JOURNAL OF PLANT PROTECTION RESEARCH Vol. 55, No. 1 (2015)

Formulation of the endophytic fungus *Cladosporium oxysporum* Berk. & M.A. Curtis, isolated from *Euphorbia bupleuroides* subsp. *luteola*, as a new biocontrol tool against the black bean aphid (*Aphis fabae* Scop.)

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Received: July 16, 2014
Accepted: February 16, 2015

Abstract: Two formulations containing culture filtrates and conidial suspensions of the endophytic fungus *Cladosporium oxysporum* Berk. & M.A. Curtis, isolated previously from stems of *Euphorbia bupleuroides* subsp. *luteola* (Kralik) Maire, were experimentally tested for their aphicid activity against the black bean aphid *Aphis fabae* Scop. found in Algeria. It was shown that invert emulsions are more effective against aphids, than using aqueous suspensions. This was especially true for formulations containing culture filtrates. The relatively insignificant mortalities obtained by formulations containing conidial suspensions indicated a low infectious potential towards the aphids. The proteolytic activity seemed to be more important than the chitinolytic activity of the fungus against the black bean aphid *A. fabae*.

Key words: *Aphis fabae*, bioformulation, *Cladosporium oxysporum*, endophytes, invert emulsion

Introduction

Faba bean (*Vicia faba* L.) is the most cultivated legume in North Africa. Algeria ranks 17th place in the world and 6th across the African continent, preceded by Ethiopia (516,000 tons), Egypt (350,000 tons), Sudan (112,000 tons), Morocco (73,000 tons), and Tunisia (45,000 tons) (Giove and Abis 2007). This crop alone occupies 58,000 ha or 43% of the area cropped with legumes in Algeria (Maatougui 1996). During the last few years, an outbreak of the black bean aphid (*Aphis fabae* Scop.) has been recorded in areas planted with the faba bean, in the southern regions of Algeria (Meradsi 2009). This aphid colonises a broad range of Fabaceae species (Bond and Lowe 1975; Cammell 1981; Cammell *et al*. 1989). Damage caused by the black bean aphid is of a notably indirect nature; by the transmission of viral diseases, such as the *Broad bean wilt fabavirus* (BBWV) and the *Faba bean necrotic yellows nanovirus* (FBNYV) (Franz *et al*. 1996, 1999; Vetten 2010).

Even if chemical treatments are applied; this solution is not satisfactory because insecticides application methods supply little control. This could be particularly seen in active substances which were not altered, thus leading to important outbreaks of aphid colonies. The recently observed insecticide resistance phenomenon, is the result.

Studies on "aphid-pathogenic" fungi and their use as a component of the biological control strategies against this aphid, are not new (Dedryver 1979; Wilding *et al*. 1986; Yeo *et al*. 2003; Shah *et al*. 2004; Assaf 2009; Guesmi-Jouini *et al*. 2010; Halimona and Jankevica 2011).

However, it should be noted that many mycotaxa may not produce the desired results because of some technical and bioecological disadvantages, including: slower action (sometimes mandatory pathogenic cycles in the host-insect), instability with respect to some physical factors (moisture, extreme temperatures, and radiations), and obstacles to using these organisms on a large scale. This is why the presentation of the biological control product in its final state, ready for use, must take into account certain essential components such as fast effectiveness and stability. Therefore, endophytic fungi may possibly satisfy the requirements aimed for by plant protectionists.

The main objective of this study was to experimentally evaluate the aphicid activities of two bioformulations prepared from *Cladosporium oxysporum* Berk. & M.A. Curtis. *Euphorbia bupleuroides* subsp. *luteola* (Kralik) Maire is a spontaneous Euphorbiaceae found in the Aurès region of Algeria. Previously, an endophytic fungus was isolated from *E. bupleuroides* subsp. *luteola*, against the black bean aphid. We hypothesised, that this fungus can be exploited in biocontrol programs against several aphids in semi-arid and arid agroecosystems.

Materials and Methods

Cladosporium oxysporum was previously isolated from healthy stems of *E. bupleuroides* subsp. *luteola*, sampled from the Chelia Mountain in the Aurès massif (eastern

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Algeria). Peripheral fragments of fungal colonies from Petri dishes containing potato dextrose agar medium (PDA) amended with tetracyclin and incubated in the dark at 24°C, were added to the Wickerham liquid medium (Hassan 2007). A relatively large fungal biomass was obtained for subsequent tests. Cultures, contained in the 500 ml Erlenmeyer flasks, were incubated at 24°C in darkness. The flasks containing the cultures were subjected to intermittent agitations with magnetic stirrers for 1 to 4 h, for two consecutive weeks.

After three days, we added glucose, at a rate of 1 g for each Erlenmeyer flask, to energize the growth and biomass gain of the fungus (Sabuquillo *et al*. 2010). The consequent fungal suspensions were recovered after serial passage in the centrifuge, followed by filtration of the supernatant through sterile Whatman paper 01 and 02. The filtrates thus obtained were stored at a low temperature (2°C) under sterile conditions for later use.

Experimental populations of targeted *A. fabae* were obtained in colonies starting from parthenogenetic-viviparous females. Aphids were reared on bean plants of unknown genotypes, planted at the campus of the University of Hadj Lakhdar, Batna, Algeria.

Two prepared bioformulations were tested on the insects' third instars. One bioformulation, an aqueous suspension consisting of the fungal culture filtrates, was incorporated in a 0.75% NaCl solution and Tween 80 (0.02%). Culture filtrates were used at four concentrations (20, 40, 60, and 80%). A so-called "invert emulsion" was also prepared. This bioformulation essentially comprises two phases embedded as 1/1: one is aqueous (including fungal fraction) whereas the other is oily, so that a water-in-oil structure is obtained. The aqueous phase contained glycerol (0.02%), and salicylic acid (0.5 g/500 ml of water) in addition to the fungal filtrate. The oily phase consists of two blended vegetable oils of low viscosity, to which Tween 80 was added as an emulsifying agent and a Bohemit clay suspension was also added. The two phases were homogenised with a magnetic stirrer for a few minutes. The same biofomulations were prepared again, but we then added a conidial suspension of different concentrations (10^5, 10^6, 10^7, and 10^8 conidia $\cdot$ ml^{-1}).

A modified ventilated chamber bioassay (Mesquita *et al*. 1996) was adopted for the experimental treatments: 20 of the third-instar-apterous aphids were kept in one Petri dish (10 repetitions) containing excised faba bean leaflets. To delay senescence of the leaflets, they were covered on the level of their excision points. For this purpose, sterile cotton containing a mineral solution was used (Mesquita *et al*. 1996; Butt and Goettel 2000).

The lids of the Petri dishes were perforated and covered with muslin. Aphids were spray-treated using a hand-operated spray bottle (Dorschner *et al*. 1991). For each bioformulation test, dishes containing untreated *A. fabae* individuals represented the control units (with the same number of repetitions). Dead insects were recorded at regular intervals (Table 1). The corrected mortality was calculated according to the formula of Abbott (1925).

In the second part of the experiment, and to show a possible relationship between aphicid enzymatic activities of the fungus, two enzymes were quantified: proteases detected using as a substrate, 0.5% gelatine (Lopez-Llorca *et al*. 2002), were included in a medium containing 1% agar (w/v) NaCl (0.3 g $\cdot$ l^{-1}), K_2HPO_4 (0.2 g $\cdot$ l^{-1}), and $MgSO_4 \cdot 7H_2O$ (0.3 g $\cdot$ l^{-1}). The extent of protease induction was determined according to Moscoso and Rosato (1987) as a ratio of the halo diameter (clear zone indicating the degradation of the substrate) and the colony diameter of the fungus.

For chitinases, fragments of the fungus colonies were added to Erlenmeyer flasks containing 150 ml of a medium. For each 1,000 ml, the medium consisted of colloidal chitin (4 g), K_2HPO_4 (0.7 g), $MgSO_4 \cdot 5H_2O$ (0.5 g), KH_2PO_4 (0.3 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), peptone (0.5 g), $MnCl_2$ (1.0 mg), and $ZnSO_4$ (1.0 mg). The medium was incubated in darkness at 25°C by undergoing regulated intermittent agitations for 30 min (150 rpm) every 2 h, for 10 days. The culture was cold-centrifuged (4°C) at 6,000 rpm for 30 min (Nguyen *et al*. 2009) and filtered through Whatman paper 2. To determine fungal chitinases, every day during the incubation period, we took a volume of 0.9 ml colloidal chitin mixed with sodium acetate (50 mM) and added it to 0.1 ml of the supernatant obtained from the fungal culture. The mixture was then incubated at 37°C for 1 h, after which an addition of 0.2 ml of NaOH (1N) was made. This mixture was centrifuged at 8000 rpm for 8 min. The supernatant was recovered for the determination of the reaction product (N-acetyl-β-D-glucosamine) by spectrophotometry using a wavelength of 535 nm every 24 h. This action was done to determine the extent of chitinolytic activity illustrated by enzymatic units. In this way, the quantity of an enzyme able to produce 1 μmol of the reduced product per hour was determined (Kuk *et al*. 2005).

Table 1. Aphicid action and lethal time 50 of bioformulations containing culture filtrates of *Cladosporium oxysporum*, against *Aphis fabae*, 48 h after treatments

Formulation	Concentration of culture filtrates [%]	Corrected mortality [%]	LT_{50} [h]
Aqueous suspension	20	20.94	93.94
	40	44.94	64.34
	60	46.95	66.33
	80	50.44	62.17
Invert emulsion	20	58.44	57.08
	40	61.45	51.99
	60	82.44	43.74
	80	91.94	36.11

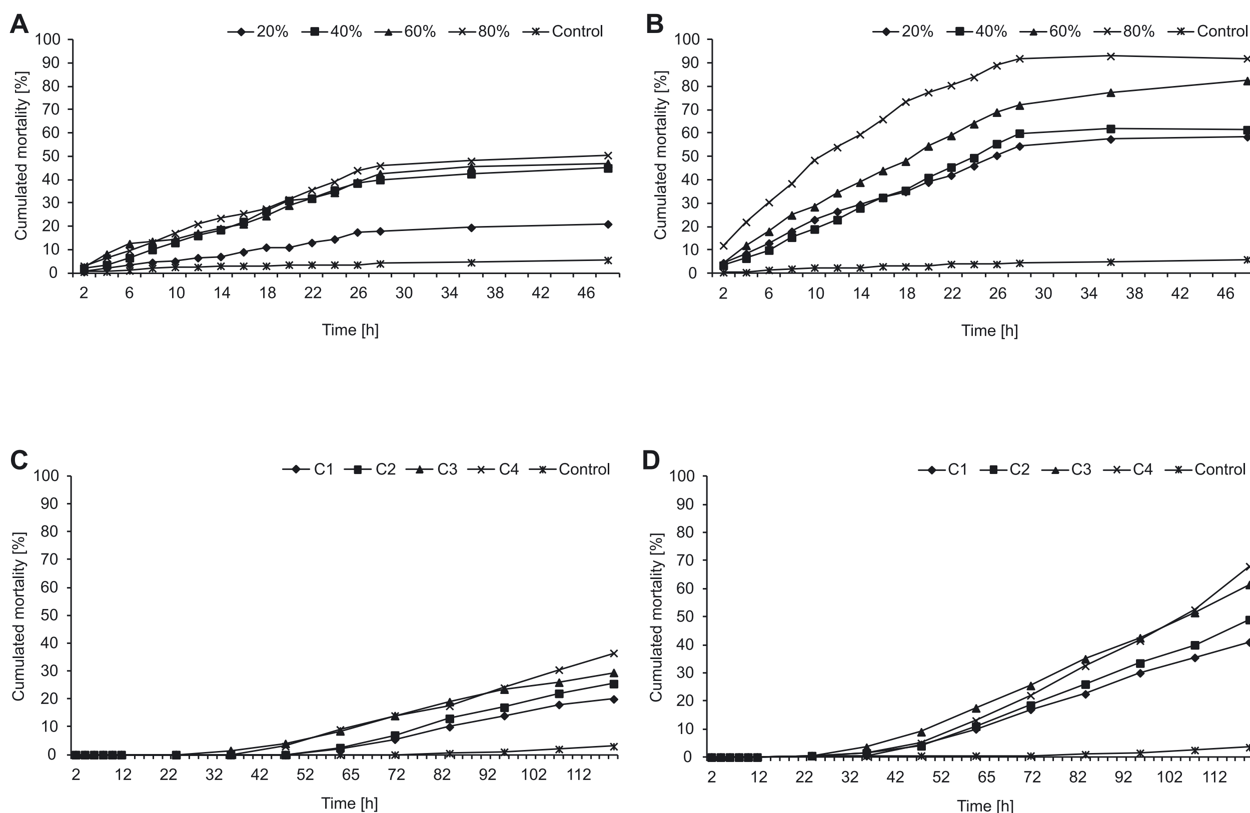

Fig. 1. Cumulated mortality of *Aphis fabae* individuals after their treatments by the prepared bioformulations containing endophytic *Cladosporium oxysporum* culture filtrate incorporated in the aqueous suspension (A) and invert emulsion (B) or conidial suspension incorporated in aqueous suspension (C) and invert emulsion (D). C1, C2, C3 and C4 represent the conidial concentration of 10^5, 10^6, 10^7 and 10^8 conidia · ml^{-1}, respectively

Analysis of variance (ANOVA) was performed to determine the possible effect of the concentrations of the biological matrix (culture filtrates and conidia) on the aphicid activity of the prepared bioformulations. Lethal concentrations 50 (LC_{50}) and lethal times 50 (LT_{50}) were calculated. Following the application of the pesticide, LT_{50} was estimated. The lethal time, LT_{50}, is the estimate of the time (in hours) it takes the experimental population of the target organism to reach a 50% mortality. It enabled us to characterise the aphicid-effect precocity of the bioformulation. Probit-logit analysis performed by XLSTAT Pro (Microsoft Office) determined the LC_{50} and LT_{50}. The probit regression models are illustrated in figures 2A–D.

Results

We recorded different mortalities 48 h after the application of the bioformulations used against *A. fabae*. These mortalities varied according to the type of bioformulation or according to the concentration of the fungal fraction for the same bioformulation (Table 1). We noted, that aphids treated by invert emulsions record the most important mortalities, from 58.44% for the lower filtrate concentration to 91.94% for the highest one (Fig. 1B). Aphids treated with the aqueous suspensions generated mortalities which were much less important. This is mainly illustrated by a mortality of only 50.44% for the highest concentration of culture filtrate (Fig. 1A).

To determine the aphicid activity of bioformulations containing conidial suspensions of *C. oxysporum* against *A. fabae*, monitoring had to be done over a prolonged period of post-treatment. The monitoring had to be done because it is normally expected that effects causing fungal infections can occur after hours or days. However, we recorded particularly low mortality rates for aphids treated with the aqueous suspensions compared to aphids treated with the invert emulsions (Table 2). In fact, mortality ranged from 19.97% for the aqueous suspension with a concentration of 10^5 conidia · ml^{-1} to only 36.47% with a concentration of 10^8 conidia · ml^{-1} (Fig. 1C). Invert emulsions gave more important aphicid action, with 40.96% of individuals treated by a bioformulation containing 10^5 conidia · ml^{-1}, and 67.96% for individuals treated by a bioformulation with 10^8 conidia · ml^{-1} (Fig. 1D).

For the invert emulsions, the LC_{50} was less, in spite of the biological substrate (Table 3). This was true even if they were correlated negatively (for the same bioformulation) to culture filtrate or the conidia concentrations. In addition, LT_{50} was relatively shorter after invert emulsion applications, compared to the aqueous suspensions (Table 2). As an example, it was 93.94 h for the lowest concentration in the culture filtrate (20%) in the case of the aqueous suspension, and 57.08 h for the same substrate concentration in the invert emulsion. The latter recorded the shortest LT_{50} of 36.11 h for the highest concentration of the culture filtrate. Furthermore, we opted for the same observations

Table 2. Aphicid action and lethal time 50 of bioformulations containing conidial suspensions of *Cladosporium oxysporum*, against *Aphis fabae*, 120 h after treatments

Formulation	Conidial concentration [· ml^{-1}]	Corrected mortality [%]	LT_{50} [h]
Aqueous suspension	1×10^5	19.97	186.35
	1×10^6	25.47	175.64
	1×10^7	29.47	180.92
	1×10^8	36.47	167.04
Invert emulsion	1×10^5	40.96	161.12
	1×10^6	48.97	152.94
	1×10^7	61.46	141.96
	1×10^8	67.96	137.20

Table 3. Lethal concentrations 50 (LC_{50}) of the designed bioformulations regarding *Aphis fabae* individuals

Culture filtrates		Conidia suspensions	
aqueous suspension	invert emulsion	aqueous suspension	invert emulsion
58.38%	16.06%	4.17×10^9 conidia · ml^{-1}	4.00×10^5 conidia · ml^{-1}

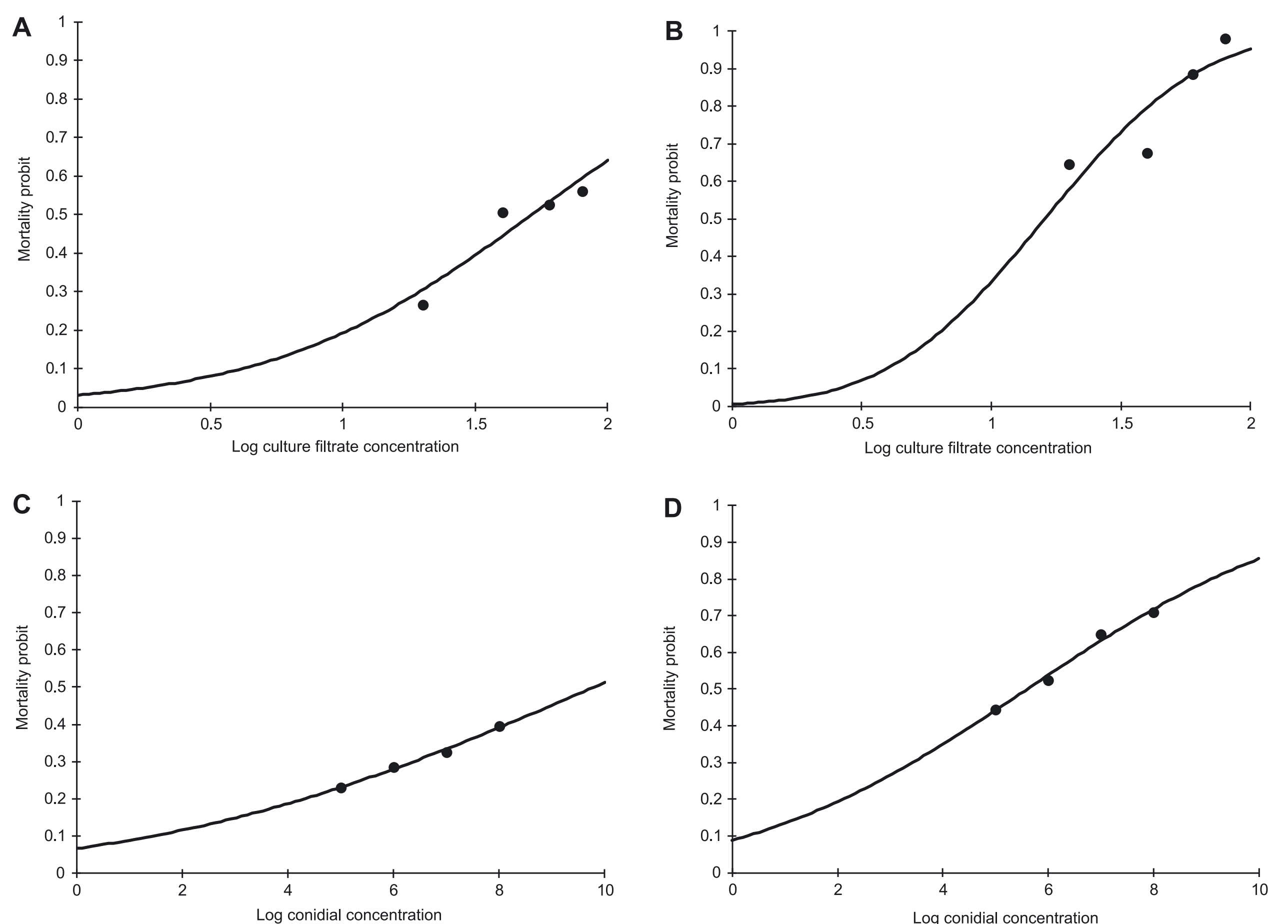

Fig. 2. Regression model of *Aphis fabae* mortality after probit-logit transformation, following treatments by *Cladosporium oxysporum* culture filtrates incorporated in aqueous suspension (A) and invert emulsion (B), conidial suspension incorporated in aqueous suspension (C) and invert emulsion (D)

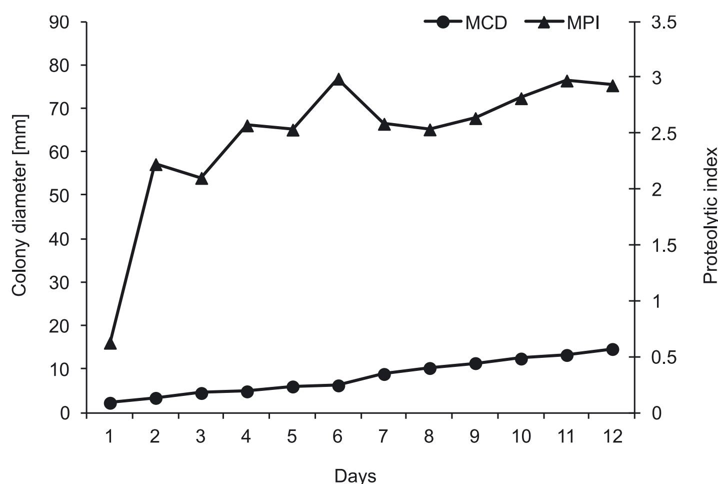

Fig. 3. Mean proteolytic index (MPI) *vs*. mean colony diameter (MCD) of *Cladosporium oxysporum*

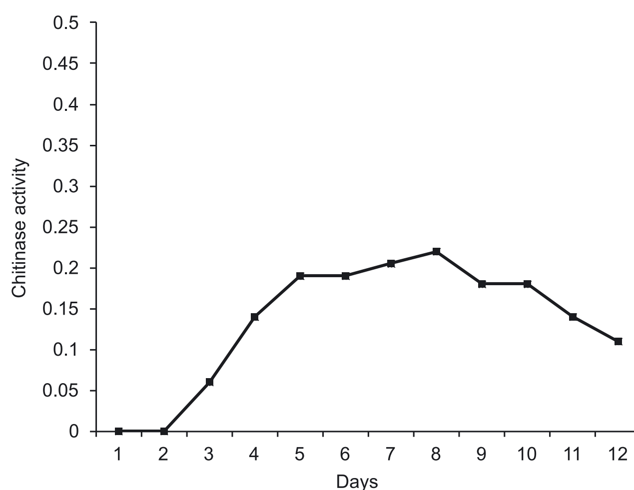

Fig. 4. Chitinase activity of *Cladosporium oxysporum* (μmol N-acetyl-β-D-glucosamine min · ml^{-1})

regarding the LT_{50} recorded for bioformulations containing fungal conidia. In fact, inverse emulsions were faster in action than aqueous suspensions, with an LT_{50} of 137.20 h and 167.04 h for the highest conidia concentration (10^8 · · ml^{-1}) incorporated into invert emulsions and aqueous suspensions, respectively (Table 2).

We evaluated the proteolytic activity of *C. oxysporum* for 12 days. Through the determination of the main proteolytic index (PI), we noticed that protease induction started from the first day (PI = 0.63) (Fig. 3) and increases quickly the 2nd day (IPM = 2.22). This gradual evolution of proteases continued until the 6th day, reaching a maximum of 2.99, and then settling on the 7th day, always exceeding 2.50. Moreover, the radial growth of colonies was significantly slow because the average diameter reached 14.6 mm, only on day 12.

In addition, the chitinolytic activity of *C. oxysporum* culture filtrates showed a relatively weak width (Fig. 4). Initially, it was only from the 3rd day then it began to reach a maximum on the 8th day (0.22 μmol N-acetyl--β-D-glucosamine · h^{-1}), only to decrease gradually until reaching 0.11 μmol · h^{-1} the last day.

Discussion

Cladosporium is very diverse. Its diversity is illustrated not only at the specific "traditional" level, but also in terms of colonised biotopes. Detailed information on the bioecological components of this genus are mentioned in the reviews of McKemy and Morgan-Jones (1990); Ho *et al*. (1999); Heuchert *et al*. (2005) and Crous *et al*. (2007). Moreover, *Cladosporium* can include some naturally entomopathogenic members, experiencing biological control potentialities against several insect pests, particularly whiteflies (Aldeghairi *et al*. 2013). Among others, *C. herbarum* (Pers.) Link is active against species belonging to the genera: *Aleurodicus*, *Bemisia*, *Aleurothrixus*, and *Dialeurodes* (Rojas *et al*. 1998),

Cladosporium sp. isolated from aphids, against the coleopteran Tenebriodidae *Alphitobius diaperinus* (Panzer) (Rezende *et al*. 2009). Earlier, Petch (1931) showed that *Cladosporium* is able to attack aphids, and so *C. oxysporum* is associated with the Homoptera (Samways and Grech 1986). In addition, endophytic forms with entomopathogenic action have been isolated from the leaves of *Coffea arabica* L. (Vega *et al*. 2008) *Schinus molle* L. (Bensaci *et al*. 2012) and *Nerium oleander* L. (Bensaci *et al*. 2013).

The treatments of *A. fabae* generated mortalities which differed according to: the bioformulation composition as well as the nature of the biological substrate. We noted that the invert emulsions showed more important aphicid action than that obtained by the aqueous suspensions. This remarkable efficiency was probably due to the particular make up of the invert emulsion's selected additives which improved the physical and chemical properties of the active biological fraction. The aqueous suspension, on the other hand, was simpler in term of added compounds.

In the beginning, the design of the biopesticides in invert emulsion formulation was obtained to control weeds and invasive plants in agricultural and aquatic ecosystems (Connick *et al*. 1991; Auld and McRae 1997), also to control plant pathogens (Batta 2003, 2004), and to a lesser degree, to control insect pests (Batta 2007). Its principle is based on the creation of a continuous oily phase within which is included a discontinuous aqueous phase. The purpose is to reduce the undesirable effects of the physical factors on the fungal filtrates, and to provide for the best conidial dispersion. In this way, the risks of volatilisation of the filtrates and loss of germination and the infectious capacity of conidia are limited.

Within the framework of the biological control of key crop pests, there have been few works which were devoted to the use of the culture filtrates of *Cladosporium* spp. By evaluating the effect of the culture filtrates of *C. echinulatum* (Berk.) G.A. de Vries and *Paecilomyces* sp. isolated from *Nerium oleander* L., against *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae), Bensaci *et al*. (2013) obtained mortalities ranging from 39 to 84% for concentrations of 25 and 100%, respectively. They also signaled that mortality is positively correlated to the culture filtrate concentration. In our study, the mortality of *A. fabae* was remarkable for inverse emulsions, indicating the compatibility of fungal filtrates with this type of preparation.

Furthermore, the use of conidial suspensions of *Cladosporium* is considered to be the most approved method by certain researchers, with regard to the proved entomo-infectious potentialities of this mycotaxon (Abdel-Baky and Abdel-Salam 2003; Saranya *et al*. 2010). Even if the mortalities obtained by the application of the formulations containing the conidial suspensions of the bioprospected fungus were much less than those obtained by use of the filtrates, it always proves that invert emulsions were more effective.

Saranya *et al*. (2010) showed that the conidial suspension of *C. oxysporum* generates a corrected mortality of 77.50% in *Aphis craccivora* Koch individuals, when the suspension was used at a concentration of 10^8 conidia · · ml^{-1} incorporated to the Teepol solution. This is contrary to our results where we noted that maximum mortality of *A. fabae* (67.96%) is obtained with the same conidial concentration but incorporated in invert emulsion. It should be noted that the fungal origin is a spontaneous plant of the Aurès region (*E. bupleuroides* subsp. *luteola*) and so we did not make a study on the natural incidence of the isolated *Cladosporium* on the naturally-associated insects to the host plant. Moreover, Abdel-Baky and Abdel-Salam (2003) found that mortality generated naturally by three species of *Cladosporium* on *A. craccivora*, *A. gossypii*, and *A. durantae* varied from 16.41 to 28.08%. It is impossible and premature to note that the tested endophyte of our study is "naturally entomopathogenic". Otherwise, it is acceptable to clarify the fact that it may adopt a "shift" strategy in its trophic behavior, which is the case for several endophytic fungi (Vega *et al*. 2008).

The values of the LC_{50} and earlier LT_{50} of invert emulsion demonstrate the effectiveness and speed of action compared to the aqueous suspensions. However, it seems that the fungus shows a very low infectious potential towards the aphids, and in fact, that is not truly desirable. The fungus probably acts by toxic bioactive metabolites rather than by the traditional way of mycoparasitic infection.

Saranya *et al*. (2010) calculated an LC_{50} of 7.4×10^5 conidia · ml^{-1} and an LT_{50} of 5.24 days, corresponding to the application of the conidial suspension of 10^8 conidia · ml^{-1} derived from *C. oxysporum* against *A. craccivora*. In addition, Abdel-Baky and Abdel-Salam (2003) showed in a laboratory test, that maximum mortalities of *A. gossypii* (37.5%) and *A. craccivora* (38%) were recorded on the third day after treatment with conidial suspensions of *Cladosporium* spp. at a concentration of 10^6 conidia · ml^{-1}. While Jeyarani *et al*. (2011) recorded an LC_{50} of 4.30×10^6 conidia · ml^{-1} and an LT_{50} of 63.80 h after mite (*Tetranychus urticae* Koch) treatments with *C. cladosporioides* suspensions.

We noted that proteolytic potential of the tested *C. oxysporum* is relatively important. This enzymatic activity regarded as paramount for fungal endophytes, is reflected by the production or the induction of proteolytic enzymes such as the proteases and the polypeptidases (Kucera 1980; Samuels and Paterson 1995). Thus, it was demonstrated that enzymatic activities and the virulence of a broad range of fungi are two closely-dependant characters (Robert and Messing-Al-Aidroos 1985; Monod *et al*. 2002). The extent of the chitinolytic activity, though, cannot inform us about the aphicid potentialities of this endophyte. Yet, it is possible to attribute the infection of aphids treated with the conidial suspensions, to an affinity to the cuticle. Samways and Grech (1986) showed that *C. oxysporum* grown in media amended with chitin, is capable of inducing major diseases in *Toxoptera citricidus* and *T. erytreae* colonies.

We believe that the endophytic fungus *C. oxysporum* isolated from *E. bupleuroides* subsp. *luteola* shows aphicid potentials that can be exploited, with the choice of a suitable formulation, not only against *A. fabae*, but other crop-harmful aphid species. Thus, we note that invert emulsion is one of the appropriate formulations for the preparation and the design of the biological products containing mycoendophytes in their final state. Such a preparation and design are appropriate in the framework of biological and integrated control of aphids in semi-arid and arid agroecosystems.

Acknowledgements

We thank Prof. Malik Laamari for his technical contribution and for providing and identifying the tested aphids.

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