تندش آسکوسپور و تشکیل چنگک *Polystigma amygdalinum* در شرایط درون شیشه‌ای و طول دوره‌ی بقای آن*

آزاده حبیبی و ضیاءالدین بنی هاشمی[1]**

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چکیده

بیماری لکه آجری برگ بادام ناشی از *Polystigma amygdalinum* از بیماری‌های مهم بادام در ناحیه خاورمیانه است. اطلاع از طول دوره‌ی بقای آسکوسپورها و شرایطی که تحت آن تندش می‌کنند، در مدیریت و پیش آگاهی بیماری مفید بوده و به درک جنبه‌های بیشتری از زیست‌شناسی و مراحل ایجاد بیماری به وسیله‌ی این قارچ زیواپرور کمک می‌کند. تندش آسکوسپور در آب مقطر و محیط کشت‌های مختلف در دماهای ۵، ۱۰، ۱۵، ۲۰ و ۲۵ درجه‌ی سلسیوس در بازه‌های زمانی ٦، ۸، ۱۰، ۲٤ و ٤۸ ساعت پس از کشت مورد بررسی قرار گرفت. نمو چنگک در *Polystigma amygdalinum* مشاهده و توصیف گردید. دما اثر معنی‌داری روی تندش و روند تشکیل چنگک داشت. تندش و تشکیل چنگک در بازه دمایی ۵ تا ۲۰ درجه‌ی سلسیوس رخ داد. درصد تندش در ۵ درجه‌ی سلسیوس بالا (٦۰٪) و در ۱۰ درجه-ی سلسیوس حداکثر (۷۰٪) بود. نور اثر معنی‌داری روی تندش آسکوسپور نداشت. آسکوسپورهای جمع‌آوری شده در طی سال‌های ۱۳۹۰ تا ۱۳۹۲ از نظر طول دوره‌ی بقا با یکدیگر تفاوت معنی‌دار داشتند. آسکوسپورهای جمع‌آوری شده در سال‌های ۱۳۹۰ تا ۱۳۹۲ در شرایط طبیعی زنده بوده و درصد تندش آن‌ها به ترتیب ۲۷، ٥٤ و ۷۰ ٪ بود. از بین محیط کشت‌های مورد بررسی، محیط کشت سیب‌زمینی-دکستروز-آگار حاوی ۰/۰۲۵ ٪ ذغال فعال بیشترین درصد تندش آسکوسپور را داشت.

کلیدواژه: لکه آجری برگ بادام، زیوا پرور، اثر دما ، اثر نور، مهارلو ، فارس

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* بخشی از پایان‌نامه دکترا ارائه‌شده به دانشگاه شیراز

** مسئول مکاتبات، پست الکترونیکی: ziabani@shirazu.ac.ir

۱- بخش گیاهپزشکی دانشکده کشاورزی دانشگاه شیراز

Ascospore germination and appressorium formation *in vitro* of *Polystigma amygdalinum* and its survival period*

A. Habibi and Z. Banihashemi[1]**

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Abstract

Ascospore germination was examined in *Polystigma amygdalinum*, incitant of red leaf blotch of almond with the goal of more detailed study of the biology and disease phases of this biotroph. Appressorium development is described for a species of *Polystigma* for the first time. Temperature had marked effect on ascospore germination and appressorium formation process. Germination and appressorium formation occurred in a temperature range of 5 to 20°C. Germination percentages were highest at 5 to 10°C. Ascospores at 25°C showed abnormal swellings and distortion of the cell content. Light had no effect on ascospore germination. The highest germination of ascospores was obtained on potato dextrose agar medium.

Keywords: *Polystigma amygdalinum*, Almond leaf blotch, ascospore germination, appressorium formation

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** Corresponding Author, Email: ziabani@shirazu.ac.ir

1. Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran, 7144165186.

Introduction

Polystigma amygdalinum P.F. Cannon , the causal agent of red leaf blotch disease of almonds, has been reported to occur in many countries (Khan, 1961; Saad and Masannat, 1997; Cımen and Ertugrul, 2007; Ghazanfari and Banihashemi, 1976). The pathogen often results in premature defoliation. Based on morphology, *P. amygdalinum* has been assumed to be a member of the order *Phyllachorales* (Cannon, 1996; Lumbsch, 2007). The pathogen overwinters as ascocarps in leaves on the soil and produces mature ascocarps by the end of winter (Ghazanfari and Banihashemi, 1976). Ascospore discharge begins at flowering and attacks the emerging leaves. The incubation period was estimated to be about 30-35 days (Banihashemi 1990). Little has done regarding the biology of *Polystigma* spp.

There is few published information on ascospore germination in *Polystigma* species and appressorium formation has not been described in any *Polystigma* spp yet. Banishashemi (1991) working on biology of *P. amygdalinum* noted that the mature ascospores obtained from overwintered leaves germinated in distilled water and water agar at 20°C and finally burst. Another report is by Suzuki *et al.* (2008) who observed the ascospore germination of *Polystigma fulvum* on water agar in which did not grow further to produce colonies on agar media. Ascospore germination and appressorium formation has been studied in other *Phyllachorales* in species of *Phyllachora* by Parbery (1963) and Dittrich *et al.* (1991). Ascospore germination and appressorium formation have been observed and described in many fungi of various ecological and phylogenetic groups.

The morphological characteristics of appressoria are reported to be distinct and used for taxonomic separation of fungal species, e.g. in the gunus *Colletotrichum* (Sutton, 1968), *Phyllachora* (Parbery, 1963), species of the *Rhytismataceae* (Osorio and Stephan, 1989) and powdery mildew species (Zaracovitis, 1966). Appressorium may therefore be a useful taxonomic criterion for species identification.

In this study experiments were conducted on germination of ascospores of *P. amygdalinum* and various factors affecting spore germination of this pathogen with the goal of obtaining the fungus in artificial culture for more detailed study of the biology and disease phases of this biotroph.

Materials and methods

Collection of Samples

Leaves of almond infected with *P. amygdalinum* containing mature ascomata were collected during March of 2013 at almond orchards of Maharlou region, Fars, Iran. The leaves stored at 4°C.

Germination of ascospores in vitro

To abtain ascospores for germination studies, the infected leaves containing *P. amygdalinum* ascostroma were crushed in distilled water on glass slides, the centrum containing ascospores transferred to sterile distilled water. Ascospore suspentions were spread over potato dextrose agar (PDA) and incubated at 5, 10, 15, 20, and 25°C. Suspensions were also mounted in hanging drops of water (Thite & Patil, 1975) on glass slides and incubated at 5, 10, 15, 20, and 25°C. The media and slides were observed at intervals of 2, 4, 6, 8, 12, 24, 48, and 72 hours after mounting. 100 spores per sample were examined with a light compound microscope. In order to compare changes in size of ascospore during germination, an average of 50 ascospores was measured before and after germination using a dino-eye microscope camera.

The following alternations in temperature were employed: i) 10°C for 24 hours changed to 20°C. ii) 10°C for 48 hours changed to 20°C.

The effect of light on ascospore germination of *P. amygdalinum* was studied by keeping media containing ascospores in black plastic bags in 20°C. Ascospore germination was examined periodically and compared to ascospore germination in the control media put at 20°C under florescent lamps.

Ascospore germination and appressorium formation on excised leaves

Ascospore germination and appressorium formation were also studied on almond excised leaves. The excised leaves were washed and surface disinfected at 95% EtOH for 10 s, 1%

NaOCl for 1 min, and then distilled H_2O for 1 min. Ascospore suspensions were spread over excised leaves, placed in moist chambers and incubated in 10 and 20°C for 10 days. The leaves were examined for a couple of days. For microscopic examinations; the leaves were cleared by a method described by Busch and Walker (1958). For recovery of viable spores from leaf surface, melted (40°C) 3% water agar applied to the leaf surface and allowed to gel (Lingappa & Lockwood, 1963). A Petri dish containing a water saturated Whatman paper was used as a moist chamber.

Media used for germination of ascospores

The media used were water agar (WA), Yeast extract agar (YEA), potato dextrose agar (PDA), corn meal agar (CMA), mung beans agar (MBA, filtrate of 100g of boiled mung beans in 1l of water), Sabouraud nutrient agar (SNA) (Dittrich *et al.*, 1991), almond leaf extract agar (AEA, 4g of almond leaves were homogenized in 20ml distilled water and then filtered with 0.2 µ Millipore filter), Almond leaf agar (ALA, 40g almond leaves were homogenized with 1000 ml distilled water). 0.3 ml 90%lactic acid was added to media. Replicates of each media containing ascospores were incubated at 5, 10, 15, 20, and 25°C. The germination of ascospores was examined by light microscope.

Results

Germination of ascospores and appressorium formation

The germination of all ascospores under study followed a similar pattern. Ascospores began to germinate within 6 hours at 5 to 20°C with the production of an unbranched non-septate germ tube. No predetermined germ pores were observed and the germ tubes emerged from any point on the ascospores. However, they were lateral in most cases (Fig 1a,b,c). Each ascospore was capable of giving rise to only one germ tube (exceptions to this were rare). Not all viable ascospores commenced germination simultaneously. In some cases, ascospores still in the ascus were also found to germinate *in situ* and the germ tubes passed through ascus wall (Fig 1d). Occasionally the ascospores seemed constricted at the middle, but no septum were seen (Fig 1e). An increase in average size of ascospores from 12.31 (±1.4) ×4.56 (±0.62) µm to 16.07 (±1.68) ×7.09 (±0.72) (µm) was observed in germinated ascospores.

Most germinated ascospores finally developed an appressorium. The appressorium initiates as a terminal swelling of the germ tube. Germ tubes could reach a length one to five times that of the ascospore and then start developing an appressorium (Fig 1f). Appressorium formation started after 12-72 hours with gradual shape and size changes and finally they became separated from the germ tube by a septum and showed their typical form (Fig 1g, h). A mature appressorium is slightly pigmented. When appressorium formation was finished, the cell content of the ascospores dissolved gradually after 3-5 days and there was no tendency to produce colonies in agar media. Very rarely a short secondary tube germinated from the appressorium.

The appressoria did not have significant differences in shape and size and were mostly uniform. The appressoria were simple and more or less globose to oval. Appressoria in hanging drops were sessile or on very short germ tube and globose (Fig 1g, h, i).

Germination of ascospores also occurred in distilled water in hanging drops without problems and at a high percentage. Appressorium formation which is the first step to infection was faster in hanging drops compared to solid media. Ascospore germination and appressorium formation in hanging drops of water started within 6-10 hours and was finished after 48 hours with the ascospore and asppressorium disappearing in the water. On PDA medium the germ tubes usually attained a considerable length before appressoria are formed, but in hangigng drops appressoria are formed immediately after a limited elongation (Fig 1i).

Ascospore germination and appressorium formation on excised leaves were studied too. Ascospores of *P. amygdalinum* were found to germinate on excised leaves of almond but with a very low percentage. No appressorium formation was observed with the methods used. No penetration onto leaves was observed.

Appressorium formation *in vitro* in *Polystigma amygdalinum* was successful.

Factors influencing the ascospore germination and appressorium formation

Temperature had marked effect on

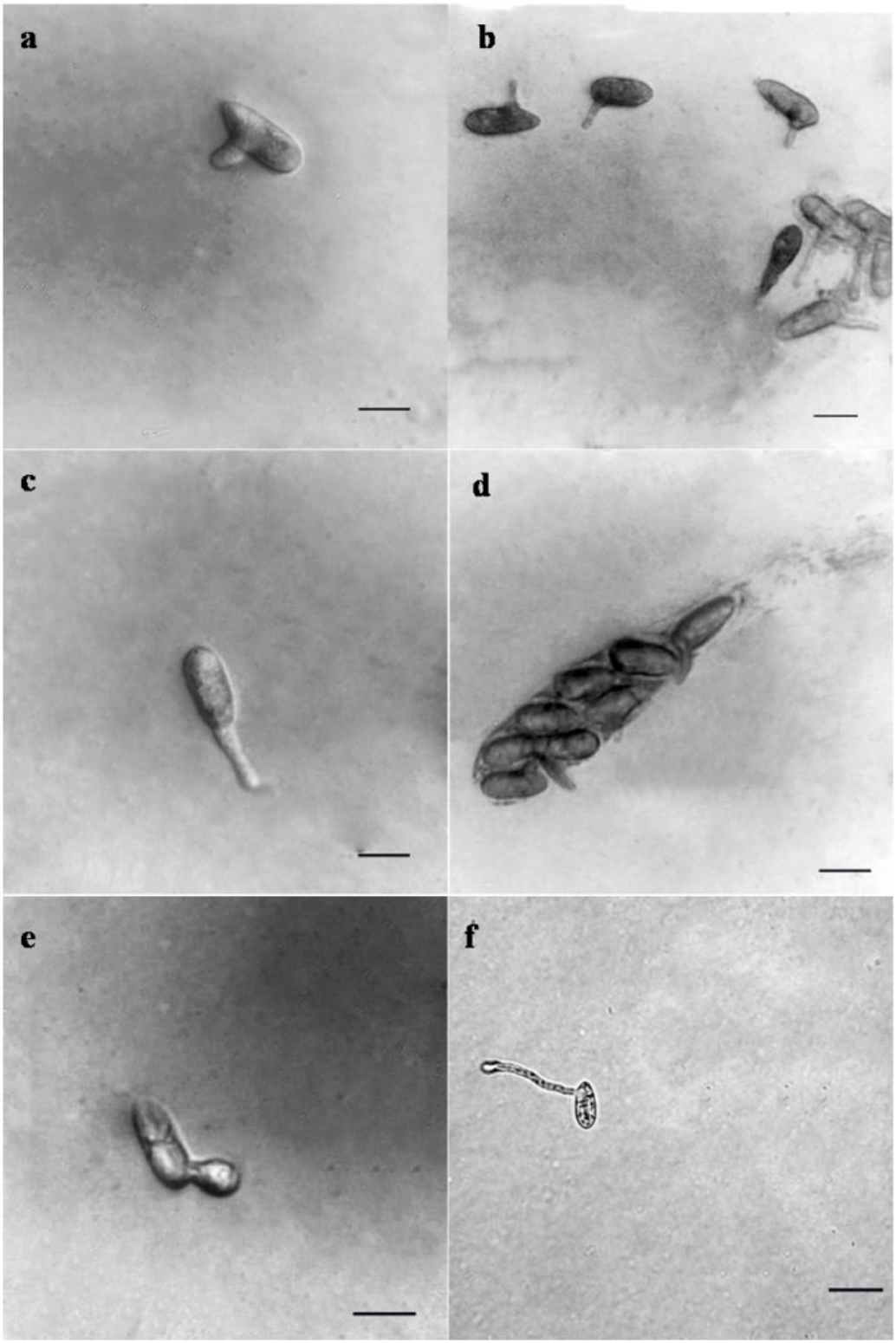
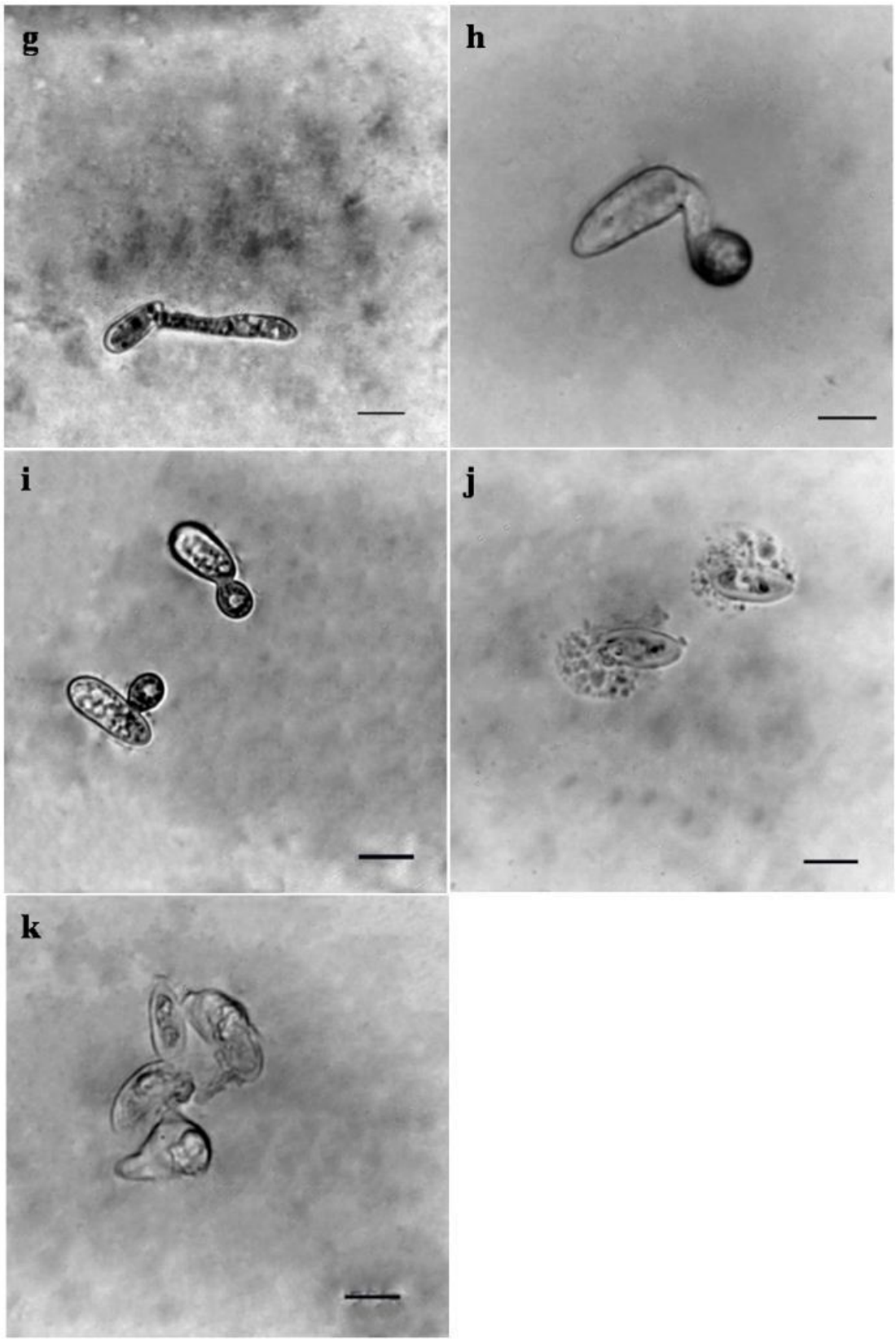


Figure 1. Germinating ascospores and appressorium development of *P. amygdalinum*. a, b, c, lateral and polar germ tubes. d, ascospore germination within ascus. e, ascospore constriction after germination. F, germ tubes could reach a length one to five times that of the ascospore. g, h, i, appressorium formation. j, k, abnormal swellings and distortion of the cell content of ascospores in 25°C



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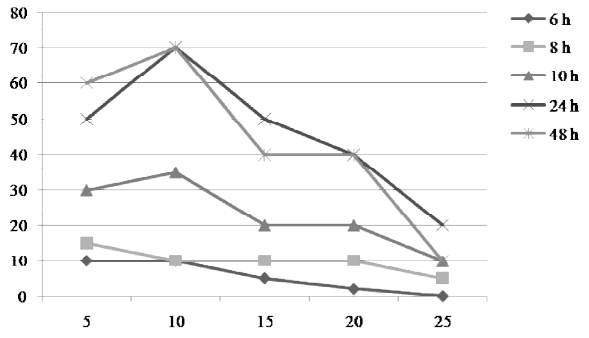

Figure 2: Effect of temperature (°C) on ascospore germination (%) of *Polystigma amygdalinum* in potato dextrose agar medium.

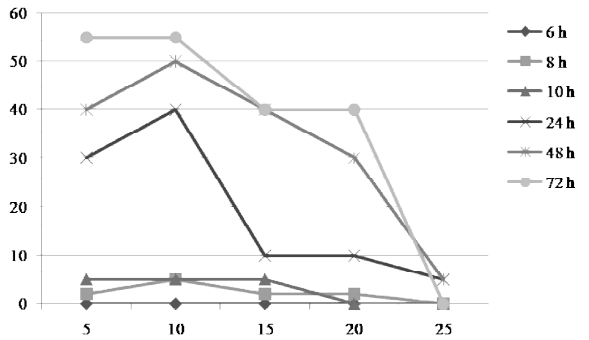

Figure 3: Effect of temperature (°C) on percent of ascospores of *Polystigma amygdalinum* that developed appressorium in potato dextrose agar medium.

germination of ascospores. Germination and appressorium formation occurred in a temperature range of 5 to 20°C. They were slower at higher temperatures. Germination percentages were highest at 5 to 10°C. 70% of the spores germinated at 10°C after 48 hours and 55% of them produced appressorium. Only 7% of the spores germinated at 25°C after 24 hours and 5% of them produced appressorium. Most ascospores at 25°C showed abnormal swellings and distortion of the cell content (Fig 1j, k) and very few ascospores survived long enough to produce germ tubes. At 5 to 10°C germ tube lengths were shorter than that at 20°C and most of appressoria were sessile. When the temperature was changed from 10°C after 24 hours to 20°C a high percentage of appressoria (60%) formed on PDA medium compared to constant temperatures.

Ascospores germinated equally under dark and light conditions used.

Germination percentage of *p. amygdalinum* ascospores varied among different media. Best results were obtained on PDA and WA. The percent of germination was very low on other media. No germination was observed on SNA, AEA and ALA. Germ tubes were longer on WA compared to that on PDA

Discussion

Although ascospore germination *in vitro* of species of *Polystigma* has been reported in *Polystigma ochraceum* and *Polystigma fulvum* (Banihashemi 1991; Suzuki *at al.* 2008), no detailed study have been done on germination patterns in this genus. The germination of ascospores of *P. amygdalinum* is to some extent similar to the pattern described for species of *Phyllachora* (Orton, 1956; Parbery, 1963; Dittrich *et al.*, 1991). We observed that *P. amygdalinum* ascospores germinate and develop appressorium at 5 to 20°C in both distilled water and agar media with the highest germination percentage at 10°C and week and abnormal germination at temperatures higher than 20°C. Orton (1956) noted a different temperature range of 25 to 27°C for ascospore germination in *Phyllachora punctum*. Our observations are partly consistent with Parbery (1963) and Dittrich *et al.* (1991) who found that the optimum temperature for *Phyllachora* species is 10 to 20°C and that at 25°C germination and appressorium formation are lower.

The optimum temperature of 10°C in which ascospore germination and appressorium formation occurs permits *P. amygdalinum* to penetrate almond leaves during early spring when the new emerging leaves are sensitive. This ability to germinate and form appressorium only at low temperatures may provide an explanation for *P. amygdalinum* associated with host leaves emergence at the beginning of the season while the mature ascocarps containing viable ascospores from previous season are present during the whole life stages of the host.

Ascospore size was increased after germination. Allen (1965) and Gottlieb (1950) stated that swelling of spores during germination is a general phenomenon. According to d'Enfert (1997) swelling is the first morphological change that can be observed during spore germination which involves water uptake as well as wall growth. It is concomitant with the restart of

numerous metabolic activities including respiration and RNA and protein synthesis and result in a cell whose diameter is two to several times that of the resting spore.

Ascospore germination within the ascus is observed by Ullasa (1969) in *Parodiella* and Orton (1956) in *Phyllachora punctum*. This was also noted during present study.

The present work is the first report on appressorium formation and its process of development in *Polystigma*. Orton (1956) saw appressorium initials in *Phyllachora punctum* and named them 'appressorium-like swellings'. Later, Parbery (1963) described the process of appressorium formation for species of *Phyllachora*. In *Polystigma*, we observed that the appressoria started as swellings at the apex of the germ tubes which were finally sessile or subtended by a germ tube from which they were separated by a septum similar to appressoria in many other species in genera such as *Phyllachora*, *Colletotrichum* and *Guignardia* (Emmett and Parbery 1975; Sutton, 1968; Skoropad, 1967). The optimum temperature for appressorium formation coincided with that for spore germinations. Although there are reports on effect of light on spore germination and appressorium formation in many fungi such as powdery mildews (Staub *et al.* 1974 and rusts (Emmett and Parbery 1975), light had no effect on spore germination and appressorium formation in *P. amygdalinum*. Ascospore germination and appressorium formation may be affected by other factors such as maturity of ascospores as noted by Osorio and Stephan (1989) in species of Rhytismataceae.

Appressorium formation was highest and fastest in hanging drops but they frequently burst in water, presumably because of high pressure potentials within the spore. Dittrich *et al.* (1991) observed the same situation in ascospore germination of *Phyllachora* species in water drops and connected it to the lack of nutrients and other essential substances. This phenomenon is also seen in other appressorium forming fungi such as *Uncinula* (Gadoury & Pearson, 1990)

Ascospores of *P. amygdalinum* germinated well on PDA medium. Nutrients available in PDA especially starch may have triggered spore germination. Foster and Wynne (1948) stated that starch neutralizes some factors unfavorable to spore germination. Inhibitors such as phenolic compounds in almond tissues (Sang *et al.*, 2002) may be an explanation for germination inhibition in ALA and AEA media. Failure of the germinated ascospores and appressoria to develop colonies in artificial media indicates its true obligate nature. Ascospores of *P. amygdalinum* were found to germinate on excised leaves of almond but with a very low percentage probably due to low availability of nutrients and possible inhibitors on leaf surface.

Future studies should refer to details of the function of appressoria in *Polystigma*. It will be necessary to overcome the resistance of this fungus to develop colonies in artificial media.

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