Commentary

The fungal endophyte dilemma

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Much has been written on endophytes and there have been many definitions of endophytes proposed. In this commentary we comment on the considerable amount of work that has been published on endophytes and detail the various definitions that have been put forward. Mycorrhizal fungi occur within roots which are symptomless and most definitions of endophytes would accommodate this ecological group. Mycorrhizae however, are not usually regarded in the same mode as endophytes. We highlight what we consider to be some of the major advances in endophyte studies and briefly comment on the occurrence of endophytes and their roles. The mutualistic role of non grass endophytes is mostly speculative and here we explore the roles of endophytes as saprobes and/or latent plant pathogens. Endophytes, especially those from medicinal plants have become the focus of research for bioactive compounds – we provide possible explanations for this. The study of endophytes is alluring and we propose reasons for this; high diversity; easy to apply statistics; easy to study. We discuss some important needs when pursuing endophyte studies. The isolation of endophytes is a method-dependent process and therefore it should be realized that in any study, the endophytes isolated will be dependent on the methodology used and intensity of the study. Direct analysis of DNA may provide an alternative method to reveal endophytes present in plant material.

Key words: DGGE, diversity, ecological role, host-specificity, methodology, mycorrhizae

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Introduction

There has been much written on fungal endophytes this year (2008) with an issue of *Fungal Biology Reveiws* and this issue of *Fungal Diversity* devoted to the subject. Several decades of research and numerous articles on endophytic fungi in plants have resulted in a plethora of knowledge of the group. The data in part however, has been biased by the methodology used, and one question that we should ask is "*how much do we really know about fungal endophytes, especially the non-grass endophytes*?" In this paper we will 1) précis the information on endophytes; especially their definition and role; 2) briefly visit natural products discovery from endophytes; 3) explain why endophyte studies appear to be alluring; 4) point out the pitfalls of endophyte studies; and 5) draw attention to the need to find new ways to study endophytes. The paper will address the mystical non-grass endophytes as much less is known concerning the role of these micro-organisms.

What are endophytes

Much has been written on endophytes; they have been defined in many ways and there have been many reviews and even books on the subject. So what is the best definition for plant endophytes? The most commonly used definition is that of Petrini (1991), "*All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host*" however, there are many alternatives (Box 1).

The term "endophyte" was introduced by De Bary and was for some time applied to "any organisms occurring within plant tissues" (De Bary, 1866)

"Mutualists, those fungi that colonize aerial parts of living plant tissues and do not cause symptoms of disease (Carroll, 1986)

"Fungi that form unapparent infections within leaves and stems of healthy plants (Carroll, 1988)

"Fungi as colonizers of the living internal tissues of their plant host" (Rollinger and Langenheim, 1993)

"All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host" (Petrini, 1991)

"A group that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Hirsch and Braun, 1992).

"Endophytes are any fungi isolated from internal symptomless plant tissues" (Cabral *et al*., 1993)

"Fungi and bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease" (Wilson, 1995)

"Infection strategy is regarded as important in the definition of the term endophyte" (Wilson, 1995)

"The term endophyte should be clearly defined in any study" (Brown *et al*., 1998)

"*True endophytes* – fungi whose colonization never results in visible disease symptoms (Mostert *et al*., 2000)

"Fungi that colonize a plant without causing visible disease symptoms at any specific moment" (Schulz and Boyle, 2005)

Box 1. Definitions of the term endophyte

In this issue of *Fungal Diversity* there are also three papers on mycorrhizae in roots. Mycorrhizae grow in symbiotic association with plants and both benefit from this association (Rinaldi *et al*., 2008). Most of the definitions of endophytes (Box 1) would accommodate mycorrhizae, however, it "*goes without saying*" that these lifestyles differ and should not be confused. The fungi associated with the roots of orchids are usually termed mycorrhizae, but the paper by Tao *et al*. (2008) reveal that "real" endophytes may also be present in the roots and other tissues of orchids. Arnold (2007) explores the problem of endophytes versus mycorrhizae and illustrate that the mycorrhizae found in roots are mostly different in taxonomic composition to those endophytes found in leaves. However, there are always exceptions to the rule; *Cenococcum geophilum* has been observed as a foliar endophyte and as a mycorrhizal associate (Arnold, 2007).

History of endophyte studies

The earliest record of mutualistic symbiosis is in the roots of the fossil tree *Amyelon radicians* from the Paleozoic era (Bacon and Hill, 1996). This is considered important not only from the standpoint of the original of endophytic symbiosis, but also for evidence that plant-fungus associations occurred very early in evolutionary terms. The major advances in endophyte studies are listed in Box 2.

- 1898 - Asymptomless endophytes were first isolated and cultured from seeds of *Lolium temulentum* (Vogl, 1898, cited by Wilson, 1996).
- 1933-1989 - This discovery prompted a series of studies in which similar asymptomatc endophytes were recorded in a wide range of grasses (Sampson, 1938; Latch *et al*., 1985; Saha *et al*., 1987; White, 1987; Clay and Leuchtmann, 1989).
- 1977-1983 - Extensive work on endophytes of conifers (Carroll *et al*., 1977; Carroll and Carroll, 1978; Carroll and Petrini, 1983)
- 1982-2000 - Significant contribution to knowledge of endophytes (Petrini and Petrini 1985; Petrini, 1991, 1996)
- 1987-1991 - Contribution to UK (and European) endophytes (Fisher and Petrini, 1987, 1990; Petrini and Fisher, 1990; Fisher *et al*., 1991).
- 1990-2000 - Extensive work on endophytes of palms (Rodrigues and Samuels, 1990; Rodrigues, 1994; Taylor *et al*., 1999; Fröhlich *et al*., 2000).
- 1996 - *Endophytic fungi in grasses and woody plants* - book on endophytes published by Redlin and Carris (1996).
- 1998 - Introduction of leaf imprint method for checking isolation protocols (Schulz *et al*., 1998).
- 2000-2008 - Significant contribution to knowledge of tropical endophytes (e.g. Suryanarayanan and Kumaresan, 2000).)
- 2000-2008 - Significant contribution to knowledge of endophytes (e.g. Arnold *et al*., 2000; Arnold, 2007)
- 2004 - Review of fungal endophytes. Topics include definitions, host-specificity, isolation techniques, modes of infection and colonization, molecular characterization and roles (Ghimire and Hyde, 2004).
- 2005 - Review, *the endophyte continuum*. Topics addressed include definitions, balansiaceous and non-balansiaceous endophytes, colonization, diversity and secondary metabolites. Much of the review focuses on mechanisms of interaction between the endophytes and host (Schulz and Boyle, 2005).
- 2008 – Issue of *Fungal Biology Reviews* dedicated to endophytes
- 2008 – Issue of *Fungal Diversity* dedicated to endophyte studies

Box 2. History of endophyte publications

Occurrence and biodiversity of non-grass endophytes

Endophytes have been isolated from all plants studied to date. Plants range from large trees (Gonthier *et al*., 2006; Oses *et al*., 2008), palms (Taylor *et al*., 1999; Fröhlich *et al*., 2000), sea grasses (Alva *et al*., 2002), and even lichens (Li *et al*., 2007). The numbers of strains and species of endophytes vary considerably and generally depend on the intensity of the study. In conclusion, 1) the numbers of endophytes (strains and species) depends on how much care, time and Petri-dishes are used in a study; a meticulous researcher will laboriously isolate thousands of strains and consequently more species; a lackadaisical researcher will achieve the opposite; 2) temperate plants yield different communities of endophytes as those from tropical plants; 3) different tissues may yield different endophyte communities and 4) we know very little about the role of endophytes (Johnston *et al*., 2006; Sieber 2007). Most endophytes isolated to date have been ascomycetes and their anamorphs, however Rungjindamai *et al*. (2008) show that several endophytes may also basidiomycetes.

Role of endophytes

When discussing the role of endophytes; the endophytes are usually categorized as clavicipitalean (grass-inhabiting) and non clavicipitalean (generally non grass-inhabiting). The functions of grass endophytes are generally better known (see Clay, 1988, 1996, 1997); whereas the role of non-grass endophytes is rather mystical. Here, we will explore the roles of non-grass endophytes.

Much has been written on the roles of non grass endophytes (Arnold, 2007; Sieber, 2007; Slippers and Wingfield, 2007). They have been implicated in mutualism, decreased herbivory, increased drought resistance, increased disease resistance and enhancement of plant growth (Fröhlich *et al*., 2000; Sieber, 2007). However, demonstration of mutualism under field conditions has been mostly inconclusive (Sieber, 2007).

Much more needs to be done to prove that endophytes have beneficial traits for plants. Koch's postulate should be performed to prove the endophyte life style, however, few studies have come close to fulfilling these requirements (Sieber, 2007). Several studies however, do indicate reduction in herbivory in

plants colonized with certain endophyte species (see Sieber; 2007).

Endophytes become primary saprobic decomposers

Several recent studies have explored relationships between endophytes and their role as saprobes (Hyde *et al*., 2007; Promputtha *et al*., 2007). The evidence is circumstantial; however, it seems likely that some (or many) saprobes are derived from endophytes (Hyde *et al*., 2007; Promputtha *et al*., 2007; Duong *et al*., 2008). If this hypothesis is correct and saprobes are derived from endophytes then it is more likely that they would be host or tissue-specific. Endophytes may have developed intimate relationships with their hosts during evolution and may be host- or even tissue-specific (Zhou and Hyde, 2001; Tao *et al*., 2008). Several studies provide evidence to support the hypothesis that saprobe host specificity in plants is dependent on internal endophytes, while others indicate that host components may regulate the endophytes within (Paulus *et al*., 2006). Whatever the reason it is clear that many endophytes in leaves (and woody tissues) are host, host genus or host family specific (Arnold, 2007) and that this specificity must depend on factors such as initial endophyte colonization and/or substances within leaves and wood (Paulus *et al*., 2006; Arnold, 2007; Hyde *et al*., 2007).

Endophytes as mutualists and latent pathogens

Some endophytes are known to be latent pathogens and much has been written on the subject (see Brown *et al*., 1998; Photita *et al*., 2004; Sieber, 2007; Slippers and Wingfield, 2007). There are numerous examples of endophytes that become pathogens (see Table 1 for some examples, Brown *et al*., 1998). Sieber (2007) states that endophytic "pathogens" have co-evolved with their hosts and are thus not highly virulent. These "pathogens" must at some time sporulate and when leaves senesce, or the plants are stressed or when the plants produce fruit that will eventually rot – this is the ideal time to sporulate. Much has been written on the subject of mutualism and latent pathogens (see Sieber, 2007) and the topics will not be addressed further.

Table 1. Some examples of endophytes that have been shown to be latent pathogens

Host	Latent pathogen	Disease	Reference
Citrus spp.	*Phomopsis citri*	Stem end rot	Wright, 1998
	Fusicoccum aesculi	Stem end rot	Wright, 1998
	Lasiodiplodia theobromae	Stem end rot	Wright, 1998
Vitis vinifera	*Phomopsis viticola*	Leaf lesions	Mostert *et al*., 2000

Discovery of natural products from endophytes

There has been considerable interest in screening endophytes for novel compounds (e.g. Kumar and Hyde, 2004; Tejesvi *et al*., 2007) and Dreyfuss and Chapela (1994) predicted that endophytic fungi are potentially a major source for new, useful metabolites. Until 2003 approximately 4,000 secondary metabolites with biological activity had been described from fungi (Dreyfuss and Chapela, 1994). Most of these metabolites are produced by so called "creative fungi" which include species of *Acremonium*, *Aspergillus*, *Fusarium* and *Penicillium*, but there has been less research on endophytes ability to produce novel metabolites. Schulz *et al*. (2002) isolated around 6500 endophytic fungi and tested their biological potential. They analyzed 135 secondary metabolites and found that 51% of bioactive compounds (38% for soil isolates) isolated from endophytic fungi were new natural products. Schulz *et al*. (2002) concluded that endophytic fungi are a good source of novel compounds and that "screening is not a random walk though a forest".

The discovery that Taxol could be produced by endophytes of the Yew tree (*Taxus* sp.) by Strobel *et al*. (1996) lead to an explosion of endophyte studies on Chinese and other medicinal plants (Tan and Zou, 2001; Strobel *et al*., 1999; Strobel, 2003; Strobel and Daisy, 2003; Tejesvi *et al*., 2007; Huang *et al*., 2008). The premise is based on the fact that endophytes have evolved with plants over a long evolutionary time (Taylor and Taylor,

2000) and that they may have changed genetic information with the plants and visa versa (Stierle *et al*., 1993). Fungal endophytes are known to produce metabolites that mimic the structure and function of host compounds (Strobel, 2002) - and can produce plant growth hormones such as gibberellins (MacMillan, 2002). Subglutinols which are produced by *Fusarium subglutinans*, an endophyte of the Chinese medicinal plant, *Tripterygium wilfordii* possess immunosuppressive properties and structural similarities to those of the active compounds in the plant (Lee *et al*., 1995; Strobel, 2002). The isolation of endophytes from medicinal plants followed by screening for bioactive compounds has become one of the main areas of endophyte research in recent years. (Kumar and Hyde, 2004; Tejesvi *et al*., 2007; Huang *et al*., 2008)

There may be an alternative explanation for the high number of bioactive compounds that have been found in endophytes (Schultz *et al*., 2002). The diversity of endophytes is high and endophytes are relatively fast growing on routinely used laboratory media. Many of the species are so-called "creative fungi" (Dreyfuss and Chapela, 1994) and produce large amount of novel compounds. It may be that the high diversity, fast growth and portion of "creative fungi" isolated as endophytes may account for the high diversity of novel compounds discovered. If saprobes were treated in a similar way, with isolation of only fast growing mostly "creative fungi" - then we might also obtain a high diversity of novel compounds.

The alluring endophytes

Considering the number of studies that have been carried out on plant endophytes - now at more than 15 papers per year (Arnold, 2007) - versus the significance of the data, one must ask the question – why are endophytes so alluring? Many of my past and present students have expressed a remarkable desire to study endophytes, usually without sound reasons. However, if a student has such enthusiasm to study a particular subject I would never without reason, hold them back. There are also many advantages to studying endophytes particularly in terms of attaining publishable

results:

1. Studies provide high taxon diversity, can be completed in the relative comfort of a laboratory with minimal fieldwork, and use a well-established traditional methodology that any motivated student can follow.
2. Most sporulating isolates are relatively easily identified (at least to genus) as they belong to less than 50 characteristic genera.
3. Various methodology can be applied to mycelia sterilia to promote sporulation; alternatively molecular methods can be utilized to identify these relatively fast growing morphotypes
4. Sophisticated statistics can be applied to the isolates which "appear" to have been derived from single random units and will satisfy the demands of any unforgiving non-fungal ecologist.
5. The relatively fast growing and "highly" diverse endophytes provide ideal tools for screening and novel compound discovery and they can easily be lodged in culture collections.

This is in contrast, for example to studies of saprobes from leaves, which require uncomfortable field work (in the tropics); the need to work lengthy hours to examine the material before it spoils; protracted microscope work and identification complexity; the intricacy and frustration in carrying out single spore isolations; the invariably slow growing strains; the criticisms in statistical analysis (how can the fruiting body represent the mycelia in the leaves), all to obtain a similarly high species diversity, albeit with greater taxonomic unpredictability. Thus if you had the choice as a student - what would you study?

Looking for endophytes

The mycologist new to fungal endophyte studies could be easily forgiven for not believing that endophytes really occur within plants. After all take a healthy leaf and surface sterilize it with some weak bleach - how can that possibly kill all epiphytes? However, there are methods to stain the leaves and view the endophytes within (see Sampson, 1938; Cabral *et al*., 1993; Alva *et al*., 2002). Johnston *et al*. (2006) used a creative technique to visualize endophytic fungi within leaves by detecting ß-

D-glucans in fungal cell walls. After looking at the micrographs in these publications even the most skeptical would accept that endophytes really do occur within living plants.

Problems with methodology

The problem in using traditional methodology to study endophytes has previously been recognized (see Duong *et al*., 2007; Hyde and Soytong, 2007). The study of endophytes is a method-dependent process (Guo *et al*., 2001). Therefore endophyte papers should always mention that there may be a problem in the methodology used. Researchers should realize that the techniques severely influence the fungal endophytes isolated. Therefore they should state the probable flaws of their study methods in manuscripts and move forward in the paper from the premise that the results are influenced by the methodology. This is better than having the reviewers point this out or no one picking it up at all!

Pilot studies are an essential component of traditional methodology used in endophytes studies (Fröhlich *et al*., 2000). Different hosts and plant organs require different sterilization times; thicker leaves require longer times than thin leaves. One must therefore establish the strength of free chlorine or other sterilants needed to make sure that all external fungi are removed from the substrate surface. Without doing this one will end up with large numbers of surface contaminants (e.g. *Aspergillus, Cladosporium, Penicillium*) if the sterilization is not effective or very few endophytes if too effecive.

Schultz *et al*. (1998) introduced a method for testing whether the surface sterilization methods efficiently eliminated epiphytes. This involves making leaf imprints on the agar surface. If no fungi grow out then the sterilization protocol can be deemed effective. Sánchez-Márquez *et al*. (2007) concluded that this is an excellent method for testing protocols for isolating endophytes and should be used in all endophyte studies. Future manuscripts should be questioned if they have not tested their protocols by using this method.

Probably the most important, yet rarely mentioned flaw in endophyte studies is the fact the pieces of plant material are placed on agar and that the endophytes isolated in any study are those that grow out (Gonthier *et al*., 2006; Devarajan and Suryanarayanan, 2006; Wei *et al*., 2007). There are numerous references to fungi being unable to grow in culture (unculturable; Duong *et al*., 2007; Seena *et al*., 2008; Tao *et al*., 2008; Zhu *et al*., 2008), commonly fungi are very slow growing (Zhu *et al*., 2008) and often fungi require specific media (e.g. *Ceratocystis*, van Wyk *et al*., 2007). Many endophytes are fastidious and will not grow on the artificial media routinely used (Guo *et al*., 2001). Thus, when we isolate endophytes by traditional methodology we conceivably only obtained the faster growing culturable fungi and it is highly probable that some or even numerous endophytes are never isolated (Guo *et al*., 2001; Duong *et al*., 2007; Hyde and Soytong, 2007). Even the addition of Rose Bengal (Frohlich *et al*., 2000) is unlikely to drastically improve this situation. This is most probably why *Colletotrichum*, *Phomopsis*, *Phyllosticta* and *xylariaceous* species are dominant endophytes and yet none of these genera commonly occur as saprobes (Wong and Hyde, 2001; Yanna *et al*., 2001; Photita *et al*., 2005). This is particularly true on palms where the dominant endophytes are *Colletotrichum*, *Phomopsis* and *Xylaria* taxa (Fröhlich and Hyde, 2000) and yet these fungi are rarely found on dead palms leaves (Fröhlich and Hyde, 2000; Taylor and Hyde, 2003; Pinnoi *et al*., 2006; Pinruan *et al*., 2007).

Any further work on endophytes resulting from traditional methodology must accept that the data has many inherent problems. This includes the fact that the biodiversity isolated is likely to be much lower than that actually present and that the endophyte communities isolated will be biased towards faster growing fungi that are capable of growing rapidly on the media used. They will comprise typical endophytes (e.g. *Colletotrichum*, *Phomopsis*, *Phyllosticta* and *Xylaria* species). Any future work would therefore be biased and therefore.

1) If statistics is applied to the data the evaluations (e.g. Frohlich *et al*., 2000; Kumar *et al*., 2004) will be unsound due to the above bias.
2) If molecular sequencing is used to identify mycelia sterilia (e.g. Lacap *et*

al., 2003; Wang *et al*., 2005; Promputtha *et al*., 2005, 2007; Sánchez-Márquez *et al*., 2007; Rungjindamai *et al*., 2008; Tao *et al*., 2008), it should be understood that only the faster growing endophytic taxa are being identified.

3) It is not wise to compare endophytic and saprobic communities as the endophytes isolated are biased towards those that are capable of growing rapidly on the media used; foliicolous saprobes on the other hand are often slow growing fungi.
4) Although endophytes can be screened for enzymes and novel compounds (e.g. Tejesvi *et al*., 2007; Ragukumar, 2008; Mitchell *et al*., 2008), the screening will not be representative of the total endophyte community present in the leaves.

Ways around the traditional methodology dilemma

Previous research into endophytes of plants may have provided incomplete data concerning endophyte biodiversity. Therefore serious thought needs to be applied to the problem of revealing the entire fungal endophyte communities present within plants. There have been several attempts to detect total fungal communities by extracting the entire host DNA (for example from leaves) with various methods to sequence individual taxa. Potentially successful methods include DNA cloning (Guo *et al*., 2000, 2001; Seena *et al*., 2008), DGGE (Duong *et al*., 2007; Tao *et al*., 2008) or T-RLFP (Nikolcheva and Bärlocher, 2004, 2005). Both DNA cloning and DGGE are labour intensive and resulted in low endophyte biodiversity. It appears that T-RLFP is only now being utilized for revealing endophyte diversity within plant tissues (ref).

Estimations of fungal communities by extraction of total DNA are further advanced in other groups, e.g. soil fungi (Schadt *et al*., 2003; Anderson *et al*., 2003; Anderson and Cairney, 2004), plant roots (Vandenkoornhuyse *et al*., 2002; Tao *et al*., 2008) and submerged leaves (Nikolcheva and Bärlocher, 2004, 2005; Seena *et al*., 2008). Seena *et al*. (2008) have used extraction of whole-community DNA, followed by amplification with fungal-specific primers and the establishment of ribosomal gene libraries to access the diversity of fungi in submerged decaying leaves. Phylogenetic analyses of randomly selected cloned sequences allow estimation of the frequencies of occurrence of various fungal groups based on their contribution to the community DNA pool (Vandenkoornhuyse *et al*., 2002; Schadt *et al*., 2003; Seena *et al*, 2008). This approach in theory, allows much greater resolution and unequivocal assignment to a taxon. Their total OTU numbers on decaying leaves in streams was high, and exceeded earlier estimates using DGGE and T-RLFP, also on leaves decaying in streams (Nikolcheva and Bärlocher, 2004, 2005).

There are potential errors in total DNA extraction; PCR-based errors have mainly been attributed to PCR biases and artefacts, and 2) data interpretation (Bidartando and Gardes, 2005; Wintzingerode *et al*., 1997). Obviously the molecular methods used are contentious and further work is needed to overcome problems. Different sequences from DGGE or T-RFLP may result in the same signal, and without further analyses the homogeneity of these sequences cannot be established (e.g. by extracting and sequencing the DNA from an individual DGGE band). The knowledge of the biases and errors in DNA extraction, followed by cloning and sequencing is also incomplete. Further progress is currently hampered by the relative scarcity of fungal and related eukaryotic sequences in databases; there is insufficient data to evaluate confidently the equivalency of percentage sequence similarities and species, genera or even families (Seena *et al*., 2008).

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