

**Somatic embryo formation by cultured ovules of
Cabernet Sauvignon grape:
Effects of fertilization and of the male gameticide toluidine blue**

by

K. RAJASEKARAN¹) and M. G. MULLINS

**Die Bildung somatischer Embryonen durch kultivierte Samenanlagen der Rebsorte
Cabernet Sauvignon:
Der Einfluß der Befruchtung und des Pollen-Gametizides Toluidinblau**

Zusammenfassung: Im Griffel von Pflanzen, die bei 27 °C (tags) und 22 °C (nachts) gehalten wurden, fand sich eine maximale Anzahl von Pollenschläuchen. Die Befruchtung erfolgte im allgemeinen etwa 24 h nach der Anthese. Die Behandlung sowohl von isoliertem Pollen wie von Infloreszenzen intakter Reben mit Toluidinblau (10—100 mg l⁻¹) verringerte die Pollenkeimung und das Pollenschlauchwachstum. Die Pollenschläuche, die das Griffelgewebe Toluidinblau-behandelter Blüten durchwuchsen, zeigten ein abnormes Erscheinungsbild. Vom Nucellusgewebe sowohl normalbefruchteter wie Toluidinblau-behandelter Blüten wurden mit gleicher Häufigkeit somatische Embryonen hervorgebracht. In allen kultivierten Samenanlagen degenerierte der Embryosack. Die Weinrebe scheint auf Toluidinblau als Induktor der haploiden Parthenogenese nicht zu reagieren.

Key words: flower, pollen, fecundation, parthenocarpy, embryo, tissue culture, toxicity.

Introduction

In many cultivars of *vinifera* grapes competence to produce somatic embryos *in vitro* is restricted to the nucellus tissues of ovules (MULLINS and SRINIVASAN 1976; SRINIVASAN and MULLINS 1980). In earlier research on somatic embryo formation ovules were taken from unpollinated and unfertilized ovules so as to avoid the possibility of confusing the formation of nucellar embryos with the formation of zygotic embryos. The present paper is concerned with the culture of fertilized ovules and of ovules from flowers treated with the male gameticide, toluidine blue. The object of the research was twofold: (i) to determine the effects of fertilization on the regenerative competence of nucellus and (ii) to investigate the possibility of inducing haploid parthenogenesis *in vitro*.

The benefits for plant breeding of haploid plants, and of the homozygous diploids derived from them, are well documented (KASHA 1974) but attempts to produce haploid grapevines by culture of isolated anthers and microspores have been unsuccessful (RAJASEKARAN and MULLINS 1979, 1983). Haploid parthenogenesis *in vitro* by culture of ovules is an alternative approach which, so far, has received little attention.

The growth of the pollen tube in the style is thought to produce a stimulus which promotes the division of haploid nuclei within the embryosac and induction of haploid embryos *in vivo* has been achieved in several species by delayed pollination, use of

¹) Present address: Department of Botany, University of Florida, Gainesville, Florida, USA.

inactivated or incompatible pollen, physical agents (X-rays, γ -rays, UV-light, temperature shocks) and chemical agents (LACADENA 1974; YANG and ZHOU 1982). Among the chemical agents is toluidine blue, a compound which is best known as a histological stain but which also acts as a gametocide by inhibiting the formation of male gametes without preventing the penetration of the pollen tube (ROGERS and ELLIS 1966). In *Populus* numerous haploid seedlings were produced following applications of toluidine blue to pistils (ILLIES 1974 a, 1974 b). In the present research studies were made of the effects of temperature and toluidine blue on pollen tube growth *in vivo* and on effects of this gametocide on pollen germination, pollen tube growth and growth of ovules *in vitro*.

Materials and methods

Test plants of *Vitis vinifera* L. cv. Cabernet Sauvignon were propagated from cool-stored cuttings (4 °C) by the method of MULLINS (1966), as modified by MULLINS and RAJASEKARAN (1981), to produce a continuous supply of vines at the inflorescence-bearing stage. These plants were used for studies on pollen tube growth *in vivo* and as the source of ovules and pollen for culture *in vitro*. The test plants were grown with natural illumination in each of five temperature-controlled compartments in a research glass-house. The day/night temperatures were as follows: 21/16 °C, 24/19 °C, 27/22 °C, 30/25 °C and 33/28 °C.

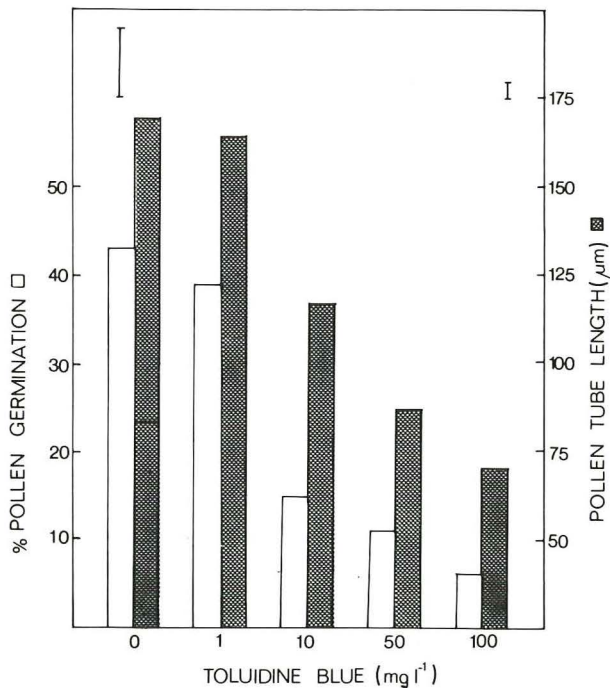


Fig. 1: Effect of toluidine blue on germination and tube growth of isolated pollen grains of Cabernet Sauvignon.

Einfluß von Toluidinblau auf Keimung und Pollenschlauchwachstum isolierter Pollenkörner von Cabernet Sauvignon.

Toluidine Blue-O (E. Merck, F.R.G.) was used in aqueous solution in studies on pollen germination and pollen tube growth *in vitro*. Fresh solutions of toluidine blue (0, 1, 10, 50 and 100 mg l⁻¹) were prepared with a germination medium as solvent, i.e. sucrose 20 %, 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 were stored at 4 °C in a desiccator for 10 d (60 % germination) and were germinated by the hanging drop (50 µl) culture method. The cultures were maintained at 26 °C with an irradiance of 4 Wm⁻². The culture period was 2 h. Mean values for percentage germination were based on counts of 200 pollen grains. Determinations of pollen tube length were made on a minimum of 30 tubes. For studies on pollen germination and pollen tube growth *in vivo* the inflorescences of test plants growing at the differing temperatures were dipped in toluidine blue solution (50 mg l⁻¹) containing 0.1 % Tween-20 as a wetting agent. Control inflorescences were treated with water containing Tween-20. Inflorescences were treated with toluidine blue on each of 4 d i.e., the 2 d before and the 2 d after anthesis. 20 pistils were sampled from each inflorescence on the 2nd d after anthesis and were used for fluorescence microscopy.

Effect of toluidine blue (50 mg l⁻¹) on pollen tube growth in pistils of Cabernet Sauvignon

Einfluß von Toluidinblau (50 mg l⁻¹) auf das Pollenschlauchwachstum in Stempeln von Cabernet Sauvignon

Temperature (°C) day/night	Number of pollen tubes growing through the pistil ¹⁾	
	Control	Treated with toluidine blue
21/16	14.2 ± 6.2	9.7 ± 4.4
24/19	15.7 ± 4.8	13.1 ± 4.7
27/22	33.3 ± 10.2	21.5 ± 4.0
30/25	18.5 ± 5.3	8.1 ± 2.6
33/28	6.9 ± 2.1	8.0 ± 2.3

¹⁾ 36 h after anthesis. Mean of 25 pistils sampled at random.

The fluorescence microscopy procedure of KHO and BAER (1968), as modified by TOMER and GOTTRICH (1975), was used to observe pollen tube growth in the pistils. Pistils were fixed (24 h) in FAA (formalin : acetic acid : alcohol : water 10 : 1 : 2 : 7), washed in water, softened (8 N NaOH for 30–60 min) and washed again with water. Finally, the pistils were stained with 0.1 % Aniline Blue-W.S. (E. Merck, F.R.G.) dissolved in 0.1 N K₃PO₄ (pH 8.2). The stained pistils were then placed on a slide in glycerine and squashed by applying pressure to the coverslip. Observations were made with a Leitz Orthoplan microscope equipped with a 200 W high pressure mercury lamp. Light at 400 nm was produced by a UG 1 excitation filter (2 mm). Photomicrographs were taken on Kodak Plus-X film with an Orthomat camera. Records were made of the numbers of pollen tubes growing through the styles in each of the sampled pistils. (Accurate measurements of the lengths of pollen tubes could not be made in these squash preparations).

For aseptic culture, a minimum of 100 ovules were excised 2 d after anthesis from normally-fertilized and toluidine blue-treated flowers from test plants growing in each of the five different temperatures. These ovules were grown in agitated liquid culture

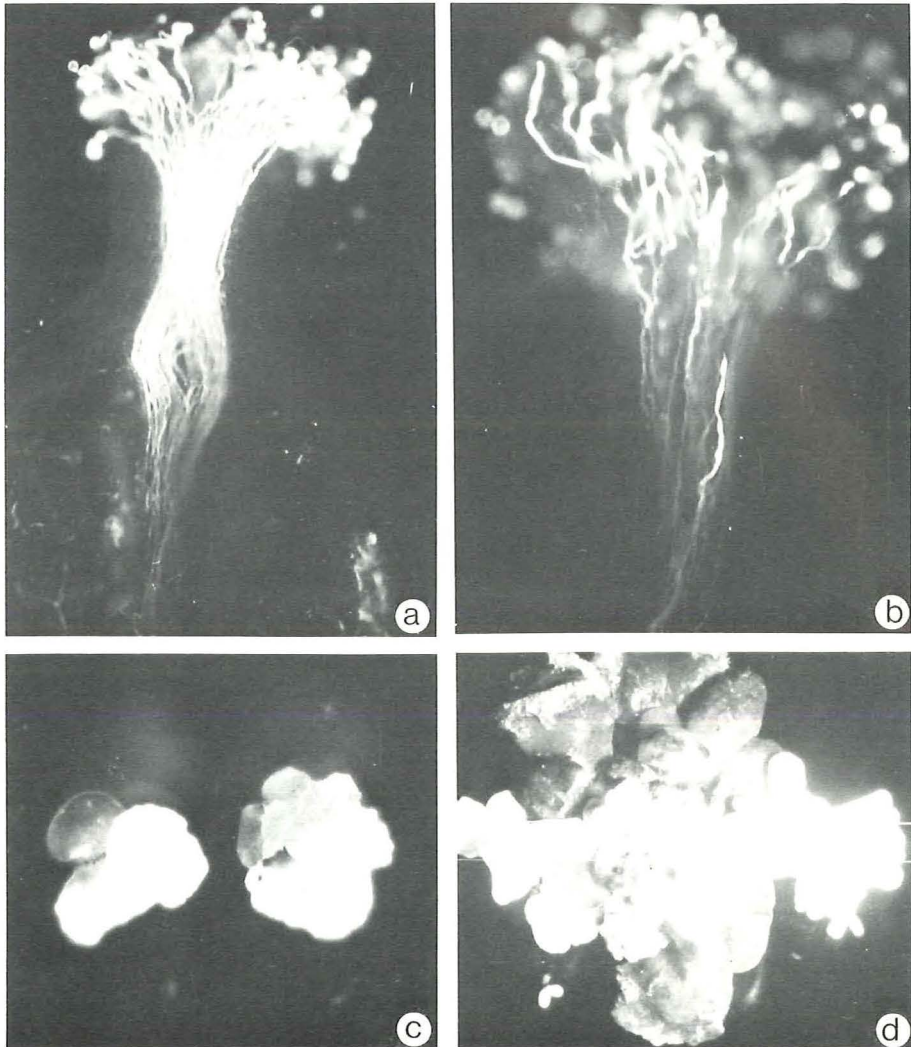


Fig. 2: Effect of toluidine blue (50 mg l^{-1}) on pollen tube growth *in vivo* and on embryo formation by cultured ovules. — a) Pollen tube growth through the style in untreated flowers from plants grown at 27°C (day) and 22°C (night) ($\times 60$). b) Pollen tube growth through the style in flowers treated with toluidine blue. The plants were grown at 27°C (day) and 22°C (night). Note the reduction in numbers of pollen tubes and discontinuous fluorescence ($\times 80$). Both a) and b) were stained with aniline blue ($0.1\% \text{ w/v}$). c) Callus production by ovules from toluidine blue-treated flowers. The ovules were grown in liquid medium (NITSCH and NITSCH 1969) supplemented with BA ($1 \mu\text{M}$), 2,4-D ($5 \mu\text{M}$) and casein hydrolysate ($0.1\% \text{ w/v}$) ($\times 15$). d) Formation of somatic embryos from cultured ovules ($\times 15$).

Einfluß von Toluidinblau (50 mg l^{-1}) auf das Pollenschlauchwachstum *in vivo* und auf die Bildung von Embryonen aus kultivierten Samenanlagen. — a) Pollenschlauchwachstum durch den Griffel unbehandelter Blüten von Pflanzen, die bei 27°C (tags) und 22°C (nachts) gehalten wurden ($60 \times$). b) Pollenschlauchwachstum durch den Griffel Toluidinblau-behandelter Blüten. Die Pflanzen wurden bei 27°C (tags) und 22°C (nachts) gehalten. Auffällig ist die verringerte Anzahl der Pollenschläuche und ihre ungleichmäßige Fluoreszenz ($80 \times$). Sowohl a) wie b) sind mit Anilinblau ($0,1\%$

by the method of SRINIVASAN and MULLINS (1980). In brief, ovules were cultured either on the basal medium of NITSCH and NITSCH (1969) or on the same medium supplemented with casein hydrolysate (CH; 0.1 % w/v), 2,4-dichlorophenoxyacetic acid (2,4-D; 5 μ M) and benzyladenine (BA; 1 μ M). For cytological studies on root tips samples were stained in Schiff's reagent according to the procedure of SALESSES (1967).

Results and discussion

Germination and pollen tube growth were both reduced when isolated pollen was cultured with toluidine blue (10–100 mg l⁻¹; Fig. 1). A symptom of toluidine blue toxicity was that many pollen tubes had swollen tips. In intact plants the germination of pollen grains on the stigmatic surface, and the numbers of tubes which penetrated the style, was affected by the temperature at which the test plants were grown. The number of pollen tubes was higher at 27/22 °C than at other temperatures (Table; Fig. 2 a). These results are in accord with the finding of STAUDT (1982) that 28 °C is the optimum temperature for pollen germination and pollen tube growth of *Vitis rupestris* in pistils of the cv. Madeleine angevine.

In control plants several pollen tubes entered the ovule within 12 h of pollination and fertilization, as evidenced by the presence of pollen tubes at the micropylar end of embryosac, generally occurred 24 h after anthesis. The number of pollen tubes growing through the style was greatly reduced at all temperatures by application of toluidine blue (50 mg l⁻¹; Table). Only a few pollen tubes reached the ovules of treated flowers and these tubes exhibited swollen tips and discontinuous fluorescence, indicating the formation of callose and loss of normal function (Fig. 2 b).

The behaviour *in vitro* of ovules from toluidine blue-treated flowers and from normally-fertilized flowers (controls) was similar. Ovules cultured in the basal medium turned black and died within 4 d of inoculation but ovules grown with the medium containing BA (1 μ M), 2,4-D (5 μ M) and CH (0.1 %) produced a greenish-white callus from the micropyle after 2–3 weeks (Fig. 2 c). This callus subsequently disintegrated to produce a suspension of cellular aggregates and single cells. After a transfer to basal medium (BA and 2,4-D omitted) the cultures derived from both treated and control flowers gave rise to large numbers of somatic embryos (Fig. 2 d). The regenerative competence of ovular callus was not affected by fertilization or by treatment with toluidine blue and somatic embryos were produced with equal frequency by ovules from both treated and untreated flowers.

Ovules were sampled for microscopy throughout the culture period and it was confirmed that the callus from which the embryos were produced arose from divisions of the nucellar calotte, a tissue located close to the micropyle (MULLINS and SRINIVASAN 1976). There was obvious degeneration of the embryosac in all of the ovules which were investigated. Plantlets produced by the embryos grown *in vitro* were found to be diploid (2n = 38).

Gew./Vol.) gefärbt. c) Kallusbildung bei Samenanlagen aus Toluidinblau-behandelten Blüten. Die Samenanlagen wurden in Flüssigmedium (NITSCH und NITSCH 1969) mit Zusatz von BA (1 μ M), 2,4-D (5 μ M) und Caseinhydrolysat (0,1 % Gew./Vol.) kultiviert (15 \times). d) Bildung somatischer Embryonen aus kultivierten Samenanlagen (15 \times).

Toluidine blue was ineffective as an inducer of haploid parthenogenesis in Cabernet Sauvignon grape. There was penetration of pollen tubes in treated flowers and observations suggest that toluidine blue prevented normal fertilization. Nevertheless, the stimulus of pollen tube growth was without effect on the embryosacs of cultured ovules. The possibility that toluidine blue might induce parthenogenesis in normally-grown grapevines cannot be dismissed but it seems likely that *Vitis vinifera* can be added to the list of unresponsive species (AL-YASIRI and ROGERS 1971; GHOSH and SHIVANNA 1977).

Summary

Maximum numbers of pollen tubes were found in styles of plants growing at 27 °C (day) and 22 °C (night). Fertilization generally occurred by 24 h after anthesis. Treatment of both isolated pollen and inflorescences of intact vines with toluidine blue (10–100 mg l⁻¹) reduced pollen germination and tube growth. Pollen tubes which penetrated the styles of toluidine blue-treated flowers appeared to be abnormal. Somatic embryos were produced with equal frequency by nucellus tissue from cultured ovules from both normally-fertilized and toluidine blue-treated flowers. There was degeneration of embryosacs in all cultured ovules. The grape appears to be unresponsive to toluidine blue as an inducer of haploid parthenogenesis.

Acknowledgement

This research was supported by the Rural Credits Development Fund, Reserve Bank of Australia.

Literature cited

- AL-YASIRI, S. A.; ROGERS, O. M.; 1971: Attempting chemical induction of haploidy using toluidine blue. *J. Amer. Soc. Hort. Sci.* **96**, 126–127.
- BREWBAKER, J. L.; KWACK, B. H.; 1963: The essential role of calcium ion on pollen germination and pollen tube growth. *Amer. J. Bot.* **50**, 859–865.
- GHOSH, N.; SHIVANNA, K. R.; 1977: Effect of toluidine blue on pollen germination and pollen tube growth. *Biol. Plant.* **19**, 360–364.
- ILLIES, Z. M.; 1974 a: Induction of haploid parthenogenesis in aspen by post pollination treatment with Toluidine-blue. *Silvae Genet.* **23**, 221–226.
- — ; 1974 b: Experimentally induced haploid parthenogenesis in the *Populus* section *leuce* after late inactivation of the male gamete with Toluidine Blue-O. In: LINSKENS, H. F. (Ed.): *Fertilization in Higher Plants*, 335–340. North-Holland Publishing Company, Amsterdam.
- KASHA, K. (Ed.); 1974: *Haploids in Higher Plants*. Proc. 1st Intern. Symp., Guelph, June 1974. University of Guelph.
- LACADENA, J. R.; 1974: Spontaneous and induced parthenogenesis and androgenesis. In: KASHA, K. (Ed.): *Haploids in Higher Plants*, 13–32. Proc. 1st Intern. Symp., Guelph, June 1974. University of Guelph.
- KHO, Y. O.; BAER, J.; 1968: Observing pollen tubes by means of fluorescence. *Euphytica* **17**, 298–302.
- MULLINS, M. G.; 1966: Test-plants for investigations of the physiology of fruiting in *Vitis vinifera* L. *Nature* **209**, 419–420.
- — ; RAJASEKARAN, K.; 1981: Fruiting cuttings: Revised method for producing test plants of grapevine cultivars. *Amer. J. Enol. Viticult.* **32**, 35–40.

- — ; SRINIVASAN, C.; 1976: Somatic embryos and plantlets of an ancient clone of the grapevine (cv. Cabernet Sauvignon) by apomixis *in vitro*. *J. Exp. Bot.* **27**, 1022—1030.
- NITSCH, J. P.; NITSCH, C.; 1969: Haploid plants from pollen grains. *Science* **163**, 85—87.
- RAJASEKARAN, K.; MULLINS, M. G.; 1979: Embryos and plantlets from cultured anthers of hybrid grapevines. *J. Exp. Bot.* **30**, 399—407.
- — ; — — ; 1983: The origin of embryos and plantlets from cultured anthers of hybrid grapevines. *Amer. J. Enol. Viticult.* **34**, 108—113.
- ROGERS, O. M.; ELLIS, J. H.; 1966: Pollen nuclear division prevented with toluidine blue in *Vinca rosea* L. *HortScience* **1**, 62—63.
- SALESSES, G.; 1967: Mise au point d'une méthode de comptage des chromosomes chez les arbres fruitiers à noyau. *Ann. Amélior. Plantes* **17**, 207—210.
- SRINIVASAN, C.; MULLINS, M. G.; 1980: High frequency somatic embryo production from unfertilized ovules of grapes. *Scientia Hort.* **13**, 245—252.
- STAUDT, G.; 1982: Pollenkeimung und Pollenschlauchwachstum *in vivo* bei *Vitis* und die Abhängigkeit von der Temperatur. *Vitis* **21**, 205—216.
- TOMER, E.; GOTTRICH, M.; 1975: Observations on the fertilization process in avocado with fluorescent light. *Euphytica* **24**, 531—535.
- YANG, H. Y.; ZHOU, C.; 1982: *In vitro* induction of haploid plants from unpollinated ovaries and ovules. *Theor. Appl. Genet.* **63**, 97—104.

Eingegangen am 13. 3. 1985

Professor M. G. MULLINS
Department of Agronomy and
Horticultural Science
University of Sydney
Sydney, NSW 2006
Australia