# Influence of Temperature and Moisture on Growth of *Gloeotinia temulenta* and Infection of Annual Ryegrass

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## ABSTRACT

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Germination, growth, and sporulation of *Gloeotinia temulenta* increased at temperatures of 5–20 C and then declined. No germination or growth occurred at 30 C. Germination of conidia slowed with decreasing water potential, although 95–100% germination occurred by 24 h at potentials of 0.0 through −4.0 MPa. Radial growth on potato-dextrose agar amended with sodium chloride, potassium chloride, or sucrose declined with decreasing water potentials through −9 to −10 MPa. Conidia were abundant with sucrose as an osmoticum but not with KCl or NaCl. Spore production on a sucrose-amended medium at 10, 15, or 20 C increased through −4 MPa and then declined through −10 MPa. Infection of annual ryegrass occurred at 15, 20, and 25 C but not at 30 C. A dew period was not required for infection when a drop of conidial suspension was placed in open flowers of annual ryegrass or when a conidia-laden exudate from infected seed was transferred to ovaries in open flowers. Production of conidia in infected seed at 20 C increased 6–16 days after inoculation.

*Additional keywords:* blind seed, *Lolium multiflorum.*

*Gloeotinia temulenta* (Prill & Delacr.) M. Wilson, M. Noble & E. Gray, causal agent of blind seed disease, is a flower-infecting pathogen of ryegrass (*Lolium* spp.) (4). Primary infection occurs from ascospores and secondary spread from conidia (2,6). The pathogen infects the ovary or developing seed and colonizes the endosperm as the seed develops (14). Infected seed may appear shrunken and rusted in color, and a pinkish slime containing abundant conidia may ooze from the infected seed (2,9). Infected seeds usually do not germinate (5).

Most of the ryegrass seed for the forage and turf grass industry in the United States is produced within the Willamette Valley of Oregon. In 1989 over 81,000 ha of annual and perennial ryegrass was harvested in the Willamette Valley (8). Until the introduction of field burning in the latter part of the 1940s, blind seed was a serious problem in this region. Recent concerns over the air pollution effects of smoke from field burning have resulted in legislation to phase out burning by 1998. The potential of increasing levels of blind seed in nonburned fields has revived an interest in this disease.

Little is known about the environmental regulation of *G. temulenta.* Infection is favored by moderate temperatures and moist conditions at the time of flowering (2,10). Neill and Hyde (9) reported that growth of *G. temulenta* on several agar media was more vigorous at 21 than at 27 C. Wright and Wilson (13) found greater numbers of conidia on a potato-dextrose-peptone medium at 22.5 C than at 18 or 21 C. However, the temperature and moisture limits for germination and growth of *G. temulenta* have not been defined. The objectives of this paper were to determine 1) the influence of temperature and water potential on germination, growth, and sporulation of *G. temulenta,* and 2) the influence of temperature and dew period on infection of annual ryegrass by *G. temulenta.*

## MATERIALS AND METHODS

**Inoculum production.** A conidial isolate of *G. temulenta* (BS-1) from an infected annual ryegrass seed collected near Brownsville, OR, in August 1989 was used in all experiments. The fungus was maintained on potato-dextrose agar (PDA) or in a greenhouse on 8- to 10-wk-old annual ryegrass (*Lolium multiflorum* Lam. 'Marshall') plants.

Flowers of 8- to 10-wk-old annual ryegrass plants were each inoculated with 5 $\mu$l of a $1 \times 10^5$ conidia per milliliter suspension of *G. temulenta.* Plants were incubated in a dew chamber at 20 C for 24 h and then maintained in a growth chamber at 20 C with a 16 h/day photoperiod at 300 $\mu$E. After 20 days, infected seeds with conidial slime were harvested. Conidia were harvested from each infected seed in 1–2 ml of distilled water. A hemacytometer was used to adjust inoculum concentration. Conidia produced on annual ryegrass were used in all germination and infection studies.

**Germination.** Four replicate 4-$\mu$l drops containing $1 \times 10^5$ conidia per milliliter were placed on glass slides (two drops per slide). Slides were placed in petri dishes (two slides per dish) containing moistened tissue paper, sealed with Parafilm, and incubated at 5, 10, 15, 20, 25, or 30 C. Slides were removed after 2, 4, 6, 8, 10, 12, 16, and 24 h, and 2 $\mu$l of 2% $CuSO_4$ was added to each conidial drop to inhibit further germination. Germination was based on the first 50 conidia encountered in each drop. Conidia with germ tubes longer than 4 $\mu$m were considered germinated. Germ tubes of the first 20 germinated conidia observed in each drop were measured under a compound microscope at 350× with an ocular reticle.

Five-microliter drops of a $1 \times 10^5$ conidial suspension were placed on 13-cm-diameter filter membranes (0.45 $\mu$m pore size). The membranes were placed on aluminum mesh screens in a 4-mm gap between 4-mm-thick layers of 2% water agar located on the top and bottom of 9-cm-diameter petri dishes. Water potential of the agar was adjusted to −1, −2, −3, −4, −5, or −6 MPa with NaCl (1). A 0 MPa treatment was included and was created by placing membranes containing conidia on saturated tissue paper. Dishes were sealed with Parafilm and incubated at 20 C. After 2, 4, 6, 8, 12, or 24 h, four replicate membranes for each water potential were stained with aniline blue (28 mg of aniline blue, 20 ml of distilled water, 10 ml of glycerol, and 10 ml of 85% lactic acid). Germination was based on the first 50 conidia encountered on each membrane.

**Mycelial growth.** Osmotic levels of PDA were adjusted with KCl, NaCl, or sucrose to −0.05 through −6 MPa (1). Agar plugs, removed with a 4-mm-diameter cork borer from the advancing

margins of 4-wk-old cultures of *G. temulenta,* were placed on osmotically adjusted PDA in 9-cm-diameter petri dishes (one plug per dish). Radial growth (mm) was measured after 4 wk of incubation at 5, 10, 15, 20, 25, or 30 C. The experimental arrangement was a three by six factorial with four replications.

**Production of conidia in vitro.** Osmotic levels of PDA were adjusted with sucrose to −0.05 through −10.0 MPa. Agar plugs (4 mm in diameter) from the advancing margins of 4-wk-old cultures of *G. temulenta* were placed on the adjusted PDA in 9-cm-diameter petri dishes (one plug per dish, four replicate plates per treatment). Conidia were collected from cultures grown on sucrose-amended PDA for 4 wk at 10, 15, or 20 C. Each dish was rinsed with 5 ml of distilled water. A rubber policeman facilitated the removal of spores. Conidia per dish were quantified using a hemacytometer.

**Infection and production of conidia in annual ryegrass.** Annual ryegrass cv. Marshall was planted in 10-cm-diameter pots containing a greenhouse potting mix. In all experiments, 6- to 8-wk-old annual ryegrass plants were used. Ten flowers on each of two seed heads on each of four replicate plants were inoculated. Unless otherwise noted, each flower was inoculated with 5 $\mu$l of a $1 \times 10^5$ conidia per milliliter suspension. Percent infected seed was determined based on 20 seeds pooled from the two seed heads on each plant.

Inoculated flowers were placed in growth chambers at 15, 20, 25, or 30 C. Temperatures at the site of infection, measured with a model HYPl hypodermic thermocouple probe attached to a HH21 digital microprocessor thermometer (Omega Engineering,

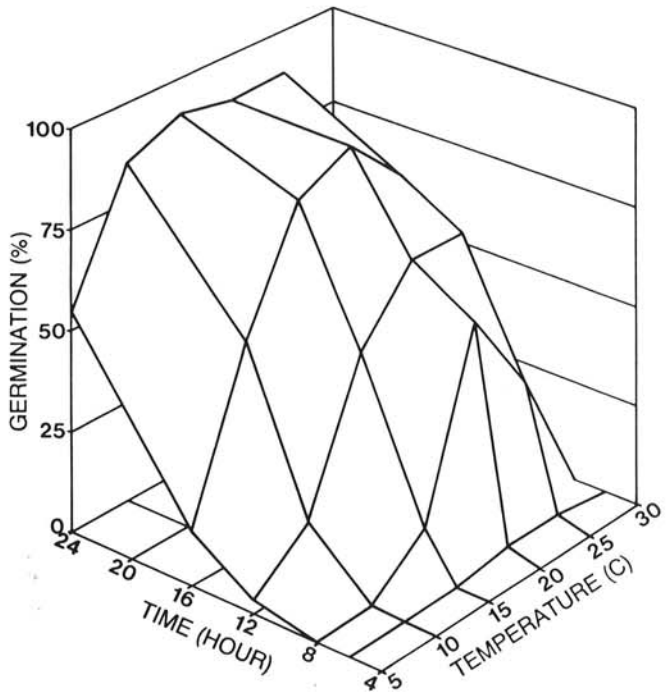


**Fig. 1.** Influence of temperature and incubation period on germination of conidia of *Gloeotinia temulenta* on glass slides.

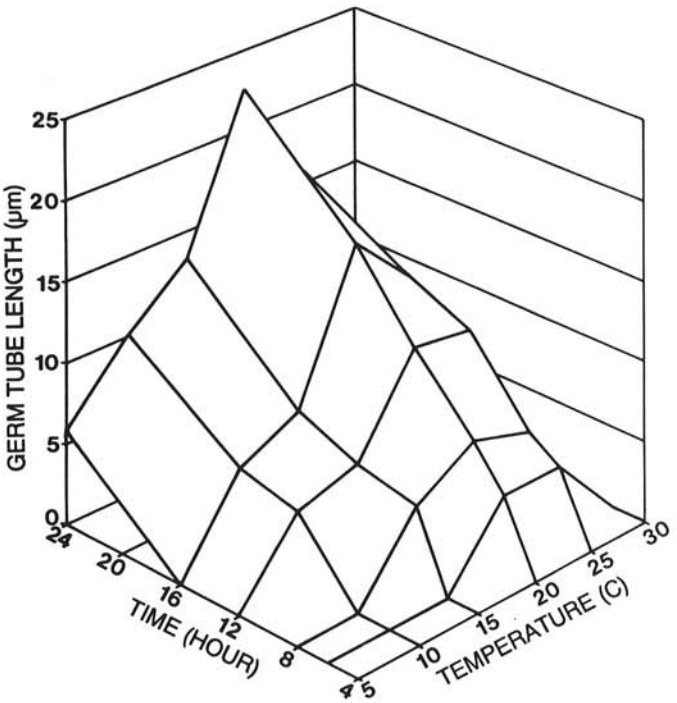


**Fig. 2.** Influence of temperature and incubation period on length of germ tubes of conidia of *Gloeotinia temulenta.*

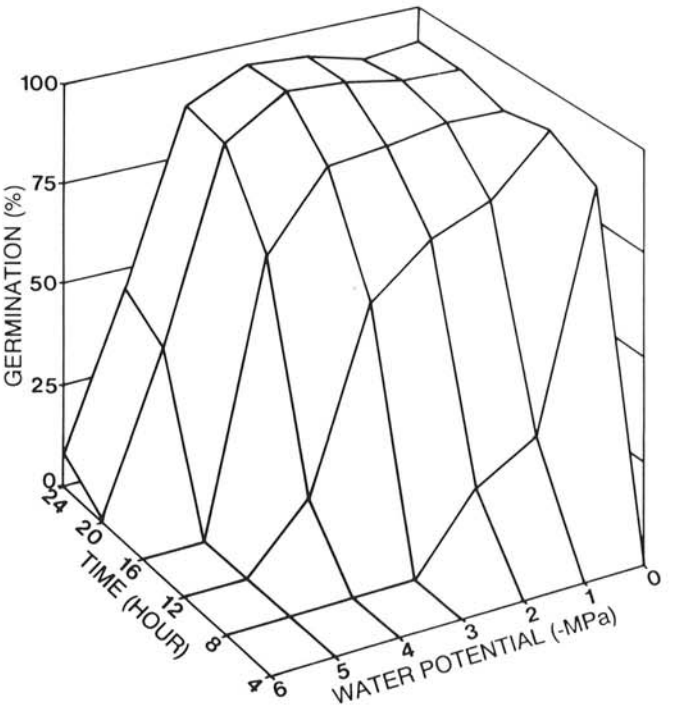


**Fig. 3.** Influence of water potential and incubation period on germination of conidia of *Gloeotinia temulenta* on micropore membranes.

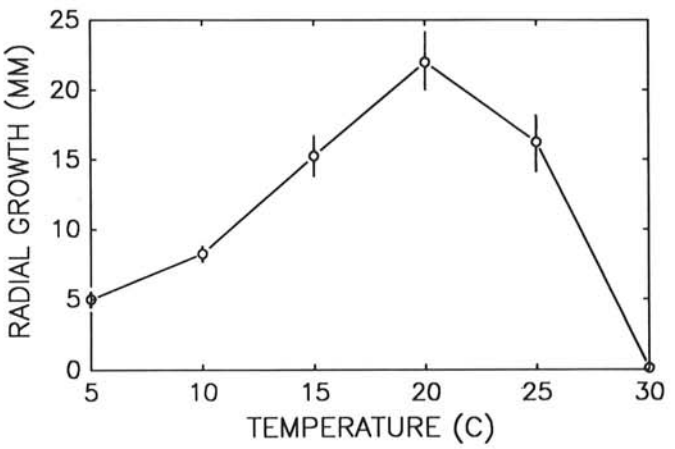


**Fig. 4.** Influence of temperature on radial growth of *Gloeotinia temulenta* after 4 wk on potato-dextrose agar. Bars represent standard deviation.

Inc., Stamford, CT), were ±1.5 C of the treatment temperature. Relative humidity within the chambers, measured with a hygrothermograph, ranged from 50 to 85%. Plants were incubated under a 9 h/day photoperiod with light intensities of 300–700 μE. After 15 days each seed was soaked in distilled water for 30 min, then the water was examined at 250× for conidia of *G. temulenta*.

To determine the influence of moisture on infection, plants were inoculated, incubated in a dew chamber at 20 C for 0, 4, 12, or 24 h, and then transferred to a growth chamber at 20 C. After 15 days, seeds were assessed for conidia of *G. temulenta*. In a separate experiment, a conidia-laden exudate from infected seed was transferred with fine tweezers to ovaries in open flowers of annual ryegrass. Plants were placed in a dew chamber for 0 or 4 h and then transferred to a growth chamber at 20 C. After 15 days, seeds were assessed for conidia of *G. temulenta*.

To determine the number of conidia in relation to days after inoculation, plants were inoculated and placed in a growth chamber at 20 C. Six days after inoculation, the number of conidia on each of 10 seeds from each of two heads from four replicate plants were determined at 2-day intervals until 18 days after inoculation.

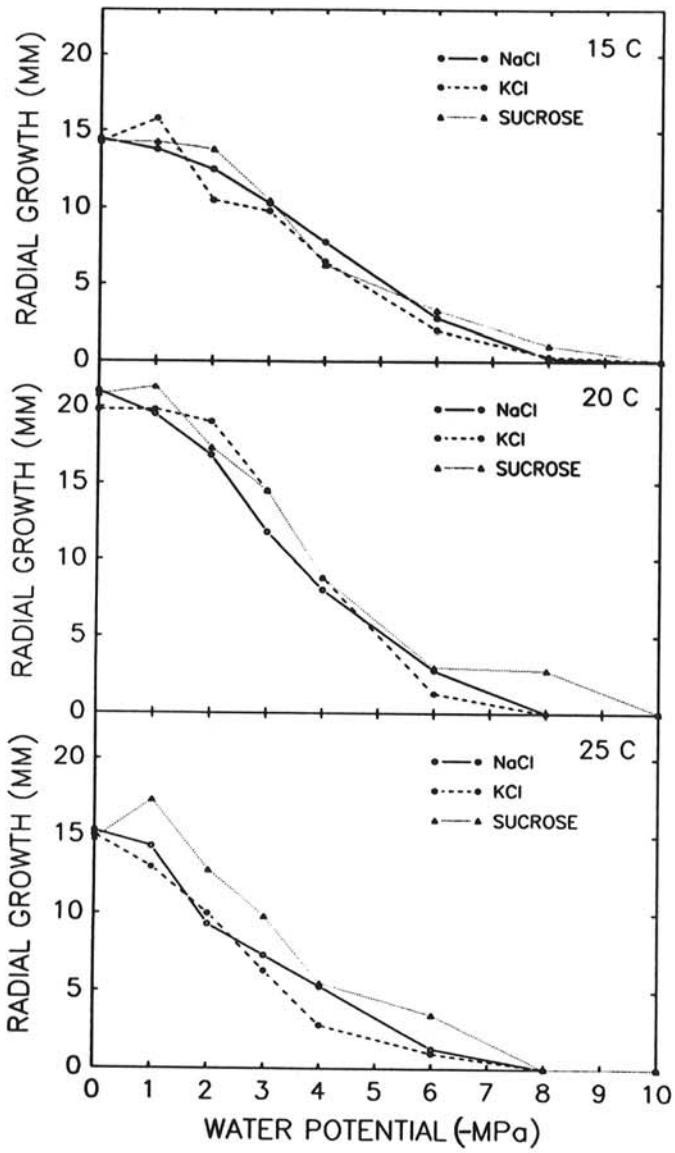


**Fig. 5.** Influence of water potential and temperature on radial growth of *Gloeotinia temulenta* on potato-dextrose agar osmotically adjusted with NaCl, KCl, or sucrose.

**Statistical analysis.** Preliminary analyses indicated that germination or germ tube data from temperature or water potential treatments were described by third-order polynomials. Surface equations were derived using multiple polynomial regression analysis. Third-order multiple polynomials were constructed based on the form $y(x,c) = c_0 + c_1x_1 + c_2x_2 + c_{11}x_1^2 + c_{22}x_2^2 + c_{12}x_1x_2 + c_{112}x_1^2x_2 + c_{122}x_1x_2^2 + c_{111}x_1^3 + c_{222}x_2^3$, where $x$ represents time, temperature, or water potential, and $c$ is the corresponding coefficient for each variable or variable combination. Parameter selection was based on an iterative process to maximize $R^2$ (REG, MAXR procedure of SAS) (11) and on examination of residuals. Models believed to most accurately represent the entire range of data, based on comparison of actual and predicted values, were selected.

Data for mycelial growth under various osmoticum and temperature treatments were subject to polynomial regression analysis. Analyses were conducted with the REG procedure of SAS (11). Model selection was based on significance of the $F$ test, coefficient of determination ($R^2$), and examination of residuals.

## RESULTS

**Germination.** Germination of *G. temulenta* was observed 6 h after inoculation at 20 or 25 C (Fig. 1). At 5 and 10 C, germination was observed after 8 h. Germination increased with increasing time through 24 h and temperature through 20 C and then declined. No germination was observed at 30 C. Germination with respect to time and temperature was described by the multiple polynomial equation $y = -14.647T + 0.804HT + 1.026T^2 - 0.021HT^2 - (6.554 \times 10^{-3})H^2T + (2.36 \times 10^{-3})H^3 - 0.019T^3 + 1.102$, where $T$ = temperature and $H$ = time ($R^2 = 0.93$).

Germ tube length increased with increasing time through 24 h and temperature through 20 C and then declined (Fig. 2). Germ tubes were not observed at 30 C. Germ tube length with respect to time and temperature was described by the multiple polynomial regression equation $y = 0.696H - 3.511T + 0.122HT + 0.258T^2 - (3.937 \times 10^{-3})T^3 + 2.341$, where $T$ = temperature and $H$ = time ($R^2 = 0.93$).

Percent germination of *G. temulenta* declined with decreasing water potential through −6 MPa (Fig. 3). A high percentage of germination (95–100%) occurred by 24 h at water potentials through −4 MPa. Germination with respect to time and water potential was described by the multiple polynomial regression equation $y = 16.295H - 0.446H^2 - 4.588W^2 + 0.029H^2W - 52.31$, where $H$ = time and $W$ = water potential ($R^2 = 0.91$).

**Mycelial growth and production of conidia.** Radial growth of *G. temulenta* increased from 5 to 20 C and then declined. Growth was not observed at 30 C (Fig. 4). On an osmotically adjusted medium at 15, 20, or 25 C, growth declined with decreasing water potential through −9 to −10 MPa (Fig. 5). Trends were similar with NaCl, KCl, and sucrose used as an osmoticum. Data were described by third-order polynomial regression equations (Table 1). Conidia and slime were observed on sucrose-amended media but not on KCl- or NaCl-amended media.

TABLE 1. Polynomial regression equations and associated $R^2$ values relating water potential ($P$, in $-MP_a$) to radial growth ($y$, in mm) of *Gloeotinia temulenta* at 15, 20, or 25 C on potato-dextrose agar amended to various water potentials with sucrose, KCL, or NaCl

| Temperature (C) | Osmoticum | Equation | $R^2$ |
|---|---|---|---|
| 15 | Sucrose | $y = -0.57P^2 + 0.04P^3 + 14.50$ | 0.96 |
| 15 | KCl | $y = -1.14P - 0.36P^2 + 0.03P^3 + 15.16$ | 0.97 |
| 15 | NaCl | $y = -0.57P^2 + 0.04P^3 + 14.32$ | 0.99 |
| 20 | Sucrose | $y = -1.93P - 0.39P^2 + 0.04P^3 + 21.96$ | 0.94 |
| 20 | KCl | $y = -0.95P^2 + 0.08P^3 + 20.54$ | 0.97 |
| 20 | NaCl | $y = -2.43P - 0.34P^2 + 0.04P^3 + 21.62$ | 0.99 |
| 25 | Sucrose | $y = -1.20P - 0.39P^2 + 0.03P^3 + 16.24$ | 0.94 |
| 25 | KCl | $y = -3.87P + 0.23P^2 + 15.83$ | 0.97 |
| 25 | NaCl | $y = -2.85P + 0.01P^3$ | 0.98 |

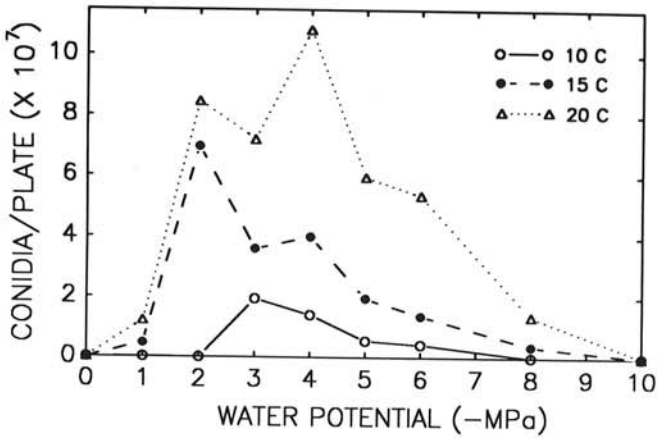


**Fig. 6.** Influence of water potential on production of conidia of *Gloeotinia temulenta* on sucrose-amended potato-dextrose agar.

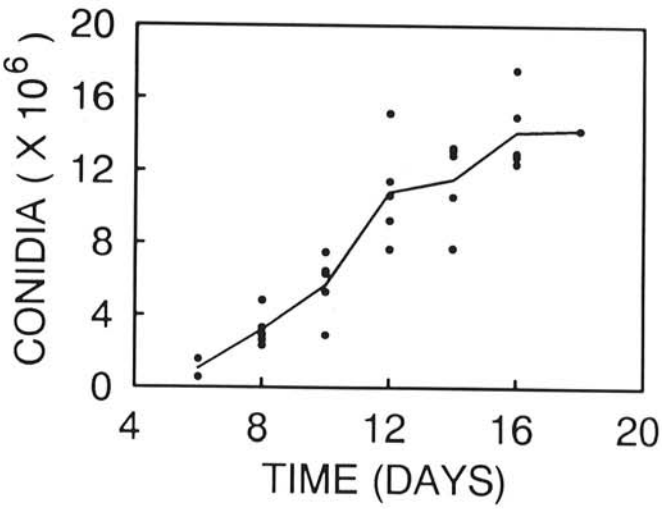


**Fig. 7.** Numbers of conidia of *Gloeotinia temulenta* produced on developing annual ryegrass seed 6 to 18 days after inoculation. Points represent means from five experimental runs. Each run included four replications (20 seeds per replication). Two runs included 6 days and one run included 18 days.

On sucrose-amended medium, conidia were abundant between −2 and −8 MPa (Fig. 6). Numbers of conidia increased with increasing temperature from 10 to 20 C.

**Infection.** Mean percentage and standard deviation of infections at 15, 20, 25, and 30 C were 85 ± 13, 91 ± 11, 81 ± 9, and 0 ± 0, respectively. Mean percentage and standard deviation of infections after dew periods of 0, 4, 12, or 24 h were 66 ± 26, 83 ± 3, 84 ± 9, and 76 ± 13, respectively. Mean percentage and standard deviation of infected seeds when a conidia-laden exudate was applied to ovaries were 94 ± 6 and 94 ± 10 under 0- and 4-h dew periods, respectively.

The number of conidia on annual ryegrass increased 6–16 days after inoculation (Fig. 7). Numbers of conidia collected 12–18 days after inoculation ranged from $7.7 \times 10^6$ to $1.8 \times 10^7$.

## DISCUSSION

The geographical distribution of *G. temulenta* includes areas of temperate grass seed production, e.g., England, Ireland, and New Zealand (3,7). Growth inhibition of *G. temulenta* at 30 C suggests that blind seed is not likely to occur in warmer climates. *G. temulenta* is favored in the Willamette Valley, where average temperature and rainfall for May (101-yr average at the Hyslop Field Station, Corvallis, OR, through 1990), when plants are flowering, are 12 C and 5 cm, respectively. Average temperature and rainfall conditions in June, during seed fill, are 17 C and 4.7 cm, respectively.

Wright and Wilson (13) observed that germination of conidia on agar increased through 72 h but was dependent on the agar medium used. Wright and Wilson reported 72 and 8% germination of conidia from potato starch-dextrose-peptone agar and malt agar, respectively, after 18 h at room temperature. I also observed inconsistent germination of conidia from culture media (*unpublished*). However, conidia from infected seed (10–15 days after inoculation) germinated readily and levels of germination greater than 90% were consistently observed. This suggests that conidia of *G. temulenta* produced on annual ryegrass may provide a more reliable source of inoculum than that produced on a synthetic substrate.

Conidial production on PDA was enhanced in the presence of sucrose. Wilson et al (12) determined that if infection occurred at the time starch was deposited in developing seeds, true blind seeds developed. In these infected seeds the fungus overwintered and produced apothecia. If infections occurred earlier in seed development, death of the ovary resulted in limited mycelial development and no apothecia. The influence of sugars, starch, or seed maturity in supporting the conidial or apothecial stage of *G. temulenta* needs to be determined.

*G. temulenta* colonizes the endosperm of ryegrass as the seed fills (12), resulting in seeds with a shrunken appearance. In some cases infected seed may appear healthy. It is not known if temperature would differentially affect the rate of seed fill and endosperm colonization. Cool temperatures during flowering may delay seed development and provide a longer window for infection. Cool, rainy conditions have been cited as most favorable for blind seed development (3,6,12). However, the role of temperature and moisture in regulating infection of annual ryegrass or production of conidia of *G. temulenta* under field conditions needs to be quantitatively defined.

The conidial slime of *G. temulenta* is water soluble, which suggests that rain splash may be the primary means of conidial dispersal. Large numbers of conidia were produced in infected seeds and production occurred over a 6- to 12-day period. Under rainy conditions, considerable inoculum could be distributed within a field.

Although rainfall may be important for dissemination of conidia, a subsequent period of free water may not be required for infection. A dew period was not required for infection when a drop of conidial suspension was placed in open flowers of annual ryegrass or when a conidia-laden exudate from infected seed was transferred to ovaries in open flowers. Moisture conditions at the ovary or stigma surface, which support germination of pollen, also may support germination of *G. temulenta.* Additional studies are needed to understand conditions at the infection site and mechanisms of infection of annual ryegrass by *G. temulenta.*

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