Molecular and phenotypic characterization of endophytic Sebacinoid strains

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vorgelegt von
M.Sc. Magdalena Basiewicz
aus Polen

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Dekan: Prof. Dr Volkmar Wolters
1. Gutachter: Prof. Dr. Karl–Heinz Kogel
2. Gutachter: Prof. Dr. Gabriele Klug

For my Parents and Sister

Dla moich Rodzicow i Siostry

Parts of this work have already been published:

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* These authors contributed equally to this work.

Papers in preparation

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1. Introduction

1.1 Rhizosphere

Rhizosphere is the zone around plant's root where the most intensive interactions between plant host and bacterial or fungal partners take place. Many fungal interaction are parasitic and can lead to diseases, the other ones are mutualistic symbioses which are beneficial to host plants. The results of microbial activity in the rhizosphere are changes in root patterns and nutrients availability to plants. Direct reactions between members of different microbial types often affect promotion of key processes assisting host's growth and health. All interactions occurring around plant roots are, at least indirectly, mediated by plant. Many naturally occurring rhizospheric bacteria and fungi are antagonistic toward pathogens (Kiely et al. 2006). They compete for colonization or infection sites as well as carbon and nitrogen sources. Moreover, pathogens can be inhibited by antimicrobial substances, such as antibiotics, secreted by rhizospheric organism. Additional, indirect mechanisms improve plant nutrition, modify root anatomy, and lead to changes in microbial community in the rhizosphere, and activation of plant defence mechanisms (Whipps 2001, Barea et al. 2005).

1.2 Endophyte

The fungi associated with plants are highly diverse, some of them are endophytes. The term fungal endophyte defines a fungus of which at least a significant part of its life cycle resides in a plant, and which colonizes tissues without causing symptoms of disease. Endophytes from rhizosphere can be easily distinguished from mycorrhizae by lacking external hyphal networks and mantels. Fungal endophytes can grow inter– and intra–cellulary as well as endo– and epi–phytically (Schulz and Boyle 2005). They are not restricted to one environment but were detected in various surroundings including those with extreme characteristic (Zhang et al. 2001).

Endophytic fungal communities adapt to different physiological conditions, in consequence they were detected in the wide spectrum of plant tissue types. Many neutral fungal endophytes are asleep pathogens which may be activated and cause infectious symptoms when the host plant is aged and/or stressed. In addition, plant's endophitic association with fungus can influence environment by determination of plant and microbial biodiversity (Clay and Holah 1999).

The endophytic microbial communities play an essential role in the physiology of host plants. Host, colonized by endophyte, often have more vigour due to secretion of plant growth–promoting substances such as indole–3–acetic acid (Ek et al. 1983, Robinson et al. 1998) or cytokines (Crafts and Miller 1974), and improvement of the hosts' absorption of nutritional nitrogen (Lyons et al. 1990) and phosphorus (Gasoni and Stegman de Gurfinkel 1997; Malinowski et al. 1999). Additionally, the endophyte partner can extensively enhance plants resistance to biotic and abiotic challenges (Latch 1993). These beneficial features have been observed in infected plants exposed to several abiotic stress such as drought (Cheplick et al. 2000), heavy metals (Monneta et al. 2001), culture medium pH lower than optimal (Lewis 2004), high salinity (Waller et al. 2005) as well as a biotic one including microbial infections (Lewis 2003, Rodriguez et al. 2004, Waller et al. 2005), insect pests (Breen 1994, Vázquez de Aldana et al. 2004) and herbivores attack (Schardl and Phillips 1997, Mandyam and Jumpponen 2005).

1.3 Sebacinales

Sebacinales belong to a taxonomically, ecologically, and physiologically diverse group of fungi in the Basidiomycota. They have been identified worldwide and form a broad spectrum of mycorrhizal types. This unique phenomenon significantly influence natural ecosystems (Weiss et al. 2004, Selosse et al. 2007). Ectomycorrhiza, orchid, ericoid, jungermannioid and cavendishoid mycorrhiza are formed by Sebacinales. Ectomycorrhiza (ECM) is an association where the fungus forms a hyphal mantle or layer around and enters into roots and grows only between cortical cells forming a Hartig net (Agrios 2005, Glen et al. 2002, Selosse et al. 2002). Fungi that colonize members of the Orchid family belong to the orchid mycorrhiza type. Orchid's protocorm cells are penetrated by fungal hyphae during the saprotrophic stage. In consequence, seedlings can continue their development (ed. Trigiano 2003). Ericoid mycorrhiza is formed between fungi, and species of the Ericaceae and Epacridaceae. Plants from these families have very fine root systems. Fungal hyphae pass through the cortical cells. In the later stadium plant cells are packed with intracellular hyphal coils (Schmid et al. 1995). Recently Kottke et al. (2003) proved that Sebacinales create symbiotic association with leafy liverworts of the subclass Jungermanniidae. Although the liverworts do not form roots, they proposed the name 'jungermannioid mycorrhiza'. During mycorrhiza growth, fungal hyphae formed coils in the stem cells. In contrast to jungermannioid mycorrhiza build by Ascomycetes no or very few ingrowths pegs were found. Cavendishoid mycorrhiza seems to be similar to ericoid

mycorrhizas because of the presence of coils in roots, an irregular mantle and weak hyphal growth between epidermal cells (Setaro et al. 2006).

Ultrastructural and microscopical characteristic placed Sebacinales within the wood–decay fungi from the order Auriculariales (Bandoni 1984). However, molecular phylogenetic analysis change Sebacinales taxonimic position (Weiss et al. 2001). Exidioid basidia without clamp connections throughout the fructifications and thickened walls of tramal hyphae were detected for both Sbacinales and Auriculariales (Wells and Oberwinkler 1982). Moreover, phytlogenetic analyses based on nuclear sequence of the large ribosomal subunit distinguish two subgroups A and B within that order which differ in their ecology (Weiss et al. 2004). Orchid mycorrhizas and ectomycorrhizas belong to subgroup A. The second subgroup is more diverse and contains ericoid, cavendishoid and jungermannioid mycorrhiza, *Sebacina vermifera* isolates from autotrophic mycorrhiza, endophytic *Piriformospora indica* and multinucleate rhizoctonia in the sense of Warcup (Weiss et al. 2004). *S. vermifera* complex is very absorbing group. They have been characterized as growth promoters. Positive influence of those isolates on barley (*Hordeum vulgare*) was demonstrated by Deshmukh et al. 2006. *S. vermifera* MAFF305830 were characterized as the best growth promoter and confered the higher reduction of powdery mildew infection. On the other hand, in similar experiments with switchgrass (*Panicum virgatum* L) the longest shoots were produced by the plants inoculated with strain MAFF305828, and the longest roots had plants colonized by the strain MAFF305830 (Ghimire et al. 2009). Those two *Sebacina vermifera* isolates were also examined in order to verify fungal development in the barley tissue. Tissue penetration patterns as well as hyphal structures observed during the expansion of these isolates were similar to those created by *P. indica*. The only differences were detected for the speed of fungal development in planta (Waller et al. 2008).

1.4 *Piriformospora indica*

Piriformospora indica belongs to the order Sebacinales and colonize roots of a broad spectrum of mono– and dicotyledonous plants including *Arabidopsis thaliana*, barley, wheat and tobacco (Sahay and Varma 1999, Varma et al. 1999, Waller et al. 2005, Serfling et al. 2007). The fungus was discovered in the rhizosphere of the woody shrubs *Prosopsis juliflora* and *Zizyphus nummularia* in the Indian Thar desert in 1997 (Varma et al. 1998). Since then, *P. indica* scientific interest increased exponentially (38 papers published to date, NCBI). Wide range of colonized species, including agronomically important plants,

makes it a very promising organism in agriculture. In contrast to AMF, the ability of creating symbiosis with *Arabidopsis thaliana* gives the opportunity for fast and effective study of the molecular basis of fungal–plant interaction.

P. indica enhances growth and yield of plant hosts, protect them against biotic (resistance to diseases) or abiotic stress (salt stress) (Rai et al. 2001, Barazani et al. 2005, Waller et al. 2006). The influences of *P. indica* on colonized plants mimic to a certain extent physiological effects of arbuscular mycorrhizal fungi. Although *P. indica* is a root endophyte, it confers resistance against leaf pathogens (Deshmukh et al. 2006). Similar to AMF, the fungus is strictly limited to the cortex, where it develops intracellular coils that are different from the arbuscules of AM fungi (Varma et al. 1999). However, by comparison to AM fungi, *P. indica* does not induce plant marker genes known to be involved in the arbuscular mycorrhiza formation as for example PT11 phosphate transporter or a gene containing peptidoglycan binding LysM domain 1 (Gutjahr et al. 2008).

Microscopic investigation of barley plants inoculated by *P. indica* chlamydospores showed fungus enters via root hairs. Germinating chlamydospores, closely attached to the rhizodermal cell walls, penetrate the subepidermal cells through intercellular spaces in within 12 to 24 hours, where they branch and continue to grow. Fungal hyphae extend their growth in rhizodermal and cortical cells at later colonization stages. The fungus also penetrates through the basal parts of root hair cells, in which bifurcated hyphae form chlamydospores (Deshmukh et al. 2006).

Further analyses were performed in order to comprehend the response of barley roots to *P. indica* colonization by transcriptional and metabolic profiling. The largest group of differentially regulated genes revealed in that study was those involved in plant defence/stress responses (Schäfer et al. 2009).

1.5 Genome estimation and sequencing

The genome comprises the total genetic information of the organism. The rapid development of sequencing technologies within last few years makes these tools commonly available and allows getting genetic information of whole organism very fast. 2487 genome sequencing projects are running (state October 2010), 827 of them being completed (http://www.ncbi.nlm.nih.gov/genomes/static/gpstat.html). The genomic information is essential for better understanding the biochemistry and molecular biology of the analyzed organisms.

The recognition of mechanisms of genetic variation in the pathogen, for instance, is essential for developing effective control measures for the disease. Identification of factors responsible for regulation of symbiotic processes (like host recognition and infection, control of host defence reaction) will help to understand fungal role in plant development and physiology. It allows also to study the ecological significance of symbioses and to comprehend the responses of organisms to their natural environments. In addition, genes involved in ecological adaptation can be clearly defined.

The genome size of ectomycorrhizal basidiomycete *Laccaria bicolor* is aprox. 65 Mb and was the largest sequenced Basidiomycete genome (Martin et al. 2008). The availability of this genome strongly contribute in deeper understanding the interaction between symbiont and plants within their ecosystem, clarify also mechanisms which are used to obtained carbon and nitrogen that are essential in plant production. *L. bicolour* genome analysis revealed a large number of small secreted proteins of unknown function. Some of them may play a role in initiating symbiosis because they are only expressed in symbiotic tissues. Lack of plant cell walls degrading enzymes was observed in *L. bicolour* genome, however, it possess enzymes which can degrade other polysaccharides, suggesting the mechanisms used to grow both in soil and in association with plants (Martin and Selosse 2008). The Perigord black truffle *Tuber melanosporum Vittad.* (Ascomycota) is the largest sequenced fungal genome (aprox. 125 Mb) published so far (Martin et al. 2010). The investigations of *T. melanosporum* genome allow better understanding of the biology and evolution of the ectomycorrhizal symbiosis as well as support identification of processes that trigger fruit body formation. Beside *L. bicolour*, *Coprinopsis cinerea* (Basidiomycota), a model organism for mushroom–forming, has also been sequenced (37 Mb) to examine multicellular development in fungi. Studies on this fungus based on DNA–mediated transformation and RNAi silencing have provided important knowledge on the regulation of mushroom fruiting, mating pheromone, and receptor signalling pathways (Stajich et al. 2010). The genome of arbuscular mycorrhizal fungus (AMF) is also analyzed. The first information about global organization of the *Glomus intraradices* genome was in 2004. Hijri and Sanders (2004) predicted *G. intraradices* genome size 14.07 ± 3.52 Mb. Since that time complete annotation and assembling is not finished. Only annotation of the mitochondrial genome (70 608 bp) is completed (Martin et al. 2008, Glomus Genome Consortium (GGC) Symposium). AMF are unique obligate symbionts. Their hyphae are coenocytic and multinucleate therefore organelles and nutrients can be transported over

long distances. Moreover, it has been shown that AMF harbour genetically different nuclei (Kuhn et al. 2001), making further analysis more complicated.

The information about genome size can provide clues to evolutionary relationship. The new genomic data can give more insights in the genetic background of analyzed fungi and allow investigating in details closely related organisms. Genus *Filobasidiella* for example, contains approximately 38 *Cryptococcus* species. Two of them: *Cryptococcus neoformans* and *Cryptococcus bacillisporus* are the casual agents of the majority of human and animal disease. The *Cryptococcus bacillisporus* genome is approximately 20 Mb, and it is organized in 14 chromosomes. The same number of chromosomes but smaller genome approx. 19 Mb has the *C. neoformans* (Loftus et al. 2005). The haploid genome of the other Basidiomycetes pathogenic fungus *Puccinia graminis*, which causes stem rust in small cereal crops such as wheat, oat, rye, and barley is estimated at 80 Mb, organized in 18 chromosomes. The genome of *Puccinia triticina*, the causal agent of leaf rust in wheat is estimated to range from 100–124 Mb.

Fungal genomes vary a lot in sizes. *Puccinia triticina* has the biggest genome size between Basidiomycetes described till now (NCBI ENTREZ genome project). On the other hand, *Malassezia globosa*, lipid–dependent yeast belonging to normal human microflora, has the smallest genome, approximately 9 Mb (Xu et al. 2007). Some pneumonia agents *Pneumocystis carinii*, *Pneumocystis carinii* f. sp. *hominis*, and *Pneumocystis carinii* f. sp. *muris*, members of Ascomycetes, have even smaller genomes 6.5–8.4 Mb (Sesterhenn et al. 2009).

Before a sequencing project of whole genome will start, its size should be estimated in order to deliver important information for proper preparation and costs prediction. There are few techniques available which can be used for fungal genome estimation such as: flow cytometry, reassociation kinetics, genomic reconstruction, pulsed field gel electrophoresis (PFGE), real–time PCR, and confocal microscope. Usually results from at least two of them are combined to ensure that prediction is accurate.

1.6 Translation elongation factor 1 alpha (TEF) and glycerol–3–phosphate dehydrogenase (GAPDH)

Translation elongation factor 1 alpha (TEF) gene encode an abundant and highly conserved protein which plays an important role in the elongation cycle of protein synthesis in eukaryotic cells (Merrick 1992). In eukaryotes, TEF is the second most profuse protein after actin, combining 1–2 % of the total protein in normal growing cells (Condeelis 1995).

It binds charged tRNA molecules and transports them to the acceptor site on the ribosome adjacent to a growing polypeptide chain. TEF can also regulate other processes by interaction with cytoskeleton and mitotic apparatus (Ichi–Ishi and Inoue 1995). Additionally, studies in the fungus *Mucor racemosus* have indicated that TEF may play a role in morphogenesis (Linz and Sypherd 1987). TEF gene can be present in multiple copies in some Ascomycota and Zygomycota, whereas in many of the analyzed Basidiomycota genomes it proved to be in single copy (see some examples in Table 1).

Table 1. Copy number of translation elongation factor 1 alpha (TEF) in some Ascomycota, Basidiomycota and Zygomycota

Taxa	Class	TEF copy number	Referencess
Ashby gossypii	Ascomycota	1	(Steiner and Philippsen 1994)
Aureobasidium pullulans	Ascomycota	1	(Thornewell et al. 1995)
Histoplasma capsulatum	Ascomycota	1	(Shearer 1995)
Metarhizium anisopliae	Ascomycota	1	(Nakazato et al. 2006)
Sordaria macrospora	Ascomycota	1	(Gagny et al. 1997)
Podospora anserina	Ascomycota	1	(Silar1994)
Podospora curvicolla	Ascomycota	1	(Gagny et al. 1997)
Trichoderma reesei	Ascomycota	1	(Nakari et al. 1993)
Arxula adeninivorans	Ascomycota	2	Rösel and Kunze 1995)
Saccharomyces cerevisiae	Ascomycota	2	(Schirmaier and Philippsen 1984)
Schizosaccharomyces pombe	Ascomycota	3	(Mita et al. 1997)
Cryptococcus neoformans	Basidiomycota	1	(Thornewell et al. 1997)
Schizophyllum commune	Basidiomycota	1	(Wendland and Kothe 1997)
Puccinia graminis f. sp. tritici	Basidiomycota	2	(Schillberg et al. 1995)
Mucor racemosu	Zygomycota	3	(Linz et al.1986)

Glycerol–3–phosphate dehydrogenase (GAPDH) is a key enzyme in both glycolysis and glycerol metabolism therefore it has a fundamental role in energy metabolism and biomass synthesis (Wei et al. 2004). The enzyme catalyzes the reduction of dihydroxyacetone phosphate to sn–glycerol 3–phosphate (Peng et al. 2010). This gene is present as single copy in many Basidiomycetes (Table 2), however there are some exceptions such as in *Agaricus bisporus* where two different genes are known.

Table 2. Copy number of glycerol–3–phosphate dehydrogenase (GAPDH)) in some Ascomycota, Basidiomycota and Zygomycota

Taxa	Class	GAPDH copy number	Referencess
Aspergillus nidulans	Ascomycota	1	(Punt et al. 1988)
Beauveria bassiana	Ascomycota	1	(Liao et al. 2008)
Saccharomyces cerevisiae	Ascomycota	1	(Sprague and Cronan 1977)
Flammulina velutipes	Basidiomycota	1	(Kuo et al. 2004)
Lentinus edodes	Basidiomycota	1	(Hirano et al. 1999)
Phanerochaete chrysosporium	Basidiomycota	1	(Harmsen et al. 1992)
Schizophyllum commune	Basidiomycota	1	(Harmsen et al. 1992)
Pseudozyma flocculosa	Basidiomycota	1	(Neveu et al. 2007)
Agaricus bisporus	Basidiomycota	2	(Harmsen et al. 1992)
Mucor racemosu	Zygomycota	3	(Wolff and Arnau 2001)

1.7 Extracellular enzymes secreted by fungi

The penetration of the external plant layers is an essential task for successful colonization of the host tissues by endophytic fungi. This effect can be obtained by either mechanical fracture of the protective tissues or by enzymatic digestion. In plant pathogens both mechanical and enzymatic components of the penetration mechanism have been at least partly demonstrated (Kolattukudy 1985, Howard et al. 1991). Based on the lifestyle and genome size of the fungus Idnurm and Howlett (2001) estimated that plant pathogenic fungi genomes consist 60–360 virulence or pathogenicity genes. Some of them are involved in the infection structure formation, synthesis of toxins or cell wall-degrading enzymes (Madrid et al. 2003, Möbius and Hertweck 2009, Werner et al. 2007). Other genes are important during establishment of a compatible pathogenic interaction. Endophytes occupy the same ecological niche as most pathogens, therefore, it can be assumed that they utilize the same strategy employed by pathogens for the penetration of the host tissues (Petrini et al. 1992). At the beginning of colonization process, endophytic fungi have to achieve at least partial degradation of cell wall. Extracellular enzymes, proteins that catalyze different types of chemical reactions, might be one of the main tools in that process. Those proteins can be divided into six main groups: oxidoreductases, lyases, hydrolases, transferases, ligases and isomerases (http://www.brenda–enzymes.org/,

Chang et al. 2009). Fungal cellulases and pectinases can be very active while plant cell wall degradation. As a response to intracellular plant protection mechanisms fungal endophytes secrete supplementary enzymes such as esterase, laccase, peroxidase and proteinase (Burke and Cairney 2002, Ramstedt and Soderhall 1983).

1.7.1 Cellulase

Cellulase belongs to hydrolases and plays important role in digestion of two major components of plant cell walls–cellulose and hemicellulose. Sequence analysis and biochemical characterization of cellulase genes have shown that many of them are multifunctional proteins. They are composed of distinct domains arranged in several combinations. Many cellulase–degrading organisms secrete several enzymes that act synergistically (Sandgren et al. 2001). Furthermore, they have evolved a battery of enzymes having different specificities with respect to endo/exo mode of action (Beguin and Aubert 1994).

1.7.2 Pectinolitic enzymes

Pectin is a complex of polysaccharides present in most primary cell walls which bind cells together by forming gel–like matrix (Wozny 2000). Fungi secrete a various number of enzymes to digest pectin which operates through different degradation pathways such as deesterification, hydrolysation or depolymerization. This huge range of activities suggests the great fungal adaptation to host tissues. Pectinases can also play a role during the establishment of ectomycorrhizal symbiosis. However, the level of enzyme production is not very high (Garcia–Romera et al. 1991, Ramstedt and Soderhall 1983). Despite that, plants produce polygalacturonase–inhibiting proteins (PGIPs) which reduce aggressive potential of pectinases and limit fungal invasion. Additionally, the host plant can influence fungal enzyme production by pectin content in cell wall. It has been demonstrated that pectin content level is higher in Dicots than in Monocots (Jarvis et al. 1988).

1.7.3 Laccase

Laccase is a blue copper protein which catalyses the reduction of O_2 to H_2O using a number of phenolic compounds as hydrogen donors (Thurston 1994). Laccase contributes to lignin degradation by oxidising free phenolic groups to phenoxy cation radicals as well as non–phenolic lignin model compounds. This enzyme is associated with morphogenesis in some Basidiomycota and Ascomycota strains (Das et al. 1997, Worrell et al. 1986,

Rehman and Thurston 1992). Additionally, it is involved in physiological processes related to pathogenesis like melanin synthesis essential for survival and longevity of fungal propagules (Bell and Wheeler 1986, Edens et al. 1999). The enzyme has been also detected in zones of mycelial contact between competing basidiomycetes (White and Boddy 1992, Iakovlev and Stenlid 2000). Subsequently, it has been suggested that laccase is involved in detoxification of phenols (Haars and Huttermann 1981) and protection against host oxidative responses (Edens et al. 1999). Many fungi secrete multiple laccase isozymes, encoded by differentially expressed genes that may fulfil different functions. *Coprinopsis cinerea* has two subfamilies of laccases with 15 and 2 nonallelic members, respectively (Kilaru et al. 2006). Five laccase genes have been identified in *Trametes villosa* (Yaver et al. 1996). *P. indica* enzyme activity in axenic culture was demonstrated using laccase specific antibody *LccCbr2* (Kellner et al. 2007)

1.7.4 Peroxidase

Peroxidases are enzymes extremely widespread and diversified, present in almost all living organisms. They play crucial role in lignin degradation. Fungi secrete two main peroxidases: lignin peroxidase (LiP) and manganese peroxidase (MnP). They are heme–containing glycoproteins which require hydrogen peroxide as an oxidant and they can be secreted in several isoenzymes form into the cultivation medium (Hatakka 1994). On the other hand plants are also able to exude peroxidases. Class III of plant peroxidases is described as group of enzymes involved in a broad range of physiological processes, including plant defence (Passardi et al. 2005, Almagro et al. 2009, Gonzalez et al. 2010).

1.7.5 Esterase

Esterases are enzymes which hydrolyze esters present in biological material of all kinds of organisms. A wide spectrum of esterases exists with different substrate specificity, protein structure, and biological function, therefore it can be assumed that they have evolved to enable access to carbon sources or to be involved in catabolic pathways (Machado and Castro–Prado 2001, Bornscheuer et al. 2002). Those enzymes do not hydrolyze long–chain fatty acid esters and prefer water–soluble substrates (Bornscheuer et al. 2002). Esterase isozyme patterns can be used for taxonomic purposes in plant–fungal interactions, and, because of their common expression in varius mycorrhizal fungi, they are also good indicators of changes in fungal activity (Sen 1990, Timonen and Sen 1998). Additionally,

esterase indicates catabolic activity in soil, which directly correlates with microbial activity (Vazquez et al. 2000).

1.7.6 Lipase

Lipases are esterases which can hydrolyse long–chain tri–aclyglycerides. Lipases can be distinguished from esterases by the phenomenon of interfacial activation–high catalytic activity which is observed only in the presence of a hydrophobic phase, a lipid droplet dispersed in water or an organic solvent. This situation is associated to the presence of a hydrophobic oligopeptide protecting the entrance to the active site. In a hydrophobic environment, the lid moves aside and the substrate can enter the binding pocket (Bornscheuer et al. 2002). The enzyme can be secreted by filamentous fungi, however the production depend on the strain, the composition of the growth medium (carbon and nitrogen sources, pH) and cultivation conditions (temperature, agitation and dissolved oxygen concentration). The enzyme is heat resistant, and plays an important role in the breakdown and mobilization of lipids within the cells of an individual as well as transfer of lipids from one organism to another (Shukla and Gupta 2007).

1.7.7 Proteinase

Proteinase belongs to a big family of proteolytic enzymes important in the metabolism of all organisms. The main plant cell component such as cellulose and other carbohydrate polymers are held together by protein linkages therefore proteolytic enzymes may also have a role in fungal invasion of the plant host (Sreedhar et al. 1999). Extracellular proteinase from ericoid mycorrhizal endophytes can degrade complex organic substrates and provide its host plants nitrogen normally unavailable to them (Leake and Read 1989). External pH regulates both activity and production of fungal proteinases (Leake and Read 1990).

1.8 Objectives

The main aim of my thesis was molecular and phenotypic characterization of seven strains belonging to the order Sebacinales. Generally, Sebacinales have been worldwide identified and comprehend a wide spectrum of lifestyles. Nonetheless, only few isolates are cultured by now. The study encompass root endophyte *Piriformospora indica*, Australian orchid mycorrhizae *Sebacina vermifera* strains and orchidaceous rhizoctonia isolate from pot cultures (multinucleate rhizoctonia DAR29830) which were described as plant growth

promoters and resistance inducer for abiotic and biotic stress. In order to better understand the relationship between Sebacinales isolates and to provide a novel genetic marker for molecular environmental analysis we investigated phylogenetic connection among *Sebacina vermifera* isolates, multinucleate rhizoctonia DAR29830, *Piriformospora indica* and three environmental samples from south Germany. Moreover, the closest related fungus to *P. indica* isolated by Williams in the 1984 from a spore of *Glomus fasiculatum* but never classified taxonomically known as multinucleate rhizoctonia was described as a new species and named as *Piriformospora glomeralium*.

In order to elucidate the molecular processes and identify the fungal factors that lead to a successful symbiosis of *P. indica* and other Sebacinales with its plant partners as well as for better understanding the mechanism of the symbiosis, the genome size of mentioned fungi was estimated. First, the techniques such as Pulsed Field Gel Electrophoresis (PFGE) and real–time PCR was establish for *Piriformospora indica* genome size estimation and further applied for the genome size determination for other fungi belonging to the order Sebacinales. Real–time PCR method relies on absolute quantification a one copy gene in genomic DNA sample. Therefore TEF gene (translation elongation factor 1 alpha) was confirmed to fulfil those conditions in all Sebacinales isolates. Furthermore, to affirm the accuracy of this approach the second gene–GAPDH (glycerol–3–phosphate dehydrogenase) was used as well. In addition, *Saccharomyces cerevisiae* was used for validation of the method. Southern blot analysis was performed to prove the copy number of GAPDH in *P. indica* genome. Moreover, a procedure for fungi protoplast preparation was developed and the best conditions for its regeneration were evaluated.

Sebacinales are successful in plant root colonization, therefore, they must secrete substances which allow them to enter into the plant organ. Extracellular enzyme can play an important role in that process, consequently, the profile of enzymes excreted by Sebacinoid strains was characterised. The special emphasis was put on *P. indica*.

2 Materials and Methods

2.1 Fungal and plant material

Piriformospora indica DSM11827 isolates were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Six *Sebacina vermifera* strains (Table 3.) were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan), multinucleate rhizoctonia DAR29830 was kindly provided by Karl–Heinz Rexer (University of Marburg, Marburg, Germany). *Rhizoctonia solani* AG8 was supplied by Timothy Paulitz from Washington State University, USA. The haploid *Saccharomyces cerevisiae* genotype BY4741, MATa (ACC. No. Y02321) and the diploid *S. cerevisiae* genotype FY1679, MATa/MATa (ACC. No. 10000D) were received from Euroscarf, Frankfurt, Germany. *S. vermifera* MAFF305837 and *S. vermifera* MAFF305835 were propagated on solid or liquid Malt–Yeast–Extract–Pepton medium (MYP) and all other Sebacinales isolates as well as *R. solani* on Complete Medium (CM, Pham et al., 2004), whereas both *S. cerevisiae* strains were grown on Yeast–Extract–Peptone–Dextrose–Adenine medium (YPAD) (Guthrie and Fink 2002). All fungi strains were grown at 24 °C in liquid cultures by shaking t 120 rpm speed.

Table 3. Sebacinales isolates

Fungus isolate	Host name
P. indica DSM11827	*Prosopis juliflora* and *Zizyphus nummularia* (woody shrubs)
S. vermifera MAFF305830	*Crytostylis reniformis* (Orchid)
S. vermifera MAFF305842	*Microtis uniflora* (Orchid)
Piriformospora glomeralium (ex multinucleate rhizoctonia DAR29830)	*Trifolium subterraneum*
S. vermifera MAFF305828	*Eriochilus cucullatus* (Orchid)
S. vermifera MAFF305837	*Caladenia dilatata* (Orchid)
S. vermifera MAFF305835	*Caladenia catenata* (Orchid)
S. vermifera MAFF305838	*Caladenia tesselata* (Orchid)

CM medium	
20x salt solution	50 ml
Glucose	20 g
Peptone	2 g
Yeast extract	1 g
Casamino acid	1 g
Microelements	1 ml
Agar–agar	15 g
dest. water	950 ml
autoclaved	

MYP Medium	
Malt–extract	7.0 g
Peptone (Soya)	1.0 g
Yeast extract	0.5 g
dest. water	1000 ml
autoclaved	

20x salt solution	
$NaNO_3$	120 g
KCl	10.4 g
$MgSO_4$ x $7H_2O$	10.4 g
KH_2PO_4	430.4 g
dest. water	1000 ml

Microelements	
$MnCl_2$ x $4H_2O$	6.00 g
H_3BO_3	1.50 g
$ZnSO_4$ x $7H_2O$	2.65 g
KI	0.75 g
Na_2MoO_4 x $2H_2O$	2.40 mg
$CuSO_4$ x $5H_2O$	130 mg
dest. water	1000 ml

YPAD	
Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Adenine hemisulphate	100 mg
Agar–agar	15 g
dest. water	1000 ml
autoclaved	

Environmental samples

Four independent environmental samples (Table 4) collected from two different areas in Germany were analyzed. DNA samples were kindly provided by Michael Weiss from Tübingen University and they belong to a poll of environmental collection encompassing

DNA isolated from root material. They were used in ITS – 28S rDNA phylogeny in Weiß et al. 2010.

Table 4. Environmental isolates

DNA sample number	host plant
15	*Lolium perenne*
65	*Medicago lupulina*
80	*Anthyllis vulneraria*
41	*Rumex acetosa*

Barley (*Hordeum vulgare* L.) cultivar Golden Promise was obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany. Barley seeds were surface–sterilized with 6 % sodium hypochloride, rinsed in water and germinated for 2 days on sterile filter paper. Afterwards, seedlings were transferred into the jars (5 seedlings/jar) and grown on liquid or solid modified plant nutrient medium (1/10 PNM) under 16h light (47 µmol m^{-2} s^{-1}) at 24 °C. In order to check enzyme production barley plants were inoculated with *P. indica* or *Piriformospora glomeralium*. Four–week old fungal mycelia were crashed using a fine blender and applied as inoculum.

1/10 PNM

1M KNO_3	0.5 ml
0.36M KH_2PO_4	1 ml
0.14M K_2HPO4	1 ml
1M $MgSO_4$ x $7H_2O$	2 ml
1M $Ca(NO_3)_2$	0.2 ml
Fe–EDTA	2.5 ml
NaCl	1 ml
Gelrite	4 g

bring to final volume 1 l with water

pH 5.6; autoclaved

Fe–EDTA

$FeSO_4$ x $7H_2O$	2.5 g
Na_2EDTA	3.36 g
water	400 ml

bring to boil

stir 30 min while cooling

bring to final volume 450 ml

For spectrophotometric enzymatic tests *P. indica* was grown on liquid 1/10 PNM with shaking 120 rpm.

2.2 Microscope analysis

Microscopic analyses were performed in order to estimate *P. indica* genome size and to measure multinucleate rhizoctonia structures. Syto 9 and propidium iodide (PI) (LIVE/DEAD® Bac Light™ Bacterial Viability Kit Invitrogen) were applied in that study for staining nuclei.

To determine the nuclear ploidy level of *P. indica*, chlamydospores were collected from 4–week–old CM–agar plates with 0.002 % Tween water. Chlamydospores were washed 3 times with 0.002 % Tween water and resuspend in 0.9 % NaCl to the final concentration of 10^9 –10^{10} spores/ml. *S. cerevisiae* (1n and 2n) cells were collected by centrifugation from 4 to 5 days–old liquid culture. In order to remove the medium, they were washed three times in 0.9 % NaCl and resuspended in the same buffer to the final concentration of 10^9 –10^{10} cells/ml. The same volume (approx. 250 µl) of *P. indica* spores and 1n or 2n *S. cerevisiae* cells suspensions were mixed together and stained with 0.5 µl of Syto 9 and PI followed by 15 minutes incubation in darkness on ice. Afterwards, excess stain was removed by washing 3 times with 0.9 % NaCl and resuspended in that buffer. The fungal material was spread onto glass slides, covered with cover glass and analyzed under confocal laser scanning microscope Leica TCS SP2 (Leica, Bensheim, Germany). Serial optical sectioning images were taken (set manually, 0.10 µm steps) for both *P. indica* and *S. cerevisiae*. Fluorescence of each section of the nucleus was measured using software provided with microscope as follow: first the area of each analyzed nucleus was marked and its fluorescence was automatically measured by software. This procedure was repeated for each section image of analyzed nucleus. Further, the histogram values of fluorescence intensity were summed up and used for genome estimation (Cano et al. 1998). *S. cerevisiae* (1n and 2n) was used as standard organism. The histogram fluorescence value of *S. cerevisiae* 2n is higher than the intencity of the haploidnucleus since fluorescence is directly proportional to the amount of DNA present. Based on that assumption the genome size of *P. indica* was estimated.

The diameter of spores as well as hyphal width, number of nuclei per cell and spore of *Piriformospora glomeralium* (ex multinucleate rhizoctonia) were analyzed under fluorescent microscope Axioplan 2 (Zeiss SMT, Oberkochen, Germany). *P. glomeralium* spores were collected as described above for *P. indica*. The *P. glomeralium* hyphal material was collected from 4–week–old liquid culture, washed few times with 0.9 % NaCl and stained as described for *P. indica* spores.

2.3 Translation elongation factor1–α gene analysis for Sebacinales isolates and environmental samples

DNA from environmental samples was amplified using the primer pair tef420f/tef420r (Table 6.) with the AccuPrime™ Taq DNA Polymerase (Invitrogen) according to the manufacturer's instructions. PCR for Sebacinales isolates were performed using the primer pairs EF1–983f/EF1–2212r, EF1–983f/EF1–1953r, EF1–983f/EF1–2218r (Table 6.). The obtained PCR products were cloned using pGEM®–T Easy Vector Systems (Promega GmbH, Mannheim, Germany) and sequenced in both directions with the M13f/r primers. Two clones from each PCR were sequenced and further analyzed.

2.4 DNA extraction

DNA was extracted from four week old liquid Sebacinales cultures and two week old *S. cerevisiae* culture using two different approaches.

Doyle & Doyle modified method followed by a CsCl centrifugation

200–300 mg frozen fungal mycelium were grinded in liquid nitrogen, and incubated in 700 µl pre–warmed to 65 ºC extraction buffer with β–mercaptoethanol for 20–30 minutes. Next, material was washed using 700 µl chloroform/isoamylalkohol (24:1) and centrifuged 13000 rpm in 4 °C for 15 min. The washing step was repeated one more time. Afterwards DNA was precipitated by adding 50 µl 10 M NH_4OAc, 60 µl 3 M NaOAc (pH 5.5) and 500 µl isopropanol. To receive high concentration of DNA, precipitation took place over night in 4 ºC. Subsequently, DNA was washed by 500 µl 70 % EtOH/10 mM NH_4OAc. After ethanol evaporatoin DNA was dissolved in TE buffer. Later CsCl– centrifugation cleaning step was performed. 10 g of CsCl was mixed with 500 µl ethidium bromide (EtBr) and 5 ml of DNA samples, further 5ml ultracentrifuge tube was fulfill with the mixture and centrifuged at 56000 rpm, 20 ºC for 24 h in Beckman XL 70 centrifuge rotor VTI 90. After centrifugation the red band DNA stained by EtBr was obtained. Genomic DNA band was collected using the needle attached to the syringe. EtBr was removed from DNA by repeated extraction using CsCl saturated 2–butanol. Later, DNA was precipitated by 1/10 volume of 3 M NaOAc and 2 volume of 100 % EtOH and incubated –20 ºC at least 1 h. DNA pellet was washed by cold 70 % EtOH. When EtOH evaporated, DNA was dissolved in TE or water.

Extraction buffer

1 M Tris–HCl	100 ml
0.5 M EDTA	40 ml
NaCl	81.82 g
CTAB	20 g
$Na_2S_2O_5$	10 g
bring to final volume 1 l with water	
autoclaved	
before use add	
ß–mercaptoethanol	2 ml

FastDNA® Spin Kit for soil (MP Biomedicals, LLC., Illkirch, France) according to the manufacturer's protocol.

2.5 Southern blot analysis

10 µg of genomic DNA was digested with 30 Units of restriction proper enzyme (Table 5.) over night (or at least 10 h) *. Digested DNA was separated on 0.8 % TAE gel. The gel run at 35 V in 4 ºC over night. After electrophoresis gel was stained with EtBr and photographed. Later the gel was washed twice in 0.25 N HCl for 15 min, rinsed with deionised water, and incubated for 15 min in solution T. Then, transferring apparatus was assembled. After over night transfer, the membrane was left for drying for 2 h in RT and later crosslink (2 x 50 s, 250 mJoule). Next membrane was washed 2 min in 2xSSC buffer and prehybridized in prehybridization buffer containing carrier DNA over night in 65 ºC. Following, the prehybridization buffer was replaced with hybridization buffer encompassing specific, radioactive–labeled probe. Hybridization process took place at least 12 h at 65 ºC. Subsequently, the membrane was washed twice with buffer I and buffer II. After washing, membrane was saran wrapped, put to the Phosphor Imager box and exposed for at least 3–4 h.

Table 5. Restriction enzymes applied for fungal, genomic DNA digestion.

organism	restriction enzymes
P. indica DSM11827	Bam HI, Hind III, SacI
P. glomeralium	Bam HI, Hind III
S. vermifera MAFF305842	Bam HI, Hind III

Solution T

0.4 M NaOH	16 g/l
0.6 M NaCl	35.06 g/l

20xSSC

3 M NaCl

0.3 M Na–citrate; pH 7.0

10 x TAE

Tris	48.4 g
acetic acid (glacial)	11.4 ml
EDTA	2.92 g
dest H2O	1 l

pH 8.5

Autoclaved

Prehybridization buffer

H_2O	15 ml
5 x HSB	6 ml
Denhardts III	3 ml
10 % SDS	3 ml

mixed together and heat to 65 ºC

add 3 ml of freshly boiled carrier DNA

5xHSB

PIPES	30.3 g

disolve in 300 ml dest H_2O pH 6.8

add

5 M NaCl	600 ml
0.5 M EDTA	40 ml

rechecked pH

adjust to 1 l with water

Autoclaved

carrier DNA

DNA sodium salt from Salmon Testes	125 mg
dest. H_2O	25 ml

heat to boiling

store at –20 °C

Denhardts III

BSA (fraction V)	4 g
SDS	20 g
Ficoll–400	4 g
PVP–360	4 g
$Na_4P_2O_7$ x10 H_2O	10 g

dissolve in 200 ml H_2O

washing buffer I

(2x SSC / 1 % SDS)

dest H_2O	800 ml
20xSSC	100 ml
10 % SDS	100 ml

washing buffer II

(1xSSC / 0.5 % SDS)

dest H_2O	900 ml
20xSSC	50 ml
10 % SDS	50 ml

Southern probe preparation

As probe was used 100 ng of DNA (PCR product specific for each analyzed fungus) in final volume 25 µl (if it was necessary 1x TE was used as dissolvent). DNA was denaturated in 95 ºC for 5 min, subsequently, cooled on ice for 5 min. Labelling beads (Amersham Ready– To–Go DNA Labelling Beads [–^{32}P] dCTP) was dissolved in 20 µl 1x TE and mixed with denaturated DNA and 5 µl α–dCTP–^{32}P and incubated 30–60 min in 37 ºC. Afterwards, the α–dCTP–^{32}P which did not incorporate to the probe was cleaned by Illustra microspin G–25 columns (Amersham). The column was vortexed very good, its tip was broken and it was centrifuged for 1min in 735 rpm in 4 ºC. Supernatant was thrown away and 50 µl of sample was loaded on the column and it was centrifuged for 2 min in 735 rpm in 4 ºC. Labelled probe was denaturated in 95 ºC for 5 min before use, nest kept 3 min on ice and mixed with pre–warmed hybridization buffer.

Hybridization buffer

H_2O	7 ml
5 x HSB	3 ml
Denhardts III	1.5 ml
10 % SDS	1.5 ml

mixed together and heat to 65 ºC

*

After digestion DNA from *S. vermifera* MAFF305830, *S. vermifera* MAFF305828 and *S. vermifera* MAFF305842 was precipitated. 1/10 volume of 3 M NaOAc pH 4.8 and 3 volume of ethanol were added to digested DNA and incubated in –70 ºC for 20 min. Following incubation DNA was spun down for 10 min, the pellet was washed with 70 % ethanol, and centrifuged one more time. DNA was air–dried and resuspend in water. Further, DNA was loaded on agarose gel and further preceded.

2.6 Genome estimation

2.6.1 Real–time PCR

Genome size was estimated using real–time PCR. This technique based on absolute quantification of one copy gene and needed standard DNA preparation. Therefore, specific PCR products were generated for the ribosomal protein S3 gene–RPS3 of the haploid and

the diploid *S. cerevisiae* as well as for translation elongation factor 1 alpha–TEF of Sebacinales and additionally glycerol–3–phosphate dehydrogenase–GAPDH for *P. indica* using the respective outer primer pairs RPS3–F1/R1 (Wilhelm et al. 2003), tef420S6f/tef420S6r for *S. vermifera* MAFF305828 as well as *S. vermifera* MAFF305842, tef420f/tef420r for *S. vermifera* MAFF305830, and *P. glomeralium* (Table 6.). Primers tef420f/tef420r and gpd383f/gpd383r were applied for *P. indica* (Table 6.). These PCR products contain the binding sites for the nested primers used in real–time PCR analysis. Standards were obtained in PCR performed in a Gene Amp® PCR System 9700 PE Applied Biosystem thermo cycler in a total volume of 25 µl containing 1x reaction buffer (DNA Cloning Service), 2.5 mM $MgCl_2$ (DNA Cloning Service, Hamburg, Germany), 0.5 U Taq DNA polymerase (DNA Cloning Service), 0.3 µM each forward and reverse primer, 200 µM each deoxynucleotide (dATP, dCTP, dGTP, and dTTP), and 50 ng genomic template DNA. After an initial denaturation step at 95 °C for 5 min, 35 cycles were performed as follow: denaturation at 95 °C for 30 s, primer annealing at temperature characteristic for each primers (Table 6.) for 30 s, elongation at 72 °C for 1min, and a final extension at 72 °C for 10 min. The PCR products were run on the agarose gel, purified using the NucleoSpin Extract II (Macherey–Nagel GmbH, Düren, Germany) and eluted in water. Quality and quantity of all purified standard DNA samples were determined by NanoDrop.

Quantitative PCR amplifications with the primer pairs PRS3–F2/R2 for both *S. cerevisiae* strains; tef150f/tef150r and gpd–f/gpd–r for *P. indica*, tef150S1r/tef150S6f for *S. vermifera* MAFF305828, tef150S1f/tef150S1r for *S. vermifera* MAFF305830 and *S. vermifera* MAFF305842, tef150f/tef150MRr multinucleate rhizoctonia were performed in 20 µl SYBR green JumpStart Taq ReadyMix (Sigma–Aldrich, München, Germany) with 350 nM oligonucleotides, using an Mx3000P thermal cycler (Stratagene, La Jolla, USA). Each run consists of series fresh made five standards (10–fold serial dilutions) and 1 µl of 2–3 different dilutions of the genomic DNA samples in 2–3 technical repetitions. PCR condition for the GAPDH gene were slightly different than for all other genes and primer's pairs: 35 cycles with 30 s at 95 °C, 1 min at 57 °C, 30 s at 72 °C and 58 °C, 1 min at 72 °C and a final extension at 72°C for 10 min. Real–time PCR performed for PRS3–F2/R2 and all tef primers were conducted: initial denaturation for 10 min at 95 °C, followed by 35 cycles with 30 s at 95 °C, 1 min at temperature characteristic for each primers (Table 6.), 30 s at 72 °C and a final extension at 72 °C for 10 min. The melting curve was examined

every run at the end of cycling to ensure amplification of only a single PCR product. Ct values were assigned by the Mx3000P V2 software (Stratagene, Heidelberg) provided with the instrument. The estimation of the genome size based on the C values was determined as described before by Wilhelm et al. (2003). In short, the size of one haploid genome (C value) was calculated from the ratio of the mass of template DNA (m–determined by UV absorbance) and the copy number of the target sequence (N–determined by real time PCR), C = m/N. The genome size was calculated by $\Gamma = (C \times N_A)/M_{Bp}$ where N_A is Avogadro's number (6.022×10^{23} mol^{-1}) and M_{Bp} is the mean molar mass of a base pair (660 g mol^{-1}).

Table 6. Sequences of primers used in that study

primer name	sequence 5'–3'	Tm
tef420f	gctgattgcgctatcctcat	55 °C
tef420r	cttgacctccttcgaccatc	55 °C
tef420S6f	gctgattgcgccattctcat	57 °C
tef420S6r	cttgttttccttggtccatc	57 °C
tef150f	tcgtcgctgtcaacaagatg	58 °C
tef150r	accgtcttggggttgtatcc	58 °C
tef150MRr	accgtcttggggttgtagcc	58 °C
tef150S1f	tcgtcgccgtcaacaagatg	58 °C
tef150S1r	acagtcttggggttgtatcc	58 °C
tef150S6f	tcgtcgcgtcaacaagatg	58 °C
EF1–983f	gcyccygghcaycgtgayttyat	62 °C
EF1–2212r	ccracrgcracrgtytgtctctcat	62 °C
EF1–1953r	ccrgcracrgtrtgtctcat	62 °C
EF1–2218r	atgacaccracrgcracrgtytg	62 °C
gpd383f	ctcgacaagtacgacccaca	55 °C
gpd383r	gcattcctgaagacgatacg	55 °C
gpd–f	gattgaaatcttggccgtca	58 °C
gpd–r	ttgccgtcctttacttcgac	58 °C
RPS3– F1	cgctgacggtgtcttctac	55 °C
RPS3– R1	cggaaacaacttcacaa	55 °C

RPS3– F2	ccaaccaagaccgaagttat	57 °C
RPS3– R2	gacagcggacaaacca	57 °C
M13f	gttttcccagtcacgac	55 °C
M13r	aacagctatgaccatga	55 °C

2.6.2 Pulsed Field Gel Electrophoresis

In order to separate fungal chromosomes on the PF agarose gel protoplasts were produced. Four–week–old fungal cultures were crashed using a fine blender. 200 ml of liquid CM were inoculated with 1 ml of homogenate and incubated for 2 days at 24 ºC with shaking. Then the mycelium was collected by filtration through sterile miracloth (Merck, Eurolab, Darmstadt, Germany), washed few times using 0.9 % NaCl and incubated 1 h at 37 ºC in a protoplasting solution. Later, protoplasts were filtered through a miracloth and washed three times with cold STC buffer. To prepare chromosomal DNA the pre–wormed protoplast suspension was mixed with equal volume of 1.8 % BioRad pulsed field certified agarose gel at 55 ºC. The solidified plugs were incubated in proteinase K buffer for 12 h and washed three times with washing buffer. This step was repeated two times. Plugs were stored in washing buffer at 4 ºC. Experiments were performed on a Bio–Rad CHEF DR III apparatus. The run conditions are detailed in Table 7. After electrophoresis gels were stained with 0.5 μg/ml of ethidium bromide and photographed. Chromosomal DNA from *S. cerevisiae* (Bio–Rad) and *Schizosaccharomyces pombe* (Bio–Rad) were used as size standards.

Protoplasting solution

Lysing Enzymes from *Trichoderma harzianum* (L1412 Sigma, Deisenhofen, Germany) 2%
SMC

STC

1.33 M Sorbitol in TC

TC

50 mM $CaCl_2$

10 mM TrisHCl pH=7.5

SMC

1.33 M sorbitol

50 mM $CaCl_2$

20 mM MES buffer

pH 5.8

Proteinase K buffer

10 mM Tris

1 mM EDTA pH 8.5

1 % Na–N–laurylsarcosinate

Table 7. PFGE running condition for each analyzed fungus. (T–temperature)

organism	condition	agarose concentration in the gel	running buffer	T
S.vermifera MAFF291366	block 1 48 h 2 V 1–1800 s angel 100°	0.8 % gel TBE	0.8xTBE	14 °C
	block 2 48 h 2 V 1–2000 s angel 106°			
	block 3 24 h 6 V 1–120 s angel 120°			
P. glomeralium	block 1 48 h 2 V 1–1800 s angel 100°	0.8 % gel TAE	0.8xTAE	4 °C
	block 2 48 h 2 V 1–2000 s angel 106°			
P. indica	block 1 69 h 2 V 1–1800 s angle 100º	0.8 % gel TAE	1xTAE	14 °C
	block 2 48 h 2 V 1–2000 s angel 106°			
S.*vermifera* MAFF305842 S.*vermifera* MAFF305828	block 1 48 h 2 V 1–1800 s angel 100°	0.8 % gel TAE	0.8xTAE	4 °C
	block 2 48 h 2 V 1–2000 s angel 106°			
	block 3 24 h 6 V 1–120 s angel 120°			

2.7 Plate enzymatic assays

Tests for extracellular enzymes activity were performed in triplicates following the methods describe in Kreisel and Schauer (1987). Mycelial plugs were cut from the edges of colonies on 7 days old culture and were used as inoculum for all plate tests. The extracellular enzymes activities were analyzed after two weeks.

2.7.1 *Cellulase activity*

Fungi were cultivated on medium enclosed 2.5 % malt extract, 1 % cellulose (SERVA, FEINBIOCHEMICA, Heidelberg, Germany) and 2 % agar. The enzyme activity was checked by spreading Lugol's solution (2 % iodine and 4 % potassium iodide in water, both Sigma, Deisenhofen, Germany). The clear area in the medium around the colony indicated cellulose degradation.

2.7.2 *Pectinase activity*

To investigate pectinase activity fungi were propagated on the plates where 0.1 % yeast extract with 1.5 % agar was enriched by 0.5 % pectin (Roth, Karlsruhe, Germany). Plates were evaluated by flooding them with 1 % solution of hexadecyltrimetylammonium

bromide (Sigma, Deisenhofen, Germany) around the growing mycelium. The clear zone around colonies suggested that fungus digested the substrate.

2.7.3 *Laccase activity*

To check laccase activity, medium contained 2.5 % malt extract and 2 % agar (MAE) was used. A dark blue coloration after 3, 24 or 72 h after spreading of 0.1 M α–naphthol (Sigma, Deisenhofen, Germany) in 96 % ethanol on the surface of the growing mycelium indicated extracellular laccase activity. Along, the laccase production was tested during interspecific interactions. For this purpose cocultures of the Sebacinoid strains with the root pathogen *R. solani* were examinated. Sebacinoid isolates grew slower than *R. solani* therefore they were precultured on MAE medium for one week before inoculation. The enzyme activity was inspected after one week co–culture as described above. Additionally laccase activity of *P. indica* and *P. glomeralium* was verified in coculture with barley roots. Barley plants were inoculated with 10^5 chlamydospores. Furthermore, barley mock–treated, autoclaved barley roots inoculated with chlamydospores and barley inoculated with autoclaved fungal mycelium were analyzed. Presence of an enzymatic activity was proved five and seven days after chlamydospores inoculation by spreading of 0.1 M α–naphthol.

2.7.4 *Peroxidase activity*

Fungi grew as described by the laccase test. After 2 weeks, attendance of peroxidase was evaluated by flooding plates with a fresh– prepared mixture of 0.4 % H_2O_2 (Roth, Karlsruhe, Germany) and 1 % pyrogallol (Sigma, Deisenhofen, Germany) dissolved in water. Plates were checked after 3, 24 or 72 h after substrate applying. A dark yellow / brown color around the mycelium indicated peroxidase activity.

2.7.5 *Protease activity*

Analyzed fungi grown on medium containing 8 % gelatine (VWR PROLABO, Darmstadt, Germany) dissolved in water at pH 6. The fungal ability to liquefy the solid media indicates proteases production. The test was read after 5, 7, 10, 12 and 14 days growth. For excluding any additional not enzymatic gelatine degradation plates were kept for 24 h at 4 °C.

2.8 Spectrophotometric enzymatic assay

For spectrophotometric assay barley plants as well as *P. indica* were grown in liquid 1/10 PNM. In order to obtain autoclaved barley roots, two weeks old barley plants were harvesting and roots were autoclaved 20 min at 120 °C. Plant material was inoculated with crashed *P. indica* mycelium. The samples were collected 1, 1.5, 2, 3, 5, 7, 10 and 15 days after inoculation. For each enzyme activity measurement, medium from a culture were assembled and filtered through miracloth. To remove the small particles like chlamydospores, it was purified once more using membrane filter with pore diameter 0.45 µm (Whatman, Dassel, Germany) as well. Subsequently, the collected material was concentrated with centrifugal devices for biomolecular separation (MACROSEP 10K OMEGA PALL Life Sciences, Mexico) according to the manufacturer's protocol. The collected supernatant was utilized for further analysis.

All tests were carried out in BioTek Synergy 2 Multi–Mode Microplate Reader.

2.8.1 *Laccase activity* (Harkin and Obst 1973)

Laccase activity was detected using 2, 2'azino–bis–3–ethylbenzthiazoline–6–sulphonic acid (ABTS) (Sigma, Deisenhofen, Germany) as a substrate in sodium tartrate buffer pH 3. The enzyme activity was measure immediately after preparing reaction mixture. The absorbance was read at 420 nm in 30 °C for 15 min. One unit of enzyme activity was defined as the amount of enzyme required for oxidation of 1 µmol ABTS in 1 min.

Reaction mixture	
0.05 M Sodium Tartrate buffer pH 3	50 µl
5 mM ABTS	100 µl
culture filtrate	100 µl

The enzyme activity was calculated using the formula below:

$$U\,L^{-1} = \frac{\Delta E_{420\,nm} \cdot V_{total} \cdot F}{V_{En} \cdot \varepsilon_{ABTS} \cdot d}$$

ΔE_{420nm} – absorbance per minute

V_{total} – the total volume of reaction mixture (0.25 ml)

F – dilution factor

V_{En} – the volume of culture (0.1 ml)

ε_{ABTS} – extension of coefficient 0.0432 L µmol^{-1} cm^{-1}

d – the distance the light travels through the material – layer thickness (0.7)

2.8.2 *Peroxidase activity* (Childs and Bardsley 1975)

Peroxidase activity was measured using a modified procedure describe for laccase activity above. The enzyme activity was checked using ABTS in sodium tartrate buffer pH 3 with hydrogen peroxide H_2O_2 (Sigma, Deisenhofen, Germany) as an additional substrate.The enzyme activity was measured immediately after preparing reaction mixture. The one unit of enzyme activity was defined as above.

Reaction mixture	
0.05 M Sodium Tartrat buffer pH 3	50 µl
5 mM ABTS	100 µl
2 mM H_2O_2	100 µl
culture filtrate	100 µl

The enzyme activity was calculated using formula below:

$$U\,L^{-1} = \frac{\Delta E_{420\,nm} \cdot V_{total} \cdot F}{V_{En} \cdot \varepsilon_{ABTS} \cdot d}$$

ΔE_{420nm} – absorbance per minute

V_{total} – the total volume of reaction mixture (0.35ml)

F – dilution factor

V_{en} – the volume of enzyme (0.1ml)

ε_{ABTS} – extension of coefficient 0.0432 L µmol^{-1} cm^{-1}

d – the distance the light travels through the material – layer thickness (0.7)

2.8.3 *Esterase activity*

Para– nitrophenylacetat (pNPA) (Sigma, Deisenhofen, Germany) was used as a substrate for esterase activity determination. The enzyme activity was measured immediately after preparing the reaction mixture. The absorbance was read at 405 nm in 30 °C for 15 min. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µmol para– nitrophenylacetat per 1 min at pH 6.5.

Reaction mixture	
80 mM potassium phosphate buffer pH 6.5	50 µl
10 mM pNPA	100 µl
culture filtrate	100 µl

The enzyme activity was calculated using formula below:

$$U\,L^{-1} = \frac{\Delta E_{405\,nm} \cdot V_{total} \cdot F}{V_{En} \cdot \varepsilon_{pNPA} \cdot d}$$

ΔE_{405nm} – absorbance per minute

V_{total} – the total volume of reaction mixture (0.25ml)

F – dilution factor

V_{En} – the volume of enzyme (0.1ml)

ε_{pNPA} – extension of coefficient 0.0183 L µmol^{-1} cm^{-1}

d – the distance the light travels through the material – layer thickness (0.7)

2.8.4 *Lipase activity* (Winkler and Stuckmann 1979)

Lipase activity was determined using 4–nitrophenyl–palmitate (4NPP) (Sigma, Deisenhofen, Germany) as a substrate in the potassium phosphate buffer pH 8.8. The enzyme activity was measured immediately after preparing the reaction mixture. The absorbance was read at 410 nm in 37 °C for 15 min. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze of 1 µmol 4–nitrophenyl–palmitate per 1 min in pH 8.8.

Substrate preparation (4NPP – buffer)	
4NPP	15 mg
isopropanol	5 ml
sonification for 5–10 s	
Deoxycholic acid Na salt (Roth, Karlsruhe, Germany)	110 mg
Gum Arabic (Roth, Karlsruhe, Germany	50 mg
potassium phosphate buffer pH 8.8	45 ml
10 min sonification	

Reaction mixture	
4NPP – buffer	100 µl
culture filtrate	50 µl

The enzyme activity was calculated using formula below:

$$U\,L^{-1} = \frac{\Delta E_{366nm} \cdot 60min}{V_{En} \cdot 15min \cdot d}$$

ΔE_{366nm} – absorbance per minute

V_{total} – the total volume of reaction mixture (0.25 ml)

V_{En} – the volume of enzyme (0.1 ml)

d – the distance the light travels through the material – layer thickness (0.7)

2.8.5 *Determination of total protein content*

The protein content of all analyzed samples was determinate using Bradford assay. The protein amount in each sample was estimated by reference to standard curve for bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany) in the range 5–120 µg/ml. All samples were analyzed in triplicate.

Reaction mixture	
Bradford solution (Roth, Karlsruhe, Germany)	200 µl
culturefiltrate / standard (BSA)	50 µl

2.9 *P. indica* protoplasts regeneration

P. indica protoplasts were prepared as described in the PFGE part. In order to examine the best condition for their regeneration few osmotic stabilizers were tested. The complex medium as well as top agar was supplemented by 0.3 M sucrose, 0.6 M sorbitol or 0.6 M mannitol. The same concentration of protoplasts was mixed with liquid top agar and spread on the bottom agar containing the same stabilizers. Regenerations took place at 28 °C and every 24 h protoplasts regeneration was checked.

As controls water and STC were included in the regeneration tests. After 7 days regeneration efficiency was compared by counting the growing colonies.

3 Results

3.1 Analysis of translation elongation factor 1 alpha gene

The translation elongation factor 1 alpha (TEF) gene was chosen for the phylogenetic study of Sebacinales. Additionally to Sebacinales isolates, three independent environmental samples, collected from two different areas in Germany, were analyzed with Sebacinales specific primers. The sequences of the two TEF gene introns were the same for all environmental clones sequenced but different from the Sebacinales isolates (Fig. 1.). The phylogenetic analysis placed them close to *P. indica* showing that closely related fungi are present in Germany (Fig. 1.). TEF phylogenetic analysis demonstrates that *P. glomeralium* (ex multinucleate rhizoctonia) is the closest related strain to *P. indica* from all the Sebacinales isolates available at present (Fig. 2.). The phylogenetic studies divided Sebacinales into three separated clades (Fig. 4.). The first clade includes *S. vermifera* MAFF 305837 and *S. vermifera* MAFF 305838, clade 2 is represented by *P. glomeralium* together with *P. indica* and the third one contains *S. vermifera* MAFF 305842, *S. vermifera* MAFF 305830, and *S. vermifera* MAFF305828.

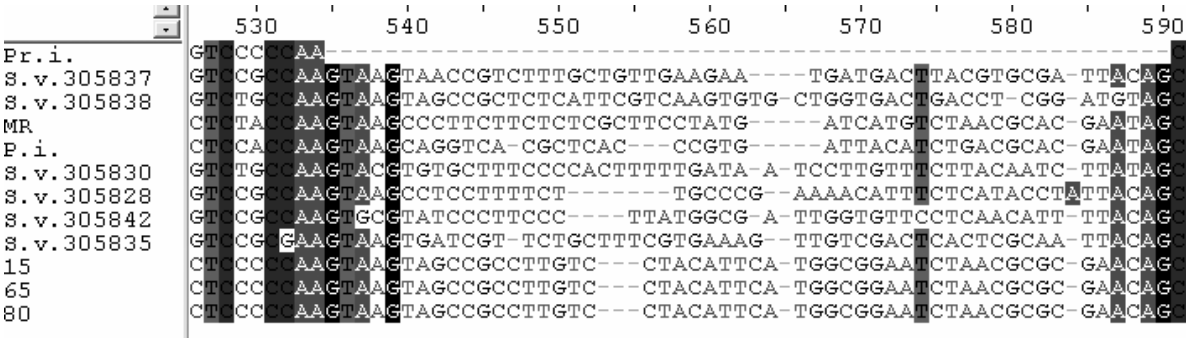

Fig. 1. Alignment of Sebacinales TEF gene including environmental samples demonstrates differences in one of introns in that gene. Pr. i.–*Protomyces inouyei*; S.v.–*Sebacina vermifera* (number indicate the strain); MR–*P. glomeralium* DAR29830; P.i.–*Piriformospora indica*; 15, 65, 80–environmental samples

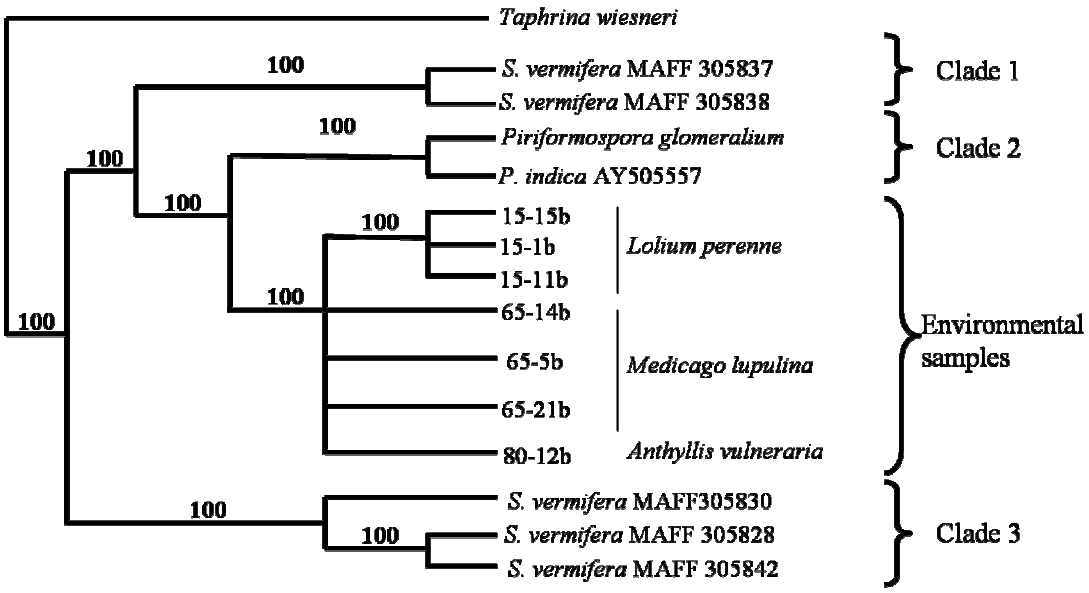

Fig. 2. *TEF* gene based phylogenetic analysis of *P. indica* related fungi

3.2 Southern blot analysis

Southern blot analyses were performed to verify copy number of the TEF gene in Sebacinales genomes. Additionally, the *P. indica* GAPDH gene was investigated. Genomic DNA digested with restriction enzymes was separated on agarose gel, transferred on nylon membrane and further hybridized with specific radioactive labelled probe. The results obtained for *P. indica* showed only one band for both analyzed genes proving that they are single copy (Fig. 3). The same enzymes combination (Bam HI, Hind III and Sac I) was implement for examination of TEF gene copy number in the other Sebacinales strains: *P. glomeralium* and *S. vermifera* MAFF305828 (Fig. 4.). *S. vermifera* MAFF 305830 have also only one copy of that gene (Zuccaro unpublished data). After genomic DNA digestion of *S. vermifera* MAFF305842 with Hind III and hybridization with specific probe multiple bands were detected. However after DNA digestion with Bam HI only one band was observed (Zuccaro unpublished data).

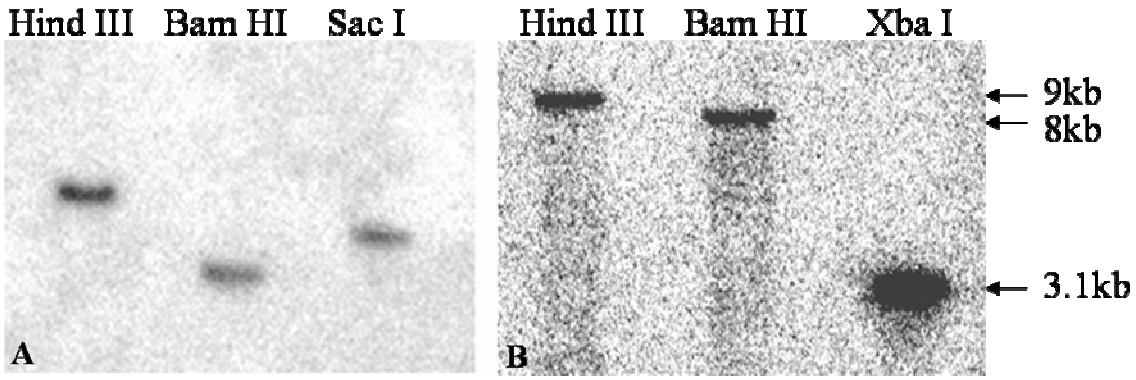

Fig. 3. Study of TEF (A) and GAPDH (B) genes copy number using southern blot approach. Genomic DNA was digested by three different enzymes and hybridized with specific radioactive labelled probe. Bam HI, Hind III and XbaI were used for TEF gene and Bam HI, Hind III and Sac I were applied for GAPDH.

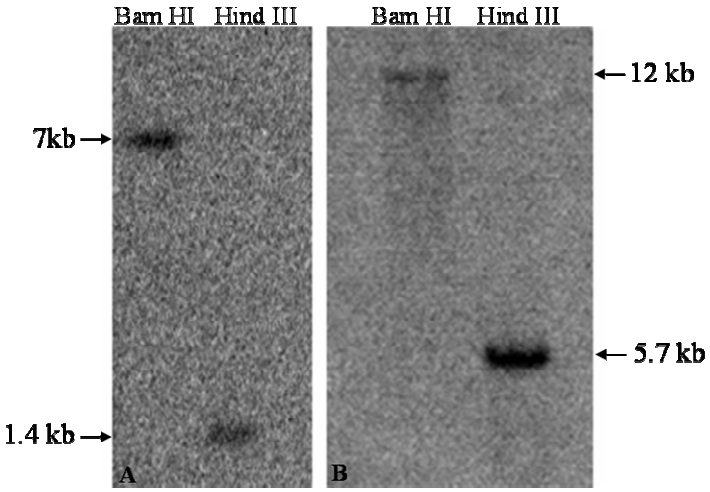

Fig. 4. Study of TEF gene copy number in *P. glomeralium* (A) and *S. vermifera* MAFF305828 (B) using southern blot approach. Genomic DNA was digested by two different enzymes–Bam HI (1), and Hind III (2) and hybridized with specific radioactive labelled probes.

Furthermore, *P. indica* chromosomes separated by PFGE were transferred onto nylon membrane and hybridized with a probe specific for GAPDH and TEF. GAPDH and TEF produce one single band on the PFGE and were located on the third and on the first chromosome respectively (Fig. 5.). The smallest band detected on the gel was verified as mitochondrial DNA (Fig. 5.).

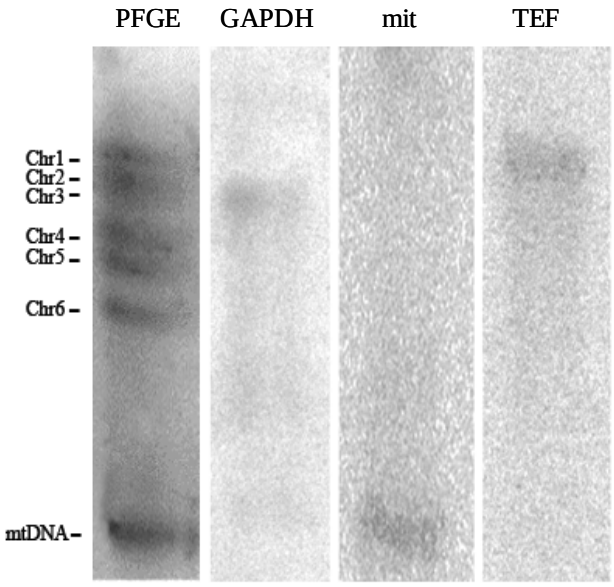

Fig. 5. Localization of GAPDH and TEF genes and identification of the mitochondrial DNA on P. *indica* chromosomes using southern blot technique. PFGE–chromosomes separated using PFGE, GAPDH, TEF–localization of analyzed genes, mit–mitochondrial DNA. Southern blot analysis was performed using specific radioactive probes.

3.3 Genome estimation

Two different techniques were used to estimate the genome size of five Sebacinoid strains: real–time PCR and Pulsed Field Gel Electrophoresis (PFGE). Additionally, confocal microscopy technique were applied for *P. indica*.

The real time PCR method based on the absolute quantification of one copy gene in genomic DNA sample. *S. cerevisiae* was chosen as control standard organism. The genome size predicted using that approach and applying primers specific for the *Saccharomyces cerevisiae* ribosomal protein S3 gene (ScRPS3) in four independent experiments was in the range of the known genome size for this organism (12 Mb, Table 8.). The efficiency of real–time PCR for *S. cerevisiae* was 94 ± 2 %. Relying on the analysis of other Basidiomycota genomes two genes TEF and GAPDH were expected to be single copy in the Sebacinales genomes. Southern blot analysis using specific probe for those two genes proved that they are single copy therefore they were applied for *P. indica* genome size calculation. Using TEF gene in eight independent real time PCR runs from CsCl purified DNA, the haploid genome size for *P. indica* was 15.6 Mb ± 2.75 (Table 8.). Using the second gene GAPDH in four independent runs the obtained genome size value of 15.3 Mb ± 3.5 (Table 8.). The real–time PCR efficiency for the TEF and GAPDH genes was 100 ± 3 % and 94 ± 2 % respectively. The *P.*

indica genome size estimation obtained from DNA samples extracted with FastDNA®SPIN Kit for soil yielded was 24 Mb ± 2.5. For the other fungi analyzed in that study only one gene TEF were applied. The genome sizes of analyzed Sebacinales isolates are presented in Table 10.

In both extraction methods the 260/280 ratio which has high sensitivity of protein contamination in DNA sample was in the optimal range of 1.9 for all analyzed fungi. However, the 260/230 ratio showed a contamination by organic compounds for the DNA extracted with the kit. The absence of both non–specific PCR products and primer–dimer accumulation were approved by the negative controls and melting curve analyses executed with each PCR.

Table 8. Sebacinales genome size estimation using real–time PCR based quantification of TEF gene and chromosomes number analysis. D&D and CsCl–genomic DNA extracted by Doyle and Doyle modified method followed by CsCl cleaning step; Kit–genomic DNA estracted using FastDNA® Spin Kit for soil. *–*S. cerevisiae* chromosomes number was not derminated in that study (Goffeau et al. 1996)

Sebacinales strain	DNA extraction method	Genome size (Mb)+/–standard deviation (Mb)	Minimal chromosomes number based on PFGE
S. vermifera MAFF305842	D&D and CsCl	12.5±2	5
	Kit	21±4	
S. vermifera MAFF 305830	D&D and CsCl	11±1.5	5
	Kit	20.7±1.9	
S. vermifera MAFF305828	D&D and CsCl	18.5±1.2	4
	Kit	26±1	
P. indica (TEF)	D&D and CsCl	15 ± 3	6–7
	Kit	24 ± 2.5	
P. indica (GAPDH)	D&D and CsCl	15.3 ± 3.5	
P. glomeralium	D&D and CsCl	15.8±2.6	5
	Kit	22±1.1	
S. cerevisiae (1n)	D&D and CsCl	10.3 ± 1.8	16*
S. cerevisiae (1n)	Kit	13 ± 1	16*
S. cerevisiae (2n)	D&D and CsCl	11.5 ± 1	16*

To separate fungal chromosomal DNA using PFGE different conditions were applied (see Table 7.). In all runs chromosomes sizes were calculated over the standards *S. cerevisiae* and *Sch. pombe*. The molecular karyotype of *P. indica* determined by that technique demonstrated a pattern of six faint chromosomal bands ranging in size from 1.3 Mb to 5.4 Mb. The genome size of the merged *P. indica* electrophoretic bands calculated from three different gels was predicted to be about 15.8 Mb ± 0.3. The appearance of chromosomes larger than 5.4 Mb was verified by extension of electrophoretic conditions (Fig. 6.). The zone, where big chromosomes were expected, was fully resolved and no additional bands were detected. The gel after PFGE indicated at least 5 chromosomal bands for *S. vermifera* MAFF305830, MAFF305842 and *P. glomeralium* and at least 4 for *S. vermifera* MAFF305828. Similar to *P. indica*, *S. vermifera* MAFF305830 and *P. glomeralium* have one big chromosome in the range of 5.4 Mb. The gels indicate that *S. vermifera* MAFF305842 and *S. vermifera* MAFF305828 have at least one chromosome bigger than the biggest chromosome of size marker–*Sch. pombe* (5.7 Mb) (Fig. 6.). Moreover, the smallest chromosome for *S. vermifera* MAFF305842 and *S. vermifera* MAFF305828 was still bigger than 2.2 Mb. *P. indica* and *P. glomeralium* have an additional small chromosome in the range of 1 Mb. The estimation of genome size relied on electrophoretic separation of chromosomes conferred a minimal size of 17 Mb for *S. vermifera* MAFF305830, 14.4 Mb for *P. glomeralium*, 22.3 Mb for *S. vermifera* MAFF305842 and 19.6 Mb for *S. vermifera* MAFF305828. The strength signal of the gel staining with ethidium bromide for *S. vermifera* MAFF305842 and *S. vermifera* MAFF305828 propose the presence of a higher number of chromosomes which were not separated under the tested conditions (Fig. 6.). Although varied condition was applied the separation was not improved.

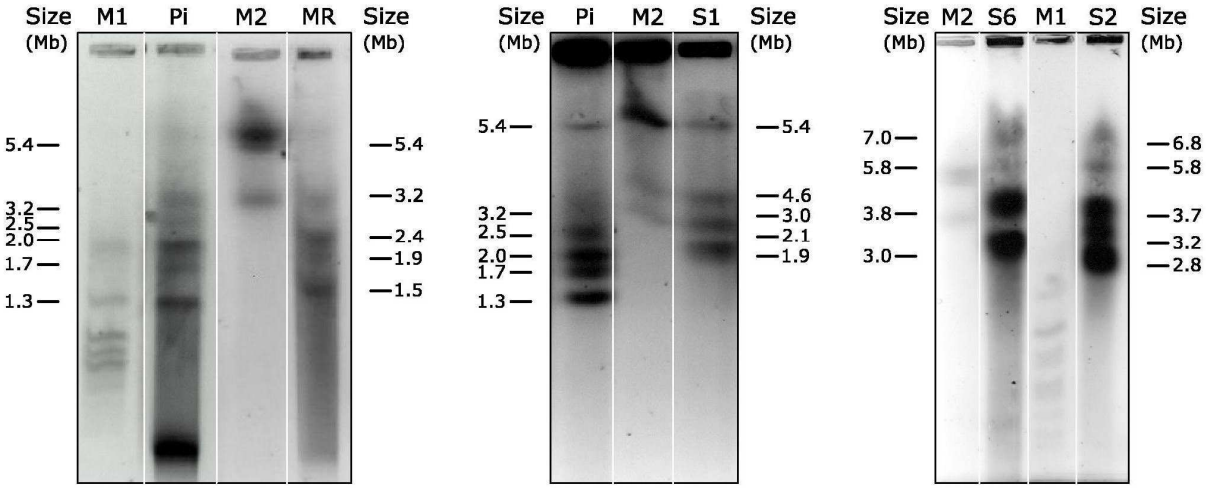

Fig. 6. Separation of *P. indica* (Pi), *P. glomeralium* (MR), *S. vermifera* MAFF305830 (S1), *S. vermifera* MAFF305842 (S2), *S. vermifera* MAFF305828 (S6) chromosomes by Pulsed Field Gel Electrophoresis (PFGE). M1–*Saccharomyces cerevisiae* (Bio–Rad) and M2–*Schizosaccharomyces pombe* (Bio–Rad) size standards.

P. indica genome size was additionally estimated using confocal scanning microscope (Fig. 7. and Fig. 8). Fluorescence histogram of 12 nuclei stained with syto 9 in chlamydospores was measured. By comparison to the fluorescence of *Saccharomyces cerevisiae* (1n and 2n) which nuclei were stained under the same condition, the genome of analyzed fungus was predicted. The value of the mean histogram for *P. indica* was placed in between that of the two *S. cerevisiae* strains suggesting that *P. indica* genome range 17–22 Mb what confirmed results obtained by pulsed field gel electrophoresis and real–time PCR.

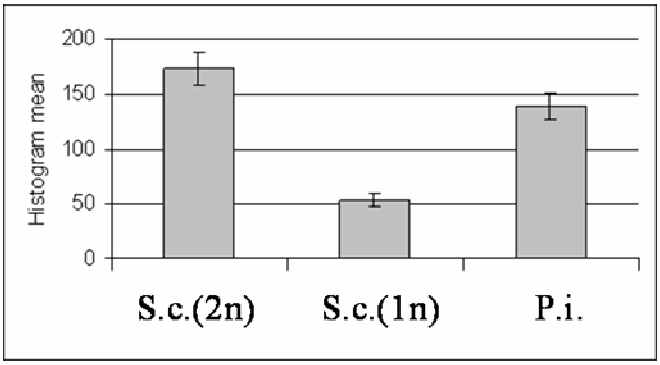

Fig. 7. Value of mean histogram fluorescence for *P. indica* and *S. cerevisiae* strains. P.i.–*P. indica*, S.c. (2n)–*S. cerevisiae* (2n) and S.c. (1n)–*S. cerevisiae* (1n)

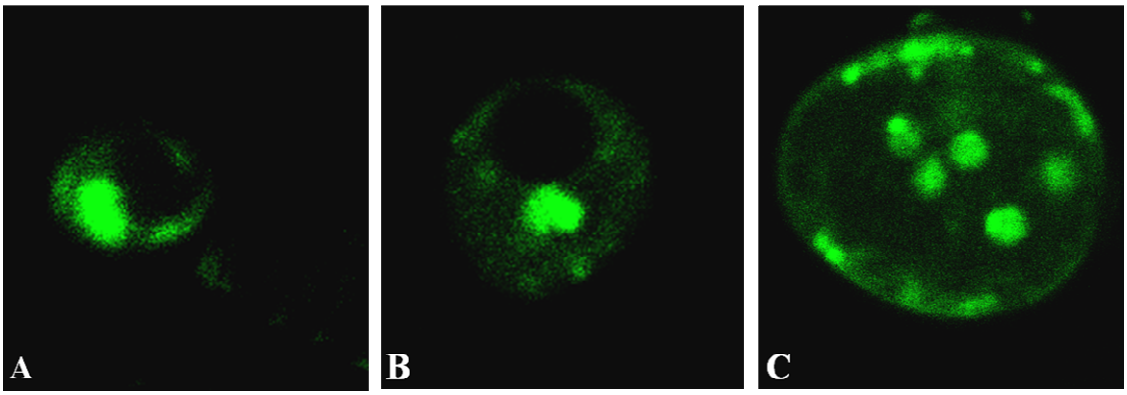

Fig. 8. Fluorescence staining by Syto 9 of *Saccharomyces cerevisiae* 2n (A) and 1n (B), and nuclei of a chlamydospore of *P. indica* (C).

3.4 Enzyme activity–plate's tests

Six *Sebacina vermifera* isolates collected from different autotrophic orchids in Australia (Warcup 1988) and *P. indica* isolated from woody shrubs in the Indian Thar desert (Varma et al. 1998) were analyzed. To study the biochemical variations between isolates, they were grown in different media to check extracellular enzyme production. The enzymes profiles of the analyzed Sebacinales strains are presented in Table 9. In fact, all of the isolates showed strong protease activity. The strongest peroxidase activity presented *S. vermifera* MAFF 305842 (Table 9. and Fig. 11.), whereas the higher amount of laccase was produced by *S. vermifera* MAFF 305830. Surprisingly, *P. glomeralium* and *P. indica* demonstrated no or small activity of those two enzymes. Nonetheless, cellulose activity was detected only for *P. indica* and *P. glomeralium* under the tested conditions (Fig. 12.). Laccase production was further investigated and all fungi were co–cultured with *Rhizoctonia solani*. *P. glomeralium* and *P. indica* did not show enzyme activity also under this condition while other Sebacinales showed strong laccase production in response to *R. solani* (Fig. 10.). Subsequently, laccase secretion of *P. glomeralium* and *P. indica* was analyzed under presence of living as well as autoclaved barley roots. The presence of barley roots affected laccase production in *P. indica* (Fig. 13.). The enzyme production was not indicated in *P. glomeralium* by nor living neither autoclaved barley roots.

Table 9. Enzymatic test (peroxidase, laccase, protease, cellulase and pectinase activity) and growth rate on MAE and gelatine of various Sebacinales isolates. +++++ high activity; + low activity; – lack of activity. The number indicate colony diameter in mm.

organism	peroxidase	laccase	protease	cellulase	pectinase	MAE [mm]	gelatine [mm]
S. vermifera MAFF 305835	++	++++	++++	–	+	10–14	32±1.4
S. vermifera MAFF 305837	++	++++	+++++	–	–	9.5±0.4	47±0.8
P. glomeralium	–	–	++++	++	–	67 ±3.6	41±3
P. indica AY505557	+	–	++++	++	–	58±1.4	29±4
S. vermifera MAFF 305830	+++	+++++	++++	–	–	60±2.5	34±3.6
S. vermifera MAFF305828	–	++++	++	+	–	32±2.7	13±0.8
S. vermifera MAFF 305842	++++	+	+++	–	–	12±1.2	23±1.4

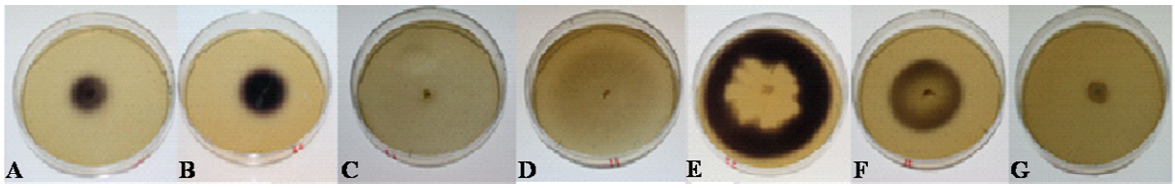

Fig. 9. Laccase plate's enzymatic test. As a substrate 0.1 M α–naphthol was used. The dark violet colour indicates enzyme activity. A–*S. vermifera* MAFF305835 B–*S. vermifera* MAFF305837, C–*P. glomeralium*, D–*P. indica* AY505557 E–*S. vermifera* MAFF 305830, F–*S. vermifera* MAFF305828, G–*S. vermifera* MAFF305842.

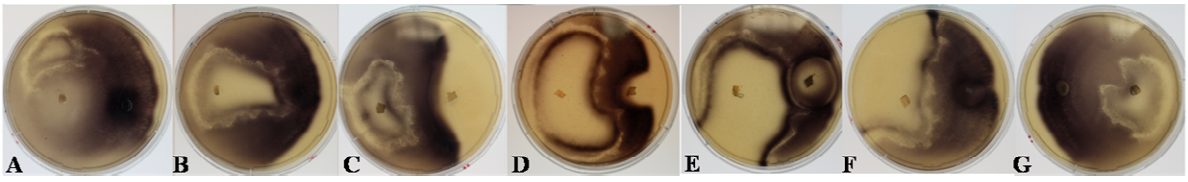

Fig. 10. Laccase production induced by co–culture with *R. solani*. On the left side of each plate *R. solani* grew and on the right Sebacinales strain. As a substrate 0.1 M α–naphthol was used. The dark violet colour indicates enzyme activity. A–*S. vermifera* MAFF305835 B–*S. vermifera* MAFF305837, C–*P. glomeralium*, D–*P. indica* AY505557 E–*S. vermifera* MAFF 305830, F–*S. vermifera* MAFF305828, G–*S. vermifera* MAFF305842.

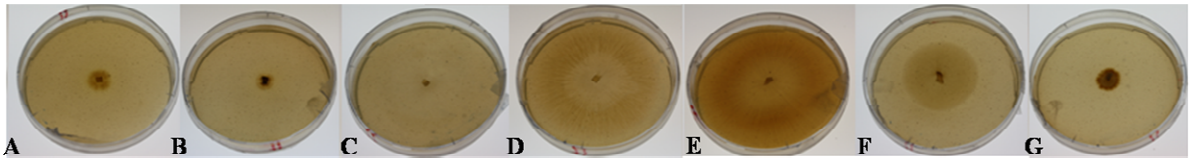

Fig. 11. Peroxidase plate's enzymatic test. As substrates were used 0.4% H_2O_2 and 1% pyrogallol. The brown colour indicates enzyme activity. A–*S. vermifera* MAFF305835 B–*S. vermifera* MAFF305837, C–*P. glomeralium*, D–*P. indica* AY505557 E–*S. vermifera* MAFF 305830, F–*S. vermifera* MAFF305828, G–*S. vermifera* MAFF305842.

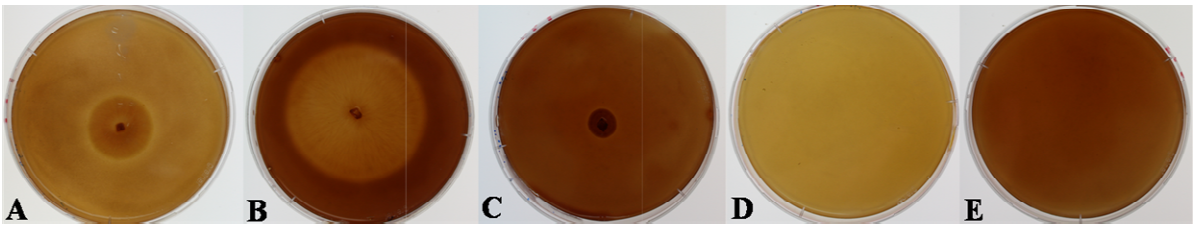

Fig. 12. Cellulase plate's enzymatic test. Fungi grew on medium containing cellulose. As substrate was used Lugol's solution. The bright zone around colonies indicates enzyme activity. A–*P. indica* AY505557, B–*P. glomeralium*, C–*S. vermifera* MAFF305842, D–positive control: MAE with cellulose treated with the lysing enzyme from *Trichoderma harzianum*, E–negative control: MAE with cellulose.

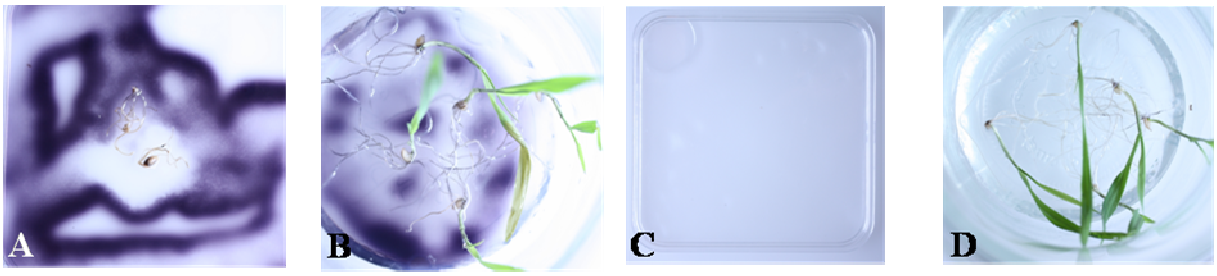

Fig. 13. *P. indica* laccase secretion induced by co–culture with *H. vulgare*. α–naphthol (0.1 M) was usedas substrate . The dark violet colour indicates enzyme activity. A–*P. indica* and autoclaved barley roots, B–*P. indica* colonizing barley roots, C–*P. indica* on 1/10 PNM, D–barley on 1/10 PNM.

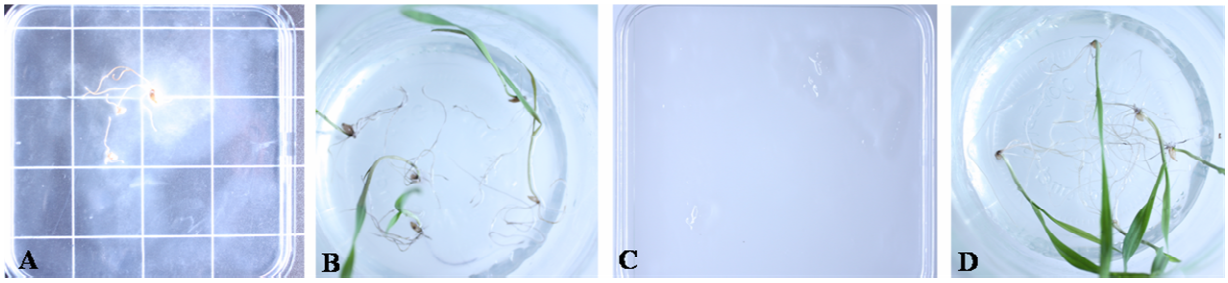

Fig. 14. Lack of laccase activity in *P. glomeralium* co–cultured with *H. vulgare*. α–naphthol (0.1 M) was used as substrate. A–*P. glomeralium* and autoclaved barley roots B–*P. glomeralium* colonizing barley roots, C–*P. glomeralium* on 1/10 PNM, D–*H. vulgare* on 1/10 PNM.

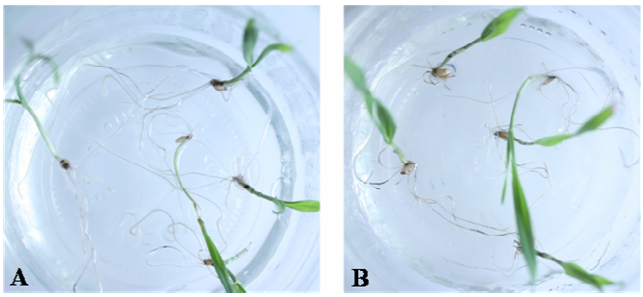

Fig. 15. Lack of laccase activity in *H. vulgare* co–cultured with autoclaved *P. indica* (A) and *P. glomeralium* (B). α–naphthol (0.1 M) was used as substrate .

3.5 Spectrophotemetric test of *Piriformospora indica*

Extracellular activity of enzyme were monitored as a function of time during growth in liquid culture (1/10 PNM). Due to differences in the scale of enzyme activity for each enzyme, their relative activity was calculated (Fig. 16a, b, c, d). With disregard to the analyzed enzymes and harvesting time point, the highest activity for each enzyme was set to 100 %. Subsequently, the enzyme activity values for other harvesting time points in each investigated condition were computed as a proportion of the highest one.

The activity of the different enzymes of *P. indica* as well as of barley grown separately did not exceed 40 % (Fig. 16a and 16c). An equal amount of enzymes activity was detected in the early time point during fungal colonization of living and decaying plant (Fig. 16b and 16d). Presence of living barley in the analyzed system induced only laccase activity which slowly increased to the highest activity at 7 days after inoculation and afterwards slowly went down (Fig. 16d and 17a). More variability in enzymes activities was detected when *P. indica* colonized decayed barley roots (Fig. 16b and 17b). High laccase production was observed

earlier (3 dai) in comparison to the case when fungus colonized living host roots. At 5th day after inoculation enzyme secretion immediately decrease and rose again on 7th day. In addition, by the second day after inoculation significant increase in activities of other enzymes were noticed. The highest lipase secretion was detected at later times–10 dai (Fig. 16b and 18d). The highest esterase production was noted after 10 days of co–culture (Fig. 16b and 18b). Peroxidase activity secreted in all inspected sets remained small and did not vary dramatically within two weeks of experiment (Fig. 16). However, considerable increase was detected at 15th day after inoculation in medium (Fig. 17c). No noticeable changes were detected in esterase and lipase activity when *P. indica* and barley were propagated alone in 1/10 PNM (Fig. 18). These results indicate that the enzymes production was predominantly associated with the presence of the symbiotic partner.

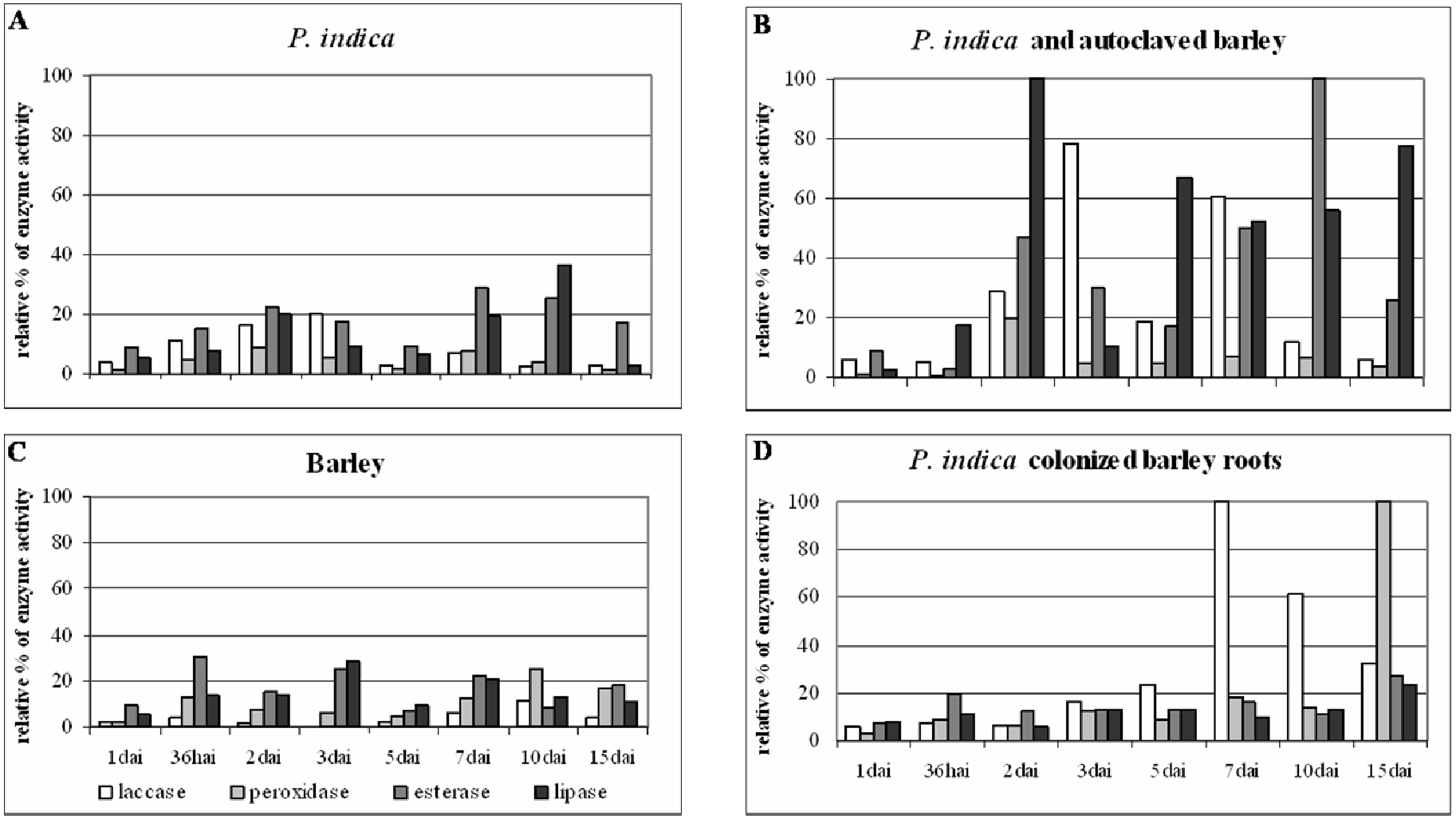

Fig. 16. Relative percentage of enzyme activity for *P. indica*, barley and *P. indica* colonizing barley roots (living and dead) cultured on 1/10 PNM during 15 days experiment period time. The experiments were repeated 3 times with similar results.

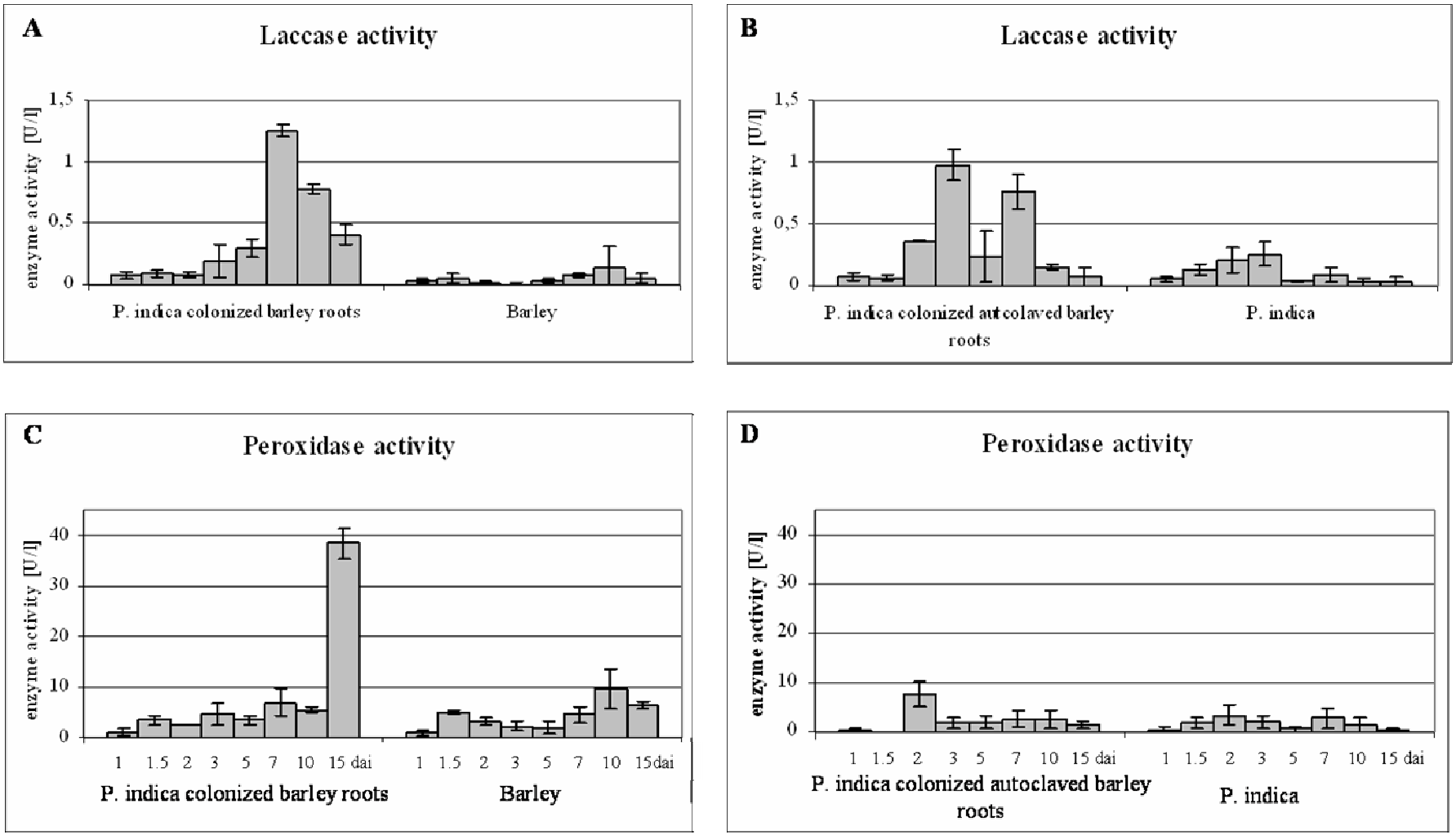

Fig 17. Variation in laccase and peroxidase activity for *P. indica*, barley and *P. indica* colonizing barley roots both (living and dead) cultured on 1/10 PNM during 15 days experiment period time. Standard deviation is calculated from 3 independent experiments.

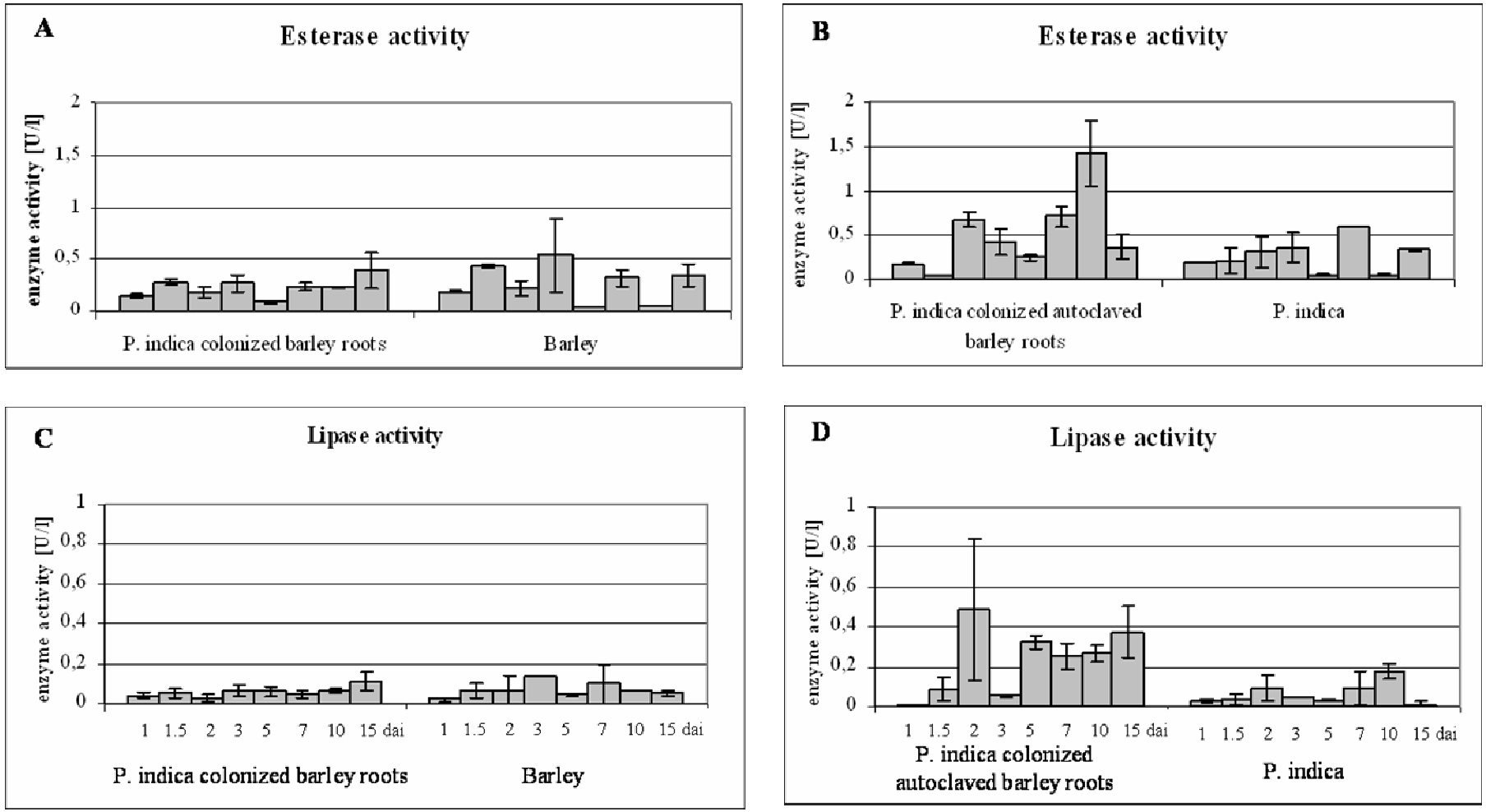

Fig 18. Variation in esterase and lipase activity for *P. indica*, barley and *P. indica* colonizing barley roots both (living and dead) cultured on 1/10 PNM during 15 days experiment period time. Standard deviation is calculated from 3 independent experiments.

3.6 *Piriformospora glomeralium* sp. nov. Zuccaro Weiss ex multinucleate rhizoctonia

The fungus was isolated by Williams in the 1984 from a spore of *Glomus fasiculatum* (Williams 1985) and can be propagated on wide range of synthetic media. On MAE colonies grew quicker than on CM, and their diameter measured after 2 weeks' growth at 24 °C was 60–70 mm and 40–50 mm, respectively. The fungal mycelium was cream–colored to pale yellow, mostly plane and submerged into the medium. The aerial mycelium was not detected. The hyphae were irregularly septate with diameter ranging from 1.6 to 2.8 μm. Multinucleate cells contained 2–6 nuclei (Fig. 19c). Chlamydospores were formed singly or in loose intercalary clusters and had mostly ring–shaped, very rare pear–shaped contained 1–10 nuclei (Fig. 19b), their diameter was similar to that of *P. indica* 8–12 μm. In older cultures plenty of chlamydospores were localized at the tip of irregularly inflated hyphae. Neither clamp connection nor sexual structures were observed. The main morophological difference between *P. indica* and the now described species is the arrangement and number of nuclei in the cells as well as the shape of the spores.

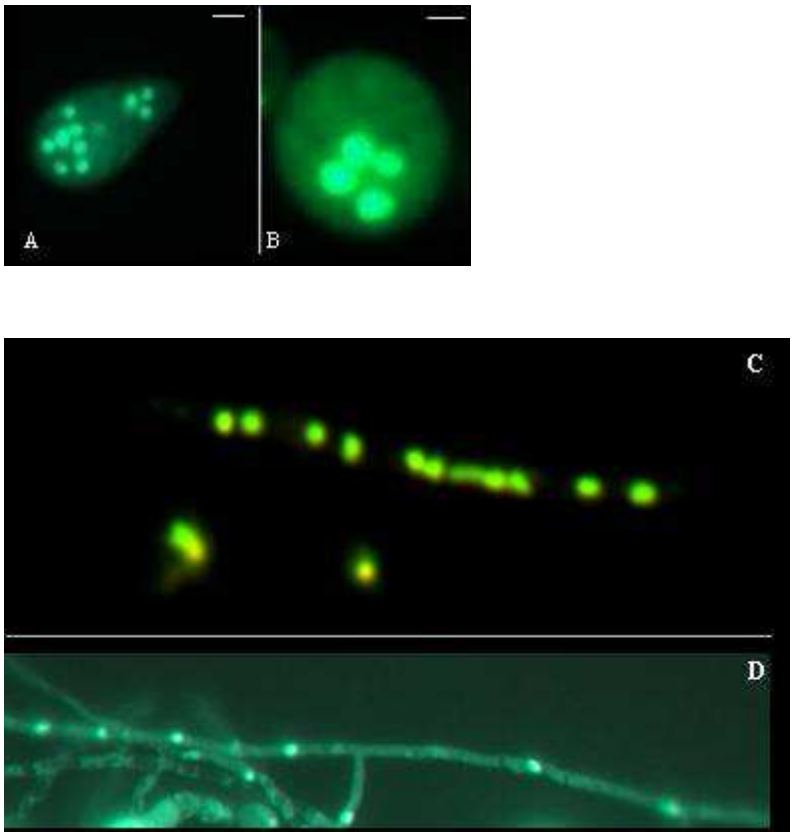

Fig. 19. A. Germinating *P. indica* spore (scale bar 4 μm), B. *Piriformospora glomeralium* spore (scale bar 2 μm), C. Hyphe stained by Syto 9 of *P. glomeralium*, and D. *P. indica*.

3.7 Protoplast regeneration

Fungal protoplasts are normally the best material for genetic transformation, therefore the best condition for protoplast preparation and regeneration was investigated. *Trichoderma harzianum* lysing enzymes were used for protoplast production from young fungal mycelia. Chlamydospores protoplastation was not successful. Three osmotic stabilizers were compared and the best regeneration was detected on medium containing 0.3 M sucrose followed by sorbitol, with colonies visible after 3 and 4 days, respectively (Fig. 20.). In addition, water was used as negative control in order to check if protoplasts solvent can influence protoplast vigor and regeneration efficiency. Protoplast regeneration of material resuspended in STC was significantly more productive than water (Fig. 21.).

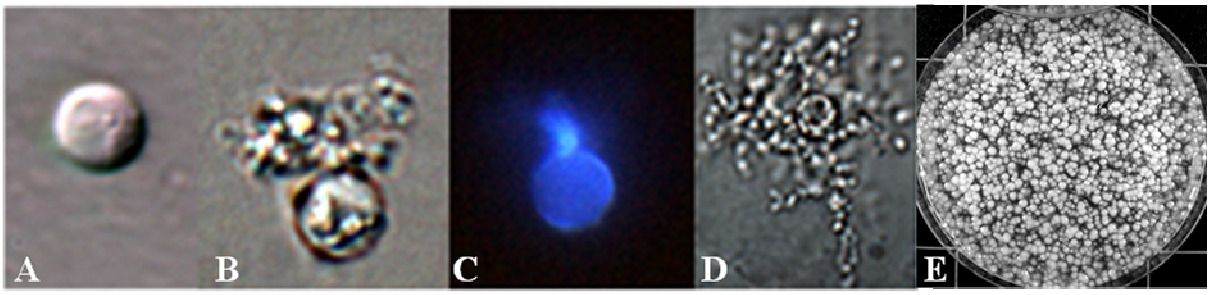

Fig. 20. Regenerationof *P. indica* protoplast. A–protoplast achieved after 60 min treatment of the young mycelium with *Trichoderma harzianum* (L1412 Sigma, Deisenhofen, Germany) lysing enzymes; B–regenerant after 24 h; B–autofluorescence of regenerant after 24 h; D–regenerants after 48 h; E–regenerants after 5 days (Zuccaro et al. 2009)

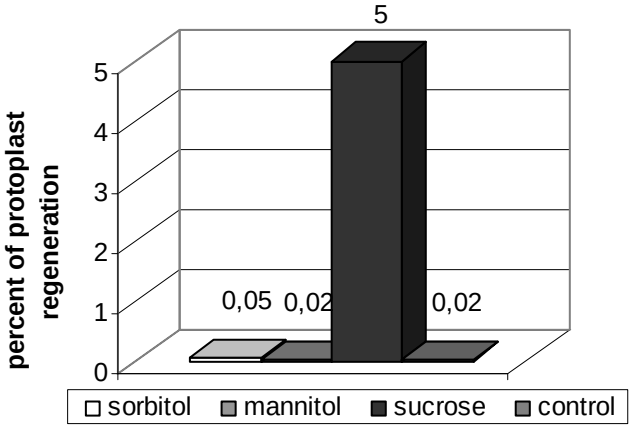

Fig. 21. Osmotic stabilizers test–Percentage of protoplast regeneration after 7 days using different stabilizers in the top agar (Zuccaro et al. 2009). Osmotic stabilizers supplemented complex medium, control–CM without any additional ingredient.

4 Discussion

Sebacinales, a worldwide distributed and very diverse group of fungi (Weiss et al. 2004), is divided into two subgroups. One includes endophytic *Sebacina vermifera* isolates, *P. glomeralium* (ex multinucleate rhizoctonia Warcup), and and *Piriformospora indica* (Selosse et al. 2007), whereas the second consists of ectomycorrhizae and endomycorrhizae species. Available isolates (*P. indica*, *P. glomeralium* and S. *vermifera* strains) confer growth promotion, disease resistance and abiotic stress tolerance to plants (Waller et al. 2005, Deshmukh et al. 2006). Moreover they are able to colonize a wide spectrum of plants including Mono– and Dicotyledons, and thus may have potential to be applied in agriculture and horticulture. Due to those reasons, it is important to isolate new closely related species in Europe. Environmental studies and phylogenetic analysis demonstrated that fungi closely related to Sebacinales isolates are present in Germany. Analyzed samples were selected from collection of DNA isolated from plant's roots of taxonomically diverse plants such as *Anthyllis*, *Medicago*, and *Lolium*. Phylogenetic analysis performed by Weiß et al. (2010) based on the Internal Transcribed Spacer region 28S nuclear ribosomal DNA ITS – 28S rDNA suggested a close relationship of those organisms with *P. indica*. Further, translation elongation factor (TEF) phylogeny was conducted. *TEF* is a conserved and strongly expressed in eukaryotic cells (Schirmaier and Philippsen 1984). Examination of the full length sequences of the *TEF* gene of *P. indica* demonstrated the presence of 8 introns (Buetehorn et al. 2000), two of them were amplified in our study. The investigated *TEF* introns were identical for all environmental clones but clearly differentiated from the laboratory isolates. The *TEF* sequences used in this study are informative of Sebacinales. In addition, phylogenetic analysis clearly divided Sebacinales isolates into three groups which most probably correspond to three different genera. Those results suggested that *TEF* genes can be used for design of specific primers for different sebacinoid groups.

4.1 Sebacinales genomes size estimation

Although Sebacinales have a positive influence on plants host, they are recently taken into consideration in genetic studies. Lack of a sexual phase make classic genetic analyses not applicable. Additionally, the number of chromosomes cannot be determined by light microscopy. However, elucidation the molecular processes and identification of the fungal

factors that lead to a successful symbiosis of *P. indica* and other Sebacinales with its plant partners is essential for better understanding the mechanism of that interaction. Analysis of sequences of whole genome seems to be the most suitable method to provide a complete story of biological networks. Although genome sequencing technologies developed very fast over last few years, some basic studies are required before, to make sequencing process fast and further analysis more efficient. Correct genome estimation is one of the most important tasks which should be performed before applying genome sequencing technologies. It is essential for sequencing costs valuation. We decided to predict genome size of five Sebacinales strains using few available molecular methods. Techniques such as: flow cytometry, reassociation kinetics, genomic reconstruction, PFGE, real–time PCR, confocal microscope can be implemented in that purpose. However, each of them has some limitation. Flow cytometry determines relative nuclear DNA content per spore and was used for genome estimation for fungi such as the basidiomycete rust fungus *Puccinia recondita* (Eilam et al. 1994), arbuscular mycorrhizal fungus *Glomus intraradices* (Hijri et al. 2004), or the etiologic agent of histoplasmosis, ascomycete fungus–*Histoplasma capsulatum* (Carr and Shearer Jr 1998). Sebacinales chlamydospores are multinucleate therefore this method cannot be applied. Reassociation kinetics, reconstruction or real time PCR based on one copy gene analysis. Genomes of a few fungi were investigated using those approaches. Genomes of the obligate Oomycetes pathogen *Bremia lactucae* (Francis et al. 1990) and the basidiomycete *Paxillus involutus* forming ectomycorrhizal symbiosis (Le Quere et al. 2002) were analysed using reassociation kinetics (reassociation rate of denatured DNA is measured under defined conditions). Reconstruction technique (the one copy gene is used as a hybridization probe) was employed for genome analysis of such organisms as *Phytophthora megasperma f. sp. glycinea* (Mao and Tyler 1991) and Ascomycetes *Colletotrichum graminicola* (Randhir and Hanau 1997). A real–time PCR based approach was established for strain 368 FY1679 of *Saccharomyces cerevisiae*, the platyfish *Xiphophorus maculatus* and *Homo sapiens sapiens* (Wilhelm et al. 2003) and applied also for such Ascomycetes fungi as: *Cladonia grayi* (Armaleo and May 2009) and *Zygosaccharomyces* species (Solieri et al. 2008). False recognition of one copy gene can be the reason of wrong genome size prediction. Additionally, real–time PCR requires very good quality and quantity of DNA. For some organisms achievement of those terms might be problematic. Too big size of chromosomes might be important barrier in PFGE

approach. Hence, the best way for correct prediction of genome size is combining few (at least two techniques), which rely on completely different assumption.

In that study, genome sizes of analyzed fungi were estimated using real–time PCR and PFGE. First, both methods were established for *P. indica* and further applied for other Sebacinales isolates. The real–time PCR approach relied on absolute quantification single copy gene in genomic DNA. Based on the presumption that *TEF* and *GAPDH* are both single copy in the *P. indica* genome, southern blot analysis of digested genomic DNA and chromosomes separated by PFGE were performed. Although in some Ascomycetes and Zygomycetes the *TEF* gene was detected in multiple copies, in almost all Basidiomycetes genomes analyzed so far only one copy of this gene was detected. The *GAPDH* gene is also present in single copy in many of Basidiomycetes. However some exceptions such as *Agaricus bisporus* with two different *GAPDH* genes are known. According to southern blot analysis, *TEF* and *GAPDH* are one copy genes. Similar assay was used for *P. glomeralium* and *Sebacina vermifera* isolates. In the genome of analyzed fungi *TEF* gene is most probably present only one time in the homokaryotic genome, therefore it was used for genome sizes estimation. Southern blot analysis performed for *S. vermifera* MAFF305842 did not give clear indication concerning the *TEF* gene copy number. Genomic DNA digested with Bam HI and hybridized with specific probe showed one band, however multiple bands were detected when Hind III enzyme was used (Zuccaro unpublished data). Those findings proposed presence of SNPs (single–nucleotide polymorphism) in TEF gene of *S. vermifera* MAFF305842. Further investigation of that gene should be performed. Additionally, for *P. indica TEF* and *GAPDH* were localized on chromosomes. *TEF* is located on first chromosome (5.4 Mb) and GAPDH on third (2.5 Mb).

Fungal DNA was isolated using two different techniques: modified method from Doyle and Doyle followed by a CsCl centrifugation and FastDNA® SPIN Kit for soil. Results are displayed in Table 8. Genome size estimated using kit extracted DNA was 30–50 % (depending on strain) bigger than with the second method for all Sebacinales isolates. Protein contaminations in samples of genomic DNA were not detected. The ratio of absorbance 260/280 was in the optimal range of 1.9 for both DNA extraction methods. However, organic compounds were present in kit extracted DNA. The ratio of absorbance 260/230 was below the optimal value for each isolate. Those findings may explain the differences in the genome size predicted using diverse methods of DNA isolation. *S.*

cerevisiae (1n and 2n) was used to validate that method. DNA from *S. cerevisiae* 1n was extracted using both method (Doyle and Doyle followed by a CsCl centrifugation and kit). The genome size predicted using primers specific for the ScRPS3 gene was in the range of the known genome size for this organism (12 Mb). Extraction method as well as the ploidy of organisms used for DNA isolation did not influence genome size estimation. Those results might suggest that Sebacinales cells contain some components which strongly interfere both with buffers used for DNA extraction either directly with DNA and inhibit extraction procedure. Without consideration of DNA extraction method, *S. vermifera* MAFF305828 seems to have the biggest genome and, *S. vermifera* MAFF 305830 the smallest one in between analyzed Sebacinales strains.

The second method implemented for genome determination was Pulse Field Gel Electrophoresis. PFGE is an effective technique for separating big fragments of DNA such as chromosomes, and is a meaningful tool for basic genetic studies, especially in lower eukaryotes such as fungi. Chromosome–sized DNA molecules of Sebacinales isolates were successfully obtained after young mycelium protoplastation and resolved by PFGE. The electrophoretic conditions permitted the separation of 6–7 chromosomal bands in the range of 1.3 to 5.4 Mb for *P. indica*. The total size of them agreed with the genome size estimated by quantitative real–time PCR. The karyotypes achieved under these conditions were reproducible. The staining intensity of the chromosome bands 3 and 5 was more intensive than other bands. The separation in this part of the gel was not satisfying. Those results displayed the possibility of attendance either heterologous chromosomes with similar or identical size or multiple copies of a homologous chromosome. Among the investigated isolates the karyotype analysis confirmed that *P. glomeralium* is the closest related fungus to *P. indica*. Similar genome size and number of chromosomes separated those two fungi from *S. vermifera* isolates. The large size of *S. vermifera* MAFF305842 and MAFF305828 chromosomes was the reason of not adequate separation. Nonetheless, it is clear that the smallest chromosomes from *P. indica* (about 1.3 Mb) and *P. glomeralium* (about 1.5 Mb) are not present in the other isolates. Specific differences in the chromosome profiles within isolates from the same clade were also evident. The genome size determinated by PFGE in an organism whose ploidy is unknown may lead to incorrect conclusions due to incapacity during separating homologous chromosomes (Torres–Guerrero 1999).

Despite the clearly diversity in the chromosome profiles among isolates, genome sizes estimated by those two techniques did not vary particularly within the clades. 8 % dissimilarity was observed between *P. indica* and *P. glomeralium* and a maximum of 28 % within the *Sebacina vermifera* strains from clade 3. Those differences might be present due to gene duplication or loss, horizontal transfer events and transposable element. *P. indica* genome size was additionally analysed using confocal scanning microscope. The staining procedure applied for chlamydospores and hyphe worked very well. However microscopic observation, in the same set conditions, such different structure like hyphae and *S. cerevisiae* cells, used as a standard organism, was not possible. Genome size estimated using that technique confirmed genome size to be in the average of 22 Mb. The dimensions of genome sizes support the thesis that sebacinoid fungi from the subclade B (Weiss et al. 2004) hold a relatively small genome. Genome sizes among known Basidiomycota ranged 25–125 Mb, with high level of repetitive DNA. Such genome sizes are characteristic for mushrooms like *Coprinopsis cinerea* (37 Mb, Stajich et al. 2010), *Schizophyllum commune* (38 Mb http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&cmd=ShowDetailView&TermToSearch=12852), *Puccinia graminis* (81 Mb, http://www.ncbi.nlm.nih.gov/sites/entrez?Db= genomeprj&cmd=ShowDetailView&TermToSearch=12848). Pathogenic Basidiomycetes possess smaller genomes. The plant pathogen *Ustilago maydis* genome is 20 Mb (Kämper et al. 2006) or that of *Cryptococcus neoformans* causing a human disease– is 19 Mb (Loftus et al. 2005). Despite small genomes, Sebacinales are free–living and non–pathogenic fungi. The TEF sequence analysis suggested already that *P. indica* introns might be small. Moreover, investigation of genomic date achieved after pyro–sequencing implying that *P. indica* has a very compact genome with very less repetition (Zuccaro et al. in prep.). Sebacinales genome size predictions additionally proved that analyzed Sebacinales strains are distinct and supported division those isolates into 3 clades.

An additional band smaller than 0.2 Mb was often observed for *P. indica* on the gel after PFGE. This band was identified using southern blot approach as a mitochondrial DNA. Normally, fungal mitochondrial genetic information size ranged from 19 kb (*Sch. pombe* (Lang et al. 1983)) to 170 kb (*Agaricus bitorquis* (Hintz et al. 1985)) and generally is located on circular, or rarely linear, double–stranded DNA molecules. It was not always detected on the gel, its presence or absence has been linked to sample preparation. Genetic information from mitochondrium was seen only for *P. indica*.

4.2 *P. indica* protoplast regeneration

P. indica can be a model organism representing Sebacinales. Genome data together with transformation system can provide a lot of information about the fungus as well as the genetic nature of symbiosis. Efficient procedure for protoplast production and proper regeneration condition for them are essential for good transformation system. In that study, the effective protocol for protoplastation was developed together with the best condition for *P. indica* protoplast regeneration. A lot of protoplastation protocols were published so far which based on the biochemical removal of the cell wall using different enzymes. Protoplast from filamentous Ascomycete *Ashbya gossypii*, for example, was obtained using zymolase (Choi 2006), mycelium from dimorphic Zygomycete *Benjaminiella poitrasii* was incubated first in protease solution, later treated with a mixture of dithiothreitol and β–mercaptoethanol and then directly suspended in the cell wall lysing enzyme mixture (Sigma lysing mixture, zymolyase and lyticase) (Chitnis and Deshpande 2002). Mixture of lysing enzyme from *Trichoderma harzianum* supported by mazerozyme, driselase and pectilyse was tested for Sebacinales, however protoplastation efficiency was not satisfied. The best effect in protoplast quality and quantity was achieved using lysing enzyme from *Trichoderma harzianum*. This commercially available substnance contains cocktail of cell wall degrading enzymes including β–glucanase with some cellulase, protease and chitinase activities. The enzymes composition allowed for very fast cell wall degradation. In consequence, exposure time of destructive enzyme activity was reduced. Protoplasts gathered in shorter exposure times to lytic enzymes have notable ability to regenerate than those which were incubated for longer time (Zhou et al. 2008). In protocol established in that study fungal mycelium was incubated with enzyme solution 1 h, whereas in instruction mentioned above from 1 h to 5 h. Additionally, the right osmotic stabilizer applied in the top agar and medium lead to the successful regeneration process. Those substances can keep the balance of interior and exterior osmotic pressure of the protoplasts, and guard them from being broken. The cell wall constitution diversifies among species, therefore different concentrations of various osmotica will be optimal for each species. A lot of chemicals including inorganic salts, sugars and sugar alcohols can be applied as osmotic pressure stabilizers (Davis 1985). Inorganic salts are more effective with filamentous fungi, and sugar and sugar alcohols with yeasts and higher plants (Lalithakumari 1996). Mannitol, sorbitol and sucrose were tested here. From among of them the best effect was observed on medium with sucrose. Furthermore, results obtained in that study showed that

STC buffer is better protoplast solvent than water. The main ingredient of that buffer, sugar alcohol–sorbitol protects protoplast against destruction.

4.3 Biochemical analysis of Sebacinales

In order to establish close interaction with the plant host, Sebacinales must overcome few barriers or natural plant defence mechanisms. The first diffculty might be plant cell wall composed of cellulose, hemicellulose, xylan and lignin. Cellulose, for example, can be digested only by fungi and some bacteria. Later, in response to invader, host is able to produce some substances with antifungal activity. We analyzed, therefore, some enzymes essential in plant cell wall degradation and later during breaking the plant defence apparatus. The diversity in enzyme activity provides clear identification that physiological processes are taking place in the analyzed environment.

To screen enzymes secreted by Sebacinales some plate's enzymatic tests were performed. Cellulase, pectinase, laccase, peroxidase and proteinase were analyzed. Although all fungi grew well in axenic culture, enzymes productions were not always detectable. Cellulose is one of the most important and very difficult digested, by other organisms, polysaccharide present in plant cell wall. Among analyzed Sebacinales strains only *P. glomeralium* and *P. indica* presented higher cellulase activity. In culture of *S. vermifera* MAFF305828 small cellulase activity was also observed. Cellobiose–the intermediate product of cellulose hydrolysis is known as an effective inhibitor of that enzyme (Walker and Wilson 1991). This phenomenon might explain the lack of enzyme activity in the test. *S. vermifera* strains might secrete cellulase, however, the cellobiose accumulation could immediately stop the further activity. Moreover, glucose was also described as enzyme inhibitor (Walker and Wilson 1991). The complete cellulolysis could break down cellulose into glucose units and, in consequence, block enzyme activity. Besides, the amount of secreted enzyme could be so faint making them not possible to identify in the applied test. Only one species – *S. vermifera* MAFF305835 secreted pectinases. Pectin–the substrate for that enzyme is present in plant cell walls next to cellulose. *S. vermifera* MAFF305835 does not produce cellulase in axenic culture but we can assume that it exudes pectinase which allows entering into the plant cell. For mycorrhiza fungi such as: *Suillus variegatus, Suillus bovinus, Piloderma croceum, Pisolithus tinctorius, Paxillus involutus* and *Amanita muscaria* no significant enhancement of pectinase activity was observed after induction with pectin, polygalacturonic acid or galacturonic acid (Ramstedt and Soderhall 1983). In

addition, minor enzyme production can be undetectable by the test (Garcia–Romera et al. 1991). On the other hand, the low activities of cellulase and pectinase secreted by investigated fungi could be an adaptation to a symbiotic life. Mycorrhizal fungi must maintain a careful balance during plant roots colonizing process. The symbiont must avoid eliciting substances which initiate host resistance reactions. Lack of cell degrading enzymes has been already observed. In the *Laccaria bicolor* genome, genes responsible for secretion of mentioned enzymes, were not detected. Probably *L. bicolor* as well as *S. vermifera* strains use different modes of root colonization. One of the possible ways is the 'crack entry' where the hyphae colonizing the root surface overrun plant tissue through breaks in the epidermis cells. Martin et al. (2008a) proposed that *L. bicolor* may use ectomycorrhiza–induced expansins for releasing the host cell wall components and promoting the growth of the hyphae in the symbiotic apoplastic space. The minimal set of hydrolytic enzymes such as polysaccharide hydrolases, lyases and pectin esterases was found also in ubiquitous pathogen *U. maydis* genome. Those findings agreed with its biotrophic lifestyle, in which minimization of damage to the host are relevant in order to avoid plant defence responses (Kämper et al. 2006). The similar mechanisms can be used by *S. vermifera* isolates. Sebacinales strains investigated in my work presented quite high proteinase activity during growth on gelatine medium. The plate enzymatic tests indicate that high production of laccase for all *Sebacina vermifera* isolates and no activity for both *P. indica* and *P. glomeralium* under experimental growth condition. Lack of laccase activity was previously observed also for other Basidiomycetes such as *Serpula lacrymans* (Score et al. 1997). Additionally, *P. indica* exudes very low amount of peroxidase, in case of *P. glomeralium* the enzyme amount was not detectable. Laccase and peroxidase belong to oxidoreductases involved in protection against host oxidative responses and are commonly secreted by fungi, especially by Basidiomycetes (Edens et al. 1999). Besides, laccase has several, very important physiological functions like detoxification of phenolic compounds. In addition, Kellner et al. (2007) analyzed laccase production by some Basidioimycetes fungi including *P. indica*. Presence of laccase activity in medium where fungus was propagated was proved using laccase specific antibody *LccCbr2*. Their results showed very weak (67 kDa), however, clear band indicating laccase activity. Moreover, *P. indica* genome analysis determined that fungus has at least two laccase genes with signal peptide (Zuccaro et al. in prep). All those findings suggested that *P. indica* has machinery necessary for the secretion of this enzyme, therefore the best conditions for laccase

production were further investigated. In addition, impact of cultural conditions on repression of the laccase gene expression was already observed (Luisa et al. 2004). The similar phenomenon could have taken place in investigated conditions for *Piriformospora* strains. Those two strains are closely related to *Sebacina vermifera* isolates, however, they are clearly divided from them. *Piriformospora* strains position on phylogenetic tree as well as origin of *Sebacina vermifera* isolates (they belong to Orchid mycorrhizae group) might explain differences in laccase secretion.

The distinctions in the enzyme profiles for Sebacinales corresponded with their phylogenetic affinity proved by both, individual and combined analyses conducted using ITS, 28S and TEF DNA sequences. Strong laccase activity demonstrated by all *Sebacina vermifera* isolates as well as lack of cellulase secretion and opposite enzyme activity in *Piriformospora* strains clearly divide those fungi in three clades.

Additionally to plate's tests, variability in *P. indica* laccases, peroxiadases, esterases, and lipases production were analyzed in order to examine fungus response to plants roots. The activity values of all measured enzymes secreted by *P. indica* were very low. The highest value (around 10 U/l) was detected for peroxidase, for other enzymes the activity did not increased over 2 U/l. Those amounts of secreted enzymes in analyzed conditions are not sufficient to use the fungus for commercial enzyme production under this growth conditions.

P. indica cultivated on 1/10 PNM demonstrated very slow growth rate as well as low, however detectable, activity of all analyzed enzymes. In fact, that medium, suitable for plants without carbon source, can be considered as minimal medium for fungus. Observation of *P. indica* implied reduction of its metabolism. Similarly to *P. indica* growing without symbiotic partner, barley without symbiont did not secret the higher amount of analyzed enzymes. All of them did not increase over 20–30 % of the highest activity, what can be consider as normal ratio for plant growing in sterile condition.

Completely different results were observed when plants (either living or dead) were present in the system. Almost all analyzed enzymes respond to decay plant material. During first 36 h after inoculation the low enzyme activity was observed. Since 2nd day post inoculation the increase of activity was identified. Only peroxidases secretion measured in those circumstances was very weak. Significant changes in enzymes production were also detected whereas the living plant partner appears in the environment.

Laccase was one of the enzymes which response was evident in that growth conditions. The enzymes variation was more visible when *P. indica* colonized decay plant roots.

On plate's tests containing MAE without any supplementary ingredients as well as on solid 1/10 PNM *P. indica* laccase activity with α–naphtol as a substrate was not observed. However, more sensitive spectrophotometric assay detected small enzyme production. In consequence of not favourable growth conditions fungus was forced to gain carbon source from its own. Hiscox et al. (2010) demonstrated higher production of this enzyme in interaction zones between *T. versicolor* and different species of asco– and basidiomycota: *Stereum gausapatum, Daldinia concentrica, Bjerkandera adusta, Fomes fomentarius, Hypholoma fasciculare* whereas no enormous fluctuations were detected in other regions. These findings suggested substantial function of the enzyme during competition between two organisms and, therefore, can have significant impact on natural environment. Coculture with *R. solani* was conducted in order to examine enzyme secretion by Sebacinales isolates in presence of competitor. Increase of laccase exudation in that system in contact zone between all scrutinized strains and *R. solani* was detecetd. Obtained results were not clear for *P. indica* and *P. glomeraliu*. The dark violet colour indicating laccase secretion was present only in contact zone, whereas on the plates with another Sbacinales strains mycelium became violet in area where contact between two fungi was limited. Those findings might suggest either enzyme activity came from *R. solani* or from both interacting partners but the enzyme secretions was strickted only to contact zone. The macro– and microscopical observation implied that *P. indica* was able to grow in presence of *R. solani* without any problem. *P. indica* mycelium grew parallel to *R. solani* and did not demonstrate any stress symptoms. Moreover, Ghahfarokhi and Goltapeh (2010) mentioned that *P. indica* is able to colonize hyphae of another fungus. This phenomenon was also observed in my study. The similar remarks were detected in coculture *P. glomeralium* with *R. solani*. Furthermore, *P. glomeralium* established more aggressive relationship whit competitor what was noticed on plates test as well as under microscope. *R. solani* growth was clearly inhibited by *P. glomeralium*. *R. solani* did not grow as fast as on plates with another Sebacinales isolates and the colour indicating laccase activity was stronger. The further analysis of that phenomenon should be performed. In tests, conducted with *P. indica* grown in presence of dead or living barley roots on solid 1/10 PNM, laccase activity was detected 5 days after inoculation in the area around roots colonized by fungus. The absence of laccase activity during axenical growth, and presence while living or dead

barley roots were present, showed inducible nature of that enzyme. Interestingly, the enzyme activity, examined with the same procedure, was not observed for *P. glomeralium*. The previous studies demonstrate that *P. indica* and *P. glomeralium* have different influence on barley. Both fungi promote plant growth and reduce disease symptoms, however *P. indica*'s positive effect is stronger (Deshmukh et al. 2006). These results do not exclude presence of laccase genes in fungus genome, they might just propose that *P. glomeralium* require other hosts for enzyme induction. Study of laccase secretion by *P. indica* colonizing living barley roots in liquid 1/10 PNM demonstrated that enzyme production slowly increased to get maximum value at 7^{th} day after inoculation. Laccase plays important role during lignin degradation as well as in neutralization of toxic phenols (Haars and Huttermann 1981) and defence against host oxidative responses (Edens et al. 1999). Microscopic investigation indicated that around third day after inoculation fungus already penetrated the plant cortex cell and further begun inter– and intra–cellular growth (Deshmukh et al. 2006). At that time laccase activity increased. When maximal activity of enzyme was observed, chlamydospores were formed within the plant root. Schaefer at al. (2009) checked plant response to *P. indica*. 459 (3 dai) and 509 (7 dai) genes were differentially regulated in *P. indica* colonized roots compared to mock–treated. Genes involved in plant defence/stress responses formed the largest group of variously regulated genes. The plant immune system answered to invaders and got feedback from fungus in higher laccase secretion. Laccase secretion was also very high when instead of living barley roots, dead ones were colonized by the fungus. The higher enzyme secretion level was observed three days after inoculation. Peroxidases activity measured in those circumstances was very weak. Similarly, lack of peroxidase with laccases as the predominant extracellular phenoloxidase was observed for white rot fungus *Pycnoporus cinnabarinus* (Eggert et al. 1996).

In presence of decay barley roots production of lipases by *P. indica* were induced in the highest degree at 2^{nd} day after inoculation Lipases are essential for catalyze the hydrolysis of ester bonds in lipids. Belcarz et al. (2005) demonstrated that, in the presence of humic acids derived from brown coal, Basidiomycete white–rot fungus *Bjerkandera adusta* synthesized significant amounts of laccase and lipase. Lipids occurred naturally in humic substances. The process of medium preparation in my study (autoclaving roots in 1/10 PNM) supports the presence of degrading plant cell wall components including lipids and can be compared to humic acids.

Esterase, which belongs to the same enzymes group with lipase, presented the highest activity while *P. indica* colonized decay too. General, those enzymes demonstrate higher activity in the rhizosphere colonized by mycorrhiza fungi than non–mycorrhizal controls (Vazquez et al. 2000). *P. indica* is not a arbuscular mycorrhiza fungus, though some mycorrhiza characteristic can be detected. In addition, one of the hypothesis concerning endophytes proposed that they become saprotrophs after the onset of senescence of host tissue. This life style modification can be due to the alteration taking place in host tissue during senescence, which allows mycelium to penetrate the epidermis and colonize host surface (Promputtha et al. 2007).

Lipase and esterase activities stopped on the level not higher than 25 % of highest activity in presence of living plant in investigated environment. Those results suggest that both enzymes are not relevant in analyzed condition.

5 Summary/ Zusammenfassung

The order Sebacinales belongs to a taxonomically, ecologically, and physiologically diverse group of fungi within the Phylum Basidiomycota. Using several molecular techniques they were detected all over the world. Few isolates, classified into the clade B, are available at the moment: the root–colonizing mutualistic fungus *Piriformospora indica,* various *Sebacina vermifera* isolates from autotrophic orchids, as well as *Piriformospora glomeralium* (ex multinucleate rhizoctonia DAR29830, Warcup). All of them were described as growth promoting and resistance–inducing fungi. In the present work, seven Sebacinales isolates of the clade B were characterized molecularly and phenotypically. In addition, the presence in Germany of fungi closely related to *P. indica* was proven.

Phylogenetic analyses conducted using DNA sequences from the 28S and the translation elongation factor 1–α gene (*TEF*) showed that analyzed Sebacinales isolates represent at least 3 distinct groups of isolates. Further, three independent environmental samples, collected from two different areas in Germany were examined. The analysis demonstrated that the sample respresented organisms closely related to *P. indica*. The analyzed introns of their TEF gene had the same sequence. This finding suggests that one genotype is present in different area of Germany which is associated with roots of taxonomically diverse plants, including *Anthyllis*, *Medicago* and *Lolium.*

Moreover, the fungal genome sizes of five sebacinoid isolates were estimated using Pulsed Field Gel Electrophoresis (PFGE) and real–time PCR based on the absolute quantification of a single copy gene (TEF). The fungi have at least 4 to 7 chromosomes and a genome size ranging from 21 to 26 Mb.

Morphological, physiological and molecular studies of the multinucleate rhizoctonia DAR29830 isolate proved that this strain is the closest related to *P. indica*, the most investigated member of the Sebacinales. Therefore it is designed in this study as a provisional new species named *Piriformospora glomeralium* sp. nov.

Seven Sebacinales strains were tested for extracellular enzyme production including peroxidase, laccase, protease, pectinase, and cellulase. In general, the enzymatic test demonstrated strong protease activity in cultures of all the analyzed fungi. The orchid mycorrhiza isolates showed stark laccase activity and lack of cellulase production in axenic culture whereas the *Piriformospora* strains were able to digest this plant cell wall component. Only one isolate proved to secrete pectinase. All Sebacinales isolates except *P.*

indica and *P. glomeralium* that were co–cultured with a fungal competitor (*Rhizoctonia solani*) generated significant amounts of laccase. The presence of a plant host, life or dead, had noticeable impact on laccase secretion by *P. indica* but no influence on P. *glomeralium* enzyme exudation. In addition, production of laccase, peroxidase, esterase, and lipase by *P. indica* in response to plant host was spectrophotometrically investigated. Diversity in laccase and lipase activity was observed mostly in presence of decay plant material.
Differences in the enzyme profile for the analyzed Sebacinales strains agreed mostly with their phylogenetic position. In addition, genome estimation as well as karyotype analysis clearly confirmed the phylogenetic study and proved that *Sebacina vermifera* should be considered as complex of isolates. Moreover, laccase secretion by *P. indica* can be induced only by the presence of the plant symbiotic partner.

Zusammenfassung

Die Ordnung der Sebacinales gehört zu einer taxonomisch, ökologisch und physiologisch diversen Gruppe von Pilzen im Phylum der Basidiomyceten. Weltweit können sie durch verschiedene molekulare Techniken detektiert werden. Wenige Isolate, klassifiziert im Untergruppe B, sind zurzeit verfügbar: der Wurzel–kolonisierende, mutualistische Pilz *Piriformospora indica*, verschiedene *Sebacina vermifera* Isolate von autotrophen Orchideen, genauso wie *Piriformospora glomeralium* (ex multinucleate rhizoctonia DAR29830, Warcup). Für alle wurde beschrieben, dass sie das Wachstum fördern und Resistenz vermitteln gegen biotischen und abiotischen Stress. In der vorliegenden Arbeit wurden sieben Sebacinales–Isolate der Untergruppe B molekularbiologisch und phänotypisch charakterisiert. Zusätzlich wurde das Vorkommen eines zu *P. indica* nahe verwandten Genotyps in Deutschland bewiesen.
Phylogenetische Analysen, unter Verwendung von DNA–Sequenzen aus 28S und dem Translationselongationsfaktor 1–α Gen (TEF), zeigten, dass diese Organismen mindestens drei verschiedene Isolat–Gruppen repräsentieren. Zudem wurden drei unabhängige Proben aus zwei verschiedenen Orten Deutschlands untersucht. Die Analyse zeigte, dass sie eng verwandt sind mit *P. indica*. Die analysierten Introns des TEF–Gens haben die gleiche Sequenz. Dies impliziert, dass ein Genotyp, welcher in verschiedenen Gegenden Deutschland vorkommt, mit den Wurzeln taxonomisch diverser Pflanzen, inklusive *Anthyllis*, *Medicago* und *Lolium*, assoziiert ist.

Darüber hinaus wurde die Genomgröße von fünf Sebacinoid–Isolaten durch Pulsed Field Gel Electrophorese (PFGE) und quantitative Real–time PCR bestimmt. Die Pilze besitzen zwischen vier und sieben Chromosomen und die Genomgröße variiert von 21 bis 26 Mb.
Morphologische, physiologische und molekulare Untersuchungen des multinuklearen Rhizoctonia Isolates zeigten, dass dieser Stamm am engsten mit *P. indica* verwandt ist und daher in dieser Abhandlung als *Piriformospora glomeralium* sp. nov. benannt wird.
Sieben Sebacinales–Stämme wurden auf extrazelluläre Ezymproduktion von Peroxidase, Laccase, Protease, Pectinase und Zellulase getestet. Generell zeigten diese Tests eine starke Peroxidase–Aktivität der Kulturen bei allen analysierten Pilzen. Die Orchideen–Mycorrhiza–Isolate zeigten eine starke Laccase–Aktivität und ein Fehlen von Zellulase–Produktion in axenischen Kulturen während die *Piriformospora*–Stämme diese Zellwandkomponente zersetzen konnten. Nur ein Isolat sekretierte Pectinase. Alle Sebacinales Isolate außer *P. indica* und P. *glomeralium* co–kultivert mit einem pilzlichen Konkurrenten (*Rhizoctonia solani*) bildeten signifikante Mengen an Laccase. Die Gegenwart des Wirts, tot oder lebendig, hatte einen bemerkenswerten Einfluss auf die Laccase–Produktion von *P. indica* und keinen Einfluss auf die Enzymexudation von *P. glomeralium*. Zusätzlich wurde die Produktion von Laccase, Peroxidase, Esterase und Lipase bei *P. indica* in Bezug auf den Wirt photospektrometrisch untersucht. Unterschiede in Laccase– und Lipase–Aktivität wurden vor allem in Gegenwart von totem Pflanzenmaterial beobachtet. Unterschiede in den Enzymprofilen der analysierten Sebacinales–Stämme korrelieren mit ihrer phylogenetischen Position. Außerdem bestätigen die Genomgrößenbestimmung und die Karyotypen–Analyse, dass *Sebacina vermifera* als ein Komplex von Isolaten angesehen werden sollte. Zudem kann die Laccase–Sekretion bei *P. indica* nur durch Anwesenheit eines symbiotischen Partners induziert werden.

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List of Abbreviations

AM	Arbuscular mycorrhizae
AMF	Arbuscular mycorrhizal fungi
bp	base pair
CsCl	Caesium chloride
D&D	DNA extracted by Doyle and Doyle followed by CsCl cleaning step
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleosidtriphosphat
dai	day(s) after inoculation
ECM	Ectomycorrhizae
EDTA	Ethylendiamintetraacetat
et al.	and others
Fig.	Figure
GAPDH	glycerol–3–phosphate dehydrogenase
kDa	Kilo Dalton
L	Litre
M	Molar
min	Minute(s)
NaCl	Sodium chloride
ng	nanogram
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
1/10 PNM	plant nutrient medium
rpm	rounds per minute
RT	Room temperature
TEF	Translation elongation factor 1 alpha

Deklaration

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus–Liebig–Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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