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RESEARCH PAPERS

Mating system and role of pycnidiospores in biology of *Polystigma amygdalinum*, the causal agent of almond red leaf blotch

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Summary. *Polystigma amygdalinum* is a serious leaf pathogen of almonds. Ascospores are believed to be the only inoculum of this pathogen. Ascocarp initials develop in infected leaves in contact with, or in close proximity to, filimentous spore-bearing bodies, which are the first to appear. While the aseptate filiform spores (scolecospores), are suspected of involvement in the sexual cycle of *P. amygdalinum*, there is no direct evidence of this. In order to study the role of the scolecospores, infected almond leaves, collected during the period from first appearance of symptoms to perithecium development, were sectioned by freezing microtome and the developmental anatomy was examined. Since ascogonia were not evident prior to the appearance of scolecospores, it was concluded that these were part of the sexual cycle of *P. amygdalinum*. This is supported by observations that perithecium initials always developed beside scolecospore fruit bodies, scolecospores failed to germinate *in vitro*, and almond leaves inoculated with them never became infected. We conclude that the filiform spores are spermatia essential to the sexual process and not asexual spores. Moreover, *P. amygdalinum* is likely to be homothallic, because single lesions in leaves infected by *P. amygdalinum*, assumed to have arisen from single ascospore infections, produced perithecia as abundantly as occurred in coalescing lesions caused by multiple ascospore infections.

Key words: mating system, scolecospores.

Introduction

Polystigma amygdalinum P.F. Cannon, the causal agent of the disease red leaf blotch of almonds, has been reported from many countries (Ghazanfari and Banihashemi, 1976; Saad and Masannat, 1997; Cimen and Ertugrul, 2007). The pathogen is widely distributed in the Mediterranean region and often causes premature defoliation of its host. While there is differing opinion on where species of *Polystigma*, including *P. amygdalinum*, belong among the unituncate ascomycetes, Cannon (1996) placed them in the order *Phyllachorales*, in the Sordariomycetidae. Habibi *et al.* (2015) considered that the genus is closely related to the *Xylariales* and placed it in the *Xylariomycetidae*. This, however, is an unresolved taxonomic question with no direct bearing on this discussion other than that numbers of species within each group appear to have similar life cycles. This in turn may well have a bearing on the development of effective disease control measures against these pathogens. Very little research has been carried out on the biology of *P. amygdalinum*, and there are unanswered questions regarding the sexual cycle and mating strategy of this pathogen.

Although information on the life cycle of *P. amygdalinum* is limited, Pearce and Hyde (2001) considered *Polystigma* and *Phyllachora* within the Sordariomycetidae, among other genera, which appear to have similar life histories, some of which exhibit only sexual reproduction. Of these, species of *Phyllachora* have been studied extensively (Parbery, 1963; Parbery and Langdon, 1963; Cannon, 1991, 1996; Pearce *et al.*, 1999; Pearce and Hyde, 2001). Many of the species in various genera produce ascospores and filiform spores, which Parbery and Langdon

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(1963) concluded were spermatia and thereby part of the sexual reproductive cycle. Pearce and Hyde (2001) reached the same conclusion, discarding the inappropriate names of asexual spores and the fruiting bodies which are widely applied to them, and using the terms spermatia and spermagonia, which are appropriate.

Following infection of almond leaves in spring with ascospores which are discharged during early February, just before flowering, until mid-May, after fruit set, the first symptoms of infection are accompanied by the formation of stromata within the host tissue, in which fruiting bodies and the filiform spores develop. The earliest disease symptoms appear mid-May and continue to develop during the summer months. The incubation period is estimated to be approximately 30–40 days under Maharlou conditions in Iran (Banihashemi, 1990) and 35–40 days under Halat conditions in Lebanon (Saad and Masannat, 1997). No secondary infection by filiform spores has been recorded. This is followed by the development of perithecia in close association with the filiform spore stage during the autumn and winter months in fallen leaves on the ground. In the following early spring, as new almond leaves emerge, ascospores are released and infect the young leaves. To date there has been no direct proof that the filiform spores found in developing colonies of *P. amygdalinum* are spermatia. However, the hypothesis that they are spermatia is supported by strong circumstantial evidence gained through the study of other species, especially in the genus *Phyllachora*. Thus the question of whether these filamentous spores associated with developing perithecia of *P. amygdalinum* are spermatia or not (Banihashimi, 1990; Saad and Masannat, 1997) has been investigated. The present study has attempted to determine if these spores are spermatia or conidia, and in either case, whether they are functional.

A further objective was to examine the mating system of *P. amygdalinum*, which has not been previously studied. According to evolutionary theory, reproductive methods adopted by organisms, sexual inbreeding or outbreeding, asexuality or parasexual reproduction, vary the rates and patterns of genome evolution over time, regulating the rate of adaption to changes in selection pressures, including host genetic variation (Whittle *et al*., 2011: Billiard *et al*., 2012) and the development of tolerance to fungicides (Milgroom, 1996). The importance of heterothallism in the production of new races of *Venturia inaequalis* able to attack new genotypes of apple was demonstrated many years ago by Keitt and Palmiter (1938). The chances of adapting to new circumstances are generally greater when sexual spores arise from a heterothallic mycelium which contain greater proportions of new genotypes than those arising from homothallic mycelium.

The main objective of the present study was to investigate the function of the filiform spores in the life cycle of *P. amygdalinum* and to follow the process of ascospore formation through examination of the developmental morphology and life history of the fungus. A further aim was to determine the mating system of this fungus by studying the reproductive outcome of colonies produced from single ascospores.

Materials and methods

Collection of samples

Almond leaves with typical red leaf blotch symptoms were collected from the Maharlou region of Fars in Iran, at 1 month intervals beginning from May 2014, when the first disease symptoms appeared, to January 2015 when perithecia formed. Samples were stored at 4°C in moistened vermiculite and moistened orchard soil to simulate overwintering conditions and favour perithecium development.

Germination and infectivity of filiform spores

To investigate the ability of the filiform spores to germinate, infected almond leaves containing colonies of *P. amygdalinum* containing only filiform spore bodies were washed in tap water and surface disinfested in 10% NaOCl for 1 min and crushed in distilled water on glass slides. Filiform spore suspensions were obtained and spread over selected media and incubated at 5, 10, 15, 20, or 25°C. The media were examined for spore germination at intervals of 2, 4, 6, 8, 12, 24, 48, and 72 h after mounting. The media used were water agar (WA) (2%), 2% yeast extract agar (YEA), potato dextrose agar (PDA), PDA amended with 0.25 g L^{-1} activated charcoal, corn meal agar (CMA), and almond leaf extract agar (AEA; 4 g of almond leaves homogenized in 20 mL of distilled water and then filtered through a 0.2 μm Millipore filter, Merck, Germany). To each medium 0.3 mL lactic acid (90%) per litre was added to suppress bacterial growth. The pH of the media was between 4 and 6.

To investigate the infectivity of the filiform spores (scolecospores), inoculum was prepared from young infected almond leaves containing colonies of *P. amygdalinum* producing only filiform spore fruit bodies. The leaves were washed in tap water, surface-disinfested in 70% EtOH for 10 s, 1% NaOCl for 1 min, then in deionized water for 1 min, and then were crushed in sterile distilled water on glass slides. The resulting suspensions were filtered through three layers of cheesecloth. The concentration of spores was adjusted to 1×10^6 filiform spores mL^{-1} using a haemocytometer. The pycnidiospores were spray-inoculated on 5–6 weeks-old almond seedlings. Plants were covered with plastic bags for 48 h to maintain high humidity around the leaves and placed in 20°C with 16 h photoperiod for a week.

Role of filiform spores in perithecium formation

To confirm the relation between the filiform spores and the ascomatal stage of *P. amygdalinum*, infected almond leaves bearing the filiform spore stage were stored in plastic containers in moistened vermiculite at 4°C to stimulate natural conditions during overwintering, when perithecium development occurs. The infected leaves were used for microtome sectioning before storage to confirm that the lesions were in the filiform spore stage. The stored leaves were examined periodically for perithecium formation during the next few months. Ascospore inoculum was prepared in the same way as the scolecospore inoculum above. Similarly, inoculations with ascospore suspensions were carried out using the same method as for scolecospore inoculations. Almond leaves were examined periodically for red leaf blotch symptoms. Leaves bearing the lesions were used for microtome sectioning and examined for spermogonium formation.

Development of *Polystigma amygdalinum* perithecium formation in sterilized vermiculite

Specimens collected in the Maharlou region of Fars, Iran were used in this anatomical study. Infected almond leaves were collected from the appearance of symptoms in May 2014 to perithecium formation, approximately 200 to 220 d later, in January 2015. Two sets of infected almond leaves with colonies at various development stages were also collected and stored, one set in moistened orchard soil, the other in moistened vermiculite, both at 4°C to simulate overwintering conditions. Anatomical comparisons of the developing fungus in each set of stored leaves were made at weekly intervals.

A small leaf portion (5 mm^2) surrounding a developing colony of *P. amygdalinum* was excised from infected leaves and frozen in Neg-50™ sectioning medium (Richard-Allan Scientific, Kalamazoo, MI, USA). Sections 6 to 18 μm thick were cut with a freezing microtome (Thermo Scientific™ HM 525 Cryostats, USA), stained in lacto phenol cotton blue and Safranin-O/fast green (Sass, 1958), and were examined using a light microscope. Observations were made of fungal structures such as filiform spores, fruiting bodies and perithecia, and any structural changes indicative of possible mating processes.

To investigate the possibility of soil microorganisms interfering with perithecium formation of *P. amygdalinum*, the following experiment was performed. Two sets of infected almond leaves were collected during summer and early autumn and stored in plastic containers, one set in moistened orchard soil, the other in moistened sterilized vermiculite, and both sets were stored at 4°C to simulate overwintering conditions. The incubated leaves were examined periodically for perithecium formation using microtome sectioning.

Perithecium formation in leaves with single lesions

The following experiment was designed to investigate the mating system of *P. amygdalinum*. Single lesions caused by the pathogen were assumed to have developed from single ascospore infections. Leaves bearing single and multiple lesions were collected separately during summer and early autumn and stored separately in plastic containers in moistened vermiculite at 4°C to simulate natural conditions for perithecium formation. The incubated leaves were examined periodically for perithecium formation and observations were recorded. One hundred lesions were examined of each group.

Genomic DNA extraction, PCR and sequencing

The relationship between the filiform spore stage and ascomatal stage of *P. amygdalinum* was examined. DNA extraction was attempted from lesions in almond leaves caused by *P. amygdalinum*, both at the

filiform spore stage and the ascomatal stage. Pseudostroma composed of plant and fungus tissue were freeze-dried and stored at -20°C. Freeze-dried tissue was homogenized using sea sand (Fluka, Germany) and plastic disposable pestles. Cells were lysed using CTAB solution (2% CTAB; 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA and 2% 2-mercaptoethanol), and DNA was extracted using DNG™-plus DNA extraction solution, following the manufacturer's instructions (Cinaclon). DNA concentrations were estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). DNA extractions were each diluted to 20 ng mL^{-1} in sterile distilled water for use as template DNA in PCR. In some cases, serial dilutions of DNA extractions were used to find the appropriate concentration for PCR, due to presumptive PCR inhibitors coming from environmental materials.

Primers PyITS1 (Green *et al*., 2004), and ITS4 (White *et al*., 1990) were used to amplify internal transcribed spacer1, 5.8S and internal transcribed spacer 2 (ITS regions) of rDNA from *P. amygdalinum*. Twenty-five µL PCR reactions contained 1× reaction buffer, 0.4 mM each primer, 200 mM dNTPs, 2.5 mM $MgCl_2$, 20 ng of DNA and 1 unit of *Taq* polymerase. PCR was carried out in a CG1-96 thermocycler (Corbett Research) and cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, then followed by 5 min at 72°C. Sequencing was performed by Tech Dragon (Korea) and sequences were deposited in GenBank. Sequences were pairwise aligned using Geneious software version 7 (Biomatters, USA). The sequences from infected almond leaves containing only filiform spore bodies were compared with those from leaves containing mature ascomata of *P. amygdalinum*.

Results

Results from the above experiments support the hypothesis that the filiform spores formed in pycnidioid structures prior to the development of proto-perithecial elements adjacent to them, are solely sexual elements. These spores failed to germinate *in vitro* and *in vivo* and when used to inoculate almond leaves under conditions conducive to infection by ascospore inoculum, no infections occurred. On the other hand, production of filiform spores always preceded the formation of ascogonia.

Development of *Polystigma amygdalinum* from colonization to sporulation

Infection and colonization

Symptoms of infection appeared as minute discolorations on the surfaces of green almond leaves about 30–40 d after ascospore release. As the lesions spread and the fungus ramified throughout the leaf mesophyll tissues (Figure 1a), the lesions became orange to reddish due to deposition of granules of orange pigment in the infected tissues. The source of the pigments is unknown. In some cases, the mesophyll within the infection court was replaced almost entirely of fungal tissue.

Filiform spore development

The onset of the orange-red pigmentation of lesions signaled the initial development of small stromata under the host leaf epidermis (Figure 1b). Hyphae branched, forming small compact masses of stromatic tissue. These stromatic bodies, with their tips pointing outward, later became flask-shaped pycnidioid bodies (Figure 1c-d). Throughout the summer spores formed in great abundance (Figure 1e), mostly within the mesophyll towards the undersurface of leaves. The lesions gradually changed to ochraceous brown, when the filiform spores were fully developed. Mature fruit bodies were flask-shaped, of dimensions $105 \pm 9 \times 96 \pm 8.8$ µm (Figure 1f). Their walls were composed of two to four cell layers and consisted of a central cavity filled with spores measuring $28 \pm 4.4 \times 1.02 \pm 0.17$ µm. These spores were filiform, hyaline, aseptate, curved at one end, and borne on sympodial generative cells (Figure 1g-h). The mature filiform spores oozed from each pycnidium and were held in a mucilaginous mass.

Perithecium development

The perithecia began development after the first filiform spores had developed. Infected almond leaves collected prior to the formation of the pycnidioid fruiting bodies and those in the first stages of development, failed to form perithecia when held under overwintering conditions (incubated at 4°C in moistened soil or vermiculite).

The first indication of perithecium initiation was deep staining ascogonial coils in late summer. Interwoven hyphae of the fungus developed into coiled, globose to oval masses, which were protoperithecia. In the early stages of perithecium development,

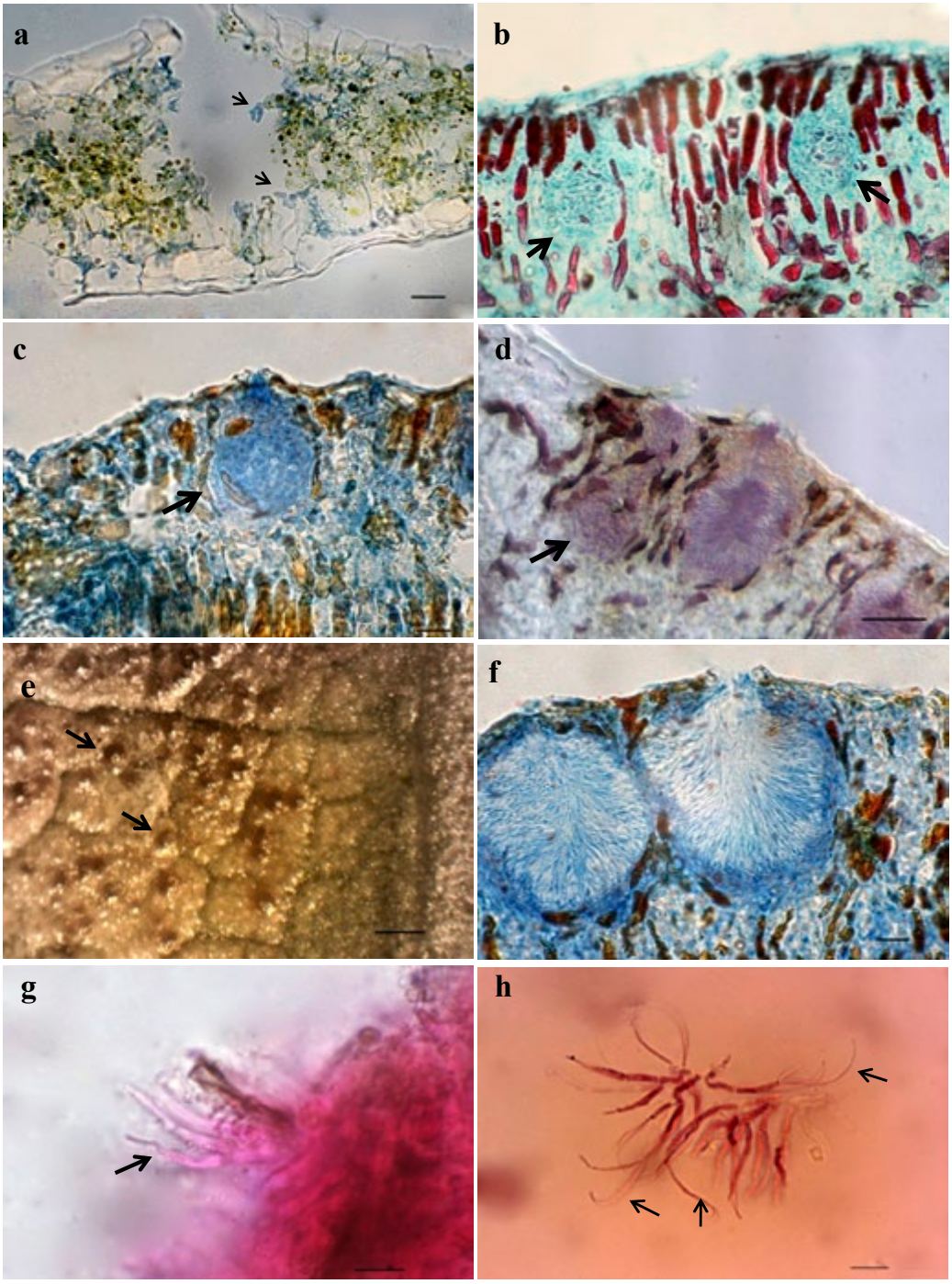

Figure 1. Stages in development of filiform spore fruit bodies in *Polystigma amygdalinum*. a, Hyphae ramifying through host tissue. b-c, Aggregation of hyphae in leaf mesophyll cells, marking initiation of development of pycnidioid bodies and young globose collection of cells. d, Further development of cell collection and vertical extension of cell collection extending through a leaf cuticle. Scale bars = 20 μm. e, Surface characteristics of a lesion with numerous pycnidioid bodies of different maturities (upper arrow indicates a mature pycnidioid body and lower arrow indicates an incipient pycnidioid body). Scale bar = 50 μm. f, Fully mature pycnidioid body filled with filiform spores, with open pore and layer of filiform spores. Scale bar = 20 μm. g, Sympodial conidiogenous cells in pycnidioid body. h, filiform spores. Scale bars = 10 μm.

it was not easy to distinguish between developing pycnidioid fruiting bodies and protoperithecia. The pycnidioid bodies developed continuously throughout summer. However, they were later differentiated through changes in cells making up the protoperithecia, in the center of which some hyphae became conspicuously larger and denser staining. Within the hyphal coils, hyphal septation was sparse. Septa were difficult to distinguish, thus the hyphae sometimes appeared non-septate. An envelope of smaller diameter hyphae formed within the outermost layers of each hyphal coil encasing the ascogonium initials in its centre (Figure 2a).

Protoperithecia were usually observed adjacent to, or in close physical contact with, the pycnidioid spore bodies (Figures 2b and 3). In some sections (Figures 2b and 3b) they appeared to be contiguous. However, evidence of direct fusion between ascogonial cells and filiform spores was not observed, although such fusions were possible across their interfaces. The outermost large cells of each central coil were suggestive of ascogenous cells which appeared as deeply stained masses (Figure 2c). No antheridia were observed. After the appearance of the first perithecium initials, protoperithecia remote from filiform spore bodies degraded (Figure 2d). The hyphal envelopes surrounding the ascogenous hyphal systems later formed the perithecial walls. As each perithecium matured, the cells composing the wall compressed.

Each developing perithecium was distinguished by a centrum composed of filiform paraphyses growing up from the base toward its apex. Cylindrical asci grew among the paraphyses towards the developing ostiole (Figure 2e). Ascus formation originated from deeply staining, thick ascogenous hyphae. Crosiers were not detected. The perithecial apex elongated and became papillate. The inner cells of the neck were replaced by very fine periphyses.

Perithecia continued to develop and mature in infected fallen leaves (Figure 2f) throughout autumn. The growing asci pushed into each perithecial cavity making space and lysing the central mass. Mature perithecia were flask-shaped containing clavate asci, which arose from basal layers of ascogenous hyphae. Each ascus developed eight ascospores as they elongated towards the periphysate ostiole. Asci in all stages of development were seen in the maturing perithecia, and asci were $71 \pm 11 \times 16 \pm 2.4$ μm. Ascospores were unicellular, oval, hyaline and thin-walled, and measured $12.3 \pm 1.4 \times 4.6 \pm 0.62$ μm (Figures 2g-h). Remnants of the spermatia were seen on the perithecial walls of young perithecia (Figure 3) suggestive of their previous association. Detailed development of asci and the ascospores were not followed.

Failure of filiform spores to germinate or infect

Filiform spores did not germinate on any medium or on almond leaves, and none of the almond seedlings which were inoculated with these spores became infected. Inoculations with ascospores resulted in infection and the development of almond leaf blotch symptoms within 4 weeks after inoculation under greenhouse conditions. The leaf lesions turned orange-red and then ochraceous-brown during 30 d and started falling off the seedlings 60 d after the appearance of the first symptoms.

The link between the filiform spore stage and the ascomatal stage

The genetic link between the filiform spore stage and the ascomatal stage of *P. amygdalinum* was confirmed by molecular analysis. The ITS regions in each stage were amplified and the DNA sequences were approx. 560 bp for both stages. Sequences were deposited in GenBank under accession nos KC756361 (filiform spores) and KC756363 (ascomata). When sequences were paired using Geneious version 7 they were identical. *Polystigma amygdalinum* produces a pseudostroma composed both of plant and fungus tissue. This problem of distinguishing the fungal from the plant DNA was solved either by using the plant-excluding primer pair PyITS1/ITS4 (Green *et al.*, 2004) or by extracting the fungal amplicons from the agarose gel. In some cases, however, special care was taken to separate only the fungal centrum tissue, which was placed in a micro-centrifuge tube for DNA extraction.

The physical association of the pycnidioid form with perithecia was observed in anatomical studies. Pycnidioid bodies and perithecia frequently developed side by side with a single wall or no wall between the two cavities. Very often, remnants of old pycnidioid structures were found still attached perithecial walls (Figure 3). Their common DNA eliminated any possibility of the two forms representing genetically different symbionts.

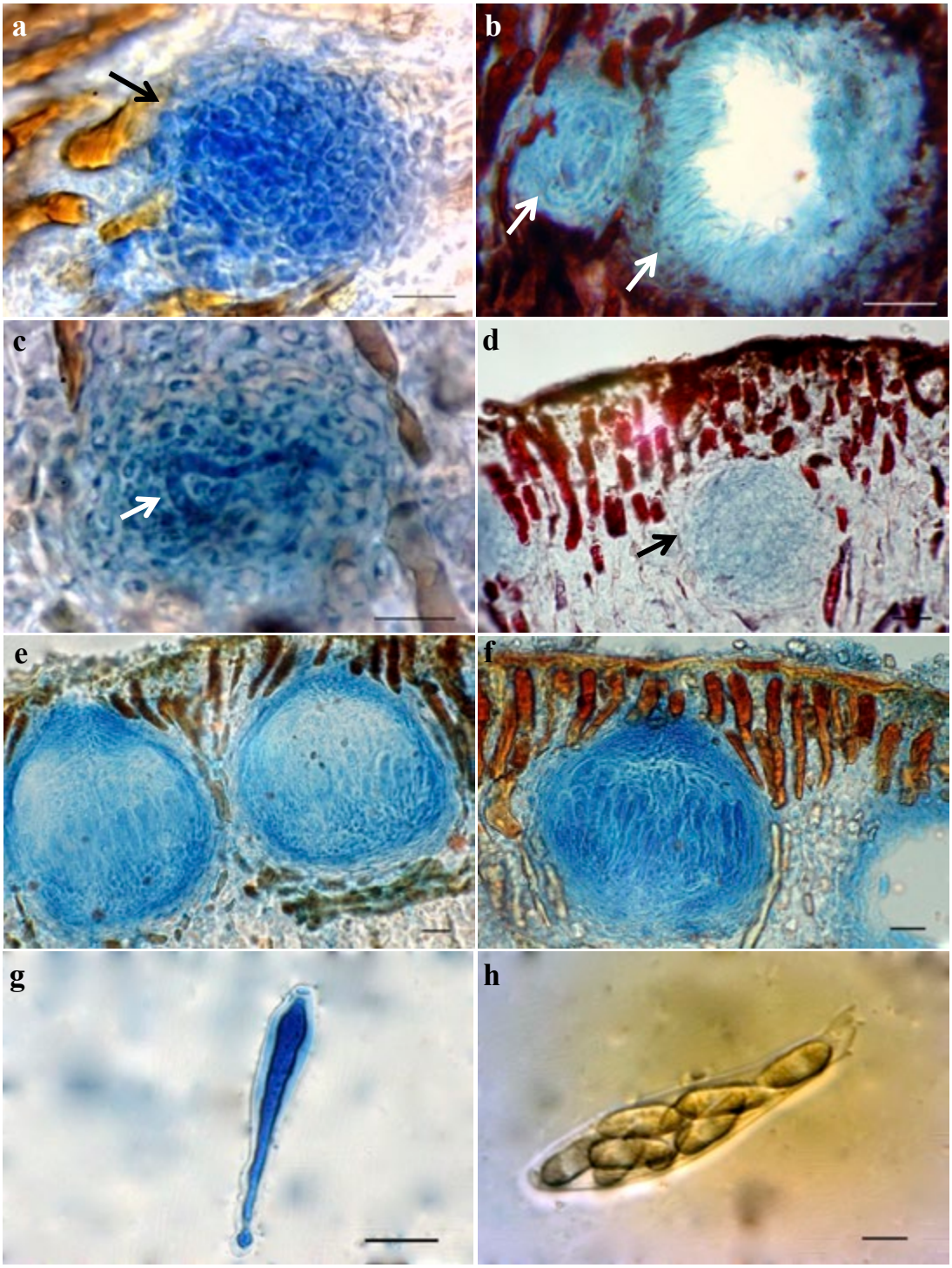

Figure 2. Stages in development of perithecia in *Polystigma amygdalinum*. a, Vertical sections through a leaf showing perithecial perimordia (or ascogonia "ascogonial coils"). b, Ascogonium fertilization by filiform spores (spermatiospores) through ascogonium-pycnidioid body interface. c, Deeply staining ascogenous cells inside the centrum. d, unfertilized degrading ascogonium. e-f, Developing perithecia and thick periphyses. g, Young ascus with an apical ring. h, Mature ascus containing eight ascospores. Scale bars = 20 µm.

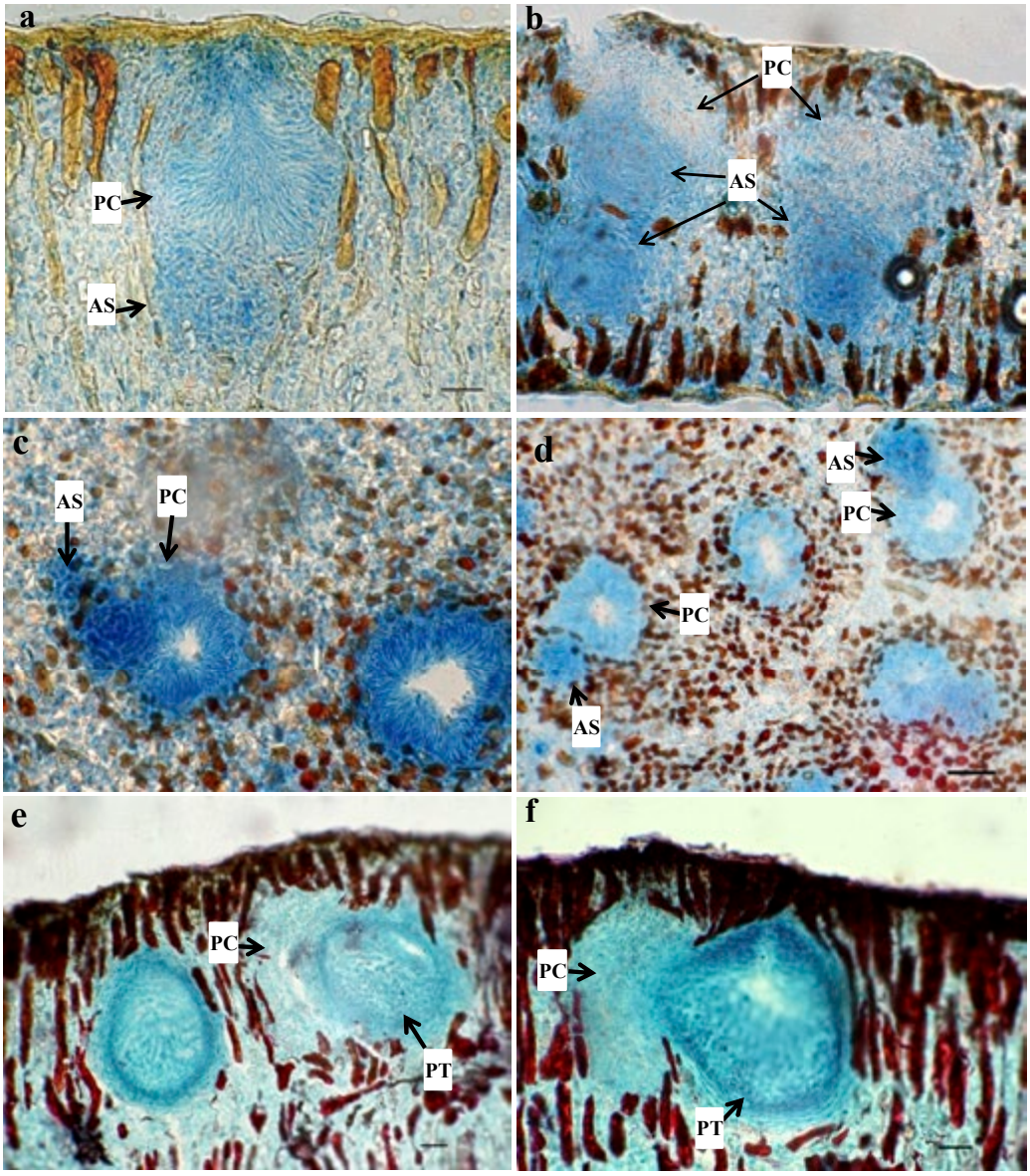

Figure 3. a-b, protoperithecia (ascogonia) in close physical association with a pycnidioid body cavity in *Polystigma amygdalinum*. c-d, Surface view of the perithecial primordia (ascogonia or protoperithecia) and close physical association with pycnidioid body cavity in *Polystigma amygdalinum*. e-f, Remnants of an old pycnidioid body were seen on the perithecial walls in *Polystigma amygdalinum*. PC: pycnidioid body; AS: Ascogonium; PT: Perithecium. Scale bars = 20 μm.

The presence of soil microorganisms did not influence perithecium formation *in vivo*. When almond leaves infected with *P. amygdalinum* were stored in plastic containers in moistened sterilized vermiculite at 4°C they produced perithecia as abundantly as leaves stored at 4°C in containers of moist non-sterilized orchard soil.

Perithecium formation in leaves with single lesions

Perithecia were produced as abundantly in all leaves bearing single lesions as in leaves with multiple and confluent lesions.

Discussion

The filiform spores of *P. amygdalinum* did not germinate *in vitro* or *in vivo* and failed to infect almond leaves. It is normally expected that conidia of plant-infecting fungi germinate and infect their hosts. Failure to germinate is a characteristic of spermatia (Higgins, 1920; Miller, 1951), but this is not necessarily sufficient reason to accept that these filiform spores function as spermatia (male sexual elements or male gametes). However, these observations support the hypothesis that the putative pycnidia are spermogonia, and are an essential part of the sexual cycle. The basic characters of spermatia, according to Higgins (1920) and Miller (1951), are their small size and inability to germinate or infect host plants.

Present observations supporting the above hypothesis, that the "pycnidial" stage preceding the development of perithecia are the prerequisite that triggers development of the perithecial initials, supports the hypothesis that they are spermagonia. This is further supported by observations that no perithecia developed in the leaves which had not developed mature putative pycnidia. Similarly, when young infected leaves were collected before the filiform stage developed and were exposed to overwintering conditions, no perithecia developed. This indicates that mature "pycnidia" are needed to initiate perithecium development. The protoperithecia only appeared once fully formed filiforms were evident.

It was also observed that the putative pycnidia were in physical contact with perithecial initials and remnants of them were frequently attached to the walls of young perithecia. Miller (1951) made similar observations while studying the development of perithecia of *Phyllachora ambrosiae*, and also reported that he saw spermogonia which mostly developed in close association with ascogonia in the one infection area. Parbery (1963) also observed a close association of "pycnidia" containing filiform spores with perithecia of *Phyllachora oryzopsidis* Theiss. & Syd. The filiform spore stage of many species of *Phyllachora* are claimed to be spermatia rather than conidia (Parbery and Langdon, 1963; Parbery, 1996; Pearce *et al*., 1999; Pearce and Hyde, 2001).

On the basis of the developmental morphology and the above evidence, we conclude that the filiform spore state of *P. amygdalinum* are not functionless structures, as has been postulated for *P. rubrum* by Blackman and Welsford (1912), but are active organs having a function in sexual reproduction as spermatia. These initiate the formation of perithecia. The process of diploidization, however, is still unclear.

While Blackman and Welsford (1912) considered spermatia of *Polystigma rubrum* to be male sexual elements that had became functionless through degeneration, Frank (1883) reported fusion between spermatia and trichogynes in *Polystigma rubrum*. However, we did not observe any trichogyne-like elements in *P. amygdalinum*, probably because in *P. rubrum* the trichogynes are larger and more obvious than in *P. amygdalinum* or that they are missing in this fungus. The divergence of the present results from results by Frank (1883) and Blackman and Welsford (1912) is probably explained by the deficiencies of the primitive techniques employed by early investigators. The spermatia in *P. rubrum* are probably also functional, but this has escaped the notice of observers. The inability to clearly distinguish sexual organs in *P. amygdalinum* is in large part a problem of discerning developmental stages in host tissues rather than in pure culture.

There are reports of other ascomycetes producing spermatia (Higgins, 1920; Kohn, 1979; Inman *et al.*, 1991). Fungi in the *Mycosphaerellaceae* produce spermatia. The small size of spermatia, and inability to germinate and infect hosts are among the proofs of spermatial function in this group (Inman *et al*., 1991). Microconidia also act as spermatia in *Botrytis cinerea* and *Sclerotinia* spp. (Kohn, 1979; Fukumori *et al*., 2004). The beta spores of *Phomopsis cinerascens* also do not germinate, and their function in the fungus life cycle is not clear (Banihashemi and Javadi, 2009).

According to Habibi *et al*. (2015), *Polystigma* spp. were close to the *Xylariales*. In this group, perithecium development is initiated under conditions which no longer favour conidium production. This association of conidia with early stages of perithecial development, the observation that they have little cytoplasm and the fact that conidia of several species germinate poorly, have led some investigators to consider them to be relictual spermatia (Rogers, 1979). Rogers (1979) stated that the ancestors of *Xylariaceae* probably had functional spermatia very similar to contemporary forms. However, over time spermatia of most *Xylariaceae* became functionless relicts or became functional propagules. Rogers and Berbee (1964), studying the developmental morphology of *Hypoxylon pruinatum* (Klotzsch) Cooke. (*Xylarials*), hypothesized that conidia served as spermatia.

The present results support the view that *P. amygdalinum* is homothallic since all single lesions, assumed to arise from single ascospores infections, produced abundant perithecia when exposed to overwintering conditions, in a manner similar to that in coalesced lesions, which also formed multiple perithecia. Little morphological or genetic variation was noted in samples of *P. amygdalinum* collected from orchards in different almond-growing regions across Iran, indicating homothallic reproduction in this fungus (unpublished data). This study provided evidence of homothallism in *P. amygdalinum*. However, the potential for homothallism in the pathogen does not mean that this is the prominent mating system of the species in nature. The prevalence of haploid selfing in this fungus in nature is hard to assess. Homothallic fungi are predicted to be predominantly outcrossing (Giraud *et al*., 2008). Studies on homothallic species have found evidence of outcrossing (*e.g. Aspergillus nidulans* (Eidam) G. Winter; Pontecorvo *et al*., 1953). However, some homothallic fungi, such as homothallic species of *Neurospora*, mainly rely on selfing because of the lack of male gamete production (Glass and Kuldau, 1992). Very few studies have investigated the outcrossing in natural populations of homothallic fungi.

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