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Inhibition of *Phytophthora* species, agents of cocoa black pod disease, by secondary metabolites of *Trichoderma* species

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Abstract Cocoa production is affected by the black pod disease caused by several *Phytophthora* species that bring, about each year, an estimated loss of 44% of world production. Chemical control remains expensive and poses an enormous risk of poisoning for the users and the environment. Biocontrol by using antagonistic microorganisms has become an alternative to the integrated control strategy against this disease. *Trichoderma viride* T7, *T. harzanium* T40, and *T. asperellum* T54, which showed in vivo and in vitro antagonistic activity against *P. palmivora*, were cultured and mycelia extracted. Inhibition activity of crude extracts was determined, and then organic compounds were isolated and characterized. The in vitro effect of each compound on the conidia germination and mycelia growth of four *P. palmivora*, two *P. megakaria*, and one *P. capsici* was evaluated. *T. viride* that displayed best activities produced two active metabolites, viridin and gliovirin, against *P. palmivora* and *P. megakaria* strains. However, no activity against *P. capsici* was observed. Besides being active separately, these two compounds have a synergistic effect for both inhibitions, mycelia growth and conidia germination. These results provide the basis for the development of a low-impact pesticide based on a mixture of viridin and gliovirine.

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Introduction

The phytopathogens from the genus *Phytophthora* are capable of causing enormous economic losses on crops worldwide. These Oomycetes are responsible for rot affecting woody ornamentals, vegetables (Kamoun et al. 2015), and trees, of which is *Theobroma cacao*.

Chocolate is produced from the beans of cocoa, cultivated over 700,000 km^2 in most tropical regions of the world. It is a major cash crop worldwide, especially in some African countries where 71% of world production of cocoa beans is produced, where one third of world production is from Ivory Coast (ICCO 2014). Cocoa production is threatened by several diseases that entail severe yield losses in many areas. Black pod causing Oomycetes are, among others, *Phytophthora palmivora* (Africa and Cuba), *P. megakarya* (Africa), and *P. capsici* (Brazil and Mexico). Cocoa pods

mature within 5 to 6 months following pollination. All the stages of pod development are susceptible to black pod disease.

Copper and metalaxyl-based fungicide chemicals are traditionally used to treat the disease. However, these negatively impact both environment and farmers' health (Acebo-Guerrero et al. 2011). In addition, the extensive use of chemicals could enhance resistance of some *Phytophthora*. Cultural methods such as the removal of diseased pods that showed relative efficiency when associated with other control methods (Ndoumbe-Nkeng et al. 2004) or the use of resistant cocoa cultivars (Nyassé et al. 2007) were reported.

The biopesticides and the related management products provide an alternative to the use of pesticides and fungicides (Seiber et al. 2014) and meet good public compliance. The biological control of some *Phytophthora* sp. was developed using fungi (Segarra et al. 2013) and bacteria (Zhou et al. 2008, Lee et al. 2013). Two mechanisms have been reported in the control of Oomycetes: (1) through production of hydrolytic enzymes especially glucanases which are able to hydrolyze the cellulose of their cell walls (Valois et al. 1996, Mishra 2010) and (2) through antibiosis mediated by few compounds (Lumsden et al. 1992, Trejo-Estrada et al. 1998, Haesler et al. 2008, El-Hasan et al. 2009, Weisshoff et al. 2014, Takeuchi et al. 2015). However, only few examples concern black pod disease (Tondje et al. 2007, Hanada et al. 2009). Endophytic fungi *Geniculospotium* sp. and *Trichoderma martiale* isolated from healthy leaves of Cocoa and from sapwood in trunks showed a potential effect against *P. megakaria* (Tondje et al. 2006) and *P. palmivora* (Hanada et al. 2008), respectively. Recently, it has been shown that *Trichoderma virens*, endophyte of leaves and roots of cocoa, reduced *Phytophthora* lesions on pods and seedlings (Sriwati et al. 2015). It was also showed that *Pseudomonas* from *T. cacao* rhizosphere has antagonistic activity against *P. palmivora (*Acebo-Guerrero et al. 2015*)*.

Previous studies assessed the biological control using fungi. Thus, collections of microorganisms from cocoa plantations were created and their ability to inhibit the growth of *P. megakaria* (Tondje et al. 2007) and *P. palmivora* (Mpika et al. 2009) were analyzed. Antagonist microorganisms were then identified as *Trichoderma* strains.

Fungi of the genus *Trichoderma*, known to produce secondary metabolites with a key role in antagonistic activities (Verma et al. 2007, Vinale et al. 2014), was used as biocontrol agents (Leng et al. 2011). Despite the effectiveness of *Trichoderma* sp., there are few reported studies on the secondary metabolites involved in the inhibition of the growth of *P. palmivora* and *P. megakarya*. Furthermore, for the use of microorganisms as biocontrol agents, there is a need to identify the active metabolites, including those with non-targeted effect (Brimner and Boland 2003). The aim of this study was to test the antibiosis mechanism and to identify the secondary

metabolites involved in the biocontrol of *P. palmivora* by *T. virens* T7, *T. harzianum* T40, and *T. asperellum* T54 previously described by Mpika (Mpika et al. 2009). The activities of metabolites on other *Phytophthora* species from various countries were also studied.

Materials and methods

General

Semi-preparative HPLC was performed using Agilent Technologies 1260 Infinity system coupled to a diode array detector with Agilent XDB-C18 column (150 × 21.2 mm). Mass spectra were recorded on an API Q-STAR PULSAR *i* of Applied Biosystem. NMR experiments were recorded on Bruker Avance III HD 400 MHz spectrometer (Wissembourg, France) equipped with a BBFO Plus Smartprobe.

Microorganisms and culture medium

P. palmivora BL7/11-2 and *Trichoderma* species were provided by the National Center for Agricultural Research (CNRA) in Ivory Coast. Other *Phytophthora* strains were kindly provided by the French Agricultural Research Organization for Development (CIRAD, Montpellier, France) (Table 1). The strains were maintained on PDA at 4 °C (*Trichoderma*) or room temperature (*Phytophthora*). V8 agar medium was prepared from V8 vegetable juice concentrate (100 ml) in distilled water (1 l) containing $CaCO_3$ (3 g) and agar (20 g). Pod glucose agar (PGA) was prepared from fresh pod (30 g l^{-1} mixed with blender), glucose (20 g l^{-1}), agar (20 g l^{-1}), and distilled water (qsp) 1 l.

Antagonist activity

All antagonist-pathogen combinations were examined on V8 agar medium in 10-cm Petri dishes. For dual cultures, a 5-mm agar plug of 5-day-old cultures of *Phytophthora* (on V8 agar) and *Trichoderma* isolates (PDA) was placed at a distance of 3 cm of each other. *Phytophthora* and *Trichoderma* isolates were inoculated the same day. For monocultures as controls, agar plugs of *Phytophthora* strains were inoculated in the center of Petri dishes. Petri dishes were incubated at 25 ± 2 °C. The growth of *Trichoderma* and *Phytophthora* spp. was surveyed daily for 7 days.

Production, purification, and characterization of secondary metabolites

Trichoderma strains were grown in Roux culture flasks containing 250 ml potato dextrose agar (PDA). Agar plugs cut from 7-day-old culture (PDA) were placed into 50 ml Falcon

Table 1 Origin of microorganisms used in this study

Pathogen strains	Geographic location	Source
P. palmivora BL7/11–2	Ivory Coast	CNRA
P. palmivora NS 487	Cameroun	CIRAD
P. palmivora GH 17	Ghana	CIRAD
P. palmivora TRI 1	Trinidad and Tobago	CIRAD
P. megakarya NS 269	Cameroun	CIRAD
P. megakarya NGR 20	Nigeria	CIRAD
P. capsici REG 2.5	French Guiana	CIRAD

tube with sterile distilled water (25 ml). After stirring (Vortex) and filtration on sterile gaze, either sterile distilled water or additional agar plugs were added to obtain a concentration of 10^7 spores ml^{-1}. Roux culture flasks were inoculated with 1 ml of these suspensions and incubated at 27 °C in dark for 5 to 15 days.

After growth, biomasses and agar were transferred in 2 l Erlenmeyer, and ethyl acetate (500 ml) was added and mechanically stirred for extraction of metabolites. After 1 h, suspensions were filtered and this was repeated twice. Organic phases were pooled, concentrated, dried (MgS0$_4$), and evaporated in vacuo at 35 °C. The red-brown residue (crude extract) was recovered and underwent various chromatographic separation techniques.

T. virens T7 metabolites

The crude extract (4.79 g) from cultures in 20 Roux flasks of *T. virens* T7 was dissolved in methanol and chromatographied on Sephadex LH20 using methanol as eluant to give a fraction 3 (2.6 g) with an inhibitory activity against *P. palmivora*. This fraction was subjected to column chromatography on silica gel by eluting with gradient of CH$_2$Cl$_2$/MeOH (98/2 to 50/50). Fractions showing similar thin-layer chromatography (TLC) profiles were combined. Five fractions were then collected. Fraction 3–1 was further separated using silica gel column chromatography, C$_6$H$_{12}$/EtOAc (60/40 to 40/60), to yield 130 mg of mixture of α-viridin **1a** and β-viridin **1b**. Fraction 3-2 was further purified by silica gel column chromatography, CH$_2$Cl$_2$/MeOH (98/2 to 50/50) to give heptelidic acid **3a** (20 mg), gliovirin **2a** (100 mg), pretrichodermamide A **2b** (13 mg), trichodermamide A **2c** (11 mg), and viridiol **1c** (7 mg). Fraction F3-3 was fractionated on a Sephadex LH-20 column eluting with MeOH to produce two sub-fractions (F3-3-1 and F3-3-2). F3-3-1 was subsequently separated on semi-preparative HPLC (colomn Agilent XDB-C18, 150 × 21.2 mm; gradient H$_2$O-TFA (0.1%) 100% to H$_2$O-TFA (0.1%)/AcN 50/50) to obtain hydroheptelic acid A **3b** (6 mg) and gliocladic acid **4** (3 mg). F3-3-2 was a mixture (11 mg) of trichoderonic acid A **5a** and a new metabolite which was named trichoderonic acid C **5b** (see Fig. S2 and S3).

Trichoderonic acid C: ^{1}H NMR (400.13 MHz, CD$_3$OD): 7,24 (1H, *d*, 3,75, H-11), 5,05 (2H, *d*, 1,86, H-3),; 4,47 (2H, *d*, 12,2, H-15), 3,6 (1H, d, 12,6, H-5), 2,7 (1H, m, H-10), 2,15 (1H, m, H-7b), 2,1 (1H, *m*, H-12), 2,09 (3H, *s*, H-17), 1,7 (1H, *m*, H-8b), 1,5 (1H, *m*, H-9), 1,4 (1H, *m*, H-8a), 1,4 (1H, *m*, H-7a), 0,99 (3H, *d*, 6,8, H-14), 0,92 (3H, *d*, 6,8, H-13). ^{13}C NMR (75,03 MHz, CD$_3$OD) 174.4 (C-4), 172.9 (C-16), 168.8 (C-1), 146.5 (C-11), 131.0 (C-2), 73.6 (C-6), 66.0 (C-3), 65.9 (C-15), 54.2 (C-5), 49.5 (C-9), 41.0 (C-10), 35.9 (C-7), 28.6 (C-17), 22.2 (C-8), 21.6 (C-14), 20.8 (C-12), 15.5 (C-13), HRESI-TOf [M + H]$^+$ 341.16.

T. harzianum T40 metabolites

The crude extract (1.2 g) from cultures of 15 days in 12 Roux flasks of *T. harzianum* T40 was chromatographied on silica gel using cyclohexane-ethyl acetate gradient. The active fraction (735 mg) was dissolved in distilled water, acidified (pH 3) with hydrochloric acid 0.1 N, and extracted with ethyl acetate. The extract was purified by chromatography on LH 20 (MeOH) and harzianic acid **6** was obtained (25 mg).

T. asperellum T54 metabolites

The crude extract (3.4 g) from cultures of 10 days in 18 Roux flasks of *T. asperellum* T54 underwent column chromatography on silica gel, by eluting with gradient of CH$_2$Cl$_2$/MeOH (98/2 to 50/50), to give mainly gliovirin **2a** (300 mg).

Evaluation of the growth inhibiting activity against *Phytophthora* species

Agar plugs (5 mm diameter) from 7-day-old cultures were centrally transferred in the plates containing V8 agar medium. Five microliters of DMSO solution of the crude extracts, chromatographic fractions and purified compounds at concentrations ranging from 0.156 to 5 mg ml^{-1} were applied on antibiogram paper disks (6 mm diameter) placed 1.5 cm near the agar plugs. All experiments were done in triplicate. The controls were obtained by applying 5 µl of DMSO. The plates were incubated at 27 °C for *P. palmivora* and at 25 °C for *P. megakaria* strains. The pathogen radial growth was measured daily and percentages of inhibition were obtained after 3 days of incubation from the following equation:

$$Inhibition\ (\%) = \frac{growth\ in\ untreated\ control - growth\ in\ treatment}{growth\ in\ untreated\ control} \times 100$$

Evaluation of the anti germination activity

Phytophthora strains were grown on V8 agar medium during 7-day cultures in culture plates (9 cm diameter). Suspensions of sporangia were prepared from these cultures with PDB (3 × 3 ml) and were refrigerated (5 °C) for 25 min and incubated in the dark at 25 °C for 30 min to induce zoospore release (Nyadanu et al. 2009). Zoospore suspensions were adjusted to 2 zoospores μl^{-1} with PDB, and 100 μl of suspensions were introduced in each well of a 96-well cell culture plate. Viridin, gliovirin, or mixture were added in DMSO solution (2 μl) to obtain final concentrations of 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 $\mu g\ ml^{-1}$. Controls were incubated with DMSO alone. All experiments were done in triplicate. The plates were incubated in orbital shaker (200 rpm) at 25 °C. The germination rate was recorded at 24 and 48 h under a phase contrast microscope (Olympus BX41). A minimal inhibitory concentration (MIC) was defined as concentration for which no germination was observed.

Confrontation zones analysis

Co-cultures of *T. virens* T7 with *P. megakarya* NS 269 on V8 agar or *P. palmivora* TRI 1 on pod agar and *T. asperellum* T54 with *P. megakarya* NS 269 on V8 agar were performed as previously described. Before recovery, the agar from the confrontation zones was excised with razor blade and extracted with ethyl acetate. After drying (Na₂SO₄) and evaporation, the crude extracts were dissolved in MeOH, filtered and analyzed in LC/MS.

Statistical analysis of data

The data collected on the growth inhibition of *Phytophthora* strains were subjected to an analysis of variance using Statistica software version 7.1. In case of significant difference, the averages were compared according to the Newman-Keuls test at 5% threshold.

Results

Bioassays in Petri dishes

The antagonisms between *Trichorderma* sp. and *P. palmivora* were essayed by plate confrontation using PDA and PGA. In both cases, the three strains of *Trichoderma* showed an effect on the growth of *P. palmivora*. This effect was not observed when assays were performed in compartmentalized Petri dishes. In contrary, *P. palmivora* was inhibited by crude ethyl acetate extracts of cultures of three *Trichoderma* on PDA.

These results suggested that the antagonistic effects involved non-volatile organic compounds, which can be purified as usual natural products.

Purification and identification of metabolites

In preliminary study, *T. virens* T7 was cultured in PDB during 20 days and crude extract of culture medium showed an activity against *P. palmivora*. However, the chromatography failed in the purification of active compounds. The main isolated products were cyclo(L-Pro-L-Val) and cyclo(L-Pro-L-Tyr), for which toxic activity against *P. infestans* was reported (Puopolo et al. 2014). Then, in order to produce and purify the metabolites produced by the *Trichoderma* species, culture conditions were first investigated using *T. virens* T7. The most active crude extract was obtained on solid culture at 25 °C in the dark. After extraction of agar and mycelium, the metabolites (Fig. 1) were purified by flash chromatography, and their activity against *P. palmivora* BL7/11-2 was investigated.

The crude extract of *T. virens* T7 solid culture was partitioned on LH 20, giving five chromatographic fractions. Among them, fractions 2 and 3 were active against *P. palmivora*. Further purification of fraction 2 yielded α-viridin **1a** and a mixture of α-viridin and β-viridin **1b**. α-Viridin **1a** is a known steroidal antifungal metabolite produced by *Gliocladium* and *Trichoderma* strains, first described in 1945 (Brian and McGowan 1945) while β-viridin **1b** is described as a result of its isomerization (Grove et al. 1965) which can occur during chromatography on silica (Avent et al. 1992). Fraction 3 gave gliovirin **2a** which is a diketopiperazine previously isolated from *Gliocladium virens* culture media (Stipanovic and Howell 1982) with an activity against *P. ultinum* and other *Phytophthora* sp. (Howell and Stipanovic 1983).

Further purification of fraction 4 allowed identifying four known metabolites. One was a reduction product of α-viridin, viridiol **2c**, which was previously described as a phytotoxin (Howell and Stipanovic 1984) and having necrotic activity (Andersson et al. 2010). The second, heptelidic acid **3a**, was a sesquiterpene produced by different strains of fungi isolated from soil samples (Itoh et al. 1980) and by fungal endophytes (Calhoun et al. 1992). It has been described with antimicrobial, against anaerobic bacteria (Itoh et al. 1980), and antiplasmodial (Tanaka et al. 1998) activities. This compound was inactive against *P. palmivora*.

The two other products were derived from gliovirin and previously isolated from *Trichoderma* sp. (Seephonkai et al. 2006), pretrichodermamide A **2b** and trichodermamide **2c**. In addition, four products were purified from fraction 5 by HPLC. Three were known metabolites, hydroheptelidic acid **3b** (Calhoun et al. 1992), gliocladic acid **4** (Itoh et al. 1982),

Fig. 1 Structures of purified metabolites
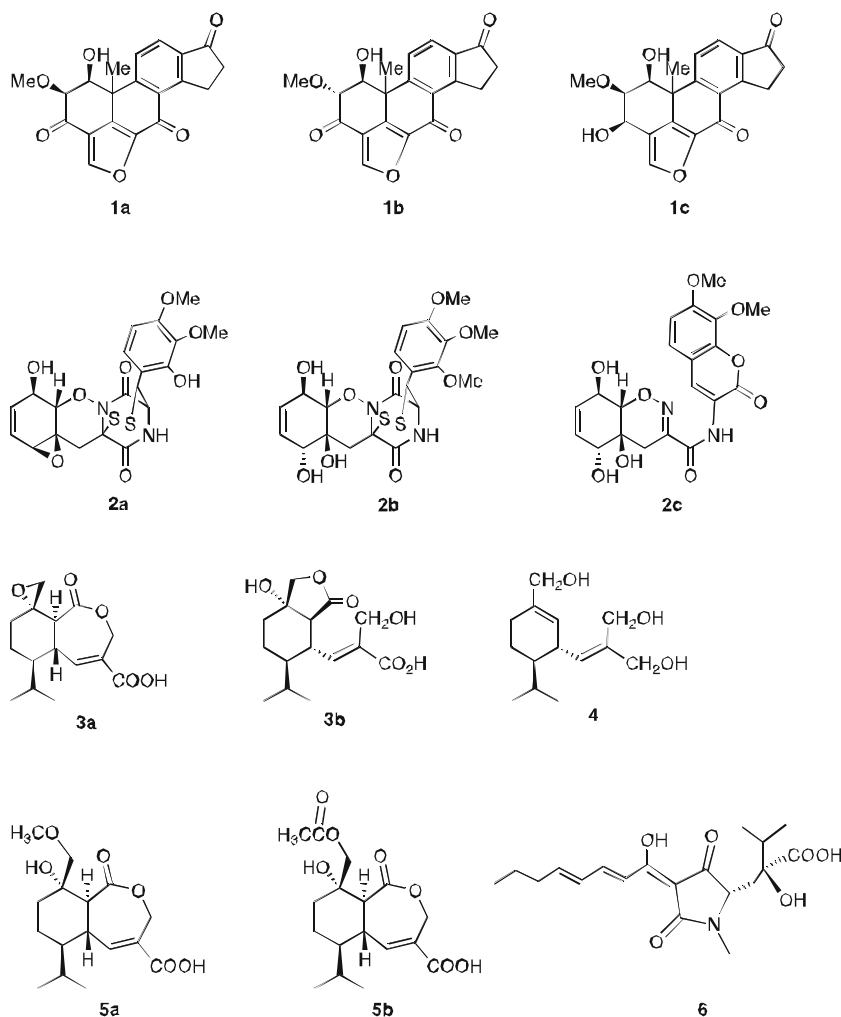
and trichoderonic acid A **5a** (Yamaguchi et al. 2010) which was obtained in mixture with the new compound **5b**. The molecular formula of **5b** was determined to be $C_{17}H_{24}O_7$ (m/z 363.14 found $[M + Na]^+$) using HR-ESIMS spectrum. Compared to that of compound **5a**, the ^{13}C NMR spectrum of compound **5b** showed one new carbonyl signal at δ 172.9 and one new methyl group at δ 28.6. These carbonyl (C-16) and methyl (C-17) groups were localized near C-15 on the basis of HMBC correlations between C-16 (δ 172.9) and H-15 (δ 4.47, d, J = 12.2 Hz) and H-17 (δ 2.09, s). Accordingly the C-16 and C-17 signals formed an acetyl group in compound **5c.** This compound was identified as a new derivative named trichoderonic acid C.

The separation of *T. harzanium* T40 extract gave one active fraction. Purification of this fraction yielded harzianic acid. It has been previously described as having an activity against *Pythium irregulare* and having a plant growth promoting effect (Vinale et al. 2009). Since it had no activity against *P. palmivora*, it was concluded that the active compound of the extract may be present in a very small amount, at least difficulty isolatable in hour hand.

Purification of the active compound from *T. asperellum* T54 extract afforded gliovirin in a good yield (300 mg, 9% of the crude extract) obtained from 18 Roux flasks.

Growth inhibiting activity

The antibiotic activities of purified compounds against seven *Phytophthora* strains (four *P. palmivora*, two *P. megakaria*, and one *P. capsici*) were determined as inhibition diameters using antibiogram paper disks soaked with solutions of the compounds (Table 2). Among them, only viridin and gliovirin were active against *Phytophthora* strains. Viridin inhibited the growth of four *P. palmivora* and *P. megakaria* NS 269 when the disk was soaked with 25 μg of product but was inactive against other *P. capsici*. Gliovirin was comparatively more active as 30% inhibition was observed with disks soaked with 6.25 μg of compound. It was also active on the mycelia growth of both *P. megakaria* but inactive against *P. capsici*.

While Mpika et al. selected *T. virens* T7 with the best antagonistic effect on *P. palmivora*, we showed that this strain produced both viridin and gliovirin. In order to study a

Table 2 Growth inhibition of *Phytophthora* strains by different concentrations of viridin, gliovirin, and equimolar mixture

	Viridin(µg/mL)		Gliovirin(µg/mL)				Viridin(µg/mL) and Gliovirin(µg/mL)				
	12.5	25	3.12	6.25	12.5	25	0.78	1.56	3.12	6.25	12.5
P. megakarya NS 269	–[a]	40 ± 5[b]	–	40 ± 5	50 ± 4	60 ± 3	–	–	–	40 ± 5	60 ± 3
P. megakarya NGR 20	–	–	–	–	47 ± 6	60 ± 5	–	–	–	40 ± 6	60 ± 5
P. capsici REG 2.5	–	–	–	–	–	–	–	–	–	–	–
P. palmivora BL7/11–2	–	27 ± 2	20 ± 2	30 ± 3	40 ± 4	70 ± 5	–	–	40 ± 4	40 ± 4	70 ± 5
P. palmivora NS 487	–	47±	–	30±	50 ±	60±	40±	40±	60±	60±	100
P. palmivora GH 17	–	20 ± 2	–	30 ± 3	40 ± 5	54 ± 4	–	–	34 ± 3	40 ± 5	47 ± 5
P. palmivora TRI 1	–	30 ± 5	–	30 ± 5	50 ± 4	67 ± 5	–	–	47 ± 4	60 ± 5	70 ± 5

Five microliters of each compound at the given concentration was applied on an antibiogram paper disk. Growth of *Phytophthora* strains were scored after 3 days of incubation, and inhibition growth is expressed as [(growth in untreated control − growth in treatment) / growth in untreated control] × 100

[a] – no inhibition

[b] In percent, the values are mean of three reading (see the "Materials and Methods" section for details)

possible synergistic effect, experiments with mixtures of viridin and gliovirin were also conducted. The same amounts of both compounds were deposited on the disks. A slight increase in inhibition was observed for *P. palmivora* and *P. megakaria* spp., especially for *P. palmivora* NS 487. However, no effect was observed for *P. capsici* which remained resistant to all of these conditions.

Anti-germination activity

The activities of purified compounds against spore germination of *P. palmivora* strains were determined. Bioassays were performed in 96-well plates by incubation of *Phytophthora* zoospores in PDB medium containing variable concentrations of the products (0.097 to 200 µg ml^{-1}). After 24 h, we observed that only viridin and gliovirin remained active. The minimum inhibitory concentrations (MICs) are reported in Table 3.

The results showed that activities were strongly dependent on the strains, and that viridin was more active than gliovirin. *P. palmivora* TRI 1 was the more sensitive to viridin with MIC ≤ 10 µg ml^{-1}. Mixtures of viridin and gliovirin were also tested as anti-germinative. Both compounds were used at the

Table 3 Minimum inhibitory concentrations (µg.mL^{-1}) of viridin, gliovirin, and equimolar mixture against germination of *P. palmivora* strains after 24 and 48 h of contact

| Strains	Viridin		Gliovirin		Viridin + gliovirin	
	24 H	48 H	24 H	48 H	24 H	48 H
P. palmivora BL7/11–2	25	50	50	100	0.78	12.5
P. palmivora NS 487	50	50	100	100	0.78	0.78
P. palmivora GH 17	12.5	50	25	25	3.12	3.12
P. palmivora TRI 1	6.25	12.5	100	100	3.12	3.12

same concentration. The results demonstrated that the activity of the mixture was much greater than each compound individually. Especially for *P. palmivora* NS 487, responding to the MIC of 50 µg ml^{-1} of viridin and 100 µg ml^{-1} of gliovirin, only 0.78 µg ml^{-1} of the mixture inhibited the germination. Moreover, the activity of the mixture was stable at 48 h, except in the case of *P. palmivora* BL7/11-2.

Since *T. virens* has been used for the biocontrol of plant pathogens, we also investigated the anti-germinative activity of these compounds against *Botrytis cinerea* and *Fusarium oxyporum*. Gliovirin exhibited no activity on these fungi while viridin showed a MIC of 25 and 12.5 µg ml^{-1} respectively, after 48-h incubation.

Detection of metabolites in the confrontation zones of fungal co-cultures

In order to verify the involvement of viridin and gliovirin in the antagonistic effect, *Trichoderma* and *Phytophthora* strains were cultured alone and in co-culture using Petri dishes containing V8 juice agar or cacao pod agar. In both media, the radial mycelial growth rates of fungi were fast and the colonies reached the edge of the plates after 5 days of inoculation. However, on V8 juice agar, colonies were compact and clearly visible (Fig. 2a) when on cacao pod agar, mycelia were thin and inconspicuous, although a significant sporulation of *Trichoderma* was observed (data no shown). In both culture media, *T. virens* T7 and *T. asperellum* T54 produced active metabolites. The growth of *P. palmivora* BL7/11-2 on cacao pod agar medium was inhibited by viridine and gliovirin (Fig. 2b). In the case of co-culture of *T. virens* T7 and *P. palmivora* TRI 1 on pod agar medium, LC/MS analysis showed the presence of gliovirin and viridin in the confrontation area before recovery.

Fig. 2 Growth inhibition of *Phytophthora sp.* **a** Antagonism between *T. virens* T7 and *P. palmivora* BL7/11-2 on V8 agar medium. **b** *P. palmivora* BL7/11-2 on pod glucose agar medium in presence of purified compound (25 μg)

A

a: *T. virens* T7,
b: *P. palmivora* BL7/11-2

B

a: DMSO, b: gliovirin, c: viridin, d: harzianic acid
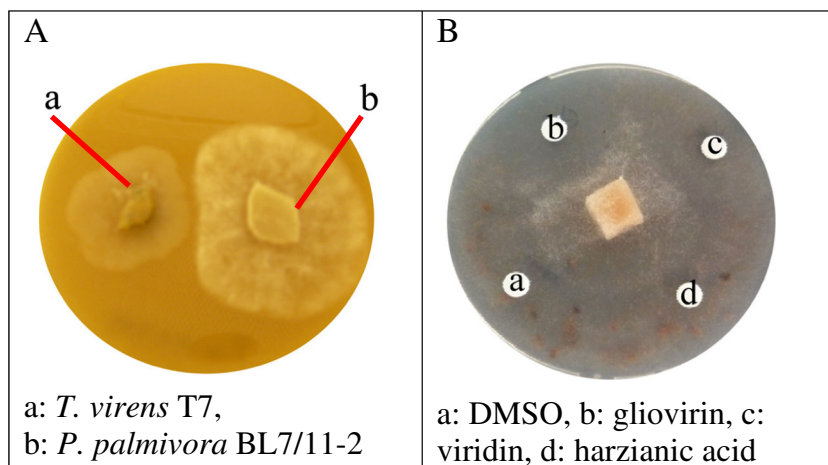
Discussion

Trichoderma were selected because of their antagonistic effect on the growth of *P. palmivora*, a causative agent of cocoa black pod disease. The *Trichoderma* strains were isolated from soil samples of a cocoa plantation in Ivory Coast by trapping, by burying fragments of pods infected with *P. palmivora* (Mpika et al. 2009). Among the *Trichoderma* isolated by Mpika, the CNRA in Ivory Coast provided us with three species *T. virens* T7, *T. harzanium* T40, and *T. asperellum* T54, and with the *P. palmivora* strain used in the previous screening study. Additional *Phytophthora* strains were isolated in other countries by the CIRAD.

The purification of crude extracts produced 12 metabolites. Among them, one was a new compound and two were already known with antagonist effects against *Phytophthora* spp., except *P. capsici*, the causing agents of the cacao black-pod disease. Viridin was reported to prevent the germination of spores of several fungi such as *B. allii*, *Colletotrichum lini*, and *F. caeruleum* at low concentrations and other strains such as *Penicillium expansum*, *Aspergillus niger*, and *Stachybotrys atra* at higher concentrations (Reino et al. 2007). It was a better inhibitor of the growth of fungi than *Pythium ultimum* (Lumsden et al. 1992). Viridin was known for its antihelminthic activity (Bacikova et al. 1965) and its antiproliferative cytostatic effects (Smith et al. 2009). Our results showed that viridin was more active in inhibiting spore germination of *P. palmivora* strains in comparison to their mycelial growth.

Moreover, gliovirin, described to be selectively active against Oomycetes (Howell and Stipanovic 1983), presented a low antigerminative activity but strongly inhibited the growth of *P. palmivora* and *P. megakarya*. This may be due to the gliovirin mechanisms of action as previously evidenced to cause protoplasm coagulation in *P. ultinum* (Howell 1982). It was shown in the epidithiodiketopiperazines like gliotoxin that the presence of the disulfide bridge (Iwasa et al. 2011)

could be one of the causes of their toxicity. Furthermore, pretrichodermamide A **2b**, an hydrolyzed-epoxide form of gliovirin, was showed to be inactive against any *Phytophthora* species. Therefore, the activity of gliovirin on the *Phytophthora* species could be due to the presence of the epoxide rather than one disulfide bridge.

A synergistic effect of viridin and gliovirin, i.e., antigerminative and growth inhibiting activities, was underscored particularly in *P. palmivora* strains. We showed the production of both these compounds as well as the synergistic effect on microorganisms cultivated on pod glucose agar medium. Similar synergies were already mentioned by previous studies, involving either two products of biological control agent like secondary metabolites and a hydrolytic enzyme (Dipietro et al. 1993, Saravanakumar et al. 2016) or one antifungal chemical and a biological control agent (Howell 1991). Here, we showed for the first time a synergetic effect against Oomycetes between two metabolites produced by one microorganism. This effect of both gliovirin and viridin not only resulted in significantly higher activities but also in the stability of activities in a long run. This latter point was important for assessing the antigerminative activity.

It is known that the biotransformation of toxic molecules is a strategy to overcome their toxicity. Furthermore, a loss of activity of gliovirin in a non-sterile soil was demonstrated, while it was preserved in a sterile soil, suggesting a biodegradation of the active molecule (Howell and Stipanovic 1983). Such biotransformations could involve the reduction of viridin into viridiol and the hydrolysis of the epoxide of gliovirine leading to inactive metabolites. We also pointed out that the growth of both tested *P. megakarya* was not inhibited in the same manner by gliovirin, but identical inhibitions (40%) were observed when treated by mixture of compounds. Further studies on the biotransformation of both compounds by phytopathogens may be necessary to explain these results.

T. virens strains are separated into two groups, "P" and "Q", on the basis of their secondary metabolites (Howell et al.

1993). The P-strains produce gliovirin and heptelidic acid and are ineffective biocontrol agents of seedling disease in cotton (Howell and Puckhaber 2005). The Q-strains, which are effective biocontrol agents of cotton seedling disease, produce gliotoxin but not gliovirin and heptelidic acid. In summary, *T. virens* T7 isolated by Mpika et al. belongs to the P group. In accordance with the synergistic effect showed in this study, the production of both the gliovirin and viridin explains the high activities observed against *P. palmivora*. Furthermore, as genes involved in the biosynthesis of secondary metabolites are usually in cluster, their expression strongly depends on the environmental factors. Our study indicated that viridin and gliovirin were co-produced by *T. virens* T7 within near-natural conditions to reach higher activity against the phyto-pathogenic competitors such as *Phytophthora* species.

Finally, our analysis on *Phytophthora* species from various parts of the world highlights the precautions for the use of *Trichoderma* species in the control of black pod disease of cocoa. Since *Trichoderma* strains used in the present work are not effective against *P. capsici*, they cannot be used as biocontrol agents in Brazil where cocoa is mainly infested by this pathogen. However, promising results were recently obtained with other strains of *Trichoderma* (Bae et al. 2016) and with *Streptomyces* sp. (Chen et al. 2016) as biological control agents against *P. capsici* on pepper and tomato plants. Likewise, in Cameroon, *P. megakarya* is the most abundant and its control could be achieved with *T. virens* T7 that produces the two effective molecules. In Ivory Coast, the first world producer of cocoa, there is a fear because *P. megakarya* is replacing *P. palmivora* in terms of populations. Hopefully, our data show once more that *T. virens* T7 could be used as a biocontrol agent over there. In addition, since both of the pathogens are sensitive to viridin and gliovirin, each one of them could be used on any one of the pathogens.

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