Tactic response of zoospores of the fungus *Phytophthora palmivora* to solutions of different pH in relation to plant infection

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Zoospores of the plant pathogen *Phytophthora palmivora* use a number of tactic responses to target specific infectible regions of host roots. Although the dominant one is believed to be chemotaxis, it has been shown that zoospores of oomycetes may also use the exogenous proton/electrical currents generated by plant roots for guidance. Since these proton currents also generate significant pH gradients in the rhizosphere, the tactic response of zoospores to changes in pH was examined. Using 'swim-in' capillary tests, zoospores of *P. palmivora* were found to be repelled by solutions of high pH and attracted to solutions of low pH, relative to a control at neutrality. This *in vitro* tactic response was generally consistent with the measured pH at sites of zoospore accumulation around intact and wounded roots. However, the endogenous pH gradient around host roots could be abolished with buffers and this treatment did not affect the extent or pattern of zoospore accumulation. Therefore, detection of root-generated pH gradients is unlikely to have a major role in the homing response of zoospores towards plant roots.

Keywords: *Phytophthora palmivora*, zoospores, chemotaxis, pH taxis, roots

INTRODUCTION

Plant roots generate electrical currents as a result of ion-conducting proteins in the cell membrane (Behrens *et al.*, 1982; Miller *et al.*, 1991; Gow *et al.*, 1992). The current is carried mainly by protons, (with minor fluxes of K^+) and appears to be regulated by Ca^{2+} (Miller & Gow, 1989a, b). Current flow establishes voltage, ionic and pH gradients in the rhizosphere that can be detected and measured with high precision voltage- and ion-selective microelectrodes. The significance of these gradients in relation to plant root growth has been discussed previously (Raven & Smith, 1974; Versel & Mayor, 1985; Rathore & Goldsworthy, 1985a, b; Toko *et al.*, 1989, 1990; Behrens *et al.*, 1982; Bjorkman & Leopold, 1987a, b; Miller & Gow, 1989a). Zoospores may sense electrical fields that are the result of these proton circulations and use them for guidance towards the root surface (Miller *et al.*, 1988; Gow *et al.*, 1992; Morris *et al.*, 1992; Morris & Gow, 1993; Gow, 1993). It is also possible that they may utilize the associated pH gradients set up by these currents in a similar manner.

The pH gradient along the length of a root can vary by 1–2 pH units depending on the external environment and position on the root. For example, by using pH-selective microelectrodes Miller *et al.* (1991) were able to show that 20 μm from the root surface *Trifolium repens* generated a pH gradient that varied from pH 5.2 (2 mm from the root tip) to 6.6 (at the root tip) depending on the nitrogen status of the surrounding medium.

Allen & Harvey (1974) and Cameron & Carlile (1980) both reported a negative chemotaxis of *Phytophthora* zoospores to a range of cations, in particular protons. Using a 'swim-out' test, negative taxis at threshold values of pH 3.8 (Cameron & Carlile, 1980) and pH 4.4 (Allen & Harvey, 1974) was reported. Similar chemo-repulsion was apparent to K^+ , Cs^+ , Mg^{2+} , La^{3+} , Li^+ and Ca^{2+} (Cameron & Carlile, 1980). In addition, Allen & Harvey (1974) showed repulsion of zoospores of *Phytophthora cinnamomi* to NH_4^+ and Na^+ . Repulsion was deduced to be dependent on an interaction between the cations and the cell membrane. The precise mechanism has not been elucidated but it is possible that it involves the effect of the ions on the membrane potential of the cell. Change in trans-membrane potential was postulated by Cameron & Carlile (1980) to cause alterations in the swimming behaviour of zoospores. They also proposed that the

Abbreviations: APW, artificial pond water; CCTV, closed circuit television; VCR, video cassette recorder.

repulsion caused by charge interactions of zoospores may also be involved in the avoidance reaction between individual zoospores at high population densities and that this may aid dispersal as zoospores are released from the sporangium (Gisi, 1983).

Preferential accumulation of zoospores at specific regions of plant roots has been well documented (Goode, 1956; Hickman, 1970; Gow, 1993). In particular, zoospores tend to accumulate at the elongation zone and wound sites. This accumulation is thought to be due primarily to chemotaxis but may involve other tactic responses such as electrotaxis, geotaxis and rheotaxis (Carlile, 1983, 1986; Deacon & Donaldson, 1993; Gow, 1993). The relative importance of these responses to root infection has yet to be determined.

Here we examine the tactic response of a population of *Phytophthora palmivora* zoospores to media of differing pH using a 'swim-in' test (Cameron & Carlile, 1978; Donaldson & Deacon, 1993). A high rate of encystment could result in zoospore accumulation due to entrapment. A method is described to determine the rate of encystment of zoospores of *P. palmivora* at acidic and alkaline pH values, in order to establish whether apparent taxis is due to enhanced encystment.

Ion-selective microelectrodes can be used to measure the pH profile of plant roots with fine spatial resolution. Using this approach we have mapped the pH gradients around intact and wounded roots of *Theobroma cacao* (a primary host of *P. palmivora*). We show that zoospores of *P. palmivora* exhibit pH taxis *in vitro* but that this tactic behaviour is unlikely to have a dominant role in the homing response of the zoospore towards plant roots.

METHODS

Assessment of tactic response. *P. palmivora* (Butler) Butler, strain P6390, was kindly supplied by Dr M. D. Coffey (University of California, Riverside, CA, USA) and grown under licence from the Scottish Ministry of Fisheries and Food (licence number PH/23/1994). Cultures were grown on modified V8 agar with a 48 h light/dark cycle according to Morris *et al.* (1992).

Zoospores were prepared in 1 mM sodium phosphate buffer at pH 7.0 at a density of 5×10^6 zoospores ml^{-1} (Morris *et al.*, 1992). Micropipettes (1 μl , Camlab) were heat-sealed at one end using a Bunsen flame. These were filled with either test or control solutions by warming the tubes in a Bunsen flame before plunging them into the relevant solution. The liquid was drawn into the tubes as they cooled. Test solutions were composed of either 1 mM sodium phosphate buffer, 1 mM citric acid/citrate buffer or 1 mM Tris/HCl, at a range of different pH values. Sodium phosphate buffer was supplemented with 100 mM HCl to bring the final pH to 4.0. Control solutions consisted of 1 mM sodium phosphate buffer at pH 7.0. The pH was measured immediately prior to each test using a digital pH meter (WPA, Cambridge, UK; model CD 620).

Taxis chambers were constructed using a glass microscope slide to which two glass strips had been glued to form a channel 5 mm wide, 25 mm long and 1 mm deep. An aliquot of zoospore suspension was placed in the channel and covered with a glass coverslip. Excess liquid on the outside of the tubes was wiped

away and the test and control capillaries were placed beneath the coverslip at either end of the channel with the open ends resting in the zoospore suspension. After 20 min, the capillary tubes were removed and the number of zoospores inside each tube was counted using an inverted microscope ($\times 100$ magnification). Results were collated by comparing the numbers of zoospores in test capillary tubes, containing buffer solutions at differing pH values, to those in control capillaries containing buffer at pH 7.0. Densities greater than 400 zoospores per microcapillary tube were prone to large counting errors, leading to unreliable comparisons with controls. Therefore, in these cases any differences between tests and controls tended to be underestimated. A paired *t*-test was used to detect significant differences between test and control values (10 replicates for each test).

A coefficient of taxis (T_c) was formulated in order to compare the relative tactic responses of zoospores to solutions of different pH. It describes the difference between the test and control densities as a function of the total number of zoospores:

$$T_c = \frac{(\text{number in test} - \text{number in control})}{(\text{number in test} + \text{number in control})} \times 100$$

Positive values of T_c indicate attraction to the test substance, whilst negative values indicate repulsion. Values approaching zero show little attraction or repulsion relative to the test material.

Encystment rate of zoospores. The relative rates of encystment of zoospores in 1 mM sodium phosphate at pH 6.0, 7.0 and 8.0 were determined using the pH taxis chamber described above. A closed circuit television (CCTV) camera (Panasonic, WV-CD 130) attached to an inverted microscope (Olympus, CK2) was used to transmit images of zoospores to a video cassette recorder (VCR) (Panasonic VHS Time-lapse recorder, AG 6720) over a 1 h period. An aliquot of zoospore suspension was poured into the central channel of the test chamber and covered with a glass coverslip. An inverted microscope was focused midway between the base of the chamber and the underside of the coverslip. A narrow depth of field was obtained by opening the condenser diaphragm of the microscope as far as possible. The intensity of light entering the video system was controlled using the electronic strobe facility of the CCTV camera. By adjusting the contrast and brightness of the recorded image from the VCR it was possible to eliminate images of zoospores outside the plane of focus. Using the freeze-frame facility of the VCR and a computer-generated overlay grid (Gray & Morris, 1992), the number of zoospores swimming in the field of view at defined time intervals was assessed. Zoospore counts in 10 frames every 15 minutes were recorded with at least a 10 s interval between frames. The rate of encystment was determined by assigning the number of zoospores at $t = 0$ as 100% and calculating the percentage of zoospores remaining, relative to the initial population, at the end of each time period.

Analyses of the turning frequency of zoospores at pH 6.0, 7.2 and 8.0, respectively, were made using the video analysis method described by Morris & Gow (1993).

Determination of the pH profile of roots. Pods of *Theobroma cacao* (cocoa) were supplied by the Cocoa Research Institute, Ghana or were kindly brought from Sri Lanka under licence by Thanuja Perera (Department of Molecular and Cell Biology, University of Aberdeen, UK). The pods contained typically 30–40 seeds. These were first washed to remove the testa and surface-sterilized by immersion in 15% hydrogen peroxide for 20 min. After several rinses in sterile water, the seeds were placed on sterile, damp filter paper in 9 cm, sealed Petri dishes and incubated at 28 °C under a 12 h light/dark cycle. After

germination, and preceding the formation of secondary rootlets, the seeds were mounted in 2 mM sodium phosphate buffer at pH 7.2 or artificial pond water (APW; Miller *et al.*, 1988) at pH 7.2 in a 9 cm Petri dish. Experiments were carried out at 27–29 °C on at least five roots. The pH profiles were determined by positioning a test electrode 50 µm from the root surface and recording the pH at 100 µm intervals along the root. Roots were wounded by nicking them with a fine scalpel blade approximately 1 mm from the root tip. Comparisons of the resulting change in pH of buffer or APW were made using a test microelectrode positioned 50 µm from the wound site at 30 s intervals.

RESULTS

Assessment of tactic response

Zoospores of *P. palmivora* prepared in media of pH 7.0 were attracted to capillary tubes containing acidic buffers and were repelled by buffers of an alkaline pH. The transition from attraction to repulsion occurred between pH 6.0 and pH 8.0 (Fig. 1) and was independent of the buffer system used (Fig. 2). Accumulation of zoospores at low pH values was often so marked as to prevent accurate enumeration of zoospores in the capillaries. Therefore, zoospore numbers greater than 400 were given an arbitrary maximal value of 400, to provide a conservative estimate of differences between the test and control capillaries. Accumulation tended to occur approximately 1–2 mm from the open end of the tube. This region may represent a critical threshold pH allowing accumulation due to entrapment.

Sodium phosphate buffer consists of sodium dihydrogen phosphate and disodium hydrogen phosphate in different ratios, depending on the required pH. Changing the relative proportions of these components also alters the concentration of sodium in the buffer. In 1 mM sodium phosphate buffer, the sodium concentration can vary from

Fig. 1. Tactic response of zoospores of *P. palmivora* to capillary tubes containing a buffer solution at different pH values. Positive values indicate attraction and negative values repulsion relative to the response at pH 7.0 (see text). Assays were carried out in 1 mM sodium phosphate buffer at the required pH and compared to a control at pH 7.0. Error bars are 95% confidence intervals for at least 10 samples.

Fig. 2. Tactic response of zoospores of *P. palmivora* to capillary tubes containing buffers at different pH values. Positive values indicate attraction and negative values repulsion relative to the response at pH 7.0 (see text). Assays were carried out in a 1 mM buffer solution at the required pH and compared to a control at pH 7.0. Three different buffer regimes were used: citric/citrate (●), sodium phosphate (□) and Tris/HCl (▲). Error bars are 95% confidence intervals for at least 10 samples.

1 to 2 mM according to buffer pH. Therefore, control assays were performed to assess any tactic responses to Na^+ . NaCl was added to 1 mM sodium dihydrogen phosphate to increase the sodium concentration to 2 mM. The relative tactic response of zoospores in this solution compared to unsupplemented disodium hydrogen phosphate was then determined. A coefficient of taxis comparing the relative numbers of zoospores in the test and control capillaries was calculated for the Na^+ supplemented buffer. A calculated value of 4.81 ± 13.47 ($\pm 95\%$ confidence limits) showed no significant difference between tests and controls lacking additional NaCl ($P = 0.38$; $n = 20$ using a paired *t*-test). These results also indicated that accumulation of zoospores was not due to osmotic differences between test and control capillaries.

Encystment rate of zoospores

Encystment rates at pH 6.0 and pH 8.0 were at least twice that at pH 7.0, taking approximately 30 min to reach 90% encystment at pH 6.0 and 8.0 compared to 60 min at pH 7.0. The encystment rate was fastest at pH 8.0 (Fig. 3).

The rate of turning of zoospores increased from 3.4 ± 2.1 turns min^{-1} at pH 8.0 to 4.6 ± 3.0 turns min^{-1} at pH 7.2 and 21.1 ± 5.8 turns min^{-1} at pH 6.0. Analysis of variance showed a significant difference ($P < 0.0001$) for the rate of turning between pH 6.0 and 7.0 and between pH 6.0 and 8.0 [$\pm 95\%$ confidence limits for 61 (pH 6.0), 123 (pH 7.0) and 61 (pH 8.0) zoospores].

At pH values below 5.0, the reduced speed of swimming of zoospores in thin films allowed observation of the turning mechanism of zoospores. Rather than using the posterior flagellum as a steering rudder (Carlile, 1983), the zoospore turned soma first during reorientation. Prior to

Fig. 3. Encystment rates of zoospores of *P. palmivora* in 1 mM sodium phosphate buffer at pH 6.0 (○), pH 7.0 (●) and pH 8.0 (■).

Fig. 5. Profile of pH change along a representative root of *T. cacao* in APW (●) and in 2 mM sodium phosphate buffer, pH 7.2 (○). Measurements were made 50 μ m from the surface at 100 μ m increments along the root from the tip.

Fig. 4. Observed method of turning of *P. palmivora* zoospores in thin aqueous films.

turning, the zoospore was observed to stop momentarily and then to change its direction by turning its body relative to its posterior flagellum. It then continued swimming in a new direction. Only after the body turned did the posterior flagellum assume its normal trailing position (Fig. 4).

Fig. 6. Change in pH at 30 s intervals, measured with a pH microelectrode 50 μ m from a wound site on a representative root of *T. cacao* in APW (●) and in 2 mM sodium phosphate buffer, pH 7.2 (○).

Root pH profile

Root-generated proton flux in APW caused a reduction in pH from an ambient value of 7.2 to approximately 6.4 at the root tip, increasing to 8.5 approaching the root hair zone of *T. cacao* (Fig. 5). In 2 mM sodium phosphate buffer, roots maintained a pH of approximately 7.2 (Fig. 5).

Wounding *T. cacao* roots caused a dramatic increase in rhizosphere pH in APW at the wound site to over 8.5 from an initial value of 6.9 and an ambient value of 7.2 (Fig. 6). Roots immersed and wounded in sodium phosphate buffer at pH 7.2 showed no gross change in pH at the wound site (Fig. 6). Qualitative observations on

Fig. 7. Roots of *T. cacao* before (a, c) and after (b, d) addition of zoospores. The patterns of accumulation around roots in 2 mM phosphate buffer at pH 7.2 (b) and distilled water (d) are similar. Bar, 1 mm.

wounded and intact roots immersed in suspensions of *P. palmivora* zoospores showed no difference in the accumulation of zoospores at the root surface in either APW or sodium phosphate buffer (Fig. 7). Thus, root-generated rhizosphere pH gradients are not necessary for normal patterns of accumulation at the root surface.

DISCUSSION

We have shown that zoospores that are first prepared in media of neutral pH are attracted to solutions of low pH and repelled by solutions at more alkaline pH. These tactic movements could be due to (a) attraction to protons and repulsion from hydroxyl ions, (b) negative taxis to hydroxyl ions but no response to moderate concentrations of protons, or (c) attraction to protons and no response to hydroxyl ions. Since the chemoreceptor systems for zoospores of *Phytophthora* and other pythiaceae species have not been characterized, it is not possible to distinguish between these hypotheses at this stage. However, if the mechanism of pH taxis involves receptor binding of free ions, the latter of these postulated mechanisms would seem most plausible since only at acid pH would moderate concentrations of protons exist in solution.

It has been shown previously that pH can affect the swimming behaviour of zoospores (Allen & Harvey, 1974; Cameron & Carlile, 1980). This may be the result of induced changes in the trans-membrane potential mediating changes in turning frequency. Such a change in membrane potential may be brought about by the interaction of cations with the negatively charged cell surface. Alternatively, changes may be due to alterations in the membrane pH gradient and hence the proton motive force. Cations approaching the membrane can exchange with those bound to the surface, or to unoccupied anionic sites. Such interactions can lead to the formation of a layer of positively charged ions adjacent to the cell surface. This structure is known as the electrical double layer (EDL) and can result in a concentration of ions at the cell surface an order of magnitude greater than that in the surrounding medium (McLaughlin, 1977). A

change of 1 pH unit could result in a transient change in theoretical trans-membrane potential of up to 59 mV at 25 °C, although this would be subject to regulatory damping. Moreover, the surface potential produced by charged lipids in the membrane is also dependent on the salt concentration of the bulk aqueous phase (McLaughlin, 1977). Thus, any alteration of membrane potential brought about by a change in pH may be heightened by the addition of salts.

The change in zoospore turning frequency when the pH was reduced is comparable to that induced by a weak external electrical field of 50 mV cm⁻¹ – a threefold increase in each case (Morris & Gow, 1993). This electrical field magnitude would be expected to cause a change in trans-membrane potential of only 0.125 mV. Only very small changes in external pH would be needed to cause an equivalent alteration in membrane potential and such small changes were not able to induce pH taxis in our experiments. It is therefore unlikely that either electro taxis or pH influence turning frequencies solely by modulating membrane potential. Many enzymic processes are affected by pH and ionic strength (Alber, 1989). Changes in the behaviour of zoospores at different pH values may also be related to cationic interactions, and indirect effects on protein structure or function. Trans-membrane potential can also be affected by other ions. For example, changes in the external concentration of K⁺ or Na⁺ ions will alter the electrochemical gradient across the membrane of a zoospore and may thus influence the trans-membrane potential of the cell. This conclusion supports those of Byrt *et al.* (1982) who suggested that the complex nature of the interactions between the zoospore membrane and its external environment is unlikely to be explained by a single mechanism brought about by changes in trans-membrane potential.

Previous observations of zoospore swimming have suggested that the posterior flagellum functions in steering (Carlile, 1983; Holwill, 1985). However, the observations described here suggest that the posterior flagellum may be responsible for zoospore swimming

stability rather than steering. The anterior flagellum may be responsible for both propulsion and changes in direction. Alternatively, bending of the proximal part of the posterior flagellum could bring about reorientation of the soma as has been described for certain chytridiomycete flagella (Holwill, 1985). According to this view, the primary role of the long, trailing, posterior flagellum is to reduce the pitch and yaw in the swimming zoospore. Swimming in thin films of liquid reduces the helical motion of zoospores and has been described as 'gliding' (Carlile, 1986). This style of swimming may be the preferred mode of movement of *Phytophthora* zoospores *in vivo* since zoospores often swim in thin films of water on leaves and soil particles.

Video analysis of zoospores has shown that the accumulation, or apparent chemoattraction, of zoospores to a buffered medium at low pH is likely to be the result of a two-stage process of entrapment. If accumulation of zoospores was due solely to encystment, then zoospores would be expected to accumulate in capillaries containing buffer at pH 8.0, since media at this pH cause increased encystment compared to media at pH 7.0. However, the numbers of zoospores in capillaries above pH 8.0 were significantly lower than those below pH 7.0 and the turning frequency was similar at these pH values.

Zoospore accumulation in capillary tubes containing low pH buffer (pH values less than 6.5) may be due to two effects. Zoospores that swim at random into the capillary will increase their turning rate, thus increasing the probability of remaining within the capillary. Increased encystment rate may then ensure further accumulation by immobilizing the zoospores (extreme orthokinesis).

The observations made by Allen & Harvey (1974) and Cameron & Carlile (1980) of a negative tactic response of zoospores to low pH may reflect differences in the taxis assays employed. These studies used a 'swim-out' test in which zoospores within a capillary tube were challenged with an external test solution. The distance which a clear zone moved along the capillary, and the boundary of this zone to the zoospore suspension, was used to determine the threshold concentration of the repellent. Carlile (1983) discussed the merits of the 'swim-in' test presented here and concluded that this method is particularly useful as a measure of attraction. Repulsion using the 'swim-in' test may be eclipsed by trapping and immobilization brought about by the test buffers. The zoospores used in the study presented here may have shown negative chemotaxis to pH values less than pH 4.0. However, this response is likely to have been masked by entrapment due to the increased rate of zoospore turning and encystment.

It is known that the endogenous proton flux around roots can be outward or inward at the elongation zone (Gow *et al.*, 1992; Gow, 1993). This proton flow results in a change in local pH. It has been shown that zoospores can respond tactically to pH (Allen & Harvey, 1974; Cameron & Carlile, 1980). This pH gradient could, in conjunction with other responses, cause local accumulation of zoospores at specific sites (Miller *et al.*, 1988). However, our data showed that the pattern and extent of zoospore

accumulation around a root was not influenced by buffers that abolished this gradient. It is likely therefore that the local pH change associated with growing roots is insufficient *per se* to account for localized accumulation of zoospores.

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