Biotransformations by endophytic fungi isolated from traditional Ecuadorian medicinal plants: Connecting ethnomedicine with biotechnology

Laura Scalvenzi

Dirección de Investigación, Universidad Estatal Amazónica
Paso lateral, km 2½ vía Napo, Puyo, Pastaza
lscalvenzi@uea.edu.ec

Abstract

Ecuador, a small country with diverse ecosystems in the Amazon, Andes and Pacific coastal regions is considered one of the 17 "megadiverse" countries, and the native ethnic groups and rural communities have a strong ethnomedical tradition in the use of native plants in healing. Traditional ethnobotanical knowledge can be used to guide biotechnological research on medicinal plants, even when the new application is an innovation only distantly related to the traditional use. Based on ethnomedicalknowledge of indigenous communities, the following plants from the Amazon and Andes regions were chosen for investigation: *Piper aduncum* (Piperaceae), *Maytenus macrocarpa* (Celastraceae), *Schinus molle* (Anacardiaceae), *Tecoma stans* (Bignoniaceae) and *Myrcianthes hallii* (Myrtaceae). The research was focused on (i) assesing the presence of endophytic fungi in the selected plants, (ii) isolating and subculturing *in vitro* pure endophytic strains, (iii) assessing the biotransformation capacity of the isolated endophytes on pure compounds (intermediates of pharmaceutical synthesis). The following compounds were chosen as substrate models for biotransformations: (+/-)-*cis*-bicyclo[3.2.0]hept-2-en-6-one, acetophenone, 1-indanone, 2-furyl methyl ketone, 2-methylcyclopentanone, 2-methylcyclohexanone, 2-methoxycyclohexanone. A total of 364 fungal strains were isolated *in vitro*; among these, five strains performed biotransformations on acetophenone to (S)-1-phenylethanol, with important yields (78-97%) and enantiomeric excess (78-100%). Three strains also yielded phenols, probably by enzymatic reactions (Baeyer-Villiger oxidations). Fifteen fungal strains yielded the 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 from (+/-)-*cis*-bicyclo[3.2.0]hept-2-en-6-one, probably as result of monooxygenase activation.

Resumen

Ecuador, un país pequeño con diversos ecosistemas en las regiones de la Amazonia, los Andes y la costa del Pacífico, es considerado como uno de los 17países "megadiversos", y los grupos étnicos nativos y las comunidades rurales tienen una

fuerte tradición etnomedicinal en el uso de plantas nativas en la curación. El conocimiento etnobotánico tradicional puedes ser usado para guiar la investigación biotecnológica en plantas medicinales, aún cuando la aplicación nueva e innovadora no está relacionada estrechamente con el uso tradicional de las plantas. En base al conocimiento etnomedicinal de las comunidades indígenas, las siguientes plantas de la Amazoníay de los Andes del Ecuador fueron elegidas para la investigación: *Piper aduncum* (Piperaceae), *Maytenus macrocarpa* (Celastraceae), *Schinus molle* (Anacardiaceae), *Tecoma stans* (Bignoniaceae) y *Myrcianthes hallii* (Myrtaceae). La investigación se enfocó en (i) determinar la presencia de hongos endofitos en las plantas seleccionadas, (ii) aislar y cultivar *in vitro* las cepas de endofitos, (iii) evaluar la capacidad de los endofitos aislados de biotransformar compuestos considerados intermedios de la sintesis de medicamentos. Los siguientes compuestos fueron investigados: (+/-)-*cis*-bicyclo[3.2.0]hept-2-en-6-one, acetophenone, 1-indanone, 2-furyl methyl ketone, 2-methylcyclopentanone, 2-methylcyclohexanone, 2-methoxycyclohexanone. 364 cepas funginas han sido aisladas. Entre ellas, cinco cepas han biotransformado el acetophenone a (S)-1-phenylethanol, con importantes rendimientos (78-97%) y excesos enantiomericos (78-100%). Tres cepas han producido también fenoles, probablemente debido a reacciones enzimáticas que catalizan las oxidaciones de Baeyer-Villiger. Quince cepas funginas han producico los lactones (-)-(1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one y (-)-(1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one a partir de (+/-)-*cis*-bicyclo[3.2.0]hept-2-en-6-one, probablemente como resultado de la activación de enzimas monooxigenasas.

Key words: ethnomedicine, Amazonian plants, Andean plants, endophyte, endophytic fungi, biotechnology, biotransformation

Introducción

Ecuador, a small country with exceptionally diverse forest ecosystems in the Amazon, Andes and Pacific coastal regions, is considered one of the 17 "megadiverse" countries (Mittermeier *et al.*, 1997; 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 study of Ecuadorian biodiversity, both under a chemical and biological point of view. The Neotropical region, including South America, contains a large percentage of the world's flora. At the same time, 80% of humankind lives in "emerging countries", basing their health needs on plant related traditional remedies (WHO, 2006).

The indigenous people of Ecuador, including Kichwa-speading communities in the Andes and Shuar and Achuar communities in the Amazon,

with their strong ethnomedical culture, constitute the background subject of this research.Several studies had been developed to determine the scientific basis of ethnomedical uses of traditional plants. In particular, the study of plant compounds and their biological activity, contributes to the development of phytochemical fingerprinting of traditional plants used by natives for ethnopharmaceutical purposes. This can be considered as a protection tool towards misappropriations of ethnomedical plants and the related knowledge. Moreover the study of new potential uses, far removed from the ethnomedical tradition, is also very interesting for science. In this sense biotechnological applications of ethno medicinal plants are extremely innovative. In particular biotransformations are a relatively new branch of biotechnology.

Biotechnology and biotransformations

Biotechnology consists in the use of live organisms, their derivatives or their biomolecularprocesses to make goods or provide services. Biotechnology is applied in several fields as agriculture, chemical industry, medicine production, health, food industry, environment and mining industry. In particular biotransformations are a relatively new branch of biotechnology. From a chemical point of view, "biotransformation" is the conversion of a chemical compound referred to as the "substrate", generally not used as a nutrient by the microorganisms, to another compound referred to as the "product", with different applications, through the enzymatic activity of biological catalysts (Bastos*et al.*, 2007). Biotransformation isa differentprocess from biosynthesis and biodegradation. Biosynthesis is an *ex novo* synthesis of complex products, catalyzed by enzymes from simple compounds such as carbon dioxide, ammonia or glucose. Biodegradation is a catabolic process, encompassing the conversion of complex compounds into different, simpler compounds. Biotransformations are increasing among biotechnological science and one of its most appreciated features is catalyzing regiospecific and stereospecific reactions underchemical (pH) and physical (temperature, pressure) conditions close to ambient the ambient condition. Moreover, biotransformations allow for the production of new products as well as improve the production of already known molecules (Giri *et al*., 2001). A huge number of studies were performed about biotransformations due to microorganisms as (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 flavour and preservative in foods and beverages.

Biotransformations, bioconversions, biodegradations and fermentations were perceived as technologies able toreplace traditional organic chemistry, due to the enthusiasm enhanced by their potential applications. Then, scientists understood that biotransformations could play above all 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 through traditional methods (complete synthesis) (Csuk *et al.*, 1991). A huge number of microorganisms are used in biotechnological applications, including endophytic ones, which are those who live inside living plants without causing disease.

Endophytes and biotechnological applications

Endophytes are bacteria or fungi living in cells of higher plant tissues, mainly located between the cell wall and membrane. Generally, clear symptoms are not induced. The most interested plant tissues are epidermis and close parenchyma. The physical and physiological relationship between host and endophyte remains really poor studied. 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, 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 mutualism 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 as the plant host, proving the fact that symbiosis could determine also a selective pressure to develop new metabolic pathways for the endophyte.

As an example, *Taxomyces andeanae,* a species-specific endophyte isolated from *Taxus brevifolia* Nutt., produces *in vitro* the alkaloid taxol, a secondary metabolite typical of the plant host. The pharmaceutical and economic importance of taxol is well known; it used in the treatment of breast cancer. 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). There are a lot of studies on biotechnology applications of fungal endophytes that improve biotechnologies in terms of increasing product yields and lower costs. The laccase enzymes, isolated from endophytes belonging to the fungal genus *Monotospora* sp. isolated from the weedy grass *Cynodon dactylon* (L.) Pers., represent a successful example for the paper industry. Laccases remove lignans from cellulose in a particular selective way, leaving cellulose fibers with a 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). Therefore, studying plants with ethnomedical importance offers the possibility to obtain pharmaceutically important chemicals through biotechnological processes. Recent studies have shown that 50% of active substances isolated from endophytic fungi were previously unknown, while for the soil microflora the same index is considerably lower (38%) (Strobel, 2003).

The aims of the research

The present study was focused on the extension of the scientific knowledge related to a group of medicinal plants from Andean and Amazonian Ecuador, focusing on potential health applications, integrated to biotechnological application of endophytic fungi isolated from these plants. The research was performed both in Ecuador (Salesian Polytechnic University, Quito), and in Italy (University of Ferrara, pharmaceutical biology labs). Based on ethnomedical knowledge of indigenous communities, the following plants from the Amazon and Andes regions were selected for this research: a) Amazonian plants: *Piper aduncum* L. (Piperaceae; common name in Ecuador "matico"); *Maytenus macrocarpa* (Ruiz & Pav.) Briq. (Celastraceae, common name "chuchuguazo"), b) Andean plants: *Schinus molle* L. (Anacardiaceae, common name "falsopepe" or "molle"); *Tecoma stans* (L.) Juss. ex Kunth (Bignoniaceae, common name "tepla"); *Myrcianthes hallii* (O. Berg) McVaugh (Myrtaceae, common name "arrayán").

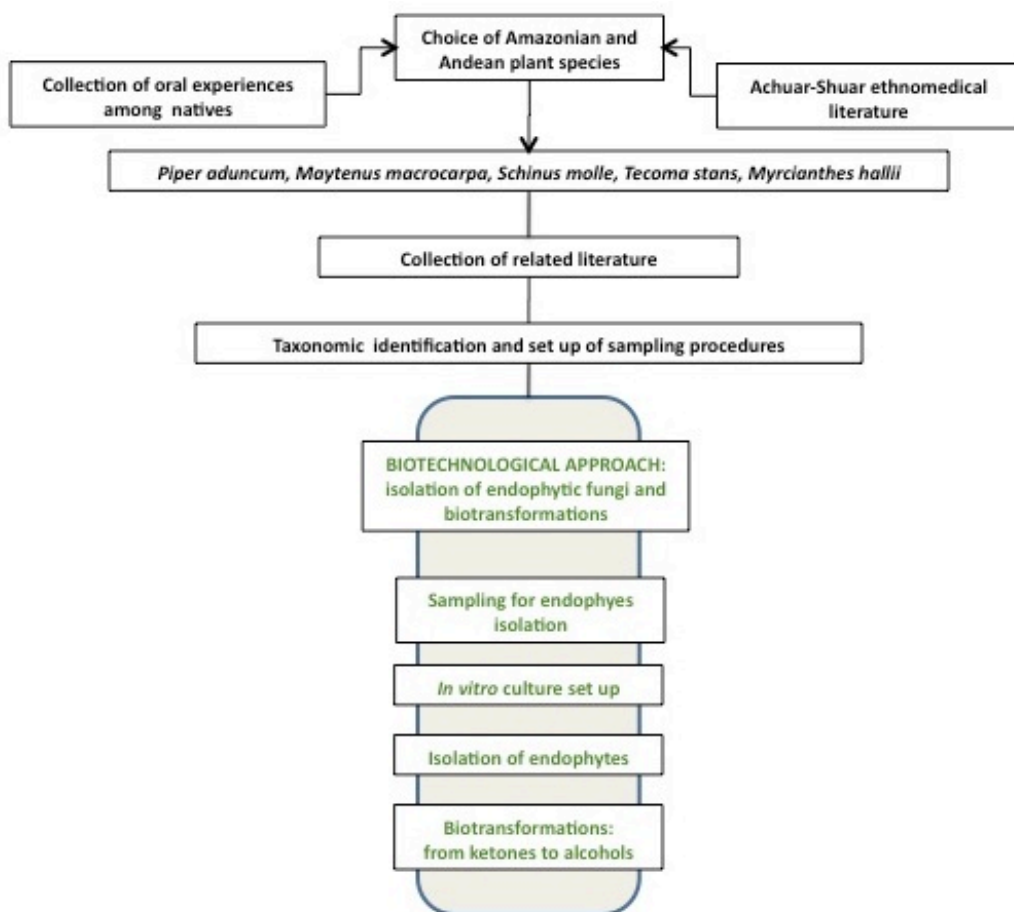The general outline of the research is shown in the diagram below (Figure 1).

Figure 1. General outline of the research.

The research was focused on biotechnology, and especially on (i) checking the presence of endophytic fungi in the selected plants, (ii) isolating and subculturing pure endophytic strains, (iii) checking the biotransformation capacity of the isolated endophytes on pure compounds. The target of the research was to identify and isolate endophytic fungi from Andean and Amazonian plant species of ethnopharmaceutical interest, estimating their biotransformation properties. Then, quality and quantity of biotransformation metabolites have been considered, focusing on oxidation products considered as the result of reaction catalyzed by monooxygenaseenzimes.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 kinds of reactions are very important in bioremediation, in the pure chemical and

pharmaceutical compounds synthesis (Urlacher *et al*., 2006).

humidity of 70% , annual precipitation 500 mm.

Materials and Methods

The research areas were the Amazonian and Andean regions of Ecuador, in particular the provinces of Morona Santiago and Pichincha, as indicated in the map (Figure 2).

The cities of Macas and Quito were chosen for collecting vegetal plant material. These areas have the following environmental conditions: a) Macas, equatorial climate at 1200 m.a.s.l., mean temperature of 22°C, relative humidity 80%, annual precipitation 3000 mm; b) Quito, Andean climate at 2500 m.a.s.l., mean temperature of 15°C, relative

Sampling and taxonomic identification of plants

The choice of the plant species was made starting from the medicinal properties attributed to plants by Amazonian and Andean indigenous communities; the ethnomedical knowledge of Natives was collected by direct interview and bibliographic documents (Kloucek *et al*., 2006). This approach gave rise to the choice of species for which knowledge and traditional uses are still mainly based on oral traditions.

Figure 2. Map of Ecuador showing Morona Santiago and Pichincha provinces.
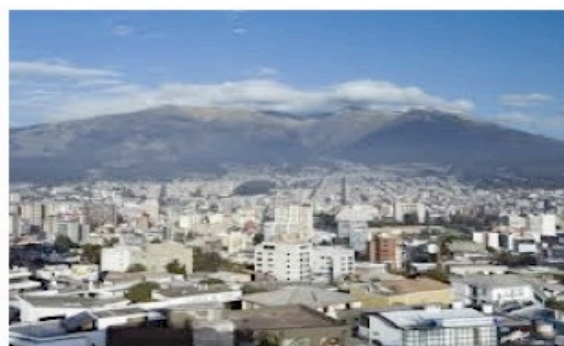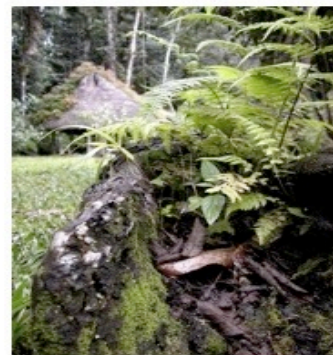
Figure 3. Images of Pichincha and Morona Santiago, the research areas.

Amazonian plant samples were collected in March and April 2007 at Wapu reserve and Sevilla Don Bosco (Morona Santiago province; 2°22''S, 78°08'W, Figure 2) and Andean plants were collected in Quito (Pichincha province; 0°15'S, 78°35'W). Three different areas have been identified (figure 3); samples of plant species were taken from these areas, in order to guarantee scientific significance of the acquiring data. The identification of plant sources was made with the help of expert Natives, while taxonomic identifications were carried out under the supervision of Marco Cerna, M.Sc., an expert in tropical botany at the Salesian Polytechnic University (UPS) and the National Herbarium of Ecuador (QCNE) in Quito.

Phytochemical and biological knowledge of selected plants

Piper aduncum, known in Ecuador as "Matico" (Figura 4), belongs to the Piperaceae family, characterized by tropical plants, usually shrubs and vines. The family includes four main genera and more than 2000 species. From an economic standpoint, the species are important as they provide black pepper (*Piper nigrum* L.), cubeba (*P. cubeba* L. f.) and kawa (*P. methysticum* G. Forst.) from which the natives of the Pacific islands get an alcoholic drink with sedative properties (Schultes, 1995). *Piper aduncum* is a

branched shrub that can reach 5 m in height. It widespread as a native plant throughout the American tropics, including the Carribbean, Mexico, and Central and South America and often acts as a weed colonizing marginal areas of urban centres (Pennington, 2004). *Piper aduncum* contains as functionally compounds terpenes (mono-, sesqui-, di-) and alkaloids. Sevral species of *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).

Figure 4. Habit and infructescence of *Piper aduncum*

Maytenus macrocarpa belongs to the Celastraceae (Figura 5), which includes about 50 genera and 800 species of plants with different habits: trees, shrubs and lianas. *Maytenus* comprises about 200 species in the American and Old World tropics (Schultes, 1995; Pennington *et al.*, 2004). *Maytenus macrocarpa* is a tree up to 25 m tall, well branched, with reddish bark, leaves entire, alternate, leathery, elliptical, light green, with very small axillary flowers (Pennington *et al.*, 2004).

The genus *Maytenus* presents a complex and relevant, but scarcely investigated, phytochemistry. It is rich in 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).

The compounds currently known to be biologically active are alkaloids, saponins, tannins, anthraquinones and glycosides. Extracts from *M. macrocarpa* have antibacterial activity towards *Escherichia coli* and antifungal activity towards *Trichophyton rubrum* (Villacres *et al.*, 1995)

In traditional Amazonian medicine, *M. macrocarpa* is used for production of a bark decoction with anti-inflammatory, antirheumatic and anti-diarrheal properties (Schultes, 1995). Recent investigations assign to the genus analgesic, anti rheumatic, tonic and antianemic activities (Rios *et al.*, 2007).

Figure 5. Leaves and bark of *Maytenus macrocarpa*

Schinus molle (Anacardiaceae) is an evergreen tree native to the inter-Andean valleys of Ecuador and Peru (Figure 6), and widely grown as an ornamental street tree in tropical and warm temperate regions such as Mexico, southern California and Australia. It reaches heights between 3 and 15 m; it is characterized by rather short trunk and fibrous dark brown bark with deep fissures. The branches are slender and pendent. The leaves are compound, 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 a terminal panicle. The fruit is a drupe similar to a pink common peppercorn in size (Barceloux, 2008). Extract of *S. molle* shows antibacterial properties towards *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Acinobacter calcoacetica* and antifungal activity towards *Aspergillus ochraceus, Aspergillus parasiticus, Fusarium*

culmorum, and *Alternaria alternatea* (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).

Figure 6. Habit of *Schinus molle* and its seeds.

Tecoma stans (Bignoniaceae) is an evergreen shrub native to the mountains of Central and South America (Figura 7), and attains 5-7 m in height. The bark has a color ranging from pale brown to gray, roughened with increasing age. The leaves are compound and imparipinnate with 2-5 pairs of leaves; the leaflets are lanceolate, about 10 cm long and with an entire margin.

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 (Pennington *et al*., 2004). Extract of *T. stans* has antibacterial activity against *P. aeruginosa* and other Gram+ and Gram- bacteria (Ramesh *et al*., 2009), as well as antifungal activity towards *Rhizoctonia solani, F. oxysporum, Penicillium expansum, A. parasiticus, Pythium ultimum* among others (Meela *et al*., 2008). An infusion of leaves is known to have diuretic properties and it is also used to treat diabetes, intestinal and stomach problems (Orwa *et al*., 2009).

Figure 7.Habit of *Tecoma stans*, flowers and bark.

Myrcianthes hallii (Myrtaceae), "Arrayán", is native to the Andean forests of Ecuador and Peru (Figure 8). The genus *Myrcianthes* comprises about 30 species of trees extending from Mexico to Chile. The inflorescence is axillary, ramiflorous and sometimes clustered at the shoot apex. Flowers have 4-5 petals and are arranged in a panicle or sometimes solitary; the stamens are numerous. The fruit is a berry crowned by the persistent calyx (Pennington *et al*., 2004). There are no reports regarding the antibacterial activity of *M. hallii*, but the aromatic leaves are used to flavor a traditional beverage in Ecuador known as "colada morada", the fruits are edible and infusions of the leaves have several traditional medicinal uses in the Andes of Ecuador (Jaramillo, 2012).

Figure 8. Flowers of *Myrcianthes hallii.*

Detection, isolation and identification of endophytic fungi

All of the plant samples were obtained from adult plants in the flowering stage. In order to have samples representative of the entire tree, samples were taken at different levels of the plant for each species: upper, middle and at the base of the stem. The plant samples included leaves, petioles, young twigs

and pieces of bark. The plant samples for endophyte isolation were immediately wrapped in a moistened paper and then immediately transported to the laboratory and stored at 4 °C. Within 48 hours all the collected material was subjected to the isolation of endophytic fungi.

The plant material was previously washed with running water and then dried. The plant samples were sanitized according to the protocol reported by Andreotti (2004). This operation eliminates the microorganisms on the external surface of the plant samples, without damaging the possible endophytes inside the plant parenchyma. The protocol consists in washing the plant material in 70% ethanol (1 min), then, dipping it in 5% sodium hypochlorite solution (5 min) followed by a final rinse in sterile distilled water (10 min). The entire operation is performed under a laminar flow hood using sterile equipment. The samples were cut into fragments of about 1cm^2 for leaves and bark, and about 2 cm^2 for branches and stems. The sample fragments were placed on the agar surface, previously autoclaved and with addition of 200mg/l of the antibiotic chloramphenicol. Four different types of culture media were used in order to isolate as many strains of endophytes as possible: Malt Agar (DIFCO), Malt Extract Agar (DIFCO) and Soy Peptone (DIFCO), Mycosel Agar (Becton Dickinson). In practice, a different

composition in culture medium generally contributes to easily noticeable differences in fungal morphology (Andreotti, 2004, Moreno 2010). A total of 64 x 3 Petri dishes were prepared for each species: 4 plates for each kind of medium, 4 plates for each sample (leaves, bark, stems, branches); each experiment was carried out in triplicate. After several days of incubation at room temperature, fungal hyphae were visible from the edge of the samples. Hyphal samples were removed and transferred to PDA dishes with the aim to obtain pure culture of each fungus. The strains with similar macroscopic characteristics – i.e. color of the mycelium and of the medium, the shape of mycelia margins – were grouped and coded. 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 taxonomic identifications of the most promising strains for biotransformations were not completed as this article went to press. However, suggestions about their classification were made on the basis of our previous experience (Andreotti, 2004; Moreno 2010).

Biotransformation activity of fungal strains

The isolated endophytes were 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 assess biotransformations were chosen for their importance as molecular patterns similar to compounds of pharmaceutical interest (Masood *et al.*, 2010; Iwaki *et al.*, 2006).

Seven different substrates were tested (Table 1): 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).

The endophytes were inoculated in PDB liquid medium (Potato Dextrose Broth, Liofilchem srl, Italy), sampling a portion of mycelium, previouslysuspended in tubes containing 2 ml of sterile water. The fungi samples were then transferred to bioreactors (20 ml sterile flasks) to assess their biotransformation capacities. Sterile flasks (20 ml) were prepared with a quantity of liquid medium corresponding to a 1:5 ratio with respect to the bioreactor volume (Andreotti, 2004; Moreno, 2010). After inoculation, 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 a globular shape. At this step the compounds to assess for biotransformations were added to the bioreactors. To the previously prepared culture broth, ketone solutions were added, obtained by dissolving 0,1 mg of ketone compounds in 1 ml DMSO (dimethylsulfoxide).
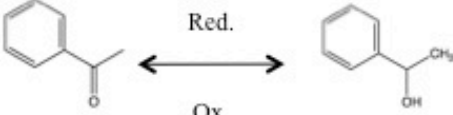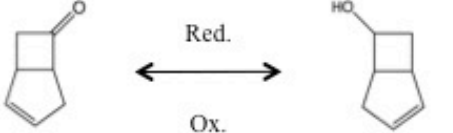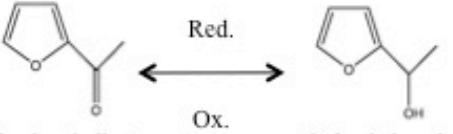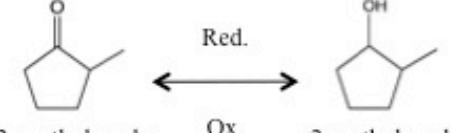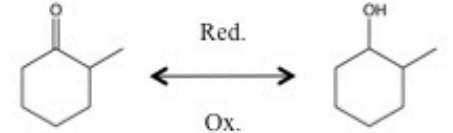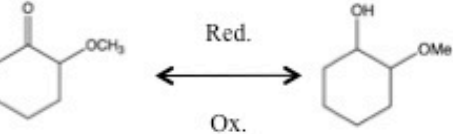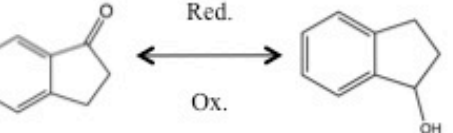
An amount of 0.2 ml of ketone solution was added to 20 ml of culture broth, for each group of endophyte. One ml of culture broth was sampled every 1, 3, 7, 10 days after inoculation to monitor biotransformations.

Each biotransformation sample was extracted immediately after inoculation 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 the ethyl acetate solvent, related in polarity to the expected alcoholic products of the substrate reduction.

Figure 9. Flasks with liquid cultures of endophytic fungi after 7 days of culturing. Note the typical globular shape.

Table 1. Ketones subjected to biotranformation and pharmaceutical selection criteria considered.

Acetophenone — Red./Ox. — Phenylethanol		The alcohol phenylethanol, obtained by the reduction of acetophenone, is used as an antiseptic, disinfectant, antimicrobial and preservatives (Masood *et al.*, 2010)
Bicycloheptenone — Red./Ox. — Bicycloheptenol		The bicycloheptenone lactones are chiral compounds with a key role for the synthesis of prostaglandins (Alphand *et al.*, 1989)
2-furylmethylketone — Red./Ox. — 2–furylethanol		The furylethanol (and its derivatives) is versatile precursor for the synthesis of natural products such as carbohydrates, alkaloids and pheromones (Kamiska *et al.*, 1996)
2–methyl cyclo pentanone — Red./Ox. — 2-methyl cyclo pentanol		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)
2–methyl cyclo hexanone — Red./Ox. — 2-methyl cyclo hexanol		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).
2-methoxy cyclo hexanone — Red./Ox. — 2-methoxy cyclo hexanol		The alcohol 2-methoxy-cyclohexanol is an important intermediate in the synthesis of chiral β-lactam antibiotics, such as penicillins (Stead *et al.*, 1996)
1-indanone — Red./Ox. — 1-indanol		The indanone is an important intermediate for the synthesis of SSRIs (Selective Serotonin Reuptake Inhibitors), compounds used for treatment of psychiatric diseases (Bõs *et al.*,1997)

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 was employed. The organic extracts of the other substrates were directly analyzed by GC. The products on TLC were assessed and detected by UV light or by spraying phosphomolybdic solution. Once the presence of biotransformed products was verified, the analyses were carried out 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; 25 m x 0,25 mm). 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 250 °C and 220 °C respectively.

Results and Discussion
Endophytic fungi

A total of 364 fungal strains were isolated from aerial parts of adult plants of *Piper aduncum*, *Maytenus macrocarpa*, *Schinus molle*, *Tecoma stans*, and *Myrcianthes hallii*, Amazonian and Andean plants known for their ethnomedical relevance. Each strain was coded with reference to its macroscopic aspect (color, colony border, texture mycelia, exudates, changes in medium color during culturing).

Then groups were established according to the similar features of the strains and coded with an alphanumerical code.

After one month of incubation, 80% of the plant samples on the plates showed the presence of emerging endophytes (Figure 10). An average of 2 fungal mycelia – easily noticeable by the different macroscopic morphology - could be detected for each positive plate.

All the isolated strains were employed to test biotransformation on pure chemicals chosen in light of their chemical structure similar to pharmaceutical drug intermediates. The taxonomic identification of the endophytes was carried out only for the strains that were most efficient in term of biotransformation capabilities.

Table 2. Plant sources, number of isolated fungal strains and code assigned to strain group.

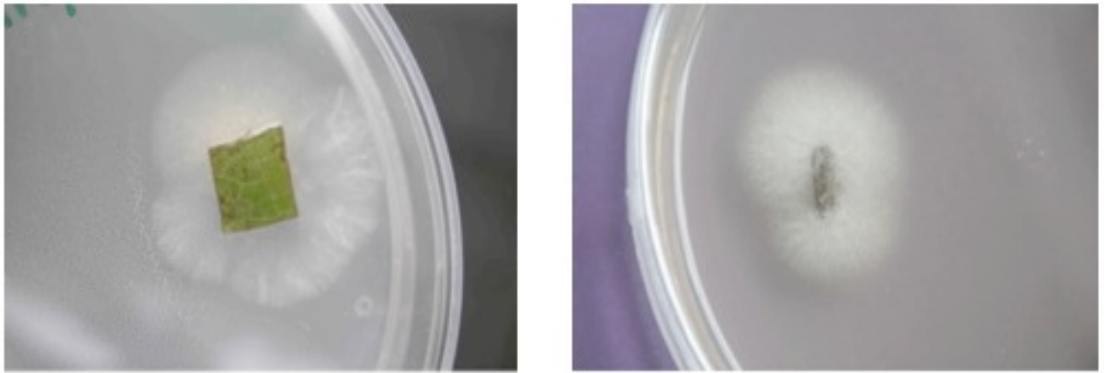| Plant Source	No. of isolated fungal strains	Code
Piper aduncum	116	from EC01 to EC65
Maytenus macrocarpa	127	
Schinus molle	28	from FE1 to FE110
Tecoma stans	32	
Myrcianthes hallii	61	
TOTAL	364	

Figure 10. Emerging fungal hyphae from plant tissues on agar medium.
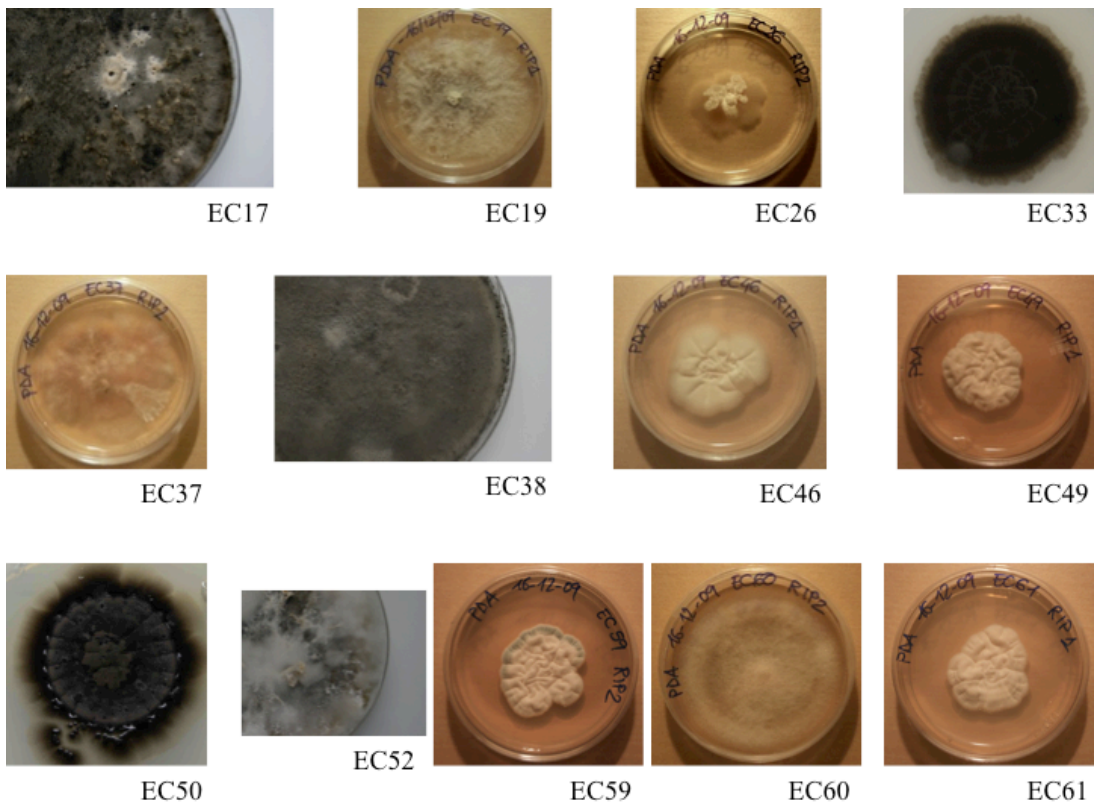

Figure 11. Selection of fungal endophytes isolated from plants with traditional ethnobotanical uses in Ecuador.

These strains were sent to the "Fungal Biodiversity Centre" at the Centraal Bureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands for identification; this work is still in progress as this article goes to press. However, a preliminary identification based on morphological aspects was carried out by the research group. In particular the preliminary determinations

indicate that EC19 and EC37 are in the genus *Fusarium*, while EC46, EC49, EC59 and EC61 are *Penicillium*.

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

Evaluation of biotransformation activity

Table 3 lists the fungal strains which performed the most relevant biotransformations, with descriptions of the macromorphology of the fungal colonies.

Endophytes isolated from *P. aduncum* and *M. macrocarpa* showed the most relevant results in terms of biotransformation capabilities. In Table 4 and Table 5 are summarized the most significant 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.

The strains EC17, EC19, EC37, EC38, EC46, EC49, EC50, EC60, EC61, FE40 and FE86 gave the best results in terms of kind of products, yield and enantiomeric excess (ee).

Figure 12. Fungal mycelia in liquid PDB culture medium.
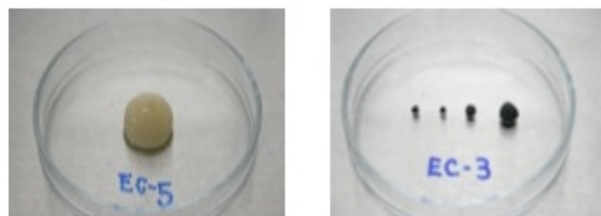
Figure 13. Detail of globular shaped mycelium grown in stirred PDBliquid medium.

Table 3. Vegetal source, plant part used for endophyte isolation, culture medium and macroscopic morphology of the fungal colonies that performed the most relevant biotransformations.

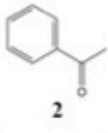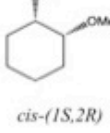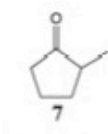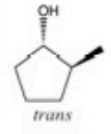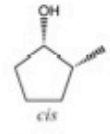| Strain	Vegetal source	Part plant	Medium*	Colony macroscopical morphology
EC17	*P. aduncum*	S	MEA	Bright pink mycelium in the center and gray-black at the colony border, cotton texture, colourless exudate. Reverse black coloured.
EC19	*P. aduncum*	S	MEA	Bright gray mycelium with circular shaped growth,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

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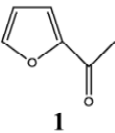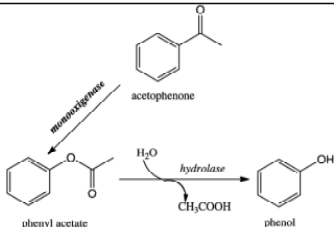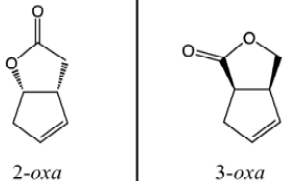L=leave
B=bark
S=stem
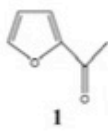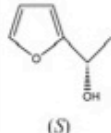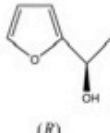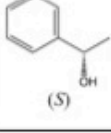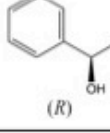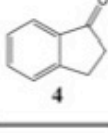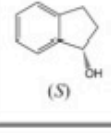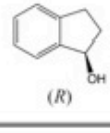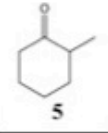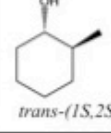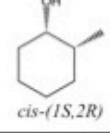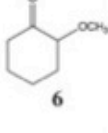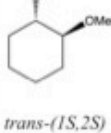
MEA= Malt Extract Agar
MA=Malt Agar
MYC= Mycosel Agar
TS=soybean peptone

Table 4. Fungal strains, biotransformation products, yields, enantiomeric excesses (ee) 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 (1S,5R,6S)	exo (1R,5S,6S)	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

Table 5. Fungal strains, biotransformation products, yields, enantiomeric excesses (ee) 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

Among all the strains, 5 of them performed biotransformations on acetophenone to (S)-1-phenylethanol, with important yields (78-97%) and enantiomeric excess (78-100%). Three strains gave also phenols, probably by enzymatic reactions (Baeyer-Villiger oxidations). 15 fungal strains gave the 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 from (+/-)-*cis*-bicyclo[3.2.0]hept-2-en-6-one, probably as result of monooxygenase activation.

Concerning the Baeyer-Villiger oxidation, on *cis*-bicyclo[3.2.0]hept-2-n-6-one, EC17, EC33, EC49 andEC61 showed the best results with total yield and enantiomericexcess (Table 5). The low production of alcohols indicate that fungal strains preferably catalyze oxidation reactions,in particular the Bayer-Villiger reaction, 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.

Final Note: Ethical Implications

The scientific publications derived from this kind of research profile – from ethnomedicine to laboratory - could be one of the starting points for a public policy of protection for indigenous peoples and rural communities as well as natural habitats in the Amazon and Andes region, against bio-piracy and misappropriation of natural sources and related knowledge by third parties. On the other hand, if traditional ethnomedical knowledge provides science with opportunities to find new sources for solutions to human

health needs, new chemicals for new drugs to treat old and new diseases, the research protocols must address the ethical implications of these values. The present research is one example of an attempt to use this approach based on traditional ethnobotanical knowledge.

Literature Cited

Alphand, V., A. Archelas & R. Furstoss. 1989. One_step synthesis of a pivotal prostaglandin chiral synthon via highly enantioselective microbiological Baeyer-Villiger type reaction. Tetrahedron Letters 30 (28): 3663-3664.

Andreotti, E. A.A. 2002-2004. Funghi endofiti come potenziale strumento di individuazione di molecole di interesse farmaceutico. Tesi di dottorato di ricerca in biocatalisi applicata e microbiologica industriale. Dipartimento delle Risorse Naturali e Culturali. Universitá di Ferrara.

Barceloux, D. G. 2008. Medical Toxicology of Natural Substances, Food, Fungi, Medicinal Herbs, Plants and venomous Animals. WILEY

Bastos Borges, K., W. De Souza Borges, M. Tallarico Pupo & P. Sueli Bonato. 2007. Endophytic fungi as models for the stereoselective biotransformation of thioridazine. Applied Microbiology Biotechnology 77: 669-674.

Bös, M., F. Jenck, J.R. Martin, J. Moreau, A.J. Sleight, J. Wichmann & U. Widmer. 1997. Novel agonists of 5HT2C receptors. J. Med. Chem 40: 2762-2769.

Csuk, R. & B. I. Glanzer. 1991. Baker's yeast mediated transformations in organic chemistry. Chemical Review 91: 49-97.

Dikshit A., A.A. Naqvi & A. Husain. 1986. *Schinus molle*: a new source of natural fungitoxicant, Applicated Environmental Microbiology 51 (5): 1085-1088.

Fukui, H., Y. Tsuchiya, K. Fujita, T. Nakagawa, H. Koshino & T. Nakata. 1997. Synthesis and biological activity of artificial analogs of mycalamide A. Bioorganic & Medicinal Chemistry Letters 7 (16): 2081-2086.

Giri A., V. Dhingra, C.C. Giri, A. Singh, O.P. Ward & M.L. Narasu. 2001. Biotransformations using plant cells, organ, culture and enzyme systems: current trends and future prospects. Biotechnology Advances 19: 175-199.

Guerrini A., G. Sacchetti, D. Rossi, G. Paganetto, M. Muzzoli, E. Andreotti, M. Tognolini, M. Maldonado & R. Bruni. 2009. Bioactivities of *Piper aduncum* L. and *Piper obliquum* Ruiz & Pavon (*Piperaceae*) essential oils from Eastern Ecuador. Environmental Toxicology and Pharmacology 27: 39-49.

Gundidza M. 1993. Antimicrobial activity of essential oil from *Schinus molle* Linn. The Central African Journal of Medicine 39(11): 231-234.

Iwaki, H., S. Wang, S. Grosse, H. Bergeron, A. Nagahashi, J. Lertvorachon, J. Yang, Y. Konishi, Y. Hasegawa & P.C.K. Lau. 2006. Pseudomonad

cyclopentadecanone monooxygenase displaying an uncommon spectrum of Baeyer-Villiger oxidation of cyclo ketones. Applied and Environmental Microbiology 72: 2707-2720.

Kaminska, J., I. Gornicka, M. Sikora & J. Gora. 1996. Preparation of homochiral (S)- and (R)-1-(2-furyl)ethanols by lipase-catalyzed transesterification. Tetrahedron Asymmetry 7 (3): 907-910.

Kloucek, P., B. Svobodova, Z. Polesny, I. Langrova, S. Smrcek & L. Kokoska. 2006. Antimicrobial activity of some medicinal barks used in Peruvian Amazon. Journal of Ethnopharmacology 111 (2): 427-429.

Masood, A.W., S. Kaul, M.K. Dhar & K.L. Dhar. 2010. GC-MS analysis reveals production of 2-phenylethanol from *Aspergillus niger* endophytic in rose. Journal of Basic Microbiology 50: 110-114.

Meela, M., L. Mdee & J.N. Eloff. 2008. Prospects for use of alien invasive weed extracts against fungal phytopathogenes. African Journal of Traditional, Complementary and Alternative medicines, Abstract of the world congress on medicinal and aromatic plants, Cape Town

Mittermeier, R.A., P.R. Gil & G.G. Mittermeier. 1997. Megadiversity: Earth's Biologically Wealthiest Nations. Conservation International, Cemex, Mexico.

Moreno Rueda M.G. A. A. 2007/2009. Biotrasformazioni di terpeni e oli essenziali con batteri e funghi isolati da frutti del genere *Citrus* della foresta amazzonica (Ecuador). Tesi di dottorato di ricerca in Biochimica, Biologia molecolare e Biotecnologie, Ciclo XXII.

Orwa, C., A. Mutua, R. Kindt, R. Jamnadass & A. Simons. 2009. Agroforestry database: a tree reference and selection guide, version 4.0. Online at: http://www.worldagroforestry.org/af/treedb/ (accessed October 2012).

Piacente, S., L.C. Dos Santos, N. Mahmood & C. Pizza. 2006. Triterpenes from *Maytenus macrocarpa* an evaluation on their anti-HIV activity. Natural Product Communications 1 (12): 1073-1078.

Rai, M. & D. Mares. 2003. Plant-derived Antimycotics. Food Products Press

Ramesh, T., V. Anusha & A.R. Kumar. 2009. Antibacterial activity of methanolic extract of roots of *Tecoma stans*. International Journal of Chemical Sciences 7(1): 6-8.

Rios, M., M. J. Koziol, H. B. Pedersen & B. Granda. 2007. Useful Plants of Ecuador. Ediciones Abya Ayala-Quito-Ecuador

Romagnoli, C. & G. Sacchetti. 2003. A new technique for the evaluation of antifungal activity of an alcohol extract of *Eugenia caryophyllata* Thunberg on *Penicillium digitatum*. In: Plant-Derived antimycotics, Current Trends and future prospects. Rai M., Mares D. Eds. Food Products Press, An Imprint of The Haworth Press, Inc., New York

Schultes, R. E. & R. F. Raffauf. 1995. The Healing Forest: Medicinal and Toxic Plants of Northwest Amazonia. Dioscorides Press, Portland, Oregon, EE.UU.

Shirota, O., S. Sekita, M. Satake, H. Morita, K. Takeya & H. Itokawa. 2004. Two new sesquiterpene pyridine alkaloids from *Maytenus chuchuhuasca*. Heterocycles 63-68.

Stead, P., H. Marley, M. Mahmoudian, G. Webb, D. Noble, Y. To Ip, E. Piga, T. Rossi, S. Roberts & M.J. Dawson. 1996. Efficient procedures for the large-scale preparation of (1S,2S)-trans-2-methoxycyclohexanol, a key chiral intermediate in the synthesis of tricyclic β-lactam antibiotics. Tetrahedron Asymmetry 7(8): 2247-2250.

Strobel, G. A. 2003. Endophytes as source of bioactive products. Microbes and Infection 5: 535-544.

Suryanarayanan, T.S., N. Thirunavukkarasu, M. B. Govindarajulu, F. Sasse, R. Jansen & T.S. Murali. 2009. Fungal endophytes and bioprospecting. Fungal Biology Reviews 23: 9-19.

Tan, R. X. & W. X. Zou. 2001. Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18: 448-459.

Urlacher, V.B. & S. Eiben. 2006. Cytochrome P450 monooxygenases: Perspectives for synthetics application. Elsevier

Wang, J.W., J. H. Wu, W. Y. Huang & R. X. Tan. 2006. Laccas production by *Monotospora* sp., an endophytic fungus in *Cynodon dactylon*. Bioresources Technology 97 (5): 786-789.