A COMPARISON OF THE ENDOPHYTIC FUNGI FROM LEAVES OF *BANKSIA INTEGRIFOLIA* AT THREE SITES ON THE EAST COAST OF AUSTRALIA

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Abstract

Fungi were isolated from healthy leaves of the xeromorphic shrub *Banksia integrifolia* at two locations from each of three sites in New South Wales. From a total of 2,399 isolates, eleven common species were found at each site. Of the eleven species, the seven that remain unidentified appear to be specific endophytes of the host species. Most of the remaining species were rare being found at only one site. While more detailed sampling may indicate a wider array of potential endophytes, many fungi appear to be occasional colonists of the host plant.

Introduction

A range of fungi exists within plants (Farr *et al.* 1989, Petrini 1991, 1996). The function of most leaf-borne fungi remains unclear: some are pathogens, while others remain asymptomatic and are referred to as endophytes. Endophytes live asymptomatically within healthy plants, for part or all of their life cycle (Petrini 1991). They can be ubiquitous, host specific or even tissue specific (Petrini 1996). The endophyte is dependent on the plant for its nutritional and environmental requirements (Petrini 1996). Some specific endophytes induce changes in host plant physiology, protect the host from pathogens and deter herbivores (Clay *et al.* 1985, Clay 1988, Clay *et al.* 1993, Tudzynski 1997). Thus the maintenance of a population of endophytes may be of ecological advantage to the host plant (Parbery 1996).

Variation in the diversity of fungi colonising a specific host may be associated with location, climate and leaf age. Plants removed from their natural habitats are exposed to an unpredictable source of inoculum. Thus the incidence of colonisation by endophytes may be much higher in areas where the plants are endemic than where introduced (Fisher *et al.* 1993). Species abundance in endophyte assemblages may differ between sites (Rollinger & Langenheim 1993). Climatic conditions, including air pollution, influence colonisation (Asai *et al.* 1998, Petrini 1991). The dynamics of the interactions in endophyte communities is also dependent on the season (Cabral 1985). Increased frequency of colonisation and species richness in a host plant are related to foliage age. The increases probably result from increased exposure to propagules (Bertoni & Cabral 1988, Fisher *et al.* 1986, Stone 1987). Thus to study the dynamics of communities of endophytes it would be necessary to sample both new and old leaves from endemic plants across their natural habitats.

Much of the information currently available on endophyte function in plants is based on research on grasses. Examination of endophyte assemblages of perennial plants, especially Australian trees and shrubs, is less well developed. Perennial plants may harbour a diversity of endophytes. Plant species growing on mineral poor soils are likely to utilise efficient mechanisms to deter or reduce herbivory (Coley *et al.* 1985), some of which may be microbial. Selection of plants from their native habitat should also result in the recovery of an endophyte population adapted to the host and habitat. Thus this study examines the diversity of endophytes of the xeromorphic plant species *Banksia integrifolia* L.f. at one time across a broad geographic range. The aim was to determine which fungi were found in this host and to detect geographic patterns in the distribution of the fungi.

Materials and Methods

Banksia integrifolia was chosen because it is native to Australia (Sedgley 1996, Taylor & Hopper 1988) where it is found in very poor sandy soils (Johnson & Burchett 1996) along the east coast of Australia. The leaves are xeromorphic. Stomata are protected by lignified hairs in deep crypts on the abaxial surface. New shoots mostly grow from December to March, though limited shoot growth continues throughout the year (Taylor & Hopper 1988). Leaves are long-lived. Little is known of the fungi found in the leaves (see for example Crous *et al.* 2000).

Samples were collected from three heathland sites in New South Wales: Sydney, the Central Coast and the North Coast, each consisting of two proximate locations. The two locations within the city of Sydney, South

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Head (33°51'00"S 151°17'00"E) and Nielsen Park (33°51'00"S 151°16'00"E), consist of remnant endemic vegetation. The mean daily maximum temperature in Sydney is approximately 21.5°C and the mean annual rainfall is 1220 mm. The locations of the Central Coast site Copacabana (33°29'30"S 151°26'00"E) and Terrigal (33°26'30"S 151°26'30"E) are adjacent to a moderately populated coastal strip. The mean daily maximum temperature is approximately 21.5°C and the mean annual rainfall is 1240 mm. The locations of the North Coast site, Corindi Beach (30°02'00"S 153°12'00"E) and Red Rock (29°59'00"S 153°13'00"E) are located on a rugged coastline and the area is sparsely populated. The mean daily maximum temperature is approximately 23.2°C and the mean annual rainfall is 1700 mm.

Three old and three young healthy leaves were randomly selected from ten trees at each location. Leaves were put into plastic bags and placed in chilled containers for transport to the laboratory where they were stored at 4°C. Leaves were processed within 48 hours of collection. Sampling commenced in July 1999 (mid winter). Collection at each site was separated by one week to allow for processing.

Entire leaves were cut into 12 segments and then surface sterilised. Following preliminary experiments to determine methods to remove epiphytes, the following procedure was used. Leaf segments were placed in sodium hypochlorite (2% Cl), which was in turn placed in a vacuum flask. The pressure in the container was reduced for one minute, during which time bubbles formed on the leaf segments. When pressure was returned to normal, the sterilising solution was presumably pulled into the stomatal crypts. The segments were further soaked in the NaOCl solution for five minutes, and then rinsed in sterile deionised H_2O. Segments were then placed on PDA containing antibiotics (3×10^{-6} g l^{-1} tetracycline, 5×10^{-5} g l^{-1} streptomycin sulphate), with all segments from one leaf per plate. Hyphae emerging from segments were subcultured onto fresh PDA, and the remnant segment discarded to avoid contaminants emerging from the leaf segment. All isolates that emerged within four weeks were subcultured. All subcultures were grown at 23°C, and where necessary under blue light to induce sporulation (Linden *et al.* 1997, Petrini & Fisher 1988). The isolates were identified where possible and otherwise grouped into morphological species (Bills 1996, Christensen 1969).

The data were analysed using three-way ANOVA to compare age, site and location.

Results

Of the total of 2399 isolates, 490 were from Sydney, 750 from the Central Coast and 1159 from the North Coast. The isolates comprised 96 species, of which 80 were from old leaves, 42 from young leaves, with 26 from both. Of the 26 species, 11 were common to the three sites though not necessarily in old and young leaves at each site. They represented 90 per cent of the isolates (Table I). No other species were found at each site. Twelve fungi were isolated from two sites, totalling 89 isolates (3.7% of total), with a maximum of only eight fungi at a particular site. The remaining fungi were mostly slow growing or late emerging isolates. Some fungi were not subcultured successfully: 18 of the emerging fungi, 15 from old leaves, did not grow following subculture. Fungi did not emerge from 12 young leaves or from one old leaf.

Data on only five of the 11 common species demonstrated homogeneous variance after transformation using log (X + 1) (Zar 1996): *Colletotrichum* sp. 1, *Nigrospora sphaerica* (Sacc.) Mason, *Pestalotiopsis* sp., Fungus 5 and Fungus 28. Between 43 and 309 isolates of *Colletotrichum* sp. 1 were obtained from sites, and age of leaf was unimportant. *Nigrospora sphaerica* and *Pestalotiopsis* sp. were more evenly distributed with reference to age, site and location. Fewer isolates of Fungus 5 were found in Sydney collections ($p < 0.05$), and colonisation of leaves increased with age. Few isolates of Fungus 28 were obtained from all sites and ages of leaf.

Discussion

The aim of this study was to establish any patterns in the distribution of endophytic fungal species from the leaves of *B. integrifolia* across a broad geographic range. The ultimate intent is to isolate widespread fungi that may be specific to *B. integrifolia*. Of the 11 species universally present, *Aureobasidium pullulans* Viala & Boyer, *Epicoccum purpurascens* Ehrenb. and *Nigrospora sphaerica* are widely associated with plants (Farr *et al.* 1989) and are thus nonspecific. Species of *Colletotrichum* may be either host specific or generalist (Farr *et al.* 1989), and some species cause disease. As all samples were isolated from apparently healthy leaves, *Colletotrichum* fulfils the requirement of an endophyte, living at least part of its life cycle asymptomatically within healthy tissues. The fungus was isolated from both old and new leaves with a trend towards more isolates from young leaves, possibly due to the increased number of other fungi in older leaves. The blastomycete is possibly an epiphyte. Yeasts are common epiphytes on a wide range of plants including this species, though

some are isolated from vascular tissues as well. The remaining five of the 11 species are unidentified, potentially host specific and warrant further investigation.

Further species isolated from two of the three sites are potentially host specific. Species isolated from the North Coast and either Sydney or the Central Coast were infrequent and slow to emerge. It is conceivable that slow growing species are present at all sites, but masked by the emergence of faster growing fungi. Some fungi isolated from Sydney and the Central Coast also emerged late and at low frequencies. These latter fungi may be geographically specific. An examination of the endophytes of other plant species at the sites would be required to determine if the fungi are located within a geographic range, or general within species within a geographic range.

The approach to isolation may have influenced the results. Use of a severe surface sterilisation, while removing most epiphytes, may have also removed some vascular endophytes. In this experiment leaf segments were discarded as fungi were subcultured from them. The purpose of this approach was to prevent fast growing fungi from overgrowing the plate and to prevent any other fungi present in the leaf segments contaminating the cultures. The approach selects for rapidly emerging and fast growing isolates and masks slow growing fungi. The approach also possibly reduces the potential for some fungi to form fruiting structures. Different fungi emerged from some leaf segments indicating that segments may be occupied by more than one fungus. Fungi that emerge late, and these were usually also slow growing, may have been discarded prior to detection.

Not all fungi that emerged from segments were successfully cultured. Either the hyphae were destroyed as they were isolated, or they were species that require a specific substratum for growth. Of the18 isolates that did not grow in culture, 15 were from old leaves. Clearly, the host is an appropriate substratum for growth, and the use of leaf fragments in culture is warranted in further investigations. However, their infrequent appearance indicates that the fungi were rare and may be ignored for the present.

The fungi were isolated at one time of the year, chosen to reflect potential newly arrived endophytes and more mature colonists. This approach does not indicate if the population changes with time or if the endophytes are interacting with one another. Further, by using large leaf fragments and discarding the fragment as soon as an isolate emerged, slower growing isolates and less competitive fungi were masked. Techniques for detecting and culturing these fungi need to be refined.

Table 1: The total number of isolates of eleven common endophytes found in young (Y) and old (O) leaves.

FUNGUS	SOUTH HEAD		NIELSEN PARK		COPACOBANA		TERRIGAL		CORRINDI BEACH		REDROCK		TOTAL
	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	
Colletotrichum sp. 1	33	23	87	115	54	36	31	12	194	115	168	87	**955**
Nigrospora sphaerica	5	22	3	5	2	13	2	19	11	16	5	33	**136**
Pestalotiopsis sp.	0	12	0	3	1	5	0	0	5	9	6	17	**58**
Ascomycete A	1	6	3	1	51	187	17	162	64	145	42	96	**775**
Aureobasidium pullulans	7	3	2	1	2	2	9	5	1	1	1	7	**41**
Colletotrichum sp. 2	3	2	0	0	5	11	0	0	9	19	2	6	**57**
Epicoccum purpurascens	0	1	0	1	7	0	0	1	1	2	2	1	**16**
Sterile brown mycelium	4	8	8	10	0	3	13	9	4	2	2	3	**66**
Sterile crusty mycelium	0	0	3	4	1	0	0	0	1	0	0	0	**9**
Blastomycete	5	6	2	3	4	1	3	1	0	1	0	0	**26**
Ascomycete B	0	1	0	0	2	2	2	3	0	3	5	9	**27**
Total	**58**	**84**	**108**	**143**	**129**	**260**	**77**	**212**	**290**	**313**	**233**	**259**	**2166**

The aim of this study was to detect geographic patterns in the distribution of fungal endophytes of *B. integrifolia*. Eleven endophytes were isolated from leaves of the host plant, of which four appear to be ubiqitous. The remaining seven species were also common in *B. integrifolia* and widely distributed. Their distribution in other plants and plant parts remain to be determined. Geographic patterns were not detected among the remaining fungi because they were rarely isolated. In future studies, the endophytes need to be identified; this will probably involve molecular markers to distinguish each fungus due to the difficulty in obtaining fruiting structures. We also wish to determine whether the fungi are specific to *B. integrifolia* and whether some of the specific endophytes play a role in plant interactions with herbivores.

References

Asai, E., Hata, K. & Futai, K. (1998). Effect of simulated acid rain on the occurrence of *Lophodermium* on Japanese black pine needles. *Mycological Research* **102,** 1316–1318.

Bertoni, M.D. & Cabral, D. (1988). Phyllosphere of *Eucalyptus viminalis* II: Distribution of endophytes. *Nova Hedwigia* **46,** 3–4.

Bills, G.F. (1996). Isolation and analysis of endophytic fungal communities from woody plants, *in* S.C. Redlin & L.M. Carris (eds), *Endophytic fungi in Grasses and Woody Plants* 31–65. APS Press, Minnesota, U.S.A.

Cabral, D. (1985). Phyllosphere of *Eucalyptus viminalis*: Dynamics of fungal populations. *Transactions of the British Mycological Society* **85,** 501–511.

Christensen, C.M. (1969). Soil microfungi of dry mesic conifer-hardwood forests in northern Wisconsin. *Ecology* **50,** 9–27.

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* **69,** 10–16.

Clay, K., Hardy, T.N. & Hammond, A.M. (1985). Fungal endophytes of grasses and their effects on an insect herbivore. *Oecologia* **66,** 1–5.

Clay, K., Marks, S. & Cheplick, G.P. (1993). Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. *Ecology* **74,** 1767–1777.

Coley, P.D., Bryant J.P. & Chapin III, F.S. (1985). Resource availability and plant antiherbivore defence. *Science* **230,** 895–899.

Crous, P.W., Summerall, B.A., Taylor, J.E. & Bullock, S. (2000). Fungi occurring on Proteaceae in Australia: selected foliicolous species. *Australasian Plant Pathology* **29,** 267–278.

Farr, D.F., Bills, G.F, Chumuris, G.P. & Rossman, A.Y. (1989). *Fungi on Plants and Plant Products in the United States* 816–837. APS Press, Minnesota, U.S.A.

Fisher, P.J., Anson, A.E. & Petrini, O. (1986). Fungal endophytes in *Ulex europaeus* and *Ulex gallii*. *Transactions of the British Mycological Society* **86,** 153–193.

Fisher, P.J., Petrini, O. & Sutton, B.C. (1993). A comparative study of fungal endophytes in leaves, xylem and bark of *Eucalyptus nitens* in Australia and England. *Sydowia* **45,** 338–345.

Johnson, K.A. & Burchett, M. (1996). *Native Australian Plants* 19–35. UNSW Press, Sydney, Australia.

Linden, H., Ballario, P. & Macino, G. (1997). Blue light regulation in *Neurospora crassa*. *Fungal Genetics and Biology* **22,** 141–150.

Parbery, D.G. (1996). Trophism and the ecology of fungi associated with plants. *Biological Reviews* **71,** 473–527.

Petrini, O. (1991). Fungal endophytes of tree leaves, *in* J.H. Andrews & S.S. Hirano (eds), *Microbial Ecology of Leaves* 179–197. Springer-Verlag, New York, U.S.A.

Petrini, O. (1996). Ecological and physiological aspects of host-specificity in endophytic fungi, *in* S.C. Redlin & L.M. Carris (eds), *Endophytic fungi in Grasses and Woody Plants* 87–100. APS Press Minnesota, U.S.A.

Petrini, O. & Fisher, P.J. (1988). A comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. *Transactions of the British Mycological Society* **91,** 233–238.

Rollinger, J.L. & Langenheim, J.H. (1993). Geographic survey of fungal endophyte community composition in leaves of coastal redwood. *Mycologia* **85,** 149–156.

Sedgley, M. (1996). *Banksia*, family Proteaceae, *in* K.A. Johnson & M. Burchett (eds), *Native Australian Plants: Horticulture and Uses* 18–35. UNSW Press, Sydney, Australia.

Stone, J.K. (1987). Initiation and development of latent infections by *Rhabdocline parkeri* on Douglas-fir. *Canadian Journal of Botany* **65,** 2614–2621.

Taylor, A.T. & Hopper, S. (1988). *The Banksia Atlas.* Australian Flora and Fauna Series: Number 8. Australian Government Publishing Service, Canberra, Australia.

Tudzynski, B. (1997). Fungal phytohormones in pathogenic and mutualistic associations, *in* G.C. Carroll & P. Tudzynski (eds), *The Mycota: Plant Relationships, Part A* 167–184. Springer-Verlag, New York, U.S.A.

Zar, J.H. (1996). *Biostatistical Analysis*, 3rd edn, 277–284. Prentice-Hall, New Jersey, U.S.A.