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# Effect of Toluidine Blue on Pollen Germination and Pollen Tube Growth

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**Abstract.** Toluidine blue is known to induce gynogenic haploids in significant numbers in *Populus*. Because the efficacy of a chemical in inducing gynogenesis depends largely on its effect on pollen germination, on pollen tube growth, and on male gamete formation, the effect of toluidine blue (0, 1, 10 and 100 mg $l^{-1}$) on these processes was studied in treated pistils of *Solanum nigrum* (4×), as well as on cultured pollen grains of *S. nigrum* and *Trigonella foenum-graecum*.

Irrespective of the time of application, toluidine blue (1 and 10 mg $l^{-1}$) had no effect on pollen germination or pollen tube growth in pistils of *S. nigrum*; at 100 mg $l^{-1}$ it invariably inhibited both the processes. Almost similar responses were elicited by cultured pollen grains. In *T. foenum-graecum* toluidine blue had no effect on pollen germination and suppressed tube growth. Gamete formation was inhibited, to various degrees, at all the concentrations tested; at 100 mg $l^{-1}$ hardly any pollen tube showed gamete formation. Based on our results, and those on other systems, the potentiality of toluidine blue as an inducer of gynogenesis has been analysed.

Toluidine blue inactivates the sperm nucleus in frogs (BRIGGS 1952) and mice (EDWARD 1954), and inhibits the division of generative nucleus in cultured pollen grains of *Vinca rosea* (ROGERS and ELLIS 1966) and *Tradescantia paludosa* (GEARHART and ROGERS 1969). In *Populus* the application of toluidine blue to pollinated pistils results in the production of as high as 43 per cent of the gynogenic haploids (ILLIES 1974a, b). Toluidine blue has therefore been considered to hold promise of being an effective chemical inducer of gynogenesis (see KASHA 1974).

In spite of the unmatched simplicity and significant success with *Populus*, the efficacy of toluidine blue in inducing gynogenesis has not yet been demonstrated in any other taxon. In tomato and corn toluidine blue has been reported to be ineffective in inducing gynogenic haploids (AL-YASIRI and ROGERS 1971). The potentiality of toluidine blue of inducing gynogenesis depends largely on its effect on the paternal reproductive material. We studied its effect on pollen germination, pollen tube growth and gamete formation both *in vivo* and *in vitro*. Investigations were conducted largely on *Solanum nigrum*; *in vitro* studies were extended to *Trigonella foenum-graecum*.

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## Material and Methods

Investigations were carried out with naturally occurring tetraploids ($n = 24$) of *Solanum nigrum* L. Flower buds were excised in the evening before anthesis, emasculated, and implanted on 1% agar plates in Petri dishes; the following day the pistils were pollinated as desired, using pollen grains collected from just-dehisced anthers.

For studies on pollen germination and pollen tube growth in the control some of the pistils were fixed at 0.5, 1, 2, 4, 8, 12 and 24 h after pollination in formalin-acetic-alcohol (FAA). The other pistils were treated with toluidine blue (Gurr, England) 0, 0.5, 1, 2 and 4 h after pollination; for each treatment toluidine blue prepared in 10% sucrose solution was used at 1, 10 and 100 mg $l^{-1}$.

For 0 h treatment, toluidine blue was applied immediately after pollination on the stigma until the solution flowed freely. For the remaining treatments the entire pistil was immersed for 15 min in the respective solutions. All the pistils were incubated under diffuse light in the laboratory (25 °C—28 °C). The pistils were fixed 24 h after pollination in FAA for about 12 h, and stored in 70% ethanol.

Pollen germination and pollen tube growth in pistils were studied with the fluorescence microscope. The pistils were cleared in 8 N NaOH for 12 h at room temperature (25 °C—28 °C) and thoroughly washed in water to remove all traces of NaOH. The cleared pistils were stained with decolorised aniline blue (0.01%) in 0.05 M $Na_2HPO_4$ (pH 8.2), and observed with a Reichert Zetopan-Binolux fluorescent microscope using exciter filter No. 2 and absorption filter No. 1.

For studies on the effects of toluidine blue on pollen germination and pollen tube growth *in vitro*, fresh solutions of toluidine blue (0, 1, 10 and 100 mg $l^{-1}$) were prepared in a germination medium (sucrose 10% + boric acid 100 mg $l^{-1}$ + calcium chloride 300 mg $l^{-1}$ + magnesium sulphate 200 mg $l^{-1}$ + potassium nitrate 100 mg $l^{-1}$ (after Brewbaker and Kwack 1963). Pollen grains collected from just-dehisced anthers were cultured in drops of 50 µl of the germination medium on a slide, and incubated in Petri plates lined with moist filter paper. The cultures were maintained at 22 °C ± ± 2 °C under diffuse light (100—200 lx) up to 12 h. Acetocarmine whole-mounts of pollen tubes were prepared to study gamete formation. Confidence interval estimates for each treatment were determined at 95% level (Campbell 1967); and a Student's 't' test was used to test the significance of the means of each toluidine blue treatment with the control (Spiegel 1972).

## Results

### Studies *in vivo*

In untreated pistils pollen grains germinated within 1 h after pollination; pollen tubes entered the stigma soon after, and in 2 h they grew through one-third the length of the style. Pollen tubes reached half the length of the style in 4 h, and up to the base of style in 8 h. Several pollen tubes entered the ovary within 12 h after pollination (Fig. 1 A, B) and in another 12 h many of them entered the ovules.

In pistils treated with toluidine blue (1 and 10 mg $l^{-1}$) immediately upon pollination or 30 min later, the majority of the pollen grains had been

washed off, because their pollen tubes would have hardly entered the stigma. Those grains which remained stuck to the stigma, however, had issued tubes that had already grown long in the style. In subjects treated with 100 mg $l^{-1}$ toluidine blue there was hardly any pollen germination.

In pistils treated subsequent to pollen germination (*i.e.* 1, 2 and 4 h after pollination), the growth of pollen tubes depended on the concentration of toluidine blue. At 1 and 10 mg $l^{-1}$ there was neither inhibition nor promotion of tube growth (Fig. 1 C); at 100 mg $l^{-1}$, tube growth was invariably inhibited (Fig. 1 D, E); in fact in no pistil did any pollen tube reach the ovary. Owing to unsatisfactory staining of the preparations of the treated pistils the effect of toluidine blue on male gamete formation could not be studied.

**Studies *in vitro***

Figure 2 presents the results of the effects of toluidine blue on pollen germination and pollen tube growth in *Solanum nigrum*. Pollen germination was inhibited drastically at 100 mg $l^{-1}$, and tube growth at 10 and 100 mg $l^{-1}$.

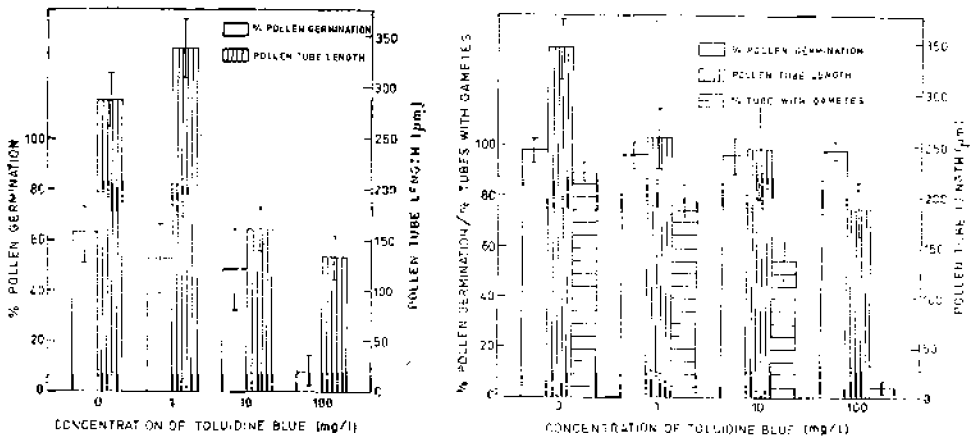


Fig. 2. Effect of toluidine blue on cultured pollen grains of *Solanum nigrum*. Culture period: 3 h. Mean values of about 200 pollen grains scored for % germination, and 50 pollen tubes scored for tube length. Vertical lines on the bars of the histograms represent confidence interval estimates at 95% level. Effect of toluidine blue on both pollen germination and tube length was significant at all the concentrations at $P = 0.975$ level.

Fig. 3. Effect of toluidine blue on pollen germination, pollen tube growth and gamete formation *in vitro* in *Trigonella foenum-graecum*. Culture period: 3 h for germination and tube length, and 10 h for gamete formation. Mean values of about 200 pollen grains (% germination), 50 and 100 pollen tubes, for tube length and gamete formation, respectively. Vertical lines on the bars of the histogram represent confidence interval estimates at 95% level. Effect of toluidine blue was not significant on pollen germination at any of the concentrations tested ($P = 0.95$), whereas the effect of the chemical was significant ($P = 0.975$ level) on pollen tube length and gamete formation at all the concentrations.

Applied 1 h after culture (by which time pollen tubes had grown *ca.* 100 μm long), toluidine blue, at 10 and 100 mg $l^{-1}$, inhibited the further growth of tubes.

Gamete formation in pollen grain cultures of *Solanum nigrum* could not be studied because the pollen tube growth ceased at 500 μm and the generative

cell failed to divide even in the controls (up to 24 h). Studies were, therefore, extended to *Trigonella foenum-graecum* in which gamete formation occurs readily in cultured pollen grains (SHIVANNA *et al.* 1974). Unlike *S. nigrum*, in *T. foenum-graecum* pollen germination was not affected by toluidine blue even at 100 mg $l^{-1}$; and tube growth was suppressed marginally (Fig. 3). However, gamete formation was invariably inhibited (Fig. 3). This inhibition was directly proportional to the concentration of the test substance. In controls over 90% tubes showed gamete formation. In treatment with 100 mg $l^{-1}$ toluidine blue the percentage of pollen tubes in which gametogenesis occurred was less than 5; the generative cell could be seen even 24 h after culture.

## Discussion

Whereas a chemical which inhibits the formation of male gametes without affecting pollen tube growth *in vivo* might be expected to stimulate gynogenesis (= parthenogenesis), our studies with toluidine blue (1 and 10 mg $l^{-1}$) have shown that the substance had no visible effect either on pollen germination or on pollen tube growth in *Solanum nigrum*. At 100 mg $l^{-1}$, however, toluidine blue suppressed both the processes. Our studies on pollen grain cultures have also yielded similar results. At none of the 3 concentrations tested did toluidine blue show any tendency to induce gynogenesis; either it had no effect on any of the three phenomena studied, or it drastically inhibited pollen tube growth in the style.

Toluidine blue failed to induce gynogenesis in tomato and maize also (AL-YASIRI and ROGERS 1971); however no information is available on pollen germination and tube growth *in vivo* in these systems. Toluidine blue has been quite effective in inducing gynogenesis in *Populus* (ILLIES 1974a, b), although its effect on pollen germination and tube growth in *Populus* has not been very different from that in *Solanum nigrum* (present investigation). In *P. tremula* when a suspension of pollen grains in toluidine blue was applied onto the stigma there was hardly any germination and only a few gynogenic haploids were recovered. When toluidine blue was sprayed on pistils after pollen germination and entry of pollen tubes into the stigma (6—24 h after pollination), it inhibited the further growth of tubes. This treatment significantly increased the frequency of haploids; up to 43% haploids were recorded in treatments given 12 h after pollination.

Gynogenesis occurs spontaneously in *Populus* with rather a high frequency and can be augmented to various degrees by several techniques such as the use of irradiated pollen (mentor pollen), heat-treated pollen, and pollen of alien species (STETTLER 1968, WINTON and EINSPAHR 1968). It appears, therefore, that in *Populus* some egg cells are stimulated to develop into embryos merely due to pollination (even without pollen germination). A treatment which permits pollen germination and entry of pollen tubes into stigma, but prevents tubes from reaching the embryo sac (as in toluidine blue treatments), is more effective in stimulating the unfertilized egg gynogenically. Thus, gynogenesis in *Populus*, is largely intrinsic rather than due to the unique effect of toluidine blue on pollen.

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*Figures at the end of the issue.*

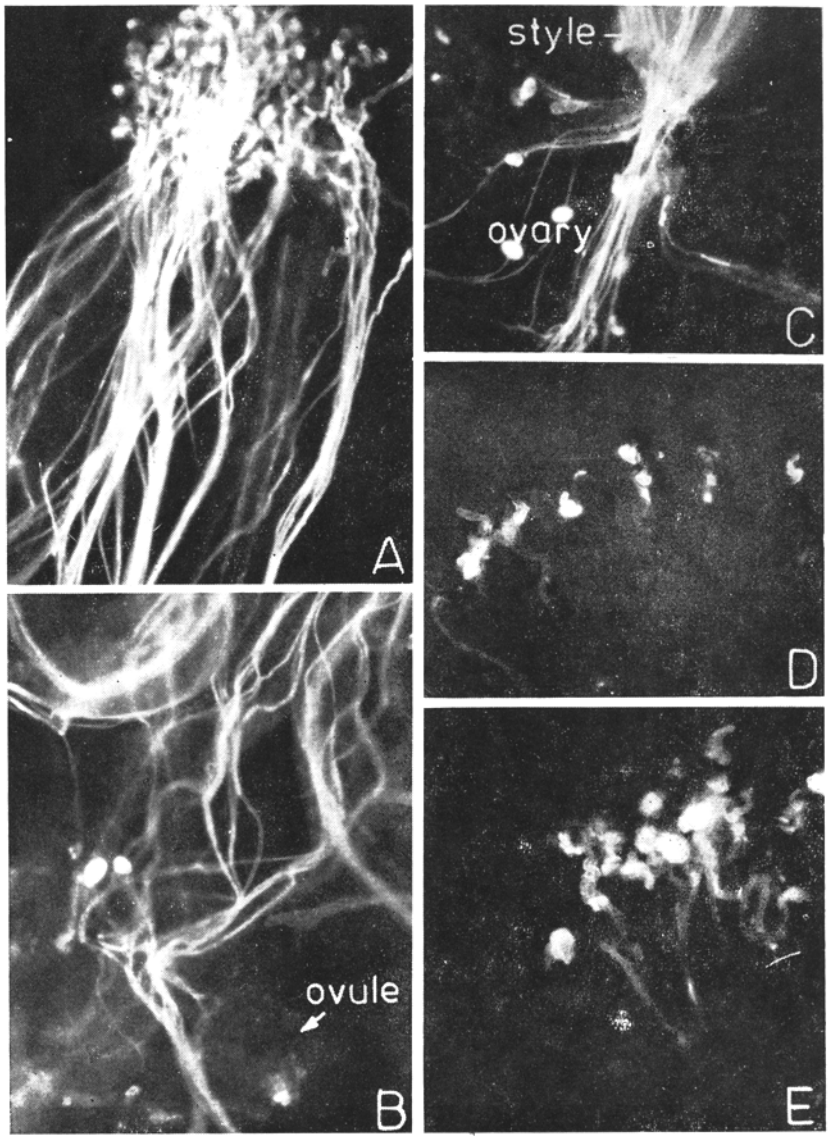


Fig. 1. Fluorescent micrographs of pistils of *Solanum nigrum* 24 h after pollination, following staining with decolorised aniline blue. *A* and *B* — controls, *C* to *E* — treated with toluidine blue. Magnification *ca.* 150×. *A:* Stigma and part of the style showing good pollen germination and tube growth. *B:* Upper part of the ovary showing entry of pollen tubes. *C:* Lower part of the style and upper part of the ovary of a pistil treated with 10 mg $l^{-1}$ of toluidine blue, 2 h after pollination. Pollen tubes have entered the ovary. *D*, *E:* Stigmatic portions of pistils treated with 100 mg $l^{-1}$ of toluidine blue, 1 and 2 h after pollination, respectively. Pollen tubes have not grown further after treatment.