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Chestnut green waste "in situ" composting for sustainable forest management: biomass composting monitoring, microbiota dynamics, and plant defence induction.

Ph.D. Dissertation
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We do not inherit the Earth from our ancestors, we borrow it from our children.

Native American

Live as if you were to die tomorrow. Learn as if you were to live forever

M. K. Gandhi

Ad Angelo e ai mie figli: Antonio e Angelica.

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Chapter 1 Introduction

1.1 The Chestnut

The *Castanea* Mill. belongs to the Eucastanum family, which also includes *Fagus* and *Quercus*. The chestnut tree is characterized by a majestic and vigorous aspect, easily reaching heights of 40 m and 1,5-2,0 m of diameter. It is possible to find trees, with a circumference up to 6-7 m, recognized as "monumental trees". *Castanea* has a very wide distribution area worldwide, where climatic conditions permit it is found in small distribution areas of Morocco, Algeria and Tunisia. It is present also in the Canary Islands, (De Magan e*t al.,* 1997), Syria and Lebanon. However the chestnut production in the world, is concentrated in Asia and Europe. The largest producer in Asia is South Korea, followed by China and Japan. In Europe, the chestnut is present in mountain region with temperate warm clime. It is cultivated between 300 up to 1200 m above sea level. Italy is the first producer of fruit followed by Turkey, Portugal, Spain, Russia and France (FAO 2000). The distribution area in Italy is influenced more by the altitude then by the latitude. Here, chestnut grows above the area of the Mediterranean evergreen scrub belt, where the climate is typified by colder winters; in the higher belt, chestnuts are replaced by beech (Pavari 1931; De Philippis 1956). Between the XI and XV centuries, chestnut cultivation grew significantly in Piedmont, Liguria, Tuscany, Campania and Calabria, but also in Lombardy, Veneto, Emilia-Romagna, Umbria and Lazio. Throughout the nineteenth century, entire populations from mountain and hilly areas rely for their survival only on chestnut products. Still, today, chestnut represents a considerable economic and social resource in many inland territories. In fact it plays a vital role, not only for the fruit and timber productions, but, above all, for the function of environmental control and hydro-geological protection.

Chestnut species present extremely strong basal shoots, this implicate fast differentiation of new plants. Therefore, consequent upon the abandonment,

chestnut fruit cultivation, can quickly evolve to other type of forest. The chestnut is especially characteristic of the associations of *Quercus*-*Castanetum*, present in the lower horizons, going upper *Quercus*-*Ostryetum* and finally *Fagetum*; numerous variants and geographic strip are described (Fenaroli 1946; Ciampi, 1956).

The chestnut can combine with different categories of undergrowth. Paci (1992) distinguishes the various stripes of the undergrowth as indicators of different human activity and type of soil. In particular grassland (*Agrostis capillaris, Molinia coerulea*, etc.), is typical of well maintained chestnut. While, chestnut tree associate with moorland (heather and ginestro) is typical of abandon. A detailed description of the chestnut habitats typical refers to classification EUNIS2 (Biondi *et al*., 2009). The woods and forests of *Castanea sativa*, including fruit crops now naturalized, are divided into following subtypes: *C. sativa* Southern Alps and Liguria Alps forest; *C. sativa* Italian-Sicilian hilly forest; *C. sativa* Sardinian-courses. This habitat include mixed forests with naturalized undergrowth. According to the latest Italian National Forests Inventory (INFC, 2005), chestnut in Italy covers 788.408 ha (9.2% area classified as high forest), in addition to 3.378 classified as other wooded lands. Chestnut represents 7.5% of the Italian forest, above all Piedmont, 21.4%, than Tuscany, Liguria, Lombardy, Calabria and Campania. The regions with major amount of fruit trees are Campania (35640 ha) and Tuscany (33964 ha). The majority of chestnut tree is conduct as root-plants (75.2%), mostly (50.8%) in the adult stage are grown in to monoplane vertical structure. Chestnut trees hosts microhabitats representing 41.5% of the woods biodiversity, compared to dry stone walls 27.4%, dens and caves 20.7%, water surface 15.6% and clearings 14% (INFC, 2005). Woods and forests contribute to soil preservation and hydro-geological protection, limiting risk of landslide, mudslides and avalanches. Thanks to their ability to reduce CO_2, woods and forest harbor varieties, specimen and native species, as well as contribute to the beauty of the landscape colors: "quality irreplaceable of the life" (art. 1 L. 353/2000).

1.2 CO_2 and greenhouse effect

The greenhouse effect is a unique natural phenomenon, which let the planet maintain and guarantees temperature conditions compatible with life. The energy that the planet receives from the sun during the day is partially filtered by the atmosphere and the residual part absorbed. During the night the land tends to re-radiate to the universe the energy received. This irradiation is opposed by a series of atmospheric spheres composed of the so called greenhouse-gases; such as water vapor, carbon dioxide, methane, ozone, present around the Earth, as a protective "glass", that tend to keep part of energy. This avoids the menace of extreme cooling (Lo Giudice *et al.,* 2007). The greenhouse effect, however, would become too heavy for the production of anthropogenic "climate-altering gases". As a result of fossil fuels utilize, the current rate of CO_2 atmospheric accumulation, seems to have no similarities in previous eras. Over the past 2000 years, as probably in the next 100 years, the concentration could double (Houghton *et al*., 2001; Keeling & Whorf, 2005). In the XX century - according to IPCC report (Intergovernmental Panel on Climate Change), the temperature increased by 0.6 °C. If greenhouse gases releases will not decrease, the planet, within the century, is destined to go through a catastrophic scenery due to a considerable increase in temperature (1.5 °C - 2.8 °C), with sea levels rising between 28-43 cm and reduction of polar ice (Lo Giudice *et al.,* 2007). Merely the CO_2 is calculated to influence the global temperature between 1 to 6 °C, depending on the different scenarios (Houghton *et al*., 2001; IPCC, 2007b). This gas, inert, colorless and odorless, naturally is present in the atmosphere. The amount of carbon on Earth is not unlimited and, similarly to water, carbon has its cycle, the carbon cycle (fig.1). Between the biosphere and the oceans and the biosphere and the atmosphere there is an intense exchange of CO_2. Under natural conditions this exchange is balanced. The humankind, however, has altered this balance as a result of the combustion of fossil fuels and deforestation, releasing supplementary CO_2 into the atmosphere. Through photosynthesis process, plants accumulate CO_2 in their own biomass by way of the

energy rich carbon compounds. Humans, animals and microorganisms, by breathing those molecules, produce CO_2. The CO_2 is released additionally through the destruction of biomass, for example by pests, forest fire, and forest cutting. In addition, fossil fuel combustion, facilitate the carbon cycle imbalance. Since the beginning of the industrialization, CO_2 concentration in the atmosphere has increased by about 30% (Nabuurs *et al*., 2007). A fourth of the extra CO_2 released is absorbed by plants and another quarter from the oceans; while, half remains into the atmosphere (Nabuurs *et al*., 2007).

Forests absorb CO_2 integrating carbon in their biomass in different manners:

in leaves, for few months;

in branches and shoots, for several years;

in trunks, for decades and centuries;

in soil, for centuries and millennia.

Ability of plants to fix carbon, for longer or shorter periods in alive or dead biomass and in soil, gives to forests and to the forestry sector, a central role in the strategies for climate change mitigation, both for the reduction of greenhouse gases emission, and for carbon stocking (Nabuurs *et al*., 2007). As a rule, forests act as carbon sinks when the balance between the carbon dioxide absorbed and emitted is positive. It becomes a source when, a disorder, for example a burning, alter the equilibrium.

The strategies for mitigation of climate change related to the forest sector, therefore tend to emphasize the reservoir role, with different means, as follows:

- forest protection and their expansion through forestation and reforestation;
- maintaining and increasing the biomass density by controlling biotic and abiotic damage (insects, pathogens, weather-climate agents, fire, etc.);
- production of goods with long life cycles, such as beam, fixtures, flooring and furniture.

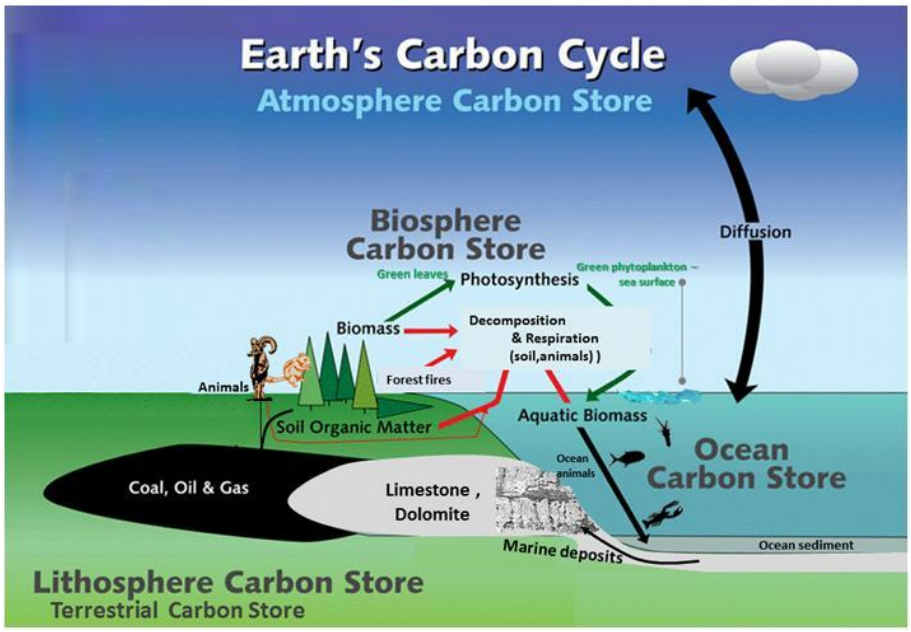

Figure 1- Earth's Carbon Cycle (www.climate-change-knowledge.org)

1.3 The role of carbon and organic matter in soil

In recent years the international scientific community is facing the issue of the role played by terrestrial ecosystems, their management, and the use of soil for containing the increase of atmospheric CO_2. Soil is the third largest carbon reservoir, it contains twice the amount of carbon in the atmosphere (Franzluebbers, 2005). This thin layer is a living part of the Earth through which lithosphere, hydrosphere, atmosphere and biosphere interact. It plays a unique role in the life of our planet because, besides adjusting the carbon cycle, regulates water, phosphorus and nitrogen cycles. The organic carbon, constitutes about 60% of the soil organic matter, plays a positive role on many soil properties and focuses, in general, in the first layers of the soil. The organic matter facilitates the aggregation of soil particles and their stability; and reduce erosion, compaction, and surface crust formation. The organic matter increases soil fertility and buffering capacities; improves microbial activity and nutrients availability such as nitrogen and phosphorus (ARPAV, 2006). The raise of organic carbon in the soil is essential to fight erosion, increase of soil fertility, and decrease the CO_2 in the atmosphere (ARPA EMR, 2004). Loss of soil organic matter is due mainly to carbon fluxes

from the soil to the atmosphere, determined by mineralization and fires (IPCC, 2000a). Lower concentrations are also consequences of intensive agricultural practices (ARPAV, 2006). Carbon content is furthermore strongly related to soil destination changes; in fact it increases significantly from arable to woody crops.

The best agricultural practices to increase carbon sink capability are those that increase biomass. This can be achieved with the following activities: conservation practices that reduce the loss of material by erosion; maintenance of organic residues to increase the coverage and amounts organic; adopt a no-till farming, conservation tillage, which improves soil structure and increase the water infiltration capacity; the growth of cover crops for increase the biodiversity and residues quality. An increase of biodiversity is equivalent to a high capacity carbon absorption and storage. The use of compost increases storage capacity of soil nutrients, equivalent to an increase of biomass production. Several years of virtuous management practices, substantially improve the carbon sink ability.

1.4 General state of atmospheric emissions and reduction targets

The increase in risks related to the problem of global warming on the economic activities and the growing sensitivity towards environmental issues, have motivated several countries to the definition of joint actions to combat climate change. In 1997 was signed the first multilateral agreement, fixing stringent and concrete commitments to achieve the objectives set by the United Nations Framework Convention on Climate Change (UNFCCC), stabilizing the amount of greenhouse gas (GHG) emitted into the atmosphere by human activities. This agreement, known as Kyoto Protocol, was signed by over 160 countries, in February 2005. Objective was the reduction of the emissions of the six greenhouse gases considered the main agents of climate change: carbon dioxide, nitrous oxide, methane, hydrofluorocarbons, perfluorocarbons, and hexafluoride sulfur.

Carbon dioxide absorption one of the major issues of the Protocol by means of:

- forests protection and expansion;

- energy efficiency improvement;
- market paradoxes adjustment;
- promotion of sustainable and extensive agriculture;
- reduction of emissions related to transport;
- promotion, dissemination and integration of the actions.

To support the Protocol implementation and reduction of the related costs were introduced flexibility mechanisms:

- emission trading, countries can sell or purchase credits or surplus from other countries;
- joint implementation, the possibility to achieve the targets set with a different distribution of obligations "Emission Reduction Units" (ERUs) carried out through specific projects to reduce emissions;
- clean development mechanisms, privates or governments providing assistance to implement projects that reduce GHG parties, can get "Certified Emission Reductions" (CERs).

The directive CE n.87/2003 (13 October 2003) of the European Parliament establishes a system for the exchange of Emission Trading System shares (ETS), an European carbon market.

The European Union in 2013 adopted Climate-Energy 20-20-20 strategy, with the obligation to reduce unilaterally its emissions by 20% by 2020 (Agende 21 Locali per Kyoto, 2009). A roadmap plans a progressive reduction of carbon and to engage in the reducing greenhouse gas emissions by 80% by 2050.

The Climatic Energy Package provides by 2020:

- 20% reduction of greenhouse gas emissions, compared to 1990 levels;
- 20% increase of renewable energy, including 10% biofuel;
- 20% increase in energy efficiency.

In 1998 the Italian Minister of Environment instituted an inter-ministerial working group, CIPE, with the task of studying and proposing a multidisciplinary action program to reduce emissions of greenhouse gases. The CIPE file fixes objective

and actions needed for 6.5% reduction of greenhouse gases controlled by the Kyoto Protocol by 2012. (Ronchi *et al.,* 2013).

The group was inspired by the definition of the actions to be taken at national importance, by the following criteria:

- reduction and absorption of greenhouse gases from agricultural and forest production;
- improved energy efficiency of the energy system, industry, and mobility of people and goods;
- encourage the development of innovative technologies, low emission and renewable sources;
- promote programs of absorption and fixation of atmospheric carbon through stable forms of increased vegetation cover, inside and outside agriculture, and increase humus in soil.

In light of these criteria, the CIPE has identified reduction emission objectives in six actions:

- increased thermoelectric efficiency (D. Lgs. n. 79/99);
- reduction of energy consumption in the transport sector;
- production of energy from renewable sources (solar, wind, biomass);
- reduction of energy consumption in the industrial /residential;
- reducing emissions in non-energy sectors, in particular; the chemical industry, livestock farming and waste management;
- reducing CO_2 emissions forest.

Italy ratified the Kyoto Protocol in 1 June 2002 n. 120 (Lumicisi *et al*., 2007). (Official Bulletin of the Piemonte region. 2004). The Minister of Environment expressed the political will to organize a "National Register of to Carbon Sinks Agro-Forestry", with decree of the 2/2/2005, (Lumicisi *et al.,* 2007).The register should monitor all soils potentially interested by forestry activities, on the Italian territory (Ciccarese *et al*., 2006). CIPE on March 8th, 2013 approved the National

Action Plan for the greenhouse gases emission reduction, the program responds to specific EU and international commitments within the programming period 2013-2020. For Italy, the reduction target is 13%, compared to 2005 levels, to be achieved by 2020. (Legge 16 aprile 1987, n. 183)

1.5 Composting

Composting process is a main technology for biomass recovery and carbon dioxide reduction in the atmosphere. In recent years a strong interest is rising for potential role of the composting in the control of the greenhouse effect and climate change.

Composting is defined as the natural aerobic process, through which environmental micro-organisms (bacteria, fungi, actinomycetes, algae and protozoa) decompose organic matter contained in plant and animal residues (Tuomela *et al.,* 2000; Maynard, 2000). From this process, the microorganisms obtain energy necessary for the maintenance of their metabolic functions, with liberation of water, carbon dioxide and heat.

This natural process occurs spontaneously, but it develops slowly. In piloted conditions, with a suitably standardized inoculum, degradation and transformation of the organic mass results considerably more efficient. In a controlled composting process it is important to create and maintain peculiar environmental conditions.

Microorganisms optimum condition depends on a few essential factors:

- Temperature;
- Oxygen;
- Porosity;
- Moisture;
- C/N ratio;
- Nutrients availability;
- pH.

Performance monitoring is especially relevant in the early stages of the process, and during the stages characterized by important microbial activity. Composting

process consist in the biological stabilization of organic waste with production of rich in: humic substances, nutrients, hygienically safe, with structuring power for soils (Silvestri, 1997). The process can be divided into two phases: first, the decomposition of rapidly rotting substances (bio-oxidation), and a second phase of transformation and synthesis of humic substances (maturation). In the first phase are oxidized soluble sugars, amino acids, proteins and organic acids with production of CO_2, water, simple sugars, amino acids and phytotoxic substances. This highly exothermic reaction, combined with the action of thermopiles bacteria, ensures the mass sanitation (Hoitink *et al*., 1996a). The process follows in a slow metabolic degradation, microbial populations attack complex carbon matter such as starch, cellulose, lignin and microorganisms vestiges. At this point, mesophilic microbial flora, bacteria, but also fungi and actinomycetes, occurs and implicate a decrease in oxygen demand. The intermediates phytotoxic compounds are transformed into CO_2, H_2O and other simple molecules, and new more complex molecules such as humic and fulvic acids are shaped (De Bertoldi *et al.*, 1985; Tilston *et al.*, 2002). Humus components confer useful characteristics to the soil: more stable materials and available nutrients, chelating properties (Chiumenti R. & Chiumenti A., 2001). A fundamental role in the humification process is played by actinomycetes, they are, in fact, responsible for the production of aromatic substances such as geosmin (especially for the compost produced by the treatment of wood and litter green), a component that confers the typical odor of forest to the finished product (Nappi, 1998). The organic matter, during the process, is also colonized by small invertebrates such as springtails, mites and centipedes that contribute to the shredding, mixing and mineralization of organic matter. They are necessary in the food chain as predators (Chiumenti R. & Chiumenti A., 2001).

1.5.1 The technologies of composting can be summarized in two main categories

Open systems: Composting occurs in piles and aerated static piles.
Closed systems: Organic waste is processed into bioreactors.

In open systems aerated static biomasses are placed in pile-like accumulations. The absence of periodic mixing, limits materials application to type that maintain a relatively stable structure during the process. To improve porosity, it is also possible to incorporated in the initial mixture, materials such as wood chips (Bulking). It is also possible to increase air distribution cumulating on grids or placing pipes at the base of the pile, tubes are connected to blowers activated periodically according to oxygen demand the pile process, Beltsville strategy (Epstein *et al*., 1976; Willson *et al*., 1980). The ventilation could be also regulated by a feedback based on temperature, Rutgers strategy (Finstein *et al*., 1980; Finstein *et al*., 1983). For the same rationale, but also to allow a uniform fermentation process the pile, maximum height is usually not taller than 2,5 m. The pile is roofed on top, with a layer of about 10 cm of natural soil, mature compost, chopped straw, or any similar matrix. This layer has the function of protecting the matrix from moisture loss and temperature isolation. In addition act as has bio-filter to decrease ammonia emissions and odors release.

Closed systems consist of vertical reactors (silos), or horizontal reactors (rotating drums) (De Bertoldi *et al*., 1985; Rynk 1992). The adoption of a particular type of system depends on several factors such as: available area, waste characteristics and amount, time request to obtain the material stabilization (Vallini, 2001).
Composting in bioreactor combines handling techniques and forced aeration. In general the organic matrix undergoes to a first homogenization and a partial transformation, while the aerobic bio-stabilization is completed later in cumulus. The matrix inside bioreactor will reach temperatures > 55°C, and air is injected

from drainpipe, in the opposite direction. Special filters prevent dispersion of odors and other volatile substances.

1.5.2 Compost

The compost outcomes by decomposition and humification of organic matter via the microbial flora naturally present in the environment. The etimology of the name "compost" originate from the Latin *compositum*, because originate from a various materials and lastly composed among a variety of reaction products.

The national legislation on fertilizers (D. Lgs. 217/06), lists three categories:

- Green Compost fertilizer (GCF);
- Mixed Compost fertilizer (MCF);
- Peat Compost fertilizer (PCF)

GCF results from treatment of waste from plants maintenance, crop residues, diverse residues of plant origin; with the exception of algae and marine plants. GCF has physical-chemical characteristics appreciable, compared to other soil improvers. Easy to use in professional horticulture, and for hobbyists, for direct planting in field, or in pots, safe if directly in contact with the root or seeds, to be immediately utilize or in mixed with peat. Conversely, soil amendment from green waste, especially if produced from woody matrices, have a contents nutrients that results poor, in particular nitrogen, phosphorus and potassium but capable of improving the physical-structural and biological properties of the agricultural soil (Centemero, 2007).

MCF is obtained from food leftovers (law n° 748/84), contributes to increase humified organic matter, relevant is the supply of nutritional elements, N, P, and K and micronutrients elements.

This ensures high performance in case of organic fertilization. It is widely used in horticulture, orchards and vineyards; other sectors are: landscaping, gardening. By contrast, the high content of soluble salts of certain compost, limits the possibility of massive use in nursery (Centemero, 2007).

PCF is obtained as results from peat and GCF mixture. Its structure, the ability to absorb and release water, as well as to retain nutrients easily assimilated by the plant, foster many of the soil biological activity (Centemero, 2007).

Physical-chemical and stability characteristics influence agronomic quality of the compost. The organic matter is the main element of the soil agronomic fertility: the soil ability to sustain plant production. Soil organic matter has a profound influence on all aspects of living organisms: plants, bacteria, fungi, and many others. It has a decisive role in the formation of the physical structure of the soil, favoring the balance between the components of the soil, air, water and solid phase. This involves a more efficient tillage, better water retention, erosion decrease. The organic molecules, because of their polyanionic nature, have a strong tendency to adsorb soluble nutrient cations (such as potassium and ammonia nitrogen) to make them available to the roots, limiting the losses of elements from the soil by leaching during rainy periods. Microorganisms metabolic activity in organic substance plays an important role in the solubilization and absorption of nutrients (Centemero *et al.,* 2004).

Compost produce hormone-like substance that effects bio-stimulants for some of the plants functions such as: germination, rooting and growth, may also foster plant stress conditions.

Several studies shows organic matter in soil enhance micro-flora and micro-fauna active in control pathogenic organisms (Vizioli, 1997). Improvement of organic matter can be very effective in control and suppression of some diseases caused by pathogens such as *Fusarium* spp. (Lewis & Papavizas, 1977; Szczech, 1999), *Phytophthora* spp. (Szczech & Smolinska, 2001), *Pythium* spp. (McKellar & Nelson, 2003; Veeken *et al*., 2005), *Rhizoctonia solani* (Papavizas & Davey, 1960; Diab *et al*., 2003), *Sclerotinia* spp. (Lumsden *et al*., 1983a; Boulter *et al*., 2002), *Sclerotium* spp. (Coventry *et al*., 2005).

According to Weller *et al*. (2002), there are two types of suppressiveness. General suppressiveness is promoted by the total microbial biomass control in the soil,

microorganisms act as bio-control agents in disease suppression, the activity results in a depletion of essential nutrients for the pathogen survival and multiplication. Some plant pathogens such as *Pythium* and *Phytophthora* spp. are controlled through the general suppressiveness (Chen *et al*., 1988b; Chen *et al*., 1988a; Mandelbaum & Hadar, 1990).

Specification suppressiveness is due to the specific activity of microorganism groups and is also transferable between soils (Hoitink *et al*., 1991; Stone *et al*., 2004). The specification suppressiveness is more qualitative compared at the general. It is based on specific effects of individuals or selected groups of antagonistic microorganisms on the pathogen, during a particular life cycle phase (Cook & Baker, 1983). The microrganism activity in the microbial community and their response at the energy reserves available in the compost are the disease control basis (Hoitink & Boehm, 1999). To regulate organisms activity is important the concentration and the availability of nutrients and carbon sources such as carbohydrates, lignin, cellulosic, chitin, lipids, etc. (Hoitink *et al*., 1997).

To explain organic matter ability to increase substrate cultivation suppressiveness, several mechanisms have been proposed:

1) increased microbial antagonists activity (Hoitink & Boehm, 1999);

2) increased resource competition (Lockwood, 1990);

3) production of fungi-toxic compounds (Smolinska, 2000);

4) induction of plant systemic resistance (Zhang *et al*., 1996; Pharand *et al.*, 2002).

The organic matter microbial community is able to be strongly competitive for degradable organic materials and nutrients (Stone *et al*., 2004). The literature suggests that compost produced outdoor in forests, or in other open environments is characterized by high microbial concentration and biodiversity, and consequently has more effective suppressive ability (Kuter *et al*., 1983).

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Chapter 2 Aims

The “National Chestnut Plan” (Piano Nazionale Castanicolo 2010/2013) highlights the need to re-educate chestnut woods towards more productive and sustainable forms, able to open new economic opportunities for this sector.

The annual practice of underbrush cleaning for harvesting preparation is a main cultivation technique in chestnut management. For this purpose, chestnut culture residues were traditionally burned on place, also to prevent accidental fire, and remove pathogen inoculum and insects. Although combustion in field is considered as illegal treatment of waste, and therefore prosecuted criminally and administratively (art. 256 D. Lgs n. 52/2006), at moment there are not economically sustainable alternatives available. Composting represents an ecologically and economically efficient technology to recover such biomass. In addition, the evaluation of compost made from chestnut cleaning and pruning residues can additional source of income for the compartment. Although composting procedures are well known, until now very few reports describe this procedure on chestnut woods remains and its use in agriculture. Studies have been conducted on pilot-scale reactors that enable easier tracking of the composting process. In contrast, field experiments do not allow strict monitoring developments in decomposition, and for the best of our knowledge, no studies are available in such conditions.

Principal object: The present study aims to create useful innovations to increase the sustainability and profitability of production, in accordance with the regulations of organic farming and environmentally friendly criteria. The proposed innovations are both of agronomical and biotechnological interest: pursuing the workers welfare in disadvantaged areas, the environmental impact reduction, and the improvement of the product quality.

Specific objects were:

a) physicochemical characterization of the fermenting biomass;
b) evaluation of bacterial and fungal populations during chestnut green waste composting;
c) agronomic evaluation of the final product;
d) nutritional characteristics induced in plant.;
e) assessment of compost suppressive activity.

Chapter 3 Composting process physicochemical monitoring and product characterization

3.1 Introduction

Compost can be considered a soil conditioner contributing to fertility, structuring, porosity, organic-matter contests, water holding capacity, and disease suppressiveness (Itavaara *et al.,* 1997; Sesay *et al*., 1997; Zucconi *et al*., 1981). The two most significant issues in a compost process, strictly linked, are original matrix physicochemical composition and existing microorganism populations.

The microbiology of the composting process involves several interrelated factors: temperature, C/N ratio, pH, water activity (aw), available nutrients, and physical state of the starting materials (Beffa *et al*., 1996b; Sartaj *et al.,* 1995). Temperature is the dominant parameter that controls microbial activity (McKinley, *et al*.,1985). There are three phases which occur through time: an initial phase, a thermophilic phase, and a curing or stabilization phase (Cook & Zentmyer, 1986). C/N ratio is a significant factor in the nutrient balance, optimum value ranges between 20:1 and 25:1 (Golueke, 1972). During the composting process, a fraction of carbon is lost as CO_2 which progressively narrows the C/N final ratio (Finstein & Morris, 1975). The acidity in the initial phase of the process is the result of organic acids formed during the fast degradation (Hellmann *et al.,* 1997). Composting increases pH from organic acid depletion, release of alkali, and accumulation of ammonia (Hellmann, *et al.,* 1997). Principal requirement for an organic material to be safely, conveniently and effectively used as a soil amendment is the degree of maturity, achieved after the treatment process (Beffa *et al.,* 1996b; Senesi & Brunetti, 1996).

Principal requirement for an organic material to be safely, conveniently and effectively used as a soil amendment is the degree of maturity, achieved after the treatment process (Beffa *et al*., 1996b; Senesi & Brunetti, 1996). Immature

compost may contain substances such as methane, ammonia, or acetic acid detrimental to plant. Even mature compost may contain substances that inhibit plant growth, such as heavy metals, salts, pesticide residues, or other toxic compounds contained in the original compost ingredients (Banegas *et al*., 2006, Garcıa, 1990; Pascual *et al*., 1997).

Respiration reveals the microbial metabolic activity. Microorganisms respiration rate is higher in presence of large amounts of bio-available organic matter. It represents an important parameter for the determination of compost stability; therefore it is used for monitoring the composting process (Raquel *et al.*, 2006).

In this chapter, physical and chemical characterization of chestnut wastes was carried out monitoring the progress and its limits, and, in the end, the stability of the final product was evalued.

3.2 Materials and Methods

The experimental site was positioned in a chestnut forests in "Regional Park of Roccamonfina Foce Garigliano", Italy (41°18'94"N., 13°55'84"E; 478 m a.s.l.).

Composting piles in three relpicates were assembled as box, 1,50 m x 1,50 m x 1,80 m (length x wight x height), made of fagots of chestnut brances, than filled in layers (fig. 1) in the following order:

1. fresh cestnut shoots;
2. wooden chipboard;
3. undergrowth (ferns, vetch, sainfoin, wild oats, horsetail grass calenzuola, curly leaves and old);
4. old leaves and curly;
5. fresh shoots;
6. wooden chipboard;
7. undergrowth;
8. old leaves and curly.

The composting bins were covered with 5 cm of local soil.

Figure 1 Composting pile and order and composition of layers in the pile

3.2.1 Sampling

Samples of 1 kg were composed from five distinct assay points in the biomass immediately after preparation (T0) and at 15, 45, 75, 105, 135, 165, 195, 225, 255, 285, 315, 345 days post accumulation (T1, T3, T5, T7, T8, T9, T10, T11, T12, T13, T14, T15 respectively). Samples were kept at controlled temperatures until the processing.

3.2.2 Physicochemical monitoring and characterization

Temperature (C°) was measured by using Thermometer 1TC HD2108.2 (Delta OHM) with specific probe (1500 mm), directly in the pile core. The pH was determined suspending samples in distillate water 1:10 (w/v). The water activity (a_w) was evaluated by using the HygroPalm 23-AW (Rotronic AG, Basserdorf, Germany).

Porosity test, water holding capacity, and respiration test of the compost were performed according to the method described by Zucconi *et al.,* (1981).

Porosity was determined as follows: the compost sample volume was measured and recorded using a graduated cylinder. Then, the compost was removed from the cylinder, which was filled with a known volume of water. At this point the compost was added, the suspension stirred, and left stay for 5 minutes to allow air bubbles to flee. At last the final volume of the compost was recorded.

Volume of compost is calculated as follows:
vol. of compost = vol. of mix (compost and water) – vol.water (70ml)

total pore volume is calculated as follows:
vol. of pore space = vol. of dray compost – vol. of compost
the porosity:

% porosity pore space = [vol of pore space/vol of dray compost] x 100

The water holding capacity is expressed as the amount of water retained for liter of soil. To determine the ability of a soil or compost to retain moisture against drainage due to the force of gravity was determined:
Water holding capacity (ml/l) = 10 x (ml water retained/100 ml sample).

Compost moisture, was determined by drying the sample at 105 °C for 24h.
Calculation
total moisture (C_M) = 100 x (S_W –S_D)/S_W
S_W (sample weight wet)
S_D (dried sample weight)

Total nitrogen content and organic nitrogen was determined by Kjeldahll method XIV.3 (D.M. 13 September 1999).

Organic carbon content was determined by volume method redox VII. 3 (D.M. 13 September 1999).

Avaiable potassium and calcium content expressed as K_2O and Ca, were determined by method XIII.5 (D.M. 13 September 1999).

Assimilable phosphorus, expressed as P_2O_5, (Olsen) method XV.3 (D.M. 13 September 1999).

3.2.3 Stability index and phytotoxicity bioassay

Respiratory test provides a measure of CO_2 rate was measured according to Zucconi *et al.* 1981. The respiration activity of the biomass was determinate measuring CO_2 production which reacts with sodium hydroxidesolution forming sodium carbonate.

The compost sample, 50% UR, was placed in a beaker 100 ml volume, and incubated in the dark at 25 °C in glass sealed containers. On the bottom was placed carbon free water to maintain the ideal humidity conditions, in the container was also placed another container containing 20ml of a sodium hydroxide solution of 1M. To determine the CO_2 production, the NaOH was titrated with HCl, in times course at 3-4-5-6 and 7days. For every fixed time interval the compost sample was left open to restore the oxygen contents and it was replaced NaOH solution. Simultaneously were set the mock samples. The data obtained at each titration, divided by the days of incubation, indicates the value of the daily breathing.

CO_2 (mg) **=** [HCl_b – HCl_c /1000 ml/l] x HCl M (mol/l) x 12g c/mol x 1000mg/g

HCl_b = ml HCl used for blank titration

HCl_c = ml HCl used for sample titration

CO_2 (mg) = (HCl_b- HCl_c) x12

Phytotoxic activity was assessed using watercress (*Lepidium sativum*) germinating seeds; the experiment was carried, as any other, in three replicates. Two milliliter

of the aqueous extract (1/10, w/v) from composts were poured on filter paper into Petri dishes, distilled water was used as control. Ten seeds of watercress were then placed on the filter paper and the dishes placed in germination chamber at 25 °C in darkness. After the incubation period (72h) the number of germinated seeds and the primary root length were measured and expressed as a percentage.
The germination index (GI) was calculated according to the formula proposed by Zucconi *et al*. (1981):

GI = [germination percentage (% G)] x [radicle length percentage (% L)] /10.00
in which:
% G = G_T/G_C X100
G_T = germination for treatment
G_C = germination for distilled water control

% L= L_T/L_Cx100
L_T = radicle lenght for treatment
L_C = radicle length for distilled water control

3.3 Results

3.3.1 Physicochemical monitoring and characterization

Physical and chemical parameters were evaluated during the all composting process. The biochemical composition of the raw materials was: UR 40.2%, organic carbon 28.9% (dry weight), total nitrogen 0.5% (dry weight), C/N ratio 57.8%.

At the beginning of the process the pile temperature was 22.5°C, rising up to 28.9°C at day 45 (T3), and then decreasing up to 20°C until 195 days (T10) of composting process. At the end of process (T15) the mature compost showed a temperature of 24°C. The trend of pile temperature was strongly influenced by climatic conditions as showed by the similar trend of the curves in the fig. 2. During the first 135 days (T8) of process, the pH values observed were quite stable since it ranged from 6.7, at the beginning (T0), to 6.3. At the end of the process (T15), in compost mature, the pH values were 6.9 (fig. 3).

Also the water activity (a_w) was quite stable, ranging from 0.92 to 0.95 (fig. 3).

345 days of composting were necessary to obtain a mature compost characterized by an UR equal to 40.8%, organic C 2.69% (dry weight), total N and organic N was respectively 0.28 % and 0.27 % (dry weight), C/N of 9.6, porosity of 58%, water holding capacity 29%, available potassium 12.767 mg kg^{-1}, calcium content 19.400 mg kg^{-1} and assimilable phosphorus to 7.6 mg kg^{-1}.

3.3.2 Stability index and phytotoxicity bioassay

The long process period was necessary to produce a very stable organic matter with no residual phototoxicity, as shown in the respiratory test, with a value of CO_2 of 1.9 mg g^{-1} well under the limit (table 1), and in the germination test indicating no inhibition with a GI of 1.1 (table 2).

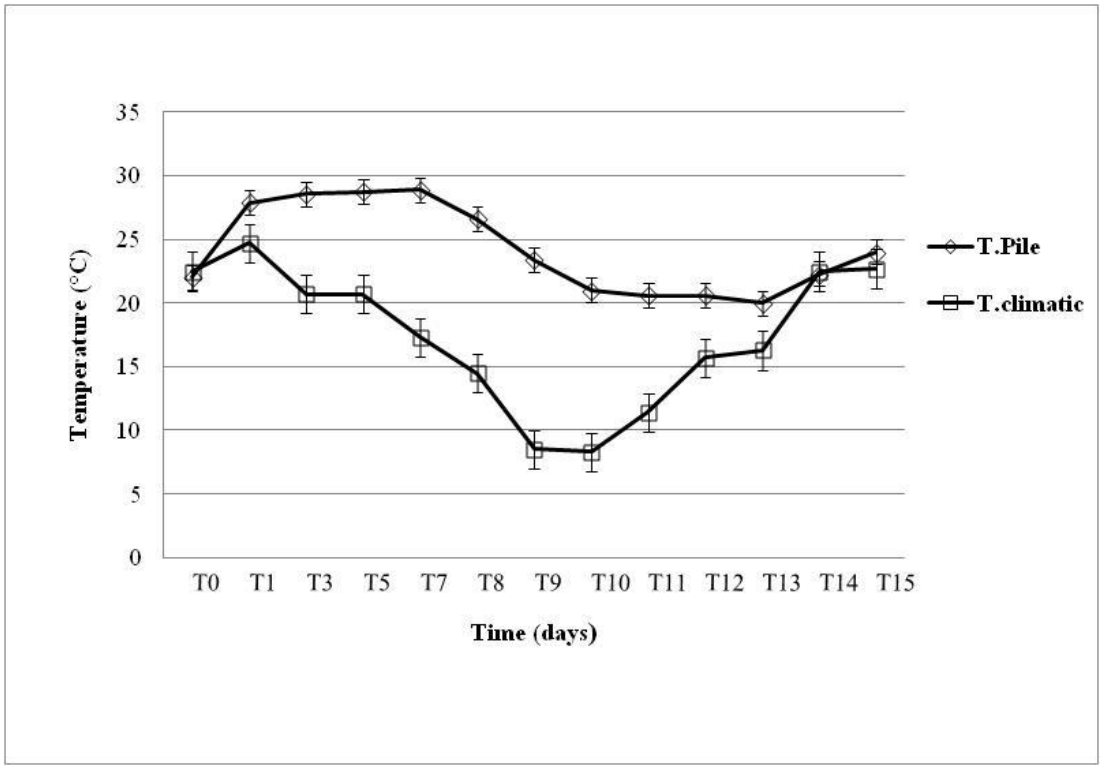

Figure 2. Trend composting temperature in relation to the climatic temperature.
The interval of time equal to 30 days until 345 days of chestnut process
The error bar means standard deviation of the data obtained from analysis of three piles.

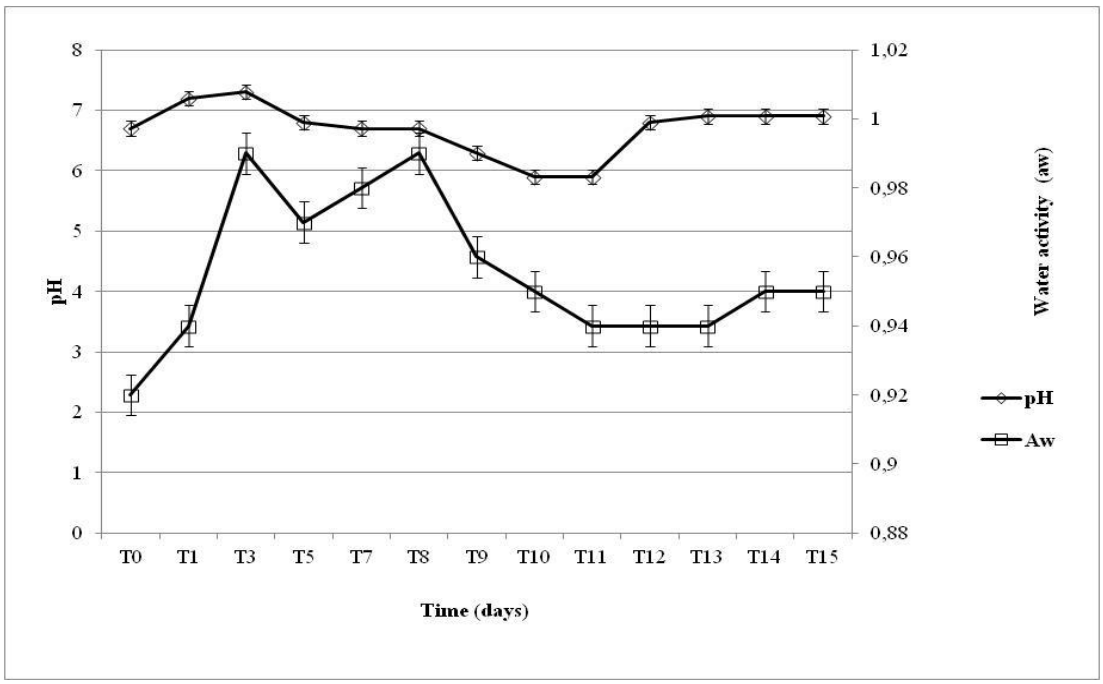

Figure 3. pH and water activity (aw) values determined during composting process
The interval of time was equal 30 days unitil 345 days of chestnut composting process.
The error bar means standard deviation of the data obtained from analysis three piles.

Table1. Compost Stability Index

Respiration rate (mg CO_2 g organic carbon/day)	**Rating**	**Trends**		
<2	Very stable	Potential odor generation ↓	Potential for inhibition of plant growth ↓	Potential for inhibition of seed germination ↓
2-5	Stable			
5-10	Moderate stable			
10-20	Unstable			
>20	Extremely unstable			

(Zucconi *et al*.,1981)

Table 2. Garden Cress Germination index

Germination index	**Rating**
1.0– 0.8	No inhibition of plant growth
0.8– 0.6	Mild inhibition
0.6 -04	Strong inhibition
< 0.4	Severe inhibition

(Zucconi *et al*.,1981)

3.4 Discussions

We can consider 345 days a long time to obtain the mature compost. It is already known that decomposition of green-waste materials is typically slow. In fact presence of a high C/N ratio, can inhibit a quick start of the composting process (Kelleher *et al*., 2002). Environmental conditions and living organisms influence composting of lignocelluloses biomass; a complex ecosystem in which autochthonous microbial communities prevail over other microorganisms because their enzymatic armory able to degrade complex molecules such as cellulose and hemicelluloses (Soares *et al.,* 2012). Moreover, since the process was carried in natural conditions, in a chestnut forest, this could result in an additional limitation due to environmental conditions such as the temperature. Periodic measurement of the temperature during composting represents an important index to monitor the process, indeed it is closely related to environmental conditions and microorganisms metabolic activities. In this work, the temperature of the pile was strongly affected by the seasonal climatic conditions showing its same trend all along the process. Although there are no common definitions for mesophilic and thermophilic phases, during composting process, generally mesophilic refers to temperatures up to approximately 40°C, and thermophilic to temperatures from 45°C up to 70°C (Miller, 1996). The temperature values detected in our experimentation suggested that the process was conducted in mesophilic conditions.

More parameters largely effective on microbial activity as pH and a_w, were monitored to comprehend the progress and its limits. The pH drop from values around 7 to about 6 after 105 days of composting, probably also in consequence of the production of organic acids by microorganisms (Guerra-Rodrìguez *et al*., 2006). The oxidative process generally occur between 3 to 11 (Guerra-Rodrìguez *et al*., 2006), with the optimal range of 5 to 8 (Sundberg *et al*., 2004). The porosity percentage observed in the final compost was 58%, while an optimal

value should be approximately 85%, and anyway not lower than 75% compost (Cattivello, 1990). Porosity involves macro and micro-pores, and depends on environmental conditions, cultural practices and crops raced. Generally the liquid phase occupy 40%, the gas phase the residual part, macro-porosity, 15 to 35% (Perelli, 2004). The water holding capacity of a soil determines its ability to sustain plant life during dry periods. Water is held in pores between soil particles and in films surrounding particles. Different types of soil retain different amounts of water, depending on the particle size and nature, and in reason of the organic matter content (Zucconi, 1981). In our chestnut mature compost the value obtained of 29% (v/v), is considered normal according to the guide for main physical parameters of growing media and soil improvers (De Boodt method - EN 13041; De Boodt *et al*., I. 1974 method). Chestnut compost presented moisture content lower than 50%, as required for green compost obtained from vegetable waste (D. Lg. no. 217, 29 April 2006). On the other hand, organic and total nitrogen and of the organic carbon were too low to classify the compost as green compost (D. Lg. no. 217, 29 April 2006). Phosphorus, one of the essential macronutrients for plant growth and development, is present in soil in two forms as organic and inorganic phosphates (Ehrlich, 1990). Key point is the conversion of insoluble phosphates compounds, both organic and inorganic, in a form accessible for plant (Igual *et al*., 2001; Rodríguez *et al*., 2006). In chestnut compost, the assimilable phosphorus is present in low amounts equal to 7.6 mg kg^{-1}.

Respiration test, after 345 days resulted 1.9 mg g^{-1}, assimilable to very stable compost (Zucconi, 1981). Seed germination tests helps to establish the efficacy of the composting process and the residual phytotoxicity (Banegas *et al*., 2006, Garcıa, 1990; Pascual *et al.,* 1997). Test chestnut compost revealed no inhibitory effects on germination: the germination index (GI) of the mature compost on *Lepidium* was 1.1, therefore the compost was be considered mature (Zucconi, 1981).

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Chapter 4 Microbial diversity: monitoring and molecular analysis

4.1 Introduction

Composting is a widely low cost used method to stabilize agricultural organic wastes through the degradation of organic materials by a consortium of microorganisms (Chen *et al*., 2014). The ecology of microbial communities involved into degration of lignocellulosic materials is very complex. It involves numerous interactions of metabolically diverse microorganisms whose activities are influenced by a wide range of environmental factors. During this bio-oxidative process a succession of different microbial populations are involved and therefore the characterization of biota present throughout the composting process as well as in the mature product is important (Cahyani *et al*., 2004). However, the knowledge on microbial dynamics and the relationship between microbial communities and degradation process is poor mainly in green waste composting (Yu *et al*., 2007). Microbial community dynamics during the composting phases for specific wastes could be investigated by molecular techniques (e.g. PCR-DGGE) that allow to directly determining the genetic diversity in different samples (Alfreider *et al*., 2002; Belyaeva *et al.,* 2012).

In this study microbiological characterization was carried out by culture-dependent and culture-independent molecular approaches. In particular, different functional groups involved in carbon cycle such as hemicellulolytic, cellulolytic and ligninolytic microorganisms, were enumerated. Moreover, PCR-DGGE was used to evaluate the succession of bacterial and fungal populations during chestnut green waste composting and in the finished compost in order to detect and characterize the dominant genera and/or species that conducted this process and their potential beneficial effects on plants.

4.2 Material and methods

4.2.1 Sampling and microbial enumeration

Composite samples of 1 kg were obtained immediately after pile preparation (T0) and after 15, 30, 45, 60, 75, 90, 105, 135, 165, 195, 225, 255, 285, 315 and 345 days of composting (T1-T15), from three different points in the pile core.

Microbiological counts were performed for T1, T3, T5, T7, T8, T9, T10, T11, T12, T13, T14, T15 samples. For analysis, an initial suspension was prepared by the addition of 20 g (w/v) of the samples to 180 ml of quarter strength Ringer's solution (Oxoid, Milan, Italy). After shaking suitable dilutions (1:10) were performed and used to inoculate different growth media. During the composting process, specific soil microbial functional groups involved in carbon cycle, as cellulolytic, hemicellulolytic and ligninolytic, were detected at 28°C by using the Surface Spread Plate Count Method. Cellulolytic and hemicellulolytic microorganisms were counted in a minimal media [1 g l^{-1} $(NH_4)NO_3$, 1 g l^{-1} yeast extract, 50 ml l^{-1} standard salt solution, 1 ml l^{-1} trace elements solution, 15 g l^{-1} bacteriological agar, at pH 7.0] containing carboxymethylcellulose (5 g l^{-1}) as sole carbon source and Remazol Brilliant Blue R to put in evidence cellulolytic activities by developing clear haloes around the colonies (Ventorino *et al*., 2010).

The medium used for hemicellulolytic populations was the minimal medium above described with xylan (5 g l^{-1}) as sole carbon source. Finally, ligninolytic microoganisms were enumerated by using LME basal medium (1 g l^{-1} KH_2PO_4, 0.5 g l^{-1}, $C_4H_{12}N_2O_6$, 0.5 g l^{-1} $MgSO_4$, 0.01 g l^{-1} $CaCl_2$, 0.01 g l^{-1} Yeast Extract, 0.001 g l^{-1} $CuSO_4$, 0.001 g l^{-1} $Fe_2(SO_4)_3$, 0.001 g l^{-1} $MnSO_4$ 16 g l^{-1} bacteriological agar) supplemented with 0.25 % (w/v) lignin as carbon source (Pointing *et al.* 1999).

Moreover, at the end of oxidative process, the compost was characterized also by enumeration of total heterotrophic aerobic bacteria on Plate Count Agar (PCA, Oxoid), composed of 1 g l^{-1} dextrose, 1 g l^{-1} of yeast extract, 5g l^{-1} of triptone, 15

g l^{-1} of agar bacteriological, pH 7.2. The plates were incubated for 3 days in aerobic conditions (Oxoid's Anairogen™ System) at 28°C.

Fungi were cultivate on plates of Malt Extract Agar (MEA, Oxoid) with chloramphenicol (100 mg l^{-1}) incubated at 28°C for 3 days.

Actinomycetes were counted on Starch Casein Agar (10 g of soluble starch, 0.30 g of casein, 2 g of KNO_3 3,2 g of NaCl, 2 g of K_2HPO_4, 0.01 g $FeSO_4$, 0.05 g $MgSO_4$, 0.02 g $CaCO_3$, 18 g agar bacteriological, 980 ml distilled water, pH 7.0) containing 100 mg l^{-1} cycloheximide (Sigma–Aldrich, Milan, Italy) to inhibit the mould growth. The plates were incubated at 28°C for 14 days (Pepe *et al*, 2013).

Free-living (N_2)-fixing aerobic bacteria were counted by MPN method using the liquid medium consisting in 10 g of mannitol, 50 ml of standard salt solution, 10 ml of soil extract, 1 ml of trace elements solution, 0.05 g of $CaCO_3$, 950 ml distilled water, pH 6.8. 5 ml of medium was distributed in tubes of 22 mm diameter, inoculated and incubated at 28°C for 14 days. Positive tubes showed a surface patina on top of the liquid medium (Pepe *et al.,* 2013).

To detect the presence of *Pseudomonas* spp., plates containing *Pseudomonas* Agar Base (Oxoid) with 10 ml l^{-1} of glycerol and CFC supplement (Oxoid), were incubated at 28°C for 3 days.

Compost hygienic quality was assessed by counting *Enterobacteriaceae* with Violet Red Bile Glucose Agar (Oxoid) by double layer pour plate method, while for faecal streptococci MPN method of count was performed by using two passages in Azide Dextrose Broth (Oxoid) and Ethyl Violet Azide Broth (Oxoid).

Conventional two-step enrichment was used for the detection of Salmonella (Pepe *et al*., 2013) using overnight incubation in buffered peptone water followed by subculture in Muller-Kauffmann Tetrathionate Broth Base (Oxoid). After an incubation for 24 h at 37°C, an aliquot of enrichment broth was streaked on plates containing selective medium Rambach agar (Merk). After 24 + 24 h of incubation at 37°C the suspect red colonies of Salmonella, were inoculated by stab in tubes containing Kligler Iron Agar slant (Oxoid), a differential medium based on the

fermentation of lactose and glucose, and on the production of hydrogen sulphide and gas. The presence of the red surface and yellow depth with gas and hydrogen sulphide production, allowed detecting the presence of Salmonella in the samples.

4.2.2 Genomic DNA extraction

Molecular analysis was performed for all collected samples. Microbial cells were desorbed from lignocellulosic matrices, and pellets were obtained prior to community DNA extraction, as previously described (Ventorino et al., 2013). Briefly, 100 g of the samples was suspended in 400 mL of acidic solution (pH 2.0), shaken at 240 rpm for 10 min, filtered and centrifuged at 10,000 rpm for 20 min. Microbial cells were washed in 45 ml of sterile phosphate buffered saline and recovered by centrifugation (6500 rpm for 20 min). The DNA was extracted from compost samples using the FastDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's specifications.

4.2.3 PCR-DGGE analysis and sequencing of the dominant bands

To amplify the bacterial community, the primers V3f (5'-CCTACGGGAGGCAGCAG-3') and V3r (5'-ATTACC GCGGCTGCTGG -3') spanning the V3 region of the 16S rDNA were used. The PCR mixture (50 µl total volume) included 5 µl DNA (50 ng), 20 picomoles of each primer, 5 nmol of each deoxyribonucleoside triphosphate, 50 nM of $MgCl_2$, 5 µl of 10x buffer and 0.5 U of *Taq* DNA polymerase (Invitrogen) (Palomba *et al*., 2011). A touchdown PCR was performed in which the annealing temperature was decreased from 65 to 55°C at a rate of 1°C every cycle followed by 20 additional annealing cycles at 55°C. A denaturation step of 94°C for 1 min was used, and extension was performed at 72°C for 3 min; a final extension step at 72°C for 10 min ended the amplification cycle (Pepe *et al*., 2013b).

To analyze the eukaryotic population, the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-

ATTCCCAAACAACTCGACTC-3') for the 26S rRNA gene were used. PCR mixture was performed as above reported. The PCR reaction included an initial denaturing step at 95°C for 3 min followed by 35 cycles of amplification (95°C for 1 min, 52°C for 1 min and 72°C for 1 min) and a final extension step at 72°C for 7 min.

According to Muyzer *et al.* (1993), a GC-clamp was added to the forward primer in all PCR reactions.

DGGE analyses were performed using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories, Milan, Italy). PCR products were loaded in a 0.8-mm polyacrylamide gel [8% (w/v) acrylamide-bisacrylamide (37:5:1)] using a denaturant gradient from 30 to 60% increasing in the direction of electrophoresis for both bacterial and fungal analysis. Electrophoresis was performed at 60 °C, at 200 V for 240 min. The gels were stained with SYBR Gold (20 min) (Invitrogen, Milan, Italy) and rinsed in distilled water (5 min).

The dominant bands were excised from the gel with sterile scalpel, transferred into 20 µl of and incubated overnight at 4°C to allow diffusion of the DNA. 2 µl of the eluted DNA was re-amplified by using the PCR conditions above described. The PCR products obtained were checked by DGGE; DNA amplified from compost samples was used as control. The products that migrated as a single band and at the same position with respect to the control were purified by QIAquick PCR Purification kit (Qiagen) and sequenced. The DNA sequences were determined and analysed as reported by Pepe *et al*. (2011). In detail, the sequences were determined by the dideoxy chain termination method by using the DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA) according to the manufacturer's instructions. The sequences were than analysed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S. A., Olivet Cedex, F) and compared to the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website

(http://www.ncbi.nlm.nih.gov), in order to determine their closest phylogenetic relatives.

4.2.4 Statistical analysis

DGGE bands were automatically detected using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England). The cluster analysis was performed by the program Phoretix 1 advanced version 3.01 after band matching. The method described by Saitou and Nei (1987) was used to obtain the correlation matrix of the DGGE patterns. The resulting matrix was used in the average linkage method by the Cluster procedure of Systat 5.2.1 to estimate the percentage of similarity (S) of microbial populations in the composting process.

4.3 Results

4.3.1 Microbial enumerations

The three functional microbial groups involved in the C cycle were present at quite high level in the mature chestnut compost even if their concentrations decreased of about 2 Log CFU g^{-1} during the chestnut composting. In fact, hemicellulolytic population was 9.04 ± 0.06 Log CFU g^{-1} at beginning but a lower level was detected at the end of the process (7.58 ± 0.003 Log CFU g^{-1}). Cellulolytic group showed also a similar trend decreasing from 9.00 ± 0.06 to 7.60 ± 0.05 Log CFU g^{-1} in the mature compost (fig. 1). Similarly, ligninolytic microorganisms showed a significant decline after 345 days of the process from 8.48 ± 0.00 up to 6.01 ± 0.002 Log CFU g^{-1} (fig. 1, table 1).

The results showed that all groups had a variable trend until 225 days (T11) of composting, especially ligninolytic group that suffered a sharp decline after 135 days (T8) followed by a new increased of the growth (T9-T11). The hemicellulolitic and cellulolytic groups increased until 75 days of composting (T5) after that they showed an inverse trend. In particular, cellulolytics continued to increase until 105 days (T7) then they declined until 195 days (T10). By contrast, hemicellulolytic group decreased until 105 days before increasing again. After 225 days the biomass entered in the stabilization/maturation phase and a gradual decrease in the concentration of all analysed functional populations was observed (table 1).

Moreover, at the end of the process, the compost was characterized by higher level of *Actinomycetes* (6.23 ± 0.003 Log CFU g^{-1}) with respect to total heterotrophic aerobic bacteria and mould that showed similar concentrations equal to 5.78 ± 0.002 and 5.77 ± 0.001 Log CFU g^{-1}, respectively (table 2).

Putative plant growth promoting rhizobacteria (PGPR) belonging to aerobic free-living (N_2)-fixing bacteria and *Pseudomonas* spp. showed lower values equal to 2.39 ±0.003 Log MPN g^{-1} and 4.78 ± 0.06 Log UFC g^{-1}, respectively.

Enterobacteriaceae and fecal streptococci were absent in 0.1 g of the sample whereas *Salmonella* spp. was undetectable in 25 g of sample (table 2).

4.3.2 Dynamic and taxonomic composition of eukaryotic communities

DGGE band patterns shown in fig.2, illustrated eukaryotic communities along the entire composting process of chestnut lignocellulosic residues.

The DGGE profiles of eukaryotic populations showed that the number of the bands during the composting process remained almost similar until 315 days (T14) after that it drastically decreased to 5 in the mature compost (T15).

Since the differences were mainly due to the different position and intensity of the bands rather than their number (fig.2), statistical analysis was carried out. Cluster analysis (fig.3) identified five major groups (cluster 1: T0; cluster 2: T1; cluster 3: T2-T9; cluster 4: T10-T14; cluster 5: T15), in which slight changes within the fungal populations were observed (similarity level from 91 to 100 %). The major cluster 3 included the samples collected from 30 to 165 days while the cluster 4 grouped the samples from 195 to 315 days. The initial chestnut material (T0), the sample after 15 of process (T1) and the mature compost (T15) could not be included in any group. In particular, cluster 1 was different from the clusters 2, 3 and 4 showing a similarity level of 50 % with cluster 2, 3 and 4; while cluster 2 was similar to cluster 3 and 4 up to 77 %. Cluster 5, represented by the final compost (T15), was very different from all others samples since its similarity value decreased to 33 % (fig.3).

To characterize the eukaryotic populations involved in the natural degradation of chestnut waste, 11 dominant bands were purified and sequenced for phylogenetic analysis. The nucleotide sequences retrieved from the profiles showed similarities to D1 region of 26S rRNA gene sequences deposited in the public database in the range of 97 to 99 % (table 3).

As reported in table 3, the majority of identified eukaryotic bands pertained to the phylum *Ascomycota*. In particular, recovered fungi included species of the genera

Penicillium, Moristroma, Paraconiothyrium, Fusarium, Cladosporium, *Aspergillus* and *Mucor* (table 3). The genera *Cladosporium*, *Penicillium, Fusarium* and the specie *Paraconiothyrium variabile* were detected in almost all composting stages and in the mature compost (table 3). The genera *Aspergillus* and *Mucor*, with the species *M. racemosus* and *M. plumbeus*, were recovered in the central phases of the composting process (T1-T12) but not in the initial chestnut materials. By contrast, the closest relative to band I, yeast *Candida railensis / Candida oleophila* with a sequence similarity value of 99 %, and the two bands (L and M) identified as *Moristroma* spp. were found only in the initial lingnocellulosic waste (T0-T1).

4.3.3 Dynamic and taxonomic composition of prokaryotic communities

As showed by the DGGE patterns (fig.4) the profiles of prokaryotic communities were very complex, especially in the initial chestnut materials (T0) where 26 different bands were enumerated. The number of the bands during the composting process remained almost similar among the sampling period ranging from 17 to 20. The number of the bands drastically decreased in the mature compost since only 10 different bands were enumerated.

Similarly to fungal results, statistical analysis of DGGE profiles evidenced five major groups (cluster 1: T0; cluster 2: T1-T5; cluster 3: T6-T9; cluster 4: T10-T14; cluster 5: T15), in which slight changes within the bacterial populations were observed (similarity level from 100% to 90%) (fig. 5). The major cluster 2 included the initial phase of the process until 75 days (T1-T5), the cluster 3 grouped the samples collected from 90 to 165 days (T6-T9) while the cluster 4 included the final phase of the chestnut composting (T10-T14). Only the initial chestnut material (T0, cluster 1) and the mature compost (T15, cluster 5) could not be included in any group. In particular, cluster 1 showed a similarity value of 57 % with the clusters 2, 3 and 4. Cluster 5, represented by the final compost (T15), was very different from all others samples since its similarity value

decreased to 35 %. The other clusters (2, 3, 4) were more similar to each other since the similarity percentage reached 74 % (fig. 5).

To characterize the bacterial populations involved in the natural degradation of chestnut waste, 17 dominant bands were purified and sequenced for phylogenetic analysis. The nucleotide sequences retrieved from the profiles showed similarities with 16S rRNA gene sequences deposited in the public database, in the range of 95 to 100 % (table 4). In table 4 were summarized the identification of the persistent bacterial taxa during composting process. The majority of the selected prokaryotic bands were affiliated with *Bacilli*, followed by *Actinobacteria*, *Flavobacteriia* and γ-*Proteobacteria* and uncultured bacteria.

The *Bacilli* were represented primarily by *Bacillus* spp. and the related genera *Brevibacillus* that were recovered at different times of the degradation process (fig. 4, table 4).

Bacteria belonging to the *Actinobacteria* class, such as *Streptomyces* spp., *Actinomyces* spp. and *Cellulomonas* spp., were found to be present during all phases of composting (T0-T15), with only exception of *Cellulomonas* spp. that was not recovered in the mature compost (T15) (fig. 4, table 4).

The genera *Flavobacterium* spp. and *Epilithonimonas tenax* representing the *Flavobacteriia* group, showed a different behaviour. In fact, the first one was recovered along the entire composting process (T0-T14) as well as in the mature compost (T15), while *Epilithonimonas tenax* was detected only in the initial phase of the composting in the chestnut waste materials (T0) (fig. 4, table 4).

Bacteria belonging to the γ-*Proteobacteria* class were recovered mainly in the mature compost. In fact, *Azotobacter* spp., *Stenotrophomonas* spp. were detected only in the finished product (T15) as well as *Pseudomonas* spp. that was detected from T8 to T15 (fig. 4, table 4).

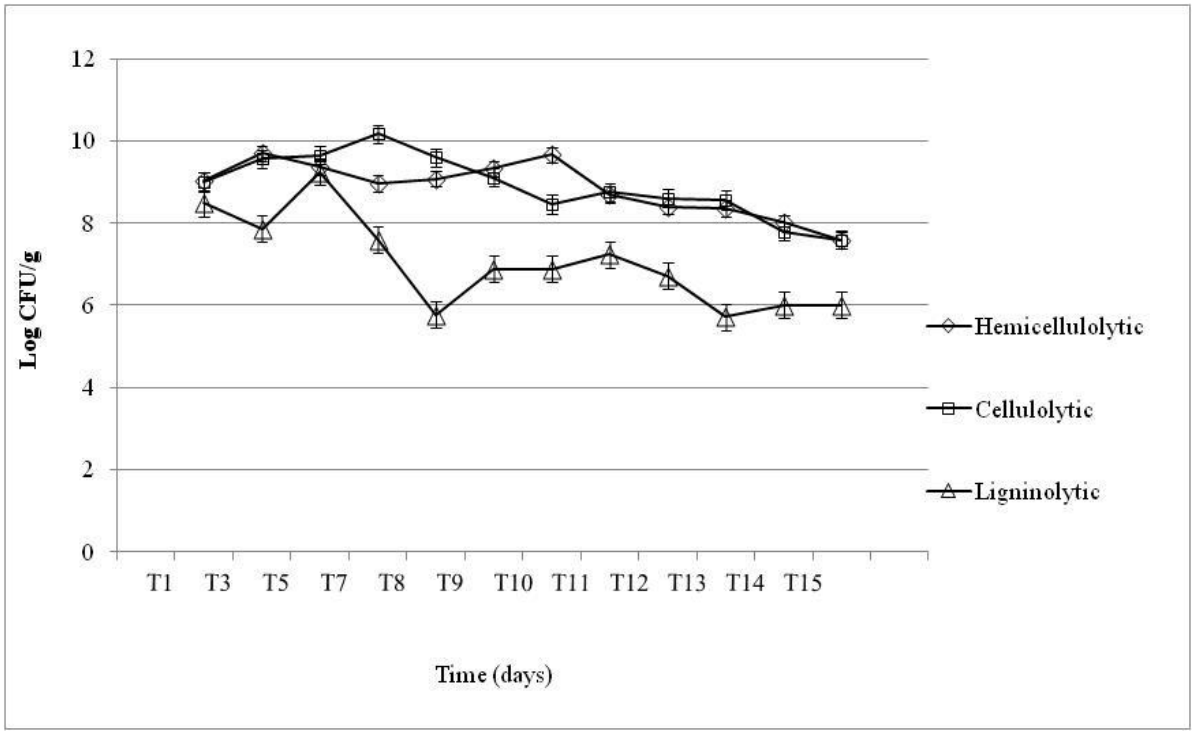

Figure 1. Dynamic of hemicellulolytic, cellulolitic and ligninolytic functional groups of the C cycle during chestnut lignocellulosic biomass. The interval of time was equal to 30 days until 345 days of chestnut composting process. The error bar means standard deviation of the data obtained from the analysis of three piles.

Table 1. Microbial contents (Log CFU g^{-1}) of hemicellulolytic, cellulolytic and ligninolytic populations during composting process. The values are means ± SD of triplicate counts.

Time sampling	**MICROBIAL FUNCTIONAL GROUP**		
(days)	*Hemicellulolytic*	*Cellulolytic*	*Ligninolytic*
T1	9.04 ± 0.06	9.00 ± 0.06	8.48 ± 0.00
T3	9.70 ± 0.03	9.56 ± 0.04	7.86 ± 0.02
T5	9.36 ± 0.08	9.65 ± 0.07	9.25 ± 0.07
T7	8.97 ± 0.21	10.18 ± 0.04	7.59 ± 0.16
T8	9.08 ± 0.30	9.60 ± 0.08	5.77 ± 0.00
T9	9.33 ± 0.01	9.11 ± 0.50	6.88 ± 0.15
T10	9.67 ± 0.53	8.46 ± 0.09	6.88 ± 0.27
T11	8.70 ± 0.08	8.75 ± 0.17	7.23 ± 0.33
T12	8.41 ± 0.16	8.61 ± 0.38	6.71 ± 0.01
T13	8.36 ± 0.02	8.56 ± 0.17	5.71 ± 0.00
T14	8.01 ± 0.01	7.79 ± 0.03	6.01 ± 0.02
T15	7.58 ± 0.003	7.60 ± 0.05	6.01 ± 0.02

Table 2. Enumeration of generic and ecophysiological microbial populations in the mature compost.The values are means ± SD of triplicate counts

Microbial groups	**Microbial contents (CFU g^{-1} or MPN g^{-1})**
Total aerobic heterotrophic bacteria	5.78 ± 0.002
Fungi	5.77 ± 0.001
Actinomycetes	6.23 ± 0.003
N_2-fixing aerobic bacteria	2.39 ± 0.03
Pseudomonadas spp.	4.78 ± 0.06
Enterobacteriaceae	Abs. in 0.1g
Fecal streptococci	Abs. in 0.1g
Salmonella spp	Abs. in 25 g

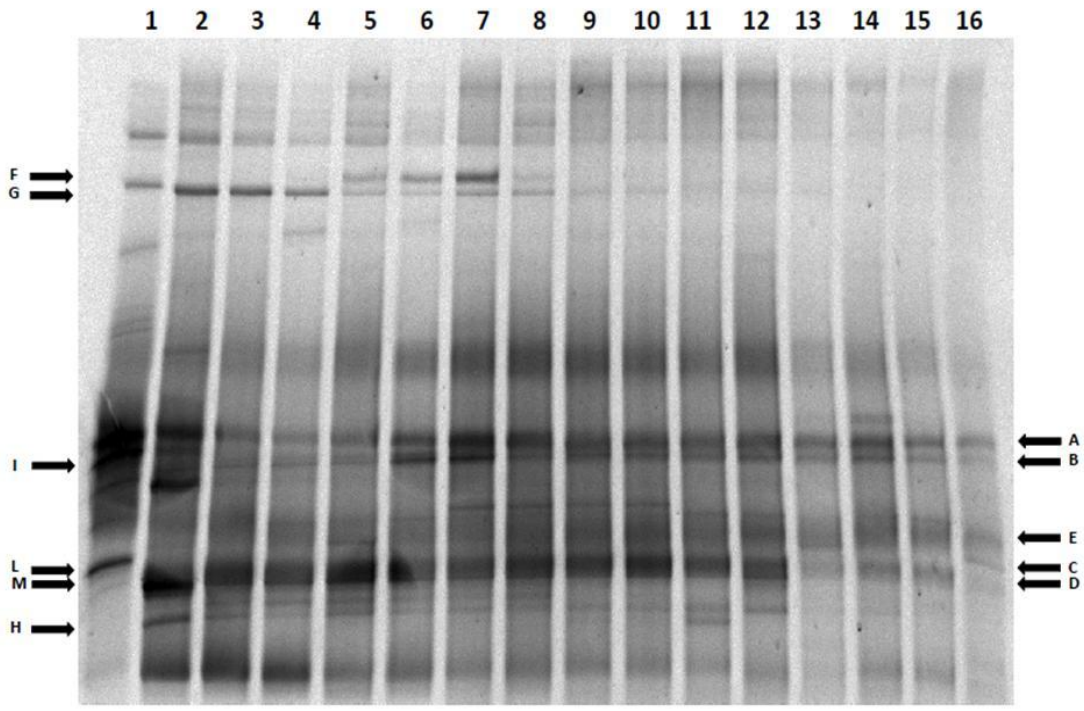

Figure 2. DGGE profiles of fungal population of chestnut waste during composting process. Lanes: 1, T0 (0 d); 2, T1 (15 d); 3, T2 (30 d); 4, T3 (45 d); 5, T4 (60 d); 6, T5 (75 d); 7, T6 (90 d); 8, T7 (105 d); 9, T8 (135 d); 10, T9 (165 d); 11, T10 (195 d); 12, T11 (225 d); 13, T12 (255 d); 14, T13 (285 d); 15, T14 (315 d); 16, T15 (345 d).

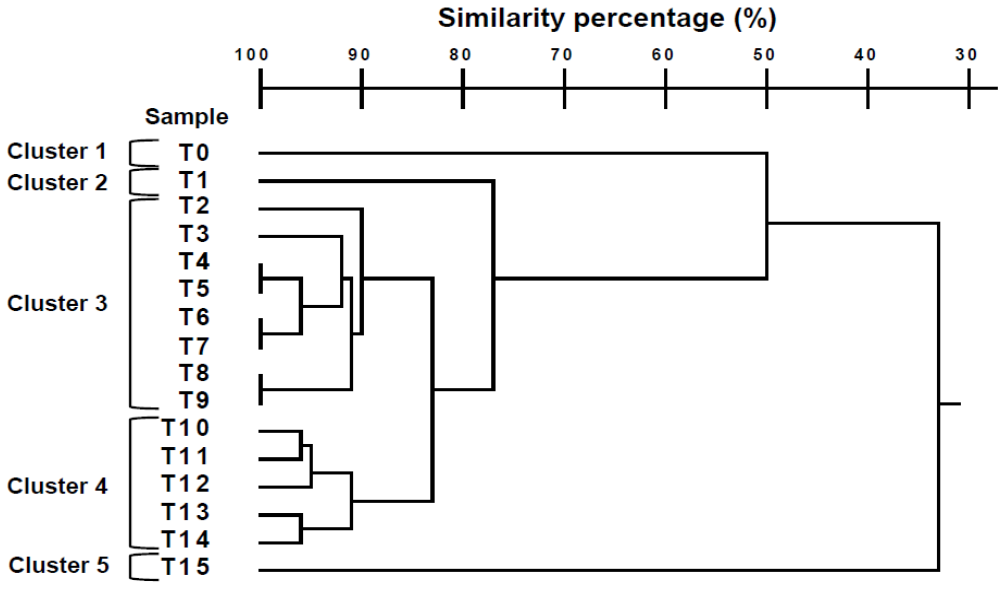

Figure 3. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the eukaryotic populations during composting of chestnut waste biomass. Sample: T0, 0 d; T1, 15 d; T2, 30 d; T3, 45 d; T4, 60 d; T5, 75 d; T6, 90 d; T7, 105 d; T8, 135 d; T9, 165 d; T10, 195 d; T11, 225 d; T12, 255 d; T13, 285 d; T14, 315 d; T15, 345 d. For the time intervals see the text.

Table 3. Identification based on the Blast comparison in the GenBank data libraries of the eukaryotic bands obtained by PCR-DGGE. Grey colour indicates the presence of the bands in the different samples.

Band[a]	Closest relative species (identity percentage)	Accession number	T0	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15
A	*Cladosporium* spp. (97%)	KJ458974.1																
B	*Paraconiothyrium variabile* (98%)	JX496139.1																
C-D	*Penicillium* spp. (98%)	FN598949.1																
E	*Fusarium* spp. (99%)	GQ505447.1																
F	*Mucor plumbeus* (98%)	HM849677.1																
G	*Mucor racemosus* (98%)	FJ345353.1																
H	*Aspergillus* spp. (97%)	GQ120973.1																
I	*Candida railenensis / Candida oleophila* (99%)	KF826531.1 / KJ095632.1																
L-M	*Moristroma quercium*/ *Moristroma japonicum* (98%)	AY254051.1/ AY254052.1																

[a] Bands are named as indicated on the DGGE gel showed in Figure 2.
[b] Accession number of the sequence of the closest relative species identified using the Blast software.

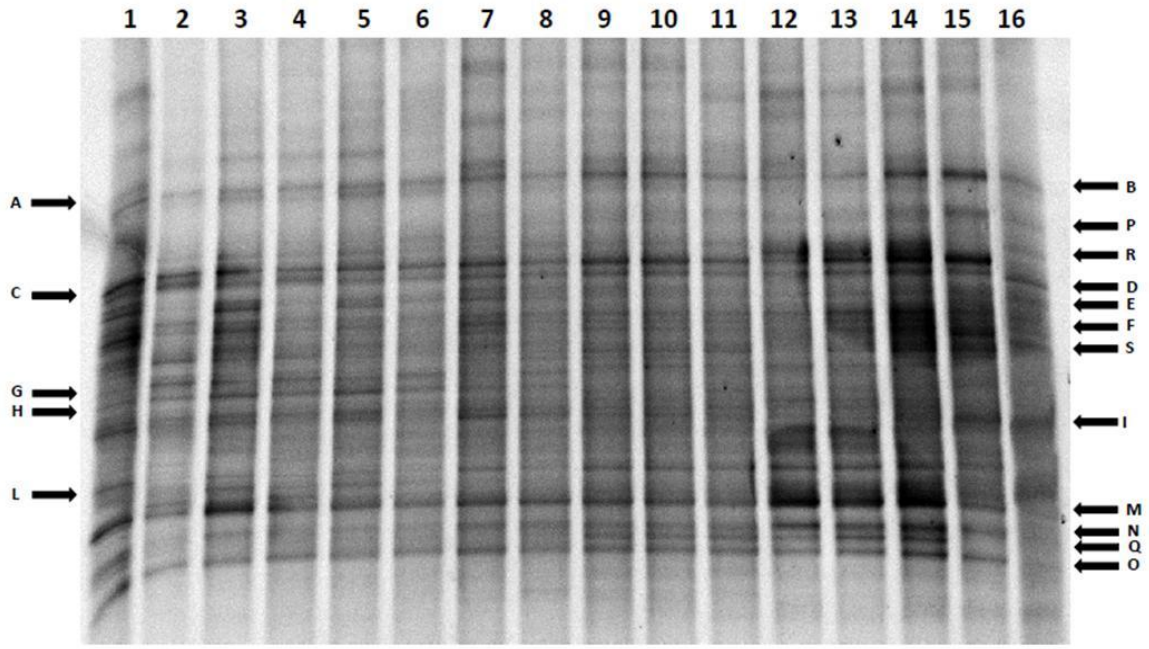

Figure 4. DGGE profiles of bacterial population of chestnut waste during composting process. Lanes: 1, T0 (0 d); 2, T1 (15 d); 3, T2 (30 d); 4, T3 (45 d); 5, T4 (60 d); 6, T5 (75 d); 7, T6 (90 d); 8, T7 (105 d); 9, T8 (135 d); 10, T9 (165 d); 11, T10 (195 d); 12, T11 (225 d); 13, T12 (255 d); 14, T13 (285 d); 15, T14 (315 d); 16, T15 (345 d).

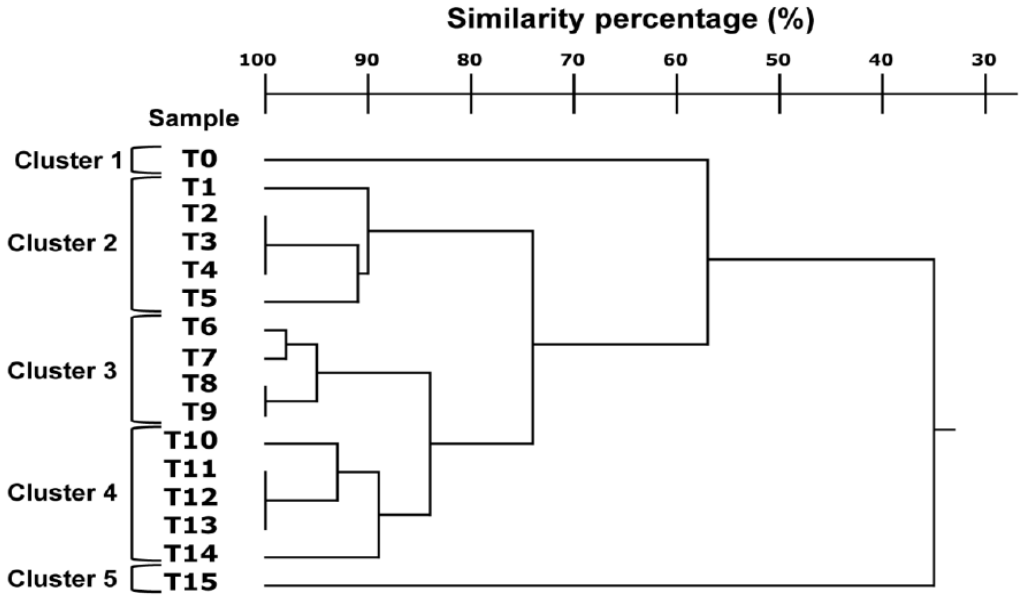

Figure 5. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the prokaryotic populations during composting of chestnut waste biomass. Sample: T0, 0 d; T1, 15 d; T2, 30 d; T3, 45 d; T4, 60 d; T5, 75 d; T6, 90 d; T7, 105 d; T8, 135 d; T9, 165 d; T10, 195 d; T11, 225 d; T12, 255 d; T13, 285 d; T14, 315 d; T15, 345 d. For the time intervals see the text.

Table 4**.** Identification based on the Blast comparison in the GenBank data libraries of the prokaryotic bands obtained by PCR-DGGE. Grey colour indicates the presence of the bands in the different samples.

Band[a]	**Closest relative species (identity percentage)**	**Accession number**	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**	**T13**	**T14**	**T15**
A	*Epilithonimonas tenax* (100%)	NR_041912.1																
B	*Streptomyces* spp. (96%)	DQ416200.1																
C	*Bacillus* spp. (95%)	AY489119.1																
D	*Bacillus megaterium* (100%)	NR_074290.1																
E	*Flavobacterium* spp. (98%)	KM046926.1																
F	*Flavobacterium* spp. (97%)	AY856849.2																
G	*Bacillus flexus* (98%)	NR_024691.1																
H	*Bacillus* spp. (97%)	KF254678.1																
I	*Actinomyces* spp. (97%)	DQ278863.1																
L	*Brevibacillus brevis* (98%)	NR_102862.1																
M	*Cellulomonas* spp. (95%)	HF954414.1																
N	Uncultered bacterium (96%)	JN802273.1																
O	Uncultured bacterium (96%)	AF534190.1																
P	*Pseudomonas* spp. (99%)	EU526849.1																
Q	Uncultered bacterium (96%)	KC852965.1																
R	*Stenotrophomonas* spp. (95%)	JN646018.1																
S	*Azotobacter* spp. (97%)	JN692282.1																

[a] Bands are named as indicated on the DGGE gel shows in Figure 4.

[b] Accession number of the sequence of the closest relative species identified using the Blast software.

4.4 Discussions

Composting is a complex aerobic microbial process in which different microbial populations ensure and maintains a healthy and balanced system. Thus, to discern the composting process is fundamental to understand the specific physiological activity of the different microbial groups that contribute to raw materials composting (Pepe *et al*., 2013). Effectiveness of the process depends mainly on the metabolic activities of these microbial groups affecting final product quality. For this reason, improving the knowledge of specific taxonomic and functional groups can allow relevant process improvements (Ryckeboer *et al.,* 2003). Because lignocellulosic materials are composed by complex molecules as hemicellulose, cellulose and lignin, different functional microbial groups with specific enzymatic activities were evaluated during the process. As previously described by Pepe *et al*. (2013), during the composting process, the microbial community would initially have grown by utilising the more accessible cellulose and hemicellulose (Van der Heijden, 2008) as demonstrated by the increase of the growth of cellulolytic and hemicellulolytic microbial groups in the first phases of the decomposition. The high incidence of these two microbial populations during the whole composting process plays an important role with respect to cellulose, that represents the principal constituent of vegetable waste and whose degradation is restricted to a narrow range of microbial enzymes as hemicellulase and cellulase (Herrmann *et al.,* 1997, Amore *et al.,* 2013). The ligninolytic population, responsible for the degradation of the more resilient lignin component, also did not show a constant trend. According to Huang and co-workers (2010) a continuous change in the microbial populations abundance during composting of lignocellulosic waste it's generally observed. The trend of functional populations detected in this research was affected by the decrease activity water and of temperature during the biooxidative process. In fact, microorganisms usually involved in the degradation of structural polymers (cellulose, hemicellulose and lignin) such as fungi and

Actinomycetes (Loveland *et al*., 2003; Amore *et al.,* 2012; Ventorino *et al*., 2015) could be negatively affected by changes of phisico-chemical parameters (Ishii *et al*., 2000). With regard to lignin decomposition, *Actinobacteria* and *Firmicutes* are the major taxa involved in its degradation (De Angelis *et al.,* 2011). Moreover, in the mature compost, the decreasing of concentration of the three functional populations, compared to the early stages of the process, was due also to deplete of assimilable fractions of the organic substrate.

As previously detected (Xie *et al*., 2003; Pepe *et al*., 2013) free-living (N_2)-fixing microorganisms in the chestnut compost reaching a microbial concentration lower than that usually found in soils. Anyway their presence in the final compost guarantees nitrogen fixation giving a fundamental support to the organic management of soils (Orr *et al.,* 2011). Moreover, the compost enriched of (N_2)-fixing bacteria could represent a compelling alternative to fertilizer addition to develop a sustainable agriculture (Xie *et al*., 2003, Pepe *et al.,* 2013).

The presence of relatively high concentration of *Pseudomonas* spp. in the mature chestnut compost is relevant since this genus is well known for its plant growth promotion activity such as preventing the invasion of soil pathogens, nitrogen fixation and improving minerals absorption (Altman *et al*., 1970; Burr *et al*., 1984; De Bertoldi *et al*., 1985; Orr *et al*., 2011; Pepe *et al.,* 2013).

However, the sanitary condition was evaluated determining the presence of *Enterobacteriaceae*, faecal enterococci spp. and *Salmonella* spp. Since these populations were not found the mature compost can be considered hygienically and sanitary safe (UNI 10780 of December 1998). In addition, the origin and the characteristics of the biomasses used represent a defined selected matrix, therefore, likely to be turned into quality compost (Chroni *et al*., 2009a; Chroni *et al*., 2009b). In fact, in chestnut compost the increase of the temperature in the mass, to reach the thermophilic phase that can ensure the sanitizing (Commission Regulation (EU) No. 142/2011), seems not necessary.

Moreover, prokaryotic and eukaryotic communities were also evaluated during the chestnut composting using molecular methods. The DGGE patterns of eukaryotic and prokaryotic populations highlighted that the number of the bands during the composting slightly varied and statistical analysis indicated that the most of the bacterial and fungal species in the chestnut compost persisted during the process period. This result could be due to the fact that the bio-oxidative process of lignocellulosic biomass is long and it was known that the decomposition of green-waste materials during composting is characteristically slow (Kelleher *et al*., 2002).

During composting process, many fungi and bacteria are involved in the degradation of complex polymeric substrates such as cellulose, hemicellulose, pectin, and lignin. In general, fungi are known as the most efficient lignocellulose degraders since their mycelial structure is a competitive advantage than bacteria (Li *et al*., 2013). During the entire chestnut composting process, the DGGE patterns of eukaryotic populations showed a predominance of *Ascomycota*, including species of the genera *Penicillium, Moristroma, Paraconiothyrium, Fusarium, Cladosporium* and *Aspergillus*. This result is in according to Cahyani *et al*. (2004) and Jurado *et al*. (2014) which reported that although different eukaryotic members occurred in the different typical stages of rice straw and lignocellulose-based composting, *Ascomycota* were ubiquitous during the entire process. Several studies reported the dominance of mesophilic fungi such as *Aspergillus*, *Fusarium* and *Mucor* (Mehta *et al*., 2014) during the later stages of composting. Different fungi belonging to *Zygomycetes* and *Ascomycetes* can degrade cellulose and hemicelluloses (Schulein, 2000). High cellulolytic activities were observed in *Fusarium* (Jahangeer *et al*., 2005), *Mucor* (Ryckeboer *et al.,* 2003; Oyeleke and Okusanmi, 2008) *Aspergillus, Cladosporium* and *Penicillium* species. Production of β-glucosidase of *Penicillium* genus was well documented (Li *et al*., 2013). Species belonging to the genera *Cladosporium*, *Penicillium* and *Mucor* isolated from compost were also able to degrade starch and lignin

(Ryckeboer *et al.,* 2003). Interestingly, in this work the occurrence of *Paraconiothyrium variabile* during the composting process was first highlighted. Although the presence of this specie in green waste composting was no previously reported, it was documented its ability to produce laccase (Asadgol *et al*., 2014) that could contribute to breakdown the lignin component in the process.

The bacterial populations involved in the composting of chestnut waste were identified by the nucleotide sequences of V3 region of 16S rRNA gene sequences. The selected prokaryotic bands were affiliated with *Bacilli*, followed by *Actinobacteria*, *Flavobacteriia* and γ-*Proteobacteria*.

According to Chandna *et al*. (2013) the *Bacilli,* represented primarily by *Bacillus megaterium*, *Bacillus flexus* and *Bacillus* spp., was the predominant taxon present throughout the whole process. On contrary *Bacilli* was not but detected in the mature chestnut compost as indeed reported by Jurado *et al*., (2014) that recovered it from compost feedstock. Usually, spore-forming bacteria such as *Bacilli* are reported as dominant species in compost material because their high thermo-tolerance allow them to survive during the thermophilic stage (Fracchia *et al*., 2006; Insam & de Bertoldi, 2007; Li *et al.*, 2013). The low incidence of spore-forming bacteria in the mature compost obtained from chestnut waste could be due to the fact that in our work the temperature of the process is lower than employed in the composting. Moreover, other mesophilic bacteria, involved in the degradation of lignocellulosic material, compete with the vegetative form of *Bacilli* for carbon source and nutrients. It is known that members belonging to *Bacillus* spp. have specific genes encoding enzymes involved in cellulose and hemicellulose degradation (Amore *et al*., 2013a; Amore *et al*., 2013b; Di Pasqua *et al*., 2013). *Brevibacillus brevis* (*Bacilli* class), no usually reported as habitant of composting piles, showed the same trend of *Bacillus* spp. since it was not recovered in the mature compost as also previously evidenced by using culture-dependent and culture-independent methods (Jurado *et al*., 2014; de Gannes *et al*., 2013) Bacteria belonging to the *Actinobacteria* class, such as *Streptomyces* spp., *Actinomyces* spp.

and *Cellulomonas* spp., secrete enzymes to efficiently degrade lignocellulosic materials under neutral to alkaline conditions (Wang *et al*., 2014). According to Jurado *et al.* (2014) and Ryckeboer *et al.* (2003), this taxon was typically present in the compost during all phases of process. Their predominance could be due to the their ability to degrade the more recalcitrant compounds after the degradation of easily degradable carbon source (Toumela *et al*., 2000). In particular, *Cellulomonas* species (de Gannes *et al*., 2013) are able to produce at least six endoglucanases and one exoglucanase (Chaudhary *et al*., 1997), High level of extracellular *endo*-1,4-ß-glucanase activity were also found for the strain *Cellulomonas flavigena* CP713 isolated from *Populus nigra* biomass (Ventorino *et al*., 2015). In addition, Akasaka and co-workers (2003) report that the predominant group involved in the degradation of rice plant residues was closely related to *Cellulomonas*. *Actinobacteria*, and in particular *Streptomyces*, is known as one of the most efficient plant biomass-degrading microbes in peat swamp forests (Kanokratana *et al*., 2011). Several *Streptomyces* spp. strains are involved in the degradation of cellulose, hemicellulose and lignin and they are reported as producers of endocellulases, laccases and peroxidases (Amore *et al*., 2012; Woo *et al.,* 2014). Franke-Whittle and co-workers (2009) recovered the presence of high levels of *Actinobacteria*, such as *Actinomyces* spp., in green waste composting and in particular in the mature compost.

The *Flavobacteriia* group was represented by the genera *Flavobacterium* spp. and *Epilithonimonas tenax*. *Flavobacterium* species are able to degrade cellulose and low molecular weight lignin fragments (Huang *et al*., 2010) and to produce cellulase capable to hydrolyze crystalline cellulose (Lednicka *et al*., 2000). In recent study, the dominance of these bacterial species is detected in different environments such as in crystalline-cellulose amended soil (Kumar & Khanna, 2014) and during the degradation of insoluble cellulose in a marine environment (Edwards *et al*., 2010). By contrast, *Epilithonimonas tenax* was detected only in

the initial material (T0) showing any involvement in the degradation process of the chestnut waste.

Bacteria belonging to the γ-*Proteobacteria* class, such as *Azotobacter* spp., *Stenotrophomonas* spp. and *Pseudomonas* spp., were recovered during the last phases of the composting process and/or in the mature compost. According to Franke-Whittle *et al*. (2014), *Azotobacter* spp. was not found in the initial substrate, but developed during composting and was found in the mature compost samples. The presence of these genera has been previously reported in mature compost bacterial communities (Danon *et al.,* 2008; Pepe *et al*., 2013a; Ryckeboer *et al.,* 2003). Members of *Pseudomonas* and *Stenotrophomonas* genera are known as efficient bacteria to degrade plant lignin, (hemi)cellulose and/or CMC (Talia *et al*., 2012; Yang *et al*., 2007; Wang *et al.,* 2013).

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Chapter 5 Agronomic evaluation and plant fitness

5.1 Introduction

Beneficial effects of organic materials on different aspects of plant health have been widely reviewed by several authors (Bayles *et al.,* 2003; Van Elsas e*t al.,* 2007; Noble *et al.,* 2005). Different works have shown that organic ammendants can increase plant growth and crop yield both in herbaceous and woody plants. The typical growth response curves reported as result from plants treated with humic substances, show progressively increased growth with increasing concentrations of humic substances, up to the critical point when the fitness ability drops down (Chen & Aviad, 1990). Hypotheses for this stimulation effect of discrete concentrations of humic substances are numerous. Most likely, this is due to ‘‘direct’’ hormo-like action on plant, together with an ‘‘indirect action’’ on soil microorganisms metabolism, nutrients uptake dynamics, and physical characteristics (Cacco & Dell'Agnola 1984; Nardi *et al*., 1988; Albuzio *et al*., 1989; Casenave de Sanfilippo *et al*., 1990; Chen & Aviad 1990; Muscolo *et al*., 1993, 1996, 1999).

As previously mentioned, plants exposed to abiotic stress, like excess of humic substance, exhibit a broad range of morphogenic responses. Despite the diversity of phenotypes, a generic “stress-induced morphogenic response” can be recognized: (a) inhibition of cell elongation, (b) localized stimulation of cell division and (c) alterations in cell differentiation status. It is hypothesized that the similarities in the morphogenic responses induced by distinct stresses, reflect common molecular processes such as production increase of Reactive Oxinen Species (ROS) (Geert *et al.,* 2007). Most common protection systems against ROS uses enzymes able to transform in less reactive and toxic compounds. If in the past ROS were considered only harmful to cells, it is now recognized that the redox regulation, involving the products of oxidative stress, is also an important factor in

the modulation of cellular activity (Allen &Tresini, 2000). In fact, in plant tissues, hydrogen peroxide acts as a signaling molecule for defense genes activationion (Levine *et al*., 1994) involving programmed cell death and Hypersensitive Response (HR) (López Huertas *et al*., 2000).

5.2 Materials and Methods

5.2.1 Effect of the chestnut compost on plant

To evaluate the effect of the chestnut compost on plant, the following parameters were considered: photosynthetic pigments concentration, leaf area, aboveground and roots biomass, crown thickness, and antioxidant activity.

Tomato (*Lycopersicon esculentum* Mill) seedlings F1 hybrid Valser, were grown by the side of the farm "Vivaio Di Maio", Francolise (CE). Plantlets were sown in different three diverse media: compost (100%), compost and neutral peat 1:1 (v/v), and neutral peat (100%) as control. For each treatment 180 tomato seeds were evaluated. Seeds germination took place in climatic chamber at 24°C and 66% UR (fig.1), three days after plantlets were repositioned in greenhouse at 18 °C for 40 days (fig. 2).

Figure1. Tomatoes seeds germination in climatic chamber

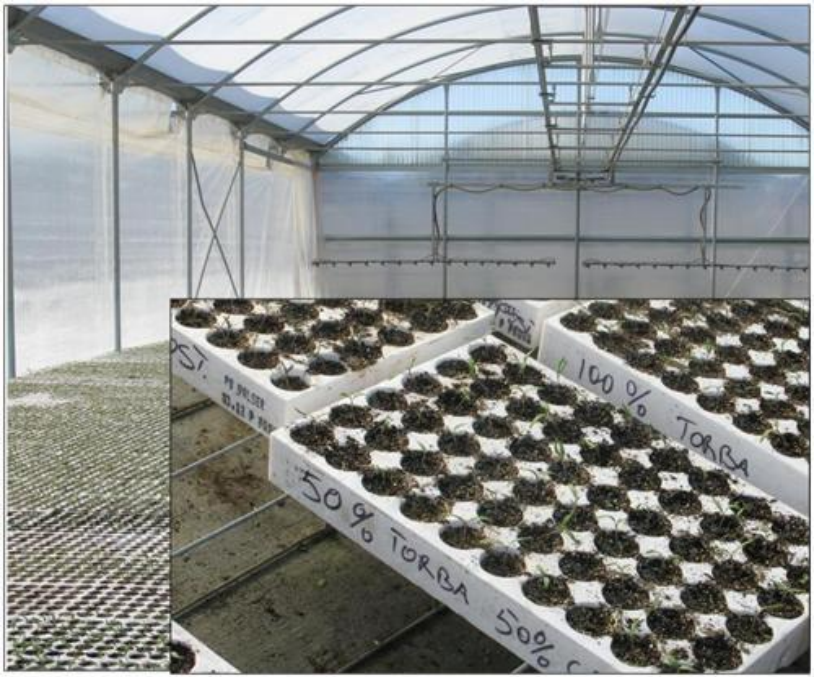

Figure 2. Tomatoes plantlets in greenhouse

The concentration of different photosynthetic pigments**,** chlorophyll a (chl a), chlorophyll b (chl b), total carotenoids and xanthophylls + beta carotene (Cx + c), was determined in leaves. 75 mg of leaves lamina, from 20 plants for each

treatment, were immersed in 2.5 mL of N-Ndimethylformamide for pigments extraction. The pigments amount was estimate by measurement of absorbance spectrophotometer (µg/g Fresh Weight): at 664 nm for chl a, at 647nm for chl b and at 480nm for carotenoids (Wellburn, 1994).

Leaf area (cm^2) was measured by image analysis using Adobe Photoshop v.16.0 of digital images acquired with Epson 10000 XL Scanner (Matthew *et al.,* 2002).

Biomass obtained in the different thesis was measured, in 40 days hold plant, as roots and green part dry weight (dw). It was also measured the plant elongation (from the soil level to the top leaf node) and of the roots. The crown thickness was measured using an electronic calliper (Atiyeh e*t al.,* 2002).

5.2.2 Determinations of antioxidant activities

Antioxidant activity was evalued on leaf tissue, 75mg fresh weight were ground to a fine powder in mortar using liquid nitrogen. Total soluble proteins were extracted using a buffer containing 0.1M potassium dihydrogen phosphate (pH 7.8), 1 mM EDTA (pH 7.0), 0.2% (v/v) Triton X-100, 2 mM dithiothreitol (DTT) and 5% (w/v) polyvinylpolypyrrolidone (PVPP).

100 µL of ascorbic acid 0.33 mM was also added when the ascorbate peroxidase activity was determined. The mixture was centrifuged at 4°C for 20 min at 14.000 *g* and the supernatant used for the enzyme assays.

Total protein content was determined as described by Bradford (1976), according to the protocol, a series of standard solutions of albumin protein bovine serum (BSA) were prepared to build a calibration line. The absorbance was measured at 595 nm.

Superoxide dismutase (SOD; E.C. 1.15.1.1) activity was assayed by determining its capacity to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) as described by Garcìa-Limones *et al*. (2002).

The assay was conducted in triplicate in a final volume of 1.5 mL, each treatment contains:

• 200 µL of extract

• 150 µL of buffer KH_2PO_4 500 mM pH 7.8

• 195 µL methionine100 mM

• 15 µL EDTA pH 7.0, 10 mM

• 70 µL NBT 1.6 mM

• 120 µL riboflavin 25 mM

The reaction began with the exposure of the mixture to four white light fluorescent bulbs, of 1000 lumens. The blue colour developed in the reaction was measured at 560 nm and the corresponding samples not exposed were used as a blank. The activity of superoxide dismutase was expressed as U mg^{-1} protein.

Catalase (CAT; E.C. 1.11.1.6) activity was determined according to Fernandez-Trujillo *et al.*(2007) in triplicate in a final volume of 1.5 mL, each treatment contains:

• 15 µL extract

• 150 µL KH_2PO_4 buffer, 50 mM at pH 7

• 340 µL H_2O_2, 20 mM

The control consisted in a mix of the total of components except for H_2O_2. The catalase reaction starts with the addition of the substrate (H_2O_2) and it was observed the decrease at 240 nm in 100 seconds, following its decomposition. The catalase activity is expressed as mol H_2O_2 min^{-1} mg^{-1} protein.

Guaiacol peroxidase (G-POD; E.C. 1.11.1.7) assays were performed according to the method described by Garcìa-Limones *et al*. (2002), the assay was conducted in triplicate in a final volume of 1.5 mL, each treatment contains:

• 20 µL extract

• 200 µL buffer KH_2PO_4 100 mM pH 7

• 469 µL guaiacol 32 mM

• 37 µL H_2O_2 88 mM

The reading was performed at 470 nm for 200 seconds, following the increase in absorbance due to the transformation of guaiacol in tetraguaiacolo mediated by the presence of the enzyme guaiacol peroxidase.

The activity of guaiacol peroxidase was expressed as nmol of tetraguaiacolo min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX; E.C. 1.11.1.11) is an enzyme belonging to the class of oxidoreductases, which catalyzes the following reaction:

$$\text{L – ascorbate} + H_2O_2 \xrightarrow{\text{APOX}} \text{dehydroascorbate} + 2\ H_2O$$

The assay was conducted in accordance with Rao *et al.* (1996) in a final volume of 1 mL, each treatment contains:

• 5 µL of extract

• 300 µL buffer KH_2PO_4 100 mM pH 7

• 6 µL of H_2O_2 0.35 mM

• 100 µL ascorbic acid 0.33 mM

The whites were constituted by the all assay components except for ascorbic acid and H_2O_2.

Once ascorbic acid and H_2O_2 were added, it was measured the decrease in absorbance at 290 nm for 50 seconds, and calculating the ΔAbs/min.

The activity of ascorbate peroxidase was expressed as mol of ascorbate ox. min^{-1} mg^{-1} protein

Ascorbate oxidase (AOX; E.C. 1.10.3.3) activity was measured recording the absorbance reduction at 265 nm as described by Garcìa-Limones *et al.* (2002).

The assay was conducted in a final volume of 1.5 mL for each treatment contains:

• 30 µL of extract

• 300 µL buffer KH_2PO_4 100 mM pH 7

• 20 µL ascorbic acid 5 mM

The whites were made of all assay components except for ascorbic acid.

After supplying ascorbic acid, it was measured the decrease in absorbance at 265 nm for 200 seconds, and calculating the ΔAbs / min.

The ascorbate peroxidase activity was expressed as nmol of ascorbate ox. min^{-1} mg^{-1} protein.

Hydrogen peroxide (H_2O_2) content was estimated after reaction with potassium iodide as described by Garcìa-Limones *et al*. (2002).

The determination involves the use of trichloro-acetic acid (TCA) 1% and 1M KI, in a volume of 2 ml, each treatment contains:

• 300 µL extract

• 1.3 ml KI 1M

• 333 µL TCA 0.1%

The samples were left in the dark for 60 minutes after which the reading was performed at 390 nm. The concentration of the sample was expressed as mol H_2O_2 g^{-1} FW.

5.2.3 Statistical analysis

One-way ANOVA followed by Duncan post hoc test for pair wise comparison of means (at $p \leq 0.05$) was used to assess the differences in plant fitness parameters. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

5.3 Results

5.3.1 Plant Fitness

Concentration of chlorophyll a in plant samples grown on compost was 594.45 µg g^{-1} F.W, lower than the concentration in 100% peat 1064.52 µg g^{-1} F.W and in mix peat and compost 733.23 µg g^{-1} F.W. Concentration of chlorophyll b and carotenoids followed analogous trends (table 1).

A table 2 show leaves expansion, plants grown on 100% compost have the lowest leaf surface, 28,04 ± 6.01 cm^2, respect to those one grown in peat the largest, 75.07 ± 5.70 cm^2, while mixed medium stimulate intermediate results, 53.23 ± 7.80 cm^2. In addition the compost-peat mix treatment showed higher values for both the plant height and roots length. Same result occurred for crown thickness: larger in the mixture thesis; while peat implicated intermediate values (table 2, fig. 3)

Table 1. Concentration of photosynthetic pigments. Vertical letters indicate statistical difference between thesis (P =0.05) Pot substrate: P, neutral peat; P.C, neutral peat + chestnut compost (1:1);C, chestnut compost.

Pot Substrate	(Ca) (µg/g FW)	(Cb) (µg/g FW)	(Cx + c) (µg/g FW)	chl a/chl b	Car/chl
C	594.45[a]± 30.25	209.80[a]± 7.41	158.73[a]± 8.72	2.82[a] ± 0.04	0.19[a] ± 8.72
P	1064.52[c]± 55.05	342.37[c]± 13.71	220.24[c]± 3.18	3.10[b]± 0.06	0.15[b] ± 8.72
P.C	733.23[b]± 51.48	247,52[b]± 21.41	175.64[b]± 8.90	2.96[a]± 0.11	0.17[b] ± 8.72

Table 2. Vegetative parameters measured in tomato plants. Vertical letters indicate statistical difference between treatments (P=0.05). Pot substrate: P, neutral peat; PC, neutral peat + chestnut compost (1:1); C, chestnut compost

Pot Substrate	Plant height (cm)	Roots length (cm)	Crown thickness (mm)	Leaf area (cm^2)	Dried biomass (g/ss)	Dried root mass (g/ss)	roots/ shoots (g)
C.	7.95± 0.81[a]	13.01±3.2[a]	2.47± 0.24[a]	28.04± 6.01[a]	21.01± 0.24[a]	6,50± 0.24[c]	0.30± 1.77[a]
P.	12.11± 1.64[b]	14.41±3.39[a]	3.54± 0.33[b]	75.07± 5.70[c]	55.40 ± 0.02[b]	9.19± 0.29[b]	0.16 ± 0.05[b]
P. C.	13.08 ± 1.12[c]	17.75±5.10[b]	3.62± 0.20[b]	53.23± 7.80[b]	55.29 ± 0.09[b]	10,33[a]± 0.45[a]	0.18 ± 0.05[ab]

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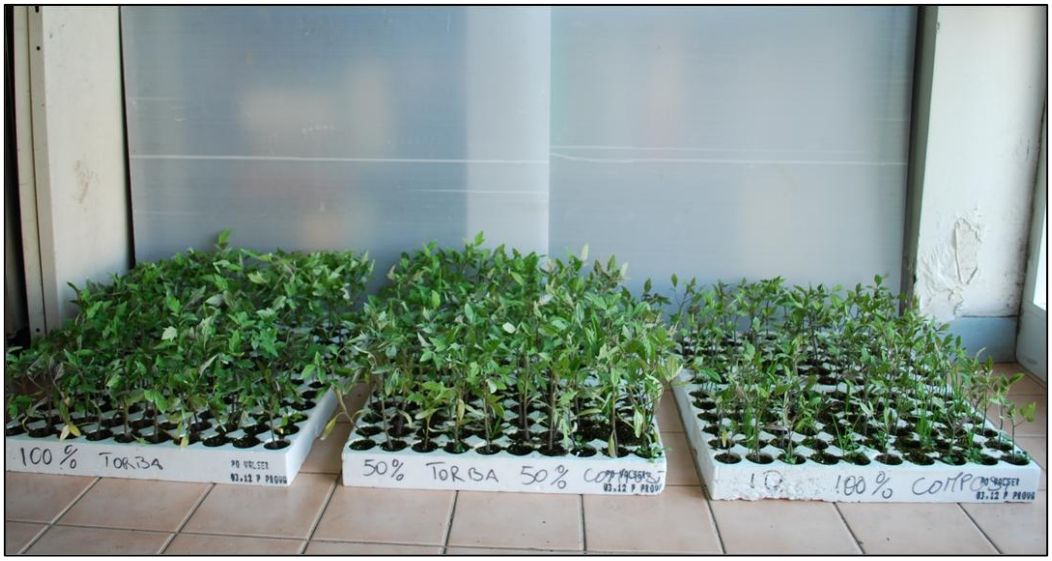

Figure 3. Tomato plants in three substrates: compost, mix medium, peat.

5.3.2 Antioxidant response

Compost as a soil supplement significantly influenced the CAT, APX and AOX activities in tomato plants. In fact, plants grown in compost showed the highest antioxidant activities, while in those grown mixed medium the lowest (table 3). The same trend was observed for H_2O_2 content since 2.49±0.02, 1.54 ± 0.05, and 1.75±0.02 μmol mg^{-1}, FW were estimated in tomato leaves grown on compost, mix medium, and peat, respectively (table 3).

Leaf G-POD activity was significantly enhanced in concomitance with the compost doses. In fact, the highest values were recorded in plants grown in compost and mixed medium, 180.33±7.48 and 187.71±5.43 nmol tetraguaicol min^{-1} mg^{-1} protein, respectively, compared to control (122.20±1.03 nmol tetraguaicol min^{-1} mg^{-1} protein). By contrast, only leaf SOD activity was unaffected (0.27±0.05 U mg^{-1} protein) when plants were grown on mixed medium (0.30±0.01 U mg^{-1} protein) and in 100% compost (0.26±0.05 U mg^{-1} protein) (table 3).

Table 3.Superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD), ascorbate peroxidase (APX) and ascorbate oxidase (AOX) activities and hydrogen peroxide (H_2O_2) content in tomato leaves. The values represent the means ± SD of three replicates of three independent experiments. Different letters after the values indicate significant differences (P =0.05)

Pot Substrate	SOD (U mg^{-1} protein)	CAT (μmol H_2O_2 min^{-1} mg^{-1} protein)	G-POD (nmol etraguaicol $min^{-1}mg^{-1}$protein)	APX (μmol AsA min^{-1} mg^{1} protein)	AOX (nmol AsA min^{-1} mg^{-1} protein)	H_2O_2 (μmol mg^{-1} FW)
C.	0.26±0.05[a]	20.29±0.84[c]	180.33±7.48[b]	3212.98±52.09[c]	1840.16±29.83[c]	2.49±0.02[c]
C.P	0.30±0.01[a]	16.18±0.47[a]	187.71±5.43[b]	1021.95±11.76[a]	262.79±3.02[a]	1.54±0.05[a]
P.	0.27±0.05[a]	17.39±0.15[b]	122.20±1.03[a]	1135.15±32.58[b]	312.57±8.24[b]	1.75±0.02[b]

5.4 Discussions

The concentration of chlorophyll (a), chlorophyll (b) and carotenoids was lower in treatments with 100% of compost. Tomato plants on 100% peat showed the highest concentration of the photosynthetic pigments, while the seedlings grown mixture compost-peat revealed an intermediate value. Carotenoids/total-chlorophyll ratio do not differentiate significantly among the treatments. Carotenoids are antioxidants, with non-enzymatic activity (Smirnoff, 2000; Foyer *et al*., 2001; Ryan *et al*., 2002). In our tests plants grown on compost, showed higher presences of hydrogen peroxide (table 3). This can explain the higher concentration of carotenoids in leaves of seedlings grown in compost.

The three treatments greatly differ in the vegetative parameters; the seedlings in compost resulted to have lower leaf surface, while those in peat showed larger leaves. The mixed substrate showed intermediate values. Leaf area is an index of plant growth that results by the interaction of plant physiology with environmental factors, including the soil characteristics. The aboveground biomass or Fresh Biomass (FBM), and analogously Dried Root Mass (DRM) are commonly measured as dry weight (Cornelissen *et al*., 2003).

Root system proliferation is a significant indication of the degree of plant adaptation, plants grown in suitable environmental conditions tend to allocate less biomass in their root systems, while plants grown in poor resource environments develop a more powerful root system (Jackson *et.al.,* 1996; Grime *et al*., 1997; Tilman, 1988).

Plants raised on compost showed a general reduction of the size of the aerial part, on the other hand the root system resulted potentiated. Those represent the first generic evidences of an induction of resistance table 1 and 2 (Heil and Baldwin 2002). Vegetative parameters, of plant raised on compost were all lower except for the root weight. This confirms that: plants grown in compost were in higher stressful conditions (table1 and 2) (Chen & Aviad, 1990).

Stress also triggers production of reactive oxygen intermediates superoxide (O_2^-) and hydrogen peroxide (H_2O_2). This oxidative burst induces several plant genes involved in cellular protection and defence, and is necessary for the initiation of host cell death in the hypersensitive disease-resistance response (HR). Additionally, infections induce antioxidant defences and particularly, different components of the ascorbate-glutathione cycle. (Gönner & Schlösser, 1993; El-Zahaby *et al.*, 1995).

In fact antioxidants, such as ascorbate, glutathione, and tocopherol interact with numerous cellular components and influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and death (De Pinto & De Gara, 2004; Potters *et al.,* 2004; Tokunaga *et al*., 2005). Most importantly they influence gene expression associated with biotic and abiotic stress responses to maximize defence. Antioxidants continuously manage ROS; growing evidence suggests a model for redox homeostasis in which ROS–antioxidant interactions mediate environmental signals to metabolic activity.

Seedlings grown in compost in which the concentration POD was high, showed a greater consistency of the vegetative tissues, compared to seedlings grown in the other two treatments. This can be explained by POD through peroxidative reaction, catalyze the cross-links between matrix components and the polymerization of lignin, thus increase the thickness and reducing the extensibility of the cell wall (Iiyama *et al*., 1994; Pomar *et al*., 2002). A negative correlation between peroxidase activity and cell elongation is, infact, widely accepted (Zheng & Van Huystee, 1992; Zarra *et al*., 1999).

In table 3 appears that the value of the POD is lower compared to other antioxidants, while that APX is the higher. In fact both APX and POD compete for H_2O_2 as substrate. Apparently APX present an H_2O_2 affinity higher than POD. (Còrdoba-Pedregosa *et al*., 1996, 2003a, b)

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Chapter 6 Compost suppressiveness

6.1 Introduction

The beneficial effect of compost application in agriculture is well recognized due to physicochemical properties and especially to the microbiological composition, numerous studies demonstrated its potential to stimulate plant growth and suppress soil-borne diseases (Martin & Brathwaite, 2012). In fact, the incorporation into the soil of microbial populations that characterize the compost could increase the soil fertility (nitrogen-fixing bacteria and nitrifiers), improve the structural stability (exopolysaccharide producers), could synthesize and excrete hormones, chelators and nutrients such as amino acids and vitamins (Boulter *et al.,* 2002) and have other effects increasing soil biological activity and quality (Buyer *et al.,* 2010). Moreover, composting has not only achieved reputation as method for resource management by sustainable nutrient recycling and as a environmental friendly alternative way to producing fertilizer, but also for providing a product which can be used to suppress soil pathogens affecting plants (Hagn *et al.,* 2008). In fact, among the biological control practices, the use of compost to suppress plant diseases has a great potential (Noble & Conventry, 2005). There are several reports that demonstrated the suppressive effect of compost from different matrices against a wide range vegetable diseases caused by a variety of soil-borne plant pathogens including *Rhizoctonia solani*, *Sclerotinia* spp., *Phytium* spp., *Fusarium* spp., *Phytophthora* spp. (Cotxarrera *et al*., 2002; Martin & Brathwaite, 2012). Suppressive characteristics of composts are due to the presence of specific antagonistic microorganisms (Huang *et al*., 2012) able to control diseases by different mechanisms including antibiotic production, parasitism, competition for nutrients, antibiosis and other extracellular enzymes, root surface colonization and induction of systemic resistance in the plants (Hoitink & Boehm, 1999). The suppressiveness varies greatly between different types of organic matter: lignin and

other recalcitrant components, decompose slowly and thus are able to support a longer activity of specific beneficial microorganisms (Hoitink & Grebus, 1994). Despite the large number of studies, there are no methods able to predict the effect of the organic substance on pathogens in soil (Erhart *et al*., 1999; Scheuerell *et al.*, 2005; Termorshuizen *et al*., 2007). Currently, a large number of studies have focused on different aspects of the organic amendments: the ability to types of organic matter to contain different plant pathogens (Abawi & Widmer, 2000; Akhtar & Malik, 2000; Litterick *et al*., 2004; Coventry & Noble, 2005), and support the work of beneficial microbes (Hoithink & Boehm, 1999); the application of the compost tea and the aqueous extracts of compost (Scheuerell & Mahaffee, 2002); eradication of pathogens during the composting process (Noble & Rob, 2004). Peat is the most utilized organic substrate for the preparation of potting mix (Carlile, 2009) because of its positive agronomic characteristics such as constant chemical and physical properties, high capacity of water retention, optimal porosity and controlled pH. However, peat is hardly ever suppressive against soil-borne pathogens such as *Rhizoctonia solani* and *Sclerotinia minor*, and fungicides are routinely used to manage damping-off diseases. Moreover, the use of peat for horticultural purpose will probably be discouraged because of its limited sustainability and negative impact on global climatic changes due to the production of greenhouse gases (Carlile, 2009). On the basis of these observations, in this study, the suppressive activity of chestnut compost to use as a soil supplement was estimated determining its effect on soil-borne plant pathogens in *in vitro* and *in vivo* experiments on tomato plants.

6.2 Materials and Methods

Chestnut compost suppressiveness activity was evaluated by *in vivo* and *in vitro* experimentation, evaluating plant induction and antagonistic activity in respect of bioticstress by *Sclerotinia minor* and *Rhizoctonia solani*.

6.2.1 *In vitro* assay

In vitro assays were performed using five different solid media: 100 % peat water extract (SM1); 50% compost water extract + 50% water (SM2); 50% peat water extract + 50% compost water extract (SM3); 30% peat water extract + 70% compost water extract (SM4); 100% compost water extract (SM5). Agar bacteriological (Oxoid, Milan, Italy) was added to media (20 g/L). Peat and compost water extract were prepared using distilled water (1:1 v/v) left shaking, 250 RPM *(*Revolutions Per Minute*),* overnight. The suspension was centrifugated 5000g and the supernatant sterilised by micro-filtration through 0.22 mm pore filters (Bonanomi *et al.,* 2007). One plaque, 5 mm in diameter, of mycelium growth of each fungus was placed in the centre of the plate. All plates, prepared in five replicates, were incubated at 28 °C. Radial mycelium colony growth was measured for 2 weeks every 24 h and the percentage of Mycelial Growth Inhibition (MGI) was calculated using the following formula (Nwachukwu E.O. & Umechuruba C.I., 2001):

$$\% \text{ MGI} = [(\text{DCO-DW}) / \text{DCO}] \times 100 \ (\%)$$

Where DCO is the mean of mycelia diameter of colonies grown in the control medium (100% peat water extract) and DW is the mean of mycelia diameter of colonies grown in the medium containing the compost water extract.

6.2.2 *In vivo* assay

To evaluate compost's effect on plant as a soil supplement and to determine the optimum rate of compost in soil, tomato seeds (*Lycopersicon esculentum* Mill) already germinated (figure1), were sown in aluminium pot (figure 2) (20x15x5) containing 1L of growing medium (15 seed pot-1) in different substrates: neutral peat as control (P), sterile compost (C.S) and compost (C). The sterilized compost was obtained by placing the compost by autoclaving at 120 °C for 30 minutes. Plantlets were positioned in greenhouse according to a fully randomized design at 27.6°C day, 22.5 °C night and inoculate with *Sclerotinia minor* (S.) and *Rhizoctonia solani* (R.). Plants were watered daily at field capacity, and monitored for 30 days; during which were performed biometric observations as: plant height, leaf area, presence of symptoms and signs. 30 days later, shoots and roots dry weights (g/plant) were determined after drying at 200°C. Also was calculated the percentage of suppression disease index (DSI) (Talibi *et al.,* 2012) as follows:

$$\% \text{ DSI} = [(NP - NM) / NP] \times 100 \ (\%)$$

Where NP is the mean of died plants in the control pots (peat) and NM is the mean of died plants in pots containing compost. For each treatment 45 tomato seedling were used. Pathogen inoculums, originally isolated from natural infections, were re-isolated from tomato at the end of the experimentation.

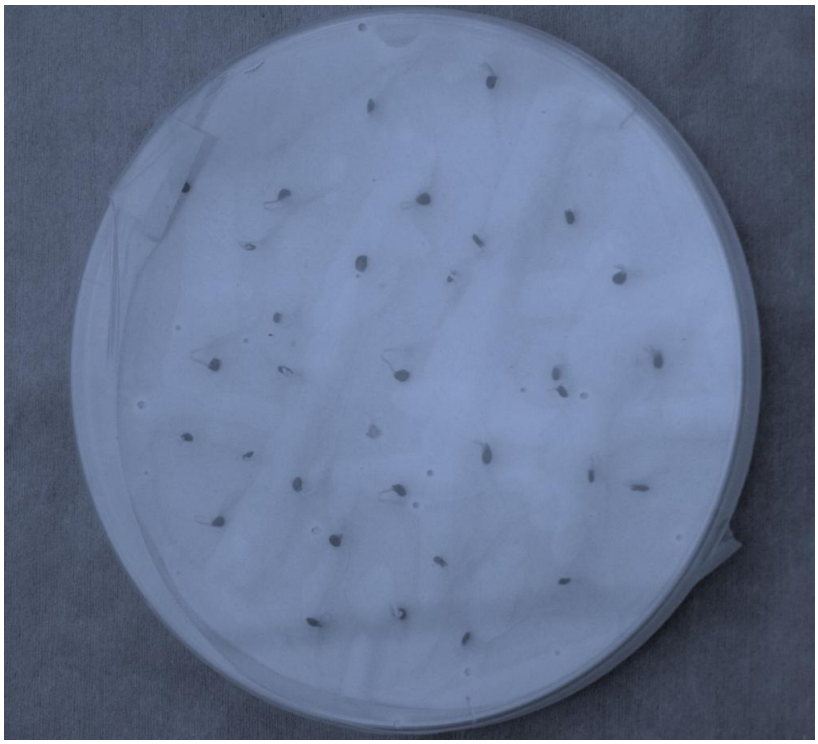

Figure1. Tomato germinated seeds

Figure 2. Tomato plants in aluminium pot

6.2.3 Statistical analysis

One-way ANOVA followed by Duncan *post hoc* test for pair wise comparison of means (at $p \leq 0.05$) was used to assess the differences in plant fitness parameters. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

6.3 Results

The compost showed a high suppressive activity against *S. minor* and *R. solani* both by *in vitro* and *in vivo* experimentations.

6.3.1 *In vitro* assay

The presence of compost water extract in the solid media used *in vitro* determined a significant reduction in the radial mycelia growth of both plant pathogens (table 1). In particular, the radial mycelia growth of *S. minor* significantly decreased from 2.48 ± 0.97 cm in the control with peat water extract (SM1) to 0.68 ± 0.18 cm in solid media containing 100% of compost water extract (SM5) showing a MGI equal to 77.35 ± 1.52 %. It is significant the positive correlation between compost water extract content and MGI of pathogen. In fact, an increasing in suppression of *S. minor* from about 67 to 77 % was observed when the concentration of water extract compost was highest (table 1). A similar trend was observed testing *R. solani* where the inhibition exerted by compost was more marked. In fact, a drastic significant decreasing of radial mycelial growth was detected (from 12.50 ± 0.13 to 1.07 ± 0.12 cm in SM1 and SM5, respectively). The MGI of *R. solani* ranged from 88-89 % in presence of compost water extract at a concentration of 50 % (SM2 and SM3) to 92.75 ± 0.56 % using 100 % compost water extract (SM5) (table 1).

6.3.2 *In vivo* assay

In both trials (*S.minor* and *R.solani*) the height of plants did not show significant differences, between tomato plants grown in, and sterile compost, while plants grown on peat, showed a lower value (table 2). Leaf area in both *S.minor* and *R.solani* treatments showed different trends. Plants grown on compost showed a leaf area of 36,98 ± 0.47 and 29.60 ± 1.00 for *S.minor* and *R.solani* respectively (table 2), higher of the plants grown on sterile compost and peat (table 2). In figures 5 and 6, were compared the leaf biomass, with mass roots, in the three assays, the histograms exemplify the differences between the three treatments. The response is equivalent for leaf biomass and mass roots. Conversely, plants treated with compost show a more efficient responce to both pathogens, this is also shown by DSI estimated on the basis of dead and healthy plants (table 2). In fact, in pots inoculated with *S. minor*, the disease suppression estimated ranged from 62.00 ± 1.68 to 70.00 ± 0.64 % using a substrate composed by compost or by sterile compost, respectively. Similar trend was observed in pots inoculated with *R. solani*, since the disease suppression ranged from 45.00 ± 0.90 to 51.00 ± 1.00% using a substrate composed by compost or sterile compost, respectively. By contrast, all plants grown on peat died after 30 days (table 2). Similar trend was observed in pots inoculated with *R. solani*: the disease suppression ranged from 45.00 ± 0.90 to 51.00 ± 1.00% on compost and sterile compost, respectively, and plants grown on peat died within 30 days (table 2).

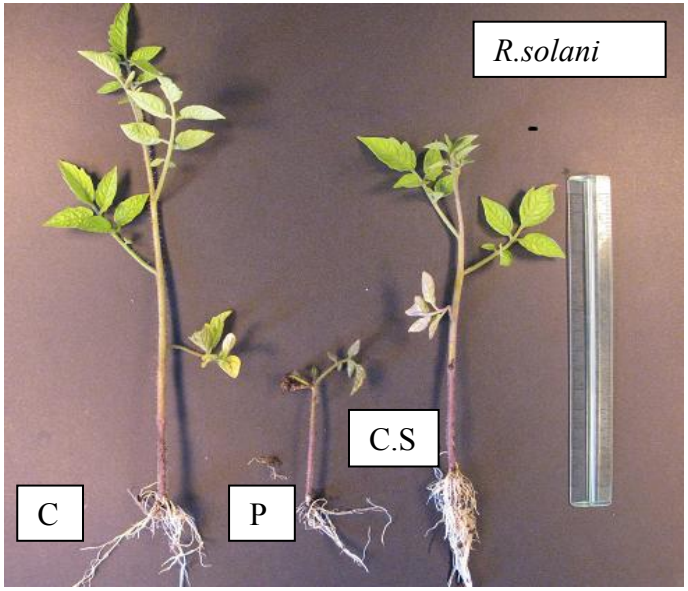

Figure 3. Tomato plants grown on three substrates inoculated with *R. solani*. C: compost 100 %; P: peat 100 C.S: sterilized compost.

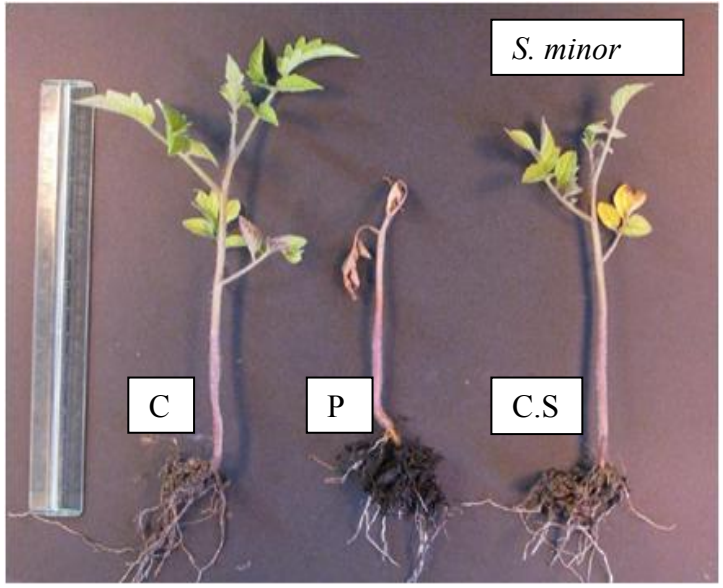

Figure 4. Tomato plants grown on three substrates inoculated with *S. minor*. C: compost 100 %; P: peat 100%; C.S: sterilized compost.

Table 1. Measurement of radial mycelial growth and percentage growth inhibition of *Sclerotinia minor* and *Rhizoctonia solani* after 15 days of incubation using different solid media.

Solid media	**Radial mycelial growth (cm)[a]**		**Mycelial growth Inhibition (%)[a]**	
	Sclerotinia minor	***Rhizoctonia solani***	***Sclerotinia minor***	***Rhizoctonia solani***
SM1	2.48 ± 0.97^{B}	12.50 ± 0.13^{C}	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}
SM2	0.95 ± 0.21^{A}	1.24 ± 0.15^{B}	68.00 ± 2.08^{B}	89.00 ± 0.70^{B}
SM3	0.79 ± 0.03^{A}	1.38 ± 0.13^{B}	67.52 ± 1.20^{B}	88.00 ± 0.35^{B}
SM4	0.78 ± 0.09^{A}	1.16 ± 0.08^{AB}	70.00 ± 1.15^{C}	90.13 ± 0.61^{C}
SM5	0.68 ± 0.18^{A}	1.07 ± 0.12^{A}	77.35 ± 1.52^{D}	92.75 ± 0.56^{D}

[a]Solid media used to evaluate suppressive compost effect in *in vitro* experiments. SM1: peat water extract (100%); SM2: compost water extract (50%) + water (50%); SM3: compost water extract (50%) + peat water extract (50%); SM4: compost water extract (70%) + peat water extract (30%); SM5: compost water extract (100%).

[b]The values represent the means ± SD of five replicates. Different letters after the values indicate significant differences ($P \leq 0.05$) on the same column

Table 2. Plant height, collar diameter, leaf area and disease suppression index (DSI) of tomato plants grown in different substrates inoculated with *Sclerotinia minor* or *Rhizoctonia solani.*

Pot substrate	**Plant height (cm)[b]**		**Leaf area (cm^2)[b]**		**DSI %**	
	Sclerotinia minor	***Rhizoctonia solani***	***Sclerotinia minor***	***Rhizoctonia solani***	***Sclerotinia minor***	***Rhizoctonia solani***
P.	6.18 ± 1.61^{B}	6.15 ± 0.82^{A}	32.97 ± 0.40^{A}	24.38 ± 0.13^{A}	0 ± 0^{A}	0 ± 0^{A}
C.S	$7{,}10 \pm 1.29^{A}$	$7.90 \pm 1{,}29^{B}$	33.75 ± 1.98^{B}	27.92 ± 0.76^{B}	70 ± 0.64^{C}	51 ± 1.00^{C}
C.	7.99 ± 1.69^{A}	7.92 ± 1.22^{B}	$36{,}98. \pm 0.47^{C}$	29.60 ± 1.00^{C}	62 ± 1.68^{B}	45 ± 0.90^{B}

[a] Pot substrate: P, neutral peat; C.S, Sterilized compost; C, chestnut compost.

[b] The values represent the means ± SD of three replicates of nine times. Different letters after the values indicate significant differences ($P = 0.05$) on the same column.

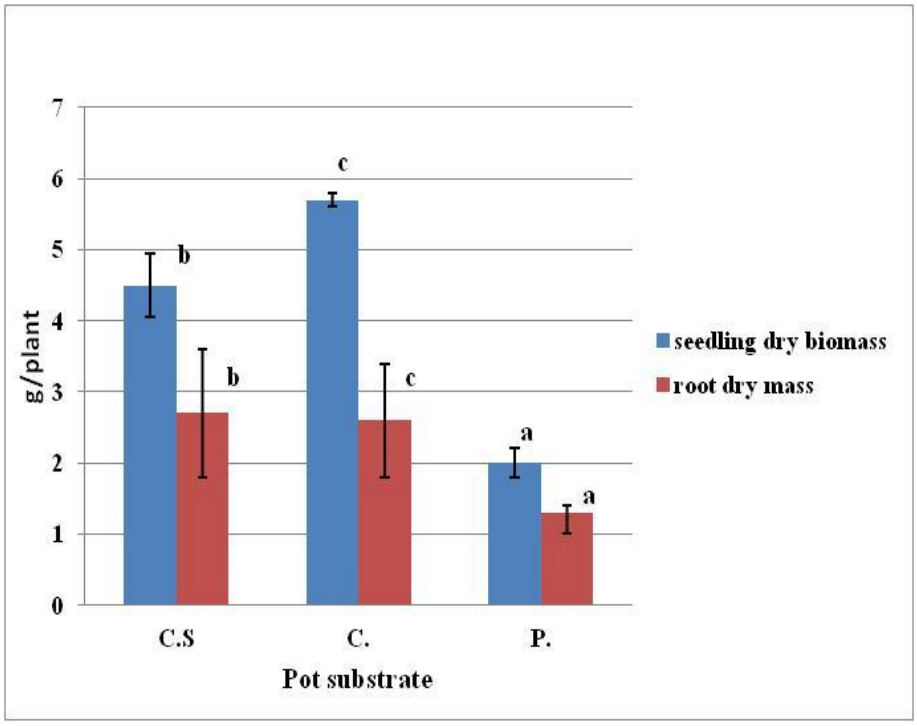

Figure 5. Suppressiveness: comparison of the seedling dry biomass an root dry biomass in tomato plants grown on three different substrate (C: compost 100%, C.S: sterilized compost, P: Paet) inoculated with *Rhyzoctonia solani*. Different letters indicate significant differences level; Duncan test, ($P < 0.05$), data are averages of three replicates (45 plants/replicates).

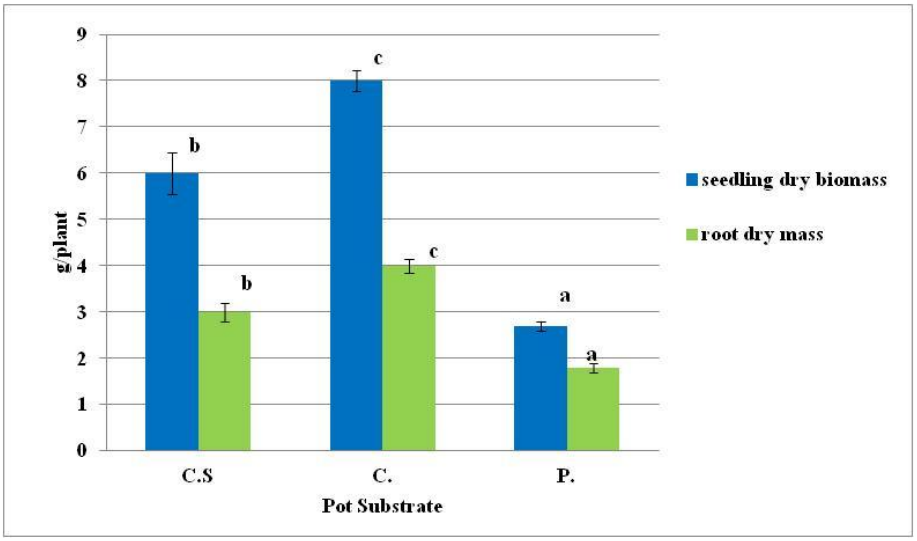

Figure 6. Suppressiveness: comparison of the seedling dry biomass an root dry biomass in tomato plants grown on three different substrate (C: compost 100%, C.S: sterilized compost, P: Paet) inoculated with Sclerotinia minor. Different letters indicate significant differences level; Duncan test, ($P < 0.05$), data are averages of three replicates (45 plants/replicates).

6.4 Discussions

Recently, on-farm composting processes have been developed to transform biomass waste of agricultural origin (Tuomela *et al*., 2000). In particular, the compost obtained from agricultural and agro-industrial wastes is more reliable of other starting matrix originated from pre-selected organic matrix composed of vegetable or food waste that are considered ‘‘clean biowaste’’ since are generally free of xenobiotics and heavy metals (Ntougias *et al*., 2008; Pepe *et al.,* 2013a). This ensures quality product, representing an interesting alternative carrier for solid-based inoculant to use in crop system (Ben Rebah *et al.,* 2007). In fact, it could be potentially able to provide an allocation in soil of microorganisms with specific and useful functions, such as suppressive effect and plant growth promotion. Since the 1970s, many studies demonstrated that compost application effectively controls many soil-borne fungal pathogens (Noble & Coventry 2005; Bonanomi *et al*., 2007).

The chestnut compost showed a high suppression activity against *S. minor* and *R. solani* plant pathogens both *in vitro* and *in vivo*. The disease suppression could be due to different mechanisms exerted by prokaryotic and eukaryotic microorganisms detected and identified in the mature compost. (Yogev *et al*., 2010). The composition of microbial communities in composts is determined by the chemistry of the starting material (Castaño *et al*., 2011). Avilés *et al.* (2011) reported that fungi, cellulolytic and oligotrophic actinomycetes, and bacteria are involved in the suppressive ability of compost obtained from agricultural and agro-industrial wastes. Several bacterial and fungal genera, such as *Bacillus*, *Fusarium* and *Penicillium*, recovered in the chestnut compost obtained in this work, have been identified as antagonists of one or more soil-borne plant pathogens (Berg *et al*., 2002; Cotxarrera *et al*., 2002; Garbeva *et al*., 2004; Avilés *et al*., 2011). *Pseudomonas* and *Stenotrophomonas* are reported as plant disease suppressive bacteria and it is well known their ability of bio-control agents (Dunne *et al.,* 2000;

Haas & Défago, 2005; Kobayashi *et al*., 2002). Also, the presence of bacteria belonging to *Streptomyces* and *Actinomyces* genera represents a positive and desirable feature in the compost since they could exert a suppressive effect with regard to plant soil-borne pathogenic microorganisms producing a wide range of different antibiotics (Franke-Whittle *et al*., 2009).

The presence of *Flavobacterium* spp. in our compost could also determine its suppressive effect (Boulter *et al.,* 2002). Krause *et al*. (1997) reported that compost pine bark inoculated with *Flavobacterium balustinum* induced suppression of diseases caused by a broad spectrum of soil-borne plant pathogens such as *Fusarium*, *Rhizoctonia solani*, *Phythophtora* and *Pythium*.

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Chapter 7 Extraction and characterization of humic substances in wheat straw co-compost including lignocellulosic fungi and metallic oxides

7.1 Introduction

Wheat straw residues are one of the major organic materials generated every year in Chile. Annually, 1.3 million tons of straw are produced and most of these residues are burnt in fields causing an environmental problem (Ruiz, 2011). Composting is considered an important way for carbon stabilization involving the partial humification process of the organic matter (OM).

Numerous studies have shown how the content of SOM (soil organic matter) depends not only on the amount of Carbon (C) input, but also the dynamics of the aggregates that, retaining and protecting them OM, allowing the storage of C in soil (Tisdall & Oades 1982; Jastrow, 1996; Six *et al*., 1998). Traditional concepts were highly focused on the role of phyllosilicates as dominating mineral surfaces for the formation of organo-mineral associations (Stevenson 1982; Theng 1979). Later, total mineral surface area has been considered as a predictor for the amount of OM that is stabilized in soils (Mayer *et al*., 1994a; Saggar *et al*., 1996; Hassink, 1997; Six *et al*., 2002).

The organic fraction associated with the mineral phase is typically regarded the most stable (Kiem & Kögel-Knabner 2002). This old OM fraction is chemically stable (Theng *et a*l., 1986). In a wide range of different soils, its concentration often correlates with the content of Fe oxides (Eusterhues, *et al*., 2003) and a short–-range order of Al silicates (Kleber *et al*., 2005; Mikutta *et al*., 2006). Such minerals provide large reactive surface areas (Eusterhues *et a*l., 2005b), which renders them most suitable for interaction with OM either via sorptive interactions or co-precipitation.

Alternative methods to stabilize C in composted residues have been developed in order to enhance the humification process and promote the carbon stabilization. Studies have noticed the use of inorganic materials such as Allophane, and Fe-Al-Mn oxides (Brunetti *et al*., 2008; Bolan *et al*., 2012; Qi *et al*., 2012), all of which interact with OM, through complex formation and catalyzation of humification; preventing its microbial decomposition and increasing C stabilization and thus C stock in soils.

The nature and properties of the soil aggregates are then determined by the quantity and quality of organic residues and humic compounds and of the degree of their interaction with soil particles (Jastrow *et al.,* 1998).On the other hand, it is very important the influence of soil biota in the formation of organic-mineral assemblages (Chenu & Stotzky, 2002). Here, the ability of microorganisms to standardize mineral surfaces to their needs by "active support preconditioning" (Bos *et al*., 1999; Dufrene *et al*., 1996) seems to promote organic-mineral associations through the deposition of extracellular polysaccharides and proteins as adhesives.

From the above, the main objective of this study was to optimize the production of chemically stabilized compost using saprophytic fungi as lignin bio-transformer and metallic oxides in order to improve the Carbon stabilization in composted wheat straw as a way to reuse a highly available material, which currently generates an environmental problem in extent areas from Southern Chile.

7.2 Materials and methods

To study the effect of the inoculation with some lignocellulosic fungi and inorganic additives in the wheat straw composting several combinations were established. For this study, the samples of analyzed composts were the following:

a) Compost with Fe_2O_3 as abiotic stabilizer and inoculated with *Trametes versicolor* (TV);

b) Compost with an allophanic soil as abiotic stabilizer and inoculated with TV;

c) Compost only inoculated with TV;

d) Control compost, without inoculation or inorganic stabilizer addition.

The experimental design corresponded to a complete randomized experiment, with three replicates in each combination.

7.2.1 Humic acids extraction

Humic acids (HA) were extracted in order to evaluate some chemical parameters. The method for this was as follows: 5 g of air-dried compost was added to 50ml of NaOH 0.5M, and shaken overnight at room temperature. Then, the dark colored supernatant was recovered by centrifugation (4500 rpm for 10 min, the HA were coagulated by means of acidifying the alkaline extract to pH 2 adding HCL 2N and separated by centrifugation (4500 rpm for 12 min). Finally, in order to minimize the ash content of HA material, purification with H_2O Milli-Q for three cycles was performed (Schnitzer,1982).

7.2.2 Composting maturity test

For this, 1g of material was extracted using 50 mL of NaOH 0.5M, shaken overnight at room temperature and centrifuged at 4500 rpm for 25 min. For determination of humification index of the composting, the absorbance of FA and HA ratios (E_4/E_6) were assayed and the absorbance of the supernatant was

measured at 472 nm and then at 664 nm (Khan *et al.*, 2009). E_4/E_6 ratio was considered as humification index of the different composting treatments.

7.2.3 Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

For the characterization we focused in samples of HA obtained from Cu-polluted soils amended with the compost inoculated with *Trametes versicolor* and supplied with Fe_2O_3. In order to determine the shape of macromolecular structures (size, degree of aggregation) of HA extracted from different soil-compost mixtures and to characterize the possible sorption or complexation of metals in HA, the Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy TEM) were used.

For SEM study, the first step was the extraction of HA from samples, by the method described by Schnitzer (1982). Then, the extracted samples were coated with gold and the observations were carried out with a Quanta 600 FEI (FEI Inc., Hillsboro, OR, USA) at 20 kV under high vacuum conditions.

For obtaining TEM images, the HA samples extracted were carbonized by hood of 25 nm in 400 mesh Copper Grids, air-dried for 24-h and visualized by means of Electron Microscopy System Hitachi H-7000. In this study also we used the spectrophotometer disperser energy (EDS) to detect some element concentrations. The system used was a JEOL JXA8600M coupled to a EDS Bruker detector, model XFLASH 5010, with resolutions 123 eVfor Mn Kα. The spectra were analyzed by QUANTAX200 (Bruker) software. Samples were worked at 15KV and 10 nA. The samples analyzed by means of SEM and TEM were: e) HA extracted form soil added with 500 ppm of Cu and amended with compost with TV and Fe_2O_3; f) HA extracted form soil added with 500 ppm of Cu and amended with compost with TV and allophanic soil; g) HA extracted form soil added with 500 ppm of Cu and amended with compost inoculated with TV; h) HA extracted of unamended soil.

7.3 Result

7.3.1 Composting maturity test

In this context, the relationship E_4/E_6 ratios decreases, in the sample with the addition of Fe and TV, the value is lower than the control as showing in the table 1.

Table 1 absorbance ratio (E4/E6) a:compost with Fe_2O_3 as abiotic stabilizer and inoculated with *Trametes versicolor* (TV); b: compost with an allophanic soil as abiotic stabilizer and inoculated with TV;c:compost only inoculated with TV; d:control compost, without inoculation or inorganic stabilizer addition.

sample	472nm	664nm	E4/E6
a	0.632	0.151	4.18
b	0.564	0.157	3.59
c	0.565	0.111	5.09
d	0.201	0.043	4.67

7.3.2 Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

SEM Images: Small particles of metal ion (60-100μm) were detected in the SEM micrographs. Obtained images showed the aggregate formation of HA wherein those molecules tended to form small spheres coalesces in dendritic structures (fig.1). In table 2 is reported the percentage of the element present in organic aggregate.

TEM images (fig.2) show the sorption and complexation at nano-scale between the organic matter and iron particles (table3) show the percentages of the elements present in organic aggregates.

Table 2. Mean % of elements mesuread SEM images. e: HA extracted form soil added with 500 ppm of Cu and amended with compost with TV and Fe_2O_3; f: HA extracted form soil added with 500 ppm of Cu and amended with compost with TV and allophanic soil; g: HA extracted form soil added with 500 ppm of Cu and amended with compost inoculated with TV; h: HA extracted of unamended soil.

Sample	C	O	Si	Fe	Cu
e (%)	41.65	48.28	2.51	1.50	0.83
f (%)	39.29	52.48	2.84	1.23	0.13
g (%)	47.87	50.10	0.81	0.27	0.03
h (%)	34.76	56.05	3.79	1.52	0.05

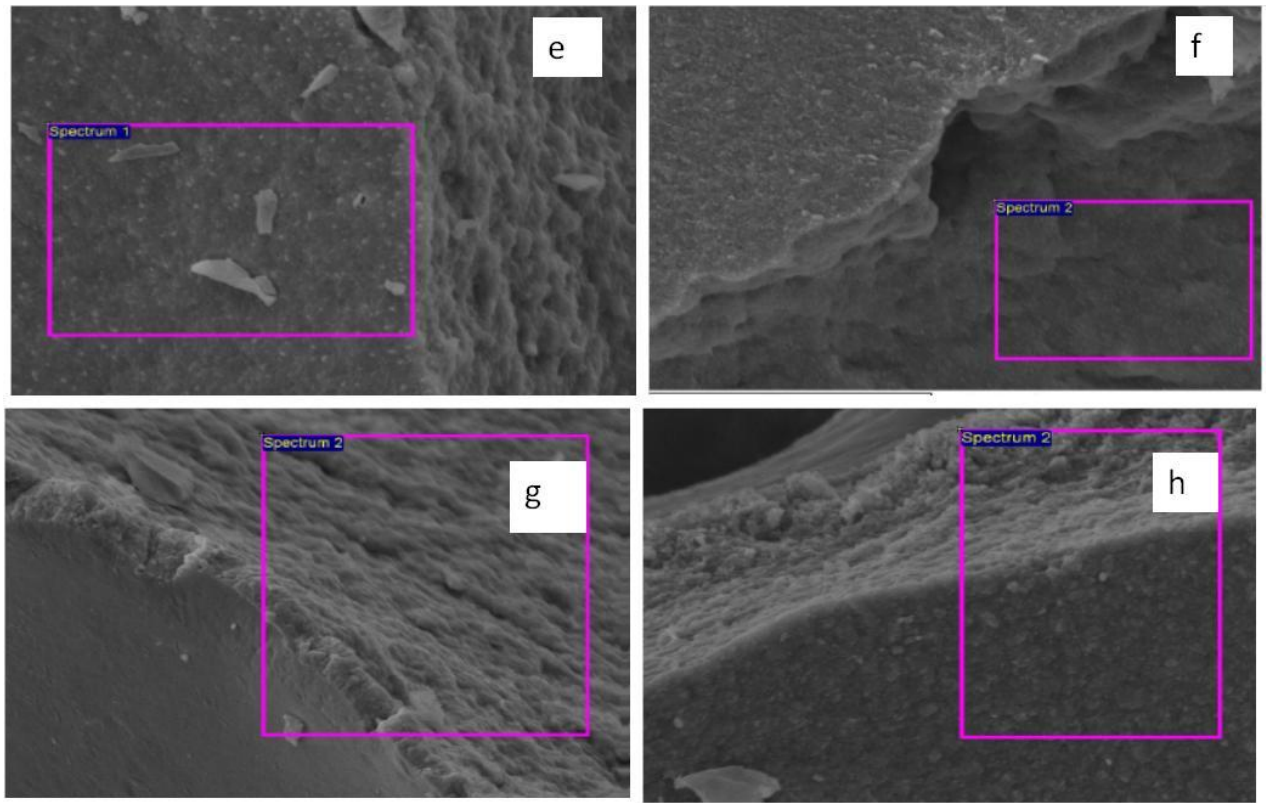

Figure1. Particular aggregates in SEM images

Table 3. Mean % of elements mesuread TEM images. e: HA extracted form soil added with 500 ppm of Cu and amended with compost with TV and Fe_2O_3; f: HA extracted form soil added with 500 ppm of Cu and amended with compost with TV and allophanic soil; g: HA extracted form soil added with 500 ppm of Cu and amended with compost inoculated with TV; h: HA extracted of unamended soil.

Sample	C	O	Na	Al	Si	S	K	Ca	Mn	Fe	Cu	Zn
e (%)	42.13	34.25	1.21	5.70	5.85	0.25	1.80	0.22	0.38	4.69	1.46	0.92
f (%)	38.21	34.84	0.81	8.19	6.68	0.33	0.52	0.15	0.27	5.21	1.57	0.98
g (%)	50.72	34.62	.038	3.37	3.23	0.48	0.21	0.08	0.26	2.68	2.05	1.25
h (%)	40.08	32.61	0.49	7.13	8.21	0.18	0.81	0.31	0.34	6.14	1.27	0.98

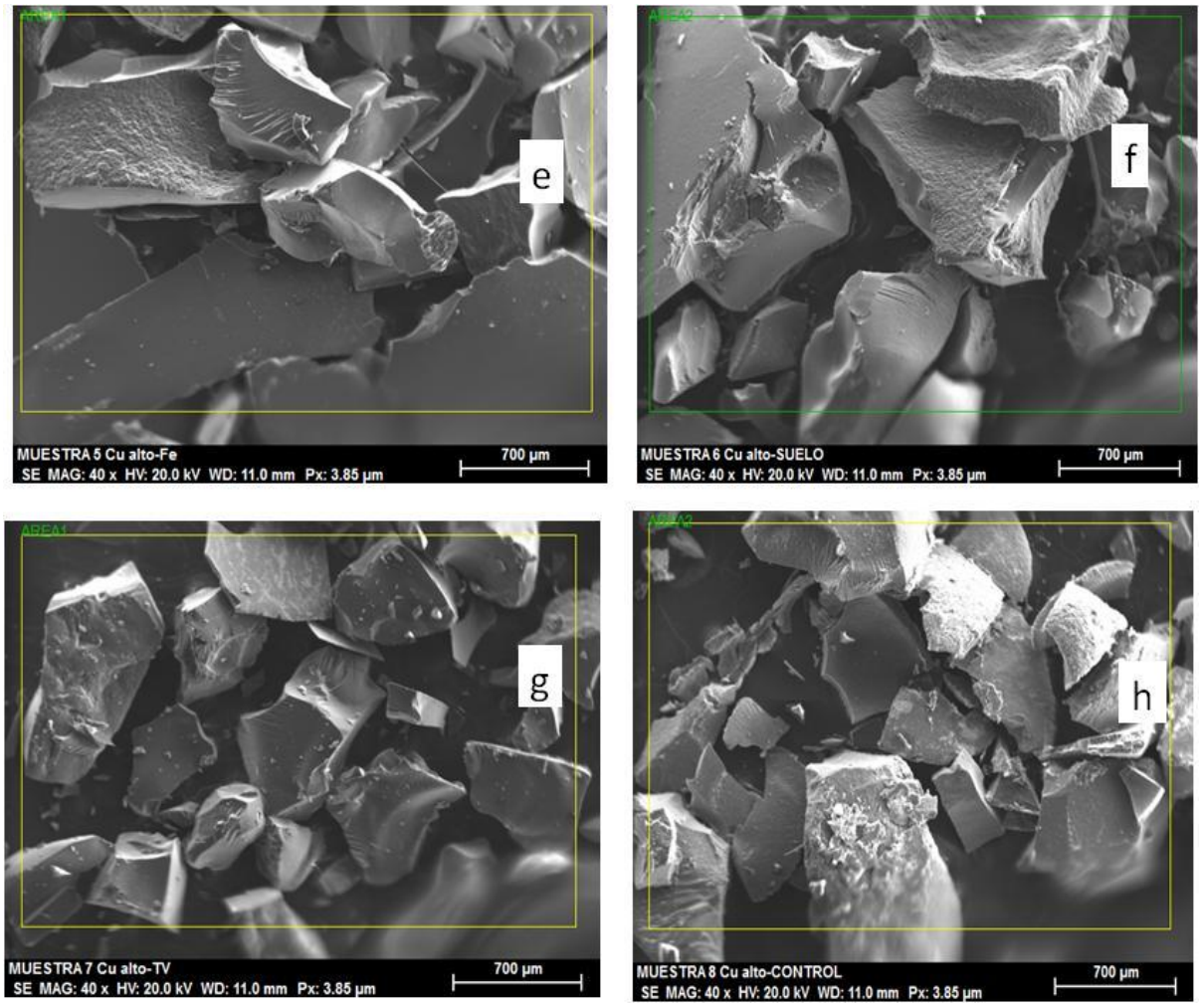

Figure 2. Particular aggregates in TEM images

7.4 Discussions

Several studies have shown that abiotic stabilization by metallic oxides as Fe, and Mn oxides can have a positive effect on stabilization and humification process (Brunetti *et al*., 2008; Qi *et al*., 2012 a; b).

The evaluation criteria used of the composting process and of the compost stability in this study, were based on physical and chemical parameters of the organic matter. The absorbance ratio (E_4/E_6) of compost is index of degree of stability of organic matter in compost (Zbytniewski & Buszewski 2005). In this context, the relationship E_4/E_6 obtained in our experiment could be explained by several mechanisms, as: the increased of high molecular weight compounds; the increased condensation and aromatization processes which produced more polycondensed humic acid during composting (Chanyasak *et al*., 1982). The results indicate that the inoculation of compost with saprophytic fungi was efficient for increase the lignocellulosic transformation. The addition of Fe_2O_3 in the composting process, favors the formation of molecules with dimensions and higher molecular weight, promoting the organic matter stabilization probably by its effect as catalyst and complexing agent. Therefore, the ratio E_4/E_6 decreases Chen *et al*., (2007) indicated that the complexation of metal ion with functional groups of HA can easily make HA to form aggregates.

Based on the above, metallic oxides have an important role in controlling the morphology, of the stable HA-ion aggregates. Fe particles tend to form nano-size aggregates with the humic substances extracted from composted wheat straw.

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Chapter 8 General Conclusions

The present doctorate thesis enlightens the composting process of biomass from chestnut woods handling. Physical, chemical parameters and their interaction with biological aspects were examined. Bacterial and fungal populations were detected and characterized during the composting process in order to identify species and their potential beneficial effects on plants. Agronomic studies were conducted to evaluate effects of the compost on plant fitness and suppressive activity.

The results of the characterization of the process showed the potential in agriculture of the mature product.

Main results of the study are:

a) The temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions. Averagely, the decomposition process occurred at low temperature;
b) The agronomic valuations allow the classification of the compost obtained as a good quality amendant;
c) Parameters analyzed for stability evaluation, respiration test and phyto-toxicity bioassay, as well as sanitary checks, indicated the product absolute suitability;
d) Parameters of plant fitness and agronomic evaluation showed that plants grown in compost activate their response to stress;
e) The suppression capability was demonstrated both in *vitro* and *in vivo*;
f) The trends of functional populations (cellulose, hemicellulose and lignin) during the bio-oxidative process were affected by the decrease of activity water and the temperature;
g) DGGE patterns of eukaryotic populations showed a predominance of Ascomycota, including species of *Penicillium, Moristroma, Paraconiothyrium, Fusarium, Cladosporium,* and *Aspergillus*. Selected

prokaryotic bands were affiliated with *Bacilli*, followed by *Actinobacteria*, *Flavobacteriia,* and γ-*Proteobacteria;*

h) Extraction and SEM-TEM characterization of humic substances from wheat straw co-compost including lignocellulosic fungi and metallic oxides, reveal the importance of metallic oxides in controlling morphology stablility of HA-ion aggregates.

This is the first time that the chestnut *in situ* composting process is characterised in all its phases. Moreover, this is the first time that the microbial dynamics during the process as well as the microbial characterization and its suppressive activity on soil-borne plant pathogens, was investigated.

The results are encouraging and indicate that biomasses from forest and agricultural origin could represent a new and underspeculated source of agriculture amendant, pathogen and pests control, income for marginal areas, carbon sequestration, and biodiversity preservation.

It is advisable that the research will keep on truck investigating the way to optimize the process to: amplify carbon sequestration, investigate the process in different matrices, and unravel the transcriptome of the plant stress response.

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Priority: IV - Specific Objective 1 - Operational Objective 4

Subproject 2. *On farm quality compost for forestry productive systems management: sustainability and plant protection*.

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Chestnut Biomass Biodegradation for Sustainable Agriculture

Valeria Ventorino, Rita Parillo, Antonino Testa, Alberto Aliberti, and Olimpia Pepe*

Biodegradation of lignocellulosic waste from chestnut residues is an important biological process of added value for this sector according to the Chestnut National Plan for agriculture. Dynamic parameters during biodegradation under natural conditions of chestnut burr, leaf, and plant biomass litter are reported in this study. Microbiological and physical-chemical characterisation of chestnut wastes was carried out to monitor this specific biodegradation, to understand the progress and limits of the process and to analyse the compost-like organic substance obtained. Physical-chemical parameters, such as temperature, pH, and water activity, were influenced by the composition of the raw materials and by seasonal climatic conditions. Moreover, microbiological monitoring was assessed by culture-dependent and independent methods. Cellulolytic, hemicellulolytic, and ligninolytic populations were counted to determine different microbial activity during biodegradation process. The functional microbial groups analysed showed different trends, but all were found at high concentrations (7 to 9 log CFU/g). In addition, PCR-DGGE was performed for bacterial and fungal populations to evaluate the microbial diversity. The similarity level during the process was generally very high, both for bacterial and fungal populations. These data are the first on suitable natural degradation for chestnut forests.

Keywords: Chestnut composting; Microbiological monitoring; PCR-DGGE; Sustainable fertilisation

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INTRODUCTION

The Italian Chestnut National Plan in Italy supports the need to guide the chestnut population toward more sustainable forms of production, characterised by a higher ability to meet the needs and interests of consumers and producers by offering new economic opportunities in agriculture. These measures are justified by the need to sequester carbon, reducing greenhouse gas emissions into the atmosphere, recovering biomass for agronomic purposes, and increasing the sustainability of agricultural productions (Marmo 2007).

The use of biomass is interesting at both the EU and national level, especially after the agreements of the Kyoto Protocol (reduction of CO_2 emissions into the atmosphere). Recently, on-farm composting processes have been developed to transform biomass waste of agricultural origin (Tuomela *et al.* 2000). The use of lignocellulosic materials in composting has several important roles, such as structural, absorptive, and biochemical changes (Hubbe *et al.* 2010). An interesting example is the composting of lignocellulosic winery wastes (Paradelo *et al.* 2013*)*. The evaluation of compost made from chestnut residues obtained from cleaning and pruning can be a source of added value for the sector. Although composting procedures are well known, very few reports

exist on the biodegradation of chestnut wood remains and their use in agriculture, and these residues are often burned (Guerra-Rodrìguez *et al.* 2006). Chestnut residue composting, related practices, and their utilisation in agriculture would be possible approaches to a sustainable management of chestnut forests. Benefits of application of biodegraded matter are well recognised. The use of compost, for instance, contributes to the following: (1) improving the soil structural stability, increasing its fertility, and recycling organic matter (humus) and nutrients in a slow release; (2) restoring microbiological diversity in soil and limiting the development of plant pathogens and plant diseases; (3) increasing biological activity and root aeration; (4) reducing the environmental impact, contributing to the degradation of pollutants, and increasing crop protection and carbon dioxide storage in soils; and (5) reducing the use of chemicals and disposal costs.

Moreover, a further relevant aspect involves mechanisms by which compost's microflora are able to contain pathogens. This consists of competition between pathogens and antagonists, antibiosis, parasitism, and induction resistance in the host plant (Hoitink *et al*. 2001). Particularly interesting is the use of compost to suppress soil-borne pathogens. This aspect has been extensively studied by several authors (De Ceuster and Hoitink 1999; Pointing 1999; Ryckeboer *et al*. 2003). The studies of biotic and agronomic components represent key points to characterise the compost (Pepe *et al*. 2013a).

Many studies have been conducted in pilot-scale reactors that enable easier tracking of the biological process: field experiments do not allow monitoring developments in biodegradation because it depends on many environmental factors. Static aerated heaps have been developed for the purpose of natural degradation of lignocellulosic waste obtained from the cleaning of chestnut. Microbiological monitoring using culture-dependent and culture-independent methods of physical-chemical parameters such as temperature, pH, and water activity have been carried out during the process of biodegradation under natural conditions. In fact, the efficiency of process stages depends on a variety of parameters, including aeration, temperature, and content of moisture in the waste. The most important aspect, however, is represented by the microorganisms involved and their functional activity (Neklyudov *et al*. 2008; Pepe *et al*. 2013a).

The present study aims to create useful innovations to increase the sustainability and profitability of production, in accordance with the regulations of organic farming and environmentally-friendly criteria. Biotechnological and agronomic innovations will contribute to the well-being of those who work in remote areas, creating less impact on the environment and improving the quality of the product. On the basis of these considerations, microbiological and physical-chemical characterisation of chestnut biomass submitted to biodegradation was carried out to investigate the bio-oxidative processes at different times and analyse the compost-like organic substance obtained from a microbial and agronomic perspective to understand the progress and limits of the process.

EXPERIMENTAL

Characteristics of the Lignocellulosic Waste

The experimental field was in Roccamonfina (province of Caserta), an area in southern Italy particularly suitable for chestnut forests. The compost-like organic substance (CLOS) was obtained from a composting bin, and the resulting materials were placed in piles. The size of the bin was 1.50 m x 1.50 m x 1.80 m (length x width x height). Fagots were placed under the bin to avoid water retention. The structure was filled with the resulting material of chestnut cleaning (without shredding), such as fresh shoots, chipboard obtained from the cleaning of stakes, undergrowth vegetation (ferns, vetch, sainfoin, wild oats, horsetail grass, and calenzuola), old leaves, and curly. All components were added in equal amounts in terms of weight (about 60 kg) and submitted to natural degradation. Finally, the vegetable biomass was covered with 5 cm of soil taken from the chestnut forest to create a sort of natural lid, which can also enhance the biodegradation process.

The degradation of lignocellulosic wastes was carried out in natural conditions for 105 days. During the whole process, the pile was never turned. Moreover, aeration was also ensured by gaps formed by lateral and basal fagots.

Sampling

Since the process was submitted to natural degradation, it was assumed that it was slow and therefore the samples were collected every two weeks. Samples of 1 kg were collected from the external (right and left side of the pile) and from the internal central part of the biomass and mixed before analyses. Sampling was performed immediately after preparation (T0) and at 15, 30, 45, 60, 75, 90, and 105 days (T1, T2, T3, T4, T5, T6, and T7, respectively) of biodegradation.

Physical-chemical Monitoring and Microbiological Count

CLOS samples were monitored using physical-chemical and microbiological parameters at T1, T2, T5, T6, and T7.

Temperature (°C) was monitored using specific sensors (1500 mm) placed in the core of the pile. The pH was also measured by resuspending 25 g of sample in 250 mL Ringer's solution. Finally, water activity (a_w) was evaluated using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany). C/N ratio was determined based on the concentration of carbon (Nelson and Sommers 1996) and of nitrogen value (Stevenson 1996).

For microbiological counting, an initial suspension was prepared by the addition of 25 g (wt/vol) of the samples to 180 mL of quarter-strength Ringer's solution (Oxoid, Milan, Italy) in 225-mL Erlenmeyer flasks. After shaking, suitable dilutions were made to the sample solution, which was then used to inoculate different solid growth media. Specific functional groups of the soil microbial community involved in the carbon cycle, such as cellulolytic, hemicellulolytic, and ligninolytic populations, were detected at 28 °C using the surface spread plate count method. Cellulolytic microorganisms were counted using a minimal medium (1 g L^{-1} $(NH_4)NO_3$, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution, 1 mL L^{-1} trace elements solution, and 15 g L^{-1} bacteriological agar, at pH 7.0) with carboxymethylcellulose (5 g L^{-1}) as the sole carbon source and Remazol Brilliant Blue R to show evidence of cellulolytic activities by developing clear haloes around the colonies (Ventorino *et al*. 2010). The medium used for hemicellulolytic population determination was the minimal medium described above with xylan (5 g L^{-1}) as the sole

carbon source. Finally, ligninolytic microorganisms were counted using LME basal medium (1 g L^{-1} KH_2PO_4, 0.5 g L^{-1}, $C_4H_{12}N_2O_6$, 0.5 g L^{-1} $MgSO_4$, 0.01 g L^{-1} $CaCl_2$, 0.01 g L^{-1} yeast extract, 0.001 g L^{-1} $CuSO_4$, 0.001 g L^{-1} $Fe_2(SO_4)_3$, 0.001 g L^{-1} $MnSO_4$, and 16 g L^{-1} bacteriological agar) supplemented with 0.25 % (wt/vol) lignin as the carbon source, as described by Pointing (1999).

DNA Extraction

Microbiological monitoring with a culture-independent method was performed for all samples (T0, T1, T2, T3, T4, T5, T6, and T7).

Microbial cells were desorbed from CLOS matrices, and pellets were obtained prior to community DNA extraction, as described by Rosewarne *et al*. (2011) with some modifications. First, 100 g of the samples was resuspended in 400 mL of acidic solution (pH 2.0), shaken at 240 rpm for 10 min, and filtered and centrifuged at 10,000 rpm for 20 min. Microbial cells were resuspended in 45 mL of sterile phosphate buffered saline (PBS), then recovered by centrifugation at 6500 rpm for 20 min. The DNA was extracted from compost samples using the FastDNA Spin Kit for Soil (MP Biomedicals) according to the supplier's recommendation.

PCR-DGGE

The primers V3f (5'-CCTACGGGAGGCAGCAG-3') and V3r (5'-ATTACC GCGGCTGCTGG -3') spanning the 200-bp V3 region of the 16S rDNA of *E. coli* were used for bacterial PCR-DGGE analysis. The PCR mixture and conditions were as previously described (Pepe *et al*. 2013b). To analyse the yeast population, the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-ATTCCCAAACAA-CTCGACTC-3') corresponding to nucleotide positions 266 to 285 on the *S. cerevisiae* 26S rRNA gene were used. The PCR mixture and conditions were as previously described (Palomba *et al*. 2011). A GC-clamp was added to the forward primer in all PCR reactions, following Muyzer *et al*. (1993).

DGGE analyses were performed using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories). PCR products were loaded in a 0.8-mm polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37:5:1)] using a denaturant gradient from 30 to 60% increasing in the direction of electrophoresis for both bacterial and fungal analysis. Electrophoresis was performed at 60 °C, initially at 50 V (5 min) and then at 200 V (240 min). The gels were stained in an ethidium bromide solution (5 min) and rinsed in distilled water (20 min).

Statistical analysis

Bands were automatically detected using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England). The cluster analysis was performed by the program after band matching; the method described by Saitou and Nei (1987) was used to obtain the correlation matrix of the DGGE patterns. The resulting matrix was used in the average linkage method by the Cluster procedure of Systat 5.2.1 to estimate the percentage of similarity (S) of microbial populations in the composting process.

RESULTS AND DISCUSSION

The site used for setting up the experiment was chosen because its climatic characteristics (400 m above sea level and north-west sun exposure) are representative of a chestnut forest. The biodegradation bin was filled with the resulting material of the chestnut cleaning, as shown in Fig. 1.

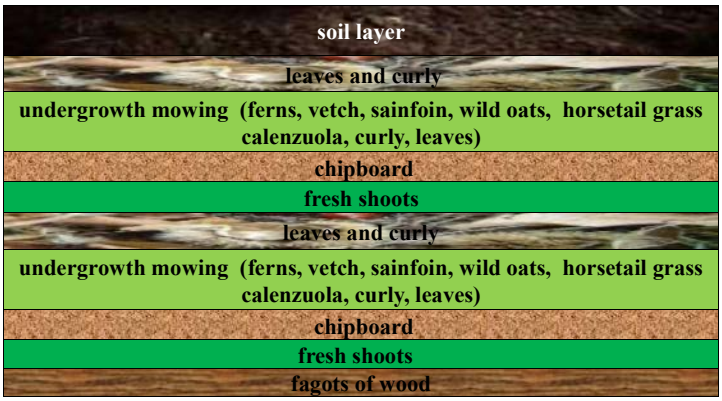

Fig. 1. Order and composition of layers in the biodegradation bin

The biochemical composition of the raw materials was: UR 40.2%, pH 4.54, organic carbon 28.9 % (dry weight), total nitrogen 0.5 % (dry weight), ammonia nitrogen 0.45 % (dry weight), C/N ratio 57.8.

The periodic measurement of the temperature during the biodegradation process is an important index to monitor the fermentation process because it is closely related to environmental conditions and to the metabolic activities of microorganisms. After 15 d, the temperature value observed in the pile was 27.1±0.06 °C (T1 sample). This value increased to 28.9±0.06 °C after 90 days of biodegradation (T6 sample). Since the biodegradation process was not carried out in controlled conditions but in a chestnut forest, it might be inferred that the temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions. In fact, as shown in Fig. 2, the temperature increased until September (28.9±0.06 °C) and underwent a sudden fall in October (26.6±0.07 °C). The detected environmental temperature showed a similar trend (Fig. 2). Although there are no common definitions of mesophilic and thermophilic phases during the composting process, mesophilic generally refers to temperatures up to approximately 40 °C, and thermophilic refers to temperatures from 45 to 70 °C (Miller 1996). The temperature values detected in this work suggest that the natural degradation process was in the mesophilic phase after 105 days.

Moreover, the detected carbon/nitrogen ratio of raw materials was very high (57.8) to reach a complete degradation. Guerra-Rodríguez *et al*. (2001a) reported that the composting of chestnut burr and leaf litter did not occur because the C/N ratio was too high and the compost never reached the thermophilic phase. In contrast, co-composting with solid poultry manure reached the temperature needed for the thermophilic phase of the process (about 60 °C) in 14 days (Guerra-Rodríguez *et al*. 2001a).

Moreover, measurements of other parameters that influenced microbial activity, such as pH and water activity (a_w), were monitored to understand the progress and limits of the process.

The pH values observed were quite stable, ranging from 6.75±0.03 to 6.8±0.02 at T1 and T7, respectively (Fig. 3). The biodegradation process should occur at a pH value from 3 to 11 (Guerra-Rodríguez *et al*. 2001b), but the best results are obtained in the range of 5 to 8 (Sundberg *et al.* 2004). Beck-Friis *et al*. (2001) reported that the change from the mesophilic to the thermophilic phase in composting corresponded to a change in pH from a range of 4.5 to 5.5 to a range of 8 to 9. In fact, in the first phase of the process, the acidic pH is due to the production of organic acid by microorganisms (Guerra-Rodríguez *et al*. 2001b), while the increase in pH up to 9 is due to ammonium production from protein degradation (Mahimaraja *et al.* 1994).

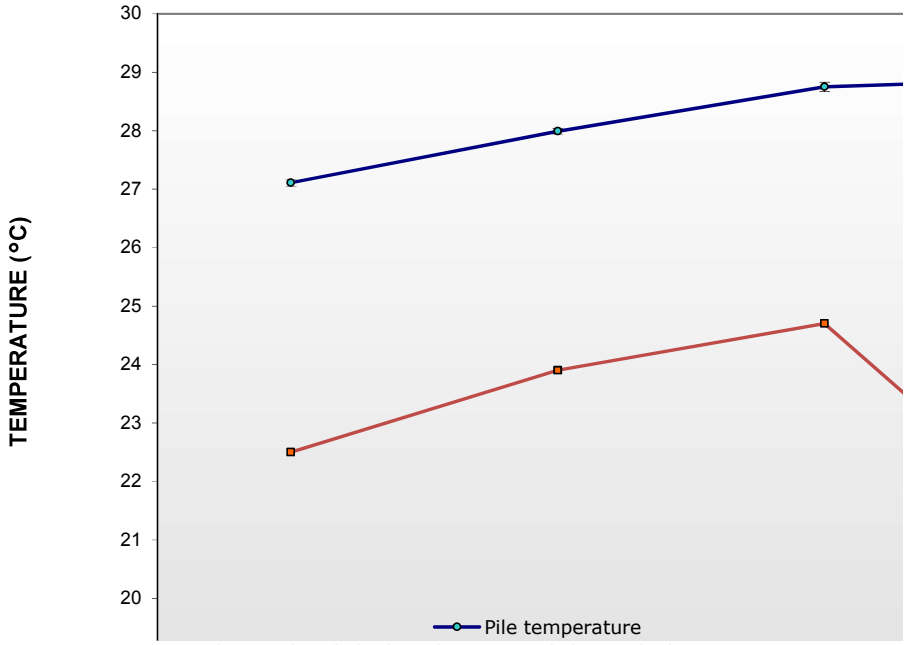

Fig. 2. Temperature values determined during the natural degradation process

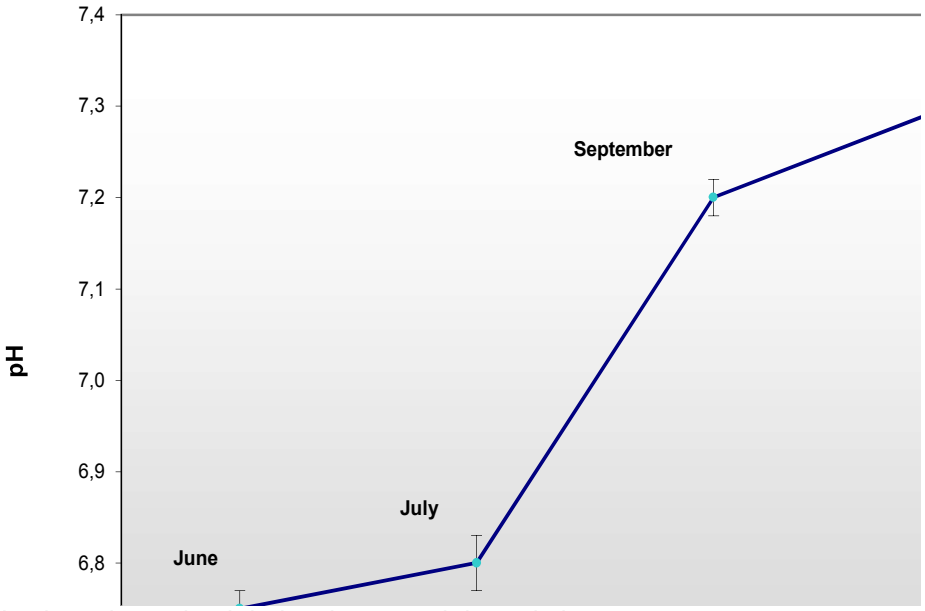

Fig. 3. pH values determined during the natural degradation process

Water activity (a_w) (water readily available for microbial metabolic activities) was also measured. The value of a_w was 0.94±0.003 after 15 d of experimentation and increased up to 0.98±0.001 after 105 days of the natural degradation process (Fig. 4).

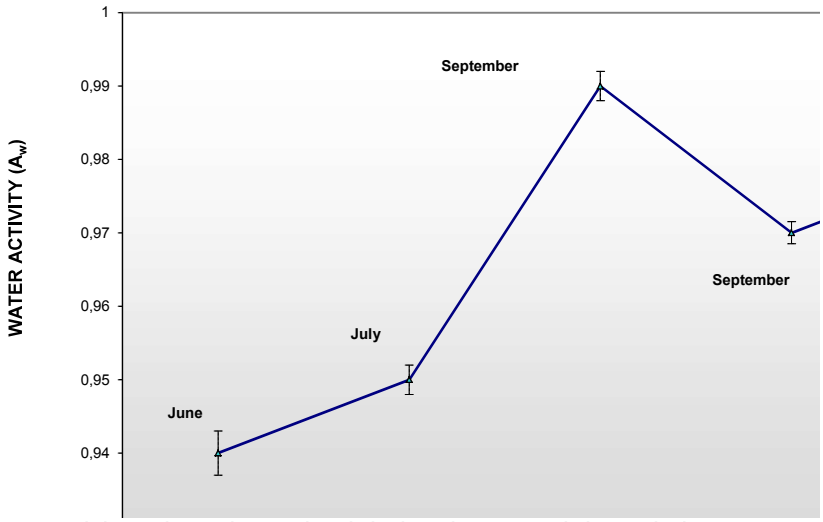

Fig. 4. Water activity values determined during the natural degradation process

Biodegradation of lignocellulosic materials is a result of the combined and sequential action of specific functional microbial groups, but little is known about the diversity of microorganisms in the carbon cycle that is specific and essential for vegetable biomass degradation. A large number of microorganisms have been studied for their potential ability of cellulose, hemicellulose, and lignin degradation in different experimental conditions (Huang *et al.* 2010a). The effectiveness of the process depends largely on the metabolic activities of microorganisms affecting the quality of the end product. For this reason, knowledge of the importance of specific functional groups can still be improved (Pepe *et al.* 2013a).

Because lignocellulosic materials are complex molecules of hemicellulose, cellulose, and lignin, three different functional microbial groups were analysed. The three functional groups involved in the carbon cycle showed different trends during biodegradation under natural conditions. Hemicellulolytic populations showed a value of about 9 log CFU g^{-1} of sample during the experimental process (Table 1). Indeed, after 105 days of biodegradation (T7), it was possible to detect an increase in cellulolytic populations, showing a concentration reaching to 10.18±0.04 log CFU g^{-1}. Observing the growth of aerobic cellulolytics, it was thought that the organic matrix possibly showed a good starting composition of cellulosic material. Pepe *et al.* (2013a) observed an increase in aerobic cellulolytics growth during composting of agro-industrial waste. The presence of hemicellulolytics and cellulolytics during the whole biodegradation process plays an important role with respect to cellulose, which represents the principal constituent of vegetable waste and whose degradation is restricted to a narrow range of microbial enzymes such as hemicellulase and cellulase (Herrmann and Shann 1997; Amore *et al.* 2013). Ligninolytic microorganisms showed an opposing trend. For this population, a significant decrease of about 1 log unit (from 8.48±0.00 to 7.59±0.16 log CFU g^{-1}) was detected (Table 1). It is probable that the decrease of temperature at T7 could negatively influence ligninolytic microbial activities, especially with regard to the fungal population.

In fact, microorganisms usually involved in the degradation of structural polymers (cellulose, hemicellulose, and lignin) such as fungi and actinomycetes (Huang *et al.* 2008; Amore *et al.* 2012) could be negatively affected by low temperature because their growth is favoured by higher temperatures (Ishii *et al.* 2000). Huang and co-workers (2010b) reported a continuous change in microbial population structure during composting of lignocellulosic waste. In particular after 3 days they detected a decrease of quinone species Q-9 associated with lignin degraders and an increase of Q-9(H2) and Q-10(H2) associated with cellulose-degrading ability.

Table 1. Microbial Contents (Log CFU g^{-1}) During Biodegradation Process

TIME SAMPLING (days)*	MICROBIAL FUNCTIONAL GROUP		
	Hemicellulolytic	*Cellulolytic*	*Ligninolytic*
15	9.04±0.06	9.00±0.06	8.48±0.00
75	9.36±0.08	9.65±0.07	9.25±0.07
90	8.58±0.03	8.64±0.19	7.34±0.19
105	8.97±0.21	10.18±0.04	7.59±0.16
* 15, T1; 75, T5; 90, T6; 105, T7.			

Microbiological monitoring with a culture-independent method was carried out during the natural degradation process. The results of the PCR-DGGE analysis performed on the samples during biodegradation are shown in Fig. 5. After 15 d, the bacterial and fungal populations showed very different profiles compared to the beginning of the process (T0). Instead, during natural degradation, the microbial community varied slightly, showing similar profiles both in bacterial and fungal populations. Ahmad *et al*. (2011) reported that the microbial community was influenced by environmental conditions and substrate characteristics, but did not change significantly during a composting process. In fact, they reported that the DGGE analysis showed similar profiles and the presence of same major bands.

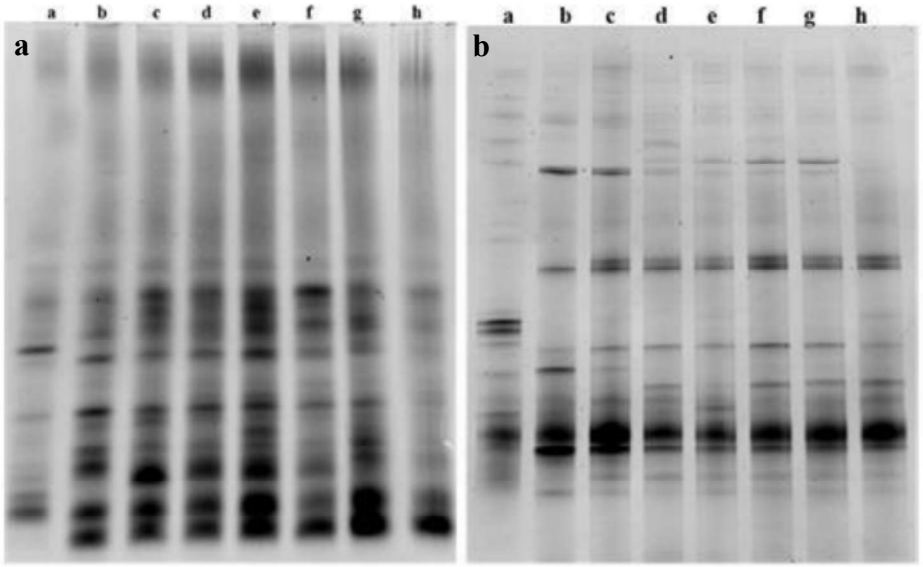

Fig. 5. DGGE profiles of bacterial (a) and fungal (b) populations during biodegradation process. Lanes: a, T0 (0 d); b, T1 (15 d); c, T2 (30 d); d, T3 (45 d); e, T4 (60 d); f, T5 (75 d); g, T6 (90 d); h, T7 (105 d)

The similarity level (S) in bacterial populations during chestnut biomass biodegradation was generally very high, showing values from 95% to 100%, except for at T0 (Fig. 6a). In fact, only at the beginning of the process were the bacterial populations very different, showing a similarity value of 60%, unlike the other sampling times. A similar trend was observed in fungal populations (Fig. 6b). In this case, the microbial diversity detected at T0 was the highest, showing a similarity value of 42% with the other times. Moreover, the levels of similarity among the fungal populations during the natural degradation varied more gradually than those detected among bacterial populations, as shown in Fig. 6.

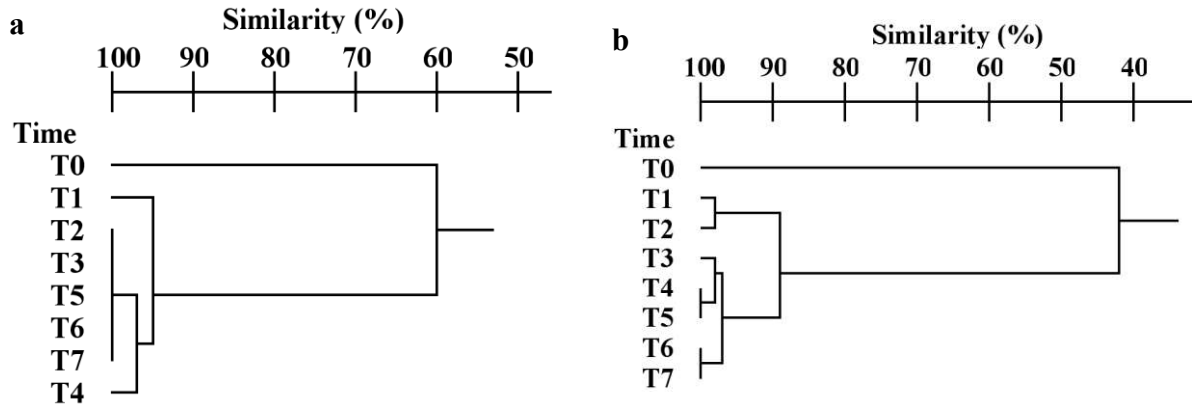

Fig. 6. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the bacterial (a) and fungal (b) populations during natural degradation of chestnut biomass

These results showed that the bacterial population was less influenced by the environmental conditions than the cellulolytic microbial functional group. In contrast, the fungal population suffered a greater variation during the biodegradation process, showing behaviour similar to a ligninolytic functional group. Because the ligninolytic activity of fungal populations is widely recognised (Feng *et al.* 2011), it is possible that the ligninolytic group was composed mainly of fungi and less so of bacteria, which exhibit more cellulolytic and hemicellulolytic activities. Despite recent findings, our results are encouraging because there have been few reports on biodegradation of chestnut biomass and there are no previous investigations about its microbiological characterisation.

CONCLUSIONS

1. The temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions.
2. Cellulolytics increased during biodegradation of chestnut biomass, while ligninolytics decreased as an effect of microbial competition and environmental conditions.
3. DGGE analysis showed similar profiles during the natural degradation process for both bacterial and fungal populations, except at the beginning of the process.
4. Because lignocellulosic biomass is also an attractive waste material for production of a wide range of high value-added products, cellulolytic, hemicellulolytic, and lignin-

lytic microorganisms should be isolated and selected for their biotechnological applications and used to improve the process.

5. To the authors' knowledge, there have been no previous investigations of the microbiological characterisation of chestnut biomass submitted to biodegradation under natural conditions. The first preliminary results are encouraging, but further tests must be carried out to improve the understanding of natural degradation of chestnut and the final product.

ACKNOWLEDGMENTS

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Microbiological and physic-chemical Characterization during chestnut Composting for sustainable Fertilization

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Abstract—**Dynamic parameters of the transformation of chestnut burr, leaf and plant biomass litter into a fertilizer by composting are reported in this study. Physic-chemical parameters, such as temperature, pH and activity water were influenced by the composition of the compost pile and by the climatic environmental conditions. Moreover, microbiological monitoring was assessed by culture and culture-independent methods. Cellulolytic, hemicellulolityc and ligninolytic populations were counted to determine the different microbial activity during the composting process. Functional microbial group analyzed showed different trend, but all were enumerated in high concentration (7-9 Log CFU/g). In addition, to evaluate the microbial diversity during composting, PCR-DGGE were performed for bacterial and fungal populations. Similarity level during composting was in general very high both in bacterial and in fungal populations. Only at the beginning of the process microbial populations were very different showing a similarity value of 60% and 42% unlike the other time of sampling for bacteria and fungi, respectively. These data are the first on composting suitable for chestnut forests.**

Keywords*; chestnut composting; microbiological monitoring; PCR-DGGE; sustainable fertilization;*

I. Introduction

Chestnut National Plan supports the need to retrain the chestnut towards more sustainable forms of production, characterized by a higher ability to meet needs and interests of consumers and producers offering new economic opportunities in agriculture.

Recently, the use of biomass is interesting both at EU and national level, especially after the agreements of the Kyoto Protocol (reduction of CO2 emissions into the atmosphere). The evaluation of compost made from chestnut residues of cleaning and pruning can be a source of added value for the sector. Although composting procedures are well known, few reports on chestnut composting were found in the literature [1]. Composting of the chestnut residues and its utilization in agriculture would be a possible approach to a sustainable management in chestnut forests. Benefits of compost application are well recognized. The use of compost contributes to :

- improve the structurural stability and increase soil fertility, recycling organic matter (humus) and nutrients in slow release;
- restore microbiological diversity in soil, limiting the development of plant pathogens and plant diseases;
- increase biological activity and root aeration;
- reduce the environmental impact, contributing to the degradation of pollutants and increasing crop protection and the storage carbon dioxide in soils;
- reduce the use of chemicals and disposal costs.

The studies of biotic and agronomic components represent keypoints to characterize the compost.

The aim of this study was to investigate fermentation processes in different times and analyze the compost obtained from a microbial and agronomic point of view.

Many studies have been conducted in pilot-scale reactors that enable easier tracking of the composting process: field experiments do not allow monitoring developments in fermentation, because it depends on many environmental factors.

To this purpose have been developed static aerated heaps for the composting of waste obtained from the cleaning of chestnut. During the process of bio-oxidation were carried out physical-chemical and microbiological monitoring by culture and culture-independent methods. In fact, the efficiency of composting stages depends on a variety of parameters, including aeration, temperature and content of moisture in the waste. The most importance, however, is represented by the microorganisms involved and their functional activity [2].

The aim of the work was the microbiological and physic-chemical characterization of a chestnut composting to understand the progress and limits of the process. An experimental field was established in Roccamonfina (province of Caserta), an area in southern Italy particularly suitable for chestnut forests.

Material and methods

A. *Caratteristics of the container composting*

The compost was obtained in a composting bin in which the resulting materials was placed in piles.

The size of the composting bin was 1.50 m x 1.50 m x 1.80 m (length x wight x height). To avoid water retain, under the box compost were placed fagots. The structure then was filled

with the resulting material of the cleaning of the chestnut (without shredding), in the following order:

1. layer of fresh shoots;
2. layer chipboard obtained from the cleaning of the stakes;
3. layer mowing the undergrowth (ferns, vetch, sainfoin, wild oats, horsetail grass calenzuola, curly leaves and old);
4. layer of leaves and curly dry year left on the ground in the chestnut.
5. layer of fresh shoots;
6. layer chipboard obtained from the cleaning of the stakes;
7. layer mowing the undergrowth;
8. layer of leaves and curly dry.

The composting bin was covered with 5 cm of soil taken in the chestnut forest.

B. *Sampling*

Samples of 1 kg were collected from the external (right and left side of the pile) and from the internal central part of biomass immediately after preparation (T0) and at 15, 30, 45, 60, 75, 90 and 105 days (T1, T2, T3, T4, T5, T6 and T7, respectively).

C. *Physic-chemical monitoring and Microbiological counts*

Compost samples were monitored by physic-chemical and microbiological parameters after T1, T2, T5, T6 and T7. Temperature (°C) was monitored by using specific sensors (1500 mm) placed in the core of the pile. The pH was also measured resuspending 25g of sample in a 250 ml Ringer's solution. Finally, water activity (aw) was evaluated by using HygroPalm23-AW (Rotronic AG, Basserdorf, Germany).

For microbiological count an initial compost suspensions were prepared by the addition of 20 g (w/vol) of the compost samples to 180 ml of quarter strength Ringer's solution (Oxoid, Milan, Italy) in 250 ml Erlenmeyer flasks. After shaking suitable dilutions were made performed in the same solution and were used to inoculate different solid media of growth. Selected populations of soil microbial community were detected at 28°C by using the Surface Spread Plate Count Method.

Cellulolytic microoganisms was were counted as described by Ventorino et al. [3].The medium used for hemicellulolytic populations was a minimal medium with xylan as sole carbon source. Ligninolytic microoganisms were enumerated as described by Pointing [4].

D. *DNA Extraction*

Microbiological monitoring by culture-independent method was performed for all compost samples (T0, T1, T2, T3, T4, T5, T6 and T7).

Microbial cells were desorbed from compost matrices and pellets were obtained prior to community DNA extraction as described by Rosewarne et al [5] with some modifications. Briefly, 100g of samples were resuspended in 400 ml of acidic solution (pH 2.0), shaken at 240 rpm for 10 min, filtered and centrifuged at 10000 rpm for 20 min. Microbial cells were resuspended in 45 ml of sterile phosphate buffered saline (PBS), then recovered by centrifugation at 6500 rpm for 20 min. The DNA was extracted from compost samples by using FastDNA Spin Kit for Soil (MP Biomedicals) according to the supplier's recommendation.

E. *PCR-DGGE*

The primers V3f and V3r, spanning the 200-bp V3 region of the 16S rRNA of E. coli [6] were used for bacterial PCR-DGGE analysis. PCR mixture and conditions were performed as described by Ercolini et al. [7].

To analyze the yeast population, the primers NL1 [8] and LS2 [9] corresponding to nucleotide positions 266 to 285 on the *S. cerevisiae* 26S RNA gene were used. PCR mixture and conditions were performed as described by Cocolin et al. [9].

In all PCR reactions a GC-clamp was added to the forward primer according to [6].

DGGE analyses were performed by using Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories). PCR products were loaded into 8% (wt/vol) polyacrylamide gels by using a denaturant gradient from 30 to 60% both bacterial and fungal analysis. Electrophoresis was performed at 60°C, initially at 50 V (5 min) and then at 200 V (240 min). The gels were stained in an ethidium bromide solution (5 min) and rinsed in distilled water (20 min).

F. *Statistical analysis*

Bands were automatically detected by using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England). The cluster analysis was performed by the program after band matching; the method described by Saitou and Nei [10] was used to obtain the correlation matrix of the DGGE patterns. The resulting matrix was used in the average linkage method by the Cluster procedure of Systat 5.2.1 in order to estimate the percentage of similarity (S) of microbial populations in composting process.

III. Results and Discussion

The site used to setting-up composting experiment was chosen because their climatic characteristics (400 m above sea level and north-west sun exposure), representative for chestnut forest.

The periodic measurement of the temperature during composting is an important index to monitor the fermentation process since it is closely related to environmental conditions and to the metabolic activities of microorganisms. After 15 d the temperature values observed in the pile was 27.1°C (T1 sample). This value increased until 26.6°C after 105 days of composting (T7 sample). The temperature values in the internal part of the pile was strongly affected by the seasonal climatic conditions. In fact, as showed in "Fig. 1", the temperature increase until September to undergo a sudden fall in October.

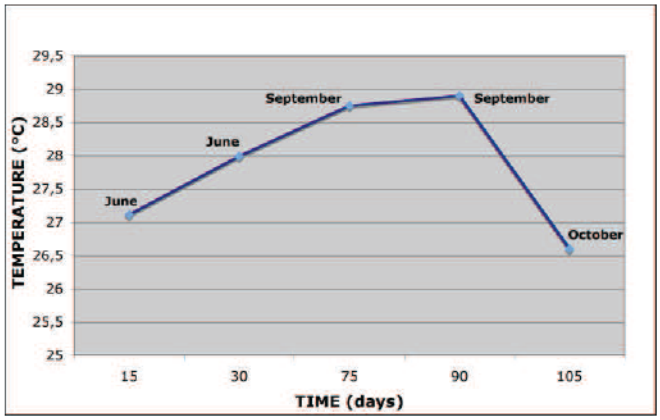

Figure 1. Temperature values determined during composting process.

Measurements of other parameters such as pH and activity water (a_w), water readily available for the microbial metabolic activities, were monitored to understand the progress and limits of the process. The pH values observed were quite stable since they range from 6.75 and 6.8 at T1 and T7 time sampling, respectively. Moreover, the value of a_w were 0.94 after 15 d of experimentation increasing up to 0.98 after 105 days of composting sampling.

The three different analyzed functional microbial groups involved in carbon cycle showed a different trend during composting. Hemicellulolytic populations showed a value of about 9 Log CFU/g of sample during experimental process (Table I). Indeed, after 105 days of composting (T7) it was possible to detect an increasing in cellulolytic populations showing a concentration reaching to 10 Log CFU/g. Observing the progress of growth of aerobic cellulolytics, it was possible suppose that the organic matrix showed a good starting composition in cellulosic material. Presence of cellulolytics during the whole composting process play an important role on the cellulose that often represents a common constituent of organic waste, whose degradation is restricted to a narrow range of cellulolytic microorganisms [11]. Ligninolytic microorganisms showed an opposite trend. In fact in this population a significant decreasing of about 1 log unit (from 8.48 to 7.59 Log CFU/g) was detected (Table I). Probably the decrease of temperature at T7 could negatively influence the ligninolytic microbial activities especially with regard to the fungal population.

TABLE I. MICROBIAL CONTENTS (LOG CFU/G) DURING COMPOSTING

Time sampling	Microbial groups		
	Hemicellulolytic	*Cellulolytic*	*Ligninolytic*
T1	9.04	9.00	8.47
T5	9.36	9.65	9.25
T6	8.57	8.64	7.34
T7	8.96	10.17	7.59

The results of the PCR-DGGE analysis performed on the samples during composting are shown in "Fig. 2".

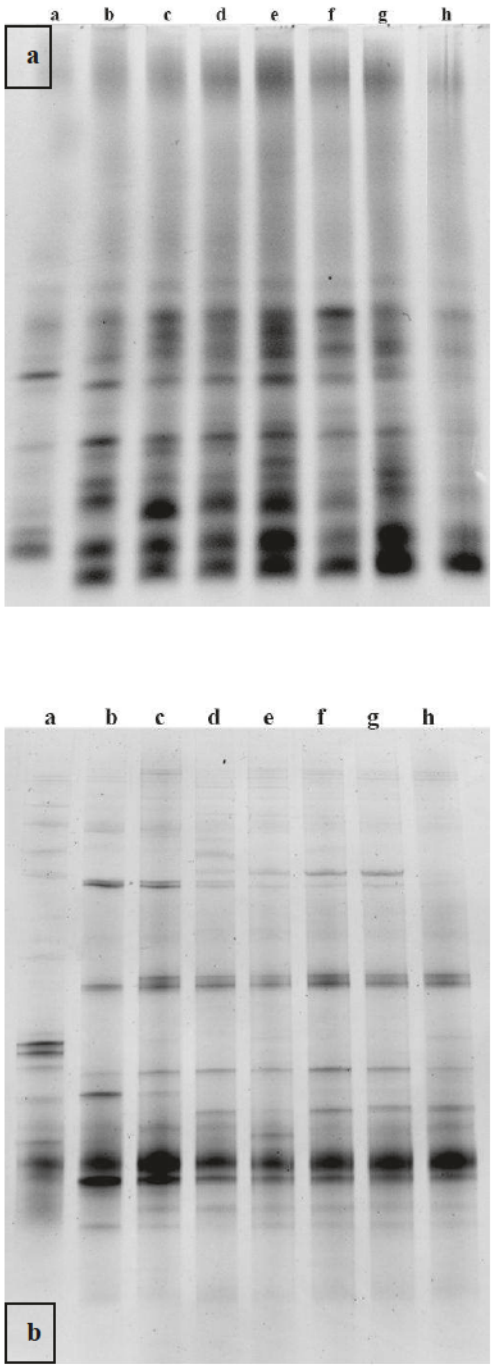

Figure 2. DGGE profiles of bacterial (a) and fungal populations during composting. Lanes: a, T0; b, T1; c, T2; d, T3; e, T4; f, T5; g, T6; h, T7.

The similarity level (S) in bacterial populations during composting was in general very high showing values from 100% to 95%, except to T0 "Fig. 3a". In fact, only at the beginning of the process the bacterial populations were very different showing a similarity value of 60% unlike the other time of sampling. A similar trend were observed in fungal populations "Fig. 3b". In this case the microbial diversity detected in T0 was most marked showing a similarity value of 42% with the other time. Moreover, the levels of similarity among the fungal populations during the composting varied more gradually than those detected among bacterial populations as shown in "Fig. 3". These results showed that the bacterial population were less influenced by the environmental conditions as the cellulolytic functional group. By contrast, fungal population suffered a greater variation during the composting showing a behavior similar to ligninolytic functional group. It is possible speculate that the ligninolytic group comprised mainly fungi than bacteria that exhibit more cellulolytic and hemicellulolytic activity.

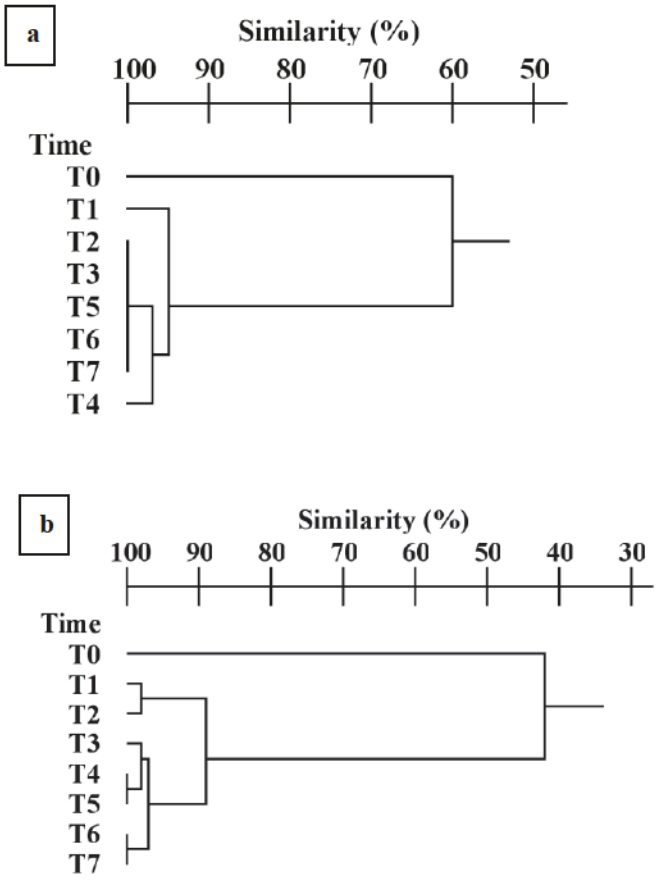

Figure 3. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the bacterial (a) and fungal (b) populations during composting.

IV. Conclusions

The present study aims to create useful innovations to increase the sustainability and profitability of production, in accordance with the regulations of organic farming and environmentally friendly criteria. Innovations are of agronomic and biotechnology: they seek to pursue the well-being of those who work in remote areas, creating less impact on the environment and improving the quality of the product.

The encouraging preliminary results are leading to program:

- test for evaluating the quality of compost against antagonists of pathogens;
- insulation tests fertilizer of active microorganisms in the promotion of plant growth, and the microbial agents able to induce the defense response in the plant;
- assays for the identification of bioactive metabolites can be used in the field of defense plant disease in organic.

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