Colonial and Morphological Characteristics of Some Microfungal Species Isolated from Agricultural Soils in Eskişehir Province (Turkey)

Semra İLHAN¹, Rasime DEMİREL², Ahmet ASAN³, Cengiz BAYÇU⁴, Engin KINACI⁵
¹Eskişehir Osmangazi University, Arts and Science Faculty, Biology Department, Eskişehir - TURKEY
²Eskişehir Osmangazi University, Graduate School of Natural and Applied Sciences, Biology Programme, Eskişehir - TURKEY
³Trakya University, Arts and Science Faculty, Biology Department, Edirne - TURKEY
⁴Eskişehir Osmangazi University, Medical Faculty, Department of Histology and Embryology, Eskişehir - TURKEY
⁵Eskişehir Osmangazi University, Agricultural Faculty, Eskişehir - TURKEY

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Abstract: Aspergillus crustosus Raper & Fennell, Eupenicillium egyptiacum (J.F.H.Beyma) Stolk & D.B.Scott, Paecilomyces ramosus Samson & H.C.Evans, and Penicillium novae-zeelandiae J.F.H.Beyma were examined for their colonial and morphological properties via visual, light and scanning electron microscopy. These species isolated from soil in different regions of Eskişehir are recorded for the first time in Turkey.

Key Words: Soil fungi, Aspergillus, Eupenicillium, Paecilomyces, Penicillium

Introduction

Microfungi are important eukaryotic micro-organisms that affect humans and the majority of living forms in different ways. Soil microfungi play an important role in the degradation of organic debris (Barnett & Hunter, 1999). In addition, they are used in industrial and food fermentation processes, and they exist commonly in different types of soils, indoor and outdoor air, food, and water. Since microfungi are found almost everywhere, they are frequently cited in species lists in ecological studies (Asan, 2004). Aspergillus Link. and Penicillium Fr. species are commonly found as contaminants in foods during drying and subsequent storage. Thus, accurate identification of Aspergillus and Penicillium and related genera at the species level is essential. Aspergillus and Penicillium are not easy to identify to the species level. To further complicate things, the taxonomy of both genera still needs work, but there appear to be fewer problems in Aspergillus than in Penicillium. Although molecular, biochemical and physiological methods are important for the systematics of these species, morphological properties are commonly used for identification (Asan, 2004).

The species of Aspergillus, Penicillium and Paecilomyces Bainer are among the most abundant and widely distributed microfungi in nature (Pitt, 1979; Christensen et al., 2000; Klich, 2002; Asan, 2004).
A number of species belonging to these genera have been isolated and identified in studies carried out in Turkey (Öner, 1970, 1973, 1974; Ekmekçi, 1975; Hasenekoğlu, 1982, 1985, 1987; Hasenekoğlu & Azaz, 1991; Hasenekoğlu & Sülün, 1990; Asan, 1997; İlhan & Asan, 2001). Morphological studies of microfungi are rare in Turkey. Eltem et al.’s work in 2004 is an important investigation about the genus Aspergillus in Turkey. Since the morphological characteristics of these genera resemble each other and there are no absolute criteria for each genus, it can be extremely difficult to distinguish the species. Pitt & Hocking (1985) discussed characteristics that could be used to differentiate Aspergillus and Penicillium from each other, and from the related genera Raperia Subram & Rajendan, Paecilomyces, Geosmithia Pitt, Nomuraea Maublanc, Eladia G.Smith, and Merimbla Pitt.

As a result of the survey, we isolated 110 species from soil. Identification of the species revealed 13 Aspergillus, 1 Eupenicillium, 4 Paecilomyces and 31 Penicillium species previously reported by our group (Demirel et al., 2005). According to Asan’s Checklist (Asan, 2004), Aspergillus crustosus Raper & Fennell, Eupenicillium egyptiacum (J.F.H.Beyma) Stolk & D.B.Scott, Paecilomyces ramosus Samson & H.C.Evans, and Penicillium novae-zeelandiae J.F.H.Beyma are recorded for the first time in Turkey. Reference strains of these soil microfungi isolates have been deposited in the Culture Collections of KUKENS (WDCM101), Centre for Research and Application of Culture Collections of Microorganisms. The purpose of this study is to contribute to the checklist of Aspergillus, Penicillium and other related species in Turkey, as well as to present macroscopic and microscopic characteristics of these species. Descriptions of 4 species which are new records for the Turkish mycoflora are presented in our study.

Materials and Methods

The research areas (Figure 1), Karacahöyük and Bahçek, are 25 km and 35 km from the centre of Eskişehir (latitude 39° 47', longitude 30° 31') towards the east, respectively. Osmangazi University experiment fields I (OGU I) and II (OGU II) are close (approx. 5 km) to the centre of Eskişehir. According to the climatologic data of the past 60 years the annual mean temperature in this province is 10.8 °C. The mean temperature of the hottest months (July-August) is 21 °C; the mean temperature of the coldest months (January-February) is -0.2 to 1.2 °C. Annual mean precipitation in the region is 25.3 mm and annual relative humidity is 67%. The climatologic data were obtained from Eskişehir Meteorology station.
The soil plate method (Waksman, 1922) was used to isolate the soil fungi from 56 composite soil samples from 4 different areas, Karacahöyük, Bahçekik, ÖGU I, and ÖGU II, in Eskişehir province in 2002 (July and October) and 2003 (January and April). Peptone dextrose agar plus Rosebengal-Streptomycine medium containing 10 g of dextrose, 5 g of peptone, 1 g of KH₂PO₄, 0.5 g of MgSO₄.7H₂O, 10 ml of (1/30,000) Rosebengal (Fluka Chemika BioChemika, Switzerland), 30 µg of streptomycin (Deva Inc., Turkey), 15 g of agar and 990 ml of distilled water was employed for the isolation of fungi.

Isolates were inoculated in Malt Extract Agar (MEA), Czapek Dox Agar (CZ), and Potato Dextrose Agar (PDA) media and incubated at 25 °C for 7 days for identification. After that colony diameters were measured. Petri dishes were first examined under a dissecting microscope (a stereomicroscope) and then under a high resolution light microscope to determine the colonial features and the morphological structures of the fungi. During determination of the morphological structures, a modified mounting medium, Lacto-Cotton Blue, as proposed by Sime & Abbott (2002), was used. Macroscopic and light photomicrographs of fungal species were obtained using a Nikon CoolPix 5000 digital camera and an Olympus microscope with a Spot In-IGHT colour digital camera, respectively.

For scanning electron microscopy (SEM), the cultures were fixed in 5% (v/v) glutaraldehyde + phosphate buffer solution for 24 h. The samples were then transferred to a graded ethanol series (50%, 70%, 90% and 100%) for 30 min each and finally to amyl acetate solution (Deo et al., 1983). Critical point dried samples were (POLARON CPD) coated with gold-palladium using a Polaron SC7620 Sputter Coater for 90 s. The coated specimens were examined in a Jeol JSM-5600 LV scanning electron microscope.

Fungi were identified to genus level according to Barnett & Hunter (1999). The isolates were identified to species level according to various mycological references as below: *Penicillium* and *Eupenicillium* species were grown on 3 different media according to Pitt (1979). Cultures were inoculated in 3 points onto Czapek Yeast Extract agar (CYA) and incubated at 3 different temperatures (5, 25 and 37 °C) for 7 days in the dark. In addition, CZ, MEA, and 25% Glycerol Nitrate agar (G25N) were used for the cultivation of *Penicillium* species (at 25 °C, for 7 days) (Raper & Thom, 1949; Pitt, 1979). *Aspergillus* species was identified according to Raper & Fennell (1965) and Klich (2002). Therefore, MEA, CZ, CYA with 20% sucrose (CY20S), CYA (at 25 and 37 °C), M40Y, and MY20 medium were prepared and *Aspergillus* culture was inoculated into each medium and incubated at 25 °C (except CYA37), for 7 days. *Paecilomyces* species were inoculated to MEA and PDA media and incubated at 25 °C for 7 days and then identified according to Samson (1974). All names of the identified species and authors were cited according to Kirk & Ansell (1992). The “Flora of British Fungi Colour Identification Chart” (CIC) was used for the colour catalogue (Henderson et al., 1969).

**Results**

According to results obtained from our previous studies, *A. crustosus* was only found in a soil sample collected from Karacahöyük in winter. *E. egyptiacum* was isolated from the Bahçekik area in summer. *P. ramosus* was one of the most abundant species and isolated from 4 different areas. *P. novae-zeelandiae* was found in 2 areas, Karacahöyük and ÖGU II, in autumn and spring (Demirel et al., 2005). The *Aspergillus*, *Eupenicillium*, *Paecilomyces* and *Penicillium* species are described below.


Colony Characteristics: Colony diameter after 7 days’ incubation on CYA at 25 °C was 10 mm. Growth was restrictedly umbonate. Conidia were sparse, olivaceous buff (CIC: 63) to grey olivaceous (CIC: 61); mycelium was white and floccose; exudate absent; soluble pigment light chestnut in colour; reverse bay (CIC: 19). Colonies on MEA were 13-15 mm in diameter, centrally umbonate, with floccose white mycelium; conidia were moderate, lemon yellow (CIC: 54) to grey olivaceous in colour; exudate and soluble pigment were absent. Reverse was chestnut (CIC: 19).

Colonies on CY20S were 9-10 mm in diameter, umbonate; mycelium was floccose; conidia were sparse to moderate, olivaceous buff in colour; exudate and soluble pigment were absent; reverse pale, light vinaceous buff (CIC: 31); margin was low, regular or irregular.

Colonies on CZ were 10-13 mm in diameter, consisting of a dense basal mycelial felt submerged and
nonsporulating in marginal area, 2 to 3 mm wide, umbonate, with floccose white mycelium; conidia were sparse, olivaceous buff; exudate and soluble pigment absent; reverse was clay pink (CIC: 30) at margin while purplish date (CIC: 22) at centre.

On CYA at 37 °C, no growth (Figure 2). Colonies on M40Y were 15 mm in diameter, plane, lemon yellow at near central area, reverse buff. Colonies were 15 to 18 mm on MY20 agar, strongly buckled and wrinkled, in colour as on M40Y agar. Hulle cells were not produced on M40Y agar.

Microscopic Characteristics: Stipes were 60-150 x 2.5-4.0 µm, smooth to slightly rough-walled, uncoloured to pale green or slightly brownish; conidial heads were columnar to radiate, 18-30 µm. Vesicles pyriform to spathulate, 6.0-14.0 µm wide, hyaline to pale green. Aspergilli were biseriate. Metulae were covering only the upper half of the vesicle, 6.0 x 2.5 µm in size; phialides were 5.0 x 2.0 µm in size, ampulliform with tapering collula. Conidia were 2.5-3.5 µm in diameter, globose to sub-globose, with wall smooth to slightly rough. Hulle cells were very abundant, globose to sub-globose, 15.0 x 20.0 µm in size, hyaline to light green en masse (Figure 2).


Colony Characteristics: Colonies on CYA (25 °C) were 22-31 mm in diameter at 7 days, radially sulcate, convolute, lightly annular, consisting of velutinous or floccose mycelium, enveloping abundant cleistothecia; margin was deep, entire or irregular; mycelium was white or off-white; conidiogenesis was inconspicuous, but after 7th day coloured light grey (CIC: 34). Exudate produced was clear to clay pink, reverse near brick (CIC: 15) to salmon, soluble pigment as reverse.

Colonies on MEA (25 °C) were 44-48 mm in diameter, other properties were similar to those on MEA. Conidia were moderate to abundant but covered by mycelium; exudate was clear; soluble pigment was absent or slightly yellow; reverse was pale to light lemon yellow (Figure 4).

Microscopic Characteristics: Hyphae were hyaline, septate, smooth-walled. Conidiogenous structures were synnematous or mononematous. Synnemata with white powdery heads were cylindrical with many side branches. Conidiophores were scattered along the synnema, 50-110 µm in length and 2.5-4.0 µm in diameter, consisting of some verticillate branches with whorls of 2 to 4 phialides. Conidiogenous cells were phialidic, consisting of a cylindrical or swollen basal portion, tapering into a long distinct neck. Phialides were 8.0-20 x 2.5-3.5 µm in size, consisting of a cylindrical portion, tapering abruptly into a long neck of 0.5-2.0 µm. Conidia were hyaline, smooth-walled, 3.5-5.0 x 1.5-3.0 µm in size, in dry, thick-walled, divergent, basipetal chains, 1 or 2-celled, pyriform, apiculate (Figure 4).


Colony Characteristics: On CYA, 25 °C, 7 days, colonies were 30-36 mm in diameter, radially sulcate, comprising a surface layer of black sclerotia, often densely packed and near the margins arranged in radial
Figure 2. *Aspergillus crustosus* A) Colonial appearance (7 days); Light microscopic appearance of B) conidial head and C) hulle cells; SEM appearance of D) conidial heads and E) hulle cell.
Figure 3. *Eupenicillium egyptiacum* A) Colonial appearance (7 days); Light microscopic appearance of B) penicilli C) cleisthotecium and D) ascus; SEM appearance of E) penicilli and F) cleisthotecium.
Figure 4. *Paecilomyces ramosus* A) Colonial appearance (7 days); Light microscopic appearance of B) conidiofor and conidia C) synnematous structure; SEM appearance of D) Phialides and tapering collula and E) branching and phialides.
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lines, consisting of floccose mycelium; margin was low, irregular; mycelium was white; conidiogenesis was sparse to moderate; conidia were en masse olivaceous buff (CIC: 64) or grey olivaceous; exudate produced was clear; soluble pigment absent; reverse dark buff to almost black especially in areas beneath sclerotia embedded in medium.

Colonies on MEA (25 °C, 7 days) were 32-35 mm in diameter, slightly sulcate, plane, consisting of velutinous or less floccose mycelium and often with sclerotial development less extensive; margin was low to deep, entire; mycelium was white, conidiogenesis was moderate, in colours similar to those on CYA; exudate and soluble pigment were absent; reverse buff, usually blackish, less beneath the sclerotia. On CYA 5 and 37 °C, 7 days, no growth.

Colonies on CZ were 15-21 mm in diameter, deeply sulcate, floccose at the margin, velutinous at the central, with margin deep and irregular; mycelium was white, conidiogenesis was light to moderate, conidia were en masse olivaceous buff; exudate produced was clear; soluble pigment absent; reverse pale. Sclerotia were borne subsurface, dark brownish green in colour, becoming black when fully formed (Figure 5).

Microscopic Characteristics: Conidiophores were borne from surface hyphae, stipes were long, 350 x 3.0 µm with rugose walls, comprising a cluster of 4 appressed metulae, 11.0 x 3.0 µm in size, apically swollen; phialides were in verticils at least 4-5 ampulliform, 6.0 x 2.0 µm with short tappered collula; conidia were subglobose to globose, 2.5-3.0 x 2.0 µm in size, slightly roughened, borne in disordered chains; sclerotia were irregular in shape and up to 140-150 µm long (Figure 5).

Discussion

According to our findings, *A. crustosus* is quite rare although *Aspergillus* species are common. Pitt (1979) reported that *E. egyptiacum* is a relatively rare soil fungus. The low coincidence of the species in soil may be related to very poor conidiogenesis. *P. ramosus* is an enthomopathogen. Although a comparatively rare species, *P. novae-zeelandiae* is widely distributed in soils and decaying vegetation (Pitt, 1979).

The most distinguishing property of *A. crustosus* is the presence of globose-subglobose hulle cells as stated by Raper & Fennell (1965). This feature was distinctly observed in our investigation. Colonies had an image consisting of a raised central area and a crusty layer of interwoven hyphae, hulle cells and conidial heads. Raper & Fennell reported that the colony of *A. crustosus* was crustlike in nature on a variety of common agar media. The colonies on M40Y agar were plane, were not crustlike in nature and had no hulle cells.

*E. egyptiacum* differ from other related species by some distinguishing features; it forms cleisthotecia which are pale, and when grown on CYA they sometime produce a brownish orange pigment in the reverse (Pitt, 1979). These features were distinctly observed during the investigation. In addition, the species showed very poor conidiogenesis on all media used.

The main characteristic of *P. ramosus* is the typically branched and erect synnemata, measuring 2.5-5.0 cm in length in natural habitat (Samson, 1974). In this study the erect synnemata were not distinguishable on MEA. However, the synnemata and typically branching were observed at microscopic investigation. The conidiophores of *P. ramosus* strongly resemble those produced in the genus *Penicillium*. The species is, however, placed in *Paecilomyces* because of its white colour, synnematus habit, and phialides that terminate into a long thin neck (Samson, 1974). On the other hand, the shape and size of *Paecilomyces* conidia differ from those of *Penicillium* conidia. *P. ramosus* conidia do not have a symmetrical shape (Figure 4).

The distinguishing feature of *P. novae-zeelandiae* is its black partially subsurface sclerotia of irregular shape (Pitt, 1979). This feature was distinctly observed on the reverse surface of the colony at the centre. In conclusion, the descriptions of some soil microfungi are compared in this paper.
Figure 5. *Penicillium novae-zelandiae* A) Colonial appearance (7 days); Light microscopic appearance of B) entire sclerotia in solid medium, C) one sclerotium and D) polygonal cells of sclerotium, E) penicilli; SEM appearance of F) penicilli and G) phialides and conidia.
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