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A new oil-based formulation of *Trichoderma asperellum* for the biological control of cacao black pod disease caused by *Phytophthora megakarya*

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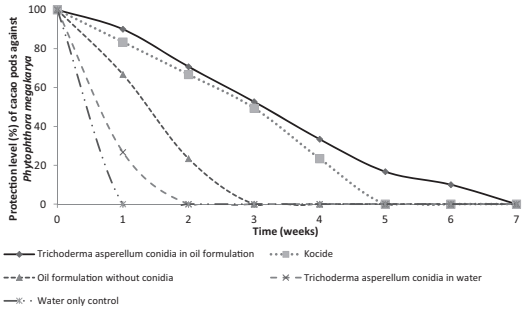
HIGHLIGHTS

- New oil-based dispersion with a half-life of *T. asperellum* conidia of 22.5 weeks.
- Complete inhibition of *P. megakarya* on sprayed detached pods.
- Enhanced rate and duration of protection on sprayed cacao pods in the field.
- 50% of pods protected 3.2 weeks after spraying in the field.
- Formulation suitable for the spraying equipment of small-scale cacao producers.

GRAPHICAL ABSTRACT

Formulated conidia of *Trichoderma* provide similar or even better protection of cacao pods against black pod disease than a conventional fungicide.



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ABSTRACT

In African cacao-producing countries, control of cacao black pod disease caused by *Phytophthora megakarya* is a priority. Introducing biological control agents as part of a *P. megakarya* control strategy is highly desirable, especially in a perspective of pesticide reduction. *Trichoderma* species are among the most used biological control agents. In Cameroon, *Trichoderma asperellum* formulated in wettable powder has produced positive effects against this disease. However, with this type of formulation, shelf-life and persistence of conidia on pods are limited. Our study therefore sought to develop a new *T. asperellum* formulation that would be more effective and better suited to the conditions of field application by small-scale producers in Cameroon. We selected a soybean oil-based oil dispersion, in which the half-life of the conidia reached 22.5 weeks, versus 5 weeks in aqueous suspension. Tested on detached pods, the formulation completely inhibited the development of the disease. When sprayed in the field on cacao clones highly sensitive to *P. megakarya*, the formulation resulted in 90% protection of treated pods after 1 week, and 50% after 3.2 weeks. The formulations exercised a measurable effect for up to 7 weeks, versus 2 weeks in the case of an aqueous conidial suspension and 5 weeks for that of a conventional fungicide (Kocide). *Trichoderma asperellum* formulated in oil dispersion has therefore great potential for the control of cacao black pod disease with less recourse to synthetic fungicides.

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Moreover, this formulation is well adapted to the types of sprayers used by small-scale cacao producers in Cameroon.

1. Introduction

Cacao, *Theobroma cacao* L., is one of the most important cash crops in numerous developing countries. World production of merchantable cacao, estimated around 4.3 million tons (ICCO, 2011), originates from 5 to 6 million producers and is essential to the livelihood of 30–40 million people throughout the world (World Cacao Foundation, 2012). Africa's two million small-scale producers account for 75% of world production (ICCO, 2011).

Cacao black pod disease, caused by several species of the Straminipile genus *Phytophthora* (Kroon et al., 2004; Tyler et al., 2006) is the most common disease of cacao causing global yield losses estimated at 30% (Guest, 2007). In Cameroon, the most widespread species is *Phytophthora megakarya* Brassier & Griffin (Mfegue, 2012), which is endemic to West Africa and present in all four of the largest cocoa producing countries in this region, Ivory Coast, Ghana, Nigeria and Cameroon (Guest, 2007). Cacao pods can be attacked all year round at all development stages. However, high-incidences occur during rainy seasons (Guest, 2007). In the absence of any crop protection measures, *P. megakarya* can sometimes cause up to 80–100% yield loss (Ndoumbe-Nkeng et al., 2004). In African cacao-producing countries, control of cacao black pod disease is thus both a necessity and a priority. Current control approaches are mainly based on strategies involving a mix of cultural and chemical control means, improved agricultural practices, the use of partially resistant and/or tolerant varieties, and biological control methods. Yet, for many, spraying of fungicides remains their principal control method.

Successes in biological control, using antagonistic agents against plant pathogens, have led to the manufacture and registration of a number of biological control products (Fravel, 2005; Kaewchai et al., 2009). Several *Trichoderma* species, such as *Trichoderma asperellum, T. harzianum, T. polysporum, T. viride* and *T. virens* have already been used successfully as biological control agents against a variety of phytopathogenic fungi (Almeida et al., 2007; Benitez et al., 2004; Harman et al., 2004; Hermosa et al., 2013; Kaewchai et al., 2009). *Trichoderma* species are also being developed as biological control agents of cacao diseases such as witches' broom (caused by *Moniliophthora perniciosa*), frosty pod rot (caused by *Moniliophthora roreri*) and black pod disease (caused by *Phytophthora* spp.) (e.g. De Souza et al., 2008; Krauss et al., 2006, 2010; Tondje et al., 2007). In the case of Witches' broom this has even led to the development of a registered biological control product, called Trichovab® (Samuels, 2004), based on *Trichoderma stromaticum*, being used in a Brazilian government funded program to control *M. perniciosa*.

In Cameroon, research efforts to develop a biological control strategy against *P. megakarya* resulted in the selection of mycoparasitic strains of *T. asperellum* (Tondje et al., 2007). These strains were formulated as wettable powders, using cassava flour as carrier, and have been tested in field trials (Deberdt et al., 2008; Tondje et al., 2007). The results of these trials, although relatively positive, also showed limitations of the formulation being used, such as a high susceptibility to wash-off due to rain and fast desiccation of the conidia when applied to cacao pods (Deberdt et al., 2008).

Improving the conidial formulation of *T. asperellum* is therefore an essential step towards optimization of this biological control strategy. Bateman and Alves (2000), recommend oil-based formulations for conidial biological control formulations because of their greater ability to adhere to the substrate. Moreover, such formulations slow down the desiccation process under conditions of fluctuating environmental factors such as temperature and relative humidity. Therefore, this study focused on assessing the feasibility of using oil dispersions as a carrier matrix for conidial formulations of *T. asperellum*. The objective is to prolong shelf-life and persistence of the biological control agent on the biological target and thus improve the protection of cacao pods against *P. megakarya*. The findings will help to optimize the practical use of *T. asperellum* in the biological control of cacao black pod disease.

2. Materials and methods

2.1. Fungal material

All experiments were performed with *P. megakarya* strain EL1. This strain was isolated from a naturally-infected cacao pod, collected in a trial plot in Eloumden (Yaoundé, Cameroon). Strain PR11 of *T. asperellum* [Genebank number EF186002] was isolated in Cameroon from a soil sample taken from within the rhizosphere of cocoyam plants (*Xanthosoma sagittifolium*) with symptoms of root rot and conserved in national and international collections as described in Begoude et al., (2007). This strain was characterized molecularly (Samuels et al., 2010), and its antagonistic activity in relation to *P. megakarya* and *Pythium myriotylum* – the pathogens respectively responsible for cacao black pod disease and cocoyam root rot disease – was ascertained (Mbarga et al., 2012; Tondje et al., 2007).

Mass production of *T. asperellum* PR11 conidia was done following the method described by Hanada et al. (2009). Conidia were produced using a solid state fermentation process with rice as the substrate. Conidia were extracted from their growth substrate using a mycoharvester, version V (http://www.dropdata.net/mycoharvester).

2.2. Formulations

2.2.1. Carrier additives

To develop the formulation matrix, several carrier additives of four different types were tested: (1) two vegetable oils (palm oil and soybean oil), (2) five emulsifying-dispersing agents (Tensiofix NTM, DB08, IW60, OC653, and Tween 20), (3) one structural agent (Tensiofix 869) and (4) one source of carbon (glucose). The Tensiofix agents were provided by the agrochemical company S.A. Ajinomoto OmniChem N.V. (www.tensiofix.com). All tested emulsifying-dispersing agents are liquid between 20 and 35 °C, have a 0.5% water content and are non-ionic, with the exception of Tensiofix IW60, which is anionic. Tensiofix 869 is a bentonite clay that comes as a powder with a maximum water content of 10%.

2.2.2. Preparation of the oil dispersions

The compositions of the different oil dispersions tested are detailed in Table 1. They were prepared by first mixing the oil with the emulsifying-dispersing agent, and then adding the structural agent and the glucose and where applicable, finally the water. The *T. asperellum* conidia were then incorporated progressively. The mixture was homogenized using a Diax 900 homogenizer (Heidolph Co., Germany) for 10 min at 5000 rpm. All formulations

Table 1
Composition (w/w) of the six different oil dispersions tested.

Ingredients of formulations	Formulations					
	1	2	3	4	5	6
Palm oil	0	0	74%	74%	0	71%
Soybean oil	74%	74%	0	0	71%	0
Tensiofix NTM	15%	15%	15%	15%	0	0
Tensiofix DB08	0	0	0	0	9%	9%
Tensiofix IW60	0	0	0	0	5%	5%
Tensiofix OC653	0	0	0	0	1%	1%
Tween 20	0	5%	0	5%	0	0
Tensiofix 869	5%	0	5%	0	0	0
Glucose	4%	4%	4%	4%	4%	4%
Water	0	0	0	0	24%	24%
Pure *T. asperellum* Conidia	2%	2%	2%	2%	2%	2%

had a final conidial concentration of 2.7×10^7 conidia ml^{-1} (Table 1). The prepared formulations were each divided over ten test tubes and stored at 25 ± 2 °C in a room with 30% relative air humidity.

2.2.3. Characterization of the oil dispersions

The pH and viscosity of each formulation were measured for each of ten test tubes, every day for ten consecutive days, using a pH-meter and a viscometer (VT-03 viscometer, Rion Co. Ltd., Japan).

An emulsification test was carried out to evaluate the stability of the formulations when mixed with water. Graduated glass test tubes were filled with 95 ml of water of moderate pH (CIPAC standard water D with 342 ppm $CaCO_3$) and kept in a 30 °C bain-marie for 24 h. Subsequently, 5 ml of a formulation was added and homogenized manually. The homogeneity of the resulting mixtures and the degree of sedimentation of the conidia were assessed every day for 10 days. Ten test tubes were used for each formulation, and the experiment was replicated three times.

A formulation was considered stable if the different variables monitored remained constant throughout the ten-day-long experiment. The formulation with the best stability and lowest viscosity was selected.

2.3. Shelf-life of T. asperellum conidia in the formulation

The viability of *T. asperellum* conidia in the formulation and in aqueous suspension was assessed over a period of 35 weeks. Each week, a sample was diluted 10^4-fold and 1 ml was spread in five Petri dishes containing Potato Dextrose Agar (PDA) (Difco Becton Dickinson, Sparks, MD) culture medium. The control was a $1{:}10^4$ dilution of the aqueous suspension of pure *T. asperellum* conidia (2.7×10^7 conidia ml^{-1}). After 12 h at 25 ± 2 °C, three sample areas of 1 cm^2 were delimited within each Petri dish and examined under a light microscope. The number of germinated conidia out of a sample of 100 conidia per sample area examined was recorded. A conidium was considered germinated when its germ tube exceeded the conidium's diameter. Incubation was pursued to further ascertain the growth potential of the formulated PR11 strain.

2.4. Effect of the carrier additives on the in vitro growth of P. megakarya

The poisoned food technique (Shekhawat and Prasad, 1971) was used. A V8 culture medium (200 ml V8 juice, 15 g agar, 3 g calcium carbonate and distilled water qsp 1000 ml) was mixed with 10% v/v of the selected formulation, yet without *T. asperellum* conidia, and used to prepare a set of Petri dishes. All formulation constituents were sterilised separately before mixing them as described in Section 2.2.2.

A 5 mm diameter implant from a five-days-old *P. megakarya* culture was then deposited in the middle of each amended Petri dish, with the mycelium-covered side in contact with the culture medium. A total of 30 replicate plates were incubated at 25 ± 2 °C for 7 days. Mycelial growth of *P. megakarya* was assessed by measuring every day the diameter of each colony along two predefined perpendicular axes. Control dishes contained *P. megakarya* cultured on unamended V8 medium. The data recorded were used to calculate the radial growth rate of each colony, expressed in mm day^{-1}, based on the slope of the linear regression of the growth curve (Begoude et al., 2007).

2.5. Protective effect of the formulation on detached cacao pods

A detached cacao pod test was carried out based on the method for evaluating the resistance of cacao clones to *Phytophthora palmivora* in the laboratory, as developed by Iwaro et al. (2000). The test was undertaken using three cacao clones: SNK10, highly sensitive to cacao black pod disease (Efombagn et al., 2011), and BBK1606 and SNK630, respectively moderately sensitive and tolerant (unpublished data). The effectiveness of the treatment with the selected formulation was compared with four other treatments: (i) the selected formulation without conidia, (ii) an aqueous suspension of conidia adjusted to a concentration of 2.7×10^7 conidia ml^{-1}, (iii) a water-only control treatment, and (iv) a conventional fungicide treatment using Kocide 2000 (a.i. copper hydroxide) (LDC Cameroun, BP 2368, Douala, Cameroon). The pods of the various cacao clones were first washed in water, and each was sprayed with one of the different treatment liquids with a hand held pressurized sprayer (mean volume used: 5 ml of formulation per pod). Treated pods were deposited in trays lined with absorbent paper and left to dry at 25 ± 2 °C for 2 h. Each one was then sprayed with 200 µl of a suspension of *P. megakarya* zoospores of the EL1 strain, adjusted to a concentration of 3×10^5 zoospores ml^{-1}. Zoospores of *P. megakarya* were obtained using the protocol as described by Tondje et al. (2006). The absorbent sheet of paper lining each tray was moistened using sterile distilled water and the trays were covered in order to create a favorable environment for the development of the disease. Four pods per clone and per treatment were used, and the experiment was replicated three times. The pods were left to incubate at 25 ± 2 °C for 4 days. The severity of the infection was scored according to the 0–7 scale developed by Iwaro et al. (2000): 0 – no visible lesion; 1 – 1 to 5 localized lesions; 2 – 6 to 15 localized lesions; 3 – over 15 localized lesions; 4 – 1 to 5 expanding lesions; 5 – 6 to 15 expanding lesions; 6 – over 15 expanding lesions; 7 – coalesced lesions. Each pod was thus awarded a sensitivity score. For each treatment, the rate of inhibition of *P. megakarya* development was calculated according to the following formula:

$$I\ (\%) = \left(1 - \frac{Cn}{Co}\right) \times 100$$

With I the mean inhibition rate in%, Cn the mean sensitivity score of the treated pods, and Co the mean sensitivity score of the water only control pods. This was done for individual clones as well as for pooled data.

2.6. Protective effect of the formulation on cacao pods in the field

The trial was set up in September 2012 in a clonal cacao plot at the IRAD Nkolbisson research station (Ten Hoopen et al., 2012), and was monitored for a period of 7 weeks. This relatively homogeneous cacao plot is made up of two sub-plots. Cacao trees in each

sub-plot were planted in 2001. Spacing between individual trees is 2.5 m × 2.5 m and maximum tree height is 3 m. Separate clones are planted along lines of approximately 30 m. Black pod disease has not yet been recorded in this particular plot. For this trial, 500 four-months-old pods were selected from 20 cacao trees of clone SNK10, 10 trees in each of the two sub-plots.

The effectiveness of the oil-based formulation was compared with the other four treatments described in Section 2.5. Each treatment was applied to 100 pods. Before application, each pod was wiped clean with 70% ethanol, left to air dry and subsequently sprayed with 10 ml of treatment liquid to incipient run-off using a hand held pressurized sprayer. For each group, every week for 7 weeks, 10 healthy pods were chosen randomly and brought to the laboratory to assess the protective effect against *P. megakarya*. For this, a droplet of 10 µl of a suspension of zoospores of the EL1 strain of *P. megakarya* adjusted to 3×10^5 zoospores ml^{-1} were deposited onto the middle of the pod. After 4 days of incubation at 25 ± 2 °C in trays lined with moistened absorbent paper, the number of healthy pods were counted for each treatment, and the corresponding protection rate was calculated by dividing the number of healthy pods by the total number of pods and multiplying by 100.

2.7. Data analysis

The evolution of the germination rate of both the formulated and non-formulated (aqueous suspension) conidia was analyzed using *Curve Expert* software, version 9.0. Data relating to the effects of the carrier additives were arc-sine-transformed prior to statistical analyses. The pod severity scores were $\log(n + 1)$ transformed prior to analysis. The analyses of variance (ANOVA) were performed using SAS software, version 9.1 (SAS Institute Inc., 2007). Whenever significant, Duncan's multiple range test was used to differentiate between means.

3. Results

3.1. Selection of the most appropriate oil dispersion

The physico-chemical characteristics of the six oil dispersions tested are shown in Table 2. When formulations 1 and 3 were mixed with water, they remained homogeneous, of a creamy-white color, and no sedimentation of the *T. asperellum* conidia other than traces was recorded during the ten-day-long experiment. In contrast, when the other four tested formulations were diluted in water and homogenized, ten minutes later the phases had separated and the *T. asperellum* conidia had all sedimented. In all formulations, pH values remained constant. Formulation 1 differed from formulation 3 in that it exhibited lower viscosity. On the basis of these physico-chemical characteristics, formulation 1 was the only one that could be considered as a good emulsion. This formulation was therefore selected for subsequent use.

3.2. Shelf-life of T. asperellum conidia in formulation 1

The germination rate of the conidia suspended in formulation 1 was 90.6 ± 2.3% after 1 week, and 20.9 ± 2% after 35 weeks. In contrast, that of the conidia in aqueous suspension was 92.4 ± 2.1% after 1 week, fell to less than 5% after just 10 weeks and was down to zero after 17 weeks. In formulation 1, the half-life of *T. asperellum* conidia (the time after which 50% of conidia were still able to germinate) was estimated at 22.5 weeks (Fig. 1). For the aqueous suspension, this point was already reached after 6 weeks. After each germination test, the viability of the *T. asperellum* conidia was further confirmed by the fact that petri-dishes became

Table 2
Characteristics of the six tested oil dispersions.

Characteristics of formulations	Formulations					
	1	2	3	4	5	6
pH	6.1	5.2	5.3	5.2	6	5.4
Viscosity (cps) [a]	106 ± 14	208.61 ± 10	3849 ± 8	90 ± 15	1620 ± 35	9250 ± 25
Conidial sedimentation [b]	Traces	Total	Traces	Total	Total	Total

[a] Viscosity measured with a viscometer. Viscosity remained practically constant from day 0 to day 10. Figures shown are the means of 10 samples of formulation ± standard deviation.
[b] Conidial sedimentation was assessed every day for the 10 days that followed the preparation of the formulations.

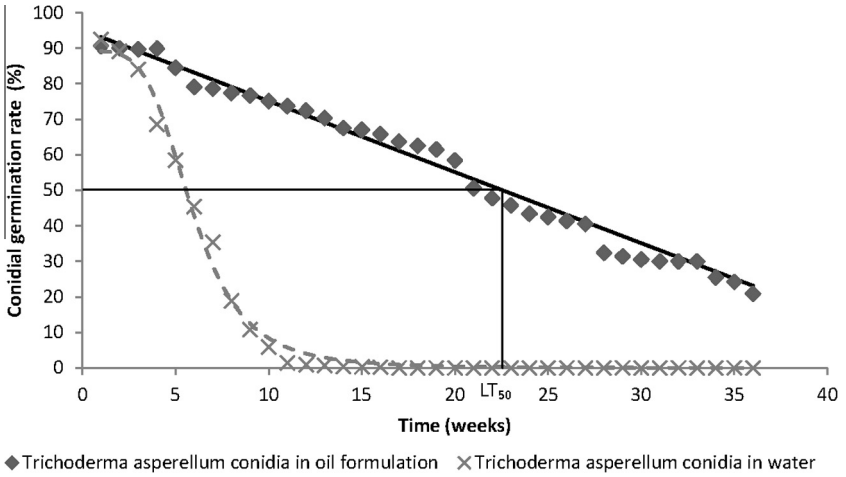

Fig. 1. Germination level of *T. asperellum* conidia over time for formulated (♦) and non-formulated conidia (▲) of *T. asperellum* PR11.

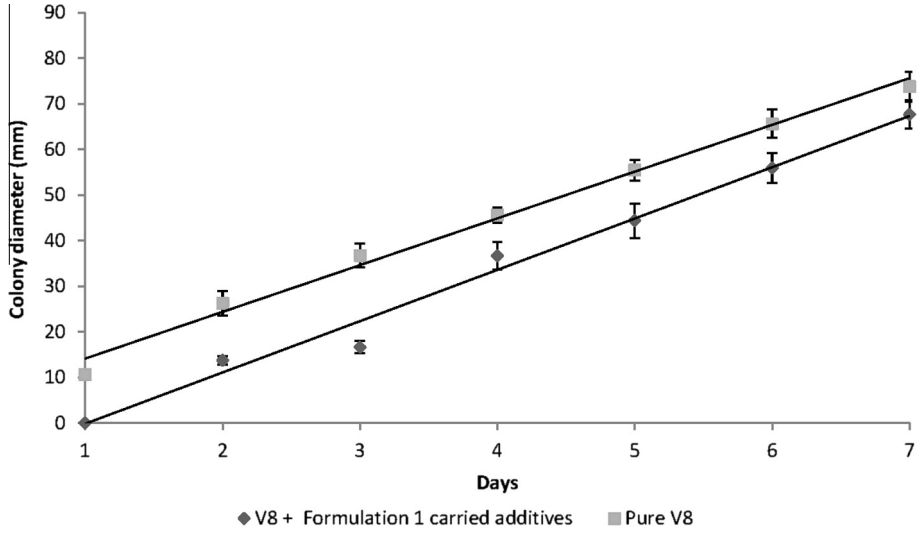

Fig. 2. Effect of the carrier additives of formulation 1 on the mycelial growth of *P. megakarya*.

completely colonized. The linear regression equation that described the decrease in germination rate of formulated conidia over time is $y = 95.1–2 * x$ ($R^2 = 0.99$). The non-linear decrease in germination rate of the conidia in aqueous suspension was best described by the Farazdaghi-Harris' model and was given by $y = 1/(0.01 + 5.8 * 10^{-6} * X^{4.3})$ ($R^2 = 0.99$).

3.3. Effect of the carrier additives of formulation 1 on the in vitro growth of P. megakarya

The effect of formulation components on *in vitro* growth of *P. megakarya* is presented in Fig. 2. In the culture medium amended with all carrier additives from formulation 1, the onset of *P. megakarya* mycelial development was delayed by 24 h. Interestingly, no other effect on subsequent growth of the pathogen was observed. The analysis of variance showed no significant difference ($P = 0.383$) for *P. megakarya* growth rate, yet revealed a highly significant difference ($P = 0.0001$) regarding the y-intercept values of the two regression lines. The linear regression equations that described *P. megakarya* growth on the unamended and amended growth media were $y = 10.3X + 3.8$ ($R^2 = 0.992$) and $y = 11.3X-11.4$ ($R^2 = 0.986$), respectively.

3.4. Protective effect of formulation 1 on detached pods

Four days after inoculation with *P. megakarya*, all pods treated with formulation 1 containing *T. asperellum* conidia were still free of disease symptoms, regardless of the cacao clone used (Table 3). For all other treatments, some *P. megakarya* lesions were found, either restricted and localized or numerous and touching, depending on the treatment administered.

The analysis of variance regarding the severity of the symptoms observed on the detached pods showed no differences between replications ($P = 0.394$) However, significant differences between cacao clones ($P < 0.0001$) as well as treatments ($P < 0.0001$) were observed. Moreover, there was a significant ($P < 0.001$) interaction between clone x treatment which would indicate that a treatment would be more or less effective depending on the clone it is applied to. Here, the interaction was due to the fact that clone BBK1606 was equally sensitive to the control treatment (water only) as SNK10 which is considered to be more sensitive, and to the fact that the formulation with *T. asperellum* conidia was 100% capable of protecting pods, irrespective of their sensibility to black pod disease. However, overall results did indeed show that SNK10 is more sensitive than BBK1606, and SNK630 is least sensitive, confirming their relative susceptibility to black pod disease as mentioned in Section 2.5. Treating the pods with formulation 1 including conidia and carrier additives resulted in the complete inhibition of the disease (Table 3). This formulation was even more effective than the synthetic fungicide used as positive control. The aqueous suspension of conidia as well as the carrier additives of formulation 1, were also able to significantly inhibit the disease in comparison with the water-only control, but formulation 1 was the only treatment that fully protected the cacao pods.

Table 3
Protective effect of formulation 1 on detached pods of three clones with differential susceptibility to *P. megakarya*.

Treatment	Clone SNK10		Clone SNK630		Clone BBK1606		All pods	
	Pod sensitivity score [a]	Black Pod inhibition rate (%)	Pod sensitivity score	Black Pod inhibition rate (%)	Pod sensitivity score	Black Pod inhibition rate (%)	Pod sensitivity score [b]	Black Pod inhibition rate (%)
Formulation 1 with conidia	$0 \pm 0^{\alpha}$	100%	$0 \pm 0^{\alpha}$	100%	$0 \pm 0^{\alpha}$	100%	0 ± 0 a	100%
Kocide	$0.8 \pm 1.2^{\beta}$	88.0%	$0 \pm 0^{\alpha}$	100%	$0 \pm 0^{\alpha}$	100%	0.3 ± 0.8 b	95.6%
Formulation 1 without conidia	$1.9 \pm 1.3^{\beta}$	72.3%	$0.3 \pm 0.5^{\alpha\beta}$	95%	$1.0 \pm 1.2^{\beta}$	85.7%	1.1 ± 1.2 c	83.3%
Conidia in aqueous suspension	$6.5 \pm 0.7^{\beta\gamma}$	6.0%	$3.5 \pm 1.6^{\beta}$	30%	$4.8 \pm 0.5^{\beta}$	32.1%	4.9 ± 1.6 d	22.0%
Water only control	$6.9 \pm 0.3^{\gamma}$	n.a.[d]	$5.0 \pm 0^{\beta}$	n.a.	$7.0 \pm 0^{\gamma}$	n.a.	6.3 ± 1.0 e	n.a.
Mean clonal sensitivity score [c]	3.2 ± 3.0 C	n.a.	1.8 ± 2.2 A	n.a.	2.0 ± 0.8 B	n.a.		

[a] Pod sensitivity scores followed by the same greek small letter do not differ at $P = 0.05$ (Duncan).
[b] Pod sensitivity scores followed by the same small letter do not differ significantly at $P = 0.05$ (Duncan).
[c] Mean clonal sensitivity scores followed by the same letter do not differ significantly at $P = 0.05$ (Duncan).
[d] n.a. = not applicable.

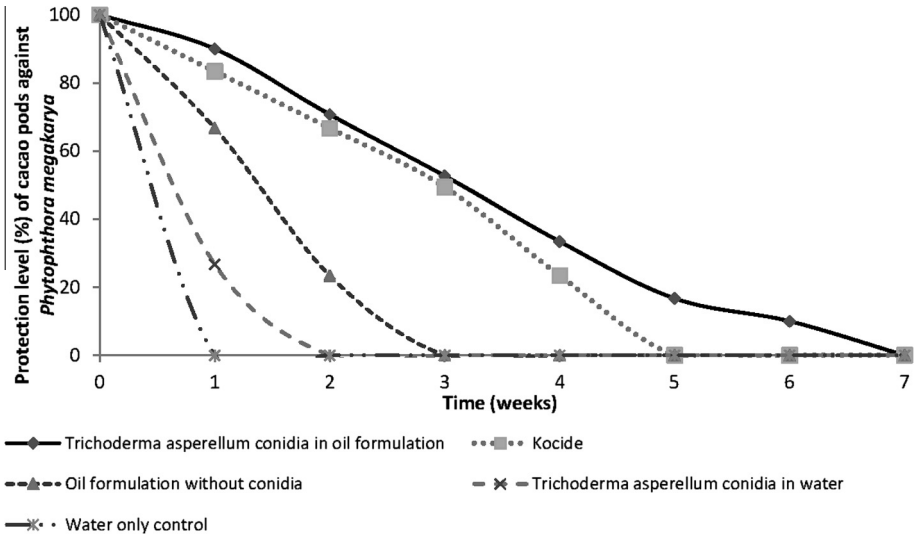

Fig. 3. Protective effect over time of formulation 1 (♦) on cacao pods in the field compared with a fungicide (■), the formulation without conidia (▲), conidia in aqueous suspension (×) and a water only control (✳).

3.5. Protective effect of formulation 1 on cacao trees in the field

The results from the field experiment showed that 90 ± 4.5% of the pods treated in the field with formulation 1 were still protected 1 week after spraying, with only 10 ± 5.5% of the pods exhibiting symptoms of black pod disease. Regarding the other treatments, after 1 week, the percentage diseased pods was 100% for the water-only control, 73.3 ± 8.2% for the aqueous suspension of *T. asperellum* conidia, 33.3 ± 8.7% for the treatment containing the carrier additives of formulation 1 (without *T. asperellum* conidia), and 16.6 ± 6.9% for Kocide 2000 (Fig. 3). The median lethal time (LT_{50}) – the time interval after which the applied product still provided a 50% protection of the pods – was approximately 3.2 weeks for formulation 1. It was around 1.5 weeks for the carrier additives (without conidia), and about 3 weeks for Kocide 2000. The protection thereafter decreased progressively with time. The longest-lasting protective effect – 7 weeks – was obtained with formulation 1.

4. Discussion

Our study is the first contribution to the development of an oil-based formulation of *T. asperellum* conidia as a biological control tool against *P. megakarya*, the causal agent of cacao black pod disease. The objectives of this study, to develop a formulation that would increase shelf life and persistence on cacao pods of formulated conidia and to improve protection against cacao black pod disease have been attained. The formulation selected was composed of soybean oil (74%), Tensiofix NTM (15%, an emulsifying-dispersing agent), Tensiofix 869 (5%, a structural agent) and glucose (4%). Our findings showed that the viability of *T. asperellum* conidia as well as their persistence on the treated pods were enhanced by this oil dispersion in comparison with the water suspension. We also showed that the application of thus formulated *T. asperellum* conidia in the field on pods of cacao clones highly sensitive to the disease have the potential to improve both the rate and the duration of the protection against *P. megakarya* in comparison with the aqueous conidial suspension and with the synthetic fungicide Kocide 2000.

About 90% of the formulations of antagonistic fungi for the biological control of plant diseases use species of *Trichoderma*, such as *T. harzianum, T. virens* and *T. viride* (Kaewchai et al., 2009). In most cases, the propagules are formulated as granules (Jin and Custis, 2011) or wettable powders (Fravel, 2005). Very few formulations use vegetable oils. To our knowledge, invert or reverse emulsions (of the water-in-oil type) are the only type of oil-based formulations of *Trichoderma* spp. conidia known to have been used so far (Batta, 2004, 2007; Wijesinghe et al., 2010, 2011). Our *Trichoderma* conidia formulated as an oil dispersion is therefore very interesting. Especially so, since it offers one important advantage compared with reverse emulsions: it is better adapted to side level knapsack (SLK) sprayers, which are the sprayers most commonly used by small-scale farmers. The conidial formulation of *T. asperellum* as a soybean oil-based oil dispersion mixes readily with water and the conidia remain in stable suspension for a long time. This is not the case of the reverse emulsions developed by Batta (2004), in which the oil and water phases can easily be discriminated. The miscibility and stability of the propagules in the formulation should facilitate their uniform distribution in the sprayer's tank when the spray liquid is prepared and the even colonization of the pods by *T. asperellum* during spraying. Reverse emulsions are applied in ultra-low volumes (Batta, 2004) with spraying equipments such as mist blowers or foggers, which many small-scale producers do not have.

In our formulation, the half-life of *T. asperellum* conidia is 22.5 weeks, i.e. 18 weeks longer than conidia in aqueous suspension. This half-life figure is close to that obtained by Batta (2004) with *T. harzianum* conidia formulated in reverse emulsion (21 weeks). Several studies have found that formulated conidia remain viable for longer periods than non-formulated conidia (Guijarro et al., 2007; Larena et al., 2007; Wijesinghe et al., 2010, 2011). This prolonged viability is thought to be related to the specific role of the various ingredients of the formulation – in this case glucose and soybean oil. The contribution of glucose to increasing the viability of microbial propagules was documented by Guijarro et al. (2007), who showed that amending a formulation with a source of carbon such as 7.5% glucose made it possible to keep *Penicillium frequentans* conidia alive for 12 months. Moreover, oils may also help to boost conidial viability by providing the micro-organisms with a food basis while regulating water availability (Paau, 1998). In this respect, it has been shown that soybean oil is among the vegetable oils that confer the best viability to *Beauveria bassiana* conidia (Mola and Afkari, 2012).

In the laboratory, treating detached cacao pods with our formulation completely inhibited the development of *P. megakarya,* whereas lesions of various sizes were observed on pods treated

with the other products, including Kocide 2000. The carrier additives used in our formulation may contribute to this protective effect, since they alone accounted for a 24 h delay in the onset of *P. megakarya* development *in vitro* and for a 83% reduction of disease development in comparison with the water-only treatment. The carrier additives included in the formulation are in theory inert ingredients in that they exhibit no capacity to control the disease in themselves. However, they may constitute a physical barrier to the infectious zoospores, and they also play a role in enhancing the effectiveness of the formulation through improving conidial viability, target coverage and wettability (Fravel, 2005).

Even though an interaction was observed between treatment and cacao pod sensibility to black pod disease, the results shown here confirm findings by Ten Hoopen et al. (2003) in that biological control and genetic disease resistance should have additive effects when both control options are applied simultaneously.

In the field, our formulation demonstrated its protective effect on the pods of cacao clones known for their susceptibility to the disease. One week after the treatment, 90 ± 4.5% of the pods were still protected. Our formulation still had 50% effectiveness after 3.2 weeks. This duration of action is practically the same as that of the *Trichoderma ovalisporum* conidial formulation used to control *M. roreri*, the causal agent of cacao frosty pod rot disease (Krauss et al., 2010). It is also similar to that of the systemic fungicides commonly employed by cacao producers in Cameroon (3 weeks). However, it is somewhat longer than that of contact fungicides such as Kocide 2000, which in practice demand a fortnightly application (Sonwa et al., 2008). Persistence probably results from the ability of *Trichoderma* species – when appropriately formulated – to colonize the cortex of the cacao pods, even when exposed to direct sunlight (Krauss et al., 2006; Ten Hoopen et al., 2003). However, effectiveness was primarily determined in the laboratory using artificial inoculations with the pathogen. Determining the effectiveness of the formulation and the persistence and viability of the *T. asperellum* PR11 spores under actual farmer-field conditions necessitates field trials, which are currently underway.

In conclusion, we selected an oil dispersion of *T. asperellum* conidia that is both stable and homogeneous, mainly constituted of soybean oil. This formulation has a positive effect on the viability and persistence of the conidia. Tested on detached pods, the formulation completely inhibited the development of the disease. When sprayed in the field on cacao tree clones highly sensitive to *P. megakarya*, the formulation performed better than a synthetic fungicide in terms of rate and duration of the protection. This *T. asperellum* in oil dispersion holds therefore potential for the control of cacao black pod disease, making it possible to rely less heavily on costly and environmentally hazardous synthetic fungicides.

This biological control approach is interesting from an environmental perspective, but also for the small-scale cacao producers of Cameroon, with the added advantage that our formulation is perfectly suited to the types of sprayers they use.

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