Ann. appl. Biol. (1993), **122**, 233–251
Printed in Great Britain

The effect of silver and other metal ions on the *in vitro* growth of root-rotting *Phytophthora* and other fungal species

By S. J. SLADE[1] and G. F. PEGG*

Department of Horticulture, University of Reading, Earley Gate, Reading, Berks RG62AU, UK

(Accepted 26 October 1992)

Summary

A range of metal ions and the oxoanion WO_4^{2-} were toxic to zoospores of *Phytophthora nicotianae parasitica* in the order: $Ag^+ >> Cu^{++} > WO_4^{2-} > Ni^+ > Co^{++} > Zn^+$. The LD_{50} for Ag^+, 0.11 μM (11.4 ppb), compared with 1.84 μM (117 ppb) for Cu^{++}. Silver was similarly toxic to a range of pathogens including *Pythium aphanidermatum, Thielaviopsis basicola* and *Fusarium oxysporum* f.spp. Most zoospores of *Phytophthora* spp. were killed by Ag^+ in the range 46 – 460 nM (5 – 50 ppb), bursting at the higher concentrations. A small sub-population of most propagules exhibited greater tolerance to silver than the whole. In 0.93 μM (100 ppb) Ag^+ 1.4% of *P. nicotianae parasitica* zoospores survived but were all killed at 500 ppb. A population of *P. cryptogea* (1.9%) surviving 0.47 μM (50 ppb) were killed at 0.93 μM (100 ppb). Zoospore cysts and germlings showed the same sensitivity to silver. Oospores were mostly killed over the range 0.23 – 0.93 μM (25 – 100 ppb) Ag^+, some surviving up to the lethal concentration of 9.26 μM (1000 ppb). Mycelium of *P. cryptogea* was generally less sensitive, with some growth occurring at 9.26 μM (100 ppb). Zoosporangiogenesis was unaffected over the range 0.47 – 4.65 μM (50 – 500 ppb). Toxicity increased with increased pH over the range 5.0 – 6.5.

Ionic silver was lost from solution during a microscope slide bioassay by binding to the glass surface. In the presence of chloride ions, colloidal AgCl formed which was equally toxic to *P. cryptogea*. Silver and AgCl were further lost from solution by colloidal agglomeration – Ostwald ripening – and by AgCl adsorption to glass. Silver, < 90 nM (10 ppb) Ag^+ as $AgNO_3$ and particles of silver chloride were both strongly attractive to zoospores of *P. cryptogea*. Spores burst or failed to germinate on entering lethal concentrations. The results are discussed in the context of the use of silver salts to control *Phytophthora* root-rot pathogens and the importance of ion availability in *in vitro* toxicity assays.

Key words: Silver, silver chloride, fungal toxicity, ionic binding, *Phytophthora*, zoospores, chemoattraction

Introduction

Plants grown in hydroponic systems using artificial soil-less substrates are especially vulnerable to diseases caused by water-borne pathogens (Price, 1976; Evans, 1979; Vanachter, van Wambeke & van Assche, 1983). The principal root-rot pathogens of tomato are *Phytophthora nicotianae* B. de Haan, var. *parasitica* (Dastur.) Waterh. (Pegg & Holderness,

[1]Present address: Pilkington Technology Centre, Hall Lane, Lathom, Ormskirk, Lancs L405UF, UK
*To whom correspondence should be addressed

1984; Holderness & Pegg, 1986) and *P. cryptogea* Pethyb. & Laff. (Pegg & Jordan, 1990; Kennedy & Pegg, 1990).

Control of pathogenic phycomycetes has long been associated with heavy metals, with copper in various forms occupying a traditional role (Kennedy & Erwin, 1961; Zentmeyer & Marshall, 1959; Halsall, 1977). Zinc and its organic and inorganic derivatives have also shown a wide spectrum of antifungal activity. Crook-root, a root-rot of watercress, caused by *Spongospora subterranea* (Wallr.) Lagerh. f.sp. *nasturtii* Tomlinson, has been effectively controlled by killing zoospores in the aqueous growing medium with Zn^{2+} slowly released from a zinc phosphate glass frit (Tomlinson, 1958), or zinc salts solutions.

The germicidal property of metallic silver has been recognised since historic times. More recently, various preparations based on silver salts or derivatives have been developed for topical use in medical and veterinary practice (Crannell, 1967). Its low mammalian toxicity led to its widespread application in the 1930s in the sterilisation of drinking water and other solutions (Krause, 1929; Brandes, 1934). The antimicrobial action was based on the natural ionisation of metallic silver, the "oligodynamic effect" (Goetz, Tracy & Harris, 1940) giving a weak aqueous solution of silver ions. This was greatly enhanced in commercial systems by low voltage electrolysis from which it was claimed 0.47 μM (0.05 ppm) Ag^{2+} would kill all bacteria within one hour (Brandes, 1934). Sokol & Klein (1975) reported growth inhibition of *Arthrobacter* sp. by silver from $AgNO_3$ or AgI at 6×10^{-9} g ml^{-1} (54 nM). The fungicidal activity of silver against plant pathogenic fungi has been known almost as long as that of copper (Raulin, 1869; Huot & Bouchardat, 1899). Most interest has centred on the possible use of silver as a foliar fungicide against aerial pathogens (Nielsen, 1942; Miller & McCallan, 1957). A high uptake of silver into the fungal spore was demonstrated by Miller, McCallan & Weed (1953), with corresponding increased permeability of the spore membrane to other compounds. Of a range of heavy metals tested, including mercury and copper, silver had the highest uptake, inhibiting germination after a contact time of one minute or less. Neilsen (1942) demonstrated the effective control of *Phytophthora infestans* on potato leaves using a range of silver salts alone and in combination with other compounds. The failure of silver to be developed as a commercial fungicide was attributed by Neilsen (1942) to problems of formulation and foliar retention of the soluble ion.

Since water can be sterilised by very low concentrations of Ag, the present study was undertaken to explore the effectiveness of Ag *in vitro* in comparison with other metals against root-rotting *Phytophthora* spp. and other species as a prelude to its possible use in the control of tomato root-rot in plants grown hydroponically in rockwool culture (Pegg & Jordan, 1990).

Materials and Methods

Fungal cultures

Fungal pathogens used in the study were: *Phytophthora nicotianae* B. de Haan var. *parasitica* (Dastur.) Waterh., *P. cryptogea* Pethyb. & Laff., *Pythium aphanidermatum* (Edson) Fitzp., *Verticillium albo-atrum* Reinke & Berth., *Thielaviopsis basicola* (Berk. & Br.) Ferraris, *Fusarium oxysporum* Schl. f.sp. *lycopersici* (Sacc.) Snyder & Hansen and *F. oxysporum* Schl. f.sp. *dianthi* (Prill & Delacr.) Snyder & Hansen. They were all pathogens of hydroponically-grown tomatoes, except for *F. oxysporum* f. *dianthi*, a wilt pathogen of carnation, *Dianthus caryophyllus* L. Cultures were from the University of Reading Department of Horticulture, except *P. aphanidermatum*, isolate 4104b, kindly provided by Dr M. W. Dick. Cultures were maintained on potato dextrose agar (PDA) at 21°C in an incubator

illuminated by a single 15 W Sylvania fluorescent lamp giving *c.* 25 lm m^{-2} and sub-cultured regularly.

Conidia for fungicide bioassays were washed off 14 day-old PDA cultures with sterile deionised water, filtered through a single layer of sterile muslin and washed three times by centrifugation at 5000 ***g*** and resuspended in sterile deionised water before diluting to the required concentration. Zoospores were produced by transferring cubes of colonised PDA to 9 cm plates containing 20 ml modified V8 medium made from V8 Juice (Campbells, Ltd) adjusted to pH 5.5 with $CaCO_3$, followed by centrifugation to remove solids and diluting 1:10 with deionised water (Menyonga & Tsao, 1966). To stimulate sporangiogenesis, 10 mg $litre^{-1}$ β-sitosterol was added before autoclaving. After 14 days at 21°C in continuous low intensity light, medium was removed from the dishes with a sterile syringe and the mycelium washed five times by resuspension in sterile deionised water (SDW). Cultures were chilled at 4°C for 20 min, followed by 1.5 h incubation at 20°C in the dark, to stimulate sporangial differentiation and zoospore release. The suspension was adjusted to appropriate concentration with SDW. Zoospores were induced to encyst in solution by shaking vigorously for 20 min. Oospores of *P. nicotianae* var. *parasitica* were induced in aqueous V8 medium by co-culturing with *P. cryptogea* and incubating in darkness for 35 days at 21°C. No *P. cryptogea* oospores were formed in the mixed culture. Oospores were removed from the mycelium by feeding the whole culture to great pond snails (*Lymnaea stagnalis* L.) in tap water. The snails were starved for 48 h before feeding with mycelium. Their faeces subsequently contained oospores and small macerated hyphal fragments. Oospores were recovered by filtering the faecal suspension in water through a double layer of muslin.

Spore bioassay

Test solutions in SDW were mixed with a concentrated spore suspension in borosilicate glass vials to give a propagule density of 1×10^5 spores ml^{-1}. A 100 μl droplet was incubated in an open cavity slide in a humidity chamber, at saturation r.h. at 20°C in darkness for 18 h. Preparation times were closely standardised to allow for the binding of metallic ions to the glass surface. The numbers of germinated and non-germinated cysts were counted in five, randomly-selected microscope fields for each of three or five replicates. Arcsin transformed data were subject to analysis of variance and statistical significance presented as standard errors or 95% confidence limits. Backtransformed values are plotted in the graphs. In some experiments, spore germination assays were carried out with the test element dissolved in a hydroponics nutrient solution (Pegg & Jordan, 1990), with an EC of 3 mS cm^{-1} and pH 5.7 using *Analar* chemicals. Changes in pH were achieved by adjusting solutions with a small volume of HNO_3 or KOH. The fungitoxicity of AgCl was assessed by mixing a dense zoospore suspension with volumes of aqueous $AgNO_3$ and KCl to the required zoospore concentration, immediately before incubation. All experiments were carried out on the same basis as the nutrient concentrations, i.e. in parts per million (ppm) or parts per thousand million (ppb). Comparative concentrations on a molar basis (μM or nM) are cited as appropriate.

Fungal growth and sporangiogenesis

The effect of Ag on vegetative growth of *P. cryptogea* was examined in a nutrient solution (Pegg & Jordan, 1990) amended with 15 g $litre^{-1}$ sucrose, 1 g $litre^{-1}$ asparagine and 25 μg $litre^{-1}$ β-sitosterol (Erwin & Katznelson, 1961) and sterilised by Millipore filtration. Three replicate conical flasks (250 ml) containing 100 ml medium and Ag as $AgNO_3$ were inoculated with 0.5 cm cubes of PDA carrying *P. cryptogea* mycelium. After 94 h incubation at 20°C, mycelial mats were washed with 100 ml water in a Buchner filter without vacuum or filter

paper and the dry weights of three replicates determined after 12 h at 85°C. Zoosporangium formation was measured by counting sporangia along the 40 mm perimeter of a 1 cm^2 × 4.7 mm deep block of PDA colonised by *P. cryptogea*, following transfer to a 5 cm Petri dish, covering with 21 ml SDW and incubating at 21°C for 40 days. Counts were based on the numbers of zoosporangia per block for each of six replicates.

Silver adsorption to glass surfaces

Experiments were carried out in 150 ml borosilicate glass conical flasks to assess the depletion of silver from solution due to binding to the glass surface. The amount of Ag remaining in solution and available to the fungus could then be determined. Flasks were washed prior to use in 1 M nitric acid and rinsed thrice in deionised water. A dilution series of $AgNO_3$ in water was prepared and five replicate 100 ml aliquots of each concentration dispensed into the flasks. Immediately afterwards, and at intervals up to 115 h, 3 ml samples were taken from each flask and stored in 5 ml borosilicate glass vials containing 0.5 ml 1% *ammonia* solution to prevent adsorption. During the experiment the flasks were not moved and were kept at 23°C in a dark room. Silver ion concentration was measured by an ARL 35000 sequential, inductively-coupled plasma, optical emission spectrophotometer (Applied Research Laboratories, Ecublens, Switzerland) using an Argon plasma. Emission was measured at a wavelength of Å3280.68. The instrument detection limit was defined as the concentration of analyte giving a signal twice the standard deviation of the background emission. Precision at this level was ± 50%. Data were analysed to find a linear relationship between the residual Ag^+ in solution at a given time and that adsorbed to the glass. The relationship between the slope of the linear plots and the duration of Ag^+ exposure to the glass was then determined.

The effect of Cl^- ions on adsorption and loss of silver from solution followed a similar protocol to that described above, except that all flasks contained 1.69 mM (60 ppm) Cl^-, as KCl solution. (Standard plant hydroponic nutrient solutions at an E.C. 3.0 mS contain 60 ppm [1.69 mM] Cl^-). The results are presented as mean concentrations with standard errors of the mean. Equations were determined relating the mean concentrations measured at each time to the initial concentration, the volume of solution and the surface area of glass in contact with the solution.

Chemotaxis

Zoospore chemotaxis was measured by "*diffusion in*", "*diffusion out*" techniques using similar apparatus to that described by Khew & Zentmyer (1973). A 25 mm × 12 mm × 2 mm chamber was constructed from cut borosilicate microscope slides. The glass pieces were held together by a thin film of petroleum jelly and a cover slip was used to create a sturdy chamber containing 0.6 ml fluid, held in place by surface tension. A 1 mm (internal diameter) soda glass capillary tube sealed at the outer end was inserted into the chamber solution and held in place by a plug of petroleum jelly. For "*diffusion out*" observations the chamber was filled with a 5×10^5 ml^{-1} *P. cryptogea* zoospore suspension in SDW with the capillary containing $AgNO_3$ solution. In the "*diffusion in*" mode, the capillary was filled with a suspension of *c.* 200 zoospores into which Ag^+ ions diffused from the chamber containing the test solution. Treatments were replicated five times and the water controls 10 times. Chemotaxis was measured as the number of encysted zoospores (dead or living) recorded after 18 h in consecutive 1 mm × 1 mm fields of view along the capillary and in a straight line away from the capillary into the chamber, using an eyepiece graticule. For "*diffusion out*" experiments a chemotaxis index (Khew & Zentmyer, 1973) was calculated from the number of zoospores in the first 1 mm^2 of the capillary divided by the number in the

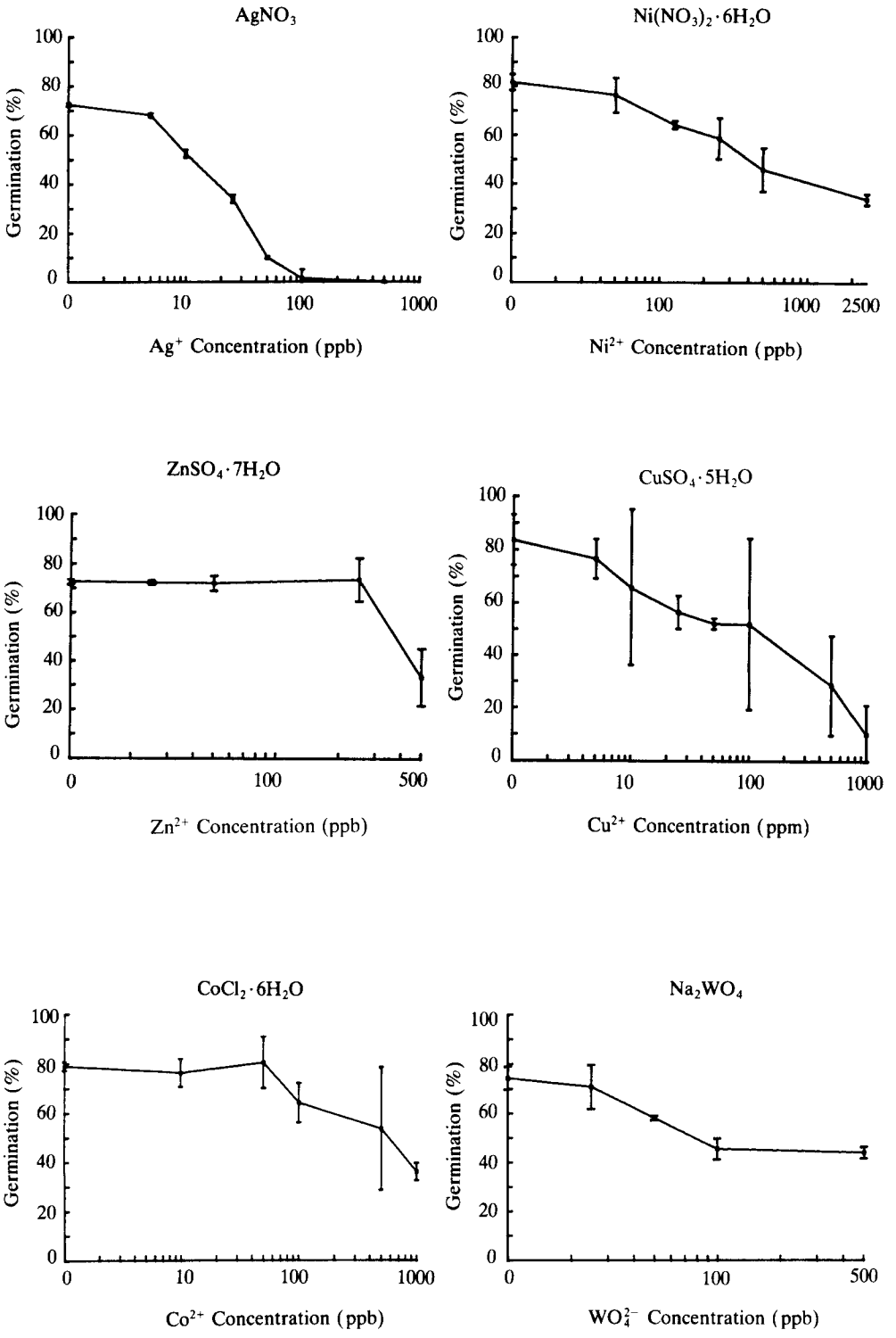

Fig. 1. The effect of fungitoxic salts on the *in vitro* germination of *P. nicotianae parasitica* zoospores. Mean percentage germination for three replicates (five for $AgNO_3$) after 18 h. Vertical bars indicate 95% confidence limits of the mean. See text for details.

same area 5 mm from the capillary tip, beyond the zone of chemotaxis. The percentage germination was analysed statistically following arcsin transformation.

Results

The effect of 10 metal ions at concentrations of 1 ppm, or greater, on the germination of *P. nicotianae* var. *parasitica* zoospores is shown in Table 1. Of these only Hg^{+} and Pb^{++} at 1.0 ppm reduced zoospore germination by 50% or more compared with the SDW controls, but in view of their high mammalian toxicity (Hammond & Foulkes, 1986; Friberg, Nordberg & Vouk, 1979), these were not studied further. Of a range of metallic ions with relatively low mammalian toxicity, suitable for investigation as hydroponic fungicides, the six most active inhibitors of zoospore germination are shown in Fig. 1. The toxicity of metal cations in order were: $Ag^{+} >> Cu^{++} > Ni^{+}$, $Co^{++} > Zn^{+}$; from the salts: $AgNO_3$, $CuSO_45H_2O$, $Ni(NO_3)_26H_2O$, $CoCl_26H_2O$ and $ZnSO_47H_2O$. The oxoanion WO_4^{2-} from Na_2WO_4 was intermediate in toxicity between Cu^{2+} and Ni^{+}. Germination was unaffected in the presence of the anhydrous sodium salts of the anions Cl^{-}, SO_4^{--} and NO_3^{-} at the highest concentrations present in the cation experiments. All the antifungal activity of the salts was attributable to the heavy metal moiety.

At concentrations greater than 0.47 μM (> 50 ppb), Ag^{+}, the most toxic cation, caused *P. nicotianae* var. *parasitica* zoospores to burst. A small population, *c.* 1.4%, of the spores viable in SDW remained viable at this concentration. Zoospore cysts responded similarly to Ag^{+} (Fig. 2*a*) but 500 ppb was lethal to cysts and motile spores. After 18 h only 47% of the oospores germinated in water. The greatest increase in oospore toxicity occurred over the range 25 – 100 ppb with a small number (< 10%) surviving up to 1000 ppb Ag^{+}, the lethal concentration (Fig. 2*b*).

The effect of silver on the germination of propagules of several root-rot pathogens, and *Fusarium oxysporum* f.sp. *dianthi* and *Fusarium oxysporum* f.sp. *lycopersici*, are shown in Fig. 3. The germination response curves for these widely differing genera were similar. Minimal lethal concentrations were: *Pythium aphanidermatum* (zoospores), 50 ppb; < *Phytophthora cryptogea* (zoospores), *Thielaviopsis basicola* and *Fusarium oxysporum* f.sp. *lycopersici*, 100 ppb; < *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *dianthi*, 500 ppb.

Table 1. *The effect of metal ions on germination of* P. nicotianae parasitica *zoospores*

Ion	Concentration (ppm)	Salt	Germination* (%) Mean	S.E. (±)
Hg	1	HgCl	30.6	3.6
Al	5	$Al_2(SO_4)_3$	73.2	4.3
Se	1	H_2SeO_4	62.0	12.6
Cr	1	$Cr_2(SO_4)_3.15H_2O$	61.6	3.9
V	1	$VOSO_4.H_2O$	54.5	8.2
Pb	1	$Pb(NO_3)_2$	35.6	10.6
Bi	5	$Bi(NO_3)_3.5H_2O$	52.1	12.2
Cd	1	$CdCl_2$	66.9	9.2
Tl	1	CH_3COOTl	67.5	11.3
Mn	2	$MnSO_4.4H_2O$	56.9	5.8
		H_2O	74.4	1.6

* Means of counts in three replicate cavity slides, after 18 h. 95% confidence interval (pooled data) = 3.5

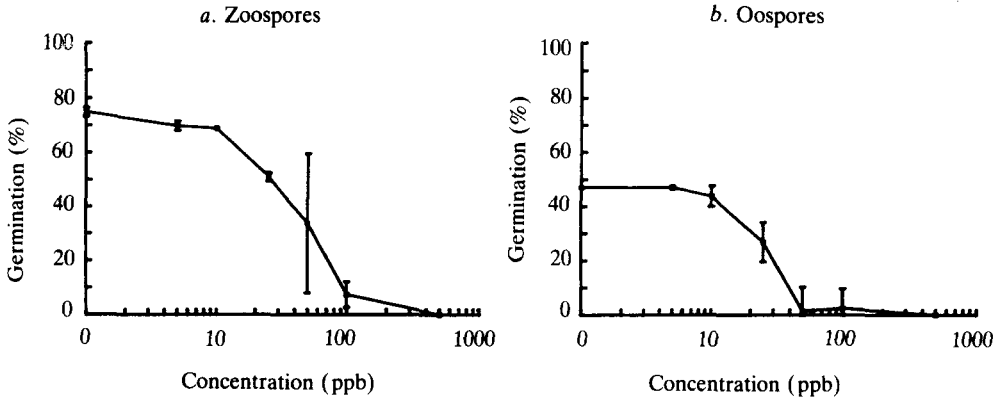

Fig. 2. The effect of silver on *in vitro* germination of *P. nicotianae parasitica* spores. Data based on means of three replicates after 18 h with 95% confidence limits.

The growth of *P. cryptogea* hyphae in the presence of Ag^+ was affected in a similar way to the spores (Table 2). The addition of 50 ppb at various times after germination resulted in the immediate killing of most cysts. A small number of germ tubes, 8.5% of the population, continued to grow for up to 18 h and exceeded the SDW control length (280.3 μM), most probably due to nutrient leakage from dead cells. Since the numbers of surviving hyphae were low, confidence limits were correspondingly high. No increase in germination in 50 ppb Ag^+ occurred (Table 3) in cysts ungerminated at the start of the experiment.

Mycelial growth in aqueous culture declined rapidly between 5 and 50 ppb (Fig. 4); unlike germ tubes, however, reduced growth was maintained up to at least 1000 ppb with abnormally small, dense colonies. Silver in concentrations of 50 – 500 ppb had no effect on zoosporangium formation.

At 25 ppb, silver fungitoxicity increased at pH above 5.5. At 37.5 ppb this effect was

Table 2. *The effect of 0.05 ppm Ag^+ on the growth of germ tubes of* P. cryptogea

	Length of germination hypha* (μm)					
	At addition		After 18 h†			
Time of Ag^+ addition (min)			Hyphae <200 μm		Hyphae >200 μm	
	Mean	S.E. (±)	Mean	S.E. (±)	Mean	S.E. (±)
0	7.4	1.1	6.9	0.1	355	39
30	19.5	0.9	20.4	1.8	347	49
75	61.5	3.5	50.4	1.3	357	27
150	83.5	4.1	91.7	5.1	442	70
210	105.9	5.7	105.4	4.5	383	38
95% Confidence interval (pooled data		8.0		7.2		104

Untreated by Ag^+: mean length 280.3 μm, 95% C.I. = 53.8 μm.

* Mean of counts in three replicate cavity slides

† At 18 h there were two populations of germlings: those killed by the Ag^+ (hyphae < 200 μm) and those that had continued to grow (hyphae > 200 μm).

See text for details.

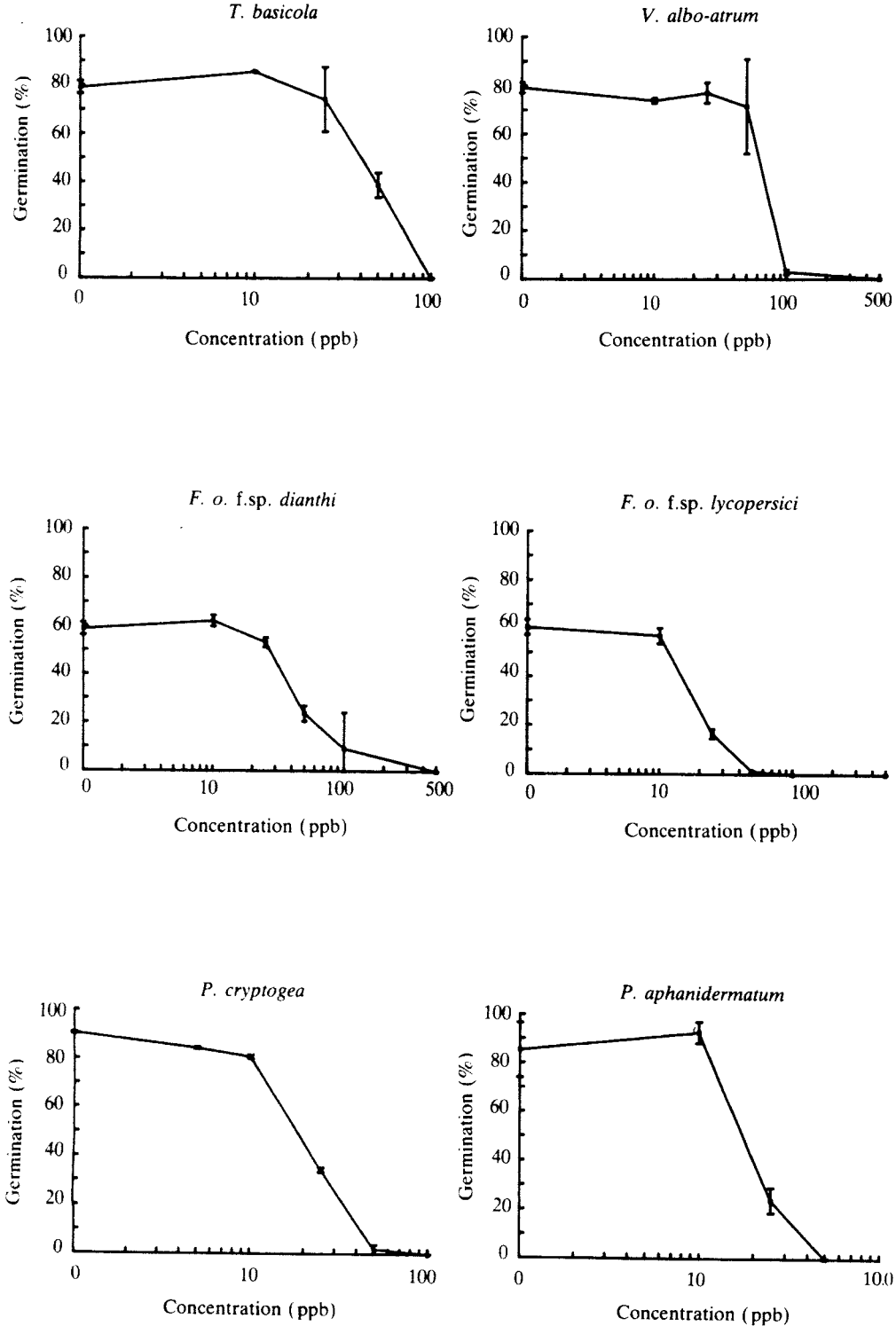

Fig. 3. The effect of silver on the *in vitro* germination of various fungal propagules.
Data represent means of three replicates (five for *P. cryptogea*) after 18 h, with 95% confidence limits.

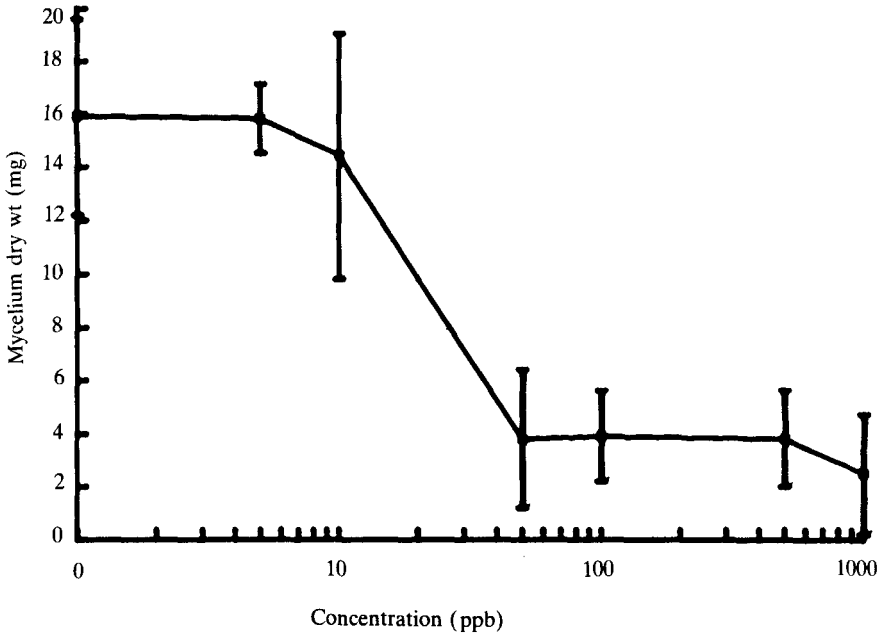

Fig. 4. The effect of silver on the mycelial growth of *P. cryptogea in vitro*.
Data represent the mean dry weights from three replicate flasks after 44 h with 95% confidence limits.
See M & M for details of the medium

enhanced. Compared with 40% germination in unbuffered solution, toxicity decreased below pH 4.8 and increased at values above this (Fig. 5).

The effect of Cl^- concentration on zoospore germination and of Cl^- on the fungitoxicity of 50 ppb Ag^+ is shown in Table 4. Increasing Cl^- concentration up to 4.22 mM (150 ppm) had no effect on zoospore viability or silver toxicity. At 200 ppm (5.63 mM) chloride concentration, zoospore germination in water was slightly stimulated, but silver toxicity was increased ($P = 0.05$). Since Ag^+ concentration is depressed by AgCl formation (in 200 ppm Cl^- there would have been 3 ppb Ag^+) antifungal activity could be attributed directly to freshly-formed, amorphous AgCl colloid.

Table 3. *The effect of 0.05 ppm Ag^+ addition on germination of zoospores of* P. cryptogea

Time of Ag^+ addition (min)	Germination* (%) At addition Mean	S.E. (±)	After 18 h Mean	S.E. (±)
0	57.3	4.6	50.1	3.4
30	85.5	2.2	76.8	2.5
75	97.3	1.4	91.1	2.5
150	92.4	2.1	93.0	7.0
210	91.9	1.7	94.1	1.2
Water control	95.0	0.2		
95% Confidence interval (pooled data)		0.9		2.8

* Mean of counts in three replicate cavity slides.

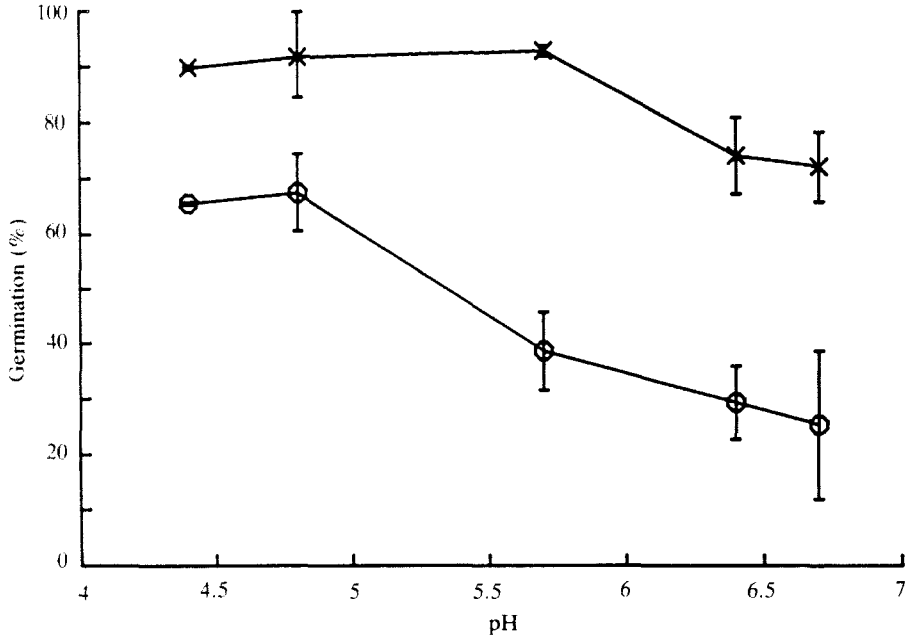

Fig. 5. Effect of pH on inhibition of germination of *P. cryptogea* zoospores by silver chloride. -○- = 37.5 ppb Ag; -×- = 25.0 ppb Ag.

Silver adsorption

The adsorption of heavy metals to glass surfaces is a well-known phenomenon (Doremus, 1973; Anderson & Rubin, 1981). Since silver was active at extremely low concentrations and the surface area/volume ratio of the cavity slide was high, the possible loss of free cations in the spore bioassay solution was an important consideration. The concentration of Ag^+ remaining in solution after various times in the borosilicate glass flasks is shown in Table 5. Data for initial concentrations of 10 and 25 ppb (0.09–0.23 μM) are not shown, since the detection limit for Ag^+ under the conditions used was found to be *c.* 30 ppb (0.28 μM). Data were analysed to determine the kinetics of the interaction between the Ag^+ ions from the $AgNO_3$ in water and the borosilicate glass surface. Only data obtained after 2 h were analysed where a significant decrease in available silver had occurred. No single

Table 4. *The effect of Cl^- concentration on the toxicity of Ag^+ to* P. cryptogea *zoospores*

Cl⁻ concentration		Germination (%)* H_2O		0.05 ppm Ag^+	
(ppm)	(mM)	Mean	S.E. (±)	Mean	S.E. (±)
200	5.63	90.1	4.0	3.2	1.4
150	4.22	90.3	1.9	6.8	3.6
100	2.82	84.8	3.4	7.4	4.2
50	1.41	85.5	1.2	6.3	0.3
0		85.9	4.0	6.7	3.6
95% Confidence Interval (pooled data)			1.1		3.2

* Means of counts in three replicate cavity slides after 18 h incubation.

Table 5. *Loss of silver from $AgNO_3$ solutions in borosilicate glass flasks**

Initial concentration (ppb)	Ag^+ concentration (ppb) after: 5 min	30 min	2 h	20 h	44 h	115 h
50	38 (±8)	48 (±9)	35 (±6)	29 (±8)	27 (±13)	25 (±13)
100	101 (±7)	92 (±8)	93 (±20)	78 (±37)	65 (±40)	52 (±18)
500	521 (±45)	500 (±60)	499 (±51)	486 (±40)	475 (±60)	424 (±33)
1000	1014 (±91)	985 (±218)	1037 (±27)	985 (±112)	975 (±69)	889 (±100)

* 100 ml $AgNO_3$ solutions incubated in 150 ml conical flasks at 23°C in darkness.
Values shown are means of five replicates. Figures in parentheses represent standard errors of the mean.

equation could be found to relate the amount of Ag^+ in solution to that bound to the glass at each sample time. Two linear relationships existed between bound and non-bound silver at each sample time. Concentrations remaining in solution were proportional to the initial concentration below 100 ppb, and decreased with time as additional binding occurred. Where initial concentrations exceeded 100 ppb, residual silver in the solution decreased with time, but the binding was independent of the initial concentration. Straight lines were fitted to the non-bound:bound Ag concentrations at 20, 44 and 115 h for initial concentrations up to 100 ppb. The slopes of the lines (m) were, 0.255, 0.524 and 0.922 cm with values of S.D. of fit of 0.04, 0.15 and 0.18 respectively.

The "surface excess", E, of Ag^+(M cm^{-1}) is

$$E = \frac{I - F}{S} V$$

Where I = initial concentration of Ag^+ in solution, F = final concentration, S = exposed glass surface area (cm^2) and V = solution volume (cm^3). The slope = m and E = Fm

Therefore 1.
$$F = \frac{I}{\left(\frac{Sm}{V}\right) + 1}$$

There was a quadratic relationship between m and exposure time t

2.
$$m = (1.420 \times 10^{-2})t + (-5.367 \times 10^{-5})t^2$$
with a S.D. of fit of 8.277×10^{-3}

Solving equation 2 gives m for any time. Equation 1 could be used to calculate the residual Ag^+ in solution for any initial Ag^+ concentration up to 100 ppb at that time. Calculated values based on actual initial concentrations and times were all within the 95% confidence interval of the measured data. The relationship was true for times < 20 h and concentrations < 100 ppb, but it was not possible to extrapolate to concentrations > 100 ppb.

From equations 1 and 2, F can be calculated for any t and I values within the above range, if the surface area of exposed glass and the liquid volume are known. The exposed surface area in the experimental flasks was 113 cm^2 and the volume 100 cm^3. Thus $V_2/100$ should be substituted for V and $S_2/113$ substituted for S. Using these terms in equation 1

Table 6. *Calculated concentration of unbound Ag^+ in $AgNO_3$ solution after 18 h incubation in borosilicate glass cavity slides*

Initial Ag^+ concentration (ppb)	(μm)	Ag^+ concentration after 18 h* (ppb)	(μm)
10	0.093	3.7†	0.034
25	0.233	9.3	0.085
50	0.465	18.6	0.171
100	0.930	37.2	0.342

* 100 ml $AgNO_3$ exposed to 0.8 cm^2 borosilicate glass.
† Calculated as described in the text.

allows the remaining Ag^+ concentration to be calculated for any volume of Ag^+ solution less than 100 ppb exposed to any borosilicate glass surface. Since temperature does not greatly affect the ion binding kinetics, the expression was true for the temperature range used in this study. The calculated Ag^+ concentrations after 18 h in the 100 μl bioassay solutions exposed to 0.8 cm^2 borosilicate glass in cavity slides are shown in Table 6, where m = 0.238 and the loss of Ag^+ from solution was 63%.

Table 7 shows the concentration of silver remaining in the samples after various times in the presence of 60 ppm Cl^-. In the presence of chloride, most silver would be in the form of colloidal AgCl. Analyses of non-bound:bound silver in KCl solution showed that unlike $AgNO_3$ solutions the relationship was non-linear but covered the whole initial concentration range. This quadratic relationship existed over a greater concentration range than seen in $AgNO_3$ solution since there was binding of AgCl colloid *already bound* to the glass. Saturation of the binding rate was not exceeded, up to at least 1000 ppb. There was a linear relationship between the square root of the surface excess (E) and the concentration remaining in solution. The slopes of the lines m were 2.2591×10^{-2}, 4.7294×10^{-2} and 0.10166 for the 20, 44 and 115 h samples respectively.

$$\text{Since } \sqrt{E} = mF \text{ and } E = \frac{(I-F)}{S}V$$

$$3. \qquad F = \frac{\frac{-V}{S} \pm \sqrt{\frac{V^2}{S^2} + 4m^2 I \frac{V}{S}}}{2m^2}$$

Table 7. *Loss of silver from solutions containing $AgNO_3$ and KCl in borosilicate glass conical flasks**

Initial concentration (ppb)	Ag^+ concentration (ppb) after: 5 min	30 min	2 h	20 h	44 h	115 h
50	†53 (±16)	48 (±9)	43 (±8)	49 (±12)	46 (±12)	52 (±24)
100	108 (±20)	92 (±9)	122 (±54)	107 (±30)	71 (±11)	65 (±14)
500	479 (±78)	500 (±60)	489 (±26)	406 (±56)	328 (±71)	181 (±163)
1000	964 (±102)	987 (±219)	1005 (±126)	751 (±100)	446 (±302)	267 (±257)

* Original solution contained 100 ml $AgNO_3$ plus KCl (60 ppm Cl^-) incubated in 150 ml conical flasks at 23°C in darkness.
† Values shown are means of five replicates with standard errors of the mean in parentheses.

Table 8. *Calculated concentration of unbound Ag from a solution of $AgNO_3$ and KCl (60 ppm Cl^-) after 18 h* incubation in a borosilicate glass cavity slide*

Initial concentration Ag^+		Free silver concentration after 18 h†		
(ppb)	(μm)	(ppb)	(μm)	Loss (%)
10	0.093	3.2	0.029	68
25	0.233	5.5	0.051	78
50	0.465	8.1	0.074	84
100	0.930	11.7	0.107	88
500	4.650	27.1	0.249	95
1000	9.300	38.6	0.355	96

* 100 μml exposed to 0.8 cm^2 borosilicate glass.
† Calculated as described in the text.

A quadratic relationship was then found between t and m:

$$m = (0.119)t + (-2.670 \times 10^{-5})t^2 \quad (4.)$$

with a S.D. of the fit of 1.640×10^{-2}

Thus solving equation 4 for any time will give m, which can be used in equation 3 to calculate the silver remaining in solution from any initial concentration up to 1000 ppb. Calculated values were within the 95% confidence limits of the measured values.

Correlation factors can be used, as above, substituting $V^2/100$ for V and $S^2/113$ for S.

The amount of non-bound silver in suspension or solution (Ag^+ or AgCl) after 18 h in a germination assay containing $AgNO_3$ and KCl (60 ppm Cl^-) in a cavity slide is shown in Table 8. In the presence of Cl^- substantial quantities of Ag were lost from the solution. Loss of Ag was not linearly related to the initial concentration and, after 18 h in 60 ppm Cl^-, losses ranged from 68% in 10 ppb to 96% in 1000 ppb.

Chemotaxis

Motile zoospores were attracted to an amorphous *c.* 0.5 mm diameter AgCl particle placed in sterile deionised water in a cavity slide (Fig. 7). This positive chemotaxis was measured quantitatively in capillary chamber experiments. The chemotaxis index calculated from zoospore distribution in *diffusion-out* experiments is shown in Table 9. A concentration

Table 9. *Chemotaxis index of the response of* P. cryptogea *zoospores to Ag^+ ions in a* diffusion-out *experiment*

Ag^+ concentration in capillary (ppb)	Chemotaxis index* Mean	(S.E.)
100	8.20	(±0.99)
10	0.90	(±0.14)
5	0.54	(±0.09)
0	0.73	(±0.08)
95% Confidence interval (pooled data)		1.6

* Based on five replicate chemotaxis chamber experiments recorded after 18 h. Standard errors of the mean in parentheses.

of 100 ppb Ag^+ in the tubes was strongly chemoattractive but no activity was shown to 10 ppb. Zoospores swam towards the capillary mouth in response to the outward diffusion of Ag^+ ions (Fig. 6*a*). In the reservoir, germination in the 1 mm^2 area 5 mm from the capillary mouth was 96.9%. (The 95% CI was ± 5.4%). Zoospores did not travel further than 4 mm into the capillary. Zoospore densities in the capillary and reservoir of an identical control apparatus using deionised water in both were the same as the outer reservoir distant from the silver source (Fig. 6*a*). A similar distribution pattern of zoospores was seen in *diffusion-in* experiments when the chamber contained 100 ppb Ag^+ and zoospores were initially present in the capillary (Fig. 6*b*). Most zoospores swam down the capillary mouth attracted

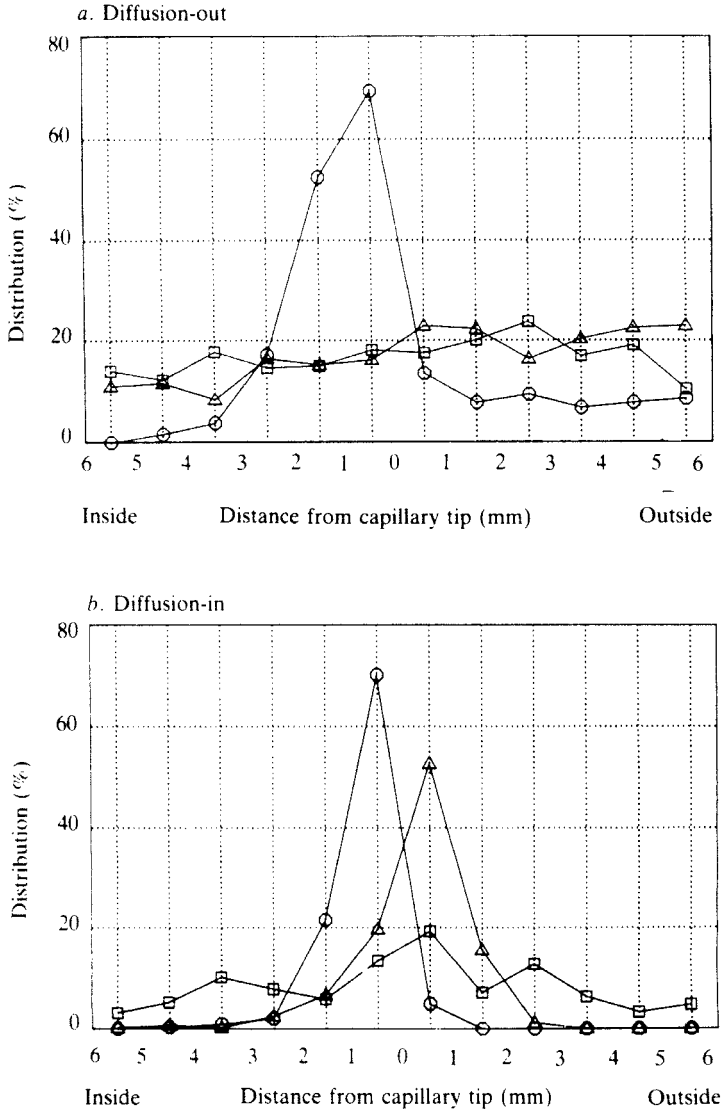

Fig. 6. The chemotactic response patterns of *P. cryptogea* zoospores to silver concentration gradients. Values shown represent the mean counts of living or dead zoospores at a point 18 h after exposure to silver. Results are based on five replicate experiments expressed as a percentage of tenfold counts of zoospores in the same location in sterile deionised water only.
–□–, 100 ppb Ag; –△–, 10 ppb Ag; –□– sterile deionised water.

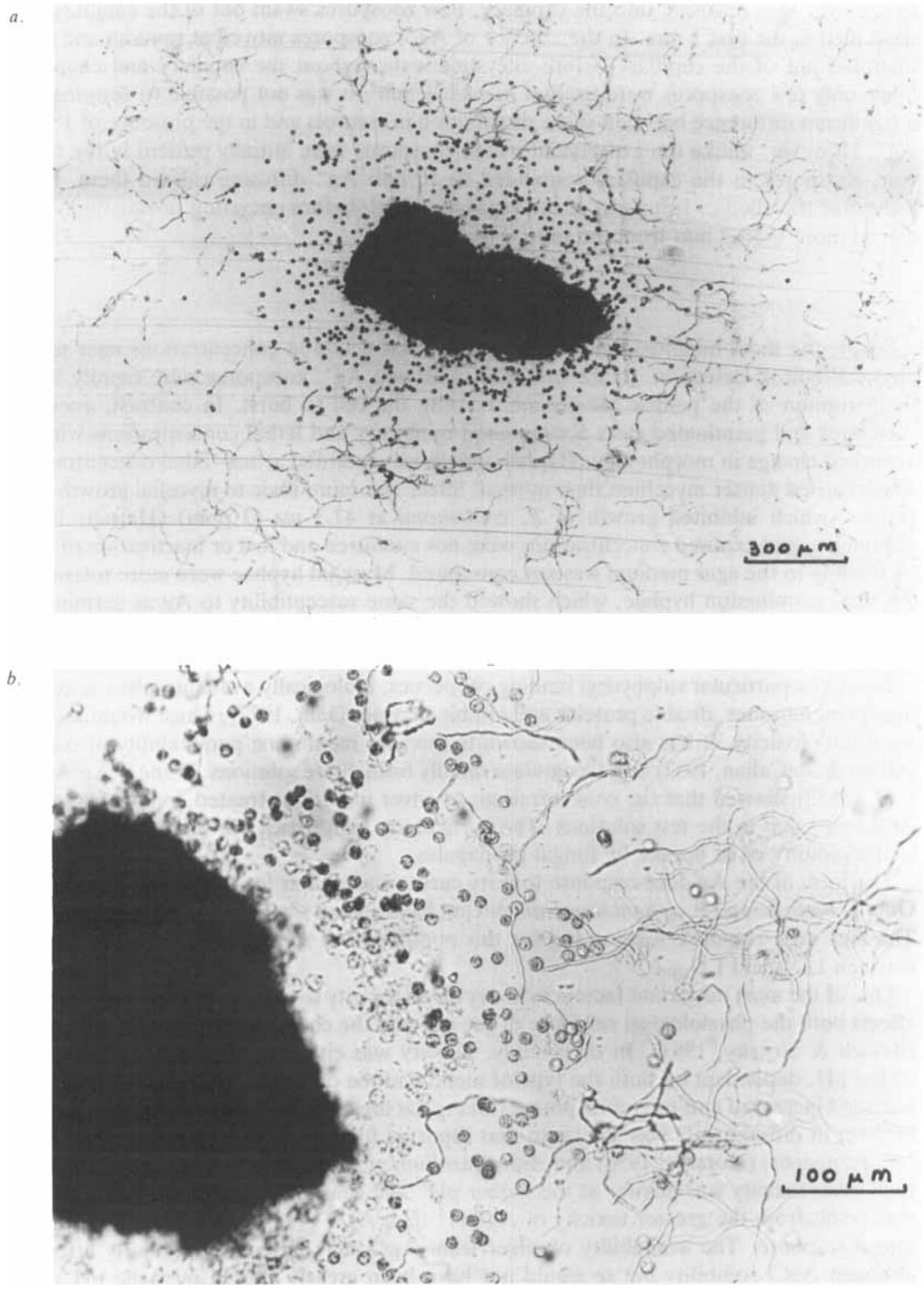

Fig. 7. *a*. Chemotaxis of *P. cryptogea* zoospores to a *c*. 0.5 mm long particle of silver chloride in sterile deionised water ×66 magnification. *b*. ×220 magnification. Burst zoospores in contact and closely adjacent to the AgCl surface, surrounded by dead zoospore cysts. Clustered germinated spores outside this zone result from attraction into weaker, non-lethal silver concentrations. Photographed after 18 h at 20°C in darkness.

by the Ag^+ ions diffusing into the capillary. Few zoospores swam out of the capillary and most died in the first 1 mm. In the absence of Ag^+, zoospores moved at random and most migrated out of the capillary before encystment throughout the capillary and chamber. Since only few zoospores were present in each 1 mm^2, it was not possible to demonstrate a significant difference between spore distribution in controls and in the presence of 10 ppb Ag^+. However, unlike the experiment where zoospores were initially present in the reservoir, zoospores in the capillary responded to 10 ppb Ag^+ diffusing toward them. Most zoospores travelled *c.* 1 mm out of the capillary mouth before encysting or bursting; none moved more than 3 mm from the tube (Fig. 6*b*).

Discussion

Silver, the most fungitoxic metal ion studied, was active at concentrations near to the physical limit of detection. In the presence of 50 ppb Ag^+, zoospores were rapidly killed by disruption of the pellicle membrane, causing the cell to burst. In contrast, encysted zoospores and germinated cysts accumulated inhibitory and lethal concentrations without a marked change in morphology. Hyphal growth was retarded at sub-lethal concentrations which caused denser mycelium than normal. Silver was more toxic to mycelial growth than copper, which inhibited growth of *P. cinnamomi* at 47.3 μM (3 ppm) (Halsall, 1977), although actual exposed concentrations were not measured and loss or inactivation of Cu^{2+} by binding to the agar medium was not considered. Mycelial hyphae were more tolerant to Ag than germination hyphae, which showed the same susceptibility to Ag as germinating zoospores. Older hyphae may have a reduced rate of uptake or have relatively fewer Ag binding sites than germination hyphae.

Due to its particular sulphydryl binding properties, biologically available silver is able to disrupt membranes, disable proteins and inhibit enzymes (Jain, 1977), which would account for it fungitoxicity. It has also been shown to increase membrane permeability of conidia (Miller & McCallan, 1957) and accumulate rapidly from dilute solutions. Using ^{110}Ag, Miller *et al.* (1953) showed that the concentrations of silver in various treated conidia was up to 2000 times that in the test solutions. The particularly high toxicity of Ag^+ may be related to the rapidity of its uptake by fungal propagules.

The form of the Ag dose-response toxicity curves was similar for all the fungi examined. Only *T. basicola* and *P. aphanidermatum* did not have a small silver-tolerant sub-population. The Ag^+ dose-response relationship for this population of *Phytophthora* was logarithmic between LD_{90} and LD_{100}.

One of the most important factors in heavy metal toxicity to microorganisms is pH, which affects both the physiological response of the cell and the chemical speciation of the metal (Babich & Stotzky, 1983). In their study, toxicity was either increased or decreased by higher pH, dependent on both the type of metal and the organism. Brandes (1934) earlier recorded increased anti-microbial potency of Ag^+ at high pH. The response of *P. cryptogea* to silver at different pH was similar to that reported for copper and *Fusarium oxysporum* f.sp. *lycopersici* (Horsfall, 1956) and *Aureobasidium pullulans* (Gadd & Griffiths, 1980). In each case, toxicity was greater at the higher pH. The cause of this effect is not clear and may result from the greater toxicity of $AgOH^+$ than Ag^+, or a direct effect of pH on the fungal response. The availability of silver from AgCl may have been similarly affected, although AgCl solubility *per se* would not have been greatly altered over the pH range assessed.

Numerous studies on the microbial toxicity of metal ions have failed to take account of potential losses due to surface adsorption (Horsfall, 1956; Byrde, 1966; Halsall, 1977). Thus test organisms remaining in suspension might be exposed to levels of toxophore considerably

lower than the initial calculated concentration. Conversely, organisms settling on, or having an affinity for, the glass surface could encounter higher concentrations.

Silver was lost from solution by two mechanisms: binding to the glass surface as Ag^+; or, forming AgCl colloid in the presence of chloride. The presence of chloride had a profound effect on silver availability due to the instantaneous formation of AgCl. Metal ions in solution bind to the silanol groups in borosilicate glass and Ag^+ adsorption may occur into several molecular layers in the glass (Doremus, 1973; Anderson & Rubin, 1981). AgCl similarly binds to silanol groups but the position is further complicated by the self-agglomeration of AgCl particles from suspension, the "Ostwald ripening" phenomenon (Shaw, 1980), causing a reduction in AgCl and hence Ag^+ from suspension and solution. Losses of Ag^+ on a mole for mole basis were therefore correspondingly higher in the presence of chloride ions. For these reasons it was not possible to store dilute silver solutions in glass containers, and bioassay solutions were therefore used immediately after preparation. Although loss of silver and AgCl by glass adsorption was substantial during the bioassay, toxicity was so great that the biological effect was negligible. Zoospore death was mostly instantaneous and the rate of Ag^+ uptake from a fresh solution into spores and hyphae would have been rapid (Miller & McCallan, 1957). Similarly, zoospores would have been killed by contact with AgCl particles in suspension. Additionally, following normal encystment in a cavity slide, sedimentation would place zoospores in direct contact with Ag^+ or AgCl bound to the glass surface. These studies highlight the potential importance of *ion* binding to surfaces in the microbial assay of toxic metals.

Particles of colloidal AgCl exhibited a unique chemoattraction. Chemotaxis has been described for various species of *Phytophthora* (Ribeiro, 1978; Cameron & Carlile, 1980; Khew & Zentmyer, 1973; Allen & Harvey, 1974), but attraction by a metal ion has not been previously reported. The most powerful chemoattractant described was isovaleraldehyde, which attracted *P. palmivora* zoospores at a concentration of 10^{-6} M, corresponding to approximately 10^{-7} M at the cell surface (Cameron & Carlile, 1981). In the present study, zoospores of *P. cryptogea* responded to 10 ppb Ag^+ (90 nM) in the chamber. The concentration detected at the zoospore surface would have been even lower. Detection in low concentration, coupled with rapid uptake, is probably related to the particular toxicity of Ag^+. It is probable that Ag^+ binds to non-specific binding sites since it is unlikely that specific binding would occur for a non-essential toxic ion. It is not clear, however, why the detection limit should be so low.

In view of the sensitivity of microbial cells to Ag^+ and the long history of its role as a microbiocide (Raulin, 1869; Krause, 1929; Brandes, 1934) it is surprising that no silver-based fungicide has been developed. Notwithstanding the affinity of Ag^+ and AgCl for glass surfaces, its broad spectrum of microbial toxicity and apparently low mammalian toxicity at 0.93 μM (0.1 ppm) would make silver a candidate for further investigation. The environment of zoosporic root-rot pathogens in hydroponic systems would seem particularly suitable for the investigation of a control method using silver. A future paper will consider this.

Acknowledgements

The authors express their gratitude to Dr B. Midmore, Department of Chemistry, University of Reading, for valuable discussions on silver chemistry.

References

Allen R N, Harvey J D. 1974. Negative chemotaxy of zoospores of *Phytophthora cinnamomi*. *Journal of General Microbiology* **84**:28–38.

Anderson M A, Rubin A J. (Eds) 1981. *Adsorption of inorganics at solid-liquid interfaces.* Michigan: Ann Arbor Science Publishers Inc. 357 pp.

Babich H, Stotzky G. 1983. Influence of chemical speciation on the toxicity of heavy metals to the microbiota. In *Aquatic Toxicology*, pp. 1–46. Vol. 13, in series *Advances in Environmental Science and Technology*. Ed. J O Nriagu. New York: Wiley Interscience.

Brandes C H. 1934. Ionic silver sterilization. *Industrial & Engineering Chemistry* **26**:962–964.

Byrde R J W. 1966. The vulnerability of fungus spores to fungicide. In *The Fungus Spore*, pp. 289–297. Ed. M F Madelin. London: Butterworths. 338 pp.

Cameron J N, Carlile M J. 1980. Negative chemotaxis of zoospores of the fungus *Phytophthora palmivora*. *Journal of General Microbiology* **120**:347–353.

Cameron J N, Carlile M J. 1981. Binding of isovaleraldehyde, an attractant, to zoospores of the fungus *Phytophthora palmivora* in relation to zoospore chemotaxis. *Journal of Cell Science* **49**:273–281.

Crannell M Y. 1967. Silver in Medicine. In *Silver Economics Metallurgy and Use*, pp. 227–234. Eds A Butts and C D Coxe. Van Nostrand. 488 pp.

Doremus R H. 1973. *Glass Science*. New York: Wiley Interscience. 349 pp.

Erwin D C, Katznelson H. 1961. Studies on the nutrition of *Phytophthora cryptogea*. *Canadian Journal of Microbiology* **7**:15–25.

Evans S G. 1979. Susceptibility of plants to fungal pathogens when grown by the nutrient film technique (NFT). *Plant Pathology* **28**:45–48.

Friberg L, Nordberg G F, Vouk V B. 1979. *Handbook on the toxicology of metals.* Amsterdam: Elsevier/North-Holland Biomedical Press.

Gadd G M, Griffiths A J. 1980. Influence of pH on toxicity and uptake of copper by *Aureobasidium pullulans*. *Transactions of the British Mycological Society* **75**:91–96.

Goetz A, Tracy R L, Harris F S. 1940. The oligodynamic effect of silver. In *Silver in Industry*, pp. 401–429. Ed. L Addicks.

Halsall D M. 1977. Effects of certain cations on the formation and infectivity of *Phytophthora* zoospores. 2. Effects of copper, boron, cobalt, manganese, molybdenum and zinc ions. *Canadian Journal of Microbiology* **23**:1002–1010.

Hammond P B, Foulkes E C. 1986. Metal ion toxicity in man and animals. In *Metal ions in biological systems. Volume 20: Concepts on metal ion toxicity*, pp. 157–194. Ed H Sigel. New York: Marcel Dekker Inc.

Holderness M, Pegg G F. 1986. Interaction of host stress and pathogen ecology on *Phytophthora* infection and symptom expression in nutrient film-grown tomatoes. In *Water, Fungi and Plants*, pp. 189–207. Eds P G Ayres and L Boddy. B.M.S. Symposium 11. Cambridge University Press. 413 pp.

Horsfall J G. 1956. *Principles of fungicidal action.* Waltham, Massachusetts: Chronica Botanica Co.

Huot T, Bouchardat G. 1899. Sur l'emploi des sels mercuriques et du nitrate d'argent en viticulture. *Revue Viticulture* **12**:528–530.

Jain M K. 1977. *Handbook of enzyme inhibitors.* UK: Wiley.

Kennedy B W, Erwin D C. 1961. Some factors influencing sporangium formation of a *Phytophthora* species isolated from lucerne in certain salt solutions. *Transactions of the British Mycological Society* **44**:291–297.

Kennedy R, Pegg G F. 1990. *Phytophthora cryptogea* root rot of tomato in rockwool nutrient culture. II. Effect of root zone temperature on infection sporulation and symptom development. *Annals of Applied Biology* **117**:537–551.

Khew K L, Zentmyer G A. 1973. Chemotactic response of zoospores of five species of *Phytophthora*. *Phytopathology* **63**:1511–1517.

Krause G A. 1929. *Neue Wege sur Wassersterilisierung.* Munich: J F Bergmann.

Menyonga J M, Tsao P H. 1966. Production of zoospore suspensions of *Phytophthora parasitica*. *Phytopathology* **56**:359–360.

Miller L P, McCallan S E A. 1957. Toxic action of metal ions to fungus spores. *Agricultural and Food Chemistry* **5**:116–122.

Miller L P, McCallan S E A, Weed R M. 1953. Rate of uptake and toxic dose on a spore weight basis of various fungicides. *Contributions from Boyce Thompson Institute* **17**:173–195.

Nielsen L W. 1942. Studies with silver compounds and mixtures as fungicidal sprays. *Memoir 248*. Ithaca, New York: Cornell University Agricultural Experimental Station.

Pegg G F, Holderness M. 1984. Infection and disease development in NFT-grown tomatoes. *Proceedings VI International Congress Soilless Culture*, pp. 493–510.

Pegg G F, Jordan M M. 1990. *Phytophthora cryptogea* root rot of tomato in rockwool nutrient culture. I. Analysis of root infection. *Annals of Applied Biology* **117**:525–535.

Price D. 1976. Nutrient-film culture. In *Annual report of the Glasshouse Crops Research Institute 1975*, p. 114. Littlehampton, UK.

Raulin J. 1869. Etudes chimiques sur la vegetation. *Annales Science Naturelle Botanique* **11**:93–199.

Ribeiro O K. 1978. *A source book of the genus Phytophthora*. USA: Lubrecht & Cramer.

Shaw D J. 1980. *Introduction to Colloid and Surface Chemistry*, p. 64. 3rd Edition. London: Butterworth & Co.

Sokol R A, Klein D A. 1975. The responses of soils and soil microorganisms to silver iodide weather modification agents. *Journal of Environmental Quality* **4**:211–214.

Tomlinson J A. 1958. Crook-rot of watercress. II. The control of the disease with zinc-fritted glass and the mechanism of its action. *Annals of Applied Biology* **46**:608–621.

Vanachter A, van Wambeke E, van Assche C. 1983. Potential danger for infection and spread of root diseases of tomatoes in hydroponics. *Acta Horticulturae* **133**:119–128.

Zentmeyer G A, Marshall L A. 1959. Factors affecting sporangial production by *Phytophthora cinnamomi*. *Phytopathology* **49**:556.

(Received 11 December 1991)