



Geliş(Received) :23/06/2018
Kabul(Accepted) :13/07/2018

Research Article
Doi:10.30708/mantar.435710

Effect of Combinations of Salt and Temperature on Morphological Characteristics of Microfungi

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Abstract

Aim of this study is investigation of effects of different salt and temperature combinations on morphological characteristics of microfungi that were isolated and identified with traditional and molecular methods from Çamaltı Saltern/İzmir. A total of 37 species as 3 species belong to *Alternaria*, 13 *Aspergillus*, 2 *Chaetomium*, 5 *Cladosporium*, 9 *Penicillium*, and 1 species belong to each species as *Acremonium*, *Arthrimum*, *Biscogniauxua*, *Diaporthe*, *Fusarium* were used in this study. Using aseptic techniques, each of the microfungi were inoculated on Mat Extract Agar medium that was added 8%, 16%, 24% NaCl. Each concentration media was incubated at 17, 27, 37 °C at 7 days. After incubation of each cultures were investigated spore formation, mycelium diameter, colony front and back colour, colony texture, colony diameters, sclerotium and cleistothecium. All results were collected in a table and photographed. As a result of the study, we showed that combination of different NaCl concentrations and temperatures have affected on colony properties. Furthermore, these effects revealed morphological differences between closely related fungi such as members of section *Versicolor*, *Aspergillus*, *Clavati*, *Circumdati*, *Flavi*, *Penicillium* subgenus *Penicillium*.

Key words: Salt, Temperature, Microfungi, Combine effect

Mikrofungusların Morfolojik Karakterleri Üzerine Tuz Ve Sıcaklık Kombinasyonlarının Etkisi

Öz: Bu çalışmanın amacı, farklı tuz ve sıcaklık kombinasyonlarının Çamaltı Tuzlası/İzmir'den izole edilen, geleneksel ve moleküler yöntemlerle tanımlanmış mikrofungusların morfolojik karakterleri üzerindeki etkilerinin araştırılmasıdır. Bu çalışmada 3 *Alternaria*, 13 *Aspergillus*, 2 *Chaetomium*, 5 *Cladosporium*, 9 *Penicillium* ve *Acremonium*, *Arthrimum*, *Biscogniauxua*, *Diaporthe*, *Fusarium* cinlerinin her birinden 1'er tane olmak üzere toplam 37 tür kullanılmıştır. Aseptik teknikler kullanılarak, mikrofungusların her biri, %8, %16, %24 NaCl eklenmiş Mat Ekstrakt Agar plaklarına aşılanmıştır. Her konsantrasyon ortamı 17, 27, 37°C'de 7 gün boyunca inkübe edilmiştir. Her kültürün inkübasyonundan sonra spor oluşumu, miselyum çapı, koloni ön ve arka rengi, koloni dokusu, koloni çapı, sklerotium ve kleistothecium varlığı incelenmiştir. Bütün sonuçlar bir tabloda toplanmış ve fotoğraflanmıştır. Çalışmanın sonucunda, farklı NaCl konsantrasyonu ve sıcaklık kombinasyonlarının koloni özelliklerine etki ettiği gösterilmiştir. Ayrıca, bu etkiler, *Versicolor*, *Aspergillus*, *Clavati*, *Circumdati*, *Flavi*, *Penicillium* subgenus *Penicillium* üyeleri gibi yakın ilişkili mikrofunguslar arasında morfolojik farklılıklar ortaya çıkarmıştır.

Anahtar kelimeler: Tuz, Sıcaklık, Mikrofungi, Kombine etki



Introduction

Microfungi are common microorganisms found in range habitats from soil, air, water and various food products to extreme environments (Krijgsheld et al., 2013; Selbmann et al., 2013; Houbraken et al., 2014; Egbuta et al., 2015; Chavez et al., 2015). In addition, they are well known as decomposer of organic materials, producer of some product in industrial and food fields, producer of important mycotoxins, reason major economic and health effects on plant, animal and human life (Asan, 2004; Shukri et al., 2015; Egbuta, 2015; Demirel, 2016; Shukri et al., 2017). Owing to their numerous impacts, studies on the identification of microfungi are vitally important.

There are numerous identification methods for microfungi based on morphological characters or molecular assays (Ciardo et al., 2007; Papagianni, 2014; Becker et al., 2014; Shukri et al., 2015). Molecular assays based on the DNA have been developing day by day and they are very successful on correct identification of microfungi. However, these assays are expensive, labour-intensive, require to interdisciplinary experiences and including a high risk for both environment and life due to using chemicals and radiation (Guarro et al., 1999; McClenny 2005; Tsui et al., 2011; Becker et al., 2014). In addition, experiences of researchers about morphological properties of investigating microfungi are necessary for correct identification, understanding of contamination, selection of PCR primers and workflow on phylogeny (Samson et al., 2010). Morphological typing methods are the phenotypic approach including macroscopic and microscopic investigation of observable characters and have been used for many years (Raper and Thom, 1949; Raper and Fennell, 1965; Pitt, 1979; Pitt, 2000; Klich, 2002; Samson et al., 2010). Morphological typing methods have critical limitations such as taking very long time, labour-intensive, effects of experiences and decisions of researchers, losing of some morphological characters and more than these factors (Guardo et al., 1999; Verweij et al., 2007; Samson et al., 2010; Sharma and Pandey, 2010; Becker et al., 2014; Demirel 2016; Hase and Nasreen, 2017). Although these negative qualifications of morphological identification techniques and continuing to improve and become more readily available of molecular identification techniques, conventional techniques based on microscopy and culture the primary laboratory tools for detecting and identification of microfungi (McClenny, 2005).

The development of new methods will ensure that traditional identification methods are improved and help to distinguish of especially closely related microfungi. Therefore, the purpose of the present study was to determination effects on phenotypic properties of microfungi of combination of salt and temperature conditions. Furthermore, we will discuss on the potential to be a conventional identification criterion of the investigated conditions.

Material and Method

Fungal Strains

The isolates that were used in this study were from a collection archived in the Eskisehir Osmangazi University, Department of Biology. All of the isolates used in this study were isolated from Camaltı Saltern/Izmir province and identified using the molecular and traditional methods in previous studies (GenBank accession numbers; KU958178, KX014873, KX022485-KX022492, KX056227-KX056238, KX426682-KX426702). Cultures were maintained at 4°C on potato dextrose agar (PDA).

Media, Growth Conditions and Determination of Growth Characteristics

A modified Malt Extract Agar (Merck 1.05398) with the different NaCl concentrations as 8, 16 and 24 % that were selected according to previous some studies (Ventosa and Arahall, 2009; Gunde-Cimerman et al., 2009, Gunde-Cimerman and Zalar, 2014) was used as the growth medium for all isolates. For the inoculation of microfungi, spore suspension was prepared in 0.1% Tween 80 and then 3 µl of spore suspension were used as three points on medium. After inoculation, the plates were incubated at 17, 27 and 37 °C (Hedi et al., 2009; Ventosa and Arahall, 2009; Gunde-Cimerman et al., 2009, Gunde-Cimerman and Zalar, 2014) for 7 days. For each of the isolates, one of the MEA without NaCl was incubated at 27 °C for 7 days and used as positive growth control. End of the incubation period, mycelial properties, colony diameter, colony colour, texture, soluble pigment, exudate and reverse colour were investigated and evaluated.

Result and Discussion

Morphological analysis in this study showed a wide range of microfungal species belong to the genera *Acremonium* (1 strain, 2.7% of total strain), *Alternaria* (3 strains, 8.1%), *Arthrinium* (1 strain, 2.7%), *Aspergillus* (13 strains, 35.1%), *Biscogniauxia* (1 strain, 2.7%), *Chaetomium* (2 strains, 5.4%), *Cladosporium* (5 strains, 13.5%), *Diaporthe* (1 strain, 2.7%), *Fusarium* (1 strains, 2.7%) and *Penicillium* (9 strains, 24.3%). All of the presented as generic concept at below. The comparing photographs of tested fungi were shared as representing genus if investigated genus have colony formatted and had more than one species.

Generic concept of *Aspergillus* members

In this study, 13 different species belong to *Aspergillus* genera were investigated and we discussed our results as section by section.

Aspergillus tamarii, *A. sclerotium* and *A. melleus* belong to *Circumdati* section were investigated. When they were compared with their controls (without NaCl, incubation at 27 °C), *A. sclerotium* and *A. melleus* showed increasing colony diameter on 8% NaCl and at 27 °C as



from 47 mm to 65 mm and from 48 mm to 60 mm, respectively. Furthermore, *A. melleus* clearly distinguished from other section members with no colony formation at the other temperatures. *A. tamarii* showed same colony diameter (as 24 mm) and same colony properties with the control on 8% NaCl at 27 °C.

A. sclerotium distinguished from other members of the section with formed a colony on same NaCl concentration at 17 °C as 20 mm and 16% NaCl at 27 °C as 13 mm, sporulation decreased from heavy to poor. When the results of the incubation on 8% NaCl at 37 °C were investigated, we determined that colony size of species clearly smaller than other conditions. In the same conditions, *A. sclerotium* showed velvety colony texture rather than cotton differently from control. Colony formation and sporulation properties prominently decreased together with increasing of NaCl concentration. Apart from that, there were not a colony on 16% NaCl at 17 and 37 °C, 24% NaCl at all temperatures.

Aspergillus versicolor and *A. sydowii* belong to *Versicolor* section are very similar in terms of morphological properties (Samson et al., 2010). As result of combination of different salt and temperature levels, *A. sydowii* (Figure 1; I-A) clearly distinguished from *A. versicolor* (Figure 1; I-B) with heavy sporulation and increasing colony diameter as 35 mm on 8% NaCl at 27 °C. Both of two species formed similar micro colony on 16% NaCl at 27 °C and there was not a colony formation at the other conditions. Section *Nidulanti* is known as one of the similar section to members of *Versicolor* section (Samson et al., 2014) and include less halotolerant members (Samson et al., 2010). We investigated *A. quadrilineatus* from this section and determined that the species formed smaller and moderately sporulated colonies (40 mm and 38 mm on 8% NaCl at 27 °C and 37 °C) than control (65 mm at 27 °C) but bigger than members of *Versicolor* section.

Other investigated species are *A. amstelodami* and *A. glaucus* belong to section *Aspergillus*. It is very clear that *A. amstelodami* showed colony formation with large colony and heavy sporulation on 8% (62 mm) and 16% NaCl (44 mm) at 37 °C, but *A. glaucus* occurred only small colony and very poor sporulation on 8% NaCl at 37 °C.

The section *Clavati* and section *Fumigati* are known as closely related members of *Aspergillus* genus as consequence of phylogeny studies (Samson et al., 2014). The control of *A. clavatus* formed 45 mm colony diameter and heavy sporulation like *A. fumigatus* that formed 70 mm colony diameter and heavy sporulation. As result of combined salt and temperature, they were clearly distinguished as *A. fumigatus* formed still large colony as 60 mm diameter and heavy sporulation while *A. clavatus* formed very small as 15 mm and moderately sporulation on 8% NaCl at 37 °C.

One of the characteristic situation were determined for *A. flavus* that is member of section *Flavi*. This species showed heavy sporulation and large colony formation as

70 mm on MEA without NaCl at 27 °C. Colony properties of this species were determined as moderately sporulation and small colony as 47 mm and 20 mm on 8% NaCl at 27 °C and 16% NaCl at 37 °C., respectively. Furthermore, *A. flavus* occurred very large colony as 75 mm on 8% NaCl at 37 °C. This shown that combined of salt and temperature have a distinctive potential.

Similarly, *A. niger* belong to section *Nigri* shown increasing of colony diameter and sporulation together with increasing of NaCl concentration and temperature. This species formed moderately sporulation, 65 and 80 mm colonies on 8% NaCl at 27 °C and 34 °C while control of this species formed 55 mm diameter colony and heavy sporulation at 27 °C.

A. terreus that is a member of section *Terrei* is a worldwide common microfungus and an important human pathogen. Already, in applied experiment condition, investigated *A. terreus* isolate has perfectly grown up at 37 °C on both 8% NaCl (68 mm) and 16% NaCl (24 mm). Similarly, this species formed strong colony formation at 27 °C on both 8% NaCl (50 mm) and 16% NaCl (11 mm). We reported as sporulation performance decreased as parallel of colony diameter. In addition, this member exhibited only micro colony formation at 17 °C on 8% NaCl. Furthermore, this species did not form a colony on 24% NaCl at all tested temperatures.

Generic concept of *Penicillium* members

In this study, 9 different species belong to *Penicillium* genera were investigated and all of them belonged to *Penicillium* subgenus *Penicillium*. Investigated species were mainly showed colony formation at 17 and 27 °C, % 8 and 16 NaCl concentration. Between NaCl concentration/temperature and sporulation degree were determined opposite interaction. In addition, we were observed equal or small size of colony than control with increasing NaCl concentration and temperature. The other common properties of investigated species, reverse colony colour, poor green and white colony colours and velvety colony texture were steady although NaCl concentration and temperature were changed.

Only *P. spinulosum* among investigated *Penicillium* members showed good colony formation at 8 % NaCl concentration and both 17 and 27 °C as 30 mm and 15 mm, respectively. *P. griseofulvum*, *P. nalgiovense*, *P. coryophilum*, *P. oxalicum*, *P. olsonii*, *P. citrinum* and *P. commune* formatted colonies at 8 and 16 % NaCl and 17 and 27 °C until 16 mm. Among *Penicillium* members, *P. griseofulvum*, *P. nalgiovense*, *P. camemberti*, *P. citrinum* and *P. commune* were distinguished with colony formation and sporulation at 8 % NaCl and 37 °C as from 7 mm to 26 mm and from poor to heavy sporulation. In addition, *P. citrinum* and *P. commune* distinctively formatted micro colony at 16 % NaCl concentration and 37 °C. In this point, *P. camemberti* and *P. commune* are of the similar species belong to *Penicillium* subgenus *Penicillium* (Samson et al.,



2010). We exhibited an option for distinguish of these two similar species. Another of the closely related species to *P. commune* (Figure 1; II-A) is *P. corylophilum* (Figure 1; II-B) and our results showed that these two species distinguished by using of parameter of micro colony formation at 16 % NaCl concentration and 37 °C of *P. commune*.

Generic concept of other microfungi

In this study, 15 different species belong to 8 genera were investigated and we discussed our results as genus by genus and comparing with controls.

The *Acremonium* genus is consist of septate hyphae giving rise to thin, for this reason, distinguish of them under microscope is difficult (Summerbell et al., 2011). The isolate *Acremonium implicatum* was investigated and this isolate formed micro colony (5 mm) with poor sporulation at only 8% NaCl concentration and 27 °C. The control formed colony diameter 20 mm and heavy sporulation. As different from control, the colony and reverse colours of this isolate changed as pale and colony texture was come to woody from bouquet at 8% NaCl concentration and 27 °C.

We investigate 3 different species of *Alternaria* genus as *A. alternata* and *A. tenuissima* members of section *Alternata* and *A. brassicicola* member of section *Brassicicola* (Woudenberg et al., 2013). *A. tenuissima* species group have often been misidentified as *A. alternata*. *A. alternata* differs by some microscopic properties such as branching conidial chains via short secondary conidiophores from apex in addition to central and basal cell of the conidium body (Samson et al., 2010). We found one more cultural differences between these two closely related *Alternaria* species. While *A. tenuissima* were not grow up on 8% NaCl and 17 °C (Figure 1; III-A), *A. alternata* formed colony diameter of 17 mm on same conditions (Figure 1; III-B). As different from control inoculation, this colony recorded as poor sporulation, pale reverse colour.

Arthrinium arundinis that is one of the endophyte present on the range substrate (Crous and Groenewald, 2013) exhibited colony formation on 8% NaCl at 27 and 17 °C. When colony properties compared with control condition, this species showed less colony diameter (to 25 mm from 75 mm), sporulation (to absent from moderately) and increased mycelium than control. In addition, *A. arundinis* distinguished with chancing colony texture to cottony from velvety.

While *Biscogniauxia mediterranea* formed colony on control condition as 75 mm colony diameter, slightly sporulation and heavy mycelium, growing on condition included NaCl at any concentration did not show.

The genus *Chaetomium* was described by Kunze based on *C. globosum* and because of the poorly information original description, identification of members of this genus was difficult and description process was repeated again and again (Wang et al., 2016). Investigated two species belong to *Chaetomium* genus as *C. globosum* and *C. subaffine*, identified species level, did not form colony while control inoculation grown up as diameter of 70 and 80 mm, respectively, heavy sporulation, brownish reverse colour, greenish colony colour and velvety texture for *C. globosum*, poor sporulation, media reverse colour, white mycelial colour dominate and woody colony texture for *C. subaffine*.

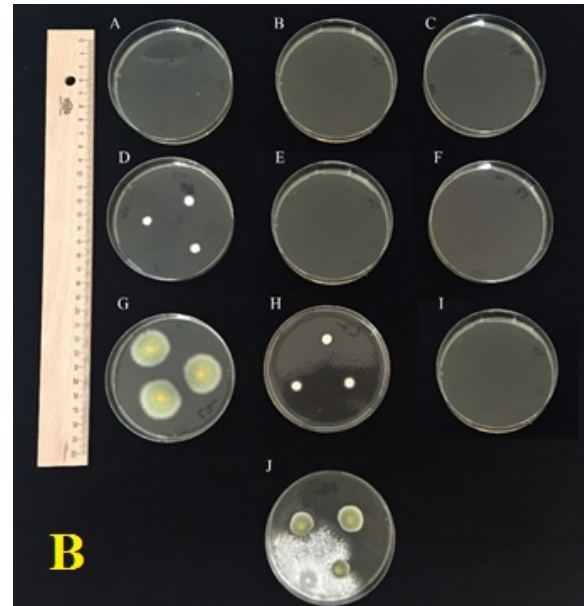
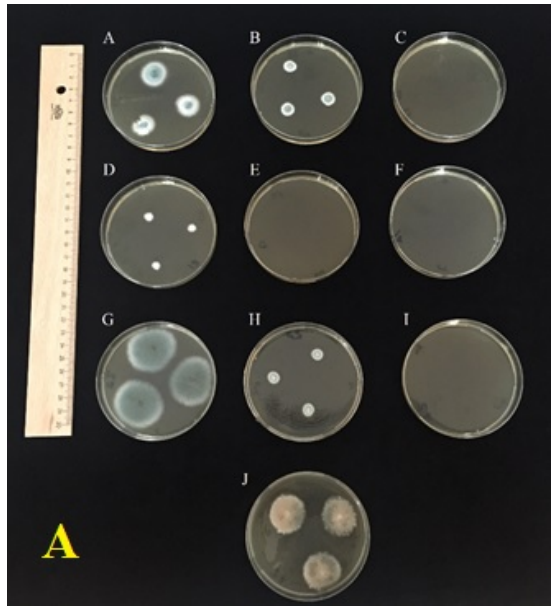
Totally five species of *Cladosporium* genus as *C. cladosporioides*, *C. herbarum*, *C. ramotenellum*, *C. sphaerospermum* and *C. variable*, were investigated. Application of different NaCl concentrations under different incubation temperatures showed that *C. variable* distinctly distinguished from other genus members with micro colony formation only on 8% NaCl at 27 °C. In addition, *C. herbarium* showed very clear distinctive result as 25 mm colony formation with moderately sporulation on 8% NaCl at 27 °C. One more clear result obtained for *C. sphaerospermum*. This species did not grown up on NaCl presence while control exhibited 11 mm diameter colony and heavy sporulation. Among investigated species, *C. ramotenellum* (Figure 1; IV-A) and *C. cladosporioides* (Figure 1; IV-B) formed colony on 8% NaCl concentration at 17 and 27 °C. When morphologically compared with control, the control exhibited colony in 14-26 mm diameter with heavy sporulation. On NaCl concentration, small colonies occurred as micro -22 mm with poor or moderately sporulation.

Diaporthe foeniculina showed growing on 8% NaCl concentration at 27 and 17 °C. When colony formation of this isolate compared with control and itself, colony diameter and sporulation dramatically decreased in the presence NaCl and at low temperature as 25 mm on control, 20 mm on 8% NaCl at 27 °C, 14 mm on 8% NaCl at 17 °C and heavy sporulation on control, moderately on 8% NaCl at 27 °C, slightly on 8% NaCl at 17 °C.

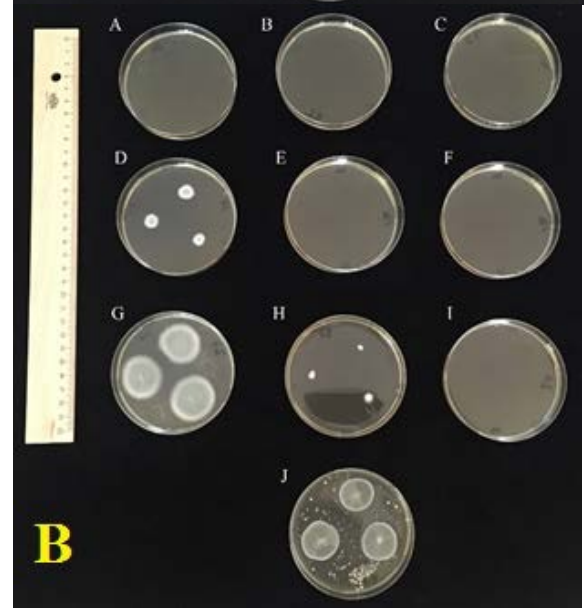
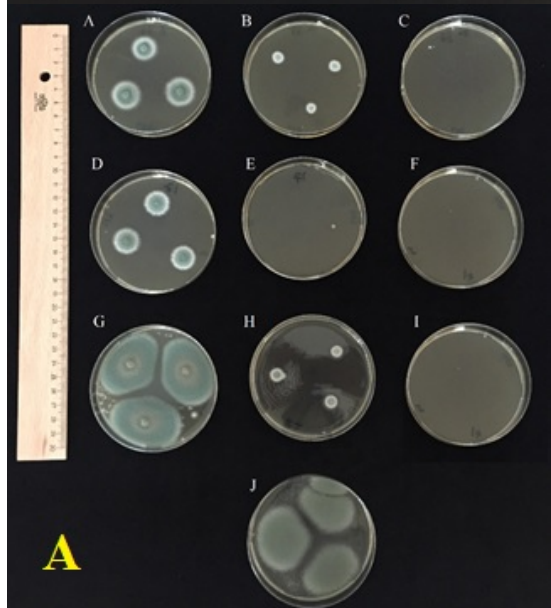
Fusarium proliferatum exhibited colony formation on 8% NaCl and at 27 and 37 °C. When we compared colony properties with control, we determined that colony diameter, sporulation and mycelial properties decreased to 22 mm from 62 mm on 8% NaCl, to slightly from heavy, to 2mm from 7 mm, respectively and to 7 mm on 8% NaCl and at 37 °C. The result indicated that colony diameter, sporulation, mycelial wideness decreased together with NaCl presence, in addition, sporulation loosed with increasing incubation temperature. For this species, both presence of 8% NaCl and incubation at 37 °C exhibited distinguish characters.



I)

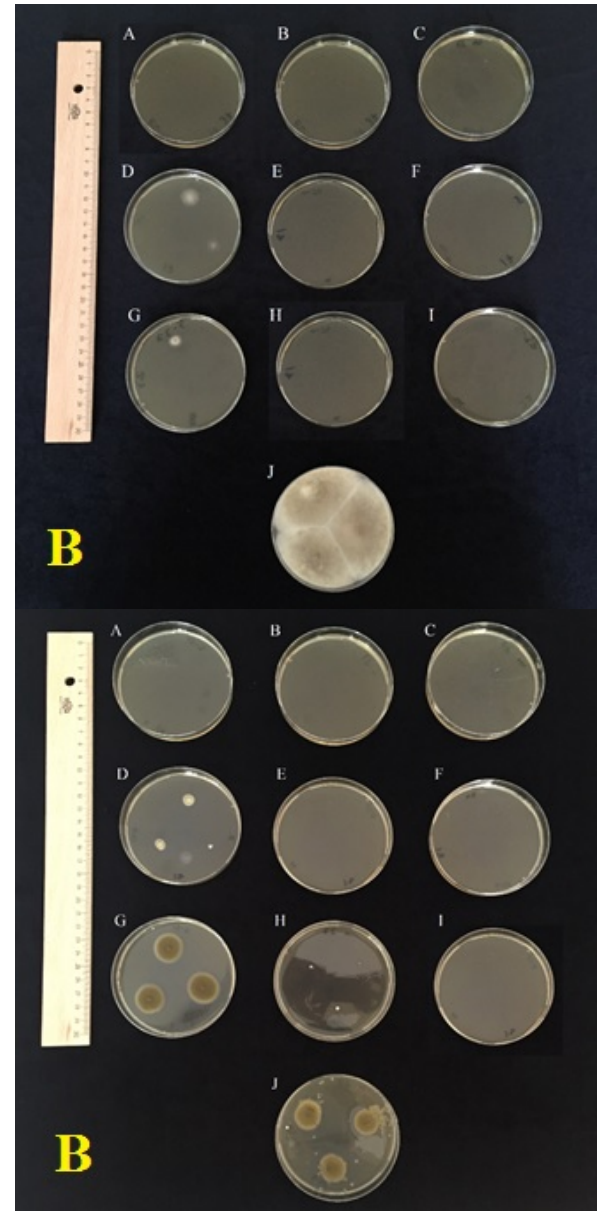
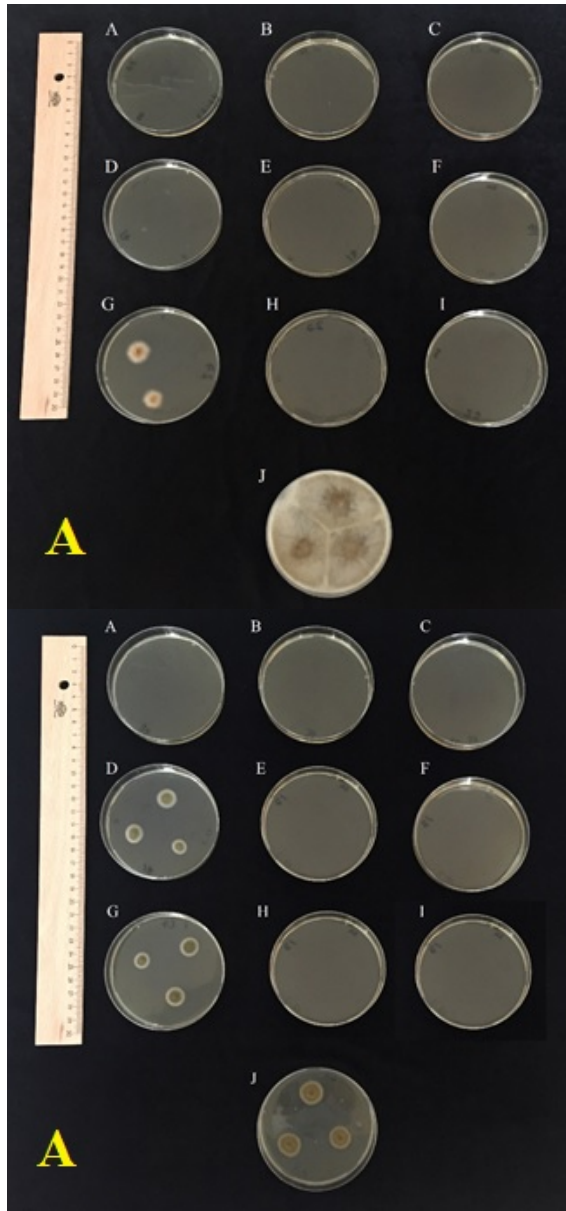


II)





III)



IV)

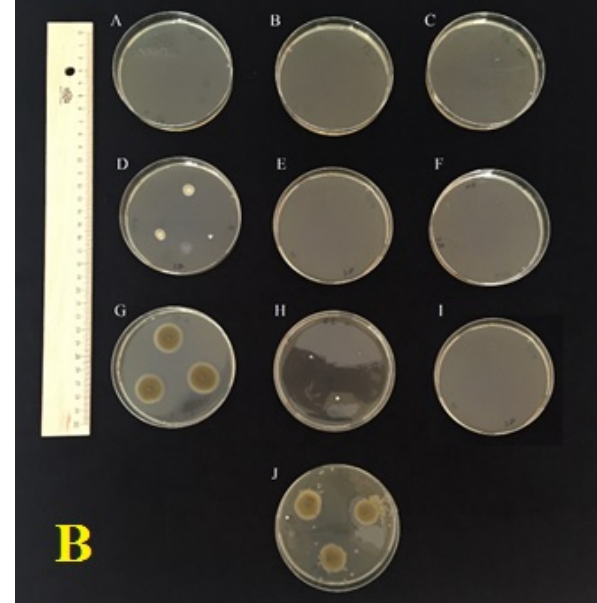
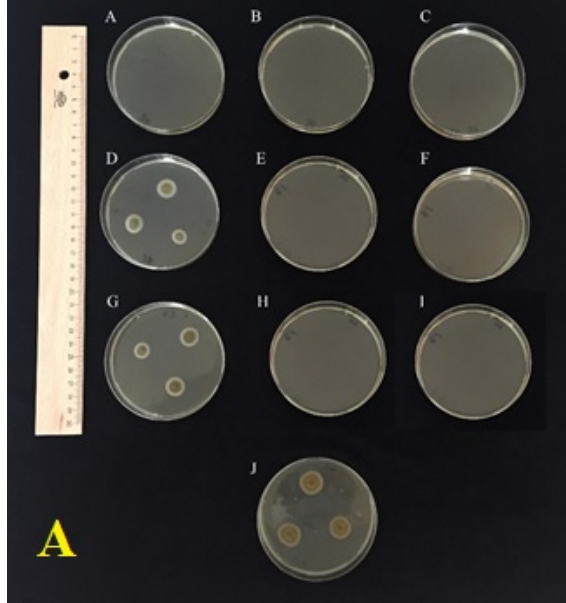


Figure 1. Colony characteristics of some tested fungi on different NaCl concentrations and temperatures; **I) A)** *Aspergillus sydowii* **B)** *Aspergillus versicolor*; **II) A)** *Penicillium commune*, **B)** *Penicillium corylophilum*; **III) A),** *Alternaria tenuissima* **B)** *Alternaria alternata*; **IV) A)** *Cladosporium ramotenellum* **B)** *Cladosporium cladosporioides* **a)** 8% NaCl, **b)** 16% NaCl, **c)** 24% NaCl at 37°C **d)** 8% NaCl, **e)** 16% NaCl, **f)** 24% NaCl at 17°C **g)** 8% NaCl, **h)** 16% NaCl, **i)** 24% NaCl at 27°C **j)** 0% NaCl at 27°C

Conclusion

Morphologically investigation and identification processes have always given successful result as dependent to researchers and used media. How many different media and incubation conditions are used, obtained results will be highly reliable. In addition, the researchers will easily reach the conclusion. In the present study showed that using of the combination of different NaCl concentration and incubation temperatures distinctly distinguish closely related fungi such as members of section *Versicolor*, *Aspergillus*, *Clavati*, *Circumdati*, *Flavi*,

Penicillium subgenus *Penicillium* and some other microfungi. Mainly, increasing of the NaCl concentration decreased colony formation and sporulation. There are very limited species that formed colony and sporulation on high NaCl concentration and at temperature. This result may have effective of distinguish, identification and description of some microfungi.

Acknowledgements

We are very grateful to project team of "Determination of Fungus Diversity in İzmir Çamaltı



Saltern Mycobiota: *Dematiaceae*, Project Number is: 201319A101” financially supported by “Eskisehir Osmangazi University, Scientific Research Projects” for

Dematiaceae isolates and research team of “Determination of Fungus Diversity in İzmir Çamaltı Salt: *Moniliaceae* and Teleomorphs.

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