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# Larvicidal activity of endophytic fungal extract of *Cochliobolus spicifer* (Pleosporales: Pleosporaceae) on *Aedes caspius* and *Culex pipiens* (Diptera: Culicidae)

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**Abstract** Endophytic microorganisms are fungi or bacteria which live inside plant tissues or organs, without causing them any harmful symptoms. Indeed, since many are also able to produce substances of biotechnological interest, they may protect the plant from insect attacks and diseases. The date palm, *Phoenix dactylifera* (Linnaeus) is a tree which has been known in Saudi Arabia for thousands of years as a staple food. The aim of the present work was to investigate the inhibitory activity of crude extracts of the fungal endophyte *Cochliobolus spicifer* (Nelson), isolated from the date palm, against *Aedes caspius* (Pallas) and *Culex pipiens* (Linnaeus) larvae. In addition, histological changes were determined in the digestive system of treated third instar larvae. The data showed that *C. spicifer* interfered with the developmental cycle of the mosquito, and had its strongest toxic effect (with 100 % mortality in third instar larvae) at concentrations of 300 ppm. This compound did not show toxicity to zebrafish. The third instar larvae treated with the active fraction showed cellular destruction and disorganization, cell spacing, and vacuolization of epithelial cells in small regions of the midgut. To the authors' knowledge, this is the first study to report the isolation of endophytic fungi *C. spicifer* from date palm trees (*P. dactylifera*). The endophytic fungal extract of *C. spicifer* proved to be a strong candidate for a natural, safe, and stable phytolarvicidal to be used in population control of *Ae. caspius* and *Cx. pipiens*.

**Keywords** *Cochliobolus spicifer* · Larvicidal activity · Mosquitoes · Zebrafish

## Introduction

Plants may serve as a reservoir of large numbers of organisms such as bacteria, insects, nematodes, protozoa, or fungi that can live endophytically within plant tissues (Müller and Döring 2009; Sieber and Grünig 2006). Endophytes are microorganisms (mostly fungi and bacteria) that inhabit plant hosts for all or part of their life cycle. Endophytic microbes can be found in apparently healthy plant tissue (Addy et al. 2005; Faeth 2002; Piercey et al. 2004; Porras-Alfaro and Bayman 2011; Saikkonen et al. 1999; Sieber 2002; Vandenkoornhuyse et al. 2002) and can be located in different plant organs such as leaves, needles, stems, or roots (Grünig et al. 2008; Sokolski et al. 2007; Verma et al. 2007). Carroll (1988) defined organisms causing asymptomatic infections within plant tissues as endophytes and excluded pathogenic fungi and special groups of mutualists such as mycorrhizal fungi. Petrini (1991) expanded Carroll's definition to include all organisms which at certain times in their life inhabit plant organs without causing any harm. The behavior of fungal endophytes can range from mutualistic (Usuki and Narisawa 2007; White and Torres 2010) to pathogenic (Tellenbach 2011; Tellenbach et al. 2011), and endophytes can switch their behavior depending on environmental factors, a characteristic described as the endophytic continuum (Schulz and Boyle 2005). Endophytic fungi are an ecologically polyphyletic group

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of highly diverse fungi, although mostly belonging to ascomycete and anamorphic fungi (Arnold 2007; Huang et al. 2001).

The presence of endophytic fungi implies a symbiotic interaction in all the photosynthetic tissues of vascular plants (Arnold et al. 2000). Fungal endophytes are hosted by nearly one million plant species, and are of biotechnological interest owing to their potential use as genetic vectors, metabolites, and biological control agents. Endophytes have been viewed as an outstanding source of secondary metabolites and bioactive antimicrobial natural products (Dorworth and Callan 1996). They are known to be able to protect their hosts against several biotic and abiotic factors, such as the attack of insects, pathogens, and herbivores (Arnold et al. 2003; Firáková et al. 2007; Mejía et al. 2008). Chobba et al. (2013) investigated the diversity of both cultivable and non-cultivable endophytic fungal floras in the internal tissues (roots and leaves) of Tunisian date palm trees (*Phoenix dactylifera* Linnaeus). They confirmed that while the roots were predominantly colonized by *Fusarium* (members of the Nectriaceae family), the leaves were essentially colonized by *Alternaria* (members of the Pleosporaceae family). In the present study, the endophyte *Cochliobolus spicifer* (Nelson) was isolated from *P. dactylifera*. *C. spicifer* is a dematiaceous hyphomycete frequently isolated from plants, which in regions with a hot and dry climate is among the most frequent airborne fungi encountered (Alcorn 1988).

Vector-borne diseases are infectious diseases transmitted to humans and animals by arthropods, of which the insect-borne are the most important, being considered priority diseases for prevention and control. These diseases include malaria, lymphatic filariasis, and arboviral diseases transmitted by mosquitoes of genera *Aedes*, *Anopheles*, and *Culex*. Many *Culex* mosquitoes, such as *Cx. quinquefasciatus* (Say), are vectors of arboviral diseases at both a global and regional level, including Rift Valley and Dengue Fever in the Arabian Peninsula. In the last decade, serious outbreaks of these two diseases occurred in the southwestern regions of Saudi Arabia near its borders with Yemen (CDC 2000; EMPRES 2000; Rathor 2009; Shoemaker et al. 2002; WHO 2004). These outbreaks were due to the proliferation of mosquito populations following heavy rains and indicated that mosquito control operations were ineffective, possibly because of the use of ineffective pesticides or because the mosquitoes had developed resistance to these insecticides. The spread of vector resistance to almost all groups of insecticides in addition to their environmental hazards and production costs have encouraged scientists to explore alternative means of pest control.

*Ae. caspius* (Pallas) is the most abundant mosquito in Al-Ahsaa, Saudi Arabia, followed by *Cx. pipiens* (Linnaeus) (Ahmed et al. 2011). *Ae. caspius* is widely distributed across different regions of Saudi Arabia such as Riyadh district (Al-Khreji 2005), the eastern district (Büttiker 1981; Mattingly and Knight 1956), and the southwestern regions (Abdullah and Merdan 1995). Omar (1996) reported that local *Cx. pipiens* mosquitoes might act as potential vectors of introduced bancroftian filariasis in Saudi Arabia. Control of such diseases is becoming alarmingly complex because of the increasing resistance in mosquitoes to many synthetic insecticides. There is, therefore, a developing interest in alternative control methods, including biocontrol using entomopathogenic fungi.

The zebrafish, *Danio rerio* (Hamilton) has become a popular experimental model in biological, toxicological, and chemical screenings during the past two decades. The zebrafish model has many advantages in laboratory research, e.g., transparent embryos, high fecundity with hundreds of embryos from each single spawning on a daily basis, low cost and space requirements for aquarium maintenance, etc. In particular, the zebrafish has emerged as an excellent toxicological model. In 2002, Nagel described a standard DarT (*D. rerio* Teratogenic) assay, in which wild-type zebrafish embryos were used to monitor several lethal and sublethal endpoints in order to evaluate the potential toxicity of chemicals at different developmental stages, with the assay covering essentially all the major organs and systems in zebrafish (Nagel 2002). This assay has since become the established zebrafish embryo test and is widely used in chemical screening as well as being recommended by the Organisation for Economic Co-operation and Development (OECD) (McGrath and Li 2008). Since insecticides are known to pose a potential threat to aquatic animals, various studies have utilized zebrafish embryos to evaluate toxicity related to these insecticides (Chandrasekar et al. 2011; Lopez-Romero et al. 2012; Mizell and Romig 1997; Osterauer and Kohler 2008; Pandey and Guo 2014; Shi et al. 2011; Xu et al. 2014; Yang et al. 2014).

The aim of this study, therefore, was to determine the toxicity of an *C. spicifer* fraction and its morphological effects on the digestive system in third instar larvae of *Ae. caspius* and *Cx. pipiens* and in zebrafish embryos.

## Materials and methods

### Mosquito culture

*Ae. caspius* and *Cx. pipiens* larvae were obtained from a colony maintained in the laboratory of the Department of Zoology, College of Science, King Saud University, without exposure to any insecticide. They were reared in a plastic tray (24 × 35 × 5 cm) containing fish feed and were kept indoors at 27 ± 2 °C, 50 ± 5 % relative humidity, and a 14:10 light/dark photoperiod. The larvae were fed daily

until they became pupae. The pupae were then transferred from the trays to a cup containing tap water and were maintained in our insectary. They were then moved into a mosquito cage where the emergent adults were fed with a 10 % glucose solution in a jar with a cotton wick. A glass Petri dish lined with filter paper and containing 100 ml tap water was kept inside the cage for oviposition.

## Collection of plant material

The date palm (*P. dactylifera*) is a multipurpose plant species, typically grown in the arid and semi-arid regions of the world, and which is globally valued for its nutritional and health-promoting fruit. The leaves of date palms obtained from Al-Oula (Medina, Saudi Arabia) were used for the investigation of endophytic fungal communities. The harvested samples were mature photosynthetic leaves, randomly chosen from different parts of plants present in the same vicinity. The samples were brought to the laboratory in sterile bags and processed immediately to reduce the risk of contamination.

## Isolation and purification of the endophytic fungus

Endophytic fungi were isolated from the date palm leaves. The isolated endophytic fungus was maintained on potato dextrose agar (PDA) slants at 30 °C. Subculturing was performed at regular monthly intervals in order to maintain the stock cultures, which were preserved at 4 °C. Starting materials for fermentation experiments were taken from an actively growing stock culture, which were then subcultured on fresh slants and incubated for 10 days at pH 7.0 and temperature 30 °C.

## Identification of the endophytic fungus by cultural and morphological methods

The identity of the endophytic fungal cultures was studied by using both cultural and morphological approaches. Cultural characteristics, such as color and the nature of the growth of the colony, were determined by visual observation. Morphological characteristics of the fungus, like mycelia, conidiophores, and conidia, were studied microscopically (using a Leica inverted microscope).

## Preparation of fungal extract

Ten plates were inoculated with approximately 6-mm disks of 4-day-old culture and kept at 30 °C for 10 days. The ten plates were then homogenized with the homogenate being transferred to a flask containing 500 ml ethyl acetate and left to stir for 10 min at 140 rpm at 30 °C. The mixture was filtered through muslin cloth and the extraction process was repeated three times. The mixture was concentrated and then centrifuged at 12,000 rpm for 5 min to remove any debris (F1). The mixture was then transferred to a round-bottomed flask, dried using a rotary evaporator at 40 °C, and stored at −20 °C until required. The same process was repeated for methanol (F2) and then an additional purification step was performed by dissolving the ethyl acetate dried extract (F1) in methanol so that two fractions were obtained. One of these fractions was soluble in methanol (F1A) whereas the other fraction was insoluble in methanol (F1B).

## FTIR analysis of bioactive fractions

The active fraction was subjected to infrared (FTIR) spectroscopy, which was performed using potassium bromide (KBr) plates (JASCO FTIR model 420, Japan).

## Larvicidal bioassay

Different concentrations of solution ranging between 50 and 300 μg/ml were prepared. Each test solution was placed in multi-well plates (12 well) and left until dried. Later, each solution was dissolved in 1 ml of tap water and tested against 10 third instar larvae (*Ae. caspius* and *Cx. pipiens*). Each experiment was conducted in triplicate and tap water was used as a negative control. The number of dead larvae was counted 24 and 48 h after exposure and the percentage of mortality was reported for the average of the three replicates.

## Histology: digestive system

A histological evaluation of the digestive system was performed using third instar larvae (treated and control) fixed in 2.5 % glutaraldehyde in a sodium cacodylate buffer (0.1 M, pH 7.4) for 4 h. They were then dehydrated with increasing concentrations of ethanol (70, 80, 90, 96, and 100 %), by immersion in each of these solutions for 15 min. Next, the larvae were embedded in Historesin JB4 and the resultant blocks were sliced using a microtome to obtain a series of 3-μm-thick sections. These sections were stained with hematoxylin–eosin, and then examined and photographed using a light microscope (Bancroft and Stevens 1996).

## Zebrafish

Wild-type zebrafish (AB/Tübingen TAB-14) were obtained from the Zebrafish International Resource Center (ZIRC, University of Oregon, OR, USA) and maintained in the animal facility at the Department of Zoology at King Saud University. The adult zebrafish were kept under the

standard laboratory conditions of 28 °C on a 14-h light/10-h dark photoperiod in fish water (FW), which consists of reverse osmosis water supplemented with a commercially available salt (0.6 % Instant Ocean salt). All experiments were carried out in accordance with the National and International Animal Use Guidelines.

### Zebrafish treatment

The wild-type (AB Tubingen) zebrafish embryos were obtained by natural pairwise mating and raised to the shield stage (6 h post-fertilization). The active fraction was resuspended in cell culture grade methanol at a stock concentration of 10 mg/ml. The synchronized staged embryos ($n = 30$–35) with chorion were exposed to serial dilution of the extracts in an embryo medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). The 0.5 % (v/v) methanol-treated embryos served as controls. The embryos remained exposed for the indicated time of treatment in an air incubator at 28 °C. The treatment was repeated at least three times with a different batch of embryos at each time. The cumulative mortalities and morphological defects were assessed at 24, 48, 72, 96, and 144 h post-fertilization.

### Microscopy and photography

All images were acquired using an Olympus stereo microscope SZX10 equipped with an Olympus DP72 camera.

### Statistical analysis

The experiment was designed as a completed random design (CRD). Data are expressed as mean ± standard error. Statistical analyses of the mortality rate were performed by analysis of $T$ test (one sample $T$ test) (SPSS 13.0 for Windows, 2004).

## Results

The fungal strain was identified using cultural and morphological approaches. After 10 days on PDA at 30 °C in the dark, colonies became gray and velutinous and were characterized as follows: reverse dark brown, conidia straight, cylindrical with rounded ends, three septate, and brown in color. Most of these morphological characteristics agree with the known features of *C. spicifer*. The cultural and morphological studies confirm that the isolate belongs to genus *Cochliobolus* and species *spicifer*. This was further confirmed, however, by sending the sample for analysis at the Mycological Center, Faculty of Science, Assiut University, Assiut, Egypt. The deposit number is AUMC10172.

In the present study, the ten plates incubated with fungus were homogenized and extracted with ethyl acetate and methanol to provide crude ethyl acetate (F1) and methanol (F2) extracts of *C. spicifer*. In the present study, the ten plates with fungus were homogenized extracts of *C. spicifer* were obtained as described in "Materials and methods". Of these extracts, the yields of F1 and F2 were 350 and 290 mg, respectively, whereas the yields of the F1A and F1B fractions (calculated as weight of dried fraction/weight of dried total extract from which the fraction is partitioned, mg/mg × 100 %) were 28.5 and 48.5 %, respectively. The ethyl acetate agar (EAA) extracts from the endophytic fungus demonstrated larvicidal activity against both mosquito species tested. The F1A fraction had a greater larvicidal activity and presented greater potentially lethal results than the methanol extracts (more polar). The methanol extract in this study had no activity against the two mosquito species tested. This finding indicates that the active larvicidal components of EAA extract of *C. spicifer* must be of low polarity.

FTIR spectroscopy is becoming increasingly important for the quality control and analysis of natural product extracts. It is a fast and non-destructive method used to authenticate crude extracts, fractions, and final products using their specific fingerprints. IR analysis of the extract revealed a peak at 1745 cm$^{-1}$ assigned to the C=O stretching vibration, which means that carbonyl compounds are present. In addition, the peaks at 2925 cm$^{-1}$ belong to the C–H stretching vibration of methyl and methylene. The absence of N–H and O–H stretching bands between 3060 and 3700 cm$^{-1}$ confirms the absence of alcohol, phenol, amide, amine, and lactam, and the absence of the aromatic C–H stretch confirms that the compound is aliphatic (Fig. 1).

All in all, these notable peaks revealed that the extract had lots of lipophilic components, such as essential oil, greases, micromolecule terpenoids, and fatty acids and their esters, which contain mostly carbonyl and alkyl functional groups.

From the above study it can be concluded that infrared spectroscopy methods can be used as a tool in the routine quality analysis of the raw materials of extracts with biological value in order to detect the presence of adulterants and common contaminants.

The percentage mortality of *Ae. caspius* and *Cx. pipiens* following treatment with different concentrations of extracts from the endophytic fungus *C. spicifer* was calculated. The ethyl acetate extract from *C. spicifer* was very effective against the third instar larvae of *Ae. caspius* and *Cx. pipiens*, resulting in mortalities of 73 and 80 %, respectively, of the larvae at a concentration of 200 ppm after 24 h, and up to 96 % of the larvae after 48 h. The F1A fraction from *C. spicifer* extract caused 100 % mortality of the

**Fig. 1** Infrared spectrum of F1A fraction of *C. spicifer* products
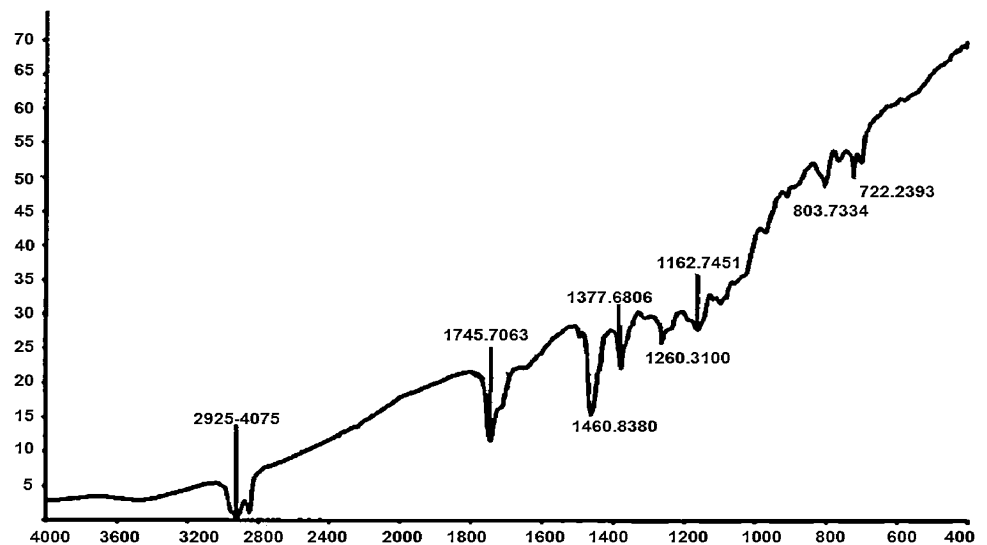


**Table 1** Larvicidal activity of different concentrations of F1A fraction of *C. spicifer* against third instar larvae of *Ae. caspius* and *Cx. pipiens*

| Mosquito species | Time | % mortality | | | | $LD_{50}$ (µg/ml) | $LD_{90}$ (µg/ml) |
|---|---|---|---|---|---|---|---|
| | | Concentration (µg/ml) | | | | | |
| | | 50 | 100 | 200 | 300 | | |
| *Ae. caspius* | 24 | $00 \pm 00$ | $13.33 \pm 3.33$ | $73.33 \pm 3.33$ | $100 \pm 00$ | 170.33 | 264.47 |
| | 48 | $16.67 \pm 3.33$ | $5.77 \pm 50$ | $96.67 \pm 3.33$ | $100 \pm 00$ | 108.18 | 184.55 |
| *Cx. pipiens* | 24 | $6.67 \pm 3.33$ | $6.67 \pm 53.33$ | $80 \pm 15.28$ | $100 \pm 00$ | 133.39 | 249.84 |
| | 48 | $23.33 \pm 3.33$ | $73.33 \pm 8.81$ | $96.67 \pm 3.33$ | $100 \pm 00$ | 84.73 | 173.15 |

Control, nil mortality

larvae at 300 ppm after 24 h, thus showing greater effectiveness against the mosquitoes.

The $LC_{50}$ and $LC_{90}$ values for the F1A fraction are summarized in Table 1. The F1A fraction from *C. spicifer* extract had a toxicity against the test larvae of *Ae. caspius* of $LC_{50} = 170.33$ µg/ml and $LC_{90} = 264.47$ µg/ml, and against *Cx. pipiens* of $LC_{50} = 133.39$ µg/ml and $LC_{90} = 249.84$ µg/ml after 24 h, respectively. After 48 h, the same fraction had a toxicity against the test larvae of *Ae. caspius* of $LC_{50} = 108.18$ µg/ml and $LC_{90} = 184.55$ µg/ml, and against *Cx. pipiens* of $LC_{50} = 84.73$ µg/ml and $LC_{90} = 173.15$ µg/ml), respectively.

Histological analysis of the *Ae. caspius* and *Cx. pipiens* larvae from the control groups showed that in the middle region of the intestine the epithelial cells appeared a little taller with the cytoplasm preserved, and with a thick brush border. This region contained some cells with a more globose appearance, similar to caliciform cells, and with a nucleus of normal appearance (Figs. 2, 3). The *Ae. caspius* and *Cx. pipiens* larvae treated with *C. spicifer* exhibited alterations in the middle intestine (Figs. 2, 3) with evidence of cell destruction, vacuolization of epithelial cells, tissue disorganization with spacing between the cells, and some rupture points of muscle tissue. There was an apparent accumulation of granules in some areas of the cytoplasm, and faint and/or absent nuclei. Some cells showed a lack of cytoplasmic borders.

In order to find out whether the extract is also toxic to vertebrates, zebrafish embryos were exposed to various concentrations (0.001–1 mg/ml) of the F1A fraction of *C. spicifer* extract. The treated zebrafish embryos were raised for up to 5 days post-fertilization to monitor any developmental toxicity. The F1A fraction of *C. spicifer* extract turned out to be very safe for zebrafish embryos and we did not notice any significant mortality or embryonic abnormality. As shown in Fig. 4, the embryos treated with F1A fraction developed and hatched normally like the control embryos. Moreover, there was no significant difference ($p = 0.3838$) between control embryos and those treated with F1A fraction in terms of mortality. The zebrafish embryos ($n = 323.33 \pm 14.529$) treated with mock 5 % v/v methanol showed around 5 % mortality, whereas 6 % mortality was observed in zebrafish embryos ($n = 330 \pm 26.457$) treated with 1 mg/ml of F1A fraction
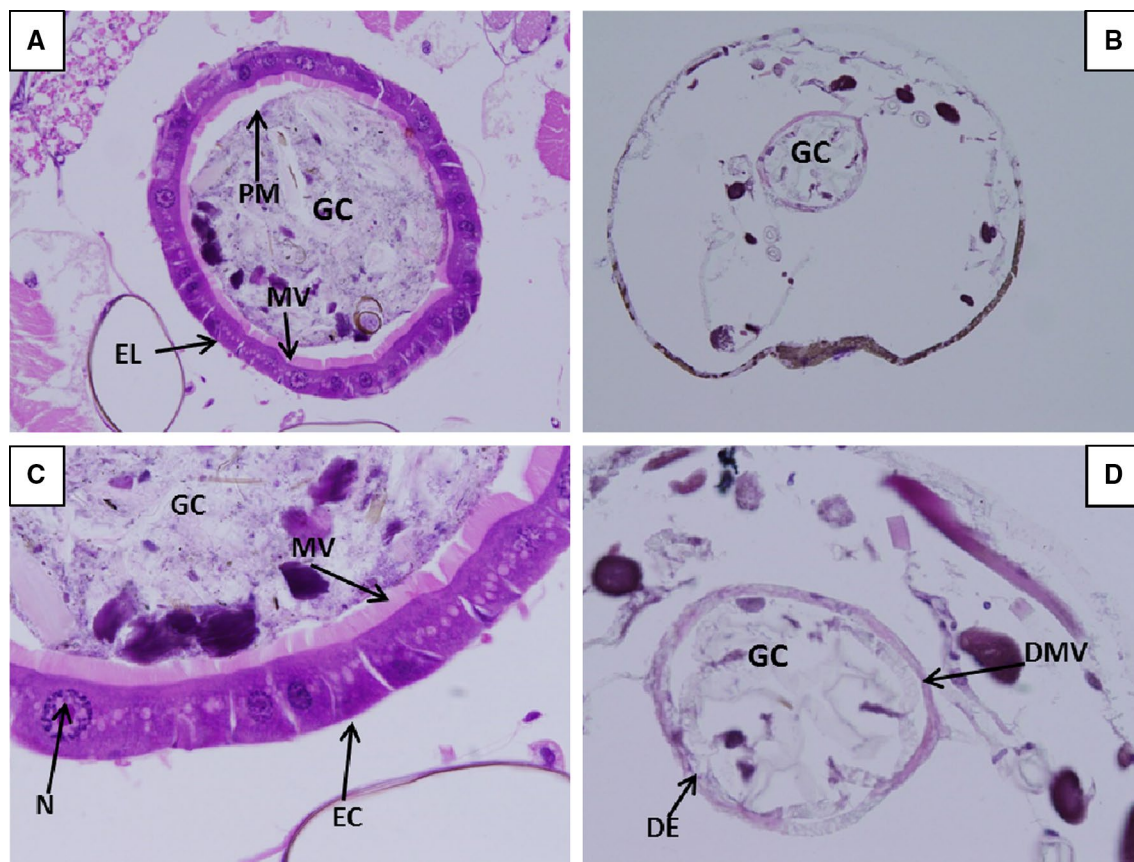
**Fig. 2** Photomicrograph of the midguts of the third instar larvae of *Cx. pipiens* maintained with food and treated with F1A fraction of *Cochliobolus spicifer*, 24 h after treatment. **a**, **c** Cross sections in midguts of untreated larvae, showing normal gut epithelial layer (*EL*) with healthy, normal epithelial cells (*EC*), peritrophic membrane (*PM*), microvilli (*MV*), nuclei (*N*), and nutritional gut contents (*GC*) filling the gut lumen. **b**, **d** Cross sections in midguts of treated larvae, showing affected gut epithelial layer with degraded microvilli (*DMV*), degenerating epithelial cells (*DE*)

of *C. spicifer*. This result shows that the F1A fraction of *C. spicifer* does not affect aquatic animals (or, at least, zebrafish embryos) and, at the tested concentration, only induces significant mortality in mosquito larvae. This fraction would, therefore, be safe for use as an insecticide to eliminate mosquito populations in infested areas.

## Discussion

There is growing interest in the use of natural insecticides to reduce the use of synthetic pesticides and avoid environmental damage. The use of larvicidal compounds involves the application of chemicals to habitats to kill pre-adult mosquitoes. This practice can reduce overall pesticide use in a control program by reducing or eliminating the need for ground or aerial chemical applications to kill adult mosquitoes (Amer and Mehlhorn 2006). The efficiency of this approach in killing larval instars of important vector species, the lack of effect on non-target organisms, and the

biological stability of extracellular metabolites make this practice a promising alternative to mycelium- and conidial-based larvicides (Vyas et al. 2007).

In this study, we isolated for the first time the fungal endophyte *C. spicifer* from date palm. Endophytic fungi represent an important and quantified component of fungal biodiversity, and are known to have an effect on plant community diversity and structure (Gonthier et al. 2006; Krings et al. 2007; Sanders 2004). A variety of relationships can exist between endophytes and their host plants, ranging from mutualism or symbiosis to antagonism or slight pathogenesis (Arnold 2007; Schulz and Boyle 2005).

Our results clearly show that the isolated *C. spicifer* fraction demonstrates high larval mortality. From the results, we can conclude that larvae of *Ae. caspius* and *Cx. pipiens* were susceptible to the compounds in the fungus extracts. Such findings could be useful in promoting research aimed at the development of new mosquito control agents, based on bioactive chemical compounds from indigenous fungus sources as an alternative to chemical larvicides.
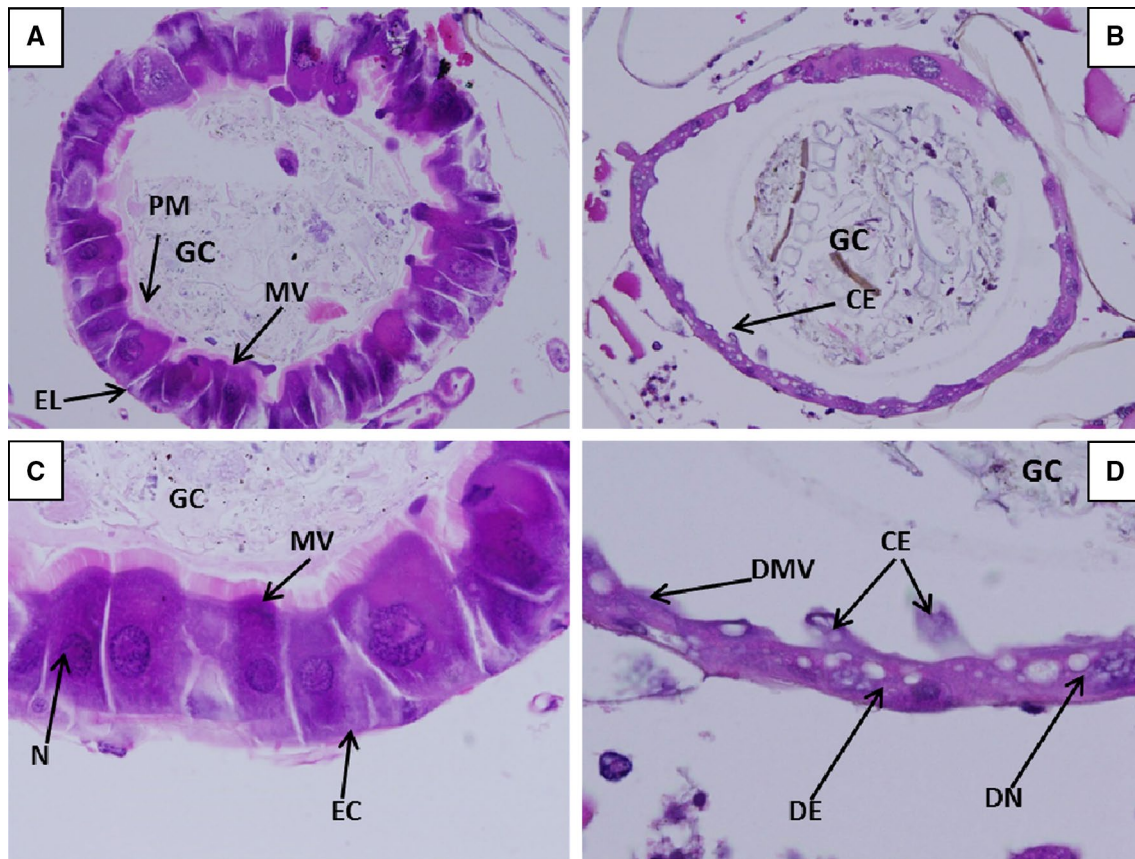
**Fig. 3** Photomicrograph of the midguts of the third instar larvae of *Aedes caspius*, maintained with food and treated with F1A fraction of *Cochliobolus spicifer*, 24 h after treatment. **a, c** Cross sections in midguts of untreated larvae, showing normal gut epithelial layer (*EL*) with healthy, normal epithelial cells (*EC*), peritrophic membrane (*PM*), microvilli (*MV*), nuclei (*N*), and nutritional gut contents (*GC*) filling the gut lumen. **b, d** Cross sections in midguts of treated larvae, showing affected gut epithelial layer, with cytoplasmic extensions (*CE*), degraded microvilli (*DMV*), degenerating epithelial cells (*DE*), degenerating nuclei (*DN*)

According to Yan et al. (2011), endophyte fungi have been shown to protect their hosts against insect pests, pathogens, and even domestic herbivores. These include *Aspergillus flavus* (Link) and *Penicillium sublateritium* (Biourge) (Webber 1981), which live and feed on the host plant and, in turn, produce functional metabolites that enhance its fitness and resistance against stresses (Rana et al. 1997). A number of species, including selected *Fusarium* sp., are reported to act as antagonists against plant pathogens. Accordingly, fungi have commonly been used in practice as inocula to improve the growth of plants and suppress pathogens (Alabouvette et al. 2009; Cao et al. 2002; Steinberg et al. 1997).

Histomorphological alterations in larvae treated with *C. spicifer* fraction, which are suggestive of the cause of death of the mosquito larvae, could be observed in the middle region of the intestine, with cellular destruction and disorganization, spacing between cells, and vacuolization of epithelial cells. Similar results were obtained in histological analyses of *Cx. nigripalpus* (Theobald) larvae infected by *Bacillus thuringiensis* (Berliner) medellin (Cry11Bb) (Moser et al. 2001) and in intestinal cells of larvae of *Ae. albopictus* (Skuse) infected with *B. thuringiensis* var. *israelensis* (Bti) (Silva et al. 2008). The authors reported as signs of infection the presence of rounded cells, cytoplasm with granules, clear or absent nuclei, and extensive cytoplasmic vacuolization of epithelial cells of the mesentery of these larvae. These histomorphological findings help us understand the toxicity of substances related to the site of action of *C. spicifer* fraction in mosquito larvae; the resultant tissue degradation hampers the survival of the larvae.

The active principle of this extract is not known; however, the safety profile of the extract in vertebrate could be attributed to its origin as a natural product. There is increasing interest in naturally derived insecticides since they do not pose a threat to animals or humans, in contrast to synthetically derived insecticides. The results reported here provide the possibility of using the endophytic fungi *C. spicifer* as a natural larvicidal product.
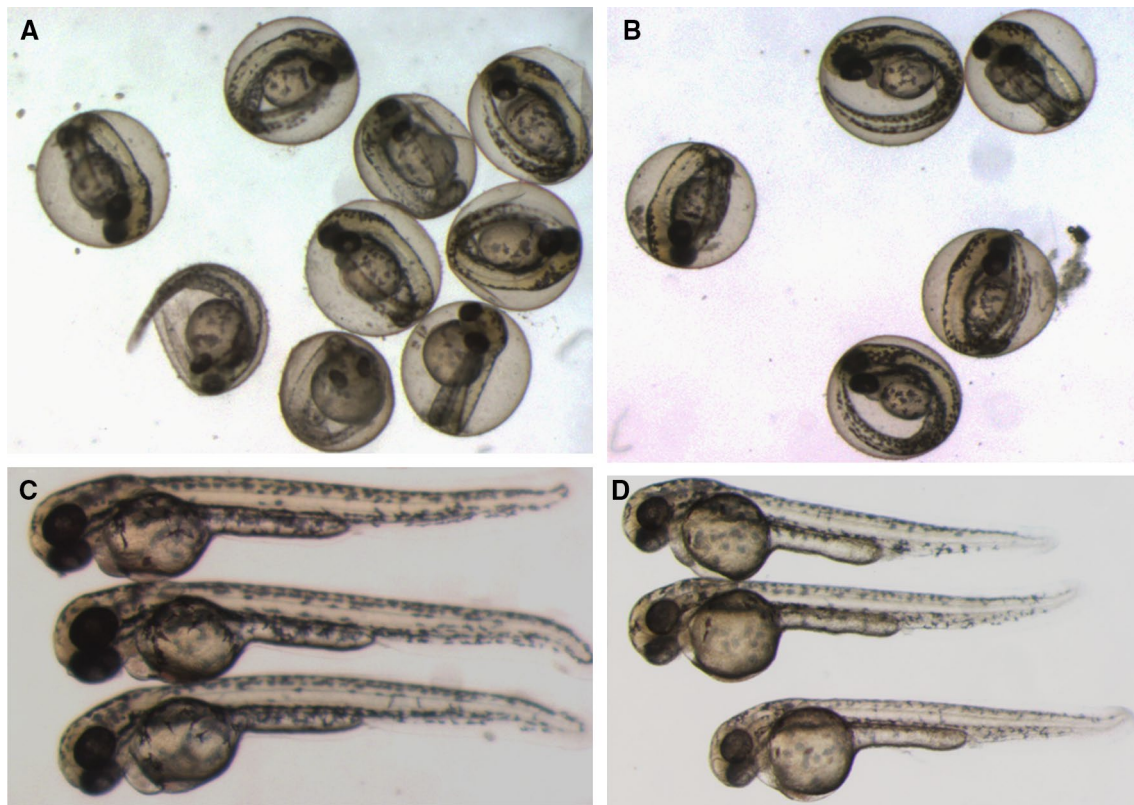
**Fig. 4** F1A fraction did not produce any teratological profile and mortalities in treated zebrafish embryos. Live images of zebrafish embryos at 48 hpf. Control (mock treated embryos) shown in chorion (**a**) and hatched (**c**). The zebrafish embryos exposed to 1 mg/ml of F1A fraction at shield stage (at 6 hpf) and photographed at 48 hpf (**b**, **d**). The treated embryos did not show any obvious developmental defect

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