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Research Article

Coevolution of *Polystigma amygdalinum* through a process of host tracking

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Abstract

Introduction: The almond tree (*Prunus dulcis*) and its wild relative, the mountain almond tree (*Amygdalus scoparia*), grew up together in the province of Fars in Iran over decades. Red leaf blotch disease caused by *Polystigma amygdalinum* is one of the most important almond diseases in the world. This research was conducted with the aim of investigating the evolution of this pathogen on its wild and domestic hosts. **Materials and Methods:** Ascospores suspension of *P. amygdalinum* isolates obtained from almond in Fars province was inoculated to almond and mountain almond seedlings in a greenhouse. The progress of the disease in the diseased leaves of mountain almond compared to almond was investigated by sectioning with a freezing microtome from the spots created on the leaves. **Results:** Red leaf blotch spots appeared on the leaves of both types of almonds. Statistical analysis of test data showed that *P. amygdalinum* isolates from almond are able to cause disease in mountain almond with significantly lower severity, and longer incubation period. **Conclusion:** The results of this research show that *P. amygdalinum*, the cause of red leaf blotch disease, have coevolved on cultivated almond through a process of host tracking.

Key words: *Amygdalus scoparia*, Host domestication, Wild almond

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مقاله پژوهشی

تکامل *Polystigma amygdalinum* از طریق روند تعقیب میزبانی

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حبیبی ا، قادری ف، بنی‌هاشمی ض (۱۴۰۱) تکامل *Polystigma amygdalinum* از طریق روند تعقیب میزبانی. دانش بیماری‌شناسی گیاهی ۱۲(۱): ۴۵-۳۶.

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چکیده

مقدمه: بادام (*Prunus dulcis*) و خویشاوند وحشی آن بادام کوهی(*Amygdalus scoparia*) طی ده‌ها سال به صورت هم‌بوم در کنار یکدیگر در استان فارس ایران رشد کرده‌اند. بیماری لکه آجری برگ ناشی از *Polystigma amygdalinum* ، یکی از بیماری‌های مهم بادام در جهان است. این پژوهش با هدف بررسی روند تکامل این بیمارگر روی میزبانهای وحشی و اهلی آن انجام شد. **مواد و روش‌ها:** آزمایش مایه‌زنی سوسپانسیون آسکوسپورهای جدایه‌های *P. amygdalinum* به دست آمده از بادام در استان فارس به گیاهچه‌های بادام و بادام کوهی در گلخانه انجام شد. پیشرفت بیماری در برگ‌های بیمار بادام کوهی در مقایسه با بادام با مقطع گیری با میکروتوم انجمادی از لکه‌های ایجاد شده روی برگها بررسی شد. **یافته‌ها:** لکه‌های آجری رنگ روی برگهای هر دو نوع بادام بروز کردند. تجزیه آماری داده‌های آزمایش نشان داد که جدایه‌های *P. amygdalinum* از بادام قادر به ایجاد بیماری در بادام کوهی با شدت معنی‌دار کمتر و دوره کمون طولانیتر هستند. **نتیجه‌گیری:** یافته‌های این پژوهش نشان می‌دهند که *P. amygdalinum* عامل لکه آجری برگ روی بادام اهلی طی روندی به نام تعقیب میزبان در طی سال‌ها تکامل یافته است.

واژگان کلیدی: اهلی سازی میزبان، بادام کوهی، *Amygdalus scoparia*

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Introduction مقدمه

The almond tree (*Prunus dulcis* (Mill.) D.A. Webb; syn. *P. amygdalus* (L.) Batsch), native to Western and Central Asia (Ladizinsky 1999, Zeinalabedini et al. 2010) is commercially grown worldwide. Many authors have suggested that cultivated almond have arose from interspecific hybridizations of wild almond species. However, the exact wild ancestor species are not identified (Ladizinsky 1999, Browicz and Zohary 1996). Twenty wild almond species have been reported in Iran (Zahedi et al. 2020). *Amygdalus scoparia* Spach (Syn. *Prunus scoparia* Spach) is one of the most valuable native wild almond species in Iran (Sabeti 1966). *A. scoparia* is the dominant wild almond species in mountain, forests, and rangelands close to cultivation areas of almond, in the Fars province of Iran.

Red leaf blotch disease of almond caused by *Polystigma amygdalinum* P.F. Cannon, is a major disease of almonds in Iran and Mediterranean countries (Saad and Masannat 1997, Banihashemi 1990, Teviotdale et al. 2002, Cimen and Ertugrul 2007, Zúñiga et al. 2018). It is an important leaf disease of almonds in many almond growing regions of Iran (Banihashemi 1990). Red leaf blotch disease causes red lesions of different shapes and sizes on almond leaves. The lesions are initially yellow and gradually turn into reddish brown and finally early defoliation occurs (Torguet et al. 2022, Zúñiga et al. 2020). *Polystigma amygdalinum* overwinters in the fallen leaves in the ground and produce mature perithecia which could infect the host in the next spring via ascospores (Zúñiga et al. 2019).

Investigations of pathogen populations from wild and cultivated hosts have increased our information about the evolution of pathogens (Stukenbrock and McDonald 2008, Gladieux et al. 2010). Emerging fungal pathogens have increased specialization to the new host because of the genetic differences between ancestral and domesticated host and the characteristics of the human made agro-ecosystems (Zhan et al. 2002; Lê Van et al. 2012). Agro-ecosystems via management practices and dense and genetically uniform populations of cultivated hosts affects pathogen populations resulting in changes in pathogenicity traits in pathogen populations (McDonald and Linde 2002). To investigate pathogenicity of *P. amygdalinum* isolates from *P. dulcis* on *A. scoparia* and the development of the disease on wild almond in greenhouse conditions cross inoculation tests were applied.

Materials and Methods مواد و روش‌ها

Samples of almond infected to *P. amygdalinum* were collected from orchards from Fars province in Iran during March 2020 and then were stored at 4°C. Seeds of cultivated almond (*P. dulcis*) and wild almond (*A. scoparia*) were stratified at 4°C for two months and sown in steamed soil. Plants were grown in a greenhouse for 20 weeks. Plants with uniform growth were chosen for subsequent inoculations. For inoculum preparation, the infected almond leaves containing *P. amygdalinum* mature ascostroma were washed under running tap water, surface-disinfested in 95% ethanol for 15 s and 1% NaOCl for 45 s, and then rinsed in sterile distilled water. Ascospores were obtained from macerated ascomata in sterile distilled water using a sterile mortar and pestel. The resulting suspension was filtered through layers of cheesecloth. The concentration of spores was adjusted to 1×10^6 ascospores ml^{-1}. Ascospore suspensions were applied to runoff onto leaves with a sprayer on 20-week-old almond and wild almond seedlings. Sterile distilled water was used for inoculation of control plants. Plants were kept at 20°C for a week.

Leaves were visually inspected for red leaf blotch symptoms after inoculations. Symptom development was inspected daily. Disease severity (percentage of infected leaves in each plant) was assessed visually.

Since *P. amygdalinum* is an obligate parasite and cannot be re-isolated from symptomatic leaves, to confirm the causal agent of the symptoms, microtome sectioning of the symptomatic leaves as well as molecular confirmation of the causal agent identity were applied. Leaves bearing symptoms were used for microtome sectioning during disease development. The leaves with pycnidial stromata were stored in sterile moistened vermiculite at 4°C to develop ascocarps.

Different pathogenic fitness measures were assessed: (i) Virulence, that is, the ability to infect the cultivated and wild host genotypes, (ii) incubation period under greenhouse conditions was determined as the time (in days) between inoculation and appearance of the first disease symptoms on leaves, and (iii) aggressiveness, that is, the severity of the disease in successful infections. Disease severity was determined in terms of the percentage of the diseased leaves. Leaves bearing red blotch lesions were examined for fungal structures formation and disease development using microtome sectioning. Six mm^2 portions of lesions were cut from infected leaves and mounted in freezing sectioning medium (Neg-50^{TM}, Richard-Allan Scientific, USA). Sections of six to ten µm thickness were prepared with a freezing microtome (Sass 1958). Fungal structures belonging to pycnidial stage and ascomatal stage were observed. Symptomatic leaves bearing mature pycnidia were stored in moistened soil at 4°C to simulate overwintering and obtain perithecia. In order to test viability of ascospores, ascospores were obtained by crushing the ascostroma on a glass slide in distilled water, spread over PDA medium incubated at 10°C according to Habibi and Banihashemi (2015).

The experiment was arranged in a completely randomized design (CRD). Statistical analysis was performed using SAS (Version 9.2; SAS Institute, Cary, NC). Diseased leaves percentages were arcsine transformed prior to analyses. The significance of differences in red leaf blotch disease severity on cultivated almond and wild almond was tested using the Proc GLM and treatment means were separated by Duncan's multiple range test.

Results یافته‌ها

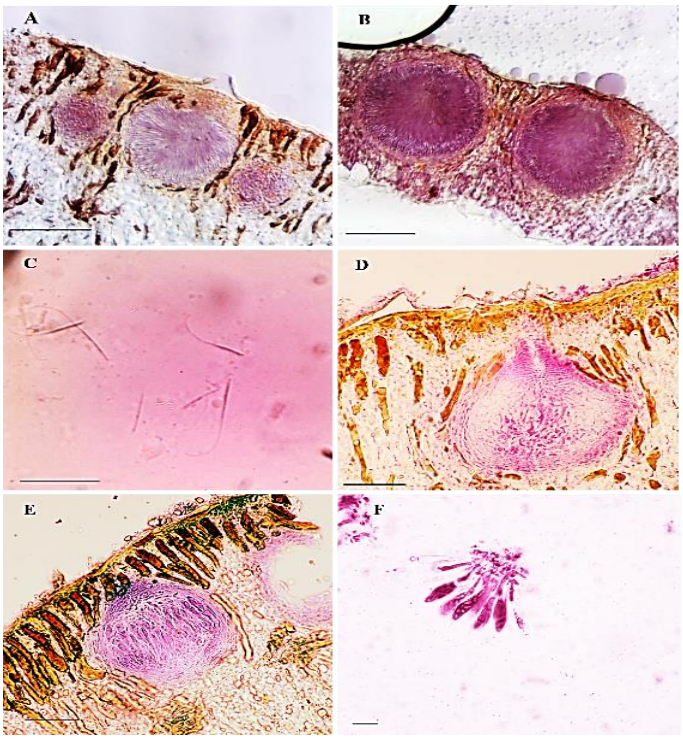
Inoculation of *P. amygdalinum* ascospores on 20-week-old almond seedlings resulted in red leaf blotch symptoms (Fig. 1). Control plants remained asymptomatic. Incubation period of the disease, *ie* the time between inoculation and appearance of the first disease symptoms on leaves, was determined 28 days under greenhouse conditions. First signs appeared as chlorotic lesions on almond leaves, which later expanded and the color changed to orange, brown and dark red. Pycnidia developed under the epidermis nine weeks (5 weeks after appearance of first symptoms) after inoculation in greenhouse. The mature pycnidia were flask-shaped, 99 (±7.5) × 90 (±3.4) µm, and the central cavity were filled with filiform pycnidiospores measuring 27.6 (±4.7) × 1 (±0.21) µm. Perithecia in overwintering leaves were first seen in week 17 after inoculation.

Red leaf blotch symptoms were visible on *A. scoparia* seedlings 39 days (incubation period) after inoculation with *P. amygdalinum* ascospores (Fig 1). First signs appeared as minute discolorations on leaves turning to reddish orange and dark brown lesions with time. Control plants remained asymptomatic. Molecular examination of the symptomatic leaves of wild almonds confirmed the causal agent of the disease as *P. amygdalinum*.

شکل ۱. A) نشانه‌های بیماری لکه آجری روی گیاهچه‌های بادام در شرایط گلخانه هشت هفته پس از مایه‌زنی (سمت راست) در مقایسه با شاهد (سمت چپ)، B) نمای نزدیک علائم بیماری لکه آجری روی گیاهچه‌های بادام، C) علائم بیماری لکه آجری روی گیاهچه‌های بادام کوهی (*Amygdalus scoparia*) در شرایط گلخانه ده هفته پس از مایه‌زنی (سمت راست) در مقایسه با شاهد (سمت چپ)، D) نمای نزدیک علائم بیماری لکه آجری روی گیاهچه‌های *A. scoparia*.

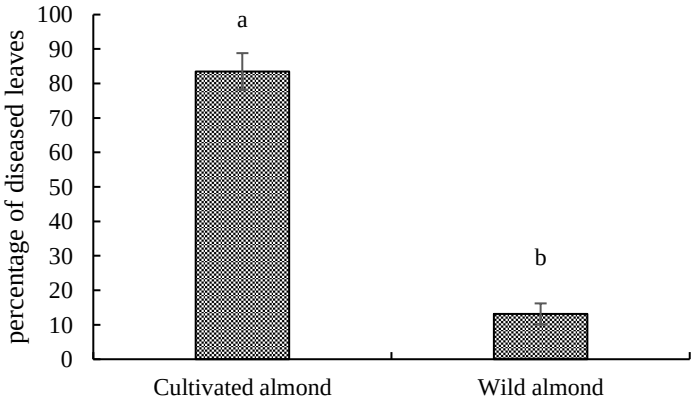
Figure 1. A) Symptoms of red leaf blotch disease on almond seedlings in greenhouse conditions after eight weeks (right) comparing to control (left); B) Close up symptoms of red leaf blotch on almond seedlings; C) Symptoms of red leaf blotch disease on wild almond (*Amygdalus scoparia*) seedlings in greenhouse after ten weeks (right) comparing to control (left). D) Close up symptoms of red leaf blotch on *A. scoparia* seedlings.

Pycnidia developed under the epidermis in week 7 after inoculation (2.5 weeks after appearance of first symptoms) in greenhouse. The mature pycnidia were flask-shaped bodies measuring 81(±2) × 76(±2.5) µm (Fig 2 a-b). Filiform pycnidiospores were 26.7(±4.1) × 0.9(±0.3) µm (Fig 2 c). The leaves that were subjected to overwintering treatment (kept in sterile moistened vermiculite at 4°C), developed young flask-shaped perithecia in week 12 after inoculation (Fig 2 d-e). Periphyses were visible in the perithecial neck (Fig 2 d). The mature perithecia contained clavate asci containing eight unicellular, oval, hyaline ascospores (Fig 2 e-f). Detailed developmental studies of the fungus were not followed, as for the purpose of this study it was not of special interest.
The severity of red leaf blotch symptoms on leaves differed significantly between *A. scoparia* and *P. dulcis* (Fig. 3).



شکل ۲. A-B) برش عرضی از برگ‌های *Amygdalus scoparia* که پیکنیدیوم‌های *Polystigma amygdalinum* را نشان می‌دهد. A) یک پیکنیدیوم و آغازه‌های پیکنیدیومی. B) پیکنیدیوم‌های کاملاً بالغ و دارای اسپورهای نخی شکل. خط مقیاس= ۵۰ میکرومتر. C) پیکنیدیوسپورهای نخی شکل. خط مقیاس= ۲۰ میکرومتر. D) پریتسیوم و پریفیزهای ضخیم. E) آسک‌های در حال تشکیل، D-E) خط مقیاس= ۵۰ میکرومتر. F) آسک‌های حاوی آسکوسپور. خط مقیاس= ۲۰ میکرومتر.

Figure 2. A-B) Vertical sections through *Amygdalus scoparia* leaves showing pycnidia of *Polystigma amygdalinum*. A) A pycnidium and a pycnidium initial. B) Fully mature pycnidia filled with filiform spores. Scale bars = 50 µm; C) Filiform pycnidiospores. Scale bar= 20 µm; D) Perithecium and thick periphyses; E) Developing asci, D-E scale bars = 50 µm; F) Asci containing ascospores. Scale bars = 20 µm.



شکل ۳. شدت لکه آجری روی برگ‌های بادام اهلی و بادام کوهی.

Figure 3. Red leaf blotch severity on leaves of cultivated almond and wild almond. Bars = standard errors.

Discussion بحث

Pathogenicity tests showed that the incubation period (IP) of *P. amygdalinum* on cultivated almond was about 28 days in greenhouse conditions. The IP in greenhouse conditions was shorter than IP in orchard conditions previously reported by other researchers. Banihashemi (1990) reported the incubation period of 4-5 weeks for *P. ochraceum* in almond seedlings exposed to ascospore discharge in almond orchard. Saad and Masannat (1997) estimated the incubation period of *P. ochraceum* approximately 35-40 days under Halat, Libanon conditions.

This is the first report of *P. amygdalinum* isolates from cultivated almond infecting wild almond (*A. scoparia*). *A. scoparia* is the dominant wild almond species growing in rangelands in almond cultivating regions of Fars province in Iran. However, *P. amygdalinum* also has been reported on one wild almond species *Amygdalus lycioides* Spash var. *horrida* (Spash) Browicz in the Lorestan province of Iran (Ershad 2009).

Inoculation of *A. scoparia* seedlings with *P. amygdalinum* ascospores resulted in red leaf blotch symptoms after 39 days. The IP of the disease on *A. scoparia* was longer than that of cultivated almond (28 days) in greenhouse conditions. The developmental studies of *P. amygdalinum* in leaves of *A. scoparia* showed that the fungus was able to successfully complete the life cycle and produce fungal structures in wild host (*A. scoparia*). After the appearance of the leaf lesions, the fungus spread throughout the leaf tissues. Flask-shaped pycnidia containing filiform spores developed after seven weeks, which compered to nine weeks in cultivated almond, was shorter. The dimensions of pycnidia were significantly different between cultivated almonds and *A. scoparia* which may be due to the differences in leaf structure and shape of the two plant species. No significant difference in filiform spores' dimensions were observed between the two pant species. All of the symptomatic leaves defoliated before Perithecia initials appearance in leaves. Perithecia development in *A. scoparia* leaves took a shorter period of 12 weeks (after inoculation) compared to Perithecia development of 17 weeks in cultivated almond.

We observed that the development of the fungal structures occurred in an interestingly short period comparing to cultivated almond in greenhouse conditions. However, the fungal structures were normal in terms of shape and developmental details according to Habibi and Banihashemi (2016). The Ascospores were viable and successfully germinated in potato dextrose agar medium at 10°C. The short life cycle of *P. amygdalinum* on *A. scoparia* may be a coping strategy for this fungus to survive in natural conditions. The short period between the appearance of the symptoms and the defoliation may be one reason that red leaf blotch is not observed or draw attention in natural populations of *A. scoparia* in Fars rangelands.

The severity of the red leaf blotch symptoms on wild almond leaves was 13.14% (±4.1) while the severity on *P. dulcis* leaves was 83.4% (±7.7). The short disease period and low aggressiveness of *P. amygdalinum* on *A. scoparia* may be due to leaf architecture and physiology and/or resistance genes in the wild host. The *P. amygdalinum* isolates showed higher pathogenic fitness on cultivated almond than wild almond.

Wild almonds seeds are bitter and toxic. Selection of sweet almond is the beginning of almond domestication. However, it is not clear that which wild almond species is the wild ancestor of sweet almond (Ladizinsky 1999). We observed that *P. amygdalinum* did not lose the ability to infect wild host *A. scoparia*. Since *P. amygdalinum* is still capable of infecting the wild host, it can be inferred that *P. amygdalinum* have coevolved on domesticated almond through a process of host tracking. Cross pathogenicity tests have been used to investigate the changes in pathogenicity traits of domesticate and wild hosts

of other pathogens. Lê Van et al. (2012) showed that the pathogen *Venturia inaequalis* have followed a process of host-tracking during the domestication of apple. At the same time aggressiveness of populations have increased in the agro-ecosystem According to this study, there is the possibility that in addition to fallen infected leaves from previous season in the orchards, *P. amygdalinum* may overwinter in neighboring *A. scoparia* rangelands as well.

Conclusion نتیجه‌گیری

We hypothesized that the red leaf blotch pathogen in Fars province, Iran, has emerged in the agro-ecosystem via host tracking process and has increased its virulence without losing the ability to infect the wild ancestral host *A. scoparia*. We can conclude that *P. amygdalinum* may overwinter in *A. scoparia* rangelands that are in located in close distances to cultivated almonds orchards in Fars province.

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References منابع

Altschul SF, Gish W, Miller W, Myers EW, Lipman, DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Banihashemi Z (1990) Biology and control of *Polystigma ochraceum*, the cause of almond red leaf blotch. Plant Pathology 39:309-315.

Browicz K, Zohary D (1996) The genus *Amygdalus* L. (Rosaceae): species relationships, distribution and evolution under domestication. Genetic Resources and Crop Evolution 43:229-247.

Cimen I, Ertugrul BB (2007) Determination of mycoflora in almond plantations under drought conditions in southeastern Anatolia project region, Turkey. Plant Pathology Journal 6:82-86.

Couch BC, Fudal I, Lebrun MH, Tharreau D, Valent B, van Kim P, Notteghem JL (2005) Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. Genetics 170:613-630.

De Vienne D, Hood M, Giraud T (2009) Phylogenetic determinants of potential host shifts in fungal pathogens. Journal of Evolutionary Biology 22:2532-2541.

Diamond J (2002) Evolution, consequences and future of plant and animal domestication. Nature 418:700-707.

Ershad D (2009) Fungi of Iran. Iranian Research Institute of Plant Protection, Tehran, Iran. (In Persian)

Gladieux P, Zhang XG, Roldan-Ruiz I, Caffier V, Leroy T, Devaux M, Van Glabeke S (2010) Evolution of the population structure of *Venturia inaequalis*, the apple scab fungus, associated with the domestication of its host. Molecular Ecology 19:658-674.

Green SJ, Freeman S, Hader Y, Mins D (2004) Molecular tools for isolate and community studies of pyrenomycete fungi. Mycologia 96:439-451.

Habibi A, Banihashemi Z (2016) Mating system and role of pycnidiospores in biology of *Polystigma amygdalinum*, the causal agent of almond red leaf blotch. Phytopathologia Mediterranea 55:98-108.

Ladizinsky G (1999) On the origin of almond. Genet. Resour. Crop Evol. 46: 143-137.

Lê Van A, Gladieux P, Lemaire C (2012) Evolution of pathogenicity traits in the apple scab fungal pathogen in response to the domestication of its host. Evolutionary Applications 5:694-704.

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology 40:349-79.

Saad T, Masannat K (1997) Economic importance and cycle of *Polystigma ochraceum*, causing red leaf blotch disease of almond, in Lebanon. Bulletin OEPP/EPPO Bulletin 27:481-485.

Sabeti H (1966) Native and Exotic Trees and Shrubs of Iran. Tehran University Press. 432p. (In Persian)

Sass JE, (1958) Botanical Microtechnique. 2nd edition. The Iowa State College Press; Constable & Co. USA; UK. 228p.

Stukenbrock EH, Banke S, Javan-Nikkhah M, McDonald BA (2007) Origin and domestication of the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. Molecular Biology and Evolution 24:398–411.

Stukenbrock EH, McDonald BA (2008) The origins of plant pathogens in agro-ecosystems. Annual Review of Phytopathology 46:75–100.

Stukenbrock, EH, Bataillon T, Dutheil JY, Hansen TT, Li R, Zala M, McDonald BA (2011) The making of a new pathogen: insights from comparative population genomics of the domesticated wheat pathogen *Mycosphaerella graminicola* and its wild sister species. Genome Research 21:2157–2166.

Teviotdale BL, Michailides TJ, Pscheidt JW (2002) Compendium of nut crop diseases in temprate zone. APS Press. 89p.

Torguet L, Zazurca L, Martínez G, Pons-Solé G, Luque J, Miarnau X (2022) Evaluation of Fungicides and Application Strategies for theManagement of the Red Leaf Blotch Disease of Almond. Horticulturae 8:501.

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp.315-322. In: MA Innis, DH Gelfand JJ, Sninsky TJ, White (eds.). PCR Protocols: A Guide to Methods and Applications., Academic Press, Inc., New York, NY, USA.

Zahedi SM, Abdelrahman M, Hosseini MS, Yousefi R, Tran, LSP (2020) Physical and biochemical properties of 10 wild almond (*Amygdalus scoparia*) accessions naturally grown in Iran. Food Bioscience 37:100721.

Zeinalabedini M, Khayam-Nekoui M, Grigorian V, Gradziel T, Martínez-Gómez P (2010) The origin and dissemination of the cultivated almond as determined by nuclear and chloroplast SSR marker analysis. Scientia Horticulturae 125:593-60.

Zhan J, Mundt CC, Hoffer ME, McDonald BA. (2002) Local adaptation and effect of host genotype on the rate of pathogen evolution: an experimental test in a plant pathosystem. Journal of Evolutionary Biology 15:634-47.

Zhang Y, Zhang S, Liu X, Wen H, Wang M (2010) A simple method of genomic DNA extraction suitable for analysis of bulk fungal strains. Letters in Applied Microbiology 51:114-118.

Zúñiga E, León M, Berbegal M, Armengol J, Luque J (2018) A qPCR-based method for detection and quantification of *Polystigma amygdalinum*, the cause of red leaf blotch of almond. Phytopathologia Mediterranea 57:257−268.

Zúñiga E, Luque J, Martos S (2019) Lignin biosynthesis as a key mechanism to repress *Polystigma amygdalinum*, the causal agent of the red leaf blotch disease in almond. Journal of Plant Physiology 236:96-104.

Zúñiga E, Romero J, Ollero-Lara A, Lovera M, Arquero O, Miarnau X, Torguet L, Trapero A, Luque J (2020) Inoculum and Infection Dynamics of *Polystigma amygdalinum* in Almond Orchards in Spain. Plant Disease 104:1239-1246.