

1 **Habitat characteristics of the rare underground orchid, *Rhizanthella***
2 ***gardneri***

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10

11 **Abstract**

12 *Rhizanthella gardneri* R. S. Rogers is an entirely subterranean mycoheterotrophic
13 orchid only known from two isolated populations within south-west Western
14 Australia. This rare species appears restricted to habitats dominated by species of the
15 *Melaleuca uncinata* complex. *Rhizanthella gardneri* purportedly forms a tripartite
16 relationship with *Melaleuca*^{*}, via a connecting mycorrhizal fungus, for the purpose of
17 carbohydrate and nutrient acquisition. Here, we quantify key climate, soil and
18 vegetation characteristics of known *R. gardneri* habitats to provide baseline data for
19 monitoring of known *R. gardneri* populations, better understand how *R. gardneri*
20 interacts with its habitat, and to identify possible new sites for *R. gardneri*
21 introduction. We found that the habitats of the two known *R. gardneri* populations
22 show considerable differences in soil chemistry, *Melaleuca* structure and *Melaleuca*

* '*Melaleuca*' in this paper refers to the various species of the *Melaleuca uncinata* complex that *Rhizanthella gardneri* is known to associate with.

1 productivity. Multivariate analyses showed that both MDS and PCA ordinations of
2 soil chemical characteristics were very similar. Individual sites within populations
3 were relatively similar in all attributes measured while overall Northern and Southern
4 habitats were distinct from each other. These results suggest that *R. gardneri* can
5 tolerate a range of conditions and may be more widespread than previously thought,
6 given that there are extensive areas of *Melaleuca* thickets with similar habitat
7 characteristics across south-western Western Australia. Variability within the habitats
8 of known *R. gardneri* populations suggests translocation of this species into sites with
9 similar vegetation may be a viable option for the survival of this species.

10

11 **Introduction**

12

13 Many orchid species are on the brink of extinction and as such intervention via
14 conservation of existing populations, identification of habitat suitable for their
15 reintroduction and restoration of degraded habitat are paramount to their continued
16 survival (Hágsater and Dumont 1996). Restoration and conservation of individual
17 species, particularly threatened species, is generally complex and requires a thorough
18 understanding of the biological systems and processes associated with both their
19 continued existence and reasons for decline (Montalvo *et al.* 1997). While
20 conservation of existing populations and habitat is of primary importance, there is
21 also an increasing need for ecological restoration and/or translocation of individuals
22 produced *ex situ* to ensure survival and proliferation of endangered species and
23 systems (Young 2000). However, habitat characteristics for many rare or threatened
24 plants are often poorly characterised, particularly in terms of fundamental ecosystem
25 properties such as productivity, soil chemistry, and nutrient cycling.

1

2 *Rhizanthella gardneri* R.S.Rogers (western underground orchid) is a critically
3 endangered orchid that is entirely subterranean and mycoheterotrophic, meaning that
4 it has no ability to photosynthesise and limited capacity to independently access soil
5 nutrient pools (Dixon and Pate 1990). *Rhizanthella gardneri* and the only other
6 member of the genus, *R. slateri* (Rupp) M.A.Clem. & P.J.Cribb (eastern underground
7 orchid), are unusual plants in that they remain subterranean during flowering (Fig. 1a)
8 – a unique trait even amongst mycoheterotrophic species (Leake 1994). It is thought
9 that *R. gardneri* is linked, via a mycorrhizal fungus (*Thanatephorus gardneri*
10 Warcup), to an autotrophic shrub (*Melaleuca*), to form a tripartite relationship of
11 nutrient and carbohydrate exchange (Warcup 1985, 1991; Dixon and Pate 1990). If *R.*
12 *gardneri* is indirectly dependent upon the surrounding shrubs for carbon and nutrients
13 then we need an increased understanding of the range in productivity and nutrient
14 availability of known habitat in order to restore, create or identify new habitat.

15

16 Habitat considerations are paramount to understanding how to best conserve and
17 restore populations of rare plant species (Fielder *et al.* 2007), including
18 mycoheterotrophic species (Leake, 1994). Habitat is the range of environments or
19 communities over which a species occurs and comprises an abstract formulation of
20 this range in terms of environmental variables and the species' limits in relation to
21 them (Whittaker *et al.* 1973). Understanding a species' limits of tolerance and
22 responses to these environmental variables is paramount to understanding how species
23 function and in predicting how they may behave in a changing environment. In the
24 case of *R. gardneri*, habitat dependence and interactions within their habitat are
25 mostly unknown, but like most plant species, dependent variables are diverse, with

1 nutrition, climate and biotic interactions probably most important. While there are
2 comprehensive climate records for all known *R. gardneri* sites, the nutritional status
3 and productivity of *R. gardneri* habitats, i.e., the capacity of the habitat to maintain
4 nutrient and carbon supply under field conditions, remains unknown.

5

6 The need for restoration of *R. gardneri* populations and surrounding habitat has
7 become increasingly apparent. Monitoring over the past 26 years has identified a suite
8 of intensifying threats to the survival and proliferation of *R. gardneri* including; small
9 population sizes, changes in rainfall distribution (Australian Bureau of Meteorology
10 2008), limited capability for recruitment due to habitat fragmentation, isolated
11 habitats in excessively cleared landscapes, possible encroachment by salinisation,
12 increased weed species competition, unsuitable fire management and herbicide aerial
13 spray drift (Brown *et al.* 1998). Populations of *R. gardneri* have purportedly been in
14 decline since monitoring began in 1979, with anecdotal evidence suggesting that
15 decreased sightings of *R. gardneri* are correlated to a decline in health of the
16 associated *Melaleuca* habitat. Declining *Melaleuca* 'health' has been associated with
17 casual observations of a reduced litter layer, diminishing ground cover of other
18 species, decreased density of thickets, limited recruitment and the yellowing of
19 foliage. However, there have been no quantitative or qualitative measurements of
20 either the structure or nutritional status of known *R. gardneri* habitats, or to what
21 extent habitat that is deemed healthy differs from that in decline. Consequently, we
22 assessed vegetation structure, productivity and soil nutrients of known *R. gardneri*
23 habitats. We sought to determine key similarities and differences among habitats to
24 help define prerequisites and limitations to the survival of *R. gardneri* and to identify
25 new *R. gardneri* populations and potential sites for *R. gardneri* introduction.

1

2 **Materials and methods**

3

4 *Study sites*

5 Field sites analysed in this study represent habitat of all known live *R. gardneri*
6 individuals during 2007. *Rhizanthella gardneri* has been found in only two areas of
7 south-west Western Australia; three sites within 30 km of Corrigin in the northern
8 wheatbelt (Northern Population) and three sites approximately 80 km east of
9 Ravensthorpe in the south-eastern wheatbelt (Southern Population) (Fig. 1c). The
10 Northern and Southern populations are 300 km apart with many areas of apparently
11 similar habitat between these sites; however, there are no records of *R. gardneri* from
12 these intervening areas. The six sites where *R. gardneri* is known to occur are
13 between one and eight hectares in area and are generally within vegetation remnants
14 directly adjacent to agricultural land. Two sites are located on private property
15 (Dallinup Creek North and South); two sites are in protected Nature Reserves
16 (Babakin and Sorenson's Reserve); and two sites are located on Unallocated Crown
17 Land (Oldfield River and Kunjin). None of the sites have been directly affected by
18 fire in the past 30 years. However, similar surrounding vegetation that has been burnt
19 in the past 30 years has rapidly and successfully regenerated.

20

21 Both the Northern and Southern sites are subject to a Mediterranean type climate
22 (wet, cool winters with hot, dry summers) as experienced by most of south-west
23 Western Australia. The Northern sites receive ~320-350 mm annual rainfall compared
24 to ~400-450 mm for the Southern sites. However, annual rainfall is quite variable and
25 it is not uncommon for any of the sites to receive > 150 mm of rain during typically

1 dry summer months (Australian Bureau of Meteorology 2008). Seasonal temperature
2 fluctuations at Northern sites range from 5-15 °C in winter and 15-32 °C in summer.
3 Climate conditions for the Southern sites are slightly milder with average winter
4 temperatures as low as 7-16 °C during winter and 14-28 °C during summer
5 (Australian Bureau of Meteorology 2008).

6

7 The Northern populations of *R. gardneri* occur in dense *Melaleuca scalena* Craven &
8 Lepschi thickets (Fig. 2b) whereas the Southern populations occur in thickets of *M.*
9 *hamata* Fielding & Gardner, *M. uncinata* R.Br. and/or a third apparently undescribed
10 species of the *M. uncinata* complex (Craven *et al.* 2004). All of the above *Melaleuca*
11 species are taxonomically similar and belong to the *M. uncinata* complex, a group of
12 species widely distributed throughout southern Australia (Craven *et al.* 2004;
13 Broadhurst *et al.* 2004). The six sites, encompassing both Northern and Southern
14 populations, although primarily thickets of *Melaleuca*, also include more open areas
15 dominated by smaller *Dryandra* spp., large exposed rocks, areas of grasses and
16 sedges, *Allocasuarina campestris* (Diels) L. A. S. Johnson thickets or occasional
17 larger *Banksia media* R.Br. and/or mallee eucalypts. However, it appears that the
18 occurrence of *R. gardneri* plants is specific to the *Melaleuca* dominated patches
19 within each site.

20

21 Soils at all six sites are generally white-grey sandy loam over a shallow heavy orange-
22 grey clay layer. The clay layer at the Dallinup Creek north and south sites (Southern
23 sites) is within a few centimetres of the surface and is hard-setting, so that water often
24 pools on the soil surface for a substantial time after heavy rainfall. However, these
25 sites are on slight slopes, which allows for some degree of drainage. Soils at Northern

1 sites are considerably sandier than the loamy soils at Southern sites, although they
2 often form hard surface crusts during prolonged dry spells, especially during summer.
3 A summary of the general site characteristics of the Northern and Southern
4 populations of *R. gardneri*, including historical information, is given in Table 1.

5

6 *Aboveground structure and productivity of Melaleuca thickets*

7 Three replicate plots of 10 m x 10 m were established within *Melaleuca* thickets at
8 each of the six sites. Shrub density, canopy cover, stem number and height were
9 measured for ten *Melaleuca* shrubs within each 10 m x 10 m plot. The biomass of
10 *Melaleuca* at each site was estimated by destructively sampling five individuals,
11 representative of the range of canopy areas, and measuring their above ground
12 biomass. Individual plants were separated into foliage and stem components, weighed
13 and a sub-sample of foliage measured for total leaf area using a Li-3100 Area Meter
14 (Li-Cor Inc, Nebraska). The sub-sample of leaves was then oven dried at 70 °C for 48
15 hours, and specific leaf area ($\text{m}^2 \text{kg}^{-1}$) was calculated for each shrub. Leaf area per
16 shrub was calculated as specific leaf area x total dry leaf mass.

17

18 Standing leaf litter was measured by collecting all litter within 50 cm x 50 cm squares
19 at 0, 50 and 100 cm distance from randomly selected *Melaleuca* shrubs from within
20 each of the three plots at each site. Litter was sorted into principal (*Melaleuca*)
21 components (leaves, fruits, twigs) and those of other species (leaves and fruit) before
22 being oven dried at 60 °C for 48 hours and then weighed.

23

24 *Melaleuca root and living fungal biomass in Melaleuca thickets*

25 Root biomass (< 2 mm diameter) was estimated from cores collected in June 2005 to

1 coincide with *R. gardneri* flowering and when soil moisture was consistently greatest.
2 Duplicate soil cores (7.5 cm diameter) were sampled 10, 25, 50 and 100 cm in a south
3 westerly direction from three randomly selected *Melaleuca* shrubs from each of the
4 three plots at each of the six sites. Cores were bulked by position within each plot
5 after separating into 0-5 cm and 5-15 cm depths for measurement of root biomass.
6 Leaf litter depth (mm) was also recorded at all sampling points. Roots were separated
7 from soil by sieving samples (< 1 mm) under running water, and hand-sorting roots.
8 Root samples were then oven dried at 60 °C for 48 hours and all roots < 2 mm
9 diameter were weighed. The gravimetric moisture content of sampled soils was
10 calculated after drying for 24 hours at 110 °C.

11

12 Living fungal biomass from a subsample of each of the 0-5 cm soil cores mentioned
13 above was estimated from ergosterol concentrations (Seitz *et al.* 1977). Duplicate
14 samples of 5-6 g of field moist soils were weighed out. One duplicate of soil was
15 amended with 100 µg ergosterol in 1 mL n-hexane-propan-2-ol (98:2 v/v) to
16 determine extraction and recovery efficiency. Ergosterol was then extracted and
17 quantified by reverse phase HPLC using the method described by Ruzicka *et al.*
18 (1995) as modified by Grierson and Adams (2000). Ergosterol recovery of ‘spiked’
19 samples (used to assess ergosterol extraction efficiency) was always greater than 75
20 %.

21

22 *Soil chemistry analysis*

23 Three samples from each of the three plots at each site were collected using 7.5 cm
24 diameter metal corers, separated into 0-2, 2-5, 5-10 and 10-20 cm depths, and bulked
25 by plot and depth prior to sieving (< 2 mm). Soils were then assayed for a range of

1 chemical attributes. The pH of 5 g fresh soil was measured after vigorously mixing
2 samples with deionised water (1:1) (Thomas 1996). Labile phosphorus was measured
3 using two techniques. First, labile inorganic P (Bray P_i) was measured according to
4 the method of Bray and Kurtz (1945). Briefly, air dry soil was mixed with an
5 extraction solution (0.03 N NH₄F and 0.1 N HCl), shaken for 45 seconds and filtered
6 immediately. Second, labile organic and inorganic hydroxide-extractable P fractions
7 were measured using the method described by Grierson and Adams (2000). Briefly,
8 10 g of soil in 50 mL of 0.1 M NaOH solutions were shaken for 16 hours after which
9 extractions were centrifuged and the supernatant removed via filtration. One aliquot
10 (OH-P_i) of the filtered supernatant was acidified with HCl, to precipitate organic
11 matter, and re-filtered. A second aliquot (OH-P_t) of the filtered supernatant was
12 digested (H₂SO₄/H₂O₂). Phosphorus in all extracts was measured using a modified
13 ascorbic acid method of Kuo (1996). Organic P (OH-P_o) was estimated as the
14 difference in P between OH-P_t and OH-P_i.

15

16 Labile inorganic nitrogen was extracted according to the method described by
17 Rayment and Higginson (1992). Briefly, 5.0 g fresh soil in 50 mL of 1M KCl was
18 shaken for 1 h and filtered before supernatant NH₄⁺-N and NO₃⁻-N concentrations
19 were determined by automated colorimetric techniques performed on a Technicon
20 Auto Analyser II (Technicon 1977).

21

22 Soil and litter samples were analysed for total N and C content (%), and δ¹⁵N and
23 δ¹³C isotope signatures, using an Automated Nitrogen Carbon Analyser-Mass
24 Spectrometer consisting of a Roboprep connected with a Tracermass isotope ratio
25 spectrometer (Europa Scientific Ltd., Crewe, UK) in the West Australian

1 Biogeochemistry Centre at the University of Western Australia. All samples were
2 standardised against a secondary reference of Radish collegate (3.167 % N, $\delta^{15}\text{N}$ 5.71;
3 41.51 % C, $\delta^{13}\text{C}$ -28.61) that was in turn standardised against primary analytical
4 standards (IAEA, Vienna). Accuracy was measured at 0.07 %, while precision was
5 measured at 0.03 %, according to the stipulations for reporting analytical error in
6 stable isotope analysis outlined by Jardine and Cunjak (2005).

7

8 *Data analysis*

9 The best models for predicting component biomass of *Melaleuca* based on canopy
10 area as the independent variable were determined after testing a number of different
11 allometric models. The allometric models that best fitted the data were chosen by
12 examining residual distributions and maximum adjusted r^2 . The behaviour of the
13 models for small diameter and "out of sampled range" trees were also carefully
14 examined. The models tested were; $y = a + bx$ and $y = a + bx + cx^2$; where x is total
15 canopy area (m^2), y is the mass of different components of sample shrubs, a , b and c
16 are constants.

17

18 Analysis of variance (ANOVA) was used to suggest site differences among
19 *Melaleuca* individual and thicket structure, biomass (*Melaleuca* aboveground,
20 *Melaleuca* fine roots (< 2mm), leaf litter and fungus) and soil nutrient measures
21 among sites. In all cases, data were first checked for normality and square root or log
22 transformed where necessary to improve homogeneity of variances. For analyses
23 where samples were divided into depths (soil analyses) or distance from *Melaleuca*
24 stems (standing litter biomass, root biomass, ergosterol concentrations), two-way
25 ANOVA was used to determine if there was significant interaction between the two

1 independent variables. Significance level for all analyses was $P \leq 0.05$ and Statview
2 5.0 software (SAS Institute 1996) was used for all statistical analyses.

3
4 Multivariate analyses were used to investigate overall similarities and differences
5 among sites. Similarity of the soil characteristics of Northern and Southern sites was
6 calculated using principal components analysis and non-metric multidimensional
7 scaling ordinations (Primer software version 6.2, Primer-E Ltd, Clarke and Gorley
8 2006). Analysis of similarity (ANOSIM) was performed to determine if samples
9 within groups were more similar than between groups. Similarity percentages
10 (SIMPER) analysis was used to determine contribution of individual variables to
11 dissimilarity of groups. Data were normalised prior to analysis and Euclidean distance
12 was used to generate the resemblance matrix for data (Clarke and Gorley 2006).

13

14 **Results**

15

16 *Aboveground structure and productivity of Melaleuca thickets*

17 *Melaleuca* shrub densities at all sites were > 3360 individuals per hectare with little or
18 no evidence of recent *Melaleuca* recruitment. Heights of *Melaleuca* were generally
19 uniform within sites (~ 2.5 m), but were in general around 0.25 m taller at the
20 Northern sites compared to the Southern sites ($P = 0.0006$; Table 2). Projected canopy
21 areas of individual *Melaleuca* shrubs ranged from 0.25 to 6.8 m². Total canopy area of
22 sites ranged from 3875 m² ha⁻¹ at Sorenson's Reserve to 10,011 m² ha⁻¹ at Dallinup
23 Creek South. However, these values are likely to be somewhat greater than total
24 cover at plot scale as individual *Melaleuca* canopies overlapped in many instances
25 (Table 2).

1
2 Canopy area of individual shrubs was a strong predictor of total shrub biomass, as
3 well as component biomass (wood versus leaves), regardless of site. Allometric
4 equations for predicting *Melaleuca* biomass did not differ either within or between
5 Northern and Southern sites, meaning that patterns of total aboveground biomass
6 allocation were consistent across all sites even though shrub size differed (Table 3).
7 Aboveground biomass per hectare of *Melaleuca* shrubs was estimated by applying the
8 plot census data. Aboveground biomass of the *Melaleuca* shrubs was significantly
9 greater at Southern sites (19,070 kg ha⁻¹) compared to Northern sites (18,300 kg ha⁻¹).
10 Shrubs also had more leaves in Southern sites, where foliage contributed 17.3 % of
11 the aboveground biomass compared to only 12.9 % at Northern sites (Table 3).
12
13 Mean leaf area was similar for *Melaleuca* from Northern (62.7 m² kg⁻¹) and Southern
14 (58.8 m² kg⁻¹) sites but was considerably more variable within Southern sites. When
15 all six sites were compared leaf area per hectare was least at Sorenson's Reserve
16 (8380 m² ha⁻¹) and greatest at Kunjin (16,534 m² ha⁻¹) with no clear distinction
17 between Northern and Southern sites (Table 2). The variability in leaf area in the
18 Southern sites may reflect different *Melaleuca* species of the *M. uncinata* complex
19 (Table 1), which are distinguished in part by leaf size and shape (Craven *et al.* 2004).
20 We attempted to quantify carbon assimilation as well as transpiration rates of
21 *Melaleuca* individuals but were unsuccessful as rates of gas exchange from the fine
22 leaves were below levels of detection of a range of infrared gas analysers. However,
23 leaf area is also an index of photosynthetic potential; while *Melaleucas* of Northern
24 and Southern sites were overall similar in their potential to supply photosynthate to a
25 mycoheterotrophic orchid, the variability within the Southern sites suggests that *R.*

1 *gardneri* can survive across a broad range and the Kunjin site, for example, may be
2 able to supply greater carbon from the host plant to any associated *R. gardneri* given
3 the much greater leaf area at that site.

4

5 Standing leaf litter at Southern sites (~0.6 – 1.5 kg m²) was about twice that of
6 Northern sites ($P = 0.0019$), reflecting the greater allocation of biomass to foliage and
7 larger total leaf area. Leaf litter accumulated directly at the base of individual
8 *Melaleuca* plants (Figure 3). *Melaleuca* leaves made up the majority of total standing
9 litter (60 % at Northern sites and 70 % at Southern sites) and was strongly correlated
10 to total standing leaf litter ($r^2 = 0.862$). When litter depth (rather than litter biomass)
11 was measured, litter depth decreased with increasing distance (10, 25, 50 and 100 cm)
12 from the base of individual *Melaleuca* plants; litter depth was also about 30 % greater
13 at Southern sites (6-15 mm) compared to Northern sites (3-12 mm) (Fig. 2).

14

15 *Melaleuca* root and living fungal biomass in *Melaleuca* thickets

16 Root (< 2mm diameter) biomass in the top 15 cm of the soil profile was similar at all
17 four sampling distances (10, 25, 50 and 100 cm) from the base of *Melaleuca* shrubs
18 (Figure 3). However, root (< 2mm diameter) biomass in the top 15 cm of soil was
19 more than twice as great at Southern sites (3.5 g cm³) compared to Northern sites
20 (Fig. 3), consistent with aboveground data and again demonstrating the higher
21 productivity of these sites. Similar trends were evident across soil depths (0-5 cm and
22 5-15 cm), where root (< 2mm diameter) biomass at Southern sites was 3.6 g cm³ (0-5
23 cm depth) and 3.3 g cm³ (5-15 cm depth), twice that of Northern sites.

24

1 Ergosterol concentration of soil samples was used as an indicator of living fungal
2 biomass (Davis and Lamar 1992) and were consistently greatest closest to *Melaleuca*
3 individuals compared to samples further away (Fig. 4). Northern sites had slightly
4 higher concentrations of ergosterol ($3.7 \mu\text{g g}^{-1}$ soil) compared to Southern sites (2.9
5 $\mu\text{g g}^{-1}$ soil). However, these data are based on a single collection made in June 2005
6 and are likely to be highly variable according to soil moisture (Grierson and Adams
7 2000).

8

9 *Soil chemistry*

10 Soils were significantly more acidic, albeit slightly, at Northern sites ($\sim\text{pH } 5.3$)
11 compared to Southern sites ($\sim\text{pH } 5.9$). Soils were ammonifying with very little nitrate.
12 Soil ammonium concentrations ($\text{NH}_4^+\text{-N}$) (0-20 cm) at Northern sites ($\sim 12 \mu\text{g g}^{-1}$)
13 were twice that of Southern sites ($\sim 6 \mu\text{g g}^{-1}$), with most ammonium concentrated in
14 the top 2 cm of soil ($\sim 22 \mu\text{g g}^{-1}$). However, total N concentrations at both Northern
15 and Southern sites were similar ($\sim 0.3\text{-}1 \text{ mg g}^{-1}$ soil), indicating likely variation in
16 organic N fractions, where more N is likely associated with greater organic matter at
17 the Southern sites. Bray- P_i was extremely low in all soils ($0.5\text{-}1.5 \mu\text{g g}^{-1}$). However,
18 OH- P_i was slightly greater at Northern sites ($1.5\text{-}3 \mu\text{g g}^{-1}$ soil; $P = 0.0061$). C:N ratios
19 of soils were significantly less ($P = 0.001$) at Southern sites (22-25) compared to
20 Northern sites (23-29). All soils were enriched in ^{15}N (1-6 ‰) and isotopic signatures
21 (both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) did not differ between Northern and Southern sites, reflecting
22 their similarity in organic matter inputs and nutrient cycling processes.

23

24 When all soil data were analysed using both MDS (Fig. 5) and PCA (data not shown)
25 analysis, individual quadrats (three at each site) separated into two distinct clusters

1 closely representing Northern and Southern sites. Both MDS and PCA ordinations
2 were very similar and showed all individual sites tended to cluster. ANOSIM analysis
3 suggested a significant ($P = 0.004$) global R value of 0.17 suggesting that Northern
4 sites were more similar than Southern sites and vice versa. SIMPER analysis showed
5 that no soil chemistry variable measured had greater than 3.2 % contribution to
6 separating Northern and Southern sites (data not shown).

7

8 **Discussion**

9

10 *Rhizanthella gardneri* is only known from two geographically distinct areas in south
11 Western Australia, which are subject to slightly different climates and also have
12 different soil properties. While there are many similarities among the habitats of
13 known populations, this study demonstrates that there is a range of conditions under
14 which *R. gardneri* grows and, presumably, is able to tolerate. In addition, the standing
15 biomass and productivity of the putative autotrophic host, *Melaleuca*, varies
16 considerably amongst Northern and Southern sites suggesting that a wide range of
17 sites could be suitable as habitat for *ex situ* material. These results also suggest the
18 possibility of additional undiscovered *R. gardneri* populations within the broader
19 distribution of the *M. uncinata s.l.* in southern Western Australia. *Melaleuca uncinata*
20 *s.l.* is common, with a distribution spanning most of southern Australia. Recent
21 reviews of *M. uncinata s.l.* taxonomy resulted in the recognition of 11 species of
22 *Melaleuca* within the group (Broadhurst *et al.* 2004; Craven *et al.* 2004), with at least
23 three (and possibly a fourth undescribed species) associating with *R. gardneri*
24 individuals. However, the concurrent presence of the mycorrhizal fungus,
25 *Thanatephorus gardneri*, within potential sites should also be assessed. Extensive

1 carefully controlled microcosm experiments or discovery of *R. gardneri* plants around
2 other species within *M. uncinata s.l.* are required to elucidate whether other species
3 can support *R. gardneri* and its mycorrhizal fungus, and even if *Thanatephorus*
4 *gardneri* is present at potential sites. Based on a combination of germination trials and
5 observations that any *R. gardneri* individuals present were always directly at the base
6 of these *Melaleuca* spp., Warcup (1985, 1991) suggested that *R. gardneri* individuals
7 are nutritionally dependent (indirectly via a connecting mycorrhizal fungus) on the
8 presence of a few specific autotrophic hosts (*M. uncinata s.l.*). However, the only
9 other *Rhizanthella* species, *R. slateri*, co-occurs with a range of tree and shrub
10 species, not *M. uncinata s.l.* (Clements 1984). While it is likely that *R. slateri*
11 associates with the same mycorrhizal fungus as *R. gardneri* (unpublished data), there
12 is no clear evidence that *R. slateri* forms tripartite relationships with surrounding
13 trees/shrubs, nor has it been determined if its mycorrhizal fungus acquires adequate
14 carbon from either organic matter or an autotrophic host to support the orchid.
15 Clearly, further research is required in order to determine if the presence of
16 *Thanatephorus gardneri* is a key characteristic for *Rhizanthella* habitat.

17

18 The density of *Melaleuca* thickets at both Southern and Northern sites is high (>3500
19 ha⁻¹) and may be an important factor in determining presence or absence of *R.*
20 *gardneri*. While *M. uncinata s.l.* often occurs as scattered individuals within a variety
21 of habitat types, *R. gardneri* plants have never been found in low density thickets or at
22 the margins of known sites where sites become more open. The density of *Melaleuca*
23 thickets measured here were only about 20 % of estimates by Dixon and Pate (1984)
24 for the Northern sites and about 80 % of that suggested for the Oldfield River site
25 (Southern site – no previous data for Dallinup Creek North and South sites).

1 Consequently, there may have been a decline in *Melaleuca* density, especially within
2 the Northern sites, which is consistent with decreasing numbers of observed flowering
3 *R. gardneri* plants (not necessarily correlating to absolute *R. gardneri* numbers) over
4 the same 24 year period. Unfortunately, no measures of *Melaleuca* biomass or canopy
5 cover were made by Dixon and Pate (1984) so we are unable to elaborate on any
6 possible secondary effects of habitat changes and how these could or did affect *R.*
7 *gardneri* populations. However, we might assume that with lower total cover that
8 there is less biomass, there has been less productivity and reduced ability to support
9 any symbionts. In addition, microclimate conditions are also likely to be different
10 with warmer and possibly drier soils in more open vegetation.

11

12 *Melaleuca* foliage biomass ($\sim 0.6 - 1.6$ tonnes C ha⁻¹) was not significantly different
13 between Northern and Southern sites or among *Melaleuca* species. However, foliar
14 nitrogen contents were significantly greater at Northern (~ 15 g kg⁻¹) compared to
15 Southern (~ 11 g kg⁻¹) sites. Foliar N content correlates strongly with rates of CO₂
16 assimilation for a wide range of C3 species (Evans 1989) suggesting that plants from
17 Northern sites were more likely to be actively sequestering carbon via photosynthesis
18 and presumably more able to support mycorrhizal symbionts. However, the relative
19 dependency of *R. gardneri* and its fungus on carbohydrates from its autotrophic host
20 relative to utilization of carbon (and nitrogen) from other sources such as organic
21 matter, remains unknown. While it is thought that *R. gardneri* obtains carbohydrates
22 and nutrients directly from its fungal partner (rather than its autotrophic host), like
23 many ectomycorrhizal fungi, the fungal symbiont associated with *R. gardneri*
24 (*Thanatephorus gardneri*) is likely to be accessing a variety of sources of carbon and
25 nutrients (Smith and Read 1997). Therefore, decomposing organic matter, soil and

1 especially leaf litter (predominantly *Melaleuca* leaves) might be important elements
2 of *R. gardneri*'s nutrition. Accumulated leaf litter was twice as great at Southern sites
3 thus representing a significantly greater carbon pool for fungi to access and which
4 may help compensate for the predicted lower photosynthetic capability and
5 subsequently reduced carbon pool of *Melaleuca* plants within Southern sites. Isotope
6 tracer studies may shed some light on the utilisation of different C and nutrient
7 sources within this ecosystem by *Melaleuca*, *Thanatephorus gardneri* and *R. gardneri*
8 (McKendrick *et al.* 2000).

9
10 Fine root (<2 mm diameter) biomass in the top few centimetres of the soil profile was
11 greatest (almost double) in Southern sites. This would presumably be attributed to the
12 greater moisture and nutrient availability at these sites (Cairns *et al.* 1997). Other
13 studies on fine root formation have found resource availability effects were species
14 specific (Lee *et al.* 2007), site specific (Swaty *et al.* 1998) and further confused when
15 specialised roots such as ectomycorrhizas were considered (Anderson and Cairney
16 2007). We know the *Melaleuca* species associating with *R. gardneri* readily form
17 ectomycorrhizas and arbuscular mycorrhizas (Warcup 1985, 1991); however, it is
18 difficult to elucidate whether mycorrhiza formation affects fine root biomass, is a
19 product of fine root biomass production or a combination of both. A microcosm study
20 by Bogeat-Triboulot *et al.* (2004) on *Pinus pinaster* Ait. showed ectomycorrhizal root
21 tip percentage remained constant regardless of decreasing moisture availability even
22 though fine root biomass increased. If these phenomena are consistent for *Melaleuca*
23 species, greater fine root biomass results in *T. gardneri* having greater access to the
24 autotrophic carbohydrate pool which might result in a greater carbon transfer to *R.*
25 *gardneri*.

1

2 *Melaleuca* biomass, density and associated properties are important habitat attributes
3 as these determine overall carbon and nutrient cycling within an ecosystem and affect
4 a range of soil chemical and physical attributes as well as influence microclimate.
5 However, *R. gardneri* is directly linked to its mycorrhizal fungus and thus, dependent
6 on the fungus' tolerances and ability to interact in these specified habitats. Given the
7 nutritional dependence of *R. gardneri* on mycorrhization and the purported specificity
8 of this association (Warcup 1985, 1991), *R. gardneri* can only survive while its fungal
9 partner is active. We found that Northern and Southern sites had similar living fungal
10 biomass. Ergosterol concentrations of soils sampled in this study were remarkably
11 high given the growth and climatic conditions, and are similar to concentrations found
12 in German grassland and forest soils (Djajakirana *et al.* 1996) and around half that of
13 soils of highly productive Jarrah (*Eucalyptus marginata* D.Don ex Sm.) forests of
14 southern Western Australia (Grierson and Adams 2000). However, it is not known
15 which species comprised most of the fungal biomass at our sites. Different fungal
16 species vary in their tolerance to drought conditions/moisture availability and it is
17 common to observe changes in ectomycorrhizal community composition (independent
18 of total fungal biomass measures) at varying moisture availability (Slankis 1974; Shi
19 *et al.* 2002). However, given the low annual precipitation at field sites in this study,
20 *Thanatephorus gardneri* probably has some degree of tolerance to reduced moisture
21 availability.

22

23 While we have characterised the known habitat of *R. gardneri*, past environmental
24 changes may have influenced current attributes; for example, it has been more than 30
25 years since any of the *R. gardneri* sites have been burnt by wildfire. However, it is

1 unknown what the natural fire frequency is in the regions studied and consequently if
2 fire is necessary for the recruitment and survival of both *Melaleuca* and *R. gardneri*.
3 Extensive land clearing has resulted in very few and small fragmented sites that have
4 been subsequently protected from fire because of proximity to agriculturally active
5 land. Nevertheless, *M. uncinata s.l.* has the ability to rapidly resprout after low
6 intensity burns (personal observations). In addition, the first *R. gardneri* plant
7 discovered intact (i.e., not during ploughing and land clearing) was found growing
8 under a ~70 cm high *Melaleuca* plant resprouting after having its aboveground
9 biomass felled (George 1980). Consequently, disturbance may have positive (or at
10 least not necessarily negative) effects on *R. gardneri*. A broad range of terrestrial
11 orchids exhibit greater recruitment and flowering after fire (Coates *et al.* 2006). For
12 example, the number of recruits of the New Zealand terrestrial orchid *Corybas carsei*
13 (Cheeseman) Hatch) tripled one year after fire and increased by a further 300 % in the
14 following year (Norton and De Lange 2003). Similarly, Coates *et al.* (2006)
15 demonstrated that the Australian terrestrial orchid *Prasophyllum correctum* D.L Jones
16 also recruited and flowered more prolifically in the few years after fire. While the
17 positive response of some terrestrial orchids to disturbance is not fully understood, it
18 is likely that increased nutrient availability (Bond and van Wilgen 1996), smoke-
19 induced triggers (Flematti *et al.* 2004) combined with reduced competition from
20 surrounding vegetation all play a role.

21

22 This research provides information useful for adaptive management for the
23 conservation of *R. gardneri*. We did not identify any single habitat characteristic that
24 explained the presence or absence of *R. gardneri*. However, given the purported
25 dependence of *R. gardneri* on its mycorrhizal fungal partner, future research must

1 consider how this fungus associates with its habitat – what conditions are promoting
2 and/or limiting its growth. What is clear from our study is that there are many areas of
3 *Melaleuca* thickets outside those described here that have similar habitat
4 characteristics. Consequently, the potential for this species to exist or have existed in
5 many parts of southern Australia is a real possibility and continued exploration of
6 potential habitat should be encouraged.

7

8 **Acknowledgements**

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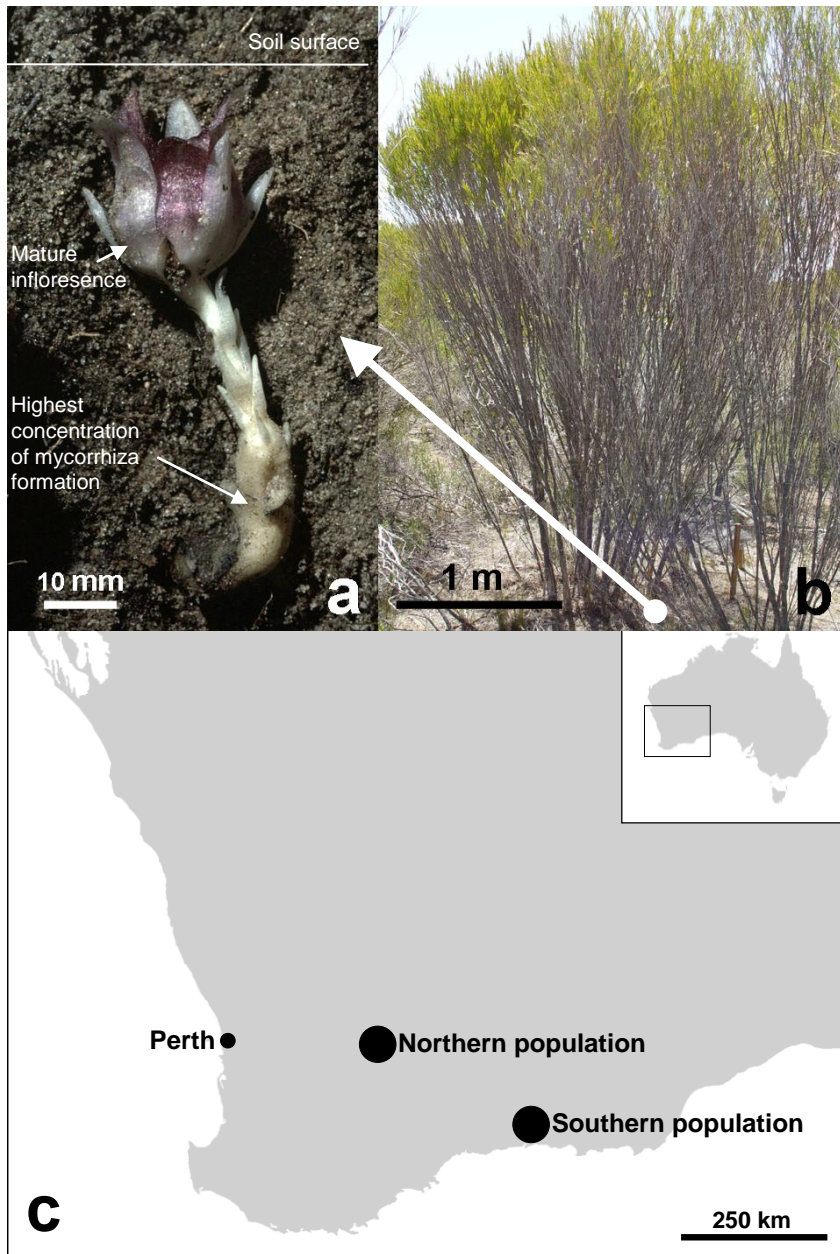
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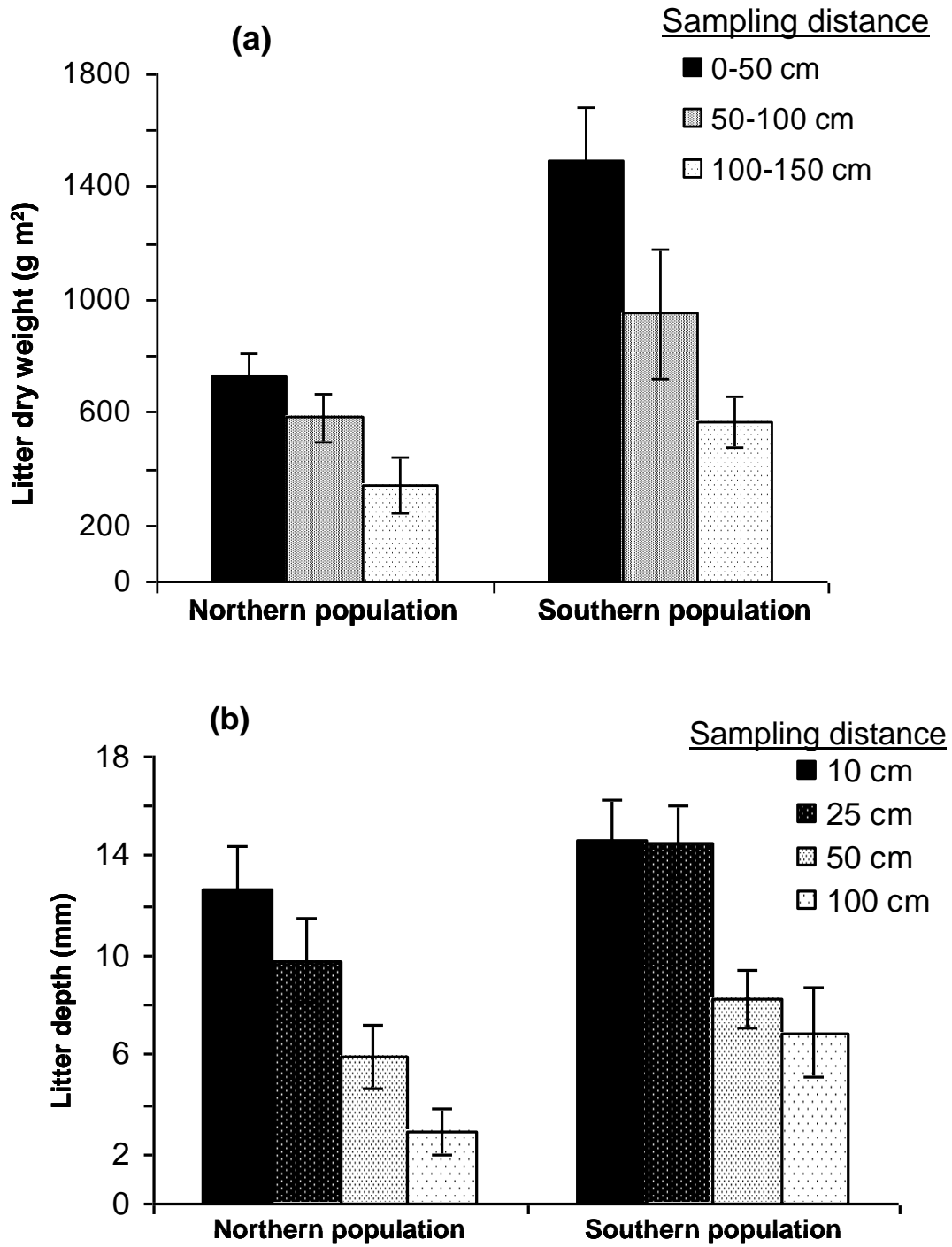
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1 **Fig. 1.** (a) Partially exposed flowering *Rhizanthella gardneri* demonstrating its
2 entirely subterranean nature. (b) *Melaleuca scalena* individual indicating where a *R.*
3 *gardneri* plant was observed (Kunjin, Western Australia). (c) Distribution of
4 currently known *Rhizanthella gardneri* populations in south-west Western Australia.
5 Each population consists of three separate sites.



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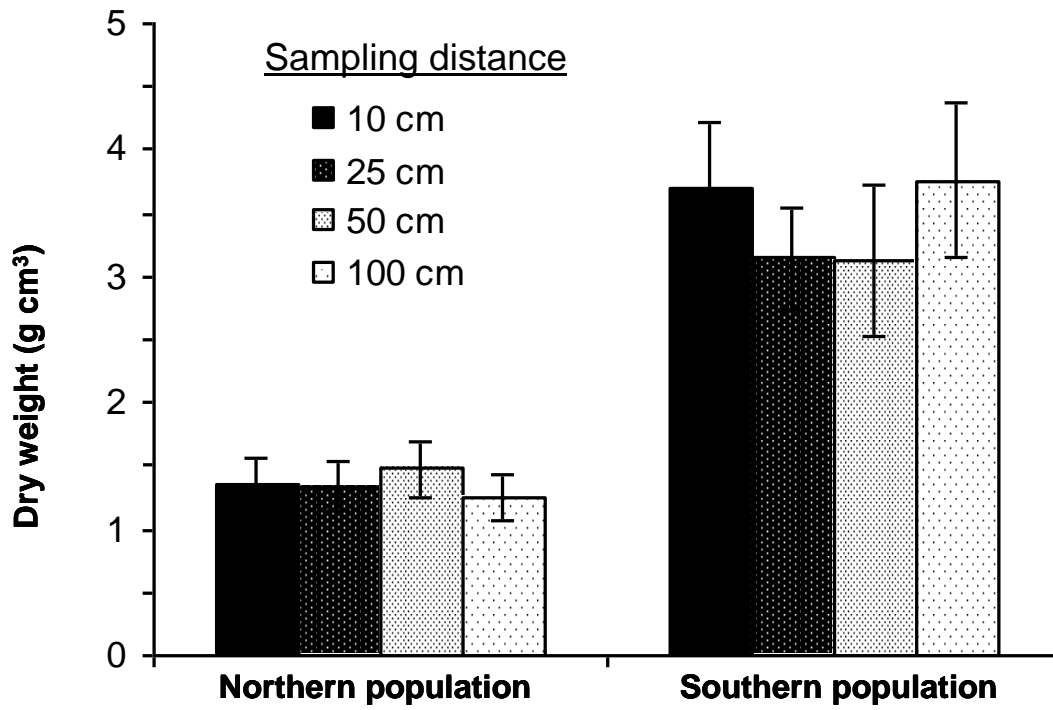
1 **Fig. 2.** (a) Total standing leaf litter of Northern and Southern populations at three
 2 distances from bases of randomly selected *Melaleuca* individuals (mean, S.E, n=9)
 3 collected November 2004. (b) Litter depth (mm) at Northern and Southern
 4 populations at four distances from bases of randomly selected *Melaleuca* individuals
 5 (mean, S.E, n=18) collected May 2006.



6

1 **Fig. 3.** Fine root (<2 mm) dry weight biomass of top 15 cm of soil profile at four
2 distances from bases of randomly selected *Melaleuca* individuals (mean, S.E, n=18)
3 collected May 2006.

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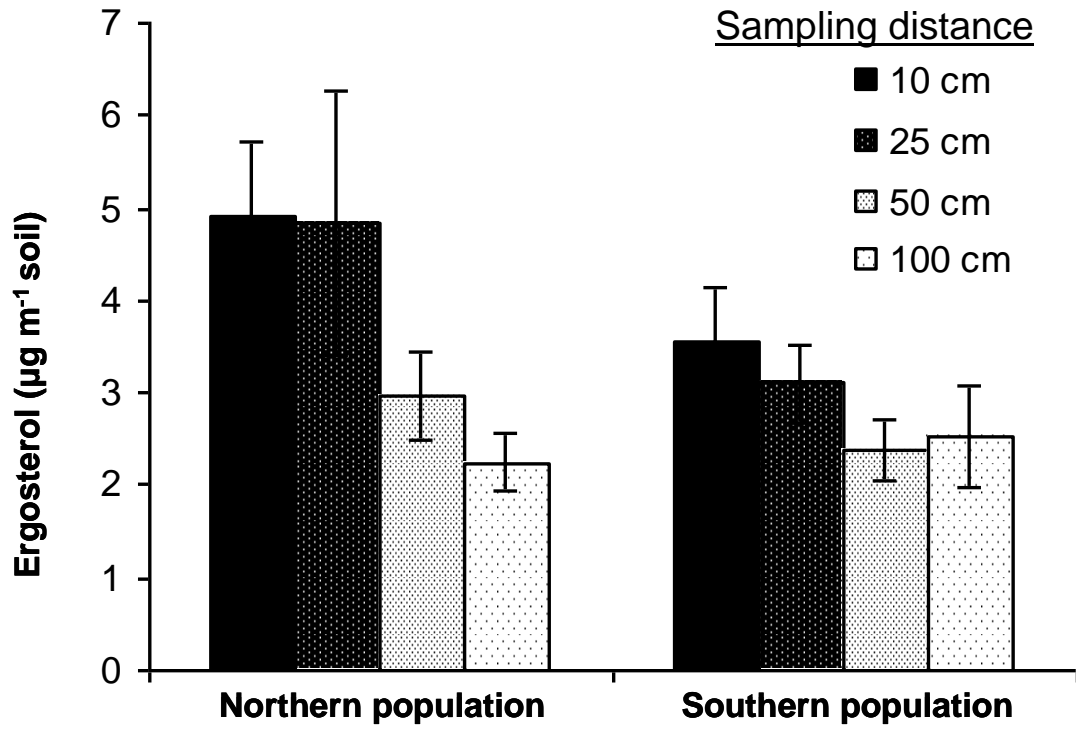


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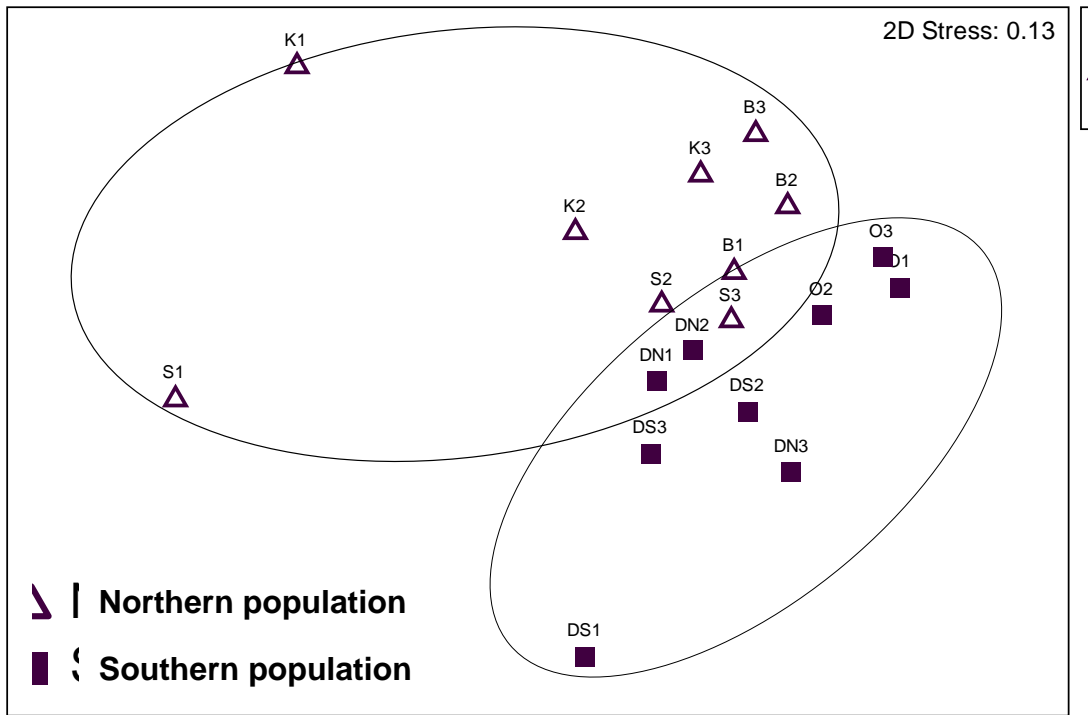
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1 **Fig. 4.** Ergosterol concentration (indicator of living fungal biomass) of top 5cm of soil
2 profile at four distances from bases of randomly selected *Melaleuca* individuals
3 (mean, S.E, n=18) collected May 2006.



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1 **Fig. 5.** Non-metric MDS ordination of soil chemistry profiles of Northern and
 2 Southern sites (n=9). Data was normalised and a resemblance matrix based on
 3 Euclidean distance was created. Letters represent site (K=Kunjin, B=Babakin,
 4 S=Sorenson's Reserve, O=Oldfield River, DN=Dallinup Creek North, DS=Dallinup
 5 creek South) and numbers represent replicate number.



6

1 **Table 1. General characteristics of known *R. gardneri* sites**

	Northern population			Southern population		
	Kunjin	Babakin	Sorenson's reserve	Oldfield River	Dallinup Creek North	Dallinup Creek South
Land allocation	Unallocated Crown Land	Nature reserve	Nature reserve	Unallocated Crown Land	Private property	Private property
Area of <i>Melaleuca</i> thicket (ha)	3	5	2	4	8	8
Predominant <i>Melaleuca</i> species (uncinata complex)	<i>M. scalena</i>	<i>M. scalena</i>	<i>M. scalena</i>	<i>M. hamata</i> , unidentified sp.	<i>M. uncinata</i>	<i>M. uncinata</i>
Other common species (shrubs/trees)	<i>Allocasuarina campestris</i>	<i>Allocasuarina campestris</i>	<i>Allocasuarina campestris</i>	<i>Banksia media</i>	<i>Eucalyptus</i> spp. mallee	<i>Eucalyptus</i> spp. mallee
Time since last fire (years)	30+	30+	30+	30+	30+	30+
Flowers observed (2000-2007)	15	40	40	25	25	3 (2004-2007)
Mean annual rainfall (mm) (1889-2006)*	358 (22%)	325 (24%)	325 (24%)	409 (20%)	442 (19%)	442 (19%)

2

3 *Coefficient of Variation (CV)

1 **Table 2. Characteristics of *Melaleuca* thickets at sites where *R. gardneri* is known**
 2 **to exist.**

	Northern population				Southern population			
	Kunjin	Babakin	Sorenson's reserve	Oldfield River	Dallinup Creek North	Dallinup Creek South		
<i>Melaleuca</i> density ha ⁻¹	380	350	337	603	443	470		
Canopy area m ² ha ⁻¹	809.4	591.5	387.5	862.3	562.6	1001.1		
Individual <i>Melaleuca</i> shrubs (n=20)								
mean height (m)	2.79	2.64	2.27	2.11	2.26	2.57		
mean canopy area (m ²)	2.13	1.69	1.15	1.43	1.27	2.13		
mean number of stems	5.18	3.85	4.40	6.95	9.90	11.45		
Aboveground biomass of <i>Melaleuca</i> individuals (n=5)								
Foliage biomass per m ² canopy								
area (kg dry weight)	0.34	0.38	0.35	0.29	0.56	0.24		
Leaf area (m ²) per kg foliage								
biomass (dry weight)	62.75	63.21	61.97	53.30	52.19	69.62		
Foliage - total N (g kg ⁻¹)	15.14	15.46	15.01	12.63	10.14	11.21		
Foliage - total C (g kg ⁻¹)	482.50	486.58	481.36	482.14	486.76	484.35		
Branch/twig/wood biomass per m ² canopy area (kg dry weight)								
	2.45	2.75	3.23	1.71	2.40	2.09		
Total aboveground biomass per m ² canopy area (kg dry weight)								
	3.90	5.83	5.41	3.51	3.90	6.01		
Aboveground biomass (kg ha⁻¹)								
Foliage	274.3	227.2	135.2	249.5	318.3	237.5		
Branch/twig/wood	1981.2	1621.2	1250.3	1471.2	1351.4	2095.2		
Total biomass								
Leaf surface area (m ² ha ⁻¹)	17203	14358	8380	13297	16609	16534		

3

4

1 **Table 3. Allometric equations used to calculate *Melaleuca spp.* (uncinata**
 2 **complex) aboveground biomass for all known *R. gardneri* sites. Equations are**
 3 **either in linear ($Y = aX + b$) or polynomial ($Y = aX^2 + bX + c$) form with Y**
 4 **relating to dry weights (kg) of; total aboveground biomass, foliage biomass or**
 5 **wood/branch biomass; and X = canopy area (m²).**
 6

	Y	Model	n	a	SE	b	SE	c	SE	r ²	p Value
	Total										
Northern population	aboveground biomass	polynomial	15	0.149	0.255	2.112	1.089	0.62	0.852	0.90	<.0001
	Foliage biomass	linear	15	3.04	0.34	0.41	0.762			0.86	<.0001
	Wood/branches biomass	polynomial	15	1.511	2.269	17.982	9.706	5.87	7.59	0.90	<.0001
	Total										
Southern population	aboveground biomass	polynomial	15	0.373	0.103	1.034	0.507	0.48	0.482	0.96	<.0001
	Foliage biomass	polynomial	15	-0.33	0.48	4.242	2.379	-0.35	2.261	0.53	0.01
	Wood/branches biomass	polynomial	15	4.067	1.063	6.101	5.248	5.14	4.988	0.96	<.0001
	Total										
All sites	aboveground biomass	polynomial	30	1.51	0.523	0.598	0.47	0.28	0.112	0.92	<.0001
	Foliage biomass	linear	30	0.282	0.041	0.057	0.093			0.63	<.0001
	Wood/branches biomass	polynomial	30	1.101	0.498	0.62	0.448	0.31	0.106	0.92	<.0001

1 **Table 4. Soil chemistry characteristics of *R. gardneri* population habitats. Soil samples are divided into depth and values represent**
 2 **means with standard error in parentheses**

	Sampling depth (cm)	pH	Total C (mg/g soil)	Total N (mg/g soil)	C:N (soil)	NH ₄ (mg/kg soil)	NO ₃ (mg/kg soil)	BKPi (µg/g soil)	OHPi (µg/g soil)	OHPo (µg/g soil)	δ ¹³ C	δ ¹⁵ N
Northern population (n = 18)	0-2	5.24 (0.11)	20.21 (3.5)	0.87 (0.17)	23.35 (1.46)	22.55 (5.20)	1.05 (0.18)	1.68 (0.31)	3.26 (0.66)	18.48 (1.86)	-25.85 (0.16)	1.13 (0.43)
	2-5	5.33 (0.09)	15.29 (4.1)	0.57 (0.17)	27.17 (1.22)	12.03 (2.84)	0.94 (0.34)	1.17 (0.17)	3.16 (0.31)	12.95 (5.46)	-25.92 (0.14)	3.78 (0.45)
	5-10	5.44 (0.12)	7.6 (0.9)	0.25 (0.02)	29.33 (1.60)	7.89 (1.18)	0.54 (0.16)	1.09 (0.11)	2.32 (0.25)	5.49 (0.20)	-26.12 (0.11)	4.88 (0.50)
	10-20	5.47 (0.17)	8.2 (2.6)	0.26 (0.06)	28.80 (1.76)	5.44 (0.82)	0.26 (0.06)	0.54 (0.08)	1.52 (0.11)	4.19 (0.32)	-26.05 (0.26)	5.54 (0.56)
Southern population (n = 18)	0-2	5.57 (0.13)	21.89 (2.9)	0.99 (0.13)	22.42 (0.62)	7.70 (1.19)	2.84 (1.97)	1.2 (0.09)	2.32 (0.91)	19.53 (2.79)	-25.90 (0.07)	1.54 (0.19)
	2-5	6.03 (0.11)	12.05 (2.5)	0.52 (0.1)	23.67 (1.26)	5.48 (0.34)	0.32 (0.19)	0.86 (0.07)	2.24 (0.34)	7.21 (0.97)	-25.95 (0.17)	3.13 (0.37)
	5-10	5.99 (0.15)	10.9 (2.5)	0.47 (0.11)	24.74 (1.61)	5.52 (0.34)	0.01 (0.04)	0.67 (0.12)	1.94 (0.23)	5.77 (0.78)	-25.84 (0.20)	4.47 (0.37)
	10-20	6.03 (0.18)	6.77 (1.3)	0.31 (0.05)	22.92 (2.22)	5.60 (0.46)	0.01 (0.04)	0.55 (0.08)	1.33 (0.16)	4.38 (0.59)	-25.89 (0.18)	5.89 (0.28)
All sites (n = 36)	0-2	5.43 (0.09)	21.05 (2.2)	0.93 (0.1)	22.89 (0.78)	15.13 (3.15)	1.94 (0.99)	1.44 (0.17)	2.79 (0.56)	19.01 (1.63)	-25.88 (0.09)	1.34 (0.26)
	2-5	5.81 (0.11)	13.67 (2.3)	0.55 (0.1)	25.42 (0.95)	8.76 (1.60)	0.63 (0.20)	1.02 (0.09)	2.70 (0.25)	10.08 (2.78)	-25.94 (0.11)	3.46 (0.29)
	5-10	5.80 (0.12)	9.25 (1.4)	0.36 (0.06)	27.04 (1.23)	6.70 (0.66)	0.27 (0.13)	0.88 (0.09)	2.13 (0.17)	5.63 (0.39)	-25.98 (0.11)	4.68 (0.30)
	10-20	5.83 (0.15)	7.49 (1.4)	0.28 (0.04)	25.86 (1.55)	5.52 (0.46)	0.13 (0.08)	0.54 (0.06)	1.42 (0.10)	4.29 (0.33)	-25.97 (0.16)	5.72 (0.31)

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