International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 24 No. 4 Nov. 2018, pp. 1834-1848

http://www.ijias.issr-journals.org/

In vitro study of the compatibility of six fungicides with two strains of *Trichoderma asperellum*, biocontrol agents used against cacao black pod disease in Cameroon

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ABSTRACT: In Cameroon, mycoparasitic strains of *T. asperellum* used in biocontrol of cocoa black pod disease have shown inconsistencies in their effectiveness. One possibility to optimize their performances is within the frames of the integrated management programme involving a combination of chemical and biological control methods. However, implementation of such an approach strongly relies on the compatibility between the biocontrol agents and the conventional synthetic fungicides. This study aimed to assess *in vitro* the compatibility between two antagonistic strains of *T. asperellum* (PR11 and PR12) and six fungicides (Ridomil, Penncozeb, Beauchamp, Nordox, Golden Blue and Kocide) approved and regularly used to control *P. megakarya*. These strains were cultured on PDA media supplemented with five different concentration levels (0, 0.01, 0.1, 10 and 100 % of the recommended field dose) of the above-cited fungicides. Effects on conidial germination, vegetative growth and conidial production were measured to evaluate the compatibility between the tested fungicides and the *T. asperellum* strains. Results showed an almost complete inhibition of conidial germination for the highest concentrations for all the fungicides. Vegetative growth and conidiogenesis were also significantly affected by the recommended field dose for all tested fungicides. The physiological parameter index used to classify compatibility showed that all tested fungicides are incompatible with both strains of *T. asperellum* at recommended field doses. According to the results reported in this study, the synthetic fungicides used to reduce the incidence of cocoa black pod disease have negative effects on both antagonistic *T. asperellum* strains PR11 and PR12. This suggests that their use in conjunction with *T. asperellum* as part of a disease control scheme would only be possible at sub-optimal concentrations.

KEYWORDS: fungicide, biocontrol agent, compatibility, cocoa, *Phytophthora megakarya*.

1 INTRODUCTION

Agriculture is one of the major sectors of the Cameroonian economy. Among the agricultural products of economic importance, cocoa is an essential income source for more than 400 000 farmers in Cameroon [1]. Cameroon is the 5th world cocoa producer with more than 230 000 tonnes produced yearly [2]. Despite this performance, this figure remains low, yet the ecological potential provides conditions for a better production. One of the reasons justifying this level of production is the prevalence of diseases; among which cocoa black pod disease caused by *Phytophthora megakarya* is the most important [3]. Annual losses in yield can reach 100 % in absence of any control measures [4].

Control strategies to reduce disease incidence mostly rely on the use of copper- and metalaxyl-based synthetic fungicides ([5]; [6]). Although effective, chemical control is costly and has negative impacts both on the environment and human health ([7]; [8]; [9]; [10]). Moreover, in spite of efforts to genetically improve the ability of cocoa trees to resist the disease, no genotype fully resistant to *P. megakarya* has been identified so far [11]. Phytosanitation, particularly removal of diseased pods, is unable to ensure full protection of the orchards [4]. Biological control, with e.g. the use of plants extracts as *Thevetia peruviana* and *Azadirachta indica* ([10];[13]), or antagonistic fungi of the genus *Trichoderma* ([114] and [15];[16]; [17];[18]), is a promising alternative that is attracting significant attention as an environmentally-friendlier way to combat cocoa black pod disease, and in particular when part of an integrated control strategy ([19]; [20] and [20]; [21]; [22]). .

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In Cameroon, mycoparasitic strains of *Trichoderma asperellum* are used as biocontrol agents against several plant pathogens ([23]; [24]; [25]; [18]). However, their effectiveness is reported to be variable ([26]; [24]). Such variability may be caused by fluctuating and unfavourable environmental conditions that could affect the efficiency of the biocontrol agent and slow down the infection process of the pathogen ([27]; [28]). Strategies involving a combination of biocontrol agents and low doses of fungicides are able to attenuate the performance variability of antagonistic strains while at the same time enhancing their efficiency ([28]). Moreover, besides improving the intrinsic capacities of the biocontrol agents, the pathogen can also be stressed and weakened through the application of sub-lethal doses of chemicals making it more sensitive to the attacks of the antagonistic organism ([29]; [30]; [31]; [32]). However, it is vital to ascertain beforehand that the concentrations of fungicides used do not impair the viability, development and virulence of the antagonistic agents [33].

In Cameroon, a wide range of synthetic fungicides are employed in cocoa production [5]. Within the perspective of coming up with a control strategy that includes beneficial microorganisms such as fungi of the genus *Trichoderma*, it is essential to examine the compatibility of biocontrol agents with those synthetic fungicides that are routinely used to control cocoa black pod disease. Therefore, this study evaluated the *in vitr*o impact of six commercial fungicides approved for *P. megakarya* control on some key stages of the life cycle (i.e. conidial germination, vegetative growth and conidiogenesis) of two *T. asperellum* strains (PR11 and PR12) also used to control the same pathogen, and thereby to assess their compatibility.

2 MATERIALS AND METHODS

2.1 FUNGUS MATERIAL

The *T. asperellum* strains PR11 and PR12 used in this study were obtained from the collection of the Regional Biocontrol and Applied Microbiology Laboratory of IRAD (Institute of Agricultural Research for Development). The strains were isolated from soil samples collected in food crop fields in Cameroon, characterized molecularly [34], and their antagonistic activities regarding *P. megakarya* and *P. myriotylum* - pathogens responsible for the black pod disease of the cocoa (*Theobroma cacao* L) and root rot disease of the cocoyam (*Xanthosoma* spp), respectively - were reported ([23]; [24]; [25]). For long-term preservation, the strains were kept in tubes on Potato Dextrose Agar medium (PDA; Merck Darmstadt, Germany) and covered with 10 ml of paraffin oil. For daily use, the strains were cultured on PDA medium in Petri dishes at ambient temperature.

2.2 EFFECT OF FUNGICIDES ON CONIDIAL GERMINATION

Six synthetic fungicides approved for use in Cameroon were tested in this study. They are listed in Table 1 along with their main specificities. The two *T. asperellum* strains were cultured in Petri dishes containing PDA medium to which was added, at different concentrations, one of each fungicide. The PDA + fungicide medium was prepared by transferring 25 ml of fungicide suspension at the relevant concentration into an Erlenmeyer flask containing 225 ml of liquid PDA cooled to 50 °C. The mixture was homogenized, and then poured into 90 mm diameter Petri dishes. For each fungicide, concentrations were prepared on the basis of the recommended field application dosage in g/l (Table 1). Thereafter, the following dilutions were tested for each fungicide (v:v; medium:fungicide): 0, 0.01, 0.1,10 and 100 % the manufacturer's recommended field dosage (X) [35]. Petri dishes without fungicide were also prepared and used as control.

Table 1. The fungicides tested in vitro and their characteristics.

Fungicide (trade name)	Active ingredient (%)	Mode and site of action	Chemical class	Recommended field dose (X)	Manufacturing company
Ridomil Gold Plus 66 (WP)	6% Metalaxyl 60% copper oxide	Systemic 1	Acylamine	3.33 g/ l	Syngenta
Penncozeb 80 (WP)	4% Metalaxyl 80% Mancozeb	Systemic 1+M	Dithiocarbamate	3.33 g/ l	Syngenta
Beauchamp 72 % (WP)	8% Metalaxyl 64% Mancozeb	Systemic 1+M	Acylamine + Dithiocarbamate	3.33 g/ l	Zhejiang heben pesticide and chemical Co
Kocide 2000 (WP)	53.8% copper hydroxide	Contact M	Fixed copper complex	4 g/ l	Du Pont de Nemours
Golden-Blue pentahydrate copper sulphate 98.5 %; (SG)	98.5 %copper sulphate	Contact M	Copper complex	6 g/ l	ADER
Nordox 75 (WG)	86 % copper oxide	Contact M	Fixed copper	2.66 g/ l	ADER

***WP**: Wettable powder; **SG**: Soluble granules; **WG**: Water-dispersible granules; **1**: single site fungicide; **M**: multi sites fungicide.*

To assess germination, 10 ml of sterile distilled water were added to a Petri dish containing a five-day-old *T. asperellum* culture. The conidia were then delicately scraped from the surface of the culture and the resulting conidial suspension was transferred into an Erlenmeyer flask along with one drop of tween 80, and then agitated in a vortex mixer for 10 s. The concentration of the suspension was then adjusted to 1.10^5 conidia/ml using a hemocytometer. After dilution, 1 ml of the suspension was sampled with a pipette and spread with a glass spatula over the PDA+fungicide. Three Petri dishes per tested concentration level were used per fungicide and per strain. Petri dishes containing the conidial suspension on fungicide-free PDA medium were used as controls. The Petri dishes were sealed with parafilm and incubated at ambient temperature. After 12 h of incubation, three 1-cm^2 areas were randomly delimited inside each Petri dish and observed under a compound light microscope at 10X magnification. At each field of view, all conidia were counted and the percentage of germination (GR) was calculated. A conidium was considered germinated when the germ tube length at least equalled conidium diameter. The whole experiment was repeated twice.

2.3 EFFECT OF FUNGICIDES ON VEGETATIVE GROWTH

In order to estimate the colonies' vegetative growth rates on artificial media, a 9-mm diameter disc of each strain of *T. asperellum,* cut from a 5-day-old culture was deposited in the centre of a Petri dish containing the media prepared as described above. Care was taken to reverse the disc so that the mycelium came into contact with the PDA-fungicide medium. The Petri dishes were then sealed with parafilm and incubated at ambient temperature. For each combination of strain, fungicide and fungicide dose, 5 Petri dishes were used and the whole experiment was conducted three times. The diameter of the colony in the centre of each Petri dish was measured daily along two perpendicular axes for 7 days. The area under the mycelial growth curves (AUMGCs), expressed in mm^2/day, was calculated for each treatment and repetition as the area under the curve of mean colony diameter over time according to the following formula:

$$AUMGC=\sum_{i=1}^{n}[(t_{i+1}-t_i)(y_{i+1}+y_i)/2]$$

Where y is the mean diameter (mm) at i^{th} observation, t_i is the time (days) at the i^{th} observation and *n* is the total of number of observations.

2.4 EFFECT OF FUNGICIDES ON CONIDIAL PRODUCTION

The number of conidia produced was estimated on the 7^{th} day post-inoculation. Ten ml of sterile distilled water were added to each Petri dish used in the experiment. The conidia were then carefully scraped off with a scalpel, and the resulting suspension was transferred to an Erlenmeyer flask containing 90 ml of sterile distilled water. This was then firmly shaken to obtain a homogeneous suspension. In order to quantify the number of conidia produced by the fungus, four 1 µl samples of the suspension were successively taken and deposited on a hemocytometer. The conidia were counted under a compound light microscope at 10X magnification, and the mean number of conidia per observation was recorded.

2.5 ASSESSMENT OF THE COMPATIBILITY OF THE SYNTHETIC FUNGICIDES WITH THE BIOCONTROL AGENTS

[33] proposed a biological index (BI) to classify the toxicological effects of chemical compounds on fungi cultured *in vitro* on a solid medium. The index is calculated using the mean germination percentage (GR), sporulation (SP) and the vegetative growth of the fungal colonies as compared with the control. BI values are computed using the following formula:

$$BI = [47 \times (VG) + 43 \times (SP) + 10 \times (GR) / 100]$$

Alves et al. (2007) classified the values of BI as below:

- BI > 66, compatible
- 42 < BI <66, moderately toxic
- BI < 42, toxic

2.6 DATA PROCESSING AND STATISTICAL ANALYSES

The data obtained from the various tests were transformed prior to statistical analyses. Arcsine, $\ln(x+1)$ and $\log_{10}(x+1)$ transformations were applied to germination, vegetative growth and sporulation data, respectively. Analyses of variance (ANOVA) of the transformed data were carried out using the SAS software (version 9.1). In case of significant differences, Student-Newman-Keuls tests were applied to classify the effects of the various factors (α=0.05).

3 RESULTS

3.1 EFFECTS OF RIDOMIL ON THE GERMINATION, VEGETATIVE GROWTH AND CONIDIAL PRODUCTION OF THE ANTAGONISTIC STRAINS OF *T. ASPERELLUM*

The measured parameters - i.e. germination, growth and conidial production - on the strains PR11 and PR12 of *T. asperellum* were affected to various degrees by the different tested doses of Ridomil. Conidial germination of both strains was affected by this fungicide. Compared to the controls, a significant reduction (P <0.0001) of the germination percentage was observed for concentrations >1% X and >10% X in PR11 and PR12, respectively (Fig. 1a and 1b). At the recommended field concentration and 12 h after inoculation germination of both strains was still totally inhibited. Regarding mycelial growth, strain PR12 did not grow at the highest concentration (100% of the recommended field dose) whereas strain PR11 grew at all tested concentrations. However, the vegetative growth of both strains was significantly reduced (P <0.0001) at concentrations ≥ 10% X (Fig. 1c and 1d). The quantity of conidia produced by the two strains followed the same trend as the vegetative growth: no conidia were produced by strain PR12 at the highest concentration, and, when compared to the control, a significant reduction of conidial production was observed for strain PR11 (Fig. 1e and 1 f). The biological index values ranged from 6.97 to 107.9 for PR11, and from 0 to 119.27 for PR12 (Table 2). Ridomil was not compatible with either strain at the recommended field application dosage and only becomes compatible at concentrations ≤ 0.1% X.

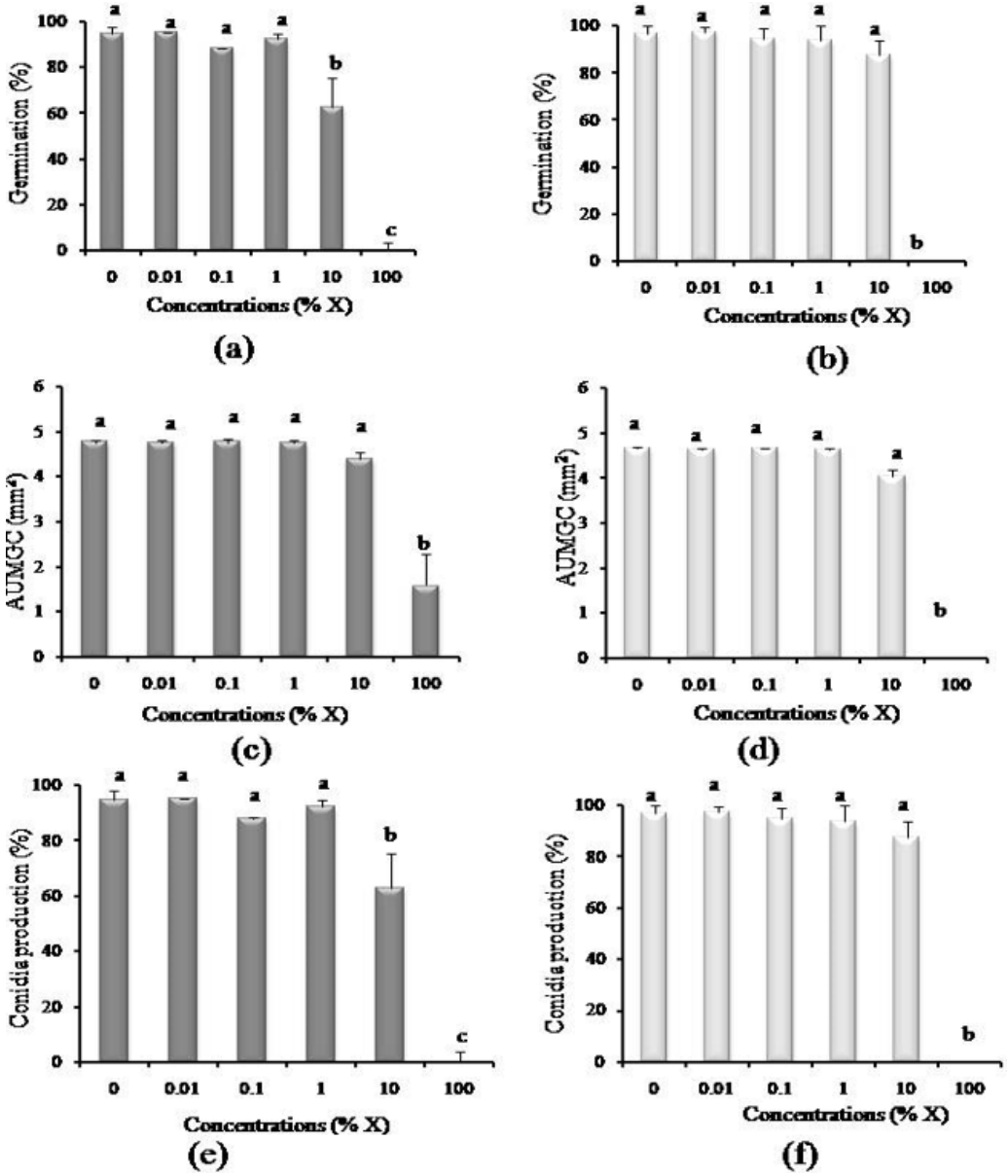

Fig. 1. Germination (a and b), vegetative growth (c and d) and number of conidia produced (e and f) by the strains PR11 and PR12 of T. asperellum on culture media amended with different concentrations of Ridomil. Bars topped by a same letter are not significantly different (P<0.05). Vertical error bars represent the standard deviation of the difference between means.

3.2 EFFECTS OF PENNCOZEB ON THE GERMINATION, VEGETATIVE GROWTH AND CONIDIAL PRODUCTION OF THE ANTAGONISTIC STRAINS OF *T. ASPERELLUM*

Germination rates of both PR11 and PR12 were affected by the various doses of the fungicide Penncozeb, but only concentrations ≥ 1% X resulted in a significant reduction (P <0.0001) of the germination percentage when compared with the controls (Fig. 2a and 2b). Mycelial growth was observed at all tested concentrations of Penncozeb for both strains, 7 days after inoculation, yet significant differences (P<0.0001) were noticed at concentrations > 0.1% X and > 10% X for PR11 and PR12, respectively (Fig. 2c and 2d). Conidial production of both strains was affected by the various concentrations, with recorded inhibition levels ranging from 8% to 65% in comparison with the controls. PR12 exhibited a significant reduction (P <0.0001) of conidial production at the recommended field concentration only, the number of conidia produced by PR11 was significantly lower than the control at all concentrations ≥ 1% X (Fig. 2e and 2f).

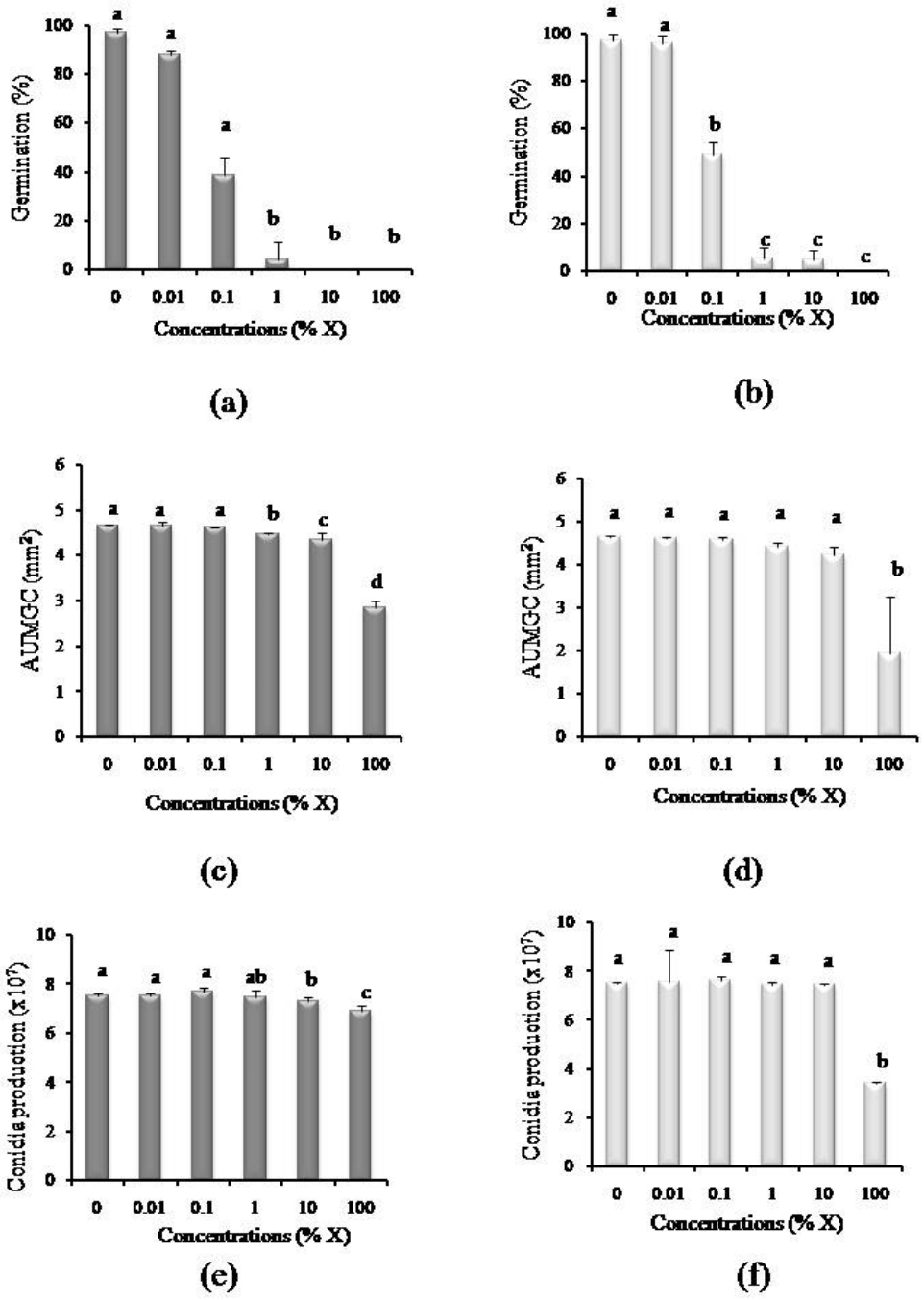

Fig. 2. Germination (a and b), vegetative growth (c and d) and number of conidia produced (e and f) by the strains PR11 and PR12 of T. asperellum on culture media amended with different concentrations of Penncozeb. Bars topped by a same letter are not significantly different (P<0.05). Vertical error bars represent the standard deviation of the difference between means.

The BI-values obtained with Penncozeb ranged from 23 to 114.43 for PR11, and from 11.53 to 105.65 for PR12 (Table 2). The lowest BI-values were obtained at the highest concentration (100% X) indicating that, this fungicide can only be considered compatible with both strains if doses are below the commercial recommendation.

Table 2. Computed values of the biological index (BI) of the tested fungicides with the strains PR11and PR12 of T. asperellum.

Fungicide	Strain	Fungicide concentration (% X)				
		100%	**10%**	**1%**	**0.1%**	**0.01%**
Ridomil	PR11	6.97	66.01	107.83	107.9	92.36
	PR12	0	77.26	90.19	119.27	113.52
Penncozeb	PR11	23	71.75	89.94	114.43	96.91
	PR12	11.53	77.1	86.03	99.23	105.65
Beauchamp	PR11	13.18	44.85	69.33	112.97	151.62
	PR12	11.99	56.23	94.12	146.41	237.08
Kocide	PR11	27.89	81.45	104.84	102.29	100.1
	PR12	26.91	118.65	146.81	170.45	190.96
Golden-Blue	PR11	0	40.78	69.76	130.88	156.24
	PR12	0	53.69	122.92	187.9	202.65
Nordox	PR11	10.02	60.82	95.66	119.46	163.71
	PR12	2.7	102.5	139.75	149.24	201.17

3.3 EFFECTS OF BEAUCHAMP ON THE GERMINATION, VEGETATIVE GROWTH AND CONIDIAL PRODUCTION OF THE ANTAGONISTIC STRAINS OF *T. ASPERELLUM*

Conidial germination of the antagonistic *T. asperellum* PR11 and PR12 strains was affected by the different concentrations, with inhibition rates ranging from 80% to 100%. In both strains, conidial germination was significantly (P <0.0001) inhibited by concentrations ≥ 1% X when compared to the controls: no conidial germination was observed 12 h after inoculation at these doses (Fig. 3a and 3b). Vegetative growth of PR11 and PR12 was significantly (P <0.0001) affected at 100% X only (Fig. 3c and 3d).

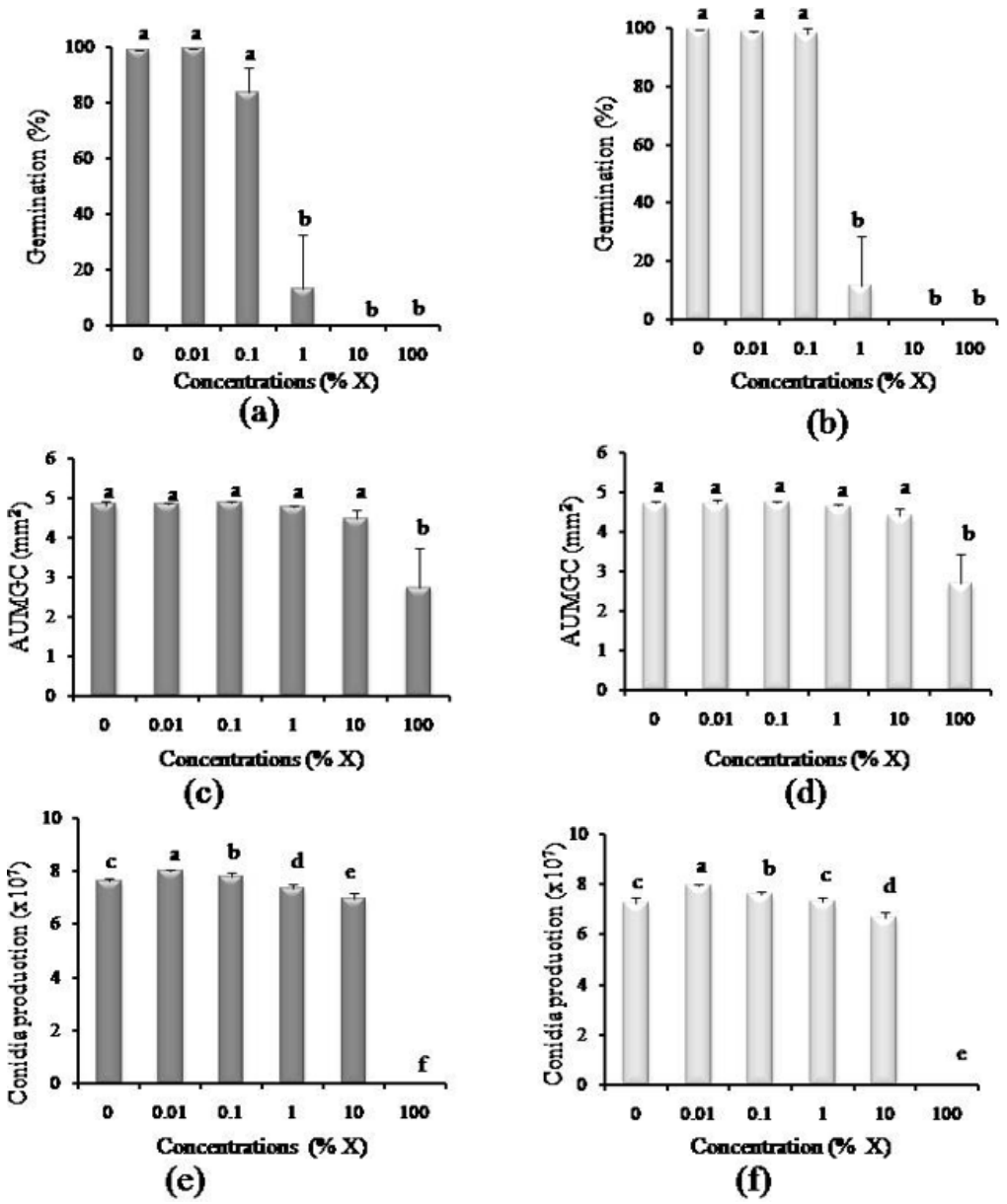

Fig. 3. Germination (a and b), vegetative growth (c and d) and number of conidia produced (e and f) by the strains PR11 and PR12 of T. asperellum on culture media amended with different concentrations of Beauchamp. Bars topped by a same letter are not significantly different (P<0.05). Vertical error bars represent the standard deviation of the difference between means.

Conidial production was recorded at all tested concentrations except the highest. At these concentrations, the analysis of variance showed that conidial production for both strains differed significantly (*P* <0.0001) from that of the controls. The highest sporulation rate was obtained at the 0.01% X dosage in both strains. The BI-values increased with decreasing concentrations of the fungicide and ranged from 13.18 to 151.62 for PR11 and from 11.99 to 237.08 for PR12 (Table 2). Beauchamp was compatible with both strains only at concentrations below 10% X.

3.4 EFFECTS OF KOCIDE ON THE GERMINATION, VEGETATIVE GROWTH AND CONIDIAL PRODUCTION OF THE ANTAGONISTIC STRAINS OF *T. ASPERELLUM*

In the culture media supplemented with Kocide, conidial germination of both strains was noticed at all tested concentrations 12 h after inoculation (Fig. 4a and 4b). However, conidial germination of PR11 was significantly (*P* <0.0001) affected at all concentrations >1% X, whereas for PR12, only the 1% X and the 100% X concentrations had a significant (*P* <0.0001) impact on the germination percentage. No negative effect on the vegetative growth of both strains cultured on media supplemented with Kocide was recorded, except at the recommended field concentration (100% X), which induced an inhibition of nearly 50% in comparison with the controls (Fig. 4c and 4d). Conidial production was observed at all concentrations for both strains. The number of conidia produced by PR11 was significantly (*P* <0.0001) higher at a concentration of 0.01% X

whereas sporulation was lowest at the highest concentrations (Fig. 4e). Similarly, for PR12, the sporulation rate was significantly (P <0.0001) higher at 0.01% X concentration and lowest in the controls and at the highest concentration tested (Fig. 4f).

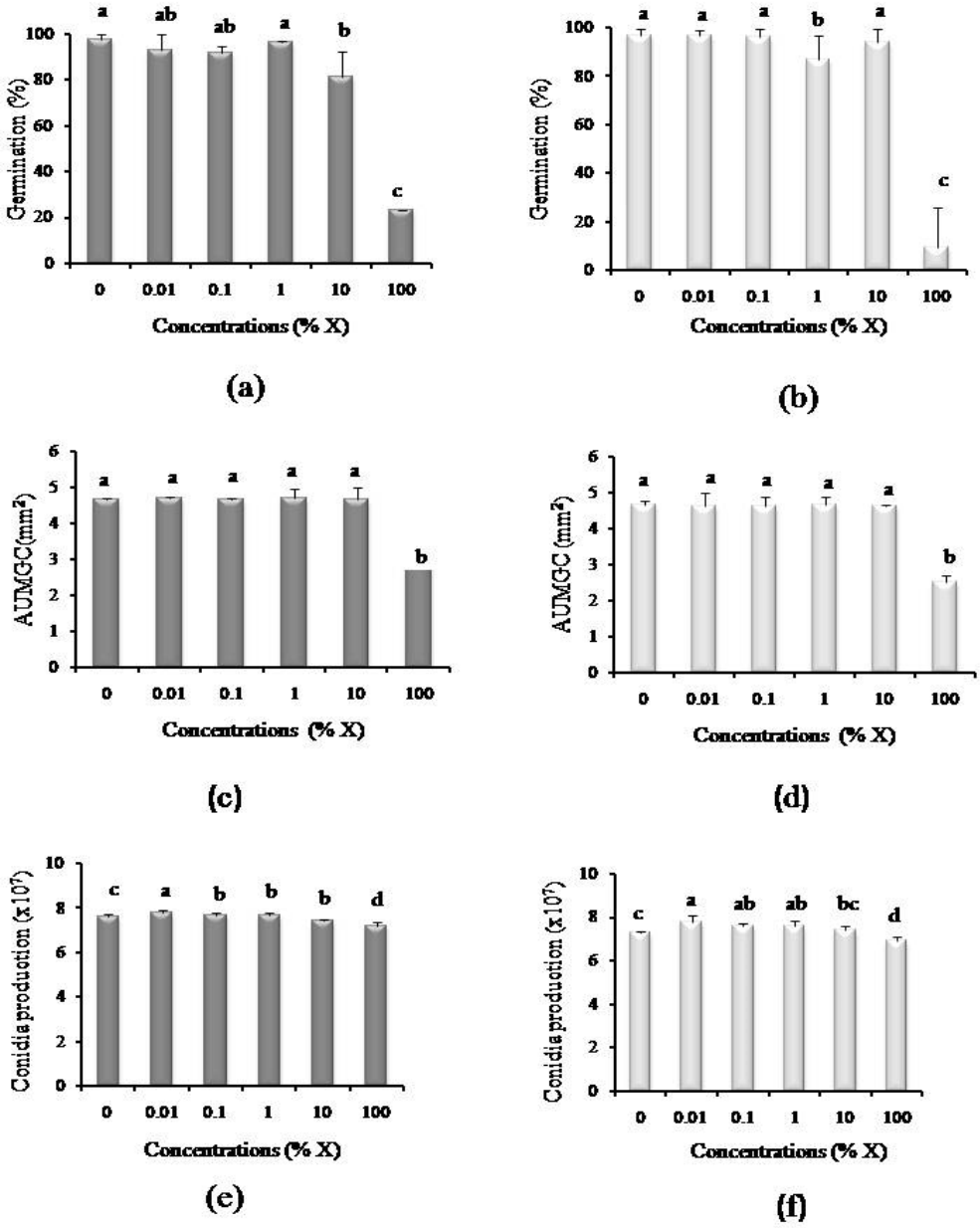

Fig. 4. Germination (a and b), vegetative growth (c and d) and number of conidia produced (e and f) by the strains PR11 and PR12 of T. asperellum on culture media amended with different concentrations of Kocide. Bars topped by a same letter are not significantly different (P<0.05). Vertical error bars represent the standard deviation of the difference between means.

The BI-values calculated for each concentration range from 27.89 to 124.77 in the case of PR11 and from 26.91 to 190.96 for PR12 (Table 2). The lowest BI-values being observed for the highest fungicide dose (100% X) indicated that Kocide is only compatible with the PR11 and PR12 when applied at concentrations below the recommended field concentration.

3.5 EFFECTS OF GOLDEN BLUE ON THE GERMINATION, VEGETATIVE GROWTH AND CONIDIAL PRODUCTION OF THE ANTAGONISTIC STRAINS OF *T. ASPERELLUM*

The germination of the *T. asperellum* strains PR11 and PR12 was significantly (P <0.0001) reduced on media containing the fungicide Golden Blue at concentrations ≥10% X, on which the inhibition varied between 20% and 100% in comparison to the controls (Fig. 5a and 5b). Similarly, the vegetative development of both strains was significantly (P <0.0001) reduced in presence of Golden Blue at concentrations ≥ 10% X, resulting in an approximately 15% to 100% inhibition in comparison with the controls (Fig. 5c and 5d). This fungicide had a significant (P <0.0001) positive effect on the conidial production of both strains at 0.01%

X and 0.1% X (Fig. 5e and 5f), but had a significant adverse effect on the conidial production of PR11 at concentrations ≥1% X, and on that of PR12 at 10% X and 100% X.

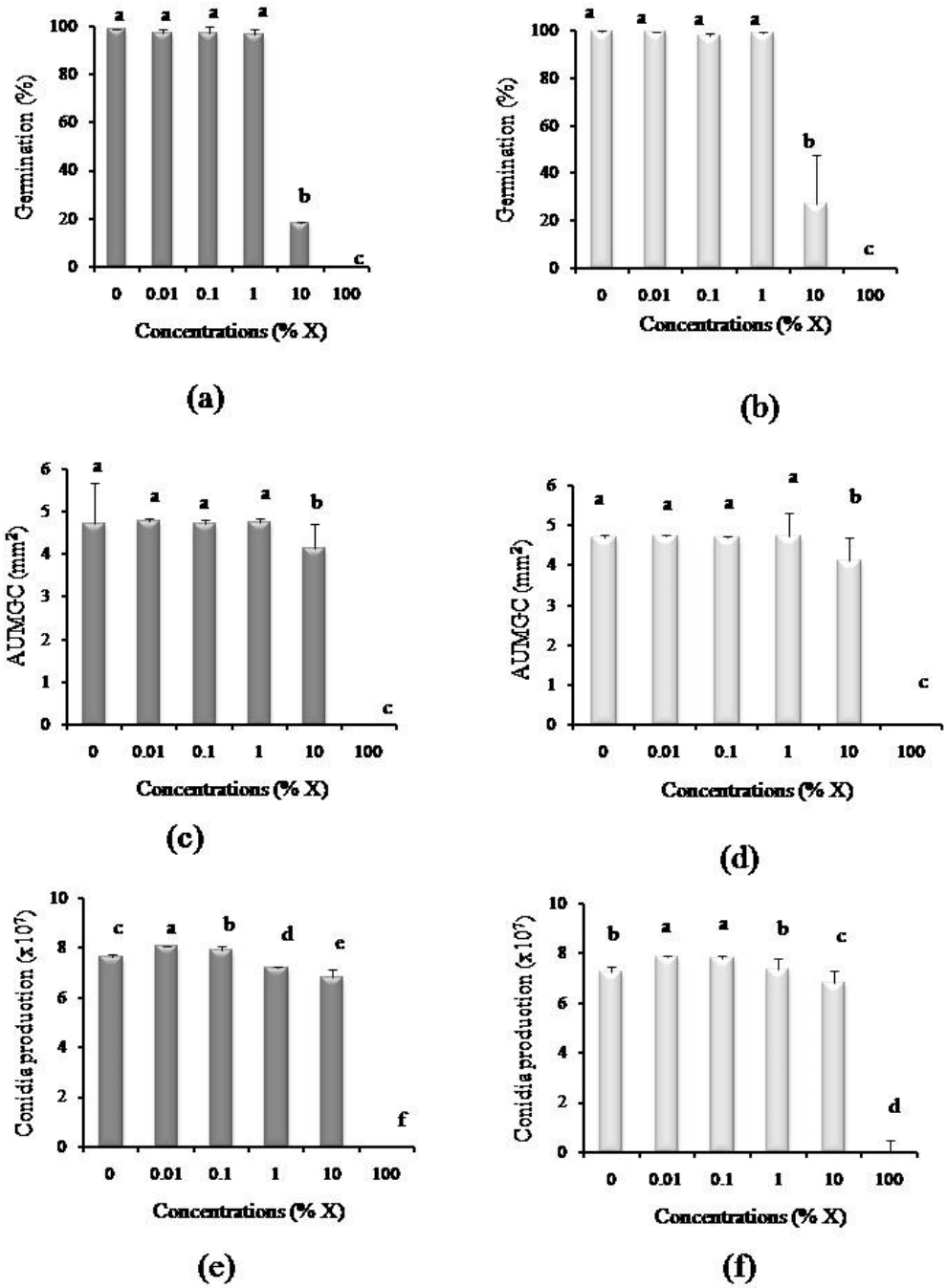

Fig. 5. Germination (a and b), vegetative growth (c and d) and number of conidia produced (e and f) by the strains PR11 and PR12 of T. asperellum on culture media amended with different concentrations of Golden Blue. Bars topped by a same letter are not significantly different (P<0.05). Vertical error bars represent the standard deviation of the difference between means.

The BI-values ranged from 0 to 156.24 for PR11 and from 0 to 202.65 in the case of PR12, indicating that concentrations ≤ 1% X only are compatible with the PR11 and PR12 strains (Table 2).

3.6 EFFECTS OF NORDOX ON THE GERMINATION, VEGETATIVE GROWTH AND CONIDIAL PRODUCTION OF THE ANTAGONISTIC STRAINS OF *T. ASPERELLUM*

The germination percentage of the strain PR11 was only significantly (P <0.0001) affected by the recommended field concentration of Nordox, at which germination was completely inhibited. Regarding strain PR12, germination was significantly (P <0.0001) reduced at the 10% X and totally inhibited at 100% X (Fig. 6a and 6b). Concerning the vegetative growth compared

to the controls, PR11 exhibited a significantly (*P* <0.0001) smaller growth on media containing ≥ 10% X, whereas the vegetative growth of PR12 was significantly (*P* <0.0001) affected only at the recommended field concentration (Fig. 6c and 6d).

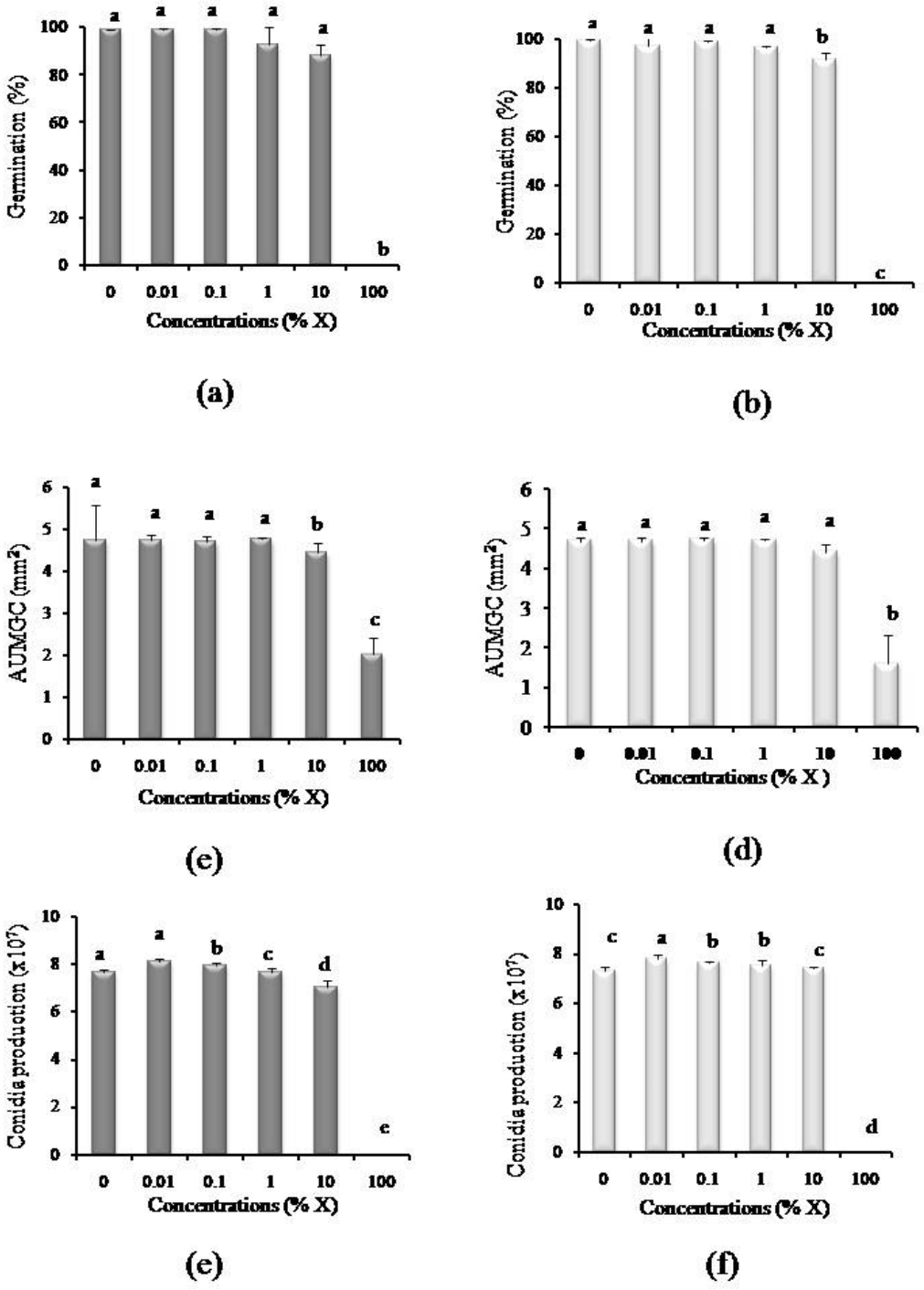

Fig. 6. Germination (a and b), vegetative growth (c and d) and number of conidia produced (e and f) by the strains PR11 and PR12 of T. asperellum on culture media amended with different concentrations of Nordox. Bars topped by a same letter are not significantly different (P<0.05). Vertical error bars represent the standard deviation of the difference between means.

A significantly (*P* <0.0001) positive effect on conidial production was noticed in the strain PR11 at 0.01% X and 0.1% X, whereas the 10% X concentration had a significant (*P* <0.0001) negative effect. For PR12, significant (*P* <0.0001) positive effects on conidial production were recorded between 0.001% X and 1% X. However, both strains exhibited a complete absence of conidial production on media supplemented with Nordox at the recommended field concentration. The BI-values varied between 10.02 and 163.71 for PR11 and between 2.70 and 201.17 for PR12. Therefore, except the recommended field concentration, all concentrations were compatible with both strains (Table 2).

4 DISCUSSION

Spraying systemic or contact synthetic fungicides is the primary component of management programmes for cocoa black pod disease in Cameroon ([5]; [6]). However, given the noxious effects of such chemical compounds on the environment and human health, developing control strategies which help reduce or in the long-term even eliminate the dependence of chemical control is necessary. An alternative such as biological control could provide additional management tools to supplement existing control means [37]. With this in mind, research is being carried out in Cameroon to develop a biological formulation with conidia of mycoparasitic strains of *T. asperellum*, as active ingredient. One of the most promising possibilities to optimize the performances of *Trichoderma* biocontrol agents is within the frame of integrated management programmes based on the combined application of physical, chemical and biological control methods ([38]; [39]). However, before introducing a *T. asperellum* bioformulation in the technological package of an integrated management programme against e.g. black pod disease of cacao, it is necessary to ascertain whether these biocontrol agents are compatible with conventional synthetic fungicides. Several examples of research assessing the compatibility of a number of *Trichoderma* spp. with low doses of commercial fungicides frequently used to control diseases on crops can be found in the literature (onions: [40]; wheat: [41]; tea: [42];[43]; soil born pathogen:[44];[45];[46];[47]; tea: [48]; mandarin-pepper-coffee: [49]; soil born pathogen: [50]). Our study is the first to explore the in vitro compatibility of *T. asperellum* with those fungicides used in cocoa production in Cameroon. Our results show that these fungicides produce very similar effects on the antagonistic strains PR11 and PR12 in terms of conidial germination, vegetative growth and conidial production,

In our study, at the field dose as recommended by the manufacturer, all fungicides tested, except Kocide, induced a complete inhibition of conidial germination in both PR11 and PR12 strains 12 h after inoculation. A significant reduction of the germination percentages was moreover observed with the fungicides Penncozeb and Beauchamp above 1% of the field dose, and above 10% with Golden Blue. Penncozeb and Beauchamp share the same active ingredient that is mancozeb (80% and 64% respectively) and their field recommended concentrations are also the same whereas Golden Blue is made up with 98.5% pentahydrate copper sulphate with the highest field recommended concentration (Table 1). The adverse effects of mancozeb on the conidial germination of *T. harzianum* were underlined by [40]. A depressed germinating ability of the conidia of some Hyphomycetes (such as *Beauveria bassiana*) was also reported by [51] in experiments with mancozeb and several copper-based fungicides. Their research uncovered the capacity of fungicides to induce a hydrophobic reaction detrimental to conidial viability and germination. The biological action of strains of *T. asperellum* tested in this study uses a mechanism essentially based on mycoparasitism ([23]; [25]). Since conidial germination is the first stage of the development cycle of these fungi, it is critical for the proper colonization of the host structures and the environment [52]. This suggests that inhibiting germination would likely have a severe impact on the effectiveness of strains of *T. asperellum* [28].

The effects of the fungicides on the vegetative development of strains PR11 and PR12 of *T. asperellum* varied according to the dose applied. Furthermore, inhibition rates increased gradually with tested concentrations. This result fully agrees with findings from previous studies involving the entomopathogenic fungus *B. bassiana* exposed to different fungicide concentrations ([53]; [54]). We also observed that the fungi's vegetative growth was less affected by the fungicides than their conidial germination. According to [55], the effects of pesticides in the environment decrease with time. Although we have no information on the stability of the tested fungicides in the culture medium used, the observed discrepancy in the reactions of the *T. asperellum* mycelia and conidia to the same concentrations of fungicide is probably partly due to differences in the duration of their exposition ([56]; [57]). Mycelia remained in contact with the fungicides for 7 days whereas conidial germination was assessed only 12 h after inoculation. Differences in conidial germination and vegetative growth sensitivity to a same chemical substance has already been reported in other Hyphomycetes such as *Verticillium lecanii* [58], *Metarrhizium anisopliae* ([59]; [54]), *Paecilomyces fumosoroseus* [56], *Dactylaria higginsii* [57], *Trichoderma harzianum* [60] and *Trichoderma viride* [50]. This suggests that the action of the fungicides used was much more static than destructive for the germination of the conidia. It is thus essential to take the time factor into consideration when assessing the effects of fungicides on fungi [56].

The production of conidia or spores is a key stage of the development cycle of fungi in general, and of fungal biocontrol agents in particular [52]. Once the mycelial colonization of the host or the environment is achieved, sporulation is the stage that determines whether the fungus is capable not only of perpetuating itself, but also of initiating secondary infections through the conidia released ([33]; [27]). Since the degree of sporulation of a given fungus partly reflects its capacity to survive, taking this parameter into consideration is crucial when conducting investigations on the effects of fungicides approved for cocoa production on biocontrol agents. In our study, the conidial production by the strains PR11 and PR12 of *T. asperellum* was practically not affected by the fungicides tested, except when these were used at their recommended field dose, in which case the levels recorded were significantly lower than in the controls. The observed deficit in the conidial production of the tested strains of *T. asperellum* is a logical consequence of the complete or partial inhibition of the conidial germination and/or mycelial development at the highest concentrations of fungicide. These results are consistent with findings from studies involving

mycorrhizal strains of *Glomus intraradices* [52], entomopathogenic strains of *B. bassiana* (([61]; [62]) and *Trichoderma harzianum* [49].

Three systemic fungicides using different modes of action were tested in this study. Ridomil is an acylalanine fungicide that affects fungi by inhibiting RNA synthesis. Penncozeb is mainly made up of mancozeb (80%), which is a dithiocarbamate fungicide and has an inhibitory action on multiple sites in fungus cells ([63]; [45]; [64]; [49]). Beauchamp is a combination of acylalanine and dithiocarbamate fungicides. While dithiocarbamates are considered non-specific, acylalanines have a moderately wide spectrum of action and are almost exclusively active against Oomycetes [51]. The impact of acylamines and dithiocarbamates on key stages (such as conidial germination and mycelial growth) of the development cycle of fungal biocontrol agents has been investigated [61]; [40]; [51]; [65]; [37]; [57]; [66]; [60]; [47]; [39]; [50]). In our study, the influence of systemic fungicides on conidial germination, mycelial growth and conidial production of the antagonistic strains PR11 and PR12 of *T. asperellum* became conspicuous only at the recommended field concentration. The most severe effects were recorded with Beauchamp, in particular on conidial germination, which was significantly inhibited at concentrations ≥ 1% X. This is consistent with the biological index values computed from our data, according to which the systemic fungicides tested are compatible with the strains of *T. asperellum* at concentrations of up to 10% of the recommended field dose.

All contact fungicides tested in this study belong to the same chemical class, that of inorganic compounds, their active ingredient being either copper hydroxide (Kocide), copper sulphate (Golden Blue) or copper oxide (Nordox). Copper-based preparations have a broad spectrum of activity and are therefore liable to have a detrimental impact on the antagonistic activity of the biocontrol agents ([62]; [49]). Among the contact fungicides tested, Golden Blue was highly fungitoxic at the commercially recommended field dose and inhibited all the monitored stages of the development cycle. Nordox and Kocide had less noxious effects. The incompatibility potential between copper sulphate and several Hypocreales species has already been reported [62]; whereas on the other hand [42], and [49] showed that *T. harzianum* is compatible with copper oxide and copper hydroxide fungicides at similar concentrations. The calculated biological index values indicated that Nordox and Kocide are both compatible with the strains of *T. asperellum* at concentrations of up to 10% of the recommended field dose, whereas Golden Blue must be further diluted to be compatible.

5 CONCLUSION

Our study shows that the synthetic fungicides used to reduce the incidence of cocoa black pod disease have detrimental effects on the strains PR11 and PR12 of *T. asperellum* antagonistic. Consequently, their application in disease management programme where *T. asperellum* is involved would only be possible if the concentrations applied are sub-optimal. Moreover, although the laboratory findings presented here cannot be expected to totally predict the effects of the fungicides under field conditions, the information obtained can nonetheless be used to develop recommendations regarding the application of synthetic fungicides. Since *T. asperellum* is considered as a biocontrol agent, tests in the real conditions of cocoa production are necessary in order to validate the results of this study.

ACKNOWLEDGMENT

The authors are grateful to the Regional Biocontrol and Applied Microbiology Laboratory, IRAD for providing the logistic as well as financial support to this research activity.

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