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Research paper

First record of *Aspergillus oryzae* as an entomopathogenic fungus against the poultry red mite *Dermanyssus gallinae*

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ABSTRACT

The poultry red mite, *Dermanyssus gallinae*, is a blood-feeding ectoparasite that affects egg-laying hens worldwide. Strategies to control this parasite have focused in the use of entomopathogenic fungi, such as *Metarhizium anisopliae*. However, only a few studies have evaluated the use of *Aspergillus oryzae* to control *D. gallinae* and none of them have employed native strains. In the work presented here, a novel entomopathogenic fungus was isolated from a dead *D. gallinae*. The results of phylogenetic analysis showed 100% similarity between the isolated strain and those of two species, *A. oryzae* and *Aspergillus flavus,* and 99.82% similarity with *A. parvisclerotigenus*, which were in the same branch of the *Flavi* section of the genus *Aspergillus*. This entomopathogenic fungus was a non-aflatoxin B1 producer, as shown by the presence of aflatoxin B1 in the conidial infection suspension. Morphological features of fungus in comparison with *A. oryzae* and *A. flavus* indicated that the isolated strain belonged to *A. oryzae*, and was named *Aspergillus* sp. Dg-1. The pathogenicity of *Aspergillus* sp. Dg-1 on *D. gallinae* at different life stages was then assessed under laboratory conditions. The experiments showed that the isolated strain significantly increased the mortality rate in adult mites, up to 24.83 ± 2.25, compared to the mortality rates in the control group, which were 15.17 ± 2.75 ($P < 0.05$). However, *Aspergillus* sp. Dg-1 did not have pathogenic effects on the second nymph stage of *D. gallinae*. Our findings demonstrate that *Aspergillus* sp. Dg-1 has pathogenic effects on *D. gallinae* in their adult stage, presenting biocontrol potential against *D. gallinae*.

1. Introduction

The poultry red mite (PRM), *Dermanyssus gallinae*, is an increasingly important pathogen in egg-laying hens in the worldwide, containing the Europe, Japan, United States, and China (Chauve, 1998; Wang et al., 2010). Recently, an epidemiological review has shown that 83% of the farms in Europe were infested by *D. gallinae*, with a prevalence of up to 94% in Germany, the Netherlands and Belgium (Flochlay et al., 2017). This extensive *D. gallinae* infestation has caused great concerns regarding its effects on birds' health and welfare. Severe infestations of *D. gallinae* can lead to very serious results such as anaemia, cannibalism and even death in some cases (Sparagano et al., 2014). In serious cases a laying hen can lose more than 3% of its blood volume every night (Emous, 2005). The remarkable economic losses caused by *D. gallinae* infestations are a consequence of the reduction in egg production and quality (Cosoroaba, 2001). Furthermore, it has been reported that *D. gallinae* may serve as a vector for a number of viral and bacterial pathogens in humans and animals, including the Western, Eastern, and Venezuelan equine encephalomyelitis viruses, the paramyxovirus that causes Newcastle disease, avian influenza A virus, and bacteria such as *Erysipelothrix rhusiopathiae* or *Escherichia coli* (Majowicz and Jscallan, 2010; Moro et al., 2011, 2009; Sommer et al., 2016).

D. gallinae suck blood mainly at night for a short period, from a few minutes to a maximum of two hours. In the rest of the time, mites reside in multi-stage colonies in cracks and crevices in the neighbourhood of its host, seeking shelter where out of reach so that chickens cannot peck and eat them, digesting its blood meal, mate and lay eggs. Their location off the host and the ensuing migratory behavior has led to the difficulty in the control of *D. gallinae*. Many controls against *D. gallinae*, including the use of silica dusts and chemical acaricides, are often applied for the control of a range of farmyard pests. It has reported that the resistance of *D. gallinae* to acaricidal drugs has emerged, such as

Abbreviations: D. gallinae, *Dermanyssus gallinae*; *A. flavus*, *Aspergillus flavus*; *A. parvisclerotigenus*, *Aspergillus parvisclerotigenus*; PDA, Potato Dextrose Agar; CIS, conidial infection suspension

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carbaryl, amitraz and permethrin (Beugnet et al., 1997; Zeman and Železný, 1985), which suggests that there is an urgent need to conduct the additional research to find more specific control strategies towards *D. gallinae*. Novel approaches for controlling *D. gallinae* include vaccines, plant-derived products and entomopathogenic fungi.

Recently, entomopathogenic fungi are currently used worldwide for their potential in the biological control of a wide range of arthropods pests (Kaoud, 2010; Steenberg and Kilpinen, 2014). In particular, *Lagenidium* spp., *Metarhizium* spp., *Beauveria* spp., *Conidiobolus* spp. and *Aspergillus* spp. have been widely tested against different pests, including mosquitoes, aphids, flies and *D. gallinae*. (Shah and Pell, 2003). Among entomopathogenic fungi, numerous insect species has been reported to be infected by *Aspergillus* spp. For example, *Aspergillus flavus* can infect many insects, such as the silkworm (*Bombyx mori*), the German cockroach (*Blattella germanica*), the desert locust (*Schistocerca gregaria*) and some coconut pests (Gupta and Gopal, 2002; Kulshrestha and Pathak, 1997; Venkatesh et al., 1975). Additionally, *A. oryzae* has been used in oriental food fermentation processes over the past 2000 years, and has potential as a biocontrol agent for locust (Zhang et al., 2015). In particular, *D. gallinae* appears susceptible to the infection by fungal isolates of *Beauveria bassiana*, *M. anisopliae*, *Trichoderma album*, and *Paecilomyces fumosoroseus* when mites were inoculated with high doses of conidia under laboratory conditions (Kaoud, 2010; Tavassoli et al., 2011). Although some entomopathogenic fungi described above have been reported to act against *D. gallinae*, it is also necessary to find a new resource of entomopathogenic fungi for the development of biological control methods against *D. gallinae*. Furthermore, researchers found that the native strains (i.e., isolated from the environment or from naturally infected hosts) of *M. anisopliae* and *B. bassiana* showed more virulent against different species of ticks in comparison with non-native strains.

As shown next, using our previously reported rearing system to produce large quantities of *D. gallinae* in controlled laboratory conditions (Wang et al., 2018), a native strain of fungi was isolated from a dead *D. gallinae.* Molecular and morphological characterization of the strain showed that the isolated strain was *A. oryzae*, and that aflatoxin B1 was not produced. We also evaluated the *in vitro* effects of the isolated strain of *A. oryzae* on adults and nymphs of *D. gallinae*.

2. Materials and methods

2.1. D. gallinae used in the experiments

D. gallinae were taken from a laboratory culture, which was originally collected from an egg-producing poultry farm naturally infested by *D. gallinae* in Pinggu district of Beijing in China, named as CBP-1. After collection, they were grown in the laboratory by feeding on chicks in the artificial climate incubator (RXZ-500B-LED, Ningbo Jiangnan Instrument Factory, China) at a constant temperature of 30 °C and 75% relative humidity (RH). Mites were divided into two groups, nymphs (i.e., deutonymphs) and adults, and finally stored at 20 °C to be used in the bioassay experiments within 24 h after collection.

2.2. Fungi origin

The fungus was originally isolated from a dead *D. gallinae* adult (as shown in Fig. 1), collected in a trap tube in 2018, and re-isolated on Potato Dextrose Agar (PDA) medium. Based on a preliminary identification, which indicated that it belongs to the genus *Aspergillus*, the fungus was named as *Aspergillus* sp. Dg-1. Single spore colonies were incubated at 28 ± 1 °C for 10 days. Spores obtained from the first subculture were used for the bioassays. The *Aspergillus* sp. Dg-1 strain was maintained on PDA and kept at 4 °C.

2.3. Amplification and sequencing of ITS region

The molecular identification of the isolated strain was carried out following a previously described PCR method. Briefly, the fungal isolate was cultured in 100 mL of sterile potato dextrose broth at 28 °C for 96 h. The mycelial mass was separated by filtration and freeze dried. DNA was extracted following the tissue protocol for the M5 Fungal Genomic DNA Kit (Azbiochem Co., LTD, Shanghai, China). The internal transcribed spacer (ITS) containing the region encoding the ITS-1, 5.8S rDNA and ITS-2 were amplified by PCR, and sequenced. The primers used were those described in previous studies (Zhang et al., 2015): forward primer (ITS-1: TCCGTAGGTGAACCTGCGG) and reverse primer (ITS-4: TCCTCCGCTTATTGATATGC), which amplified a 595-bp region. Polymerase chain reaction cycling conditions were as follows: initial denaturing at 95 °C was performed for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The PCR products were examined on 1.5% agarose gels, with a DNA ladder (Tiangen Biotech Co., LTD, Beijing, China). The amplified PCR products were sequenced by a commercial company (Sangon Biotech Co., LTD, Shanghai) using an ABI 3730XL DNA Analyzer. ITS sequence from *Aspergillus* sp. Dg-1 was edited using ChromasPro or ContigExpress (Vector NTI, Thermo Fisher Scientific, Waltham, USA). For ITS sequence, primer sites were manually removed to avoid sequence errors.

The genetic similarity of the isolated strain *Aspergillus* sp. Dg-1 to other sequences in the GenBank was determined by performing the BLAST searches (GenBank) using the rDNA sequences of the ITS regions. The nucleotide sequences of ITS were aligned using ClustalX and exported into MEGA6 software. Subsequently, the phylogenetic trees were constructed by the neighbour joining (NJ) method with the Kimura two-parameter (K2P) option. A bootstrap analysis was conducted with 1000 replications in the NJ analysis.

2.4. Morphological analysis

For macromorphological observations, the isolate was inoculated on a PDA plate at 28 °C and the colonies at different stages were observed and photographed. Two types of microscopes were used for micromorphological observations. The general morphological features of the colonies were observed under a stereomicroscope (SteREO-Discovery.V12, Zeiss Microscopy). Conidial heads, flask-shaped vesicles and spores were photographed using a microscope (Olympus, Japan).

2.5. Detection of aflatoxin B1

The presence of aflatoxin B1 was detected by an enzyme linked immunosorbent assay. According to manufacturer's instructions provided by the aflatoxin B1 test kit (Weide Weikang Biotechnology Co., LTD, Beijing, China), the extraction and detection of aflatoxin B1 were conducted.

2.6. Laboratory bioassay on D. gallinae

The conidial infection suspension (CIS) of *Aspergillus* sp. Dg-1 was obtained by culturing the strain on PDA at 28 °C for 3 weeks. It has been reported that the mortality of *Locusta migratoria* caused by *A. oryzae* was dose-dependent (Zhang et al., 2015). Thus the highest concentration of *Aspergillus* sp. Dg-1 that could be prepared (1×10^9 conidia/mL, based on preliminary study), was chosen to evaluate the effects of fungi on mites. Conidia were harvested by adding sterile distilled water (dH_2O) containing 0.1% Tween 80 to the plates and washing the plates. The suspension containing conidia was collected, and the turbidity of suspension was adjusted spectrophotometrically (Biosan DEN 1) to an optical density of 10 McFarland (1×10^9 conidia/mL), as reported. Then, the amount of conidia used in the experiment was evaluated by quantitative plate counts of CFU/mL in PDA.

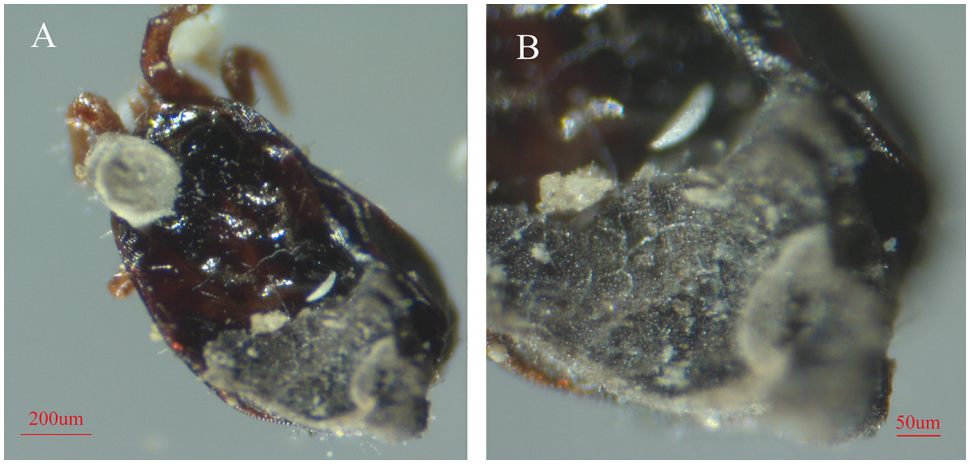

Fig. 1. A dead *D. gallinae* adult infected by fungi originally isolated from the incubator. Scale bar of A = 200 μm; Scale bar of B = 50 μm.

Table 1
Laboratory bioassay data in treated (TG) and control (CG) groups.

Groups	Life stage	Number of individuals	Sex*	Exposure dose of fungi	Temperature and relative humidity	Duration of exposure
TG	Adult	20	Female	1×10^9 conidia/mL CIS	25 ± 1 °C, 80 ± 5% RH	10 days
	Nymph	20	–	1×10^9 conidia/mL CIS	25 ± 1 °C, 80 ± 5% RH	10 days
CG	Adult	20	Female	dH_2O plus 0.1% Tween 80	25 ± 1 °C, 80 ± 5% RH	10 days
	Nymph	20	–	dH_2O plus 0.1% Tween 80	25 ± 1 °C, 80 ± 5% RH	10 days

* Both adults and nymphs were tested in the bioassays. Specifically, females were used in the adult subgroup, while sex was not determined in the nymph subgroup due to the difficulty of sex discrimination in nymphs. The experiments were done in triplicate.

A total of 120 adults and 120 nymphs were tested in the bioassays, which consisted of two groups of *D. gallinae*, one treated group (TG) (CIS of 1×10^9 conidia/mL) and one control group (CG). Each group was composed of two subgroups with twenty mites (i.e., adult and second nymph mites) (Table 1). Specifically, females were used in the adult subgroup, while sex was not determined in the nymph subgroup due to the difficulty of sex discrimination in nymphs (Mul et al., 2009). The experiment was carried out according to a previously described method (Immediato et al., 2015). Briefly, mites were placed into the bioassay chambers composed of Petri dishes (60 mm diameter) which includes filter paper of the same diameter. The filter paper was soaked with 0.2 mL of CIS (1×10^9 conidia/mL) in the treated group and with 0.2 mL of sterile distilled water plus 0.1% Tween 80 in the control group. The mites were placed on the filter paper soaked with either CIS or sterile distilled water plus 0.1% Tween 80. After that, the bioassay chambers were covered with a lid, sealed with parafilm to avoid the escape of mites, and then incubated at 25 ± 1 °C and 80 ± 5% RH. There were three replicates for each treatment. For the duration of the experiment, the mites were continuously exposed to *Aspergillus* sp. Dg-1 in the bioassay chambers. The observation period was 10 days, following a previously described method (Tavassoli et al., 2008). Therefore, the mortality of *D. gallinae* was recorded and evaluated daily for 10 days in CGs and TGs. Mites were considered as dead if they showd no reaction and movement after the repeated mechanical stimulation by three different examiners with an entomological pin. In each group the dead mites was cultured on the PDA medium to verify the presence of viable fungus. The death caused by fungal infection was checked according to Koch's postulate (Immediato et al., 2015).

2.7. Statistical analysis

All experiments were repeated three times. The mortality rates of *D. gallinae* between control groups and treated groups at 10 days were compared using Student's *t*-tests, with 5% significance ($P < 0.05$). The analyses were performed using IBM SPSS statistics 20.0.

3. Results

3.1. Amplification and sequencing of the ITS region

Molecular identification of the isolated strain was conducted using the phylogenetic analysis. The size of the amplified PCR products of ITS was confirmed by agarose gel electrophoresis, which was consistent with the expected sizes of the ITS (595 bp) (Fig. 2A). The ITS rDNA region of *Aspergillus* sp. Dg-1 was sequenced and then the ITS sequence was deposited in the GenBank database with the accession number MK674278. Before performing Blast search in Genbank, primer sites were manually removed to avoid sequence errors. In the ITS alignment, the ITS sequence of *Aspergillus* sp. Dg-1 was compared with those available sequences in the GenBank using BLAST. The results indicated that the ITS sequence of *Aspergillus* sp. Dg-1 was 100% similar to those of several strains belonging to the two species *A. oryzae* (i.e. MK120548.1) and *A. flavus* (i.e. MK087749.1), and was 99.82% identical with *A. parvisclerotigenus* (i.e. KY689161.1).

The NJ tree was built by using MEGA6 software with the ITS sequence of *Aspergillus* sp. Dg-1 and the ITS sequences of accepted species within the *Flavi* section of the genus *Aspergillus* in the GenBank (Varga et al., 2011). As shown in Fig. 2B, the phylogenetic tree and the estimated evolutionary divergence of *Aspergillus* sp. Dg-1 in comparition with top matches from results of BLAST indicated that *Aspergillus* sp. Dg-1 is closely related to the strains of *A. oryzae*, *A. flavus* and *A. parvisclerotigenus*. According to the results of BLAST searches and the phylogenetic tree, *Aspergillus* sp. Dg-1 might be a strain of one of these three species: *A. oryzae*, *A. flavus* and *A. parvisclerotigenus*.

3.2. Aflatoxin B1 detection and morphological identification

Aflatoxin B1 was not detected in the conidial infection suspension of *Aspergillus* sp. Dg-1 (data not shown), indicating that *Aspergillus* sp. Dg-1 is a non-aflatoxin B1 producer. For this reason, *Aspergillus* sp. Dg-1 was not considered a strain of *A. parvisclerotigenus*, which is a typical

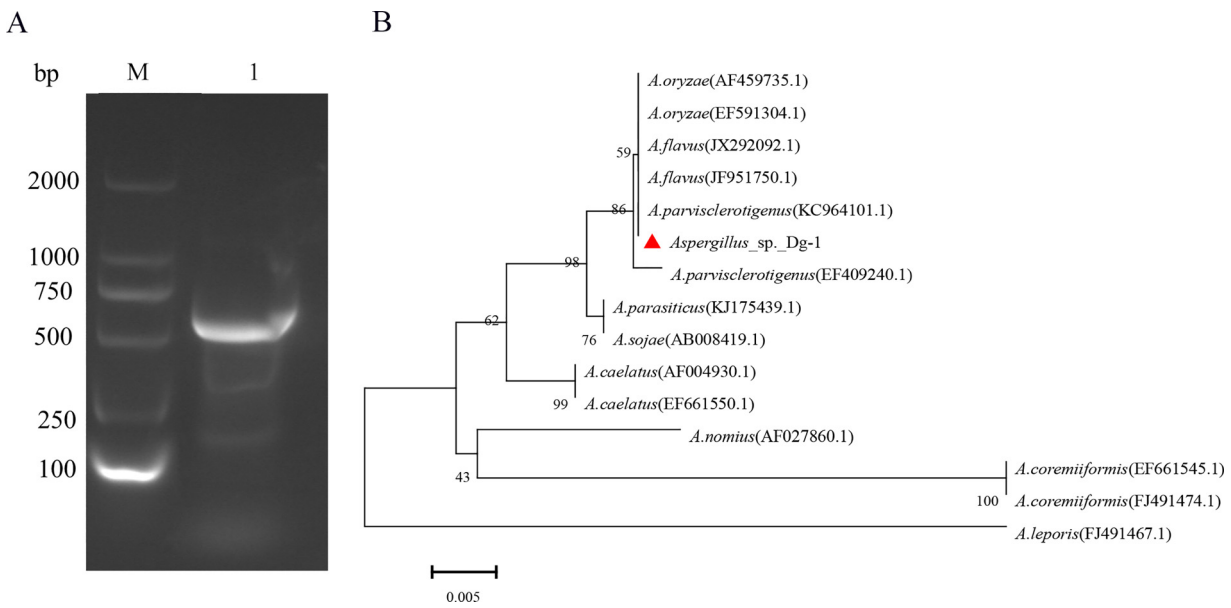

Fig. 2. PCR products of ITS (A) and neighbour-joining tree based on ITS sequence data of *Aspergillus* sp. Dg-1 (B). A. PCR amplicons of ITS in *Aspergillus* sp. Dg-1. M: 2000 bp DNA ladder; 1: ITS gene. B. Numbers above branches are bootstrap values and numbers after each strain indicate the GenBank accession numbers. Only values above 70% are indicated.

aflatoxin B1 producer.

The morphological analysis of the isolated strain was then constructed to make sure whether *Aspergillus* sp. Dg-1 belonged to *A. oryzae* or *A. flavus*. *Aspergillus* sp. Dg-1 grew on PDA at 28 °C for 10 days and the colony exhibited light-yellow and olive-green (Fig. 3A). We observed that the aerial hyphae had directly grew out of the PDA medium (Fig. 3B). The conidiophores stipe was finely roughened and hyaline, and the conidial head was subglobose to loosely columnar (Fig. 3C and D). Most of the surface of the flask-shaped vesicles was shown to be fertile (Fig. 3C). What's more, metula were not evident and the conidia grew directly on the phialide in chain-like conformations (Fig. 3E). The conidia were globose to subglobose with smooth outer walls, and the diameter was 3.5 to 5 μm (Fig. 3F). Comparing the important morphological features from *A. flavus*, *A. oryzae* and *Aspergillus* sp. Dg-1, the morphological features of the isolated strain suggests that *Aspergillus* sp. Dg-1 is likely a strain of *A. oryzae*.

3.3. Laboratory bioassay on D. gallinae

Spores of *Aspergillus* sp. Dg-1 covering mites and the surface of the

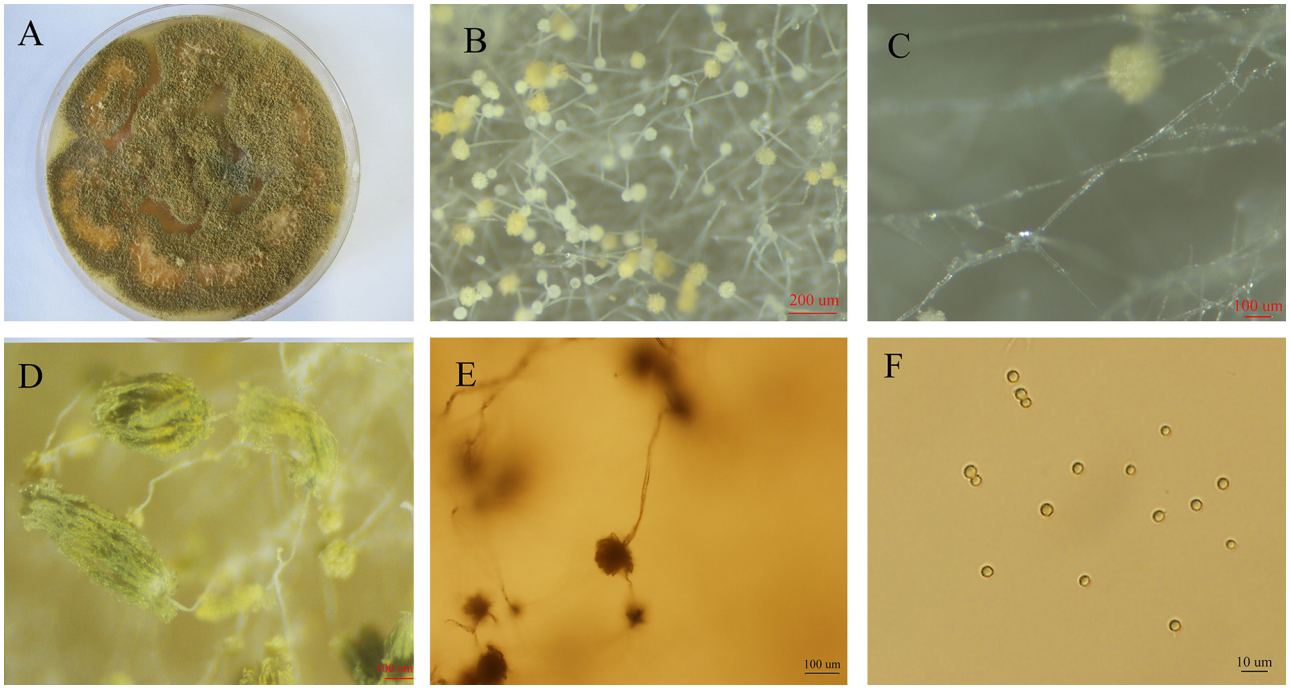

Fig. 3. Morphology of *Aspergillus* sp. Dg-1. *Aspergillus* sp. Dg-1 grown on PDA medium at 28 °C for 3 days (B and C) or 10 days (A and D-F). A. Colonies with a dark yellow-green color. B. Aerial hyphae directly grew out of the PDA medium. C. The conidiophores stipe was hyaline and finely roughened. D and E: Different types of conidial heads and flask-shaped vesicles. F. Conidia with a smooth outer wall. Scale bar of B = 200 μm; Scale bar of C–E = 100 μm; Scale bar of F = 10 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

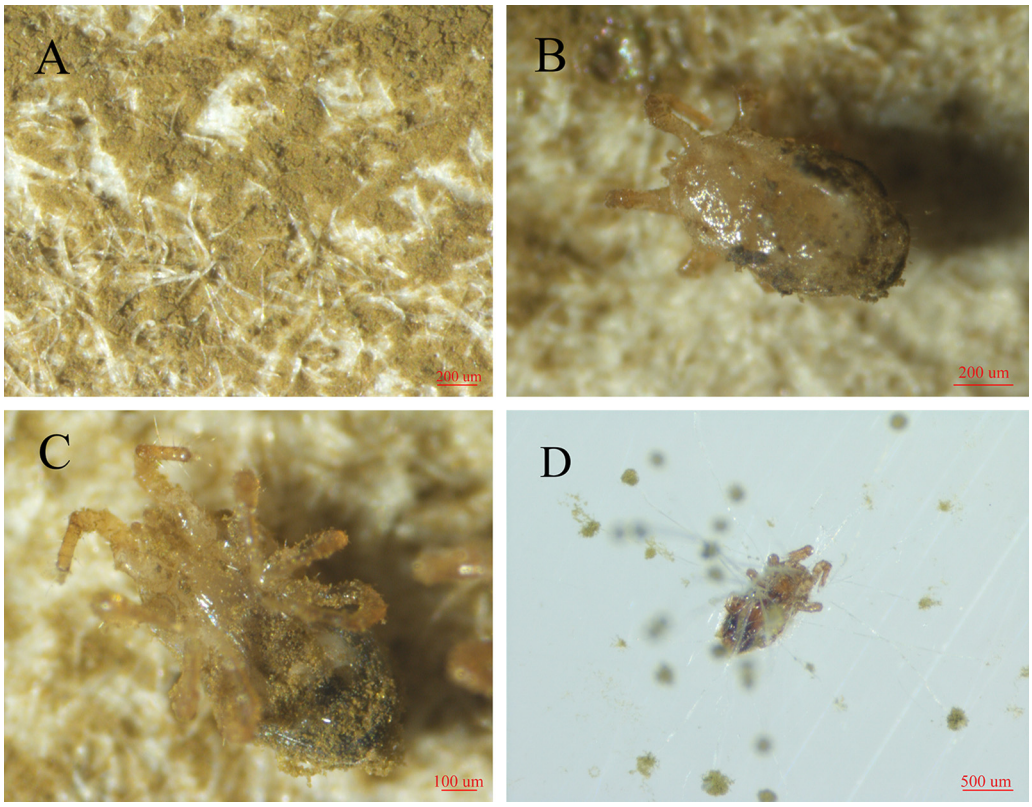

Fig. 4. Spores of *Aspergillus* sp. Dg-1 covering mites and the surface of filter paper. The spores on the filter paper and mites were observed under a stereomicroscope. A. The spores on the filter paper. B. The spores adhered to the back of adult mites. C. The spores adhered to the abdomen of adult mites. D. The dead mite from treated group cultured on PDA. Scale bar of A and B = 200 μm; Scale bar of C = 100 μm; Scale bar of D = 500 μm.

filter paper can be seen in the pictures in Fig. 4. Fungal spores of *Aspergillus* sp. Dg-1 spread evenly over the filter paper (Fig. 4A). We found that many spores adhered to the abdomen and back of adult mites (Fig. 4B and C), as well as deutonymphs (data not shown), providing the opportunity for the fungus to act against the mites.

The *in vitro* effect of *Aspergillus* sp. Dg-1 against nymphs and adults of *D. gallinae* is presented in Figs. 4D and 5 . The mortality rates of adults and nymphs in all groups increased after the treatment (Fig. 5A). More specifically, the mortality rates of adults in the treated group were higher than those in the control group from 2 to 9 days after infection, but there was no statistically significant difference. However, as shown in Fig. 5B, the mortality rates of adult mites 10 days after infection in the treatment group (24.83 ± 2.25%) were significantly higher ($P < 0.05$) than those in the control group (15.17 ± 2.75%). The mortality rates of deutonymphs 10 days after infection in the treated group were 20.33 ± 2.52%, which were slightly higher than those in the control group, with 19.67 ± 2.52%, but not significantly different, indicating that *Aspergillus* sp. Dg-1 did not have a significant killing effect on the second nymph stage of *D. gallinae*. Furthermore, adult mites showed a significantly higher susceptibility to *Aspergillus* sp. Dg-1 infection than deutonymphs. Every single dead mite from any of the groups was cultured on PDA. White fungal mycelium started to emerge on the surface of nymphs and adults of *D. gallinae* in the treated group on day 3. Fertile conidiophores began to appear at day 5, but only on the surface of dead mites (Fig. 4D). Under stereomicroscope observation and on culture, no fungal growth was observed on the mites in the control group. The results from this bioassay indicate that *Aspergillus* sp. Dg-1 had a pathogenic effect on *D. gallinae* in their adult stage.

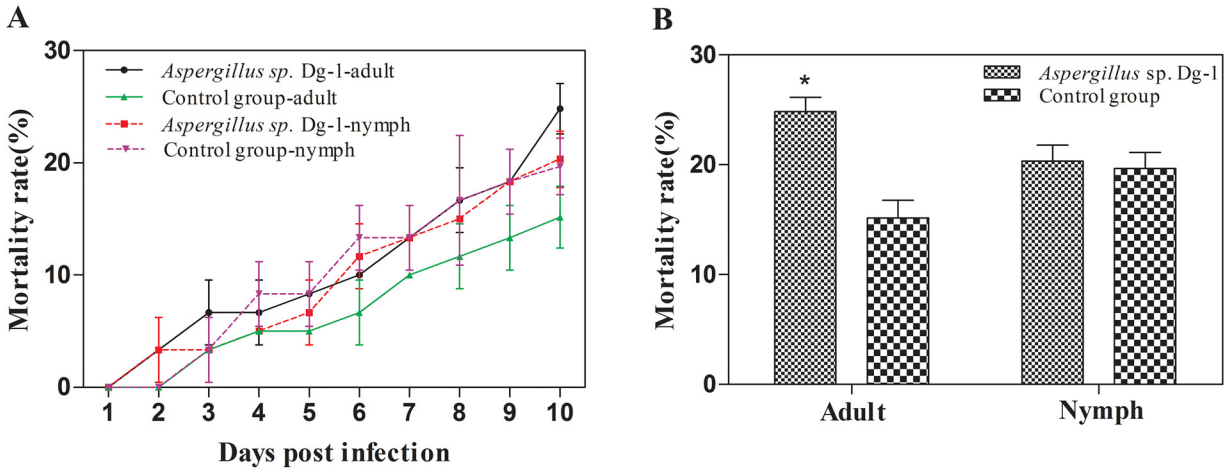

Fig. 5. Mortality rate of *D. gallinae* at different days after infection of *Aspergillus* sp. Dg-1 (A) and 10 days after infection (B). The bioassay chambers containing mites and fungi were incubated at 25 ± 1 °C and 80 ± 5% RH. The mortality of *D. gallinae* was recorded and evaluated daily for 10 days in CGs and TGs. All values are shown as mean ± SD from three independent experiments.

4. Discussion

With increasing resistance of *D. gallinae* to synthetic acaricides and changes in legislation and production practices affecting large areas of the globe, *D. gallinae* poses an ever-increasing threat to global poultry production. Effective control of *D. gallinae* is therefore potentially important not only in the poultry sector, but also in numerous other sectors, including human health. Biological control is commonly adopted in the control of pests in crops and forests. However, it has only recently begun to be developed for control of *D. gallinae*. It has been reported that *D. gallinae* is susceptible to infection by fungal isolates of *B. bassiana*, *M. anisopliae*, *T. album*, and *P. fumosoroseus* when mites are inoculated with high doses of conidia under laboratory conditions (Kaoud, 2010; Tavassoli et al., 2011). Nevertheless, finding new sources of entomopathogenic fungi is also necessary for the development of biological control agents against *D. gallinae*.

In the present study, conducted in controlled laboratory conditions, we designed a number of experiments to test the infectivity against *D. gallinae* of a novel "native" strain of a fungi isolated from a dead mite. The identification of the isolated strain was carried out by combining a comparative sequence analysis of ITS, morphological analysis and aflatoxin B1 detection. It has been found that the gene of ITS1-5.8S rDNA-ITS2 was an appropriate site for the identification of *Aspergillus* strains, however it could not provide enough sensitivity to distinguish individual species (Balajee et al., 2007). Morphology and physiology analyses are both traditional methods in the identification of fungi; however, neither physiological nor morphological characteristics alone can reliably distinguish closely related species (Klich et al., 2001). Generally, several combined methods are used in fungal identification (Geiser et al., 2007). In the present study, the results of molecular identification indicated that *Aspergillus* sp. Dg-1 is likely a strain of *A. oryzae*, *A. flavus*, or *A. parvisclerotigenus*. Based on the general morphological features, we conducted that *Aspergillus* sp. Dg-1 belongs to the *Flavi* section of the genus *Aspergillus* (Samson and Pitt, 1985), and this was in accordance with the results of molecular identification. Thus, *Aspergillus* sp. Dg-1 may be *A. flavus* or *A. oryzae*. There are some important phenotypic differences between *A. oryzae* and *A. flavus*. However, several features, including colony colors, colony textures and conidia surfaces were consistent with *A. oryzae.* Therefore, we considered that this novel entomopathogenic fungus of *D. gallinae* was likely a strain of *A. oryzae*.

The present study was performed to evaluate how effectively the isolated strain of entomopathogenic fungus *Aspergillus* sp. Dg-1 could be used against poultry red mite, *D. gallinae*, *in vitro*. The pathogenicity and bioassay data showed that, mortality of *D. gallinae* began on the 6th day after infection with *Aspergillus* sp. Dg-1. The cadavers of *D. gallinae* were put in the moist Petri dishes, and then in the following days the hyphal growth and sporulation of the fungus were emerged and observed. These results suggested that the "native" strain tested herein was virulent towards *D. gallinae*, therefore being of potential use for the control of this mite. It has been previously reported that the insecticidal mechanisms of entomopathogenic fungi include the failure of vital physiological functions of the pests caused by fungal toxins and the nutritional depletion induced by an increase of the hyphal mass in the insect's hemocoel (Bidochka et al., 2012). Generally, the host can be killed by the toxins produced by fungal within the first 3–5 days, and the cadaver decomposes by bacterial action, since the life cycle of fungus cannot be completed within such a short period of time (GökçE and Er, 2003). However, the nutritional depletion induced by extensive fungal growth requires a relatively long time with hyphae emerging through the intersegmental regions of the integument within 2 days after death under proper conditions (GökçE and Er, 2003). Consequently, it might be the depletion of nutrients and not the toxic secondary metabolites that produced by the *Aspergillus* sp. Dg-1 what might have caused the *D. gallinae*'s mortality. The pathogenicity of *Aspergillus* spp. to different insect species has been evaluated previously, indicating that this fungus has considerable potential as a microbial control agent for the management of pests. Two strains of *A. flavus* (VGCN9E and VGC2P) were effective against larvae of the mosquito *Aedes fluviatilis* (Schlein et al., 1985). Zhang et al., in a study on the sublethal effects of *A. oryzae* on third-instar locusts, revealed that the mortality was dose-dependent, and showed that *A. oryzae* XJ-1 may have biocontrol potential against locusts (Zhang et al., 2015). Therefore, the strain *A. oryzae* has potential as a biocontrol agent for this pest; though further studies will be necessary to systematical assess its full potential.

It is interesting to note that the adults showed a significantly higher susceptibility than deutonymphs to the infection of *Aspergillus* sp. Dg-1. The reduced mortality of nymphs, compared with adult stage mites, was also previously reported for *D. gallinae* treated with *B. bassiana* and for *R.sanguineus s.l.* treated with *M. anisopliae* or *B. bassiana* (Immediato et al., 2015; Samish et al., 2001). Recently, host species, developmental stage, age, nutritional status and sex have frequently been reported to affect the susceptibility of insect to fungal infection (Dimbi et al., 2003; Maniania and Odulaja, 1998), while feeding status and host age are among the most important physiological characteristics. In the current study, the feeding status of adults in the laboratory bioassay was post-blood feeding, and was the same for the deutonymphs. Thus, the difference in susceptibility of adults and deutonymphs to the fungus might be due to the host age. It is well known that the exoskeleton of acari is made of chitin, a tough and resilient polymer (Pritchard et al., 2015). In an unmodified state, chitin is translucent and comparatively flexible, often seen in the larval stage of *D. gallinae*. The polymerization of chitin is triggered by hormones, and mixed with various protein families and phenolic compounds, creating a sclerotized layer. Sclerotized cuticles can be identified by a brown/yellowish area, often covering the whole outer part of the adult body, and is replaced during each moulting stage, as it cannot be extended during mite growth (Pritchard et al., 2015). Therefore, along with the change in mite external morphology, the composition of cuticles in larvae, nymphs and adults might be different. The outer part of the *D. gallinae* exoskeleton, which known as the epicuticle, contains a layer of wax, which limits the loss of water, and a cement layer, which protects the cuticle from external abrasion (Pritchard et al., 2015). The difference of susceptibility of adults and deutonymphs might be due to a different composition of cuticle in nymphs and adults, thereby influencing the penetration of fungi. Indeed, the lipid composition of arthropod's cuticles selectively affects the germination of conidia and the formation of appressoria, which are important factors in the interactions between entomopathogenic fungi and their arthropod hosts (Cafarchia et al., 2015). What's more, the presence of exuvium at the nymphal stage might be able to influence the adhesion of conidia to the body surface of nymphs, thereby influencing and reducing the infection efficiency of *Aspergillus* sp. Dg-1 (Wu et al., 2014).

The limits of the currently used conventional control methods, especially the development of *D. gallinae* resistance to synthetic acaricides, is the main reason for the use of the entomopathogenic fungi to control *D. gallinae* (Chauve, 1998). However, the use of entomopathogenic fungi as control method also has some limitations, such as lower pathogenicity compared with chemical pesticides, and the need of specific storage temperatures of the fungi spores at low temperature (4 °C) (Jenkins et al., 1998). In addition, the activity of fungi requires specific environmental conditions (e.g., very high humidity or free water), which may limit their efficacy when deployed against *D. gallinae* in the field (Sparagano et al., 2014). As found in our study, fungi were able to affect *D. gallinae*, but the multiplication rate of the fungi was too low to reduce the *D. gallinae* population effectively, as previously reported for *D. gallinae* treated by *B. bassiana* (Immediato et al., 2016). As reported, both entomopathogenic fungi and essential oils (EOs) have potential for control of *D. gallinae*, with some limitations in their use: the acaricidal effect of EOs is rapid, but short-lived, while that of fungi is slow, but long-lasting. Davide Immediato et al. first

determined the non-toxic dose of *Eucalyptus globulus*, *Eucalyptus citriodora*, *Thymus vulgaris* and *Eugenia caryophyllata* essential oils for *B. bassiana*, and selected a 0.2% (v/v) concentration of *E. globulus* essential oil, which was then added to the CIS of *B. bassiana*, and their combined action against *D. gallinae* was evaluated by using laboratory bioassays. They found that the combination had greater efficacy compared to *E. globulus* or *B. bassiana* alone against the adult stage, and displayed an earlier acaricidal effect towards both nymphs and adults (Immediato et al., 2016). Therefore, the delayed effect of the fungi alone may be overcome by combining entomopathogenic fungi and essential oils (EOs). The effect of *Aspergillus* sp. Dg-1 in combination with EOs against *D. gallinae* requires further work, along with studies in practical applications in poultry farms.

5. Conclusions

The current study provides the first systematic report of the activity of a "native" isolated entomopathogenic fungus against *D. gallinae*. The fungus was identified as *Aspergillus oryzae*. The isolated strain was virulent against *D. gallinae* in the adult stage, and appears to be of potential use for the control of infestations caused by this mite. Further studies of systematically assessing its full potential in controlling *D. gallinae* are necessary to be conducted. Such studies should contain optimising the method of production, assessing the tolerance and stability of the conidia produced, and testing the efficacy of the fungi in combination with EOs against *D. gallinae* in the field, while assessing any potential effects on non-targeted organisms.

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