

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

Eskişehir Yöresindeki Tarım Topraklarından İzole Edilen Bazı Mikrofungus Türlerinin Koloni ve Morfolojik Özellikleri

Özet: *Aspergillus crustosus* Raper & Fennell, *Eupenicillium egyptiacum* (J.F.H.Beyma) Stolk & D.B.Scott, *Paecilomyces ramosus* Samson & H.C.Evans, ve *Penicillium novae-zeelandiae* J.F.H.Beyma koloni ve morfolojik özellikleri açısından çıplak gözle, ışık ve taramalı elektron mikroskobu ile incelenmiştir. Eskişehir'de farklı bölgelerden alınan toprak örneklerinden izole edilen bu türlerin Türkiye için yeni kayıt olma olasılığı yüksektir.

Anahtar Sözcükler: Toprak fungusları, *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* Bainier 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 Bahçecik, 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.

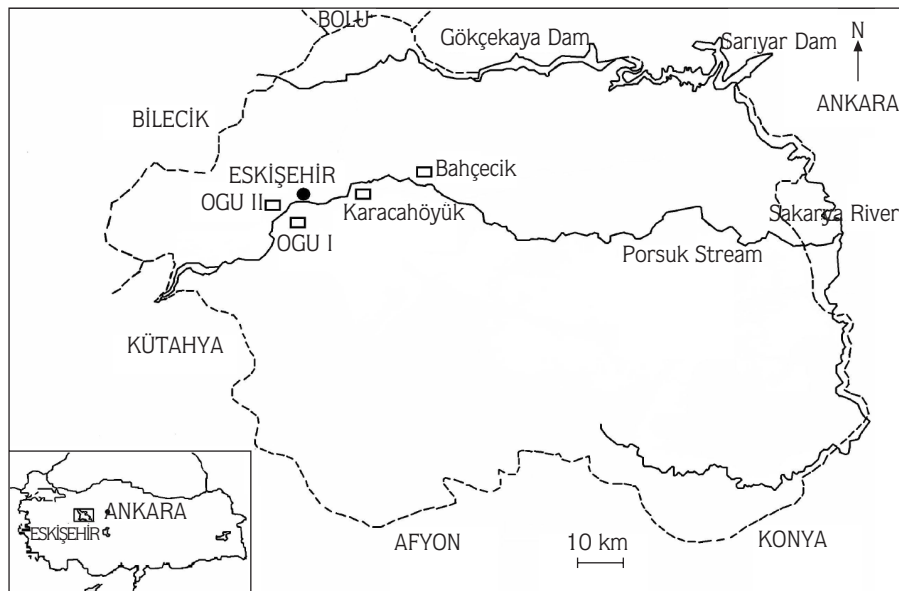


Figure 1. Map of investigation area.

The soil plate method (Waksman, 1922) was used to isolate the soil fungi from 56 composite soil samples from 4 different areas, Karacahöyük, Bahçecik, OGU I, and OGU 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 KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 Bahçecik 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 OGU II, in autumn and spring (Demirel et al., 2005). The *Aspergillus*, *Eupenicillium*, *Paecilomyces* and *Penicillium* species are described below.

Aspergillus crustosus Raper & Fennell, The Genus *Aspergillus*: 530 (1965).

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: 23).

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 spatulate, 6.0-14.0 µm wide, hyaline to pale green. Aspergilli were biserial. 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).

Eupenicillium egyptiacum (C.F.H.Beyma) Stolk & D.B.Scott, Persoonia 4: 401 (1967).

Anamorph: *Penicillium nilense* Pitt, The Genus *Penicillium* and its teleomorph states *Eupenicillium* and *Talaromyces* (London): 145 (1980) [1979]

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 cleistotesia; 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 21-25 mm in diameter at 7 days, radially sulcate, plane, slightly centrally umbonate, consisting of floccose white mycelium; conidiogenesis was inconspicuous, exudate was clear and soluble pigment absent; reverse pale or yellow.

On CYA, 5 °C and 37 °C, 7 days, no growth. Colonies on CZ (25 °C, 7 days) were similar in morphology to those on CYA25 (Figure 3).

Microscopic Characteristics: Cleistothecia were 200-300 µm in diameter, pseudo parenchymatous, maturing within 3 weeks, asci borne in chains, 6.0-10.0 µm in size. Ascospores were broadly ellipsoidal, 3.0 x 2.5 µm in size, smooth walled and slightly furrowed. Stipes were 155 x 3.0 µm in size and bearing biverticillate or occasionally terverticillate penicilli, smooth walled Rami 5.0 x 2.5 µm; metulae 10.0 x 2.5 µm, each metula had 4 phialides; phialides 7.5 x 25 µm in size, ampulliform, with gradually tapering collula. Conidia were globose, 2.5 µm in diameter, smooth walled, borne in disordered chains (Figure 3).

Paecilomyces ramosus Samson & H.C.Evans, Samson, Stud. Mycol. 6: 44 (1974).

Colony Characteristics: Colonies on MEA (25 °C, 7 days) were 44-48 mm in diameter, low, plane, with floccose white mycelium; conidia were sparse, white to lemon yellow; exudate was clear; soluble pigment was lemon yellow; reverse was luteus to lemon yellow in colour.

On PDA colonies were 43-44 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 synnematosus or mononematosus. 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).

Penicillium novae-zeelandiae J.F.H.Beyma, Antonie van Leeuwenhoek 6: 273 (1940).

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

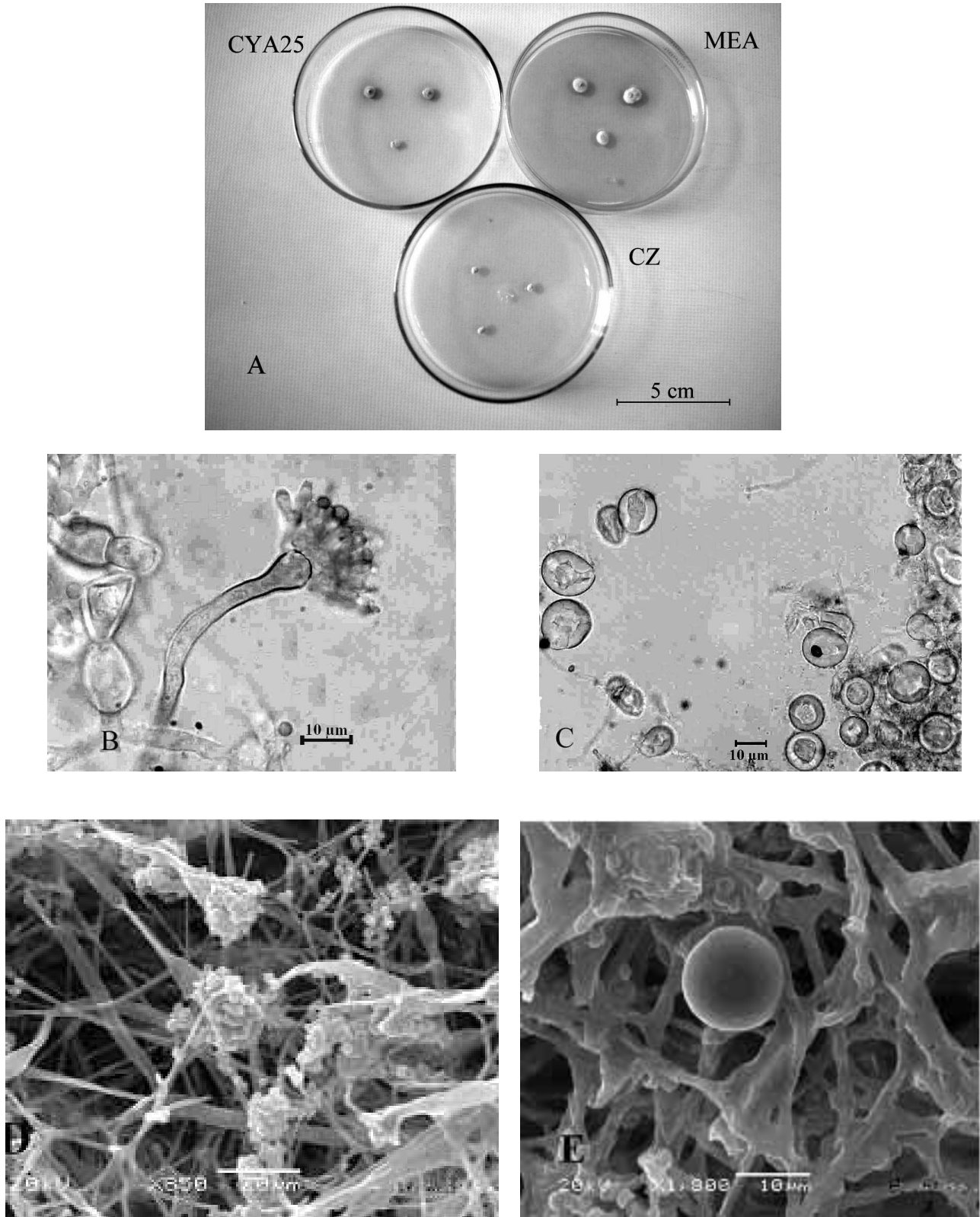


Figure 2. *Aspergillus crustosus* A) Colonial appearance (7 days); Light microscopic appearance of B) conidial head and C) hulle cells; SEM appearance of D) conidial heads and E) hulle cell.

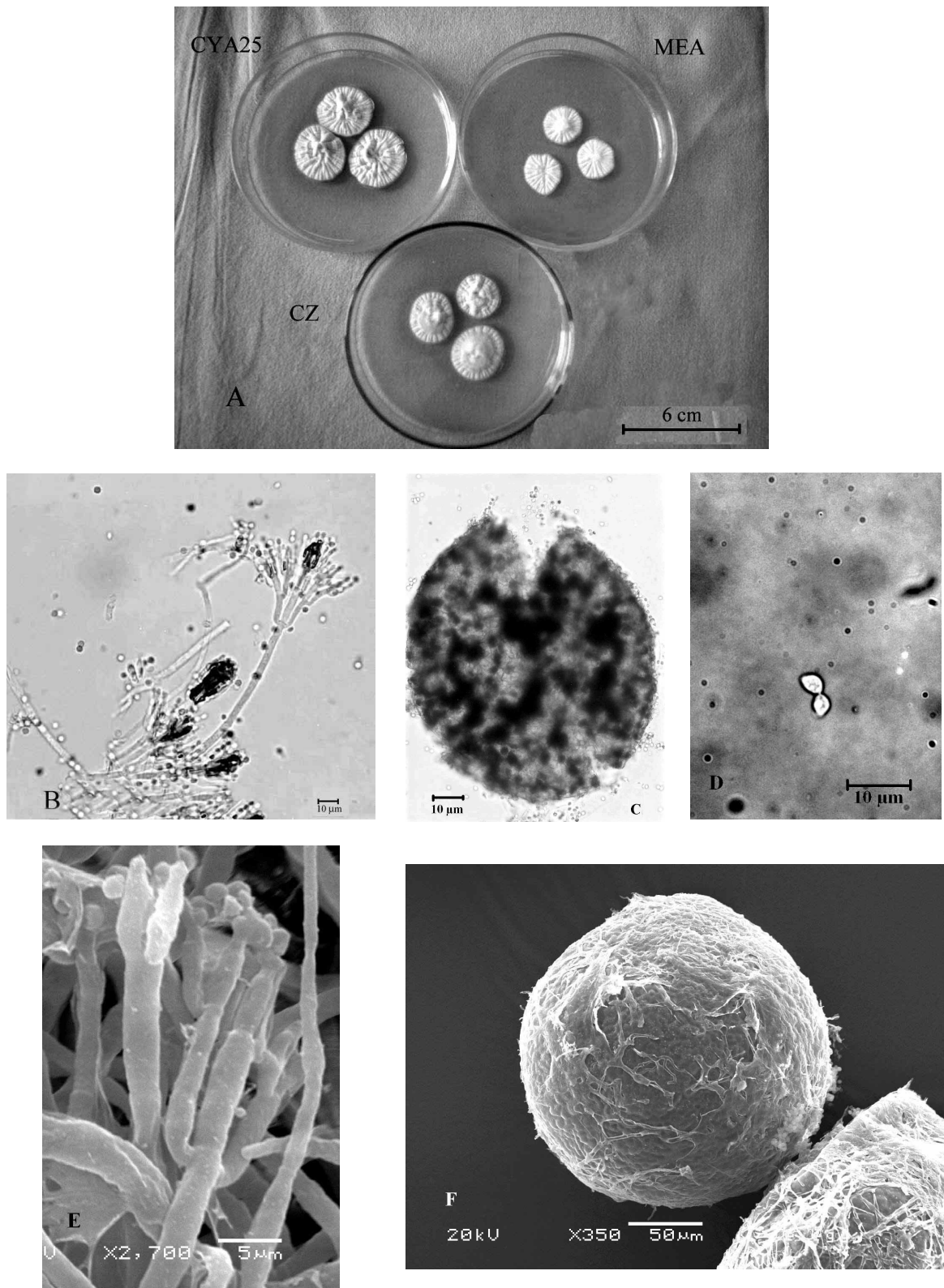


Figure 3. *Eupenicillium egypticum* A) Colonial appearance (7 days); Light microscopic appearance of B) penicilli C) cleistothecium and D) ascus; SEM appearance of E) penicilli and F) cleistothecium.

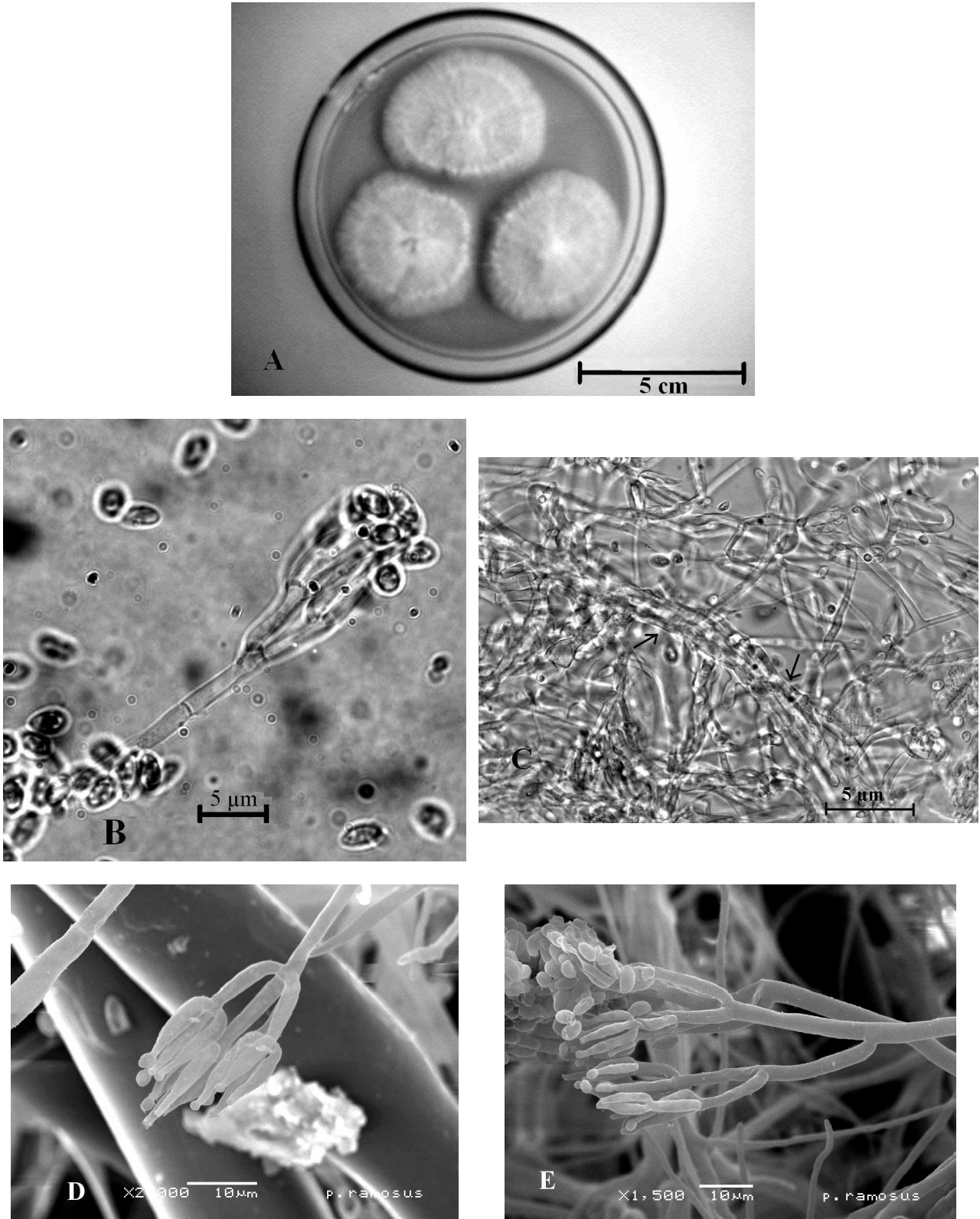


Figure 4. *Paecilomyces ramosus* A) Colonial appearance (7 days); Light microscopic appearance of B) conidiofor and conidia C) synnematosus structure; SEM appearance of D) Phialides and tapering collula and E) branching and phialides.

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 tapered 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

The species belonging to the genera *Aspergillus* and *Penicillium* exist in greater numbers and more frequently than the other species in soil. In the checklist of mycoflora in Turkey, Asan (2004) reported that there were 200 *Aspergillus* species and 116 *Penicillium* species isolated from different regions of Turkey. The numbers include *P. novae-zeelandiae*, which were isolated in this study. In the same aforementioned checklist, 11 *Eupenicillium* and 10 *Paecilomyces* species were reported for Turkey, including *E. egyptiacum* and *P. ramosus* (Asan, 2004).

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 entomopathogen. 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 intervoven 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 cleistothecia 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, synnematous 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.

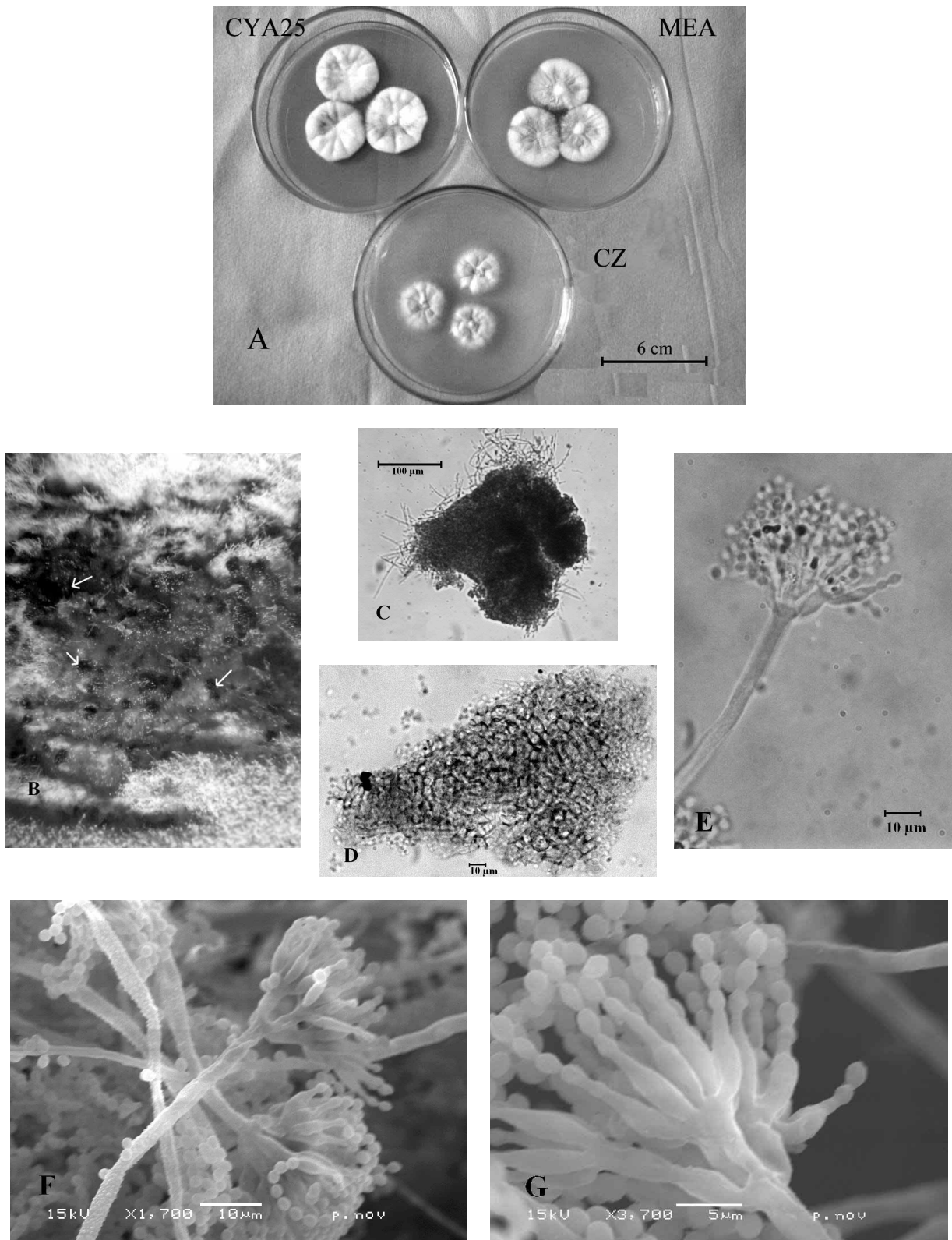


Figure 5. *Penicillium novae-zelandiae* A) Colonial appearance (7 days); Light microscopic appearance of B) entire sclerotia in solid medium, C) one sclerotium and D) polygonal cells of sclerotium, E) penicilli; SEM appearance of F) penicilli and G) phialides and conidia.

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