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AMAZONIAN PLANTS FROM ETHNOMEDICINE THROUGH PHARMACEUTICAL BIOLOGY APPROACHES: A PhD EXPERIENCE IN CONNECTING FOREST WITH LABORATORY

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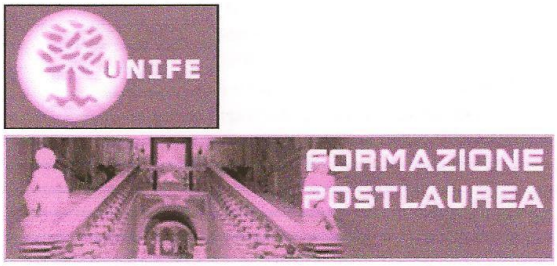

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a Severo e Padre Silvio

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1. PhD BACKGROUND AND PRELIMINARY CONSIDERATIONS

The present PhD research has its background in a cooperation and development project organized and sponsored by University of Ferrara, VIS-ONG (Volontariato Internazionale per lo Sviluppo), MAE (Ministero degli Affari Esteri) and Salesian Polytechnic University of Quito (Ecuador).

At the beginning, I attended to this project as VIS-ONG and MAE volunteer, with the specific target to set up strategies to valorize Amazonian ethnomedicine with cultural and economic relapses on the Natives Shuar and Achuar people.

I had been living in Ecuador since 2002 until 2008, and in the first part of that period I started to link productive relationships among Natives, Fundación Chankuap (an Amazonian cultural association supported by Salesian) and University of Ferrara with the aim to market Amazonian essential oils through "Fair Trade", with the quality control check and validation by the pharmaceutical biology lab of the University of Ferrara.

Considering the context where I worked as volunteer and the cited partners, it has been started a parallel wider research project with the aim to valorize, also through biotechnological strategies, Amazonian ethnomedical sources. Since this step on, the activities and studies had been starting as PhD.

Quito and Macas, the latter a small town at the border of Amazonian forest, were the places where I lived and performed my studies. A part from Quito, with its university and laboratories, any places adequate to perform *in vitro* activities were instead lacking in Macas. Thus, with the help of VIS and Salesian associations, I contribute to organize and set up a small laboratory in Macas where to perform *in vitro*

protocols. This first step, and first year of PhD, was devoted to this aim and to the sampling of plant material to isolate endophytes.

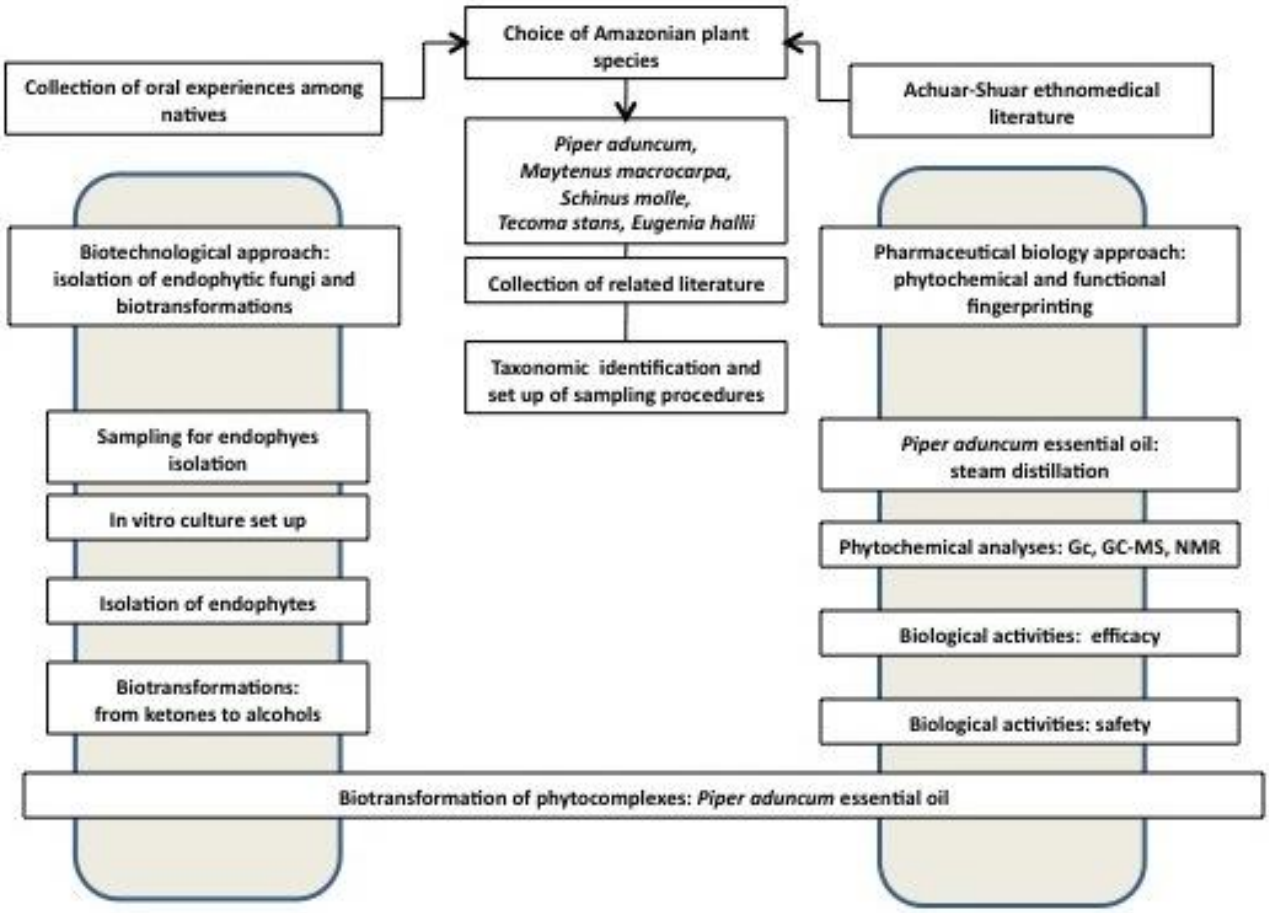
The second part of the PhD was performed at the University of Ferrara, processing biotransformations employing isolated endophytes and analyzing, under a phytochemical and biological point of view, the plant species chosen for the research. In this thesis, it has been reported only the phytochemical and biological characterization of one of the plants studied. However, before the official starting of the PhD and during its development, I contribute to chemically and biologically characterize other plant species, some of them part of this PhD. In synthesis, as conclusive paragraph of the thesis, all the publications to which I contributed for their realization are reported. For some of them, I could not take part as official author because of conflict of interest due to my fellowship contract with ONG-VIS and MAE.

Finally, and in light of this experience, it is my opinion that scientific publications derived from this kind of research profile – from ethnomedicine to laboratory – could be one of the starting points for a protection policy toward Natives and Amazonian Nature, against bio-piracy and mis-appropriation of natural sources and related knowledge by third parties. In fact, if traditional knowledge, such that ethnomedical, gives to science the opportunities to find new sources for human beings progress, new chemicals for new drugs to treat old and new diseases, on the other hand, research needs to be performed with ethical respect to these values. My Ecuadorian experience as volunteer and the present PhD research would try to give a little example of this approach.

2. STRATEGY AND GOALS

The goal of this PhD research would be the extension of the scientific knowledge related to a group of medicinal plants from the Amazon region of Ecuador, focusing on potential health application. The general outline of the research is summarized in the diagram below, which will be further discussed in the "Material and Methods" and "Results" sections.

Research outline



The research has been performed following two complementary approaches: biotransformations and chemical and biological fingerprinting (pharmaceutical biology approach). About biotransformations, the target is to identify and isolate endophytic fungi from Amazonian plant species of ethnopharmaceutical interest, estimating their biotransformation properties and, when possible, the specific identity. Then, quality and

quantity of biotransformation metabolites, focusing on oxidation products considered as the result of reaction catalyzed by monooxygenase have been considered.

In particular, Baeyer-Villiger reactions have been considered, catalyzed by flavoenzymes that catalyze oxidation and enantioselective reactions, converting linear and cyclic ketones into esters and lactones respectively. These kind of reactions are very important in bioremediation, in the pure chemical and pharmaceutical compounds synthesis (Urlacher *et al*., 2006).

The pharmaceutical biology approach was instead focused on phytochemical and biological characterization of Amazon plants. The biological activity has been developed with the aim to determine 1) efficacy and safety properties, through antimicrobial, antioxidant, antitrombotic, antinociceptive, mutagenic and mutagen-protective tests, and the scientific basis of ethnomedical uses.

In more detail, the research has been developed through three phases:

PHASE I:

1) Collecting of ethnomedical information and choice of plant specie to study;
2) Botanical source taxonomical identification to be checked for the presence of endophytes;
3) Endophytic fungi isolation and subculturing;

PHASE II:

1) Grouping endophytic fungi in light of macroscopical features of the colony;
2) Endophyte biotransformations to test pure compounds;
3) Endophyte biotransformations on plant extract and on the most abundant compounds.

PHASE III:

1) GC-FID and GC-MS analysis of possible biotransformation product;
2) chemical and biological characterization of plant derived extract: *P. aduncum* essential oil.

3. INTRODUCTION

Is it a problem of symbiosis?

During the evolution, organisms have needed to adapt themselves to the environment, developing relevant and different interactive abilities with other organisms. The symbiosis is the most common relationship that predicts the existence of an organism in close and continuing association with other life forms. Depending on the kind of association, the symbiosis can be roughly divided into two categories: ectosymbiosis (the microorganisms remain outside the "host" cells) and endosymbiosis.

90% of terrestrial plants live in association with soil fungi; the most common association is known as mycorrhizae. The mycorrhizae gives rise to trophic and protective effects. The host plant usually improves water and soil nutrient absorption due to the presence of symbionts. An example is the *Fabaceae* family in which bacteria belonging to *Rhizobium* genus penetrate the host root, leading to the development of nodules in which they are able to fix atmospheric nitrogen, making it available for the host; in return, the host provides carbon to symbiont microorganisms.

The symbiosis, as well as offering an improvement in trophic level, is also providing protective effects to the host, often in organs far from where the symbiosis is histologically and anatomically present.

The symbiotic fungi help to prevent pathogens attacks, to protect from soil abiotic stress and also to facilitate the intake of plant essential nutrients, such as phosphorus, iron, calcium and aluminium (Selosse *et al*., 2004).

The symbiosis is known as mutual when both organisms benefit from the relationship; while it is known as parasitic when just a single member takes advantage. It needs to be remembered that environmental factors may alter the quality of symbiosis, inducing, for exemple the change from mutualistic symbiosis to a parasitic interaction. An endophyte lives within a host plant (generally at subcuticular level) establishing a temporary biotrophic relationship with the host, and infecting its tissues without giving any symptoms at least for a certain period of time. Once the symbiosis is established, the natural selection, forced by the ecology, determines the success of the physiological interaction. The result is a high specialization of both the organisms; the symbiont loses its ability to live outside the host becoming increasingly specific to the partner choice. This is easily found in species from biomes with high levels of endemism and peculiar environmental features such as the Amazon forest. This PhD has taken place from study of Amazonian plant species belonging to the ethnomedicine of southern Ecuador

peoples, joining in a wider project focused on promoting the self-development of some indigenous communities in Ecuador, through training and technical assistance for productive and conservative use of biodiversity. The project has been sponsored by the Italian NGO VIS (Volontariato Internazionale per lo Sviluppo), in collaboration with the Centre of International Development and Cooperation of the University of Ferrara and the UPS (Salesian Polytechnic University, Quito-Ecuador). Among all these research actors, I contribute to the wider research project as collector figure of ethnic and scientific information and, successively as PhD student with the laboratory research following described. My PhD research in this context presented hard difficulties, mainly related to the lack of adequate structures, places and financial resources. This circumstance involved the extention of the time needed to achieve results. However, notwithstanding difficulties, I am particulary proud of my job because of the fact that other then the scientific results achieved, ethical approaches in making research has never been forgot, with the whising that applicative perspectives, which could take place from laboratory evidences, would be useful also for Amazonian people and biodiversity.

Ecuadorian colleagues from UPS; L. Scalvenzi in Amazonian forest during sampling; Achuar woman during ethnomedical interview



ENDOPHYTES

The word “endophyte” indicates bacteria or fungi living in cells of higher plant tissues, mainly located between cell wall and membrane. Generally, clear symptoms are not induced. The most interested plant tissue are epidermis and close parenchyma. The physical and physiological relationship between host and endophyte remains uninvestigated in most cases or really poor studied (Strobel, 2003). Since my research regards endophytic fungi, it has to be stressed that the endophytic fungi mainly belong

to Deuteromycetes and Ascomycetes. The endophytes, if detected, have been isolated from all plants organs, without exceptions. However, it has to be taken in consideration that during the ontogenetic senescence period of the plant, some endophytic fungal species were observed to become saprophytes (i.e. *Cladosporium* and *Penicillium)*; this is probably due to the weakening of the plant defense capacity. Due to this fact, it has been frequently ascertained that saprophytes have been mistaken with endophytes during the *in vitro* isolation procedures from fresh plant parts (Petrini, 1993).

Many endophytic genera as *Phomopsis*, *Phoma*, *Colletotrichum* and *Phyllosticta* have a wide hosts range and seemed to have developed the capacity of colonize several taxonomically unrelated plants, suggesting their biological evolution ability to adapt and overcome different kinds of host defences. In general, the endophyte-plant host first interaction phases are poorly known, till now. One of the several reasons of this lacking is surely the fact that it is a relatively new field of the biological and ecological knowledge. However, some interaction phenomena are described in many aspect with appreciable details; such as the case of *Colletotrichum gloeosporioides*, a typical phytopathogen which is able to become endophyte, in some environmtental conditions or physiological status, without producing syntoms in plant host. The reason of this particular ecological different behaviour is not still disclosed, but many suggestions are reported in related literature (Suryanarayanan *et al*., 2009 and reference therein). Other research aspect, (i) as the different phytoalexins synthesis profile and pathway in plants before and after symbiosis, (ii) the different expression of plant cell apoptosis, and (iii) other metabolic differences expressed both by host and endophyte with reference with the different species and numbers of endosymbiotic fungi are in progress but still far to be completely clarified. Common conclusions at this step, regard the fact that environmental conditions strongly affect the genetic and the physiological expression of the symbiosis by both the organisms involved independently by the specie of plant and endophytes. Certainly, the study of these interactions enriches tht possibility to have new pharmaceutical molecules, taking advantages of the particular metabolic expression of the symbiosis which could determine different biosynthetic capacity in producing secondary metabolites both by plant and endophyte (Suryanarayanan *et al*., 2009).

Some authors observed that, the mutualistic relationship plant-endophyte seems to consist in the constant physiological oscillation between parasitic and pathogenic condition. In other words, in some ontogenetic and ecological conditions, it is not already clear, what are the conditions inducing the fungus to become an ecological

enrichment for the plant or a vector of plant pathology. However, when the metabolic expression of the plant host and the endophyte is determined by a real symbiosis, a greater resistance to biotic and abiotic stress has been also observed (Strobel, 2003).

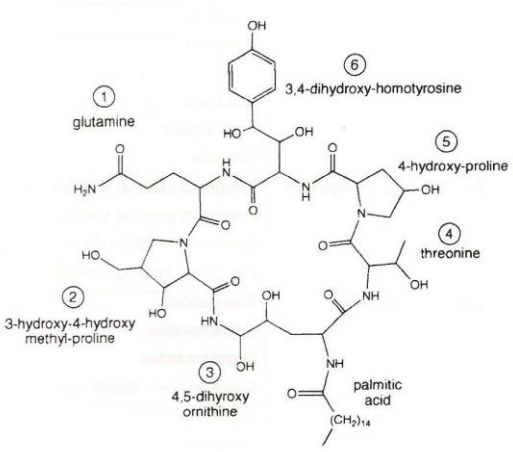
In some cases, a strong mutualistic relationship between plant and endophyte has been observed in the specie-specific expression of the symbiosis; i.e. the metabolic expression of the endophyte is strictly related to taxonomical characters of plant and endophyte. As metabolic expression of this aspect, it could be stressed that some endophytes isolated *in vitro* produce the same metabolite of the plant host, proving the fact that symbiosis could determine also a selective pressure to develop new metabolic pathways for the endophyte. As example, *Taxomyces andeanae* a specie-specificendophyte isolated from *Taxus brevifolia,* produces *in vitro* the alkaloid taxol, secondary metabolite typical of the plant host. It is known the pharmaceutical and economic importance of taxol, together with the importance to have the same chemical through different production chains, from those synthetic to those biotechnological. The biotechnological perspective meets the possibility to lower costs of the anti-cancer drug production saving the environment – in fact, pharmaceutical taxol needs semi-synthetic steps – and enhancing eco-friendly production strategy limiting solvent use (Suryanarayanan *et al*., 2009; Tan *et al.*, 2001).

Therefore, studying plants with ethnomedical importance offers the alluring possibility, to obtain pharmaceutically important chemicals through biotechcnological strategy. Recent studies have shown that 50% of active substances isolated from endophytic fungi were previously unknown, while for the soil microflora the same index considerably lower (38%) (Strobel, 2003).

In 2002, a study about the tropical plant *Kennedia nigricans,* used by the Northen Australia aborigens as ethnomedical remedy for skin wounds, led to detect munumbicins: secondary metabolites that demonstrated important and broad spectrum antibiotic activity. The antibiotic molecules were found to be typical of the plant genus *Kennedia* and represent the secondary metabolites derived from biosyntethic pathway actived by phytopathogen attack. Fresh samples of aerial parts of the plant showed the presence of bacterial endophytes belonging to *Streptomyces* sp. The endophytes were then *in vitro* isolated and cultured in bioreactors with appropriate culture media and the munumbicin antibiotic chemicals have been obtained with interesting yield. The fact that the isolated endophytes may to produce munumbicins probably represents the result of mutualistic metabolism developed by the symbiosis (Castillo *et al.*, 2002).

A similar example is represented by cryptocandin, an antibiotic compound produced by the endophytic fungus *Cryptosporiopsis quercina* isolated from the tree species *Tripterigeum wilfordii*, an endemic medicinal plant from Eurasia. Cryptocandin contains several hydroxylated amino acids including the recently discovered 3-hydroxy-4-hydroxy-methyl-proline, a molecule chemically similar to two antibiotic families: echinocandins and pneumocandins. Cryptocandin is also active against several plant pathogenic fungi, *Sclerotinia sclerotiorum* and *Botrytis cinerea* included. The molecule is currently being tested also against human dermatophytes, including those causing diseases to nails (Strobel *et al.*, 2003).

Molecular structure of Cryptocandin



The mutualism reveals a big interest in applied research when it is responsible of production of secondary metabolites that potentially can be considered as resource for agro-pharmaceutical industries, which are always looking for new food supplements molecules for the treatments of old and new diseases.

In synthesis, the study of (i) the mutualism between plant and endophyte, (ii) of the secondary metabolism expressed by symbiosis as well as (iii) the biotransformation abilities of endophytes, becomes particularly interesting in terms of laboratory and industrial development. Even more interesting under a research point of view is the fact that the present study in particular involves Amazonian plants, always at the center of scientific interest because of the lack of related literature and often interesting source of new molecules for disease treatments. The wide biodiversity expressed as chemodiversity is the result of the particular Amazonian ecosystem, that evidence a

high biological competition leading the evolution pressure to enhance secondary metabolism specialization.

ENDOPHYTIC FUNGI AND BIOTECHNOLOGICAL APPLICATIONS

Fungi are used in many industrial processes including the production of pharmaceutical enzymes, vitamins, polysaccharides, pigments, lipids and glycolipids. Some of these compounds are synthetically produced, while others may be obtained by biotechnological processes. Fungi are not only used as catalysts for fermentation processes, but also in biotransformations that are becoming increasingly important for "fine" chemistry industry interested in obtaining pure isomers instead of racemic solutions (Adrio *et al*., 2003).

There are a lot of studies on biotechnology applications of fungal endophytes in many fields including that pharmaceutical and phytoiatric, improving biotechnologies in terms of increasing product yields and lower costs. An example is represented by the production of cellulolytic enzymes by endophytic fungi. The xylans are polysaccharides belonging to the categories of hemicelluloses, characterized by the presence of xylose as the most abundant monomer. The xylans are abundant in plant cell walls and are classified as fibers, known to be not-hydrolysable by human digestive enzymes pools. In contrast, mono and/or oligosaccharides are more easily hydrolysable and in-depth studied fot their contribute as supplements for diabetes prevention. From these premises, it looks particulary interesting the fact that from a different numbers of endophytes isolated from Japanese plants (i.e *Aconitum yesoense, Gnaphalium japonicum Ilex crenata*), it was found that 90% of them were able to produce the enzyme xylanase, which is essential to hydrolyse xylans from plants obtaining oligosaccharides, xylose and omologous, for food supplement industry (Suto *et al.*, 2002).

A similar example with analogous industrial relapses is represented by the enzymes laccases, isolated from endophytes belonging to the fungal genus *Monotospora* sp isolated from the weed *Cynodon dactylon*. Laccases remove lignans from cellulose in a particular selective way, leaving cellulose fibers with an high purity. This evidence represents a relevant biotechnological perspective for paper industry, bioremediations, bio-fuels production and pharmaceutical industry (i.e. excipients as microcrystalline cellulose) (Wang *et al*., 2005).

Interesting biotechnological applications of endophytic fungi are also related to crop protection. The fungus *Muscodor crispans* was recently isolated from *Ananas ananassoides* grass species that grows wild in Bolivian Amazon. *M. crispans* produces volatile organic compounds known by the acronym of VOCs (Volatile Organic Compounds typical of the plant host), as well as other species of the genus *Muscodor*, mainly characterized by propanoic acid with interesting antibiotic properties. In fact, the VOCs are active towards a wide range of plant pathogens such as fungi *Pythium ultimum*, *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum* and *Mycosphaerella fijiensis* (causal agent of black Sigatoka, widespread disease that affects *Musa paradisiaca* plants) and the bacterium *Xanthomonas axonopodis* pv. *citri* (citrus bacterial cancer). In addition, VOCs also have shown antibiotic activity against the human pathogens *Yersinia pestis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Mitchell *et al.*, 2010).

BIOTRANSFORMATIONS

From a chemical point of view, "biotransformation" is the conversion of a chemical compound called "substrate" in another one called "product" through the enzymatic activity of biological catalysts. These bioreactions are due to pools of enzyme occurring in microorganisms (fungi and/or bacteria), individual cells and/or organelles, that could be also extracted and isolated. The biotransformation can transform substrates, chemicals not used as nutrient by the microorganisms, in different compounds, with different application perspective (Bastos Borges *et al.*, 2007).

The chemical compounds that can be considered as substrates for applicative biotransformations are, for example, aromatic compounds, steroids, alkaloids, coumarins, terpenes. The biotransformation is different from biosynthesis and biodegradation. The first is essentially an *ex-novo* synthesis of relatively complex products, catalyzed by enzymes from simple compounds such as carbon dioxide, ammonia or glucose. Biodegradation consists in a catabolic process, that means the conversion of complex compounds into other simpler forms (Giri *et al*., 2001).

Biotransformations are increasing among biotechnological science and one of its most appreciated features is catalyzing regio and stereospecific reactions in chemical (pH) and physical (temperature, pressure) conditions close to those environmental. Moreover, biotransformations allow to obtaine new products as well as to improve the production of already known molecules (Giri *et al*., 2001).

BIOTRANSFORMATIONS AND THEIR EVOLUTION

Throughout history, many different microorganisms have been employed to transform natural resources into processed products, mainly for food and health uses. Obviously, the chemical and biological mechanisms that allow the transformation remained unknown. Only since the last three centuries the scientific research in microbiology, chemistry and biotechnology has allowed us to study and better understanding many aspects at the basis of the biotransformation processes. A huge number of studies were performed about biotrasformations due to microorganisms: (i) sugar fermentation by *Saccharomyces cerevisiae* cells; (ii) conversion mechanism of alcohol to citric acid by *Bacterium xylinum;* (iii) conversion of lactose to lactic acid by *Lactobacillus bulgaricus* and (iv) the sucrose conversion to citric acid by *Aspergillus niger,* used as flavor and preservative in foods and beverages. Biotransformations, bioconversions, biodegradations and fermentations were perceived as technologies able to replace traditional organic chemistry, due to the enthusiasm enhanced by their potential applications. Then, scientists understood that biotransformations can play above all a support and synergy roles for organic chemistry, rather than its substitution. In fact, biotransformations were - and still are - used to facilitate specific steps of semi-synthesis and synthesis of chemical reactions, difficult to perform throught traditional methods (complete synthesis) (Csuk *et al.*, 1991).

Following steps of biotransformation researches considered in the study selection and characterization of microbial strains, followed by the development of technology proper to mantain and optimize the microorganism cultures. These studies also highlighted the importance of secondary metabolism products, especially for pharmaceutical field, such as penicillin and cephalosporin, produced by fungi belonging to the genera *Penicillium* and *Cefalosporium* respectively (Davies *et al.*, 1989).

MICROORGANISMS AND ENZYMES

Biotransformations are chemical reactions catalyzed by enzymes isolated or inside cells (microorganisms or tissues). The biotransformation research aims to discriminate the use of isolated enzymes or cells with reference to chemical perspective (i.e. stereoselectivity yields) or economic point of view (costs and market perspective).

Both approaches have advantages and disadvantages; some of them are shown in the chart below:

Advantages and disadvantages of catalityc system

CATALITYC SYSTEM	ADVANTAGES	DISADVANTAGES
MICROORGANISM	Cheaper	Experience and equipment for microbiological biotransformation are required
		Both biomass removal and product separation are rather long
	Cofactor already present	Possibility of undesidered reactions
		Use of organic cosolvents poorly tolerated by microorganisms
ISOLATED ENZYMES	Simple equipment	Expensive
	Easy control of the process	Adding cofactors or recycling derived molecules is needed
	Easy product separation	
	High stereo selectivity	
	Good tolerance to cosolvents	

The "International Union of Biochemistry" (IUB) classifies enzymes as six main classes according to the catalyzed reaction, as shown in the following chart:

Classification of enzymes according to catalyzed reactions

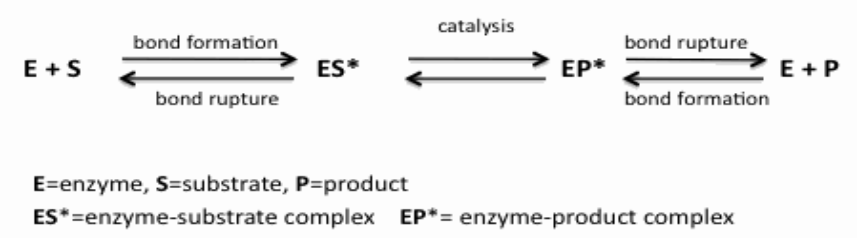
CLASS OF ENZYMES	CATALYZED REACTIONS
Oxidoreductase	Catalysis of redox reactions. That is removal or addition of H with variation of the carbon oxidation state [by removing a couple of electrons (or one H) from a donor chemical (reduction)]. These enzymes always need a specific cofactor (NAD+/ NADH), which acts as acceptor/donor of electrons. The following enzymes are oxidereductase: - hydrogenase: transferring of H_2; - oxidase: electron transferring to O_2; - oxygenase: oxygen transferring; - peroxidase: electron transferring to peroxide.
Transferase	Transferring of atoms from a donor to an acceptor: i.e. transaminases and trans carboxylase.
Hydrolase	Hydrolyzing bonds between C and a different element in presence of H_2O: i.e. protease, amylase, lipase, esterase, etc.
Lyase	Catalyzing of addition or removal of chemical groups with double bonds, C=C, C=O, C=N (excluding peptide bond): i.e. aldolase, decarboxylase.
Isomerase	Isomerization catalysis reactions and transferring reactions within a molecule. The most important is glucose-isomerase.
Ligase	Catalysis of covalent bonds between two molecules

Biotransformations can be performed through the different methods following described:

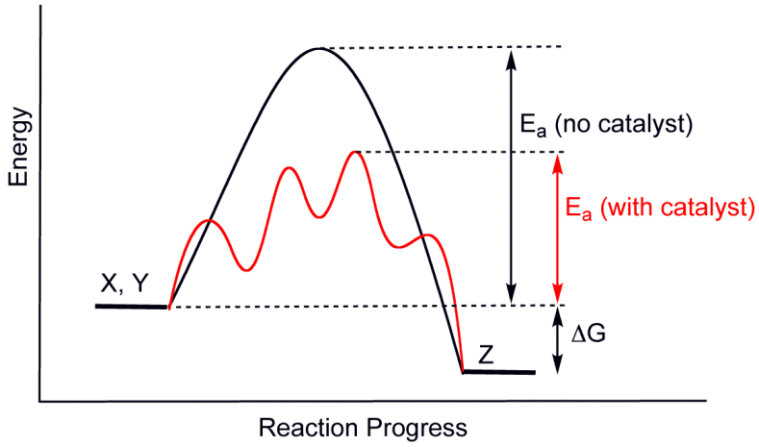
A) Isolated enzymes:

In order to use enzymes as biotransformation tools in industrial processes and scientific research, they have to be extracted, isolated from the source and purified. Despite the isolated enzymes are considered the best catalysts for high purity compounds production, their industrial use is limited by high costs of purification. Moreover, their use requires cofactors that need to be recycled (Yamada *et al*., 1988).

Biotransformations through isolated enzymes



Energy-reaction progres diagram showing reactions performed with and without catalyst.



A biocatalyst must have the following properties in order to better perform their function:

- Maintain unchanged its structure able to reply several times the same reaction;
- Be significantly effective in limited quantities;
- Do not modify the reaction balance;

- Specificity in catalizing the reaction (Ricciotti, 1987).

The most relevant advantages in using isolated enzymes are:

- Catalytic efficiency: the reactions catalyzed occur at a speed 10^8-10^{10} higher than that of non-enzymatic reactions;
- Eco-friendly environmental relapses due to their complete biodegradability and to low concentrations required;
- Chemical and physical environmental conditions: enzymes plays at temperatures around 30 °C, pH values close to 7.0 and environmental pressure. These conditions are extremely soft if compared to those needed by some organic synthesis processes;
- Highly selective reactions: enzymes catalyze specific reactions discriminating three types of selectivity:

  a) *Chemoselectivity:* ability to react with a specific functional group even if other groups are present;

  b) *Regioselectivity*: selectivity towards two or more identical functional groups located in different molecular regions;

  c) *Stereoselective*: ability to turn a chiral compound in a single stereoisomer, or to react with one isomer of a racemic mixture.

Below is reported a list of "false myths" regarding enzymes and their biotechnological applications:

- Isolated enzymes act only in their natural substrate. A lot of enzymes, especially hydrolase (esterases, lipases, proteases, etc.), accept a wide range of substrates. For example, lipases hydrolyzing triglycerides perform also the production of esters from alcohols (Pagani, 1998);
- Isolated enzymes catalize reactions only in aqueous medium. This is true for numerous enzyme classes, but there are exceptions, including lipases. In fact, lipases catalize reactions in lipid-water interphase in cells, and this justify the fact that some lipases catalize biotrasnformations in toluene and ethers as solvent (Pagani, 1998);

Isolated enzymes are extremely sensitive to some environmental conditions. The use of stabilization mechanisms, such as immobilization, crystallization and cross-linking, enable enzymes to be used for several production cycles (Pagani, 1998);

- Isolated enzymes are too expensive for biotransformation processes. It is surely true but not always. In particular, the set up of biotransformation processes has surely high costs at starting point of the industrial chain, but they will be then lower, because for example, the possibility to recicle the enzymes (Rasor *et al*., 2001).

Biological catalysts have also disadvantages:

- Denaturation. This occurs when chemical and physical denaturing agents are present: extreme pH values (acid or basic), not adequate temperature and pressure, particular organic solvents;
- Inactivation. In some cases, once plated catalysis, enzymes tend towards inactive forms, making difficult their recycling;
- Inhibition. Some enzymes are sensitive to the products amount (feed back); to avoid this phenomena, continuing removal of the products from the bioreactor is required.

Since the studies of Buchner (1897), it is clear that enzymes can be active also outside living cells in a proper environment. This has encouraged research on isolation and purification of enzymes from biosources. The availability of pure enzymes has provided an increasing application in bioremediation food processing, textiles, paper industries, biofuels productions, diagnostics and in synthesis of pure chemicals.

B) Microorganisms:

The use of microorganisms in biotransformations is increasing because of many aspects, the most relevant of them is that microorganisms represent a multi-enzymatic system, and, therefore, potentially able to perform different kinds of biotransformations on the same substrate. Other aspects have been reported in more detail the above cited table. The choice of the microorganism is obviously crucial for the success of the biotransformation. At present, the endophytic fungi have not yet been considered, due to above discussed lack of knowledge about their physiology and metabolism (Bastos *et al*., 2007).

The ratio among biomass (microorganisms), medium culture volume and substrate concentrations may vary with reference to microorganism kind (fungi or bacteria) and

to taxonomical classification. Generally, considering fungi as biotransforming tools, the biomass/medium volume ratio is 1:5, but experimental optimizations are needed, such as for what concerns substrate concentration which obviously needs to be lower than cytotoxic levels (Andreotti, 2004; Moreno Rueda, 2010).

The sampling timetable for monitoring biotransformation needs to be set with reference to the different ontogenetic profile of the microorganisms, and may vary from hours to days. Each sampling will be done paying attention to separate biomass from medium solution (filtration, centrifugation) and chemically analyzed. The best performing biotrasnformations are those in which products are completely dissolved in medium, avoiding of storage by microorganisms.

In summary, the most critical points are the following:

- The substrates need to be water soluble; otherwise emulsifier (DMSO, Twin, etc.) is requested;
- Products extraction could be difficult because of different products that could occur in the same experiment;
- In the same biotransformation process, undesired chemicals could be produced due both to enzymatic and chemical reactions (Giri, 2001).

C) Culture of plant cells:

A large number of active ingredients for pharmaceuticals, cosmetics, food and agro-chemicals are extracted from plants: below are listed some problems regarding their chemical production by plant cell culture bioreactors:

- Great metabolic variability mainly due to genetic variability and environmental conditions;
- Plant cells have wall and vacuole: the wall represents a physical barrier to the intake of the substrate in case of endocellular biotrasnformations. Moreover, the cell wall, since it is composed by the polymer cellulose, could seize substrates limiting the biotransformation yield. The vacuole could represent an endocellular storing structure for biotransformation products, making more difficult the extraction procedures. Plant cells could be more useful for biotransformations as protoplasts, i.e. cells without walls.

The biotechnological importance of plant cells is, above all, the capacity to produce a large number and variety of secondary metabolites. The pharmaceutical importance of

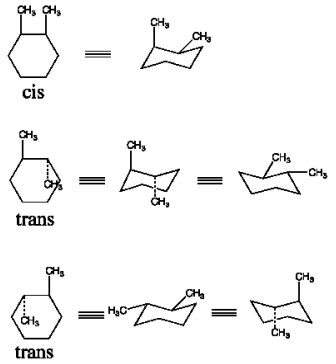
plant secondary metabolites drived the biotechnological research towards the selection of high producing plants (cultivars) and the set up of cell cultures (bioreactors) producing secondary metabolites with applicative importance (Giri *et al*., 2001).

CHEMISTRY OF BIOTRANSFORMATIONS

Chemistry of biotransformation is obviously related to physical and chemical characteristics of the substrates. Above all, stereochemistry is particulary relevant, in particular with reference to pharmaceutical perspectives, where only enantiomeric compounds display activity. Stereochemistry (from the greek *isos*=equal, *mèros*=compound and *stereos*=space) implies stereoisomers: compounds that present the same molecular structure but different spatial configuration, characterizing **enantiomers** and **diastereoisomers.** Enantiomers are defined as molecules whose spatial structures are non-overlapped (chiral chemicals) mirror images of each other and determine the spinning of polarized light plane in an opposite way by the single chemical (optical isomers). With reference to the direction of polarized light rotation, enantiomers are defined as right-handed (+) or left-handed (-). Racemate or racemic mixture is defined as 1:1 ratio of gropued enantiomers (+/-). Enantiomally pure is called a racemic mixture composed by only one of possible enantiomers + or -. Enantiomeric excess (e.e.) is referred instead to a racemic mixture in which enantiomers ratio exceed in behalf of one isomer. As a consequence, biotransformations producing enantiomerically pure products or racemic mixture with important enantiomeric excess promoting the interesting isomer, represent a real biotechnological perspective for those applicative areas, as that pharmaceutical, in which isomers represent the real requested products, otherwise extremely different and expensive to obtain through classical laboratory synthesis.

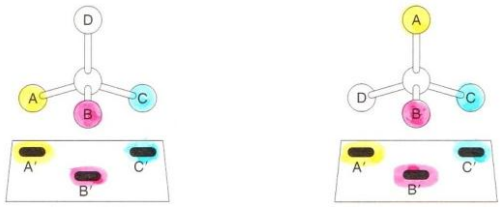
The **diastereoisomers** are instead not-overlapping chemicals but they are not enantiomers, since their images are not identical due to chemical substitute groups localizated on the same size (*cis*) or on the opposite one (*trans*).

Isomers *cis* and *trans* of a molecule



As already mentioned, stereochemistry plays an important role in biological efficacy of chemicals, especially in pharmaceutics. In 1933, Easton and Stedman suggested that the different biological activities between enantiomers were the result of a selective interaction with the receptor (enzyme). The hypothesis stated that those interactions required at least three points of contact with the receptor.

Only the receptor (enzyme) A has the functional groups A, B, C aligned with the corresponding binding sites of the potentially active isomer



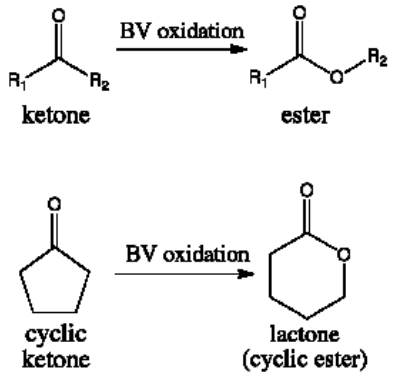
ENZYME A – Receptor surface ENZYME B - Receptor surface

In Figure 3, A-B-C are shown the functional isomer groups that can interact with the complementary sites on the surface of the receptor (enzyme A'-B'-C'). Just one enantiomer has the adequate spatial configuration to interact with the corresponding sites of the receptor. The inhability to obtain the same interactions for the other enantiomer explains its reduced or absent biological activity.

Baeyer-Villiger oxidations through microorganisms

In the late nineteenth century, Adolf von Baeyer and Victor Villiger displayed the chemical processes at the base of the conversion of ketones into esters, as well as cyclic ketones into lactones (cyclic esters).

Baeyer-Villiger oxidation mechanisms



The enantiomerically pure lactones are important structures required in the synthesis of prostaglandins and nucleosides. In this type of oxidation, ketones react with peracids producing a very unstable intermediate. Although this reaction would be useful for the lactones and esters synthesis, the use of reagents such as peracids has a negative environmental relapse.

In 1948, different fungi were found to perform *in vitro* Baeyer-Villiger (BV) oxidations and, in light of this evidence, following researches pointed out the biotransformation capacity of fungi to produce aflatoxins, as well as the production capacity of toxins by shellfish, steroids and iridoids by plants and their cell cultures. Monooxigenase were found to be the enzyme class mainly responsible of all the BVs observed in different biological system and, in many cases, able to produce regio- and enantioselective products (Kamerbeek *et al*., 2003; Wright *et al*., 1996).

BIOTRANSFORMATION PHARMACEUTICAL TARGETS

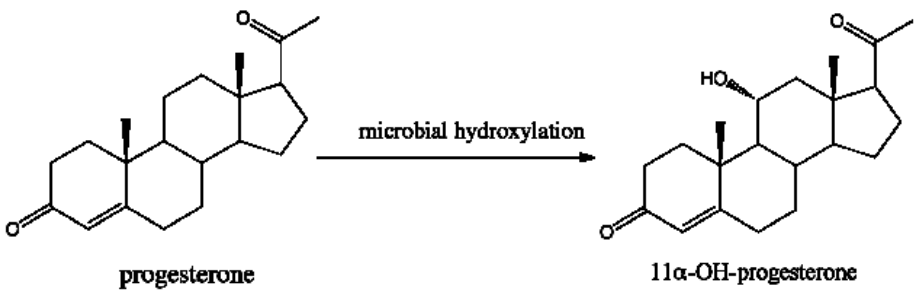
In the last decades, biotechnologies have reached an increasing attention by pharmaceutical research and industry. One of the main reasonis linked to the fact that biotechnologies, once set up as productive chain, could result a relative cheap productive strategy with the possibility to control stereoselective reactions to obtain highly purified enantiomers. As known, the efficacy of many chemical drug depends on their stereospecificity, difficult and expensive to reach through classical chemical synthesis. Biotechnologies, such as biotrasnformations, could be the useful tool for reaching with high yields, highly purified stereoisomers, limiting or avoiding side effects due to non-pharmaceutical isomers. Following are reported some examples about these problems:

- The penicillamine: S-form used as antibiotic while R-form resulted hepatotoxic;
- The sadly known thalidomide can: S-form has teratogenic effects, R-form results sedative (Fabro *et al*., 1967; Icard et al., 2003; Moreno Rueda, 2010).

The results achieved by the pharmaceutical industry through biotechnology are also due to advances in genetics and molecular technologies, that have led increasing availability of new biocatalysts suitable for application on industrial scale. The molecular complexity of active pharmaceutical ingredients is increasing and the synthesis of new active pharmaceutical ingredients (APIs) needs advanced intermediates (AI - Advanced Intermediate), requiring highly selective catalysts. Therefore, enzymes are particularly useful for these purposes in accordance with their chemo, regio and stereo selectivity (Panke *et al.,* 2005).

Given these assumptions, it is easy to understand the big relevance that biotechnologies, related to isolated enzymes, microorganisms and/or cell cultures have for production of new pharmaceuticals. Furthermore, how already stressed, it could be often less expensive obtaining organic compounds through biotechnologies rather than through chemical synthesis. In this regard, it should be remembered the results achieved in the synthesis of pharmaceutical cortisone, a steroid hormone used against rheumatoid arthritis, allergic diseases, asthma, inflammatory diseases in general, cerebral edema, lymphomas, *Lupus erythematosus* and other immunitary diseases, through progesterone hydroxylation (a cortisone precursor), introducing an oxygen on the C_{11} by *Rhizopus arrhizu* (Peterson *et al*., 1952; Bruni, 1999). Thus, in 1952, the "biological" synthesis of cortisone through the biotechnological use of a fungus was reached. The great therapeutic demand, gave a strong impulse to cortisone chemical synthesis, but it required several and very expensive steps with a final rather low yield.

Microbial hydroxylation of progesterone



progesterone 11α-OH-progesterone

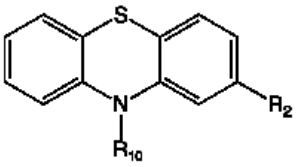
In Italy, a quarter of the pharmaceutical costs concern cardiovascular substances (Istituto Superiore di Sanita', 2007). The statins are drugs that inhibit endogenous cholesterol synthesis by acting on the HMG-CoA reductase (3-hydroxy-3-methylglutarylCoA reductase), the enzyme that converts the 3-hydroxy-3-methylglutarylCoA in mevalonic acid (cholesterol precursor). The research about statin synthesis was improved thanks to the huge interest on cholesterol-reducing drugs. At least six different synthetic pathways are involved in statin biocatalysts. It has been shown that the intermediate production for the synthesis of atorvastatin, one of the most popular statin, can be done through enzymes isolated from *Bacillus megaterium* and *Candida magnoliae*, with a yield enantiomeric excess equal to 99% (Panke *et al*., 2005). The sale of drugs for gastrointestinal disorders follows, by sales volume, the sale of cardiovascular ones. Several studies find new agents with antimicrobial activity threat *Helicobacter pylori* related diseases. In this regard at the University of Nanjing, China, 32 endophytic fungi were isolated from the herbaceous plant *Cynodon dactylon* belonging to the family *Poaceae* (Li *et al.*, 2005); among the 32 isolated fungi, 16 showed high activity against *H. pylori*. In particular, a specie belonging to the genus *Aspergillus*, was able to produce four active secondary metabolites: helvolic acid, monomethylsulochrin, ergosterol, hydroxy-epidiossi-ergostadiene. The corresponding MIC (Minimum Inhibitory Concentration) were respectively 8, 10, 20 and 30 μg/ml; the MIC of the reference antibiotic ampicillin was 2 μg/ml (Li *et al.*, 2005).

Bacteria are also used in biotechnological synthesis of drugs. From *Escherichia coli*, it is possible to isolate shikimic acid, a precursor of many molecular structures responsible of pharmaceutical effects such as *oseltamivir*, the active compound used as antiviral in Tamiflu^{TM} drug (Hoffmann-La Roche and Gilead Sciences), suggested for the treatment of flu. Oseltamivir presents three chiral centers difficult to reproduce by chemical syntesis. Shikimic acid can be isolated in large quantities only from plants or synthetically produced, and this could represent a negative element for ecological sustainability of resources used. However, the overexpression of the genes responsible of the shikimic acid synthesis by *E. coli* in bioreactors has led to the accumulation of high amounts of the chemical equal to 20 g/l (Rasor *et al.*, 2001).

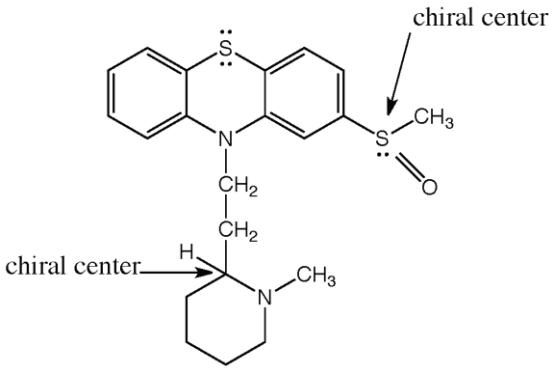
Thus, biotechnologies in general, and biotransformations in particular, are valuable tools for production of enantiomerically pure compounds, and also for supporting and improving research and industrial production in pharmaceutical field. The

biotransformation of **thioridazine** (THD) produced by the endophytic fungi isolated from the plant genus *Viguiera* (Family *Asteraceae*), belongs to the neuroleptic phenothiazines chemical family usually used in the treatment of psychosis. By chemical point of view, phenothiazines are distinguished by their different lateral chains (R_2 e R_{10}).

The neuroleptics family of phenothiazines, to which thioridazine belongs



Phenothiazine



Thioridazine (THD)

The biotransformation of THD by endophytes from *Viguiera* spp. shows how it is possible to imitate the metabolism of the hepatic enzyme CYP1A2/CYP3A4 or CYP2D6[1], that are responsible for drug degradation. In fact, the biotechnological importance and ability of the tested endophytes was to "imitate" the metabolism of mammals producing high amounts of degradation intermediates, showing that this microbial system is a viable alternative for studying thioridazine biotransformation improving knowledge about effects and efficacy on humans (Bastos *et al*., 2007).

TERPENES AND BIOTRANSFORMATIONS

Terpenes occur widely in Nature, mainly as constituents of essential oils in plants. More than 20,000 individual terpenoids are known at present, making them the largest group of studied natural products. Terpenes have increased research attention because of their

[1] The "cytochrome P450 monooxygenase" is a family of hepatic enzymes responsible for oxidation and degradation of drugs. The different enzymes are described by the abbreviation CYP (cytochrome P450), followed by an number indicating the immediate family (CYP1, CYP2, CYP3, etc.) and then by a letter specifying the subfamily (CYP1A, CYP2C, etc. .) (Williams, 2005).

role in prevention and therapy of several diseases, in their activity as antimicrobial agents and as building blocks for the synthesis of many applicative useful compounds.

Among terpenes, monoterpenes are C_{10} volatile organic compounds (VOCs) naturally produced by many plant species representing important constituents of numerous essential oils under qualitative, quantitative and functional point of view. Even though chemical synthetic process still represents the cheapest technology for monoterpenes industrial production, in recent years a significant preference towards biotransformation processes has been carried out by using microbial cells as biocatalysts for the production of fine chemicals. Biotransformation processes regarding terpenes constitute a relatively recent research subject. The importance of this biotechnological approach resides in the possibility to produce enantiomerically pure compounds under mild reaction conditions (de Carvalho *et al.*, 2006). Moreover, the use of cells as biocatalysts is considered an eco-friendly technology. Under a normative point of view, molecules obtained by such bioprocesses are labeled as "natural" or "natural flavor" (USA, EU) (Ponzoni *et al.*, 2008).

ANALYTICAL TECHNIQUES

Chromatography

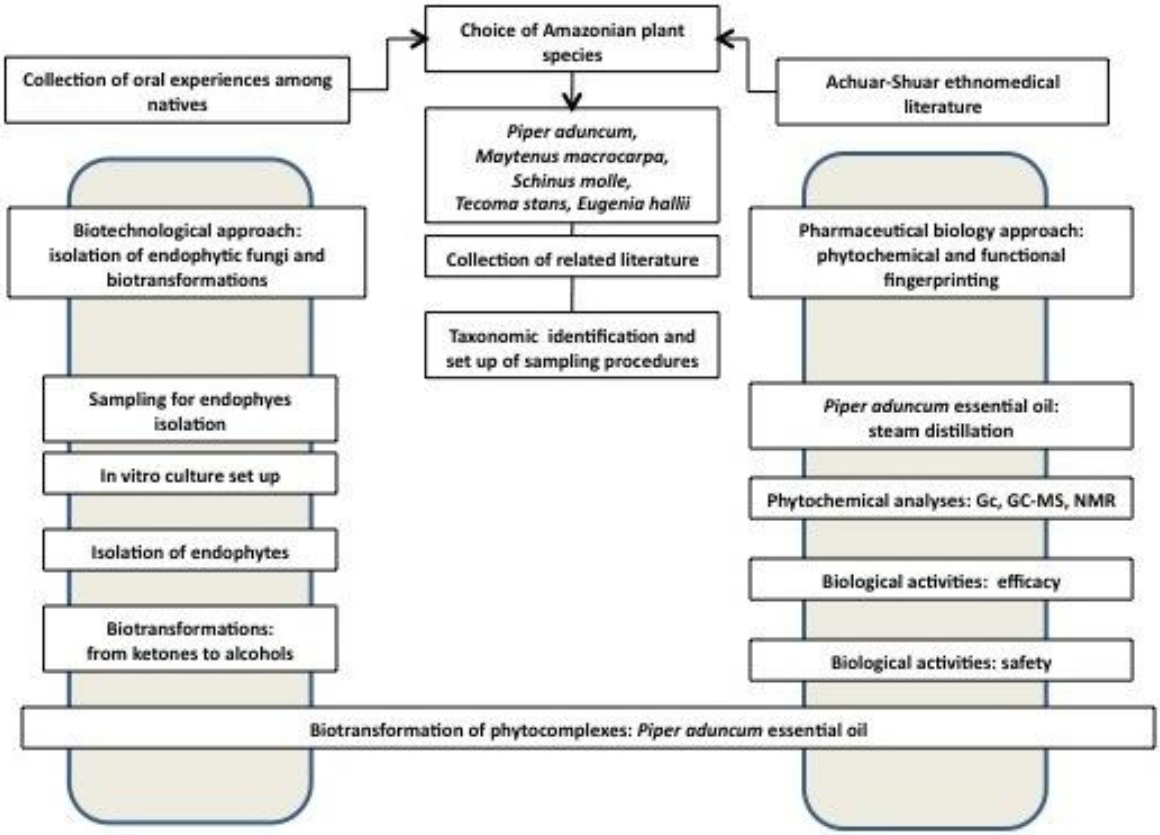
The need to separate individual compounds from mixtures has led to the development of separation techniques, which discriminate between physical and chemical differences between the molecules. In this research, TLC (Thin Layer Chromatography) and GC (Gas Chromatography, FID and MS) were considered as main chromatographic tools to achieve analitycal data about biotransformations and pharmaceutical biology characterization of Amazonian plants. NMR analysis were also used where appropriate.

4. MATERIALS AND METHODS

RESEARCH PREMISES

The research premises and the materials and methods paragraphs are summarized in the following Scheme 1, which reports in more detail what planned for the PhD study:

Scheme 1 – Research plan:



THE RESEARCH AREA AND KEYS FOR CHOOSING THE PLANT SPECIES

The criteria of botanical sources selection are extremely relevant for increasing the opportunities to isolate new microorganisms and new active compounds for applicative uses such as pharmaceuticals. Thus, the choice should consider some notable aspects such as high biodiversity of the origin ecosystems, ethnobotanical aspects, the endemisms and their longevity (Saikia, 2008).

Considering these premises, the following botanical sources were chosen as the subject of the research with general reference to the above cited considerations, to the opportunities to collect fresh and available plant material limiting biological degradation, and with particular interest to the ethnomedical importance which these species have for Shuar and Achuar people, the ethnic groups typical of the Amazonian Ecuador areas where the first part of the researches have been performed.

Thus, the availability of the plant material constitutes one of the most important factor to guarantee an easy performing research and reproducibility of the data achieved. In fact, the work during the first year consisted also in setting up a laboratory allowing to perform the first steps of the research, as already pointed out in the introduction paragraphs (Scheme 1).

The research area is the Amazonian region of Ecuador. In particular, it is evidenced in the following map the circled region, just to the border of the Amazonian forest, in which the first part of the research has been performed.

Map of Ecuador and research area (circled)

The Amazon is the ecosystem with the highest biodiversity in the world and Ecuador is considered one of the 17 "megadiverse" countries (Rai *et al*., 2003). It is well known that South America is a promising region for the study of the health potential of plants as sources of new pharmaceutical treatments. The presence of a strong ethnomedical tradition leads the research toward an in-depth and developing study of amazonian biodiversity, both under a chemical and biological point of view. The large part of the higher plant species grows in South America, amazonian forest in particular, and, in parallel, 80% of humankind lives in "emerging countries", basing their health needs on plant related traditional remedies (WHO-OMS). Furthermore, the choice of the species has been made starting from the medicinal properties attributed to plants by Amazonian indigenous people, Achuar and Shuar in particular; the ethnomedical knowledge of Natives has been collected by direct interview and bibliographic documents (Kloucek *et al*., 2006 and references therein).

This approach gave rise to the choice of species, whose knowledge and traditional uses are still and mainly based on oral hand on.

Thus, the species subject of the present research are:

a. *Piper aduncum* (Fam. Piperaceae, common name in Ecuador "Matico");
b. *Maytenus macrocarpa* (Fam. Celastraceae, common name in Ecuador "Chuchuguazo");
c. *Schinus molle* L. (Fam. Anacardiaceae, common name in Ecuador "falso pepe");
d. *Tecoma stans* (L.) H., B. et K. (Fam. Bigoniaceae, common name in Ecuador "Tepla");
e. *Eugenia hallii* Berg. (Fam. Myrtaceae, common name in Ecuador "Arrayan")

Piper aduncum - MATICO

Botanical classification (Missouri Botanical Garden)
Common name: MATICO

Branch: ***Magnoliophyta*** Class: ***Magnoliopsida*** Order: ***Piperales*** Family: ***Piperaceae*** Genus: ***Piper*** Specie: ***P. aduncum***	**Synonyms - among the existing 129, here following are reported the most cited:** *Piper aduncifolium* Trel. *Piper anguillaespicum* Trel. *Piper angustifolium* Lam. *Artanthe celtidifolia* (Kunth) Miq.

Botanical description

Piper aduncum belongs to Family *Piperaceae* characterized by tropical plants, usually shrubs and vines. The family includes four main genus (*Lepianthes, Peperomia, Piper, Macropiper*) and more than 2000 species. From an economic standpoint, the species are important as they provide the pepper *Piper nigrum* (black pepper), the cubeba (*P. cubeba*) native of Java, used as a spice and as a urinary antiseptic as essential oil and kawa (*P. methysticum*) from which the natives of the Pacific islands get an alcoholic drink with sedative properties (Schultes, 1995). The species *P. aduncum* is a branched shrub that can reach 5 m in height. It is native from the Caribbean and it is now widespread throughout the tropical area, often acts also as a weed colonizing marginal areas of urban centres (Guerrini *et al*., 2009).

P. aduncum adult plants



Phytochemical and biological acknowledgement

Compounds currently known to be functionally characterizing traditional health properties: terpenes (mono-, sesqui-, di-), alkaloids

Health traditional remedies and bioactivities: Several species of the genus *Piper* are used in traditional medicine for their antiseptic, insecticidal and antibiotic properties. An infusion made with leaves and roots is used to treat diarrhea, nausea, genital and urinary infections, and also to control the bleeding in haemorrhage. The essential oil is known to have insecticidal properties, molluscicides and antibacterial activity (Guerrini *et al*., 2009).

Maytenus macrocarpa - CHUCHUGUAZO

Botanical classification (Missouri Botanical Garden)
Common name: CHUCHUGUAZO

Branch: ***Magnoliophyta***	**Synonymous:**
Class: ***Magnoliopsida***	*Celastrus macrocarpus* Ruiz & Pav.
Order: ***Celastrales***	*Haenkea macrocarpa* Steud.
Family: ***Celastraceae***	*Haenkea multiflora* Ruiz & Pav.
Genus: ***Maytenus***	*Maytenus multiflora* (Ruiz & Pav.) Loes
Specie: ***M. macrocarpa***	*Maytenus tarapotensis* Briq

Botanical description

Maytenus macrocarpa belongs to the family *Celastraceae*, which includes about 50 genera and 800 species of plants with different habit: tree, shrub and climbing. The *Maytenus* genus is characterized by higher plant evergreens and climbing plants, is present in tropical South America and western India (Schultes, 1995). The species *M. macrocarpa* is a tree up to 25 m tall, well branched, with reddish bark, leaves entire, alternate, leathery, elliptical, light green, very small axillary flowers.

Adult plants and leaves of *Maytenus macrocarpa*

Phytochemical and biological acknowledgement

Compounds currently known to be functionally characterizing traditional health properties: alkaloids, saponins, tannins, anthraquinones, glycosides, cardiotonic
Health traditional remedies and bioactivities:
- toxicity in *Artemia salina*: DL50=8,76 ppm (bark);
- antibacterial activity towards *Escherichia coli;* low performance (not significant) against *Staphylococcus aureus*, *Klebsiella spp.*, *Salmonella enteriditis*, *Pseudomonas aeruginosas*, *Proteus vulgaris* and *Salmonella typhi;*
- antifungal activity towards *Trichophyton rubrum;* low performance (not significant) against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (data from Villacres *et al*., 1995).

The genus *Maytenus* presents a complex and relevant – but scarcely investigated – phytochemistry; it is rich of particular compounds including the macrocyclic alkaloids, which are closely similar to fungal substances, known as ansa-macrolide and generally characterized by strong antibiotic properties, named chuchuhuanine (Shirota *et al*., 2004) and laevisine (Piacente *et al.*, 1999).
In traditional Amazonian medicine, it is used for the production of bark decoction with anti-inflammatory, antirheumatic and anti-diarrheal properties (Schultes, 1995). Further and recent investigations assign to the genus analgesic, antirheumatic, tonic and antianemic activities (Rios *et al*., 2007).

Schinus molle- FALSO PEPE

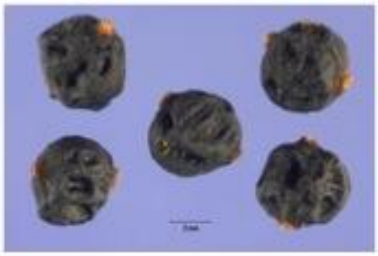
Botanical classification (Missouri Botanical Garden)
Common name: FALSO PEPE

Branch: ***Magnoliophyta*** Class: ***Magnoliopsida*** Order: ***Sapindales*** Family: ***Anacardiaceae*** Genus: ***Schinus*** Specie: ***S. molle***	**Synonyms:** *Schinus areira* L. *Schinus huygan* Molina

Botanical description

The *Schinus molle* is an evergreen tree, native to South America where it spontaneously grows, spread from southern Mexico to northern Chile. It reaches heights between 3 and 15 m; it is characterized by rather short woody stem with deep cracks; the bark is dark brown. The branches are slender with drooping posture. The leaves are composed, narrow and lance-shaped, smooth and deep green with a characteristic smell similar to that of pepper, if rubbed. The hermaphrodite flowers are small, grouped in terminal panicle dangling orange. The fruit is a drupe similar to a pink common peppercorn in size (Barceloux, 2008).

Seeds, adult plant and leaves of *S. molle*


Phytochemical and biological acknowledgement

Compounds currently known to be functionally characterizing traditional health properties: terpenes (mono-, sesqui-)
Health traditional remedies and bioactivities:
- toxicity: no literature reports
- antibacterial activity against *Klebsiella pneumoniae, Alcaligenes faecalis, Pseudomonas aeruginosa, Acinetobacter calcoacetica, Escherichia coli, Beneckea natriegens, Citrobacter freundii, Serratia marcescens, Bacillus subtilis, Brochonthrix thermosphacata;*
- antifungal activity against *Aspergillus ochraceus, Aspergillus parasiticus, Fusarium culmorum, Alternaria alternata*
(data from Gundidza, 1993)

The essential oil obtained by steam distillation of fresh leaves of *S. molle* is reported to have a significant antifungal activity against the most common fungi detectable in food spoiling. The toxicity of the essential oil persists even at temperature above 80°C and over 90 days of storage, but decreased significantly when autoclaved. Its chemical composition includes 50 different compounds (Dikshit, 1986).

***Tecoma stans*- TEPLA**

Botanical classification (Missouri Botanical Garden)
Common name: TEPLA

Branch: ***Magnoliophyta***
Class: ***Magnoliopsida***
Order: ***Lamiales***
Family: ***Bignoniaceae***
Genus: ***Tecoma***
Specie: ***T. stans*** (L.) Juss. ex Kunth

Synonymous:
Bignonia stans L.
Stenolobium stans Seem.

Botanical description

Tecoma stans is an evergreen shrub native of Central and South America, which reaches a height of between 5 and 7 m. The bark has a color ranging from pale brown to gray, roughened with increasing age. The leaves are composed and imparipinnate with 2-5 pairs of leaves; at the end the leaves are lanceolate in shape, about 10cm long and with a tight edge. The flowers are clustered at the distal part of the branches, trumpet shaped with yellow corolla. The fruits are narrow and slightly flattened, about 20 cm long; they are green when immature, and pale brown at maturity, growing on the plant for several months.

Tecoma stans adult plant. Blooming young stems; blooming adult plant with flowers and bark (particular); leaves.



Phytochemical and biological acknowledgement

Compounds currently known to be functionally characterizing traditional health properties: phenols, flavonoids, alkaloids (Alonso-Castro *et al.*, 2010)

Health traditional remedies and bioactivities:
- toxicity: no literature reports
- antibacterial activity against *Pseudomonas aeruginosa* (Ramesh *et al.*, 2009) and other Gram + and Gram- bacteria (Binutu *et al.*, 1994);
- antifungal activity against *Rhizoctonia solani, Fusarium oxysporum, Penicillium janthinellum, P. expansum, Aspergillus parasiticus, Colletotrichum gloeosoariiodes, Trichoderma harzianum, Pythium ultimum, Phytophthora nicotiana;* average value of the MIC, for all the mushrooms considered, equal to 0.55 mg/ml (Meela *et al.*, 2008).

An infusion of leaves is known to have diuretic properties and it is also used to treat diabetes, stomach and intestinal problems (Orwa *et al.*, 2009).
The alkaloid tecomina seems to be responsible of hypoglycemic activity of traditional preparations (Costantino *et al.*, 2003).

***Eugenia hallii*- ARRAYAN**

Botanical classification (Missouri Botanical Garden)
Common name: ARRAYAN

Branch: ***Magnoliophyta***
Class: ***Magnoliopsida***
Order: ***Myrtales***
Family: ***Myrtaceae***
Genus: ***Eugenia***
Specie: ***E. hallii***

Synonymous:
Myrcianthes hallii (O. Berg) McVaugh
Myrteola hallii (O. Berg) Kausel
Amyrsia hallii (O. Berg) Kausel

Botanical description

Eugenia is a genus of evergreen trees and shrubs. It has a tropical and sub-tropical distribution, especially diffused in the Andean region, Caribbean, Brazil, New Caledonia and Madagascar. Several species are grown as ornamental plants for their attractive foliage, and edible fruits, even if the production is not adequate to a large marketing.

Phytochemical and biological acknowledgement

Compounds currently known to be functionally characterizing traditional health properties: terpenes

Health traditional remedies and bioactivities:
- toxicity: no literature reports
- antibacterial activity: no reports regarding in particular the specie *E. hallii*. Other studies performed on essential oil from species of the same genus, including *E. caryophylli* (cloves), *E. brasiliensis* (Grumichama), *E. umbelliflora* and *E. caryophyllata* showed antibacterial activity against *S. aureus*, *P. aeruginosa, E. coli* and against the most resistant strain lines of *S. aureus* (Magina *et al*., 2009);
- antifungal activity. no reports regarding in particular the specie *E. hallii*. Other studies about the essential oil of *E. caryophyllata* has shown activity against fungal plant pathogen *Botrytis cinerea* (Wilson *et al*., 1996); same results were achieved with aqueous and alcoholic extracts from *E. caryophyllata* seeds against *C. albicans*, *Cryptococcus neoformans*, *A. flavus*, *A. fumigatus*, *A. niger*, *Rhizopus* sp., *Trichophyton rubrum*, *T. Mentagrophytes*, *Microsporum gypseum* and *Penicillium digitatum* (Chandrasekaran *et al*., 2004; Romagnoli and Sacchetti, 2003).

THE BEGINNING: ***a general outline of the research***

Among the beginning steps of the research, one of the most relevant consisted in collecting ethno-pharmaco-botanical information directly from members of the Shuar and Achuar ethnic groups by informal interview, together with the usual bibliographic web research done, in part, within the laboratory of the Salesian Polytechnic University (Macas and Quito) and completed at the University of Ferrara. The informal interviews were taken in the local communities in the Amazonian forest where plant traditional knowledge is still very strong. I met native women, who allowed me to visit their medicinal gardens and share knowledge with them. My aim was to valorise and collect the ancestral knowledge directly from its best guardians: native peoples. I had wide acceptance, due to my activity as international volunteer in partnership with native representatives.

Shuar and Achuar ethnic groups met during interviews

Information on plants was obtained through casual conversations: about 20 inteviews with Natives mainly keepers of the Shuar and Achuar ethnomedical culture (about 30-35 years old on average). All the persons interviewed treat any kind of diseases with

plants and their preparation. Attention has been paid to spontaneity and to the weighting of each information to avoid ambiguous classification. The criteria employed to collect etnomedical information were taken considering the suggestions reported by Ealler (1993).

Scientific news about the species considered are few and limited but the ethnobotanical knowledge needs to be translated into a scientific language through performing phytochemical studies and cross-linking acquired data with healthy perspectives (Rai *et al*., 2003): this was one of my PhD main and general purpose. However, for what concerns pertinent bibliographic data, biological evidences, a part from the "GRAS" characterization (Generally Recognized As Safe) of these drugs, are still expressed with *in vitro* models and thus still far from a full healthy evidence and a detailed clinical and chemical efficacy. Besides the cross linking of phytochemical and biological data, to stress the biotechnological imprinting of my PhD research, it has been considered of great interest to investigate the fungal endophyte populations of selected plants, then estimating, by an healthy projection, their biotechnological potential and perspectives in general, biotransformations in particular.

The isolation of endophytic fungi was performed from specific explants, as reported by classical biotechnological related methods (Lu *et al*., 2000), requiring, however, experience and considerable time consuming, both in relation to the quality and quantity of isolated fungi and to the laboratory conditions in Amazonian Ecuador, related to a research strategy and planning defined in a development and cooperation context between Italian Institutions (VIS, Volontariato Internazionale per lo Sviluppo; MAE, Ministero degli Affari Esteri; University of Ferrara) and the Ecuadorian ones (Salesian Polytechnic University, Quito and Fundacion Chankuap, Macas).

Salesian Polytechnic University in Quito

This context, which mainly characterized the first year of PhD, has imposed a dual purpose: the first, substantially different from a classical way to perform PhD, linked to the needs to set up and develop laboratories and instrumentation lacking, and the second to perform the planned research. The first aspect, obviously, constitutes a limit in a practical and conceptual sense of performing a traditional PhD; however, the same allowed to live new experiences for new frontiers of research, strictly connected to Cooperation and Development activities, cross-countries programmes to which my PhD belongs together with voluntary associations. This aspect characterizes the research of ethical aspects, eco-friendly and sustainable profiles.

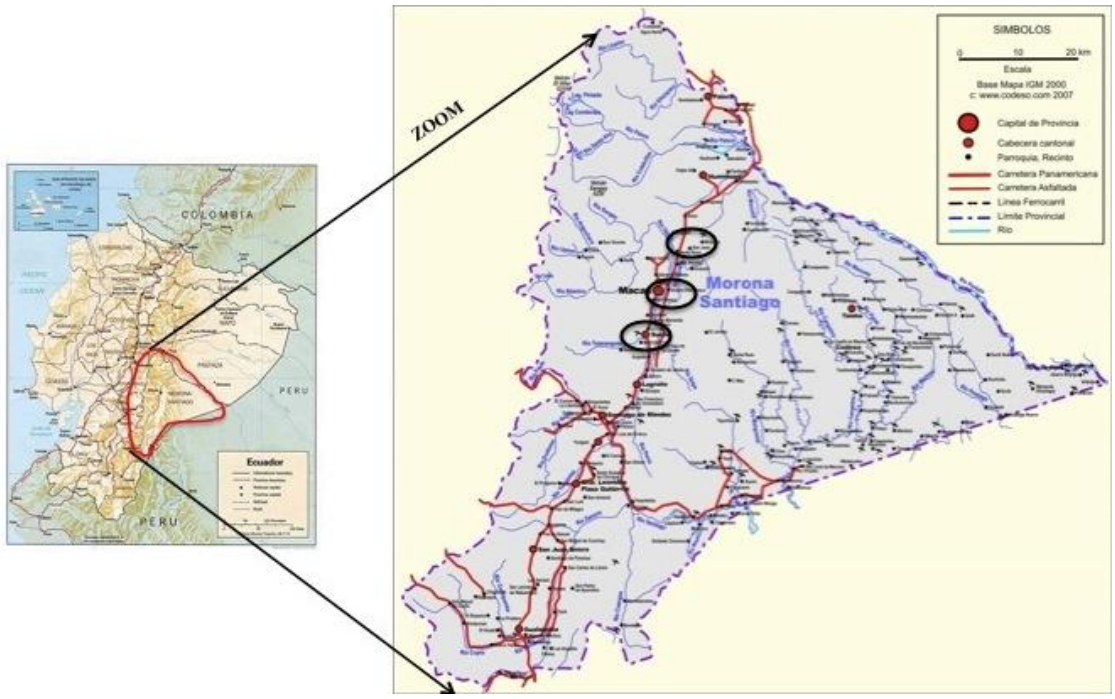
In fact, the greatest biodiversity is concentrated in "developing" countries and represents one of the greatest opportunities for the development of life sciences in general, and of the pharmaceutical ones in particular.

From these bases, it started the research characterizing my PhD.

Sampling and taxonomic identification of the plants

Samples were collected from the above mentioned adult plants in March and April 2007 at Wapu reserve and Sevilla Don Bosco (Morona Santiago District, Ecuador; Long 78°08"W/ Lat 2°22"S; see above figure). Three different areas have been identified from which samples of each specie were taken, in order to guarantee scientific significance of the acquiring data. The chosen areas were similar for geographic and environmental conditions, at 1200mt above the sea level with equatorial climate (see following maps).

Map of Morona Santiago District, areas of plant collecting (circled)



The sampling was particularly critical for the environmental conditions of the context: temperature (about 22°C) and humidity (about 80%) which can easily determine a rapid degradation of the biological samples, general problems due to the specificity of the place: the amazonian forest, so amazing but at the same time so unpredictable under many points of view (i.e. the lack of pathways), could become sometimes also dangerous.

The identification of plant sources in wild environment has been made with the help of expert Natives, while taxonomic characterization has been performed under the supervision of Prof. Marco Cerna, an expert in tropical botany of the Salesian Polytechnic University (UPS) at the National Herbarium of Ecuador, near Quito, where authentic specimens have been then stored.

The taxonomic identification followed a classic taxonomic study based on the comparison between wild samples and those authentic deposited in the herbarium, by macro- and microscopical observations and morphometric evaluations of the blooming parts of the plants, young and mature leaves, shoots and fruits. For all these operations a Leica ZOOM 2000 microscope was used. Samples specimens characterizing each specie have been also deposited at the "Centro de Investigacion y Valoraciòn de la Biodiversidad ” (CIVABI) of the UPS of Quito.

Dried specimens were deposited at the Department and Evolution University of Ferrara with the following code: POB001-*P. aduncum*, CHU1-*M. macrocarpa*, SCH001-*S. molle*, TST001-*T. stans*, EUH001-*E. hallii*

Images of Morona Santiago District environment

The plant samples were characterized by picked leaves, petioles, young twigs and bark samples (see following figures).

Sampling example: bark of *Maytenus macrocarpa* (Chuchuguazo)

The sampling of all the chosen plant species has been made on adult blooming plants.

In order to have samples representative of the entire tree, sampling has been made at different levels of the plant for each specie: upper, middle and at the base of the stalk. Roots were omitted because do not used as traditional crude drug. Leaves and petioles were manually removed; young branches and stem barks were instead sampled employing an adequate cutter. The cutter was previously washed and sterilized with 70% alcohol solution order to limit possible contaminations.

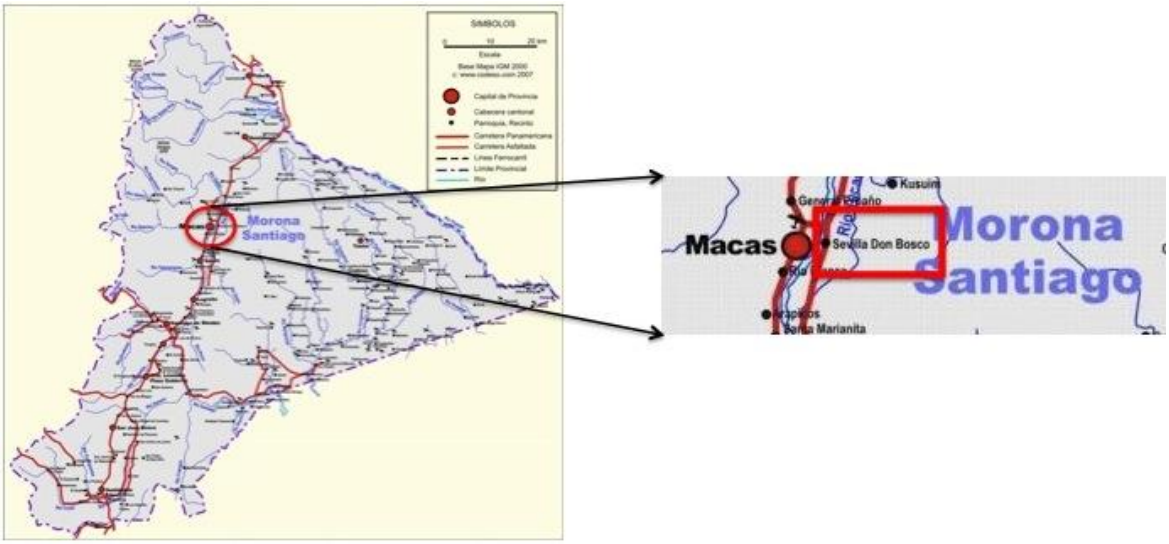
The collected material was immediately wrapped in a moistened paper and then immediately transported to the laboratory (about 1h flight) and stored at 4 °C. Within 48 hs all the collected material was subjected to the isolation of endophytic fungi. This last phase was characterized by processing the samples using the modified method of Petrini (Petrini, 1993). Then, the treated samples were transferred in *in vitro* supports (Petri plates) in order to allow growth and following isolation of endophytes.

BIOTECHNOLOGICAL APPROACH: ISOLATION OF ENDOPHYTIC FUNGI AND BIOTRANSFORMATIONS

The Sevilla Don Bosco laboratory

This phase of the research took place in Sevilla Don Bosco, 2 km faraway from Macas (Ecuador), a small town at the border of the amazonian forest (see map below), that means in a structure which I practically contribute to built and organize as laboratory: the laboratory has been built in relation to a Cooperation and Development project, coordinated both by the "University Center for International Development Cooperation" of the University of Ferrara and by the CIVABI "Centro de Investigación y Valoración de la Biodiversidad" of the Salesian Polytechnic University, Quito.

Location of Sevilla Don Bosco laboratory



CIVABI in Sevilla Don Bosco: colleagues and I outside the laboratory just before inauguration.

Inside the laboratory: departments of microbiology and phytochemistry

Isolation of endophytic fungi: general procedures

Before the inoculation of plant samples, the laboratory equipment was prepared as indicated in the following table:

Laboratory material used

Ethanol 70%	/	Sigma Aldrich
Sodium hypochlorite 5%	/	Sigma Aldrich
MA (Malt Agar)	malt extract (20g) agar (15g) distillated water (1000ml)	DIFCO
MEA (Malt Extract Agar)	malt extract (20g) peptone (1g) glucose (20g) agar (15g) distillated water (1000ml)	DIFCO
TS (Soy peptone)	bactosoytone (10g) glucose (20g)	DIFCO
MYCOSEL AGAR	fitone (10g) glucose (20g) ciclo-heximide (0,4g) distillated water (1000ml)	Becton Dickinson

Each medium, prepared according to label claims, was autoclaved for 15' at 121 °C, and then, after cooling at 45 °C, 200 mg/l of the antibiotic chloramphenicol (Sigma-Aldrich) to inhibit bacterial growth were added. The mediums, still liquid, have been transferred into Petri dishes at a rate of 30 ml/plate. When mediums were solidified, the Petri dishes were kept at 4 °C until their use.

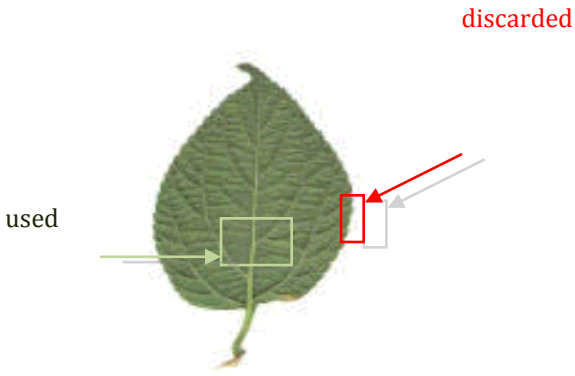
Four different types of culture media were used (see table above) in order to isolate as many strains of endophytes as possible, following both literature indications and laboratory experiences of the Italian research group to which I collaborate. In fact, a different composition in culture medium generally contributes to an easily noticeable fungal growth by assessed morphological aspects (Andreotti, 2004, Moreno Rueda 2010).

Samples set up for inoculum

The plant material was previously washed with running water to remove coarse impurities and then dried with paper towels. Then, we proceed to the plant matrix sanitization according to the protocol reported by Andreotti (2004; see scheme below). This operation allows to kill the microorganisms on the outer surface of the plant samples, without damaging the possible endophytes inside the plant parenchymas.

The protocol used is outlined below:

The plant material was washed in ethanol 70% (1 min); then, dipped in sodium hypochlorite solution (5%; 5 min) and finally rinsed in sterile distilled water (10 min). The whole operation is performed under a laminar flow hood using sterile equipment. The samples were then cut with sterile cutter until obtaining bits of about $1cm^2$ in size for leaves and barks, and about $2cm^2$ for branches and stems. In fact, the different plant samples and the different tissues which characterize the plant material to be processed need particular size to make easier the outflow of the possible endophytes (Andreotti, 2004, Moreno Rueda 2010 and references therein). For this reason, in fact, leaves were deprived of the outer edge and cut into square pieces, taking care to choose pieces of tissue around the midrib, while branches, stems and bark were deprived of the coriaceous outer tissues (Petrini *et al*., 1992). See scheme below (leaves).

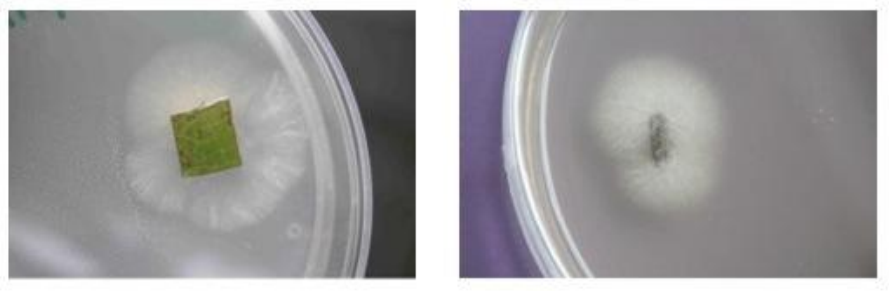


Inoculum of plant samples for checking endophytes

Considering the high volume of plant material for inoculum, the Petri dishes were carefully and adequately marked to easily trace them for the plant specie, the kind of samples (part of the plant), kind of culture medium, date. For each plant specie 64x3 plates were set up, considering 4 plates for each kind of medium, 4 plates for each plant sample (leaves, bark, stems, branches), and the the fact that the experiment has been performed in triplicate.

The plant tissue bits have been lied on the agar surface of the plate, making them properly adhere to the culture medium: this condition promotes the emission of fungal hyphae (Andreotti, 2004, Moreno Rueda 2010 and references cited) (see figure below).

P. aduncum leave and branch samples laid on PDA; endophyte fungal hyphae are clearly spurting out from the edges of the samples



Once prepared as described, the plates were kept at 24 ºC (1 month). Every 48hs the plates were checked in order to verify the presence of endophytic fungi proliferation.

Isolation of endophytic fungi

The isolation is a very crucial step because it had to be done mainly with reference to general morphological discrimination of endophytic fungi, eventually grown. After the one month incubation period, a percentage of 80% plates showed the presence of emerging endophytes. On average of 2 fungal mycelia – easily noticeable by the different macroscopical morphology - could be checked. See figure below.

Plate in which almost three different mycelia could be distinguished.
Stem samples of *P. aduncum;* MEA medium. Isolation in laboratory

The plates showing endophytic mycelia have been processed by progressive subculturing to isolate the strains as pure cultures (see figure below). The subculturing of the mycelia has been made inoculating fungal explants on PDA, chloramphenicol enriched (200 mg/L). The plates were kept at room temperature as stated by appropriate protocols (Andreotti, 2004, Moreno Rueda 2010).

Each pure strain has been coded with a serial number as reference of the above mentioned parameters, plus the number of subculturing and a brief morphological description. A pattern of this way to proceed is summarized below:

Example scheme of the pattern used to catalog isolated pure endophyte strains.

Legends: Mat=Matico, S=stem, MEA=Malt Extract Agar, MA=Malt Agar, TS=bacterial soy peptone, MYC=Mycosel Agar

Strain No.	Plant	Vegetal matrix	MEA	MA	TS	MYC	No. of times	Colony description
077	Mat	S			X		1	Colony diameter: 2cm. Mycelium: white and pale opalescent, sporulated in the center.

Description of endophytic fungi mycelium

The systematic identification and classification considers features as macroscopic morphology, texture of mycelium, color, the reverse of the colony, the presence of

exudates and the color of the grown medium. Those microscopic, istead, refer to the kind of hyphae (more or less septed), the kind of fruiting body, etc. However, as practical criteria for beginning to characterize and isolate fungi as the same or different, the strains with similar macroscopical characteristics – i.e. color of the mycelium and of the medium, the shape of mycelia margins – were grouped and coded as shown in the following table:

Plant	No. isolated strains	Codes
Piper aduncum *Maytenus macrocarpa*	235	from **EC01** to **EC65**
Schinus molle *Tecoma stans* *Eugenia hallii*	129	from **FE1** to **FE129**

Identification of endophytic fungi

All the isolated fungal strains were not taxonomically described. However, it could be reasonable to suggest that each fungi do not necessarily correspond to a different specie. However, in light of our starting biotransformative targets, the characterization of each strain as morphologically different from the others, was a sufficient criteria to proceed for checking the biotechnological capacities. The strains that gave the best results in terms of biotransformation activity have been sent to the Fungal Biodiversity Centre of the Central Bureau Voor Schimmelcultures (CBS) in Utrecht, the Netherlands to be taxonomically identified. The identification will be performed evaluating phenotypic aspects of the colony and/or DNA analysis of fungal organism. Unfortunately, the taxonomic identification of the most performing strains for biotransformations (see appropriate "Results and Discussion" section) are not still completed. However, suggestions about their classification could be made on the basis of our previous experiences (Andreotti, 2004; Moreno Rueda 2010 and reference cited).

Pure cultures of isolated endophytic fungi

Storing of fungal strains

After obtaining the endophytic fungi pure culture on PDA, I proceeded to the storing phase, in order to keep them viable in time to realize the following biotransformation steps of the research. For each pure fungal strain, definitely isolated in Petri dishes and checked for regular growth after subculturings, 5 cylindrical ($\approx 1 cm^3$) samples were taken under sterile conditions. The number of samples reflects the need to preserve the strains and to have abundant mycelium to perform following biotransformations studies at the same time. Each cylindrical samples was characterized by agarized medium and growing mycelium. The sampling of each Petri dishes was randomized, but growing mycelium (vegetative growth phase) at the border of the plate, and fruiting mycelium, in the center of plate generally (reproductive phase of groth) have been taken from each Petri. Each sample has been then placed in vials with screw cap containing 20 ml of sterile distilled water (Figure below). The endophyte samples were then stored at room temperature. The procedure was performed in order to have identical samples for the CIVABI labs (Quito), Sevilla Don Bosco (Macas) labs, and for the University of Ferrara. In order to transfer the isolated strains at the Pharmaceutical biology and biotransformations labs of the University of Ferrara, each strain was grown in slant tubes with PDA medium. To export all the strains in Italy, all legal documentations have been produced.

Strains subculturing and storing: tools and laboratory material; sterile operations

Biotransformation activity of fungal strains

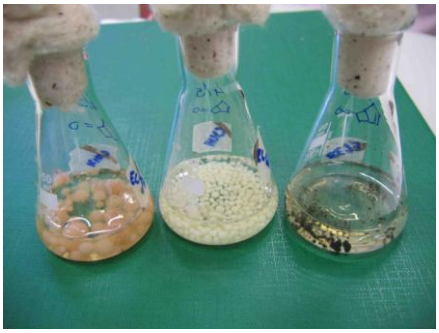
The isolated endophytes were then tested *in vitro* for biotransformation capacities on various chemicals with the specific aim to evaluate biocatalytic reactions with regio and stereo selective results. The chemicals employed to check biotransformations (see following table) were chosen for their importance as molecular pattern similar to that pharmaceutical interest compounds (Masood *et al.*, 2010; Iwaki *et al*., 2006).

In vitro *cultures set up for biotransformations*

The endophytes were inoculated in PDB liquid medium (Potato Dextrose Broth, Liofilchem srl, Italy), sampling a portion of mycelium from the respective slants, prepared as above described, suspended in tubes containing 2 ml of sterile water as preliminar step before transferring the fungi samples in bioreactors (20 ml sterile flasks) to check their biotransformation capacities. Sterile flasks (20 ml) were prepared with a liquid medium quantity corresponding to a 1:5 ratio with respect to bioreactors volume (Andreotti, 2004; Moreno Rueda, 2010). After inoculum, the flasks were incubated at 27°C and maintained under constant shaking (120rpm). After 7 days, the fungi reached an adequate biomass to perform biotransformations, showing typical mycelia with globular shape (see figure below). At this step of the research, the compounds to check

for biotransformations, named “substrates” from this point on, were added to bioreactors.

Flasks with liquid cultures of endophytic fungi after 7 days of culturing.
Note the typical globular shape

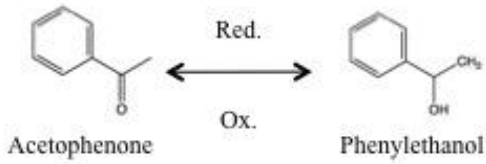
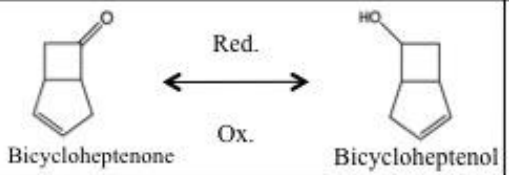
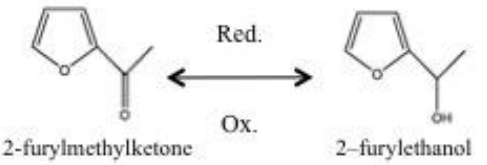
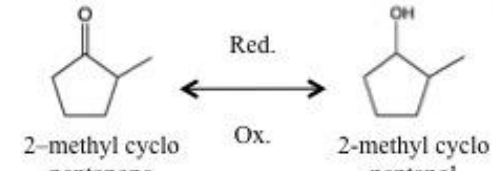
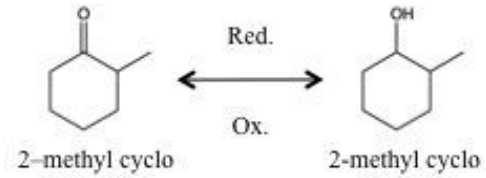
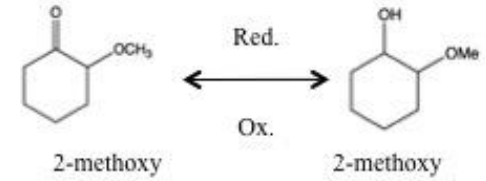
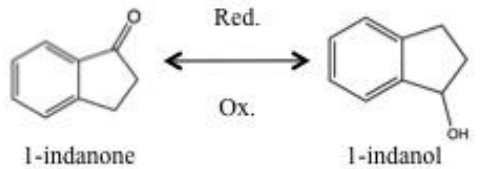


Biotransformations: single compounds or phytocomplexes as substrate?

The biotransformations have been performed focusing on reduction of ketones, mainly because of the following reasons: i) the experiences of the pharmaceutical biology and biotransformations lab on checking and monitoring these particular kind of bioreactions; ii) the fact that the reduction of prokiral carbonyl groups producing enantiomerically pure alchools is one of the most diffused biotransformations capacities among microorganisms; iii) and finally, the fact that the enantiomerically pure alchools are very useful as chiral auxiliaries in organic chemistry, in analytical applications and for the synthesis of compounds such as pheromones, perfumes, flavorings and pharmaceutical useful chemicals (Pedrini *et al*., 2009).

The substrates tested were chosen because of their importance as precursors of molecules considered as key intermediates of reactions for the synthesis of active pharmaceutical compounds. The following table shows the seven substrates tested and the biotransformations eventually expected: 2-furylmethylketone (Fluka), acetophenone (Aldrich), *cis*-bicyclo-[3,2,0]-hept-2-en-6-one (Fluka), 1-indanone (Fluka), 2-methyl-cyclohexanone (Aldrich), 2-methoxy-cyclohexanone (Aldrich), acetylfuran (Fluka), 2-methyl-cyclopentanone (Aldrich)

Ketones subjected to reduction screening and pharmaceutical selection criteria considered

	The alcohol phenylethanol, obtained by the reduction of acetophenone, is used as an antiseptic, disinfectant, antimicrobial and preservatives (Masood *et al*., 2010)
	The bicycloheptenone lactones are chiral compounds with a key role for the synthesis of prostaglandins (Alphand *et al*., 1989)
	The furylethanol (and its derivatives) is versatile precursor for the synthesis of natural products such as carbohydrates, alkaloids and pheromones (Kamiska *et al*., 1996)
	Mono-cyclic ketones are molecules poorly studied in the biotransformation despite their wide presence in nature (steroids, vegetable oils, secondary metabolites of plants, etc.) (Iwaki *et al*., 2006)
	The lactone 3-methyl-2-oxa-1-cyclohexanone is used in the synthesis of analogues micalamide A, natural compounds produced by the marine sponge of the genus *Mycale* with cytotoxic, anticancer and antiviral properties (Fukui *et al*., 1997).
	The alcohol 2-methoxy-cyclohexanol is an important intermediate in the synthesis of chiral β-lactam antibiotics, such as penicillins (Stead *et al*., 1996)
	The indanone is an important intermediate for the synthesis of SSRIs (Selective Serotonin Reuptake Inhibitors), compounds used for treatment of psychiatric diseases (Bōs *et al*.,1997)

DMSO (dimethylsulfoxide); for each 20 ml of culture broth, 0.2 ml of the solution were added. One ml of culture broth was sampled every 1, 3, 7, 10 days after inoculum to monitor biotransformations. Each biotransformation sample has been - immediately after sampling – extracted by adding ethyl acetate (1ml) and anhydrous sodium sulfate (Na_2SO_4). The vigorous shaking of the mixture allowed the dissolving of possible bioreaction products from the broth solution to ethyl acetate solvent, polarly related to the alcoholic products of the substrates reduction expected.

Checking for biotransformed products: TLC and GC

The organic extract was initially analyzed by silica gel TLC, using hexane-ethyl acetate 5:1 as eluent for acetophenone, indanone and acetylfuran; while a mixture of petroleum ether and diethyl ether 7:3 for the bicycloeptenone has been employed. The organic extracts of the other substrates were directly analyzed by GC. The products on TLC were checked and detected by UV light or by spraying phosphomolybdic solution. Once verified the presence of biotransformation products, the analyses have been performed by gas chromatography (GC 6000 Vega Series 2-Carlo Erba). The chromatographic analyses were processed using a capillary column (MEGADEX OV 1701 containing dimethyl-n-pentyl-β-cyclodextrin; 25m x 0.25mm). Helium (80 kPa) was used as carrier gas; air (100 kPa) and hydrogen (50 kPa) were used for flame ionization detector. Injector and detector temperatures were respectively 250 °C and 220 °C.

The following table shows the temperature scale up for the different substrates and the retention times observed for the possible biotransformation products.

GC set up programme: temperatures scale up and retention times (RT)

Substrate	T1 (°C)	T2 (°C)	Gradient (°C/min)	RT biotransformation products (min)
Acetophenone	130	200	2	6,1 = acetophenone 8,5 = (R)-1 phenyletanol 8,7 = phenol 8,9 = (S)-1-phenyletanol
2-furyl methyl ketone	80	200	1	12,8 = 2-furyl methyl ketone 21,9 = (*R*)-2 furyl ethanol 22,1 = (*S*)-2 furyl ethanol
1-indanone	125	200	2	15,2 = indanone 16,6 =(*S*)-1-indanol 16,7 =(*R*)-1-indanol
Bicyclo heptenone	100-120	120-200	1,5 5	8,3 = (-) (1*S*,5*R*)-bicycloheptenone 8,8 = (+) (1*R*,5*S*)-bicycloheptenone 14,3 = *endo*-(1*R*,5*S*,6*R*)-bicycloheptenol 14,4 = *endo*-(1*S*,5*R*,6*S*)-bicycloheptenol 16,1 = *exo*-(1*S*,5*R*,6*R*)-bicycloheptenol 16,9 = *exo*-(1*R*,5*S*,6*S*)-bicycloheptenol 21,3 = (-) (1*R*,5*S*)-3-oxabicycloheptenone 21,6 = (+) (1*R*,5*S*)-2-oxabicycloheptenone 21,9 = (+) (1*S*,5*R*)-3-oxabicycloheptenone 22,1 = (-) (1*S*,5*R*)-2-ossabicycloheptenone
2-methyl cyclo pentanone	70 (12' isotermal)	70-80 80-200	2 10	10,6 = 2-methylcyclopentanone 10,8 = 2-methylcyclopentanone 18,8 = *cis*-2-methylcyclopentanol 19,0 = *cis*-2-methylcyclopentanol 19,3 = *trans*-2-methylcyclopentanol 19,6 = *trans*-2-methylcyclopentanol
2-methyl cyclohexanone	80-110	110-200	1,5 5	11,8 = *(S)*-2-methylcyclohexanone 12,0 = (*R*)-2-methylcyclohexanone 16,2 = *trans*-methylcyclohexanol 17,0 = *cis*-methylcyclohexanonl 18,1 = *trans*-(1*S*,2*S*)-acetylderivative 18,6 = *cis*-(1*S*,2*R*)-acetylderivative 19,2 = *cis*-(1*R*,2*S*)-acetylderivative 19,4 = *trans*-(1*R*,2*R*)-acetylderivative
2-methoxy cyclohexanone	70	200	5	11,7 = (*S*)-2-methoxycyclohexanone 11,8 = (*R*)-2-methoxycyclohexanone 12,2 = *trans*-(1*S*, 2*S*)-2-methoxycyclohexanol 12,4 = *trans*-(1*R*, 2*R*)-2-methoxycyclohexanol 12,9 = *cis*-(1*S*, 2*R*)-2-methoxycyclohexanol 13,0 = *cis*-(1*R*, 2*S*)-2- methoxycyclohexanol

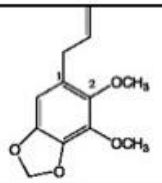
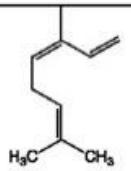
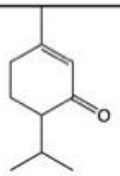
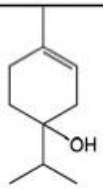
Once verified the biotransformation capacities of the endophytes isolated from the ecuadorian plant species it has been planned the biotransformation capacity of the best performing microorganisms on prochiral carbonyl compounds and also on phytocomplexes derived from the same plant species ethnomedically known for their health properties. In fact, besides the study of the biotransformation capacities of the

isolated endophytes, it would also point out the study of the scarcely known phytocomplexes of the investigated plants both under a phytochemical and functional point of view (see framework scheme of the research). The object of this approach would be that of verifying both the biotransformation capacities on phytocomplexes, and to that on a single pure compound, checking the yield and quality of the products. A further development would be, in case of biotransformations, to check the bioactivity of the transformed phytocomplexes in comparison with that expressed by natural ones. However, this latter perpective goes over the project hypothesized in PhD framework, but the results here achieved certainly represent its starting point for future development.

Therefore, the research here presented considers the essential oil of *Piper aduncum*, since the specie is that most investigated in our laboratories till now. The other species will be studied following the same research pattern. For the procedures adopted to obtain *P. aduncum* essential oil see the appropriate section (see page 55). On the bases of the latter considerations and evidences, the following table shows the endophyte selected. The only endophytes isolated from *P. aduncum* able to transform prochiral carbonyl groups were considered.

Considering the essential oil composition (see pag.79), it has been tested the biotransformation of the whole essential oil and also of the single pure compounds quantitatively most representative of the oil. Thus, the single compounds tested were: cis-ocimene, (-) terpinen-4-ol (Fluka), and (+/-) piperitone (Extrasynthese) (see the molecular scheme below).

The most abundant compounds of *Piper aduncum* essential oil tested for biotransformations

Dillapiol	
trans - ocimene	
Piperitone	
Terpinen-4-ol	

The endophytic strains used to check biotransformation of *P. aduncum* essential oil were all isolated from the same plant. In particular, they were:

Fungal strain	*Plant part*
EC19	Stem
EC46	Bark
EC49	Stem

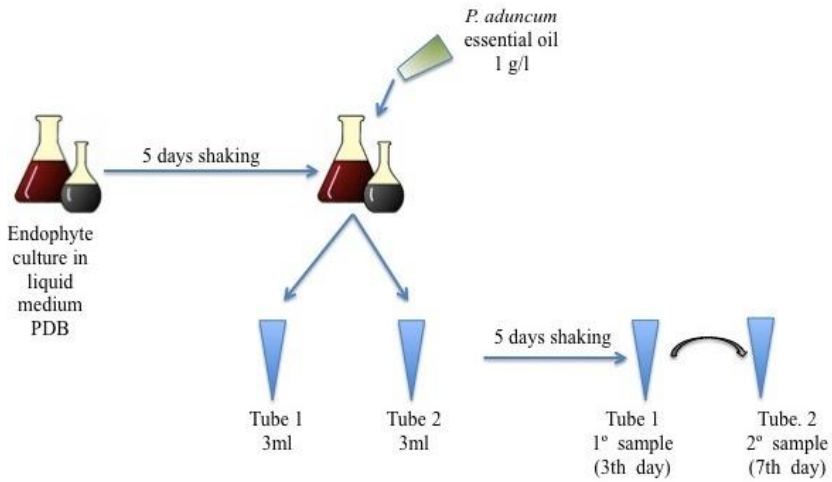
As samples of the functional importance of the selected compounds and their chemical characteristics, few bibliographic news detected about are following reported.

In fact, recent studies have shown that dillapiol inhibits the biosynthesis of aflatoxin G1 produced by *Aspergillus parasiticus*, very known chemicals to be extremely dangerous to human health typically occurring in foodstuffs (cereals, oil seeds, etc.) in post-harvest chains (Razzaghi-Abyaneh *et al.*, 2007). Piperitone also showed antifungal activity inhibiting the growth of *Aspergillus flavus*, an aflatoxin producer fungus (Cárdenas Ortega N. *et al.*, 2005).

Ocimene is instead a monoterpene present in a wide variety of plants and fruits, generally as racemic mixture of the two stereoisomers, *cis* and *trans*.
Terpinen-4-ol is a natural monoterpenic alcohol, known to be one of the main components of the essential oil extracted from nutmeg (*Myristica frangrans*), and from the "Tea Tree" (*Melaleuca alternifolia*). The essential oil extracted from the leaves of the Tea Tree is known to have antibacterial and antifungal properties that ensure the commercial success of the phytocomplex (Russell *et al*., 2002).

Fungal coltures were inoculated in 20 ml of liquid medium PDB (Potato Dextrose Broth, Liofilchem srl, Italy), previously autoclaved (121 °C, 15min), and incubated for 5 days at 27 °C, under shaking (120rpm), as previously described. At the end of the fungal growth period, *P. aduncum* essential oil was added in amount of 1 g/l, previously dissolved in DMSO (dimethyl sulfoxide), in sterile tubes with scraw cap sealed with parafilm, to prevent the loss of the most volatile compounds, together with appropriate fungal biomass quantity. The amount of biomass takes into account both the possibility to record the biotrasformation fungi activity, without causing toxicity. In particular, this amount corresponds to 1 cm^3 of mycelium (grown on solid agar medium) in 20 ml of broth medium. For each fungal strain to test, two sterile tubes were set (3ml): one to be examined at the 3rd day of incubation, while, the second, at the 7th day. The procedure is shown in the following scheme and it has been set at Pharmaceutical biology laboratories of the University of Ferrara during the performing of my PhD research and related ones (Moreno Rueda, 2010). The protocol represents the most effective strategy to limit, under threshold significance, the loss of most volatile compounds (Moreno Rueda, 2010).

Biotransformation protocol adopted for whole essential oil



In order to verify the presence of biotransformed product within colture broths, the extraction was done using 1.5 ml of ethyl ether (Fluka) as solvent in 3 ml of culture broth; anhydrous sodium sulphate (Fluka) was employed in case ether extraction anhydrification would be requested. It was of crucial importance to operate with the aim to obtain extracts with a biotransformation products concentration compatible with their clear detection at GC. As further contribute to limit the volatility of the potential transformed products, each extraction mixture has been obtained with refrigerated solvent, kept at -18°C for 10 min, and then vortexed. After this period, the extracts were immediately analyzed by GC following the same previously described methods and conditions.

It was observed that the potential loss of biotransformated products by evaporation, during the incubation period, is not detectable at GC confirming the accurancy of the biotransformation protocol adopted (Moreno Rueda, 2010).

PHARMACEUTICAL BIOLOGY APPROACH: PHYTOCHEMICAL AND FUNCTIONAL FINGERPRINTING

As already stated, the skills and experiences of the research group about biotransformations, phytochemistry and health functional profiles drived to expand my PhD research towards the study of the phytocomplexes obtained from the plants object of the present study. In light of the ethnomedical news collected in the field about the preparations, health purposes and cultural importance of each specie among Shuar and Achuar people, *P. aduncum* was considered first. *P. aduncum* is here exclusively reported also as research pattern for all other species, a key guide to perform similar researches. In this way, it is our opinion that one of the main goals of the PhD, i.e. to form new researchers, has been reached. Thus, the studies about plant species from the Amazon fall under the planning investigation of the research group, which aims to develop a phytochemical fingerprinting of plants used by indigenous peoples for ethnopharmaceutical purposes.

Vapour steam distillation apparatus: on the left, that used at "Centro de Acopio" Foundation Chankuap, Macas; and on the right that used at Wasakentsa reserve, amazonian forest



In addition to what reported in the summarized scheme about the plant (see page 27) some Pipers are also employed in folk medicine as analgesics in pain management, toothache and wound treatment (Gatti, 1985). From a phytochemical standpoint, safrole, dillapiol, myristicine and similar methylenedioxyphenyl derivatives have been frequently detected in sensible amounts in the genus (Parmar *et al*., 1997) and many of these compounds are known to be toxic (Buchanan, 1978) and liable to induce DNA alteration through different mechanisms, often involving hepatic microsomial

bioactivation (Dietz and Bolton, 2007). Notwithstanding their widespread traditional and commercial use, Piper essential oil have been scarcely evaluated for their mutagenic/genotoxic properties and received little attention on this regard, despite the renowned toxicological profiles of some of the aforementioned substances.

PHYTOCHEMICAL EXTRACTION

Distillation and chemical characterization of the *Piper aduncum* essential oil

The essential oil was obtained by steam distillation of aerial parts of *Piper aduncum* supplied by Fundaciòn Chankuap (Macas, Ecuador) and collected at blooming January 2006 from wild plants growing in three different locations on the outskirts of Wasakentsa reserve in eastern Ecuador (77°15″W/2°35″S) and positively identified by the National Herbary of "Pontificia Universidad Catolica del Ecuador" (J. Jaramillo). Dried specimens were deposited at the Department of Biology and Evolution, University of Ferrara, Code POB001.

Essential oil distillation

Essential oil was isolated by a 3 h hydrodistillation of 7 kg of *P. aduncum* aerial parts in a stainless steel distiller equipped with a commercial Clevenger apparatus. Essential oil yield was determined on a volume to dry weight basis. The values for essential oil yield of three distinct distillations corresponding to the three different samplings were averaged. The essential oil samples were stored in glass vials with Teflon-sealed caps at 2±0.5 ◦C in the absence of light.

Gas chromatography

Essential oil sample was analyzed and the relative peak areas for individual constituents averaged. The relative percentages were determined using a Thermo- Quest GC-Trace gas-chromatograph equipped with a FID detector and a Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25mm; length, 30 m; film thickness, 0.15 m). Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, Carrier (Helium) flow rate 1ml/min and split ratio 1:50. Oven temperature was initially 55 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 15 min. One microliter of each sample dissolved in CH_2Cl_2 was injected. The

percentage composition of the oil was computed by the normalization method from the GC peak areas, without using correction factors.

GC/mass spectrometry analysis

Essential oil constituents were then analyzed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library. The constituents of the volatile oils were identified by comparing their GC retention times, KI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in the literature (Adams, 2001). A Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25mm; length, 30 m; film thickness, 0.15 μm) was used. Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, Carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55 °C and then raised to 100 °C at a rate of 1°C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 15 min. One microliter of each sample dissolved in CH_2Cl_2 was injected. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 Amp; scan rate, 1 scan/s; mass range, 29–400 Da; trap temperature, 150 °C, transfer line temperature, 300 °C. In order to determine the Kovats index of the components, a mixture of alkenes (C_8–C_{24}) was added to the essential oil before injecting in the GC–MS equipment and analyzed under the same conditions as above.

NMR Spectroscopy

^{13}C NMR spectrum was recorded at 100.58MHz and at temperature of 303K with a Varian Gemini-400 spectrometer. The essential oil was dissolved in $CDCl_3$ (70mg/0.8 ml) into a 5mm NMR and solvent signalwas used for spectral calibration (central line of triplet at 77.0 ppm). Chemical shifts (ppm) and peak attribution were made according with those of literature (Kubeczka, 2002), SDBS and Sigma–Aldrich NMR spectra databases and pure standard or mixture of these (dillapiol, -terpinene, *cis*-ocimene for *P. aduncum*). *Cis*-ocimene, *trans*-ocimene, -terpinene, 4-terpineol, piperitone and dillapiol were identified via ^{13}CNMR. DEPT, ^{1}H and bidimensional NMR experiments were used to attribute signals for compounds in low proportions (Rezzi *et al*., 2002; Agnihotri *et al*., 2005; Guerrini *et al*., 2006).

Biological activities

All the following biological activities of *P. aduncum* essential oil were compared to those achieved with synthetic positive controls and commercial *Thymus vulgaris* essential oil, in order to provide a direct reference with an essential oil reputed for its antioxidant and antibacterial properties. Data reported for each assay are the average of three determinations of three independent experiments. Biological activities (antifungal and antibacterial activity) of *P. aduncum* essential oil were performed by employing the standard disks diffusion technique according to a previously described methodology (Guerrini *et al.*, 2006). The culture media and conditions employed for ATCC strains were in accordance with American Type Culture Collections protocols.

Antibacterial activity

Mother cultures of each microorganism were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating from the mother cultures Petri dishes with proper sterile media with the aim of obtaining the microorganisms concentration of 10^5 and 10^6 CFU/ml for yeasts and bacteria, respectively. Aliquot of dimethyl sulphoxide (DMSO) was added to the essential oil and different amounts of the solution deposited on sterile paper disks (6mm diameter, Difco) in order to obtain a 0.01–0.5 mg/ml concentration range. The lowest concentration showing a clear zone of inhibition was taken as the minimum inhibitory concentration (MIC). Controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution.

Antifungal activity

Cultures of the fungi were grown on potato dextrose agar (PDA, Difco,Detroit, MI, USA) for phytopathogens and on sabouraud dextrose agar (SDA, Difco, MI, USA) for dermatophytes. Antifungal activity was checked by dissolving the extracts in DMSO and aseptically added them to sterile media at 45 °C in order to obtain the two concentrations of 50, 100 and 500 g/ml. The DMSO concentration in the final solution was adjusted to 0.3%. Controls were set up with equivalent quantities of DMSO. The cultures were obtained by transplanting mycelium disks (diameter 10mm) from a pure mother culture in stationary phase on thin cellophane sheets at 26±1 °C until the logarithmic growth phase was reached. Subsequently the cultures were transferred to Petri plates with media containing extracts diluted to the above-mentioned final

concentrations. The fungal growth was daily evaluated by measuring the diameter of the cultures for five (phytopathogens) or eight (dermatophytes) days from the treatment onset (kept as 0 time). There were three replicates for each treatment.

Antioxidant activity

Radical scavenging and antioxidant properties were performed in different assays, namely DPPH (1,1-diphenyl-2-picrylhydrazyl) bleaching test, ABTS (2,2 - azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) test and Photochemilumiscence according to previously described methods (Sacchetti *et al*., 2005; Scartezzini *et al*., 2005).

Platelet aggregation studies

Blood from male Guinea pig was obtained by cardiac puncture after CO_2 euthanasia, collected in plastic tubes and anticoagulated with sodium citrate 3.8% 1 part citrate: 9 part blood. After centrifugation for 15 min at 180×g to obtain platelet rich plasma (PRP), the remaining blood was centrifuged again 10 min 2000 g to obtain platelet poor plasma (PPP). PRP from guinea pig was used to perform aggregation in the aggregometer PAP-4D (Biodata, Horsham, PA, USA) following Born's turbidimetric method (Born, 1962). Aggregation was recorded as the percent change in light transmission: the baseline was set using PRP and maximal transmission using PPP. PRP was preincubated at 37 °C for 5min with solvent (DMSO, final concentration 0.5%) or the compound under study before addition of the platelet aggregatory agent. Maximal aggregation was induced stimulating platelets with 3 μM ADP, 50 μM arachidonic acid (AA) or 1 μM U46619. Tests were performed within 3h to avoid platelet inactivation. The effects of test compounds and aspirin were expressed as percent inhibition compared with control samples. DMSO at 0.5% did not interfere with platelet aggregation.

Antinociceptive activity

The writhing test was performed according to Koster's method (Koster *et al.*,1959). Briefly, vehicle or 100mg/kg essential oil swere orally administered to mice1h before intraperitoneal injection of 0.2 ml of 0.6% acetic acid. After treatment with the algogen agent, mice were placed in observational chambers and the number of writhes of each mouse was counted over a period of 30 min.

Platelet aggregation and antinociceptive activities have been performed at Department of Pharmacological Science of University of Parma; for these experiments, a special thanks goes to Prof. M. Tognolini.

Essential oil mutagenic activity

Essential oil dissolved in DMSO was tested with *Salmonella typhimurium* strains TA98 and TA100 (100 μl per plate of a fresh overnight culture) with and without the addition of 0.5 ml of a 5% S9 exogenous metabolic system (S9 mix), using plate incorporation assay (Maron and Ames, 1983). The concentrations of the test samples used were 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2} and 10^{-1} g plate^{-1}. The plate for negative control contained 100 μl of DMSO, with or without S9 mix. The positive control plates for the first contained 2 μg plate^{-1} of 2-aminoantracene for both TA98 and TA100 strains. The positive control plates without S9 mix contained 2 μg plate^{-1} of 2-nitrofluorene for TA98 strain and 1 μg plate^{-1} of sodium azide for TA100 strain. A sample was considered mutagenic when the observed number of colonies was at least 2-fold over the spontaneous level of revertants (Maron and Ames, 1983). The colonies were counted manually after 48 h of incubation at 37 °C using a Colony Counter 560 Suntex (Antibioticos, Italy). Lyophilized post-mitochondrial supernatant S9 mix (Aroclor 1254-induced, Sprague–Dawley male rat liver in 0.154M KCl solution), commonly used for the activation of promutagens to mutagenic metabolites, was purchased fromMolecular Toxicology, Inc. (Boone, NC, USA). Before its use, the S9 mixwas filtered through a 0.45 μm Millipore disposable filter.

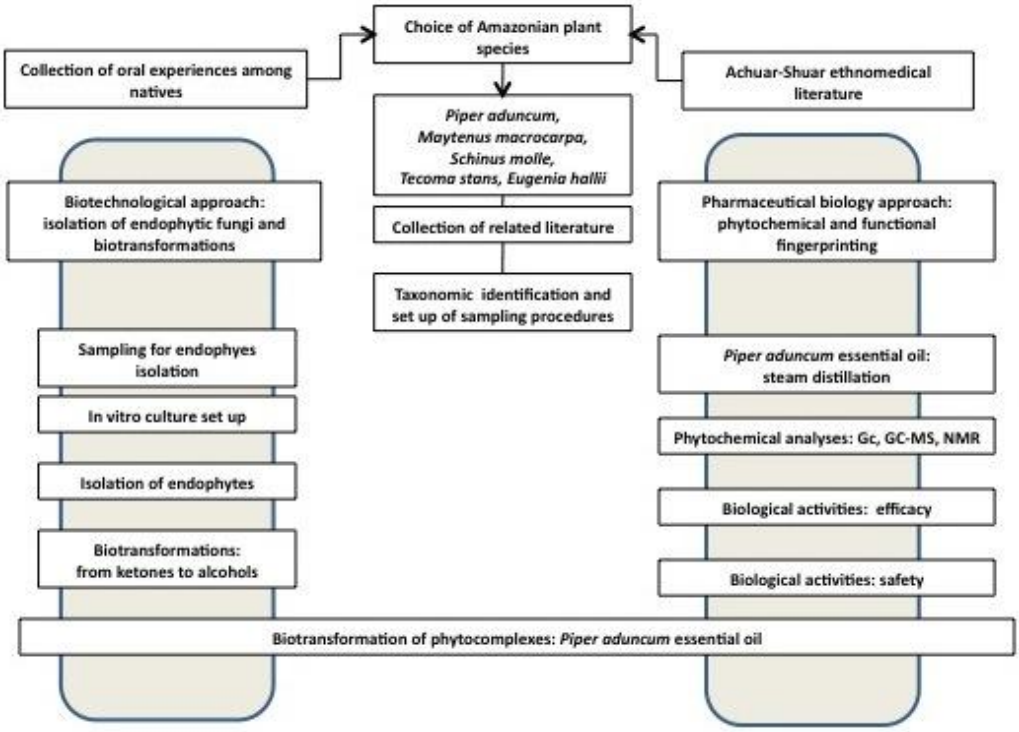
Antimutagenic activity and toxicity

The inibithory effect of essential oils (10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2} and 10^{-1} μg plate^{-1}) on mutagenic activity of direct acting mutagen 2- nitrofluorene (2 μg plate^{-1}) and sodium azide (1 μg plate^{-1}), was examined in plate incorporation assay, derived from mutagenicity test as described by Maron and Ames (1983) with some minor modifications, using tester strain TA98 and TA100, respectively. The inhibitory effect of essential oil on mutagenic activity of the indirectly acting mutagen 2-aminoanthracene (2 μg plate^{-1}) was examined in plate incorporation assay, using tester strain TA98 and TA100 with S9 mix. The inhibition rate for mutagenic was calculated according to the formula: inhibition rate (%) = (A−B)×100/A, where A are revertants in

positive control, and B are revertants in the extract sample, having subtracted the spontaneous revertants. The significant differences ($P \leq 0.05$) between the means of revertants per plate of the samples in relation to the mutagens were calculated using the post hoc Tuckey honest significant difference test (HSD) (Spjotovoll and Stoline, 1973). This statistical test has been already applied to antimutagenic activity of extracts of natural substances in the *Salmonella*/microsome assay by Horn and Vargas (2003). A critical point, affecting the outcome of the interaction between an antimutagen and a testing bacterial strain is the overlapping of the citotoxic and antimutagenic dose concentration. In other worlds, it is important to confirm that the dose-dependent disappearance of the mutant colonies is not a result of cell-killing. For this purpose a simple survival assay for the treated bacteria must be performed to evaluate a cytotoxic lowest effective dose (LED) or a highest uneffective dose (HUD). In order to verify the toxicity of the analysed samples on bacterial cells and evaluate the HUD, a toxicity test was performed (Maron and Ames, 1983). A fresh 15-h culture was diluted 10^5 times to give a $1–2\times10^4$ bacteria ml^{-1} solution. The test samples at several concentrations (10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1} μg $plate^{-1}$) diluted in DMSO, mixed with 2ml of molten top agar, were plated with 0.1ml of the diluted culture. Histidine/biotin agar plates were enriched with 10 μmoles of L-histidine and 0.05 μmoles of biotin by incorporating these nutrients into the soft agar overlay. Triplicate plates were poured for each dose of solution. The colony-forming units (CFU) were assessed after the plates were incubated at 37 °C for 48 h and compared with that of control where no test samples were added. The final criterion used to interpret the results of significant decrease in the number of *Salmonella* revertants was statistical analysis ($P \leq 0.05$) in the Tuckey HSD test combined with HUD comparison. When the modulator dose concentration is statistically effective and it ranges below or coincide with HUD, the samples were considered to present sign of the effect (antimutagenicity). HUD for *P. aduncum* is hardly derivate by visual estimation, therefore we fixed it at the lower dose just before LED. LED for *P. aduncum* was statistically evaluated, with Poisson assumptions, by using the stepwise collapsing of the homogeneous control and dose counts (Khromov-Borisov *et al.*, 2000). All data were analyzed for homogeneity of variance using Levene's test (a robust test against normality). All computations were made by employing the statistical software SPSS Ver. 10.0 and personal developed software (for estimation of LED).

5. RESULTS AND DISCUSSION

Results are described according to the following chart, which deals with biotransformation and pharmaceutical biology approaches.



PhD research was performed as part of a wider project supported by the "Centre of International Development and Cooperation" of the University of Ferrara. This PhD has started with two main targets: 1) the taxonomic identification of selected plants and isolation of endophytic fungi (at UPS-CIVABI, Ecuador); 2) the biotransformation properties of isolated endophytes and phytochemical and functional characterization of plant sources from which endophytes have been isolated (at University of Ferrara, Italy).

BIOTECHNOLOGICAL APPROACH

Plant sources and endophytic fungi

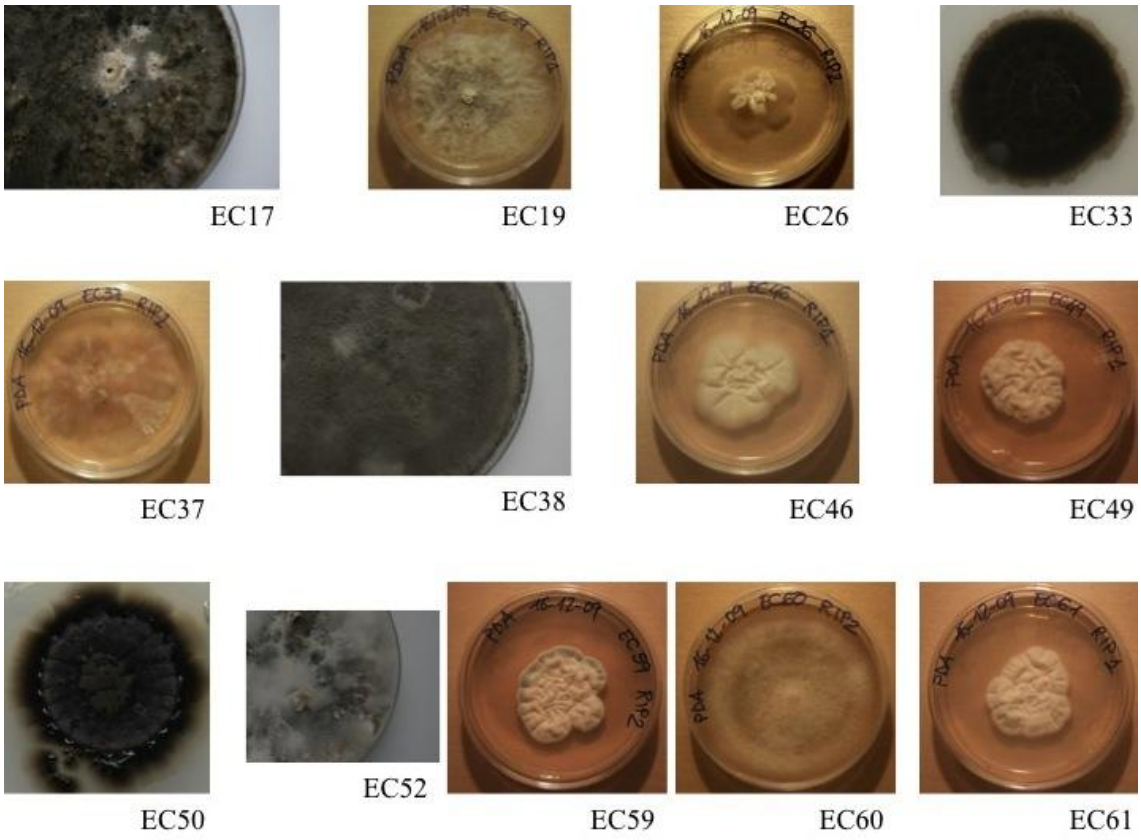
The plant species considered for their ethnomedical importance among Natives on the basis of literature and interview were *Piper aduncum* (Matico), *Maytenus macrocarpa* (Chuchuguazo), *Schinus molle* (Falso pepe), *Tecoma stans* (Tepla), *Eugenia hallii* (Arrayan). From sampling the aerial parts of adult plants were isolated endophytes through biotechnological methods. A total of 364 fungal strains were isolated. Each strain was coded with reference to its macroscopic (colour, colony border, texture mycelia, exudates, changes in medium colour during culturing). Then groups were considered for those strains with similar features (see table below):

Table 1 - Plant source, number of isolated endophytic fungi from each species and identification code

Plant Source	**No. of isolated fungal strains**	**Code**
Piper aduncum	116	from **EC01** to **EC65**
Maytenus macrocarpa	127	
Schinus molle	28	from **FE1** to **FE110**
Tecoma stans	32	
Eugenia hallii	61	
TOTAL	**364**	

After the one month of plant samples incubation, a percentage of 80% plates showed the presence of emerging endophytes. An average of 2 fungal mycelia – easily noticeable by the different macroscopical morphology - could be checked for each positive plate.
The following figure shows a selection of most relevant fungal strains, then tested for biotransformations on pure chemicals and on *Piper aduncum* essential oil.

Figure 1 - Selection of fungal strains isolated from Amazonian plants



All the isolated strains were employed to test biotransformation, both on pure chemicals chosen in light of their chemical structure similar to pharmaceutical drug intermediates and on *P. aduncum* essential oil (i.e. a phytocomplex: terpene mixture). For taxonomic identification of endophytes, performed successively for those most efficient biotransformers, the help of "Fungal Biodiversity Centre" of Central Bureau Voor Schimmelcultures (CBS) have been requested and it is still in progress. However, my contribute to the identification is here reported and it is represented by the association of endophytes to the genus with reference to the most coherent morphological aspect. In particular my suggestions lead to conclude that EC19 and EC37 belong to *Fusarium* genus, while EC46, EC49, EC59 and EC61 to *Penicillium* one.

Fungal strains cultivated in liquid culture medium PDB (Potato Dextrose Broth) showed a different macroscopical morphology if compared with ones grown in solid culture medium PDA (Potato Dextrose Agar). In fact, fungal mycelia acquired a globular shape when stirring in liquid medium. Thus, fungi showed different size, morphology and colour if compared to ones grown in solid medium as shown in figures below.

Figure 2 - Fungal mycelia in liquid PDB culture medium

Figure 3 - Detail of globular shaped mycelium grown in stirring PDB liquid medium

EVALUATION OF BIOTRANSFORMATION ACTIVITY

Biotransformation of pure chemicals

In the table below the fungal strains which performed the most relevant biotransformating results.

Vegetal source, isolated plant part, culture medium and macroscopical morphology of fungal colonies

Strain	Vetegal source	Part plant	Medium*	Colony macroscopical morphology
EC17	Matico (*P. aduncum*)	S	MEA	Bright pink mycelium in the center and gray-black at the colony border, cotton texture, colourless exudate. Reverse black coloured.
EC19	Matico (*P. aduncum*)	S	MEA	Bright gray mycelium with circular shaped growth, slightly cotton texture, gray-green central pigmentation, jagged colony border, slightly red-pigmented agar. Reverse dark red-black coloured.
EC26	Matico (*P. aduncum*)	L	MA	White mycelium, gray-black when ripe, irregular with coloured patches, slightly powdery, jagged colony border, orange-pigmented agar. Reverse black coloured.
EC33	Chuchuguazo (*M. macrocarpa*)	B	MYC	White-gray mycelium in the middle, light gray at colony border. Reverse gray-black coloured.
EC37	Chuchuguazo (*M. macrocarpa*)	L	MEA	White-pink mycelium, slightly cotton, jagged colony border, non-homogeneous. Reverse white coloured with dark pink center.
EC38	Chuchuguazo (*M. macrocarpa*)	S	MYC	Gray mycelium, cotton texture, high growth. Reverse dark gray coloured.
EC46	Chuchuguazo (*M. macrocarpa*)	B	MA	Light gray mycelium, lighter on colony border.
EC49	Matico (*P. aduncum*)	S	MEA	White mycelium, compact, defined colony border. Reverse beige coloured.
EC50	Matico (*P. aduncum*)	S	MYC	Black mycelium, slightly powdery, defined colony border, black-pigmented agar. Reverse black coloured.
EC52	Matico (*P. aduncum*)	S	TS	Mycelium white, not defined colony border. Reverse beige coloured.
EC59	Matico (*P. aduncum*)	L	MEA	Mycelium light gray, compact, wrinkled, defined border, slightly powdery. Reverse beige coloured.
EC60	Matico (*P. aduncum*)	L	MEA	Light green mycelium, slightly. Reverse beige-green .
EC61	Matico (*P. aduncum*)	L	TS	Gray-white mycelium, compact, wrinkled, defined border. Reverse beige coloured.
FE86	Tepla (*T. stans*)	B	MEA	Black mycelium. Orange-pigmented agar. Reverse dark red and black coloured.

*− isolation culture medium
L=leave
B=bark
S=stem
MEA− Malt Extract Agar
MA=Malt Agar
MYC= Mycosel Agar
TS=soybean peptone

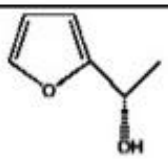
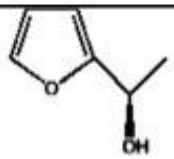
The biotransformation have been processed on the following chemicals because of their importance as molecular precursors in the synthesis of active pharmaceuticals compounds (Masood *et al.*, 2010; Alphand *et al.*, 1989; Kamiska *et al.*,1996; Iwaki *et al.*, 2006; Fukui *et al.*, 1997; Stead *et al.*, 1996; Bös *et al.*,1997). The chemicals were: 2-furyl methyl ketone, acetophenone, *cis*-bicyclo[3.2.0]hept-2-en-6-one, 1-indanone, (+/-)-2-methyl-cyclohexanone, (+/-)-2-methoxycyclohexanone, (+/-)-2-methylcyclopentanone.

The results are following reported for each compounds with reference to the best performing endophyte strains. All the chemicals results have been achieved through GC-MS, GC-FID analysis. Detection and identification were also supported by chromatographic comparison with data achieved with chemicals pure standards (commercial or related and completely characterized in our laboratory).

- **2-FURYL METHYL KETONE**

Fungal strains which showed a the ketones reduction were those reported in Table 2. In particular, EC17, EC19, EC37, EC49 and EC61 strains reduced 2-furyl methyl ketone to the corresponding (*S*)-1-(2-furyl)-ethanol, but with low yield percentage and enantiomeric excess (e.e.). In fact, EC19 produced the best yield (91%) but a low e.e. (20%). In contrast, EC49 showed an interesting e.e. (66%) with low yield (10%). EC46, EC52, EC53, EC55 and EC60 produced an enantiomeric mixture with abundance of (*R*)-1-(2-furyl)-ethanol enantiomer, giving rasonable yield (between 20% and 63%) but low e.e. (between 11% and 52%). Only EC60 is able to reduce 2-furyl methyl ketone with good yield (20%) and e.e. (52%). On the other hand FE40 and FE86 gave the highest e.e. but a low yield of *S* enantiomer equal to 26% and 6% respectively. No compounds were found to be considered as products of Baeyer-Villiger oxidation.

Table 2 - 2-furyl methyl ketone biotransformation products

 *1*	Time (days)	 *2* **S yield %** (ee%)	 *3* **R yield%** (ee%)
EC17	10	**5** (56)	
EC19	7	**91** (20)	
EC37	10	**17** (59)	
EC46	10		**63** (18)
EC49	10	**10** (66)	
EC52	10		**58** (15)
EC53	10		**46** (20)
EC55	10		**40** (11)
EC60	10		**20** (52)
EC61	10	**55** (45)	
FE40	7	**26** (100)	
FE86	7	**6** (100)	

1 2-furyl methyl ketone, **2** (*S*)-1-(2-furyl)-ethanol, **3** (*R*)-1-(2-furyl)-ethanol

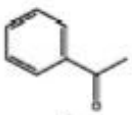
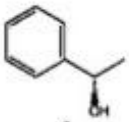
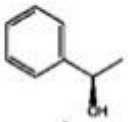
Biotransformation products were chemically confirmed by GC-MS. The assignement of alcohol absolute configuration was determined by GC-FID equipped with proper chiral column and by comparison with standards previously isolated in our laboratory and completely identified by chemical and physical properties (Pedrini *et al*., 2009).

- **ACETOPHENONE**

The reduction of acetophenone carbonilic group was widespread among tested fungal strains, as shown in table below. Some of them produced the (*S*)-1-phenylethanol enantiomer with interesting yield (comprised between 78% and 98%) and e.e. (between 78% and 100%). In contrast, the *R*-enantiomer was produced with low abundance. A significant number of strains gave good yield

comprised between 40% and 98%, and enantiomeric excesses between 66% to 100%. In particular, the production of (*S*)-1-phenylethanol, both in terms of yield and e.e., was better performed by the strains EC17, EC19, EC35, EC37, EC61, while EC38, EC46, EC50, EC59, EC65 gave the (*R*)-1-phenylethanol. The best yield and e.e. of *R*-form were recorded by strains EC38 and EC50 with values of 48% (yield) – 86% (e.e.) and 49% (yield) – 84% (e.e.) respectively.

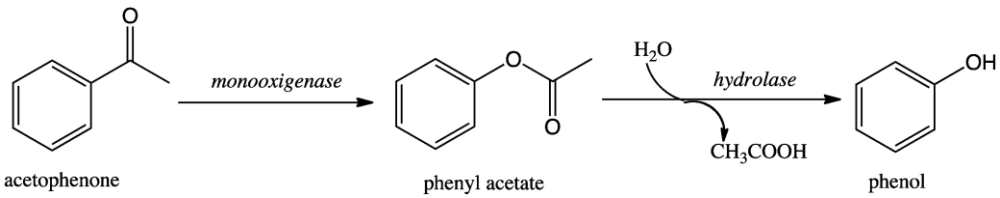
Table 3 - Acetophenone biotransformation products

 *1*	Time (days)	 *2* ***S* yield%** (ee%)	 *3* ***R* yield%** (ee%)	 *4* **yield %**
EC 17	7	**82** (100)		
EC 19	7	**97** (100)		
EC 26	10	**78** (78)		
EC 35	10	**98** (96)		
EC 37	10	**88** (94)		
EC 38	10		**48** (86)	**22**
EC 46	10		**32** (87)	
EC 47	10	--	--	**4**
EC 49	10	**43** (91)		
EC 50	10		**49** (84)	
EC 59	10		**33** (83)	
EC 60	7	**44** (59)		**13**
EC 61	7	**91** (92)		
EC 65	10		**40** (82)	
FE 86	7	**36** (66)		

1 acetophenone, **2** (S)-1-phenylethanol, **3** (R)-1-phenylethanol, **4** phenol

Concerning the Baeyer-Villiger oxidation, only EC38, EC47, EC60 were able to convert acetophenone to phenol according to the reaction sequence showed in Chart below. The phenol production is most probably the result of phenyl acetate hidrolysis by an hydrolase enzyme: yields were between 4% and 22%.

Scheme 2 – Acetophenone towards phenol bioconversion



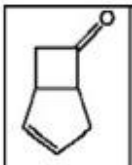
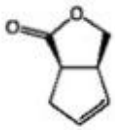
Biotransformation products were then chemically confirmed by GC-MS. The assignement of alcohol absolute configuration was determined by GC-FID equipped with proper chiral column and by comparison with standards previously isolated in our laboratory and completely identified by chemical and physical properties (Pedrini *et al*., 2009).

- ***cis*-BICYCLO[3.2.0]HEPT-2-EN-6-ONE**

Tested fungal strains catalyzed two different chemical reactions on *cis*-bicyclo[3.2.0]hept-2-en-6-one: the reduction of the carbonyl group and the addition of an oxigen atom.

The ketone was poorly reduced in terms of yield (between 3% and 39%) but e.e. were the highest detected amoung all the screened compounds (Table 4).

Table 4 - Biotransformation products of *cis*-bicyclo[3.2.0]hept-2-en-6-one

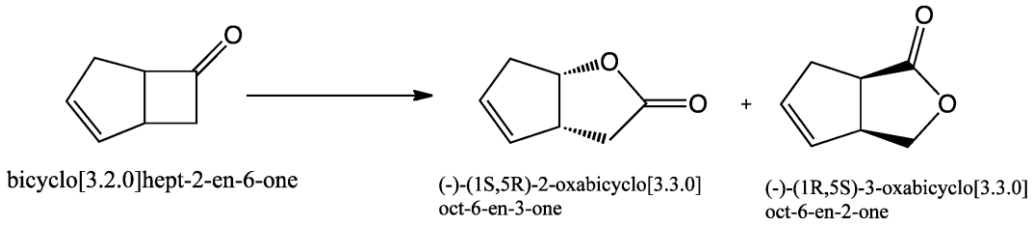
 **cis** ***1***	Time (days)	 ***2*** *endo* **yield%** (ee%)	 ***3*** *exo* **yield%** (ee%)	 ***4*** **yield %** (ee%)	 ***5*** **yield %** (ee%)
EC17	3	**39** (87)	**3** (100)		
EC17	10		**22** (100)	**10** (55)	**18** (92)
EC19	10	**10** (68)		**67** (44)	**18** (5)
EC26	10	**7** (-70)*	**3** (100)	**66** (18)	**21** (66)
EC33	10	**8** (-14)*	**18** (100)	**5** (100)	**23** (78)
EC37	7			**82** (5)	**18** (-25)*
EC38	7	**34** (53)	**51** (98)	**17** (13)	**2** (0)
EC46	10		**12** (82)	**55** (30)	**28** (57)
EC49	10		**12** (82)	**53** (37)	**30** (61)
EC52	10	**29** (1)	**18** (100)	**6** (14)	**3** (0)
EC53	10		**11** (100)	**57** (29)	**28** (61)
EC55	10		**11** (100)	**57** (28)	**28** (62)
EC59	10		**12** (90)	**56** (30)	**25** (52)
EC60	10	**6** (11)	**23** (90)	**14** (30)	**10** (62)
EC61	10		**11** (75)	**56** (30)	**29** (62)
EC64	10		**12** (83)	**57** (26)	**27** (61)
FE86	7	**21** (95)	**14** (100)		

1 ***cis***-bicyclo[3.2.0]hept-2-en-6-one **2** *endo*-(1S,5R,6S)-bicyclo[3.2.0]hept-2-en-6-ol, **3** *exo*-(1R,5S,6S)-bicyclo[3.2.0]hept-2-en-6-ol, **4** (-)-(1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one, **5** (-)-(1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one

*: the "minus" sign indicates that the enantiomeric excess is in favor of the other enantiomer, there is an inversion configuration

EC17, EC33, EC38 and EC52 gave good results both in terms of yield (between 18% and 51%) and e.e. (between 53% and 100%) with reference to substrate reduction to *exo*-(1*R*,5*S*,6*S*)-bicyclo[3.2.0]hept-2-en-6-ol. Only EC17 gave good yield and e.e. of *endo*-(1*S*,5*R*,6*S*)-bicyclo[3.2.0]hept-2-en-6-ol at 3rd day. FE86 produced interesting yields and e.e. both for *exo* and *endo* alcohols (14% yield-100% e.e. and 21% yield-95% e.e). Concerning the Baeyer-Villiger oxidation, EC17, EC33, EC49, EC53, EC55, EC61 and EC64 showed the best results about yield% and e.e.% (Table 4). The low production of alcohols allowed to understand that fungal strains preferably catalyze oxidation reactions, Bayer-Villiger in particular leading to the production of the two lactones (-)-(*1S,5R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (-)-(*1R,5S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one.

Scheme 2 – Baeyer-Villiger oxidation. Biotrasformation of *cis* bicyclo[3.2.0]hept-2-en-6-one into lactones: (-)-(*1S,5R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (-)-(*1R,5S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one



bicyclo[3.2.0]hept-2-en-6-one

(-)-(1S,5R)-2-oxabicyclo[3.3.0] oct-6-en-3-one

(-)-(1R,5S)-3-oxabicyclo[3.3.0] oct-6-en-2-one

Thus, monooxigenase enzymes are preferelly activated compared to other oxido-reductive enzymes. This is a starting phase to deepen studies aimed at enhancing yields and e.e. of lactones.

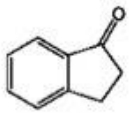
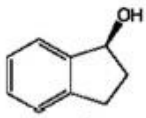
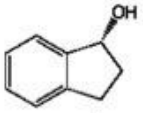
Biotransformation products were chemically confirmed by GC-MS. The assignment of alcohol absolute configuration was determined by GC-FID, by comparing with standards previously isolated in our laboratory and completely identified by chemical and physical point of view (Pedrini *et al*., 2009).

The absolute configuration of lactones was defined by comparison with Fluka commercial standards (-)-(*1S,5R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one, (+)-(*1R,5S*)-2-oxabicyclo[3.3.0]oct-6-en-3-one, (-)-(*1R,5S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one.

➢ 1-INDANONE

The 1-indanone biotransformation did not give interesting results, since alcohol yields were very low. However, remarkable results were those achieved about the e.e. checked with the highest value (Table 5). In particular, 5 strains (EC33, EC36, EC38, EC60 and EC64) reduced 1-indanone into (*S*)-indanol and (*R*)-indanol alcohol, with yield between 1% and 4%. Therefore, notwithstanding the low yield, the fungal strains showed a comproved enantioselectivity with a 100% e.e..

Table 5 – 1-indanone biotransformation products

 *1*	Time (days)	 *2* **S yield%** (ee%)	 *3* **R yield%** (ee%)
EC33	10	**2** (100)	
EC36	10	**4** (100)	
EC38	10		**3** (40)
EC60	10	**2** (63)	
EC64	10		**1** (100)

1 1-indanone, *2* (S)-indanol, *3* (R)-indanol

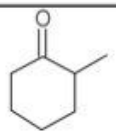
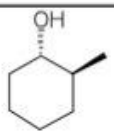
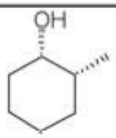
As for the previous reported data, biotransformation products were chemically confirmed by GC-MS. The assignement of alcohol absolute configuration was determined by GC-FID equipped with proper chiral column and by comparison with standards previously isolated in our laboratory and completely identified by chemical and physical properties (Pedrini *et al*., 2009).

➢ 2-METHYLCYCLOHEXANONE

Biotransformation of (+/-)-2-methyl-cyclohexanone gave good results both in terms of yield and enantiomeric excess (Table 6). It has been observed an higher production of *trans*-2-methylcyclohexanol if compared with *cis*-form yield. Strains that gave the best yields and enantiomeric excesses of the alcohol were EC38 and EC60. Only EC26 and EC37 produced the *cis* enantiomer with higher e.e. (56% and 61% respectively).

The identification of isomers *cis* and *trans* was carried out by GC-FID injection of an alcohol mixture obtained by chemical reduction (with sodium borohydride) of (+/-)-2-methyl-cyclohexanone. This reaction produced 64% and 36% of *trans* and *cis* isomers, as consequence of the steric hindrance of methylic group (Noyce *et al*., 1950).

Table 6 – 2-methyl-cyclohexanone biotransformation products

 *1*	Time (days)	 *2* *trans* **yield%** (ee%)	 *3* *cis* **yield%** (ee%)
EC19	10	**47** (73)	**33** (90)
EC26	10	**35** (70)	**56** (75)
EC37	3	**20** (75)	**61** (88)
EC38	10	**81** (98)	**2** (95)
EC46	10	**45** (89)	**43** (73)
EC49	10	**44** (90)	**44** (70)
EC59	10	**57** (81)	**27** (52)
EC60	7	**65** (93)	**10** (90)
EC61	10	**47** (78)	**40** (82)

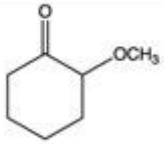
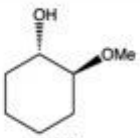
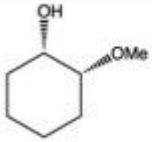
1 (+/-)-2-methyl-cyclohexanone **2** *trans*-(1S,2S)-2-methylcyclohexanol
3 *cis*-(1S,2R)-2-methylcyclohexanol

Biotransformation products were chemically confirmed by GC-MS. The assignement of alcohol absolute configuration was determined by GC-FID equipped with proper chiral column and by comparison with standards previously isolated in our laboratory and completely identified by chemical and physical properties (Pedrini *et al*., 2009).

- **2-METHOXYCYCLOHEXANONE**

In general the enantiomer *trans*-2-methoxycyclohexanol was preferably produced rather than *cis* form (Table 7). In particular, EC37 and EC60 strains showed the highest yield and enantiomeric excesses of this enantiomer, while, EC19, EC26, EC46, EC59 and EC61 produced the *cis*-2-methoxycyclohexanol with important enantiomeric excesses (comprised between 74% and 93%).

Table 7 – 2-methoxycyclohexanone biotransformation products

 *1*	Time (days)	 *2* *trans* **yield%** (ee%)	 *3* *cis* **yield%** (ee%)
EC19	10	**82** (46)	**18** (91)
EC26	10	**82** (33)	**18** (93)
EC37	7	**65** (78)	**3** (100)
EC38	10	**54** (47)	**45** (28)
EC46	10	**86** (25)	**13** (74)
EC49	10	**88** (14)	**8** (59)
EC59	10	**88** (22)	**12** (74)
EC60	7	**50** (80)	**43** (57)
EC61	10	**86** (23)	**14** (79)

1 (+/-)-2-methoxycyclohexanone, ***2*** *trans*-(1S,2S)-2 methoxy-cyclohexanol, ***3*** *cis*-(1S,2R)-2-methoxycyclohexanol

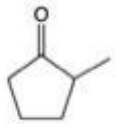
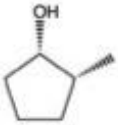
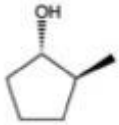
The identification of *cis* and *trans* isomers was carried out by GC-FID injecting an alcohols mixture obtained by chemical reduction (with sodium borohydride) of (+/-)-2-methoxycyclohexanone in methanol and it was confirmed by the NMR data (Roberts, 1997; Bocca *et al*., 2006). The absolute configuration was determined through column chromatography (petroleum ether/diethyl ether 10:2 as eluent) afforded the *trans* isomer from EC37 and *cis* isomer from EC26. The experimental optical rotation of isolated *trans*-(1S,2S)-2-methoxy-cyclohexanol - $[\alpha]_D$= + 50 (C=2, CH_2Cl_2) - was in accordance with literature data - $[\alpha]_D$= + 69.5 (C=2, CH_2Cl_2) at 96% of enantiomeric excess (Fukazawa,1993).

The experimental optical rotation of isolated *cis*-(1S,2R)-2-methoxy-cyclohexanol was negative in accordance with literature data (Okuma *et al*., 2003).

➢ 2-METHYLCYCLOPENTANONE

EC38, EC49 and EC59 strains evidenced high values in yields and e.e. with report to reduction of (+/-)-2-methylcyclopentanone. In particular, EC49 and EC59 produced an enantiomeric excess equal to 100% both for *trans* and *cis* alcohols, but the determination of absolute configuration is still in progress.

Table 8 – (+/-)-2-methylcyclopentanone biotransformation products

 *1*	Time (days)	 *2* *cis* **yield%** (ee%)	 *3* *trans* **yield%** (ee%)
EC19	7	**4** (76)	**19** (87)
EC26	10	**4** (55)	**20** (84)
EC37	7	0	**13** (100)
EC38	10	**20** (19)	**58** (-84) *
EC46	7	**46** (-35)*	**34** (-21) *
EC49	10	**55** (-100)*	**31** (-100) *
EC59	10	**52** (-86)*	**34** (-100) *
EC60	7	**3** (28)	**7** (-12) *
EC61	7	**9** (21)	**28** (85)

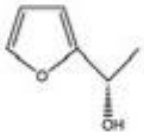
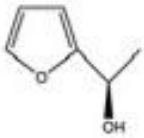
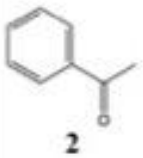
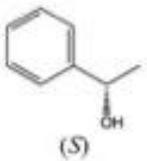
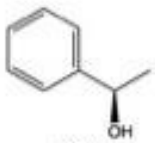
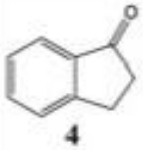
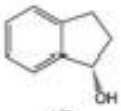
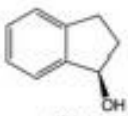
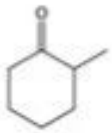
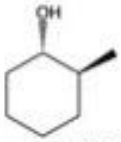
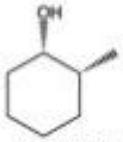
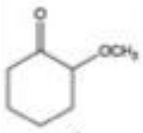
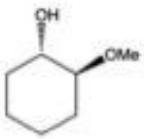
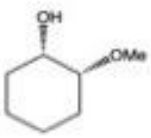
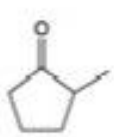
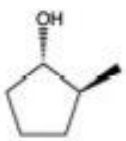
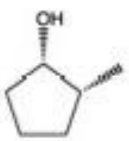
1 (+/-)-2-methylcyclopentanone, *2* *cis*-(1*S*,2*R*)-2-methylcyclopentanol or *cis*-(1*R*,2*S*)-2-methylcyclopentanol, *3* *trans*-(1*S*,2*S*)-2-methylcyclopentanol or *trans*-(1*R*,2*R*)-2-methyl-cyclopentanol
*: the "minus" sign indicates that the enantiomeric excess is in favor of the other enantiomer, there is an inversion configuration

The identification of isomers *cis* and *trans* was carried out by GC-FID injecting a cocktail of alcohols obtained by chemical reduction (with sodium borohydride) of (+/-)-2-methylcyclopentanol; this reduction produced 75% and 25% of isomers *trans* and *cis* respectively (Umland *et al.*, 1956).

Further studies are in progress to determine the absolute configuration of enantiomers.

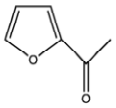
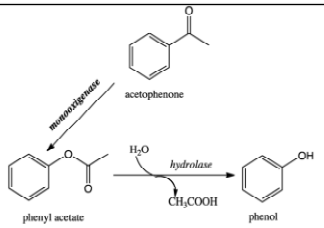
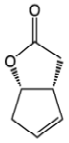
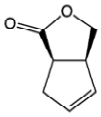
Below are reported two tables summarizing the most significative results obtained in the biotransformation of pharmaceutical ketones: the first table is referred to alcohols (reduction products), the second to the Baeyer-Villiger oxidation products.

Table 9 – Fungal strains, products, yields, enantiomeric excesses of pharmaceutical ketones biotransformation

Substrate	Endophyte strain	Time (days)	Biotransformation products		Yield % (ee%)	
 **1**	EC37 EC49 EC60 FE40 FE86	10 10 10 7 7	 (*S*)	 (*R*)	**17** (59) **10** (66) - **26** (100) **6** (100)	- - **20** (52) - -
 **2**	EC17 EC19 EC37 EC38 EC50 EC61	7 7 10 10 10 7	 (*S*)	 (*R*)	**82** (100) **97** (100) **88** (94) - - **91** (82)	- - - **48** (86) **49** (84) -
 **3**	EC17 EC38 EC52 FE86	3 7 10 7	 *endo* (1*S*,5*R*,6*S*)	 *exo* (1*R*,5*S*,6*S*)	**39** (87) **34** (53) **29** (1) **21** (95)	- **51** (98) **18** (100) **14** (100)
 **4**	EC36 EC38	10 10	 (*S*)	 (*R*)	**4** (100) -	- **3** (40)
 **5**	EC19 EC37 EC38 EC46 EC49 EC60	10 3 10 10 10 7	 *trans*-(1*S*,2*S*)	 *cis*-(1*S*,2*R*)	**47** (73) **20** (75) **81** (98) **45** (89) **44** (90) **65** (93)	**33** (90) **61** (88) **2** (95) **43** (73) **44** (70) **10** (90)
 **6**	EC19 EC26 EC37 EC60 EC61	10 10 7 7 10	 *trans*-(1*S*,2*S*)	 *cis*-(1*S*,2*R*)	**82** (46) **82** (33) **65** (78) **50** (80) **86** (23)	**18** (91) **18** (93) **3** (100) **43** (57) **14** (79)
 **7**	EC19 EC37 EC46 EC61	7 7 7 7	 *trans*	 *cis*	**19** (87) **13** (100) **34** (-21) **28** (85)	**4** (76) - **46** (-35) **9** (21)

1 2-furyl methyl ketone, **2** acetophenone, **3** *cis*-bicyclo[3.2.0]hept-2-en-6-one, **4** 1-indanone, **5** 2-methylcyclohexanone, **6** 2-methoxycyclohexanone, **7** 2-methylcyclopentanone

Table 10 – Fungal strains, products, yields, enantiomeric excesses of Baeyer-Villiger oxidation products

Substrate	**Endophyte strain**	**Time (days)**	**Biotransformation products**		**Yield %** (ee%)	
 **1**	EC38 EC60	10 7			**22** **13**	
 **2**	EC17 EC33 EC49 EC61	10 10 10 10	 2-*oxa*	 3-*oxa*	**10** (55) **5** (100) **53** (37) **56** (30)	**18** (92) **23** (78) **30** (61) **29** (62)

1 2-furyl methyl ketone, **2** *cis*-bicyclo[3.2.0]hept-2-en-6-one

Phytochemical analysis and biotransformation of *Piper aduncum* essential oil

Steam distillation of Ecuadorian *P. aduncum* aerial parts provided an essential oil yield of 8.0 ml/kg (0.8%), higher than that reported for Brazilian *P. aduncum* (2.8 ml/kg) (Roseli *et al.*, 2009). The table below shows chemical composition of *P. aduncum* essential oil:

Table 11 - Chemical composition of *Piper aduncum* essential oil

Compound[a]	Method	RT	RI[b]	*P. aduncum* (%[c])
α-Thujene	RI, MS[d]	5.223	930	0.35
α-Pinene	RI, MS	5.395	939	1.35
Camphene	RI, MS	5.848	954	0.05
β-Pinene	RI, MS	6.935	979	1.27
Myrcene	RI, MS	7.867	991	0.73
α-Phellandrene	RI, MS	8.295	1003	1.06
3-Carene	RI, MS	8.578	1005	0.07
α-Terpinene	RI, MS, NMR[e,f]	8.953	1017	0.82
p-Cymene	RI, MS	9.367	1025	0.73
Limonene	RI, MS	9.544	1027	1.6
1,8-Cineole	RI, MS	9.610	1031	1.3
Z-Ocimene	RI, MS, NMR[e]	10.534	1037	2.23
E-Ocimene	RI, MS, NMR[e]	11.197	1050	10.39
γ-Terpinene	RI, MS, NMR[f]	11.569	1060	2.42
Sabinene hydrate *cis*	RI, MS	12.011	1070	0.42
Isoterpinolene	RI, MS	13.449	1088	0.12
Terpinolene	RI, MS, NMR[f]	13.558	1089	0.82
p-Cymenene	RI, MS	13.997	1093	0.69
Linalool	RI, MS	14.830	1097	1.82
Sabinene hydrate *trans*	RI, MS	15.232	1122	0.24
allo-Ocimene	RI, MS	17.222	1132	0.36
Camphor	RI, MS	17.478	1146	0.05
Terpinen-4-ol	RI, MS, NMR[e]	20.933	1177	3.14
α-Terpineol	RI, MS	22.349	1189	0.06
cis-Piperitol	RI, MS	22.744	1196	0.05
trans-Piperitol	RI, MS	24.169	1208	0.13
Piperitone	RI, MS, NMR[e]	28.632	1253	8.47
Safrole	RI, MS, NMR[f]	32.858	1287	–
δ-Elemene	RI, MS	38.118	1338	0.08
Methyl decanoate	RI, MS	38.358	1345	–
α-Ylangene	RI, MS	40.549	1375	0.08
α-Copaene	RI, MS	41.901	1377	0.27
β-Bourbonene	RI, MS	42.675	1388	–
β-Cubebene	RI, MS	43.816	1388	0.05
β-Elemene	RI, MS	44.161	1391	0.45
α-Gurjunene	RI, MS	45.463	1410	0.13
β-Caryophyllene	RI, MS	46.271	1419	2.57
β-Copaene	RI, MS	47.582	1432	0.09
Aromadendrene	RI, MS	48.396	1441	0.05
α-Humulene	RI, MS	50.021	1455	0.65
γ-Muurolene	RI, MS	53.341	1480	1.01
Bicyclogermacrene	RI, MS	55.093	1498	1.45
α-Muurolene	RI, MS	56.063	1500	0.07
δ-Amorphene	RI, MS	56.554	1512	0.14
Cubebol	RI, MS	56.975	1515	0.84
Nonadecane	RI, MS	57.562	1517	–
Myristicin	RI, MS	57.834	1519	0.89
Humulene epoxyde II	RI, MS	61.638	1608	0.18
Dillapiol	RI, MS, NMR[f]	62.985	1678	45.92
Total				**95,66**

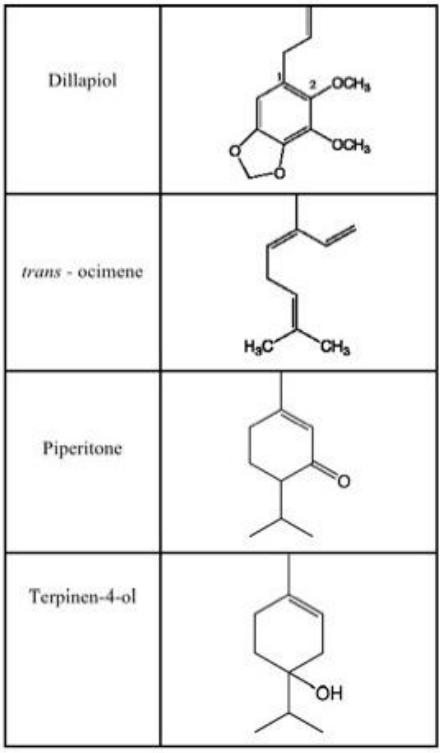
a Compounds are listed in order of elution from Varian FactorFour VF-5ms
b KI, Retention indices calculated on Varian FactorFour VF-5ms
c RA%, relative area percentage (peak area relative to total peak area %, calculated on Varian FactorFour VF-5ms
d Mass spectrometry
e Nuclear magnetic resonance
f *Piper aduncum*

46 compounds accounting for 95.66% of the total were respectively identified. *P. aduncum* essential oil has been thoroughly evaluated and different profiles have been described from different parts of the world. *P. aduncum* is seemingly presenting some degrees of chemical polymorphism. Different chemical profiles are known: (i) one from Bolivia in which the main constituent is 1,8-cineole (40%), (ii) one from Panama rich in sesquiterpenes like β-caryophyllene and aromadendrene, (iii) a chemotype frequently found in the Americas, South East Asia and Oceania, in which dillapiol is predominant (30–90%) (Guerrini *et al*., 2009) in accordance to Amazon Brazilian chemotype as reported by Roseli (2009). Some Brazilian accessions are rich in linalool or nerolidol and surprisingly devoid of phenylpropenes (Guerrini *et al*., 2009).

P. aduncum essential oil from eastern Ecuador proved to be abundant in dillapiol, as often reported from the Amazon. Piperitone (8.47%), *trans*-ocimene (10.39%) and terpinen-4-ol (3.14%) were also present.

The ^{13}C-NMR confirms the presence of the main components of *P. aduncum* essential oil, that is *cis*-ocimene, *trans*-ocimene, γ-terpinene, terpinen-4-ol, piperitone and dillapiol.

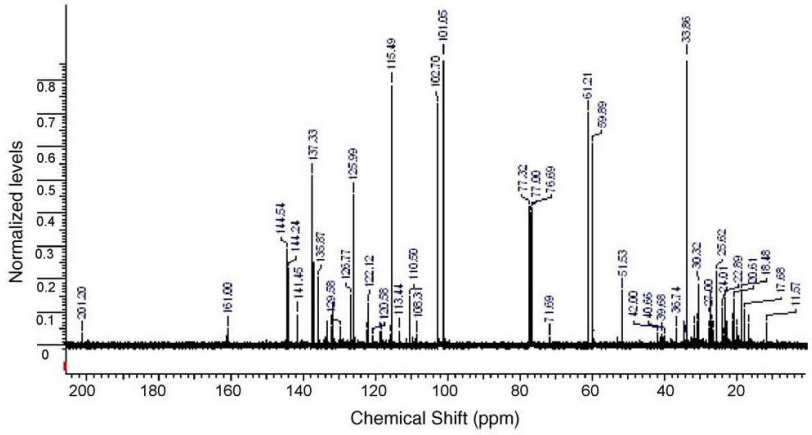
Scheme 4 - Main compounds of *P. aduncum* essential oil



For fingerprinting purposes, the NMR characterization is a powerful tool useful to discriminate between different cultivars, chemotypes and hybrids. It is also useful for identification, quality control and adulteration of drugs (Guerrini *et al*., 2009 and reference therein).

The fungal strains EC19, EC46 and EC49 were tested on dillapiol, *trans*-ocimene, piperitone, terpinen-4-ol as the most abundant compounds checked with essential oil. The same endophyte were tested for biotransforming capacity on the whole essential oil. The biotransformation activity was recorded on two compounds: piperitone and *trans*-ocimene.

Figure 4 - ^{13}C NMR of *P. aduncum* essential oil



a/ Dillapiol

The biotransformation of dillapiol gave products in not significant amount.

b/ Ocimene

The biotransformation of *trans*-ocimene (commercial mixture of 75% *trans*-ocimene and 25% limonene) afforded good results with all strains tested, especially with EC19. The biotransformation products were: dimethyl-octatetraene, dimethyl-octatrienol, dimethyl-octadiendiol.

The GC-MS analysis identified two isomers of the dimethyl-octatetraene which actually showed different retention times. Investigations are in progress to define the absolute configuration of compounds.

Table 12 – Biotransformation products of *trans*-ocimene, obtained by EC19, EC46, EC49 strains

Fungal strains	Time (days)	Yield% **octatetraene** isomer 1	Yield% **octatetraene** isomer 2	Yield% **Octatrienol** isomer 1	2	3	Yield% **octadiendiol**
EC19	3	21	26	13			39
	7	6	6	0			88
EC46	3	1	2	-	17	32	-
	7	11	10	-	14	39	-
EC49	3	5	4	-	23	22	-
	7	-	27	-	35	39	-

After 3 days from inoculum, EC19 produced dimethyl-octatetraene with a 47% yield (considering the both isomers), and dimethyl-octadiendiol with a 39% yield. During biotransformation process, octatetraene was obtained by dehydrogenation of *trans*-ocimene followed by the production of dimethyl-octadiendiol through an hydratation reaction on 2 double bound with a yield of 88% (Table 13). EC46 and EC49 instead catalyzed the hydratation just on 1 doble bound giving the two isomers of dimethyl-octatrienol, as emerged by the different retention times of the EC19 product analysis. Chromatograms and spectra of reaction products are reported below. Determination of relative stereochemistry is still in progress.

Figure 5 – Chromatograms of biotransformation of *trans*-ocimene obtained by EC19 strain, at 3rd and 7th day from inoculum

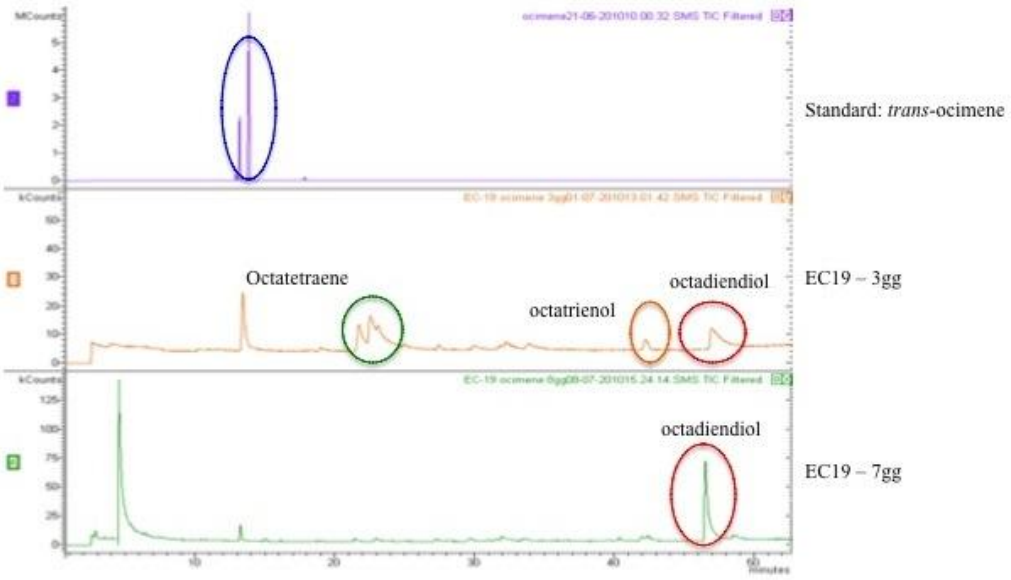
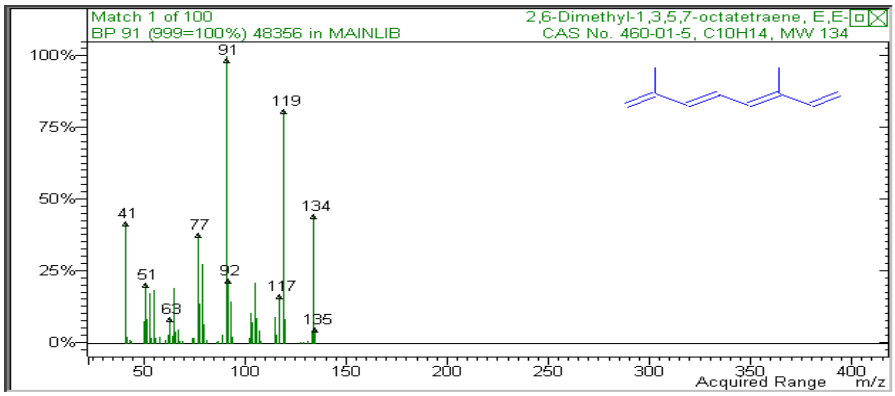


Figure 6 – MS spectrum of dimethyl-octatetraene



EC46 and EC49 produced preferably dimethyl-octatrienol as shown below.

Figure 7 – Chromatograms showny biotransformation of *trans*-ocimene obtained by EC46 and EC49, at 3rd e 7th day from inoculum

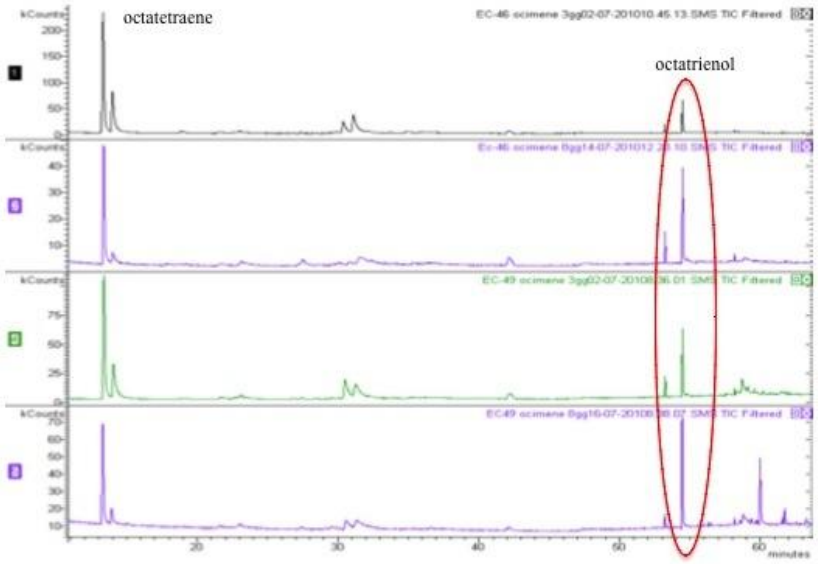


Figure 8 – MS spectrum of dimethyl-octadiendiol

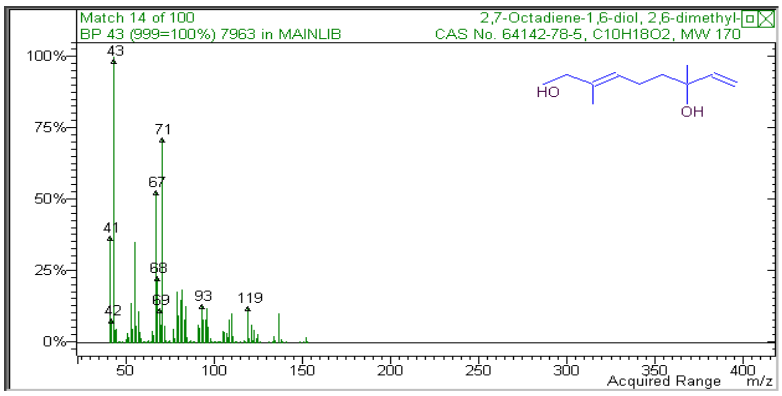
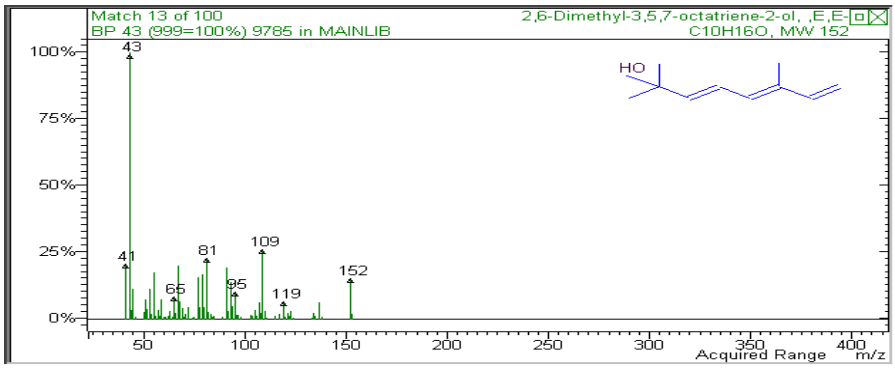


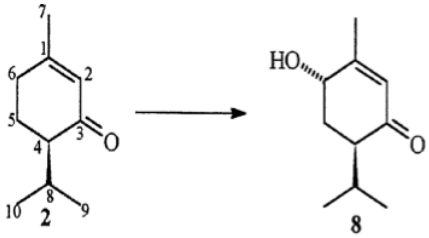
Figure 9 - MS spectrum of dimethyl-octatrienol



c/ Piperitone

Figure 10 illustrates the hydroxylation of (-)-(4*R*)-piperitone (**2**), which produced (4*R*,6*S*)-*trans*-6-hydroxy-piperitone (**8**), as investigated by van Dik (1997). During the first screening with (+/-) piperitone only hydroxylation products were obtained: i.e. a mixture of *trans*-6-hydroxy-piperitone and *cis*-6-hydroxy-piperitone.

Figure 10 – Hydroxylation reaction of (-)-(4R) piperitone



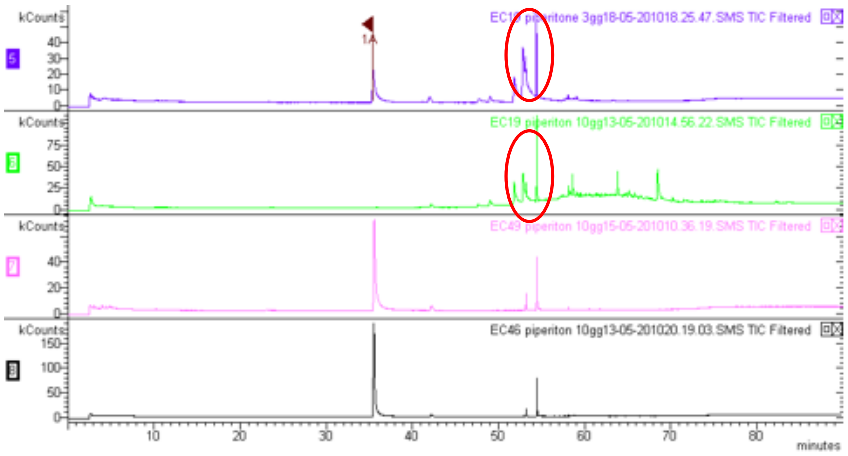
The production of *cis*-6-hydroxy-piperitone (yield 18%) and *trans*-6-hydroxy-piperitone (yield 50%) were observed after 3 days from inoculum. Moreover, after 10 days the (+/-) piperitone completely disappears and good yields of enantiomers *trans* and *cis* of hydroxy-piperitone were recorded.

Table 13 – Biotransformaion products, yields and enantiomeric excess of (+/-) piperitone

(+/-) piperitone	Time (days)	Yield*%* ***cis*** 6-hydroxy-piperitone	Yield*%* ***trans*** 6-hydroxy-piperitone
32%	3	18%	50%
0%	10	40%	60%

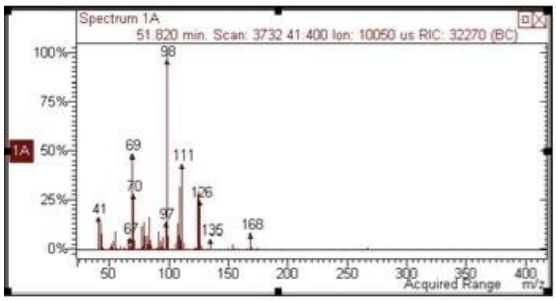
Below chromatograms showing the (+/-) piperitone biotransformation products by EC19, EC46, EC49 are reported. Already since the 3rd day of inoculum, EC19 showed to be able to produce the highest abundance of *cis*-6-hydroxypiperitone and *trans*-6-hydroxypiperitone (peaks red circled). Analysis are still in progress to definitely confirm the absolute configuration of the compounds.

Figure 11 – Chromatograms of EC19, EC46 and EC49 concerning piperitone biotransformation

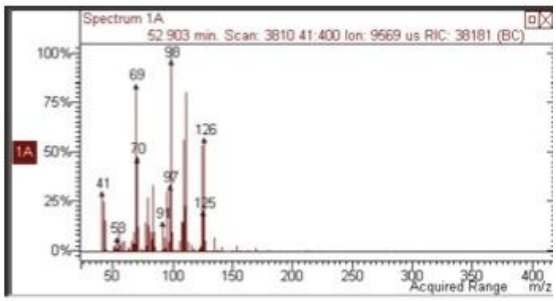


Below are reported spectra of reaction products. Data are confirmed by literature spectra (Van Dik *et al*., 1998)

Figure 12 - Experimental spectra of: *cis*-hydroxypiperitone and *trans*-hydroxypiperitone



cis-hydroxypiperitone experimental spectrum



trans-hydroxypiperitone experimental spectrum

d/ Terpinen-4-ol

At 3rd and 7th day after inoculum, GC-MS and GC-FID analysis did not evidence interesting products abundance.

e/ Biotransformation on whole *Piper aduncum* essential oil

No biotransformation products have been checked with report to the experiment with *P. aduncum* essential oil, with the sole exception of reaction detected on some monoterpenes (ocimene and piperitone) showing low yield.

This could be due by the high abundance of dillapiol which would mask the possible capacity of other less abundant compounds to activate enzymatic pools.

PHARMACEUTICAL BIOLOGY APPROACH

Among all the plant species considered, *Piper aduncum* was chosen as starting point for the pharmaceutical biology approach. Before the starting of my PhD, in light of the background of the research project, *Maytenus macrocarpa* was been already explored with the same aim (see final chapter with published paper attached). The research pattern adopted for *M. macrocarpa* and *P. aduncum* will be performed for the remaining plant species, whose chemicals and biological characterization is still in progress.

***P. aduncum* plant material and essential oil**

Fresh aerial parts of adult plants of P. aduncum were distilled direct in the forest with an apparatus shown in the figure below. The extraction yield was of 8.0 ml/kg (0.8%).

BIOLOGICAL ACTIVITY: EFFICACY

Given the lack of information regarding biological activities of *P. aduncum* essential oil from amazonian Ecuador and the presence of putative noxious substances, evaluation of their mutagenicity and a number of biological activities were performed. Essential oils can be topically applied or orally administered and careful examination of possible mutagenic properties is required to confirm and assure the safety of their use. Moreover, some evidences of possible genotoxic activities exerted by essential oils are emerging. Notwithstanding the traditional use of *P. aduncum* and its growing commercial availability in the worldwide market of natural remedies, the mutagenic/antimutagenic behaviour of its essential oil have not been investigated before. It should be emphasized that tradition in use is by no means warrants of safety, particularly regard to mutagenicity/carcinogenicity, where a complex set of cause–effect relationships, signs

and symptoms is involved and not easily recognized by the population (Guerrini *et al.*, 2009).

Antibacterial activity

Regarding the antimicrobial properties of the oil, it must be noted that was scarcely active if compared with *T. vulgaris* essential oil against both Gram positive (*Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus foecalis*) and Gram negative (*Klebsiella oxytoca*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*) bacteria. Such data are seemingly discordant with those available in the literature suggesting a lower effectiveness of our Ecuadorian sample.

P. acundum essential oil showed a lower antibacterial activity than that expressed by the reference *T. vulgaris* essential oil against all tested bacterial strains. The sole interesting result regarded the activity against *E. coli* (0.23 mg/ml) with values similar to that expressed by T. vulgaris (0.06 mg/ml), even if almost four time higher.

Table 14 - Antibacterial activity expressed in MIC mg/ml of *P. aduncum* essential oil compared with *T. vulgaris* and Chloramfenicolo

	Piper aduncum MIC (mg/ml)	**Thymus vulgaris** MIC (mg/ml)	**Cloramfenicolo** MIC (μg/ml)
Gram -			
Escherichia coli ATCC 4350	0,23	0,06	2,50
Pseudomonas aeruginosa ATCC 17934	5,24	0,18	5,00
Klebsiella oxytoca ATCC 29516	>5,24	0,40	1,25
Proteus mirabilis ATCC 29852	5,24	0,40	2,50
Gram +			
Enterococcus foecalis ATCC 29212	>5,24	0,11	5,00
Micrococcus luteus ATCC 9622	5,24	0,11	1,25
Bacillus subtilis ATCC 7003	5,24	0,11	2,50
Staphylococcus aureus ATCC 29213	>5,24	0,11	2,50

Antifungal activity

The efficacy of the oil on fungal strains was noticeable compared to microbial strains. *P. aduncum* essential oil was active in a dose-dependent manner against both dermatophytes and phytopathogens, inducing complete inhibition of *Magnaporthe grisea* and *Trichophyton* species at 500mg/ml. It also exerted a good efficacy at lower doses (50 μg/ml) against *M. grisea*, evidencing a potency three times higher than

Triciclazole. Such activity fits with previous antifungine evaluations of Matico essential oil rich in dillapiol and allylbenzenes (Guerrini *et al.*, 2009).

Table 15 - Antifungal activity of Matico essential oil (EO), expressed as growth inhibition %, compared with antifungal synthetic compounds: triciclazole, piraclostrobin, boscalid

PHYTOPATOGENS	***Magnaporthe grisea* ATCC 64413** % inhibition			***Pythium ultimum*** % inhibition			***Botrytis cinerea* ATCC 48339** % inhibition		
	50 µg/ml	100 µg/ml	500 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml
EO *Piper aduncum*	**62**	**83**	**98**	**4**	**25**	**57**	**16**	**24**	**70**
Triciclazole	24	62	94						
Pyraclostrobin				100	100	100			
Boscalid							89	100	100

DERMATOPHYTES	***Trichophyton mentagrophytes*** CBS % inhibition			***Trichophyton tonsurans*** CBS % inhibition			***Nanizzia cajetani*** IHME 3441 % inhibition		
	50 µg/ml	100 µg/ml	500 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml
EO *Piper aduncum*	/	**59**	**100**	/	**46**	**100**	/	**25**	**84**
Ketoconazole	100	100	100	100	100	100	100	100	100

Antioxidant activity

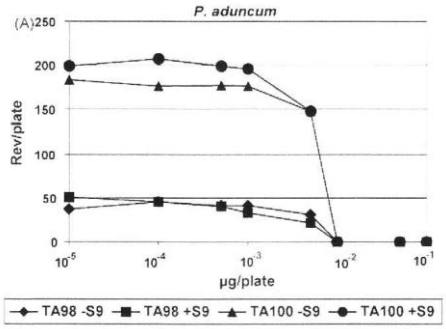
The *P. aduncum* essential oil was not active as radical scavenger at 20 µl/ml in DPPH and ABTS assays and only in photochemiluminescence an antioxidant activity comparable to Trolox was obtained (3.7 mmol Trolox/g).

BIOLOGICAL ACTIVITY: SAFETY

Mutagenic activity

By using the plate incorporation assay, no mutagenic activity of *P. aduncum* essential oil was detected. The activity was investigated on *S. typhimurium* tester strains TA98 and TA100, either with or without S9 activation.

Figure 13 - Mutagenic activity of *P. aduncum* essential oil on *S. typhimurium* TA98 e TA100, with and without S9 activation



Lack of mutagenic activity may be some how surprising, given the known hepatocarcinogenic activity of safrole and other allylbenzenes, yet in literature are reported questionable results about safrole mutagenicity by using Ames *Salmonella* reversion assay. Dorange (1977) reported that safrole was mutagenic in strain TA1535 in the presence of liver enzymes. On the other hand, Wislocki (1977) reported that safrole was not mutagenic in strain TA100 or in TA1535. They observed, however, that the safrole metabolite (safrole-2′,3′-epoxide) was mutagenic in both strains TA100 and TA1535 even in the absence of S9. Our negative result was in agreement with Sekizawa and Takayuki (1982), who used strains TA98 and TA100 with and without metabolic activation. Moreover, it must be underscored that these authors tested pure chemicals rather then evaluating a phytocomplex as an essential oil, in which different substances are or may be simultaneously active, and may lead to different result than the administration of a single substance. Essential oil is utilized *in toto* and thus its evaluation as a phytocomplex is more representative of its veritable effects (Guerrini *et al.*, 2009).

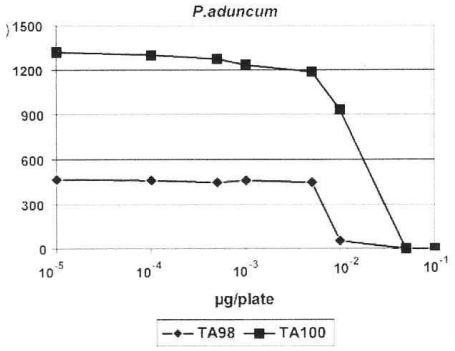
Cytotoxic activity

The cytotoxic activity of P. aduncum essential oil have been determined with refernce to the results emerged by TA98 and TA100 strains. The cytotoxic dose of *P. aduncum* essential oil was 10^{-2} μg/plate for both strains, while 10^{-3} μg/plate was determined as the highest uneffective dose (HUD): both the values resulted useful for mutagen-protective assays.

Mutagen-protective activity

P. aduncum essential oil when tested for mutagen-protective efficacy in the Ames *Salmonella*/microsome assay has not shown any significant statistical effects of increasing amounts on the activity of directly acting mutagens 2-nitrofluorene and sodium azide. In *P. aduncum* HUD (Highest Uneffective Dose) for toxic effect has been settled at 10^{-3} μg plate^{-1} for both TA98 and TA100. Significant off-set differences from revertants of 2-nitrofluorene and sodium azide ($P<0.05$) was, for both chemicals and according to Tuckey HSD test, settled at 10^{-2} μg plate^{-1}.

Figure 14 - Antimutagenic effect in *S. typhimurium* TA98 and TA100 of *P. aduncum* essential oil, assessed without metabolic activation.



Antiplatelet activity

The essential oil of *P. aduncum* was moderately active in the antiplatelet assays performed on three inducers of aggregation: ADP, arachidonic acid (AA) and U46619, a stable tromboxane A_2 agonist. If compared with essential oils previously screened the activity of *P. aduncum* was not as high as those provided by oils rich in phenypropenes, but nevertheless not negligible.

Table 16 - Antiplatelet activity of essential oil of *P. aduncum* compared with ADP, AA and U46619

	IC50 (µg/ml)		
	ADP	AA	U46619
OE *Piper aduncum*	**196** (169-218)	**149** (105-210)	**168** (155-182)

IC50=inhibition concentration
ADP=Adenosine diphosphate
AA= arachidonic acid
U46619= tromboxane A2 (vasoconstrictor)

Antinociceptive activity

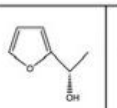
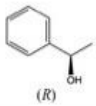
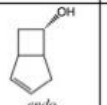
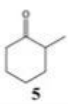
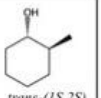
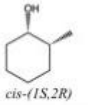
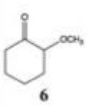
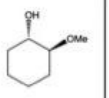
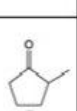
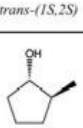
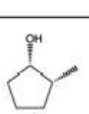
In view of the purported analgesic properties ascribed to *P. aduncum* in folk medicine, a preliminary test was conducted in order to evaluate the possible contribution of essential oil to noci-ception. The test, however, gave negative results.

Table 17 - Antinociceptive activity of *P. aduncum* essential oil

	100 mg/kg os	
	Hot plate	Edema
OE *Piper aduncum*	**inactive**	**inactive**

6. CONCLUSIONS

- 364 endophytic fungal strains have been isolated from *Piper aduncum*, *Maytenus macrocarpa*, *Schinus molle*, *Tecoms stans*, *Eugenia hallii* Amazonian plants known for their ethnomedical relevance.
- The strains have been then tested for biotransformations; in particular, reduction products of 2-furylmethylketone, acetophenone, *cis*-bicyclo-[3,2,0]-hept-2-en-6-one, 1-indanone, 2-methyl-cyclohexanone, 2-methoxy-cyclohexanone, acetylfuran, 2-methyl-cyclopentanone were investigated.
- The pure chemicals have been chosen because of their molecular relevance as precursors of synthetic pathways to produce pharmaceutical drugs.
- EC17, EC19, EC37, EC38, EC46, EC49, EC50, EC60, EC61, FE40 and FE86 gave the best results in terms of biotransforming capacity. The following results (see table below) in terms of kind of products, yield and enantiomeric excess have been obtained with reference to each strain.

Substrate	Endophyte strain	Time (days)	Biotransformation products		Yield % (ee%)	
 1	EC37 EC49 EC60 FE40 FE86	10 10 10 7 7	 (*S*)	 (*R*)	**17** (59) **10** (66) - **26** (100) **6** (100)	- - **20** (52) - -
 2	EC17 EC19 EC37 EC38 EC50 EC61	7 7 10 10 10 7	 (*S*)	 (*R*)	**82** (100) **97** (100) **88** (94) - - **91** (82)	- - - **48** (86) **49** (84) -
 3	EC17 EC38 EC52 FE86	3 7 10 7	 *endo* (1*S*,5*R*,6*S*)	 *exo* (1*R*,5*S*,6*S*)	**39** (87) **34** (53) **29** (1) **21** (95)	- **51** (98) **18** (100) **14** (100)
 4	EC36 EC38	10 10	 (*S*)	 (*R*)	**4** (100) -	- **3** (40)
 5	EC19 EC37 EC38 EC46 EC49 EC60	10 3 10 10 10 7	 *trans-(1S,2S)*	 *cis-(1S,2R)*	**47** (73) **20** (75) **81** (98) **45** (89) **44** (90) **65** (93)	**33** (90) **61** (88) **2** (95) **43** (73) **44** (70) **10** (90)
 6	EC19 EC26 EC37 EC60 EC61	10 10 7 7 10	 *trans-(1S,2S)*	 *cis-(1S,2R)*	**82** (46) **82** (33) **65** (78) **50** (80) **86** (23)	**18** (91) **18** (93) **3** (100) **43** (57) **14** (79)
 7	EC19 EC37 EC46 EC61	7 7 7 7	 *trans*	 *cis*	**19** (87) **13** (100) **34** (-21) **28** (85)	**4** (76) - **46** (-35) **9** (21)

1 2-furyl methyl ketone, **2** acetophenone, **3** *cis*-bicyclo[3.2.0]hept-2-en-6-one,
4 1-indanone, **5** 2-methylcyclohexanone, **6** 2-methoxycyclohexanone,
7 2-methylcyclopentanone

- The strains taxonomical identification is still in progress with the contribute of CBS (Fungal Biodiversity Centre - Netherlands). However, macroscopical and microscopical evidences lead to suggest EC19 and EC37 genus as belonging to *Fusarium* genus, and EC46, EC49, EC59 and EC61 belonging to *Penicillium* one.
- The biotransformation products were all identified by comparison with pure standards by GC. However, structural analyses are still in progress to definitely confirm the suggestions. *P. aduncum* essential oil has been examined through pharmaceutical biology approach being chemically and biologically investigated. Dillapiol, *trans*-ocimene, piperitone and terpinen-4-ol were checked as most abundant.
- Biotransformations on the whole essential oil and on its prevalent compounds were performed providing results probably influenced by dillapiol, since it is one of the most abundant chemicals and probably masks biotransformation on other minor compounds. Biotransformations on *trans*-ocimene gave dimethyl-octatetraene, dimethyl-octatrienol and dimethyl-octadiendiol products. In particular, EC19 showed good results (at 3rd day of inoculum 46% of dimethyl-octatetraene, at 7th day 88% dimethyl-octadiendiol). Biotransformations on piperitone gave *cis*-6-hydroxypiperitone and *trans*-6-hydroxypiperitone. In particular, EC19 showed the highest abundance of both enantiomers.
- Biological activities of whole *P. aduncum* essential oil as those antimicrobial, antifungal and antioxidant were performed. Poor results with respect to that antimicrobial were checked; exceptions could be stressed about the antifungal activity against dermatophytes and phytopathogens, including complete inhibition of *Magnaporthe grisea* and *Trichophyton* species. Antioxidant activity was very low even if PCL displayed results comparable to that of the control. Mutagen and cytotoxic properties were performed characterizing the essential oil of its safety limits, giving also suggestions about its anti-mutagenic capacities. Antinociceptive and platelet aggregation assays completed the functional properties performed for the *P. aduncum* essential oil.
- Finally, all the biotransformation results achieved could be useful, notwithstanding in-depth studies are needed, for possible following patents regarding those strains able to produce pharmaceutically interesting chemicals (as pure drugs or intermediates).

- Moreover, the functional profile of the *P. aduncum* essential oil expressed as bioactivities (safety and efficacy) supports ethnomedical knowledge and use about the plant specie by Natives as cicatrizant and antimicrobial crude drug. The results here reported suggest also the possible employ of the essential oil as preservative ingredient for health supplements.

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8. FINAL NOTE

Before the official starting of this PhD and during its development, I contribute to chemically and biologically characterize other plant species, some of them part of this PhD, as well as their derivatives from Ecuadorian ethnomedicine. In synthesis, as conclusive paragraph of the thesis, all the publications to which I contributed for their realization are reported. For some of them, I could not take part as official author because of conflict of interest due to my fellowship contract with ONG-VIS and MAE.

1) Antimutagenic, antioxidant and antimicrobial properties of *Maytenus krukovii* bark.
Fitoterapia 77, 538-545, 2006.

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2) Ecuadorian stingless bee (*Meliponinae*) honey: a chemical and functional profile of an ancient health product.
Food Chemistry 114, 1413-1420, 2009.

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3) Chemical fingerprinting and bioactivity of Amazonian Ecuador *Croton lechleri* Müll. Arg. (*Euphorbiaceae*) stem bark essential oil: a new functional food ingredient?
Ms. Ref. No.: FOODCHEM-D-10-02644R1 Food Chemistry

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4) Bioactivities of *Piper aduncum* L. and *Piper obliquum* Ruiz & Pavon (*Piperaceae*) essential oils from Eastern Ecuador.

Environmental Toxicology and Pharmacology 27, 39-48, 2009.

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5) Ecuadorian *Citrus* petitgrain oils control through phytochemical and functional approaches.

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