Fungi isolated from the endorhizosphere of halophytic plants from the Red Sea Coast of Egypt

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Endorhizosphere fungi were isolated from roots of halophytic plants collected in the intertidal region (*Avicennia marina*), salt marshes (*Arthrocnemum macrostachum*, *Halocnemum strobilecium*, *Limonastrum monopetalum Zygophyllum album* and *Z. simplex*) and salt affected land (*Tamarix nilotica*, *Zilla spinosa* and *Z. coccineum*) of the Red Sea Coast of Egypt. Twenty-three taxa in addition to Mycelia Sterilia and unidentified yeast species were recovered in low numbers. With the exception of *Chaetomium hamadae* (Ascomycetes) all endophytes were mitosporic taxa. The most common species were *Alternaria alternata*, *Cladosporium cladosporioides* and *Penicillium chrysogenum*. The majority of isolates were of rhizosphere origin, with the reminder being endophytic in origin. Correspondence analysis indicated that some fungi occurred more often on specific hosts. Species of the genus *Zygophyllum* and individuals of *Avicennia marina* from different sites co-ordinate closely with colonizing fungi. *Alternaria alternata* was found to be restricted to species of *Zygophyllum*, whereas *Papulaspora immersa* exclusively colonised *Limonastrum monopetalum*. *Alternaria alternata*, *Chaetomium hamadae*, *Conoplea olivacea*, *Conoplea* sp., *Papulaspora immersa* and *Trimatostroma* sp., were the fastest growing species in culture. The endophyte isolates were able to degrade cellulose, glucose, maltose, pectin, starch, sucrose and xylan. Conversely, tannin repressed the growth of the majority of taxa.

Key words: carbon source, detrended correspondence analysis, endophytes.

Introduction

The rhizosphere can hypothetically be divided into two main parts, the ecto- and endorhizosphere. The ectorhizosphere is the outer surface of the root, while the region inside the root is known as the endorhizosphere. Most fungi colonizing the ectorhizosphere are able to invade the endorhizosphere by direct penetration of the rhizodermis via occasional wounds (Old and Nicholson, 1975), and indirectly through stomata (Verhoeff, 1974). These taxa may therefore be isolated as endophytes using traditional endophyte isolation techniques (Guo *et al.*, 1998). There are also intercellular spaces within the roots that may protect internal fungi from surface sterilization and these fungi

may also be isolated as endophytes. Thus, the term endophyte refers to organisms colonizing hosts intracellularly. Endophytes were initially recorded as outgrowths from wheat leaves (Unger, 1833) and their identification was a matter of speculation at the turn of last century (Vogl, 1898). Endophytes comprise a heterogeneous group of microorganisms; primarily ascomycetes and their anamorphs (Petrini, 1986; Petrini *et al.*, 1992).

Research on endophytes has mainly been concerned with parasitic *Clavicipitaceae* which occur within grasses (Bacon *et al.*, 1977; White *et al.*, 1996). Cryptic or symptomless fungal infection of non grass hosts, also termed "endophytes" have been recorded from deciduous trees and shrubs (Petrini and Fisher, 1990; Lodge *et al.*, 1996), angiosperm parasites (Suryanarayanan *et al.*, 2000), bamboo (Umali *et al.*, 1999), lichens (Petrini *et al.*, 1990), palms (Taylor *et al.*, 1999; Fröhlich *et al.*, 2000), and mangroves (Southcott and Johnson, 1997; Suryanarayanan *et al.*, 1998). Recently endophytes of roots have been studied. They are predominantly soil fungi (Fisher *et al.*, 1991a,b; Holdenrieder and Sieber, 1992) that are frequently isolated as endophytes from leaves and bark (Bills and Polishook, 1991; Peláez *et al.*, 1998). Mitosporic taxa (hyphomycetes) are predominant colonizers of roots of conifers (Summerbell, 1989; Holdenrieder and Sieber, 1992), orchids (Currah *et al.*, 1990), alpine and subalpine plants (Stoyke *et al.*, 1992), ericaceous plants (Douglas *et al.*, 1989), *Cupressaceae*, *Betulaceae* and *Fagaceae* (Ahlich and Sieber, 1996) and seedlings (Wilcox and Wang, 1987). Ascomycetes have also been isolated from rhizoids of liverworts (Duckett and Read, 1995). A variety of sterile endophytes have been also isolated from Ericales (Duckett and Read, 1995). Phylloplane fungi have also frequently been isolated as endophytes (Cabral *et al.*, 1993).

Research on endophytes has previously concentrated on temperate woody plants, whereas until recently very little information was available on endophytes of tropical plants (Brown *et al.*, 1998; Taylor *et al.*, 1999; Umali *et al.*, 1999; Fröhlich *et al.*, 2000). Likewise, fungal endophytes in extreme environments, such as deserts, marine environments and salt marshes, are poorly known (Suryanarayanan *et al.*, 1998). Only few studies have been conducted on endophytes of xerophytes (Muhsin and Zwain, 1989) and halophytes (Fisher and Petrini, 1987). Although the history of endophytes date back 4400 years to grass seeds found in the tomb of a fifth Dynasty Egyptian Pharaoh (Lindau, 1940) and discovered by Vogl (1898) in seeds of *Lolium temulentum*, no subsequent work has been undertaken in this field in Egypt. To our knowledge, this is the first investigation providing data on the biodiversity and substrate utilization of endophytes from roots of Red Sea halophytes.

Materials and methods

Habitat

The study areas were located on the Egyptian coast of the Red Sea. The climate is arid, rainfall is Mediterranean (winter), daily temperature maxima is approximately 27 C in May, humidity is about 50% and tidal range is small. The aridity increases the rate of the evaporation from the marsh soil, the low precipitation leads to insufficient salt leaching and accumulation of salts in surface crusts. The coast mainly consists of vertically raised fossil cliffs with a series of small sheltered lagoon and bays with minimal wave action. These trap sediments and contain patches of sand and mud. This intertidal zone is particularly harsh with very high solar heating and desiccation stress, and a very low tidal range. These are favorable habitats for the growth of mangrove and other coastal vegetation. Mangroves or "Shura" (local name) are mainly represented by *Avicennia marina* in Ras Mohammed and areas from Abu-Shar southward to Halibe where *Rhizophora mucronata* are intermixed with *A. marina*. The areas on the land side are adjacent to sublittoral salt marshes that are exposed to sporadic inundation by tides and have poor vegetation cover. The general characteristics of Red Sea environment and selected habitats were discussed in detail in El-Morsy (1999). Plants collected for investigation are those studied by El-Morsy (1999) for ectorhizosphere fungi and include *Halocnemum strobilecium* from Abu-Shar; *Zygophyllum album* from Wady Safaga; *Z. coccineum* from W. Abu-Hamra El-bahri; *Z. simplex* from W. Gemal; *Arthrocnemum macrostachum* and *Limonastrum monopetalum* from Sherm luli; and *Tamarix nilotica* and *Zilla spinosa* both from Wady Shagra.

Sampling procedures and isolation methods

Plants were collected in May 1997. Ten individuals of each plant species were collected from the same location. Anchored roots of *Avicennia marina* were collected. Roots of each plant species were cut, placed in clean plastic bags, transported to the laboratory, and then stored in refrigerator at 4° C. Samples were processed within 48 h. Stored roots were dissected into small segments (approximately 5 × 20 mm^2), scrubbed under tap water and surface-sterilized by sequential immersion in 5% hypochlorite for 10 minutes, followed by 75% ethanol for 30 s. Surface sterilised segments from each root sample (4 segments/plate) were selected, laid out on 2% malt extract agar (MEA) and potato-dextrose agar (PDA) in seawater (salinity = 43 g/L), amended with 0.5 gm streptomycin, 2000 units penicillin G per plate and 0.3 g/L Rose Bengal. Seawater was used to provide halic microenvironment for emerged hyphae and to remove non halotolerant species. Ten plates were used for each root. The plates were then incubated at room temperature (20-25 C) for 15-30 d.

Developing colonies were then subcultured on 2% MEA slopes for further investigation.

Classification of fungi

Sporulating isolates was identified using various media for identification. Non sporulating strains were grouped as Mycelia Sterilia according to similarities in colony morphology (Taylor *et al.*, 1999). Voucher slides of all fungi identified were prepared and are held in the author's herbarium.

Cultural studies

To assess the endophytic isolates for their ability to utilize cellulose, glucose, maltose, pectin, starch, sucrose, tannin and xylan, Czapekes-Dox medium was used. Sucrose was substituted with either 10g of glucose and starch (ADWIC); maltose and tannin (Riedel-De Haën AG Seelze-Hannover); Cellulose, Citrus pectin (Polygalacturonic acid methyl ester) or xylan (Sigma). Plates were inoculated with 8 mm diam. mycelium plugs cut from the margins of actively growing colonies on 3% MEA (Oxoid) and incubated at 28 C for 10 d. Three replicates were tested for each species.

Data analysis

Community ordination was tested by detrended correspondence analysis (DCA). The analysis was performed using Canoco: a Fortran program version 2.1 (Ter Braak, 1988). This analysis builds upon the frequency of colonization of fungi in each host species (Table 1). The relative frequency of colonization was calculated as the number of species isolated from each plant divided by the total number of plants. The isolated species were classified as very frequent (> 20%) frequent (10-20%) or infrequent (<10%) as adapted from Tan and Leong (1989).

Results

Biodiversity

Twenty-three fungal taxa were isolated from all samples in addition to Mycelia Sterilia and unidentified yeasts (Table 1). With the exception of *Chaetomium hamadae* (Ascomycetes), all identified isolates were mitosporic taxa. *Penicillium chrysogenum* (45%), *Alternaria alternata* (27%) and *Cladosporium cladosporioides* (27%) were very frequent species.

The relationship between the endophytes and host plant species was evaluated using DCA. The ordination diagram (Fig. 1) shows the relationship between the ordination axes with eigenvalues of 0.962 and 0.687, where plants and microfungi are arranged on the basis of their frequency scores on the two

Table 1. Mean number and percentage of ocurrence of fungi isolated from the endorhizosphere of halophytes collected from the Red Sea coast of Egypt.

Fungal species	Plant species											% of occurrence	Remarks
	AMG	AMB	AMS	AMA	HST	LIM	TAN	ZAL	ZCO	ZSI	ZIL		
Acremonium strictum Gams	0	0	0	0	0	0	5	0	0	0	2	18%	F
Acremonium sp.1	0	0	0	0	0	0	6	0	0	0	0	9%	I
Acremonium sp.2	0	0	0	0	0	0	0	0	0	0	4	9%	I
Acremonium sp.3	0	0	0	0	0	0	0	0	0	0	5	9%	I
Alternaria alternata (Fres.) Keissler	0	0	0	0	0	0	0	6	5	4	0	27%	V
Aspergillus carneus (Van Tiegh) Bloohwitz	0	0	0	0	0	0	1	0	0	0	0	9%	I
A. niger Van Tiegh	0	1	0	0	2	0	0	0	0	0	0	18%	F
A. sulphureus Fres.	0	0	0	0	0	0	1	0	0	0	0	9%	I
Cladosporium cladosporoides (Fres.) de Varies	8	5	4	0	0	0	0	0	0	0	0	27%	V
Cl. sphaerospermum Penz.	0	4	4	0	0	0	0	0	0	0	0	18%	F
Nigrospora sphaerica (Sacc.) Mason	0	0	0	0	3	0	1	0	0	0	0	18%	F
Papulaspora immersa Hots.	0	0	0	0	0	7	0	0	0	0	0	9%	I
Penicillium levitum Raper and Fennel	0	1	0	0	0	0	0	0	0	0	0	9%	I
P. chrysogenum Thom	0	1	0	0	0	0	0	2	1	1	1	45%	V
P. purpurogenum Stoll.	0	0	0	0	3	0	0	0	0	0	0	9%	I
Scopulariopsis brevicaulis (Sacc.) Bain.	0	4	0	0	0	0	0	0	0	0	0	9%	I
Stachybotrys chartarum (Ehrenb.ex Link)	6	0	0	0	0	0	0	0	0	0	0	9%	I
Conoplea sp.	0	0	0	4	2	0	0	0	0	0	0	18%	F
Conoplea olivacea Fres.	0	0	0	4	2	0	0	0	0	0	0	18%	F
Sterile mycelia	0	0	0	0	4	0	0	0	0	0	0	9%	I
Trimmatostroma sp.	0	0	0	0	0	0	5	0	0	0	0	9%	I
Verticillium chlamydosporium Goddar	0	0	0	2	0	0	0	0	0	0	3	18%	F
V. cycolsporum (Grove) Mason and Hughes	1	5	0	0	0	0	0	0	0	0	0	18%	F
Yeast	0	0	0	0	0	0	0	0	0	0	1	9%	I
Chaetomium hamadae Udagawa	1	6	0	0	0	0	0	0	0	0	0	18%	F

AMB =*Avicennia marina* (from Abu-Shar), AMS =*A. marina* (from W. Safaga), AMG =*A. marina* (from W.Gemal), AMA = *A. macrostachyum*, HST= *H. strobilaceum*, ZAL= *Z. album*, ZCO= *Z.coccineum*, ZSI= *Z. simplex* , LIM= *L. monopetalum*, TAN= *Tamarix nilotica*, ZIL= *Zilla spinosa*, V = very frequent, F = frequent and I = infrequent.

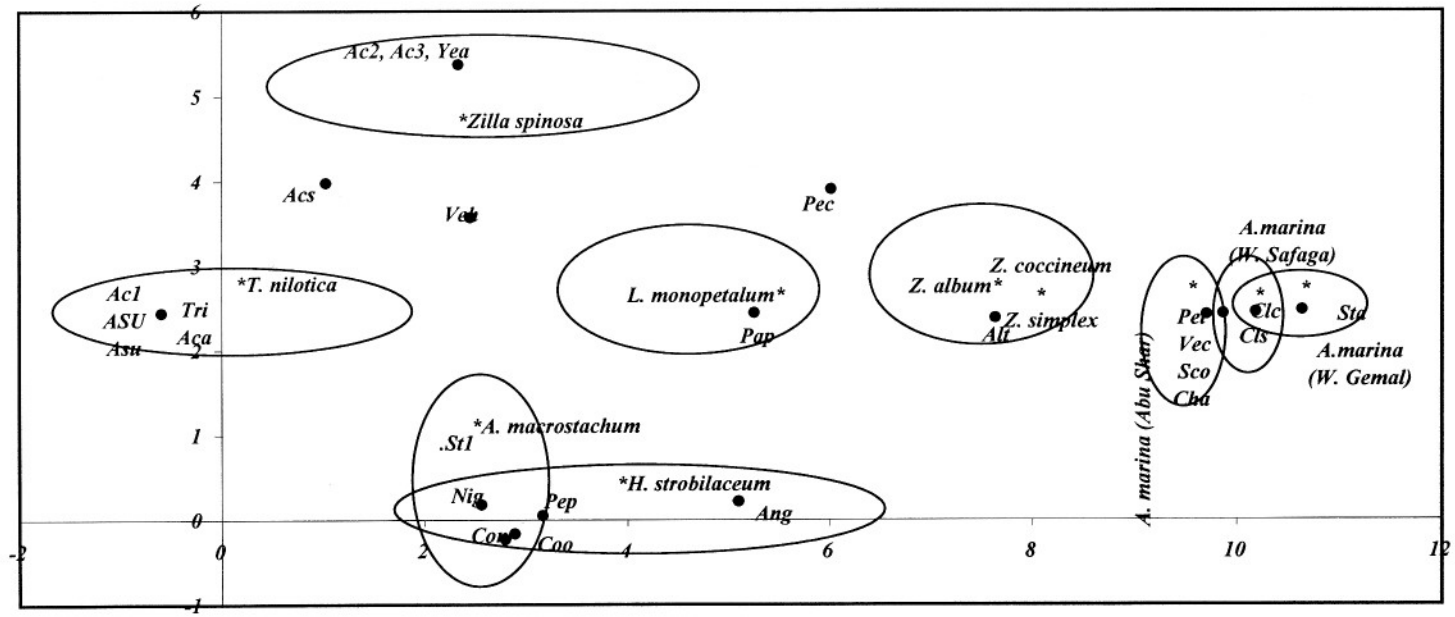

Fig. 1. Correspondence analysis. Ordination of fungal species (•) and their hosts (*), based upon frequency of occurrence (eigen values = 0.962 and 0.687). (Abbreviation: Ac1 = *Acremonium* sp. 1; Ac2 = *Acremonium* sp. 2; Ac3 = *Acremonium* sp. 3; Acs = *Acremonium strictum;* Alt = *Alternaria alternata*; Aca = *Aspergillus carneus*; Ang = *A. niger*; Asu = *A. sulphureus*; Cha = *Chaetomium hamadae*; Clc = *Cladosporium cladosporoides*; Cls = *Cl. sphaerospermum*; Con = *Conoplea* sp.; Coo = *Conoplea olivacea*; Nig = *Nigrospora sphaerica*; Pap = *Pa. immersa*; Pec = *Penicillium chrysogenum*; Pel = *P. levitum*; Pep = *P. purpurogenum*; Sco = *Scopulariopsis brevicaulis*; Sta = *Stachybotrys chartarum*; St1 = sterile mycelia 1; Tri = *Trimmatostroma* sp.; Veh = *Verticillium chlamydosporium*; Vec = *V. cycolsporum*; Yea = Yeast).

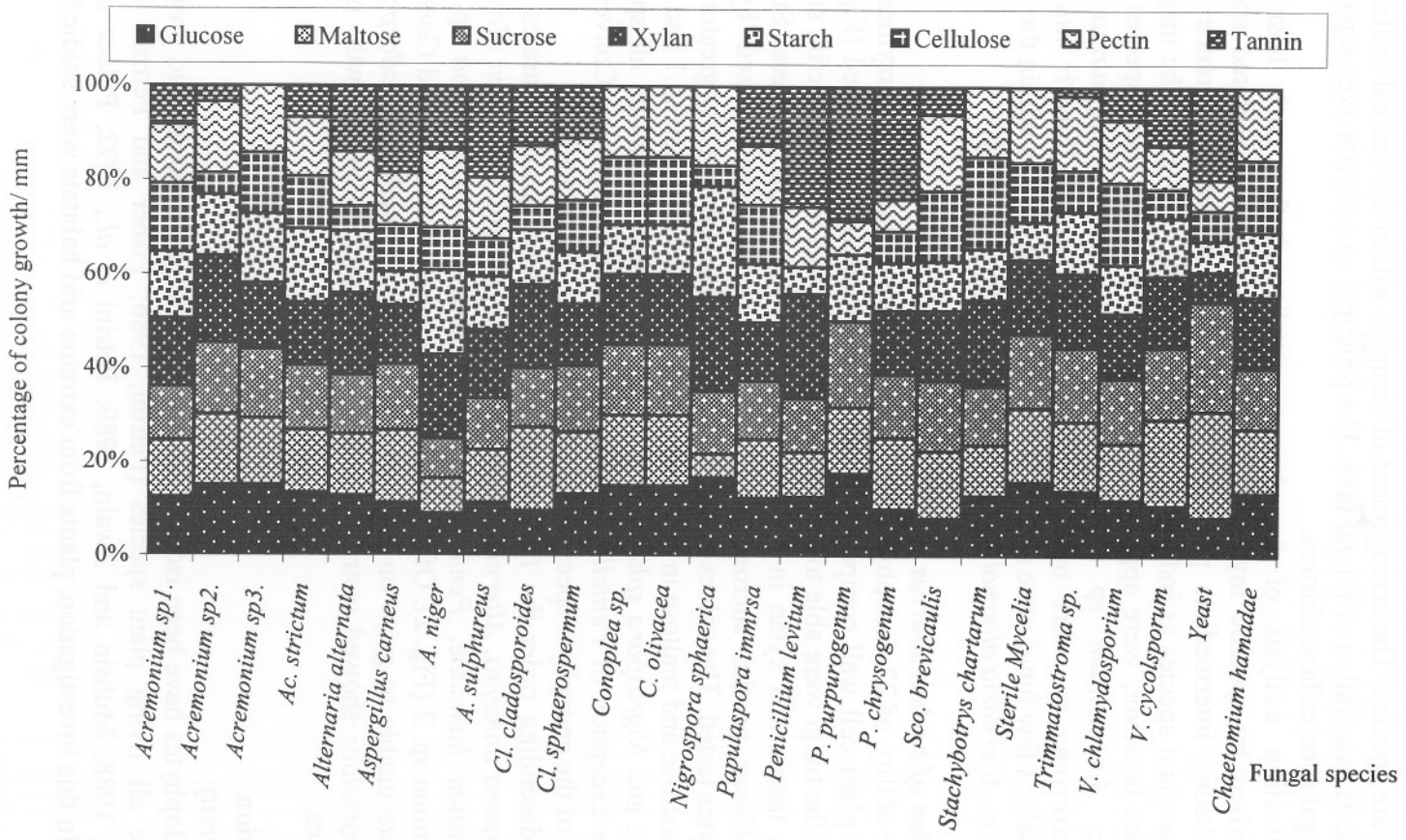

Fig. 2. Growth of endophytic fungi on glucose, xylan, starch, cellulose, maltose and tannin Czepeks-Dox media.

axes. The relative distance of a species to a plant indicates the relative abundance of that species on that particular plant. Species on the edges are usually rare species. The arrangement of samples within the second ordination axis reflects association of fungal taxa. The principal co-ordinate axes separated nine fungal species/host clusters.

Ordination analysis of fungal scores indicated that each host was characterised by specific mycota with only few frequently isolated species always taking intermediate positions. Some fungi showed recurrence on a particular plant species as indicated by their proximity in Fig. 1. The majority of species however, were apparently restricted to a single plant species. For example, *Acremonium* sp. 1, *Aspergillus carneus*, *A. sulphureus* and *Trimmatostroma* sp. were only associated with roots of *Tamatix nilotica*. Conversely, a few fungi were restricted to different host species within the same genus, e.g. *Alternaria alternata* was confined to *Zygophyllum* spp.

Utilization of carbon sources

The ability of 23 endophyte species to produce extracellular enzymes that degrade plant cell wall components and storage materials was tested (Fig. 2). Most of the fungi were able to utilize cellulose, glucose, maltose, pectin, starch, sucrose, tannin and xylan in Czapek's-Dox agar to variable degrees. Starch, xylan, *Citrus* pectin, sucrose, maltose and glucose were utilised by all endophytes tested. The values of utilised glucose were occasionally greater than that of sucrose and maltose in the same species, e.g. *Acremonium* sp.1 and sp.3, *A. niger*, and *Nigrospora sphaerica*. Cellulose was also utilised by all species with the exception of *Penicillium levitum* and *P. purpurogenum*. Conversely, only 17 of the endophyte species were able to utilize tannic acid.

In desending order the fastest growing species on most substrates were *Papulaspora immersa*, *Alternaria alternata*, *Conoplea* sp., *Conoplea olivacea*, *Chaetomium hamadae*, *Penicillium chrysogenum*, *Trimmatostroma* sp. and *Acremonium* sp. 2 (Fig. 2). Of these fungi *Chaetomium hamadae* and *Conoplea* spp. were unable to grow on tannic acid medium. Conversely, *Cladosporium cladosporioides* showed restricted growth (10-12 mm colony growth) on all substrates.

Discussion

Biodiversity

Endophytes have been isolated from a wide range of species and probably colonize all living plant species (Petrini, 1986; Fisher and Petrini, 1987; Carroll, 1988; Muhsin and Zwain, 1989; Petrini *et al.*, 1992; Peláez *et al.*, 1998). In this investigation, plants from extreme arid habitats were studied. The

number of species isolated was small and consisted primarily of mitosporic taxa, mycelia sterilia and one ascomycete species (*Chaetomium hamadae*). The low fungal diversity obtained may be due to the use of seawater media for isolation and the harsh habitats occupied by the hosts (El-Morsy, 1999). Mitosporic taxa have previously been found to be the primary colonizers of roots (Wilcox and Wang, 1987; Summerbell, 1989; Holdenrieder and Sieber, 1992; Stoyke *et al.*, 1992; Ahlich and Sieber, 1996). Ascomycetes (Duckett and Read, 1995) and Mycelia Sterilia (Duckett and Read, 1995) were infrequently isolated.

The endophytes isolated in this study are typically of ectorhizosphere fungi (El-Morsy, 1999), with the exception of a few uncommon species. The dominant species were *Alternaria alternata*, *Cladosporium cladosporioides* and *Penicillium chrysogenum*. These are widely distributed common non specific saprobes.

The endophytes isolated here are not restricted to roots. *Acremonium* spp., *Alternaria alternata*, *Cladosporium* spp. and *Nigrospora sphaerica* have previously been isolated from the stems, leaves, twigs and bark of several hosts (Bettuci and Alonso, 1997; Peláez *et al.*, 1998; Bettuci *et al.*, 1999).

Utilization of carbon sources

The colonisation of plants by fungi involves enzymatic digestion of the main constituents of plant cell wall. Thus, colonization of certain hosts is dependent upon their mechanisms of penetration. Lignin, cellulose and hemicellulose are the main components of the primary and secondary cell walls. Lignin is ordinarily found in a complex with cellulose and hemicellulose (Fengel and Wegner, 1983), where xylan is covalently bound to lignin, in the cell wall. Cooper (1984) pointed out that phytopathogens produce a diverse range of enzymes capable of degrading plant cell wall components. It is assumed that endophytes occupy the same ecological niche as most pathogens and produce the enzymes necessary for the colonization of plant tissues (Petrini *et al.*, 1992). Therefore, fungi occupying the endorhizosphere must be able to use up the most important structural carbohydrates such as starch and cellulose. The utilisation of starch however, may be limited to a small number of endophytes (Sieber, 1989).

In the present study endophytes readily assimilated pectin, xylan and tannin which indicate their ability to produce pectinases, xylanases and phenolases. Xylanase production indicates an ability to degrade lignin. The biodegradation products are readily assimilated by endophytes that are hydrolysed maltose, sucrose and glucose as power sources allowing them to penetrate the weakened cell walls.

Alternaria alternata, *Chaetomium hamadae*, *Conoplea olivacea*, *Conoplea* sp., *Papulasporas immersa* and *Trimatostroma* sp. had the highest growth rates on almost all substrates. Similar studies on endophytes from coniferous foliage (Carroll and Petrini, 1983), wheat seeds (Sieber, 1989) and grasses (White *et al.*, 1991) have also shown that endophytes are able to utilise most of the substrates forming hosts walls.

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