Mycologia Iranica 4(2): 121 – 129, 2017 Original Article DOI: 10.22043/mi.2017.117424

Some aspects of biology and host range of *Biscogniauxia mediterranea*, one of the causal agent of oak charcoal disease

D. Safaee ✉
Plant Protection Research Department, Kermanshah Agricultural and Natural Resources Research and Education Center, AREEO, Kermanshah, Iran

S. A. Khodaparast
Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

M. Mirabolfathy
Plant Pathology Research Department, Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

M. Sheikholeslami
Plant Protection Research Department, Kermanshah Agricultural and Natural Resources Research and Education Center, AREEO, Kermanshah, Iran

Abstract: Field surveys in Zagros forests located in western Iran were done to study some aspects of biology and host range of *Biscogniauxia mediterranea* during 2014-2016. The ability of its conidia and ascospores to cause the disease was evaluated under greenhouse condition. Ascospore viability and development of anamorph and teleomorph were surveyed under different conditions. The pathogen host range was determined by field observations, laboratory and greenhouse tests. Results showed that ascospores unlike conidia produced colony and caused disease by inoculation into the lesion in *Quercus brantii* seedlings. Ascospore germination was decreased as much as 17.3% under forest conditions after one year. Extent necrosis in the inner bark and wood tissues of inoculated adult *Q. brantii* trees was induced however no fungal stroma has appeared for three years. Monopartite black stroma lacking teleomorph was formed beneath the outer bark of inoculated excised branches on which conidia were produced. Teleomorph was formed only on one *Q. infectoria* excised branch under natural-like condition. Teleomorph and anamorph have not produced on inoculated *Q. brantii*, *Q. infectoria* and *Q. libani* seedlings for 34 months. In the forest, the sign of charcoal disease was seen only on *Q. brantii* and *Q. infectoria* trees that the latter is a new host for it. Necrosis appeared in the inner bark of inoculated excised branches of *Amygdalus* sp. *Acer monspessulanum*, *Cerasus microcarpa*, *Pistacia* sp. and *Rosa* sp. but not in *Crataegus* sp. and *Ulmus* sp. Pathogenicity of *B. mediterranea* was demonstrated for the first time on seedlings of *Q. infectoria* and *Q. libani.*

Key words: Ascospore, *Quercus*, Zagros forests, biology, pathogenicity

INTRODUCTION

Zagros forests (ZFs) extend from northwest to southeast of Iran and their total area is about 4 million hectares (Sabeti 1994). These forests play an important role in protecting soil, water and the environment. Human activities such as using forest wood for fuel, conversion of forests to agricultural lands and other purposes and also unconditional grazing of livestock have noticeably destroyed some parts of ZFs. Moreover, deleterious effects of pests, plant diseases and parasites and also severe drought have increased by, increasing the concentration of the micron-sized dust and increasing their establishment time in different areas of ZFs, different adverse effects on the plant's physiology and forest trees have observed. It is believed that the weakening of trees and shrubs in recent years due to successive droughts has led to increase their susceptibility to pests and diseases in these areas.

Most of the trees in the Zagros mountains are mainly from oak (*Quercus* L.), including *Q. brantii* Lindl., *Q. infectoria* Oliv. and *Q. libani* Oliv. (Jazirehi & Ebrahimi Rostaghi 2003). Charcoal disease caused by *Biscogniauxia mediterranea* (De Not.) Kuntze has been reported as the most common

Submitted 2 Feb. 2017, accepted for publication 3 Oct. 2017
✉ Corresponding Author E-mail: dasafaee@Yahoo.com

and important disease of oak trees in Zagros forests (Mirabulfathy 2013). In subsequent studies, Mirabolfathy et al. (2013) showed that in some Zagros areas, the signs macroscopically similar to that of charcoal disease on some oak trees are related to the species *Obolarina persica* Mirabolfathy, Ju, Hsieh & Rogers. Following this research, Safaee et al. (2017) designed a multiplex PCR using two pairs of specific primers to detect and identify the two species in oak tree tissue before the disease sign appears.

Biscogniauxia mediterranea has been in the center of attention in most of the oak forests over the world (Jurc & Ogris 2006, Vasilyeva et al. 2007, Whalley 1996, Vanini & Valentini 1994). Most important aspects of the pathogenicity of *B. mediterranea* are the relationship between the role of drought stress and the disease outbreak (Vannini et al. 2009, Vanini & Valentini, 1994, Vannini & Scarascia Mugnozza, 1991, Ragazzi et al. 2001, Linaldeddu et al. 2011, Luchi et al. 2005), the molecular identification of the cause and tracing of the disease before the sign appears (Mazzaglia et al. 2001, Luchi et al. 2005, Safaee et al. 2017).

Vanini et al. (1999) Study the genetic structure of *B. mediterranea* using Random Amplified Polymorphic DNA (RAPD) showed that there is a great genetic diversity within the population derived from a single stroma and among various isolates collected from a limited area, therefore, they proposed a heteroathallic mating type system for the fungus.

Biscogniauxia mediterranea has a long endophytic stage in which the infected trees have no symptoms. The fungus acts in an aggressive manner under drought stress and high abnormal temperature. The considerable effect of water content on the activity of *B. mediterranea* has been proved. It is believed that the development of the fungus in the endophytic phase depends on the reduction of the host water potential (Vannini & Scarascia Mugnozza 1991).

Vannini et al. (1996) investigated the factors influencing the release and germination of *B. mediterraneua* (*H. mediterraneum*) ascospores on Turkish oak (*Q. cerris*) under natural and laboratory conditions and concluded that ascospore release and germination depend on the presence of free water or high relative humidity ,however they did not test ascospore pathogenicity.

In Spain, Martin et al. (2005) investigated the correlation between wrong operations for the harvesting of cork oak trees (*Q. suber*) and perforation caused by beetles and contamination to *B. mediteranea* in oak forests. They realized that there is a strong correlation (r = 0.86) between wrong harvesting operations and holes made by beetles (*Cerambyx* spp.), and also between the damage caused by beetles and contamination to *B. mediteranea* (r = 0.91).

Toxins extraction and identification from *B. mediterranea* implemented by Evidente et al. (2005) and the results showed that in addition to the two previously known toxins, phenylacetic acid and 5-methylmellein, this fungus produces another toxin called biscopyran, which is non-specific and makes epinasty in *Q. suber*.

Lack of research and information about the biology of *B. mediterranea* caused some difficulties in the biology of this species, however, the role of ascospore and conidium in pathogenicity, the survival duration (viability) of ascospores, development of sexual and asexual stages and host range of the species were investigated.

MATERIALS AND METHODS

Fungal Isolates

Samples of stromata containing perithecia of charcoal disease agents were collected from different regions of ZFs in Kurdistan, Kermanshah, Ilam and Lorestan provinces during 2014-2016. Pure cultures were obtained from single ascospore. To this, the contents of few adjacent perithecia were added to 1 ml of sterilized water by a sterile needle and after shaking, 10 µL of the suspension was spread over the WA medium. After 24 to 48 hours, one germinated ascospore was transferred to a PDA plate. All isolates were kept on slant PDA tubes at 4°C.

Identification of oak charcoal disease agent

To identify the species, the characteristics of the teleomorph (stroma, perithecium, ascus and ascospore) were investigated and compared with descriptions of the genus and species in valid mycological references (Ju & Rogers 1996, Ju et al. 1998, Ju & Rogers 2001). To confirm the morphological identification of the species based on molecular characteristics, a pair of specific primers MED1 (forward: 5′-GCTATAAGGGATCACCGCC TC-3′) and MED2 (reverse: 5′-TGCAGAAGCGAGA GTGTGG-3′), designed by Mazaglya et al. (2001) were used. DNA from colonies of single spore isolates was extracted by using Chelex 100 (BIORAD, USA) based on the method of Hirata & Takamatsu (1996) and its concentration was determined using a spectrophotometer (Epoch 2, BioTek, USA). Polymerase chain reaction was performed in 200-µl microtubes containing 25 µl mixtures of reaction. The reaction mixture consists of a PCR buffer (1X), 0.2 mM of dNTPs, 2 mM of $MgCl_2$, 0.6 µM of each of the MED1 and MED2 primers, 0.8 units of hot start Taq DNA polymerase (Thermo Scientific, Lithuania) and 2 ng of DNA extracted from each isolate. Moreover, a negative control (reaction mixture without DNA) and a positive control (reaction mixture containing DNA extracted from an identified *B. mediterranea*) were used. The reaction was performed in a thermocycler device (BIO-RAD, USA). The thermal program including 36 cycles was performed as follows. An

initial denaturing at 95°C for 4 min, two cycles of denaturing at 95°C for 40 s, annealing at 62°C for 30 s, extension at 72°C for 30 s, two cycles of 95°C for 40 s, 61°C for 30 s, 72°C for 30 s, 32 cycles of 95°C for 40 s, 60°C for 30 s, 72°C for 30 s and a final extension at 72°C for five min. Amplified DNA fragments and a 100-bp DNA marker (Sinaclon, Iran) were passed through Agarose gel 1.5% (Sinoclon, Iran) containing DNA safe stain (Sinoclon, Iran) in an electrophoresis device. After electrophoresis, the DNA bands in the agarose gel were visualized and photographed using the UV-gel documentation system (UVITEC, France).

Determination of the host range Based on the field observation: During investigating charcoal disease on oak trees (*Quercus* spp.), its possible presence on other forest species including *Crataegus* spp., *Amygdalus* spp., *Acer monspessulanum*, *Cerasus microcarpa*, *Pistacia* spp., *Rosa* spp., *Ulmus* sp., and *Pyrus* spp. was also considered.

Based on inoculation on excised branches: According to Safaee et al. (2015), from healthy trees of *Quercus* spp., *Crataegus* spp., *Amygdalus* spp., *Cerasus microcarpa*, *Pistacia* spp., *Rosa* spp., *Ulmus* sp., and *Pyrus* spp. in the areas without charcoal disease, some branches with a diameter of about 2 to 4 cm and a length of about 1 to 1.5 meters were cut and transferred to the laboratory. These branches were segmented into pieces of 30 cm in length and were first washed with tap water and then completely immersed in 0.5% sodium hypochlorite for 10 min and then soaked in sterilized water for 15 min. Inoculation was performed with and without making a wound in the bark. The wound was made in the medial of the excised branch by removing one square centimeter of bark. A 5-mm diameter mycelium plug of the fungus was placed into each wound and the inoculated area wrapped with Parafilm. In none-wounded treatments, after placement of the inoculum on the bark surface, it was covered in the same above manner. In control treatments, autoclaved colony plugs were used. The experiment was conducted as a factorial based on a completely randomized design. The treatments consisted of, 1) with wounding 2) without wounding and 3) control treatments in 5 replications (each replication included two excised branches). The inoculated excised branches were placed in a sterilized plastic container and held in an incubator at 25 ± 1°C. The length of necrosis tissue under the bark was measured after a month and used for comparison. To re-isolate the pathogen, fragments from the margin of healthy and diseased tissue were removed aseptically and cultured on PDA containing streptomycin sulfate.

Based on seedling inoculation: Regarding the importance of oak species, the pathogenicity test of *B. mediterranea* on seedlings of three species of oak (*Q. brantii*, *Q. infectoria* and *Q. libani*) which are most frequent in Zagros forests, was conducted on 20-month-old seedlings in 3.5-liter plastic pots containing forest soil. An experiment was conducted as factorial on the basis of a completely randomized design at five replications, each replication including two pots and each pot including one seedling. Inoculation of seedlings was performed by placing a plug of the fungal colony into the wounded stem (in bark-wounded treatments) and on intact stem surface (in non-wounded treatments), then the inoculated area wrapped with Parafilm. The autoclaved inoculum was used in controls. The pots were held in the greenhouse for two months after inoculation, and then necrosis extension in inner bark was evaluated. Pathogenicity proof was carried out by re-isolation of the fungus.

Pathogenicity and survival

Preparation of ascospores and conidium suspension: Of the sixteen stromatic samples containing live ascospores, six samples were randomly selected. Ascospores of three pieces of each sample were collected according to Vannini et al., (1996). The outer layer of the pieces (about 2 × 2 centimeters in size) was removed from the perithecial stroma so that the perithecium opening appeared. Then these pieces were set on a sterilized wet tissue paper into Petri dishes and placed in an incubator at 8°C. After 48 hours, the ascospores released on the inner surface of the Petri dishes were collected by adding sterile distilled water and pipetting, resulting in a concentration of about 10^6 ml^{-1}.

Of the sixteen single spore isolates of *B. mediterranea*, six isolates were randomly selected and cultured on oatmeal agar (OMA) for production of conidia. The cultures were placed under a 12-hour light/dark condition with a mixture of fluorescent light and near ultraviolet at 25± 3°C for 10 days. Conidia were washed from the colony surface with sterilized distilled water, filtered through glass wool to remove mycelial fragments. The concentrations of conidia and small passed mycelial fragments (SPMFs) were determined by microscopic count with a hemocytometer which resulted in concentrations of 10^5 and 50 ml^{-1} conidium and SPMFs respectively.

The rate of ascospore and conidium germination: The amount of 5 µl of ascospore suspension of six above samples were spread on PDA, MEA and WA media with three replications and incubated at 25°C for 48 hours. Fifty ascospores were randomly observed microscopically (Olympus-BH2, Japon) at 400X at each replication on plates. When germ tube emerged from ascospores, they were regarded as germinated ascospores.

According to this method, the rate of conidium germination of six above conidium suspensions was calculated.

Inoculation of seedlings with ascospores and conidia: Eighty seedlings of 20 months old seedlings of *Q.brantii*, approximately equal in height, were inoculated with each ascospore and conidia just as the previous method for seedling inoculation except that inoculum was a suspension of ascospores or conidia

at concentrations of 10^6 and 10^5 ml^{-1} respectively. Pathogenicity assessment was performed in the same way as before.

Survival of ascospores: In the protected zone of Bisotun (Kermanshah province), three *Q.brantii* trees containing stroma of *B. mediterranea* were marked. From each tree, a branch containing stroma was cut so that half of the stroma remained on the tree and the other half was transferred to the laboratory. Ascospore suspensions were prepared from each of them, and their rate of germination was calculated and considered as initial germination. After that, we split the stroma-bearing branches and put a part of them in the paper bags and kept in the room condition and hung the other part on a tree in the yard of Kermanshah Agricultural and Natural Resources Research and Education Center (KANRREC), which provided almost conditions similar to the presence of stroma on the trees under natural conditions. After one year, the percentage of germination (secondary germination) of ascospores on marked oak trees, the hung parts in the yard and the parts in paper bags were estimated, and their mean differences were compared by Duncan's test. The percentage reduction of germination after one year was calculated with the following formula:

Ascospore germination = [(Percent of primary germination - Percent of secondary germination)/Percent of primary germination] × 100

Development of sexual and asexual stages

The time of the onset of the symptoms after penetrating *B. mediterranea* in the oak tissue, the rate of expansion in the tissue and the development of a sexual and asexual form of the fungus were studied under the following conditions.

Under field conditions: In one of the forested regions of Kermanshah province, in which charcoal disease was prevalent, we selected 12 *Q. brantii* trees with trunk diameter around 17-21 cm at breast height, in an area of about 2000 m^2. These trees did not have stroma of charcoal disease and also its causal agent was not isolated from their tissue. In six of them, a piece of trunk bark (approximately 5 × 5 cm) was removed with a chisel (sterilized with alcohol and flame between each use). Then some of the inoculum (colony of *B. mediterranea* on a PDA of about 9 cm^2 in size) was placed on the wood surface and the removed bark was returned to its place so that the inoculum was set between the tissue of the inner bark and the wood. The inoculated spot was first covered with wet autoclaved cheesecloth and then with parafilm. Six other trees were inoculated with autoclaved fungus colony as a control. These trees were surveyed in autumn, winter, spring, and summer every 15, 30, 10 and 20 days, respectively during 24 months. In these periods, we investigated the possibility of forming of the stromata containing perithecia and/or the emergence of the colonies bearing conidia in the site of inoculation and surrounding tissues. By removing the tissue at 5 cm intervals from the margin of the inoculated wound, the extension of necrosis in the tissue was assayed and some samples for re-isolation of the fungus were provided. At the end of the experiment, the outer bark was removed around the inoculation site until the necrosis continued to measure the longitudinal extension (along the trunk). Also, we sampled some inner bark and wood tissues in the opposite side of the inoculation site to detect possible invasion of the fungus across the trunk.

On the excised branches: Three excised branches of each *Q. brantii* and *Q. infectoria* were inoculated by *B. mediterranea* as mentioned above and kept in the incubator at 25°C for one month. Subsequently, all of them were placed below a wired cloth box (Fig. 1) in the garden of the KANRREC beside which several pieces bearing *B. mediterranea* perithecia were placed. At the above-mentioned intervals, their status was examined. After 24 months they were individually placed in a paper bag and kept at room temperature.

In greenhouse: Ten 2-year-old seedlings of *Q. brantii* were inoculated with *B. mediterranea* by placing the mycelial plug into the wound on the stem as earlier mentioned and five seedlings were used as a control. The inoculated plants were held under normal conditions in the greenhouse. All the treatments were monitored almost every week for 34 months to record fungal and disease development. At the end of the experiment, re-isolation of the fungus from the margin of the healthy and infected tissues was performed.

Fig. 1. The excised branches of *Q. brantii* and *Q. infectoria* inoculated by *B. mediterranea* and placed on sand surrounded by a wire cloth.

RESULTS

Identification of fungal isolates

Thirty-one stromatic samples were collected from oak trees in Kurdistan, Kermanshah, Ilam and

Lorestan provinces whose teleomorph characteristics were consistent with the description of *B. mediterranae*. Of these, the stromata of the seven specimens were rotting and their ascospores were not able to germinate. Of the remaining specimens, 24 single spore isolates were obtained. Specific primers (MED1 and MED2) were able to produce fragments of 377 bp in a polymerase chain reaction mixture containing DNA of identified isolates, and their morphological identification was confirmed.

Determination of the host range

Signs of charcoal disease (black stroma containing perithecia of *B. mediterranea*) were observed only on *Q. brantii* and *Q. infectoria*. While in some sampled regions, the charcoal disease sign on oak trees was prevalent, it was not observed on other forest tree species including *Crataegus* spp., *Amygdalus* spp., *Acer monspessulanum*, *Cerasus microcarpa*, *Pistacia* spp., *Rosa* spp., *Ulmus* sp. and *Pyrus* spp.

In the inner bark tissue of the excised branches of *Amygdalus* sp., *Acer monspessulanum*, *Cerasus microcarpa*, *Pistacia* sp. and *Rosa* sp., *B. mediterranea* created extensive brown necrosis with a length of about 8-15 centimeters, but the disease did not occur in *Crataegus* sp. and *Ulmus* sp. from the margins between healthy and necrotic tissue, the fungus was reisolated and according to this method, its pathogenicity was proved mentioned above species.

Biscogniauxia mediterranea created brown necrosis in the inner bark tissue of *Q. brantii*, *Q. infectoria* and *Q. libani* seedlings only in bark-wounded treatments. Extension of necrosis was significantly different in the treatments contained disease symptoms compared to control. Although *Q. infectoria* was more sensitive to the disease compared to *Q. libani*, it was not statistically significant (α = 0.05).

Germination of conidia and ascospores

The average of ascospores germination of three isolates of *B. mediterranea*, collected from different locations was varied from 37.2 to 95.8%, (Table 1). The growth of the mycelium produced by the ascospores continued and resulted in the production of colonies. Only 1.1 percent of conidia (0-1.1 percent) germinated (Table 2), however, the growth of the germ tube stopped quickly and its length did not exceed that of the conidium. The small segments of the mycelium which were approximately the same size as conidia and passed through the filter, germinated and formed colonies.

Table 1. The average of ascospore germination percentage of three isolates of *Biscogniauxia. mediterranea* on three different culture media.

Isolate	Media		
	WA	MEA	PDA
1	59.1	63.4	62.5
2	92.5	91.7	95.8
3	35.4	40.6	37.2

Table 2. The average of conidium germination percentage of three isolates of *Biscogniauxia. mediterranea* on three different culture media.

Isolate	Media		
	WA	MEA	PDA
1	0	0	0
2	0	0	0
3	0.8	1.1	0.2

Pathogenicity ability of ascospore and conidium

Ascospore inoculation of *Q. brantii*s seedlings caused fungal infection and invasion of the inner bark tissue in bark-wounded treatments. The pathogenicity of *B. mediterranea* ascospores was approved by re-isolating the fungus from the margin between diseased and healthy tissue.

It should be noted that ascospores did not produce disease in wound-free treatment. The incidence of necrosis by inoculation of ascospores was significantly higher than that of the control and the necrosis caused by the conidium (α = 0.01).

Of the 10 seedlings that were inoculated with conidium suspension in the wound on stems, only one seedling showed disease symptoms, however, none of the wound-free treatments developed the disease.

Survival of ascospores

Reduction of germination percentage of *B. mediterranea* ascospores after one year under the condition of placing stroma containing ascospores in the natural state (on the oak tree in forest conditions), similar to natural condition (hanging in the yard of KANRREC) and in the paper bag in the room was 17.3%, 13.5% and 7.4%, respectively, and the difference between initial and secondary germination of two first cases was significant at α=0.05 (Table 3).

Table 3. Mean comparison of initial germination percentage of ascospores with their germination percentage after one year under forest conditions, similar to the forest and in-room condition by Duncan multiple range tests (α = 0.05).

Condition	Germination*	
	Initial	After one year
Forest	92.3[a]	76.3[b]
Similar to the forest	92.3[a]	79.8[b]
Room	92.3[a]	85.5[ab]

* Difference among means with the same letter is not statically significant at α = 0.05

Development of sexual and asexual stages

Biscogniauxia mediterranea caused brown to dark brown necrosis in the inner bark of *Q. brantii* trees and gradually extended along the trunk length. Necrosis penetration depth often expanded to the center of the trunk and its width was about twice as the width of the inoculation site (about 5 cm). The fungus was re-isolated five months after inoculation, from 7 to 10 centimeters and two years later, from 40 to 60 centimeters distance from the inoculation site along the length of the trunk. On the opposite side of the inoculated area, the symptoms of the disease were not detected in both inner bark and wood. In all

examined trees, no signs and symptoms were seen on the surface of the outer bark under which inner bark had become necrotic, and had a normal appearance. There was a highly significant difference between treatments with a control.

From the inoculation site on the excised branches, the symptom of the disease began as brown to dark brown necrosis in the inner bark and wood and it extended along the length, width (approximately half of the cross-section periphery) and depth (up to center) of excised branches. Some white colonies were usually formed at two ends of the excised branches but at this stage, the bark surface had no signs and symptoms of the disease. Seven months after inoculation, white colonies containing conidia of *B. mediterreanea* emerged around the wounded site of inoculation. Under the outer bark, a thin layer (about 200 µm) of black stroma without teleomorph emerged, which gradually extended and an olivaceous brown mass of conidiophores and conidia formed on it, in this stage some parts of outer bark was peeling from lower tissue (Fig. 2 a). The growth of the germ tube of these conidia (Fig. 2 b) on PDA and WA media stopped quickly like those from a pure culture. One year after inoculation, the excised branches were investigated on which no teleomorph appeared. Then they were kept in the paper bag in room conditions. Two months later, we examined them again and found that a two-part stroma containing perithecia of *B. mediterranea* had been formed on one of the excised branches of *Q. infectoria.* (Fig. 3.).

From the inoculation site, the color of the inner bark and surface of wood tissue began to turn brownish to dark brown and extended on both sides of the inoculation site along the seedling stem. The progression of the necrosis within the stem breadth was about six millimeters (nearly twice the width of the wound site). Conidiophores, conidia and stroma of *B. mediterranea* have not been formed on seedlings since inoculation time (for 34 months).

DISCUSSION

Of the 23 stromatic samples of *B. mediterranea* collected from different regions, seven of them did not contain live ascospores. The texture of these stromata was rotten and easily crushed due to the effects of environmental conditions and the activity of other microorganisms. Although inappropriate environmental conditions have a significant effect on decreasing the survival rate of ascospores, aging alone reduces the life of ascospores, the rate of decline under natural conditions and storage conditions in the room after one year was 17.3 and 7.4 percent respectively. Therefore, in field surveys, the status of stroma should be considered in terms of active or inactive. Despite touring in different areas of the Zagros forests, including areas with severe contamination to charcoal disease, the sign of the disease was only observed on two species of oak (*Q. brantii* and *Q. infectoria*). This is the first report of charcoal disease with the cause of *B. mediterranea* on *Q. infectoria* under natural conditions.

Biscogniauxia mediterranea created brown necrosis in the inner bark tissue of *Q. brantii, Q. infectoria* and *Q. libani* seedlings that its pathogenicity on *Q. infectoria* and *Q. libani* is a new report. Therefore, although no charcoal disease has ever been reported under natural conditions in *Q. libani*, these forests are potentially susceptible to this disease. Of course, there may have been a charcoal disease in the *Q.* libani trees, however, our sampling has not shown it.

The fungus-induced disease in excised branches of *Amygdalus* spp., *Cerasus microcarpa*, *Pistacia* spp. and *Rosa* spp, so these trees may be considered as potential hosts for the charcoal disease.

But with this method, no disease was caused on *Crataegus* spp. and *Ulmus* sp. Due to the fact that *B. mediterranae* did not cause the disease on the excised branches of *Crataegus* sp. and *Ulmus* sp., it seems that these trees can be considered in afforestation programs in contaminated areas. However, it is necessary that their seedling resistance in greenhouse conditions and the resistance of adult trees under field conditions be assayed.

In the present study, ascospore germination of *B. mediterranea* isolates resulted in colony formation. A very small number of their conidia (0-1.1%) germinated (Table 3), but their germ tube was not longer than the length of the conidium and therefore no colony formed. Based on the present study, the pathogenicity of ascospores of *B. mediterranea* on *Q. brantii* seedlings was approved for the first time in greenhouse conditions, although in the same condition, their conidia were not pathogenic. It should be noted that from 10 inoculated seedlings inoculated by *B. mediterranea* conidia into the wounded bark, only in one seedling the disease symptoms appeared which statistically does not play a role in the pathogenicity of *B. mediterranea*. Considering that the conidia were not able to produce colonies and on the other hand the complete removal of the small mycelial segments from the suspension was not feasible. Therefore, the disease appeared in the seedling may be due to the presence of the small mycelial segments in the suspension of conidia. Griffin et al. (1992) studied the pathogenicity of *Hypoxylon mammatum* (a close species to *B. mediteranea*) in *Populus tremuloides* in the United States. They concluded that ascospores play a major role in the pathogenicity and distribution of *H. mammatum* and the growth of conidial germ tube on the culture medium is stopped and does not result in the production of colonies. Inoculation of *B. mediterranea* on mature trees and seedlings of *Q. brantii* in forest and greenhouse conditions respectively showed that the fungus established in the oak tissue and extended in the inner bark and also a bit in woody tissue but in neither of them the fungus teleomorph was produced after 28 months. It may be possible that this species has a heterothallic system and/or teleomorph forming needs special conditions.

Fig. 2. *Biscogniauxia mediterranea*. a. An excised branch of *Q brantii* inoculated by *B. mediterranea* and kept under natural like condition on which a thin black layer of stroma and an olivaceous brown mass of conidiophores and conidia are visible; b. Germinating conidia that the growth of their germ tube has stopped. — Scale bars: a = 2 cm & b = 10 µm.

Fig. 3. *Biscogniauxia mediterranea*. a. Formation of a two-part black stroma containing perithecia of *B. mediterranea* on the excised branch of *Q. infectoria*; b. Stroma with more magnification — Scale bars = 2 cm.

Vannini et al., 1999 using a RAPD marker, indirectly suggested a heterothallic mating system for *B. mediterranea*. Ghasemi Esfahlan et al. (2016) crossed five isolates of *B. mediterranea* on the oak stem water agar culture medium, which did not lead to forming sexual stage, due to the possibility that the isolates were the same mating type or unsuitable conditions for the formation of teleomorph. Results of the present study showed that 24 isolates of this species did not form teleomorph singly on the PDA and OMA media under alternating dark/light conditions which also indicates heterothallism. Of course, it is obvious that this subject requires an independent and coherent research to resolve the ambiguities. Formation of anamorph has also limitations in the conditions of Zagros forests, and it seems to be associated with high relative humidity. Since in the spring and only when the covers (parafilm and cloth) were removed on inoculation sites, the anamorph was observed on the margin of necrotic tissue. The covering provided a humid microclimate under which was wet. In late spring when air changed into dry condition, the anamorphs gradually disappeared and did not appear in the following year on the uncovered sites. On the seedlings, the anamorph of this species did not occur at all stages of the experiment. Contrary to Ju et al. (1998), who had suggested that the conidia act as a source of contamination, the results of this study showed that conidia cannot form colonies and did not cause disease in the greenhouse experiment, thus conidia have no function in the distribution of charcoal disease.

The difference in the production of colony, conidia, and stroma (without perithecium) on the excised branches compared with trees and seedlings is undoubted because of the physiological differences between living tissues and dying ones like excised branches. Vannini et al. (2009) showed that the growth of *B. mediterranea* increased with decreasing in tissue water content. It is clear that gradually drying of excised branches reduces the amount of water in their tissues, which results in more activity and change in the behavior of the fungus.

At the end of the experiment, it was found that on one of the excised branches of *Q. infectoria*, inoculated with *B. mediterranea*, a two-part stroma containing perithecium was formed. Considering the fungus seems heterothallic, it is not clear how the mating happened. At the site of placing the inoculated excised branches, samples from the branches containing stromata of *B. mediterranea* were also placed, so it is likely that their conidia had played the role of sexual sporidium and caused fertilization of the opposite mating type on the excised branch. Another possibility is that the branch from which the cut-off shoot was made had already been latently infected in the forest to the opposite mating type. On the other hand due to the fact that the emergence of teleomorph occurred when the excised branch was completely dried, so drying of the host tissue may

also be a necessary condition for the formation of sexual form. Clearance of this issue, however, needs further investigations.

ACKNOWLEDGEMENTS

We should thank Dr. Hoshmand Safary for statistical analyses of the data.

REFERENCES

Evidente A, Andolfi A, Maddau L, Franceschini A, Marras F. 2005. Biscopyran, a phytotoxic hexasubstituted pyranopyran produced by Biscognauxia mediterranea, a fungus pathogen of cork oak. Journal of Natural Products 68:568–571.

Ghasemi–Esfahlan S, Arzanlou M, Babai–Ahari A. 2016. Detection of Biscogniauxia mediterranea, the causal agent of charcoal rot disease on oak trees in Arasbaran forests and evaluation of its pathogenicity on oak under glasshouse conditions. Iranian Journal of Plant Pathology 52:217–230.

Grifin D H, Quinn KE, Gilbert GS, Wang CJK, Rosemarin S. 1992. The role of Ascospores and conidia as propagules in the disease cycle of Hypoxylon mammatum. Phytopathology 82:114–119.

Jazirehi MH, Ebrahimi Rostaghi M. 2003. Silviculture in Zagros. Teharan, Iran, University of Tehran Publication, Tehran, Iran.

Ju YM, Rogers JD. 1996. A revision of the genus Hypoxylon. APS, St. Paul, MN, USA.

Ju, Y M, Rogers JD, San Martin F, Granmo A. 1998. The genus Bicogniauxia. Mycotaxon 66:1–98.

Ju YM, Rogers JD. 2001. New and interesting Biscogniauxia taxa, with a key to the world species. Mycological Research 105:1123–1133.

Jurc D, Ogris N. 2006. First reported outbreak of charcoal disease caused by Biscogniauxia mediterranea on Turkey oak in Slovenia. Plant Pathology 55: 299–299.

Linaldeddu BT, Sirca C, Spano D, Franceschini A. 2011. Variation of endophytic cork oak–associated fungal communities in relation to plant health and water stress. Forest Pathology 41: 193–201.

Luchi N, Capretti P, Pinzani P, Orlando C, Pazzagli M. 2005. Real–time PCR detection of Biscogniauxia mediterranea in symptomless oak tissue. Letters in Applied Microbiology 41: 61–68.

Martı´n J, Cabezas J, Buyolo T, Pato´n D. 2005. The relationship between Cerambyx spp. damage and subsequent Biscogniauxia mediterranum infection on Quercussuber forests. Forest Ecology and Management 216: 166–174.

Mazzaglia A, Anselmi N, Gasbarri A, Vannini A. 2001. Development of a Polymerase Chain Reaction (PCR) assay for the specific detection of Biscogniauxia mediterranea living as an endophyte in oak tissue. Mycological Research 105: 952–956.

Mirabolfathy M. 2013. Outbreak of charcoal disease on Quercus spp. And Zelkova carpinifolia trees in forests of Zagros and Alborz mountains in Iran. Iranian Journal of Plant Pathology 49: 77–79.

Mirabolfathy M, Ju YM, Hsieh HM, Rogers JD. 2013. Obolarina persica sp. nov., associated with dying Quercus in Iran. Mycoscience 54: 315–400.

Ragazzi A, Moricca S, Capretti P, Dellavall I, Mancini F, Turco E. 2001. Endophytic fungi in Quercus cerris: isolation frequency in relation to phenological phase, tree health and the organ affected. Phytopathologia Mediterranea 40: 165–171.

Sabeti H. 1994. Forest, trees and shrubs of Iran. 2nd ed., Iran, University of Science and Technology Publication, Tehran Iran.

Safaee D, Khodaparast S A, Mirabolfathy M, Mousanejad S. 2015. Comparison of excised twig with seedling to assay the pathogenicity of Biscogniauxia mediterranea on Persian oak (Quercus brantii) seedlings. Proceeding of 2nd Iranian Mycological Congress, University of Tehran.

Safaee D, Khodaparast S A, Mirabolfathy M, Mousanejad S. 2017. A multiplex PCR–based technique for identification of Biscogniauxia mediterranea and Obolarina persica causing charcoal disease of oak trees in Zagros forests. Forest pathology 47: DOI: 10.1111/efp.12330.

Vannini A, Lucero G, Anselmi N, Vettraino AM. 2009. Response of endophytic Biscogniauxia mediterranea to variation in leaf water potential of Quercus cerris. Forest Pathology 39: 8–14.

Vannini A, Mazzaglia A, Anselmi N. 1999. Use of random amplified polymorphic DNA (RAPD) for detection of genetic variation and proof of the heterothallic mating system in Hypoxylon mediterraneum. European Journal of Forest Pathology 29: 209–218.

Vannini A, Paganini R, Anselmi N. 1996. Factors affecting discharge and germination of ascospores of Hypoxylon mediterraneum. Eurpean Jouranl of Forest Pathology 26: 12–24.

Vannini A, Scaracia Mugnozza G. 1991. Water stress: a predisposing factor in the pathogenesis of Hypoxylon mediterraneum on Quercus cerris. Eurpean Jouranl of Forest Pathology 21: 193–201.

Vanini A, Valentini R. 1994. Influence of water relations on Quercus cerris–Hypoxylon mediterraneum interaction: a model of drought–induced susceptibility to a weakness parasite. Tree Physiology 14: 129–139.

Vasilyeva LN, Stephenson SL, Miller AN. 2007. Pyrenomycetes of the Great Smoky Mountains national park. IV. Biscogniauxia, Camaropella, Camarops, Camillea, Peridoxylon and Whalleya. Fungal Diversity 25: 219–231.

Whalley AJS. 1996. The xylariaceous way of life. Mycological Research 100: 897–92.

جنبه‌هایی از زیست شناسی و دامنه میزبانی *Biscogniauxia mediterranea* عامل بیماری زغالی بلوط

داریوش صفایی[1]✉، سیداکبر خداپرست[2]، منصوره میرابوالفتحی[3] و مهیار شیخ الاسلامی[1]

۱– بخش تحقیقات گیاهپزشکی، مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی استان کرمانشاه، سازمان تحقیقات، آموزش و ترویج کشاورزی، کرمانشاه، ایران

۲– گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه گیلان، رشت، ایران

۳– بخش بیماری شناسی گیاهی، موسسه تحقیقات گیاپزشکی کشور، سازمان تحقیقات، آموزش و ترویج کشاورزی، تهران، ایران

چکیده: بررسی‌های میدانی برای مطالعه زیست شناسی و تعیین دامنه میزبانی *Biscogniauxia mediterranea* در جنگل‌های زاگرس در غرب ایران طی سال‌های ۱۳۹۳ تا ۱۳۹۵ انجام گرفت. در شرایط گلخانه توانائی بیماریزائی کنیدیوم و آسکوسپور ارزیابی شد و قدرت بقاء آسکوسپور و پدید آمدن آنامورف و تلئومورف در شرایط متفاوت بررسی گردید. دامنه میزبانی بر اساس مشاهدات در عرصه و عملیات آزمایشگاهی و گلخانه‌ای تعیین شد. نتایج نشان داد که آسکوسپورها می‌توانند در پوست داخلی ساقه *Quercus brantii* نکروز ایجاد کنند اما کنیدیوم‌ها قادر نیستند. مقدار تندش آسکوسپورها در شرایط جنگل پس از یک سال به میزان ۱۷/۳٪ کاهش یافت. نکروز گسترده در پوست داخلی و چوب درختان بلوط ایرانی مایه زنی شده ایجاد شد اما پس از گذشت سه سال استرومای قارچ روی آنها تشکیل نشد. استرومای سیاه یک قسمتی و فاقد تلئومورف در زیر پوست خارجی شاخه‌های بریده‌ی مایه زنی شده پدید آمد که روی آن توده‌ای از کنیدیوم‌ها قرار داشت. تلئومورف فقط روی یک شاخه بریده‌ی *Q. infectoria* در شرایط مشابه جنگل تشکیل شد. تلئومورف و آنامورف روی دانهال‌های مایه‌زنی شده‌ی *Q. brantii*، *Q. infectoria* و *Q. libani* با گذشت ۳۴ ماه ایجاد نشد. علامت بیماری زغالی در شرایط جنگل فقط روی درختان *Q. brantii* و *Q. infectoria* دیده شد که گزارش میزبانی گونه اخیر، جدید است. مایه‌زنی شاخه بریده‌ی گونه‌های *Amygdalus* sp.، *Acer monspessulanum*، *Cerasus microcarpa*، *Pistacia* sp. و *Rosa* sp. موجب نکروز در بافت پوست داخلی شد اما در گونه‌های *Crataegus* sp. و *Ulmus* sp. بیماری رخ نداد. در این بررسی برای نخستین بار بیماریزایی *B. mediterranea* روی دانهال‌های *Q. infectoria* و *Q. libani* نشان داده شد.

کلمات کلیدی: آسکوسپور، *Quercus*، جنگل‌های زاگرس، زیست شناسی، بیماریزایی

مکاتبه کننده: داریوش صفایی Email: dasafaee@yahoo.com

تاریخ دریافت: ۱۳۹۵/۱۲/۱۸ تاریخ پذیرش: ۱۳۹۶/۰۷/۱۱