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Fungal species isolated from *Quercus castaneifolia* in Hyrcanian Forests, North of Iran

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ABSTRACT

Kavosi MR, Feridon F, Hajizadeh G. 2013. Fungal species isolated from Quercus castaneifolia *in Hyrcanian Forests, North of Iran. Biodiversitas 14: 61-66.* In order to isolate and identify of fungi associated with *Quercus castaneifolia* seed, sampling carried out in Shast-Kalate, Ghorogh, Loveh and Golestan forest. Collected seeds sterilized and then separated sections including: outer section of seed (crust) and inner seed section (endosperm). Each section of seed tissue is cultured on potato dextrose agar media. After sub-culture and providing of the fungi pure cultures, various species isolated and identified by spores characteristics, their size and color, including: *Aspergillus flavus*, *A. niger*, *Curvularia affinis*, *Trichoderma harzianum*, *Trichothecium roseum*, *Eurotium rubrum*, *E. amstelodami*, *Penicillium implicatum*, *P. fellutanum*, *Diplodia* sp. *Nigrospora gossypi*, *Alternaria alternata* , *Fusarium oxysporum* and *Beltrania santapaui*. The most frequency of fungus in Shast-Kalate forest was *P. implicatum* by 74% of frequency within seed section, the most frequency in Ghorogh and Loveh Forest was *P. fellutanum* with 63 and 66% of frequency within seed section respectively and the most frequency in Golestan forest was *B. santapaui* by 51% of frequency outer seed section. The result showed diversity of the fungi on the outer seed section is higher than within seed section. The results also showed during several isolation a saprophyte fungus always could be finding on the acorn seeds. This is the comprehensive report on fungi associated with *Quercus castaneifolia* seed in Hyrcanian forest, North of Iran.

Key words: crust, endosperm, fungal, Hyrcanian forest, *Quercus castaneifolia*, seed

INTRODUCTION

Forest tree seeds continuously are affected by physical and physiological disturbance which most of these diseases caused by fungi. Health and growth ability sapling considerably depend on seed quality (Mittal and Mathur 1998). Most of fungi associate with seeds of forest trees are molds which expand on surface of seed and sometime they are inner pollution factor (Huss 1956). Effect of mold on seeds is that they seem health apparently but they originally have spoiled on basis of vitality considerable (Shea 1957). Recently known that all seeds contain microscopic fungi spores whether on surface of seed or inside of seed (Singh and Mathur 1993). Urosevic (1961) was specified that some of fungi spores have germinated and after growing and mycelium penetrating, it influences in to the cotyledon which through have nourished from germs.

Fungus associate with seed can cause weakness of seed germination directly and indirectly and can dispose these seeds to earthborn pathogen fungi attack (Gibson 1957). Healthy seed in forest for natural regeneration is important issues which future life forest depends on it. Disease and damaged seeds even under suitable environmental condition cannot have desired regeneration for forest survival or cannot cause specific species. The trees appeared from damaged and diseased seeds have slight growth and seeds produced by these trees will be had low vitality (Rai and Mamatha 2005).

The purpose of this study was identification of inner and outer fungi of Chestnut-leaved oak (*Quercus castaneifolia*) seed and specifying their frequency in Golestan province forests.

MATERIALS AND METHODS

Sampling site

In this research four forest regions in Golestan province, north of Iran including (i) Shast-Kalate Research and Education Forest, (ii) Ghorogh Forest Park, (iii) Loveh Research Forest, and (iv) Golestan National Park were selected (Figure 1). In each region, four trees were chosen randomly, and 25 seeds of each tree were selected randomly. Collected seeds have been settled in new and sterile bags and after recording region specification and collection date, they transferred to laboratory and settled in a place with suitable temperature and ventilation. In finally, four samples of 25 kinds of seeds of each region were collected.

Isolation and purification seed fungi

For fungus isolation, seeds were divided less segments and also inner (crust) and outer (endosperm) portion. Separation of inner portion from outer portion of seed

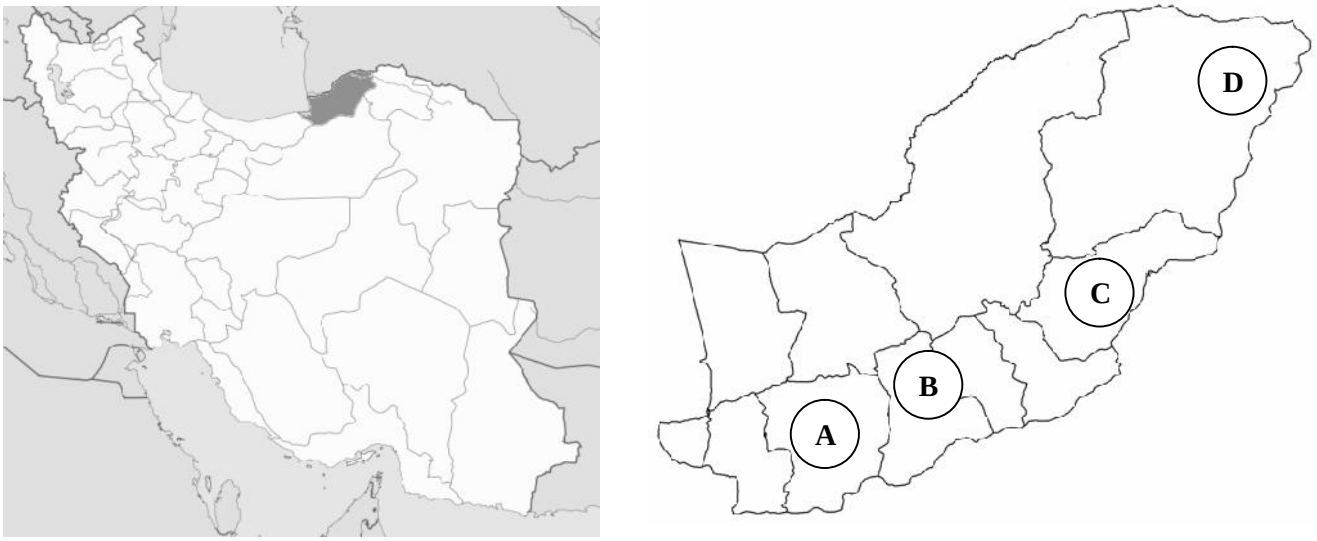


Figure 1. Location of the study site inside the Hyrcanian forests of Golestan Province, North of Iran, including: A. Shastkalate Research and Education Forest, B. Ghorogh Forest Park, C. Loveh Research Forest, and D. Golestan National Park.

due to comparing mentioned two portion fungi was conducted. After seed was divided less segment, its surface was disinfected with 0.5% sodium hypochlorite and ethanol during 1-2 minutes and was washed three times with sterile Distilled water and settled in to sterile filter paper for desiccating. Then segments of inner and outer portion of seed separately and with four repetitions on nutrition medium of potato-dextrose-agar (PDA) extract contain lactic acid and preserved in incubator in 25±1°C. After three days, grown fungi were subculture on medium and hereby fungi become sterilization.

Identification of fungi

Identification of fungi genus after their growing on the seed segments was used valid reference of Barnett and Hunter (1998) and Ellis (1976) and their classification on basis of Eriksson (2006) and Alexopoulos et al. (1996). For identification of species was used various medium and valid reference. Further species were identified and purified on PDA medium and 25°C in absolute darkness. For some of species like *Fusarium* which do not know spore Carnation leaf-piece Agar (CLA) medium and optical period LD 12:12 in 25°C according to Nelson et al. (1983) and Saremi (1998) method were used. For *Alternaria*, for denoting spore number in spore chain, LD 16:8 optical period in 20-23°C and Agar-Water (AW) medium in addition to PDA were used according to Dingra and Sinclair (1995) method and on basis of Ellis (1971, 1976) cognition key. About *Trichoderma*, LD 12:12 optical period and 25°C according to Dingra and Sinclair (1995) method and, Kubicek and Harman (1998) cognition key were used. Pitt (1997, 2000) cognition keys was applied for identification of *Eurotium*, *Aspergillus*, *Penicillium* fungi. Litvinov (1967) and Ellis (1976) description the identification of *Trichothecium*, *Curvularia*, *Beltrania* and *Nigrospora* fungi and Barnett and Hunter (1998) for identification *Diplodia* fungus.

This identification was on basis of various criteria such as presence or absence of septum; shape and size of ascus; ascospore; conidia and phialid, kind of ascospore; number of ascospore in each ascus; number of conidia laid on conidiophore or phialid; being one or more cellular of ascospore and conidia; in some species, presence or absence of metulae; diameter growth of colonies; colonies color and made.

RESULTS AND DISCUSSION

Species specification

Results of this study showed that all seeds polluted with one or more species of separated fungi which most of them were imperfect fungi or *Ascomycetes*. After sterilization and specification of thallus and colonies, 12 species including: *Nigrospora gossypii*, *Aspergillus flavus*, *A. niger*, *Trichoderma harzianum*, *Alternaria alternate*, *Trichothecium roseum*, *Fusarium oxysporum*, *Beltrania santapaui*, *Penicillium implicatum*, *Eurotium rubrum*, *Curvularia affinis* and *Diplodia* sp. become isolation and identification on *Quercus castaneifolia* seeds that frequency and description of specification of each one in detail is following in Table 1. All identification fungi on *Q. castaneifolia* seeds were reported from Hyrcanian Forests, North of Iran for the first time.

Alternaria alternata (Fr.) Keissl.

Colonies usually was approximately olivaceous to black and sometimes grey with pubescent appearance on PDA medium and 25°C. Colonies diameter growth after three days was 3-3.5 cm (Figure 2A). Conidiophores were partly small with 7-10×43-50 µm dimensions, simple and branched, approximately brown and even surface. Conidia were formed on WA medium in 6 to 17 fold chains (Figure 2C) and ovoid to obclavate or pear form and contain

Table 1. Fungi frequency percent of inner and outer portion of oak seed in four regions in Golestan province

Fungi	Regions							
	Shast-kalate		Ghorogh park		Loveh		Golestan park	
	I	O	I	O	I	O	I	O
Alternaria alternata	0	0	20	30	13	0	16	0
Diplodia sp.	0	23	0	0	0	0	0	0
Aspergillus flavus	27	46	0	8	0	0	5	3
Aspergillus niger	11	16	16	14	0	0	0	0
Curvularia affinis	39	0	0	0	0	0	0	0
Eurotium rubrum	0	35	0	34	0	0	0	0
Nigrospora gossypii	0	0	0	17	0	15	0	0
Penicillium implicatum	74	31	39	26	39	18	11	6
Beltrania santapaui	0	0	0	0	0	56	0	51
Fusarium oxysporum	0	0	0	0	0	35	0	19
Trichothecium roseum	0	41	13	5	0	0	0	0
Trichoderma harzianum	0	7	0	0	0	0	0	0

Note: I = inner, O = outer

surface covered by tiny tubers. Conidia have 2 to 7 transverse walls and 2 to 4 vertical walls and 12-34×6.5-12.5 µm dimensions and the end of the conidium nearest the conidiophore was round while it tapers towards the apex with 2.5-4 µm width (Figure 2B).

***Diplodia* sp.**

Colonies was specified with whitish yellow on PDA medium and 25°C (Figure 3A). Pycnidia were black, individual, spherical and stomatous (Figure 3B). Conidiophores were tiny and simple and conidia were dark, bicellular, 5-7 µm, elliptical or ovoid and in some of them there was curve (Figure 3C).

Aspergillus flavus Link

Colonies on PDA medium and 25°C was olive to lime green with a cream reverse. Colonies has fast diameter growth, after three days it was about 5.2 cm and has woolly to cottony texture which contain small granular (Figure 4A). Hyphae have light and septum. Conidia were settled on vesicle radially or perpendicular. Conidiophores were coarse and colourless and up to 800 µm length and 15-20 µm width. Vesicles were spherical to semi spherical (20-40 µm) and phialids (3-4×8-12 µm) covered approximately all surface of vesicle. Conidia were 3-6 µm, even, tiny and spherical to semi spherical (Figure 4B).

Aspergillus niger Tiegh. nom. cons.

Colonies at first was white but due to producing conidia become black and the reverse side seemed light yellow or pail on PDA medium and 25°C which during the growth, they produced radial gaps on medium (Figure 5A). Colonies diameter growth after three days was 4.3 cm. Hyphae had transparent and septum that conidia were settled on vesicle radially. This species produced metulae. Conidiophores were long (400 to 3000 µm), even and transparent that were dark in tip and end to bubble or cell of spherical vesicle (30-75 µm). Metulaes and phialids cover all surface of vesicle. Conidia were brown to black, uneven and with tuber, spherical and 4-5 µm (Figure 5B).

Curvularia affinis Boedijn.

Colonies was black to dark greenish black on PDA medium and 25°C which in white margins, colonies texture was cotton and its diameter growth after three days was 4.4 cm (Figure 6A). Conidiophores mostly were simple and had spores that formed with two sympodial geniculate. Conidia were 2 to 4 cells, 23-33×8-14 µm, fusiform and curve so that the central cell was typically darker and enlarged compared to the end cells in the conidium and the swelling of the central cell usually gave the conidium a curved appearance (Figure 6B).

Eurotium rubrum Jos. König etal Bainier & Sartory

Colonies on PDA medium and 25°C was reddish orange that in white margin, colonies texture was cotton and its diameter growth after three days was 3.5 cm (Figure 7A). Cleistothecia were occurred spherical to nearly elliptical form and with yellow colour (Figure 7B). Ascuses were almost egg form to elliptical, 12-13 µm and they had a tiny and unstable wall. Ascospores were unicellular, oblate (like a flattened sphere) and have equatorial ridges, thus resembling pulleys, 4.4-5×6.2-6.8 µm, elliptical, yellow with even margin and with eight fold form in to the Ascus (Figure 7C).

Nigrospora gossypii Jacz.

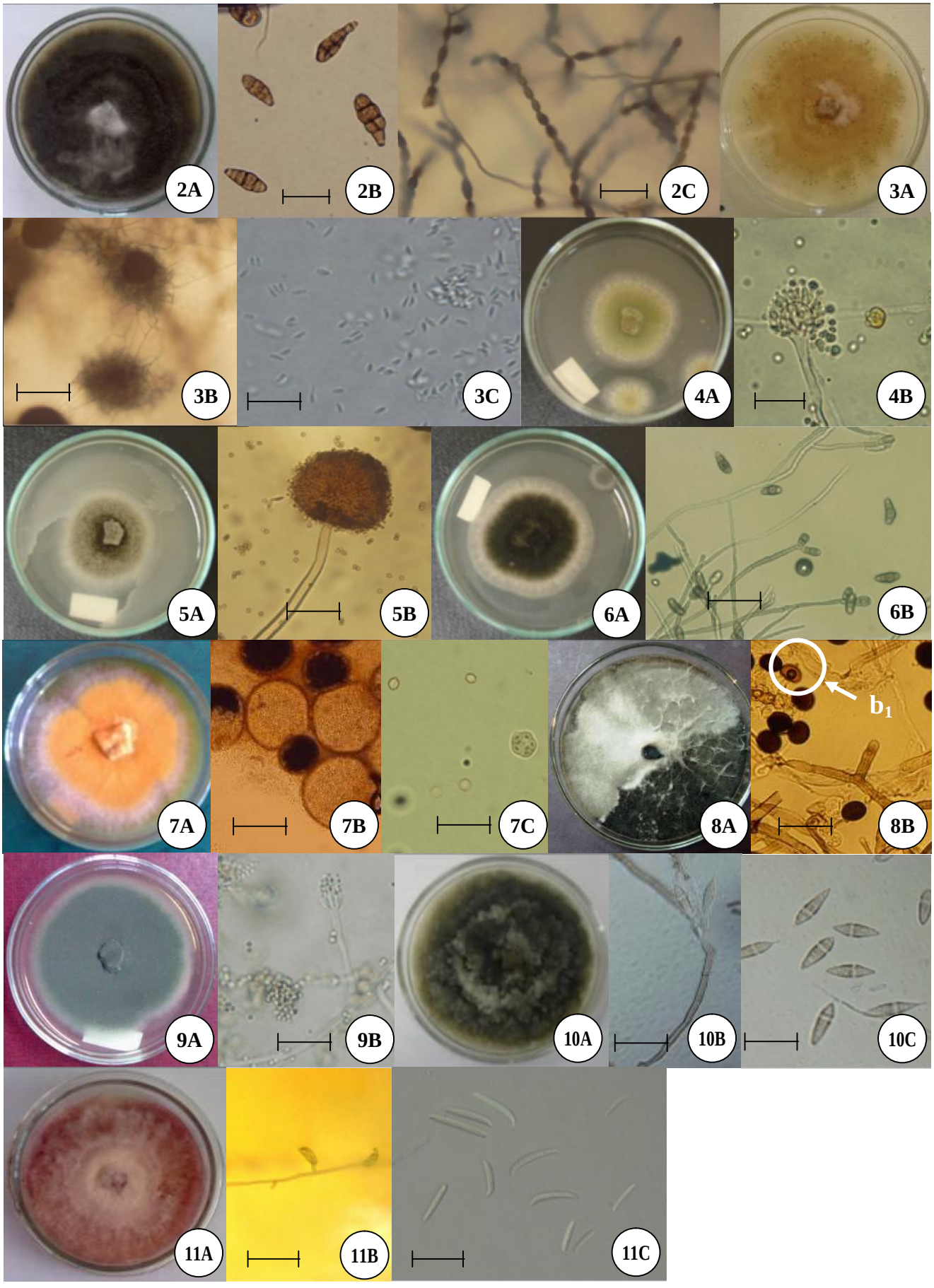
Colonies on PDA medium and 25°C was dark grey with small and large while point in its background which were basically cotton form that stick fungus mycelium to top of the container. It's colour was black grey and approximately dark-blue behind of container and colonies diameter growth after 3 days was 7-7.5 cm (Figure 8A). Conidiophores were simple, transparent and were settled vertically on mycelium which their length was 10-12.5 µm. Conidia were black, unicellular and semi spherical and partiy elliptical form with even surface and flat section that their size was 11-15 µm. This fungus also had middle chlamydospores (Figure 8B).

Penicillium implicatum Biourge

Colonies on PDA medium and 25°C at first was cotton white that finally will become powdery blue-green (Figure 9A). Colonies diameter growth after 3 days was 2.6 cm. Conidiophores were out of growth mycelium individually and ended to phialids. Conidiophore height was 25-50 µm and phialids length was 8-10 µm. Conidia were spherical, dark green, 2-3.5 µm, unicellular, and were formed from chains which youngest conidia settled in base of chain (Figure 9B).

Beltrania santapaui Pirozynski & Patil

Colonies on PDA medium and 25°C was grayish dark brown which behind of container was light grey and colonies growth on PDA medium after three days was 3.5 cm (Figure 10A). Conidiophores were simple and had septum which at the end, they were branched and conidia were formed on each of these branches (Figure 10B). Conidiophore length was 87.5 µm and conidia were seen


2A
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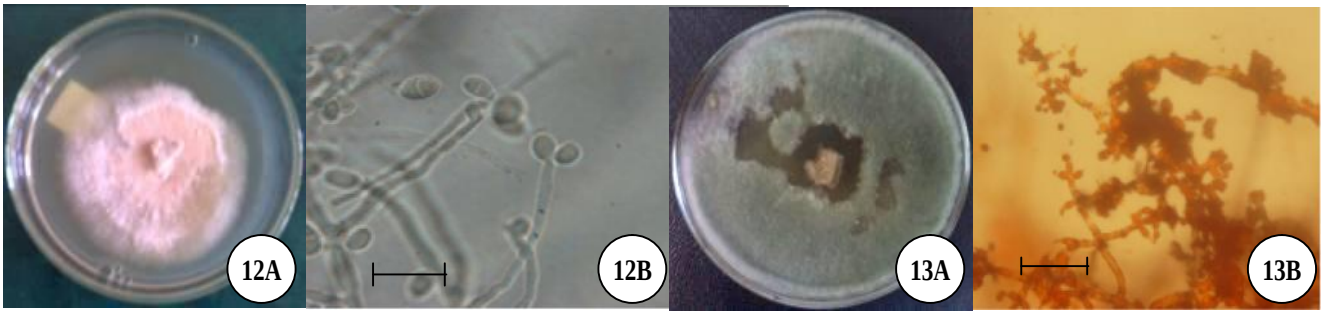

Figure 1. *Alternaria alternata* (a: colony on PDA medium, b: conidia, c: fold chains on WA medium). Bar = 300 µm
Figure 3. *Diplodia* sp. (a: colony on PDA medium, b: pycnidia, c: conidia). Bar = 300 µm
Figure 4. *Aspergillus flavus* (a: colony on PDA medium, b: conidiophore and conidia). Bar = 300 µm
Figure 5. *Aspergillus niger* (a: colony on PDA medium, b: conidiophore and conidia). Bar = 20 µm
Figure 6. *Curvularia affinis* (a: colony on PDA medium, b: conidiophore and conidia). Bar = 20 µm
Figure 7. *Eurotium rubrum* (a: colony on PDA medium, b: cleistothecia, bar = 150 µm, c: ascus and ascospore). Bar = 30 µm
Figure 8. *Nigrospora gossypii* (a: colony on PDA medium, b: conidiophore and, b_1 : conidia). Bar = 20 µm
Figure 9. *Penicillium implicatum* (a: colony on PDA medium, b: conidiophore and conidia). Bar = 20 µm
Figure 10. *Beltrania santapaui* (a: colony on PDA medium, b: conidiophore and conidia, bar = 45 µm, c: conidia). Bar = 30 µm
Figure 11. *Fusarium oxysporum* (a: colony on PDA medium, b: monophialide, bar = 75 µm, c: macroconidia and microconidia). Bar = 30 µm
Figure 12. *Trichothecium roseum* (a: colony on PDA medium, b: conidiophore and conidia). Bar = 20 µm
Figure 13. *Trichoderma harzianum* (a: colony on PDA medium, b: conidiophore and conidia). Bar = 20 µm

bicellular and elliptical form with one appendage that was dark brawn. Conidia size without appendage was equal to 5-7.5×15-21 µm and its length was 2.5-3.5 µm (Figure 10C).

Fusarium oxysporum Schltdl.

Colonies diameter growth was measured 3.1-3.8 cm on PDA medium and 25°C, its color at first was light pinkish white and finally become violet that its center was lighter and its margin was dark violet. Mycelium was cotton and scatter that were condensed by growth completion. Behind of container in the margin was dark violet and in the center was opaque orange (Figure 11A). Middle chlamydospores were formed frequently on mycelium. Macroconidia on abundant sporodochia that were sickle from and partly longitude, most of them had three tiny septum and their length was 3-5×24-30 µm (Figure 11C). Macroconidia and also Microconidia on short and individual phialides were formed which microconidia were false-heads on these phialides (Figure 11B). Microconidia were most of time egg form or longitude elliptical unicellular or kidney form (Figure 11C).

Trichothecium roseum (Pers.) Link

Colonies approximately grow up rapidly. Its diameter growth after three days on PDA medium and 25°C was 2.8 cm and was whitish light pink and partly powder form (Figure 12A). Until first conidia produce, conidiophores were not separation from growth section hyphae. They were vertical, without branch and most of time they had septum nearby base of conidiophore. Two conidia were formed alternatively and with overlap in tip of conidiophore. Conidia were bicellular, elliptical or pear form with joint place to curve, transparent, even to partly coarse and were 11-16×7-10 µm (Figure 12B).

Trichoderma harzianum Rifai

Colonies on PDA medium had rapid growth which at first was cotton white but after 2 days was approximately light green (Figure 13A). hyphae had septum were branched and 2.5-5.5 µm diameter. Chlamydospores at the end or in the middle of hyphae were elliptical to fusiform with even wall and 8.5-10×5-7.5 µm diameter. Conidiophores were branched that through end of conidiophore, length of these branches were smaller. Phialids were short, bar form, on the base they were narrower than middle area and on top of its conic, its dimensions were 5-7.5×2.5-3.5 µm. Conidia individually collected at the end of Phialids and were egg form to spherical with even wall and 2.5-3.4 µm dimensions (Figure 13B).

Discussion

In this study, specified that *Penicillium* fungus rather than identified fungi have more frequency which its species had most frequency in all range site. This genus along with fungi such as *Fusarium* and *Trichoderma* caused for discolor of seeds (El-Gali 2003).

Fungi grow both on seed crust and on seed cotyledon but a variety of fungi of seed crust are more than cotyledon. In inward section, *Penicillium* had further frequency in all regions whilst *Trichoderma* become isolation on seed crust. This is corresponded to Winston (1956) and El-Gali (2003) studies which isolated *Penicillium*, *Fusarium* and *Trichoderma* fungi on Red Oak (*Quercus rubra*) seeds.

Dorsey et al. (1962) separated *Penicillium* on seeds of *Q. velutina* and *Q. rubra*. *Aspergillus* and *Penicillium* are genera that have generality in color change of cotyledon and even seed crust and finally causal lesion and crack on seed crust (Swiecki et al. 1991). In Swiecki studies,

Fusarium oxysporum and *Trichothecium* sp. obtain on seed and seedling of *Q. macrocarpa* in northern California which similar to our study.

In this study, isolated *Fusarium* that was isolated on *Q. alba* and *Q. macrocarpa* seeds by Vozzo (1984). Agbaba and Gradecki (2005) isolated *Ciboria batschiana*, *Phomopsis quercella*, *Fusarium* sp. *Ophiostoma* sp. *Penicillium* sp. *Trichothecium roseum*, and *Trichoderma viride* from *Q. pubescens* seeds which *Fusarium*, *Penicillium*, *Trichoderma* genus and *Trichothecium roseum* species is corresponded to our study.

Tiberi et al. (2002) isolated *Fusarium solani*, *Fusarium eumartii*, *Verticillium dahliae*, *Diplodia mutila* and *Phomopsis quercina* from oaks seed of Italy which *Diplodia* and *Fusarium* is observed in our study. Gallego et al. (1999) isolated *Fusarium oxysporum* seen in our study from *Q. ilex* seed for testing of being pathogen the fungi. Santos et al. (2005) and Merouani et al. (2001) separated many fungi on *Q. suber* seed which among them can be referred to *Penicillium implicatum*, *Trichoderma harzianum*, *Trichothecium roseum*, *Fusarium oxysporum*, *Diplodia mutila*, *Aspergillus niger*, *Aspergillus flavus* and *Alternaria alternata* that whole of these species were seen in our study. Also some studies conducted on fungi along with major forest trees that fungus similar our study was including *Alternaria*, *Fusarium*, *Aspergillus*, *Penicillium*, *Trichoderma*, and *Beltrania* (Vladimir et al. 2005; Swapna and Nagaveni 2008).

CONCLUSION

The results show that all acorn seeds collected were infected with one or more species of fungi have been isolated which are often classified to *Ascomycetes* fungi. Since the length of oak seed dormancy and physiological process is very long period. This could be due to opportunistic fungi such as contact with the surface of the seed coat and the seed easily reach and thereby is prevented from germinating. This is the comprehensive report on fungi associated with *Quercus castaneifolia* seed in Hyrcanian forest, North of Iran.

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