Phenotypic and molecular characterization of the causal agent of chafer beetle mortality in the wheat fields of the Kurdistan province, Iran

Keivan Karimi[1]*, *Mahdi Arzanlou*[1], *Asadollah Babai Ahari*[1], *Mostafa Mansour Ghazi*[2]

[1] Plant Protection Department, Agricultural Faculty, University of Tabriz, P.O. Box 5166614766, Tabriz, Iran
[2] Plant Protection Department, Agricultural and Natural Resources Research Center of Kurdistan, P.O. Box 714, Sanandaj, Iran

Abstract: We report the first case of chafer beetle [*Anisoplia austriaca* (Herbst 1783)] mortality caused by *Actinomucor elegans* var. *elegans* in wheat fields of the Kurdistan province, Iran. For three years, dead larvae of *Anisoplia austriaca* were collected from wheat fields of the Kurdistan province. Similar isolates of a fast-growing fungus were recovered from all samples. The fungal isolates were identified as *A. elegans* var. *elegans* based on morphological and cultural characteristics. The identity of the species was further confirmed using sequence data of the ITS (Internal Transcribed Spacer) region of ribosomal DNA. Koch's postulates were fulfilled by the inoculation of the larvae of *A. austriaca* and *Galleria mellonella* (Linnaeus, 1758) (as the model insect) using the spore suspension of *A. elegans* var. *elegans*. The viability of sporangiospores was evaluated using a spore dilution technique on germination medium. The results on the pathogenicity (100% mortality in *A. austriaca* larvae) and viability tests (germination: 95.45%) demonstrated that *A. elegans* var. *elegans* can be considered as a potential biocontrol agent against the chafer beetle. Field experiments are still required to evaluate the capacity of *A. elegans* as a biological control agent.

Key words: *Actinomucor elegans* var. *elegans*, biocontrol, chafer beetle, mortality

Introduction

Of all the cereals, wheat (*Triticum aestivum* L.) is the most valuable and strategic crop. Wheat serves as a staple food commodity in major parts of the world, especially in the Middle East region including Iran. Wheat production is affected by many biotic and abiotic factors. Diseases (e.g. rusts, fusarium head blight) and pests (e.g. sun pest) can cause a significant reduction of the yield.

Wheat chafer beetle [*Anisoplia austriaca* (Herbst, 1783)*, Scarabidae] is one of the important pests of wheat. As an adult, this beetle feeds on plant pollen or immature plant seeds, and as larvae feed on wheat roots (Jameson *et al*. 2007). These feeding practices result in yield losses. In general, scarab beetles are likely to increase in significance because of, for one, the decreasing availability of conventional chemical insecticides. Unfortunately, there are very few microorganisms commercially available that can effectively and economically replace the broad spectrum chemical insecticides used against these pests (Klein and Kaya 1995). Based on these facts, and due to the large beetle population, and wheat damage caused by the chafer beetle on wheat in the Kurdistan province, the aim of the present study was to survey the natural enemies of the chafer beetle in wheat fields in the Kurdistan province, Iran. Dead larvae of chafer beetle were frequently observed in wheat fields. The causal agent was characterized using morphological and molecular data. The pathogenic potential of this agent was further evaluated on healthy larvae of *A. austriaca* and *Galleria mellonella* (Linnaeus, 1758) in laboratory conditions.

Materials and Methods

Monitoring and microorganisms isolation

A monitoring operation was carried out from the beginning of March to the end of July during three years: 2011, 2012, and 2013, in fields. Multiple wells were excavated by shovel randomly, and dead larvae were collected (Fig. 1A, B, C) and taken to the laboratory. Samples were stored at –20°C until processing. For the isolation of potential pathogenic agents, frozen dead larvae were defrosted at room temperature for several minutes, and surface sterilised using 70% alcohol for 1.5 min. Then, the larvae were subsequently immersed in a 1% sodium hypochlorite solution for 30 s, rinsed in sterile distilled water three times, and dried on sterile paper towels. Potato Dextrose Agar (PDA, Fulka, Hamburg, Germany) and Nutrient Agar (NA, Merck, Darmstadt, Germany) media were used for the isolation of likely fungal and bacterial pathogens,
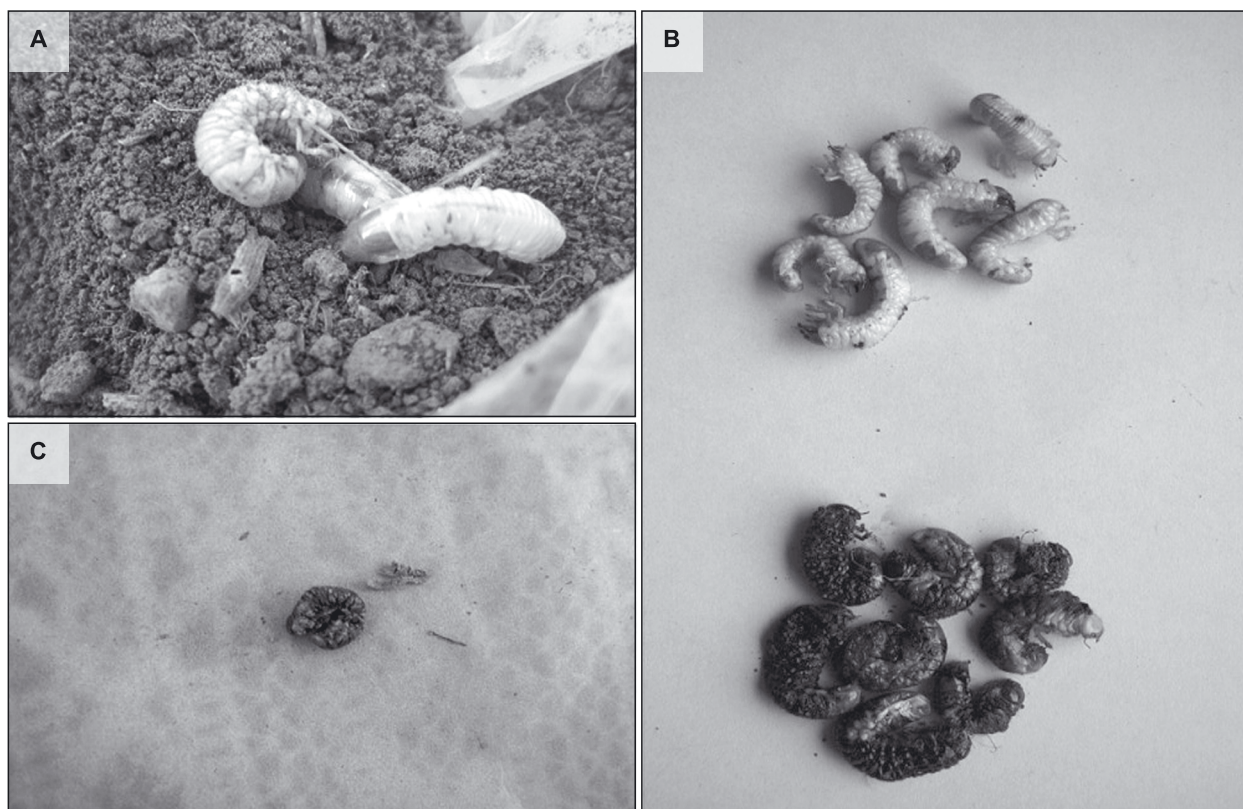
*Corresponding address:
Keivankarimi@tabrizu.ac.ir

Fig. 1. Chafer beetle mortality caused by *Actinomucor elegans* var. *elegans* in wheat fields of Kurdistan province, Iran: A – healthy larvae; B and C – naturally infected larvae of *Anisoplia austriaca* collected from wheat fields

respectively. For the isolation of the bacteria, disinfected larvae were squashed in sterile distilled water using a sterile scalpel. The resulted suspension was streaked on NA and cultures were incubated at 27°C for 48 h. For the isolation of fungal pathogens, disinfected larvae were divided into three parts: the head, chest, and abdomen, and cultured on PDA, then incubated for 48 h at 30°C. Pure cultures were deposited in the Culture Collection of Tabriz University (CCTU) for further investigations. Also the fresh culture of CCTU 1815 and CCTU 1816 isolates were deposited at the Iranian Fungal Culture Collection (IRIPP), Tehran, under collection number of IRAN 2361C and IRAN 2362C respectively.

Morphological description

Cultural characteristics including shape, color, and growth rate of the fungal colony were determined on different culture media including PDA, Czapek solution agar (Merck, Darmstadt, Germany), and Synthetic Nutrient Agar (SNA). Microscopic observations were made using the slide culture technique set up as explained by Arzanlou *et al*. (2007). For microscopic structures, 30 measurements were made per relevant microscopic feature at ×1000 magnification using an Olympus BX41 light microscope. For the measurements of each structure, 95% confidence intervals were derived. Photographs of microscopic fungal structures were captured using an Olympus digital DM-21 high definition color camera mounted on the above-mentioned light microscope.

Pathogenicity test (Koch's postulates)

Koch's postulates were fulfilled using spore suspension and injection methods as described by Cotter *et al*. (2000) and Sun and Liu (2008). For the spore-suspension method, the recovered fungus was grown on PDA for 4 days at 26°C. Spore suspension was obtained by flooding cultures with sterile water containing 0.05% Tween 80. Mycelium bodies and spores were separated by sterile glass wool. Spore concentration was adjusted at 1×10^6 spores $\cdot$ $\cdot$ ml^{-1}, by hemocytometer. Using forceps, the instar larvae of *A. austriaca* and *G. mellonella* (as the model insects) were dipped in spore suspension by forceps for 5 s, and then placed into 9 cm Petri dishes which contained moistened filter paper (Whatman No. 2). For *Galleria* larvae, an artificial diet (including flour 600 g, bee wax 60 g, yeast 150 g, honey 300 g, and glycerol 250 ml) was also added to the Petri dishes. The Petri dishes were sealed by parafilm and kept in darkness at 25°C. For the controls, instar larvae were dipped in sterile distilled water containing 0.05% Tween 80. For the injection test, 20 µl of spore suspension was injected into the haemocoel through the hindmost left proleg of *G. mellonella* larvae only (due to presence of prolegs) by a diabete syringe needle with a diameter of 0.23 mm (Avapezeshk, Tehran, Iran). For the control, distilled water was injected instead of spore suspension. An additional control, as untreated larvae, was also included. Mortality rate was recorded at 24 h intervals. Larval death was investigated by the lack of motion of larvae in response to stimulation. Each Petri dish contained five larvae.

DNA extraction, sequencing and analysis

Fresh mycelia were harvested from a five-day-old fungal colony grown on PDA. Genomic DNA was extracted using the protocol of Möller *et al*. (1992). Sequence data from the ITS (Internal Transcribed Spacer) region of ribosomal DNA was used to further confirm the identity of the causal agent. The primers V9G (de Hoog and van den Ende 1998) and LR5 (Vilgalys and Hester 1990) were used to amplify the 3′ end of the 18S rRNA gene, ITS1, 5.8S rDNA, ITS2, and the first approximately 900 bp of the 5′ end of the 28S nrRNA gene. For amplification, polymerase chain reaction (PCR) was performed on a Bio-Rad PCR System. The reaction mixture contained 1× PCR buffer, 1 mM MgCl$_2$, 60 µl of 1 mM dNTPs, 0.2 pM of each primer, 0.5 U of Taq polymerase, 0.5 µl DMSO, and 10–15 ng of fungal genomic DNA. The final reaction volume was adjusted to 15 µl by adding sterile distilled water. The thermal cycling condition consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, followed by a final extension cycle at 72°C for 7 min. The amplicon was sequenced using PCR primers and two additional internal primers, ITS4 (White *et al*. 1990) and LR0R (Rehner and Samuels 1994) in order to obtain good quality sequence over the entire length of the amplicon. A BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA) was used, and analyses were done on an ABI Prism 3700 (Applied Biosystems, Foster City, CA). The sequence for the ITS region was subjected to a Megablast search at NCBI's GenBank nucleotide database. Sequences with high similarity were downloaded from GenBank and aligned together with the sequence obtained in this study. Sequence alignment was carried out by using the ClustalW algorithm implemented in MEGA 5 (Tamura *et al*. 2011). Phylogenetic analysis was performed using the maximum likelihood method. A model of nucleotide substitution was selected using MEGA 5 software as Tamura 3-Parameters (T92) (Tamura *et al*. 2011). Bootstrap analysis was performed with 10,000 replicates. The phylogenetic tree was rooted to *Mucor heimalis* (GenBank accession number KF876829.1).

Viability of spores

The viability of fungal spores was determined by direct enumeration of the germinated spores on medium in Petri dishes containing 16 g agar, 0.5 g yeast extract, 0.1 g Tween 80, and 20 mg Benlate 50 WP per liter. The prepared medium was poured into 9 cm Petri dishes in a thin layer (< 5 mm). Spore suspension (100 µl) was poured and spread on the medium after incubation for 24 h at 26°C in the darkness. Several plugs of the medium were removed and a drop of lactophenol was added on the surface of each plug. The plugs were then covered with a cover slip. Germination of over 90% of the spores on the medium surface was considered as a viable culture (Costa *et al*. 2001). The germination percentage of spores was calculated using the following formula:

$$\% \text{ of germinated spores} = (x / x + y) \times 100,$$

where: x – germinated spores, y – ungerminated spores.

Results

Samples and isolates

In the first and second monitoring, three and eight infected larvae were collected from wheat fields, respectively (Fig. 1B). White, fast-growing fungal colonies grew from all of the samples on PDA culture medium after 2 days of incubation. No growth was observed on NA culture medium after one month of incubation. All of the isolates showed similar cultural and morphological characteristics. The isolate CCTU 1815 was selected for molecular identification and the pathogenicity test.

Morphological descriptions

Colonies on PDA and Czapek's agar fast-growing, filled a 9 cm diameter Petri dish in 4 days at 26°C. Woolly, floccose colonies forming abundant aerial mycelia, reached the lid of the Petri dish. But no growth was observed at 37°C. Colony colour on the surface was initially white to grey on both media, with age colour became cream to olive-buff on PDA, and grey on Czapek agar. Colony colour in reverse: white to pale olive-buff and often as cloud-like spots, this appeared to be as a result of rhizoid formation on the medium. Yellow oil droplets were recorded on aerial mycelia. Stolons (5 to 22 µm in diameter) and rhizoid were present (Fig. 2G). Branched rhizoids produced abundantly. Sporangiophore originated from stolons (without opposite rhizoid) or rhizoid (Fig. 2I), highly variable in length and width and ending to a sporangium or a set of sporangia. Sporangia formed as primary and secondary in a verticillate manner (Fig. 2B). Primary sporangia were bigger than secondary sporangia. Separation from secondary sporangia was by a cross wall and secondary sporangia separated from sporangiophore and each other by cross walls (Fig. 2B). Sporangia globose to subglobose, buff-color to bright brown, 13 to 75 µm in diameter (Fig. 2A), deliquescent or persistent and smooth or spiny, produced on branched and unbranched sporangiophores (Fig. 2C and F). Columellae present in different sizes and shapes, columella of the large spornagia elongate-oval to pyriform, hyaline to faintly coloured, columellae of the secondary sporangia globose to dorsiventrally-flattened. Collarette was often seen (Fig. 2E). Apophysis was not seen. Sporangiospores were smooth to slightly roughened, hyaline, globose to elliptical (3 to 8 µm diam) (Fig. 2H). Pleomorphic chlamydospores especially formed in older culture as intercalary single or in short chains, forming an inter-hyphal connection or sporangiophore upon germination (Fig. 2D). Zygospores were absent.

DNA extraction, PCR amplification and sequencing of the ITS region

After PCR and sequencing of the ITS region, a megablast search at GenBank database showed 100% similarity with the ITS sequences of other *A. elegans* (Accession numbers: FJ176396.1, AM745429.1) and one strain of *A. elegans* var. *meitauzae* (Accession number: AY492087.1). Next, Phylog-
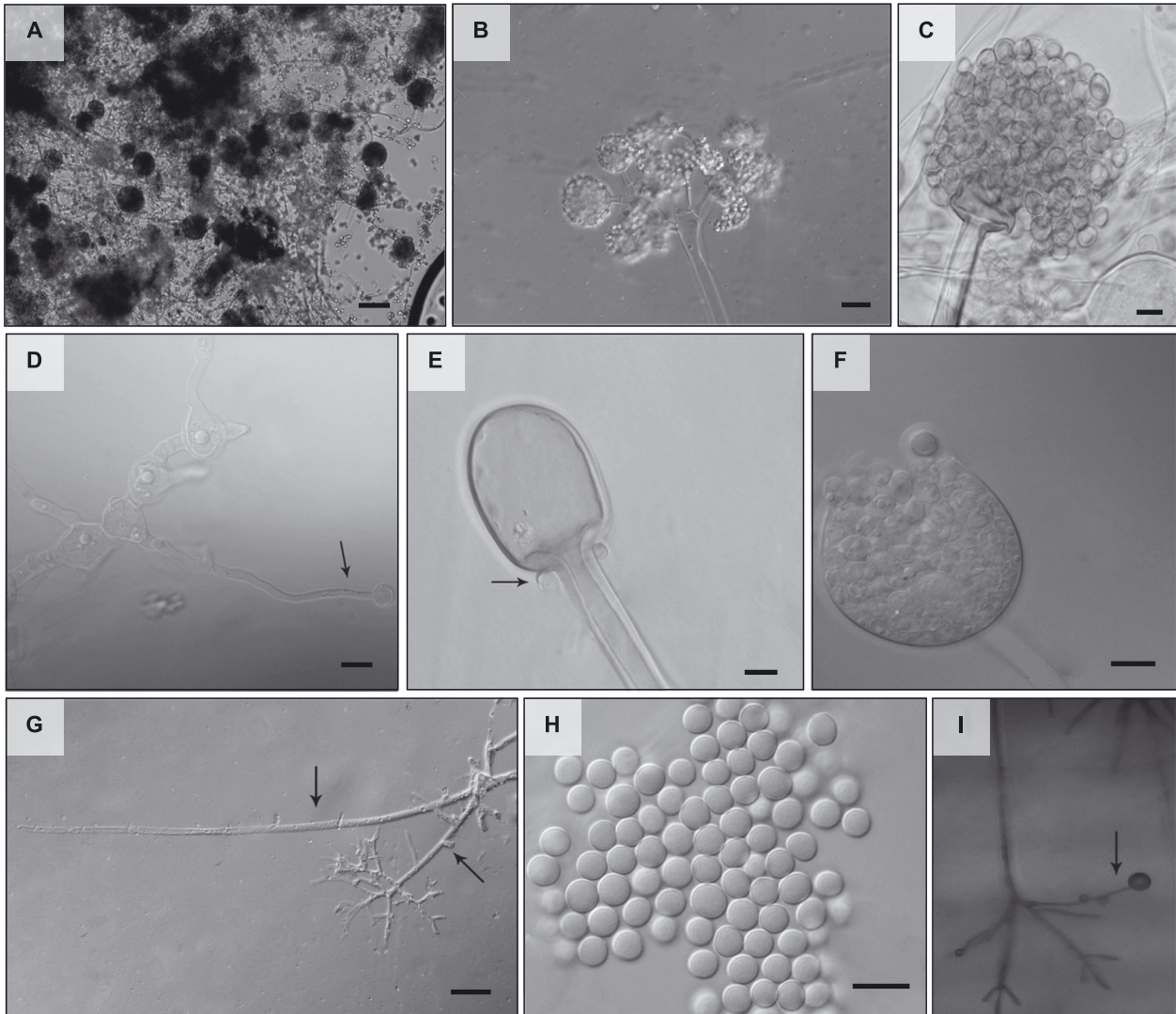
Fig. 2. *Actinomucor elegans* var. *elegans:* A – sporangia on PDA; B – primary and secondary sporangia along with sporangiophore; C – sporangium and columella; D – chlamydospores and branched sporangiophores originating from chlamydospores; E – columella and conspicuous *collarette* underneath (shown by arrow); F – ruptured sporangium along with released sporangiospores and visible columella; G – rhizoid (lower arrow) and stolon (upper arrow); H – sporangiospores; I – sporangium arising from rhizoid. Bars: A – 50 μm; B – 20 μm; C, D, E, F, H – 10 μm; G – 100 μm
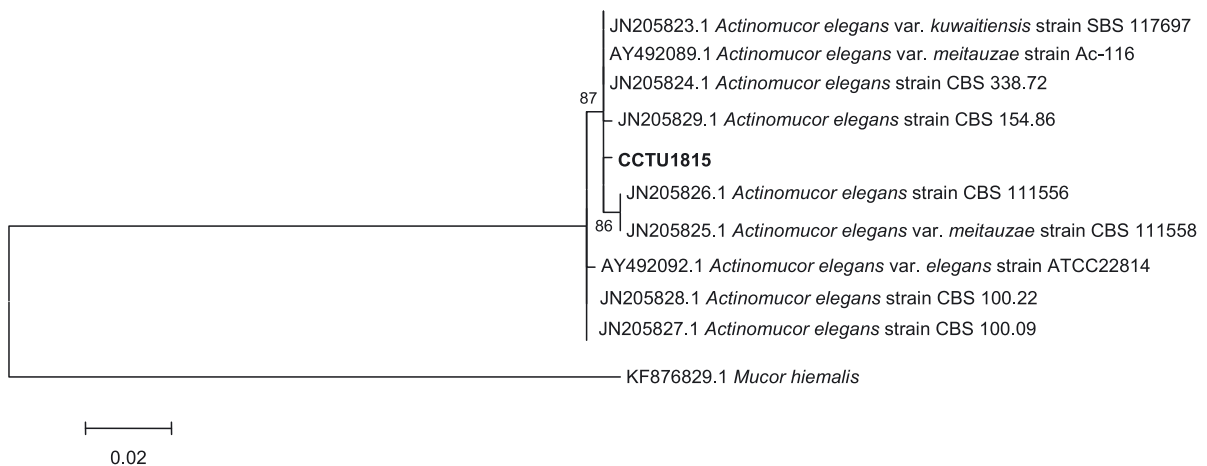

Fig. 3. A maximum likelihood phylogenetic tree based on sequence data from ITS-rDNA region. The scale indicates the number of substitutions per 100 nucleotides. Support for branches was assessed by bootstrap resampling of the data set with 10,000 replications. Tree was rooted by *Mucor hiemalis* (GenBank accession number KF876829.1). The sequences in boldface text (CCTU 1815) was generated in this study

Table 1. Pathogenicity assay of *Actinomucor elegans* var. *elegans* on instar larvae of *Anisoplia austriaca* and *Galleria mellonella*

Methods	*Anisoplia austriaca*			*Galleria mellonella*		
	dead	alive	MP	dead	alive	MP
Immersion method	5	0	100%	1	4	20%
Injection method	–	–	–	1	4	20%

MP – Mortality percentage
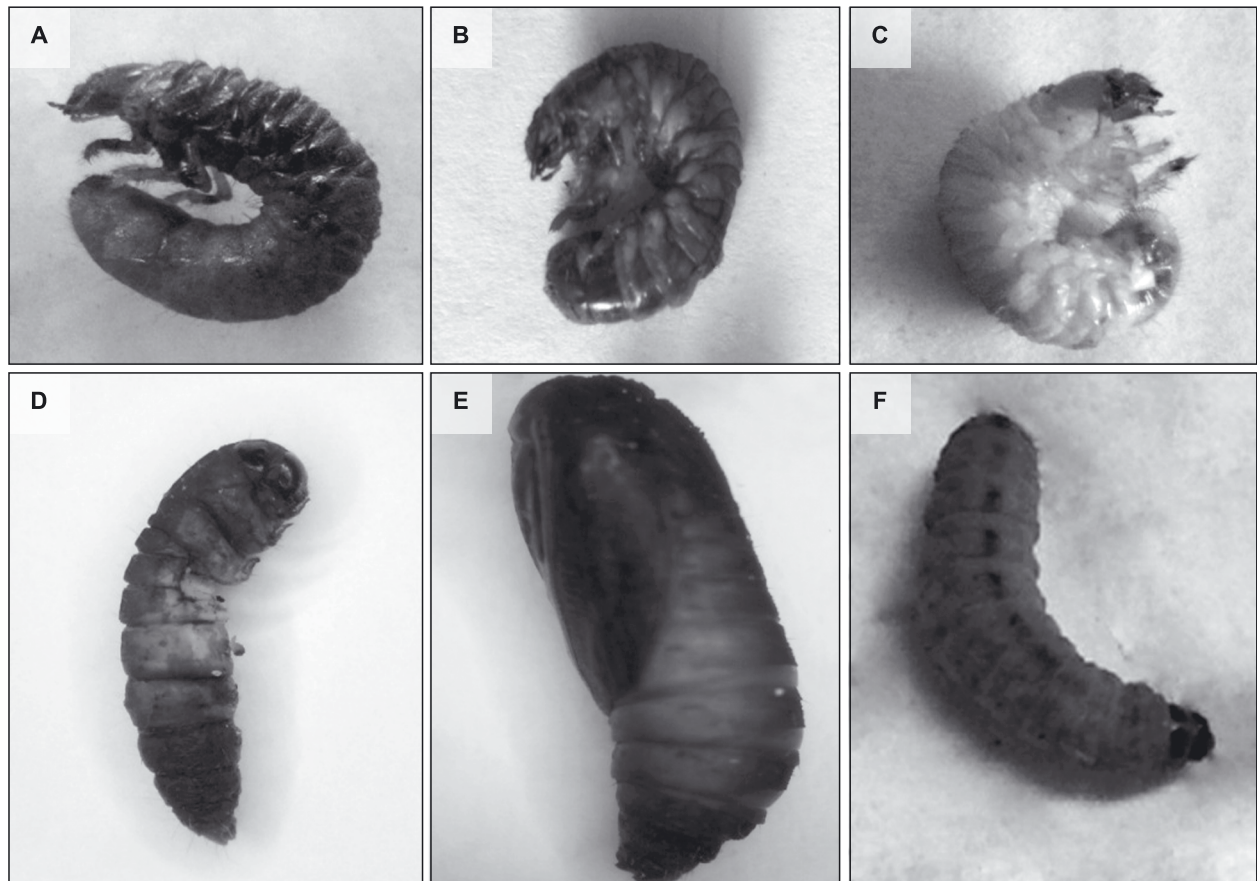
Fig. 4. Pathogenicity assay of *Actinomucor elegans* var. *elegans* on instar larvae of *Anisoplia austriaca* and *Galleria mellonella:* A and B – dead larvae of *A. austriaca* using spore suspension method; C – control (intact larva of *Anisoplia austriaca*); D – dead larva of *G. mellonella* using injection method; E – dead pupa of *G. mellonella* using spore suspension; F – control (intact larva of *G. mellonella*)

Table 2. Results of viability test on *Actinomucor elegans* var. *elegans*

Replications	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	G	UNG	G	UNG	G	UNG	G	UNG	G	UNG
Replication 1	33	3	29	1	21	1	22	–	24	2
Replication 2	29	–	30	–	21	2	25	–	32	2
Replication 3	28	2	21	2	23	–	28	–	24	4
MS	94.99%		95.98%		95.58%		100%		90.7%	
Total mean					95.45%					

G – Germinated; UNG – Ungerminated; MS – Mean per sample
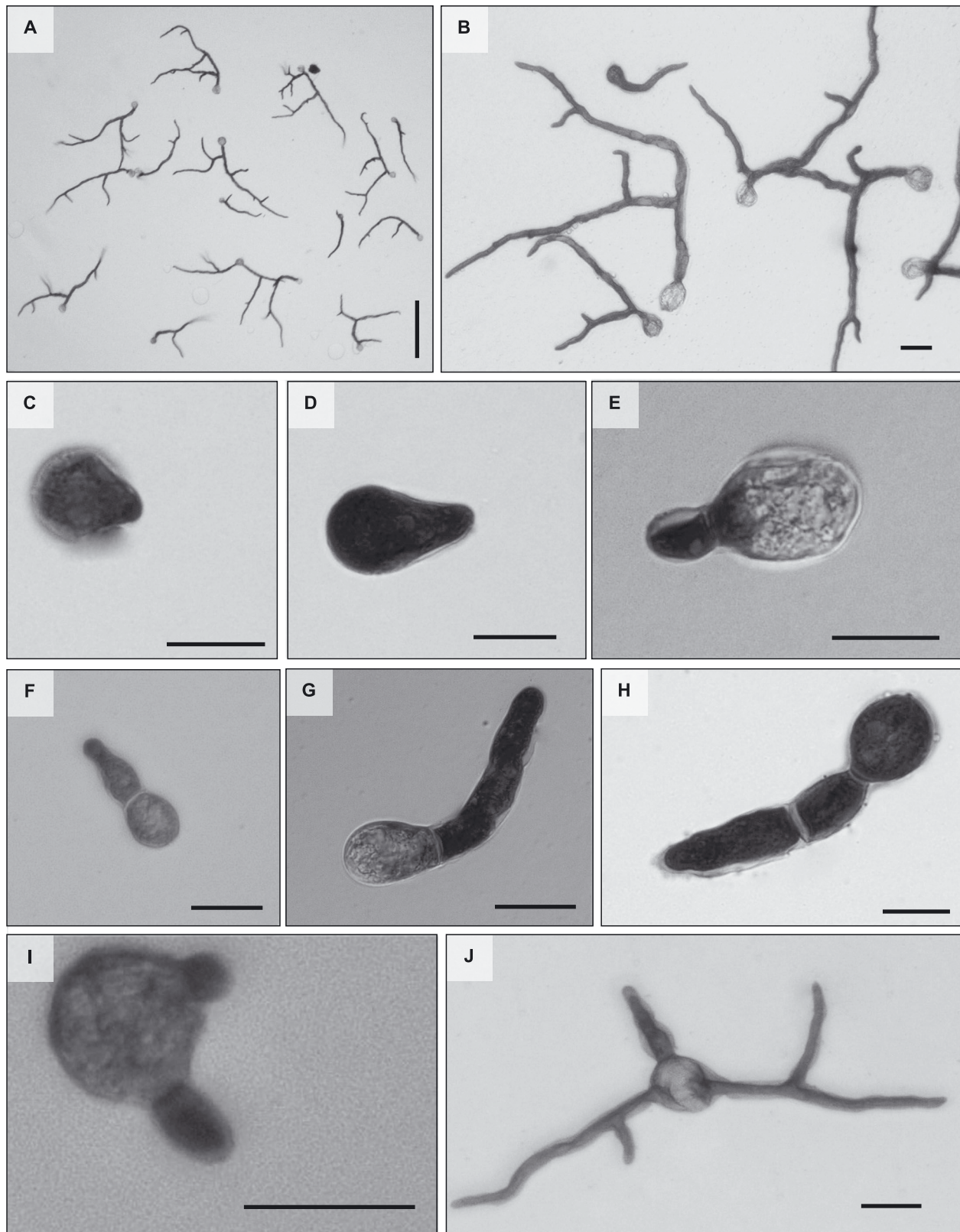
Fig. 5. Viability test of *Actinomucor elegans* var. *elegans* isolates: A and B – germinated sporangiospores after 16 h on yeast extract agar culture media; C–J – different patterns of sporangiospore germination among *Actinomucor elegans* var. *elegans* isolates. Bars: A – 100 µm; B, F, I and J – 20 µm; C, D, E, G and H – 10 µm

eny inferred using the sequence data of ITS region generated in this study, clustered our isolate together with other strains taken from Genbank including *A. elegans*, *A. elegans* var. *meitauzae*, and *A. elegans* var. *kuwaitiensis* in high support value (Fig. 3).

Pathogenicity assay

Treatment of *A. austriaca* larvae with spore suspension resulted in a 100% mortality rate 4 days after inoculation. However, the mortality rate for *G. mellonella* larvae was

lower than the mortality rate for *A. austriaca* in both suspension and injection methods. Only 20% of the larvae died after 12 days when the injection method was used and after 16 days when the suspension method was used (Table 1; Fig. 4). To satisfy Koch's postulates, the dead and inoculated samples in all of the treatments were cultured and *A. elegans* were isolated again from all the samples, except the controls.

Viability test

Germination of the sporangiospores was examined on yeast extract agar medium (Table 2). Our results revealed a viability mean of 95.45% (> 90%) for *A. elegans* isolates. Sporangiospores were enlarged and swollen nearly two to three fold (Fig. 5A to J) and some of them germinated in two or three directions (Fig. 5I and J).

Discussion

The results of the pathogenicity assay revealed that *A. elegans* was highly pathogenic on *A. austriaca* larvae while on *G. mellonella* larvae, *A. elegans* showed low pathogenicity. The lower mortality rate on *G. mellonella* larvae is intriguing, which might indicate that *A. elegans* isolates from *A. austriaca* are host specific. To support this hypothesis, new experiments have to be designed to further evaluate the pathogenicity of *A. elegans* on other insect species.

The morphological identification of *A. elegans* was further confirmed using sequence data from the ITS-rDNA region. Based on cultural and morphological characteristics, on PDA and Czapek's agar, the fungal isolates were identified as *A. elegans*. The observations were in full agreement with *A. elegans* features that were previously re-evaluated by Benjamin and Hesseltine (1957). ITS sequence data clearly differentiates *Actinomucor* from the other morphologically similar genera, such as *Mucor, Rhizopus, Rhizomucor*, and *Absidia*, yet, ITS region does not discriminate varieties of *A. elegans* from each other (Walther *et al*. 2013). In a phylogeny inferred from ITS sequence data, strains with the characteristics of var. *meitauzae* intermingled with other varieties of nearly all parts of the tree, and only a number of the strains belonging to var. *elegans* clustered together with the ex-type strain of var. *elegans* in a well-supported clade (Walther *et al*. 2013). A combination of morphological and cultural characteristics such as shape, size, ornamentation of the sporangiospores; ability to growth on Czapek's agar and maximum growth temperature (Zheng and Liu 2005; Khan *et al*. 2008) have been used to distinguish the varieties of *A. elegans*. On Czapek's agar, the var. *meitauzae* and var. *kuwaitiensis* show reduced growth. Contrary to earlier reports (Jong and Yuan 1985), maximum growth temperature is of little help in discriminating the varieties (Zheng and Liu 2005; Khan *et al*. 2008). The morphological features of our isolates were in agreement with the description of *A. elegans* var. *elegans*: growing on Czapek's agar, sporangiospores with a size ranging from 3 to 8 μm, and an inability to grow at 37°C.

One of the important aspects of biocontrol agents is their spore viability. Spore viability is an innate virulence factor that has a prominent role in determining the pathogenicity of fungal pathogens of insects. For example, reduced conidial viability of *Beauveria bassiana* has been correlated with the decrease in the mortality rate of the insect host, due to *B. bassiana* infection (Soetopo 2004). Since our isolates had spore viability higher than 90%, the cultures were considered viable (Milner *et al*. 1991) (Table 2). Moreover, the other criterion for a spore to be considered viable is the length of the germ tube being twofold of the spore diameter or spore swelling (Herlinda 2010). This characteristic was observed in our experiments (Fig. 5). High spore viability subsequently increases the probability of a viable spore contact by an insect pest when the activity of pest around its environment is after the application (Costa *et al*. 2001). Swollen or even strongly swollen sporangiospores were recorded in this study. This phenomenon has been observed and reported in some studies for sporangiiferous fungi such as *Rhizopus oryzae* and *Nothadelphia mortierellicola* (Waldorf and Diamond 1985; Degawa and Gams 2004).

Mucoralean fungi are ubiquitous, predominantly saprobic soil organisms on decaying organic material. Some species, though, are known to parasite plants, fungi, and animals (Hoffmann *et al*. 2013). *Actinomucor elegans* var. *elegans* is apparently ubiquitous and has been isolated from different hosts and substrates such as rhizosphere of various plants (oat, barley, corn, wheat, alfalfa, and hop), mouse and rabbit dung, nests, feathers and fecal of free-living birds (Phalip *et al*. 2006; Domsch *et al*. 2007). Moreover, *A. elegans* var. *elegans* along with *A. elegans* var. *kuwaitiensis* have been reported as the etiologic agent of mucormycosis in humans in several cases (Davel *et al*. 2001; Khan *et al*. 2008; Tully *et al*. 2009; Mahmud *et al*. 2012). Another variety, *A. elegans* var. *meitauzae* has industrial significance producing various enzymes such as proteolytic and lipolytic enzymes. This variety is frequently used for the production of popular fermented soybean foods including Sufu and Chao, and as a mould starter (Han *et al*. 2003). Given the results of this study, *A. elegans* var. *elegans* can be considered as a potential biocontrol agent against the chafer beetle, owing to its fast growing behavior, abundant production of propagules, and soil inhabitant nature. *Actinomucor elegans* has previously been isolated from dead bees and termites; however, there is no supplementary data available on the pathogenicity of *A. elegans* on these hosts (Batra *et al*. 1973; Zoberi and Grace 1990). To the best of our knowledge, this is first report on the occurrence and pathogenicity of *A. elegans* var. *elegans* on the chafer beetle.

References

Arzanlou M., Groenewald J.Z., Gams W., Braun U., Shin H.D., Crous P.W. 2007. Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera. Studies in Mycology 58: 57–93.

Batra L.R., Batra S.W.T., Bohart G.E. 1973. The mycoflora of domesticated and wild bees (Apoidea). Mycopathologia et Mycologia Applicata 49 (1): 13–44.

Benjamin C.R., Hesseltine C.W. 1957. The genus *Actinomucor*. Mycologia 49: 240–249.

Costa H.S., Robb K.L., Wilen C.A. 2001. Increased persistence of *Beauveria bassiana* spore viability under high ultraviolet-blocking greenhouse plastic. HortScience 36 (6): 1082–1084.

Cotter G., Doyle S., Kavanagh K. 2000. Development of an insect model for the in vivo pathogenicity testing of yeasts. FEMS Immunology & Medical Microbiology 27 (2): 163–169.

Davel G., Featherston P., Fernández A., Abrantes R., Cristina C., Rodero L., Sztern C., Perrotta D. 2001. Maxillary sinusitis caused by *Actinomucor elegans*. Journal of Clinical Microbiology 39 (2): 740–742.

de Hoog G.S., Gerrits van den Ende A.H.G. 1998. Molecular diagnostics of clinical strains of filamentous Basidiomycetes. Mycoses 41 (5–6): 183–189.

Degawa Y., Gams W. 2004. A new species of *Mortierella*, and an associated sporangiiferous mycoparasite in a new genus, *Nothadelphia*. Studies in Mycology 50 (2): 567–572.

Domsch K.H., Gams W., Anderson T.H. 2007. Compendium of Soil Fungi. IHW-Verlag, Eching, Netherlands, 43 pp.

Han B.Z., Ma Y., Rombouts F.M., Nout M.J.R. 2003. Effects of temperature and relative humidity on growth and enzyme production by *Actinomucor elegans* and *Rhizopus oligosporus* during sufu pehtze preparation. Food Chemistry 81 (1): 27–34.

Herlinda S. 2010. Spore density and viability of entomopathogenic fungal isolates from Indonesia, and their virulence against *Aphis gossypii* Glover (Homoptera: Aphididae). Trop Life Sciences Results 21 (1): 11–19.

Hoffmann K., Pawłowska J., Walther G., Wrzosek M., de Hoog G.S., Benny G.L., Kirk P.M., Voigt K. 2013. The family structure of the *Mucorales*: a synoptic revision based on comprehensive multigene-genealogies. Persoonia 30: 57–76.

Jameson M., Mico E., Galante E. 2007. Evolution and phylogeny of the scarab subtribe Anisopliina (Coleoptera: Scarabaeidae: Rutelinae: Anomalini). Systematic Entomology 32 (3): 429–449.

Jong S.C., Yuan G.F. 1985. *Actinomucor taiwanensis* sp. nov., for manufacture of fermented soybean food. Mycotaxon 23: 261–264.

Khan Z.U., Ahmad S., Mokaddas E., Chandy R., Cano J., Guarro J. 2008. *Actinomucor elegans* var. *kuwaitiensis* isolated from the wound of a diabetic patient. Antonie Van Leeuwenhoek 94 (3): 343–352.

Klein M.G., Kaya H.K. 1995. *Bacillus* and *Serratia* a species for scarab control. Memórias do Instituto Oswaldo Cruz 90 (1): 87–95.

Mahmud A., Lee R., Munfus-McCray D., Kwiatkowski N., Subramanian A., Neofytos D., Carroll K., Zhang S.X. 2012. *Actinomucor elegans* as an emerging cause of Mucormycosis. Journal of Clinical Microbiology 50 (3): 1092–1095.

Milner R.J., Huppatz R J., Swaris S.C. 1991. A new method for assessment of germination of *Metarhizium* conidia. Journal of Invertebrate Pathology 57 (1): 121–123.

Möller E.M., Bahnweg G., Geiger H.H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Results 20 (22): 6115–6116.

Phalip V., Hatsch D., Laugel B., Jeltsch J.M. 2006. An overview of fungal community diversity in diseased hop plantations. FEMS Microbiology Ecology 56 (2): 321–329.

Rehner S.A., Samuels G.J. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycological Research 98 (6): 625–634.

Soetopo D. 2004. Efficacy of selected *Beauveria bassiana* (Bals.) Vuill. isolates in combination with a resistant cotton variety (PSB-Ct 9) against the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Ph.D. thesis, University of the Philippines Los Banos, Philippines.

Sun B.D., Liu X.Z. 2008. Occurrence and diversity of insect-associated fungi in natural soils in China. Applied Soil Ecology 39 (1): 100–108.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28 (10): 2731–2739.

Tully C.C., Romanelli A.M., Sutton D.A., Wickes B.L., Hospenthal D.R. 2009. Fatal *Actinomucor elegans* var. *kuwaitiensis* infection following combat trauma. Journal of Clinical Microbiology 47 (10): 3394–3399.

Vilgalys R., Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172 (8): 4238–4246.

Waldorf A.R., Diamond R.D. 1985. Neutrophil chemotactic responses induced by fresh and swollen *Rhizopus oryzae* spores and *Aspergillus fumigatus* conidia. Infection and Immunity 48 (2): 458–463.

Walther G., Pawłowska J., Alastruey-Izquierdo A., Wrzosek M., Rodriguez-Tudela J.L., Dolatabadi S., Chakrabarti A., de Hoog G.S. 2013. DNA barcoding in Mucorales: an inventory of biodiversity. Persoonia 30: 11–47.

White T.J., Bruns T.D., Lee S.B., Taylor J.W. 1990. Amplification and sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315–322. In: "PCR-Protocols and Applications, a Laboratory Manual" (N. Innis, D. Gelfand, J. Sninsky, T.C. White, eds.). Academic Press, New York, USA.

Zheng R.Y., Liu X.Y. 2005. *Actinomucor elegans* var. *meitauzae*, the correct name for *A. taiwanensis* and *Mucor meitauzae* (Mucorales, Zygomycota). Nova Hedwigia 80 (3–4): 419–431.

Zoberi M.H., Grace J.K. 1990. Fungal associated with the subterranean termite *Reticulitermes flavipes* in Ontario. Mycologia 82 (3): 289–294.