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Selection for tolerance to copper during pollen formation in *Mimulus guttatus* Fischer ex DC

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Abstract. In *Mimulus guttatus*, copper tolerance is determined largely by a single gene and is expressed in both the sporophyte and microgametophyte. This study explores the extent to which selection during pollen formation affects copper tolerance in the sporophytic generation. Two sets of plants heterozygous for copper tolerance, produced by reciprocal crosses between different copper-tolerant or sensitive families, and the plant on which the original observations were based, were cloned and grown in control or copper-supplemented solutions. Pollen viability and the number of tolerant progeny produced in backcrosses to sensitive plants were compared. In addition, the effect of copper treatment on pollen viability in vitro was compared for plants tolerant, sensitive and heterozygous for copper tolerance. The extent to which in vitro pollen viability decreased in response to copper treatment corresponded to the copper tolerance of the pollen source. When grown with added copper, four of the five plants showed significant reductions in pollen viability, ranging from 18% to 48% of control values. The reductions in pollen viability were correlated with an increase in tolerant progeny ($r = 0.679$, $P = 0.004$). Increases in tolerant progeny could be large, ranging from 119% to 170% of that of controls, but were usually smaller than was predicted from the reductions in viable pollen. In addition, plants derived from reciprocal crosses differed significantly in the extent to which pollen viability was decreased and sporophytic tolerance increased. Thus, while selection during pollen formation could increase sporophytic tolerance, sporophytic factors, perhaps including cytoplasmic or epigenetic ones, moderated the effectiveness of pollen selection for copper tolerance.

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Key words: Pollen selection – Pollen viability – Copper tolerance – *Mimulus guttatus*

Introduction

Many of the same genes are expressed in the diploid sporophyte and haploid microgametophyte of flowering plants (see Mascarenhas 1989, 1990; Ottaviano and Mulcahy 1989 for recent reviews). Thus, the possibility that selection during the microgametophytic stage of the plant life cycle could affect sporophytic traits has received considerable attention (Mulcahy 1979; Zamir 1983; Hormaza and Herrero 1992). While many of the genes expressed in both phases of the life cycle relate to general metabolic functions (Brewbaker 1971; Ottaviano et al. 1980; Weeden 1986), there are indications that genes relating to the tolerance of certain environmental stresses are also expressed in the sporophyte and microgametophyte (see Hormaza and Herrero 1992 for a recent review). Thus, pollen selection could affect sporophytic stress tolerance.

One period when pollen appears to be particularly sensitive to environmental stress is during pollen formation (the period following meiosis of the pollen mother cell until the development of the mature pollen grain just prior to anthesis). Stress associated with unfavorable microclimate (Jones 1976), drought (Woodell et al. 1977) or low nutrients (Schlichting 1986), temperature extremes (Zamir and Vallejos 1983; Patterson et al. 1987; Ahmed et al. 1992), air pollutants (Wolters and Martens 1987) and copper deficiency (Graham 1975) reduce pollen viability or pollen production. Even when stress is not sufficient to reduce pollen viability, stress may affect the

vigor and, consequently, the competitive ability of pollen grains (Young and Stanton 1990; Shivanna et al. 1991).

Previous studies with a single individual of *Mimulus guttatus* that was heterozygous for tolerance to copper indicated that selection during pollen formation could increase sporophytic tolerance to copper. In this case, growing plants in a copper-supplemented solution resulted in a 40–45% decrease in viable pollen and a 79% increase in tolerant progeny compared to control values (Searcy and Mulcahy 1986). Since copper tolerance in this species is determined primarily by a single major gene and is dominant (Macnair 1983), it appeared as if the decrease in pollen viability and the increase in tolerant progeny were closely correlated. Several other studies using either high temperatures (Mulinix and Iezzoni 1988) or a herbicide (Sari Gorla et al. 1989) have also found that selection during pollen formation affects sporophytic tolerance. However, there are also reports of little, if any, effect on sporophytic tolerances when stress was applied during pollen formation in vivo (Zamir and Vallejos 1983) or just after pollen dehiscence in vitro (Hodgkin 1990). Some of this variation may be due to the particular stress applied. However, there are indications that the sporophytic tissue has an important role to play in pollen viability (Ottaviano et al. 1982, 1988). Indeed, subsequent observations on pollen from different plants of *Mimulus guttatus*, that were heterozygous for tolerance to copper indicated considerable variability in the reduction in pollen viability in response to copper treatment. Therefore, the present investigation was undertaken.

In this study, the fluorochromatic reaction (FCR) (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison et al. 1984) was used to monitor pollen viability following copper treatment. The cell membrane has an important role to play in copper tolerance in *M. guttatus* (Strange and Macnair 1991), so the fluorochromatic reaction, which depends on membrane integrity (Heslop-Harrison et al. 1984), should be a particularly useful way to identify differences in copper tolerance in pollen grains. Comparisons were made for plants tolerant, sensitive or heterozygous for copper tolerance in vitro, and in vivo for plants heterozygous for copper tolerance. The effectiveness of selection during pollen formation was assessed by comparing the relative decrease in pollen viability of the treated clones to the relative increase in tolerant progeny produced when pollen from treated plants was used in crosses to sensitive plants.

Materials and methods

Most of the plants used in this study were grown from seed obtained from Dr. M. Macnair, University of Exeter, England. The stocks were produced by three generations of crossing heterozygous, copper-tolerant plants to a sensitive population from Stinson Beach, Calif. (see Macnair 1981 and Strange and Macnair 1991 for details). Tolerant plants from the last generation were selfed to produce the homozygous copper-tolerant (Macnair's families, J and B) and sensitive plants (Macnair's families, F and D) used in this study. Plants heterozygous for copper tolerance were produced by crosses among these families. Thus, the plants differed in copper tolerance, but otherwise shared the same genetic background. In addition, one plant (Plant 5 = C-T) heterozygous for copper tolerance which had been grown from seed collected at Copperopolis, Calif. was used. It had been included in previous studies (Searcy and Mulcahy 1986). All plants were screened to confirm their copper tolerance.

Effect of copper on pollen viability in vitro

These experiments were done in 1989 using one copper-tolerant and one sensitive plant as well as several plants heterozygous for tolerance to copper. These plants were grown in a standard potting soil.

For each individual, pollen was pooled from several flowers. Pollen was placed in 0.5 ml polypropylene centrifuge tubes and hydrated (Shivanna and Heslop-Harrison 1981) for 30 min at 20 °C. Next, 200 µl of germination medium containing 0.7 *M* sucrose, 1.62 m*M* H_2BO_3 and 20 m*M* MES (Sigma), pH 5.5, was added to one tube (control), and the same medium plus 0.5 m*M* $CuSO_4 \cdot 5H_2O$ was added to the other (treatment). Calcium was omitted to prevent germination since germinating pollen grains showed a range of intensities of fluorescence making scoring difficult. In addition, by omitting calcium, the contrast between fluorescing and non-fluorescing grains was enhanced. Tubes were placed on a rotary shaker at 20 °C for 2 h. Finally, fluorescein diacetate stock solution diluted with germination medium was then added to the pollen samples. After 10–15 min, two 50-µl samples were removed, and the percentage of brightly fluorescing pollen grains was scored on coded samples for 200 grains in each drop. Differences in response to the treatments were compared with a two-way analysis of variance using Systat (Wilkinson 1988). Data were transformed using the arcsine square root transformation prior to analysis.

Effects of copper on pollen viability in vivo

Five plants heterozygous for tolerance to copper were used as pollen sources. Plants 1 and 2 were derived from reciprocal crosses between one set of copper-tolerant (family J) or sensitive (family F) parents, and 3 and 4 from reciprocal crosses with a different set of parents, (families B and D). For both pairs, reciprocal crosses were between full sibs of the same family rather than between the same two individuals. Experiments with plant 5 were conducted as described below but were done in 1989. Crosses using pollen from this plant were made to a full sib of one of the sensitive plants used in the backcrosses for plants 1–4.

Treatment of the pollen sources

Each pollen source was vegetatively cloned to obtain two plants. One plant was grown in quartz sand in one-quarter strength Hoagland's solution alone (control), the other was grown in the same solution to which 2 ppm copper was added. The treatment was started on March 19 and terminated May 10 1991. Pollen was collected and crosses made between April 29 and May 10. Plants were maintained in a greenhouse and watered as needed with the appropriate solution.

Pollen from treated and control clones were collected the same day from freshly dehisced anthers. Some pollen was used to make crosses to copper-sensitive plants which were grown in a standard potting soil. The remainder was placed in 0.5 ml

polypropylene centrifuge tubes, hydrated for 30 min at 20 °C, then suspended in the standard germination medium described above but to which fluorescein diacetate (Heslop-Harrison et al. 1984) had been added. The sample was thoroughly mixed and after about 10 min, two 50-µl samples of the suspension were added to a single slide. Pollen was scored on coded samples for 200 pollen grains in drop. Each experiment was repeated 3–5 times. The effect of treatment and pollen source on the proportion of viable pollen was analyzed with a two-way analysis of variance using Systat (Wilkinson 1988). Data were transformed with the arcsine square root transformation prior to analysis.

Progeny tests

Approximately 50 seedlings were tested from four pairs of capsules resulting from crosses using pollen from the treated or control clone of each pollen source (two pairs of capsules for plant 5). Seedlings from 36 capsules were tested in all. Because of space considerations all seeds could not be planted at the same time. However, capsules produced from each treated and control clone and its reciprocal were started at the same time. After 2 weeks, 55–60 seedlings were transplanted to 18 × 36 cm trays and grown for an additional 4–6 weeks. The plants were cut at soil level, trimmed to a uniform size and suspended in 5 l of 0.5 ppm copper, added as $CuSO_4 \cdot 5H_2O$, and 0.5 g/l $Ca(NO_3)_2$ (Macnair 1983). At this concentration, copper-sensitive plants fail to root or produce short discolored roots, while tolerant plants produce normal looking roots that are usually longer than 2 mm. Testing was done under fluorescent lights with 12-h days and a temperature of 20–23 °C. The solutions were aerated continuously. After 6 days, cuttings were removed and the length of the longest root measured.

For each pollen source, differences in the number of tolerant and sensitive progeny produced by pollen from treated and control clones were tested using a 2 × 2 contingency chi-square test. For each pollen source, the four separate 2 × 2 chi-square tests were combined into a single test using the methods given in Everitt (1977). The square root of each chi-square was calculated and given a sign depending on the direction of the difference in the proportion of tolerants in the two treatments. These chi-squares are approximately normally distributed with a mean of zero and a unit standard deviation. The Z statistic,

$$Z = \sum_{i=1}^{g} \chi_i g^{-1/2}$$

where g is the number of independent 2 × 2 tables, provides an overall test of the null hypothesis that no consistent differences in proportion of copper-tolerant and sensitive progeny occur. The significance of Z was tested using tables of the standard normal distribution.

Results

In vitro pollen response to copper treatment

When treated in vitro (Fig. 1), pollen from copper-tolerant and sensitive sources showed a highly significant difference in response to copper treatment (pollen source × treatment; $F_{1,8} = 46.2$, $P < 0.001$). The percentage of viable pollen from the sensitive source decreased to about 12% of the control value, while pollen from the copper-tolerant source showed a slight but not statistically significant increase in the percentage of brightly fluorescing pollen. Pollen from heterozygous sources (Fig. 2), treated in vitro, also showed significant decreases in fluorescent pollen (ANOVA, copper treatment: $F_{1,12} = 27.9$, $P < 0.001$). These decreases, which ranged from 32–48% of the control value, appeared to be intermediate to those of the copper-tolerant or sensitive source.

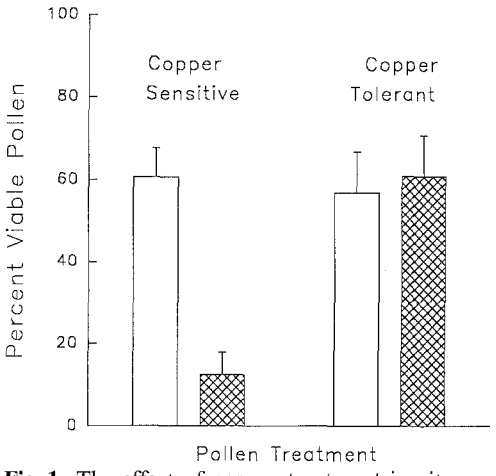

Fig. 1. The effect of copper treatment in vitro on the percent viable pollen from plants homozygous for their respective tolerances. Percent viable pollen was measured after 2 h in standard germination medium (*open bars*), or after 2 h in the same medium to which 0.5 m*M* copper was added (*hatched bars*). Means are the average of two separate trials (200 grains each) for tolerant plant and three trials for the copper-sensitive plant. *Vertical bars* are the standard errors

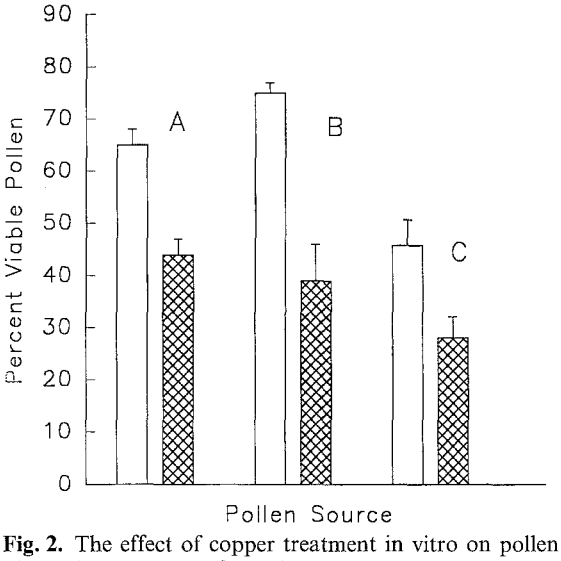

Fig. 2. The effect of copper treatment in vitro on pollen from plants heterozygous for tolerance to copper. Heterozygous *plant A* was produced by a cross between a copper-tolerant female (family J) and copper-sensitive male (family D). Heterozygous *plant B* was produced by a cross between a copper-sensitive female (family F) and a copper-tolerant male (family B). *Plant C* is plant 5 and was collected from Copperopolis, Calif. (see text). Means are the average of two separate trials for plants A and B and five for plant C. *Open bars* are the control; *hatched bar*, copper-treated sample. *Vertical bars* are the standard errors

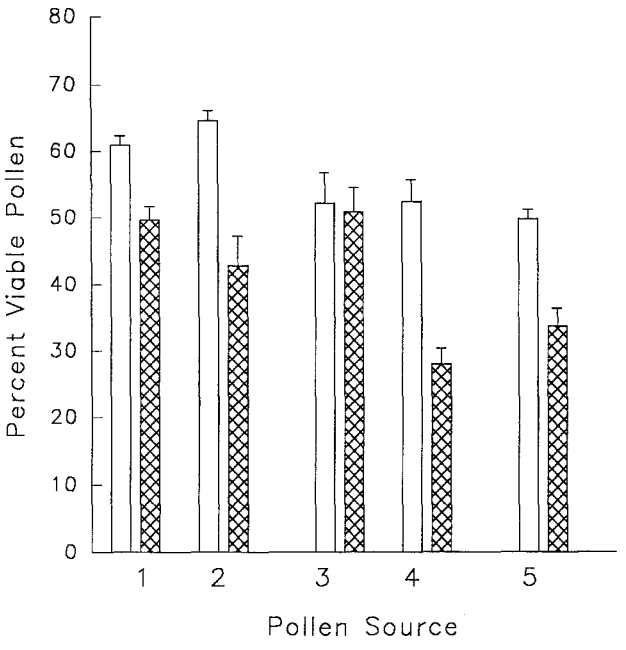

Fig. 3. The effect of copper treatment in vivo on the percent viable pollen for plants 1–5. These plants are all heterozygous for tolerance to copper. Pollen viability was measured at anthesis for plants cloned and grown in one-quarter strength Hoagland's solution alone (*open bars*) or in Hoagland's solution supplemented with 2 ppm copper (*hatched bars*). *Plants 1* and *2* were produced by reciprocal crosses between families J, copper tolerant, and F, copper sensitive. *Plants 3* and *4* were produced by reciprocal crosses between families B, copper tolerant and D, copper sensitive. For plants 1 and 3 the female parent was copper tolerant, and for plants 2 and 4 the female parent was copper sensitive

Table 1. The number of copper-sensitive (non-rooting, NR) or copper-tolerant (rooting, R) seedlings/capsule following crosses using pollen from plants cloned and grown in control or copper-supplemented conditions. Crosses were made to two copper sensitive plants

	Treatment of the pollen source				Square root chi-square	Direction[a] +/−
	Control		2 ppm copper			
	NR	R	NR	R		
Plant 1	27	20	24	25	0.691	+
(T × S)	29	24	35	18	1.420	−
	27	25	30	24	0.140	−
	31	20	31	22	0.057	+
Plant 2	35	25	29	26	0.366	+
(S × T)	35	18	29	23	1.160	+
	31	23	25	26	0.741	+
	26	27	22	30	0.481	+
Plant 3	25	27	28	24	0.588	−
(T × S)	22	30	28	25	1.080	−
	22	26	32	22	1.360	−
	20	32	18	34	0.407	+
Plant 4	17	35	13	40	0.925	+
(S × T)	26	27	27	25	0.238	−
	34	18	27	27	1.600	+
	24	24	13	39	2.580	+
Plant 5	37	19	16	20	4.192	+
	19	09	12	16	3.540	+

[a] + indicates an increase in tolerant progeny compared to the control

In vivo pollen viability of treated and control plants heterozygous for tolerance to copper

Pollen viability was also reduced relative to the untreated control clones (Fig. 3) when heterozygous plants 1–4 were grown with added copper. However, an analysis of variance indicated a significant treatment × pollen source interaction, so each clone was tested separately with an analysis of variance. Clones 1 ($F_{1,18}=19.7$, $P<0.001$), 2 ($F_{1,12}=22.1$, $P<0.001$) and 4 ($F_{1,12}=35.5$, $P<0.0001$) showed significant decreases in viable pollen compared to control values. Pollen viability was also significantly reduced in Plant 5 ($F_{1,6}=110$, $P<0.0001$) when grown with added copper.

An interesting pattern emerged when the decrease in pollen viability for the two pairs of plants derived from reciprocal crosses was compared (Fig. 3). For both pairs of pollen sources, whether the maternal parent was copper tolerant (T) or sensitive (S) had a significant effect on the decrease in pollen viability when the plants were grown in a copper-supplemented solution [(plants 1 and 2, treatment × source: $F_{1,28}=4.74$, $P=0.038$) and for the second pair (plants 3 and 4, treatment × source: $F_{1,30}=8.53$, $P=0.007$)]. In the first pair, both plant 1 (T × S) and 2 (S × T) showed a decrease in viable pollen when grown in copper-supplemented solutions. However, viable pollen decreased only by 19% when the pollen source was (T × S) but by 34% when the source was (S × T). For the second pair, pollen viability was not significantly reduced when plant 3 (T × S) was grown with added copper but for its reciprocal, plant 4 (S × T), the percentage of viable pollen declined to 48% of the control value when grown with added copper.

Effect on the sporophytic generation

If the decrease in pollen viability was specific for tolerance to copper, I expected an increase in copper-tolerant progeny comparable to the decrease in viable pollen. This appeared to be true of plant 5 (Fig. 3; Table 1). In this case, a decline in viable pollen of 34% relative to the control produced a highly significant increase ($Z=2.8$, $P=0.01$) of 70% in copper-tolerant progeny. However, for plants 1–4, although there was a significant correlation ($r=0.679$; $P=0.004$) between the decrease in viable pollen and the relative increase in proportion of

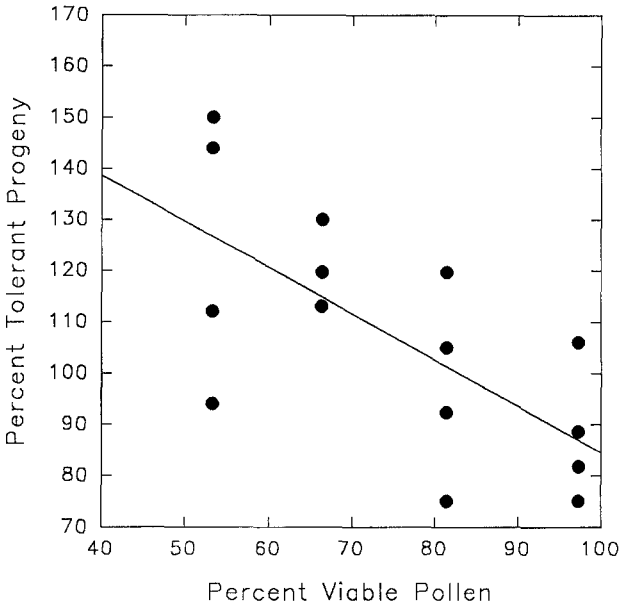

Fig. 4. Percent viable pollen and proportion of tolerant progeny produced from plants 1–4. Both percent viable pollen and proportion of tolerant progeny are expressed as a percentage of the control value

copper-tolerant progeny (Fig. 4), the decline in viable pollen accounted for just under half of the variance ($r^2 = 0.462$). Pollen from treated plants produced significant increases in tolerant progeny for plant 2 ($Z = 1.6$, $P = 0.05$) and plant 4 ($Z = 2.5$, $P < 0.01$) compared to the untreated controls (Table 1). As with the reduction in viable pollen, plants derived from reciprocal crosses differed in response to treatment when the numbers of tolerant progeny were compared. For reciprocals 3 and 4 there was no significant difference in numbers of tolerant progeny produced when the pollen came from control plants, but there was a highly significant difference when the pollen came from treated plants (Fishers exact test: 6.04; $P = 0.007$). The same pattern was apparent for reciprocals 1 and 2, but the difference in numbers of tolerant progeny when plants were treated was not statistically significant (Fishers exact test 2.02, $P = 0.07$). Altogether, increases in number of tolerant progeny were found in seven out of eight capsules for treated pollen sources in which the female parent had been copper sensitive (plants 2 and 4) but in only three out of eight capsules for treated pollen sources in which the female parent had been copper tolerant (plants 1 and 3) (Table 1).

Discussion

The difference in fluorescent and, presumably viable pollen (Shivanna and Heslop-Harrison 1981; Heslop-Harrison et al. 1984), following copper treatment in vitro suggests that copper affected the membrane integrity of pollen from copper-sensitive but not copper-tolerant plants of *Mimulus guttatus*. These results are consistent with the observation of Strange and Macnair (1991) that differences in the cell membrane are likely involved in copper tolerance in this species. In their study, copper increased the leakage of potassium from cells of the root tips of sensitive but not tolerant plants. In addition, since pollen from heterozygous sources showed intermediate reductions in viable pollen, it appeared that pollen grains within a single plant differed with respect to copper tolerance as predicted if copper tolerance were independently expressed in the microgametophyte. Since differences in the effect of copper on membranes may also be involved with copper tolerance in *Silene cucubalus* (De Vos et al. 1989), it would be interesting to see if this effect also extended to pollen in this species as well.

Although it seems likely that the mechanism of copper tolerance in pollen and sporophyte are the same, a higher concentration of copper was used in the experiments with pollen than in those producing differences in potassium leakage in roots (Strange and Macnair 1991). Other studies have also reported that a higher concentration of toxic agents was required to produce an effect in pollen (Bino et al. 1988; Searcy and Mulcahy 1990). However, at least in this case, the concentration of copper may have been higher than necessary to produce a measurable difference. Concentrations of copper similar to those used by Strange and Macnair (1991) reduce the germination level, of pollen from sensitive sources by at least 50% while having no significant effect on the germination of pollen from tolerant sources (Searcy and Mulcahy 1986). The concentration used for these experiments was chosen because tolerant pollen can recover at least some of its ability to germinate at this concentrations, while pollen from sensitive sources does not (Searcy 1984).

The *in vitro* studies indicated that copper treatment could produce reductions in viable pollen depending on the copper tolerance of individual pollen grains. However, comparison of both the reduction in pollen viability and the increase in copper-tolerant progeny indicated that the pollen response *in vivo* was complex. For plant 5, most of the decrease in pollen viability appeared to be related to copper tolerance. In this case, a 34% reduction in viable pollen resulted in a 70% increase in copper-tolerant progeny. This is similar to the previously reported response of the same plant (Searcy and Mulcahy 1986). However, for plants 1–4, the increases in tolerant progeny were smaller than expected based on decreases in pollen viability. For example, when pollen viability was decreased to almost 50% of the control value, rather than producing all copper-tolerant progeny, the proportion of tolerant progeny only increased 22%. This suggests that some of the decrease in pollen viability was not

directly related to the copper tolerance of the individual pollen grains. Although there were no obvious effects on the parent plant of growing in copper-supplemented conditions, pollen development can be affected by stress, which shows very little effect on the sporophytic generation (Harry and Graham 1981).

What was surprising was that pollen viability and the proportion of copper-tolerant progeny differed in response to treatment between plants derived from reciprocal crosses. Differences in plants derived from reciprocal crosses are frequently attributed to cytoplasmic factors (Grun 1976). Indeed, cytoplasmic effects on pollen viability and stress tolerance are well known (Grun 1976; Laughnan and Gabay 1983). However, differences in copper tolerance for reciprocal crosses have not been reported for *M. guttatus* (Allen and Sheppard 1971). More importantly, all of the reciprocals used in this study shared the same cytoplasmic background. Thus, at this point, it is difficult to interpret the differences observed in terms of cytoplasmic effects.

It is also possible that the differences between reciprocals in response to copper treatment reflect individual variation in copper tolerance among the heterozygous plants. The pollen sources were not true reciprocals. That is, they were not produced by reciprocal crosses between the same two individuals but were reciprocal crosses between different full sibs. Nevertheless, the pollen sources did not appear to differ between reciprocals under control conditions either in pollen viability (Fig. 2, plants 1 and 2, or plants 3 and 4) or in the proportion of tolerant plants produced (Table 1). In addition, each pair of reciprocal pollen sources was selected after screening for copper tolerance, and the particular individuals selected were chosen because they produced roots of comparable length (1.5–2.0 cm long) in the same solution used to test the progeny. Thus, there appeared to be very little indication of differences between reciprocals at the sporophytic level.

Thus, in *Mimulus guttatus*, microgametophytic selection during pollen formation was effective at increasing copper tolerance in the sporophytic generation. The mechanism appeared to be a differential loss of viability that depended on the copper tolerance of individual pollen grains. However, the effectiveness of selection varied among individuals. For some plants, the effectiveness of selection was weakened by a general reduction in viable pollen when the sporophyte was treated. For others, there were also indications of buffering from a direct response to environmental stress. This could involve some epigenetic effect and is an area that could use further investigation. Clearly, the results based on small numbers of sporophytes should be interpreted cautiously. Nevertheless, for some plants of *M. guttatus*, selection during pollen formation had large effects on the proportion of tolerant progeny in the sporophytic generation. In these cases, selection was 2–10 times more effective than at other times during the reproductive cycle (Searcy and Macnair (1993))

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