

Research Article

## Shot hole disease, survival and pathogenicity of the causal agent on stone fruit trees in Northeast Iran

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**Abstract:** Shot hole caused by *Wilsonomyces carpophilus* is one of the main constraints to prune fruit production in Iran particularly in Khorasan Razavi province. It causes foliage shot hole in spring and early summer; fruit-spotting and cankers on limbs and twigs during autumn rains. The fungus was isolated from the lesions of twigs and was purified on PDA. The pathogenicity and virulence on detached twigs of stone fruit tree species was examined *in vitro*. Virulence of the pathogen as measured by lesion length was significantly different among the different host species, showing the nectarine as the most susceptible species. In contrast to other hosts, sour cherry did not show any canker on shoots or twigs and disease progress was just as tissue colonization by the fungus hyphae. However, other species such as prune, cherry, apricot and almond did not show significant differences. The results of bud and shoot evaluations indicated that the fungus overwinters as hyphae and conidia in buds, and in the form of hyphae as well as thick-walled globular chlamydospores in twigs. Additionally, viability of recovered conidia ranged from 33 to 90% throughout the dormant season. A better understanding of disease cycle and survival mode of the fungus will help to manage and prevent the disease.

**Keywords:** Shot-hole disease, overwintering, virulence, stone fruit shoots

### Introduction

Shot hole disease is one of the major foliar diseases of *Prunus* species worldwide which is caused by several pathogens and particularly by *Wilsonomyces carpophilus* (Adaskaveg *et al.*, 1990). The fungus was first observed on peach trees in France in 1843, later in Africa, Asia, Europe, North, Central and South Americas, Australia and Oceania (Văcăroju *et al.*, 2008). The fungus causes local lesions on stone fruit trees that are economically important

hosts. The most limiting factor to their production is shot-hole disease. On peach, nectarine and apricot perennial infections of current-season twigs are more common than in other *Prunus* hosts (Ogawa and English, 1991). A number of peach diseases such as peach canker, constriction disease, constriction canker in the Midwest and Mid-Atlantic States, shoot blight in Georgia and dieback in Europe have been reported to have symptoms similar to shot hole (Cayley, 1923; Cohoon and Daines, 1956; Guba 1953; Selby, 1898) that may cause confusion in apparent identification of shot hole disease. Twig infections are catastrophic in shot hole because of devastation of buds. The disease causes blackish spots on twigs in close vicinity of buds. Spots gradually enlarge and

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become brown in color with purple margin. The spots occur beneath flowering buds in spring. Plant's sap suddenly stops in infected plants resulting in drying of twigs and blooms (Ashkan and Asadi, 1971). The disease is most harmful in very cool and wet conditions of spring, although it can occur and cause damage at any time during prolonged wet weather (Evans *et al.*, 2008). *W. carpophilus* overwinters as hyphae and conidia in the cankers on twigs and inside of scales in infected buds. In early spring, under suitable environmental conditions conidia germinate (Highberg and Ogawa, 1986). Then, the sporodochia and spores are formed, inoculum builds up and conidia spread on leaves, twigs and buds. Infection of these organs is critical for overwintering of the fungus in stone fruit trees. It is important to conduct exact studies about overwintering of the fungus. The objectives of the present study were to show the importance of the causal agent, pathogen survival in buds and twigs, inoculum buildup and disease severity on shoots for determination of fungicides application times.

## Materials and Methods

### Symptomatology and isolation of fungus from infected tissues

Infected shoots of peach, apricot, nectarine, prune and almond trees were collected from different orchards in Khorasan Razavi province, Iran, during 2007-2008. The symptoms were tan spots with purple to brownish margins that resulted in long canker on twigs (Fig. 1). Infected buds were darker than healthy buds. Shoots with canker were thoroughly washed under tap water, cut into segments (3-4 mm) from margin of healthy and infected tissues and surface-disinfested by immersing in NaOCl 1% for 5 min. Tissue pieces were transferred onto potato dextrose agar (PDA) plates and kept in an incubator at 22 °C for a week in dark, and then a week in

light condition according to Shaw *et al.* (1990) procedure.

### Overwintering assay of *W. carpophilus* in buds

Overwintering of *W. carpophilus* inside buds of some stone fruit trees was examined based on Highberg and Ogawa (1986) procedure with a little modification. One hundred and fifty flower and leaf buds were collected individually from infected twigs of peach, nectarine, apricot, almond and prune during winter 2007. The buds were removed from shoots, and all inner and outer bud scales were removed. The remaining scales of 150 buds were chopped and placed in a glass centrifuge tube containing 3 ml of sterile distilled water. The tube contents were subsequently vortexed for 30-40 min, centrifuged at 3000 rpm for 5 min and remixed for 2-3 min to suspend. Conidial suspension obtained from the bud scales was removed with a Pasteur pipette and transferred to a clean centrifuge tube. The entire washing procedure was repeated three times for each sample, and then suspensions containing conidia and mycelial pieces were mixed and centrifuged for 3 min at 5000 rpm. Pelleted materials were resuspended in 0.5 ml of sterile distilled water and spread onto 1% water agar plates. Plates were incubated at 25 °C for a week in dark condition. The number of germinated conidia after 24h in each sample was recorded using an Olympus microscope at 10X.

### Identification

To identify species of *W. carpophilus* based on Adaskaveg *et al.* (1990), one week old culture of the fungus grown on PDA in dark at 25 °C was used. Based on morphological and physiological characters and optimal temperatures for growth of the culture, all the isolates were identified as *W. carpophilus* and used in pathogenicity test.



**Figure 1** Symptoms of shot hole disease on peach shoots in current season (left) and last season (right).

### Pathogenicity

An isolate of *W. carpophilus* (Astan Qods Peach Fruit; APF1) was selected to pathogenicity test of twigs. Before each trial, percentage of spore germination was determined on water agar medium 1%. The test was carried out on detached shoots from young trees of Torouque Research Station according to Uddin *et al.* (1997) procedure with a little modification. Shoots of current season were collected from healthy young trees of nectarine, *Prunus persica* cv. Sunking, apricot, *Prunus armeniaca* cv. Shahrudi, sour cherry, *Prunus cerasus* cv. Erdi, prune, *Prunus domestica* cv. Golden Drop and almond, *Prunus amygdalus* cv. domestic grown in Torouque Research Station. Shoots were cut to fragments of 10-30 cm in length. Shoots were thoroughly washed under tap water for 15 min, blotted dry and surface-disinfested by ethanol 70% for 15 sec and then immersed in NaOCl 1% for 20 min. After blotter drying the shoots were again surface disinfested with ethanol 70% for 5 sec. From sites of inter two buds shoots were dissected horizontally using a sterile blade. A 10-20 mm superficial V-shaped incisures was made at a 45° angle at the dissected area to expose the tissue under the bark. An agar plug culture of the fungus the size of incisures was placed under the bark. The inoculation site was sealed with sterile moist cheesecloth according to the procedure described by Dhingra and Sinclair (1985). Control shoots were treated similarly but with sterile blocks of PDA. Six shoots were selected from each species. Each shoot was usually inoculated in three points. Control shoots for each cv. were separately placed in a sterile moist

chamber kept at 15 °C for two days and then at 25 °C for 30 days in growth chamber. The inoculated shoots were arranged in a completely randomized design with 15 replicates for each species (5 treatments of stone fruit species). Ten days after inoculation, cheese cloths were removed. The disease severity was evaluated by measuring the length of each spot at 10, 20, and 30 days after inoculation. Data were subjected to analysis of variance using SAS (version 9.1) and means were compared by LSD test ( $\alpha = 0.05$ ).

### Results

#### Isolation and description of the pathogen

*Wilsonomyces carpophilus* was isolated from overwintering inoculum of different varieties of stone fruit trees as hyphae, conidia and globular chlamydospores. Colonies grown on PDA medium at the beginning were colorless and greenish brown to olive, conidiophores macronematous, usually short and packed closely together forming pulvinate sporodochia, subhyaline to olivaceous brown, 4-10 × 10-37.5 μ, smooth or verrucose. Chlamydospores were pale brown to dark in color, and formed terminally or intercalary as solitary or in chains of three to four and rarely more. Conidia cylindrical, clavate, ellipsoidal or fusiform, truncate at base, with 1-6 (mostly 3-5) transverse septa and occasionally 1-2 oblique septa, 7.5-17.5 × 22.5-67.5 μ long.

In addition, a number of other fungi including *Alternaria* sp., *Penicillium* and *Cladosporium* spp. were isolated from spots on twigs.

The results of spore counts in buds of different stone fruit trees and their viability are shown in Table 1.

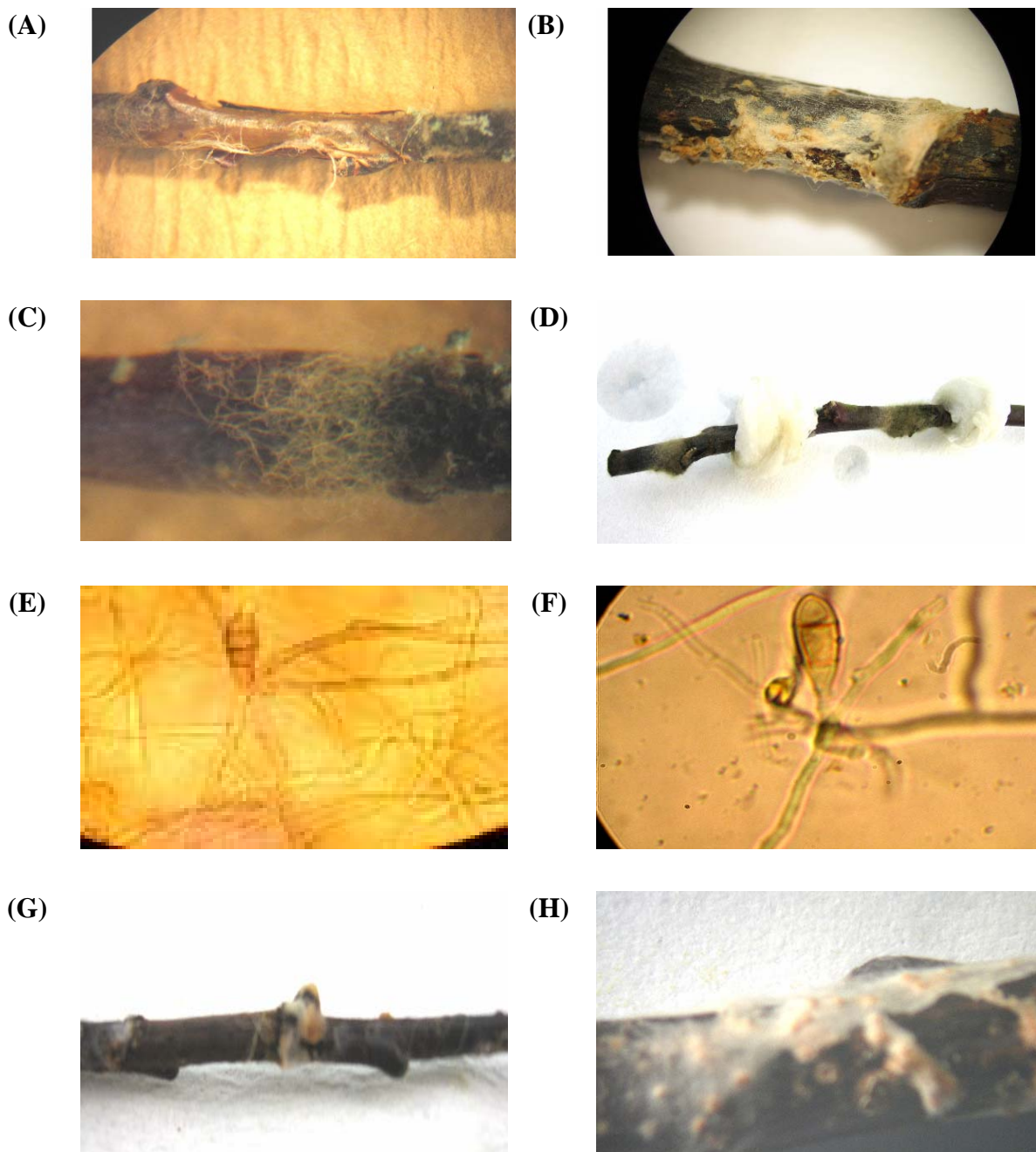
**Table 1** Number and viability of overwintering conidia in buds of *Wilsonomyces carpophilus* in washings from buds of different stone fruit trees.

Host	Collection	Date and site	Number spores	Spore germination (%)	Other isolated fungi
Almond cv. Domestic	Dec 2006	Torouque	0	0	<i>Alternaria</i> sp.
Apricot (cv. unknown)	Feb 2007	Zarkesh Kal	10	90	<i>Alternaria</i> sp.
Peach cv. Missouri	Feb 2007	Torouque	6	0	<i>Stemphylium</i> sp., <i>Alternaria</i> sp.
Almond cv. Domestic	Feb 2007	Torouque	0	0	<i>Alternaria</i> sp.
Apricot cv. Shahrudi	Feb 2007	Torouque	3	66.66	-
Nectarine cv. Quota	Feb 2007	Ghods Astan orchards (1)	0	0	<i>Cladosporium</i> sp., etc.
Peach cv. Amestan	Feb 2007	Ghods Astan orchards (2)	0	0	Unknown spores
Apricot cv. Lasgerdi	Feb 2007	Ghods Astan orchards (2)	8	50	<i>Alternaria</i> sp., <i>Stemphylium</i> sp., <i>Cladosporium</i> sp., etc.
Peach (cv. unknown)	Mar 2007	Zarkesh Kale	739	91.47	<i>Stemphylium</i> sp., <i>Alternaria</i> sp., <i>Ulocladium</i> sp., <i>Cladosporium</i> sp., etc.
Peach cv. Platycarpa	Mar 2007	Ghods Astan orchards (2)	6	33.33	-
Prune cv. Compote	Mar 2007	Ghods Astan orchards (2)	0	0	<i>Cladosporium</i> sp.

### Pathogenicity test

Germination percentage of conidia was 80% on 1% water agar medium. Six to seven days post inoculation the disease symptoms appeared as tan to brown and purple brownish spots on inoculation sites of detached shoots. Small cavities were formed around inoculation sites of detached shoots of Nectarine and Shahrudi apricot. The cavity size progressed on the entire bark around the inoculated sites, and finally the infested bark wrinkled. Buds in those sites were hindered in growth (Fig. 2-A). In all inoculated shoots except almond cv. Domestic, surfaces of buds and shoots in close vicinity of inoculation sites were covered by fungal white hyphae (Fig. 2-H). The appearance of white hyphae

was more frequent around buds (Fig. 2-C, D). The hyphae were compressed in some areas as a flat layer, and seemed white to orange in color (Fig. 2-B, I). As the size of spots increased the active growth of inoculated shoot stopped while the control shoots showed active growth and produced young leaves. Thirty days after inoculation of the fungus, the longest spots (20 mm) appeared on cherry shoots. Appearance of infection in some replications was just as hyphae instead of lesions. The results of analysis of variance revealed a significant difference in lesion length among the five species (Table 2).



**Figure 2** Symptoms of *Wilsonomyces carpophilus* on shoots of different stone fruit trees. A: Apricot shoot bark showing wrinkle at inoculated sites, B and H: Tissue colonization by the fungus, C, D: The appearance of white hyphae around buds, E, F: conidia formation at colonized sites, G: The appearance of white hyphae on shoots on two-sides of inoculated sites.

Of course, sour cherry cv. Erdi was exception. It produced small restricted spots covered by fungal hyphae. Nectarine was placed in one group (A) and the other species in another group (B) (Table 3).

Microscopic identification of white hyphae and colonies of the bark surface of inoculated detached shoots confirmed that *W. carpophilus* was the disease causal agent (Fig. 2-E, F).

**Table 2** Analysis of variance of lesion length in different stone fruit species inoculated by *Wilsonomyces carpophilus* *in vitro*.

Source	df	MS	Pr > F
Host	4	116.54	0.0242
Error	70	38.87	
Corrected Total	74		

**Table 3** Means comparison of lesion length of *Wilsonomyces carpophilus* (APF1) inoculated on shoots of five stone fruit species and recorded 30 days after inoculation.

Host	N	Lesion length (mm) <sup>1</sup>
Nectarine	15	12.467a
Prune	15	7.400b
Apricot	15	7.267b
Almond	15	7.000b
Cherry	15	4.933b

<sup>1</sup> Means followed by the same letters in a column are not significantly different (LSD test,  $P < 0.05$ )

## Discussion

Results showed that viable conidia of *W. carpophilus* were associated with apparently healthy dormant blossom buds throughout the 2007-2008 dormant seasons in orchards. This observation together with results obtained from pathogenicity test on shoots support the hypothesis that *W. carpophilus* conidia contribute to the overwintering of the fungus and disease development on stone fruit trees (Shaw *et al.*, 1990). The infection is spread by conidia. In dry conditions conidia remain viable for several months but cannot be detached or spread by the wind. Rain is necessary for their dispersal. In humid conditions, they can germinate at highly varying temperatures above 2 °C, which accounts for the winter infection of buds. Temperature and duration of wet periods

during the inoculation influence the development of shot-hole disease on leaves of the *Prunus* species caused by *W. carpophilus* (Văcăroiu *et al.*, 2009). Results of study on the presence of spores inside buds (Table 1) showed that conidia associated with dormant buds on higher twigs of the trees were being washed down by rain onto dormant buds on lower branches of the tree. For this reason, samplings that mainly were conducted from available lower buds in the tree can't be indicative of actual population of overwintering conidia. Also, in areas and at periods of high rainfall, greater conidial populations are observed in buds. The insignificant increase in number of conidia detected in bud samples collected in the Zarkesh Kal orchard over time can be explained in terms of both climatic and disease conditions that existed within the orchard during the dormant period. Also, some decrease in number of conidia was detected in bud samples collected in other areas where overwintering form is as hyphae in buds. However, studies on inoculation of almond buds with this fungus by Highberg and Ogawa (1986) indicated that the greatest increase in numbers of detected conidia occurred during the period between bud swell and early pink bud stage of bloom. Our evaluation of infected trees revealed that when healthy buds were in pink stage, both hyphae and conidia were observed in the blighted buds, but where *W. carpophilus* overwintered only as hyphae in infected twigs and blighted buds on almond, any infection of leaves and fruit was not observed in spring. Also, in comparison with other hosts, sour cherry did not show any cankers on shoots or twigs although its buds were infected and blighted as was confirmed by laboratory tests of shoots. This suggests that the buildup of inoculum levels that occur during

Rain fall is an important component in the shot hole disease cycle. The conidia formed on infected leaves during rainy periods in fall contribute not only to the overwintering population of the fungus but also provide a ready source of primary inoculum for spring infections. Unlike the situation observed for

other multiple-cycle diseases, the amount of primary inoculum present for initial infections appears to be an important factor in development of shot hole disease. Infection periods are determined by duration of moisture conditions and the temperature. At cooler temperatures, longer periods of moisture are required (Shaw *et al.*, 1990). Yousefi *et al.* (2010) observed that conidium germination started at a temperature of 1 °C (1-2%), the optimal temperature was recorded between 12 and 24 °C (25-80%) and it decreased to 5% at 30 °C. Germination of spore was recorded starting from 1 °C, the highest colony growth rate was reached at 20 °C and decreased again at 35 °C. The conidia generated by inoculum source are transported by rain and infect flowers and young leaves. After penetration of the infection hypha, the fungus produces intercellular mycelium. From this mycelium, loosely packed cushions of hyphal cells emerge to the surface, and give rise to conidia (Văcăroiu *et al.*, 2008). Results of this study indicated that the fungus overwinters as hyphae and conidia in buds and as hyphae in twig spots. These results are in conformity with those of Highberg and Ogawa (1986). Also, overwintering as chlamyospore in spots of twigs was in conformity with the results of Koul and Naarain (1983). Chlamyospores which are formed in natural conditions may act as a source of inoculum for the pathogen. According to Highberg and Ogawa (1986), if conidia were found to survive the dormant season in association with healthy dormant buds, a control program aimed at preventing fall buildup of inoculum could reduce the amount of primary inoculum for the following spring because conidia may be deposited on bud scales of healthy dormant buds during fall rains and remain there viable until the next season. Fungicidal tests conducted by Wilson (1937) for destruction of *W. carpophilus* conidia in infected buds showed that 80-90 percent of conidia on twigs were destroyed after a week. However orchard studies have shown that great numbers of conidia in spring were produced from these sprayed buds with fungicides

because winter spraying of fungicides is not effective on hyphae inside of host tissue (Ashkan and Asadi, 1971). This indicated the survival ability of the pathogen for several years inside twig cankers and infected buds under unfavorable environmental conditions. The pathogenicity test on different species of stone fruits by *W. carpophilus* showed a little constriction associated with wrinkle in inoculated sites in Peach and Nectarine species which caused wilting and death of the shoot. This was in conformity with the observed results of Uddin *et al.* (1997) for infection by *Phomopsis* sp. on peach. Uddin *et al.* (1997) also, indicated that rapid development of disease from wounded dormant buds may be due to easy penetration and colonization of the wood, a conclusion that was confirmed by our examination of pathogenicity on inoculated shoots. Ahmadpour *et al.* (2009) isolated from infected leaves, fruits and twigs of different Prunus species (apricot, almond, peach, nectarine, plum, sweet cherry and sour cherry) hyphae of the fungus *W. carpophilus* but its importance in disease cycle was not mentioned. According to Evans *et al.* (2008), the disease is most harmful in extended cool and moist periods in spring, while it can occur and cause damage at any time and any season during long lasting wet weather. This is the first documentation of the pathogenicity of *W. carpophilus* on shoots of stone fruit species *in vitro*.

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## بیماری غربالی، بقا و بیماریزایی عامل بیماری روی درختان میوه هسته‌دار شمال شرق ایران

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**چکیده:** بیماری غربالی با عامل بیماری *Wilsonomyces carpophilus* یکی از عوامل محدودکننده تولید میوه درختان میوه هسته‌دار در ایران به ویژه در خراسان رضوی می‌باشد. این بیماری باعث ریزش برگ‌ها در بهار و اوایل تابستان، لکه‌دار شدن میوه و ایجاد شانکر در سرشاخه‌ها در طی بارندگی‌های پاییزه می‌شود. قارچ عامل بیماری از لکه‌های سرشاخه‌ها جدا شد و روی محیط کشت PDA خالص‌سازی گردید. بیماریزایی قارچ روی شاخه بریده گونه‌های مختلف درختان میوه هسته‌دار در شرایط آزمایشگاه مورد بررسی قرار گرفت. در مقایسه با سایر میزبان‌ها، آلبالو هیچ شانکر یا لکه‌ای بر روی سرشاخه نشان نداد و علائم بیماری منحصر به کلنیزه کردن بافت میزبان به وسیله هیف‌های سفید رنگ قارچ می‌شد. شدت بیماری به وسیله اندازه‌گیری طول لکه‌ها در میان ارقام تفاوت معنی‌داری داشت. به طوری که بیشترین حساسیت به آلودگی را سرشاخه‌های شلیل داشتند اما گونه‌های گیلاس، زردآلو، آلو و بادام اختلاف معنی‌داری با هم نداشتند. نتایج ارزیابی جوانه و سرشاخه مشخص کرد که قارچ به صورت هیف و کنیدی در جوانه و هیف و هم‌چنین کلامیدوسپورهایی با دیواره ضخیم در سرشاخه‌ها زمستان‌گذرانی می‌کند. هم‌چنین جوانه‌زنی کنیدی‌های یافت شده در فصل خواب، ۳۳ تا ۹۰ درصد بود. اطلاعات به‌دست آمده از چرخه بیماری و نحوه بقای قارچ می‌تواند به مدیریت و پیشگیری این بیماری کمک مؤثری نماید.

**واژگان کلیدی:** بیماری غربالی، زمستان‌گذرانی، شدت بیماریزایی، شاخساره درختان میوه هسته‌دار