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The parallel expression of metal tolerance in pollen and sporophytes of *Silene dioica* (L.) Clairv., *S. alba* (Mill.) Krause and *Mimulus guttatus* DC.

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Summary. The purpose of this paper was to determine if heavy metal tolerance was expressed in pollen and if its expression was correlated with the tolerance of the pollen source. Clones of *Silene dioica*, tolerant to zinc, closely related but nontolerant *S. alba* and clones of *Mimulus guttatus* tolerant and sensitive to copper were grown in the greenhouse in either standard potting soil or nutrient culture. Pollen was collected shortly after dehiscence, hydrated, and tested over a broad range of metal concentrations. The tolerance of the pollen source was determined by comparing root growth in solutions with and without heavy metals. In both *Silene* species and *M. guttatus*, the tolerance of the parental clone was expressed in its pollen. Pollen from tolerant individuals was able to germinate and grow at concentrations of metals which markedly inhibited pollen from nontolerants.

Key words: Pollen – Gene expression – Metal tolerance – *Silene* – *Mimulus guttatus* – Copper – Zinc

Introduction

In angiosperms, genes are expressed in both the diploid sporophyte and the haploid microgametophyte or pollen (Zamir 1983). Estimates based on studies of tomato (Tanksley et al. 1981) and *Tradescantia* (Willing and Mascarhenas 1984) indicate that the genes expressed in pollen may represent up to 60% of genes expressed in the sporophytic phase. Because of large population sizes and haploid genotypes, pollen might provide an effective tool to screen for sporophytic characteristics. In addition, if the same genes are expressed (transcribed and translated) in both pollen and sporophyte, pollen selection could modify gene frequencies in subsequent generations (Mulcahy 1975, 1979). Thus, characteristics which are expressed in pollen are of interest, both for understanding a potentially adaptive mechanism of plant evolution, and for plant breeding.

One important area in which the pollen response may parallel that of the sporophyte is in its response to environmental stress. Parallel responses to temperature stress (Herrero and Johnson 1980; Zamir et al. 1981), ozone (Feder 1968, 1981), and salinity (Eisikowitch and Woodell 1975; Sacher et al. 1983) have been reported. In at least one case (Zamir et al. 1982), temperature sensitivity appears to be the result of the same genes being expressed in both pollen and sporophyte.

This paper compares the response of pollen from plants tolerant to zinc or copper with that of pollen from nontolerant ecotypes or from closely related species. It attempts to determine of the metal tolerance of the parent plant is expressed in its pollen. Copper and zinc are frequently found in high quantities in plant tissues (Antonovics et al. 1971) so that pollen might experience metal stress. Furthermore, studies on the physiology of metal tolerance (Mathys 1980; Thurman 1981), have suggested a number of mechanisms which could be active in pollen.

Plants were chosen to represent bi- and trinucleate pollen since these contrasting pollen types differ physiologically (Brewbaker and Majumder 1961; Hoekstra and Bruinsma 1978). Pollen was tested by measuring in vitro percent germination and pollen tube growth over a broad range of metal concentrations. This permitted determination of toxic concentrations and provided a test for possible requirements for heavy metals, as indicated for some tolerant plants (Allen and Sheppard 1971; Antonovics et al. 1971).

Materials and methods

Materials

Mimulus guttatus DC. (binucleate pollen). Plants differing primarily in copper tolerance were grown from seed obtained from M. R. Macnair, University of Exeter, England. Two families were used in this study; Macnair's 5587/24 (M24-NT), derived from selfed seed of a nontolerant individual, and 5587/22 (M22-T), derived from selfed seed of a tolerant individual (Macnair, personal communication). Details of the crosses involved in the generation of these stocks are available in Macnair (1981 and 1983). One individual from each family was cloned and used in all experiments. In addition, several families of plants were grown from seed collected by Richard Kesseli, University of California, Davis, at an abandoned copper mine at Copperopolis, California. Details of this location are in Allen and Sheppard (1971).

Silene (trinucleate pollen). Zinc tolerant *Silene dioica* (L.) Clairv. (SD-T) was collected at a lead-zinc mine site in Wales, ordinance survey location (SN 68 28 32). Individuals were maintained in the greenhouse at the University of Massachusetts for 3 years prior to any testing. Only female plants of a nontolerant *S. dioica* flowered during the course of these experiments. Therefore, comparisons were made to closely related and interfertile (Baker 1948; McNeill 1978) *S. alba* (Mill.) Krause (SA-NT) which was grown from seed collected in Leverett, Massachusetts.

Determining the tolerance of pollen sources

Mimulus. Copper tolerance in the parental clones was confirmed by comparing root growth of unrooted cuttings in solutions of 0.5 g/l $Ca(NO_3)_2$ to that in 0.5 g/l $Ca(NO_3)_2$ + 0.5 ppm Cu (Macnair 1983). Tests were made in a growth chamber with 16-h days, 20 °C, 75% relative humidity, and a light intensity of 240 $\mu E \cdot m^{-2} \cdot s^{-1}$. The solutions were aerated continuously and changed every 2 days. The length of the longest root was measured after 6 days for 6 cuttings from each clone.

Silene. Because the zinc tolerance of neither *S. dioica* nor *S. alba* had been studied, root growth was tested over a range of metal concentrations using ramets which had been removed from the root crown and rooted in Perlite. Four ramets from each clone were randomly assigned to one of 4 treatments and placed in a frame suspended over 15 l plastic containers so that only the roots and extreme lowest and of the stem were in contact with the solution. Following a 4 day pretreatment in 1/10 strength Hoagland's solution (macronutrients, Hoagland and Snyder 1933; micronutrients, Johnson et al. 1957), the length of the 5 longest roots on each plant was measured from a point marked on the stem. Next, solutions containing different concentrations of zinc in 0.5 g/l $Ca(NO_3)_2$ (see Fig. 1) were added and root lengths were measured again after an additional 6 days. Difference in growth of the 5 longest roots was considered an estimate of zinc tolerance. Plants were maintained in a growth chamber with 14-h days, 22 °C, 75% relative humidity, and a light intensity of 240 $\mu E \cdot m^{-2} \cdot s^{-1}$. Solutions were aerated continuously, and changed on day 3 of the experiment. Data from two replicates (3 for 2.5 ppm Zn) were combined and analyzed using an AVOVA in SPSS (Nie et al. 1975) with clones and zinc concentrations as the main effects.

Determining metal tolerance in pollen

All plants from which pollen was taken were grown without added metals. Pollen from both genera was collected within a few hours of being shed and used the same day. Following a ½-h hydration (Shivanna and Heslop-Harrison 1981) at 20 °C, *Mimulus guttatus* pollen was grown for 3-h at 20 °C in 0.20 ml of solution in ½-ml polypropylene centrifuge tubes placed on a rotary shaker. Growth was stopped by freezing the entire sample. Pollen from *Silene* was grown for 3-h at 25 °C in 0.020–0.030 ml drops on a microscope slide in a covered petri dish. Humidity was maintained in the petri dish by a piece of moistened filter paper. Growth was stopped by letting the drops dry, which took 5–10 min. Pollen concentration in both cases was approximately 2 mg/ml.

Pollen was grown in liquid media containing 1.62 mM H_3BO_3, 1.27 mM $Ca(NO_3)_2$, 15 mM MES (Sigma), pH 5.5, and either 0.4 M sucrose (*Silene*), or 0.8 M sucrose (*Mimulus*). Metals were added as $ZnSO_4 \cdot 7H_2O$ or $CuSO_4 \cdot 5H_2O$. All solutions were made with deionized, distilled water.

Percent germination was scored on coded samples for 400 pollen grains at each concentration. Since stress is known to induce the extrusion of short pollen tubes (Stanley and Linskens 1974), pollen was recorded as germinated only when the pollen tube length was twice the diameter of the pollen grain. Pollen tube length was measured at each concentration using an ocular micrometer for 50 pollen tubes of *Mimulus*, or 25 pollen tubes if germination was very low, and 25 tubes in *Silene*.

Pollen viability in all the clones of *Mimulus* was low, and ranged from 30–60%. Therefore, pollen quality was independently assessed using fluorescein diacetate (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison et al. 1984). Germination was then expressed as a percent of potentially viable pollen. After correction, germination percentages in *Mimulus* ranged from 30–70% so were not transformed (Steel and Torrie 1980). Percent germination data from *Silene* ranged from 20–30% and were transformed using the arcsin transformation prior to analysis. Data were analyzed using ANOVA and ONEWAY programs in SPSS (Nie et al. 1975). Duncan's multiple range tests were used in the latter to partition significant differences among means ($P < 0.05$) in either germination or pollen tube length.

Results

Tolerance of pollen sources to copper or zinc

A comparison of each *Mimulus* clone used as a pollen source to root in solutions with and without 0.5 ppm Cu is shown in Table 1. The tolerant (M22-T) and Copperopolis (C-T) clones produced healthy roots in both solutions. In the solution with 0.5 ppm Cu, the nontolerant (M24-NT) clone failed to produce any roots, or produced short roots less than 2 mm long, which, unlike roots of the tolerant clones, had turned brown by day 6.

Silene. The average increase in root length of the tolerant (SD-T) and nontolerant (SA-NT) pollen sources at different zinc concentrations is shown in Fig. 1. *Silene alba* (SA-NT) roots were unable to grow at concentrations of zinc above 2.5 ppm. At the three highest concentrations, SA-NT roots became necrotic, and no new roots were initiated. In contrast, roots grew over the entire range of zinc concentrations tested in the tolerant (SD-T) clone collected from a lead/zinc mine site. At concentrations of 2.5 ppm zinc and above, a considerable proportion of root growth in SD-T appeared to involve production of new primary and lateral roots. An analysis of variance showed that *S. alba* and *S. dioica* differed significantly in root growth ($F=40.4$, $df=1$, $P < 0.001$) but the interaction factor (clones × zinc concentration) was not significant ($F=1.84$, $df=4$, $P < 0.13$). This could be interpreted to indicate that the clones SD-T and SA-NT did not differ in zinc tolerance (see Wilkins 1978). However, measuring just root elongation appeared to underestimate the zinc tolerance in *S. dioica*. Furthermore, when grown in nutrient solutions containing 5 or 10 ppm zinc for extended periods, shoots of SD-T showed no sign of injury, whereas those of SA-NT became stunted and chlorotic.

In vitro tolerance of pollen to copper or zinc

Mimulus. A comparison of germination and pollen tube length of M22-T and M24-NT is shown in Fig. 2. An analysis of variance of pollen germination showed that increasing copper concentration had a significant effect on percent germination in both clones, but that the difference in response of M22-T and M24-NT was highly significant ($F=5.38$, $df=5$, $P < 0.001$). In the tolerant clone, maximum pollen germination was at 0.1 µM Cu. There was an obvious reduction in percent

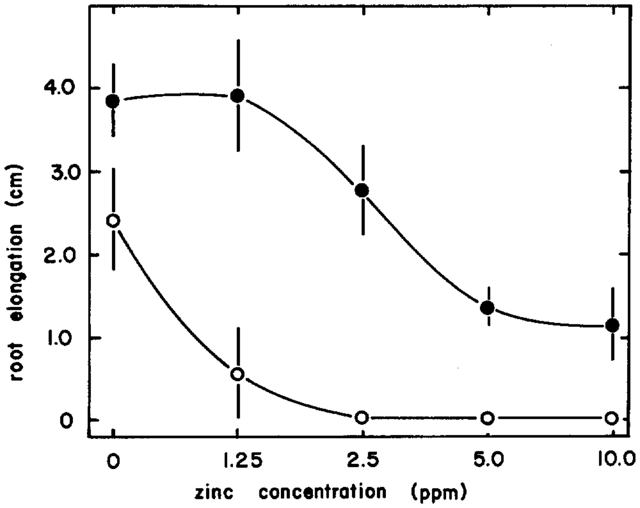

Fig. 1. Root elongation (cm) of *S. dioica* (SD-T, ● – ●) and *S. alba* (SA-NT, ○ – ○) at different zinc concentrations in 0.5 g/l $Ca(NO_3)_2$. Each point is the average of 2 (3 for 2.5 ppm Zn) experiments. The vertical bars are the standard errors

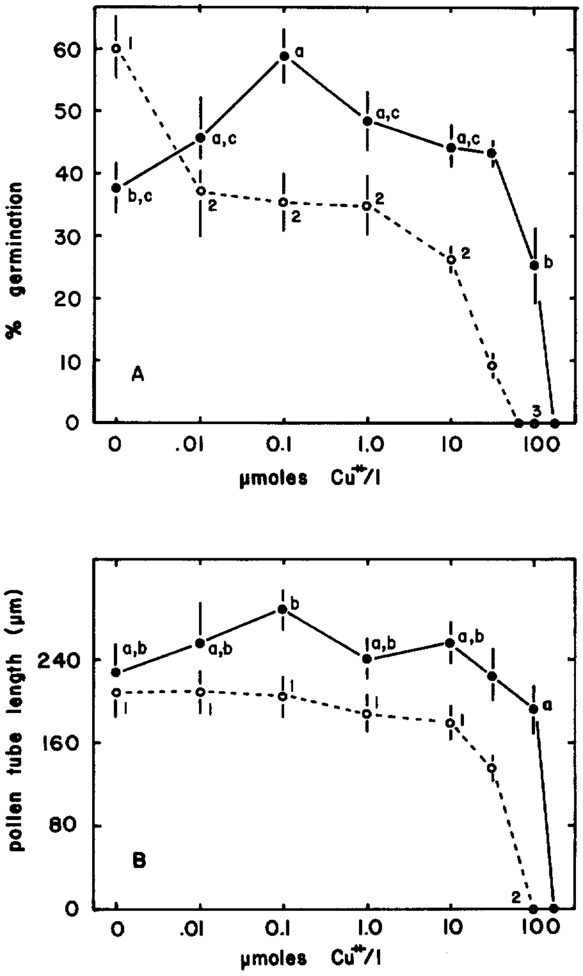

Fig. 2A, B. The effect of increasing copper concentration on percent germination and tube length of pollen from tolerant (M22-T) and non-tolerant (M24-NT) clones of *M. guttatus*. **A** Pollen germination (percent of potentially viable pollen); **B** Average pollen tube length (µm). M22-T (● – ●) M24-NT (○ – ○). M24-NT had 60% and M22-T 30% potentially viable pollen. Data are based on 5 complete replicates, done over a period of 2 years. *Note:* Data at 50 µM Cu were not included in the statistical analysis. Means within each clone with the same letter or number are not significantly different using Duncan's multiple range test ($\alpha=0.05$). Vertical bars are the standard errors

Table 1. Comparison of root length in solutions with, and without, added copper for each clone of *Mimulus guttatus* used as a pollen source. Data are the average length (cm) of the longest root in each solution. ($n=6$) The standard errors are in parentheses

Clones	Solutions	
	Control	0.5 ppm Cu
Nontolerant (M24-NT)	2.20 (0.18)	< 0.20
Tolerant (M22-T)	2.10 (0.31)	1.53 (0.14)
Copperopolis (C-T)	6.40 (0.23)	4.30 (0.17)

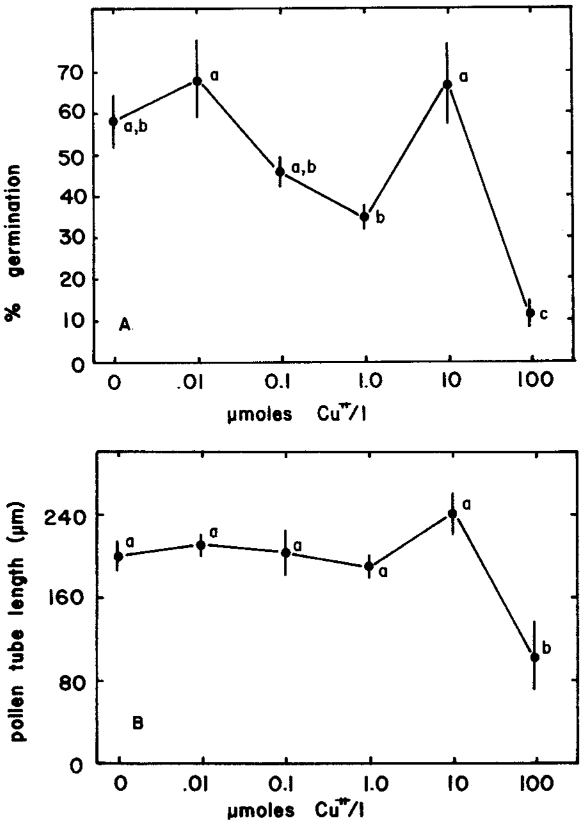

Fig. 3.A, B. The effect of increasing copper concentration on percent germination and tube length of pollen from the Copperopolis clone (C-T) of *M. guttatus*. **A** Percent germination (percent of potentially viable pollen); **B** Pollen tube length (µm). The data are the means of 4 replicates from one individual and 2 from a second. Fifty percent of the pollen was potentially viable. Means with the same letter are not significantly different using Duncan's multiple range test ($\alpha = 0.05$). Vertical bars are the standard errors

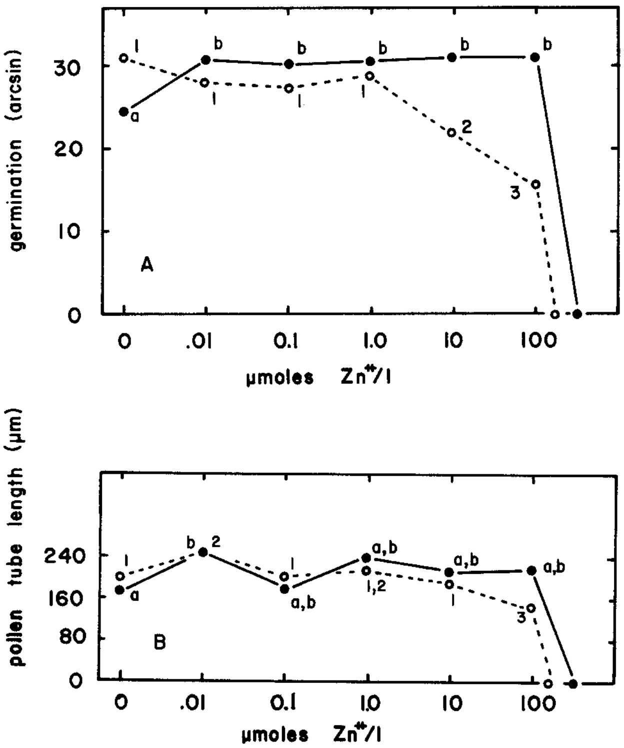

Fig. 4A, B. The effect of increasing zinc concentration on percent germination and tube length of pollen from *S. dioica* (SD-T) and *S. alba* (SA-NT). **A** Germination (arcsin); **B** Pollen tube length (µm). *S. dioica* (SD-T, ●–●), *S. alba* (SA-NT, ○–○). Potential pollen viability was about 90% in both species. The data for *S. alba* are the means of 6 replicates for all concentrations. For *S. dioica*, data are the means of 6 replicates at all but 0.01 and 0.1 µM Zn which are the means of 2 replicates. *Note:* Data at concentrations above 100 µM Zn were not included in the statistical analysis. Means within each clone with the same number or letter are not significantly different using Duncan's multiple range test ($\alpha = 0.05$)

germination at copper concentrations above 50 µM, but, even at 100 µM Cu, percent germination in the tolerant clone was not significantly different from that without added copper. In contrast, germination in pollen from nontolerant (M24-NT) source was significantly reduced by the addition of even 0.01 µM Cu. However, between 0.01 and 10 µM Cu, percent germination was insensitive to increasing copper. Copper became toxic at concentrations above 10 µM, and there was no germination at 100 µM Cu.

One-way analyses of variance on pollen tube growth showed that tube length in the tolerant pollen did not differ over the entire range of concentrations tested. In the nontolerant, there was no significant difference in tube length from 0–10 µM Cu (Fig. 2B). However, there was a highly significant difference ($F = 3.49$, $df = 5$, $P < 0.009$) when pollen tube lengths from M22-T and M24-NT were compared over equal increases in concentration from 0–100 µM Cu.

Copperopolis clone. In vitro pollen germination was tested for three families grown from seed collected at Copperopolis, California. All had germination maxima at concentrations other than 0 copper. Two individuals from one family (C-T) were selected for further study on the basis of an unexpected germination pattern shown in Fig. 3A. Pollen germination had two distinct peaks. These results indicate the possibility an individual with two sub-populations of pollen; one with a germination maximum at 0.01 µM Cu and no, or a very low, requirement for copper, and one with a germination maximum at 10 µM Cu and a requirement for copper. There was no corresponding pattern when tube length was measured (Fig. 3B). A one way analysis of variance indicated that mean tube length did not differ significantly from 0 to 10 µM Cu. As was the case with pollen of the nontolerant clone, copper became toxic above 10 µM Cu, but, unlike nontolerant pollen, some pollen grains did germinate at 100 µM Cu.

Silene. Low percent germinations are characteristic of trinucleate pollen in liquid culture (Brewbaker and Majumder 1961). A comparison of germination and pollen tube length of pollen from *S. dioica* and *S. alba* is shown in Fig. 4.

Silene dioica (SD-T) pollen showed a significant increase in percent germination with the addition of zinc, indicating a possible requirement for that element (Fig. 4 A). Germination was completely inhibited at a concentration of 500 μM. In *S. alba*, SA-NT, zinc had no significant effect on pollen germination up to 1.0 μM. However, germination declined steadily at higher concentrations, and was completely inhibited at 250 μM Zn. The results indicated that increasing zinc concentration from 10 to 100 μM reduced germination in (SA-NT) but produced no significant effect on pollen from SD-T.

For SA-NT, pollen tube growth rate (Fig. 4 B) appeared to be less sensitive to zinc than did pollen germination since germination was significantly reduced at 10 μM but there was no significant decrease in tube length until 100 μM Zn.

An analysis of variance comparing SD-T and SA-NT pollen response to equal increases in zinc concentration, (0 to 100 μM) indicated that, using either pollen germination ($F = 11.24$, $df = 5$, $P < 0.001$) or pollen tube length ($F = 2.82$, $df = 5$ $P < 0.025$) as a criterion, the two species differed significantly in their response to zinc.

Discussion

The data indicated that zinc tolerance in *Silene dioica* and *S. alba*, and copper tolerance in *Mimulus guttatus* is expressed in pollen and that its expression is related to that of the pollen source. Pollen from tolerant sources germinated and grew at concentrations of metal ions which markedly inhibited pollen from nontolerant sources. In this study, the expression of metal tolerance did not depend on whether the pollen was binucleate (*Mimulus*) or trinucleate (*Silene*).

Although percent germination appeared more sensitive to increasing metal ion concentration than did pollen tube length, the general response appeared to be one of relative insensitivity to increasing concentrations of heavy metals over a broad range, followed by an abrupt decline once a critical or threshold concentration had been reached. Thus, the pollen response resembled yield curves for dry matter production in response to heavy metals (Davis and Beckett 1977).

The percent germination response of pollen from the Copperopolis clone (C-T) of *M. guttatus* did not follow the general pattern. Instead, the data indicated the presence of two sub-populations of pollen with different germination requirements. In addition, the significant decrease in germination in the nontolerant clone of *M. guttatus* (M24-NT) at 0.01 μM Cu may also indicate a subpopulation of pollen that is very sensitive to copper. However, this drop in germination could also represent a less viable subpopulation of pollen within which any stress would reduce germination. That the ability of pollen tubes to grow did not always reflect the ability of pollen to germinate may indicate that if pollen grains germinated, they were often able to grow well. However, the apparent differential sensitivity may also be a function of the way in which germination and tube length were scored. Only pollen with tubes twice the diameter of the pollen grain were considered germinated and thus measured for tube length, so that germination as recorded here, actually included a growth component.

In both *Mimulus* and *Silene*, pollen from the tolerant clones germinated better with added metals than without, indicating a requirement for heavy metals might be present. However, a metal requirement was not demonstrated by root growth experiments, although some copper tolerant *M. guttatus* appear to have such a requirement (Allen and Sheppard 1971).

An association between sporophytic and gametophytic tolerance to heavy metals, as shown here, is of interest for at least two reasons. First, irrespective of the mechanism of expression, an association between the response of pollen and source to heavy metals might be used to screen for sporophytic resistance or sensitivity. Pollen provides a large sample which can be tested in a variety of conditions. Furthermore, testing can be done in hours rather than weeks or months. In addition, since pollen has most of the metabolic pathways (Stanley 1971) and many enzymes (Brewbaker 1971) of the more complex sporophyte, but a relatively simple developmental system, it might be useful in studying some aspects of the mechanisms of heavy metal tolerance.

Second, if metal tolerance in the pollen is a result of post-meiotic gene expression, microgametophytic selection might help account for the rapid development (Wu et al. 1975; Bradshaw 1982) of populations tolerant to heavy metals. The data in this paper did not permit a clear distinction as to whether metal tolerance in pollen was controlled by the genome of the pollen source, the sporophyte, being imposed on the gametes, as in sporophytic self-incompatibility systems (Heslop-Harrison et al. 1973; de Nettancourt 1977), or if the same genes are being expressed in both phases of the life cycle. In *Silene*, the germination percentage was too low to study the possibility of one or several sub-populations. However, data from the Copperopolis clones of *M. guttatus* indicated the existence of two sub-populations, implying gametophytic control. This problem is currently under study.

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