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## Evidence for the role of phytophagous insects in dispersal of non-grass fungal endophytes

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Fungal endophytes were isolated from the leaves of *Calotropis gigantea* (milkweed). *Colletotrichum gloeosporioides* was most frequently isolated over the 24 month study period as an endophyte and it contributed to about 45% of the total number of endophyte isolates obtained. *Poeciloceris pictus* (painted grasshopper) which feeds on the leaves of milkweed neither avoided nor preferred milkweed leaves coated with a spore suspension of *C. gloeosporioides*. Three of the endophytes tested passed through the gut of the insect without being digested and retained their viability. This suggests that phytophagous insects could serve as an agent for the dispersal of non-grass fungal endophytes in plant communities such as tropical forests.

**Key words:** fungal endophytes, endophyte transmission, phytophagous insects

### Introduction

Non-clavicipitaceous endophytes cause discrete and symptomless infections in the aerial tissues of plants and survive within these tissues at least for part of their life cycle. Unlike the clavicipitaceous endophytes that are restricted to grasses and are often transmitted vertically, the non-clavicipitaceous endophytes infect a broad range of plant hosts belonging to disparate groups and families and are transmitted horizontally. These endophytes are taxonomically restricted to the ascomycetes and their anamorphs and span several ecological functional groups such as mutualists (Redman *et al.*, 2002; Arnold *et al.*, 2003), commensals (Deckert *et al.*, 2001) and latent pathogens (Sinclair and Cerkauskas, 1996; Photita *et al.*, 2004, 2005; Gonthier *et al.*, 2006; Suryanarayanan and Murali, 2006).

Recent studies have shown that the leaves of tropical plants are densely colonized by endophytes although their species richness varies with the type of the tropical plant communities (Arnold *et al.*, 2003; Suryanarayanan *et al.*,

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2003; Kumar and Hyde, 2004; Promputtha *et al.*, 2005; Wang *et al.*, 2005). Compared to the data that have accumulated on the biology of clavicipitaceous endophytes, the information that we have on non-clavicipitaceous endophytes is less. There are also very few experimental studies on the role phytophagous insects in the dissemination of such endophytes. Here, we discuss the possible role of the Painted grasshopper (*Poeciloceris pictus*) in passively aiding the dispersal of foliar fungal endophytes of milkweed (*Calotropis gigantea*). This plant belongs to the family *Asclepiadaceae*; it is a large shrub found throughout India in dry, waste grounds. All parts of this plant are used as medicine in the indigenous system of medicine, the Ayurveda (Warrier *et al.*, 1994). *Poeciloceris pictus* is one of the many insects associated with *Calotropis gigantea*. This grasshopper feeds on the leaves of milkweed and, at times, entirely defoliates the plant.

## **Materials and methods**

### ***Sampling of leaves for endophytes***

Mature, healthy leaves of *Calotropis gigantea* plants growing in the city of Chennai were screened for endophyte presence on a monthly basis for 24 consecutive months (January 2001-December 2002). Every month, 40 leaves were collected, washed thoroughly in running tap water, and from each leaf, three segments (0.5 cm<sup>2</sup>) were cut from the apical, middle and basal portions of the midrib and screened.

### ***Surface sterilization and isolation of endophytes***

The 120 leaf segments obtained each month were surface sterilized by dipping them in 70% ethanol for 5 seconds, followed by treatment in sodium hypochlorite (4% available chlorine) for 60 seconds and finally rinsed in sterile water for 10 seconds (Dobranic *et al.*, 1995). Of these, 100 surface sterilized segments were plated on Potato Dextrose Agar medium contained in Petri dishes. Ten segments were placed equidistantly in each Petri dish containing 20 ml of the medium amended with chloramphenicol (150 mg/L<sup>-1</sup>). The Petri dishes were sealed with Parafilm<sup>TM</sup> and incubated in a light chamber for 21 days at 26°C (Bills and Polishook, 1992). The light regime provided was 12 hours light: 12 hours darkness from cool white, daylight fluorescent lamps (Suryanarayanan *et al.*, 1998). The colonization frequency (CF%) was calculated following the method of Hata and Futai (1995).

***Testing the ability of grasshopper to transmit endophytes***

Adult insects collected from *Calotropis gigantea* plants growing in the city of Chennai, were kept in sterile containers and starved for 48 hours. Batches of insects kept in separate chambers were then fed with surface sterilized leaves of *C. gigantea* (control) or surface sterilized leaves coated with a spore suspension of *Colletotrichum gloeosporioides* (the most frequently isolated endophyte in the present study) or *Chaetomium* sp. isolated previously as an endophyte from the leaves of *C. gigantea*. Leaves coated with a cell suspension of a yeast strain (AS 8) isolated as an endophyte from the leaves of *C. gigantea* were also used as feed. Both *Chaetomium* sp. and AS 8 were isolated infrequently as endophytes from milkweed leaves. A 0.5 ml of a spore suspension containing  $10^6$  spores/ml of the fungi or yeast cells was applied on the upper surface of a mature, healthy and apparently symptomless leaf of *C. gigantea* and used as feed. Surface sterilized leaves without fungal coatings served as a control. The faecal pellets of these insects were collected separately and processed in two ways. In the first method (direct plating method), ten pellets for each treatment were inoculated directly on PDA medium amended with Chloramphenicol. In the second method (maceration method), 10 faecal pellets were macerated using a sterile glass rod in 10 ml of sterile water and 1 ml of the macerate was decanted into a Petri dish to which warm PDA medium (with Chloramphenicol) was added. The Petri dishes were sealed with Parafilm and incubated in a light chamber at 26 °C for 21 days.

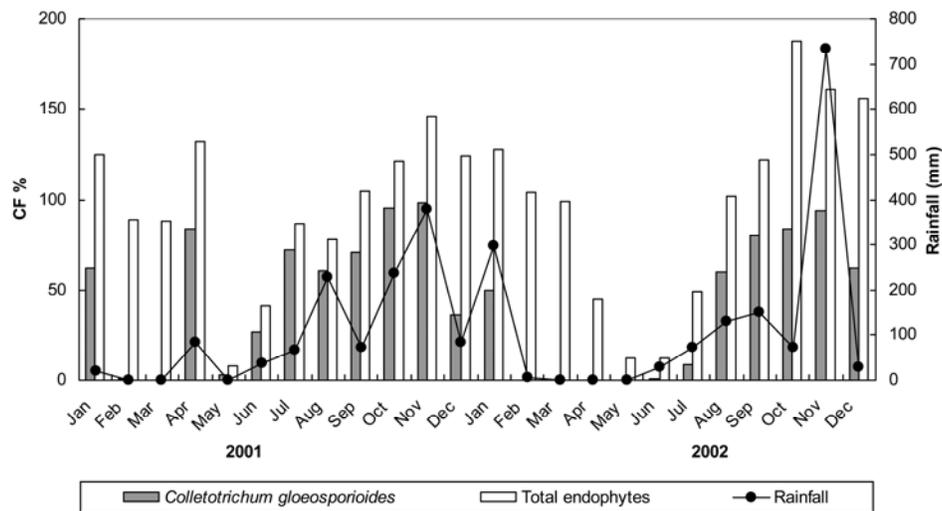
***Test for endophyte preference by insects***

Preference for endophyte-treated leaf tissue by grasshoppers in their diet was tested as follows using one of the endophyte species. Thoroughly washed leaves of *C. gigantea* were cut into square or circular segments (4 cm<sup>2</sup>). One-hundred and twenty circular leaf segments dipped in a conidial suspension of endophytic *Colletotrichum gloeosporioides* were presented to 10 insects that have been starved for 48 hours. Treated segments were randomly placed in the insect chamber along with 120 untreated square segments of similar size that served as a control. Alternatively, square segments were treated with fungal cells and circular segments were provided as control feed. The number of leaf segments consumed was counted after 48 hours of incubation.

## Results and discussion

Although studies on endophytes of woody plants have confirmed that these fungi could be extremely diverse (Arnold *et al.*, 2000), only a few recent investigations have looked at the ecology of these fungi and their association with their hosts (Fröhlich and Hyde, 1999; Kumaresan and Suryanarayanan, 2002; Arnold and Herre, 2003; Suryanarayanan and Thennarasan, 2004). In the present study, we found that the CF% of the endophytes increased with precipitation (Fig. 1). Such a positive correlation between endophyte infection and precipitation has been observed for a few hosts (Rodrigues, 1994; Wilson and Carroll, 1994; Suryanarayanan *et al.*, 1998). This could be a result of enhanced dispersal and germination of fungal spores favoured by precipitation (Wilson, 2000). Although there was an appreciable increase in the number of endophyte isolates that were obtained from *C. gigantea* leaves sampled during the wet season, this increase was mainly due to increased colonization by the dominant *Colletotrichum gloeosporioides* (Fig. 1). This indicated that though the wet season was favourable for endophyte infection, the leaves recruited no new species. *Colletotrichum* species are generalists among endophyte genera and colonize many plant hosts (Brown *et al.*, 1998; Kumar and Hyde, 2004). Further studies on the endophyte status of tropical plant communities during different seasons should improve our knowledge, especially since wet tropical forests are hyperdiverse with reference to endophytes (Arnold *et al.*, 2000).

Very few studies have looked into the actual source of inoculum and spread of foliar endophytes of woody plants. They are believed to be transmitted horizontally by spore dispersal (Bayman *et al.*, 1998; Arnold and Herre, 2003; Fröhlich *et al.*, 2000). Rainfall has been suggested to be the major factor involved in the dispersal of the propagules of many of these endophytes (Wilson, 2000). Wilson (2000) demonstrated that endophyte infections were reduced substantially or totally lacking in leaves that were shielded experimentally from rainfall. Since non-grass foliar endophytic fungi are also encountered commonly on leaf and wood litter (Van Bael *et al.*, 2005), it is generally assumed that the endophytes fruit on such senesced tissues (Wilson, 2000). Consequently, such litter could be a source of endophyte inoculum (Stone, 1987; Carroll, 1995). Although insects are thought to aid in the dispersal of endophytes (Wilson, 2000; Van Bael *et al.*, 2005), this aspect has not been approached experimentally. In the present study, the foliar endophytes passed through the gut of grasshoppers without being digested and retained the capacity to grow. More colonies of an endophyte were observed on agar medium seeded with the faecal pellets of insects that had fed on that particular endophyte-coated leaves (Table 1).



**Fig. 1.** CF (%) of endophytes and *Colletotrichum gloeosporioides* isolated during a 24 month study period.

There are a few cases of non-clavicipitaceous endophytes functioning as insect antagonists and offering natural protection to their hosts (Webber, 1981; Carroll, 1991; Wilson, 2000). However, Faeth (2002) contends that very few of the horizontally transmitted endophytes should function as defensive mutualists in deterring insect herbivory because the insects may be involved in the dissemination of endophytes; thus, selection for insect tolerance or facilitation of herbivory is in order rather than for insect resistance (Faeth and Hammon, 1997). In the present study, the grasshoppers did not show any avoidance of endophytes as they fed equally on both endophyte-coated and control leaf segments when presented together as feed. Monk and Samuels (1990) isolated several saprobic and plant pathogenic fungi from the faecal pellets of grasshoppers of Indo-Malayan rainforests. They also isolated anamorphic states of *Xylaria*, a common endophyte of dicotyledonous plants and concluded that grasshoppers are involved in the transmission of endophytes.

Isolation of common endophytic fungi from insect faeces is however, not conclusive proof for insect transmission of such fungi especially since many of them, including anamorphs of *Xylaria* spp. also occur as saprobes. We now show that grasshoppers aid in the dispersal of non-clavicipitaceous endophytic fungi. It is likely that other phytophagous insects also are passive transmitters of endophytic fungi in tropical plant communities. Insect folivory could influence positively the diversity of foliar endophytes by way of injury-assisted endophyte colonization (Arnold as cited in Van Bael *et al.*, 2005). Our results

show that phytophagous insects may also aid in the transmission of endophytes by increasing the inoculum load due to their faecal pellets serving as organic nutrient source for the fungi to sporulate. Similar results on insect transmission of endophytic fungi have also been obtained by Stan Faeth, Arizona State University, USA (personal communication). Detailed studies could provide data on the distribution of endophytic fungi in tropical plant communities.

**Table 1.** Colonization frequency (%) of endophytes isolated from the faecal pellets of *P. pictus* fed with surface sterilized leaves of *C. gigantea* or surface sterilized leaves coated with spores/cells of its foliar endophytes.

Endophyte	I		II		III		IV	
	D	M	D	M	D	M	D	M
<i>Alternaria</i> sp. 1	1						1	
<i>Aspergillus flavus</i>							3	
<i>Aspergillus niger</i>							1	
<i>Aspergillus</i> sp. 6			5	3	3	1	1	
<i>Chaetomium</i> sp.					12	22		
<i>Cladosporium</i> sp. 1	1							11
<i>Colletotrichum gloeosporioides</i>			11	64				
<i>Penicillium</i> sp. 1	1	1	3					
<i>Phoma</i> sp.	1							
<i>Sporormiella intermedia</i>	1							
AS 128		1						
AS 8	13	+		+		+	14	+
AS 9	3	+						+
AS 10	1							

D-Direct plating; M -Macerate; + - Present

I-surface sterilized leaves; II-surface sterilized leaves coated with *Colletotrichum gloeosporioides* conidia; III-surface sterilized leaves coated with *Chaetomium* sp. spores; IV-surface sterilized leaves coated with yeast (AS 8) cells.

Dominant and ubiquitous endophytic fungi such as species of *Colletotrichum* (Wright *et al.*, 1997; Lu *et al.*, 2004), *Pestalotiopsis* (Jeewon *et al.*, 2004) and *Phyllosticta* (Baayen *et al.*, 2002; Pandey *et al.*, 2003; Okane *et al.*, 2003) are generalists with a wide host range. Furthermore, Novotny *et al.* (2002) contend that most phytophagous insects of tropics are generalists. It would thus be worthwhile to address the question whether herbivory and fungal resistance to digestion by insects together shaped the evolution of generalists among non-clavicipitaceous endophytes and phytophagous insects.

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