FUNGAL LEAF MYCOTA OF SELECTED AROMATIC PLANTS IN NORTH QUEENSLAND

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Abstract

The phyllosphere, endophytic and pathogenic mycota of pepper, cinnamon and tumeric growing in close proximity in a tropical location were studied. Increasing washing cycles up to ten did not significantly alter the number of colonies recovered except in cinnamon which showed reduced numbers recovered beyond seven washes. The isolated fungi were both pathogenic and saprophytic. The fungal genera most frequently isolated from the plant samples by the spread plate method were *Aspergillus*, *Cladosporium, Colletotrichum* and *Nigrospora*. The leaf piece method of isolation most frequently recovered *Cladosporium*, *Colletotrichum*, *Nigrospora*, *Pestalotiopsis*, *Phoma* and non-sporing dematiaceous fungi. Three of these species isolated by the leaf piece method (*Colletotrichum gloeosporioides* on pepper, *Pestalotiopsis* cf. *versicolor* on cinnamon, and *Phoma* sp. on tumeric) are putative pathogens. The diversity of species isolated by washing was less than half that isolated by the leaf piece method. Recovery of species solely by the leaf piece plate method was presumably because these fungi were present primarily inside leaves rather than growing and fruiting abundantly on the leaf surface.

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Introduction

The microbial diversity of phylloplane communities is influenced by plant age, species, micro- and macro-habitat, changes to environmental regimes and position of leaf on the plant (Kinkel 1997; Talley *et al*. 2002; Behrendt *et al*. 2004). Plant genera growing in close proximity have their own characteristic mycota (Kinkel 1997) which is conditioned by the nature of the plant exudates, microclimate and by other members of the mycota (Goodman *et al*. 1986; Lucas & Knights 1987; Osono & Mori 2004). There is evidence that the germination behaviour of some members of the mycota may be influenced by the genetic constitution of the host plant (Goodman *et al*. 1986). In host plants where little selective pressure has been exerted on microbes, through directed plant breeding activities, a balance has been achieved with potential pathogens (Prell & Day 2001; Agrios 2005).

The study of the filamentous fungal flora on leaves is challenging on account of the limitations of the methods available (Lacey 1988). We chose to assess the diversity of fungi on or in the leaves of aromatic plants (black pepper, cinnamon and tumeric) growing in close proximity by using two indirect methods of isolation. This provided means to increase the ability to isolate members of the leaf mycota, to compare fungal diversity between plant species, and most importantly to isolate putative plant pathogens for later

work and to obtain estimates of relative loads of phyllosphere fungi. No specific attempt was made to differentiate fungi growing inside leaves (endophytes – as defined by Wilson 1995 – unapparent and asymptomatic) from epiphytes.

Materials and Methods

Leaves were taken from black pepper (*Piper nigrum* L.), cinnamon (*Cinnamomum zeylanicum* Blume) and tumeric (*Curcuma domestica* Val.) growing in close proximity to each other from the L. & L. Pepperfarm near Silkwood, North Queensland.

Leaf mycota. Leaf pieces of pepper, cinnamon, and tumeric plant leaves (10 x 10 mm) were washed several times with sterile distilled water to assess their active fungal populations (Shipton *et al*. 1981). Washing schedules were 2, 4, 7, and 10 times (two replicates each). Twelve pieces of 100 mm^2 leaflets (four pieces from one leaf) were placed in Universal bottles (two replicates). This meant that six separate leaves per plant were assessed. Leaf pieces were washed by shaking for 2 minutes first with one change of 10 mL sterile surfactant Tween 80 (2 mL/ litre water) using a Griffin Flask Shaker. This was followed by additional changes of sterile distilled water (10 mL).

Spread plate assessment was used to recover fungal fragments and spores (Black 1999). Three aliquots (0.25 mL) were plated out from nominated washing on to one-sixth strength Czapek-Dox Yeast Extract agar (CDYE agar - Warcup 1955) adjusted to pH 4.6 to restrict bacterial growth (three replicates; antibiotics used in preliminary tests were ineffective). Wash water recovered at each of the nominated wash cycles was plated separately so that figures reported represent estimated recoveries for that cycle. Finally, four pieces of washed leaflets (3 replicates) from each washing schedule were plated (leaf plate method; Black 1999) onto CDYE agar; this meant that 12 leaf pieces were used in assessments. Plates were incubated for 5 days at 28° C. The plates were examined for fungal colonies daily and isolates generally taken on day 5. Each colony was counted and the figures were recorded as colony forming units (cfu's) per mL after applying the appropriate multiplication factor.

Cultures of *Bipolaris*, *Exserohillum*, *Colletotrichum*, *Curvularia*, *Pestalotiopsis*, and *Phoma* were sent to the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, for more detailed identification.

Data analysis. The effect of serial washing schedules on fungal colonization was analysed by one-way analysis of variance after establishing the suitability of the data for the test (Zar 1999).

Results and Discussion

Colony counts of leaf mycota. Both bacteria and fungi were isolated from pepper, cinnamon, and tumeric leaves. Several pigmented bacteria dominated the bacterial population but these numbers were not assessed nor their identity established as the purpose of the study was to isolate the mycota. Fokkema & van den Heuvel (1986) and Lindow & Elliott (2000) mentioned that large bacterial populations, as epiphytic bacteria, commonly colonize plant leaf surfaces. Each method of recovery utilized had its own limitations. This study formed part of a larger exercise where the efficacy of surface sprays was being established. The leaf piece method was selected to enable the fungi of primary interest (leaf surface fungi) to be isolated. Leaf pieces (10 x 10 mm) were selected for reasons of ease of size standardization. The leaf wash recovery method complimented this method. Thus, under these circumstances no definitive data are available on the location of the fungi isolated by the leaf piece method; whether they came from the inside or outside the leaf. It is acknowledged that recent evidence (not available when this study was commenced) suggests that smaller leaf pieces would have been more appropriate to maximize numbers of fungi isolated (Gamboa *et al*. 2002). We believe that the potential disadvantages of using a recovery medium with a low pH, which encouraged slow growth, were compensated for by using the wash recovery method of isolation. This method largely avoided the problem of colony overgrowth. Generally the colonies were well distributed on the culture plates. In our preliminary tests, antibiotic additions (penicillin and streptomycin) to the growth medium failed to give adequate control

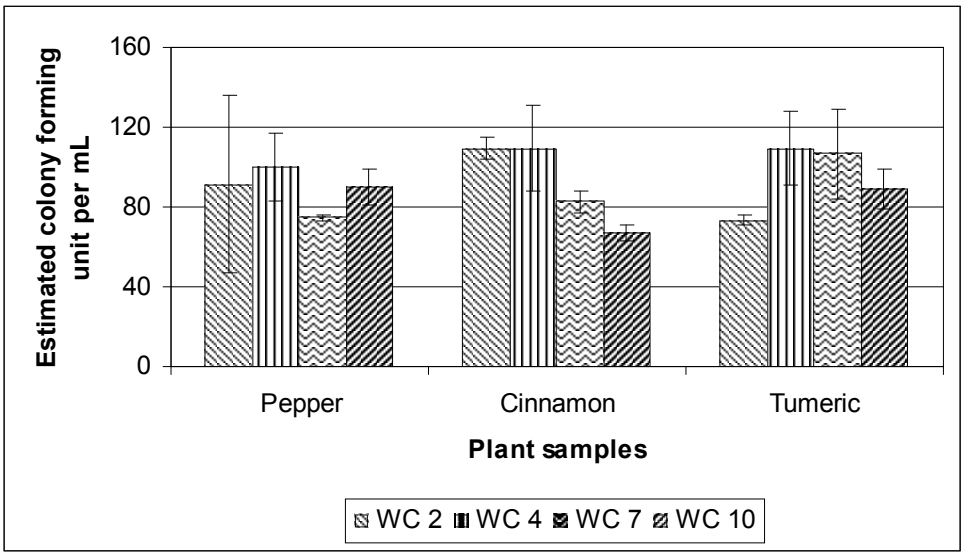

Figure 1. Numbers of fungi isolated from pepper, cinnamon, and tumeric leaves after subjecting them to various washing cycles. Standard error bars are shown. WC = washing cycle.

of bacterial overgrowth, making isolation of a range of slow growing fungi difficult. The developing fungal colonies were counted and the results are expressed as estimated colony forming units per mL (Fig. 1). The number of isolated fungal colonies remained largely unchanged with the number of washing cycles (up to ten tested) in all of the plant samples except cinnamon, which decreased after the seventh wash cycle (Fig. 1). There was suggestive evidence that the number of species was richer at the fourth wash cycle in contrast to the second (Table 1). This perhaps indicates the loosening effect of hydration on mucilaginous exudates associated with hyphae and sporulation structures. The leaves did not fragment noticeably during the process. The data show that fungi (spores and hyphal fragments) can be washed from a leaf surface, but with some apparent difficulty. Washing procedures are most likely to favour the recovery of sporulating fungi and those genera with fragile hyphae. The figures say nothing about relative biomass of the respective isolates and give no information about possible endophytic behaviour.

Fungi isolated from pepper, cinnamon, and tumeric leaves. Twenty-four distinct fungal species were isolated from pepper, cinnamon, and tumeric plants. Methodological changes may have yielded more isolates especially through the use of smaller leaf pieces (Gamboa *et al*. 2002). Some species were isolated infrequently, such as *Bipolaris spicifera* (Bainier) Subramanian, *Colletotrichum orbiculare* (Berk. & Mont.) Bailey & Jeger, *Fusarium* spp., *Nigrospora sphaerica* Aim. and some *Curvularia* species as recorded in Table 1.

The fungal genera most frequently isolated (those recovered with frequencies greater than 10% on more than one occasion) from aromatic plant species by the spread plate method were *Cladosporium*, *Colletotrichum* and an unknown dermatiaceous fungi (pepper); *Aspergillus* and *Cladosporium* (cinnamon); *Cladosporium*, *Nigrospora* and *Phoma* (tumeric). The leaf piece method of isolation most frequently led to the recovery of *Cladosporium* and *Colletotrichum* (pepper); *Cladosporium*, *Helminthosporium*, *Nigrospora* and *Pestalotiopsis* (cinnamon); *Cladosporium*, *Exerohilum*, *Nigrospora, Pestalotiopsis* and *Phoma* (tumeric). Two distinct dematiaceous morphotypes were identified from pepper and one from cinnamon. Tumeric yielded two distinct hyaline, non-sporing morphotypes. Clearly, the method of isolation gives different yield patterns. The leaf piece method recovered mycoflora on and in the leaf, but did not distinguish between these sites. Many species were not recovered by the spread plate method, which assayed for mycoflora growing and sporulating on the leaf surface,

Table 1. Fungal isolation frequencies (%) from leaves of pepper, cinnamon and tumeric. Fungi were isolated using two methods after subjecting them to up to ten washes.

Fungi isolated	**Washing cycle**							
	2	**4**	**7**	**10**	**2**	**4**	**7**	**10**
Pepper	**Spread plate**				**Leaf piece method**			
Cladosporium sp.	67.0	28.9	54.3	14.1	12.5	8.3	8.3	0
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.	0	33.3	33.3	33.3	87.5	95.8	91.7	87.5
C. orbiculare	0	0	0	0	0	0	0	8.3
Curvularia sp.	0	0	0	0	8.3	8.3	4.2	0
Fusarium sp. 1	0	13.2	0	0	0	0	0	0
Fusarium sp. 2	0	0	0	0	4.2	0	0	0
Nigrospora sphaerica	0	0	0	0	4.2	0	4.2	4.2
Pestalotiopsis cf. *versicolor*	0	0	0	0	0	0	8.3	0
Trichoderma sp. 1	0	0	0	0	4.2	0	0	0
Trichoderma sp. 2	0	0	0	0	0	0	8.3	0
Unknown dematiaceous 1	15.8	15.1	54.3	30.1	50.0	58.3	50.0	12.5
Unknown dematiaceous 2	0	0	0	0	8.3	20.8	12.5	12.5
Cinnamon	**Spread plate**				**Leaf piece method**			
Aspergillus sp.	25.5	30.9	26.3	11.1	8.3	0	0	4.2
Cladosporium sp.	14.7	41.8	37.4	55.5	12.5	0	12.5	12.5
Curvularia sp.	0	0	0	0	0	4.2	0	0
Curvularia inaequalis (Shear) Boedijn	0	0	13.6	0	0	0	0	0
Helminthosporium sp.	0	0	0	0	16.7	12.5	25	20.8
Nigrospora sp.	0	0	0	0	29.2	25	33.3	16.7
Pestalotiopsis cf. *versicolor* (Speg.) Steyaert	0	0	0	16.7	100	100	100	100
Rhizopus sp.	0	0	0	0	4.2	0	0	0
Trichoderma sp.	0	0	0	0	8.3	0	20.8	0
Unknown dematiaceous	0	0	0	0	4.2	0	0	0
Tumeric	**Spread plate**				**Leaf piece method**			
Bipolaris spicifera	0	0	0	0	0	0	0	4.2
Cladosporium sp.	30.8	61.7	61.4	60.3	54.2	41.7	50.0	41.7
Curvularia sp.	0	0	0	0	0	0	8.3	0
Exserohilum macginnisii A.A. Padhye & Ajello	0	0	0	0	4.2	12.5	8.3	20.8
Nigrospora sp.	38.8	14.7	27.9	12.5	25.0	25.0	29.2	12.5
Paecilomyces sp.	0	0	0	0	4.2	0	4.2	0
Pestalotiopsis sp.	0	0	0	0	37.5	29.2	33.3	62.5
Phoma sp.	0	16.7	0	16.7	91.7	95.8	83.8	95.8
Rhizopus sp.	0	0	0	0	8.3	8.3	12.5	4.2
Trichoderma sp.	0	0	0	0	0	8.3	20.8	8.3
Unknown hyaline 1	0	0	0	0	12.5	0	20.8	4.2
Unknown hyaline 2	0	0	0	0	0	4.2	0	4.2

for example, *Curvularia*, *Nigrospora*, *Pestalotiopsis* and *Trichoderma* from pepper, *Helminthosporium, Nigrospora*, *Rhizopus*, *Trichoderma* and an unknown dematiaceous fungus from cinnamon and *Bipolaris*, *Curvularia*, *Exersohilum*, *Paecilomyces*, *Pestalotiopsis*, *Rhizopus*, *Trichoderma* and two unknown hyaline fungi from tumeric. Twenty-two of the twenty-five species provisionally identified were recovered by the leaf piece method. There is no reason to imagine that either *Rhizopus* or *Trichoderma* were contaminants as the leaf pieces were plated after the washing procedure was finalized under biohazard cabinet conditions. The wash aliquots effectively acted as a control and did not contain evidence of the presence of either genus. In support of the proposal that at least one genus may be a phyllosphere fungus on tumeric is the report from Japan that *Trichoderma* was recovered consistently on leaves of *Swida controversa* (Hemsley) Sojak

as an epiphyte during selected portions of the growing season (Osono & Mori 2005). It has been reported with less regularity as an endophyte from *Madhuka nerifolia* (Moon) H. J. Lam. (Raviraja *et al*. 2006).

Members of the genera *Colletotrichum*, *Pestalotiopsis*, and *Phoma* dominated the leaf piece isolations from pepper, cinnamon and tumeric, respectively. They are common pathogens (Barnett & Hunter 1972; Agrios 2005). Other fungal genera isolated, such as *Cladosporium*, *Curvularia*, *Fusarium* and *Helminthosporium,* have been reported previously to be the most common resident fungi isolated from medicinal plants under field conditions (Aziz *et al*. 1998). *Trichoderma* is recognized as a successful saprophytic fungus, common in soil, besides being reported as a parasite on other fungi (Barnett & Hunter 1972; Agrios 2005). However, it has been claimed that it may grow epiphytically or endophytically (Osono & Mori 2005; Raviraja *et al*. 2006). *Aspergillus* and *Rhizopus* are recognized as the most common contaminant fungi of stored plant materials (Aziz *et al*. 1998) and as soil inhabitants.

The fungi *C. gloeosporioides*, *Pestalotiopsis* sp., and *Phoma* sp. are presumed as a major cause of the disease on pepper, cinnamon, and tumeric on account of their frequent occurrence and their known behaviour on other host plants. In separate tests the pathogenicity of *C. gloeosporioides* was confirmed on pepper. Potentially some of the fungi isolated may have been endophytes but this point was not clarified. A range of genera have been suggested in this category including a number of *Alternaria*, *Cladosporium*, *Curvularia, Fusarium*, *Nigrospora* and *Trichoderma* isolates (Raviraja *et al*. 2006; White & Backhouse 2006; Kim *et al*. 2007). However, very few isolates have been established with certainty as being endophytes according to the definition adopted in this paper (unapparent and asymptomatic). It is assumed commonly that if fungi grow after the application of a surface sterilization schedule then they are endophytic. This does not follow since refuge may be found on the leaf enabling survival. More rigorous testing after the style of Mucciarelli (2002) is required before a microorganism may be classified with confidence as an endophyte.

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