U. PORTO

FC FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

ciimar
Centro Interdisciplinar
de Investigação
Marinha e Ambiental

U. PORTO
FC FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Study of the microbiome of the fluoroacetate producing plant *Dichapetalum cymosum*: Investigation of microbial community

Zita Diana Marinho Couto

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto

Mestrado em Genética Forense

2015/2016

FC

Study of the microbiome of the fluoroacetate producing plant *Dichapetalum cymosum*: Investigation of microbial community

Zita Diana Marinho Couto
Mestrado em Genética Forense
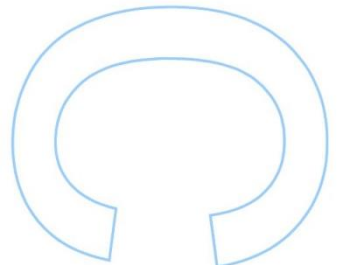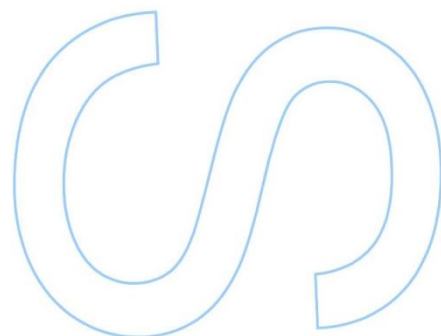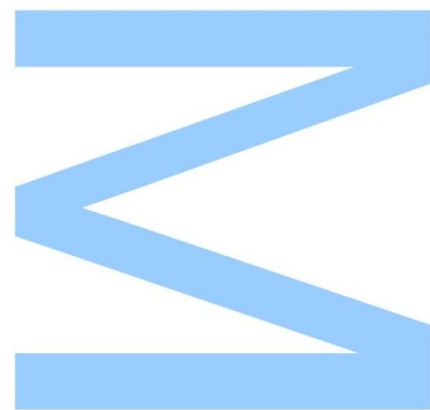Departamento de Biologia
2015/2016

Orientador
Maria de Fátima Carvalho, PhD, Investigadora no Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Coorientador
Filipe Pereira, PhD, Investigador no Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

U. PORTO

FC FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

ciimar
Centro Interdisciplinar
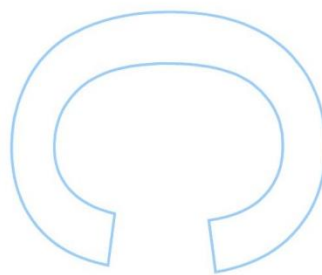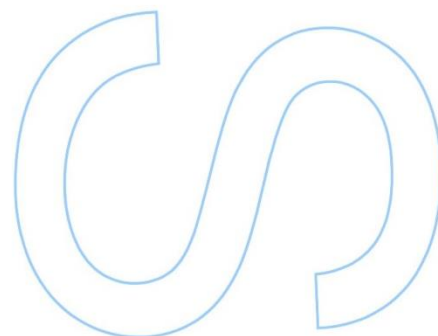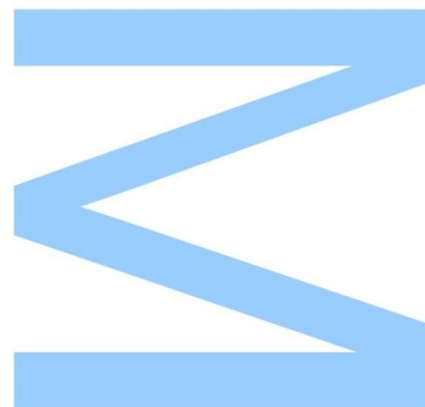de Investigação
Marinha e Ambiental

Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Dissertação de candidatura ao grau de Mestre em Genética Forense submetida à Faculdade de Ciências da Universidade do Porto.

O presente trabalho foi desenvolvido no Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) sob a orientação científica da Doutora Maria de Fátima Carvalho e coorientação do Doutor Filipe Pereira.

Dissertation for applying to a Master's Degree in Forensic Genetics, submitted to the Faculty of Sciences of the University of Porto.

The present work was developed at Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) under the scientific supervision of Maria de Fátima Carvalho, PhD and co-supervision of Filipe Pereira, PhD.

Acknowledgments

A special thanks to my supervisor, Maria de Fátima Carvalho for having me accepted in this project, which allowed a journey through the world of microbiology. I also thank her for her patience, kindness and prompt clarification of doubts, as well as the shared knowledge.

I also thank my co-supervisor, Filipe Pereira for the recommendations, availability and the shared knowledge.

I also would like to thank Diogo Alexandrino, Inês Ribeiro and Patrícia Duarte for all the help in the laboratory.

Last but not the least, I thank the teams of Ecobiotec and LEGE, for the support in the experiments.

Resumo

Os compostos fluorados com origem natural são muito raros. Estes compostos são produzidos por algumas plantas tropicais e subtropicais e por um pequeno número de microrganismos pertencentes ao filo Actinobacteria. O fluoroacetato (FA) é o composto fluorado natural mais comum, produzido biologicamente. Algumas plantas capazes de produzir FA já foram identificadas, embora o mecanismo pelo qual produzem este composto fluorado ainda não é conhecido. No entanto, a biossíntese de compostos fluorados pelo número limitado de espécies de Actinobacteria com esta capacidade já foi completamente elucidada, sendo mediada por uma enzima única, fluorinase, que catalisa a síntese da ligação C-F.

A planta arbustiva *Dichapetalum cymosum* é uma das plantas conhecidas produtoras de FA, e é considerada um risco para animais e pessoas que vivem nas suas proximidades, devido à presença de níveis elevados de FA nos seus tecidos. Encontra-se ainda aberta a questão se são as plantas ou os seus microrganismos simbiontes os responsáveis pela biossíntese de FA e, por isso, a investigação do microbioma destas plantas produtoras de FA é uma abordagem importante para ajudar a responder a esta questão.

Neste contexto, este estudo teve como objetivo investigar a diversidade microbiana associada à planta *Dichapetalum cymosum* e identificar a possível presença do gene da fluorinase na comunidade microbiana desta planta. Para isso, a comunidade microbiana cultivável associada a esta planta, com especial foco no filo Actinobacteria, foi isolada, tendo sido também realizada uma análise metagenómica de forma a obter uma visão geral da comunidade microbiana associada a esta planta.

Amostras de caule, folhas e solo foram recolhidas da planta *D. cymosum* em Pretória, África do Sul. Para aumentar as probabilidades de isolamento de microrganismos pertencentes ao filo Actinobacteria, foram usados três meios seletivos e um meio não seletivo, juntamente com vários pré-tratamentos, o que permitiu a obtenção de 206 isolados. O DNA genómico foi extraído pelo método de fenol-clorofórmio e a identificação dos isolados obtidos foi realizada por sequenciação do gene 16S rRNA, para os isolados bacterianos, e por sequenciação da região ITS, para os isolados de fungos. Não foi possível identificar todos os isolados, mas os resultados até agora obtidos mostraram que as estirpes microbianas isoladas

encontram-se distribuídas entre os filos Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes e Ascomycota.

A análise metagenómica só foi possível ser realizada para a amostra de solo recolhida na vizinhança da planta *D. cymosum*, devido à fraca qualidade do DNA extraído das amostras de folhas e caules. O filo Actinobacteria foi o mais abundante (37%) na amostra analisada, tendo sido a ordem Actinomycetales a mais representativa. Alguns géneros de fungos foram também identificados, sendo os géneros *Phyllosticta, Boeremia* e *Knufia* os mais abundantes.

Para identificar a potencial presença do gene da fluorinase no microbioma da planta *D. cymosum*, foram concebidos seis primers específicos para este gene. A eficiência destes primers foi testada com duas espécies de *Streptomyces,* que possuem o gene da fluorinase, *S. cattleya* e *S. xinghaiensis*. Não foi obtida amplificação do gene da fluorinase com o DNA extraído a partir das folhas e caule de *D. cymosum* e do solo na sua proximidade. A presença do gene da fluorinase foi também investigada em todas as estirpes de Actinobacteria isoladas neste estudo e até agora identificadas.

No geral, este estudo demonstrou uma elevada diversidade de microrganismos associados com a planta *Dichapetalum cymosum* e investigações adicionais sobre a possível presença do gene da fluorinase no microbioma desta planta produtora de FA poderão ajudar a esclarecer o mecanismo pelo qual estas plantas produzem este composto fluorado natural.

Palavras-chave: *Dichapetalum cymosum;* Fluoroacetato; Fluorinase; Actinobacteria.

Abstract

Fluorinated compounds with a natural origin are very rare. These compounds are known to be produced by some tropical and sub-tropical plants and by a few microorganisms belonging to the phylum Actinobacteria. Fluoroacetate (FA) constitutes the most common natural fluorinated compound biologically produced. A number of plants capable of producing FA have been identified, though the mechanism by which these produce this fluorinated compound is not yet known. However, biosynthesis of fluorinated compounds by the limited number of actinobacterial species with this capacity has been completely elucidated and is mediated by a unique enzyme, fluorinase, which catalyzes the synthesis of the C-F bond.

The shrubby plant *Dichapetalum cymosum* is one of the known FA producing plants, and is considered a hazard to animals and people living nearby due to the presence of high levels of FA in its tissues. It is still open the question whether the plants or its symbiotic microorganisms are responsible for the biosynthesis of FA and, thus, the investigation of the microbiome of these FA producing plants is an important approach to help answering this question.

In this context, this study aimed to investigate the microbial diversity associated with *Dichapetalum cymosum* and identify the possible presence of the fluorinase gene in the microbial community of the plant. For this, the culturable microbial community associated with the plant, with special focus in Actinobacteria, was isolated and a metagenomics analysis was conducted to obtain a whole view of the microbial community associated with this plant.

Stems, leaves and soil samples were collected from *D. cymosum* in Pretoria, South Africa. To increase the chances of isolating microorganisms belonging to the phylum Actinobacteria, three selective media and one non-selective medium, together with various pretreatments were used, which allowed to obtain 206 isolates. Genomic DNA was extracted by the phenol-chloroform method and identification of the obtained isolates was performed by 16S rRNA gene sequencing, for the bacterial isolates, and by sequencing of the ITS region, for fungal isolates. It was not possible to identify all isolates but the results so far obtained showed that the isolated microbial strains were distributed among the phyla Actinobacteria, Proteobacteria, Firmicutes, Bacterioidetes and Ascomycota.

Metagenomics analysis could only be performed for the sample of soil collected in the vicinity of *D. cymosum*, due to the poor quality of the DNA extracted from the leaf and stems samples. Actinobacteria constituted the most abundant (37%) bacterial phylum with the order Actinomycetales being the most representative. Some fungal genera were also identified with the genera *Phyllosticta, Boeremia* and *Knufia* being the most abundant.

To identify the potential presence of the fluorinase gene in the microbiome of *D. cymosum*, six primers specific to this gene were designed. The efficiency of these primers was tested with two *Streptomyces* species known to carry the fluorinase gene, *S. cattleya* and *S. xinghaiensis*. No amplification of the fluorinase gene was obtained with the DNA extracted from leaves and stems of *D. cymosum* and from the soil in its proximity. The presence of the fluorinase gene was also investigated in all actinobacterial strains isolated in this study and so far identified.

Overall, this study demonstrated a high diversity of microorganisms associated with the plant *Dichapetalum cymosum* and further investigations on the potential presence of the fluorinase gene in the microbiome of this FA producing plant may help clarifying the mechanism by which these plants produce this natural fluorinated compound.

Keywords: *Dichapetalum cymosum;* Fluoroacetate; Fluorinase; Actinobacteria.

Table of contents

List of tables

List of figures

List of abbreviations

4 - FT	4–fluorothreonine
5' - FDA	5'–fluoro–5'–deoxyadenosine
BLAST	Basic Local Alignment Search Tool
bp	Base pair (s)
DNA	Deoxyribonucleic acid
EDTA	Ethylene–diaminetetraacetic acid
FA	Fluoroacetate
ITS	Internal transcribed spacer region
kb	Thousand base pairs
NC	Negative control
NCBI	National Center for Biotechnology Information
No.	Number
nt	Nucleotide (s)
OligoCalc	Oligonucleotide Properties Calculator
OTU	Operational taxonomic unit
PC	Positive control
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PET	Positron emission tomography
RH	Raffinose – Histidine agar
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAM	S–(5'–adenosyl)–L-methionine
SCN	Starch Casein Nitrate agar
SDS	Sodium dodecyl sulfate
STE	Sodium chloride – Tris - EDTA
SYBR	(Z)-4-((3-Methylbenzo[d]thiazol-2(3H)-ylidene)methyl)-1-propylquinolin-1-ium 4-methylbenzenesulfonate
TWYE	Tap Water Yeast Extract agar
UV	Ultraviolet
YEME	Yeast Extract – Malt Extract medium

I. Introduction

1. Natural halogenated compounds

After many years of skepticism from the scientific community, it is now well established that nature is responsible for the production of a myriad of halogenated compounds (Fowden and Robinson, 1968; Gribble, 2002). The astonishing increase in the number of natural halogenated compounds is a result of the exploration of a variety of environments, especially oceans, due to the urgent need to search for natural products as potential sources of new drugs (Gribble, 2003).

All halogenated compounds bear at least one halogen atom in their structure. The halogen elements hitherto known are fluorine (F), chlorine (Cl), bromine (Br), iodine (I) and the most recent discovered astatine (At). These five elements share a common feature, in which they all present seven external electrons (Fig. 1), being commonly incorporated synthetically in biological molecules, except astatine (At), which constitutes the rarest element with natural occurrence and is radioactively unstable.

Figure 1. Halogen elements (fluorine - F, chlorine - Cl, bromine - Br, iodine - I and astatine - At) arranged in the Periodic Table, displaying the shared feature of seven external electrons. Adapted from (Gribble, 2004).

Most of the natural halogenated compounds contain chlorine or bromine: from ca. 4500 naturally occurring halogenated compounds identified so far, ca. 2320 are chlorinated, 2050 brominated, 110 iodinated and 20 fluorinated (Gribble, 2004; Walker and Chang, 2014).

The sources of these compounds vary from biogenic and abiogenic origins. Biogenic organohalogens are produced by a variety of bacteria, fungi, lichens, plants, insects, animals and even humans. On the other hand, abiogenic organohalogens are formed by forest fires, volcanoes and other geothermal processes (Gribble, 2002; Gribble, 2004).

1.1. Natural fluorinated compounds

Fluorine is the most abundant halogen in the Earth's crust, occurring in higher concentrations (270-740 ppm) than chlorine (10-180 ppm) (Harper et al., 2003). In spite of its high abundance, fluorine has a very low bioavailability due to the fact that this element is almost insoluble in water: sea water only contains 1.3 ppm of fluorine in contrast to 19 000 ppm of chlorine (Harper and O'hagan, 1994; Walker and Chang, 2014). Despite the low concentrations of bioavailable fluorine in both marine and terrestrial environments, inorganic fluorine can be accumulated in significant quantities by living organisms, for example, 10% of the dry weight of the marine sponge *Halichondria moorei* , corresponds to fluorine (Gregson et al., 1979), and the terrestrial plant belonging to the genus *Camelia* accumulates up to 300 ppm of fluorine (Venkateswarlu et al., 1965).

However, organically bound fluorine is very rare in nature. It has been identified only in a small number of tropical and sub-tropical plants and in a few bacteria belonging to the phylum Actinobacteria. Unlike biological fluorinated molecules, synthetic bond fluorine is very common and has a wide commercial application, being present in pharmaceutical and agricultural products, plastics, surfactants, refrigerants, etc. (Maienfisch and Hall, 2004; Thayer, 2006).

Compared with the abundance of other natural halogenated compounds, the main reason for the shortage of biogenic fluorinated compounds lays in the unique chemical characteristics of fluorine, since its properties are very distinct from the other halogens. Fluorine is the most electronegative element, and due to this property it has a high electron withdrawal capacity, creating a highly polarized C-F bond with one of the strongest bonds known in nature. These properties together with its poor nucleophilic character, high heat of hydration and high redox potential prevent the activation of the fluorine atom and its participation in biological reactions. The latter property constitutes the major factor restricting the participation of fluorine in biochemical processes mediated by haloperoxidases, the major route by which organohalogenated

compounds are thought to be formed in nature (Neidleman and Geigert, 1986; Harper and O'hagan, 1994; Neumann et al., 2008).

Presently, about 20 fluorinated natural compounds are known, including fluoroacetate, fluorocitrate, fluoroacetone, several ω-fluorofatty acids, nucleocidin and 4-fluorothreonine (Fig. 2).
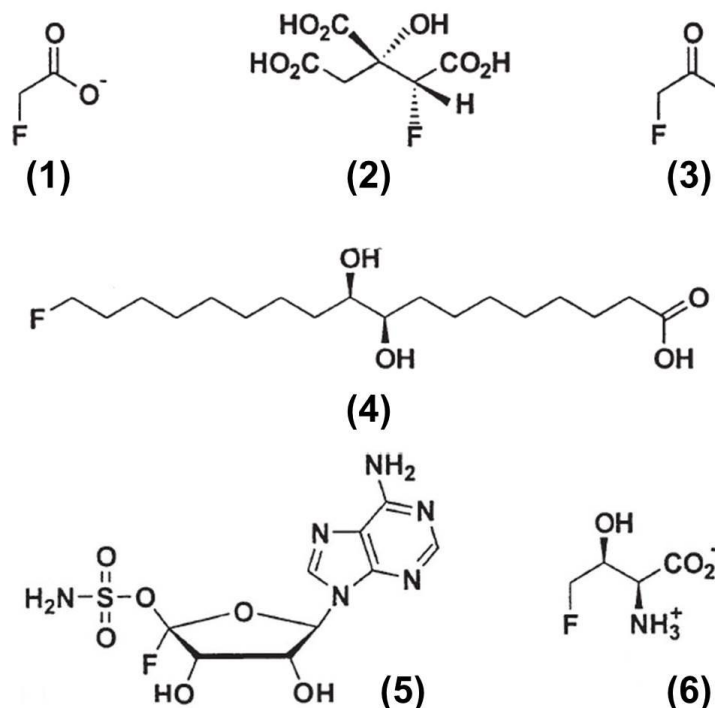
Figure 2. Chemical structure of the fluorinated natural compounds currently known. (1) Fluoroacetate; (2) Fluorocitrate; (3) Fluoroacetone; (4) ω-Fluorofatty acids; (5) Nucleocidin; (6) 4-fluorothreonine. Adapted from (Harper et al., 2003).

Some of these compounds are produced by plants while others have a bacterial origin, namely bacteria belonging to the genus *Streptomyces* (O'Hagan and Harper, 1999; Harper et al., 2003). Fluoroacetate (FA) is the most common natural organofluorine compound known and it was firstly discovered by Marais in the South African plant *Dichapetalum cymosum* (Marais, 1943; Marais, 1944). This compound was described to be metabolized *in vivo* to fluorocitrate, fluoroacetone or ω-fluorofatty acids (Harper et al., 2003). However, recent studies showed that fluoroacetone is probably a misidentified natural product (O'Hagan and Deng, 2014).

The first organofluorine compound to be isolated from a bacterial source was nucleocidin, produced by *Streptomyces calvus* isolated from a soil sample in India (Thomas et al., 1957). Later, while attempting the maximization of an antibiotic production, FA and 4-fluorothreonine were discovered to be produced by *Streptomyces cattleya* (Sanada et al., 1986).

2. Fluoroacetate producing plants

2.1. Plant species and geographical distribution

In 1943, Marais isolated FA from the shrubby plant *Dichapetalum cymosum* (Fig. 3), commonly known by the inhabitants as gifblaar (poison leaf), due to its toxicity. In spring, the young leaves of the plant were described to contain up to 2500 ppm of FA (Harper et al., 2003). The immature leaves, shoots, seeds and flowers are particularly toxic and are considered a hazard to livestock (Steyn, 1928; Meyer and Grobbelaar, 1990; Kellerman et al., 1996).

Figure 3. *Dichapetalum cymosum.*

Since this discovery, many other tropical and sub-tropical plants have been identified (Hall, 1972) and found to accumulate FA, presumably for defense purposes. Most of these plants belong to the *Dichapetalum* genus, as for example, *D.heudelotti*, *D. stuhlmannii* and *D. toxicarium* (O'Hagan and Harper, 1999; Harper et al., 2003) .

In Tanzania, the FA producing plant *Dichapetalum braunii* was also identified and found to produce the highest concentrations of FA known in plants, with young leaves with up to 8000 ppm of this compound (O'Hagan et al., 1993). In other continents, such as Australia and South America, other plants that contain FA have also been identified. In Australia, two plant species are among the most toxic ones, *Oxylobium parviflorum* and *Gastrolobium bilobum* containing up to 2600 ppm of FA in their leaves and 6500 ppm in their seeds (Aplin, 1971; Twigg et al., 1996). In South America, *Palicourea marcgravii* accumulate concentrations up to 5000 ppm of FA (Hall, 1972). Another plant

was also found to contain FA in India, *Cyamopsis tetragonolobus,* but with lower levels, 10 ppm (Vartiainen and Gynther, 1984).

In a more recent survey, several other plants, mainly distributed in Africa, Australia and South America, and belonging to families such as Fabaceae, Rubiaceae, Bignoniaceae, Malpighiaceae and Dichapetalaceae were found to contain FA (Lee et al., 2014).

2.2. Fluoroacetate production pathway by plants

The pathway for FA synthesis in FA producing plants has not yet been elucidated (Walker and Chang, 2014). Some authors hypothesized that the roots of these plants are able to absorb FA from the surrounding soil and transporting it to the aerial parts (Hall and Cain, 1972), with no synthesis of this compound occurring in the plant. In fact, *D. cymosum* has an extensively networked root system but it is not clear if the roots had evolved to assimilate FA from the soil (O'Hagan and Harper, 1999).

The limiting step in the biosynthesis of FA relies in the formation of the C-F bond and, back in 1992, Meyer and O'Hagan believed in the existence of an enzyme capable of removing the hydration of the fluorine ion (Meyer and O'Hagan, 1992). Over the years, many authors proposed possible mechanisms for C-F bond biosynthesis in plants. In 1967, Peters proposed that fluorophosphate could be an intermediate of FA biosynthesis and that the former compound could be produced during glycolysis by a fluorokinase (Peters, 1967). Later, Peters suggested that FA biosynthesis could occur through intermediates like volatile fluorocarbons derived from ethylene (Peters, 1973). Vickery and colleagues defended that FA could be formed by a haloperoxidase reaction, but this seems very unlikely due to the thermodynamic impossibility of fluorine activation (Vickery et al., 1979). Mead and Segal believed that fluoropyruvate could act as an intermediate of FA biosynthesis and it was demonstrated that cultures of *D.cymosum* were capable of converting fluoropyruvate into FA (Mead and Segal, 1972; Meyer and O'Hagan, 1992).

Thus, many authors tried to explain the pathway of FA synthesis in producing plants, but until now it remains unclear, and doubts still exist whether this compound is actually produced by plants or by microorganisms colonizing them (Harper et al., 2003; Walker and Chang, 2014).

2.3. Toxicity

FA is a highly toxic compound due to the blockage of the tricarboxylic acid cycle (Krebs cycle) caused by its *in vivo* conversion to fluorocitrate, in which FA is metabolized to fluoroacetyl-CoA and then to (2R, 3R) – fluorocitrate. This last compound is the only toxic form, for the others stereoisomers are non-toxic. Thus, is a potent inhibitor of the enzyme aconitate hydratase that converts fluorocitrate into 4-hydroxy-*trans*-aconitate, blocking citrate metabolism, and leading to its accumulation in the living tissues (Peters et al., 1953; Lauble et al., 1996; Lee et al., 2014) (Fig.4).
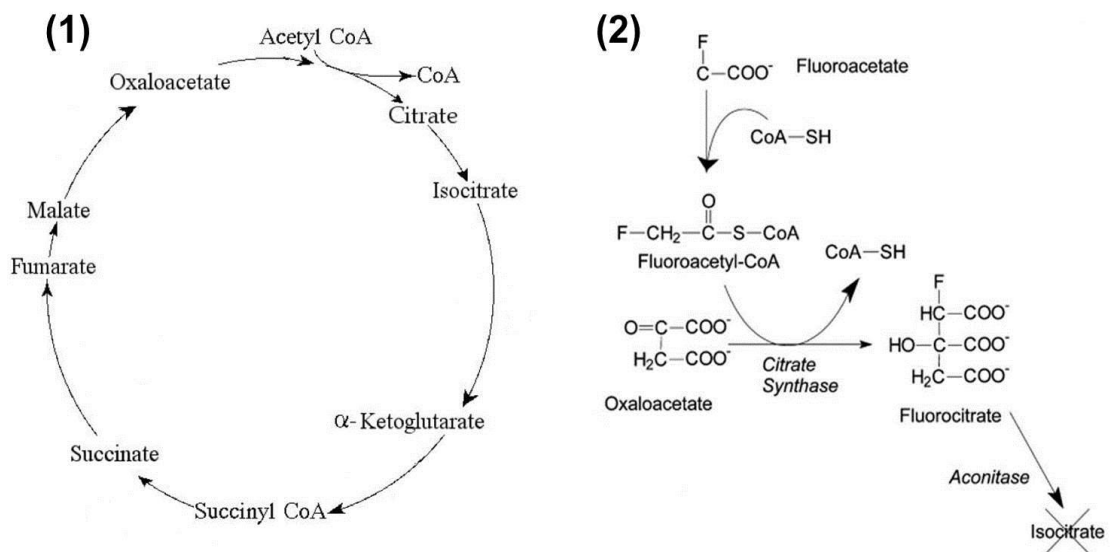
Figure 4. Normal tricarboxylic acid cycle (Krebs cycle) (1); Partial tricarboxylic acid cycle (Krebs cycle) highlighting aconitate inhibition due to the incorporation of fluoroacetate into the cycle (2). Adapted from (Lee et al., 2014).

It seems also that fluorocitrate can inhibit citrate transport across the mitochondrial membrane, and a study indicated that this could lead to a higher toxicity than the accumulated fluorocitrate in the living tissues (Kun et al., 1977).

FA producing plants, like *D. cymosum,* had to develop some resistance mechanisms in order to resist to the toxicity of FA. In fact, there are some theories that attempt to explain this resistance, however none of them truly elucidate the resistance of the plants to autointoxication (O'Hagan and Harper, 1999). Some authors defend that the synthesis of acetyl-CoA is located in the chloroplasts instead of mitochondria, thus becoming impossible the formation of fluorocitrate (Kuhn et al., 1981). Others explain that these plants do not produce fluorocitrate due to a lower enzymatic affinity to fluoroacetyl-CoA (Eloff and Von Sydow, 1971) and other authors demonstrated the

existence of an enzyme capable of hydrolyzing fluoroacetyl-CoA (Meyer and O'Hagan, 1992).

D. cymosum is considered a hazard to livestock due to FA poisoning, as a result of the ingestion of these plants (Kellerman et al., 1996). The ingestion of FA producing plants affects the central nervous system of mammalians and can lead to convulsions and heart attacks (Goncharov et al., 2006). However, many animals that cohabit in areas where FA producing plants grow revealed some tolerance to this compound. Indeed tolerance to this poison has been demonstrated in insects, reptiles, birds and even mammals (Lee et al., 2014). One famous example is the caterpillar of the moth *Sindrus albimaculatus,* which consumes portions of *D. cymosum,* appearing to defluorinate FA and to accumulate it as a form of defense (Meyer and O'Hagan, 1992).

3. Fluoroacetate producing microorganisms

3.1. Actinobacteria

More recently, FA was also found to be produced by five bacteria: *Streptomyces cattleya* (Fig. 5A), *Streptomyces xinghaiensis, Streptomyces sp MA37, Nocardia brasiliensis* (Fig. 5B) and *Actinoplanes sp N902-109,* though in the latter two species only the genetic potential to produce this compound has been demonstrated (O'Hagan et al., 2002; Deng et al., 2014; Huang S., 2014).
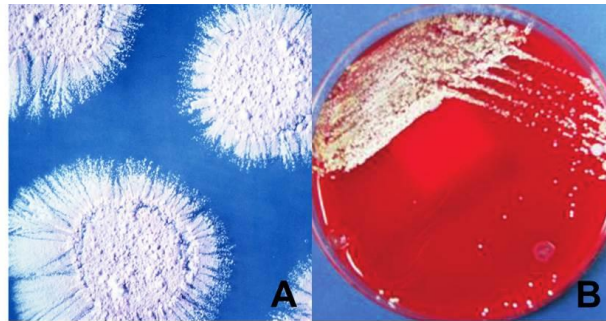
Figure 5. *Streptomyces cattleya* (A). *Nocardia brasiliensis* (B).

It is interesting to notice that all these five bacteria belong to the phylum Actinobacteria. This phylum is one of the largest taxonomic units among the major lineages recognized until now within the domain Bacteria (Ventura et al., 2007; Barka et al., 2016).

Actinobacteria are Gram-positive bacteria with a high content of guanine and cytosine in their genome, and are distributed in both terrestrial and aquatic environments, like *Streptomyces spp., Micromonospora spp.* and *Rhodococcus spp.* They can also be found as plant symbionts (e.g., *Frankia spp.*), plant commensals (e.g., *Leifsonia* spp.) pathogens (e.g., *Corynebacterium spp., Tropheryma spp., Mycobacterium spp., Nocardia spp., Propionibacterium spp.*) and gastrointestinal commensals (e.g., *Bifidobacterium spp.*) (Ventura et al., 2007).

Many of these microorganisms, and in particular *Streptomyces spp.,* are saprophytic, soil organisms with the ability to form spores, especially under stress conditions, such as limitation of exogenous nutrients. *Streptomyces* species play key roles in soil ecology, mainly because of their ability to hydrolyze many polysaccharides, such as cellulose, xylan and other natural macromolecules and of their capacity to

produce a myriad of bioactive substances. In fact, soil populations are commonly dominated by the genus *Streptomyces* which like other Actinobacteria are mostly mesophilic, with optimal growth temperatures between 25 ºC and 30 ºC (Barka et al., 2016).

Actinobacteria are known producers of plenty of bioactive secondary metabolites and, thereby, are of great importance to the field of biotechnology, with their metabolites having many applications in the industry, medicine and agriculture. Among these, *Streptomyces spp.* are prolific producers of antibiotics, which makes them crucial in the fight against resistant pathogens. Examples include actinomycin isolated from *Streptomyces antibioticus*, streptothricin produced by *Streptomyces lavendulae* and streptomycin originated from *Streptomyces griseus*. A wide range of other secondary metabolites with different bioactivities is also produced, as insecticides, like macrotetrolides produced by *Streptomyces aureus*, anthelmintic agents, like ivermectin produced by *Streptomyces avermitilis,* antifungals, like validamycin isolated from *Streptomyces hygroscopicus* var.limoneus, among others (Barka et al., 2016).

Actinobacteria also play many important roles in plant-associated microbial communities and endophytic Actinobacteria, which colonize plant tissues without causing disease, may be essential for the development of certain plants (Wilson, 1995; Bacon and Hinton, 2007). For example, *Frankia spp.* is found in the roots of some non-leguminous plants, such as *Alnus spp.,* forming a symbiotic association and allowing these plants to grow in soils with poor nitrogen levels (Pawlowski and Demchenko, 2012).

Endophytic Actinobacteria have been isolated from different plants, with the most frequently isolated species belonging to the genera *Microbispora, Nocardia, Micromonospora* and *Streptomyces.* Studies have demonstrated that these microorganisms benefit the growth of their plant host and plant defenses and improve the protection of the plant against several pathogens (Barka et al., 2016). Although endophytic Actinobacteria have been less investigated it becomes important to study these microorganisms due to their capacity to produce key compounds for plant health, development and defense, as well as natural compounds for human applications (Brader et al., 2014).

3.2. Fluoroacetate production pathway by Actinobacteria

Unlike FA producing plants, the mechanism by which the five Actinobacteria, *S. cattleya, S. xinghaiensis, Streptomyces sp* MA37, *N. brasiliensis* and *Actinoplanes sp* N902-109, biosynthesize FA is already known, being mediated by a unique enzyme, fluorinase, responsible for the formation of the C-F bond (O'Hagan, 2002; Deng et al., 2014; Huang et al., 2014).

The first fluorinase was discovered by O'Hagan and colleagues (O'Hagan et al., 2002; Deng et al., 2004; O'Hagan, 2006), who demonstrated in a partially purified protein extract from *S. cattleya* that the enzyme fluorinase was responsible for the biosynthesis of FA through the initial conversion of fluoride ion and S-(5'-adenosyl)–L-methionine (SAM) into 5'-fluoro-5'-deoxyadenosine (5´-FDA). Further reactions culminated in the production of FA and of a second fluorinated metabolite, 4-fluorothreonine (4-FT) (Fig. 6).
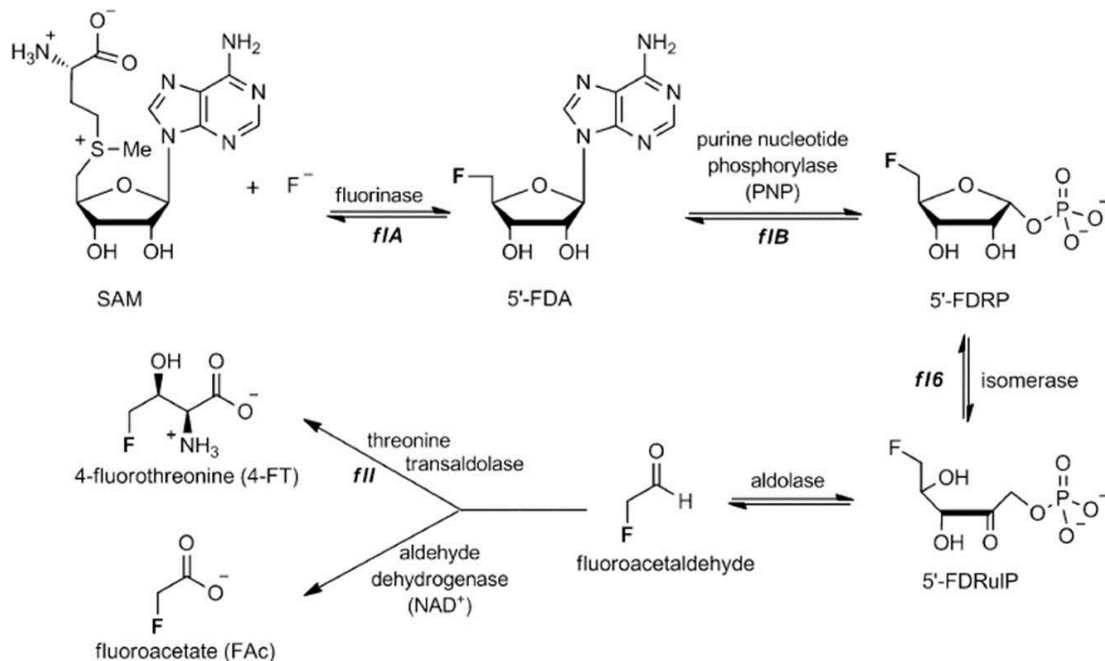
Figure 6. Biosynthesis of fluoroacetate and 4 - fluorothreonine in *Streptomyces cattleya*. The metabolic pathway initiates through the action of the fluorinase enzyme which converts fluoride ion and S-(5'-adenosyl)–L-methionine (SAM) into 5'–fluoro-5'-deoxyadenosine (5´-FDA). Adapted from (Deng et al., 2014).

Later, three new fluorinases were identified by genome mining in *Streptomyces sp* MA37*, Nocardia brasiliensis* and *Actinoplanes sp* N902-109*,* having a homology of 80% to 87%, to the original fluorinase isolated from *S. cattleya* (Deng et al., 2014). In the same year another fluorinase was identified in a marine bacterium, *S. xinghaiensis* NRRL B-24674, by genome sequencing (Huang et al., 2014).

3.3. Biotechnological potential of fluorinase

The discovery and identification of microorganisms capable of biosynthesizing fluorinated compounds is a promising tool for the fluorine industry. Such discoveries may allow the design of enzymes, pathways and organisms that can handle fluorine. This is highly important given the increasing commercial significance of fluorinated compounds. The modern medicine sector constitutes an example of this, with organofluorine compounds being found in up to 15% of the commercialized pharmaceutical products (Eustáquio et al., 2010).

Thus, the recent discovery of the fluorinase gene reveals to be very promising for the production of fluorinated products through genetic engineering, avoiding many of the drawbacks associated with the chemical synthesis of these compounds, such nonselectivity, toxicity and reactivity of the employed fluorination reagents (Eustáquio et al., 2010; Thayer, 2006). This enzyme is already being used to produce [^{18}F]-labelled radiotracers for positron emission tomography (PET), an imaging technique frequently employed in the diagnostic of tumors and brain malfunctions (Deng et al., 2006; Schirrmacher et al., 2007; Onega et al., 2010; Dall'Angelo et al., 2013; O'Hagan and Deng, 2014; Thompson et al., 2015).

4. The use of FA with potential forensic implications

Synthetic FA is commercially available as a rodenticide, best known as 1080 compound or sodium fluoroacetate (Fig. 7). It was developed during World War II and was introduced in the USA in 1946 (Chenoweth, 1949).
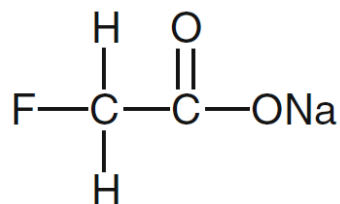
Figure 7. Chemical structure of 1080 compound or sodium fluoroacetate.

Nowadays, 1080 compound continues to be widely used in baiting programs, in order to protect agricultural production and native fauna and flora. This compound can be incorporated into many different forms of bait and is a non-volatile, odorless, tasteless and water soluble toxin (Goh et al., 2005).

Due to its extreme toxicity, this compound is only licensed in few countries. In USA it is used against coyotes that prey on sheep and goats, while in Australia and New Zealand, the largest users of 1080, it is used to kill unwanted introduced species, such as foxes, feral dogs and cats, possums, among others. This compound was additionally reported to be used in Japan, Mexico and Israel (Proudfoot et al., 2006; Marks et al., 2014; Lee et al., 2014).

There are several risks associated with the use of 1080 to control animal populations as, due to the high water solubility of this compound, it is susceptible to contaminate underground waters and kill non-target species (Sherley, 2004; Goh et al., 2005; Eason et al., 2011; Eason et al., 2013)

The exposition of humans to sodium fluoroacetate can occur mainly *via* drinking of contaminated water, ingestion of the 1080 baits and consumption of contaminated food (Eason et al., 2011). Nonetheless, the risk of secondary poisoning by 1080 in meat intended for human consumption is low, due to its highly solubility in water and its short half-life in livestock. Studies demonstrated the rapid elimination of sub-lethal doses of this compound (Egekeze and Oehme, 1979; Eason et al., 1994; Gooneratne et al., 1995; Twigg et al., 2002). In fact, 1080 would not persist in livestock for more than a

few days since it is highly soluble in water and therefore it is quickly metabolized and excreted (Eason et al., 2011). However, in poisoning carcasses the persistence of 1080 can last for several months increasing the risk of killing non-target species that feed on the carcasses (Eason et al., 2013). Some cases of accidental poisoning by sodium fluoroacetate have been reported despite being uncommon, with dogs being extremely sensitive to this compound (Proudfoot et al., 2006; Eason et al., 2011; Eason et al., 2013).

FA can also be used as a poison for criminal purposes with the intention of causing injury or death (Sherley, 2007; Twigg and Parker, 2010). Thus, the existence of rigorous methods for the detection of sodium fluoroacetate is highly important, as there is the possibility of criminal use. Thereby, the development of accurate and efficient methods to detect FA or the species responsible for its production will be important for forensic investigations dealing with the criminal use of this dangerous poison.

II. Aims

The aim of this thesis was to investigate the microbial flora associated with the FA producing plant, *Dichapetalum cymosum*. The main goal was help elucidating whether the plant or its microbial symbionts are responsible for the biosynthesis of FA. The thesis had the following specific objectives:

- Isolation of the culturable community of microorganisms associated with the FA producing plant, *D. cymosum,* with special emphasis in Actinobacteria;

- Investigation of the microbial diversity of *D. cymosum* through culturable independent techniques, namely metagenomics analysis;

- Identification of the possible presence of the fluorinase gene in the microbiome of the *D.* cymosum plant.

III. Materials and methods

1. Sampling of *Dichapetalum cymosum*

Samples of stems and leaves of the FA producing plant *D. cymosum* and of soil in its vicinity were collected in the beginning of December 2015, in Pretoria, South Africa. It was not possible to collect root samples of this plant as they are usually found at ca. 10 m deep.

The samples were transported to the laboratory by air, at room temperature, and processed 4 days after their harvest.

2. Isolation of culturable bacterial community associated with *D. cymosum* plants

The culturable bacterial community associated with *D. cymosum,* with special emphasis in Actinobacteria, was analyzed in samples of stems and leaves of the plant as well as in its surrounding soil. Both epiphytic and endophytic communities were analyzed in the stems and leaves samples.

Culture media used in the study included one general, non-selective medium, Plate Count Agar (PCA) (Liofilchem, Italy), and three media selective for Actinobacteria: Starch Casein Nitrate agar (SCN), Tap Water Yeast Extract agar (TWYE) and Raffinose-Histidine agar (RH). The composition *per liter* of the selective media was the following:

- Starch Casein Nitrate agar: starch, 10 g; casein, 0.3 g; KNO_3, 2 g; NaCl, 2 g; K_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 17 g; dH_2O 1 L (Küster and Williams, 1964);
- Tap Water Yeast Extract agar: yeast extract, 0.25 g; K_2HPO_4, 0.5 g; agar, 18 g; tap water 1 L (Coombs and Franco, 2003);
- Raffinose-Histidine agar: raffinose, 10 g; L-histidine, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 15 g; dH_2O 1 L (Vickers et al., 1984).

All media were autoclaved at a temperature of 121 ºC for 20 min, after which selective media was supplemented with nalidixic acid (50 mg/L), against Gram-negative bacteria, and cycloheximide (50 mg/L), to inhibit fungal growth, and PCA was supplemented with cycloheximide (50 mg/L).

2.1. Isolation of epiphytic bacteria

For the isolation of epiphytic bacteria from *D. cymosum*, samples of stems and leaves were washed with deionized water (dH$_2$O), dried in a laminar flow chamber and cut aseptically into pieces of about 2 cm long. These pieces were then plated, in duplicate, on the selective media SCN, TWYE and RH and on the non-selective medium PCA and incubated in a growth chamber (VWR® Digital mini incubater, VWR®, Portugal) at a temperature of 28 ºC, for 2 to 8 weeks.

2.2. Isolation of endophytic bacteria

For the isolation of endophytic bacteria, samples of stems and leaves of *D. cymosum* were first subjected to a surface sterilization procedure. The protocol used was adapted from Qin et al. (2009) and Zhao et al. (2011) and consisted in the following steps: samples were initially washed with dH$_2$O, after which were transferred to tubes containing ca. 50 mL of dH$_2$O and sonicated for 10 min at 150 W to remove soil particles and loosely adhered microorganisms; samples were then submitted to a sequential surface sterilization procedure consisting in the immersion of the samples for 10 min in a solution of NaOCl (5%), followed by a 5 min wash in dH$_2$O, a 5 min wash in 75% of ethanol, a 5 min wash in dH$_2$O, a 10 min wash in 10% NaHCO$_3$ and, finally, a three times rinse (2 min each) in dH$_2$O. All sterilization steps were performed in a laminar flow chamber.

For each sterilised sample, the effectiveness of surface sterilization was checked by plating 100 µL of the final rinse water onto PCA medium and incubating the plates at 28 ºC for a 8 weeks period.

Surface-desinfected tissues of stems and leaves were dried in a laminar flow chamber and aseptically cut into pieces of about 2 cm long. For stem samples, outer tissues were aseptically removed using a sterile scalpel. The resulting fragments were then subjected to one of the following pretreatments:

- Pretreatment 1: Fragments of stems and leaves were plated without additonal treatment, in duplicate, on the selective media SCN, TWYE and RH and on the non-selective medium PCA, and incubated at 28 ºC for 2 to 8 weeks.

- Pretreatment 2: Fragments of stems and leaves were aseptically crumbled into small fragments, using a sterile mortar, and plated, in duplicate, on SCN, TWYE, RH and PCA media. Plates were incubated at 28 ºC for 2 to 8 weeks.

- Pretreatment 3 (Adapted from Qin et al., 2009): Fragments of stems and leaves were mixed in a sterile mortar with 0.01 g of sterile CaCO3, which modifies the pH to a more alkaline environment favoring the growth of *Actinobacteria*, and then transferred to a Petri dish, to which 2 mL of deionized water were added to keep the sample moisty. Petri dishes were incubated at a temperature of 28 ºC, for 5 weeks. After this period, samples were serially diluted to 10^{-3} with a 0.85% saline solution and plated, in duplicate, on SCN, TWYE, RH and PCA. Plates were incubated at 28 ºC for 1 to 2 weeks.

2.3.   Isolation of soil bacteria

For the isolation of the culturable bacterial community, with special emphasis on the actinobacterial community, from the soil samples collected in the vicinity of *D. cymosum* plants, 4 pretreatment methods were used.

Pretreatment 1 consisted in suspending 1 g of soil in 9 mL of a 0.85% saline solution and vortexing for 1 min at maximum speed. The resulting suspension was then serially diluted to 10^{-3} with saline solution and each dilution was plated, in duplicate, in the selective media SCN, TWYE and RH. Plates were incubated at 28 ºC for 2 to 8 weeks.

Pretreatment 2 consisted in making a soil suspension as in pretreatment 1 and incubating it at 60 ºC for 5 min, which selects *Actinobacteria* since this group of microorganisms have the ability to form spores, thus resisting to this temperature. The suspension was then diluted and plated as described in pretreatment 1.

Pretreatment 3 was similar to pretreatment 2 except that instead of incubating soil suspension at 60 ºC, this was mixed with nalidixic acid (1 mg/L) and cycloheximide (1 mg/L) and incubated at 28 ºC for 30 min.

Pretreatment 4 consisted in mixing in a sterile mortar 1 g of soil with 0.5 g of sterile CaCO3. The mixture was then transferred to a Petri dish to which 3 mL of deionized water were added. The Petri dish was incubated at 28 ºC during 5 weeks, after which the mixture was diluted and plated as described in pretreatment 1 and the plates were incubated for 1 to 2 weeks.

2.4. Purification and preservation of the different microbial isolates

Microbial colonies grown on the different media were analyzed based on their morphological characteristics, such as form, elevation, margin, dimension and brightness. Colonies with distinct morphologies were purified by repetitive streaking onto the respective growth media.

The different isolates were preserved in glycerol (final concentration in the culture, 18%), at -80 ºC, for further studies. Colonies of each isolate were picked up with a 10 µL loop (whenever possible, a full loop was collected) and suspended in 1 mL of saline solution (0.85%) to which 270 µL of a 85% glycerol solution was added.

2.5. Extraction of DNA for identification of the microbial isolates

For the extraction of DNA from the different microbial isolates, colonies of each isolate were picked up with a loop and suspended in 500 µL of sterile ultrapure water. The biomass was preserved at -20 ºC until DNA extraction.

Genomic DNA was extracted using a standard phenol-chloroform method (Sambrook et al., 1989) with some modifications. Five hundred microliters of STE buffer 1x (Sodium Chloride–Tris-EDTA, pH 7.8 ± 0.2) were added to the collected biomass of each isolate, together with 25 µL of sodium dodecyl sulfate (SDS) 20%. The mixtures were then incubated at 95 ºC for 10 min, and placed afterwards at -80 ºC for 5 min. The samples were then treated with 10 µL of Proteinase K (20 mg/mL) and incubated overnight at 56 ºC, in a thermomixer *(Eppendorf Thermomixer® compact, Eppendorf*, USA) with agitation at 300 rpm. After the incubation period, 20 µL of NaCl (5 M) were added to the tubes and the mixtures were transferred to new 2 mL tubes containing *Phase Lock gel* (*5PRIME*, Germany). Then, 575 µL of phenol:chloroform:isoamyl alcohol solution (25:24:1) (*SIGMA*, USA) were added and mixed by inverting the tubes. Tubes were centrifuged at 14.000 g for 3 min and the supernatants were collected into new 2 mL tubes containing *Phase Lock gel*. Equal volume of chloroform:isoamyl alcohol solution (24:1) (*SIGMA*, USA) was added to each tube and mixed by inversion. After centrifugation (14.000 g, 3 min), the top aqueous layer containing the DNA was collected and transferred to 2 mL Eppendorf tubes. One

milliliter of ethanol 96%, at -20 ºC, was added and the tubes were placed at -80 ºC for at least 15 min. After centrifugation (14.000 g, 15 min, 4 ºC), the 96% ethanol was discarded and the DNA pellets were washed with 1 mL of 70% ethanol, which was eluted after centrifugation (14.000 g, 5 min). The DNA pellets were air dried under a laminar flow chamber and resuspended in 50 µL of DNA-free sterile H$_2$O and stored at -20 ºC.

All DNA extractions were performed in sterile workspaces and negative controls were always carried out to verify the non-existence of contaminants in any stage of the extraction.

2.6.　　　Identification of the microbial isolates

Taxonomic identification of the different microbial isolates was performed through amplification of the 16S ribosomal RNA (rRNA) gene, for bacterial isolates, and of the internal transcribed spacer region (ITS), for fungal isolates.

16S rRNA gene was amplified using the universal primers 27F (5'–AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTAC GACTT- 3') (Lane, 1991; Weisburg et al., 1991), and the ITS region was amplified with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (White et al., 1990). The PCR mixtures included in a final volume of 10 µL: 5 µL of *Taq PCR Master Mix Kit* (*Qiagen*, Germany), 2 µL of DNA-free sterile H$_2$O, 1 µL of forward primer (2 µM), 1 µL of reverse primer (2 µM) and 1 µL of template DNA. Some reactions were carried out in a final volume of 20 µL. In all PCRs, the template DNA was used with a dilution of 10x.

All PCR mixtures were carried out in sterile workspaces and negative controls were always performed.

PCRs were performed in a thermocycler (*Veriti™ Thermal Cycler, Applied Biosystems,* Portugal) and the PCR conditions used are described in Table1.

Table 1. PCR conditions used for the amplification of the 16S rRNA gene, for bacterial isolates, and of the internal transcribed spacer region (ITS), for fungal isolates

Cycle step	Bacterial isolates			Fungal isolates		
	Temperature	Time	No. of cycles	Temperature	Time	No. of cycles
Initial extension	95 ºC	15 min	1	95ºC	15 min	1
Denaturation	94 ºC	30 s		94ºC	30 s	
Annealing	48 ºC	90 s	30 - 35	56ºC	90 s	35
Extension	72 ºC	2 min		72ºC	2 min	
Final extension	72 ºC	10 min	1	72ºC	10 min	1

The PCR products were analyzed in 2% agarose gels stained with *SYBR Safe* (*ThermoFisher Scientific*, USA). Amplified DNA was visualized under UV light (*Molecular Imager® ChemiDocTM XRS Imaging System, Bio Rad*, USA) with the software *Image Lab* v4.1 (*Bio Rad*, USA).

Sanger sequencing of the amplified DNA fragments was performed at GATC Biotech (European Genome and Diagnostics Centre, Constance, Germany) and I3S (Institute for Research and Innovation, Porto, Portugal), with the same primers used in PCR amplifications.

For the treatment of the sequences provided by GATC and I3S, the forward and reverse sequences were assembled using the *Geneious* v4.8.2 software and the extracted consensus sequence was used as query in the *Basic Local Alignment Search Tool* (BLAST - http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the database nucleotide collection, of the National Center for Biotechnology Information (NCBI), using a *Max Target Sequences* of 1000. In some cases, the database EzTaxon (http://www.ezbiocloud.net/eztaxon/database) was also used.

3. Metagenomics analysis of the microbial community of *D. cymosum*

3.1. Extraction of DNA from samples of stems and leaves

Samples of stems and leaves of *D. cymosum* were aseptically reduced to a fine powder in a mortar, using liquid nitrogen. Aliquots of tissues powder were then used for DNA extraction for metagenomics analysis.

DNA was extracted from 0.2 g of the powder resultant from the stem samples and from 0.6 g of the powder resultant from the leaves, using the SDS-based method (Maropola et al., 2015). Briefly, RNase A (50 µg/mL, final concentration) and 500 µL of lysozyme buffer (25 mM Tris-HCl; 50 mM glucose; 10 mM EDTA; 25 mg/mL lysozyme) were added to the powdered samples and vortexed for 20 s. The mixtures were incubated at 37 ºC for 1 h and treated afterwards with proteinase K (1 mg/mL, final concentration) at 37 ºC for 1 h. SDS (1%, final concentration) was then added, mixed by inverting the tubes and incubated at 65 ºC, for 30 min. Tubes were centrifuged (14.000 g, 2 min) and the supernatants collected into new tubes. An equal volume of phenol was added, mixed by inversion and centrifuged (10.000 g, 1 min). The supernatant containing the DNA was collected and the phenol extraction was repeated once more. An equal volume of chloroform/isoamyl alcohol solution (24:1) was added, mixed by inversion and centrifuged (10.000 g, 10 min). The top aqueous layer containing the DNA was transferred into new tubes that were placed on ice and an equal volume of ice-cold isopropanol was added to the tubes and incubated at 4 ºC, for 20 min. After centrifugation (10.000 g, 5 min), the isopropanol was discarded and the DNA pellets were air dried under a laminar flow chamber. Then, the DNA pellets were washed twice with 250 µL of 70% ethanol, which was removed after centrifugation (10.000 g, 5 min). DNA pellets were air dried under a laminar flow chamber and stored at -20 ºC.

For metagenomics analysis, DNA pellets were shipped to Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China, where metagenomics analysis were performed.

3.2.   Extraction of DNA from soil samples

DNA from soil samples collected in the neighborhood of *D. cymosum* plants was extracted from 0.5 g of soil, using the commercial kit, PowerSoil® DNA Isolation Kit from MOBIO Laboratories, Inc., according to the manufacturer's instructions.

The extracted DNA was then shipped for metagenomics analysis, as previously described.

3.3.   Metagenomics analysis

Metagenomics analysis was performed using the Illumina MiSeq sequencing. The primers pair 515F–907R (12 bp sample – specific adaptor sequence was attached to the 5' – end of the primer 515F) were used for the amplification of the V4–V5 region, for the 16S rRNA gene, while for the 18S rRNA gene, the primers were EUK528F-EUK706R.

PCR was performed with TaKaRa Premix (TaKaRa, Japan) in a final volume of 50 µL: 25 µL of TaKaRa Premix Taq, 18.5 µL of PCR water, 2 µL of each primer (10 mM) and 2.5 µL of template DNA. The amplification was performed at 94 ºC for 3 min, followed by 30 cycles at 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 45 s and a final extension at 72 ºC for 5 min. PCR products were verified on 1.2% agarose gel stained with Goldview and were purified using a MiniBEST DNA Fragment Purification Kit Ver. 3.0 (TaKaRa, Japan). Then, PCR products were quantified by a NanoDrop ND – 1000 spectrophotometer and mixed at an equimolar ratio.

Subsequently, the library was established using TruSeq Nano DNA LT Sample Prep Kit Set A (24 samples) and sequencing followed the manufacturer's protocol by MiSeq Reagent Kit v3 (600 cycles).

The data was then processed using QIIME (Caporaso et al., 2010) and UPARSE (Edgar, 2013) and by unique barcode the sequences were demultiplexed allowing no mismatch. Each sample was joined by FLASH (Magoč and Salzberg, 2011), using default parameters. The high quality reads were clustered and filtered to avoid chimeras (similarity of 97%) with UPARSE, while reads with lower quality were discarded. Then, one representative sequence of each operational taxonomic unit (OTU) was chosen and taxonomic identified, using SILVA 123 database (Quast et al., 2013), with assign_taxonomy.py, in QIIME.

4. Investigation of the presence of the fluorinase gene in the microbiome of *D. cymosum* plants

4.1. Primer design

PCR primers were designed in order to screen the presence of the fluorinase gene in the microbiome of the *D. cymosum* plants. All sequences of the fluorinase gene available in GenBank were downloaded, and belonged to the following six microorganisms:

- *Actinoplanes sp.* N902-109 - NC_021191.1
- *Nocardia brasiliensis* ATCC 700358 - CP003876.1
- *Streptomyces sp.* MA37 - HG428740.1
- *Streptomyces xinghaiensis* - HG975299.1
- *Streptomyces cattleya* - AJ581748.1
- *Streptomyces cattleya* NRRL 8057 = DSM 46488 - NC_016111.1

The sequences were aligned using the *Muscle* software (Edgar, 2004) available in the *Geneious* program.

Four conserved regions were selected in the multiple sequence alignment for the placement of six PCR primers, as shown in Figure 8.
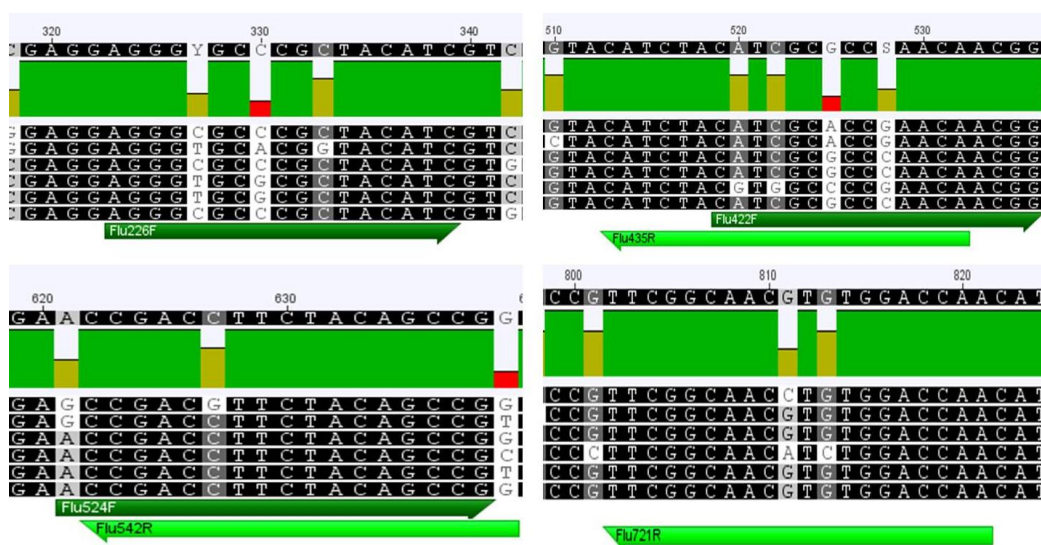
Figure 8. Conserved fluorinase regions used for the design of forward (Flu226F, Flu422F and Flu524F) and reverse (Flu435R, Flu542R and Flu721R) primers, for the amplification of the fluorinase gene.

With the *Geneious* program and also with the help of the Oligo Calc website (http://biotools.nubic.northwestern.edu/OligoCalc.html), the primers were tested in order to have a size between 17 and 25 nt and a melting temperature between 58 ºC and 60 ºC, in order to avoid the formation of secondary structures and/or primer-dimers. Also, the primers were designed to have a GC content of 50% to 60% and to avoid repeats of the nucleotides guanine and cytosine more than three times (Table 2).

Table 2. Sequences of the primers designed for the amplification of the fluorinase gene and respective characteristics

Primer's name	Primer's direction	Sequence (5' - 3')	Melting temperature (°C)
Flu226F	Forward	AGGGCGCCCGCTACATC	59.8
Flu422F	Forward	CATCGCGCCCAACAACGG	60.8
Flu524F	Forward	ACCGACCTTCTACAGCCG	58.4
Flu435R	Reverse	TGTTGGGCGCGATGTAGATG	60.5
Flu542R	Reverse	CCGGCTGTAGAAGGTCGG	60.8
Flu721R	Reverse	TTGGTCCACACGTTGCCGAA	60.5

4.2.   Efficiency of the designed primers

The specificity of the designed primers was tested with two bacterial strains encoding the fluorinase gene *(Streptomyces cattleya* JCM 4925 and *Streptomyces xinghaiensis* JCM 16958), provided by the Japan Collection of Microorganisms (JCM) (http://jcm.brc.riken.jp/en/).

The lyophilized cultures were revived, according to the JCM instructions, with 0.3 to 0.5 mL of rehydration fluid and grown in YEME medium (yeast extract, 0.3 g; malt extract, 0.5 g; peptone, 0.5 g; glucose, 1 g; $MgCl_2$, 0.1 g; dH_2O, 100 mL)(Kieser T., 2000) supplemented with nalidixic acid (25 µg/mL). DNA from both strains was extracted from 1 mL of bacterial culture using the MasterPure™ Gram Positive DNA Purification Kit (epicentre®), according to the manufacturer's instructions.

The efficiency of the three primer sets was tested by PCR in mixtures containing in a final volume of 5 µL: 2.5 µL of *Taq PCR Master Mix Kit* (*Qiagen*, Germany), 0.5 µL of DNA-free sterile H_2O, 0.5 µL of forward primer (2 µM), 0.5 µL of reverse primer (2 µM) and 1 µL of template DNA. The amplification was performed at 95 ºC for 15 min, followed by 30 cycles at 94 ºC for 30 sec, 56 ºC for 90 sec, 72 ºC for 2 min, and a final extension at 72 ºC for 10 min. The PCR products were visualized as previously described.

4.3. Analysis of the fluorinase gene in the microbiome of *D. cymosum*

In order to detect the presence of the fluorinase gene in the microbiome of *D. cymosum* plants, a multiplex PCR was performed with the primers set Flu226F, Flu435R, Flu524F and Flu721R (Fig. 9).
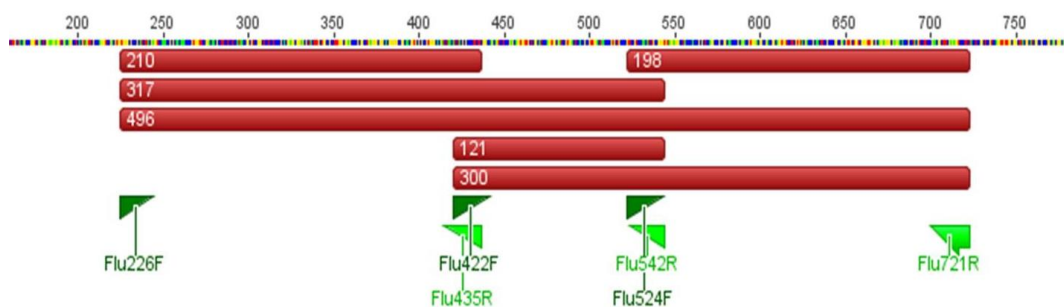
Figure 9. Primers Flu226F, Flu422F, Flu524F, Flu435R, Flu542R and Flu721R, as well as expected size of the amplified products.

Template DNA for the multiplex PCR was obtained in a similar way as the DNA for metagenomics analysis, except that the extracted DNA was resuspended in 20 µL of DNA-free sterile H_2O before being stored at -20 ⁰C. The DNA samples tested for the presence of the fluorinase gene included DNA extracted from stems and leaves of *D. cymosum*, from soil in its vicinity and from all actinobacterial strains isolated in this study and so far identified.

In order to analyze the quality of the DNA extracted from the samples of stems, leaves and soil, amplification of the 16S rRNA gene with the primers set 27F and 1492R was performed in these samples.

PCRs mixtures contained (in a final volume of 5 µL): 2.5 µL of *Taq PCR Master Mix Kit* (*Qiagen*, Germany), 1 µL of DNA-free sterile H_2O, 0.5 µL of primer mix (each primer at 2 µM) and 1 µL of template DNA. The amplification was carried out at 95 ⁰C for 15 min, followed by 40 cycles at 94 ⁰C for 30 sec, 48 ⁰C for 90 sec, 72 ⁰C for 2 min and a final extension at 72 ⁰C for 10 min.

In order to confirm the presence of the fluorinase gene in the amplified products and to discard the hypothesis of non-specific binding, the bands corresponding to amplified products were excised from the 2% agarose gel, using a sterile scalpel, and transferred to sterile Eppendorfs. These were then frozen at -80 ⁰C for at least 20 min, followed by defrosting at 42 ⁰C, after which the gel bands were transferred to sephadex columns, a

gel filtration matrix. The sephadex columns were previously assembled in Eppendorfs with 800 μL of sephadex and centrifuged at 2.200 g for 4 min, to pack down the sephadex, in order to prepare the filtration matrix. The amplified products in the gel bands were then filtrated by centrifugation (4.400 g, 10 min), and the sephadex columns were discarded. Afterwards, 3 μL of glycogen (20 μg/μL), 50 μL of ammonium acetate (10 M) and 500 μL of 100% ethanol were added to the Eppendorfs, centrifuged (14.000 g, 5 min) and the supernatant was discarded. The resultant pellets were washed with 1 mL of 70% ethanol, which was eluted after centrifugation (14.000 g, 5 min), air dried under a laminar flow chamber and resuspended in 12 μL of DNA-free sterile H_2O. The pellets were then stored at -20 ºC.

IV. Results and discussion

1. Culturable microbial community associated with the fluoroacetate producing plant *D. cymosum*

In order to investigate the culturable microbial diversity associated with the FA producing plant, *D. cymosum*, with special emphasis in the actinobacterial community, both epiphytic and endophytic bacteria were studied, as well as the microbial community of the soil in the vicinity of the plant. With this objective, several pretreatments were applied to the samples and a combination of selective and non-selective growth media was employed. Using this approach, a total of 206 isolates were obtained. Figure 10 shows examples of some of these isolates.
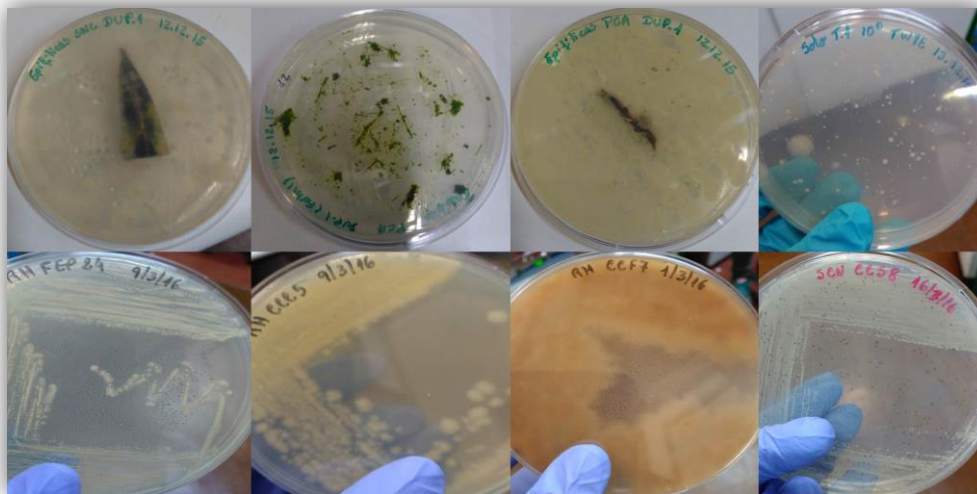
Figure 10. Examples of microbial isolates obtained from samples of leaves and stems of D. *cymosum* and from soil in the vicinity of the plant.

The experimental procedure targeting the isolation of the epiphytic microbial community of *D. cymosum* led to the isolation of 55 strains, with 21 strains being obtained from leaves and 34 from stems. The different pretreatments used for the isolation of endophytic microorganisms led to the recovery of 86 isolates, with 45 being obtained from leaves and 41 from stems. Sixty five isolates were obtained from soil samples (Fig. 11).
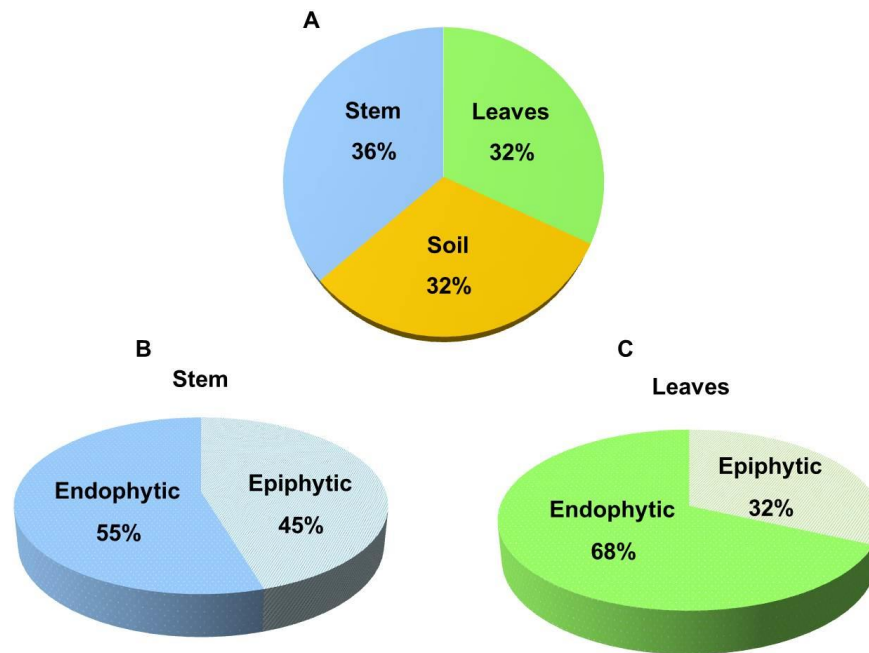
Figure 11. Percentage of isolates obtained from stems and leaves of *D. cymosum* and from the soil in its vicinity (A); Percentage of endophytic and epiphytic isolates obtained from stems (B) and leaves (C) of *D. cymosum*.

From Fig. 11, it is possible to observe that a higher number of endophytic microorganisms were isolated compared to epiphytic microorganisms. However, the surface sterilization protocol used, which targeted the elimination of epiphytic bacteria and the selection of endophytic microorganisms, revealed not to be 100% effective, as some microbial growth was observed on some of the PCA plates inoculated with the final rinse water resultant from the surface sterilization of samples of leaves and stems of *D. cymosum* (7% of the total isolates obtained in the study). The fact that the surface sterilization protocol was not completely effective may have been due to the characteristics of the leaves and stems of the studied plant, especially the latter ones. Stems samples collected from *D. cymosum* plants were very rigid and appeared to have many cavities, which could serve as sheltering for many microorganisms, thus allowing their survival to the sterilization procedure. Due to the obtained results, the endophytic isolates recovered in this study cannot be all considered endophytes.

Several studies reported the effectiveness of the surface sterilization procedure adopted in this study (Qin et al., 2009; Zhao et al., 2011), nonetheless the application of this procedure by Coombs and Franco (2003), resulted in some growth (<5%) from sterilized root tissues and, according to the authors, this may have been due to an

inadequate removal of soil particles. In fact, the stems samples from *D. cymosum* carried some soil particles and even with a sonication step may not have been sufficient for their removal, which may have led to a not completely efficient surface sterilization procedure.

In this study, the investigation of the culturable microbial community associated with *D. cymosum* plants mainly targeted microorganisms belonging to the phylum Actinobacteria, since the production of fluorinated compounds, in particular FA, has only been identified, so far, in this group of microorganisms. Thus, three culture media selective for these microorganisms (SCN, TWYE and RH) were used in order to improve the isolation efficiency of these microorganisms. At the same time, a non-selective medium (PCA) was also used in order to have a general profile of the microbial communities associated with this plant. Only for soil samples, this latter medium was not used due to the expected high microbial diversity present in this type of matrix. The distribution of the obtained isolates in the different culture media is illustrated in Figure 12.
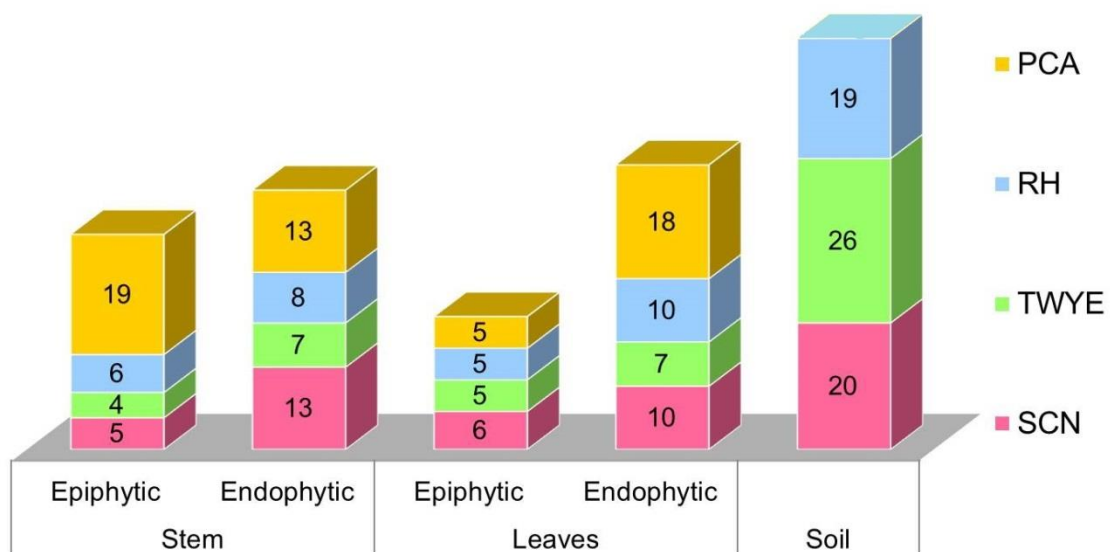
Figure 12. Number of isolates obtained in each of the different culture media.

For stems and leaves samples, the majority of the obtained isolates was recovered from PCA (39% of the total isolates obtained from these samples) and SCN media (24% of the total isolates obtained from these samples). Concerning soil samples, a higher number of isolates was obtained from TWYE medium.

With regard to the pretreatments carried out for the investigation of endophytic and soil microbial communities, the highest number of isolates was obtained with pretreatment 1 (Fig. 13).
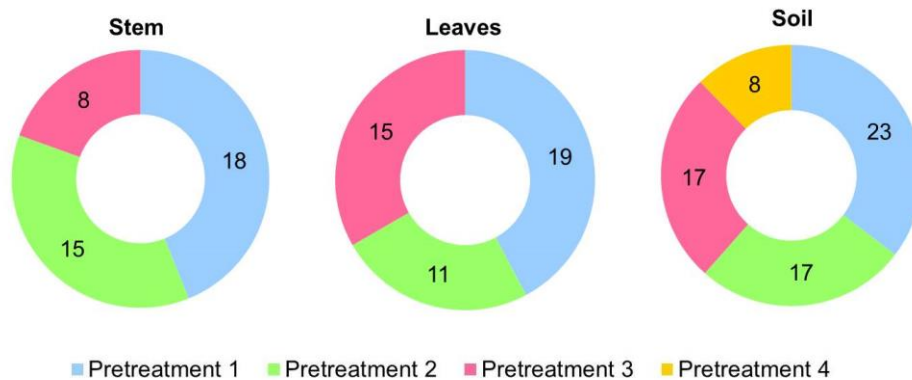
Figure 13. Number of endophytic and soil microbial isolates obtained in the different pretreatments carried out.

Though the comparison of the different pretreatments employed was not the objective of this study and lacks adequate statistic data, the obtained result is not surprising given the fact that samples subjected to pretreatment 1 were not exposed to any selective pressure. In the case of soil samples, pretreatment 4 showed a high selective effect, being isolated only 8 microbial strains by application of this pretreatment.

All the isolated strains were identified through sequencing of the 16S rRNA gene, for the bacterial isolates and ITS region, for fungal isolates. The extraction of genomic DNA from each of the 206 isolates was conducted using a standard phenol-chloroform method, which is widely employed in different types of samples, such as environmental samples (Stach et al., 2003), saliva and animal tissues (Pereira et al., 2010).

16S rRNA gene sequencing has been widely used for several years to analyze phylogenetic and taxonomic relationships among bacteria (Woese, 1987; Weisburg et al., 1991). The functional stability of this gene, together with the presence of conserved regions, its occurrence in all bacteria and its easy sequencing, makes 16S rRNA gene highly valuable for studying the phylogeny and taxonomy of bacteria (Coenye and Vandamme, 2003). On the other hand, for the identification of fungi, sequencing of the ITS region has been largely used due to its higher degree of variation than other regions in the rRNA gene, allowing the selective amplification of fungal sequences (White et al., 1990; De Beeck et al., 2014).

A set of PCR primers extensively used in the identification of a wide range of bacterial strains, 27F and 1492R (Weisburg et al., 1991; Lane, 1991), and fungal strains, ITS1 and ITS4 (White et al., 1990) were applied in order to amplify the taxonomically relevant DNA regions.

After PCR amplification, the amplified products were analyzed in a 2% agarose gel in order to confirm the success of the amplification reactions and if the amplified products had the expected size, i.e. about 1500 bp for bacterial strains and 600 bp for fungal strains, as showed in Figure 14.
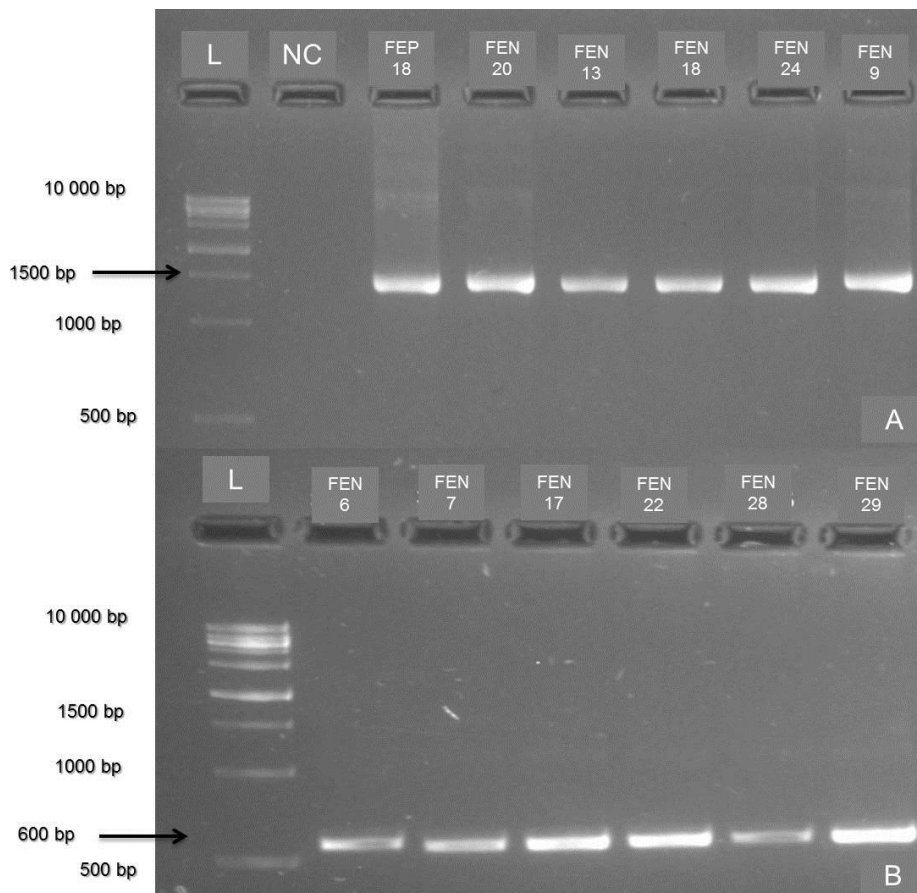
Figure 14. Example of a 2% agarose gel containing amplified DNA products with the expected size, 1500 bp for the bacterial isolates FEP18, FEN20, FEN13, FEN18, FEN24 and FEN9 (A) and 600 bp for the fungal isolates FEN6, FEN7, FEN17, FEN22, FEN28 and FEN29 (B). L – Ladder 1kb. NC – Negative control.

Taxonomic identification of the microorganisms was performed by comparing the obtained consensus sequences with those available in the NCBI and EzTaxon databases (Fig. 15).

A

B

Description	Max score	Total score	Query cover	E value	Ident	Accession
Curtobacterium flaccumfaciens pv. flaccumfaciens partial 16S rRNA gene, strain HPR-2	1275	1275	100%	0.0	100%	HE716950.1
Curtobacterium sp. MOLA 1589 16S ribosomal RNA gene, partial sequence	1269	1269	100%	0.0	99%	KM274017.1
Curtobacterium flaccumfaciens strain EB90 16S ribosomal RNA gene, partial sequence	1269	1269	100%	0.0	99%	KP209415.1

C

Taxonomy	Accession	Pairwise Similarity (%)	Diff/Total nt	Completeness (%)
Bacteria; Actinobacteria; Actinobacteria_c; Micrococcales; Microbacteriaceae; Curtobacterium; Curtobacterium flaccumfaciens;	AJ312209	99.86	1/690	100
Bacteria; Actinobacteria; Actinobacteria_c; Micrococcales; Microbacteriaceae; Curtobacterium; Curtobacterium pusillum;	AJ784400	99.71	2/690	100
Bacteria; Actinobacteria; Actinobacteria_c; Micrococcales; Microbacteriaceae; Curtobacterium; Flavobacterium oceanosedimentum;	EF592577	99.71	2/688	97.6

Figure 15. Taxonomic identification of the isolate FEN 13. Consense sequence of the amplified 16S rDNA obtained by Software *Geneious* v4.8.2 (A). BLAST result (B). EzTaxon result (C).

It was not possible to obtain an amplified DNA product for some isolates, regardless the PCR conditions used. This failure may have been due to inefficiency of the primers used, bad quality of the extracted DNA or the presence of some inhibitor resulting from the phenol-chloroform extraction. In other cases, some isolates were not identified due to the low quality of the electropherograms. In fact, the use of a single combination of PCR primers and a DNA extraction technique does not allow the recovery of all species present in a sample, limiting the knowledge of the microbial diversity (Hong et al., 2009). The fact that, in this study, only an extraction method was employed and one pair of primers was used for bacterial or fungal strains, could have limited DNA extraction or amplification. Therefore, different combinations of PCR primers and extraction methods may lead to the identification of the remaining isolates.

The taxonomic results of the isolates so far identified are indicated in Table 3. From the 206 strains isolated in this study, 100 were taxonomically identified, with 72 isolates being bacteria and 28 being fungi.

Table 3. Taxonomic identification of some of the microbial isolates recovered from the leaves and stems of the FA producing plant, *D. cymosum*, and from the soil in its vicinity

Sample	Origin	Isolate	Taxonomic identification	Similarity	Phylum	Originally isolated from
Stem	**Epiphytic**	CEP11	*Microbacterium sp.*	99%	Actinobacteria	-
		CEP28	*Microbacterium sp.*	100%	Actinobacteria	-
		CEP1	*Bacillus sp.*	99%	Firmicutes	-
		CEP2	*Brevibacillus reuszeri*	99%	Firmicutes	**Soil** (Shida et al., 1995)
		CEP3	*Paenibacillus pabuli*	99%	Firmicutes	**Soil** (Nakamura, 1984)
		CEP4	*Bacillus simplex*	100%	Firmicutes	**Soil** (Priest et al., 1988)
		CEP5	*Paenibacillus sp.*	99%	Firmicutes	-
		CEP6A	*Paenibacillus elgii*	100%	Firmicutes	**Roots of *Perilla frutescens*** (Kim et al., 2004)
		CEP6B	*Paenibacillus elgii*	99%	Firmicutes	**Roots of *Perilla frutescens*** (Kim et al., 2004)
		CEP7	*Paenibacillus pinihumi*	99%	Firmicutes	**Rhizosphere of *Pinus densiflora*** (Kim et al., 2009)
		CEP8	*Cohnella boryungensis*	99%	Firmicutes	**Soil from west coast of the Korean peninsula** (Yoon and Jung, 2012)
		CEP9	*Bacillus bataviensis*	98%	Firmicutes	**Agricultural soil** (Heyrman et al., 2004)
		CEP10	*Bacillus niacini*	98%	Firmicutes	**Soil** (Nagel and Andreesen, 1991)
		CEP19	*Cohnella sp.*	96%	Firmicutes	-

CEP24	*Bacillus sp.*	100%	Firmicutes	-
CEP25	*Bacillus sp.*	99%	Firmicutes	-
CEP26	*Bacillus sp.*	100%	Firmicutes	-
CEP27	*Bacillus sp.*	99%	Firmicutes	-
CEP13	Rhizobium sp.	99%	Proteobacteria	-
CEP17	*Novosphingobium lindaniclasticum*	99%	Proteobacteria	**Dumpsite contaminated with hexachlorocyclohexane** (Saxena et al., 2013)
CEP33	*Rhizobium sp.*	99%	Proteobacteria	-
CEP35	*Rhizobium sp.*	99%	Proteobacteria	-
CEP34	*Pedobacter kyungheensis*	98%	Bacteroidetes	**Soil of a ginseng field** (Yang et al., 2012)
CEP29	*Fusarium neocosmosporiellum*	99%	Ascomycota	**Cotton, watermelon and cowpea** (Smith, 1899)
CEP31	*Fusarium neocosmosporiellum*	99%	Ascomycota	**Cotton, watermelon and cowpea** (Smith, 1899)
CEP15	*Mucor sp.*	99%	*Incertae sedis*	-
Endophytic				
CCC2	*Microbacterium foliorum*	99%	Actinobacteria	**Phyllosphere of grasses** (Behrendt et al., 2001)
CCC4	*Microbacterium foliorum*	99%	Actinobacteria	**Phyllosphere of grasses** (Behrendt et al., 2001)
CEN1	*Bacillus sp.*	99%	Firmicutes	-
CEN2	*Paenibacillus polymyxa*	99%	Firmicutes	**Soil** (Ash et al., 1993)
CEN3	*Bacillus sp.*	99%	Firmicutes	-
CEN13	*Bacillus sp.*	99%	Firmicutes	-

CEN14	*Bacillus sp.*	100%	Firmicutes	-
CEN19	*Paenibacillus pabuli*	99%	Firmicutes	**Soil** (Nakamura, 1984)
CEN20	*Bacillus sp.*	99%	Firmicutes	-
CEN15	*Enterobacter sp.*	99%	Proteobacteria	-
CEN16	*Ochrobactrum grignonense*	99%	Proteobacteria	**Agricultural soils and wheat roots** (Lebuhn et al., 2000)
CEN18	*Pseudomonas azotoformans*	99%	Proteobacteria	**Cereal grains** (Iizuka and Komagata, 1963)
CEN22	*Achromobacter mucicolens*	99%	Proteobacteria	**Human clinical samples** (Vandamme et al., 2013)
CEN27	*Rhizobium sp.*	99%	Proteobacteria	-
CEN40	*Rhizobium sp.*	99%	Proteobacteria	-
CCC1	*Methylobacterium dankookense*	100%	Proteobacteria	**Water** (Lee et al., 2009)
CCC6	*Novosphingobium lindaniclasticum*	99%	Proteobacteria	**Dumpsite contaminated with hexachlorocyclohexane** (Saxena et al., 2013)
CCC9	*Ochrobactrum grignonense*	99%	Proteobacteria	**Agricultural soils and wheat roots** (Lebuhn et al., 2000)
CEN11	*Fusarium oxysporum*	100%	Ascomycota	**Soil** (Schlechtendal, 1824)
CEN30	*Fusarium oxysporum*	100%	Ascomycota	**Soil** (Schlechtendal, 1824)
CEN31	*Fusarium oxysporum*	100%	Ascomycota	**Soil** (Schlechtendal, 1824)

		CEN41	*Talaromyces assiutensis*	99%	Ascomycota	**Egyptian soil** (Samson and Abdel-Fattah, 1978)
Leaves	**Epiphytic**	FEP18	*Microbacterium sp.*	100%	Actinobacteria	-
		FEP16	*Leucobacter chromiiresistens*	99%	Actinobacteria	**German soil** (Sturm et al., 2011)
		FEP24	*Streptomyces sp.*	100%	Actinobacteria	-
		FEP15	*Bacillus sp.*	99%	Firmicutes	-
		FEP17	*Bacillus cereus*	100%	Firmicutes	**Air** (Frankland and Frankland, 1887)
		FEP2	*Sphingomonas phyllosphaerae*	100%	Proteobacteria	**Phyllosphere of *Acacia caven*** (Rivas et al., 2004)
		FEP11	*Cyphellophora sp.*	97%	Ascomycota	-
		FEP14	*Cyphellophora sp.*	97%	Ascomycota	-
	Endophytic					
		FEN13	*Curtobacterium flaccumfaciens*	100%	Actinobacteria	**Beans** (Hedges, 1922)
		FEN18	*Curtobacterium flaccumfaciens*	99%	Actinobacteria	**Beans** (Hedges, 1922)
		FEN23	*Curtobacterium flaccumfaciens*	99%	Actinobacteria	**Beans** (Hedges, 1922)
		FEN24	*Arthrobacter sp.*	99%	Actinobacteria	-
		FEN26	*Streptomyces sp.*	100%	Actinobacteria	-
		FEN27	*Nocardioides simplex*	100%	Actinobacteria	**Soil** (O'Donnell et al., 1982)

FEN30	*Cellulomonas cartae*	100%	Actinobacteria	**Soil** (Stackebrandt and Kandler, 1980)
FEN31	*Streptomyces sp.*	100%	Actinobacteria	-
CCF14	*Microbacterium sp.*	100%	Actinobacteria	-
FEN9	*Paenibacillus pabuli*	99%	Firmicutes	**Soil** (Nakamura, 1984)
FEN10	*Bacillus sp.*	99%	Firmicutes	-
FEN12	*Bacillus cereus*	100%	Firmicutes	**Air** (Frankland and Frankland, 1887)
FEN16	*Bacillus sp.*	99%	Firmicutes	-
FEN19	*Bacillus sp.*	99%	Firmicutes	-
FEN20	*Bacillus sp.*	99%	Firmicutes	-
FEN21	*Bacillus sp.*	99%	Firmicutes	-
FEN11	*Achromobacter animicus*	99%	Proteobacteria	**Human clinical samples** (Vandamme et al., 2013)
CCF7	*Brevundimonas sp.*	100%	Proteobacteria	-
FEN1	*Alternaria sp.*	100%	Ascomycota	-
FEN7	*Alternaria sp.*	100%	Ascomycota	-
FEN17	*Alternaria sp.*	100%	Ascomycota	-
FEN22	*Alternaria sp.*	100%	Ascomycota	-
FEN25	*Alternaria sp.*	100%	Ascomycota	-
FEN29	*Alternaria sp.*	100%	Ascomycota	-
CCF1	*Alternaria sp.*	99%	Ascomycota	-
CCF2	*Alternaria sp.*	100%	Ascomycota	-
CCF3	*Alternaria sp.*	100%	Ascomycota	-
CCF4	*Alternaria sp.*	100%	Ascomycota	-
CCF5	*Alternaria sp.*	100%	Ascomycota	-

CCF6	*Alternaria sp.*	100%	Ascomycota	-
CCF8	*Alternaria sp.*	100%	Ascomycota	-
CCF9	*Alternaria sp.*	99%	Ascomycota	-
CCF10	*Alternaria sp.*	99%	Ascomycota	-
CCF12	*Alternaria sp.*	100%	Ascomycota	-
CCF13	*Alternaria sp.*	100%	Ascomycota	-
CCF16	*Aspergillus sp.*	100%	Ascomycota	-
Soil				
CCS1	*Arthrobacter globiformis*	99%	Actinobacteria	**Soil** (Conn, 1928)
CCS6	*Arthrobacter globiformis*	99%	Actinobacteria	**Soil** (Conn, 1928)
CCS8	*Arthrobacter globiformis*	98%	Actinobacteria	**Soil** (Conn, 1928)
CCS10	*Streptomyces sp.*	99%	Actinobacteria	-
CCS3	*Methylobacterium oxalidis*	99%	Proteobacteria	**Leaves of *Oxalis corniculata*** (Tani et al., 2012)
CCS5	*Methylobacterium oxalidis*	99%	Proteobacteria	**Leaves of *Oxalis corniculata*** (Tani et al., 2012)
CCS7	*Methylobacterium sp.*	99%	Proteobacteria	-
S62	*Beauveria bassiana*	99%	Ascomycota	**Soil** (Vuillemin, 1912)

The identification results presented in Table 3 show that the isolated strains belong to the bacterial phyla Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes and to the fungi phylum Ascomycota. Though the experimental approach and selective media employed in this study mainly focused the isolation of bacteria belonging to the phylum Actinobacteria, it is normal to obtain other groups of microorganisms as isolation methods are not completely selective for a particular microbial group. For instance, the antibiotics added to the isolation media are not 100% effective against Gram-negative bacteria and eukaryotic microorganisms. Also, there is the possibility of some isolates being the same microorganism due to the fact that similar identification results were obtained, which may reduce the actual number of isolates recovered.

Figure 16 presents the distribution over the taxonomic rank phylum of the isolates so far identified, obtained from samples of stems and leaves of *D. cymosum* and from the soil in its vicinity.
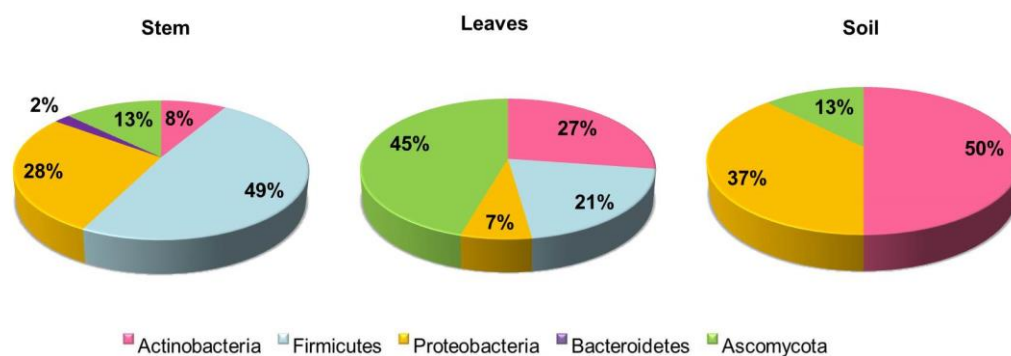
Figure 16. Distribution by phylum (%) of the identified isolates obtained from stems and leaves of *D.cymosum* and from soil in its neighborhood.

Stems of *D. cymosum* showed to have a high percentage of Firmicutes (49%), with the majority of the identified microorganisms recovered from these samples belonging to this phylum, followed by Proteobacteria (28%), Ascomycota (13%) and Actinobacteria (8%). On the other hand, leaves of *D. cymosum* are apparently rich in fungi belonging to the phylum Ascomycota (45%), though many of the recovered isolates may be the same microorganism. In terms of bacteria, the phylum Actinobacteria was the most represented in these samples (27%) followed by Firmicutes (21%). Soil samples collected in the vicinity of *D. cymosum* seem to be very rich in Actinobacteria, with 50% of the microorganisms so far identified belonging to this phylum, while 37% of the isolates were identified as belonging to the phylum Proteobacteria.

As observed in Figure 16, with the experimental approach used in this study and based on the identification results obtained so far, the phyla Actinobacteria, Firmicutes and Proteobacteria were the most significant in terms of microorganisms recovered from the samples of stems and leaves of *D. cymosum* and from the soil in its proximity. Microorganisms belonging to these phyla have been commonly isolated from roots, stems and leaves of other plants as well as from soil samples (Xia et al., 2013; Qin et al., 2009; Qin et al., 2010; Qin et al., 2015; Cui et al., 2014).

The distribution of the identified isolates in terms of genus is represented in Figure 17.
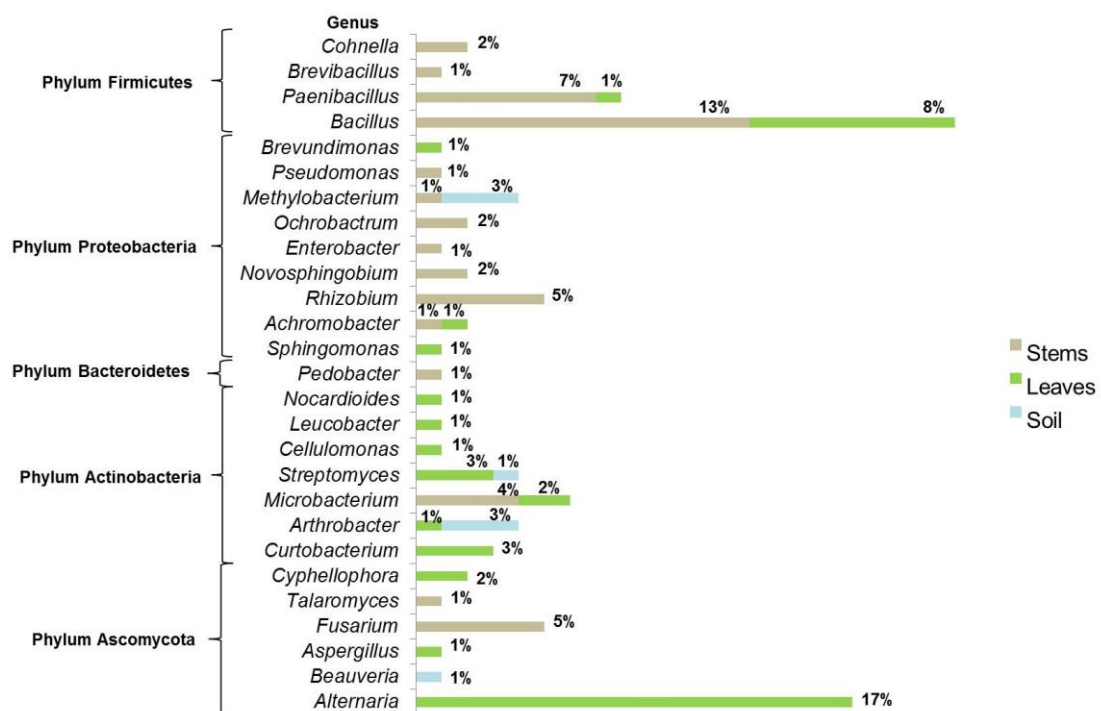
Figure 17. Distribution by genus (%) of the identified isolates obtained from stems and leaves of *D. cymosum* and from the soil in its vicinity.

Regarding the stems and leaves samples obtained from *D. cymosum,* the bacterial genera that mostly represented the analyzed tissues with the adopted experimental approach, were *Bacillus* (21%), *Paenibacillus* (8%), *Microbacterium* (6%), and *Rhizobium* (5%). The genus *Bacillus* is found in a myriad of environments and it is highly related with induced systemic resistance in plants (Kloepper et al., 2004; Choudhary and Johri, 2009). This genus is very common in the rhizosphere of various species of plants, including cotton (Reva et al., 2002), beans (Kumar et al., 2012; Wahyudi et al., 2011), grass (Saile and Koehler, 2006), rice (Madhaiyan et al., 2010),

among others. It is also found in the phyllosphere of plants (Kucheryava et al., 1999) and as an endophytic microorganism (Bai et al., 2002; Cho et al., 2002). *Paenibacillus* like the genus *Bacillus* is also widely distributed, being found in plant roots, soil (Berge et al., 2002) and as an endophyte (Ulrich et al., 2008). In this study, these two genera were isolated from both stems and leaves samples of *D. cymosum*. Members of the genus *Microbacterium* are also widely distributed and have been isolated from diverse matrixes such as soil, plants and water (Krishnamurthi et al., 2012). This genus has been isolated from the phyllosphere of sugar beet and wheat (Legard et al., 1994; Thompson et al., 1993), as well as from the phyllosphere of grasses (Behrendt et al., 2001). The species *Microbacterium foliorum* was isolated in this study from stem samples of *D. cymosum* as an endophytic microorganism, however this species was reported to be originally isolated from the phyllosphere of grasses (Behrendt et al., 2001), suggesting that the strains isolated in this study can actually be epiphytic bacteria that may have resisted to the surface sterilization procedure. The genus *Rhizobium* is frequently associated with the formation of nodules on the roots of plants, forming a symbiotic relationship that allows the assimilation of nitrogen by the plant (Gage, 2004; Masson-Boivin et al., 2009). These bacteria can also grow and live freely in the soil or induce the formation of nodules in stems, which explains the results obtained in this study, due to the fact that *Rhizobium* sp. was isolated from stem samples of *D. cymosum*.

Since the present study focused on the isolation of microorganisms belonging to the phylum Actinobacteria, it was expected to obtain a higher percentage of these microorganisms, as the isolation methods employed were selective for this microbial group. However, it is important to notice that only about 50% of the isolates have been so far identified, and that the number of Actinobacteria may increase once all of these isolates are identified. Isolates belonging to this phylum were predominantly identified as *Microbacterium* sp., *Curtobacterium* (3%) and *Streptomyces* (3%). The genus *Curtobacterium* has been found both in epiphytic (Elbeltagy et al., 2000) and endophytic (Ercolani, 1991; Huang et al., 2006) tissues of plants. In this study, *Curtobacterium flaccumfaciens,* a species highly related with the bacterial wilt of beans, was isolated as an endophyte from the leaves of *D. cymosum*.

It is well known that the genus *Streptomyces* represents the largest antibiotic producers (Chater, 2006), and that these microorganisms are commonly found in soil (Cui et al., 2014). However an increasing number of studies have been showing that these bacteria can also be recovered from a variety of environments, including marine environments. In fact, *Streptomyces* have already been found in fishes, molluscs, seawater, sediments, sponges, among others (Pimentel-Elardo et al., 2010; Dharmaraj,

2010). This genus has also been recovered as endophytes of different plant tissues, such as tomatoes roots (Cao et al., 2004), several medicinal plants (Qin et al., 2009; Zhao et al., 2011; Qin et al., 2015), tropical plants (Janso and Carter, 2010), among others. Endophytic *Streptomyces* are more frequently recovered from root tissues, but can also be present in leaves. In this study, several *Streptomyces* species were found in the leaves of *D. cymosum.*

The bacterial genera that mostly represented the soil collected in the proximity of *D. cymosum* were *Arthrobacter* (3%) and *Methylobacterium* (3%). Microorganisms belonging to the *Arthrobacter* genus are commonly found in soil (Jones and Keddie, 2006), but have also been found in the phyllosphere of various plants (Scheublin and Leveau, 2013) and as endophytes of stems of cotton (Mcinroy and Kloepper, 1995) and black pepper (Aravind et al., 2009). In the present study, the species *Arthrobacter globiformis* was recovered from the soil collected in the neighborhood of *D. cymosum*, and one isolate identified as *Arthrobacter sp.* was found as an endophyte of *D. cymosum* leaves. The genus *Methylobacterium* is also very common in soil (Cao et al., 2011), but can be also found as well in water (Lee et al., 2009) and in plants, as epiphytic or endophytic microorganisms (Tani et al., 2012; Knief et al., 2012; Araújo et al., 2002). In fact, the *Methylobacterium* species isolated in this study were recovered from the soil in the proximity of the plant *D. cymosum* and from its stems.

The experimental approach used in this study also led to the isolation of some fungal strains and it is interesting to notice that the majority of the identified fungi belong to the genus *Alternaria* (17%), that was recovered from the leaves of *D. cymosum* (Fig. 17), and is known as a pathogenic endophytic fungus (Akimitsu et al., 2014). It was also possible to isolate from the stems of *D. cymosum,* one of the top 10 fungal pathogens, *Fusarium oxysporum* (Dean et al., 2012), that comprehended 5% of the total genera identified. It is not known if these microorganisms were exerting a pathogenic effect in the plant, as the leaves and stems used for microbial analysis apparently had, at a first sight, a healthy appearance.

2. Metagenomics analysis of the microbial community associated with *D. cymosum*

Nearly 99% of microorganisms in the environment cannot be cultured in the laboratory, which leads to approximately 1% that can be cultivated under laboratory conditions, deeply limiting the knowledge of microbial communities in their natural environments (Schloss and Handelsman, 2005). In this context, metagenomics analysis of leaf and stem samples of *D. cymosum* and of soil in its proximity was performed in order to get a whole view of the microbial community associated with these plants.

However, it was only possible to obtain metagenomics results for the sample of soil, as the DNA extracted from stems and leaves did not have enough quality for the metagenomics analysis, probably due to the presence of substances derived from the plant that inhibited the PCR.

The metagenomics results obtained for the soil sample are presented in Figures 18, 19 and 20 and Table 4.

Figure 18. Relative abundance (%) of bacterial phyla obtained by metagenomics analysis of a soil sample collected in the neighborhood of *D. cymosum* (only orders with a relative abundance > 0.2% are shown).

As observed in Figure 18, there is a wide diversity of bacterial phyla associated with the soil in the vicinity of the FA producing plant *D. cymosum*. The major phylum present in the soil is Actinobacteria, with an abundance of 37%, followed by Proteobacteria, representing 17% of the whole community. Interestingly, the bacterial strains isolated in this study from the same soil sample, and so far identified, also belong to these two phyla.

Figure 19 shows that the 37% of microorganisms belonging to the phylum Actinobacteria are distributed in five orders.
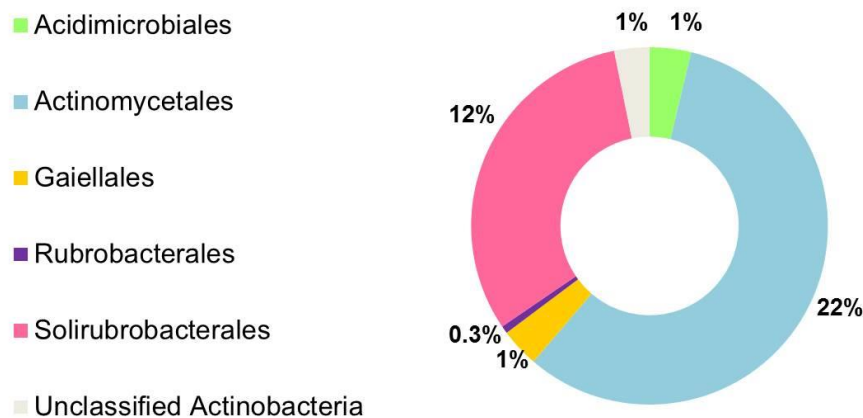
Figure 19. Relative abundance (%) of Actinobacteria orders obtained by metagenomics analysis of a soil sample collected in the neighborhood of *D. cymosum*.

Actinomycetales showed the highest relative abundance (22%) followed by Solirubrobacterales (12%). The actinobacterial strains isolated in this study from the soil in the vicinity of *D. cymosum* plants also belong to the Actinomycetales order.

Considering the 17% of microorganisms belonging to the phylum Proteobacteria, Figure 20 shows that these are distributed by 6 major orders.
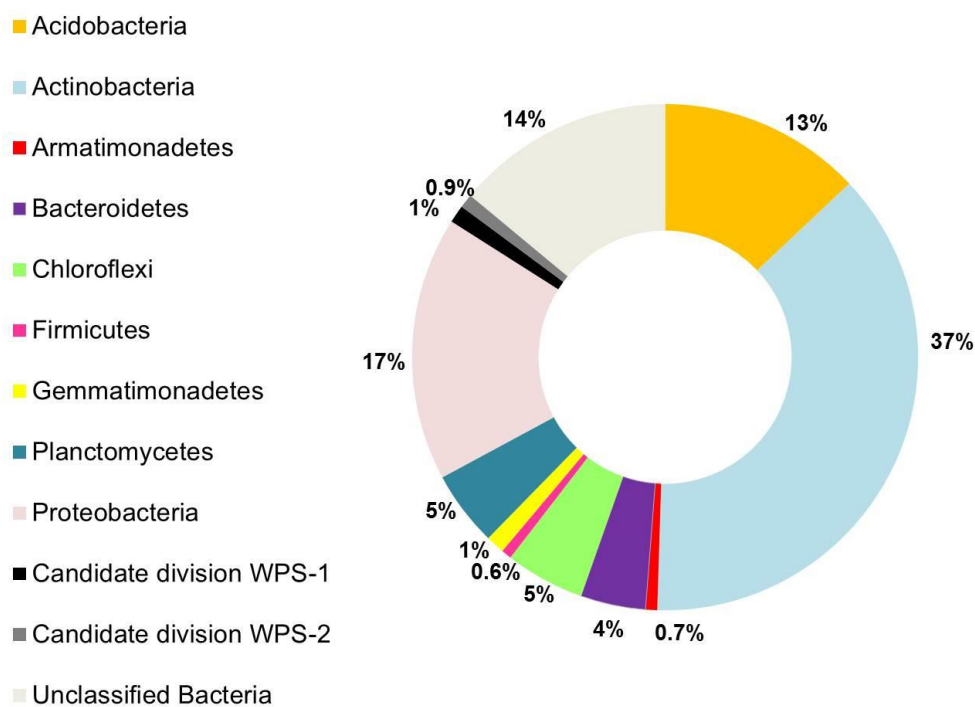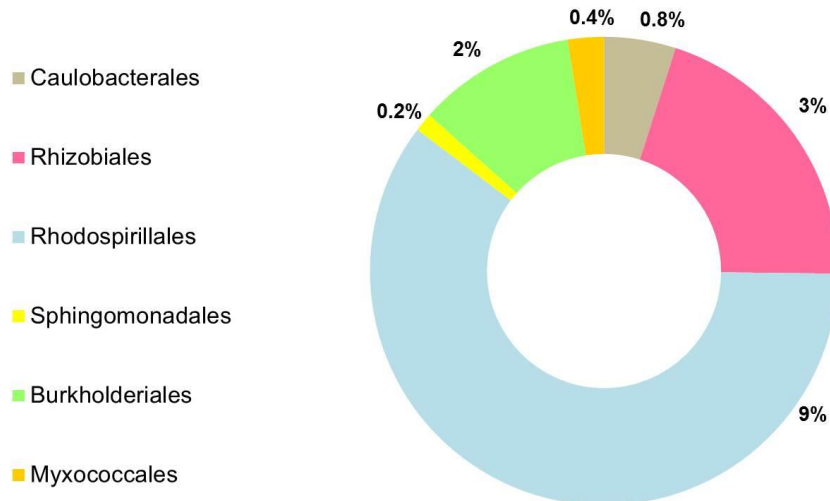
Figure 20. Relative abundance (%) of Proteobacteria orders obtained by metagenomics analysis of a soil sample collected in the neighborhood of *D.cymosum* (only orders with a relative abundance > 0.2% are shown).

The order Rhodospirillales showed the highest relative abundance (9%) followed by Rhizobiales (3%) and Burkholderiales (2%). All the other orders were represented with a relative abundance of less than 0.2%. The Proteobacteria strains isolated in this study from the soil sample of *D. cymosum* and identified as belonging to the *Methylobacterium* genus are also integrated in the Rhizobiales order.

Other metagenomics analyses of microbial communities present in soils have shown that, likewise the soil sample analysed in this study, Actinomycetales, Rhodospirillales, Rhizobiales and Burkholderiales are the most representative, being found in a myriad of environments, such as forest, tundra, rainforest and agricultural soils (Montana et al., 2012; Verastegui et al., 2014).

Metagenomics analysis of the soil sample in the vicinity of *D. cymosum* allowed the identification of the bacterial genera *Blastococcus, Geodermatophilus, Mycobacterium, Pseudonocardia, Gaiella, Flavisolibacter, Ktedonobacter* and *Gemmatimonas* (Table 4)*. None of these genera has been so far recovered from this soil sample through culturable dependent analysis. Also, several genera of fungi have been identified, with *Phyllosticta, Boeremia* and *Knufia* constituting the most abundant ones. It is interesting to notice that all of these genera include pathogenic microorganisms. *Phyllosticta* is a plant pathogen known to cause various fruit diseases, as the citrus black spot (Wikee et al., 2011; Glienke et al., 2011). Recently, the genus *Boeremia* was also reported to be associated with the black spot disease in plants (Li et al., 2015), while the genus *Knufia* is a conidial fungi that forms black colonies and it is related with the black knot disease (Hutchison et al., 1995).

Table 4. Metagenomics profile of a soil sample collected in the neighborhood of *D. cymosum* showing the relative abundance of each taxonomic group in the soil (relative abundances below 1% were not considered)

Phylum	Class	Order	Family	Genus	Relative abundance (%)
Acidobacteria	Acidobacteria	Unclassified Acidobacteria			10.37
Actinobacteria	Actinobacteria	Acidimicrobiales	Unclassified Acidimicrobiales		1.12
		Actinomycetales	Geodermatophilaceae	*Blastococcus*	1.05
				Geodermatophilus	2.36
				Unclassified Geodermatophilaceae	1.09
			Mycobacteriaceae	*Mycobacterium*	2.22
			Pseudonocardiaceae	*Pseudonocardia*	1.57
			Unclassified Actinomycetales		8.99
		Gaiellales	Gaiellaceae	*Gaiella*	1.31
		Solirubrobacterales	Unclassified Solirubrobacterales		10.89
	Unclassified Actinobacteria				1.04
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Flavisolibacter*	1.61
				Unclassified Chitinophagaceae	2.13
Chloroflexi	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	*Ktedonobacter*	1.51
		Unclassified Ktedonobacteria			2.47
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	1.20
Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	*Unclassified Planctomycetaceae*	4.23
Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclassified Rhizobiales		1.44
		Rhodospirillales	Acetobacteraceae	*Unclassified Acetobacteraceae*	7.11
			Unclassified Rhodospirillales		1.13

Candidate division WPS-1	Unclassified candidate division WPS-1				1.13
Unclassified Bacteria					13.93
Opisthokonta	Nucletmycea	Fungi	Dothideomycetes	*Capnodiales*	1.01
				Pleosporales	1.35
				Uncultured	1.26
			Eurotiomycetes	*Chaetothyriales*	1.49
				Eurotiales	1.24
			Leotiomycetes	*Helotiales*	3.23
			Botryosphaeriaceae	*Phyllosticta*	8.82
			Chaetomiaceae	*Unclassified Chaetomiaceae*	7.25
			Didymellaceae	*Boeremia*	8.12
			Herpotrichiellaceae	*Phialophora*	1.43
			Incertae Sedis	*Knufia*	12.37
			Lophiostomataceae	*Myrothecium*	2.78
				Lophiostoma	1.33
			Nectriaceae	*Unclassified Nectriaceae*	1.57
			Trichocomaceae	*Aspergillus*	1.60
			Unclassified Fungi		10.80

3. Presence of fluorinase gene in the microbial community associated with *D. cymosum*

The possible presence of the fluorinase gene in the microbiome of the FA producing plant *D. cymosum* was investigated by PCR using primers designed within the conserved region of fluorinase gene. The sequence from *Streptomyces cattleya* NRRL 8057 = DSM 46488 - NC_016111.1 was used as reference, since this is the first microorganism known to have the capacity to produce the enzyme fluorinase. Four conserved regions were found in the alignment which allowed the design of six primers, three forward (Flu226F, Flu422F and Flu524F) and three reverse (Flu435R, Flu542R and Flu721R).

In order to confirm the specificity of the designed primers, each pair of primers was tested individually by conducting a PCR with two bacterial strains, known to carry the fluorinase gene (*Streptomyces cattleya* JCM 4925 and *Streptomyces xinghaiensis* JCM 16958) (Fig. 21).
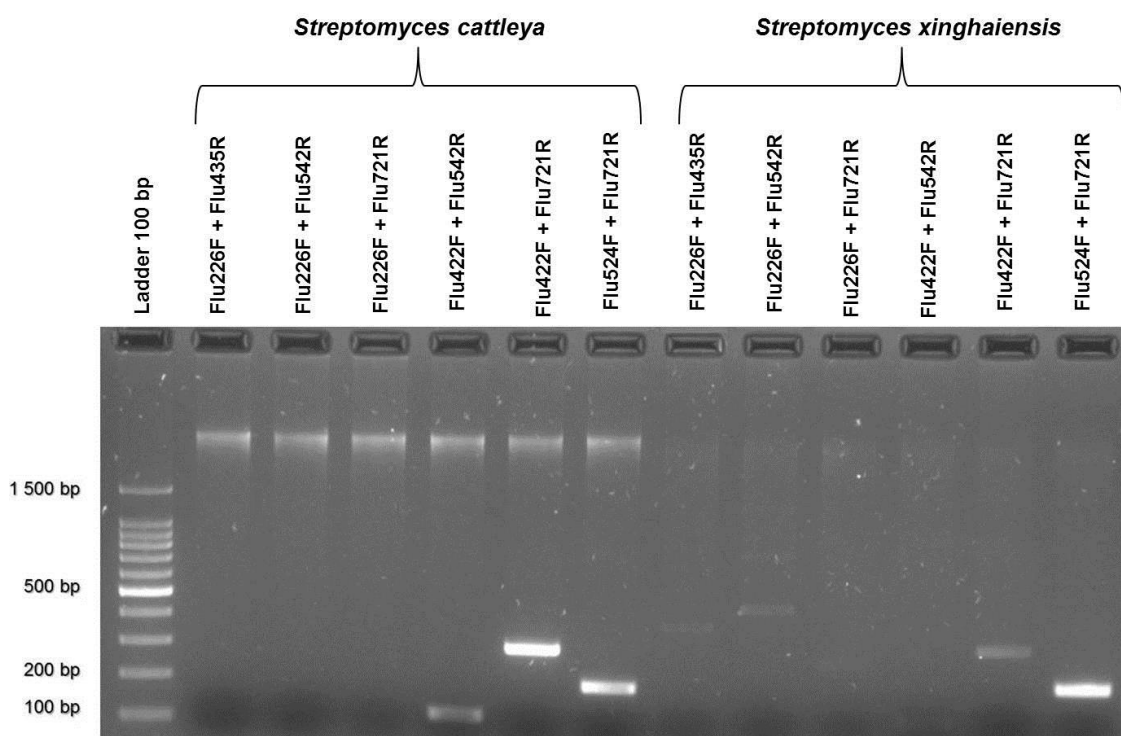
Figure 21. Agarose gel with PCR products obtained with the designed fluorinase primers (Flu226F, Flu422F, Flu524F, Flu435R, Flu542R and Flu721R) and DNA (with dilution 1/10) of *Streptomyces cattleya* and *Streptomyces xinghaiensis*.

As observed in Figure 21, the primers Flu422F, Flu524F and Flu721R seemed to be more efficient since there was amplification in both bacterial species.

Nonetheless, in order to investigate the presence of the fluorinase gene in the microbiome of *D. cymosum*, the primers set Flu226F, Flu435R, Flu524F and Flu721R were used in a multiplex PCR. The use of the primer set Flu226F-Flu435R instead of Flu422F-Flu721R, was due to the attempt to amplify the whole fluorinase gene, as the primer set Flu524F-Flu721R was already amplifying almost half of the gene.

The multiplex PCR was performed with DNA extracted from samples of stems and leaves of *D. cymosum* and from the soil in its proximity. The quality of the DNA samples was analyzed by amplification of the 16S rRNA gene with the primers 27F-1492R. The results obtained are illustrated in Figure 22.
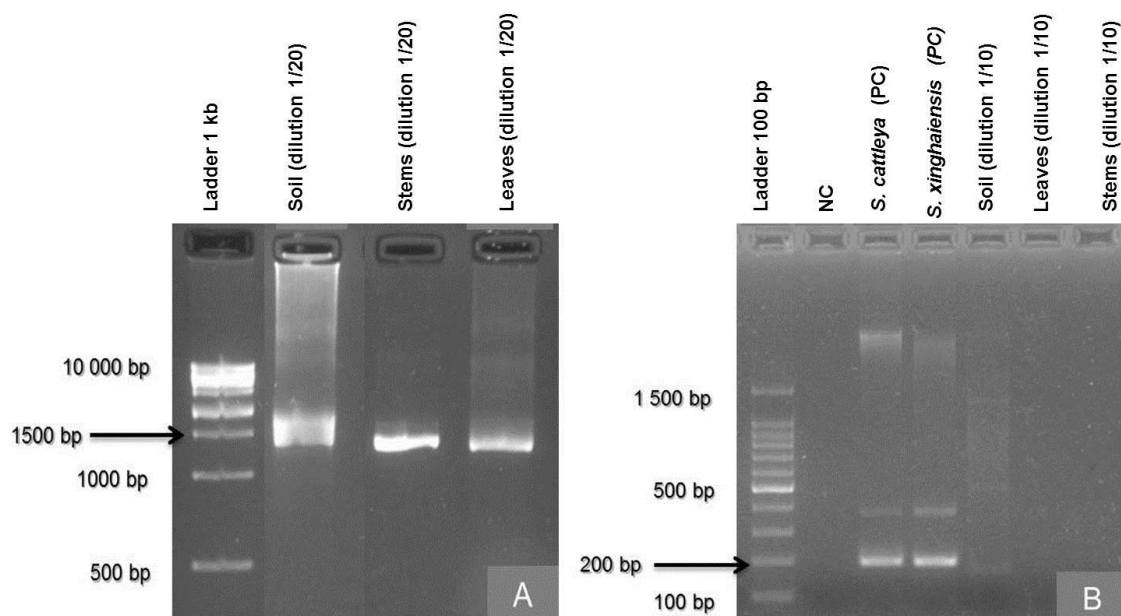
Figure 22. Agarose gel with PCR products obtained with DNA samples of leaves and stems of *D. cymosum* and of soil in its vicinity, as well as DNA (with dilution 1/10) of *Streptomyces cattleya* and *Streptomyces xinghaiensis.* (A) PCR with 27F and 1492R primers. (B) Multiplex PCR with the primers Flu226F, Flu435R, Flu524F and Flu721R; NC – Negative control; PC – Positive control.

Amplification with the 16S rRNA gene primers, 27F and 1492R, showed that the DNA extracted from the samples of soil, stem and leaves of *D. cymosum* had enough quality for the analysis of the presence of the fluorinase gene, since there was amplification of bacterial DNA on those samples. However, the results obtained with the multiplex PCR did not indicate the presence of the fluorinase gene in the analyzed

samples of *D. cymosum*. Nonetheless, further experiments should be conducted, with different primers, conditions and/or DNA from additional samples of this plant.

The multiplex PCR was also performed with all actinobacterial strains isolated from *D. cymosum*, since the presence of the fluorinase gene has been so far found in Actinobacteria. The results revealed that from all the identified microorganisms belonging to this phylum, only the isolate FEN 24 showed amplified products with the expected size, as illustrated in Figure 23.
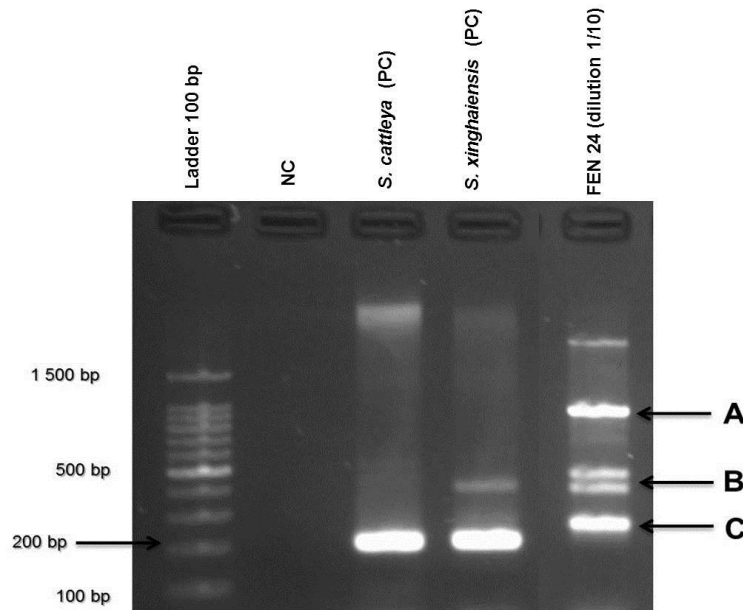
Figure 23. Agarose gel with PCR products obtained from the DNA (with dilution 1/10) of *Streptomyces cattleya*, *Streptomyces xinghaiensis* and the isolate FEN 24, with the primers Flu226F, Flu435R, Flu524F and Flu721R; NC – Negative control; PC – Positive control; A, B and C – Bands sent for sequencing.

In order to confirm the presence of the fluorinase gene in the isolate FEN 24 and to discard the hypothesis of non-specific binding, DNA from the agarose gel (bands A, B and C) was collected and then sequenced, with the primers Flu226F and Flu721R. Unfortunately, the amplified products revealed to be non-specific, and thus are not relative to the fluorinase gene.

V. Conclusions and future perspectives

FA is produced only by some tropical and sub-tropical plants and by a few microorganisms belonging to the phylum Actinobacteria. While the mechanism by which the FA producing plants produce FA is not yet elucidated, the biosynthesis of FA by the limited number of actinobacterial species is already known and is mediated by a unique enzyme, fluorinase. It is not known whether FA producing plants or their microbial symbionts are responsible for the biosynthesis of FA and, thus studying the microbiome of these plants is an important approach to help clarifying this issue.

In this context, the present study contributed to increase the knowledge on the microbiome of the FA producing plant, *Dichapetalum cymosum.* The investigation of the microbial community associated with this plant mainly focused in microorganisms belonging to the phylum Actinobacteria, since the production of FA has only been identified so far in these bacteria.

With the experimental approach used, the isolated microorganisms mainly belonged to the bacterial phyla Actinobacteria, Firmicutes and Proteobacteria and to the fungi phyla Ascomycota, which are common phyla associated with plants. The genera *Alternaria, Bacillus, Paenibacillus, Rhizobium, Microbacterium, Curtobacterium, Arthrobacter, Streptomyces* and *Methylobacterium* were mostly identified. Nonetheless, several of the isolated strains were not yet identified and thus, subsequent studies can show a higher diversity of phyla associated with this plant.

Metagenomics analysis of the soil sample collected in the neighborhood of *D. cymosum* plant revealed that the phyla Actinobacteria (37%) and Proteobacteria (17%) and the orders Actinomycetales (22%), Rhodospirillales (9%) and Rhizobiales (3%) were present in higher abundance, and in fact several bacterial strains belonging to these taxonomic ranks were isolated using a culture-based approach.

The investigation of the presence of the fluorinase gene in the microbiome of the FA producing plant *D. cymosum* revealed to be so far unfruitful, however further studies with the primers designed for the fluorinase gene may provide evidence on the role of the microorganisms in the production of FA. Subsequent research with other FA producing plants, different pretreatments, primers and DNA extraction protocols can also increase the number of isolates and, thus can help to clarify whether the microbial symbionts or the plants are responsible for the production of FA.

VI. References

Akimitsu, K., Tsuge, T., Kodama, M., Yamamoto, M. & Otani, H. 2014. *Alternaria* host-selective toxins: determinant factors of plant disease. *Journal of general plant pathology,* 80**,** 109-122.

Aplin, T. 1971. Poison plants of Western Australia: the toxic species of the genera *Gastrolobium* and *Oxylobium. J. Agric. West. Austral,* 12**,** 12-18.

Araújo, W. L., Marcon, J., Maccheroni, W., Van Elsas, J. D., Van Vuurde, J. W. & Azevedo, J. L. 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Applied and environmental microbiology,* 68**,** 4906-4914.

Aravind, R., Kumar, A., Eapen, S. & Ramana, K. 2009. Endophytic bacterial flora in root and stem tissues of black pepper (*Piper nigrum* L.) genotype: isolation, identification and evaluation against *Phytophthora capsici. Letters in applied microbiology,* 48**,** 58-64.

Ash, C., Priest, F. G. & Collins, M. D. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie van Leeuwenhoek,* 64**,** 253-260.

Bacon, C. W. & Hinton, D. M. 2007. Bacterial endophytes: the endophytic niche, its occupants, and its utility. *Plant-associated bacteria.* Springer.

Bai, Y., D'aoust, F., Smith, D. L. & Driscoll, B. T. 2002. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Canadian Journal of Microbiology,* 48**,** 230-238.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y. & Van Wezel, G. P. 2016. Taxonomy, physiology, and natural products of Actinobacteria. *Microbiology and Molecular Biology Reviews,* 80**,** 1-43.

Behrendt, U., Ulrich, A. & Schumann, P. 2001. Description of *Microbacterium foliorum* sp. nov. and *Microbacterium phyllosphaerae* sp. nov., isolated from the phyllosphere of grasses and the surface litter after mulching the sward, and reclassification of *Aureobacterium resistens* (Funke et al. 1998) as *Microbacterium resistens* comb. nov. *International journal of systematic and evolutionary microbiology,* 51**,** 1267-1276.

Berge, O., Guinebretière, M.-H., Achouak, W., Normand, P. & Heulin, T. 2002. *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food. *International journal of systematic and evolutionary microbiology,* 52**,** 607-616.

Brader, G., Compant, S., Mitter, B., Trognitz, F. & Sessitsch, A. 2014. Metabolic potential of endophytic bacteria. *Current opinion in biotechnology,* 27**,** 30-37.

Cao, L., Qiu, Z., You, J., Tan, H. & Zhou, S. 2004. Isolation and characterization of endophytic *Streptomyces* strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. *Letters in applied microbiology,* 39**,** 425-430.

Cao, Y.-R., Wang, Q., Jin, R.-X., Tang, S.-K., Jiang, Y., He, W.-X., Lai, H.-X., Xu, L.-H. & Jiang, C.-L. 2011. *Methylobacterium soli* sp. nov. a methanol-utilizing bacterium isolated from the forest soil. *Antonie van Leeuwenhoek,* 99**,** 629-634.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K. & Gordon, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods,* 7**,** 335-336.

Chater, K. F. 2006. *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 361**,** 761-768.

Chenoweth, M. 1949. Monofluoroacetic acid and related compounds. *Pharmacological reviews,* 1**,** 383-424.

Cho, S. J., Park, S. R., Kim, M. K., Lim, W. J., Ryu, S. K., An, C. L., Hong, S. Y., Lee, Y. H., Jeong, S. G. & Cho, Y. U. 2002. Endophytic *Bacillus* sp. isolated from the interior of balloon flower root. *Bioscience, biotechnology, and biochemistry,* 66**,** 1270-1275.

Choudhary, D. K. & Johri, B. N. 2009. Interactions of *Bacillus* spp. and plants–with special reference to induced systemic resistance (ISR). *Microbiological research,* 164**,** 493-513.

Coenye, T. & Vandamme, P. 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiology Letters,* 228**,** 45-49.

Conn, H. 1928. *Type of Bacteria Abundant in Productive Soils, But Apparently Lacking in Certain Soils of Low Productivity.*

Coombs, J. T. & Franco, C. M. 2003. Isolation and identification of actinobacteria from surface-sterilized wheat roots. *Applied and environmental microbiology,* 69**,** 5603-5608.

Cui, H., Yang, X., Lu, D., Jin, H., Yan, Z., Chen, J., Li, X. & Qin, B. 2014. Isolation and characterization of bacteria from the rhizosphere and bulk soil of *Stellera chamaejasme* L. *Canadian journal of microbiology,* 61**,** 171-181.

Dall'angelo, S., Bandaranayaka, N., Windhorst, A. D., Vugts, D. J., Van Der Born, D., Onega, M., Schweiger, L. F., Zanda, M. & O'hagan, D. 2013. Tumour imaging by Positron Emission Tomography using fluorinase generated 5-[18 F] fluoro-5-deoxyribose as a novel tracer. *Nuclear medicine and biology,* 40, 464-470.

De Beeck, M. O., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J. & Colpaert, J. V. 2014. Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PLoS One,* 9**,** e97629.

Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R. & Ellis, J. 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular plant pathology,* 13**,** 414-430.

Deng, H., Cobb, S. L., Gee, A. D., Lockhart, A., Martarello, L., Mcglinchey, R. P., O'hagan, D. & Onega, M. 2006. Fluorinase mediated C–18 F bond formation, an enzymatic tool for PET labelling. *Chemical communications***,** 652-654.

Deng, H., Ma, L., Bandaranayaka, N., Qin, Z., Mann, G., Kyeremeh, K., Yu, Y., Shepherd, T., Naismith, J. H. & O'hagan, D. 2014. Identification of fluorinases from *Streptomyces* sp MA37, *Norcardia brasiliensis*, and *Actinoplanes* sp N902-109 by Genome Mining. *ChemBioChem,* 15**,** 364-368.

Deng, H., O'hagan, D. & Schaffrath, C. 2004. Fluorometabolite biosynthesis and the fluorinase from *Streptomyces cattleya. Natural product reports,* 21**,** 773-784.

Dharmaraj, S. 2010. Marine *Streptomyces* as a novel source of bioactive substances. *World Journal of Microbiology and Biotechnology,* 26**,** 2123-2139.

Eason, C., Gooneratnel, R., Fitzgerald, H., Wright, G. & Frampton, C. 1994. Persistence of sodium monofluoroacetate in livestock animals and risk to humans. *Human & experimental toxicology,* 13**,** 119-122.

Eason, C., Miller, A., Ogilvie, S. & Fairweather, A. 2011. An updated review of the toxicology and ecotoxicology of sodium fluoroacetate (1080) in relation to its use as a pest control tool in New Zealand. *New Zealand Journal of Ecology***,** 1-20.

Eason, C., Ross, J. & Miller, A. 2013. Secondary poisoning risks from 1080-poisoned carcasses and risk of trophic transfer—a review. *New Zealand Journal of Zoology,* 40**,** 217-225.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research,* 32**,** 1792-1797.

Edgar, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods,* 10**,** 996-998.

Egekeze, J. O. & Oehme, F. W. 1979. Inorganic and organic fluoride concentrations in tissues after the oral administration of sodium monofluoroacetate (compound 1080) to rats. *Toxicology,* 15**,** 43-53.

Elbeltagy, A., Nishioka, K., Suzuki, H., Sato, T., Sato, Y.-I., Morisaki, H., Mitsui, H. & Minamisawa, K. 2000. Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. *Soil science and plant nutrition,* 46**,** 617-629.

Eloff, J. & Von Sydow, B. 1971. Experiments on the fluoroacetate metabolism of *Dichapetalum cymosum* (Gifblaar). *Phytochemistry,* 10**,** 1409-1415.

Ercolani, G. 1991. Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. *Microbial Ecology,* 21**,** 35-48.

Eustáquio, A. S., O'hagan, D. & Moore, B. S. 2010. Engineering Fluorometabolite Production: Fluorinase Expression in *Salinispora tropica* Yields Fluorosalinosporamide†. *Journal of natural products,* 73**,** 378-382.

Fowden, L. & Robinson, R. 1968. The Occurrence and Metabolism of Carbon-Halogen Compounds [and Discussion]. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* 171**,** 5-18.

Frankland, G. C. & Frankland, P. F. 1887. Studies on some new micro-organisms obtained from air. *Philosophical Transactions of the Royal Society of London. B,* 178**,** 257-287.

Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews,* 68**,** 280-300.

Glienke, C., Pereira, O., Stringari, D., Fabris, J., Kava-Cordeiro, V., Galli-Terasawa, L., Cunnington, J., Shivas, R., Groenewald, J. & Crous, P. 2011. Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. *Persoonia-Molecular Phylogeny and Evolution of Fungi,* 26**,** 47-56.

Goh, C., Hodgson, D., Fearnside, S., Heller, J. & Malikides, N. 2005. Sodium monofluoroacetate (Compound 1080) poisoning in dogs. *Australian veterinary journal,* 83**,** 474-479.

Goncharov, N. V., Jenkins, R. O. & Radilov, A. S. 2006. Toxicology of fluoroacetate: a review, with possible directions for therapy research. *Journal of Applied Toxicology,* 26**,** 148-161.

Gooneratne, S., Eason, C., Dickson, C., Fitzgerald, H. & Wright, G. 1995. Persistence of sodium monofluoroacetate in rabbits and risk to non-target species. *Human & experimental toxicology,* 14**,** 212-216.

Gregson, R., Baldo, B., Thomas, P., Quinn, R., Bergquist, P., Stephens, J. & Horne, A. 1979. Fluorine is a major constituent of the marine sponge *Halichondria moorei. Science,* 206**,** 1108-1109.

Gribble, G. W. 2002. Naturally occurring organofluorines. *Organofluorines.* Springer.

Gribble, G. W. 2003. The diversity of naturally produced organohalogens. *Chemosphere,* 52**,** 289-297.

Gribble, G. W. 2004. Amazing organohalogens: Although best known as synthetic toxicants, thousands of halogen compounds are, in fact, part of our natural enviornment. *American scientist,* 92**,** 342-349.

Hall, R. 1972. The distribution of organic fluorine in some toxic tropical plants. *New Phytologist,* 71**,** 855-871.

Hall, R. & Cain, R. 1972. Organic fluorine in tropical soils. *New phytologist,* 71**,** 839-853.

Harper, D. & O'hagan, D. 1994. The fluorinated natural products. *Natural product reports,* 11**,** 123.

Harper, D. B., O'hagan, D. & Murphy, C. D. 2003. Fluorinated natural products: Occurrence and biosynthesis. *Natural Production of Organohalogen Compounds.* Springer.

Hedges, F. 1922. A bacterial wilt of the bean caused by *Bacterium flaccumfaciens* nov. sp. *American Association for the Advancement of Science. Science,* 55**,** 433-434.

Heyrman, J., Vanparys, B., Logan, N. A., Balcaen, A., Rodríguez-Díaz, M., Felske, A. & De Vos, P. 2004. *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. *International journal of systematic and evolutionary microbiology,* 54**,** 47-57.

Hong, S., Bunge, J., Leslin, C., Jeon, S. & Epstein, S. S. 2009. Polymerase chain reaction primers miss half of rRNA microbial diversity. *The ISME journal,* 3**,** 1365-1373.

Huang, H., Erickson, R., Yanke, L., Chelle, C. & Mündel, H.-H. 2006. First report of the purple variant of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, causal agent of bacterial wilt of bean, in Canada. *Plant Disease,* 90**,** 1262-1262.

Huang, S., Ma, L., Tong, M. H., Yu, Y., O'hagan, D. & Deng, H. 2014. Fluoroacetate biosynthesis from the marine derived bacterium *Streptomyces xinghaiensis* NRRL B-24674. *Organic & Biomolecular Chemistry* 12: 4828–4831.

Huang S., M. L., Tong M.H., Yu, Y., O'hagan D. And Deng H. 2014. Fluoroacetate biosynthesis from the marine-derived bacterium *Streptomyces xinghaiensis* NRRL B-24674. *Organic & Biomolecular Chemistry 12: 4828–4831.*

Hutchison, L. J., Untereiner, W. A. & Hiratsuka, Y. 1995. *Knufia cryptophialidica* gen. et sp. nov., a dematiaceous hyphomycete isolated from black galls of trembling aspen (Populus tremuloides). *Mycologia*, 902-908.

Iizuka, H. & Komagata, K. 1963. New species of *Pseudomonas* belonged to fluorescent group (studies on the microorganisms of cereal grains. Part V). *J Agric Chem Soc,* 37, 137-141.

Janso, J. E. & Carter, G. T. 2010. Biosynthetic potential of phylogenetically unique endophytic actinomycetes from tropical plants. *Applied and environmental microbiology,* 76, 4377-4386.

Jones, D. & Keddie, R. M. 2006. The genus *Arthrobacter. The prokaryotes.* Springer.

Kellerman, T. S., Naudé, T. & Fourie, N. 1996. The distribution, diagnoses and estimated economic impact of plant poisonings and mycotoxicoses in South Africa.

Kieser T., B. M., Buttner Mj, Chater Kf & Hopwood Da 2000. Practical *Streptomyces* Genetics. . *John Innes Foundation.*

Kim, B.-C., Lee, K. H., Kim, M. N., Kim, E.-M., Rhee, M.-S., Kwon, O.-Y. & Shin, K.-S. 2009. *Paenibacillus pinihumi* sp. nov., a cellulolytic bacterium isolated from the rhizosphere of Pinus densiflora. *The Journal of Microbiology,* 47, 530-535.

Kim, D.-S., Bae, C.-Y., Jeon, J.-J., Chun, S.-J., Oh, H. W., Hong, S. G., Baek, K.-S., Moon, E. Y. & Bae, K. S. 2004. *Paenibacillus elgii* sp. nov., with broad antimicrobial activity. *International journal of systematic and evolutionary microbiology,* 54, 2031-2035.

Kloepper, J. W., Ryu, C.-M. & Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology,* 94, 1259-1266.

Knief, C., Dengler, V., Bodelier, P. L. & Vorholt, J. A. 2012. Characterization of *Methylobacterium* strains isolated from the phyllosphere and description of *Methylobacterium longum* sp. nov. *Antonie van Leeuwenhoek,* 101, 169-183.

Krishnamurthi, S., Bhattacharya, A., Schumann, P., Dastager, S. G., Tang, S.-K., Li, W.-J. & Chakrabarti, T. 2012. *Microbacterium immunditiarum* sp. nov., an actinobacterium isolated from landfill surface soil, and emended description of

the genus *Microbacterium*. *International journal of systematic and evolutionary microbiology,* 62**,** 2187-2193.

Kucheryava, N., Fiss, M., Auling, G. & Kroppenstedt, R. M. 1999. Isolation and characterization of epiphytic bacteria from the phyllosphere of apple, antagonistic in vitro to *Venturia inaequalis*, the causal agent of apple scab. *Systematic and applied microbiology,* 22**,** 472-478.

Kuhn, D. N., Knauf, M. & Stumpf, P. 1981. Subcellular localization of acetyl-CoA synthetase in leaf protoplasts of *Spinacia oleracea. Archives of biochemistry and biophysics,* 209**,** 441-450.

Kumar, P., Dubey, R. & Maheshwari, D. 2012. *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiological research,* 167**,** 493-499.

Kun, E., Kirsten, E. & Sharma, M. L. 1977. Enzymatic formation of glutathione-citryl thioester by a mitochondrial system and its inhibition by (-) erythrofluorocitrate. *Proceedings of the National Academy of Sciences,* 74**,** 4942-4946.

Küster, E. & Williams, S. 1964. Selection of media for isolation of *streptomycetes. Nature,* 202**,** 928-929.

Lane, D. J. 1991. 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics (Stackebrandt, E., Goodfellow, M., Eds.)***,** pp.115-175 Wiley, Chichester, UK.

Lauble, H., Kennedy, M., Emptage, M., Beinert, H. & Stout, C. 1996. The reaction of fluorocitrate with aconitase and the crystal structure of the enzyme-inhibitor complex. *Proceedings of the National Academy of Sciences,* 93**,** 13699-13703.

Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. 2000. Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* 50**,** 2207-2223.

Lee, S.-W., Oh, H.-W., Lee, K.-H. & Ahn, T.-Y. 2009. *Methylobacterium dankookense* sp. nov., isolated from drinking water. *The Journal of Microbiology,* 47**,** 716-720.

Lee, S. T., Cook, D., Pfister, J. A., Allen, J. G., Colegate, S. M., Riet-Correa, F. & Taylor, C. M. 2014. Monofluoroacetate-containing plants that are potentially toxic to livestock. *Journal of agricultural and food chemistry,* 62**,** 7345-7354.

Legard, D., Mcquilken, M., Whipps, J., Fenlon, J., Fermor, T., Thompson, I., Bailey, M. & Lynch, J. 1994. Studies of seasonal changes in the microbial populations on the phyllosphere of spring wheat as a prelude to the release of a genetically modified microorganism. *Agriculture, ecosystems & environment,* 50**,** 87-101.

Li, Y., You, M., Finnegan, P., Khan, T., Lanoiselet, V., Eyres, N. & Barbetti, M. 2015. First report of black spot caused by *Boeremia exigua* var. exigua on field pea in Australia. *Plant Disease,* 99**,** 580-587.

Madhaiyan, M., Poonguzhali, S., Kwon, S.-W. & Sa, T.-M. 2010. *Bacillus methylotrophicus* sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil. *International journal of systematic and evolutionary microbiology,* 60**,** 2490-2495.

Magoč, T. & Salzberg, S. L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics,* 27**,** 2957-2963.

Maienfisch, P. & Hall, R. G. 2004. The importance of fluorine in the life science industry. *CHIMIA International Journal for Chemistry,* 58**,** 93-99.

Marais, J. 1943. The isolation of the toxic principle potassium cymonate from gifblaar *Dichapetalum cymosum* (Hook) Engl. *Onderstepoort Journal of Veterinary Science and Animal Husbandry,* 18**,** 203-206.

Marais, J. 1944. Monofluoroacetic acid, the toxic principle of "gifblaar" *Dichapetalum cymosum* (Hook) Engl. *Onderstepoort Journal of Veterinary Science and Animal Industry,* 20**,** 67-73.

Marks, C. A., Edwards, I., Obendorf, D., Pereira, F. & Hall, G. P. 2014. Did 'precautionary'1080 baiting have a realistic potential to eradicate Red Fox (*Vulpes vulpes*) in Tasmania without in situ monitoring data? *Ecological Management & Restoration,* 15**,** 196-203.

Maropola, M. K. A., Ramond, J.-B. & Trindade, M. 2015. Impact of metagenomic DNA extraction procedures on the identifiable endophytic bacterial diversity in *Sorghum bicolor* (l. Moench). *Journal of microbiological methods,* 112**,** 104-117.

Masson-Boivin, C., Giraud, E., Perret, X. & Batut, J. 2009. Establishing nitrogen-fixing symbiosis with legumes: how many *rhizobium* recipes? *Trends in microbiology,* 17**,** 458-466.

Mcinroy, J. A. & Kloepper, J. W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and soil,* 173**,** 337-342.

Mead, R. & Segal, W. 1972. Fluoroacetic acid biosynthesis: a proposed mechanism. *Australian journal of biological sciences,* 25**,** 327-334.

Meyer, J. M. & Grobbelaar, N. 1990. The determination, uptake and transport of fluoroacetate in *Dichapetalum cymosum. Journal of plant physiology,* 135**,** 546-549.

Meyer, J. M. & O'hagan, D. 1992. Conversion of fluoropyruvate to fluoroacetate by *Dichapetalum cymosum. Phytochemistry,* 31**,** 499-501.

Montana, J. S., Jiménez, D. J., Hernández, M., Ángel, T. & Baena, S. 2012. Taxonomic and functional assignment of cloned sequences from high Andean forest soil metagenome. *Antonie van Leeuwenhoek,* 101**,** 205-215.

Nagel, M. & Andreesen, J. 1991. *Bacillus niacini* sp. nov., a nicotinate-metabolizing mesophile isolated from soil. *International Journal of Systematic and Evolutionary Microbiology,* 41**,** 134-139.

Nakamura, L. 1984. *Bacillus amylolyticus* sp. nov., nom. rev., *Bacillus lautus* sp. nov., nom. rev., *Bacillus pabuli* sp. nov., nom. rev., and *Bacillus validus* sp. nov., nom. rev. *International Journal of Systematic and Evolutionary Microbiology,* 34**,** 224-226.

Neidleman, S. L. & Geigert, J. 1986. *Biohalogenation: principles, basic roles, and applications*, Halsted Press.

Neumann, C. S., Fujimori, D. G. & Walsh, C. T. 2008. Halogenation strategies in natural product biosynthesis. *Chemistry & biology,* 15**,** 99-109.

O'donnell, A. G., Goodfellow, M. & Minnikin, D. E. 1982. Lipids in the classification of *Nocardioides*: reclassification of *Arthrobacter simplex* (Jensen) lochhead in the genus *Nocardioides* (Prauser) emend. O'Donnell et al. as *Nocardioides simplex* comb. nov. *Archives of Microbiology,* 133**,** 323-329.

O'hagan, D., Perry, R., Lock, J. M., Meyer, J. M., Dasaradhi, L., Hamilton, J. T. & Harper, D. B. 1993. High levels of monofluoroacetate in *Dichapetalum braunii*. *Phytochemistry,* 33**,** 1043-1045.

O'hagan, D., Schaffrath, C., Cobb, S. L., Hamilton, J. T. & Murphy, C. D. 2002. Biochemistry: biosynthesis of an organofluorine molecule. *Nature,* 416**,** 279-279.

O'hagan, D., Schaffrath, C, Cobb, Sl, Hamilton, Jtg, and Murphy, Cd 2002. Biosynthesis of an organofluorine molecule. *Nature,* 416: 279.

O'hagan, D. 2006. Recent developments on the fluorinase from *Streptomyces cattleya*. *Journal of fluorine chemistry,* 127**,** 1479-1483.

O'hagan, D. & Deng, H. 2014. Enzymatic fluorination and biotechnological developments of the fluorinase. *Chemical reviews,* 115**,** 634-649.

O'hagan, D. & Harper, D. B. 1999. Fluorine-containing natural products. *Journal of Fluorine Chemistry,* 100**,** 127-133.

Onega, M., Domarkas, J., Deng, H., Schweiger, L. F., Smith, T. A., Welch, A. E., Plisson, C., Gee, A. D. & O'hagan, D. 2010. An enzymatic route to 5-deoxy-5-[18F] fluoro-d-ribose, a [18 F]-fluorinated sugar for PET imaging. *Chemical Communications,* 46**,** 139-141.

Pawlowski, K. & Demchenko, K. N. 2012. The diversity of actinorhizal symbiosis. *Protoplasma,* 249**,** 967-979.

Pereira, F., Carneiro, J., Matthiesen, R., Van Asch, B., Pinto, N., Gusmão, L. & Amorim, A. 2010. Identification of species by multiplex analysis of variable-length sequences. *Nucleic acids research,* 38**,** e203-e203.

Peters, R. 1967. Some adventures in biochemical pharmacology. *Record of chemical progress,* 28**,** 197.

Peters, R. 1973. Keynote and historical perspective of organic fluorides in plants. *Fluoride;(United States),* 6.

Peters, R., Wakelin, R. & Buffa, P. 1953. Biochemistry of fluoroacetate poisoning the isolation and some properties of the fluorotricarboxylic acid inhibitor of citrate metabolism. *Proceedings of the Royal Society of London B: Biological Sciences,* 140**,** 497-506.

Pimentel-Elardo, S. M., Kozytska, S., Bugni, T. S., Ireland, C. M., Moll, H. & Hentschel, U. 2010. Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. *Marine drugs,* 8**,** 373-380.

Priest, F. G., Goodfellow, M. & Todd, C. 1988. A numerical classification of the genus *Bacillus. Microbiology,* 134**,** 1847-1882.

Proudfoot, A. T., Bradberry, S. M. & Vale, J. A. 2006. Sodium fluoroacetate poisoning. *Toxicological reviews,* 25**,** 213-219.

Qin, S., Li, J., Chen, H.-H., Zhao, G.-Z., Zhu, W.-Y., Jiang, C.-L., Xu, L.-H. & Li, W.-J. 2009. Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *Applied and environmental microbiology,* 75**,** 6176-6186.

Qin, S., Miao, Q., Feng, W.-W., Wang, Y., Zhu, X., Xing, K. & Jiang, J.-H. 2015. Biodiversity and plant growth promoting traits of culturable endophytic actinobacteria associated with Jatropha curcas L. growing in Panxi dry-hot valley soil. *Applied Soil Ecology,* 93**,** 47-55.

Qin, S., Zhu, W.-Y., Jiang, J.-H., Klenk, H.-P., Li, J., Zhao, G.-Z., Xu, L.-H. & Li, W.-J. 2010. *Pseudonocardia tropica* sp. nov., an endophytic actinomycete isolated from the stem of *Maytenus austroyunnanensis. International journal of systematic and evolutionary microbiology,* 60**,** 2524-2528.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. & Glöckner, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research,* 41**,** D590-D596.

Reva, O. N., Smirnov, V. V., Pettersson, B. & Priest, F. G. 2002. *Bacillus endophyticus* sp. nov., isolated from the inner tissues of cotton plants (Gossypium sp.). *International journal of systematic and evolutionary microbiology,* 52**,** 101-107.

Rivas, R., Abril, A., Trujillo, M. E. & Velázquez, E. 2004. *Sphingomonas phyllosphaerae* sp. nov., from the phyllosphere of *Acacia caven* in Argentina. *International journal of systematic and evolutionary microbiology,* 54**,** 2147-2150.

Saile, E. & Koehler, T. M. 2006. *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. *Applied and environmental microbiology,* 72**,** 3168-3174.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. *Molecular cloning*, Cold spring harbor laboratory press New York.

Samson, R. & Abdel-Fattah, H. 1978. A new species of *Talaromyces* and a discussion of some recently described taxa. *Persoonia-Molecular Phylogeny and Evolution of Fungi,* 9**,** 501-504.

Sanada, M., Miyano, T., Iwadare, S., Williamson, J., Arison, B., Smith, J., Douglas, A., Liesch, J. & Inamine, E. 1986. Biosynthesis of fluorothreonine and fluoroacetic acid by the thienamycin producer, *Streptomyces cattleya*. *The Journal of antibiotics,* 39**,** 259-265.

Saxena, A., Anand, S., Dua, A., Sangwan, N., Khan, F. & Lal, R. 2013. *Novosphingobium lindaniclasticum* sp. nov., a hexachlorocyclohexane (HCH)-degrading bacterium isolated from an HCH dumpsite. *International journal of systematic and evolutionary microbiology,* 63**,** 2160-2167.

Scheublin, T. R. & Leveau, J. H. 2013. Isolation of *Arthrobacter* species from the phyllosphere and demonstration of their epiphytic fitness. *Microbiologyopen,* 2**,** 205-213.

Schirrmacher, R., Wangler, C. & Schirrmacher, E. 2007. Recent developments and trends in 18F-radiochemistry: syntheses and applications. *Mini-Reviews in Organic Chemistry,* 4**,** 317-329.

Schlechtendal, D. D. 1824. Flora Berolinensis. Pars secunda. *Cryptogamia. Berolini. 284pp*.

Schloss, P., Handelsman, J. 2005. Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. Genome biology, 6:229.

Sherley, M. 2004. The traditional categories of fluoroacetate poisoning signs and symptoms belie substantial underlying similarities. *Toxicology letters,* 151**,** 399-406.

Sherley, M. 2007. Is sodium fluoroacetate (1080) a humane poison? *ANIMAL WELFARE-POTTERS BAR THEN WHEATHAMPSTEAD-,* 16**,** 449.

Shida, O., Takagi, H., Kadowaki, K., Udaka, S., Nakamura, L. K. & Komagata, K. 1995. Proposal of *Bacillus reuszeri* sp. nov., *Bacillus formosus* sp. nov., nom. rev., and *Bacillus borstelensis* sp. nov., nom. rev. *International Journal of Systematic and Evolutionary Microbiology,* 45**,** 93-100.

Smith, E. F. 1899. Wilt disease of cotton, watermelon, and cowpea (Neocosmospora nov. gen.).

Stach, J. E., Maldonado, L. A., Ward, A. C., Goodfellow, M. & Bull, A. T. 2003. New primers for the class Actinobacteria: application to marine and terrestrial environments. *Environmental Microbiology,* 5**,** 828-841.

Stackebrandt, E. & Kandler, O. 1980. *Cellulomonas cartae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* 30**,** 186-188.

Steyn, D. 1928. Gifblaar poisoning: a summary of our present knowledge in respect of poisoning. Pretoria: Government Printer and Stationery Office.

Sturm, G., Jacobs, J., Spröer, C., Schumann, P. & Gescher, J. 2011. *Leucobacter chromiiresistens* sp. nov., a chromate-resistant strain. *International journal of systematic and evolutionary microbiology,* 61**,** 956-960.

Tani, A., Sahin, N. & Kimbara, K. 2012. *Methylobacterium oxalidis* sp. nov., isolated from leaves of *Oxalis corniculata*. *International journal of systematic and evolutionary microbiology,* 62**,** 1647-1652.

Thayer, A. M. 2006. Fabulous fluorine. *Chem. Eng. News,* 84**,** 15-24.

Thomas, S., Singleton, V., Lowery, J., Sharpe, R., Pruess, L., Porter, J., Mowat, J. & Bohonos, N. 1957. Nucleocidin, a new antibiotic with activity against Trypanosomes. *Antibiotics Annual 1956-1957***,** 716-721.

Thompson, I., Bailey, M., Fenlon, J., Fermor, T., Lilley, A., Lynch, J., Mccormack, P., Mcquilken, M., Purdy, K. & Rainey, P. 1993. Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (*Beta vulgaris*). *Plant and Soil,* 150**,** 177-191.

Thompson, S., Onega, M., Ashworth, S., Fleming, I. N., Passchier, J. & O'hagan, D. 2015. A two-step fluorinase enzyme mediated 18 F labelling of an RGD peptide for positron emission tomography. *Chemical Communications,* 51**,** 13542-13545.

Twigg, L. & Parker, R. 2010. Is sodium fluoroacetate (1080) a humane poison? The influence of mode of action, physiological effects, and target specificity. *Animal Welfare,* 19**,** 249-263.

Twigg, L. E., King, D. R., Bowen, L. H., Wright, G. R. & Eason, C. T. 1996. Fluoroacetate content of some species of the toxic Australian plant genus, *Gastrolobium*, and its environmental persistence. *Natural Toxins,* 4**,** 122-127.

Twigg, L. E., Martin, G. R. & Lowe, T. J. 2002. Evidence of pesticide resistance in medium-sized mammalian pests: a case study with 1080 poison and Australian rabbits. *Journal of Applied Ecology,* 39**,** 549-560.

Ulrich, K., Stauber, T. & Ewald, D. 2008. *Paenibacillus*—a predominant endophytic bacterium colonising tissue cultures of woody plants. *Plant Cell, Tissue and Organ Culture,* 93**,** 347-351.

Vandamme, P., Moore, E. R., Cnockaert, M., De Brandt, E., Svensson-Stadler, L., Houf, K., Spilker, T. & Lipuma, J. J. 2013. *Achromobacter animicus* sp. nov., *Achromobacter mucicolens* sp. nov., *Achromobacter pulmonis* sp. nov. and *Achromobacter spiritinus* sp. nov., from human clinical samples. *Systematic and applied microbiology,* 36**,** 1-10.

Vartiainen, T. & Gynther, J. 1984. Fluoroacetic acid in guar gum. *Food and chemical toxicology,* 22**,** 307-308.

Venkateswarlu, P., Armstrong, W. & Singer, L. 1965. Absorption of fluoride and chloride by barley roots. *Plant physiology,* 40, 255.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F. & Van Sinderen, D. 2007. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiology and Molecular Biology Reviews,* 71**,** 495-548.

Verastegui, Y., Cheng, J., Engel, K., Kolczynski, D., Mortimer, S., Lavigne, J., Montalibet, J., Romantsov, T., Hall, M. & Mcconkey, B. 2014. Multisubstrate isotope labeling and metagenomic analysis of active soil bacterial communities. *MBio,* 5**,** e01157-14.

Vickers, J., Williams, S. & Ross, G. 1984. A taxonomic approach to selective isolation of *streptomycetes* from soil. *Biological, Biochemical and Biomedical Aspects of Actinomycetes,* 553-561.

Vickery, B., Vickery, M. L. & Kaberia, F. 1979. A possible biomimetic synthesis of fluoroacetic acid. *Cellular and Molecular Life Sciences,* 35**,** 299-300.

Vuillemin, P. M. 1912. *Beauveria,* nouveau genre de *Verticilliacées. Bulletin de la Société Botanique de France,* 59**,** 34-40.

Wahyudi, A. T., Astuti, R. P., Widyawati, A., Mery, A. & Nawangsih, A. A. 2011. Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. *Journal of Microbiology and Antimicrobials,* 3**,** 34-40.

Walker, M. C. & Chang, M. C. 2014. Natural and engineered biosynthesis of fluorinated natural products. *Chemical Society Reviews,* 43**,** 6527-6536.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology,* 173**,** 697-703.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* 18**,** 315-322.

Wikee, S., Udayanga, D., Crous, P. W., Chukeatirote, E., Mckenzie, E. H., Bahkali, A. H., Dai, D. & Hyde, K. D. 2011. Phyllosticta—an overview of current status of species recognition. *Fungal Diversity,* 51**,** 43-61.

Wilson, D. 1995. Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos***,** 274-276.

Woese, C. R. 1987. Bacterial evolution. *Microbiological reviews,* 51**,** 221.

Xia, Y., Greissworth, E., Mucci, C., Williams, M. A. & De Bolt, S. 2013. Characterization of culturable bacterial endophytes of switchgrass (*Panicum virgatum L.*) and their capacity to influence plant growth. *GCB Bioenergy,* 5**,** 674-682.

Yang, J.-E., Shin, J.-Y., Park, S.-Y., T. Mavlonov, G., Yi, E.-J., Lee, E.-H., Lee, J. M. & Yi, T.-H. 2012. *Pedobacter kyungheensis* sp. nov., with ginsenoside converting activity. *The Journal of general and applied microbiology,* 58**,** 309-316.

Yoon, J.-H. & Jung, Y.-T. 2012. *Cohnella boryungensis* sp. nov., isolated from soil. *Antonie van Leeuwenhoek,* 101**,** 769-775.

Zhao, K., Penttinen, P., Guan, T., Xiao, J., Chen, Q., Xu, J., Lindström, K., Zhang, L., Zhang, X. & Strobel, G. A. 2011. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in *Panxi plateau*, China. *Current microbiology,* 62**,** 182-190.