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Growth, Sporulation, Conidial Germination and Lethal Temperature of *Paraphoma radicina*, A Fungal Pathogen of Alfalfa (*Medicago sativa*) Root Rot

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Abstract: In 2020, alfalfa root rot, a disease caused by *Paraphoma radicina*, was identified in Inner Mongolia, China, where it seriously affected alfalfa crop yields. Conditions for in vitro growth, sporulation and conidial germination of *P. radicina* are poorly understood, limiting further studies. In this contribution, we evaluated the suitability of different media, carbon and nitrogen sources, as well as temperature and pH for *P. radicina* in vitro growth and germination. In addition, the temperature sensitivity of these cultures was assessed. *Paraphoma radicina* growth and sporulation were most vigorous on the ARDA medium, reaching the maximum growth and sporulation rates after 4 weeks of incubation. All carbon and nitrogen sources supported growth, but none induced sporulation. The best carbon and nitrogen sources for growth were mannitol and peptone, respectively. Conidial germination was observed in the 4 to 35 °C temperature range, with an optimum temperature of 25 °C. The germination rate was highest at pH 7, and more than 50% of conidia germinated after 38 h of incubation at 25 °C. On the other hand, temperatures above 55 °C (10 min) and 41 °C (10 min) proved lethal for the mycelial and conidial forms of the pathogen, respectively. These results can provide clues to the environmental conditions amenable for *P. radicina* infection of alfalfa crops and, on the whole, a better understanding of pathogenicity.

Keywords: *Paraphoma radicina*; growth; sporulation; conidial germination; lethal temperature

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1. Introduction

Fungal root rot is a common disease in alfalfa crops in several areas of the world, including the USA, Canada, Australia, Iran and China [1–5]. Root rot induces discoloration of the infected tissues and subsequent development of black and necrotic areas, often leading to alfalfa plant death. A variety of plant pathogenic fungi has been reported to induce root rot in alfalfa, such as *Fusarium* spp. [1,6], *Pythium* sp. [7,8], *Phytophthora* sp. [9,10], *Mycoleptodiscus terrestris* [11,12], *Microdochium tabacinum* [13], *Rhizoctonia solani* [14] and *Phoma sclerotioides* [2]. *Paraphoma radicina* was reported to be a new pathogen causing alfalfa root rot in Inner Mongolia, China, and pathogenicity experiments showed that *P. radicina* is significantly detrimental to alfalfa growth [15].

Paraphoma radicina is the type species of the genus *Paraphoma*, although it was originally named *Phoma radicina* [16]. The fungus was initially described in isolates obtained from cysts of *Heterodera glycines* in North Carolina, USA, in soybean field soils [17]. Subsequently, it was also recorded on *H. glycines* in Shenyang, China [18]. *Paraphoma radicina* was previously reported as a saprophytic species on the roots of herbaceous and woody plants [16]. However, in a previous publication, we showed this species to be able to infect

alfalfa roots and cause severe root rot [15]. Pycnidia of *P. radicina* are commonly present on black necrotic root tissue of diseased alfalfa. Pycnidia formed on the diseased plant tissue were found to contain many viable conidia, which are believed to serve as inocula in alfalfa infection. Since the teleomorph of the fungus is still unknown [19], it is considered that conidia play an important role in the spread of the disease and, therefore, conidial germination may be an important factor for successful infection.

Conidial germination, sporulation, colonization and mycelial growth of fungi are greatly influenced by nutritional and environmental factors [20–22]. However, information on the influence of these factors on the biological and physiological characteristics of *P. radicina* is currently limited. The effects of temperature and pH on mycelial growth have been previously evaluated [15], where it was found difficult to induce conidiation of *P. radicina* in vitro, which may limit further studies on effective disease control. In the present study, our objectives were to determine the effect of (i) culture media on mycelial growth and conidial production, (ii) carbon and nitrogen sources on growth and (iii) temperature and pH on conidial germination, and to determine the lethal temperature of *P. radicina*. The outcome of the research described here will contribute to a greater understanding of the etiological agent of the alfalfa root rot epidemic to guide strategies for preventing the spread of this disease and mitigate potential crop yield losses in China.

2. Materials and Methods

2.1. Biological Material

Isolates of *P. radicina* were obtained from root tissues of diseased alfalfa plants in Chifeng city, Inner Mongolia, China. The type isolate of *P. radicina* (LYZ187) used in all experiments was that previously obtained [15]. Pure cultures of *P. radicina* were grown on potato dextrose agar (PDA) at 25 °C and L:D 0:24 h for four weeks for later use as inocula. Mycelial plugs (5 mm diameter) were aseptically cut from the margins of actively growing colonies with a sterile cork borer and transferred to a new PDA Petri dish. Plates were inoculated by placing plugs face down on each dish, with five replicates per treatment. The dishes were sealed with laboratory film (Parafilm^{TM}, Oshkosh, WI, USA) and randomly arranged in a 25 °C incubator.

2.2. Mycelial Growth and Sporulation of P. Radicina on Different Agar Media

The growth of *P. radicina* was assessed on eight different media (Table 1). All agar media were autoclaved at 121 °C for 20 min prior to being poured into individual Petri dishes. Colony shape and color were observed in the first week either by the eye or under a dissecting microscope. Photographs were taken with a Handheld Canon DS126391 camera and colony features were recorded. Colony diameters were measured weekly for four weeks, and growth rates were calculated as the colony diameter with less than five millimeters of mycelial plugs. After one week of incubation, five milliliters of sterilized distilled water were added to each plate, and the colony surface was gently scraped with a sterilized glass spreader to dislodge conidia. The resulting suspension was filtered through sterile gauze, and the procedure was repeated with another five milliliters of water. The combined conidial suspension volume was measured, and the concentration of conidiospores was estimated with a hemacytometer. The collection of conidiospores was repeated after four weeks of incubation [23].

Table 1. Test media and the preparation method.

Media Name	Ingredients and Preparation Method [1,2]
Potato dextrose agar (PDA)	200 g fresh potato, 20 g dextrose, distilled water 1000 mL
Oatmeal agar (OA)	30 g oatmeal (boiled in distilled water for 1 h, then filtered through 2-layer gauze), distilled water 1000 mL
Pine needle agar (PNA)	100 g fresh pine needle (take the filtrate after boiling for 1 h), 10 g dextrose, 5 g peptone, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, distilled water 200 mL
Malt extract agar (MEA)	30 g malt extract, 3 g peptone, distilled water 1000 mL
Potato carrot agar (PCA)	20 g fresh potato, 20 g fresh carrot, distilled water 1000 mL
Cherry decoction agar (CHA)	200 g fresh cherry (extract the juice with a juicer, and boiled in distilled water for 30 min, then filtered through 2-layer gauze), distilled water 1000 mL
V-8 juice agar (V8A)	3 g $CaCO_3$, 200 mL V-8 juice
Alfalfa root decoction agar (ARDA)	100 g clean alfalfa root (cut the root into small pieces, boiled for 30 min and filtered), distilled water 1000 mL

[1] Each 1000 mL media contained 17 g agar. [2] PDA, OA, MEA, CHA and V8A were prepared as described by Crous et al. [24]. PNA as defined by Wu [25]. PCA was prepared according to the method of Li and Nan [26]. ARDA was self-prepared.

2.3. Effects of Carbon and Nitrogen Sources on Growth

For carbon source suitability experiments, nine carbon compounds were assessed for their ability to support mycelial growth. The basal medium consisted of 20 g of dextrose, 5 g of KNO_3, 2 g of Na_3PO_4, 1 g of $MgSO_4$, 17 g of agar and up to 1000 mL of distilled water; the other eight carbon sources were sucrose, mannitol, fructose, D-xylose, lactose, soluble starch, maltose and cellulose. Dextrose in the basal medium was replaced with 20 g of each carbon source to prepare individual formulations to be tested [26]. Growth rates, measured as the colony diameter, were monitored for four weeks.

Seven nitrogen compounds were assessed for their ability to support mycelial growth: Peptone, urea, ammonium nitrate, ammonium chloride, glycine, sodium nitrate and ammonium sulfate. Five grams of each nitrogen source were added to the basal media (20 g of dextrose, 2 g of Na_3PO_4, 1 g of $MgSO_4$, 17 g of agar and up to 1000 mL of distilled water) to prepare individual formulations to be tested. Inoculated media were incubated in the dark at 25 °C.

2.4. Effect of Temperature and pH on Conidial Germination

To determine the optimal temperature for conidial germination of *P. radicina*, conidial suspensions in sterile distilled water were adjusted to 1.0×10^6 spores/mL with a hemacytometer. Using the slide technique [27], a 10 µL drop of a conidial suspension was placed in a cavity slide using a micropipette. The slide was then placed onto the well of a 90 mm plastic Petri plate on top of a moist filter paper to maintain the humidity level. The plates were sealed with parafilm and incubated (L:D 0:24 h) at 4, 10, 15, 20, 25, 28, 30 or 35 °C, respectively. Treatments were replicated three times. Conidial germination was monitored every four hours through microscopic observation at ×400 magnification using an Olympus CX31 microscope (Olympus, Tokyo, Japan). Samples were observed continuously for 38 h until there were no new conidia germinating. The conidial germination rate was determined by counting conidia in 30 randomly selected fields on each plate. A conidium was considered germinated if the germ tube was at least one-half the length of the conidium.

To assess the effect of pH on conidial germination, sterile water was adjusted to pH 4, 5, 6, 7, 8, 9 or 10 by the addition of 0.1 M NaOH or HCl prior to the preparation of conidial suspensions. These suspensions were allowed to germinate while being kept moist using the slide technique described above. All Petri dishes were incubated at 25 °C in the dark. Observation and evaluation of the conidial germination were carried out as described above.

2.5. Lethal Temperature of Mycelium and Conidia

Mycelial plugs (5 mm diameter) were placed in a sterilized test tube, and then the tubes were placed in a thermostat water bath at 40–55 °C (temperature gradient: 1 °C) for 10 min (preheating: One minute). After cooling, the plugs were taken out and placed face down on PDA media dishes. All plates were incubated for one week at 25 °C in the dark to evaluate mycelial growth capability.

To determine the lethal temperature for conidia, 2 mL of a conidial suspension was taken into sterilized test tubes and subjected to heat treatment as described above. Conidial viability was determined by measuring the germination rate after 34 h in cavity slides as described [28].

2.6. Data Analysis

Growth rate, sporulation and conidial germination rate data were subjected to analysis using SPSS Statistics 21 analysis software (SPSS Inc., Chicago, IL, USA) using descriptive statistics, one-way ANOVA and the Duncan test with $p \leq 0.05$ as the significance threshold. The effects of carbon and nitrogen sources on growth were statistically analyzed by the least significant difference (LSD) and Duncan tests ($p \leq 0.05$).

3. Results

3.1. Suitability of Media for Growth and Sporulation

P. radicina was able to grow on all media. Aerial mycelia were compact, floccose or cottony after one week of cultivation on PDA, PNA, MEA, CHA and V8A. Conversely, marginal hyphae were sparse on OA, PCA and ARDA, where they were concentrated at the center of the colonies, and the hyphae grew first vertically and then horizontally. On OA, the colony color appeared pale luteous, with a pale olivaceous grey mycelium (Figure S1).

After four weeks of incubation on PDA, OA, PNA, MEA, PCA, CHA, V8A and ARDA media dishes, growth rates ranged from 0.21 to 0.30 cm/d, with the largest growth rate observed on ARDA. Growth rates were in the order ARDA > OA > V8A > CHA > MEA > PCA > PNA > PDA, where the growth rates on ARDA, OA and V8A were significantly greater than those measured in other media ($p \leq 0.05$) (Table 2).

Table 2. Mean (±SEM) growth rates and sporulation of *Paraphoma radicina* on eight different media grown at L:D 0:24 h and 25 °C and measured one and four weeks after inoculation.

Media	4 Weeks Growth Rate (cm/d)	Sporulation (10^6 Conidia/mL)	
		1 Week	4 Weeks
PDA	0.21 ± 0.08 c [1]	/ [2]	1.18 ± 0.48 b
OA	0.29 ± 0.01 a	/	/
PNA	0.23 ± 0.01 bc	/	/
MEA	0.24 ± 0.03 b	/	/
PCA	0.23 ± 0.00 b	/	/
CHA	0.25 ± 0.00 b	/	/
V8A	0.28 ± 0.01 a	/	/
ARDA	0.30 ± 0.01 a	1.20 ± 0.37	17.85 ± 7.35 a

[1] The growth rate on each media was calculated by dividing the colony diameter by the number of days. Means with different letters in the same column are significantly different according to Duncan's test at $p \leq 0.05$. [2] / = No sporulation.

The fungus produced no conidia on OA, PNA, MEA, PCA, CHA and V8A after four weeks, but did sporulate on PDA and ARDA. On ARDA, the sporulation was 1.20×10^6 conidia/mL after one week and 17.85×10^6 conidia/mL after four weeks. On the other hand, no conidia were produced on PDA after one week, but 1.18×10^6 conidia/mL were recorded after four weeks. The sporulation was significantly greater on ARDA than PDA after four weeks of incubation ($p \leq 0.05$) (Table 2).

3.2. Effects of Carbon and Nitrogen Sources on Growth

All carbon sources supported the growth of *P. radicina*, but vigor varied substantially between media with different carbon sources. From one to four weeks, the colony diameter increased significantly with the incubation time in all carbon sources tested (Table 3). The diameter of the *P. radicina* colonies was the largest on the lactose medium after one week; this was significantly greater when compared with other carbon sources. After four weeks, this fungus exhibited the largest colony diameter on mannitol, soluble starch, lactose and sucrose, and the least vigorous growth on cellulose. Growth on cellulose was the least vigorous. After four weeks, *P. radicina* growth on mannitol, soluble starch, lactose, sucrose and D-xylose was significantly greater than on the other media. Out of the nine compounds tested, the carbon source best supporting *P. radicina* growth was mannitol, while cellulose was barely utilized by this fungus (Table 3).

Table 3. Mean (±SEM) colony diameter (cm) of *Paraphoma radicina* measured weekly for four weeks on different carbon sources and at L:D 0:24 h and 25 °C.

Carbon Source	Colony Diameter (cm)			
	1 Week	2 Weeks	3 Weeks	4 Weeks
Dextrose	2.47 ± 0.06 d [1]	4.33 ± 0.35 c	6.43 ± 0.32 b	7.27 ± 0.06 a
Sucrose	3.00 ± 0.00 c	5.40 ± 0.20 b	7.47 ± 0.06 a	7.73 ± 0.21 a
Mannitol	2.97 ± 0.06 d	5.80 ± 0.10 c	7.50 ± 0.20 b	7.83 ± 0.15 a
Fructose	2.67 ± 0.12 c	4.93 ± 0.60 b	7.03 ± 0.06 a	7.47 ± 0.15 a
D-Xylose	2.83 ± 0.15 d	5.53 ± 0.21 c	7.10 ± 0.53 b	7.70 ± 0.10 a
Lactose	3.23 ± 0.21 c	5.80 ± 0.52 b	7.30 ± 0.26 a	7.77 ± 0.06 a
Soluble starch	3.00 ± 0.00 d	5.50 ± 0.10 c	7.53 ± 0.06 b	7.77 ± 0.06 a
Maltose	2.87 ± 0.06 d	4.87 ± 0.21 c	6.47 ± 0.15 b	7.10 ± 0.10 a
Cellulose	2.40 ± 0.10 d	4.47 ± 0.06 c	6.37 ± 0.12 b	6.93 ± 0.15 a
LSD $_{(p<0.05)}$ [2]	0.181	0.544	0.420	0.216

[1] Treatment means with different letters across the four assessment periods are significantly different according to Duncan's test at $p \leq 0.05$. [2] Data in the same column are separated by the least significant different (LSD) ($p \leq 0.05$).

The ability of *P. radicina* to grow on media with different nitrogen sources was variable. Growth on the peptone medium was significantly greater than those on other nitrogen sources after one week. The colony diameter of *P. radicina* was also the largest on the peptone medium after four weeks of incubation, and was significantly greater than on glycine and urea, but not significantly different from other nitrogen sources (Table 4). The nitrogen source best supporting the growth of *P. radicina* was peptone, while glycine and urea were deficient sources for fungal growth.

Table 4. Mean (±SEM) colony diameter of *Paraphoma radicina* on seven different nitrogen sources grown at L:D 0:24 h and 25 °C, measured one and four weeks after inoculation.

Nitrogen Sources	Colony Diameter (cm)	
	1 Week	4 Weeks
Peptone	3.00 ± 0.00 c [1]	7.07 ± 0.15 a
Urea	1.67 ± 0.06 d	6.23 ± 0.15 a
Ammonium nitrate	2.67 ± 0.06 c	6.73 ± 0.12 a
Ammonium chloride	2.53 ± 0.06 c	6.77 ± 0.06 a
Glycine	3.00 ± 0.10 b	5.17 ± 0.81 a
Sodium nitrate	2.77 ± 0.06 c	6.70 ± 0.17 a
Ammonium sulfate	2.60 ± 0.10 c	6.70 ± 0.20 a
LSD$_{(p<0.05)}$ [2]	0.121	0.591

[1] Treatment means with different letters across the two assessment periods are significantly different according to Duncan's test at $p \leq 0.05$. [2] Data in the same column are separated by the least significant different (LSD) ($p \leq 0.05$).

The fungus produced no conidia on either of the nine carbon sources media or on the seven nitrogen source media tested in our experiments.

3.3. Effect of Temperature and pH on Conidial Germination

Conidia of *P. radicina* were able to germinate in the range of 4–35 °C, but the time of the onset of germination varied with temperature. Regardless, conidial germination onset was not observed earlier than 14 h at any temperature tested. Indeed, conidial germination was initiated at 18 h after incubation at 20, 25 and 28 °C, while the onset of germination was at 22 h, 26 h and 30 h at 30, 15 and 35 °C, respectively; lastly, initial germination was observed after 34 h at 4 °C and 10 °C. The conidial germination rate reached a maximum after 34 h at all temperature treatments and remained constant afterward (Table 5). When compared within any set time, percentages of conidial germination increased in the 4 to 25 °C range, where a maximum was observed, and then decreased with increasing temperature. After incubation for 38 h at 25 °C, germination reached a maximum of 52.36%, which was significantly greater than that observed at other temperatures. In contrast, germination rates at 4 and 35 °C were 1.14% and 1.92%, respectively, significantly smaller than those observed in other treatments (Table 5). Therefore, the observed optimum temperature for conidial germination of *P. radicina* was determined as 25 °C.

Table 5. Conidial germination rate (%) of *Paraphoma radicina* at varying temperatures and measured across seven time intervals.

Temperature (°C)	Incubation Time (h)						
	14	18	22	26	30	34	38
4	0.00 ± 0.00 a [1]	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 e	0.00 ± 0.00 d	1.14 ± 0.89 e	1.14 ± 0.89 e
10	0.00 ± 0.00 a	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 e	0.00 ± 0.00 d	8.22 ± 3.11 d	8.22 ± 3.11 d
15	0.00 ± 0.00 a	0.00 ± 0.00 c	0.00 ± 0.00 d	1.98 ± 1.73 de	4.36 ± 2.28 d	14.30 ± 2.42 c	14.30 ± 2.42 c
20	0.00 ± 0.00 a	4.13 ± 2.03 b	6.50 ± 4.18 c	11.31 ± 5.13 c	17.07 ± 5.22 bc	25.71 ± 2.75 b	25.71 ± 2.75 b
25	0.00 ± 0.00 a	14.27 ± 3.81 a	22.48 ± 4.51 a	34.32 ± 6.66 a	44.25 ± 12.72 a	52.36 ± 4.74 a	52.36 ± 4.74 a
28	0.00 ± 0.00 a	6.77 ± 3.64 b	14.49 ± 4.81 b	18.88 ± 2.13 b	21.93 ± 6.19 b	24.88 ± 5.03 b	24.88 ± 5.03 b
30	0.00 ± 0.00 a	0.00 ± 0.00 c	4.85 ± 3.57 c	6.86 ± 1.96 cd	12.88 ± 6.27 c	17.16 ± 2.07 c	17.16 ± 2.07 c
35	0.00 ± 0.00 a	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 e	1.65 ± 1.42 d	1.92 ± 1.50 e	1.92 ± 1.50 e

[1] Means with different letters in the same column are significantly different according to Duncan's test at $p \leq 0.05$.

Conidia of *P. radicina* had the highest germination rate at 25 °C, with the onset of germination at 18 h. On the other hand, the germination rate displayed no changes after 38 h, compared with the previous time set. Therefore, those conditions were chosen to assess the effect of pH on the conidial germination rate. Conidia of *P. radicina* were able to germinate across all pH levels tested. Both after 18 h or 38 h of incubation in sterile water at different pH, the germination rate increased as pH values increased from pH 4 to pH 7, with a maximum rate observed at pH 7. The germination rate then decreased as the pH increased from pH 7 to pH 10. Furthermore, at their maxima, after 18 h and 38 h of incubation, the values observed (43.83 % and 57.92 %, respectively) were significantly greater than those found at any other pH (Table 6). Therefore, the optimum pH value for conidial germination of *P. radicina* was determined to be pH 7.

Table 6. Conidial germination rate (%) of *Paraphoma radicina* at seven pH values at 18 and 38 h. Grown at 25°C and L:D 0:24.

pH	Incubation Time (h)	
	18	38
4	19.78 ± 4.92 b [1]	21.96 ± 3.44 c
5	20.58 ± 1.69 b	22.41 ± 3.33 c
6	20.93 ± 1.99 b	24.13 ± 5.36 c
7	43.83 ± 6.03 a	57.92 ± 5.90 a
8	6.25 ± 1.59 c	36.24 ± 1.97 b
9	3.14 ± 0.53 cd	28.35 ± 7.55 c
10	1.54 ± 0.44 d	26.03 ± 10.05 c

[1] Means with different letters in the same column are significantly different according to Duncan's test at $p \leq 0.05$.

3.4. Lethal Temperature of Mycelium and Conidia

Mycelia of *P. radicina* were able to grow after 10 min of heat treatment at temperatures in the range of 35 to 54 °C. Nevertheless, mycelia were found to be unable to grow at temperatures greater than or equal to 55 °C (Table S1). On the other hand, conidia were found to be able to germinate after 10 min of heat treatment in a water bath at 35 and 40 °C only, with observed germination rates of 15.12% and 6.77%, respectively. However, in our hands, conidia no longer germinated when the temperature was greater than or equal to 41 °C (Table S1). Thus, the lethal temperatures for *P. radicina* mycelial growth and conidial germination determined were 55 °C (10 min) and 41 °C (10 min), respectively.

4. Discussion

The establishment of a successful parasitic relationship between fungal pathogens and their hosts is influenced to varying degrees by environmental conditions [20,29]. Therefore, the elucidation of the factors influencing fungal growth, sporulation and conidial germination is of practical importance to devise better disease control strategies.

In earlier research, the aerial mycelium of *P. radicina* was described as pale olivaceous on OA medium and the colony color as pale luteous due to the production of a diffusible pigment [16]. Our initial results after evaluating the colony characteristics of *P. radicina* on various media agreed with those previously reported. In the present study, we evaluated the effects of eight media on conidia production of *P. radicina* and demonstrated that it was able to produce pycnidia and conidia only on PDA and ARDA media, while few conidia were generated on PDA. This is in agreement with earlier studies where it was also reported that pycnidia and conidia of *P. radicina* were not produced abundantly on the PDA medium [17]. Furthermore, in this study, we found that conidia of *P. radicina* were not produced on OA. Conversely, previous authors mentioned that the fungus produced abundant pycnidia on OA [16]. This discrepancy is likely due to differences in culture conditions. Our research showed that *P. radicina* displayed the greatest growth rate and sporulation on the ARDA medium, indicating that ARDA is the optimal medium for *P. radicina* culture.

This research also showed that *P. radicina* was able to use all tested carbon and nitrogen sources. After four weeks of incubation, the fungus showed significantly that the most vigorous growth was attained when mannitol was used as a carbon source, and when peptone was used as a nitrogen source. These attributes have been found in other fungal pathogens. For example, the growth rate of *Embellisia astragali* was reported to be greatest on mannitol and peptone after four weeks [26]. External nutrients can promote conidia production in some pathogenic fungi. Thus, *Verticillium alfalfa* displayed excellent sporulation on lactose and starch, and the greatest sporulation rate on peptone [30]. Furthermore, when the carbon nutritional requirements of *Phoma medicaginis* were studied, it was found that monosaccharides and disaccharides were nearly equivalent in the production of pycnidia, but superior to polysaccharides [31]. The same study reported that the formation of pycnidia and conidia was favored on nitrate more than ammonium nitrogen sources in

P. medicaginis. However, we found no conidia production by *P. radicina* on any of the tested carbon and nitrogen sources. This may indicate that conditions required for sporulation are more demanding compared to other fungi.

Our research showed that the optimal temperature for conidial germination of *P. radicina* was 25 °C. There was little germination at temperatures below 5 °C or above 35 °C. These results were similar to those in earlier reports where other species of fungi from alfalfa were studied [30,32]. In comparison, *P. radicina* mycelia can grow across a wide range of temperatures from 5 to 35 °C, with an optimal temperature in the range of 25 to 30 °C [15]. This finding suggests that *P. radicina* temperature requirements for conidial germination are in general agreement with those for mycelial growth. Of interest was the result showing that the lethal temperature for mycelial growth was greater than that of the conidia. This may be due to the formation of chlamydospores within the mycelium during incubation.

Fungal conidial germination was found early to be influenced by the hydrogen ion concentration in the growth medium [33]. In the present study, we observed that *P. radicina* conidia germinated at pHs ranging from pH 4 to pH 10, with the optimal at pH 7. In a previous report, we found that the optimal pH for mycelial growth of this fungus was within the range of pH 8 to pH 9, and that the growth was slow when the pH of the culture medium was <pH 4 or >pH 10 [15]. Despite this difference in the optimal pH for conidial germination versus mycelial growth, these results indicate that both excessive acidity and alkalinity are detrimental to the growth and conidial germination of *P. radicina*.

5. Conclusions

Our study has identified several factors that affect the growth, sporulation, and conidial germination of *P. radicina* and further defined the lethal temperature for mycelial growth and conidial germination of the fungus. The results indicated that ARDA was the most suitable medium for the growth and sporulation of *P. radicina*. Mannitol and peptone were the best sources of carbon and nitrogen, respectively. The optimal temperature for conidial germination was 25 °C, and the germination rate showed the maximum at pH 7. The lethal temperatures for *P. radicina* mycelial growth and conidial germination were 55 °C (10 min) and 41 °C (10 min), respectively. We believe our findings will contribute to the knowledge of the biological and physiological characteristics of *P. radicina* and assist in the development of disease control measures to mitigate alfalfa crop losses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12091501/s1. Figure S1: Morphology and characters of *P. radicina* after one week of cultivation on eight media; Table S1: Effect of temperature on *P. radicina* germination and mycelium growth.

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