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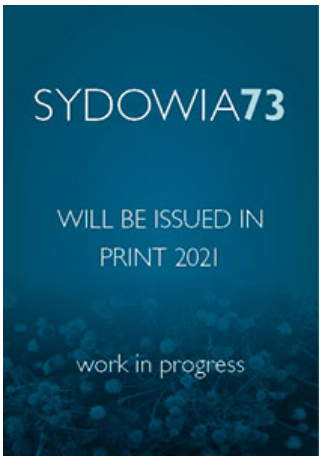
There are very few studies on endophyte communities of freshly consumed leaves. This is vital as some endophytes which continue to survive in detached leaves belong to mycotoxin producing genera. We studied the endophyte community of betel leaf, which is eaten fresh by a huge population, especially in Asia. Young and mature leaves of betel vine were screened for their endophyte assemblage. In addition, mature leaves collected during dry and wet seasons and those stored in the market were studied for endophyte presence. The entire leaf was infected with endophytes. Though endophyte colonization frequency (CF %) increased with leaf age, their species composition did not vary. The CF % of most of the endophytes was low (0.7 %–8.7 %) while that of Colletotrichum sp. 1 was high irrespective of leaf age or season (46 %–78.67 %). The CF % of Fusarium pallidoroseum increased with leaf age and storage period; the presence of F. mangiferae increased with storage time. The two Fusarium endophytes isolated from betel leaf produce an array of mycotoxins including beauvericin, enniatin, nivalenol, fusarenon X, equisetin, HT2 toxin, diacetoxyscirpenol and neosolaniol. This study underscores the importance of investigating the endophyte communities of leafy vegetables, especially those having relatively longer shelf life.

Keywords: Fusarium mangiferae, Fusarium pallidoroseum.



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Fungal endophytes of betel leaves: the need to study mycotoxin-producing endophytes in leafy vegetables

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There are very few studies on endophyte communities of freshly consumed leaves. This is vital as some endophytes which continue to survive in detached leaves belong to mycotoxin producing genera. We studied the endophyte community of betel leaf, which is eaten fresh by a huge population, especially in Asia. Young and mature leaves of betel vine were screened for their endophyte assemblage. In addition, mature leaves collected during dry and wet seasons and those stored in the market were studied for endophyte presence. The entire leaf was infected with endophytes. Though endophyte colonization frequency (CF %) increased with leaf age, their species composition did not vary. The CF % of most of the endophytes was low (0.7 %–8.7 %) while that of *Colletotrichum* sp. 1 was high irrespective of leaf age or season (46 %–78.67 %). The CF % of *Fusarium pallidoroseum* increased with leaf age and storage period; the presence of *F. mangiferae* increased with storage time. The two *Fusarium* endophytes isolated from betel leaf produce an array of mycotoxins including beauvericin, enniatin, nivalenol, fusarenon X, equisetin, HT2 toxin, diacetoxyscirpenol and neosolaniol. This study underscores the importance of investigating the endophyte communities of leafy vegetables, especially those having relatively longer shelf life.

Keywords: *Fusarium mangiferae*, *Fusarium pallidoroseum*.

Endophytes (bacteria and fungi), a constant component of the plant microbiome, are nonpathogenic and survive in living tissues of plants. There are many study addressing the ecology (Govinda Rajulu et al. 2013, Rajamani et al. 2018), diversity (Gazis & Chaverri 2010, Suryanarayanan et al. 2018), and application potential (Schulz et al. 2002, Suryanarayanan et al. 2009, Paranetharan et al. 2018) of fungal endophytes residing in the leaves of terrestrial plants. However, there are very few studies on fungal endophytes of green leafy vegetables or edible fresh leaves (Kumaresan et al. 2013). Our earlier study showed that *Fusarium mangiferae* and *F. pallidoroseum* endophytes isolated from betel leaves produce large amounts of several mycotoxins (Thirumalai et al. 2013). To evaluate the health risk posed by mycotoxins, fungal endophyte communities of young, mature and stored betel leaves were determined to discern the presence of potential mycotoxin producers.

Betel (*Piper betle* L.) is a tropical, perennial, evergreen climber propagated asexually for harvesting its leaves. The fresh, green leaves of betel vine are consumed (usually along with betel nut and quicklime) on a daily basis by nearly 600 million people of Bangladesh, China, India, Indonesia, Malaysia, Myanmar, Nepal, Pakistan, Philippines, South Africa, Sri Lanka, and Thailand (Sharma et al. 1996, Kumar et al. 2010). India supports nearly 55000 ha of betel cultivation (Das et al. 2016). Betel leaf is known for its medicinal properties which include anti-bacterial, anti-fungal, anti-inflammatory, hepatoprotective, anti-cancer and anti-leishmanial effects (Nadkarni & Nadkarnai 2007, Misra et al. 2009, Kumar et al. 2010, Paranjpe et al. 2013). Furthermore, betel leaf chewing is considered as an effective method of drug delivery through buccal mucosa (Shojaei 1998). Betel quid chewing (betel leaf, areca nut and slaked lime – with or without tobacco) alters the oral microbiome (Hernandez et al. 2017) and excessive consumption, even without tobacco, can be a cause of oral cancer (Amarasinghe et al. 2010, Guha et al. 2014).

Mycotoxins are secondary metabolites produced by several fungi and exhibit mutagenic, carcinogenic, or teratogenic effects (Omotayo et al al. 2019). They pose a great health safety risk in developing countries (Shephard 2008). Considering the health risk posed by mycotoxins, fungal endophyte communities of young, mature stored betel leaves were

determined to primarily discern the fate of mycotoxin producing endophytes in them. To our knowledge, this is the first investigation on fungal endophytes of betel leaf.

Materials and methods

Fresh, symptomless leaves from two-year-old vines (Sirugamani 1 variety) cultivated near the town of Thiruvannamalai (Lat. 12.2, Long. 79.10), Tamilnadu, southern India were processed within 24 h of collection. Sixty young (6–10 day old, pale green) and mature (20–25 day old, dark green) leaves from sixty individual plants were studied for their fungal endophyte assemblage. Furthermore, mature leaves collected during the wet and dry season as well as leaves stored for 2, 4, or 6 days in the market for selling were screened for endophytes.

The leaves were washed in sterile water and from each leaf, three segments (0.5 cm^2 each) were cut from the apical, middle, and basal portion. The segments were surface sterilized by dipping them in 70 % ethanol for 5 s, followed by treatment with NaOCl (4 %) for 90 s and rinsed in sterile distilled water for 10 s (Suryanarayanan et al. 1998). Of these, 150 segments were plated on Potato Dextrose Agar (PDA) medium amended with Chloramphenicol (150 mg/l) to isolate endophytes. The effectiveness of surface sterilization was confirmed as follows (Schulz et al. 1998): the surface sterilized tissue segments were gently pressed on to the isolation medium in a Petri dish, removed and the Petri dishes were incubated and observed for fungal growth. The absence of any fungal growth of any fungi confirmed the effectiveness of the procedure.

To induce sporulation in the fungi to aid identification, the Petri dishes were incubated in a light chamber fitted with daylight fluorescent lamps. They were exposed to 2200 Lux of light (12 h : 12 h Light : Dark) for 21 days (Suryanarayanan 1992). The fungi growing out of the tissue segments were identified based on culture characteristics. The colonization frequency (CF%) of an endophyte species in the leaf was calculated as follows: CF %= (N_{col}/N_{tot}) x 100, where, N_{col} and N_{tot} are the number of segments colonized by each endophyte and the total number of segments observed respectively (Hata & Futai 1995).

Representative leaf sampling of leaf is usually recommended to study foliar endophytes. However, since the entire betel leaf is chewed, we decided to study the distribution of endophytes in the entire leaf blade. This was done by recording endophytes which grew out in the isolation medium from successive larger segments of a mature leaf (Suryanarayanan et al. 2002). Applying the nested series of expanding quadrat method to the data obtained by this method (Condit et al. 1996), the species area curves were constructed (Suryanarayanan et al. 2002). A coefficient of similarity was also calculated (Carroll & Carroll 1978) for determining the overlap of the endophyte assemblage after 6 days of leaf storage. All cultures are preserved in distilled water (Ellis 1979, Suryanarayanan 2019) in VINSTROM's culture collection. In additon, *Fusarium pallidoroseum* NFCCI (Accession No: 3031) and *Fusarium mangiferae* NFCCI (Accession No: 3032) were deposited in the National Fungal Culture Collection of India, Pune.

Results

Screening tissue segments of an entire young or mature leaf collected during the wet or dry season indicated the entire lamina was uniformly infected by 12 to 16 endophytic fungi (Fig. 1). A comparison of the endophyte status of young and mature leaves showed that although the total colonization frequency (CF %) of endophytes was higher in old leaves, the species composition of endophytes did not vary with leaf age (Tab. 1). *Alternaria* sp., *Cercospora* sp., *Colletotrichum* sp. 1, *Colletotrichum* sp. 2, *Corynespora* sp., *Drechslera* sp., *Fusarium pallidoroseum*, *Glomerella* sp., *Humicola* sp. and *Lasiodiplodia theobromae* which were absent or present in low CF % in young leaves were present or exhibited a higher CF % in old leaves (Tab. 1). The CF% of most of the endophytes was low while that of only one species viz. *Colletotrichum* sp. 1 was high irrespective of the leaf's age or season of sampling (Tab. 1). The CF % for the leaf was higher in the dry

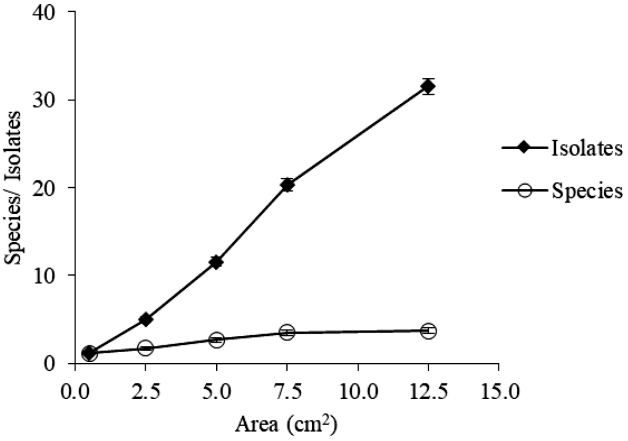

Fig. 1. Species area curve for foliar endophytes of *Piper betle* (Readings are mean of fifteen observations obtained from three leaves. Bars show standard error)

Tab. 1. Colonization Frequency (CF %) of fungal endophytes isolated from leaves of *Piper betle* sampled during the wet and dry seasons.

Endophyte	Wet		Dry
	Young leaf	Mature leaf	Mature leaf
Alternaria sp.	0.0	3.3	4.0
Cercospora sp.	1.3	12.0	7.3
Cladosporium sp.	0.0	0.0	2.7
Colletotrichum sp. 1	63.3	68.7	46.0
Colletotrichum sp. 2	0.0	2.7	5.3
Colletotrichum sp. 3	0.0	0.0	0.7
Corynespora sp.	0.0	0.7	2.0
Curvularia lunata	0.0	0.0	7.3
Drechslera sp.	0.7	6.0	1.3
Fusarium pallidoroseum	2.7	8.7	34.0
Fusarium mangiferae	1.3	1.3	0.0
Glomerella sp.	0.7	1.3	0.0
Guignardia sp.	1.3	0.0	0.0
Humicola sp.	0.0	4.0	2.7
Lasiodiplodia theobromae	0.7	4.7	0.7
Nigrospora oryzae	0.0	0.0	5.3
Nodulisporium sp.	1.3	0.0	1.3
Paecilomyces sp.	0.7	0.0	0.0
Phoma sp.	1.3	0.7	0.0
Phomopsis sp.	0.0	0.0	3.3
Phyllosticta capitalensis	1.3	1.3	0.7
Total No. of species	12	13	16
Total CF %	76.6	115.4	124.6

(124) than in the wet season (115). *Cladosporium* sp., *Colletotrichum* sp. 3, *Curvularia lunata*, *Nigrospora oryzae* and *Phomopsis* sp. were present as endophytes only in the leaf collected during the dry season. *Fusarium mangiferae*, *Glomerella* sp. and *Phoma* sp. were present as endophytes in mature leaves of the wet season were absent in the dry season. The CF % of *Alternaria* sp., *Corynespora* sp. and *Paecilomyces* sp. decreased with storage period while that of *Colletotrichum* sp.1, *F. pallidoroseum*, *F. mangiferae*, *Humicola* sp. and *Nodulisporium* sp. increased with storage time (Tab. 2).

Discussion

Foliar endophytes are known to survive as saprotrophs in detached leaves (Korkama-Rajala et al. 2008, Vo íšková & Baldrian 2013, Reddy et al. 2016). It was essential to know if our procedure of sampling the tip, middle and the basal portion of the leaf represented the entire endophyte assemblage of an entire leaf. A species area curve confirmed that the sampling was adequate since with increasing leaf area, only the number of endophyte isolates but not of the species increased (Fig. 1). The colonization of betel leaf by endophytes was uneven as one fungus viz. *Colletotrichum* sp. 1 was dominant and the rest of the species showed low frequency of colonization (Tab. 1). Such a trend has been observed for foliar endophytes of many tropical plant species (Suryanarayanan et al. 2018); culture based studies have shown that *Colletotrichum*, among a few other genera, colonizes a wide range of tropical plants as foliar endophyte (Suryanarayanan et al. 2011, Vaz et al. 2018). Since *Colletotrichum* spp. cause anthracnose of betel vine (Maiti & Sen 1979) and since pathogenic fungi are known to survive as symptomless endophytes in plants (Suryanarayanan & Murali 2006), it would be of interest to know if any of the *Colletotrichum* species carried by betel leaf as endophyte is a latent pathogen. The increased colonization (CF %) by endophytes in older leaves is consistent with earlier findings that aged leaves carry more endophytes when compared to young

Tab. 2. CF % of fungal endophytes in stored mature leaves of *Piper betle* (sampled during wet season).

Endophyte	Days of storage			
	0 day	2 days	4 days	6 days
Alternaria sp.	4.67	2.67	2.67	2.67
Cercospora sp.	9.33	7.33	7.33	9.33
Cladosporium sp.	0.67	0.67	0.0	0.0
Colletotrichum sp. 1	63.33	68.67	74.67	78.67
Colletotrichum sp. 2	1.33	0.0	0.0	0.0
Colletotrichum sp. 3	1.33	0.0	0.0	0.0
Corynespora sp.	12.00	2.67	2.67	2.00
Curvularia lunata	1.33	0.0	0.0	0.0
Drechslera sp.	5.33	2.00	3.33	1.33
Fusarium pallidoroseum	5.33	7.33	10.67	14.67
Fusarium mangiferae	2.67	0.0	3.33	5.33
Guignardia sp.	0.0	0.0	1.33	0.0
Humicola sp.	1.33	4.00	3.33	5.33
Lasiodiplodia theobromae	2.67	6.00	2.67	2.00
Nigrospora oryzae	0.0	5.33	0.0	2.67
Nodulisporium sp.	0.67	0.67	2.00	2.67
Paecilomyces sp.	4.00	1.33	1.33	0.0
Phomopsis sp.	8.67	1.33	2.00	2.00
Phyllosticta capitalensis	1.33	0.0	1.33	0.0
Total No. of species	17	13	14	12
Total CF%	126.0	110.0	118.6	128.6

leaves (Taylor et al. 1999, Suryanarayanan & Thennarasan 2004) owing to their larger surface area and longer exposure to endophyte inoculum (Carroll et al. 1977, Bertoni & Cabral 1988). Although more endophytes were isolated from mature leaves, the absence of more endophyte species in mature leaves compared to young leaves, as well as the presence of fewer endophyte species in it even during the wet season which favors fungal spore dispersal and infection (Bahnweg et al. 2005, Suryanarayanan et al. 2011) is intriguing. Furthermore, the overlap between the endophyte assemblage of excised leaves after 0 and 6 days of storage as measured by a coefficient of similarity index was as high as 69 % suggesting that excised leaf resisted colonization by other saprotrophic fungi. This view is not untenable as betel leaves are rich in antifungal chemicals (Guha 2006), which are known to inhibit more than 100 species of fungi (Guha 2006, Ali et al. 2010). Such antifungal compounds in the leaves could favour antibiotic resistant species. Resident endophytes could also inhibit infection by other fungal species by producing antifungal compounds (Wicklow et al. 2005, Mohandoss & Suryanarayanan 2009).

Although freshly consumed leaves of betel have been studied for its *Salmonella* and *E. coli* contaminations (McLauchlin et al. 2018), there is no information on their fungal endophyte status. We reported earlier that the betel leaf endophytes *Fusarium mangiferae* produces large amounts of nivalenol, fusarenon X and equisetin and *F. pallidoroseum* elaborates large amounts of beauvericin and trace amounts of enniatin (Thirumalai et al. 2013). In the present study, we observed that stored betel leaf which is sold in the market for direct consumption continued to support some of its mycotoxin producing endophytes such as *Alternaria* (Chagas et al. 2016) and *Fusarium* (Brown et al. 2008); the CF % of *F. pallidoroseum* and *F. mangiferae* in it increased suggesting that these fungi grow under storage conditions. Species of *Fusarium* are known to produce several mycotoxins which include both 'traditional' 'emerging' mycotoxins. The former are those which 'have been assessed and monitored in relative depth' (Schaarschmidt & Fauhl-Hassek 2019) and the latter, which includes beauvericin, are 'neither routinely determined, nor legislatively regulated' though 'evidence of their incidence is rapidly increasing' (Vaclavikova et al. 2013).

Mycotoxins pose risks to human and animal health (Schatzmayr & Streit 2013, Malachova et al. 2014). Though we did not attempt to quantify the mycotoxins in our basic study, it underscores the importance of investigating the endophyte community of leafy vegetables, especially those having relatively longer shelf life, for their mycotoxin production. Though low mycotoxin levels in food is permissible, it is important to note that climate change is predicted to increase significantly mycotoxin production by fungi (Medina et al. 2015). Since some mycotoxins are antimicrobial (Wang & Xu 2012), and since the interaction between fungi could alter mycotoxin levels (Combès et al. 2012), more detailed studies are needed to know if mycotoxins influence endophyte composition in betel leaves. Betel leaves are rich in various secondary metabolites such as volatile compounds, organic acids, alkaloids, steroids, fatty acids, phenols and terpenes (Valentão et al. 2010); since endophyte infection could induce novel secondary metabolites in leaves (Hartley et al. 2015), it would be of further interest to study the influence of endophyte presence on the secondary metabolite spectrum of betel leaves.

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