Effects of soil temperature, inoculum density, and incubation time on red root rot of vetch.

A re-analysis of data in "The Root and Stem Rot Disease of Vetch", a thesis leading to an award of Master of Science in Agriculture in March 1967.

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Abstract

Field observations on the effects of sowing date and cropping history on red root rot of *Vicia sativa* cv. Golden Tares were corroborated with laboratory and glasshouse experiments. Optimum temperature for disease development in the glasshouse was 25.7°C, while that for the pathogen in culture was 30.5°C. The pathogen formed *gemmae* in culture that constituted infective colony forming units (cfu). An inoculum density of 2500 cfu ml^{-1} was found in severely infested soil. Disease severity increased with inoculum density in the range of 16-4000 cfu ml^{-1}, inversely in proportion with the calculated average distance between cfu. At an average soil temperature of 17.4°C, red root rot remained mild on inoculated plants even with 4000 cfu ml^{-1}. A mathematical model incorporating the effects of soil temperature, inoculum density, and incubation time described 95% of the variation in disease severity.

Introduction

In 1964, a new root rot disease was identified in common vetch (*Vicia sativa* cv. Golden Tares) grown on red ferrosols of the far north coast of New South Wales, Australia (Allen 1967). Root rot occurred more severely in fields sown in February-March than in April-May, and more commonly in fields that had been sown to vetch previously than in those planted for the first time. The pathogen was an unnamed fungus (*isolate number 48*), later accessioned as *DAR14088* in the New South Wales State Government, Plant Disease and Mycology Herbarium. It was characterized by mycelial branches called *gemmae* that constituted infective, colony-forming units (cfu). Spores, sporophores and sclerotia have not been observed in pure cultures or within infected root tissues.

Vetch root rot was renamed as *red root rot* by Allen (1972) and this name was used in relation to a similar disease of *V. sativa* cv. Morava caused by *Atractiella rhizophila* (Allen et al. 2020). Both Golden Tares vetch and *DAR14088* have become extinct, and nucleic acid sequences amplified from the dried herbarium specimen have not been diagnostic for *A. rhizophila* or any other fungus. However, the morphology, histopathology, and host range of *DAR14088* correspond with *A. rhizophila* from Morava vetch. A novel feature of *A. rhizophila* was that it was pathogenic to seedling vetch (Allen et al. 2020) when previously considered only an endophyte or possible symbiont of the root microbiome (Bonito et al. 2017; Vélez et al. 2017).

Allen (1967) reported a partial analysis of epidemiological data from laboratory and glasshouse experiments to corroborate field observations on sowing date and cropping history. The partial analysis examined the effects of soil temperature (T), inoculum density (φ), and incubation time (Δt_m) on measured disease severity ($Y_{t.measured}$). Allen (1967) used linearizing transformations to analyze the data to a limited extent. Subsequently, non-linear mathematical models were developed to describe the data and iterative least squares optimization (ILSO) procedures were used to estimate model constants[1]. The present purpose is to update details of epidemiological analyses.

Methods and Results

Extent of natural infestation

Samples of red ferrosols were taken from fields affected by red root rot. Samples from the same fields were disinfested by aerated steam at 60°C for 30 minutes (Baker 1957) and used to dilute naturally infested soil in a 10-fold series up to 7 levels. At each level, five sub-samples of 1 litre each were transferred to glasshouse containers and sown with 5 Golden Tares seed. After 40 days incubation in a glasshouse, the root systems were washed, examined for red root rot symptoms, and the most probable number method (Fisher and Yates 1963) was used to estimate inoculum density in cfu ml^{-1} of soil. The most severely infested soil contained 2500±150 cfu ml^{-1}.

Glasshouse experiments

Two complementary experiments were conducted in a glasshouse during 1965. Each experiment involved 3 water baths, each with eight 5 litre containers of potting media amended either with homogenized potato dextrose agar (PDA) or homogenized mycelium of *DAR14088* amid an equivalent concentration of PDA. The glasshouse air temperature was >15°C and <30°C, and the potting media consisted of equal parts by volume of peat moss and sandy loam.

- Experiment 1. Water baths were set at 26.8°C, 23.4°C, or 17.4°C, representing average soil temperatures on the far north coast of New South Wales in January, February-March, and April-May, respectively (Anonymous 1957), with an average precision of ±0.3°C. Uniformity experiments had previously established the confounding effects of water bath position and temperature setting did not affect results significantly. Potting media temperatures were measured daily at 9am and 5pm, and the daily records were averaged over the whole experimental period ($T_{average}$). There were six containers with potting media amended only with PDA homogenate and 18 containers each with 4000±250 cfu ml^{-1} of potting media. Ten Golden Tares vetch seed were sown in each container and the number of emergent epicotyls was counted in each container for the first nine days after sowing. One

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[1] The *Solver* add-in of Microsoft Excel, Redmond, Washington, United States of America, was used in model development. Subsequently, *R* software from the Institute for Statistics and Mathematics, Vienna, Austria, was used for estimating model constants and standard errors.

inoculated container was selected randomly from each tank at 10, 15, 20, and 30 days after sowing and the seedling roots were assessed for disease severity (Table 1). After 40 days, vetch seedlings in all remaining containers were assessed for disease and dried to constant weight at 110°C. Treatment effects on dry matter yield were examined by analysis of variance.

- Experiment 2 involved the same three water tanks as in Experiment 1, all set at $T_{average}$ of 26.8°C. Two containers in each tank had potting media amended with PDA only (designated *non-inoculated*), two with *DAR14088* mycelium at a rate of one PDA plate colony per container (*inoculated high-density*), two with 0.1 PDA plates per container, and two with 0.01 plates per container. The inoculation series was prepared using *non-inoculated* potting media as the diluent. The *inoculated high density* potting media contained 1600±100 cfu ml^{-1} of potting media. Containers were sown and placed randomly in the three water tanks. Epicotyl emergence was monitored as in Experiment 1. After incubation for 14 and 21 days, the vetch plants from two containers of each of the three inoculum densities, and two non-inoculated containers, were selected at random and assessed for disease as before (Table 2). After incubation for 35 days, the vetch plants in all remaining containers were assessed for disease, dried to constant weight, and subjected to analysis of variance as in Experiment 1.

Disease severity assessment

Allen (1967) assessed disease severity using an ordinal scale involving both quantitative and qualitative elements. The categories ranged from *no disease* (category “0”) to *plants killed by root rot and foot rot* (category “6”). This scale was revised to remove qualitative elements of lesion size and position and then recalibrated to known proportions of root tissue with disease symptoms. The mean of individual plant assessments was recalculated for each container, using the mid-point interpolation method of Chiang et al. (2017), and carried forward to parametric analyses.

Table 1. *Experiment 1*. Red root rot disease severity measurements ($Y_{t.measured}$) of Golden Tares vetch plants in 24 containers inoculated with 4000 colony forming units (cfu) of *DAR14088* per ml of potting media, or not inoculated, and incubated for 10, 15, 20, 30, or 40 days, at 17.4℃, 23.4℃, or 26.8℃. The measured $Y_{t.measured}$ is the mean of assessments made on 10 observed plants.

Container identification	Incubation time (Δt_m *days*)	Incubation temperature ($T_{average}$ °C)	Inoculum density ($cfu.ml^{-1}$)	Y_t measured
1	10	17.4	4000	0.10
2	15	17.4	4000	0.13
3	20	17.4	4000	0.20
4	30	17.4	4000	0.30
5	40	17.4	4000	0.21
6	40	17.4	4000	0.26
7	40	17.4	0	0
8	40	17.4	0	0
9	10	23.4	4000	0.24
10	15	23.4	4000	0.39
11	20	23.4	4000	0.42
12	30	23.4	4000	0.67
13	40	23.4	4000	0.73
14	40	23.4	4000	0.77
15	40	23.4	0	0
16	40	23.4	0	0
17	10	26.8	4000	0.30
18	15	26.8	4000	0.52
19	20	26.8	4000	0.62
20	30	26.8	4000	0.67
21	40	26.8	4000	0.77
22	40	26.8	4000	0.77
23	40	26.8	0	0
24	40	26.8	0	0

Table 2. *Experiment 2*. Red root rot disease severity measurements ($Y_{t.measured}$) of Golden Tares vetch plants in 24 containers inoculated with 16, 160, or 1600 colony forming units (*cfu*) of *DAR14088* per ml of potting media, or not inoculated, then incubated for 14, 21, or 35 days at 26.8℃. The measured $Y_{t.measured}$ is the mean of assessments made on 10 observed plants.

Container identification	Incubation time (Δt_m *days*)	Incubation temperature ($T_{average}$ °C)	Inoculum density ($cfu.ml^{-1}$)	$Y_{t\text{-}measured}$
1	14	26.8	0	0
2	14	26.8	16	0.03
3	14	26.8	160	0.13
4	14	26.8	1600	0.28
5	14	26.8	0	0
6	14	26.8	16	0.03
7	14	26.8	160	0.14
8	14	26.8	1600	0.33
9	21	26.8	0	0
10	21	26.8	16	0.15
11	21	26.8	160	0.28
12	21	26.8	1600	0.55
13	21	26.8	0	0
14	21	26.8	16	0.16
15	21	26.8	160	0.35
16	21	26.8	1600	0.56
17	35	26.8	0	0
18	35	26.8	16	0.23
19	35	26.8	160	0.54
20	35	26.8	1600	0.57
21	35	26.8	0	0
22	35	26.8	16	0.28
23	35	26.8	160	0.56
24	35	26.8	1600	0.61

Model development and application

Effect of incubation temperature on the radial growth rate of DAR14088 in pure culture

Figure 1 illustrates data from Allen (1967) on the measured radial growth rates of *DAR14088* ($G_{Tmeasured.PDA}$) after incubation in PDA plates at ten constant temperatures (T) from 10-39°C. Growth was evident on plates at 15°C and 37°C after incubation for seven days. The data were analyzed using an adapted Gaussian function:

$$G_{Tmeasured.PDA} = G_{Topt.PDA} \exp\left[-\left[\frac{(T - T_{opt.PDA})}{K_{x.PDA}}\right]^2\right] \qquad \text{(Equation 1)}$$

where $G_{Topt.PDA}$ was the maximum radial growth rate of *DAR14088* in PDA at the optimum temperature ($T_{opt.PDA}$) and $K_{x.PDA}$ was the temperature dispersion constant for incubation temperatures above ($K_{above.PDA}$) and below ($K_{below.PDA}$) the optimum temperature. Model constants were $G_{Topt.PDA}$=3.3±0.3 mm d^{-1} at $T_{opt.PDA}$=30.5±0.6°C, $K_{above.PDA}$=4.2±0.6°C and $K_{below.PDA}$=11.5±0.9°C. Equation 1 was not sufficiently accurate at the tails of the distribution, so the cardinal values were determined by observation as indicated above.

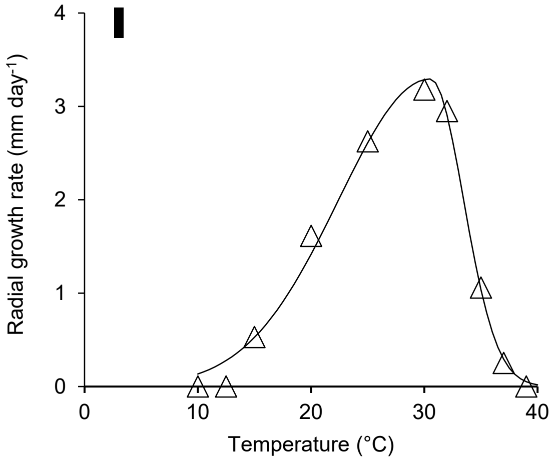

Figure 1. Radial growth rates of *DAR14088* in potato dextrose agar (points) at 10 constant temperatures compared with values predicted by Equation 1 and constants estimated by iterative least squares optimization (line).

Effects of incubation soil temperature on vetch epicotyl emergence and growth in the glasshouse

The root systems of establishing Golden Tares vetch seedlings involved a taproot and several orders of secondary roots, including adventitious roots that developed from epicotyl tissue in contact with the potting media. In Experiment 1, the time until 50% of epicotyls emerged ($E_{50\%}$) was estimated as 6.4 days at 17.4°C, 5.2 days at 23.4°C, and 4.4 days at 26.8°C, with an average precision of ±0.5 days. The product ($T_{average} E_{50\%}$) was a constant 114±5 degree-days. The dry matter yield after incubation for 40 days averaged 13±1 mg plant^{-1} for all treatments except for plants incubated at 26.8°C in the presence of 4000 cfu ml^{-1} of *DAR14088* where yields averaged only 9±1 mg plant^{-1} and were significantly less than other treatments ($P<0.05$). An identical result was obtained in Experiment 2 with respect to the incubation temperature of 26.8°C. Yield differences related to inoculum density were not statistically significant ($P>0.05$) where the average dry weight after 35 days was 11±1 mg plant^{-1}. The plants incubated at 17.4°C in the presence of 4000 cfu ml^{-1} of *DAR14088* developed only mild RRRD symptoms and lesions remained localised.

Effects of incubation soil temperature, inoculum density and incubation time on disease severity

All vetch plants in the inoculated containers developed red root rot to some degree whereas no plants developed disease symptoms in the non-inoculated containers. Two of 20 plants died in containers with 4000 cfu ml^{-1} when incubated at 26.8°C for more than 30 days, while other plants in this treatment became chlorotic and lost leaves. Plants in other containers survived with apparently healthy stems and leaves.

Measured disease severity was plotted over Δt_m for both Experiment 1 and Experiment 2 (Figure 2). In Experiment 1 (constant φ, variable $T_{average}$), disease symptoms became apparent after a delay period dependent on $T_{average}$, increased in severity over time, and reached an asymptote correlated with $T_{average}$. In Experiment 2 (constant $T_{average}$, variable φ), disease severity increased after a common delay period and tended towards the same asymptote but at rates dependent on inoculum density.

Severe disease, defined arbitrarily as $Y_t>0.5$, occurred approximately 21 days after sowing when plants were incubated at 26.8°C with 4000 and 1600 cfu ml^{-1}, and after 35 days with 160 cfu ml^{-1} (Figure 2). Disease symptoms remained mild at 26.8°C with 16 cfu ml^{-1}, although symptoms were still developing 35 days after sowing. Plants incubated at 17.4°C developed only mild disease symptoms with 4000 cfu ml^{-1} and symptom severity attained an asymptote within 40 days of sowing. Lesions at 17.4°C remained localized as streaks illustrated by Allen et al. (2020).

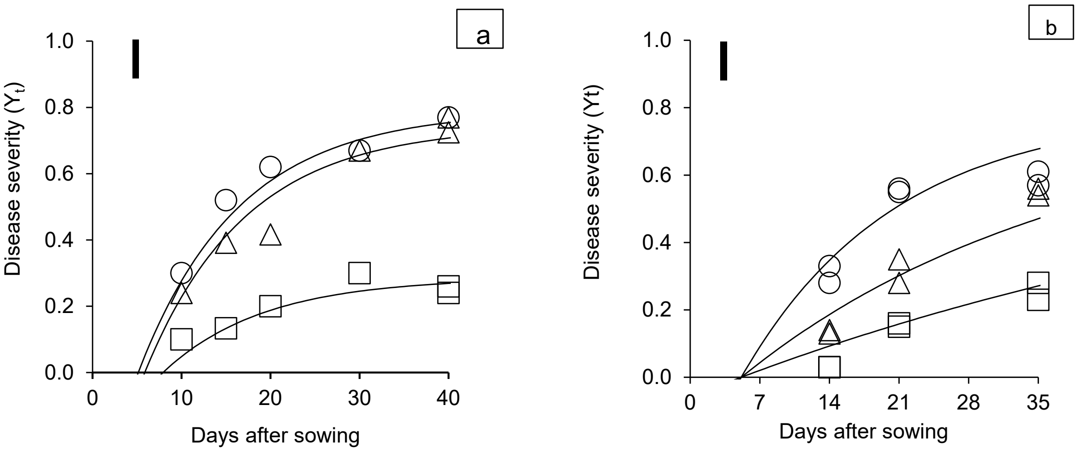

Figure 2. Relationships between red root rot disease severity (Y_t) and days after sowing Golden Tares vetch seed into potting media inoculated with *DAR14088*, presenting $Y_{t.measured}$ (points) and $Y_{t.estimated}$ from Equation 8 (lines). Each point is the mean Y_t of 10 measured plants. The vertical bar indicates the 95% confidence interval of model estimates.

a) *Experiment 1.* The points are measured data for average soil temperatures of 17.4°C (□), 23.4°C (Δ), and 26.8°C (○) with an inoculum density of 4000 cfu ml^{-1} while the lines were drawn using model constant estimates involving all 36 data sets.

b) *Experiment 2.* Replicate points are measured data for inoculum densities of 1600 cfu ml^{-1} (○), 160 cfu ml^{-1} (Δ), and 16 cfu ml^{-1} (□), each incubated at 26.8°C, while the lines were drawn using model constant estimates involving all 36 data sets.

Model development

A biological model of disease progression appropriate to red root rot in glasshouse containers was proposed by Gilligan (1990):

$$Y_t = F_u\left[1-\exp(-\beta(\Delta t_m - \Delta t_\delta))\right] + \varepsilon \qquad \text{(Equation 2)}$$

where, in addition to variables already defined, F_u was an upper asymptote attained, Δt_d was a delay or incubation period between root exposure and disease symptom development, β was a rate constant, and ε was a random error. Earlier, Gilligan (1979) had defined the rate constant in a rhizosphere model as:

$$\beta = N_\varphi \lambda \qquad \text{(Equation 3)}$$

where N_φ was the number of cfu within a defined rhizosphere zone and λ was the proportion of cfu that succeeded in infecting a host cell within the rhizosphere per day. In the case of red root rot, the rhizosphere was best described by a linear zone without a radius and in particular the average distance between cfu (ϕ or the inverse of N_{φ}) so that:

$$\phi = \sqrt[3]{\sqrt{2}/\varphi} \qquad \text{(Equation 4)}$$

and consequently:

$$\beta = \lambda/\phi \qquad \text{(Equation 5)}$$

Equation 4 was derived from Hales (2005), assuming inoculated cfu were in hexagonal close packed array within the potting media. The variable ϕ was measured in cm when inoculum density was expressed as cfu ml^{-1}. The upper asymptote attained ($Y_{t.max}$) was correlated with $T_{average}$ so that:

$$F_u = Y_{t.max} exp\left[-\left(\frac{T_{average} - T_{opt.host}}{K_{below.host}}\right)^2\right] \qquad \text{(Equation 6)}$$

where $T_{opt.host}$ was the optimum temperature for disease development in the host and $K_{below.host}$ was the dispersion constant relevant to $T_{opt.host}$ as in the Gaussian function described in Equation 1. The delay constant was also correlated with the average incubation soil temperature so that:

$$\Delta t_{\delta} = K_{\delta}/T_{average} \qquad \text{(Equation 7)}$$

where K_d was a thermal energy constant with units of degree-days.

In summary, the biological model for red root rot disease severity measured in Experiments 1 and 2 was:

$$Y_{t.measured} = Y_{t.max} exp\left[-\left(\frac{T_{average} - T_{opt.host}}{K_{below.host}}\right)^2\right]\left[1 - exp\left(\frac{\lambda}{\phi}\left[\Delta t_m - \left(K_{\delta}/T_{average}\right)\right]\right)\right] + \varepsilon \qquad \text{(Equation 8)}$$

The dependent variable ($Y_{t.measured}$) was the mean of ten seedling assessments per container, and the independent variables ($T_{average}$, Δt_m, and ϕ) were determined experimentally. Other independent variables ($T_{opt.host}$, $K_{below.host}$, λ, and K_d) were estimated by ILSO and the error term (ε) was indicated by the residual least square after ILSO.

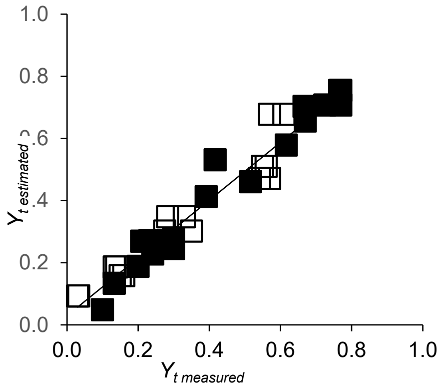

Figure 3. Correlation of disease severity estimated by Equation 8 ($Y_{t.estimated}$) on disease severity measured ($Y_{t.measured}$) in Experiment 1 (■) and Experiment 2 (□), with a linear trendline (Equation 9).

Estimation of constants for Equation 8

The progressions of $Y_{t.measured}$ with increasing Δt_m were well described by Equation 8 when fitted to data for inoculated containers in both Experiment 1 and 2 (Figure 3). Model constants estimated by ILSO were $Y_{t.max}$=0.80±0.05, $T_{opt.host}$=25.7±0.9°C, $K_{below.host}$=8.2±0.6°C, λ=0.0063±0.0007 day^{-1} and K_d=137±10 degree-days. The relationship:

$$Y_{t.estimated} = 0.93\,Y_{t.measured} + 0.03 \qquad \text{(Equation 9)}$$

had a correlation coefficient of 0.95 (n=36; P<0.001), a residual standard error of 0.05 and a 95% confidence interval of model estimates of 0.10. Deviations from Equation 9 were normally distributed, and the mean deviation was not significantly different from zero (P<0.05). Equation 8 described the two experiments almost equally (Figure 3). The probability of any of the model constants occurring by chance was P<0.01.

Discussion

Growth of *DAR14088* in PDA was favoured by high temperatures, with growth in the range of 15-37°C and an optimum of 30.5°C. Disease development was also favoured by high soil temperatures as observed in February-March sowings, with an optimum of 25.7°C. The Gaussian function (Equation 1) well described the responses of the pathogen to incubation temperature in PDA and gave reliable estimates of $T_{opt.PDA}$ and $K_{x.PDA}$ with five reference temperatures above and below the optimum. The same function was used to estimate $T_{opt.host}$ and $K_{below.host}$ (Equations 6 and 8) for a convenient comparison with Equation 1. However, these estimates are approximate because of the limited range of incubation temperatures set experimentally. Red root rot did not develop significantly at 17.4°C and this result was consistent with field evidence from April-May sowings (Allen 1967).

In the glasshouse, the delay period of the disease was shorter at high temperatures than at low temperatures. Evidence from epicotyl emergence ($E_{50\%}$), which indicates the earliest time that roots can be exposed to cfu, correlated with the delay period estimated from Equation 8. This estimate was based on an average over the whole experimental period instead of the single emergence estimate. A major effect of $T_{average}$ was on the extent of disease development, with asymptotes indicating an equilibrium was attained between the rates of symptom development and root growth. This equilibrium was correlated with the relative growth rate of *DAR14088* at the incubation temperature. It is thought that symptoms developed on young root tissue in a repeated process while new roots were produced. Each cycle apparently slowed as the available susceptible tissue became limiting, only to be repeated when new roots developed. Also, temperature may have affected the rate of symptom development while properties such as age-dependent disease resistance were developing (Raftoyannis and Dick 2002). In the glasshouse, red root rot was favoured by a high inoculum density at sowing. This was consistent with field evidence on cropping history. A novel feature of red root rot modelling (Equation 8) was that inoculum density affected the rate constant in proportion with numbers of cfu encountered by extending roots. Effects of treatment on the rate constant are unusual (Gilligan 1990).

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