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FUMIGANT TOXICITY OF SOME VOLATILE BOTANICAL SUBSTANCES AGAINST THE WHEAT PEST *SITOPHILUS ORYZAE* AND TWO SEED-BORN FUNGI, *ASPERGILLUS WESTERDIJKIAE* AND *FUSARIUM GRAMINEARUM*

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ABSTRACT

Bioassays were performed to determine insecticidal and antifungal activity of volatile botanical substances, allylisothiocyanate (AITC) and ethyl formate (EtF), against the rice weevil, *Sitophilus oryzae*, and two mycotoxigenic fungi, *Aspergillus westerdijkiae* and *Fusarium graminearum*. Insect mortality rate was determined after 24 h exposure time in airtight enclosure. Antifungal activity was quantified either through fumigation toxicity assay (micro-atmosphere test) by measuring conidia germination inhibition and mycelium growth reduction rate after 72 h exposure time, or after incorporation of active compounds to fungi culture medium (agar diffusion plate method). A comparison with antifungal efficacy of clove oil, a less volatile botanical with remarkable antimicrobial properties, was also carried out with the latter method. Fungal growth reduction rate was determined according to dose mortality relationship model fit by a logistic regression. The lethal concentration of AITC vapour phase leading to 95% mortality of *S. oryzae* (LC₉₅) was 9.9 $\mu\text{L.L}^{-1}$ compared with lower effect for EtF with LC₉₅ determined at 52.1 $\mu\text{L.L}^{-1}$. For clove essential oil (EO), only the LC₅₀ could be accurately determined after 24 h exposure time which one was observed at 311.5 $\mu\text{L.L}^{-1}$ (vs. 6.3 $\mu\text{L.L}^{-1}$ for AITC LC₅₀). AITC exhibited both antifungal and sporicidal activity on the two fungal species. AITC LC₉₉ for fungal growth total inhibition of *A. westerdijkiae* and *F. graminearum* was 14.4 and 7.8 μL per Petri dish culture, respectively, whereas it was 630 and 320 $\mu\text{L.L}^{-1}$ of culture medium with clove EO. These results demonstrated that AITC may be an efficient fumigant helpful for sanitation of empty storage facilities or for preservation of grain stored in unsafe condition with a risk of fungal growth. However, these encouraging data must be consolidated by tests on grain and sorption studies.

Key words: Allylisothiocyanate, Clove oil, Ethyl formate, Fumigant activity.

INTRODUCTION

The search for new molecules with fumigant properties is essential in Europe in response to recent phasing out of active substances of common use in stored grain protection or stored-product warehouses disinfestation (Ciesla et al., 2008). During the last decade, several natural volatile substances were proposed as potential alternatives to these banned pesticides such as

methyl bromide, dichlorvos and malathion with more or less success. Allyl-isothiocyanate (AITC) is one of the predominant glucosinolates in cultivated *Brassicaceae* spp. (rape, radish, cabbage, mustard, celery, etc.). Insecticidal properties of AITC are well known for a long time but to date, this insecticidal activity is not practically exploited for stored grain protection according to toxicological properties and grain sorption behavior of vapor phase. Ethyl formate (EtF), a natural compound occurring in barley and beer with known fumigant activity, is registered in Australia for disinfestation of dried fruits and on grain, mixed with CO₂ (Ciesla et al., 2008; Rouzes et al., 2008). It was demonstrated that EtF is effective against stored grain pests (Muthu et al., 1984). Thus, ethyl formate (EtF) having appreciable vapor pressure at ambient temperature, the formulation of EtF in CO₂ as carrier gas was studied and developed as an alternative to phosphine treatments in grain farm storage facilities, especially where phosphine-resistant insect strains were identified (Haritos et al., 2003; 2006; Ryan and Bishop, 2003; Ren et al., 2005).

Beside this insecticidal activity, vapour phase of EtF and AITC was demonstrated with a promising antifungal activity against seed-borne fungi of *Aspergillus* or *Penicillium* genera (Mari et al., 2003) claimed to potentially limit fresh fruit decay rate after harvest (Wang et al., 2010). There are also numerous studies demonstrating antifungal properties of AITC for bio-fumigation of soil and suppression of several plant pathogenic fungi from black mustard (*Brassica nigra*) or wild radish (*Raphanus raphanistrum*) culture plough in (Mayton et al., 1996; Sarwar et al., 1998). The disinfestation of storage structures and empty bins after cleaning is difficult to carry out with liquid antimicrobial compounds and fungitoxic activity of AITC or EtF in vapour phase may be useful when all parts of empty storage bins cannot be easily accessible for thorough cleaning before uploading a new harvest. For both stored grain disinfestation and empty bins antifungal treatment, the joint antifungal and insecticidal activity of these two volatile phytochemicals may be interesting for large grain bulks stored with poor control means with grain re-hydration risks and a lack of grain cooling equipment. These situations are relatively common with large grain bulks stored in 'flat' storage facilities, where *in situ* fumigation is the single disinfestation acceptable method.

The *in vitro* antifungal and insecticidal activities of AITC and EtF were investigated in order to determine the susceptibility of vapour phase exposure of the two kinds of target organisms: a stored grain primary pest, *Sitophilus oryzae* (L.) and two mycotoxigenic fungi: *Aspergillus westerdijkiae* Frisvad and Samson (= *A. ochraceus* NRRL 3174, Wilhelm strain) and *Fusarium graminearum* (Schwabe).

MATERIALS AND METHODS

Bioassay with grain insects

Tested compounds and application method

Mustard oil (AITC 95%) and pure EtF (Sigma, St. Quentin Fallavier, France) were tested for their fumigation toxicity in hermetic glassware of 237 mL capacity (Fig. 1). Insects (25 *S. oryzae* adults per replicate, 4 replicates per treatment) were placed on a filter paper at the bottom of the jar, the top hermetically sealed by a lid perforated by a 6 mm diameter hole in its centre, hermetically stopped with a rubber septum (Cardiet et al., 2011). The test substance was injected by a micro-syringe (Hamilton, Bonaduz, Switzerland) through the rubber septum into a cotton plug stick to the lid inside the vessel. The dose of compounds in the fumigation test was from 2 to 40 $\mu\text{L.L}^{-1}$. Water (40 $\mu\text{L.L}^{-1}$) was used for the untreated control.

Insecticidal activity determination

Mortality of insects after 24 h exposure time in airtight enclosure was assessed and related to the dose per volume ($\mu\text{L}\cdot\text{L}^{-1}$). The lethal concentration 50% (LC_{50}) and 95% (LC_{95}) were calculated according to the model of correlation dose / mortality rate fit by a four-parameter logistic regression. The determination of lethal concentrations leading to 50% and 95% mortality rate and the standard deviation at this critical level were performed by XLstat data processing software (Addinsoft, France).

Antifungal screening bioassay

Tested compounds and application method on fungi culture

Mustard oil (95% AITC) and pure EtF were tested for conidia germination inhibition rate and fungal growth reduction rate for the two seed-contaminating and mycotoxigenic fungi: *F. graminearum* and *A. westerdijkiae*. A series treated with clove essential oil (EO) (Xeda International, Aubagne, France), a non-volatile botanical extract registered for use as a fungicide for sanitation of empty fresh fruit storage facilities (Bompeix et al., 2009), was added in the test as a reference compound. The fungi species were bred on two culture media: potato dextrose agar (PDA) and Czapek yeast extract agar (CYA) for *F. graminearum* and *A. westerdijkiae*, respectively. The germination inhibition and growth reduction related to the dose of bioactive compounds (AITC, EtF and clove EO) was determined through micro-atmosphere diffusion bioassay in Petri dish and through agar diffusion bioassay (see Cardiet et al., 2011) enabling fungal growth inhibition zone measurement according to untreated condition. The fungal growth inhibition rate was assessed for the more active compound (AITC) in comparison to clove EO by agar diffusion test: a dose of tested substance was deposited on a 6 mm diameter 'cellulose test disk' and placed at the surface of PDA medium covered by a mixture of 1 mL spore suspension added to 4 ml PDA (1%) culture medium, *i.e.* 10^6 spore per Petri dish of 90 mm in diameter. Taking into account the high volatility of the tested compound (AITC), each series (dose) was enclosed in a hermetic glass jar (1.5 L capacity) during the incubation period of 72 h before control of fungal growth inhibition rate.

The germicide activity of AITC and clove EO on the spores of the two fungal species was assessed from fungi culture medium in Petri dish (90 mm in diameter), inoculated by a spore suspension and incubated during 16 h allowing spore germination in normal conditions. Then, the Petri dishes were returned upside down and the dose of tested substances deposited on a cellulose disk (6-mm in diameter) placed inside the dish lid (Cardiet et al., 2011). The dose range in the test for conidia germination inhibition was from 0.04 to 40 μL for mustard oil (AITC) and 31 μL to 1 mL for clove EO. All treatments were done in four Petri dishes (replicates) per dose, each series enclosed in a hermetic glass jar to avoid vapour transfer between series during incubation period at 25°C.

Quantification of fungal spore germination and growth inhibition rate

The effects of the active substances were checked up to 72 h after the assay begun. Fungal spore germination or total inhibition was visually measured, allowing the assessment of critical inhibitory concentrations (IC_{50} and IC_{99}) of AITC and EtF per Petri dish.

RESULTS

Insecticidal activity

The three compounds differed greatly in their ability to kill *S. oryzae* adults when used as a fumigant in a hermetic chamber and after 24 h exposure time. The regression equation of

mortality rate with the dose of tested compounds allowed to deduce the CL_{50} and CL_{95} (Table 1). There was observed that AITC had the most potent fumigant effect after 24 h exposure compared to EtF and that the fumigant effect on *S. oryzae* of clove EO was very poor after 24 h. Thus, CL_{50} after 24 h exposure was observed at 6.3, 36.2 and 311.5 $\mu\text{L.L}^{-1}$ for AITC, EtF and clove EO, respectively. The CL_{95} was in the same order: 9.9 and 52.1 $\mu\text{L.L}^{-1}$ for AITC and EtF respectively. CL_{95} for clove EO used as a fumigant could not be reached at the maximum dosage of 1 mL.L^{-1} . The confidence interval at 95% either at CL_{50} or at CL_{95} was low and the determination coefficient was very highly significant, especially for the logistic regression model with four parameters (Table 1). The logistic curve fit cannot be obtained with clove EO according to its very low vapor pressure compared to the two other volatile compounds (Figs. 1-3).

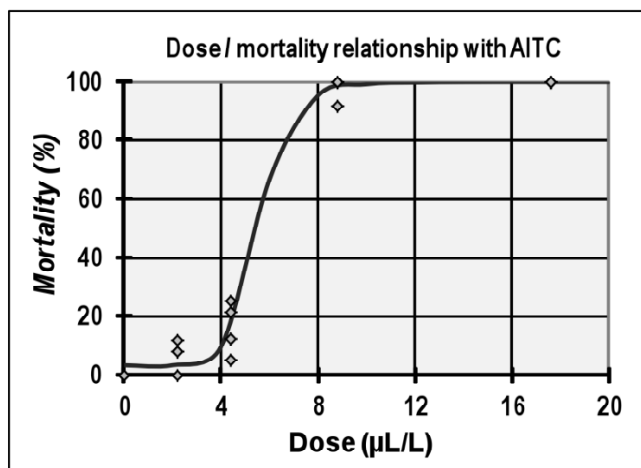


Fig. 1- Logistic regression of mortality rate of *S. oryzae* adults exposed during 24 h to different doses of AITC vapour in a hermetic enclosure (4 replicates of 25 insects per series).

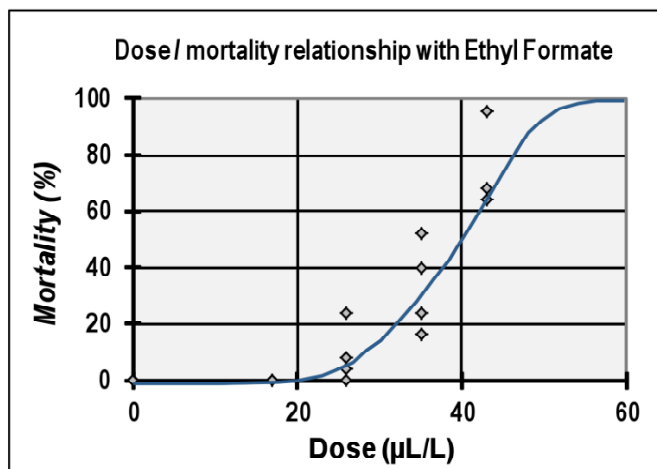


Fig. 2- Logistic regression of mortality rate of *S. oryzae* adults exposed during 24 h to different doses of ethyl formate vapour in a hermetic enclosure (4 x 25 insects per series).

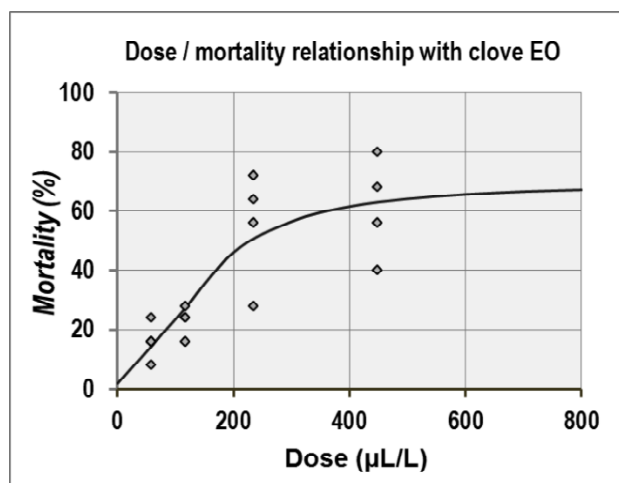


Fig. 3- Logistic regression of mortality rate of *S. oryzae* adults exposed during 24 h to different doses of clove oil vapour in a hermetic enclosure (4 x 25 insects per series).

Table 1. Logistic model of regression of *S. oryzae* mortality rate with the dose of AITC, EtF and clove EO in vapour phase and critical dose / mortality rate ratios determination

Substances	Logistic or polynomial regression model	R ²	LC ₅₀	CI 50% lower limit	CI 50% upper limit	LC ₉₅	CI 95% lower limit	CI 95% upper limit
AITC	$y = 100.086 + (3.461 - 100.086) / (1 + (x / 5.548)^{8.179})$	0.989	6.3	5.9	6.8	9.9	9.2	10.9
Ethyl formate	$y = 0.0011 x^2 - 0.0385 x + 0.3392$	0.902	38.2	36.9	39.8	52.1	49.2	56.3
Clove EO	$y = 69.434 + (1.866 - 69.434) / (1 + (x / 145.422)^{1.988})$	0.809	311.5	269.3	367.3	IND	IND	IND
IND =	indeterminable							

Antifungal activity

Fungal growth and in vitro development inhibition

From the results of micro-atmosphere test method, it was shown that the seed-borne fungal species *A. westerdijkiae* was more tolerant to AITC in vapour phase than the hydrophilic plant pathogen *F. graminearum*. Thus, from the probit conversion of growth rate inhibition measurements (vs. untreated control), IC₅₀ was determined by statistical analysis at 7.1 and 3.1 µL for *A. westerdijkiae* and *F. graminearum*, respectively; whereas CI₉₉ (assimilated to minimum inhibition concentration, MIC) was determined at 14.4 and 7.8 µL per culture plate (Table 2 and Fig. 4).

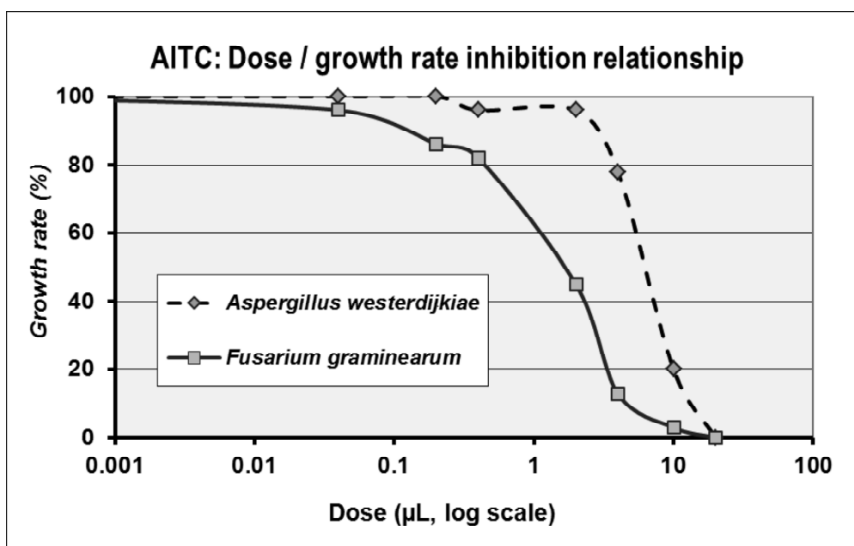


Fig. 4- Logistic regression of mycelial growth of the mycotoxigenic fungi *A. westerdijkiae* and *F. graminearum* exposed to vapour of AITC in Petri dish cultures (4 replicates per series)

Table 2. Regression of mycelial growth of *A. westerdijkiae* and *F. graminearum* grown on agar medium with AITC dose exposure as a fumigant: determination of CL₅₀ and CL₉₉ and their confidence interval ($P \leq 0.05$)

Fungus	CL ₅₀ (µL per dish)	Lower limit	Upper limit	CL ₉₉ (µL per dish)	Lower limit	Upper limit
<i>Aspergillus westerdijkiae</i>	7.07	6.5	7.74	14.4	12.9	16.2
<i>Fusarium graminearum</i>	3.05	2.22	3.81	7.83	6.5	8.84

The sum of the doses used in each treatment (4 fungi culture plates in a glass vessel of 1.5 L) allowed the calculation of the effective dose in vapour phase inducing complete inhibition of fungal growth of the two fungi. Thus MIC was assessed at 60 µL for 1.5 L container, *i.e.* 40 µL.L⁻¹. The treatment with clove oil led to antifungal efficacy at much higher dose than AITC according to the very low vapour pressure developed by clove essential oil. Thus, IC₅₀ with clove EO treatment was observed at 218 and 133 µL.L⁻¹ of agar culture substrate (Table 3 and Fig. 5).

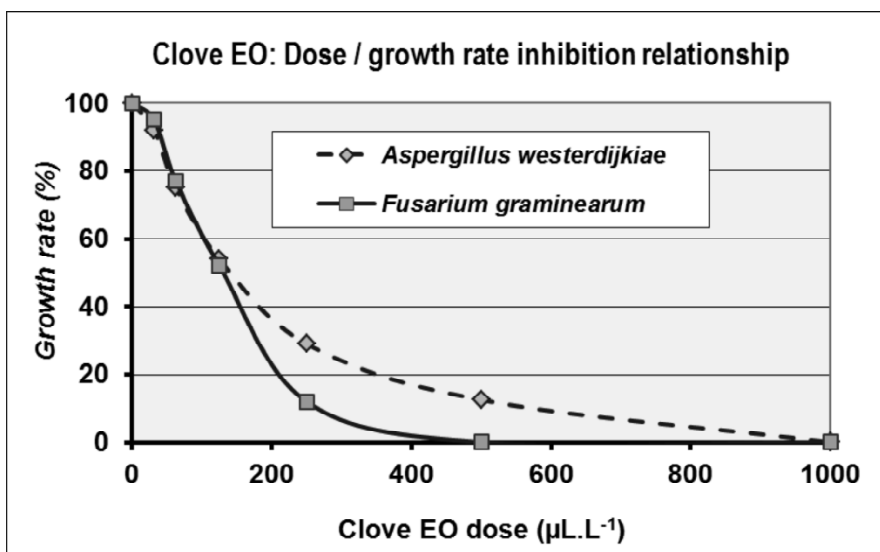


Fig. 5- Logistic regression of mycelial growth of the mycotoxigenic fungi *A. westerdijkiae* and *F. graminearum* exposed to clove oil dilution in Petri dish cultures (4 replicates per series).

Table 3. Regression of mycelium growth diameter of *A. westerdijkiae* and *F. graminearum* grown on agar medium with clove oil dose incorporated in *in vitro* culture medium (µL.L⁻¹): determination of CL₅₀ and CL₉₉ and their confidence interval (P ≤ 0.05)

Fungus	CL ₅₀ (µL)	Lower limit	Upper limit	CL ₉₉ (µL)	Lower limit	Upper limit
<i>Aspergillus westerdijkiae</i>	218	199	265	630	557	739
<i>Fusarium graminearum</i>	133	125	169	320	303	387

Fungal spore germination inhibition

The complete inhibition of spore germination of the two fungi species was observed through micro-atmosphere diffusion test at 1 µL of AITC per culture plate, *i.e.* about 3 µL.L⁻¹ of air in the fungal spores exposure chamber (Fig. 6).

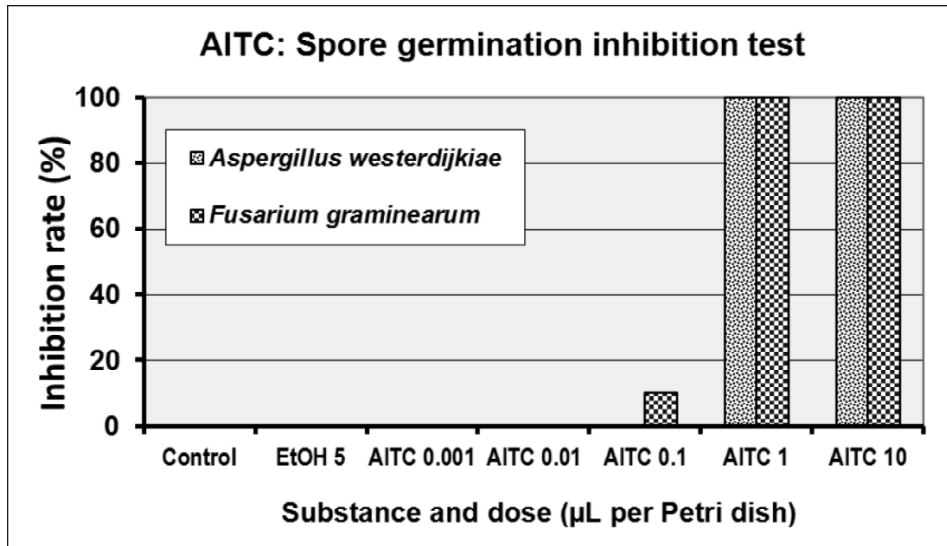


Fig. 6- Regression of spore germination rate of spores of *A. westerdijkiae* and *F. graminearum* on culture medium in Petri dish exposed to progressive dose of AITC in vapour phase (micro-atmosphere diffusion test) – 4 replicates per series.

As a reference with clove EO incorporation to fungal culture medium, only 50% inhibition of fungal spore germination was observed at a concentration of 15 µL.L⁻¹ agar culture substrate (Fig. 7).

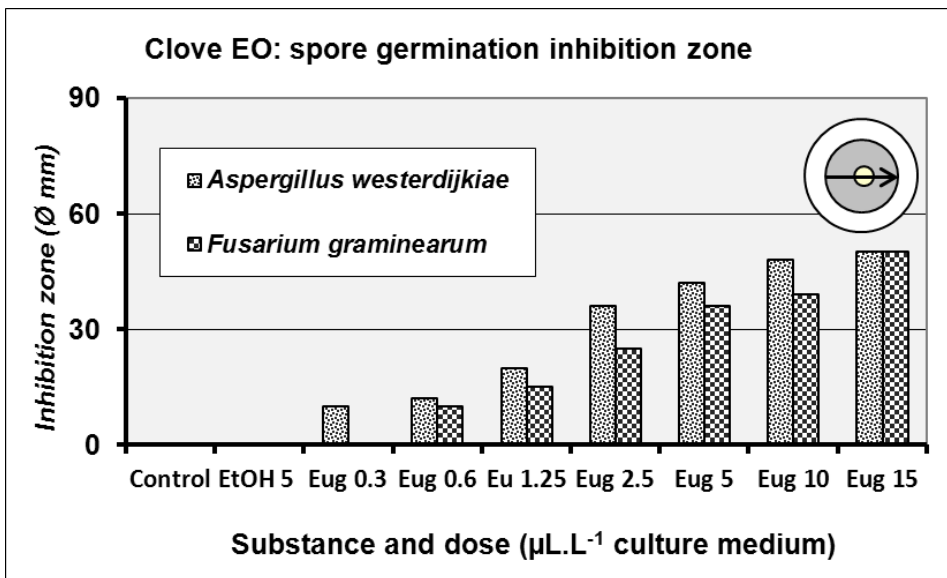


Fig. 7- Regression rate of germination rate of spores of *A. westerdijkiae* and *F. graminearum* on culture medium in Petri dish spiked with increasing doses of clove oil (agar diffusion bioassay) – 4 replicates per series.

DISCUSSION

The mustard oil major compound, allylisothiocyanate (AITC) exhibited remarkable activity both against grain insect pest *S. oryzae* (adult stage) and against spore germination and mycelial growth of the two mycotoxigenic fungi *A. westerdijkiae* and *F. graminearum*. At a concentration in vapour phase of $40 \mu\text{L.L}^{-1}$, the germination of fungal spore of the two fungi is completely inhibited and adults of the rice weevil are killed after 24 h exposure at less than $10 \mu\text{L.L}^{-1}$.

The insecticidal activity of AITC is five times above the one of ethyl formate (LC_{95} of EtF observed at $52 \mu\text{L.L}^{-1}$ to be compared to $10 \mu\text{L.L}^{-1}$ for AITC). The sporicide activity of AITC against the seed-borne and pathogenic fungi tested in the present study is remarkable at a dose as low as $40 \mu\text{L.L}^{-1}$ air volume. These antifungal properties should be developed for the fumigation of empty storage facilities or grain bins with a good airtightness in situations where the access to thorough cleaning and insecticide treatment before loading with grain is complicated or impossible in some locations (e.g. inside perforated aeration duct). Generally, these “uneasy cleanable” points are accumulating broken grain, dust and impurities and are often humid and favourable to fungal development and secondary insect pests population multiplication. The use of AITC for the sanitation of empty grain bin or sealable grain storage facilities when access inside is difficult will be profitable for efficient limitation of the risks of infestation or contamination of the new sound harvest coming into these storage facilities. For grain disinfestation purposes, the use of AITC as a new fumigant is more problematic. The combination of AITC and EtF was tentatively proposed in Australia and in France some years ago (Ciesla et al., 2008), but the barrier of registration was not overcome today. New advances on the joint antifungal and insecticidal activity of AITC will require more studies with infested grain and mouldy grain aiming at a confirmation of the present promising results.

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