

**Identification of fungi**

The fungi associated with seedling was distinguished based upon the morphological characteristics of their cultures in PDA medium including growth rate, mycelial texture, colony density, colony color both at the upper surface and the lower surface, zonation, and production of pigments. In addition, with the aid of a light microscope, hyphae septation and branching as well as size, shape, or color of conidiophores, phialides and conidia were also observed. This assessment of characteristics was used in taxonomic keys for identification of fungi (Barnett, 1998; Kiffer, 1997).

**Analysis**

The occurrence of *T. asperellum* in treated and untreated cacao seedling tissues was analyzed without any transformation. A T-test was then used for evaluating significant differences between means in the two. While for co-occurring dominant fungal endophytes, statistical analysis was not done, because sometimes the number of morphospecies in treated and untreated was different.

**RESULTS**

**Reisolation of *Trichoderma asperellum* from cacao seedling tissues**

*Trichoderma asperellum* was reisolated from all inoculated seedlings at all sampling times. In the uninoculated seedlings, *Trichoderma* was either not detected or occurred at a level that was far below that observed in the inoculated seedlings (Figure 1, 2 and 3). Therefore, we are confident that the *Trichoderma* that we isolated is the one that was inoculated.

A sampling of seedling tissues weekly over four weeks post-inoculation through foliar spraying showed that *T. asperellum* was capable of rapidly moving from the site of inoculation to stems and roots of the seedlings (Figure 1). The percentage of colonization dropped over time in each of the tissues and four weeks post-inoculation *T. asperellum* was isolated from just 4.0% of leaf pieces and 24.0% of the stem pieces; it was not recovered from root pieces (Figure 1).

Following stem infusion, isolations were made weekly over four weeks post-inoculation as described above (Figure 2). *T. asperellum* colonized the stem progressively and moved slowly into roots, but did not rise into leaves. The colonization...
Fig. 1. Colonization of *Trichoderma asperellum* in leaf (A), stem (B), and root (C) tissues at one, two, three, and four weeks post-inoculation through foliar spraying. Means of colonization at the same time followed by the same letter are not significantly different according to T-test (P<0.05)
percentage of stems rose from 36% one week post-inoculation to a maximum of 84% three weeks post-inoculation and then fell to 28% after four weeks. On the other hand, colonization of roots increased steadily from 0% one week post-inoculation to a maximum of 32% four weeks post-inoculation. In control seedlings, *Trichoderma* was observed to colonize 4.0% of stem and 4.0% root tissues one and two weeks post-inoculation.

Application of *T. asperellum* through soil drenching demonstrated that this fungus could reach, penetrate and colonize roots and then rise to colonize stems and leaves. However, there were no clear patterns of recovery of *T. asperellum* from leaves (12%, 16%, 24%, 16%) and stems (8%, 40%, 8%, 24%), respectively, over four weeks post-inoculation beyond the fact that a certain percentage of these tissues remained colonized by the endophyte. On the other hand, colonization of root pieces reached a maximum one week post-inoculation and remained steady until the percentage colonization dropped four weeks post-inoculation (28%, 24%, 28%, 16% respectively). As with leaves and stems, *T. asperellum* could always be reisolated from a certain percentage of plants. In control seedlings, *Trichoderma* was found to colonize 4.0% of root tissues three weeks post-inoculation.

**Fungi associated with cacao seedlings and their competitiveness with *T. asperellum***

**Fig. 2.** Colonization of *Trichoderma asperellum* in stem (B) and root (C) tissues at one, two, three, and four weeks post-inoculation through stem infusion. The fungus was not detected in leaf tissues. Means of colonization at the same time followed by the same letter are not significantly different according to T-test (P≤0.05)

Fig. 3. Colonization of *Trichoderma asperellum* in leaf (A), stem (B) and root (C) tissues at one, two, three, and four weeks post-inoculation through soil drenching. Means of colonization at the same time followed by the same letter are not significantly different according to T-test ($P<0.05$).
Roots, stems, and leaves of the plants that had been inoculated with *T. asperellum* by the respective methods were sampled for the presence of fungi other than *T. asperellum*. These fungi were characterized by their morphological and cultural characters and classified into morphospecies on these bases. The occurrence of these fungi in tissues of inoculated seedlings was compared to their occurrence in uninoculated seedlings. These fungi were ambient in the environment in which the cacao seedlings were incubated. Infection of the seedlings by them was incidental. In general incidental dominant fungi were more numerous in tissues of the uninoculated, control seedlings than in those that had been inoculated with *T. asperellum* over three weeks post-inoculation, but instead four weeks post inoculation (Figure 4 and 5) This pattern was changed in leaves, stems, and roots following soil drenching where higher colonization of dominant fungi in treated seedlings was begun earlier (Figure 6).

In two months old seedlings that were inoculated with *T. asperellum* by foliar spraying, *Fusarium* 1 and 3 were dominant in stems and root and a small numerous in leaves. Others, including *Fusarium* 2 and 4, and *Rhizoctonia* were recovered from roots, and *Aspergillus* was isolated a few times from leaves. In the presence of *T. asperellum*, in general colonization of the two dominant *Fusarium* in the stem and root tissues

![Bar chart](chart.png)

**Fig. 4.** Dominant fungal endophytes colonizing stem (B) and root tissues (C) of two-month-old seedling at one, two, three, and four weeks after Trichoderma asperellum inoculation through foliar spraying. +, with *T. asperellum*; -, without *T. asperellum*
Fig. 5. Dominant fungal endophytes colonizing leaf (A), stem (B) and root tissues (C) of four-month-old seedling at one, two, three, and four weeks after *Trichoderma asperellum* inoculation through stem infusion. +, with *T. asperellum*; -, without *T. asperellum*
increased respectively from 4.0% one week post-inoculation to respectively 28.0% and 16.0% four weeks post-inoculation. Contrarily, in the control of same tissues, the colonization of these fungi decreased from respectively 48.0%, and 20.0% one week-post inoculation to respectively 32.0%.

Fig. 6. Dominant fungal endophytes colonizing leaf (A), stem (B) and root tissues (C) of five-month-old seedling at one, two, three, and four weeks after *Trichoderma asperellum* inoculation through soil drenching. +, with *T. asperellum*; -, without *T. asperellum*
and 4.0% four weeks post-inoculation.

In four-month-old seedlings used for stem infusion, *Rhizoctonia, Verticillium, Lasiodiplodia 1, Aspergillus*, and three unidentified fungi were found, but the most abundant was *Lasiodiplodia 1* and morphospecies 1 and 3. (Figure 5). Colonization of these dominant endophytes were more numerous in seedling treated than those not treated four weeks post-inoculation of *T. asperellum*. The pattern of its recovery in leave, stem, and root tissues of seedling treated in this time was 20.0%, 24.0%, and 24.0% respectively, while in the control was 16.0%, 8.0%, and 20.0% respectively (Figur 2).

In five-month-old seedling leaves used for soil drenching, we isolated fungi including *Lasidioplidia 3, and 2, Lasiodiplodia 1*, and *Aspergillus*. The same fungi were also isolated from stems and roots with exception of *Aspergillus*. However, the most dominant was the first four. In the presence of *T. asperellum*, these dominant endophytes did not observe to colonize leaf tissues one-week post-inoculation, while in the control, it is observed 28.0% of colonization. Conversely, two, three and four weeks post-inoculation, these endophytes were more numerous in those treated with colonization of 52.0%, 24.0%, and 44.0% respectively than in the control with colonization of 40.0%, 12.0%, and 36.0% respectively. In stem tissues treated, dominant endophytes were less numerous one week and two weeks post-inoculation with colonization of 8.0% and 12.0% respectively than the control with colonization of 12.0% and 44.0% respectively, while to the contrary three and four weeks post-inoculation these endophytes were numerous in those treated with colonization of 52.0% and 20.0% respectively than the control with colonization of 32.0% and 0.0% respectively. The dominant endophytes in root tissues treated were just observed numerously three weeks post-inoculation with the colonization of 40.0% compared to 36.0% in control (Figure 6).

**DISCUSSION**

The results presented here demonstrate the ability of an endophytic culture *T. asperellum* to spread quickly from its point of inoculation in the leaf, vascular cambium and root to all other parts of cacao seedlings. Previous studies showed also that *Trichoderma* can be re-isolated as the endophyte from all seedling tissues, but in general after application through seeds and roots under gnotobiotic conditions (Bailey et al. 2006; Bailey et al. 2008; Rosmana et al., 2015). Our trial was made in an unsterilized environment in a green house but the control seedlings typically were not infected with any *Trichoderma*. Our results indicate that horizontal transmission is not important in the spread of *Trichoderma* endophytes.

Association of *Trichoderma* with root systems has been extensively studied and is well characterized (Harman, 2000; Harman et al., 2004). An intense study of root colonization by *T. asperellum T-203* showed their hyphae curl around root hairs and form swellings similar to appressoria (Yedidia et al., 2000). In early attachment, hydrophobin proteins are involved and then swollenin protein plays a role in loosening the root cell wall by expanding cellulose fibers, therefore extenuating the action of a vast arsenal of cell wall degrading cellulases (Brotman et al., 2008; Yedidia et al., 2000) and finally, intercellular penetration to a depth of a few cells into the root cortex (Harman et al., 2004). However, few studies have considered the interaction between *Trichoderma* and aerial parts of plants. Many studies show that *Trichoderma* is found in leaves, pods, branches, and stem bark (e.g., Hanada et al., 2010; Rosmana et al., 2015; Rubini et al., 2005). However, the mechanism by which *Trichoderma* enters plants through their aerial parts remains a mystery. Indeed, plant defenses such as the cuticle that covers the surface of leaves, including their stomata, hidatode, and trichomes, can inhibit penetration by *Trichoderma*. Rabdocline parkeri, an endophyte of douglas fir, produces fine penetration hyphae that penetrate the surface of healthy leaves (Stone, 1988), while *Cladosporium cladosporioides* and *Alternaria alternata*, endophytes of *Juncus* spp., infect leaves through stomata (Cabral et al., 1993). One study has shown that a *Trichoderma* can colonize glandular trichomes and form swellings resembling appressoria (Bailey et al., 2009), indicating the fungus can probably penetrate them. In this research, we sprayed *T. asperellum* onto young leaves where the trichomes were still abundant. *Trichoderma* may also be able to penetrate the stem.
surface. It has been suggested that *Trichoderma* penetrates the stem through the thin periderm, lenticels, leaf scars or scars of bud scales (Baum et al., 2003; Ouellette et al., 1995; Stone, 1987) and also stem trichomes (Bailey et al., 2009). But here, we inoculated *T. asperellum* through infusion directly into the vascular cambium, making it easier for the *Trichoderma* to penetrate into the plant. *Trichoderma harzianum* is capable of penetrating the wood of grapevines from treated pruning wounds (Harvey and Hunt, 2006).

When *Trichoderma* was inoculated through the soil, this fungus could be re-isolated from xylem, bark, apical meristem, stem trichomes, and to a lesser degree from leaves (Bailey et al., 2008; Bailey et al., 2009). This indicates that *Trichoderma* can spread from roots to other parts of cacao tissues through the xylem. We always found *T. asperellum* not only in roots but also in stems and leaves from the first week to the fourth-week post-inoculation through soil drenching. Therefore, our finding support xylem as being the facilitator of long distance spread of *Trichoderma*. When we applied *T. asperellum* through foliar spraying, we found it also in leaves, stems, and roots, but colonization tended to decrease with the time and even four weeks post infection *Trichoderma* was found in leaves but no longer in roots. This demonstrates that *Trichoderma* can descend from leaves to roots, probably through the phloem. When we inoculated with *T. asperellum* into the vascular cambium through stem infusion, we found it only in the stems and roots but not leaves, suggesting its spread through the vascular cambium or phloem.

At emergence from seed, cacao tissues are endophyte-free. They are subsequently contaminated by diverse endophytes from spore sources in the environment.

Within two until three weeks a few species that are consistently dominant members of the assemblage heavily colonize the leaves and they are joined a larger number of exceedingly rare fungi (Arnold et al., 2003; Herre et al., 2005; Mejia et al., 2008). Our results support these findings by showing that endophytic fungi from the two-month-old cacao seedlings that were used for foliar spraying of *Trichoderma* were dominated by *Fusarium*1 and 3, which colonized stems and roots. While, the endophytic fungi isolated from four-month-old cacao seedlings that had been inoculated through the vascular cambium were dominated by *Lasiodiplodia* 1 and two unidentified fungi in leaves, stems, and roots. The endophytes found in five month old seedlings that were inoculated with *T. asperellum* through soil drenching were dominated by *Lasiodiplodia* 2, 3, and 4, and *Paecilomyces* and colonizing leaves stems and roots.

*Trichoderma asperellum* did not limit seedling tissue colonization by all dominant endophytic fungi mentioned above. In general, they were less numerous at the initial phase of *Trichoderma* presence and turned to become more numerous colonization than the control at next phase of up to four weeks (Figure 4 and 5). Moreover, when the number of dominant endophytes increased in five-month-old seedlings, we observed markedly the more numerous colonization especially in leaf and stem tissues treated than in untreated one (Figure 6). It is apparently that *Trichoderma* could enhance them to colonize seedling tissues. With this fact, we suggest that in cacao disease suppression, *T. asperellum* can jointly to make a consortium with dominant fungal endophyte present in certain period of cacao development phase. *Fusarium* species are very diverse in cacao, and many studies show that fungus can serve as a control agent of cacao diseases (Arnold et al., 2003; Hanada et al., 2010). While, no report concerning the use of *Lasiodiplodia* for controlling the disease on cacao and so far six morphospecies have been found (A. Asman, unpublished data). *Paecilomyces* have also known can inhibit *Phytophthora palmivora*, the causal agent of Phytophthora pod rot on cacao (Adebola and Amadi, 2010). Some species of *Fusarium* and *Lasiodiplodia* can cause disease on cacao such as dieback (Adu-Acheampong, 2011, del Castillo et al., 2016, Rosmana et al., 2013), but the disease is just expressed in stress conditions (Burgess and Bryden, 2012; Müllen et al., 1991; Rosmana et al., 2013). Therefore, their presence if any in assemblage will support apparently to compete with the major pathogen.

Even in the presence of endophytic fungi mentioned above, *T. asperellum* was able to consistently colonize over four weeks the seedling leaves, stems, and roots, especially following soil drenching. The ability of *Trichoderma* to colonize roots has been used as a selectable trait (Harman et al., 2004). However, its presence at the same time
in leaves and stems would offer localized effects either via direct pathogen inhibition or via localized induction of host defensive pathways (Aneja et al., 2006, Bailey et al., 2006), therefore Trichoderma would be capable of reducing cacao diseases, most of which act on above-ground tissues. In addition, the capacity to regulate co-occurrence dominant fungal endophytes, it would reinforce Trichoderma for more persistent in the suppression of the diseases.

We conclude that Trichoderma asperellum can be applied through foliar spraying, stem infusion, soil drenching. These three methods can be used as an option and can be adapted according to age and phase development of cacao for efficiency. Colonization of this fungus in leaves, stems, and roots did not cause a shift, but more to a regulation in the colonization of fungal endophyte community and this could potentially be used to develop a method in crop management.

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