Crook root disease of watercress: investigations into zoospore attraction, diagnostics, and phylogeny

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CROOK ROOT DISEASE OF WATERCRESS: INVESTIGATIONS INTO ZOOSPORE ATTRACTION, DIAGNOSTICS, AND PHYLOGENY

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ABSTRACT

The plasmodiophorid organism, *Spongospora subterranea* f. sp. *nasturtii*, is the causal agent of crook root disease of watercress (*Rorippa nasturtium-aquaticum*). The only current control measure is zinc, used such that levels do not exceed 0.075μg ml^{-1} in effluent water. Laboratory results indicated that zinc acts by actively lysing zoospores of *S. subterranea*, but does not cause 100% mortality at field concentrations. At 8μg ml^{-1}, around 20 % of zoospores were seen to survive compared to controls. Of other cations investigated as potential control agents, none were as effective as zinc at equivalent levels. Only cobalt was capable of significant zoospore lysis.
Attraction of zoospores to watercress roots and total root extracts appeared to be non-specific when compared to attraction to tobacco (*Nicotiana rustica*), tomato (*Lycopersicon esculentum*), and mustard (*Brassica napus*). Results implied that a general plant attractant was present, and that this did not mask a more specific attractant.

A molecular, PCR-based diagnostic test was developed for *S. subterranea* f. sp. *nasturtii*. The internal transcribed spacer (ITS) and 18S ribosomal DNA were used to design specific PCR primers, capable of detecting DNA directly from zoospores. As little as 5ng genomic DNA or 100 zoospores were required as template. Efforts to develop a sampling technique for zoospores in watercress beds were successful based on washing root material followed by PCR.

DNA analysis also identified a Chytridiomycete-like organism as being consistently associated with crooked roots. However, preliminary studies showed that it did not have a direct role in crook root disease.

The 18S ribosomal DNA sequence was used to infer phylogeny of *S. subterranea* f. sp. *nasturtii*, and plasmodiophorids generally, by performing parsimonious and distance-based analyses. Within the plasmodiophorid grouping, *S. subterranea* f. sp. *nasturtii* appeared to be closely related to *S. subterranea* f. sp. *subterranea* and *Plasmodiophora brassicae*, based on 270 bases at the 3' end of the gene, whilst *Polymyxa* species aligned on a separate branch. Based on a complete 18S ribosomal DNA analysis, plasmodiophorids seemed to form a discrete taxonomic grouping, not closely linked to either protists or fungi.

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Table of contents

1. Introduction

Watercress

Watercress, *Rorippa nasturtium-aquaticum*, is a diploid (2n=32), perennial, aquatic plant, belonging to the family Brassicaceae. Two other species exist, the tetraploid (2n=64) *Rorippa microphyllum*, and the sterile triploid (2n=48) *Rorippa sterilis* (Airy Shaw, 1951), but only the diploid is widely cultivated (Ryder, 1979). It is thought that watercress is native to the Mediterranean (Ryder, 1979), where it has been widely used for medicinal purposes for over two thousand years. More recently it has been grown in the Americas, South Africa, and Oceania, having most likely been introduced from Europe (Howard and Lyon, 1952). It was first cultivated in the U.K. (Kent) early in the 19th Century.

Figure 1.1 Watercress growing at a commercial farm.

Watercress (Figure 1.1) is grown in shallow flowing water in areas of chalk or limestone, where fresh water is available directly from springs or can be pumped from boreholes. The flow rate through watercress beds is around 2000 litres m^{-2} hr^{-1}, and one hectare of watercress requires approximately 11 million litres of water each day. The beds in which watercress is grown are shallow rectangles usually 5-9 metres wide, and 50-70 metres in length, separated from each other by concrete walkways (M.A.F.F., 1983). They generally have a gravel base, covering bedrock, with water entering at the top of a bed, and passing down a gradient of approximately 1 in 360 to the outlet. The flow rate and depth of the water can be controlled using sluices. The spring water supplies most of the plants nutrients, of which nitrogen is a particularly high requirement, although it is sometimes necessary to add supplementary potassium and phosphorus (M.A.F.F., 1983).

Plants have a basal root system anchoring them in the gravel base, while nutrients are largely absorbed through adventitious roots, which float freely (Cumbus and Robinson, 1977). The adventitious roots are attached to a section of the hollow stem below water, whilst above the surface, the stem may reach 10-60 cm (although commercially, plants are harvested at no greater than around 15 cm). The aerial section of stem gives rise to leaves, which are pinnate, dark green, and glossy, traits which are selected for their commercially attractive characteristics (Rothwell, 1983). Late flowering is also an important commercial characteristic, as the plant grows more vigorously prior to flowering. If allowed to flower, watercress is self-compatible, but may be cross-pollinated also (Howard, 1976).

Larger scale farms often grow watercress from seeds, which are generally sown in peat in controlled glasshouse conditions. The seedlings are transplanted to beds after 7-10 days (Rothwell, 1983), and the flow rate may be reduced until plants become established. Smaller commercial farms often allow plants to regenerate from cuttings. Seed selection has been the major method by which new lines of watercress have arisen, as commercial scale breeding has not been carried out.

Plants may be grown all year as the water temperature entering the beds is generally constant at around 10°C. A rapid turnover of just six weeks from planting to harvesting is possible in the summer months, with 16 weeks more usual in winter when the plants grow less vigorously (Casey and Smith, 1994). In winter, the constant water temperature helps protect the plants from frost damage, although plastic coverings are sometimes used, particularly if the crop is close to harvesting. Traditionally, harvesting is carried out by hand, with sprigs cut at the stem base to allow regeneration, but mechanical cutters are becoming more widely used. After harvesting, beds may be cleared, and replanted, or plants may be left to regenerate from stubble via axillary buds.

The harvested cress is maintained at 3°C and 100% humidity for as long as possible prior to sale (Rothwell, pers. comm.) The leaves and stalks are either bunched or packed in plastic, stored on ice, and transported directly to supermarkets. Watercress is used in salads, soups, sandwiches, and as a garnish. The leaves are rich in ß-carotene, vitamin C and calcium, with 100g of watercress supplying 300%, 100% and 30% respectively, of the recommended daily adult intake of these nutrients (National

Research Council, 1989). The pungent, slightly bitter flavour of watercress is derived partly from the volatile compound ß-phenethyl isothiocyanate (Cumbus, 1975). Total fresh weight production of watercress has been estimated at 13.5 kg m^{-2} per annum (Casey and Smith, 1994). The current value of the U.K. crop exceeds £25 million per annum retail sales value (Rothwell, pers comm.), from about 87 ha of land, most of which is located in Dorset, Hamphire and Wiltshire. Vitacress Salads Ltd. account for about 70% of sales, the Watercress Company 25%, and the remainder is from smaller, independent growers.

Of the diseases affecting watercress, crook root and associated viruses are the most damaging. Aphid species are known to transmit turnip mosaic virus, which may be controlled using disease-free seed (Tomlinson, 1974). The only serious direct insect damage is caused by the mustard beetle, *Phaedon cochliariae*, which may be controlled using insecticidal applications (M.A.F.F. 1983).

Crook root disease

Crook root disease was first observed in 1947, in a watercress bed in Wiltshire, from where it spread rapidly to all growing areas of the U.K. The origin of the disease is uncertain. Infected plants exhibit swollen, brittle roots, which are often curved so that they resemble a shepherd's crook. Plants also exhibit stunted growth, and chlorotic leaves (Figure 1.2). Over time, the roots may rot and the plants lose anchorage in the bed. Symptoms usually first appear in October, most commonly near the bed outlet (Tomlinson 1958a), and then infection spreads back up the bed, persisting until April, by which time, in severe outbreaks, whole beds may be lost to the disease.

Figure 1.2 Crook root symptoms observed on watercress in the field.

The causal organism was identified as a member of the Plasmodiophorales, by observations on plasmodia, zoosporangia, and spore balls in infected roots (Spencer and Glasscock, 1953). Tomlinson (1958b), identified the pathogen as *Spongospora subterranea* Wallr. (Langerh.) f. sp. *nasturtii*, based on observations of spore balls, which are irregularly channelled, sponge-like masses of resting spores, and on zoospores, zoosporangia, and plasmodia.

The plasmodiophorids

The group of organisms to which *Spongospora subterranea* f. sp. *nasturtii* belongs have been variously referred to as Order Plasmodiophorales and Family Plasmodiophoraceae (Sparrow, 1960) and Phylum Plasmodiophoromycota (class Plasmodiophoromycetes) (see Barr, 1992). Much of the confusion over naming these

organisms has arisen due to uncertainty regarding their phylogenetic status, which is reviewed in a later section. Where a formal grouping is not required, this author will use the informal term, plasmodiophorid, to describe these organisms as adopted by Braselton (1995).

Life histories

The first plasmodiophorid life cycle to be elucidated in detail was that of *Plasmodiophora brassicae*, the causal agent of clubroot of cruciferous plants. This was described by Ingram and Tommerup (1972), and is shown diagrammatically in Figure 1.3.

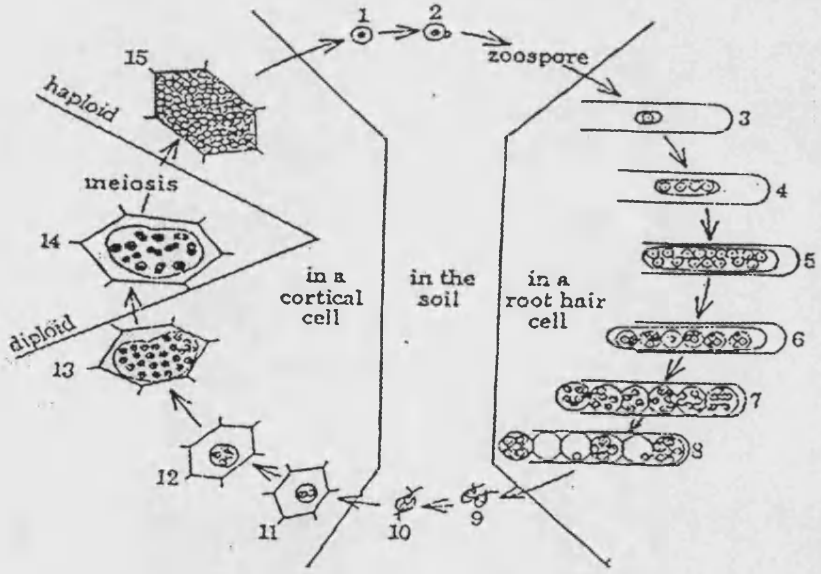


Figure 1.3 *Generalised life-cycle of Plasmodiophora brassicae* (Ingram and Tommerup, 1972).

All plasmodiophorids are obligate biotrophs (Webster, 1980), although some dual culturing successes have been reported. Ingram (1969) managed to maintain

Plasmodiophora brassicae on host callus tissue for at least 18 months, while *Woronina pythii* is apparently easy to maintain in culture with host *Pythium* species (Dylewski, 1987). Limited success in maintaining growth of *Plasmodiophora brassicae* on ¼ strength Ringers agar, pre-treated with *Escherichia coli* has been reported (Arnold *et al.*, 1996).

Plasmodiophorids survive as resting spores when away from hosts, or when conditions are not suitable for infection. These tend to form aggregates, known as spore balls or cystosori, which, in the case of *Spongospora subterranea* f. sp. *nasturtii*, generally appear as sponge-like masses, perforated by irregular channels (Tomlinson, 1958c). *Plasmodiophora brassicae* resting spores contain 25.1% chitin by dry weight, and high levels of protein (33.6%) and lipids (>17%), which may help to account for their ability to survive for many years before germinating, a feature common to plasmodiophorids (Moxham and Buczacki, 1983).

Specific requirements for germination have not been established for any plasmodiophorid. Merz (1993), working on *Spongospora subterranea* f. sp. *subterranea*, concluded that root exudates were involved, although germination in response to non-host species such as wheat, suggested that the stimulus was non-specific. In contrast, Ingram and Tommerup (1972), stated that *Plasmodiophora brassicae* was capable of germinating on glass slides in the absence of host tissue, although water must always be present (Harrison *et al.*, 1997).

Germinated resting spores release single zoospores (Ingram and Tommerup, 1972). In *P. brassicae,* these are typically 3-5µm in diameter (Ingram and Tommerup, 1972), whilst those of *S. subterranea* f. sp. *nasturtii* may be 2.4-6µm (Tomlinson, 1958b).

Zoospores are generally ovate, although subject to changes in shape (Tomlinson, 1958b), bounded by a single cell membrane, and possess two whiplash flagella, of unequal length, which are important for cell motility (Merz, 1992). Zoospores released from resting spores are often referred to as primary, because they initiate what is known as primary infection (MacFarlane, 1958). In some plasmodiophorids, including *Spongospora subterranea* f. sp. *nasturtii*, primary infection is the only infectious stage which has been recorded (Claxton *et al.*, 1996).

Primary zoospores swim or are carried through water to host roots, where they adhere, and encyst. In *Spongospora subterranea* f. sp. *nasturtii*, encystment was not confined to particular root zones (Claxton *et al.*, 1996).

Penetration has been observed in *S. subterranea* f. sp. *nasturtii* (Claxton *et al.*, 1996), using light and electron microscopy, and appears to be similar to that in *P. brassicae*, as described by Aist and Williams (1971). In *P. brassicae*, zoospores attach at a point opposite the flagella, and form spherical, walled cysts 2-3 minutes after attachment. The membrane invaginates, forming a cavity known as the Rohr, in which a dense, bullet shaped structure known as the Stachel is formed. After two and a half to three and a half hours, penetration begins, with an adhesorium forming at the end of the Rohr, and the Stachel penetrating the host cell wall. The parasite is injected, and is swept away in the cytoplasmic stream after a few seconds (Aist and Williams, 1971).

The uninucleate protoplast is now separated from the host by a membrane 'envelope', the thickness of which is reported to vary between genera. Aist and Williams (1971) observed a seven-layer envelope in *P. brassicae*, whilst *Sorosphaera*, and

Membranosorus species have just a single layer separating them from host cytoplasm (Braselton and Miller, 1975; Braselton, 1983). Uninucleate plasmodia undergo repeated nuclear division (Ingram and Tommerup, 1972) followed by cleavage to form uninucleate zoosporangia, a stage which has been observed in *S. subterranea* f. sp. *nasturtii* (Clay and Walsh, 1990). In *S. subterranea* f. sp. *nasturtii* these zoosporangia have been seen to differentiate into 4-8 zoospores after 2 or 3 mitotic divisions (Clay and Walsh, 1990). The zoospores are referred to as secondary (MacFarlane, 1958), although studies on *S. subterranea* f. sp. *subterranea* suggest that they are morphologically indistinguishable from primary zoospores (Merz, 1997). These secondary zoospores may be released back into the environment, but some evidence suggests that they are capable of infecting deeper into the host. Aist and Williams (1971), observed release and encystment of *P. brassicae* secondary zoospores within root hair cells, and secondary infection has been claimed for *S. subterranea* f. sp. *subterranea* (Diriwächter and Parbery, 1990), and *Polymyxa betae* (Barr and Asher, 1996). Some authors have suggested that uninucleate myxamoebae are able to move from cell to cell and could be responsible (Claxton *et al.*, 1996; Mithen and Magrath, 1992).

Virus transmission

Both *Polymyxa* and *Spongospora* are known to transmit viruses to host plants (Campbell, 1996). Transmission of these viruses is *in vivo*, the particles being retained within zoospores and resting spores.

S. subterranea f. sp. *nasturtii* is responsible for vectoring two watercress viruses, watercress chlorotic leaf-spot virus (WCLV) (Tomlinson and Hunt, 1987), and watercress yellow spot virus (WYSV) (Walsh *et al.*, 1989). Symptoms of the two diseases are indistinguishable, and are described as bright yellow spots on the leaves of infected plants (Tomlinson and Hunt, 1987). Although Tomlinson and Hunt were unable to obtain virus particles of WCLV, Walsh *et al.* (1989) were able to demonstrate that WYSV was a rod-shaped virus. It was distinct from WCLV on the basis of number of spots per leaf, recovery of virus particles from hosts, and lack of reaction when antisera to WYSV was tested against purified preparations of watercress showing symptoms of WCLV.

Spongospora was implicated as the host for both viruses largely on the evidence from transmission experiments where plants containing viable zoospores and virus were able to transmit both diseases, but treatments which killed zoospores prevented transmission of viruses (Tomlinson and Hunt, 1987). This was not confirmed by Walsh *et al.* (1989), who were able to transmit high levels of WYSV, even when *Spongospora* zoospores were treated with 7µg/ml zinc, which should have been lethal (see below). They also found WYSV particles free in water, suggesting that an absolute requirement for *Spongospora* as a vector may not be the case.

Some growers consider these viruses to be a greater threat than crook root itself (R. Gibbs, pers comm.), and an ELISA test has been developed for WYSV (Walsh *et al.*, 1989). This showed that occurrence of WYSV and crook root were closely correlated, adding to the circumstantial evidence linking the two diseases (Walsh and Phelps, 1991).

Current options for control of crook root disease

The infection cycle of *S. subterranea* f. sp. *nasturtii* may be completed within 7-10 days in favourable conditions (Tomlinson, 1958b). Hence, inoculum levels may build up very rapidly, and watercress crops severely reduced. Tomlinson (1958a) discovered that addition of zinc to water resulted in reduction of crook root disease levels. 0.5 $\mu g\ ml^{-1}$ zinc sulphate ($ZnSO_4$) or 0.2g zinc oxide (ZnO) in 350ml water were enough to prevent infection of watercress, and the effect was shown to be caused by lysis of the zoospores. 0.5 $\mu g\ ml^{-1}$ zinc sulphate was shown to reduce the number of roots infected to just 0.03% in laboratory tests, compared to 12.5% in distilled water, and 13% in 0.1 $\mu g\ ml^{-1}$ zinc sulphate.

Since then zinc has been used widely in these two forms to control crook root, either as drip feeds, or by allowing the chemicals to dissolve into the water at a steady rate. No other control measure has been shown to be effective, aside from calcium bicarbonate at 540$\mu g\ ml^{-1}$ (Tomlinson 1958c), and this was uneconomic due to the input of carbon dioxide required to maintain this concentration.

The current limit on the levels of zinc which are allowed to leave watercress beds is 0.075 $\mu g\ ml^{-1}$, due to concerns over the effect of zinc on ecological communities downstream, such as reduced feeding rates in the freshwater shrimp, *Gammarus pulex* (Roddie *et al.*, 1992). It has also been demonstrated that zinc does not remove *S.subterranea* f. sp. *nasturtii* from beds completely (Gooding, 1985). Therefore optimisation and replacement of zinc are current research priorities.

Control measures for other plasmodiophorids are of limited help, as the economically important pathogens are all soil-borne. Control of *S. subterranea* f. sp. *subterranea* has been investigated with consideration of factors including crop rotation, use of resistant varieties, and fungicidal treatment of tubers and soil. No satisfactory control methods have been found so far (Harrison *et al.*, 1997). *Plasmodiophora brassicae* may be controlled by soil drainage and alteration of soil pH (Buczacki, 1983), or by fungicides such as pentachloronitrobenzene (Arie *et al.*, 1998). Recently, a chemical released by *Phoma glomerata*, known as epoxydon was shown to be effective against resting spores of *P. brassicae* (Arie *et al.*, 1998). In another study, crabshell meal was proven to be effective against resting spores of the Chytridiomycete fungus *Synchytrium endobioticum*, which causes potato wart (Hampson and Coombes, 1991). The effect was due to encouragement of chitinolytic bacteria in soil, which degraded cell walls of the pathogen as well as the meal. Unfortunately, both of these control methods may be less effective in an aquatic environment, due to lack of persistence of the control agent.

Host resistance

Resistance mechanisms have not been fully characterised for any plasmodiophorid-plant interaction. Most work has been carried out on *P. brassicae*, where recently a resistance gene has been located in *Arabidopsis thaliana* (Arbeiter *et al.*, 1999), following work which demonstrated that the resistance was pathotype specific, dominant, and monogenically inherited (Fuchs and Sacristán, 1996). This was in contrast to resistance in *Brassica oleracea*, which was described as partly recessive,

and partly additive (Voorrips, 1995). Scott (1985), obtained four distinct pathotypes of *P. brassicae*, and the presence of multiple pathotypes is likely to reflect complex genetics of resistance.

The presence of pathotypes of *S. subterranea* f. sp. *nasturtii* has not been investigated, and disease resistance has been mostly studied by generation of somaclonal variants of watercress. Arnold *et al.* (1995), were able to create somaclones of watercress with increased resistance to crook root, but large scale screening revealed no significant disease resistance in 833 somaclones tested (Claxton *et al.*, 1998).

Zoospore taxis and encystment

Zoospores of fungi and protists respond to a range of environmental stimuli, at various stages of their life cycles. For pathogenic organisms, potential stages for both host-specific and non-specific responses include germination, taxis, encystment, and penetration. Zoospores must find and attach to host surfaces in significant numbers to colonise successfully. To do this they often have been shown to detect, and respond to gradients of attractants produced by plant roots, and by compounds tested *in vitro*. Such responses are known as taxes, and zoospores generally show taxis through alteration of their swimming pattern, and motility (see Deacon and Donaldson, 1993). Whether or not zoospores display chemotactic behaviour, they must recognise and encyst on host surfaces. Although this often involves surface features (Deacon and Donaldson, 1993; Mitchell and Deacon, 1986a, 1986b; Estrada-Garcia *et al.*, 1990), factors involved in chemotaxis are sometimes also involved in encystment.

Encystment involves many chemical and ultrastructural changes in the zoospore, often

including changes of shape, flagella retraction, and secretion of cell wall material (Deacon, 1988; Byrt *et al.*, 1982a).

Much of the published literature on taxis and encystment has involved studies on Oomycetes (see for example, Deacon and Donaldson, 1993). However, other groups of organisms including green algae (e.g. Ermilova and Gromov, 1988), cryptophytes (Lee *et al.*, 1999), bacteria (e.g. Pandya *et al.*, 1999), Chytridiomycetes (e.g. Orpin and Bountiff, 1978), Archaea (Suk Yu and Alam, 1997), and slime molds (Thomas and Peterson, 1990) have been examined. Studies on taxis in Plasmodiophoromycete species have been limited, although Claxton (1996), in preliminary studies was able to induce taxis of zoospores of *S. subterranea* f. sp. *nasturtii* to watercress root extracts, and to roots of a range of plant species.

Several forms of taxis may be exhibited by zoospores, including chemotaxis, electrotaxis, rheotaxis (Katsura and Miyata, 1971), geotaxis (Cameron and Carlile, 1977), phototaxis (Hegemann and Musgrave, 1991), magnetotaxis (Torres de Araaja *et al.*, 1986), and aerotaxis.

Evidence for chemotaxis of zoospores and consideration of compounds responsible

To date, it is still not known precisely which factors are responsible for zoospore taxis for any zoosporic parasite *in vivo* (Deacon and Donaldson, 1993).

Root exudates were implicated in attracting zoospores when it was observed that zoospores of *Phytophthora* spp. encysted at a distance from roots in a gradient related fashion (Zentmyer, 1961).

In vitro, a wide range of chemicals have been demonstrated to affect zoospore behaviour, but when used in combination, results are often conflicting. In 1978, Cameron and Carlile noted that in *Phytophthora* and *Pythium*, no specific substance had been shown to be as attractive as root exudates, suggesting that additive or synergistic effects may operate.

Amino acids

Glutamic acid and aspartic acid have been reported to attract zoospores, and to cause encystment. Khew and Zentmyer (1973) reported that zoospores of *Phytophthora* species showed a directionally oriented attraction to these two amino acids at 10-300μM, and that attraction was greater than to alanine, and methionine, although sensitivity was shown to vary between *Phytophthora* species. *Phytophthora cinnamomi*, has been shown to be attracted to 1mM aspartic acid and glutamic acid (Cahill and Hardham, 1994a). Donaldson and Deacon (1993a) also found glutamic acid and aspartic acid to be chemoattractants, at concentrations of 1mM and 4mM respectively, causing significant accumulation of *Pythium aphanidermatum* zoospores around assay chambers containing the appropriate amino acid. Glutamic and aspartic acids are capable of inducing encystment of *Pythium aphanidermatum* at 5mM and 6mM respectively, whereas other amino acids were not effective, and 40mM background levels did not prevent 7mM glutamic acid or aspartic acid causing encystment (Donaldson and Deacon, 1993a).

Other amino acids implicated as chemotactic inducers include alanine, asparagine, and glutamine, which were observed to attract some *Pythium* species at 10mM levels

(Donaldson and Deacon, 1993a) or less with Morris and Ward (1992) reporting attraction of *Pythium irregulare* to 2μM asparagine, and attraction of *Phytophthora medicaginis* to 1μM levels. Asparagine and glutamine are known to cause encystment of *Phytophthora cinnamomi* at 20mM (Byrt *et al.*, 1982a). Alanine and methionine are frequently reported as chemoattractants of zoospores (e.g. Lee *et al.*, 1999). Work on *Phytophthora cinnamomi* identified that L-methionine was an attractant, but that the D isomer was not (Cahill and Hardham, 1994a), suggesting specificity of a binding site. Aspartic acid was found to induce taxis in either configuration, however.

Synergistic or additive effects of amino acids have been observed. When supplied at 10mM each, lysine and arginine were found to attract zoospores of *Pythium aphanidermatum* to a much greater extent than separately (Jones *et al.*, 1991). Conversely, this study also showed that phenylalanine was a weak attractant on its own, but not an attractant at all in combination with alanine, valine, leucine and methionine (all at 10mM), suggesting some sort of antagonism.

Carlile (1983) suggested that zoospores would benefit from having receptors for several attractants. If receptors for a particular signal became saturated before reaching the host, other receptors would enable the zoospore to continue detecting chemical gradients.

Carbohydrates

Attraction of the fungus *Aphanomyces cochlioides* to sugar beet seedlings was observed in response to glucose and fructose exudates as early as 1966 (Rai and

Strobel, 1966). Khew and Zentmyer (1973) reported that sugars were attractants of *Phytophthora* species, but did not specify individual compounds. In *Pythium*, three species were reported to be positively chemotactic toward mannose at 10mM, whilst *Pythium aphanidermatum* also responded to sucrose and maltose at 10mM (Donaldson and Deacon, 1993a). A species dependent response to D-glucose, and L-fucose was seen. Jones *et al.* (1991), reported that a combination of seven sugars caused strong chemotaxis in *Pythium aphanidermatum* when each was supplied at 10mM, whilst glucose and galactose alone did not induce chemotaxis at 10mM.

In some studies sugars have been found to be relatively unimportant. *Phytophthora cinnamomi* was shown not to respond to 12 sugars tested, at concentrations up to 100mM (Cahill and Hardham, 1994a). Sugars appear not to cause encystment (Donaldson and Deacon, 1993a; Byrt *et al.* 1982a).

More complex organic molecules implicated in chemotaxis of zoospores include pectin, shown to cause chemotaxis in *Phytophthora cinnamomi* at 100μg ml^{-1} (Cahill and Hardham, 1994a), in which species it also causes encystment at 500μg ml^{-1} (Byrt *et al.*, 1982a), as does polygalacturonic acid (PGA) at 500μg ml^{-1}. PGA also induced encystment in *Pythium aphanidermatum* (Jones *et al.*, 1991).

Phenolics, organic acids and volatiles

It has been suggested that volatile compounds can diffuse faster and further than non-volatiles, and so could be very effective chemoattractants *in vivo* (Allen and Newhook, 1973). Khew and Zentmyer (1973) listed phenolics and organic acids as attractants of *Phytophthora* species. More recent work has revealed that 10mM levels

of chemicals including ferulic acid, and caffeic acid can attract *Phytophthora cinnamomi*, whilst as little as 0.1mM isocaproic acid can induce *P. palmivora* taxis (Cameron and Carlile, 1978). Isocaproic acid, and other organic acids were shown to be effective when un-ionised only. Hence, they act only when in a volatile state. *P. palmivora* was also attracted to 0.001mM isovaleraldehyde, 1mM isobutanol, 1mM isopropanol, and 5mM ethanol, amongst other compounds. *P. cinnamomi* responded to 1mM isovaleraldehyde, and 25mM ethanol, methanol, and isopropanol (Cahill and Hardham, 1994a). *Pythium aphanidermatum*, however was seen only to respond to 25mM butanol, and not ethanol or acetaldehyde (Jones *et al.*, 1991). In the case of isovaleraldehyde, a specific receptor was determined, using displacement techniques, confirming a definitive role in chemotaxis *in vivo* (Cameron and Carlile, 1981).

Plant roots contain and release groups of compounds known as flavonoids and isoflavonoids into their environment (Graham, 1991). Attraction to a flavonoid known as cochliopholin A (5-hydroxy-6,7-methylenedioxyflavone) was demonstrated to *Aphanomyces cochlioides*, when isolated from spinach roots (Horio *et al.*, 1992). Levels of 1nM were sufficient to cause chemotaxis. In a study on *Phytophthora sojae*, a soybean pathogen, two isoflavones, diadzein, and genistein, caused chemotaxis of this species, specifically, at levels down to 10nM (Morris and Ward, 1992). These same authors also demonstrated that the isoflavones could cause zoospore encystment, once the source of taxis had been reached, and also in situations where these chemicals were added directly to active zoospore suspensions.

Cations

Cations have rarely been implicated in causing zoospore taxis, but are often involved in encystment. One exception was *Chlamydomonas reinhardtii*, which showed taxis toward cobalt chloride at less than 1mM, and manganese sulphate at 1-10mM (Hirschberg and Rodgers, 1978). In *Pythium*, divalent cations (Ca, Ba, Mg, Mn, Sr), were shown to block alteration of zoospore swimming patterns, at concentrations up to 500μM (Donaldson and Deacon, 1993b). Calcium channel blockers, chelators, and ionophores had similar effects. Addition of chelators such as EGTA, or channel blockers such as verapamil, were followed by addition of known amino acid attractants. These could no longer induce chemotaxis, indicating that Ca^{2+} ions were important in zoospore motility. Monovalent cations did not have any effect.

Calcium ions were shown to be involved in induction of encystment in *Phytophthora parasitica* (vonBroembsen and Deacon, 1997; Warburton and Deacon, 1998), with Warburton and Deacon observing that transmembrane Ca^{2+} fluxes were associated with the ability to encyst. Ersek *et al.* (1991), also demonstrated the importance of calcium, when they found that lithium ions at 100mM could immobilise *Phytophthora infestans* zoospores, but that subsequent encystment was dependent on duration of treatment, and addition of Ca^{2+}.

Byrt *et al.* (1982b), working on *Phytophthora cinnamomi*, stated that calcium ions could act as promoters of encystment, but that Fe^{3+} at 20μM, and Mn^{2+} at 50μM, were active at lower concentrations. The role of intracellular calcium signalling was not examined in this case.

Autoaggregation

Calcium is also thought to be involved in the phenomenon of autoaggregation, in which, at high densities, zoospores exhibit taxis to each other. Suggested functions for this phenomenon include the recruitment of zoospores to potential infection sites, and also as a potential survival mechanism (Reid *et al.*, 1995). Thomas and Peterson (1990), working on *Achlya heterosexualis* proposed that zoospores were initially attracted by a host signal, which was reinforced by autoaggregation. Porter and Shaw (1978), reported that autoaggregation of *Phytophthora dreschleri* occurred in response to an uncharacterised chemotactic stimulant.

Calcium involvement in autoaggregation of *Phytophthora palmivora* was suggested on the basis that zoospore accumulation and autoaggregation around roots was reduced in the presence of the calcium channel blocker verapamil (Reid *et al.*, 1995). The precise role of calcium was unclear however, since zoospores of *Phytophthora* were unable to attract zoospores of three *Pythium* species tested. Were calcium the signalling molecule *per se*, it might be expected that a wide range of zoospores would respond. Amongst the plasmodiophorids, reference has been made to 'rafts' of *S.subterranea* f. sp. *subterranea* zoospores swimming slowly, which could be an example of autoaggregation (Harrison *et al.*, 1997).

Non-chemotactic host location

Chemotaxis is not always a significant factor in host location. Studies on the interactions between marine oomycetes (*Halophytophthora* spp.), on submerged leaves of red mangrove (*Rhizophora mangle*), led to the conclusion that water flow

was the major influence on host location, and not chemotaxis (Newell and Fell, 1992). This was based on the observation that zoospores accumulated on experimental leaf discs more readily in turbulent conditions, than when discs were sheltered from water flow. The authors conceded that an alternative explanation for the results might involve negative chemotaxis to tannins released by mangroves.

Factors other than chemicals are known to affect encystment of zoospores. Agitation was shown to induce encystment of *Phytophthora* zoospores (Ho and Hickman, 1967b). In *Spongospora subterranea* f. sp. *nasturtii* this appears not to be the case (Claxton, 1996). However, temperature is a factor which does influence encystment in *S. subterranea* f. sp. *nasturtii*, with it being more rapid and pronounced at 10°C, than at either 15°C or 20°C (Claxton *et al.*, 1995).

Electrotaxis

Plant roots generate electric currents (for example, see Miller *et al.*, 1991). Protons are responsible for transmitting the currents, which enter roots at the meristem and exit around mature root tissue (Miller *et al.*, 1991). Currents are generated by transmembrane potentials, which are important in pH regulation, transport of nutrients, turgor and wall plasticity regulation in cells. The current around growing root tips may have particular importance in regulation of root polarity (Miller *et al.*, 1991).

Electrotaxis of zoospores was observed when *Phytophthora palmivora* var. *nicotianae* was found to accumulate at cathodes (Troutman and Wills, 1964). Plant roots generally have field strengths in the region of 0.5V/m, rising to 2-5V/m around

wound sites, and many early studies incorporated electric fields in excess of this (Morris *et al.*, 1992). More recent work has incorporated field strengths of a physiologically relevant range. Zoospores of *Phytophthora palmivora* were demonstrated to be attracted toward the anode of an electrotaxis chamber in which care was taken to ensure that results were not due to electrolytic effects on pH, oxygen or chemical gradients (Morris *et al.*, 1992). At physiological field strengths, electrophoretic and electroosmotic drift of zoospores was not found to influence results either. Subsequently, *Pythium aphanidermatum* zoospores were shown to be cathodic, while two other *Pythium* species were anodic (Morris and Gow, 1993). The effect of the fields was seen to be on the frequency of turning of the zoospores, and the polarity effect was related to the charge on the flagella. It has been suggested that electrotaxis may work in conjunction with chemotaxis in host location, possibly enabling the distinction between living and dead host tissue.

Site of zoospore attachment on roots

Roots may be divided into several zones. A meristematic zone occurs at the tip, whilst behind this is the zone of elongation. Further back, root hairs develop (Taiz and Zeiger, 1991). Zoospores of *Phytophthora cinnamomi* were found to accumulate preferentially around the zone of elongation of avocado seedling roots (Zentmyer, 1961). Ho and Hickman (1967a) reported similar findings with *Phytophthora megasperma* var. *sojae*, as did Chi and Sabo (1978), with *Phytophthora megasperma* on alfalfa. Chi and Sabo (1978) found that zoospores were not attracted to root hairs, and very few encysted at the root apex. These findings may relate to chemical release

from the zone of elongation, and to an avoidance of rapidly dividing cells at the root apex, which could reduce ability to colonise. Wound sites are also known to attract zoospores, as demonstrated with *Phytophthora parasitica* var. *nicotianae* on tobacco (Dukes and Apple, 1961).

Goldberg *et al.* (1989) were unable to find a consistent zone of attraction in *Pythium dissotocum*. They found attraction to root caps of cotton and cucumber, root hairs and elongation zone in maize, and the whole root of lettuce. In the case of *Plasmodiophora brassicae* on cabbage roots, infection through both the root epidermis, and root hairs has been observed (Williams *et al.*, 1971). Root hairs are also sites of attachment in *Polymyxa betae* (Dahm and Buchenauer, 1993).

Host specificity

Specificity of attraction has rarely been demonstrated conclusively. Zentmyer (1961), reported that although zoospores of *Phytophthora cinnamomi* were attracted to avocado, they were not to tomato or tobacco. However, weaker attraction to plants including pea, was observed. *Phytophthora citrophthora* was attracted to citrus roots, but not avocado, implying some form of species specific interactions. Chi and Sabo (1978) found attraction of *Phytophthora megasperma* to alfalfa seedling roots, but not to non-host plants.

Goldberg *et al.* (1989) demonstrated specific attraction of *Pythium* species to cotton roots. A non-pathogenic species was not attracted. Both fungi responded to the same amino acids *in vitro*, however, and so the explanation of specificity may be due to the release of antagonists as well as attractants from roots. *Pythium catenulatum* (the

non-pathogen) responded to glutamic acid, but this was much reduced in the presence of background glycine or histidine, both non-attractants (Donaldson and Deacon, 1993a). *Pythium dissotocum* (the pathogen) was unaffected by them.

The glucosinolate-myrosinase system

Potential specific chemoattractants in watercress might include glucosinolates and their derivatives. Glucosinolates are thioglucosides, or sulphur-linked glucosides (Rodman, 1981), with a general structure as shown in Figure 1.4.

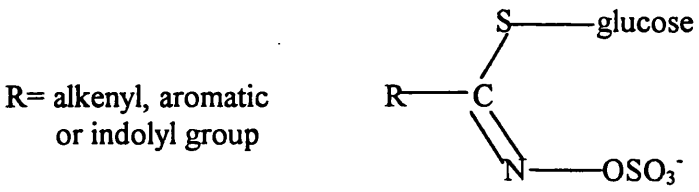


Figure 1.4 *General structure of a glucosinolate* (Rodman, 1981).

As many as 100 different glucosinolates are known (Halkier and Du, 1997), with the vast majority occurring in crucifers, where up to 400 species have been shown to possess them (Rodman, 1981). Every member of the Cruciferae thus far tested had proven positive for glucosinolates. Up to 15 different glucosinolates (differing in the side-chain) have been isolated from individual species.

Currently, glucosinolates are of interest scientifically, as potential anticarcinogens, including phenylethyl glucosinolate in watercress (Hecht *et al.*, 1999; Mithen *et al.*,

1998). Their benefits to the plant have largely been attributed to defence against pathogens.

When tissues are damaged, enzymes known as myrosinases are activated, which convert glucosinolates to volatile derivatives including isothiocyanates, thiocyanates, and nitriles, as shown in Figure 1.5 (Bennett and Wallsgrove, 1994).

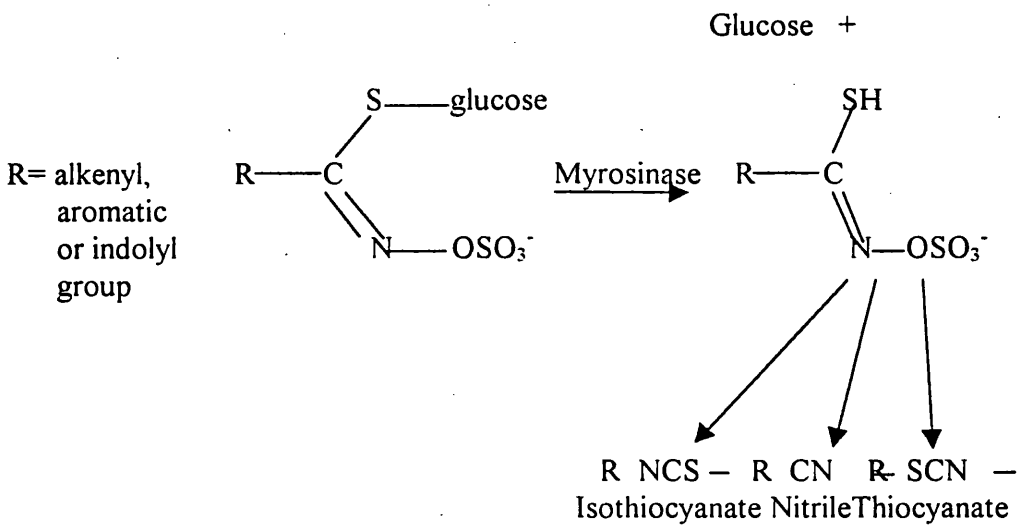


Figure 1.5 Glucosinolate breakdown via the enzyme myrosinase.

These derivatives have been shown to have activity against fungi and insects both in preventing infection, and as antifeedants (see for example Doughty *et al.*, 1996; Bennett and Wallsgrove, 1994).

In some cases, glucosinolates have been shown to act as attractants. In brassicas, for example, they are known to act as stimulants for oviposition in the root flies *Delia radicum* and *Delia floralis* (Baur *et al.*, 1996). Bennett and Wallsgrove (1994) report

on several studies implicating glucosinolates and their derivatives as inducing feeding behaviour in insects, and in attracting parasitoids of these pests.

The role of glucosinolates in susceptibility of brassicas to plasmodiophorids

Susceptibility of Chinese cabbage (*Brassica campestris* var. *pekinensis*) to *Plasmodiophora brassicae* has been shown to correlate with glucosinolate content (Ludwig-Muller *et al.*, 1997). Levels of indole glucosinolates increased after infection in susceptible varieties. The glucosinolate content correlated with development of clubroot symptoms, with higher content varieties showing greater symptoms. This may also be connected with jasmonic acid levels, which have also been shown to increase in clubbed roots of Chinese cabbage (Grsic *et al.*, 1999). Ludwig-Muller *et al.* (1996) found that glucosinolates did not correlate with actual initiation of infection, however, and evidence for glucosinolates as attractants of *P. brassicae* has not been presented.

The predominance in crucifers and their structural variability coupled with their volatility, and known roles both as attractants and repellents to pests and pathogens, make glucosinolates and their derivatives candidates as potential specific attractants of *Spongospora subterranea* f. sp. *nasturtii*.

Detection of plasmodiophorids

As plasmodiophorids are obligate biotrophs, it has not been possible to detect and quantify them by plate counts, as is frequently done for active cells in environmental samples (Amann *et al.*, 1995). Traditional methods for detecting plasmodiophorids

have utilised bait plants to attract pathogens. An example of this was reported by Flett (1983), where sterile tomato seedlings were incubated in bottles containing soil samples in solution. The soil was potentially contaminated with *S. subterranea* f. sp. *subterranea* resting spores. After 21 days, seedlings were killed, and root sections stained with Phloxine B to detect sporangia of *Spongospora.* The sporangia were distinguished from other plasmodiophorids by size and shape, although no other species or genera were observed. The major problems with such a method are that it requires considerable space and biomass, and that the method is time consuming.

A method for assessing over 100 samples in a day was developed for *Plasmodiophora brassicae* resting spores (Thornton *et al.*, 1991). This was based on an assay where chitin in resting spore walls was degraded by alkaline digestion, and the resulting N-acetylglucosamine detected colorimetrically. Although this method had the advantage of speed over baiting methods or direct microscopical observation (which often requires skilled identification), there were drawbacks. Firstly, host compounds interfered with readings to a small extent, and this effect was thought likely to vary between host species, resulting in slight underestimation of spore numbers. Secondly different strains of *P. brassicae* may differ in the chitin composition of their resting spores.

More recently, attention has turned to development of molecular detection techniques. Immunological detection has been investigated for both *S. subterranea* f. sp. *subterranea* and *P. brassicae*. In 1989, Lange and co-workers developed antisera to *P. brassicae*, which reacted to antigens on the surface of resting spores. This technique was found to be able to differentiate between *P. brassicae* and

Polymyxa, and was able to detect as few as 310 spores. Wakeham and White (1996) were able to detect as few as 100 resting spores using polyclonal antisera. It appeared that polyclonal antibodies were detecting components of the spore wall, which were released during sample preparation. If soils were probed directly using an enzyme-linked immunosorbent assay (ELISA), however, spores were not detectable.

Early work on antigenic detection of *S. subterranea* f. sp. *subterranea* had similar difficulties. Harrison *et al.* (1993) reported that as few as 0.08 spore balls ml^{-1}, could be detected from potato tuber extracts, but that unrealistically high numbers needed to be present in soil to allow detection. Similarly, antibodies raised against zoospores of *S. subterranea* were capable of detecting just 3 zoospores in an ELISA well plate, but soil samples were negative (Wallace *et al.*, 1995).

Walsh *et al.* (1996), were able to improve detection of *S. subterranea* spore balls in soil to a limit of 100 spore balls/ gram of soil. However, quantification was unreliable below 2000 spore balls per gram of soil. A bioassay based on bait plants was sensitive at lower spore concentrations, but was time consuming, and the multiplication of *S. subterranea* during the assay could not be ruled out.

Miller *et al.* (1989) used soybean leaf discs, rather than whole plants, in flooded soils to trap *Phytophthora megasperma* f. sp. *glycinea*, before carrying out ELISA.

In the Oomycetes, diagnostic kits have been developed for *Phytophthora* and *Pythium* (Werres and Steffens, 1994). These have been based on monoclonal antibodies, but still suffer from problems such as cross-reactivity, interference of fungicides, and a lack of correlation with biomass. An example of such a kit is provided by Ali-Shtayeh *et al.* (1991), whereby water samples containing

Phytophthora or *Pythium* were filtered through 0.45μm pores, heated for a few minutes, and the extracts then used in an ELISA test.

Generally speaking, antibody based techniques tend to encounter problems with specificity, sensitivity in the environment, and are often specific to particular stages of life-cycles (Ward *et al.*, 1994). DNA-based testing may overcome these drawbacks.

DNA-based detection systems

Early DNA-based diagnostic tests for micro-organisms were mostly for bacteria, and involved DNA-DNA hybridisation techniques (e.g. Sayler *et al.*, 1985), or the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987), followed by a DNA probing stage, such as dot-blotting (Steffan and Atlas, 1988). Studies on fungi followed rapidly, however. Oligonucleotide probes were used to detect strains and clones of filamentous fungi by DNA fingerprinting (Meyer *et al.*, 1991), whilst PCR was used to identify ectomycorrhizal fungi (Gardes *et al.*, 1991). Initial PCR-based studies tended to use degenerate primers to amplify DNA, with all possible base combinations present which would be required to make the amino acid sequence expected (e.g. Zehr and McReynolds, 1989). As DNA sequences became more widely available, specific oligonucleotide primers could be designed for well conserved DNA regions - the so-called universal primers (Olsen, 1988). Identification and separation of organisms using random primers, known as randomly amplified polymorphic DNA (RAPD's) has also proven successful in many studies. Möller and Harling (1996) used RAPD's to distinguish between European races of *Plasmodiophora brassicae*. RAPD analysis is frequently sensitive to biological

contaminants, however, and requires large amounts of template DNA for the PCR (Garbelotto *et al.*, 1996). Möller and Harling (1996) used purified, sterile, resting spores of *P. brassicae* for their work. RAPD's have proven useful as an initial step, prior to design of specific primers, based on RAPD differences seen (e.g. Schilling *et al.*, 1996).

An alternative approach has been to amplify sequences using designed primers, before cutting the products with restriction enzymes to generate band profiles on a gel. McKay and Cooke (1997) used this technique to amplify and cut a conserved region of the ß-tubulin gene in the silver scurf pathogen of potato, *Helminthosporium solani*. Benzimidazole resistant strains were identified by a point mutation in the codon sequence following PCR, and this was exploited using a restriction digest, such that only sensitive isolates had their DNA cleaved. In *Plasmodiophora brassicae*, this technique was reversed, in a study in which *Eco RI* was used to digest *P.brassicae* genomic DNA, before construction of a DNA library (Buhariwalla *et al.*, 1995). High copy number plasmid inserts of *P. brassicae* were identified by screening with sheared *P. brassicae* DNA. These were then used to design primers for PCR. A similar approach was used to obtain *Polymyxa betae* sequences (Mutasa *et al.*, 1993).

A technique has recently been developed whereby genomic DNA is enzymically digested, followed by selective PCR amplification of fragments, known as amplified fragment length polymorphism (AFLP) (Majer *et al.*, 1996).

Drawbacks of RFLP's include the requirement for several steps, which makes the process time-consuming (Garbelotto *et al.*, 1996). AFLP has proven to be quicker, reproducible, and covers the entire genome, but still has disadvantages including loss

of restriction sites, insertions, deletions, and different fragments of the same size are not accounted for (Majer *et al.*, 1996).

Randomly generating DNA sequence information or profiles may be problematic when dealing with obligate biotrophs such as *Spongospora*, in that host DNA is likely to be treated at the same time and so may complicate results.

The generation of specific PCR products removes this problem. To obtain specific primers it is often necessary to use more universal primers, followed by cloning and sequencing of products to separate organisms, or to compare infected and healthy host DNA profiles to find pathogen DNA (Chen and Chen, 1995).

Mutasa *et al.* (1993) obtained a 1.8kb fragment from *Polymyxa betae*, which was used to design specific primers for PCR detection of the pathogen in seedling roots. This was successful, but if infection levels were low, PCR products could not be viewed by conventional ethidium bromide (EtBr) stained agarose gels. One solution to this was to blot PCR products onto a membrane, and Southern probe with the original 1.8kb probe. Southern blotting is a time-consuming procedure however, and the authors devised a nested PCR approach, in which following an initial PCR with specific primers, a second set of primers was designed within this sequence. These were then used to reamplify from the low levels of PCR product initially obtained. Nested PCR has also recently been used to diagnose a phytoplasma present in New Zealand flax (Andersen *et al.*, 1998). This was achieved by PCR using universal primers for phytoplasma 16S ribosomal DNA, followed by PCR of these products using a second primer pair). Single-tube nested PCR was recently used to detect as little as one resting spore of *P. brassicae* in a gram of soil (Ito *et al.*, 1999).

A slightly simpler approach may be employed in which a sample from the original PCR is reamplified using the same initial primers (e.g. Pillai *et al.*, 1991). However, this strategy has often resulted in the presence of non-specific reaction products, and primer dimers (Pillai *et al.*, 1991). These artifacts are reduced by the use of a second set of primers (Mutasa *et al.*, 1995). Mutasa *et al.* (1996), were able to devise nested primers which could be used in the same reaction as the initial primers, and were able to detect *Polymyxa betae* DNA from as little as 1pg total genomic DNA from infected sugar beet roots.

PCR-based diagnostic tests have been applied to detecting single products from pathogens closely associated with host tissues. RoeckelDrevet *et al.* (1999), were able to develop specific oligonucleotide probes from one cloned polymorphic DNA fragment of *Plasmopara halstedii*. These were capable of amplifying DNA directly from sunflower (the host species) tissues. Using a similar approach, Wille *et al.* (1999) used genotype-specific primers flanking variable length loci to distinguish diagnostically *Epichlöe* endophytes within plant tissues.

Adaptation and extension of basic PCR methodology has resulted in a range of techniques for detecting DNA, including *in situ* PCR (e.g Johansen, 1997), subtractive hybridisation (e.g. Clapp *et al.*, 1995), and real-time PCR (e.g. Böhm *et al.*, 1999), which may allow increased sensitivity, specificity, or rapidity of sample analysis. Although the approaches described above are all capable of identifying specific DNA molecules of interest, diagnostic tests should ideally be quantitative. Rongsen and Liren (1997) reviewed major techniques of quantitative PCR. One method is limiting dilution of template DNA until no product is seen in a reaction.

This is then compared to an external standard based on amplification of a known amount of the same target. Another method involves use of an unrelated standard template in the same PCR reaction as the template of interest, with a different set of primers for each, in a series of reactions until the same intensity of product is seen with known and unknown starting templates. An alternative approach involves the production of a competitor DNA molecule, with the same primer binding sites as the unquantified template. The advantages of this system over the others are that the PCR templates are in the same reaction tube, and are affected equally by the PCR conditions, and that the need for multiple samples is removed. It has been suggested, however that this method can lead to the formation of heterodimers, and so some authors have preferred the use of heterologous competitors (e.g. Nicholson *et al.*, 1997). Examples of the design of homologous competitor method include Diviacco *et al.* (1992), and the use of such competitors include quantification of *Epichlöe* endophytes in grass tissue (Groppe and Boller, 1997).

Many of the above techniques have been applied to investigation of ribosomal DNA.

Ribosomal DNA

Ribosomal RNA genes and associated DNA have been frequently used for the detection of specific DNAs. A ribosomal DNA repeat unit from a typical eukaryote is shown in Figure 1.6. Such units are usually repeated tandemly in the genome for several hundred copies and in many organisms these repeat units exist in high copy number (Hillis and Dixon, 1991). Their usefulness is enhanced by the fact that different regions of the repeat unit evolve at different rates. The 16-18S (or small

subunit) rRNA gene is one of the most slowly evolving sequences known, and can be used when comparing ancient evolutionary events (Hillis and Dixon, 1991). The internal transcribed spacer (ITS) rDNA, on the other hand, has no known functional role, and evolves relatively rapidly, so that it can be used to separate closely related species, or even populations within a species.

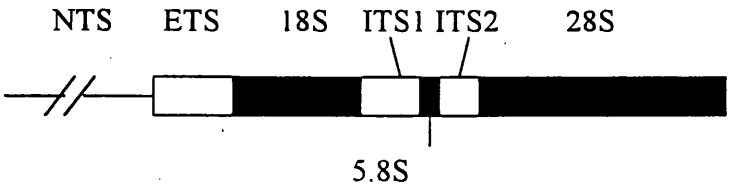


Figure 1.6 A typical eukaryote ribosomal DNA repeat unit (Hillis and Dixon, 1991).
NTS = non-transcribed spacer ETS = external transcribed spacer
ITS = internal transcribed spacer

As these spacer regions are flanked by conserved sequences (the rRNA genes) then the design of primers to amplify this region has been fairly straightforward, and a range of ITS primers have been used. Some are universal primers, such as ITS1, 2, 3, 4, and 5, capable of amplifying DNA from a wide range of species (White *et al.*, 1990). White *et al.* (1990) have also designed a range of primers to amplify small subunit rDNA (the NS primers).

As more sequence information has been obtained, more specific primers have been developed, such as the fungal specific primer ITS 1F, and the Basidiomycete specific primer ITS 4B (Gardes and Bruns, 1993). The divergent sequences exhibited by the ITS regions make them attractive targets for isolating specific species of interest from a mix of initial DNA samples.

The ITS region has been used to study plasmodiophorid sequences. Ward *et al.* (1994), used ITS5 and ITS4 to amplify *Polymyxa* isolates, before using restriction enzymes to digest the PCR products, and differentiating *Polymyxa graminis* isolates based on band profiles. Further sequencing of plasmodiophorid rDNA, including some of the 3' end of the small subunit gene, has enabled genus-specific *Polymyxa* and species-specific *P. graminis* primers to be developed (Ward and Adams, 1998). Amplification of *S. subterranea* f. sp. *subterranea* has been achieved using specific primers, following initial amplification of the ITS using more general primers (Bulman and Marshall, 1998). The authors found that PCR amplification was inhibited by humic acids from soil, when present in samples. The use of columns containing polyvinylpolypyrolidone (PVPP), dilution of samples, and the use of nested PCR helped to alleviate the problem. Bell *et al.* (unpublished.), have reported that treatment of soil samples with a bead beater, followed by cetyltrimethylammonium bromide, and then Sephadex and PVPP, yields DNA samples sufficiently pure for PCR. They have developed a quantitative PCR based approach, using a competitor template, for specific detection of *S. subterranea* f. sp. *subterranea* in soil samples.

The use of ribosomal DNA in phylogenetics

The variable rates of evolution shown by ribosomal DNA have proven useful in reconstructing the phylogeny, or evolutionary history, of organisms.

The ITS rDNA has been used to infer the phylogeny of closely related organisms. Examples include Wesson *et al.* (1993), who analysed populations of *Ixodes* species (some of which are carriers for Lyme disease). Using ITS rDNA, it was possible to

compare individuals between species, between populations, and within populations, and the authors were able to conclude that *I. scapularis* and *I. dammini* were not distinct from each other. Zambino and Szabo (1993) similarly analysed strains and *formae speciales* of *Puccinia* and found relationships contradicting some previous taxonomic studies. ITS rDNA phylogeny has also been used at the level of clustering species within genera, and separating or merging genera. Cooke and Duncan (1997), used ITS rDNA to examine the relationships between nine *Phytophthora* species. Kretzer *et al.* (1996) were able to delineate the Basidiomycete genera *Boletinus* and *Fuscoboletinus* from the genus *Suillus sensu lato* using ITS rDNA sequences.

As the small subunit rRNA gene evolves much more slowly than the ITS rDNA it has been used to determine the phylogeny of more distantly related groups of organisms, although the region has sometimes been variable enough to permit its use in determining species status, as demonstrated with *Lactobacillus* (Back *et al.*, 1996). Ahrén *et al.* (1998) used 18S rDNA to find the relationships between a range of nematode-trapping fungi, whilst Ward and Adams (1998) have used the 3' end of the gene to investigate plasmodiophorid phylogeny.

Other regions of the rDNA have been less widely used. The large subunit rRNA gene is more variable than the small subunit (Hillis and Dixon, 1991), and to date has mainly been used for investigating closely related taxa (Van der Auwera and De Wachter, 1998). The non-transcribed spacers (NTS) evolve more rapidly than the ITS (Hillis and Dixon, 1991), limiting their use to very closely related organisms, whilst the 5.8S gene generally has a combination of being invariable, but too short to provide useful phylogenetic information (Hillis and Dixon, 1991).

As well as the rDNA regions themselves, insertion sequences known as introns have been used to investigate molecular evolution. Those found in rDNA are of a type known as group I introns, which are capable of self-splicing (Cech, 1988). Introns have been shown to evolve independently from their host genomes, and can distribute themselves by horizontal transfer between organisms, a process which may be mediated by either reverse splicing or *via* action of site-specific homing endonucleases (Holst-Jensen *et al.*, 1999). The fact that they do not exhibit solely vertical transmission makes them invalid for inferring host phylogeny, but it is of interest that the only plasmodiophorid small subunit rDNA to be sequenced (*P. brassicae*), contained three such introns (Castlebury and Domier, 1998).

Methods for inferring phylogeny

The basic concepts and methodologies for analysing molecular evolution have been well reviewed (e.g. Page and Holmes, 1998; Swofford and Olsen, 1990).

The sequence of the DNA molecule to be analysed (e.g. 18S rDNA) is first obtained from a range of organisms, spanning the number of taxa required for the study (dependent on the depth of evolutionary history being examined). Many sequences can now be accessed from web-based databases, such as GenBank and the European Molecular Biology Laboratory (EMBL)..

These sequences are aligned, using both computer programs and manual judgement. For estimation of phylogenies, not all base positions within a sequence provide useful information. If all sequences share the same base at the same position, then this will provide no useful information about the variation between them. Sequence data can

also provide misleading information. Insertions and deletions, for example, require care, as one evolutionary event in which several bases are gained or lost, may result in a computer based alignment considering that each base that is present/absent represents a single, separate evolutionary event. Regions of DNA which do not align well are generally excluded from phylogenetic analyses, as the penalties for misalignment may be far greater than for non-alignment (Swofford and Olsen, 1990). Other considerations when building a picture of molecular evolution, include the ratio of transitions to transversions, the secondary structure of the molecule, site to site variation in evolutionary rate, and multiple evolutionary events.

Some models of nucleotide substitution assume that base changes occur with equal frequency (Jukes-Cantor model), whereas others such as Kimura's 2 parameter model take account of the fact that transitional changes are more common than transversions (Page and Holmes, 1998).

Ribosomal RNA forms complex 'stem and loop' structures which may affect the possibility of change of state for some nucleotide positions, leading to a site to site variation in rate of change (Kumar and Rzhetsky, 1996). The distribution of change in the small subunit rDNA was found to be gamma distributed with a size and shape parameter value equal to 0.76 (Kumar and Rzhetsky, 1996). This value may be incorporated into phylogenetic calculations.

Phylogenetic data is usually represented as a tree. These may either be rooted, with all species present descended from an ancestor at the base of the tree, or unrooted, with no fixed start time (Swofford and Olsen, 1990). Rooted trees are directional, with the direction corresponding to evolutionary time (Page and Holmes, 1998).

Trees may be built using several methods, which may use discrete or continuous input characters, and clustering (stepwise addition of sequences) or optimality (trees are given a score of 'fitness' to original data) criteria (Page and Holmes, 1998). Popular tree-based methods include parsimony (which has many variants), maximum likelihood, and distance based methods. The basis of the methods is indicated in Figure 1.7.

	Distances	Nucleotides
Clustering	**UPGMA** **Neighbour joining**	
Optimality	**Minimum evolution**	**Max. parsimony** **Max. likelihood**

Figure 1.7. An indication of some common tree-building methods, and the criteria on which they are based. Adapted from Page and Holmes (1998).

Parsimony produces a tree which involves the least amount of evolutionary change based upon the sequences supplied (Page and Holmes, 1998). It analyses each nucleotide position separately, and is a discrete, character based, method, using optimality criteria. Likelihood requires an explicit evolutionary hypothesis to be formulated beforehand, and then trees are produced, which are assessed for their likelihood of fitting the original data (Page and Holmes, 1998). It is also a discrete method, based on optimality criteria.

Distance methods treat the sequences as a whole. Many are clustering methods. Firstly a pairwise distance matrix is produced based upon overall differences between

two sequences chosen as a starting point (see Figure 1.8.). Then these are tested against the next sequence, and the differences scored, and so on. From this matrix of evolutionary differences between sequences, an evolutionary history can be constructed as a tree. There are several methods of building distance related trees, such as neighbour-joining, Fitch-Margoliasch, and Unweighted Pair Group Method with Arithmetic Means (UPGMA). Some of these methods assume a molecular clock (e.g. UPGMA), which means that all sequences evolve at equal rates, whereas others (e.g. neighbour joining) do not.

All the methodologies described have individual strengths and weaknesses, and are all reliant on receiving accurate input data. Trees which are produced by these methods may be checked for sampling error by a variety of methods, of which the bootstrap is the most widely used. Bootstrapping works by sampling randomly with replacement from sites within the whole sequence length being analysed (Page and Holmes, 1998). Each round of sampling (the number of which is defined by the user), results in a tree, and the percentage of trees on which a particular divergence of species occurs is the bootstrap value for that split. Values of greater than 70% are generally accepted as being significant (Lake and Moore, 1998).

Many computer packages are available for phylogenetic reconstruction. Among the most popular are PAUP (phylogenetic analysis using parsimony) and PHYLIP [Phylogeny Inference Package] (Lake and Moore, 1998).

sequences *distances*

sites

	1	T A A A A A A				
	2	T T A A A A T			2	2
sequences	3	A A A A A A A	sequences	3	1	3
					1	**2**

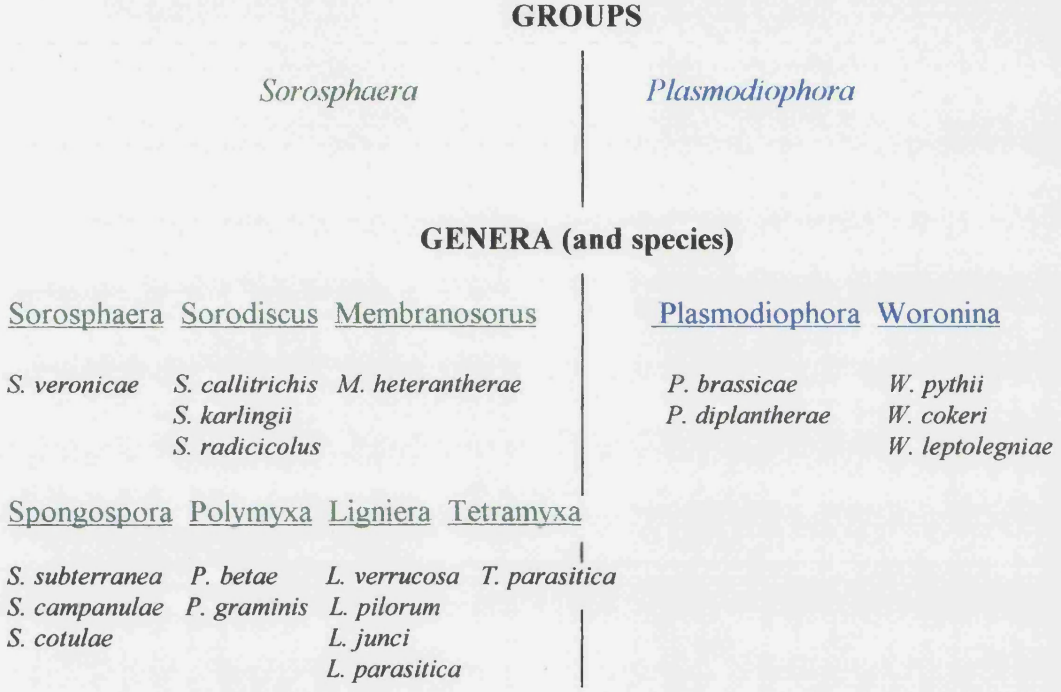
Figure 1.8. The three sequences on the left-hand chart are converted in distance values on the right. For instance, at the first nucleotide position, sequence 1 and 2 are identical, and score 0 difference, whereas 1 and 3, and 2 and 3 differ, and hence score 1. The cumulative score is indicated in the distance matrix. Note that this example does not need to account for a transition/transversion ratio. Adapted from Page and Holmes (1998).

Taxonomic status of the plasmodiophorids

The defining character of the plasmodiophorids is cruciform nuclear division (Braselton, 1995; Buczacki, 1983). This has been described as "a type of mitotic division in which a persistent nucleolus is elongated perpendicularly to the metaphase plate of chromatin, and end-to-end centriolar pairs occur at each pole" (Braselton, 1995).

Genera within the plasmodiophorids have been distinguished on the basis of number and morphology of resting spores in groups [sporosori] (Braselton, 1995), and more recently, by ultrastructural examination of host-parasite boundaries, chromosome numbers (karyotypes), and other nuclear characteristics, such as synaptonemal complex length, and definition [clarity] (Braselton, 1995, 1992, 1990, 1983; Bryan *et al.*, 1996; Robbins and Braselton, 1997; Braselton and Miller, 1975).

Based on such characteristics, a splitting of the plasmodiophorids into two groups has been proposed (Braselton, 1995). The *Plasmodiophora* group contains this genus and *Woronina*, whilst the *Sorosphaera* group includes all other genera. The known genera (10) and a sample of species (35 are recognized), are listed in Figure 1.9.



The final genus, Octomyxa has not been isolated for many years (Braselton, 1998), but *O. brevilegniae* was described by Pendergrass (1950).

Figure 1.9. The groups (as suggested by Braselton (1995)), genera and selected species of plasmodiophorids.

Ward and Adams (1998), were able to recognise *Polymyxa* and *Ligniera* as distinct taxa, using pairwise distance methods applied to the ITS rDNA, but relationships with other plasmodiophorids were unclear.

The wider taxonomic status of the plasmodiophorids is less certain. DeBary (1884), considered that they were protozoans, whilst Karling (1968) placed them with the

lower fungi. Barr (1983), reviewed the taxonomic status of a range of taxa, and considered that the plasmodiophorids most probably had a recent protozoan ancestor. Analysis of the NS7-NS8 region of the small subunit rDNA using both distance and parsimony resulted in plasmodiophorids grouping separately from both fungi and protozoans (Ward and Adams, 1998). Castlebury and Domier (1998) tested the full 18S rDNA sequence of *Plasmodiophora brassicae*, using likelihood, distance, and parsimonious techniques. There was found to be little relationship with the true fungi (Chytridiomycetes, Zygomycetes, and higher fungi), and the authors concluded that the taxonomic grouping should be with the protists, although an exact lineage was unclear. Ward and Adams (1998) found that database searches (EMBL and GenBank), for comparisons with *Polymyxa* rDNA, matched the DNA most closely with Oomycetes and diatoms.

Aims of the project

It was thought possible that zoospores of *Spongospora subterranea* f. sp. *nasturtii* might show taxis to host specific root exudates. Such attractants could be used to develop alternative control measures for crook root disease. One aim of the project was to investigate this possibility. Even if no specific attractant were occurring, it would be desirable to test possible replacement chemicals for zinc, as crook root control agents.

Due to concerns over the use of zinc, it is desirable to rationalise its use. A project aim was to develop a diagnostic, PCR-based test for *S. subterranea* f. sp. *nasturtii*.

Levels of *S. subterranea* in watercress beds could then be calculated and used to determine whether addition of zinc would be necessary.

A final aim of the project was to use molecular phylogenetic techniques to understand better the relationship between *S. subterranea*, the plasmodiophorids generally, and other organisms.

2. Chemotactic responses of *S. subterranea* f. sp. *nasturtii* zoospores and investigations into potential replacements for zinc as a control measure

2.1 MATERIALS AND METHODS

Growth and collection of plant material

Healthy and diseased watercress plants were harvested from beds at Hurd's Spring Valley Watercress farm, near Warminster (ST 868405). Crooked and healthy roots were removed manually, transported to the laboratory in autoclave bags, and either used immediately, or stored at -20°C for molecular work.

All other plant species used were grown from seed, in Levington C2 compost, under glasshouse conditions (16 hour daylength, 20°C). Plant roots were removed after six weeks for immediate use in subsequent experiments.

Preparation of root extracts for chemotaxis experiments

Extracts were prepared using a method based on Claxton (1996). Roots of healthy appearing watercress, and roots of glasshouse grown species, were washed under tap water, and then ground to a powder in $N_2(l)$ in a pestle and mortar. The fine powder was transferred to a $25cm^3$ Universal, and its mass ascertained. MilliQ water was then added such that 1ml of water was used for each gram of powder. The root and solvent mixture was incubated at 37°C, and 100rpm (Gallenkamp orbital incubator), for 2 hours, followed by 5 minutes on ice, before being filtered through muslin. The filtrate was retained, and stored at 5°C until required, in a sealed Universal.

Root extracts were mixed in a 1:1 ratio with 2% low melting point agarose (SeaPlaque agarose®, FMC BioProducts), which had been cooled to less than 50°C beforehand.

Design of chemotaxis assay chambers

Equipment for analysing chemotaxis has generally involved setting up potential chemoattractants in capillary tubes, which are then placed in contact with suspensions containing the organism being studied (see Adler, 1973; Khew and Zentmyer, 1973; Palleroni 1976).

The chambers in this study were based on a modified version of Khew and Zentmyer (1973). One end of 2µl diameter capillaries (Camlab) were placed into an agarose and root extract mixture, and the liquid gel taken up until it filled the capillary tube. Control capillaries were filled with 2% low melting point agarose only.

Chemotaxis chambers were designed as in Figure 2.1. Shallow Petri dishes, 5cm in diameter or their lids, were modified by cutting four grooves in the sides. Each of these was placed within the base of a larger, 9cm diameter Petri dish, the base of which was lined with damp paper towels, to maintain humidity during experiments. A 22mm^2 glass cover slip was placed in the centre of the shallow Petri dish, and 300µl of a 1 x 10^5 ml^{-1} zoospore suspension pipetted onto it. One end of a capillary tube containing root extract was placed in this suspension, with the other end resting in the grooves cut into the shallow Petri dish. On some occasions, several tubes containing different root extracts were used in one such dish. A control tube containing agarose only was placed in all dishes. Distances between mouths of

capillaries were difficult to control precisely, but tubes were kept about 1 cm apart to reduce interference of potential chemoattractants. Once capillary tubes were in position, a second cover slip was placed on top of the suspension, and the assay chambers stored at 5°C until taxis was assessed.

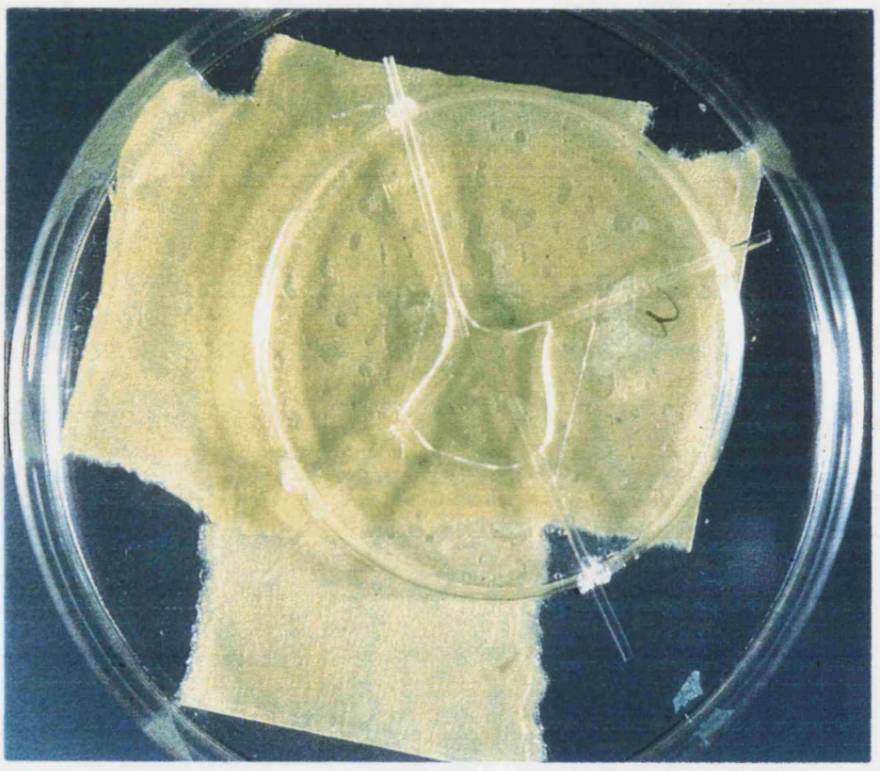


Figure 2.1 Chemotaxis assay chamber for *S. subterranea* f. sp. *nasturtii*.

Preparation of zoospore suspensions from crooked roots

Diseased watercress plants were rinsed in tap water, and crooked roots removed. These were placed in 9cm Petri dishes, containing 10ml nutrient solution (Table 2.1). Dishes were covered with laboratory film (Parafilm M), and stored at 5°C in darkness.

The number of zoospores released from these roots was assessed daily from 72 hours onwards until at least 2 x 10^5 ml^{-1} were obtained. Assessment of numbers was carried out by taking five replicate samples of 40µl from each dish, and counting zoospores using a haemocytometer. Zoospores were diluted using nutrient solution if the concentration exceeded experimental requirements.

Measurement of zoospore taxis to capillary tubes

After a set period of time, dependent on the experiment being carried out, the shallow Petri dishes were removed from assay chambers, and viewed under a x10 or x20 objective lens (Olympus BH-2 microscope). Zoospores which had entered the mouths of capillary tubes, and which had either encysted or were still motile, were counted, and recorded.

TABLE 2.1 Nutrient solution composition (Arnold *et al.*, 1994)

Macronutrients

Element	Chemical Compound	Stock solution ($g\ l^{-1}$)	Nutrient solution ($mg\ l^{-1}$)
N	$NaNO_3$	30.35	10.0
P	$NaH_2PO_4.2H_2O$	12.59	5.0
K	KCl	4.68	5.0
Ca	$CaCl_2.2H_2O$	18.33	10.0
Mg	$MgCl_2.6H_2O$	8.36	2.0

Micronutrients

Element	Chemical Compound	Stock Solution ($g\ l^{-1}$)	Nutrient Solution ($mg\ l^{-1}$)
B	H_3BO_3	0.0286	0.01
Cu	$CuSO_4.4H_2O$	0.002	0.001
Mn	$MnSO_4.H_2O$	0.0406	0.02
Mo	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0009	0.001
Zn	$ZnSO_4.7H_2O$	0.011	0.005
Fe	$C_{10}H_{12}O_8N_2FeNa.H_2O$	3.286	1.0

To create nutrient solution from stocks, such that composition resembles watercress bed water (pH 7.0), 2ml of both micromutrient and macronutrient stock were added to 1 litre of distilled water.

Attraction of zoospores to root extracts over time

The time taken for zoospores to respond chemotactically to root extracts was examined. Root extracts were prepared from watercress, tobacco (*Nicotiana rustica*), tomato (*Lycopersicon esculentum*), and mustard (*Brassica napus*). These were mixed in a 1:1 ratio with low melting point agarose, and placed in chemotaxis chambers, each chamber containing one capillary tube with extract in, and a control capillary. Attraction to these extracts was recorded at 4, 8, 12, 24, and 48 hours after setting up. At least 10 replicates of each treatment were made.

Investigation of potential attraction to leaf extracts

Plant leaf extracts were prepared in the same manner as root extracts, and were tested for ability to attract zoospores. Watercress root extract was tested at the same time, and recordings made after 6, 24, and 48 hours.

Effects of incorporating multiple root extracts in single assay chambers

An experiment was set up in which more than one root extract was used in each assay chamber. This involved setting up two or more capillary tubes per chamber, plus an agarose control tube. Some chambers incorporated two extracts, and some three (mustard was not tested). All possible permutations of watercress, tobacco, and tomato were tested. Additional controls, using just one root extract per chamber were set up for comparison of results. Counts were made after 6, 24, and 48 hours.

Preparation of a dilution series of root extracts

Root extracts were prepared as previously described. Once filtered through muslin, they were diluted in MilliQ water to create final extract:water ratios of 1:1, 1:10, 1:100, 1:1000, or 1:10 000. Extracts of watercress, tobacco, tomato, and mustard roots were tested for ability to attract zoospores at each of these dilutions, in assay chambers, with root extracts from all species in single chambers. Agarose control tubes were incorporated as previously described. Chambers containing only watercress extract at the appropriate concentration were set up to compare use of single, and multi-extract chambers. Chambers with watercress extract at 1:1 dilution with water were also examined in direct comparison to chambers containing diluted extracts. Zoospores were counted at 6, 24 and 48 hours after set up. Replicates for each treatment numbered between 15-20.

Measurement of zoospore attraction to intact roots

Roots of watercress, tobacco, mustard, and tomato plants were excised approximately 3cm behind the tip. These were then placed in chemotaxis assay chambers, replacing capillary tubes. Empty capillaries were used as controls.
In some experiments one plant species only was used per chamber, with at least five replicate chambers. In others, two roots of different species were used to observe whether specific attraction to one species would take place. The combination investigated was watercress with tobacco, with 10 replicates. Assay chambers were stored at 5°C and counts were made after 6 and 24 hours. 250μm segments were counted, starting at the root tip, and working backward for 2.5mm, or until root hairs obscured viewing.

Attraction of zoospores to watercress bed water

50ml samples of water were collected from the outflows of diseased and healthy appearing watercress beds. Samples were also collected from bed inlets. These were used to fill 2μl capillary tubes in a 1:1 mixture with low melting point agarose. MilliQ water was mixed with agarose as a control. Chemotaxis assays were set up as described previously. Counts were made after 3 and 24 hours, on at least 10 replicates.

Attraction of zoospores to chemicals released from intact roots

Healthy, single watercress plants were removed from watercress beds, and placed in 50ml plastic tubes containing nutrient solution. Water samples (1ml) were removed at

1, 3, 6, 12, 24, 48, and 72 hours after set up. These samples were mixed 1:1 with low melting point agarose, and used in chemotaxis assays. Nutrient solution was mixed with agarose to form a control. Counts of zoospores were made after 3, 6, and 24 hours, using at least 10 replicates.

Effects of organic molecules on zoospore taxis and encystment

1µl capillaries were filled with 20mM solutions of various amino acids (methionine, glutamine, gluatamic acid, aspartic acid, alanine, asparagine, lysine), and sugars (galactose, sucrose, glucose). Pectin was used at 1.8mg ml^{-1}, and polygalacturonic acid at 360µg ml^{-1}. The pH of the solutions was recorded. The capillaries were placed in taxis chambers containing 200µl of 3 x 10^5 ml^{-1} zoospore suspension, and attraction was recorded after 2 hours. 20mM solutions (pectin and PGA as above), were used in repli-plates to assess encystment within a 200µl suspension containing 3 x 10^5 ml^{-1} zoospores in wells of plastic repli-plates. Percentage encystment (non-motile cells) was estimated after 2 hours, and compared to that in plates containing nutrient solution or MilliQ water only.

Effect of initial zoospore density on taxis and encystment

To assess the possible effect of initial zoospore concentration on zoospore taxis, a range of zoospore densities was prepared, from 1 x 10^4 ml^{-1}, to 4 x 10^5 ml^{-1}. Watercress root extract (at a 1:1 ratio with water) was prepared, and zoospores placed in identical chemotaxis assay chambers, with zoospore concentration the only variable. Counts of attraction to extracts were made after 6 and 24 hours, from 8

replicates. In addition, 40µl samples of suspension were removed at these times, and counts of total cell number, and percentage of non-motile spores, were made from 4 replicates.

Effects of zinc on zoospore taxis, encystment, and lysis

The aim of this experiment was to determine how zinc affects zoospores, and at what concentrations. Solutions of zinc sulphate ($ZnSO_4.7H_2O$) at a range of concentrations were prepared, along with solutions of zinc chloride ($ZnCl_2$), and zinc acetate ($(CH_3COO)_2Zn.2H_2O$). These were added to 1 x 10^5 ml^{-1} zoospore suspensions in chemotaxis chambers containing watercress root extract (1:1), such that the final concentration of zinc ranged from 0.025µg ml^{-1} to 10µg ml^{-1}. Zinc acetate and zinc chloride, were used at 1µg ml^{-1} of zinc only. Control chambers contained no zinc. Assessments of zoospore attraction, total cell number (viability), and percentage motility were made as previously described, after 6 and 24 hours.

Zinc pulsing effects on development of crook root and zoospore survival

In order to assess the frequency at which it is necessary to apply zinc as a control measure, zinc pulsing tests were performed. Watercress plants were grown from sterile seed (English Dark Green Watercress, collected in 1992), by sowing directly into nutrient solution (Table 2.1). After 2 weeks, seedlings were transferred to 50ml test tubes containing 45ml nutrient solution. 5ml of 2 x 10^5 ml^{-1} zoospore suspension was added to this. A pulse of 0.075µg ml^{-1} zinc was supplied after a set time (0, 30, 60, 120, 180, or 240 minutes), for a 15 minute period to simulate the situation in the

field. After this, seedlings were washed, and floated in 50ml fresh nutrient solution. Plants were incubated at 10°C in a Fisons 600G3/THTL Growth cabinet with 8 hours daylength for 7 days. Nutrient solution was then changed, and plants incubated for a further 7 days before crook root symptoms were assessed visually. Ten seedlings were assessed for each pulse time. Counts of zoospores present in the nutrient solution were also made at this time.

Zoospore motility and viability in response to cations

Zoospore encystment was investigated in the presence of a range of cations (copper, potassium, calcium, lanthanum, ammonium, sodium, zinc, magnesium, manganese, lithium, caesium, and cobalt). To determine encystment, 1ml of test solution containing a cation at 0.5, 5, or 50mM was added to 200µl suspension containing 3 x 10^5 ml^{-1} zoospores in wells of plastic repli-plates. Percentage encystment (non-motile cells) was estimated after 2 hours, and compared to that in plates containing nutrient solution or MilliQ water only.

Viability of zoospores was assessed by pipetting 2ml of zoospore suspensions containing 2 x 10^5 ml^{-1} zoospores into 5cm diameter Petri dishes. To these was added one of the following solutions in which the final cation level was 2µg ml^{-1}.

Manganese ($MnCl_2.4H_2O$)

Manganese ($MnSO_4.7H_2O$)

Ammonium (NH_4NO_3)

Aluminium ($AlK(SO_4)_2.12H_20$)

Cobalt ($CoCl_2.6H_2O$)

Zinc ($ZnSO_4.7H_2O$)

Controls were nutrient solution only. All dishes were stored in darkness at 5°C. After 2 hours, the number of zoospores remaining was estimated by counting zoospores using a haemocytometer. Fifteen replicates were examined for each treatment.

Effects of cobalt on zoospore taxis, encystment, and lysis

The effects of cobalt on zoospores compared to zinc, was investigated further in order to ascertain whether cobalt might be as effective or more effective than zinc against zoospores of *S. subterranea* f. sp. *nasturtii*.

1×10^5 ml^{-1} zoospore suspension was added to chemotaxis chambers supplied with watercress root extract at 1:5 ratio (w/v) with water. Cobalt (as cobalt chloride) or zinc (as zinc sulphate) were incorporated at 2µg ml^{-1} or 4µg ml^{-1} of the cation. Control chambers had nutrient solution only. Zoospore attraction was counted after 3 and 24 hours, from at least 10 replicates.

Effects on zoospore viability were assessed at concentrations ranging from 0.5µg ml^{-1} to 8µg ml^{-1} of cobalt or zinc, by adding appropriate amounts to Petri dishes containing zoospores, as detailed earlier for a range of cations. Counts were made after 2 hours, with the proportion of non-motile cells also recorded, and at least 15 replicate dishes were used.

In some dishes, a combination of cobalt and zinc ions at up to 1μg ml^{-1} each were supplied in an attempt to ascertain whether synergistic effects occurred in affecting zoospore number.

Statistical analysis of data

The significance of differences in attraction between treatments (such as different root extracts) was tested using Tukey's honest significant difference test (Snedecor, 1956). This test assesses the significance of differences between multiple means, on a pairwise basis, based on ANOVA. Results were considered for significance at the 95% conficence interval.

Some data, such as the relationship between zoospore behaviour and initial density, was also subjected to correlation analysis. Analysis was carried out using Minitab 12 for Windows.

2.2 RESULTS

Zoospore attraction to plant root extracts

The species chosen for testing as root extracts were selected after preliminary tests on a wide range of species, which implied that tobacco root extract might be more attractive to zoospores than watercress, and tomato less attractive. Mustard is a brassicaceous plant like watercress, tomato is a host for *Spongospora subterranea* f. sp. *subterranea*, whilst tobacco is from the Solanaceae and is not a known host for *Spongospora* (Claxton, 1996).

Root extracts were more attractive than agarose controls, although *Spongospora subterranea* f. sp. *nasturtii* zoospores showed no specific attraction to watercress root extract at any time-point tested over a 48 hour period, when root extracts were supplied as a 1:1 mix with low melting point agarose (see Figure 2.2). Although tobacco appeared to be the most attractive root extract, and tomato the least, the data were quite variable, and no significant difference could be found at the 5% significance level.

Spongospora zoospores were 3-4μm in length, which is in agreement with the 2.4-6μm described by Tomlinson (1958b), and were slightly ovate. Encysted zoospores appeared to be more spherical, with a darker, clearly defined outline, presumably the cyst wall.

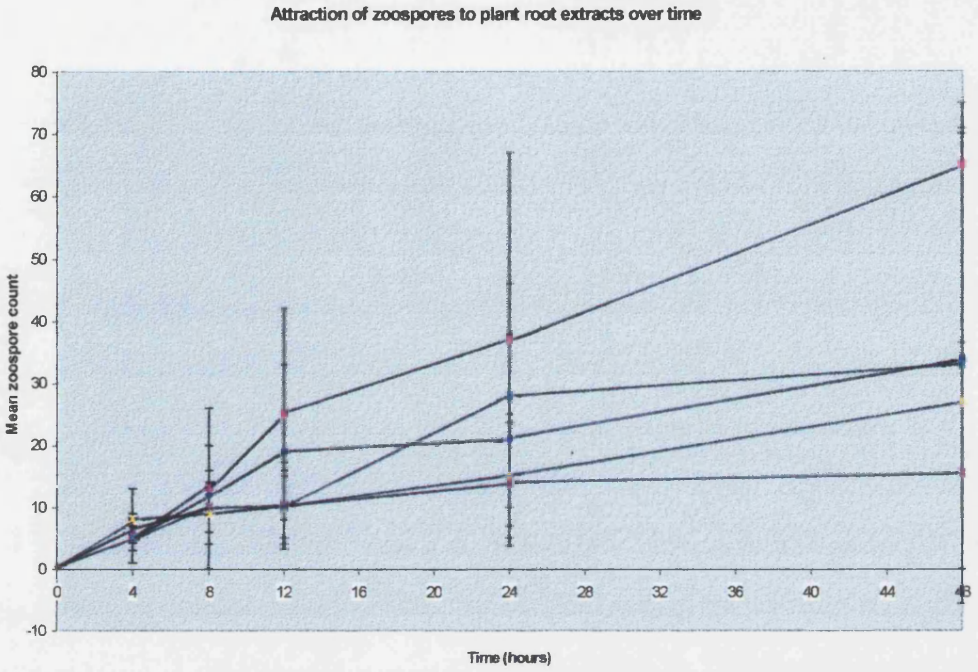


Figure 2.2 Mean attraction of zoospores to plant root extracts mixed 1:1 with agarose, over time. Results are means of at least 10 replicate counts. Error bars indicate standard deviation from means.

The number of zoospores attracted increased over time, and relationship with the x-axis describes a curve, with the rate of zoospore attraction decreasing over time. Zoospore counts were not made before 4 hours, due to time constraints when setting up many replicate dishes. Counts were not made after 48 hours, due to desiccation of zoospore suspension.

Investigation of attraction of zoospores to leaf extracts

To attempt to determine if root extracts were attractive due to a root-specific compound, extracts from leaves were investigated.

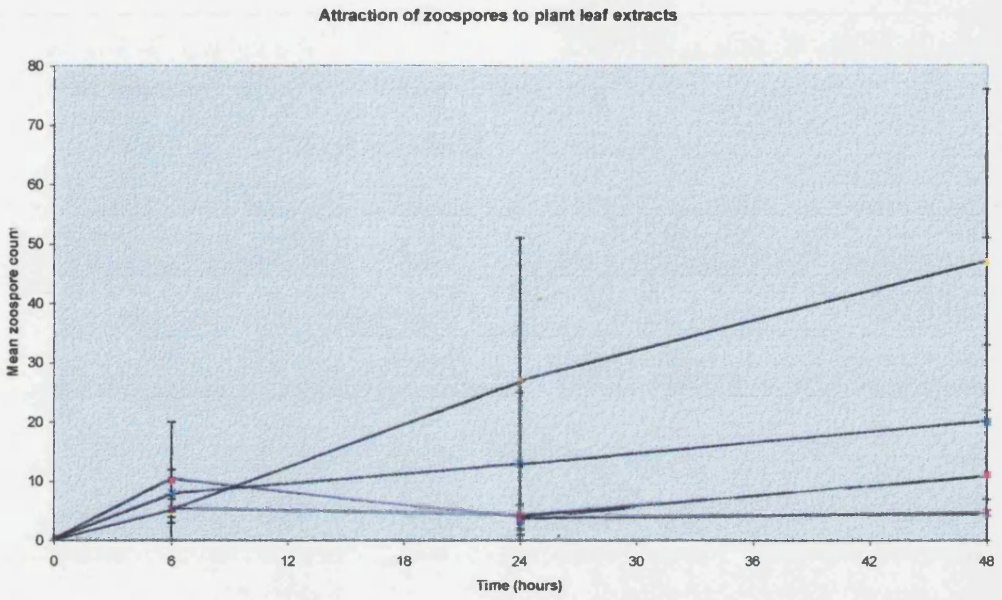


Figure 2.3 Mean attraction of zoospores toward plant leaf extracts mixed 1:1 with agarose, over time. Results are means of 10 replicates. Error bars indicate standard deviation from the mean.

Figure 2.3 indicates that leaf extracts appear to attract zoospores of *Spongospora subterranea* f. sp. *nasturtii* as much as root extracts. Both watercress root extract and watercress leaf extract attracted a mean of 20 zoospores after 48 hours. Attraction to root extracts was seen largely within the first 24 hours however, whereas with watercress leaf extract little attraction was seen until after 24 hours. This was not true of tobacco leaf extract which attracted zoospores steadily throughout, and was significantly more attractive than watercress leaf extract after 24 hours. Tomato leaf extract was relatively unattractive to zoospores. Leaf extracts were more attractive than agarose controls, indicating something within the extract is acting as a chemoattractant.

Zoospore attraction to root extracts in assay chambers containing many different extracts

Results presented thus far describe attraction to extracts when only extracts from one plant species was present. Chambers containing multiple extracts were set up in an attempt to ascertain whether zoospores would show preferential taxis to one plant species over another.

The results are presented in Tables 2.1 and 2.2, and in Figure 2.4. Some root extracts appeared to be more attractive than others, but the differences were not significant, or consistent between combinations.

At 48 hours, watercress was more attractive than tobacco and tomato extracts, but less attractive than tobacco when tomato extracts were omitted from the taxis chambers. Where watercress and tomato extracts were used in combination, in

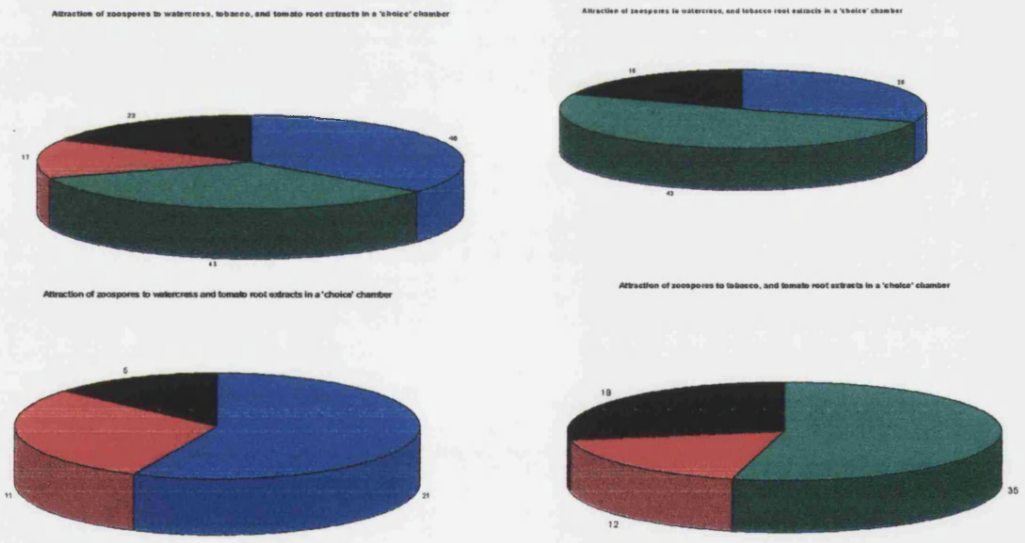
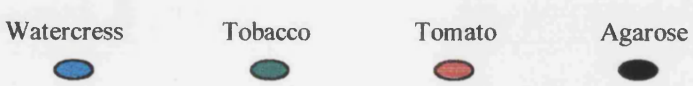


Figure 2.4 Diagrammatic representation of the proportion of zoospores attracted to root extracts after 24 hours in 'choice' chambers. Numbers on the pie charts indicate number of zoospores attracted to that extract. Results are means of 15-20 replicates.



the absence of tobacco, watercress was significantly more attractive than agarose after 24 and 48 hours, as was tomato at 48 hours, when tested at the 0.05 significance level using Tukey's comparison of multiple means. Agarose was not significantly less attractive than root extracts in other combinations tested.

In conclusion it appears that no specificity to watercress is demonstrated.

TABLE 2.1 Mean zoospore attraction to root extracts mixed 1:1 with agarose in a chamber containing a range of extracts. Results are means of 15-20 replicates, and are taken from counts after 6 hours.

Watercress	Tobacco	Tomato	Agarose
21	24	12	17
19	23	--	9
12	--	14	10
--	39	10	16

TABLE 2.2 Mean zoospore attraction to root extract mixed 1:1 with agarose in a Chamber containing a range of extracts. Results are means of 15-20 replicates, and are taken from counts after 48 hours.

Watercress	Tobacco	Tomato	Agarose
51	41	21	20
23	42	--	9
27	--	20	6
--	40	22	14

Attraction of zoospores to root extracts diluted in water

To determine if highly concentrated root extracts contained levels of general attractants such as amino acids, which might mask specific attraction, dilution series of extracts were prepared.

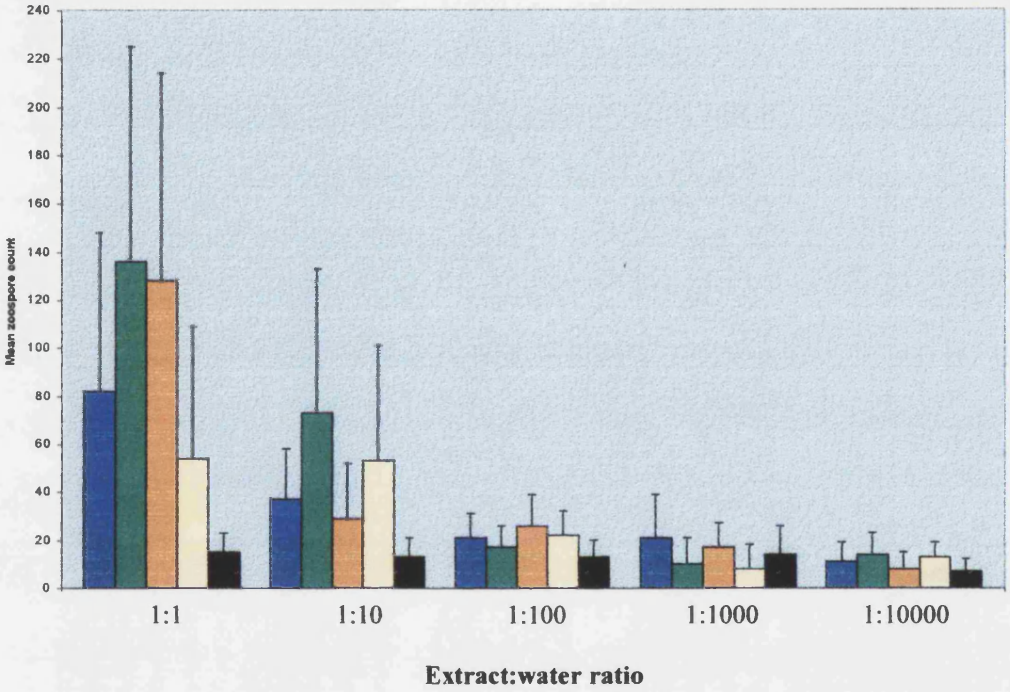


Figure 2.5 Attraction of zoospores to root extracts at a range of concentrations. Each series of bars represents a concentration of extract, while each bar within a series identifies the plant species. Results are means of 15-20 replicates, and are taken from counts after 24 hours. Error bars indicate standard deviation from the mean.

Watercress Tobacco Tomato Mustard Agarose

Figure 2.5 illustrates that no specificity to watercress root extracts was seen at any concentration at which root extracts are supplied. Watercress was never significantly more attractive than other species tested (Tables 2.3 and 2.4), except when compared to tobacco and mustard at 1:1000 dilution, after 24 hours. Tobacco was frequently

the most attractive root extract tested at higher concentrations, being significantly more attractive than tomato at 1:10 and 1:100 dilutions, and more so than watercress at 1:10 dilution. Diluting root extracts appeared to decrease chemotaxis of zoospores - i.e. taxis was dose dependent. When root extracts were diluted 100-fold or more, they were no longer more attractive than agarose controls, indicating that chemotactic factors had passed below a threshold level at which they were effective. Table 2.4 re-emphasises that there was rarely a significant difference between attraction of zoospores to extracts diluted 10 times than to extracts more dilute than this. The fact that dilution of extracts does have a real effect on zoospore taxis, was shown by the comparison of watercress root extracts at 1:1 with water, with more dilute watercress root extracts, when set up in the same experiments (Table 2.5). Significant differences were seen using Tukey's test at the 0.05 significance level.

TABLE 2.3 Significant differences between zoospore attraction to root extracts at comparable dilutions, at the 0.05 significance level, when means of all treatments were compared. ">" = "more attractive than". Tob= tobacco, Tom = tomato, Wat = watercress, Mus = mustard.

Dilution factor (extract:water)	6 hours	24 hours	48hours
1:1			
1:10	Tob > Tom	Tob > Tom Tob > Wat	
1:100		Tom > Tob	
1:1000		Wat > Tob Wat > Mus	
1:10000			

TABLE 2.4 Significant differences in zoospore attraction to root extracts of a given species dependent on dilution factors in water as indicated by the figures in the chart (1 = 1:1, 10 = 1:10 etc). The left hand number in each case is the more attractive concentration). Means of all dilutions were compared statistically, and significance limits set at 0.05.

Extract	6 hours counts	24 hours counts	48 hours counts
Watercress	1>10	1>10	
	1>100	1>100	1>100 10>100
	1>1000	1>1000	1>1000 10>1000
	1>10000	1>10000	1>10000 10>10000
Tobacco	1>10		
	1>100	1>100	
	1>1000	1>1000	
	1>10000	1>10000	
Tomato	1>10	1>10	
	1>100	1>100	
	1>1000	1>1000	1>1000
	1>10000	1>10000	
Mustard	1>10		
	1>100		
	1>1000	1>1000 10>1000	
	1>10000	1>10000 10>10000	

Incorporating more than one extract into a chemotaxis chamber, was shown to be a valid technique, as attraction to watercress extracts in chambers containing multiple root extracts, was not significantly different to attraction to watercress extract alone, at comparable dilutions. The major conclusion from this experiment is that it appears that no specific attractant is operating in watercress.

Attraction of zoospores to intact roots

Root extracts represent an artificial system, and it was necessary to confirm data obtained, by investigating zoospore attraction to intact roots.

Zoospores were observed to encyst on the surface of roots, in the same manner as with root extracts. Counts at any 250μm segment were generally comparable to, or lower than those to root extracts in capillary tubes, where counts had been over distances of less than $5\mu m^3$. This indicates that root extracts were a much more concentrated source of chemoattractants.

No particular region of roots was significantly more attractive than others (see Figure 2.6 for example), in any species tested. Watercress was not shown to be more attractive than other species tested, either separately (Table 2.5), or in combination in a single assay chamber (Table 2.6). Attraction to roots was greater than to control capillaries, indicating that roots are releasing signals which zoospores perceive.

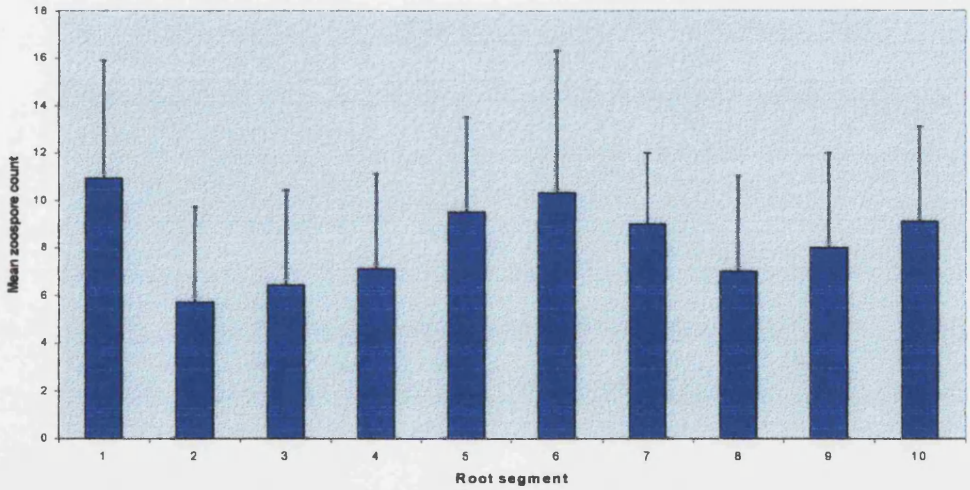


Figure 2.6 Attraction of zoospores to watercress root sections is indicated by the bar heights. The x-axis scale indicates each 250μm section of root measured, where 1 = 0-250μm from the root tip, and 2-10 = subsequent distances back from the tip. Counts are mean of 10 roots, and were made after 24 hours.

TABLE 2.5 Zoospore attraction to plant roots.

Each species tested was compared to a watercress root standard, and a blank capillary tube control. Results are means of 10 sections per root, over a sample of 5-10 roots/species (i.e. total = 50-100 replicates). Counts were made after 24 hours.

Mean zoospore attraction to 250µm root section		
Plant species	Watercress control	Capillary control
Watercress **8.3**		**1.25**
Tobacco **10.3**	**9.2**	**2.6**
Tomato **6.8**	**7**	**3**
Mustard **5.2**	**6.1**	**3**

TABLE 2.6 Zoospore attraction to roots of watercress and tobacco, when placed in the same assay chamber. Figures presented are means of 10 root sections per root and are based on 24 hour counts. 5 replicate chambers were examined.

Mean zoospore attraction to 250µm root section	
Watercress	Tobacco
3.7	**3.6**
10.3	**4.3**
2.8	**4.7**
4.9	**4.7**
9.3	**5.9**

Attraction of zoospores to watercress bed water

The density of plants in commercial watercress beds is very high, reaching tens of plants per square metre. Such a concentration of plants could release a large amount of root exudates into the water, which may result in a concentration gradient down the bed. To test this, water samples from various sites within beds, were mixed with low melting point agarose, and potential chemoattractiveness was investigated. Zoospores did not show significantly greater taxis to water from the outflow of infected beds than they did to water from the ouflow of healthy beds, inlets of beds, or MilliQ water (see Figure 2.7).

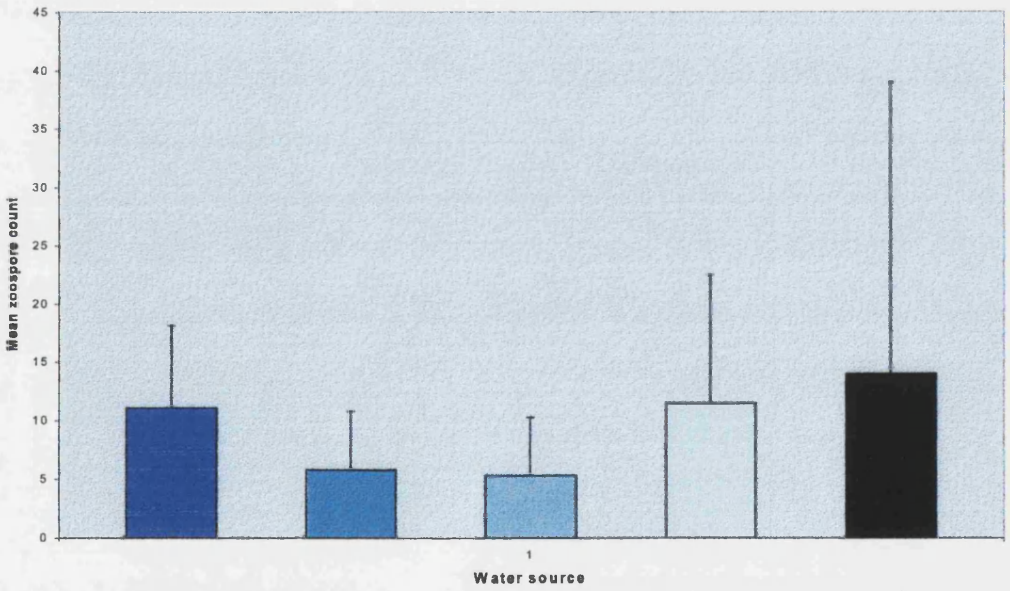


Figure 2.7 Zoospore attraction to watercress bed water . Results are means of 10 replicates, and were taken after 24 hours. Error bars indicate standard deviation from the mean.

DO = Outlet water of bed with crook root HO = Outlet water of bed without obvious crook root

I = Inlet water MQ = MilliQ water Ag = Agarose

Some of the agarose controls in chambers for testing attraction to MilliQ water gave counts much higher than expected, due to a few very high counts, resulting in an increase in the mean. The variation is reflected in the large error bars on Fig. 2.7. The attraction of zoospores to water samples was generally lower than to root extracts.

Attraction of zoospores to chemicals released from intact roots

In a closed environment it is possible that root exudates may build up over time, until the amount in a given volume of water is enough to elicit significant chemotaxis. This experiment was designed to give an indication of rate of release, and distance over which attractants are active.

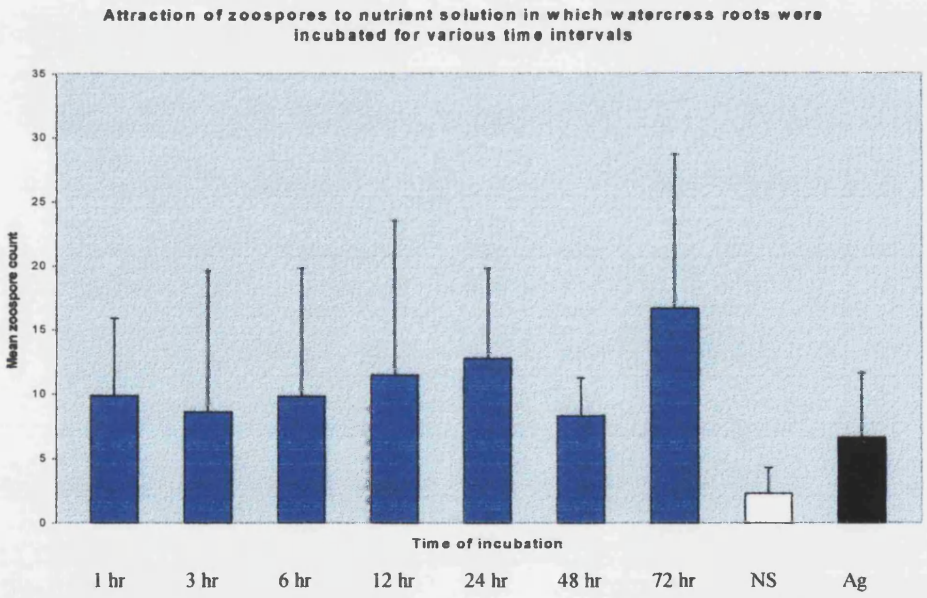


Figure 2.8 Zoospore attraction to nutrient solution is indicated by the bars on the graph. NS = nutrient solution control, Ag = agarose only control.
Results are means of 10 replicates, and are taken from readings after 24 hours following set up of the chemotaxis chambers. Error bars indicate standard deviation from the mean.

It was necessary to leave watercress plants in nutrient solution for 72 hours before the solution was significantly more attractive to zoospores than was a nutrient solution + agarose control (Figure 2.8), or was agarose alone. Significance was at the 5% level, and was observed with counts taken after 3, 6, and 24 hours following setting up of chambers. Attraction to samples taken after 72 hours was significantly different to those taken after 48 hours, but not at any other time point, even as early as 1 hour. The differences at 72 hours, and 48 hours sampling times were only observed when zoospores were counted 3 or 6 hours after sampling, and were not observed in 24 hour counts.

Preliminary results on the effect of organic molecules on zoospore taxis and encystment

Amino acids and carbohydrates have been shown to be stimulants of zoospore taxis and encystment (Deacon, 1988). As they are ubiquitous plant root compounds, it is possible that some of these could act as chemoattractants to *S. subterranea* zoospores.

A selection of results is presented in Table 2.7, with the full data set in Appendix B. Based on a single assay for encystment and two for taxis, aspartic acid, methionine, glutamine, and pectin appeared to attract zoospores at levels in excess of that seen with attraction to nutrient solution or MilliQ water controls. Aspartic acid was the most attractive amino acid, with a mean of 11 zoospores attracted, compared to 5 in controls. Glutamic acid was no more attractive than controls. Polygalacturonic acid attracted a mean of 1 zoospore, and sugars also appeared to attract equal numbers or

fewer zoospores than controls. This may be as a result of increased encystment due to these chemicals. Polygalacturonic acid (PGA) was effective in causing zoospore encystment (100%), as were all three sugars tested (glucose, sucrose, galactose). Correlation between attraction to amino acids, and encystment due to them was found to be weakly negative, implying that there is a weak relationship, whereby increasing zoospore attraction to organic molecules is correlated with decreasing encystment levels caused by organic molecules. All compounds tested were more effective in inducing encystment than nutrient solution.

TABLE 2.7 Effects of organic molecules on taxis and encystment of *S. subterranea* f. sp. *nasturtii* zoospores. Results are from a single test for encystment, and two replicates for taxis. Tests for taxis and encystment were performed in separate experiments.

Organic molecule tested (20mM)	No. of zoospores attracted	Percentage zoospore encystment (non-motile cells)
Aspartic acid	11	Not tested
Glutamic acid	4	Not tested
Polygalacturonic acid **	1	100
Galactose	5	83
Nutrient solution	5	20
MilliQ water	5	77

** PGA supplied at 560µg ml^{-1}

Effect of initial zoospore density on taxis and encystment

Behaviour of zoosporic organisms is reported to be affected by the density at which they occur (Reid *et al.*, 1995). At higher densities, greater accumulation of zoospores may occur, due to the phenomenon known as autoaggregation. Chemotaxis of zoospores at various initial concentrations was investigated in order to ascertain whether initial density could influence results.

Correlation values of 0.71 (6 hour readings), and 0.92 (24 hours), between initial density and zoospore attraction, confirmed that an increase in initial zoospore density results in an increase in zoospore attraction to watercress roots (Table 2.8). After 6 hours, however, the gradient was just 0.19, and after 24 hours, 0.45, indicating a less than directly proportional relationship. Table 2.8 shows the effect of initial zoospore density on taxis, encystment and survival after 24 hours. Results obtained from 6 hour counts were very similar. The number of zoospores surviving at higher densities was lower than anticipated if a directly proportional relationship with density were expected, and the proportion of cells which encysted appeared to decrease with increasing density.

TABLE 2.8 Number of zoospores of *Spongospora subterranea* f. sp. *nasturtii* counted in nutrient solution in chemotaxis assay chambers, and the proportion of these which were non-motile. Count expected was equivalent to the initial density of zoospores. Counts are means of 4 replicates.

Initial cell density	Mean zoospore attraction (24 hr)	Expected count of viable cells (24 hr)	Actual mean count	Proportion non-motile
$4x10^5ml^{-1}$	24	40	7.33	32%
$2x10^5ml^{-1}$	15	20	8.25	58%
$1x10^5ml^{-1}$	9	10	7	86%
$5x10^4ml^{-1}$	12	5	0.3	100%
$1x10^4ml^{-1}$	4	1	2.5	100%

Effects of zinc on zoospore taxis, encystment and lysis

Tomlinson (1958a) found 0.5µg ml^{-1} zinc sulphate to be effective in killing zoospores of *S. subterranea* f. sp. *nasturtii*, by reducing levels of infection in the laboratory to 0.03% of roots. As 0.5µg ml^{-1} zinc sulphate is equivalent to 0.11µg ml^{-1} zinc, it would be anticipated that 0.1µg ml^{-1} zinc in trials would be effective in killing zoospores. As Table 2.9 indicates, this has not been confirmed in this project.

TABLE 2.9 Zoospore survival and motility in solutions containing zinc, measured after 3 and 24 hours. Results are means of at least 6 replicates.

Zinc content of solution ($\mu g\ ml^{-1}$)	Mean cell count (3 hours)	% non-motile
None	8.25	61
0.025	3.67	66
0.050	3.92	62
0.075	3.00	50
0.1	3.08	51
0.1 (as chloride)	3.17	37
0.1 (as acetate)	3.17	53
0.5	6.50	60

Zinc content of solution ($\mu g\ ml^{-1}$)	Mean cell count (24 hours)	% non-motile
None	3.67	50
0.025	4.67	45
0.050	5.56	38
0.075	3.83	50
0.1	4.00	44
0.1 (as chloride)	5.17	29
0.1 (as acetate)	3.33	35
0.5	3.42	41

Zoospores appeared to be steadily reduced in number, with increasing zinc levels, but even at 0.5$\mu g\ ml^{-1}$ zinc, greater than 50% of zoospores were still present. Even at 8$\mu g\ ml^{-1}$ zinc (see Figure 2.12) total kill was not achieved. After 24 hours, total cell number decreased even in nutrient solution alone, whereas after 3 hours, significantly more zoospores remained in nutrient solution than in all other solutions tested, bar 0.5$\mu g\ ml^{-1}$zinc. This was not reflected in cell motility.

Attraction of zoospores to watercress root extract decreased with increasing zinc supply, (Figure 2.9). Although zinc chloride, and zinc acetate at 0.1$\mu g\ ml^{-1}$ appeared

to support greater taxis of zoospores than zinc sulphate at 0.1μg ml^{-1}, this was not statistically significant.

Nutrient solution was not seen to support greater chemotaxis of zoospores to root extract than any concentration of zinc tested. The only significant differences were between 0.5μg ml^{-1} zinc, and agarose controls at 3 and 24 hour readings (not shown on Figure 2.9), and between 0.05μg ml^{-1} and 0.5μg ml^{-1} zinc at 3 hour readings. In both cases addition of 0.5μg ml^{-1} zinc resulted in lower levels of zoospore attraction.

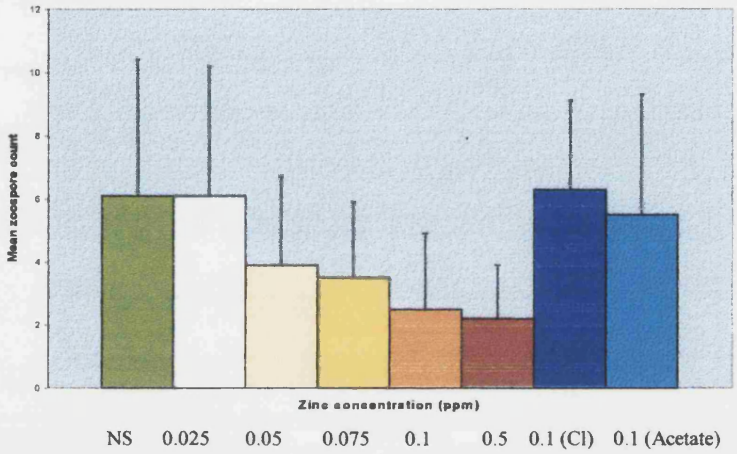


Figure 2.9 Zoospore attraction to watercress root extracts in the presence of zinc. Results are means of 10 replicates, and are based on 24 hour counts. Error bars indicate standard deviation from mean values.

Zinc pulsing effects on development of crook root and zoospore survival

Zinc may be supplied to watercress beds either continually or as a pulse delivered at regular time intervals. A test was set up using seedlings grown under conditions simulating those of a watercress bed (daylength, nutrients, pH, temperature), which were exposed to zoospores for periods of time prior to a zinc 'pulse'.

Although a proportion of the seedlings died in this experiment, crook root symptoms on roots were not observed: it is not possible to conclusively correlate the death or survival of the seedlings with the interaction of zinc and *Spongospora*.

TABLE 2.10 Survival of watercress seedlings exposed to *S.subterranea* f. sp. *nasturtii* for a given time, before addition of 0.075µg ml^{-1} zinc as a 15 minute pulse. 10 seedlings were used per treatment.

Zoospore exposure time (minutes)	% mortality of seedlings	Zoospore recovery (per ml)
0	10	0
30	40	2500
60	80	0
120	70	10000
180	70	2500
240	90	7500

Of two plants available for controls without zinc addition, 1 died with recovery of 15000 zoospores ml^{-1} from solution, whilst the other was healthy and no zoospores were recovered.

When zoospores were allowed 60 minutes or greater in which to infect seedlings, before zinc addition, mortality of seedlings was around 70-90%, compared to a marked decline in mortality if zinc was added after less than 60 minutes (Table 2.10). The number of zoospores recovered after 14 days (the end of the experiment)

from the solution in which the plants had been incubated, tended to correlate positively with seedling mortality (Table 2.10). The two control plants showed a correlation between number of zoospores recovered, and health of the plants – the solution in which zoospores were observed, was the one in which the seedling had died.

Preliminary results on the effect of cations on zoospore encystment

Cations have previously been demonstrated to affect zoospore encystment and motility in genera such as *Phytophthora* (Byrt *et al.*, 1982b). In this study cations were investigated at a range of concentrations based on Byrt *et al.* (1982b).

TABLE 2.11 Effect of cations on the encystment of zoospores of *S. subterranea* f. sp. *nasturtii*. Compounds were chlorides unless otherwise stated.

Cation	Encystment of zoospores		
	50mM	5mM	0.5mM
Cu^{2+}	85	70	50
$Cu^{2}SO_4^{2-}$	100	100	95
K^+	100	75	75
$K^{+}H_2PO_4^-$	95	65	60
Ca^{2+}	75	75	70
La^{3+}	100	95	99
NH_4^+	90	50	75
Na^+	95	70	80
$Na^{+}NO_3^-$	90	72	70
Zn^{2+}	100	100	100
$Zn^{2+}SO_4^{2-}$	100	70	95
Mg^{2+}	80	55	70
Mn^{2+}	95	75	75
Li^+	55	50	70
Cs^+	100	70	80
Co^{2+}	90	60	75
Nut. soln	55	MilliQ	75

Most of the cations caused encystment of zoospores above the 40% level seen in nutrient solution standards (Table 2.11). Copper ions (as copper sulphate), and lanthanum ions seemed particularly effective, inducing nearly 100% encystment at 0.5-50mM. Calcium, zinc and lithium ions also induced encystment to a slightly lesser degree. This test does not account for the proportion of cells which were lysed. Results are from one replicate only and must be interpreted with caution.

Effect of cations on zoospore survival

The cations which were tested were selected as follows. Manganese was used due to it having two soluble salts (sulphate and chloride), which were easily accessible. Cobalt was another divalent cation, ammonium a monovalent cation, and aluminium trivalent.

Cobalt and zinc caused significant lysis of zoospores at 2μg ml^{-1} compared to all other cations tested, and nutrient solution (Figure 2.10). Aluminium caused some lysis, but this was not significant. The anion involved, appeared not to effect zoospore lysis, as shown by the similarity of results using manganese sulphate and manganese chloride.

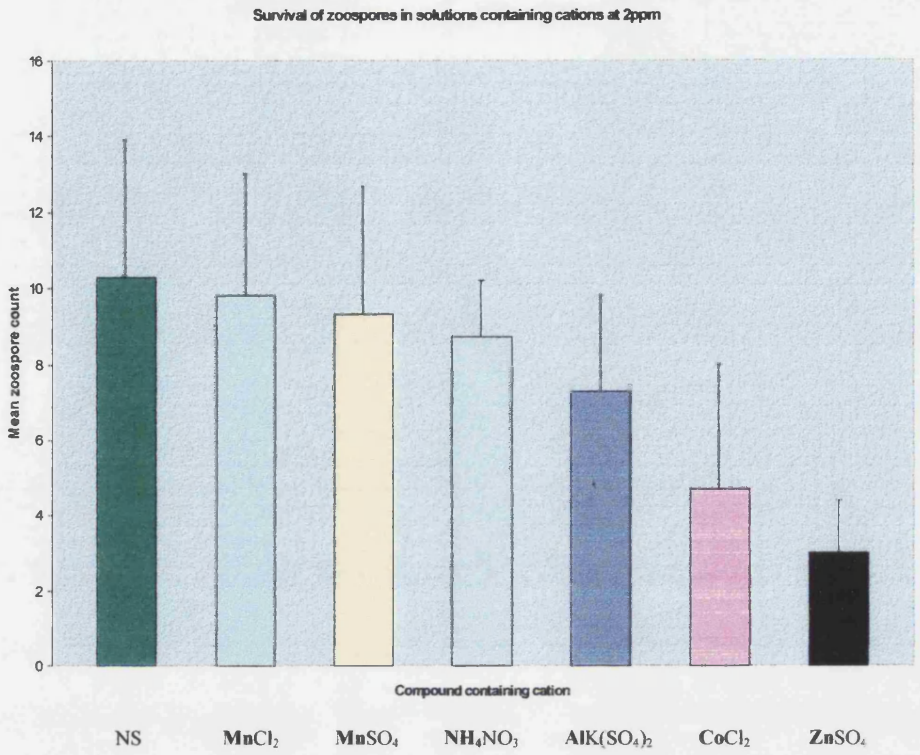


Figure 2.10 Zoospore lysis in solutions containing cations of interest (indicated in bold type). Lysis was assessed by counting zoospores after 2 hours, and comparing to nutrient solution (NS) controls. Results are mean of 15 replicates. Error bars indicate standard deviation from means.

Effect of cobalt on zoospore taxis, encystment and survival

Following the positive effect of cobalt on zoospore lysis, it was tested alongside zinc. Using 2μg ml^{-1} zinc or cobalt, zoospore attraction to watercress root extract could be significantly reduced (Figure 2.11), which correlates well with the effects of 2μg ml^{-1} cations on cell lysis, already presented. A summary of statistical significance of results for the effects of cations, including zinc and cobalt is given in Appendix A.

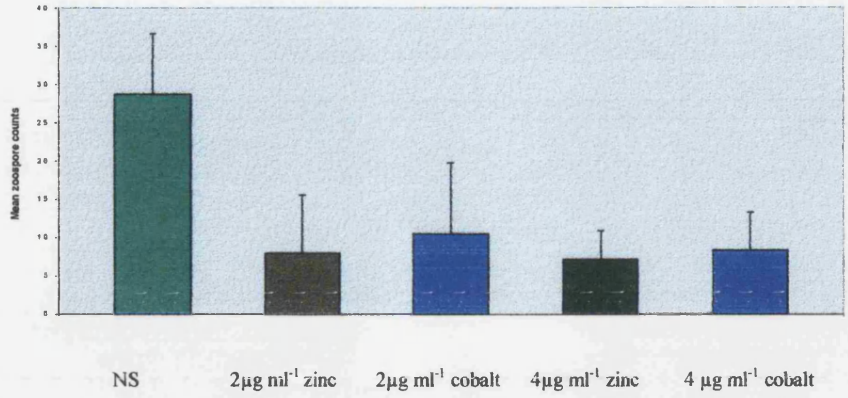


Figure 2.11 Attraction of zoospores to watercress root extract in the presence of zinc or cobalt ions. The results are means of 10 replicates, and are recorded after 24 hours. Error bars indicate standard deviation from the mean.

However, at concentrations ranging from 0.5 –8µg ml^{-1} cobalt was never as effective as equivalent zinc concentrations in lysing zoospores (Figure 2.12).

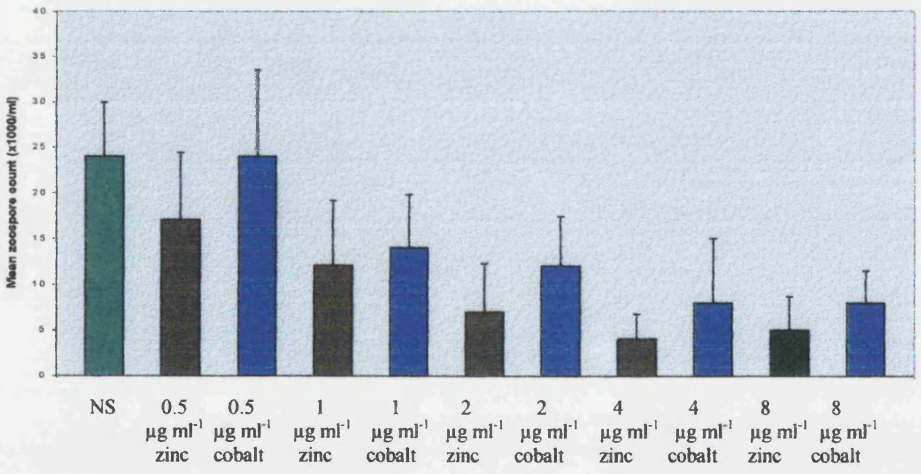


Figure 2.12 Zoospore lysis caused by zinc or cobalt cations. Bars indicate the number of zoospores counted after 2 hours in the appropriate solution. Results are means of 15 replicates. Error bars indicate standard deviation from the mean.

Zinc caused significant zoospore lysis at as little as 1μg ml^{-1}, whereas cobalt was not effective below 2μg ml^{-1}. Although zinc was never significantly more effective than cobalt at equivalent levels, it was clearly at least as effective. Cobalt at higher concentrations was not significantly more effective when compared to zinc at lower concentrations, whereas the reverse situation was sometimes true (for example, 2μg ml^{-1} zinc was significantly more effective than 0.5μg ml^{-1} cobalt).

Synergistic effects of zinc and cobalt on zoospore lysis were not seen (Figure 2.13).

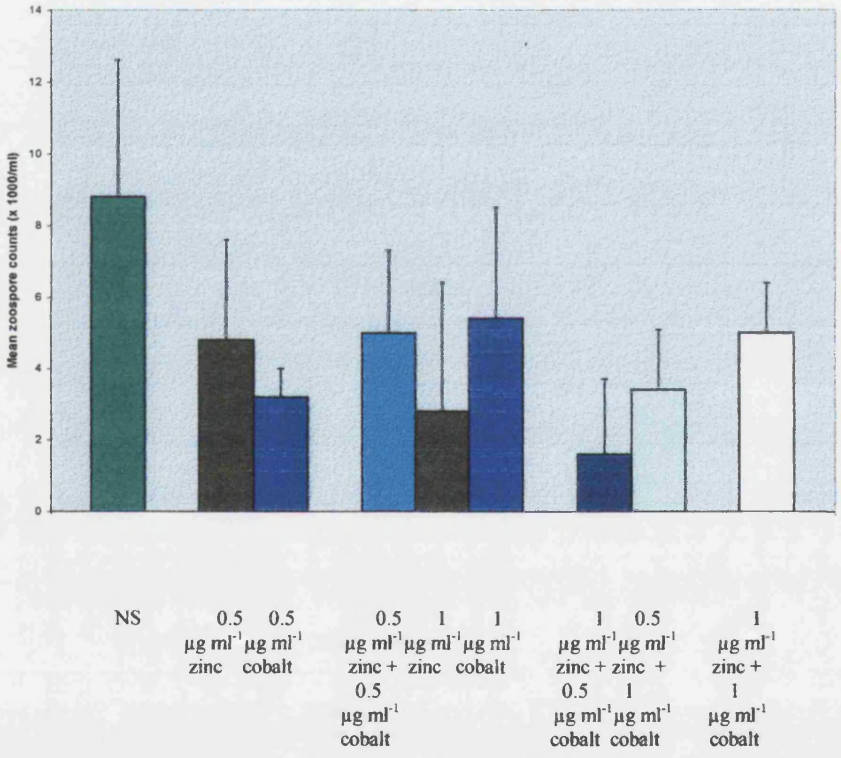


Figure 2.13 Effect of combinations of zinc and cobalt ions on zoospore lysis. Results are means of 10 replicates and were taken after 2 hours. Error bars indicate standard deviation from the mean.

2.3 DISCUSSION

Attraction of zoospores to plant roots and root extracts

The results presented have shown that attraction of *S. subterranea* f. sp. *nasturtii* zoospores to root extracts was non-specific. Testing extracts from different plant species either in the same or separate assay chambers, revealed few significant differences, and results from serial dilution of extracts showed that specific attraction to watercress roots was not operating (Figure 2.5).

Generally, tobacco was seen to induce a greater chemotactic response than watercress or mustard, which in turn were more attractive than tomato (e.g. Figs.2.2, 2.4, 2.5). This implies that tobacco either contains higher levels of a general attractant than the other species tested, and/or different, more attractive compounds. Tobacco leaf extracts were also more attractive to zoospores than other leaf extracts tested (Figure 2.3), further implying that a general chemoattractant is operating. Claxton (1996) investigated zoospore attraction to roots of a range of species and concluded tobacco (*Nicotiana rustica*) to be more attractive than other species tested, including two varieties of watercress. As tobacco is not known to produce glucosinolates (Kiddle *et al.*, 1999), the group of compounds demonstrated as signalling molecules specific to the Cruciferae (Rodman,1981; Bennett and Wallsgrove, 1994), it is highly unlikely that these specific compounds account for chemotaxis of *S. subterranea* zoospores. In view of the results obtained, profiling of root extracts to look for specific molecules was not carried out, due to the complex mixture of chemicals likely to be obtained.

The dilution series of root extracts indicated a dose-dependent response on chemotaxis (Figure 2.5), and the results strongly reduced the possibility that a watercress-specific attractant might be present, as attraction was not greater to watercress than other species at any concentration tested. These results were confirmed by using intact roots, where zoospores were seen to migrate to both host and non-host roots (Tables 2.5 and 2.6). Specificity of infection must operate at a later stage, although it should be noted that in this study no plasmodia were observed within roots. Claxton (1996) observed *Spongospora* plasmodia inside roots of watercress, but not a range of other species examined, including tobacco (*Nicotiana rustica*), mustard (*Brassica nigra*), and tomato (*Lycopersicon esculentum* cv. Roma). Unlike *Phytophthora* (Zentmyer, 1961; Ho and Hickman, 1967a; Chi and Sabo, 1978), *S. subterranea* zoospores did not appear to be attracted to a specific root zone, confirming findings of Claxton (1996) on a range of plant species (Figure 2.6), including watercress. This reduces the likelihood that factors such as electrotaxis are involved, because currents generate fields which show polarity at different zones of the root (Miller *et al.*, 1991), which would lead to spatial differences in zoospore attraction.

Figure 2.2 and the combined data from Tables 2.1, 2.2 and Figure 2.4 indicate that the rate of zoospore attraction was greatest within the first 3-6 hours after being exposed to root extracts, and that this rate declines toward 24 and 48 hours.

The rapid zoospore response indicates that the putative chemoattractants tested were not present at levels high enough to repel zoospores, as has been observed in the case of *Phytophthora palmivora* in response to low molecular weight cations (Cameron

and Carlile, 1980), and also with *Pythium aphanidermatum* in response to butyraldehyde (Jones *et al.*, 1991). Maceration of roots to create root extracts was thought likely to result in release of a much higher concentration of potential chemoattractants than would be released from intact roots, making zoospore taxis easier to observe. When a 1:1 ratio of root extract (see Materials and Methods) and agarose was used, chemotaxis of zoospores was generally within the range of 10-100+ zoospores reaching the mouths of capillary tubes within 24 hours. The description of the zoospore response as 'rapid' should be considered in the context of the time course covered by these experiments. In other studies, zoospore attraction has been observed within minutes, such as 20 minutes in the case of *Pythium* and *Phytophthora* spp. (Donaldson and Deacon, 1993a). However, in preliminary work on chemotaxis in *Spongospora subterranea* f. sp. *nasturtii*, Claxton (1996), recorded results after 2-3 hours.

As zoospores are thought to be unable to take up nutrients in most species (Deacon and Donaldson, 1993), then the attraction to root extracts seen, must be caused by signalling molecules. The decline in rate of attraction over time, may be due to loss of signal due to the volatile state of these attractants (e.g. aldehydes, phenolics), or possibly because of diffusion of molecules from the capillary tubes into the surrounding zoospore suspension. Zoospores were observed to encyst away from capillary tubes, and whether this is due to a reduction in the chemotactic gradient, or simply a survival response of zoospores unable to locate a host, it would still account for the drop in rate of attraction. At 5°C, zoospores of *S. subterranea* f. sp. *nasturtii*

were observed to remain motile in nutrient solution for at least 3 days before declining in number, and so zoospore survival should not account for the results. The fact that of at least 20 000 zoospores released per assay chamber, less than 1% generally reached the source of chemoattraction, may relate in part to encystment away from the source, and also in part to the distance of up to 1cm that zoospores furthest away from the source were required to travel. Zoospores furthest from the capillary tube containing root extract, were closest to the agarose control tube in experiments where only two tubes were used. This may account for the residual attraction to agarose control tubes, which was generally seen to be less than attraction to root extracts.

Effects of organic molecules on zoospore taxis and encystment

Organic molecules, including amino acids and volatiles have been shown to affect zoospore taxis (e.g. Donaldson and Deacon, 1993a; Cameron and Carlile, 1978). Preliminary investigations on the chemoattractiveness of organic molecules suggested that none were as attractive as root extracts, as was found to be the case in *Pythium* and *Phytophthora* species (Cameron and Carlile, 1978). Possibly the amino acid or carbohydrate component of root exudates is not important in taxis, or a mixture of molecules, perhaps at different concentrations to that tested here, are required. Cameron and Carlile (1978), proposed that additive or synergistic effects operate between known attractants, or unknown attractants are involved (or both). The molecules which were more attractive than water or nutrient solution in the current study were generally those that have been reported as chemoattractants for other

zoosporic organisms, such as methionine (Lee *et al.*, 1999), which induced a reduction in stopping and turning behaviour of a *Chroomonas* (marine cryptophyte) species at less than 1μM concentrations. Jones *et al.* (1991) found methionine to be insignificant in attracting *Pythium aphanidermatum* zoospores, however, at 10mM concentration, a value closer to the 20mM used in the current study. Aspartic acid and glutamine have been demonstrated as attractants for *Pythium* and *Phytophthora* (Donaldson and Deacon, 1993a; Cahill and Hardham, 1994a). Donaldson and Deacon (1993a) showed that 10mM L-aspartate attracted 9.4 times as many zoospores of *Pythium aphanidermatum* to capillaries compared to control capillaries containing phosphate-buffered water, with 10mM glutamine shown to be capable of inducing chemotaxis of zoospores of *Pythium species* (Donaldson and Deacon, 1993a). However, Donaldson and Deacon (1993a) also found glutamic acid and asparagine to be chemoattractants, which was not seen to be the case with preliminary results. Pectin has been demonstrated as an attractant for *Phytophthora cinnamomi* at 100μg ml^{-1} (Cahill and Hardham, 1994a), but at 500μg ml^{-1} it acted as an inducer of encystment (Byrt *et al.*, 1982a). Estrada-Garcia *et al.* (1990) also found significant encystment of *Pythium aphanidermatum* in response to 612μg cm^{-3} pectin on slides. These levels are lower than used to obtain results presented here (Table 2.7 and Appendix B).

Sugars and polygalacturonic acid (PGA) appeared to induce increased encystment, rather than chemotaxis. PGA has been shown to cause encystment in both *Pythium* and *Phytophthora* species (Jones *et al.*, 1991; Byrt *et al.*, 1982a), at equivalent concentrations to those used in the current study. Effects may be related to pH of the

solutions containing organic molecules, although there was not conclusive data to support this. Pectin and PGA both had pH <4, but whereas pectin attracted zoospores, PGA did not. Also, the sugars tested had pH >5, but were almost as effective inducers of encystment as PGA.

Diffusion of signalling molecules from roots

The concentration of potential chemoattractants in water was too low to induce significant chemotaxis (Fig 2.7). The flow rate of water through watercress beds is around 2000 litres m^{-2} h^{-1}, and so root exudates will be constantly diluted. Results for zoospore attraction to agarose controls, and to MilliQ water were higher than expected, but considering the rest of the data, there was a visual trend (not statistically significant) whereby water from outlets of diseased beds was more attractive than outlet water from healthy beds, which in turn was more attractive than inlet water (Figure 2.7). This would imply that a watercress root exudate is present as an attractant, but that this might be augmented by higher concentrations, or extra signals from diseased roots. Plasmodiophorid infection clearly has effects on host cell behaviour. In *P. brassicae* infected cabbage (*Brassica oleracea* var. *capitata*), it was observed that root hair cells had enlarged nucleoli compared to non-infected cells (Williams *et al.*, 1971), implying increased metabolic activity.

An attempt was made to estimate how long roots would have to be left in nutrient solution before the solution became attractive to zoospores (Figure 2.8). After 72 hours, some significant attraction of zoospores was seen, adding further evidence for the involvement of root exudates in chemotaxis. However, the experiment was

flawed, in that the solution is likely to become anaerobic quite rapidly, which may result in stressed plants, and atypical root behaviour, including release of stress-related chemicals such as ethylene (Beyer *et al.*, 1984).

The effect of zoospore density on behaviour

If zoospores were to show the phenomenon of autoaggregation (Thomas and Peterson, 1990; Reid *et al.*, 1995), then initial density may be important in determining the number of zoospores responding to potential attractants. Table 2.8 indicates that density does not seem to be important. The gradient is less than 1, which would be the value expected if attraction were directly proportional to initial density. If autoaggregation were to be operating, the gradient would be expected to be more exponential. The possibility that zoospores are able to communicate with each other was still suggested from some of the results obtained. Firstly, the proportion of zoospores encysting, seemed to decrease as initial density increased (Table 2.8). As the only difference in the experimental chambers was the number of zoospores, this suggests that a factor relating to them is responsible. Possibly, at higher concentrations of zoospores, signalling between them does occur, whilst at lower concentration signals are not transmitted, and the few zoospores present encyst as a result. Thomas and Peterson (1990) reported aggregation of *Achlya* zoospores in the absence of exogenous signals, indicating the potential for zoospore-zoospore communication. Secondly, many results in the work on zoospore taxis, showed high levels of variability in zoospore counts, usually by the time the first readings were taken after 3-6 hours (e.g. Fig. 2.2). This could be due to initial small differences in

zoospore attraction between replicates, being exacerbated by recruitment of zoospores. Thomas and Peterson (1990) reported this type of effect also when working on *Achlya heterosexualis*, with initial responses to host signals being augmented by autoaggregation. Chemotactically induced autoaggregation has also been observed in *Pythium* and *Phytophthora* species, where it was possibly due to genus-specific factors (Reid *et al.*, 1995).

The response of zoospores to cations

Zinc has been the sole control measure for crook root since its ability to lyse zoospores was discovered (Tomlinson, 1958a). The precise mode of action is unknown, and the effects of other cations have not been thoroughly investigated. Claxton (1996), examined the effects of copper and manganese (as sulphates) on zoospores, at concentrations up to 150μM, and found that manganese had no effect, whilst copper was only effective at the highest concentration tested. As Figure 2.10 indicates, magnesium, ammonium, and aluminium were not effective in controlling zoospores at 2μg ml^{-1}, and the use of chloride, sulphate, and nitrate suggests that anions do not have a role in zoospore lysis. Anions have been investigated for their effects on *Phytophthora cinnamomi*, and aside from acetate and fluoride, were ineffective (Byrt *et al.*, 1982b). These authors also found aluminium to have little effect on zoospores, and although some loss of viability was seen with magnesium and ammonium ions, this was at 30mM concentrations, much higher than tested here on *S. subterranea*.

In the current study, cobalt ions (Co^{2+}) did have a significant effect on reducing numbers of *Spongospora* zoospores, as did zinc at 2µg ml^{-1}. Further investigations (see Figures 2.11 and 2.12), showed that cobalt was not as effective as zinc. Cobalt has not been widely investigated as a control agent for fungi or protists. Karamushka *et al.* (1997) found that cobalt had a toxic effect on *Saccharomyces cerevisiae*, but that the effect was less than caused by equivalent concentrations of zinc. They observed that zinc was taken up by cells, whereas cobalt salts were not, which might account for differences in toxicity. The effects of zinc and cobalt on *S. cerevisiae* were mediated by calcium or magnesium, suggesting competition for cell surface sites.

Zinc was not observed to be as effective at control of *S. subterranea* as reported by Tomlinson (1958a), who reported 0.5µg ml^{-1} zinc sulphate (0.11µg ml^{-1} zinc) to be enough to lyse zoospores. In this study, zoospore lysis was not significant below 1µg ml^{-1} zinc (see Figure 2.12). Claxton (1996) also found zinc to be ineffective below 150µM, which is over 100 times greater than concentrations added in the field. Lysis of zoospores by zinc was confirmed by the fact that cell number was reduced in the presence of zinc, but that percentage encystment was not (Table 2.9). Survival was shown to be independent of the anion associated with zinc.

Attraction of zoospores to watercress root extract was not inhibited by zinc, except when cell number was significantly reduced (Figure 2.9). It may be that once encysted, zoospores are not then as vulnerable to zinc. Calcium bicarbonate at 540µg ml^{-1} was reported to control crook root disease (Tomlinson 1958c), but even at 1000 µg ml^{-1}, it did not affect zoospores directly. If zinc were to compete for calcium

binding sites at the cell membrane as postulated for *Saccharomyces cerevisiae* (Karamushka *et al.*, 1997), then an effect on zoospore swimming ability might be expected as calcium is known to be important in control of this function (Deacon and Donaldson, 1993).

Zinc does appear to reduce crook root infections, as reported by Tomlinson and Hunt (1987), where 0.1μg ml^{-1} zinc in outflow water correlated with no crook root in the watercress bed, whilst untreated beds in the same trial had 96% infection. This author has often observed widespread crooked roots in zinc treated beds, even though the aerial parts of plants appeared healthy. Gooding (1985) reported that despite zinc usage, 80% of commercial watercress beds are infected with *S. subterranea* f. sp. *nasturtii* to some extent. Preliminary studies on the effect of zinc on the viability of zoospores appeared to show that zoospores require less than 60 minutes to penetrate host roots (Table 2.10), although the results were based on mortality as opposed to direct symptoms of crook root. The results, if due to control/susceptibility to crook root, are not in accordance with those of Adams and Swaby (1988), who concluded that *Polymyxa graminis* zoospores were immobilised by 10μg ml^{-1} zinc ions when added at up to 3 hours after zoospore exposure to host roots. Claxton (1996) observed that *S. subterranea* f. sp. *nasturtii* cysts were present on roots 48 hours after inoculation. Therefore, zinc should have come into contact with encysted zoospores whenever it was added in the current study. Again, this suggests that it may be that *S. subterranea* zoospores are only vulnerable to zinc prior to enystment. Walsh *et al.* (1989) investigated the transmission of watercress yellow spot virus (WYSV), which was thought to be vectored by *S. subterranea*. When *Spongospora*

infected roots were incubated with healthy roots, virus symptoms were obtained. With the addition of 7μg ml^{-1} zinc, some virus transmission was still observed. The conclusion drawn was that the virus may not have an absolute requirement for *S. subterranea* as a vector, but in the light of results from this PhD., an alternative possibility may be the inefficiency of zinc in controlling *Spongospora*. As Figure 2.12 indicates, a small proportion of zoospores were observed after incubation in 8μg ml^{-1} zinc. This suggests that some level of resistance or tolerance may operate in the *Spongospora* population, or that factors influencing zoospore susceptibility in the field were not replicated in the laboratory.

3. Development of a molecular diagnostic test for *Spongospora subterranea* f. sp. *nasturtii*

3.1 MATERIALS AND METHODS

Genomic DNA extraction

Plant and pathogen DNA were obtained using the method of Raeder and Broda (1985). Plant material or plasmodiophorid spore balls (cystosori) were ground in liquid nitrogen in a pestle and mortar. 500mg of the resulting powder was placed in a 1.5ml tube and mixed with 500µl of DNA extraction buffer.

DNA extraction buffer: 200mM Tris-HCl buffer (pH 8.5)

250mM NaCl

25mM EDTA

0.5% sodium dodecyl sulphate

350µl phenol and 150µl chloroform were then added, and the suspension mixed thoroughly, before centrifugation at 13 000g for 60 minutes (Eppendorf 5415 C bench centrifuge). The upper layer of liquid was then removed to a new, sterile tube, taking care not to carry over any cell debris. 25µl of 20mg ml^{-1} RNAse A was added, and the mixture incubated at 37°C for 10 minutes, before addition of 1 volume of chloroform. A second centrifugation step was performed at 13 000g for 10 minutes, and the upper layer subsequently pipetted to a sterile tube. DNA was precipitated by addition of 0.54 volumes of propan-2-ol, with repeated inversion of the tube. DNA was allowed to settle for 20 minutes at -20°C, before centrifugation at 7 000g for 2

minutes. The alcohol was removed, taking care not to disturb the remaining pellet, which was then washed with 70% ethanol, before a further centrifugation at 7 000g for 2 minutes. Again the alcohol was decanted off, and the pellet allowed to air-dry. The DNA was then resuspended in 50µl MilliQ water, and stored at -20°C until required. Quantification of DNA was performed by running a 5µl sample on an agarose gel (as described later), with a 200ng λ DNA standard (Kramel Biotech.).

Initial attempts to PCR amplify *Spongospora subterranea* f. sp. *nasturtii* ITS rDNA

Conditions for amplification of crooked root genomic DNA (containing plant and pathogen DNA), were optimised using the Taguchi method (Cobb and Clarkson, 1994), with nucleotides (dNTP's), magnesium ion (Mg^{2+}) concentration, and oligonucleotide primer concentration varied until the optimum was identified.

The ribosomal primers used were ITS 4 (White *et al.*, 1990), and ITS 1F (Gardes and Bruns, 1993), which are classed as universal, and fungal-specific, respectively. These primers bound at sites flanking the ITS rDNA, as indicated in Figure 3.1.

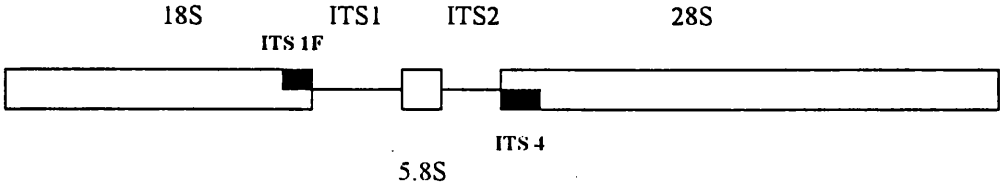


Figure 3.1 Overview of the binding sites of primers ITS 1F and ITS 4.

PCR was performed in a PTC-100™ Programmable Thermal Controller (MJ Research Inc.), with an initial denaturation step of 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 42°C for 60 seconds, and 72°C for 60 seconds. A final extension step of 72°C for 5 minutes concluded the PCR. For all PCR reactions, cycling parameters, and reaction components were optimised to suit the particular primers and DNA samples being investigated, and are listed in the appropriate Results sections. All PCR reactions included a negative control, in which components were added as above, except that no template DNA was introduced.

Agarose gel electrophoresis

PCR products and genomic DNA samples were visualised using agarose gel electrophoresis. 1% agarose (GibCo BRL) was melted into 0.5 x TBE buffer prepared from 5x stock solution.

5x TBE Buffer: (per litre) 54g Tris base
27.5g orthoboric acid
20ml 0.5M EDTA (pH 8.0)

Ethidium bromide was added, to a final concentration of 0.4µg ml^{-1}. The DNA samples were mixed with 4µl loading buffer, and run alongside suitable markers, such as λ DNA for quantification, or 100 base pair ladder (Pharmacia), for size determination, in 0.5 x TBE buffer, at 80-100V d.c.

Loading buffer: (in water) 0.25% bromophenol blue
0.25% xylene cyanol FF
15% Ficoll (Type 400:Pharmacia)

DNA was viewed under UV illumination, using a UVP WhiteUV Transilluminator, and photographed using Grab-IT 2.0 (Synoptics Ltd.), linked to a Sony UP-D680 E Digital Graphic Printer. Useful images were stored, and optimised in PaintShopPro 4.12 (JASC, Inc.).

DNA purification

DNA bands visualised on agarose gels, were retained using a Qiaex® II Gel Extraction kit (Qiagen). DNA bands of interest were excised from the gel, and placed in a buffer mixture at 50°C for the purpose of dissolving the agarose, and binding the DNA to a silica substrate. The suspension was then centrifuged briefly at 13 000g, and the pellet washed repeatedly to remove agarose contaminants and salts. The DNA was re-eluted into 20µl MilliQ water, and stored at -20°C.

Automated sequencing of DNA

Once purified, amplified DNA was generally sequenced directly, although on some occasions, DNA was cloned into a plasmid vector (see later).

For PCR products, 30-40ng of DNA was mixed with 3.2pmol of one of the oligonucleotide primers used in the PCR, in a total volume of 6µl, which was made up by the addition of MilliQ water. For cloned products, approximately 200ng DNA was used in the same volume.

The DNA was sequenced using an ABI 377 automated sequencer, at Bath University, following the dideoxy chain termination procedure (Sanger *et al.*, 1977), with fluorescent dyes (PE Biosystems). The original PCR primer was used to generate a

single-strand template. This was then used with sequencing primers, and modified dNTP's (PE Biosystems) to generate the final products, detected as fluorescent patterns. The results were analysed as text files (Microsoft Word), and as chromatograms (ABI files).

Sequence similarity searching

Sequences in text form were submitted to a BLAST (Basic Local Alignment Similarity Tool) (Altschul *et al.*, 1990) search using the NCBI GenBank database. BLAST was performed either by 'blind' searching for matches, or by aligning DNA of interest against other specific sequences on the database (for instance, those of taxonomically close organisms).

Output from the searches was obtained as a list of sequences most closely matching the input sequence, based on overall sequence alignment (performed with a gap penalty of 11, and a cost of 1 per residue gap), and on E-values, which indicate the chance of a similarity score obtained for a match, being achieved by chance (Brenner, 1998).

Cloning of PCR products

PCR products were cloned into pGEM®-T Easy plasmid vectors (Promega), according to the manufacturers instructions, except that an insert:vector ratio of 5:1 was used (S. Screen, pers. comm.).

Ligated vectors were then transformed into *Escherichia coli* competent cells (JM109, Promega), according to the manufacturer's instructions. Transformed colonies were

identified by colour on plates treated with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) and ampicillin, due to inserts causing inactivation of a part of the ß-galactosidase coding region.

DNA was recovered from these colonies using the alkaline lysis procedure (Sambrook *et al.*, 1989). Individual colonies were selected, placed in 2ml liquid broth containing 4µl of 50mg ml^{-1} ampicillin, and incubated at 37°C and 100rpm overnight in an orbital incubator (Gallenkamp). 1.5ml of this was then centrifuged at 13 000g for 5 minutes, and the pellet air-dried. This was mixed with 100µl of solution I, followed by 200µl solution II, and finally 150µl solution III.

Solution I:	Solution II:	Solution III:
50mM glucose	0.2M NaOH	5M potassium acetate (60%)
25mM Tris-HCl	1% SDS	Glacial acetic acid (11.5%)
10mM EDTA		Water (28.5%)

After another centrifugation at 13 000g for 5 minutes, the supernatant was retained, and the DNA precipitated with 2 volumes of 100% ethanol. The DNA was then resuspended in MilliQ water.

Southern hybridisation of DNA samples

DNA samples to be probed were run on an agarose gel, and blotted overnight. The apparatus was set up as shown in Figure 3.2.

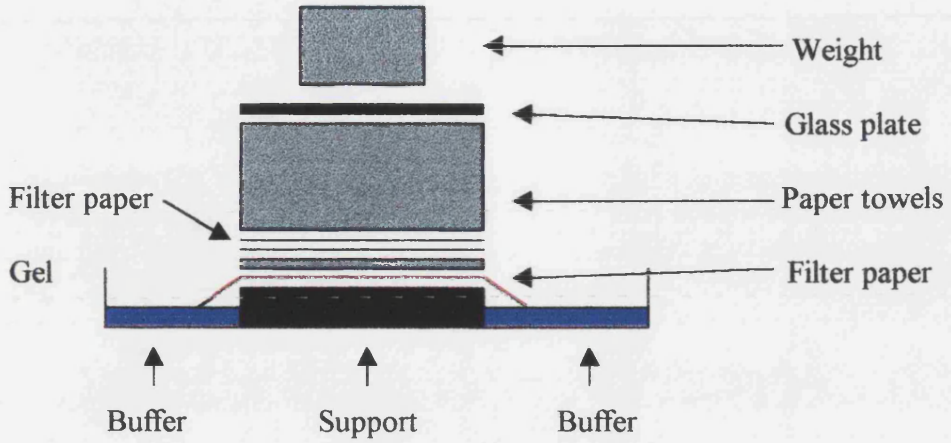


Figure 3.2 Southern hybridisation apparatus. The buffer was 0.4M NaOH.

Hybond-N (Amersham) nylon membranes were soaked in 0.4M NaOH, whilst the gel containing the DNA was denatured for 30 minutes in 0.4M NaOH also. The blotting tank was set up to transfer DNA from the gel to the membrane at room temperature by overnight capillary transfer using 0.4M NaOH as the solvent. Following this, the blot was dismantled, the membrane washed briefly in 0.4M NaOH, dried, and wrapped in SaranWrap film (Dow). DNA was fixed to the membrane by UV illumination for 3 minutes.

Blots were prepared for hybridisation by incubating for 1 hour in prehybridization buffer, at 65°C, to which 0.5ml of denatured 1mg ml^{-1} herring sperm DNA had been added.

Prehybridization buffer: (25ml) 6.25ml 20x SSC

1.25ml Denhardt's reagent

1.25ml 10% SDS.

To make the probe, DNA was labelled with ^{32}P, using an oligolabelling kit (Pharmacia). The reaction volume of 50µl contained 39µl labelled d-CTP + denatured PCR product (at 20µCi, and 200ng respectively), and 1µl Klenow fragment (5-10 Units), as well as 10µl reagent mix. The mixture was incubated at 37°C for 30 minutes, before a final denaturation at 95°C for 5 minutes.

The probe was then added to the prehybridization mixture, and hybridised overnight. The membrane was subsequently washed twice in 2x SSC (from 20x stock).

20x SSC: (per litre) 175.3g sodium chloride

88.2g sodium citrate (pH 7.0)

0.1% SDS

The initial wash was 10 minutes at room temperature, then once in 1x SSC, 0.1% SDS for 15 minutes at 65°C, and if a strong signal was still present, once in 0.1x SSC, 0.1% SDS for 15 minutes at 65°C.

The membrane was wrapped in SaranWrap, and exposed to autoradiographic film (Kodak Scientific Imaging film) at -70°C.

Investigations on zoosporic organisms associated with watercress seed

In an attempt to generate disease free watercress for studies on infection by *Spongospora*, English Dark Green watercress seed collected in 1992 from Vitacress Salads Ltd., was sown into 1 litre of nutrient solution (see Table 2.1 for composition),

in a plastic container, and left to germinate under laboratory conditions.

Approximately 90% of seed germinated within one week, and plants were allowed to develop for two weeks from germination, before use in experiments.

Routine monitoring during growth revealed the presence of zoospores. To remove these from subsequent trials, seed was soaked briefly in distilled water, followed by a solution of 0.5% sodium hypochlorite (containing less than 5% free chlorine) for 15 minutes. The seed was then thoroughly rinsed with distilled water, before sowing.

The identity of the zoosporic organism(s) was investigated by PCR and electron microscopy.

Zoospores were harvested from the nutrient solution surrounding the seedlings, and approximately 1500 of them were amplified using either ITS1F/4, or ITS5/POL (see Results), as primer combinations (conditions as detailed earlier for ITS1F/4).

Products were identified by band size on EtBr stained agarose gels.

For low temperature scanning electron microscopy, zoospores in nutrient solution (containing germinated seedlings) were investigated.

Prepared samples were plunged into nitrogen slush at -210°C, and then placed on the stage of a cooled, evacuated Cryotrans 1500 Low Temperature machine (Oxford Instruments, UK), attached to a Jeol 6310 SEM (Jeol, Tokyo). Samples were then transferred to the microscope stage, which was maintained at -180°C by nitrogen gas. Sublimation of frost at -80°C for 5 minutes followed, before reduction of the temperature to -120°C to allow coating with gold. Micrographs were obtained at about 10kV. Washed seeds were examined without using the low temperature

Cryotrans 1500 instrument, and were gold labelled with a conventional sputter coater (Edwards S150B).

3.2 RESULTS

Genomic DNA extracted by the method of Raeder and Broda (1985) was generally of sufficient quality for subsequent use in PCR.

PCR amplification using primers ITS 1F and ITS 4

The primer pair ITS 1F/4 amplified two products from crooked root total genomic DNA, under the following conditions: 10x NH_4^+ reaction buffer (Bioline), 2mM Mg^{2+} (Bioline), 0.06 mM dNTP's each (Bioline), 10pmol primers (LifeTechnologies), 20-50ng template DNA, and 2.5Units *Taq* polymerase (BioTaq™, Bioline), in a total volume of 25µl.

The bands on an EtBr stained agarose gel appeared to be approximately 650 and 750 base pairs, with the former present in higher quantity (Figure 3.3).

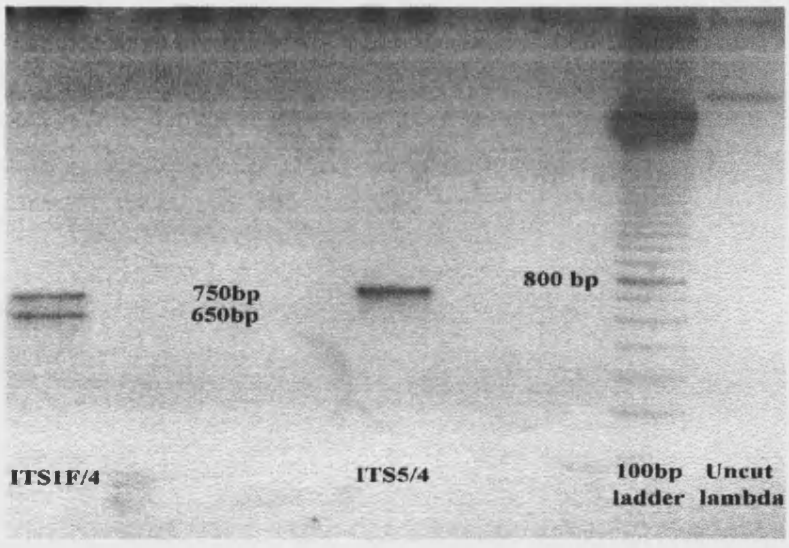


Figure 3.3 PCR products from crooked root genomic DNA using the primers indicated. Uncut lambda DNA allows an estimate of product quantity. 200ng lambda DNA was loaded here.

ITS 5/4, a Universal primer combination, amplified only a single product of approximately 750 base pairs. When sequenced, and compared using BLAST, this DNA was shown to be watercress (*Rorippa nasturtium-aquaticum*) ITS rDNA (Hurd's Summer cress).

The 650 base pair band obtained with ITS 1F/4, was assumed to be from a species other than watercress, due to the use of the fungal specific primer ITS 1F in the PCR. When sequenced (Figure 3.4), this DNA was seen to be 681 base pairs in length and had greatest homology to the Basidiomycete fungi, *Volvariella volvacea* and *Omphalina obscurata*, largely within the 5.8S rDNA (Table 3.1).

```
1   GGCGGCCGCG  GGAATTCGAT  TCTTGGTCAT  TTAGAGGAAG  TAAAAGTCGT
51  AACAAGGTTT  CCGTAGGTGA  ACCTGCGGAA  GGATCATTAA  TAAATGCTTC
101 ATTGCATCCA  CCTTTTGTGC  ACCGTAAATT  TTCACTTAAA  CTTTGTCTGA
151 TTTATATCTG  TTCTGAAAGG  AACATGAATT  GTAATCTAAT  ACAACTTTTA
201 ACAATGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG  CGAAATGCGA
251 TAAGTAATGT  GAATTGCAGA  ATTCAGTGAA  TCATCGAATC  TTTGAACGCA
301 TATTGCGCTC  TCTGGCATTC  CGGAGAGCAT  GCCTGTTTGA  GTCTTGGTAC
351 CAATCTGGTG  TAACCTTTGT  TATTAAGAAT  AGAACTTTTT  ATAAAAAAAC
401 TGCATTGAGA  CGTCTTGATA  AGTAAAATTG  TTAAGTGTCT  TAAAATACAA
451 TAGTACAGCT  ATAGCTCACC  CTCTTTGGAG  TAATATGTGA  CCTGAAAGGG
501 TAACTCTATT  TCTCCTCGTT  GGGAAGACTT  GTTGCTAGAA  GTTTTTGATT
551 GCTGAACGTC  AAAGGCGGCA  TTCAATTGTT  CGAGTAACGA  CGAACTTAAA
601 AATTCAAGCT  CAAATCAGGT  AGGATTACCC  GCTGAACTTA  AGCATATCAA
651 TAAGCGGAGG  AATCACTAGT  GAATTCGCGG  G
```

Figure 3.4 ITS rDNA sequence of a PCR product consistently obtained from crooked root DNA using the primers ITS 1F and ITS 4.

TABLE 3.1 BLAST sequence similarity results for ITS1F/4 PCR product from crooked roots. The sequence comparison was over 594 bases.

GenBank accession number	Species showing similarity	BLAST similarity score (bits)	E-value
U15973	*Volvariella volvacea*	246	3e-63
U66448	*Omphalina obscurata*	246	3e-63
AF042431	*Tremella mycophaga*	244	1e-62
AF042418	*Tremella neofoliacea*	244	1e-62
AF042417	*Tremella foliacea*	244	1e-62

Sequence similarity was found almost exclusively within the 5.8S rDNA.

Design of specific primers within putative *S. subterranea* f. sp. *nasturtii* ITS rDNA

Following apparent amplification of *Spongospora subterranea* f. sp. *nasturtii* ITS rDNA, it was necessary to attempt to design specific primers, on which a diagnostic test could be based.

ITS 1F and ITS 4 were used to amplify plasmodiophorid DNA from either infected plant material or resting spores supplied by SCRI (J. Claxton and J. Duncan). These samples contained *Polymyxa graminis, Polymyxa betae, Ligniera* spp., and *Spongospora subterranea* f. sp. *subterranea*. Watercress DNA and *Plasmodiophora brassicae* DNA were obtained at Bath University, using the techniques described in Materials and Methods. The sequences obtained were aligned with that obtained from crooked root DNA using ITS 1F/4 primers.

Alignment was carried out using the PileUp program for determining pairwise sequence similarity scores, with default penalties of 5 for creation of a gap, and 0.3

for each extension of it. These alignments were then searched by eye for regions where the putative *S. subterranea* f. sp. *nasturtii* sequence showed maximum dissimilarity to the other sequences, and these regions were used to design potentially specific primers. In designing primers, the program PrimerCalc (Molecular Sensors Ltd.) was used to check primers for potential dimerisation, runs of unsuitable nucleotides, and self-annealing problems. Primers selected included the forward primers SSNF1 and SSNF2, and reverse primers SSNB1 and SSNB2, the sequences and binding positions of which are indicated in Appendix B.

Optimal PCR conditions were calculated for the primer combinations SSNF1/B1 and SSNF2/B2 using Taguchi methods as described by Cobb and Clarkson (1994).

SSNF1/B1:	SSNF2/B2:
0.05mM dNTP's each	0.05mM dNTP's each
3mM Mg^{2+}	1mM Mg^{2+}
30pmol primers	20pmol primers
2.5U *Taq* polymerase	2.5U *Taq* polymerase

Other components were as with ITS 1F/4 reactions. Initial denaturation at 94°C (2 minutes), was followed in both primer combinations by 30 cycles of 94°C for 20 seconds, 60°C for 120 seconds, and 72°C for 24 seconds. These cycling parameters were calculated using the program FirstStep™ 1.0.1 (Molecular Sensors Ltd.).

PCR products were visualised and sequenced as described in Materials and Methods.

Primer pairs SSNF1/B1 and SSNF2/B2 were shown to be effective in amplifying DNA isolated from various geographic locations.

Design of improved primers for *Spongospora subterranea* f. sp. *nasturtii* ITS rDNA amplification

DNA sequences of plasmodiophorid ITS rDNA sequences of *Polymyxa, Plasmodiophora, Ligniera,* and *S. subterranea* f. sp. *subterranea* (Ward and Adams, 1998) failed to match with the sequences obtained in this study thus far. The fungal specific primer, ITS 1F was aligned with the published sequences (Ward and Adams, 1998) using GenBank, and as Figure 3.5 indicates, was shown to match well with *Olpidium brassicae*, a Chytridiomycete comparison species used by Ward and Adams (1998), but not to plasmodiophorids. Therefore it was assumed that the DNA amplified from crooked roots using ITS1F/4 was unlikely to be *S. subterranea* f. sp. *nasturtii* DNA.

A

```
ITS 1F:          1   cttggtcatttagaggaagtaa 22
                     ||||||||||||||||||||||
O. brassicae:  320 cttggtcatttagaggaagtaa 341
```

B

```
ITS 1F:          1   cttggtcatttagaggaagtaa 22
                      ||||  |  |||||  ||||  |
P. brassicae:  321 tttgggctcttagaagaaggag 342
```

Figure 3.5 Alignment of primer ITS 1F with putative binding sites *in Olpidium brassicae*, a Chytridiomycete fungus, and with *Plasmodiophora brassicae* a plasmodiophorid species.

The BLAST similarity search, taking these published sequences into account, also indicated a closer homology of the crook root associated DNA to *O. brassicae* than to plasmodiophorids (Table 3.2).

TABLE 3.2 Sequences showing similarity to ITS1F/4 PCR product from crooked roots, with more recent data base submissions than indicated on Table 3.3. The sequence comparison was over 681 bases.

GenBank accession number	**Species showing similarity**	**BLAST similarity score (bits)**	**E-value**
Y12830	*Olpidium brassicae*	347	3e-93
AB015699	*Amanita hemibapha*	262	1e-67
U85795	*Athelia bombacina*	250	4e-64
AB015701	*Amanita pantherina*	246	7e-63
AB015700	*Amanita muscaria*	246	7e-63

Sequence similarity was mainly within the 5.8S rDNA region.

Three putative plasmodiophorid specific primers were designed based on these published sequences. These were:

PLA - 5' gga gtg gtc gaa ctt cat taa att t 3' (position 299-323 of GenBank Accession Y12831)

POL - 5' tct tac ctc atc tga gat ctt g 3' (position 803-782 of GenBank Accession Y12824)

LIG - 5' gca cac atc tgg gga atg gtt t 3' (position 54-75 of GenBank Accession Y12828)

Amplification of ITS rDNA from *S. subterranea* f. sp. *nasturtii* and other plasmodiophorids

Primers designed by analysis of the published plasmodiophorid sequences (Ward and Adams, 1998) were successful in amplifying plasmodiophorid DNA, using the same PCR conditions as for ITS1F/4. Figure 3.6 indicates the sequence obtained from amplifying from crooked root DNA using ITS5, a universal primer (White *et al.*, 1990) and POL. The size of the complete fragment was around 510bp. Table 3.3 shows that the BLAST similarity search of this DNA matches it most closely to *S. subterranea* f. sp. *subterranea* ITS rDNA, but that there are significant differences (Figure 3.6). POL did not appear to be suitable for binding to *Plasmodiophora brassicae*, based on sequence information (Ward and Adams, 1998), and therefore PLA/ITS4 was used in combination to amplify its ITS rDNA. Likewise, with *Ligniera* amplification, the combination LIG/ITS4 was used.

```
1    GTGGAAGTAA AAGTCGTAAC AAGGTTTCCG TAGGTGAAGA ACCTGCGGAA
51   GGATCATTAA CACTGATGTC GGCCTCGGCC GACGCACCCC CAAACAACTT
101  GTGAATTTCA TGACCGTTTG TCATTTTTAA CAATCAAAAA TACAAACCTA
151  ACGAAAAACA ATAACCAATA TACAACTCTT AGCAATGGAT ATCTTGGTTC
201  CCACAACGAT GAAGAACGCA GCGAAATGCG ATACGTAATG CGAATTGCAG
251  AATTCAGTGA ATCATCAAAT CTTTGAACGC AAGTTGCGCT TTCGAGATAT
301  CCTTGAAAGC ATGCCTCTTT GAGTATCGGT TTCCATGTTT GCAATTCGTT
351  TACGAAACTG CAAGTGAATG AGTTTGCCAG AGTCAAATCT GTCTGCCTCG
401  AAATGCAATC GGCCGCAAGT CAAACTGCAC TCGTACTTTT CGACACACAA
451  GTCGAGCGCG GGCGGCCAAC AAACCTTTAC CAATCAAGAT CTCAGATGAG
501  GTAGGAAT
```

Figure 3.6 ITS rDNA of *Spongospora subterranea* f. sp. *nasturtii*, amplified using primers ITS5 and POL.

TABLE 3.3 Database sequences showing similarity to *S. subterranea* f. sp. *nasturtii* ITS rDNA. Sequence comparisons were made over 508 bases in total.

GenBank accession number	Species showing similarity	BLAST similarity scores	E-value
AF102820	*S. subterranea* f. sp. *subterranea*	301	1e-79
AF102819	*S. subterranea* f. sp. *subterranea*	301	1e-79
AF104308	*S. subterranea* f. sp. *subterranea*	301	1e-79
Y12829	*S. subterranea* f. sp. *subterranea*	301	1e-79
AF231027	*P. brassicae*	268	2e-69
Y12826	*P. graminis*	268	2e-69
Y12831	*P. brassicae*	268	2e-69
Y12824	*P. graminis*	256	7e-66
Y12825	*P. graminis*	250	4e-64
Y12827	*P. betae*	234	2e-59
Y12828	*Ligniera* sp.	204	2e-56

PLA/ITS4 was capable of amplifying *Plasmodiophora brassicae* from clubbed roots, LIG/ITS4 amplified *Ligniera* from infected plant material supplied (M.Adams, IACR-Rothamstead) and ITS5/POL was successful in amplifying DNA from *Polymyxa betae* (M. Adams), *Polymyxa graminis* (M. Adams) and *S. subterranea* f. sp. *subterranea* resting spores (K. Bell, SCRI) using supplied material also (Figure 3.7). PCR of *S. subterranea* f. sp. *subterranea* DNA, following the genomic DNA extraction method of Raeder and Broda (1985) on spore balls, was not always successful.

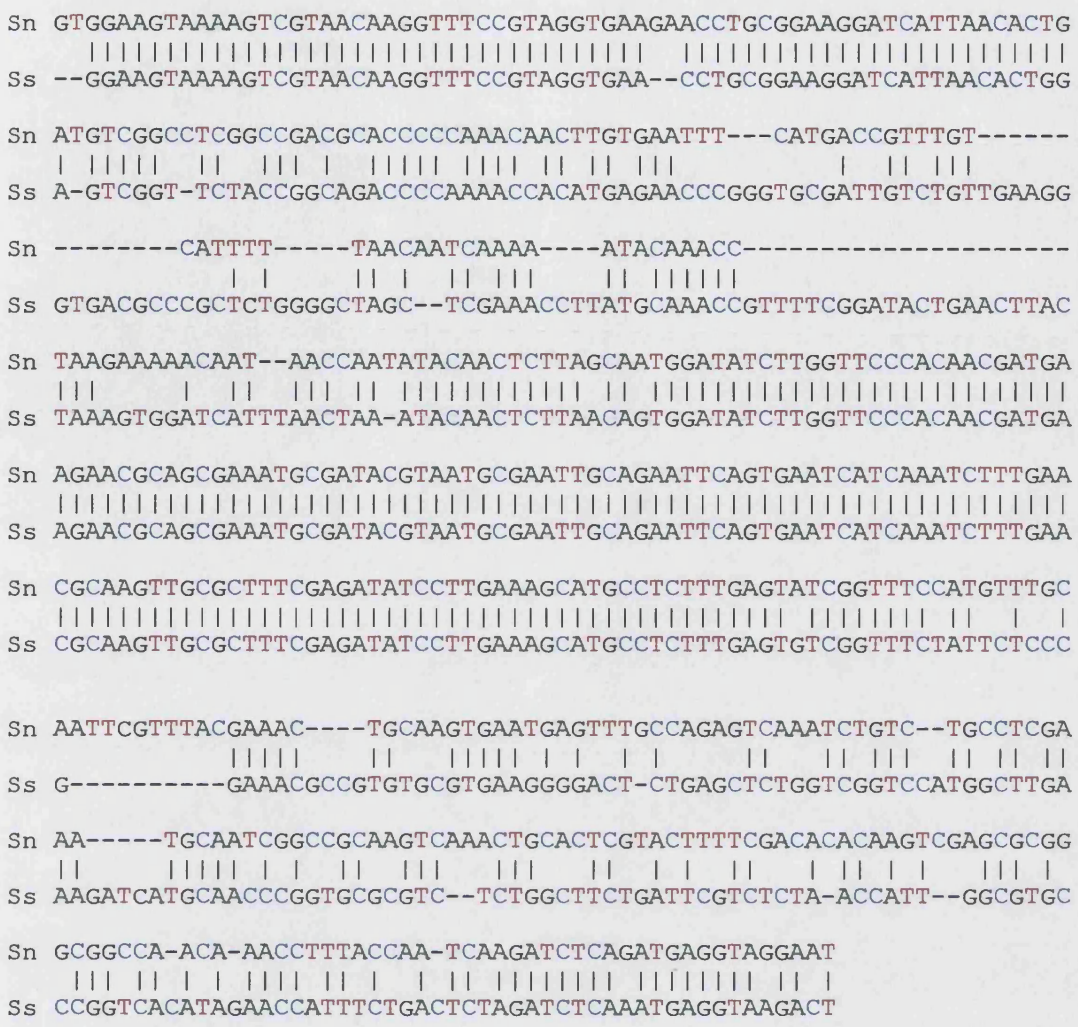


Figure 3.7 Alignment of *S. subterranea* f. sp. *nasturtii* ITS rDNA (Sn) with *S. subterranea* f. sp. *subterranea* ITSrDNA (Ss) using ClustalW alignment. A gap opening penalty of 10, and gap extension penalty of 0.05 were incorporated.

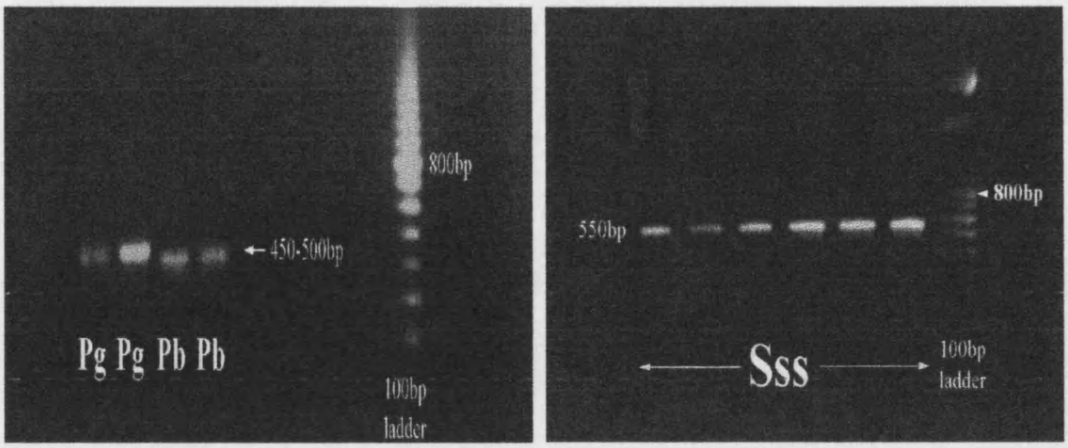


Figure 3.8 Amplification of *Polymyxa graminis* (Pg), *Polymyxa betae* (Pb), and *Spongospora subterranea* f. sp. *subterranea* (Sss), using the primer designed for plasmodiophorids (POL), in combination with ITS5.

Testing for geographic diversity of *S. subterranea* f. sp. *nasturtii* ITS rDNA

It was necessary to examine the geographic conservation of *S. subterranea* ITS rDNA, so that any specific primers designed would be suitable for a diagnostic test on *S. subterranea* f. sp. *nasturtii* samples from all U.K. watercress farms. Crooked roots were sampled from the following locations.

TABLE 3.4 Sites from which crooked root samples were collected for analysis of *S. subterranea* f. sp. *nasturtii* ITS rDNA diversity

Site (and samples taken)	Address
Warminster (4 samples - B, F, G, H)	Hurd's Spring Valley Watercress, Hill Deverill, Warminster, WILTS.
Bere Regis (BR)	Vitacress Salads, Doddings Farm, Bere Regis, DORSET.
Dorking (DK)	R. Coe & Sons, Abinger Hammer, Dorking, SURREY.
St. Mary Bourne (SM)	Vitacress Salads, St.Mary Bourne, HANTS.

Genomic DNA was extracted, and amplified using ITS5/POL.

PCR products were obtained from all sites tested (Figure 3.9 - Warminster samples not shown here) using ITS5/POL as the primer combination. The PCR products appeared equal in size when run on 1% agarose gels, except on one occasion with a DNA sample from Bere Regis where multiple bands were observed. Subsequent sequencing indicated that the products of expected size (510bp) were identical from all locations. The possibility that contamination of PCR components had occurred, leading to the same DNA being amplified in all cases, was ruled out, as negative controls, lacking only template DNA during PCR, showed no amplification.

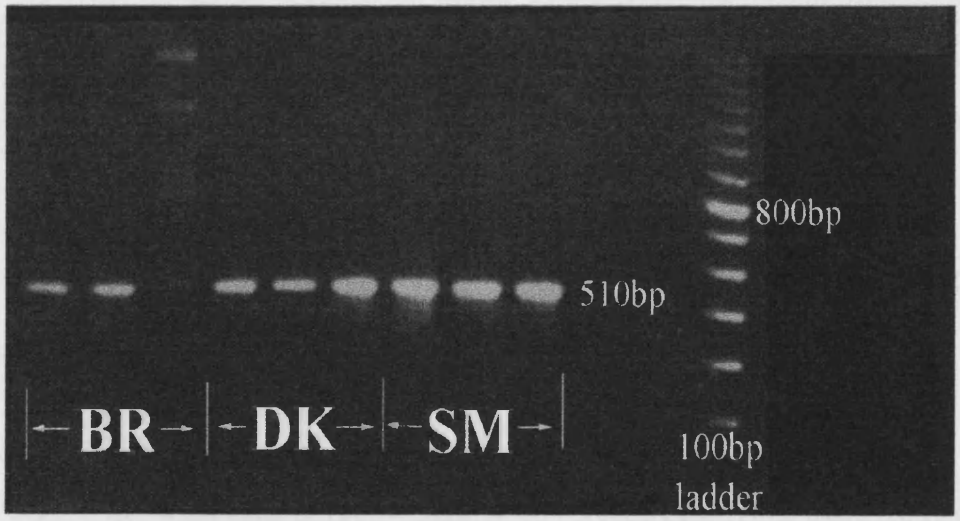


Figure 3.9 Amplification of *S. subterranea* f. sp. *nasturtii* ITS rDNA using primers ITS5/POL. DNA samples from sites at Bere Regis (BR), Dorking (DK), and St.Mary Bourne (SM). Multiple bands can be seen with one BR sample.

Design of specific primers for *S. subterranea* f. sp. *nasturtii* DNA

Following confirmation of sequence conservation of the *S. subterranea* ITS rDNA, the sequence was aligned with other plasmodiophorid ITS rDNA's, using the program PileUp (Figure 3.10), in the manner described previously. Several regions were found to be unique to *S. subterranea* f. sp. *nasturtii*, on either side of the 5.8S rDNA gene.

```
Ssn   GTGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACACT
Sss   -------------------------TTCGTTAGGTGAACTGCGGAAGGA-CATTAACACT
PBr   GTGTGCGTCACATGCACATATACG-TTCCGTAGGTGAACCTGCGGAAGGATCATTAACACA
Pg    GAGGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGCGTT
Pb    GAGGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGC---
Lig   ---GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGC---
Chyt  GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTA-----

Ssn   GATGTCGGCCTCGGCCGACGCAC----CCCCAAACAAC--------------------TTG
Sss   GAGTCGGTTCTACCGGCAGACCCAAAACCACATGAGAACCTGGGTGCGATTGTCTGTTGAA
PBr   GTGGGCGGCCCTAGCGCTGCATC----CCATATCCAACCCCATGTGAACCGGTGACGTGCG
Pg    GAATGGTTGTTGCCATTTCGTAAAAATGTGGATCGTCTCTGTTGCTGGATACCGGGATGGA
Pb    -------------------GTTATGGGATTTTGTAATCCTTTGGTTAAATGTGGAATTTGA
Lig   ---------ACACATCTGGGGAATGGTTTCCTTTGGCTCTATGTGGACATTGGGTAATGTG
Chyt  ------------------------------------------------ATAAATGCTTCAT

Ssn   [redacted]TGTCATTTTTAACAATCAAAAATACAAA-----CCTAACGAAA-
Sss   GGGTGACGCCCGCTCTGGGGCTAGCTCGAAACCTTATGCAAACCG-----TATTACTGAAC
PBr   GCGACTCCAGCTGCGTGTTTCATTTTCGAACCATCCTAGCCGAAA-----CACGACTAAAG
Pg    ACGCCCTCGTGGTGGTTCTTGTCTTTCACGAATTGGATCAAAACGGTGGCTAAAGTCGAAT
Pb    ACAAGTGACTTGGTAATCTTTACGAATTGGAAACGT----------------------T
Lig   TCAGCGCGGTGGTTCCTTTTTGGAAACGGGTTATGTAT-----------TTATCGAAGGCG
Chyt  TGCATCCACCTTTTGTGCACCGTAAATTTTCACTTAAACTTTGTCTGATTTATATCTGTTC

Ssn   ------AACAATA------ACCAATATACAACTCTTAGCAATGGATATCTTGGTTCCCACA
Sss   TTACTAAAGTGGATCGTTTAACTAAATACAACTCTTAACAGTGGATATCTTGGTTCCCACA
PBr   TTCCATACATACA------TACATGTTACAACTCTTAGCAATGGATATCTTGGTTCTCACA
Pg    TGGTTTCATAACCTACAAACAATATATACAACTCTTAGCAATGGATATCTTGGTTCCCACA
Pb    TGGCTTATGGATACATTTATAAAAAATACAACTCTTAGCAATGGATATCTTGGTTCCCACA
Lig   CTCTTATGGAAATTGCATCAACAACGAACAACTCCTAGCAATGGATATCTTGGTTCCCGCA
Chyt  TGAAAGGAACATGAATTGTAATCTAATACAACTTTTAACAATGGATCTCTTGGCTCTCGCA

Ssn   ACGATGAAGAACGCAGCGAAATGCGATACGTAATGCGAATTGCAGAATTCAGTGAATCATC
Sss   ACGATGAAGAACGCAGCGAAATGCGATACGTAATGCGAATTGCAGAATTCAGTGAATCATC
PBr   ACGATGAAGAACGCAGCGAACTGCGATACGTAGTGCGAATTGCAGAATTCAGTGAATCATC
Pg    ACGATGAAGAACGCAGCGAAATGCGATACGTAATGCGAATTGCAGAATTCAGTGAATCATC
Pb    ACGATGAAGAACGCAGCGAAATGCGATACGTAATGCGAATTGCAGGACTCAGTGAATCATC
Lig   ACGATGAAGAACGCAGCGAAATGCGATACGTAGTGCGAATTGCAGAATTCAGTGAATCATC
Chyt  TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
```

```
Ssn   AAATCTTTGAACGCAAGTTGCGCTTTCGAGATATCCTTGAAAGCATGCCTCTTTGAGTA
Sss   AAATCTTTGAACGCAAGTTGCGCTTTCGAGATATCCTTGAAAGCATGCCTCTTTGAGTG
PBr   AAATCTTTGAACGCAAGTTGCGCTTCCGAGATACCCTTGGAAGCATGCCTCTTTGAGTG
Pg    GAATCTTTGAACGCAAGTTGCGCTTTCGAGA-GCCCTCGGAAGCATGCCTCTCTGAGTA
Pb    GAATCTTTGAACGCATGTTGCGCTTTCGAGA-GCCCTCGGAAGCATGCCTCTCTGAGTA
Lig   GAATCTTTGAACGCAAGTTGCGCTCGGGAGA-GCCCTTCCGAGCATGCTTCTTTGAGTG
Chyt  GAATCTTTGAACGCATATTGCGCTCTCTGGCATTCC-GGAGAGCATGCCTGTTTGAGTC

Ssn   TCGGTTTCCATGTTTGCAATTCGTTTACGAAACTGCAAGTGAATGAGTTTGCCAGAGTC
Sss   TCGGTTTCTATTCTCCCGGAAACGCCCTGTGCGTGGAAGGGGACTA----------TG
PBr   TCGGTTTC---TGTTCGTTTGCGCGCCCGCGCGCGCAAGACAATGAGCTTTGCTGCGGC
Pg    TCGGTTTCC-CATCTCACACCCGGTGTGGAGAATGAGGGTCGGGCGC-----------
Pb    TCGGTTCCTATATCACA----TCTTGATGTGGACAATGAGAGTCG-------------
Lig   TCGGTTTCCATCTCTCACCCCCCCGGGTGAGGACAGTGAGAGTCGGG----------
Chyt  TTGGTACCAATCTGGTGTAACCTTTGTATTAAGAATAGAACTTTTTATAAAAAAACTGC

Ssn   AAATC-TGTCTGCCTCGAAATGCAATCGGCCGC-AAGTCAAACTGCACTCGTACTTTTC
Sss   AGCTCTGGTCGGTCCATGGCTTGAAAGATTATCCAACCGGTGCGCGTCTCTGGCTTCTG
PBr   ATAGCTTGAACGAAGCGACCCGGGATCGTGCGCGCATTGCTGATACCCTCGAGAATGCC
Pg    ----CTCGGCGCCGGTCCCTTCAAATCAGGCCGGCCTGGTCAAGTCCATCGGATTGCTG
Pb    ------ATTCGTTCGTCTCTTGAAATCATGTCGGCAACCGAAAGTCCATCGGATTCTTG
Lig   ----GCGGTCGCCCTCTCTTGAAATTCGAGGCCGTAAGTCCAAGCGATTTCTGGGAAGC
Chyt  ATTGAGACGTCTTGATAAGTAAAATTGTTAAGTGTCTTAAAATACAATAGTACAGCTAT

Ssn   G--------ACACACAAGTCGAGCGCGGGCGGCCAACAAACCTTTACCAATCAAGATCT
Sss   ATTCGTCTCTANCCATTGGCGTGCCCGGTCNTATAGAACCATTTTTTGACTCTAGATCT
PBr   GGAGCGGTCCCCGCCGATCTGCGCGCAATCGCAGATCGACACACACACATCAAAGATCT
Pg    GTACGATAGTCCGTCATGGCGCAAGGAACCACTTGG---------------CAAGATCT
Pb    GAACGATAATCCGCCATGGCGCCCG----AACCATA---------------CAAGATCT
Lig   GTAACACAGTCGTTCATGGCAC-ACCAACCACACAC---------------ACAGATCT
Chyt  AGCTCACCCTCTTTGGAGTAATATGTGACCTGAAAGGGTAACTCTATTTCTCCTCGTTG

Ssn   CAGATGAGGTAGGAAT
Sss   CAAATGANGTAAGACT
PBr   CAAATGAGGTAGGATT
Pg    CAGATGAGGTAAGACT
Pb    CAGATGAGGTAAGATT
Lig   CAAATGAAGTAAGACT
Chyt  GGAAGACTTGTTGCTG
```

Figure 3.10 Multiple sequence alignment of plasmodiophorid ITS rDNA sequences using PileUp. The ITS rDNA of the Chytrid-like organism associated with crooked roots is also included. *Ssn* = *S. subterranea* f. sp. *nasturtii*, *Sss* = *S. subterranea* f. sp. *subterranea* (from spore balls supplied by SCRI), *PBr* = *P. brassicae*, *Pg* = *P. graminis* (sample F38, from infected plant material supplied by IACR-Rothamstead), *Pb* = *P. betae* (sample F41, same source as *Pg*), *Lig* = *Ligniera* sp. (sample F69, same source as *Pg*), Chyt = unidentified Chytrid-like organism. Putative specific primer binding sites for *Ssn* are indicated by colour coded regions, SPO1, NS4, SPO2, SPO1. SPO1amplifies from left to right, the other right to left on this diagram.

Specific primers were designed within this region, in the manner previously described.

These were:

SPO 1 - 5' ttg tga att tca tga ccg tt 3' (forward primer)

SPO 2 - 5' agg aga cag att tga ctc t 3' (reverse primer)

SPO 4 - 5' gta cga gtg cag ttt gac tt 3' (reverse primer)

A further forward primer (SSN18) was created, based on an apparently unique region of the 18S rDNA sequence of *S. subterranea* f. sp. *nasturtii*. BLAST searches using this primer revealed no matches to other 18S rDNA sequences. The sequencing of the 18S rRNA gene and the location of this primer are described in more detail in Chapter 4.

SSN18 - 5' att atc tcc gga tag ttc ttg ga 3'

This was tested in combination with SPO2 and SPO4, as well as NSA, which was designed to bind at the 5' end of the ITS rDNA.

NSA 5' gtc atg aaa ttc aca agt t 3'

PrimerCalc (Molecular Sensors Limited) was used to assess the suitability of potential primers selected. The binding sites of primers selected for testing is shown in Figure 3.10.

Testing putative *S. subterranea* f. sp. *nasturtii* specific primers

1. *Optimisation with DNA samples*

Optimal PCR conditions were established for all primer pairs, for amplification of DNA from crooked root total genomic DNA using Taguchi analysis as described by Cobb and Clarkson (1994). For SPO1/2 and SPO1/4 these conditions were:

0.1mM dNTP's each, 2mM Mg^{2+} ions, 30pmol primers and

1U *Taq* polymerase.

All other components were as described for ITS1F/4 amplifications. The cycling parameters were an initial denaturation of 94°C for 4 minutes, followed by 30 cycles of 94°C for 20 seconds, 58°C for 60 seconds, and 72°C for 24 seconds.

For SSN18/SPO2, SSN18/SPO4 and SSN18/NSA, optimal conditions varied slightly with the reverse primer used, but under the cycling conditions used for SPO1/2 and SPO1/4 combinations, were generally: 0.1mM dNTP's each, 2mM Mg^{2+}, 30pmol primers, 1U *Taq* polymerase.

Primers were subjected to a BLAST search in order to establish their likelihood for binding to non-*Spongospora* DNA. Although individual primers showed some similarity to a few sequences on GenBank (usually cosmids), in combination, no primer pair tested matched well with potential contaminants.

2. *Testing ability of primers to amplify DNA from various geographic locations*

The ability of primer pairs to detect *S. subterranea* DNA from a range of sites was assessed.

TABLE 3.5 Samples on which putative *S. subterranea* f. sp. *nasturtii* specific primers were tested, and how PCR products were identified as *S. subterranea*

Primers used	Sites and samples tested	Identified by product being of expected size	Identified by sequencing
SPO1/2	**Warminster** (samples A, B, E, F, G, H) **Dorking** (DK) **Bere Regis** (BR) **St. Mary Bourne** (J, K, L, SM)	All 11	DK + SM
SPO1/4	**Warminster** (A, B, F, G, H) **Dorking** (DK) **Bere Regis** (BR) **St. Mary Bourne** (J, K, L, SM)	All 11	
SSN18/SPO2	**Warminster** (B, G, H) **Bere Regis** (BR) **St. Mary Bourne** (L, SM)	All 6	Warminster (G)
SSN18/SPO4	As SSN18/SPO2	All 6	
SSN18/NSA	As SSN18/SPO2	All 6	

All primer combinations tested were capable of detecting *Spongospora* DNA from total genomic DNA taken from various sites at a single farm (J. Hurd, Warminster), and from a range of other farms. Figure 3.11 illustrates the consistency of size of product obtained using SPO1/2, SPO1/4, and SSN18/SPO2. Lack of DNA in

negative controls indicated no carry over of DNA from sample to sample. Sequencing of products obtained with SPO1/2, and SSN18/SPO2, confirmed that the DNA was from *S. subterranea*.

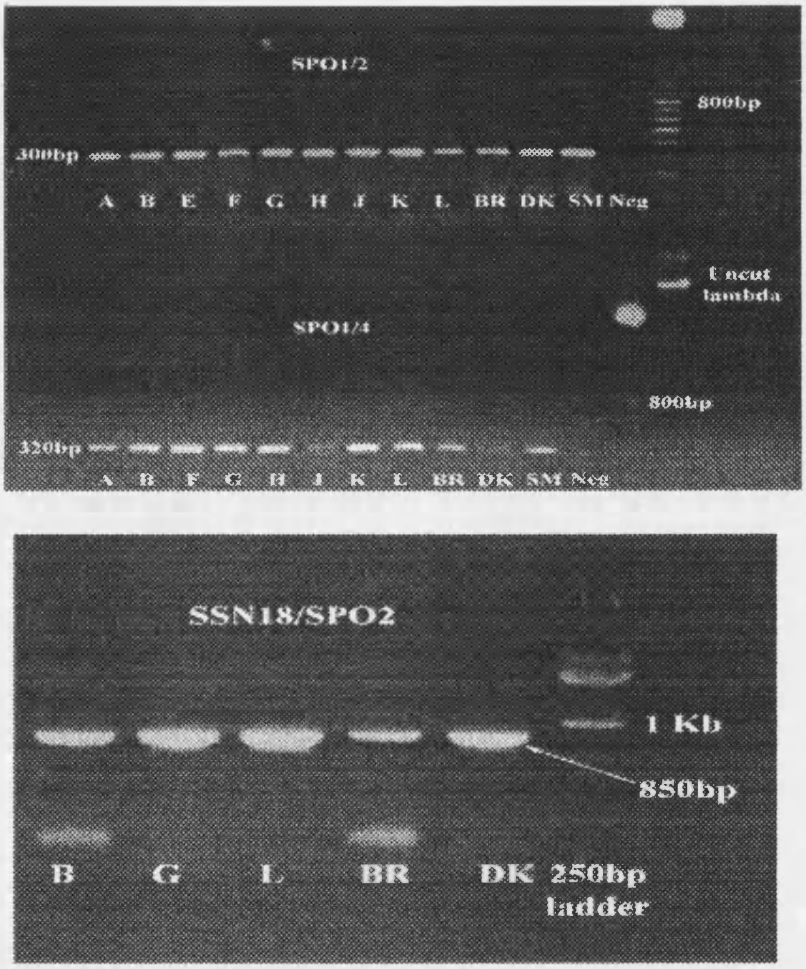


Figure 3.11 PCR amplification of *S. subterranea* f. sp. *nasturtii* rDNA using genomic DNA from material collected from a range of UK locations, as listed in Table 3.5.

3. *Testing the specificity of primers*

All primer pairs thus far tested were used in PCR reactions with genomic DNA from a range of organisms to check their specificity in amplifying *S. subterranea*. All DNA samples examined were first confirmed as being viable for PCR amplification by using

primers known to work with the organisms in question (e.g. IT5/4 for wheat, ITS5/POL for *P. brassicae*). DNA sources were:

Total crook root genomic DNA, *Spongospora subterranea* f. sp. *nasturtii* ITS rDNA, watercress (*Rorippa nasturtium-aquaticum*), *Spongospora subterranea* f. sp. *subterranea*[1], *Polymyxa graminis*[2] (+ plant material), *Polymyxa betae*[2] (+ plant material), *Ligniera* spp.[2] (+ plant material), *Plasmodiophora brassicae* (+ plant material), *Saccharomyces cerevisiae, Kluyveromyces* spp., ITS 1F/4 PCR product previously isolated from crooked roots (Chytrid-like organism), *Trichoderma harzianum, Agaricus bisporus, Verticillium dahliae, Metarhizium anisopliae, Triticum* spp.

1 = *S. subterranea subterranea* spore balls supplied by K. Bell and J. Duncan (SCRI)
2 = Infected plant material supplied by M. Adams (IACR-Rothamstead).
P. graminis = sample F 38 (see Ward and Adams, 1998)
P. betae = sample F 41
Ligniera sp.= sample F 69

SPO1/2 and SPO1/4 were tested at an annealing temperature of 54°C initially, and then again at 60°C annealing temperature. PCR products were visualised on agarose gels under UV light, and photographed.

SPO1/2 and SPO1/4 were not found to be specific to *S. subterranea* DNA when an annealing temperature of 54°C was employed. SPO1/2 showed some amplification of *S. cerevisiae*, and *T. harzianum*, while SPO1/4 were capable of amplifying *P. brassicae, S. cerevisiae, T. harzianum*, and a *Kluyveromyces* species. When the annealing temperature was raised to a level such that these DNA's were not amplified (60°C), the conditions were found to be too stringent to allow PCR of

S. subterranea.

SSN18/SPO4, and SSN18/NSA were also able to amplify DNA other than *S. subterranea* under conditions listed earlier for these primers (including a *Ligniera* spp. and a *Kluyveromyces* spp.). With an annealing temperature of 58°C, and under cycling parameters listed earlier, SSN18/SPO2 amplified DNA only from *S. subterranea* (Figure 3.12). In all tests, control reactions with no DNA showed no amplification.

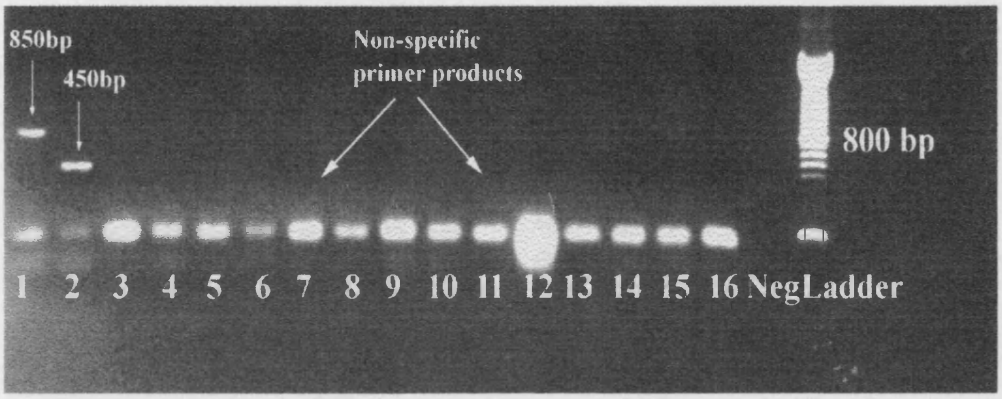


Figure 3.12 Testing the ability of SSN18/SPO2 primer pair for ability to amplify from a range of template DNA's.
1 = crook root total genomic DNA 2 = *Spongospora subterranea* f. sp. *nasturtii* ITS rDNA
3 = watercress (*Rorippa nasturtium-aquaticum*) 4 = *Spongospora subterranea* f. sp. *subterranea*
5 = *Polymyxa graminis* 6 = *Polymyxa betae* 7 = *Ligniera* sp. 8 = *Plasmodiophora brassicae*
9 = *Saccharomyces cerevisiae* 10 = *Kluyveromyces* sp. 11 = ITS 1F/4 PCR product of chytrid-like organism associated with crooked roots 12 = *Trichoderma harzianum* 13 = *Agaricus bisporus*
14 = *Verticillium dahliae* 15 = *Metarhizhium anisopliae* 16 = *Triticum* sp. Neg = negative control

As SSN18 was within the 18S rDNA, it was not possible for this primer to bind to purified ITS rDNA from the unidentified chytrid-like organism associated with crook root (amplified with ITS1F/4). However, SPO2 alone did not produce a product, whereas it did with *S. subterranea* ITS rDNA, indicating specificity. In addition the

amplification product from total genomic crook root DNA, was purified and sequenced, and confirmed as *S. subterranea* f. sp. *nasturtii*.

4. *Testing the ability of primers to amplify DNA directly from zoospores*

Once primers had been tested for specificity, it was necessary to test their sensitivity in amplifying DNA under controlled conditions (i.e. known amounts of template). Following a chance observation that SSN18/NSA showed amplification of *S. subterranea* DNA (multiple products), in the absence of addition of magnesium to the PCR mix, the use of low levels of magnesium was investigated.

Figure 3.13 shows the results of PCR reactions incorporating 0.1mM dNTP's, 20pmol primers, 1-2 Units *Taq* polymerase, and magnesium ion concentration of 0.5mM. 1500 zoospores were used as template, with primer pairs SPO1/2, SSN18/SPO2, and SSN18/NSA. The T_A was 60°C.

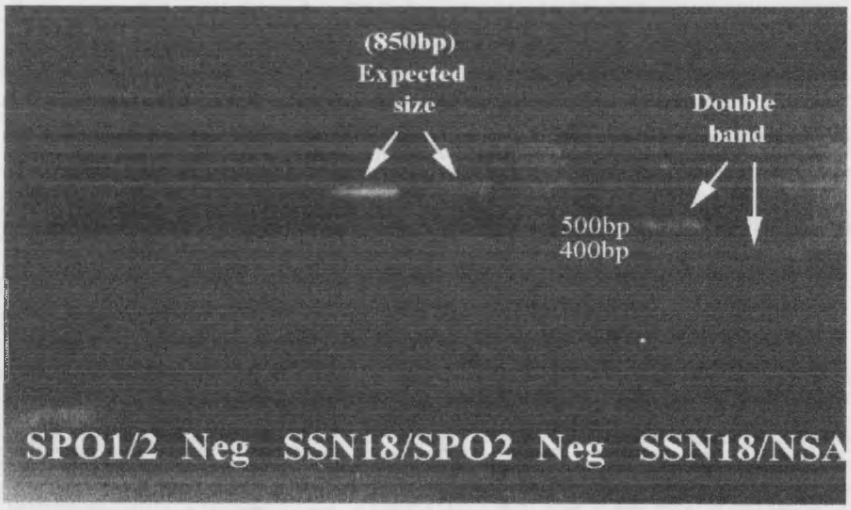


Figure 3.13 Attempts to amplify *S. subterranea* f. sp. *nasturtii* rDNA directly from zoospores using Mg2+ at 0.5mM only. Each primer pair indicated was tested in duplicate reactions, with negative controls (Neg) also run.

SPO1/2 failed to produce any product, SSN18/NSA produced faint double band products, including the expected 500bp band, whilst SSN18/SPO2 gave single band products of 8-900 base pairs, using 0.5mM or 1mM magnesium ion concentration. The results were consistently reproducible.

SSN18/SPO2 was selected as the most promising primer pair tested, and was eventually shown to produce strongest amplification of DNA with a T_A of 57°C. With a range of zoospore numbers optimal conditions were:

0.1mM dNTP each, 1mM Mg^{2+}, 20pmol primers, 0.5U *Taq* polymerase, with other components as ITS1F/4 reactions listed previously.

The cycling parameters were an initial 94°C for 4 minutes, then 30 cycles of 94°C for 40 seconds, 57°C for 45 seconds, and 72°C for 90 seconds. A final extension step of 72°C for 5 minutes, concluded the reactions.

Amplification of DNA was seen to be possible from as little as 5ng total genomic DNA, and 350 zoospores (Figure 3.14). Results with zoospores were variable, but amplification of DNA from less than 1000 was consistently possible.

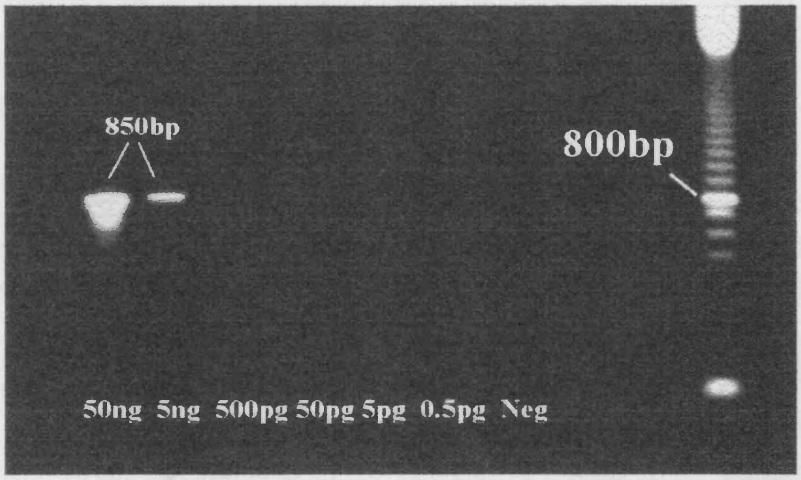


Figure 3.14 Amplification of *S. subterranea* f. sp. *nasturtii* DNA using SSN18/SPO2 PCR primers. The template DNA amount for each PCR reaction is indicated at the base of each lane.

TABLE 3.6 Summary results of testing of various primer pairs for ability to amplify *S. subterranea* f. sp. *nasturtii* DNA. SSN18/SPO2 was the most successful combination tested.

Primer pair	**Optimisation with DNA samples**	**Ability to amplify DNA from various locations**	**Specificity with *S. subterranea***	**Ability to amplify from zoospores**
SPO1/2	Successful	Successful	Unsuccessful	Unsuccessful
SPO1/4	Successful	Successful	Unsuccessful	Unsuccessful
SSN18/SPO2	Successful	Successful	Successful	Successful
SSN18/SPO4	Successful	Successful	Unsuccessful	Not tested
SSN18/NSA	Successful	Successful	Unsuccessful	Partial success

Development of field sampling techniques, and detection of *S. subterranea* f. sp. *nasturtii*

Following assessment of the ability of SSN18/SPO2 to amplify zoospores from lab-based samples, preliminary attempts were made to develop a field-based sampling procedure. Several techniques were initially employed to trap zoospores from watercress beds, before PCR with SSN18/SPO2 under conditions previously listed. Five basic sampling techniques were investigated.

1. Membrane filters with a pore size of 0.8 or 1.2µm (MilliPore) were attached to plastic rods, which were anchored in a watercress bed, near the outflow. Water then flowed past the filter, with the aim of trapping zoospores by random contact. Occasional success was obtained after allowing water to flow over the surface for 2-3 hours.

2. A second method was similar to the first, but the membrane was attached to the inside of a Buchner funnel by Vaseline, such that water flow was more directed than in method number one. PCR results were negative following this procedure.

3. Watercress root extracts were prepared as previously described in Chapter 2, and mixed 1:1, 1:10, or 1:1000 with low melting point agarose. 1ml of the mixture was then placed in a Bijou bottle, and this was placed in the watercress bed, with the mouth of the tube facing the direction of flow, such that zoospores would be washed into the tubes. PCR results were negative following this procedure.

4. One litre water samples were taken from the bed outflow, and returned to the laboratory, where they were filtered through a membrane filter, either passively, or under vacuum. A second, related, approach was to try and bait zoospores in the water samples, in the same way as in the field (though without directional water flow), with tubes removed for PCR at time-points from 1 minute up to 24 hours. PCR results were negative following both approaches.

5. Forty grams of plant material from the bed was returned to the laboratory, and washed for one hour in 1 litre of distilled water. The plant material was then removed, and the water either filtered or baited as in method four.

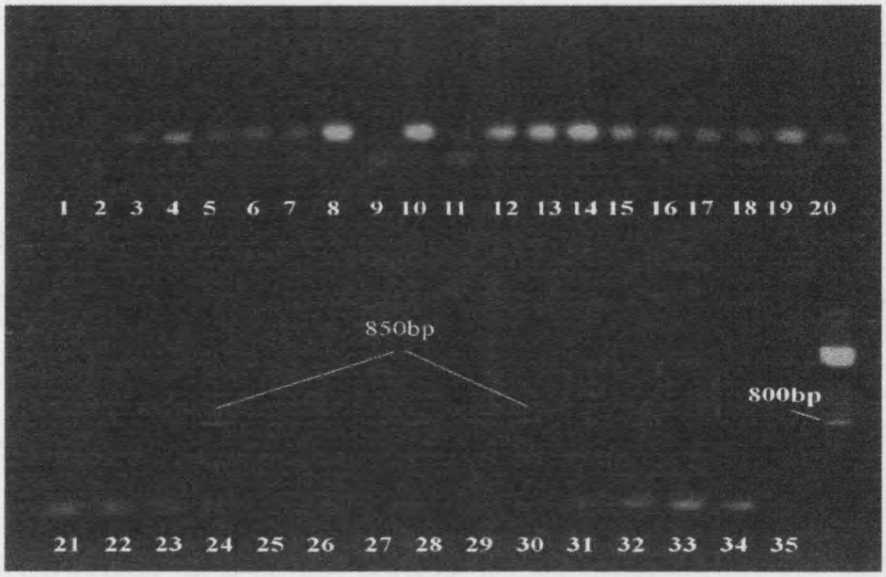


Figure 3.15 Attempts to amplify zoospores of *S. subterranea* by PCR from field-collected samples. Sampling techniques are indicated by lane numbers on the gel photograph.

Lane 1 = Filter placed in bed for 1 hour
2 = " " " " " 2 hrs
3 = " " " " " 3 hrs
4 = " " " " " 6 hrs
5 = " " " " " 24 hrs
6 = 1:1 root extract:agarose bait in bed for 1 hr
7 = " " " " " " " " 2 hrs
8 = " " " " " " " " 3 hrs
9 = " " " " " " " " 6 hrs
10 = " " " " " " " " 24 hrs
11 = 1:10 " " " " " " " 1 hr
12 = " " " " " " " " 2 hrs
13 = " " " " " " " " 3 hrs
14 = " " " " " " " " 6 hrs
15 = 1:1000 " " " " " " " 1 hr
16 = " " " " " " " " 2 hrs
17 = " " " " " " " " 3 hrs
18 = " " " " " " " " 6 hrs
19 = 1:1000 root extract:agarose bait in bed for 24 hrs
20 = Agarose only bait for 1 hour
21 = " " " " 2 hrs
22 = " " " " 3 hrs
23 = " " " " 6 hrs
24 = 40g roots → wash for 1 hr → filter
25 = 1 litre of water (outflow) → filter
26 = Negative control
27 = 40g roots + 1:1 bait for 1 hr → PCR
28 = " " " " " 2 hr → "
29 = " " " " " 3 hrs → "
30 = " " " " " 6 hrs → "
31 = 1 litre of water + 1:1 bait for 1 hr
32 = " " " " " " " 2 hrs
33 = " " " " " " " 3 hrs
34 = " " " " " " " 6 hrs
35 = 1000 zoospores in 1 litre + 1:1 bait for 1 hour.

Successful samples were obtained by following method 5 above, and baiting zoospores (see Figure 3.15), but consistent PCR products were only achieved when the following procedure was adopted.

40 grams of plant material was removed from diseased watercress beds, and incubated in distilled water for 1 hour. The roots were then removed, and the water filtered through 1.2μm cellulose ester filters. The filters were then rinsed in up to 1ml of MilliQ water, and 10μl of this added to a PCR mix.

Modifications made to this method included changing the volume of water in which roots were washed (40g in 1l, 20g in 100ml, 20g in 1l, 10g in 100ml, 10g in 20ml), and also the washing time (1min, 10 min, 30min, 60min, 120min, 180min, 6 hours, and 24 hours). It was possible to generate PCR products from an original 20g of plant material washed in 100ml distilled water (see Figure 3.15). It was sometimes possible to obtain positive results with washing times of as little as 1 minute for the plant material, but 1 hour was effective on 14 out of 15 occasions tested with 40g in 1l or 20g in 100ml starting material. The presence or absence of PCR products and the microscopic observation of zoospores in samples collected did not correlate in a quantifiable manner (Table 3.7).

TABLE 3.7 Correlation between number of zoospores in samples collected from watercress beds and estimated numbers detected by PCR (compared to known zoospore concentrations).

Week sampled	**Zoospore count**	**Estimated limit of zoospore detection by PCR**
Oct 14, 1999	2.5×10^4 ml^{-1}	2×10^4 ml^{-1}
Nov 11, 1999	$3.5 \times 10^4 ml^{-1}$	3×10^3 ml^{-1}
Nov 18, 1999	2×10^4 ml^{-1}	0

The brightness of EtBr stained bands on agarose gels was compared to those generated by PCR of known amounts of zoospores in control reactions. Band brightness did not always reflect the expected number of zoospores, and occasionally PCR bands were not detected, although zoospores were observed.

To test the limits of detection of the system, known amounts of zoospores released from crooked roots were diluted and filtered, before PCR. The results are presented in Figure 3.14, and it can be seen that as few as 100 zoospores were detectable.

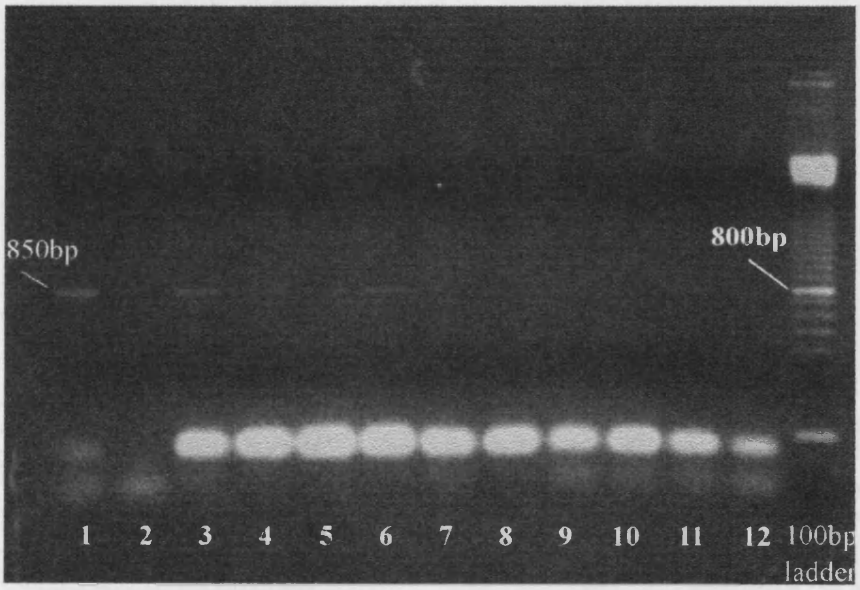


Figure 3.16 PCR from *S. subterranea* f. sp. *nasturtii* zoospores using SSN18/SPO2 primers.

Lanes 1and 2 = 20g roots washed in 100ml dH2O► filtered

Lane 3 = 2500 zoospores in 1 litre ►filtered
Lane 4 = 1000 " " " " "
Lane 5 = 500 " " " " "
Lane 6 = 100 " " " " "
Lane 7 = 50 " " " " "

Lane 8 = 20 zoospores in 1 litre► filtered
Lane 9 = 10 " " " " "
Lane 10 = 5 " " " " "
Lane 11 = 1 " " " " "
Lane 12 = Negative control

Accurate quantification of *S. subterranea* DNA present in field samples was attempted using the LightCycler - DNA Master SYBR Green I system (Roche). Total genomic crooked root DNA was used to optimise the LightCycler system with respect to the amount of magnesium required for successful PCR amplification, with a total of 3mM proving optimal. The SSN18/SPO2 PCR product was successfully cloned into a pGEM-T Easy plasmid vector (Promega), and plasmid copy number quantified by UV spectrometry at 260nm. Assuming that all plasmids contained an insert, the estimate of DNA concentration was 30-40ng μl^{-1}. Using 20µl reaction volumes, containing $MgCl_2$, H_2O, 20pmol SSN18 and SPO2, and 2µl DNA Master SYBR Green I (containing fluorescent dye, and DNA polymerase), a dilution series of plasmid insert DNA was amplified. The results are shown in Figure 3.17.

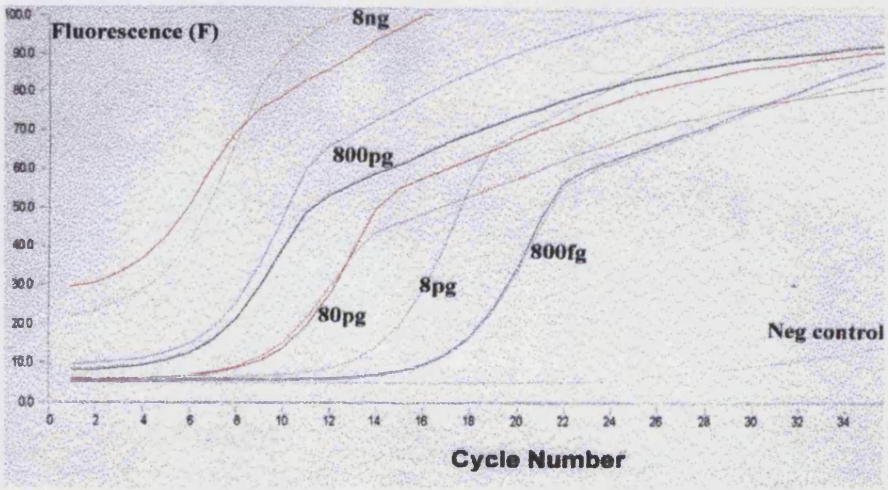


Figure 3.17 Output from PCR reaction using a LightCycler-DNA Master SYBR Green I system (Roche). The number of PCR cycles is indicated along the x-axis, with amount of double-stranded DNA produced indicated by fluorescence of SYBR Green on the y-axis. The template DNA for the reaction was *S. subterranea* SSN18/SPO2 PCR product cloned into a plasmid vector, and was amplified using the same primers. An estimate of the starting concentration of DNA is shown on the graph, with each mass tested in duplicate.

Amplification of DNA from zoospores was not possible using the LightCycler system, either due to the presence of inhibitors, or the presence of too few *S. subterranea* zoospores in samples.

Confirmation of identity of PCR products from field samples

Amplification of DNA from zoospores often resulted in very faint bands on agarose gels. Losses of DNA during subsequent purification meant that sequencing of DNA was not a suitable means of confirming the identity of the PCR product.

Southern hybridisation was used as a more sensitive technique.

A DNA probe was designed by amplification of the ITS II region of *Spongospora* DNA, using the primer pair SPO3/POL.

SPO3 - 5' tgt ttg caa ttc gtt tac gaa 3'

The 200bp probe itself was sequenced, and confirmed as *S. subterranea* f. sp. *nasturtii* DNA. Using this probe it was possible to detect DNA amplified from field samples with SSN18/SPO2.

As Figure 3.18 indicates, the probe was able to bind to PCR products from field samples, and gave a stronger signal than when using agarose gel electrophoresis. Interestingly, the presence of a second PCR product is revealed, which was only occasionally seen on EtBr stained gels. As the probe clearly hybridises to this product, which is approximately half the size expected (around 400 base pairs), then it must be *S. subterranea* DNA. The ratio of the two products appears to be relatively constant, with the full-length product always present in greater quantity. Double

bands were almost never detected on agarose gels, following amplification from DNA samples rather than zoospores.

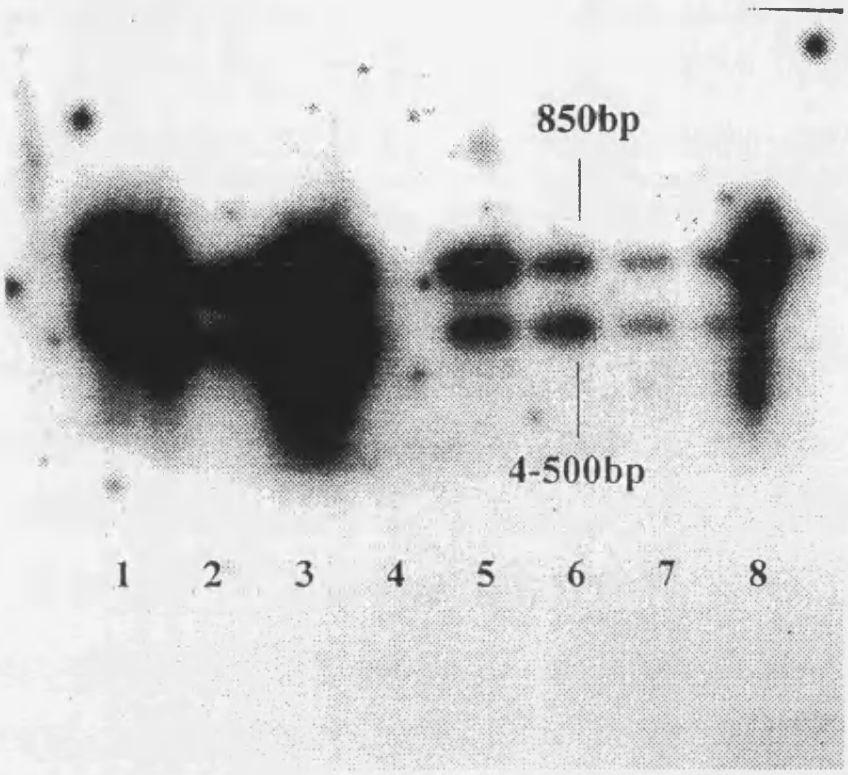


Figure 3.18 Southern hybridisation using a *Spongospora subterranea* f. sp. *nasturtii* specific probe to detect and identify PCR products amplified from zoospores.

Lanes 1and 2 = 40g roots washed in 1 litre dH_2O ► filtered ► PCR

Lane 3 and 5 = 20g " " " " " " " "

Lane 6 = 10g " " " " " " " "

Lane 7 = 5g " " " " " " " "

Lane 8 = Positive control from PCR reaction with genomic crook root DNA

Lane 4 = No samples

Elucidation of the role of the Chytridiomycete organism in crook root disease

The Chytridiomycete organism associated with crooked roots was already seen to be associated with all samples thus far analysed for ITS rDNA between October and April at J. Hurd's Spring Valley Watercress, near Warminster.

The geographic diversity of the fungus was demonstrated by amplification using ITS 1F/4 of eight samples from J.Hurd's farm, and three samples from other sites (St.Mary Bourne, Hants., Dorking, Surrey, and Bere Regis, Dorset). On some occasions, PCR of Bere Regis samples resulted in multiple PCR products (see Appendix D.), whilst on other occasions, only one product, of the expected size was observed. Sequencing of ITS 1F/4 PCR products from different farms confirmed the lack of geographic variation between populations.

To determine whether the unidentified contaminant species was significantly involved with crook root disease, samples of crooked roots were collected and treated in one of two ways. Some roots were washed thoroughly in tap water, while a second group was not. Total genomic DNA was extracted from both sets, as described previously, and quantified by agarose gel electrophoresis, alongside known amounts of uncut lambda DNA (Kramel Biotech). This DNA was denatured, and mixed in a 1:1 ratio with 20x SSC, before being pipetted onto a pre-wetted nylon membrane (Hybond-N; Amersham) in 2µl aliquots. Separate membranes were used for washed and unwashed root DNA, and duplicate membranes were prepared. Repeated additions of aliquots, allowed a dilution series of each treatment to be obtained, from 1µg down to 50ng. The DNA was subsequently denatured again in a solution of 1.5M NaCl and 0.5M NaOH, before neutralising in a solution containing 1.5M NaCl, 0.5M Tris-HCl (pH

7.2), and 0.001M EDTA. The DNA was fixed to the air-dried membrane using UV illumination for 3 minutes. As well as crook root DNA, ITS-rDNA from both *S. subterranea* and the unidentified Chytridiomycete was diluted 5000-fold, and spotted onto the membrane. 200ng watercress genomic DNA was used as a negative control. Washed and unwashed root DNA was probed using a *S. subterranea* specific probe (see earlier) prepared by the method described in the Material and Methods section of this Chapter, using SPO3/POL as primers. A probe for the unidentified organism was prepared in the same manner, using primers 305F and 461B (see Appendix C. for binding sites).

305F 5' acc aat ctg gtg taa cct ttg ta 3'

461B 5' caa gtc ttc cca acg agg aga aat a 3'

Probes were labelled with ^{32}P, and hybridised as previously detailed.

With the unwashed root DNA, both *S. subterranea* f. sp. *nasturtii* and the chytrid were clearly detectable from as little as 50ng starting material. The *S. subterranea* appeared to give a stronger signal than the Chytridiomycete DNA, although results were obscured somewhat at higher DNA concentrations, and are not shown here. The stronger signal with *S. subterranea* most likely reflected the presence of more DNA, but could also be due to higher binding capacity of this probe. With washed roots, *S. subterranea* was still detected strongly (Figure 3.19), and results were seen from 50ng starting material. The signal from the chytrid blot was much weaker, undetectable below 500ng total DNA. This suggests that there was much less Chytridiomycete species DNA present in washed roots than there was *S. subterranea*.

Hybridisation to watercress DNA was absent, apart from perhaps a faint trace, which could be due to the presence of very low levels of chytrid DNA.

The specificity of the probes was demonstrated by using each of them to bind to ITS rDNA of both *S. subterranea* and the chytrid. Each probe was capable of hybridising only to its corresponding PCR product, and not to the other species (Figure 3.20).

To examine further any role that the Chytridiomycete might have in crook root disease, genomic DNA collected in July 1998 was amplified with ITS 1F/4 and the products sequenced. These were found to be from the chytrid, and were present in DNA obtained from healthy plants.

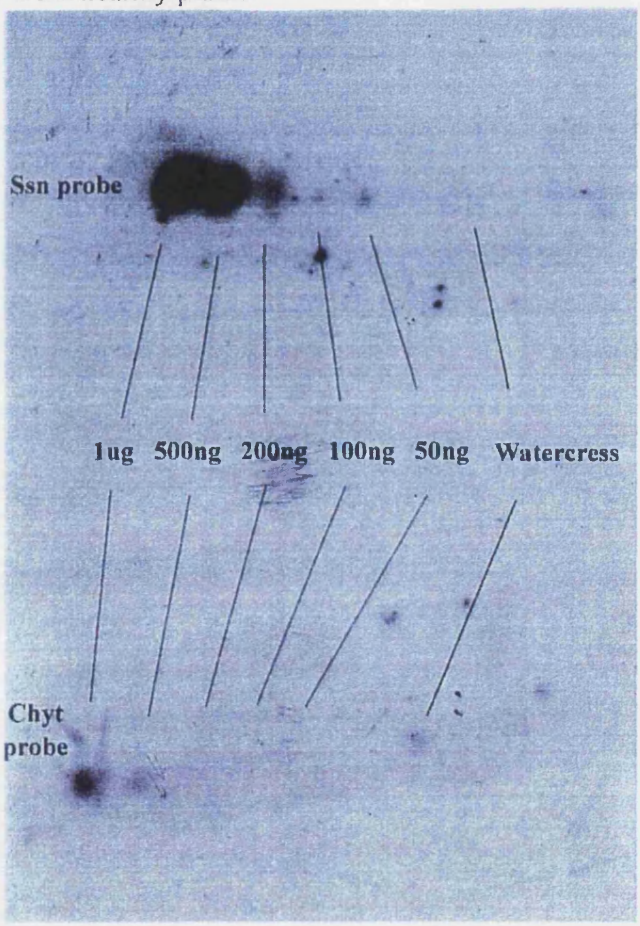


Figure 3. 19 Southern hybridisation of genomic DNA from washed crooked roots, using probes specific to either *S. subterranea* f. sp. *nasturtii* or to the chytrid-like organism associated with crooked roots.

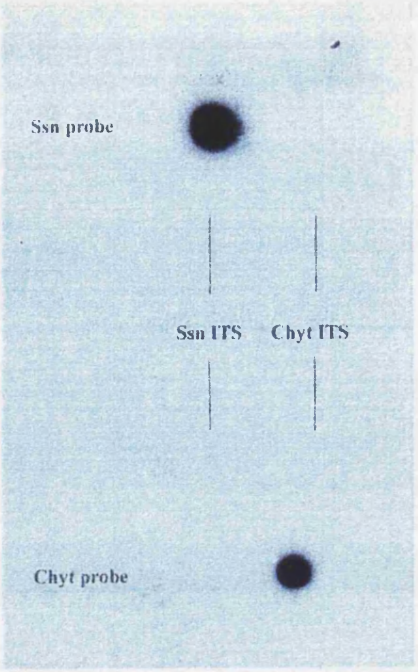


Figure 3. 20 Testing the specificity of *S. subterranea* f. sp. *nasturtii* and chytrid probes by attempting to hybridise each probe to the other's target sequence.

Investigation into zoospores associated with seedlings grown in the laboratory

Microscopic observation revealed zoospores in nutrient solution surrounding watercress seedlings, which had been germinated in the laboratory. The seed had been collected in 1992 by J. Claxton, and were sowed directly into nutrient solution, in which up to 2×10^5 ml^{-1} zoospores were then observed within 2 days of seed germination. Pre-treatment of the seeds with sodium hypochlorite eliminated the appearance of any zoospores, implying that the contaminant was either associated with the seed surface, or with plant debris found amongst the seeds.

PCR of the zoospores with chytrid and *S. subterranea* f. sp. *nasturtii* specific primers revealed that both were associated with the seed, but that the chytrid zoospores appeared to be present in much higher quantity. Sequencing of the Chytridiomycete PCR product confirmed its identity, whilst the presence of *S. subterranea* was

confirmed by amplification of genomic DNA using SSN18/SPO2 as primers (Figure 3.21).

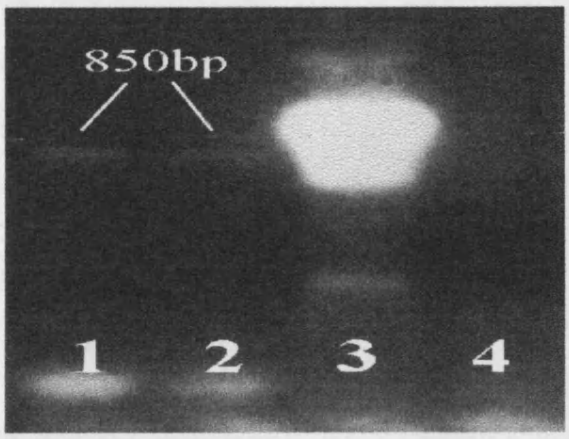


Figure 3.21 PCR amplification of zoospores associated with watercress seed using SSN18/SPO2 as primers (Lanes 1 + 2). Lane 3 indicates the same reaction with genomic crooked root DNA as template. Lane 4 shows a negative control reaction.

Under light microscopy (x 100), the majority of the zoospores, assumed to be the chytrid, were 6-7µm in length, slightly cone-shaped, with an apparently posterior flagellum/flagella, and a granular cytoplasm.

SEM was used in an attempt to visualise both resting spores and zoospores associated with seed. A possible spore ball (group of resting spores) washed off of the seeds was seen, but it was perhaps more regular and crystalline than expected, and also high in calcium, although irregular channels could be seen, as observed previously with *S. subterranea* (Hutchison and Kawchuk, 1998). Zoospores released into the nutrient solution surrounding seedlings, were not well observed using electron microscopy. However, Figure 3.22 shows structures resembling zoospores (see Bryngelsson *et al.*, 1988 on *P. brassicae*), but lacking the adhesorium of

S. subterranea f. sp. *nasturtii* observed by Claxton (1996), except on a few occasions, as in Figure 3.23, where one is possibly present.

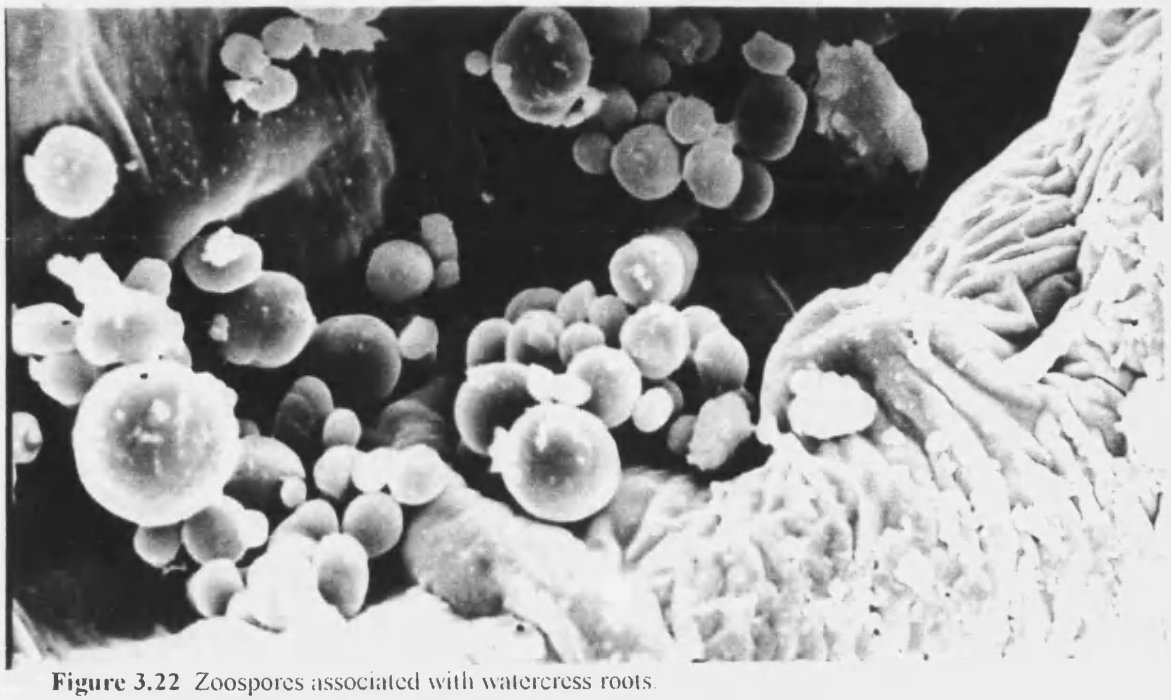


Figure 3.22 Zoospores associated with watercress roots.

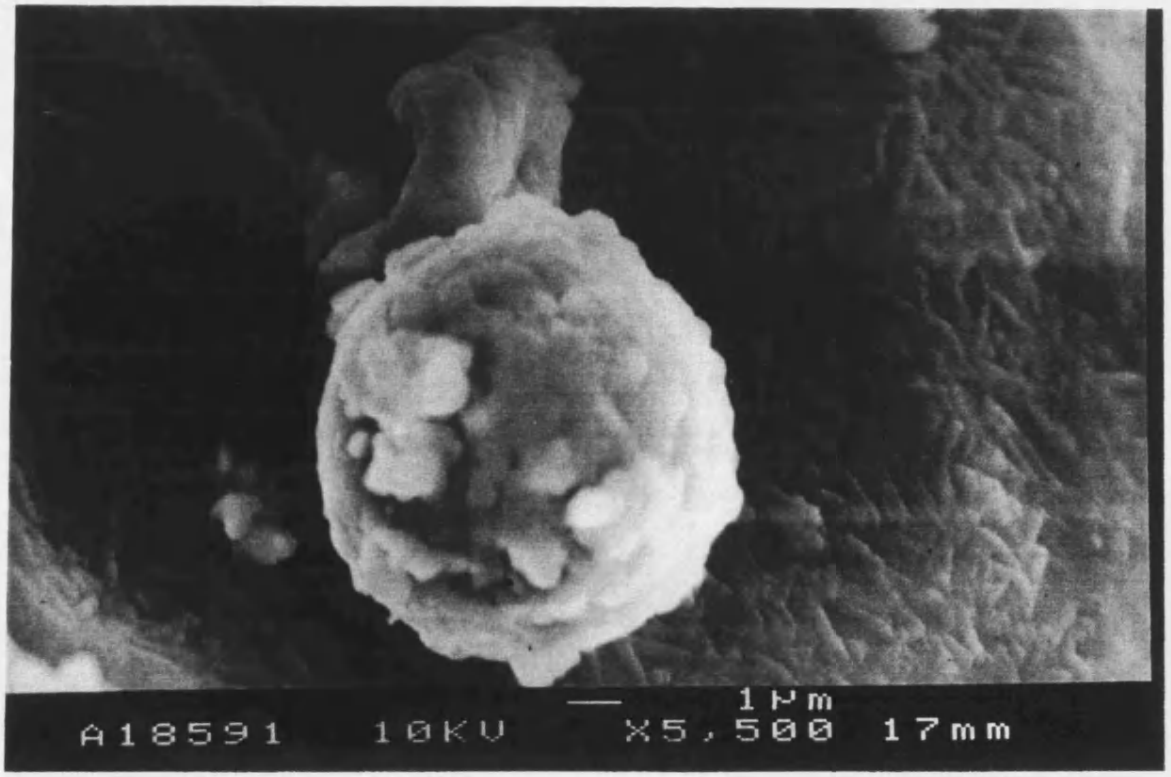


Figure 3.23 Zoospore associated with watercress roots, showing a possible adhesorium.

3.3 DISCUSSION

This study has used both 18S and ITS rDNA data from *Spongospora subterranea* f. sp. *nasturtii* to design specific primers capable of detecting *S. subterranea* in field collected samples. The laboratory procedures could be completed within one day and preliminary tests indicated that it was possible to use the postal system to send samples from farms to laboratories for testing. A two day transit at ambient temperatures did not prevent PCR amplification of *S. subterranea* f. sp. *nasturtii* zoospores (results similar to those shown in Figures 3.15 and 3.16), making the whole test a 3 day process on average. The zoosporic life-cycle of *S. subterranea* can be completed within 7 days (Tomlinson, 1958b), and so the ability to inform farmers of results within 3 days of sampling should allow prevention of all but a few early season infections, and allow optimisation of subsequent zinc usage.

Field-based diagnostic testing

Field-based diagnostic tests have been developed for microbes found in water and soil, but these have generally utilised serological reactions rather than analysis of DNA. Cahill and Hardham (1994b) developed a monoclonal antibody attached to *Phytophthora cinnamomi* cysts, which were incorporated into soil bound to a nylon membrane. Results indicated that the detection limit was 250 zoospores ml^{-1} soil-water suspension. Ali-Shtayeh *et al.* (1991), described a very similar sampling technique to that used for *S. subterranea* f. sp. *nasturtii*, where water samples containing either *Phytophthora* or *Pythium* were filtered through a membrane with a

pore size small enough to retain spores of the pathogen. They also found that sensitivity of the test was dependent on the amount of water that could be passed through the filters. Following filtration, samples were tested by ELISA, not PCR, but there seems no reason why the technique could not be adapted for PCR. Certainly, the diagnostic test developed for *S. subterranea* f. sp. *nasturtii* has advantages over the perceived problems with antibody kits, which Werres and Steffans (1994) described as including cross-reactivity, and lack of correlation with biomass.

In developing the field sampling stage of the diagnostic test, several approaches were investigated. Ideally, perhaps, a field-based filtration system would be employed, but this was not successful in trials (see Figure 3.15), maybe due to a low level of zoospores in free-flowing water. The lack of inoculum obtained from water samples collected at bed outlets, compared to those from washed plant material, strongly suggests that zoospores remain very closely associated with the plant surfaces between infection cycles. The fact that zoospores are bounded only by a fragile membrane would certainly help to account for such behaviour.

Some PCR-based diagnostic tests have been developed for material collected on site, and then treated in the laboratory, as in this study. The water-borne human pathogen, *Cryptosporidium parvum*, has been detected down to a limit of one cyst in up to 100 litres of water, using PCR, followed by digoxygenin labelling (Hallier-Soulier and Guillot, 1999). One major consideration with this technique was that very large volumes of water were required for filtering, which may also apply to tests for *S. subterranea* if sampling from water. A second requirement of the Hallier-Soulier and Guillot (1999) method was that the *Cryptosporidium* oocysts were first trapped

by binding to anti-*Cryptosporidium* molecules attached to magnetic beads. Thus, the technique was not purely based on PCR. DNA-based detection of soil-borne fungi has been carried out, but has often involved DNA extraction procedures, purification of samples, and techniques such as slot-blot hybridisation, rather than PCR (e.g. Harvey and Ophel-Keller, 1996; Whisson and Herdina, 1995). Sensitive, PCR based detection has been demonstrated for soil-borne pathogens of potato. Cullen *et al.* (unpublished) were able to extract DNA of *Streptomyces scabies, Helminthosporium solani* and *Colletotrichum coccodes* from soil, and developed a multiplex, nested PCR for all three pathogens, capable of detecting as few as 3 spores per gram of soil.

The single product quantitative PCR-based approach has advantages over other detection methods. It is more rapid than procedures such as Southern blotting, RAPD analysis, or nested PCR for example. It is also generally more sensitive and specific than approaches such as ELISA (for example, see Wakeham and White, 1996).

Diagnostic testing for plasmodiophorids

The diagnostic test developed for *S. subterranea* f. sp. *nasturtii* based on a single PCR reaction direct from environmental samples, appears to be the most straightforward for any plasmodiophorid so far. Bulman and Marshall (1998) designed a pair of primers within the ITS rDNA of *S. subterranea* f. sp. *subterranea* which specifically distinguished this organism from *S. subterranea* f. sp. *nasturtii*, *P. brassicae, Sorosphaera veronicae* and *P. graminis* amongst others. The *S. subterranea* spores were present in scabs heavily contaminated with soil, and it was first necessary to use a spin column containing PVPP to remove PCR contaminants.

The DNA then had to be diluted 20-fold before PCR was successful. The final protocol could detect DNA from as few as 20 sporosori. More recently, Faggian *et al.* (1999) have used DNA from ribosomal DNA genes and spacers to specifically amplify *P. brassicae* DNA. The authors reported that detection limits were 1000 spores in 1g of potting mix, and that detection from soil was possible. DNA-based techniques have also been applied to detection of *Polymyxa* species. Mutasa *et al.* (1996) reported the development of a nested PCR approach for the specific detection of *P. betae* from sugar beet roots. It was first necessary to perform a DNA extraction procedure, before performing a 35 cycle nested PCR. As little as 1pg of total starting DNA could be detected, but this required Southern hybridisation or colorimetric analysis, with 10pg the limit of detection by standard agarose gel electrophoresis. The actual initial amount of *P. betae* in environmental samples was not quantified. Ward and Adams (1998) have recently developed primers for the specific identification of *Polymyxa graminis* isolates, whilst Möller and Harling (1996) used RAPD analysis to distinguish races of *P. brassicae*.

An alternative approach to detection of plasmodiophorids has been to develop polyclonal or monoclonal antibodies for plasmodiophorid detection. Such methods have generally proven unreliable. Walsh *et al.* (1996) were able to detect as few as 100 spore balls of *S. subterranea* f. sp. *subterranea* per gram of soil using polyclonal antisera, whereas Bulman and Marshall (1998) detected 20 sporosori per $2cm^3$ of powdery scab infected tissue using PCR. In the current study, *S. subterranea* f. sp. *nasturtii* was detectable from as little as 500mg root material, and from less than 1000 zoospores, consistently. Detection of the pathogen based on zoospores, rather

than resting spores has the advantage that the material being assayed (DNA or spore components) is almost certainly from a living cell. Walsh *et al.* (1996), and Harrison *et al.* (1993) have both reported an inability to distinguish between live and dead resting spores of *S. subterranea* f. sp. *subterranea* using immunological techniques. Wakeham and White (1996) reported similar limits of detection to Walsh *et al.* (1996), when using ELISA, indirect immunofluorescence, or dip-stick assays for *P. brassicae* detection in soil samples. Such techniques are unlikely to be as sensitive as DNA-based approaches (Bulman and Marshall, 1998), which potentially only require one zoospore or resting spores for diagnosis.

Development of a diagnostic test for *S. subterranea* f. sp. *nasturtii* and other plasmodiophorids is complicated by their obligate biotrophy. For many fungal pathogens, it may be possible to allow them to sporulate after sample collection. MacKay and Cooke (1997) followed such an approach in detection of *Helminthosporium solani*. With protistan pathogens too, culturing of organisms is often possible (e.g. Cooke and Duncan, 1997).

Potential application of real-time PCR

The speed of detection of *S. subterranea* in filtered samples may be enhanced by use of quantitative real time PCR. This technique has already been used in specific diagnosis of human inherited disorders, for instance in the detection of point mutations (Matsubara *et al.*, 1999). In another study, quantitative detection of herpes simplex virus DNA from clinical samples was possible from as little as 10 copies of viral DNA (Ryncarz *et al.*, 1999), illustrating the sensitivity of the technique.

Although the time available for the present study allowed only limited progress to be made, quantitation of zoospore DNA should be feasible. Using the target sequence cloned into a plasmid vector it was possible to follow PCR reactions with predetermined template concentrations. SYBR Green fluorescence in the presence of dsDNA permitted a curve to be plotted of PCR product against number of cycles (see Figure 3.17). Following completion of the reaction, products could be melted by raising the temperature from 70°C to 90°C, and a melting curve plotted. By plotting a further graph of the negative derivative of the fluorescence (F) against temperature (T) (i.e. -dF/dT), melting "peaks" can be produced (Rasmussen *et al.*, 1998). The specific PCR products will melt at a specific T_M, whilst non-specific primer products will melt at lower temperatures, and over a wider range. By relating the relative areas under the melting peaks, quantification of PCR products is possible. So the reaction curves and melting peaks produced by amplified standard DNA could be used to calculate an unknown amount of starting template in another PCR reaction run on the same machine at the same time. A similar protocol was followed by Woo *et al.* (1999) to specifically identify *Leptospira biflexa* 16S rDNA products from melting curve data.

The advantages over conventional PCR include the removal of a need for a competitor template DNA, and the fact that the whole procedure can be carried out in less than 1 hour. The LightCycler™ cannot distinguish whether PCR products produced are those expected. Steuerwald *et al.* (1999) working on gene expression in human oogenesis and embryogenesis noted that SYBR Green I dye labelled both

specific and non-specific products. It would still be necessary to run checks (agarose gels) at present.

Use of disease indices in development of diagnostic testing

Ideally, the diagnostic test for *S. subterranea* f. sp. *nasturtii* should be correlated to a disease index, such that the amount of zoospore DNA detected can be related to symptoms in the field, and a threshold limit can be decided upon. This research remains to be carried out. Such a disease index could be based on visual observations of symptoms, for example numbers of crooked roots, or plants showing leaf chlorosis, or by making use of a system such as that used by Flett (1983), whereby the number of *S. subterranea* f. sp. *subterranea* sporangia within tomato root hairs was examined microscopically to give an index value. Disease indices are commonly based on visible symptoms, such as plant health (e.g. Venuto *et al.*, 1999) or plant yield (e.g. Piccinni *et al.*, 2000).

Considerations for a diagnostic test at the laboratory development stage

Prior to the field sampling trials, several aspects of the laboratory testing had proven critical. The choice of primers was partly determined by elimination of other unsuitable primers based on specificity and sensitivity. Factors such as annealing temperatures (T_A), and magnesium ion concentration were important in development of ideal PCR conditions. Lowering the annealing temperatures allowed the primers to bind to non-target sequences. Setting annealing temperatures too high resulted in no PCR products at all, probably because the temperature was too close to the

oligonucleotides T_M. Magnesium levels were also dependent on the DNA source, and target sequence to be amplified. Magnesium acts as a cofactor for the enzyme *Taq* polymerase. When amplifying from zoospores, very low levels (0.5 mM) of Mg^{2+} were required (see Figure 3.13), suggesting that zoospores or watercress bed water may contain higher levels of magnesium than standard total genomic DNA template mixes. However, Arnold *et al.* (1994) devised a nutrient solution based on watercress bed water, and the concentration of magnesium ions in this can be calculated to be around 0.01mM, which is only one fiftieth of that used in PCR of zoospores. Many authors have reported the importance of magnesium ion concentration. Khandka *et al.* (1997), investigated the effect of RAPD reaction conditions for a range of organisms, and concluded that magnesium concentration optima varied between them. Most studies where magnesium concentration has been explicitly considered, suggest that low levels are inhibitory to PCR (e.g. Rychlik, 1995). It is thought that humic compounds in water or soil samples may chelate magnesium, requiring additional input into PCR reactions (Rodgers *et al.*, 1993). This was not found to be the case with the primers tested in the current study.

Use of a primer within the 18S rDNA had advantages over an ITS rDNA primer pair. The 18S rDNA is much more conserved than the ITS rDNA (Hillis and Dixon, 1991), and so the sequence will evolve more slowly, increasing the time over which the primer will remain universally viable, and specific. No other plasmodiophorid detection system has been developed using small subunit rDNA, but the gene has been utilised in other studies. Reinhold-Hurek and Hurek (1997) used 16S rDNA to identify isolates of the bacterial genus *Azoarcus* using PCR. Allsopp *et al.* (1997)

used variable regions of 16S rDNA of the rickettsial parasite, *Cowdria ruminantium* to design primers capable of identification to the genotype level. Other studies have used the conserved nature of the small subunit rDNA to design general primers for PCR amplification, followed by RFLP to identify particular species of interest (e.g. Back *et al.*, 1998). This is a more time-consuming approach, however, and could potentially amplify a wide range of organisms in the initial PCR.

Explanations for the amplification of two products from *S. subterranea* f. sp. *nasturtii* zoospores

When using SSN18/SPO2 to amplify DNA from zoospores, a much smaller product (4-500bp) was sometimes amplified in addition to the anticipated size product of 850bp. Several explanations could be offered for this, including the possibility that the primers were binding DNA from species other than *S. subterranea* f. sp. *nasturtii*. However, Southern hybridisations using a *S. subterranea* f. sp. *nasturtii* -specific probe, showed that the smaller product represented *S. subterranea* (Figure 3.18). A second possibility was that the smaller product represents a subpopulation of *S. subterranea* with a large deletion between the two primer binding sites. As the effect was not observed with genomic DNA as template, and as such a size of deletion would probably involve a functional part of the rDNA, this explanation was also considered unlikely. The most plausible explanation may be that one of the primers was able to bind at a second site within the amplified region, at a lower efficiency, and that PCR conditions for amplifying from zoospores favoured this

result. BLAST searches of SSN18 and SPO2 sequences against the target sequence did not reveal any such homology elsewhere in the amplified sequence.

The secondary product appeared to be present in lower quantity, and was reasonably proportional in intensity to the expected size product. If this is actually the case, then there would be little problem for development of a diagnostic test, once the relative proportions were established. If real-time PCR were used and melting peaks obtained were to be at the same temperature for both products, then again no quantification problems would be presented, as both products would appear as one peak in the data. Complications of analysis could arise if the proportions of the two products vary from reaction to reaction, for example, due to variable primer binding during early PCR cycles, and if, in addition, the smaller product were to melt at a lower temperature than the larger. This would require careful analysis of real time PCR data.

Inefficiency of ITS fungal specific primers in amplifying from *S. subterranea* f. sp. *nasturtii* DNA

Initial amplification of *S. subterranea* ITS rDNA was complicated by the inability of ITS 1F, a primer designed to be specific to fungi (Gardes and Bruns, 1993), to bind the DNA effectively. It is possible that in a pure *S. subterranea* f. sp. *nasturtii* sample, the primer pair ITS1F/4 may be effective, but the presence of DNA from other species in total genomic samples resulted in selection against *Spongospora* amplification. ITS4 and ITS5 were designed for the purpose of amplifying fungal DNA (White *et al.*, 1990), but proved capable of generating PCR products from watercress. Other authors have also found these primers capable of amplifying plant

DNA (Ward and Akrofi, 1994). ITS1F/4 was also able to amplify plant DNA, but to a much lesser extent, which confirms findings of Gardes and Bruns (1993).

Conservation of ITS rDNA between populations of *S. subterranea f. sp. nasturtii*

The apparent 100% geographic conservation of *S. subterranea* f. sp. *nasturtii* ITS rDNA in samples tested (Figure 3.9), when amplified with ITS5/POL, was perhaps surprising given the low selective pressure on maintaining ITS sequences. An example of this has been provided by examination of isolates of *Polymyxa graminis* (Ward and Adams, 1998). They found considerable variation between isolates sequenced, although the three isolates for which ITS rDNA sequences were presented differed either in host species or continent of origin (U.K. on barley, U.K., on oats, Indian, on sorghum). Despite the sequence variation detectable by eye, the isolates apparently still grouped well during phylogenetic analysis. Crook root disease has only been recognised for 50 years, and so this may not have been long enough for variations to evolve. In contrast to the studies on *P. graminis*, independent submissions of *S. subterranea* f. sp. *subterranea* ITS rDNA to sequence databases, from groups working in the U.K. and New Zealand, revealed very little sequence variation between geographic isolates. Bulman and Marshall (1998) reported no sequence variation between most isolates collected from Europe and Australasia. Another possibility for *S. subterranea* f. sp. *nasturtii* is that movement of materials such as watercress seed between growing sites, results in transmission of *S. subterranea* resting spores on a regular basis, so that geographically isolated populations do not really exist. This would also explain the well conserved ITS rDNA

of the crook root associated Chytridiomycete species. Seed transfer does not occur regularly within the industry however (S. Rothwell, pers. comm.). Despite the use of primers based on well conserved flanking regions of the ITS rDNA in both species, it is remotely possible that only a proportion of the populations are amplified by these primers, were variation to occur within the primer binding sites. This would also account for geographic conservancy in the PCR products.

The occasional occurrence of multiple PCR products when using ITS1F/4 or ITS5/POL on material collected at Bere Regis, Dorset, was interesting (Figure 3.9). Perhaps, as suggested earlier, ITS1F/4 does amplify low levels of *S. subterranea*, and this is responsible for the multiple banding pattern observed on agarose gels of ITS1F/4 PCR products. Potential explanations include the possibility that mutations exist within the rDNA, which allow the primers to bind at multiple sites. A second possibility is that insertion sequences are present within a proportion of the populations. However, if a sub-population of the chytrid or *S. subterranea* were harbouring an insertion sequence it would be likely to be observed in all PCR reactions using DNA from this site. This was not the case, and so a third explanation could be the presence of other organisms at this site only, to which the primers can bind. This is unlikely to account for multiple bands though. A further option is that the primer binding sites may be affected for at least one of ITS1F/4 and ITS5/POL, resulting in reduced efficiency of binding at the target site, and some binding at other positions of the rDNA. None of the explanations above seems entirely satisfactory. Using ITS4, a portion of the 1.2kb product obtained using ITS1F/4 was sequenced.

The sequence was pure (see Appendix D.), but no matches to any database sequence could be found.

The role of a crook root associated Chytridiomycete in development of the disease and symptoms

The ability of ITS1F/4 to amplify consistently DNA with high homology to a Chytridiomycete fungus, suggested that it may have a role in crook root disease. This was strongly refuted by the results of Southern hybridisation using DNA from unwashed and washed crooked roots (Figure 3.19). Washing roots clearly removed most of the chytrid DNA indicating that only very low levels at most are present within watercress roots. A PCR result showing this organism to be present during the summer months, when crook root symptoms are absent, substantiates this argument. Chytridiomycete fungi can parasitise a wide range of hosts, including animals, plants, other fungi and protists (Powell, 1993). On the evidence in this study, a parasitic or endophytic lifestyle within either watercress or *S. subterranea* seems improbable. A more plausible hypothesis is that the chytrid is either feeding off the surface of the watercress plant, or is living within another associated organism, such as an alga or arthropod, commonly found in watercress beds. Evidently, either ITS1F/4 binds this species DNA more tightly than to that of any other fungi present, or there are no/very few other fungi present. *Fusarium* species have been found in industrial flowing water systems (Elvers *et al.*, 1998), whilst aquatic hyphomycetes are known to occur in river systems (Maamri *et al.*, 1998), so it appears likely that aquatic fungi would be present in watercress beds. Chapter 4 elaborates on sequence data obtained from this

organism, but the closest sequence alignment to the 18S rDNA was *Spizellomyces acuminatus*, a soil-inhabiting Chytridiomycete (Barr, 1984), who gave no indication of host parasitism for this organism. Due to their general lack of economic significance, and apparent scientific interest (Powell, 1993), the vast majority of chytrid fungi probably remain uninvestigated using molecular techniques, and so the species found in this study may require the use of classical morphology and sub-cellular characteristics for identification.

The possibility remains that this organism could be responsible for vectoring viruses associated with crook root, especially as WCLV has never been successfully isolated from *S. subterranea* (Tomlinson and Hunt, 1987). However, it was observed by Tomlinson and Hunt (1987) that crook root and virus outbreaks were closely correlated. Chytridiomycetes, especially the genus *Olpidium* are known to be capable of transmitting viruses (Campbell and Sim, 1994), but would need to penetrate into the host first (Campbell, 1996).

Zoospores of both *S. subterranea* f. sp. *nasturtii* and predominantly the chytrid were observed in suspension, after germination of 7 year old watercress seed (see Figures 3.21-3.23). Bleaching of seed material removed the ability to detect these zoospores or their DNA, indicating that they were superficially associated with the seed. This emphasises the potential for the distribution of zoosporic organisms from one watercress growing area to another, particularly as the life span of resting spores can be many years. The Chytridiomycete *Synchytrium endobioticum*, for example, may survive for 40 years in a resting spore state (Powell, 1993), and Harrison *et al.*,(1997) reported that *S. subterranea* f. sp. *subterranea* may survive for many years in soil.

4. Phylogenetic analysis of *S. subterranea* f. sp. *nasturtii* and other plasmodiophorids based on ribosomal DNA sequences

4.1 MATERIALS AND METHODS

DNA samples were isolated, amplified, purified, and sequenced as described in Chapter 3. The sequence information was required for subsequent phylogenetic analysis of *S. subterranea* f. sp. *nasturtii* and plasmodiophorids generally. Detailed information on sequences obtained is presented in the results section of this Chapter. Sequences obtained were treated as follows.

Sequence alignment

Multiple sequence alignments were carried out using ClustalW (Thompson *et al.*, 1994), accessed from the EBI website[1]. Sequences were aligned using default input parameters including penalties of 10 for gap opening and 0.05 for gap extension. Positions at which nucleotides aligned scored 1, whilst a mismatch scored 0. The gap penalty settings were set to penalise gap creation, but the penalty for increasing gap size was very slight in comparison to opening a gap. General recommendations for gap penalties have yet to be established (Swofford and Olsen, 1990). The matrix used to create the alignment was from the BLOSUM series, which is recommended as the superior method for detecting base similarities (Thompson *et al.*, 1994). Following alignment, sequences were checked by eye for any obvious errors. When intending to

1= **www.ebi.ac.uk**

use subsequently the phylogenetic inference techniques of parsimony, and distance methods within the PHYLIP package (Felsenstein 1993) sequences were converted to Phylip format, and introduced in an interleaved format to the PHYLIP page on the Pasteur Institute website[2].

Distance-based methods for tree generation

Tree generation using distances required initial calculation of a distance matrix, using the program DNADIST, from PHYLIP. A Kimura-2-parameter model whereby transitions and transversions are differentially weighted was incorporated, with a transition/transversion ratio of 2.0, as transitions are generally more common than transversions (Page and Holmes, 1998). Bootstrapping was undertaken with 100 resamples for most small datasets, and 1000 for more complex assemblages of sequences. After production of a distance matrix, trees were inferred using three different programs within PHYLIP.

1. NEIGHBOR.

This is an implementation of the Neighbor Joining method (Saitou and Nei, 1987). This method successively clusters nodes based on distances, starting with the least divergent (Swofford and Olsen, 1990). These terminal groups are then removed from the tree, leaving the node that joined them at the terminus. This node is then linked to the next closest species and so on until only two nodes are left, separated by a single branch. A molecular clock is not assumed. The input order of sequences was jumbled twice to determine whether input order affected the order of tree branching.

2= bioweb.pasteur.fr

Bootstrapping of 100 or 1000 resamplings was used to provide confidence on results.

2. FITCH.

This estimates phylogenies using the Fitch-Margoliash criterion, and some least-squares criteria. One of the ways in which FITCH differs from Neighbor is in the allowance for distance errors (Felsenstein, 1993). The FITCH algorithm works along the following lines for species A and B.

$$\text{Sum of squares} = \text{Sum of } \frac{(\text{Observed distance between A-B minus the expected distance})^2}{\text{Observed distance A-B}^{P}}$$

The aim is to find the minimum sum of squares. The power P was set to 2, and this allows smoothing out of variation of sums from small and large observed and expected differences. Neighbor effectively sets P=0, meaning that large observed-expected difference score much more than small ones. This is only suitable if the measurement error is expected to be the same for small and large distances. No assumption is made of a molecular clock.

The input order of data was jumbled twice, and negative branch lengths were not allowed as they have no meaning biologically. Bootstrapping of data was carried out.

Both NEIGHBOR and FITCH allow for the assumption that the data being analysed is additive, due to the fact that DNADIST, used to construct the distance matrices accounts for evolutionary reversals, and parallelism (Felsenstein, 1993). The other major assumption, that each distance is measured independently of others may not

hold. Neighbor-joining using NEIGHBOR is a faster method than FITCH, but trees may not be as accurate (Felsenstein, 1993).

3. BIONJ.

This is an improved form of the neighbour-joining algorithm for molecular sequence data (Gascuel, 1997). The selection of the two closest species distances, followed by removal of these, to create a single node, and so forth is the same procedure as used in Neighbor. However, BioNJ has an addition to its algorithm, which allows variances of distance estimates to be accounted for. At each step in building a tree, the step which minimises the variance of the new distance matrix will be selected. This procedure ensures that estimates improve as the tree is successively 'pruned'. The program is an improvement on Neighbor if substitution rates exceed 0.1 per site, or when they vary among lineages.

Parsimony

Parsimonious tree construction was carried out using DNAPARS from PHYLIP. It produces trees showing the least amount of evolutionary change based upon the sequences supplied (Page and Holmes, 1998).Each nucleotide position is considered independently. The sequence input order was jumbled twice. Bootstrapping was performed, with either 100 or 1000 resamplings.

Consensus trees were constructed from all tree-building methods used, with treefiles created from the programs above being entered into the program CONSENSE

(Felsenstein, 1993), if this step was required to compute a consensus tree. This program builds a tree containing monophyletic groups which occur most frequently in the data supplied. Groups which occur with less frequency, and which contradict tree topology formed by higher frequency groupings, will be rejected.

Trees produced were redrawn using Adobe Photoshop 4.0.1. The branch lengths on these trees do not correspond to evolutionary distance.

4.2 RESULTS

Amplification and sequencing of 18S rDNA

The small subunit ribosomal DNA sequence of *S. subterranea* f. sp. *nasturtii* was chosen for phylogenetic analysis, because of its slow rate of evolution (Hillis and Dixon, 1991). Automated sequencing could not be performed accurately for sequences over 600-700 bases in length, and long fragments are harder to amplify than shorter fragments (White *et al.*, 1990). Therefore it was necessary to amplify the ss rDNA in stages. The PCR conditions and primers used are listed in Appendix D., but the overall scheme is represented in Figure 4.1.

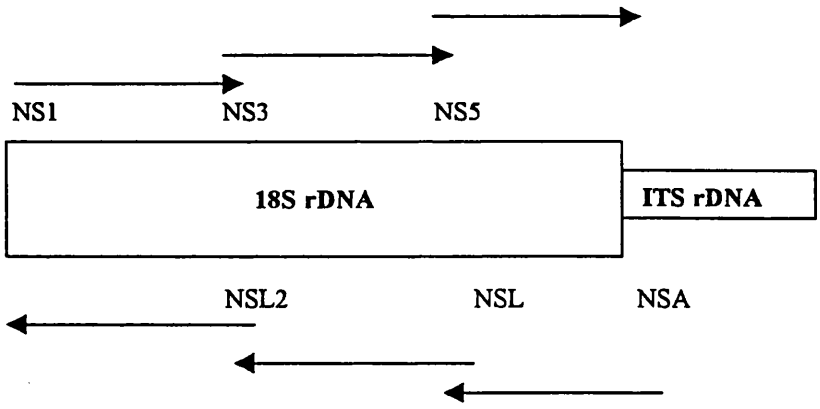


Figure 4.1 Schematic representation of binding sites of primers used to amplify *S. subterranea* f. sp. *nasturtii* 18S rDNA.

Initially, use of NS5, a Universal primer, in combination with NSA, a primer designed to be specific to *Spongospora subterranea*, was successful in amplifying approximately 600 base pairs of the 18S rDNA. When sequenced, the closest database match was *Plasmodiophora brassicae*, the only other plasmodiophorid

where the complete 18S rDNA sequence is known (Castlebury and Domier 1998). The 3' end of this gene has been sequenced for some other plasmodiophorids, including *Polymyxa* spp. (Ward and Adams, 1998), and good homology to these was also seen.

The sequence of NS5/NSA PCR product contained 3 small regions of DNA of less than 100 bases, which appeared to be unique to *S. subterranea*. The primer NSL was designed from one such region, and used in combination with the universal primer, NS3 to isolate the next upstream segment of the gene. Following sequencing, this middle section of the gene showed closest similarity to *P. brassicae* on the GenBank database. No other plasmodiophorid matches were seen, as no others have been sequenced in this region. Following BLAST alignment, another specific primer, NSL2, was designed within this region, and used in combination with NS1 (universal) located at the 5' end of the gene, to complete the 18S rDNA amplification and sequencing. A small gap remaining after sequencing the NS5/NSA PCR product was deduced by comparison with the *P. brassicae* sequence, and a lack of overlap with forward and reverse sequences. This gap was filled using specifically designed primers NS5-1 and NSA-1. The complete gene sequence is shown in Figure 4.2, and has been deposited in GenBank (accession number AF245217). An illustration of homology to other sequences is shown in Table 4.1. The DNA was sequenced in both orientations, and overlapping fragments were aligned with each other using BLAST. Any anomalies were corrected by careful examination of sequence chromatograms.

The primer SSN18 was designed by analysis of a DNA region which appeared to be substantially different from *P. brassicae* and all other closely related species. The

primer binding site is indicated in Figure 4.2 and Appendix F. When the SSN18 primer sequence was used to search the GenBank database for possible matches, no significant similarities were found. This primer was subsequently used in design of the diagnostic test as previously described in Chapter 3.

TABLE 4.1a BLAST alignment results for *S. subterranea* f. sp. *nasturtii* 18S rDNA. Sequence comparison was over 1835 bases.

GenBank accession number	**Species showing similarity**	**BLAST similarity score (bits)**	**E-value**
U18981	*Plasmodiophora brassicae*	801	0.0
AF123596	*Bolidomonas mediterranea*	509	e-142
Y10570	*Odontella sinensis*	494	e-137
AF123595	*Bolidomonas pacifica*	490	e-136
M87330	*Stephanopyxis* cf. *Broschii*	482	e-133

TABLE 4.1b Groups of organisms showing closest similarity to *S. subterranea* f. sp. *nasturtii* 18S rDNA. The top 48 BLAST matches are considered.

Groups of species	**Number of matches**
Plasmodiophorids	1
Stramenopiles	17
Alveolates	1
Scaly green flagellates	6
Ascomycetes	13
Cryptomonads	1
Green algae	9

```
1    CCTGGTTGAT CCTGCCAGTA GTGATATGCT TGTTTCAAAG ATTAAGCCAT
51   GCATGTCTAA GTATAAGCGA ACTTATACAG TGAAACTGCA AATGGCTCAT
101  TAAAACAGTT TGAATTTATT TGATGGATGT ACGAAAGCAC TACATGGATA
151  TCCGTCGAAA TTCGAGAGCT AATACATGCA ACAACGCCCG ACCGTTCGCC
201  GTAGGGTTGT ATTTATTGGA TACAAAACCC ATGACCTGGC AACAGGTTTT
251  CCTTGCTGAT TCACAATAAC TGATCGGATC GCGGCTTCGG ATGCGATAGG
301  TCATTCATCA TTCTGCCCTA TCACCTTTCG ATGGTTCTGT ACTGGACAGC
351  CATGGCTTTA ACGGGTAACG ATAGAATCAC GGTTCGGTTC CGGGGAGGGC
401  GCTTGAGAAT TGGAGACCAC ATCTAAGGAA GGNCAGCAGG CGCGCAAATT
451  ACCCAATCCT GACTCAGGGA GGTAGTGACA AAAAATAACA ATACCGGATC
501  TTTTTGGGTC TGGTAATTGG AATGAGAACA AGTTAAACCC ATTATCGAGG
551  ATCCATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TACCAGCTCC
601  CAGTAGCGTA TATTAAAGTT GTTGCGTTAA AAAGCTCGTA GTTGGACTTG
651  TGTGTGTTGC TGTTCAGCGG TCTGATTCAA AAGAGTGTTA CGACATGCGG
701  CAGTGGCGCC GTAAAATCTT CAACTGGATC GCAACTGGAC TTAATTGCCT
751  GGTTGCAGTC ATTCGGTTGA TCGTTTACTG TGAGAAAATT AGAGTGTTCA
801  AGGCAGGCAT TATTGCAATT GAATATGTTA GCATGGAATA ATAAGATAAG
851  GCTTTCGCGT TTATTTTGTT GGTTTCTAGA TCGGAAGTAA TGATTGATAG
901  GGACAGTTGG GGGTGCTAGT ATTCAGCGGC CAGAGGTGAA ATTCATGGAT
951  TCGCTGAAGA CTAACTTATG CGAAAGCATT CACCAAGGAT GTCCTCTTTA
1001 ATCAAGAACG AAAGTTGGGG GATCGAAGAC GATCAGATAC CGTCGTAGTC
1051 TCAACCATAA ACTATGTCGA CTAGGGATTG GCAGGTGTTT TTCAATTAAG
1101 ACTCTGTCAG CACCTTATGA GAAATCAAAG TGTTTGGACT CTTGGGGAAG
1151 TATGGTCGCA AGGCTGAAAC TTAAAGGAAT TGACGGAAGG GCACCACCAG
1201 GAGAGGAACT GCGGCTTAAT TTGACTCAAC ACGGGAAAAC TTACCAGGTC
1251 CAGAGATTAG AGGATTGACA GATTGAAGCT CTTTCTTGAT CACTTCGGTG
1301 GTGGTGCATG GCCGTTCTTA GTTGGTGGAG TGATTTGTCT GGTTAATTCC
1351 GTTAACGAAC GAGACCTCAG CCTGCTTTTG TANTTCCGAT TATCTCCGGA
1401 TAGTTCTTGG AACTTCTTAG AGGGACTATG TGTTTTTCGC ACATGGAAGT
1451 TTGAGGCAAT AACAGGTCTG TGATGCCCTT AGATGTTCTG GGCCGCACGC
1501 GCGTTACAAT GTGTGGTTCA ACGAGTTTTT TTCTTGGTCG AAAGGCCTGG
1551 ATAATCTTCT GAAATCCACA CGTGCTGGGG CTTGCGGCTT GCAACTAGCC
1601 GCACCAACGA GGAATTCCTA GTAGACGCAA GTCATCAACT TGCATCGATT
1651 ACGTCCCTGC CCTTTGTACA CACCGCCCGT CGCTCCTACT GATTGAATGC
1701 TCCGGTGAAA CGTCGGGAGA GCGCTTCGAT ATCAGCAATG GTTGAAGAGT
1751 GCTCGAACTT CCTTAAATTG TAGTATTTAG AAGAAGGAGA AGTCGTAACA
1801 AGGTTTCCGT AGGTGAACCT GCGGAAGGAT CATTA
```

Figure 4.2 *Spongospora subterranea* f. sp. *nasturtii* 18S rDNA sequence. The location of a putative small intron is highlighted, and the sequence of primer SSN18 is underlined.

In *P. brassicae*, the 18S rDNA contains three Group I introns (Castlebury and Domier, 1998), but *S. subterranea* f. sp. *nasturtii* appeared to lack these, with sequences matching closely if introns were removed from the alignments. The presence of small introns, characterised by the 5' to 3' motif **GTNN(G/A)Pyr...TPurCTAAC...PyrAG** (P. Bridge, pers comm.) was investigated, and one potential candidate was located, beginning at position 936 on the gene sequence (Figure 4.2).

In addition to analysis of the *S. subterranea* f. sp. *nasturtii* 18S rDNA, attempts were made to sequence the gene from other species, including the unidentified chytrid-like organism associated with crooked roots. Almost the entire 18S rDNA (see Appendix F.) was sequenced from this organism using primers NS1, 2, 3, 5, and 8 (White *et al.*, 1990) for amplifying fungal DNA. The closest sequence similarity overall was to *Spizellomyces acuminatus*, a Chytridiomycete species, belonging to the Order Spizellomycetales[1] (see Table 4.2). Members of this Order are often found in soil, and also include *Olpidium* species. *O. brassicae* was found to be most similar to the ITS rDNA of this organism in BLAST searches (see Table 3.2). Other close matches to the 18S rDNA were to anaerobic Chytridiomycetes of the genus *Neocallimastix*, which are unlikely to be encountered in watercress beds, and *Endogone pisiformis*, a Zygomycete, which would produce non-septate hyphae, and thick-walled zygospores (Martin and Hine, 1999), not seen in this study.

1 = **http://zoosporic-fungi.dmc.maine.edu/Spizello.htm**

Although *S. acuminatus* ss rDNA was the closest matching sequence on the GenBank database, there were differences between the two sequences, suggesting that the organism associated with watercress was not this particular species.

TABLE 4.2 BLAST similarity results for 18S rDNA of a chytrid-like organism associated with crooked roots of watercress. The input sequence was 1509 bases.

GenBank accession number	Species showing similarity	BLAST similarity score (bits)	E-value
M59759	*Spizellomyces acuminatus*	726	0.0
X58724	*Endogone pisiformis*	710	0.0
M59761	*Neocallimastix* sp.	686	0.0
X80341	*Neocallimastix frontalis*	686	0.0

Attempts to amplify the 18S rDNA of *S. subterranea* f. sp. *subterranea* were partially successful. PCR products were obtained using the primer pair NS3 (fungal universal), and POL (used to amplify *S. subterranea* f. sp. *subterranea* ITS rDNA). Sequencing of this region using NS3 and NS8 produced a partial sequence, and design of a specific primer spNS5 within this, enabled around 1.2 kb to be amplified and sequenced from the 3' end of the gene (Figure 4.3). The greatest homology found using GenBank, was to *P. brassicae*, and good alignment with the *S. subterranea* f. sp. *nasturtii* sequence previously obtained, was also seen (Appendix E.). Attempts to complete the 18S rDNA sequence using primers NS1/2 were unsuccessful.

```
1    CANTGGNTGC CNNNCNNCNG GTGGNCNGCC NAAAAGTTGT TGCAGTTAAA
51   AAGCTCGTAG TTGGACTTGT GTGCATGGGA ACTCGCGGTC TTCGTCCAAA
101  AGGGCGTTAC GACATGCGGT TCCTGTGCCG TAAAATCTTC AACTGGATCG
151  CACCTGGTCT TGATTGGCTG GGCGCTGTCA TTCGGTTGAT CGTTTACTGT
201  GAGAAAATTA GAGTGTTCAA GGCAGGCATG ATTGCAATTG AATATGTTAG
251  CATGGAATAA TAAGATAAGG CTTCTGCTTT ATTTTTTTGG TTTCTAGAGT
301  GGAAGTAATG ATTGATANGG ATAGTTGGGG GTGCTAGTAT TCAGNGGCCA
351  NANGTGAAAT TCATGGCTTC GCTNAAGACT AACTTATGCN ANAGCATTCA
401  CCAAGGACGT CCTCTTTAAT CAANAACGAA AGTTGGGGGA TCGAAGACGA
451  TCAGATACCG TCGTAGTCTC AACCATAAAC TATGTCGACT AGGGATTGGC
501  GGTCGTTTTT ATTATAAGAC TCCGTCAGCA CCTTATGAGA AATCAAAGTG
551  TTTGGACTCT TGGGGAAGTA TGGTCGCAAG GCTGAAACTT AAAGGAATTG
601  ACGGAAGGGC ACCACCAGGA GTGGAGCCTG CGGCTTAATT TGACTCAACA
651  CGGGAAAACT TACCAGGTCC AGAGATGTAC AGGATTGACA GATTGAAGCT
701  CTTTCTTGAT CACTTCGGTG GTGGNGCATG GCCGTTCTTA AATTGGTGGA
751  GTGATTTGTC TGGTTAATTC CGTNAANGAN CGAGACCTCA GCCTGCTTTT
801  GTANTCTGGC CTATCCTCCG GGCTATGTTT CCAACTTCNT ATANGGACTA
851  TCNATTTTCA NTCGATGGAA NGTTTNACGC AANAACAGGT CTGTGATNCC
901  CTACGCACTG NGCGNTACAA TGNNTGGTTC AACGAGTNNT TNACTTGGNT
951  TGAGAGGCNT GGTTAATNTT TTGAAANCCA AGCGTGCTTG GGCTTGCCTC
1001 TTGCAACTAG AGGCACCAAC GAGGAATTCC TAGTAGACGC AAGTCATCAA
1051 CTTGCATCGA TTACGTCCCT GCCCTTTGTA CACACCGCCC GTCGCTCCTA
1101 CTGANTGAAT GCTCCGGTGA AACGTCGGGA GAATGCGCCT GCGATCAGCA
1151 TTGGTCCAGG TGTGGGNNCA CTTCCTTAAT TTTGGGTTTT TAGGGCCAGG
1201 CAAANNAANA TATNT
```

Figure 4.3 Partial 18S rDNA sequence of 18S rDNA from *Spongospora subterranea* f. sp. *subterranea*. The sequence shows the 3' end of the gene, with around 600 bases at the 5' end remaining to be sequenced. Spore balls of *S. subterranea* were kindly supplied by J.Duncan and J. Claxton at SCRI.

Phylogenetic analyses

In reconstructing the phylogeny of *S. subterranea* f. sp. *nasturtii*, three questions were addressed.

1. How does *S. subterranea* relate to other plasmodiophorid species and genera?

2. Are *S. subterranea* and other plasmodiophorids more closely related to fungi or protists?

3. How are plasmodiophorids placed on a more wide-ranging 'tree of life'?

Use was made of both distance and character based methods, with results tested statistically by the bootstrapping procedure.

Comparison of *S. subterranea* f. sp. *nasturtii* to other plasmodiophorids using 18S and ITS rDNA

18S rDNA sequenced from *S. subterranea* f. sp. *nasturtii* and *S. subterranea* f. sp. *subterranea* was compared to other plasmodiophorids. Species used to construct trees were (Accession numbers in brackets):

S. subterranea f. sp. *nasturtii*, *S. subterranea* f. sp. *subterranea*, *Plasmodiophora brassicae* (U18981), *Polymyxa graminis* (Y12826), *Polymyxa betae* (Y12827), and *Olpidium brassicae* (Y12830).

As *P. graminis*, *P. betae* and the Chytridiomycete outgroup species *O. brassicae* have been sequenced only within the NS7-NS8 region, phylogenetic analysis was restricted to about 270bp of DNA within this region. The *P. brassicae* sequence contained an intron within this region and this was excluded from the alignments and

tree-building methods as this may not reflect the true phylogeny of the organism.

Alignment of the NS7-8 region using ClustalW (Figure 4.4) indicated that the DNA is highly conserved between species and *formae speciales*.

```
Pg    CCTTAGATGTTCTGGGC---CGCAC-GCGCGCTACAATGCTAGGTTCAACGAGT----CTGCG
Pbe   CCTTAGATGTTCTGGGC---CGCAC-GCGCGCTACAATGCTAGGTTCAACGAGT----ATCGG
Ssn   CCTTAGATGTTCTGGGC---CGCAC-GCGCGTTACAATGTGTGGTTCAACGAGT----TTTTT
Sss   -CCTAGATATNTTNNAGGAACGCACTGGGCGTTACAATGTGTGGTTCAACGAGT----GNTTG
PBr   CCTTAGATGTTCTGGGC---CGCAC-GCGCGCTACACTGCTGGGTTCAACGAGTCCGAACCGT
Obr   CCTTAGATGTTCTGGGC---CGCAC-GCGCGCTACACTGATGAAGTCAACGAGT-----TTAT

Pg    A--CTTGGCCGAGAGGCCTGGTTAATCTTGTGAAATCCTAGCGTGCTTGGGCTTGCCTCTTGC
Pbe   A--CTTGGTCGAGAGGCCTGGTTAATCTTTTGAAATCCTAGCGTGCTTGGGCTTGCCTCTTGC
Ssn   T--CTTGGTCGAAAGGCCTGGATAATCTTCTGAAATCCACACGTGCTGGGGCTTGCGGCTTGC
Sss   A--CTTGGNTGAGAGGCCTGGTTAATCTTTTGAAATCCAAGCGTGCTTGGGCTTGCCTCTTGC
PBr   A--CTTGGCTGAAAGGCCTGGTTAATCTTTTGAAATCCCAGCGTGCTTGGGCTTGCCTCTTGG
Obr   AACCTTGGCCGGAAGGTCTGGGTAATCTTTTGAAACTTCATCGTGCTGGGGATAGTCCATTGC

Pg    AACTAGAGG-CACCAACGAGGAATTCCTAGTAGACGCAGGTCATCAACCTGCATCGATTACGT
Pbe   AACTAGAGG-CACCAACGAGGAATTCCTAGTAGACGCAGGTCATCAACCTGCATCGATTACGT
Ssn   AACTAGCCG-CACCAACGAGGAATTCCTAGTAGACGCAAGTCATCAACTTGCATCGATTACGT
Sss   AACTAGAGG-CACCAACGAGGAATTCCTAGTAGACGCAAGTCATCAACTTGCATCGATTACGT
PBr   AACTAGAGG-CACCAACGAGGAATTCCTAGTAGACGCAAGTCATCAACTTGCATCGATTACGT
Obr   AATTATTGGACTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGT

Pg    CCCTGCCCTTTGTACACACCGCCCGTCGCTTCTACCGATTGGTCGTCCCGGTGAAC-AATCGG
Pbe   CCCTGCCCTTTGTACACACCGCCCGTCGCTTCTACCGATTGGTCGTCCCGGTGAAT-GATCGG
Ssn   CCCTGCCCTTTGTACACACCGCCCGTCGCTGCTACTGATTGAATGCTCCGGTGAAA-CGTCGG
Sss   CCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACTGATTGAATGCTCCGGTGAAA-CGTCGG
PBr   CCCTGCCCTTTGTACACACCGCCCGTCGGTCCTACTGATTGAGTGCTCCGGTGAAAATGTCGG
Obr   CCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTTCGG

Pg    ---GAGGGCGCGCACCAG--GTCAGC
Pbe   ---GAGGGCGCGCTGCTG--ACCAGC
Ssn   ---GAGAGCGCTTCGAT---ATCAGC
Sss   ---GAGAATGCGCCTGCG--ATCAGC
PBr   ---GAGATTGCTC--GCG--ATCAGC
Obr   ATTGGAGGTAGGTTGCTGGCAACAGC
```

Figure 4.4 ClustalW alignment of a 260-270bp segment of the NS7-8 rDNA region from plasmodiophorid species. The settings for alignments included a penalty of 10 for opening gaps and 0.05 for extension of gaps.

Pg = *Polymyxa graminis* (Ward and Adams, 1998), *Pbe* = *Polymyxa betae* (Ward and Adams, 1998) *Ssn* = *Spongospora subterranea* f. sp. *nasturtii*, *Sss* = *Spongospora subterranea* f. sp. *subterranea*, *PBr* = *Plasmodiophora brassicae* (Ward and Adams, 1998), *Obr* = *Olpidium brassicae* (Ward and Adams, 1998).

Approximately 85-90% of nucleotide positions were conserved between plasmodiophorids when compared on a pairwise basis (Table 4.3).

S. subterranea f. sp. *nasturtii* and *S. subterranea* f. sp. *subterranea*, for example, differ at 38 positions (of which 5 involve unidentified bases), whilst *S. subterranea* f. sp. *nasturtii* and *P. brassicae* differ at 39 positions.

TABLE 4.3a Comparison of NS7-8 ss rDNA sequences of plasmodiophorids as aligned using ClustalW. Positions where bases do not align are indicated in bold, and the number of these which are transversions in brackets. Any differences accounted for by N's are indicated in addition to the total in italics

	Pbr	*Ssn*	*Sss*	*Pg*	*Pbe*	*Ob*
	Total differences (of which Transversions) *Unidentified nucleotides*					
Pbr	----------	**39** (17)	**35** (12) *5*	**39** (15)	**36** (10)	**73** (21)
Ssn		----------	**33** (14) *5*	**42** (22)	**38** (19)	**67** (25)
Sss			----------	**41** (14) *5*	**39** (11) *5*	**79** (25) *5*
Pg				----------	**12** (7)	**71** (26)
Pbe					----------	**67** (19)
Ob						----------

TABLE 4.3b Scores for sequence similarities, based on Table 4.1a, where transitions score 1, transversions 2, gaps 1, and N's 0.1. Closest matches to each organism are shown in bold.

	Pbr	*Ssn*	*Sss*	*Pg*	*Pbe*	*Ob*
Pbr	----------	56	**47.5**	54	**46**	94
Ssn		----------	**47.5**	64	57	92
Sss			----------	55.5	50.5	104.5
Pg				----------	**19**	97
Pbe					----------	**86**
Ob						----------

TABLE 4.3c Scores for sequence similarities based on Table 4.1a, where transitions score 1, transversions score 1, gaps score 1, and N's score 0.

	Pbr	*Ssn*	*Sss*	*Pg*	*Pbe*	*Ob*
Pbr	----------	39	**35**	39	36	73
Ssn		----------	**33**	42	38	**67**
Sss			----------	41	39	79
Pg				----------	**12**	71
Pbe					----------	**67**
Ob						----------

Based on the nucleotide data supplied, all distance-based methods tested grouped the two *formae speciales* of *Spongospora* together at the tree tip (Figures 4.5-4.8), although never with unequivocal bootstrap support. Using the closely related BioNJ and Neighbor programmes from the PHYLIP package, and also FITCH, *S. subterranea* f. sp. *subterranea* paired with *S. subterranea* f. sp. *nasturtii* at a terminal tree node, but without strong bootstrap support (from 57-71% in the three tests applied). *P. brassicae* grouped with *S. subterranea*, although again there was a lack of good bootstrap support for grouping these three species separately from the others. Values ranged from 64% of resamplings using Neighbor, to 69% with FITCH. *Polymyxa graminis* and *Polymyxa betae* were always seen to cluster together, although as just stated, their relationship as being distinct from the other plasmodiophorids was statistically uncertain. The outgroup, *O. brassicae* was chosen as a common zoosporic plant pathogen, which had shown ITS rDNA homology to an organism associated with crooked watercress roots. It was always seen to be clearly divergent from the plasmodiophorids on the basis of this 18S rDNA data, except on the Neighbor tree (Figure 4.6), where the bootstrap value was only 72%.

In common with distance-based analyses, parsimonious analysis of the data using DNAPARS resulted in the two *S. subterranea formae speciales* grouping together (Figure 4.8). These appeared to diverge after *P. brassicae*, but with less than the 100% confidence seen when grouping *Polymyxa* species together. Overall tree topology was identical with all tree-building algorithms employed.

The ITS rDNA sequence of *S. subterranea* f. sp. *nasturtii* was obtained by PCR using primers as described in Chapter 3, and aligned with published DNA sequences using PileUp (Figure 3.10). Attempts were made to use this data to produce phylogenetic trees, but the level of nucleotide variability between sequences meant that relationships could not be inferred with confidence.

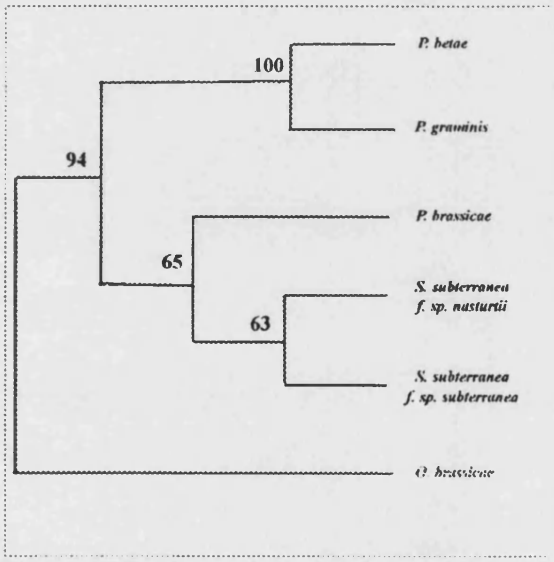


Figure 4.5 Phylogenetic consensus tree produced using BioNJ (PHYLIP) on NS7-8 18S rDNA sequences aligned using ClustalW.
Black = plasmodiophorid species
Grey = Chytridiomycetes
Numbers indicate bootstrap support for nodes from 100 resamplings.

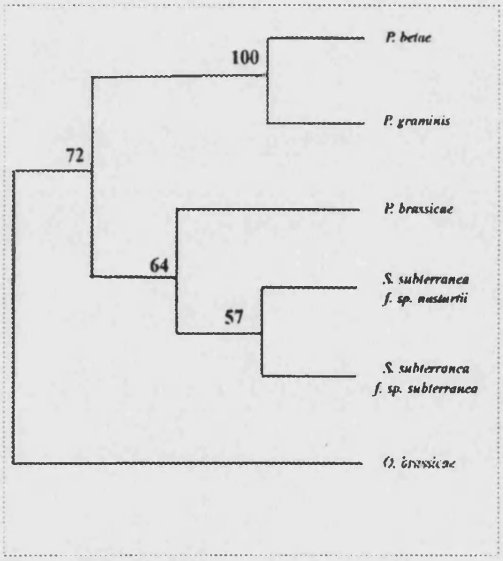


Figure 4.6 Phylogenetic consensus tree produced using Neighbor (PHYLIP) on NS7-8 18S rDNA sequences aligned using ClustalW.
Black = plasmodiophorid species
Grey = Chytridiomycetes
Numbers indicate bootstrap support for nodes From 100 resamplings.

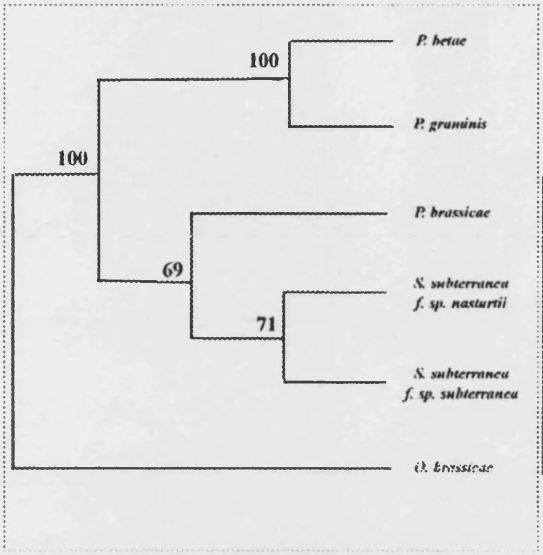


Figure 4.7 Phylogenetic consensus tree produced using FITCH (PHYLIP) on NS7-8 18S rDNA sequences aligned using ClustalW.
Black = plasmodiophorids
Grey = Chytridiomycetes
Numbers indicate bootstrap support for nodes from 100 resamplings.

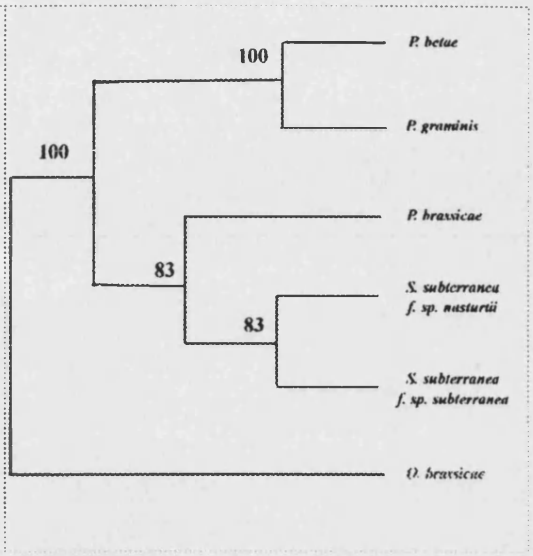


Figure 4.8 Phylogenetic consensus tree produced using DNAPARS (PHYLIP) on NS7-8 18S rDNA sequences aligned using ClustalW.
Black = plasmodiophorids
Grey = Chytridiomycetes
Numbers indicate bootstrap support for nodes from 100 resamplings.

Determination of plasmodiophorid phylogeny using 18S rDNA

The entire 18S rDNA had previously been sequenced for an isolate of *P. brassicae* (Castlebury and Domier, 1998), and has now been completed for *S. subterranea* f. sp. *nasturtii*. A BLAST search for species showing homology to *S. subterranea* 18S rDNA, revealed that most close matches were from protists (Table 4.1), particularly from a grouping known as the stramenopiles (Patterson, 1989). Bit scores were in the region of 3-500, which was much lower than the match with *P. brassicae* which

scored 801. On the assumption that such sequences may still be the most closely related to *S. subterranea* f. sp. *nasturtii*, a range of them were included for inferring phylogeny. *S. subterranea* f. sp. *nasturtii* has traditionally been classified as a fungus (Sparrow, 1960), and to test the protistan/fungal evolution, fungi were also included in alignments and phylogeny reconstruction.

Species selected were:

Stramenopiles *Asterionellopsis glacialis* (X77701), *Bolidomonas mediterranea* (AF123596), *Fragilaria striatula* (X77704), *Odontella sinensis* (Y10570), *Phytophthora megasperma* (X54265), *Stephanopyxis broschii* (M87330), *Tessellaria volvocina* (U73219), *Thalassiosira rotula* (X85397).

Alveolates: *Obertrumia. georgiana* (X65149).

Green algae: *Pycnococcus provasolii* (X91264, coccoid green alga CCMP1205 (U40921).

Plasmodiophorids: *Plasmodiophora brassicae* (U18981), *Spongospora subterranea* f. sp. *nasturtii*.

Chytridiomycetes: *Spizellomyces acuminatus* (M59759).

Ascomycetes: *Eupenicillium javanicum* (U21298), *Kluyveromyces yarrowi* (X89528), *Taphrina deformans* (X69852).

Slime mould: *Dictyostelium discoideum* (X00134).

Some close matching BLAST sequences were excluded as they either lacked the complete 18S rDNA sequence, had too many ambiguous bases, or simply to ensure that the analysis was not entirely composed of very similar species. For this last

reason, a range of taxa were incorporated. *Dictyostelium discoideum* was used as an outgroup in tree construction as the slime moulds are believed to be fairly primitive (Van de Peer *et al.*, 1993).

The trees produced by different phylogenetic methods were superficially very similar. Figures 4.9, 4.10, and 4.11 indicate the consensus trees produced using the tree building programs BioNJ, FITCH, and DNAPARS, respectively. *P. brassicae* and *S. subterranea* f. sp. *nasturtii* always grouped together with absolute confidence from bootstrapping. The plasmodiophorids were seen to diverge near the base of the trees, after *D. discoideum*, and before all the other taxa, from which they were clearly distinct. On 824 BioNJ trees, 750 FITCH trees, and 970 trees produced by parsimonious reconstruction, the plasmodiophorids were excluded from any other groupings on the tree, following the divergence of the slime mold. As with NS7-8 analysis, introns had been removed from *P. brassicae* 18S rDNA prior to sequence alignment, as no introns were observed in the other sequences used. Alignment and parsimony of sequences with *P. brassicae* introns included, resulted in tree topologies identical to those with introns removed.

Aside from the plasmodiophorids, many other species grouped together on the trees (Figures 4.9-4.11) with very strong bootstrapping support. All the currently recognised taxa were clearly divergent from each other. The order in which taxa diverged from each other varied slightly with the different methods used. Using BioNJ (Figure 4.9), stramenopiles appeared to diverge later than the alveolate, whereas this was reversed when FITCH was used to construct trees (Figure 4.10). Parsimony also suggested that the alveolates diverged later than stramenopiles

(Figure 4.11), although in no case was there much confidence for placing the two taxa in these positions on the tree. For example, only 433 resamplings using FITCH grouped the alveolates with the green algae, and true fungi, separately from the stramenopile lineage, and using parsimony, on only 537 out of 1000 times did the alveolates form a separate lineage to the stramenopiles/green algae/fungi. There was complete bootstrapping support for grouping all of the stramenopiles as a monophyletic unit with all methods used. The green algae appeared to form a grouping with the fungi, before branching off. On distance and parsimonious trees, this occurred prior to fungal diversification. In all methods there was good support for Chytridiomycetes branching earlier than the Ascomycetes. Amongst the Ascomycetes, *T. deformans* diverged before *K. yarrowi* and *E. javanicum*. There was a clearly defined branching order among the stramenopiles, with *P. megasperma* diverging first, followed by *T. volvocina* and then *B. mediterranea*. *Dictyostelium discoideum* was clearly unrelated to the other species.

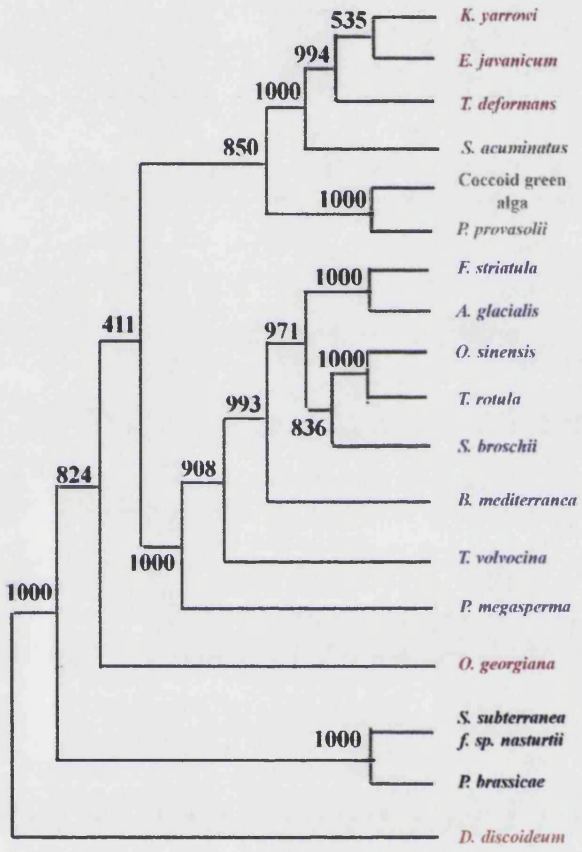


Figure 4.9 Phylogenetic reconstruction of 18S rDNA data aligned by ClustalW, using the program BioNJ. Numbers indicate bootstrap support for nodes from 1000 resamplings.

Orange = slime mold
Black = plasmodiophorids
Red = alveolate
Blue = stramenopiles
Green = *Viridiplantae*
Maroon = Ascomycetes
Grey = Chytridiomycete

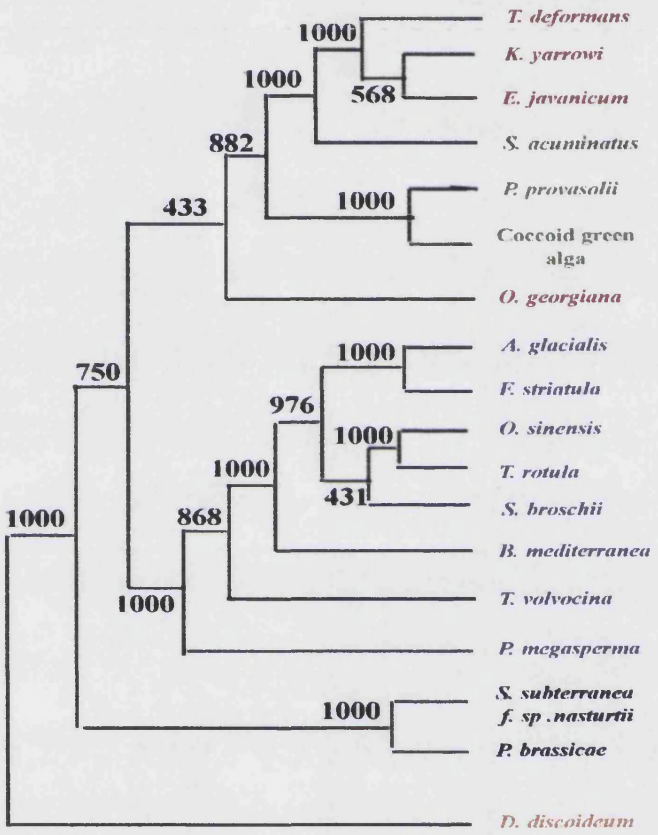


Figure 4.10 Phylogenetic reconstruction of 18S rDNA data aligned by ClustalW, using the program FITCH. Numbers indicate bootstrap support for nodes from 1000 resamplings.

Orange = slime mold
Black = plasmodiophorids
Red = alveolate
Blue = stramenopiles
Green = *Viridiplantae*
Maroon = Ascomycetes
Grey = Chytridiomycete

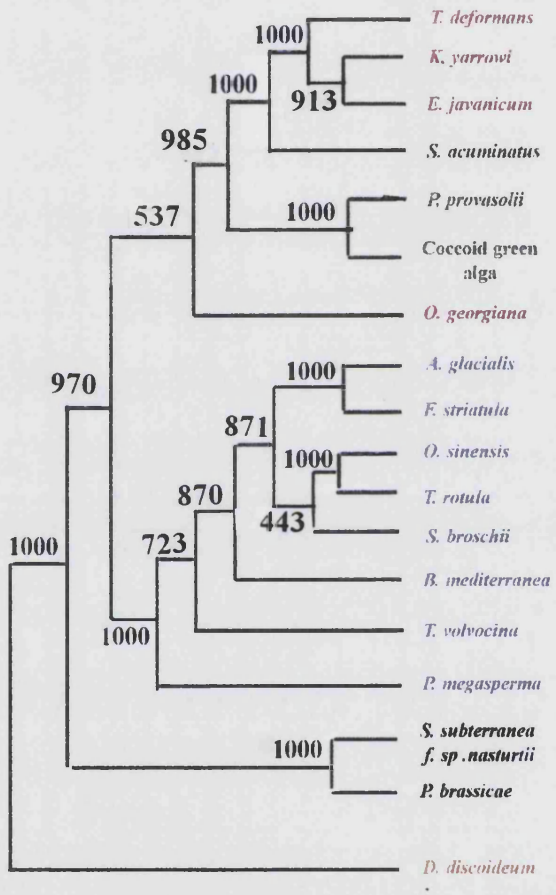


Figure 4.11 Phylogenetic reconstruction of 18S rDNA data aligned by Clustal W, using the program DNAPARS. Numbers indicate bootstrap support for nodes from 1000 resamplings.

Orange = slime mold
Black = plasmodiophorids
Red = alveolate
Blue = stramenopiles
Green = *Viridiplantae*
Maroon = Ascomycetes
Grey = Chytridiomycetes

Having sequenced approximately two thirds of the *S. subterranea* f. sp. *subterranea* 18S rDNA (Figure 4.3), attempts were made to include this in phylogenetic reconstructions of protistan and fungal species. It was first necessary to cut the other sequences so that they started and ended at the same point as the *S. subterranea* f. sp. *subterranea* partial gene sequence. *S. broschii, T. volvocina, P. provasolii,* coccoid green alga, and *T. deformans* were omitted from the trees.

In the results shown earlier (Figs. 4.5-4.8) using the NS7-8 region of the 18S rDNA, *S. subterranea* f. sp. *nasturtii* and *S. subterranea* f. sp. *subterranea* were most similar to each other. Using approximately two thirds of the 18S rDNA, this trend was not seen. The PHYLIP programs BioNJ (Figure 4.12), FITCH (Figure 4.13), and DNAPARS (Figure 4.14) all placed *S. subterranea* f. sp. *subterranea* and *P. brassicae* together at a terminal tree node, with *S. subterranea* f. sp. *nasturtii* branching off one node earlier. Confidence in this grouping ranged from 73.2% using neighbor-joining algorithms, to 84.2% with parsimony. There was 100% support for grouping the plasmodiophorids as separate from all other taxa, and they were placed near the base of the trees, diverging after *D. discoideum*. The number of taxa used to produce these trees was less than that previously (Figs. 4.9-4.11), and this resulted in generally stronger bootstrapping support across the trees. For example, using BioNJ, the lowest confidence level found was that for groupings amongst the plasmodiophorids, with all other tree nodes receiving greater than 85% support. The branching order for the rest of the taxa on these trees was not greatly different to that obtained using the entire 18S rDNA. Using BioNJ (Figure 4.12) and DNAPARS

(Figure 4.14), the alveolate species, *O. georgiana*, was seen to diverge after the stramenopiles, whereas previously the reverse had been found. Support for this change was good using BioNJ (87.7%), but not parsimony (51.7%).

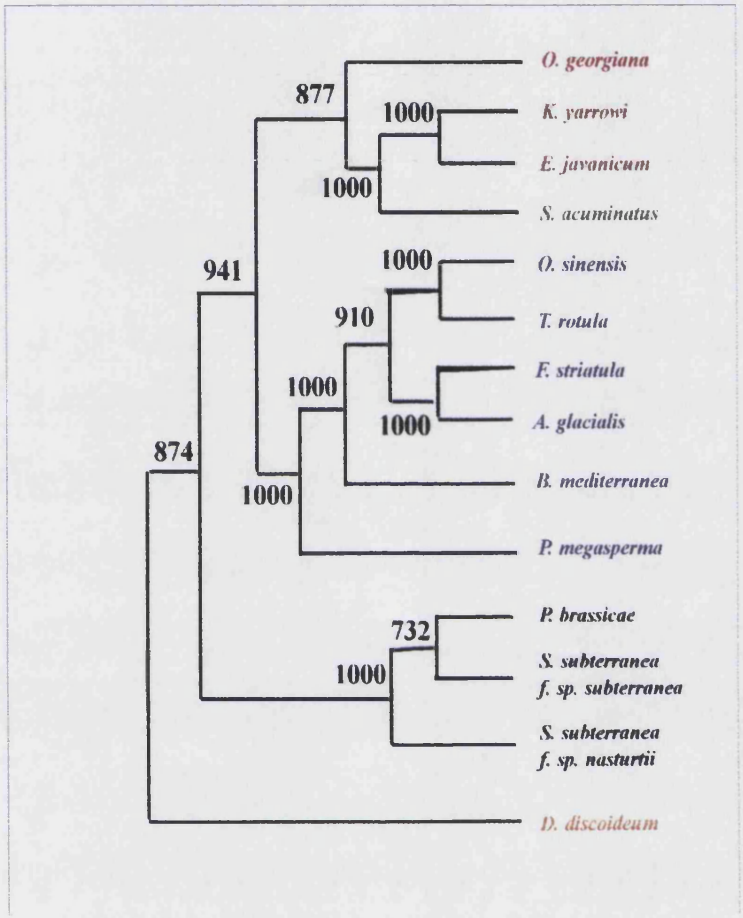


Figure 4.12 Phylogenetic reconstruction of approximately 1.2kb 18S rDNA sequences aligned using ClustalW. The tree was constructed using BioNJ (PHYLIP). Numbers indicate bootstrap support for nodes from 1000 resamplings.

Orange = slime mold	Black = plasmodiophorids	Red = alveolate
Blue = stramenopiles	Maroon = Ascomycetes	Grey = Chytridiomycetes

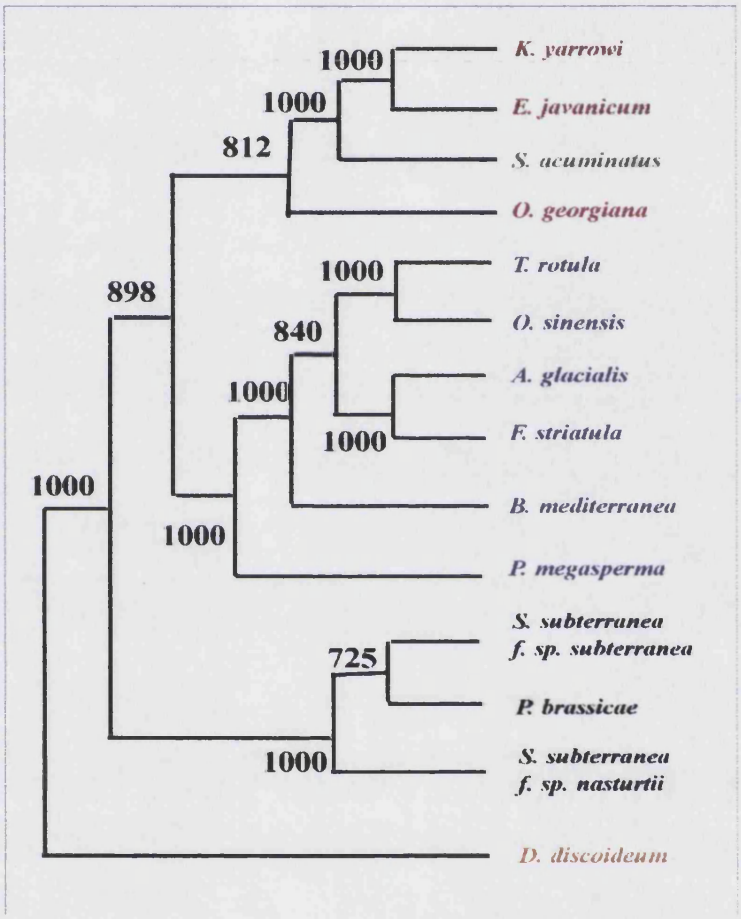


Figure 4.13 Phylogenetic reconstruction of approximately 1.2kb of 18S rDNA sequences aligned using ClustalW. The tree was constructed using FITCH (PHYLIP). Numbers indicate bootstrap support for nodes from 1000 resamplings.

Orange = slime mold	Black = plasmodiophorids	Red = alveolates
Blue = stramenopiles	Maroon = Ascomycetes	Grey = Chytridiomycetes

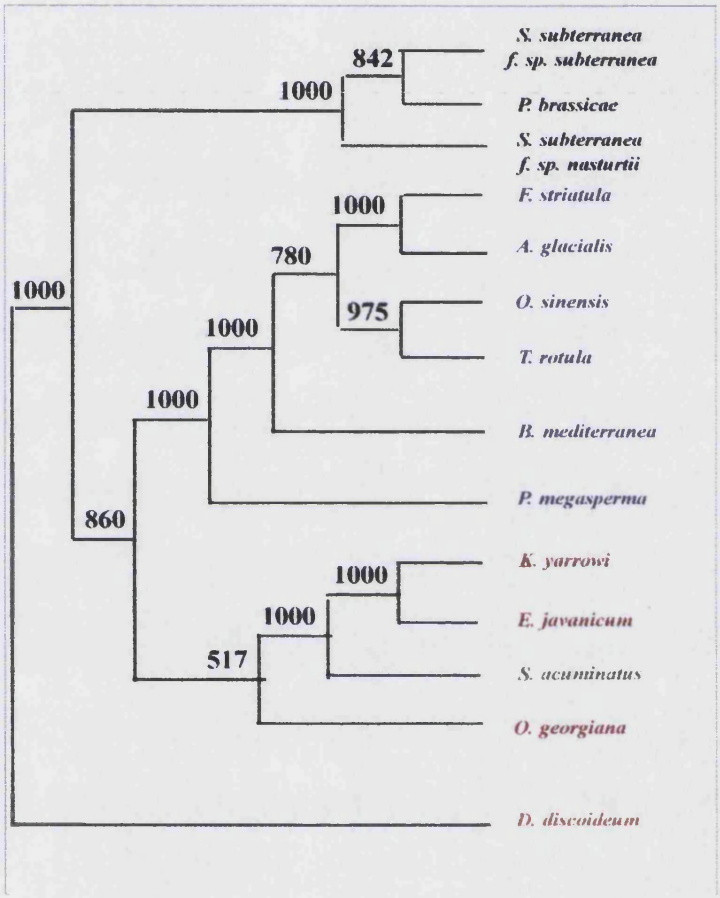


Figure 4.14 Phylogenetic reconstruction of approximately 1.2 kb of 18S rDNA sequences aligned using ClustalW. The tree was constructed using DNAPARS. Numbers indicate bootstrap support for nodes from 1000 resamplings.

Orange = slime mold	Black = plasmodiophorids	Red = alveolates
Blue = stramenopiles	Maroon = Ascomycetes	Grey = Chytridiomycete

Placing plasmodiophorids on trees containing a wider range of Kingdoms

Following phylogenetic analysis of fungal and protistan species, an attempt was made to incorporate a wider range of taxa including red algae (*Rhodophyta*) and green algae (*Viridiplantae*).

Species added included:

Red algae: *Carpopeltis phyllophora* (U33124), *Gracilaria gracilis* (M33638)

Chlorarachniophytes: *Gymnochlora stellata* (AF076171).

Cryptomonads: *Guillardia theta* (X57162)

Due to time constraints, only a parsimonious tree was produced using these species, and bootstrapping was limited to 100 resamplings of the data. The results are presented in Figure 4.15. The plasmodiophorids grouped together with 100% confidence, but appeared to be closely linked to the Chlorarachniophyte species *Gymnochlora stellata* on 81 out of 100 trees sampled. Also, the plasmodiophorid lineage was no longer seen to diverge early on the tree, but appeared to have evolved more recently from *G. stellata*, which in turn had split off from the stramenopile grouping. However, support at deeper tree nodes was poor. On only 37/100 trees was the stramenopile/plasmodiophorid/chlorarachniophyte assemblage separated from the alveolate/fungal/cryptomonad/green algae grouping, and these groups as a whole were separated from the red algae on only 34/100 occasions. To illustrate the level of uncertainty that this indicates, the red algae diverged after the plasmodiophorids and

G. stellata on 23/100 resamplings, and grouped separately from stramenopiles and alveolates on a further 21 resamplings.

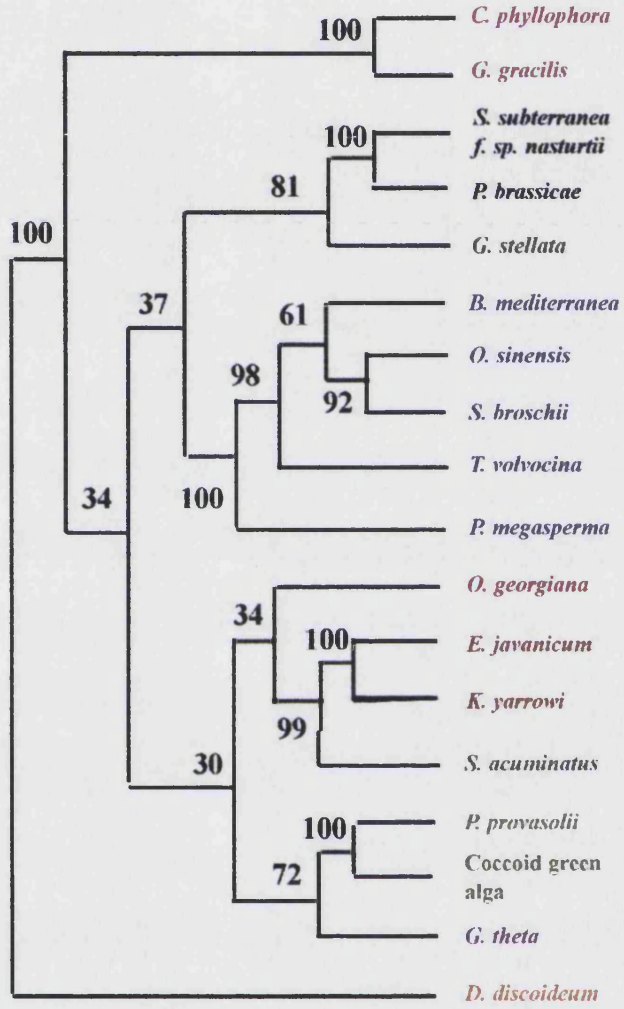


Figure 4.15 Phylogenetic reconstruction using 18S rDNA sequences aligned by ClustalW. The tree was produced using DNAPARS. Numbers indicate bootstrap support for nodes from 100 resamplings.

Orange = slime mold	Black = plasmodiophorids	Red = alveolate
Blue = stramenopiles	Maroon = Ascomycetes	Grey = Chytridiomycete
Green = *Viridiplantae*	Purple = cryptomonad	Pink = Rhodophytes

Dark green = Chlorarachniophyte

To determine the relationship between the plasmodiophorids and the Chlorarachniophyta, an 18S rDNA tree was constructed using parsimony (DNAPARS), incorporating a range of protistan and algal species, including two additional representatives of the Chlorarachniophyta, *Lotharella vacuolata* (AF054890), and *Chlorarachnion reptans* (X70809). The resulting consensus tree (Figure 4.16) did not support the link between plasmodiophorids and Chlorarachniophytes, with the plasmodiophorids branching off first, and the Chlorarachniophytes separated from all other species at the next tree node. Confidence limits were not good for these two splits, or at many other nodes on the tree.

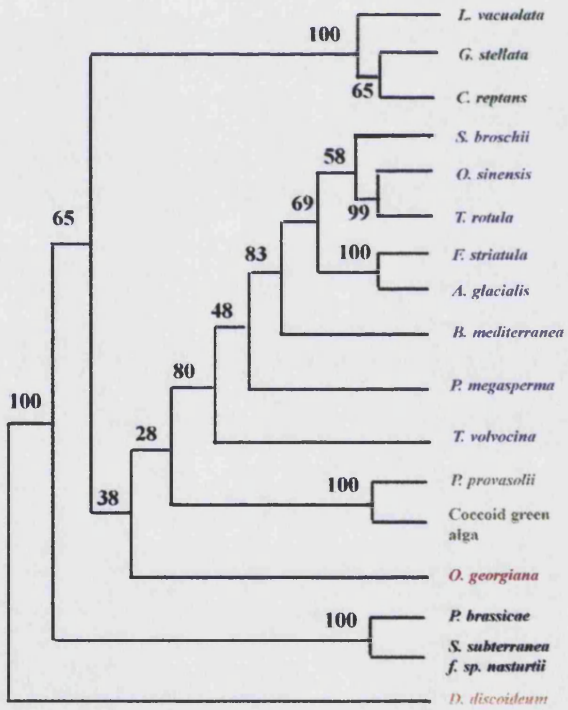


Figure 4.16 Phylogenetic reconstruction using 18S rDNA data aligned by ClustalW. The tree was produced using DNAPARS. Numbers indicate bootstrap support from 100 resamplings.
Orange = slime mold Black = plasmodiophorids Red = alveolate Blue = stramenopiles
Maroon = Ascomycetes Grey = Chytridiomycete Dark green = Chlorarachniophytes

TABLE 4.4 Summary of tree-building methods used and results relating to *S. subterranea* f. sp. *nasturtii* (*Ssn*). *Sss* = *S. subterranea* f. sp. *subterranea*, *Pbr* = *P. brassicae*.

DNA and species analysed	**Parsimony**	**BioNJ**	**Neighbour**	**FITCH**
NS7-8	*Ssn* and *Sss* together	As parsimony	As parsimony	As parsimony
18S rDNA protists/ fungi	*Ssn* closest to *Pbr*, then no other close relatives	As parsimony		As parsimony
18S rDNA with *Sss*	*Sss* closest to *Pbr* then *Ssn*. No other close relatives	As parsimony		As parsimony
18S rDNA wider taxa	*Ssn* closest *Pbr* then *G. stellata* (on only one of two consensus trees)			

4.3 DISCUSSION

Plasmodiophorid phylogeny based on NS7-8 rDNA

The plasmodiophorid phylogeny inferred in the results was generally in agreement with traditional classifications based on morphology, and with previous examples of molecular studies. Data analysis of around 260 bases at the 3' end of the small subunit rDNA (Figures 4.5-4.8, and Table 4.3) indicated that the two *formae speciales* of *S. subterranea* were the most closely related, and that these two organisms together with *P. brassicae* form a group distinct from *Polymyxa*. Ward and Adams (1998) also examined the NS7-8 rDNA region, with an extra 80-90 bases completing the 3' end compared to the current study. Using Neighbor, DNAPARS and FITCH, they also found good bootstrapping support for separation of *P. brassicae* from *Polymyxa* species of plasmodiophorids, and some confidence (78% using Neighbor) for recognition of *Polymyxa graminis* and *Polymyxa betae* as separate species. The issue of speciation of *Polymyxa* cannot be considered here as only one isolate of each species was analysed. Inference of relationships among plasmodiophorids using NS7-8 ss rDNA did identify them as a monophyletic grouping with *Olpidium brassicae* clearly divergent. This is in agreement with the findings of Ward and Adams (1998). Ward and Adams (1998) also examined the ITS rDNA of a range of plasmodiophorids including *S. subterranea* f. sp. *subterranea*, but were unable to resolve relationships amongst them, although *Spongospora* or *Ligniera* was always seen to group with *P. brassicae* rather than *Polymyxa* provided that the alignment incorporated only *Spongospora* or *Ligniera*. Preliminary studies on ITS rDNA

phylogeny in the current study failed to find consistent and confident tree topologies, perhaps due to a lack of conserved nucleotides amongst the ITS rDNA. The variability of ITS rDNA is illustrated by Cooke and Duncan (1997), whose studies on the ITS 1 rDNA of *Phytophthora* isolates revealed that perhaps only 10% of positions were conserved amongst more than 50% of 17 isolates examined. Wee *et al.* (1996) have recommended that phylogenetic inference should be based on regions of 1000bp or greater, and that sequences should diverge by 5-15%, although they do advocate the use of ITS rDNA for lower taxonomic levels. Ward and Adams (1998) also acknowledged that the whole of the 18S rDNA would be more appropriate for assessing true phylogenetic relationships, although the NS7-8 region was felt to be suitable for giving a general indication of relationships. In the current study, the lack of absolute bootstrap support for the close relationship between *S. subterranea* f. sp. *nasturtii* and *S. subterranea* f. sp. *subterranea* may relate in part to the fact that the sequence of *S. subterranea* f. sp. *subterranea* had several ambiguous bases in it. This may influence both sequence alignment (see Table 4.3) and subsequent bootstrapping of phylogenies, when mismatches are encountered. The lack of certainty over separation of *Polymyxa* from the other plasmodiophorid species compared to results of Ward and Adams (1998) could also result from the shortness of sequence used if there are more phylogenetically useful characters are present in the remaining 80-90 bases of the NS7-8 region examined by Ward and Adams. If transversions are weighted as twice transitions over the 80-90 bases, then *P. brassicae* varies from the *Polymyxa* isolates examined here, by almost three times as much as they vary from each other. Also Ward and Adams used more than one isolate of each species which

may add confidence for them grouping separately. They also examined a smaller range of plasmodiophorid species overall. Finally, the parameters set for multiple sequence alignment by Ward and Adams (1998) were more lenient for gap creations (5), but harsher for gap extension (0.3), than in this study, which will lead to differences in alignment of sequences. Calculations of similarity between species following ClustalW alignment of this DNA region (Table 4.3) indicated that *S. subterranea* f. sp. *subterranea* was almost as closely linked to *P. brassicae* as to *S. subterranea* f. sp. *nasturtii*. Similar numbers of nucleotide substitutions were observed, with a higher proportion of transversion events between *S. subterranea* f. sp. *subterranea* and *S. subterranea* f. sp. *nasturtii* than between *S. subterranea* f. sp. *subterranea* and *P. brassicae*. *S. subterranea* f. sp. *nasturtii* and *P. brassicae* were more distantly related by these criteria. It is evident that determination of initial parameters for tree building will be critical to inferring the correct tree topology. If transversions are weighted as less likely than transitions, then there is greater likelihood of inferring that *S. subterranea* f. sp. *subterranea* and *P. brassicae* are most closely related. Intuitively, transversions might be expected to be more common than transitions, as there are two possible state changes with the former and only one with the latter, but the reverse is typically the case (Page and Holmes, 1998). Such considerations apply to rDNA, and other authors including Van der Auwera and DeWachter (1998) and Leipe *et al.* (1996), have used a transition/transversion ratio of 2.0 in assessing rDNA phylogeny. Other factors, such as the importance given to opening of gaps, and extension of gaps may also influence tree topology, regardless of the actual biological significance they carry. ClustalW allows for consideration of

gap parameters (Figure 4.4), when aligning sequences. Gaps cannot be simply ignored (Swofford and Olsen, 1990), as two sequences that are identical except for gaps are clearly not the same. Also gaps are sometimes inserted to improve alignment of remaining nucleotides, and so ignoring them makes sequences unrealistically homologous. On the other hand the processes leading to insertions and deletions are different to substitutions, and so treating gaps as a 5th nucleotide state may not be valid either (which is what PHYLIP programs did).

Plasmodiophorid phylogeny based on the entire 18S rDNA and incorporating a range of protists and fungi

The BLAST sequence similarity matches with the entire *S. subterranea* f. sp. *nasturtii* 18S rDNA clearly indicated that *P. brassicae* 18S rDNA was the most similar sequence (Table 4.1). Other matches were mostly from stramenopiles with a few examples of green algae and Ascomycetes. The monophyletic taxon of stramenopiles was first suggested by Patterson (1989). This grouping of protists includes oomycetes, chrysophytes, phaeophytes, synurophytes, diatoms, xanthophytes, bicosecoids, and slime nets (Sogin and Silberman, 1998; Sogin and Patterson, 1999). All organisms within this grouping possess tripartite tubular hairs, or are descended from such species (Leipe *et al.*, 1996). The hairs enable reversal of swimming direction compared to naked flagella (Cahill *et al.*, 1996). Whilst BLAST searches do not infer any evolutionary relationships (they merely report a similarity score and an error value relating to the probability of matches occurring by chance [Altschul *et al.*, 1990]) they do provide a starting point for phylogenetic analysis. Ward and Adams

(1998) used a BLAST search for comparison of *Polymyxa* and *Plasmodiophora* isolates, and also found that closest matches were to stramenopiles, particularly oomycetes and diatoms (75% similarity). Many of the close matches seen to *S. subterranea* f. sp. *nasturtii* in the current study were also diatoms (such as *Odontella sinensis*).

Phylogenetic examination of the entire 18S rDNA led to the conclusion that the plasmodiophorids form a discrete taxonomic group, not closely related to either protists or fungi (Figures 4.9-4.11). In the past there has been considerable uncertainty regarding the taxonomic treatment of plasmodiophorids. Some authors considered that they exhibit characteristics of fungi. Buczacki (1983) argued that the presence of chitin in resting spores walls inferred relationship to the Chytridiomycetes. Barr (1992) considered that there was no justification for placing the plasmodiophorids amongst the fungi and suggested earlier (Barr, 1983) that they should be treated as protists. Clay and Walsh (1997) described the flagellar apparatus of *S. subterranea* f. sp. *subterranea*, and found that the transition zone between the kinetosome and flagellum was similar to ciliated protozoa, but not other zoosporic fungi, providing further argument for removing plasmodiophorids from the fungal Kingdom.

There was good statistical support for the plasmodiophorids diverging after *D. discoideum*, but before the radiation of other groups (Figures 4.9-4.11). This was in agreement with the hypothetical tree produced by Barr (1983) to account for plasmodiophorid evolution, on which the plasmodiophorids were assigned a separate lineage to true fungi, and oomycetes (Figure 4.17).

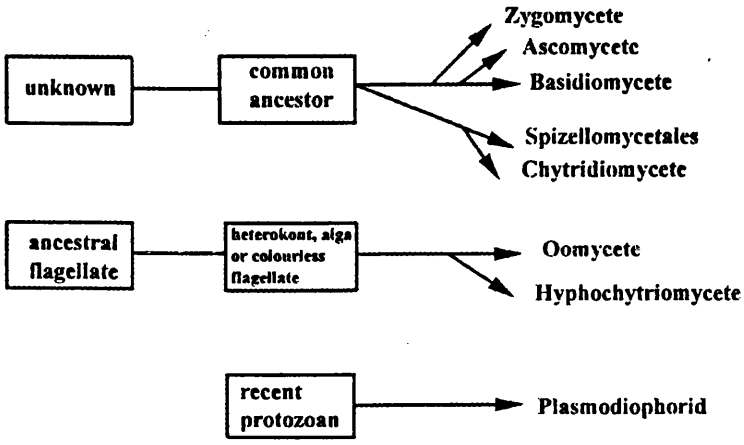


Figure 4.17 Schematic representation of fungal, stramenopile and plasmodiophorid evolution. Adapted from Barr (1983).

The separate grouping of the plasmodiophorids was also found by Ward and Adams (1998) using only the NS7-8 region of the ss rDNA, and they concluded that their results supported the fact that plasmodiophorids were not closely allied to the fungi, but that their precise evolutionary relationships were unresolved. Castlebury and Domier (1998) concluded that *P. brassicae* was a sister group to the alveolates based on maximum likelihood analysis, although confidence for this arrangement was not high and was not found using neighbour-joining or parsimony. Alveolates have been considered a sister group to the stramenopiles (Van de Peer *et al.*, 1996), and are characterised by a system of submembrane alveoli (Sogin and Silberman, 1998). Castlebury and Domier (1998) eventually concluded that plasmodiophorids had no close relationship to any other group of organisms tested. They found that

P. brassicae was placed within the crown group of eukaryotes, where the currently recognised major lineages abruptly diverge from each other. Results presented in this current study do not refute this analysis, and it may be that the plasmodiophorids do have relationships with other groups, but that further analysis is required. Wainwright *et al.* (1993) observed that the nodes for each of the crown groups (e.g. fungi, stramenopiles, plants), are separated by less than five nucleotide differences per thousand bases, making the precise branching order difficult to determine.

Phylogenetic relationships inferred among protists and fungi using 18S rDNA

Van de Peer and De Wachter (1997) attempted to resolve relationships among crown taxa, but on the basis of 18S rDNA many evolutionary relationships remained obscure. This was apparent in the current study, whereby the precise branching order of the stramenopiles and alveolates was variable depending on the tree-building method used (Figures 4.9-4.11). Van de Peer and De Wachter (1997) considered the stramenopiles and alveolates to be sister groups, diverging simultaneously from a common ancestor. A similar suggestion could be made from the results of the tree compiled using FITCH and DNAPARS in this current study (Figures 4.10 and 4.11). Cavalier-Smith (1993) did not support this view, placing alveolates in Kingdom Protozoa, and stramenopiles in a separate Kingdom Chromista, largely on the basis of the location of chloroplasts in autotrophic representatives. As other members of the Kingdom Protozoa were not seriously examined in the current study, it is not possible to criticise this taxonomy. Based on minimum evolution analysis, Kumar and Rzhetsky (1996) suggested that the stramenopiles and alveolates diverged prior to

fungi and green plants. This view was only supported in the current study by a BioNJ tree, with low confidence (Figure 4.9). FITCH and parsimony analysis suggested a simultaneous split from fungi and green plants.

The trees produced in Figures 4.9-4.11 show good agreement with previous studies at lower taxonomic levels than kingdoms and phyla. For example, using BioNJ, FITCH or DNAPARS algorithms, it was seen that the oomycete *Phytophthora megasperma* diverged prior to the other stramenopiles, a feature also observed by Van de Peer *et al.* (1996), Leipe *et al.* (1996), Saunders *et al.* (1995) and Bhattacharya *et al.* (1992), amongst others when examining ss rDNA data. The recognised classes of stramenopiles also group well on the trees in Figures 4.9-4.11. *Odontella sinensis*, *A. glacialis*, *F. striatula*, *T. rotula* and *S.* cf. *broschii* are all members of the Bacillariophyta (diatoms) and were seen to form a cluster at the tips of the branches, diverging after the oomycetes, chrysophyte (*T. volvocina*), and bolidophyte (*B. mediterranea*). Studies by Van de Peer *et al.* (1996), Leipe *et al.* (1996), Saunders *et al.* (1995) and Bhattacharya *et al.* (1992) placed the Chrysophyta as more recently diverged than the Bacillariophyta, but they did not use *T. volvocina* in their studies. Leipe *et al.* (1996) were unable to separate the order of divergence of these two classes if greater than 50% bootstrapping values were required for a combined analysis using maximum likelihood, maximum parsimony and neighbour joining. Their results concurred with the current study in placing autotrophic stramenopile classes nearer the tree tip than heterotrophs (oomycetes). Amongst the fungi, divergence of Chytridiomycetes before the radiation of Ascomycete species

(Figures 4.9-4.11), is well supported in the literature (e.g. Van de Peer and De Wachter, 1997; Van de Peer *et al.*, 1993).

Relationships between plasmodiophorids and a wider range of taxa

Attempts to further investigate the phylogeny of the plasmodiophorids by incorporating a wider range of taxa in analyses presented unexpected results. Figure 4.15 showed that there was good support for grouping plasmodiophorids with a chlorarachniophyte alga, *Gymnochlora stellata*. The chlorarachniophytes have generally been placed amongst early diverging branches in the stramenopile cluster (Van der Peer and De Wachter, 1997; Cavalier-Smith *et al.*, 1994), which could conceivably place them closer to the plasmodiophorids than many other organisms examined here. The direct relationship between chlorarachniophytes and plasmodiophorids was not retained when further chlorarachniophyte species were added to the tree (Figure 4.16), with the three chlorarachniophyte species forming a monophyletic grouping, separate from all other lineages (as was the case with plasmodiophorids). Chlorarachniopytes have an unusual biology, in that they retain the remnants of an endosymbiont nucleus within their cells (Palmer and Delwiche, 1996), which codes for rRNA. The origin of this endosymbiont is thought to be common to the cryptomonads also, and both may be related to the red algae (Van de Peer and De Wachter, 1997; Palmer and Delwiche, 1996; Cavalier-Smith *et al.*, 1994). The direct lineage connecting plasmodiophorids and chlorarachniophytes suggested in Figure 4.15 is unlikely to be true as chlorarachniophytes are photosynthetic organisms, whereas plasmodiophorids are not. Figure 4.15 suggests

that this would require an evolutionary pathway involving acquisition of an endosymbiont, then a loss of all trace of this organism to evolve plasmodiophorids, whilst only the endosymbiont nucleus is lost to give rise to stramenopiles containing chloroplasts. However, such an explanation may be required to account for the phylogenetic placement of oomycetes (Cavalier-Smith *et al.*, 1994). An alternative hypothesis would be independent acquisition of plastids by chlorarachniophytes and stramenopiles [and other taxa not considered here] (Sogin and Silberman, 1998), but this would not account for plasmodiophorids evolving from chlorarachniophytes. Chlorarachniophyte divergence after plasmodiophorids is a theoretical possibility and is suggested by Figure 4.17. A plasmodiophorid ancestor could have phagocytosed an endosymbiont, giving rise to chlorarachniophytes, which then lost their endosymbiont nucleus to form other crown groups. This would require prior evolution of algal species however, which does not fit with plasmodiophorid evolution based on ssrDNA in this study or those of Ward and Adams (1998), and Castlebury and Domier (1998).

The effect on plasmodiophorid phylogeny when a partial 18S rDNA of *S. subterranea* f. sp. *subterranea* was added to analyses

Trees constructed using approximately 1200 bases at the 3' end of the ssrDNA, and incorporating *S. subterranea* f. sp. *subterranea* in addition to *P. brassicae*, *S. subterranea* f. sp. *nasturtii*, and a range of protists, and fungi, suggested that *S. subterranea* f. sp. *subterranea* was more closely related to *P. brassicae* than to

S. subterranea f. sp. *nasturtii* (Figures 4.12-4.14). Although the bootstrapping values for this arrangement were not highly convincing, neither were those for grouping the two *formae speciales* of *S. subterranea* together using NS7-8 rDNA (Figures 4.5-4.8). The results in Figures 4.12-4.14 could conceivably arise as a result of distortion of alignments due to a number of anomalous base sequences within the *S. subterranea* f. sp. *subterranea* sequence, or because of the weightings given to parameters for alignments and tree-building. It may also be the case that *Plasmodiophora* and *Spongospora* are similar genera within the plasmodiophorids, as suggested by the analyses of Ward and Adams (1998). Braselton (1995) considered that they belonged within separate groups amongst the plasmodiophorids, due to *P. brassicae* having narrower central regions of the synaptonemal complex, smaller nuclear volume, and a multi-layered host-parasite membrane. The two *formae speciales* of *S. subterranea* were originally created by Tomlinson (1958b) when examining crook root of watercress. This division was based on similarity in resting spores, and the formation of aggregates of spores into so-called spore balls. Since this time there has been little in the way of direct comparison between the two *formae speciales*. Braselton (1992) examined only *S. subterranea* f. sp. *subterranea* when defining the species with respect to synaptonemal complexes and haploid chromosome number. It was noted by Braselton (1992) that *S. subterranea* f. sp. *subterranea* has thin host-parasite boundaries during early sporogenic development, whereas *S. subterranea* f. sp. *nasturtii* has a much thicker interface at this stage (Clay and Walsh, 1990). Clay and Walsh (1997) also reported no serological cross-reactions between the two *formae speciales*. Therefore the similarity between

S. subterranea f. sp. *subterranea* and *P. brassicae* based on 18S rDNA may not necessarily represent an anomalous result. In the current study, it should be noted that only one isolate of each of these plasmodiophorids has been assessed in this study, and it would be beneficial to have complete sequences of more plasmodiophorids for a more comprehensive analysis.

Insertion sequences in plasmodiophorid 18S rDNA

Spongospora subterranea f. sp. *subterranea* and *S. subterranea* f. sp. *nasturtii* both differ from *P. brassicae* in the lack of group I introns within the 18S rDNA. Group I introns occur relatively commonly in the ribosomal DNA of algae, fungi and slime molds, with at least one record within the stramenopiles (Holst-Jensen *et al.*, 1999; Van de Peer and De Wachter, 1998). They are frequently found in parasitic or symbiotic species (Nishida *et al.*, 1998), and in moist habitats (Holst-Jensen *et al.*, 1999). Johansen *et al.* (1992), have suggested that introns may be acquired through phagocytic behaviour. All of the above characteristics apply to the plasmodiophorids. Introns are thought to be capable of horizontal gene transfer, as well as vertical transmission (Nishida *et al.*, 1998), as fungal phylogeny does not always reflect intron phylogeny reconstruction, but this may be a rare event as it can be traced amongst lineages which diverged a long time ago (Holst-Jensen *et al.*, 1999). As stated in the results section, phylogenetic trees produced with *P. brassicae* introns retained did not have altered tree topology from those where they were omitted. Ward and Adams (1998) omitted *P. brassicae* introns from their phylogenetic analysis, whereas

Castlebury and Domier (1998) do not state that they did. Assuming that intronic regions were included in their tree-building procedures, this did not cause *P. brassicae* to show affinity with any other taxonomic groupings.

There are two explanations for the presence of introns in *P. brassicae* 18S rDNA, but not in *S. subterranea*. One is that *P. brassicae* acquired introns at some point after diverging from *S. subterranea*. The other is that *S. subterranea* had introns, but has since lost them. Bhattacharya *et al.* (1996) suggest that this is a common event, based on the observation that in groups of closely related species group I introns are often present in some, but not all representatives. The first hypothesis is supported by the trees shown in Figures 4.12-4.14, where acquisition of introns in *P. brassicae* requires one step, and loss in *S. subterranea* requires two. If in reality, *P. brassicae* diverged prior to *S. subterranea,* either explanation for intron presence or absence is equally parsimonious. Within *Polymyxa* species, no introns were seen in the NS7-8 region (Ward and Adams, 1998). If no introns are present in the entire 18S rDNA of *Polymyxa*, then Figures 4.5-4.8 suggest that the most likely hypothesis may be acquisition of introns by the *P. brassicae/ S. subterranea* group, followed by loss in *S. subterranea*.

A region of DNA with characteristics of a small insertion sequence found in *S. subterranea* f. sp. *nasturtii* (Figure 4.2), was also seen in *P. brassicae* and in *S. subterranea* f. sp. *subterranea*, where variation by at least one nucleotide variation was seen. Stenroos and De Priest (1998) have suggested that these might represent degenerate group I introns, although they concede that these sequences are known to sometimes occur at positions where no full length introns are known. BLAST

matches to organisms using the *S. subterranea* f. sp. *nasturtii* 18S rDNA did not reveal any introns at this position in similar species.

Methodological considerations

It could be argued that the assumptions made in calculating phylogeny in this study have been oversimplified. Kumar and Rzhetsky (1996) have reported that oversimplified models may lead to poor relationships among higher-order phylogenies. In addition to incorporating the Kimura-2-parameter model to weight transitions and transversions, they also introduced a correction for variation in site-to-site substitution rates across the ss rDNA. Such variation may arise from functional constraints in parts of the gene (Yang, 1996), and corrections may be introduced into algorithms to account for this. Models not accounting for this phenomenon (such as used in this current study) may lead to underestimation of substitutions at fast-evolving sites, and hence underestimation of distances between sequences (Yang, 1996). Also, if transitions are common, those at fast-evolving sites may be ignored in the analysis, and hence transitions will be underestimated. The ss rDNA molecule does have a distinct secondary structure, and hence functional constraints on some nucleotide positions (Van de Peer and DeWachter, 1997; Neefs *et al.*, 1991), and future studies should correct for this.

Future work

Future studies need to concentrate on amplifying complete 18S rDNA from more isolates of more plasmodiophorid species and genera if phylogenetic relationships are

to be successfully resolved. It may also be useful be advantageous to include a wider range of taxa in inferring phylogenetic relationships. Wee *et al.* (1996), make the point that when inferring phylogeny of an organism, particularly at higher taxonomic levels, it is important to sample from more than one DNA sequence, and this should be considered with regard to the plasmodiophorids.

In view of the economic importance of many plasmodiophorid species, their phylogenetic history may have relevance regarding novel control measures. As Sogin and Silberman (1998) state, phylogeny allows identification of appropriate model systems for studying disease processes.

5. General Discussion

This study is the first detailed assessment of the molecular biology of *S. subterranea* f. sp. *nasturtii* at the level of DNA sequence data. Means of detection of *S. subterranea* f. sp. *nasturtii* since the 1950's have been limited to observation of symptoms of crook root, leading to a reactionary use of zinc, rather than calculated addition. Utilising specific DNA regions within the 18S rDNA and ITS rDNA, it has been possible to develop a diagnostic PCR-based test for the pathogen. This test works effectively with infected root material collected from the field, then washed in water, which is then filtered to trap zoospores. Additional research is needed before the test could become commercially viable. Firstly, it would be much more efficient and less subjective to trap zoospores directly in effluent water at farms. This would require filtration of large, as yet unquantified, volumes of water, and use of some form of suction on site. Development of such a procedure would allow standardisation of sample volume, and removal of subjective sampling of plant material. The determination of the volume of water needed for detection of zoospores would provide a reference point for studies into correlation of *S. subterranea* levels and disease symptoms. If zinc use is to be rationalised, such quantification would be important, although environmental factors, such as temperature, may affect the relationship between pathogen density and disease symptoms. Quantification of *S. subterranea* zoospores should be possible using real-time PCR, which would speed up analysis, and could also reduce costs of commercial testing, once initial investment in equipment has been made.

The use of the 18S rDNA region for phylogenetic analysis has helped in increasing understanding of plasmodiophorid taxonomy. Although wider affinities of the group remain uncertain, relationships within the plasmodiophorids have become clearer, and it was seen that based on a small section of the 18S rDNA, *S. subterranea* f. sp. *nasturtii* has similarity to both *S. subterranea* f. sp. *subterranea* and *P. brassicae.* It should be noted that the models used to infer phylogeny may not necessarily be the most effective, based on the data supplied, and that further treatments (such as accounting for secondary structure) may give variations on the results obtained. Future challenges will include analysis of further species of plasmodiophorids using molecular data, and analysis of a wider range of genes to confirm rDNA-based findings. For many plasmodiophorid genera, molecular data are virtually non-existent (e.g. *Tetramyxa, Woronina*), and true understanding of phylogeny based on DNA sequences cannot be complete until such gaps are filled. It is also important not to ignore the good work that has been carried out using classical morphology, ultrastructural studies, and karyotyping of these organisms, when considering molecular phylogenies.

There was some confusion based on the 18S rDNA, as regards the relationship between *S. subterranea* f. sp. *nasturtii* and *S. subterranea* f. sp. *subterranea.* Techniques such as subtractive hybridisation, which selectively removes identical DNA sequences may be of assistance in identifying how these organisms differ.

One of the aims of this research project was to improve control measures for crook root disease of watercress. Concern over the environmental impacts of zinc, the only current control agent, mean that its use is tightly regulated, and make rationalisation or replacement a priority for the industry. No successful attempt to replace zinc as a control measure has been made since its introduction in the 1950's.

An assessment of zoospore response to plant root chemicals suggested that a non-specific exudate from watercress roots was acting as an attractant. Had a specific molecule been identified, it could have formed the basis for a new control strategy. By flooding watercress beds with such a chemical it is conceivable that zoospore chemotaxis to roots could be affected. Identification of an attractant could also have led to further research into production of watercress lines with reduced production of attractant, or into the mechanisms by which zoospores detect and respond to chemical gradients.

Watercress is the only known host for *S. subterranea* f. sp. *nasturtii*, and Claxton (1996) demonstrated that penetration of roots of tobacco, tomato, and mustard did not occur. Therefore, although no specificity is found in attraction to roots, there must be incompatibility in non-hosts at some point. This could be related to an inability to penetrate non-hosts, or to host resistance once plasmodia are injected. Such interactions have been well-studied in many systems, and techniques such as detection of specific mRNA production could be used to follow disease progression in watercress and non-hosts. Identification of host-specific factors could be used for development of control measures, either biochemical, or based on genetic modification of hosts. A drawback with the latter approach is that watercress genetics

are poorly understood, and no serious breeding programmes have ever been undertaken.

Efforts to find a crook root control agent as effective as zinc were unsuccessful. Other divalent cations tested were not as effective at lysing zoospores, leading to the conclusion that there must be a specific effect caused by the zinc ion (Zn^{2+}), perhaps at a membrane binding site, or after transportation into cells. The precise mode of action of zinc is unresolved, and as watercress is rich in zinc, it should be considered possible that as well as directly killing zoospores it may also act to improve host resistance. This study showed that lysis of zoospores was not as effective as might be expected in the field, suggesting that factors such as zinc accumulation in plants may be important. Zinc accumulation could be an alternative explanation for why disease outbreaks often start near bed outflows, and spread back toward the inlet. It may be assumed that zoospores will accumulate at greater density as they are washed down the beds, but it is also possible that zinc will accumulate nearer the inlet, where it is supplied. Measurement of zinc accumulation should be straightforward to carry out. Control of *S. subterranea* f. sp. *nasturtii* zoospores is likely to be less efficient than targeting resting spores, which present a considerable inoculum potential over the summer months in the beds. It would be desirable to carry out research into resting spore elimination, as this is a stage of the life-cycle about which little is known. Triggers for germination of resting spores have not been determined, and are potentially a major opportunity in disease control, such as by blocking germination, if chemical components of the process could be identified. Another possibility may be to

induce germination during summer months, or in empty beds, following which zoospores could be eliminated by zinc.

It would also be useful to confirm that *S. subterranea* f. sp. *nasturtii* is the major vector for WCLV and WYSV, the two major viruses purportedly vectored by *S. subterranea* f. sp. *nasturtii*. A quantitative diagnostic approach could be developed allowing correlation between *Spongospora* and viral DNA.

6. References

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APPENDIX A

Summary of statistical analyses for results presented in Chapter 2. Sections for which statistical analysis was not carried out are excluded. Method of analysis was ANOVA, with Tukey's test applied to assess significance of results. If no significance was found, this is stated, otherwise a summary table is shown. Differences between treatments and agarose/capillary controls are not included.
">" implies significantly more than (for whatever is being tested e.g. lysis)

NS = nutrient solution

Zoospore attraction to plant root extracts - no significant differences

Investigation of attraction of zoospores to leaf extracts
> = more attractive than

24 hour counts	Species tested			
	Watercress	Tobacco	Tomato	Watercress root
Watercress (W)				
Tobacco (Tob)	Tob > W			
Tomato (Tom)	None	None		
Watercress root (Wr)	None	None	None	

No significant differences at 3 or 6 hour counts.

Zoospore attraction to root extracts in assay chambers containing many extracts - results presented in Chapter 2.

Attraction of zoospores to root extracts diluted in water - results presented in Chapter 2.

Attraction of zoospores to intact roots - no significant differences

Attraction of zoospores to watercress bed water - no significant differences

Attraction of zoospores to chemicals released from intact roots
> = more attractive than

3 and 6 hour counts	Time at which samples were taken (hours)							
	1	3	6	12	24	48	72	NS
1								
3	None							
6	None	None						
12	None	None	None					
24	None	None	None	None				
48	None	None	None	None	None			
72	None	None	None	None	None	72>48		
NS	None	None	None	None	None	48> NS	None	

No significant differences at 24 hour counts, except 72hr>nutrient solution.

Effect of initial zoospore density on taxis and encystment - see Chapter 2 for correlation results.

Effect of zinc on zoospore taxis, encystment and lysis > = more attractive than
a) taxis

3 hours	Concentration of zinc (μg ml^{-1})							
	NS	0.025	0.05	0.075	0.1	0.5	0.1 (as Cl)	0.1 (as acetate)
NS								
0.025	None							
0.05	None	None						
0.075	None	None	None					
0.1	None	None	None	None				
0.5	None	None	0.5> 0.05	None	None			
0.1 Cl	None	None	None	None	None	None		
0.1 acetate	None	None	None	None	None	None	None	

No significant differences after 24 hours.

b) Lysis > = more lysis than

3 hours	Concentration of zinc (μg ml^{-1})							
	NS	0.025	0.05	0.075	0.1	0.5	0.1 (as Cl)	0.1 (as acetate)
NS								
0.025	0.025> NS							
0.05	0.05> NS	None						
0.075	0.075> NS	None	None					
0.1	0.1> NS	None	None	None				
0.5	None	None	None	None	None			
0.1 Cl	0.1> NS	None	None	None	None	None		
0.1 acetate	0.1> NS	None	None	None	None	None	None	

No significance after 24 hours

NS (24 hours) > NS (3 hours)

c) Motility - no significance at 3 or 24 hours.

Effects of cations on zoospore survival
> = more lysis than

	Cation tested						
	Mn chloride	Mn sulphate	NH_4^+	Al	Co	Zn	NS
Mn chloride							
Mn sulphate	None						
NH_4^+	None	None					
Al	None	None	None				
Co	Co>Mn	Co>Mn	Co>NH_4	None			
Zn	Zn>Mn	Zn>Mn	Zn>NH_4	Zn>Al	None		
NS	None	None	None	None	Co>NS	Zn>NS	

Effect of cobalt on zoospore taxis, encystment and survival
➢ = more attractive than

a) taxis

24 hours	Concentrations of cations (μg ml^{-1})				
	NS	2 (Zn)	4 (Zn)	2 (Co)	4 (Co)
NS					
2 (Zn)	NS>Zn				
4 (Zn)	NS>Zn	None			
2 (Co)	NS>Co	None	None		
4 (Co)	NS>Co	None	None	None	

b) lysis

> = more lysis than

2 hr	Concentration of cations (μg ml^{-1})										
	NS	0.5 (Zn)	0.5 (Co)	1 (Zn)	1 (Co)	2 (Zn)	2 (Co)	4 (Zn)	4 (Co)	8 (Zn)	8 (Co)
NS											
0.5 (Zn)	None										
0.5 (Co)	None	None									
1 (Zn)	Zn> NS	None	None								
1 (Co)	None	None	None	None							
2 (Zn)	Zn> NS	None	Zn> Co	None	None						
2 (Co)	Co> NS	None	None	None	None	None					
4 (Zn)	Zn> NS	4Zn >0.5	Zn> Co	None	None	None	None				
4 (Co)	Co> NS	None	None	None	None	None	None	None			
8 (Zn)	Zn> NS	8Zn >0.5	Zn> Co	None	None	None	None	None	None		
8 (Co)	Co> NS	8Co >0.5	None	None	None	None	None	None	None	None	

No significant differences seen with regard to cell lysis when zinc and cobalt were used in combination.

APPENDIX B

Effectiveness of organic molecules as zoospore chemoattractants, and their effect on zoospore encystment, as measured by cell motility. Results are means of two replicates for zoospore attraction, and one test for encystment. Tests for chemotaxis and encystment were performed in separate experiments.

Test chemical (20mM)	No. of zoospores attracted	No. zoospores counted in repli-dish compartment	% non-motile
Aspartic acid	11	Not tested	Not tested
Alanine	7	Not tested	Not tested
Methionine	9	52	65
Glutamine	7	45	69
Pectin *	7	100+	70
Proline	7	25	68
Histidine	4	37	76
Nutrient solution	5	100+	20
MilliQ water	5	26	77
Glycine	4	19	63
Asparagine	4	40	95
Galactose	5	36	83
Isoleucine	2	29	48
Phenylalanine	2	62	60
Sucrose	2	20	85
Glutamic acid	4	Not tested	Not tested
Polygalacturonic acid **	1	100+	100
Glucose	3	55	89

* Pectin supplied at 1.8 mg ml^{-1}

** PGA supplied at 560µg ml^{-1}

APPENDIX C

ITS rDNA sequence of the chytrid-like organism associated with crooked roots, indicating the biding position of primers designed to be specific for this organism. The primer sequences are also listed.

```
1   GGCGGCCGCG  GGAATTCGAT  TCTTGGTCAT  TTAGAGGAAG  TAAAAGTCGT
51  AACAAGGTTT  CCGTAGGTGA  ACCTGCGGAA  GGATCATTAA  TAAATGCTTC
101 ATTGCATCCA  CCTTTTGTGC  ACCGTAAATT  TTCACTTAAA  CTTTGTCTGA
151 TTTATATCTG  TTCTGAAAGG  AACATGAATT  GTAATCTAAT  ACAACTTTTA
201 ACAATGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG  CGAAATGCGA
251 TAAGTAATGT  GAATTGCAGA  ATTCAGTGAA  TCATCGAATC  TTTGAACGCA
301 TATTGCGCTC  TCTGGCATTC  CGGAGAGCAT  GCCTGTTTGA  GTCTTGGTAC
351 CAATCTGGTG  TAACCTTTGT  TATTAAGAAT  AGAACTTTTT  ATAAAAAAAC
401 TGCATTGAGA  CGTCTTGATA  AGTAAAATTG  TTAAGTGTCT  TAAAATACAA
451 TAGTACAGCT  ATAGCTCACC  CTCTTTGGAG  TAATATGTGA  CCTGAAAGGG
501 TAACTCTATT  TCTCCTCGTT  GGGAAGACTT  GTTGCTAGAA  GTTTTTGATT
551 GCTGAACGTC  AAAGGCGGCA  TTCAATTGTT  CGAGTAACGA  CGAACTTAAA
601 AATTCAAGCT  CAAATCAGGT  AGGATTACCC  GCTGAACTTA  AGCATATCAA
651 TAAGCGGAGG  AATCACTAGT  GAATTCGCGG  G
```

SSNF1 = CCTTTTGTGCTCCGTAAATTTTCA SSNB1 = ACTTCTAGCAACAAGTCTTCCC

SSNF2 = As SSNF1, with CA at the start SSNB2 = ACCCTTTCAGGTCACATATTACTCC

305F = ACCAATCTGGTGTAACCTTTGTTA 461B = CAAGTCTTCCCAACGAGGAGAAATA

APPENDIX D

Sequence obtained from a DNA band isolated from multiple PCR products when amplifying DNA from crooked roots using ITSIF/ITS4. The sequencing primer was ITS 4. No similar sequences were found using GenBank. The length is 364 bases.

CNGAGGTCGGATAAGTGATCAAAGTACATGAACAGTTTATGTGCCAACAATATACAAAACAGAATCCGTAA
ATCACGTAACAGAACACAACTTATTAAGCTGTGATTCCGAAACAATAAAAGTCCTTTACAGAAACCAGATT
GTNATAATCAATATTGTTAACGAAACCGACATCATAATTAATCGANACGAATCGAATAGGAAATACTCGAA
ACGAATCTAGTCTCCAACTTTTTACAGTCGGAGGTAGTCATCAGCCAATTAACGCGTCAAACTACTATCAN
ATTAAAGAGGCACTCGGTGAGAGTGAANCCCTCANTNCACTACTATTGGNCGTNATGGNNAGTATTNTGCC
GACCCTCAT

APPENDIX E

PCR reactions carried out to obtain *S. subterranea* f. sp. *nasturtii* 18S rDNA.

1. Primers NS5 and NSA.

NS5 = AACTTAAAGGAATTGACGGAAG

NSA = GTCATGAAATTCACAAGTT

1mM dNTP's each, 3mM Mg^{2+}, 30pmol primers, 2.5U *Taq* polymerase.

Cycling parameters: 94°C for 20 seconds, 50°C for 120 seconds, 72°C for 120 seconds.

2. Primers NS3 and NSL.

NS3 = GCAAGTCTGGTGCCAGCAGCC

NSL = TCCAAGAACTATCCGGAGATAAT

1mM dNTP's each, 3mM Mg^{2+}, 30pmol primers, 2.5U *Taq* polymerase.

Cycling parameters: 94°C for 30 seconds, 60°C for 60 seconds, 72°C for 120 seconds.

3. Primers NS1 and NSL2.

NS1 = GTAGTCATATGCTTGTCTC

NSL2 = AACACACACAAGTCCAACTA

1mM dNTP's each, 3mM Mg^{2+}, 20pmol primers, 2.5U *Taq* polymerase.

Cycling parameters: 94°C for 20 seconds, 57°C for 120 seconds, 72°C for 60 seconds.

4. Primers NS5-1 and NSA-1.

NS 5-1 = CCGCACTCGCGTTACAATGTG

NSA-1 = ACAAAGGGCAGGGACGTATTC

1mM dNTP's each, 3mM Mg^{2+}, 10pmol primers, 2.5U *Taq* polymerase.

Cycling parameters: 94°C for 20 seconds, 58°C for 120 seconds, 72°C for 60 seocnds.

APPENDIX F

```
Ssn --CCTGGTTGATCCTGCCAGTAGTGATATGCTTGTTTCAAAGATTAAGCCATGCATGTCT 58
PBr ACCCTGGTTGATCCTGCCAGTAGTCATATGCTTGTTTCAAAGATTAAGCCATGCATGTCT 60
Sss ------------------------------------------------------------

Ssn AAGTATAAGCGAACTTATACAGTGAAACTGCAAATGGCTCATTAAAACAGTTTGAATTTA 118
PBr AAGTATAAGCGAACTTATACAGTGAAACTGCGGATGGCTCATTAAAACAGTTTGAATTTA 120
Sss ------------------------------------------------------------

Ssn TTTGATGGATGTACGAAAGCACTACATGGATATCCGTCGAAATTCGAGAGCTAATACATG 178
PBr TTTGATGGATGTACGCGAGT-CTACATGGATATCCGTAGTAATTCTAGAGCTAATACATG 179
Sss ------------------------------------------------------------

Ssn CAACAACGCCCGACCGTTCGCCGTAGGGTTGTATTTATTGGATACAAAACCCATGACCTG 238
PBr CAGGAACGCCCGACC-CTCGCGGAAGGGCTGTATTTATTGGATACAAAAACCA-AACCTG 237
Sss ------------------------------------------------------------

Ssn GCAACAGGTTTTCCTTGCTGATTCACAATAACTGATCGGATCGCGGCTTCGGATGCGATA 298
PBr GCAACAG-TTTCGTTTGTTGATTCACAATAACTGATCGGACCGTTGCCTCGGCAGCG-CA 295
Sss ------------------------------------------------------------

Ssn GGTCATTCATCATTCTGCCCTATCACCTTTCGATGGTTCTGTACTGGACAGCCATGGCTT 358
PBr GGTCATTCAACATTCTGCCCTATCAGCTTTCGATGGTTGTGTAGTGG-CA-CCATGGCCG 353
Sss ------------------------------------------------------------

Ssn TAACGGGTAACGATAGAATCACGGTTCGGTTCCGGGGAGGGCGCTTGAGAATTGGAGACC 418
PBr CAACGGGTAACGG-AGAATCGGGGTTCGATTCCGGAGAGGGAGCTTGAGAATTGGCTACC 412
Sss ------------------------------------------------------------

Ssn ACATCTAAGGAAGGNCAGCAGGCGCGCAAATTACCCAATCCTGACTCAGGGAGGTAGTGA 478
PBr ACATCTAAGGAAGGC-AGCAGGCGCGCAAATTACCCAATCCTGACTCAGGGAGGTAGTGA 471
Sss ------------------------------------------------------------

Ssn CAAAAAATAACAATACCGGATCTTTTTGGGTCTGGTAATTGGAATGAGAACAAGTTAAAC 538
PBr CAAAAAATAACAATACCGGTTCTTGTTAGGACTARTAATTGGAATGAGAACAAGTTAAAC 531
Sss ------------------------------------------------------------

Ssn CCATTATCGAGGATCCATTGGAGGGCAAGTCTGGTG---------------------- 574
PBr CTATTATCGAGGATCCATTGGAGGGCAAGTCTGGTGGACGATTCGACAGTGTCTGTTTGC 591
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr TGTTGGCGCCAGAGATAGTACGGCTTCTCTTTTAAAGAAGTTATGCGTGCTAGTCGAGCA 651
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr GCCCAATCTAGTTGTGGGCTGGCTGCCGGCGAGGTAACCTGGTACGAGGAAGGCTTAACG 711
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
```

```
PBr GATTTTTGATAATCCTATGCTAATCTCGTGGCGAGCCTGTGCCGAAGCGATTCGGGCATA 771
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr GAGGCCGTCGTAACGCGCGGAAAGGTACCGGGCGGGCTTCCACAGTCCGCTCAAGGAACG 831
Sss ------------------------------------------------------------
Ssn ------------------------------------------------------------
PBr TGCTAATCCCATTCGAAAGAAAATGCCTGATCGACTGGTAGCGCCCACAACGCAAAGGAC 891
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr GATCGTGCAGTGTGTGGCGCGTGTGATGGAGGAATGCCATCAGACGAGACCCGGTATATG 951
Sss ------------------------------------------------------------

Ssn ----CCAGCAGCCGCGGTAATACCAGCTCCCAGTAGCGTATATTAAAGTTGTTGC-GTTA 629
PBr TTGGCCAGCAGCCGCGGTAATTCCAGCTCC-AATAGCGTATATTAAAGTTGTTGCAGTTA 1010
Sss GTGGN---CNGCC----NAA-------------------------AAGTTGTTGCAGTTA 28

Ssn AAAAGCTCGTAGTTGGACTTGTGTGTGT-TGC-TGTTCAGCGGTCTG-ATTCAAAAGAGT 686
PBr AAAAGCTCGTAGTTGGACTTGTGTGCCTGCGCGTGTTCAGCGGTCTGCGTTCAAAAGAGC 1070
Sss AAAAGCTCGTAGTTGGACTTGTGTGCAT--GGGAACTC-GCGGTCTTCGTCCAAAAGGGC 85

Ssn GTTACGACATGCGGCAGTGGCGCCGTAAAATCTTCAACTGGATCGCAACTGGACTTAATT 746
PBr GTTACGACATGCG--CGCGG-GCCGTACAATCTTCAACTGGATCGCGTCTGGTCTTGATT 1127
Sss GTTACGACATGCGGTTCCTGTGCCGTAAAATCTTCAACTGGATCGCACCTGGTCTTGATT 145

Ssn GCCTGGTTGCAGTC-ATTCGGTTGATCGTTTACTGTGAGAAAATTAGAGTGTTCAAGGCA 805
PBr GACTGG--GCTGCCTATTCGGTTGATCGTTTACTGTGAGAAAAGTAGAGTGTTCAAGGCA 1185
Sss GGCTGGGCGCTGTC-ATTCGGTTGATCGTTTACTGTGAGAAAATTAGAGTGTTCAAGGCA 204

Ssn GGCATTATTGCAATTGAATATGTTAGCATGGAATAATAAGATAAGGCTTTCGCGTTTATT 865
PBr GGCATTATTGCAATTGAATATGTTAGCATGGAATAATAAGATAAGGCTTTCGC-TCTATT 1244
Sss GGCATGATTGCAATTGAATATGTTAGCATGGAATAATAAGATAAGGCTTCTGC-TTTATT 263

Ssn TTGTTGGTTTCTAGATCGGAAGTAATGATTGATAGGGACAGTTGGGGGTGCTAGTATTCA 925
PBr TTGTTGGTTTCTAGGGCGCA-GTA-TGATGAATAGGGATAGTTGGGGGTGCTAGTATTCA 1302
Sss TTTTTGGTTTCTAGAGTGGAAGTAATGATTGATANGGATAGTTGGGGGTGCTAGTATTCA 323

Ssn GC-GGCCAGAGGTGAAATTCATGGATTCGCTGAAGACTAACTTATGCGAAAGCATTCACC 984
PBr GCCGGCCAGAGGTGAAATTCATGGATTCGCTGAAGACTAACTTATGCGAAAGCATTCACC 1362
Sss GN-GGCCANANGTGAAATTCATGGCTTCGCTNAAGACTAACTTATGCNANAGCATTCACC 382

Ssn AAGGATGTCCTCTTTAATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTC 1044
PBr AAGGATGTCCTCTTTAATCAAGAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTC 1422
Sss AAGGACGTCCTCTTTAATCAANAACGAAAGTTGGGGGATCGAAGACGATCAGATACCGTC 442

Ssn GTAGTCTCAACCATAAACTATGTCGACTAGGGATTGGCAGGTGTTTTTCAATTAAGACTC 1104
PBr GTAGTCTCAACCATAAACTATGTCGACTAGGGATTGGCGGTCGTTTTTTATTGTAAGACTC 1482
Sss GTAGTCTCAACCATAAACTATGTCGACTAGGGATTGGCGGTCGTTTTTTATTATAAGACTC 502
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Ssn TGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGAGGAAC---------------- 1209
PBr TGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAACCATGCGTGTTCATGC 1602
Sss TGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGC---------------- 607

Ssn ------------------------------------------------------------
PBr CCCGCCGCGAGCAGCATCCGCTCCGAATAGCAGCTGGAAACAGTGAGGTGGGTCGGGTCC 1662
Sss ------------------------------------------------------------
Ssn ------------------------------------------------------------
PBr TTGTAAAACAAACCTGCTAGTGCCGACGGACGAGTTCGTCGACACGACATCGTCAAATTG 1722
Sss ----------------------------------------------C------------ 608

Ssn ------------------------------------------------------------
PBr CGGGAACCTCCTAACACTTCACCTACCAAGTGCTGCTACGCGTGTGCGCAAAAGCACGGC 1782
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr CGAGCTAATCACCTGGGTATGGTAACAACGGTGAAGATATATCATGGACGATCCGCAGCC 1842
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr AAGTCCTACGTCGAAGTTCATTCGATATGGAACGGGTTCAGAGACTAGATGGCGGTGGGC 1902
Sss ------------------------------------------------------------

Ssn ------------------------------------------------------------
PBr CTCGCGCCTCGCTTAAGATATAGTCCAATCCGGTCCAGCGCAAAAGCGCCGGCAACGATA 1962
Sss ------------------------------------------------------------

Ssn ---------TGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGAGAT-TA 1259
PBr TTGGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGAGATGTA 2022
Sss ---------TGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGAGATGTA 659

Ssn GAGGATTGACAGATTGAAGCTCTTTCTTGATCACTTCGGTGGTGGTGCATGGCCGTTCTT 1319
PBr GAGGATTGACAGATTGAAGCTCTTTCTTGATCACTTCGGTGGTGGTGCATGGCCGTTCTT 2082
Sss CAGGATTGACAGATTGAAGCTCTTTCTTGATCACTTCGGTGGTGGNGCATGGCCGTTCTT 719

Ssn AG-TTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTTT 1378
PBr AG-TTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCTACCTGCTTT 2141
Sss AAATTGGTGGAGTGATTTGTCTGGTTAATTCCGTNAANGANCGAGACCTCAGCCTGCTTT 779

Ssn TGTANTTCCGATTATCT-CCGGATAGTTCTTGGAACTTCTTAGAGGGACTATGTGTTTTT 1437
PBr -GTAGTTATGCCTATGC-CTAG---GTCCGTG--ACTTCTTAGAGGGACTATCGGTGTGG 2194
Sss TGTANTCTGGCCTATCCTCCGGGCTATGTTTCCAACTTCNTATANGGACTATCNATTTT- 838

Ssn C-GCACATGGAAG-TTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCG 1495
PBr CAGCCGATGGAAG-TTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCG 2253
Sss CANTCGATGGAANGTTTNACGCAANAACAGGTCTGTGATNCCCT---ACGCACTGNGC-- 893

Ssn CACGCGCGTTACAATGTGTGGTTCAACGAGTTT----TTTTCTTGGTC-GAAAGGCCTGG 1550
PBr CACGCGCGCTACACTGCTGGGTTCAACGAGTCCGAACCGTACTTGGCT-GAAAGGCCTGG 2312
Sss -------GNTACAATGNNTGGTTCAACGAGTNN----TTNACTTGGNTTGAGAGGCNTGG 902

Ssn ATAATCTTCTGAAATCCACACGTGCTGGGGCTTGCGGCTTGCAACTAGCCGCACCAACGA 1610
PBr TTAATCTTTTGAAATCCCAGCGTGCTTGGGCTTGCCTCTTGGAACTAGAGGCACCAACGA 2372
Sss TTAATNTTTTGAAANCCAAGCGTGCTTGGGCTTGCCTCTTGCAACTAGAGGCACCAACGA 1002
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Ssn GGAATTCCTAGTAGACGCAAGTCATCAACTTGCATCGATTACGTCCCTGCCCTTTGTACA 1670
PBr GGAATTCCTAGTAGACGCAAGTCATCAACTTGCATCGATTACGTCCCTGCCCTTTGTACA 2432
Sss GGAATTCCTAGTAGACGCAAGTCATCAACTTGCATCGATTACGTCCCTGCCCTTTGTACA 1022

Ssn CACCGCCCGTCGCTCCTACTGATTGAATGCTCCGGTGAAA-CGTCGGGAGAGCGCT---- 1725
PBr CACCGCCCGTCGGTCCTACTGATTGAGTGCTCCGGTGAAAATGTCGGGAGATTGCTCGCG 2492
Sss CACCGCCCGTCGCTCCTACTGANTGAATGCTCCGGTGAAA-CGTCGGGAGAATGCGC--- 1078

Ssn ------------------------------------------------------------
PBr ATCAGCAGGAGTGGCAACTTCATTAAATTTGGGCTCTTAGAAGAAGGAGAAGTCGTAACA 2552
Sss ------------------------------------------------------------

Ssn --------------TCGATATCAGCAA-------------TGGT---------------- 1742
PBr AGGTTTCCGTAGCGTCGTCGTCGGCGACCGCTACAGGAGCTGGTCCTTCCACACGGTCAT 2612
Sss --------------CTGCGATCAGCAT-------------TGGT---------------- 1135

Ssn ---------------------------------TGAA----------------------G 1747
PBr CGCCCGGGAAGCCTTAGCAGCCGGAAACGGTGCTGAACGCGACTTTGTCAAAAAACAACG 2672
Sss ---------------------------------CCAG----------------------G 1140

Ssn AGT--GCTCGAA-----------------CTT--------CCTTAAATTGTAG------- 1773
PBr AGTCAGCTTGAATGCTAGTGTGTCGCGCGCCTATGCGACACGTTAAATTGCGGGGACCCT 2732
Sss TGTGGGNNCA-------------------CTT--------CCTTAATTTTGGG------- 1166

Ssn ------------------------------------------------TATT--------- 1777
PBr GAAGCTTGTCTGCCAATCGCCCTGGGTATGGTAACAACGGACAAGGATGTTATATATGGG 2792
Sss ------------------------------------------------TT----------- 1168

Ssn ---------------------------------------------TAGAA------------ 1782
PBr TGATCCGCAGCCAAGTCCTACGTCGAATCGCGCATGTTCGATATGGAACGGGTTCACAGA 2852
Sss ---------------------------------------------TT--------------- 1170

Ssn ------------------------------------------------------------GA 1784
PBr CTAGATAGCAGTGGGTGGCACACACGTGTGTCGCTTAAGATATAGTCGGTCTCGTGCCGA 2912
Sss ------------------------------------------------------------

Ssn A------GGAGAAGT----------------------CGTA------------ACAAGG 1803
PBr AACGCACGGGGAGGTGATCGACGCTCTTTCGTGTCGCTCGTATTCGACAACTGCACAGTG 2972
Sss ------------------------------------------------------TAGGG 1175

Ssn T-------------------------TTCCGTAGGTGAACCTGCGGAAGGATCATTA 1835
PBr TGCGTCACATCGCCATCATGATGACGTTCCGTAGGTGAACCTGCRGAAGGATCA--- 3026
Sss C-----------------------------CAGGCAAANN----AANATATNT--- 1195
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Primer SSN18 sequence is highlighted.

APPENDIX G

Partial sequence of 18S rDNA of the chytrid-like species associated with crooked roots.

The sequence is 1509 bases in length, and lacks around 95 bases between the highlighted nucleotides (according to BLAST matches).

ACACAGTGAANGGGCCAAGGGCTCATTAAAACATTTANAGTTTATTTGATAGTACTTTACAACTTGG
ATACCCGTGGTAATTCTAGGAGCCAATACATGCGTTAAAANCCCGACTTTTTGAAGGGATGTATATA
TTAGATAAAAAACCAATGCCAGCAATGGCTTTCTTGGTGATTCATAATAACTTTTCGAATCGTATGN
CCTTGTGTNGACGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTN
CCATGGTTTTAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC
CACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAA
TAACAATACAGGGCCCTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGTTT
TGATCTGGCTGAGGTGTCGGCCGCAAGGTCTGTACTCCTTGGCTGGATCTTTCCTTCTGGAGAGCCA
TTATGCCATTTATTTGGTGTTTTGGGGATCCAGGACTTTTACCTTGAAAAAATTAGAGTGTTTAAAG
CAGGCATACGCTTGAATACATTAGCATGGAATAATAGAATAGGACTTTGGTTCTATTTTGTTGGTTT
CTAGGACCGAAGTAATGATTAATAGGGATAGTTGGGGGCATTAGTATTTAATTGTCAGAGGTGAAAT
TCTTGGATTTATGAAAGACTAACTTCTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAAC
GAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAANCTATGCCGACTAGGG
ATCGGNCGATGTTATTTTATTGACTCCGTTCGGCACCTTATGAGAAATCAAAGTTTTTGGGTTCCGG
GGGGATTCTGGTCGCAAGGCTGAAACTTATTGACTCAACACGGGGAAACTCACCAGGTCCAAGACAT
GGTAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACNAACGAGACCTTAACCTGCTAAATAGTTACGCG
AATTCTTATTCGTGGCCAACTTCTTATAGGGACTGTTGATGTTTAATCAACGGGAAGTTTGAGGCAA
TAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGTCAACGAGT
TTATAACCTTGGCCGGAAGGTCTGGGTAATCTTTTGAAACTTCATCGTGCTGGGGATAGTCCATTGC
AATTATTGGACTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCANCTCGCGTTGATTACGTCCCT
GCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTTCGGATTGGAGG
TAGGTTGCTGGCAACAGCAACCAGCTTCCCGAGAA