Mucor circinelloides as an entomopathogenic fungus on melon weevil (Acytopeus curvirostris persicus) from Iran

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Abstract

Melon weevil, *Acytopeus curvirostris persicus* Thompson (Col.: Curculionidae), is one of the most important pests of melons that is spread in the Middle East countries. Through sampling from different parts of south Khorasan province, in April to October, a species belonging to the genus Mucor was isolated from mycoses adult melon weevil of *A. curvirostris persicus*. Identification of fungi was performed using morphological and molecular features and pathogenicity test was done to fulfill Koch's postulates. To perform pathogenicity test, two purified isolates of the mentioned fungus with adjusted spore concentration were sprayed as suspension on the larvae and adult melon weevil. Based on results of morphological data and sequencing of ITS region of ribosomal DNA, fungal specimens was identified as *Mucor circinelloides*. The results on the pathogenicity (50% mortality on melon weevil larvae and 33% on adults, 10 days after inoculation) demonstrated that *M. circinelloides* can be considered as a potential biocontrol agent against the melon weevil. Field experiments are still required to evaluate the capacity of genus *Mucor circinelloides* as a safe biological control.

Introduction

The order Mucorales represents a phylogenetically ancient group of fungi comprising predominantly saprotrophs inhabiting soil, dung and dead plant material, as well as several parasites on plants and on other fungi. Mucoralean fungi are also known to be involved in human infection. Mucormycoses are still very rare, but their incidence is increasing in hosts with severe immune or metabolic impair- ment, e.g. due to hemomalignancy, hematopoietic stem cell transplantation or un controlled ketoacidotic diabetes mellitus (Skiada et al. 2013).

The order Mucorales in the sense of Spatafora et al (2016) comprise 261 species in 55 genera.Twenty-eight of the species have been described since 2000. Thirty-eight out of these 261 species have been reported to be involved in human infections (Benny et al., 2016). Preliminary estimates suggest that the Chytridiomycota or zoosporic fungi diverged from the progenitor of the Zygomycota in the Ordovician (Berbee, 1996) and that these taxa are very likely nonmonophyletic, Perhaps reflective of their putative polyphyly,the Zygomycota comprise 10 morphologically and ecologically diverse orders that include endo- and ectomycorrhizal symbionts of vascular plants (Redecker et al 2000), obligate symbionts of aquatic insect larvae entomopathogens (Jensen et al 1998), obligate mycoparasites (Tanabe et al 2000), and the ubiquitous saprobes of the order Mucorales noted for their intricatel beautiful reproductive morphology. An eleventh order of Zygomycota, the Amoebidiales, has recently been shown to be nested within the protozoa (Benny and O'Donnell 2000, Ustinova et al 2000).

The genus *Mucor* is currently made up of 76 accepted species (12 species are known to be involvoled in human infection) and is by far the largest genus in the Mucorales. Several molecular studies revealed the polyphyly of *Mucor* (O'Donnell et al., 2001., Voigt et al., 2001). *Mucor* was previously characterized by the formation of sporangia and equally shaped suspensors, as well as the absence of apophysis, rhizoids (root-like hyphae), and sporangiola. Recently, it was shown that *Mucor* species are able to form rhizoids

(Walther et al., 2013., Wagner et al., 2019). This explains the misclassification of several *Mucor* species in the genus *Rhizomucor* due to the formation of rhizoids in culture. Sequence analyses also revealed that taxa with an apophysis, such as *Mucor durus* (syn. *Circinella rigida*), and with sporangiola, such as *Mucor ctenidius* (syn. *Backusella ctenidia*), belong to the genus *Mucor*. Based on Walther et al., all *Zygorhynchus* species were transferred to genus *Mucor* (Walther., 2013).

Melon weevil, *Acytopeus curvirostris* persicus Thompson (Col.: Curculionidae), is one of the most important pests of melons that is spread in the Middle East countries (Mohammadpour, 2013) that attacks members of Cucurbitaceae (Mohammadpour., 2015). Its wild host watermelon is *Citrullus colocynthis* (commonly is known as the colocynth, bitter apple, bitter cucumber, desert gourd, egusi, or vine of Sodom), which grows in salt and gypsum lands. Due to themulti-generational nature of the pest, farmers use large amounts of insecticides during the growing season for control (Mohammadpour et al., 2013). In this study, we examined effect of *Mucor circinelloides* as biocontrol fungus on *A.curvirostris* .

Materials and Methods

Microorganisms isolation and Culture conditions:

During January and February , mycosed adults of melon weevil beetles (*A. curvirostris* persicus) were collected from melon and watermelon fruit infested with larvae, or adults, from the Mohamadiah region, Birjand,South Khorasan Province, Iran. Surface strelizition done by 1% sodium hypochlorite for 3 min, 70% ethanol for 3 min and then were rinsed with sterile water three times (Lacey and Brock., 1997). After disinfection, dead adult cultured on to Potato Dextrose Agar medium and Water Agar and incubated in 25° c and dark for 5 days. Conidial concentrations of 1×10^3, 1×10^4, 1×10^5 and 1×10^6 conidia/ml were applied in 1 ml aliquots to the test adult and larvae. Positive controls for the tested fungi came from a laboratory. Each experiment was replicated 5 times with 10 number of larva or adult for each replicate. The entire experiment was conducted twice. (Majumbar et al., 2008). A moistened filter paper was placed in each container (plastic Petri dish) for maintaining high relative humidity. After 7 days, watermelon weevils and larvae were checked for mortality and the number of dead insects was recorded. In order to fulfillment of Koch's postulates, each sample of tested larva or adult were reculture in PDA.

Morphological description

Morphological features of fungus including shape,,color and growth rates of fungus colony were investigated onto PDA(potato Dextrose Agar), WA(Water Agar) and SMA(Syntethic Mucor Agar).

Microscopic structure (25 measurements) were observed with slide culture technique using Olympus BX-40 light microscope.

Genomic DNA extraction:

Genomic DNA was extracted using a modified Chelex method with an initial step of grinding the mycelia in liquid nitrogen from two fungal specimens (Walsh et al., 1991). Further identification was confirmed by sequencing the ITS region of rDNA using the primers ITS4 (TCCTCCGCTTATTGATATGC) & ITS5 (GGAAGTAAAAGTCGTAA CAAGG). Fungal isolates were cultured on PDA medium, after 3 days, 100mg of mycelium of fungus was scraped with stril scalpel, homogenized with nitrogen liquid then transferred to microtube containing 200ml 8% chelex 100 suspension. The Tubes were vortexed, ancubated in 56° C for 12 hours and bathing in boiling water for 7 mins. Extract were vortexed, then, again heating in boiling water for 8 mins and centrifuged at 12000 rpm for 5 minutes.

The polymerase chain reaction (PCR) was performed using the Thermocycler, (BIO-RAD Model,USA) with the final reaction volume of 25 µL, containing 4.4 micro-liters mixture of *taq* DNA polymerase enzyme, $MgCl_2$, PCR buffer, dntps, 1 µL of each forward-reverse primer (10 pmol), and 5 µL of template DNA. PCR amplification was carried out under the following conditions: initial denaturation at 94ºC for 3 min; followed by 30 cycles of 94ºC denaturation (45 s), 54ºC annealing for 45 s, and 72ºC extension for 2 min and a final extension at 72ºC for 5 min. The reaction products were stored at -20°C. A volume of 25 microliter of each PCR product with each of the forward-reverse primers at a concentration of 10 pm (picomole) was sent to Bioneer company, South Korea for sequencing. The samples were sequenced by automated Sanger method using the PCR purification kit. To enhance the accuracy of the sequencing, each sample was sequenced with both forward and reverse primers. The ITS rDNA-based alignment was performed using CLUSTALW program implemented in the MEGA v. 5 software and Phylogenetic tree construction was performed using MEGA v. 5 software for Minimum Evolution (ME) tree based on Tamura-Nei model with 1000 bootstrap replications.

Results and Discussion

A fungus with the features of *Mucor circinelloides* was isolated from the mycosed adults of *A. curvirostris* persicus on PDA. at 38°c Colonies of *Mucor* on SMA grow rapidly and fill the petri dishes after 8 days, the texture was cotton-candy like, the color of the colony at first is white initially and turns grey to yellowish brown. Rhizoids very abundant and varios form presents, chlamydospores which are single, in group and chains, terminal or intercalary, colomellae very variable and irregular shape formed as pyriform ,globose, subglobose, ovoid and conical. Apophysis presents. Hyphae branching, nonseptate when young and hyaline, sporangiospore in varios form, Sporangiophores are irregularly branched and end in sporangia at their apices. Sporangia are brown in color and round in shape , 20-50 µm in diameter. Sporangiospores are small, unicellular, and round to ellipsoidal in shape, 3-4 µm in diameter. Measuring of maximum temperature of growth shows that fungal isolate is non thermophilic fungus. Maximum temperature reaching to 40°c . Zygospre unknown (Fig 2).

A nucleotide blast search in the NCBI database (http://www.*ncbi*.nlm.nih.gov/) showed that the sequence in this study had 100% homology with *Mucor circinelloides* (Gene Bank ID :MT603942) isolates. Based on Wagner et al., *Mucor circinelloides* and its relatives were recently taxonomically revised resulting in the recognition of its classical formae as independent species (*M.*

circinelloides sensu stricto , *M. griseocynanus*, *M. janssenii*, and *M. lusitanicus*), the acceptance of *M. velutinosus* as a discrete species and the description of several new *Mucor* species.

In the *Mucor circinelloides* relationship, protein-coding genes such as rpb1 have a much higher resolution power than ITS, but reference sequences of these genes are usually lacking (Wagner et al., 2019). A disadvantage of using ITS sequencing for identification is that ITS copies differ slightly in some taxa and direct sequencing becomes impossible Also of note, in some genus like *Syncephalastrum* some strains have two clearly differing types of ITS-sequences (vitale et al., 2012). Alternatively, LSU can be used for identification and has the advantage that direct sequencing is nearly always possible. To date and our knowledge, this region resolves all mucoralean species, but the sequence differences among the species are relatively small.

Classification of genus *Mucor* has undergone many changes by progressive DNA assay. Some species were transfred to this genus in recent years, for examples All mesophilic species of *Rhizomucor*, including *Rm. chlamydosporus, Rm. endophyticus*, *Rm. Regularior* (syn. *Rm. variabilis var. regularior*), and *Rm. variabilis* were transferred to *Mucor* based on molecular data. In its current classification, *Rhizomucor* only harbours thermophilic species with maximum growth temperatures above 50°c (in older studies, 40°c was maximum temp for *Rhizomucor* species) and minimum growth temperatures below 20 °c .

To our knowledge, this is the first record of *Mucor circinelloides* infecting a member of *Coleoptera*. Three fungal isolates caused high mortality ranging from 33% (on adults beetles) to 50 % on (larvae) by 10 days after inoculation (Figure 2). According to preliminary investigations, fungal isolates also were spray on Cucurbitaceae plants and any symptoms didn't occur in this plants.

To date, there are few reports about entomopathogenic fungi from Curculionidae family. Keller et al., isolated the fungal pathogen *Zoophthora phytonomi* as the pathogen of *Phytonomus punctatus* that belongs to family Curculionidae (Keller et al., 2007). Based on Batta, , 2011, formulation of *Zoophthora radicans* (zygomycetes: entomophtorales) can play an important role in controlling *Plutella xylostella* moth . Recently, various researchs in the field of Curculionidae entomopathogenic fungus have been carried out. Carillo et al (2014), were tested isolates of *Isaria fumosorosea* and *Beauveria bassiana* on the redbay ambrosia beetle, *Xyleborus glabratus* (Coleoptera: Curculionidae). Their results showed that using any of the biopesticides resulted in death of all RAB females, B. bassianakilled RAB females faster and no significant differences were observed in the mortality of beetles exposed to entomopathogenic fungi by dipping in a fungal suspension or walking on treated avocado bolt.

Apart from zygomycetes fungi that have been isolated frequently as entomopathogenic causes from different hosts so far, in 2012, Batta succeeded to isolate *Fusarium avanaceum* from rice beetle insect (*Sitophilus oryzae* L.: Curculionidae, Coleoptera). According to his results, the mentioned fungi had 94% pathogenicity on the adult rice weevils.

Mucor circinelloides is a filamentous fungus by nature and is most commonly found in soil, plants and decaying fruits. This genus is associated with both biotechnology industry and human health (Carvalho et al., 2015; Lee et al., 2014). As previously mentioned, in *Mucor* 12 species are known to cause infection in humans and animals, but comprehensive antifungal susceptibility (AFS), only exist for *M.circinelloides*, *M. indicus* and *M.ramosissimus* (Wagner et al., 2019)

Members of the *Mucor circinelloides* complex (MCC) as defined by Walther et al. (2013) are among the *Mucor* species that are most frequently isolated from clinical sources (Walther et al. 2013). Taxonomy of *M.circinelloides* and its close relatives (MCC) is still based on morphology and mating behavior. In recent years molecular equipment has big role in taxonomy of mucorales, despite this it seems Using various markers (ITS, LSU, rpb1, cfs,..), mating test and determination of maximum growth temperatures, is the most reliable method for calssifying Mucorales. based on latest classification (wagner et al., 2019),some genus *Rhizomucor* are transferred to *Mucor*. and has been reported as fungus infecting human and animal but seems to be more common in inducing animal than human mycoses (Franks& Guiducci.,1953). Research shows that fungi can be used as biocontrol agent of Coleoptreran insects. This is the first report of genus *mucor circinelloides* for mycobiota of Iran and entomopathogenic fungus on melon weevil which is deposited in Iranian fungal culture collection under access number IRAN 2237C.

According promising results achieved by efficacy of the fungus on results on larvae and adult weevils, the fungal isolates seem to be applied in the future as biocontrol agents of weevil pest. However, the effects of this fungus on other insects have not been studied and interaction between fungi and other organisms, pests and harmful insects, beneficial insects and their host that more research is needed.

Conclusion

Despite huge economic damage of melon weevil in Iran, A suitable biocontrol method has not yet been found to control this pest. Results of this study showed that pathogenic fungus, *mucor circinelloides*, has potential to biocontrol melon weevil in laboratory conditions, but more researchs in needed to make sure it is safe to consumer.

Declarations

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Ethics approval and consent to participate: The present study is part of a Master dissertation in Plant Pathology approved by the University of Birjand, Birjand, Iran.

Consent for publication: Applicable

Competing interests : The authors declare no competing interests as defined by Springer or other interests that might be provided be influence the results and/ discussion reported in this paper.

Availability of data and materials : The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors' contributions: Narges sepasi,Mehdi jahani,Mohammadreza Mirzaei and Kazem Mohammadpour, Conceived and designed the analysis, collected the data, contributes data or analysis tools, performed the analysis and wrote the paper. All authors reviewed the manuscript.

Informed Consent: Informed consent was obtained from all individual participants included in the study.

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Figures

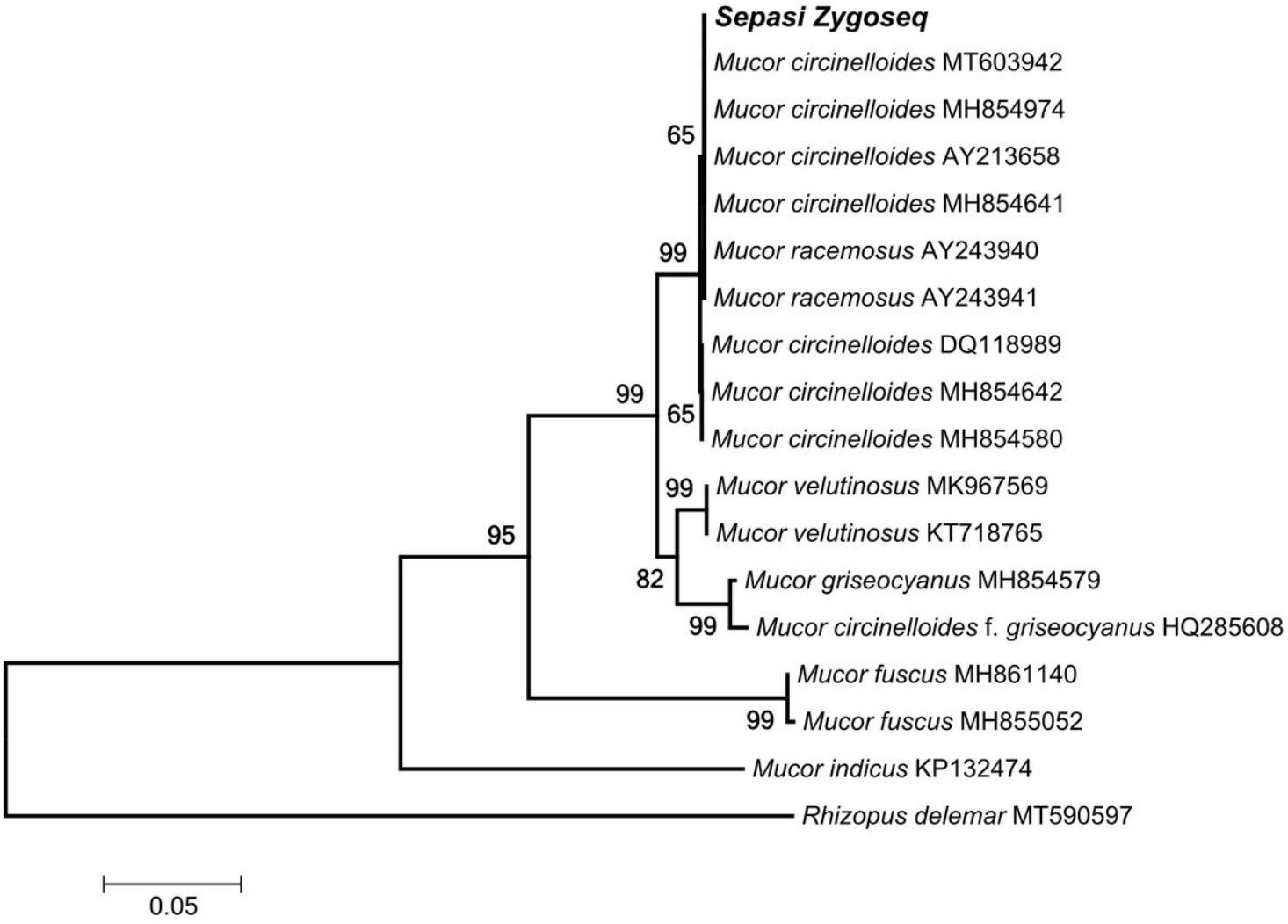

Figure 1

Minimum Evolution (ME) phylogenetic tree based on sequences data from ITS-Rdna region. Support for branches was assessed by bootstrap resampling of data set with 1000 replications. The sequences that named **Sepasi Zygoseq**was generated in this study.

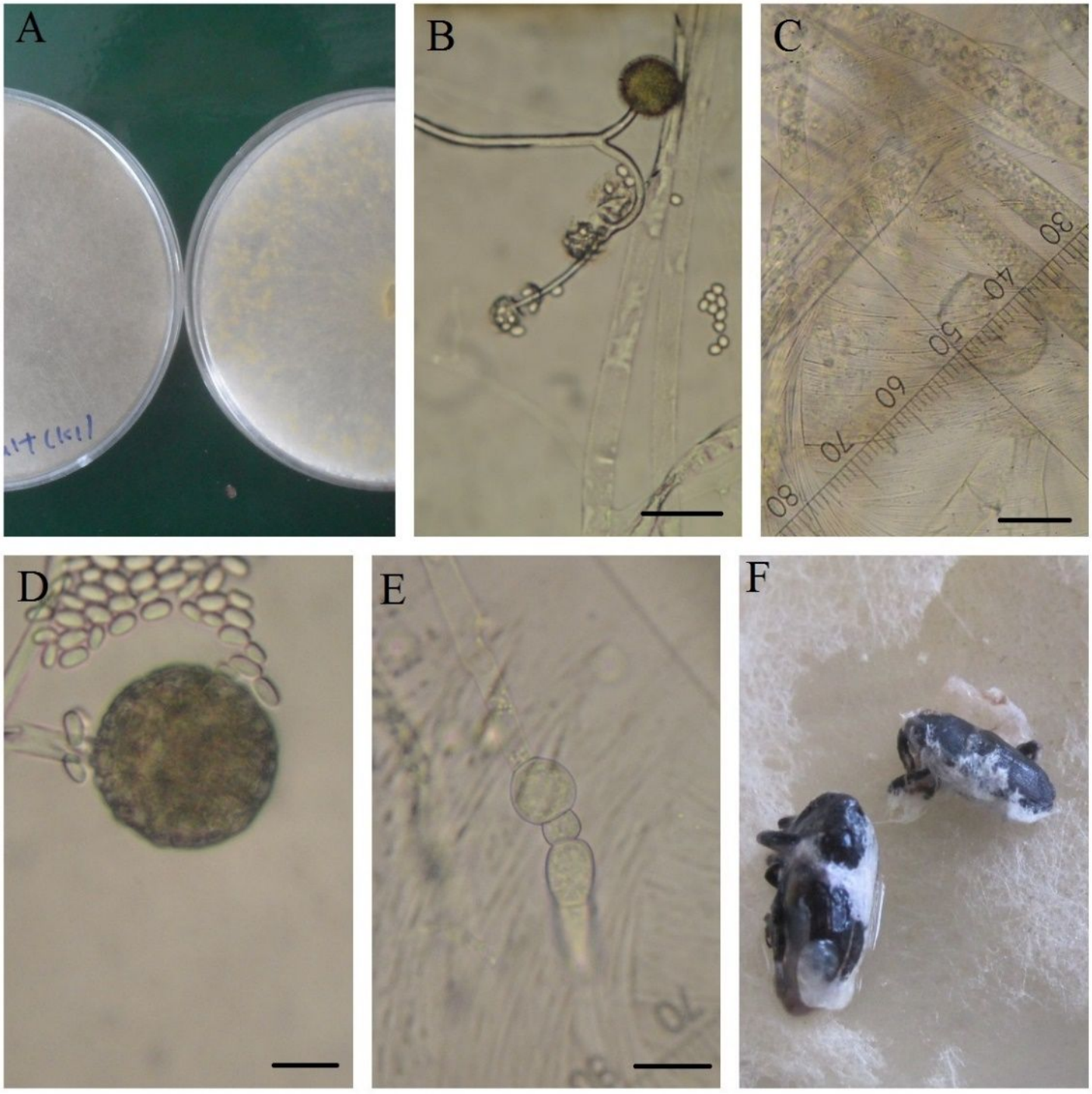

Figure 2

A.Front and Reverse of 7days old culture of *M. circinelloides* on SMA. B.Sporangiophore with Sporangia. C.Columella. D.Sporangia and Sporangiospores. E.Intra-hyphal Chlamydospore. F. Adults of *A.corvirostris* parasite by *M. circinelloides.* Bar: 20 µm