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ELECTROLYTE LEAKAGE FROM PLANT AND FUNGAL TISSUES AND DISRUPTION OF LIPOSOME MEMBRANES BY α-TOMATINE

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Key Word Index—Liposomes, electrolyte loss, membrane disruption, sterols, 3β-hydroxysterols, α-tomatine, tomato, fungal pathogens

Abstract—α-Tomatine, a pre-formed antifungal compound from tomato tissue was found to disrupt liposome membranes containing a 3β-hydroxy sterol. Liposome membranes contaning sterols lacking a 3β-hydroxy sterol were resistant to α-tomatine. α-Tomatine caused electrolyte leakage from a number of plant tissues with the exception of tomato and potato which contain a low amount of free sterol. Sterol substitution may explain why tomato tissue is able to withstand high concentrations of α-tomatine.

Fungal pathogens of tomato were generally more resistant to α-tomatine than non-pathogenic fungi (as determined by electrolyte loss). Electrolyte leakage from *Phytophthora megasperma* was found to be dependent upon incorporation of sterol into mycelium *P. megasperma* cultured on sterol free medium was more resistant to α-tomatine

INTRODUCTION

α-Tomatine, a steroidal glycoalkaloid compound with antimicrobial properties, is found in the tomato plant and other solanaceous species [1] α-Tomatine was first purified from tomato sap [2] and since then several attempts have been made to correlate the levels of α-tomatine with disease resistance [3].

Evidence suggests that α-tomatine is not important as a primary varietal resistance determinant to the vascular wilt fungi *Fusarium oxysporum* f. sp. *lycopersici* [4] and *Verticillium albo-atrum* [5] although α-tomatine is present at concentrations in resistant and susceptible cultivars [4–7] adequate to inhibit both fungi *in vitro* [4, 5, 8]. However, both *F oxysporum* f sp. *lycopersici* (Fol)† [4] and *V. albo-atrum* [5] are able to colonize tomato tissue containing fungitoxic levels of α-tomatine. Additionally, α-tomatine content of both resistant and susceptible cultivars of tomato increases following inoculation with either Fol [4] or *V. albo-atrum* [5]. Either fungal pathogens such as *F. oxysporum* f. sp *lycopersici* (Fol) do not come into contact with α-tomatine when they colonize susceptible tomato plants or the α-tomatine present is detoxified [5, 9]

Other evidence suggests that some fungi are able to colonize α-tomatine-containing tomato tissue by lowering the pH of the infection site [10] The membrane lytic effects of α-tomatine are pH dependent and the optimum pH for membrane disruption is between 6.0 and 7.0. At an acidic pH α-tomatine is in the protonated form and lacks the ability to disrupt membranes [11].

α-Tomatine will attack a number of eukaryotic cells including most fungi [12], mammalian red blood cells [3, 13], protozoa [14] and plant organelles [15] Prokaryotic cells are essentially unaffected by α-tomatine. α-Tomatine causes an irreversible leakage of electrolytes from fungal cells [16] indicating that, like other saponins [17], it may affect membrane integrity α-Tomatine has been suggested to attack membranes firstly as a weak surfactant by virtue of its amphipathic properties [18] and secondly by complexing with 3β-hydroxy sterols, a property demonstrated *in vitro* [19]. Mutants of *Fusarium solani* with a reduced membrane sterol content are less sensitive to α-tomatine [20] while Roddick and Drysdale [11] demonstrated that the disruption of artificial membranes by α-tomatine was dependent upon the amount of sterol in the membrane All three sterols used by Roddick and Drysdale [11] were in the free alcohol form and showed similar sensitivities to disruption by α-tomatine.

If α-tomatine acts on membrane sterols *in vivo* then tomato membranes must be protected from the effects of the glycoalkaloid since it is present in some organs of tomato at concentrations in excess of 1 mg/g fresh weight [6, 7]. Tomato membranes contain an exceptionally high level of substituted sterols [21], i e sterols lacking a free 3β-hydroxyl group. This substitution is predominantly in the form of steryl glycosides and acylated steryl glycosides and may explain why tomato membranes are tolerant of endogenous α-tomatine.

Pathogenic fungi of tomato tend to be more resistant to α-tomatine than are non-pathogenic fungi [8, 10] Since α-tomatine binds *in vitro* to sterols [22], the susceptibility or resistance of fungi to α-tomatine may be due to differing membrane sterol content The principal fungal sterol is ergosterol which is predominantly in the free alcohol form [23, 24]. Pythiaceous fungi (e.g. *Phytophthora megasperma*) are able to grow in the absence of sterols [23] but when sterols are supplied exogenously they will incorporate them into their membranes [25]. Thus it is possible to have two cultures of a pythiaceous

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†The abbreviation Fol is used for this organism throughout the remainder of the text

fungus that are identical in every respect except membrane sterol content. Pythiaceous fungi are therefore ideally suitable for experiments to study the importance of membrane sterols.

This paper describes the leakge of electrolytes from a number of cell systems incubated with α-tomatine and the use of liposome vesicles mimicking tomato and fungal membranes to test the hypothesis that α-tomatine acts by binding membrane-located 3β-hydroxy sterols.

RESULTS

Disruption of sterol-containing liposomes by α-tomatine

The ability of α-tomatine to disrupt sterol containing membranes was investigated using phosphatidylcholine liposomes prepared with different sterols containing a 3-hydroxyl group (Table 1). Liposomes prepared with sterols containing a 3β-hydroxyl group (i.e. cholesterol, ergosterol, sitosterol and 5α-cholestan-3β-ol) were sensitive to α-tomatine disruption. Liposomes prepared with a sterol containing a 3α-hydroxyl (i.e. 5β-cholestan-3α-ol) were resistant. A 3β-hydroxyl group therefore appears to be essential for the action of α-tomatine. In order to confirm this observation, liposomes were also prepared with a number of commercially available sterols lacking a hydroxyl group on any part of the sterol molecule viz. cholesteryl acetate, cholesteryl 3β-methyl ether, cholesteryl 6β-methyl ether, cholest-5-en-3-one, cholesteryl palmitate, 5α-cholestane, 5β-cholestane. All such preparations were also found to be resistant to α-tomatine.

Liposomes were prepared with different proportions of free to substituted sterols using cholesterol and cholesteryl glucoside (Table 2). As the proportion of cholesteryl glucoside increased in the sterol mixture so the liposomes became more resistant to the action of α-tomatine.

Liposomes were then prepared from crude lipid extracts of tomato and *F. oxysporum* f. sp. *lycopersici*

Table 1. Effect of α-tomatine on peroxidase leakage from phosphatidylcholine liposomes prepared with sterols containing a hydroxyl group

Sterol	Treatment	Total peroxidase activity*	%Activity in supernatant	%Activity in pellet
Cholesterol†	Control	2.7	17.6	82.4
	α-tomatine	4.3	73.2	26.8
Ergosterol†	Control	1.4	18.3	81.7
	α-tomatine	2.8	75.1	24.9
Sitosterol†	Control	0.8	24.0	76.0
	α-tomatine	1.8	90.6	9.4
5α-Cholestan-3β-ol†	Control	2.2	20.7	79.3
	α-tomatine	3.8	81.3	18.7
5β-Cholestan-3α-ol‡	Control	1.9	21.7	78.3
	α-tomatine	4.1	12.2	87.8

*Expressed as $\Delta A_{420}/\text{sec} \times 10^3$. Liposomes were treated with 150 μM alkaloid at pH 7.2 for 1 hr. Values are means of four replicates.

†Sterol containing a 3β-hydroxyl group.

‡Sterol containing a 3α-hydroxyl group.

Table 2. Effect of α-tomatine on peroxidase leakage from phosphatidylcholine liposomes with different ratios of free substituted sterols

% Cholesterol in liposome membrane	% Cholesteryl glucoside in liposome membrane	Treatment	Total peroxidase activity*	%Activity in supernatant	%Activity in pellet
0	100	Control	1.3	17.8	82.2
		α-tomatine	1.9	18.8	81.2
10	90	Control	0.8	17.5	82.5
		α-tomatine	0.9	24.0	76.0
30	70	Control	0.8	20.6	79.4
		α-tomatine	1.1	43.3	56.7
50	50	Control	1.2	25.8	74.2
		α-tomatine	2.1	51.6	48.4
100	0	Control	0.8	19.6	80.4
		α-tomatine	1.5	71.5	28.5

*Expressed as $\Delta A_{420}/\text{sec} \times 10^3$. Liposomes were treated with 150 μM alkaloid at pH 7.2 for 1 hr. Values are means of four replicates.

Liposomes prepared with crude lipid extracts tended to be leaky even in the absence of α-tomatine indicating that the membranes were imperfect. Nevertheless the membranes formed from the three extracts, tomato seedlings, 12-week-old tomato leaves and Fol all appeared to be resistant to the action of α-tomatine

Electrolyte loss from cells incubated in the presence of α-tomatine

In these experiments electrolyte loss from tissues incubated with α-tomatine is expressed as a percentage of the total possible loss, the latter (i.e 100%) being determined by addition of chloroform at the end of each experiment. Data from control experiments were subtracted form test material

Disks of cucumber mesocarp tissue were found to lose electrolytes when incubated with α-tomatine while disks of green tomato fruit pericarp and six-week-old tomato stem slices were resistant (Fig 1) Electrolyte leakage was determined for leaf disks cut from a number of different plants with differing proportions of free sterols [21]. Potato (12% free sterols) and tomato (10% free sterols) leaf disks were both more resistant to the action of α-tomatine than tobacco (50% free sterols) or *Nicandra physaloides* (54% free sterols) leaf disks (Fig 2)

Washed fungal material (1 g wet weight) was incubated with α-tomatine and the electrolyte leakage monitored with time Fungal pathogens of tomato (*Botrytis cinerea*, *Verticillium albo-atrum*) grown in shake culture were found to be more resistant to α-tomatine than non-pathogenic fungi (*Alternaria tenuis*, *Ascochyta pisi*, *Fusarium graminearum* and *Penicillium expansum*) (Fig 3). *Fusarium solani*, pathogenic for ripe tomato fruit but non-pathogenic for α-tomatine-containing green fruit [20], showed intermediate sensitivity to α-tomatine

As it has been suggested that α-tomatine may act by binding to membrane sterols [1, 11, 12], electrolyte leakage was then investigated in a fungus lacking membrane sterols, namely *Phytophthora megasperma* [23] *Phytophthora megasperma* will incorporate sterols into its membranes if supplied in the medium [25]. Electrolyte loss due to α-tomatine damage was determined for *P megasperma* mycelium grown in the presence of increasing concentrations of cholesterol (Fig 4). Sterol-contain-

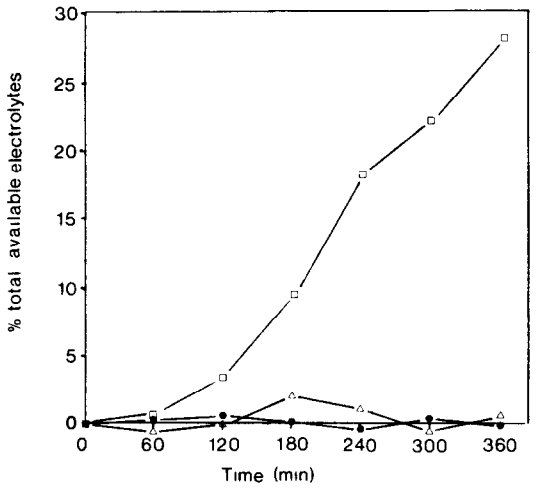

Fig 1 Leakage of electrolytes from cucumber mesocarp (□), green tomato pericarp (Δ) and tomato stem disks (●) incubated with α-tomatine (150 μM)

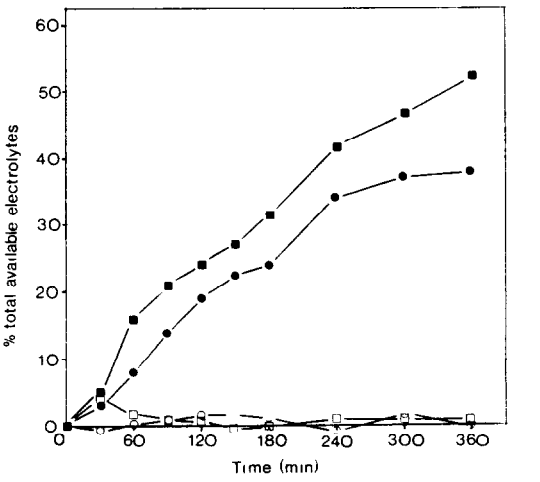

Fig 2 Leakage of electrolytes from leaf disks of tomato (○), potato (□), tobacco (●) and *N. physaloides* (■) incubated with α-tomatine (150 μM)

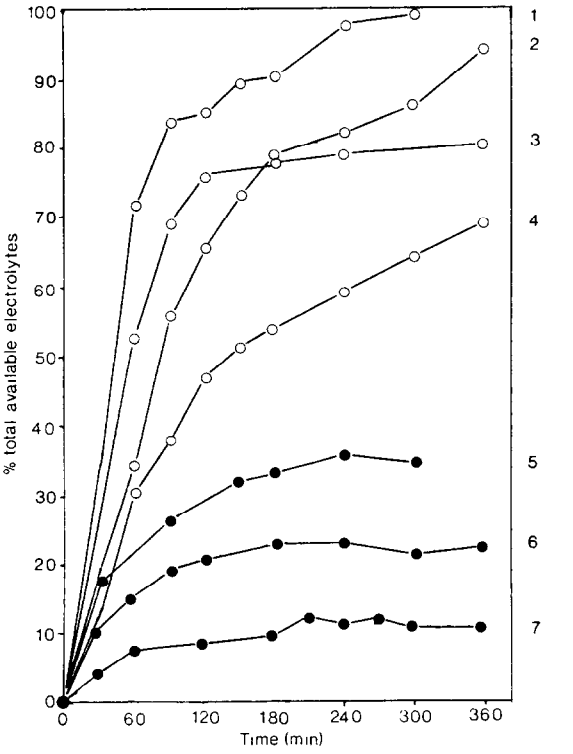

Fig 3 Leakage of electrolytes from fungal material (1 g wet weight) incubated with α-tomatine (150 μM) (1) *A pisi*, (2) *F graminearum*, (3) *P expansum* (4) *A tenuis*, (5) *F solani*, (6) *V albo-atrum*, (7) *B cinerea* Pathogens of tomato (●), Non-pathogens of tomato (○)

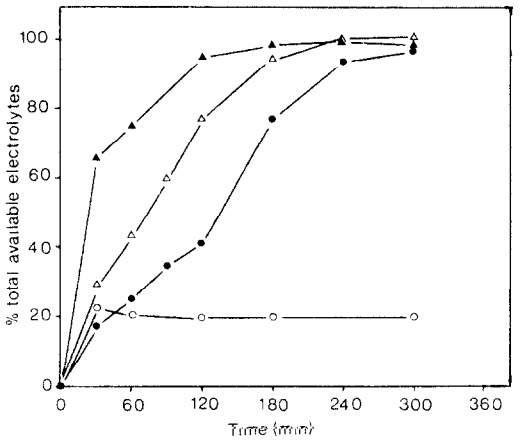

Fig 4 Leakage of electrolytes from *P megasperma* mycelium cultured with increasing concentrations of exogenous sterol and incubated with α-tomatine (150 μM) 0 mg/l cholesterol (○), 10 mg/l cholesterol (●), 15 mg/l cholesterol (Δ), 20 mg/l cholesterol (▲)

ing mycelium was found to be more susceptible to α-tomatine damage then sterol-free mycelium

DISCUSSION

The membrane disruptive effects of α-tomatine upon liposome vesicles is dependent upon the proportion of sterol incorporated into the liposome membrane [11] Liposomes prepared with the most abundant sterol (in the free 3β-hydroxyl form) from animal cells (cholesterol), fungal cells (ergosterol) and plant cells (sitosterol) all showed similar sensitivities to disruption by α-tomatine as did those liposomes prepared with 5α-cholestan-3β-ol Liposome vesicles prepared with a sterol lacking a free 3β-hydroxyl group were found to be resistant to α-tomatine The fact that α-tomatine-containing tomato tissue contains a low proportion of membrane sterols in the free alcohol form [21] may explain why tomato tissue itself is able to withstand α-tomatine concentrations in excess of 1 mg/g fresh weight [6, 7]

In tomato tissue the degree of sterol substitution is dependent upon the organ and age of the plant. In 12-week-old leaves 90% of the sterols are substituted, while in 4-week-old stem tissue only 47% of sterols are substituted [21] In tomato seedlings, approximately 80% of the sterols are substituted This substitution is predominantly in the form of steryl glycosides and acylated steryl glycosides and the principal sugars involved are glucose and galactose [21] Liposomes prepared with lipid extracts from tomato seedlings and 12-week-old tomato leaves appeared to be resistant to the action of α-tomatine However, liposomes prepared with plant lipid extracts exhibited a low incorporation of the peroxidase enzyme and tended to be leaky even in the absence of α-tomatine, as were liposomes prepared from a Fol lipid extract Since such lipid extracts were a crude preparation of total lipids from plant material, components of non-membrane origin may have been present in the extract which interfere with the formation of intact membranes

α-Tomatine caused a leakage of electrolytes from disks of cucumber pericarp while both tomato mesocarp from unripe fruit and tomato stem disks were resistant Tomato leaf disks and potato leaf disks which both contain low amounts of free sterol [21] (10% respectively) were more resistant to α-tomatine than leaf disks of either tobacco or *N physaloides* which contain 50 and 54% free sterol respectively [21]

If the glycoalkaloids from potato, α-solanine and α-chaconine [26] attack membranes in the same way as α-tomatine, then the low free sterol content in potato leaves may explain why potato, like tomato, is able to withstand high concentrations of glycoalkaloids

Schlosser [17], demonstrated that digitonin and filipin would only cause leakage of amino acids from mycelium of a pythiaceous fungus, *Pythium ultimum* cultured in the presence of sterol Mycelium lacking sterol, i e cultured in the absence of exogenous sterol was resistant to the action of digitonin and filipin Similarly, in this study α-tomatine has been demonstrated to cause electrolyte loss from *Phytophthora megasperma* mycelium containing sterol while mycelium of *P megasperma* lacking sterol was more resistant

Fungal pathogens of tomato examined in this study grown in shake culture (i e *B cinerea*, *V albo-atrum* and *F solani*) were found to be more resistant to α-tomatine than non pathogenic fungi (i e *A nisi*, *A tenuis*, *F graminearum* and *P expansum*) This work is in agreement with the earlier work of Arneson and Durbin [8] in which the authors compared the relative sensitivities of different fungi to α-tomatine on the basis of inhibition of growth on agar plates

If the hypothesis is correct that α-tomatine is antimicrobial by virtue of its ability to bind membrane located 3β-hydroxy sterols [12], then the relative sensitivities of different fungi to α-tomatine may be due to differences in membrane sterol content The ability of some fungi to colonize α-tomatine containing tomato tissue, present at fungitoxic levels as determined *in vitro* may be dependent upon the ability of the pathogen to alter its sterol content *in vivo* Fungal sterol content has been extensively studied in the yeast *Saccharomyces cerevisiae* [27] and until the advent of the sterol biosynthesis inhibiting fungicides in the last 20 years [28], studies on the sterol composition of other fungi have received little attention Further research is required to determine the sterol composition of fungi non-pathogenic and pathogenic to tomato in order to confirm the hypothesis that α-tomatine sensitivity is correlated with possession of membrane located 3β-hydroxy sterols

Membrane sterol content of a tomato fungal pathogen is likely to be different *in vivo* from that found *in vitro* Sterol content is affected by cultural conditions and the physiological state of the fungus [24, 29, 30] The oxygen content of the tomato vascular system has been estimated as being as little as 0 8 ppm [31] Sterol biosynthesis involves an oxygen requiring post-squalene stage [32] Thus under anaerobic conditions the sterol biosynthetic pathway terminates at squalene without the formation of sterols. The optimum oxygen concentration for the formation and activation of squalene epoxidase in *Saccharomyces cerevisiae* has been estimated to be 0 1% [33] It is therefore possible that within the vascular tissue of a tomato plant, where oxygen is scarce, sterol biosynthesis by vascular wilt pathogen such as *V albo-atrum* or Fol is retarded

The role of α-tomatine in preventing fungal colonization, if any, in the tomato plant is not understood Why

tomato pathogens, such as Fol and *V albo-atrum* are able to colonize α-tomatine containing tomato tissue at inhibitory concentrations (as determined *in vitro*) [4, 5] is not known It may be that α-tomatine is important in preventing the colonization of tomato tissues by other less specialized fungi that are not normally pathogenic for tomato [8].

EXPERIMENTAL

Preparation of liposomes The method employed to prepare cationic liposomes was a modification of the method of Roddick and Drysdale [11] The lipid materials comprising 25 mg phosphatidylcholine (Sigma-Type VII-E from frozen egg yolk), 5 mg sterol and 1 mg stearylamine (Sigma) were sonicated in an aqueous phase (0 75 ml) consisting of horse radish peroxidase (Sigma Type II) (5 mg) and Bovine Serum Albumin (25 mg) in phosphate buffer (0 2 M, pH 7 2) as previously described [11]. Commercially available sterols were obtained from Sigma and cholesteryl glucoside was a gift from Dr N Bagget, Department of Chemistry, University of Birmingham, U K For the preparation of liposomes from plant tissues, freeze dried material was homogenized in $CHCl_3$–MeOH (2 1) (5 ml/g dry weight, 4°, 2 min) The extract was filtered (Whatman No 1) and taken to dryness in a preweighed flask The resulting material was dissolved in $CHCl_3$–MeOH (9 1) (1 ml per 5 mg of material) and an equivalent of 30 mg of material added to a fresh flask and taken to dryness under vacuum at room temp The resulting film was re-dissolved in an aqueous phase (0.75 ml) consisting of 5 mg horse radish peroxidase and 25 mg BSA in Pi buffer (0 2 M, pH 7 2). Liposome vesicles were then prepared as described above.

Preparation of α-tomatine solution for liposome preparations and electrolyte loss studies α-Tomatine (Sigma) (40 mg) was dissolved in HCl (0 1 M, 0 4 ml) and made up to 20 ml with deionised, double distilled H_2O Control solns consisted of 0 002 M HCl in the absence of α-tomatine

Treatment of liposomes with α-tomatine and assessment of membrane damage. The method employed was a modification of Roddick and Drysdale [11] The reaction mixture consisted of 3 65 ml Tris–HCl buffer (0 05 M, pH 7 2), 25 μl liposome suspension and 0 3 ml of α-tomatine or control soln. Reaction mixtures were incubated (25°, 1 hr) and the pellets and supernatants separated by centrifugation (18 000 *g*, 4°, 1 hr) The supernatant was decanted (4 ml) and the pellet was resuspended in 4 ml Tris–HCl buffer (0 05 M, pH 7 2 The extent of membrane damage was determined by assay of the peroxidase activity in the supernatant and pellet fractions of the assay mixture after incubation A 50 μl aliquot of either supernatant or resuspended pellet was transferred to a 10 mm spectrophotometer cuvette containing 2 5 ml of pyrogallol (BDH) (0 025 M soln in 0 01 M Pi buffer, pH 7.2), 0.25 ml H_2O_2 (BDH) and either 0.3 ml H_2O for supernatant or 0 3 ml Triton/X-100 for pellets The absorbance of the mixture (420 nm) was continuously recorded and the change in absorbance per second calculated as an index of enzyme activity. Enzyme activity was expressed using the following formula, ΔA_{420}/sec × 1000

Electrolyte loss studies Electrolyte loss studies were carried out at 20° using a conductivity meter fitted with an immersion type probe (Radiometer, Copenhagen) At the end of each experiment $CHCl_3$ (0 1 ml per 12 ml) was added and the conductance measured after 2 hr to determine total electrolyte loss

(i) Fungal mycelium. Cultures (100 ml, 96 hr) were washed (4 ×) by centrifugation (1000 *g*, 15 min) using MOPS–KOH buffer (Sigma) (0 001 M, pH 7 0, conductance = < 25 μS). Tomatine (0 9 ml) was added to 1 g wet wt of washed culture in 10 1 ml of buffer while 0 002 M HCl (0 9 ml) in the absence of α-tomatine was added to control mycelium The conductance was measured at intervals of time and control data subtracted

(ii) *Plant tissues.* Fifteen disks (8.0 mm diam) of plant tissue were cut using a No 4 cork borer Tomato stem disks were cut from the lower stem of 12-week-old tomato plants (cv Homestead 24) Tissue disks were washed in four 10 ml vols of distilled H_2O (2 min each) Before use the disks were blotted on absorbent paper Disks were then added to MOPS–KOH buffer (0 001 M, pH 7.0, 22 2 ml) in a tissue culture jar (Sterilin) α-Tomatine (1.8 ml) was added to test material while 0 002 M HCl (1 8 ml) in the absence of α-tomatine was added to control material. The conductance was recorded and control data substracted from experimental data

Cultivation of plant material Seeds of tobacco and *Nicandra physaloides* were sown in compost trays and allowed to germinate in a greenhouse (25°) Seedlings were transplanted to 12 5 cm pot (3 weeks after sowing) in Arthur Bowers peat-based compost. Plants were maintained in the greenhouse Tomato plants were cultivated as previously described [4]

Maintenance and culture of fungi. Stock cultures of *Phytophthora megasperma* were stored on french bean agar plates at 4° When required for electrolyte loss studies, an agar plug (5 mm) was transferred to a 500 ml conical flask containing 50 ml of sucrose asparagine synthetic medium supplemented either with or without cholesterol [34] Cultures of *P megasperma* were grown without agitation. Other fungi were stored on slopes of Vogels salt soln supplemented with 2% sucrose and 2% agar at 4° [35]. When required, fungi were grown in Vogels salt soln supplemented with 2% sucrose (50 ml) in 250 ml conical flasks with agitation (25°)

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