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Rhizophlyctis rosea (Rhizophlyctidales, Chytridiomycota) in soil: frequency, abundance and density of colonization of lens paper baits

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With 2 figures and 6 tables

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Abstract: The distribution of *Rhizophlyctis rosea* was examined in soils from 22 locations including disturbed and undisturbed habitats in eastern Australia. Thalli of *Rh. rosea* were observed on baits from 60% of the sites (67% agricultural and 33% natural soils). Within the disturbed habitats, samples from four sites that experience different temperature ranges were assessed for frequency, abundance, number of thalli and density of colonization of the baits. Lens paper baits were placed into Petri dishes with sterile deionized water and air-dried soil and incubated for up to four days at 20, 35 or 40°C, and at 20°C after freezing (-15°C) and heating (80°C) the soil. The abundance, frequency, number of thalli and density of colonization varied among the samples analysed, with the greatest abundance, number of thalli and density for Pitt Town Bottoms and the highest frequency for Pitt Town Bottoms and Narrabri soils. All isolates grew and released zoospores after heat and freezing treatments. Freezing soil before baiting increased the number of thalli and density of colonization of baits, while heating decreased the frequency and abundance of *Rh. rosea*.

Key words: distribution, *Rhizophlyctidales*, ruderal, soil ecology, temperature, zoosporic true fungi.

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Introduction

Zoosporic true fungi (Blastocladiomycota and Chytridiomycota) are common inhabitants of soil, however, they are difficult to culture and identify and have often been omitted from ecological and biological studies (Ellanskaya et al. 2000, Gusmão et al. 2001). Members of the orders Spizellomycetales and Rhizophlyctidales have been observed frequently from soil (Barr 1984, Lozupone and Klein 2002, Letcher et al. 2008) and may play an important role in the decomposition of plant structural polymers, such as cellulose, hemicellulose and pectin, therefore contributing to the global carbon cycle (Gadd 2004). Thus, a clearer understanding of the factors regulating the distribution, abundance and mechanisms of survival and recovery of these fungi is needed.

Many soil microorganisms have adapted to wide temporal and spatial fluctuations of stressful environments. Temperature is an important abiotic factor affecting the distribution and activities of fungi in soils. Most fungi are mesophilic and grow at temperatures ranging from 5 to 35°C, thus they can be found in both cold (Zak and Wildman 2004) and hot habitats such as Australian soils exposed to sunlight during the summer (McGee 1989, Gleason et al. 2004, Commandeur et al. 2005). Zoosporic true fungi probably survive unfavorable extremes in temperature in the soil as resistant structures (Sparrow 1960) and then recover and resume growth either as soon as clement conditions return or after dormancy of the resistant structures is broken. The mechanism of recovery of zoosporic true fungi from resistant structures remains unclear.

Rhizophlyctis rosea (de Bary and Woronin) A.Fisch. (Rhizophlyctidales, Chytridiomycota) is readily identifiable by its distinctive morphological characteristics (Sparrow 1960, Karling 1977). This fungus is one of the most frequently observed zoosporic fungi in cropping soils (Booth 1971a, b, c, Karling 1976, Letcher et al. 2004a, Sparrow and Dogma 1973) where it may play an important role in the degradation of cellulosic debris (Willoughby 1998). *Rh. rosea* was previously observed in soil samples from agricultural sites and national parks and reserves in Australia (Letcher et al. 2004b) and appeared to be slightly more common in disturbed (e.g., cropping systems) than in natural soils (Willoughby 1965), indicating adaptation to stressors, such as a wide range of temperatures, associated with cultivation of soil.

We focused on *Rh. rosea* in a preliminary study of the distributional patterns of zoosporic true fungi in soil ecosystems because this species is widespread in soils and is easy to identify microscopically (Gleason et al. 2004). We used traditional microscopic techniques to determine the distribution of the fungus because we wished to clarify whether resistant structures of the fungus are dormant. The greater recovery of *Rh. rosea* from disturbed than from natural soils may indicate that a quiescent state is formed at the higher temperatures in these soils followed by a rapid recovery as conditions improve, that is, the fungus may have a ruderal strategy. Although molecular techniques indicate the presence of DNA of an organism, they do not indicate functional state of an organism.

The aims of this study were: (*i*) to assess the presence of the *Rhizophlyctis rosea* in agricultural and natural soils in eastern Australia; *(ii)* to estimate abundance, frequency

of colonization, number of thalli and density of colonization of baits of *Rh. rosea* in four agricultural soils that experience different temperature regimes; and (*iii*) to compare the abundance, frequency of colonization, number of thalli and density of colonization of baits in response to different temperature treatments in the laboratory. We expected *Rh. rosea* to *(i)* be more common in disturbed than undisturbed habitats; *(ii)* survive stressful conditions as quiescent structures; and *(iii)* colonize baits to different degrees (i.e., abundance, frequency, number of thalli and density) under different temperature treatments in the laboratory.

Materials and methods

COLLECTION SITES: The presence of *Rh. rosea* was assessed from forty soil samples collected from 22 different locations (Fig. 1 and Table 1) between January and September 2003. In January 2009 four soils from New South Wales and Queensland (Australia) that experience different temperature regimes (Table 2) were selected for quantitative measurements: (*i*) Biloela, QLD: herb (e.g., basil, coriander) fields; (*ii*) Narrabri, NSW: cotton-wheat rotation fields from the Australian Cotton Research Institute in Wee Waa; (*iii*) Pitt Town Bottoms, NSW: corn fields; and (*iv*) Robertson, NSW: potato fields. Climate conditions (temperature and rainfall) were characterized according to the Bureau of Meteorology, Commonwealth of Australia (http://www.bom.gov.au./climate).

SAMPLING AND LABORATORY ANALYSIS: Procedures in the preliminary study were as described by Gleason et al. (2004). For the quantitative study, three haphazardly distributed samples of soil (300-400 g) were collected at each of four sites. Samples were transported to the laboratory in sealed plastic (Ziploc®) bags and stored at 6°C until processed. Soil organic carbon content was analysed using a Vario max CNS analyser (Elementar electronics, Germany). The pH of the samples (in deionized water and $CaCl_2$) was measured with a pH meter. Air-dried subsamples of soil were either kept at 20°C, frozen at -15°C or heated in an oven (Thermoline, Australia) at 80°C for 4 d. Three grams of soil were placed in plastic Petri dishes with 25 mL sterilized de-ionized water and baited with five sterilized Whatman® lens cleaning tissue disks (5 mm in diameter) (Marano and Steciow 2006). We chose lens paper disks as baits because sporangia of *Rh. rosea* were easily seen on the fibers of lens paper disks and the disks remained on the surface of the water.

Air-dried soil samples were baited and then incubated at 20°C (untreated control) for 3 d, 35 and 40°C for 2 d and at 20°C after freezing or heating. Incubation was terminated before discharge so that small, second generation thalli were not included in the counts. The growth of *Rh. rosea* was also assessed at 37°C in three replicates of each air-dried sample.

Each Petri dish was considered as a sample unit. Five sample units were prepared from each soil sample and a total of 300 Petri dishes with 1500 baits (60 sample units for each sampled soil at each temperature) were analysed.

DATA ANALYSIS: Baits were examined microscopically for thalli identified as *Rh. rosea* by morphological characteristics (Sparrow 1960, Karling 1977). The presence of thalli on each bait and in each Petri dish were recorded. Distributional patterns in terms of frequency, abundance and number of thalli and density of colonization of baits were assessed: (*i*) Frequency of colonization (FC) = (number of sample units colonized by *Rh. rosea*/number of sample units examined) × 100 (Figuereda and Barata 2007); (*ii*) Abundance (A) = (number of colonized lens paper discs/number of lens paper discs used) × 100; (*iii*) Number of thalli (sporangia) = number of thalli for each disk; and (*iv*) Density of colonization of baits (D) = number of thalli recorded/number of disks analysed × area of each disk). Frequency of colonization and abundance were calculated according to Marano and Steciow (2006) whereas number of thalli and density of colonization of baits according to Willoughby (1998) and Marano et al. (2006).

We assigned *Rh. rosea* populations to five groupings of the Braun-Blanquet scale: 100–80.1% occurrence = ubiquitous; 80–60.1% occurrence = common; 60–40.1% occurrence = often present; 40–20.1% occurrence = scarce; and 20–0.1% occurrence = rare (Kershaw 1973; Letcher and Powell 2001; 2002).

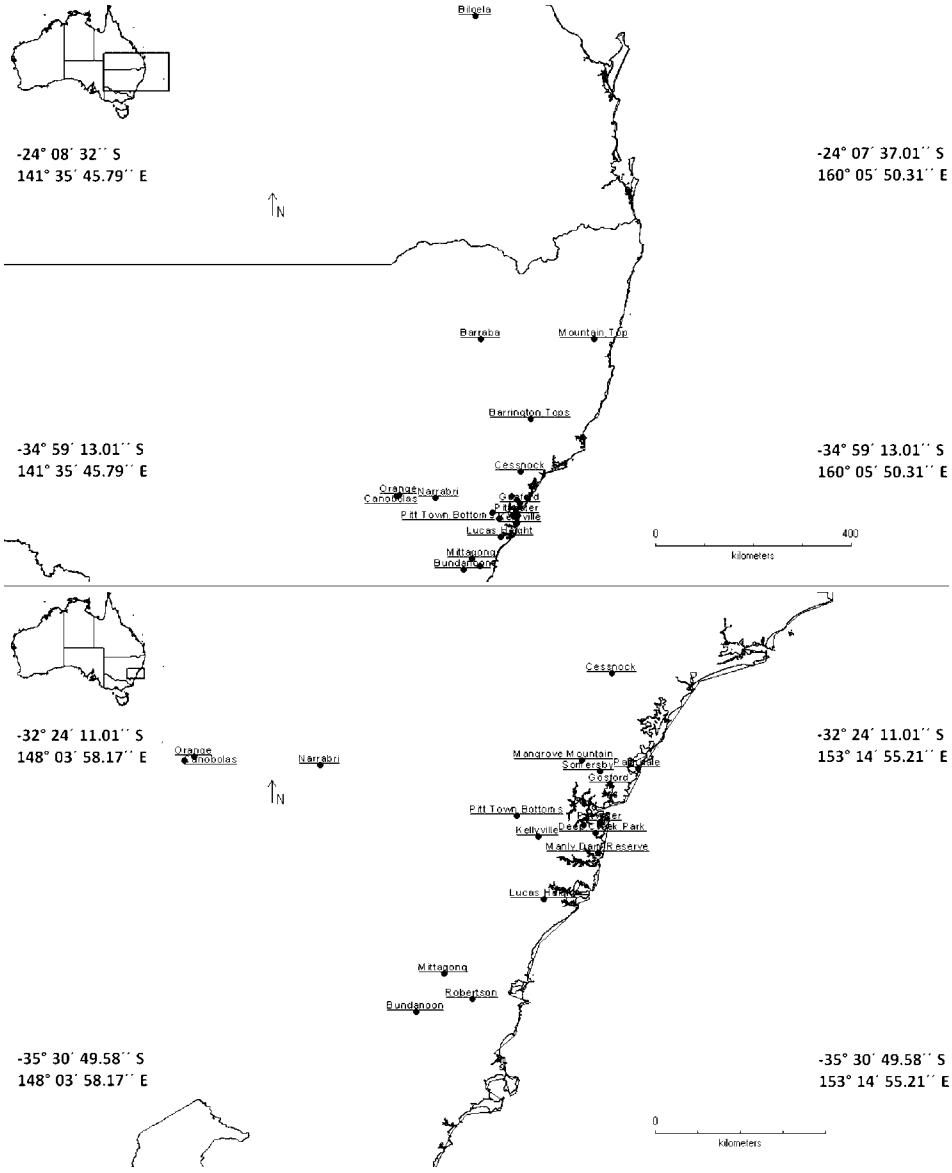

Fig.1. Maps indicating the geographic distribution of the sampled locations. Maps were constructed using the software DIVA-GIS® (version 7.1.7.2).

Normal distribution of the data was tested by the Shapiro-Wilk test (Shapiro and Wilk 1965). Chi-squared test (Plackett 1983) was performed to assess whether the presence of this fungus was associated with disturbance (i.e. cultivation) of soil. We explored the differences of frequency, abundance, number

Table 1. Distribution of *Rhizophlyctis rosea* in agricultural (AS)/ disturbed (DS) and natural soils (NS) from various locations. NSW: New South Wales, QLD: Queensland. The soils selected for further analyses are indicated in grey. + indicates its presence in a particular soil.

Locations	Geographical reference	Type	Vegetation	Presence
Biloela	QLD, 24.40°S, 150.51°E	AS	Herbs field	-
			Herbs field	-
			Basil Field	+
Narrabri	NSW, 30.33°S, 149.77°E	NS	Riparian	+
			Bush	-
		AS	Cotton-cotton rotation field	-
			Cotton-wheat rotation field	+
			Cotton-vetch rotation field	+
Mountain Top	NSW, 30.38°S, 152.72°E	AS	*Citrus*	+
Barraba	NSW, 30.38°S, 150.62°E	NS	Bush	-
Barrington Tops	NSW, 32.02°S, 151.27°E	NS	Snow gums	+
Cessnock	NSW, 32.83°S, 151.35°E	AS	Grapes field	+
Orange	NSW, 33.28°S, 149.10°E	NS	Riparian	+
		AS	Grapes field	+
			Apples field	+
			Cherries field	+
Mangrove Mountain	NSW, 33.30°S, 151.19°E	AS	Lettuce field	+
Palmdale	NSW, 33.33°S, 151.37°E	AS	Blueberries field	-
			Grapes field	+
		NS	Rainforest	-
		NS	Wet sclerophyll forest	+
Canobolas	NSW, 33.35°S, 149.01°E	NS	Snow gums	+
Somersby	NSW, 33.37°S, 151.28°E	NS	*Banksia* dry sclerophyll forest	-
Gosford	NSW, 33.42°S, 151.34°E	NS	Rainforest	-
			Angophora forest	-
Pitt Town Bottoms	NSW, 33.58°S, 150.83°E	AS	Cabbage field	+
			Lettuce field	+
			Corn field	+
			Capsicum field	+
			Squash field	+
Pittwater	NSW, 33.62°S, 151.28°E	NS	*Casuarina* forest	+
Ku-ring-gai Chase	NSW, 33.65°S, 151.20°E	NS	Dry sclerophyll forest	-
Deep Creek Park	NSW, 33.70°S, 151.27°E	NS	Bush	+
Kellyville	NSW, 33.73°S, 151.35°E	AS	Tomatoe field	-
Manly Dam Reserve	NSW, 33.80°S, 151.28°E	NS	Bush	-
Lucas Height	NSW, 34.02°S, 151.00°E	DS		-
Mittagong	NSW, 34.45°S, 150.45°E	AS	Grapes field	-
Robertson	NSW, 34.59°S, 150.59°E	AS	Potatoe field	+
Bundanoon	NSW, 34.66°S, 150.30°E	NS	Dry sclerophyll forest	-

of thalli and density of colonization of baits among the soil samples with the Kruskal-Wallis test (Kruskal and Wallis 1952). A one-way ANOVA (Fisher 1925) was employed to analyse the differences in the frequency, abundance, number of thalli and density of colonization of *Rh. rosea* on baits subjected to different temperature treatments in the laboratory. Pearson's correlation test (Sokal and Rohlf 1995) was performed to assess the relationship between biological parameters (frequency, abundance and density of colonization of baits) and soil factors (pH and organic carbon).

Table 2. Climate conditions at the sampling sites. Data from the closest stations (http://www.bom.gov.au/index.shtml).

	Max Annual Temp Range (C)	Min Annual Temp Range (C)	Mean Max Temp-Jan 2009 (C)	Mean Min Annual Temp (C)	Total Rain -fall Jan 2009 (mm)	Total Annual Rainfall (mm)
Biloela	27–30	12–15	32	20.9	110	535.8
Narrabri	24–27	12–15	34.6	20.7	19.8	482.6
PTB[1]	21–24	6–9	32	16.5	18.2	578.2
Robertson	18–21	0–3	28.5	12.7	12.4	525.6

[1]Pitt Town Bottoms

Table 3. Mean pH and organic carbon (OC) content (%) of the soils at the sampling sites. SD: standard deviation.

	Mean pH (water)	Mean pH ($CaCl_2$)	Mean OC (%) ± SD
Biloela	7.30	6.70	2.33 ± 0.08
Narrabri	8.3	7.6	0.94 ± 0.06
Pitt Town Bottoms	5.88	5.63	2.21 ± 0.11
Robertson	4.83	4.41	4.24 ± 0.24

Table 4. Total and mean number of thalli (± SD) and density of colonization of baits of *Rhizophlyctis rosea* populations in the soils incubated at 20°C (without pre-treatment). SD: standard deviation. ** indicates differences of statistical significance (P > 0.05).

	Total number of thalli	Mean number of thalli (± SD)	Density of colonization (thalli/mm^2)
Biloela	846	11 ± 22.4	2
Narrabri	1431	20 ± 41.3	4
Pitt Town Bottoms	4824**	69 ± 59.1**	13**
Robertson	1075	15 ± 31.4	3

Results

We found *Rh. rosea* in 24 of the soils sampled from 13 locations (FC: 60%); 67% were from agricultural soils and 33% from natural ones (Table 1). Although numerically more thalli were found from agricultural soils, these differences were not statistically significant ($P < 0.05$, n: 1, χ^{2obs}: 3.63).

Climate conditions and soil characteristics

Biloela has a hot climate with summer dominant rainfall (Table 2), contrasting with Robertson, which is cool and has rainfall evenly distributed over the seasons. Robertson soil had the lowest pH values and the highest carbon content and Narrabri had the highest pH and the lowest carbon content (Table 3).

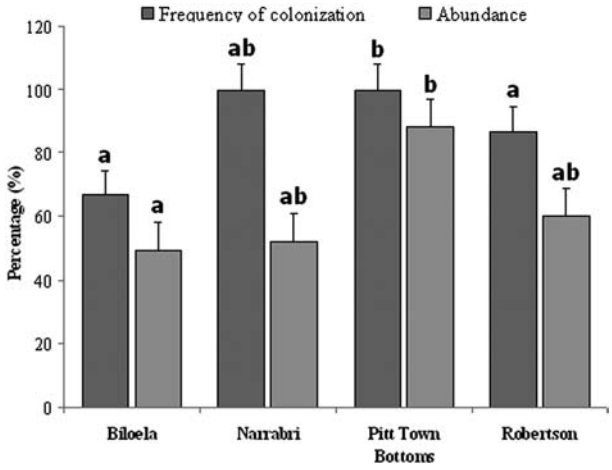

Fig. 2. Frequency of colonization and abundance of *Rhizophlyctis rosea* in the soils analyzed. Error bars: SE. The same letters above bars indicate that the values do not differ significantly as determined by Kruskall Wallis test ($P < 0.05$).

Distribution in the four soils analysed

R. rosea occurred in all samples from the four agricultural soils. After incubation at 20°C, we found *Rh. rosea* in 88.3% of the dishes and 62.3% of the baits were colonized. We recovered 8176 thalli; therefore the density of colonization of baits placed with the agricultural soils was 17.2 thalli/mm^2. Abundance, frequency, number of thalli and density of colonization differed significantly ($P > 0.05$) among the four soil types. The highest frequency of colonization was obtained from Pitt Town Bottoms and Narrabri soils (93%). The greatest abundance, number of thalli and density of colonization of baits were for Pitt Town Bottoms. *Rh. rosea* was less frequently isolated from Biloela soils (Fig. 2 and Table 4).

Distribution in relation to the temperature of incubation

No thalli of *Rh. rosea* grew in any of the soil samples that were incubated at 40°C; only bacteria colonized the baits at this temperature. At 37°C thalli were baited only from the Biloela and Pitt Town Bottoms soils. Frequency and abundance of *Rh. rosea* decreased after treatment of soils at 80°C. The density of colonization of baits increased after exposure of the soils to -15°C (Table 5). Depending on the soil, freezing or heating before incubation produced significant increases or decreases in *Rh. rosea* thalli. The frequency of colonization and abundance of this species did not differ significantly when samples were pre-treated in comparison to the control (20°C), except for Pitt Town Bottoms soils at 80°C. After heating, the density of colonization of baits from Narrabri increased and the frequency and abundance from Pitt Town Bottoms soils decreased. The number of thalli and density of colonization from Pitt Town Bottoms increased after freezing whereas the density of colonization decreased when Robertson soil was not pre-treated (Table 6).

Table 5. Frequency of colonization, abundance, number of thalli and density of *Rhizophlyctis rosea* after different temperature treatments. *indicates pre-treated samples.

Temperature (C)	Frequency of colonization	Abundance	Number of thalli	Density of colonization (thalli/mm^2)
-15* then 20℃	88.3	64.3	20287	43.1
20	88.3	62.3	8176	17.2
35	86.7	66	6013	12.7
80* then 20℃	70	46	4210	14

Distribution in relation to soil factors

No significant correlation was found between all biological parameters and soil factors ($P > 0.05$).

Discussion

Distribution in soils

Rh. rosea was widely distributed in eastern Australia. The fungus attached to and grew rapidly on cellulosic baits (lens paper) placed with a wide range of disturbed and undisturbed soils. Cultivation of soil did not significantly increase detection of this fungus. *Rh. rosea* was ubiquitous in all of the four agricultural soils, but was abundant in only some of them. Even though the greatest abundance, number of thalli, and density of colonization of baits of *Rh. rosea* were found in soil from Pitt Town Bottoms, our data showed relatively high densities of colonization of baits in soil from Narrabri and Biloela. Thus, although high temperature and disturbance of the soil may influence the presence of other zoosporic true fungi, they did not inhibit the survival of *Rh. rosea* in these soils from warmer habitats.

Adaptation to stressful conditions

Fungi are able to occupy niches in stressful environments by adopting different ecological strategies (Dix and Webster 1995): *(i)* competitive (C-selected); *(ii)* stress-tolerant (S-selected), and *(iii)* ruderal (R-selected). Strategies may change under different environmental conditions or during different stages in the life cycle of a fungus (Boddy and Wimpenny 1992). Some zoosporic true fungi have short generation times completing their life cycle in 48 hours (Ward 1939) and would most likely be ruderals. In natural environments explosive growth rates of zoosporic true fungi have often been observed when the temperature warms or when new substrates become available for colonization (Sparrow 1960, Gleason and Macarthur 2008). In this study cellulose baits became covered by thalli of *Rh. rosea* after incubation for 2 d at 35°C and 3 d at 20°C. Some isolates of *Rh. rosea* still grew and released zoospores at temperatures up to 37°C and all survived as dry propagules at 80°C for 3 d. After a 2–3 d incubation following freezing or heating all thalli were large and easily recognized. Populations of *Rh. rosea* appeared to tolerate extremes of temperature

Table 6. One-way analysis of variance (ANOVA) for each of the soils analysed. Independent variable: temperature treatment, Dependent variables: frequency of colonization, abundance, number of thalli and density of colonization of *Rhizophlyctis rosea*. ** indicates differences of statistical significance ($P > 0.001$).

Temperature (℃)	Soil sample	Frequency of colonization	A bundance	Number of thalli	Density of colonization (thalli/mm^2)
-15*	Biloela	3.7	13	598	20.7
20		3.3	12.3	282	2.33
35		3.7	10.7	252.3	2
80*		3	11.3	242	13.7
-15*	Narrabri	4	11.7	350.7	14
20		5	13	477	3.7
35		3.7	17.7	383	3
80*		5	15	682	27.3**
-15*	Pitt Town Bottoms	5	22	4894.3**	196**
20		5	22	1608	13
35		5	23	1086	8.7
80*		1**	2**	13.3	0.3
-15*	Robertson	5	17.7	372.7	15
20		4.	15	358.3	2.7**
35		4.3	18	283	2.33**
80*		5	17.7	372.7	15

*indicates pre-treated samples.

when dry and responded rapidly to changing environmental conditions. Even so, we consider the fungus to have predominantly ruderal characteristics because it rapidly colonizes cellulose baits. If *Rh. rosea* is ruderal, then major changes in abundance may be associated with specific stages in the cycle of growth of the fungus. Support for a ruderal strategy comes from the lack of any soil factor associated with aspects of fungal emergence, multiplication and distribution. Relative abundance measures therefore may have little relevance to the determination of overall presence of the fungus in each of the soils.

Although the fungus tolerated stressful temperatures when dry, recovery of growth is not necessarily an indication of a "stress" tolerance strategy. The absence of increased recovery of thalli after temperature stress indicates that resistant structures do not have a temperature-induced dormancy; the structures are likely to be quiescent indicating an absence of specific mechanisms that enable tolerance of stressful temperatures.

The lack of thalli in any of the soils incubated at 40°C indicates an absence of adaptation to the local temperature regime. Biloela experiences much higher temperatures than Robertson, and if *Rh. rosea* had adapted to local conditions it might be expected to germinate during summer temperatures when the soil was moist. The absence of local adaptation of *Rh. rosea* across a wide geographic range is puzzling. The fungus is globally distributed; given that it is found in soil, it may disperse

readily, either carried in soil with the movement of agricultural materials or blown by the wind when agricultural soils are bare and dry. The fungus appears to be ruderal and thus may be well adapted to disturbance. The lack of local adaptation might be due to constant and ongoing dispersal of the fungus. The hypothesis of recent expansion coinciding with agriculture is supported for the arbuscular mycorrhizal fungus *Glomus mosseae* (Rosendahl et al. 2009). This study compared molecular data of isolates from around the globe. As the *Rh. rosea* complex is globally distributed, readily isolated, and sequence information is available, a similar evaluation of on-going dispersal could be developed for *Rh. rosea*.

Final considerations

Rh. rosea was recovered readily from a wide range of soils and following storage for short periods in dry soil at high or low temperatures. The fungus readily grew after dry storage. Abundance along the east coast of Australia was variable with the variation possibly due to the ruderal strategy adapted by the fungus and that the fungus is readily dispersed. We cannot conclude that the fungus favors disturbed or undisturbed soils.

Tolerance of temperature stress appears to be limited within the zoosporic true fungi (Gleason et al. 2004, 2005). The capacity to recover rapidly from a period in dry soil regardless of the temperature would enable rapid use of the nutrients made available with the changing conditions. Thus *Rh. rosea* would benefit from slow changes in the environment that are found in soil, enabling completion of the life cycle before the onset of inclement conditions.

The lack of differences in response to temperature indicates the distribution of very similar fungi along the east coast of Australia. While speculative at this stage, the capacity for the fungus to rapidly initiate colonies from resistant structures, complete the life cycle and form resistant structures, tolerate high and low temperatures when as a resistant structure, indicate mechanisms that might enable the fungus to be distributed with soil, and help explain the apparent widespread distribution in disturbed habitats.

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