EFFECT OF CATIONS ON GERMINATION & GERM TUBE DEVELOPMENT OF PUCCINIA CORONATA UREDOSPORES 1, 2
H. MELVIN COUEY 3 & FREDERICK G. SMITH

Previous work in this laboratory (20) showed that germ tubes of Puccinia coronata uredospores would form structures in vitro resembling those found in infected host plants (18). This so-called vesicle formation occurred on Difco gelatin in the pH range of 6.2 to 6.9 on addition of 14 to 22 \times 10^{-8} \text{M} \ Zn. Under these conditions germ tube length was reduced, and in some cases the percentage germination was lowered. Other metals, including Cd, Cu, Fe, Mg, Ca, Mo, Co, and Mn, did not replace Zn.

Several preliminary observations in Sharp’s work (19) and in the present study indicated gelatin contained substances affecting the action of Zn on germ tube growth and differentiation. A. The extent of vesicle formation varied with different gelatin samples. B. The concentration of Zn required increased with the gelatin concentration. C. A water extract of gelatin with added Zn stimulated vesicle formation on an agar substrate at pH 6.3 to 6.5 D. When gelatin was purified by water extraction, adding Zn failed to induce vesicle formation, and Zn inhibited germination at much lower concentrations. This paper reports further study of the action of Zn and gelatin under better defined conditions and presents evidence that both germination and vesicle formation are subject to cation effects.

MATERIALS & METHODS

Uredospores were mixed race collections of Puccinia coronata Corda var. avenae F. and L. from both the field and greenhouse. The germination substrate ordinarily was 4 ml of 2% Difco gelatin in 6 cm petri dishes. Spores were applied from a small cyclone duster (22). With liquid substrates, spores were added to 2 ml volumes in 5 ml beakers by transferring loops of spores floating on water. The germination vessels were covered loosely or placed in a moist chamber and incubated at 20 to 21°C in the dark. Under these conditions, no evidence was observed of the self inhibition of germination reported for P. graminis tritici uredospores (2, 6).

Germination counts were made after about 12 hours and vesicle formation counts after 36 to 40 hours. A spore was counted as germinated if the germ tube length was greater than the spore diameter. The formation of a spindle-shaped substomatal vesicle was the minimum requirement for counting as vesicle formation since a distinct appressorium was sometimes missing at high Zn concentrations. Five fields of 35 to 50 spores each were examined per plate. Ordinarily, a single plate for each treatment was used, and the experiments were repeated several times. Statistical analysis was not regularly applied, but previous experience (21) and several analyses of replicate experiments indicated that a difference of about 10 to 12 in percentage germination was significant at the 5% level.

Only samples of Difco gelatin which supported vesicle formation were used. Ion-exchange purification was carried out first with a carboxylate resin (Amberlite IRC-50) in the Na form and later with a mixed bed column of a strong acid and strong base resins (Amberlite IR-120 and IRA-400) in the H and OH forms, respectively. Both systems maintained a pH near enough to neutrality to avoid gelatin hydrolysis. The columns were held at 40 to 45°C to prevent solidification of the gelatin solutions. Flow rates and quantities of gelatin were kept well below the exchange capacity of the columns. The mixed bed system was more efficient in cation removal, and the effluent gelatin was close to the isoelectric point. Preparations from the carboxylate column will be referred to as “cation-free” gelatin though the gelatin cations actually were replaced by Na ions. The preparation from the mixed bed column is called deionized gelatin.

RESULTS

EFFECT OF CATION EXCHANGE TREATMENT OF GELATIN ON GERMINATION AND VESICLE FORMATION: The first evidence that other cations affected the action of Zn was observed with cation-free gelatin as substrate. When the native gelatin cations were replaced by Na ions, Zn was more toxic and by itself no longer would induce vesicle formation (table I and fig 1). The percentage germination in the absence of added Zn also was reduced on the treated gelatin (fig 1). Adding Ca or Mg reduced the toxicity of Zn and restored its ability to promote vesicle formation (table I). A mixture of the two cations usually was more effective than either one alone. Since the level of vesicle formation under these conditions was roughly equivalent to that observed on the corresponding whole gelatin, it appeared that cations other than Ca and Mg were not required.

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2 Journal Paper No. 3880 of Iowa Agricultural and Home Economics Experiment Station, Ames. Project No. 1258. These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Iowa State University, NR 103-109.
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Fig. 1. Effect of Zn concentration on germination at pH 6.5 on whole, cation-free, and deionized gelatins.
Fig. 2. Effect of Ca and Mg on Zn inhibition of germination at pH 6.5 on deionized gelatin.
Fig. 3. Relation of Ca concentration to Zn inhibition of germination at pH 7 on deionized gelatin.
Fig. 4. Germination on whole gelatin and deionized gelatins as a function of pH.
Fig. 5. Germination on deionized gelatin as a function of pH and Ca concentration.
Fig. 6. Germination on whole gelatin as a function of pH and Zn concentration.
Fig. 7. Germination on deionized gelatin as a function of pH and Zn concentration.
Fig. 8. Germination on deionized gelatin as a function of pH and Zn and Ca concentration.
Fig. 9. Vesicle formation on glycine solutions as a function of glycine and calculated free Zn concentration.


Table I

<table>
<thead>
<tr>
<th>Mg ( \times 10^{-4} \text{ M} )</th>
<th>Ca ( \times 10^{-4} \text{ M} )</th>
<th>Germination</th>
<th>Vesicle formation</th>
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<td>( 7.5 )</td>
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\( Zn = 18 \times 10^{-5} \text{ M, pH} = 6.5 \)

**Effect of Cation Interaction in Germination on Deionized Gelatin:** With deionized gelatin as substrate, Zn was even more toxic than with cation-free gelatin (fig 1). This was attributed to the more complete removal of antagonistic ions. Adding sufficient Ca or Mg reduced the toxic action of Zn, and again an equimolar mixture of the two was more effective than either one alone (fig 2). Adding Ca or Mg also raised the percentage germination in the absence of added Zn at pH 6.5 (fig 2) but not at pH 7.0 (fig 3). This difference suggested that H ion toxicity occurred at the lower pH in absence of antagonistic cations. It appeared that the interaction of Ca and Zn could be studied at pH 7 without the complication of H ion toxicity. In the representative experiment in figure 3 about a 100-fold increase in Ca was required to balance the effect of a 4-fold increase in Zn, and \( 10^{-3} \text{ M} \) Ca did not fully reverse the effect of only \( 6 \times 10^{-5} \text{ M} \) Zn.

In the early stages of this work the pH used routinely was 6.4 to 6.5, the level found optimum for vesicle formation by Sharp and Smith (20). However, it became clear that deionization of the gelatin changed the ionic environment to such an extent that it was necessary to reexamine the influence of pH on both germination and vesicle formation. No other buffer was added since the gelatin itself provided adequate buffering over the desired range. On whole gelatin some germination occurred from pH 4 to 10 with maximum percentage from about pH 5 to 9. With deionized gelatin the acid branch of the pH response curve was shifted to higher pH values, and a narrower range of maximum germination from about pH 7 to 9 was obtained (fig 4). This shift in pH response explained the depressed germination due to deionization observed earlier (fig 1) since the pH of 6.5 used at that time is on the shoulder of the curve. Since this change in pH response apparently was due to removal of soluble cations from the gelatin, the effect of adding Ca or Mg was investigated. These cations shifted the acid branch of the curve back to lower pH's (fig 5). The effect increased from \( 10^{-5} \text{ M} \) Ca (indistinguishable from the control, fig 4) to about \( 10^{-3} \text{ M} \) Ca. Similar results were obtained with Mg. The high level of Ca (fig 5) also inhibited germination in the alkaline region, causing a difference from the control curve similar to that between curves on deionized and whole gelatin. However, this effect of Ca was not as consistent or as large as that in the acid region.

Next, the effect of Zn on the pH-germination curves was investigated. It proved to be toxic throughout the pH range with both whole and deionized gelatin (fig 6 & 7). With whole gelatin (fig 6) Zn was relatively more toxic at higher pH's, so the pH optimum for partially inhibited germination was about 6. With deionized gelatin (fig 7) Zn was much more toxic than with whole gelatin over the broad pH range of 4 to 9 as well as at the pH of 6.5 used earlier. The region of maximum germination (pH 7–9) was the same both with and without Zn, indicating that Zn toxicity was not greatly influenced by H ion concentration. Adding Ca to deionized gelatin containing Zn (fig 8) shifted the pH range of maximum germination back to the acid side and decreased the toxicity of Zn (cf. fig 7). These effects of added Ca were similar, though not identical, to the differences in pH-germination curves for whole and deionized gelatin containing toxic levels of Zn. It was concluded that Zn toxicity was influenced more by metallic cation impurities in the gelatin than by H ions.

Although the influence of other cations on H and Zn toxicity was not studied extensively, Mn and Ba at \( 10^{-3} \text{ M} \) gave effects like Ca and Mg, but K and Na did not. It is possible, therefore, that a number of polyvalent cations may influence the action of H and Zn on uredospore gelatin.

**Effect of Cations on Vesicle Formation with Deionized Gelatin:** The failure of Zn to induce vesicle formation in the pH range 6.2 to 6.9 (20) after cation removal from gelatin in the present work prompted a reexamination of the effect of H ion concentration on germ tube differentiation. A few germinating uredospores (1–2 %) formed vesicles on the Zn-deionized gelatin substrate at pH 8 and 9 but not at lower pH's. Adding Ca, Mg, or mixtures of the two increased vesicle formation at pH 8 to about the level found on whole gelatin. These experiments also indicated that the required concentration of added cation was dependent on the Zn concentration. The most consistent results were achieved with \( 10^{-4} \text{ M} \) Ca and 4 to \( 18 \times 10^{-5} \text{ M} \) Zn. Table II summarizes a typical experiment comparing vesicle formation as a function of H ion concentration on whole and deionized gelatin. The pH range permitting vesicle formation on this whole gelatin sample was 6.5 to 7.5.
Deionized gelatin was studied. It was found with 0.0 M pH 8.0 did not differ. Percentage estimates indicated was near 20% of germination. Some estimated that about 20% of the germinating uredospores were undergoing typical germ tube differentiation. No vesicle formation occurred without addition of both Zn and glycine, but Ca or Mg was not required. As on deionized gelatin, the pH optimum was near 8. This pH was used in subsequent experiments.

To clarify the role of the glycine, the effect of the amino acid concentration on the Zn level required for vesicle formation was examined. The data in table III show that increasing the glycine concentration raised the level of Zn required. Similar results were obtained with alanine and valine. The free Zn concentration was calculated according to equations given by Rabin and Crook (16) using affinity constants determined by Maley and Mellor (15). A graph of vesicle formation as a function of free Zn and glycine concentrations is shown in figure 9. Vesicle formation was essentially independent of amino acid concentration from 0.01 to 0.1 M. Graphs for alanine and valine were similar. It was concluded the amino acids were acting primarily, if not exclusively, as Zn buffers. In this way a reservoir of Zn could be maintained without reaching the toxic level of free Zn.

The lack of a Ca or Mg requirement for vesicle formation on amino acid solutions as compared with deionized gelatin posed an interesting question that seemed to involve Zn toxicity and factors affecting it. At pH 8 on 0.001 M tris buffer, Zn inhibited germination throughout the concentration range (10^{-5} M or greater of total Zn) that induced vesicle formation on amino acid solutions. However, if Ca or Mg was added to antagonize Zn toxicity, some vesicle formation occurred under the following conditions: A. 10^{-4} M Ca + 10^{-5} M Zn, B. 10^{-4} to 10^{-5} M Mg + 1 to 2 × 10^{-5} M Zn, or C. Ca and

### Table II

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<th>Substrate</th>
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Percentage vesicle formation on basis of germinated spores
* Indicates conditions permitting less than 20% germination

### Table III

| Glycine M | Zn (× 10^{-5} M) | 0   | 1   | 2   | 3   | 4   | 6   | 8   | 10  | 12  | 16  | 20  | 24  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 120 | 140 | 160 |
|-----------|------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 0.01      |                  | 0   | +   | +   | +   | ±   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0.02      |                  | 0   | 0   | +   | +   | +   | ±   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0.04      |                  | 0   | 0   | 0   | +   | +   | +   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0.06      |                  | 0   | 0   | 0   | 0   | +   | +   | +   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0.08      |                  | 0   | 0   | 0   | 0   | +   | +   | +   | +   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |
| 0.10      |                  | 0   | 0   | 0   | 0   | 0   | +   | +   | +   | +   | 0   |     |     |     |     |     |     |     |     |     |     |

+ Indicates vesicle formation, 0 no vesicle formation, ± variable results
Mg at \(5 \times 10^{-4} \text{M} \) each + 1 to \(4 \times 10^{-5} \text{M} \) Zn. There was some indication that vesicle formation was dependent on the relative concentrations of Zn and the protective cation (Ca or Mg), but this relationship requires further study. Similar results were obtained with low concentrations of amino acids at pH 8. For example, on 0.001 M glycine addition of protective cations reduced Zn toxicity, and vesicle formation occurred under the following conditions: A. \(10^{-3} \text{M} \) Mg + 3 to \(4 \times 10^{-5} \text{M} \) Zn and B. Ca and Mg at \(5 \times 10^{-4} \text{M} \) each + \(4 \times 10^{-5} \text{M} \) Zn. These experiments suggested that a certain minimum level of Zn was required for vesicle formation, but in the absence of antagonistic cations or binding agents this concentration completely inhibited germination. This conclusion led to the idea that an amino acid might substitute for Ca or Mg in permitting vesicle formation on deionized gelatin. However, no success was achieved in several attempts with various levels of glycine and Zn.

**Discussion**

This study presents evidence that the growth and differentiation of *P. coronata* germ tubes are sensitive to the interaction of Zn, H, and Ca or Mg ions. With deionized gelatin as substrate it could be demonstrated that divalent cations such as Ca and Mg reduce Zn inhibition of germination. Such antagonistic action of Ca and Mg toward toxic cations like Zn has been reported for a number of microorganisms including several fungi (1.11, 12, 14) though apparently not previously for the rust fungi. A significant interaction between H ions and Ca and Mg ions also was established on deionized gelatin. This type of cation interaction has not been widely observed with fungi perhaps because it is obscured by cations in the nutrients or substrates commonly used. However, similar shifts by alkaline earth cations in the pH-respiration curve of yeast (17) and the pH-growth curve of a fungus colony (13) have been reported.

What do the observed pH effects suggest about the nature of the uredospore germination process? From one point of view, the shift in the acid branch of the curve toward lower pH values may be regarded as antagonism of H ion toxicity by Ca and Mg. The roughly bell-shaped nature of these curves indicates that the germination process is controlled by dissociation of acidic groups at the cell surface. For germination to occur, some groups with ionization constants between pH 5 and 6 must be in the dissociated form while other groups with constants around pH 10 must be in the undissociated form. To explain the shift in the acid branch of the curve with increasing concentrations of Ca or Mg, one may assume that binding these cations causes an increase in the acidity of the low pH dissociating groups as has been shown with proteins (10). The validity of this viewpoint must be tested further. More extensive data are needed, particularly with substrates other than gelatin, and more direct methods must be employed to establish whether these cation effects are actually equilibrium phenomena. If this mechanism of cation interaction in the germination process can be substantiated, it would suggest that bound Ca, Mg, or similar cations may be required for growth perhaps as constituents of metallo-enzymes as MacLeod and Snell (14) have proposed in similar work on Lactobacilli. Hydrogen and Zn ions then may act by displacing and controlling the activity of the native cations.

This work also has demonstrated that the ability of Zn to induce vesicle formation by germinating uredospores of *P. coronata* is greatly influenced by other constituents of the substrate. On whole gelatin (20) this phenomenon was restricted to the pH range of 6.2 to 6.9. When the gelatin was purified to remove ionic material, Zn alone was not effective. On addition of Ca or Mg and lowering of the H ion concentration to a pH of about 8, vesicle formation was restored. This higher pH requirement also was observed with amino acid solutions and in some unreported experiments with agar as substrate. Hydrogen ions as well as the divalent cations seemed to affect the activity of the Zn in inducing vesicle formation. Experiments with amino acid solutions provided further evidence that suitable control of Zn activity would result in germ tube differentiation. On these substrates no addition of Ca or Mg was necessary. The amino acids appeared to maintain the appropriate free Zn concentration by acting as Zn buffers. With a low amino acid concentration or with tris buffer, however, adding Ca or Mg was necessary to reduce the toxicity of Zn sufficiently to permit vesicle formation. It may be concluded that Zn alone is capable of inducing vesicle formation in germinating uredospores of *P. coronata* if its activity is properly regulated.

Any consideration of the mechanism of Zn action in inducing germ tube differentiation must recognize the concomitant reduction in germ tube growth and spore germination. This suggests that other factors inhibiting germ tube growth might induce differentiation. This possibility has not been tested extensively, but experience so far indicates that it is unlikely. The specificity of Zn remains to be established; however, since other germ tube growth inhibitors have not been studied over as wide a range of conditions as has Zn.

Some remarkable reports have appeared on factors affecting germination and germ tube differentiation of *P. graminis tritici*. Allen (3) and French, Massey, and Weintraub (7) have shown that a distillate from a uredospore extract would retard germ tube growth and induce vesicle formation. The latter group (8) also have isolated pelargonaldehyde from the distillate and reported that it is active. In addition, Fuchs and Gaertner (9) have observed marked stimulation of vesicle formation by cysteine and glutathione in the presence of iron compounds on a mineral-salts-glucose-silica gel substrate. Finally, Emge (5) has shown that vesicle formation can be
induced on various artificial substrates by the proper sequence of light and temperature conditions. These recent reports along with Dickinson’s earlier germination studies (4) on artificial membranes and the work in this laboratory would indicate that a variety of factors can induce rust germ tube differentiation. It remains to be seen how much species differ in response to these various treatments and whether or not any common mechanism is involved.

**Summary**

Evidence is presented that the cation composition of artificial substrates influences the germination and germ tube differentiation of uredospores of *Puccinia coronata* Corda var. avenae F. and L. Zinc and H ion toxicities were antagonized by added Ca and Mg. Hydrogen ions and the alkaline earth cations affected the ability of Zn to induce differentiation of infection structures. However, Zn alone was effective if its activity was properly controlled by amino acids acting as metal buffers. It is suggested that the observed cation interactions in germ tube growth and differentiation may involve displacement of native cations.

**Literature Cited**


