Effects of Cations on Germination of Urediniospores of *Uromyces phaseoli*

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ABSTRACT

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Urediniospores of *Uromyces phaseoli* races 39 and 40 germinated better in unpurified tap water than in ultrapure laboratory water. Experiments with freeze-dried residue of tap water dissolved in ultrapure water suggested that the tap water contained a component that stimulated spore germination. When ions were removed from the tap water with ion-exchange resins, only cation-exchange resins decreased spore germination. Analysis of the tap water demonstrated that the major inorganic cations present were Ca^{2+}, K^+, Mg^{2+}, and Na^+. Spores of both races were incubated for 3–4 hr with solutions containing varying amounts of these cations. Germination was measured with a microscope to view 100 spores for each of five replicates. Calcium, at concentrations between 0.1 and 3 mM, was found to have a stimulatory effect proportional to the amount of cation present. Concentrations of calcium greater than 3 mM had little or no additional effect on germination. Spore germination in the presence of Ca^{2+} was comparable to that of tap water. Magnesium had a lesser effect on spores with statistically significant increases in germination requiring higher cation concentrations of about 1 mM. Maximum germination in the presence of Mg^{2+} was about 30% that of calcium. The monovalent cations tested at concentrations of 0.1 to 8 mM had little stimulatory effect on germination.

Germination is a critical stage in the life cycle of a fungus whereby the spore shifts from a low metabolic resting state to an active growth phase. This transformation is an intricate process requiring that numerous conditions be met. These include environmental and nutritional factors (8,11), developmental factors such as spore maturation and spore age (8,11), and chemical factors involving substances inhibitory or stimulatory to germination (5,9).

Ongoing studies in this laboratory (2,3) are examining the mechanism by which a component of *Bacillus subtilis* (Ehrenberg) Cohn controls bean rust caused by *Uromyces phaseoli* (Pers.) Wint. Urediniospore germination is inhibited on bean leaves treated with the bacterial component. While studying this process in vitro, we found we could not achieve good germination of urediniospores unless tap water was used as a germination fluid. Although there are reports (6,9) regarding germination of urediniospores of *U. phaseoli* in distilled water, we were unable to achieve satisfactory germination without adding certain cations. Although it has been reported that various cations have inhibitory or developmental effects on germination, this is the first report of which we are aware that demonstrates the stimulatory effect that certain cations have on spore germination. A preliminary report was published (1).

MATERIALS AND METHODS

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), potassium chloride (KCl), and sodium chloride (NaCl) were obtained from Baker Chemical Co., Phillipsburg, NJ. Calcium chloride ($CaCl_2$) and Tris were obtained from Sigma Chemical Co., St. Louis, MO. All chemicals were reagent grade. The ultrapurified water (Type I reagent grade) used routinely in this study was treated by reverse osmosis and deionization with a system from Hydro Water Services, Rockville, MD. Tap water was obtained from the hot-water tap and cooled to room temperature.

Production and storage of urediniospores. Urediniospores of races 39 and 40 were maintained on bean cultivar Lake Shasta and Pinto 111, respectively. Three plants were grown per 10-cm pot under greenhouse conditions at 78 ± 8 C. Seedlings 5–7 days old were sprayed with an aqueous 0.01% Tween 20 solution containing about 20,000 spores per milliliter. The seedlings were placed in the dark in a dew chamber for 16 hr at 18 C and then returned to the greenhouse. Spores were collected from young uredinia 7–10 days after inoculation and either used immediately or stored in a freezer at −20 C.

Ion-exchange treatment of water. Tap water was subjected to cation and anion exchange using Bio-Rad 50W-X8 (H^+ form, 100–200 mesh) and AG 1-X8 (OH^- form, 100–200 mesh) resins, respectively (Bio-Rad, Richmond, CA). Glass pasteur pipets fitted with glass wool near the tip were half-filled with resin, about 2 ml, and flushed with distilled water. Twelve milliliters of tap water was added to the columns, and the last 10 ml was collected and assayed for its ability to support germination.

Spore germination bioassay. Two methods were used to determine spore germination. A subjective rating was used in all preliminary experiments. As previously described (3), *U. phaseoli* urediniospores were washed free of their self inhibitor by washing three times for 10 min in ultrapurified water. Washed spores were transferred to microtiter plate wells containing 350 μl of the test solution. Plates were covered, wrapped with plastic, and placed in a dew chamber at 20 C and 99% humidity without light overnight. All treatments were duplicated, and controls for these experiments usually consisted of tap water and ultrapurified water. The rating system used for this assay was subjective: 0 = no germination, 1 = occasional germ tube, 2 = 10% germination, 4 = about 25% germination, 6 = about 50% germination, 8 = about 75% germination, and 10 = >90% germination. A rating of less than 4 indicated poor germination.

A more quantitative bioassay was used to determine the effects of various cations on spore germination. Inorganic salts to be

tested were made in 1 mM Tris-HCl, pH 7.0, at concentrations ranging from 0.05 to 10 mM with ultrapurified water. Controls consisted of tap water, Tris buffer, and water. Spores, prepared in the same manner as above, were transferred to microtiter plate wells containing 350 μl of the test solution. Plates were wrapped with aluminum foil and incubated at 22 C without light for 3–4 hr. At least 100 spores from each well were then counted by viewing through a light microscope at 100×. Spores were counted as germinated if a germ tube was visible.

Experiments with each inorganic salt were done at least twice on each urediniospore race, and each concentration had five replicates. Data were analyzed using version 5 of the Statistical Analysis System (SAS). The data were transformed with the arc sine square root of percent germination. Analyses were performed with both raw and transformed data, and the logarithm base 2 ($\log_2$) of the ion concentration. The Ca^{2+} data seemed to approximate a negative exponential function, and the Mn^{2+} data seemed to approximate an exponential function. We fit these data to the appropriate function using the NLIN procedure of SAS.

Analysis of test solutions. Analyses of tap water and ultrapurified water as well as samples of the inorganic stock solutions were performed with a Spectra Span 3B argon plasma emission spectrophotometer (Spectrametrics, Inc., Andover MA). Samples of tap water were collected on several days to check for significant fluctuations in its composition.

RESULTS

In preliminary experiments, germination of urediniospores of race 40 incubated overnight on ultrapurified water was <10% compared with >90% germination on tap water. To determine if a germination inhibitor might have been picked up during processing of the ultrapurified water, samples of tap water were freeze-dried and the residue suspended with equal volumes of ultrapurified water. Spores germinated well with a germination rating of 8 (Table 1). Spores were incubated on tap water that had been passed through ion-exchange resins; <10% germination occurred with water treated with the cation-exchange resin, and >90% germination occurred with the anion-exchange resin (Table 2). The pH of all solutions tested was 5–5.8, which was not inhibitory to spore germination. To ensure that the water samples that passed through the cation-exchange column had not eluted any inhibitory materials from the column, the eluant was mixed with an equal volume of tap water, which resulted in >50% germination.

To determine the inorganic cations that might be present in the tap water, five samples of tap water were collected and analyzed. The most prevalent cations were: Ca^{2+}, 341 ± 13 μM; Mg^{2+}, 135 ± 5 μM; K^+, 74 ± 2 μM; and Na^+, 235 ± 4 μM. Samples of purified water registered no detectable levels of cations in the μM range.

To determine if the inorganic cations found in tap water affect germination, spores of two races were incubated for 3–4 hr on buffered solutions containing varying concentrations of salts of the individual cations. Spore germination on the 1 mM Tris buffer control varied between spore collections but ranged 5 to 10%. Germination on tap water varied between 45 and 55% between spore collections. Additional spores germinated after the 3- to 4-hr incubation time; however, the elongating germ tubes from previously germinated spores made quantitation difficult. The percent germination readings determined after 3–4 hr of incubation correlated well with the subjective observations made after 16 hr, when maximum germination had been achieved.

We performed analyses with both the raw and transformed data. It is common practice to transform percentage data with the arc sine square root transformation. However, Table 3 shows that the regression models that were significant were significant to a high degree. We also plotted the residuals from the regression analyses, and using the raw data did not result in any patterns; the residuals were random. Therefore, we are presenting the data and analyses for the untransformed germination data.

TABLE 1. Ability of urediniospores of *Uromyces phaseoli* to germinate in tap and ultrapurified water that was freeze-dried and resuspended in water from either source

Water source		Germination rating[a]
Freeze-dried	Final suspension	
Ultrapurified	Tap	8
Ultrapurified	Ultrapurified	2
Tap	Ultrapurified	8
Tap	Tap	10

[a] The rating system used for this assay was subjective: 0 = no germination, 2 = <10% germination, 6 = about 50% germination, 8 = about 75% germination, and 10 = >90% germination.

TABLE 2. Effects of ion-exchange resins on the ability of ultrapurified and tap water to support urediniospore germination

Water source	Ion-exchange resin[a]	Germination rating[b]
1. Ultrapurified	Cationic	1
2. Tap	Cationic	0
3. Ultrapurified	Anionic	2
4. Tap	Anionic	10
5. Ultrapurified	No treatment	1
6. Tap	No treatment	10
7. Mixture (50:50, treatments 1 and 5)	No further treatment	7

[a] Ultrapurified and tap water were subjected to cation and anion exchange using Bio-Rad 50W-X8 (H^+ form, 100–200 mesh) and AG 1-X8 (OH^- form, 100–200 mesh) resins, respectively.

[b] The rating system used for this assay was subjective: 0 = no germination, 1 = occasional germ tube, 2 = <10% germination, 4 = about 25% germination, 6 = about 50% germination, 8 = about 75% germination, and 10 = >90% germination.

TABLE 3. Regression of spore germination[a] by two races of *Uromyces phaseoli* on concentration of various metallic ions

Ion	Race	Ion concentration[b]			Log_2 ion concentration		
		Intercept[c]	Slope[d]	R^2[e]	Intercept	Slope	R^2
CA	39	9.23	41.58	0.81***	45.44	9.15	0.87***
	40	22.04	23.23	0.46***	40.74	3.86	0.38**
MG	39	5.47	17.58	0.60***	20.53	3.87	0.56***
	40	7.66	5.49	0.43***	12.21	1.00	0.35**
NA	39	12.17	0.76	0.28**	13.70	1.08	0.09ns
	40	6.87	1.58	0.56***	8.83	2.82	0.28**
K	39	6.91	1.03	0.01ns	8.32	0.56	0.07ns
	40	5.72	6.26	0.34**	11.43	1.31	0.37**

[a] Germination was transformed using the arc sine square root of percent spore germination.

[b] The independent variable in the regression equation, either the concentration of the ion or the base 2 logarithm of the ion concentration.

[c] The intercept of the regression line.

[d] The regression coefficient, or slope, of the regression equation.

[e] The coefficient of determination, an estimate of the amount of the variation explained by the model. Asterisks indicate the significance of the model: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq -0.001$, and ns = not significant.

The results of the regression analyses of spore germination are shown in Table 3. The data were quite variable, which is reflected in the relatively low coeffecients of determination. Spore germination by both races increased proportionally over that in buffer controls in response to increasing amounts of calcium (Fig. 1). Linear regression of spore germination was significant for both races and demonstrated steep slopes (Table 3). Regression of spore germination on the logarithm base 2 ($\log_2$) of calcium concentration improved the coefficient of determination for race 39 but not for race 40. The plot for race 40 increased more rapidly at the low end of the concentration scale than race 39 did; consequently, we analyzed the data with the NLIN procedure of SAS using the model for the negative exponential:

$$\text{Percent germination} = B_0 * (1 - e^{(-B_1 * [Ca])}).$$

Parameter estimates for B_0 and B_1 for race 39 are 47.98 and 3.08, respectively. For race 40, the estimates of B_0 and B_1 are 36.78 and 18.80, respectively. Race 40 was more sensitive to the addition of small amounts of calcium. The 95% confidence bands resulting from these models are plotted in Figure 2. The confidence bands demonstrate some overlap, indicating that the relationship between spore germination of *U. phaseoli* spores and calcium is one of rapid increase as the calcium concentration increases until a plateau is reached. Urediniospores of race 40 were germinated in calcium solutions ranging from 0 to 16 mM to verify the negative exponential model derived from calcium concentrations up to 1 mM. These data were analyzed with the NLIN procedure of SAS and yielded similar B_0 and B_1 coeffecients. Therefore, we concluded that the data are adequately described by a negative exponential function, i.e., a sharp rise in germination that approaches an upper limit. The relationship to $\log_2$ is also good, particularly for race 39. With our data, however, the negative exponential seems to provide a better general relationship.

Spore germination of both races increased as the magnesium sulfate concentration increased (Fig. 3). However, the data for race 40 were variable, resulting in a relatively shallow slope and a low coefficient of determination, even though the regression was significant (Table 3). The $\log_2$ transformation of Mg^{2+} concentration did not improve the linear relationship.

Monovalent cations had little effect on spore germination. Although the regression of spore germination on Na^+ concentration was statistically significant, the slopes were so shallow as to make the relationship of no consequence (Table 3).

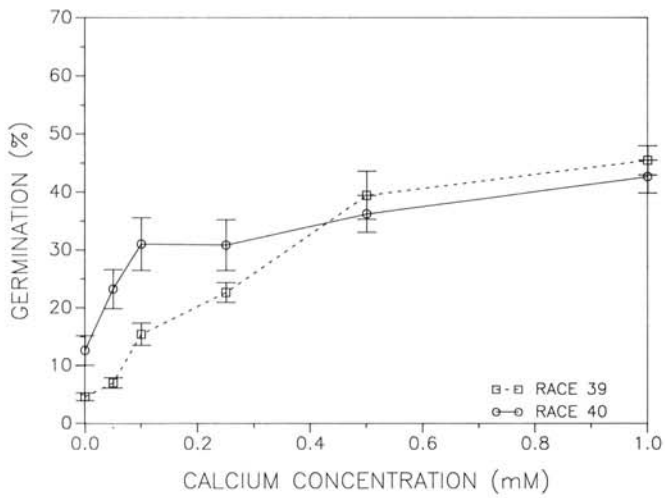

Fig. 1. Effects of calcium on germination of urediniospores of *Uromyces phaseoli*. Spores were incubated in 1 mM Tris, pH 7, containing calcium chloride. Each data point represents the average of five replicates in which 100 spores were counted. Bars indicate the standard error of the mean.

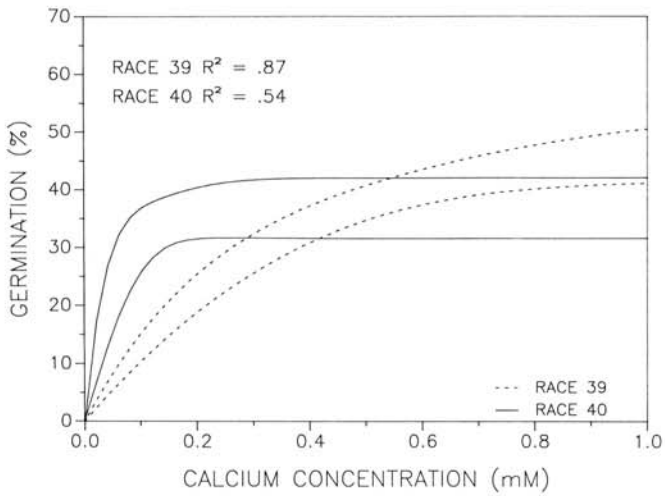

Fig. 2. Plot of the 95% confidence bands of the negative exponential relationship of spore germination of urediniospores of *U. phaseoli* to calcium ions. The general equation is: percent germination = $B_0 * (1 - e^{(-B_1 * [Ca])})$, where B_0 and B_1 are 47.98 and 3.08, respectively, for race 39 and 36.78 and 18.80, respectively, for race 40. The R^2 values are the coefficients of determination for the models.

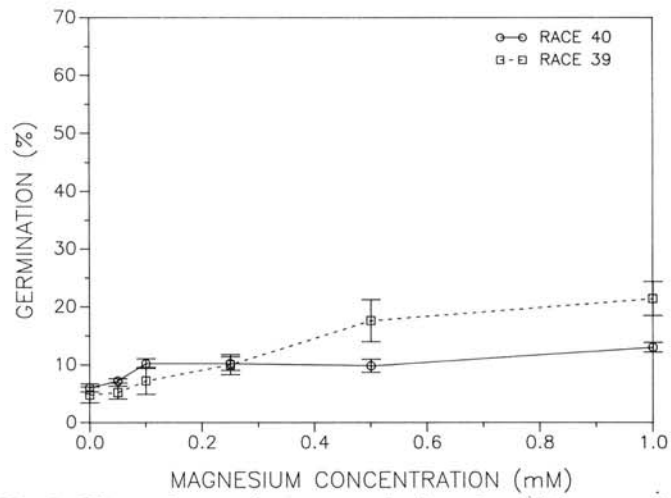

Fig. 3. Effects of magnesium on germination of urediniospores of *Uromyces phaseoli*. Spores were incubated in 1 mM Tris, pH 7, containing magnesium sulfate. Each data point represents the average of five replicates in which 100 spores were counted. Bars indicate the standard error of the mean.

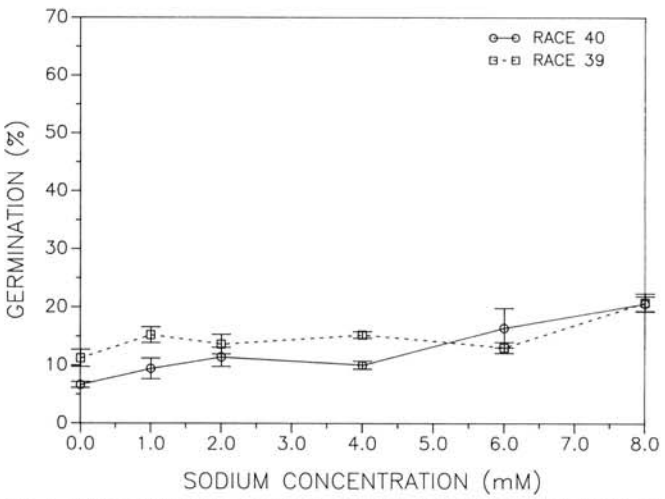

Fig. 4. Effects of sodium on germination of urediniospores of *Uromyces phaseoli*. Spores were incubated in 1 mM Tris, pH 7, containing sodium chloride. Each data point represents the average of five replicates in which 100 spores were counted. Bars indicate the standard error of the mean.

This conclusion is borne out by Figure 4. The apparent significance of the model incorporating $\log_2$ of the Na^+ concentration for race 40 is probably the result of the response of germination to concentrations above 2 mM (Fig. 4).

Likewise, the effect of potassium on spore germination was of little consequence, although the regression analyses for race 40 indicates otherwise (Table 3). However, this is probably due to the relatively low germination in ultrapure water (Fig. 5). In addition, the total range of germination was less than 10%.

Preliminary tests to determine if other divalent cations might affect spore germination showed that manganese sulfate inhibited spore germination. To determine if Mn^{2+} was actually inhibitory, spores of race 40 were incubated on solutions containing 4 mM calcium chloride plus 0–1,000 μM manganese sulfate. Concentrations greater than 300 μM appeared to inhibit spore germination (Fig. 6). The data were analyzed with the NLIN procedure of SAS, and the general exponential model was significant with a coefficient of determination of 0.50. The equation is:

$$\text{Germination} = 42.45 * e^{(-0.00119*[\text{Mn}])}$$

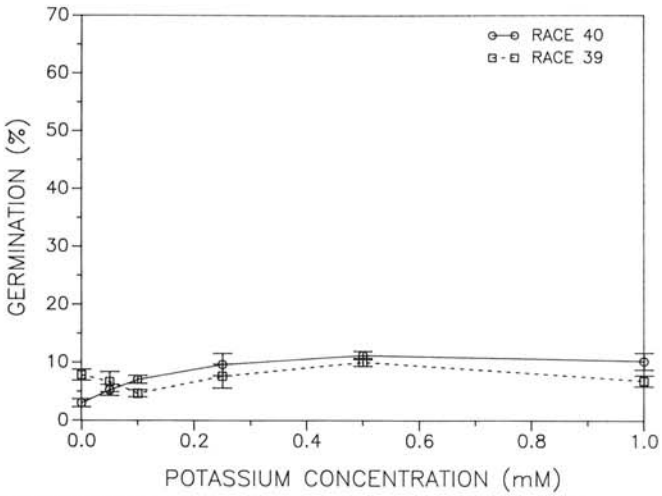

Fig. 5. Effects of potassium on germination of urediniospores of *Uromyces phaseoli*. Spores were incubated in 1 mM Tris, pH 7, containing potassium chloride. Each data point represents the average of five replicates in which 100 spores were counted. Bars indicate the standard error of the mean.

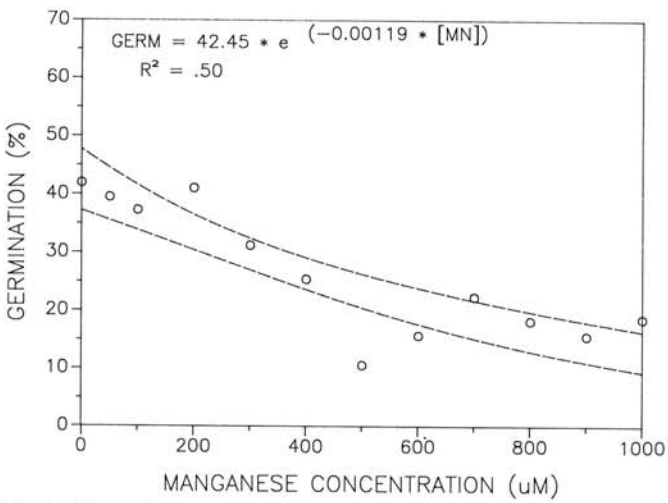

Fig. 6. Effects of manganese on spore germination. Spores were incubated on 1 mM Tris, pH 7, containing 4 mM calcium chloride plus varying concentrations of manganese chloride ranging from 0 to 1 mM. Dashed lines indicate the 95% confidence band for the model. Open circles indicate the mean germination of five replicates of 100 spores.

DISCUSSION

Our study resulted from an initial observation that urediniospores of *U. phaseoli* germinate well on tap water but not on ultrapurified laboratory water. Preliminary experiments in treating the tap water with ion-exchange resins suggested that urediniospores produced under our conditions and asssayed under our bioassay conditions require cations for germination. Germination of urediniospores on buffered purified water was stimulated in the presence of calcium, the major cation found in the tap water. The percentage of spores that germinated was proportional to the concentration of calcium, suggesting that calcium is required for germination. Other inorganic cations found in the tap water were tested and found not to be as effective as calcium in stimulating germination. Divalent cations appeared more effective than monovalent cations. There appears to be some specificity with calcium preferred over magnesium; manganese is inhibitory.

Little has been published on essential inorganic requirements for urediniospore germination. Most of the work has concentrated on the toxic effects of cations used in fungicides. Couey and Smith (4) found that calcium and magnesium reduced the toxic effect of zinc on urediniospores of *Puccinia coronata*. Richardson and Thorn (12) reported that copper and other heavy metal ions added to distilled water stimulated germination of high densities of conidia from *Glomerella cingulata*. Their results suggest that this apparent stimulation was due to the toxic effect of copper on some spores, causing a release of nutrients requisite for germination of the surviving spores. Gupta and Saxena (7) studied the effects of certain salt solutions on germination of conidia of *Erysiphe cichoracearum* and chlamydospores of *Ustilago cynodotis*. They found zinc sulfate most toxic followed by calcium nitrate, sodium nitrate and copper sulfate.

Although there have been reports of urediniospore germination in distilled water (6,9), our results strongly indicate certain inorganic cations are neccessary for germination. This apparent inconsistency could be due to either the purity of the water used in previous studies and/or the manner in which the urediniospores were produced. In the latter case, it is conceivable that because of different cultural practices in watering host plants, the spore wall may adsorb varying amounts of cations from the leaf surface.

It appears from this study that calcium, whether from exogenous or endogenous sources, plays an important role in *U. phaseoli* urediniospore germination. It is interesting that similar results are noticed in H^+/Ca^{2+} exchange in oat coleoptile tonoplasts (13). Divalent cations at concentrations in the micromolar range affect H^+/Ca^{2+} exchange in tonoplast vesicles; Ca^{2+} is more stimulatory than Mg^{2+} while Mn^{2+} is inhibitory. In light of increasing evidence that Ca^{2+} acts as a second messenger in plants to regulate numerous physiological processes, it is feasible that Ca^{2+} may play a similar role in regulating spore germination (10).

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