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Fungal endophyte diversity and community patterns in healthy and yellowing leaves of *Citrus limon*

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ARTICLE INFO

Article history:
Received 1 August 2012
Revision received 2 January 2013
Accepted 23 January 2013
Available online 4 April 2013
Corresponding editor:
James White Jnr

Keywords:
Chlorosis
Citrus diseases
Colletotrichum gloeosporioides
Competitive antagonism
Foliar endophytes
ITS rDNA
Mycosphaerella
Non-pathogenic
Tropics

ABSTRACT

Fungal endophytes on citrus plants have been little studied, and the effects of citrus diseases on their incidence and diversity have not been addressed. In this study, we examined the foliar fungal endophytes of *Citrus limon* in the vicinity of Yaoundé, Cameroon, with emphasis on the differences between endophyte communities in healthy and yellowing leaves. From 82.3 % of the 480 leaf fragments, a total of 482 isolates were recovered and analysis of *ITS* sequences revealed 20 phylotypes. All fungal endophytes were ascomycetes and, except for one species, were common plant pathogens. *Mycosphaerella* and its anamorphs (34.2 % of all isolates), and *Colletotrichum gloeosporioides* (50.4 % of all isolates), were isolated most frequently. Mycosphaerellaceous species dominated in healthy leaves, and were absent from yellowing leaves. *C. gloeosporioides* was isolated significantly more frequently from yellowing than healthy leaves. Yellowing leaves had a significantly higher overall infection frequency but, in contrast, the least species diversity. Difference in the endophyte assemblages of healthy and yellowing leaves suggests that yellowing of leaves may facilitate the incidence of certain endophytes and impose growth inhibition on others.

Introduction

Citrus spp. are often affected by abiotic and biotic diseases causing heavy losses in fruit yields (Sagaram *et al.* 2009; Glienke *et al.* 2011; Wang *et al.* 2012). Biotic diseases are associated with different organisms, including fungi such as zygomycetes, ascomycetes and basidiomycetes (Wright 1998; Baayen *et al.* 2002; Glienke-Blanco *et al.* 2002; Pretorius *et al.* 2003; Wulandari *et al.* 2009; Glienke *et al.* 2011; Wang *et al.* 2012). Leaf spots and chlorosis are often symptoms of diseased citrus plants, the latter characterizing pathogenic as well as non-pathogenic diseases (Teixeira *et al.* 2005). One of the most widespread and devastating diseases is Huanglongbing (HBL), which is associated with the bacterium *Candidatus liberibacter* (Bastianel *et al.* 2005; Teixeira *et al.* 2005). Salient symptoms on HLB-infected plants include blotchy mottling and vein yellowing of all or part of the leaves (Sijam *et al.* 2008). However, abiotic stress caused to citrus plants by drought and nutrient deficiency (iron, manganese, potassium or zinc) induces leaf

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1754-5048/$ – see front matter
http://dx.doi.org/10.1016/j.funeco.2013.01.004

chlorosis similar to that found in HLB disease. In general, chlorosis caused by HLB, as well as nutrient deficiency, leads to important changes in the physiology and ecological niche of the leaves. This environmental alteration may influence the diversity and interactions of other organisms inhabiting leaf tissues, such as endophytic fungi (Kriel *et al.* 2000).

Endophytic fungi live their entire life cycle inside plant tissues without causing apparent symptoms of infection, and form associations with all plants (Arnold & Lutzoni 2007; Hyde & Soytong 2008; Ghimire *et al.* 2011; Rocha *et al.* 2011; Douanla-Meli & Langer 2012; Tadych *et al.* 2012). Their ecological significance has been well studied, but remains incompletely understood. They compete with pathogens for the same ecological niches and improve the interactions and defence systems of their host plants (Vicari *et al.* 2002; Arnold *et al.* 2003; Holmes *et al.* 2004; Rubini *et al.* 2005; Tondje *et al.* 2006; Zhang *et al.* 2009; Ghimire *et al.* 2011). Endophytic fungi also benefit plants under drought or nutrient stress (Maki 2006; Wäli *et al.* 2008; Foyer & Shigeoka 2011; Hamilton *et al.* 2010; Hamilton & Bauerle 2012) and, in turn, their incidence frequency and community assemblages are influenced by nutrient variation in host tissues (Gosling *et al.* 2006; Larkin *et al.* 2012). On the other hand, fungal endophyte species are frequently reported as pathogens on the same or different hosts, and thus may be pathogens in a latent phase of their life cycle (Romero *et al.* 2001; Photita *et al.* 2004; Slippers & Wingfield 2007; Cheplick & Faeth 2009). Therefore, characterizing fungal endophytic communities and their interactions is crucial to understanding fungal diseases of the host plant and is a prerequisite for best management practice.

Globally, in contrast to the intensively studied fungal pathogens of citrus plants (Baayen *et al.* 2002; Ezeibekwe & Unamba 2009; Glienke *et al.* 2011; Wang *et al.* 2012), the diversity of their fungal endophytes remains poorly known (Wright 1998; Glienke-Blanco *et al.* 2002; Durán *et al.* 2005). It is well known that endophyte diversity is shaped by the host identity (Higgins *et al.* 2007; Johnston *et al.* 2012), and also that the endophyte community depends on geographic situation (Saikkonen 2007; Thomas *et al.* 2008; Hoffman & Arnold 2008). The latter combined with environmental factors and fitness of host plant (Gundel *et al.* 20011; Zimmerman & Vitousek 2012), should be considered for estimating the overall endophyte diversity. *Citrus limon* that originated in Asia is currently commercially grown in all warm regions of the world. Available data on its fungal endophyte diversity are based on limited studies from Brazil (Glienke-Blanco *et al.* 2002; Durán *et al.* 2005) and South Asia (Wright 1998), and show a general low diversity and difference in communities of foliar endophyte in the two geographical regions. No study so far has assessed the effects of leaf disease on foliar endophyte communities of *C. limon*. In the present study, our objectives were to: (1) examine the assemblage of foliar endophyte fungi of *C. limon* with focus on material from tropical Africa; (2) conduct a comparative study of fungal endophyte communities in green healthy leaves and yellowing leaves in order to verify whether yellowing of lemon leaves influences the fungal endophytes; and (3) assess the possible interactions among these endophytes.

Materials and methods

Sampling site and plant material

Lemon leaves were collected on 12 Feb. 2012 in the vicinity of Yaoundé, Central Region of Cameroon (750 masl; N 3° 52′, E 11° 31′), which is characterized by a warm and humid climate, with an annual mean temperature of 25 °C and total annual mean precipitation of 1 747 mm. Citriculture is not practised in this area, but plantations and domestic gardens contain lemon and orange trees, which are usually not managed. Mostly, plants showing branches with both healthy and yellowing leaves were sampled. Plants were considered suitable for sampling when leaves had no other disease symptom than yellowing leaves. In each plantation or domestic garden, 24 trees were randomly selected and two leaves were collected from branches accessible at a standing height from ground level. Healthy and yellowing leaves from the same tree represented one collection. Leaves were kept in paper bags at 4 °C and processed within 48 hr.

Isolation and culture of fungal endophytes

Leaves were initially washed with tap water, then surface-sterilized by dipping in 96 % ethanol for 1 min, in bleach solution containing 3.5 % sodium hypochlorite for 5 min, in 96 % ethanol for 30 s and finally were rinsed twice (5 min each) in deionized and autoclaved water. To confirm whether the sterilization process was successful, the final rinse water was plated onto 2 % malt-extract agar (MEA); there was neither fungal nor bacterial growth after 2 weeks of incubation at 25 °C.

Using a flame-sterilized scalpel, 10 leaf fragments (or samples) of 2 × 5 mm were cut per leaf for a total of 480 samples. Five samples were placed on each MEA plate with 1 % tetracycline added to inhibit bacterial growth. Plates were incubated at 25 °C in darkness for 4 weeks and were checked daily for hyphal growth. Emerging hyphae were transferred onto a new MEA plate. Pure isolates were further grown on MEA and PDA (potato dextrose agar; Roth, Germany) at 25 °C under a regime of 12 hr darkness/12 hr cool white fluorescent light to study cultural characteristics such as growth rate, conidiation, diffusing pigment, surface texture and aerial hyphae. All isolates are preserved in the private culture collection of the Ecology Laboratory at the University of Kassel, Germany.

Identification of fungal endophytes

Data, recorded from culture and microscopy, were used to group isolates into morphotaxa. Representative isolates of morphotaxa were chosen for DNA extraction and sequencing. Genomic DNA extraction, PCR amplification of the internal transcribed spacer (*ITS1-5.8S-IT2*) regions with primers ITS1f and ITS4 (White *et al.* 1990) and sequencing conditions were the same as those used by Douanla-Meli & Langer (2012). Sequences were assembled using Sequencher® 5.0 (Gene Codes, Ann Arbor, MI) and deposited in GenBank with the corresponding accession numbers JX436777—JX436807.

Morphotaxa were provisionally identified from morphology when diagnostic characters were evident. *ITS* rDNA sequences were analyzed to verify and confirm identification where possible. Generated sequences were initially assembled into contigs based on ≥98 % similarity using Sequencher® 5.0. A representative sequence of each contig was analyzed by a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/; accessed 15.12.2012) to test for similarity with sequences present in the GenBank database and the first three sequences with high similarity were downloaded. For species appearing in the top three hits, sequences of type, ex-type or ex-epitype, if available in GenBank, were used for comparison and included in phylogenetic reconstruction as suggested in Ko Ko *et al.* (2011). Accession numbers of additional sequences from GenBank are provided in the phylogenetic tree.

Sequences were automatically aligned using the online version of MAFFT v. 6 (http://mafft.cbrc.jp/alignment/server/index.html) under the default settings and optimized in SeAl v2.0a11 (Rambaut 2002). Maximum parsimony (MP) analyzes in PAUP* 4.0b10 (Swofford 2002) used the following settings: 1 000 random stepwise addition sequences, TBR branch swapping algorithm, and MAXTREES set to autoincrease, retaining 100 trees after each replicate. Bootstrap support values (BSS) were calculated with 1 000 replicates (Felsenstein 1985). The best-scoring Maximum likelihood (ML) tree was estimated in RAxML 7.2.6 (Stamatakis 2006) using raxmlGUI (Silvestro & Michalak 2012), under the GTRGAMMAmodel of sequence evolution. Robustness of nodes was tested using 1 000 replicates bootstrap analysis. Endophyte morphotaxa were assigned to phylotypes with respect to the contig assemblage and topology of the phylogenetic tree.

Dual-culture experiments

Dual-culture tests were conducted to verify whether antagonist activity exists between dominant taxa in healthy and yellowing leaves. Agar disks (4 mm diameter) from 5-d-old colonies of antagonist isolates were placed 40 mm apart on 90 mm diameter PDA plates. Slow-growing isolates were also plated 2 or 4 weeks before the other antagonist. Control plates contained a mycelial disk of one isolate only. Experiments were conducted in a Completely Randomized Design with four replicates. Plates were incubated in the dark at 25 °C, and after 3 d the colony radius (R1) of each isolate towards its antagonist was measured every day for 10 d. Competitive interactions were interpreted using the rating scale of Badalyan *et al.* (2002) and estimated based on the percentage inhibition of radial extension (PIRG) (Jinantana & Sariah 1997) calculated as follows: $PIRG = R_2 - R_1 / R_2 \times 100$, with R_2 being the radius of the control colony.

Detection of HLB bacteria in lemon leaves and origin of yellowing of lemon leaves

As the type of yellowing observed in the field varied and could not be unambiguously assigned either to abiotic stress or HLB disease (Supplementary Fig S1), detection of *C. liberibacter* in healthy and yellowing leaves was performed. HLB bacteria cannot be cultured, both detection and identification rely exclusively on molecular methods, such as conventional and Real-Time PCR (Hung *et al.* 2004; Li *et al.* 2009). DNA extraction and PCR amplification were conducted as described in Coletta-Filho *et al.* (2005). To detect the three known *Liberibacter* species, we used three sets of PCR primers specific to amplification of *16S* rDNA of *Liberibacter*, *OA1/OI2c* and *OI1/OI2c* for *Ca. L.* africanus and *Ca. L.* asiaticus respectively (Jagoueix *et al.* 1996), and *GB1/GB3* for *Ca. L.* americanus (Teixeira *et al.* 2005).

Data analysis

For statistical analysis, each collection was considered as a repetition. Infection frequency (IF) was determined for both healthy and yellowing leaves in each collection, as follows: $IF\ (\%) = N_i/N_s \times 100$, where N_i is the number of samples yielding at least one isolate, and N_s is the total number of samples investigated. Similarity of species composition between healthy and yellowing leaves was estimated using the Jaccard index (J) and Sørenson index (S) (Magurran 2004) calculated using Estimate S Win version 8 (Colwell 2006). Data were analyzed by one-way analysis of variance (ANOVA) and a Duncan's test was used to determine significant differences ($p < 0.05$) between the means.

Results

Detection of HLB bacteria and origin of yellowing of lemon leaves

Samples of healthy and yellowing lemon leaves were all negative in the three primer sets tested. The negative outcome of this test implied that yellowing leaves result from non-pathogenic disease caused by either nutrient deficiency or drought stress.

Frequency and diversity of culturable endophytic fungi of lemon leaves

We obtained a total of 482 isolates, with an overall infection frequency of 82.3 % and isolation rate of 1.0, because some samples yielded more than one endophytic isolate. All isolates were grouped into 26 morphotaxa. Distribution of isolates among morphotaxa was highly heterogeneous, with few common and many rare morphotaxa (Table 1). A total of 21 OTUs were identified using *ITS* sequences of representative isolates. MP and ML trees were largely congruent and MP strict consensus tree yielded a similar topology to the optimal best ML tree (−1 nL = 7 941.891507). In Fig 1, one of the MP trees (TL = 1 540 steps; CI = 0.492; RI = 0.927; RC = 0.456) presents the phylogenetic relationships of lemon foliar fungal endophytes. Six major clades were resolved and lemon fungal endophytes assigned to 20 phylotypes. All phylotypes belong to the Ascomycota, in three orders of Dothideomycetes (40 % of the isolates) and three orders of Sordariomycetes (60 % of the isolates). Identification of completely named morphotaxa was corroborated on phylogenetic tree by the resolution with sequences from ex-type or ex-epitype cultures, and many incomplete names must be treated with caution (Fig 1).

Xylariaceous species were rare, represented by *Xylaria* (one single isolate) and *Pestalotiopsis* (1.9 % of all isolates), from which morphology and *ITS* analysis distinguished four

Table 1 – Number of isolates, infection frequency and distribution of different phylotypes of fungal endophytes between healthy and chlorotic leaves of *C. limon*

Phylotypes	Total number of isolates	Number of isolates and IF (%)	
		Healthy leaves ($n = 360$)	Yellowing leaves ($n = 120$)
A.	248	104 (28.8 %)D	144 (95.8 %)E
B.	20	12 (3.3 %)C	8 (6.6 %)B
C.	1	1 (0.28 %)A	0 (– %)A
D.	3	2 (0.56 %)A	1 (0.83 %)A
E.	2	2 (0.56 %)A	0 (– %)A
F.	2	2 (0.56 %)A	0 (– %)A
G.	1	1 (0.28 %)A	0 (– %)A
H.	1	1 (0.28 %)A	0 (– %)A
I.	2	2 (0.56 %)A	0 (– %)A
J.	3	2 (0.56 %)A	1 (0.83 %)A
K.	1	1 (0.28 %)A	0 (– %)A
L.	1	1 (0.28 %)A	0 (– %)A
M.	61	61 (10.4 %)B	0(– %)A
N.	9	9 (2.5 %)C	0 (– %)A
O.	2	2 (0.56 %)A	0 (– %)A
P.	47	47 (9.1 %)B	0 (– %)A
Q.	34	34 (6.9 %)B	0 (– %)A
R.	12	12(3.3 %)C	0 (– %)A
S.	29	27 (7.5 %)C	2 (1.7 %)A
T.	3	3 (0.83 %)A	0 (– %)A
20	482	326 (71 %)	156 (98 %)

Mean IF values of a phylotype between HL and CL with the same letter are not significantly different by Duncan's test ($p < 0.05$). IF values were calculated from the number of samples yielding at least one isolate of fungal endophyte.

phylotypes. *Mycosphaerella* and their asexual states (34.2 % of all isolates) constituted one dominant group and included six phylotypes (Table 2). *Passalora loranthi* (9.8 % of all isolates) and Mycosphaerellaceae sp. 2 (12.65 % of all isolates) were isolated particularly frequently. *Colletotrichum* isolates were grouped into six morphotaxa and yielded three phylotypes. *Colletotrichum gloeosporioides*, which had the highest recovery (50.4 % of all isolates) of the endophytic assemblage, also showed high morphological variation in four morphotaxa (Supplementary Fig S5). However, *ITS* sequences from all morphotaxa had less than 1 % base pair differences, formed a unique OTU and clade including the type sequence. *Colletotrichum boninense* was recovered only in small numbers, while the last *Colletotrichum* morphotaxon, which included only a single isolate, was not fully identified. Botryosphaeriaceae isolates (6 % of all isolates) were grouped into two morphotaxa; both were, however, identified as one phylotype, *Phyllosticta capitalensis*. Isolates related to *Diaporthe/Phomopsis* species (1.5 % of all isolates) belonged to four OTUs and resolved into four phylotypes. Sequences of the three *Phoma*-like isolates formed one OTU, and they obviously represent the same species.

Endophytic fungal communities of healthy and yellowing leaves

We examined 360 samples from healthy leaves and 120 samples from yellowing leaves. Each leaf showed endophytic colonization, but IF varied between healthy and yellowing leaves. Yellowing leaves were significantly more colonized ($p < 0.05$) than healthy leaves (Table 1). The average number of endophyte isolates per sample was 0.92 and 1.3 for healthy leaves and yellowing leaves, respectively. Endophyte species richness of healthy leaves was higher than that of yellowing leaves. All 20 phylotypes identified were found in healthy leaves (Table 1) and the endophytic assemblage of yellowing leaves comprised only five phylotypes ($J = 0.23$, QS = 0.37). *Mycosphaerella* and allied anamorphic species were the dominant endophytic group in healthy leaves, and no isolate was recovered in yellowing leaves. Among non-mycosphaerellaceous fungi, *C. gloeosporioides* was isolated at levels of statistical significance ($p < 0.05$) more frequently from yellowing leaves than from healthy leaves (Table 1, Fig 2). In contrast, IF of all other phylotypes decreased in yellowing leaves.

Interactions among fungal endophytes

More than one endophyte isolate was recovered from 33 % to 10.5 % of healthy leaf and yellowing leaf samples, respectively. In healthy leaf samples, there was dominance of mycosphaerellaceous fungal species coexisting and with *P. capitalensis*. Multiple isolates from some yellowing leaf samples were indeed culture variants of the same phylotype. The highest number (five) of culture variants was obtained for *C. gloeosporioides*. Co-infection in yellowing leaf samples occurred only between *Colletotrichum* and *Pestalotiopsis*.

In dual culture, the pairing of *C. gloeosporioides* with mycosphaerellaceous taxa yielded variable antagonistic effects. With simultaneous plating, *C. gloeosporioides* had a significantly ($p < 0.05$, compared to control) higher inhibitory effect, PIRG 30–65 %, on mycosphaerellaceous isolates. After mycelial contact, *C. gloeosporioides* overgrew mycosphaerellaceous isolates and suppressed their growth (Supplementary Fig S2). Mycosphaerellaceous isolates, which had been plated a week in advance, were similarly overgrown without initial deadlock. Interestingly, inverse competitive interactions occurred when mycosphaerellaceous fungi were plated 4 weeks in advance. The growth of *C. gloeosporioides* was significantly ($p < 0.05$) reduced in the range of 20–45 %, and a deadlock occurred at distance. Colonies of *C. gloeosporioides* were mostly sparse, poorly sporulating, and in particular formed a darker pigmented marginal zone (Supplementary Fig S3). Light microscopic examination of the dark zones revealed consistent hyphal modifications, including cell shortening, swelling, distortion and pigmentation (Supplementary Fig S4). However, malformed hyphae inoculated onto a new PDA plate were able to revitalize and produce normal sporulating colonies of *C. gloeosporioides*.

Discussion

Endophytic colonization and effects of yellowing in C. limon leaves

All examined lemon leaves were densely colonized and IF values characterizing the endophytic community profile were

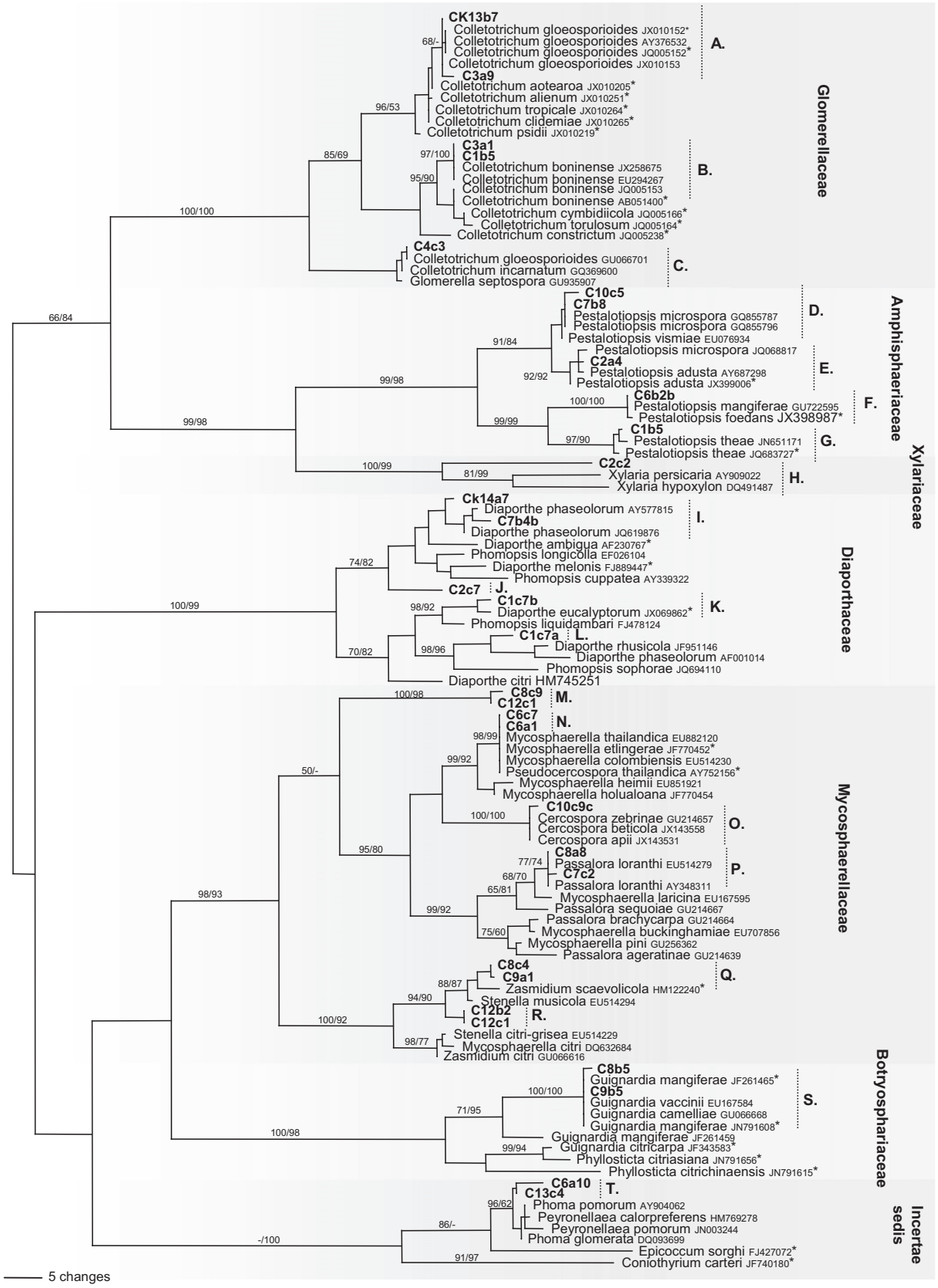

Fig 1 – Phylogenetic relationships of fungal endophytes isolated from healthy and yellowing leaves of *Citrus limon*, as shown by one of the most parsimonious trees constructed based on ITS1-5.8S-ITS2 sequences. Number above branches indicate MP and ML bootstrap values > 50 % respectively. In bold case are isolate codes of fungal endophytes from leaf tissues of *C. limon*. A–T designate the identified phylotypes. Sequences of type, ex-type or ex-epitype cultures from GenBank are marked with an asterisk. The tree was rooted by midpoint.

Table 2 – Molecular identification and classification of fungal endophytes isolated from healthy and yellowing leaves of *Citrus limon*

Selected isolates	GenBank accession numbers	Query coverage and identity (%), highest match in GenBank			Reference	Identified phylotypes	Family, order
C3a9	JX436790	Fungal endophyte	HM537032	100/99	He *et al.* 2012	A. *Colletotrichum gloeosporioides*	G, Phy
Ck13b7	JX436791	*Colletotrichum gloeosporioides*	JQ580704	100/99		–	–
C1b5a	JX436792	*Colletotrichum boninense*	JX258772	100/100		B. *Colletotrichum boninense*	–
C3a1	JX436793	Fungal endophyte	HM537031	100/100	He *et al.* 2012	–	–
C4c3	JX436794	*Colletotrichum gloeosporioides*	GU066701	99/100		C. *Colletotrichum* sp.	—
C7b8a	JX436800	*Pestalotiopsis microspora*	EU935587	100/99	Wu *et al.* 2009	D. *Pestalotiopsis microspora*	A, Xyl
C10c5	JX436801	*Pestalotiopsis pallidotheae*	JQ676182	99/99		—	—
C2a4	JX436802	*Pestalotiopsis* sp.	HQ608091	97/99	Rodrigues *et al.* 2011	E. *Pestalotiopsis adusta*	—
C6b2b	JX436803	Uncultured Xylariales	GU056016	100/99		F. *Pestalotiopsis foedans*	—
C1b5b	JX436804	*Pestalotiopsis theae*	HQ832793	97/99		G. *Pestalotiopsis theae*	—
C2c2	JX436805	Fungal sp.	FJ612855	93/99	U'ren *et al.* 2009	H. *Xylaria* sp.	X, Xyl
Ck14a7	JX436797	*Diaporthe* sp.	FJ799938	95/99	Van Bael *et al.* 2009	I. *Diaporthe phaseolorum*	D, Dia
C7b4b	JX436798	*Diaporthe* sp.	FJ799938	100/99	—	–	—
C2c7	JX436799	*Diaporthe* sp.	FJ799941	97/98	—	J. *Phomopsis* sp.	—
C1c7b	JX436795	*Diaporthe eucalyptorum*	JX069862	99/99	Crous *et al.* 2012	K. *Phomopsis eucalyptorum*	—
C1c7a	JX436796	Fungal endophyte	HM537034	100/97	He *et al.* 2012	L. *Diaporthe* sp.	—
C12c1	JX436786	Mycosphaerellaceae sp.	JN601144	80/97		M. Mycosphaerellaceae sp. 1	M, Cap
C8c9	JX436787	*Ramichloridium cerophilum*	EU041798	100/91	Arzanlou *et al.* 2007b	—	—
C6c7	JX436777	*Mycosphaerella thailandica*	EU882120	99/100	Cheewangkoon *et al.* 2008	N. *Mycosphaerella thailandica*	—
C6a1	JX436778	*Mycosphaerella thailandica*	EU882120	99/100	—	—	—
C10c9c	JX436779	*Cercospora* cf. *zinniae*	JX143759	100/99	Groenewald *et al.* 2013	O. *Cercospora* sp.	—
C8a8	JX436780	*Mycosphaerella laricina*	EU167595	100/98	Simon *et al.* 2009	P. *Palassora loranthi*	—
C7c2	JX436781	*Palassora loranthi*	EU853479	99/99		—	—
C8c4b	JX436782	Uncultured fungus	HM572246	100/99	Singh *et al.* 2011	Q. *Zasmidium scaevolicola*	—
C9a1	JX436783	*Stenella mucicola*	EU514294	100/98	Arzanlou *et al.* 2008	—	—
C12b2	JX436784	Uncultured fungus	JF497135	100/99	Singh *et al.* 2011	R. Mycosphaerellaceae sp. 2	—
C12c10	JX436785	Uncultured fungus	GU370737	100/98	—	—	—
C9b5	JX436788	Fungal endophyte	JQ743587	100/100	Wong *et al.* 2012	S. *Phyllosticta capitalensis*	B, Bot
C8b5	JX436789	Fungal endophyte	EF419973	99/100	Hoffman & Arnold 2008	—	—
C6a10	JX436806	Uncultured ascomycete	EU489902	97/99		T. Phoma sp.	Is, Ple
C13c4	JX436807	*Peyronellaea pomorum*	JN003244	99/99		—	—

Family: A = Amphisphaeriaceae; B = Botryosphaeriaceae; D = Diaporthaceae; G = Glomerellaceae; Is = Incertae sedis; M = Mycosphaerellaceae; X = Xylariaceae. Order: Bot = Botryosphaeriales; Cap = Capnodiales; Dia = Diaporthales; Phy = Phyllachorales; Ple = Pleosporales; Xyl = Xylariales.

very similar to those obtained from some tropical plants (Fröhlich *et al.* 2000; Crozier *et al.* 2006; Thomas *et al.* 2008; Rakotoniriana *et al.* 2008). In previous studies on citrus fungal endophytes, Glienke-Blanco *et al.* (2002) found an 81 % IF in tangerine plants in Brazil, but Durán *et al.* (2005) reported lower values of 69.7–72.3 % IF from Argentinian lemon leaves. In contrast to the above-mentioned studies, our sampling was limited geographically and temporally, and the accumulation curve (not shown) was not asymptotic, indicating that endophyte diversity of lemon leaves was not fully recovered.

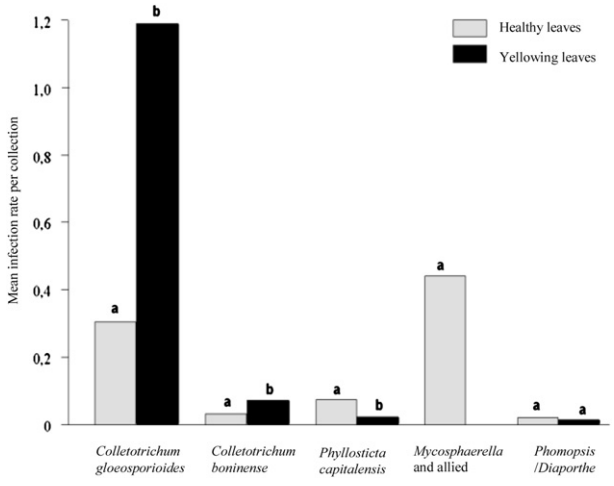

Fig 2 – Infection frequencies of fungal endophytes from healthy and yellowing leaves of *Citrus limon*. Means within a phylotype with the same letter are not significantly different by Duncan's test ($p < 0.05$).

Interestingly, as hypothesized, the IF and species composition of fungal endophytes were significantly different between healthy and yellowing leaves. Probable explanations for the higher IF in yellowing leaves relate to physiological changes in leaf tissues and the endophyte–host plant and fungal interactions. Leaf yellowing is an important plant problem, which, as assumed in this study, may be caused by nutrient deficiency or drought stress, and negatively affects photosynthetic processes. This was to some extent, demonstrated for Rapeseed (Baryla *et al.* 2001; Zhu *et al.* 2005). On the other hand, many studies have pointed out that endophyte–host plant interactions respond to these disturbances by enhancing either the accumulation of mineral nutrients by the plant (Lyons *et al.* 1990; Hamilton *et al.* 2010; Mei & Flinn 2010) or resistance to drought stress (Maki 2006; Spiering *et al.* 2006; Bayat *et al.* 2009; Hamilton & Bauerle 2012). It is also recognized that these interactions are mediated by means of fungal metabolites (Kuldau & Bacon 2008) that are, however, also active against other fungi (Christensen 1996; Campanile *et al.* 2007; Zhang *et al.* 2009; Sumarah *et al.* 2011).

Despite the decline in incidence of most species in yellowing leaves, IF increased significantly, but solely as a result of the quadrupled incidence of *C. gloeosporioides*. This species may be advantageous for yellowing leaves in coping with physiological disturbances, or is simply tolerant of environmental changes (Lappalainen *et al.* 1999). Since fungal endophytes receive nutrition and protection from the host plant, their incidence can be lowered or even hindered by physiological variation within host tissue (Lappalainen *et al.* 1999; Kriel *et al.* 2000). This may explain the alteration of species composition in yellowing leaves that implies growth inhibition of *Mycosphaerella* and allied species. Additionally, inhibitory activity among endophytes affects the patterns of their within-tissue distribution and composition (Hata *et al.* 2002; Bandara *et al.* 2006; Rakotoniriana *et al.* 2008). In this context, we noted the *in vivo* mutual exclusion between *C. gloeosporioides* and other endophytes. Wright (1998) found *C. gloeosporioides* to be an important niche competitor with stem end rot fungi on citrus. It is likely that its competitive ability also extends to endophytic species, as confirmed by the results of *in vitro* dual culture in the present study. However, old grown mycosphaerellaceous isolates had a significant antagonistic activity towards *C. gloeosporioides*, possibly through late production of diffusible secondary metabolites. It, therefore, seems that an increasing frequency of *C. gloeosporioides* in yellowing leaves may also result from the absence of mycosphaerellaceous fungi; further studies are, however, necessary to test this hypothesis.

Diversity and ecology of foliar endophytic fungi of C. limon

Endophytic diversity recovered from lemon leaves in Cameroon was two times higher than that found in Argentina (Durán *et al.* 2005). In addition, a comparison of the species composition in endophytic fungal assemblages showed that, except for one common species, the recovered endophytic communities were rather different between the two countries. As found in previous studies (Wright 1998; Durán *et al.* 2005), *C. gloeosporioides* was also the dominant endophyte in Cameroon lemon leaves. This species has a broad ecological diversity and host range (Freeman *et al.* 1998; Cannon & Simmons 2002; Rojas *et al.* 2010; Lima *et al.* 2011; Weir *et al.* 2012) and is an anthracnose pathogen of many hosts, including citrus plants (Sonoda & Pelosi 1988; Kuramae-Izioka *et al.* 1997; Wright 1998; Benyahia *et al.* 2003; Lima *et al.* 2011). Two further *Colletotrichum* species were newly recovered from lemon leaves, namely *Colletotrichum* sp. and *C. boninense*. The latter, similarly to *C. gloeosporioides*, is endophytic, but is also a frequent pathogen causing anthracnose on various host plants (Moriwaki *et al.* 2003; Lubbe *et al.* 2004; Tarnowski & Ploetz 2010; Silva-Rojas & Ávila-Quezada 2011; Damm *et al.* 2012). Co-occurrence of the three species on lemon leaves indicates that the *Colletotrichum* diversity on citrus plants may be higher than expected based on current knowledge (Kuramae-Izioka *et al.* 1997; Damm *et al.* 2012). Although a pathogenicity test with *Colletotrichum* isolates on lemon leaves was not conducted in the present study, it is worth noting that, during field sampling, anthracnose symptoms were not observed. This confirms that *Colletotrichum* were isolated either in their endophytic lifestyle or as latent pathogens (Brown *et al.* 1998; Wright 1998; Photita *et al.* 2004).

Surprisingly, mycosphaerellaceous fungi were the most diverse and second most frequently isolated endophytic group, including *Mycosphaerella* and its anamorphs. *Mycosphaerella* species are saprobic or pathogenic on a wide range of hosts (Goodwin *et al.* 2001; Crous *et al.* 2006, Crous 2009; Arzanlou *et al.* 2007a), but may also occur as symptomless endophytes (Wright 1998; Crous 2009). *Mycosphaerella citri*, *Mycosphaerella horii* and *Mycosphaerella lagenniformis*, known to be associated with citrus fruit and leaf spot diseases (Wright 1998; Pretorius *et al.* 2003), were not found in the present study. Anamorphic mycosphaerellaceous species recovered as endophytes on lemon leaves are common leaf blight pathogens of several plant species (Goodwin *et al.* 2001; Crous *et al.* 2006, Crous 2009; Arzanlou *et al.* 2007b), but *Cercospora*, which is an agent of fruit and leaf spot, is the only one known

to be a citrus pathogen (Pretorius *et al.* 2003). *Passalora* and *Zasmidium* have not been reported as citrus endophytes.

Although host preference in mycosphaerellaceous species, at least for pathogen species, is high (Pretorius *et al.* 2003; Crous *et al.* 2006; Arzanlou *et al.* 2008), we found species previously described from other host plants as endophytes in lemon leaves. Numerous isolates had a high affinity for, and also clustered with, *Passalora loranthi* and *Zasmidium scaevolicola*, originally described as pathogens on *Musa* and *Scaevola taccada*, respectively (Arzanlou *et al.* 2008; Shivas *et al.* 2010). Likewise *Mycosphaerella* isolates were identical in terms of percentage to sequences of *Mycosphaerella thailandica*, but also *Mycosphaerella colombiensis* and *Mycosphaerella etlingerae*, and their type sequences all clustered in the same highly supported clade in our *ITS* analysis. In Crous *et al.* (2011), *ITS* and *LSU* sequences were identical for the three species, but with combined actin, calmodulin and histone datasets, *M. thailandica* was resolved as a distinct species from *M. colombiensis* (Crous *et al.* 2004). The ecology seems to be important for differentiating the three species. Both *M. thailandica* and *M. colombiensis* were described as leaf spot pathogens, the first on *Acacia mangium* and the second on *Musa* sp. and *Eucalyptus*, whereas *M. etlingerae* was found on dead leaves of *Etlingera elatior* (Crous *et al.* 2004 2011). Some further lemon mycosphaerellaceous isolates were not satisfactorily identified, because the sequences did not match with those of known species in GenBank, and further analysis is needed to confirm whether they represent new species.

Another specific pattern evident for the endophytic assemblage in lemon leaves is the low IF and diversity of xylariaceous species, four species represented by 1.9 % of all isolates, in contrast with the prevalence found in leaves of many other tropical plants (Bayman *et al.* 1998; Santamaria & Bayman 2005; Douanla-Meli & Langer 2012; Linnakoski *et al.* 2012). Durán *et al.* (2005) found 0.9–1.7 % IF for Xylariaceae in Argentinian lemon leaves. In the leaves of *C. sinensis* collected from our study area, xylariaceous fungi also had (at 1 %) the lowest frequency (unpublished data). An explanation of the paucity of endophytic xylariaceous fungi on citrus plants may be more due to the influence of the host plant on the endophytic assemblage than the geographic environment or isolation method (Hoffman & Arnold 2008; Johnston *et al.* 2012). Xylariaceous species recovered in the present study, *Pestalotiopsis* and *Xylaria*, are ubiquitous (Suryanarayanan *et al.* 2002; Douanla-Meli & Langer 2012). If *Xylaria* are mainly saprobic or endophytic, *Pestalotiopsis* additionally occur as plant pathogens (Chang *et al.* 1997; Wei *et al.* 2007), but no species is to date known as a pathogen on citrus plants. On the other hand, of the isolated *Diaporthe/Phomopsis* and *Phyllosticta* species, none is pathogenic for citrus. *P. capitalensis*, previously isolated (also as *G. mangiferae*) as a non-pathogenic endophyte from citrus plants (Baayen *et al.* 2002; Glienke-Blanco *et al.* 2002; Glienke *et al.* 2011), was confirmed to be a common lemon foliar endophyte.

In conclusion, this study, although based on a relatively limited sampling, has demonstrated that fungal endophytes densely inhabit lemon leaves and that the species composition would appear to be very variable due to factors that remain to be determined. Our results support the hypothesis that the yellowing of leaves affect foliar endophytic communities, and that interactions among endophytes may also underlie the difference in species composition and structure observed between healthy and yellowing leaves. Importantly, it remains to be determined whether pathogenic and non-pathogenic chlorosis of lemon leaves lead to the same effects. Although this study has increased the known diversity of lemon foliar endophytes, other methods than culture need to be applied if the total diversity is to be determined. Further investigations aimed at a better understanding of the precise role of lemon fungal endophytes, as well as their interactions, could be beneficial to biological control of many species of pathogens of this plant.

Acknowledgements

We are grateful to Dr. BR Nana Nbendjo (University of Yaoundé I, Cameroon) for helping to collect lemon leaves. Ulrike Friedling and Carola Weißkopf are acknowledged for technical assistance. We also thank the two anonymous reviewers who greatly improved the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2013.01.004.

REFERENCES

Arnold AE, Lutzoni F, 2007. Diversity and host range of foliar endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**: 541–549.

Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA, 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences USA* **100**: 15649–15654.

Arzanlou M, Abeln ECA, Kema GHJ, Waalwijk C, Carlier J, Vries I de, Guzmán M, Crous PW, 2007a. Molecular diagnostics for the Sigatoka disease complex of banana. *Phytopathology* **97**: 1112–1118.

Arzanlou M, Groenewald JZ, Gams W, Braun U, Shin H-D, Crous PW, 2007b. Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera. *Studies in Mycology* **58**: 57–93.

Arzanlou M, Groenewald JZ, Fullerton RA, Abeln ECA, Carlier J, Zapater M-F, Buddenhagen WI, Viljoen A, Crous PW, 2008. Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. *Persoonia* **20**: 19–37.

Baayen RP, Bonants PJM, Verkley G, Carroll GC, van der Aa HA, de Weerdt M, van Brouwershaven IR, Schutte GC, Maccheroni Jr W, Glienke de Blanco C, Azevedo JL, 2002. Non-pathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). *Phytopathology* **92**: 464–477.

Badalyan SM, Innocenti G, Garibyan NG, 2002. Antagonistic activity of xylotrophic mushrooms against pathogenic fungi of cereals in dual culture. *Phytopathologia Mediterranea* **41**: 200–225.

Bandara WMMS, Seneviratne G, Kulasooriya SA, 2006. Interactions among endophytic bacteria and fungi: effects and potentials. *Journal of Biosciences* **31**: 645–650.

Bayat F, Mirlohi A, Khodambashi M, 2009. Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. *Russian Journal of Plant Physiology* **56**: 510–516.

Baryla A, Carrier P, Franck F, Coulomb C, Sahut C, HAvaux M, 2001. Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: causes and consequences for photosynthesis and growth. *Planta* **212**: 696–709.

Bastianel G, Garnier-Semancik M, Rnaudin J, Bové JM, Eveillard S, 2005. Diversity of "*Candidatus liberibacter* asiaticus", based on the *omp* gene sequence. *Applied and Environmental Microbiology* **71**: 6473–6478.

Bayman P, Angulo-Sandoval P, Báez-ortiz Z, Lodge DJ, 1998. Distribution and dispersal of *Xylaria* endophytes in two tree species in Puerto Rico. *Mycological Research* **102**: 944–948.

Benyahia H, Jrifi A, Smaili C, Afellah M, Timmer W, 2003. First report of *Colletotrichum gloeosporioides* causing withertip on twigs and tear stain on fruit of citrus in Morocco. *New Disease Reports* **7**: 20.

Brown KB, Hyde KD, Guest DI, 1998. Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. *Fungal Diversity* **1**: 27–51.

Campanile G, Ruscelli A, Luisi N, 2007. Antagonistic activity of endophytic fungi towards *Diplodia corticola* assessed by in vitro and in planta tests. *European Journal of Plant Pathology* **117**: 237–246.

Cannon PF, Simmons CM, 2002. Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia* **94**: 210–220.

Chang TH, Lim TH, Chung BK, Kim BS, 1997. Studies on cultural characteristics *Pestalotiopsis theae* causing leaf blight on oriental persimmon tree. *Korean Journal of Plant Pathology* **13**: 232–238.

Cheewangkoon R, Crous PW, Hyde KD, Groenewald JZ, To-Anan C, 2008. Species of Mycosphaerella and related anamorphs on Eucalyptus leaves from Thailand. *Persoonia* **21**: 77–91.

Cheplick GP, Faeth S, 2009. *Ecology and Evolution of the Grass-endophyte Symbiosis*. Oxford University Press, USA.

Christensen MJ, 1996. Antifungal activity in grasses infected with *Acremonium* and *Epichloe* endophytes. *Australasian Plant Pathology* **25**: 186–191.

Coletta-Filho HD, Takita MA, Targon MLPN, Machado MA, 2005. Analysis of 16S rDNA sequences from citrus huanglongbing bacteria reveal a different "Ca. Liberibacter" strain associated with citrus disease in Sao Paulo. *Plant Disease* **89**: 848–852.

Colwell RK, 2006. *Estimates: statistical estimate of species richness and shared species from samples, version 8*. http://viceroy.eeb.uconn.edu/EstimateSPages/EstSUsersGuide/. EstimateSUsersGuide.htm (accessed: 09.07.12).

Crozier J, Thomas SE, Aime MC, Evans HC, Holmes KA, 2006. Molecular characterisation of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. *Plant Pathology* **55**: 783–791.

Crous PW, 2009. Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs. *Fungal Diversity* **38**: 1–24.

Crous PW, Groenewald JZ, Pongpanich K, Himaman W, Arzanlou M, Wingfield MJ, 2004. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. *Studies in Mycology* **50**: 457–469.

Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ, 2006. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. *Studies in Mycology* **55**: 99–131.

Crous PW, Tanaka K, Summerell BA, Groenewald JZ, 2011. Additions to the *Mycosphaerella* complex. *IMA Fungus* **2**: 49–64.

Crous PW, Summerell BA, Shivas RG, Burgess TI, Decock CA, Dreyer LL, Granke LL, Guest DI, Hardy GE, Hausbeck MK, Hüberli D, Jung T, Koukol O, Lennox CL, Liew EC, Lombard L, McTaggart AR, Pryke JS, Roets F, Saude C, Shuttleworth LA, Stukely MJ, Vánky K, Webster BJ, Windstam ST, Groenewald JZ, 2012. Fungal Planet description sheets: 107–127. *Persoonia* **28**: 138–182.

Damm U, Cannon PF, Woudenberg JHC, Johnston PR, Weir BS, Tan YP, Shivas RG, Crous PW, 2012. The *Colletotrichum boninense* species complex. *Studies in Mycology* **73**: 1–36.

Douanla-Meli C, Langer E, 2012. Diversity and molecular phylogeny of fungal endophytes associated with *Diospyros crassiflora*. *Mycology: An International Journal on Fungal Biology* **3**: 175–187.

Durán EL, Ploper LD, Ramallo JC, Piccolo Grandi RA, Hupper Giancoli ÁC, Azevedo JL, 2005. The foliar fungal endophytes of *Citrus limon* in Argentina. *Canadian Journal of Botany* **83**: 350–355.

Ezeibekwe IO, Unamba CIN, 2009. Pathogens associated with citrus fruit rots in Imo State of Nigeria. *Journal of American Science* **5**: 129–132.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Foyer CH, Shigeoka S, 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology* **155**: 93–100.

Freeman S, Katan T, Shabi E, 1998. Characterization of *Colletotrichum* species responsible for anthracnose disease of various fruits. *Plant Disease* **82**: 596–605.

Fröhlich J, Hyde KD, Petrini O, 2000. Endophytic fungi associated with palms. *Mycological Research* **104**: 1202–1212.

Ghimire SR, Charlton ND, Bell JD, Krishnamurthy YL, Craven KD, 2011. Biodiversity of fungal endophyte communities inhabiting switchgrass (*Panicum virgatum* L.) growing in the native tallgrass prairie of northern Oklahoma. *Fungal Diversity* **47**: 19–27.

Glienke-Blanco C, Aguilar-Vildoso CI, Vieira MLC, Barroso PAV, Azevedo JL, 2002. Genetic variability in the endophytic fungus *Guignardia citricarpa* isolated from citrus plants. *Genetics and Molecular Biology* **25**: 251–255.

Glienke C, Pereira OL, Stringari D, Fabris J, Kava-Cordeiro V, Galli-Terasawa L, Cunnington J, Shivas RG, Groenewald JZ, Crous PW, 2011. Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. *Persoonia* **26**: 47–56.

Goodwin SB, Dunkle LD, Zismann VL, 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. *Phytopathology* **91**: 648–658.

Gosling P, Hodge A, Goodlass G, Bending GD, 2006. Arbuscular mycorrhizal fungi and organic farming. *Agriculture, Ecosystems and Environment* **113**: 17–35.

Groenewald JZ, Nakashima C, Nishikawa J, Shin H-D, Park J-H, Jama AN, Groenewald M, Braun U, Crous PW, 2013. Species concepts in *Cercospora*: spotting the weeds among the roses. *Studies in Mycology* **75**: 115–170.

Gundel PE, Garibaldi LA, Martínez-Ghersa MA, Ghersa CM, 2001. Neotyphodium endophyte transmission to Lolium multiflorum seeds depends on the host plant fitness. *Environmental and Experimental Botany* **71**: 359–366.

Hamilton CE, Bauerle TL, 2012. A new currency for mutualism? Fungal endophytes alter antioxidant activity in hosts responding to drought. *Fungal Diversity* **54**: 39–49.

Hamilton CE, Dowling TE, Faeth SH, 2010. Hybridization in endophyte symbionts alters host response to moisture and nutrient treatments. *Microbial Ecology* **59**: 768–775.

Hata K, Atari R, Sone K, 2002. Isolation of endophytic fungi from leaves of *Pasania edulis* and their within-leaf distributions. *Mycoscience* **43**: 369–373.

He X, Han G, Lin Y, Tian X, Xiang C, Tian Q, Wang F, He Z, 2012. Diversity and decomposition potential of endophytes in leaves of a Cinnamomum camphora plantation in China. *Ecological Research* **27**: 273–284.

Hoffman MT, Arnold AE, 2008. Geographic locality and host identity shape fungal endophyte communities in cupressaceous trees. *Mycological Research* **112**: 331–344.

Holmes KA, Schroers HJ, Thomas SE, Evans HC, Samuels GJ, 2004. Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin of South America. *Mycological Progress* **3**: 199–210.

Hung TH, Hung SC, Chen CN, Hsu MH, Su HJ, 2004. Detection by PCR of *Candidatus liberibacter* asiaticus, the bacterium causing citrus greening in vector psyllids: application to the study of vector-pathogen relationship. *Plant Pathology* **53**: 96–102.

Hyde KD, Soytong K, 2008. The fungal endophyte dilemma. *Fungal Diversity* **33**: 163–173.

Jagoueix S, Bové JM, Garnier M, 1996. PCR detection of the two „*Candidatus*" Liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes* **10**: 43–50.

Jinantana J, Sariah M, 1997. Antagonistic effect of Malaysian isolates of *Trichoderma harzianum* and *Gliocladium* Viren on Sclerotium rolfsii. *Pertanika: Journal of Tropical Agricultural Science* **20**: 35–41.

Johnston PR, Johansen RB, Williams AF, Paula Wikie J, Park D, 2012. Patterns of fungal diversity in New Zealand Nothofagus forests. *Fungal Biology* **116**: 401–412.

Ko Ko TW, Stephenson SL, Bahkali AH, Hyde KD, 2011. From morphology to molecular biology: can we use sequence data to identify fungal endophytes? *Fungal Diversity* **50**: 113–120.

Kriel WM, Swart WJ, Crous PW, 2000. Foliar endophytes and their interactions with host plants, with specific reference to the Gymnospermae. In: Callow JA (ed), *Advances in Botanical Research*. Academic Press, London, pp. 1–34.

Kuldau G, Bacon C, 2008. Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. *Biological Control* **46**: 57–71.

Kuramae-Izioka EE, Lopes CR, Souza NL, Machado MA, 1997. Morphological and molecular characterization of *Colletotrichum* spp. From citrus orchards affected by postbloom fruit drop in Brazil. *European Journal of Plant Pathology* **103**: 323–329.

Larkin BG, Hunt LS, Ramsey PW, 2012. Foliar nutrients shape fungal endophyte communities in Western white pine (*Pinus monticola*) with implications for white-tailed deer herbivory. *Fungal Ecology* **5**: 252–260.

Lappalainen JH, Koricheva J, Helander ML, Haukioja E, 1999. Densities of endophytic fungi and performance of leafminers (Lepidoptera: Eriocraniidae) on birch along a pollution gradient. *Environmental Pollution* **104**: 99–105.

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee I-M, Duan Y, Levy L, 2009. Multiplex real-time PCR for detection, identification and quantification of „*Candidatus liberibacter* solanacearum" in potato plants with zebra chip. *Journal of Microbiological Methods* **78**: 59–65.

Lima WG, Sposito MB, Amorim L, Gonçalves FP, de Filho PAM, 2011. *Colletotrichum gloeosporioides*, a new causal agent of citrus post-bloom fruit drop. *European Journal of Plant Pathology* **131**: 157–165.

Linnakoski R, Puhakka-Tarvainen H, Pappinen A, 2012. Endophytic fungi isolated from *Khaya anthotheca* in Ghana. *Fungal Ecology* **5**: 298–308.

Lyons PC, Evans JJ, Bacon CW, 1990. Effects of the fungal endophyte *Acremonium coenophialum* on nitrogen accumulation and metabolism in Tall Fescue. *Plant Physiology* **92**: 726–732.

Lubbe CM, Denman S, Cannon PF, Groenewald JZ, Lamprecht SC, Crous PW, 2004. Characterization of Colletotrichum species associated with diseases of Proteaceae. *Mycologia* **96**: 1268–1279.

Magurran AE, 2004. *Measuring Biological Diversity*. Blackwell Science, Oxford, UK.

Maki CS, 2006. *Diversidade e potencial biotecnológico de fungos endofíticos de cacau (Theobroma cacao L.)*. PhD thesis, Universidade de São Paulo, Piracicaba.

Mei C, Flinn BS, 2010. The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. *Recent Patents on Biotechnology* **4**: 81–95.

Moriwaki J, Sato T, Tsukiboshi T, 2003. Morphological and molecular characterization of *Colletotrichum boninense* sp. nov. from Japan. *Mycoscience* **44**: 47–53.

Photita W, Lumyong S, Lumyong P, McKenzie EHC, Hyde KD, 2004. Are some endophytes of *Musa acuminata* latent pathogens? *Fungal Diversity* **16**: 131–140.

Pretorius MC, Crous PW, Groenewald, Braun U, 2003. Phylogeny of some cercosporoid fungi from *Citrus*. *Sydowia* **55**: 286–305.

Rakotoniriana EF, Munaut F, Decock C, Randriamampionona D, Andriambololoniaina M, Rakotomalala T, Rakotonirina EJ, Rabemanantsoa C, Cheuk K, Ratsimamanga SU, Mahillon J, El-Jaziri M, Quetin-Leclercq J, Corbisier AM, 2008. Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves. *Antonie van Leeuwenhoek* **93**: 27–36.

Rambaut A, 2002. *Sequence Alignment Editor. Version 2.0*. Department of Zoology, University of Oxford, South Parks Road, Oxford OXI 4JD, U.K.

Rocha DOL, Reis GM, da Silva VN, Braghini R, Teixeira MM, Correa B, 2011. Molecular characterization and fumonisin production by *Fusarium verticillioides* isolated from corn grains of different geographic origins in Brazil. *International Journal of Food Microbiology* **145**: 9–21.

Rodrigues A, Mueller UG, Ishak HD, Bacci Jr M, Pagnocca FC, 2011. Ecology of microfungal communities in gardens of fungus-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine ants in Central Texas. *FEMS Microbiology Ecology* **78**: 244–255.

Rojas EI, Rehner SA, Samuels GJ, Van Bael S, Herre EA, Cannon P, Chen R, Pang J, Wang R, Zhang Y, Peng Y-Q, Sha T, 2010. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panamá: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia* **102**: 1318–1338.

Romero A, Carrion G, Rico-Gray V, 2001. Fungal latent pathogens and endophytes from leaves of *Parthenium hysterophorus* (Asteraceae). *Fungal Diversity* **7**: 81–87.

Rubini MR, Silva-Ribeiro RT, Pomella AWV, Maki CS, Araújo WL, dos Santos D, Azevedo JL, 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao L.*) and biological control of *Crinipellis perniciosa*, causal agent of Witches' Broom Disease. *International Journal of Biological Sciences* **1**: 24–33.

Saikkonen K, 2007. Forest structure and fungal endophytes. *Fungal Biology Reviews* **21**: 67–74.

Sagaram US, DeAngelis KM, Trivedi P, Andersen GL, Lu S-E, Wang N, 2009. Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using PhyloChip arrays and 16S rRNA gene clone library sequencing. *Applied and Environmental Microbiology* **75**: 1566–1574.

Santamaria J, Bayman P, 2005. Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). *Microbial Ecology* **50**: 1–8.

Shivas RG, McTaggart AR, Young AJ, Crous PW, 2010. *Zasmidium scaevolicola*. Fungal Planet 47. *Persoonia* **24**: 132–133.

Sijam K, Ahmad K, Kamaruzaman S, Habibuddin H, Jugah K, Syed OSR, 2008. Occurrence and spread of *Candiidatus*

liberibacter asiaticus, the causal agent of Huanglongbin disease of citrus in Malaysia. *Research Journal of Agriculture and Biological Sciences* **4**: 103–111.

Silva-Rojas HV, Ávila-Quezada GD, 2011. Phylogenetic and morphological identification of *Colletotrichum boninense*: a novel causal agent of anthracnose in avocado. *Plant Pathology* **60**: 899–908.

Singh P, Raghukumar C, Verna P, Shouche Y, 2011. Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach. *Microbial Ecology* **61**: 507–517.

Slippers B, Wingfield MJ, 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Review* **21**: 90–106.

Silvestro D, Michalak I, 2012. raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity & Evolution* **12**: 335–337.

Simon UK, Groenewald JZ, Crous PW, 2009. Cymadothea trifolii, an obligate biotrophic leaf parasite of Trifolium, belongs to Mycosphaerellaceae as shown by nuclear ribosomal DNA analyses. *Persoonia* **22**: 49–55.

Sonoda RM, Pelosi RR, 1988. Characteristics of *Colletotrichum gloeosporioides* from lesions on citrus blossoms in the Indian River area of Florida. *Proceedings of the Florida State Horticultural Society* **101**: 36–38.

Spiering MJ, Greer DH, Schmid J, 2006. Effects of the fungal endophyte, *Neotyphodium lolii*, on net photosynthesis and growth rates of perennial ryegrass (*Lolium perenne*) are independent of in Planta endophyte concentration. *Annals of Botany* **98**: 379–387.

Stamatakis A, 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Sumarah MW, Kesting JR, Sørensen D, Miller JD, 2011. Antifungal metabolites from fungal endophytes of *Pinus strobus*. *Phytochemistry* **72**: 1833–1837.

Suryanarayanan TS, Murali TS, Venkatesan G, 2002. Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. *Canadian Journal of Botany* **80**: 818–826.

Swofford DL, 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods), Version* 4. Sinauer, Sunder-land, Massachusetts.

Tadych M, Bergen MS, Johnson-Cicalese J, Polashock JJ, Vorsa N, White Jr JF, 2012. Endophytic and pathogenic fungi of developing cranberry ovaries from flower to mature fruit: diversity and succession. *Fungal Diversity* **54**: 101–116.

Tarnowski TLB, Ploetz RC, 2010. First report of *Colletotrichum boninense*, *C. capsici*, and a *Glomerella* sp. as causes of postharvest anthracnose of passion fruit in Florida. *Plant Disease* **94**: 786.

Teixeira DC, Saillard C, Eveillard S, Danet JL, da Costa PI, Ayres AJ, Bové J, 2005. '*Candidatus liberibacter* americanus', associated with citrus Huanglongbing (greening disease) in São Paulo State, Brazil. *International Journal of Systematic and Evolutionary Microbiology* **55**: 1857–1862.

Thomas SE, Crozier J, Aime MC, Evans HC, Holmes KA, 2008. Molecular characterisation of endophytic morphospecies associated with the indigenous forest tree, *Theobroma gileri*, in Ecuador. *Mycological Research* **112**: 852–860.

Tondje PR, Hebbar KP, Samuels G, Bowers JH, Weise S, Nyemb E, Begoude D, Foko J, Fontem D, 2006. Bioassay of *Genicolosporium* species for *Phytophthora megakarya* biological control on cacao pod husk pieces. *African Journal of Biotechnology* **5**: 648–652.

U'ren JM, Dalling JW, Gallery RE, Maddison DR, Davis EC, Gibson CM, Arnold AE, 2009. Diversity and evolutionary origins of fungi associated with seeds of a neotropical pioneer tree: a case study for analysing fungal environmental samples. *Mycological Research* **113**: 432–449.

Van Bael SA, Fernandez-Marin H, Valencia MC, Rojas EI, Wcislo WT, Herre EA, 2009. Two fungal symbioses collide: endophytic fungi are not welcome in leaf-cutting ant gardens. *Proceeding of Biological Sciences* **276**: 2419–2426.

Vicari M, Hatcher PE, Ayres PG, 2002. Combined effect of foliar and mycorrhizal endophytes on an insect herbivore. *Ecology* **83**: 2452–2464.

Wäli PR, Helander M, Nissinen O, Lehtonen P, Saikkonen K, 2008. Endophyte infection, nutrient status of the soil and duration of snow cover influence the performance of meadow fescue in sub-artic conditions. *Grass and Forage Science* **63**: 324–330.

Wang X, Chen G, Huang F, Zhang J, Hyde KD, Li H, 2012. Phyllosticta species associated with citrus diseases in China. *Fungal Diversity* **52**: 209–224.

Wei JG, Xu T, Guo LD, Liu AR, Zhang Y, Pan XH, 2007. Endophytic *Pestalotiopsis* species associated with plants of Podocarpaceae, Theaceae and Taxaceae in southern China. *Fungal Diversiy* **24**: 55–74.

Weir BS, Johnston PR, Damm U, 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology* **73**: 115–180.

White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: a guide to methods and applications*. Academic Press Inc, New York, pp. 315–322.

Wong M-H, Crous PW, Henderson J, Groenewald JZ, Drenth A, 2012. Phyllosticta species associated with freckle disease of banana. *Fungal Diversity* **56**: 173–187.

Wu MD, Li GQ, Jiang DH, 2009. First report of Pestalotiopsis microspora causing leaf blight of Reineckea carnea in Central China. *Plant Disease* **93**: 667.

Wulandari NF, To-Anun C, Hyde KD, Duong LM, de Gruyter J, Meffert JP, Groenewald JZ, Crous PW, 2009. *Phyllosticta citriasiana* sp. nov., the cause of Citrus tan spot of *Citrus maxima* in Asia. *Fungal Diversity* **34**: 23–39.

Wright JD, 1998. *The Role of Endophytes in Citrus Stem End Rots*. PhD thesis, University of Hong Kong, Hon Kong.

Zhang DX, Nagabhyru P, Schardl CL, 2009. Regulation of a chemical defence against herbivory produced by symbiotic fungi in grass plants. *Plant Physiology* **150**: 1072–1082.

Zimmerman NB, Vitousek PM, 2012. Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proceedings of the National Academy of Sciences* **109**: 13022–13027.

Zhu R, Macfie SM, Ding Z, 2005. Cadmium-induced plant stress investigated by scanning electrochemical microscopy. *Journal of Experimental Botany* **56**: 2831–2838.