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TESI DI DOTTORATO DI RICERCA

[Apple Proliferation disease: insights on the phenomenon of 'recovery' and use of fungal endophytes for phytoplasma control]

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LIST OF ABBREVIATIONS

ACO: Aminocyclopropane-1 carbo- xylate oxidase

AFLP: Amplified fragment length polymorphism

AGO: Argonaute protein

AOS2: Allene oxide synthase 2

AP: apple proliferation

AY-WB: Aster yellows witches' broom

BLAST: Basic Local Alignment Search Tool

BN: Bois Noir

bp: base pair

Ca. P. mali: *Candidatus* Phytoplasma mali

CaSy: Callose synthase

CMT: Chromomethylase

CSS: Corn stunt spiroplasma

Ct: Threshold cycle

CTAB: Cetyltrimethylammoniumbromid

cv: Cultivar

D: Diseased

DAD: diode array detector

DME: DEMETER

DML: DEMETER-like

DNA: Desoxyribonucleid acid

DRM: Domain rearranged methyltransferase

E: Efficiency

EBS-Medium - medium for bacteria

EDTA: Ethylene-diamine-tetra-acetate

EE: ethyl acetate

EP: Endophyte

ERG: C2 domain containing protein

F: Fraction

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GU: genomic unit

H: Healthy

HPLC: high performance liquid chromatography

IOM: International Organization of Mycoplasmology

ISR : interspace regions

ITS: internal transcribed spacer

JA : Jasmonic acid

kb: kilobase pairs

m/z: mass-to-charge ratio

MET: Cytosine-5-methyltransferase

MIC: minimum inhibitory concentration

min: minute

MLOs: Mycoplasma-like organisms

MNE: Mean Normalized Expression

MS: mass spectroscopy

NMR: nuclear magnetic resonance

NPR: Non-expressor Pathogenesis-related protein

nt: not tested

OPDA: oxophytodienoic acid

OPR12: Oxophytodienoate reductase12

OY-M: onion yellows M strain

PCR: polymerase chain reaction

PI II: Proteinase inhibitor II

Pol: polymerases

PP: Phloem protien

PR: Pathogenesis-related protein

qRT-PCR: reverse transcription-PCR

R: Recovered

RdDM: RNA-directed DNA methylation pathways

rDNA: ribosomal DNA

RDR: RNA-Dependent RNA polymerase

RFLP: Restriction fragment length polymorphism

ROS1: Repressor of silencing1

rp: Ribosomal protein

rpm: rotations per minute

SA: Salicylic acid

SAR: Systemic acquired resistance

SE: standard error

SEO: Sieve Element Occlusion

sp.: species

TAE-buffer: Tris-acetate-EDTA buffer

TEM : Transmission Electron Microscopy

Tris: Tris-(hydroxymethyl)-aminomethan

UV: ultraviolett

XAD: adsorber resin

YM-Medium: yeast-malt-glucosemedium

ZM½: Sugar-Malt-medium

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ABSTRACT

Phytoplasmas are insect-transmitted, phloem-restricted pathogens that can cause devastating losses in crops of economical importance such as fruits, vegetables, ornamental or weeds. Apple proliferation (AP) disease is considered as one of the most important disease in all apple production areas generally and in Italy especially. AP disease is associated with the phytoplasma '*Candidatus* Phytoplasma mali' ('*Ca*. P. mali'), which belongs to the 16SrX group. Spontaneous remission of AP symptoms in plants that previously were symptomatic is called 'recovery'. Recovery of apple trees from AP were studied by gene expression analyses of several plant defense-related genes. Real Time-PCR analyses demonstrated that genes were differentially expressed in apple leaf tissue according to the plants' state of health.

Since phytoplasma diseases are not curable, the fungal endophyte *Epicoccum nigrum*, previously reported as biocontrol agent or resistance inducer against different pathogens, inoculated in apple plants, maintained in semi-field conditions, as a possible strategy for the control of AP disease.

In this study, the elucidation of plant–phytoplasma–endophyte relationships performed by defense-related gene expression analyses and ultrastructural observations on leaf tissues. Real Time-PCR analyses demonstrated that tested genes were differentially expressed in apple leaf tissue following endophyte inoculation in the shoot. Phytoplasma concentration was quantified by real-time PCR in endophyte-treated and untreated plants: preliminary results revealed that '*Ca*. P. mali' was less concentrated in the treated plants. Ultrastructural observations revealed that in endophyte-treated plants, cytological changes, such as abundant callose depositions and P-protein aggregations in the sieve elements, occurred.

Thirteen endophytic strains of *E. nigrum* were cultivated (submerged culture) in two liquid media (YM and ZM ½) and the crude extracts were then analyzed by HPLC-DAD/MS. The molecular phylogenetic affinities among the different strains were also analyzed using ITS and partial ß-tubulin gene sequences.

HPLC-DAD/MS metabolite profiles for the thirteen isolates grown the two different culture media revealed a variation in the secondary metabolites profiles. Using the ZM ½ medium resulted in more complex metabolite profile and more variability of the produced metabolites.

Preliminary ultramicroscopical observations performed on leaf tissues from AP-infected cuttings of the model plant *Catharanthus roseus*, treated with the most active fungal secondary metabolites, revealed ultrastructural modifications in some phytoplasma cells and cytological defense reactions in the host plant tissues.

The results of this study shown that *E. nigrum* is a highly diverse fungal endophyte, producing a broad range of bioactive secondary metabolites. Moreover, this study might represent a first step in the clarification of plant–phytoplasma–endophyte relationships to find possible strategies for phytoplasma diseases control.

THE PURPOSE OF THIS STUDY

In this study we aimed to improve knowledge about apple plants and '*Candidatus* Phytoplasma mali' interactions and, in particular, about the spontaneous phenomenon of 'recovery', which is not fully understood yet. About recovery, we concentrated the experiments on expression analyses of apple genes coding for special phloem proteins, named sieve element occlusion (SEO) proteins, involved in phloem response to stress. Moreover, expression levels of genes coding for pathogenesis-related proteins and jasmonate-pathway marker enzymes were analysed. Genes involved in DNA methylation and demethylation were also studied as epigenetic markers of 'recovery'.

As it was demonstrated that 'recovery' could be explained through the involvement of a systemic acquired resistance (S.A.R) in the apple plant (Musetti *et al.*, 2004), it was hypothesized that endophytic micro-organisms could be involved in resistance being established in the host. Iin this study we also investigated the interactions intercurring between the apple fungal endophyte *Epicoccum nigrum* and *Malus domestica*. Using combined approache, we aimed to verify if the preventive endophyte inoculation in apple plants could trigger defense reactions in the host against '*Ca*. P. mali'.

Furthermore, different *E. nigrum* endophytic isolates were characterized using integrated molecular and chemical approaches. Antagonistic tests against different cultivable microorganisms were performed to verify if the fungal extracts would be bioactive. A test against phytoplasmas was set up *in vivo*, using the model plant *Catharanthus roseus*.

INTORDUCTION

The phytoplasmas

Phytoplasmas are insect-transmitted, phloem-restricted pathogens that can cause devastating losses in crops of economical importance such as fruits, vegetables, ornamental or weeds (Lee *et al.*, 2000). Phytoplasmas are characterized by lacking a rigid cell wall, they are small in size (±500 nm in diameter) surrounded by a three-layered cell membrane. They are variable in shape (pleomorphic), presenting round, elongate and dumbbell forms (Figure 1) (Anderson *et al.*, 2001).

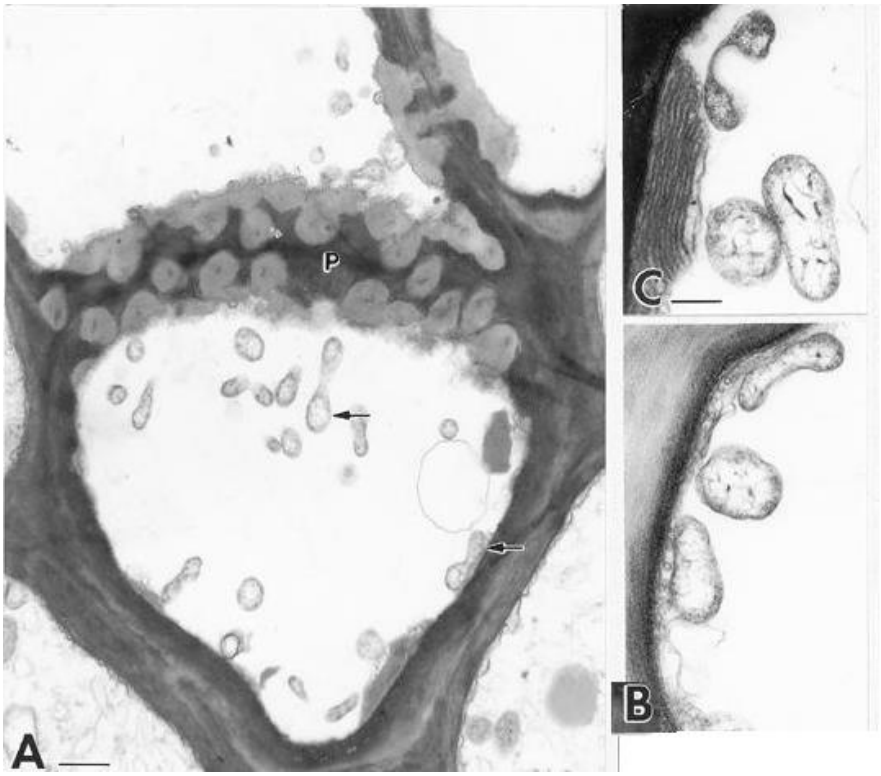


Figure 1. Transmission electron micrographs of phytoplasma cells within a vascular bundle rhizome: A, sieve element with sieve plate (P), where phytoplasmas with variable shapes are shown. Selected dumbbell-shaped phytoplasma cells are indicated by arrow. In B and C, phytoplasma cells are visible at higher magnification. Bar represents 500 nm in A, and 200 nm in B and C (Anderson *et al.*, 2001).

Comparing to bacteria, phytoplasmas are characterized by possessing a small genome lacking genes coding the majority of the essential metabolic pathways, so they are obligate parasite. Considerable variation in genome size from 660 to 1,130 kilobases (kb) was observed (Marcone *et al.,* 1999) with low content of G+C, thus varying from 23 to 29.5 mol% (Firrao *et al.,* 1996). Most phytoplasmas contain one or two circular double-stranded chromosomal DNA molecules (Neimark and Kirkpatrick, 1993). However, some phytoplasmas, including '*Candidatus* Phytoplasma mali', possess linear chromosomes (Kube *et al.,* 2008).

Up to date, only four phytoplasma genomes were fully sequenced. These include two strains belonging to *'Candidatus* Phytoplasma asteris', the onion yellows M (OY-M) strain (Oshima *et al.,* 2004), and the aster yellows witches' broom (AY-WB) strain (Bai *et al.,* 2006); a strain of '*Candidatus* Phytoplasma australiense' (Liefting *et al.,* 2006; Tran-Nguyen *et al.,* 2008) and a strain of *'Candidatus* Phytoplasma mali' (Kube *et al.,* 2008).

Attempts to culture axenically phytoplasma have hitherto always failed, so phytoplasma have been always known to be uncultivable in cell-free medium, which restricts their biological, chemical and physiological studies, as well as the genome sequencing and functional genomics studies. However, recently a successful axenic culture of a phytoplasma on a specific commercial medium was demonstrated, and the identity of the organism was confirmed by PCR/RFLP analyses and sequencing of phytoplasma-specific genes (Contaldo *et al.,* 2012). However, further optimization is needed in order to obtain sufficient colony growth for future studies of this organism.

Taxonomic classification of phytoplasma

Four decades ago it was demonstrated that yellows symptoms present on diseased plants were not always associated to viral infections. Doi *et al.,* (1967) demonstrated by transmission electron microscope, the presence of prokaryotes similar to the mycoplasmas affecting animals and humans, in the phloem of such plants. Those prokaryotes were named Mycoplasma-like organisms (MLOs). In 1992, the phytoplasma working team, during the 9th Congress of the International Organization of Mycoplasmology (IOM), named 'phytoplasma' the MLOs found in plant tissues (Firrao *et al.,* 2004). Since then phytoplasmas were considered to be a new class of plant pathogens, and they were subjected to several new classifications as new strain groups were discovered.

After phytoplasma discovery, several systems based on phytoplasma biological characteristics, were adopted for phytoplasma classification, but about two decades later molecular-based analyses were proven to be more accurate and reliable than biological criteria (Lee *et al.,* 2000). Many marker gene sequences were used as bases for phytoplasma classification, such as the ribosomal protein gene (Lee *at. al.,* 1998a), the internal spacer region 16S-23S (ISR), the elongation factor Tu (Schneider *et al.,* 1997), the secY gene (coding for a subunit of the translocation system Sec) (Lee *et al.,* 2004) and other genes. Currently, phytoplasma differentiation and classification are mainly dependent on restriction fragment length polymorphism (RFLP) analysis of 16S rRNA gene, which is widely used (Wei *et al.,* 2007 and 2008). Based on similarity coefficients derived from RFLP analyses, phytoplasma were grouped first into nine distinct 16S rRNA (16Sr) groups and fourteen subgroups (Lee *et al.,* 1998b). New groups and subgroups were assigned on similarity coefficients of collective RFLP patterns (Lee *et al.,* 1998b). The scheme is often updated and the groups then were expanded as new strains are discovered. Currently they are 19 groups and 50 16Sr-subgroups, which are described so far (Figure 2) (Wei *et al.,* 2008).

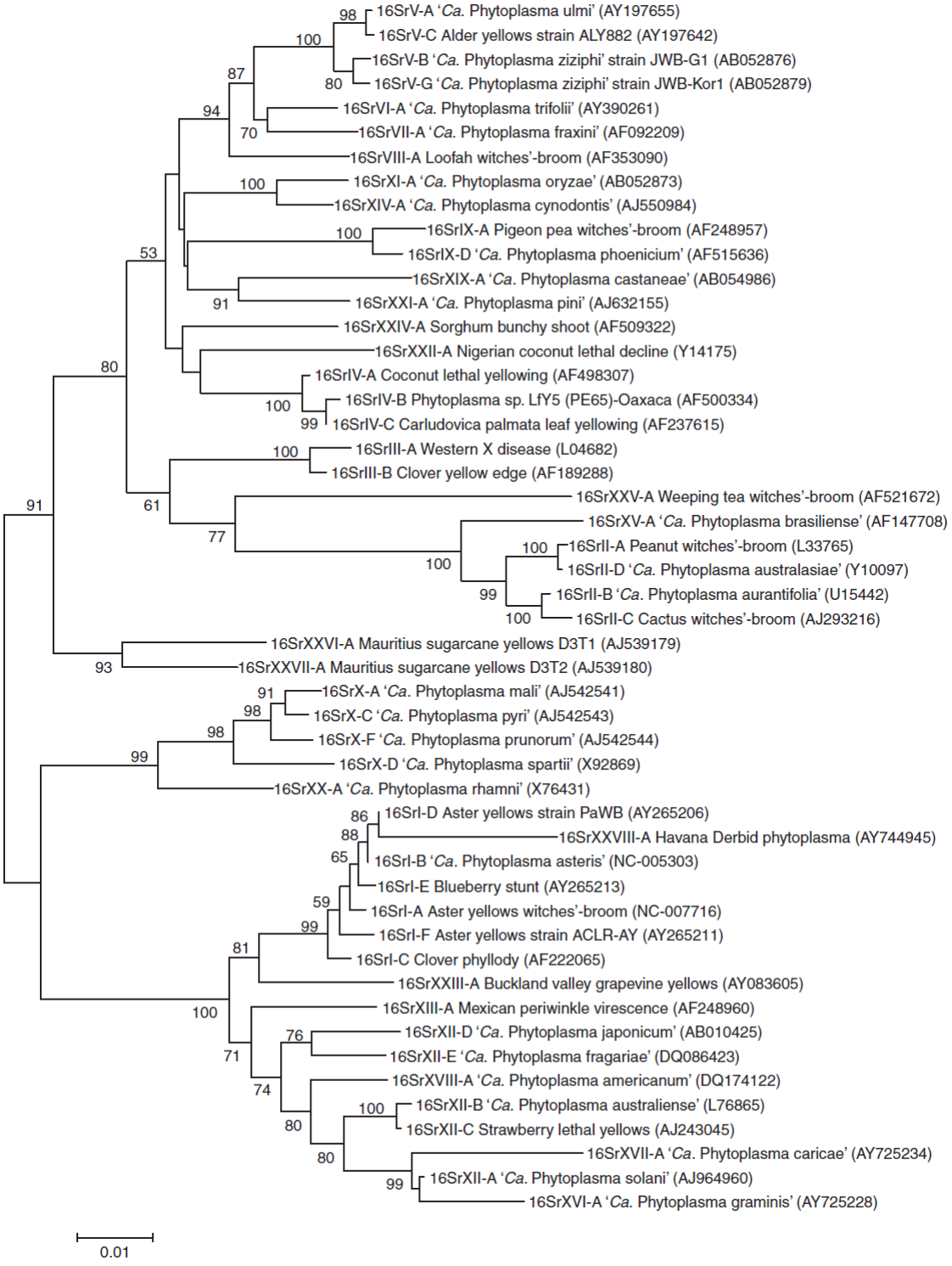


Figure 2. Dendrogram, constructed by the neighbor-joining method, showing the phylogenetic relationships amongst all the 28 phytoplasma groups identified by Wei *et al.*, (2008).

Phytoplasma life cycle and phytoplasma-insect interaction

Phytoplasmas complete their life cycle in two distinct hosts belonging to two distinct kingdoms, *Animalia* and *Plantae* (Figure 3). In fact, they have the ability to profit from the nutrients available in the phloem sieve tubes in plant and in insect hemolymph. Insects are the vectors, which spread phytoplasma in nature.

As phytoplasmas are phloem-restricted pathogens, they are transmitted to plants by phloem-feeding insects, primarily leafhoppers, planthoppers, and psyllids (Weintraub and Beanland, 2006). Phytoplasma life cycle starts when the insect vector acquires the phytoplasma as it is feeding the phloem sap, a phase termed as acquisition feeding phase. The phytoplasma diffusion into the insect tissues can take about three weeks before phytoplasma titre reaches an infectious level which is known as the latency period (Christensen *et al.,* 2005) during which the insect vector is not able to transmit the phytoplasma to another healthy plant. After latency, the vector becomes competent to inject the phytoplasma into the phloem of a healthy plant, feeding on the phloem sap, in a process known as inoculation feeding. Phytoplasmas then colonize the phloem tissue until they reach the titre that the symptoms would appear on the plant.

The phytoplasma-insect interaction is very complex and variable; several studies aimed to elucidate the relationship between phytoplasma and insect in term of specificity. Insect vector specificity plays a key role in the epidemiology of these pathogens and phytoplasmas are usually transmitted by a narrow range of vector species (Galetto *et al.,* 2013). However, numerous phytoplasmas, such as AY and WX strains, are transmitted by several different insect species. On the other hand a single vector species may transmit two or more phytoplasmas and an individual vector can be infected with two or multiple phytoplasma strains (Weintraub and Beanland, 2006). In another study it was reported that four different species of leafhoppers are likely capable of transmission corn stunt spiroplasma (CSS) concluding that, in this case, the interaction is not as specific (Ebbert *et al.,* 2001). To date there is no solid prove for insect-phytoplasma specificity and research is ongoing to elucidate the phytoplasma-insect interaction.

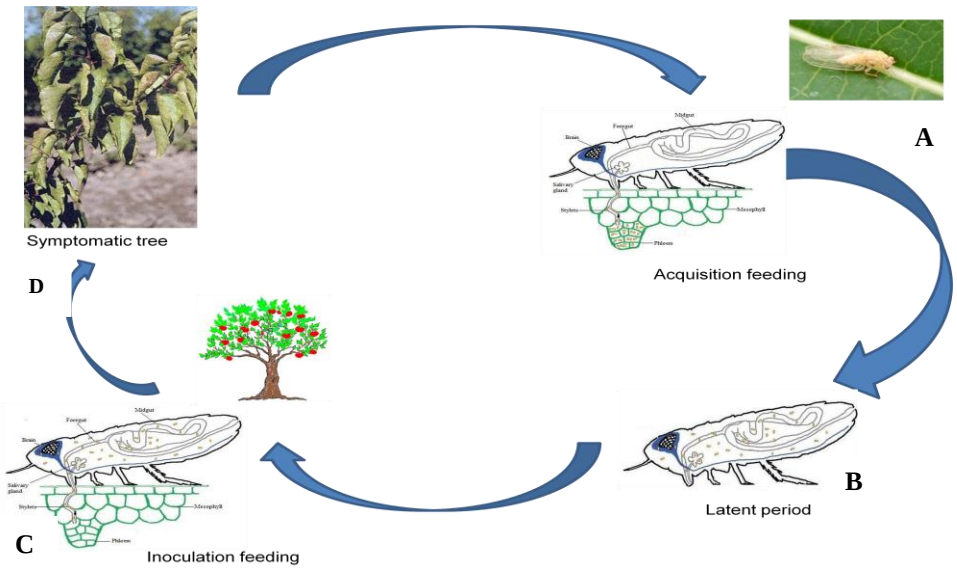


Figure 3. Phytoplasma life cycle (A), A healthy leafhopper feeds on a phytoplasma-infected plant (acquisition feeding). (B and C), Latency period, during which the phytoplasmas multiply within the insect, is necessary before the insect is capable to transmit phytoplasmas to a healthy plant (inoculation feeding). (D), Colonization and spread of phytoplasmas in the phloem of the host plant is accompanied by the appearance of disease symptoms.

Phytoplasma-plant interaction

Several studies have tried to explain the molecular and physiological changes occurring in the host plants upon phytoplasma infection. Cytological modifications, such as callose deposition and sieve tube collapse in phytoplasma infected woody plants were reported since the seventies (Braun and Sinclair, 1978). However, the physiological significance of these ultrastructural modifications was not completely explained. Musetti *et al.*, (2013), performed *in vivo* observations of *Vicia faba* sieve elements by confocal laser scanning microscopy, demonstrating that phytoplasma infection leads to sieve-tube occlusion, impairing phloem functions by triggering Ca^{2+} influx into the sieve elements, conferring phloem protein (forisome) dispersion, callose deposition, and probably cell wall thickening.

Gene expression studies gave also new insights to plant-phytoplasma interactions. Genes coding for proteins involved in photosynthesis, sugar transport, stress response, and phytosterol synthesis were found differentially expressed in *Catharanthus roseus* upon phytoplasma infections (Jagoueix-Eveillard *et al.*, 2001).

In another study, using microarray technique, Albertazzi *et al.,* (2009) demonstrated that the expression levels of few hundred genes were altered in Bois Noir (BN) infected grapevine plants.

A drastic inhibition of genes coding for enzymes of photosynthesis, transcription of Calvin-cycle enzymes, lipid and phenylpropanoid metabolism, was reported. On the other hand, induction of genes involved in cell wall reinforcement as well as expression of a Myb-transcription factor, belonging to a gene family that has a role in defense response, has been also reported.

Gene expression levels in healthy and '*Candidatus* Phytoplasma mali' infected leaf tissues were analysed by cDNA-Amplified Fragment Length Polymorphism (AFLP) technique (Moser *et al.,* 2007), demonstrating that genes associated with stress response, electron transport and protein degradation and modification were differentially expressed upon phytoplasma infection. Other studies used quantitative real-time-PCR analysis to compare the expression gene levels in healthy and phytoplasma-infected plants. As phytoplasmas are phloem-limited prokaryotes, phloem-specific protein expression patterns were studied with particular interest in phytoplasma-infected plants to elucidate the role of these specialized proteins in plant defense processes and in the infection establishment in phytoplasma-infected plant comparing with the healthy ones (Musetti *et al.,* 2010; 2011a). A significant induction in callose synthase transcripts, in apple trees and grapevines infected with AP and BN respectively (Hren *et al.,* 2009; Musetti *et al.,* 2010; Santi *et al.,* 2013) reported.

Moreover, expression levels of genes coding for Pathogenesis-Related (PR) proteins in several phytoplasma-infected plants were evaluated (Landi and Romanazzi, 2011; Ahmad and Eveillard, 2011) together with the protein accumulation (Zhong and Shen, 2004, Margaria and Palmano, 2011), suggesting that these proteins could be involved in the plant response to the phytoplasma diseases, as already demonstrated for other types of pathogens (Gau *et al.,* 2004; Bonasera *et al.,* 2006; El-kereamy *et al.,* 2011). The discovery of phytoplasma effector proteins (Bai *et al.,* 2009; Sugio *et al.,* 2011) could give a boost to the studies of the mechanisms involved in plant–phytoplasma interactions.

The recovery phenomenon

"Recovery" is defined as the spontaneous remission of the disease symptoms in plants that previously showed them. It was described in grapevines, apple and apricot trees infected by phytoplasma (Musetti *et al.,* 2007; 2010). In apple trees and grapevines "recovery" is associated with the disappearance of phytoplasma from the crown (Carraro *et al.*, 2004).

Interestingly, Osler *et al.*, (2000) demonstrated that recovered plants can be re-infected in nature but with a lesser extent, indicating that some resistance mechanism could be involved in the phenomenon.

In this context, the interest about "recovery" becomes not only scientificially interesting, but also important from a practical point of view. In fact this phenomenon could play a role in the management of phytoplasma diseases of perennial woody crops, as phytoplasma diseases are not curable and presently, the unique possible method to contrast their spread is the vector control.

The physiological basis of "recovery" is not completely known, however, during the last years, studies have been carried out with the aim to add new insight about the phenomenon. Cytochemical analyses revealed that it is accompanied by biochemical changes in the phloem. In fact, it was demonstrated that recovered plants are able to accumulate H_2O_2 in the sieve elements, a stable reactive oxygen species whose antimicrobial as well as signaling roles are well known. It was also observed that in these plants the activities of two main enzymatic H_2O_2 scavengers (catalase and ascorbate peroxidases) are selectively and steadily down-regulated (Musetti *et al.,* 2004, 2005, and 2007). The variation of sieve-element oxidative status leads to modifications of phloem protein (P-protein) conformation and in phloem occlusion expression patterns. An anomalous accumulation of callose and protein, associated with the up-regulation of callose synthase- and P-protein- coding genes, was observed in the sieve elements of recovered apple trees (Musetti *et al.*, 2010), supporting the hypothesis that recovered plants are able to develop resistance mechanisms depending on Ca^{2+} signal activity (Musetti *et al.*, 2010, 2013).

Recovery occurrence in individuals that showed severe symptoms for several years, suggests that additional mechanisms could be responsible of this phenomenon.

DNA methylation is an important epigenetic mark involved in diverse biological processes in plants. A process in which a methyl group is added to the cytosine bases of DNA to form 5-methylcytosine (He *et al.,* 2011). Changes in DNA methylation patterns were recently suggested to be an adaptative response that may modulate gene expression (He *et al.,* 2011).

Global DNA methylation level modification and changes have been reported in several studies. DNA methylation levels in plants were modified in response to environmental stimuli and to biotic stresses (Steward *et al.,* 2002). Changes in DNA methylation have been reported during the interaction between Tobacco Mosaic Virus and tobacco plants as well as in Pseudomonas-infected Arabidopsis (Wada *et al.,* 2004; Pavet *et al.,* 2006). As regards phytoplasma diseases, Ahmad *et al.,* (2012) reported that DNA methylation is involved in the down-regualtion of floral development genes in stolbur-infected tomatoes.

To determine possible relationships between recovery from the phytoplasma infection and epigenetic mechanisms, the methylation status of genomic DNA from healthy, infected and recovered leaf tissues of apple trees would be analyzed.

"Recovery" is a complex phenomenon still not fully understood even from the epidemiological point of view. The difficulty of studying "recovery" is probably derived from the complexity of the trinomial: plant species/phytoplasma /insect vectors. As a current opinion, the permanent or stable "recovery" is considered a minimum asymptomatic period of two-three years after the expression of the phytoplasma disease symptoms (Maixner *et al.,* 2011).

In the case of grapevine yellows, phytoplasmas were not detectable by nested-PCR in the canopy of recovered grapevines cvs. Prosecco, Chardonnay, Pinot Noir, Barbera, (Osler *et al.,* 2003; Morone *et al.,* 2007). This suggests that recovered plants are not source of inoculum for the potential spreading of the disease.

"Recovery" also occurs in apple trees, in which phytoplasmas remain confined in roots (Carraro *et al.,* 2004); also in this case, the epidemiological risk related to the presence of recovered plants is not relevant because vectors are not able to acquire the pathogen from the roots (Seemüller *et al.,* 2008).

Research on the stability of "recovery" over the time are still in progress; preliminary results on grapevines recovered from "Bois Noir" disease showed that the probability of these plants to become infected and to show again the disease symptoms is 4.9% in 5 years; the percentage of the new symptomatic plants in the same period is 8.4% (Ermacora *et al.*, 2012).

"Recovery" is a natural, spontaneous event, still not experimentally reproducible. The explanation of the phenomenon is not exactly known, but the hypothesis that the recovery phenomenon is due to the expression of an acquired resistance by the plants is confirmed.

Recovered plants exhibit similar behavior to healthy plants, but with the important advantage of the resistance acquisition towards the disease. The main difficulty to include "recovery" as an integrated measure for phytoplasma disease management consists in the evaluation of the expected rate of spontaneous remission of symptoms under specific environmental conditions. However, in some areas, natural "recovery" occurrence, limiting the disease spread may be fruitful for phytoplasma disease control.

The apple proliferation disease, associated with '*Candidatus* Phytoplasma mali'

Apple proliferation (AP) disease is considered among the most important diseases in all apple production areas worldwide. AP disease is associated with the phytoplasma '*Ca.* P. mali', which belongs to the 16SrX group (Torres *et al.*, 2005).

As all other phytoplasmas, '*Ca.* P. mali' is transmitted in nature by the phloem feeding insect vectors such as leafhoppers and psyllids belonging to the genus *Cacopsylla* in a persistent-propagative manner (Mattedi *et al.*, 2008). Among these, there are *C. picta* and *C. melanoneura*. Even though these are known to be the main vectors in nature, other insects could transmit the '*Ca.* P. mali' under laboratory conditions (Weintrau and Beanland, 2006). Tedeschi and Alma (2006) reported the transmission of '*Ca.* P. mali' to healthy apple seedlings using the leafhopper *Fieberiella florii*.

Typical symptoms in infected apple plants are: stunting, leaf yellowing or reddening, reduced leaf size and enlarged stipules (Figure 4), shortening of internodes forming the so called witches' brooms. Symptoms can affect also flowers, including virescence, phyllody and sterility and reduced flower size and number, fruit malformation and reduction in size and quality.

Figure 4. Typical symptoms of apple proliferation in an infected apple plant. (A), enlarged stipules, (B), witches' brooms and (C), fruit malformations.

Molecular diagnosis of '*Candidatus* Phytoplasma mali'

'Candidatus Phytoplasma mali' was first detected in 1990 (Bonnet *et al.,* 1990) by DNA hybridization technique, using a hybridization probe developed from AP-infected periwinkle plants, then used to detect the phytoplasma in its natural host *Malus domestica.* Later, rapid progresses was made in phytoplasma diagnosis using rapid, sensitive and efficient techniques, such as the polymerase chain reaction (PCR) amplification of part of the 16S ribosomal RNA gene (Firrao *et al.,* 1994). Ever since different diagnosis techniques have been applied, more specific primers have been designed using ribosomal and not ribosomal sequences to detect '*Ca*. P. mali' and to distinguish it from the closely related phytoplasma ('*Ca*. P. pyri' and '*Ca*. P. prunorum') (Lorenz *et al.,* 1995). Recently new approaches, based on the faster and more sensitive real-time PCR technique, allow specific detection of all '*Ca*. P. mali' strains, distinguishing them from the other genetically related phytoplasmas (Baric and Dalla-Via, 2003).

Fungal endophytes: a promising strategy for phytoplasma disease control

As phytoplasma diseases are not curable and their control is exclusively based on vector control, the use of endophytic microorganisms could represent an alternative strategy against phytoplasma. In fact, the actual concept of management and control of plant diseases implies the application of modern measures compatible with the environment, the cultural essential for the crops and economic thresholds. Fungal endophyte strains have been isolated from grapevines and apple plants grown in areas where "recovery" was recurrent. Some of these endophytes, such as *Epicoccum nigrum* and *Aureobasidium pullulans*, raised our interest in particular because these species were already reported as biocontrol agents or resistance inducers (Martini *et al.,* 2009). Recent research activities, performed using the model plant *Catharanthus roseus* infected with '*Ca*. P. mali', demonstrated that reduction in symptom severity and lower phytoplasma titre in host tissues occur when the plants were previously inoculated with an endophytic strain of *E. nigrum* (Musetti *et al.,* 2011b).

In the next section, an overview of fungal endophytes and their potential as secondary metabolite producers and biocontrol agents is reported.

The fungal endophytes: an overview

Fungal endophytes are microorganisms that live within plant tissues without causing disease symptoms (Porras-Alfaro and Bayman, 2011). The name 'endo' implies the location (i. e. 'into the plant') of the microorganisms rather than their type, which could be fungi or bacteria. Endophytic fungi are taxonomically and ecologically heterogenous groups of organisms, mainly belonging to the Ascomycotina and Deuteromycotina (Petrini *et al.*, 1992).

Fungal endophytes groups

Fungal endophytes consist of three basic ecological groups: the mycorrhizal fungi, the balansiaceous or 'grass endophytes', and the non-balansiaceous taxa (Schulz and Boyle, 2005).

Mycorrhizas are multifaceted associations comprising diverse morphological, functional and evolutionary categories (Brundrett, 2004). Brundrett (2004) distinguished mycorrhizal from endophytic interactions, mycorrhizas having synchronized plant-fungus development and nutrient transfer at specialized interfaces. These fungi are referred to as 'dark septate endophytes' (DSE)

Balansiaceous endophytes or Grass endophytes form a distinctive group of closely related fungi with ecological requirements and adaptation discrete from those of other endophytes (Petrini, 1996). They are vertically transmitted through the seeds (Bacon and White, 2000) and produce a diverse array of secondary metabolites such as toxic alkaloids including the anti-insect alkaloids peramine and lolines, and the anti-vertebrate alkaloids lolitrem B and ergovaline (Schardl, 2001).

In contrast, **Non-balansiaceous endophytes** are diverse, both phylogenetically and with respect to life-history strategy. Most of these fungi belong to the Ascomycota (Petrini 1986). The status of the interaction between endophyte and host may be transient. The stability or variability of the asymptomatic interaction depends on numerous factors (Schulz and Boyle, 2005).

Endophyte functional roles

Fungal endophytes have many ecological functional roles; moreover they could change their functional role during its life cycle (Porras-Alfaro and Bayman, 2011). Endophytes could include latent pathogens, saprotrophs, and mycorrhizal fungi and rhizobia at early stages of colonization. The different functional roles are illustrated in figure 5.

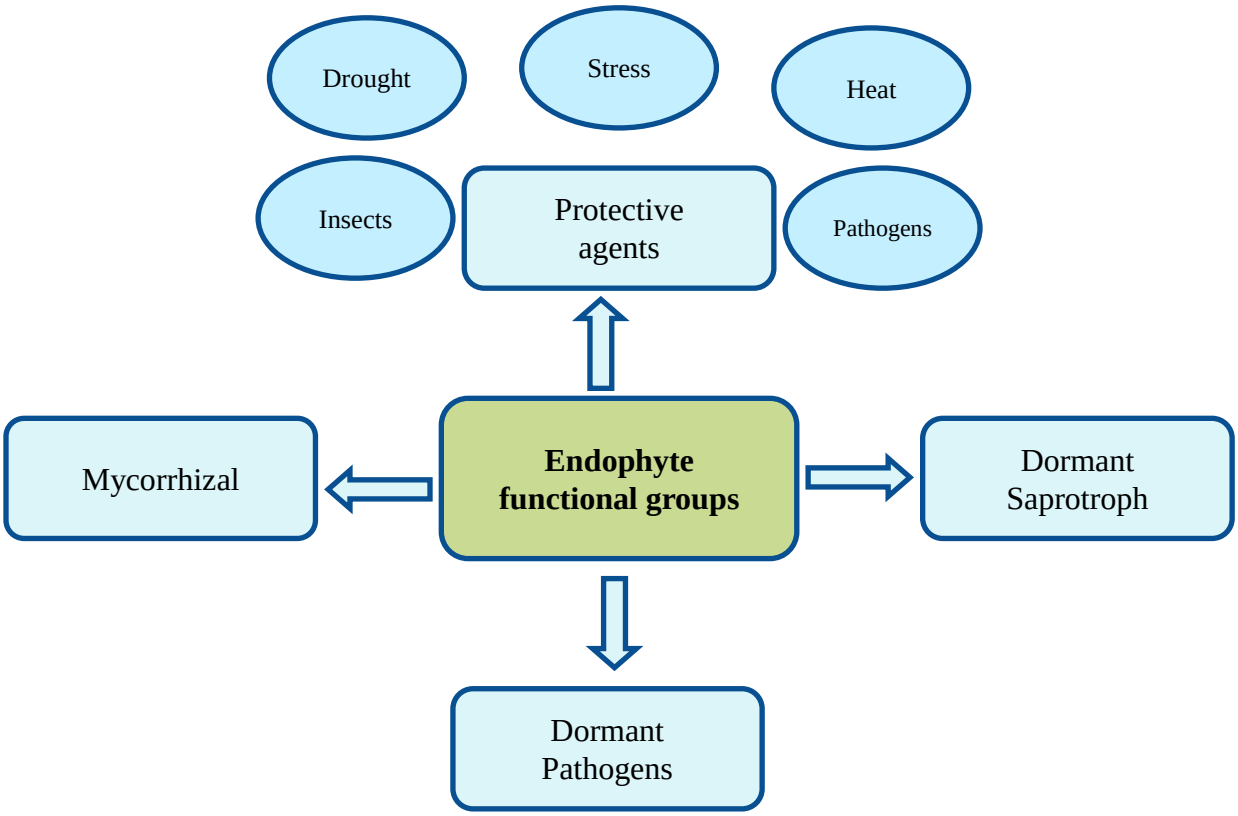


Figure 5. Functional roles that endophytes may take or switch between during their life cycle in response to the host or the environmental changes (Porras-Alfaro and Bayman, 2011).

Endophytes as mycorrhizal fungi

Fungal endophyte colonization may also be intracellular without causing any nutrition disruptions to host roots. Little is known about root endophytes because they cannot sporulate in culture medium so the use of molecular techniques is necessary for their identification and characterization (Porras-Alfaro *et al.*, 2008). They coexist with mycorrhizal fungi and they can be easily distinguished from mycorrhizal fungi becuase their hyphae are darkly pigmented due to the presence of melanin. Dark septate endophytes (DSE) can form a mutualistic symbiosis with plant and provide the plant with nitrogen as the case of Chinese cabbage (Usuki and Narisawa 2007). Root endophytes also can increase the plant biomass and activate specific metabolic pathways that facilitate nutrient transfer. So, according some authors, the functional overlap between the endophytes and the mycorrhizal in colonization, nutrient supplies and mutualism suggests that fungal endophytes could act as mycorrhizal fungi (Porras-Alfaro and Bayman, 2011).

Endophytes as latent pathogens

Fungal endophytes infect their hosts without causing visible disease symptoms, but this definition only refers to fungi at the moment of detection without regard for the future status of the interaction. Schulz and Boyle (2005) hypothesized that there is no neutral interactions, but rather that endophyte-host interactions involve a balance of antagonisms, and there is a degree of virulence, which enables the fungus infection. Both fungal endophytes and pathogens are producing secondary metabolites, which are toxic to the plant host but only fungal pathogens are able to suppress and overcome plant defense reaction, while endophytes are able to tolerate the plant defense, achieving the balanced antagonism status. This status might be broken, for example, in case of severe stress for the host plant, so that endophyte can become pathogen (Schulz *et al.,* 1990). Molecular studies of the ITS region of fungal endophytes, isolated from woody plants, demonstrated that they are closely related to described pathogenic fungi (Ganley *et al.,* 2004).

Authors hypothesized that these fungal endophytes are parasites in a latent phase within the host or less-pathogenic lineages derived from pathogens. Environmental and habitat-specific conditions and the stress situation or change in gene expression patterns could shift towards mutualism between the plant and endophytes to parasitism (Johnson and Oelmüller, 2009), which makes endophytes as latent pathogens.

Endophytes as saprotrophs

Literature also describe some endophytes as latent saprotrophes that become active upon host death. Saprotrophes start growing as soon as on the senescent tissue, gaining competitive advantages over the other saprotrophes. In order to elucidate the phylogenetic relationships between endophytes and saprotrophes, ribosomal DNA-based sequence comparison was made (Promputth *et al.,* 2007) and molecular results suggest there are fungal taxa that possibly exist as endophytes and saprotrophs, implying that fungal endophytes can change its ecological role and adopt the saprotrophe role.

Phoma medicaginis was found to switch from the endophytic to the saprotrophic period following the death of infected host tissue (Weber *et al.,* 2004), in which a rapid colonization of host tissue and sporulation were observed within 9 day on dead plant material upon incubation in a moist chamber.

Endophytes as protective agent

Numerous studies and literature discussed the mutualism and the profit that endophytes provide to the host plant. Endophytes can make plants more effective at tolerating and degrading xenobiotic compounds in the soil. Endophytes influence plant establishment, resistance to environmental stress and survival, moreover it is expected that they could influence the response of plants and ecosystems to climate change (Porras-Alfaro and Bayman, 2011). Arachevaleta *et al.,* (1989) reported the benefits derived from association between the endophyte *Acremonium coenophialum* with its host plant, resulting in growth stimulation, improved survival, and drought tolerance.

Pathogen control using endophytic microbes was applied for a broad range of pathogens on many hosts (Fernando *et al.,* 2005; Minerdi *et al.,* 2009). Many mechanisms involved were described to explain the protective capability; some of them were reviewed in Alabouvett *et al.,* (2009). The authors proposed direct interaction between the endophyte and the pathogen by parasitism, competition for the nutrients and for the colonization of the host tissue.

Other mechanisms were also suggested; the most interesting was the resistance induction in the host plant (Rowan and Latch, 1994; Schulz *et al.,* 1999; Vu *et al.,* 2006; Musetti *et al.,* 2011b). Fungal endophytes from the genus *Neotyphodium* have significant impacts on herbivores because they produce toxic secondary metabolites with repellent action, enhancing resistance of their plant hosts by providing additional chemical defenses. The interaction is highly variable depending on the host and fungal endophyte genotype, insect species, as well as upon environmental factors such as soil water and nutrient availability (Hartley and Gange, 2009).

Epicoccum nigrum and *Epicoccum nigrum* diversity

Among fungal endophytes, we are particularly interested to *Epicoccum nigrum* because, as reported above, it demonstrated activity against '*Ca*. P. mali' on the experimental host *C. roseus*, maintained in greenhouse (Musetti *et al.,* 2011b). *Epicoccum nigrum* Link (syn. *E. purpurascens*) is an anamorphic ascomycete distributed worldwide (Fàvaro *et al.,* 2011). *E. nigrum* is genotypically and phenotypically highly variable, and integrated methods been applied to study the diversity among collection isolates around the world (Arenal *et al.,* 2002). Each individual technique allows differentiation of the isolates to some degree and the information obtained by each technique could be considered as complementary.

The integration of molecular and morphological approaches as well as the study and the characterization of the fungal secondary metabolites are considered to be powerful for strain differentiation. According to morphological and genetic variation *E. nigrum* presents two genotypes that may comprise more than one species (Fàvaro *et al.,* 2011).

Epicoccum nigrum as biocontrol agent

Epicoccum nigrum is known to be a biological control agent, effective against many pathogens and it produces a variety of secondary metabolites with important biological activities as well as biotechnological application. *E. nigrum* conidial formulations have been used in peach and nectarine orchards to control pathogenic fungi belonging to *Monilinia spp*. (De Cal *et al.*, 2008; Mari *et al.*, 2007; Torres *et al.*, 2005). Culture broth of *E. nigrum* was also used to evaluate the effectiveness in the control of the powdery mildew in different crops, such as okra, cucumber and squash (Derbalah *et al.*, 2011; Derbalah, and Elkot, 2011). As a biological herbicide *E. nigrum* was found to have effect on early stage growing weed in rice culture (Motlagh, 2011). In addition it was tested against *Sclerotinia sclerotiorum* in sunflowers (Pieckenstain *et al.*, 2001) and against *Pythium* in cotton (Hashem and Ali, 2004). Beside field trails, *E. nigrum* was used *in vitro* to evaluate the activity against selected pathogens under control conditions. *E. nigrum* showed activity against *Fusarium avenaceum, F. graminearum,* and *F. oxysporum,* limiting the growth of these pathogens in culture plates (Ogórek and Pląskowska, 2011).

It was also tested for activities against *Rhizoctonia solani* AG3 (Lahlali and Hijri, 2010), *Leucostoma cincta* (Biggs and Alm, 1991), *Sclerotinia sclerotiorum* (Zhou and Reeleder, 1989), *Colletotrichum gloeosporioides* (Pandey *et al.*, 1993), *Colletotrichum kahawae* (Guerra-Guimarães *et al.*, 2007), *Botrytis cinerea* (Peng and Sutton, 1991), *Monilinia laxa* (Larena *et al.*, 2004) and to some bacteria, such as *Pseudomonas* spp., *Bacillus subtilis* and *Enterobacter cloacae* (Punja, 1997) proving its ability to control a wild range of fungi and bacteria.

Screening for biologically active metabolites

Resistance to antibiotics is an emerging serious problem worldwide; therefore novel antibiotics will be necessary to combat resistant pathogens. Since the discovery of penicillin from *Penicillium spp*. in 1928, fungi were considered to be a rich source for antibiotics. Endophytic fungi are a good source of novel secondary metabolites however; the biological activities and thus the produced metabolites are associated with the respective biotope and/or the host (Schulz *et al.*, 2002).

Several strategies were set up in order to discover novel antibiotics; the most used method is based on culturing the microorganisms, starting from their natural habitats or from their hosts, and then culturing them on the most suitable culture media. Secondary metabolite biosynthesis was known to be responsive to environmental cues, including the carbon and nitrogen source, ambient temperature, light and pH (Keller *et al.*, 2005).

Several tests and assays were discribed to evaluate the activity of endophyte strains *in vitro* for antimicrobial activity. To determine the best sources of novel, biologically active metabolites, endophytic fungi follow preselection of the isolates, according to taxon and metabolic profiles (Schulz *et al.*, 2008). About the antagonistic potential of *E. nigrum, E. nigrum* reduced *C. paradoxa* and *F. verticillioides* radial growth by more than 50% (Fàvaro *et al.*, 2012).

Another strategy for obtaining biologically active fungal secondary metabolites (which is also used in this study) is to culture the endophyte strains on the proper medium, then to extract and evaluate the secondary metabolites profile by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD).

The screening for the biological activity of the crude extracts can be done by the serial dilution assay and by the determination of the minimum inhibitory concentrations, as described by Andrews (2001).

Chapter 1

STUDY OF RECOVERY FROM APPLE PROLIFERATION DISEASE (AP)

MATERIALS AND METHODS

Starting from the data published by Musetti *et al.*, in 2004 and 2010 about the interactions occurring between '*Ca*. P. mali' and apple trees, and in particular, about the "recovery" phenomenon in previously-infected plants, new analyses have been performed to gain new insights into the phenomenon.

Plant materials and phytoplasma detection

Plant materials for gene expression study were obtained from an experimental organic apple orchard (cv. Florina), which was established in 1988 in Friuli Venezia Giulia region, North East Italy, where serious epidemic of AP occurred. The orchard had been checked at least three times per year for presence of AP symptoms. Three groups of plants, AP-symptomatic diseased, (D), healthy (H) and recovered (R), were selected in the orchard. Recovered plants resulted asymptomatic during the previous four consecutive years and healthy plants had been asymptomatic since planting.

Phytoplasma detection in randomly sampled leaves was carried out by means of polymerase chain reaction (PCR). A phytoplasma DNA enrichment procedure (Ahrens, 1992) was used to extract DNA from 1 g of fresh leaf material; PCR was performed using ribosomal fO1/rO1 AP group-specific primers (Lorenz *et al.,* 1995). Amplification conditions were 35 cycles each at 95°C for 30 s, at 55°C for 75 s, and at 72°C for 90 s. Amplification products were analyzed by direct agarose gel electrophoresis.

RNA extraction from apple leaves from apple orchard

Total RNA was extracted from frozen tissues using a cetyl-methyl ammonium bromide based method described by Gasic *et al.,* (2004).

The extracted RNA was treated with RNase-free DNase I (Promega Italia, Milano, Italy) according to the manufacturer's protocol. Following digestion, nucleotides were removed from RNA using a G50 Sepharose buffer exchange column (GE Healthcare Europe, Milano, Italy). RNA concentration and integrity were checked with a Lambda 3B spectrophotometer (Perkin Elmer Italy, Cologno Monzese, Italy) before and after DNase I digestion.

Only RNA samples with a 260/280 ratio (an index of protein contamination) between 1.9 and 2.1 and a 260/230 ratio (an index of reagent contamination) greater than 2.0 before and after DNase I digestion were used for cDNA synthesis. The quality of RNA samples was also assessed by electrophoresis on 1% formaldehyde agarose gels.

cDNA synthesis and gene expression analyses

The transcripts of the identified genes were amplified by RT-PCR using a pool of RNAs extracted from D, H, and R leaves as described above. First-strand cDNA was synthesized from 3 µg of total RNA by Expand Reverse Transcriptase (Roche Diagnostics, Milano, Italy) according to manufacturer's protocol. The expression of the cDNAs were analyzed by quantitative real-time PCR (qRT-PCR) in leaves from three individual trees for each of the D, H, and R groups, collected in September, when typical AP symptoms were visible. cDNA was ten-fold diluted for qRT-PCR analyses. RT-PCR analyses were performed in a Bio-Rad CFX96 Real Time PCR System using Sso Fast EvaGreen SuperMix (Bio-Rad Laboratories, Inc., USA). Three technical replicas were analyzed for each biological replica.

Reaction mixture of 10 µl was prepared as follows:

- 5 µl Fast EvaGreen SuperMix
- 0.3 µl forward primer (0.3 µM)
- 0.3 µl revers primer (0.3 µM)
- 0.4 µl H_2O
- 4 µl cDNA templets

The thermal profile comprised three segments: (i) 95°C for 3 min; (ii) 39 cycles of 5 s denaturation at 95°C, 10 s annealing and extension at 60°C (amplification data collected at the end of each extension step); and (iii) dissociation curve consisting of 5 s incubation from 65°C to 95°C, at 0.5 °C increment intervals followed by plate reading, as illustrated in the scheme in the figure 6.

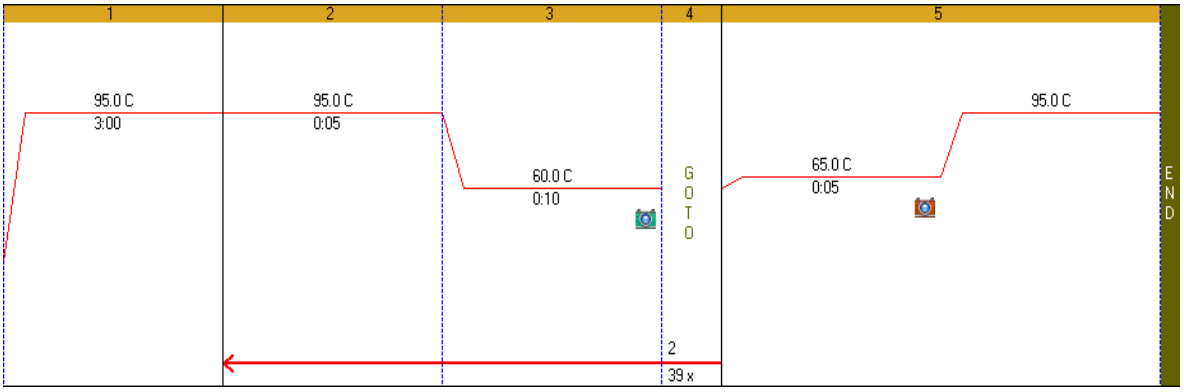


Figure 6. Schematic representation of the q Real-time PCR reaction conditions used for gene expression analyses

Five-point standard calibration curves of several dilutions from cDNA (corresponding to 10, 5, 2, 1, and 0.5 ng of the initial RNA used for cDNA synthesis) were used for PCR amplification efficiency calculation of each primer pair used. The efficiency (E) was calculated as described by Pfaffl (2001). Primers pairs with amplification efficiency in the range of 96-104 % were chosen. Raw Ct values were transformed to relative quantities using the formula: $MNE = (E_{reference})^{Ct,\ mean} / (E_{target})^{Ct,\ mean}$, where MNE = Mean Normalized Expression (Muller *et al.*, 2002), and E = 2.

The expression level of each target gene was normalized on the expression level of the reference gene GAPDH, once the expression stability of some candidate reference genes had been evaluated by the software program NormFinder (Musetti *et al.*, 2010).

Genes under study were fallen into four gene groups:

- Phloem proteins: sieve element occlusion genes (SEO) a and b;
- Pathogenesis related proteins (PRs): PR1, PR2, PR5 and PR8;
- Jasmonic Acid pathway related genes: AOS2, 12-OPR3 and PI II;
- DNA Methylation (MET1, MET2, DRM2, DRM8, CMT3, PolV, PolIV 1, PolIV 2, RDR2, AGO4, DME and Demethylation (DLM and DML3) genes.

The expression levels of genes of interest were compared both separately on three individual plants for each experimental group (H, R, and D) and by considering the average of the three plants from each group.

Pairwise comparisons between the means of the three groups (H, D, and R) were analyzed using the In Stat GraphPad software package (La Jolla, CA, USA) using Kruskal-Wallis non-parametric method (Dunn's multiple comparison tests') to evaluate the significant differences among the groups under study. The mean square error was computed as mean of the variances of the three groups, which was performed with Microsoft Office Excel®.

Candidate gene identification and primer design

Sequence of *Malus domestica* genes under study were retrieved from the NCBI database for genes which have been previously completely sequenced, whereas genes which are not completely sequenced, sequences were retrieved from TIGR Apple Gene Index databases by TBLASTN with homologous sequences of *Arabidopsis thaliana.* Primer 3 software was used for primer picking. Sequences of each primer pair along with the accession numbers are listed in Table 1. Efficiency and specificity of the primer pairs were determined in real-time RT-PCR experiments as described in the previous section and melting curves were used to verify the amplification specificity.

Primer name	Oligonucleotide sequence (5'- 3')	Sequence accession number	References	Primer specificity
Md PR1a for	GCTCAGCCCTAATACAATCCTCTC	CV524932.1	(Bonasera *et al.*, 2006)	Pathogenesis-related protein 1a coding gene
Md PR1a rev	TACCCCCACTACTGCACCTCACT		(Bonasera *et al.*, 2006)	
Md PR2 for	CTTCACAGTCACCATCTTCAACA	AY548364.1	(Bonasera *et al.*, 2006)	Pathogenesis-related protein 2 coding gene
Md PR2 rev	GGTGCACCAGCTTTTTCAA		(Bonasera *et al.*, 2006)	
Md PR5 for	GGCAGGCGCAGTTCCACCAG	DQ318213.1	(Bonasera *et al.*, 2006)	Pathogenesis-related protein 5 coding gene
Md PR5 rev	GACATGTCTCCGGCATATCA		(Bonasera *et al.*, 2006)	
Md PR8 for2	CGTTCCCGGATACTCACCTA	DQ318214.1	present work	Pathogenesis-related protein 8 coding gene
Md PR8 rev2	ACCGTCTGCATACTGGCATT		present work	
Md NPR1 for3	CAGGTTGGAAGCCCTTATGA	EU624123.1	present work	Non-expressor Pathogenesis-related protein coding gene
Md NPR1 rev3	ACAAATGAGGGAGGTCGTCA		present work	
Md AOS2 for	GGGAGAAGCTGTTGAAGCAC	TC59991	present work	Allene oxide synthase 2 coding gene
Md AOS2 rev	TCCAGCACACTGTTTGTTCC		present work	
Md OPR12 for	GACAGGGAAGATGGGAACAA	TC87763	present work	Oxophytodienoate reductase12 coding gene
Md OPR12 rev	CTTCGGCAAGTCTGGGTTAG		present work	
Md PI2 for	TGGTGGAGAAAACAATTCGTC	TC65477	present work	Proteinase inhibitor II coding gene
Md PI2 rev	AGTGCCTGGTTAGCGAAAAA		present work	
GAPDH for	TGACAGGTTCGGAATTGTTGAG	CN929227	(Musetti *et al.*, 2010)	Glyceraldehyde-3-phosphate dehydrogenase coding gene
GAPDH rev	CCAGTGCTGCTAGGAATGATG		(Musetti *et al.*, 2010)	
fO1	CGGAAACTTTTAGTTTCAGT	JN555598.1	(Lorenz *et al.*, 1995)	*Candidatus* Phytoplasma mali 16S ribosomal RNA gene
rO1	AAGTGCCCAACTAAATGAT		(Lorenz *et al.*, 1995)	
MdSEOa For	ATGCTAGGCATAGCAC	HM162887	(Rüping *et al.*, 2010)	Sieve Element Occlusion a coding gene
MdSEOa Rev	TCAATGATGCGCGTTGATGG		(Rüping *et al.*, 2010)	
MdSEOb For	ATGCTAGGTCTAGCAAAC	HM162888	(Rüping *et al.*, 2010)	Sieve Element Occlusion b coding gene
MdSEOb Rev	TTAGTGATGTGCAGTAGG		(Rüping *et al.*, 2010)	
rpAP15 For	TGC TGAAGCTAATTTGGC	EF193366.1	(Martini *et al.*, 2010)	Ribosomal protein coding gene
rpAP15 Rev	CCCATGAATATTAACCTCCT		(Martini *et al.*, 2010)	
qMd-ACO For	CCAGAATGTCGATAGCCTCGT	Y14005	(Baric *et al.*, 2011)	1-aminocyclopropane-1 carbo-xylate oxidase coding gene
qMd-ACO Rev	GGTGCTGGGCTGATGAATG		(Baric *et al.*, 2011)	

MET1 for	AAGAAAATGCGAGGGGAGAT	EU273287.1	present work	Cytosine-5-methyltransferase 1 coding gene
MET1 rev	CTCTGGCAGCGAATGAGATT		present work	
MET2 for	GCATCCTGAGCAAGATAGGG	TC91512	present work	Cytosine-5-methyltransferase 2 coding gene
MET2 rev	CCGCTGAGCCTGTAAAAATC		present work	
DRM2 for	TATGTTTCCCAGTGGCATCA	EU273288.1	present work	Domain rearranged methyltransferase 2 coding gene
DRM2 rev	TCTTCAGAGGGACACCAAGC		present work	
DRM8 for	AACGAGACAGACTGGGCACT	TC84878	present work	Domain rearranged methyltransferase 8 coding gene
DRM8 rev	CGGCTGCTGACAAATGATAA		present work	
CMT3 for	GCAGTTCCGTATGCGAGTTT	EB142093.1	present work	Chromomethylase3 coding gene
CMT3 rev	ACACCCCTTGAAACAACGTC		present work	
DML for.	TTGGCAAGTTGTGTGACGAT	TC77505	present work	DEMETER-like coding gene
DML rev.	TGTTCGGCATGGTATCAAGA		present work	
DME for	CATTGAGGAACCATCATCACC	Moser, unpublished data	present work	DEMETER coding gene
DME rev	TTTGCATATCGGGAAGGAAC		present work	
DML3 for	AAACCGCCAATCCCTTCA	Moser, unpublished data	present work	DEMETER-like 3 coding gene
DML3 rev	GCCATCAGTCTCTGCTTTCTGT		present work	
AGO4 for	GAAAGGGTGCTGGAAGAAGA	Moser, unpublished data	present work	Argonaute protein 4 coding gene
AGO4 rev	AGGACCATCAGGTTCACTGG		present work	
RDR2 for	GTTTGCGGTCGGATACAGTT	Moser, unpublished data	present work	RNA-Dependent RNA polymerase 2 coding gene
RDR2 rev	CAAATGGTATTGCGCTTGTG		present work	
PolIV 1 for	TTCTGTGGGTTGCGAGAAG	Moser, unpublished data	present work	Largest subunit of polymerases Iva coding gene
PolIV 1 rev	CTGACTGAGGGCAGCAGATT		present work	
PolIV 2 for	CGGCAACATCATTCTTCAAC	Moser, unpublished data	present work	Second largest subunit of polymerases Iva coding gene
PolIV 2 rev	GCCATTCATTGTCTCCCTTC		present work	
PolV for	GCAGAGAAAGTCGCACCAA	Moser, unpublished data	present work	Largest subunit of polymerases IVb coding gene
PolV rev	TAGGTGAATCCCACCCAGAC		present work	
MDPP2-1 for	TGAGACCGCATACCTGACAC	FN395067	(Musetti *et al.*, 2010)	Phloem protien 1 coding gne
MDPP2-1 rev	ATTCGGCAACTCGGACATAAC		(Musetti *et al.*, 2010)	
MDPP2-2 for	AGATGTATGCTGGCTAGATGTG	FN395068	(Musetti *et al.*, 2010)	Phloem protien 2 coding gne
MDPP2-2 rev	AGGGAGAGTGAGGCTGAAGTTG		(Musetti *et al.*, 2010)	
MDPP2-3 for	CGTAGAACTGGCACCTGATG	FN395069	(Musetti *et al.*, 2010)	Phloem protien 3 coding gne
MDPP2-3 rev	CGGAGCATTGGACGGAAC		(Musetti *et al.*, 2010)	
MDERG1 for	CCGAGGGTAGAACAGGATTATG	FN395070	(Musetti *et al.*, 2010)	C2 domain containing protein coding gene
MDERG1 rev	GGAGTCAGCCCAGATTCAAGC		(Musetti *et al.*, 2010)	

MDCALS1 for	CATCGGTTCCGACTCTTGC	FN395071	(Musetti *et al.*, 2010)	Callose synthase 1 coding gene
MDCALS1 rev	GCCTCTGCTGAATGCTTGG		(Musetti *et al.*, 2010)	
MDCALS2 for	GCATTCTTGGCTTGGTTTCC	FN395072	(Musetti *et al.*, 2010)	Callose synthase 2 coding gene
MDCALS2 rev	CATTATTGTTGGACGAGTGAGG		(Musetti *et al.*, 2010)	
MDCALS3 for	AGTAATTGTCATGGCTCCTGTG	FN395073	(Musetti *et al.*, 2010)	Callose synthase 3 coding gene
MDCALS3 rev	AAGAATCGTGACCTCTGAATGC		(Musetti *et al.*, 2010)	
MDCALS4 for	AATGACACCTGTGGCAGTATTA	FN395074	(Musetti *et al.*, 2010)	Callose synthase 4 coding gene
MDCALS4 rev	GTCTAAACCGTAATCCAACTCC		(Musetti *et al.*, 2010)	
MDCALS5 for	AGAAACTCCGTCTATGGGCATC	FN395075	(Musetti *et al.*, 2010)	Callose synthase 5 coding gene
MDCALS5 rev	GCAGCCTTATAGCCTTCCATTA		(Musetti *et al.*, 2010)	

Table 1: Primer sequences used for the quantitative polymerase chain reaction (qRT-PCR) with the references sited when the sequence were used from previously designed gene specific primers.

RESULTS

Detection of '*Candidatus* Phytoplasma mali' in apple trees (healthy, diseased and recovered)

PCR analysis, performed using group-specific primers for AP, confirmed the presence of phytoplasmas in all the leaves of diseased (D) apple trees, but not in those of healthy (H) or recovered (R) plants, confirming that the remission of AP symptoms is associated to the disappearance of phytoplasmas from plant leaves, as previously observed (Carraro *et al.*, 2004; Musetti *et al.*, 2004).

Expression analysis of cDNA coding for Sieve Element Occlusion (SEO) proteins

A previous study on recovery of apple trees from AP revealed changes in gene expression of five genes, three genes coding for callose synthases and two genes coding for phloem proteins (PP2 like) which were found remarkably up-regulated in recovered trees (Musetti *et.al.*, 2010). qReal-time PRC confirmed an alternation in the expression level of the two genes coding for SEO: as regards SEOa, a higher expression level (about twice) was recorded in recovered leaves comparing to healthy ones. However, there was no significant difference in the expression level in diseased comparing to healthy leaf tissues. The expression level of SEOb showed similar trend as SEOa, but with slight significant difference disease samples comparing to healthy (Figure 7).

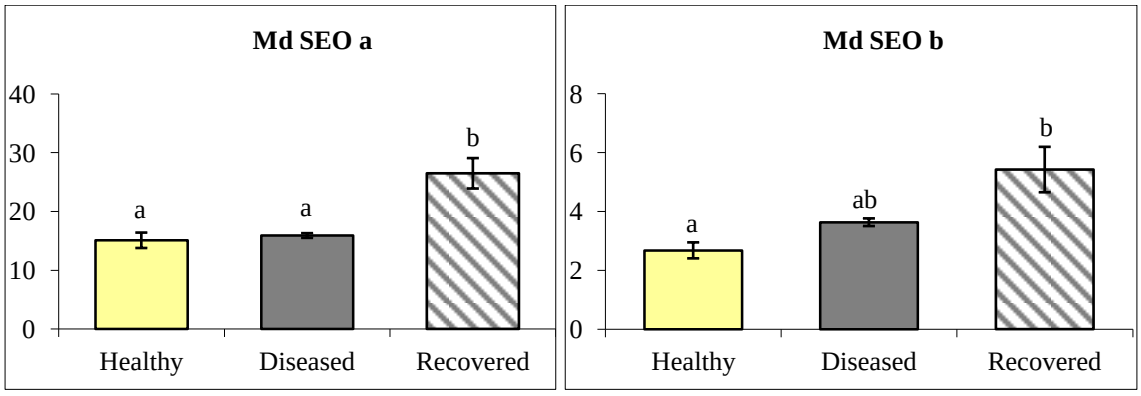


Figure 7. Expression level of sieve element occlusion (SEO) genes in leaves from healthy, diseased and recovered plants. Relative expression levels by quantitative real-time reverse transcription polymerase chain reaction of two genes coding SEOa and SEOb based on the average of three plants for each of the three experimental groups (healthy, diseased and recovered). Normalized values of relative expressions of the four genes are given as means ± SE. Different letters denote significant differences at $P \leq 0.01$

Expression analysis of cDNA coding for Pathogenesis Related (PR) proteins

Expression levels of MdPR 1, MdPR 2, MdPR 5, and MdPR 8 are reported in Fig. 4. qRT-PCR analyses demonstrated that the diverse genes were differentially expressed in apple leaves according to the phytosanitary status of the plants. In particular, MdPR 1 and MdPR 2 were 2.5 times higher in D plants than in H plants and more than two times higher than in R plants. MdPR 5 was 6-fold induced in D plants compared to H plants and 2.5-fold to R plants (Fig. 4). It is noteworthy that the level of expression of PR1 and PR5 genes in R leaves stand in between the levels in H and D plants. The transcription level of MdPR 8 was similar in the D and R plants being on average, about two times comparing to H plants (Figure 8).

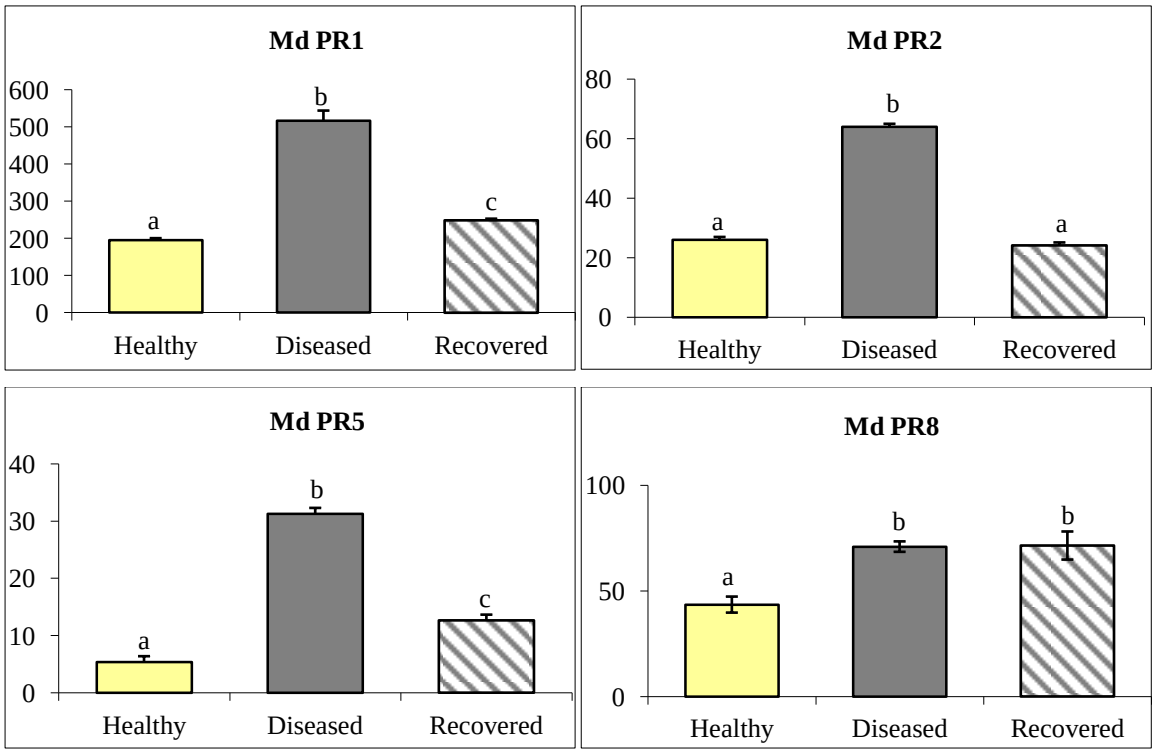


Figure 8. Expression level of pathogenesis related (PR) genes in leaf tissues from healthy, diseased and recovered plants. Relative expression levels by quantitative real-time reverse transcription polymerase chain reaction of PR1, PR2, PR5 and PR8 based on the average of three plants for each of the three experimental groups (healthy, diseased and recovered). Normalized values of relative expressions of the four genes are given as means ± SE. Different letters denote significant differences at $P \leq 0.01$

Expression analysis of cDNA coding for Jasmonic Acid (JA) signaling pathway genes

Regarding the JA-marker genes, the expression level of three genes coding key enzymes of the JA pathway, namely AOS 2, 12-OPR 3 and PI II were evaluated. AOS catalyzes the first step in the biosynthesis of JA from lipoxygenase-derived hydroperoxides of free fatty acids, while OPR enzymes convert the precursor 12-oxophytodienoic acid (OPDA) to JA (Schaller *et al.*, 2005). The expression of PI II has been extensively characterized as a final event in the JA-induced signal transduction cascade, especially in tomato plants (Sivasankar *et al.*, 2000). 12-OPR 3 and PI II were significantly repressed in the leaves of D apple plants comparing to the H plants (Figure 9). On the other hand, these genes tended to have higher expression levels in R plants compared to the D ones. The trend in gene expression level of the JA signaling pathway was the contrary compared to the trend of PR genes (related to the Salicylic acid pathway). This confirmed the cross-communication between SA-pathway and JA-pathway, as the two pathways play main role in plant defence mechanesism (Spoel *et. al.*, 2003).

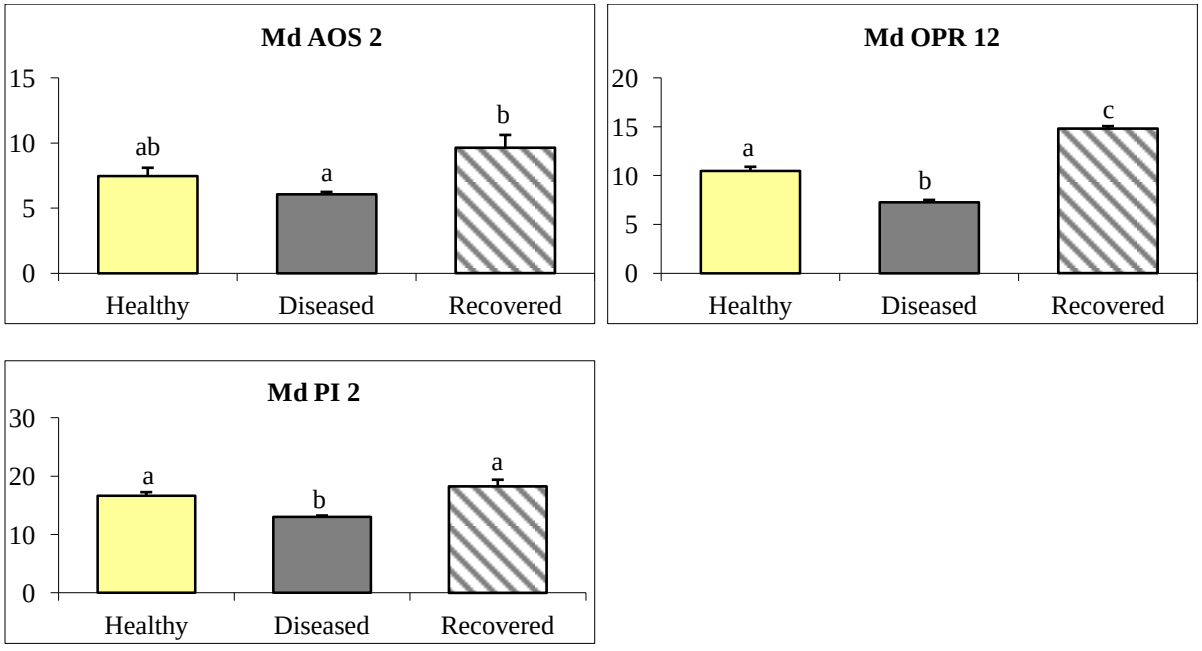


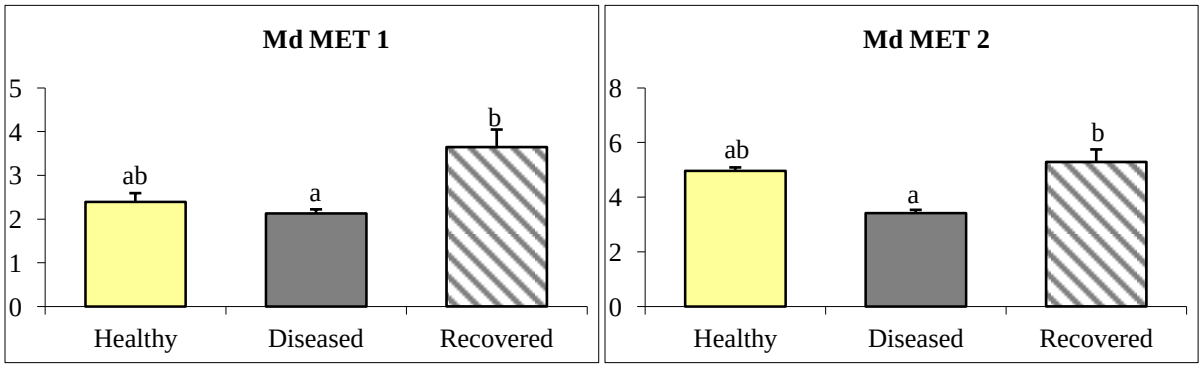
Figure 9. Expression level of jasmonic acid (JA) genes in leaf tissues from healthy, diseased and recovered plants. Relative expression levels by quantitative real-time reverse transcription polymerase chain reaction of JA signaling pathway, allene oxide synthase 2 (AOS2), oxophytodienoate reductase (12-OPR 3), and JA-inducible proteinase inhibitor II (PI-II)based on the average of three plants for each of the three experimental groups (healthy, diseased and recovered). Normalized values of relative expressions of the four genes are given as means ± SE. Different letters denote significant differences at $P \leq 0.01$

Expression analysis of cDNA coding for DNA methylation and demethylation genes

DNA methylation is involved in diverse biological processes in plants (He *et al.,* 2011).

To investigate the effect of '*Ca*. P. mali' infection on methylation status of the host-plant DNA, the expression of genes involved in cytosine methylation was studied by qRT-PCR. There are two classes of DNA methyltransferases currently characterized: first, ***de novo* class** enzymes that create new methylation marks on the DNA, and second, a **maintenance class** that recognizes the methylation marks on the parental strand of DNA and transfers new methylation to the daughter's strands after DNA replication (Finnegan *et al.,* 1998). Transcript levels of five distinct DNA methyltransferases, (methyltransferases, MET1 and MET2; chromomethylases, CMT3; and domain rearranged methyltransferases, DRM2 and DRM8) already described in *Arabidopsis* (Finnegan *et al.,*1998; Teyssier *et al.,* 2008) were determined in leaves of healthy, diseased and recovered apple trees. Gene expression analyses demonstrated that the diverse genes are differentially expressed in apple leaves according to the phytosanitary status of the plants.

Gene expression levels are reported in (Figure 10). The expression of the DNA methyltransferase genes was not significantly altered upon AP infection except the CMT 3 gene whose expression resulted significantly down-regulated compared to healthy and recovered plants. In leaf tissues from recovered trees the expression of MET1 and MET2 were confirmed to be significantly up-regulated, between 1.5-1.75 folds, comparing to the diseased plants.



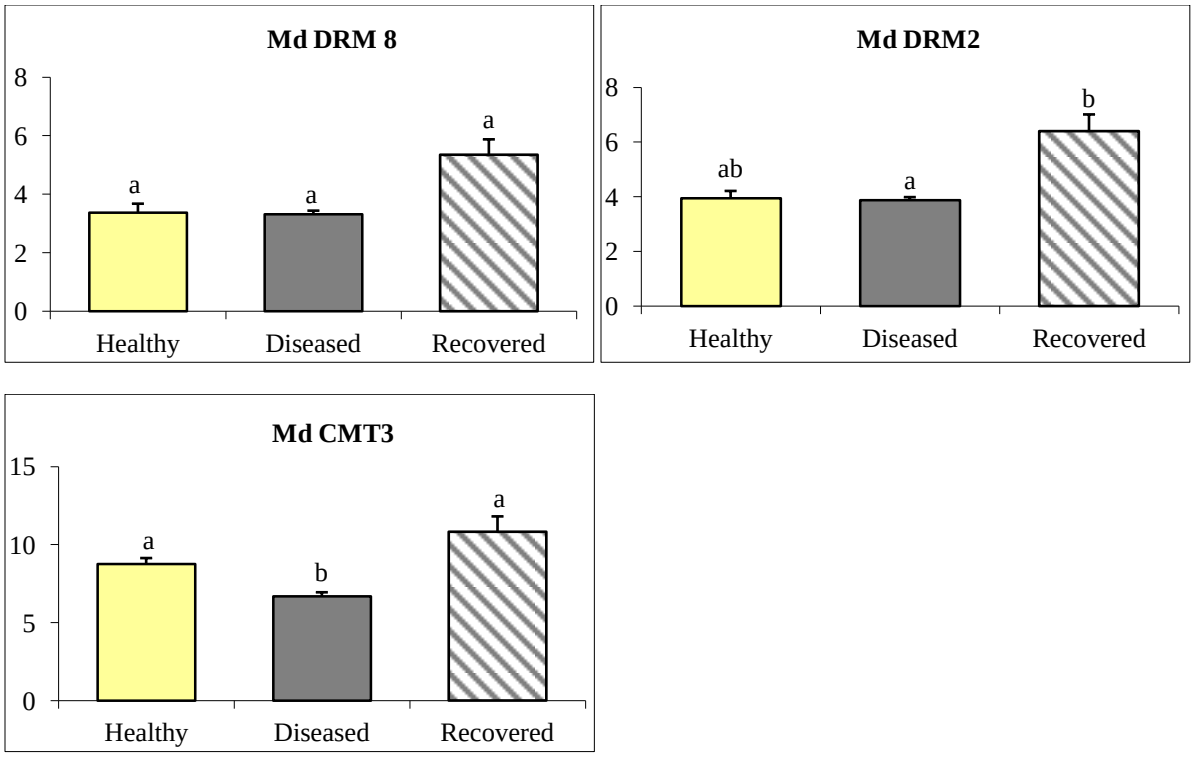


Figure 10. Expression level of genes coding for methyltransferases in leaf tissues from healthy, diseased and recovered plants. Relative expression levels by quantitative real-time reverse transcription polymerase chain reaction of methylation coding genes (MET1, MET2, CMT3, DRM 2 and DRM 8) based on the average of at least four for each of the three experimental groups (healthy, diseased and recovered). Normalized values of relative expressions of the four genes are given as means ± SE. Different letters denote significant differences at $P \leq 0.01$

Expression analyses of genes involved in *de novo* methylation were not comparable to expression pattern found in methyltransferase genes. Analyses revealed a significant suppression in AGO4, Pol IV and Pol IV2 gene expression levels in diseased leaf tissues, while there were no significant changes in the expression level of RDR2 and Pol V genes among the different plant groups (H, D and R).

In the recovered plant the expression profile of *de novo* methylation genes were not totally in accordance to the trend showed by the methyltransferase genes. The expression analysis revealed that all tested genes, except the RDR2, whose expression level resulted similar in the leaf tissue from the three plant groups, tended to be down-regulated in leaf tissues from recovered plants comparing to the healthy plant. The expression profiles of *de novo* methylation genes are illustrated in Figure 11.

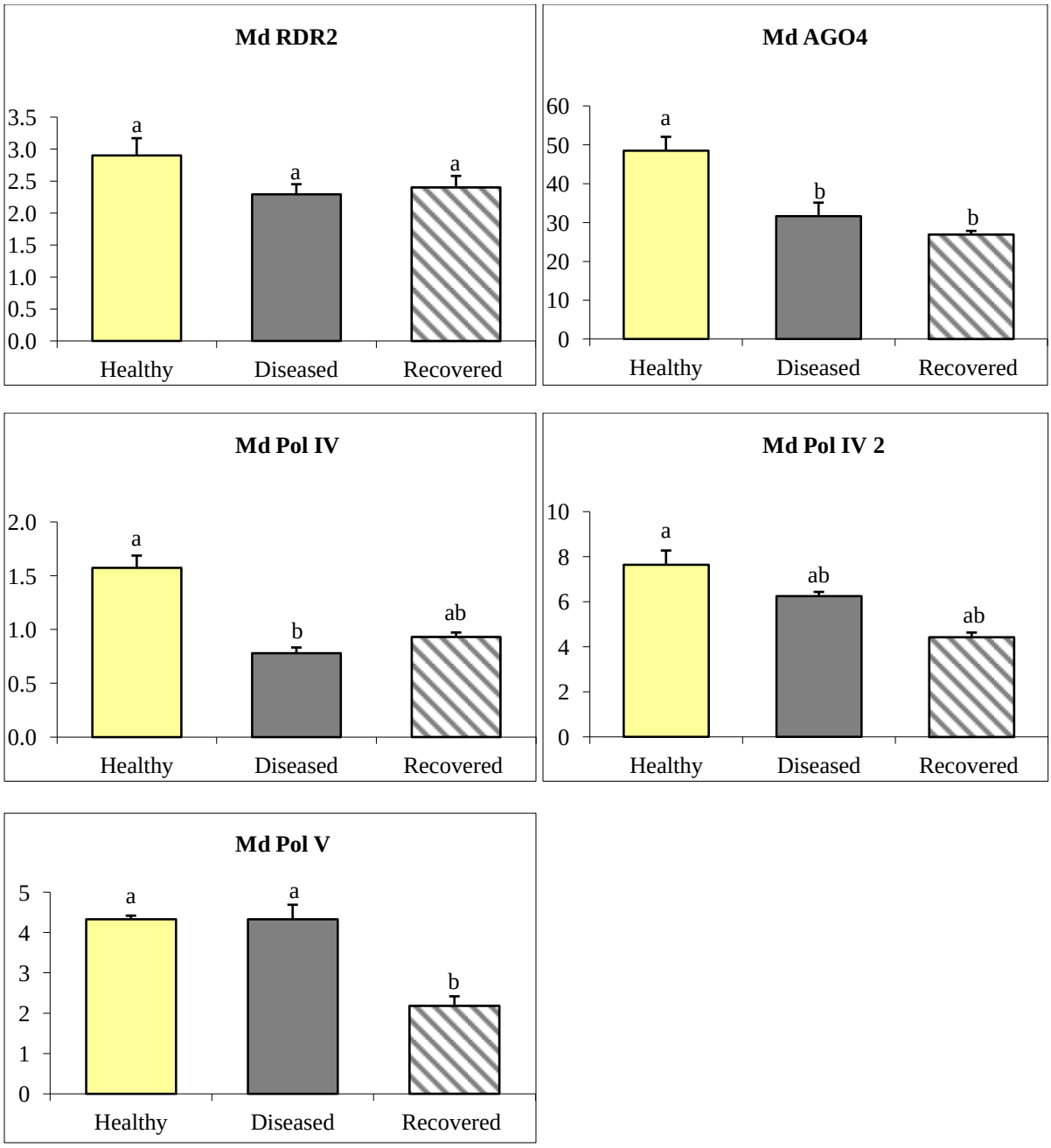


Figure 11. Expression level in *de novo* methylation genes in leaf tissues from healthy, diseased and recovered plants. Relative expression levels by quantitative real-time reverse transcription polymerase chain reaction of *de novo* methylation genes (RDR2, AGO4, Pol IV, Pol IV2 and Pol V) based on the average of at least four plants for each of the three experimental groups (healthy, diseased and recovered). Normalized values of relative expressions of the four genes are given as means ± SE. Different letters denote significant differences at $P \leq 0.01$

As regards the expression levels of the three demethylation genes, they were variable in the expression level in the different plant groups and different isoforms (*i.e.* DML and DML3) showed a variable trend (Figure 12). The expression level of DME was unchanged in leafe tissue from healthy and recovered plants but it was down-regulated in the diseased ones.

DML and DML3 genes showed similar expression trend in leafe tissue from diseased and recovered plants, but as regards leafe tissue from healthy plants, DML was up-regulated while DML3 resulted down-regulated.

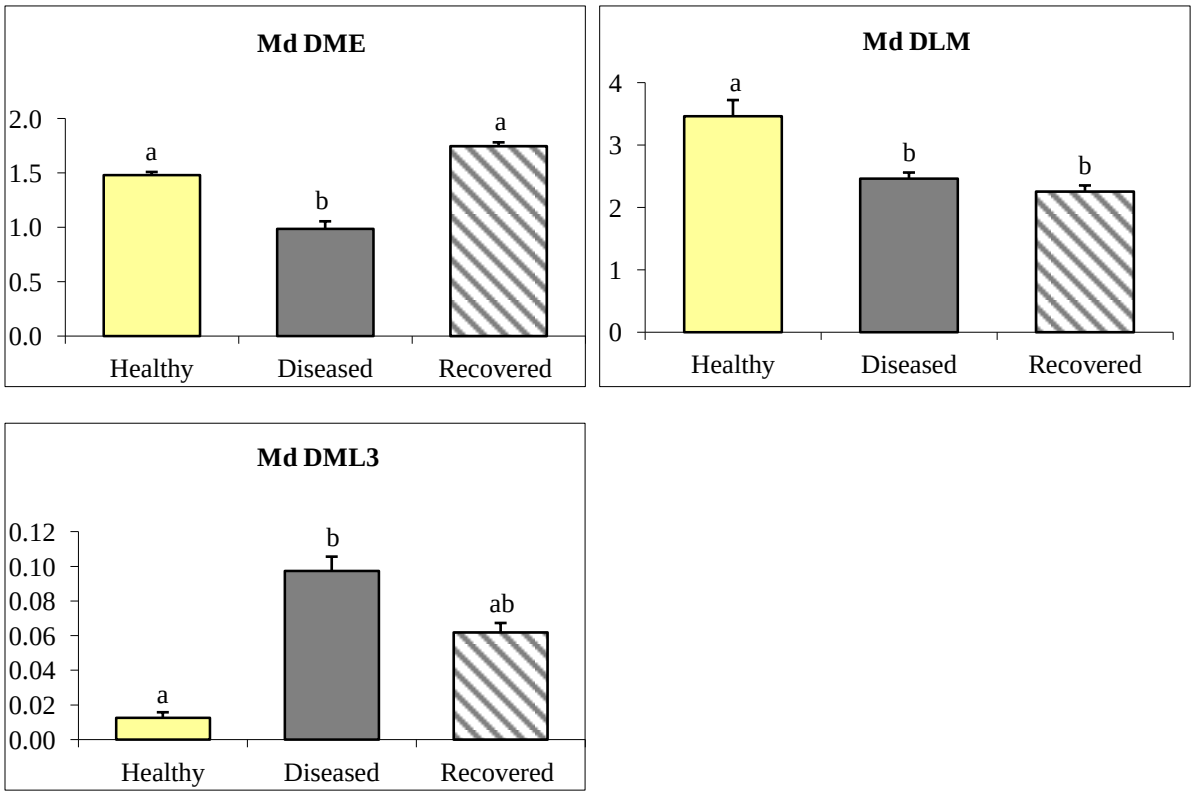


Figure 12. Expression level in demethylation genes in leaves from healthy, diseased and recovered plants. Relative expression levels by quantitative real-time reverse transcription polymerase chain reaction of demethylation coding genes (DME, DML and DML3) based on the average of at least four plants for each of the three experimental groups (healthy, diseased and recovered). Normalized values of relative expressions of the four genes are given as means ± SE. Different letters denote significant differences at $P \leq 0.01$

DISCUSSION

Recovery phenomenon: *Malus domestica* from apple proliferation disease

'Candidatus Phytoplasma mali' belong to the 16SrX group considered to be a guarantee phytoplasma, being the causal agent of the apple proliferation (AP) disease (Torres *et al.*, 2005) which causes devastating losses and represents a critical issue in apple production area. Little is what we know about plant-phytoplasma interaction despite the several studies that have been attempted to explain the physiological and biochemical changes in the host plant challenged by phytoplasma infection. Different approaches have been applied to study this interaction using molecular, biochemical or microscopic techniques.

As curative methods against AP are not available, the disease control is only based on insect vector control, infected crop eradication and weed control. Plants infected by phytoplasmas have been reported to show spontaneous remission of symptoms, a phenomenon called "recovery", which is associated with the disappearance of the pathogen from the plant crown (Osler *et al,.* 2000; Carraro *et al.*, 2004; Musetti *et al.*, 2004 and 2010). The physiological basis of "recovery", as well as the molecular signals and mechanisms involved in the onset and establishment of the phenomenon are still far from being elucidated. Comparative studies performed on leaf phloem tissue of apple plants showing different phytosanitary status, *i.e.* healthy-never symptomatic (H), symptomatic-diseased (D) and recovered (R), demonstrated an anomalous accumulation of callose and proteins in the phloem of R apple plants associated with the up-regulation of callose synthase- and phloem protein-coding genes, supporting the hypothesis that R apple plants were able to develop resistance mechanisms depending on Ca^{2+} signal activity (Musetti *et al.*, 2010).

In this thesis we performed further analyses for other specialized calcium-powered phloem proteins, named sieve element occlusion (SEO) proteins. These proteins are characteristic of the sieve elements of *Fabaceae*, where they are aggregated in giants bodies, the forisomes; but gene encoding SEO resulted widespread among dicots (Rüping *et al.*, 2010), suggesting an important role in the sieve element preservation from wounds and biotic stresses.

Comparing the expression patterns of SEO proteins in H, D and R apple trees, an up-regulation in leaf tissues of R plants has been found. This result reinforces the assumption that phloem proteins could have an important role in the plant defense processes and in the establishment of 'recovery' in apple plants (Musetti *et al.*, 2010).

Plant-pathogen interactions can result in the activation of numerous other mechanisms of local and systemic defences. PR proteins are generally considered to be defence proteins whose function is to prevent or limit of the invasion or spreading of the pathogens in plants. However, the mode of action of such proteins and their contribution to resistance has not been elucidated in many cases (van Loon *et al.,* 2006). In plants belonging to the genus *Malus*, MdPR 1, MdPR 2, MdPR 5 and MdPR 8 were proposed as candidate genes involved in plant response to attacks by pathogens *E. amylovora* and *V. inaequalis* (Bonasera *et al.*, 2006; Malnoy *et al.,* 2007). In this work we demonstrate that the above mentioned genes are involve in the response of apple plants to AP infection which also results in the over-expression of these genes in infected apple trees, thus confirming that these proteins are not pathogen-specific, but are determined by the reaction of the host plants.

As regarding R apple plants, results revealed an increment in transcript abundance of PR 1, PR 5 and PR 8 in comparison to H individuals. This finding gives reason for the activation of plant defense and could explain the fact that R plants are more resistant than H plants to new phytoplasma infections (Osler *et al.*, 2000). PR 1 proteins are those which are most abundantly produced after pathogen infection, and following the accumulation of the transcript occurring during pathogen attack. As previously reported, these proteins are strongly induced by SA (Durrant and Dong, 2004). Patui *et al.*, (2012) reported data about a significant increase of SA content in D apple leaf tissues compared to H or R tissues, while JA content was higher in R plants than H.

Increased expression level of PR 5, associated to a PR 5 protein accumulation, has been found in grapevine infected by Flavescence dorée and in *Chrysanthemum carinatum* infected by Onion Yellows (Margaria and Palmano, 2011; Zhong and Shen, 2004). El-kereamy *et al.,* (2011) suggested that in addition to known direct antifungal/antibacterial activity for the PR 5 proteins, the same proteins may have additional function in plant cells, correlated to the synthesis and accumulations of phytoalexins. In this regard, Landi and Romanazzi (2011) hypothesized that recovery could be connected with metabolic processes linked to phytoalexin production and accumulation.

The occurrence of JA-related defence response in plant/phytoplasma/vector relationships was recently illustrated by Sugio *et al.,* (2011) in *Arabidopsis thaliana.* These authors reported that JA synthesis is down-regulated in Aster Yellows-infected Arabidopsis compared to the healthy plants and that phytoplasma effectors are able to target JA-mediated plant response. In particular, these authors demonstrated that SAP 11, an AY phytoplasma effector, targets JA synthesis via interaction with a plant transcription factor and modulates plant defense responses by reducing JA production to the advantage of the AY insect vector. The observed repression of the JA- pathway marker genes in apples affected by AP would confirm Sugio *et al.,* (2011).

On the other hand the up-regulation of AOS, catalyzes the first step in the biosynthesis of JA from lipoxygenase-derived hydroperoxides of free fatty acids, while OPR enzymes convert the precursor 12-oxophytodienoic acid (OPDA) to JA (Schaller *et al.,* 2005) in recovered tree confirmed the resulted obtained by Patui *et al.,* (2012) that recovered tree found to contained higher accumulation if JA in their tissue. Several studies have reported that jasmonate (JA) signaling pathway plays a crucial role in mediating antiherbivore defense responses in plants (Thaler *at al.,* 2004; Ye *et al.,* 2013). The activation of JA signaling pathway in recovered plant therefore provide protection against insect herbivore and perhaps it would provide also protection against phytoplasma insect vectors which also could play role in the resistant of recovered tree against new phytoplasma infection.

In conclusion, the different transcription profiles found for the examined genes in AP diseased and recovered apple plants allow us to hypothesize that plant defence could take place in at least two subsequent phases, regulated by two different pathways. That during the spread of pathogens in the host tissues and development of disease and symptoms, SA is involved in the prompt response to disease (Patui *et al.*, 2012) inducing PR gene up-regulation, thereby antagonizing JA-dependent defences; in fact, in the diseased apple trees, two of the tested JA-pathway genes were significantly repressed compared to healthy ones, with the consequence that JA production is impaired in plants during the development of symptoms. On the other hand, inhibition of the PR genes and activation of the JA-related defence mechanism via JA gene up-regulation occur in recovered comparing to diseased plants.

Given that recovery is a natural, spontaneous event not reproducible artificially, explanation of the phenomenon is not simple. It has been hypothesized that different defence signals are finely tuned in plants (Beckers and Spoel, 2006) and that SA and JA pathways could be simultaneously activated, resulting in an enhanced resistance for the host, compared to either defence response alone (van Wees *et al.,* 2000). This result demonstrates for the first time that two diverse sets of defence genes are involved in the interaction between apple plants and '*Ca.* P. mali' and those they are differentially expressed during phytoplasma infection or recovery.

DNA methylation is an important epigenetic marker of heritable changes in gene expression that occur without a change in the DNA sequence (Rival *et al.*, 2010). DNA methylation is a relatively stable epigenetic mark. Nevertheless it is still dynamically regulated. Plants, as well as animals, share some common mechanisms for the regulation of DNA methylation and demethylation. Both in plants and animals, maintenance of DNA methylation depends on the maintenance DNA methyltransferases during DNA replication.

Two distinct classes of DNA methylation enzymes have been characterized: class one which is a maintenance class that recognizes the methylation marks on the parental strand of DNA and transfers new methylations to the daughter's strands after DNA replication. The second class of enzymes belongs to the RNA-directed DNA methylation pathways (RdDM), also called *de novo* methylation pathway. The mechanism of RdDM remains not completely understood. During the annotation process of *A. thaliana* genome special DNA-dependent RNA polymerase genes were discovered. They found have function in RNA-mediated transcriptional silencing and heterochromatin formation and play an essential role in RdDM, a process by which 24-nucleotide small-interfering RNAs (siRNAs), direct the *de novo* cytosine methylation of complementary DNA sequence (Haag and Pikaard, 2011). The discovery of new component from DNA methylation pathway shows the importance of this epigenetic role not only to transposon silencing but also to expression regulation of genes important for plant physiology and development. The model for dynamic regulation of RdDM of the two methylation classes both maintenance and *de novo* methylation have been characterized and described in Matzke *et al.*, (2007). The two classes were shown to work cooperatively in a complex pathway, which contributes to the global methylation in the plant. The schematic presentation in Figure 13 shows the dynamic methylation pathway in plant and the component of RdDM (*de novo* methylation), or maintenance methylation and DNA active demethylation.

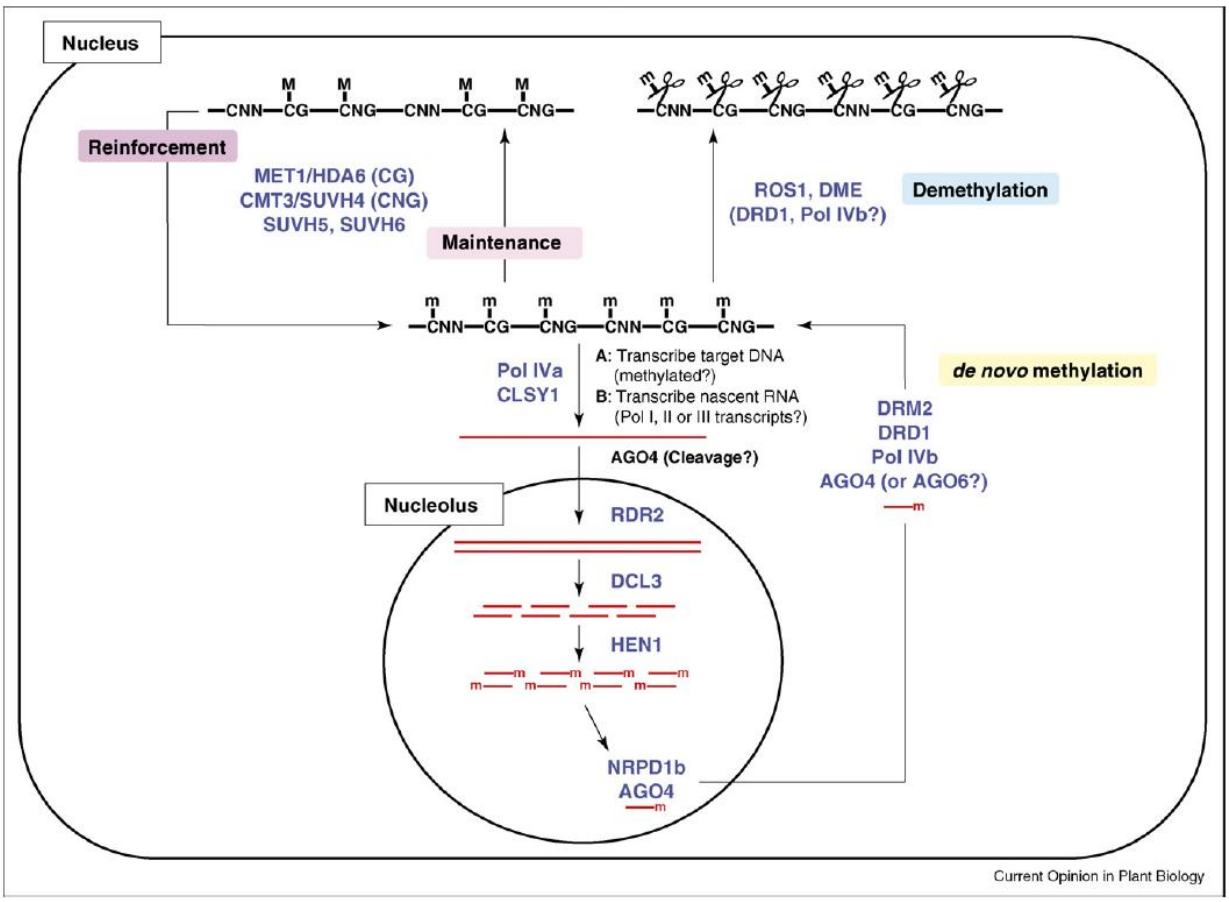


Figure 13. A model for dynamic regulation of RdDM. explain the mechanisims and show the gene involve in both maintenance and *de novo* methylation also DNA demethylation (Matzke *et al.*, 2007).

DNA methylation and have been reported to play critical roles in regulating gene expression in response to biotic and abiotic stress (Wada *et al.*, 2004; Pavet *et al.*, 2006; Choi and Sano, 2007; Boyko and Kovalchuk, 2008). It has been reported that DNA methylation levels in plants are modified in response to environmental stimuli (Steward *et al.*, 2002) and to biotic stresses. Changes in DNA methylation have been reported during the interaction between Tobacco Mosaic Virus and in tobacco plants as well as in *Pseudomonas*-infected *Arabidopsis* (Wada *et al.*, 2004; Pavet *et al.*, 2006).

As regards phytoplasma diseases, Ahmad *et al.,* (2012) reported that the expression level of methylase and demethylase genes was globally down-regulated in tomato plants infected with stolbur isolate PO. Our data, in general, are in accordance with this report, as a down-regulation of the tested methylase genes and of two out three demethylase genes, was found in D leaf tissues compared to the controls. Moreover, in this work, for the first time, we demonstrated an involvement of the DNA methylation pathway in the establishment of recovery of apple plants from AP infection. Last but not least, our analyses support a recent report by Osler and co-workers (Plant Disease, in press). These authors demonstrated that resistance exhibited from R plants, when they are exposed again to phytoplasma infection, is transmissible from plant to plant by chip-grafting, supporting the hypothesis that in recovered plants epigenetic changes occurred and that they are due to a graft-transmissible memory.

Chapter 2

USE OF FUNGAL ENDOPHYTES IN THE CONTROL OF APPLE PROLIFERATION DISEASE

MATERIALS AND METHODS

Epicoccum nigrum and '*Candidatus* Phytoplasma mali' interaction on the natural host plant (*Malus domestica*)

To study the interaction between the fungal endophyte *Epicoccum nigrum* and '*Candidatus* Phytoplasma mali' in the natural host plant, *Malus domestica,* forty young (four years old) apple trees, Golden Delicious (GD) cultivar, were selected from nursery and set in pots and maintained in semi-field environment in early summer of 2011. The forty plants were divided into four studying groups, ten plants each.

- Group one: plants inoculated with the endophytes *E. nigrum* (EP+).
- Group two: plants inoculated with the endophyte *E. nigrum* and *'Ca.* P. mali' (EP+, AP+).
- Group three: plants inoculated with '*Ca.* P. mali' (AP+).
- Group four: plants were set as Control (C).

Epicoccum nigrum culture and apple tree fungal enophyte inoculation

Epicoccum nigrum strain was obtained from the collection maintained at the Department of Agricultural and Environmental Science, University of Udine, Italy (Martini *et al.*, 2009). For inoculation in apple trees, the strain H2F1, previously tested in apple proliferation-infected *Catharanthus roseus* plants (Musetti *et al.*, 2011b), was used. The fungus was routinely grown in potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 24^{o}C under dark condition. Twenty apple trees were randomly selected for *E. nigrum* inoculation. Three internodal cuts in three different branches were made using a grafting knife and immediately an agar plug containing actively growing mycelium of *E. nigrum* was applied on each sectioned stem (EP+ plants). Inocula were then coated with parafilm to avoid drying.

'*Candidatus* Phytoplasma mali' infection of apple trees

Four days after fungal inoculation, twenty apple plants, ten plant inoculated with the fungal endophyte and ten not inoculated were ioculated with '*Ca.* P. mali' AP 15 strain, by bud-grafting (EP+ AP+ plants and AP+ plants, respectively). The original source of inoculum was obtained from a symptomatic apple plant from the field.

First evaluation about AP symptom on the AP+ and EP+AP+ plants were performed in June and September 2012 and repeated in the same months in 2013.

Interactions of *Epicoccum nigrum* and *Malus domestica*

The interactions between the apple plants and the endophyte *E. nigrum* have been investigated using an integrated approach including molecular and ultrastructural studies.

The molecular studies were performed by analysing the changes in the expression levels of some plant defense-related genes and other groups of genes, which have been analysed for recovery studies over three years.

Three sample collections from all apple tree groups (EP+, AP+, EP+AP+, C) were made in 2011, three days after the *E. nigrum* inoculation, ten days after phytoplasma inoculation and the last sample collection was made two months after phytoplasma inoculation. Two sample collections were made also in 2012, in spring (April) and in autumn (September), and last sampling was made in autum 2013, when the phytoplasma symptoms were visible on the plants.

The investigated genes were fallen into four groups:

- Phloem proteins which include genes coding for Phloem proteins (PP2), two genes coding for sieve element occlusion (SEO) proteins and five genes coding for callose synthases (CaSy);
- Pathogenesis related proteins (PRs): NPR1, PR1, PR2, PR5 and PR8;
- Jasmonate (JA)-pathway marker genes: AOS2, 12OPR-3 and PI2;
- DNA Methylation (MET1, MET2, DRM2, DRM8, CMT3) and Demethylation (DLM) genes.

Total RNA extraction, cDNA synthesis and gene expression analyses

Total RNA was extracted from frozen apple leaf tissues using RNeasy Kit (Qiagen) according to the manufacturer's instructions with minor modifications. The extractions were carried out in 2 ml eppendorf tube as follows:

- grind the tissue with liquid nitrogen thoroughly in a mortar and pestle;
- transfer about 100 mg of the powder to a liquid nitrogen-cooled 2 ml microcentrifuge tube;
- add immediately 800 µl of prepared RLT lysis buffer (1 ml RTL buffer, 10 µl ß-mercaptoethanol and 25 mg of PVP);
- vortex vigorously for 30 second and add 1/10 of the volume to the lysate tissue sarkosyl solution;
- incubate for 10 min in water bath at 56 ^{o}C;
- centrifuge for 5 min at 300g and transfer the lysate QIAshredder spin Colum.

Further steps were performed according to manufacturer's protocol.

RNA concentration and purity were checked using Lambda 3B spectrophotometer (Perkin Elmer Italy, Cologno Monzese, Italy). Only RNA samples with a 260/280 ratio (an index of protein contamination) between 1.9 and 2.1 and a 260/230 ratio (an index of reagent contamination) greater than 2.0 were proceed for cDNA synthesis.

First-strand cDNA was synthesized from 1 µg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol.

Real Time-PCR analyses were performed as described in the prvious chaptere using Bio-Rad CFX96 Real Time PCR System using Sso Fast EvaGreen SuperMix (Bio-Rad Laboratories, Inc., USA).

The sequences of each primer pair along with the accession numbers are listed in Table 1.

Diagnosis and quantification of '*Candidatus* Phytoplasma mali' in apple leaf tissues

'Candidatus phytoplasma mali' was detected and quantified in leaf material from EP+AP+ and AP+ plants in autum when typical AP symptoms were well visible on infected plants. Total DNA was extracted from 1 g of infected apple midribs using CTAB extraction method (Martini *et al.,* 2009). Midribs were cut from randomly chosen leaves and each sample was extracted in duplicate (two biological replicas). The presence of '*Ca.* P. mali' (AP 15 strain) in the apple trees was verified using the SYBR® Green I real-time PCR technology using the primer pairs AP15f/ AP15r (Martini *et al.,* 2010).

The '*Ca*. P. mali' quantification was expressed in genome units (GU) per ng of plant DNA (Marzachì and Bosco 2005). The 1-aminocyclopropane-1-carboxylate oxidase (ACO) was chosen as target for the amplification of plant DNA (Baric *et al.,* 2011). The standard curve was established diluting plasmid pGEM-Easy Vector (Promega, Madison, WI, USA) containing rp genes of AP15 strain; 1:10 serial dilutions of the plasmid starting from (1 ng/µl to 1 fg/µl were prepared in 20 ng/µl of total DNA from leaf tissues of healthy apple plants. To quantify plant DNA, a standard curve was prepared with 1:10 serial dilutions of total DNA from leaf tissues of healthy apple plants starting from 50 ng/µl to 5 pg/µl. RT-PCR analyses were performed in a Bio-Rad CFX96 Real Time PCR System using Sso Fast EvaGreen SuperMix (Bio-Rad Laboratories, Inc., USA).

Reaction mixture of 20 µl was prepared as follows:

- 10 µl Fast EvaGreen SuperMix;
- 0.6 µl forward primer (0.3 µM);
- 0.6 µl revers primer (0.3 µM);
- 6.8 µl H_2O;
- 2 µl cDNA templets (20 ng/ µl).

The thermal profile included three segments: (i) 95°C for 2 min; (ii) 40 cycles of 15 s denaturation at 94°C, 15 s annealing and extension at 56°C (and 60 ^{o}C for plant standard curve) and extrusion of 20 s at 72 ^{o}C and final extension for 5 min at 72 ^{o}C (amplification data collected at the end of each extension step); and (iii) dissociation curve consisting of 5 s incubation at 65°C to 95°C, at 0.5 °C increment intervals followed by plate reading, as illustrated in the scheme in Figure 14.

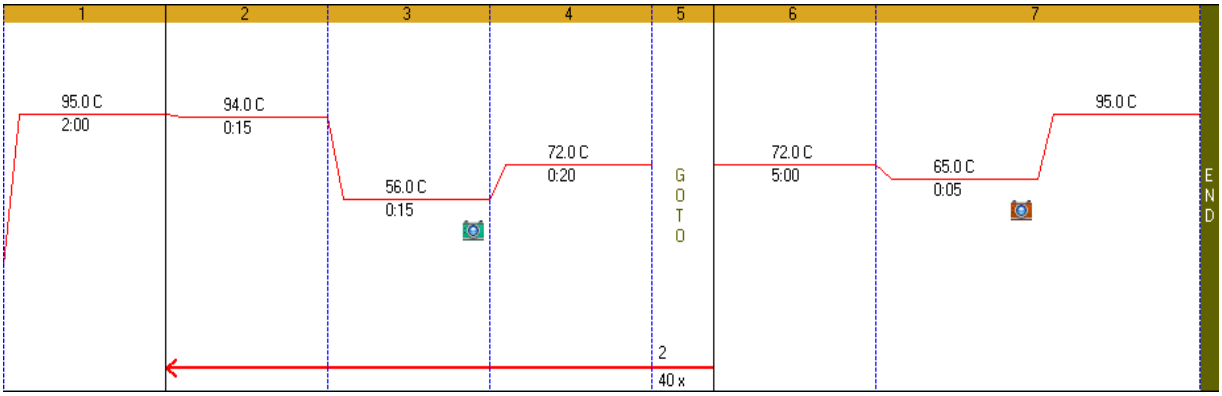


Figure 14. Schematic representation of the qReal-time PCR reaction conditions used for the diagnosis and quantification of *'Candidatus* Phytoplasma mali'.

Transmission Electron Microscopy (TEM)

Randomly chosen leaves were sampled from the four group plants (E+, E+AP+, AP+ and C) and processed for Transmission Electron Microscopy (TEM) observations. Leaf tissues were excised and cut into small portions (3x3 mm) and further proceed as follows.

Sample fixation was performed in three steps:

- primary fixation for 2 hours in a solution of 3 % glutaraldehyde at 4 ^{o}C;
- washing for 30 min with 150 mM sodium phosphate buffer (pH 7.0) at 4 ^{o}C;
- post-fixation with 1% osmium tetroxide (w⁄v) for 2 h at 4 ^{o}C.

Later the samples were dehydrated in ethanol as follows:

- ethanol 25% for 20 min at 4 ^{o}C;
- ethanol 50% for 20 min at 4 ^{o}C;
- ethanol 75% overnight at 4 ^{o}C;
- ethanol 100% for 60 min at 4 ^{o}C;
- propylene oxide for 20 min at 4 ^{o}C.

After, the samples were gradually embedded in Epon /Araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA), as follows:

Propylene oxide-resin mixture 1:1 for 1 hour at 4°C;

Propylene oxide-resin mixture 1:3 overnight at 4°C.

The resin was prepared as follows:

- Araldite (Fluka) 10 ml
- Epon 812 (Fluka) 12.5 ml
- Dodecenyl Succinic Anhydride (DDSA) (E.M.S.) 30 ml
- Epon accelerator DMP-30 Trisdimethylaminomethylphenol (Fluka) 0.8 ml

The sample then were left to dry over a parafilm then they were placed in the polymerization box, covered with the resin, and let to polymerize in the oven at 40 ^{o}C for 24 hours, followed by 60 ^{o}C for 72 hours.

Several serial ultrathin sections (60-90 nm) from each sample group were prepared by the ultramicrotome, using a diamond knife. The sections were collected on copper grids and stained with 3% uranyl acetate for 20 min in the oven at 40 ^{o}C, washed with distilled water and then stained by lead citrate for 10 min at room temperature. The samples then were observed under a Philips CM 10 (FEI, Eindhoven, the Netherlands) TEM, operating at 80 kV.

Effects induced by active secondary metabolites extracted from *Epicoccum nigrum* on phytoplasmas in *Catharanthus roseus* cuttings

Small cuttings (about 8 cm in length) were taken from both symptomatic AP-infected and healthy *Catharanthus roseus* plants. The cutting were then immersed in 50 ml falcon tube filled with 30 ml solutions of sterile distilled water containing 5 mg of purified secondary metabolite of bioactive properties extracted from the *E. nigrumm*, and crude extracts previously dissolved in 2 ml methanol. Solution of sterile water containing the same amount of methanol used for metabolite dissolution was used as a negative control; each treatment was performed in three replicas for both healthy and AP-infected plants.

All the cuttings were maintained at 20 °C, under natural light/dark photoperiod for 7 days. After incubation period, vein samples were removed from three leaves of each cutting and subjected to fixation and embedding for transmission electron microscope (TEM) observations, according to the method described earlier.

RESULTS

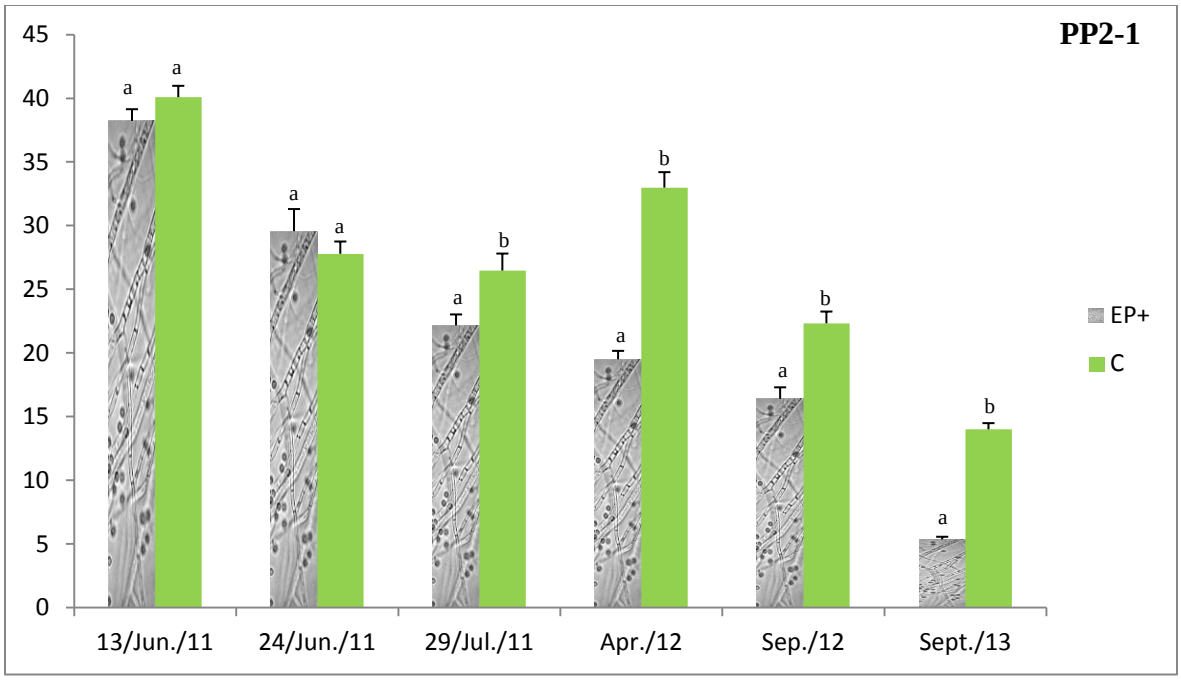
Epicoccum nigrum induces the expression of defense-related gene in *Malus domestica*

The study of the interactions occurring between apple plant and *E. nigrum* was performed by analyzing expression patterns of sellected defense-related genes in *Malus domestica*, following the inoculation with the fungal endophyte. Ultrastructural modifications were studied as well by TEM, in both inoculated (EP+) and not-inoculated control (C) plants.

Expression analysis of genes coding for Phloem Proteins-2 (PP-2), Sieve Element Occlusion (SEO) proteins and Callose Synthases (CaSy)

Expression profiles of genes coding for phloem proteins (PP2-1, PP2-2, PP2-3 and ERG1) over the three years' time period are illustrated in Figure 15. The genes have revealed a different expression pattern within the two different plant groups, EP+ and C, over the time. The expression level of two out four phloem protein transript (PP2-2 and ERG1), were significantly up-regulated in apple leaf tissues three days after inoculation with *E. nigrurm*. Later, a balance in the level of expression was restored. PP2-1 and PP2-3 showed the same trend of expression over the time, resulting in general, down-regulated in EP+ leaf tissues compared to the control.

The trend was different for the two genes coding for SEO proteins a and b (Figure 16). Unlike PP2, there was not significant alteration in the expression patterns. Which suggest that these specialized proteins were not involved in the interaction under study, which shows that the intracellular fungal colonization does not affect the plant cells or photoassimilates translocation in plant, which is the main function of SEO protiens.


PP2-1
45
40
35
30
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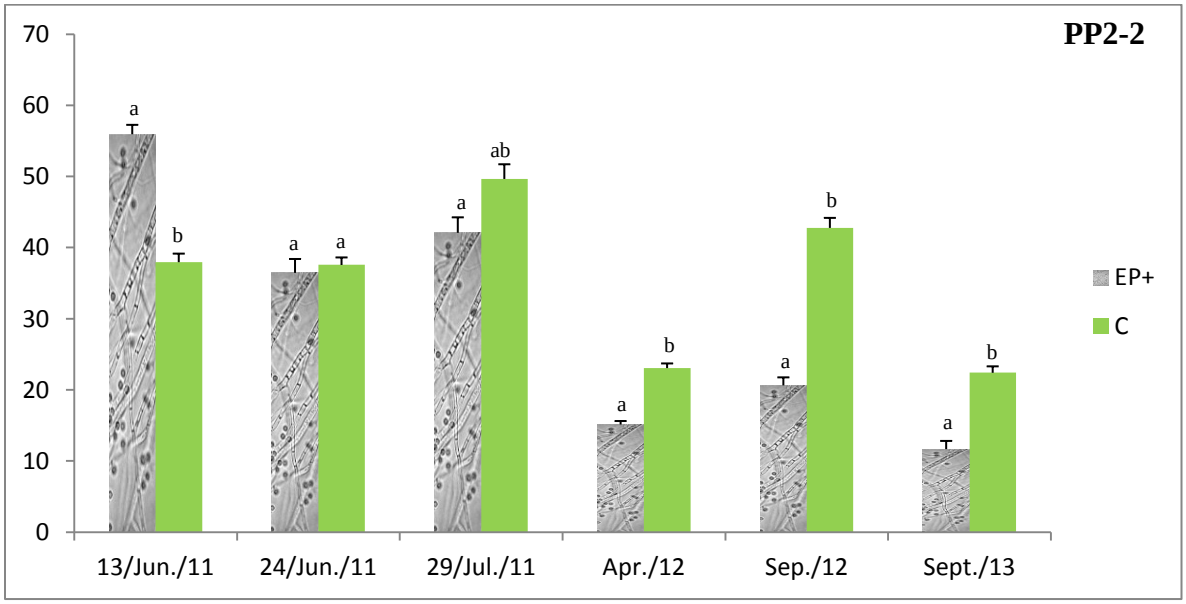
24/Jun./11
29/Jul./11
Apr./12
Sep./12
Sept./13
EP+
C


PP2-2
70
60
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a
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a
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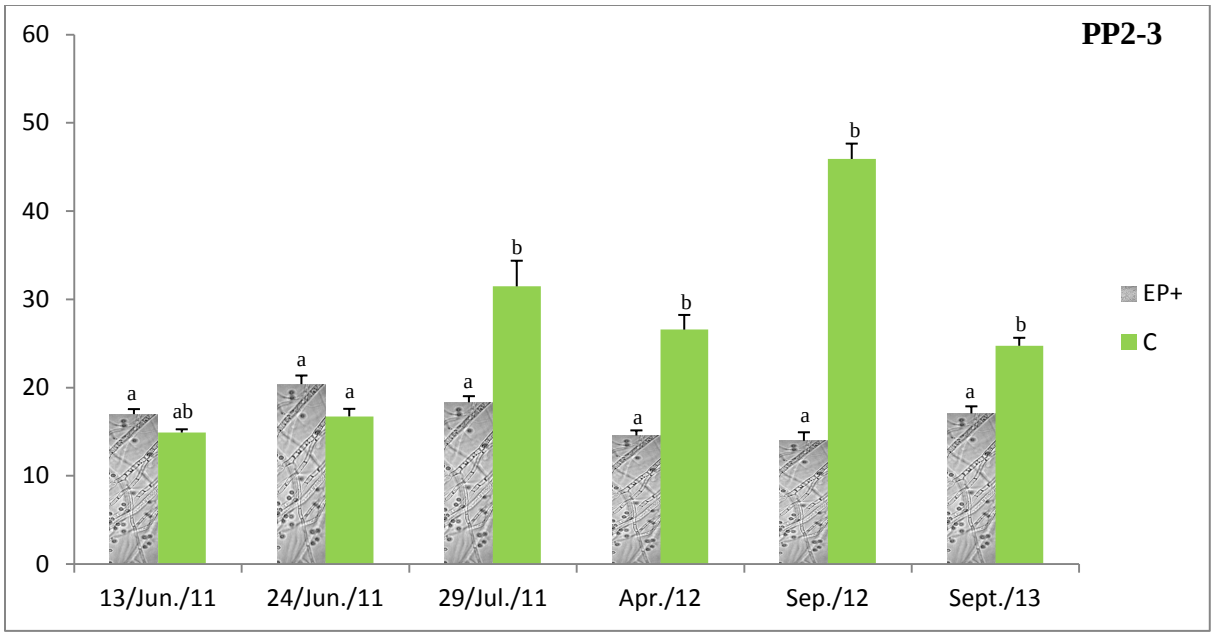
Sept./13
EP+
C



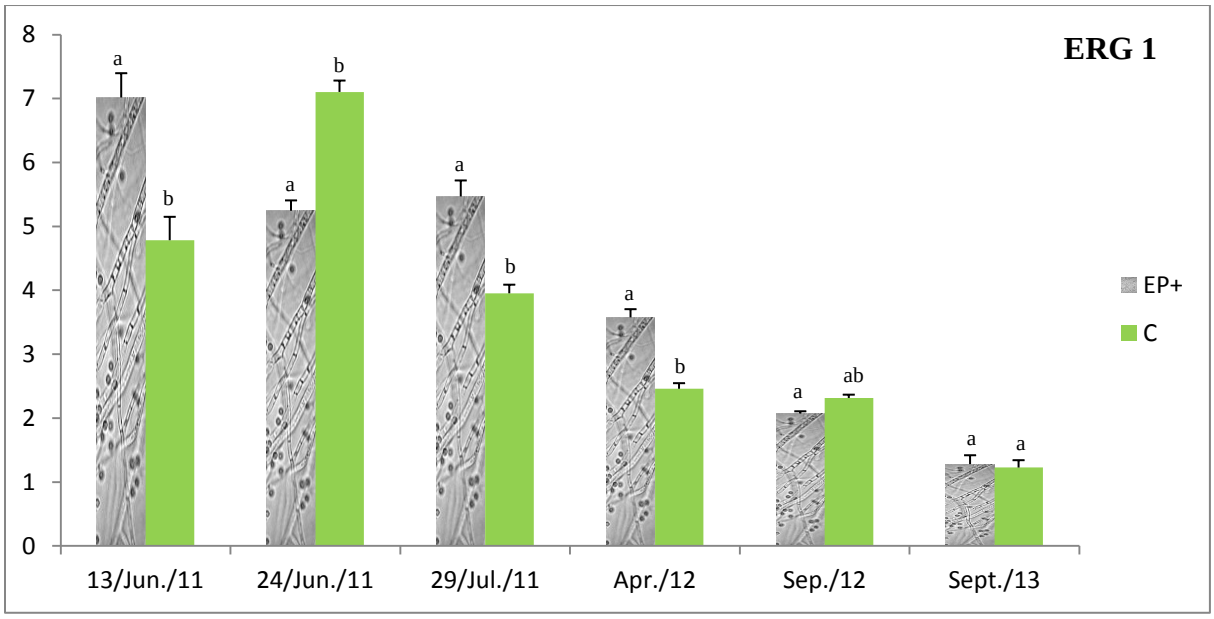


Figure 15. Gene expression analysis of *Malus domestica* Phloem Proteins 2 (PP2-1, PP2-2, PP2-3) and ERG 1 genes. Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the genes of interest were normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at $P \leq 0.01$

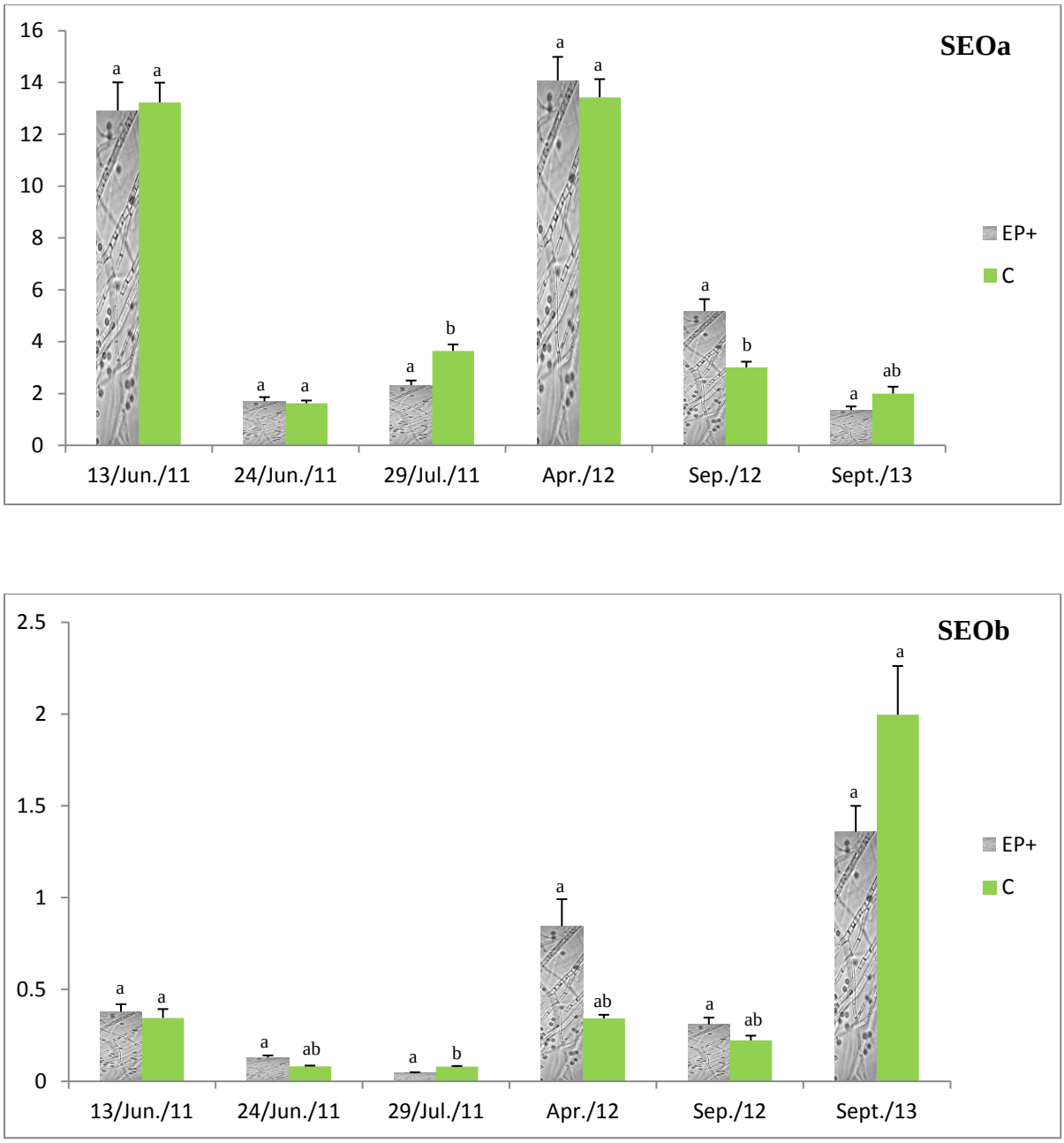
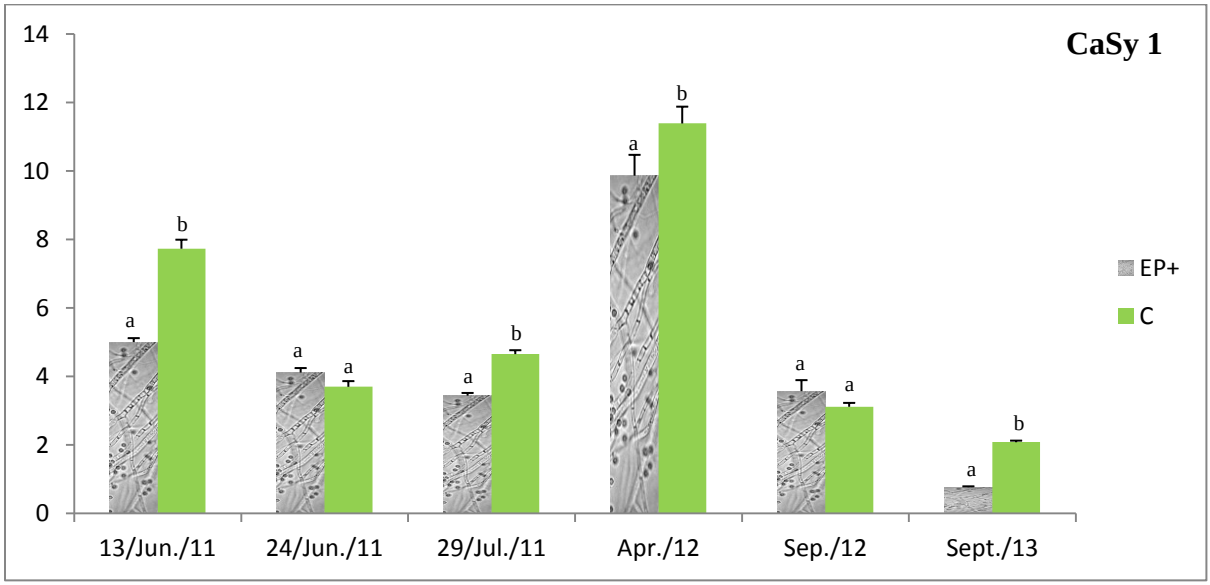
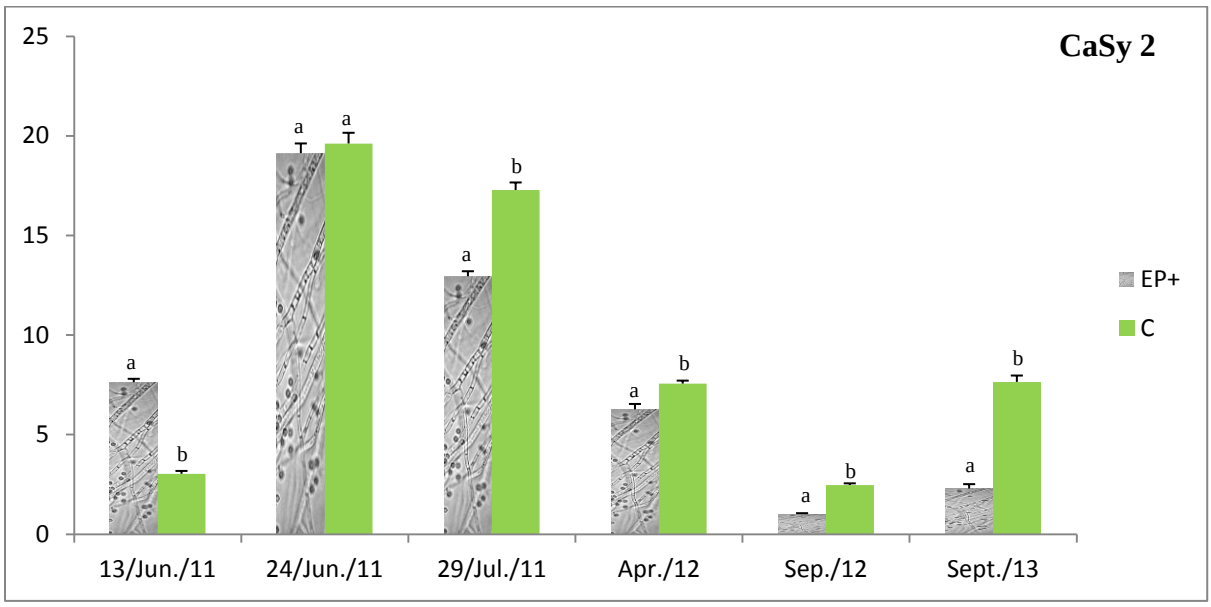
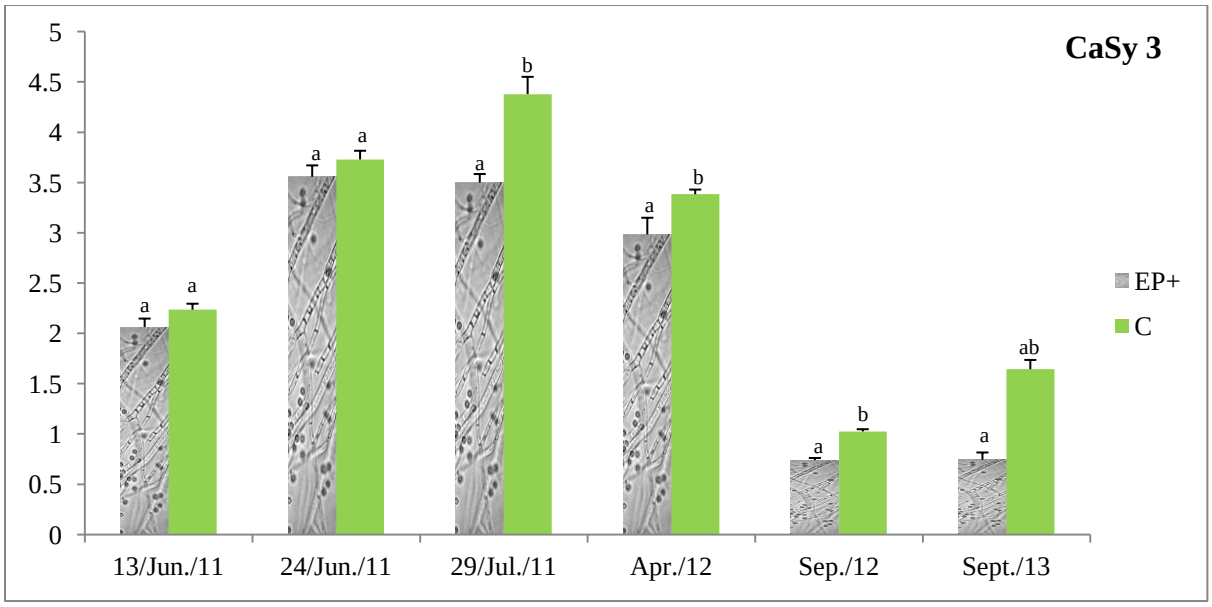


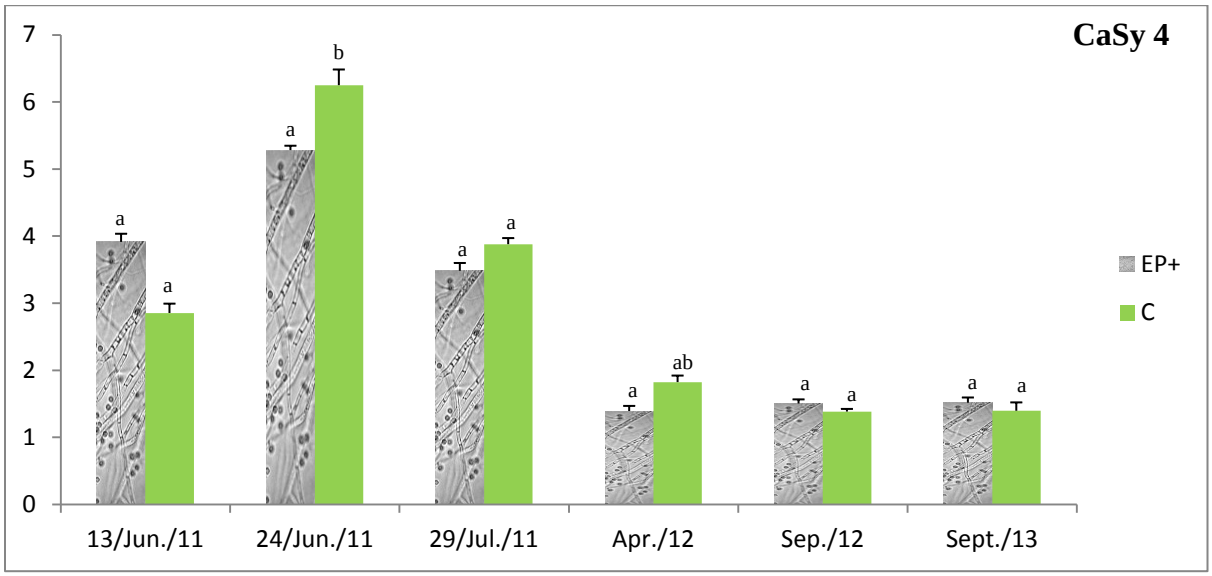
Figure 16. Gene expression analysis of *Malus domestica* sieve element occlusion (SEO) a and b genes. Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the genes of interest were normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at $P \leq 0.01$

Gene expression analyses of five genes coding for callose synthase (CaSy1, 2, 3, 4 and 5) were also analyzed (Figure 17). In comparison with C samples, CaSy 2, CaSy 4 and CaSy 5 were up-regulated in EP+ leaf tissues three days after endophyte inoculation later the expression levels tended to decrease even it resulted in significant down-regulated in CaSy2 and Casy4 upon the fungal inoculation. CaSy 1 and CaSy 3, in other hand, resulted in up-regulated in EP+ leaf tissues after fifty days after endophyte inoculation, then maintaining a high level of expression over the time (in particular CaSy 3).






CaSy 3
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a a
a a
a b
a b
a b
a ab
13/Jun./11
24/Jun./11
29/Jul./11
Apr./12
Sep./12
Sept./13
EP+
C


CaSy 4
7
6
5
4
3
2
1
0
a a
a b
a a
a ab
a a
a a
13/Jun./11
24/Jun./11
29/Jul./11
Apr./12
Sep./12
Sept./13
EP+
C

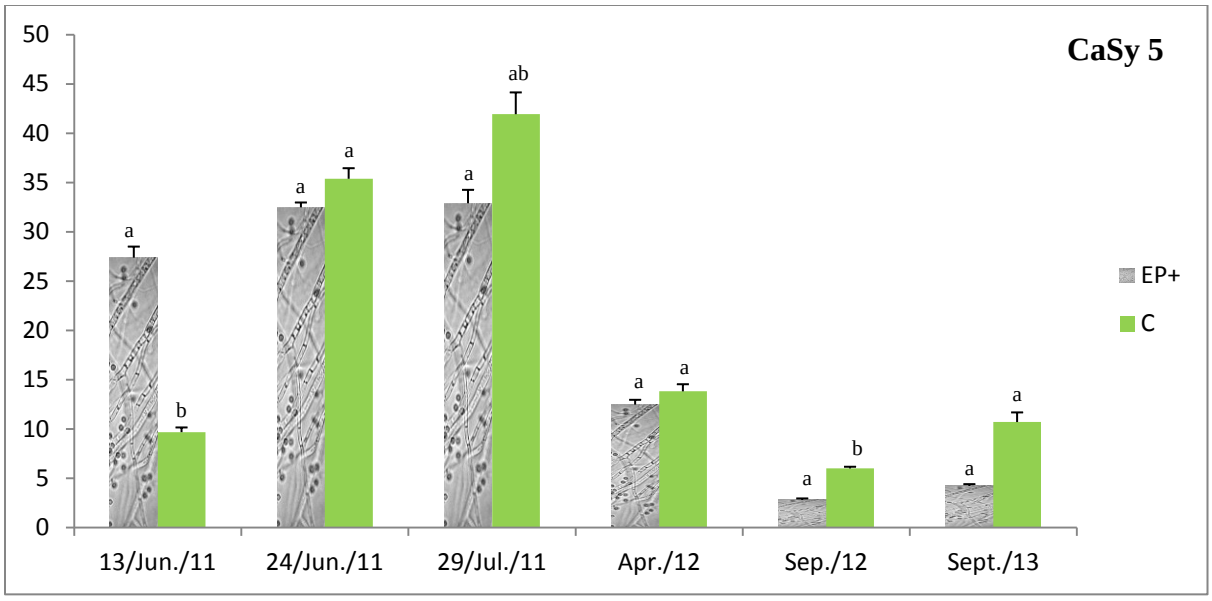


Figure 17. Expression analysis of *Malus domestica* callose synthase genes (CaSy 1, 2, 3, 4 and 5). Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the genes of interest were normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at $P \leq 0.01$

Expression analysis of genes coding for Pathogenesis Related (PR) proteins

Nonexpressor of PR 1 gene (NPR1) is known to have a basic role in systemic acquired resistance (SAR) because it is the key gene for SA-mediated SAR in *Arabidopsis*. Overexpression of this gene in *Arabidopsis* and rice results in increased disease resistance and elevated expression of pathogenesis-related (PR) genes (Chern *et al.*, 2005; Malnoy *et al.*, 2007). The expression patterns of the homolog gene in *Malus domestica* have been studied over the time to examine whether the endophyte *E. nigrum* would have effect on its expression as a trigger for SAR.

Real-time PCR analyses demonstrate that in endophyte-treated plants (EP+) a significant up-regulation of NPR1 gene was induced in leaf tissues three days after endophyte inoculation. Afterwards *NPR1* expression level tended to be balanced in EP+ and C leaf tissues (Figure 18) both ten and fifty days after endophyte inoculation.

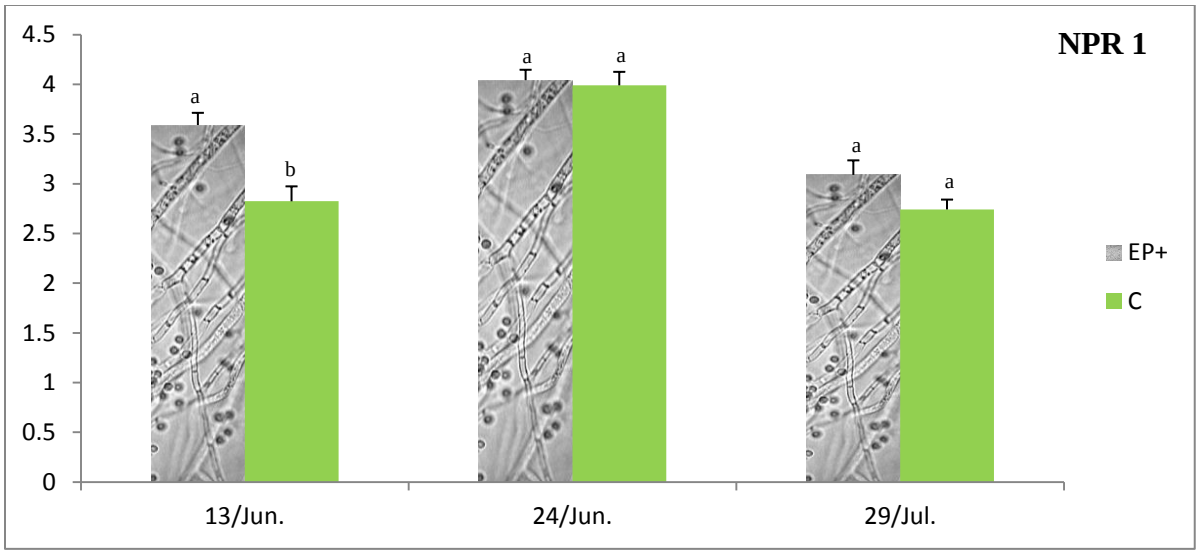
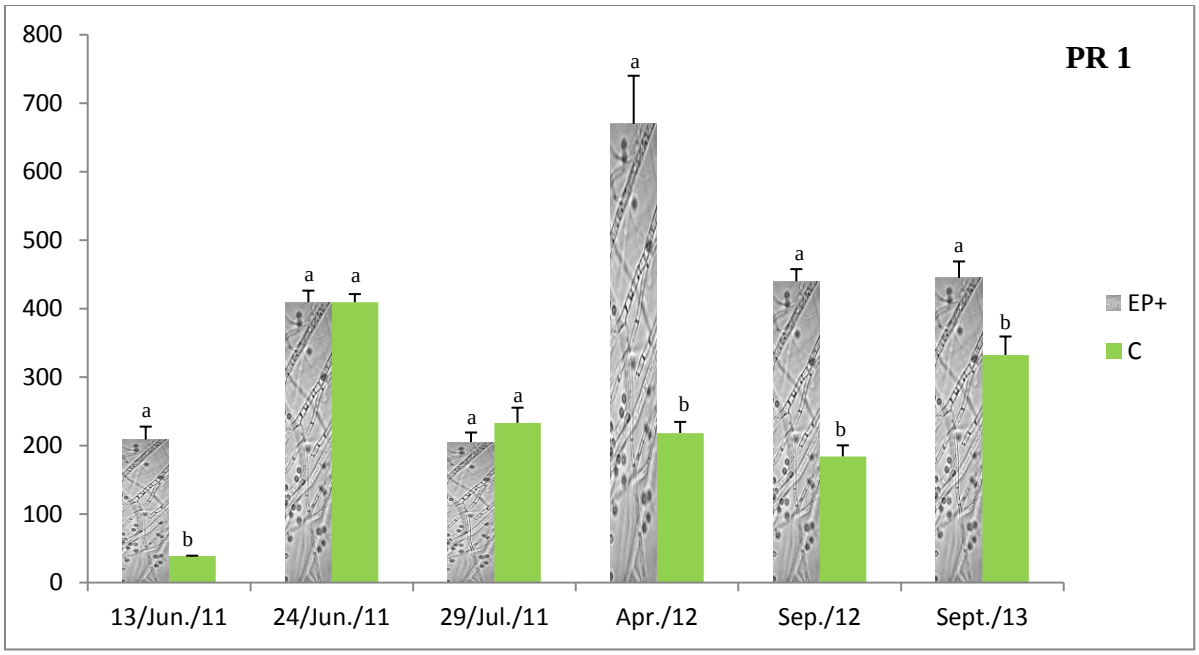


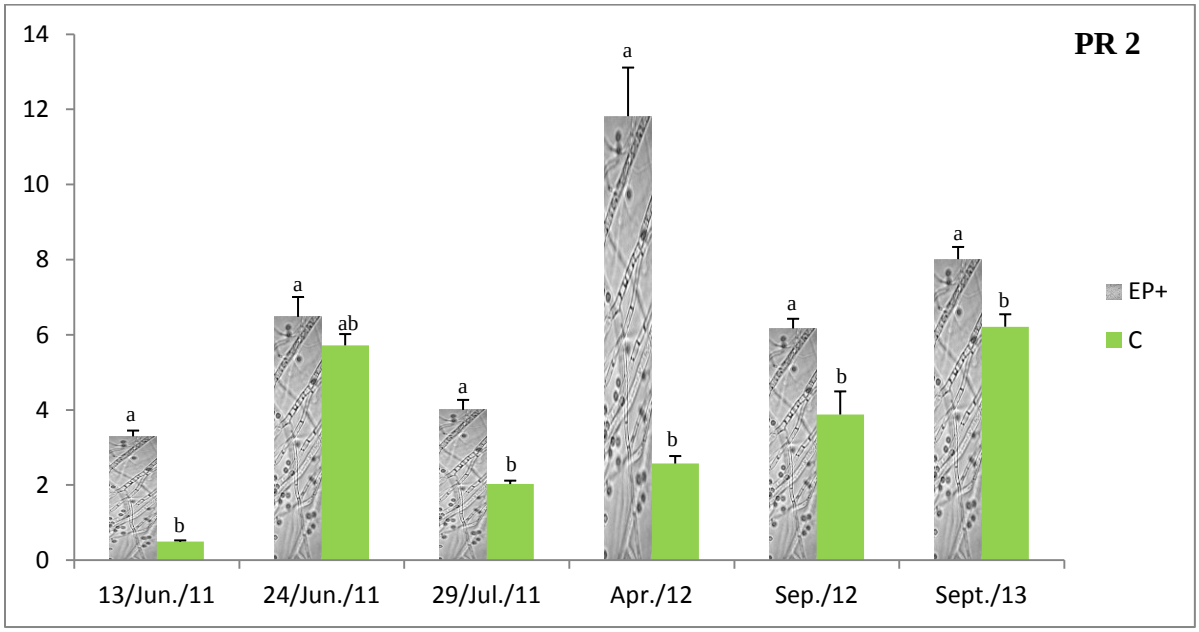
Figure 18. Gene expression analysis of *Malus domestica* Nonexpressor of PR (NPR1) gene. Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the gene of interest was normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at $P \leq 0.01$

Four genes coding for pathogenesis-related (PR) proteins, PR 1, PR 2, PR 5 and PR8 have been studied comparing their relative level of expression in leaf tissues of EP+ and C apple plants (Figure 19). Expression profiles of the selected genes revealed different expression patterns. In general the four PR tested genes resulted in up-regulation over the time in variable extent.

In particular upon endophyte inoculation, PR1 resulted in a significant induction of about 6 folds at 3 days after endophyte inoculation comparing to controls plants. The expression levels of the induced genes tend to return analogous to control plants two weeks and two months after endophyte inoculation. In the following growing seasons the PR1 expression patterns return to be remarkably up-regulated in EP+ leaf tissues.

The expression profile of *PR2* demonstrated a significant induction three days after endophyte inoculation (6.6 folds), and it sustained the trend over the time with variable extent. Similar trend were also recorded in the expression profile of PR5 and PR8, induced three days after endophyte inoculation 3 folds and 2 folds respectively.


PR 1
800
700
600
500
400
300
200
100
0
a
b
a
a
a
a
a
b
a
b
a
b
EP+
C
13/Jun./11
24/Jun./11
29/Jul./11
Apr./12
Sep./12
Sept./13


PR 2
14
12
10
8
6
4
2
0
a
b
a
ab
a
b
a
b
a
b
a
b
EP+
C
13/Jun./11
24/Jun./11
29/Jul./11
Apr./12
Sep./12
Sept./13

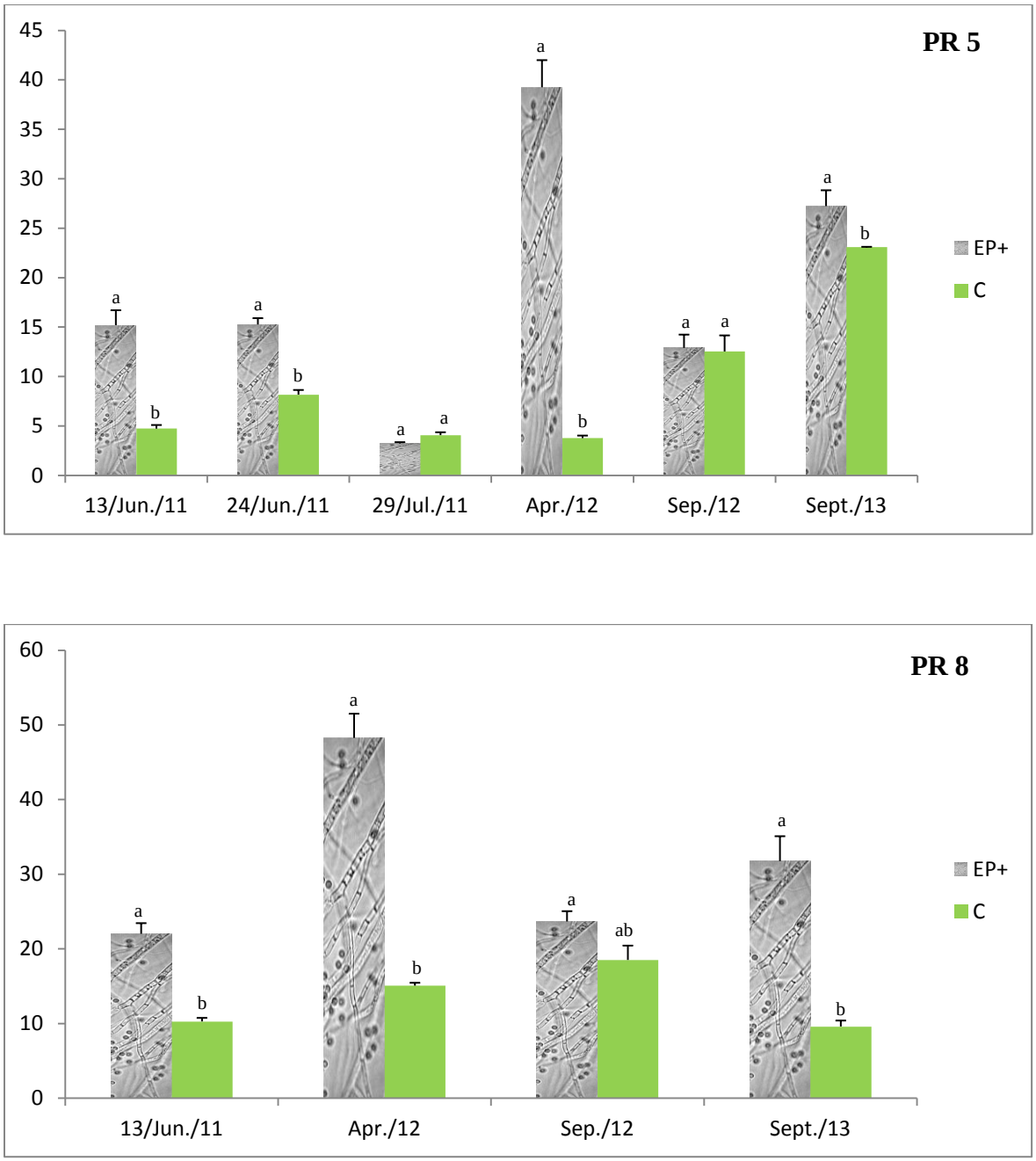
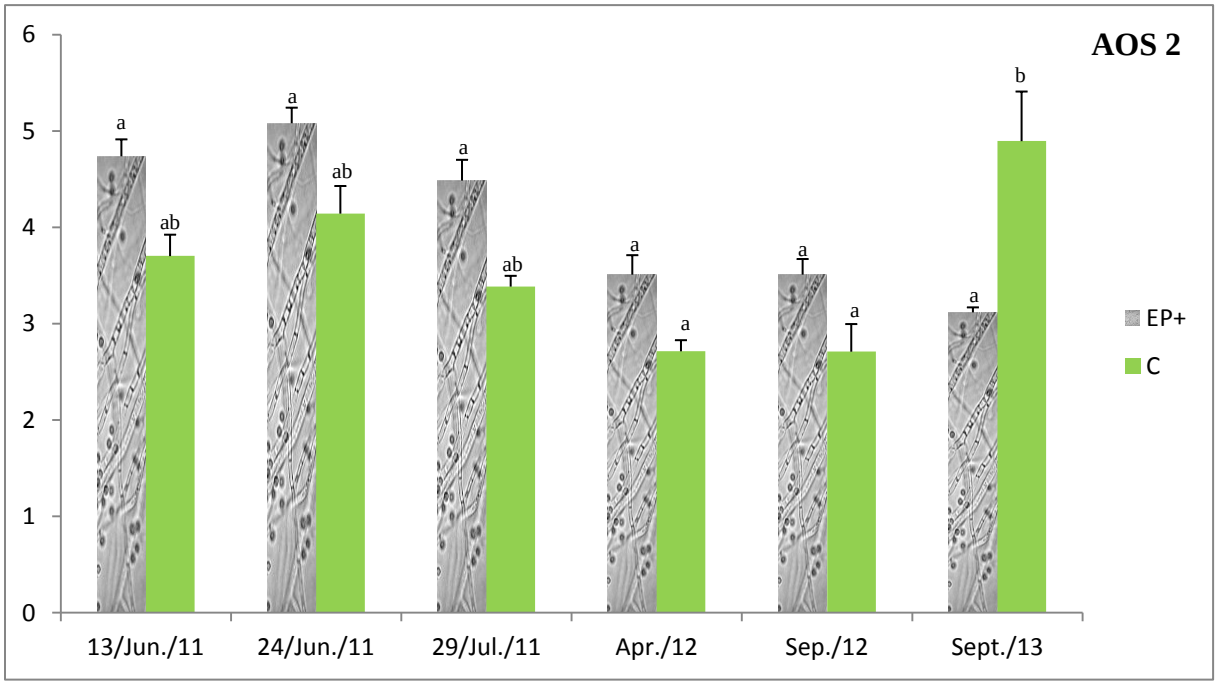


Figure 19. Expression analysis of *Malus domestica* Pathogeneses-Related (PR) protein genes PR1, PR2 PR5 and PR8. Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the genes of interest were normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at P≤0.01

Expression analysis of genes related to Jasmonic Acid (JA) pathway

The expression of apple genes coding for three JA-pathway marker enzymes, Allene Oxide Synthase 2 (AOS 2); 12-Oxyphytodienoate reductase 3 (12-OPR 3); JA-inducible Proteinase Inhibitor II (PI II), were investigated comparing their relative expression levels in apple leaf tissues from both plant groups (EP+ and C). The results are shown in Figure 20.

The expression profile of AOS 2 gene resulted in slight alteration in expression level in the plants from both groups, three days after endophyte inoculation untill late autumn of the season 2011. The expression level trend was generally towards up-regulation in leaf tissues from EP+ plants. However, in September 2013 a significant down-regulation in the expression level of AOS2 was recorded. Regarding the other genes, down-regulation was confirmed in the expression level of 12-OPR3 and PI II genes in leaf tissues from EP+ plants comparing to the control, however, no significant differences were recorded.



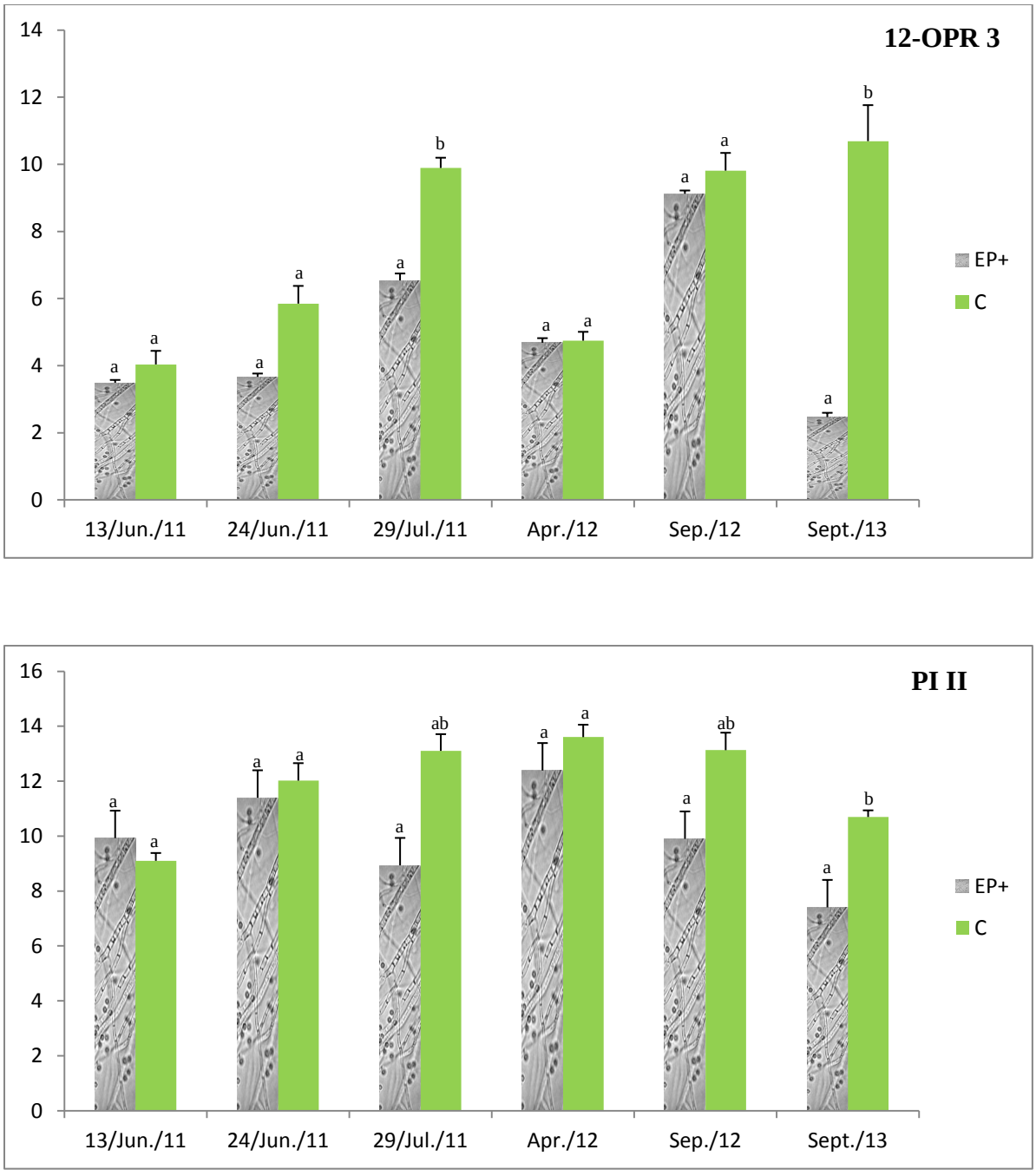


Figure 20. Gene expression analysis of *Malus domestica* Three jasmonate (JA)-pathway marker enzymes, Allene Oxide Synthase 2 (AOS 2); 12-Oxyphytodienoate reductase 3 (12-OPR 3); JA-inducible Proteinase Inhibitor II (PI II). Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the genes of interest were normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at $P \leq 0.01$

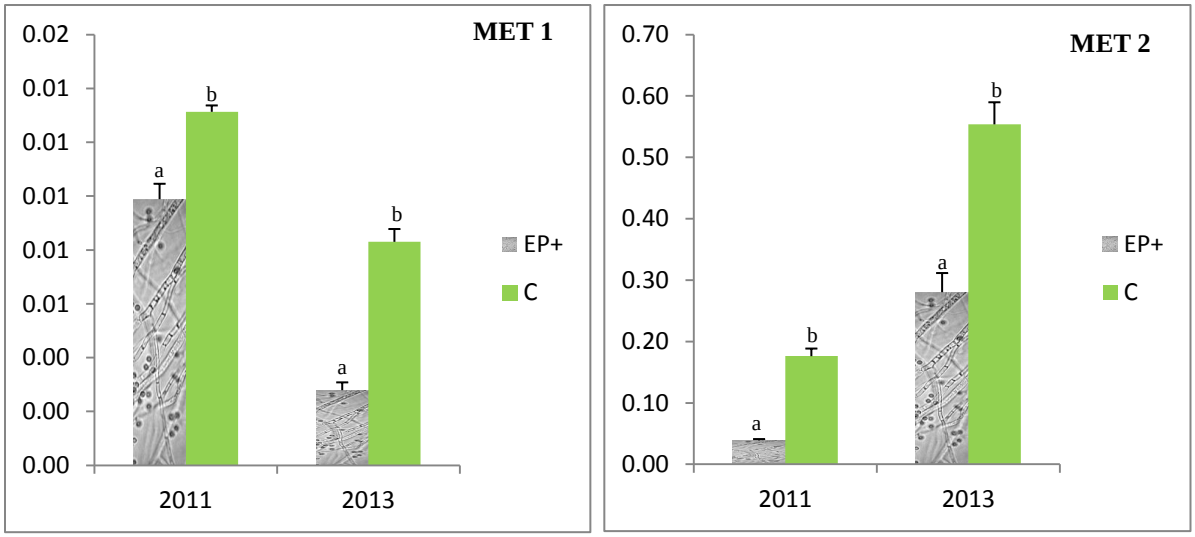
Expression analysis of genes coding for DNA methylation and demethylation enzymes

Upon *E. nigrum* inoculation and on the first growing season, delays in flowing phenomenon were observed in all individuals that have been inoculated with the endophyte but not in control plants (Figure 21). DNA methylation status has a major role in plant development and it has been reported to influence the fruit development stage in tomato plant (Teyssier *et al.*, 2008). In another study preformed on phytoplasma disease in tomato (Ahmad *et al.*, 2013) the DNA methylation enzymes were found to be generally down regulated in tomato plants challenged with phytoplasma, suggesting that DNA methylation might be involved in the regulation of floral gene expression.

To study the methylation status in the both *E. nigrum* inoculated and control plants (EP+ and C) the gene expression profile of five genes coding DNA methylation enzymes were studied, Cytosine-5-methyltransferase 1 and 2 (MET 1 and MET 2), Domain rearranged methyltransferase 2 and 8 (DRM2 and DRM8) and Chromomethylase3 (CMT 3) coding genes was investigated. In addition the Demeter-like (DML) gene involved in DNA demethylation pathway was also studied.

Figure 21. Differences in flowering status between *Epicoccum nigrum* inoculated individuale plants (EP+) and the control group (C). Flowering delay was observed in all EP+ individuals (left), while flowers were present in the control individuals (right).

Expression profile of the genes coding for DNA methylation enzymes showed the same trend for both growing seasons (early summer Jun. of 2011 or late summer Sept. of 2013). The analysis revealed down-regulation in all genes coding for DNA methylation enzymes in apple leaf tissues, except DRM8, which did not show significant alteration. The down-regulation in the expression was varying among the different genes, as for example CMT3 and MET2 genes were remarkably down-regulated in EP+ leaf tissues, 12 folds and 5 folds respectively, comparing to tissue from control plants. In late summer of the 2013, EP+ plants tended to maintain the same trend previously observed, except DRM8 gene in which down-regulation was recorded aftere there were no significant alteration previously (figure 22). On the other hand, and regarding demethylation gene, up-regulation in DML DML expression was recorded in EP+ plants compared to control in early summer 2011. However, a contrary trend was recorded in late summer 2013.



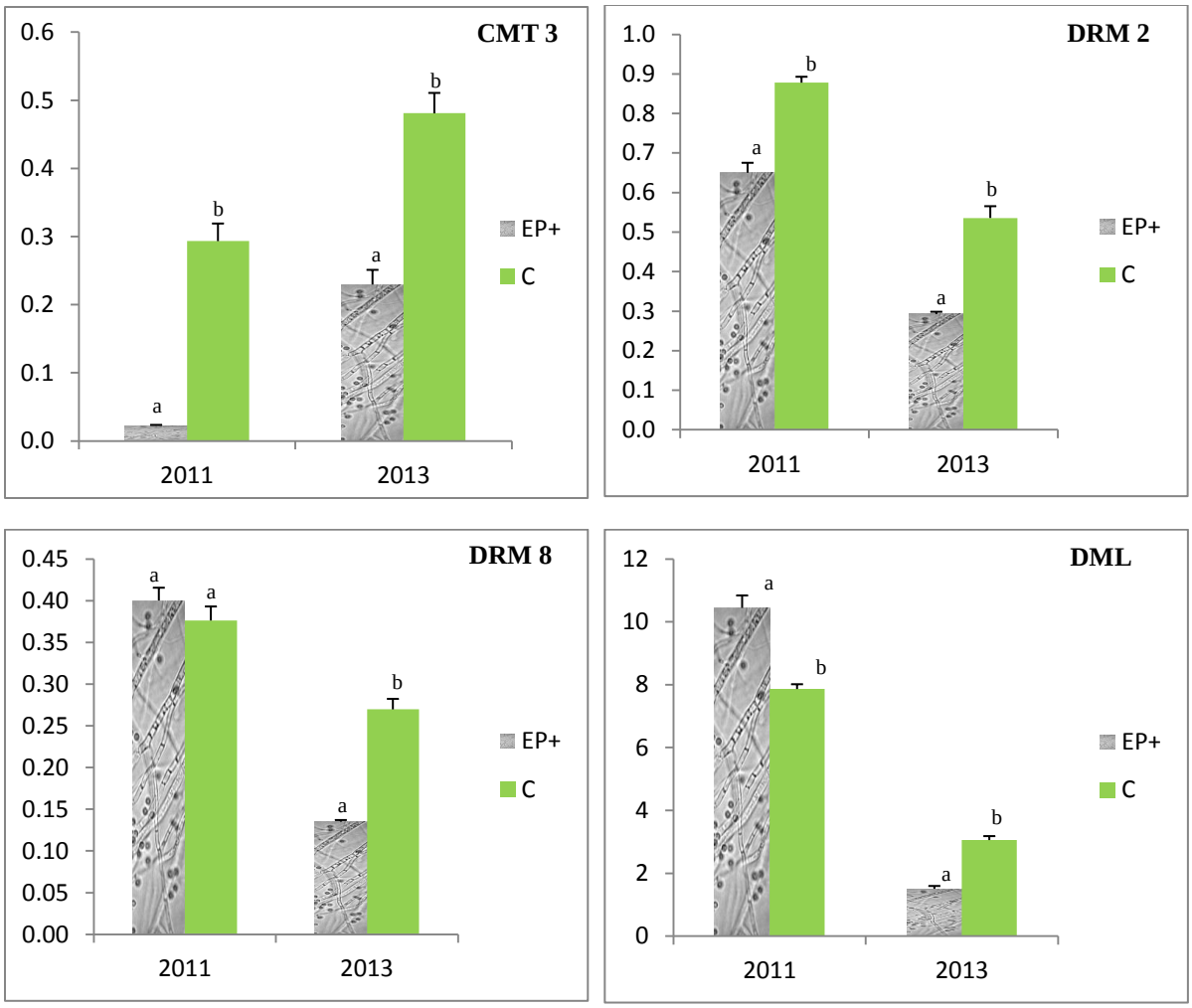


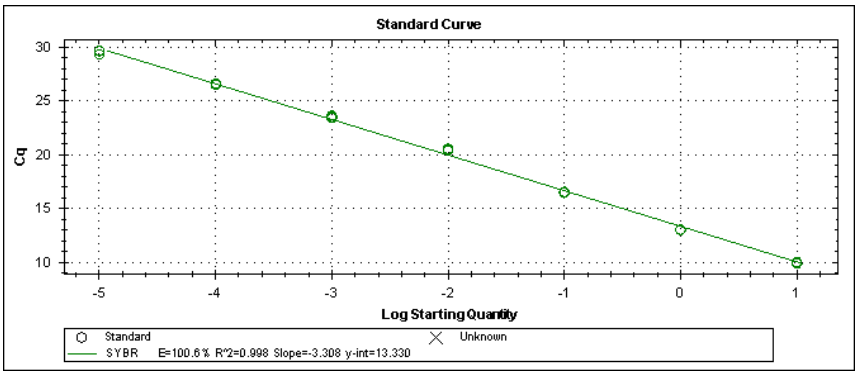
Figure 22. Expression analysis of *Malus domestica* genes coding for DNA methylation enzymes, Cytosine-5-methyltransferase 1 and 2 (MET 1 and MET 2), Domain rearranged methyltransferase 2 and 8 (DRM2 and DRM8) and Chromomethylase3 (CMT 3), and for Demeter-like (DML) gene, involved in DNA demethylation pathway. Mean expression values from five individuals for each plant group (EP+ and C) plus standard errors are shown. Expression levels of the genes of interest were normalized to GDPH. Statistical comparisons were made using an ANOVA Tukey-Kramer Multiple Comparisons Test to evaluate significant differences. Different letters denote significant differences at $P \leq 0.01$.

'*Candidatus* Phytoplasma mali' diagnosis and quantification in *Epicoccum nigrum*-treated and untreated plants

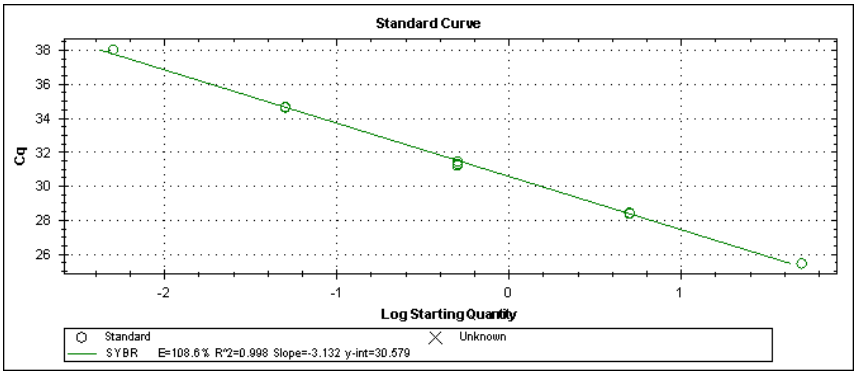
'*Candidatus* P. mali' presence was assessed by real-time PCR technique performed on the total DNA extracted from leaf phloem tissue. Analyses were performed using leaf tissues from plants showing typical AP symptoms (enlarged stipules and witches'brooms). Three plants showed symptoms in summer 2012, one year after the phytoplasma inoculation by grafting, two belonging to AP+ group and one belonging to EP+AP+ group. Symptoms were persisting also in summer 2013. Despite the low number of symptomatic plants, which is normal situation when young individuals are grafted (Osler *et al.*, 1993), a comparison between the phytoplasma titre in the two plant groups were made, to have a preliminary idea about *E. nigrum* treatment efficiency against '*Ca*. P. mali'.

The quantification of *'Ca.* P. mali' in all samples was made by the standard curve quantification method. Standard curve of *'Ca.* P. mali' DNA based on serial dilutions of cloned rpl22-gene diluted in 20 ng/µl) of total DNA from healthy apple plant was obtained by plotting cycle threshold (Ct) vs log of starting quantity (copy number of rp-gene).

Quantification real-time PCR slope of *'Ca*. P. mali' in healthy plant DNA indicated PCR efficiency was close to 100%, irrespective of the host DNA background. For the quantification of infection level in plant, ACO gene served to normalize the data. Standard curve of healthy apple DNA was obtained by plotting Ct vs. log of starting quantity (ng). Slope of real-time PCR for the quantification of plant DNA indicated PCR efficiency close to 100% (108%) (Figure 23).



A



B

Figure 23. Standard curves of '*Candidatus* Phytoplasma mali' DNA (A) and plant DNA (B) obtained by plotting Ct vs log of starting quantity.

Both real-time PCRs showed a high linear dependence between the two variables with coefficient of correlation equal to 1.00 and 1.08, respectively (Figure 23). Moreover, the two real-time PCRs yielded a single PCR product (no artifact bands or primer–dimers) as demonstrated by melting curve analysis.

Infection level of *'Ca*. P. mali' (expressed as *'Ca.* P. mali' GU per ng apple DNA) in AP+ and EP+AP+ apple plants ranged from $3.1*10^3$ to $1.5*10^3$ respectively (Figure 24). These preliminar results demonstrated that *'Ca.* P. mali' concentration in EP+AP+ sample was lower about twice as much as it was for AP+ samples.

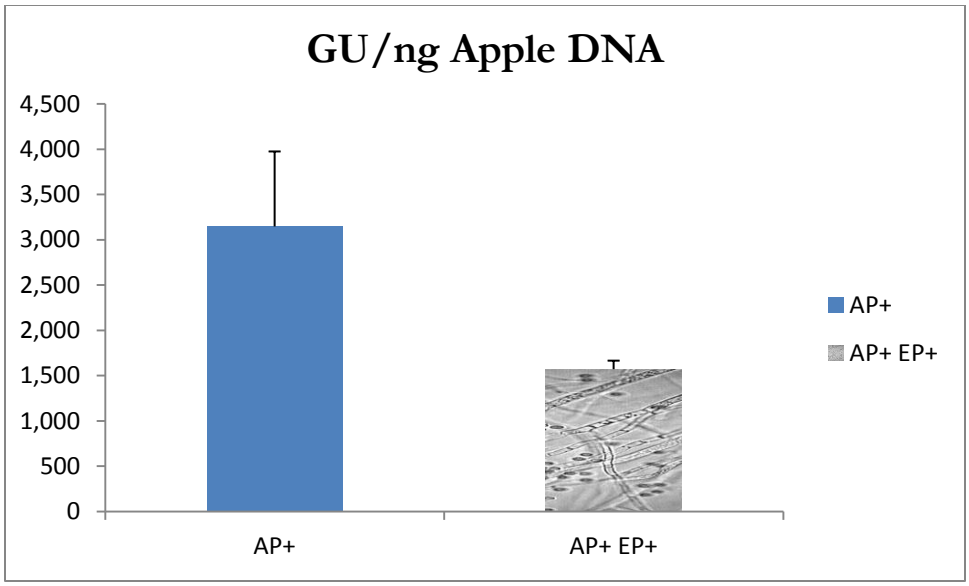


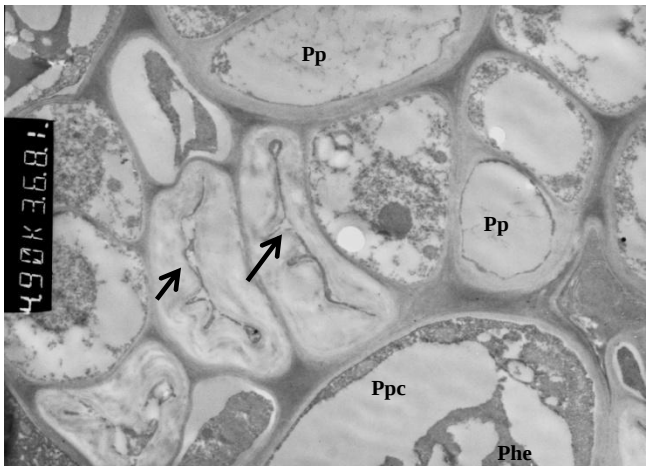
Figure 24. Quantity of *'Candidatus* Phytoplasma mali', expressed in GU per ng of apple DNA, in AP+ and EP+AP+ *Malus domestica.* Error bars represented calculated experimental error.

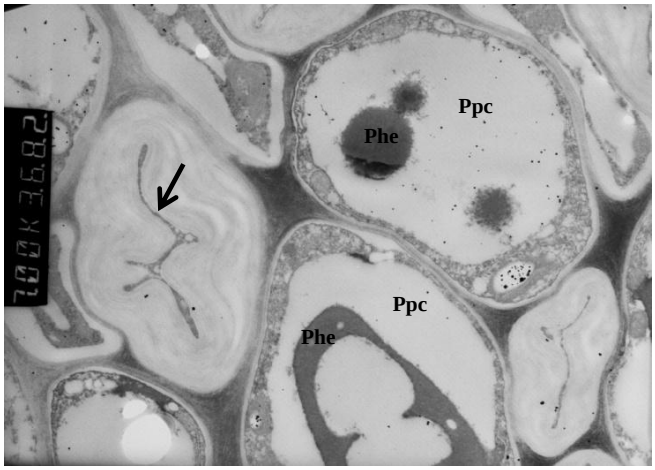
Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) observations were performed in leaf tissues from the four plant groups (EP+, AP+, EP+AP+ and C) to verify if the preventive endophyte inoculation on apple plants would affect host and/or phytoplasma ultrastructure. Leaf samples were collected in September 2012, when AP symptoms appeared on some plants belonging to AP+ and EP+AP+ groups. Also samples from asymptomatic plants, belong to the same group were analyzed as a control. TEM observations performed in C and EP+ apple leaf material showed that tissues were well preserved and phytoplasmas were not observed.

Ultrastructural characteristics of leaf tissues from control apple plants

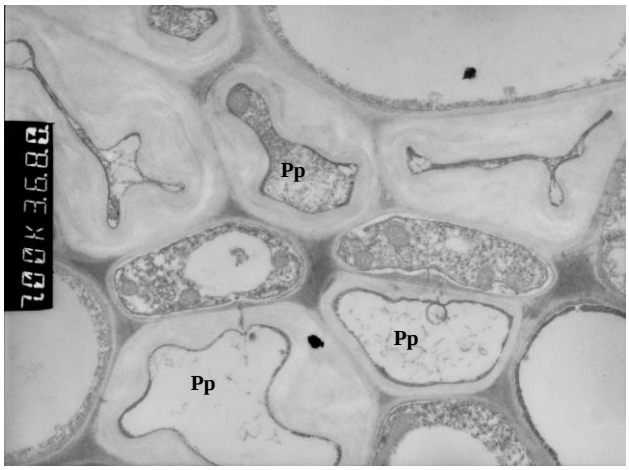
In the phloem of C plants (Figs. 3681 and 3682), narrow sieve elements, typical of apple phloem, were visible (3681, 3682, arrows). Dispersed fibrils of phloem protein (Pp) were visualized in the sieve tube lumen (Fig. 3681, Pp) and in the phloem parenchymal cells (Fig. 3682, ppc) vacular phenolic inclusions were present (Fig. 3682, Phe).

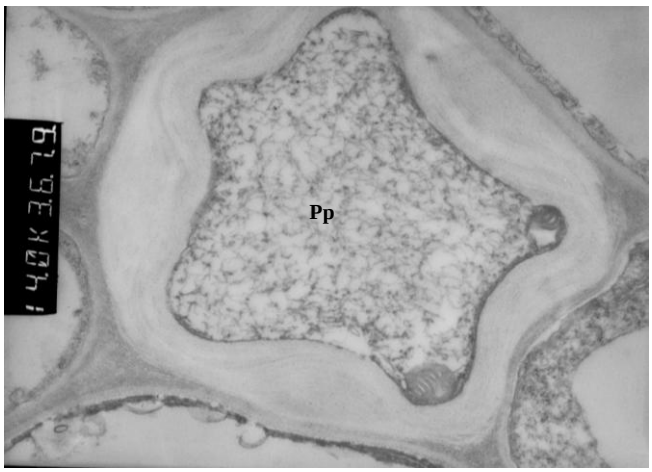


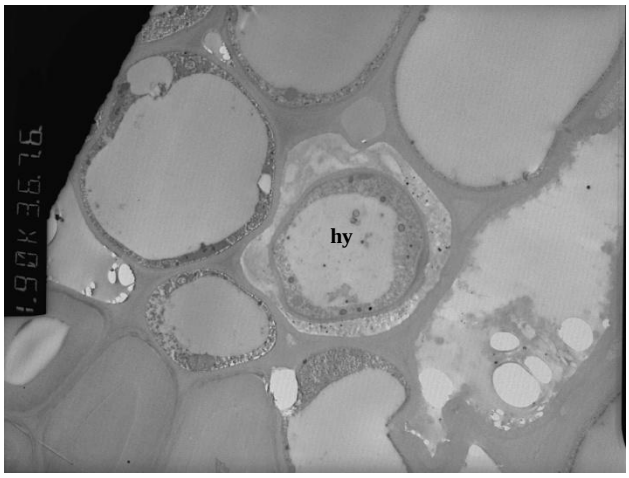


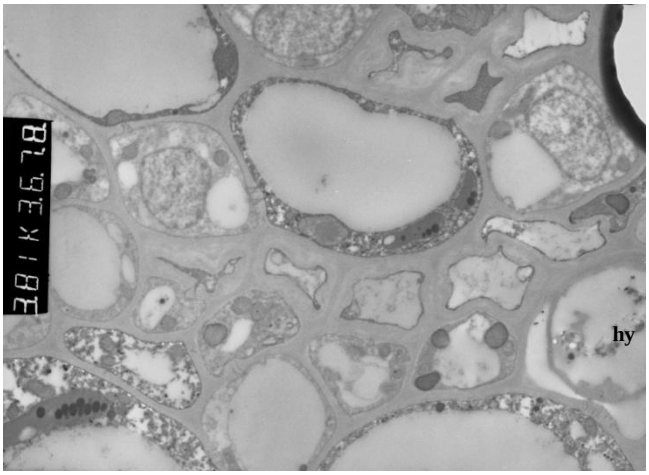
Ultrastructural characteristics of leaf tissues from EP+ apple plants

In leaf tissues from EP+ trees, numerous sieve elements presented accumulations of P-protein in the lumen (Figs. 3680, 3679, Pp), sometimes in the proximity of the sieve plates. Abundant callose deposits at sieve plates were not observed (not shown). In the phloem parenchymal cells, hyphae were found (Figs. 3676 and 3678, hy), whose presence is not associated to ultrastructural modifications of the host cells.



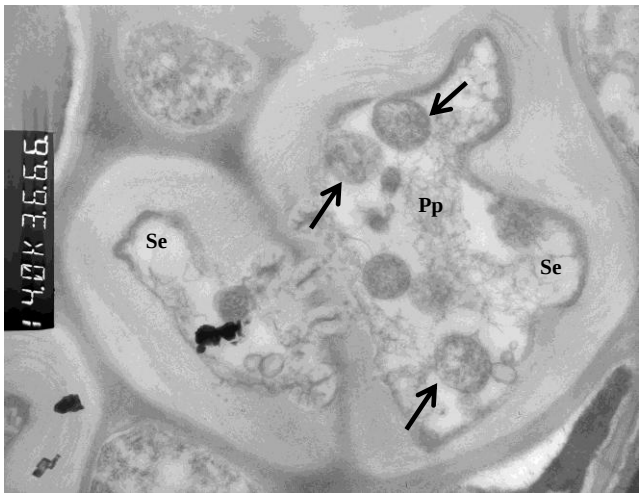


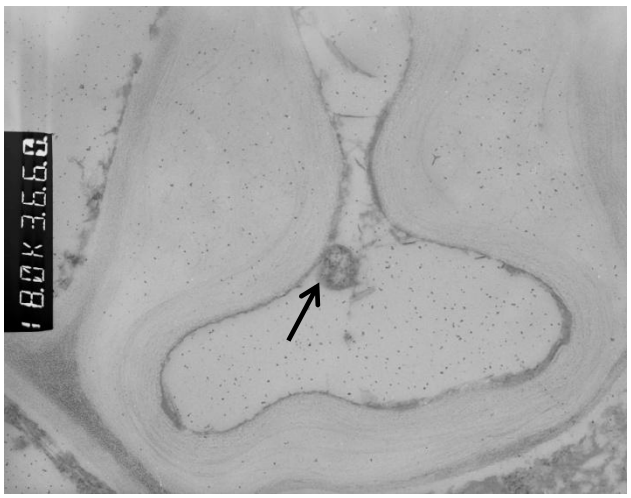


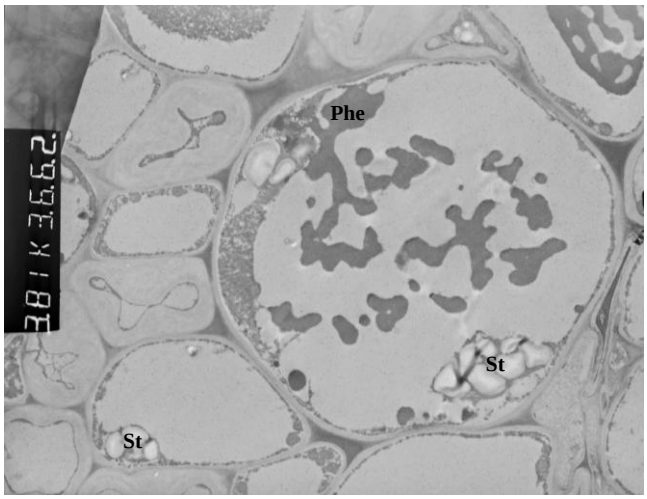


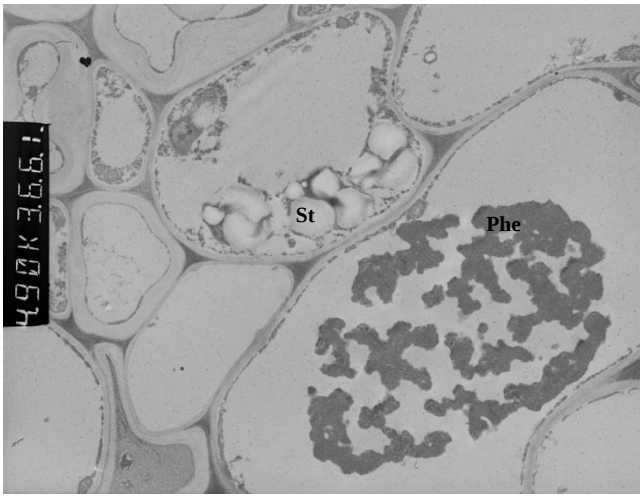
Ultrastructural characteristics of leaf tissues from AP+ apple plants

In both AP+ and EP+ AP+ leaf tissues, TEM observations revealed the presence of phytoplasmas (Figs 3666, 3660 and 3664). In leaf tissues from AP+ plants showing disease symptoms, phytoplasmas appeared typically pleomorphic in shape (Fig. 3666 and 3660, arrows). They were localized in the sieve elements (Fig. 3666, se) associated to phloem proteins (Fig. 3666, Pp). Big vacuolar phenolic inclusions and starch accumulation were observed in phloem parenchymal cells (Figs. 3361, 3362, Phe and St).

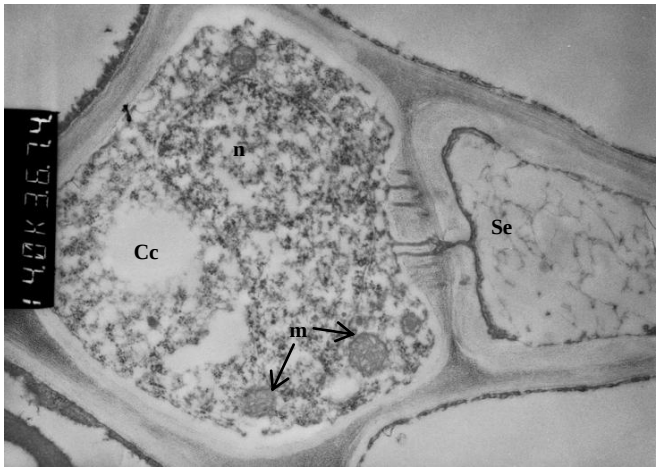


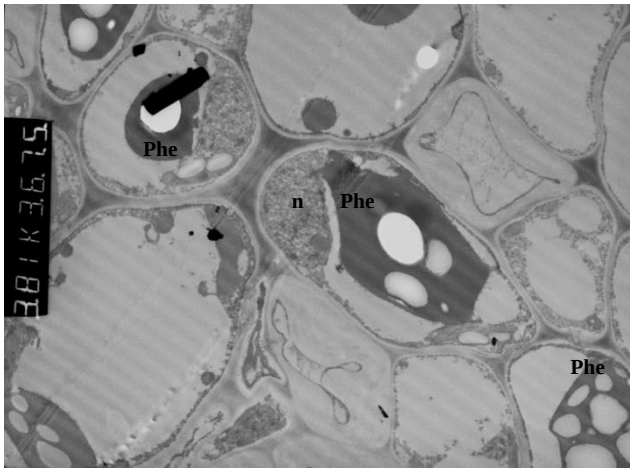






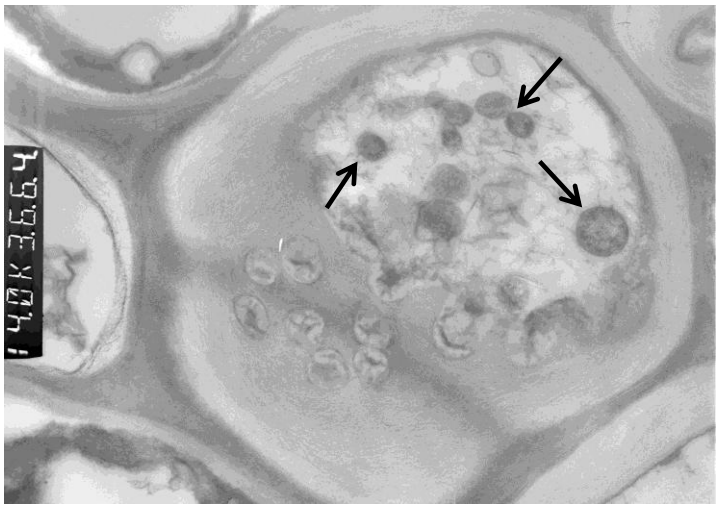
In plants belonging to the same group but not exhibiting symptoms, phytoplasmas were not visualized inside the sieve elements (Fig. 3674, se; CC: companion cell, n: nucleus, m: mitochondrion), however big phenolic vacuolar inclusions were found in the phloem parenchyml cells (Fig. 3675, Phe).





Ultrastructural characteristics of leaf tissues from EP+AP+ apple plants

In EP+AP+ leaf tissues phytoplasmas were visualized only in the phloem of the unique plant showing symptoms in September 2012. Phytoplasmas did not show modified ultrastructure compared to those found in AP+ leaf tissues (Fig. 3664, arrows).

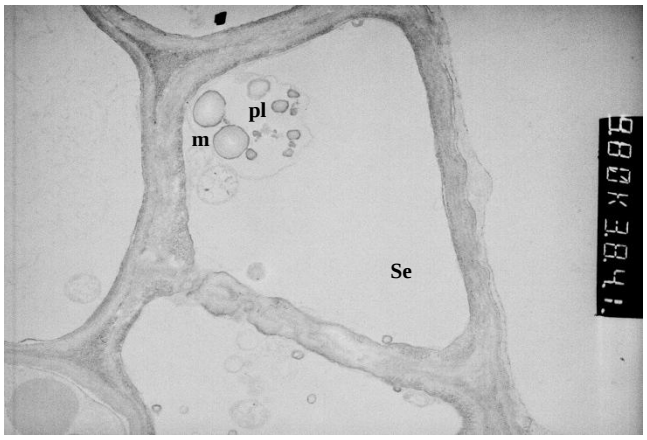


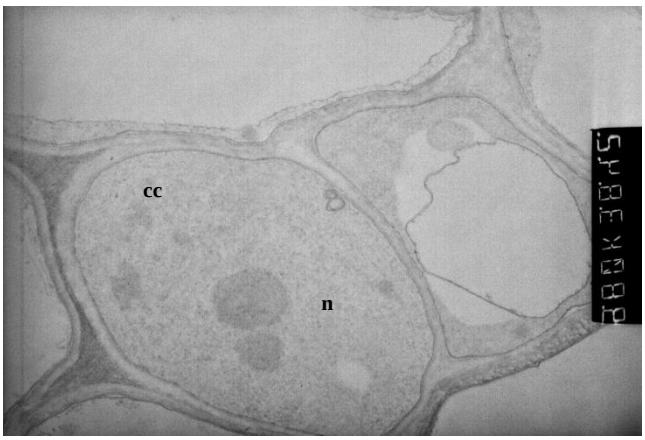
Asymptomatic plants belonging to the same group did not show phytoplasmas inside sieve elements. Sieve elements did not show significant ultrastructural modification with exception of P-protein, present in agglutinated forms, as described above for tissues from EP+ plants.

Effects induced by active secondary metabolites extracted from *Epicoccum nigrum* on phytoplasmas on *Catharanthus roseus* cuttings

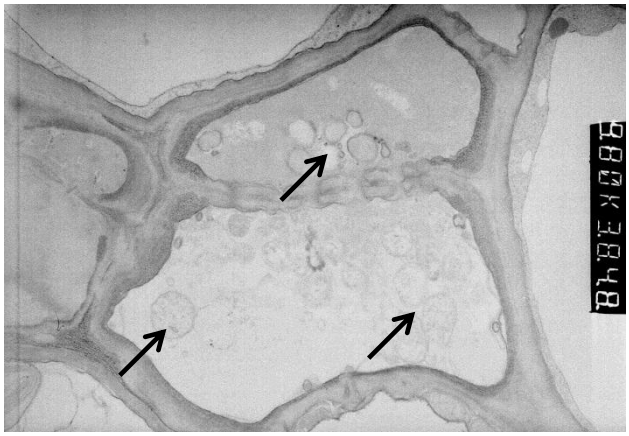
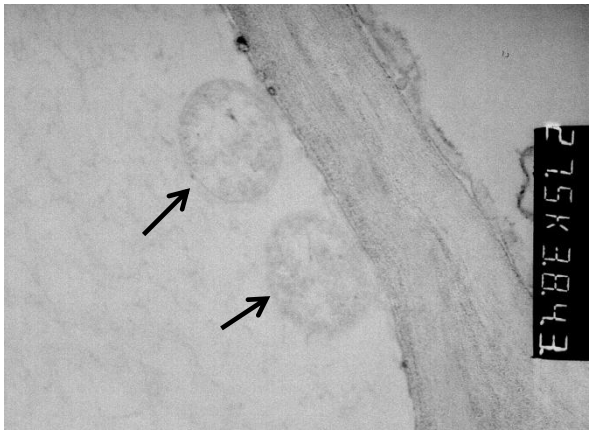
Since phytoplasmas have been always resisting growing in axenic culture, to evaluate the activity and the antagonistic effect of metabolites produced by the endophyte *E. nigrum*, a preliminary assay was set up and performed using cuttings from infected-symptomatic *Catharanthus roseus* plants, as previously described by Musetti *et al.,* (2007). Two secondary metabolites with bioactive properties extracted from the *E. nigrum* were sellected to be evaluated against phytoplasmas in *C. roseus* leaf tissue.

Four days after incubation in *E. nigrum* metabolite suspension, *C. roseus* cuttings did not show visible symptoms or lesions in both treatments. TEM observations revealed that metabolites treatment did not affect *C. roseus* leaf ultrastructure, as cytological modifications were not visible in sieve elements (Fig. 3841, se) and in companion cells (Fig. 3842, cc. n: nucleus; m: mitochondrion, pl: plastids).

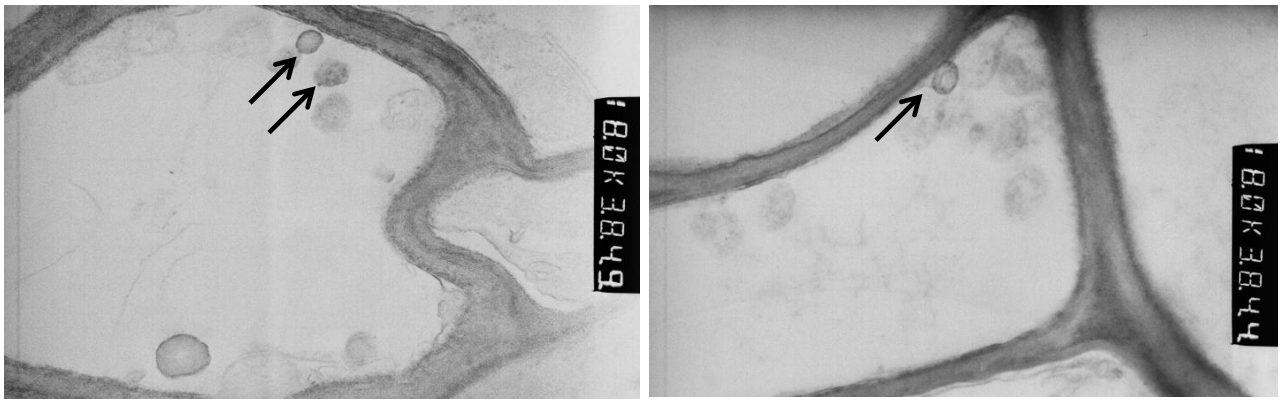




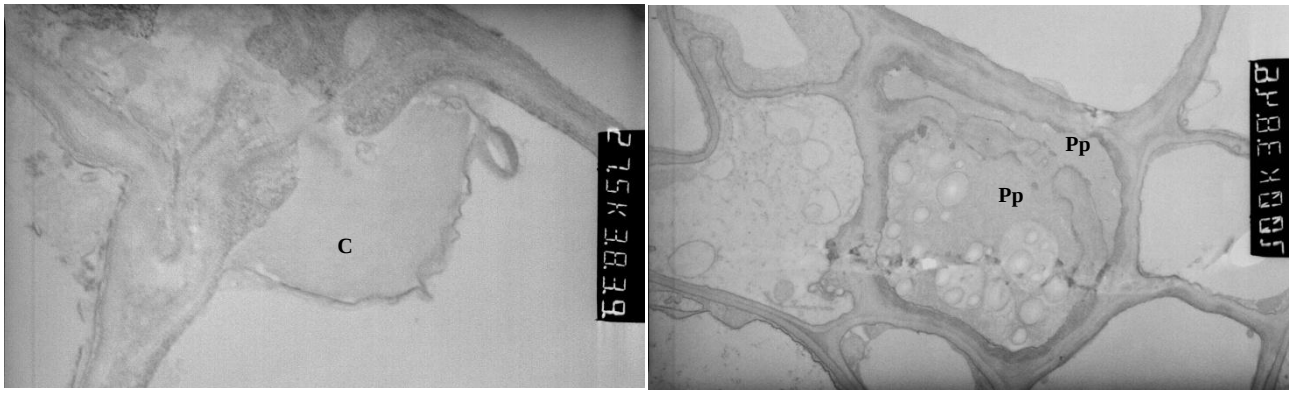
In AP-infected phloem tissues, untreated and treated with *E. nigrum* metabolites, TEM observations revealed the presence of phytoplasmas (Fig. 2848, 2843, arrows).

However, in the treated cuttings (form both treatments), several phytoplasmas showed modified ultrastructure, such as irregular and distorted shape, presence of cytoplasm confined at the periphery of the cell, not well distinguishable cell membrane (Figs. 3849 and 3844, arrows).



Moreover, plant cytological changes, suggesting the activation of defence response, were observed. Abundant callose depositions (Figs. 3839, C) were found in sieve elements, as well as P-protein plugs (Fig. 3846, Pp) particularly occluding sieve plates.



DISCUSSION

Epicoccum nigrum induces the expression of defense-related gene in *Malus domestica*

Endophytes are micro-organisms living inside host plants without causing disease symptoms or visible injury. Most of endophytes exhibit positive effect for the host plant by promoting growth, improving resistance to multiple stresses and protecting them against pathogens and insects. The possibility to use endophytes as biocontrol agents or resistance-inducers against phytoplasmas was recently reported. Musetti *et al.,* (2011b) reported that an endophytic strain of *E. nigrum,* inoculation in *Catharanthus roseus,* experimentally infected with '*Ca*. P. mali', reduced symptom severity and phytoplasma titre inside the plant, inducing ultrastructural modifications both to the phytoplasma and to the host. In this work the endophyte *E. nigrum* was inoculated in *M. domestica*, the natural host plant of '*Ca*. P. mali', as a possible strategy for AP control.

Inoculation of *E. nigrum*, in apple trees, did not induce any disease symptoms (i.e. necrosis, rot) or other visible stress-related responses, confirming the typical endophytic life-style in the host.

The interaction between *E. nigrum* and the apple plant were studied by evaluating the expression levels of several selected genes, already tested by studying 'recovery' in apple trees (Musetti *et al.,* 2010 and the present study). Among the genes coding for different phloem proteins (PP2-1, PP2-2, PP2-3, ERG 1, SEOa and SEOb) only PP2-2 and ERG 1 expression levels were found significantly up-regulated in EP+ plants three day after endophyte inoculation. Interestingly PP2-2 and ERG 1 were reported to be up-regulated in apple trees exhibiting 'recovery' status (Musetti *et al.,* 2010). Molecular data regarding phloem proteins were found to correspond with TEM observations.

Phloem protein aggregation was reported in phytoplasma-infected tobacco plants treated with elicitors (Lherminier *et al.,* 2003), as well as in other pathosystems (Elad and Evensen 1995; Goleki *et al.,* 1999). The aggregation of P-protein, which shifts from the unpolymerized to the polymerized form in sieve tubes, is one of the first responses of phloem cells to pH modification (Goleki *et al.,* 1999), which can be correlated with different causes, among which is pathogen attack (Knoblauch *et al.,* 2001).

Callose synthase genes 2 and 5 were demostrated to be up-regulated upon endophyte inoculation and, interestingly, the latter resulted over-induced in apple trees exhibiting 'recovery' (Musetti *et al.,* 2010). Callose synthesis is probably the first key event among defence plant cell modifications (Skou *et al.,* 1984), which may result in reinforcments of the cell wall at the attempted site of pathogen penetration, in providing a medium for the deposition of toxic compounds, and in impeding nutrient transfer from host to pathogen. Particularly, callose synthase 5 shows high homology with the *Arabidopsis CalS7*, which is responsible for callose deposition in the phloem (Xie *et al.,* 2011), the site of phytoplasma infection.

The achievement of induced resistance against not curable diseases (such as phytoplasma) is considere to be an important issue. SAR is a long-lasting phenomenon and appears to be effective against a broad spectrum of pathogens (Durrant and Dong 2004). It can be correlated to the expression of PR genes in plants. We demonstrated that four PR genes (PR1, PR2, PR5 and PR8) were over expressed in EP+ plants, supporting the hypothesis of resistance mechanism activation in EP+ plants.

As regards JA-pathway related enzymes, the antagonistic effect of PR gene expression on JA signaling was reported (Spoel *et al.,* 2003), which explained the suppression of JA markers in EP+ plants.

In conclusion, endophyte fungal inoculation could result in the priming expression of a set of stress-related genes or eliciting stress hormone production compared with uncolonized plants (Sherameti *et al.,* 2008). Growth-promoting phytohormones have been isolated from the culture medium of an endophytic strain of *E. nigrum* (Rowan and Latch 1994). In addition, the capability of the fungus of producing numerous secondary metabolites, with antibiotic properties, is throughly documented (Hill *et al.,* 1999; Elmer *et al.,* 2001).

DNA methylation was reported to play critical roles in regulating gene expression in response to biotic stress in *Arabidopsis*, tobacco and tomato (Wada *et al.,* 2004; Pavet *et al.,* 2006; Ahmad *et al.,* 2012). Methylation has also an important role in plant development: in fact, loss of DNA methylation function provokes a range of abnormalities in *Arabidopsis* (Finnegan *et al.,* 1996).

Moreover, it was reported that reduction in DNA methylation in *Arabidopsis* alteres leaf shape, increases cauline leaf number, and causes delays in the onset of flowering (Kakutani *et al.,* 1995; Kakutani *et al.,* 1998).

E. nigrum also demonstrated influence on the DNA methylation in apple leaf tissues, as genes involved in DNA methylation resulted in down-regulation in EP+ plants. The suppression of the DNA methylation genes could explain the delaying in flowering in all EP+ individual plants. However, these results are considered to be preliminary, since further analyses are needed to clearfy more the influence of the endophyte on changing the DNA methylation *in planta*.

Effect of *Epicoccum nigrum* inoculation on '*Candiatus* Phytoplasma mali' titres in AP-infected apple trees

Previous study performed on the periwinkle plants demonstrated that *'Ca.* P. mali' concentration in endophyte treated plants was reduced to about 2.8 times comparing to the untreated ones (Musetti *et al.,* 2011b). We performed phytoplasma quantification analyses in EP+AP+ and AP+ plants, by quantitative real-time PCR. Even though the low number of symptomatic plants which is not valuable to obtain outcome with statistic significance, the quantification results gave us a preliminary but encouraging indication about the possibility that *E. nigrum* treatment could affect '*Ca*. P. mali' in apple plants.

Effects of *Epicoccum nigrum* secondary metabolites on '*Candidatus* Phytoplasma mali' in *Catharanthus roseus* cuttings

Two of purified secondary metabolites of bioactive properties were chosen to be tested against phytoplasmas as they resulted particularly active as described in serial dilution assay (next chapter).

Given the difficulties to obtain phytoplasmas in axenic culture, a preliminary assay was set up using cuttings from infected-symptomatic *Catharanthus roseus* plants.

The obtained results, even if still preliminary, demonstrated that *E. nigrum* secondary metabolites induced ultrastructural changes both in phytoplasma-infected *C. roseus* tissues and in the pathogen. The described ultrastructural modifications indicated an enhancing of defence response in the host. The presence of P-protein in its aggregate state could prevent the phytoplasma spread inside the host plant, as already discussed previously. In endophyte-treated cuttings, the described modifications appeared more evident than those observed in untreated ones.

Phytopasmas also showed modified morphology under endophyte treatment. Agglutinations and degeneration of phytopasma cells also observed in tomato plants treated with arbuscular-mycorrhizal fungi, and correlated with mycorrhizal hormone activity (Lingua *et al.*, 2002).

Eventhough further investigations are necessary to give insight on the relationship among phytoplasma and endophytes, the preliminary results reported in this study could offer new perspectives phytoplasma disease control and management.

Chapter 4

STUDIES ON THE SECONDARY METABOLITES OF *EPICCOCUM NIGRUM* AND ACTIVITY AS BIOCONTROL AGENT *IN VITRO*

MATERIALS AND METHODS

Culture media used for cultivation

Yeast Malt Glucose (YM)-Medium

Malt extract (Carl Roth GmbH & Co.KG).....10.0 g

Ohly® Kat Yeast extract (Ohly GmbH).....4.0 g

Glucose (Cerestar).....4.0 g

Distilled water up to.....1.0 L

pH.....6.3

Bacto™ Agar (BD Diagnostic Systems) .....20.0 g

Zucker-Malz-Medium (ZM½):

Molasses (Nordzucker AG).....5.0 g

Oatmeal (Herrnmühle Reichelsheim) .....5.0 g

Sucrose (Carl Roth)..... .....4.0 g

Mannitol (AppliChem).....4.0 g

D-Glucose (Cerestar).....1.5 g

Calciumcarbonate ($CaCO_3$) (AppliChem).....1.5 g

Lactalbumin-Hydrolase (Edamin) (Oxoid Limited)..... 0.5 g

Ammonium sulfate, $(NH_4)2SO_4$ (Carl Roth).....0.5 g

pH..... 7,2

Distilled water up to .....1.0 L

EBS-Medium

Casein Peptone (Marcor) ..... 5.0 g

D-Glucose (Cerestar).....5.0 g

Meat extract (Carl Roth) .....1.0 g

Ohly® Kat Yeast extract (Ohly GmbH).. .....1.0 g

50 mM HEPES BUFFERAN® buffer .....11.9 g

Distilled water up to .....1.0 L

pH..... 7,0

Epicoccum nigrum culture plate

Twenty one *Epicoccum nigrum* isolates obtained from grapevine were cultivated on solid YM Petri agar plates. The plates were incubated in an incubation room at 22 ^{o}C in dark conditions until the mycelium was covering at least two-thirds of the plate (10-14 days). The mycelium was later used for DNA extraction and morphological/phylogenetic studies, and preserved in the strain collection. From the twenty-one isolates thirteen isolates were selected and sub-cultured on YM agar plates for further analyses.

DNA isolation

DNA isolation was done by scraping about 0.1 g of mycelium with a fine needle. The mycelium was placed in a 1.5 ml tube (with screw tap). The mycelium was homogenized with 6-8 ceramic beads (Precellys ® Ceramic Beads, Ø 1.4 mm, Rohmand Haas Deutschland GmbH) and 400 µl of lysis buffer. DNA extraction was performed using the Invisorb Spin ® Plant mini kit (STRATEC Molecular GmbH). Mycelium homogenization was performed in a homogenizer at 6000 rpm for 40 s; tubes were then incubated for 30 min on heating block at 65 ° C. Further extraction steps were performed according to the manufacturer's instructions. Nucleic acid concentration and purity were checked by ImplenNanoPhotometer®.

DNA extraction was preceded by polymerase chain reaction (PCR) amplification. ITS region and β-tubulin gene were amplified to verify isolate identity and to compare the sequences. Primers used for ITS region amplification were ITS1F (5`-CTTGGTCATTTAGAGGAAGT AA-3`) according to (Gardes and Bruns, 1993) and ITS4 (5`-TCCTCCGCTTATTGATAT GC-3`), according to (White *et al.,* 1990). T1 (5`-AACATGCGTGAGATTGTAAGT-3`) and T22 (5`-TCTGGATGTTGTTGGGAATCC-3`) primers pair were used for β-tubulin gene amplification (O`Donnell & Cigelnik 1997). The figure 25 shows the location of the primers.

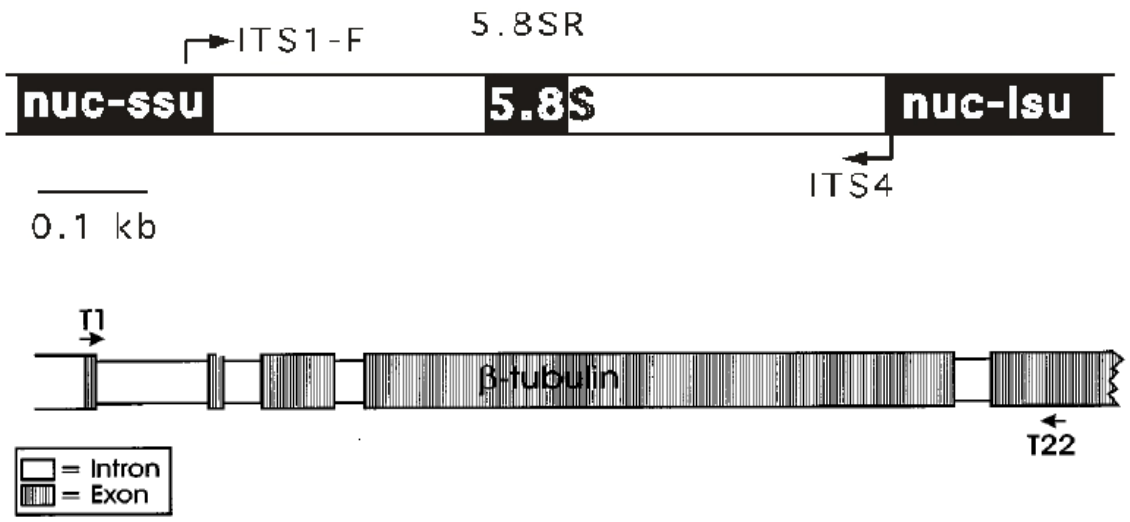


Figure 25. Maps of the nuclear ITS (top) and β-tubulin genes (bottom); the arrow shows the location of the primers used in the amplification (O`Donnell & Cigelnik 1997)

A reaction mixture of total volume of 50 µl was set in 0.2 ml PCR tubes, composed of 19 µl PCR grade water, 1 µl of each forward and reverses primers, 25 µl JumpStart ™ Taq ReadyMix ™ (Sigma- Aldrich Chemie GmbH) and 4 µl of isolated total DNA. The PCR reaction was performed using PCR Thermocycler. The amplification started with an initial denaturation step for 5 min at 95 ° C, followed by 34 cycles of 30 sec denaturation at 94 ° C, 30 s, annealing at 52 ° C and 2 min elongation at 72 ° C. The final elongation step was for 10 min at 72 ° C.

Subsequently, the PCR product quality and the DNA integrity were analyzed by gel electrophoresis. A 0.8% agarose gel in TAE buffer (Roche Applied Science) was used, the gel was run at 80 V for 40 min and the bands were then visualized under UV light. 1kb DNA Ladder (New England Biolabs GmbH) was used as marker.

ITS and β-tubulin sequencing, sequence blasts and isolate comparison

PCR products of expected sizes were subsequently purified using a PCR Clean-Up Kit Nucleo Spin® Gel and PCR (Macherey-Nagel GmbH & Co. KG) following the manufacturer's instructions. The purified PCR products were analyzed for sequencing at the Helmholtz Center for Infection Research (HZI).

To verify the identity of the different fungal isolates, sequence Fasta format files were generated using the software SeqMan ™ II (5.05. DNASTAR) for ITS and β-tubulin gene and the sequences were blasted by using BLAST tool available online in GeneBank web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A compression alignments for the ITS and β-tubulin gene sequences were performed using ClustaW tool available online at EMBL-EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Phylogenetic analysis

Sequences of the ITS region and β -tubulin gene from the 13 isolates were compared by phylogenetic analysis. The sequences were aligned using CLUSTAL, the sequences of ITS region (GU014950, GU014946) and β–tubulin (GU563404, GU563403) of the two reference strains of *Epicoccum nigrum* CBS 161.73 and CBS 318.83 respectively were used in the alignments. The ITS and β–tubulin gene sequences of *Phaeosphaeria nodorum* were used as outgroup (AF250830 and AY786336, respectively). The phylogenetic trees were calculated using Mega5 (Tamura *et al.,* 2011) using Neighbour joining method, confidence (bootstrap 1000) was used to estimate the stability of the obtained phylogenetic trees.

Secondary metabolite extraction from *Epicoccum nigrum* isolates

To study the difference in the production of secondary metabolites, submerged fementation of thirteen selected isolates were cultured in two different culture media (YM and ZM $_{1/2}$). Each fungal mycelium was inoculated in 200 ml Erlenmeyer flask containing 50 ml of culture medium using 4 agar disks (7 mm in diameter) containing actively growing mycelia, using a sterile loop tip.

The flasks were then placed in an incubation chamber at 22° C for 7-9 days, on shaker at 140 rpm. The glucose level was measured using glucose test strip (Macherey-Nagel GmbH & Co.KG): the color change of the test strip indicates the glucose level in the media, expressed in mg/dl. In case of high glucose level, the cultures were kept for further incubation until the completly glucose consumption and the pH around 8 (slightly basic).

Secondary metabolites were extracted from culture media with an equal volume of ethyl acetate (50 ml) (Avantor Performance Materials, Center Valley PA. USA). The flasks then were incubated in ultrasonic bath at 40° C for 30 min. The culture then was transferred into separating funnel to split the hydrophilic from the hydrophobic compounds. The filtrates were then separated into two phases: the aqueous phase (lower part), which would be discarded, and the organic phase which is containing the metabolites. The organic phase then was filtered again through anhydrous sodium sulfate (Na_2SO_4) to remove water residual. The extracts obtained then were dried using a rotary evaporator at 40° C and then dissolved in methanol (Merck KGaA) at the concentration of 2 mg/ml.

High performance liquid chromatography/ diode array- mass spectrometry (HPLC/DAD-MS)

Part of the extracted samples from the different isolates (60 µl) was set to a concentration 2 mg/ µl and then was analyzed with the High Performance Liquid Chromatography/ diode array-Mass Spectrometry (HPLC/DAD-MS). The HPLC was performed on an Agilent 1260 Infinity series HPLC system with Amazon Ion-Trap MS (Bruker) [Column: 50×2.1 mm, Acquity UPLC BEH C-18, 1.7 mm (Waters); Solvent A: H_2O with 0.1% formic acid; Solvent B: acetonitrile with 0.1% formic acid; Gradient: 5% B for 0.5 min, increasing to 100% B in 19.5 min and continued at 100% B for 5 min, Flowrate = 0.6 mL/min; UV detection 200-500 nm]. The data were evaluated using the program "Data Analysis" available by Bruker.

Solid-plate cultures extraction and HPLC/DAD-MS

The thirteen selected isolates of *E. nigrum* were subcultured on YM agar plates and incubated for about 2 weeks until the mycelia were well grown (covering the entire plate surface). The agar was then cut in small pieces and transferred into a glass bottle (500 ml in volume) containing 300 ml of ethyl acetate for secondary metabolite extraction and incubated in ultrasonic bath for 30 min at 40 °C. The extracts were dried using a rotary evaporator at 40° C and then dissolved in methanol at the concentration of 2 mg/ml. Samples were analysed by HPLC/DAD-MS as described in the previous section.

Screening for antimicrobial bioactivity and serial dilution assay

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). MIC technique was used to determine and evaluate the bioactivity of crude extracts of *E. nigrum* isolates grown in two different culture media (MZ ½ and YM).

Crude extract stocks were prepared by dissolving the dried extract in methanol at a concentration of 4.5 mg/ml. The inocules were prepared using overnight culture of microorganisms used in the MIC test, Gram-negative bacteria (*Escherichia coli*- K 12), Gram-positive bacteria (*Bacillus subtilis*- DSM 10), filamentous fungi (*Mucor plumbeus*- MUCL 49355) and yeasts *(Saccharomyces cerevisiae*- DSM 1333). The bacteria were cultured in EBS medium, while yeasts and fungi in YM medium. The overnight cultures were prepared in 250 ml Erlenmeyer flasks flasks each filled with 100 ml of the proper culture medium and inoculated with 150 µl of a thawed stock of the corresponding strains and incubated overnight at 30° C on a shaker for *S. cerevisiae*, *M. plumbeus* and at 37 ° C for *B. subtilis*, *E. coli*. Cell counting was performed using the hemocytometer (0.1 mm depth, Neubauer) under microscope; the cell number per ml was adjusted to 6.7 x 10^5 for the bacteria and yeast and for *M. plumbeus* spore suspension cell number was adjusted to 2.7 x 10^7 cells per ml.

The assay was performed in 96-well microtiter plates (TPP Techno Plastic Products AG). Each well of the plate was filled with 150 µl of each microorganism solution, in the first row an additional 130 µl of the inculcated culture media plus 20 µl of the crude extract dissolved in methanol (90 mg) were added; a 150 µl from the fitst row then were transferred to the next row and so on, achieving a serial dilution up to 0.7 mg of tested crude extract per well at the last row. So the Minimum Inhibitory Concentration (MIC) would be determined. A Colum was left as a negative control and filled with methanol.

As positive controls, penicillin was used against *B. subtilis*, ciprofloxacin against *E. coli* and cycloheximide against *M. plumbeus* and *S. cerevisiae*.

The plates were incubated for 24 hours at 30° C on a shaker and the evaluation of the microorganism growth inhibition was performed visually.

Ten-liter fermentation and secondary metabolites extraction

The isolate 13059 was selected for further investigation. Four agar disks of active mycelium grown on YM agar plates were cut out and propagated in 2.5 L ZM ½ medium in 5 L Erlenmeyer flasks, as inoculum 250 ml of well-grown ZM ½ shake culture was used for each flask.

The flasks were placed on the shaker and incubated at 22° C at 140 rpm for 7-9 days, and the glucose level and pH were checked and adjusted as described earlier.

For secondary metabolite extraction, initially the mycelium was filtrated and separated from the culture broth using a sieve; the extraction was performed on both mycelium and the culture broth separately as illustrated in the scheme in Figure 26.

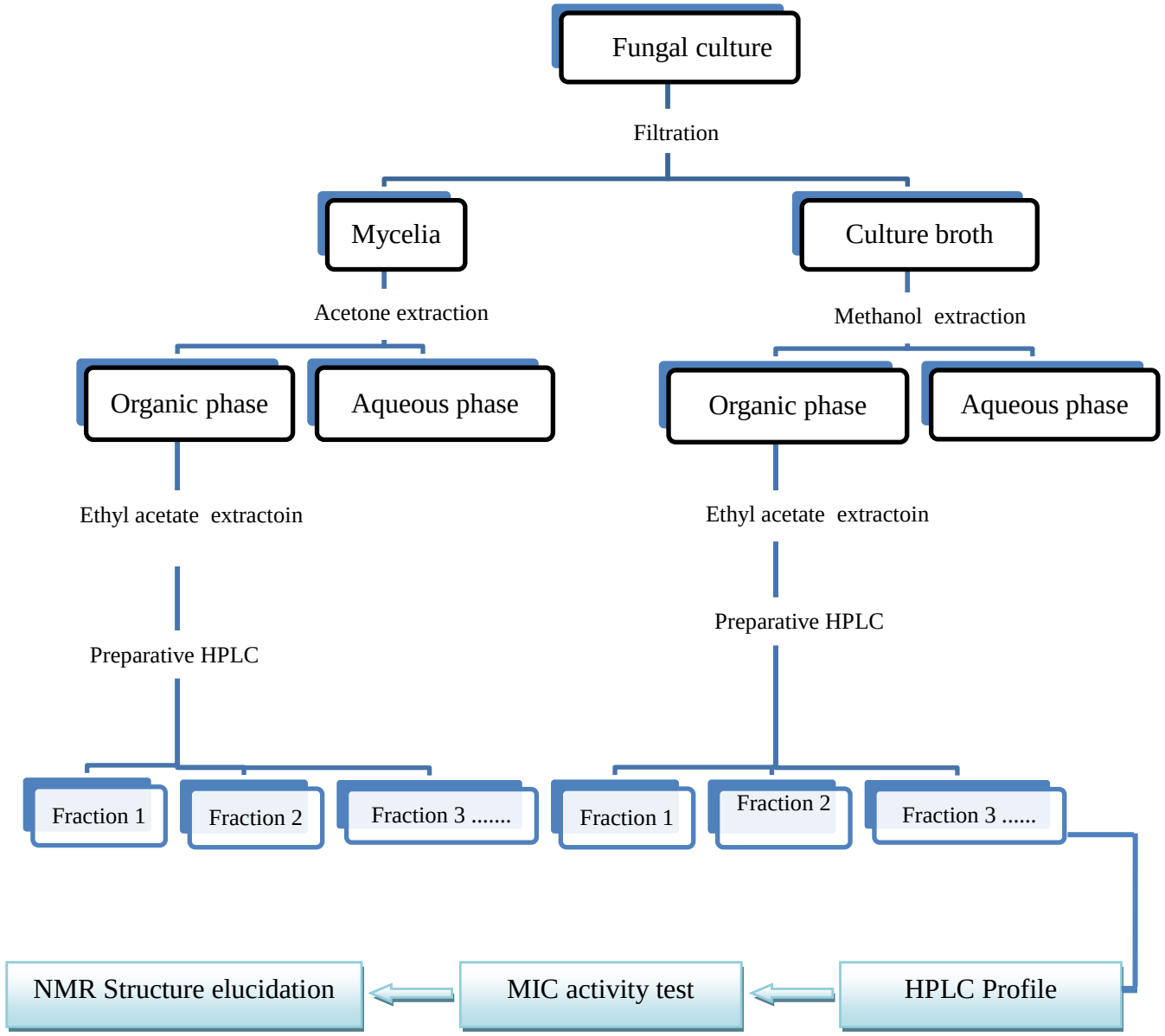


Figure 26. Work flow for extraction and downstream processing of secondary metabolites from *Epicoccum nigrum*

The mycelia were extracted twice with 500 ml acetone in an ultrasonic bath at 40° C for 30 min each time, the acetone was later dried with a rotary evaporator at 40° C. The remaining aqueous phase was extracted then with an equal volume of ethyl acetate as described earlier; the dried crude extracts were kept at - 20° C till further proceeding.

Extraction form the culture broth was prepared by transferring the filtrated broth into a large container (15 liter). About 400 g of Amberlite® XAD16 (Sigma-Aldrich) were added to the broth and incubated overnight under stirring at 60 rpm under the fume-hood, as illustrated in Figure 27. The resin later was harvested from the broth using a sieve; the harvested resin was extracted twice with methanol in ultrasonic bath at 40 °C for 30 min each. The methanol elute then was evaporated with a rotary evaporator at 40 ^{o} C, the remaining aqueous phase was extracted with an equal volume of ethyl acetate as descried earlier, the dried crude extract was kept at -20 ^{o} C till further proceeding.

Part of the dried extracts of both the mycelium and the filtrate was then dissolved in methanol and analyzed by High Performance Liquid Chromatography coupled to diode array - mass spectrometry (HPLC/DAD-MS) as described in HPLC/DAD-MS section.

Figure 27. Secondary metabolite extraction from the culture broth using the Amberlite® XAD16

Preparative HPLC, metabolite fractionation and determination of minimum inhibitory concentration (MIC) values

Preparative HPLC was performed on the crude extracts from mycelium and the culture broth separately using a Gilson GX270 Series HPLC system [column 125x40 mm, Nucleodur C18 ec, 7 μm (Macherey-Nagel).

The dried extracts were dissolved in HPLC grade acetonitrile (Avantor Performance Materials) and Millipore water in 1:1 ratio. Mobile phase, solvent A composed of Millipore water with 0.5% glacial acetic acid and mobile phase B composed of acetonitrile with 0.5% glacial acetic acid, pre-filtered.

This method is suited well, first to remove the highly polar compounds and then to elute the less polar compounds ensuring good separation and higher resolution peaks. To ensure this, the rato of solvent B was gradually increased over time.
The compositions of the mobile phase gradients over the time span are listed in table 2 and 3.

Time course (min.)	% composition of solvent A	% composition of solvent B	Duration (min.)
5	60	40	10
15	50	50	3
18	45	55	10
28	38	62	3
31	34	66	25
56	10	90	2
58	0	100	2
60	0	100	Stop fraction collection

Table 2. Solvent gradient for the fractionation of the mycelium extract

Time course (min.)	% composition of solvent A	% composition of solvent B	Duration (min.)
5	60	40	35
40	35	65	10
50	15	85	3
53	0	100	Stop fraction collection

Table 3. Solvent gradient for the fractionation of the culture filtrate extract

The sample was injected automatically and the pumps were set at a flow rate of 25 ml per min. The fractions were collected in 18 ml collecting tubes; fractions corresponding to a single peak were combined later on in a round flask and evaporated using the rotary evaporator. Isopropanol was added to facilitate water evaporation.

The collected dried fractions were re-dissolved in methanol to a concentration of 2 mg/ml and 60 µl of the obtained solutions were analyzed by HPLC/DAD-MS to evaluate the purity of the isolated fractions, as described previously.

Later, the purified fractions were used to determine the MIC value and to locate the active fractions; the serial dilution assay was done using the above test organisms, i.e. *Bacillus subtilis Escherichia coli, Mucor plumbeus* and *Saccharomyces cerevisiae* in microtiter plates as described earlier.

Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy is a complex technique to analyze the molecular structure of compounds. After the preperative HPLC fractionation, fractions were dissolved with 500 µl of methanol-D4 (Sigma-Aldrich) and subsequently submitted for structure elucidation. The spectra were recorded using Bruker instruments (Avance III 500, ^{1}H 500 MHz, ^{13}C 100 MHz) and Avance III 700 with cryogenic equipment (^{1}H 700 MHz, ^{13}C 100 MHz). Structure elucidation was performed by Dr. K. Wittstein at HZI.

RESULTS

DNA isolation, its and β-tubulin PCR amplification and gel electrophoresis

DNA extracted from the isolates was adjusted to an average concentration of 1.5 µg /µl, resulting in a ratio between the readings of absorbance A260/A280 A260/A230 of above 2, indicating good quantity and quality of the extracted DNA.

The results of polymerase chain reaction (PCR) amplification performed on the extracted DNA of the 13 isolates, using the primer pairs ITS1F, ITS4 produced a band with a size 570 bp (the expected size) and the amplification of β-tubulin gene using the primers pairs T1, T22 produced a fragment of about 1200 bp. The PCR products were run in 0.8% agarose gel, visualized under UV light, which revealed a single visible band for each product, corresponding to the expected amplified size (Figures 28 and 29).

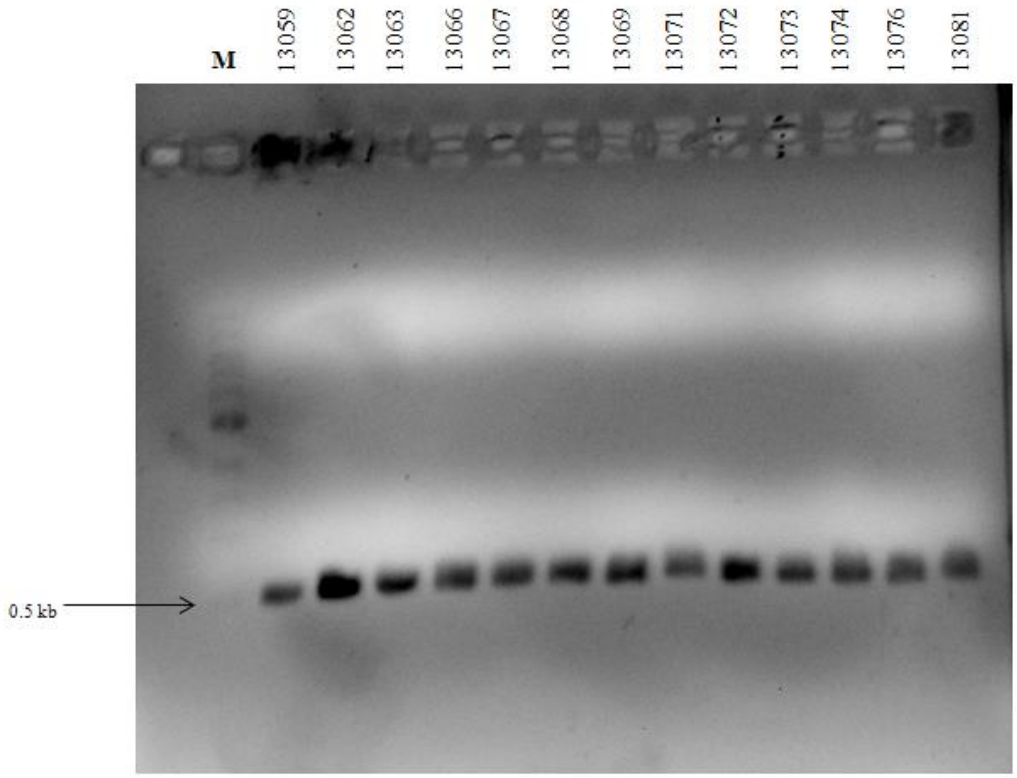


Figure 28. 0.8 % agarose gel electrophoresis of the ITS region PCR amplification from the different *Epicoccum nigrum* DNAs, using the universal primers (ITS1F, ITS4). M is 1 kb DNA ladder (New England Biolabs) used as marker.

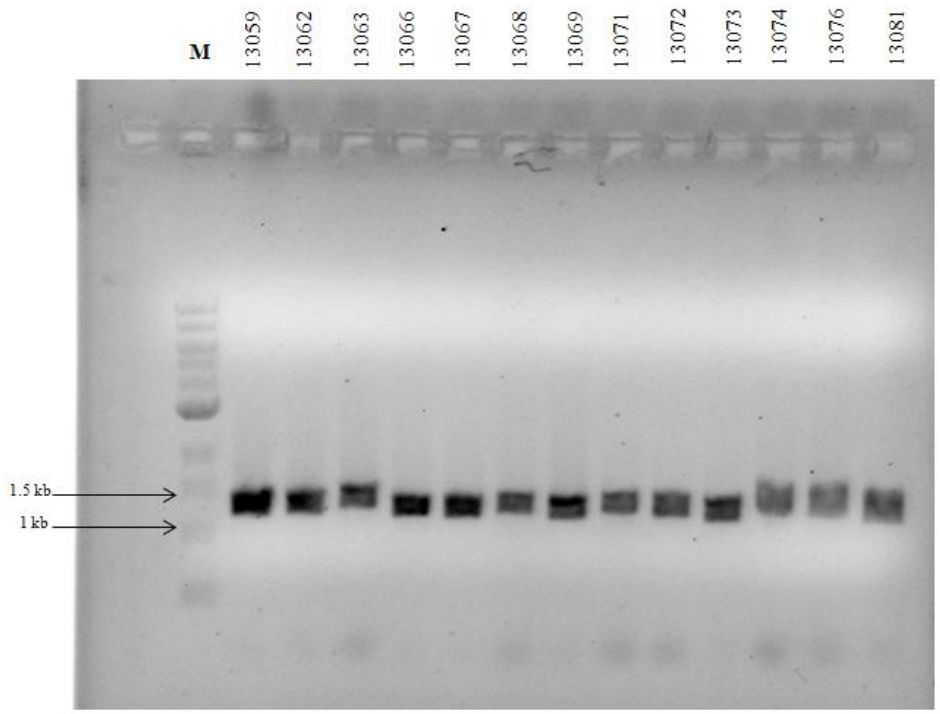


Figure 29. 0.8 % agarose gel electrophoresis of the β-tubulin PCR amplification of the different *E. nigrum* DNAs using the universal primers (T1, T22). M is 1 kb DNA ladder (New England, Biolabs) used as marker.

ITS and β-tubulin sequencing, sequences blast and isolates identification

Both ITS region and β-tubulin gene sequences were aligned with reference sequences from GenBank. The ITS regions showed a 100% identity to *Epicoccum nigrum* except the isolates 13071 and 13081 which resulted to belong to *Stemphylium solani* and *Phoma* spp. respectively. ITS sequences strongly suggest that *Epicoccum nigrum* and *Phoma epicoccina* are the same biological species; however, such simple comparsion needs careful interpretation (Arenal *et al.*, 2002). Using the BLAST for β-tubulin sequencing, several isolates showed similarity to *Phoma nigrificans*, except the isolate 13071 which resulted in *Phaeosphaeria nodorum* as best match, as well as β-tubulin BLAST gave a low query coverage with the data from GenBank and that because sequences of β-tubulin from *E. nigrum* are not available in data base, also *Phoma nigrificans* and *Epicoccum nigrum* are related therefore using apolythetic approach is necessary for closely related species classification and differentiation. BLAST results are summarized in Tables 4 and 5, the sequences alignments are listed in the appendices.

Isolate code	ITS length (bp)	ITS blast	Identity %	Accession number	Reference
13059	556	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13062	577	*Epicoccum nigrum*	100 %	HQ115657.1	Gorfer *et al.*, 2011
13063	563	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13066	556	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13067	561	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13068	558	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13069	560	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13071	595	*Stemphylium solani*	99%	AF203449.1	Mehta *et al.*, 2002
13072	573	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13073	559	*Epicoccum nigrum*	100%	FN868456.1	Botella and Diez, 2011
13074	561	*Phoma herbarum*	99%	JQ282910.1	Li *et al.*, 2012
13076	563	*Epicoccum nigrum*	100%	FJ424249.1	Larena and Melgarejo, 2009
13081	572	*Phoma sp.*	99%	HQ630999.1	Shrestha *et al.*, 2011

Table 4: Summary of the BLAST results of the isolate ITS region. Matching percentages, accession numbers and references are listed.

Isolate code	β-tubulin length (bp)	β-tubulin blast	Identity %	Accession number	Reference
13059	1192	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13062	1133	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13063	1031	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13066	1191	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13067	1186	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13068	1126	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13069	1196	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13071	1171	*Phaeosphaeria nodorum*	90%	AY786339	Malkus *et al.*, 2004
13072	1182	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13073	1141	*Phoma nigrificans*	95%	AY749030.1	Voigt *et al.*, 2005
13074	1145	*Phoma nigrificans*	93%	AY749030.1	Voigt *et al.*, 2005
13076	1189	*Phoma herbarum*	93%	AY749025.1	Voigt *et al.*, 2005
13081	NA	NA	NA	NA	NA

Table 5: Summary of the BLAST results of the sequenced β-tubulin genes. Matching percentages, accession numbers and references are listed.

Phylogenetic analysis

The phylogenetic relationships between the endophyte isolates of *E. nigrum* and *Phoma* were examined using sequences of the ITS region and the partial sequences of ß-tubulin gene by the Neighbour joining method analysis. Phylogenetic analysis based on the complete sequences of ITS region, with the ITS sequence of *Phaeosphaeria nodorum* as outgroup, revealed that both reference strains CBS 318.83 and CBS 161.73, were clustered in the same calde with most isolates including the *Phoma* and a second clade, which included only one isolate 13071(figure 30).

The phylogenetic analysis using the ß-tubulin distinguished the isolates into two distinct clades (figure 31); the first clade included both reference strains but also included the out-group strain. The second clade included only the isolate 13063.

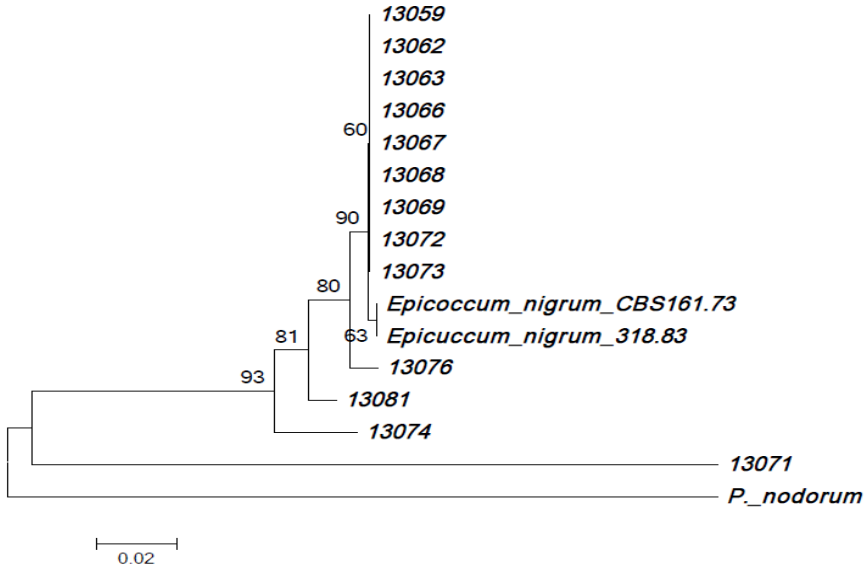


Figure 30. Phylogenetic tree using sequences of ITS region from the different isolates using neighbour joining method (Bootstrap 1000, Software Mega5) values are shown next to relevant nodes. Two *E. nigrum* reference strains (CBS 318.83 and CBS 161.73) and *P. nodorum* as outgroup sequence.

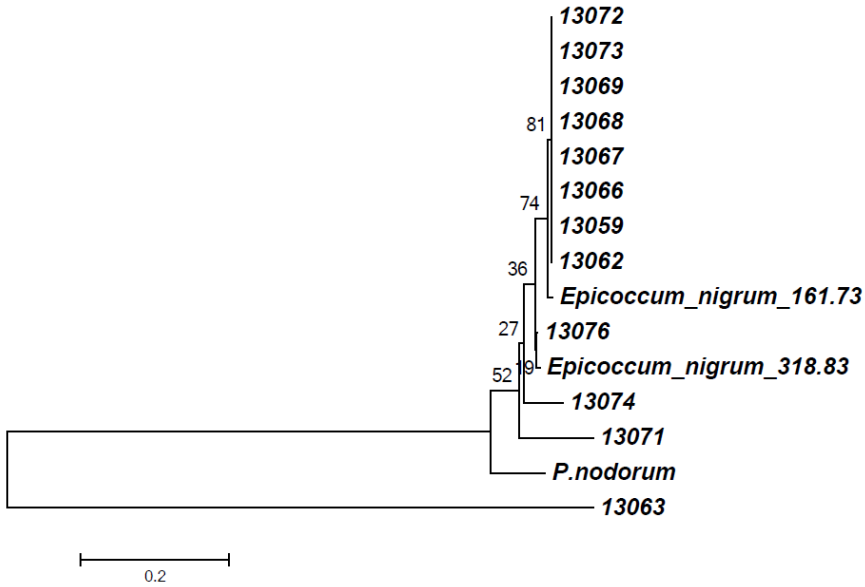


Figure 31. Phylogenetic tree using sequences of β-tubulin gene from the different isolates using neighbour joining method (Bootstrap 1000, Software Mega5) values are shown next to relevant nodes. Two *E. nigrum* reference strains (CBS 318.83 and CBS 161.73) and *P. nodorum* as outgroup sequence.

Secondary metabolite extraction from *Epicoccum nigrum* isolates

Different amounts (ranging between 1-8 mg) of crude extracts were extracted from small scale shaking flasks, from both YM and ZM ½ cultures. The quantities extracted were not related to the culture media; rather they were related to the isolate genotypes.

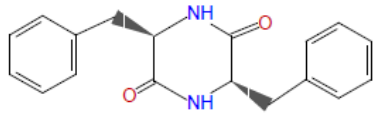
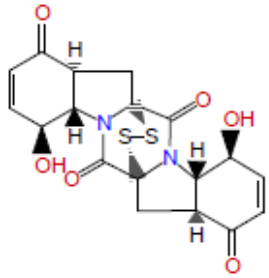
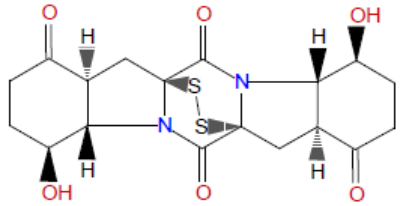
High performance liquid chromatography/ diode array - mass spectrometry (HPLC/DAD-MS)

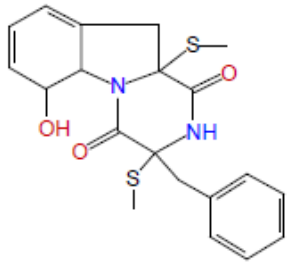
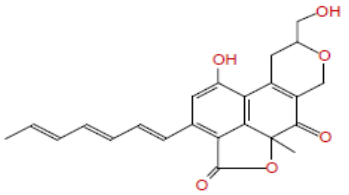
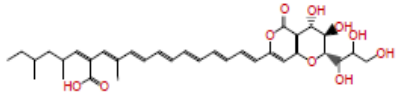
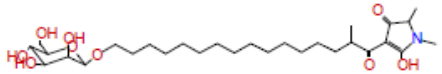
HPLC/DAD-MS metabolites profiles of the crude extracts from the thirteen isolates from both culture media were generated to study the difference in the secondary metabolite profiles. The chromatograms were generated using Diode Array detection recording UV/visual spectra over wavelength range (200-600 nm) are shown in the appendices.

The HPLC/DAD-MS analysis was used to evaluate the diversity in the secondary metabolites produced by the different isolates under study in different culture media. Isolates grown in complex ZM ½ medium produced complex metablic profile containing more peaks in the chromatogrames.

On the other hand the similarities between the metabolite profiles produced by the same isolate grown in two different media were evaluated, depending on the molecular weight of the metabolites generated by mass spectrometry. MS data revealed that majority of the metabolites are not medium specific, therefore they were produced in the same isolate in both media; some metabolites were medium specific since different metabolites were produced in different medium stype.

The HPLC/DAD-MS profiles revealed the production of several secondary metabolites. The tentatively identified metabolites are listed in table 6, where the molecular weight, name, empirical formula and absolute structure are listed. The majority of the metabolites produced are unidentified yet.

Molecular mass of the Identified compounds (g/mol)	Name	Empirical formula	Productive Isolates grown in YM culture media	Productive Isolates grown in ZM ½ culture media	Structure	Reference
294.34	3,6-Dibenzyl-2,5-piperazinedione	$C_{18}H_{18}N_2O_2$	13059, 13081	-		Baute *et. al.*, 1973
420.45	Epicorazine A	$C_{18}H_{16}N_2O_6S_2$	13071, 13081	13076		Deffieux *et. al.*, 1987
424.49	Rostratin B	$C_{18}H_{20}N_2O_6S_2$	13066, 13069, 13073	13063, 13067, 13068, 13072		Tan *et. al.*, 2004

402.53	3822-B	$C_{20}H_{22}N_2O_3S_2$	-	13071		Mizogami *et al.*,1991
210.31	Epirodin	$C_{16}H_{18}$	13063, 13066, 13068, 13069, 13071, 13073, 13081	13063		Ikawa *et. al.*, 1977
394.41	*Epicoccum purpurascens* naphthopyran	$C_{23}H_{22}O_6$	13059, 13062, 13066, 13067, 13069, 13073, 13074	13059, 13062, 13066, 13067, 13068, 13069, 13073, 13074,		Assante *et. al.*, 1978
612.70	Orevactaene	$C_{34}H_{44}O_{10}$	13073, 13076	13068, 13069, 13076, 13081		Shu *et. al.*, 1997
557.71	Epicoccamide	$C_{29}H_{51}NO_9$	13059, 13076	-		Wright *et.al.*, 2003

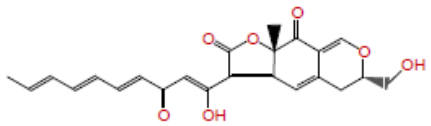
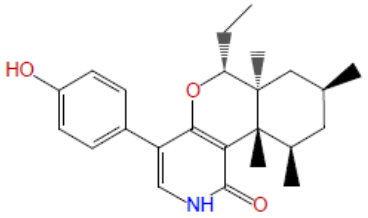
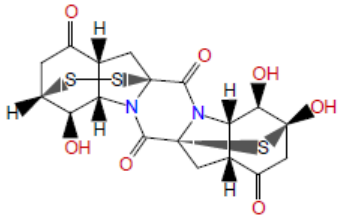
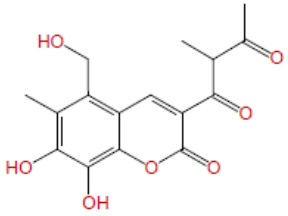
410.41	Epicocconone	$C_{23}H_{22}O_7$	13059, 13062, 13066, 13067, 13069, 13072, 13074	13059, 13062, 13063, 13067, 13068, 13069, 13072		Bell and Karuso, 2003
381.50	Epipyridone	$C_{24}H_{31}NO_3$	13059	13062		Wanguna and Hertweck, 2007
470.54	Epicoccin	$C_{18}H_{18}N_2O_7S_3$	13076,13081	13076,13081		Zhang *et. al.*, 2007
320.29	Epicoccalone	$C_{16}H_{16}O_7$	13068	13071		Wangun *et. al.*, 2008

Table 6: The tentatively identified metabolites extracted from the submerged culture of the endophytic isolates in two different media

Solid-plate cultures extraction and HPLC/DAD-MS

HPLC/DAD-MS metabolite profiles of the thirteen isolates extracted from YM agar plates were generated. The HPLC/DAD-MS profiles served to compare the secondary metabolite profile produced in the solid state culture, with that produced in the submerged culture.

The chromatograms were generated using Diode Array detection recording UV/Visual spectra over wavelength range (200-600 nm) and they are shown in the appendices. HPLC/DAD-MS profiles of the secondary metabolites shown differences among the isolates when different genotypes or different culture methods (solid or submerged) were compared. Only three metabolites were produced in both solid state and submerged culture (these metabolites have molecular mass 294, 424 and 612).

The HPLC/DAD-MS profile reveled also the diversity of the secondary metabolites among the different isolates; only three metabolites were produced by all isolates. These metabolites have molecular masses of 294, 367 and 291), therefore different genetypes revealed different secondary metabolites profiles. Five metablites produced by the different isolates were tentatively identified. Metabolites are listed in table 7, where the molecular weight, name, empirical formula and structure are also listed.

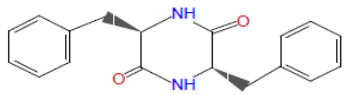
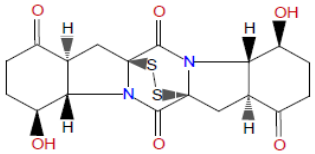
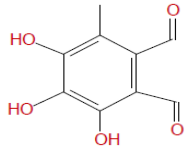
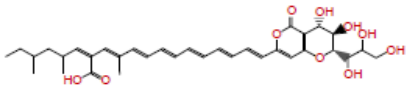
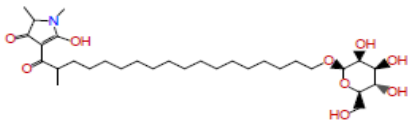
Molecular mass of the Identified compounds (g/mol)	Name	Empirical formula	Productive Isolates grown in YM agar plate	Structure	Reference
294.34	3,6-Dibenzyl-2,5-piperazinedione	$C_{18}H_{18}N_2O_2$	13059, 13062, 13063,13067		Baute *et. al.*, 1973
424.49	Rostratin	$C_{18}H_{20}N_2O_6S_2$	13072, 13081		Tan *et. al.*, 2004
196.15	Flavipin	$C_9H_8O_5$	13076		Nitao *et. al.*, 2001
612.70	Orevactaene	$C_{34}H_{44}O_{10}$	13073, 13076 13081		Shu *et. al.*, 1997
585.77	Epicoccamide D	$C_{31}H_{55}NO_9$	13076		Wangun *et. al.*, 2007

Table 7: The tentatively identified metabolites extracted from the endophytic isolates grown on the YM agar plates

Screening for antimicrobial activity and serial dilution assay

The evaluation of the biological activity of the 13 isolates was based on the minimum inhibitory concentration (MIC) values. The MIC values were determined for all crude extracts from isolates grown in both culture media, as above reported.

All the 13 isolates showed activity against *S. cerevisiae, M. plumbeus* and *B. subtilis*t but not against *E. coli*. The efficiency against the tested microorganisms was vary depending on the genotype and the growth conditions (medium).

E. nigrum isolates showed a significant activity against *S. cerevisiae,* the highest activities were for the isolates 13059 grown in ZM ½ medium and the isolate 13072 in YM medium. The values were 5.6 µg/ml and 11.5 µg/ml respectively, as compared to 0.5 µg/ml for the antibiotic cycloheximide which was used as positive control. The other MIC values are shown in table 8.

Isolate code	molecular ID	YM Culture (µg/ml)	ZM ½ Culture (µg/ml)	Positive control (µg/ml)
13059	*Epicoccum nigrum*	45	5.6	0.5
13062	*Epicoccum nigrum*	90	90	0.5
13063	*Epicoccum nigrum*	na	11.5	0.5
13066	*Epicoccum nigrum*	22.5	45	0.5
13067	*Epicoccum nigrum*	na	22.5	0.5
13068	*Epicoccum nigrum*	90	11.5	0.5
13069	*Epicoccum nigrum*	45	45	0.5
13071	*Epicoccum nigrum*	90	90	0.5
13072	*Epicoccum nigrum*	11.5	22.5	0.5
13073	*Epicoccum nigrum*	na	45	0.5
13074	*Phoma herbarum*	90	90	0.5
13076	*Epicoccum nigrum*	45	na	0.5
13081	*Dothiorella gregaria*	90	90	0.5

Table 8: The minimum inhibitory concentration (MIC) values of antimicrobial activity of the tested isolates against *Saccharomyces cerevisiae* the numbers are expressed in µg/ml of the crude extract (na- no activity).

Despite the fact that none of the isolates showed an activity against the Gram-negative bacterium (*E.coli*), the isolates 13059 in ZM ½ medium and 13072 in YM medium showed the highest activities against the Gram-positive bacterium (*B. subtilis*). The MIC value was 2.81 µg/ml for both isolates as comparing to 90 µg/ml for the positive control (penicillin). The other isolates showed activity at least in one medium type, except the isolate 13081 which showed no activity. The other MIC values are shown in the table 9.

Isolate code	molecular ID	YM Culture (µg/ml)	ZM ½ Culture (µg/ml)	Positive control (µg/ml)
13059	*Epicoccum nigrum*	22.5	2.81	90
13062	*Epicoccum nigrum*	45	22.5	90
13063	*Epicoccum nigrum*	na	5.625	90
13066	*Epicoccum nigrum*	11.5	45	90
13067	*Epicoccum nigrum*	45	11.5	90
13068	*Epicoccum nigrum*	45	5.625	90
13069	*Epicoccum nigrum*	22.5	11.5	90
13071	*Epicoccum nigrum*	45	90	90
13072	*Epicoccum nigrum*	2.81	11.5	90
13073	*Epicoccum nigrum*	45	11.5	90
13074	*Phoma herbarum*	90	45	90
13076	*Epicoccum nigrum*	90	90	90
13081	*Dothiorella gregaria*	na	na	90

Table 9: The minimum inhibitory concentration (MIC) values of antimicrobial activity of the tested isolates against *bacillus subtilis* the numbers values are expressed in µg of the crude extracts (na- no activity).

E. nigrum isolates showed showed a significant activity against *M. plumbeus*, the highest activity was 11.5 µg/ml comparing to 22.5 µg/ml for the positive control cycloheximid. The other MIC values are listed in the table 10 for the thirteen isolates. The microtiter plate's photos are supplied in the appendices.

Isolate code	molecular ID	YM Culture (µg/ml)	ZM ½ Culture (µg/ml)	Positive control(µg/ml)
13059	*Epicoccum nigrum*	22.5	22.5	22.5
13062	*Epicoccum nigrum*	22.5	22.5	22.5
13063	*Epicoccum nigrum*	22.5	22.5	22.5
13066	*Epicoccum nigrum*	22.5	22.5	22.5
13067	*Epicoccum nigrum*	90	45	22.5
13068	*Epicoccum nigrum*	90	90	22.5
13069	*Epicoccum nigrum*	45	22.5	22.5
13071	*Epicoccum nigrum*	90	90	22.5
13072	*Epicoccum nigrum*	45	90	22.5
13073	*Epicoccum nigrum*	90	45	22.5
13074	*Phoma herbarum*	11.5	11.5	22.5
13076	*Epicoccum nigrum*	11.5	45	22.5
13081	*Dothiorella gregaria*	22.5	22.5	22.5

Table 10: The minimum inhibitory concentration (MIC) values of antimicrobial activity of the tested isolates against *Mucor plumbeus.* The values are expressed in µg of the crude extracts.

Fermentation of *Epicoccum nigrum* in 10 liters scale and secondary metabolites extraction

Fermentation of *E. nigrum* in 10 liters scale fermentation of the selected isolate in the proper medium depending on the biological activity of the crude extracts from the thirteen isolates (13059 in ZM ½) resulted in higher amount of the crude extracts produced from both mycelium and the culture broth (865 and 267 respectivly).

Preparative HPLC, fractionation and determination of MIC values

Preparative HPLC was used to fractionate both mycelium and culture broth crude extracts (Figure 32). Fifteen distinctive peaks were assigned on the mycelium chromatogram and 13 from the culture broth. The first peaks from the chromatogram (highly polar compounds) were not considered in fractions collection. The retention times expressed by (min) for each fraction are listed in the table 11.

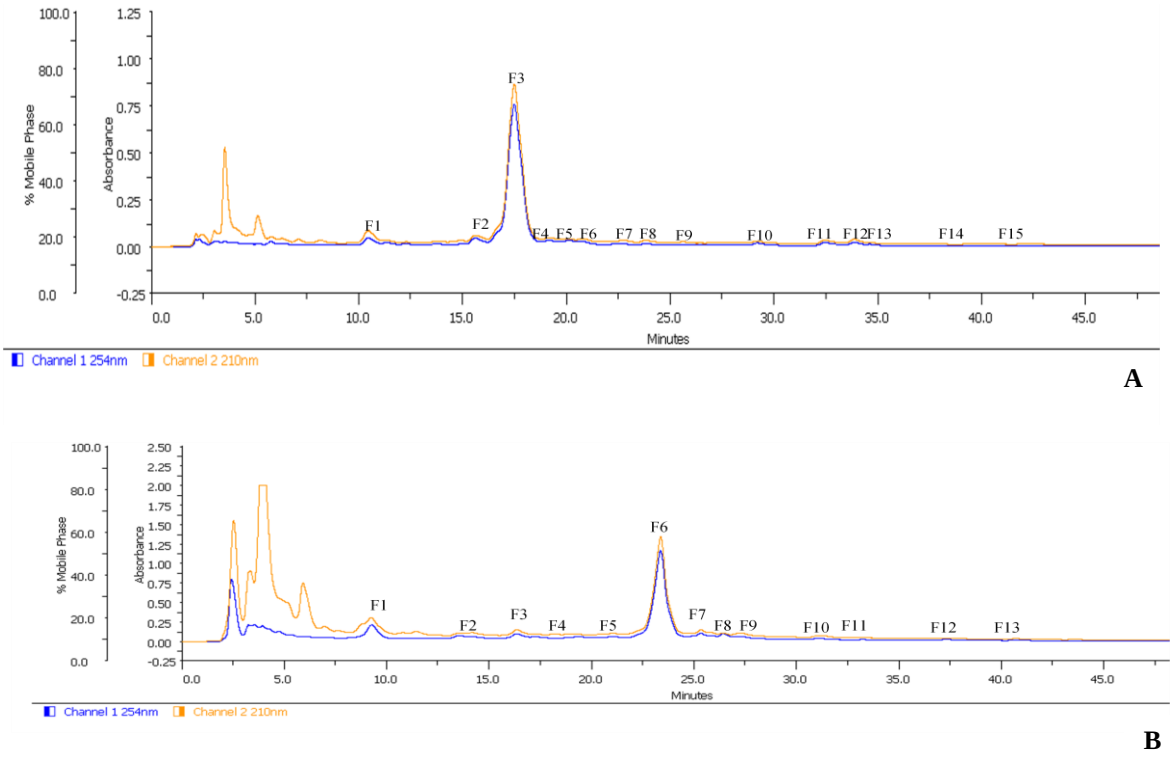


Figure 32. Chromatograms obtained by application the preparative HPLC from the mycelium crude extract (A) and culture broth crude extract (B). The fractions obtained from the preparative HPLC are indicated on the chromatogram.

Each fraction then were further analyze by HPLC/DAD-MS to be assessed for purity as described earlier, from the chromatogram generated by the HPLC analysis 3 fraction from the mycelium (F7, F11 and F14) shown to be the most pure fractions collected, and 5 fractions from the culture broth (F2, F3, F8, F11 and F13) which were subjected to NMR analysis. The chromatograms of these fractions are supplied in the appendices. The mass of the main peak in each fraction are listed in the table below. The serial dilution assay was performed using the testing organisms (*B. subtilis*, *E. coli*, *M. plumbeus* and *S. cerevisiae*) revealed the MIC value for each fraction.

Only two fractions from the mycelium (F12 and F13) showed activity against *S. cerevisiae* while there was no any activity noticed in the culture broth fractions. When the crude extracts of the isolate 13059 did not show any activity against *E.coli* most of fractions purified showed bioactivity up to 5 µg/ml. The same activity also had been demonstrated against *B. subtilis* and *M. plumbeus* as well. The MIC values for the fractions are listed in table 11.

Fraction	R.T (min)	Mean peak mass	*S. cerevisiae* (µg/ml)	*E.coli* (µg/ml)	*B. subtilis* (µg/ml)	*M. plumbeus* (µg/ml)
1	10.9	406	na	40	na	na
2	16.3	474	na	na	na	20
3	17.2	394	na	60	60	30
4	19.4	394	na	20	40	20
5	21	392	na	5	10	20
6	21.7	410	na	20	20	20
7	24	384	na	40	40	10
8	25.8	392	na	20	40	5
9	26.4	317	na	20	40	5
10	31	336	na	20	20	20
11	32.9	410	na	10	10	10
12	33.2	410	40	5	5	10
13	33.5	410	40	5	5	10
14	40	497	na	na	na	10
15	43	497	na	na	na	10

Table 11: The minimum inhibitory concentration (MIC) values of antimicrobial activity of the mycelium extract fractions from the isolate 13059 against S. *cerevisiae*, *E.coli*, *B. subtilis* and *M. plumbeus* the numbers values are expressed in µg.

Fraction	R.T (min)	Mean peak mass	*S. cerevisiae* (µg/ml)	*E. coli* (µg/ml)	*B. subtilis* (µg/ml)	*M. plumbeus* (µg/ml)
1	9.27	394	na	na	na	15
2	14.2	362	na	na	na	10
3	17.3	392	na	40	na	10
4	19.4	348	na	na	na	10
5	21	394	na	na	na	20
6	23.38	394	na	15	60	na
7	25.35	394	na	10	0.65	na
8	26.47	392	nt	nt	nt	na
9	27.29	410	nt	nt	nt	na
10	31	392	nt	nt	nt	na
11	33.25	678	nt	nt	nt	na
12	37.37	343	nt	nt	nt	na
13	40.74	378	nt	nt	nt	na

Table 12: The minimum inhibitory concentration (MIC) values of antimicrobial activity of the culture broth extracts fractions from the isolate 13059 against S. *cerevisiae, E.coli, B. subtilis* and *M. plumbeus* the numbers values are expressed in µg. (na- no activity, nt- not tested)

Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) is a phenomenon that occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field.NMR is considered as a routine technique that chemists use to determine the structure of complicated molecules. NMR is used in a wide range of application in chemistry, biology and medicine. The NMR spectroscopic data was recorded with a BrukerAvance III 500 (^{1}H 700 MHz, ^{13}C 100 MHz) and Avance III 700 with cryogenic equipment (^{1}H 700 MHz, ^{13}C 100 MHz) by the internal HZI NMR service HRESIMS: m/z $[M+H]^{+}$ 395.148915. Structure elucidation performed by K. Wittstein at the HZI. The structures along the NMR spectra are illustrated in figure 33.

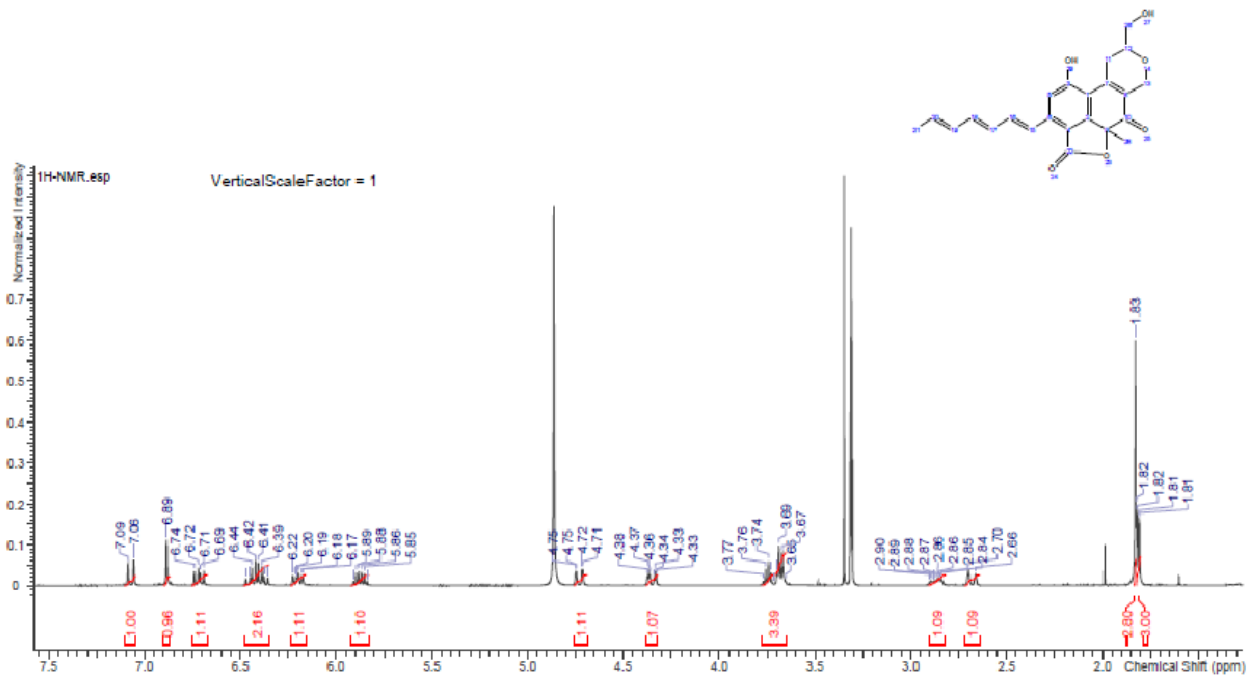


Figure 33. Proton Nuclear magnetic resonance (NMR) spectrum and structure proposal of fraction 5 from the preparative-HPLC performed on the mycelium crude extract of E. nigrum, isolate 13072. Data anaylsis was performed by Dr. K. Wittstein, HZI.

DISCUSSION

Diversity of *Epicoccum nigrum*

Morphology and reproductive biology analysis provide the basis for the identification and classification of fungal species, but phylogenetic approaches have become popular for the recognition of species (Taylor *et al.*, 2000). *E. nigrum* is an endophytic fungal species that shows a great phenotypic and genotypic variability (Arenal *et al.*, 2002).

Sequence blast of the thirteen isolates revealed that the *E. nigrum* endophytic strains can be assigned to *E. nigrum* or *Phoma* spp. using the ITS region. The ß-tubulin partial sequences, showed highest homology to *Phoma nigrificans.* A previous study using the ITS region sequences suggested that *E. nigrum* and *Phoma epicoccina* belong to the same biological specie (Arenal *et al.*, 2002). Walter *et al.*, (2012) reported in a study on *Hypocrea caerulescens* sp. that several species of the section *Trichoderma* (comprise 43 species) ITS sequences were identical or nearly identical. Most of the *E. nigrum* isolates resulted not distinguishable when the alignment of ITS region sequences was used since the ITS sequences were also identical or nearly identical. Furthermore, many of the sequences deposited as in GenBank and culture collection of *E. nigrum* strains should be reclassified.

The genetic relationships between endophytic isolates of *E. nigrum* from were examined using sequences of the ITS region of rDNA and partial sequence of ß-tubulin gene using neighbour joining method. The phylogenetic analysis showed that *E. nigrum* could be also clustered in a different manner. The result suggests that the classification of *E. nigrum* is not a single variable species but complex species.

Rather small number of the analysed isolates and the fact that only two gene loci were so far considered makes the phylogenetic analysis not fully complete, therefore more individuals, and possible, additional genes, will have to be analysed for a better clarification of the phylogenetic affinities among *E. nigrum* strains. The most important point will be to select and include type or epitype strains of *E. nigrum* and associated genera and species in such studies, which was beyond of scope of the present thesis.

Most studies on classification and characterization of endophytic fungi were conducted by using a combination of morphological and molecular methods. Nevertheless the combination of more different approaches (polythetic or polyphasic approach) is necessary for better characterization of the closely related stains, as previously reported for other fungal endophytes with complex taxonomy (Polizzotto *et al.,* 2012).

HPLC based techniques have progressed considerably in the past decade, providing analytical tools for further work in this field (Stadler *et al.,* 2001). The Hypoxyloideae were evaluated by a morphological and HPLC- based chemotaxonomic survey (Stadler and Fournnier, 2006), Garcia *et al.,* (2011) reported that fatty acid profiles showed complementarily with phylogenetic findings and therefore might be regarded as a significant tool for the chemo-taxonomic classification of myxobactetria. Three new species of Hypoxylon (*Xylariaceae*) were recognized by combinations of morphological characters and secondary metabolites profiles based on HPLC/DAD-MS as well as by comparison of ITS and partial ß-tubulin DNA sequences with related taxa (Kuhnert *et al.,* 2013).

HPLC/DAD-MS revealed the chemical profiles of the extracts from the different culture media used for growth of *E. nigrum.* In particular, the complex medium ZM ½ resulted in more complex metabolites profile compared to other medium. The data from mass spectrometry (MS) showed the variation in the metabolites, even though the HPLC chromatograms did not show the same degree of variability. Only few of these metabolites were identified and know.

A list of the tentatively identified metabolites is reported in the Results section. Known metabolites do not exceed one tenth of the metabolites detected by HPLC-MS. Some of the metabolites resulted produced by the diverse isolates, others were strains specific, produced only by specific strains, or resulted culture-dependent, so they were produced by different isolates but only on specific medium. However, specificity of the production needs to be studied unsing a broad range of culture media and also follwoing the time course of production.

Analyses of extracts from mycelia grown on YM media on agar plate showed that most of endophytic strains are able to produce further diverse secondary metabolites on this medium, in particular when isolates were grown in submerged culture.

However, MS data revealed that two metabolites with the molecular mass of 196.15 and 585.77, which tentatively identified as Flavipin and Epicoccamide respectively, were produced exclusively in the isolate 13076 grown on the agar plat but not from the submerged media.

Flavipin and Epicoccamide are two metabolites of important use. Flavipin was reported to have antifungal activity and antagonistic properties to plant-parasitic nematodes (Bamford *et al.,* 1961; Nitao *et al.,* 2001), while Epicoccamide has weak to moderate cytotoxicity to human leukemia cell lines (K-562) (Wright *et al.,* 2003 and Wangun *et al.,* 2007).

E. nigrum shown the capability of producing a vast variability in secondary metabolites: the metabolite production was coordinated according to the isolate genotype or to the growing medium (YM or ZM ½), or to the mode of fermentation (submerged or solid state). The results indicate that using more type of media, *E. nigrum* strains could be able to produce new and different metabolites with higher potential in the production of novel metabolites with bioactive properties. However, the identified metabolites are still few in comparison to the total secondary metabolites produced. This fact could indicate abundancy of novel bioactive metabolites to be discovered.

Screening for antimicrobial activity

Epicoccum nigrum is known to be active as biocontrol agent against important plant pathogens (Elmer *et al.,* 2001), due to its productivity of a wild range of secondary metabolites with antibiotic properties. For example, Flavipin is an antifungal metabolite (Bamford *et al.,* 1961) antagonistic to plant-parasitic nematodes (Nitao *et al.,* 2001), Epicorazine A and Epicorazine B are two antibiotics active against *Staphylococcus aureus* (Deffieux *et al.,* 1978), Epirodin is a pigment with antibiotic properties (Ikawa *et al.,* 1978), Epicoccins A–D (Zhang, *et al.,* 2007) showed modest antimicrobial activity, Epicoccarines A, B and Epipyridone (Wanguna and Hertweck, 2007) showed antibacterial activity against Gram positive bacteria, such as *Mycobacterium vaccae*.

In addition to the antibiotic properties, other compounds with antioxidant activity (Abdel-Lateff *et al.,* 2003). Rostratins A-D (Tan *et al.,* 2004) showed cytotoxicity *in vitro* against human colon carcinoma, Orevactaene displayed inhibitory activity against HIV replication (Shu *et al.,* 1997); similar effects on HIV replication exhibited by epicoccins G-H and diphenylalazines A (Guo *et al.,* 2009). Epicoccamide A (Wright *et al.,* 2003) and Epicoccamide B-D (Wangun *et al.,* 2007), exhibited weak to moderate cytotoxicity to human leukemia cell lines (K-562). Epicoccalone was described as a new inhibitor of the serine protease (Wangun *et al.,* 2008), and the Telomerse inhibitor, D8646-2-6 (Kanai *et al.,* 2007). Novel compounds and fluorescent pigments, useful in biotechnological applications, also were isolated and characterized. Epicocconone is a fluorescent compound with a potential utility in cell tracking, flow cytometry, microscopy, or protein detection and multicolor staining applications (Bell and Karuso, 2003). It has been also used in protein staining in polyacrylamide gel and electroblots (Coghlan *et al.,* 2005), as natural non-toxic cell staining in living or fixed cells (Choi *et al.,* 2006) and it been used in real-time monitoring of protein digestion *in situ* (Karuso *et al.,* 2007). An intensive search for new and more effective agents to deal drug-resistant problem is under way and endophytes represent a novel source of potentially useful medicinal compounds (Strobel, 2003). *E. nigrum* isolates exhibited antifungal and antibacterial activity with variable sensitivity. The activity was only not dependent on the isolate genotype but also on the culture medium in which the isolate was grown.

HRMS-NMR spectroscopy

Characterizations of some fration from the fungal crude extract structure by NMR and HRMSThe NMR techniques evaluated by Dr. K. Wittstein at HZI, result proposed the sturcut of what was previously *E. nigrum* naphthopyran. Further NMR investigations are in pending to elucidate more metabolites from endophytic strains of *E. nigrum*, which could be novel antibiotic.

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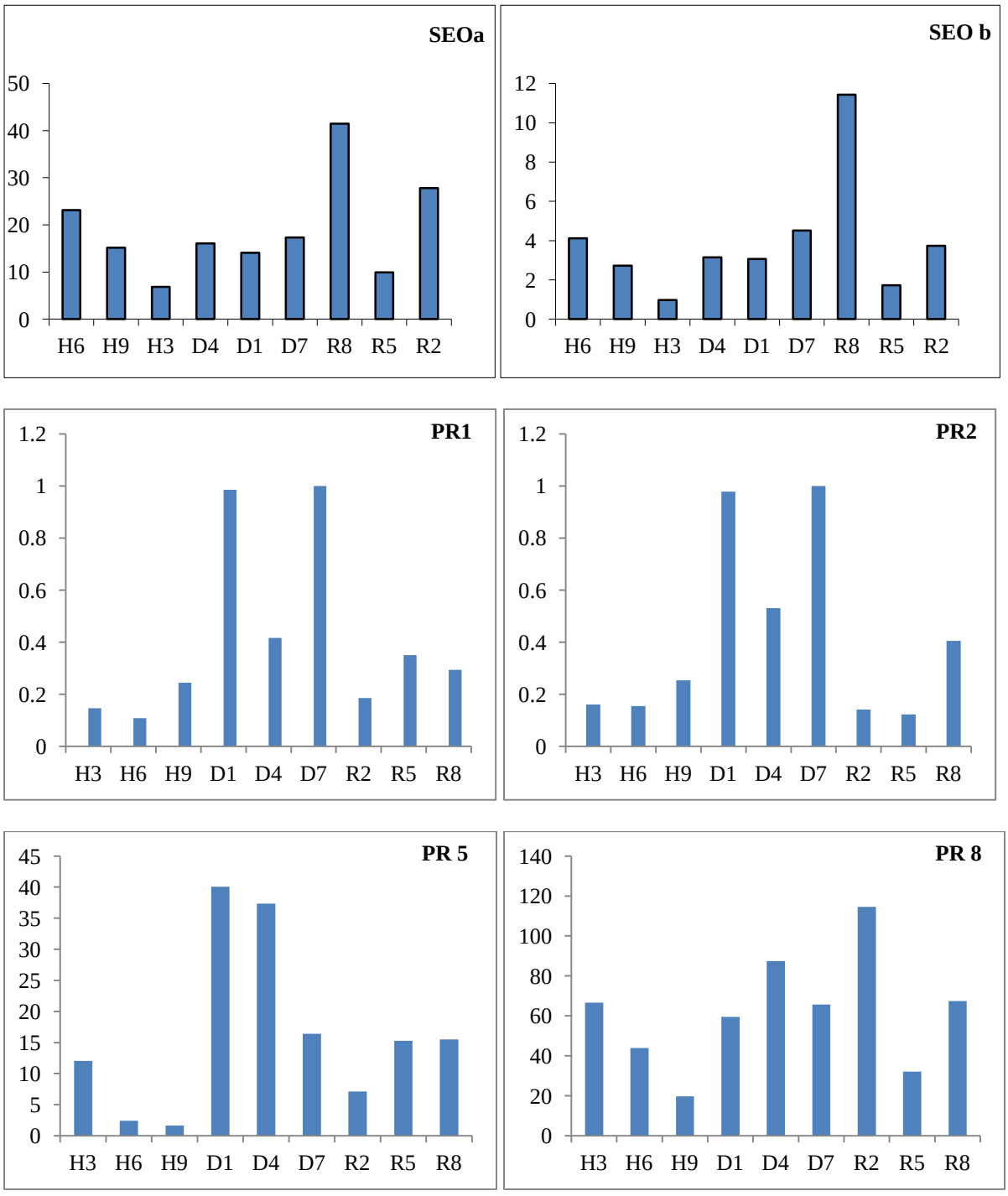
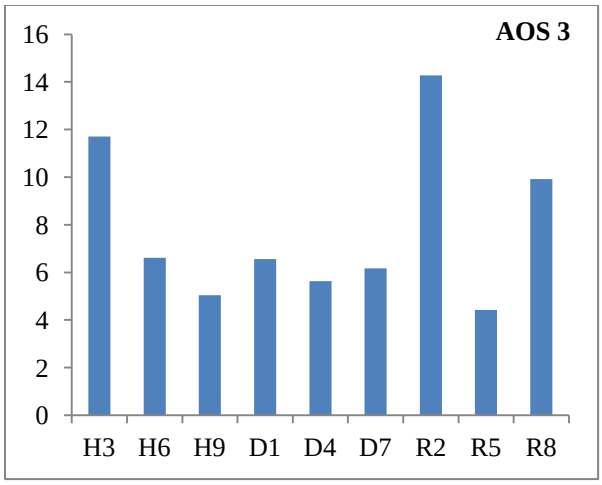
APPENDICES

App. 1: relative quantification by qRT-PCR of the expression levels of genes grups used in recovery study

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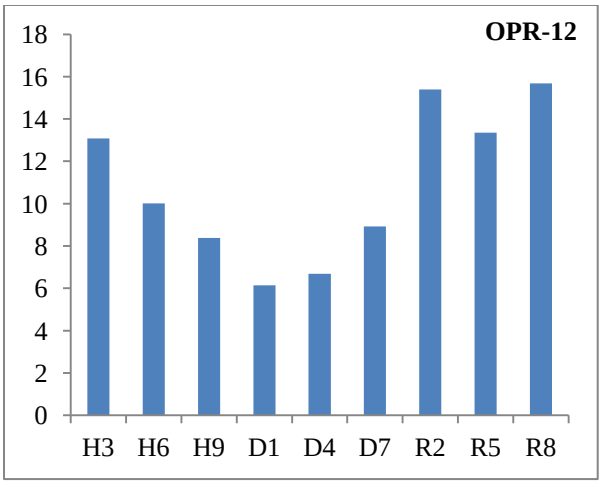
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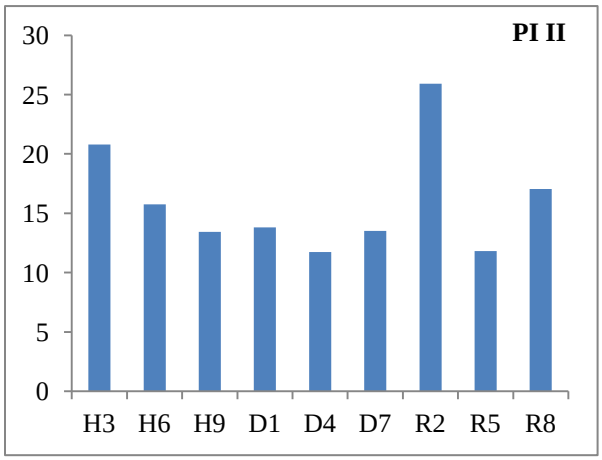
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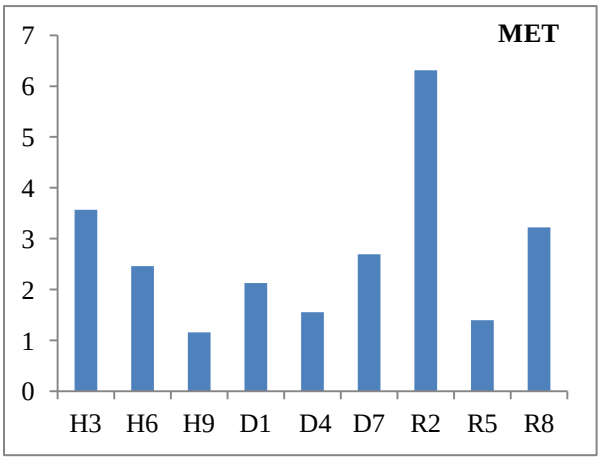
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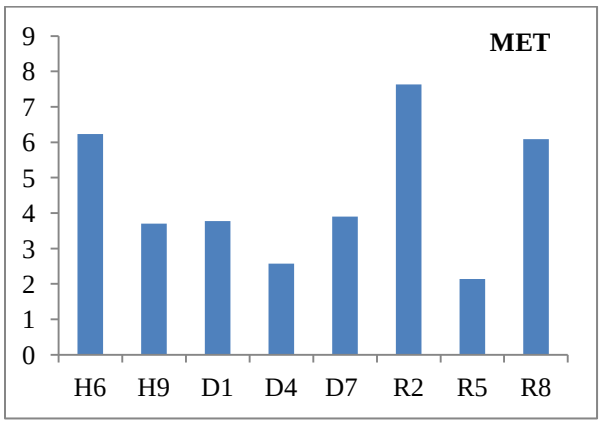
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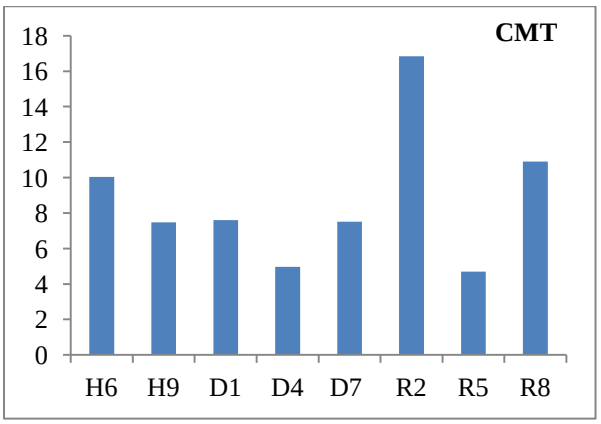
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14
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10
8
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2
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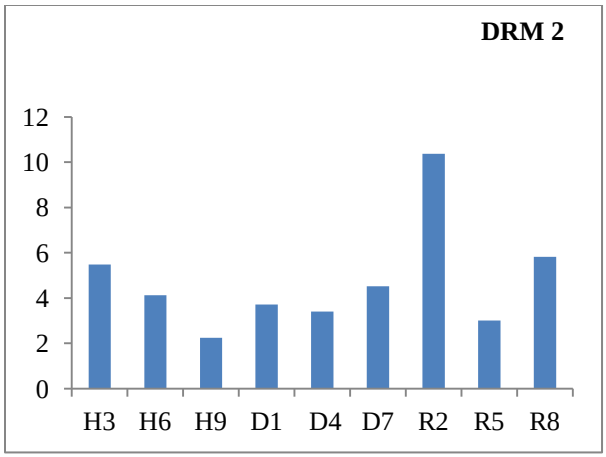
H6 H9 D1 D4 D7 R2 R5 R8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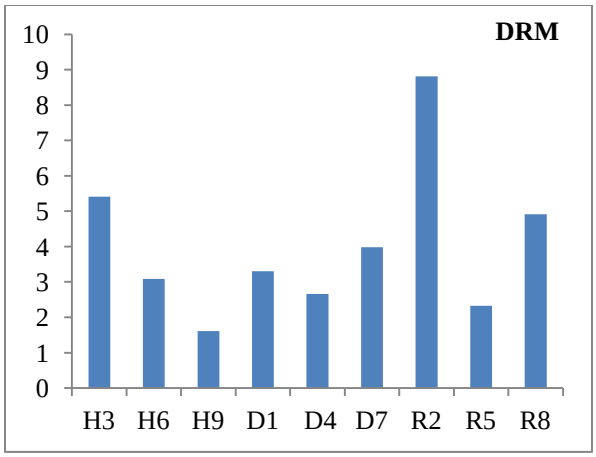
H3 H6 H9 D1 D4 D7 R2 R5 R8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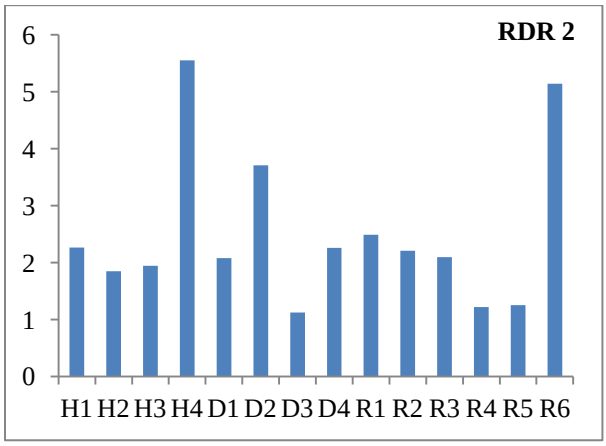
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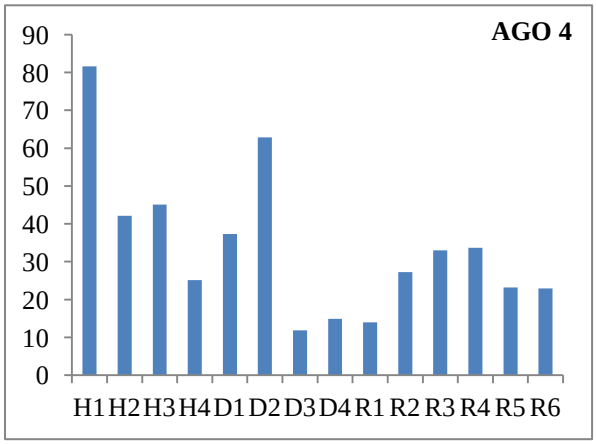
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80
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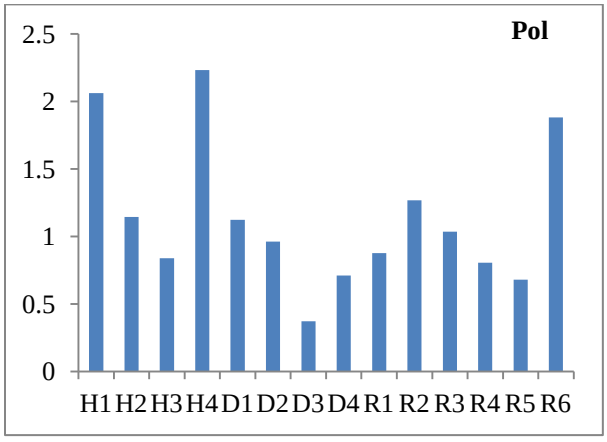
H1 H2 H3 H4 D1 D2 D3 D4 R1 R2 R3 R4 R5 R6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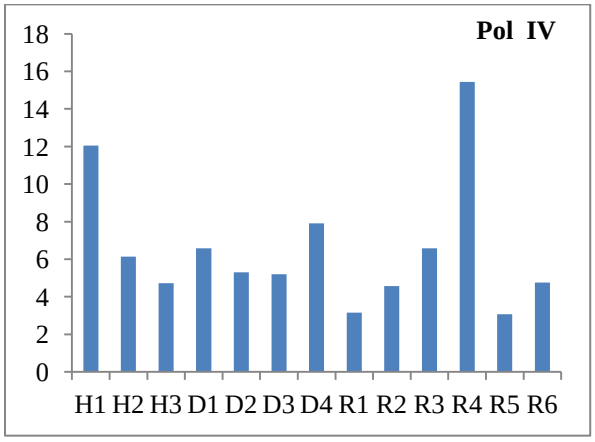
2
1.5
1
0.5
0
H1 H2 H3 H4 D1 D2 D3 D4 R1 R2 R3 R4 R5 R6


Pol IV
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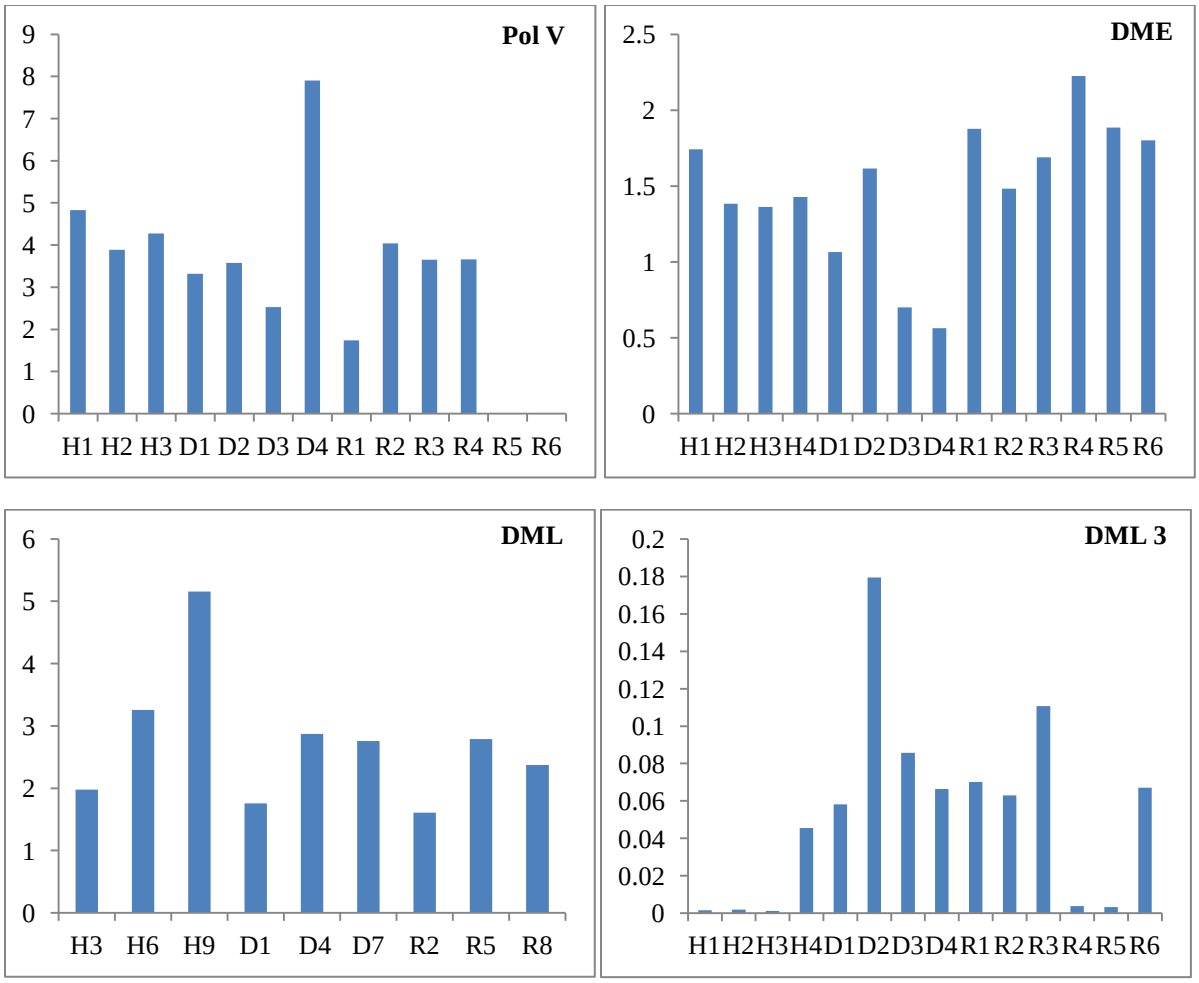
16
14
12
10
8
6
4
2
0
H1 H2 H3 D1 D2 D3 D4 R1 R2 R3 R4 R5 R6



Relative quantification by qRT-PCR of the expression levels of genes groups used in recovery study in healthy (H), diseased (D) and recovered (R) plants. cDNA samples (three biological replicas of at least three plants for each diseased, healthy and recovered trees) were tested in triplicate and normalized using the geometric average of the reference genes GAPDH (Genbank accession CN929227). Before normalization the raw Ct values were transformed into relative quantities.

App. 2: Comparison of nucleotide sequences of ITS region and the partial sequences of β-tubulin gene of *Epicoccum nigrum* obtained from this study

CLUSTAL 2.1 ITS region multiple sequence alignment

```
13066           ----------------GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 44
13067           -------------GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 47
13072           ---TGGTCCATTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 57
13059           ---------------GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 45
13062           TCTTGGTCCCTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 60
13073           ----------TTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 50
13063           --------CCTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 52
13068           -------------GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 47
13069           -----------TAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 49
13076           ---------CTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 51
13081           ----------TTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 50
13074           -----------TTGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 49
13071           ----------TTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAG 50
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13066           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 79
13067           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 82
13072           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 92
13059           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 80
13062           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 95
13073           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 85
13063           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 87
13068           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 82
13069           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 84
13076           GGATCATTACCTA----------GAGTTT---------------GTGGACTTCGGTCTGC 86
13081           GGATCATTACCTA----------GAGTT----------------GTAGGCTTTG-CCTGC 83
13074           GGATCATTACCTA----------GAGTCA---------------GTGGGCTTTG-CCTGC 83
13071           GGATCATTACACAATATGAAAGCGGGTTGGGACCTCACCTCGGTGAGGGCTCCAGCTTGT 110
                ********** *          * **                 *  * **      **

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13072           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 148
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13062           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 151
13073           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 141
13063           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 143
13068           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 138
13069           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 140
13076           T---ACCTCTTACCCATGTCTTTTGAGTACCTT-CGTTTCCTCGGCGGGTCCGCCCGCCG 142
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13074           T---ATCTCTTACCCATGTTTTATGAGCAC-TTACGTTTCCTCGGTGGGTTCGCCCACCG 139
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13072          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 203
13059          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 191
13062          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 206
13073          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 196
13063          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 198
13068          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 193
13069          ATTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACATAAT 195
13076          GTTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACTTAAT 197
13081          ATTGGACAA--TT-TAAACCATTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACTTAAT 193
13074          ATTGGACCA--CT-TAAACCCTTT-GCAGTTTTGTAATCAGCGTCTGAAAA-A-CTTAAT 193
13071          CCAGGACCAAACCATAAACCTTTTTGTAATTT--GCAATCAGCGTCAGTAAACAATGTAAT 228
                  **** *      ***** *** * * **  * ********** * *** *   ****


13066          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 250
13067          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 253
13072          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 263
13059          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 251
13062          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 266
13073          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 256
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13074          GCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGC 373
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13072          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 442
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13063          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 437
13068          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 432
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13074          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 432
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13062          GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 556
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13071          ATCCATCAAGACCACATTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTG 582
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13067          AACTTAAGCATATCAATA----------- 561
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13069          AACTTAAGCATATCA-------------- 560
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13074          AACTTAAGCATATCCATA----------- 561
13071          AACTTAAGCATAT---------------- 595
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CLUSTAL 2.1 β-tubulin gene multiple sequence alignment

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13073           ----------TTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 50
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13072           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 92
13059           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 80
13062           GGATCATTACCTA----------GAGTTT---------------GTAGACTTCGGTCTGC 95
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13076           GGATCATTACCTA----------GAGTTT---------------GTGGACTTCGGTCTGC 86
13081           GGATCATTACCTA----------GAGTT----------------GTAGGCTTTG-CCTGC 83
13074           GGATCATTACCTA----------GAGTCA---------------GTGGGCTTTG-CCTGC 83
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13074           T---ATCTCTTACCCATGTTTTATGAGCAC-TTACGTTTCCTCGGTGGGTTCGCCCACCG 139
13071           CTGAATTATTCACCCATGTCTTTTGCGCACTTCTTGTTTCCTGGGCGGGTTCGCCCGCCA 170
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13076           GTTGGACAACATT-CAAACCCTTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACTTAAT 197
13081           ATTGGACAA--TT-TAAACCATTT-GCAGTT--GCAATCAGCGTCTGAAAA-AACTTAAT 193
13074           ATTGGACCA--CT-TAAACCCTTT-GCAGTTTTGTAATCAGCGTCTGAAAA-A-CTTAAT 193
13071           CCAGGACCAAACCATAAACCTTTTTGTAATT--GCAATCAGCGTCAGTAAACAATGTAAT 228
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13067          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 253
13072          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 263
13059          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 251
13062          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 266
13073          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 256
13063          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 258
13068          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 253
13069          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 255
13076          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 257
13081          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 253
13074          AGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 253
13071          TATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT 288
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13067          GCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC 313
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13062          GCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC 326
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13063          GCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC 318
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13071          GCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC 348
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13066          GCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGC 370
13067          GCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGC 373
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13081          GCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGC 373
13074          GCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGC 373
13071          GCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGC 408
               **** ***********  ****************************** ******* ***

13066          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 429
13067          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 432
13072          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 442
13059          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 430
13062          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 445
13073          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 435
13063          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 437
13068          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 432
13069          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 434
13076          TTGGTGTTGGGTGTTTTGTCTCGCCTCCGCGCGCAGACTCGCCTTAAAACAATTGGCAGC 437
13081          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTCAAAACAATTGGCAGC 432
13074          TTGGTGTTGGGTGTTT-GTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGC 432
13071          TTGGTGTTGGGCGTCT----TTGTCTCT-CACG-AGACTCGCCTTAAAATGATTGGCAGC 462
               ********** ** *    * * ***  *  * ********** ****  *********
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13066           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 485
13067           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 488
13072           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 498
13059           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 486
13062           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 501
13073           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 491
13063           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 493
13068           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 488
13069           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 490
13076           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 493
13081           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 488
13074           CGGCGTATTGATTTCGGAGCGCAGTACA--TCTCGCGCTTTGCACTCATAACGAC--GAC 488
13071           CGACCTACTGGTTTCGGAGCGCAGCACAATTCTTGCACTTTGAATCAGCCTTGGTTGAGC 522
                ** * ** ** ************* ***  *** ** ***** *        *      *

13066           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 540
13067           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 543
13072           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 553
13059           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 541
13062           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 556
13073           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 546
13063           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 548
13068           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 543
13069           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 545
13076           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 548
13081           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 543
13074           GTCCA---AAAGTACATTTTTAC--ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTG 543
13071           ATCCATCAAGACCACATTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTG 582
                 ****   * *  ********    *** ********************************

13066           AACTTAAGCATATCAA------------- 556
13067           AACTTAAGCATATCAATA----------- 561
13072           AACTTAAGCATATCAATAGG--------- 573
13059           AACTTAAGCATATCA-------------- 556
13062           AACTTAAGCATATCGCATATC-------- 577
13073           AACTTAAGCATATC---------------- 560
13063           AACTTAAGCATATCA-------------- 563
13068           AACTTAAGCATATCA-------------- 558
13069           AACTTAAGCATATCA-------------- 560
13076           AACTTAAGCATATCA-------------- 563
13081           AACTTAAGCATATCAATAGGCCGGAGGAA 572
13074           AACTTAAGCATATCCATA----------- 561
13071           AACTTAAGCATAT----------------- 595
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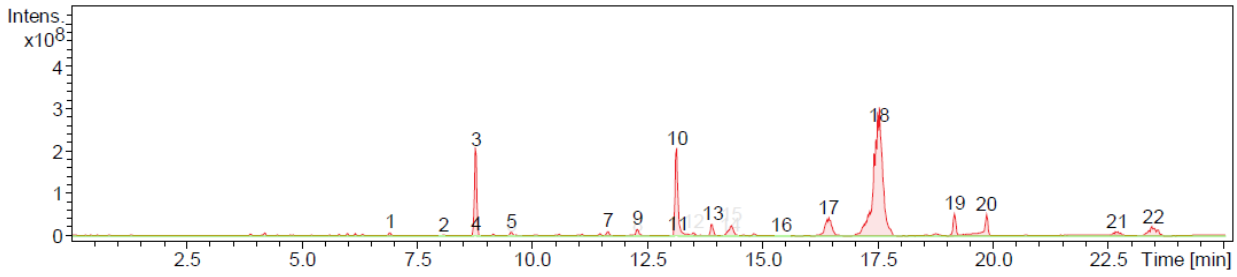
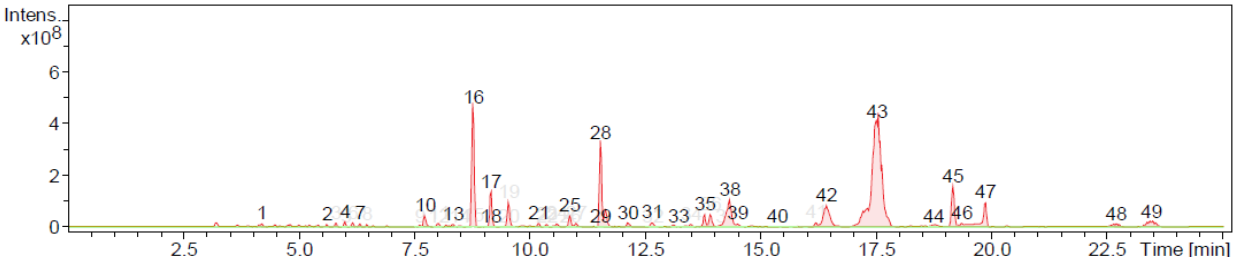
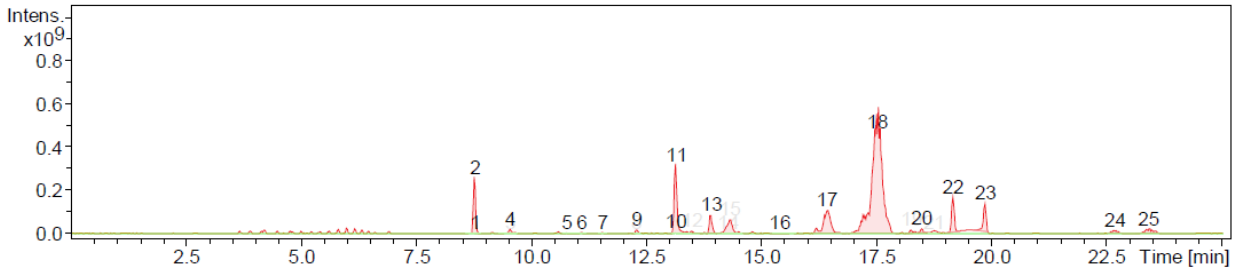
App. 3: The chromatograms of secondary metabolite profiles of *Epicoccum nigrum* isolates grown in submerged culture



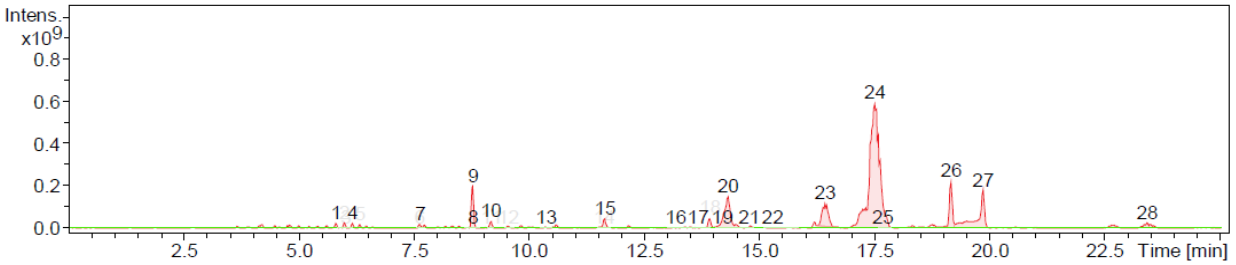
Metabolite profile of the Isolate 13059 grown in YM media



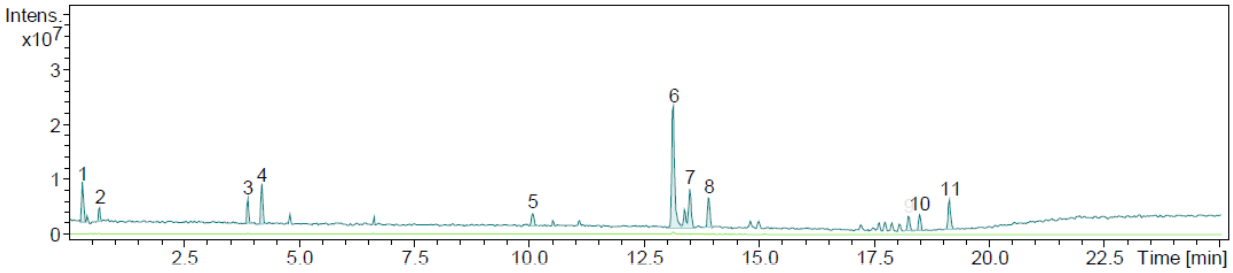
Metabolite profile of the Isolate 13059 grown in ZM



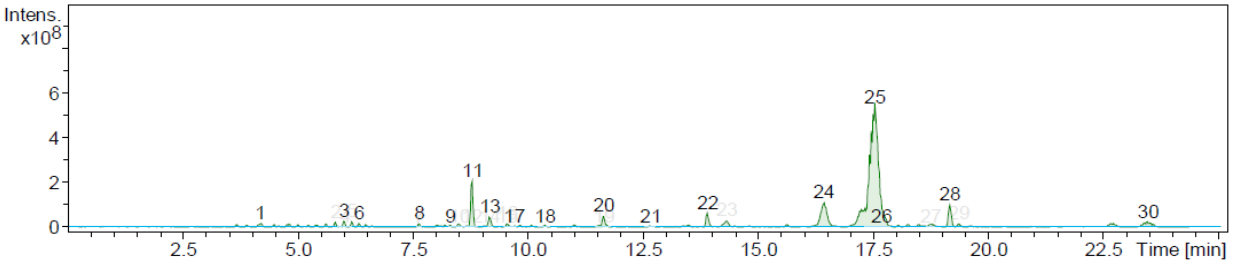
Metabolite profile of the Isolate 13062 grown in YM



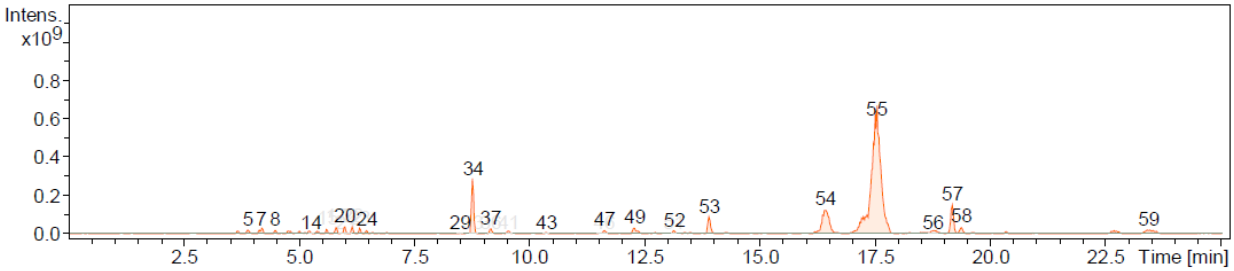
Metabolite profile of the Isolate 13062 grown in ZM ½



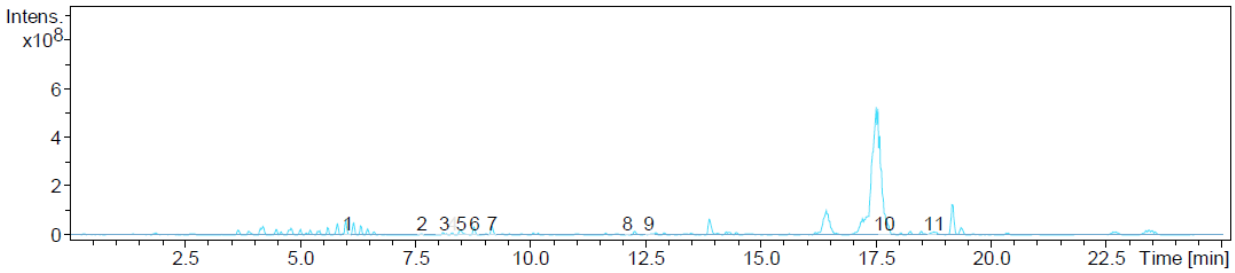
Metabolite profile of the Isolate 13063 grown in YM media



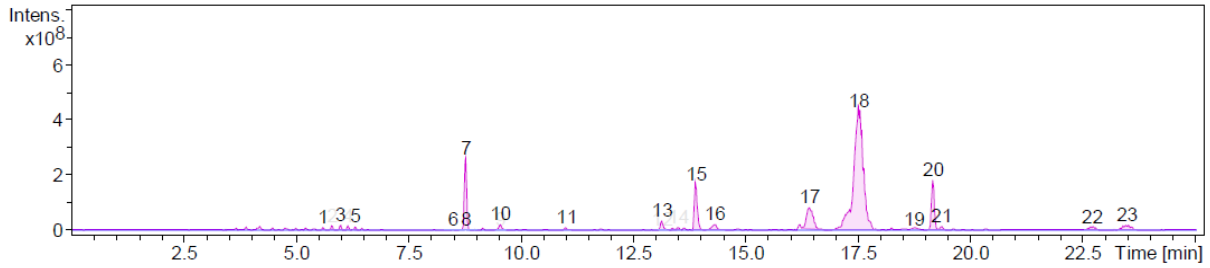
Metabolite profile of the Isolate 13063 grown in ZM ½ media



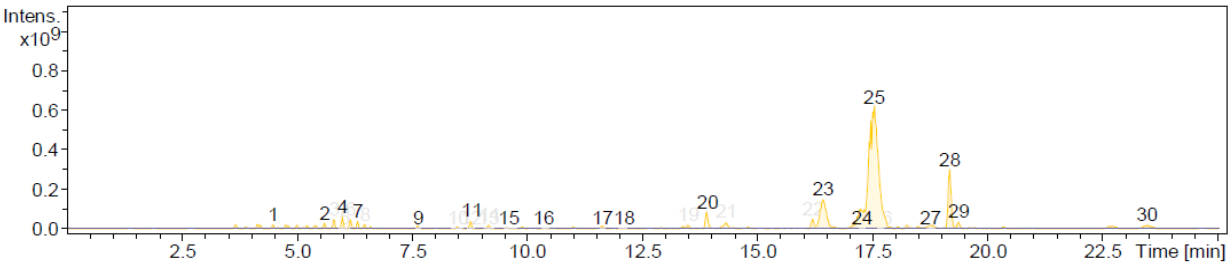
Metabolite profile of the Isolate 13066 grown in YM



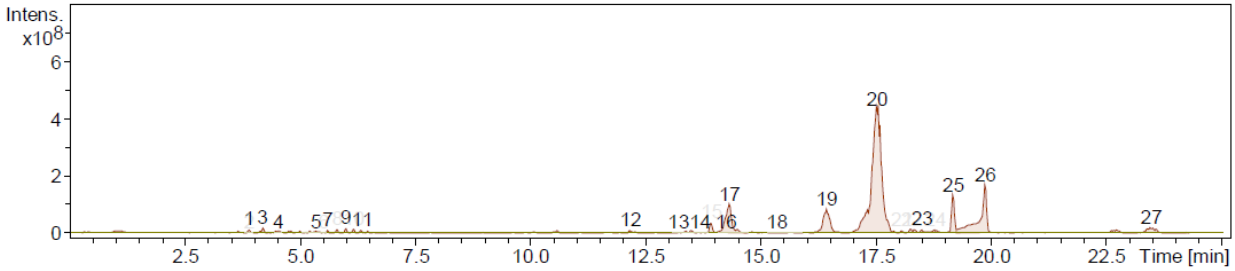
Metabolite profile of the Isolate 13066 grown in ZM



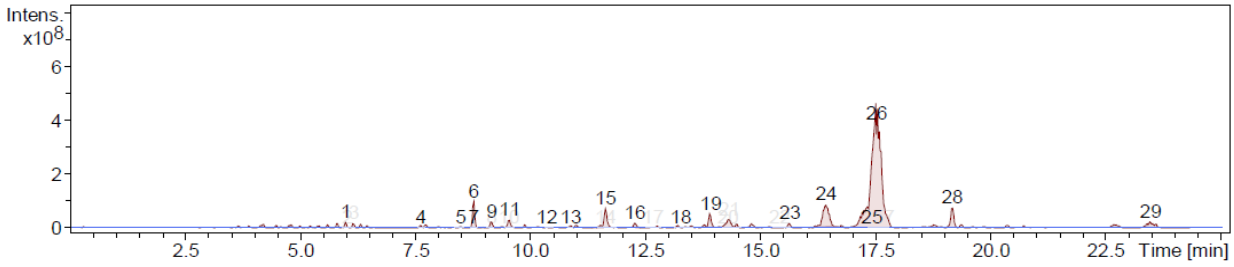
Metabolite profile of the Isolate 13067 grown in YM



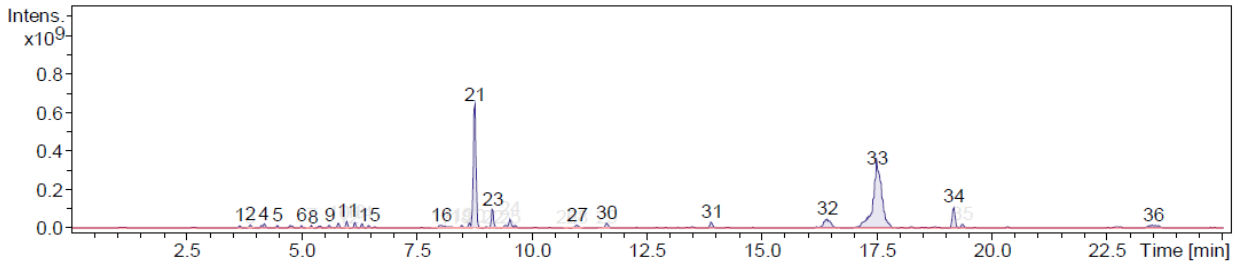
Metabolite profile of the Isolate 13067 grown in ZM ½



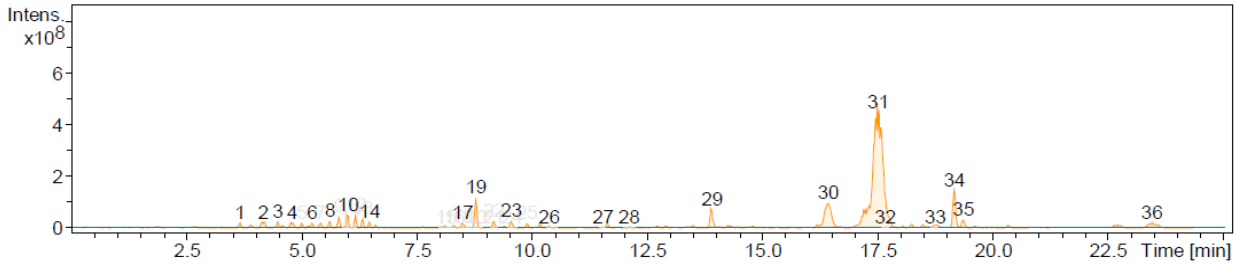
Metabolite profile of the Isolate 13068 grown in YM



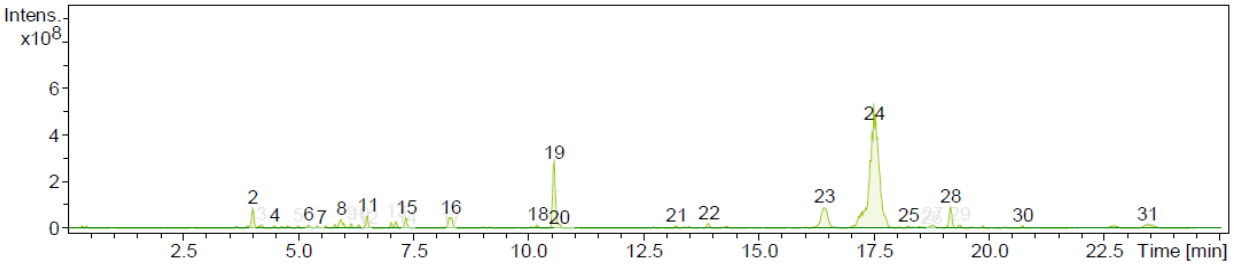
Metabolite profile of the Isolate 13068 grown in ZM ½



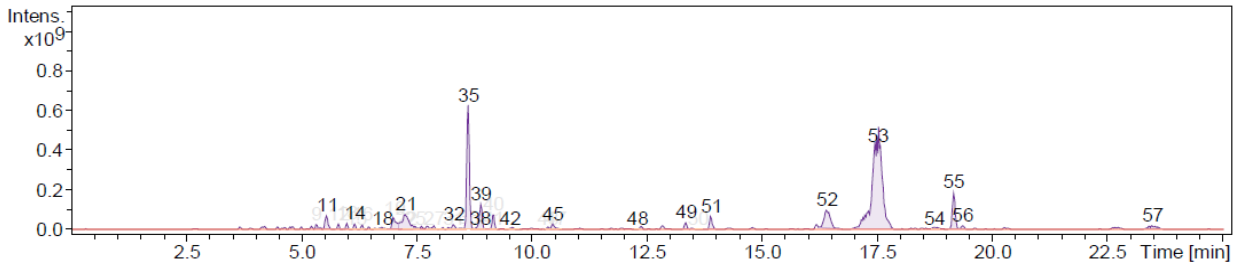
Metabolite profile of the Isolate 13069 grown in YM media



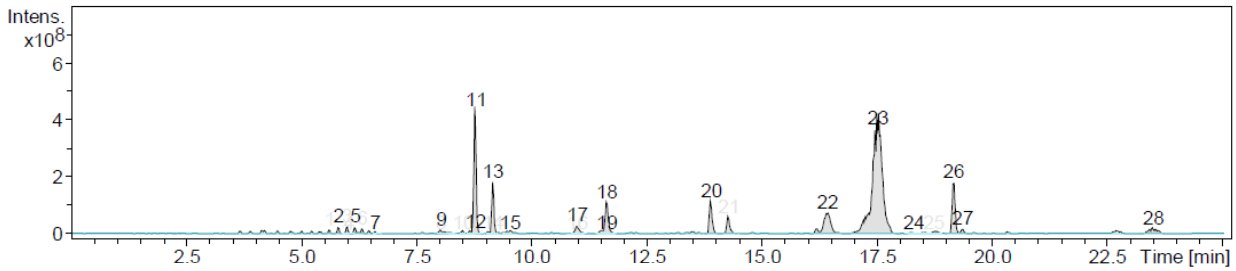
Metabolite profile of the Isolate 13069 grown in ZM ½ media



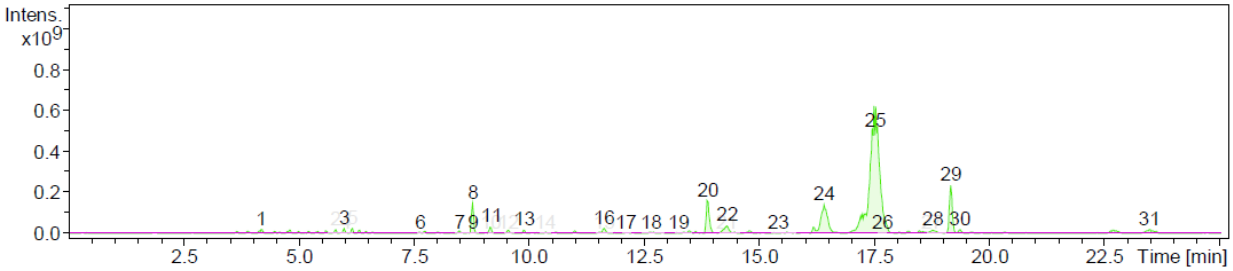
Metabolite profile of the Isolate 13071 grown in YM



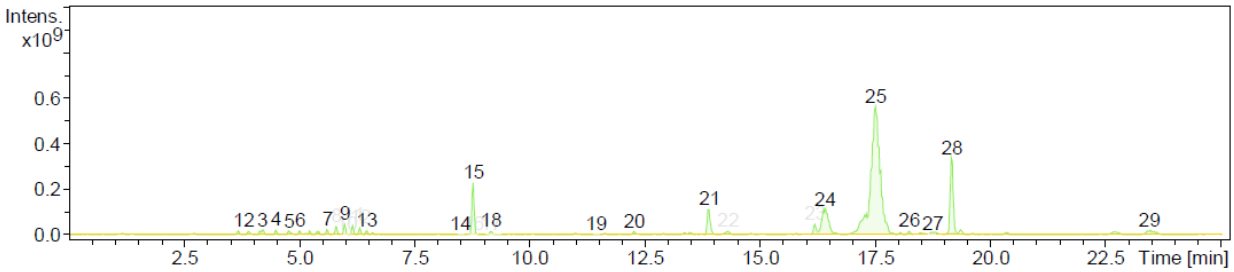
Metabolite profile of the Isolate 13071 grown in ZM ½ media



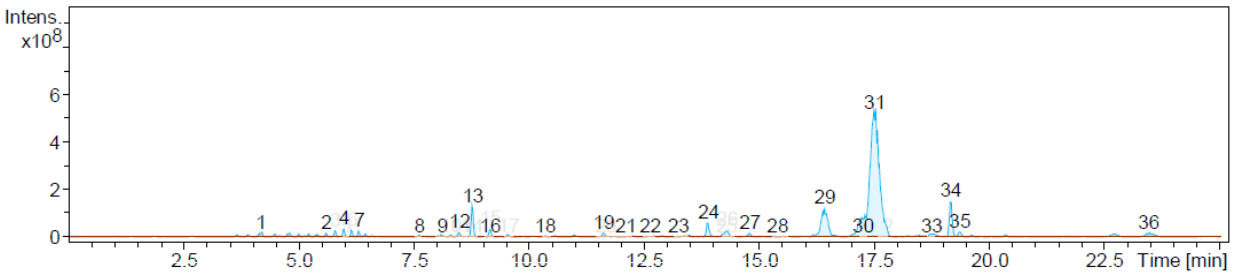
Metabolite profile of the Isolate 13072 grown in YM media



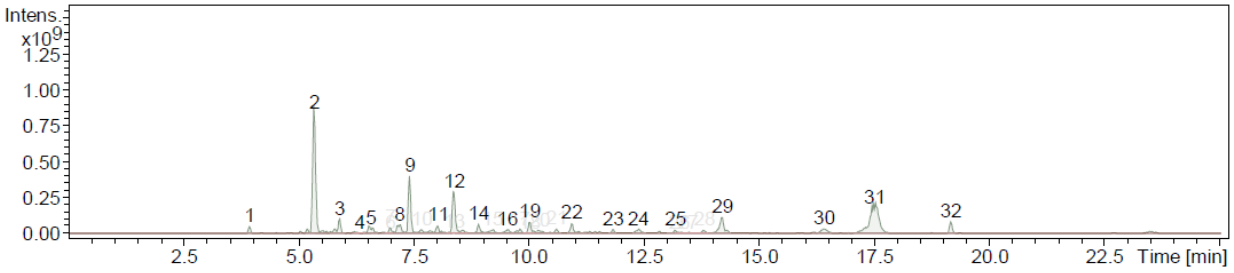
Metabolite profile of the Isolate 13072 grown in ZM ½



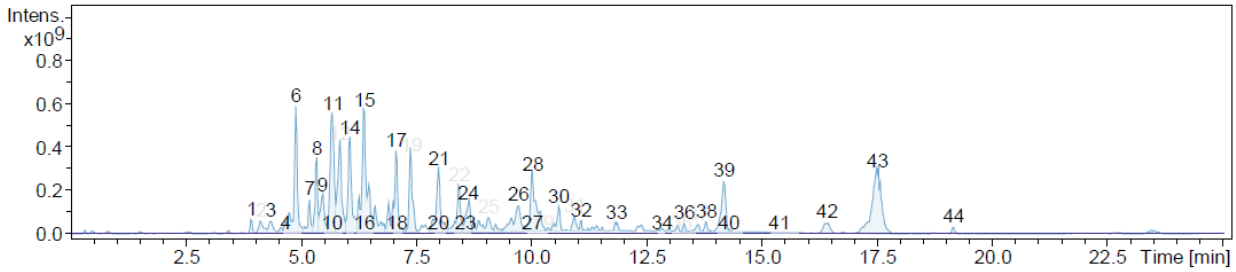
Metabolite profile of the Isolate 13073 grown in YM media



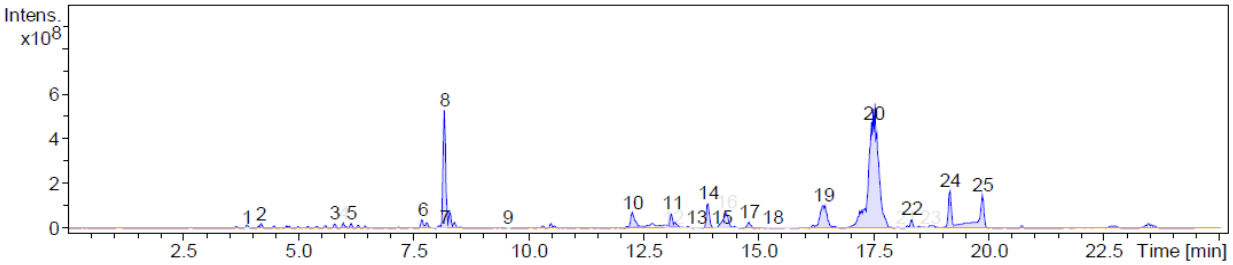
Metabolite profile of the Isolate 13073 grown in ZM ½



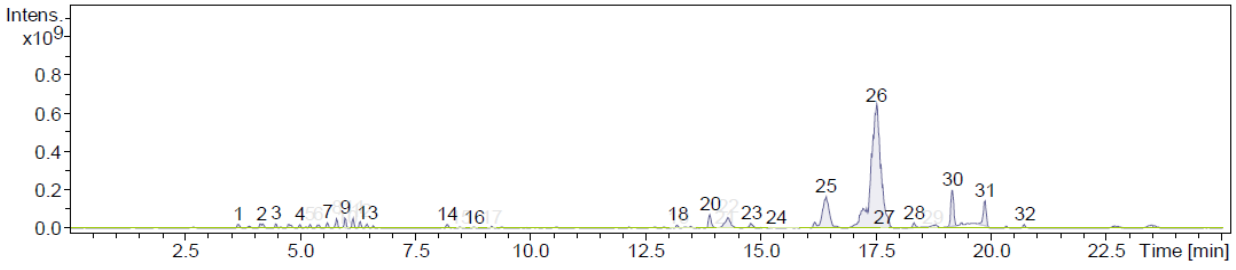
Metabolite profile of the Isolate 13074 grown in YM



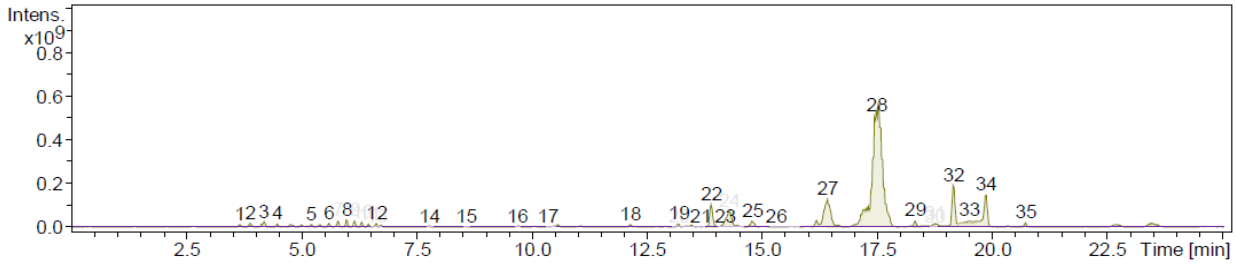
Metabolite profile of the Isolate 13074 grown in ZM ½ media



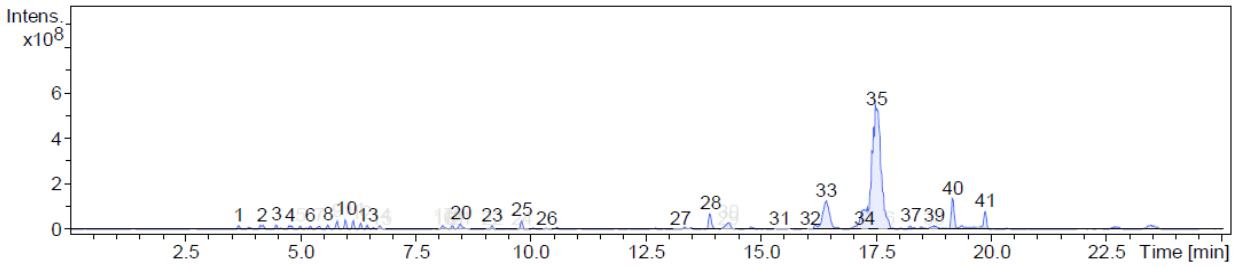
Metabolite profile of the Isolate 13076 grown in YM media



Metabolite profile of the Isolate 13076 grown ZM ½



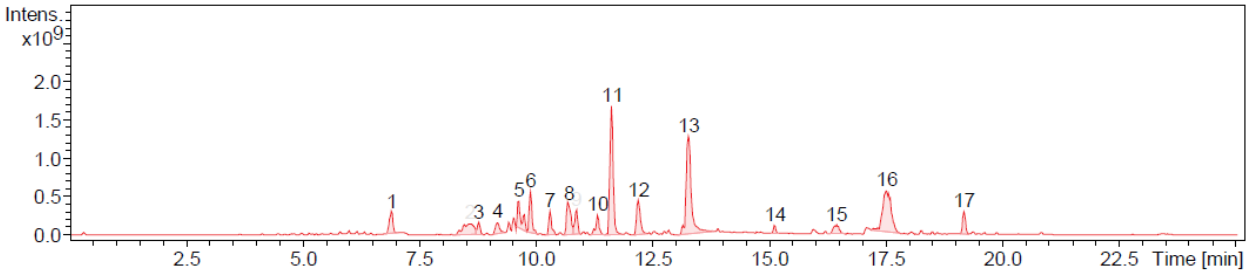
Metabolite profile of the Isolate 13081 grown YM media



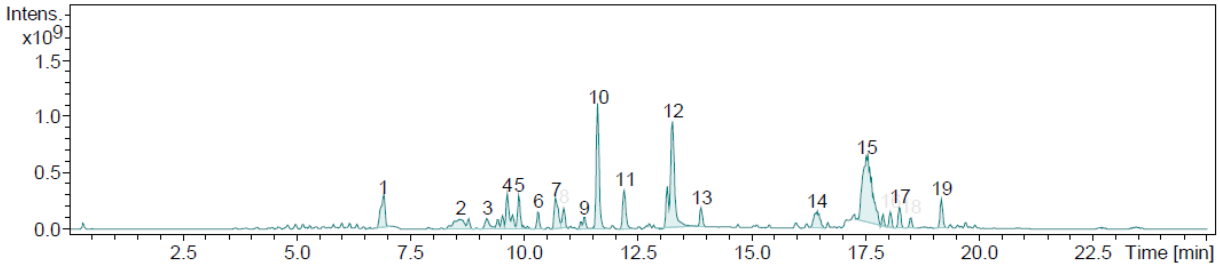
Metabolite profile of the Isolate 13081 grown ZM ½ media

The chromatograms of Secondary metabolite profiles of *Epicoccum nigrum* isolates gorwn in submerged culture, detected by HPLC/DAD-MS (Column: 50×2.1 mm, Acquity UPLC BEH C-18, 1.7 mm (Waters); Solvent A: H_2O with 0.1% formic acid; Solvent B: acetonitrile with 0.1% formic acid flow rate = 0.6 mL/min; UV detection 200-500 nm) Isolate name and medium type are listed underneath.

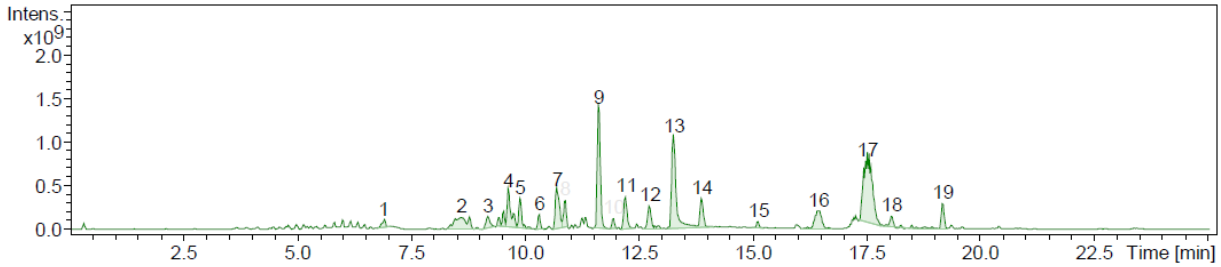
App. 4: The chromatograms of secondary metabolite profiles of *Epicoccum nigrum* isolates grown on solid-plate cultures



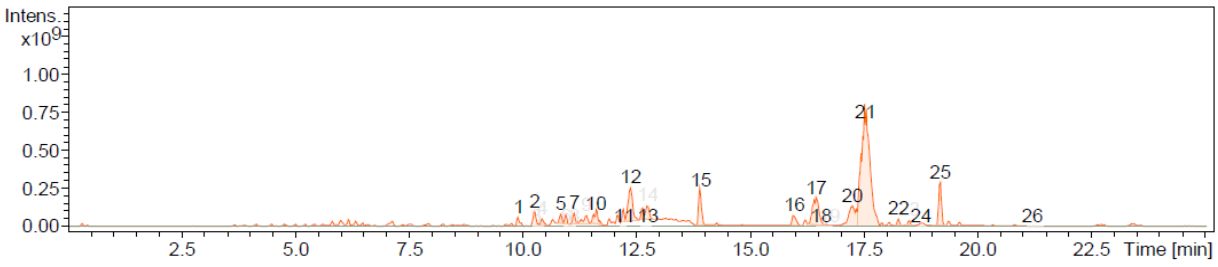
13059 Isolate metabolite profile



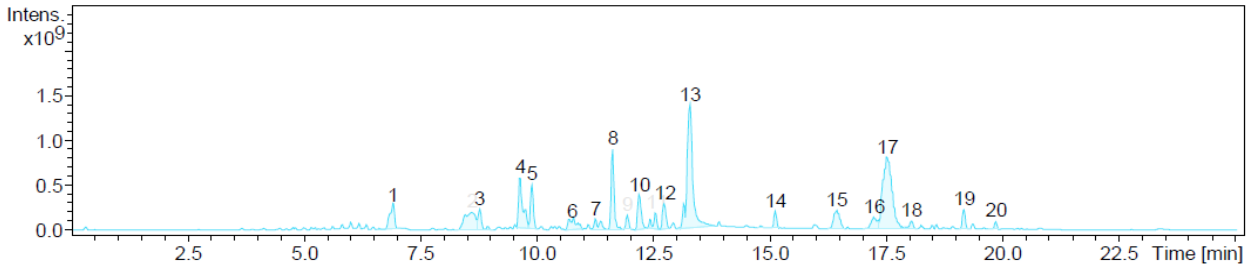
13062 Isolate metabolite profile



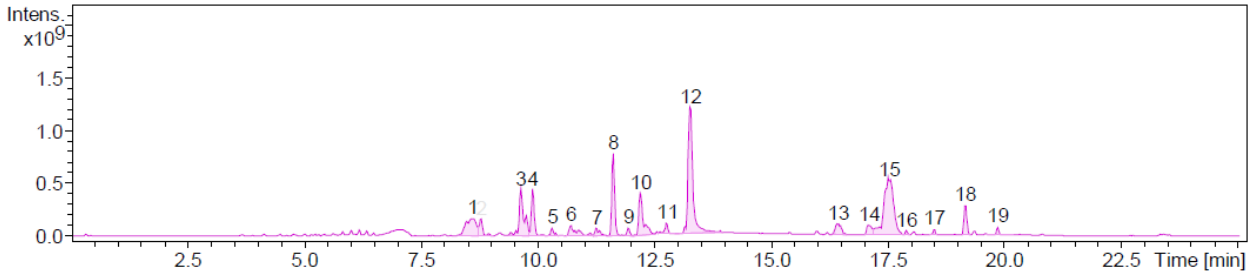
13063 Isolate metabolite profile



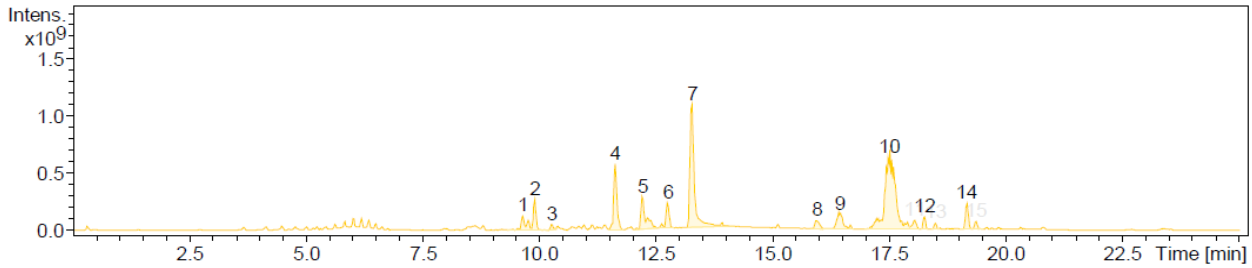
13066 Isolate metabolite profile



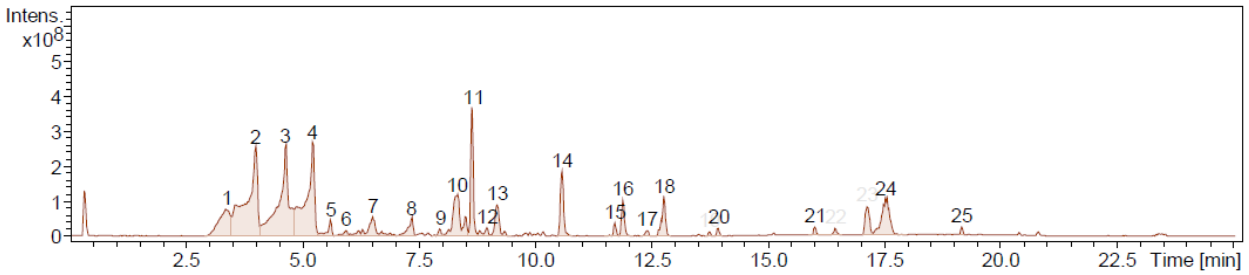
13067 Isolate metabolite profile



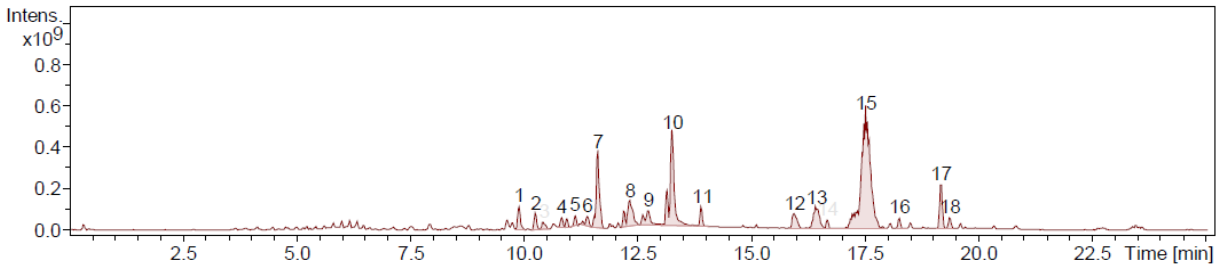
13068 Isolate metabolite profile



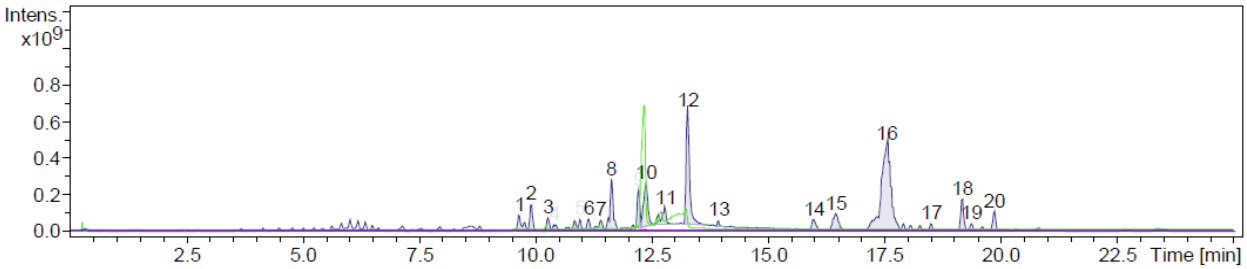
13069Isolate metabolite



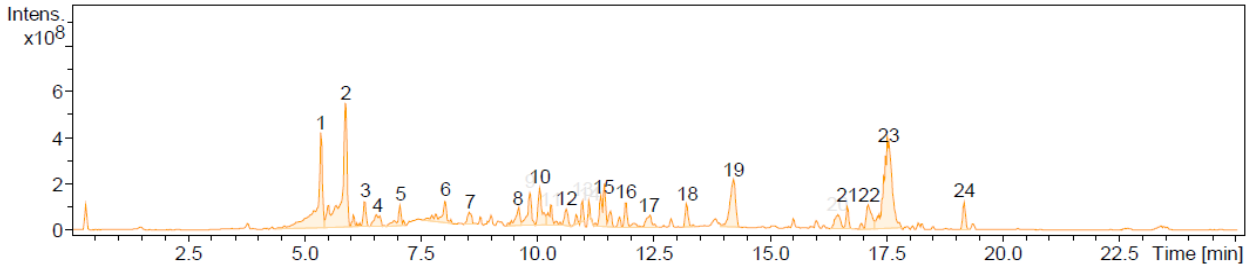
13071 Isolate metabolite profile



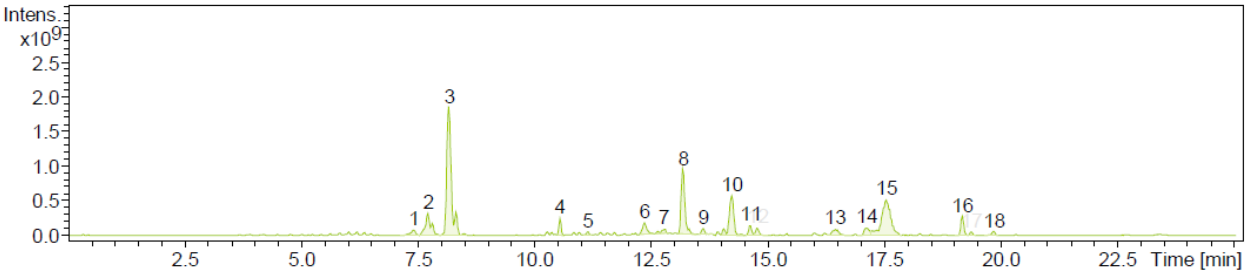
13072 Isolate metabolite profile



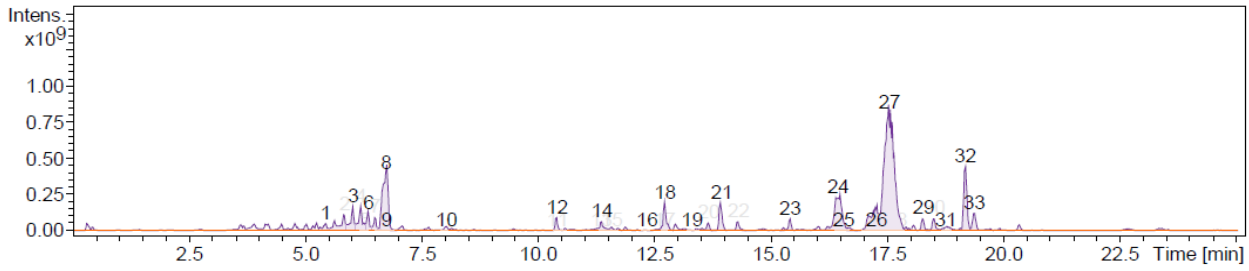
13073 Isolate metabolite profile



13074 Isolate metabolite profile



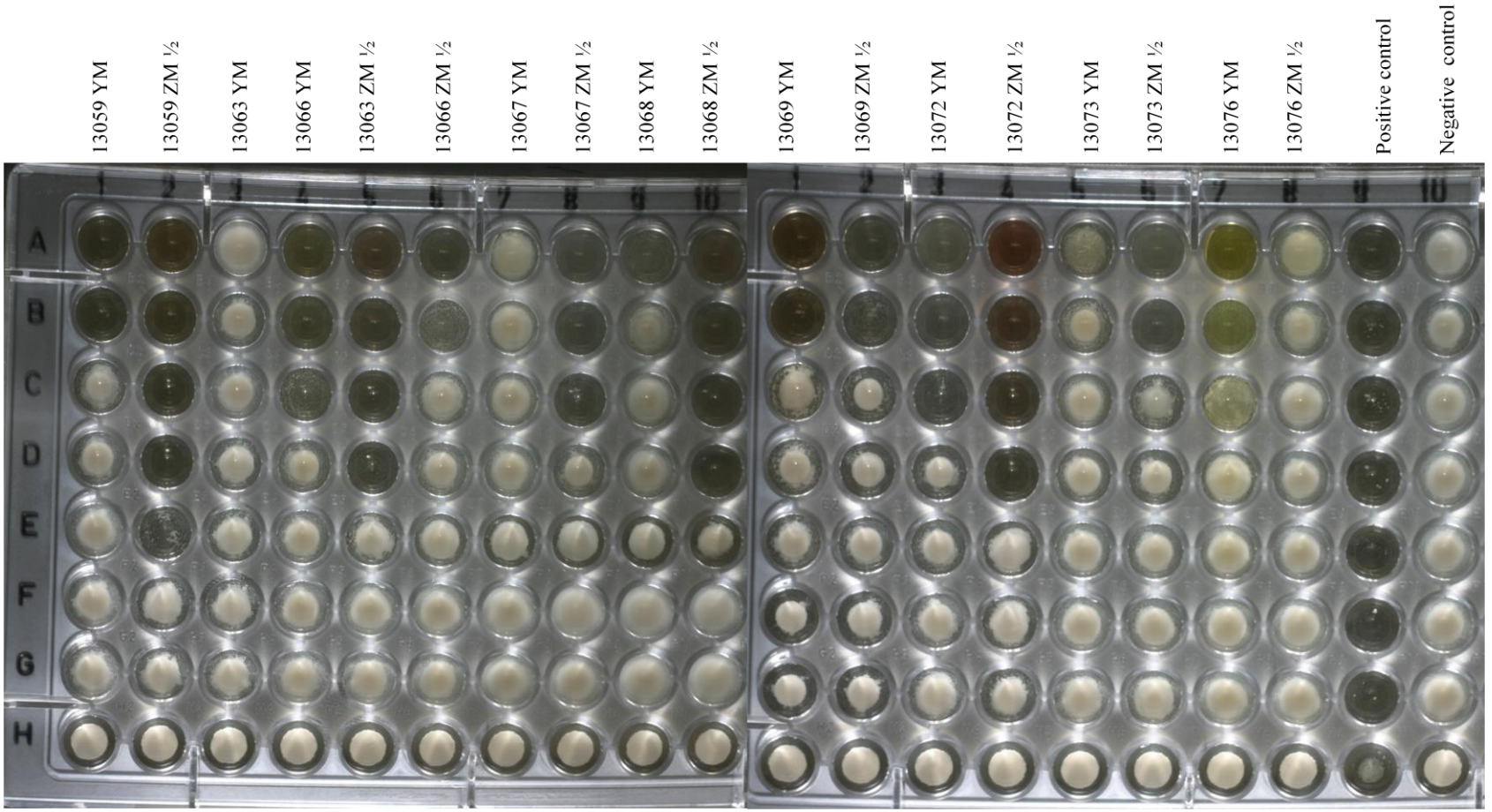
13076 Isolate metabolite profile

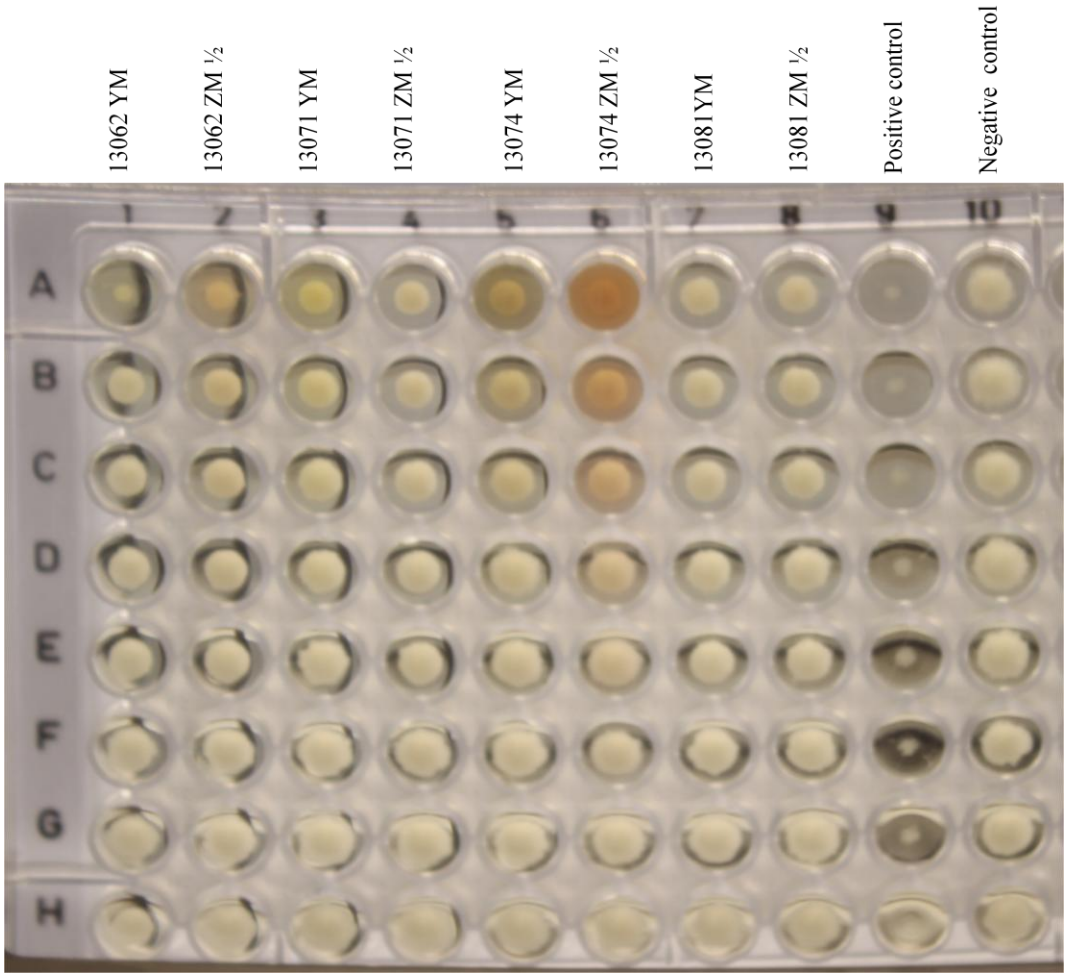


13081 Isolate metabolite profile

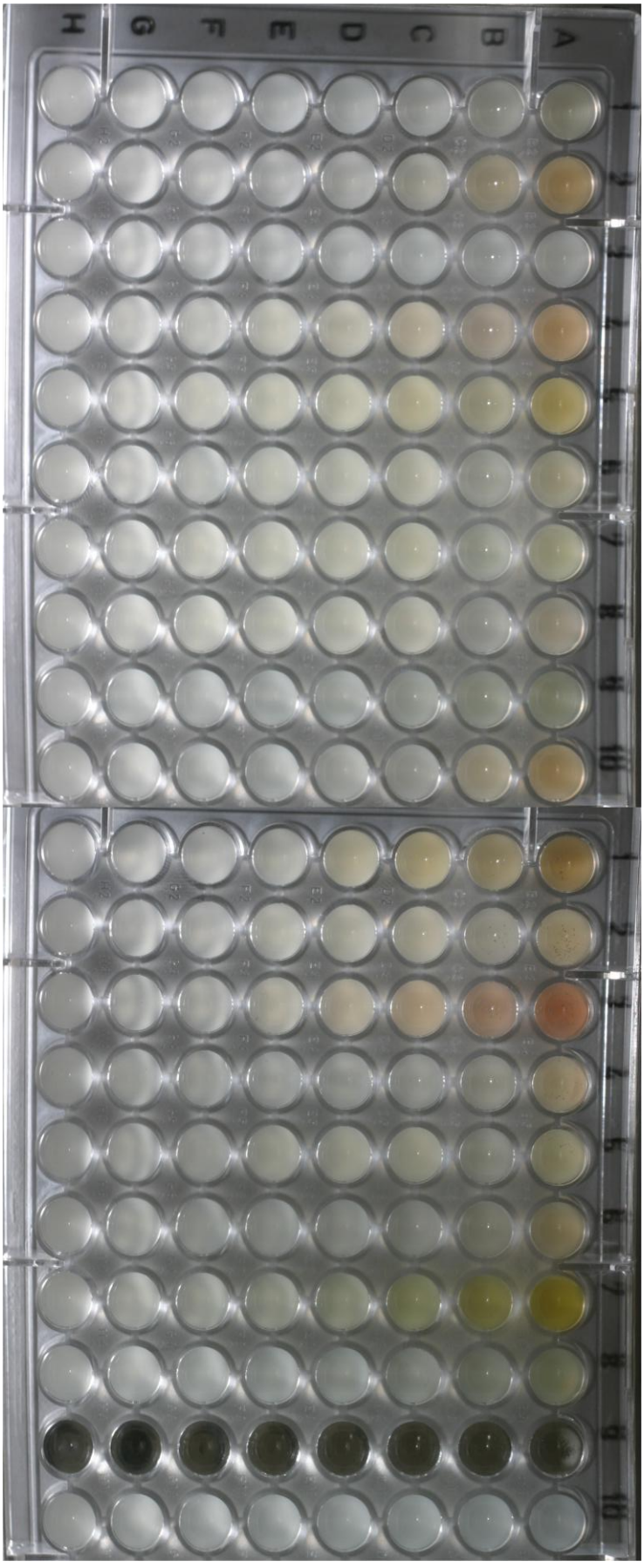
The chromatograms of Secondary metabolite profiles of *Epicoccum nigrum* isolates gown on Solid-plate cultures detected by HPLC/DAD-MS (Column: 50×2.1 mm, Acquity UPLC BEH C-18, 1.7 mm (Waters)a; Solvent A: H_2O with 0.1% formic acid; Solvent B: acetonitrile with 0.1% formic acid flow rate = 0.6 mL/min; UV detection 200-500 nm) Isolate name is listed underneath.

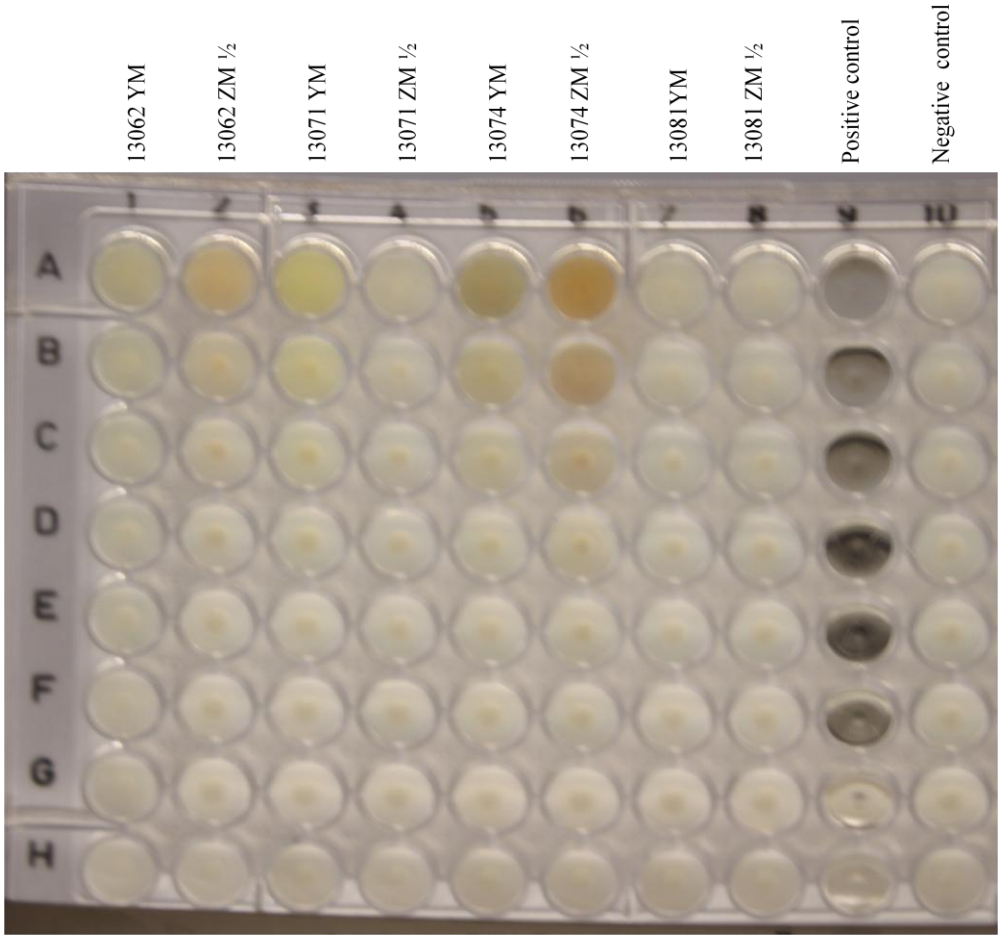
App. 5: Serial dilution assay microtitre plates



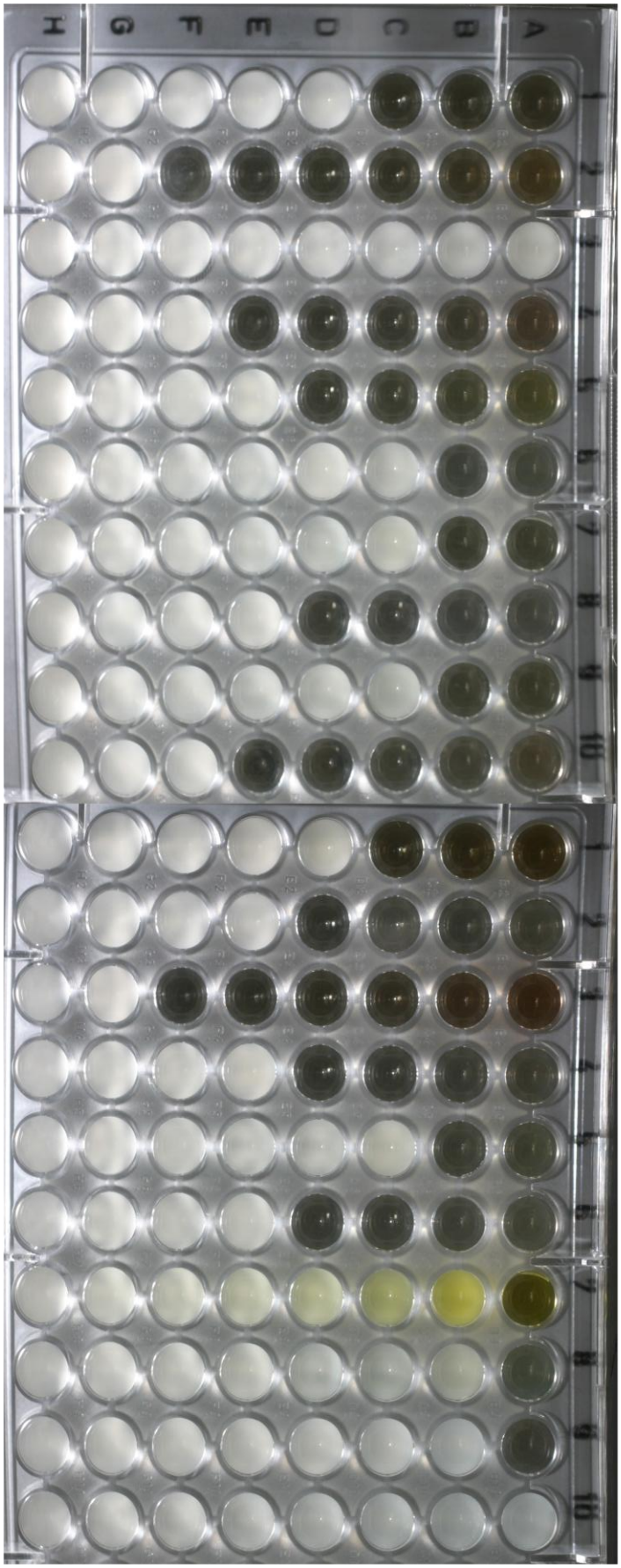


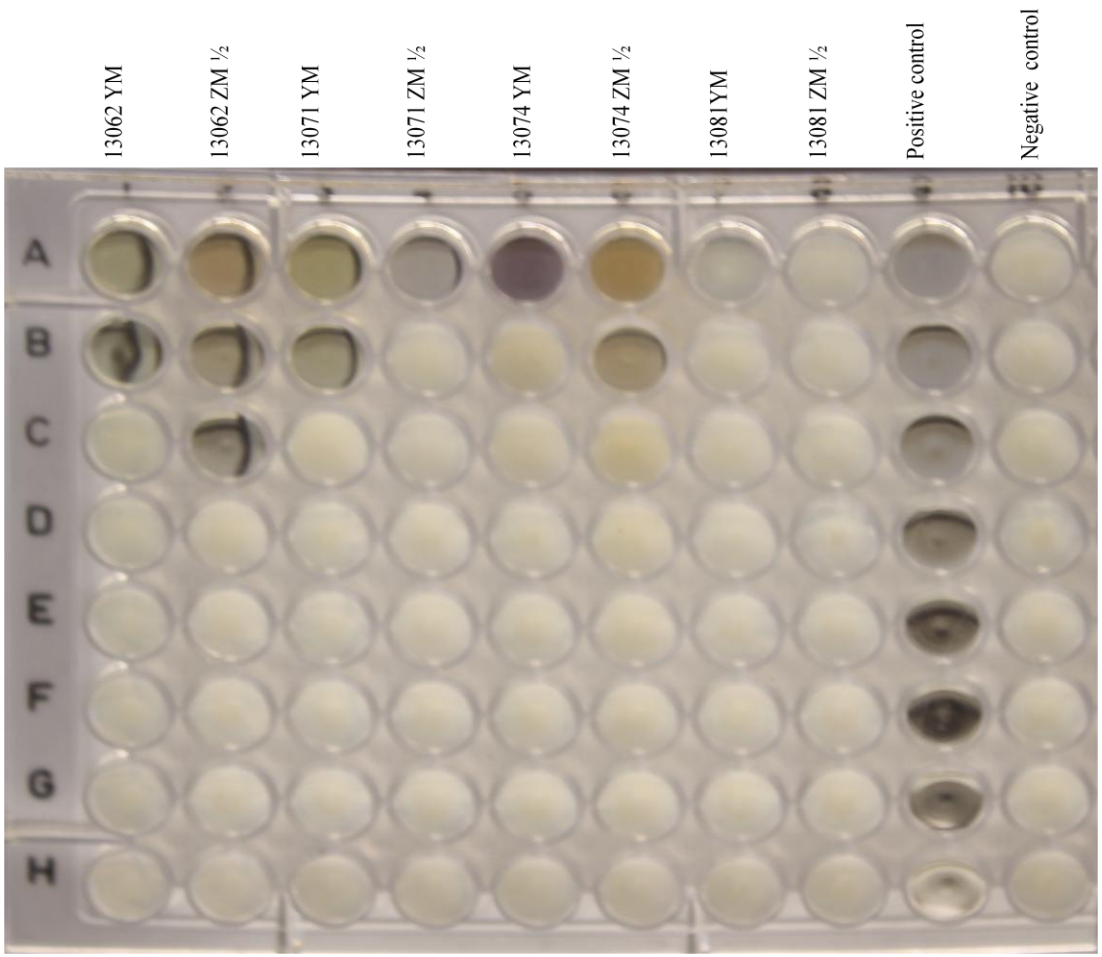
Microtiter plates result of the serial dilution assay of crud extract of *E. nigrum* isolates against *saccharomyces cerevisiae* after 24 hours of incubation. Turbid indicate to intensive yeast growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.


H
G
F
E
D
C
B
A
13059 YM
13059 ZM ½
13063 YM
13063 ZM ½
13066 YM
13066 ZM ½
13067 YM
13067 ZM ½
13068 YM
13068 ZM ½
13069 YM
13069 ZM ½
13072 YM
13072 ZM ½
13073 YM
13073 ZM ½
13076 YM
13076 ZM ½
Positive control
Negative control

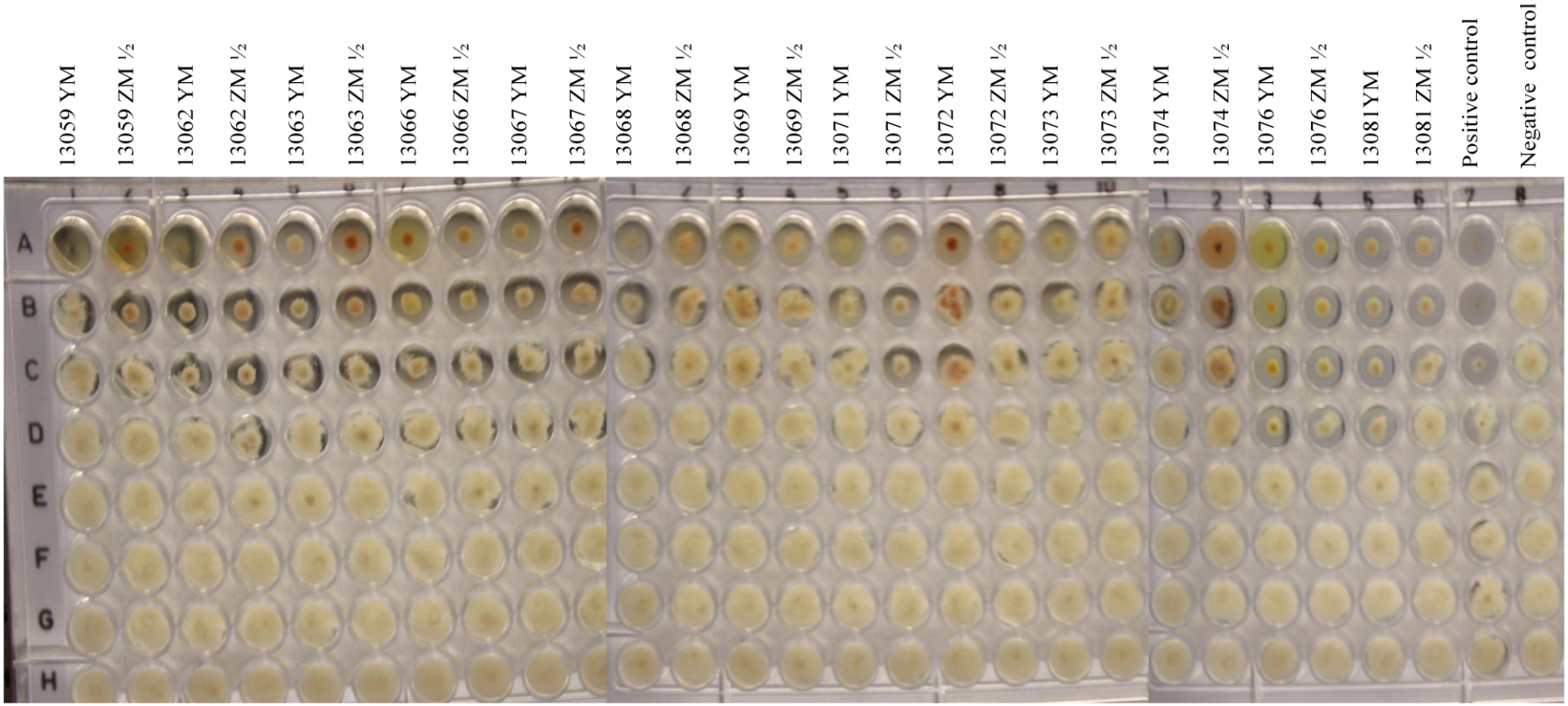


Microtiter plates result of the serial dilution assay of crud extract of *E. nigrum* isolates against *Escherichia coli*. after 24 hours of incubation. Turbid indicate to intensive bacterial growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.

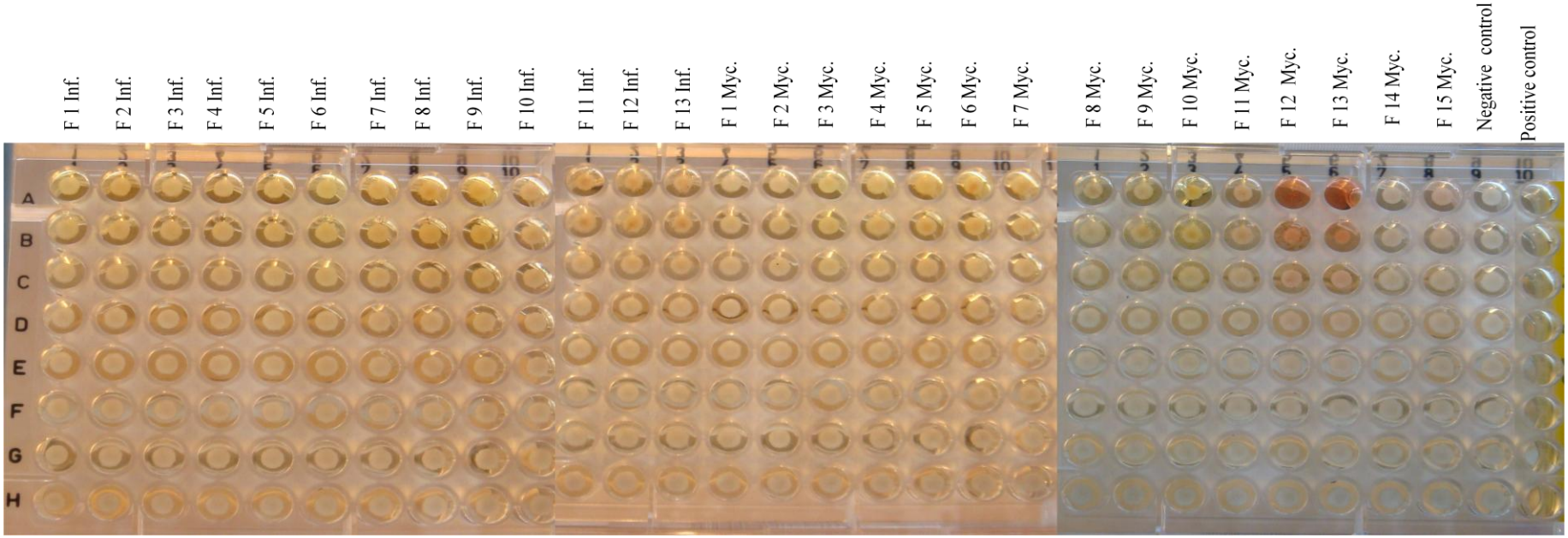

H
G
F
E
D
C
B
A
1
2
3
4
5
6
7
8
9
10
13059 YM
13059 ZM ½
13063 YM
13066 YM
13063 ZM ½
13066 ZM ½
13067 YM
13067 ZM ½
13068 YM
13068 ZM ½
13069 YM
13069 ZM ½
13072 YM
13072 ZM ½
13073 YM
13073 ZM ½
13076 YM
13076 ZM ½
Positive control
Negative control



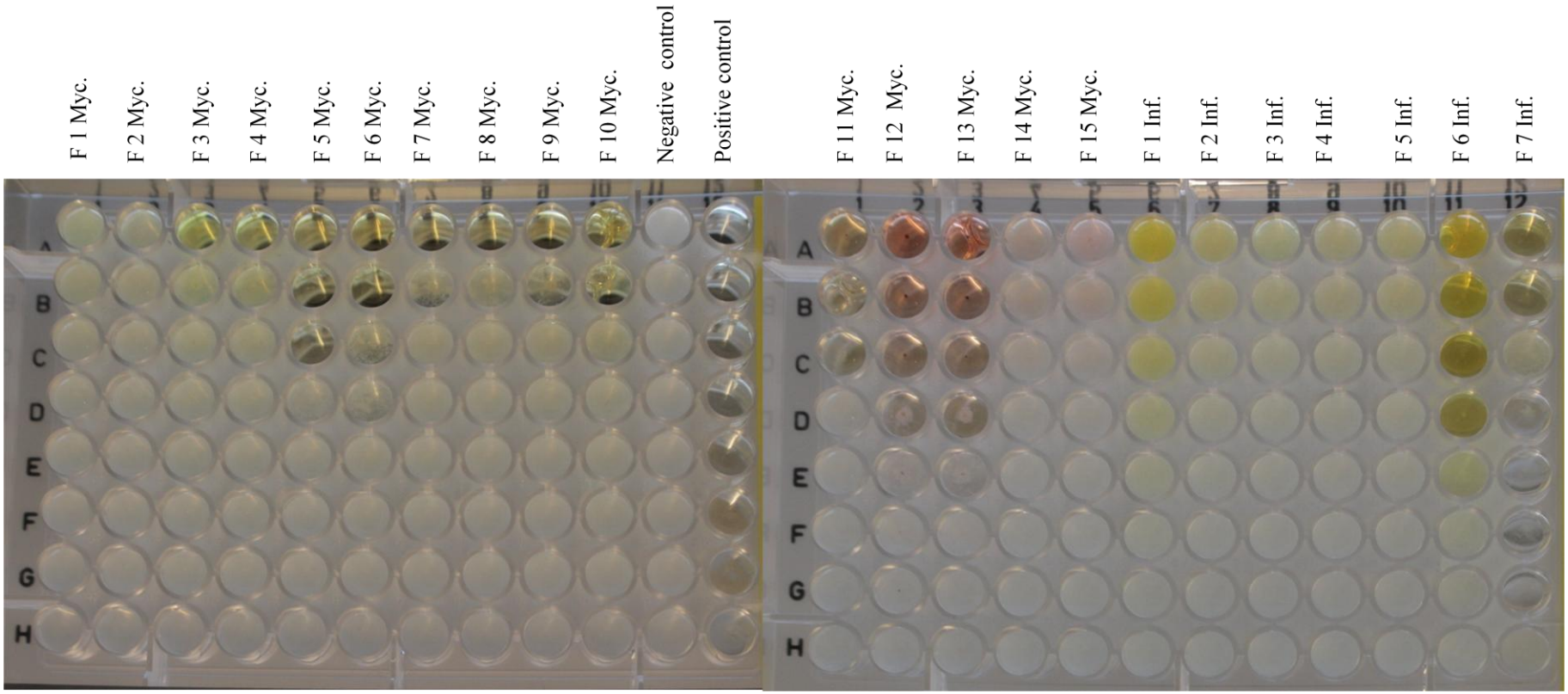
Microtiter plates result of the serial dilution assay of crud extract of *E. nigrum* isolates against *Bacillus subtilis* after 24 hours of incubation. Turbid indicate to intensive bacterial growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.



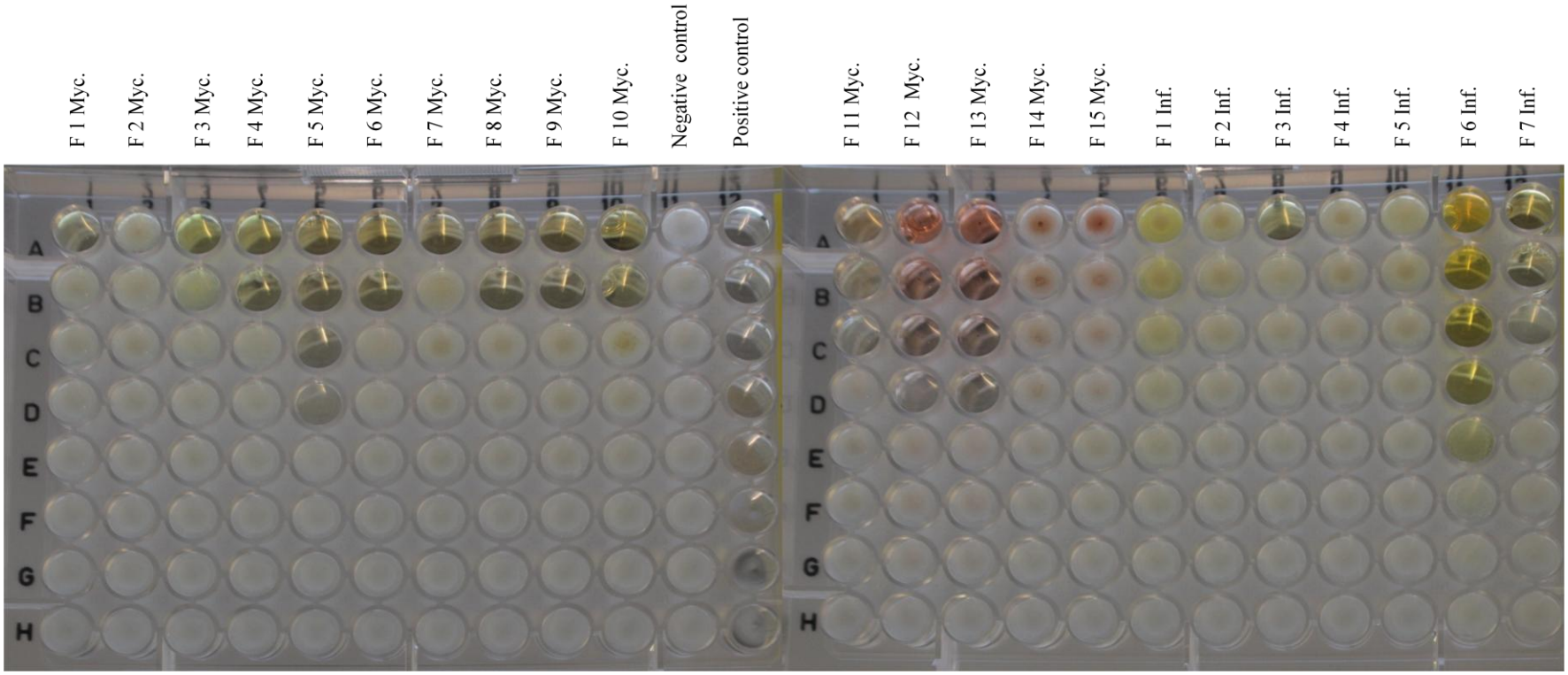
Microtiter plates result of the serial dilution assay of crud extract of *E. nigrum* isolates against *Mucor plumbeus* after 24 hours of incubation. Turbid indicate to intensive fungal growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.



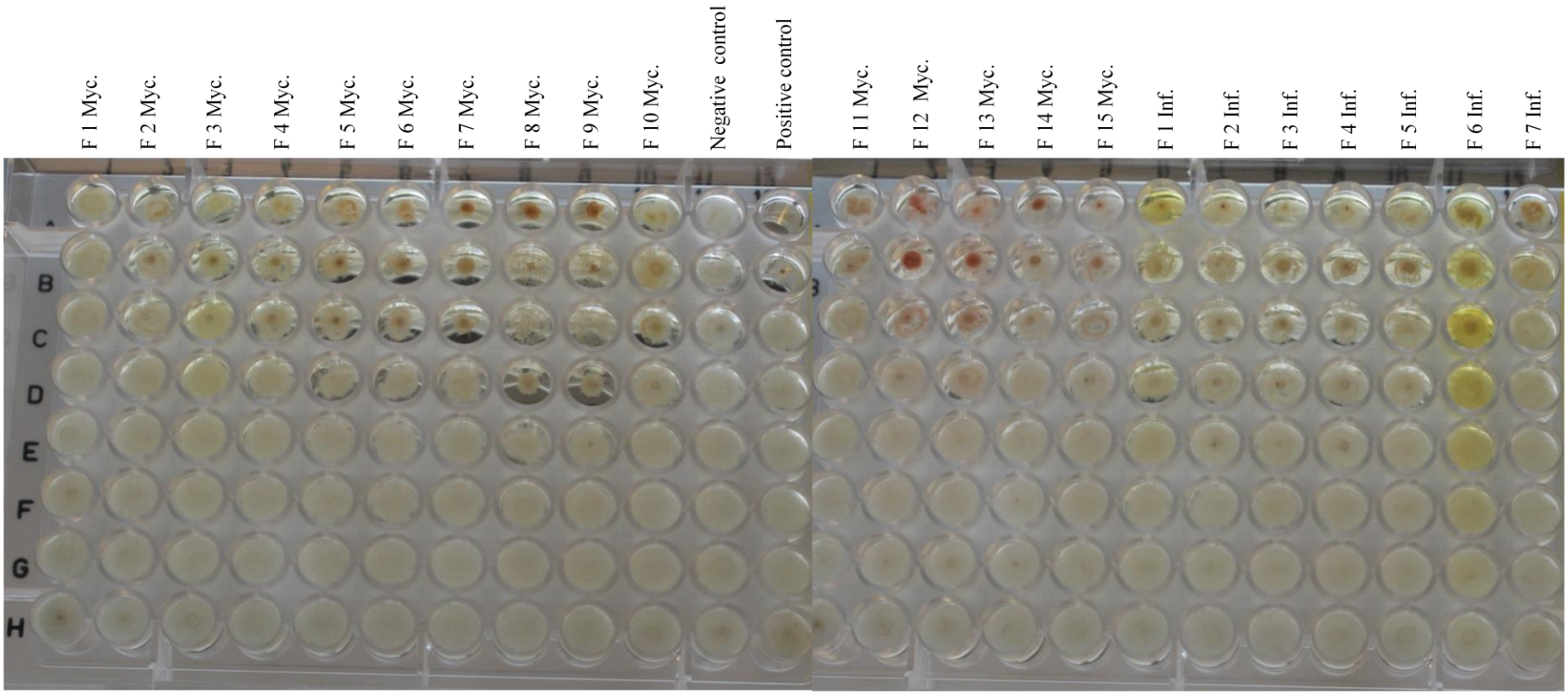
Microtiter plates result of the serial dilution assay of mycelium and culture broth extracts fractions from the isolate 13059 of *E. nigrum* against *saccharomyces cerevisiae* after 24 hours of incubation. Turbid indicate to intensive fungal growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.



Microtiter plates result of the serial dilution assay of mycelium and culture broth extracts fractions from the isolate 13059 of *E. nigrum* against *B. subtilis* after 24 hours of incubation. Turbid indicate to intensive fungal growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column



Microtiter plates result of the serial dilution assay of mycelium and culture broth extracts fractions from the isolate 13059 of *E. nigrum* against *E.coli* after 24 hours of incubation. Turbid indicate to intensive fungal growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.



Microtiter plates result of the serial dilution assay of mycelium and culture broth extracts fractions from the isolate 13059 of *E. nigrum* against *M. piriformis* after 24 hours of incubation. Turbid indicate to intensive fungal growth and clear wells indicate growth inhibition (concentration of the extracts 4.5mg/ml), isolate name and medium type are indicated at the top of each column.