Etiology

The Effect of Free Water on the Potential Germinability of Cold-Dormant Urediniospores of *Puccinia graminis* f. sp. *tritici*

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ABSTRACT

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Germination of fresh urediniospores of *Puccinia graminis* f. sp. *tritici*, race 56, always was greatly reduced after storage at −196 C ("cold-dormant" spores). Immersing cold-dormant spores in water or buffer solution for 1–2 h at room temperature caused irreversible injury to a majority of the population but, contrary to published reports, up to 32% of the spores survived as judged by subsequent germination tests on water agar. When cold-dormant spores were heat-shocked at 40 C for 5 min before incubation on water agar at 18 C, their germinability was comparable to that of fresh nonfrozen spores of the same lot. Following liquid immersion of cold-dormant spores, subsequent heat-shocking did not reverse dormancy; if heat-shocked in liquid, further injury to spores occurred, but drying spores before heat-shock provided protection against further damage. Both freshly collected and cold-dormant spores, regardless of preincubation treatments, germinated at higher levels on water agar than in water or buffer medium during 2-h incubation at 18 C in the dark.

Spores of many fungi, including urediniospores of several species that cause rust in cereals, exhibit low germinability after chilling to temperatures below freezing (2,4,5). Fresh urediniospores of *Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn. chilled to temperatures from −1.1 to −196 C could be restored to germination levels as high as nonchilled control spores by either a heat-shock for 5 min at 40 C or by vapor-phase hydration for 16–24 h at 20 C (2). It now is standard practice in many laboratories to store *P. g. tritici* spores in liquid nitrogen refrigerators. When required for use in studies after removal from the liquid nitrogen refrigerator, the spores are given a heat-shock treatment to restore germinability. It has been reported that if cold-dormant urediniospores are suspended in a liquid medium before being heat-shocked, irreversible damage is inflicted on the spores (6,11). These workers suggested that the damage was osmotic in nature and resulted in death to all cold-dormant spores thus treated (6,11). Preliminary work in our laboratory, however, indicated that not all cold-dormant urediniospores of *P. g. tritici* contacting water before a heat shock are damaged. In some cases an appreciable portion of the spores survive and are capable of germinating. As a result of these inconsistencies, further work was undertaken in our laboratory to examine critically the effects of moisture and temperature. The early studies on cold dormancy (2,4,5) had determined spore viability only on agar medium; the later studies reporting the lethal effect of liquid immersion on cold-dormant spores (6) used primarily liquid media although agar was used in a few tests. The present studies compared germinative capabilities of both fresh and cold-dormant heat-shocked and non-heat-shocked spores on liquid and agar media. The effects of various dehydration and heat-shocking treatments subsequent to liquid immersion of cold-dormant spores were also investigated. The data obtained could modify our present understanding of the extent to which eventual germination of cold-dormant spores is affected by free water, a factor of significance to urediniospore survival through the winter and hence epidemiology of stem rust of wheat in temperate regions.

MATERIALS AND METHODS

Spores. Urediniospores of *P. g. tritici* race 56 were produced on Baart wheat in the glasshouse. Moisture content of spore lots harvested varied from 14 to 20%. "Fresh" spores used in experiments were harvested from infected plants with a cyclone collector (3) and hydrated for 18–24 h in a water-saturated atmosphere at 21–23 C. "Cold-dormant" spores were aliquots from a stock of urediniospores produced as a single lot in the glasshouse and stored for 3–12 mo in a liquid nitrogen refrigerator at −196 C. Before use in the specific experiments described below, some cold-dormant spores were heat-shocked by suspending them enclosed in a watertight vessel in a water bath at 40 ± 0.5 C for 5 min.

Fresh, cold-dormant heat-shocked, and cold-dormant non-heat-shocked spores were seeded in a settling tower directly onto 1% water agar, into distilled H_2O containing 0.01% Tween 20 (v/v), or into a mixed calcium and potassium phosphate buffer solution at pH 7.0 (7). Glass-distilled deionized water was used in the preparation of all media. Preliminary studies had shown that the addition of Tween 20 to water prevented clumping of spores, thus providing a more uniform suspension; also, germination was shown to be unaffected by this wetting agent at the concentration used. The spores seeded directly onto agar were immediately incubated in the dark for 2 h at 18 C, then placed in a formaldehyde-saturated atmosphere to halt biological activity. The treatment of the spores in liquid media is described below for each of three experiments.

Experiment 1. Fresh, heat-shocked, and non-heat-shocked spores were each treated as shown in Table 1. Two concentrations of spores in H_2O were used: the low concentration was 0.2 mg of spores per ml H_2O and the high concentration was 5 mg of spores per ml H_2O. The suspensions were shaken in 50-ml Erlenmeyer flasks (30 ml of suspension/flask) on a rotary shaker at 125 rpm under diffuse light at room temperature for 2 h. The spores were removed from the H_2O by pipette and placed onto water agar or into fresh H_2O contained in plastic 35- × 9-mm petri dishes. Spores on agar of some plates were killed immediately with formaldehyde, and the remaining spores in other plates on agar and in H_2O incubated for two additional h before killing. The killed spores from each treatment (three replicate plates) were examined microscopically at 100× to determine germination percentages. A spore was considered germinated if the germ tube was equal to or longer than the minor diameter of the parent spore. Four hundred or more spores were examined in each of the three subsample plates (1,200 or more spores per treatment); if "zero" germination was found, then the total number of spores observed in that treatment was increased to 6,000 to verify that no germination had occurred. This experiment was conducted three times.

Experiment 2. Only cold-dormant spores were used in this study (Table 2). Heat-shocked and non-heat-shocked spores were placed directly onto water agar, into the buffer medium (1 mg of spores per ml medium), or into H_2O (1 mg of spores per ml H_2O). Spores in liquid medium were shaken as described for experiment 1, except that the shaking time was 1 h. After shaking, spores were placed onto water agar, into fresh H_2O, or into fresh buffer medium. In addition, non-heat-shocked spores from the H_2O- and buffer-shake treatments were heat-shocked either in their respective liquids (wet) or dried by filtration and then heat-shocked. Following the heat-shock, the spores were further incubated for 2 h on water agar, H_2O, or buffer medium. Sampling, killing of spores with formaldehyde, and determination of germination percentages were performed as described in experiment 1. This experiment was conducted two times.

Experiment 3. Heat-shocked and non-heat-shocked spores were seeded directly onto water agar or into H_2O; other spores were placed into H_2O and shaken for 1 h (Table 3). After shaking, heat-shocked spores were placed onto water agar or into H_2O. After shaking, non-heat-shocked spores were collected by filtration and divided into three groups. One group of spores was deposited directly onto agar, into H_2O, or into H_2O followed by heat-shock. The second group was dried under vacuum for 30 min, and the third group was dried for 60 min. Following the drying, spores were placed directly onto agar or into H_2O, or heat-shocked for 5 or 3 min and then placed onto water agar or into H_2O. Sampling, killing of spores, and determination of germination percentages were as described in experiment 1. This experiment was conducted two times.

Data analyses. Raw data (percentages) were coded by adding "1" to each value to eliminate zero values then transformed to arcsine. Each experiment was analyzed separately. Transformed data were used to perform an analysis of variance and Tukey's multiple range test ($P = 0.05$). Nontransformed data are shown in the tables for comparison of treatment means. Each experiment was repeated two or more times, yielding similar results in each case.

RESULTS

Experiment 1. Germination data (Table 1) indicated that 2-h shaking in water significantly increased germinability of fresh and heat-shocked spores, compared with their germinabilities when incubated directly on agar. The high concentration of cold-dormant spores germinated better (25%) after shaking in H_2O before incubation on agar than did those incubated directly on agar (14.8%). Fresh and heat-shocked spores, shaken at low concentration in H_2O, germinated at levels of 5.1 and 9.8%, respectively, when incubated an additional 2 h in H_2O; this

TABLE 1. Germination percentages of fresh and cold-dormant urediniospores of *Puccinia graminis* f. sp. *tritici* incubated on water agar or in water after preincubation treatments

Treatment number	Spore source	Heat shock	Preincubation treatment	Incubation medium	Germination (%)[a]
1	Fresh	No	Hydrated 18–24 h	Water agar	72.1 e
2			5 mg/ml, shake 2 h in H_2O	Water agar	94.7 f
3			5 mg/ml, shake 2 h in H_2O	Water	0.3 a
4			0.2 mg/ml, shake 2 h in H_2O	Water agar	93.4 f
5			0.2 mg/ml, shake 2 h in H_2O	Water	5.1 b
6	Cold-dormant	Yes	None	Water agar	81.2 e
7			5 mg/ml, shake 2 h in H_2O	Water agar	96.3 f
8			5 mg/ml, shake 2 h in H_2O	Water	0.0 a
9			0.2 mg/ml, shake 2 h in H_2O	Water agar	93.3 f
10			0.2 mg/ml, shake 2 h in H_2O	Water	9.8 bc
11		No	None	Water agar	14.8 cd
12			5 mg/ml, shake 2 h in H_2O	Water agar	25.1 d
13			5 mg/ml, shake 2 h in H_2O	Water	0.0 a
14			0.2 mg/ml, shake 2 h in H_2O	Water agar	7.9 bc
15			0.2 mg/ml, shake 2 h in H_2O	Water	0.0 a

[a] Means of three replicates; means followed by the same lowercase letter are not significantly different ($P = 0.05$) according to Tukey's multiple range test.

difference was not significant. A few of the low-concentration spores (4%) had germinated by the time they were removed from the shaker and killed.

TABLE 2. Germination percentages of cold-dormant urediniospores of *Puccinia graminis* f. sp. *tritici* incubated on water agar, in water, or in buffer after preincubation treatments

Treatment number	Heat shock	Preincubation treatment	Incubation medium	Germination (%)[a]
1	Yes	None	Water agar	84.2 h
2		None	Water	37.0 fg
3		None	Buffer	47.0 g
4		Shake 1 h in H_2O	Water agar	84.4 h
5		Shake 1 h in H_2O	Water	13.0 e
6		Shake 1 h in H_2O	Buffer	27.0 f
7		Shake 1 h in buffer	Water agar	81.1 h
8		Shake 1 h in buffer	Water	35.1 f
9		Shake 1 h in buffer	Buffer	31.7 f
10	No	None	Water agar	6.3 c–e
11		None	Water	0.7 a–c
12		None	Buffer	2.3 a–d
13		Shake 1 h in H_2O	Water agar	32.0 f
14		Shake 1 h in H_2O	Water	2.7 a–d
15		Shake 1 h in H_2O	Buffer	4.1 b–d
16		Shake 1 h in H_2O, HS[b] wet	Water agar	0.0 a
17		Shake 1 h in H_2O, HS[b] wet	Water	0.0 a
18		Shake 1 h in H_2O, HS[b] wet	Buffer	0.0 a
19		Shake 1 h in H_2O, dry, HS	Water agar	32.0 f
20		Shake 1 h in H_2O, dry, HS	Water	4.3 c–e
21	No	Shake 1 h in H_2O, dry, HS	Buffer	4.9 c–e
22		Shake 1 h in buffer	Water agar	29.9 f
23		Shake 1 h in buffer	Water	11.0 de
24		Shake 1 h in buffer	Buffer	3.3 a–d
25		Shake 1 h in buffer, HS wet	Water agar	1.3 a–c
26		Shake 1 h in buffer, HS wet	Water	2.2 a–d
27		Shake 1 h in buffer, HS wet	Buffer	0.9 a–c
28		Shake 1 h in buffer, dry, HS	Water agar	29.9 f
29		Shake 1 h in buffer, dry, HS	Water	0.6 a–c
30		Shake 1 h in buffer, dry, HS	Buffer	1.9 a–d

[a] Means of three replicates; means followed by the same lowercase letter are not significantly different ($P = 0.05$) according to Tukey's multiple range test.
[b] HS indicates heat-shock.

Fresh spores (high concentration) germinated very poorly (0.3%) during a 2-h incubation in H_2O after the shaking treatment. Cold-dormant spores (high concentration), both heat-shocked and non-heat-shocked, did not germinate during a 2-h incubation in H_2O after a 2-h shaking in H_2O.

Experiment 2. Results where only cold-dormant spores were used are shown in Table 2. For spores initially receiving a heat-shock, there were no significant differences in the germination percentages whether spores were placed directly onto water agar and incubated 2 h (84.2%), or placed in buffer medium or H_2O and shaken for 1 h before being placed on water agar (81.1 and 84.4%, respectively). Compared with germination on agar, germinability was significantly reduced when spores were incubated in H_2O or buffer medium after shaking for 1 h in either H_2O or buffer medium. Less than 0.5% of spores germinated during the 1-h period on the shaker.

Non-heat-shocked spores germinated at 6% when placed directly onto agar, 0.7% when placed directly into H_2O, and 2.3% when incubated directly in buffer; these differences were not significant. Shaking non-heat-shocked spores in H_2O or in the buffer medium for 1 h before placing the spores on water agar significantly increased the germination percentages. Non-heat-shocked spores shaken in H_2O or in buffer medium for 1 h and then transferred to water agar had 32 and 29.9% germination, respectively (not significantly different). Germination was significantly lower if spores were shaken in either liquid and then placed in H_2O or buffer medium to test germination.

When non-heat-shocked spores were shaken in H_2O or buffer medium for 1 h and then heat-shocked while still in their respective liquids ("wet"), germination was low no matter how the spores were further treated. If, however, non-heat-shocked spores were shaken in H_2O or buffer medium, removed from the liquids and heat-shocked dry before incubation on agar, the germination percentages were similar to those of non-heat-shocked spores incubated directly on agar after the initial shaking in the liquids.

Experiment 3. Non-heat-shocked spores when placed, without shaking, in H_2O for 2 h had a 1.7% germination rate and when placed directly on water agar had a 4.2% germination rate, a nonsignificant difference (Table 3). Shaking non-heat-shocked spores in H_2O for 1 h, then filtering the spores from the H_2O suspension and incubating these spores on water agar, resulted in an increase in germination to 7.3%. Drying the spores under vacuum for 30 or 60 min and placing them into H_2O or onto

TABLE 3. Germination percentages of cold-dormant urediniospores of *Puccinia graminis* f. sp. *tritici* incubated on water agar or in water after preincubation treatments

Treatment number	Heat shock	Preincubation treatment	Incubation medium	Germination (%)[a]
1	Yes	None	Water agar	56.4 g
2		None	Water	13.9 e
3		Shake 1 h in H_2O	Water agar	58.0 g
4		Shake 1 h in H_2O	Water	25.9 f
5	No	None	Water agar	4.2 b–d
6		None	Water	1.7 a–c
7		Shake 1 h in H_2O	Water agar	7.3 de
8		Shake 1 h in H_2O	Water	4.2 b–d
9		Shake 1 h in H_2O, HS[b] 5 min wet	Water	0.0 a
10		Shake 1 h in H_2O, dry 30 min	Water agar	4.8 cd
11		Shake 1 h in H_2O, dry 30 min	Water	1.6 a–c
12		Shake 1 h in H_2O, dry 30 min, HS 5 min	Water agar	1.3 a–c
13		Shake 1 h in H_2O, dry 30 min, HS 5 min	Water	1.1 ab
14		Shake 1 h in H_2O, dry 30 min, HS 3 min	Water agar	2.4 a–c
15		Shake 1 h in H_2O, dry 30 min, HS 3 min	Water	0.2 a
16		Shake 1 h in H_2O, dry 60 min	Water agar	1.5 a–c
17		Shake 1 h in H_2O, dry 60 min	Water	0.9 a–c
18		Shake 1 h in H_2O, dry 60 min, HS 5 min	Water agar	2.4 a–c
19		Shake 1 h in H_2O, dry 60 min, HS 5 min	Water	0.1 a
20		Shake 1 h in H_2O, dry 60 min, HS 3 min	Water agar	1.8 a–c
21		Shake 1 h in H_2O, dry 60 min, HS 3 min	Water	0.6 ab

[a] Means of three replicates; means followed by the same lowercase letter are not significantly different ($P = 0.05$) according to Tukey's multiple range test.
[b] HS indicates heat-shock.

water agar, or drying for 30 or 60 min followed by either a 3- or 5-min heat-shock at 40 C did not improve the germination. The best germination of non-heat-shocked spores was with spores shaken in H_2O for 1 h and then placed on water agar. As in experiments 1 and 2, all cold-dormant heat-shocked spores germinated at significantly higher levels than non-heat-shocked spores, regardless of pretreatments, when incubation was on water agar.

In all three experiments, the treatment resulting in the highest percentage of spore germination was the 1- or 2-h shaking of heat-shocked spores in H_2O followed by incubation on water agar (96.3, 84.4, and 58.0% for experiments 1, 2, and 3, respectively).

DISCUSSION

In each of our studies, it was clearly demonstrated that not all cold-dormant spores were "irreversibly damaged" when immersed in liquid water or buffer solution for 1 or 2 h without prior heat-shock or vapor-phase hydration; 6–32% of such spores subsequently germinated on water agar and 2.7–4.2% germinated in H_2O. This finding is not in agreement with the results of earlier workers (6,11) using the same organism; according to their published data, they never observed any germination with cold-dormant spores once the spores had contacted liquid media, regardless of how the spores were subsequently treated. Our findings do agree with results of a study with *P. striiformis* (9) in which cold-dormant urediniospores did germinate in H_2O (although at very low percentages) without prior heat-shock or hydration.

After cold-dormant spores were immersed in liquid, a subsequent heat-shock with the spores suspended in liquid resulted in zero or extremely low germination, regardless of whether final incubation was on agar or in H_2O or buffer medium. This corroborates earlier reported results (11). If, however, the spores were collected from the immersion liquid on filter paper and dried for various lengths of time before heat-shock, up to 32% germination occurred upon subsequent incubation on water agar (Tables 2 and 3). This level of germination was not better than that obtained with spores placed directly on agar from the immersion liquids without heat-shock. Apparently, drying the spores after liquid immersion protects them from further damage during subsequent heat-shock, compared with spores heat-shocked while suspended in liquid, but does not reverse the cold-dormant state (compare in Table 2 the cold-dormant heat-shocked spores incubated directly on agar that germinated at 84.2% with the 32% germination of the non-heat-shocked spores incubated on agar after a 1-h immersion in H_2O, drying, and heat-shocking).

In the present studies, when buffer medium was used in place of H_2O in the various treatments, slightly better germination resulted in most but not all comparisons (Table 2).

Where the two concentrations of spores were compared, the higher concentration resulted in lower germination (0% in two instances) than did the lower concentration when final incubation was in H_2O. Perhaps carryover of more self-inhibitor (1) of germination occurred with spores from the higher concentration.

Fresh spores that germinated at 72% when incubated directly on water agar (Fig. 1) germinated at 5% or less when incubated in H_2O for 2 h after a 2-h shaking in H_2O. But when spores from the 2-h shaking were incubated on water agar, their germinability was actually higher than that observed on direct agar incubation. In this instance, the poor germination in H_2O was not caused by injury associated with previous chilling of spores. Insufficient oxygen may be partially responsible for the discrepancy; however, less than 1% of the fresh spores germinated after 2 h of shaking in H_20 (where oxygen should be less limiting) whereas fresh spores on agar for 2 h had reached a germination level of 72%. Cold-dormant heat-shocked spores behaved very much like fresh spores when subjected to the same treatments, as noted in previous studies (2,4–6).

It is clear from the present study and the literature (2,8,10,12,13) that considerable variation in germinative capacities exists among different spore lots. In the data reported here, 14.8, 6.3, and 4.2% of the cold-dormant non-heat-shocked spores germinated when incubated directly on water agar (as high as 65% has been obtained in studies in our laboratory). Obviously, within each spore lot ("population") there is a group of individuals that either does not become "cold-dormant" during storage at −196 C or has the innate capacity to germinate from the cold-dormant state at room temperature without prior heat-shock or vapor-phase hydration. It would be interesting to learn why these spores behave differently.

No obvious ultrastructural differences were found among fresh, cold-dormant, and heat-activated spores before each was immersed in liquid medium (11). Possibly injurious changes were noted only with cold-dormant spores beginning by 5 min after putting them in liquid medium; severe disruption of lipid inclusions and disorganization of the cristae of the mitochondria were among the changes observed. Rehydration in liquid medium resulted in greatly increased leakage of metabolites from cold-dormant spores, compared with the leakage associated with either fresh or with cold-dormant spores that had been heat-shocked before immersion in liquid (7). Physical changes in the lipoprotein of the cytoplasmic membranes were postulated to explain the extent and generalized nature of the leakage from cold-dormant spores in liquid. Based on the leakage observations, respiration measurements, and studies of incorporation of isotopic carbon, it was concluded that permeability changes are the primary cause of damage to cold-dormant spores (when immersed in liquid medium) and are followed by metabolic injury (6).

There is no question that a large proportion of the potentially viable urediniospores in most lots of cold-dormant spores are rendered incapable of germination by immersion in water. The intriguing question is why some of the spores in such cold-dormant spore lots are not irreversibly damaged by liquid immersion. What is different about these spores that do not behave as cold-dormant spores even after prolonged storage at −196 C? There must be a fundamental difference between the "injury prone" and the "injury resistant" segments of each cold-dormant spore population. There are undoubtedly variations in maturity among urediniospores in any collected spore lot; it is theoretically conceivable that spores at different levels of "physiological maturity" might respond differently to freezing and subsequent liquid immersion.

Reversible cold-dormancy is not confined to *P. g. tritici;* it has been found in urediniospores of other species of *Puccinia* (2). How possession of this characteristic might increase the probability of survival of an organism is unclear. *P. g. tritici* has a hardy survival mechanism in the teleutospore. In the absence of the alternate host over extensive areas, however, the teleutospore leads to a biological dead-end, and survival dependence would shift to the urediniospore. Urediniospores are not normally considered long-term survival propagules, their longevity is thought of in terms of days or weeks. There is ample evidence, however, that freezing does not harm urediniospores, hence cold per se would not prevent carryover of urediniospores as viable inoculum from fall to spring in the temperate zone (in our laboratory we have obtained 60% and higher germination with spores stored at −196 C for 12 yr). Alternating periods of freezing and thawing of dry urediniospores of *P. g. tritici,* up to 15 cold-warm cycles, did not adversely affect germinability, nor did the rates of cooling and thawing (2,4). We are aware of no quantitative data on the effects of alternating periods of wetting and drying in conjunction with simulated winter-spring temperature fluctuations. If, as our studies indicated, some fraction of the cold-dormant population after being thawed to room temperature (even if wetted) was capable of germination, then the possibility of early infection from overwintering spores must be seriously considered.

Although immersion of spores in liquid media has been studied, actually it is difficult to suspend urediniospores in water. Usually a wetting agent and agitation are necessary. In nature, urediniospores may experience something closer to hydration rather than total immersion in liquid droplets. If so, then reversal of cold-dormancy may be the rule in urediniospores in spring and early

summer at air temperatures normally encountered.

An understanding of the combination of effects of temperature and water on germinability of urediniospores and the physiological reasons for these effects could have implications allowing a better understanding of the potential for urediniospores of *P. g. tritici* and similar spores to survive in temperate climates. The data presented here suggest that under certain conditions a few urediniospores might survive to cause early infection. Early stem rust establishment could lead to greater disease loss than generally expected.

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