# Ph.D Thesis

# Diversity and Antagonistic Activity of Endophytic Fungi from Sweet Cherry and Pepper

**Neda Haddad Derafshi**

Supervisors:

Dr. Krisztián Halász

Prof. Dr. Noémi Lukács


**Department of Plant Physiology and Plant Biochemistry**

**Budapest Corvinus University**

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| **Name:** | Doctoral School of Horticultural Sciences |
| **Field:** | Crop Sciences and Horticulture |
| **Head of Ph.D School:** | Dr. Magdolna Tóth<br>Academic Professor, DSc<br>Department of Fruit Sciences, Faculty of Horticultural Science, Corvinus University of Budapest |
| **Supervisors:** | Dr. Noémi Lukács<br>Academic Professor, DSc<br>Department of Plant Physiology and Plant Biochemistry, Faculty of Horticultural Science, Corvinus University of Budapest<br><br>Dr. Krisztián Halász<br>Assistant Professor, Ph.D<br>Department of Plant Physiology and Plant Biochemistry, Faculty of Horticultural Science, Corvinus University of Budapest |

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| Prof. Dr. Tóth Magdolna<br>Head of the Ph.D School | Prof. Dr. Noémi Lukács<br>Supervisor | Dr. Krisztián Halász<br>Supervisor |

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## 1. Introduction

Synthetic agents, widely used for medicinal and industrial purposes, are also essential to reduce economical losses caused by pathogens in agriculture. Due to their effect on nature and to the health risks of their application reduction of their use would be highly advisible. Recently, there has been an emerging attempt to explore nature-friendly compounds which could substitute for chemically synthesized products. A growing body of research have perceived plants as „bio-factories" of potentially valuable bioactive compounds, but slow growing rate and harvesting of rare, endangered species pose a risk in themselves, therefore this approach is not generally applicable. This is why alternative sources are outmost essential, since organic synthesis of natural products is not yet economically feasible and cost-effective (Pereira & Castro, 2014). By searching for new potential sources of novel bioactive molecules special attention has been given to symbiotic microorganisms that are associated with a wide-range of plant species and are termed as „endophytes"(Guo *et al.*, 2008).

Endophytic symbionts including bacteria and fungi live within plant tissues without causing any obvious negative effects and have been found in every plant species examined to date. It became evident that endophytes are rich sources of bioactive natural products, and many different agents have been isolated from these microorganisms with promising applications in development of natural drugs and other industrial products. According to Berdy (Berdy, 2005) more than 20,000 bioactive metabolites are of microbial origin. Fungi are among the most important groups of eukaryotic organisms that are well known for producing many novel metabolites which are directly used as drugs or function as lead structures for various bioactive products. The success of several drugs such as the antibiotic penicillin from *Penicillium sp.*, the immunosuppressant cyclosporine from *Tolypocladium inflatum* and *Cylindrocarpon lucidum*, the antifungal agent griseofulvin from *Penicillium griseofulvum* fungus, the cholesterol biosynthesis inhibitor lovastatin from *Aspergillus terreus*, and *β*-lactam antibiotics from various fungal taxa, has shifted the focus of drug discovery from plants to these microorganisms (Gunatilaka, 2006); (Kock *et al.*, 2001); (Stadler & Keller, 2008). In addition to the importance of fungal endophytes and their metabolites in development of new pharmaceutical compounds, studies in recent decades have suggested a positive impact on the host plant as well.

Endophytic fungi were first studied in plants in temperate regions, but recently these studies were extended to tropical plants as well. All plants maintain associations with fungal endophytes and epibionts. These associations between fungi and plants are generally a cryptic phenomenon in Nature. Fungal endophytes may inhabit tissues of roots, stems, branches, twigs, bark, leaves, petioles, flowers, fruit, and seeds, including xylem of all available plant organs. These fungi are alleged to affect the ecology of plants, by frequently enhancing the capacity of host plants to survive and resist environmental and biological stresses through mechanisms that are only partially understood. It is also believed that endophytes have important roles in plant protection, acting against herbivores, insects and pathogens of the host and may also increase plant resistance to pathogens and biotic and abiotic stresses (Ahlholm *et al.*, 2002); (Kogel *et al.*, 2006). However, association between plants and micro-symbionts has been controversial. Symbiosis between a fungus and a plant is a widespread phenomenon in nature. The outcome of such an interaction can vary in a seamless manner from mutualism to parasitism. In most cases, the host plant does not suffer; in fact it often gains an advantage from colonization by a fungus. This benefit is based on a fine-tuned balance between the demands of the invader and the plant response. If the interaction becomes unbalanced, disease symptoms may appear or the fungus is excluded by induced host defense reactions. Symbioses of plants with beneficial or neutral endophytes share many common attributes with plant interactions with pathogens. Recent findings derived from studies on host- endophytic fungi interactions have improved our understanding of causative factors that determine symbiotic or parasitic behavior of endophytes. Besides available data could help to infer how plants avoid exploitation by detrimental parasites but benefit from mutualistic endophytes (Faeth & Fagan, 2002); (Kogel *et al.*, 2006).

Contribution of endophytes to biological functions of the host plant was primarily studied in cool season grasses, i.e. grasses that most actively grow in the spring and fall when the soil temprature is about 18°C or lower. Endophyte-infected grasses tend to be comparatively more vigorous, especially under conditions of minimal fertilization and irrigation. Infected plants were found to produce greater numbers of tillers and roots making them more drought-tolerant, more competitive with weed species, to recover more rapidly from injury, and to be generally more persistent in the field. The higher performance was particularly notable under stressful conditions, such as high temperature or nutrient and water deficiency (Clay, 1992). The result of the endophytes presence is a grass that is highly suitable for medium to low input situations.

Endophyte-infested grasses have also shown high resistance to foliar-feeding insects. Biologically active alkaloids were found only in infested grasses. The insecticidal effects produced by these compounds may deter insects from feeding or cause "antibiosis" effects which alter the life cycle of the insect (Clay, 1992); (Leuchtmann, 1992). Among fungi-derived alkaloids, the effect of loline alkaloids (saturated 1-aminopyrrolizidines with an oxygen bridge) that occur almost exclusively in many grasses associated with fungal endophytes of the genera *Epichloë* or *Neotyphodium,* on the host resistance against aphids has been well investigated (Westendorf *et al*., 1993).

While systemic endophytes in grasses of agronomic importance have been systematically studied, the interactions between host plants and endophytes in natural populations and communities are poorly understood. The emerging picture from the limited studies of horizontally (spore) transmitted endophytes in plants suggests that (i) they are very abundant and common as localized infections in all types of plants, ranging from algae to angiosperms; (ii) they are extremely diverse, particularly in longer-lived woody plants, and (iii) have the same attributes as other macro-communities, including seasonality, sequential changes, dominant and rare, and/or generalist and specialist species (Faeth & Fagan, 2002); (Saikkonen *et al.*, 2004).

It has been documented that higher non-grass plants furnish complex, multilayered, spatially and temporally diverse habitats that support species-rich assemblages of microorganisms. Microfungi are dominant components of those communities, colonizing foliar and twig surfaces (epiphytes), internal tissues of foliage (foliar endophytes), young and old bark (bark endophyte), roots, fruits, flowers, seed and wood (xylem endophytes and wood decomposers). Increasing interest in cryptic occupation of internal tissues of healthy plants by endophytic micro-fungi has led to a growing awareness that higher plants likely harbor a reservoir of undiscovered fungi (Arnold *et al.*, 2007); (Rodriguez *et al.*, 2009 b). It is assumed that the structure of endophytic assemblages within the same species may vary not only due to the geographical differences, but also to changes in climate conditions in the region (Arnold *et al*., 2003). Considering the presence of endophytes in every known plant species, such characteristics make fungal endophytes as one the most diverse components of the biomass that are dynamically being modified to adjust to the environmental changes and to host physiology (Aly *et al.*, 2011). It is estimated that there are approximately 1 million fungal endophyte species worldwide (Ganley *et al.*, 2004); however, only a fraction has been described and explored to date.

Hungary has an eminent potential for horticultural production due to its geographical situation and agro-ecological conditions. Along with other Central and Eastern European countries, Hungary has started to improve the state‟s capacities for attending the world market of horticultural products. In the last two decades sweet cherry (*Prunus avium*) received an increasing attention. As most other fruit plants, sweet cherry is grafted. To produce high quality products for the fresh market vigorous rootstocks well adapted to the regional climate and soil condition are needed, this is why different strains of *P. mahaleb* have been introduced as rootstocks in Hungary (Gyeviki *et al*., 2008). To develop the cultivation of sweet cherry in large scales an integrated research program, from basic to applied science, is fundamentally needed to improve productivity of domestic species and their resistance to stresses and natural pathogens. Fungal endophyte assemblages associated with their host plant may have an influence on pathophysiology of the host. As there has been no data regarding the biodiversity of endophytic fungi and their association with sweet cherry, the present study was carried out to obtain the following objectives:

1- Determining the biodiversity of endophytic fungi in sweet cheryy grafted on different *P. mahaleb* rootstocks

2- Identification of potentialy host-specific or or tissue-specific (leaf, twig, and root) strains and their dynamic changes during the growing season.

3- Evaluation of anti-microbial activities of isolated fungal endophytes.

In additon to sweet cherry we also investigated the endophytic fungi of pepper under different growing conditions and in different cultivars. My task in the latter studies was to help to start the cultures and to monohyphenate / monosporulate the individual strains and establish the strain collection.

## 2. Literature Review

### 2.1. Definition of endophytes

During the past 30 years the terms endophyte and endophytic fungi have appeared frequently in the mycological literature to describe the internal mycota of living plants. Although the origin of the term "endophyte" can be traced back to the nineteenth century, its contemporary meaning is different from the original one. Today most commonly used definition is that of Petrini *et al.* (Petrini *et al.*, 1992). In the broadest sense, endophytic fungi are fungi that colonize living plant tissue without causing any immediate, overt negative effects (Hirsch & Kapulnik, 1998). This definition includes virtually the entire spectrum of symbiotic interactions in which fungi and plants participate: parasitism, commensalism, and mutualism. For grass hosts (primarily *Poaceae*), the word endophyte has been used to denote a particular type of systemic, nonpathogenic symbiosis. Grass endophytes provide their hosts with a number of benefits, such as protection against herbivory and pathogens, and thereby increase their fitness (Saikkonen *et al*., 2004). Taxonomically these fungi are primarily *Neotyphodium* anamorphs of *Balansiae* (*Clavicipitaceae*); they colonize leaf, culms, and root tissues of species of cool-season grasses extensively and are transmitted in their hosts‟ seeds. Sporulation on the host is suppressed completely, and host and fungus function together essentially as a single organism. These symptomless endophytes of *Lolium*, *Festuca*, and other genera of pooid grasses are interspecific hybrid strains derived from *Epichloë* species that cause partial or complete host sterility (Moon *et al.*, 1999).

One of the early publications describing an endophytic fungus was by Freeman in 1904, where he has made reference to four other papers on endophytes that were published in 1898. This paper described a fungus in Persian darnel - an annual grass that today is considered a troublesome weed by many wheat farmers (Schardl *et al.*, 2004); (Schulz & Boyle, 2005). Between 1930-1990, several discoveries prompted a series of studies in which similar asymptomatic endophytes were recorded in a wide range of grasses (Rodriguez *et al.*, 2009 a). More recent reports describe European endophytes, endophytes of palms, grasses and woody plants (Alvarez-Loayza *et al*., 2011); (Petrini *et al*., 1992); (Schulz & Boyle, 2005).

## 2.2. Ecology

Many of the fungi commonly reported as endophytes are regarded as minor or secondary pathogens by forest pathologists. Their common occurrences in both healthy and diseased tissues underscore the uncertainty of boundaries separating endophytes, facultative pathogens, and latent pathogens. Pathogenic fungi capable of symptomless occupation of their hosts in part of the infection cycle ,"quiescent infections" (Selosse *et al.*, 2004), and strains with impaired virulence can be considered endophytes (Schardl & An, 1993) as well as a variety of commensal saprobic and mutualistic fungi that have cryptic, non-apparent patterns of host colonization. Fungi described as "endophytic" characteristically exhibit a prolonged, inconspicuous period in which growth and colonization cease temporarily, resuming after a physical or maturational change in the host (Stepniewska & Kuzniar, 2013); (Zuccaro *et al.*, 2014). This episodic growth is a defined feature of endophytes, whether they ultimately are considered commensal saprobes, latent pathogens or protective mutualists. Although such a definition may seem too broad, most fungal biologists agree that the species composition of the internal mycobiota is distinct for various hosts, organs, and tissues although some species of endophytic infections also may be found in the epiphytic or rhizosphere mycobiota (Saikkonen *et al.*, 2004).

Endophytic fungi are polyphyletic; mostly belonging to ascomycetes and to anamorphic fungi (Aly *et al.*, 2011); (Arnold *et al.*, 2007). There are nearly 300,000 plant species on earth and each individual plant is host to one or more endophytes, and many of them may colonize only certain hosts. It has been estimated that there may be as many as one million different endophytic fungal taxa, thus endophytes may be hyper diverse (Strobel & Daisy, 2003); (Petrini *et al.*, 1992). Endophytes occur in almost all known plants such as algae (Hawas *et al.*, 2012), mosses and lichens (U'Ren *et al.*, 2010), liverworts (Pressel *et al.*, 2008), ferns and fern allies (Higginbotham *et al.*, 2013); (Del Olmo-Ruiz & Arnold, 2014), numerous angiosperms and gymnosperms, including tropical palms, broad-leaved trees (Moricca & Ragazzi, 2008); (Arnold & Herre, 2003); (Clay, 1992); (Petrini *et al.*, 1992), diverse herbaceous annuals, and many deciduous and evergreen perennials, in all known plant-growing regions from xeric to mesic temperate and tropical environments and from extreme arctic to alpine, temperate, tropical and boreal forests (Petrini *et al.*, 1992).

## 2.3. Biology

Endophytes may be transmitted either vertically (from parent to offspring) or horizontally (from individual to unrelated individual). Vertically transmitted fungal endophytes are asexual and transmit via fungal hyphae penetrating the host's seeds (e.g., *Neotyphodium*). Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutualistic. Conversely, horizontally transmitted fungal endophytes are sexual and transmit via spores that can be spread by wind and/or insect vectors. Since they spread similarly to pathogens, horizontally transmitted endophytes are often closely related to pathogenic fungi, although they are not pathogenic themselves (Selosse *et al*., 2004). It is generally believed that variations in sexual reproduction and modes of transmission can cause variations in symbiotic traits of plant-fungus interaction. These differences among endophytes, in concert with biotic and abiotic environmental factors, are likely to have implications for genotypic diversity, generation time, spatial and temporal distribution of endophytes and the nature of host-fungus interplay.

Life history traits, such as the mode of transmission, largely determine the spatial and temporal distribution of endophytes (Saikkonen *et al*., 2004). Vertically transmitted grass-endophytes usually produce considerable mycelial biomass within the host, sometimes throughout the whole plant and always along the stem to developing flower heads and seeds. The generation time of vertically transmitted grass-endophytes is relatively long, often covering several grass generations. In contrast, abundance and diversity of horizontally transmitted endophytes in plants accumulate throughout the growing season, mostly in foliage (Faeth & Sullivan, 2003); (Saikkonen *et al.*, 2004). Individual endophyte infections are localized and the mycelial biomass remains very low relative to plant biomass. Spores are usually dispersed from senescent and abscised leaves, and thus the lifespan of foliage limits the lifespan of most endophytes inhabiting woody plants. The spatial and temporal patterns of endophytes differ not only between grasses and trees, but also between evergreen and deciduous trees (Arnold *et al.*, 2007).

### 2.3.1. Reproduction and transmission mode of endophytic fungi

Reproductive and transmission modes of endophytic fungi are often used synonymously to refer to their spread within the host and among the population of host plants. They are, however, clearly different processes, whereby reproduction mode specifies the sexual or asexual characteristics of the process, while the mode of transmission describes mechanisms by which

fungal infections are distributed. So far, there are two known transmission modes for fungal endophytes:

Fungal hyphae may grow clonally into host seeds and are thereby transmitted to offspring of infested plants which is commonly termed as vertical transmission. Alternatively, the fungus may produce spores and promote horizontal transmission. To fully understand the ecological and evolutionary consequences of these life history strategies, however, it is essential to recognize that fungi may produce either mitotic asexual or meiotic sexual spores. Thus, asexual reproduction of fungi is possible through vertical transmission via host seeds and horizontal transmission by spores, or possibly hyphae, whereas sexual reproduction requires production of sexual spores and is therefore always horizontal (Tadych *et al.*, 2014). The reproductive and transmission mode of the fungus appears to be adapted to the life history of the host, particularly the growth pattern, expected lifetime, and age of sexual maturity of the plant. The vast majority of ecological literature on fungal endophytes associated with grasses has focused on two related fungal genera, *Neotyphodium* and *Epichloë*. Both of them occur as systemic infection (i.e,. growing throughout the host plant to developing inflorescence and seeds), and are transmitted vertically from maternal plants to offspring. *Neotyphodium* endophytes are assumed to be strictly vertically transmitted, and thus, considered „trapped" in the host plant (Clay, 1992); (Eaton *et al.*, 2011). In contrast, *Epichloë* endophytes can also be transmitted sexually by spores (Clay, 1992); (Schardl *et al*., 2004). However, contagious spread should not be ruled out even in *Neotyphodium* endophytes because they produce asexual conidia on growth media and on living plants (di Menna *et al.*, 2012); (White & Torres, 2010). Recent evidence indicates horizontal transmission in natural grass populations (Tadych *et al.*, 2014). Foliar endophytes of woody plants are non-systemic and transmitted horizontally by spores from plant to plant, usually causing highly restricted local infections. Endophytes of woody plants have also been detected in seeds and acorns (Petrini *et al*., 1992), but vertical transmission of woody plant endophytes is probably rare (Saikkonen *et al*., 2004). Many tree-endophytes also produce asexual spores, and horizontal transmission and sexual reproduction of some fungal species is likely to result in relatively higher genotypic diversity in populations of fungal endophytes in trees than in grasses. Reproduction and transmission modes are well recognized as important factors related to the epidemiology and evolution of virulence in parasite and pathogen interactions (Herre *et al.,* 1999); (Herre *et al.*, 2007). Mode of transmission, pattern of endophyte infections, architecture

and lifespan of the host and the fungus likely affect the probability of endophyte-plant interactions occurring along the continuum from antagonistic to mutualistic interactions (Clay, 1992); (Tsai *et al.*, 1994). Saikkonen *et al*. (Saikkonen *et al.*, 2002) suggested that exclusively vertically transmitted asexual grass endophytes are more likely to fall nearer the mutualistic end of the interaction continuum compared with mixed strategy (both vertically and horizontally) or only horizontally transmitted endophytes. However, strict vertical transmission does not guarantee mutualistic interactions with the host (Clay, 1992); (Faeth, 2009); (Saikkonen *et al.*, 2004).

### 2.3.2. Partner fidelity and evolution of virulence

Evolutionary theory predicts that vertical transmission should align the interests of partners toward mutualistic associations, whereas horizontal transmission, with increased opportunities for contagious spread, should promote the evolution of increased virulence (Clay, 1992); (Herre *et al.* 1999). Most empirical literature on endophytes generally supports this theory. Interactions between *Neotyphodium* endophytes and grasses represent an extreme form of partner fidelity, because the fungus spreads only with seeds of infected plants, and thus it is fully dependent on the host plant for survival and reproduction. *Neotyphodium* interactions are often found as mutualistic, lending support to the theory. In contrast, other grass endophytes, such as some *Epichloë* species, with mixed modes of transmission, may incur severe costs to the host by producing fungal sexual structures (stromata) in the plant inflorescences thereby decreasing seed production of the host plant. In general, endophytes that are transmitted horizontally by spores are often either neutral or parasitic (Ahlholm *et al*., 2002); (Saikkonen *et al.*, 2004) , even though these endophytes as well were originally proposed as defensive mutualists against rapidly evolving herbivores (Faeth & Shochat, 2010). Although vertically transmitted endophytes appear to be selected for lowered virulence, their interactions with grasses do not necessarily remain mutualistic and evolutionary stable for several reasons. First, costs and benefits of the partners are not symmetric, even in mutualistic plant-endophyte symbioses. The symbiosis is critical for long-term survival and reproduction of the fungus, which has presumably lost the independent phase of its life cycle. Alternatively, the fungus may only minimally increase plant survival and reproduction. Recent empirical evidence suggests in some environments and for some endophyte-host combinations, that endophytes may reduce host growth and reproduction, further skewing the relative cost and benefits of association between partners (Ahlholm *et al.*, 2002);

(Sullivan & Faeth, 2004). Another important destabilizing factor is the mismatch between genetic diversity of the host grass and asexual endophytes. Asexual, vertically transmitted endophytes, such as *Neotyphodium*, have greatly reduced genetic diversity and exhibit very low gene flow in natural populations (Faeth & Sullivan, 2003). Increased benefits of endophyte are typically manifested through increased production or diversity of endophytic alkaloids (Faeth & Sullivan, 2003). The consequence of this strategy is that the majority of vertically transmitted endophytes of native grasses may only be weakly mutualistic, such that genetically limited haplotypes can persist over time in an ever-changing (genetically) host background. Endophyte-host associations that are strongly mutualistic (i.e., great benefits) may also be highly harmful in terms of high or diverse alkaloid production. Faeth and Fagan (Faeth & Fagan, 2002) reviewed the literature and found far fewer native grass-endophyte associations that were highly toxic to herbivores than expected based upon estimated species of grasses infected with *Neotyphodium*, contrary to prevailing ideas of endophytic mutualisms. The strategy of many seed-borne endophytes may be: do little harm but provide few benefits. In fact, when genetic diversity of the host grass is low, more mutualistic associations are expected because more constant plant genotypic backgrounds appear generation after generation. This appears exactly the case in agronomic grasses such as tall fescue and perennial ryegrass, well known for high and diverse alkaloid production that inhibits herbivores. Cultivars of these agronomic plants are highly inbred and exhibit much lower genetic diversity than their native counter-parts (Saikkonen *et al*., 2004); (Saari *et al.*, 2010).

### 2.4. Impacts of plant-endophyte interactions on endophyte diversity

Natural selection operates on heritable properties of individuals, and sexual reproduction promotes genetic variability through outcrossing, permitting rapid response to changing selection pressures (Ahlholm *et al.*, 2002); (Muller & Krauss, 2005). Sexual reproduction also removes accumulating deleterious mutations (Muller & Krauss, 2005). Thus in theory, although loss of sexual reproduction may provide short-term benefits, it should increase probability of extinction of plant mutualistic fungi. Interestingly, however, in about 20% of all known fungi, including *Neotyphodium* endophytes, sex has never been observed in nature, although some of them may be very old (Schardl, 2001). There are two hypotheses that may explain how asexual endophytes may be able to cope with changing selection pressures. First, fitness of fungus is intertwined with

the fitness of the host plant. As for C-endophytes, although only one fungal genotype is transmitted vertically to seed progeny, novel genetic combinations of vertically transmitted endophytes and their hosts are formed regularly through sexual reproduction of hosts. Thus, the fungus may be buffered by its outcrossing host that evolves rapidly enough in the face of environmental changes. Recent evidence also indicates the importance of interactive effects of fungal and plant genotypes, which affect the mutual fitness of the fungus and the host plant. Faeth and fagan (Faeth & Fagan, 2002) found that plant genotype rather than endophyte haplotype or environmental conditions determined mainly the mycotoxin levels within the examined population of Arizona fescue (*Festuca arizonica*).

Species interactions, even obligate mutualisms, are generally accepted as being based on mutual exploitation rather than reciprocal altruism, with sanctions imposed against overexploitation by either partner (Rowan & Knowlton, 1995). Theory predicts that sporulating endophytes should range from negative to positive in their interactions with host plants, and that contagious spreading should favor less-mutualistic interactions ((Faeth & Fagan, 2002); (Saikkonen *et al.*, 2004). However, the costs of systemic and vertically transmitted endophytes have been underestimated in earlier literature, the costs of harboring endophytes were assumed to be negligible (Faeth & Sullivan, 2003). Clearly, systemic *Epichloë* endophytes that form stromata which surround and destroy developing inflorescences (choke disease) during the sexual phase of the fungus, are obviously costly and act parasitically (Meijer & Leuchtmann, 2001); (Moon *et al.*, 1999); (Ahlholm *et al*., 2002). The cost of systemic endophytic infections in native grasses have been overlooked because the vast majority of studies have been conducted under enriched resource environments, either in agronomic environments or green-houses using fertilized standard potting soil, and agronomic grass cultivars (Faeth & Fagan, 2002). According to life history theory, competition for limited resources is assumed to result in negative correlations (i.e. trade-offs) between competing functions, such as growth, reproduction, maintenance, and defense (Hamilton *et al.*, 2010).

Nonclavicipitaceous (NC)- endophytes are highly diverse, representing a polyphyletic assemblage of primarily ascomycetous fungi with diverse and often poorly defined or unknown ecological roles. NC- endophytes have been recovered from every major lineage of land plants and from all terrestrial ecosystems, including both agro-ecosystems and biomes ranging from the tropics to the tundra (Arnold *et al*., 2007). NC- endophytes can be differentiated into three

functional classes based on host colonization patterns, mechanism of transmission between host generations, *in planta* biodiversity levels and ecological function (Table 2.1). Although all three classes have broad host ranges, Class 2 endophytes may grow in both above- and below-ground tissues. By contrast, Class 3 and 4 endophytes are restricted to above-ground tissues and roots, respectively. Colonization of host tissues also differs: Class 3 endophytes form highly localized infections, while Class 2 and 4 endophytes are capable of extensive tissue colonization. In general, the diversity of Class 2 endophytes in individual host plants is quite limited, whereas the diversity of Class 3 endophytes within a host plant or tissue can be extremely high, e.g. >20 species recorded from a single tropical leaf (Arnold *et al.*, 2003). The diversity of Class 4 endophytes within individual plants has not been sufficiently evaluated. Differences in *in planta* biodiversity of Class 2 and 3 endophytes may reflect differences in host colonization and transmission patterns: although members of both classes are transmitted horizontally, Class 2 endophytes also are transmitted vertically via seed coats, seeds or rhizomes (Cannon & Simmons, 2002); (Arnold *et al*., 2007).

Class 2 endophytes comprise a diversity of species, all of which are members of the *Dikarya* (*Ascomycota* or *Basidiomycota*). Most belong to the *Ascomycota*, with a minority of *Basidiomycota*. Members of the former are restricted to the *Pezizomycotina*, wherein they represent several classes. Class 2 endophytes within the *Basidiomycota* include a few members of the *Agaricomycotina* and *Pucciniomycotina*. Class 2 endophytes are distinct from the other NC- endophytes because in general they colonize roots, stems and leaves; are capable of forming extensive infections within plants; are transmitted via seed coats and/or rhizomes; have low abundance in the rhizosphere; confer habitat-adapted fitness benefits in addition to nonhabitat-adapted benefits; and typically have high infection frequencies (90–100%) in plants growing in high-stress habitats (Rodriguez *et al.*, 2009 a) (Table 2.1).

**Table 2.1**: Symbiotic criteria used to characterize fungal endophytes classes.

| | Clavicipitaceous | Non-clavicipitaceous | | |
|---|---|---|---|---|
| | **Class 1** | **Class 2** | **Class 3** | **Class 4** |
| **Criteria** | | | | |
| **Host range** | Narrow | Broad | Broad | Broad |
| **Tissue(s) colonized** | Shoot & Rhizome | Shoot, Root & Rhizome | Shoot | Root |
| ***In planta* colonization** | Extensive | Extensive | Limited | Extensive |
| ***In planta* biodiversity** | Low | Low | High | Unknown |
| **Transmission** | Vertical & Horizontal | Vertical & Horizontal | Horizontal | Horizontal |
| **Fitness benefits*** | NHA | NHA & HA | NHA | NHA |

*Non-habitat-adapted (NHA) benefits such as drought tolerance and growth enhancement are common among endophytes regardless of the habitat of origin. Habitat-adapted (HA) benefits result from habitat-specific selection pressure such as pH, temperature and salinity (*Rodriguez et al*., 2009 *a*).

In comparison to clavicipitaceous (C)- endophytes, available data about diversity and life history of NC-endophytes is scarce. A limited number of reports appeared describing fitness benefits conferred to plants by Class 2 endophytes. One of the clearest examples was reported in 1995 about the brown alga *Ascophyllum nodosum* which requires the fungus *Mycophycia ascophylli* for normal growth and development. In addition to being required for the normal development of some plants, some Class 2 endophytes increase plant root biomass and confer tolerance to a variety of biotic and abiotic stresses such as disease, drought, desiccation, heat and salinity (Rodriguez *et al*., 2009 a). Class 2 endophytes, like all endophytes, colonize plants via infection structures such as appressoria or by direct penetration of plant tissues via hyphae (Ernst *et al.*, 2003). Growth through plant tissues is dominantly intercellular with little to no impact on host cells (Ernst *et al.*, 2003); (Rodriguez *et al.*, 2009 a). While there may be a low level of sporulation or appressorial formation in healthy plants these fungi rapidly emerge and sporulate during host senescence (Weber *et al.*, 2005); (Rodriguez *et al.*, 2009 a). There have not been sufficient ecological studies to permit full understanding of the distribution and abundance of Class 2 endophytes in the rhizosphere. However, some species such as *Phoma spp*, (Newsham, 2011) are known to occur in soils at high abundance while others (*Fusarium culmorum*, *Colletotrichum magna* and *Curvularia protuberate*; (Rodriguez *et al.*, 2008) are present at very

low abundance. Analysis of soil fungi typically involves making soil suspensions, dilution plating and enumerating colony-forming units; therefore, it is difficult to discern the ability of endophytes to propagate in soil (Selosse *et al.*, 2004); (Rodriguez *et al.*, 2009 a). Although these characteristics highlight horizontal transmission, Class 2 endophytes often are transmitted vertically: they can be passed from maternal plants via seed coats (Rodriguez, *et al*., 2009 a). Interestingly, culturable Class 2 endophytes can grow on a variety of simple media. The prevalence of these fungi in plant hosts and their abundance in soils suggest that some of them are unable to compete outside hosts, while others may have multiple lifestyles (symbiotic or saprophytic) (Rodriguez *et al.*, 2009 a).

Like Class 2 endophytes, the majority of Class 3 endophytes are members of the *Dikaryomycota* (*Ascomycota* or *Basidiomycota*), with a special concentration in the *Ascomycota*. Class 3 endophytes are distinguished on the basis of their occurrence primarily or exclusively in above-ground tissues, horizontal transmission, the formation of highly localized infections, the potential to confer benefits or costs on hosts that are not necessarily habitat-specific and extremely high *in planta* biodiversity (Rodriguez *et al*., 2009 a); (Table 2.1). Class 3 endophytes include the hyper-diverse endophytic fungi associated with leaves of tropical trees (Arnold & Lutzoni, 2007); (Gamboa *et al.*, 2002), as well as the highly diverse associates of above-ground tissues of nonvascular plants, seedless vascular plants, conifers, and woody and herbaceous angiosperms in biomes ranging from tropical forests to boreal and Arctic/Antarctic communities (Petrini *et al.*, 1992); (Rodriguez *et al*., 2009 b). In addition to occurring within photosynthetic tissues, Class 3 endophytes are found in flowers and fruits, as well as in asymptomatic wood and inner bark (Verma *et al.*, 2007); (Rodriguez *et al*., 2009 a). Class 3 endophytes are especially notable for their high diversity within individual host tissues, plants and populations. For example, apparently healthy leaves in tropical forests contain numerous, independent infections, rather than systemic or otherwise extensive growth of hyphae (Arnold & Herre, 2003); (Arnold *et al*., 2003). The biomass resulting from any given infection is very low, such that each leaf represents a densely packed mosaic of diverse endophyte species (Rodriguez *et al*., 2009 b). In tropical forests in central Panama, where 100% of mature leaves of diverse trees and shrubs typically contain endophytes, individual leaves may harbor up to one isolate per 2 $mm^2$ of leaf tissue and frequently contain dozens of species. Different leaves on the same tree may have quite distinctive assemblages of endophytic fungi (Arnold *et al.*, 2003); (Gamboa *et al.*, 2002).

Individual plants may harbor hundreds of species, and plant species across their native ranges may be inhabited by thousands of species. This tremendous diversity, showcased in some tropical plants and localities, is not exclusively a tropical phenomenon: plants in temperate and boreal communities also harbor an astonishing richness of Class 3 endophytes. For example, Higgins *et al*. (2007) identified >50 species among the examined 280 boreal and arctic endophyte isolates. Although horizontally transmitted, Class 3 endophytes are typically distinct from pathogens associated with the same host species (Ganley *et al*., 2004) and from epiphyllous fungi even on the same leaves (Santamaria & Bayman, 2005), but their distinctiveness from saprotrophic communities is debated (Selosse *et al.*, 2009). Class 3 endophytes are rarely isolated from seeds (Arnold *et al.*, 2003); (Ganley *et al*., 2004).

The diversity of Class 3 endophytes raises several questions regarding their ecological roles. Indeed, most recent studies of Class 3 endophytes have focused on characterizing bioactive strains or enumeration of species, leaving aside the potential ecological roles of these fungi *in planta* (Arnold *et al*., 2003) or their evolutionary implications for plants, although it is hardly possible to set up general rules given the occurrence of tens to hundreds of phylogenetically diverse endophytic fungi within the foliage of a single host (Rodriguez *et al*., 2009 b); (Arnold & Herre, 2003). Class 3 endophytes reproduce by hyphal fragmentation and/or by the production of sexual or asexual spores on dead or senescent tissue (Herre *et al*., 2007). Spores and hyphal fragments may be released passively, by herbivores or by physical disturbances such as wind or rain (Rodriguez *et al.*, 2009 b). Some, such as *Phyllosticta sp*., produce slimy spores that rely at least in part on rain for dispersal, while the *Ingoldian* fungi produce spores that depend on water for dispersal and infection (Selosse *et al*., 2009). In general, seedlings raised under sterile conditions do not contain culturable Class 3 endophytes, highlighting a key difference relative to Class 2 endophytes (which may be transmitted vertically). Colonization by Class 3 endophytes proceeds rapidly given the presence of airborne inoculum and high relative humidity or wetting of leaf surfaces by dew, rain or fog (Arnold & Herre, 2003).

Class 4 endophytes were primarily described by Merlin in 1922 as brown to black pigmented fungi associated with terrestrial plant roots. Presently, these fungi are referred to as „dark septate endophytes“ (DSE) and are grouped together as Class 4 endophytes. In general, Class 4 endophytes are primarily ascomycetous fungi that are conidial or sterile and that form melanized structures such as inter- and intracellular hyphae and microsclerotia in the roots. DSE have little

host or habitat specificity; they have been reported in association with ≈600 plants including plants that are non-mycorrhizal, from Antarctic, Arctic, alpine, sub-alpine, and temperate zones, as well as from African coastal plains and lowlands, and from some tropical ecosystems (Jumpponen *et al.* 1998). DSE are often found in boreal and temperate forests associated with the fine roots of trees and shrubs, especially of conifers (Reininger & Sieber, 2012). These fungi are not thought to be pathogenic, as they are observed on healthy fine roots, and in this context, may be referred to as endophytes. DSE are found worldwide, are prevalent in high-stress environments, and appear to be ubiquitous and abundant across various ecosystems. Collectively, these observations suggest that DSE may play an important role in the ecophysiology of plants. However, little is still known about the role of these elusive fungal symbionts (Rodriguez *et al*., 2009 a). DSE appear to represent a large and interesting class of endophytes that have still not been well defined taxonomically and/or ecologically. Presently, the presence of asexual, darkly pigmented, septate endophytes in plant roots is the primary criterion for DSE designation. Class 4 endophytes were found associated with 587 plants species representing 320 genera and 114 families. Colonization studies were conducted using five described anamorphic taxa of DSE (*Chloridium paucisporum*, *Leptodontidium orchidicola*, *Phialocephala dimorphosphora*, *Phialocephala fortinii* and *Phialophora finlandia*) under natural and experimental conditions (inoculation of root systems in pots). Collectively, these DSE species had a large host range and/or lacked host specificity (Jumpponen *et al.*, 1998); (Mandyam *et al.*, 2010); (Mandyam *et al.*, 2013). Because of the presence of DSE in soils and plant roots, transmission is most likely horizontal and proceeds by mycelial fragmentation and dispersal of conidia (Jumpponen *et al.*, 1998). Although anamorph–teleomorph connections have not yet been identified for most DSE, the possibility of sexual reproduction should not be discounted (Rodriguez *et al*., 2009 a).

### 2.5. Pathophysiological aspects of plant-endophyte symbiosis

A variety of relationships exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic effects. Recent studies of endophytic fungi and their relationships with host plants have elucidated that plant-endophyte mutualism has not only a crucial role in biological functions of both parties, but directs the ecophysiology of host plant and their symbionts to enhanced ability to adapt environmental stresses throughout the evolutionary time (Chaudhari *et al.*, 2014). Although there has been

extensive research in plant stress responses, it is not known why so few species are able to colonize high stress habitats. However, plant stress research rarely takes into consideration a ubiquitous aspect of plant-fungi symbiosis. It has been indicated that fitness benefits conferred by mutualistic fungi contribute to or are responsible for plant adaptation to stress (Schulz & Boyle, 2005); (Selosse *et al.*, 2004). Collectively, mutualistic fungi may confer tolerance to drought, metals, disease, heat, and herbivory, and/or promote growth and nutrient acquisition. It has become clear that at least some plants are unable to endure habitat-imposed abiotic and biotic stresses in the absence of fungal endophytes (Schardl & An, 1993).

### 2.5.1. Host protection against herbivores

Much has been published on the highly specific nature of grass-endophyte symbiosis and the effects of fungal alkaloids on vertebrate and invertebrate herbivores. Pervasive systemic colonization of host tissue with endophyte hyphae ensures that herbivores, whether large mammals or small arthropods, encounter fungal metabolites while consuming the plant tissues. Most clavicipitaceous endophytes enhance resistance of hosts to insect feeding (Stepniewska & Kuzniar, 2013). Tintjer & Rudgers (2006) found that deterrence of insect herbivory depends on the fungal strain and growth stage of the plant. Other studies have provided evidence for anti-nematode activity of Class 1 endophytes as well (Strobel & Daisy, 2003). However, research has also shown that some Class 1 endophytes do not provide insect or nematode resistance to host plants, and have highlighted the importance of examining native plants under natural conditions in determining endophyte-conferred benefits (Saikkonen *et al*., 2004). Because of several examples where endophytes do not appear to provide defensive benefits to host plants, some investigators have questioned the tendency to classify C-endophytes as defensive mutualists (Hawas *et al*., 2012). There is evidence that Class 4 endophytes may also involved in host defense against herbivores by production of secondary metabolites (Rodriguez *et al*., 2009 a).

### 2.5.2. Effects to stress tolerance of the host plant

All plants are known to initiate complex biosynthetic responses to elevated temperatures. These involve the synthesis of heat shock proteins and antioxidant systems as well as adjustments in osmotic potential and membrane lipid composition (Pressel *et al*., 2008). Interaction between host plant and endophytic fungi may cause a drastic enhancement of plant resistance to heat stress as shown in the *Dichanthelium-Curvularia* system. Laboratory and field studies revealed that the plant *Dichanthelium lanuginosum* is only able to grow in geothermal

soil at temperatures as high as 57º C when it harbors the endophytic fungus *Curvularia sp.* infected with a dsRNA virus (U'Ren *et al.*, 2010).

Although all plants respond to water deficit, only a few species are drought-tolerant not showing detrimental impacts of water stress (Higginbotham *et al*., 2013). However, there are numerous reports describing drought tolerance conferred to plants by fungal symbionts (Del Olmo-Ruiz & Arnold, 2014). The mechanism of symbiont conferred drought tolerance is not known, although it is thought to involve osmotic adjustments and/or altered stomatal activity. Some mycorrhizal fungi can also confer salt tolerance. The physiological basis of fungal-conferred salt tolerance has not been investigated but this appears to be a generalized phenomenon occurring in several plant species including banana, tomato and lettuce. It is a methodological problem, that research is usually focused on one plant-fungus interaction and not on the complex symbiotic partnerships that are more common in nature (Moricca & Ragazzi, 2008).

It is assumed that at least some Class 2 endophytes are mutualistic, conferring positive fitness benefits to hosts while also obtaining nutrition for growth and reproduction from host tissues, and avoiding abiotic stress via symbiosis. Class 2 endophytes commonly increase plant biomass under stressful conditions, while plants infected by multiple Class 3 endophytes typically show no observable change in growth rate, biomass accumulation, root/shoot ratio, or other easily quantifiable characteristics following inoculation under *in vivo* conditions (Arnold *et al*., 2003).

### 2.5.3. Induction of plant resistance to pathogens

It has been recently demonstrated that endophyte fungal have the ability to protect the host from diseases and to limit the damage caused by pathogen microorganisms. Many fungal endophytes produce secondary metabolites and some of these compounds are antifungal and antibacterial strongly inhibiting the growth of other microorganisms including plant pathogens (Arnold *et al*., 2003). It has been implied that some fungal species may switch between pathogenic and mutualistic life styles under certain circunstances (Clay, 1992); (Arnold *et al*., 2003). In addition, a single fungal isolate can express pathogenicity in certain plant species, and commensalism or mutualism in others. When non-symbiotic plants respond to pathogen challenge activation of their defense response is slower and weaker than that of their symbiotic counterparts, suggesting that communication between host and symbiont increases the ability of plants to perceive a pathogen and rapidly activate its defense systems. Some studies indicated

that Class 3 endophytes can be mutualistic, despite the fact that several aspects of their ecology (i.e. high diversity within hosts and horizontal transmission) are more frequently associated with parasitic or pathogenic lifestyles (Rodriguez *et al*., 2009 a). The outcome of plant-pathogen-endophyte interaction probably depends on the endophytic niche. Endophytic recognition and colonization may lead to rapid occupation of the ecological niche and leave no space for pathogens, that could be the common and main reason for the protective action of endophytes (Petrini *et al*., 1992).

### 2.6. Agricultural use of endophytic fungi

In addition to providing ideal research systems for testing ecological and evolutionary theory, endophytes also have broad economic applications. Since endophytes can affect virtually every type of plant-plant, plant-pathogen, and plant-herbivore interaction (Selosse *et al*., 2004) (Petrini *et al*., 1992), any human activities (agriculture, deforestation, pollution, etc.), which alter diversity of endophyte-plant interactions, may have unpredictable effects on population dynamics and community structure of plants, pathogens and herbivores in terrestrial ecosystems. Direct anti-herbivore properties of endophytes (particularly in grasses) have already been exploited, for example in:

1. Biocontrol through developing natural pesticides or improvement of herbivore-resistant cultivars by introducing biologically active (e.g. high mycotoxin producing) fungal strains into cultivars (Tadych *et al*., 2014). On the other hand, colonization of plants with nonpathogenic fungi and bacteria can lead to induced systemic resistance (ISR) in the host plant. Induced resistance is a plant-mediated biocontrol mechanism whereby the biocontrol agent and the phytopathogen do not make physical contact with one another. Plants react to the presence of a pathogen with a rapid expression of defense-related genes. Thus in addition to economic value, endophytes may lower investments in chemical pest control by providing environmentally friendly and energy-efficient biocontrol, and help consumers to avoid remnants of chemical pesticides in the crop (Tsai *et al*., 1994).

2. Economic value may also arise from understanding harmful effects in agricultural production. Mycotoxins cause decreased weight gain of livestock and animal disorders. In this context, endophytic fungi have been largely ignored in European grass-ecosystems although

most pasture grasses used in the northern hemisphere are of Eurasian origin and infected with endophytes (Saikkonen *et al.*, 2004).

3. Alternative fungal strains which do not produce mycotoxins harmful to vertebrates but increase plant growth, seed production, seed germination rate and stress tolerance can be used to increase productivity when introduced to the cultivars used as forage. This has already been accomplished for some tall fescue and perennial ryegrass cultivars (Rodriguez *et al.*, 2009 b).

4. As a direct result of the role that secondary metabolites of endophytic fungi may play in Nature, they may ultimately be shown to have applicability in medicine. Endophytic fungi very frequently occur in herbs and in medicinal plants. Many studies investigate their effect on secondary metabolites and the possibility of their use to produce rare and expensive products such as taxol (Saikkonen *et al*., 2002).

### 2.7. Occurrence and biodiversity of endophytic fungi

Although many studies illustrate endophyte diversity in different ecologies, there is no reliable estimation of the number of endophytic species, of their host- and tissue-selectivity, since environmental factors have a complex effect on these features. Most endophytes isolated to date have been *Ascomycetes* and their anamorphs; however, several endophytes belonging to *Basidiomycetes* have also been observed, but their colonization rate varied greatly. Some of the common endophytes not only exist in a broad range of plant species, but also have different relative frequency in every host. In contrast, some other endophytic fungi have high specificity for the host plant (Eaton *et al*., 2011); (Ahlholm *et al*., 2002).

Available data regarding biodiversity of fungal endophytes mainly refer to species which have been isolated from plants in temperate and tropical regions. As a general overview, endophytes isolated from tropical plant species are highly diverse indicating relatively lower host-specificity in comparison to that in temperate zones.

Methods are currently used for identification of endophytic fungi include morphological studies and determination of phylogenic relationships between isolated morphotaxa. The nuclear ribosomal RNA (rRNA) cistron has been used for fungal diagnostics and phylogenetics for more than 20 years. The eukaryotic rRNA cistron consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a unit by RNA polymerase I. Posttranscriptional processes split the cistron, removing two internal transcribed spacers (ITS). These two spacers, including the 5.8S gene, are

usually referred to as the ITS region (Schardl, 2001). The 18S nuclear ribosomal small subunit rRNA gene (SSU) is commonly used in phylogenetics, and although its homolog (16S) is often used as a species diagnostic for bacteria, it has fewer hypervariable domains in fungi. The 28S nuclear ribosomal large subunit rRNA gene (LSU) sometimes discriminates species on its own or combined with the ITS. Eukaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays, located at the chromosomal sites known as nucleolar organizing regions (NORs) (Faeth & Fagan, 2002). Each repeat unit consists of a transcribed region (having genes for 18S, 5.8S and 26S rRNAs and of the external transcribed spacers ETS1 and ETS2 and a non-transcribed spacer (NTS) region. In the transcribed region, internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary**.** Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (up to 30000 per cell) of rDNA repeats. This makes the ITS region an interesting subject for evolutionary/phylogenetic as well as bio- geographic investigations (Faeth & Fagan, 2002); (Faeth, 2009).

Higgins *et al*. (Higgins *et al.*, 2007) examined endophytic fungi associated with 11 *Poaceae* species in a lowland tropical forest at Barro Colorado Island, Panama, and suggested prevalent host generalism in tropical forest grasses. In another study at the same region 418 endophyte morphospecies, most of which (59%) originated from a single isolate, were isolated from leaves of *Heisteria concinna* and *Ouratea lucens*, and researchers suggested spatial heterogeneity of endophytes in tropical forests (di Menna *et al.*, 2012). Molecular sequence data from 1403 endophyte strains showed that endophytes increase in incidence, diversity and host range from arctic to tropical sites. Endophyte communities from higher latitudes constituted of relatively few species whereas tropical endophyte assemblages were dominated by a small number of classes with a very large number of endophytic species (di Menna *et al*., 2012). From leaves of coffee trees (*Coffea arabica*) in Puerto Rico a total of 821 endophyte colonies were isolated and grouped into 131 morphospecies. The four most common non-sporulating strains were identified by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region as *Xylaria* (two isolates), *Botryosphaeria* and *Guignardia*. Of the most common genera, *Pestalotia* and *Botryosphaeria* were significantly higher represented among epiphytes, while *Colletotrichum*,

*Xylaria*, and *Guignardia* were significantly more common as endophytes. Surprisingly, more morphospecies occurred as endophytes than as epiphytes in leaves of coffee tree (Faeth & Shochat, 2010).

Bernardi-Wenzel *et al*. (2010) discussed that one or two endophyte species are frequently predominant in a specific host, while other isolates are rare. The close association between endophytes and plant species, with a high degree of specialization of interactions, is a possible indication that the species have evolved together. According to their report, genera *Alternaria*, *Cochliobolus, Diaporthe*, *Epicoccum, Guignardia, Phoma,* and *Phomopsis,* were identified; rDNA sequence analysis showed intra-species variability among endophyte isolates of the genus *Phomopsis* sp., in Brazilian *Luehea divaricata* Mart. (*Tiliaceae*) (White & Torres, 2010). Association of *Phomopsis, Diaporthe, Dothideomycete*, and *Cordyceps* genera with *Trichilia elegans* (*Meliaceae*) and *Cochliobolus, Alternaria, Curvularia, Phomopsis, Diaporthe* and *Phoma* with *Sapindus saponaria* (Herre *et al*., 1999) has been also evaluated showing the predominance of *Phomopsis sp.* in these host plants (Herre *et al*., 2007) .

According to Davis and Shaw (2008), differences between endophytic communities may correlate with geographic distances rather than with host phylogeny. Therefore identification of fungal endophyte populations in various geographical and ecological regions is necessary to infer more comprehensive view of endophyte diversity worldwide. They collected liverworts in North Carolina, Washington, Idaho, British Columbia, Germany and New Zealand and identified endophytes using culture-based and molecular methods. They reported that 53 – 88% of the major lineages of filamentous *Ascomycetes* recovered belonged to the *Xylariales* and there was no significant difference in species richness between regional endophyte communities, however, North Carolina and New Zealand had richer communities than Germany and the Pacific Northwest. The authors assumed that this pattern reflects lower per-host endophyte density and prevalence of a common, shared sequence group in Germany and the Pacific Northwest. They also tested regional and host specificity of the isolates and reported that endophyte floras of hosts within a geographic area are more similar to one another than to those of closely related hosts in different locations (Faeth, 2009).

A similar situation has been observed in temperate and arctic regions. A study on 4 species of the carnivorous pitcher plant genus *Sarracenia*: *S. minor*, *S. oreophila, S. purpurea*, and *S. psittacina* collected from savanna and temperate zones in North America resulted in isolation of

twelve taxa of fungi, 8 *Ascomycota* and 4 *Basidiomycota.* Authors stated that their study was the first prooving that *Coniothyrium*/*Paraconiothyrium*, *Penicillium*, *Cryptosporiopsis*, *Phomopsis* and *Colletotrichum spp*. are endophytes of *Sarraceniaceae* and the first report describing fungal endophytes in leaves of a carnivorous plant family. The isolation of *Colletotrichum spp*. from multiple *Sarracenia* individuals of all 4 species at locations 300+ miles apart and in different years, led to the conclusion that at least this fungal genus is a true pitcher plant endophyte (Herre *et al*., 1999). Another report from temperate zone (South Korea) described endophytes in leaf and root samples of *Taraxacum coreanum* (white dandelion). Of the 72 isolates recovered, 39 were from leaves and 33 from roots with an isolation frequency of 54% and 46%, respectively. Based on ITS sequence analysis, 72 isolates were classified into 19 genera of which 17 were *Ascomycota* and 2 *Basidiomycota*. Diverse genera were found, dominated by *Alternaria*, *Cladosporium*, *Fusarium* and *Phoma*. Out of the 19 genera, *Apodus*, *Ceriporia*, *Dothideales*, *Leptodontidium*, *Nemania*, *Neoplaconema*, *Phaeosphaeria*, *Plectosphaerella* and *Terfezia* were new to Korea. Seventy two isolates were screened for antifungal activity, of which 10 isolates (14%) were found active at least against one of the tested fungi (Sullivan & Faeth, 2004). Osono and Masuya (2012) examined the diversity and species composition of endophytic fungi in leaves of 11 species of *Betulaceae*, with reference to climatic, tree species, and seasonal variations. A total of 186 fungal isolates were obtained from 190 leaves collected in a subalpine forest, a cool temperate forest, and a subtropical forest in Japan, and the most frequent taxonomic units were found as *Muscodor sp*. and *Nemania sp.* in *Xylariaceae*, followed by *Gnomonia sp*., *Glomerella acutata*, *Apiosporopsis sp*., *Asteroma sp*., and *Cladosporium cladosporioides*. They assumed that the seasonal changes in composition of fungal endophytes assemblages in leaves of *Betula* was higher in subalpine forests than in cool temperate forests (Faeth & Sullivan, 2003).

### 2.8. Current status of the issue in the area of the present study

Yet, there is a lack of information regarding the features of endophytic fungal communities in different host plants in Europe. Available data are principally originating from studies on characterization of bioactive products of endophytes with industrial or medicinal applications. Nonetheless, understanding the composition and dynamics of endophytic assemblages and impacts of host-specificity and tissue-colonization of these symbionts on physiology, is fundamental to improve the existing knowledge about the bioecology of plant-endophyte

mutualism and is required to pave the lane toward finding novel bio-agents with pesticidal, medicinal and industrial applications.

Located in Central Europe, Hungary possesses a promising natural phyto-geographical condition. The state‟s floristic feature encompasses Central European Circumboreal Region within Boreal (Holarctic) Kingdom and according to The World Wide Fund for Nature (WWF), the territory of Hungary belongs to the eco-region of Pannonian mixed forests. The land of Hungary includes:

a) the Little Hungarian Plain (tectonic basin) (Kisalföld) and Transdanubia which encloses Lake Balaton and Lake Hévíz, the largest lake in Central Europe and the largest thermal lake in the world, respectively.

b) the Great Hungarian Plain (Alföld) that includes the largest natural grass land in Europe (Hortobágy National Park) and foothills of the Carpathians.

Almost a fifth of the country is forested, however, only 10 percent is natural forest. Hungary is home to some 2200 flowering plant species although many of them are thought to be immigrant species because of the state‟s topography and the transitional climate.

Despite the fact that research on endophytic fungi associated with plants has gained much interest the available data is rather insufficient about endophytes in Hungary. Among recent papers, one report described the identification of *Coelomycetous* fungi classified in *Ascochyta*, *Phoma*, and *Phyllosticta* which are known as pathogens of soybeans in Hungary (Faeth & Fagan, 2002). Gonda *et al*. (2013) demonstrated the contribution of endophytic fungi to metabolite stability/instability in leaves of a medicinal plant (*Plantago lanceolata L*.) and showed the association of genera *Epicoccum*, *Bipolaris*, *Cladosporium*, *Leptosphaerulina*, *Aspergillus*, *Eurotium* and *Penicillium* with this host plant. Data are also available on orchid-associated mycorrhizal fungi where the authors claimed that they had isolated *Ceratobasidiaceae*, *Epulorhiza 1*, *Epulorhiza 2* and *Sebacinaceae* from Hungarian orchids (Faeth & Fagan, 2002). Recent attempts for isolation and identification of dark septate endophytic fungi from the root of invasive and native plants obtained from semiarid grasslands in Hungary have apparently resulted a similar picture of endophytic communities associated with host plants to that in other regions with the same climate around the globe. Fungi belonged to Ascomycota were the most dominant isolated from sampled plants in these studies (Knapp & Kovács, 2010); (Knapp *et al*., 2012); (Knapp *et al*., 2015).

## 2.9. Endophytic fungi in Capsicum

Pepper (*Capsicum annuum L.*) is an important vegetable as well as spice crop, used worldwide for domestic and commercial purposes. It is a rich source of antioxidants, vitamin C, pro-vitamin A, E, and B. Pepper is being seen as a "Hungaricum" in Hungary, i.e. as an agricultural product associated with Hungarian culture and economy since centuries. Pepper is a constant part of the diet consumed daily in relatively large quantities not only in cooked dishes, but also as raw vegetable and spice.

It was shown that endophytic fungi may confer protection to plants against different pathogens and pests. This is also the case with *Capsicum*. Bae *et al*. (2010) were able to protect pepper against *Phytophtora capsici* by using *Trichoderma ovalisporum*, *T. theobromica*, *T. hamatum*, *T. stilbohypoxyli* or *T. caribaeum* var. *aequatoriale* originally isolated from different plants (not from pepper). At the end of their experiments 26-60% of the *Trichoderma*-treated plants were free of symptoms, while in the control only 0-10% stayed healthy. The presence of endophytic fungi such as *Nigrospora*, *Aspergillus* and *Coniothyrium* can also inhibit growth, virility and reproduction of *Aphis gossypii* and some of the fungal strains seem even fit to be utilized for biological protection (Hernawati *et al*., 2011). Martinuz *et al*. (2012) demonstrated that *Fusarium oxysporum* Fo162 and *Rhizobium etli* G12 strains can induce systemic resistance to *Aphis gossypii.* Some authors described protection of chili pepper against *Meloidogyne incognita* by combined use of *Pasteuria penetrans* and *Paecilomyces lilacinus* (Chaudhary & Kaul, 2011). Three different endophytic fungal strains (*Penicillium resedanum*, *Cladosporium cladosporioides*, and *Paraconiothyrium sp.*) were isolated from pepper plants. They improved plant growth and protected plants against pathogenic attack and environmental stresses (Paul *et al*., 2012); (Marasco et al., 2012); (Khan *et al*., 2012); (Khan *et al.,* 2013).

A systematic study to identify endophytic fungi in *C. annum* was carried out by Paul *et al*. (2011) in Korea. They collected samples from *C. annuum* leaves, stem and root in 3 different phenophasids during the year. Out of 900 organ samples they obtained about 480 fungal isolates belongig to 21 genera. The following genera were identified by sequencing the ITS regions: *Alternaria*, *Aspergillus*, *Bionectria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Cordyceps*, *Fusarium*, *Geomyces*, *Gnomonia*, *Heterobasidion*, *Irpex*, *Paeccilomyces*, *Papulaspora*, *Penicillium*, *Peyronellaea*, *Phanerochaete*, *Phialophora*, *Phlebiopsis*, *Plectosphaerella* and *Xylaria*. The *Colletotrichum* genus was found most frequently (18.92%), followed by *Fusarium*

(18.71%), *Alternaria* (7.48%) and *Penicillium* (6.24%). The occurrence and frequncy of induvidual fungus species varied by the phenophases, but *Alternaria*, *Fusarium* and *Cladosporium* species were present in all developmental stages of pepper (Paul *et al*., 2011).

## 3. Materials and Methods

Methods for studying patterns of infection and colonization by endophytic fungi are essentially the same as those used in the study of fungal plant pathogens (Sia Ede *et al*., 2013). In mycobiotic surveys, host tissues are sampled methodically, and the spatial and temporal distributions of the fungal colonists encountered are described using methods to determine the patterns of the endophytic distribution according to host genera and families, habitat types, infection frequencies related to foliage age, host distribution, and temporal and spatial variations of endophyte infections. Host species, host-endophyte interactions, interspecific and intraspecific interactions of endophytes, tissue types and ages, geological and habitat distributions, types of fungal colonization, culture conditions, and selective media all can influence the efficiency of a sampling strategy for detection and enumeration of endophytic fungi.

In the current study, first four different species of cherry (*Prunus. sp*) were selected due to their horticultural importance and recent attempts for their application in nursery and orchard trials to find candidate rootstocks for quality control and release in Hungary.

### 3.1. Locality and sampling strategy of the study

Samples were selected randomly from 110 trees among 180 sweet cherry trees grown in the orchard of Corvinus University of Budapest, Soroksár, nearby the city of Budapest lies between 19° 07‟ 00‟‟E and 47° 24‟ 00‟‟N geographical coordinates. Trees were planted at a spacing of 4x2 m, resulting in a density of 1,250 trees $ha^{-1}$. Orientation of the rows was north-south. The same individual plants were subjected to the study in all sampling periods. The study was conducted in autumn 2008, spring 2009 and autumn 2009.

Tissue samples from leaf, twig and root of trees (cultivar Péter grafted on different rootstocks) were collected. Trees were nearly 8 years old when sampled for the first time, and rootstocks originated from four different species: *Prunus mahaleb* L. (Érdi V., Bogdány, SL64, Egervár, Korponay, SM11/4, CEMANY and Magyar rootstocks), a variety of *P. avium* (Vadcseresznye)

and *P. fruticosa* (Prob) and from a hybrid inbred rootstock of *P.cerasus* and *P.canescens* (Gisela 6) (Hrotko and Magyar, 2004).

### 3.2. Tissue preparation

Samples were obtained from root, twig and leaf of each individual tree and were collected in plastic bags. Although rapid changes in endophyte colonization probably do not occur immediately following collection, all samples were handled carefully and processed as quickly as possible. Samples were air-dried to remove any surface moisture before transport or storage. During transport, samples were kept cool and dry.

Samples were transported to the laboratory at the Department of Plant Physiology and Biochemistry, Faculty of Horticultural Sciences, Corvinus University of Budapest (Hungary). In the laboratory, all samples were washed thoroughly by detergent under running tap water. Surface sterilization of plant material was carried out using chlorine bleach (NaOCl) diluted in water to concentrations of 2–10% to treat the specimens. After being sunk in 96% ethanol for 1 min, tissue samples were washed by dipping into hypochlorite 3% solution for 10 min and then were sunk again for 1 min in 96% ethanol. Procedure was followed by washing the samples twice with sterile distilled water for 5 min.

Size of the sampling unit and surface sterilization procedures vary according to the preferences of the investigator, the species of host plant, and host tissue type sampled. A pilot study prior to commencement of main sampling procedure was fulfilled to optimize the techniques for tissue preparation with higher efficiency and obtaining larger numbers of endophytic fungi from each sample. Accordingly, it became clear that the smaller the sampling unit was, the greater the recovery of diverse species/genotypes could be achieved. Also, conversely, the larger the sampling unit was taken, the greater the potential existed to miss rare or slow-growing species and to recover mixed genotypes of the same species. Thus, two sections from different parts of each tissue compartment, from leaf as 0.5 cm in diameter each and from twig and root two sections as 0.5 cm in length, were randomly cut for isolation of endophytic fungi on the selected media.

### 3.3. Primary isolation of endophytic fungi

Routine mycological media are suitable for primary isolation, sub-culturing and identification of endophytic fungi. Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA), 1-2%, were used as pre-culturing media. Each 10 pieces of a single segment were transferred into PDA plates supplemented with 250 mg/l amoxicillin, 250 mg/l cephalexin and 100 mg/l chloramphenicol. Plates were incubated at 22°C and colonies were observed after 1-2 weeks. Fungi with rapid growth were sub-cultured onto media without inhibitors to enhance normal sporulation. Optimal incubation conditions varied according to the provenance of the host tissue and therefore some cultures were incubated for two to three weeks to let the slow-growing fungi emerge. Plates were sealed with Parafilm to prevent desiccation of the media, and were incubated in a growth chamber with a humidity control.

### 3.4. Single spore isolation of endophytic fungi

Single spore isolation of endophytic fungi provides an opportunity to obtain certain fungal subcultures from polyspore isolates when it is important to identify the origin and molecular phylogeny of the mycelia. The use of fungal cultures obtained from single spore isolations is fundamental to the identification of fungi in term of morphological species concept, as these cultures exhibit extra specific characteristics and anamorph-telomorph connections. In phylogenetic studies which include both morphological and molecular traits single spore cultures are essentially required.

In the present study, single spore isolation of endophytic fungi was performed according to the method described by White *et al.* (1990). Accordingly, 20 g/l water agar was used as sporulation medium in sterile Petri dishes. After autoclaveing at 121°C for 20 min penicillin (0.5 g/l) was added when the temperature of agar was about 50°C, then the medium was distributed into 90 mm Petri dishes inside a laminar flow cabinet. By employing a fine sterile sampler, spores were picked up from the surface of an individual colony. Different isolation methods were employed based on differences between fungal isolates according to the type of their fruiting bodies. Fungi with closed fruiting bodies such as *Ascomycetes* with cleistothecia or perithecia and *Coelomycetes* with pycnidia were removed from the substrate surface. Fungi with cup shape fruiting bodies as like as *Ascomycetes* with apothecia and *Coelomycetes* with acervuli, were transferred directly by removing the whole fruiting body. Spores from *Basidiomycetes* with gills

were obtained by removing a few segments of gills and finally when no sporulation was detected, subcultures were prepared by transferring single thread of hyphae (single hyphae) into PDA plates.

To overcome the problem of bacteria or yeast contamination and prevent the transfer of wrong species, in addition to using antibiotics, spore masses were also diluted in sterile water. A glass container was sterilized using ethanol 70% and wiped with a towel on which ethanol 70% had been sprayed. A sterilized pipette was then used to transfer about 6 drops of sterilized water into the container and spore masses obtained as explained above were added to make a spore suspension. This homogenous spore suspension was finally transferred onto the water agar plates. A permanent slide was also prepared by using a drop of each spore suspension to check whether the correct fungus had been selected.

The procedure was followed by incubation of the plates at 22°C for 24 hours. Germinated spores were detected by microscope examination and then one single spore was picked up and transferred into another PDA plate. Isolates were incubated at 25°C and were checked frequently till their colony diameter was about 1-2 centimeters.

### 3.5. Morphological study

Cultures on both PDA and MEA media were assessed according to their morphology. Colony appearance, mycelium color and structure, shape of conidiomata, conidia and conidiophore (size, color, ornamentation, etc.) and characters of conidiogenous cells were observed for morphological classification of isolated fungi using a light microscope with 5X, 10X and 40X objective lenses for magnification.

### 3.6. PCR amplification of ribosomal internal transcribed spacer regions

In the present study, amplification of the fungi ITS region was performed using fungal domain specific ITS1 and ITS4 primers. Total DNA was extracted from fungal components by applying a modified CTAB (cetyltrimethylammonium bromide) method (Faeth & Sullivan, 2003). After pre-heating at 65°C in water bath, 15 µl mercapto-ethanol + 2% polivynil-pyrrolidone solution was added to the samples. CTAB buffer containing 2% CTAB, 0.1 M Tris-HCl (pH 7.0-8.0), 20 mM EDTA and 1.4 M NaCl, previously heated at 65°C, was mixed with the content of each tube to the total volume of 600µl. Samples were incubated at 65°C for 30 min and supernatants were

collected after centrifugation for 15 min at 12 000 g. Protein content was depleted by adding 600 µl chloroform to each tube followed by centrifugation at 12 000g for 8 . The procedure was repeated one more time, then 900 µl of 96% ethanol was added to each tube and samples were incubated at -20°C for 2 hours. After centrifugation at 12 000xg for 15 min, pellets were washed by 70% ethanol and resolved in 100 µl of sterile Milli-Q water.

The PCR reaction mixture consisted of 5 µl fungal DNA, 2.5 µl 10x loading buffer, 0.5 µl 10 mM dNTP mix, 15.6 µl sterile Milli-Q water, 0.4 µl Taq polymerase and 0.5 µl each of the forward and the reverse primers ITS1 5‟-TCCGTAGGTGAACCTGCGG-3‟ and ITS4 5‟-TCCTCCGCTTATTGATATGC-3‟ (White *et al*., 1990) in a total reaction volume of 25µl. Amplification was performed in a Thermal Cycler (BioRad T100 THERM) and PCR conditions were 15 min at 95°C followed by 40 cycles at 95°C for 1 min, 30 sec at the annealing temperature and 72°C for 1min. Aliquots of each amplified product were electrophoretically separated on a 2% agarose gel in 1x TAE buffer and visualized using ethidium bromide under UV illumination..

PCR amplicons were recovered using the kit. Isolates whose sequences had a similarity greater than 95% were considered to belong to the same species. Sequence-based identifications were made by searching by FASTA algorithms the EMBL/Genbank database of fungal nucleotide sequences. The criterium for species identification was an ≥97% identity to the database sequence, genera were positively identified when the sequence match reached 96.9 – 95.0%. When the similarity was less than 95%, the isolate was considered as unidentified.

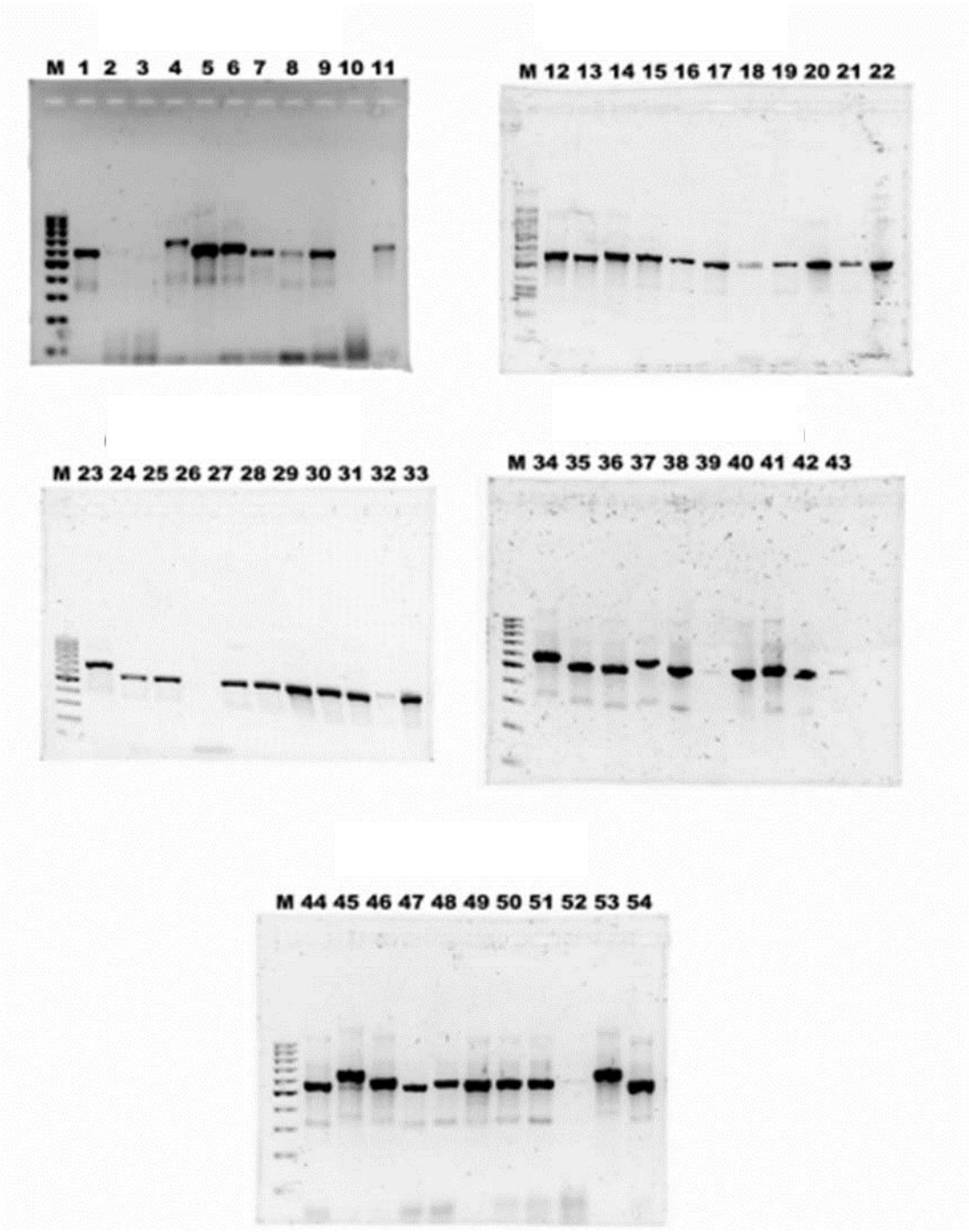



**Figure 3.1**: PCR amplicons of 54 isolated fungal strains. M: 100 kBp DNA marker (Fermentas). From the BSc thesis of Tímea Pósa (2009).

### 3.7. Analysis of endophyte – pathogen antagonism by dual culture method

As it was previously described, endophytic fungi may compete with other, potentially pathogenic microorganisms that colonize the host. There is some evidence that the endophyte-pathogen interplay may result in decreased virulence or even depletion of the pathogen and such may confer host resistance. Different methods can be applied to investigate endophyte-pathogen antagonism (Knowlton & Rohwer, 2003). Of the many mechanisms of pathogen inhibition, production of antimicrobial compounds is the most easily detected and widely studied. Antimicrobial compounds are produced in either volatile or nonvolatile forms. The nonvolatile compounds are often detected using simple plate assay. Contrary, the extraction, characterization and identification of volatile compounds produced by fungal endophytes can only be studied using more sophisticated means, such as with the use of gas-chromatography and/or mass-spectrophotometry (Rowan & Knowlton, 1995). Moreover, studies on beneficial inhibitory volatile metabolites are relatively new. Nevertheless, the role of volatile inhibitory compounds in biological control remains to be investigated and may have tremendous potential as the volatile compounds can be entrapped or immobilized in bio-formulations and manipulated for applications in the field or for post-harvest disease management (misting, spraying and droplets). Applications using volatile metabolites also exclude the introduction of viable cells, eliminating the unnecessary or accidental introduction of foreign microbes into the environment.

In the present study, a dual culture method was applied to assess the antagonism of endophytic fungi against pathogens. Two relevant pathogens of sweet cherry were used: *Agrobacterium tumefaciens* strain, kindly provided by Ernő Szegedi from FVM Vine and Wine Research Institute (Kecskemét, Hungary) and *Monilia laxa,* obtained from Géza Nagy at the Department of Plant Pathology, Corvinus University of Budapest, Hungary.

Two culture media were examined, Malt Yeast-Extract Agar (MYEA) and Potato-Dextrose Agar (PDA), of these PDA was found to be more adventageous and was therefore. Fungi which had faster growth on culture plates were selected for antagonism test. With the help of a sampler needle, an inoculum of the selected endophyte and of the pathogen were aseptically planted 40 mm far from each other on a Petri plate (d = 90 mm) containing 30 ml fresh PDA medium. At the same time, inocula of the endophyte and the pathogen were placed separately on a PDA-containing Petri dish as controls. All plates were incubated at 28°C for 7 days.

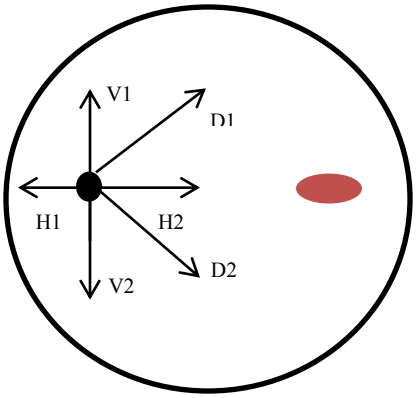



**Figure 3.2:** Assessment of radial growth of a desired colony in dual-cultured plate is schematically demonstrated in this Figure. In brief, colony extension was measured in three dimensions (horizontal: H, vertical: V, and diagonal: D) in two directions and mean value of each measurement was used for analytical studies.

Six replicates were used for each plate. Growth inhibition was tested by measuring the radial growth of each colony in three directions (horizontal, diagonal and vertical) every day during a period of 15 days after inoculation and calculated by applying the following formula:

$$\text{Growth Inhibition (GI) (\%)} = [(DC - DP)/DC]\text{x}100$$

where DC = diameter of control, and DP = diameter of pathogen colony dual cultured with endophyte. Values were calculated as the averages of all achieved data.

Radial growth measurements of each colony in three directions on an actual day were used also to calculate the growth rate (GR) of colonies. The growth rate value was defined as the slope of measurements logarithmic curve during 15 days and was expressed in mm/day.

### 3.8. Statistical analysis

Data derived from the present study were assessed with an emphasis on diversity of endophytic fungi isolated from different cherry rootstocks. Leaves, twigs and roots as three different tissue compartments from each rootstock, were also examined to indicate the anatomical distribution pattern of isolated endophytes. Differences in number and diversity of fungal endophytes recovered from cherry rootstocks in three distinctive periods when sampling process had been performed (autumn 2008, spring 2009 and autumn 2009, hereafter: season I, season II and season III, respectively), were also considered for data analysis. Accordingly, relative frequency (RF) of

various species was defined as the proportion of recovered colonies belonging to an identified endophytic fungus compared to the total number of isolates during a particular season.

Infection frequency in different organs was expressed as the percentage of endophyte-bearer explants among the cultured specimens from a particular tissue and also by dividing the number of isolated colonies from each organ to the total number of tissue explants, which were defined as colonization rate (CR) and isolation rate (IR), respectively. Relative abundance and species richness were calculated for diversity analysis. Shannon-Weaver index ($e^{H'}$) was used to compare the distribution of endophyte species in all examined tissue compartments of every rootstock in the 3 sampling times. Simpson‟s diversity index (D) was calculated representing the diversity of endophytic assemblages on different rootstocks.

Endophytic fungal colonies which could not be characterized by morphological or molecular methods, were marked as unidentified and were omitted from analytical calculations.

A two-way variance analysis (ANOVA) was applied to determine the significance of growth inhibitory effect between all groups. Student T test was also used for comparing the growth factors of a group with corresponding control sample. All data were expressed as the mean value (mm) of total measurements for six replicates.

$P \leq 0.001$ was determined as the level of significance.

### 3.9. Sampling, cultivation and identification of endophytic fungi in Capsicum annuum L.

As described in Chapter 2.9, endophytic fungi also occur in pepper (*Capsicum annuum* L.). We investigated two pepper cultivars (Hó F1 and Kárpia F1) and from these cultivars 8 organs such as roots, shoot, leaves, pedicles, pericarp at different developmental stages and seeds. Samples were taken in 3 replicates four times during the vegetation period in 2013 (in April, May, August and October). The plants lived in open field on original sandy soil with drip irrigation or in greenhouse on rockwool. By the sampling in April and May plants were in the seedling stage, such only root, leaf and shoot samples could be collected. Samples were separately put into plastic bags and pre-cultivation started in 6 hours after sample collection.

Surface sterilization was performed by soaking the corresponding organ for 1 min in 70% ethanol, after that for 10 min in 20% hypochlorite, and finally again for 1 min in 70% ethanol. After surface sterilization samples were washed by dipping into sterile distilled water. Tissue samples of 5 mm were cut under sterile conditions. We put 9 pieces from each organ sample on

PDA pre-culturing medium supplemented with 1 g/l chloamphenicol. We incubated our samples in 90 mm Petri dishes at room temperature in dark for 2 weeks. After this period we surveyed and evaluated the frequency of the outgrowing colonies. Then representative colonies were selected for further work. Small pieces from these cultures were transferred under sterile conditions to fresh PDA plates and were then monosporulated or monohyphated. my personal contribution to this part of the project was mainly in this last step.

Identification of endophytic fungi was done by PCR and sequencing and selected strains were also investigated for their ITS regions (Faeth & Sullivan, 2003).

## 4. Results

A total of 9823 tissue segments (inocula), 3397 inocula from roots, 3233 inocula from twigs and 3193 inocula from leaves of all cherry rootstocks were examined while 1614, 2530 and 1037 inocula showed fungal endophyte infection in cultures from roots, twigs and leaves, respectively (Table 4.1).

All isolates were primarily identified by morphological characteristics and then were subjected to single spore isolation process where distinguished culture of every individual colony was prepared and phylogenetically examined by molecular experiments.

**Table 4.1**: Overall pattern of isolation of endophytic fungi in tissue samples obtained from cherry rootstocks.

| Tissue | Total number of inocula | Number of infected inocula | Colonization rate % (CR) | Total number of fungi isolates | Isolation rate (IR) |
|---|---|---|---|---|---|
| **Root** | 3397 | 1614 | 47.5 | 1944 | 0.57 |
| **Twig** | 3233 | 2530 | 78.3 | 3427 | 1.06 |
| **Leaf** | 3193 | 1037 | 32.5 | 1216 | 0.38 |
| **Total** | 9823 | 5181 | 52.7 | 6587 | 0.67 |

In term of methodology, biodiversity of endophytic fungi in 11 rootstocks of *Prunus* sp., was assayed by a multidimensional analysis regarding host-specificity, temporal changes and colonization outlines dependent to the harboring tissue and results were combined to achieve a comprehensive insight about fungal endophytes assemblages associated with examined plants.

### 4.1. Identified endophytic fungi associated with Prunus sp., rootstocks

Regardless the analytical values which would help to evolve biodiversity and composition of endophyte infection in examined host plants, results derived from the present study provided brand new information about fungal microorganisms that may contribute to host-endophyte symbiosis in cherry rootstocks within the locality where this study took place.

Among 6587 isolates obtained from roots, twigs and leaves of cherry rootstocks, 6072 isolates were taxonomically identified to the level of genus or species following the single-spore isolation and a number of 519 colonies remained unidentified (marked as unknown).

Table 4.2 contains the listed names of overall fungi taxa isolated from tissue samples of cherry rootstocks. Among isolated genera, two species of *Alternaria* (*Alternaria* sp.1 and *Alternaria* sp.2) with a total of 1931 colonies for *A.* sp.1 and 1473 colonies for *A.* sp.2 had the first and the second largest number of colonies isolated from cherry trees. In contrast, *Ceratobasidium* sp.1 and *Ceratobasidium* sp.2 had the minimum number of colonies (4 and 15 colonies, respectively) among other isolates and were observed only in root samples.

In root samples, however, *A.* sp.1 exhibited the highest frequency as 605 colonies of this fungus were isolated from root samples. *A.* sp.2 composed the largest population of endophytes with 1349 isolated colonies (although this isolate was not observed in the root) and the lowest number of isolated colonies belonged to *Glomerella acutata* (21 colonies) which was detected only in twig samples. Similar to root samples, A.sp.1 with 502 isolated colonies was the most frequent fungus in leaves while *Pyronema* sp., with 10 colonies showed the minimum frequency in this tissue. Along with *Pyronema* sp*.,* two other identified isolates, *Rosellinia* sp*.,* and *Xylaria digitata* were only observed in leaf samples.

### 4.2. Diversity of identified endophytic fungi in different sampling periods

Collection of tissue samples from cherry rootstocks was accomplished in three time periods: autumn 2008 (season I), spring 2009 (season II), and autumn 2009 (season III). According to the

results, the total species richness of *Prunus mahaleb* rootstocks (Bogdány, SL64, SM11/4, Egervár, Korponay, Magyar, CEMANY, and Érdi V) was higher than other rootstocks. Average number of distinctive species isolated from *Prunus mahaleb* rootstocks was 12.5 in season I, 10.1 in season II, and 10.1 in season III. During season I, the largest number of species was isolated from SM11/4 (16 species) while Magyar rootstock harbored 9 different species in composition of endophytic fungi associated with this rootstock. Species richness of *Prunus mahaleb* rootstocks in season II ranged from 10 species (SM11/4 rootstock) to 13 species that were detected on Korponay, Bogdány, and Érdi V rootstocks. During season III, the maximum number of species (12 species) was isolated from Korponay, while SL64 had the minimum species richness among other *Prunus mahaleb* rootstocks (8 species) in this sampling period. The difference of species richness between *Prunus mahaleb* rootstocks in three sampling periods was not significant.

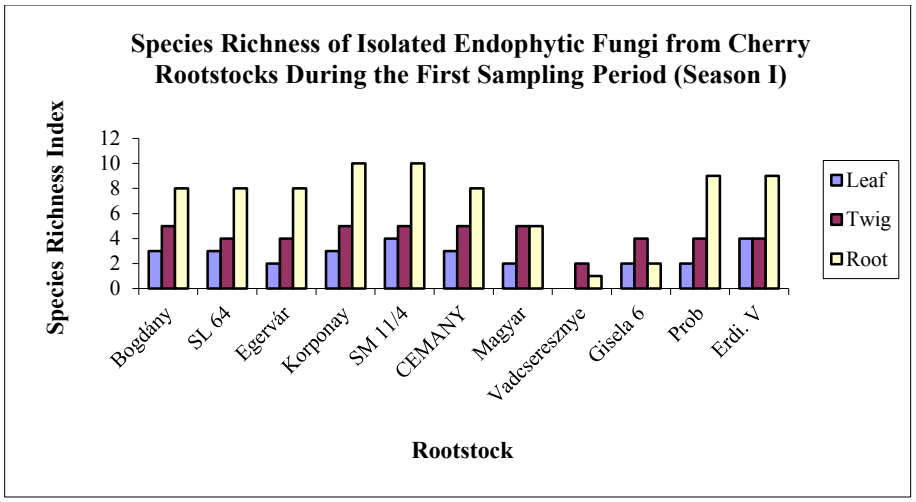
Although a number of 12 different species was isolated from Prob rootstock (*Prunus fruticosa*) in season I but the species richness had a significant decrease ($P \leq 0.05$) in season II and season III (7 species, and 8 sepcies, respectively). Gisela6 (*Prunus cerasus, Prunus canescens*) showed a relatively low species richness with no difference in all three sampling periods (6 species, 7 species, and 8 species respectively). During season I, the lowest species richness was observed in Vadcseresznye (*Prunus avium*) (3 isolated species), but this index had an increase ($P \leq 0.05$) in season II (10 species) and in season III (7 species).

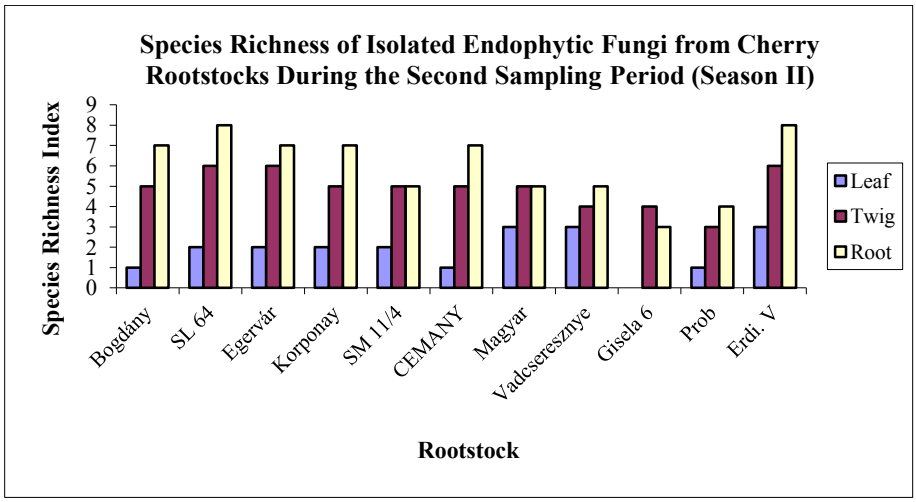
As a conclusion, average species richness was the highest in season I (11 species) but had a slightly fall in season II (10.7 species) in compare with season III (9.5 species). However, this difference was not significant. *Prunus mahaleb* rootstocks harbored the most heterogeneous endophyte communities with almost the same species richness in all seasons, but other rootstocks were associated with endophytic fungi community which showed comparatively less species richness. Figure 5.1 demonstrates species richness in different tissue compartments of examined rootstocks in three sampling periods. Collectively, root samples had the richest endophytic fungi assemblages regarding the number of identified species in season I (average 7.1 species, maximum= 10 species, isolated from Korponay and SM11/4 rootstocks, minimum= 1 species, isolated from Vadcseresznye rootstock). During the season II, root samples harbored again more distinct species (average 6 species, maximum= 8 species, from Érdi V and SL64 rootstocks, minimum= 3 species, isolated from Gisela6 rootstock), whereas species richness index showed

no difference between root and twig as a consequence of increase in number of different species isolated from twig samples in this season (average 4.9 species, maximum= 6 species, isolated from Érdi V, Egervár, and SL64 rootstocks, minimum= 3 species, isolated from Prob rootstock). Distribution of different endophyte species during the season III had a shift toward predominant species richness in twigs (average 6.3, maximum= 8 species, isolated from Korponay and Érdi V rootstocks, minimum= 5 species, isolated from SL64, CEMANY, Vadcseresznye, and Gisela6 rootstocks). Leaf samples had the lowest species richness in all sampling periods ($P<0.05$). Abundance of identified endophytic fungi on different tissue compartments of each individual rootstock during three sampling periods has been summarized in an Appendix section.

**Table 4.2:** Endophytic fungi isolated and identified from tissue samples of cherry rootstocks.

| *Order* | *Genus/*Species | *Best matching Acc. Nr. in GenBank* | *Number of isolated colonies* | | | *Total* |
|---|---|---|---|---|---|---|
| | | | ***Root*** | ***Shoot*** | ***Leaf*** | ***Total*** |
| Pleosporales | *Alternaria* sp.1 | KM215624.1 | 605 | 824 | 502 | **1931** |
| | *Alternaria* sp.2 | KM274131.1 | 0 | 1349 | 124 | **1473** |
| | *Paraphoma* sp. | JN903931.1 | 213 | - | - | **213** |
| | *Embellisia* sp. | JN859368.1 | 7 | 72 | - | **79** |
| Hypocreales | *Fusarium solani* | EU314982.1 | - | 59 | - | **59** |
| | *Fusarium oxy*sporum | LC055797.1 | 11 | - | - | **11** |
| | *Fusarium* sp.3 | JQ929907.1 | 44 | 23 | 252 | **319** |
| | *Fusarium* sp.1 | KM817213.1 | 163 | 110 | - | **273** |
| | *Fusarium* sp.2 | HQ607944.1 | 22 | 270 | - | **292** |
| | *Acremonium* sp. | JQ717326.1 | 133 | - | - | **133** |
| | *Neonectria radicicola* | AJ875317.1 | 23 | - | - | **23** |
| | *Acremonium furcatum* | JN596334.1 | 15 | - | - | **15** |
| Incertae sedis | *Epicoccum nigrum* | JX981489.1 | 28 | 404 | 110 | **542** |
| | *Rhizopycnis vagum* | KF494166.1 | 15 | - | - | **15** |
| Botryosphaeriales | *Macrophomina phaseolina* | KF531825.1 | - | 88 | - | **88** |
| Phyllachorales | *Glomerella acutata* | JX392857.1 | - | 21 | - | **21** |
| Capnodiales | *Davidiella* sp. | JF449792.1 | 323 | 0 | 16 | **339** |
| Cantharellales | *Ceratobasidium* sp.1 | KF176563.1 | 4 | - | - | **4** |
| | *Ceratobasidium* sp.2 | KJ489319.1 | 15 | - | - | **15** |
| Eurotiales | *Aspergillus niger* | KJ365316.1 | 17 | - | - | **17** |
| Sordariales | *Chaetomium* sp. | KP101188.1 | - | 59 | - | **59** |
| Helotiales | *Botrytis cinerea* | CP009808.1 | - | 50 | - | **50** |
| Diaporthales | *Phomopsis* sp. | EF432295.1 | - | 24 | - | **24** |
| Xylariales | *Rosellinia* sp. | KF293984.1 | - | - | 50 | **50** |
| | *Xylaria digitata* | GU322456.1 | - | - | 13 | **13** |
| Pezizales | *Pyronema* sp. | FN868474.1 | - | - | 10 | **10** |
| Unknown | - | | 306 | 74 | 139 | **519** |
| **Total** | | | **1944** | **3427** | **1216** | **6587** |



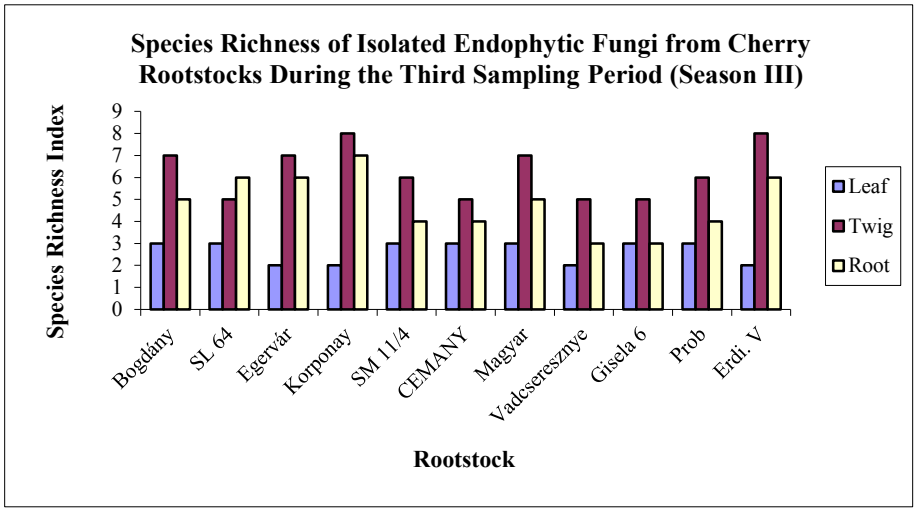




**Figure 4.1:** Species richness of endophytic fungi associated with different tissue compartments of examined cherry rootstocks has been demonstrated in three sampling periods.

As an average, overall species richness of leaf samples from cherry rootstocks was 2.5 species, ranged from no isolated fungus in Vadcseresznye rootstock to 4 species in SM11/4 and Érdi V, during season I. Maximum value of species richness during the second season in leaves was observed in Magyar, Vadcseresznye and Érdi V rootstocks (3 species), while no isolate was obtained from leaf samples of Gisela6 rootstock in season II (average species richness= 1.8 species from leaves in season II). As it has been shown in Figure 4.1, species richness in leaf had no significant change in season III in compare with other seasons but leaf specimens were significantly more infested during this season. The average species richness of leaf in season III was 2.6 species while maximum number of species (3 species) was detected on Bogdány, SL64, SM11/4, Magyar, CEMANY, Gisela6, and Prob rootstocks. Minimum number of species isolated from leaf samples in season III was observed in Egervár, Korponay, Érdi V, and Vadcseresznye rootstocks (2 species).

Diversity of endophytic fungi was also determined by Shannon-Weaver ($e^{H"}$) and Simpson (D) diversity indices. Both indices take into account not only the species richness but the abundance of species in a community. Values of $e^{H"}$ and D were calculated separately for each rootstock according to the total composition of isolated endophyte communities in three seasons. Evenness (Simpson"s index) of isolated endophytic fungi from each rootstock has been demonstrated in Figure 4.2. As a sum, the evenness of different species in all rootstocks was comparatively higher in season II than that in other seasons. The higher value of $e^{H"}$ shows either diversity of species or equitable distribution of isolated colonies among the species. In this sense, it is expected to defy Simpson index (D) which measures the probability whether two randomly selected individual colony in an endophyte community belong to the same species, however they refer to different concepts of diversity. Accordingly, Simpson index for assessment the diversity of fungal endophytes in each rootstock verified the occurrence of more heterogeneous endophyte assemblages on cheryy rootstocks during season II. In general, endophyte communities associated with rootstocks belonged to *Prunus mahaleb* were relatively more diverse particularly during the second sampling period in compare with other cherry rootstock examined in the present study.

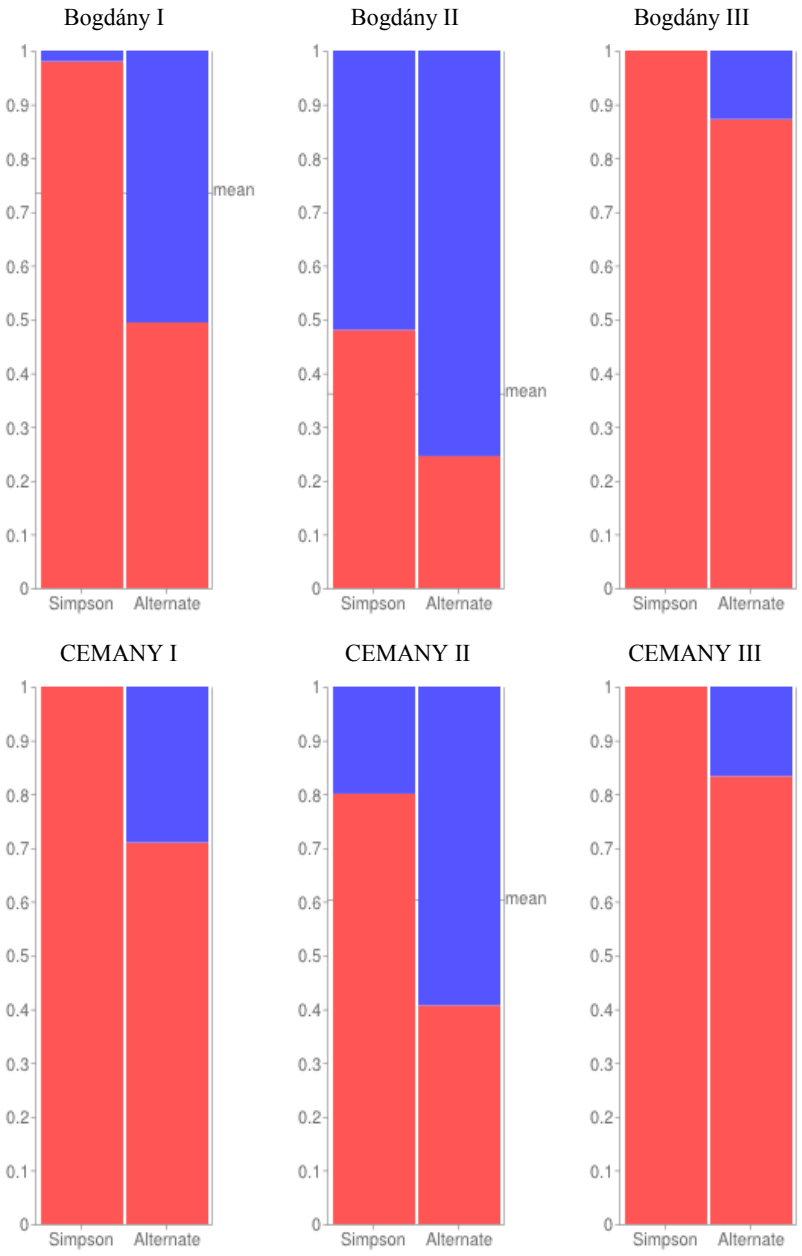



**Figure 4.2:** Probability of two randomly selected endophyte fungi to be of similar species has been demonstrated by Simpson index (red zone) versus the effective number of diverse species (blue zone) for each rootstock during three sampling periods (I, II, and III). The weighted arithmetic value (mean) indicates the average proportional abundance of species when the community of identified endophytes is diverse (mean= 0 refers to a completely diverse, and M=1 refers to a homogeneous community).

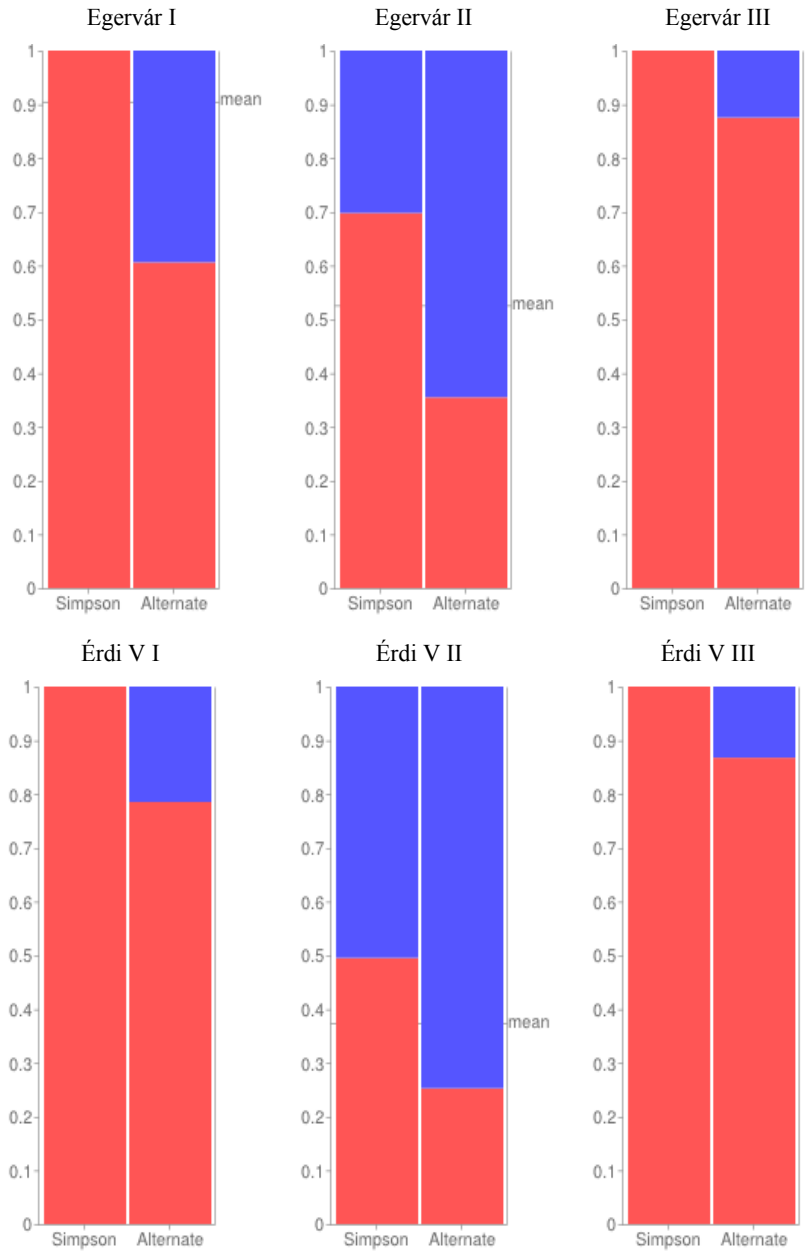
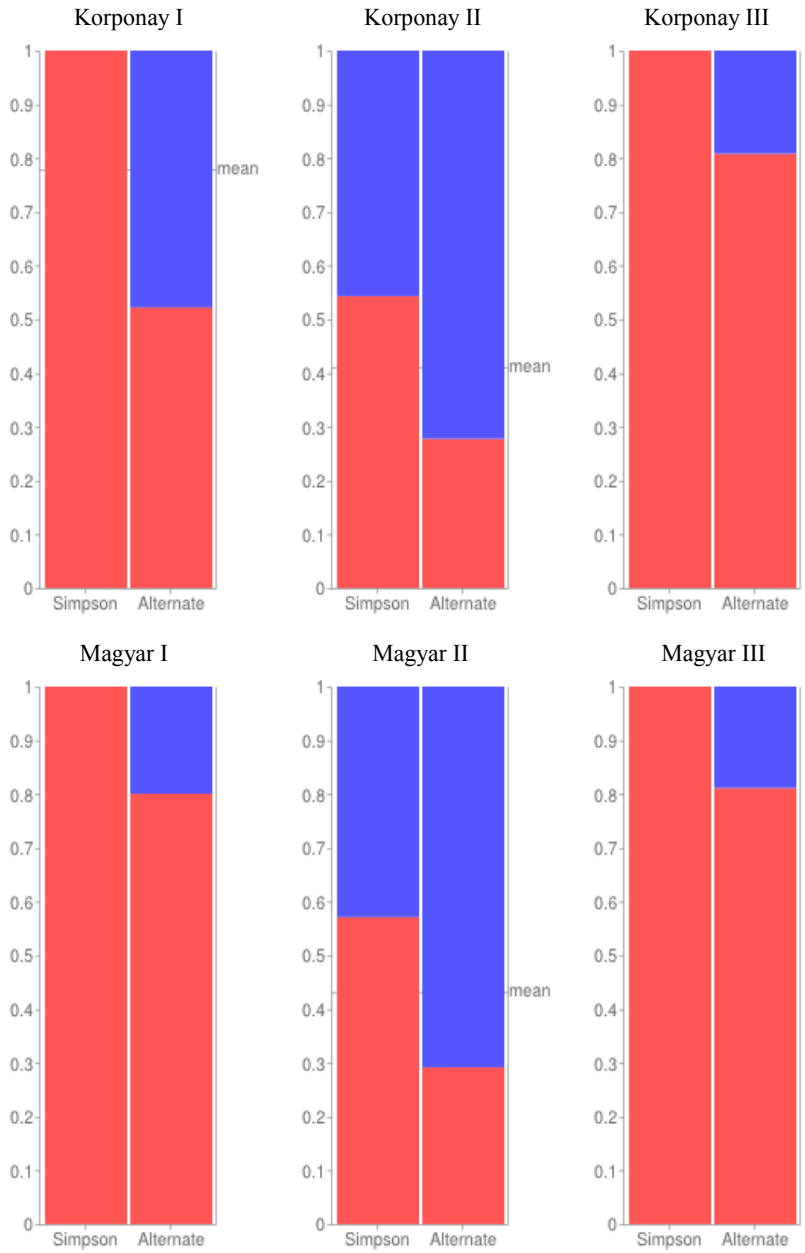



**Figure 4.2:** Continued.

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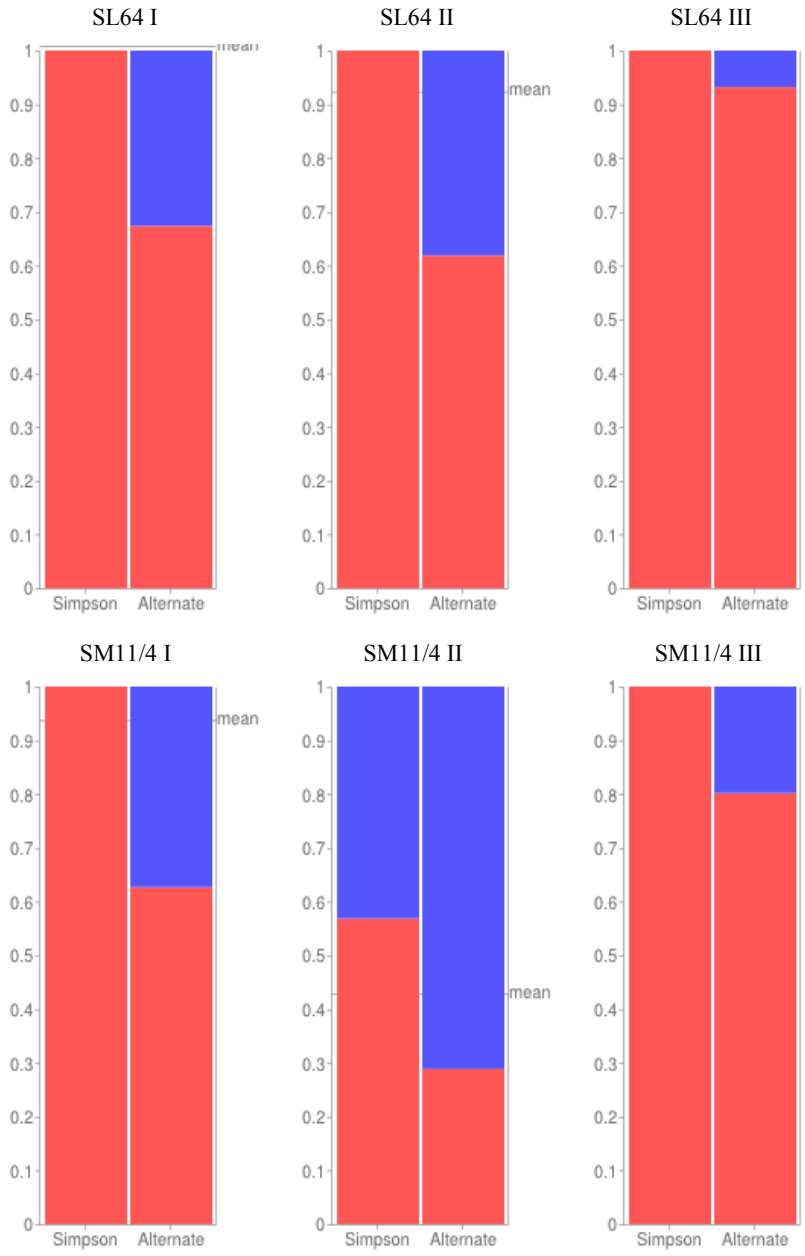


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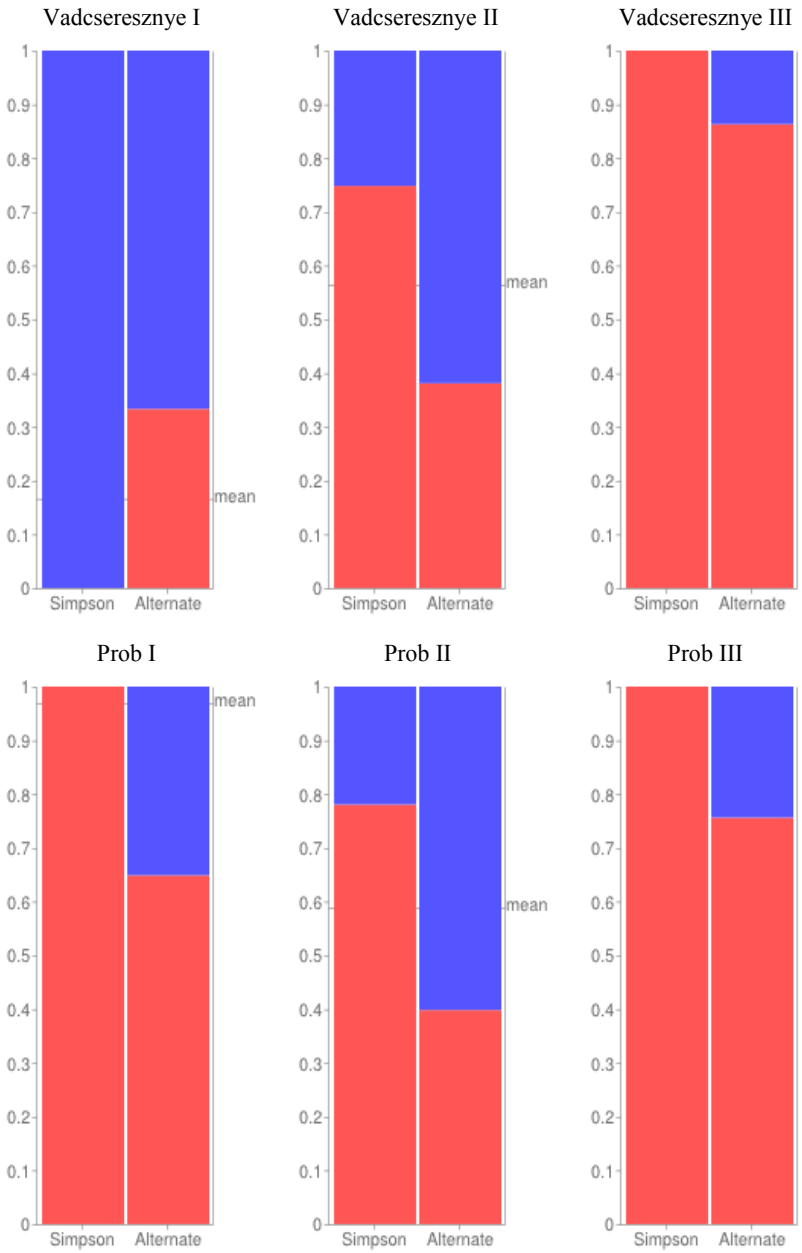



**Figure 4.2:** Continued.

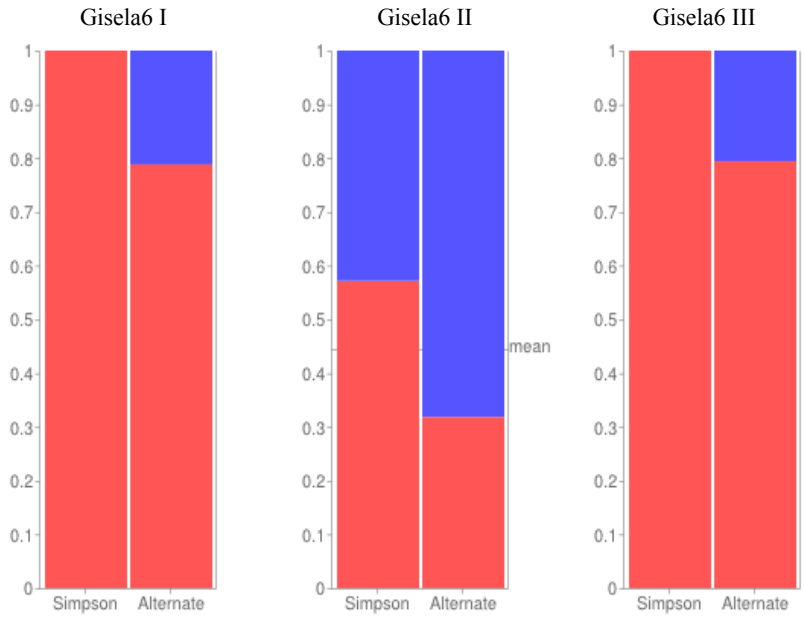



**Figure 4.2:** Continued.

### 4.3. Pattern of colonization and distribution of endophytic fungi on examined cherry rootstocks

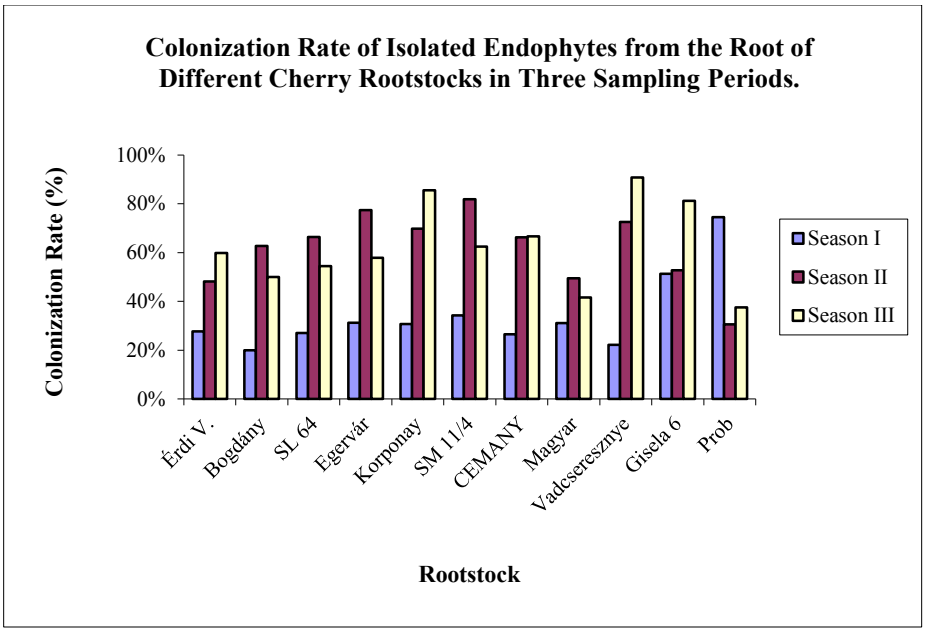
Although root samples obtained from cherry rootstocks demonstrated the highest average of species richness in compare with other tissues, twig was found as the most infected histological compartment in examined host plants. To analyze the rate of infection in specimens, the proportion of infested inocula in total number of tested tissue explants (CR) and the rate of isolated colonies from every explant (IR) were applied. Accordingly, twig was found as the most infected tissue compartment (CR=79%, IR=1.06) while root harbored less endophyte infection (CR=54%, IR=0.62) than twig. As an overall, leaf samples showed the lowest colonization and isolation rates in the present study (CR=33%, IR=0.38) ($P<0.05$). Indices that show the rate of infection are assumed to be corresponding, but further analysis indicated subsequent changes in colonization and isolation rates consistency based on the host species and the time periods when samples were obtained.

All rootstocks of *Prunus mahaleb* showed the same pattern regarding the distribution and colonization rate of endophytic fungi in their roots during three sampling periods. Both colonization rate and isolation rate were relatively low in season I but had increase in season II and remained unchanged in season III ($P \leq 0.05$). Among other rootstocks, only Vadcseresznye had the same pattern as *Prunus mahaleb* rootstocks. The feature of endophyte infection in root, however, was detected comparatively different in other rootstocks. Root samples obtained from Prob rootstock had the highest colonization rate in season I, but had a descending rate in season II and season III ($P \leq 0.05$). Such a result for Gisela6 showed a different temporal alteration in infection rates when no significant change was observed between these rates in season I and season II, although trees that were sampled from this rootstock showed significantly higher infection rates during season III ($P < 0.05$). Changes in isolation rate calculated for root samples had the same pattern as their colonization rate in all rootstocks during three seasons, although Gisela6 the average number of isolated endophyte colonies from every inocula had a decrease from season I to season II and again a sudden increase occurred regarding this rate in season III ($P < 0.05$). Prob rootstock had the highest colonization and isolation rate in root samples during season I (CR=74%, IR=0.8) and the minimum colonization rate of root was detected in Bogdány (CR=20%) but isolation rate was the lowest in Vadcsresznye root samples among other rootstocks (IR=0.2) in this season. During season II, SM11/4 rootstock showed the maximum infection rate (CR=82%, IR=1.22) in root, while Prob rootstock had the lowest colonization rate (CR=31%) and Gisela6 had the lowest number of isolated colonies from root (IR=0.06). Vadcseresznye and Gisela6 had the highest colonization and isolation rates in root samples obtained from these rootstocks (CR=91%, and IR=1.03, respectively) in season III while the lowest values of these rates were calculated for root samples of Prob (CR=38%) and Magyar (IR=0.4) during the last season (Figure 4.3).

Changes in infection burden of twig samples obtained from cherry rootstocks indicated the same equation according to the colonization and isolation rates in three sampling periods. Both values for all rootstocks were relatively high in season I and season III (with no significant difference) and significantly low ($P < 0.05$) in season II. Maximum infection rate of twig samples was found in Vadcseresznye (CR=100%, IR=1.5) and Korponay rootstock had the minimum rate (CR=67%, IR=0.95) during season I. A descending change in infection rates of twig samples was observed in season II when colonization for twig samples obtained from Prob (CR=57%) was the

highest and Gisela6 (CR=33%) showed the minimum rate. Interestingly, Gisela6 showed the highest isolation rate (IR=1.03) for twig samples while the lowest rate was calculated for twig samples from SL64 rootstock (IR=0.54) during season II. CEMANY and SM11/4 (both from *Prunus mahaleb L,* rootstocks) had the highest rates of colonization (CEMANY, CR=100%) and isolation (SM11/4, IR=1.6) from twig samples obtained from these rootstocks in season III. Moreover, Prob and Magyar rootstocks had the lowest colonization rate (CR=77%) and isolation rate (IR=1.16) in twigs during the last season, respectively (Figure 4.4).

As it was mentioned previously, leaf samples from cherry rootstocks had the lowest infection rates in compare with other tissues in all sampling seasons, although these rates showed temporal changes in leaf according to the rate of fungal endophytes infection in three different sampling periods of the present study. In brief, colonization and isolation rates had a significant decrease during season II in compare with season I ($P<0.05$), but showed a drastic increase in season III ($P<0.05$) when both indices were observed remarkably higher than the first two seasons. Such a pattern was detected in all examined rootstocks. During season I, the maximum colonization and isolation rates were detected for leaf samples from Érdi V (CR=24%) and Bogdány (IR=0.27), respectively, while Vadcseresznye rootstock bearded no endophyte infection in its examined leaf samples (CR=0%, IR=0). Vadcseresznye rootstock showed the highest rates (CR=9%, IR=0.09) of infection in leaf among other rootstocks in season II and no infection was detected on Gisela6 (CR=0%, IR=0), during the second season. In season III, SL64 rootstock showed the maximum colonization rate (CR=100%) in the leaf and Érdi V had the highest isolation rate (IR=1.18) while corresponding values were found the lowest in SM11/4 (CR=63%) and Gisela6 (IR=0.7) rootstocks during the third sampling season (Figure 4.5).



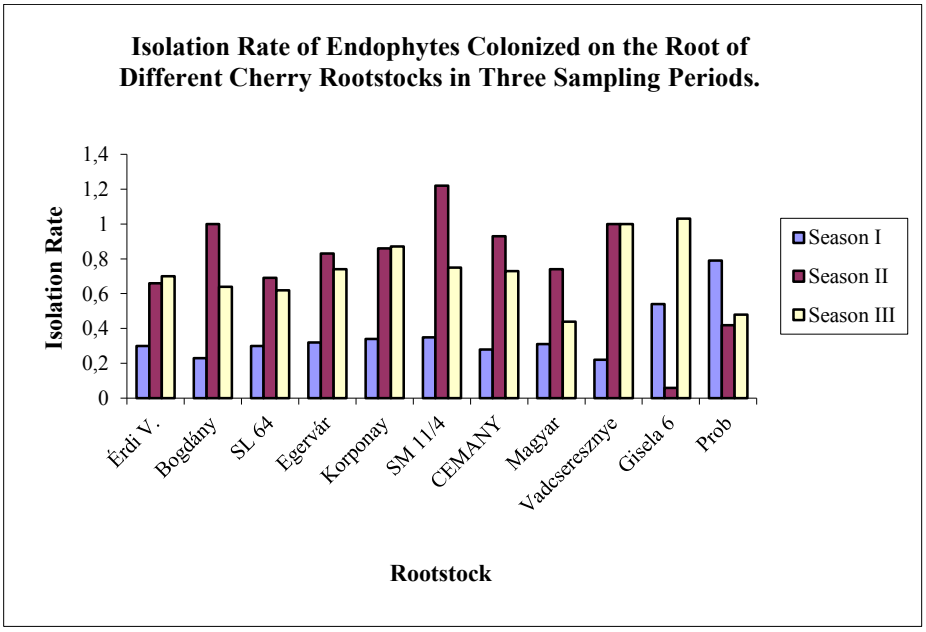
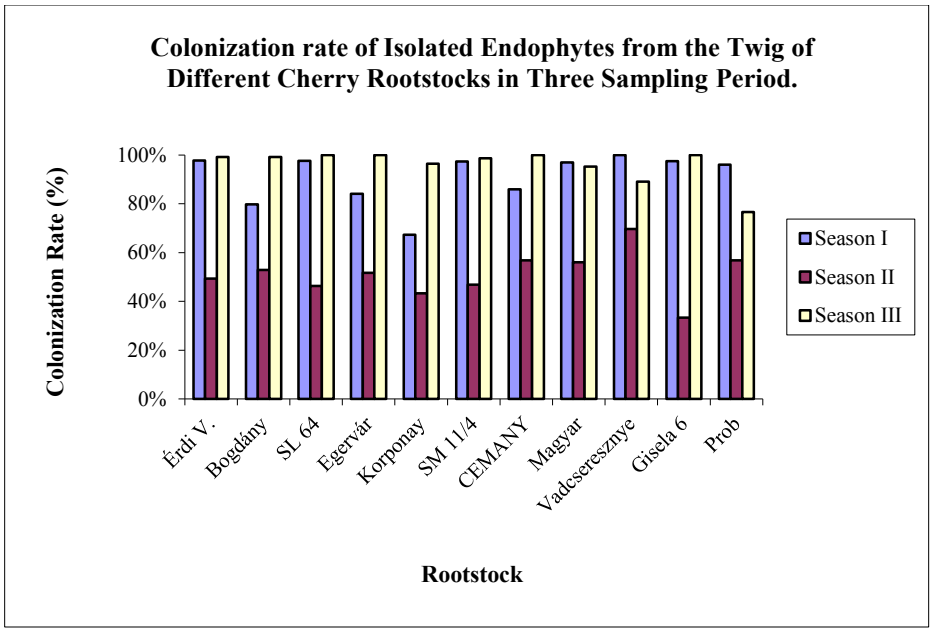




**Figure 4.3:** Colonization and isolation rates of endophytic fungi infection in root samples obtained from cherry rootstocks in three different sampling periods.



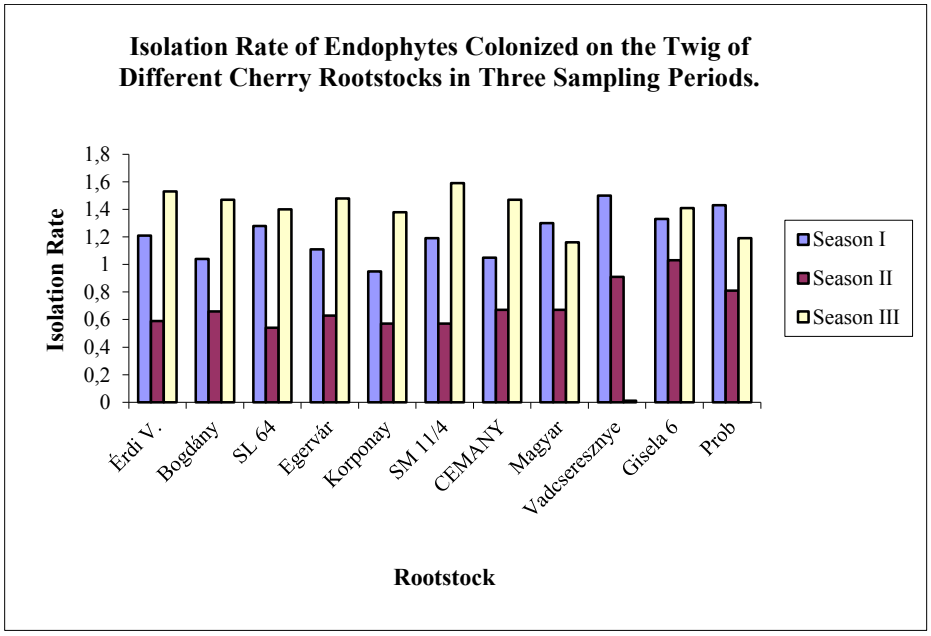




**Figure 4.4:** Colonization and isolation rates of endophytic fungi infection in twig samples obtained from cherry rootstocks in three different sampling periods.

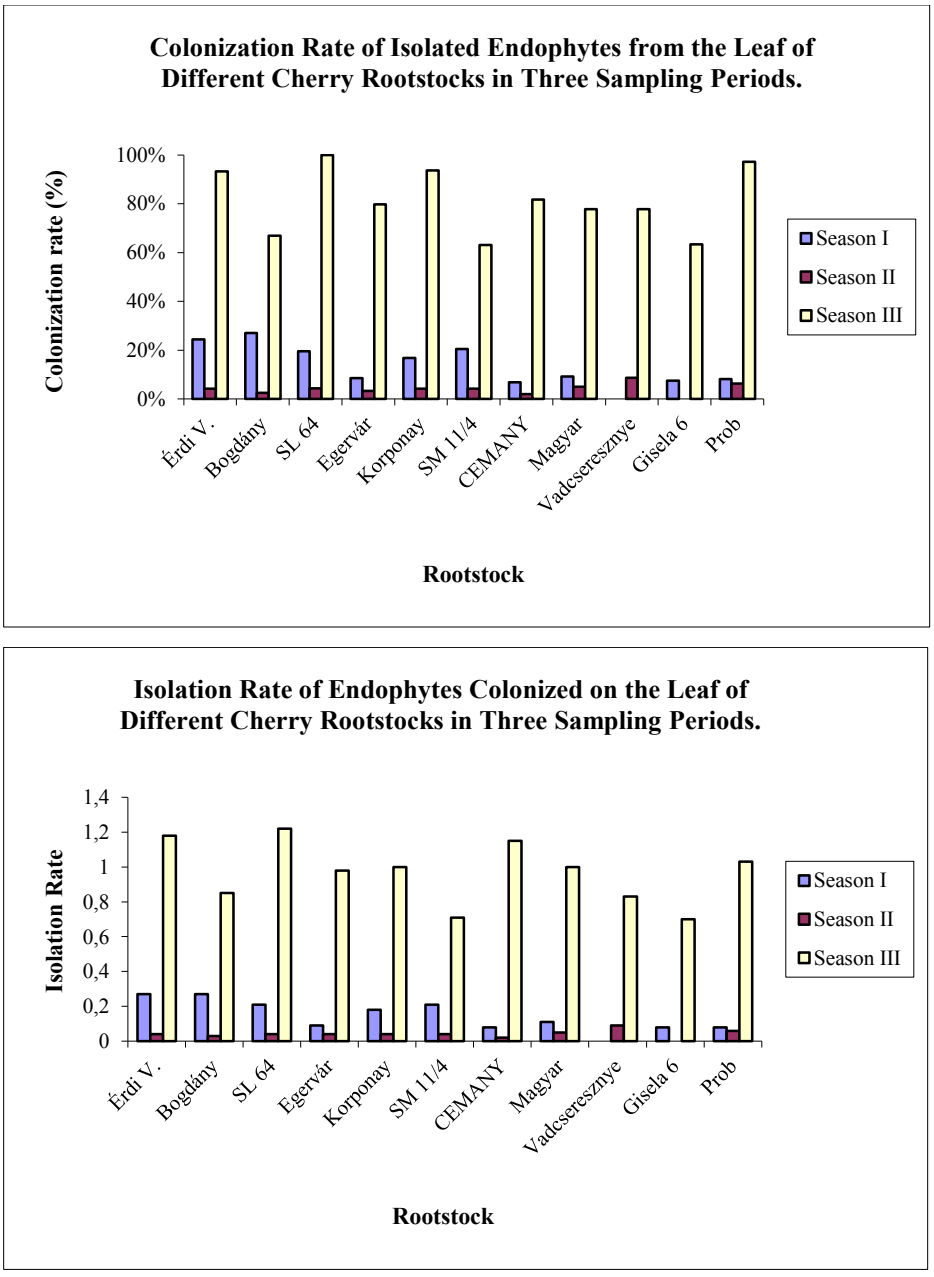



**Figure 4.5:** Colonization and isolation rates of endophytic fungi infection in leaf samples obtained from cherry rootstocks in three different sampling periods.

### 4.4: Relative frequency of identified endophytic fungi on cherry rootstocks

Regardless the host species, harboring tissue and the sampling period, overall results achieved by the present study indicated that *Alternaria* sp.1 (RF=28.11%) and *Alternaria* sp.2 (RF=23.65%) were the first and the second most frequent endophytic fungi associated with examined cherry rootstocks. On the other hand, identified endophytic fungi of the genus *Fusarium* contained the most diverse species (5 different species) composition among other taxa. *Ceratobasidium* sp.1 had the lowest abundance (RF=0.06%) in general structure of endophytic fungi communities on cherry rootstocks. Relative frequency of identified fungi that composed endophyte assemblages on examined cherry rootstocks has been schematically demonstrated in Figure 4.6.

As the most abundant species, relative frequency of *Alternaria* sp.1 and *Alternaria* sp.2 was assessed for each individual host. According to the results, Korponay rootstock had the most frequent population of *Alternaria* sp.1 (RF=32.27%) while the less abundant occurrence of this species was indicated on Prob (RF=19.9%). Interestingly, *Alternaria* sp.2 showed reverse pattern of frequency on recently mentioned hosts, as the maximum relative frequency of this species was found in Prob (RF=30.71%) and Korponay bearded the less frequent population of *Alternaria* sp.2 (RF=18.9%) within the composition of endophytic fungi associated with this rootstock. The ratio of *Alternaria* sp.1/*Alternaria* sp.2 occurrence, however, showed no difference between examined rootstocks (Figure 4.7).

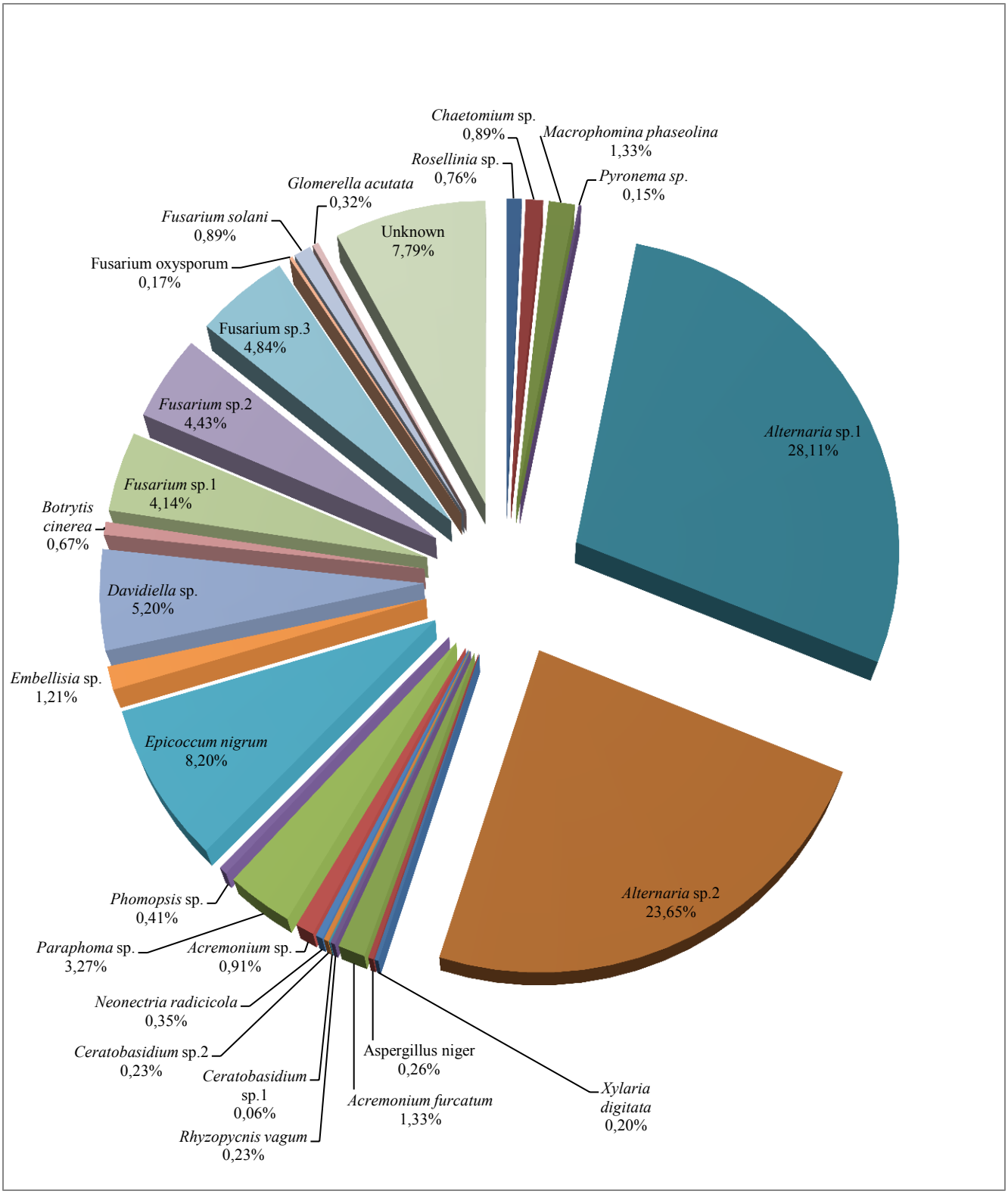



**Figure 4.6:** Overall relative frequency of endophytic fungi on cherry rootstocks.

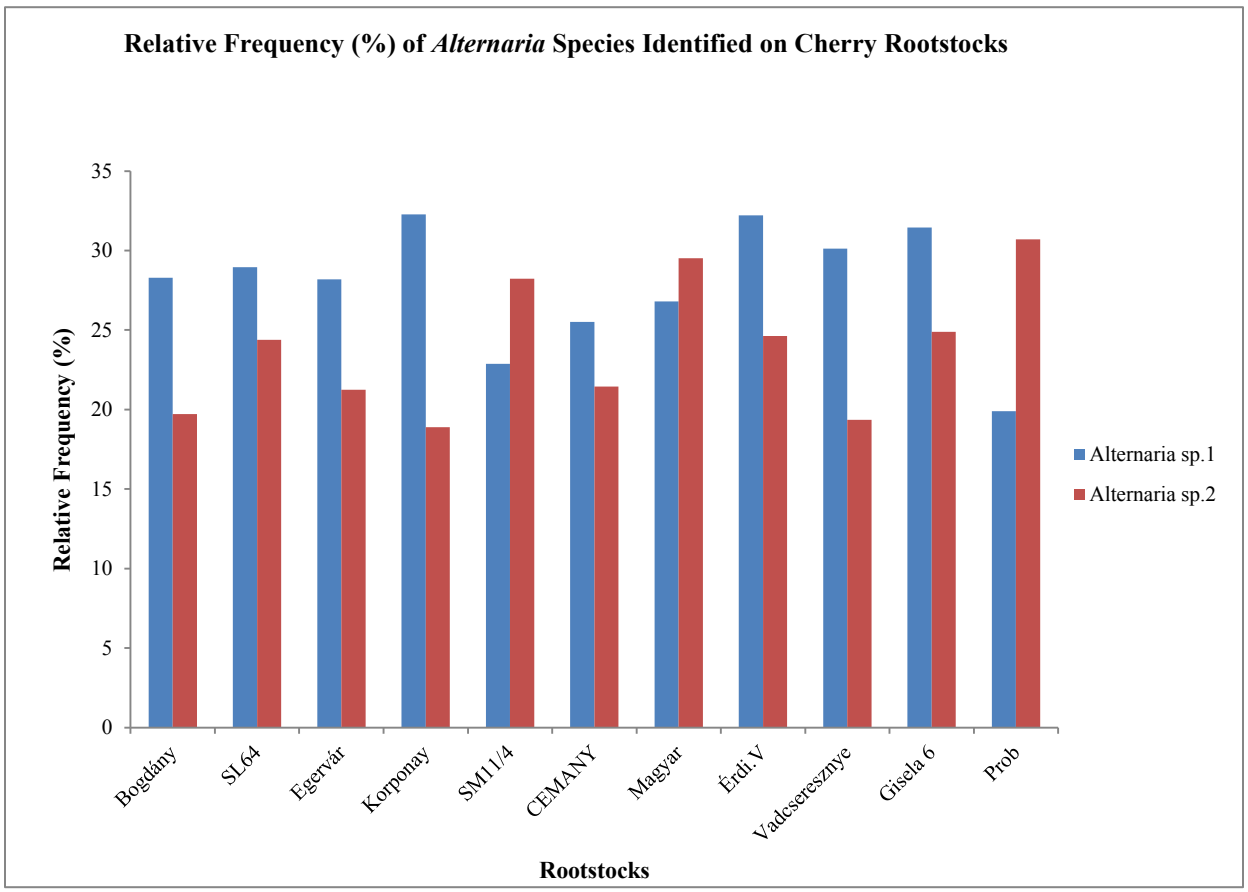



**Figure 4.7:** A Comparison of relative frequency values corresponding occurrence of two largest populations of identified fungi species composed endophytes assemblages on cherry rootstocks.

According to the results, identified endophytic fungi exhibited some levels of specificity when their frequency was assessed on different cherry rootstocks. *Alternaria* sp.1, *Alternaria* sp.2, *Paraphoma* sp., *Macrophomina phaseolina*, *Fusarium* sp.1, *Fusarium* sp.2, *Fusarium* sp.*3*, *Fusarium solani*, *Epicoccum nigrum*, and *Davidiella* sp., were identified on all rootstocks. As well, *Phomopsis* sp. and *Embellisia* sp. were detected on all hosts except on Vadcseresznye rootstock. *Chaetomium* sp. also colonized all rootstocks but was not found on Gisela6 and Prob. Some endophytes were only observed on *Prunus mahaleb* rootstocks while had no occurrence on other cherry species. As an instance, *Aspergillus niger* was detected on Bogdány, SL64, Egervár, Korponay, and Érdi V, and was not frequent on other rootstocks. Besides, *Rhizopycnis vagum* was detected on SL64, Egervár, SM11/4, CEMANY, and Érdi V. *Ceratobasidium* sp.1 was only observed on Bogdány, Egervár, CEMANY, and Érdi V. a species identified as *Pyronema* sp. was only detected on SM11/4 rootstock. Relative frequency of identified fungal endophytes in different rootstocks has been shown in Figure 4.8.

Although some endophytic fungi demonstrated tendency to colonize a specific tissue compartment in examined hosts, but it seemed to be changeable in different sampling periods. Accordingly, leaf samples of all rootstocks were mostly colonized by *Alternaria* sp.2 (RF=54.89%) while *Alternaria* sp.1 had no occurrence in leaf during season I. *Rosellinia* sp. (RF=27.17%), *Xylaria digitata* (RF=1.63%), and *Pyronema* sp. (RF=5.43%) were exclusively observed in leaf during season I. *Davidiella* sp. was also detected in leaves with relatively low frequency in the first sampling period (RF=5.98%). Such a pattern was slightly different observed in leaf during season II when *Alternaria* sp.2 as the largest fungal population (RF=66%) and *Xylaria digitata* (RF=20%) and *Davidiella* sp. (RF=10%) were the only species identified in this tissue. In season III, *Alternaria* sp.1 was the predominant species found in leaves (RF=50.66%) that along with *Epicoccum nigrum* and *Fusarium* sp.*3* composed the endophyte communities in this tissue (Figure 4.9).

Twig samples showed the same structure of endophytic fungi community as the most abundant fungus was *Alternaria* sp*.*2 (RF=69.36%) in season I, *Alternaria* Sp.2 (RF=67.57%) in season II, and *Alternaria* sp.1 (RF=56.55%) in season III, identified in this tissue. During season I, other endophyte as like as *Chaetomium* sp., *Epicoccum nigrum*, *Fusarium* sp.*3*, *Fusarium solani*, and *Macrophomina phaseolina* were found in twig.

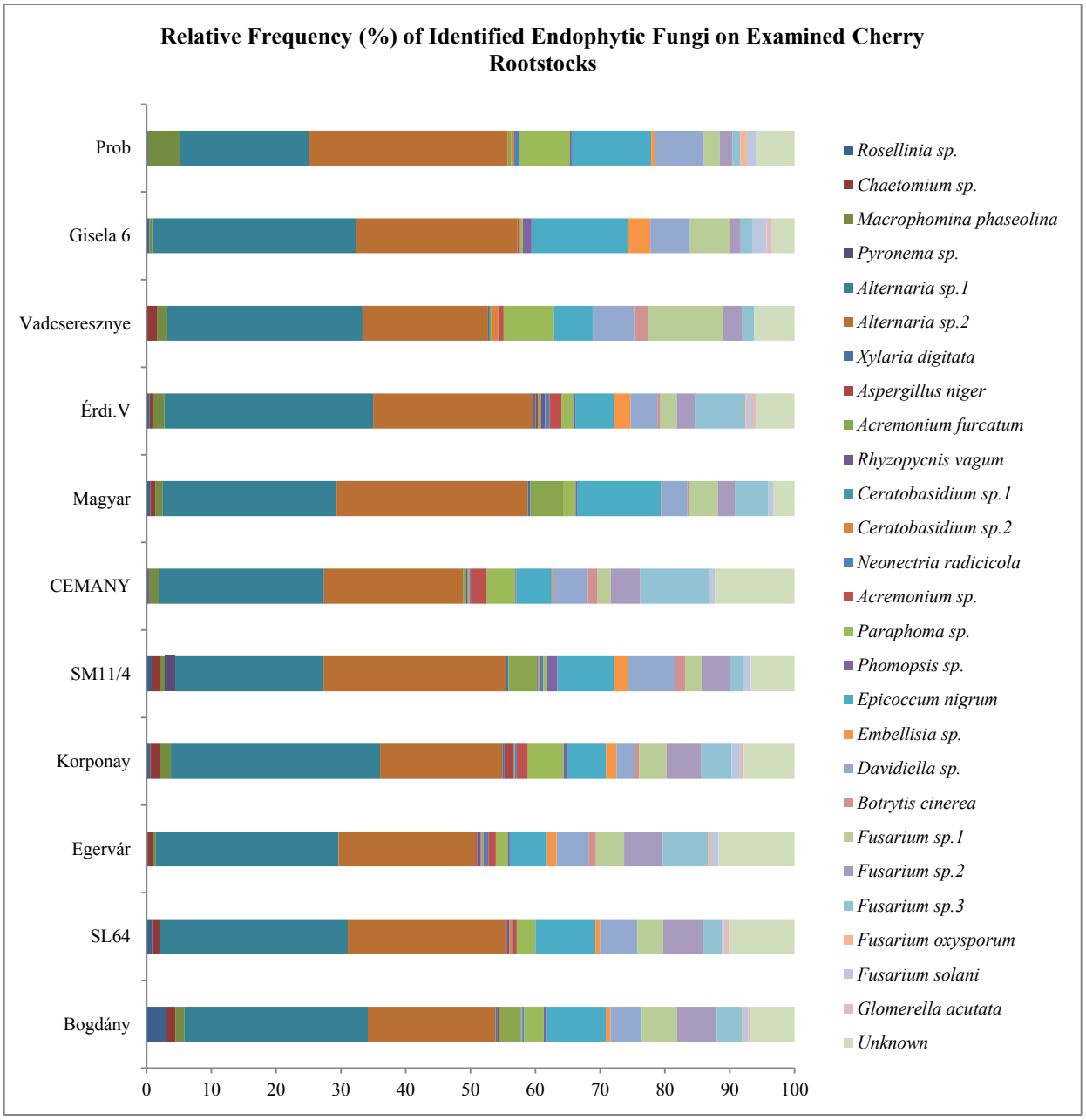



**Figure 4.8:** Overall relative frequency (%) of identified endophytic fungi on examined cherry rootstocks.

*Chaetomium* sp. and *Macrophomina phaseolina* with relatively higher frequency than the previous season along with *Epicoccum nigrum* were detected in twig of cherry rootstocks during season II. Endophytic fungi identified in twig samples from cherry rootstocks were more diverse and included species such as *Phomopsis* sp., *Epicoccum nigrum*, *Embellisia* sp., *Davidiella* sp., *Botrytis cinerea*, *Fusarium* sp.1, *Fusarium* sp.2, and *Glomerella acutata* (Figure 4.10).

Endophyte infection of root had different structure in compare with other tissue samples and showed more divergent composition during three sampling periods. During season I, *Davidiella* sp., was the most abundant fungus in the root (RF=18.47%). Although this species had higher frequency in season II (RF=2638%) than season I, but Alternaria sp.1 was the predominant species found in roots during the second season (RF=33.06%). *Davidiella* sp., had no occurrence in roots during season III. Either *Alternaria* sp.1 or *Alternaria* sp.2 was observed in root samples during season I and season II, although their abundance did not have the same ration in the first two seasons. During season I, *Alternaria* sp.2 had higher frequency (RF=14.25%) while in season II, abundance of this isolate was much lower (RF=2.4%). *Alternaria* sp.1 was also the most frequent species in root during season III (RF=29.25%), while *Alternaria* sp.2 was not detected in root of cherry rootstocks during this season (Figure 4.11).

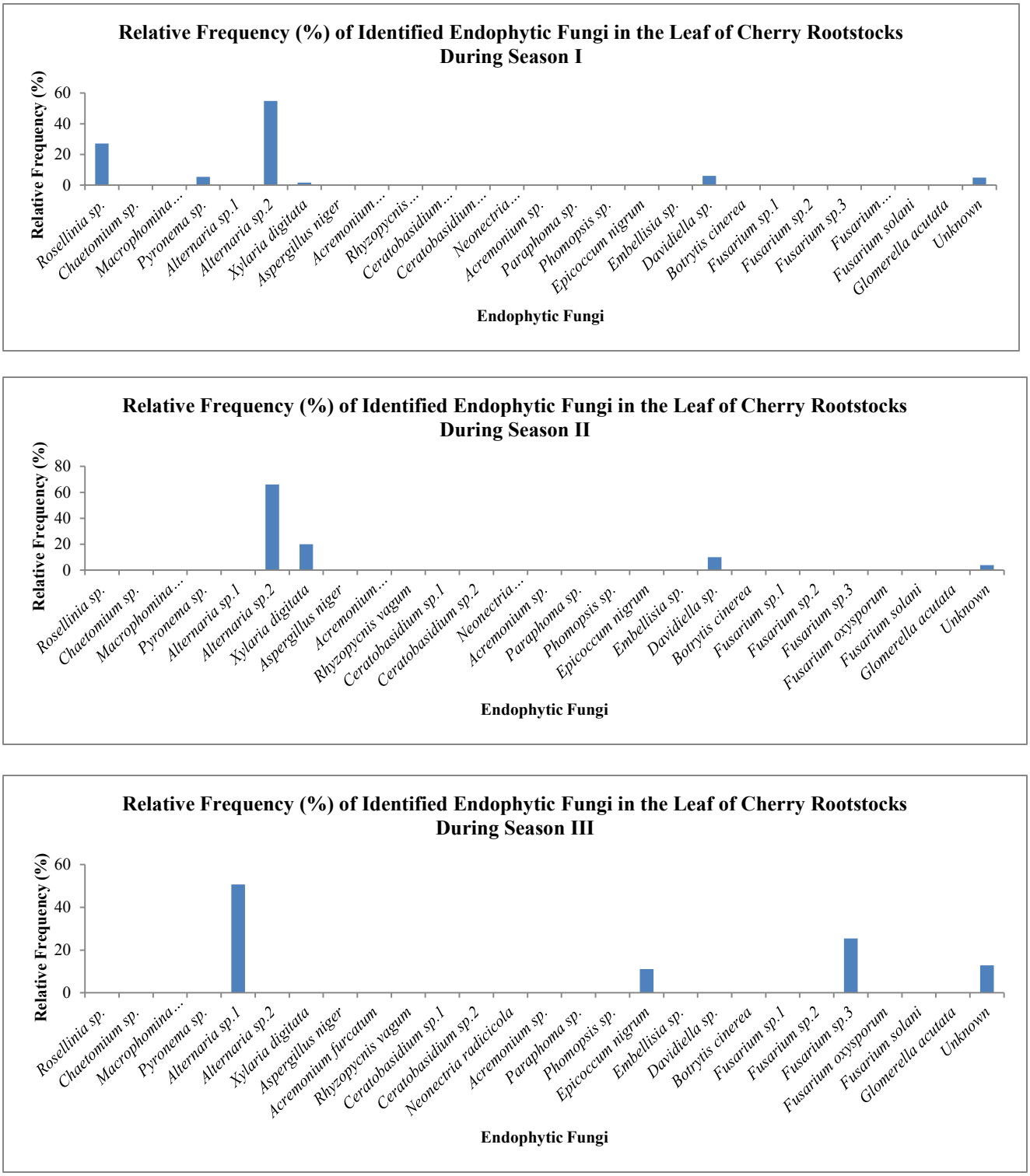



**Figure 4.9:** Overall relative frequency of endophytic fungi in the leaf of cherry rootstocks in different sampling periods.

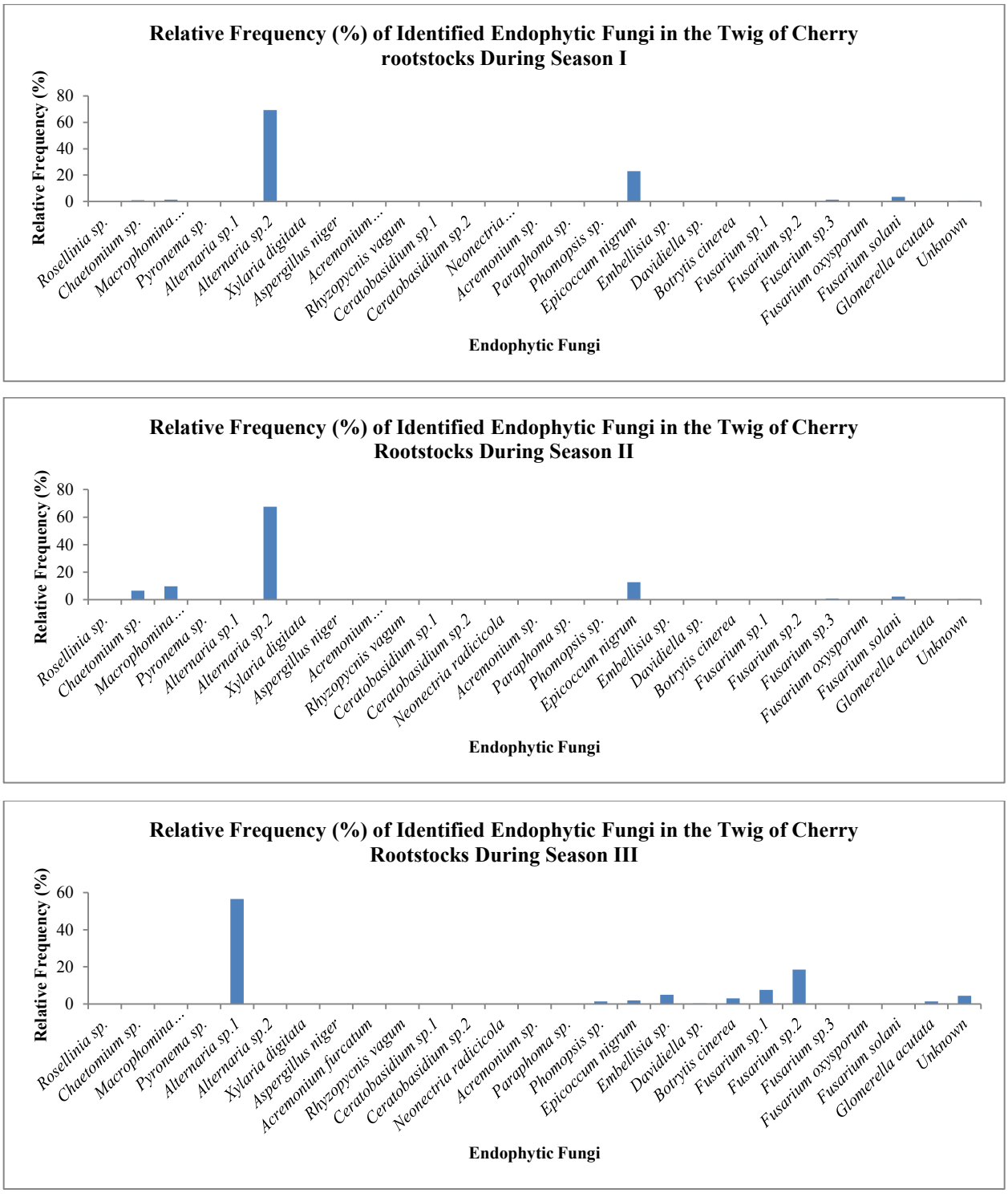



**Figure 4.10:** Overall relative frequency of endophytic fungi in the twig of cherry rootstocks in different sampling periods.

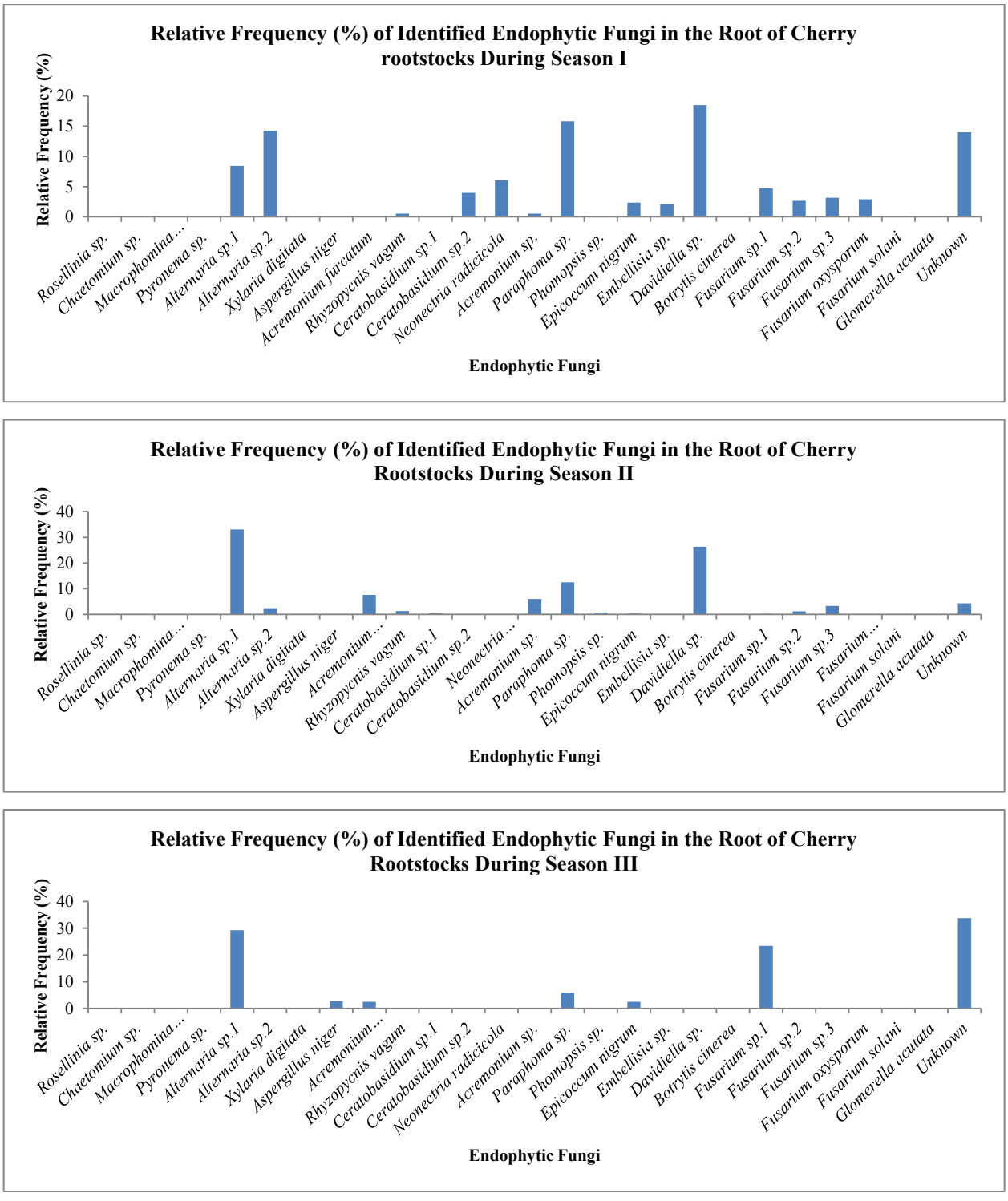



**Figure 4.11:** Overall relative frequency of endophytic fungi in the root of cherry rootstocks in different sampling periods.

### 4.5. Endophytic fungi associated with Capsicum annuum L.

The aim of this project was the identification of endophytic fungi living in pepper to find out whether significant cultivar dependent differences can be observed. We also investigated the effect of environment and cultivation conditions, therefore both cultivars, „Hó" and „Kárpia", were cultivated in greenhouse as well as in open field. Several students took part in the project, I was partly involved in starting the fungal cultures, but my main task was to prepare and establish single spore / single hyphae cultures from selected isolates to have well defined, stable cultures for DNA ITS-sequence based identification of individual fungal strains. Samples originated from 8 different organs and were collected in April, May, August and October. Cultivation and first morphological categorization of endophytic fungi was essentially the same as in case of sweet cherry. Single spore cultures were initiated from 118 isolates selected from the endophytes of samples collected in August and October. Methodology applied for identification of endophytic fungi associated with pepper was as the same as that was used for cherry.

I would like to summarize briefly the final results of our team, stating that the quantitative evaluation of the frequency of endophytic fungi was mainly done by Csaba Borbély and is described in his diploma thesis (Borbely, 2014). Andras Bärnkopf was mainly responsible for the ITS sequence based identification of single spore fungal isolates, detailed results were presented in his diploma thesis (Bärnkopf, 2013). The first question analyzed was whether colonization by endophytic filamentous microfungi arises during the vegetation period. The data are summarized in Figure 4.12 (taken von Borbely (2014) with permission) and clearly show that considerably more endophytes were isolated at the end of the vegetation period, i.e. in August and in October than in April and May, the difference between the frequencies in May and August or May and October are significant for both cultivars at $\alpha<0,1\%$. Although we isolated more endophytic fungi from the cultivar „Hó" than from „Kárpia" (Figure 4.12) at all sampling times, statistically significant difference ($\alpha<0,1\%$) was only found in October. When colonization rates of field-grown plants were compared to those in greenhouse, the former were always significantly higher for both cultivars (Borbely, 2014).

Fungal genera and species were identified on the basis of their ITS1+5.8S RNA+ITS2 sequences. 50 strains from our collection were identified in this way. As in sweet cherry, we observed that the potentially pathogenic *Alternaria* genus occurred most frequently in pepper, members of this genus were detected in every organ. Figure 4.13 shows the occurrence of

different morphotypes in the samples collected in October. In addition to *Alternaria* 4 further morphotypes were very common: *Cladosporium*, *Verticilium*, *Acremonium* and the yet unidentified group DB (Figure 4.13); (Bärnkopf, 2013); (Borbély, 2014). From the root we also isolated *Plectosphaerella*, *Colletotrichum*, *Paecilomyces*, *Penicillium* and *Fusarium* strains, while in the shoot *Cladosporium*, *Acremonium*, *Chaetomium* and *Lewia* strains were identified.

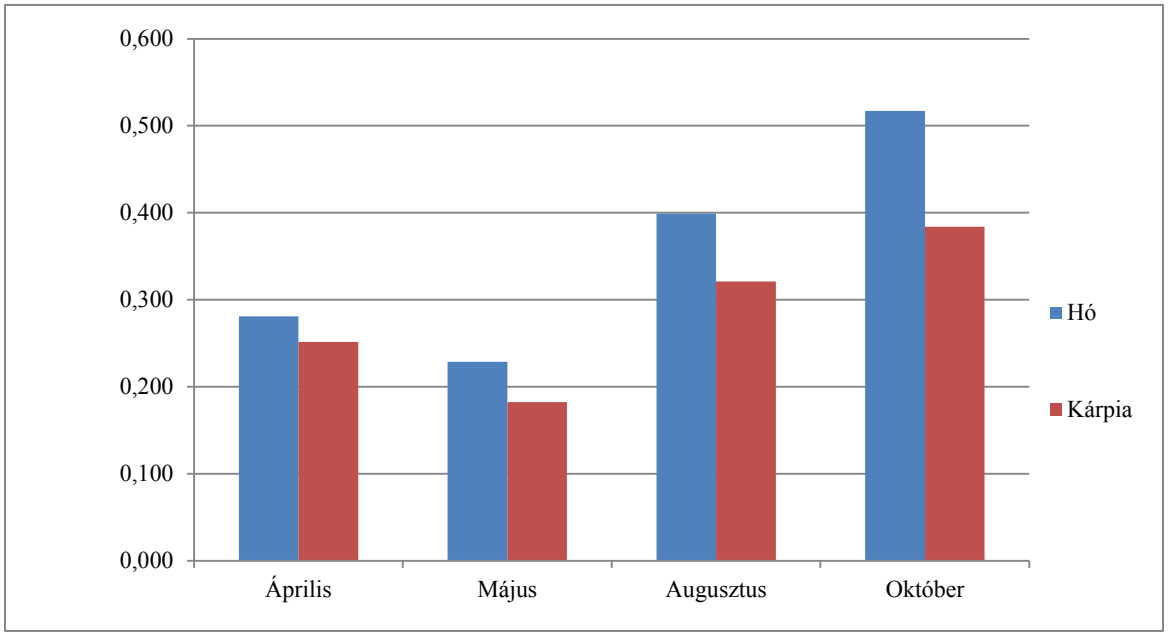



**Figure 4.12:** Change of colonization rates during the vegetation period. Colonization rates = colonized samples/all samples. The results demonstrate enhanced colonization towards the end of vegetation period, and higher colonization of the cultivar „Hó" compared to „Kárpia". (Figure from Borbely, 2014).

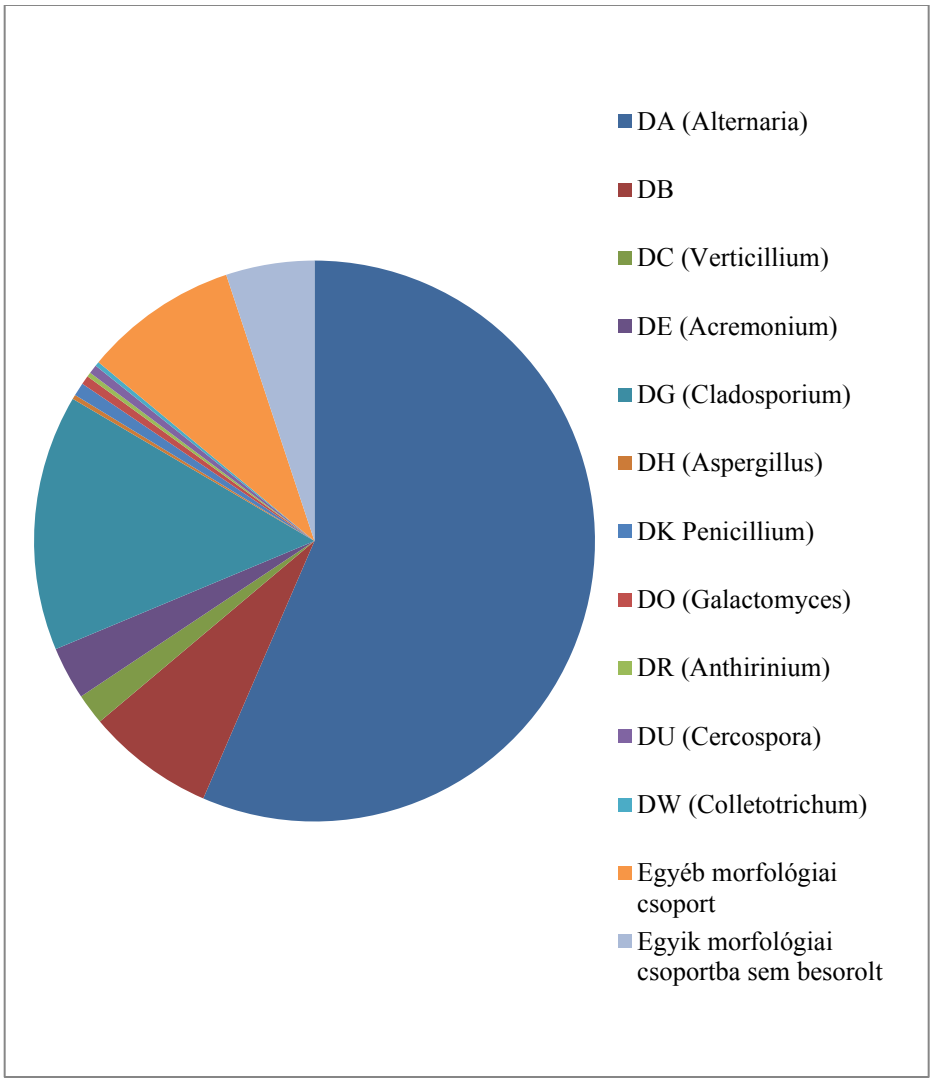



**Figure 4.13:** Colonization by different morphotypes in pepper in October. Of all endophytic fungi identified *Alternaria* is by far the most common, followed by *Cladosporium*. (From: Borbely, 2014)

Our results indicate that greenhouse-grown and field-grown peppers harbour different endomycota. We also observed differences between individual plant parts and seasonal difference in the samples of the same plant. Plant parts of the cultivar „Hó" usually showed higher colonisation rate by endophytic fungi than those of „Kárpia". As expected, colonization rates were higher in field-grown plants and in older organs. Highest colonisation frequency was found in old leaves and in stalks of fruits. Several strains belonging to the Alternaria genus were detected; members of this genus occurred in every plant part. From the root we also isolated Plectosphaerella, Colletotrichum, Paecilomyces, Penicillium, Rhizopycnis, Pyrenochaeta and Fusarium strains, while in the shoot Cladosporium, Acremonium, Chaetomium, Myrothecium, and Verticillium strains were identified. Young and older leaves showed Acremonium, Alternaria, Cercospora, Xylaria and Penicillium strains. Pericarp was colonized mainly with Alternaria, while Arthrinium, Galactomyces, Penicillium, Acremonium, Cladosporium, Chaetomium and Lewia were isolated from fruit stalks (Table 4.3).

### 4.6. The evolutionary relationship of identified fungal endophyte taxa

The evolutionary history was inferred using the UPGMA method (Sneath & Sokal, 1973). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1987) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1987). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 42 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 240 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2007). Figures 4.14 and 4.15 demonstrate the overall evolutionary relationship between isolated morphotaxa from cherry and peppr, repectively.

**Table 4.3:** Features of endophytic fungi morphotaxa isolated from pepper (*Capsicum annuum*)

| Number | Isolated fungi | Cultivar | Organ | Growing site |
|---|---|---|---|---|
| P4 | Alternaria sp. | Kárpia | leaf | greenhouse |
| P6 | Alternaria sp. | Kárpia | roots | greenhouse |
| P7 | Alternaria sp. | Kárpia | roots | greenhouse |
| P9 | Plectosphaerella cucumerina | Hó | roots | greenhouse |
| P10 | Paecilomyces sp. | Hó | roots | greenhouse |
| P13 | Alternaria sp. | Hó | roots | greenhouse |
| P20 | Penicillium adametzii | Kárpia | roots | greenhouse |
| P22 | Cladosporium sp. 1 | Hó | shoot | greenhouse |
| P28 | Xylaria sp. | Kárpia | young leaf | open field |
| P31 | Fusarium nematophilum | Kárpia | roots | open field |
| P33 | Lewia infectoria | Kárpia | pedicle | open field |
| P34 | Acremonium sp. | Kárpia | young leaf | greenhouse |
| P35 | Lewia infectoria | Kárpia | old leaf | greenhouse |
| P36 | Alternaria sp. | Kárpia | old leaf | greenhouse |
| P37 | Cladosporium sp. 2 | Kárpia | pedicle | greenhouse |
| P38 | Penicillium steckii | Kárpia | roots | greenhouse |
| P40 | Rhizopycnis vagum | Hó | roots | open field |
| P42 | Fusarium oxysporum | Hó | roots | open field |
| P43 | Colletotrichum aff. coccodes | Hó | roots | open field |
| P45 | Chaetomium globosum | Hó | pedicle | open field |
| P49 | Alternaria sp. | Hó | young pericarp | open field |
| P50 | Aspergillus sp. | Hó | young pericarp | open field |
| P54 | Cladosporium sphaerospermum | Hó | pedicle | greenhouse |
| P55 | Acremonium sp. | Hó | old leaf | greenhouse |
| P57 | Acremonium sp. | Hó | pedicle | greenhouse |
| P58 | Acremonium sp. | Hó | pericarp | greenhouse |
| P59 | Cladosporium sphaerospermum | Hó | old pericarp | greenhouse |
| P60 | Myrothecium sp. | Hó | shoot | greenhouse |
| P62 | Chaetomium sp. 2 | Hó | old pericarp | greenhouse |
| P63 | Acremonium sp. | Hó | shoot | greenhouse |
| P64 | Pyrenochaeta sp. | Kárpia | roots | open field |
| P76 | Penicillium olsonii | Hó | pedicle | greenhouse |
| P82 | Alternaria sp. | Kápia | young pericarp | greenhouse |
| P84 | Penicillium olsonii | Kárpia | old leaf | greenhouse |
| P86 | Verticillium dahliae | Kárpia | shoot | open field |
| P87 | Acremonium sp. | Kárpia | old leaf | greenhouse |
| P89 | Galactomyces geotrichum | Kárpia | pedicle | greenhouse |
| P90 | Cercospora sp. | Kárpia | old leaf | greenhouse |
| P93 | Arthrinium sp. | Kárpia | pedicle | open field |
| P95 | Alternaria sp. | Kárpia | young pericarp | open field |
| P96 | Alternaria sp. | Kárpia | old pericarp | open field |
| P97a | Alternaria sp. | Kárpia | old pericarp | open field |

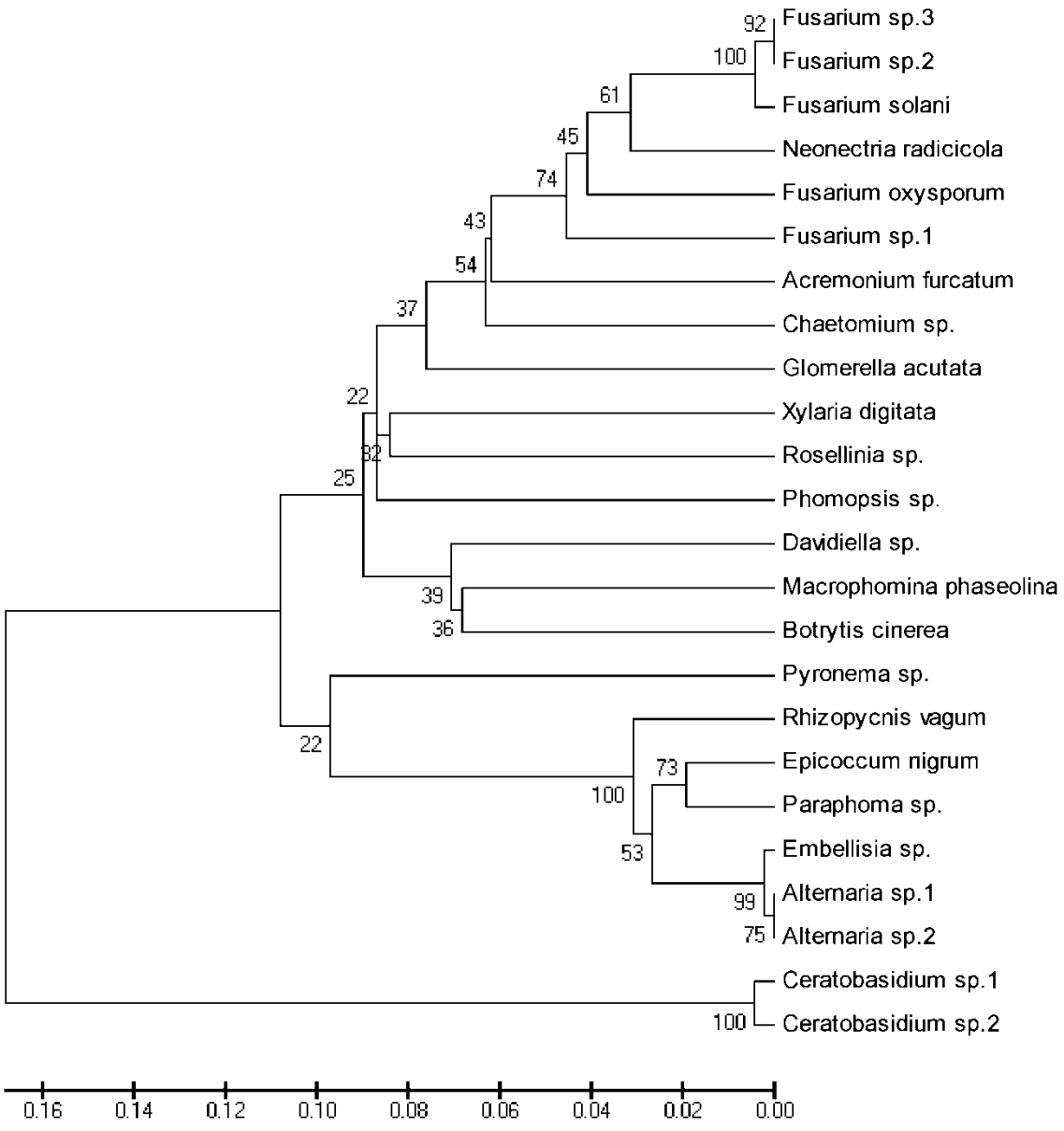



**Figure 4.14:** Phylogeny tree showing the revolutionary relationship between identified fungal endophytes from cherry.

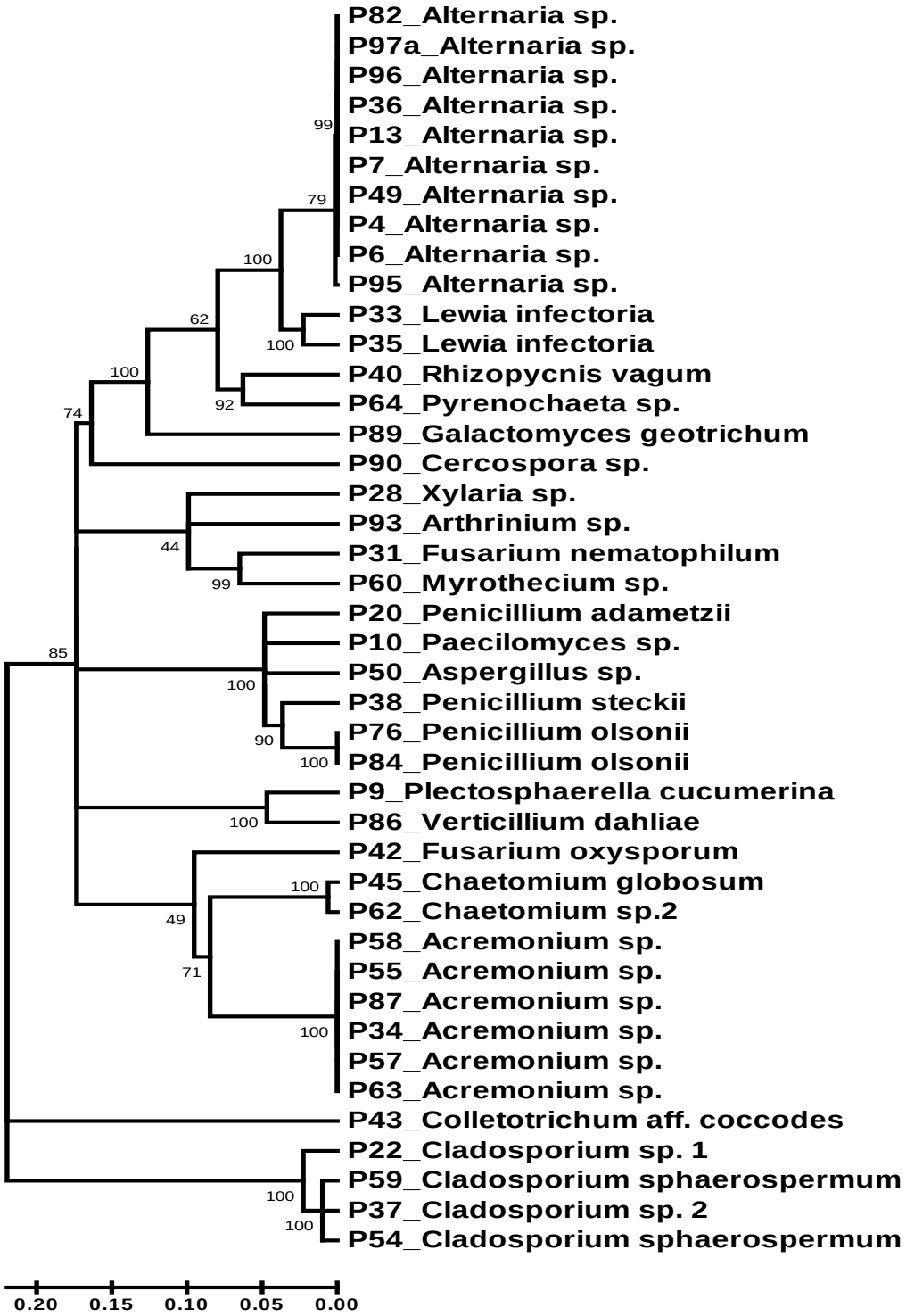



**Figure 4.15:** Phylogeny tree showing the revolutionary relationship between identified fungal endophytes from pepper.

### 4.7. Dual-culture of identified endophytic fungi with selected pathogens and potential antagonistic activities

According to the results, endophytic fungi communities isolated from cherry rootstocks included 27 different species. All identified taxa were subjected to a pilot study to investigate their potential antagonistic activity against two selected plant pathogens, *Agrobacterium tumefaciens* and *Monilia laxa* using dual-culture method on Malt yeast-Extract Agar (MYEA) and Potato-Dextrose Agar (PDA) media. Due to the aftermath of MYEA cultures, the pilot study yielded that examined fungi species have remarkably more vigorous growth on PDA. Thus this culture medium was chosen for further investigation about anti-pathogenic traits of isolated fungal endophytes.

Furthermore, amidst 27 taxa included in antagonistic activity test, 7 species showed competent colony expansion and development in dual-cultures with pathogens: *Botrytis cinerea*, *Alternaria* sp.1*, Rhizopycnis vagum, Epicoccum nigrum, Embellisia* sp.*, Fusarium oxysporum* and *Ceratobasidium* sp1. Therefore, recently mentioned endophytes were applied for dual-culture with pathogens and other species were omitted from the test. Each endophytic isolate was cultured separately with *Agrobacterium tumefaciens* and *Monilia laxa* in two different Petri dishes and single cultures of endophyte, *Monilia laxa*, and *Agrobacterium tumefaciens* were used as controls. All cultures were prepared in 6 replicates and radial growth of colonies was measured within 15 days.

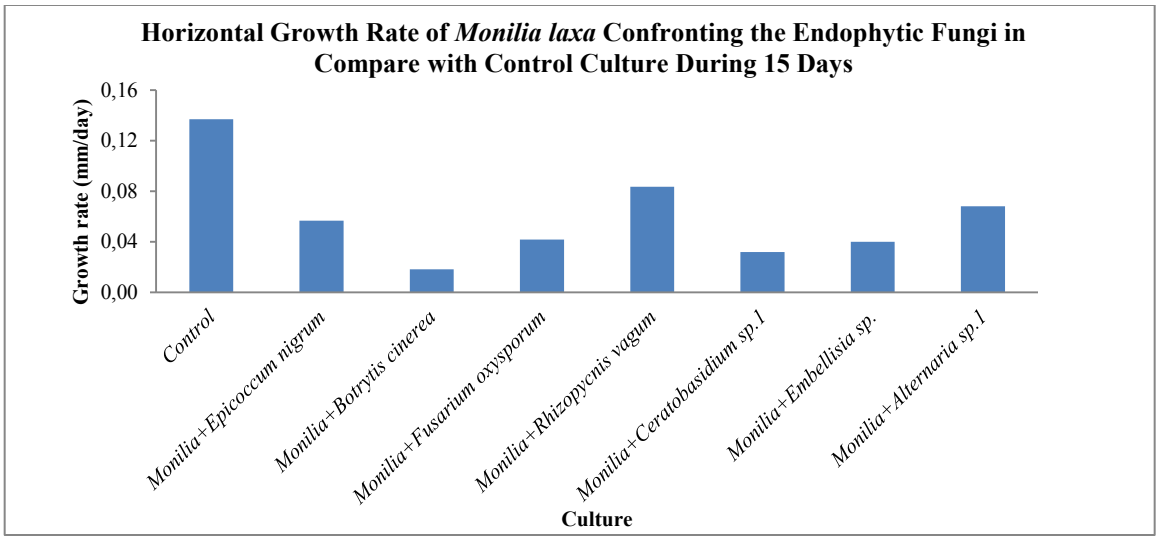
In control plates, *Monilia laxa* exhibited a competent growth rate on PDA with average vertical expansion as 0.2 mm/day, and 0.1 mm/day and 0.3 mm/day growth rate in diagonal and horizontal directions, respectively. *Agrobacterium tumefaciens*, however, had a relatively less vigorous growth rate on PDA in control plates where colonies had almost the same expansion towards each measured directions during 15 days (~0.07 mm/day). Details of radial growth measurments for all media cultures corresponding to antagonistic tests have been demonstrated in an Apendix section.

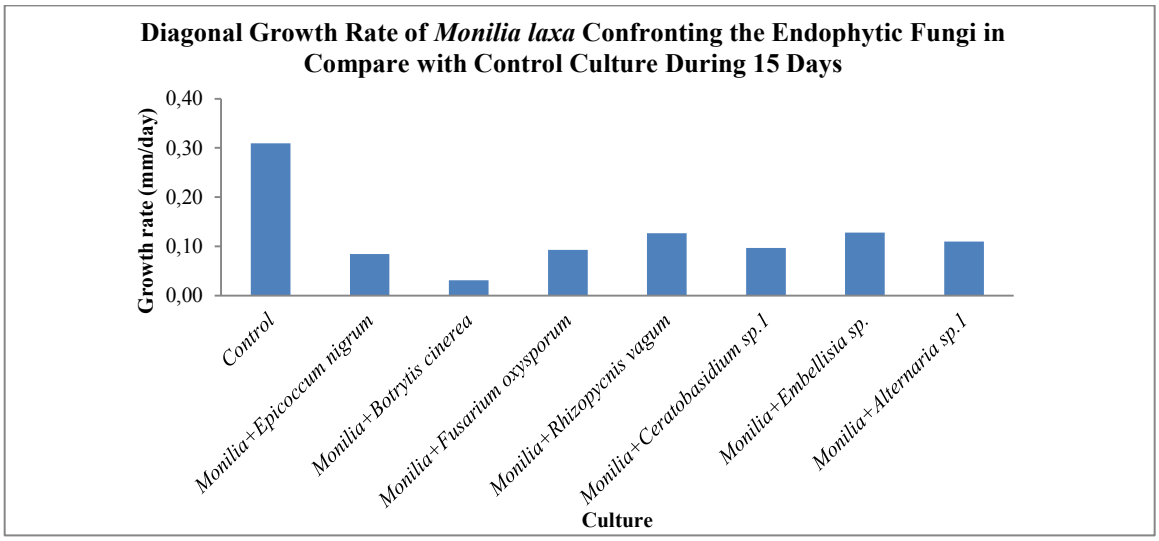
Primary evaluation of antagonistic effect between endophytes and the selected pathogens demonstrated the potential ability of the candidate fungi species to inhibit the radial growth of both *Monilia laxa* and *Agrobacterium tumefaciens* on PDA cultures. Inhibition of growth was assayed by calculating growth rate (GR) and growth inhibition (GI) indices and results were tested regarding the significance of the differences by a one-way ANOVA test. Endophytes

which were nominated for antagonistic test had the most vigorous growth on PDA, therefore colonies could last long enough to measure growth factor in dual-cultures with the pathogen. All values were defined as mean of measurements for six replicates of every dual-culture and single culture of pathogens and each endophyte were used as controls.

As a conclusion, a decline in growth rates of both *Monilia laxa* and *Agrobacterium tumefaciens* was observed in all dual-cultures after the incubation period (15 days), however neither endophytic fungus showed any defect in growth rate while confronting the pathogen in compare with control samples. According to the growth rate, *Monilia laxa* had comparatively less radial extension toward horizontal and diagonal dimensions but such a difference was not found in vertical growth of the pathogen (Figure 4.16). Expansion of *Agrobacterium tumefaciens* colonies was dominated in all measured dimensions while confronting the endophyte in dual-cultures (Figure 4.17).

Radial growth of colonies was applied to assess the growth inhibition value in dual-cultures. All measurements were recorded from day 0 to day 15, and results were compared. Accordingly, *Alternaria* sp.1 had the strongest antagonistic effect on *Monilia laxa* while *Fusarium oxysporum* showed a relatively feeble effect on this pathogen particularly against vertical extension of the colonies. In compare with other endophytes, *Fusarium oxysporum* had also weaker antagonistic effect on growth of *Agrobacterium tumefaciens* although other fungi showed almost the same growth inhibitory effect in dual-cultures with this pathogen (Figure 4.18 to Figure 4.24).



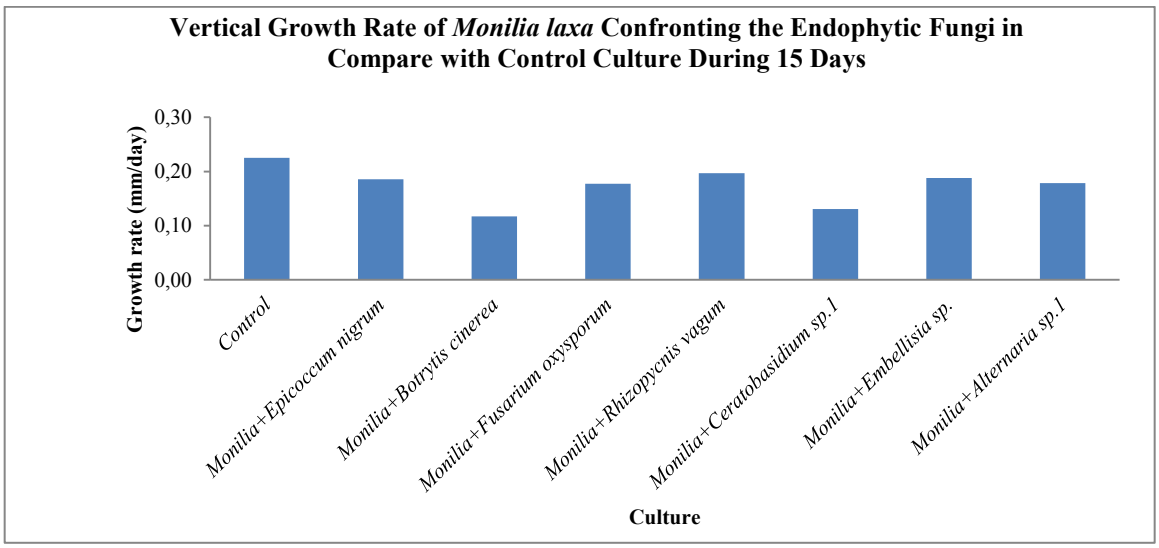
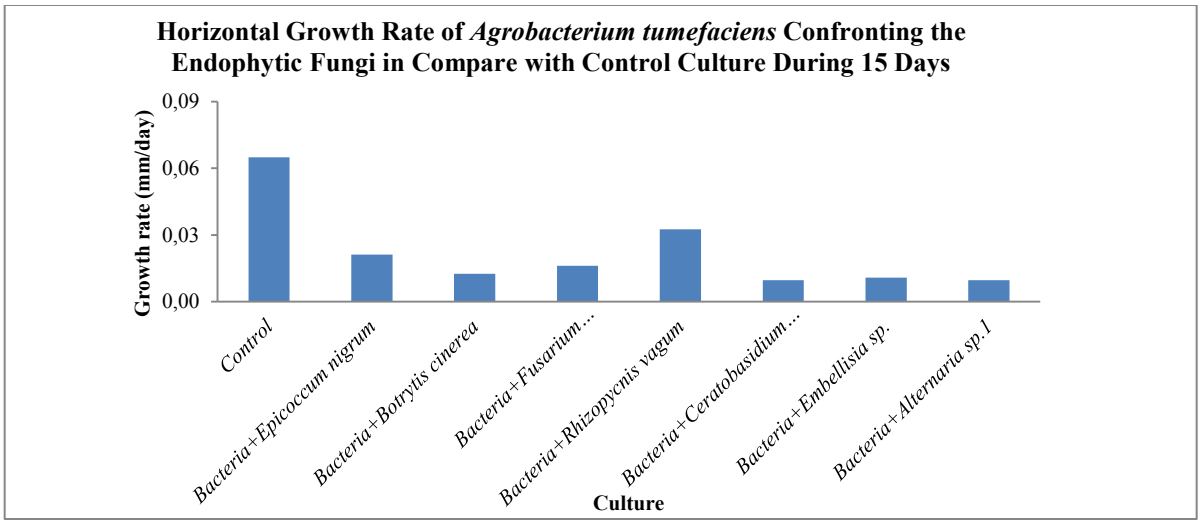
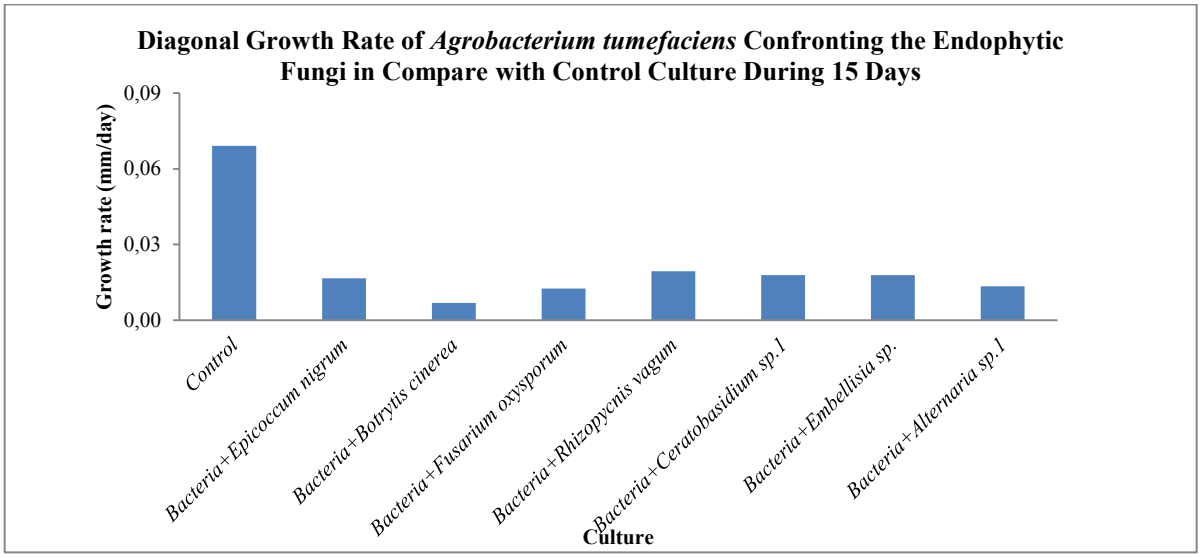




**Figure 4.16:** Growth rate of *Monilia laxa* colonies in dual-cultures with endophytic fungi.



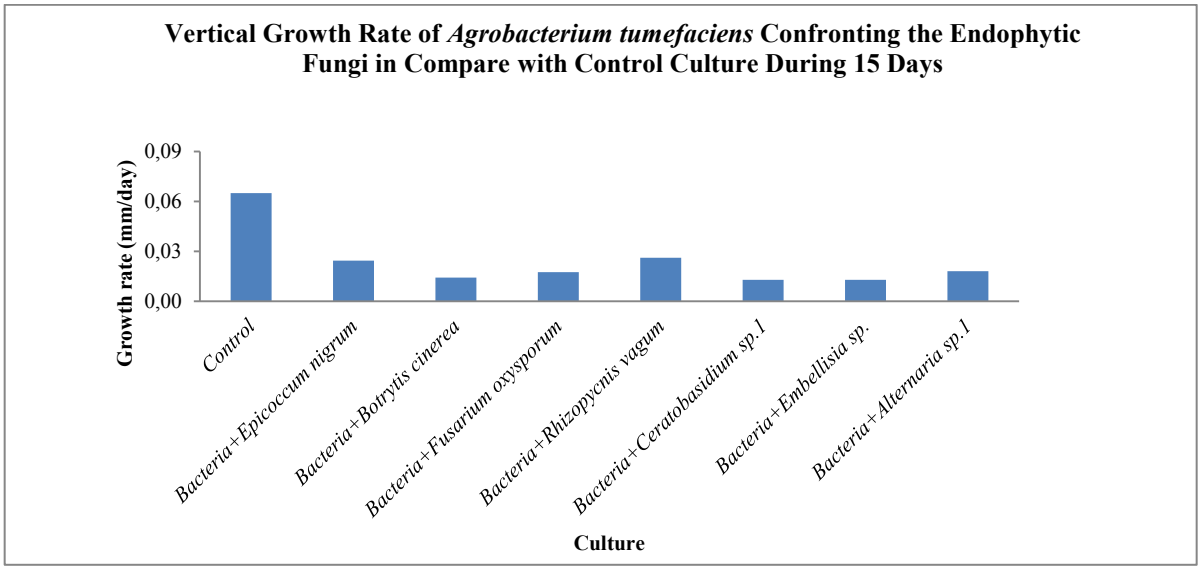
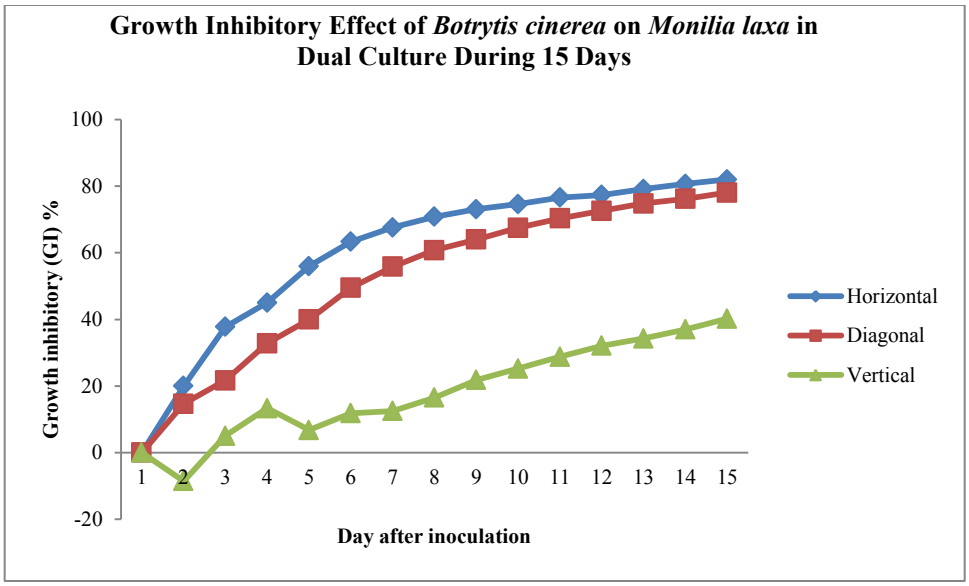




**Figure 4.17:** Growth rate of *Agrobacterium tumefaciens* in dual-cultures with endophytes.



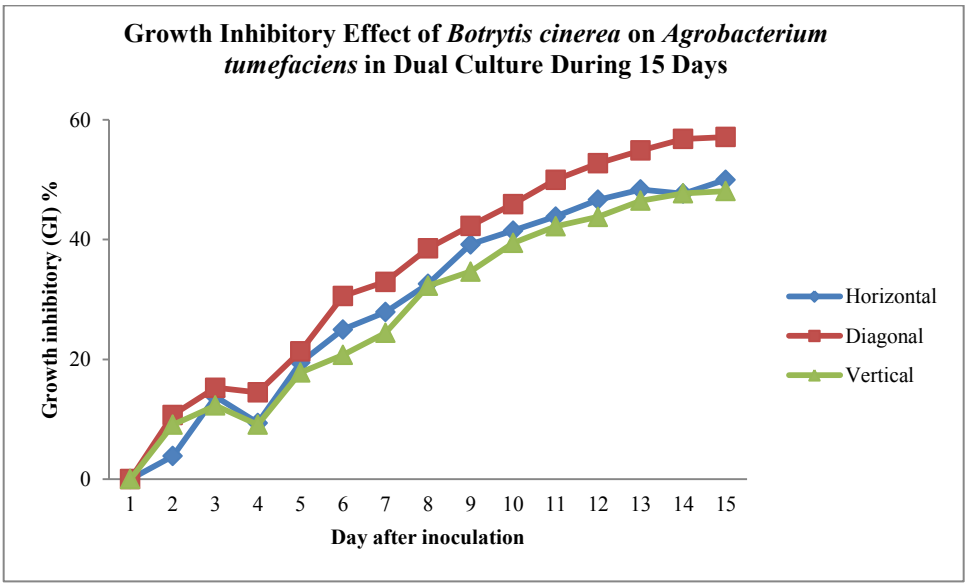
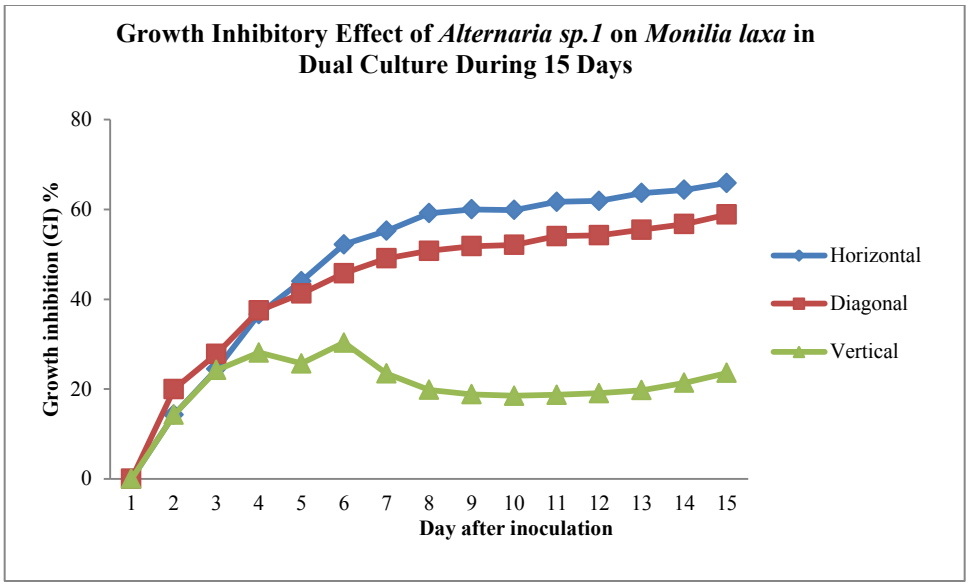




**Figure 4.18:** Growth inhibitory effect of *Botrytis cinerea* on selected pathogens in dual-cultures during 15 days.



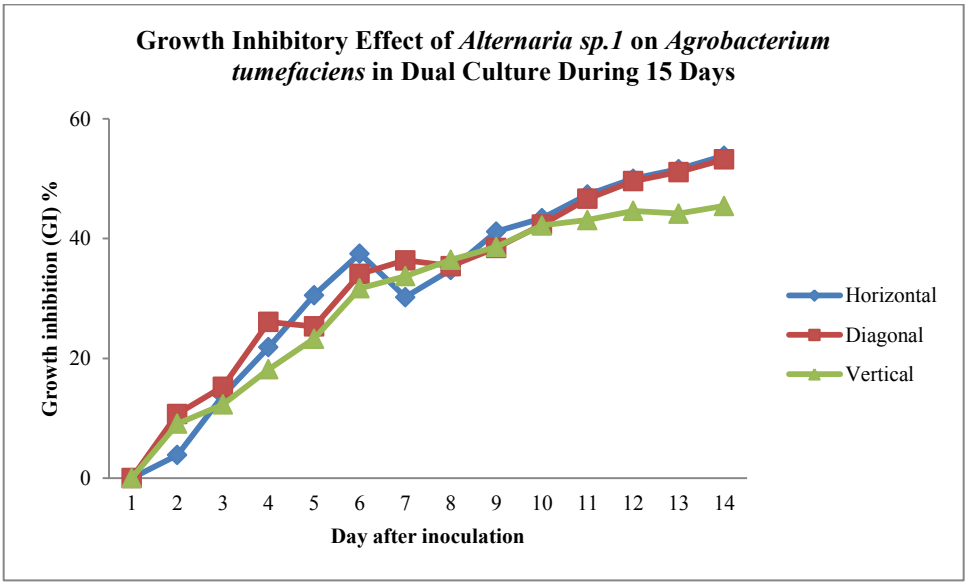




**Figure 4.19:** Growth inhibitory effect of *Alternaria* sp.1 on selected pathogens in dual-cultures during 15 days.

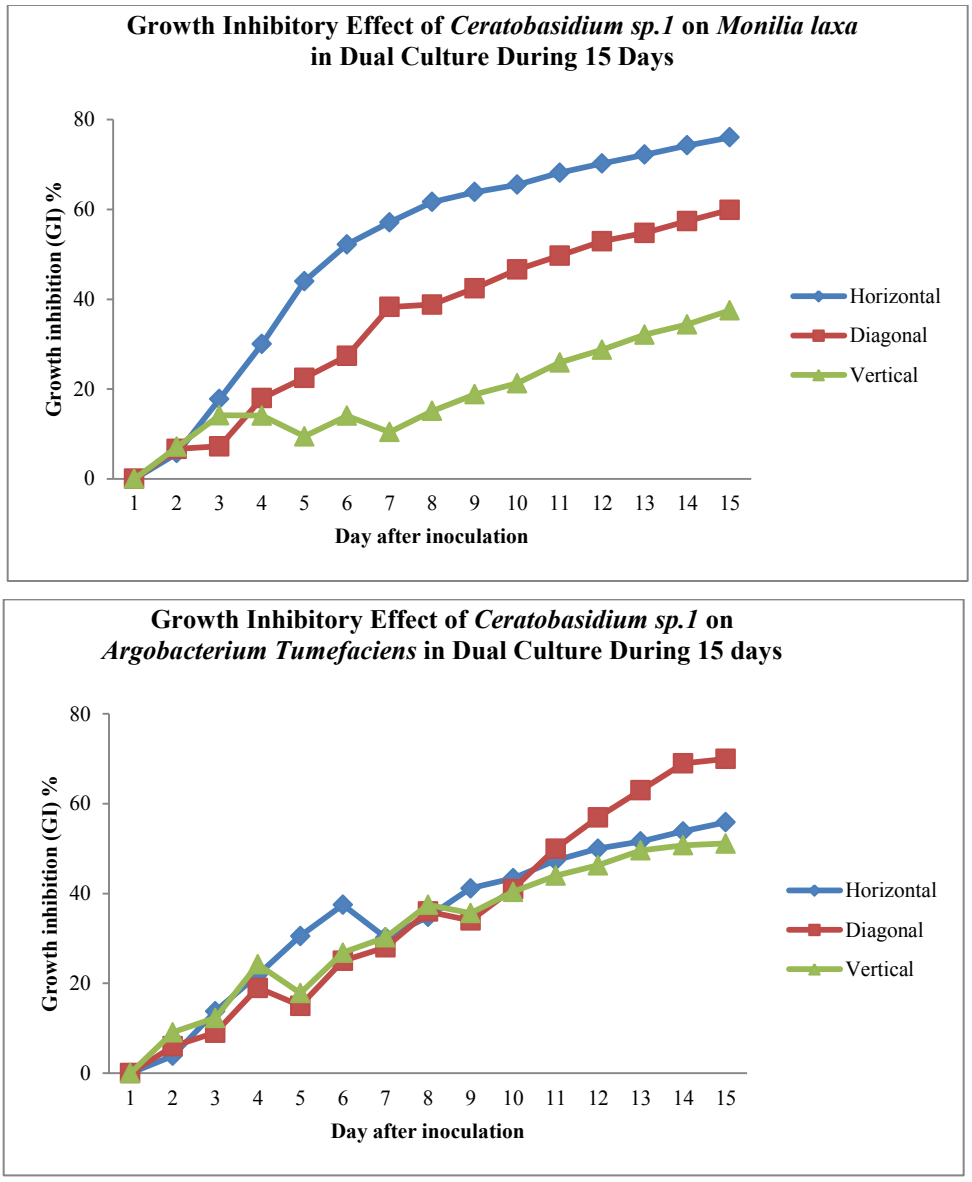
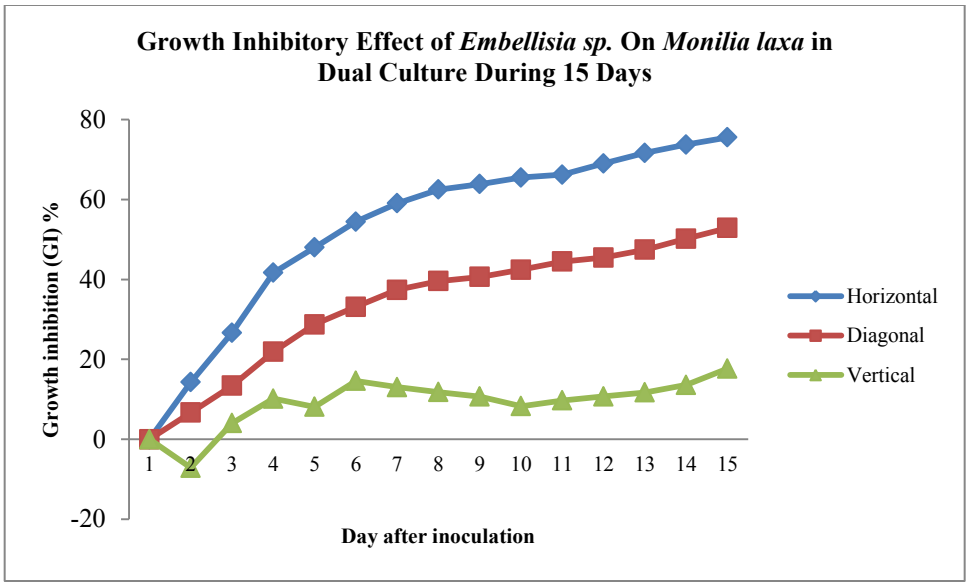



**Figure 4.20:** Growth inhibitory effect of *Ceratobasidium* sp.1 on selected pathogens in dual-cultures during 15 days.



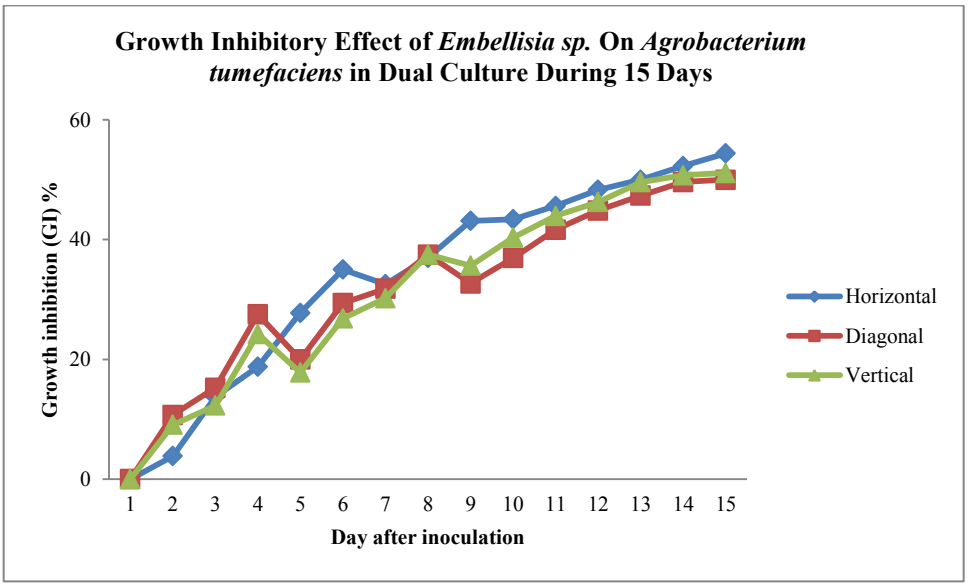
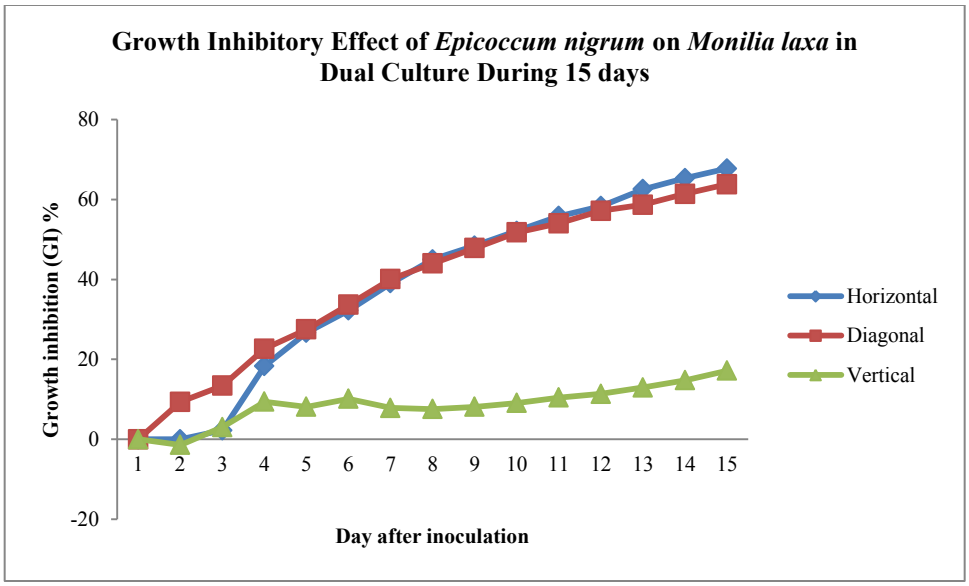




**Figure 4.21:** Growth inhibitory effect of *Embellisia* sp. on selected pathogens in dual-cultures during 15 days.



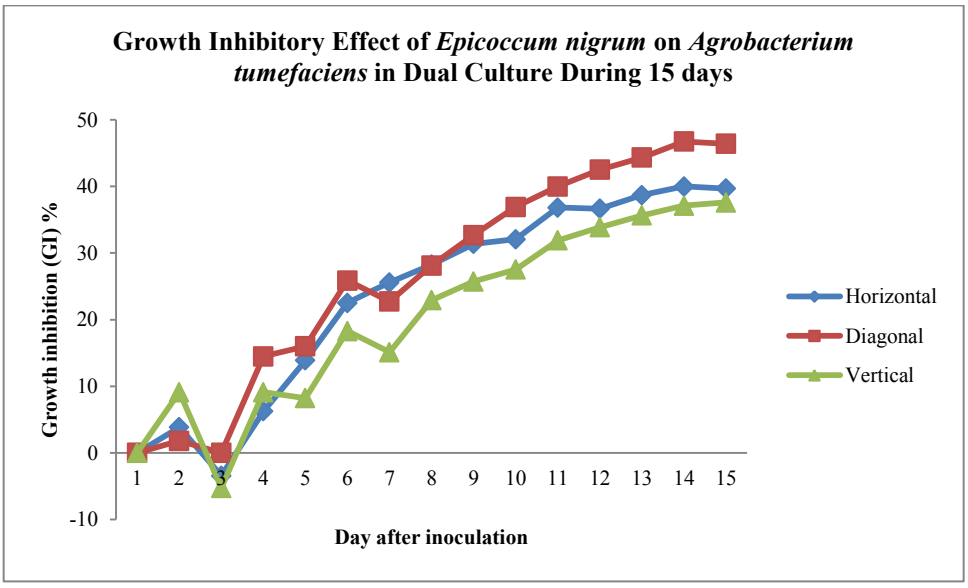
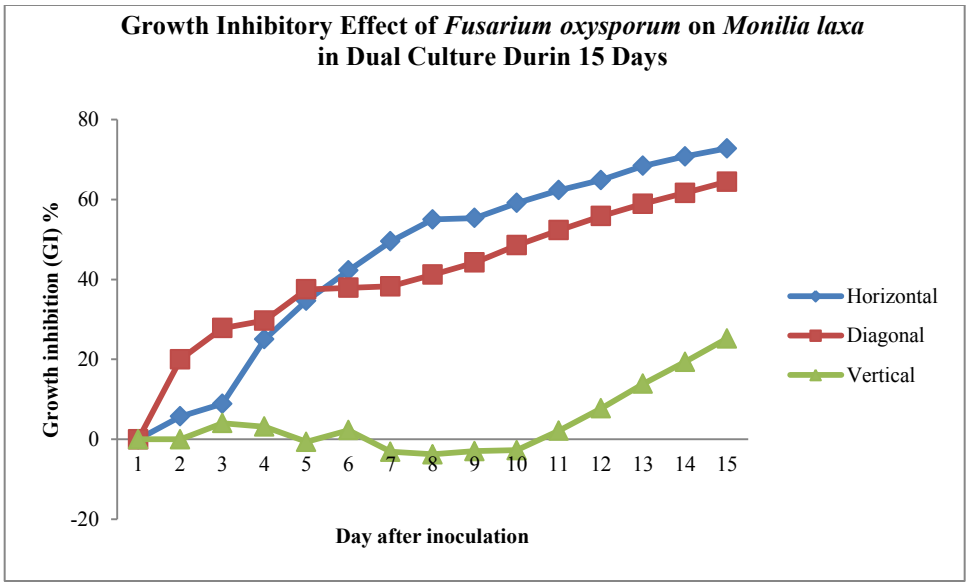




**Figure 4.22:** Growth inhibitory effect of *Epicoccum nigrum* on selected pathogens in dual-cultures during 15 days.



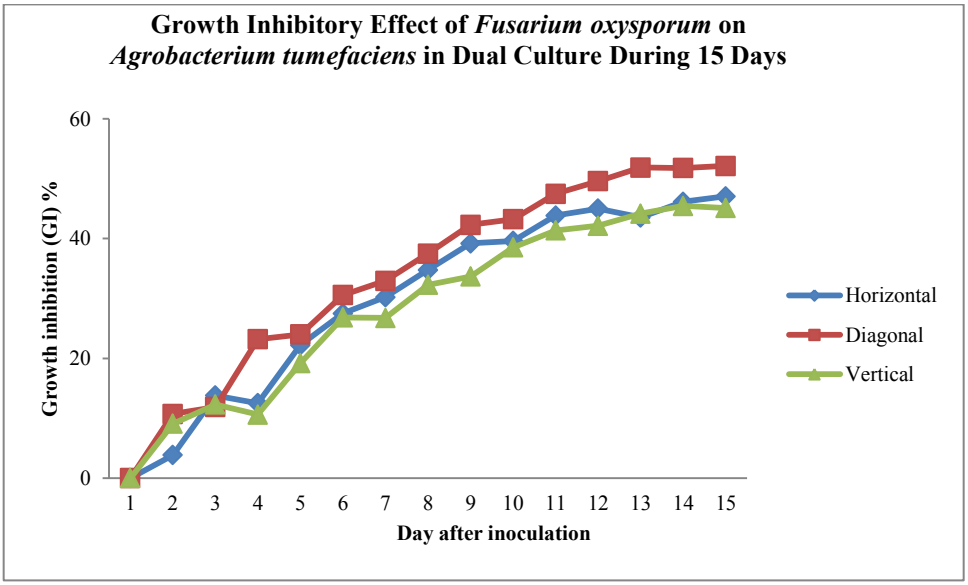
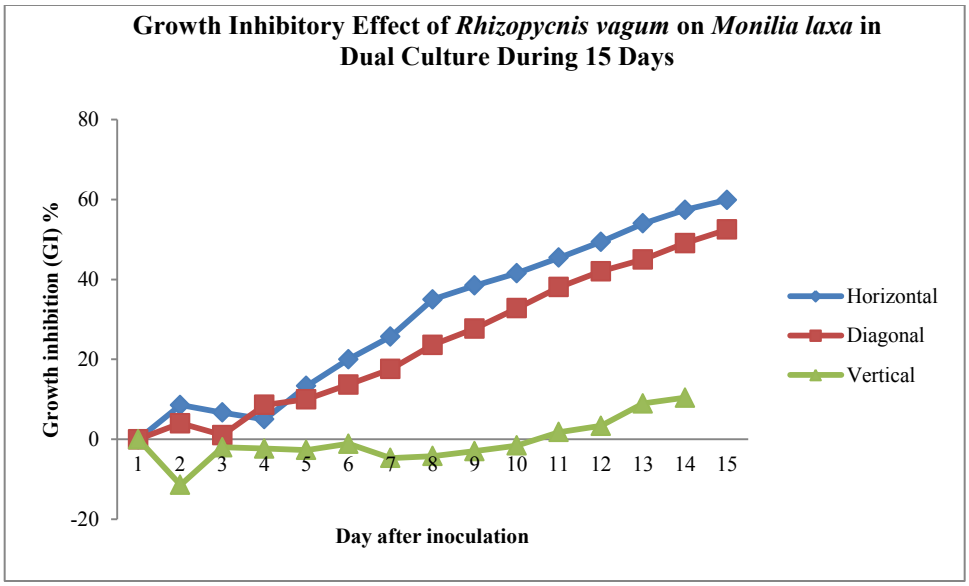




**Figure 4.23:** Growth inhibitory effect of *Fusarium oxysporum* on selected pathogens in dual-cultures during 15 days.



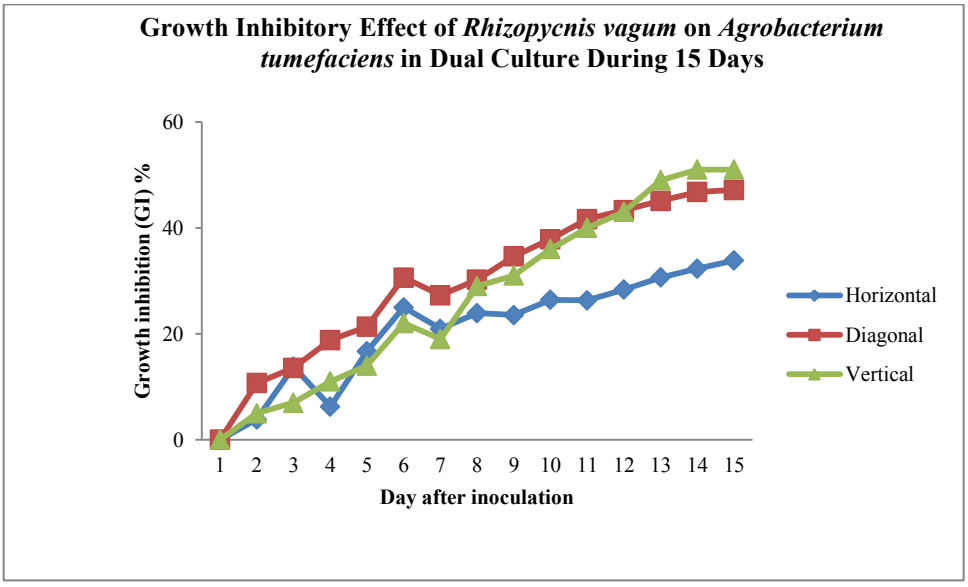




**Figure 4.24:** Growth inhibitory effect of *Rhizopycnis vagum* on selected pathogens in dual-cultures during 15 days.

Analytical data confirmed the primary results derived from *in vitro* assays about growth inhibitory effects of nominated endophytic fungi on selected pathogens. The mean values of all measurements were taken into account for calculating the variance (mean square) of radial growth of an individual colony on PDA in three dimensions (horizontal, diagonal, and vertical). Achieved data was applied to examine the significance of alterations in radial growth of every colony on PDA during the incubation time (15 days) in single cultures (control) and in dual-cultures where endophyte and pathogen confronted.

Collectively, *Monilia laxa* appeared more competent in dual-cultures with endophytes in compare with *Agrobacterium tumefaciens*. In general, restriction in colony growth of *Monilia laxa* was significant (P=0) in horizontal and diagonal directions but no difference was found in vertical growth of the pathogen colonies in deal with endophyte in dual-cultures. On the other hand, comparative analysis revealed that differences between endophytic fungi regarding their capability to inhibit horizontal growth of *Monilia laxa* was significantly higher than that for diagonal growth of the pathogen. Changes in horizontal growth of this pathogen ranged from relatively weak when cultured with *Rhizopycnis vagum* (mean±variance=1.39±0.17 mm) to the most regressed growth in dual-culture with *Botrytis cinerea* (mean±variance=0.68±0.01 mm) on PDA. The inequality in growth inhibition effect between nominated endophytes was less significant concerning their ability to diminish diagonal growth of *Monilia laxa* colonies. Although *Botrytis cinerea* showed a relatively strong inhibitory effect on diagonal growth (mean±variance=0.92±0.03 mm) of the pathogen, differences between other endophytes was not significant. No significant difference was found between endophytic fungi regarding their capability to inhibit vertical growth of *Monilia laxa* on PDA (Table 4.4) (Figure 4.25).

All examined endophytes had significant inhibitory effect on *Agrobacterium tumefaciens* growth on PDA with no significant difference toward three dimensions. Nonetheless, the outcome of inhibition was found different between endophyte taxa. According to the results, horizontal growth of the pathogen had the maximum regression while confronted *Ceratobasidium* sp.1, *Embellisia* sp., and *Alternaria* sp.1 on the media and the mean value of horizontal regression of the pathogen colonies was equal in dual-cultures with mentioned fungi (mean± variance= 0.56± 0.003 mm). In contrast, *Rhizopycnis vagum* had the weakest effect on horizontal extension of the pathogen (mean±variance=0.71±0.022 mm). As well, this fungus had relatively weaker effect on diagonal (mean±variance=0.66±0.006 mm) and vertical

(mean±variance=0.71±0.01 mm) growth of *Agrobacterium tumefaciens* in compare with other fungi, however variance of radial growth inhibition toward diagonal and vertical dimensions had no significant difference between dual-cultures of this pathogen with selected endophytic fungi (Table 4.5) (Figure 4.26).

**Table 4.4**: Two-way ANOVA test results of the average growth inhibition effect studied by dual-culture of tested endophytic fungi with *Monilia laxa*.

| Radial Growth | Variance | Sum Square | Df* | Mean Square | Mean Square Between** (F) | P value |
|---|---|---|---|---|---|---|
| Horizontal | Within | 27.671 | 112 | 0.247 | 16.235 | 0.000 |
| | Between | 28.109 | 7 | 4.016 | | |
| **Total** | | **55.78** | **119** | **-** | | |
| Diagonal | Within | 47.909 | 112 | 0.428 | 8.158 | 0.000 |
| | Between | 24.428 | 7 | 3.49 | | |
| **Total** | | **72.337** | **119** | **-** | | |
| Vertical | Within | 72.657 | 112 | 0.649 | 0.942 | 0.477 |
| | Between | 4.277 | 7 | 0.611 | | |
| **Total** | | **76.934** | **119** | **-** | | |

* df- Degree of freedom

** Mean Square Between shows the mean square (variance) of inhibition effect between different dimensions. F≥1 when the test is significant.

**Table 4.5**: Two-way ANOVA test results of average growth inhibition effect studied by dual-culture of tested endophytic fungi with *Agrobacterium tumefaciens*.

| Radial Growth | Variance | Sum Square | Df* | Mean Square | Mean Square Between** (F) | P value |
|---|---|---|---|---|---|---|
| Horizontal | Within | 1.853 | 112 | 0.017 | 13.415 | 0.000 |
| | Between | 1.554 | 7 | 0.222 | | |
| **Total** | | **3.407** | **119** | **-** | | |
| Diagonal | Within | 1.867 | 112 | 0.017 | 14.894 | 0.000 |
| | Between | 1.738 | 7 | 0.248 | | |
| **Total** | | **3.606** | **119** | **-** | | |
| Vertical | Within | 1.942 | 112 | 0.017 | 11.246 | 0.000 |
| | Between | 1.365 | 7 | 0.195 | | |
| **Total** | | **3.307** | **119** | **-** | | |

* df- Degree of freedom

** Mean Square Between shows the mean square (variance) of inhibition effect between different dimensions. F≥1 when the test is significant.

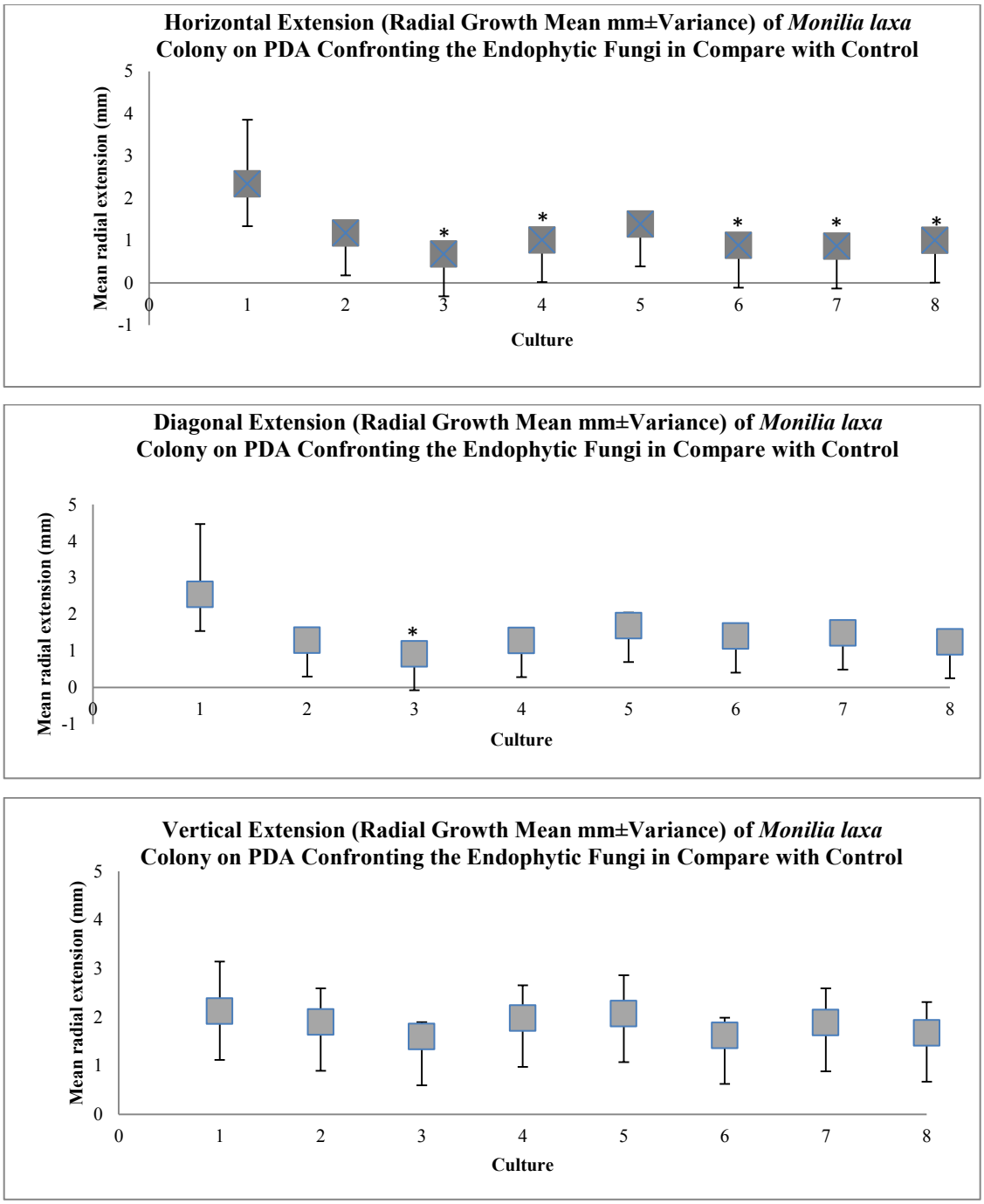



**Figure 4.25:** Radial growth (mean mm± variance) of *Monilia laxa* colonies in 1: single culture (control), and dual-cultures with 2: *Epicoccum nigrum*, 3: *Botrytis cinerea*, 4: *Fusarium oxysporum*, 5: *Rhizopycnis vagum*, 6: *Ceratobasidium* sp.1, 7: *Embellisia* sp., and 8: *Alternaria* sp.1, has been demonstrated in this Figure. Significance of the radial growth change has been indicated by (*). Bars show the variance of the radial growth of the pathogen in each dual-culture.

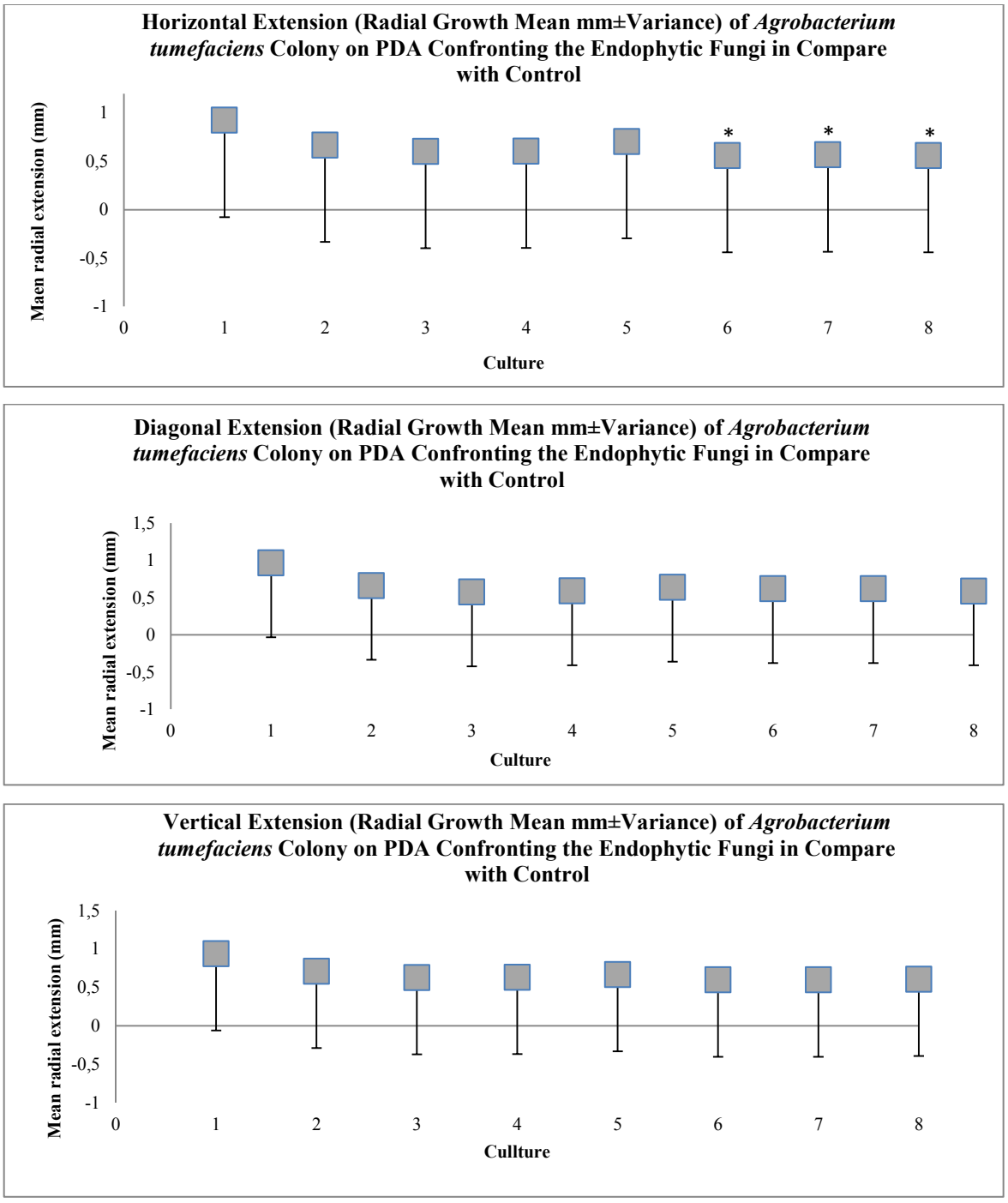



**Figure 4.26:** Radial growth (mean mm±variance) of *Agrobacterium tumefaciens* colonies in 1: single culture (control), and dual-cultures with 2: *Epicoccum nigrum*, 3: *Botrytis cinerea*, 4: *Fusarium oxysporum*, 5: *Rhizopycnis vagum*, 6: *Ceratobasidium* sp.1, 7: *Embellisia* sp., and 8: *Alternaria* sp.1, has been demonstrated in this Figure. Significance of the radial growth change has been indicated by (*). Bars show the variance of the radial growth of the pathogen in each dual-culture.

As a sample demonstration figures 4.27 and 4.28 display growth inhibitory effect of *Epicoccum nigrum* on examined pathogens on PDA medium.

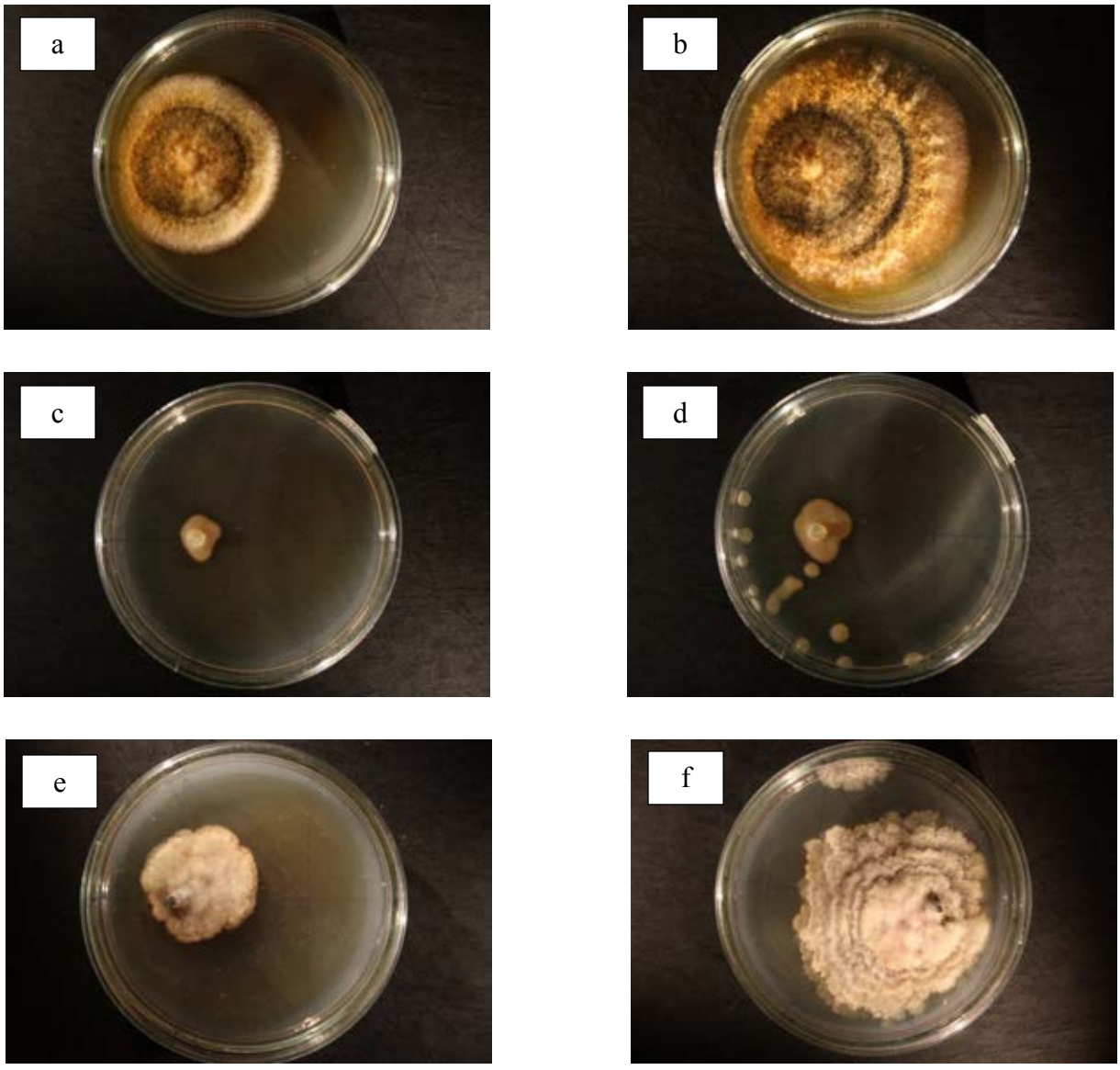



**Figure 4.27:** Single- culture of *Epicoccum nigrum* **a)** after 8 days and **b)** after 15 days, *Agrobacterium tumefaciens* **c)** after 8 days and **d)** after 15 days, and *Monilia laxa* **e)** after 8 days and **f)** after 15 days on PDA.

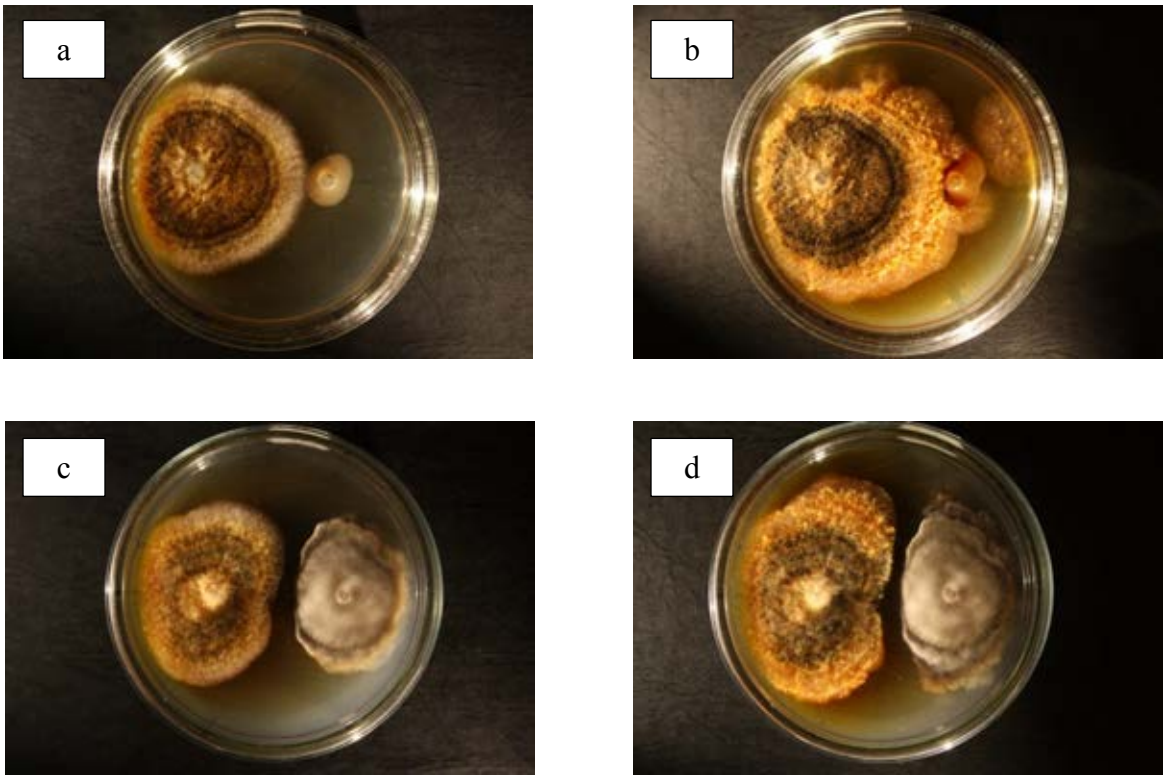



**Figure 4.28:** Dual- culture of *Epicoccum nigrum* vs *Agrobacterium tumefaciens* **a)** after 8 days and **b)** after 15 days, and *Epicoccum nigrum* vs *Monilia laxa* **c)** after 8 days and **d)** after 15 days on PDA.

### 4.8. Novel scientific achievements by the present study

Based on the assigned goals and analytical approaches of the present study, our findings were entirely unique as there has been no study as comprehensive and descriptive as ours on the biodiversity of fungal endophytes associated with the examined hosts in Hungary as well as in the Central Europe yet. The most important highlights of our achieved data are listed as follow:

1. The present study for the first time showed the phenotypic and phylogenic interrelations of endophytic fungi assemblages grafted on *Prunus Sp.*

2. Biodiversity of isolated endophytes was analyzed on different rootstocks of *Prunus Sp.* By application of statistical indices such as Simpson‟s diversity index and Shannon-Weaver index which could reliably demonstrate the host-dependent features of identified fungi colonized these species.

3. Results of the present Study were also analyzed according to the tendency of isolated fungal endophytes to colonize a particular anatomical compartment in order to represent the tissue-specific traits of these symbionts on the examined host species.

4. Based on the sampling procedures, we were also able to assess the impact of temporal changes on the biodiversity of isolated endophytic fungi associated with the examined host species.

5. All the above mentioned analyses were cumulatively considered to indicate the significant variations in host-specific and tissue-specific characteristics of isolated fungal endophytes according to the temporal changes in the sampling area.

6. We also examined the impact of harboring tissue, the host species and cultivation conditions on fungal endophytes assemblages associated with *Capsicum annuum* L.

7. Results of this study for the first time showed the antagonistic effects of fungal symbionts colonizing cherry rootstocks on growth of potential pathogens in vitro. As efficiently applied criteria, the growth rate toward three dimensions and the inhibitory effect of fungus-pathogen dual cultures were defined for all assays and the results were comprehensively discussed.

## 5. Discussion

The present study provided valuable data regarding biodiversity and impact of determinant factors on composition of fungal endophyte communities that attribute symbiotic micro-flora of widely used cherry rootstocks in the region. It has been demonstrated that endophytic fungi can profoundly influence different aspects of plant pathophysiology and actively participate bio-mechanisms by which plant growth and regeneration is controlled. Thus understanding the structure of endophyte assemblages is required for further approaches in order to improve the agro-economic status of the fruit production and to find novel applications of these microorganisms and their metabolites in horticultural, or even medicinal, studies. Results achieved by the present study were applied for an analytical assessment to explicate the composition of endophytic fungi assemblages on sweet cherry and pepper in Hungary. Moreover, the antagonistic activity of isolated endophytes with vigorous growth on PDA was assessed *in vitro* against to common plant pathogens. Data derived from such a study could be useful for further investigation about the effect of these symbionts in pathophysiology of the host and their contribution in host resistance against pathogens.

Biodiversity of endophytic fungi associated with host plant and structure of endophyte communities are dynamically convertible depending on plant physiology, environmental stresses, their interplay with other parasitic or pathogenic microorganisms and bio-geographical factors (Arnold *et al*., 2007). Endophytes also tend to be host-specific that can change the prevalence of endophytic taxa in a particular plant genus between different species. As well, divergent endophyte composition may be harbored in host organs due to the histological differences and availability of nutrients by which endophyte colonization is conducted in a tissue-specific manner within a distinctive host species (Arnold *et al*., 2007). Along with temporal changes in the niche where both host plant and endophyte inhabit, host species and harboring tissue are consequently the most important factors modulating endophyte diversity (Arnold & Herre, 2003) (Arnold *et al*., 2007). Therefore, the present study was conducted with defined methodology so that could be applied to obtain such data and infer a comprehensive view about composition of fungal endophyte communities associated with sweet cherry and pepper.

### 5.1. Characterization of endophytic fungi isolated from cherry rootstocks

As a sum, 26 morpho-taxa were classified among a total of 6587 isolated colonies from cherry rootstocks. Some species primarily identified based on the morphology were later subsumed with a different species name due to their phylogeny. Moreover, fungi with unique ITS sequence which did not have acceptable resemblance to existed data, were identified as unknown.

Although differences were observed in composition of endophyte assemblages between host individuals, terrestrial and spatial accumulation of the endophytes and their occurrence due to the temporal changes, an overall view could be inferred to characterize genera that orchestrate the endophytic fungi populations associated with cherry rootstocks. The majority of identified species (24 species) were from the phylum *Ascomycota*. Only two species of the phylum *Basidiomycota* were isolated from cherry rootstocks. From *Ascomycota*, the most diverse species belonged to *Sordariomycetes* while *Dothideomycetes* were the most frequent fungi isolated from cherry rootstocks. *Sordariomycetes* were mostly *Hypocreales* (5 species), but this class also included *Phyllachorales* (1 species), *Sordariales* (1 species), *Diaporthales* (1 species), and *Xylariales* (2 species). The most abundant fungi (*Alternaria sp.1* and *Alternaria sp.2*) (Paleosporales) along with other less frequent fungi: *Davidiella sp.* (Capnodiales), *Macrophomina phaseolina* ( Botryosphaeriales), *Epicoccum nigrum* and *Rhizopycnis vagum* belonged to class *Dothideomycetes,* however taxonomy of the last two species is not thoroughly assigned yet (Incertae sedis). Such a structure of endophytic fungi assemblages is identical to formerly suggested endophytic association with woody plants and implies the potential contribution of class 2 endophytes in symbiotic micro-flora of cherry rootstocks (Faeth & Fagan, 2002). *Basidiomycota* are reported to have rare evenness in plants. According to the results, two species of Agaricomycetes (*Ceratobasidium sp.1* and *Ceratobasidium sp.2*) were the less abundant fungi identified on examined cherry rootstocks (Faeth & Sullivan, 2003).

Class 3 endophytes are usually considered as highly diverse fungi that are harbored in above ground tissues of the host. Former studies have suggested the community of class 3 endophyte mostly comprises *Agaricomycetes*, and with less frequency may contain *Eurotiomycetes* and Pezizomycetes (Faeth & Sullivan, 2003). From *Eurotiomycetes*, *Aspergillus niger* (Eurotiales) was identified on cherry rootstocks, whereas had occurrence only in root samples. A species belonged to Pezizomycetes (*Pyronema sp.*) was also identified only in leaf with comparatively

low frequency. *Botrytis cinerea* was the only genus of *Leotiomycetes* isolated from cherry rootstocks and was only observed in twig.

In general, class 2 endophytes are recognized by their broad host range and horizontal transmission in hosts. Nonetheless, self-experience on isolation of fungi from canola seed and other studies on different plant species show that air-born fungi may colonize the seed (vertical transmission) and thus may maintain transmission both within and between plant generations (Faeth & Sullivan, 2003). Moreover, these endophytes are thought to enhance the host tolerance against drought, temperature and salt and may help the host to resist against pathogens. the greatest proportion of fungal metabolites which have industrial or medicinal applications and are currently isolated and used in biological control, have been found in class 2 endophytes (Faeth & Sullivan, 2003); (Berdy, 2005). Feature of endophytic fungi assemblages on cherry rootstocks, as the results showed, highly resembles the association of class 2 and class 3 endophytes that indicates the transmission mode of the fungi and their impact on biotic and abiotic tolerance of the host. Nonetheless these endophytes also possess another trait which could be taken into account to realistically conclude their relationship with host plants. Either class 2 or class 3 endophytic fungi may confer benefits to the host in a habitat-adapted manner, a characteristic that make the host- endophyte interaction fluctuate in a range of symbiosis to parasitism and they may also turn to pathogens under particular circumstances (Aly *et al.*, 2011); (Faeth & Sullivan, 2003).

To characterize the nature of host plant-endophyte relationship, it is essential to determine potential pathogenicity of these symbiotic inhabitants and their role in plant diseases. Nearly all class 2, as well as class 3 endophytes were primarily identified as plant pathogens. *Alternaria sp.*, as the most frequent endophytic genus found in cherry rootstocks, is known as a major plant pathogen with a widespread distribution; however study has documented the endophytic life style of this genus in different plants. *Embellisia sp.*, has been shown to induce bulb canker in garlic and causes formation of black soot on alliums (Faeth & Fagan, 2002). *Neonectria radicicola* is reported to cause ginseng root rot (Hamilton *et al*., 2010) but this species has been also isolated as an endophyte harbored in roots of *Pinus sylvestris* (Hamilton *et al*., 2009). *Rhizopycnis vagum*, is a recently described coelomycetous fungus that contributes to vine decline of muskmelone in Central and North America, although this species has been isolated from healthy tissues of cacao (*Theobroma cacao L*.) in Brazil (Meijer & Leuchtmann, 2001).

*Macrophomina phaseolina* is recognized as an opportunistic fungus that may transform from a saprophyte to a pathogen particularly in high temperature or water stress and causes root rot in different plant species worldwide (Faeth & Sullivan, 2003). *Glomerella acutata* develops on naturally infected fruit in two forms: conidial (*Colletotrichum acutatum*) and the ascigerous (*Glomerella acutata*), but it endophytic association with plant in tropical and temperate regions has been reported (Rodriguez *et al*., 2009 a); (Ernst *et al*., 2003). *Davidiella sp.*, formerly known as a pathogen inducing fruit rot in date palm, has been indicated to colonize host plants as an endophyte from Antarctica to temperate flora (Weber *et al*., 2005); (Selosse *et al*., 2004); (Newsham, 2011). *Aspergillus niger* and *Chaetomium sp.*, are mainly considered as black mold or dark-walled mold (dematiaceous) with pathogenic properties for plant and human, but have been widely used in food industries and producing biocontrol agents due to their metabolites. Reports are scarce about the endophytic life style of recently mentioned fungi species (Rodriguez *et al*., 2008); (Rodriguez *et al.*, 2009 a); (Arnold & Lutzoni, 2007).

Phytopathogens are conventionally categorized into three groups based on their habitat and the way they obtain their nutrient supply: biotrophic, hemibiotrophic, and necrotrophic. Among isolated endophytes from cherry rootstocks, *Botrytis cinerea* is known as a necrotrophic fungus and usually is attributed with grey mold, although it has been recently demonstrated to have endophytic traits in different plant species (Gamboa *et al*., 2002). *Phomopsis sp*., is also a facultative plant pathogen which has been recently isolate as an endophyte from different hosts (Verma *et al*., 2007); (Arnold & Herre, 2003). From *Xylariales*, the greatest number of *Xylaria sp.*, has been found in tropics. This fungus normally lives in dead tropical woody plants and may colonize healthy tissue (Higgins *et al.*, 2007); (Ganley *et al*., 2004); (Santamaria & Bayman, 2005). Whereas *Xylaria digitata* was identified as an endophyte associated with cherry rootstocks, this species had relatively low frequency in the host. *Ceratobasidium sp.*, was the only genus of Basidiomycota isolated from cherry rootstocks. These fungi are commonly facultative pathogens and have also saprotrophic life style. Species belong to this genus have been isolated as endophytes mainly from host in the temperate regions (Selosse *et al*., 2009); (Herre *et al*., 2007); (Rodriguez *et al.*, 2009 b).

The most diverse fungi in orchestration of endophytic communities associated with cherry rootstocks were *Fusarium sp*. Such a result may imply the ability of this genus to colonize host tissue with no detectable symptom, however these fungi are normally considered as plant

pathogens. There is a body of evidence that confirms symbiotic life style of *Fusarium sp.*, with plants (Jumpponen *et al.*, 1998); (Kaplan *et al.*, 2013); (Reininger & Sieber, 2012) that altogether they highlight the importance of these fungi in bio-ecosystem and their impact on plant pathophysiology. Some endophytes isolated from cherry rootstocks have formerly been shown to be beneficial to the host. *Acremonium sp.*, and *Epicoccum nigrum*, as two widespread fungi, have been described as endophytes with ability to improve the host resistance against pathogens (Mandyam *et al*., 2013); (Paul *et al*., 2012); (Marasco *et al*., 2012).

The most striking outcome of this study was the isolation and identification of two fungi species on the leaf of cherry rootstocks (only during season I) which according to the best of our knowledge have not been reported so far as resident endophytes of leaves at least in temperate climate. *Rosellinia sp.*, (Sordariomycetes) and *Pyronema sp.*, (Pezizomycetes) were found only in leaves in the first sampling period and had no occurrence on other tissues in that season or on all examined specimens during the next two seasons, suggesting that these fungi may colonize the host incidentally in a tissue-specific manner in early stages of the foliar growth.

## 5.2. Impact of host species, colonized tissue, and temporal changes on biodiversity of endophytic fungi

### 5.2.1. Sweet cherry

All cherry trees subjected to the present study, were selected from an orchard nursery. Thus such a sampling procedure did not allow analyzing the biodiversity of endophytes in different habitats. Nonetheless, by considering the regional ecosystem of the sampling locality and other factors like quality of soil and environmental conditions, results of this study can not only demonstrate the status of endophytic fungi infection in cherry rootstocks throughout the state but also can be extended to a broader geographical range in Central and Eastern Europe.

According to the results, endophytic communities associated with rootstocks belonged to *Prunus mahaleb L,* were comparatively more diverse and distinctive species had higher occurrence on these rootstocks during a particular sampling period, although the general feature of endophyte assemblages on cherry rootstocks showed more tendencies to vary depending on the host organ and seasonal changes than the host species. Perhaps less diverse endophytic assemblages were observed on some rootstocks like Vadcseresznye or Gisela6, but disparities in infection rates and species richness in different tissues of these individual hosts during three sampling periods suggested mild impact of host species in composition of endophyte

communities and their diversity in cherry rootstocks. Consequently, it remains to be elucidated whether the association of endophytic fungi can favor vegetative growth, precocity, adaptability to different environmental conditions, disease tolerance, and productivity of the examined cherry rootstocks. Furthermore, the probability of host-endophytes interaction to switch from mutualism to antagonism and changes in transmission mode of the fungi under the influence of host physiology and other stresses is to be studied.

Infection burden on the host tissues and diversity of endophytic fungi showed more correlation to the organ where they were colonized and the time when tissue specimens were obtained. However, some degree of tissue-specificity was detected in distinctive fungi, but colonization pattern and distribution of endophytes encompassed the pivotal role of temporal changes in host physiology that more likely can induce alteration in the feature of these symbionts communities.

The first and the third sampling procedures were fulfilled in early autumn time, with almost one year interval between two periods, prior to defoliation of the trees, when the second sampling period was carried on in mid spring during the flowering time. Differences concerning the infection rate and composition of endophytes assemblages between these two periods perhaps demonstrate the effect of annual changes and aging process in the host on biodiversity of fungal endophytes. Nevertheless, host species may have an impact on endophyte divergence, more probably based on the differences in growth habit, vegetation characteristics and their ability to maintain efficient adaptivity to environmental stresses.

Results of the present study showed that roots of *Prunus mahaleb L*, rootstocks were less infected in season I and then infection rate raised during the next two seasons. This index was found different in Gisela6 which showed high rate of infection in the root during season III. The highest infection in the root was detected during season I in Prob but decreased in the last two seasons. It is generally believed that the root in woody plants is the main habitat of soil-borne microorganisms and usually harbors more diverse symbiotic fungi and bacteria than spatial organs of the host. It may explain the higher diversity of endophytic fungi in cherry rootstocks however decline in number of isolated species and increased infection burden occurred in roots of examined rootstocks can indicated that endophytic fungi horizontally transmit between individual hosts and colonize the root while incidental species are depleted through the aging process and taxa which are capable to adapt their niche perpetuate distribution and dominantly

amplify the infection rate in the tissue. Seasonal changes in physiological functions of the root rather affect endophyte continuum toward occurrence of more competent and prevalent fungal endophyte than infection with more diverse and incidental species. It is not clear that how these resident symbionts confer benefits (i.e. absorbance of water and nutrients, growth vigor, and vulnerability to pathogens) to the root when their share of photosynthesis is supplied by the host plant.

Temporal changes of infection rate in twigs were similar in all rootstocks and exhibited high colonization and isolation rates in season I and season III, however these indices comparatively had decline in season II. On the other hands, the number of isolated taxa had no difference between the first two seasons and rose during the last sampling period. Altogether these findings indicate that biodiversity of endophyte infection in the twig is more likely under the influence of annual biochemical and physiological changes in this organ. It can be assumed that aged twigs are preferentially colonized by endophytes that travel through water and nutrient transportation system from the root to foliage during the time of plant vegetative growth. Moreover, twigs may be less vulnerable to endophyte infection probably due to the physiological processes occur during simultaneous vegetative and reproductive growth of the host.

As for leaves, results showed a remarkable increase in infection burden during season III in compare with the first two seasons. Perhaps such findings indicate that the infection burden and diversity of fungal endophyte flora on leaves of cherry rootstocks differs in accordance to the aging process in foliar tissues but has less correlation to the annual changes. In addition, tendency of some isolated fungi to exclusively colonize the leaf and occurrence of other taxa that as well were accommodated in twigs and roots can highlight the existence of air-born fungi in the studied ecosystem, although soil-born endophytes can infest the leaf via water and nutrient transportation system.

As a matter of fact, these outcomes can be confirmed by existing knowledge about aspects of endophytic fungi biodiversity in terrestrial plants. Former studies have also suggested that species composition of endophyte assemblages and infection frequencies vary according to host species, site characteristics, such as elevation, exposure, and associated vegetation, tissue type; and tissue age (Khan,*et al*., 2012); (Ahlholm *et al*., 2002). For large woody hosts, growth stage and position in the canopy also may affect distribution. In addition to the core group of species consistently isolated as endophytes from any given host, surveys of plant hosts for endophytes

invariably generate long lists of incidental species that are not known to sporulate on the host (Aly *et al*., 2011); (Khan *et al.*, 2012). Each incidental species often is represented only once or twice in several hundred samples. In general, the number of rare and incidental species isolated is proportional to the intensity of sampling; distribution of rare species is influenced more by site than by host (Khan *et al*., 2012); (Arnold *et al.,* 2007). Variation in species assemblages on the same host at different sites usually is attributable to recovery of incidental species with more disjunctive distributions. Pronounced seasonal differences in colonization frequencies might be predicted for climates with a distinct wet/dry seasonal cycle. Therefore species composition and relative abundances in endophyte assemblages may reflect spatial distributions as well as sampling times (Arnold *et al*., 2007).

Studies on foliar and stem endophytes have been repeatedly verified that overall infection frequencies increase with the age of host organs or tissues (Parsa *et al.*, 2013); (Arnold *et al*., 2007). This is best observed from evergreen plants or plants with long-lived foliage but is also apparent to a lesser degree in deciduous trees and annuals. In term of methodology, however, it is essential to distinguish between systemic endophtes and non-systemic epiphytes to assess the colonization pattern and tissue-specificity of endophytes (Arnold *et al*., 2007). The infection domain of endophytes has a profound effect on sampling efficiency for species diversity. Unfortunately there is a lack of information about non-systemic, non–seed-borne fungi. Dominant non-systemic endophytes known today are generally familiar epiphytes, such as *Alternaria sp.*, *Cladosporium sp.*, and *Epicoccum sp.*, or typical pathogen of grass hosts (Rodriguez *et al*., 2009 a); (Provorov, 2009); (Porras-Alfaro & Bayman, 2011) . The differences between systemic infections and those of limited domain should be taken into account for plotting an efficient sampling strategy particularly when isolation of greater number of species and/or assessment of tissue-specific biodiversity of endophytes is subjected. No sampling method has been established yet that would be reliable to apply for detection of systemic and epiphytic fungi and selection of sample units in majority of published studies is apparently arbitrary and is highly variable. If sample units are not appropriate to the microscopic scale of infections, undue bias will be introduced and then inferences regarding species dominance and diversity drawn from those may be uncertain as a consequence (Arnold & Herre, 2003); (Rodriguez *et al*., 2009 a).

An important notion is also worth considering about protocols are used for collection and preparation endophyte samples. So far, methods applied to detect and quantify the endophytic fungi have been mainly based on common isolation from surfaced-sterilized tissue sections and culture techniques. For inventories of species occurrences and diversity, that is presently the most practical approach, although some species (e.g., obligate biotrophs) may be undetected or underrepresented. Isolates that fail to sporulate in culture may need to be characterized by other means. Presumably smaller sections of plant tissues and multiple cultures yield more accurate estimation of fungal infection frequencies (Watrud *et al*., 2006). Also, conversely, larger sampling units have greater potential to miss rare or slow-growing species and to recover mixed genotypes of the same species.

### 5.2.2. Capsicum annuum L.

In our experiments 42 endophytic fungal strains were successfully identified in pepper (Bärnkopf, 2013). They belong to 19 genera: *Alternaria*, *Cladosporium*, *Penicillium*, *Acremonium*, *Chaetomium*, *Fusarium*, *Lewia*, *Arthirinium*, *Cercospora*, *Colletotrichum*, *Galactomyces*, *Myrothecium*, *Paecilomyces, Plectosphaerella*, *Pyrenochaeta*, *Rhizopycnis*, *Verticillium* and *Xylaria*. 10 of these genera were also showed to be present in pepper by Paul *et al.* (2011): *Alternaria*, *Aspergillus, Bionectria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Plectosphaerella* and *Xylaria*. We also identified *Acremonium* sp. as a quite frequently occurring endophyte, but this was not found by the Korean research group. On the other hand, 11 of their strains were not positively identified in our experiments up to now (Bärnkopf, 2013). This discrepancy may originate from the completely different geographical region of cultivation, but also from the fact that neither Paul and coworkers‟ studies (Paul *et al*., 2011) nor ours cover the whole range of the endophytes. The success of detection of endophytic fungi depends on many factors. This shows the necessity to use NGS sequencing methods for endophyte identification to gain more detailed information on the endophyte population present.

Colonization of pepper plants did show several characteristic features. Colonization was higher at the end of vegetation period and under open field conditions, but we have also observed cultivar and organ specific differences. Of our cultivars „Hó‟ seemed to be higher colonized than „Kárpia‟ and the fungi preferred old leaves, fruit stalk and roots in comparison to other organs.

### 5.3. Pathogen growth inhibition by endophytic fungi

Recently, many scholars focus on the study of endophytes as biocontrol agent of pathogen and insects and successfully applied endophytes to plant protection. It is suggested that endophytic fungi confer resistance to plant pathogens by means of different mechanisms such as direct and indirect inhibition of pathogenic microorganisms and ecological effects (Li *et al*., 2007); (Zhi-Lin *et al*., 2012). The common method to evaluate antagonistic activity of endophytic fungi against plant pathogens is the In Vitro co-culture with pathogens and endophytes, or comparison of the survival rate of plant inoculated with fungal endophytes with endophyte-free plants (In Planta methods). Despite growing number of studies, existing knowledge about potential mechanisms by which endophytic fungi limit pathogen damage and protect their hosts from diseases, is still so poor.

In the present study, seven endophytic species with more vigorous growth on the media were selected for dual cultures with *Monilia laxa* and *Agrobacterium tumefaciens*. As it was demonstrated by results, all species showed a range of inhibition and growth factors of pathogens in all plates were collectively impaired in compare with controls. In all plates, endophytic fungi had higher radial growth rate and their confrontation with pathogens had not only no influence on colony expansion and development but also in the case of some species induced improved growth rate to the endophyte. Presumably, being obliged competing other microorganisms inflicts enhanced growth and intensifies colony development of the endophytic fungi in order to acquire more nutrient resources and extend the life span of the species. Such an inference may explain a possible mechanism by which endophytic fungi induce immunity to the host as they contend other microorganisms in colonized tissues and inhibit the growth of potential pathogens. These findings remark the ability of endophytic fungi to directly inhibit the pathogen. Many fungal endophytes produce secondary metabolites and some of these compounds are antifungal and antibacterial which strongly inhibit the growth of other microorganisms including plant pathogens. For example, altersetin, a new alkaloid isolated from endophytic *Alternaria* spp., showed antibacterial activity against several pathogenic gram-positive bacteria (Hellwig *et al*., 2002). However, as low concentration of these substances are excreted by endophytes in host plant, the effects of antibiotics to pathogens In Vivo need to be assessed. As well, many microorganisms produce and release lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemi-cellulose and DNA

(Choudhary *et al*., 2014). When endophytes colonize on the plant surface, they produce enzymes to hydrolyze plant cell walls. As a result, these enzymes also have the function to suppress plant pathogen activities directly and have the capability of degrading the cell walls of fungi and oomycetes. Although, the enzymes may not be of most importance in antagonism, they may contribute to antagonistic activity through a combination of mechanisms (Choudhary *et al*., 2014). Endophytic fungi are also thought to induce host resistance to pathogen by activating the plant natural defenses. During the long term evolution, plants have developed two types of innate resistance: non-specific (general) resistance and specific resistance. The first form is effective against several pathogenic species, while the latter can resist infection of one or a few pathogenic strains (Barna *et al*., 2003). Since fungal endophytes may evolve from plant pathogenic fungi, plant defense could be triggered by fungal endophytes like pathogens. Actually, the defense of plant associated with endophytes is increased through resistance enhancement and secondary metabolites production. As an example, *Fusarium solani* isolated from root tissues of tomato elicited systemic resistance against the tomato foliar pathogen *Septoria lycopersici* and triggered pathogenesis-related proteins (PR) genes, PR5 and PR7 expression in roots (Kavroulakis *et al*., 2007). *Epicoccum nigrum* which was found in cherry rootstocks and had growth inhibitory effect on examined pathogens in dual-cultures is also reported to contribute enhanced root growth and generation of antifungal compounds in sugarcane. Antimicrobial activity of *Epicoccum sp.*, is attributed to production of antibiotics such as epicorazines A-B, epirodines A-B, flavipin, epicoccines A-D, epipiridones, and epicocarines. In particular, flavipin and epicorazines A-B have been associated with *Epicoccum nigrum* biocontrol activity against *Monilia sp*. (Favaro *et al*., 2012).

Furthermore, it has been implied that endophytic fungi association can stimulate plant resistance more rapidly and enhances the production plant secondary metabolites. These metabolites have an important role in plants adaptation with their environment. However, studies refer to the plant secondary metabolism mediated by the fungal endophytes is still on the early stage. As an instance, a strains of *Fusarium oxysporum* could up-regulate the expression of glutelin A (GLUA) and salicylic acid-inducible PR-1a genes in tomato root which promoted tissue resistance against Fusarium wilt (Aime *et al.*, 2013). It seems that co-culturing with endophytic elicitor is an alternative way to enhance plant secondary metabolites and increase plant resistance. Mechanism of endophytic elicitor induced plant secondary metabolites

production is similar to stimulation of plant resistance. Colonization of fungal endophytes results in the secretion of hydrolase of plant cell to limit the growth of fungi, thus, fragments of endophytes, acting as elicitor, are produced by hydrolization. The elicitors such as lipopolysaccharides, polysaccharides and glycoprotein will stimulate plant defense and plant secondary metabolites, which would suppress pathogens attack efficiently. However, determination of how endophytes can survive in high concentration of secondary metabolites in host plant is still unknown (Ming *et al*., 2013).

Arguably, fungal endophyte association with host plants can shift between pathogenic and symbiotic under the particular circumstances (Kaplan *et al*., 2013). It is assumed that horizontally transmitted fungi may primarily generate parasitism, but in case emergence of infection by other microorganisms, apply mechanisms to eliminate the rival and sustain normal status of the host through life-history trade-offs with growth/survival. Thus they can perpetuate their dominance in the adapted niche that in turn indirectly confers benefit to the host and decreases the pathology of diseases (Faeth, 2009); (Arnold *et al*., 2003). Although screening of antimicrobial activity of endophytic fungi isolated from cherry rootstocks was performed by using In Vitro assays, such a life-history could be evolved for some species. For instance, *Botrytis cinerea* had a comparatively high antagonistic effect on pathogen growth in dual cultures, whereas it has been rather introduced as a pathogenic fungus in different hosts. These types of fungi probably tend to colonize the host tissue as parasites and rapidly occupy the ecological niche and leave no space for pathogen which would be the main reason that fungal endophytes inhibit pathogen infection in plants. Such a property of fungal endophyte along with the host reactions against endophytes (i.e. secretion of lignin and other cell-wall deposits to limit the endophyte growth which as a shelter protects endophyte from pathogen invasion) and their capability to parasitize pathogen organisms (hyperparasite endophytes) can be considered as the ecological effects of endophytic fungi on plant resistance to pathogens (Petrini *et al*., 1992); (Grosch *et al*., 2006).

**Summary**

Endophytic symbionts including bacteria and fungi live within plant tissues without causing any obvious negative effects and have been found in every plant species examined to date. It became evident that endophytes are rich sources of bioactive natural products with promising applications in development of pharmaceutical and industrial compounds. In addition studies in recent decades have suggested all plants maintain associations with endophytes and epibionts while such a symbiotic relationship is generally a cryptic phenomenon in Nature. Fungal endophytes may inhabit tissues of roots, stems, branches, twigs, bark, leaves, petioles, flowers, fruit, and seeds, including xylem of all available plant organs. These fungi are alleged to affect the ecophysiology of plants, by frequently enhancing the capacity of host plants to tolerate different environmental stresses through mechanisms that are only partially understood. It is also believed that endophytes have important roles in plant protection by enhancing the plant resistance against herbivores, insects and pathogens. Therefore, understanding the pattern of endophytic infection and diversity of these symbionts in hosts within a certain biogeographical niche is of a fundamental importance for improving the existing knowledge of plant physiology. Besides, any attempt to expound the impact of the plant-endophyte interaction on biological resistance of host plants against biotic and abiotic stresses can lead to apply more efficient strategies for increasing the quality and quantity of agricultural and horticultural products.

In the current research work, colonization pattern of fungal endophytes on different rootstocks of sweet cherry (Prunus spp) grafted on cultivar Péter including Érdi V., Bogdány, SL64, Egervár, Korponay, SM11/4, CEMANY and Magyar rootstocks (of *Prunus mahaleb* L.,) , two varieties of *P.avium* (Vadcseresznye) and *P.fruticosa* (Prob) and hybrid inbred rootstock of *P.cerasus* and *P.canescens* (Gisela 6), was studied by application of morphological and molecular phylogeny methods. Three anatomical tissue compartments (roots, twigs and leaves) of above-mentioned rootstocks were collected during three different sampling periods (autumn 2008, spring 2009 and autumn 2009) and endophytic fungi were isolated by single-spore methods and primarily identified according to their morphology. Phylogeny of the isolated fungi was determined by application of PCR using ribosomal internal transcribed spacer (ITS) region sequence. Furthermore, endophytes with more vigorous growth on culture media were used for dual-cultures with two selected pathogens (*Monilia laxa* and *Agrobacterium tumefaciens*) to

elucidate their antagonistic effect on one another. The same method was applied for isolation and identification of endophytic fungi associated with pepper (*Capsicum annuum* L.) cultivars Hó F1 and Kárpia F1.. Samples were obtained from the root, shoot, leaves, pedicles, pericarps at different vegetative stages and seed of pepper plants from open field (grown on sandy soil) and the greenhouse (grown on rockwool). This process was performed in three replicates for four times during the vegetation period (April, May, August and October, 2013).

As a sum, 26 morpho-taxa were classified among a total of 6587 isolated colonies from cherry rootstocks. The majority of identified species (24 species) were from the phylum Ascomycota. Two species of *Alternaria spp* (*Paleosporales*) were the most frequent isolates while *Fusarium spp* showed the highest diversity in fungal assemblages on cherry rootstocks. The highest species richness was detected in roots while twig samples showed the the highest infection burden in cherry rootstocks. This study was the first to report isolation and identification of *Rosellinia sp*., (*Sordariomycetes*) and *Pyronema sp*., (*Pezizomycetes*) from trees in temperate climate only detected on leaves in the first sampling period and had no occurrence on other tissues in that season or on all examined specimens during the next two seasons. Collectively, however some degrees of tissue-specificity was observed in composition of fungal assemblages identified on cherry rootstocks, results of this study suggested that the biodiversity of endophytes is more likely affected by annual climate alterations, temporal changes in the host physiology and the process of aging. As well, species richness and relative frequency of fungi were found comparatively higher in *Prunus mahaleb* L, although host-specificity seemed to affect the biodiversity of endophytes on cherry trees less significantly than seasonal changes. As for the growth inhibitory test, all examined endophytes showed antagonistic effect on growth and development of the targeted pathogens while *Alternaria sp.1* and *Fusarium spp*. were the strongest and the weakest antagonists to pathogen growth, respectively. As an overall, 42 fungal endophyte strains belonged to 19 genera were isolated from *Capsicum annuum*. Results showed the dominant occurrence of *Alternaria sp*., and as well colonies belonged *Acremonium sp*., were also isolated from different tissue compartments of pepper. Colonization rate of endophytic fungi showed alteration due to the cultivar, harboring tissue and growth condition of the host. Cultivar Hó F1 showed the higher colonization rate compared to Kárpia F1. The highest infection burden was found at the end of vegetation period under the open field condition. Endophytic fungi

showed higher tendency to old leaves, fruit stalks and roots of examined pepper plants. The inhibitory effect of endophytes on pathogens growth has been discussed in detail.

As a conclusion, fungal endophytes isolated from cherry rootstocks demonstrated a considerable diversity which is seemingly sustained by the physiological traits of the organ they inhabit and temporal climate changes that influence the species richness and frequency of these symbionts in their hosts. Moreover, these endophytes can likely contribute to the host resistance against potential pathogens.

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## Appendix

### A1. Biodiversity of endophytic fungi associated with Prunus sp., rootstocks

Composition of endophytic fungi community in different rootstocks of *Prunus* sp*.,* demonstrated various structures either between host species or in temporal and tissue-specific manner between isolated colonies from an individual host.

#### A.1.1. Biodiversity of endophytic fungi in Prunus mahaleb L., rootstocks

Bogdány, Érdi V, SL64, SM11/4, Egervár, Korponay, CEMANY, and Magyar were rootstocks of *Prunus mahaleb* L., examined in the present study.

From Bogdány, a total of 700 colonies were isolated from root, twig and leaf specimens and the highest infection burden was observed in season III (collectively, 336 colonies were isolated at this season). During all three sampling periods, from all identified fungal endophyte species in this study, 22 species were isolated from this rootstock. Maximum species richness in season I was observed in root samples (8 species), in root samples in season II (7 species) but in twig samples in season III (7 species) (Table A.1). The evenness of species, indicated by Shannon-Weaver index was comparatively the highest in Season II ($e^{H'}$=1.71), while Simpson index was expectedly the lowest (D=0.48) in the same season for Bogdány, demonstrating the highest diversity of the endophytic fungi assemblages on this rootstock during the season II. The highest abundance was detected for *Alternaria* sp.1 and *Alternaria* sp.2 that along with *Epicoccum nigrum* (with comparatively lower abundance) were isolated from all tissue compartments of this rootstock. The largest number of *Alternaria* sp.1 colonies was found on Twig samples in season III and *Alternaria* sp.2; however was the most abundant on twig samples in season I. *Rosellinia* sp*.,* (RF=70%), *Xylaria digitata* (RF=67%) and *Alternaria* sp.1 (RF=55%) were the most frequent species on leaf samples in season I, season II and season III, respectively. *Rosellinia* sp*.,* was only isolated from leaf samples of this rootstock. *Alternaria* sp.2 was the most frequent species obtained from twig samples in season I (RF=66%) and season II (RF=67%), while this species was not observed in twigs during season III and instead, *Alternaria* sp.1 (RF=47%) was the most frequent fungi in twigs from season III. The pattern of frequency in root samples during season I was dominated by *Alternaria* sp.1 (RF=24%) and *Paraphoma* sp*.,* (RF=24%). Alternaria sp.1 was also the most frequent fungi in root during season II (RF=34%) but was not isolated from this tissue in season III. In the last sampling period, *Pyronema* sp*.,* (RF=28%)

showed the highest frequency among other isolates in root samples from Bogdány rootstock. According to the results, during season I and season III, explants collected from twigs of Bogdány, demonstrated the highest colonization rate (CR=80%, and CR=99%, respectively) along with the highest isolation rate (IR=1.04, and IR=1.47, respectively). The highest colonization rate in the root was observed in season II (CR=63%) and in the leaf during season III (CR=67%). As a sum, leaf samples exhibited the lowest colonization and isolation rates in compare with other tissue specimens in this rootstock.

In total, 869 fungal endophyte colonies were isolated from Érdi V rootstock. The largest number of isolated colonies was observed in twig samples during season III (199 colonies isolated). Leaf samples during the season I showed the lowest number of isolated colonies (6 colonies isolated), but in season III a number of 159 colonies was obtained from this tissue. The most abundant species in this rootstock were *Alternaria* sp.1 (280 isolated colonies) and *Alternaria* sp.2 (214 isolated colonies) while the first species was obtained from all tissue compartments in season III (120 isolated colonies from twigs, 82 isolated colonies from leaves and 28 isolated colonies from roots) (RF=60%, RF=52%, and RF=36%, respectively) and only from root samples (50 isolated colonies) during season II, but no isolate of *Alternaria* sp.1 was observed in tissue samples from this rootstock in season I. on contrary, *Alternaria* sp.2 was only isolated from all tissue samples in season I and from leaves and twigs, but not roots, in season II. No colony of this species was isolated from this rootstock in season III. Among all identified species, *Rosellinia* sp., was only isolated from leaf samples during the season I (4 isolated colonies) (Table A.2). The highest species richness on Érdi V rootstock was observed in root during the season I (9 species) while leaf samples showed the lowest richness according to the isolated species and in season III, only two different species were isolated from leaves. The evenness of identified species in season II was the highest in compare with other seasons ($e^{H''}$=1.69) and the most entropy in endophyte communities was calculated in season III ($e^{H''}$=0.41) when Simpson index showed the highest diversity of endophytic fungi associated with Érdi V rootstock (D=1.73). During the season III, twigs and leaves showed a comparatively high colonization rate (CR=99%, and CR=93%, respectively), however this index was much lower in season II particularly in leaf samples (4.3%). Accordingly, the highest isolation rate was observed in leaves and twigs during season III (IR=1.18, and IR=1.53, respectively). Root

samples from this rootstock showed the moderate range of colonization, from CR=28% in season I to CR=60% in season III, and isolation rate from IR=0.3 in season I to IR=0.7 in season III.

A total number of 898 endophytic fungi colonies were isolated from SL64 rootstock. Maximmum infestion of this rootstock was found on twigs collected in season III (237 isolated colonies). The lowest endophyte burden was indicated in leaves collected during season II (7 isolated colonies), although 165 colonies were collectively isolated from leaves in season III. The most frequent species was *Alternaria* sp.1 identified in all tissue compartments during season III: 139 colonies from twigs (RF=59%), 96 colonies from leaves (RF=58%), and 16 colonies from roots (RF=18%), however this species was not observed in all tissue samples from SL64 during the season I. *Alternaria* sp.2 also showed a reletively high frequency in this rootstock, but was mostly isolated from tissue samples in season II: 5 colonies from leaves (RF=71%), 57 colonies from twigs (RF=77%), and 23 colonies from roots (RF=24%). During season I, this species was isolated from leaves (16 isolated colonies, RF=57%) and twigs (118 isolated colonies, RF=73%), but was not observed in season III. During the season I, 8 colonies of *Rosellinia* sp*.,* were obtained from leaf samples (Table A.3). whereas the infection burden on root samples was comparatively moderate, endophytes colonized on root samples showed the highest species richness in all sampling periods (8 species in season I and season II, 6 species in season III). The highest diversity in endophytic fungi communities was calculated in season II ($e^{H''}$=0.96), but the index for season III showed less diverse endophytic assemblages on this rootstock ($e^{H''}$=0.23). Simpson index for season II and season III was D=1.23 and D=1.86, respectively. Explants obtained from twigs and leaves from SL64 during the third sampling periods were entirely infected (CR=100%). However, this pattern was observed in twigs during season I (CR=98%), but had an increase in season II (CR=46%) and was found relatively low in leaves during season I and season II (CR=20%, and CR=4%, respectively). Accordingly, the highest isolation rate was found in twig and leaf samples durin season III (IR=1.4, and IR=1.2, respectively).

From SM11/4 rootstock, a number of 634 colonies were isolated showing the highest infection burden on root samples during season II (141 isolated colonies) but less number of fungi colonies on leaves in that season (5 isolated colonies). During the first season, the largest number of colonies was isolated from twigs (133 isolated colonies) while this rate was almost constant during next seasons (73 isolated colonies in season II and 121 isolated colonies in

season III). Regarding other tissue samples, the number of isolated colonies was relatively flactuated. For instance, from leaved 32 colonies in season I, 5 colonies in season II and 54 colonies in season III were isolated. The most frequent species were *Alternaria* sp.1 and *Alternaria* sp.2, predominantly composed the endophyte cummunities associated with SM11/4 rootstock. *Alternaria* sp.1 was isolated from root in season II (60 isolated colonies, RF=43%), and from leaf (20 isolated colonies, RF=37%), twig (54 isolated colonies, RF=45%), and root (11 isolated colonies, RF=31%) samples in season III, but was not observed in season I. in season I, alternaria sp.2 was predominant and found in leaf (16 isolated colonies, RF=50%), twig (103 isolated colonies, RF=77%), and root (7 isolated colonies, 18%) samples. During season II, the recent species was only found in leaves (3 isolated colonies, RF=60%) and twigs (50 isolated colonies, RF=68%) and was not observed in season III. five isolated colonies of *Rosellinia* sp., was observed only in leaf samples during season I (Table A.4). the highest sepecies richness was found in root during the season I (10 species) while leaf samples in season II beared the lowest number of species (2 species) in this rootstock. Diversity indices demonstrated the most divergent endophytic infection in season II ($e^{H''}$=1.49, D=0.57) and the most homogenic structure of endophyte assemblages during season III ($e^{H''}$=0.58, D=1.6) associated with SM11/4 rootstock. Twig samples were the most infected tissue compartments of this rootstock as the highest colonization rate was observed in twigs during the first (CR=97%, IR=1.2) and the third (CR=99%, IR=1.6) sampling periods, however indices remarking infection rate showed an decrease in season II (CR=47%, IR=0.6) in twigs. Leaf samples had also the same temporal infection pattern but the highest colonization rate and isolation rate in roots were measured during the season II (CR=82%, IR=1.2).

Experiment on Egervár rootstock yielded a total number of 692 endophyte colonies where the largest number of isolates were detected in twig samples. In total, 125 isolated colonies during season I, 74 isolated colonies during season II, and 169 isolated colonies during season III were obtained from twigs. In the first two sampling periods, leaves showed a very low rate of infection, although in season III, 105 colonies were isolated from leaf samples of this rootstock. As a consequence, either twigs or leaves showed the maximum infection burden during the third season, whereas root samples were mostly infested in season II (a total of 95 colonies were isolated from root samples). The most abundant species on this rootstock was *Alternaria* sp.1 ( a total of 195 isolated colonies) which was onserved in all tissue samples during season III: 43

isolated colonied on leaf (RF=41%), 83 isolated colonies on twig (RF=49%), and 19 isolated colonies on root (RF=27%), but it was only isolated from root samples during season I and season II (17 isolated colonies, RF=43%, and 33 isolated colonies, RF=35%, respectively). *Alternaria* sp.2 had a slightly lower abundance (a total of 147 isolated colonies), however it was isolated from examined tissue samples of leaves and twigs in season I: 7 isolated colonies from leaf (RF=70%), and 84 isolated colonies from twigs (RF=67%), and in season II: 4 isolated colonies from leaf (RF=80%), and 52 isolated colonies from twig (RF=70%) with relatively high frequency but was not observed in root samples. The recent species was not detected on tissue samples during season III. *Rosellinia* sp*.,* was isolated only from leaf samples (1 colony) during the season I (Table A.5). As a result, the highest species richness was measured in root during the first two seasons (8 and 7 sepcies, respectively) but twig samples in season III harbored the highest number of species (7 species) in compare with other tissue compartments. The most diverse endophyte communities colonizing Egervár rootstock were detected in season II ($e^{H''}$=1.42, D=0.7), but diversity indices showed that the endophytic fungi associted with this rootstock had relativley the most homogenous structure in season III, according to the existence of different taxa ($e^{H''}$=0.4, D=1.8). During season I, the highest colonization and isolation rates were measured in twigs (CR=84%, IR=1.1), while such a pattern shifted on root during season II when these indices showed the highest values for this tissue (CR=77%, IR=0.8). during the first two seasons, leaves showed relatively low colonization and isolation rates (CR=7%, IR=0.1, and CR=3%, IR=0.04, respectively). In season III, however, explants from twigs were the most infected tissue specimens of this rootstock (CR=100%, IR=1.5) and besides, leaf samples also exhibited higher colonization and isolation rates (CR=80%, IR=1) in compare with previous sampling periods. Root samples had the lowest infection rates during the season III (CR=58%, IR=0.7).

According to the results, 688 fungal endophyte colonies were isolated from Korponay rootstock. The adundance of isolated colonies was found the highest in twigs during season I (107 isolated colonies) and season III (154 isolated colonies), but the largest number of colonies was isolated from roots (102 isolated colonies) in the second sampling period. The infection burden of leaf in this rootstock had a drastic increase in season III (111 isolated colonies) in compare with the first two seasons. Collectively, twig was found as the most infected tissue compartment in Korponay rootstock while leaf samples had the lowest infection burden in this

host. The most frequent species was *Alternaria* sp.1 (222 isolated colonies) that was detected on all samples during season III: 69 colonies isolated from leaf samples (RF=62%), 97 colonies isolated from twigs (RF=63%), and 18 colonies isolated from roots (RF=21%), but only was obtained from root samples in season II (38 isolated colonies, RF=37%) and was observed on neither of the tissue samples during season I. In contrast, *Alternaria* sp.2 was detected in season I on leaves (13 isolated colonies, RF=68%), twigs (66 isolated colonies, RF=62%), and on roots (4 isolated colonies, RF=10%), and in season II on leaf (4 isolated colonies, RF=80%) and twig (43 isolated colonies, RF=63%), but had no occurance in season III. *Rosellinia sp*., was just isolated from leaf samples during season I (4 isolated colonies) (Table A.6). Root samples of this rootstock bearded the richest species divesity during season I and season II (10 species and 7 species, respectively) but in season III the highest number of species was identified in twig samples (8 species). In all seasons, the lowest species richness was detected in leaves. Diversity indices also showed that the endophytic fungi associated with Korponay rootstoch were comparatively the most divergent in season II and had the less diversity in season III ($e^{H''}$=1.64, D=0.54, and $e^{H''}$=0.53, D=1.62, respectively). During season I and seaon III, the highest colonization and isolation rates were calculated in twigs (CR=67%, IR=1, and CR=96%, IR=1.4, respectively) while these values were the highest in root samples in season II (CR=70%, IR=0.9). Leaves showed comparatively low values of infection in season I and season II (CR=17%, IR=0.2, and CR=4%, IR=0.04, respectively), but indices had increase (CR=94%, IR=1) during season III.

From CEMANY rootstock, a total of 592 colonies were isolated and identified during three sampling periods. In season I and season III, the largest number of isolated colonies was obtained from twigs (97 isolated colonies and 134 isolated colonies, respectively), while in season II, root samples had the highest infection burden (91 isolated colonies). In the first two seasons, leaf samples showed a very low infection burden (7 isolated colonies and 2 isolated colonies, respectively) but in season III a total of 107 colonies were isolated from leaves of this rootstock. *Alternaria* sp.1 was the most frequent species (151 isolated colonies) obtained only from root samples (26 isolated colonies, RF=29%) in season II, but from all tissue samples in season III (23 isolated colonies from leaf, RF=21%, 84 isolated colonies from twig, RF=63%, and 18 isolated colonies from root, RF=37%). This fungus was not observed in season I. the second most frequent species, *Alternaria* sp.2 (127 isolated colonies), was detected in leaves (4

isolated colonies, RF=57%), twigs (67 isolated colonies, RF=69%, and in roots (3 isolated colonies, RF=37%) during the season I and in leaf (2 isolated colonies, RF=100%) and in twig (51 isolated colonies, RF=75%) but not from root during the season II. This fungus, however, was not observed in season III. *Rosellinia* sp*.,* was detected on leaf samples during season I (2 isolated colonies) (Table A.7). As a sum, the highest species richness was found in root. Although this value was the highest in root during season I (8 species) and season II (7 species), but in season III more species were isolated from twigs (5 species) in compare with root samples (4 species). Leaf samples obtained from CEMANY rootstock showed the lowest species richness in all seasons. Diversity indices demonstrated the highest diversity in endophyte communities during season II ($e^{H''}$=1.27, D=0.8) and less diverse composition of the identified endophytes assemblages during season III ($e^{H''}$=0.5, D=1.7). Whereas, the rate of infection was proportionally higher in root samples (CR=66%, IR=0.9) during season II in compare with leaf and twig, the most remarkable colonization and isolation rates were calculated in twigs during season I (CR=86%, IR=1.1) and season III (CR=100%, IR=1.5) among all tissue samples. Infection rates in leaf samples from CEMANY rootstock, although were the lowest in the first two seasons (CR=7%, IR=0.1, and CR=2%, IR=0.02, respectively), showed a striking increase in season III (CR=82%, IR=1.2).

A total number of 515 colonies were isolated from Magyar rootstock. Although in general, twig samples had the highest infection burden but in season II the largest number of colonies was isolated from root samples (75 isolated colonies). The number of isolated colonies was comparatively low in leaf samples during the first two seasons (11 isolated colonies and 5 isolated colonies, respectively), while 81 colonies were isolated from this tissue in season III. *Alternaria* sp.2 was the most frequent fungus isolated from this rootstock (152 isolated colonies). This species was found only in root (30 isolated colonies, RF=40%) in season II, but was isolated from all tissue compartments (35 isolated colonies from leaf, RF=43%, 65 isolated colonies from twig, RF=68%, and 8 isolated colonies from root, RF=26%) during season III. *Alternaria* sp.2 was not detected on Magyar rootstock in season I. with a slightly lower abundance, *Alternaria* sp.1 was the second most frequent species on this rootstock isolated in season I from leaf (7 isolated colonies, RF=64%), twig (87 isolated colonies, RF=67%), and from root (7 isolated colonies, RF=33%) and in season II from leaf (2 isolated colonies, RF=40%), and from twig (49 isolated colonies, RF=73%) and had no occurance in root during

this season. Moreover, the recent species was not detected on Magyar rootstock during season III. *Rosellinia* sp., was isolated (3 colonies) from leaf only during the first season (Table A.8). Species richness was higher in root samples in compare with the other tissues (8 species and 7 species were identified from root samples during season I and season II, respectively), but this value was the highest in twig samples during season III (5 species). Diversity of endophytic fungi associated with Magyar rootstock was almost the same in season I and season III, however indices showed more diversity in season II ($e^{H'}$=1.53, D=0.57). In all three seasons, the highest colonization and isolation rates were found in twig samples. As a sum, 97% of twig explants (CR) were infected during season I and an avarage of 1.3 fungi colonies (IR) were isolated from each explant during the season. These values for twig samples in season II (CR=56%, IR=0.7) and season III (CR=95%, IR=1.2) also were relatively higher than the other tissue compartments of Magyar rootstock. Colonization rate and isolation rate in leaves were calculated as CR=9.2% , IR=0.1 in season I and CR=5%, IR=0.05 in season II, although a drastic increase was detected in the rate of infection for leaf samples during the third season (CR=78%, IR=1).

**Table A.1:** Abundance of isolated colonies from identified fungal endophyte species associated with Bogdány rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 21 | - | - | - | - | - | - | - | - | **21** |
| **Chaetomium sp.** | - | 2 | - | - | 8 | - | - | - | - | **10** |
| **Macrophomina phaseolina** | - | - | - | - | 10 | - | - | - | - | **10** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | 6 | - | - | 37 | 55 | 83 | 17 | **198** |
| **Alternaria sp.2** | 7 | 78 | - | 1 | 52 | - | - | - | - | **138** |
| **Xylaria digitata** | - | - | - | 2 | - | - | - | - | - | **2** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | 2 | **2** |
| **Acremonium furcatum** | - | - | - | - | - | 23 | - | - | - | **23** |
| **Rhyzopycnis vagum** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.1** | - | - | - | - | - | 1 | - | - | - | **1** |
| **Ceratobasidium sp.2** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Neonectria radicicola** | - | - | 2 | - | - | - | - | - | - | **2** |
| **Acremonium sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Paraphoma sp.** | - | - | 6 | - | - | 11 | - | - | 4 | **21** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 3 | - | **3** |
| **Epicoccum nigrum** | - | 32 | 3 | - | 7 | - | 12 | 9 | 1 | **64** |
| **Embellisia sp.** | - | - | 1 | - | - | - | - | 4 | - | **5** |
| **Davidiella sp.** | 1 | - | - | - | - | 33 | - | - | - | **34** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium sp.1** | - | - | - | - | - | - | - | 23 | 15 | **38** |
| **Fusarium sp.2** | - | - | - | - | - | 1 | - | 42 | - | **43** |
| **Fusarium sp.3** | - | 1 | 2 | - | - | 1 | 23 | - | - | **27** |
| **Fusarium oxyporum** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Fusarium solani** | - | 5 | - | - | 1 | - | - | - | - | **6** |
| **Glomerella acutata** | - | - | - | - | - | - | - | 2 | - | **2** |
| **Unknown** | 1 | - | 3 | - | - | 3 | 10 | 9 | 22 | **48** |
| **Total** | **30** | **118** | **25** | **3** | **78** | **110** | **100** | **175** | **61** | **700** |

**Table A.2:** Abundance of isolated colonies from identified fungal endophyte species associated with Érdi V rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 4 | - | - | - | - | - | - | - | - | **4** |
| **Chaetomium sp.** | - | - | - | - | 4 | - | - | - | - | **4** |
| **Macrophomina phaseolina** | - | - | - | - | 16 | - | - | - | - | **16** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 50 | 82 | 120 | 28 | **280** |
| **Alternaria sp.2** | 25 | 125 | 12 | 3 | 49 | - | - | - | - | **214** |
| **Xylaria digitata** | 2 | - | - | 2 | - | - | - | - | - | **4** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | 3 | **3** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | 4 | **4** |
| **Rhyzopycnis vagum** | - | - | 1 | - | - | 4 | - | - | - | **5** |
| **Ceratobasidium sp.1** | - | - | - | - | - | 1 | - | - | - | **1** |
| **Ceratobasidium sp.2** | - | - | - | - | - | - | - | - | - | **-** |
| **Neonectria radicicola** | - | - | 5 | - | - | - | - | - | - | **5** |
| **Acremonium sp.** | - | - | - | - | - | 16 | - | - | - | **16** |
| **Paraphoma sp.** | - | - | 2 | - | - | 5 | - | - | 9 | **16** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 3 | - | **3** |
| **Epicoccum nigrum** | - | 31 | - | - | 9 | 1 | | 9 | 2 | **52** |
| **Embellisia sp.** | - | - | 1 | - | - | - | - | 21 | - | **22** |
| **Davidiella sp.** | 6 | - | 3 | 1 | - | 26 | - | - | - | **36** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 4 | - | **4** |
| **Fusarium sp.1** | - | - | 3 | - | - | - | - | 7 | 12 | **22** |
| **Fusarium sp.2** | - | - | - | - | - | - | - | 24 | - | **24** |
| **Fusarium sp.3** | - | 2 | 2 | - | 2 | 1 | 61 | - | - | **68** |
| **Fusarium oxyporum** | - | - | 2 | - | - | - | - | - | - | **2** |
| **Fusarium solani** | - | 4 | - | - | 2 | - | - | - | - | **6** |
| **Glomerella acutata** | - | - | - | - | - | - | - | 6 | - | **6** |
| **Unknown** | 1 | - | 8 | - | - | 2 | 16 | 5 | 20 | **52** |
| **Total** | **38** | **162** | **39** | **6** | **82** | **106** | **159** | **199** | **78** | **869** |

**Table A.3:** Abundance of isolated colonies from identified fungal endophyte species associated with SL64 rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 8 | - | - | - | - | - | - | - | - | **8** |
| **Chaetomium sp.** | - | - | - | - | 10 | - | - | - | - | **10** |
| **Macrophomina phaseolina** | - | - | - | - | 1 | - | - | - | - | **1** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | 9 | - | - | - | 96 | 139 | 16 | **260** |
| **Alternaria sp.2** | 16 | 118 | - | 5 | 57 | 23 | - | - | - | **219** |
| **Xylaria digitata** | - | - | - | - | - | - | - | - | - | **-** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | 1 | **1** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | - | **-** |
| **Rhyzopycnis vagum** | - | - | - | - | - | 4 | - | - | - | **4** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | 4 | - | - | - | - | - | - | **4** |
| **Neonectria radicicola** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Acremonium sp.** | - | - | 1 | - | - | 4 | - | - | - | **5** |
| **Paraphoma sp.** | - | - | 4 | - | - | 16 | - | - | 6 | **26** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Epicoccum nigrum** | - | 37 | - | - | 4 | 2 | 35 | 3 | 2 | **83** |
| **Embellisia sp.** | - | - | - | - | - | - | - | 6 | - | **6** |
| **Davidiella sp.** | 2 | - | 9 | 1 | - | 40 | - | - | - | **52** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium sp.1** | - | - | 2 | - | - | - | - | 19 | 15 | **36** |
| **Fusarium sp.2** | - | | - | - | - | 1 | - | 54 | - | **55** |
| **Fusarium sp.3** | - | 3 | 3 | - | 1 | 2 | 17 | - | - | **26** |
| **Fusarium oxyporum** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium solani** | - | 2 | - | - | 1 | - | - | - | - | **3** |
| **Glomerella acutata** | - | - | - | - | - | - | - | 7 | - | **7** |
| **Unknown** | 2 | 1 | 7 | 1 | - | 5 | 17 | 9 | 49 | **91** |
| **Total** | **28** | **161** | **40** | **7** | **74** | **97** | **165** | **237** | **89** | **898** |

**Table A.4:** Abundance of isolated colonies from identified fungal endophyte species associated with SM11/4 rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 5 | - | - | - | - | - | - | - | - | **5** |
| **Chaetomium sp.** | - | 2 | - | - | 6 | - | - | - | - | **8** |
| **Macrophomina phaseolina** | - | - | - | - | 5 | - | - | - | - | **5** |
| **Pyronema sp.** | 10 | - | - | - | - | - | - | - | - | **10** |
| **Alternaria sp.1** | - | - | - | - | - | 60 | 20 | 54 | 11 | **145** |
| **Alternaria sp.2** | 16 | 103 | 7 | 3 | 50 | - | - | - | - | **179** |
| **Xylaria digitata** | - | - | - | 2 | - | - | - | - | - | **2** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium furcatum** | - | - | - | - | - | 27 | - | - | 1 | **28** |
| **Rhyzopycnis vagum** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Neonectria radicicola** | - | - | 4 | - | - | - | - | - | - | **4** |
| **Acremonium sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Paraphoma sp.** | - | - | 3 | - | - | - | - | - | 1 | **4** |
| **Phomopsis sp.** | - | - | - | - | - | 7 | - | 3 | - | **10** |
| **Epicoccum nigrum** | - | 19 | - | - | 11 | - | 22 | - | 3 | **55** |
| **Embellisia sp.** | - | - | - | - | - | - | - | 14 | - | **14** |
| **Davidiella sp.** | 1 | - | 9 | - | - | 36 | - | - | - | **46** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 10 | - | **10** |
| **Fusarium sp.1** | - | - | 6 | - | - | - | - | 10 | - | **16** |
| **Fusarium sp.2** | - | - | 4 | - | - | - | - | 24 | - | **28** |
| **Fusarium sp.3** | - | 1 | 1 | - | 1 | 4 | 5 | - | - | **12** |
| **Fusarium oxyporum** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Fusarium solani** | - | 7 | - | - | - | - | - | - | - | **7** |
| **Glomerella acutata** | - | - | - | - | - | - | - | - | - | **-** |
| **Unknown** | - | 1 | 2 | - | - | 7 | 7 | 6 | 20 | **43** |
| **Total** | **32** | **133** | **39** | **5** | **73** | **141** | **54** | **121** | **36** | **634** |

**Table A.5:** Abundance of isolated colonies from identified fungal endophyte species associated with Egervár rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 1 | - | - | - | - | - | - | - | - | **1** |
| **Chaetomium sp.** | - | - | - | - | 6 | - | - | - | - | **6** |
| **Macrophomina phaseolina** | - | - | - | - | 3 | - | - | - | - | **3** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | 17 | - | - | 33 | 43 | 83 | 19 | **195** |
| **Alternaria sp.2** | 7 | 84 | - | 4 | 52 | - | - | - | - | **147** |
| **Xylaria digitata** | - | - | - | - | - | - | - | - | - | **-** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | 1 | **1** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | - | **-** |
| **Rhyzopycnis vagum** | - | - | - | - | - | 4 | - | - | - | **4** |
| **Ceratobasidium sp.1** | - | - | - | - | - | 1 | - | - | - | **1** |
| **Ceratobasidium sp.2** | - | - | 2 | - | - | - | - | - | - | **2** |
| **Neonectria radicicola** | - | - | 5 | - | - | - | - | - | - | **5** |
| **Acremonium sp.** | - | - | - | - | - | 8 | - | - | - | **8** |
| **Paraphoma sp.** | - | - | - | - | - | 9 | - | - | 4 | **13** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 2 | - | **2** |
| **Epicoccum nigrum** | - | 29 | 1 | - | 8 | - | - | 1 | 1 | **40** |
| **Embellisia sp.** | - | - | 1 | - | - | - | - | 9 | - | **10** |
| **Davidiella sp.** | - | - | 8 | 1 | - | 25 | - | - | - | **34** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 8 | - | **8** |
| **Fusarium sp.1** | - | - | - | - | - | - | - | 9 | 21 | **30** |
| **Fusarium sp.2** | - | - | - | - | - | - | - | 41 | - | **41** |
| **Fusarium sp.3** | - | 7 | 1 | - | 1 | 2 | 38 | - | - | **49** |
| **Fusarium oxyporum** | - | - | 3 | - | - | - | - | - | - | **3** |
| **Fusarium solani** | - | 5 | - | - | 3 | - | - | - | - | **8** |
| **Glomerella acutata** | - | - | - | - | - | - | - | - | - | **-** |
| **Unknown** | 2 | - | 2 | - | 1 | 13 | 24 | 16 | 23 | **81** |
| **Total** | **10** | **125** | **40** | **5** | **74** | **95** | **105** | **169** | **69** | **692** |

**Table A.6:** Abundance of isolated colonies from identified fungal endophyte species associated with Korponay rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 4 | - | - | - | - | - | - | - | - | **4** |
| **Chaetomium sp.** | - | 4 | - | - | 6 | - | - | - | - | **10** |
| **Macrophomina phaseolina** | - | 5 | - | - | 7 | - | - | - | - | **12** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 38 | 69 | 97 | 18 | **222** |
| **Alternaria sp.2** | 13 | 66 | 4 | 4 | 43 | - | - | - | - | **130** |
| **Xylaria digitata** | 1 | - | - | 1 | - | - | - | - | - | **2** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | 10 | **10** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | 1 | **1** |
| **Rhyzopycnis vagum** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | - | - | - | - | - | - | - | **-** |
| **Neonectria radicicola** | - | - | 2 | - | - | - | - | - | - | **2** |
| **Acremonium sp.** | - | - | 1 | - | - | 11 | - | - | - | **12** |
| **Paraphoma sp.** | - | - | 8 | - | - | 23 | - | - | 7 | **38** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 3 | - | **3** |
| **Epicoccum nigrum** | - | 23 | 2 | - | 8 | - | - | 4 | 5 | **42** |
| **Embellisia sp.** | - | - | 5 | - | 0 | - | - | 6 | - | **11** |
| **Davidiella sp.** | - | - | 8 | - | - | 12 | - | - | - | **20** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 4 | - | **4** |
| **Fusarium sp.1** | - | - | 2 | - | - | 2 | - | 9 | 16 | **29** |
| **Fusarium sp.2** | - | - | 2 | - | - | 10 | - | 25 | - | **37** |
| **Fusarium sp.3** | - | - | 1 | - | - | 5 | 26 | - | - | **32** |
| **Fusarium oxyporum** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium solani** | - | 6 | - | - | 3 | - | - | - | - | **9** |
| **Glomerella acutata** | - | - | - | - | - | - | - | 4 | - | **4** |
| **Unknown** | 1 | 3 | 4 | - | 1 | 1 | 16 | 2 | 26 | **54** |
| **Total** | **19** | **107** | **39** | **5** | **68** | **102** | **111** | **154** | **83** | **688** |

**Table A.7:** Abundance of isolated colonies from identified fungal endophyte species associated with CEMANY rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 2 | - | - | - | - | - | - | - | - | **2** |
| **Chaetomium sp.** | - | 1 | - | - | - | - | - | - | - | **1** |
| **Macrophomina phaseolina** | - | 3 | - | - | 5 | - | - | - | - | **8** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 26 | 23 | 84 | 18 | **151** |
| **Alternaria sp.2** | 4 | 67 | 3 | 2 | 51 | - | - | - | - | **127** |
| **Xylaria digitata** | - | - | - | - | - | - | - | - | - | **-** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | 3 | **3** |
| **Rhyzopycnis vagum** | - | - | - | - | - | 1 | - | - | - | **1** |
| **Ceratobasidium sp.1** | - | - | - | - | - | 1 | - | - | - | **1** |
| **Ceratobasidium sp.2** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Neonectria radicicola** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Acremonium sp.** | - | - | - | - | - | 15 | - | - | - | **15** |
| **Paraphoma sp.** | - | - | 14 | - | - | 11 | - | - | 1 | **26** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 1 | - | **1** |
| **Epicoccum nigrum** | - | 22 | - | - | 9 | - | 1 | - | - | **32** |
| **Embellisia sp.** | - | - | - | - | - | - | - | 1 | - | **1** |
| **Davidiella sp.** | 1 | - | 6 | - | - | 25 | - | - | - | **32** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 9 | - | **9** |
| **Fusarium sp.1** | - | - | 3 | - | - | - | - | - | 9 | **12** |
| **Fusarium sp.2** | - | - | 4 | - | - | - | - | 23 | - | **27** |
| **Fusarium sp.3** | - | - | 1 | - | 1 | 6 | 55 | - | - | **63** |
| **Fusarium oxyporum** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium solani** | - | 3 | - | - | 2 | - | - | - | - | **5** |
| **Glomerella acutata** | - | - | - | - | - | - | - | - | - | **-** |
| **Unknown** | - | 1 | 4 | - | - | 6 | 28 | 16 | 18 | **73** |
| **Total** | **7** | **97** | **37** | **2** | **68** | **91** | **107** | **134** | **49** | **592** |

**Table A.8:** Abundance of isolated colonies from identified fungal endophyte species associated with Magyar rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 3 | - | - | - | - | - | - | - | - | **3** |
| **Chaetomium sp.** | - | 2 | - | - | 2 | - | - | - | - | **4** |
| **Macrophomina phaseolina** | - | 2 | - | - | 4 | - | - | - | - | **6** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 30 | 35 | 65 | 8 | **138** |
| **Alternaria sp.2** | 7 | 87 | 7 | 2 | 49 | - | - | - | - | **152** |
| **Xylaria digitata** | - | - | - | 2 | - | - | - | - | - | **2** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | | **-** |
| **Acremonium furcatum** | - | - | - | - | - | 23 | - | - | 3 | **26** |
| **Rhyzopycnis vagum** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | - | - | - | - | - | - | - | **-** |
| **Neonectria radicicola** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Paraphoma sp.** | - | - | 5 | - | - | 4 | - | - | 1 | **10** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 1 | - | **1** |
| **Epicoccum nigrum** | - | 36 | 1 | - | 9 | - | 20 | 1 | - | **67** |
| **Embellisia sp.** | - | - | - | - | - | - | - | 1 | - | **1** |
| **Davidiella sp.** | - | - | 6 | 1 | - | 13 | - | - | - | **20** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 1 | - | **1** |
| **Fusarium sp.1** | - | - | 1 | - | - | - | - | 13 | 9 | **23** |
| **Fusarium sp.2** | - | - | - | - | - | - | - | 14 | - | **14** |
| **Fusarium sp.3** | - | - | - | - | - | 4 | 22 | - | - | **26** |
| **Fusarium oxyporum** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium solani** | - | 2 | - | - | 2 | - | - | - | - | **4** |
| **Glomerella acutata** | - | - | - | - | - | - | - | - | - | **-** |
| **Unknown** | 1 | - | 1 | - | 1 | 1 | 4 | - | 9 | **17** |
| **Total** | **11** | **129** | **21** | **5** | **67** | **75** | **81** | **96** | **30** | **515** |

**A.1.2. Biodiversity of endophytic fungi associated with other Prunus sp., rootstocks**

From *Prunus avium*, a rootstock with high prevalence in Hungarian niche (Vadcseresznye) was selected for the present study. According to the results, a total number of 372 fungal endophyte colonies were isolated from tissue samples of Vadcseresznye rootstock. The infection burden in the first season was relatively low as 30 colonies from twig samples and only 4 colonies from root samples were isolated and leaf samples showed no infection during season I. The rate of infection had an increase during the second and the third seasons. In season II, the largest number of colonies was isolated from roots (80 isolated colonies) but this amount reduced to 65 isolated colonies in season III. During the last sampling period, twig samples were more infected in compare with the other two tissue compartments as 84 isolated colonies were obtained from twigs in season III. Although leaf had the lowest infection burden in all seasons, but the number of isolated endophytes from leaf samples had a significant increase as 7 colonies in season II and 30 colonies in season III were isolated from these specimens. *Alternaria* sp.1 and *Alternaria* sp.2 were the most frequent species among other identified endophytes associated with Vadcseresznye rootstock; however the pattern of tissue colonization and temporal occurrence of these species showed a significant difference. Collectively, *Alternaria* sp.1 was only isolated from root samples in season II (21 isolated colonies, RF=27%) and from leaf (24 isolated colonies, RF=80%), twig (42 isolated colonies, RF=50%), and from root (25 isolated colonies, RF=38%) during season III. This species was not detected on Vadcseresznye rootstock in season I. On the other hand, *Alternaria* sp.2 was only observed on twigs during season I (20 isolated colonies, RF=67%) and during season II was isolated from leaf (4 isolated colonies, RF=57%) and from twig (48 isolated colonies, RF=67%), and had no occurrence in season III (Table A.9). Species richness in this rootstock was comparatively low in compare with other rootstocks during all seasons as the highest value in season I was demonstrated for twig (2 species) and in season II for root (5 species) and in season III for twig (5 species). Diversity indices also showed significantly homogeneous endophyte communities particularly in season III, whereas the infection burden was higher in this season in compare with other sampling periods ($e^{H'}$=0.4, D=1.72). Furthermore, despite a very low infection rate in season I, endophytic fungi species isolated from Vadcseresznye rootstock appeared to be highly diverse ($e^{H'}$=1.1, D=0). During the season I, no endophyte was found in leaf samples but colonization rate in twig explants showed a very high value in this season (CR=100%, IR=1.5). In the next two seasons,

root samples demonstrated the highest colonization and isolation rates (in season II: CR=73%, IR=1, and in season III: CR=91%, IR=1) while these value for twig were remained almost unchanged. Infection in leaf had a sever change from uninfected in season I to a high rate of colonization (CR=78%, IR=0.8) in season III.

A total of 407 colonies were isolated from Prob rootstock (*Prunus fruticosa*) during three sampling periods, while the highest infection burden was detected on twig in all seasons (109, 66, and 50 isolated colonies, respectively). In the first season 75 colonies were isolated from root samples, but the infection burden drastically decreased in root as in season III only 19 colonies were obtained from this tissue. On contrary, the number of isolated colonies in leaf was the lowest during the first two seasons (6, and 5 isolated colonies, respectively) but in season III increased to 37 isolated colonies, showing a temporal change in infection burden of leaves in this rootstock. The most frequent species was *Alternaria* sp.2 (125 isolated colonies) which was observed in season I on leaf (5 isolated colonies, RF=83%), twig (72 isolated colonies, RF=66%), and on root (4 isolated colonies, RF=5%), and in season II on leaf (5 isolated colonies, RF=100%) and on twig (39 isolated colonies, RF=59%). This species was not detected on root during the second season and had no occurrence in season III. *Alternaria* sp.1 was the second most frequent species which was found only in root (15 isolated colonies, RF=38%) during season II, and in all tissue samples: leaf (25 isolated colonies, RF=68%), twig (36 isolated colonies, RF=72%), and root (5 isolated colonies, RF=26%), in season III, but was not observed in season I. One colony of *Rosellinia* sp*.,* was detected in leaf samples from Prob rootstock during season I (Table A.10). The number of species identified on this rootstock was higher in the first season in compare with other sampling periods (17 identified species), while 9 species were harbored on root. In season II, 8 different species were identified on all specimens and the highest number (4 species) was found in root. During the last season, twig samples showed more species richness (a total of 6 identified species) in compare with other tissue compartments. Diversity of endophytic fungi on Prob rootstock was calculated the highest during season II ($e^{H''}$=1.17, D=0.78) and less diversity was observed in season III ($e^{H''}$=0.67, D=1.51). Twigs had the highest colonization and isolation rates during all seasons. In season I, root samples also showed comparatively high colonization and isolation rates (CR=74%, IR=0.8), however these value decreased in season II (CR=31%, IR=0.4) and remained almost constant through season III (CR=38%, IR=0.5). Leaf samples which had the lowest colonization and isolation rates among

other tissue specimens, (CR=8%, IR=0.1 for season I, and CR=6%, IR=0.1 for season II), showed a sudden increase in season III (CR=97%, IR=1).

Gisela6 rootstock, known as a hybrid rootstock of *Prunus cerasus* and *Prunus canescens*, was also examined regarding the association of endophytic fungi on different tissue compartments in the present study. As a sum, 407 colonies were isolated from this rootstock and the highest infection burden was observed in twig samples during all seasons (109, 66, and 50 isolated colonies, respectively). Infection burden showed a gradual decrease in root and sudden increase in leaf from season I to season III. In the first season, 75 colonies were isolated from root samples but this amount was 40 isolated colonies in season II and 19 isolated colonies in season III. Contrarily, the number of isolated colonies from 6 in season I ascended to 37 in season III for leaf samples. The most frequent species in Gisela6 rootstock was *Alternaria* sp.1 (72 isolated colonies) which was detected only in root (7 isolated colonies, RF=32%) during season II, but had occurrence on all tissues: 30 isolated colonies (RF=71%) in leaf, 21 isolated colonies (RF=55%) in twig, and 14 isolated colonies (RF=42%) in root, during the third season. This species was not detected on any specimen from different tissues of Gisela6 rootstock in season I. The second most frequent species was *Alternaria* sp.2, with no occurrence in season III, but was observed in leaf (1 isolated colony, RF=33%), twig (29 isolated colonies, RF=55%), and in root (17 isolated colonies, RF=85%) in season I and during season II was only isolated from twig (10 isolated colonies, RF=56%). *Rosellinia* sp*.,* was isolated only from leaf samples (one colony) during season I (Table A.11). The endophytes communities associated with this rootstock showed a comparatively low species richness in all seasons. The highest number of identified species was found in twigs: 4 species in season I, 4 species in season II, and 5 species in season III, while leaf had the less species richness: 2 species in season I, no identified species in season II, and 3 species in season III, among other examined tissues. The most heterogeneous endophyte assemblage associated with Gisela6 rootstock was observed in season II ($e^{H''}$=1.43, D=0.57), although diversity indices showed slight diversity in fungal endophyte communities during season I and season III. In season I, the highest values of colonization and isolation rates were calculated in twig (CR=98%, IR=1.3), but these indices decreased in season II (CR=33%, IR=1). In season III, 100% of explants obtained from twig were infected (CR=100%) and as an average at least one endophyte colony (IR=1) was isolated from every explants. Root samples had almost constant infection rates (CR=51%, IR=0.5 in season I, and CR=53%, IR=0.1 in

season II), but in season III 81% of explants (CR=81%) were infected and at least one endophytic colony (IR=1) was isolated from every inocula of this tissue. Leaf samples also had significant changes according to the colonization and isolation rate values. In season I, these values were CR=8% and IR=0.1, for leaf samples and in season II were very low (close to 0), but showed an increase to CR=63%, IR=1 in season III.

**Table A.9:** Abundance of isolated colonies from identified fungal endophyte species associated with Vadcseresznye rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Chaetomium sp.** | - | - | - | - | 6 | - | - | - | - | **6** |
| **Macrophomina phaseolina** | - | - | - | - | 6 | - | - | - | - | **6** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 21 | 24 | 42 | 25 | **112** |
| **Alternaria sp.2** | - | 20 | - | 4 | 48 | - | - | - | - | **72** |
| **Xylaria digitata** | - | - | - | 1 | - | - | - | - | - | **1** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | 1 | **1** |
| **Rhyzopycnis vagum** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | 4 | - | - | - | - | - | - | **4** |
| **Neonectria radicicola** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium sp.** | - | - | - | - | - | 3 | - | - | - | **3** |
| **Paraphoma sp.** | - | - | - | - | - | 29 | - | - | - | **29** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Epicoccum nigrum** | - | 10 | - | - | 12 | - | - | - | - | **22** |
| **Embellisia sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Davidiella sp.** | - | - | - | 1 | - | 19 | - | 4 | - | **24** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | 8 | - | **8** |
| **Fusarium sp.1** | - | - | - | - | - | - | - | 19 | 24 | **43** |
| **Fusarium sp.2** | - | - | - | - | - | - | - | 11 | - | **11** |
| **Fusarium sp.3** | - | - | - | - | - | 4 | 3 | - | - | **7** |
| **Fusarium oxyporum** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium solani** | - | - | - | - | - | - | - | - | - | **-** |
| **Glomerella acutata** | - | - | - | - | - | - | - | - | - | **-** |
| **Unknown** | - | - | - | 1 | - | 4 | 3 | - | 15 | **23** |
| **Total** | **0** | **30** | **4** | **7** | **72** | **80** | **30** | **84** | **65** | **372** |

**Table A.10:** Abundance of isolated colonies from identified fungal endophyte species associated with Prob rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 1 | - | - | - | - | - | - | - | - | **1** |
| **Chaetomium sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Macrophomina phaseolina** | - | 6 | - | - | 14 | - | - | - | - | **20** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 15 | 25 | 36 | 5 | **81** |
| **Alternaria sp.2** | 5 | 72 | 4 | 5 | 39 | - | - | - | - | **125** |
| **Xylaria digitata** | - | - | - | - | - | - | - | - | - | **-** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | 2 | **2** |
| **Rhyzopycnis vagum** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | 2 | - | - | - | - | - | - | **2** |
| **Neonectria radicicola** | - | - | 3 | - | - | - | - | - | - | **3** |
| **Acremonium sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Paraphoma sp.** | - | - | 17 | - | - | 12 | - | - | 3 | **32** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 1 | - | **1** |
| **Epicoccum nigrum** | - | 24 | 2 | - | 13 | - | 10 | 1 | - | **50** |
| **Embellisia sp.** | - | - | - | - | - | - | - | 2 | - | **2** |
| **Davidiella sp.** | - | - | 21 | - | - | 10 | - | - | - | **31** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium sp.1** | - | - | 1 | - | - | - | - | 1 | 8 | **10** |
| **Fusarium sp.2** | - | - | - | - | - | - | - | 8 | - | **8** |
| **Fusarium sp.3** | - | - | 1 | - | - | 3 | 1 | - | - | **5** |
| **Fusarium oxyporum** | - | - | 4 | - | - | - | - | - | - | **4** |
| **Fusarium solani** | - | 6 | - | - | - | - | - | - | - | **6** |
| **Glomerella acutata** | - | - | - | - | - | - | - | - | - | **-** |
| **Unknown** | - | 1 | 20 | - | - | - | 1 | 1 | 1 | **24** |
| **Total** | **6** | **109** | **75** | **5** | **66** | **40** | **37** | **50** | **19** | **407** |

**Table A.11:** Abundance of isolated colonies from identified fungal endophyte species associated with Gisela6 rootstock in three sampling periods.

| Fungi Genera | Season I | | | Season II | | | Season III | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | *Leaf* | *Twig* | *Root* | |
| **Rosellinia sp.** | 1 | - | - | - | - | - | - | - | - | **1** |
| **Chaetomium sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Macrophomina phaseolina** | - | - | - | - | 1 | - | - | - | - | **1** |
| **Pyronema sp.** | - | - | - | - | - | - | - | - | - | **-** |
| **Alternaria sp.1** | - | - | - | - | - | 7 | 30 | 21 | 14 | **72** |
| **Alternaria sp.2** | 1 | 29 | 17 | - | 10 | - | - | - | - | **57** |
| **Xylaria digitata** | - | - | - | - | - | - | - | - | - | **-** |
| **Aspergillus niger** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium furcatum** | - | - | - | - | - | - | - | - | - | **-** |
| **Rhyzopycnis vagum** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.1** | - | - | - | - | - | - | - | - | - | **-** |
| **Ceratobasidium sp.2** | - | - | - | - | - | - | - | - | - | **-** |
| **Neonectria radicicola** | - | - | - | - | - | - | - | - | - | **-** |
| **Acremonium sp.** | - | - | - | - | - | 1 | - | - | - | **1** |
| **Paraphoma sp.** | - | - | 1 | - | - | - | - | - | - | **1** |
| **Phomopsis sp.** | - | - | - | - | - | - | - | 3 | - | **3** |
| **Epicoccum nigrum** | - | 19 | - | - | 4 | - | 10 | - | 1 | **34** |
| **Embellisia sp.** | - | - | - | - | - | - | - | 8 | - | **8** |
| **Davidiella sp.** | - | - | - | - | - | 14 | - | - | - | **14** |
| **Botrytis cinerea** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium sp.1** | - | - | - | - | - | - | - | - | 14 | **14** |
| **Fusarium sp.2** | - | - | - | - | - | - | - | 4 | - | **4** |
| **Fusarium sp.3** | - | 3 | - | - | - | - | 1 | - | - | **4** |
| **Fusarium oxyporum** | - | - | - | - | - | - | - | - | - | **-** |
| **Fusarium solani** | - | 2 | - | - | 3 | - | - | - | - | **5** |
| **Glomerella acutata** | - | - | - | - | - | - | - | 2 | - | **2** |
| **Unknown** | 1 | - | 2 | - | - | - | 1 | - | 4 | **8** |
| **Total** | **3** | **53** | **20** | **0** | **18** | **22** | **42** | **38** | **33** | **229** |

## A.2. Radial growth measurements for antagonistic activity of selected fungal endophytes

Two pathogens, *Monilia laxa* and *Agrobacterium tumefaciens*, were chosen for examining the growth inhibitory effect of nominated endophyte in vitro. The growth rate of these pathogens on PDA while were solely cultured has been shown in Figure A.1.

*Ceratobasidium* sp.1 was the most vigorous species regarding its higher growth rate among endophytic fungi examined in this study. In single cultures, colonies of this fungus had averagely 0.7 mm/day, 0.8 mm/day, and 0.8 mm/day expansion to horizontal, diagonal, and vertical directions, respectively. Nonetheless, a drastic recline in development of endophyte colonies was observed after day 8. Dual-culture of this endophyte with *Monilia laxa* significantly ($P<0.05$) decreased the pathogen horizontal (0.03 mm/day) and diagonal (0.1 mm/day) growth on PDA but had no significant effect on vertical growth rate of the pathogen. Accordingly, the growth inhibition index showed that the pathogen colony expansion toward horizontal and diagonal directions were 76% and 60% lower than its growth in single culture plates, respectively. In compare with single cultures, diagonal and vertical growth of the endophyte showed the same pattern of recline in dual-cultures with *Monilia laxa,* but the horizontal growth extended even to day 15 in these plates. *Agrobacterium tumefaciens* also showed a significant decrease in colony expansion toward all directions when confronted *Ceratobasidium* sp.1 in dual cultures (horizontal GR=0.01 mm/day, diagonal GR=0.02, and vertical GR=0.01, $P<0.05$). Diagonal expansion of the bacterial pathogen showed GI=70% decrease in dual cultures while restriction of pathogen growth in horizontal and vertical directions was calculated as GI=56% and GI=51% of the observed colony development in single cultures, respectively. No difference was observed according to features of the endophyte colonies and their growth on PDA between single cultures of this endophyte and dual-cultures with *Agrobacterium tumefaciens* (Figure A.2).

*Alternaria* sp.1 in control cultures showed a medium growth rate (horizontal GR=0.3 mm/day, diagonal GR=0.3 mm/day, and vertical GR=0.2 mm/day), which did not significantly differ in dual cultures with both pathogens. Growth of *Monilia laxa* colonies, however, significantly decreased in horizontal (GR=0.07 mm/day, GI=66%, $P<0.05$) and diagonal (GR=0.1 mm/day, GI=59%, $P<0.05$) when were cultured in the same plate with *Alternaria* sp.1. The same pattern of inhibition was detected in dual-cultures of this endophyte with *Agrobacterium tumefaciens*. According to the results, growth rate of the bacterium was weakened significantly in horizontal (GR=0.01 mm/day, GI=54%) and diagonal (GR=0.01

mm/day, GI=53%) and vertical (GR=0.02 mm/day, GI=46%) ($P<0.05$) directions when encountered the endophyte in dual-cultures (Figure A.3).

*Botrytis cinerea* showed a promising growth rate in single cultures during the first 9 days of incubation, although colonies had a sudden recline after this period in their vertical extension. Such a weakened growth was observed in *Botrytis cinerea* colonies toward other directions after day 13. Being cultured in the same plate with pathogen organisms, the growth pattern of the endophyte remained unchanged, whereas decrease in vertical growth of the endophyte colonies was postponed to day 13-14 and did not occur in diagonal and horizontal directions when confronted with *Monilia laxa* and *Agrobacterium tumefaciens* in dual-cultures. On contrary, growth factors of both pathogens were significantly lower in dual-cultures in compare with their controls. Botrytis cinerea had the most sever effect on horizontal and diagonal growth rates of *Monilia laxa* among other examined endophytes. According to the results, the recent pathogen showed restricted growth in dual-cultures with the endophyte as its colonies had GR=0.02 mm/day in horizontal direction (GI=82%), GR=0.03 mm/day in diagonal direction (GI=78%), and GR=0.1 in vertical direction (GI=40%) in this cultures. The value of P was $<0.05$ for all analysis. This endophyte also had strong growth inhibitory effect on *Agrobacterium tumefaciens* on PDA and growth indices of the pathogen were GR=0.01 mm/day in horizontal direction (GI=50%), GR=0.007 mm/day in diagonal direction (GI=57%), and GR=0.01 mm/day in vertical direction (GI=48%) with acceptable value of significance $P<0.05$ (Figure A.4).

Although *Embellisia* sp.*,* showed a decline in vertical growth of the colonies after 15 days in control cultures, but in dual-cultured plates had almost similar growth rate in all direction during the incubation period. This fungus had significant growth inhibitory effect on *Monilia laxa* in every directions (horizontal GR=0.04 mm/day, GI=76%, diagonal GR=0.1, GI=53%, $P<0.05$) except vertical growth of the pathogen in which the decrease in extension of the colonies was not significant. On the other hand, this endophyte restricted the growth of *Agrobacterium tumefaciens* toward all directions: horizontal GR=0.01 mm/day, GI=54%, diagonal GR=0.02 mm/day, GI=50% and vertical GR=0.01, GI=51% ($P<0.05$) in dual-cultures (Figure A.5).

*Epicoccum nigrum* had almost the same (~0.3 mm/day) growth rate in all directions in single cultures, however the vertical expansion of its colonies was impaired at the end of incubation period. In dual-cultures with *Monilia laxa*, the endophyte colonies grew vertically as well as to other directions even after 15 days. Cultured with *Agrobacterium tumefaciens*, the endophyte,

however, showed the same growth pattern as that in single cultures. Antagonistic effect of this fungus on horizontal (GR=0.06 mm/day, GI=68%) and diagonal (GR=0.08, GI=64%) growth (but not on vertical growth) of *Monilia laxa* was found significant ($P<0.05$). Besides, this endophyte significantly inhibited the growth of *Agrobacterium tumefaciens* in every direction: horizontal GR=0.02 mm/day, GI=40%, diagonal GR=0.02 mm/day, GI=46%, and vertical GR=0.03 mm/day, GI=38%, although the overall growth inhibitory effect of this fungus against the bacterial pathogen was weaker than other examined endophytic fungi (Figure A.6).

*Fusarium oxy*sp*orum* in single cultures had a dramatic recline between day 9 and day 15 according to the expansion of colonies in every direction. Dual-culture with pathogen microorganisms only resulted to an amplified horizontal growth of the endophyte colonies, but impaired development of colonies was observed for other directions. This endophyte had significant growth inhibitory effect on *Monilia laxa* as in dual-cultures resulted in decreased growth factors of the pathogen: horizontal GR=0.04 mm/day, GI=73%, diagonal GR=0.1 mm/day, GI=64%, vertical GR=0.2 mm/day, GI=25% ($P<0.05$). These values were also significantly lower than control cultures for *Agrobacterium tumefaciens* when confronted *Fusarium oxy*sp*orum*: horizontal GR=0.02 mm/day, GI=47%, diagonal GR=0.02 mm/day, GI=47%, and vertical GR=0.03 mm/day, GI=51% ($P<0.05$) (Figure A.7).

No change was observed in growth pattern of *Rhizopycnis vagum* between dial-cultures with pathogens and control plates. This fungus had promising growth on PDA, although its expansion toward vertical direction declined after 11 days of incubation. The antagonistic effect of this endophyte against *Monilia laxa* caused significant decrease in growth rates of the pathogen colonies toward horizontal (GR=0.08 mm/day, GI=60%), and diagonal (GR=0.1 mm/day, GI=52%, $P<0.05$), but not vertical direction. Although this endophyte showed the lowest growth inhibitory effect against horizontal extension of *Agrobacteriun tumefaciens* in dual-cultures among other examined endophytes, but significantly restricted the pathogens growth toward all directions ($P<0.05$): horizontal GR=0.03 mm/day, GI=34%, diagonal GR=0.02 mm/day, GI=47%, and vertical GR=0.03 mm/day, GI=51% (Figure A.8).

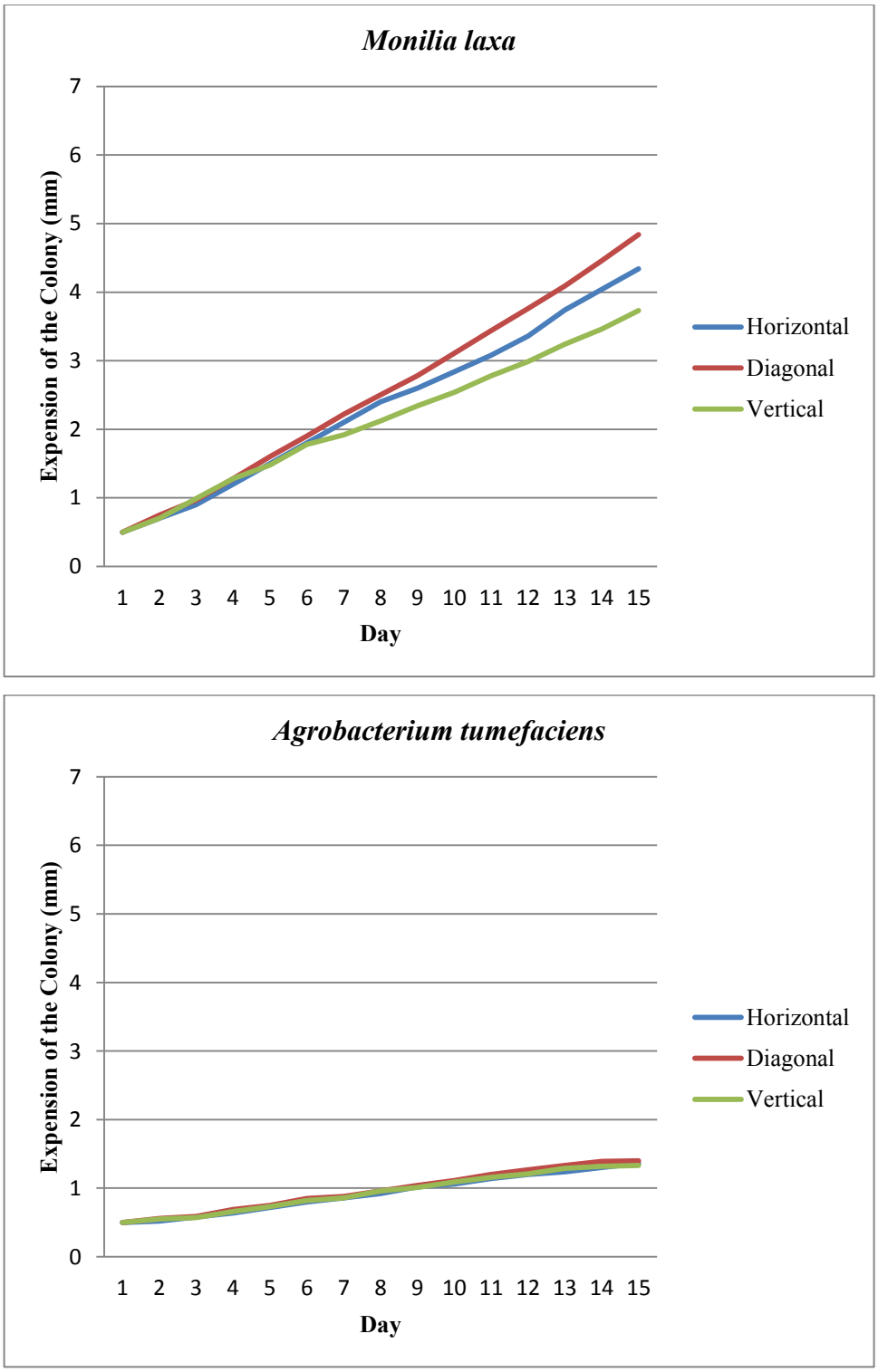


**Figure A.1:** Growth rate (mm/day) of pathogenic organisms applied for antagonistic activity tests in single culture plates (control samples).

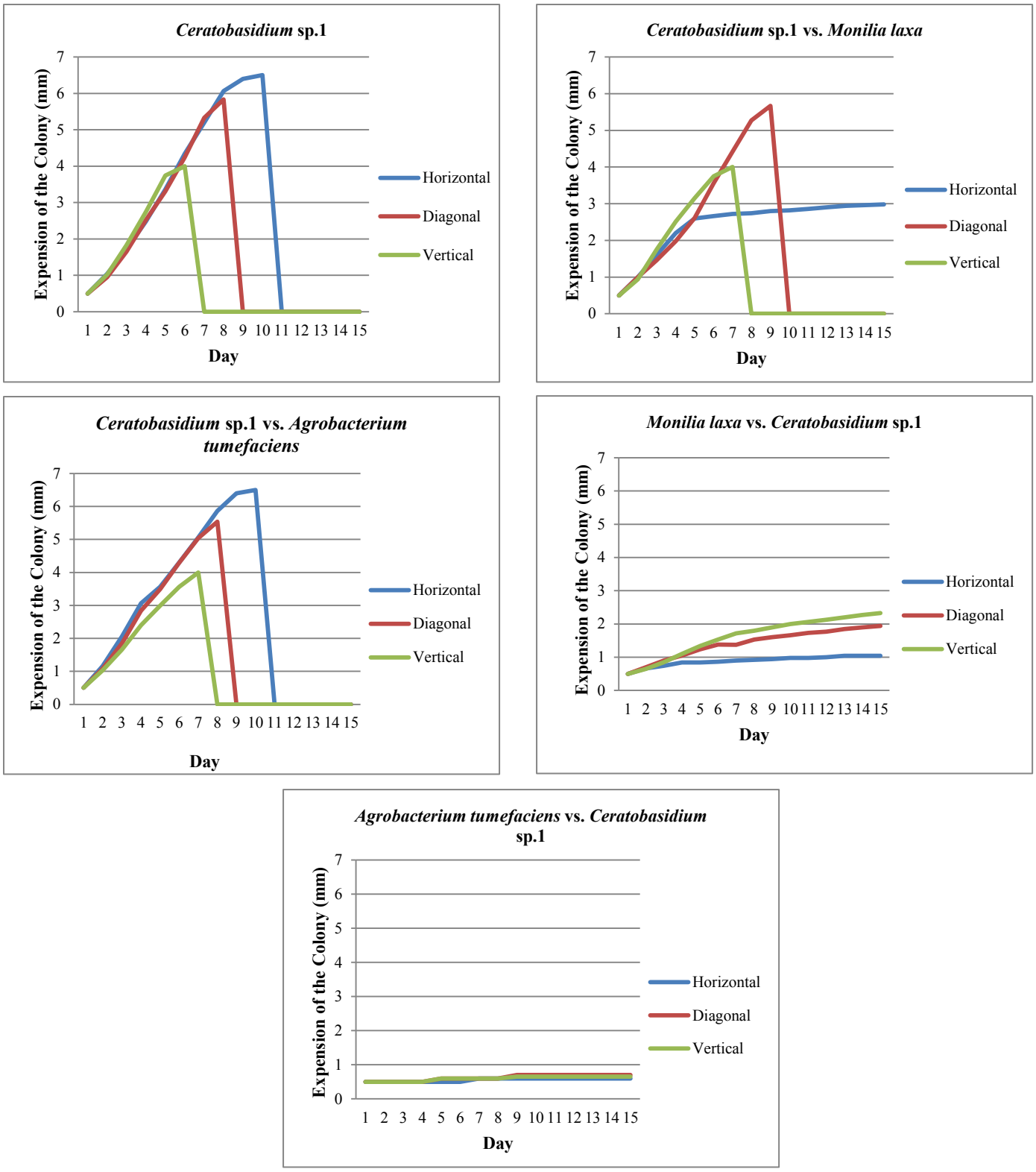


**Figure A.2:** Growth rate of colonies, measured in three directions, in single cultures of *Ceratobasidium* sp.1 and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

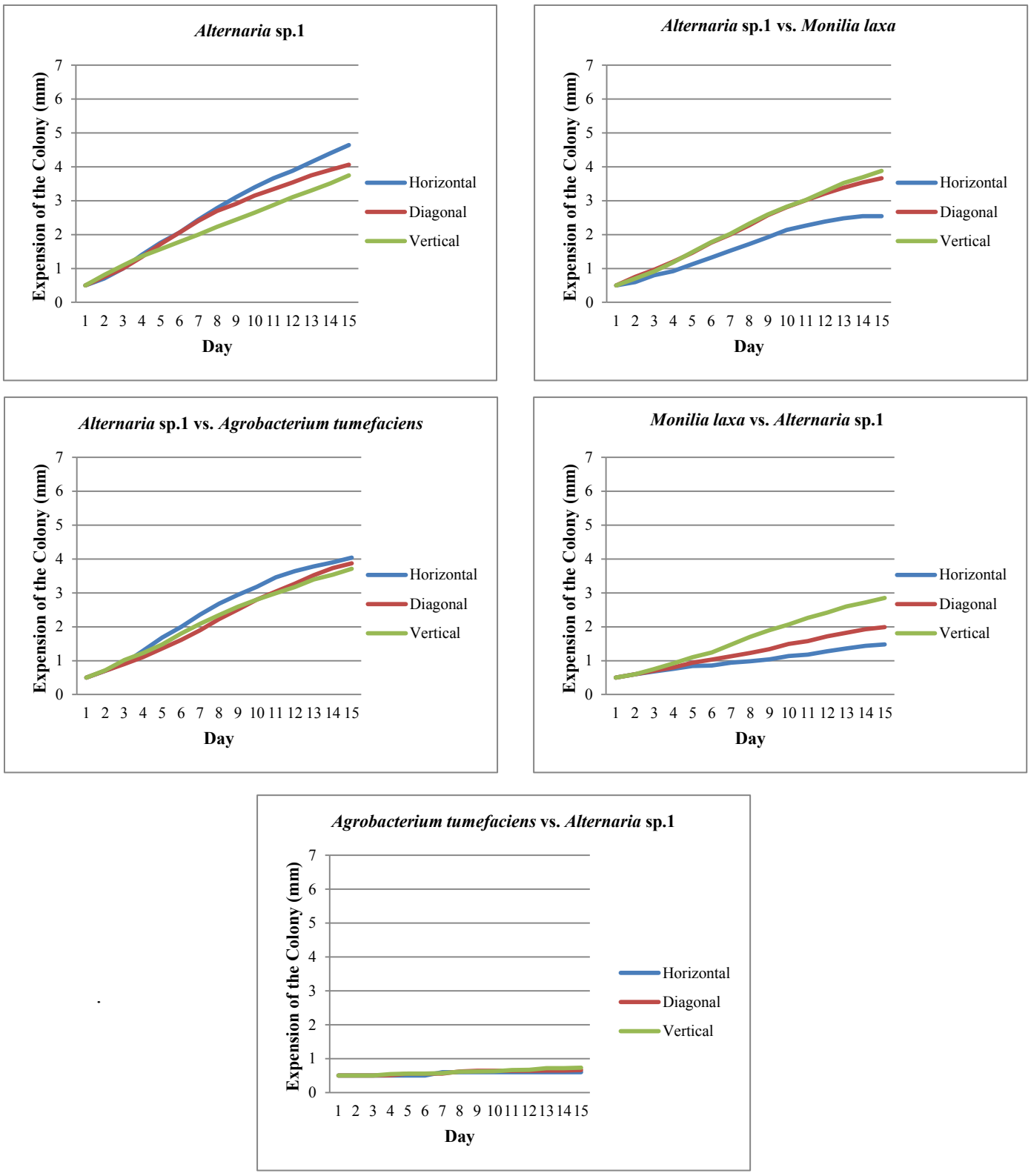


**Figure A.3:** Growth rate of colonies, measured in three directions, in single cultures of *Alternaria* sp.1 and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

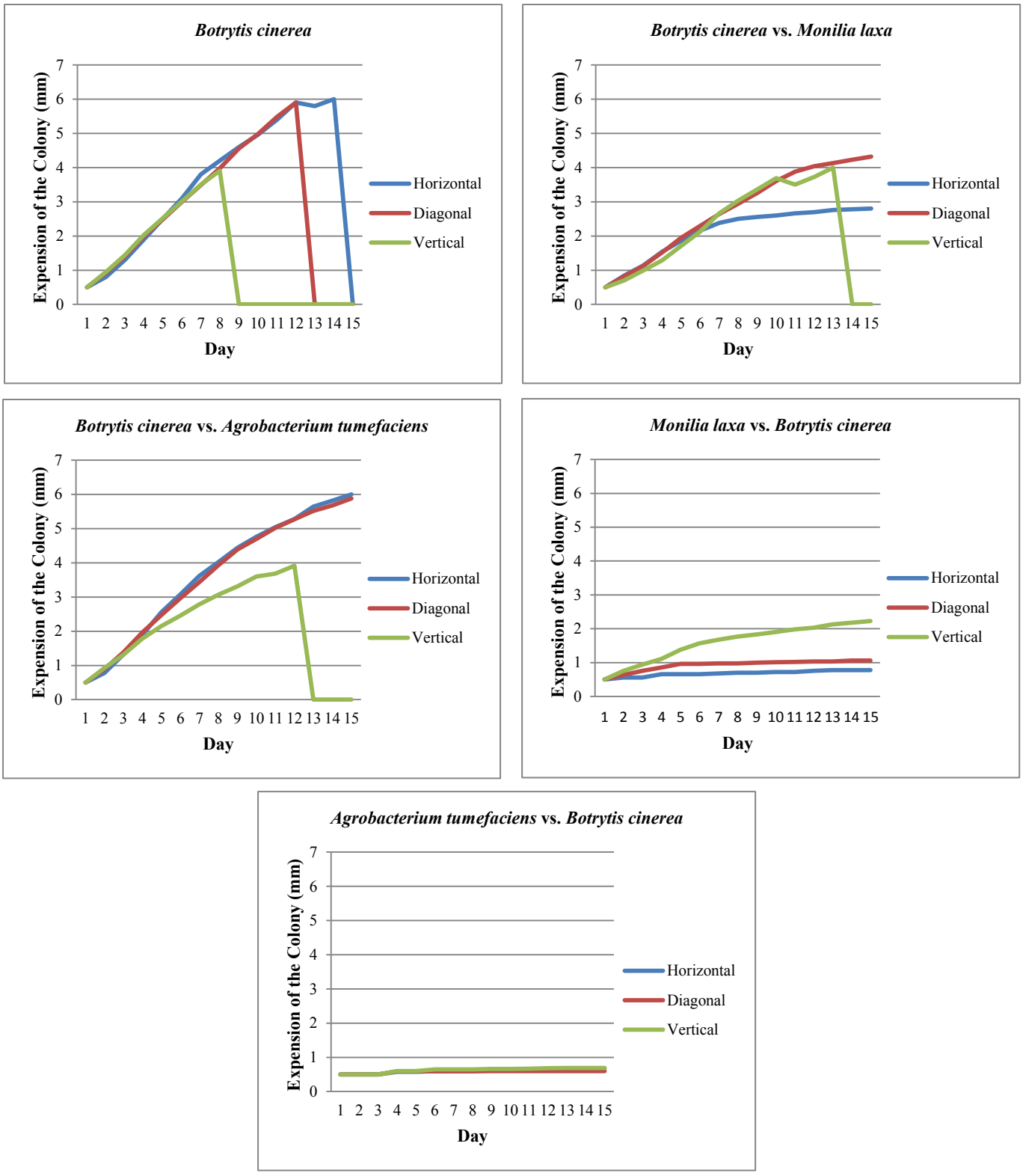


**Figure A.4:** Growth rate of colonies, measured in three directions, in single cultures of *Botrytis cinerea* and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

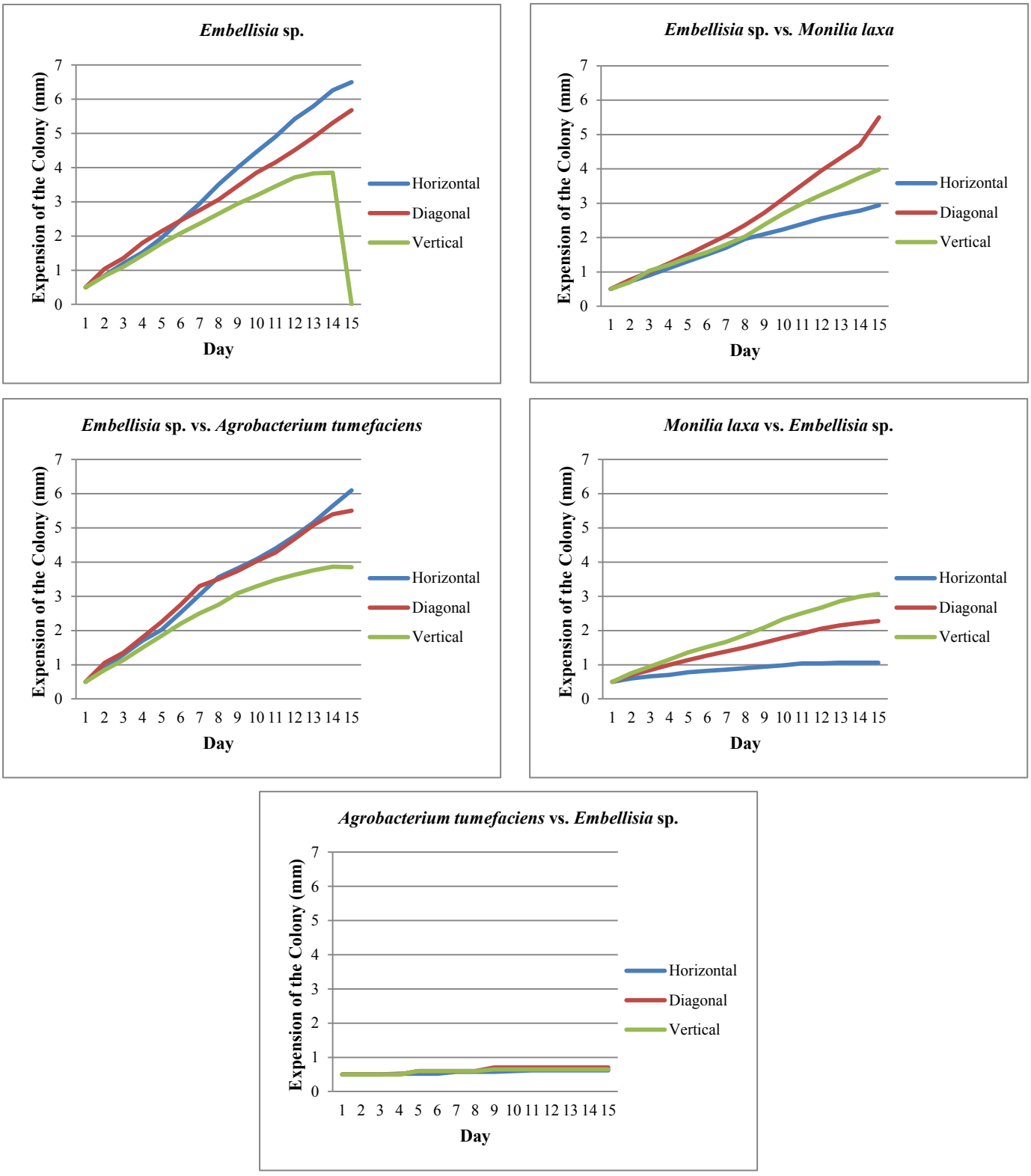


**Figure A.5:** Growth rate of colonies, measured in three directions, in single cultures of *Embellisia* sp., and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

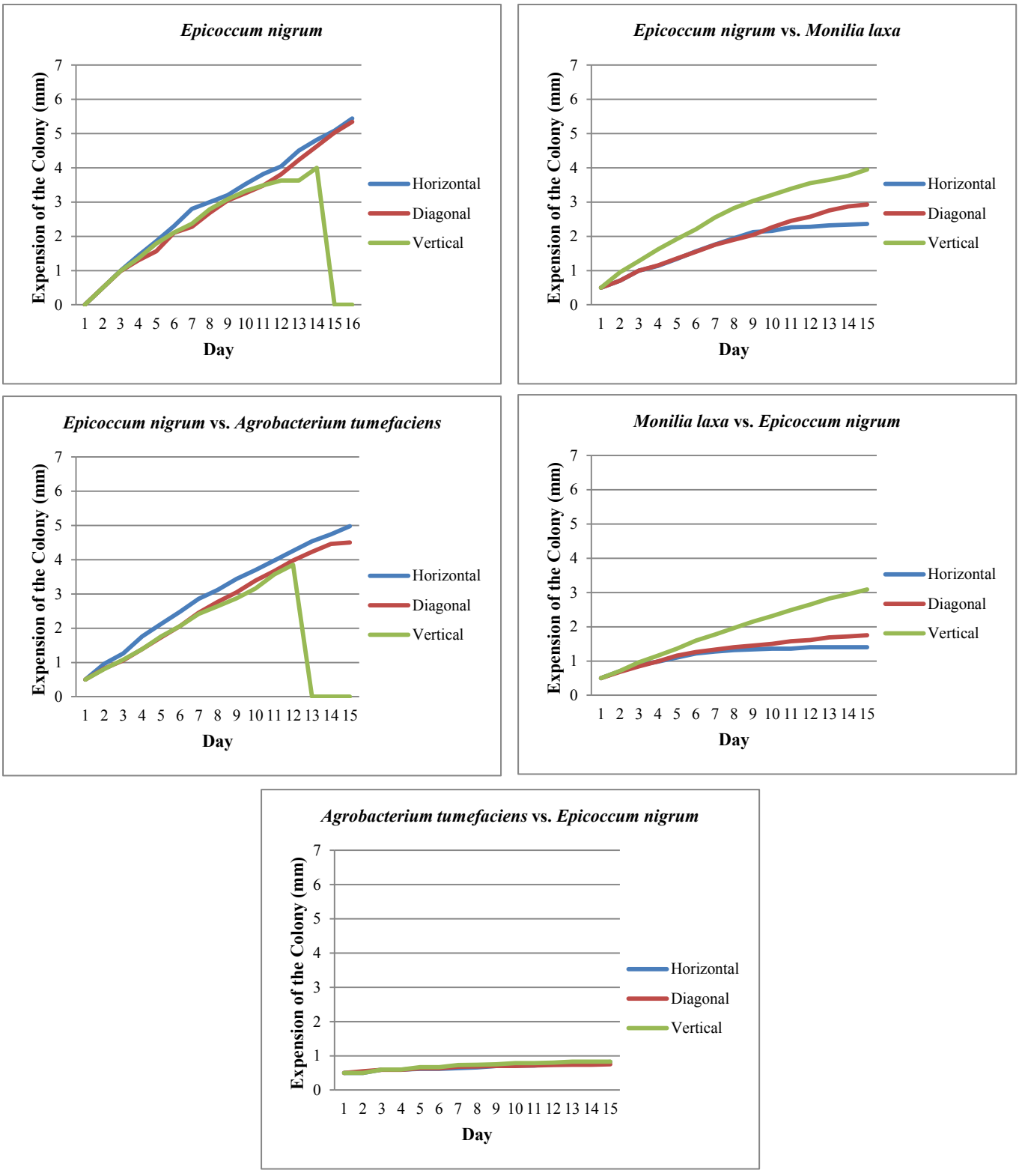


**Figure A.6:** Growth rate of colonies, measured in three directions, in single cultures of *Epicoccum nigrum* and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

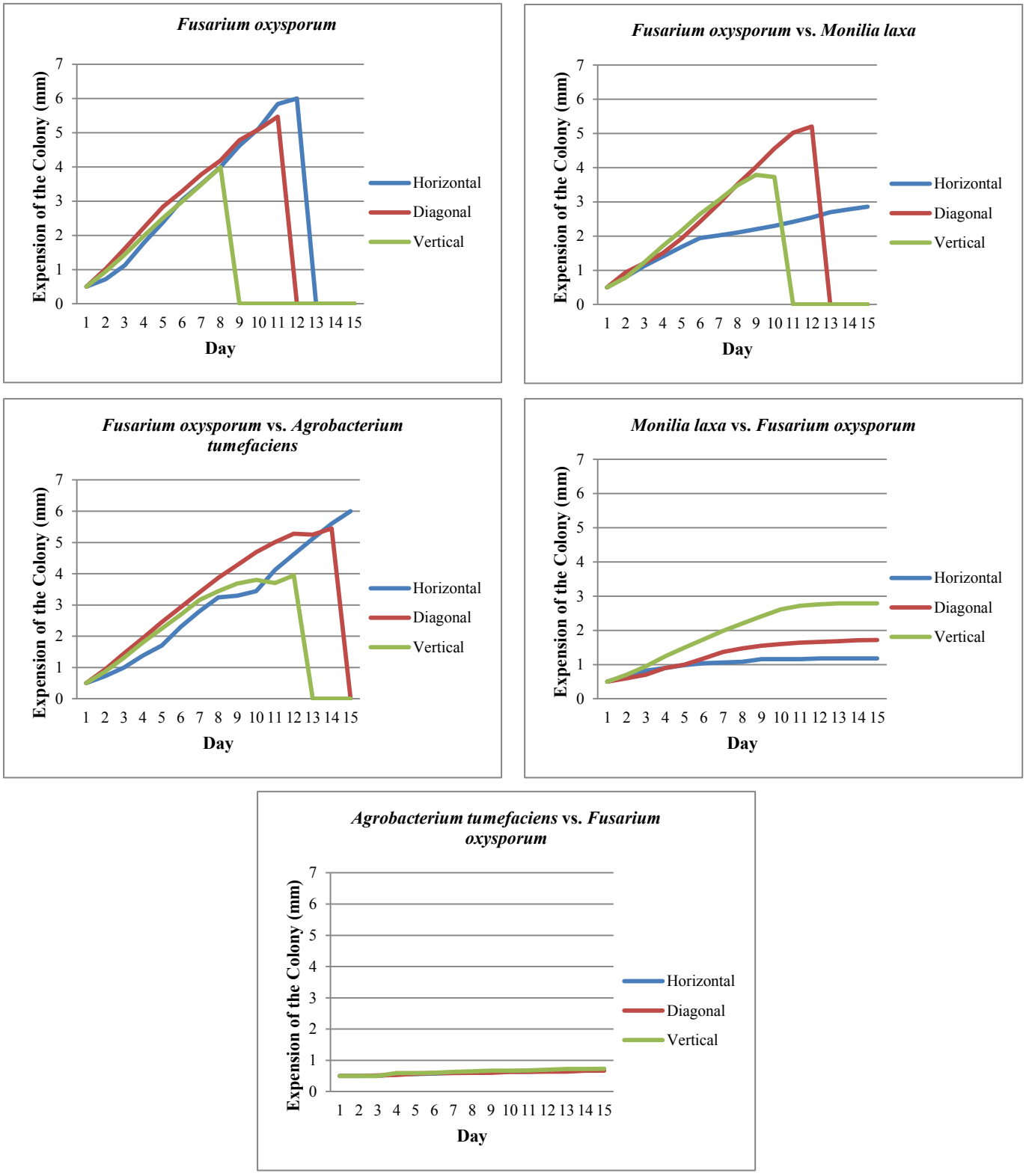


**Figure A.7:** Growth rate of colonies, measured in three directions, in single cultures of *Fusarium oxysporum* and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

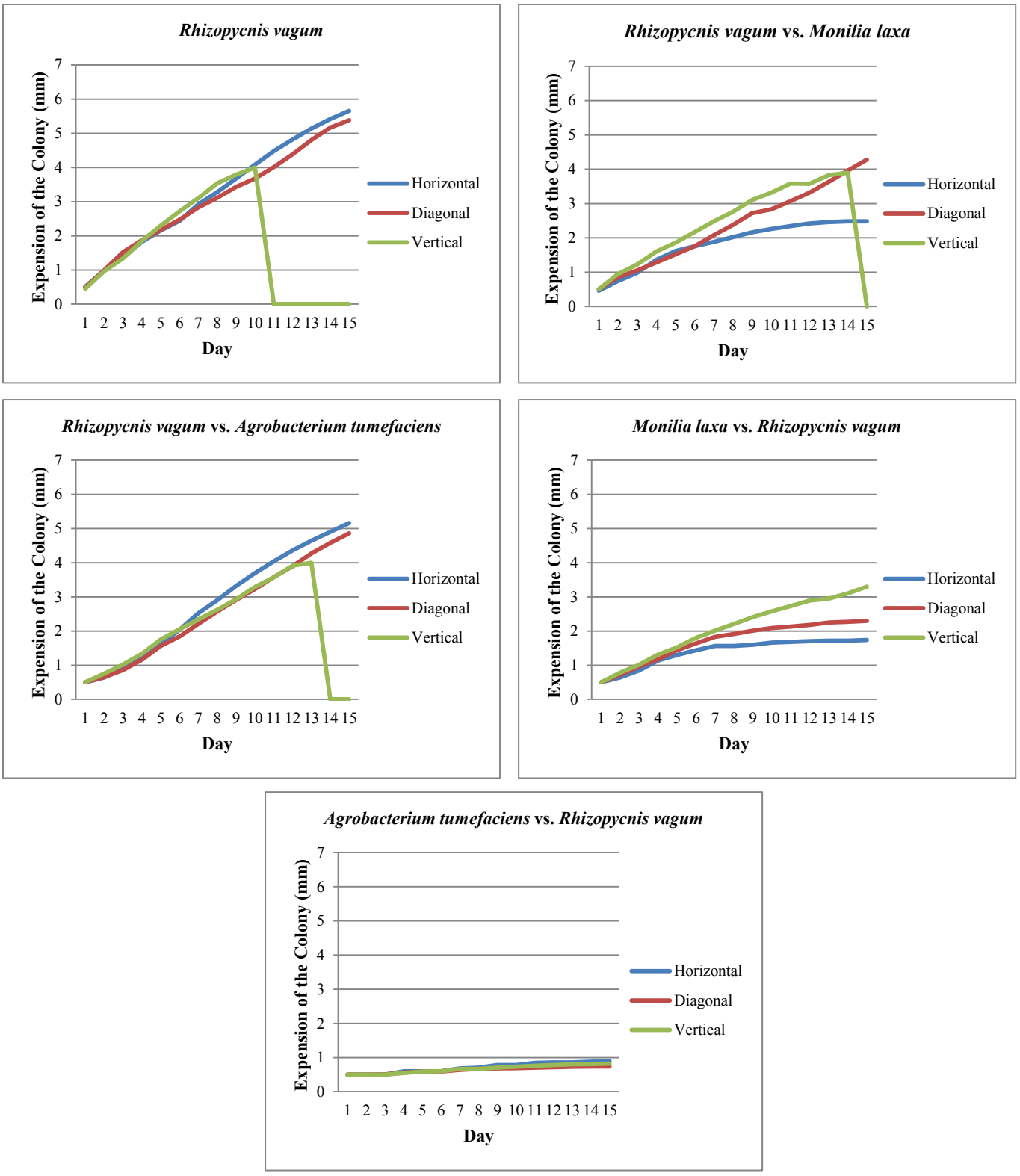


**Figure A.8:** Growth rate of colonies, measured in three directions, in single cultures of *Rhizopycnis vagum* and dual-cultures of this fungus with selected pathogenic organisms, has been demonstrated in this Figure.

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