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Testate Amoebae Communities in the Rhizosphere of *Rhododendron ponticum* (Ericaceae) in an Evergreen Broadleaf Forest in Southern Spain

Martin VOHNÍK[1,2], Zuzana BURDÍKOVÁ[3] and David M. WILKINSON[4]

[1]**Department of Mycorrhizal Symbioses, Institute of Botany ASCR, Průhonice, Czech Republic;** [2]**Department of Plant Experimental Biology, Faculty of Science, Charles University in Prague, Prague, Czech Republic;** [3]**Department of Biomathematics, Institute of Physiology ASCR, Prague, Czech Republic;** [4]**School of Natural Science and Psychology, Liverpool John Moores University, Liverpool, UK**

Abstract. Testate amoebae (TA) are an important part of soil microbial communities and in certain ecosystems they may represent a substantial proportion of total microbial biomass. Their distribution and abundance is driven by various abiotic factors (e.g., pH, organic matter, soil moisture, soil/water chemistry) but comparatively less is known about the role of biotic interactions. TA often co-occur with Ericaceae, a ubiquitous plant family inhabiting acidic soils with poor nutrient status. Ericaceae can significantly change soil properties through production of recalcitrant litter and possibly also due to root exudates and activities of root-inhabiting fungi; this may result in profound modifications of microbial communities. A recent study from northwest England showed that the invasive ericaceous shrub *Rhododendron ponticum* may significantly modify communities of soil TA. Here, we investigate the effect of pH, organic matter, and *R. ponticum* presence on TA communities within the native range of the ericaceous shrub at two sites in southern Spain and compare our results with the previous study from NW England. At the Spanish sites, organic matter content, *R. ponticum* presence and pH affected occurrence and abundance of several TA species; *R. ponticum* presence and organic matter content were highly correlated and explained most of the observed variability in TA communities (i.e. the effect of the *R. ponticum* rhizosphere). *R. ponticum* rhizosphere affected especially TA with relatively large tests, i.e., *Cyclopyxis eurystoma*, *Phryganella acropodia* and *Trigonopyxis arcula*. Interestingly, *T. arcula* was also positively associated with *R. ponticum* in the previously studied British sites. The rhizosphere of the ericaceous shrub appears to have a positive effect on testate amoebae taxon richness at the two studied autochthonous Spanish sites but may reduce taxon richness in the sites in Britain where *R. ponticum* is an introduced species. Such possible positive/negative effects of native/invasive species, as well as other plant guilds, on TA communities clearly deserve further investigation.

Key words: Testaceans, *Trigonopyxis*, invasive species, rhizosphere ecology, root associated fungi, ericoid mycorrhiza.

Address for correspondence: Martin Vohník, Institute of Botany ASCR, Lesní 332, CZ-25243 Průhonice, Czech Republic. Tel.: +420271015331; E-mail: vohnik@ibot.cas.cz

INTRODUCTION

Testate amoebae (TA) are protists in which the single cell is enclosed within a shell – usually referred to as a test. Although polyphyletic, TA seem to form a reasonably uniform ecological grouping. The morphology of their shells allows them to be identified to a range of different morphospecies (e.g. Ogden and Hedley 1980), making them useful taxa for investigating changes in soil microbiology as they can be enumerated by direct counting (giving population data as well as just species lists) without having to resort to culturing or molecular methods (Wilkinson and Mitchell 2010). Composition of TA communities is influenced by a wide range of abiotic factors, but organic content, pH, soil moisture and water table depth seem to play an especially important role (Charman and Warner 1992, Ekelund *et al.* 2001, Booth and Zygmunt 2005, Opravilová and Hájek 2006, Mieczan 2009, Jassey *et al.* 2011). Several authors investigated the importance of various biotic factors but their effects seem to be less clear (or harder to explain straightforwardly) than abiotic factors (Ingham and Massicotte 1994, Timonen *et al.* 2004, Krashevska *et al.* 2008, Vohník *et al.* 2009, Vohník *et al.* 2011). TA are abundant and potentially important in various soil processes especially in *Sphagnum*-dominated peatlands and in organic soils in general (Gilbert *et al.* 1998, Wilkinson and Mitchell 2010, Lara *et al.* 2011).

Ericaceae (the heath family) are a specialized plant group ubiquitous on nutrient poor acidic soils of heathlands, peatlands and mire complexes as well as in ground layers of many temperate and boreal forests (Specht 1979, Read *et al.* 2004). They form dense root systems functionally dominated by hair roots with inflated rhizodermal cells which host ericoid mycorrhizal (ErM) fungi (Read 1996). These mycobionts were suggested as a major component of Ericaceae success in nutrient impoverished soils (Read and Perez-Moreno 2003). Indeed, ErM fungi developed an effective enzymatic apparatus to scavenge nutrients from various complex substrates such as peptides, proteins, chitin, fungal mycelium and plant-mycorrhizal necromass (Bajwa and Read 1985; Bajwa *et al.* 1985; Kerley and Read 1995, 1997, 1998), hence changing nutrient flows in soils below Ericaceae. Additionally, ericaceous plants contribute to the characteristic chemistry of soils they inhabit by production of recalcitrant litter low in nitrogen and phosphorus (Read *et al.* 2004); they seem to have the slowest litter-decay rate among all mycorrhizal plants (Read 1991).

Humans have altered the distribution of a large number of plant species, introducing them into parts of the world where they do not naturally occur. Some of these introduced plants have become major pest species in their new ranges, radically altering the native vegetation. Examples include Brazilian pepper (*Schinus terebinthifolius* Raddi) and kudzu vine (*Pueraria montana* var. *lobata* (Willd.) Maesen and S. Almeida) in Florida, USA (Silvertown 2005) and Spanish cedar (*Cedrela odorata* L.) in the Galapagos Islands (Hambler 2004). Introduced plant species are now viewed as a major conservation problem in many parts of the world – often because the introduced species comes to dominate the local vegetation to the detriment of native species (Hambler 2004). In many parts of the world the vegetation is now dominated by non-native species – a spectacular example being parts of Ascension Island in the tropical South Atlantic where almost all the plant species are introduced (Wilkinson 2004). Ecologists were slow to study such 'novel ecosystems,' however such systems have been attracting a growing amount of attention over the last decade as their global significance has become apparent (Hobbs *et al.* 2006, Mascaro *et al.* 2012).

The shrub *Rhododendron ponticum* L. (Ericaceae) is an important invasive plant species in Britain and Ireland which has been described as 'a menace to conservation' (Rackham 1986) and is the most important non-native invasive plant threatening Sites of Special Scientific Interest in Wales, UK (Thomas and Dines 2010). Intriguingly the origin of the plants introduced to Britain appears to be Spain (Milne and Abbott 2000) where the shrub is a relatively rare and declining species of conservation concern (Mejías *et al.* 2002, 2007). *R. ponticum* was originally introduced to Britain, as an ornamental plant, around 1763 and by 1849 the species was producing viable seeds and starting to colonize the wider countryside (Dehen-Schmutz *et al.* 2004). It has become widely established in woodland, heathlands, bogs and grasslands within Britain – in all of these habitats it is now usually considered to be a major conservation problem (Cross 1975, Thomson *et al.* 1993, Dehen-Schmutz *et al.* 2004). In many British woodlands it has come to dominate the shrub layer, creating a novel ecosystem with a very different light climate and soil chemistry on the woodland floor compared to *Rhododendron* free woodland (Cross 1975, Thomas and Dines 2010, Wilkinson, personal observation).

In both Britain and its native Spain *R. ponticum* is a plant of mainly acidic soils and its litter pro-

duces a mor humus (Cross 1975, Mejías *et al*. 2007). *R. ponticum* effects soil ecology and has been recorded as reducing the numbers of a range of earthworm species – although the species affected appear to vary between sites (Cross 1975). A number of different chemicals have been implicated in the effects of *Rhododendron* on both soil invertebrates and plants; these include polyphenols and cyanidin (Cross 1975, Wurzburger and Hendrick 2007). Sutton and Wilkinson (2007) showed that *R. ponticum* presence significantly affected TA communities in woodland soils in northwest England. However, they were unable to compare these data with soils from under native *R. ponticum* in Spanish woodlands because of an absence of any published data.

In this paper we describe TA communities from two Spanish autochthonous *R. ponticum* sites, investigate how they are affected by soil pH, organic matter content and *R. ponticum* presence and compare our data with those obtained by Sutton and Wilkinson (2007). We use TA as model organisms to investigate the extent to which the free living microbial communities are affected by an ericaceous shrub within and outside its autochthonous range, which may contribute to understanding of the potential for introduced plant species to produce novel soil ecosystems.

MATERIALS AND METHODS

The effect of *R. ponticum* on TA communities was investigated within its native range in two adjacent stream valleys near Puerto de Galiz, Spain. Valley 1 (440 m a. s. l.; N 36°33.604′, W 5°35.758′) was dominated by *Quercus suber* L. with *R. ponticum* undergrowth while Valley 2 (438 m a. s. l.; N 36°33.590′, W 5°35.661′) was dominated by *R. ponticum* shrubs. Valley 1 was more open, dry and shallow than Valley 2 (Figs 1 and 2, respectively). Sampling in Spain took place in mid-February 2008. We additionally re-sampled two British sites previously investigated by Sutton and Wilkinson (2007) – Stockton's Wood and Childwall Wood – to assess the extent to which the taxonomical approach taken in this study corresponded to that of Sutton and Wilkinson (2007). Sampling in England took place in late June 2008. At each locality, TA communities were sampled in pairs, soil samples were taken below *R. ponticum* shrubs (R+) and in close vicinity without *R. ponticum* (R–). There were five sampling micro-sites in each Valley in Spain and two micro-sites in Stockton's Wood and Childwall Wood, England. It should be noted that the extent of our investigation was limited by the fact that the Spanish TA samples were collected as an additional aspect of a main study focused on fungi associating with roots of European autochthonous rhododendrons (Vohník and Albrechtová 2011).

The sampling procedure followed Sutton and Wilkinson (2007). Briefly, the litter layer was gently removed and a soil sample was extracted from the substrate beneath [O-horizon, considered to contain the greatest number of microbes and invertebrate animals (Bardgett 2005)]. The samples were sealed in plastic tubes, transported to the laboratory and stored in a fridge until processed. Soil pH values were determined using standard methods; 1:2.5 soil-water suspensions being prepared for pH measurement based on 10 g < 2 mm soil to 25 ml deionised water (Hesse 1971). Organic matter content was determined by loss-on-ignition (at 450°C) following Avery and Bascomb (1982).

Samples were sieved (250 µm) and concentrated by centrifugation at 3500 rpm before being mounted on a microscope slide. TA tests were counted at a magnification of 400×, the majority (approximately 2/3) of samples being identified using an Olympus BX60 microscope. TA spectra were evaluated by two people (Z. B. and D. M. W.); therefore to standardize the taxonomy seven hours were devoted for searching the slides originating from each soil sample by both researchers working together in the same laboratory at the start of the project. Live and empty tests were not differentiated as differences in color vision between Z. B. and D. M. W. made the usual staining approach problematic.

The data on TA communities are presented as frequency data, rather than as estimates on numbers per unit volume (or weight) of soil. In this we follow 'the majority of recent testate amoebae studies' (Payne *et al.* 2012), although we note that testate concentration data may have added additional information. To our knowledge, however, none of the methods used to collect such data have been rigorously tested for TA. Multivariate analysis was used to estimate the effect of *R. ponticum* presence, soil organic matter content and pH on assemblages of TA found at the two Spanish sites. Unconstrained ordination was evaluated with detrended correspondence analysis (DCA) which suggested the use of linear models. Constrained ordination was evaluated with redundancy analysis (RDA) testing all parameters combined together with the sampling sites as covariates while partial constrained ordinations were computed with all parameters separately. Additionally, we tested the effect of *Rhododendron* with sampling sites and organic matter as covariates, the effect of organic matter with sampling sites and *Rhododendron* as covariates, and the effect of *Rhododendron* + organic matter with sampling sites as covariates. Significance of the analyses was evaluated with Monte Carlo permutation tests. All analyses were performed in CANOCO ver. 4.56 (ter Braak and Šmilauer 2002).

RESULTS AND DISCUSSION

In total across all the studied localities we detected 22 TA genera with 60 species (+ one unidentified) in 2041 specimens (Table 1). More TA species-rich and abundant communities were detected in the two stream valleys in Puerto de Galiz where we identified 21 TA genera with 54 species in 1599 specimens while the two UK sites hosted only 14 TA genera with 21 species in 442 specimens. Although more species were recorded in the Spanish samples the ratio of species to total individuals was higher in the UK samples, which is consistent with the textbook decline in the rate at which

Fig. 1. A photo showing characteristics of the first sampling site in Puerto de Galiz, Spain (Valley 1; for details see Materials and Methods).

Fig. 2. A photo showing characteristics of the second sampling site in Puerto de Galiz, Spain (Valley 2; for details see Materials and Methods). The *Rhododendron* cover at the UK sites was much denser than at these Spanish sites.

Table 1. Summary of testate amoebae spectra at two localities in Puerto de Galiz, Spain and Childwall Wood and Stockton's Wood, UK.

		Puerto de Galiz 1		Puerto de Galiz 2		Childwall Wood		Stockton's Wood	
		R+	R–	R+	R–	R+	R–	R+	R–
Rhododendron presence									
average pH		5.96	7.06	5.36	6.38	n.a.	n.a.	n.a.	n.a.
average organic matter content		20.0%	10.1%	31.0%	16.7%	40.0%	42.8%	36.5%	71.5%
CERCOZOA									
Amphitrema	*wrightianum*	2							
Assulina	*muscorum*							2	
	seminulum			3					
Corythion	*dubium*	13	2	5	3			5	1
	pulchellum			1				1	4
Euglypha	*compressa*	1	1	6				2	3
	cristata	2		6	3				
	ciliata			2				1	3
	rotunda	34	24	31	35	1	6	11	15
	strigosa	1		2	6		1	6	9
	tuberculata	9	6	6	21				
Pseudodifflugia	*gracilis*	2			1				
Sphenoderia	*fissirostris*	2		2	1				
	lenta	2	1	5	8				
Tracheleuglypha	*arcalla*	1							
Trachelocorythion	*dentata*	7	3	9	8	2	4	8	5
	pulchellum		1	2					
Trinema	*complanatum*	12	11	44	25	4	11	12	18
	enchelys	30	12	32	35	9	16	15	18
	lineare	54	38	36	45	2	9	6	21
AMOEBOZOA									
Arcella	*arenaria*			1					
	bathystoma			1					
	catinus	4							
	conica			1					
	discoides							1	
	hemisphaerica		1						
	vulgaris			1		3		1	
Bullinularia	*indica*	3	1	6	3				1

Genus	Species	Puerto de Galiz 1		Puerto de Galiz 2		Childwall Wood		Stockton's Wood	
Centropyxis	*aerophila*	12	4	6	6	10		16	4
	aculeata	5	1	22	10				
	cassis	6		3	10				
	constricta					1	9		
	elongata				1				
	laevigata			4					
	minuta	9	3	14	12	3	7		
	orbicularis	6		6	3				
	plagiostoma	2	3	3	2				
	platystoma	4	3		1	1			
	strigosa	2		1					
Cryptodifflugia	*oviformis*	1				1		1	2
	sacculus	1		1					
	sp.	6		3	7				
Cyclopyxis	*arcelloides*	1		8		2	9	6	1
	eurystoma	48	25	67	35	7	3	7	
	kahli	19	18	34	20	9			1
Difflugia	*ampulla*			1	1				
	brevicola	4	2		1				
	bryophila	2	2	1	5				
	lucida			1					
	penardi	8	2	14	8				
	pulex	7	6	5	11				
Heleopera	*petricola*	1			1				
	sylvatica	1				1			
Hyalosphenia	*ovalis*		1						
Nebela	*subflava*			2		26	25	20	
	collaris						5		
	tincta					1			
Phryganella	*acropodia*	113	36	82	48	3	5		
Plagiopyxis	*declivis*			2					
Schoenbornia	*humicola*		1		1				
Trigonopyxis	*arcula*	20	1	67	7	11	2	7	
Total count per locality		454	212	548	385	97	112	122	111

new species are recorded as sampling effort increases (e.g. Southwood and Henderson 2000). In addition this may also be influenced by a lower number of microsites screened in the UK habitats. *Amphinema, Pseudodifflugia, Sphenoderia* and *Trachelocorythion* (Cercozoa) and *Bullinularia, Difflugia, Nebela, Plagiopyxis* and *Schoenbornia* (Amoebozoa) were detected only in Spain while two species of *Nebela* (Amoebozoa) were only detected in UK. The most diverse genera detected in our study comprised *Centropyxis* (with 11 species detected), *Arcella* (7), *Euglypha* (6) and *Difflugia* (6) while the most abundant genera were *Trinema* (515 specimens), *Cyclopyxis* (319), *Phryganella* (287), *Euglypha* (254) and *Centropyxis* (215). The most abundant species were the potentially mycophagic/bacteriophagic *Phryganella acropodia* (287 individuals) followed by *Trinema lineare* (211) and *Cyclopyxis eurystoma* (186) (Table 1). At the same UK sites, Sutton and Wilkinson (2007) detected 8 TA genera with 15 species; our study thus added *Assulina, Tracheleuglypha, Arcella, Cryptodifflugia* and *Phryganella* to the list of TA genera occurring in Childwall Wood and Stockton's Wood. Some additional taxa were to be expected as the data in Sutton and Wilkinson (2007) were mainly collected by a biologist inexperienced in testate identification, as discussed in the original paper. Otherwise, the taxonomical approach employed here and in Sutton and Wilkinson (2007) appeared to be very similar. TA communities associated with the broadleaf *R. ponticum* in Spain were also generally richer than those detected in the rhizospheres of the small-leaved *Rhododendron hirsutum, Rhododendron luteum* and *Rhododendron kotschyi* in Slovenia and Romania by Vohník *et al.* (2009).

The separate RDA analyses showed significant effects of *Rhododendron* presence (13.2% of the total variability of the observed TA communities; $F = 2.815$, $p = 0.032$), organic matter content (16.4%; $F = 3.756$, $p = 0.008$) and soil pH (10.1%; $F = 2.001$, $p = 0.050$). In the RDA analysis testing all three parameters, the effect of *Rhododendron* presence strongly correlated with the first ordination axis, which explained 17.8% of the variation in the observed TA communities (Fig. 3). A test of significance of the first canonical axis was highly significant ($F = 3.285$, $p = 0.020$) but significance of all canonical axes was only marginally significant ($F = 1.383$, $p = 0.062$). With organic matter content as a covariate, the effect of *Rhododendron* presence was not significant ($F = 0.380$, $p = 0.8680$); similarly, the effect of *Rhododendron* was not significant with pH as a covariate ($F = 1.037$, $p = 0.2440$). Such a situation typically oc-

curs when testing highly correlated variables – separate analyses indicate their (sometimes high) significance but when tested with the respective covariate, their effects are non-significant (Lepš and Šmilauer 2000). The RDA analysis testing the effect of *Rhododendron* + organic matter showed that most of the explainable TA variability in this study was due to a combined influence of these apparently correlated variables (17.0%; $F = 1.939$, $p = 0.020$) which is further discussed as the effect of the *Rhododendron* rhizosphere.

Figure 3 shows that there were many TA species positively associated with the *R. ponticum* rhizosphere, especially *Cyclopyxis eurystoma, Phryganella acropodia* and *Trigonopyxis arcula*. Congruently, Sutton and Wilkinson (2007) detected a positive relationship between *R. ponticum* and *Trigonopyxis arcula* in the UK habitats; on the other hand, *Cyclopyxis eurystoma* and *Phryganella acropodia* were missing in their samples (these two species were also rare in the UK samples in this study). In the current study *Trigonopyxis arcula* was commoner in the *Rhododendron* soils at all four sites studied (two Spanish and two UK). Although this gives a very small sample size, the Mann-Whitney U-test is potentially applicable to studies with as few as four samples (Fowler *et al.* 1998) and when applied to these data shows a significant positive effect of the presence of *Rhododendron* on the population size of this species of TA ($U = 0.500$, $p = 0.029$). However caution should certainly be applied in interpreting such a result due to a small sample size.

Sutton and Wilkinson (2007) found a statistically significant tendency for the non-*Rhododendron* soils in the UK to be more species rich than the *Rhododendron* ones, even when soil organic matter was controlled for (which was not possible in the current study). On the other hand, in the Spanish sites where *R. ponticum* is native, we found greater species richness in the *Rhododendron* rhizosphere (Table 1). Therefore the current study – combined with the data from Sutton and Wilkinson (2007) – is suggestive of the idea that while *R. ponticum* has a diverse TA fauna associated in its native range it may reduce TA diversity when growing as an introduced species in Britain. However, more samples (especially a greater number of sites in its native range) would be required to fully demonstrate this pattern.

Only a few studies investigated the effect of plants on TA communities and most of them detected a highly significant effect of the vegetation type; for example, Trappeniers *et al.* (2002) showed that another ericoid
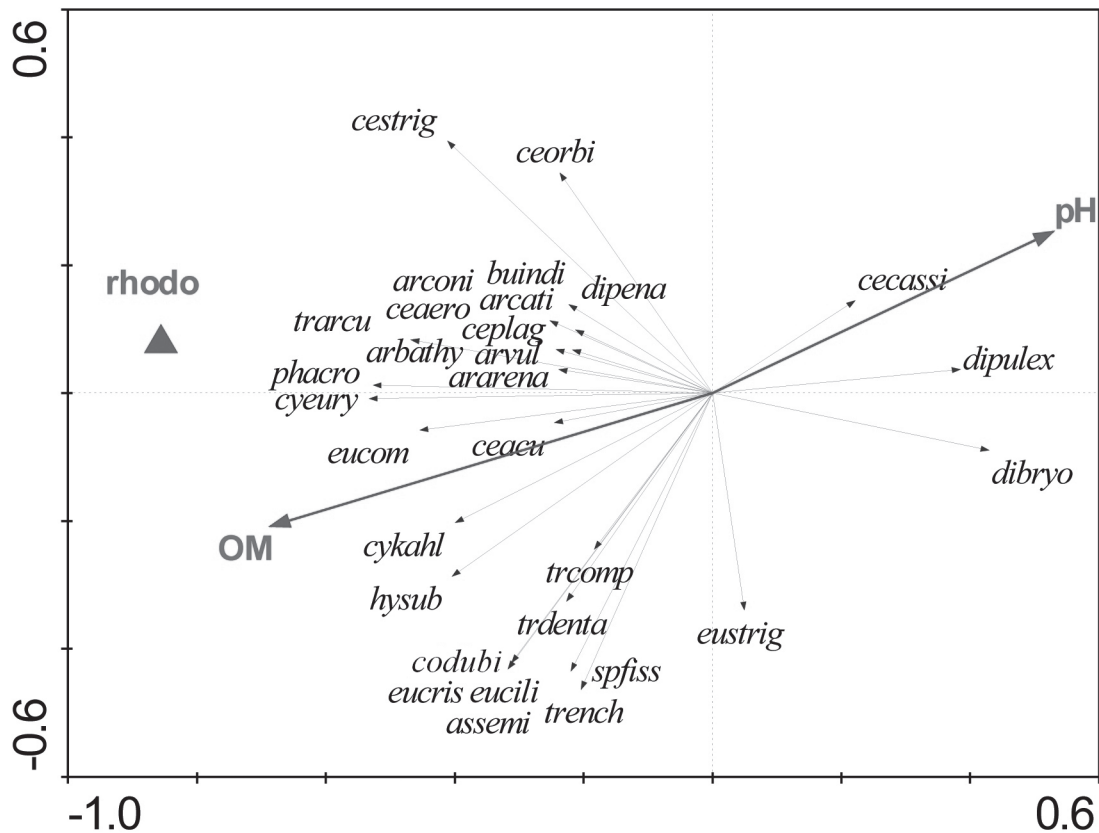
Fig. 3. The effect of *Rhododendron ponticum*, organic matter content and pH on occurrence and abundance of testate amoebae in Puerto de Galiz, Spain. We used RDA analysis of the effect of all variables (*Rhododendron* – rhodo, organic matter content – OM) with sample sites as covariates followed by Monte Carlo permutation test. The presence of *R. ponticum* strongly correlated with the first ordination axis, which explained 17.8% of the variation. A test of significance of the first canonical axis was highly significant ($p = 0.020$) but significance of all canonical axes was only marginally significant ($p = 0.062$). Only species with > 5% response to the screened factors are included in the figure. Abbreviations of testate amoebae species: **arbathy** – *Arcella bathystoma*, **trarcu** – *Trigonopyxis arcula*, **arconi** – *Arcella conica*, **trcomp** – *Trinema complanatum*, **arcati** – *Arcella catinus*, **trdenta** – *Tracheleuglypha dentata*, **arvul** – *Arcella vulgaris*, **trench** – *Trinema enchelys*, **assemi** – *Assulina seminulum*, **buindi** – *Bullinularia indica*, **ceacu** – *Centropyxis aculeata*, **ceaero** – *Centropyxis aerophila*, **cecassi** – *Centropyxis cassis*, **ceplag** – *Centropyxis plagiostoma*, **ceorbi** – *Centropyxis orbicularis*, **cestrig** – *Centropyxis strigosa*, **codubi** – *Corython dubium*, **cyeury** – *Cyclopyxis eurystoma*, **cykahl** – *Cyclopyxis kahli*, **dibryo** – *Difflugia bryophila*, **dipena** – *Difflugia penardi*, **dipulex** – *Difflugia pulex*, **eucili** – *Euglypha ciliata*, **eucom** – *Euglypha compressa*, **eucris** – *Euglypha cristata*, **eustrig** – *Euglypha strigosa*, **hysub** – *Hyalosphenia subflava*, **phacro** – *Phryganella acropodia*, **spfis** – *Sphenoderia fissirostris*.

mycorrhizal plant, *Vaccinium uliginosum*, positively influenced the occurrence of *Difflugia globulus* (morphologically similar to *Phryganella acropodia*) and *Centropyxis aerophila* var. *sphagnicola*. Such a positive relationship between TA with relatively large tests and rhizospheres of highly mycotrophic plants is intriguing and points at possible direct and/or indirect interactions between TA and plants through their root-associated fungi. It is of interest that *Phryganella acropodia* was the most abundant TA species detected in Scots pine (*Pinus sylvestris*) litter microcosms by Vohník *et al.*

(2011) and that the authors found a highly positive spatial correlation between an ascomycetous saprotrophic fungus colonizing the litter and this TA species. *P. acropodia* has been suggested as a typical mycophagous species (Coûteaux 1985, Ogden and Pitta 1990) but Vohník *et al.* (2011) pointed out that at least in some cases it may feed rather on hyphal exudates or bacteria associated with the hyphosphere than directly on the fungal mycelium.

Invasiveness of some plant species in certain habitats may be at least in part due to their interactions with
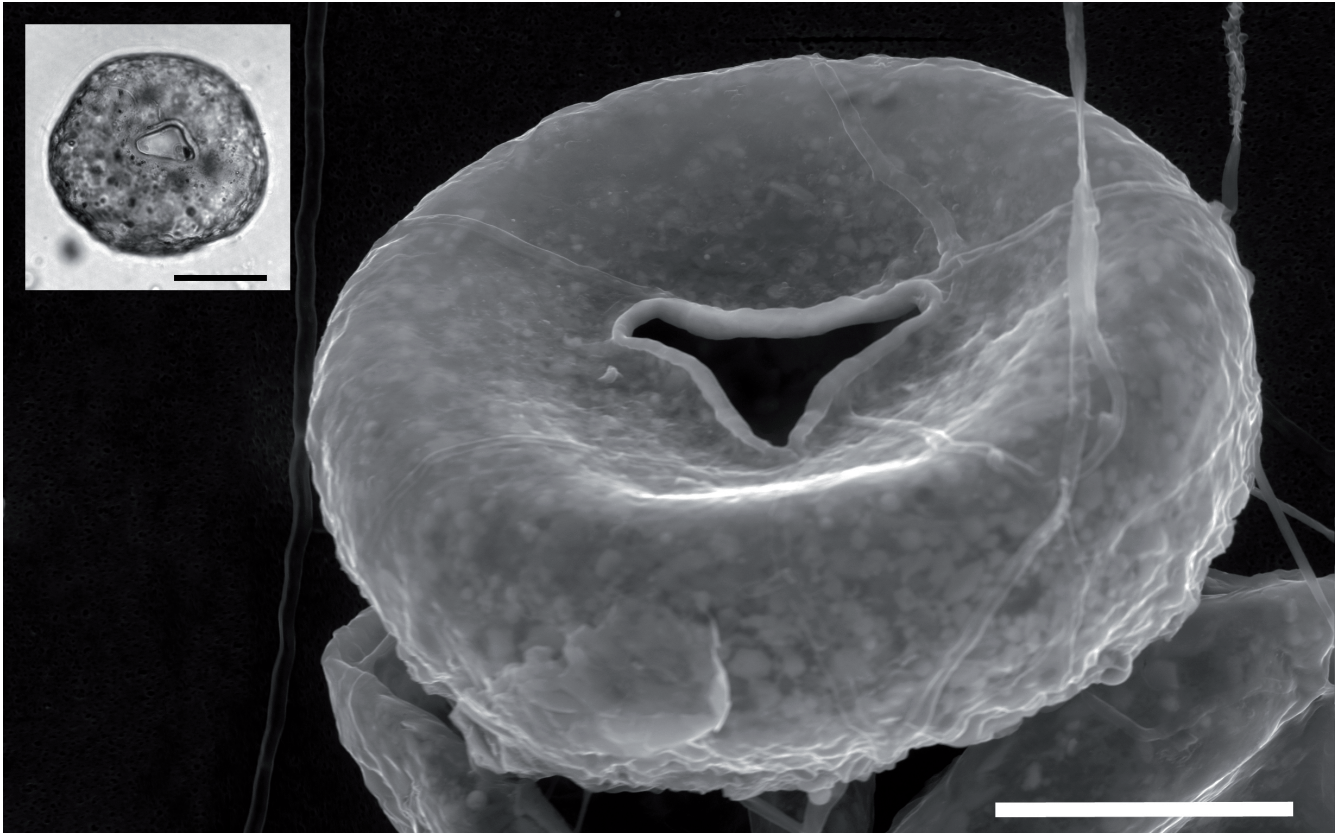
Fig. 4. A combined SEM/light microscopy picture of *Trigonopyxis arcula*. This testate amoeba species was proposed as a possible indicator of *Rhododendron*-affected soils by Sutton and Wilkinson (2007). Some hyphae of soil fungi are attached to the shell of the SEM-depicted specimen. Bars: 50 µm.

soil biota (Klironomos 2002). It appears that the invasive introduced *R. ponticum* in Britain is making its rhizosphere TA community more similar to that found below the native *R. ponticum* in Spain. Differences in the development of TA communities could potentially provide a way of monitoring changes in the soils once the invasive *Rhododendron* has been cleared from a site as part of conservation management schemes. Our current work, along with that of Sutton and Wilkinson (2007), suggests that TA with large shells (e.g. *Trigonopyxis arcula*), may be useful indicators of such *Rhododendron*-affected soils. In this context it may be useful that *Trigonopyxis arcula* is a particularly easy taxon of TA to identify (Fig. 4), even by a non-specialist.

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