ATTACHMENT 3

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Phytotoxic Metabolites from *Drechslera wirreganensis* and *D. campanulata*

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Abstract. Phytotoxic components detected by a bean (*Vicia faba*) leaf assay were isolated from extracts of dried mycelia and liquid culture filtrates of *D. wirreganensis* and *D. campanulata* by preparative thin layer chromatography. Mass spectrometric examination of chromatographically homogeneous phytotoxic fractions was consistent with the presence of cytochalasin B and dihydrocytochalasins in both fungi. Cytochalasin B was present at 10.3 g kg^{-1} dry weight of the mycelium for *D. wirreganensis* and 7.7 g kg^{-1} for *D. campanulata*. In addition, differences were observed in the minor cytochalasin components. In *D. wirreganensis*, a more hydrophobic cytochalasin derivative with an ion at m/z 675 was present. In *D. campanulata* a component of similar hydrophobicity was present, but with an ion at 481 m/z. Measurable inhibition of wheat seed germination by cytochalasin B was only observed at or above 70 µM in 1% dimethyl sulfoxide solution. The formation of bi-nucleate cells, a common feature in mammalian cells treated with cytochalasin B is reported in barley root tips. Differences in lesion morphology caused by these two related pathogenic fungi are also associated with differences in types and amounts of the phytotoxic components they produce.

Additional keywords: bi-nucleate plant cells, Cytochalasin B, dihydrocytochalasin, *Pyrenophora semeniperda*, toxic metabolites.

Introduction

D. wirreganensis Wallwork, Lichon & Sivanesan is a cereal pathogen recorded in Australia (Wallwork *et al.* 1992) and in South Africa (personal communication). The fungus closely resembles the graminaeceous pathogen, *D. campanulata* (Lév.) Sutton [syn. *Pyrenophora semeniperda* (Britt. and Adam) Shoem.] by producing conspicuous conidia-bearing synnemata and by inducing small spot lesions on grass leaves similar to the ring spots caused by the latter pathogen. *D. wirreganensis* has been isolated from barley, wheat and grass weed species (Wallwork *et al.* 1995).

In addition to small ring spots, *D. wirreganensis* frequently causes much larger brown blotch lesions that expand and may cause whole leaves to die. Wallwork *et al.* (1995) suggested that these large lesions may be caused by toxic metabolites. This symptom is commonly referred to as "Wirrega blotch". Symptoms induced by *D. wirreganensis* are distinguishable by the presence of a hole or tear in the middle of the lesions.

Wallace (1959) demonstrated the phytotoxicity of culture filtrates of *D. campanulata* to seedlings of wheat and oats. By contrast, (Campbell *et al.* 1996) found no effect of culture filtrates on the germination of wheat and brome grass seeds. The same pathogen was reported to cause mycotoxicosis on sheep and dairy cattle that grazed on infected green oats in South Africa (Van Der Westhuizen *et al.* 1985). Recently, (Evidente *et al.* 2002) have isolated and characterised cytochalasin B (CB, $C_{29}H_{37}NO_5$) as the major cytochalasin along with six other minor cytochalasins on *P. semeniperda* grown on wheat kernels.

This investigation was conducted to identify and chemically characterise and compare the phytotoxic compound(s) produced by *D. wirreganensis* and *D. campanulata*. It also aimed to determine whether these chemicals could play a role in disease development, particularly in symptom formation.

Materials and Methods

Filtrate production

Spores of *D. wirreganensis* and *D. campanulata* were germinated on potato dextrose agar (PDA, 39 g L^{-1}) for 18-20 h and transferred singly onto Petri dishes containing water agar (WA, 20 g L^{-1}). The cultures were incubated in the dark for 48 h at 20-22°C after which pieces of autoclaved barley leaves (2 cm) were arranged around the growing fungus. The plates were further incubated for 4 weeks at 15:20°C night:day temperature with 12 h photoperiod (combination of 8-watt black light and cool white lights). A small amount of sterile distilled water was added into the plates and the spores were scraped with a camel's hair brush. The resulting spore suspension was passed through a 250-µm sieve and the spore number adjusted to 1 x 10^4 conidia/mL.

Liquid Glucose Yeast Medium (GYM) (Paterson and Bridge 1994) composed of 10 g glucose, 5 g yeast extract, 1mL zinc solution, 1 g $NH_4H_2PO_4$, 1 mL copper solution, 0.2 g KH_2PO_4 and 0.2 g $MgSO_4$ x $7H_2O$ per L distilled water was used for filtrate production. The medium was dispensed in 500-mL Schott bottles and autoclaved for 20 min at 121°C.

One mL spore suspension was added per 100 mL medium and the culture bottles were covered with aluminium foil and incubated at 20-22°C for 7 days. On the 8th day, the cultures were shaken vigorously to break the mycelia and were further grown for 22 days at the same temperature conditions with 10 h photoperiod under fluorescent lights. The cultures were shaken on alternate days to minimise the clumping of mycelia.

Extraction, purification, detection and chemical characterisation

All liquid cultures were sieved through 8 layers of sterile cheesecloth at 30 days after subculture. The filtrates were passed through gravity filtration using #541 Whatman filter paper and were serially extracted (6 times) with chloroform and diethyl ether. Each filtrate was concentrated to dryness in a rotary vacuum evaporator (Büchi, model EL 131) at 40-43°C and re-dissolved in methanol (1 mL methanol /100 mL extract).

The mycelia were dried overnight in a 40°C oven. A 100-mg sample of each fungus was finely ground in an acid-proof mortar and the resulting powder soaked in 30 mL 50:50 (v/v) Chloroform: Methanol (CM) in a glass-capped container and left on a 120 rpm-shaker at 20-22°C and 12 h photoperiod under fluorescent lights for 5 days. The mixture was filtered on a #541 Whatman filter paper and the eluate concentrated to dryness in a Speedvac refrigerated vacuum concentrator and re-dissolved in 2 mL methanol.

Thin Layer Chromatography

Preparative thin layer chromatography (TLC) was performed with plastic backed sheets of Merck Silica $\text{Gel}_{254.}$ For quantitative separations, 0.5–0.6 mL of each mycelial methanol concentrate was applied as a 7 cm band alongside a 25 µg CB standard. To maximise resolution, crude extracts were developed 3 times with solvent A (5:4:1, v/v/v ethyl acetate:hexane:formic acid). Fluorescing or absorbing bands were detected under long (366 nm) and short (254 nm) wavelengths using a portable UV lamp (Mineral Lamp™, model UV GL-25). Each band was eluted, concentrated and re-dissolved in 1 mL of 50:50 (v/v) CM and checked for biological activity using the faba bean leaf bioassay. Biologically active bands were re-purified by the same procedure and checked for with solvent B (6:3:1, v/v/v, hexane: diethylamine: formic acid). Cytochalasin B and related species were detected by dipping the sheet in a mixture of 9:1 (v/v) alcohol/acidified *p*-anisaldehyde reagent (Paterson and Bridge 1994). The acidified plates were heated at 100°C until dark blue cytochalasin bands appeared. Biologically active and homogeneous fractions were examined by mass spectrometry and the concentration of CB was determined by polarimetry.

Mass Spectroscopy

Homogeneous phytotoxic bands of *D. wirreganensis* and *D. campanulata* were characterised by Mass Spectroscopy at the Australian Wine Research Institute, Waite Campus, South Australia, with a Finnegan Triple Stage Quadruple Mass Spectrometer using a direct insertion probe using both electron impact ionisation and chemical ionisation in both positive and negative ion modes.

Polarimetry

Polarimetric measurements were made with a Perkin Elmer 141 polarimeter using 100 mm path length microcell of 0.6 ml capacity. Measurements were made at both 589 and 365 nm using sodium and mercury vapour lamps. For CB concentrations, the rotation of an approximately 0.08% w/v (0.8 g L^{-1}) reference sample of commercial CB in methanol was determined at 589 nm using the literature (Rothweiler and Tamm 1966) value of the specific rotation as $[\alpha]_D = +83$ to determine the precise concentration and the larger specific rotation for the same sample at 365 nm was calculated as $[\alpha]_{365} = +111.3$. The concentration of aliquots of the chromatographically homogeneous CB samples Dw1 and Dc1 were measured at 365 nm and converted to concentration using the appropriate specific rotation.

Bioassay

Faba bean leaf: the phytotoxicity of the crude filtrates and purified extracts was assayed using the leaf drop bioassay technique (Kohmoto 1992). All assays were conducted using leaves of faba bean (*Vicia faba* L.) variety Fiord unless otherwise stated.

Freshly harvested leaves were washed in 1:1.5 (v/v) methanol and sterile distilled water for one minute, rinsed 3 times with sterile distilled water, wrapped in sterile tissue papers and dried under a current of air in a laminar flow cabinet. Leaves were cut into halves and the abaxial side scratched using a broken capillary glass. Lightly scratched, but non-punctured leaf pieces were arranged (abaxial side up) on a clean urethane sponge lined with moistened sterile cheesecloth in a plastic container.

Hydrophilic aqueous culture filtrates (50–100 μL) were applied directly to the scratched area. The cellulose-adsorbed hydrophobic fractions were added onto a drop of sterile distilled water previously placed on the scratched area. Aliquots of sterile distilled water were added whenever the droplets showed desiccation during the 8 days incubation period at 20-22°C and 10 h photoperiod under fluorescent lights. Phytotoxicity was scored based on the amount of necrotic area on 3 leaf pieces per treatment. The following rating scale was adopted: **-** = none; **+/-** = less than 10% of leaf area; **+** =10-20%; **++** = 20-40%; **+++** = 40-60%; **++++** = 60-80% and **+++++** = 80-100%.

Seed germination test: Seeds of wheat variety Machete were surface sterilised in 1% NaOCl and dried under a laminar flow. Twenty seeds were placed in 55-mm Petri plates (2 replicates) and 4 mL of commercial CB in 1% aqueous dimethyl sulfoxide (DMSO) with final concentrations of 70,140 and 280 µM were added. Further additions of these solutions were made when needed. Root length was measured after 94 h incubation on a 50-rpm shaker at 20-22°C with 10 h photoperiod under fluorescent lights. Data were analysed using the Analytical Software Statistix version 7.1 for Windows 95, 98, NT and 2000.

Bi-nucleate cell detection

Surface sterilised seeds of barley cultivar Skiff were soaked overnight in sterile distilled water and 20 seedlings having approximately the same length of radicle were transferred to 55-mm plastic Petri plates lined with sterile filter paper. Four mL of the above CB solutions were added and the plates were incubated for 48 h at 20-22°C. Approximately 1 cm root tips were excised and fixed in 45% acetic acid, squashed and stained following the Feulgen method (Purvis *et al.* 1964). Binucleate cells were observed under bright field on an Olympus Vanox-T photomicrographic microscope.

Determination of apparent molecular size

Five ultracentrifuge tubes [Activon^{TM}, 5000- and 10,000 nominal molecular cut off (nmco)] were filled with 400 µL of culture filtrate (concentrated 20 times in rotavapor) of each fungus and centrifuged three times at 17,900 rcf for 30 min each run. The filtrate and retentate were bioassayed in a dilution series corresponding to 1:0, 1:2, 1:4, 1:8 and 1:16.

Results

The concentrated crude extracts from the liquid culture filtrates of *D. wirreganensis* and *D. campanulata* induced various degrees of necrosis on all test plants (Table 1). Necrotic lesions on faba bean cultivar Fiord were black, round and large (up to 15 mm in diameter). Lesion size decreased with the dilution of the test filtrate. Lesions on all grass hosts were small (2-8 mm), round to elongate and grey to black in colour. The more sensitive reaction exhibited by faba bean and the ease of handling the leaves during assay made this plant the bio-indicator of choice. The minimal detectable leaf-drop dose was 0.07 µg of CB.

Phytotoxicity was detectable up to a dilution of 1:4 with *D. campanulata* and 1:16 with *D. wirreganensis*. Rapid loss of activity beyond this dilution was attributed to the presence of relatively low concentrations of the phytotoxic principle in the filtrates. Initial bioassay of crude ultrafiltrates (Table 2) indicated that the phytotoxic metabolites produced by *D. wirreganensis* and *D. campanulata* had an apparent molecular size of less than 5000 nominal mass.

Table 3 correlates the phytotoxicity of bands detected by the *p*-anisaldehyde reagent for cytochalasins and mass spectral parameters of the major components present in TLC-purified extracts of *D. wirreganensis* and *D. campanulata*. The most polar phytotoxic compound in extracts of both *D. wirreganensis* (Rf 0.42) and *D. campanulata* (Rf 0.41) was also the darkest staining band with the acidified *p*-anisaldehyde reagent for cytochalasins (Fig 1).

Insert Fig 1 here

It exhibited a positive molecular radical ion at m/z 479. Mass spectral comparisons (as similarity indices) to authentic CB (1.00) were Dw1 (0.94) and Dc1 (0.91). Dw1 and Dc1 were also indistinguishable in their chromatographic properties and biological activity (Table 3). Quantification of eluants from the cytochalasin TLC band by polarimetry indicated that *D. wirreganensis* (10.3 g kg^{-1} dry mycelia) contained a higher concentration of CB than *D. campanulata* (7.7 g kg^{-1} dry mycelia).

The characteristic mass spectrometric ions of active components 1-5 in Fig 1 are listed in Table 3.

Cytochalasin B at 70, 140 and 280 µM in 1% aqueous DMSO reduced root elongation of wheat seedlings (Table 4), as a linear function ($y = -0.1144x + 41.488$, $R^2 = 0.9875$) of the CB concentration (x).

Binucleate cells (Fig 2) were observed on squashed root tips of barley seedlings grown in 1% DMSO solutions containing 280 µM CB.

Insert Fig 2 here.

Discussion

This investigation was conducted to characterise by mass spectrometry and to identify phytotoxic components produced by *D. wirreganensis* and *D. campanulata* and if possible to relate these compounds to symptoms typical of each pathogen. The current studies demonstrate that the major hydrophobic phytotoxic metabolites produced by both pathogens are of a nominal molecular cut off (nmco) of less than 5000 daltons and belong to the cytochalasin group.

The cytochalasins (cytos= cells, chalasis = relaxation) bring about a wide range of cytological effects (Rothweiler and Tamm 1966). Of these, CB is the most widely studied and has been extensively used for cytological investigations (Carter 1967; Copeland 1973; Lin and Spudich 1974; Tamm 1978; Thomas 1978; Thomas *et al.* 1973) but has also been considered less toxic than related cytochalasins (Betina *et al.* 1972; Bottalico *et al.* 1989; Carter 1967). In addition to a recent report by Evidente *et al.* (2002), CB has been previously extracted from various phytopathogenic fungi such as *Drechslera dematioidea* (Aldridge *et al.* 1967) and *Phoma exigua* strain 298, *P. exigua* var. *exigua* on potatoes (Scott *et al.* 1975), *Hormiscium* spp. on tomatoes (Pribela *et al.* 1975) and *Aschochyta heteromorpha* on chickpea (Capasso *et al.* 1988). Evidente *et al.* (2002) did not observe the presence of dihydrocytochalasin in *D. campanulata* extracts, but noted the biological activity of synthetic 21,22-dihydrocytochalasin B (Bottalico *et al.* 1990). Differences observed in the types of minor cytochalasins, which were produced by *D. campanulata*, as reported by Evidente *et al.* (2002) can probably be ascribed to the different types of culture methods (liquid culture in the current study *versus* seed culture, Evidente *et al.* 2002). In general there is good agreement in the facts that CB is the major component and the concentrations of CB necessary to observe root inhibition effects.

All of the phytotoxic compounds detected in the current work, and the dihydrocytochalasin derivative are relatively hydrophobic compounds, which exhibit some degree of amphiphilicity due to the presence of hydroxyl substituents. They are readily isolated from both 30-day old culture filtrates and the dried mycelia of *D. wirreganensis* and *D. campanulata*. The necessity for amphiphylic solvents such as ethanol or DMSO to bring these hydrophobic molecules with low aqueous solubility into solution and the usefulness of dispersal on cellulose for an effective plant bioassay, may explain the previously reported lack of phytotoxicity of CB (Betina *et al.* 1972; Bottalico *et al.* 1989; Carter 1967). The bioassay in the current study used CB evaporated onto cellulose powder and placed in intimate contact with the leaf throughout the assay. This protocol maximises the surface area allowing diffusion of relatively hydrophobic molecules to the lipophilic plant cell walls in an aqueous environment. Naturally occurring detergent-like molecules such as saponins may speed up the rate of diffusion and contribute to the observed phytotoxic effects.

All active components including Dc2 (Rf 0.51) and Dw2 (Rf 0.49) from *D. campanulata* and *D. wirreganensis* showed the characteristic base peak benzylium ion at m/z 91. In addition, Dc2 and Dw2 showed ions at m/z 481($M^{+\cdot}$), 463($M^{+\cdot}$-H_2O), (445($M^{+\cdot}$-$2H_2O$) indicative of the presence of a dihydrocytochalasin B.

Only one band (Dw2) with an ion at m/z 481 was detectable in the extracts of *D. wirreganensis*. However, *D. campanulata* bands Dc2 and Dc4 both exhibited ions at m/z 91 and 481. The much greater mobility of Dc4 (Rf 0.66) compared to Dc2 (Rf 0.51) is probably not consistent with a simple isomeric difference, but may suggest rapid thermal loss of a hydrophobic substituent on the mass spectrometer probe to yield a dihydrocytochalasin radical fragment ion.

The biologically active band (Dw4) of *D. wirreganensis* found at Rf 0.62, with a series higher mass ions up to 675 m/z is suggestive of a different type of hydrophobically-substituted CB.

Given that the concentration of CB differs only marginally between *D. wirreganensis* and *D. campanulata*, it is possible that the slight qualitative differences in symptoms between *D. wirreganensis* and *D. campanulata* are due to differing phytotoxic properties of the substituted cytochalasin (Dw4) unique to *D. wirreganensis* and the putative substituted dihydrocytochalasin (Dc4) unique to *D. campanulata*.

Crystalline cytochalasin B, indistinguishable in all its physico-chemical properties from an authentic sample when used at concentrations up to 280 µM in 1% aqueous DMSO, reduced root elongation of wheat seedlings (Table 4) as a linear function of CB, but did not completely stop germination. CB at 100 - 200 µg mL^{-1} (209 - 418 µM) in 2% DMSO reduced the rate of elongation in wheat coleoptile and maize root (Pope *et al.* 1979). Consequently, it is unlikely that CB alone, is the postulated phytotoxin involved in the total inhibition of wheat seed germination by natural infections of *D. campanulata* as reported previously (Wallace 1959).

The detection of binucleate cells is a well documented feature (Carter 1967; Krishan and Ray-Chaudhuri 1969; Ridler and Smith 1968) in the cytology of aberrant cytochalasin- induced cell division in animal cells. Aberrant plant cell behaviour observed by earlier researchers at significantly lower CB concentrations include: rapid cessation of cytoplasmic streaming in pollen tubes of *Lilium longiflorum* in solutions containing 0.1µg mL^{-1} (0.21 µM) (Herth *et al.* 1972); complete inhibition of cytoplasmic streaming in *Chara* at a concentration of 25 µg mL^{-1} (52.2 µM) (Williamson 1975) and inhibition of root hair growth in lettuce at 5-10 µg mL^{-1} (10.4 – 20.8 µM) CB (Sawhney and Srivastava 1974).

The original observation of binucleate cells in onion tissue treated with 0.5 – 25 µg mL^{-1} (1.04 – 52.2 µM) of CB (Palevitz 1980) was disputed by Thomas *et al.* (1973). It is possible that plant cuticular waxes may adsorb much of the applied hydrophobic CB, making detection of the binucleate cells very dependent upon the amount of lipid present in the particular cell walls of the tissue being investigated, thereby making the detection problematic at low concentrations of CB.

It is noteworthy that in the current study, very large concentrations (280 µM) of CB were required to detect the effects on barley root tips (Fig 2). Nevertheless, binucleate cell formation has been a very valuable tool in animal cytological work (Carter 1967; Thilly *et al.* 1978). The detection of binucleate cells in the root tips of barley seedlings in the current study supports the conclusion that part of the phytotoxicity of both *D. wirreganensis* and *D. campanulata* is associated with these cytochalasin species.

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Table 1. Necrosis induced by crude filtrates of *D. wirreganensis* and *D. campanulata* on various plant species.

Pathogen/ Dilution	Faba bean	*Phalaris* sp.	Wheat	Franklin barley	Weeah barley
D. wirreganensis					
1:0	+++++	+++	+++	+++	+
1:2	+++++	-	-	+	-
1:4	++++	-	-	-	-
1:8	+++	-	-	-	-
1:16	++	-	-	-	-
D. campanulata					
1:0	+++++	++	++	++	+
1:2	+++++	-	-	++	-
1:4	++++	-	-	+	-
1:8	-	-	-	-	-
1:16	-	-	-	-	-

Table 2. Necrosis induced by crude ultrafiltrates[1] of *D. wirreganensis* and *D. campanulata* on faba bean leaves.

Dilution	*D. wirreganensis*		*D. campanulata*	
	10000 nmco	5000 nmco	10000 nmco	5000 nmco
1:0	+++++	+++++	+++++	+++++
1:2	+++++	++++	+++++	++++
1:4	++++	++++	+++++	++++
1:8	++++	+/-	++++	+/-
1:16	+/-	-	+/-	-
SDW	-	-	-	-

[1] Four mL concentrated crude culture filtrates in Activon™ ultrafilters (10000 or 5000 nominal molecular cut off, nmco) were centrifuged three times at 17900 rcf for 30 min each run.

Table 3. Correlation of biologically active bands detected from TLC-purified extracts[1] of culture filtrates and mycelia of *D. wirreganensis* and *D. campanulata* with m/s spectra.

Fungus/Bioactive Band	Necrotic rating	Nominal Rf value[2]	Colour Reaction	Base Peak	Apparent DI/EI molecular ions
D. wirreganensis					
Dw-1	+++++	0.42	Blue	m/z 91	M/z 479
Dw-2	+++	0.49	Blue	m/z 91	M/z 481
Dw-3	+++++	0.56	Blue		
Dw-4	++++	0.62	Blue	m/z 91	M/z 675
Dw-5	+++	0.84	Blue		
D. campanulata					
Dc-1	+++++	0.41	Blue	m/z 91	M/z 479
Dc-2	+++	0.51	Blue	m/z 91	M/z 481
Dc-3	+++	0.57	Blue		
Dc-4	+++++	0.66	Blue	m/z 91	M/z 481
Dc-5	++++	0.80	Blue		
Cytochalasin B	+++++	0.42	Blue	M/z 91	M/z 479

[1] TLC developed in 5:4:1 (v/v/v) ethyl acetate: hexane: formic acid; re-dissolved in 50:50 (v/v) Chloroform:Methanol and re-purified in 6:3:1 (v/v/v) hexane: diethylamine: formic acid. TLC dipped in 9:1 (v/v) alcohol/acidified *p*-anisaldehyde reagent and heated at 100°C.

[2] Nominal Rf values refer to separation distance divided by a constant solvent front distance. They signify relative migration rates after a multiple elution (3x) sequence.

Table 4. Germination of wheat in different concentrations of cytochalasin B dissolved in 1% DMSO.

Cytochalasin B (μM)	Mean root length (mm)
0 (1%DMSO)	42.9a
0 (Water)	40.7a
70	34.5b
140	30.7b
280	18.6c

Means followed by the same letter are not significantly different according to LSD (T) test at P=0.05, n=40.

Fig 1. TLC comparison of the bioactive bands (numbers 1-5) produced by *D. wirreganensis* (Dw, lanes 1 and 2) and *D. campanulata* (Dc, lanes 4 and 5) with commercial reference cytochalasin B (CB, lane 3). The purified CB isolated from *D. wirreganensis* and *D. campanulata* is shown in lanes 1 and 5, respectively.

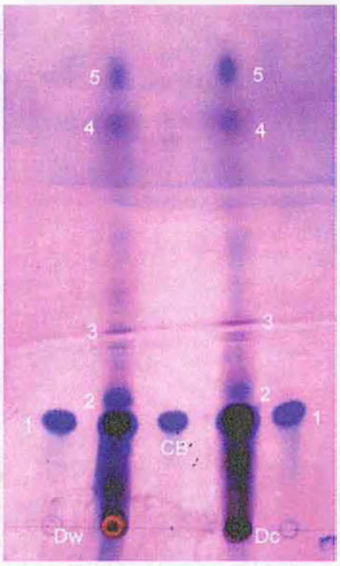
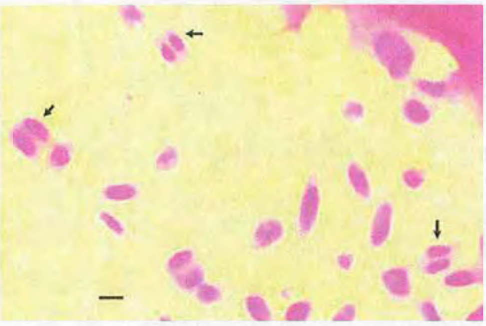


Fig 2. Binucleate cells (arrows) on root tips of barley germinated for 48 h in cytochalasin B at 280 µM (bar = 5µm).



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