Identification and bioactive properties of endophytic fungi isolated from phyllodes of *Acacia* species

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Endophytes are microorganisms that exist within the tissues of living plants. Generally the relationship between the plant and its endophytes is symbiotic whereby the endophytes colonise the internal tissues of the plant without any adverse effects on the host. In the past two decades, there has been growing interest in endophytes and their origins, their biodiversity, endophyte-host interactions, their role in ecology and the characterisation of their secondary metabolites. However, the sheer diversity of plant-endophyte relationships means that only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology. The genus *Acacia* comprises over 1300 species of which nearly 1000 are found in Australia. *Acacia* species are used widely as food (e.g. seeds are ground into flour and the gum is edible) and the wood has been traditionally made into clubs, spears, boomerangs and shields. Various species are used as narcotics and painkillers, to treat headaches, cold and fevers, as antiseptics and bactericides and to treat skin disorders by the indigenous people of Australia. While there is some information available about the medicinal properties of *Acacia*, there is no information about the endophytic microorganisms of these plants. With increased need for new bioactive compounds with medical, industrial or biotechnological applications, we have investigated the bioactive properties of fungal endophytes of *Acacia* species. Specifically, we have isolated endophytic fungi from the phyllodes of *Acacia baileyana*, *Acacia podalyriifolia* and *Acacia floribunda*. These were classified as *Aureobasidium, Chaetomium* and *Sordariomycetes* through genetic analysis of ribosomal RNA genes. The bioactivity of the fungal endophytes was examined and a number of isolates exhibited antibacterial and antifungal properties. Other isolates also exhibited amylase activity and were thus able to hydrolyse starch. This study showed that fungal endophytes are readily isolated from the phyllodes of *Acacia* species and that these exhibit promising bioactive properties. Thus, endophytes from Australian native plants may be a useful source of novel bioactive compounds.

Keywords: Endophyte; *Acacia*; bioactivity; enzymes; antibacterial

1. Introduction

Endophytes are micro-organisms that reside in the internal tissues of living plants without causing any apparent negative effects. Generally the relationship between the plant and its endophytes is one of a symbiotic nature whereby the endophytes colonise the internal tissues of the plant [1-4]. There is growing interest in endophytes and their origins, their biodiversity, endophyte-host interactions, their role in ecology and the characterisation of their secondary metabolites [5-6]. However, only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology [7]. Worldwide, the threat posed by diseases such as cancers and infections is ever-increasing [3,7,8]. For example, the emergence and increasing incidence of antibiotic resistant bacteria has resulted in an urgent need for new antimicrobial drugs with novel modes of action. Clearly, there is a need for new and beneficial compounds that can provide relief against ailments and diseases [7]. It has become apparent that an enormous and relatively untapped source of biological diversity is represented by microbial endophytes which are a promising source of novel natural products for use in medicine, agriculture, and industry. However, escalating rates of environmental degradation and spoilage of land and water resources caused by climate change, the use of toxic insecticides and the release of industrial effluent have led to a loss of biodiversity, especially of plant species. It is highly likely that vegetation loss is associated with the loss of many as yet undiscovered endophyte species. The range of chemicals produced by endophytes is very diverse. Like their host plants, they synthesise a wealth of secondary metabolites which are not directly involved in the metabolism of the micro-organisms but play a role in the fitness and survival of themselves and their hosts [9,10]. These functional metabolites include alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols and phenolic acids, and peptides. Some species produce novel antimicrobial agents (e.g. cryptocandin from *Cryptosporiopsis quercina*), other produce potent anti-cancer compounds (e.g. taxol from *Taxomyces andreanae*) and yet others produce compounds that can be utilized industrially, such as enzymes and solvents [7]. There are few reports of endophytes from Australian native plants and even fewer reports of the bioactive compounds produced by them. This study addresses this scientific knowledge gap and explores whether Australian plant endophytes, in particular those isolated from *Acacia* species, are a natural resource with the potential to yield useful biologically-active compounds. The genus *Acacia* belongs to the family Mimosaceae and consists of over 1300 species, of which nearly 1000 are found in Australia [11]. The acacias, commonly known as wattles, are prevalent in the arid, semi-arid and dry sub-tropical regions of Australia, although they can be found in a wide range of differing habitats

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from the coastal to the sub-alpine regions, including areas of high rainfall and arid inland areas. *Acacia* species are used widely as foods (e.g. seeds are ground into flour and the gum is edible) and the wood has been reportedly made into clubs, spears, boomerangs and shields [11]. With respect to medicinal uses, various species are still used as narcotics and painkillers, to treat headaches, cold and fevers, as antiseptics and bactericides and to treat skin disorders by the indigenous people of Australia (Table 1). There is limited information available about the endophyte species of *Acacia*. Recently, Mohali *et al.* [13] isolated and identified a new species of *Fusicoccum*, named *F. stromaticum*, from the branches and stems of *Acacia mangium* trees from Venezuela. This species has not yet been found in other parts of the world.

Table 1 Traditional Medicinal Uses of *Acacia* by Australian aborigines*

Species	Traditional Medicinal use	Active constituents
Acacia auriculoformis	Antiseptic cleanser; treatment for allergy rash	Possibly saponins
Acacia beaverdiana	Narcotic (mixed with tobacco and chewed)	Alkali present in ash release alkaloids in tobacco enhancing the narcotic effect
Acacia bivenosa subspecies *wayi*	Bark soaked or boiled in water and the decoction used as a cough medicine	Not known
Acacia cuthbertsonii	Bark was used for the relief of toothache and rheumatism	Not known
Acacia decurrens	Decoction of bark used for extreme dysentery	Tannins
Acacia falcate	Embrocation of bark used to cure skin diseases	Tannins
Acacia holosericea	Mashed roots soaked in water and infusion drunk for laryngitis	Not known
Acacia implexa	Embrocation of bark used to cure skin diseases	Tannins
Acacia leptocarpa	Mashed green phyllodes soaked in water and the infusion applied to sore eyes	Not known
Acacia melanoxylum	Hot infusion of roasted bark used to bathe rheumatic joints	Bark is rich in tannins
Acacia monticola	Used for coughs and colds; drunk or used in bathing	Not known
Acacia tetragonophylla	Cleaned inner bark soaked in water and infusion drunk as a cough medicine; leaves chewed for dysentery; ashes from bark-free wood used as an antiseptic; points of pungent phyllodes inserted under warts	Not known
Acacia transluscens	Leaves and twigs mashed in water and the liquid used to bathe skin sores and applied to the head for headaches	Not known, probably tannins

* Information taken from Kjaerstad *et al.* [12]

This aim of this study was to isolate and identify endophytic fungi from the phyllodes of *Acacia baileyana*, *A. floribunda* and *A. podalyriifolia* using morphology examination and sequence analysis of the ITS region of the ribosomal RNA genes. A further aim was to determine if the endophytes produced bioactive compounds by testing their ability to inhibit the growth of bacteria and fungi and hydrolyse starch.

2. Materials

2.1. Isolation of endophytes

Endophytic fungi were isolated from the phyllodes of *A. baileyana*, *A. floribunda* and *A. podalyriifolia*. The plants were collected from various sites within Melbourne, Australia, and their identities were verified by John Reid (Identifications Botanist, Royal Botanic Gardens, Melbourne). The phyllodes were removed from the plants and the surface tissue was sterilised by immersion in 70% ethanol for 5 minutes, followed by washing in sterile water. After air-drying, the tissues were aseptically cut into small pieces, placed onto water agar (1% agar in water) plates and incubated at 30°C for up to 7 days to allow for hyphal growth from within the plant tissue. Surface sterilised and un-dissected tissues were also placed onto water agar plates to ensure that sterilisation procedures had been effective. Hyphal material was transferred to Potato Dextrose Agar (PDA; Becton Dickinson) plates and incubated at 30°C for 7 days to allow the growth of mycelium. The microscopic features of the fungi were observed after staining with lactophenol cotton blue.

2.2. Sequence analysis of Internal Transcribed Spacer (ITS) region

Endophytes were transferred to Potato Dextrose Broth (PDB; Becton Dickinson) and incubated with shaking (180 rpm) at 30°C for 7 days. Mycelia were collected by centrifugation and DNA was extracted using the ZR Fungal/Bacterial DNA Kit (ZYMO Research, USA). Purified DNA was subjected to PCR amplification using primers ITS1 and ITS4, which produce an amplicon of approximately 550 bp of the ITS region. Amplicon DNA was purified and submitted to the Australian Genome Research Facility (Melbourne) for sequencing using Big Dye Terminator version 3.1 and a 3730xl capillary sequencer (Applied Biosystems). The sequence data were analyzed using the BLAST software (BLASTN) available at the National Center of Biotechnolgy Information (NCBI) web site (http://wwwncbinihgov/) to determine the identity of the endophyte.

2.3 Antimicrobial assays

Endophyte cultures were grown in PDB as described above and the supernatants were filtered through 0.45µm pore size filters to remove fungal mycelia and spores. The filtrates were used directly in antimicrobial testing. Test bacteria, *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were grown in Nutrient Broth (Oxoid), while fungi, *Candida albicans* (Food Science Australia Culture Collection 5580), were cultivated in PDB. Antimicrobial assays were performed in Nutrient Agar (NA; Oxoid) or PDA plates using the plate-hole diffusion method as previously described (Palombo and Semple, 2001) with the exception that wells made in agar were 12 mm in diameter and 200µL of endophyte culture filtrate were added to each test well. PDB was used as a negative control, while ampicillin (25 µg/ml) was used as a positive control in the antibacterial assays. NA plates were incubated at 37°C for 24 hours while PDA plates were incubated at 30°C for 24 hours.

2.4 Assay for amylase activity

To test for production of amylase, endophytes were inoculated onto PDA supplemented with 1% starch and incubated at 30°C for 7 days. The plates were then flooded with iodine and observed for clear zones around the fungal colony, which indicated amylase activity.

3. Results

3.1 Isolation and identification of endophytes

Six endophytes were isolated from the phyllodes of the three *Acacia* specimens that were collected (Table 2). Overall, the endophytes were classified into three different fungal genera, with *Aureobasidium* and *Chaetomium* each being isolated from two plant species. *A. floribunda* yielded the most endophyte diversity with all three fungal genera being isolated from this plant. Interestingly, the ITS sequences of the two isolates of *Aureobasidium pullans* were not identical suggesting some level of genetic diversity in the endophytes associated with the closely related plants, *A.baileyana* and *A. floribunda*. Microscopic examination of fungal mycelia revealed the presence of spores in all of the isolates, confirming that all endophytes belonged to the phylum *Ascomycota*.

Table 2 Identification of endophytes based on sequence of ITS DNA

Isolate	Source	Size of ITS amplicon (bp)	Closest match in GenBank	Percentage identity
S6E3	*Acacia baileyana*	523	*Aureobasidium pullulans*	100
S7E1	*Acacia podalyriifolia*	515	*Chaetomium* sp.	99
S7E2	*Acacia podalyriifolia*	515	*Chaetomium globosum*	99
S8E2	*Acacia floribunda*	520	*Chaetomium globosum*	99
S8E3	*Acacia floribunda*	525	*Sordariomycetes*	98
S8E6	*Acacia floribunda*	532	*Aureobasidium pullulans*	99

3.2 Antimicrobial activity

Endophyte culture filtrates were assessed for their antimicrobial activities against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria, and a yeast (*Candida albicans*). Four of the endophytes showed activity against *E. coli*, two showed anti-fungal activity, while none were active against *S. aureus* (Table 3). Endophyte S7E2 (*Chaetomium globosum*) showed both antibacterial and anti-fungal activity. It was interesting to note that the other *C. globosum* isolate (S8E2) did not exhibit any antimicrobial activity. Given that this endophyte was isolated from a different *Acacia* species, it suggests that isolates from diverse host species are fundamentally different at the genetic/biochemical level. The lack of activity against *S. aureus* was unexpected, given that plant-derived natural products often show better activity against Gram positive bacteria [14]. Nonetheless, the results of this study indicated that the antimicrobial metabolites produced by active endophytes may have specific application where the control of Gram negative bacteria and/or fungi is desired.

Table 3 Antimicrobial activity of *Acacia* endophytes

Isolate	Activity against*		
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
S6E3	-	+ (30)	-
S7E1	-	+ (33)	-
S7E2	-	+ (25)	+ (36)
S8E2	-	-	-
S8E3	-	+ (23)	-
S8E6	-	-	+ (25)

* Numbers in brackets indicate sizes of zones of inhibition (mm)

3.3 Amylase production

Endophytes were cultivated on PDA-starch plates. After staining with iodine, amylase activity was detected in three of the isolates, S6E3 (*Aureobasidium pullulans*) and S8E3 (*Sordariomycetes*) (Table 4 and Fig. 1). The two isolates of *Aureobasidium pullulans* (S6E3 isolated from *Acacia baileyana* and S8E6 isolated from *Acacia floribunda*) both exhibited amylase activity suggesting that they have similar biochemical properties. However, the fact that these endophytes exhibited different antimicrobial activities indicated that they are not genetically identical fungi. This was reflected by genetic analysis of the ITS amplicons which did not exhibit the same percentage identity with the closest match in GenBank (Table 1).

Table 4 Amylase activity of *Acacia* endophytes

Isolate	Amylase activity
S6E3	+
S7E1	-
S7E2	-
S8E2	-
S8E3	+
S8E6	+

Fig. 1 Endophyte S8E6 (*Aureobasidium pullulans*) grown on PDA plus starch and stained with iodine. A zone of starch hydrolysis is observed around the fungal colony.

4. Discussion

There is an increasing need for new bioactive compounds that can be used in medicine, industry and agriculture. Historically, many such compounds have been isolated from the natural environment, particularly plants, and many of the drugs available commercially are derived from plant-based chemicals. While plants have been a major source of new lead compounds for drug discovery, attention has more recently turned to endophytes as these microorganisms are seen as having great potential as sources for new bioactive compounds [3]. While much of the interest in endophyte bioactive compounds has been in drugs for medicinal use, compounds that may have industrial or agricultural applications are also gaining attention. In particular, amylase is an important enzyme that is used in numerous applications in a variety of industries and there is growing interest in amylases with a wider spectrum of biological properties that can function at diverse pH and temperature ranges [15]. The need for new antimicrobial agents, in general, comes from the increasing rates of resistance to antimicrobial agents, such as antibiotics [16]. This problem extends beyond the clinical application of antimicrobial drugs and many microorganisms of agricultural concern are also known to have acquired resistance to commonly used antimicrobial chemicals [17]. In addition, the community desire for products that are organic has increased interest in natural methods of pathogen control. Thus, there is a growing need for new, environmentally-friendly antimicrobial agents that may be used safely in agriculture to control plant pathogens and spoilage organisms post-harvest [18].

In this study, we isolated and characterised endophytes from three representatives of the genus *Acacia*. Species of *Acacia* are widely distributed in the Australian environment and many species have been used traditionally by the aboriginal people of Australia [19]. While much is known about the phytochemistry of the genus [20], little information is available about the endophyte biology. Two new endophytic *Fusicoccum* species collected in Venezuela one of which, *F. stromaticum*, was isolated from various *Acacia* species have recently been described [13]. There have been few studies of the endophyte populations of Australian native plants. An extensive survey of ectomycorrhizal-associated *Ascomycota* isolated from *Eucalyptus regnans*, *Nothofagus cunninghamii* and *Pomaderris apetala* in Tasmania identified fungi from the orders *Helotiales*, *Hypocreales*, *Chaetothyriales* and *Sordariales* [21]. Investigation of the endophytes from the stems of Australian native *Gossypium* species (*G. australe, G. bickii, G. nelsonii* and *G. sturtianum*) identified 17 genera, including *Phoma, Alternaria, Fusarium, Dichomera, Phomopsis, Scopulariopsis, Truncatella,Botryodiplodia (B. theobromae), Aureobasidium, Aspergillus, Pestalotiopsis, Cladosporium, Exserohilum, Bartalinia, Monochaetia and Myrothecium* [22]. The potential for discovery of bioactive compounds from Australian traditional medicinal plants has been demonstrated by the description of novel broad-spectrum antibiotics from an endophytic *Streptomyces* isolated from the snakevine plant *(Kennedia nigriscans*), which was used traditionally as an antiseptic to treat open, bleeding wounds [23]. To our knowledge, there have been no descriptions of endophytes from Australian *Acacia*.

The endophytes isolated in this study all belong to the phylum *Ascomycota* and members of these species have been previously identified as endophytes of other plants [24,25,26]. While these have not been extensively tested for their bioactive properties, an endophytic *Chaetomium globusum* has been shown to successfully protect wheat plants from the pathogen *Pyrenophora tritici-repentis*, the causal agent of tan spot disease [27]. Given that the *Chaetomium globusum* isolated from *Acacia podalyriifolia* in this study displayed antimicrobial activity, it would be of interest to determine whether pathogens of economically important agricultural plants are inhibited by this endophyte. If so, this fungus, or its metabolites, may be useful in biocontrol of plant pathogens. The observation that enzyme activity was detected in half of the endophyte species tested in this study encourages further investigation of other potentially useful enzyme activities, such as laccase [28] and chitinase [29]. In addition, further investigation of the amylase activity detected in this study would be useful to determine whether the enzymes produced by the endophytes possess favourable properties, such as temperature and pH tolerance. Similarly, it would be useful to extend the antimicrobial testing to other microorganisms of medical or agricultural importance.

In conclusion, our study has shown that fungal endophytes were readily isolated from Australian native plant species and that limited bioactivity screening indicated that the majority of such species displayed potentially useful chemical and biochemical properties. Further investigation of these and other isolates may yield novel compounds that may have practical applications in a variety of biotechnological areas.

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