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Impatiens Pollen Germination and Tube Growth as a Bioassay for Toxic Substances

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Pollen of *Impatiens sultanii* Hook F. germinates and forms tubes rapidly at 25°C in a simple medium containing 111.0 ppm $CaCl_2$, 13.6 ppm KH_2PO_4, and 1000 ppm boric acid. Calcium, potassium, and boron are essential for germination and tube growth, but sucrose is not required. Pollen tubes grow with equal rapidity in liquid medium or on a medium solidified with 1% agar. Tube growth rates are linear for 1 hr. When different pollen sources or clonal sources are utilized, no variation in pollen tube growth is observed, and pollen from individual flowers remain viable for 26 hr. Formaldehyde inhibits pollen germination, tube production, and tube lengths at 7.5-10 ppm. With 2,4-dichlorophenol, pollen germination and tube production is inhibited at 0.5-20 ppm, while tube growth is inhibited significantly at 25 ppm. A biphasic inhibition of germination and tube formation occurs with *p*-cresol with a low level of inhibition occurring at 40-60 ppm and a higher one at 100-125 ppm. Tube lengths were inhibited at 150 ppm *p*-cresol. Acrylamide and dioctyl phthalate have no measureable effect upon pollen germination and tube growth.

Introduction

Pollen germination and tube growth of some flowering plants are sensitive to toxic substances found in the environment. Certain insecticides (*1*), herbicides (*2, 3*), toxic gases, SO_2 (*4, 5*), O_3 (*6, 7*), NO_2, acrolein, and formaldehyde (*8*) severely inhibit both pollen germination and tube elongation. Except for the fact that pollen from different flowers of the same plant often give variable results (*9-12*), this critical reproductive process of flowering plants does possess great potential as an important bioassay for suspected toxic substances. The objective of this investigation has been to explore this potential utilizing the pollen of Impatiens.

Materials and Methods

Flowering plants of *Impatiens sultanii* Hook F. were maintained in the greenhouse as a source of pollen and cuttings for the establishment of clonal populations of plants. Flowers with dehisced anthers were removed from plants and maintained on a moist filter paper in a petri dish at 25°C for the duration of the experiment. Using a dissecting needle, pollen was transferred from the anthers to a drop of the basal medium containing 111.0 ppm $CaCl_2$, 13.6 ppm KH_2PO_4, and 1000 ppm boric acid on a depression slide, dispersed in the liquid, and covered with a cover glass. Usually one flower would provide enough pollen to complete an entire experimental series. To retard evaporation of the incubation medium, the depression slide was kept in a closed plastic box containing moist paper toweling in a growth chamber with a light intensity of 6 J/m^2-sec at 25°C. Since the pollen tubes continued to elongate after the 15-min incubation period, photomicrographs taken in a random manner were the only effective method of terminating an experiment. The number of pollen grains photographed and analyzed ranged from 25 to 190.

Percentages were determined by counting the total number of grains, the number of germinated grains, grains producing tubes longer than 40 μm and lysed grains from projected 35 mm negatives. With little attention to pollen tube orientation, pollen tubes first were traced on a sheet of paper from the negatives. Curved tubes were subdivided along their lengths into small linear portions. Using

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a lighted tracing table, all tubes then were transferred to graph paper (10 mm to 1.0 cm), and by rotating the graph paper, the individual linear portions of a curved tube were arranged end to end along the vertical axis of the graph paper. Tube lengths were counted in millimeters with 1.0 mm equalling 10 μm of pollen tube. The mean tube lengths and range of variation as a standard deviation were calculated.

To investigate the growth of pollen tubes on a solid medium, 1% agar was added to the basal medium and autoclaved. Several drops of the hot medium were dispensed on depression slides, and pollen was inoculated onto the surface of the cooled solidified medium and incubated as described previously. The effects of some selected toxic substances upon pollen germination and tube growth were investigated by adding various concentrations of methanol-free formaldehyde, 2,4-dichlorophenol, *p*-cresol, acrylamide, and dioctyl phthalate to the aqueous basal incubation medium.

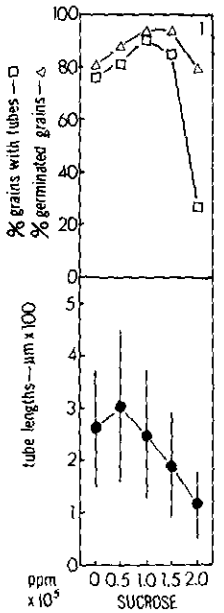


FIGURE 1. Effect of various concentrations of sucrose on pollen germination, tube production, and tube lengths.

Results

Basal Medium

During preliminary experiments, Impatiens pollen was incubated in a slightly modified medium containing 10^5 ppm sucrose, 1109.9 ppm $CaCl_2$, 6.8 ppm KH_2PO_4, and 100 ppm boric acid which was originally developed for Tradescantia by Mascarenhas (*13*). Since this medium supported pollen germination and tube elongation, the individual components were varied in an attempt to maximize the response by Impatiens pollen.

The addition of sucrose at various concentrations to the medium had little effect upon pollen germination and no significant effect upon the final length of the tubes (Fig. 1). Only 2.0×10^5 ppm sucrose caused a substantial reduction in the percentage of pollen forming tubes. Pollen incubated in a medium without sucrose did not produce any multiple tubes. However, 15, 24, 27, and 13% of the pollen formed from two to four tubes when grown in medium containing 0.5, 1.0, 1.5, or 2.0×10^5 ppm sucrose, respectively. In order to eliminate multiple tubes and standardize the growth response of tubes, sucrose was deleted from the medium.

With no calcium in the medium, few pollen grains germinated and only 2% produced tubes (Fig. 2). Maximal germination and tube production was achieved in a medium containing 111.0 ppm $CaCl_2$. Whereas these two growth parameters were reduced substantially with 1109.9 ppm $CaCl_2$, the final tube lengths achieved on this medium did not vary significantly from the medium containing 111.0

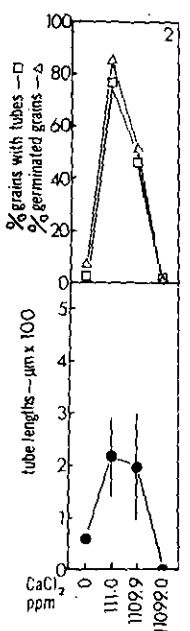


FIGURE 2. Effect of various concentrations of $CaCl_2$ on pollen germination, tube production, and growth.

ppm $CaCl_2$. Germination and tube production was completely inhibited in medium containing 11099.0 ppm $CaCl_2$.

When KH_2PO_4 was deleted from the medium, few grains germinated and even fewer developed tubes (Fig. 3). However, there was little difference in the response of the pollen when the concentra-

tion of KH_2PO_4 was varied from 1.36 to 13.6 ppm. Potassium was more important for pollen germination and tube growth than phosphate. There was no difference in pollen germination and tube growth when potassium was added as KCl at a concentration equal to that of 6.81 ppm KH_2PO_4.

When boric acid was deleted from the medium, only 33% of the pollen germinated and only 29% produced short tubes (Fig. 4). When 10, 100, or 1000 ppm boric acid was added to medium, a marked increase in the percentage of grains germinating and producing tubes occurred. However, the final tube lengths achieved in medium containing 1000 ppm boric acid were significantly better than those in medium containing the other two concentrations of boric acid. Higher concentrations of boric acid severely inhibited germination, tube production and growth.

A more sophisticated series of experiments were conducted to determine the interrelationship between the essential components of the medium (Fig. 5). Varying three concentrations of KH_2PO_4 or two concentrations of $CaCl_2$ and keeping the concentration of boric acid constant had little effect upon pollen germination, tube production and final tube lengths. However, when the concentration of boric acid was varied, an interesting pattern emerged. There was a reduction in most cases in pollen germination, tube production and final tube lengths when 10 ppm boric acid was added to the various media.

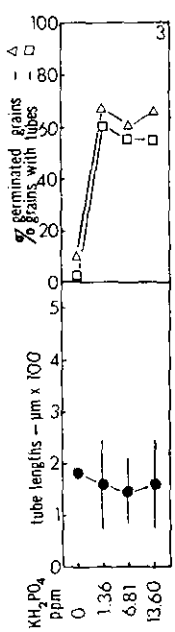


FIGURE 3. Effect of various concentrations of KH_2PO_4 on pollen germination, tube production, and growth.

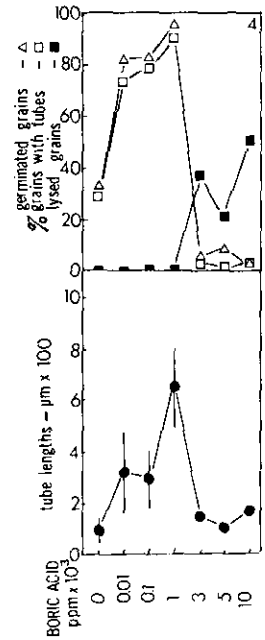


FIGURE 4. Effect of various concentrations of boric acid on pollen germination, grain lysis, tube production, and tube lengths.

For Impatiens pollen, the best basal medium contained 111.0 ppm $CaCl_2$, 13.6 ppm KH_2PO_4, and 1000 ppm boric acid.

Solid Medium

When pollen was sown on the basal medium solidified with 1% agar, there were fewer germinated grains and tubes produced and more lysed grains than in the liquid medium (Fig. 6). However, there was no significant difference in the lengths of pollen tubes.

Temperature, Light, and Darkness

When pollen grains were incubated in the basal medium at 10°C, only 49% of the grains germinated and only 39% of them produced tubes (Fig. 7). At 15°C, 57% of the pollen germinated and 54% produced tubes. However, as the temperature was increased to 20, 25, or 30°C, most of the pollen germinated and formed tubes. At 35°C only 6% of the grains germinated and formed tubes, while 53% of them lysed. Final tube lengths increased as the temperature increased, achieving a maximum length at 25 or 30°C with more variation in tube lengths occurring at 30°C than at 25°C.

Incubation of the pollen in the dark resulted in only a slight increase in the percentage of germi-

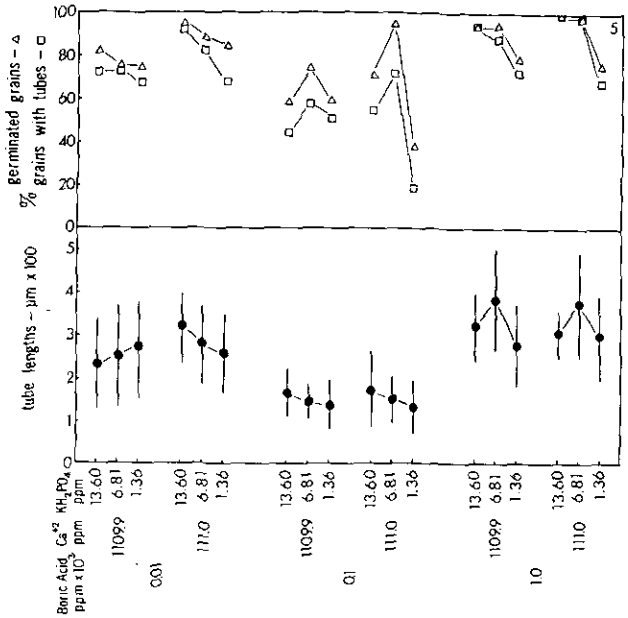


FIGURE 5. Effect of various concentrations of KH_2PO_4, $CaCl_2$, and boric acid on pollen germination, tube production, and growth.

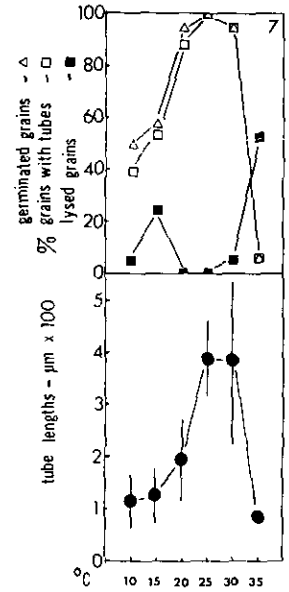


FIGURE 7. Effect of various temperatures on pollen germination, grain lysis, tube production, and growth.

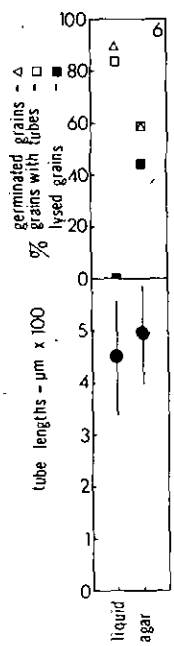


FIGURE 6. Germination, lysis, tube production and growth of pollen in a liquid or on a solid medium.

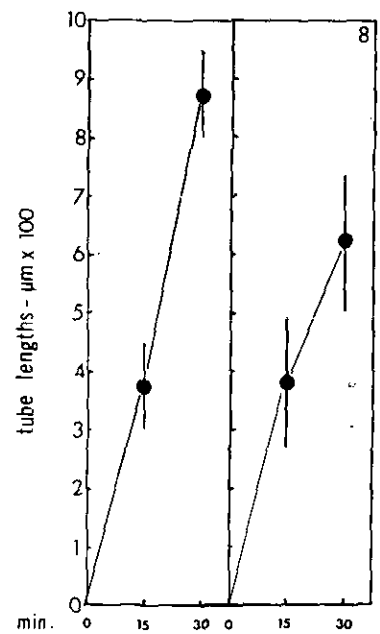


FIGURE 8. Growth of pollen tubes incubated up to 30 min.

nated grains and tube production but had no effect upon the final length of the tubes.

Growth Rates

Two pollen preparations were photographed after 15 min and then incubated for an additional 15 min (Fig. 8). The rate of pollen tube elongation was linear or nearly so during the extended incubation period. For one additional preparation, the incubation period was extended to 2 hr (Fig. 9). The rate of pollen tube growth was linear for the first hour but then declined during the second hour. Furthermore, the variability in the final tube lengths

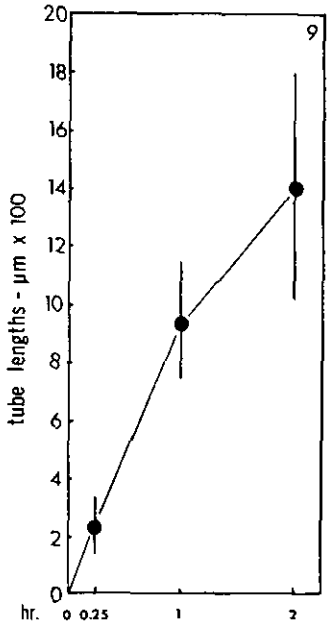


FIGURE 9. Growth of pollen tubes incubated up to 2 hr.

increased dramatically as the length of incubation period was extended.

Viability of Pollen

Pollen was gently removed from a flower at various times after anthesis and incubated in the basal medium (Fig. 10). At anthesis, pollen germination and tube production was low; however, there was considerable improvement by 3 hr after anthesis. Tubes achieved similar lengths during the first 10 hr after anthesis but then increased, reaching a maximum at 26 hr. However, the variability of the tube lengths also was very large at 26 hr. By 28 hr, no pollen germinated and formed tubes.

Variation among Plants and Clones of Plants

Five different Impatiens plants were used to establish clones. One clone produced inviable pollen and was discarded. When individual members of any one clone were compared, variation in the percentage of germinated grains and those forming tubes was evident; however, most of the germinated pollen did form tubes (Fig. 11). There was no significant difference in the final lengths of the pollen tubes when individual members of any particular clone was compared or when clones 2, 3, 4, 5, and 6 are compared to one another. The pollen of clone 1 germinated and produced tubes at a much slower rate, the final lengths being those achieved

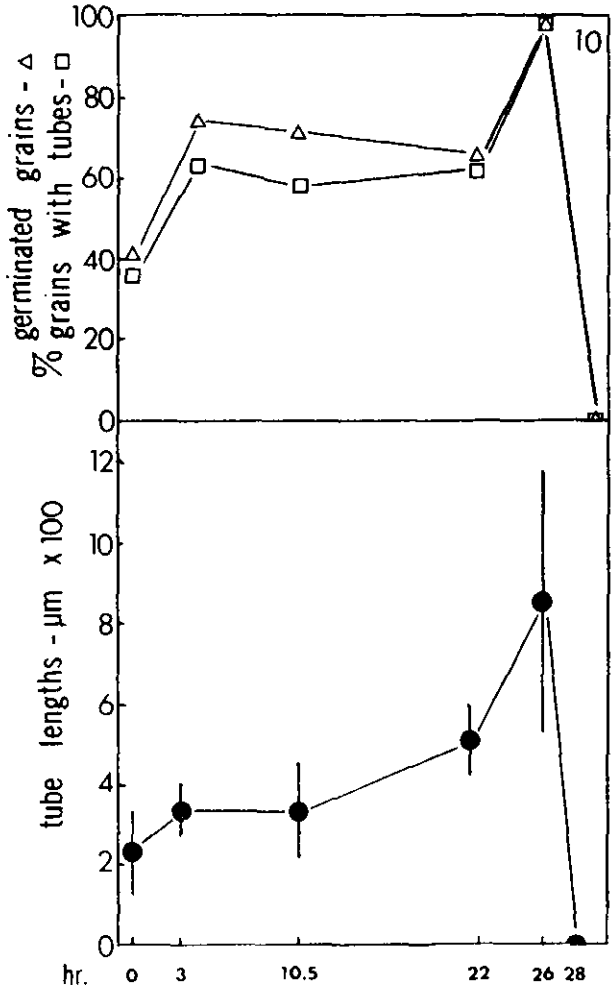


FIGURE 10. Germination, tube production, and growth of pollen removed from a flower at various times after anthesis.

after 30 min of incubation and not the customary 15 min.

Selected Toxic Substances

Formaldehyde—Little variability in the sensitivity of the pollen to formaldehyde was observed when different pollen sources were used (Fig. 12). The addition of 1 ppm formaldehyde to the basal medium had little effect upon germination, tube formation or final tube lengths for both pollen sources. With 5 ppm. there was a slight decline in pollen germination and tube production and an increase in the percentage of lysed grains (8-12%). There was no significant difference in the final tube lengths, but 16-27% of those tubes lysed after achieving some growth. With one pollen source, (Fig. 12*a*) 7.5 or 10 ppm formaldehyde resulted in a further decline in pollen germination and tube

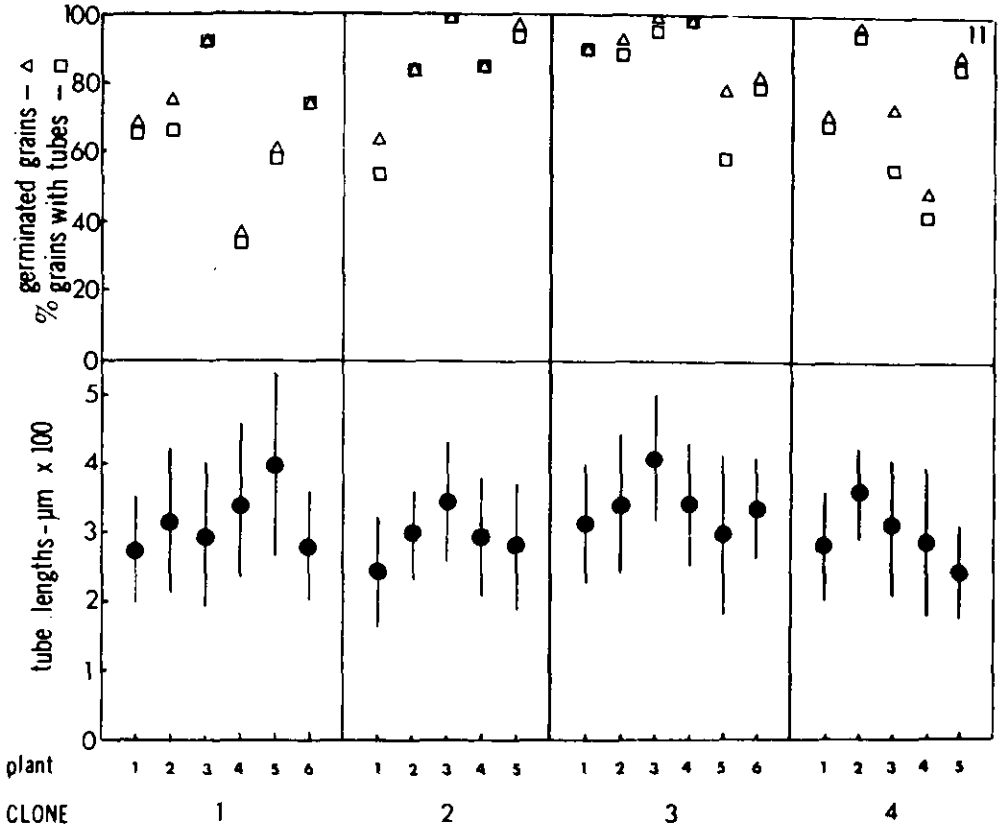


FIGURE 11. Germination, tube production, and growth of pollen from different plants and clones of plants.

formation even though slightly more germination and tube production occurred in medium containing 10 ppm than 7 ppm formaldehyde. With the other pollen source, (Fig. 12*b*), germination and tube formation was uniformly low at both of these concentrations. The percentage of lysed grains in 7.5 ppm formaldehyde was only 6% for one pollen source and 29% for the other, but at 10 ppm, the percentage of lysed grains was 35 and 70% for each source. With both sources, there was a significant reduction in final length of the pollen tubes at 7.5 ppm formaldehyde. Even elongating tubes did not sustain growth. From 38 to 50% and from 57 to 100% of the tubes lysed with 7.5 and 10 ppm formaldehyde. respectively. A 50% reduction in pollen germination, tube production and tube lengths occurred at a concentration of 7.5-10 ppm formaldehyde (Table 1).

2,4-Dichlorophenol. With 2,4-dichlorophenol, there was some variation in the sensitivity to the toxic substance when different sources of pollen were utilized (Fig. 13). The pollen from some plants was sensitive to the substance, while pollen from others was less so. With sensitive pollen (Fig. 13*a*), the addition of 0.5, 1.0, or 5.0 ppm 2,4-dichlorophenol to the basal medium inhibited germination and tube production and caused 42-51% of the pollen to lyse but did not significantly inhibit tube growth. When the concentration of 2,4-dichlorophenol was 7.5 ppm, 30% of grains lysed. 46% germinated, but only 3% produced tubes. With 10 ppm, there was a further reduction in the percentage of germinated pollen to 10% and an increase in the percentage of lysed grains to 71%. With 7.5 and 10 ppm, the final tube lengths of the few tubes produced did not differ from those of the control. With less sensitive pollen (Fig. 13*b*), a significant decline in germination and tube production occurred when 20 ppm 2,4-dichlorophenol was added to the basal medium. The percentage of lysed grains (75%) also increased dramatically at that concentration, but final tube lengths did not significantly differ from that of the control. A concentration of 25 ppm 2,4-dichlorophenol completely inhibited tube production and growth. For both pollen sources, 50% inhibition in germination and tube production occurred between 0.5 and 20 ppm while tube growth was inhibited at 25 ppm (Table 1).

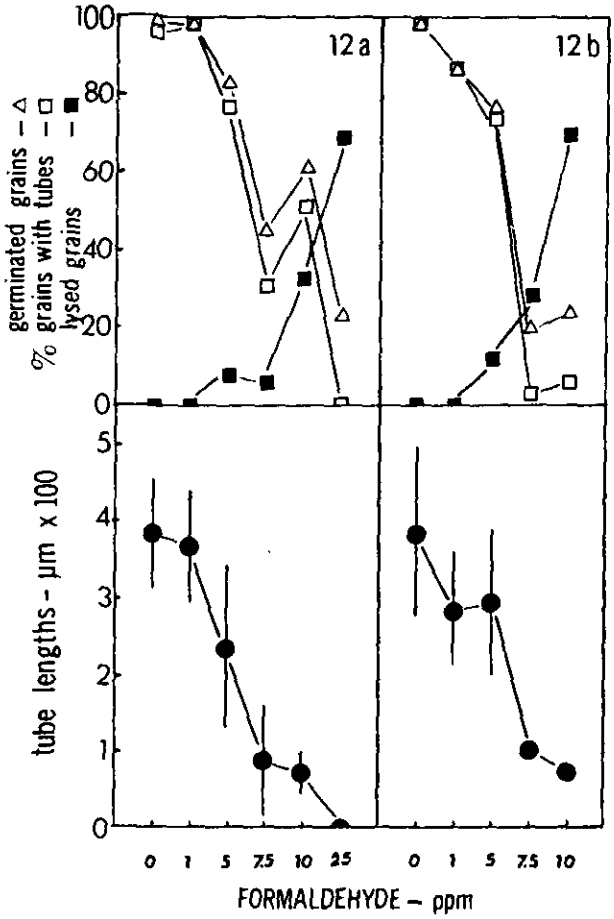


FIGURE 12. Effect of various concentrations of formaldehyde on pollen germination, grain lysis, tube production, and growth.

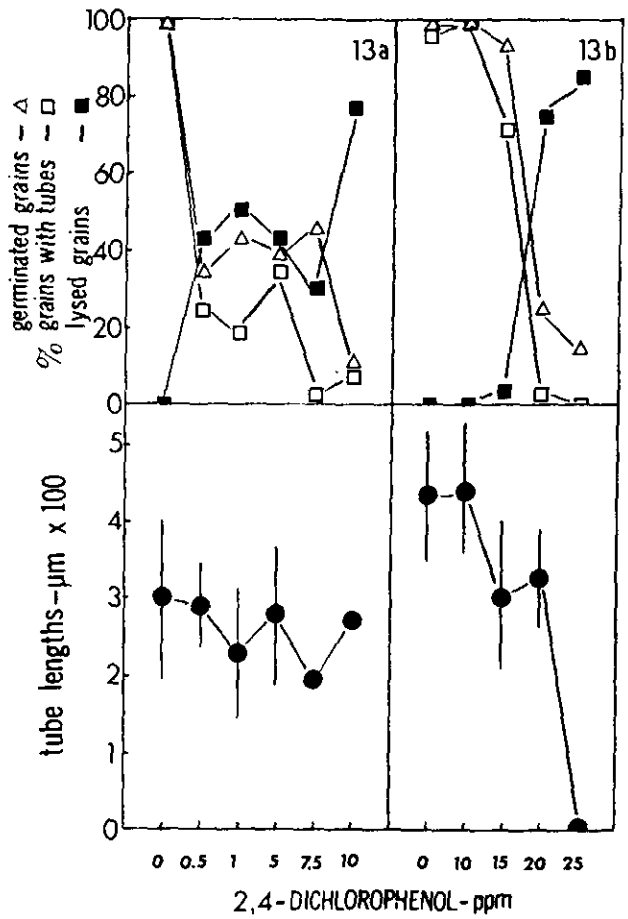


FIGURE 13. Effect of various concentrations of 2,4-dichlorophenol on pollen germination, grain lysis, tube production, and growth.

Table 1. Concentrations at which at least 50% inhibition of pollen germination, tube production and tube growth occurred.

	Level of 50% inhibition, ppm		
	Germination	Tube production	Tube length
Formaldehyde	7.5-10	7.5-10	7.5
2,4-Dichlorophenol	0.5-20	0.5-20	25
p-Cresol	100	40-60	150

Cresol. The addition of 10 ppm cresol to the basal medium had little effect upon pollen germination, tube production and growth (Fig. 14). When 40, 60, or 80 ppm cresol was added to the basal medium, some comparable results were obtained. From 51 to 58% of the grains germinated and from 23 to 35% produced tubes, but the percentage of lysed grains was variable, ranging from 2 to 34%. When the concentration of cresol was 100 or 125 ppm, a second decline in germination and tube formation was observed, and the percentage of lysed grains increased to 62-74%. With 150 ppm cresol, 21% of the pollen germinated, but none produced tubes. The final tube lengths did not differ from the control except at a concentration of 150 ppm cresol.

In other experiments, the first inhibition of germination and tube formation occurred at 60 ppm cresol and the second at 125 ppm. A 50% inhibition in germination, tube production and tube growth occurred at 100, 40-60, and 150 ppm cresol. respectively (Table 1).

Acrylamide. When acrylamide was added to the basal medium at concentrations ranging from 10 to 2000 ppm, there was no significant effect upon germination, tube formation, or tube growth (Fig. 15).

Dioctyl Phthalate. When pollen was exposed to nearly saturated or saturated solutions of dioctyl phthalate (0.1 and 1.0 ppm), there was no notice-

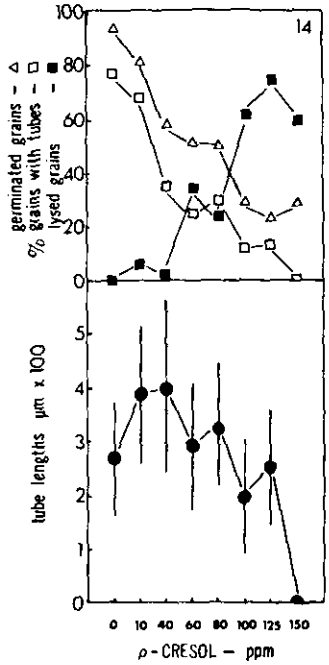
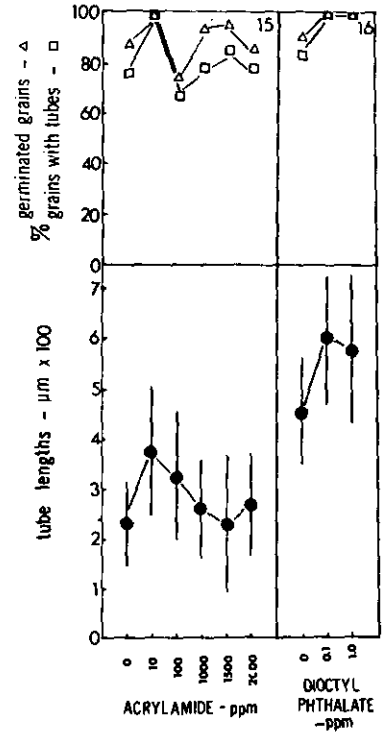


FIGURE 14. Effect of various concentrations of *p*-cresol on pollen germination, tube production, and growth.



FIGURES 15 and 16. Effect of various concentrations of acrylamide and dioctyl phthalate upon germination, tube production, and growth.

able effect upon germination, tube production or final tube lengths (Fig. 16).

Discussion

Jost (*14*) first suggested that the role of sugar during pollen germination and tube growth was to regulate the osmotic pressure of the incubation medium. However, O'Kelley (*15*) did find that the pollen of Lonicera, Tecoma, and Nicotiana incubated for 2 hr could take up sugar and utilize it during respiration. Since most concentrations of sucrose had no effect upon the germination of Impatiens pollen, tube production and growth, an osmoticum apparently is not required. Only 2×10^5 ppm sucrose inhibited the formation of tubes without having an effect upon germination and tube growth. Since sucrose had little effect, it is unlikely that sucrose was being utilized to any great extent.

The importance of boron for pollen germination and tube growth initially was recognized by Schmucker (*16, 17*), but its physiological role was unknown until Dickinson (*18*) reported that boron binds in a reversible manner to growth related sites in pollen tubes. However, he could not determine whether such sites were cytoplasmic or at the cell surface. Impatiens pollen also required boron although some germination, tube production and growth occurred in its absence. On several occasions, the addition of a high concentration of boron (1000 ppm) to the basal medium significantly enhanced tube elongation. This concentration was ten times higher than that utilized by many investigators (*13, 15, 18-20*).

It also has long been known that calcium is essential for pollen tube growth (*21, 22*). With Impatiens, pollen grains germinated and produced tubes only when calcium was present in the basal medium at concentrations of 1109.9 or 111.0 ppm. Higher concentrations of $CaCl_2$ (11099.0 ppm) were completely inhibitory.

Mascarenhas (*13*) added KH_2PO_4 to his medium for Tradescantia pollen. With Impatiens, few pollen grains germinated and produced tubes unless KH_2PO_4 was present in the basal medium. However, K^+ and not $PO_4^{\equiv}$ was the essential ion regulating germination and tube growth. Weisenseel and Jaffe (*23*) have shown that K^+ entered the tips of growing Lilium pollen tubes. Presumably, influx of K^+ was also important for the tube growth of Impatiens pollen.

Selected toxic substances affected Impatiens pollen germination, tube production and elongation in different ways. Acrylamide and dioctyl phthalate had no measurable effect upon pollen germination and tube growth. On the other hand, formaldehyde

proved to be very toxic, inhibiting germination, tube production and growth at a concentration of 7.5-10 ppm. Masura, Syozo, and Sabaro (*8*) also found that formaldehyde would markedly inhibit the elongation of Lilium pollen tubes at a concentration of 2.4 ppm. With 2,4-dichlorophenol, differential sensitivity was observed with respect to the source of the pollen. With one source of pollen, germination and tube production was inhibited at the concentration of 0.5 ppm, but with another pollen source, this inhibition occurred at 20 ppm. With both pollen sources, germination and tube production was inhibited at a lower concentration of 2,4-dichlorophenol than were tube lengths. With cresol, a biphasic inhibition of germination and tube formation occurred with the lower level of inhibition occurring when 40-60 ppm was added to the basal medium and the higher one at 100-125 ppm. As with 2,4-dichlorophenol, germination and tube formation were inhibited at lower concentrations of cresol than were tube lengths.

Impatiens pollen is an excellent bioassay for toxic substances. Plants grow well in the greenhouse and can be maintained continuously in the flowering state. Cuttings can be taken and rooted for the establishment of clones. The basal medium and the method of preparation is simple and the incubation period short. Since the medium contains no sugars, the possibility of bacterial and fungal contamination is reduced. Pollen tubes grow equally as well in a liquid medium or on an agar surface. There is little variation in the response of pollen from different plants or from clones of the same plant. Since a single flower is a prodigious source of pollen, a complete series of experiments can be accomplished from one pollen source. Any variation due to the viability of the pollen can be eliminated by quickly testing the pollen source for adequate germination and tube growth. If inadequacies are observed, that pollen source can be discarded and another one tested before any suspected toxic substances are tested. The only significant detraction from the bioassay is that pollen tubes seldom grow straight. However, this problem can be overcome by utilizing photomicrography and tracing techniques. Finally, Impatiens pollen exhibits a differential sensitivity to different toxic substances.

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