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# **Sudden death of *Theobroma cacao* L. caused by *Verticillium dahliae* Kleb. in Peru and its *in vitro* biocontrol**

## **Muerte repentina de *Theobroma cacao* L. causado por *Verticillium dahliae* Kleb. en el Perú y su biocontrol *in vitro***

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## Abstract

In the Atusparia sector, Tocache, of the San Martín region in Peru, two-year-old plants of *Theobroma cacao* L. (Malvaceae) of the CCN-51 clone showed for the first time in January 2010, symptoms of leaf yellowing, wilt and dieback, with an internal obstruction of the vascular system of the plant. To isolate the fungus, diseased tissues were cultured in Petri dishes containing potato dextrose agar oxytetracycline, and the identification was based on morphological and microscopic analyses and by the Sanger sequencing. Pathogenicity tests were carried out on three-months-old *T. cacao* ICS-1 clone plants and were inoculated with a conidia suspension of  $1 \times 10^7$  cfu/mL employing two inoculation methods

(*Drench* to the soil and stem puncture). Likewise, the antagonistic capacity (mycoparasitism and antibiosis) was established *in vitro* with endophytic *Trichoderma* isolates on the pathogen. With both inoculation methods symptoms of wilt of different degrees of severity were observed and the causal agent of sudden death in *T. cacao* was confirmed to be the fungus *Verticillium dahliae* Kleb. BLAST sequence analysis revealed that the isolate showed 100% homology with *V. dahliae* sequences in GenBank. *Trichoderma* TE-91 was identified as the most aggressive mycoparasite because its metabolites inhibited completely the mycelial growth of the pathogen.

**Key words:** biological control, endophyte, wilt, *Trichoderma*

## Resumen

En el sector Atusparia, Tocache, de la Región de San Martín, Perú, plantas de *Theobroma cacao* L. (Malvaceae) clon CCN-51 de dos años de edad presentaron, por primera vez en enero de 2010, síntomas de amarillamiento en hojas, marchitez y muerte, e internamente obstrucción del sistema vascular. Para el aislamiento del agente causal, tejidos enfermos fueron sembrados en placas Petri que contenían medio papa dextrosa agar oxitetraciclina, cuya identificación fue con base en un análisis morfológico, microscópico y secuenciación de Sanger. Las pruebas de patogenicidad se hicieron en *T. cacao* clon ICS-1 con edad de tres meses, inoculadas con una suspensión de conidias de  $1 \times 10^7$  ufc/mL por dos métodos de inoculación (*Drench*

al suelo y punción en el tallo). La capacidad antagónica (micoparasitismo y antibiosis) se determinó *in vitro* con aislamientos de *Trichoderma* endófito sobre el patógeno. En ambos métodos de inoculación, se observaron síntomas de marchitez de diferentes grados de severidad y se confirmó que el agente causal de la muerte súbita del *T. cacao* fue el hongo *Verticillium dahliae* Kleb. El análisis de la secuencia BLAST reveló que el aislamiento mostró un 100% de homología con las secuencias de *V. dahliae* en GenBank. El *Trichoderma* TE-91 fue identificado como el micoparásito más agresivo, debido a que sus metabolitos inhibieron completamente el crecimiento micelial del patógeno.

**Palabras clave:** control biológico, endófito, marchitez, *Trichoderma*

## Introduction

*Theobroma cacao* L. (Malvaceae) is a plant species with its center of origin found in the humid forests of South America; its beans and derivatives are the basic input for chocolate, cosmetics and for the pharmaceutical industry (Zhang & Motilal, 2016). However, there are three major diseases that limit global cocoa production, including brown rot (caused by *Phytophthora* spp.), Moniliasis (*Moniliophthora roreri*) and witch's broom (*Moniliophthora perniciosa*) (Bailey & Meinhardt, 2016). These phytopathogens are able to limit significantly its commercial production and reduce yields by up to 20 %, e.g. the decline in cocoa production in 2012 was estimated at 1.3 million tonnes of dry beans (Bailey & Meinhardt, 2016). Moreover, another disease that severely limits production in Uganda, Brazil and Colombia is the sudden death of *T. cacao* plants caused by *Verticillium dahliae* Kleb (Resende, Flood, & Cooper, 1994).

The species *V. dahliae* and *V. albo-atrum* are highly pathogenic as they cause wilt in more than 200 plant species, including economically important crops (Pegg & Brady, 2002; Schnathorst, 1981). Solely *V. dahliae* results in yields losses of up to 80 % and its control is a challenge, because its pathogenicity is highly variable and it has a high survival capacity (Wang et al., 2016).

Currently, there is no biocontrol management or treatment for infected plants, and no effective fungicides are available to control wilt caused by *Verticillium*; basically, prevention is the only effective method to avoid losses (Yildiz & Benlioglu, 2010; Yuan et al., 2017). Furthermore, the available control and prevention measures are not completely successful when applied individually. Therefore, the only way to effectively control the disease in the field is through an integrated management aimed to reduce the disease to coexistence levels (López-Escudero & Mercado-Blanco, 2011).

A promising ecological strategy within the integrated disease management is the use of endophytic fungi of the crop of interest, as they are taxonomically and biologically diverse, they colonize the internal

tissues of plants without causing visible symptoms and they can improve plant growth and health. Additionally, they can induce the production of compounds and can activate some genes involved in plant defense responses to biotic and abiotic factors (Bae et al., 2009; De Souza et al., 2008; Hanson & Howell, 2004). Among the main endophytes used as biological control agents are some species of the *Trichoderma* genus considered as natural antagonists of phytopathogens, that have widely been used in agriculture, and contribute to the improvement of plant growth and development (Guédez, Cañizález, Castillo, & Olivar, Harman, 2006, Junaid, Dar, Bhat, Bhat, & Bhat, 2013, Toghueo et al., 2016). In *T. cacao*, recent studies have shown that endophytic fungi can limit damage by *Moniliophthora perniciosa*, *M. roreri* and *Phytophthora palmivora* (Bailey et al., 2008; De Souza et al., 2008; Mejía et al., 2008).

In Peru, staff from Instituto de Cultivos Tropicales (ICT) conducted an expedition to the upper Amazon region in 2008, in order to search for endophytes that have coevolved with pathogens, obtaining a diverse collection. These researchers collected 126 *Trichoderma* isolates from stems and leaves (Márquez et al., 2010). However, only a small number of *Trichoderma* spp. isolates of the collection have been studied to establish the endophytic and biocontrol capacities related to main cacao pathogens (Arévalo, Canto, Leon, & Meinhardt, 2010a; Arévalo, Canto, Leon, Meinhardt, & Cayotopa, 2010b; Leon et al. 2010a; Leon, Rojas, Rodríguez, Arévalo, & Márquez, 2010b).

Currently, *V. dahliae* has not been confirmed as being present in Peru. Therefore, the causal agent was isolated from two-year-old *T. cacao* clone CCN-51 plants collected in Atusparia, Tocache sector of the San Martín region of Peru, that showed for the first time in January 2010 symptoms of leaf yellowing, generalized wilt and dieback, as well as internal obstruction of the vascular system. According to the aforementioned, the aim of this study was to identify and characterize the causal agent of the sudden death of *T. cacao* plants and establish the *in vitro* antagonistic capacity of endophytic *Trichoderma* isolates on the causal agent.

## Materials and methods

### Location of the experiment

This research was carried out in the nursery and in the phytopathology laboratory of the experimental station Juan Bernito of ICT, located in the district of La Banda de Shilcayo, province and region of San Martín, Peru, at 76°00'18" W and 06°00'28" S, at an altitude of 315 m.a.s.l. The temperature, relative humidity and precipitation were 25 ° C, 73 % and 1,188 mm, respectively.

### Plant material

For the isolation and identification of the causal agent of the sudden death of *T. cacao*, samples of affected tissues with death symptoms and plants with "sudden death" typically affected by *Verticillium* were collected in January 2010 on a two-year-old CCN-51 cacao plantation of the Atusparia sector, in the district and province of Tocache in the San Martín region of Peru. In this plantation, 5 % of the plants exhibited symptoms of leaf yellowing, widespread wilting and plant death. When performing transverse cuts of the stem, internal vascular occlusion was observed as points, meanwhile in the longitudinal cuts, brown striae were observed in the vascular system. Samples were taken to the ICT Phytopathology Laboratory for their isolation and identification.

### Isolation and identification

Isolation of the causative agent was obtained from segmented tissues of the stem, root and neck of diseased plants. The segments were washed with water and disinfested with 1 % sodium hypochlorite for 10 minutes to remove surface microorganisms; in addition, they were washed with sterile distilled water and placed on paper towels for drying. From each segment, 1 cm portions were cut and planted in Petri dishes containing potato dextrose agar medium with oxytetracycline (PDAO), and incubated for 7 days at 25 ° C (Resende, Flood, & Cooper, 1995). The plates were subjected to artificial illumination at a

room temperature of 28 ° C  $\pm$  0.57 ° C and a relative humidity of 70 %  $\pm$  1.71 % for 10 days  $\pm$  1 to favor fungus sporulation. The taxonomic identification of the fungus was made by observing the macroscopic morphological characteristics of the colony (appearance and coloration) as well as the microscopic morphological characteristics (type and size of vegetative and reproductive structures). The keys published by Barnett and Hunter (1998), Barron (1968) and Watanabe (2010) were used for identification of the pathogen.

Further, the identification of the isolates was performed using Sanger's sequencing. For this purpose, the isolates were cultivated in flasks containing 50 mL of potato dextrose broth (PDB, 24 g/L) at 25 ° C until the mycelium colonized the surface of the broth. The mycelium was washed, filtered and stored at -20 ° C until DNA extraction was carried out. The mycelium was triturated in liquid nitrogen and the DNA was extracted using the DNeasy PowerPlant Pro kit (Qiagen, Germany), following the manufacturer's instructions. Partitions ITS1/ITS4 (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'; ITS4: 5'-TCCTCCGCTTATTGATATGC-3') (White, Bruns, Lee, & Taylor, 1990) were used to amplify DNA. The PCR mixture was prepared using Master Mix 2x PCR (Promega, U.S.A.), in a final volume of 25  $\mu$ L containing Milli-Q water, 1x MasterMix PCR, 0.5  $\mu$ M of each 5' to 3' partitions (forward or direct), 3' to 5' (reverse) partitions and 2  $\mu$ L of cDNA.

The PCR was performed on a Techne TC-412 thermal thermocycler (Techne). The conditions employed were 94 ° C for 2 minutes, followed by 35 cycles of 94 ° C for 10 seconds, 54 ° C for 30 seconds and 72 ° C for 1 minute, with a final elongation phase of 72 ° C for 10 minutes. Amplicons were monitored by agarose gel electrophoresis at 1 % prior to purification, using the QIAquick PCR purification kit (Qiagen, Germany). Amplicons were sequenced in both directions using Sanger's sequencing (GATC Biotech, Konstanz, Germany). Sequences were trimmed and aligned with the BioEdit Sequence Alignment Editor (Hall, 1999) and compared with the accessions of *V. dahliae* in GenBank using the Nucleotide BLAST tool.

### Pathogenicity test

To carry out the pathogenicity test, *V. dahliae* inoculum was grown from a young culture in PDAO medium. Three discs of fungus colonized medium (6 mm of diameter) were placed in 100 mL of potato broth with 2% dextrose, and incubated under agitation at 110 rpm at room temperature (27 °C) for one week, to favor the development of the fungus. To remove the mycelia formed and to obtain the spore suspension used as inoculum, the broth was filtered through four layers of sterile gauze. The inoculum obtained was adjusted to a concentration of 10<sup>7</sup> cfu/mL (Resende et al., 1995). For the inoculation of the pathogen, 40 plants from three-month-old ICS-1 *T. cacao* seeds (Resende et al., 1994, 1995) grown on sterile soil contained in black polyethylene bags were used. The soil was autoclaved at 121 °C and 15 lb/in<sup>2</sup> for two consecutive days, for 45 and 30 min (Ormeño-Orrillo & Dávila, 1999). Two inoculation methods were evaluated: a) via drench to the soil with 50 mL of the conidia suspension (10<sup>7</sup> cfu/mL) around the root of 20 plants (Resende et al., 1995); b) puncture in the stem of 20 plants employing a sterile needle. Wounds were made in four points on the stem up to a depth of 2 mm (two on the bud of the cotyledonal leaves and two on the bud of the first true leaves), inoculating 5 µL of conidia suspension into each localized wounds (Trapero, Díez, Rallo, Barranco, & López-Escudero, 2013). Plants were maintained

in the greenhouse of the ICT Phytopathology Laboratory at a temperature of 28 °C ± 0.57 °C, at a relative humidity of 70% ± 5.71% and soil conditions at field capacity (Resende et al., 1995).

On the other hand, the severity of the disease was evaluated with a scale of 0 to 6 (Villarreal-Zaballos, Feng, Iglesias, du Toit, & Correll, 2012), where 0 = no symptoms; 1 = between 0 and 10% of chlorosis or necrosis in the plant; 2 = between 10 and 25% of chlorosis or necrosis; 3 = between 25 and 50% chlorosis or necrosis; 4 = between 50 and 75% of chlorosis or necrosis; 5 = between 75 and 100% chlorosis or necrosis, and 6 = dead plant. Pathogen reevaluation was performed from sections of plant tissue sections (root and stem) in which the symptoms of the disease were observed, following the same isolation methodology described previously.

### Endophytic *Trichoderma* (ET)

Endophytic *Trichoderma* (ET) isolates used in this study were obtained from the ICT collection. Ten ET isolates (table 1) with endophytic (Arévalo et al., 2010b) and antagonistic capacities against *Moniliophthora perniciosa*, *M. roreri* and *Phytophthora palmivora* (Arévalo et al., 2010a; Leon et al., 2010a, 2010b), were selected to establish the antagonistic capacity against *V. dahliae*.

**Table 1.** Origin of endophytic *Trichoderma* (ET) isolates obtained from the stem and leaves of native cacao genotypes from localities of the Alto Amazonas region in Peru during 2008

N.º	Isolate	Organ	Locality	Species
1	ET-17	Stem	Pastaza	<i>T. brevicompactum</i>
2	ET-22	Stem	Ungumayo	<i>T. virens</i>
3	ET-50	Stem	Urituyacu	<i>T. harzianum</i>
4	ET-55	Stem	Urituyacu	<i>T. harzianum</i>
5	ET-68	Stem	Charupa	<i>T. harzianum</i>
6	ET-72	Stem	Ungurahui	<i>T. atroviride</i>
7	ET-76	Stem	Ungumayo	<i>T. harzianum</i>
8	ET-91	Leaves	Charupa	<i>T. asperellum</i>
9	ET-103	Leaves	Urituyacu	<i>T. aggressivum</i>
10	ET-126	Stem	Ungurahui	<i>T. atroviride</i>

Source: Elaborated by the authors

## Antibiosis

Soluble inhibitory metabolites were produced in three culture broths and the antibiosis test was adapted to the method described by Bailey et al. (2008). *Trichoderma* isolates were cultured in Petri dishes of 90 cm in diameter, containing malt extract agar (MEA) culture medium and incubated at 25 °C ± 0 °C for five days. From these cultures a suspension of conidia was obtained by addition of sterile distilled water (SDW) to each plate. For this test, three culture broths were used: 1) based on minimum salts (MIN), 2) malt extract (ME), and 3) potato sucrose (PS). For each broth, 1 mL of conidia suspension (10<sup>6</sup> cfu/mL) was added per 100 mL; then, it was incubated at 25 °C under agitation at 110 rpm for one week. Mycelia were filtered through a layer of sterile cotton and then centrifuged for 20 minutes at 2,500 rpm. The supernatant was stored at -20 °C until it was used.

Before being incorporated into the medium, the centrifuged filtrate was placed in a water bath at 90 °C ± 0 °C for two hours to inactivate the enzymes; then, an equal volume of PSA medium with solidified agar (3%) was added to the Petri dishes. A 4-mm-diameter broth slice of medium with the developed pathogen from a five-day-old colony was placed in the center of the Petri dish. Inhibition of the mycelial growth of the pathogen was recorded when the control covered the entire box (Osorio Hernández et al., 2016).

## Mycoparasitism

For this test the precolonized plaque method described by Bailey et al. (2008) was used. A 2.5 × 0.5 cm piece of inoculum was extracted from a young culture of each *Trichoderma* isolate and located at one end of the plates containing PDAO medium precolonized by *V. dahliae*. Three plates were used as replicates. The Petri dishes were incubated at 25 °C under darkness conditions. Two weeks later the fungi were re-established. A total of eight samples (medium disks) of each box or replica were removed with a 5 mm diameter

punch, from the point where the pathogen was planted. These samples were located in Petri dishes with PDAO medium and incubated at 25 °C under darkness conditions; after 14 days the growth of *Trichoderma* and of the pathogen was observed, and the colonization percentage of *Trichoderma* biocontrol on medium disks with the pathogen was established.

## Statistical analysis

Antibiosis and mycoparasitism tests were established under a completely randomized experimental design. Ten treatments were evaluated and corresponded to selected endophytic *Trichoderma* isolates. Three replicates per treatment were performed. The parameters evaluated were inhibition percentage of mycelial growth and colonization by *Trichoderma* of mycelium precolonized by *V. dahliae*. The percentage data was transformed angularly (arcsine  $\sqrt{X}$ ) to confirm normality and homogeneity of variances as recommended by Montgomery (2008). An analysis of variance (ANDEVA) and the Duncan contrast test with a level of significance of 0.05% were carried out in the statistical software InfoStat version 2008 (Di Rienzo et al., 2008).

## Results and discussion

### *In situ* symptoms

Figure 1 shows the different symptoms observed in sudden death in a two-year-old CCN-51 *T. cacao* plantation located in the Atusparia sector, district and province of Tocache, Peru. Initially, flaccidity, epinasty, chlorosis, and necrosis of upper leaves are observed, and finally, wilting of the entire plant. When a cross-section of the upper limb was performed, necrotic points of the vascular system were identified and, in a longitudinal section, necrotic striae showing blackened infected vessels in the wood were observed. Similarly, necrotic rings were detected in the cross-section of the root of affected plants.



**Figure 1.** Symptoms of sudden death of cocoa in a plant of *T. cacao* CCN-51 clone located in the Atusparia sector, district and province of Tocache, Peru a. Yellowing, epinasty and wilting; b. Discoloration of the vascular system; c. Necrotic points throughout the vascular system; d. Necrotic striae in a longitudinal cut of the branch; and e. Necrotic rings in a cross section of the root.

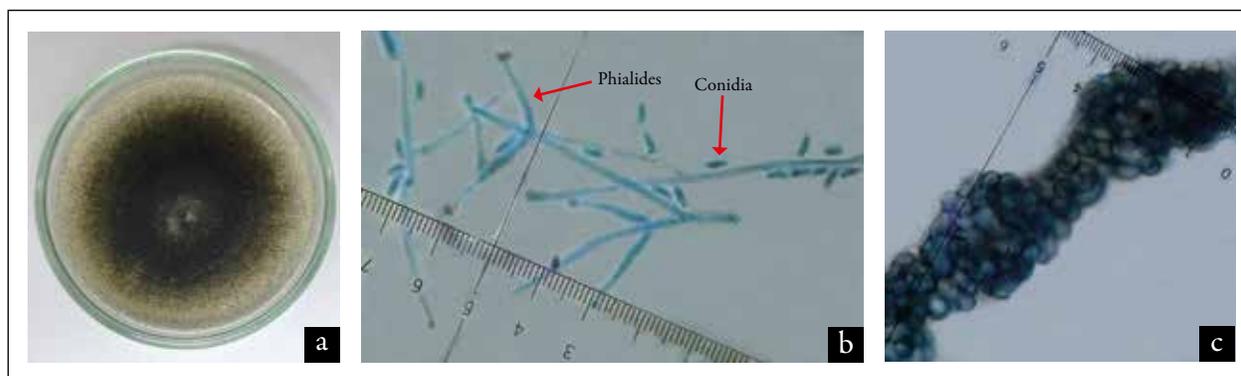
### Morphological and molecular identification of *Verticillium*

The coloration of the colony in PDAO medium was grayish to blackish due to microsclerotia formation (figure 2a). In the mycelium immersed in the middle and spase, with the help of a stereoscope, conidia immersed in mucilage were clearly observed as small water droplets. These colony characteristics correspond to those observed in the genus *Verticillium* (Barnett & Hunter, 1998; Barron, 1968).

The conidiophores are hyaline, erect and carry a mass of conidia apically on each verticillated phialide in 2-3 fertile portions (figure 2b).

Conidia are hyaline, smooth-walled, ellipsoidal, unicellular, non-septated, and measure on average  $6.7 \pm 1.4 \mu\text{m} \times 2.9 \mu\text{m} \pm 0.5 \mu\text{m}$ . In figures 2a and 2c, microsclerotes immersed in the PDAO culture medium regularly distributed throughout the colony, are blackish brown in color, in chains and aggregated, rounded and with a thin cell wall. The microscopic morphology characteristics mentioned place the pathogen as belonging to the species *V. dahliae* (Inderbitzin et al., 2011; Watanabe, 2010).

BLAST sequence analysis revealed that the *Verticillium* isolate showed 100% homology to the *V. dahliae* sequences in GenBank (accession number NCBI: NR\_126124.1 and HQ206718.1) and *V. longisporum* (HQ206832.1).



**Figure 2.** Macroscopic colony and microscopic morphology of vegetative and reproductive structures of *Verticillium dahliae* Kleb. a. The 15-days-old colony in potato dextrose agar (PDA) medium; b. Conidia and verticillial phialides (40 X); c. Microscleriosis (40X).

**Pathogenicity**

With the two methods of inoculation the Koch postulates were achieved and the causal agent of the sudden death of *T. cacao* was confirmed to be the species *V. dahliae*. Both the drench to the soil and the stem puncture inoculation methods using suspensions of *V. dahliae* conidia were able to reproduce wilting symptoms in 28 young inoculated cacao plants. However, the incubation period in the plants inoculated using the drench to the soil method was longer, and in which the first symptoms of leaf flaccidity followed by chlorosis and necrosis of the upper leaf apex were observed

in ten plants (50 %) 30 days after inoculation (DAI) with the pathogen (figure 3a); furthermore, ten days later the first symptoms of necrosis in the leaf area of the upper leaves (figure 3b) with a severity of  $55\% \pm 1.70$  were perceived. However, with the stem puncture method, the first symptoms were observed 10 DAI (figure 3c) in 18 plants ( $90\% \pm 2.37$ ), showing symptoms similar to those obtained with the drench to the soil method. The progression of the disease was severe; four days after observing the first symptoms, epithelia, wrinkling and necrosis of all leaves of the plant (figure 3d) occurred, and two days after that, some plants died showing a severity of  $80\% \pm 2.38\%$  (table 2).

**Table 2.** Incidence and severity of sudden death of *T. cacao* by *V. dahliae* employing two inoculation methods at the Juan Bernito experimental station of ICT, district of La Banda de Shilcayo, province and region of San Martín, Peru, in 2010

Inoculation method	Incubation period	Incidence (%)	Severity (%)
Drench to the soil	30 days	$50 \pm 0.42$	$55B \pm 1.70$
Stem puncture	10 days	$90 \pm 0.24$	$80A \pm 2.38$

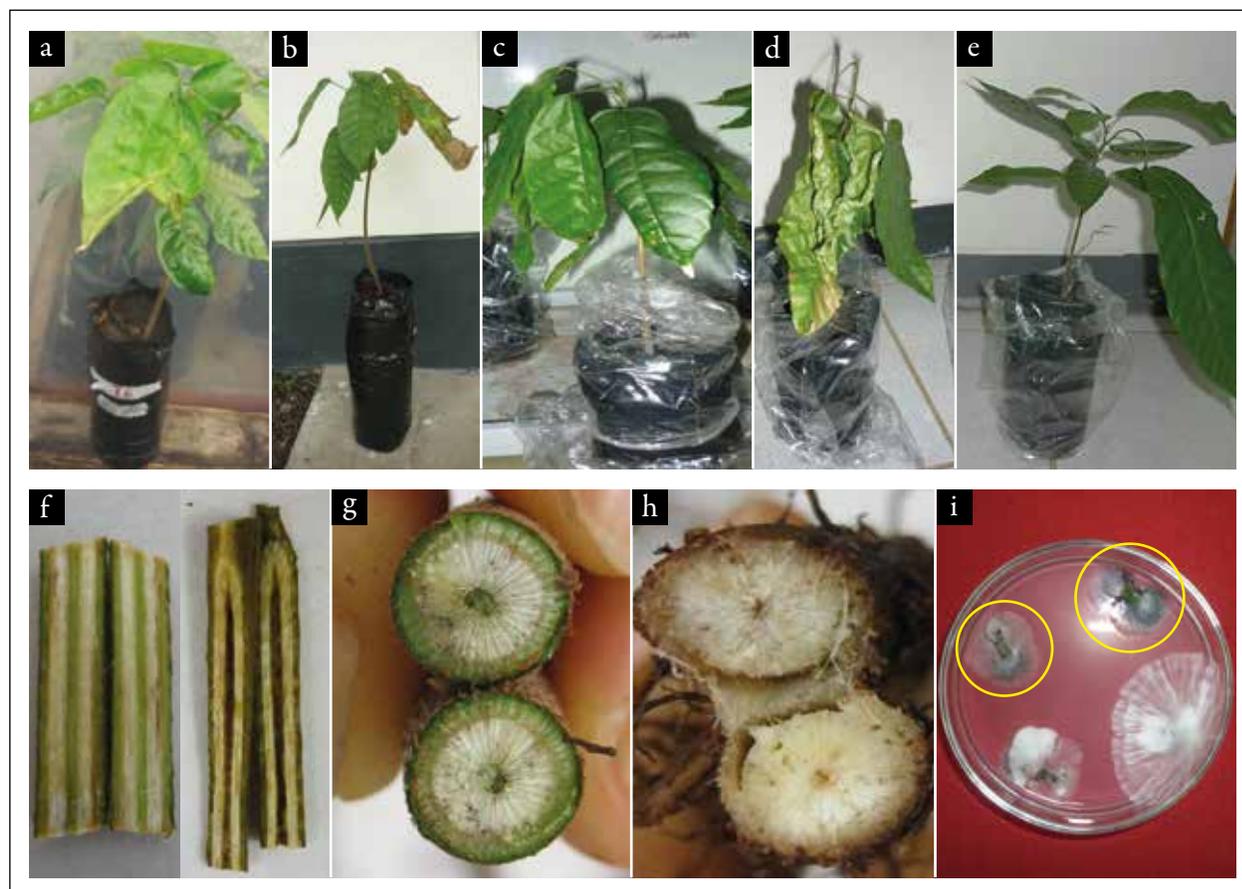
Note: Average data of 20 plants for severity per inoculation method. Different letters indicate significant differences according to the Duncan test ( $p \leq 0.05$ ).

Source: Elaborated by the authors

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In both methods, longitudinal and transverse cuts to the stem were made, and the presence of striae and necrotic points, respectively, in the entire vascular system (figures 3f and g) were identified. The pathogen was inoculated in PDAO medium from sections of the upper stem tissue of 10 and 18 affected plants (figure 3i), which were inoculated

using the drench to the soil and stem puncture methods, respectively, and from 12 inoculated asymptomatic plants. Plants that showed 80% severity were not able to resume growth unlike plants that showed minor symptoms, that were able to resume their growth.



**Figura 3.** Symptoms of pathogenicity with *V. dahliae* in plants of *T. cacao* ICS-1 clone employing the drench to the soil inoculation method. a. Wilt; b. Necrosis caused by the stem puncture inoculation method; c. Chlorosis; d. Wilt; e. Healthy plant; f. Stem showing necrotic striae; g and h. Necrotic points in the vascular system; i. Pathogen observed in PDAO medium (inside the yellow circles).

The current study comprises the first report that shows the presence of the sudden death of cacao plants in Peru, corroborating with the Koch's postulates that *V. dahliae* is the causal agent of the disease. Of the 40 plants inoculated with the pathogen, 50% and 90% showed typical symptoms of the disease with the drench to the soil and the stem puncture inoculation methods, respectively. This disease caused by *V. dahliae* was

reported in Uganda in 1965 (Emechebe, Leakey, & Banage, 1971; Leakey, 1965), and in Brazil it has become a serious problem in the states of Bahia and Espírito Santo, where 85% of the cocoa is produced (Resende et al., 1995). In addition, *T. cacao* in north-eastern Brazil was considered as a favorite host for *Verticillium*, because all Brazilian isolates of *T. cacao* were reported as pathogenic and destructive (Resende et al., 1994).

In this study, *V. dahliae* infections were studied in three-month-old cacao plants of the ICS-1 clone using the drench to the soil and the stem puncture methods, finding that the first symptoms were observed 30 days with the first method. On the contrary, Resende et al. (1994) reported the first symptoms at an earlier development stage, i.e. after only 20 days, and after 36 days, the disease rate was higher causing wilt in a small number of ICS-1 clone plants. Probably, this difference in days is due to the climatic conditions found in each location, as the temperature ( $28\text{ }^{\circ}\text{C} \pm 0.57\text{ }^{\circ}\text{C}$ ) and the relative humidity ( $70\% \pm 5.71\%$ ) were different from those reported by Resende et al. (1994) (temperature of  $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  and relative humidity of  $60\% \pm 10\%$ ). However, the progression of the disease was rapid and showed more severe symptoms in 90% of the seedlings inoculated by stem puncture, which agrees with what Resende et al. (1995) reported. These last authors used the same inoculation method, inoculum concentration and cacao plant genotypes (ICS-1 clone), observing the first symptoms after 10 days, which probably shows that the isolated pathogen of Peru has the same behavior as the one from Brazil. In addition, with the re-isolation of the pathogen, Koch's postulates were fulfilled and internal distribution within the plant was established.

Likewise, Trapero et al. (2013) indicated that inoculation through stem injection in young olive seedlings (40 days old) was the most effective inoculation method to begin the selection process of olive genotypes resistant to the wilting due to

*Verticillium*, constituting an effective method that saves work, space and resources. This method was also used to isolate cacao *V. dahliae* in *Abelmoschus esculentus* Medik and *Gossypium hirsutum* L. (Malvaceae), which induced similar symptoms on artificially inoculated *T. cacao* seedlings (Emechebe et al., 1971).

#### Antagonistic capacity of endophytic *Trichoderma* against *V. dahliae*

In the antibiosis test, the endophytic *Trichoderma cacao* isolates were different in their ability to prevent the mycelial growth of *V. dahliae* in PSA medium with mixtures of inhibitory metabolites (table 3). The metabolites obtained in PS medium had a higher effect on the mycelial inhibition (29.1%) of *V. dahliae*, followed by MIN (22.2%) compared to the metabolites obtained in ME medium, which showed the lowest percentage (16.6%). According to the mean culture media data, metabolites of the isolates TE-91, TE-17 and TE-126 prevented the growth of the pathogen with 66.5%, 55.2% and 51.9%, respectively, compared to the other isolates that inhibited less than 17.2%. Figure 4 shows that metabolites obtained from the TE-91 isolate in MIN broth prevented completely the mycelial growth of *V. dahliae*, followed by TE-17 with 77.2% and 75.0% in PS and ME media, respectively; additionally, TE-126 with 72.8% and 70.5% in PS and ME media, respectively, and TE-22 with 44.3% in MIN. The other isolates had a low percentage of inhibition, i.e. less than 13.5%.



Figure 4. Inhibition of mycelial growth of *V. dahliae* with soluble inhibitory metabolites of endophytic *Trichoderma*.

**Table 3.** Effect of inhibitory metabolites of endophytic *Trichoderma* isolates obtained in three culture broths on the percentage of mycelial inhibition of *V. dahliae*.

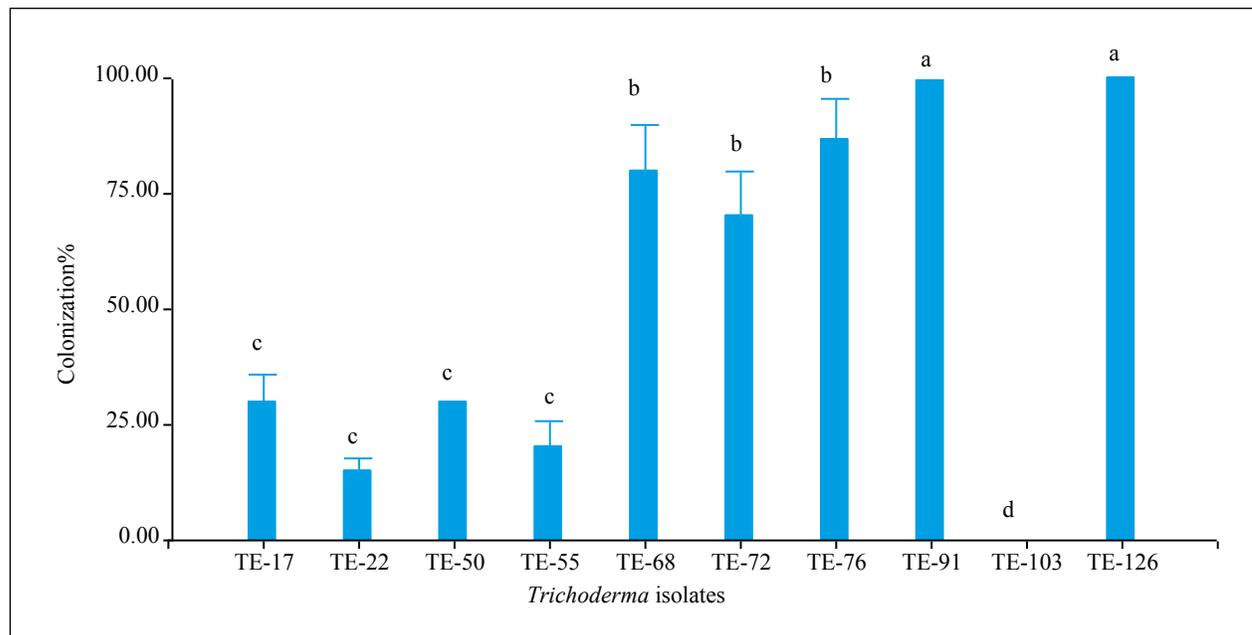
Treatments	MIN			CPS			CEM			Average	
TE-17	13.56	± 0.97	C	77.27	± 0.69	B	75.02	± 2.74	A	55.28	B
TE-22	44.34	± 3.59	B	5.14	± 1.21	CD	2.30	± 0.36	BC	17.26	C
TE-50	11.59	± 2.60	CDE	6.87	± 1.93	CD	1.46	± 1.36	CD	6.64	D
TE-55	8.36	± 1.55	DE	6.08	± 0.94	CD	2.51	± 0.85	BC	5.65	D
TE-68	8.36	± 1.40	CDE	4.20	± 0.45	CD	2.72	± 1.15	BC	5.09	D
TE-72	5.55	± 0.73	E	10.25	± 0.75	C	3.34	± 1.69	BC	6.38	D
TE-76	8.78	± 1.23	CDE	4.85	± 0.62	CD	1.98	± 1.28	BCD	5.21	D
TE-91	100.00	± 0.00	A	99.50	± 0.19	D	0.00	± 0.00	D	66.5	A
TE-103	8.64	± 2.77	CDE	3.92	± 0.50	D	6.05	± 1.16	B	6.20	D
TE-126	12.43	± 1.52	CD	72.89	± 1.09	B	70.56	± 1.95	A	51.96	B
<b>Average</b>	<b>22.2</b>		<b>B</b>	<b>29.1</b>		<b>A</b>	<b>16.6</b>		<b>C</b>		

Note: MIN: Broth based on mineral salts; PS: potato sucrose broth; ME: Malt extract broth. Averages followed by different letters indicate significant differences between treatments according to the Duncan test ( $p \leq 0.05$ ). Values to the right of the mean indicate the standard error.

Source: Elaborated by the authors

In the myoparasitic test, the ANOVA showed significant differences between the endophytic *Trichoderma* isolates ( $p = 0.05$ ) in the colonization of *V. dahliae* (figure 5). The isolates TE-91 and TE-126 were the most aggressive mycoparasites of *V. dahliae* with 100% colonization on the pathogen, followed by TE-68, TE-72 and TE-76 with 80%, 70% and 87% colonization, respectively, compared to isolates TE-17, TE-22, TE-50 and TE-55, which colonized *V. dahliae* in percentages lower than 30%.

Antibiosis, the production of antimicrobial compounds, mycoparasitism and the feeding of a fungus by another organism are some of the mechanisms of *Trichoderma* species that provide protection to plants against pathogens (Junaid et al., 2013; Martínez, Infante, & Reyes, 2013). The colonization and production of soluble inhibitory metabolites of endophytic *Trichoderma* isolates obtained from different media varied in their ability to mycoparasite and inhibit the mycelial growth of *V. dahliae* in CCC medium, respectively. This agrees with the results reported by Bailey et al.



**Figure 5.** Percentage of endophytic *Trichoderma* colonization on *V. dahliae*. Different letters indicate significant differences according to the Duncan test ( $p \leq 0.05$ ).

Source: Elaborated by the authors

(2008), whose *Trichoderma* isolates varied greatly in their ability to produce inhibitory compounds of *M. roseri* and in their ability to parasitize these. In this study, *Trichoderma asperellum* (TE-91) proved to be the most aggressive mycoparasite that colonized and completely inhibited mycelial growth of the pathogen. This is probably due to a combined action between secondary metabolites such as terpenoids, which this species of *Trichoderma* releases into the pathogen, that inhibits germination and the synergistic interactions of hydrolytic enzymes such as chitinases, glucanases and proteases that degrade the cell wall of the pathogen until it causes its death (Lorito, Farkas, Rebuffat, Bodo, & Kubicek, 1996; Sivasithamparam & Ghisalberti, 1998; Zeilinger, Gruber, Bansal, & Mukherjee, 2016). In addition, isolates of *Trichoderma* species have been reported as biocontrollers of *V. dahliae* (Carrero-Carrón et al., 2016; Gao, Jun, Pan, Wu, & Zhang, 2007; Jabnoun-Khiareddine, Daami-Remadi, Ayed, Mahjoub, 2009; Meng, Tang, Huang, Ye, & Kan, 2007) and *Phytophthora megacarya* (Mbarga et al., 2014). Furthermore, culture broths have an influence on the production of inhibitory metabolites such as non-ribosomal peptides (pept antibiotics, siderophores and diketopiperazines), such as gliotoxin and gliovirine, polyketides, terpenes, pyrones and isocyanate metabolites, which vary with the species

of fungi and the environmental conditions (Zeilinger et al., 2016). The metabolites of isolates TE-17, TE-91 and TE-126 obtained in PS culture were the ones that inhibited most of the mycelial growth of *V. dahliae* and belong to the species *T. brevicompactum*, *T. asperellum* and *T. atroviride*, respectively. At the same time, TE-126 also proved to be a very aggressive mycoparasite, to such an extent that the pathogen could not be recovered. *Trichoderma atroviride*, reported as a mycoparasite, responds to a number of external stimuli, produces hydrolytic enzymes in the presence of a fungal host and wraps around the host hyphae (Harman, 2006). In the same way, Degenkolb, Gräfenhan, Nirenberg, Gams and Brückner (2006) analyzed the formation of polypeptide antibiotics from four isolates of *T. brevicompactum* selected for their potential antagonism against regressive death. Isolates TE-50, TE-55, TE-68 and TE-76 belonging to the *T. harzianum* species inhibited the mycelial growth of the pathogen in the antibiosis test. However, TE-68 and TE-76 turned out to be aggressive mycoparasites. Bailey et al. (2008) indicated that *T. harzianum* isolates tend to have moderate to low antibiosis activity, and moderate to high mycoparasitism activity. This is probably due to the parallel formation of hydrolytic enzymes, together with a group of membrane-activating

polypeptides called peptabiotics, whose synergistic action induces an important role in mycoparasitism between *T. harzianum* and its fungal host (Lorito et al. 1996; Schirmböck et al., 1994).

The selection of *Trichoderma* strains with good endophilic capacity, in addition to other attributes such as mycoparasitism, antibiosis or induced resistance, can greatly improve the possibilities of developing strategies for biological control of cacao diseases (Bailey et al., 2008).

## Conclusions

The presence of the species *V. dahliae* is reported for the first time in Peru, resulting in the unexpected or sudden death of cacao plants. Metabolites extracted from endophytic *Trichoderma* isolates in PS broth had a higher effect on *V. dahliae* mycelial inhibition. The isolation of TE-91 in MIN and PS culture media showed very good *in vitro* biocontrol capacity due to their ability to completely inhibit the mycelial growth of *V. dahliae*, followed by TE-17 and TE-126 obtained in PS and ME culture media, respectively. Also, isolates TE-91 and TE-126

were the most aggressive mycoparasites for the *V. dahliae* species. These results indicate that the use of *Trichoderma* could be a biological alternative to consider in the integrated management of the cacao sudden death disease.

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## Disclaimer

The authors state that there are no conflicts of interest that might compromise the validity of the results presented in this research.

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