

Influence of relative humidity and temperature on development of *Didymella rabiei* on chickpea debris

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Didymella rabiei grew saprophytically on pieces of artificially and naturally infected chickpea stem debris under artificial incubation conditions, and formed pseudothecia and pycnidia. The extent of growth was not significantly affected by temperature of incubation within the range 5–25°C, but was significantly reduced as relative humidity (RH) decreased from 100% to 86%, when no growth occurred. Pseudothecia matured at 10°C and constant 100% RH, or at 5 and 10°C and alternating 100%/34% RH. Under these conditions, pseudothecial maturation, assessed by a pseudothecia maturity index, increased over time according to the logistic model. For temperatures higher than 10°C or RH lower than 100%, pseudothecia either did not form ascospores, or ascospores did not mature and their content degenerated. When pseudothecia that initially developed to a given developmental stage were further incubated at a constant 100% RH, temperature became less limiting for complete pseudothecial development as the developmental stage was more advanced. Pycnidia of the fungus developed and formed viable conidia in all environmental conditions studied, except at 86% RH. However, the density of pycnidia formed and the number of viable conidia per pycnidium were significantly influenced by temperature, RH and the type of debris (artificially or naturally infected) used.

Introduction

Ascochyta blight, caused by *Ascochyta rabiei*, can devastate chickpea (*Cicer arietinum*) crops in many regions of the world when cool, wet weather occurs during the growing season (Saxena & Singh, 1984; Nene & Reddy, 1987). The annual economic losses caused by the disease in the Palouse region of eastern Washington and northern Idaho alone can exceed US \$1 million (Kaiser & Muehlbauer, 1989). Ascochyta blight is also one of the most important limiting factors for autumn–winter chickpea sowings, which otherwise can significantly increase seed yield because of reduced abiotic stresses and low incidence of fusarium wilt (*Fusarium oxysporum* f.sp. *ciceris*) (Saxena & Singh, 1984; Trapero-Casas & Jiménez-Díaz, 1986). *A. rabiei* causes necrotic lesions on all above-ground parts of the plant at any stage of crop development; these often cause breaking of stems and death of the plant parts above

the affected zone (Nene & Reddy, 1987). Within necrotic tissue, pycnidia develop and produce conidia that may contribute to secondary infections if suitable environmental conditions occur (Nene & Reddy, 1987).

Pseudothecia of *Didymella rabiei* (= *Mycosphaerella rabiei*), the teleomorphic stage of *A. rabiei*, were first discovered by Kovachevsky on overwintering chickpea debris in Bulgaria (Kovachevski, 1936). Subsequently, the teleomorph was reported in Russia (Gorlenko & Bushkova, 1958), Greece (Zachos *et al.*, 1963), Hungary (Kövics *et al.*, 1986), the United States (Kaiser & Hannan, 1987), Spain (Jiménez-Díaz *et al.*, 1987) and Syria (Haware, 1987). *D. rabiei* is heterothallic, with a bipolar diallelic mating incompatibility system (Wilson & Kaiser, 1995). Kaiser (1995) has shown that fertile pseudothecia developed on naturally infected debris from a number of chickpea growing countries in northern Africa, west Asia and eastern and western Europe, indicating the widespread distribution of the two mating types in nature. Airborne ascospores are a major form of primary inoculum for epidemics of ascochyta blight. Pseudothecial development begins in the autumn and winter, and ascospores of the fungus are discharged into the air from the end of winter through the spring. Ascospore maturation typically peaks by the beginning of the spring, and ascospore liberation decreases drastically or is nil by the beginning of summer. After exhaustion of

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pseudothecia, no more ascospores are produced and pseudothecial walls degenerate. New pseudothecia do not develop on the infected chickpea debris during the next crop season (Trapero-Casas & Kaiser, 1992; Navas-Cortés *et al.*, 1995; Trapero-Casas *et al.*, 1996).

The role of the teleomorph of *D. rabiei* as overwintering inoculum for ascochyta blight, its importance in the long-distance dissemination of the pathogen and the increasing genetic diversity in the pathogen, have increased the need to assess the influence of the environmental factors required for its formation. However, research on this subject has been very limited (Trapero-Casas & Kaiser, 1992). Development of the teleomorphic stage of *D. rabiei* has been observed in chickpea debris but never in infected plants or when the fungus has been cultured on artificial media (Kovachevski, 1936; Kóvics *et al.*, 1986; Trapero-Casas & Kaiser, 1992). Moisture is essential for the initiation and development of pseudothecia (Trapero-Casas & Kaiser, 1992). If moisture does not limit pseudothecial development, a minimum temperature range of 5–10°C is required for pseudothecial maturation. However, the simultaneous effects of temperature and moisture on pseudothecial development have not been quantified. The objective of this study was to determine quantitatively the influence of relative humidity (RH) and temperature on the development of *D. rabiei* and production of pseudothecia and pycnidia of the fungus on chickpea debris.

Materials and methods

Chickpea debris

Two types of chickpea stem debris from the highly susceptible cultivar Blanco Lechoso (Trapero-Casas & Jiménez-Díaz, 1984) were used in all the experiments: (a) debris from naturally infected plants; and (b) sterilized debris from healthy plants, which was artificially infested. In July 1988, infested stem debris was gathered from a chickpea crop in the Córdoba province that had been subject to a severe attack of ascochyta blight in the spring of that year. The debris was stored in sacks in a dry place at room temperature until used. The dried infested stems were cut into 6-cm-long pieces that had at least two necrotic lesions with pycnidia of *D. rabiei*. Stem pieces were washed in running tap water for 60 min, surface-disinfested in 1% NaClO for 5 min, and then drained on sterilized filter paper. Dry chickpea stems were collected from healthy plants growing in a field free from ascochyta blight in the Córdoba province in July 1988. These stems were cut into 6-cm-long pieces and sterilized with propylene oxide. Sterilized stem pieces were infested with *D. rabiei* by dipping them for 20 min in a 1 : 1 conidial suspension (10^5 conidia mL⁻¹) derived from the sexually compatible, monoascosporic isolates ATCC-76501 and ATCC-76502 (Trapero-Casas & Kaiser, 1992). The infested stem pieces were then dried on sterilized filter paper.

Temperature and moisture treatments

Pieces of stem debris, artificially or naturally infected with *D. rabiei*, were suspended on a grill 15 mm above a film of distilled water or over a saturated saline solution inside hermetically sealed plastic boxes (21.5 cm long × 15.5 cm wide × 9.0 cm deep) in darkness at constant temperatures of 5, 10, 15, 20 or 25 ± 1°C. For each incubation temperature, the relative humidity (RH) in the boxes was held constant at either 100, 98, 95 or 86%, or fluctuating between 100% and 34% at weekly intervals. Following each weekly dry period, debris was moistened in sterile water for 1 h before subsequent exposure to 100% RH. Levels of RH in the boxes were provided by distilled water (100%) and saline solutions of K₂SO₄ (98%), Zn SO₄ × 7H₂O (95% at 5 and 10°C), KNO₃ (95% at 15, 20 and 25°C), KCl (86%) and Mg₂CL × 6H₂O (34%) (Winston & Bates, 1960). Treatment combinations were maintained for 13 weeks, except for debris incubated at 5, 10 and 15°C with constant 100% RH, or alternating 100%/34% RH for which experiments lasted 25 weeks.

Assessment of fungal development in chickpea debris

Samples consisting of three stem segments were collected from each treatment combination at weekly intervals beginning with the second week of incubation. Sampled stem pieces were assessed for pseudothecial development and ascospore production. To assess pseudothecial development, 20 pseudothecia from each of three stem pieces were dissected from the tissue, squashed in lactophenol-acid fuchsin, and microscopically examined to determine their developmental stages. Pseudothecial development was assessed according to a 1–7 scale of pseudothecial maturity in which 1 = stromatic pseudothecial initial; 2 = pseudoparaphyses filling the lumen of the pseudothecium; 3 = appearance of asci arising among pseudoparaphyses; 4 = asci formed but contents not differentiated; 5 = asci with ascospores being formed or completely formed and mature, very few pseudoparaphyses remaining; 6 = empty or half-empty asci and released ascospores; and 7 = empty pseudothecium, all ascospores discharged and some asci detected (Trapero-Casas & Kaiser, 1992). For each sample, a pseudothecial maturity index (PMI) was calculated as the weighted average of the observed stages using the expression

$$PMI = \sum n_i(st_i)/N,$$

in which n_i = number of pseudothecia in the developmental stage i , st_i is the value of the pseudothecial developmental stage (1–7), and N = total number of pseudothecia assessed. To estimate ascospore production in pseudothecia, several strips of tissue colonized by pseudothecia were placed on 2% water agar (WA: 20 g agar, 1 L deionized water) until they covered an area of 4 cm². The WA block was then attached to the inner surface of the lid of a petri dish and the ascospores were allowed to discharge down on to WA, or into sterilized

water, in darkness for 24 h at 20°C. The discharged ascospores were then counted.

Regression analyses were carried out to describe pseudothecial development during each weekly incubation period as a function of time for each treatment. The increase in PMI was fitted to the logistic model by nonlinear regression analysis conducted by Marquardt's compromise method using the software package Plot IT 3.12 (Scientific Programming Enterprises, Haslett, MI). The coefficient of determination (R^2), the standard error, the significance of the estimated parameters and the pattern of residuals plotted against expected values were used to indicate the goodness-of-fit of data to the model (Campbell & Madden, 1990). The estimated values and confidence intervals ($P=0.05$) for the PMI asymptote parameter and the rate of PMI increase over time (r) were used to compare PMI progress of different treatments statistically (Campbell & Madden, 1990).

The number of fungal fruiting bodies (pycnidia and pseudothecia) per cm^2 of tissue (fruiting body density) and number of spores produced were measured in pieces of stem debris sampled 4 and 8 weeks after incubation to account for possible time effect on fungal reproduction. To determine fruiting body density, stem pieces were observed with a dissecting microscope at $\times 80$. Fruiting bodies in 10 microscope fields (1.91 mm^2 each) from each of three stem pieces were removed and characterized as pycnidia or pseudothecia of *D. rabiei* with a compound microscope. To determine conidial production, 20 pycnidia were removed from each of three stem pieces, placed in a drop of sterile water, macerated with a pestle to obtain a homogeneous conidial suspension, and a serial dilution was spread evenly on acidified WA (AWA: 0.25 mL of 85% lactic acid per litre of WA) in petri dishes. The dishes were incubated at 20°C in darkness for 10 days and the number of *D. rabiei* colonies growing on the agar plates counted. The number of conidia per pycnidium was estimated by the mean number of colonies of *D. rabiei* formed per pycnidium in the macerates, assuming that each colony developed from a single conidium. The production of asci and ascospores per pseudothecium was determined for 60 selected pseudothecia in which the asci appeared to be mature, with the ascospores clearly differentiated (stage 5 of pseudothecial development). Pseudothecia were squashed on microscope slides, stained with lactophenol-acid fuchsin and examined under the microscope.

Samples of the naturally infected and inoculated stem debris were subjected to a split-split plot design of five temperatures and five moisture levels in which temperatures were main plots and RH levels were subplots. Data were analysed by standard analysis of variance (ANOVA). Analyses of density of fruiting bodies and number of conidia per pycnidium were performed with observations made after 4 and 8 weeks of incubation as blocks. Analysis of the number of asci per pseudothecium was carried out with the 60 sampled pseudothecia as replications. There was variance heterogeneity between the two types of chickpea debris (artificially or naturally

infected) according to Barlett's test of equal variances. Therefore, separate analyses were performed for each type of debris. Temperature \times relative humidity significant interactions were analysed by partitioning the interaction sum of squares. Trend comparison procedures, based on orthogonal polynomials for treatments with equal intervals for temperature, were used. Additionally, orthogonal single-degree of freedom contrasts were computed to test the effect of the relative humidity treatments. Data were analysed using the software package STATISTIX 4.1 (Analytical Software, Roseville, MN, USA).

Effect of temperature on subsequent development of immature pseudothecia

Pieces of infected stem debris in which pseudothecia of *D. rabiei* had developed to a given stage were selected to determine the effect of temperature on subsequent pseudothecial development. The experiment was performed using two types of stem debris and incubation conditions. (a) Sterilized stem fragments from healthy chickpeas were artificially infested as described above and incubated over a film of sterilized water in darkness inside hermetically sealed plastic boxes at 8°C and 100% RH, conditions favourable for pseudothecial development (Trapero-Casas & Kaiser, 1992). Then, when pseudothecia reached the average stages of development (PMI 1.5, 3.5 and 5) that occurred after 4, 8 and 10 weeks of incubation, respectively, stem debris was subjected to various temperature treatments. (b) Naturally infected chickpea stem debris was placed on the soil surface of a 4×3 m plot in a field at Granada, southern Spain, on 6 October 1988. Debris was retained in place by a 5-mm-mesh nylon/net attached to the soil to prevent dispersal of the stem pieces by wind. Pieces of stem debris were examined periodically for pseudothecial development. On 1 December, 15 February and 15 March, stem debris colonized with pseudothecia at the average stage of development of PMI 1.5, 3.5 and 5, respectively, was brought into the laboratory and subjected to various temperature treatments.

Selected stem pieces, placed at 8°C and 100% RH or located in the field plot at Granada, were placed inside hermetically sealed plastic boxes and further incubated at constant 15, 20 and 25°C and 100% RH. Samples of three stem fragments were collected from each treatment combination at weekly intervals for 9, 8 and 5 weeks for PMI values of 1.5, 3.5 and 5, respectively. Pseudothecial development in the sampled fragments was assessed as described above.

Results

Production of pseudothecia and pycnidia in chickpea stem debris

Both pseudothecia and pycnidia developed in most of the treatment combinations in which chickpea stem

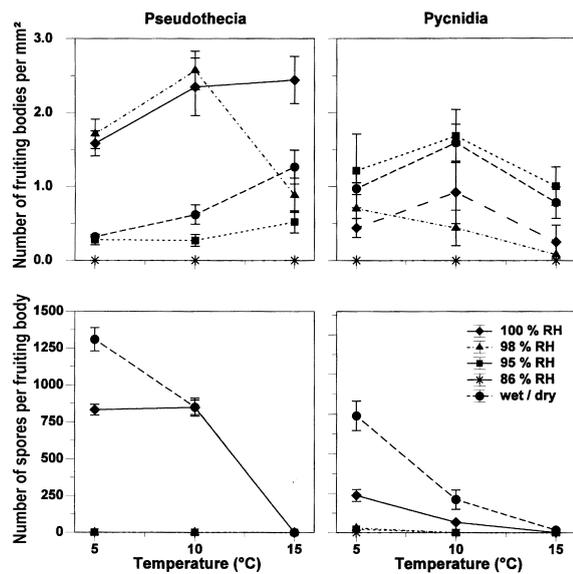


Figure 1 Influence of temperature and relative humidity on production of pseudothecia and pycnidia of *Didymella rabiei* on naturally infected chickpea stem debris. Density of fruiting bodies was determined on 60 microscope fields at $\times 80$ magnification (1.91 mm^2 each). The number of viable conidia produced per pycnidium was determined in 120 pycnidia by means of dilution plating on acidified water agar. The number of ascospores was determined from 60 pseudothecia which contained clearly differentiated ascospores. Vertical bars are standard error of the mean. Chickpea debris was incubated at constant temperatures (5, 10 and 15°C). For each incubation temperature, relative humidity was constant and adjusted to 100, 98, 95 and 86%, or 100% (wet) and 34% (dry) at weekly intervals. Following a dry period of 34% RH, debris was moistened in sterile deionized water for 1 h before the next exposure.

debris was colonized by *D. rabiei*. Relative humidity was the factor that most strongly influenced fungal development in stem debris. Fungal development was nil at 86% RH and very limited at 95% RH, irrespective of temperature and type of debris (Figs 1 and 2).

Overall, a larger density of fruiting bodies formed in artificially infested debris (Fig. 2) than in naturally infected debris (Fig. 1); such a difference was due to the high density of pycnidia in the former. No significant differences ($F=2.09$, $P=0.1490$) were found between numbers of pseudothecia on the two types of debris. However, a significantly higher ($F=51.87$, $P<0.0001$) number of pycnidia developed on artificially infested debris. At 20 and 25°C , empty or nondifferentiated fruiting bodies developed on stem debris incubated at 100% and 100%/34% RH, and 98 and 95% RH, respectively (data not shown).

For both artificially and naturally infected chickpea debris, the density of pseudothecia on stem debris incubated at 100% RH or 100%/34% RH showed a positive linear trend with increase in temperature (Table 1; Figs 1 and 2). A negative linear trend was observed for pseudothecial density in artificially infested stem debris incubated at 95% RH (Table 1; Fig. 2). A quadratic

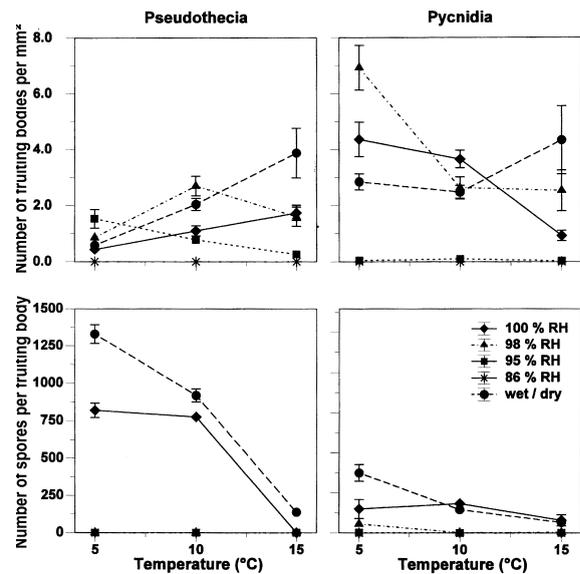


Figure 2 Influence of temperature and relative humidity on production of pseudothecia and pycnidia of *Didymella rabiei* on artificially infested chickpea stem debris. Density of fruiting bodies was determined on 60 microscope fields at $\times 80$ (1.91 mm^2 each). The number of viable conidia produced per pycnidium was determined in 120 pycnidia by means of dilution plating on acidified water agar. The number of ascospores was determined from 60 pseudothecia which contained clearly differentiated ascospores. Vertical bars are standard error of the mean. Chickpea debris was incubated at constant temperatures (5, 10 and 15°C). For each incubation temperature, relative humidity was constant and adjusted to 100, 98, 95 and 86%, or 100% (wet) and 34% (dry) at weekly intervals. Following a dry period of 34% RH, debris was moistened in sterile deionized water for 1 h before the next exposure.

trend with increasing temperature occurred for pseudothecial density on artificially infested debris at 98% RH (Table 1; Fig. 2). Overall, the density of pseudothecia in naturally infected stem debris averaged over temperatures of incubation was significantly higher at 100% RH ($P<0.0001$) than that at a lower relative humidity or with alternating wet/dry periods (Table 2; Fig. 1). However, the density of pseudothecia in artificially infested debris was not significantly affected by relative humidity (Table 2; Fig. 1).

On naturally infected stem debris, the highest density of pycnidia developed at 10°C and at all relative humidities except for constant 98% RH; therefore, most of the variability was explained by a quadratic trend with increase in temperature (Table 1, Fig. 1). At 98% RH, pycnidial density showed a negative linear trend with increase in temperature (Table 1, Fig. 1). On artificially infested debris, the density of pycnidia was highest at 5°C , and showed a significant negative linear trend with increase in temperature (Table 1, Fig. 2). On debris, the density of pycnidia, averaged over temperature of incubation, was higher at 100% RH than at alternating 100%/34% RH. No significant differences between the two moisture treatments were found for

Table 1 Partitioning of temperature × relative humidity sum of squares (SS) of production of pseudothecia, pycnidia and spores of *Didymella rabiei* on chickpea stem debris

Relative humidity (%) ^a	Source of variation	Density of fruiting bodies ^b		Spores produced per fruiting body	
		Psd % of SS	Pyc % of SS	Asc/Psd ^c % of SS	Con/Pyc ^d % of SS
Naturally infected chickpea debris					
100	Linear	83.2	7.0	97.1	93.7
	Quadratic	16.8	93.1	2.9	6.3
98	Linear	24.0	99.2	–	75.1
	Quadratic	76.0	0.8	–	24.9
95	Linear	72.3	9.0	–	76.2
	Quadratic	27.7	91.0	–	23.8
Wet/dry	Linear	95.7	5.1	73.6	93.3
	Quadratic	4.3	94.9	26.4	6.7
Artificially infested chickpea debris					
100	Linear	99.9	89.8	96.9	44.3
	Quadratic	0.1	10.2	3.1	55.7
98	Linear	16.3	76.6	–	71.7
	Quadratic	83.7	23.4	–	28.3
95	Linear	98.9	5.1	–	94.2
	Quadratic	1.1	94.9	–	5.8
Wet/dry	Linear	99.5	57.1	78.9	92.7
	Quadratic	0.5	42.9	21.1	7.3

^a For each incubation temperature, relative humidity (RH) was constant and adjusted to 100, 98, and 95%, or 100% (wet) and 34% (dry) at weekly intervals. Following a dry period of 34% RH, debris was moistened in sterile deionized water for 1 h before the next exposure to 100% RH.
^b The number of pseudothecia (Psd) and pycnidia (Pyc) formed on debris was determined on 60 stereo microscopic fields at ×80 (1.91 mm²/each).
^c The number of ascospores per pseudothecium (Asc/Psd) was determined for 60 pseudothecia at the developmental stage of asci with differentiated ascospores (pseudothecia maturity index = 5).
^d The number of viable conidia per pycnidium (Con/Pyc) was determined for 120 pycnidia. Pycnidia were macerated in sterile water and diluted suspensions were plated on acidified water agar.

naturally infected debris (Table 2; Figs 1 and 2). Pycnidia developed to a higher density at 98% RH than at 95% RH in artificially infested stem debris, but the opposite occurred in naturally infected debris (Table 2; Figs 1 and 2).

Spore production in pseudothecia and pycnidia

Pseudothecia of *D. rabiei* in artificially or naturally infected stem debris formed mature asci and ascospores only when debris was incubated at 5 or 10°C and

Table 2 Analysis of variance, with partitioning of relative humidity sum of squares (SS) of production of pseudothecia, pycnidia and spores of *Didymella rabiei* on chickpea stem debris incubated at 5, 10 or 15°C and different relative humidity levels^a

Source of variation	Density of fruiting bodies ^b				Spores produced per fruiting body			
	Psd		Pyc		Asc/Psd ^c		Con/Pyc ^d	
	F	P	F	P	F	P	F	P
Naturally infected chickpea debris								
Constant 100% vs wet/dry	21.04	<0.0001	2.11	0.1006	16.59	0.0001	22.31	<0.0001
100% vs (98%, 95%)	17.04	<0.0001	0.85	0.4739	–	–	5.30	0.0028
98% vs 95%	33.50	<0.0001	5.16	0.0023	–	–	0.01	0.9982
Artificially infested chickpea debris								
Constant 100% vs wet/dry	1.78	0.1511	5.57	0.0013	66.81	<0.0001	2.48	0.0685
100% vs (98%, 95%)	0.27	0.8481	2.03	0.1107	–	–	15.62	<0.0001
98% vs 95%	4.06	0.0087	27.34	<0.0001	–	–	0.27	0.8462

^a For each incubation temperature, relative humidity (RH) was constant and adjusted to 100, 98, and 95%, or 100% (wet) and 34% (dry) at weekly intervals. Following a dry period of 34% RH, debris was moistened in sterile deionized water for 1 h before the next exposure to 100% RH. Data were averaged for the three incubation temperatures.
^b The number of pseudothecia (Psd) and pycnidia (Pyc) formed on debris was determined on 60 stereo microscopic fields at ×80 (1.91 mm²/each).
^c The number of ascospores per pseudothecium (Asc/Psd) was determined for 60 pseudothecia at the developmental stage of asci with differentiated ascospores (pseudothecia maturity index = 5).
^d The number of viable conidia per pycnidium (Con/Pyc) was determined for 120 pycnidia. Pycnidia were macerated in sterile water and diluted suspensions were plated on acidified water agar.

Table 3 Pseudothecium developmental stage^a reached by *Didymella rabiei* pseudothecia when colonized chickpea stem debris was incubated at different levels of temperature and relative humidity^b

Type of chickpea debris	Relative humidity (%)	Temperature (°C)				
		5	10	15	20	25
Naturally infected	100	3	7	2*	1*	1*
	98	1	1	1*	1*	1*
	95	1	1	1	1	1
	86	— ^c	—	—	—	—
	Wet/dry	6	6	2*	1*	1*
Artificially infested	100	4	7	3*	1*	1*
	98	1	1	1*	1*	1*
	95	1	1	1	1	1
	86	—	—	—	—	—
	Wet/dry	6	7	6*	1*	1*

^a Pseudothecia developmental stage: 1, stromatic pseudothecial initial; 2, pseudoparaphyses filling the lumen of the pseudothecium; 3, appearance of asci arising among pseudoparaphyses; 4, asci formed but contents not differentiated; 5, asci with ascospores being formed or completely formed and mature; 6, empty or half-empty asci and released ascospores; and 7, empty pseudothecium, all ascospores discharged. Values followed by an asterisk indicate those treatments for which pseudothecia degenerated without fully developing.

^b For each incubation temperature, relative humidity (RH) was constant and adjusted to 100, 98, and 95% for 13 weeks; or 100% (wet) and 34% (dry) at weekly intervals for 25 weeks. Following a dry period of 34% RH, debris was moistened in sterile deionized water for 1 h before the next exposure to 100% RH.

^c Experimental treatments where fungal growth was not observed.

constant 100% RH, or alternating 100%/34% RH. Asci and ascospores also formed when artificially infested debris was incubated at 15°C and alternating 100%/34% RH (Table 1; Figs 1 and 2). Overall, the number of ascospores produced on artificially infested and naturally infected stem debris did not differ significantly ($F=0.29$, $P=0.5893$). For both types of debris, the number of ascospores produced per pseudothecium decreased linearly with increasing incubation temperature (73.6–97.1% of the total variance) (Table 1; Figs 1 and 2). Also, for both types of debris, the number of ascospores produced per pseudothecium was significantly higher ($P<0.0001$) in pseudothecia on stem debris incubated at alternating 100%/34% RH compared with those incubated at constant 100% RH (Table 2; Figs 1 and 2).

Overall, the number of viable conidia produced per pycnidium was not significantly affected ($F=0.54$, $P=0.4636$) by the type of stem debris (i.e. artificially or naturally infected). For both types of chickpea stem debris used, the number of viable conidia produced per pycnidium decreased with a linear trend as temperature increased for all levels of relative humidity, except for pycnidia on artificially infested debris incubated at constant 100% RH for which conidial production was not significantly influenced by the incubation

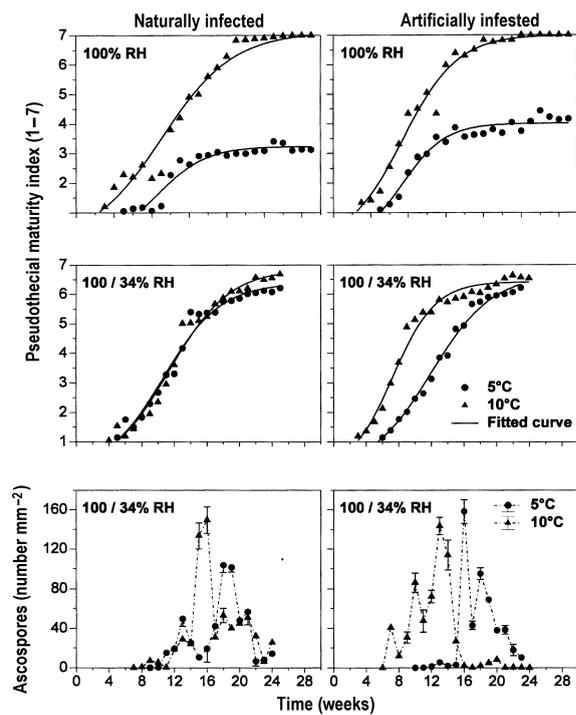


Figure 3 Development of *Didymella rabiei* on chickpea stem debris under two moisture and temperature conditions. For each week and treatment, at least 60 pseudothecia and 4 cm² of highly infested tissue were used to estimate the pseudothecial maturity index and the number of ascospores discharged, respectively. Vertical bars are the standard error of the mean.

temperature (Table 1; Figs 1 and 2). For both types of debris, conidial production, averaged over temperature of incubation, was highest ($P<0.0001$) at alternating 100%/34% RH than at constant 100% RH (Table 2, Figs 1 and 2). The average number of viable conidia produced per pycnidium at a constant relative humidity of less than 98% RH was significantly lower ($P<0.005$) than that produced at constant 100% RH (Table 2; Figs 1 and 2).

Pseudothecial maturation

Pseudothecial development and maturation were influenced mainly by temperature and relative humidity (Table 3). Pseudothecial maturation occurred only at 10°C and constant 100% RH, and at 5 and 10°C and alternating 100%/34% RH (Table 3). At 5°C and 100% RH, pseudothecia contained asci that did not fully mature, as asci did not differentiate ascospores after 24 weeks of incubation (Table 3). For all other treatment combinations studied, either pseudothecia development did not proceed beyond the initial stages (PMI = 1–2) or pseudothecial contents degenerated (Table 3), except for those on artificially infested chickpea stem debris incubated at 15°C and alternate 100%/34% RH, on which 6% of the sampled pseudothecia formed mature asci and ascospores (Table 3).

Table 4 Nonlinear regression analysis for fitting the increase of the maturity index of *Didymella rabiei* pseudothecia over time to the logistic model

Chickpea debris	Relative humidity (%)	Temperature (°C)	Parameter estimates ^a						Statistics ^b	
			A	SE (A)	B	SE(B)	r	SE(r)	R ²	SSR
Naturally infected	100	5	3.235	0.077	28.513	2.098	0.389	0.013	0.925	1.1361
		10	7.000	0.000	11.170	0.122	0.265	0.088	0.968	3.5067
	wet/dry	5	6.377	0.104	21.031	0.527	0.285	0.004	0.978	1.4380
		10	6.859	0.114	21.972	0.298	0.271	0.002	0.980	1.7956
Artificially infested	100	5	4.009	0.064	26.112	2.157	0.438	0.014	0.954	0.8759
		10	7.000	0.000	13.399	0.079	0.347	0.088	0.980	1.8090
	wet/dry	5	6.674	0.092	23.538	0.440	0.263	0.003	0.991	0.5177
		10	6.420	0.077	16.172	0.316	0.381	0.005	0.982	1.2747

^aA, asymptotic value of pseudothecial maturity index; B, constant of integration; r, rate parameter; SE, standard error for the parameter estimates. ^bR², coefficient of determination; SSR, final sum of squares of residuals.

For those treatment combinations that gave rise to mature pseudothecia, the increase of PMI over time was described appropriately by the logistic model (Fig. 3). The estimates for the asymptote parameter were significantly lower when either artificially or naturally infected chickpea stem debris was incubated at 5°C and constant 100% RH (Table 4; Fig. 3). The rate of PMI increase over time was faster for pseudothecia developed

on chickpea stem debris incubated at 5°C and constant 100% RH as compared with those incubated at alternate 100%/34% RH, irrespective of the type of debris (artificially or naturally infected) (Table 4; Fig. 3). Minor differences were observed for the estimates of the asymptote or rate parameter for the remaining experimental combinations (Table 4).

The effect of temperature on further development of immature pseudothecia

The effect of temperature on the further development of pseudothecia varied with the pseudothecial developmental stage at which treatments were applied and with the type of debris (artificially or naturally infected) on which pseudothecia formed (Table 5; Fig. 4). In pieces of stem debris that served as controls (PMI = 0 at initiation of the experiment), pseudothecia formed at all incubation temperatures but only reached full development when incubated at 5 and 10°C. At higher temperatures (15, 20 and 25°C), pseudothecial contents degenerated after a few weeks of incubation, and no asci or ascospores differentiated. When pieces of stem debris with pseudothecia developed to PMI stage 1.5 (pseudothecial lumen containing pseudoparaphyses) were used, pseudothecia reached full development on debris incubated at 5 and 10°C. However, in the same debris, pseudothecial contents degenerated when debris was incubated at 15, 20 or 25°C. At 15°C, most pseudothecia remained at PMI stage 3 (asci differentiation) and a very low percentage of these reached subsequent developmental stages. No further pseudothecial development occurred on debris incubated at 20 and 25°C, as pseudothecial contents degenerated after 4 and 5 weeks of incubation, respectively (Fig. 4).

When pseudothecia were allowed to develop to initial PMI stage 3.5 (asci differentiation) and then incubated at several temperatures, pseudothecia continued to develop to PMI stage 6 (empty or half-empty asci and released ascospores) after 9 and 5 weeks of incubation at 5 and 10°C, respectively. Pseudothecia incubated at 15,

Table 5 Final development of *Didymella rabiei* pseudothecia when colonized chickpea stem debris with pseudothecia at the indicated pseudothecial developmental stage was further incubated at given temperatures and 100% relative humidity for 8 weeks

Chickpea debris ^a	Temperature (°C)	Pseudothecial developmental stage ^b			
		PMI=0	PMI=1.5	PMI=3.5	PMI=5
Naturally infected	5	2 ^c	5	5	6
	10	4	6	7	7
	15	*	*	7	7
	20	*	*	7	7
	25	*	*	7	7
Artificially infested	5	2	4	5	6
	10	4	4	6	6
	15	*	*	7	7
	20	*	*	7	7
	25	*	*	7	7

^aArtificially infested: debris was incubated at 8°C and constant 100% relative humidity (RH) until a pseudothecial developmental stage was reached. Naturally infected: debris was incubated under field conditions until a pseudothecial developmental stage was reached.

^bDebris with pseudothecia at the given stage of pseudothecial maturity index (PMI) developed under artificial or natural conditions was further incubated at constant temperature and 100% RH.

^cPseudothecia developmental stage: 1, stromatic pseudothecial initial; 2, pseudoparaphyses filling the lumen of the pseudothecium; 3, appearance of asci arising among pseudoparaphyses; 4, asci formed but contents not differentiated; 5, asci with ascospores being formed or completely formed and mature; 6, empty or half-empty asci and released ascospores; and 7, empty pseudothecium, all ascospores discharged; *treatments for which pseudothecia degenerated without fully developing.

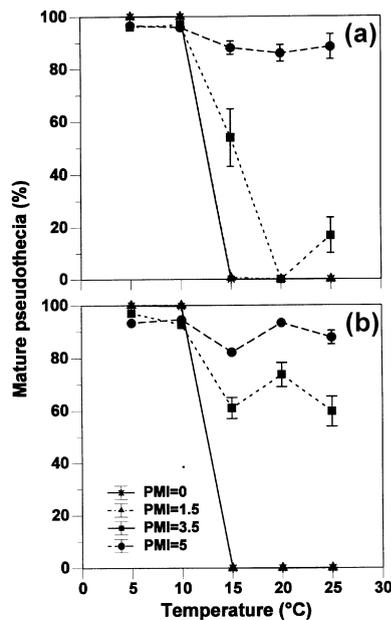


Figure 4 Effect of temperature on the percentage of mature pseudothecia of *Didymella rabiei* on chickpea stem debris with pre-formed immature pseudothecia. Debris which was initially incubated for pseudothecia to reach a pseudothecial maturity index (PMI) of 1.5, 3.5 or 5 was further incubated at given temperatures and 100% relative humidity for 8 weeks. (a) Naturally infected chickpea debris initially incubated under field conditions. (b) Artificially infested chickpea debris initially incubated at 8°C and constant 100% RH. Vertical bars are the standard error of the mean.

20 or 25°C also completed their development. However, at these temperatures, mature pseudothecia intermixed with degenerate pseudothecia accounted for 26–100% of all pseudothecia formed (Fig. 4).

On debris where pseudothecia had already reached PMI stage 5 (asci fully developed containing mature ascospores), pseudothecia completed full development in less than 4 weeks at all incubation temperatures studied. However, the time period required to complete full development decreased as incubation temperature increased. A low percentage (7–17%) of degenerate pseudothecia occurred on debris incubated at 20 and 25°C (Fig. 4).

Discussion

Previous work by Trapero-Casas & Kaiser (1992) indicated that high moisture availability is the essential factor for saprophytic growth and induction of pseudothecial development of *D. rabiei* on infected chickpea debris. Our results confirm that high moisture, either constant 100% RH or alternate 100%/34% RH, is a fundamental limiting factor for development of pseudothecia and pycnidia of *D. rabiei* on infected chickpea stem debris. The development of the anamorphic stage of the fungus was influenced by relative humidity to a lesser extent than the teleomorphic stage, since pycnidia

with viable conidia formed at all levels of relative humidity for which fungal growth was observed. Moisture is a determining factor for growth and sporulation of most fungi in plant tissues and crop debris, and even alternating dry and moist periods may be particularly favourable for sporulation of some pathogens (Müller, 1979). Pseudothecia of *Venturia inaequalis* develop only within a range of 78–100% RH or with alternating dry and moist periods (O'Leary & Sutton, 1986). Similarly, conidiogenesis in *Pyrenophora tritici-repentis* requires more than 85% RH and reaches an optimum at 100% RH (Platt & Morrall, 1980). Conversely, development of pseudothecia of *Mycosphaerella ligulicola* is favoured by low moisture or alternating moist and dry periods (McCoy *et al.*, 1972).

If moisture requirements are satisfied, temperature does not seem to be a limiting factor for the development of *D. rabiei* fruiting bodies on chickpea debris. However, cool temperatures (5–15°C) are required for sporulation and seem to be a critical factor for maturation of *D. rabiei* pseudothecia on chickpea debris. While pseudothecial differentiation may be initiated at temperatures within 5–25°C, further development is regulated by cool temperatures. Optimum temperatures for pseudothecial maturation are between 5 and 10°C. Temperatures above 15°C are detrimental for development, as pseudothecia either remained at the initial stages of differentiation at low relative humidity or their contents degenerated and no ascospores were produced at high relative humidity. Our results agree with those of Trapero-Casas & Kaiser (1992) who showed that pseudothecia of *D. rabiei* may start to develop on infected chickpea debris within a wide range of temperatures (5–25°C), but about 50 days were required for maturation of most pseudothecia and ascospores at 5–10°C. According to these authors, only a low number of pseudothecia matured at 15°C and all pseudothecia degenerated at 20 and 25°C. The longer period required for ascospore maturation in our experiments, as compared with that in the work of Trapero-Casas & Kaiser (1992), may be due to differences in the methods used to establish moisture levels, as well as in the duration and frequency of the dry period used by the two laboratories.

Although temperature influences the whole process of pseudothecial maturation on chickpea debris infested by *D. rabiei*, its significance for the process diminishes as the pseudothecial developmental stage advances. Thus, temperatures lower than 15°C are required for pseudothecial development from an initial stage without differentiated asci. However, once ascus and ascospore differentiation are initiated, further pseudothecial development seems to be less dependent on temperature. Temperature is also essential for pseudothecial production by other fungi. Several authors indicate that pseudothecia of *V. inaequalis* degenerate and ascospore production declines at temperatures above 24°C (O'Leary & Sutton, 1986). In *P. tritici-repentis*, the rate of pseudothecial maturation is highest at 15°C and,

as with *D. rabiei*, pseudothecial development is delayed at temperatures suboptimal for mycelial growth (20–25°C); however, the final number of pseudothecia formed is not influenced by temperatures within 10–30°C (Summerell & Burgess, 1988).

Our results on the influence of temperature on the development of *D. rabiei* pycnidia on infected chickpea stems differ from data concerning mycelial growth and pycnidial formation on artificial growth media, which are optimum at 15–20°C with lower and upper thresholds of 5 and 30°C, respectively (Kaiser, 1973; Zachos *et al.*, 1963). In our study, neither pycnidia nor conidia formed on stem debris incubated at temperatures above 15°C, irrespective of moisture level and type of debris (artificially or naturally infected) used. However, Kaiser (1973) obtained pycnidia with viable conidia on inoculated chickpea stems incubated at 10–30°C, with an optimum temperature of 20°C for pycnidial formation. These differences might be due to variation in the nature and composition of growth substrate (artificial growth media and chickpea debris), as well as to the period of incubation. Also, viability of conidia formed in pycnidia on chickpea debris could be affected by the long exposure to moderate or high temperatures (20 or 25°C), as well as to activity of saprophytic fungi which extensively colonize chickpea debris incubated on moist soil at 15, 20 and 25°C (Navas-Cortés *et al.*, 1995).

Development of *D. rabiei* pseudothecia on naturally infected chickpea debris was delayed compared with that on artificially infested debris, although the same final developmental stage was achieved on both types of debris. Similarly, pycnidial density and conidial production per pycnidium were significantly lower on naturally infected debris than on artificially infested debris. These differences, which were larger at lower temperatures and relative humidity, might relate to the energy available for active growth of the fungus on the infected debris. In artificially infested debris, all nutrients available in the sterilized substrate can be used for active fungal growth, whereas in naturally infected debris some nutrients in the substrate may have been used during the survival stage or by other organisms, and additional nutrients may be required for the fungus to renew active growth. This difference in the available energy of each substrate is likely to determine the ability of *D. rabiei* to grow and reproduce under suboptimal moisture and temperature conditions.

That *D. rabiei* requires specific environmental conditions for the formation of the teleomorph may explain why the sexual stage of the fungus rarely occurs in many dry chickpea-growing regions of the world (Kaiser, 1973; Nene & Reddy, 1987). However, it also suggests that the teleomorphic stage is well adapted in regions where overwintering chickpea debris is exposed to cool temperatures and sporadic humidity (Trapero-Casas & Kaiser, 1992). Most field reports on the occurrence of the teleomorph agree with this suggestion (Gorlenko & Bushkova, 1958; Zachos *et al.*, 1963; Haware, 1987; Kaiser & Hannan, 1987; Navas-Cortés, 1992;

Trapero-Casas & Kaiser, 1992; Navas-Cortés *et al.*, 1995; Trapero-Casas *et al.*, 1996).

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References

- Campbell CL, Madden LV, 1990. *Introduction to Plant Disease Epidemiology*. New York, NY: John Wiley & Sons, Inc.
- Gorlenko MV, Bushkova LN, 1958. Perfect stage of the causal agent of ascochytirosis of chickpea (in Russian). *Plant Protection, Moscow* **3**, 60 (abstract in *Review of Applied Mycology*, **37**, 695).
- Haware MP, 1987. Occurrence of perfect state of *Ascochyta rabiei* in Syria. *International Chickpea Newsletter* **17**, 29–30.
- Jiménez-Díaz RM, Navas-Cortés JA, Trapero-Casas A, 1987. Occurrence of *Mycosphaerella rabiei*, the teleomorph of *Ascochyta rabiei* in Andalucía. *Proceedings of the 7th Congress of the Mediterranean Phytopathological Union, 1987*. Granada, Spain: Consejería de Agricultura y Pesca de la Junta de Andalucía, 124–5.
- Kaiser WJ, 1973. Factors affecting growth, sporulation, pathogenicity, and survival of *Ascochyta rabiei*. *Mycologia* **65**, 444–57.
- Kaiser WJ, 1995. World distribution of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, on chickpea (Abstract). *Phytopathology* **85**, 1040.
- Kaiser WJ, Hannan RM, 1987. First report of *Mycosphaerella rabiei* on chickpeas in the Western Hemisphere. *Plant Disease* **71**, 192.
- Kaiser WJ, Muehlbauer FJ, 1989. An outbreak of *Ascochyta* blight of chickpea in the Pacific Northwest, USA, in 1987. *International Chickpea Newsletter* **18**, 16–7.
- Kovachevski IC, 1936. The blight of chickpea (*Cicer arietinum*), *Mycosphaerella rabiei* n.sp. (in Bulgarian). Sofia, Bulgaria: Ministry of Agriculture and National Domains, Plant Protection Institute.
- Kövics G, Holly L, Simay EI, 1986. An ascochytirosis of the chickpea (*Cicer arietinum* L.) caused by *Didymella rabiei* (Kov.) vs. Arx: Imperfect *Ascochyta rabiei* (Pass.) Lab. in Hungary. *Acta Phytopathologica et Entomologica Hungarica* **21**, 147–50.
- McCoy RE, Horst RK, Dimock AW, 1972. Environmental factors regulating sexual and asexual reproduction by *Mycosphaerella ligulicola*. *Phytopathology* **62**, 1188–95.
- Müller E, 1979. Factors inducing asexual and sexual sporulation in fungi (mainly Ascomycetes). In: Kendrick B, ed. *The Whole Fungus*. Ottawa, Canada: National Museum of Natural Sciences, 265–72.
- Navas-Cortés JA, 1992. *El teleomorfo de Ascochyta rabiei (Pass.) Labrousse en España: Detección, desarrollo y su papel en la epidemiología de la Rabia del Garbanzo* (*Cicer*

- arietinum L) (PhD thesis). Córdoba, Spain: University of Córdoba.
- Navas-Cortés JA, Trapero-Casas A, Jiménez-Díaz RM, 1995. Survival of *Didymella rabiei* in chickpea straw debris in Spain. *Plant Pathology* **44**, 332–9.
- Nene YL, Reddy MV, 1987. Chickpea diseases and their control. In: Saxena MC, Singh KB, eds. *The Chickpea*. Oxon, UK: CAB International, 233–70.
- O'Leary AL, Sutton TB, 1986. The influence of temperature and moisture in the quantitative production of pseudotecia of *Venturia inaequalis*. *Phytopathology* **76**, 199–204.
- Platt HW, Morrall RAA, 1980. Effects of light intensity and relative humidity on conidiation in *Pyrenophora tritici-repentis*. *Canadian Journal of Plant Pathology* **2**, 53–7.
- Saxena MC, Singh KB, (eds) 1984. *Ascochyta Blight and Winter Sowing of Chickpeas*. The Hague, the Netherlands: Martinus Nijhoff/Dr W. Junk Publishers.
- Summerell BA, Burgess LW, 1988. Factors influencing production of pseudothecia by *Pyrenophora tritici-repentis*. *Transactions of the British Mycological Society* **90**, 557–62.
- Trapero-Casas A, Jiménez-Díaz RM, 1984. Siembras tempranas y Rabia del garbanzo. *Actas III Congreso Nacional de Fitopatología*. Puerto de la Cruz, Tenerife, Spain: Sociedad Española de Fitopatología, 95.
- Trapero-Casas A, Jiménez-Díaz RM, 1986. Influence of sowing date on Fusarium wilt and Ascochyta blight of chickpea in Southern Spain. In: O'Keefe LE, Muehlbauer FJ, eds. *Poster Abstracts, International Food Legume Conference*. Moscow, Idaho, USA: College of Agriculture, University of Idaho, 11.
- Trapero-Casas A, Kaiser WJ, 1992. Development of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, on chickpea straw. *Phytopathology* **82**, 1261–6.
- Trapero-Casas A, Navas-Cortés JA, Jiménez-Díaz RM, 1996. Airborne ascospores of *Didymella rabiei* as a major primary inoculum for Ascochyta blight epidemics in chickpea crops in southern Spain. *European Journal of Plant Pathology* **102**, 237–45.
- Wilson AD, Kaiser WJ, 1995. Cytology and genetics of sexual incompatibility in *Didymella rabiei*. *Mycologia* **87**, 795–804.
- Winston PW, Bates DH, 1960. Saturated solutions for the control of humidity in biological research. *Ecology* **41**, 232–7.
- Zachos DG, Panagopoulos CG, Makris SA, 1963. Recherches sur la biologie, l'épidémiologie et la lutte contre l'antracnose du pois-chiche. *Annales de l'Institut Phytopathologique, Benaki* **5**, 167–92.