University of Naples Federico II

Department of Agricultural Sciences

Improvement and Management of Agro-forestry Resources

BIO-EFFECTORS: REDUCING THE IMPACT OF MINERAL FERTILIZERS IN AGRICULTURE BY APPLICATION OF MICROBIAL AND HUMIFIED PRODUCTS

Ph.D. Thesis

Hiarhi Monda

Tutor: Prof. Riccardo Spaccini

Co-Tutor: Prof. Alessandro Piccolo

Coordinator: Prof. Guido D'Urso

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SUMMARY

Sustainability in natural ecosystems is dependent on chemical and biological balance in the soil which is mainly governed by the cross-interaction of microbial communities with humic matter and plant roots, a concept that can be directly expressed as the micro-bio-humeome in the rhizosphere. The development of efficient tools to manage these key factors could offer to modern agriculture a way to improve productivity and reduce the reliance on chemical fertilizers. The research described in this thesis is focused on the increase of efficiency of the most promising microbial bio-effectors (i.e. plant growth promoting microorganisms PGPMs) and active natural organic compounds derived from recycled biomasses with the final goal to contribute to the sustainable intensification of agriculture, by developing a viable alternative to mineral fertilisers.

A series of humic materials were obtained from different composted agricultural biomasses: artichoke compost (C-CYN), an artichoke/fennel compost (C-CYNF), a tomato/woodchips compost (C-TOM), in order to evaluate their bioactivity on maize seeds germination and seedlings growth in initial hydroponic experiments. Both water-extractable organic matter (WEOMs) and humic acids (HA) isolated from the different *on-farm* composts were characterized in details for their chemical properties and molecular composition. Experiments in growth chamber highlighted the relationship with the capacity of isolated humic materials to increase rate of germination of maize seeds and growth promotion of maize plantlets. The carbohydrate-rich CYN-WEOM was the material that produced the greatest germination rate and plant growth development.

A greenhouse pot experiment was conducted with maize plants and a phosphorus deficient soil to verify the capacity of different phosphate solubilising microorganisms (*Trichoderma harzianum* and *Bacillus amyloliquefaciens*), to increase P availability from two mineral fertilizers (triple superphosphate and rock phosphate) and from two organic amendments (composted cow/buffalo manure and composted horse manure). The ability of each bio-effectors to promote maize plant

growth and nutrient uptake and their effect on the microbial community through the analysis of phospholipids fatty acids (PLFA) and neutral lipids fatty acids (NLFA) was investigated. Mineral fertilizers produced the best performances but evidence is provided that the addition of composted manure and microorganisms positively affected indigenous mycorrhizal fungi population and plant fitness when compared to control. However, None of the used PGPM per se appeared capable to provide a feasible alternative to mineral fertilizers.

A second greenhouse experiment verified the potential synergistic effect of different combination of bio-effectors towards the increasing efficiency of plant nutrient uptake. Two phosphate solubilizing bacteria, *Pseudomonas* spp. proradix® and *Bacillus amyloliquefaciens*, a mixture of two arbuscular mycorrhizal fungi (AMF), *Glomus mosseae* and *Rhizophagus irregularis*, and a humic acid (HA) extracted from manure compost were combined in order to study the best combination when composted manure were used as a P-source. Best synergistic effects were obtained with the combination of phosphate solubilizing microorganisms, AMF and humic acid. Furthermore, soil analysis of PLFA biomarkers and DGGE molecular fingerprint provided an insight into microbial communities changes and composition. It was revealed the increased development of AMF and the simultaneous depletion of saprophytic fungi when plants were co-inoculated with *B. amyloliquefaciens* and HA.

Although strong evidence supports the functional significance in plant growth promotion of phosphate solubilising microorganisms in laboratory experiments and knowledge on specific mechanisms of various BEs has accumulated, field experiments represent the final step in order to verify the application of new technologies. A field experiment was carried out to assess the potential efficacy of a phosphate solubilizing bacteria, *Pseudomonas* spp. proradix® in combination with economically feasible quantity of organic amendments (cow/buffalo composted manure) with the final goal to evaluate the differences compared to mineral fertilizers (triple superphosphate, TSP). Preliminary results evidenced only a slight decrease, in plants treated with composted manure, of grain yield and nutrient content, especially when microbial strains were concomitantly

added to soil. These promising results support the idea that new sustainable fertilization technology are possible and only needs to be optimized relying on the current acquired knowledge.

The outcomes achieved by this dissertation provide valuable information on the dynamics of the multi-partite interactions involved in the rhizosphere and highlight the role of humic substances in sustaining microbial activity and possibly driving plant microbes cross-communication. The combination of different bio-effectors is thus confirmed as a potential biotechnological tool to overcome mineral fertilization systems.

CHAPTER I

INTRODUCTION

The global rural population that lives on land farming are responsible for more than half of the world's food production (FAO, 2012). Since ancient times, indeed, agriculture has represented the most important source providing nourishment, as well as fibers and fuels, leading a global economy based on the production of high quality, safe and affordable food and products for an ever-increasing worldwide population, which, according to United Nations evaluations, is projected to reach 8.9 billion by 2050 (Cohen, 2001). This means to satisfy an increased demand in food supply with a consequent depletion of soil and natural resources. Although many basic practices of agriculture have remained the same over the times, farming techniques have changed dramatically in the last 50 years. Modern farmers have had a greater awareness of the concepts of cost and profit focusing on much higher efficiency and business management. To maintain incomes, there has been a trend of agricultural specialization and fusion of small plots of land in large estates, as a result the size of the companies have grown, reaching an average of 18.4 hectares (1997). Due to economies of scale, farmers were able to increase the yield and productivity using techniques that lead to automation process and intensification of the agro-chemicals application. Production methods have been then standardized and the average yield of crops has increased more than 55% compared to the '50s, the decade that is generally considered the beginning of the Green Revolution (Ray et al., 2013).

However, agricultural productions, as well as every human activity, entail negative impacts on the environment: deforestation, water flow channeling, chemicals use are just some of the several practices involved in generating the progressive erosion of soil with a resulting decreased fertility

level, the reduction of wild fauna habitats, the declining of biodiversity and the raised emission of greenhouse gases.

The report of the International Resource Panel of the United Nations Environment Program published in 2010 states that agriculture and food consumption are considered among the most important factors that generate strong pressures on the environment. Although Green Revolution has brought great improvements, the price paid in terms of environmental damage is still very high; the massive use of mineral fertilizers depletes the organic matter of the soil, the excessive use of pesticides has a negative impact on the health of farmers and consumers, the continuous withdrawal of water leads to a reduction of water reserves, and the constant use of monocultures has negative effects on biodiversity. Is therefore required a radical change in production systems that takes advantage of the most advanced research and innovation. The primary goal for future agriculture is to guarantee long-term food security and to meet the growing world food demand by sustainable cropping strategies capable to reduce the external inputs. In order to achieve these targets, innovative technologies that suit a more efficient management of the limited natural resources, such soil, reserves of mineral nutrients, energy and water, are needed and required to preserve biodiversity and reducing the environmental impact of agricultural production. Because of its systemic nature the studies on sustainability are not limited to separately analyze individual components, but need to extend the investigation field to the multiple interactions between subjects and assess the consequent effects. Research is therefore called to pursue multiple objectives in a multi and interdisciplinary manner.

Regarding these challenges, an improved understanding and utilization of biological processes supporting soil fertility, healthy plant growth and resource efficiency are required too (Uphoff et al., 2006). In this context, the interactions between plant, soil and microorganisms represents an important starting point to recognize and improve processes underlying the uptake of nutrients, the optimization of microbial activity, the root and shoot growth and suppression of pathogens. Approaches to manage this complex network of interactions are regarded as promising alternatives

to improve crop yield as well as decrease or even replace conventional agrochemical use and arise as a major challenge of this project.

To keep soil fertility with an eco-friendly approach, is essential an in-depth knowledge of the physical, chemical and biological processes that provide nutrients essential for the healthy growth of nutritive crops in plant available forms (Garnett and Godfray, 2012). Mineral nutrients are commonly supplied above the harvest compensation levels due to the loss from agricultural system by leaching, erosion, or quickly conversion into unavailable forms, or in gaseous form. In addition, they need to be replaced continuously to balance the quantities removed with the harvest and soil organic matter that is degraded during the growing season. Sophisticated and interdependent nature of plant interactions with multiple components of the rhizosphere environment could potentially shift to an approach that emphasizes the "built-in" inherent self-sustaining strengths of agro-ecosystems. Plant roots respond dynamically to their specific soil environment and play an active role in the spatial or chemical acquisition of nutrients and water (Neumann and Römheld, 2002). Furthermore, plants nourish mutual interactions with a wide variety of soil microorganisms, mediated in the close proximity of their roots, named "rhizosphere", which may assist or hamper the functions of the root, thus representing an important determinant of soil fertility (Watt et al., 2006; Marschner, 2012; Neumann and Römheld, 2012).

One of the most important interactions between plants and microorganisms is represented by mycorrhizal symbiosis: about 90% of plants establish with this class of fungi a mutually beneficial partnership. Mycorrhizae play a key role by gathering essential mineral nutrients and transporting them into the plant, increasing its growth. In turn, the plant provides carbohydrates as a source of energy for their fitness. Moreover, phytohormones and other signaling compounds of plant and microbial origin are involved in the intra- and inter-specific communication between roots, shoots and associated microorganisms (Neumann and Römheld, 2002). A wide range of natural compounds has beneficial plant growth effects.

Humic substances extracted from geochemical sources, such as lignite, or recycled biomass, such as

compost, have been shown to enhance plant performances by direct or indirect growth stimulatory mechanisms, such as phytohormonal effects (Piccolo et al., 1992; Zandonadi et al., 2007; Canellas et al., 2008). The benefit of these bioeffectors can be attributed to diverse, but poorly defined modes of action, including inhibitory, as well as stimulatory effects on plant-associated microbial populations (Canellas and Olivares, 2014; Ramos et al., 2015).

In this context, increasing the field efficiency of interactions between the most promising plant growth promoting microorganisms (PGPMs) and/or active natural compounds (i.e. bio-effectors) represents the final goal to contribute to the ecological intensification of agriculture, by developing a viable alternative to mineral fertilizers.

OBJECTIVES

The general objective of this work was to relate the effects of microbial bio-effectors and well characterized chemical bio-stimulants to the performance of maize plants grown in P-deficient soils growth in either growth-chamber or greenhouse or field experiments. The synergistic bioactivity of bioeffectors, bio-stimulants and inorganic and organic P fertilizers was assessed not only by following germination rate of maize seeds, growth of maize plantlets and plant nutrient uptake, but also by evaluating the effects on the microbial communities of soils though chemical and biotechnological methods.

The final goal of this doctoral research was further to understand the relationship between bioeffectors/biostimulants with the mechanisms involved in plant growth, and, thus, contribute to the economically sustainable, environmental-friendly and ecological intensification of agriculture.

CHAPTER II

Experimental Developments

2. 1 Molecular characteristics of water-extractable organic matter from different composted biomasses as related to effects on seed germination and early growth of maize

Background concept

Dissolved organic matter is widely recognized to influence soil biological activity. Water-phase properties such as pH and ionic strength determine organic matter solubility, whereas its interaction with clay minerals affects the sorption/desorption equilibrium between the dissolved phase and the solid phase of soil organic matter.

Crop residues can be used to produce green compost that is rich in nutrients and humified organic material. An easy and fast way to take advantages of compost properties is to extract water soluble organic matter and test its properties on plant germination and growth.

Development

Water-extractable organic matter (WEOMs) was isolated from different *on-farm* compost (C-CYN = composted artichoke residues, C-CYNF = composted 43.5% artichoke, 23.5% fennel residues, C-TOM = composted tomato residues, C-MSW = commercial urban-waste compost).

A detailed characterization, by means of NMR ^{13}CPMAS, ^{1}H NMR and FTIR-ATR spectroscopy and thermo-gravimetric analysis (TGA, DSC), were used to study WEOMs chemical-physical properties.

The application of WEOMs on germination and early growth of maize seedlings was tested for their potential use as biostimulants. Phenological data and chlorophyll content were evaluated. Multivariate statistical analysis (PCA) was used to asses relationship between WEOMs properties and bioactivity.

Results

Best performances were obtained with the application of CYN and TOM WEOMs. We found the aromatic components to influence mainly the root length and architecture, while the most hydrophilic compounds are associated with the increase in biomass. A flexible conformational structure, due to the large content of carbohydrates, could facilitate solubility of bioactive molecule capable to boost biological activity on plant growth.

2.2 Biostimulation of humic acids isolated from different *on-farm* composts. Relationship between chemical structure and biological effects on maize early growth

Background concept

Investigation about the nature of humic substances is of primary importance in order to understand the mechanisms by which supramolecular humic aggregates influence plant physiology and

biochemistry. Humic acids (HA) are most studied humified organic matter fraction because of their reactivity and biostimulation properties on plant growth. Isolation of HA from compost could represent an alternative to non-renewable sources such as soils, peat and leonardite.

Development

HA were isolated from different *on-farm* compost (HA-CYN = composted artichoke residues, HA-CYNF = composted 43.5% artichoke, 23.5% fennel residues, HA-TOM = composted tomato residues, HA-CAV = composted cauliflower residues).

A detailed characterization, by means of NMR ^{13}CPMAS, ^{1}H NMR and FTIR-ATR spectroscopy and thermo-gravimetric analysis (TGA, DSC), were used to study HA chemical-physical properties.

The application of HA at three different concentrations (25, 50 and 100 ppm C L^{-1}) was tested on early growth of maize seedlings. Phenological data and chlorophyll content were evaluated. Analysis of variance (ANOVA) and multivariate statistical analysis (PCA) were used to compare means and assess relationship between HA properties and bioactivity.

Results

All assayed HA favored plant growth in a very similar way, favoring relatively large responses at low application rates. Correlations between chemical-physical characteristics of HA and stimulation of plant growth suggest that bioactivity is to be more related to the strength with which humic molecules are mutually associated. The balance between hydrophobic/hydrophilic domains was responsible for the observed dose-response effect.

2.3 Synergistic effects of phosphate solubilizing bacteria, AMF and humic acids enhance plant growth in a compost-based organic farming

Background concept

Sustainable agriculture requires inter-disciplinary studies to address new understanding of the complex multifaceted environment in which chemical and biological soil-plant-microbe interactions occur. Investigation on the relationship between plant growth promoting microorganisms, arbuscular mychorrhizal fungi and humic acids in the rhizosphere could help the development of new bio-formulations capable to increase plant nutrients uptake and contribute to an eco-friendly management of fertilizers and, at the same time, reduce the reliance on agro-chemicals.

Development

The combination of two phosphate solubilizing bacteria (PSB), *Pseudomonas* proradix® and *Bacillus amyloliquefaciens*, a mixture of two arbuscular mycorrhizal fungi (AMF), *Glomus mosseae* and *Rhizophagus irregularis*, and a humic acid (HA) extracted from green compost were applied to grow maize plants in a greenhouse pot-trial using cow/buffalo composted manure as source of phosphorus (P). Synergistic effects on plant growth were evaluated by measuring phenological parameters (fresh and dry biomass, fresh and dry shoots and roots, plant height). Total plant P was evaluated colorimetrically and total nitrogen content by means of Kjeldahl digestion method. Analysis of variance (ANOVA) were used to assess significant differences among treatments.

To investigate the specificities of plant microbial interactions in soil, phospholipid fatty acids (PLFA) and denaturing gradient gel electrophoresis (DGGE) techniques were used. Principal component analysis (PCA) was used to evaluate PLFA biomarkers distribution, whereas

permutation test based on Pearson's correlation coefficient was used to detect changes in the microbial functioning and dynamics.

Results

Best absolute bioactivity on plant growth were obtained with combination of *B. amyloliquefaciens*, AMF and HA. PLFA and DGGE analysis of soils showed a significant variation in both bacterial and fungal community structure induced by co-inoculation. A key role in the synergistic response was attributed to the ability of HA to provide metabolic carbon source and to increase plant exudation of organic acids, that, in turn, triggered the association and colonization of lateral roots by beneficial microbial communities.

RESEARCH IN PROGRESS I

2.4 Effects on P availability to plants and soil microbial community composition in a greenhouse treatments with organic and inorganic P sources and bioinoculants.

A greenhouse pot experiment was conducted with maize plants treated with different phosphate solubilising microorganisms (*Trichoderma harzianum* and *Bacillus amyloliquefaciens*) in a phosphorus deficient soils, when different type of P-fertilizers were used (mineral fertilizers: triple super phosphate and rock phosphate; organic fertilizers: composted cow/buffalo manure and composted horse manure).

We investigated the ability of each bio-effector to promote maize plant growth and nutrient uptake and evaluated the effects on the soil microbial community through PLFA and NLFA analysis.

Preliminary results:

Mineral fertilizers were most effective on plant growth but evidence is provided that the addition of composted manure and microorganisms enhanced the indigenous mycorrhizal fungi population and plant fitness, as compared to control .

Further analyses to be still conducted: shoot P, N, Ca, K, Mg, Cu, Fe, Mn and Zn concentration, mycorrhizal root colonization, PLFA, available soil P.

RESEARCH IN PROGRESS II

2.5 The effect of *Pseudomonas* spp. treatment on maize (*Zea mays L.*) growth, yield and phosphorus uptake in field conditions under mineral and low-input organic fertilizers.

Aim of this study was to investigate plant growth and agricultural productivity in a low input and organic farming. Field experiment from June to October 2014 was conducted to compare the mineral with the organic fertilization (triple super phosphate and cow/buffalo composted manure, respectively) in presence of a plant growth promoting rhizobacteria (*Pseudomonas* spp., Proradix®). Maize plants were harvested at four different period (30d, 60d, 90d and 120d) and the phenological parameters measured.

Preliminary results:

Best plant growth results were obtained with mineral fertilizer, as expected, but plants treated with composted manure showed only a slight decrease in grain yield and nutrient content, especially when microbial strains were concomitantly added to soil. Our results promise to cast the basis of a new fertilization technology whereby compost as organic fertilizer is used in combination with plant growth promoting microorganisms.

Further analyses to be still conducted: shoot P, N, Ca, K, Mg, Cu, Fe, Mn and Zn concentration, mycorrhizal root colonization, PLFA, available soil P.

CHAPTER III

Molecular characteristics of water-extractable organic matter from different composted biomasses as related to effects on seed germination and early growth of maize

Keywords: Compost water extracts; Humic substances; Bioactivity; Seedling growth; NMR ^{13}CPMAS; IR DRIFT; TGA

Abstract: Water-extractable organic matter (WEOM) was obtained from composts made out of residues of artichoke (C-CYN), artichoke/fennel (C-CYNF), tomato/woodchips (C-TOM) and a commercial municipal solid waste (C-MSW). WEOMs were used as bio-stimulants of both maize seeds germination and maize seedling growth. Composts and their extracts were characterized by spectroscopic (^{13}C-CPMAS- and ^{1}H-NMR, FTIR-ATR) and thermal methods (TGA, DSC). CYN-WEOM was the only active material to increase germination rate and primary and lateral roots length, whereas activity of other extracts was not significantly different from control. Conversely, all WEOM extracts had positive effects on plant growth. The effective bioactivity of CYN-WEOM appeared to be correlated to a large degree of aromaticity and content of methoxyl groups, while that of TOM-WEOM was associated not only to preservation of lignin residues but also to the presence of carbohydrates. Our results confirm that the presence of aromatic components mainly affect root length and architecture, especially when in combination with more hydrophilic structures, thereby allowing the conformational flexibility required for the hormone-like molecules to exert their bioactivity on biological surfaces. This work also highlighted that the WEOMs from compost from horticultural biomasses were more effective that those obtained from mainly alkyl

but equally hydrophobic municipal organic wastes, which depressed germination rate, while still improving plant growth.

1. Introduction

The composting process is a low-cost and sustainable technology to recycle organic biomasses, while its use in agriculture has become popular as alternative to chemical fertilizers and to soil pathogens treatements (Veeken and Hamelers, 2002; Pane et al., 2013). The biochemical transformation of organic biomasses leads to a process of humification that resembles that occurring in soil, by which apolar compounds progressively accumulate in hydrophobic domains excluded from water and microbial activity (Piccolo, 2002; Spaccini et al., 2002; Spaccini and Piccolo, 2007a). Nevertheless, a fraction of organic matter can be still extracted from compost in aqueous solutions (WEOM) and, as dissolved organic matter (DOM), may be operationally defined as the organic matter passing a filter pore size of 0.4-0.6 μm (Herbert and Bertsch, 1995; Zsolnay, 1996; Puglisi et al., 2010).

DOM and WEOM are composed by an a heterogeneous mixture of medium-polar molecules which arrange in relatively complex supramolecular associations (Piccolo, 2002; Nebbioso and Piccolo, 2013). The humic molecules in WEOM reflect the biological and chemical transformation of organic matter (Chefetz, 1998), influence soil biological activity (Flessa et al., 2000), and interact with metals and organic pollutants (Romkens and Dolfing, 1998; Nebbioso and Piccolo, 2009; Smejkalova et al., 2009). While a number of studies has followed WEOM changes during composting (Said- Pullicino and Gigliotti, 2007; Maia et al., 2008; Shao et al., 2009), only recently a rising attention has been devoted to the role of humic molecules from compost as a source of bioactive compounds (Canellas et al., 2002; Ramos et al., 2015).

It is well-established that natural organic matter exerts significant and direct influences on plant growth, affecting morphological, physiological and biochemical processes on seed germination, cell

differentiation, ion uptake and overall plant growth (Nardi et al., 2007; Canellas and Olivares, 2014; Vaccaro et al., 2014). As in the case of humic substances, compost WEOM at high concentration is recognized to promote plant growth by acting on membrane bound H+ -ATPase in the root system increasing nitrate uptake (Pinton et al., 1999; Canellas and Olivares, 2014). A compost obtained with citrus biomass residues and its WEOM were found to exert an hormone-like activity on melon seedlings, even though the effect of the water extract was significantly lower (Bernal-Vicente et al., 2008). Furthermore, WEOM was believed to contain unidentified chemical properties, such low molecular weight compounds, that appear to play a role as biocontrol agent and induce systemic resistance in plants (Weymann et al., 1995; Cronin et al., 1996; Zhang et al., 1998).

However, there is still a relative little information on the relationship between WEOM chemical composition and its biological activity on plant growth. In fact, a detailed molecular characterization is an essential requirement to elucidate the interaction between the intrinsic structural complexity of compost extracts and its plant biochemical activity. Several properties, such as pH and ionic strength of the aqueous extract, determine the WEOM solubility (Chantigny, 2003), while the extract concentration can affect the strength of the supramolecular arrangement of humic molecules (Piccolo, 2002; Smejkalova and Piccolo, 2008). The environmental reactivity of WEOM has been recently related to the hydrophobic/hydrophilic ratio of molecular components (Dobbss et al., 2010; Canellas et al., 2012; Canellas ad Olivares, 2014), although the impact of this ratio on bioactivity has not yet been extensively studied, and existing information are fragmented. In fact, compost WEOM may be expected to exert an important role in improving soil fertility by promoting plant growth.

The aim of this work was then to obtain detailed information on the molecular composition of WEOM from different compost types and relate it to the effect on plant bioactivity.

2. Materials and methods

2.1 Compost and water-extractable organic matter (WEOM)

The composts used in this study were obtained through a 45-days on-farm composting process (active or thermophilic phase) of static piles of chipped plant residues under forced aeration, followed by a two months-curing period. The different on-farm composts were selected on the basis of their composition and identified as: 1. C-CYN = 78.0% artichoke, 20% woodchips and 2% mature compost as starter; 2. C-CYNF = 43.5% artichoke, 23.5% fennel, 11.0% escarole residues, 20% woodchips and 2% mature compost as starter; 3. C-TOM = 50.0% tomato residues, 48% woodchips and 2% mature compost as starter; 4. C-MSW = a 1-year old commercial compost made of urban-waste purchased from Gesenu (Perugia,Italy). An aliquot of each compost was freeze-dried before chemical analyses..

WEOM was obtained from each compost as it follows. 100 g of each air-dried compost was suspended in 1000 ml of distilled water and mechanically shaken for 24 h. The suspension was then centrifuged at 2500 rpm for 15 min and finally filtered through a 0.45 μm Whatman filter. An aliquot of each WEOM extract was freeze-dried before further analytical analysis.

Some physical and chemical properties of the four composts and WEOM samples are shown in Table 1. The electrical conductivity (EC) and the pH were measured by conventional methods. The pH was determined in water (1:10 w/v), as well as the electrical conductivity (EC) (1:10 w/v). The C and N concentrations were measured in composts and WEOM samples by an element analyzer (CHNS, Fison Instruments EA).

2.2 NMR Spectroscopy of composts and WEOM

A 300 MHz Bruker Avance spectrometer, equipped with a 4 mm wide-bore MAS probe, was used to run solid-state spectra of compost samples. Each fine-powdered sample was packed into a 4 mm zirconium rotor, stoppered by a Kel-F cap and spun at a rate of 13000±1 Hz. In particular, ^{13}C-

NMR spectra were acquired through the Cross-Polarization Magic-Angle-Spinning (CPMAS) technique, by using 2 s of recycle delay, 1 ms of contact time, 30 ms of acquisition time and 4000 scans.

A 400-MHz Bruker Avance spectrometer, equipped with a 5-mm Bruker BBI (Broad Band inverse) probe, was employed to conduct liquid-state NMR spectra of water-soluble extracts from compost. Each sample (5.0 mg mL^{-1}) was dissolved in deuterated water (D2O) and placed into a 5.0-mm quartz tube. ^{1}H-NMR spectra were acquired with 2 s of thermal equilibrium delay, 90° pulse length ranging within 8.5 and 9.5 μs, 32768 time domain points, and 64 transients. The residual water signal at around 4.7 ppm was suppressed by adopting the on-resonance pre-saturation technique (~57 dB power attenuation). All spectra were processed by using both Bruker Topspin Software (v.2.1, Bruker Biospin, Rheinstetten, Germany) and MestReC NMR Processing Software (v.4.8.6.0, Cambridgesoft, Cambridge, Massachusetts, USA).

2.3 FTIR–ATR Spectroscopy of WEOM

Infrared (IR) spectra were recorded on a Perkin-Elmer Frontier Fourier Transform Infrared Spectrometer using an attenuated total reflection (ATR) device equipped with a diamond/ZnSe crystal. About 2 mg grinded powders were put on the crystal device and the contact was obtained by applying a strength of about 150 N on the sample. Each spectrum was subjected to 32 scans with the resolution of 4 cm^{-1} from 4000 to 400 cm^{-1} region. The sample was scanned five times and the average of these spectra was analyzed.

2.4 Thermogravimetric analysis and differential scanning calorimetry

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves were obtained by air combustion of approximately 10 mg of each studied sample in a simultaneous

thermal analyzer (STA 6000-Perkin Elmer). The initial and final temperatures were 30°C and 700°C, respectively, with an increasing temperature rate of 10°C min^{-1}.

Plant material and bioactivity assays

2.5.1 Germination test

The germination tests were conducted in a growth-chamber at 25°C in the dark by setting relative humidity at 85%. Fifteen maize seeds (*Zea mays* L. cv. 30.21, Limagrain) were placed on a filter paper in Petri dishes (9 cm diameter) and moistened with 10 ml of either distilled water (control) or one of the WEOM extracts. All treatments were replicated 5 times. After 5 days of incubation, germination rate, length of coleoptile, and of primary and lateral seminal roots were evaluated. The percentages of relative seed germination (RSG), relative primary root growth (1RRG), relative secondary root growth (2RRG) and germination index (GI) were calculated as follows:

$$\text{RSG (\%)} = \frac{\text{n° seeds germinated WEOM}}{\text{n° seeds germinated control}} * 100$$

$$\text{RRG (\%)} = \frac{\text{mean root lenght WEOM}}{\text{mean root lenght control}} * 100$$

$$\text{GI (\%)} = \frac{\text{RSG}}{\text{1RRG} + \text{2RRG}} * 100$$

2.5.2 Growth of maize seedlings

A bioactivity assay on seedling growth was conducted in the growth-chamber under the following parameters: 75% of humidity, 20-27°C temperature range and a photoperiod comprising 8 h of darkness and 16 h of light. Maize seeds (*Zea mays* L. var. 30.21, Limagrain) were soaked in distilled water for one night and germinated in the dark at 25°C on filter paper moisted with

distilled water. After germination, maize seedlings (four days old) with uniform size, shape and healthy aspect were selected and transferred into 15 mL tubes filled with a modified Hoagland solution (Hoagland and Arnon, 1950) composed of: 40 µM KH_2PO_4, 200 µM $Ca(NO_3)_2$, 200 µM KNO_3, 200 µM $MgSO_4$, 10 µM FeNaEDTA, 4.6 µM H_3BO_3, 0.036 µ M $CuCl_2 \cdot 2H_2O$, 0.9 µ M $MnCl_2 \cdot 4H_2O$, 0.09 µ M $ZnCl_2$, 0.01 µ M $NaMoO_3 \cdot 2H_2O$. After 8 d from transplanting, plantlets were treated as it follows: 1. control with only nutrient solution, 2. addition of the WEOM extract diluted 1:10 into the nutrient solution. Plants were harvested 72 h after the application of WEOM treatments. Fresh and dry weight of shoots and roots and root length were measured.

The chlorophyll content was determined as it follows: 9 mm of fresh foliar tissue from each plant were ground in liquid nitrogen, homogenized in 5 ml of pure acetone, and then transferred into 15 ml glass centrifuge tubes to be centrifuged at 3000 x g for 5 min at 8°C. The supernatant absorbances at 645 and 663 nm were measured using a spectrophotometer and the concentrations of Chl a, Chl b, total Chl (Chl a + b) were calculated according the method of Lichtenthaler (1987) and expressed in mg of pigment per cm^{-2} of leaf fresh weight.

Roots of seedlings and plantlets from both germination and growth tests, respectively, were scanned with an Epson Perfection V700 modified flatbed scanner and measurements of their length were achieved by the WinRhizo software, version 2012b (Regent Instruments, Inc.).

2.6 Data analysis

Data obtained were subjected to one-way analysis of variance (ANOVA) and tested for significance with Tukey´s HSD test in SPSS software. Statistical significance was defined for $p<0.05$. In order to evaluate the possible bioactivity of WEOM on plant growth, we applied principal component analysis to phenological and analytical data as exploratory tool to identify which variables mostly affect the result of WEOM application and to predict how they correlate compost and extract chemical features.

3. Results

3.1 Chemical composition of WEOM

The main chemical characteristics of compost and WEOM samples are reported in Table 1. MSW-WEOM showed the highest pH and EC values, whereas the latter parameter was the lowest for CYN-WEOM. Both C-MSW and MSW-WEOM were richest in carbon and nitrogen, resulting in the lowest H/C ratio. Conversely, C-CYN and CYN-WOEM had the largest C/N ratio, followed by C-CYNF and CYNF-WOEM.

3.1 Solid-state ^{13}C NMR spectra of composts

The ^{13}C-CPMAS-NMR spectra of compost samples (Fig. 1) indicated a predominance of O-alkyl carbons (60-110 ppm) due mainly to mono- and polysaccharides (Spaccini and Piccolo, 2009). Spectral integration of C-CYN, C-TOM, C-CYNF and C-MSW samples revealed that this region accounted for 54.8, 51.8, 46.6 and 54.3% of total signal area, respectively (Table 1). In particular, the intense signal at 72 ppm is ascribable to signals overlapping of C-2, C-3, and C-5 carbons in the pyranosidic structures of cellulose and several hemicelluloses, whereas the 104 ppm signal is commonly attributed to sugars anomeric carbons. The large NMR resonances between 0 and 45 ppm are assigned to methylene and methyl groups in alkyl chains deriving from lipids, such as plant waxes and polyesters. The broad signal in the 45-60 ppm region may be due to both methoxyl carbons, in guaiacyl and syringyl units of lignin fragments, and C-α in oligo- and polypepetides. Aromatic carbons in lignin residues and other aromatic biomolecules are responsible for the 116-160 ppm region of the spectra. The signals included in the downfield 145-160 ppm region typical of quaternary aromatic carbons, may be assigned to lignin aromatic C bound to either hydroxyl or methoxyl groups. However, the abundance of aromatic compounds was relatively low in all

compost samples (Fig. 1 and Table 2). The neat signal at 174 ppm corresponded to carbonyl carbons of acid, ester, and amide functional groups.

Integration of ^{13}C spectra allowed to calculate the distribution of different carbons and derive structural indexes, such as alkyl-C/O-alkyl-C (A/OA), aromaticity (ARM), hydrophobicity (HB/HI), and Lignin ratio (LigR) (Table 2). In particular, the A/OA, ARM and HB/HI indexes are commonly related to the biochemical stability of different organic materials. In fact, their relatively large values indicate an increased process of organic matter stabilization, as associated to selective accumulation of hydrophobic compounds (Spaccini and Piccolo, 2007a). On the other hand, the LigR ratio between hydroxyl and methoxyl substituents suggests the contribution of lignin structures in compost.

The most stabilized hydrophobic compost appeared to be C-CYNF, with the largest HB/HI ratio and ARM index (Table 2). A concomitant lowest LigR valuest indicated the largest preservation of lignin residues C-CYNF. Conversely, non-lignin C-O compounds were predominant in C-TOM followed by C-CYN, as displayed by larger LigR values, and suggested a relative greater presence of proteinaceous moieties. The smallest ARM and largest A/OA values for C-MSW showed that this compost reached a rather high hydrophobic stabilization without a too large lignin contribution.

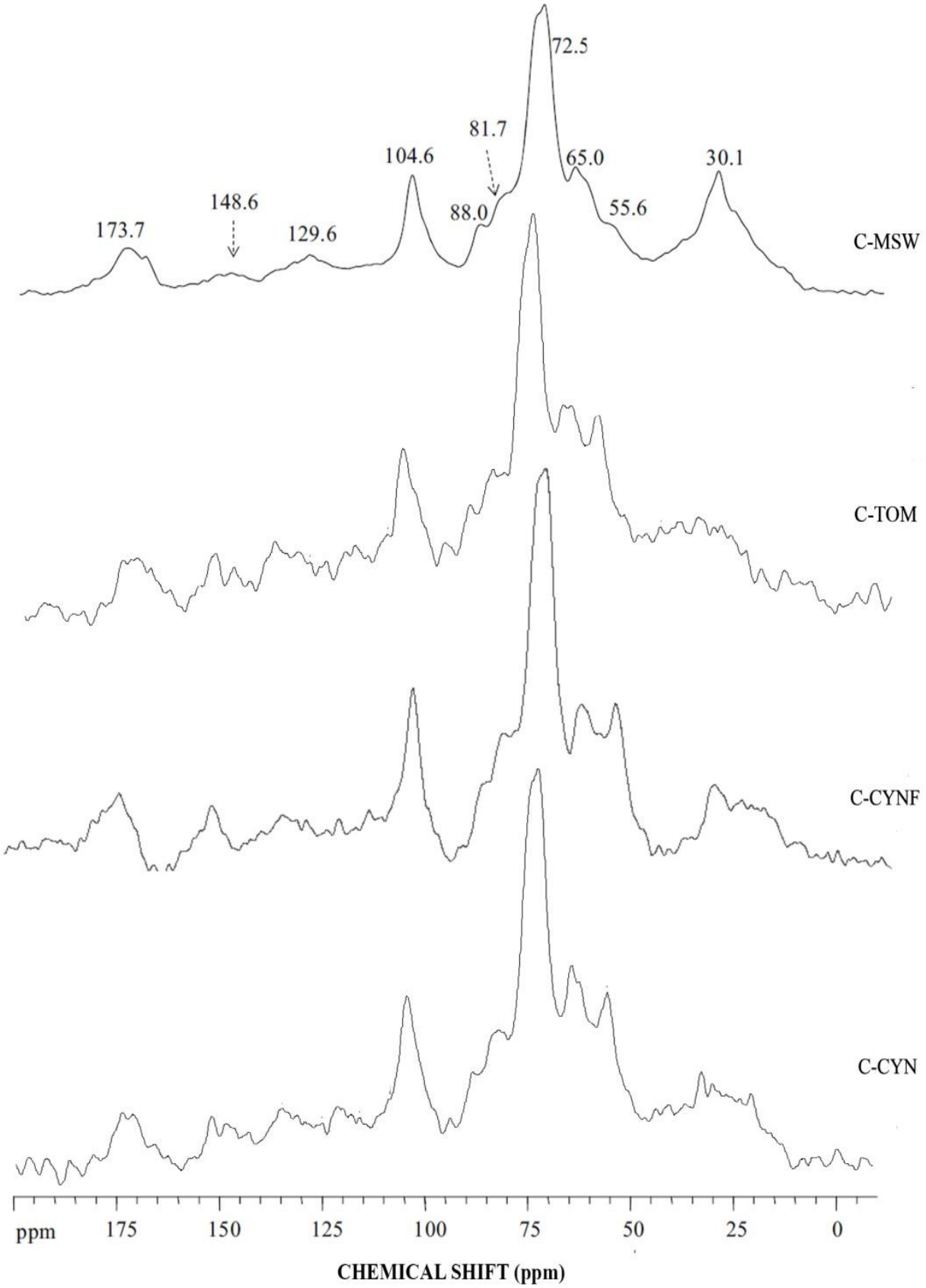

Figure 1. ^{13}C CPMAS NMR spectra of composts.

Table 1. Main characteristics of composts and WEOMs. Total C, N and H are given as % dry matter. C/N and H/C calculated as atomic ratios.

	C-CYN	CYN-WOEM	C-CYNF	CYNF-WOEM	C-TOM	TOM-WOEM	C-MSW	MSW-WOEM
pH		8		8.29		8.3		8.62
E.C. (dS m^{-1})		2.03		3.02		2.97		3.61
Total C (%)	24.13±1.2	21.55±1.1	22.44±1.8	19.45±1.4	18.86±0.8	16.55±1.3	32.19±1.1	31.2±1.1
Total N (%)	1.32±0.0	1.35±0.0	1.52±0.2	1.34±0.2	1.29±0.0	1.32±0.0	2.43±0.1	2.37±0.0
Total H (%)	4.86±0.0	4.43±0.0	4.8±1.1	4.47±0.1	3.37±0.1	3.17±1.1	4.7±0.1	4.2±0.1
C/N	21.6	18.69	17.2	17.05	17.0	14.57	17.87	15.38
H/C	2.4	2.47	2.6	2.75	2.2	2.31	1.75	1.61

Table 2. Relative contribution (%) of main C structures over chemical shift regions (ppm) and ratios between relative abundance of grouped C molecular types assessed by ^{13}C CPMAS-NMR spectroscopy of the WEOM samples obtained from different organic source materials.

	^{13}C NMR regions						^{13}C NMR structural indexes			
	Carboxyl-C	Phenol-C	Aryl-C	O-Alkyl-C	Methoxyl-C	Alkyl-C				
Sample	*190-160*	*160-145*	*145-110*	*110-60*	*60-45*	*45-0*	HB/HI[a]	A/OA[b]	ARM[c]	LigR[d]
C-CYN	4.04	3.2	12.11	54.82	10.77	15.03	0.44	0.27	0.23	3.36
C-CYNF	9.3	5.03	13.48	46.57	9.57	16.09	0.53	0.35	0.33	1.9
C-TOM	3.49	2.97	11.43	51.88	12.85	17.33	0.46	0.33	0.22	4.31
C-MSW	5.2	2.9	9	53.3	8.3	21.4	0.50	0.40	0.19	2.86

Different letters in the same column indicate significant differences at $P<0.05$ (Tukey's test)
a)HB/HI=hydrophobicity index=[Σ (0–45) + (110–160)/ Σ (45–60) + (60–110) + (160–190)]
b) A/OA=alkyl/O-alkyl ratio (0–45)/(60–110)
c) ARM = aromaticity index [(110–160)/Σ (0–45) + (60–110)]
d) LigR = Lignin ratio (45–60)/(140–160).

Solution-state ^{1}H NMR spectroscopy of WEOM

^{1}H-NMR spectra of WEOM extracted from compost samples (Fig. 2) showed three main resonance regions: alkyl (aliphatic components, 0-3 ppm), O-Alkyl (prevalently oligo- and polysaccharides, 3-5 ppm) and aromatic (mainly lignin components, 6-8 ppm) (Simpson et al., 2011). In the alkyl spectral region, three intense peaks are assigned to methyls (0.82 and 0.98 ppm, mostly related to terminal methyl groups in lipid chains), and methylene protons of alkyl chains (1.24 ppm) (Piccolo et al., 1990). The signals resonating between 1.7 and 2.7 ppm are assigned to protons bound to electron withdrawing groups such as carbonyl or aromatic groups. The broad peaks resonating at downfield frequencies in the 3.7-3.8 ppm interval are attributed mainly to methoxyl groups in lignin residues. The multitude of signals between 6.5 and 8 ppm are referred to aromatic moieties. The integration of signals provided the percent proton distribution in the different regions of WEOM samples (Table 3). The ^{1}H-spectra of all WEOM samples suggested a similarity in compositions, except for the MSW-WEOM that was the least aromatic and most aliphatic of all samples. Conversely, CYN-WEOM appeared as the most aromatic among these water-soluble organic materials, partially following the characteristics of the original compost.

Table 3. Assignments of ^{1}H NMR and H distribution (%) of WEOM.

Sample	ALKYL-H *3-0 ppm*	H-CO / H-CN *5-3 ppm*	ARYL-H *8-6 ppm*
CYN-WEOM	45.93	36.85	17.21
CYNF-WEOM	45.78	40.41	13.82
TOM-WEOM	45.53	41.91	12.56
MSW-WEOM	57.21	35.77	7.01

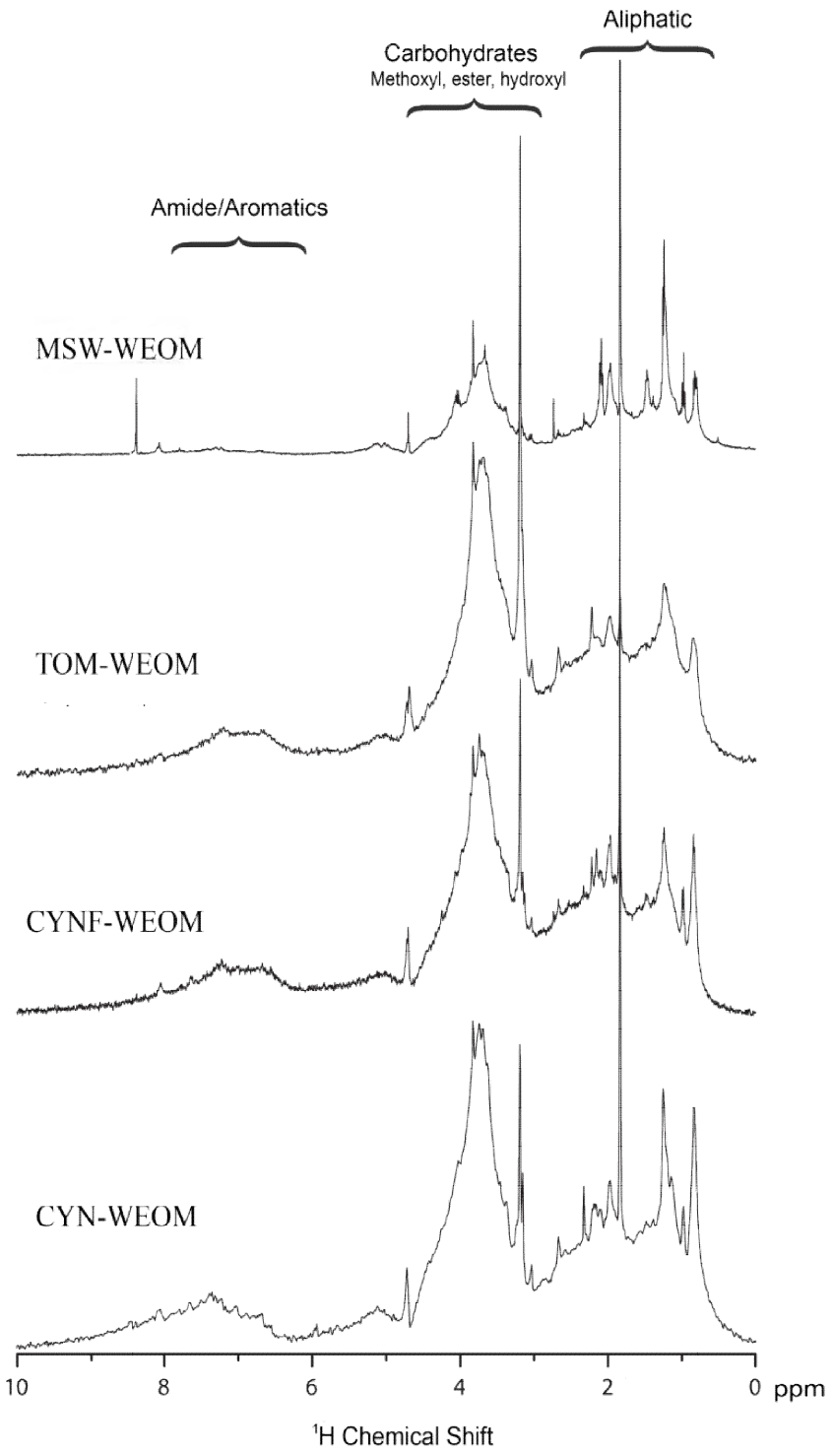

Figure 2. ^{1}H NMR spectra of WEOMs.

3.3 Infrared Spectroscopy of WEOM

The ATR-IR spectra of WEOM (Fig. 2) showed a large absorption band between 3500 and 2500 cm^{-1} which indicates the presence of both phenol and alcoholic O–H groups and N-H proteinaceous moieties. In particular, the peaks centred at around 2930 cm^{-1}, 2850 cm^{-1} and 1450 cm^{-1} represent the symmetrical and asymmetrical stretching and bending vibrations of methylene groups of aliphatic structures, such as fatty acids, waxes and various other aliphatics.

The bands centred at around 1660–1620 cm^{-1} and 1590 cm^{-1} are currently associated with amide I and amide II bonds of peptidic material (Piccolo and Stevenson, 1982). Concomitantly, the signal at 1590 and 1380 cm^{-1} in MSW-WEOM and TOM-WEOM can be attributed to carboxylate ions in aliphatic acids. The band at 1514 cm^{-1} may be due to C-C stretching vibrations in aromatic moieties of lignin (Brunow et al., 2001). The 1420-1460 cm^{-1} shoulder appears related to aliphatic C–H deformations and aromatic ring vibrations, whereas the peak at 1400 cm^{-1} is generated by both the C-O stretching of phenolic OH and the C–H deformation of CH_2 and CH_3 groups. The weak band at 1260 cm^{-1} shown by CYNF-WEOM and TOM-WEOM spectra may be produced by guaiacyl ring breathing, as well as by amides or ethers. Carbohydrates content was revealed by peaks at 1105 and 1030 cm^{-1}, assigned to C-O stretching bonds in both polyalcoholic and glycosidic functional groups, which were particularly intense in the spectrum of CYN-WEOM followed by that of TOM-WEOM. Differences in bands intensity but similar absorptions at around 2800-2500 cm^{-1} and 1600 cm^{-1} suggest the presence of lipid compounds in all water-soluble organic materials. Peptides and carbohydrates were noticeable in CYNF-WEOM and TOM-WEOM, and, to a lesser extent, in MSW-WEOM.

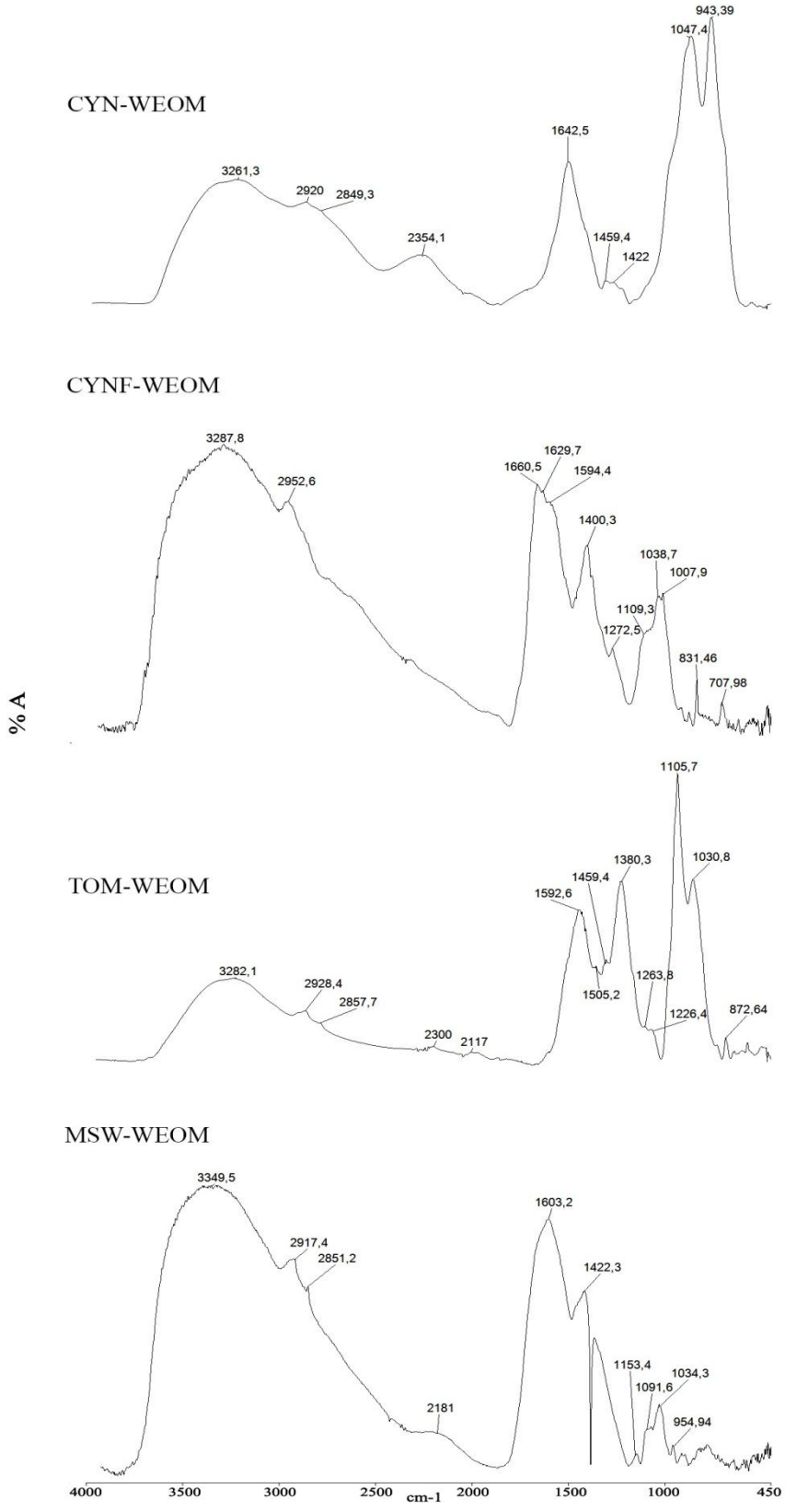

Figure 2. FTIR-ATR spectra of WEOMs.

3.4 Thermal properties of WEOM

TG curves (Fig. 3A) indicated that MSW-WEOM produced the largest weight loss (70%) with the raise in temperature, while CYN-WEOM and CYNF-WEOM behaved similarly, accounting for a 60% weight loss, and TOM-WEOM showed the least weight losses of around 45%. Thermograms revealed two distinct shoulders attributed to moisture evaporation and combustion of aliphatic biodegradable compounds, such as carbohydrates (50-300°C), and to thermal degradation of aromatic and heterocyclic structures (350-550°C).

Although minor differences arose among samples, DSC curves reflected the different thermal behavior of the water-extracted OM (Fig. 3B). In fact, MSW-WEOM featured the most intense DSC signal, probably due to its larger C content (Table 2), with the first peak centered at 320°C and the second one at around 480°C. Conversely, CYN-WEOM and CYNF-WEOM curves more closely resembled those of humic substances (Campanella et al., 1990) with a main peak around 520°C and a shoulder at 550°C. However, these materials lacked a peak around 300°C, that was instead found in TOM-WEOM, though shifted to a lower temperature when compared to MSW-WEOM. CYN-WEOM and CYNF-WEOM shared a similar thermal decomposition of aromatic moieties, which may be considered as an index of biological stability (Dell'Abate et al., 2000; Gómez et al., 2007), while the exothermic peak at lower temperature, shown by MSW-WEOM and TOM-WEOM, suggested biolabile structures, such as carbohydrates, simple lipids and amino acids.

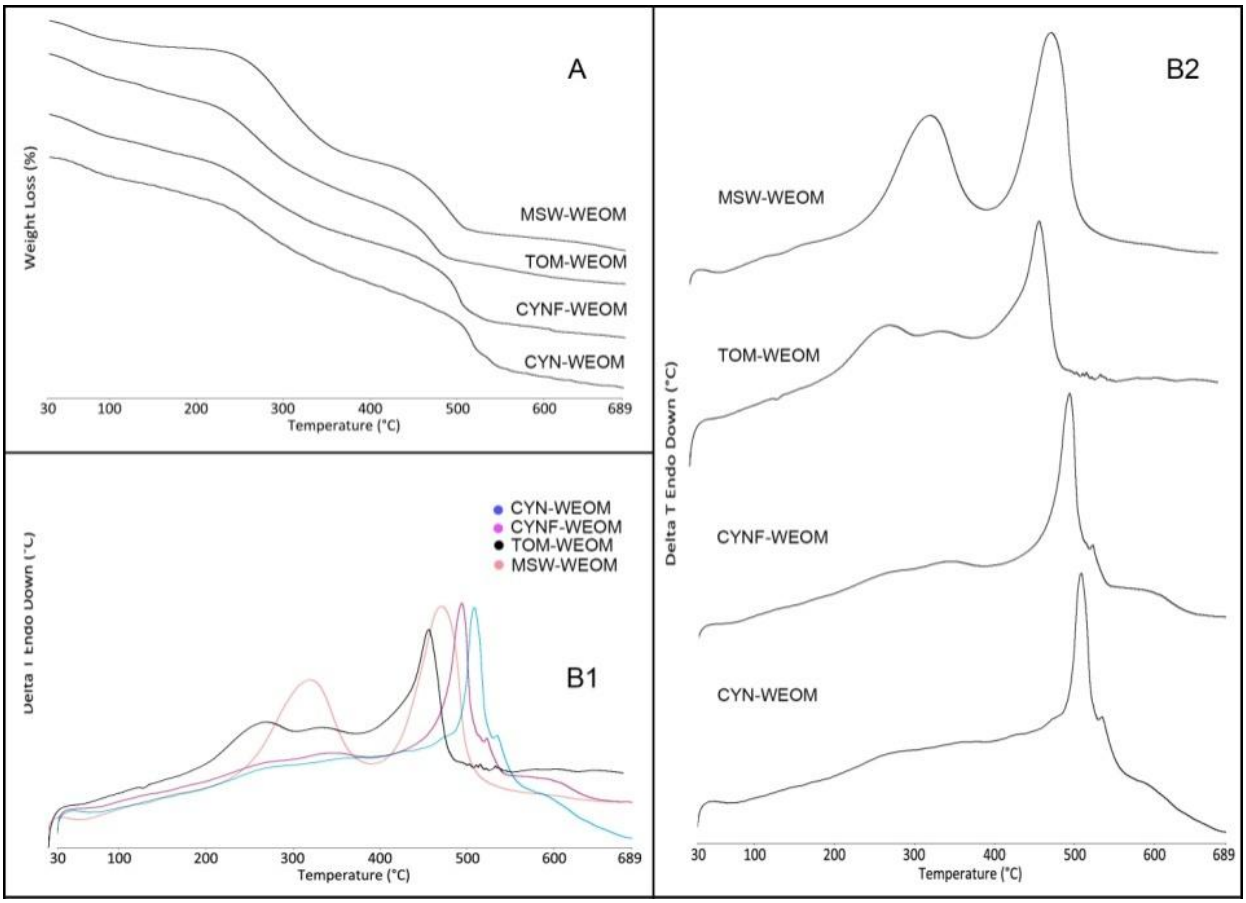

Figure 3. (A) TG curves. (**B)** DSC curves.

3.2 Effects of C-WEOM on maize germination and early growth:

The effects of WEOM samples on root, shoot and coleoptile lengths are shown in Table 4. Maize seeds treated with WEOM extracts showed that the germination rate (GI) was positively affected by CYN-WEOM and, to a lesser extent, TOM-WEOM. In fact, the GI values for CYN-WEOM were significantly larger than control and resulted in the variation of both primary and secondary roots elongation in seedlings, while the still positive GI values of TOM-WEOM were related only to germination rate. No such effects were noticed for other WEOMs, while CYNF-WEOM and MSW-WEOM even induced a significant inhibition of germination and root elongation. A significant effect on the coleoptile elongation was also shown by the CYN-WEOM treatment.

Application of WEOM significantly affected the growth of maize plantlets (Table 5). Total fresh biomass increased when plants were treated with each WEOM, whereas root and shoot dry weights did not show significant differences. The most bioactive product was TOM-WEOM that showed the largest effect on total fresh and dry biomass. Although MSW-WEOM treatment also increased fresh shoot weight, a negative effect was observed on dry root weight when compared to control. A significantly great increase in total root length was observed with the CYN-WEOM and TOM-WEOM treatments, while the chlorophyll content was positively affected by all WEOM extracts.

Table 4. RSG, RRG, GI percentage and coleoptile length of maize seed as affected by WEOM*****.

	RSG (%)	***1RRG (%)***	***2RRG (%)***	***GI (%)***	***COLEOPTILE***
	91.18	132.23	122.63	115.92	119.79
CYNF-WEOM	70.56	88.41	84.77	62.38	84.76
TOM-WEOM	117.26	100	100	103.66	84.75
MSW-WEOM	79.47	53.66	55.44	43.39	80.64

* % in respect to control expressed as 100%.

Table 5. Phenological parameters measured for each WEOM treatment. Different letters indicate significant difference at P<0.05 (Tukey's test).

	FRESH WEIGHT				DRY WEIGHT				ROOT LENGHT	CHL
WEOMs	Tot. Biomass	Shoot	Root	S/R ratio	Tot. Biomass	Shoot	Root	S/R ratio	cm	mg/cm2
Control	1.84 ± 0.09 d	0.84± 0.09 d	1± 0.06 a	0.84± 0.09 d	0.17± 0.07 b	0.07± 0.07 c	0.10± 0.004 ab	0.7± 0.13 bc	200.2±14.1 c	54.23±2.73 b
CYN	2.15 ± 0.09 b	1.12± 0.01 bc	1.03± 0.07 a	1.08± 0.07 bc	0.18± 0.08 a	0.08± 0.07 ab	0.10± 0.002 ab	0.8± 0.09 b	272.1±12.45 a	80.41±3.02 a
CYNF	2.04 ± 0.07 c	1.08± 0.007 c	0.96± 0.07 b	1.12± 0.09 b	0.19±0.07 a	0.08±0.07 ab	0.11±0.016 ab	0.72± 0.09 bc	223.5±20 b	75.72±2.31 a
TOM	2.35 ± 0.1 a	1.2± 0.01 ab	1.04± 0.06 a	1.15± 0.07 b	0.18±0.07 a	0.06±0.07 d	0.12±0.008 a	0.5± 0.09 c	279±26.31 a	71.38±5.78 a
MSW	2.13 ± 0.21 c	1.24± 0.01 a	0.89± 0.08 c	1.39± 0.11 a	0.18±0.07 a	0.09±0.12 a	0.09±0.009 b	1 ± 0.13 a	229.2±13.01 b	78.55±6.24 a

3.3 Multivariate analysis (PCA)

Multivariate analysis, such as Principal Component Analysis (PCA), is commonly employed to correlate chemical composition of humic substances to their origin or bioactivity on plant growth (Smejkalova et al., 2008; Canellas et al., 2012; Aguiar et al., 2013; Silva et al., 2013; Costa et al., 2016). PCA was used here to relate the chemical characteristics of WEOM extracts to their effects on maize seed germination and maize plant growth. The resulting PCA bi-plot (Fig. 4) showed a separation among treatments that follows the different chemical composition of WEOM. Along the first PC (34.51% of total variance) MSW-WEOM was neatly separated from the rest of WEOM according to the content of aromatic (negative loadings) and alkyl moieties (positive loadings). The second PC (29.80% of total variance) clearly separated CYN-WEOM from TOM-WEOM and CYNF-WEOM treatments, according to the positive loadings of hydrophobicity and aromaticity, and negative loadings of the LigR ratio and carbohydrate content (NMR signals in the 110-60 ppm range.

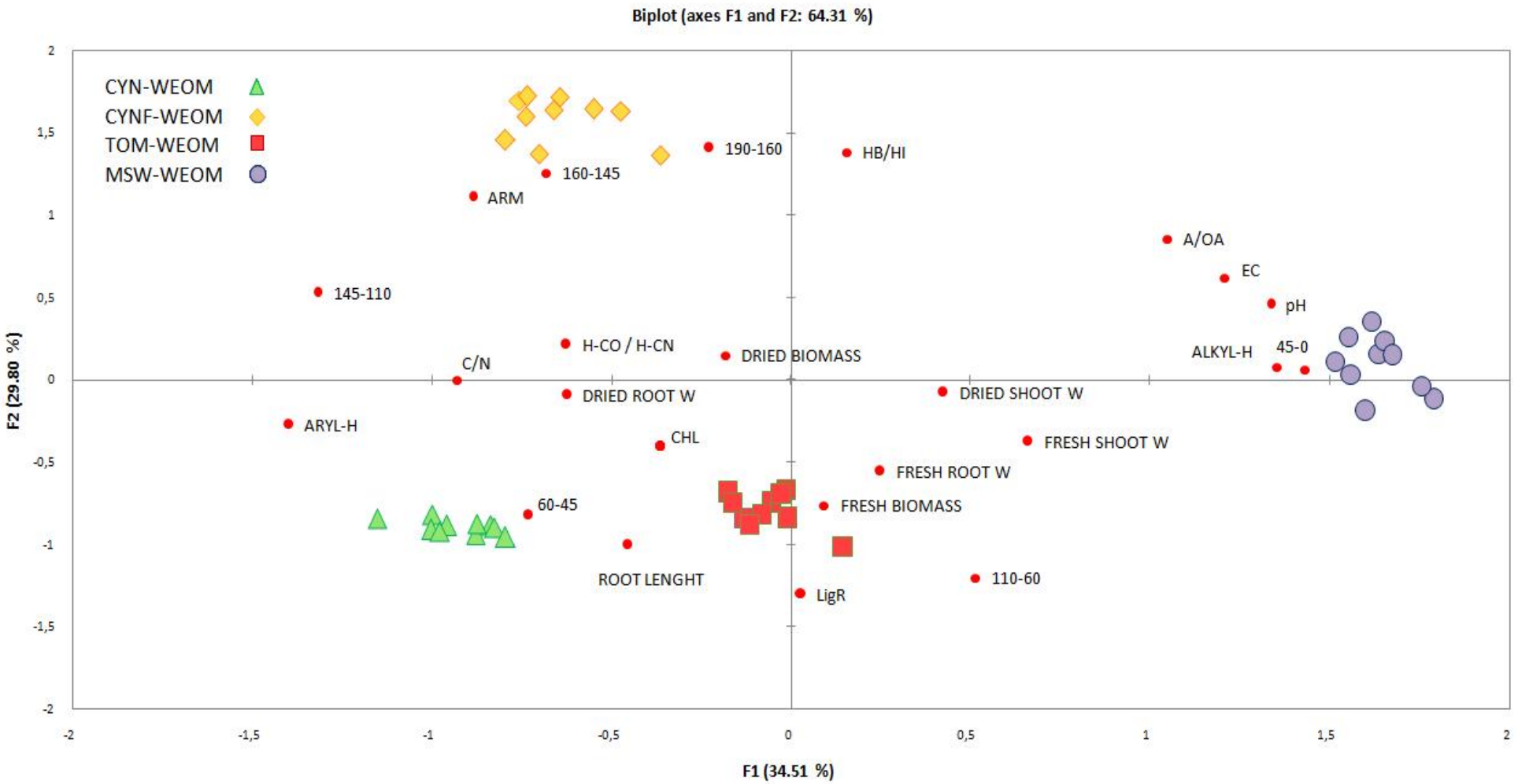

Figure 4. PCA bi-plot of compost and WEOMs chemical characteristics correlated with germination test and bioactivity assay.

4. Discussion

The treatment of maize seedlings with water-soluble organic matter extracted from different on-farm composts revealed that the physiological activities related to seed germination and plantlet growth were affected by the specific chemical composition of the compost water-soluble extracts.

The water-soluble CYN-WEOM isolated from a compost made mainly from artichoke biomasses showed a significant positive effect on the elongation of coleoptile in the germination test and the increase in root length in the plant growth assay. This effect appeared to be due to its content of lignin (60-45 ppm), aromatic (160-110 ppm) and carbohydratic (ATR-IR spectra) residues, as well as to the degree of aromaticity (ARM). The positive bioactivity of TOM-WEOM from compost made of tomato residues was similarly correlated to its lignin ratio, with a greater incorporation of plant derived carbohydrates, but a lesser preservation of aromatic structures than for CYN-WEOM.

The MSW-WEOM isolated from compost obtained from municipal organic wastes retained a relative large amount of lipid compounds that conferred a great alkyl hydrophobicity that made this water-soluble organic matter the least effective or even phytotoxic for the emergence of seedlings. This behaviour could also be ascribable to large E.C. values, although the soluble salts concentration in MSW-WEOM was still within the non phytotoxic range (Hoekstra et al., 2002). However, contrary to performance in germination, MSW-WEOM produced a rapid shoot development, although accompanied with a poor rooting capabilities. The positive effect on fresh shoot biomass could be attributed to an adaptation of plants to the stress condition.

Several authors reported that aliphatic and aromatic components along with a large hydrophobicity are capable to induce lateral root emergence and stimulate plant growth (Canellas et al., 2009, 2010; Aguiar et al., 2013; Martinez-Balmori et al., 2014), while other results also argued that the hydrophilic and readily available components in humic matter promote a more efficient diffusion of bioactive molecules at cellular membrane level (Nardi et al., 2007; Canellas et al., 2008).

In this study we observed that the influence on root length and architecture of the WEOMs were mainly related to the content of aromatic components, while the hydrophilic constituents of WEOMs were more preferably associated with the biomass increase. It is plausible that the decomposition of lignin biopolymers in the original source material may have promoted a preferential accumulation of small and active aromatic fragments, which allowed a prompt plant physiological response due to their hormone-like activity (Piccolo et al., 1992; Quaggiotti et al., 2004). This action should also be in association with a large content of oligo- and poly-saccharides that ensure the mobility of bioactive molecules throughout the hydrophobic supramolecular associations. In fact, the combination of aromatic and carbohydrates structures observed mainly in CYN-WEOM and TOM-WEOM may confer the conformational structure of these two extracts the flexibility for the hormone-like molecules to exert their biological activity on plant growth (Vaccaro et al., 2009; Canellas and Olivares, 2014).

4. Conclusion

Compost made with mixture of agricultural residues was proved here to be an adequate source of water extractable organic matter that may be usefully employed as biostimulants for plant growth. However, not all water-extractable organic materials were equally effective in their bioactivity. In fact, this depended on chemical composition of the WEOM that, in turn, was a function of the specific biomasses used in the composting process. Here we found that the effects on plant growth were due to the intrinsic molecular composition of each material, that was, above all, related to the content of aromatic hormone-like structures, as well as the hydrophobic/hydrophilic balance of components in the WEOM. Further studies are required to increase knowledge on the structure-activity relationship in order to finally set up an useful technology to exploit the bio-stimulation properties of humic matter and contribute to a sustainable agriculture.

CHAPTER IV

Biostimulation of humic acids isolated from different *on-farm* composts. Relationship between chemical structure and biological effects on maize early growth

Keywords: Humic substances; Bioactivity; Seedling growth; Bioassay; NMR ^{13}CPMAS; ^{1}H NMR; IR DRIFT; TGA

Abstract: The use of compost is here proposed as a source of humic substances capable to boost plant growth. Humic acids (HAs) were isolated from different on-farm composts obtained from agricultural waste biomasses: 1. artichoke compost (HA-CYN), 2. artichoke/fennel compost (HA-CYNF), 3. tomato/woodchips compost (HA-TOM), 4. a cauliflower compost (HA-CAV). In this work, we investigated the effects of humic acids at three different concentration rates (25, 50 and 100 ppm C L^{-1}) on maize (*Zea mays*) seedling growth and elucidated the relationship between bioactivity and chemical features of humic substances. Humic extracts were characterized by spectroscopic (^{13}C CPMAS NMR and ^{1}H NMR, FTIR-ATR), elemental and thermal analysis (TGA, DSC). All the assayed humic acids favored plant growth, in a very similar pattern with relatively large responses at low application rates, therefore evidencing a dose-response effect. In addition, structural analysis revealed the predominance of aromatic character in HA-CYN and HA-TOM, the latter probably due to phenolic compounds. HA-CAV showed a large hydrophobic characteristic mainly derived from aliphatic components. Bioactivity seems to be related to the capacity of humic acids to change their conformational structure in solution, potentially releasing active molecules that directly or indirectly influence plants development.

1. Introduction

During the last century, the world growing population has increased the global food supply demand rapidly leading to the intensification of agricultural management practices and exploitation of natural resources with serious impacts on environment such as loss of biodiversity and decline of soil fertility. Current challenges faces the need of different agricultural approaches capable to minimize unsustainable farming strategies and promote ecological intensification models in order to improve crop yield by optimizing nutrient-use efficiency while reducing external agrochemical inputs (Tittonell, 2014).

Within this context, humic substance-derived products (HS) represent a powerful technology since they are recognized as a key component of soil fertility capable to influence chemical and biological properties of the rhizosphere (Nardi et al. 2005; Sparling et al., 2006). HS derive from the biodegradation of plant and animal residues transformed by chemical and biochemical reactions during decay into a mixture of complex polydispersed materials with unique features.

The most reactive fraction of organic matter are humic acids (HAs) which are operationally defined by their solubility properties in acidic solution. They can be extracted from a variety of sources, including lignite, peat and soil, but their use can contribute to the depletion of these natural resources. The alternative use of compost as a source of HA have several advantages for both environmental recycling possibilities and sustainable agricultural practices by reducing the reliance on chemical inputs (Ayuso et al., 1996; Castaldi et al., 2004; Kowaljow et al., 2007; Pascual et al., 2007) and by the increase in humified organic matter (Piccolo and Mbagwu, 1997; Spaccini et al., 2008).

Because of their beneficial effects on physical and chemical properties of soil as well as the effect on promoting plant growth, a rising attention of farmers and producers led to the development of a growing market of HS as plant bio-stimulators. Although there is significant evidence that HS can help improving soil fertility as well as influencing plant productivity (Piccolo et al., 1992; Canellas

and Olivares, 2014), the mechanisms by which HS exert their effects are still not well understood.

It has long been known that HS can display auxin-like activities (Bottomley, 1917; Pinton et al., 1997; Muscolo et al., 1999). Canellas et al (2015) suggested that HS increase the amount of plasma membrane H+-ATPase which in turns acidifies the apoplast inducing, through the mechanism of acidic growth, the cell wall elongation and the development of lateral roots, resulting in the enhancement of nutrient uptake (Quaggiotti et al., 2004; Zandonadi et al., 2007). On the other hand, Muscolo et al. (2013) highlighted that the simple presence of auxin in the HS isn't sufficient to justify the biological responses and possibly other signaling molecules are involved in mediating HS effects. Nevertheless, uncertainties arise when trying to investigate the role of chemical composition on the stimulation of plant growth. In particular a topical research approach is to investigate whether the conformational properties of HS, such as molecular size of humic suprastructures as well as the hydrophobic/hydrophilic ratio, could be involved in the mechanism of biostimulation (Nardi et al. 2000; Canellas et al., 2009; Dobbs et al., 2010).

The complexity of these materials, indeed, make difficult to define structurally their physiochemical properties that change according to the source and environmental conditions (Trevisan et al., 2010a). As a consequence, the biological effect of HS result not predictable and the lack of detailed knowledge on the composition makes difficult to identify the relationship between the structure and activity of these substances.

Considering the potential benefits of HS, it´s important to add information on their chemical structure because the larger amount and conformational arrangements of different chemical components of humic materials, may confer to each humic substance distinct effective activity, making their molecular characterization an unavoidable requirement.

Moving from these assumptions, we report a detailed characterization of humic acids extracted from different on farm composts trying to investigate the correlation with plant growth with the final goal to contribute to the actual knowledge of HS chemistry and plant biological response.

2. Materials and methods

2.1 Humic acids extraction

The composts used in this study were obtained from composted plant residues through 45-days on-farm composting (active or thermophilic phase) of chipped raw organic materials, with forced aeration of static piles, followed by a bimonthly-curing period. The different on-farm composts were selected on the basis of their mixture composition and identified as: 1. C-CYN = 78.0% artichoke, 20% woodchips and 2% mature compost as starter; 2. C-CYNF = 43.5% artichoke, 23.5% fennel, 11.0% escarole residues, 20% woodchips and 2% mature compost as starter; 3. C-TOM = 50.0% tomato residues, 48% woodchips and 2% mature compost as starter; 4. C-CAV = composted cauliflower residues. Humic acids were obtained through the following extraction procedure: an aliquot of 100 g of each air-dried compost (2 mm sieved) was suspended in 500 ml 0.1 mol L^{-1} NaOH and 500 ml 0.1 mol L^{-1} $Na_4P_2O_7$ and mechanically shaken for 24 h. The suspension was then centrifuged at 7000 rpm for 20 min and glass wool filtered. The extraction was repeated 2 times (1h agitation step). The suspension was acidified to pH 1.5 with 6 mol L^{-1} HCL to allow the precipitation of humic acids. After 24h, the samples were centrifuged at 4000 rpm for 20 min and humic acids collected and dialysed against deionized water using 1-kD cutoff spectrapore membrane until the electrical conductivity resulted lower than 0.5 dS m^{-1}. Humic acids were then freeze-dried for further analytical characterization. Compost and humic samples were characterized for their elemental content using a Fison EA 1108 Elemental Analyzer. The resulting compost ash content was less than 3%.

2.2 FTIR–ATR Spectroscopy of composts and HAs

Infrared (IR) spectra were recorded on a Perkin-Elmer Frontier Fourier transform infrared spectrometer using attenuated total reflection (ATR) device equipped with a diamond/ZnSe crystal. About 2 mg grinded powder were put on the crystal device and the contact was obtained applying a

strength of about 150 N on the sample. Each spectrum was subjected to 32 scans with the resolution of 4 cm^{-1} from 4000 to 400 cm^{-1} region. The final average spectra was derived by the acquisition of five interferograms.

2.3 NMR Spectroscopy of composts and HAs

A 300 MHz Bruker Avance spectrometer, equipped with a 4 mm wide-bore MAS probe, was used to perform solid-state analyses of compost and humic samples. Each fine-powdered sample was packed into a 4 mm zirconium rotor, provided with a Kel-F cap, and spun at a rate of 13000±1 Hz. The, 13C NMR spectra were acquired through Cross-Polarization Magic-Angle-Spinning (CPMAS) technique by using 2 s of recycle delay, 1 ms of contact time, 30 ms of acquisition time and 4000 scans. According to literature, five chemical shift regions were assigned to the main organic functional groups: 0–45 ppm (aliphatic C), 45–60 ppm (O-substituted alkyl C), 60-110 ppm (O-alkyl-carbon), 110–145 ppm (aromatic C), 145-160 (O-aryl-C), 160–190 ppm (carbonylic C).

The area of each of the regions was determined by integration (MestreNova 6.2.0 software, Mestre-lab Research, 2010), and expressed as percentage of the total area. In order to summarize the structural composition of organic materials, four dimensionless indices were calculated following from the relative areas of NMR spectra:

HB/HI hydrophobicity index: the ratio (Alkyl-C +Aryl-C + Phenol-C)-to-(O-Alkyl-C +Carbonyl-C), i.e. the ratio of signal intensity in the intervals (0-45 ppm +110-160 ppm) over that in the intervals (60-110 ppm -160+190 ppm).

A/OA alkyl index: the ratio between Alkyl-C-to-O-Alkyl-C i.e. of signal intensity in the 0-45 ppm over that in the 60-110 interval.

ARM aromaticity index: represents the relative contribution of aromatic components in respect to aliphatic compounds (110-160 ppm)/(0-60ppm+60-110 ppm+160-190 ppm).

LigR lignin ratio: derive from the comparison of signal area of Methoxyl-C in respect to the

Phenolic- C region (45-60ppm/145-160ppm).

A 400-MHz Bruker Avance spectrometer, equipped with a 5-mm Bruker BBI (Broad Band inverse) probe, was employed to perform liquid-state NMR measurements of compost humic extracts. Each sample (5.0 mg mL- 1) was dissolved with deuterated water (D2O) and placed into a 5.0-mm quartz tube. ^{1}H NMR spectra were acquired with 2 s of thermal equilibrium delay, 90° pulse length ranging within 8.5 and 9.5 μs, 32768 time domain points, and 64 transients. The residual water signal at ˜4.7 ppm was suppressed by adopting the on-resonance pre-saturation technique (˜57 dB power attenuation).

2.4 Thermogravimetric analysis and differential scanning calorimetry of HAs

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves were obtained by air combustion of approximately 10 mg of each sample in a simultaneous thermal analyzer (STA 6000-Perkin Elmer). The initial and final temperatures were 30°C and 700°C, respectively, with an increasing temperature rate of 10°C min^{-1}.

2.5 Plant growth bioactivity assays

Maize seeds (*Zea mays L.)* were soaked in tap water overnight and germinated on wet filter paper on petri dishes in the dark at 25°C. Independent bioactivity assays were conducted in the growth chamber setting the parameters as follow: 75% of humidity, 21-27°C temperature range and photoperiod of 8h of darkness and 16h of light. Five-days old maize seedlings of uniform size were transferred into 15 ml tubes containing deionized water and grown for 5 days afterward water was replaced with 25, 50 and 100 ppm C L^{-1} solution of each different humic extract, except for the control. Treatments were replicated 5 times, each treatment consisted of 10 plants and the entire experiment lasted 15 days. By the end of the test, fresh and dry weights of shoots and roots were recorded and roots collected were scanned at 300 dpi for the evaluation of root lengths by

WhinRhizo scan software. Finally, to determine the chlorophyll content, 9 mm of fresh foliar tissue from each plant were ground in liquid nitrogen, homogenized in 5 ml of pure acetone, and then transferred into 15 ml glass centrifuge tubes, in order to centrifuge them at 3000 x g for 5 min at 8°C. The absorbance of the supernatant at 645 and 663 nm was measured using a spectrophotometer and the concentrations of Chl a, Chl b, total Chl (Chl a + b) were calculated according the methodology of Lichtenthaler (1987) and expressed in mg of pigment per cm^{-2} of leaf fresh weight.

2.6 Data analysis

Data obtained were subjected to normality test (Shapiro-Wilk test) to evaluate the distribution of the samples, means values were compared using one-way analysis of variance (ANOVA) and tested for significance with Tukey´s HSD test in SPSS software (IBM SPSS Statistics). Statistical significance was defined for $p<0.05$. Multivariate principal component analysis (PCA) was used to correlate chemical-physical properties of humic acids with phonological parameters obtained from biological assay. Data were transformed normalizing values to control and expressed as percentage setting control as 0%.

3. Results and Discussion

3.1 Elemental analysis

The elemental composition, C/N ratio and H/C ratio of humic acids and parental composts are summarized in Table 1. The carbon and nitrogen content were larger in all extracted humic samples as compared to corresponding compost source, as expected by the larger organic matter amount and lower mineral content usually found in organic extracts in respect to bulk composts. The high C/N values of the original composts indicated that plant residues are the major contributors to the formation of humic substances (Chai et al., 2007; Campitelli et al., 2008). The lower C/N ratios

found in HAs may be related with the preferential incorporation of soluble low molecular weight materials, such as peptides, in respect to the polymeric components (e.g. polysaccharides) left in residual biomasses. This finding is confirmed by the decrease of H/C values of HA extracts, related with the relative improvement of aromatic compounds and the lower content of aliphatic structures such as polysaccharides.

Table 1. Elemental composition of composts and humic extracts. Total C, N and H are given as % dry matter. (C/N and H/C calculated as atomic ratios).

	Unit	C-CYN	HA-CYN	C-CYNF	HA-CYNF	C-TOM	HA-TOM	C-CAV	HA-CAV
Total C	%	24.13±1,2	29.10±2.2	22.44±1.8	35.62±1.4	18.86±0.8	38.36±	32.19±3.8	32.50±7.5
Total N	%	1.32±0.0	2.52±0.0	1.52±0.2	3.00±0.0	1.29±0.0	2.91±0.2	2.43±0.0	2.62±0.1
Total H	%	4.86±0.0	3.79±0.2	4.8±1.1	5.29±0.1	3.37±0.1	4.9±0.0	4.7±0.1	4.26±0.0
C/N Ratio		21.6	13.5	17.2	13.8	17.0	15.4	15.4	14.5
H/C Ratio		2.4	1.6	2.6	1.8	2.2	1.5	1.8	1.6

3.2 Infrared Spectroscopy

FTIR–ATR spectra of HAs and original composts are shown in Figure 1. The absorption band around 3000-3500 cm^{-1} is shown by all spectra and is attributed to -OH stretching vibrations in alcohols, phenolic or carboxylic acids. The shoulders at 2920 and 2852 cm^{-1}, weakly present in HA-CYN, are commonly attributed to the symmetric and asymmetric stretching vibrations in -CH2 and -CH groups of aliphatic chains. The presence of lipidic material is suggested by the shoulder at around 1709 cm^{-1}, related to C=O double bonds of carboxyl groups, and the peak at 1220 cm^{-1}, assigned to C-O carbonyl group vibrations in carboxylate acidic function. These vibrations were more evident in humic extracts compared to original composts, although slightly visible as shoulders in HA-CYN, whereas they assume a sharp slope in HA-TOM and HA-CYNF, suggesting a more lipidic character derived from plant material such as fatty acids and waxes. The prominent band absorption at 1630 cm^{-1} and the shoulder around 1550 cm^{-1} could result from the overlapping absorptions corresponding to amide I and amide II bonds, respectively (Piccolo and Stevenson, 1982). However a possible contribution of C=C ring vibrations, indicative of aromatic compounds, could not be excluded (Niemeyer et al., 1990), as further indicated by the peak centered at 1509 cm^{-1} in HA-TOM associated with the vibrational C=C stretching of aromatic moieties (Brunow et al., 2001; Carletti et al., 2010). Moreover the weak vibrations of aryl C-H stretching, around 3030 cm^{-1}, in the IR spectra of HAs, suggested the incorporation of aromatic moieties. HA-CAV and HA-CYNF and HA-TOM share quite similar absorption bands in the region 1450-1320 cm^{-1}, commonly assigned to, respectively, aliphatic CH2 bending vibrations (1450 and 1420 cm^{-1}), and to either, phenolic OH or C-OH deformation of COOH and COO^{-} symmetric stretching. On the other hand the peak at 1320 cm^{-1} may derive from the deformation vibrations of CH groups of O-alkyl functions of cellulose and hemicelluloses derivatives (Pandey and Pitman, 2003; Grube et al., 2006; Spaccini et al., 2009). Indeed, the broad band centered at 1030 cm^{-1} and signals at 1120 and 1220 cm^{-1}, assigned to C-O stretching of carbohydrates, suggested a significant level of polysaccharides

in almost all samples except for HA-CYNF that showed weaker C-O absorptions (Inbar et al., 1989).

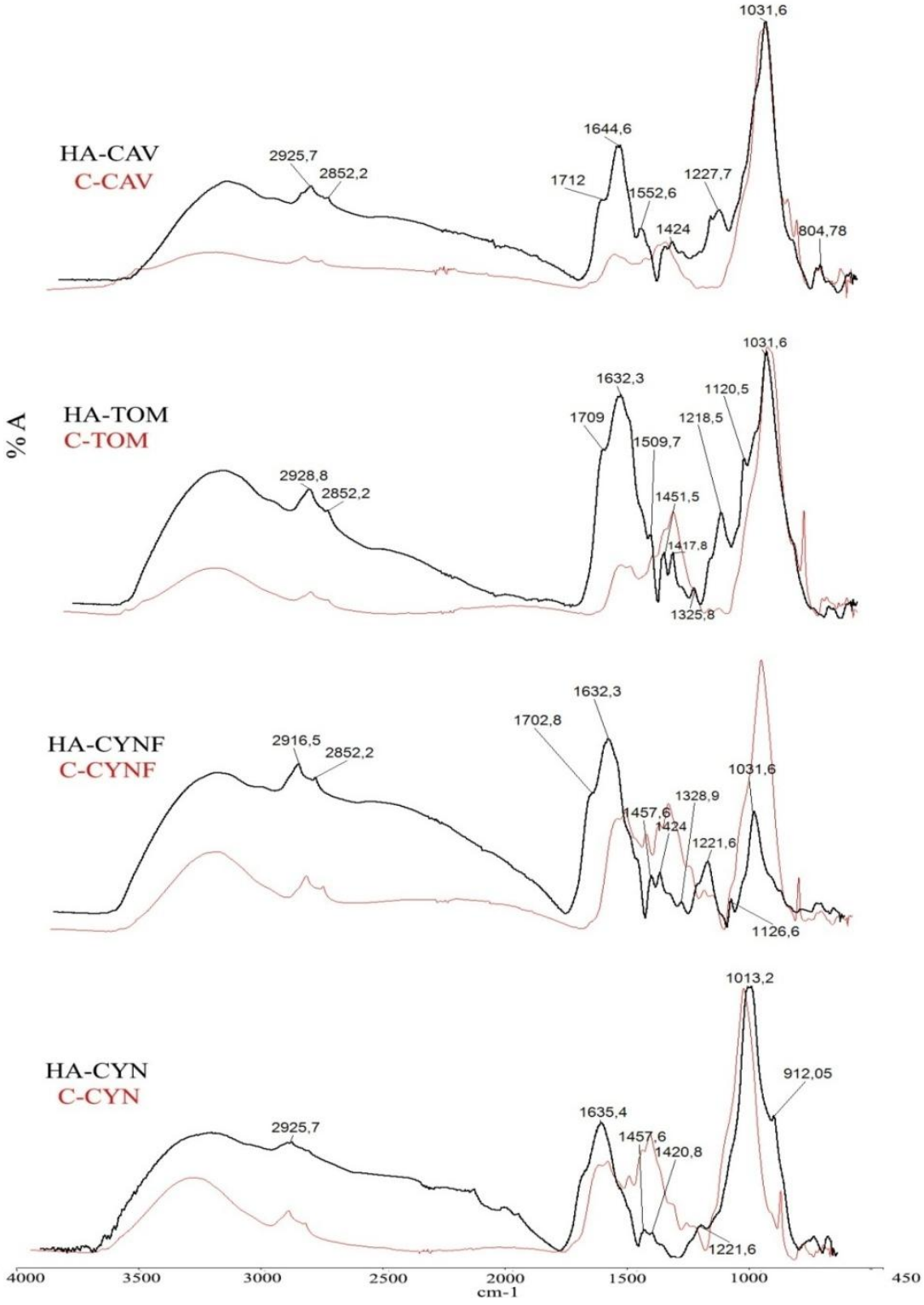

Figure 1. FTIR-ATR spectra of humic acids and parental composts.

3.3 Solid-state ^{13}C NMR spectra of composts and HAs

The 13C NMR spectra of both HAs and corresponding composts are shown in Fig. 2, while the data in Table 2 gives the relative carbon distributions among main functional groups over the chemical shift regions. The broad resonance in the 0-45 ppm range, carrying distinct signals around 16, 23 and 30 ppm, reveal the incorporation of methyl and methylenic group in alkyl chains from various lipid compounds such as fatty acids waxes and biopolyesters (Conte et al., 1997; Keeler et al., 2006). The alkyl-C region account for 31.37% of the total area in HA-CAV spectrum, whereas it constitutes only a minor contribution in HA-CYN, HA-CYNF and HA-TOM, accounting for 17.24%, 20.01% and 20%, in the order. An overall increase of aliphatic content was found in the NMR spectra of HAs compared to composts, thereby suggesting the preferential inclusion of alkyl hydrophobic components. The peaks in the 45-60 ppm chemical shift region are representative of methoxy substituent in lignin structures as well as of C-N bonds of aminoacids and peptidic moieties (Spaccini et al., 2009). In particular, the sharp peak at 55 ppm and the shoulder at 60 ppm may be assigned to either O-CH3 and O-CH2 substituents in aromatic ring and side propylic chain of lignin monomers, respectively (Hatfield et al., 1987). The different resonances in the O-alkyl-C region (60-110 ppm) are currently assigned to monomeric units in oligo and polysaccharides of plant tissue. The intense signal around 72 ppm corresponds to the overlapping resonances of carbon 2, 3, and 5 in the pyranoside structures in cellulose and hemicelluloses, whereas the signal at 105 ppm is the specific mark of anomeric carbon 1 of glucose chains (Wilson, 1987; Robert et al., 1998; Jhonson et al., 2005). The spectra of humic extracts, highlight a decrease of carbohydrates derivatives, which percentage ranged from 23.44% to 34.39%, in respect to the large amount of O-alkyl-C functional groups (> 50%) shown by bulk composts (Table 2). Conversely a significative increase of aromatic components in all humic materials, is revealed by the broad band in the aryl /olefinic C region (110-145 ppm), assigned to the overlapping resonances of unsubstituted and C-substituted carbons in aromatic rings. The signals shown in the phenolic region (145-160 ppm) are

related to O-substituted ring carbon in aromatic structures of lignin molecules and polyphenol compounds. The resonances included in the 148-155 ppm range are usually assigned to carbon 3, 4, and 5 in the aromatic ring in lignin components, carbon 3 and 5 being coupled to methoxyl substituent. Finally, the broad signal resolved at 174 ppm indicate the content of carboxyl carbon in different functional groups like fatty acids, esters amide carbons, etc.

The main differences in molecular composition between bulk composts and humic extracts may be summarized by the evaluation of structural indices (Table 2). The hydrophobicity index (HB/HI), aromaticity (ARM) and alkyl-C/O-alkyl-C (A/OA) ratios are currently used to assess the biochemical stability of different organic materials. Larger values are associated to selective accumulation of recalcitrant compounds and, thus, to progressive stabilization of organic biomasses (Piccolo et al., 2005). The lignin ratio (LigR) highlights the relations between methoxyl and phenol carbons and may be used to either evaluate the relative contribution of lignin-methoxyl substituent and that of C-N carrying moieties as well as to discriminate between signals owing to lignin and those characteristic to other phenolic compounds (Spaccini and Piccolo 2012). The values of HB/HI, ARM and A/OA clearly indicate that all HAs are characterized by larger hydrophobicity in respect to corresponding composts, with the concurrent contribution of both alkyl-C and aromatic components (Table 2). Among humic materials, HA-CAV showed the larger hydrophobic characteristic mainly related to the incorporation of aliphatic compounds. Specific composition for the various organic materials were outlined by the evaluation of LigR ratio. The possible contribution of peptidic moieties to the 45-60 ppm spectral region is revealed by the larger LigR levels found in C-CYN and C-TOM samples, while the lower ratio shown by C-CYNF compost suggest the presence, in the aromatic region, of phenolic compounds not derived from lignocellulosic structures (i.e. tannins, resins etc.). An opposite shift is found for the corresponding humics extracts where an improved incorporation of lignin moieties is revealed by the lower LigR values of HA-CYN and HA-TOM, while the raised index level observed for the HA-CYNF indicate a preferential extraction and inclusion of C-N bearing soluble molecules (De Marco et al, 2012).

Table 2. Relative contribution (%) of main C structures over chemical shift regions (ppm) and ratios between relative abundance of grouped C molecular types assessed by ^{13}C CPMAS-NMR spectroscopy of the WEOM samples obtained from different organic source materials.

	Carboxyl-C	Phenol-C	Aryl-C	O-Alkyl-C	Methoxyl-C	Alkyl-C	Structural indices			
Sample	*190-160*	*160-145*	*145-110*	*110-60*	*60-45*	*45-0*	HB/HI[a]	A/OA[b]	ARM[c]	LigR[d]
C-CYN	4.04	3.2	12.11	54.82	10.77	15.03	0.43	0.27	0.22	3.36
HA:CYN	6.72	5.21	22.07	34.39	14.37	17.25	0.8	0.5	0.52	2.76
C-CYNF	9.3	5.03	13.48	46.57	9.57	16.09	0.53	0.35	0.21	1.9
HA-CYNF	5.77	4.89	20.07	33.22	16.04	20.01	0.82	0.6	0.47	3.28
C-TOM	3.49	2.97	11.43	51.88	12.85	17.33	0.46	0.33	0.29	4.31
HA-TOM	11.69	6.56	19.67	28.71	13.37	19.99	0.86	0.7	0.53	2.04
C-CAV	4.33	3.51	12.85	54.93	9.52	14.84	0.45	0.27	0.35	2.71
HA-CAV	7.10	4.53	21.02	23.44	12.51	31.37	1.26	1.63	0.48	2.22

Different letters in the same column indicate significant differences at $P<0.05$ (Tukey's test)

a)HB/HI=hydrophobicity index=[Σ (0–45) + (110–160)/ Σ (45–60) + (60–110) + (160–190)]

b) A/OA=alkyl ratio (0–45)/(60–110)

c) ARM = aromaticity index [(110–160)/Σ (0–45) + (60–110)]

d) LigR = Lignin ratio (45–60)/(140–160).

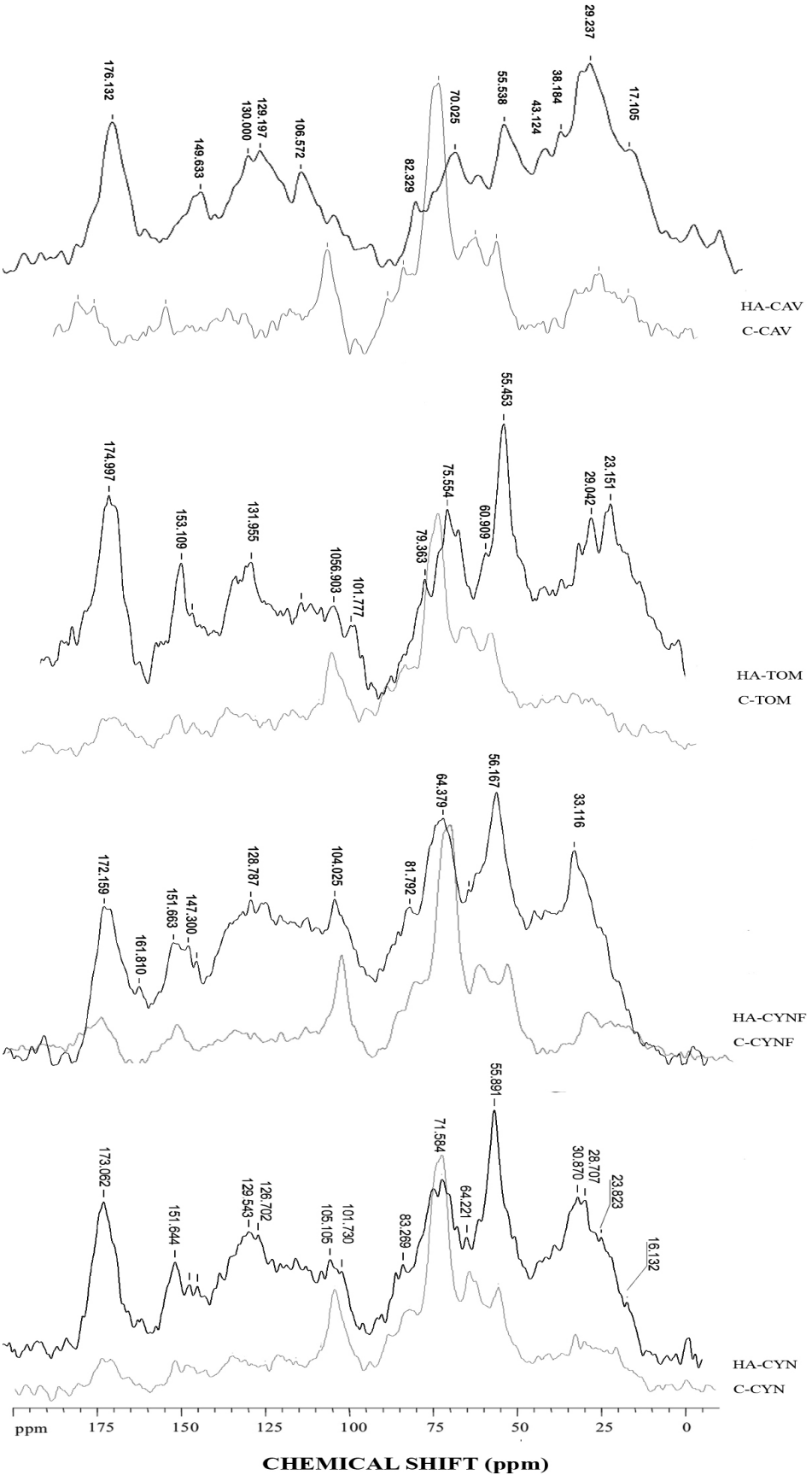

Figure 2. ^{13}C CPMAS NMR spectra of humic acids and composts.

3.4 Solution-state ^{1}H NMR spectroscopy of HAs

In Figure 3 are shown the proton spectra of humic acids analyzed by liquid state NMR. Most of the proton peak broadness is due to the establishment of weak interactions among humic domains which reduce the proton mobility thus affecting their peak shape. In line with literature, the attribution of principal proton signals may be performed by dividing each spectrum into three main regions: alkylic region (mostly aliphatics components, 0-3 ppm), O-Alkyl region (prevalently oligo- and polysaccharides, 3-5.5 ppm) and aromatics region (mainly lignin components, 6.5-8.5 ppm) (Thorn et al., 1989; Ma et al., 2001; Adani and Ricca, 2004; Simpson et al., 2011) (Table 3). From a qualitative point of view, such spectra appear almost similar to each other, except for several minor differences. The alkyl region shows four preponderant resonances including the signal at around 0.79 ppm, ascribable to methyl protons, the signal at 1.20 ppm, corresponding to methylene protons in alkyl chains (Malcom, 1990), the signals at around 1.83 and 1.95 ppm, attributable to CHn-protons generally bound to electron withdrawing groups, such as carbonyls, carboxyls or aromatic rings. The peaks covering the range 3.2-4.3 ppm may be prevalently ascribed to hydroxylated groups in saccharidic domains as well as it is expected that a significant contribution in the range 3.3-3.8 ppm is provided by methoxyl groups mostly related to lignin residues. Relatively intense peaks are visible in the aromatic region included within 6.3-8.3 ppm. In particular, the 3 intense signals resonating at 6.48, 6.88 and 7.25 ppm denote the predominance of aromatic rings bound to activating substituents such as hydroxyls and methoxyls. In semi-quantitative terms, the comparison between different HAs did not show major differences (Table 3). However, these results highlight the preponderance of alkyl and saccharidic structures over aromatic moieties.

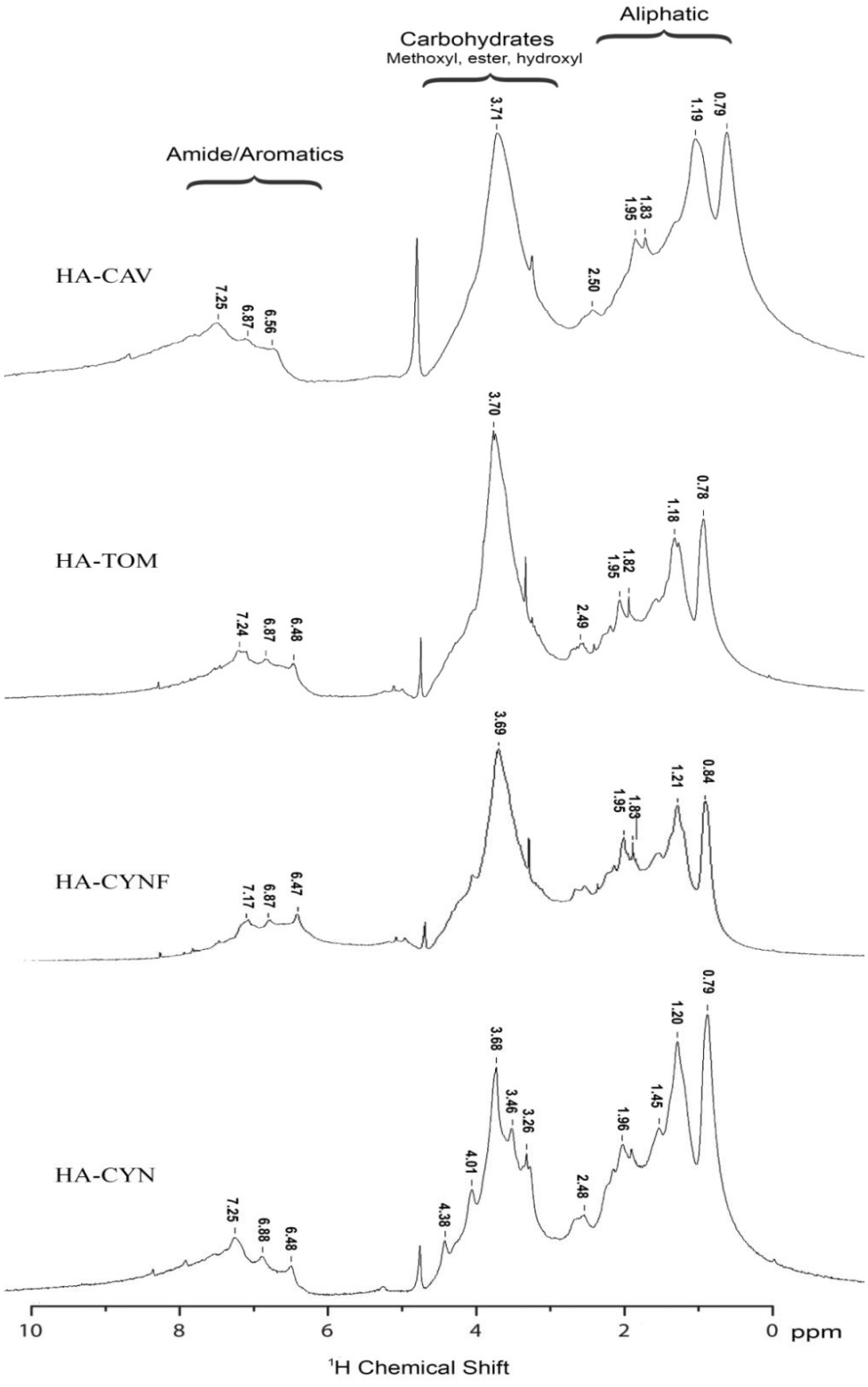

Figure 3. ^{1}H NMR spectra of humic acids.

Table 3.

Assignments of ^{1}H NMR and H distribution (%) of humic acids.

Sample	ALKYL-H *3-0 ppm*	O-ALKYL-H *5-3 ppm*	ARYL-H *8-6 ppm*
HA-CYN	56.33	28.58	15.09
HA-CYNF	56.66	35.43	7.91
HA-TOM	47.9	35.85	16.25
HA-CAV	53.69	29.61	16.7

3.5 Thermogravimetric analysis of HAs

The TG and DSC curves of HAs are reported in Figure 4. The thermal profile of TG curves (Fig. 4 A) indicate that largest weight loss occurred in HA-CYNF (91.1%), followed by HA-CYN (90.3%), HA-TOM (69.3%) and HA-CAV (65.1%). DSC curves better describes the behavior of the HAs correlating weight loss with the thermal sample degradation (Fig. 4 B1, B2).

The thermal curves of HA-CYNF and HA-TOM are characterized by two exothermic peaks, the first one corresponding to the progressive degradation of mostly labile components like carbohydrates and aliphatic compounds such as free lipids and amino acids (300 °C) (Wesolowsky and Erecinska, 2005; Fernandez et al., 2012). The large aromatic content of all HAs is further indicated by the common second exothermic peak at high temperature (350-500°C) attributed to the combustion of complex aromatic compounds such as lignin and/or other polyphenols (Plante et al., 2009). HA-CAV shows a small shoulder at 500°C that can result from the breakdown of easily biodegradable aromatic structures (El Ouaqoudi et al., 2014). Furthermore, the shifted second exothermic peak to higher themperatures (600°C) for HA-CAV and HA-CYN as compared to HA-TOM and HA-CYNF curves may be related with different conformational arrangement and structural stability of these samples.

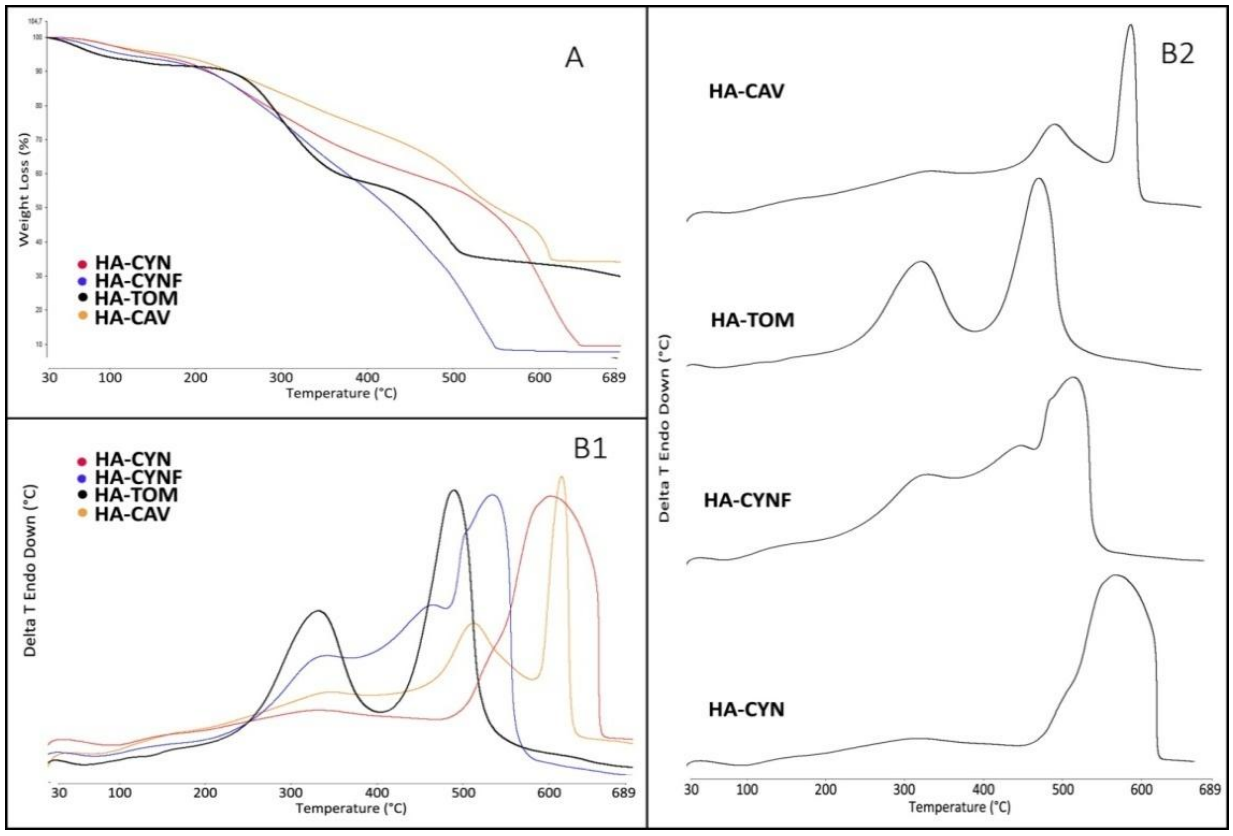

Figure 4. (A) TG curves and (**B1, B2)** DSC curves of humic acids.

3.6 Plant growth bioassay

Phenological results of the application of HAs at different concentration on maize plant growth are summarized in Figures 5-8. The plants most affected by the addition of humic acids resulted those treated with the less concentrated solutions of humates in all treatments, except for those treated with HA-TOM (Fig. 7). As the concentration rise, the stimulation effect shows the tendency to decrease and to produce, as in the case of HA-CAV, negative effects on the natural seedlings development (Fig. 6).

Compared to control, HA-CYN at 25 ppm increased biomass by 24.08% (+21% dry weight), as well as plant height and root length by 14.33% and 15.34%, respectively (Fig. 5). Even though higher concentration of HA-CYN (50 ppm and 100 ppm) increased biomass and root weight, they did not differ significantly when compared to control treatment. Moreover, the highest

concentration induced a negative effects on plants that showed a reduced root length (-3.34%) (Fig. 5). A similar trend was observed for plants treated with HA-CAV (Fig. 6). All plant growth parameters were increased with the application of HA-CAV 25 ppm and decreased proportionally when the concentrations of humic acids was higher. In particular, HA-CAV 100 ppm negatively affected the chlorophyll content (-9.72%), therefore suggesting a kind of photosynthetic pathway dysfunction. For maize seedlings treated with HA-TOM, significant increase was observed when highest concentrated solution was supplied, contrarily to previously described humic acids (Fig. 7). The most affected parameters were dry biomass (+18.91%), root weight (+30.34%) and root length (+17.94%). HA-TOM at lower concentration also induced a better development of plantlets when compared to control, even if significant differences were less pronounced. Plant growth was increased consistently by treatments with HA-CYNF at all concentrations used in the experiment (Fig. 8). In particular, best performances were achieved by HA-CYNF at 25 and 50 ppm, meanwhile, lower, but still significant biostimulation effect was observed with the addition of HA-CYNF 100 ppm.

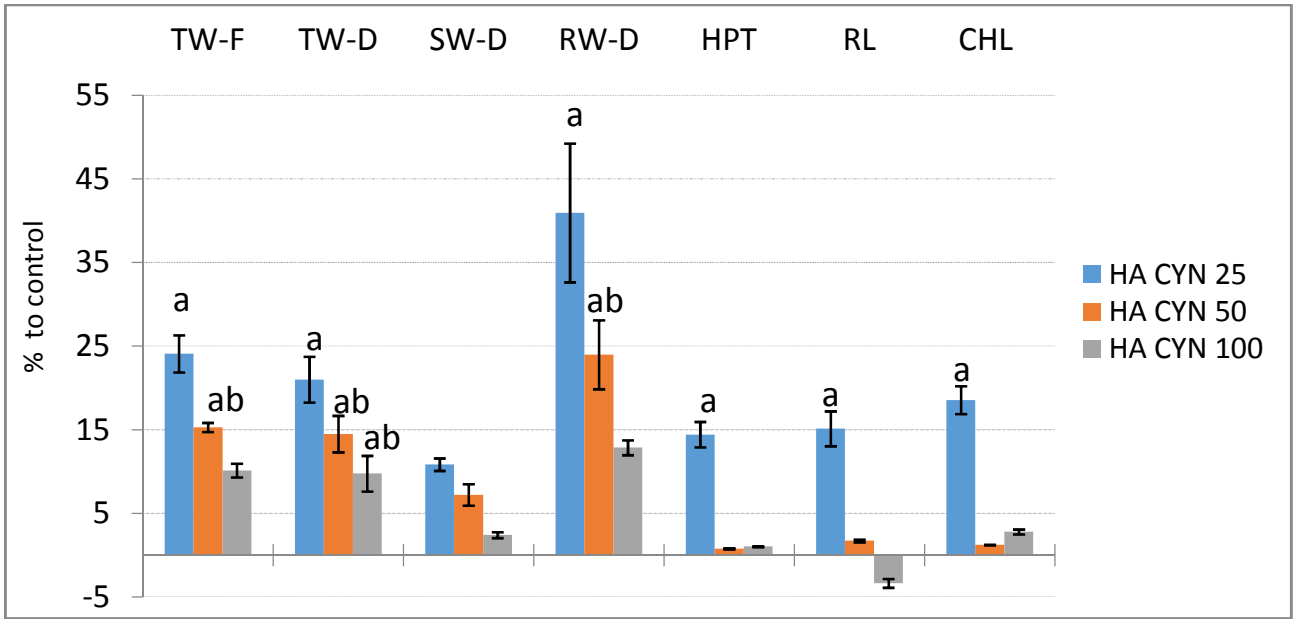

Figure 5. Effects of humic acids (artichoke) at different concentration on plantlets growth parameters. TW-F=Total weight fresh, TW-D=Total weight dry, SW-D=Shoot weight dry, RW-D=Root weight dry, HPT=Plant height, RL=Root length, CHL=Chlorophyll. Values expressed in % respect to control. Columns (mean ± S.D.) followed by different letters indicate significant difference at $P<0.05$ (Tukey's test).

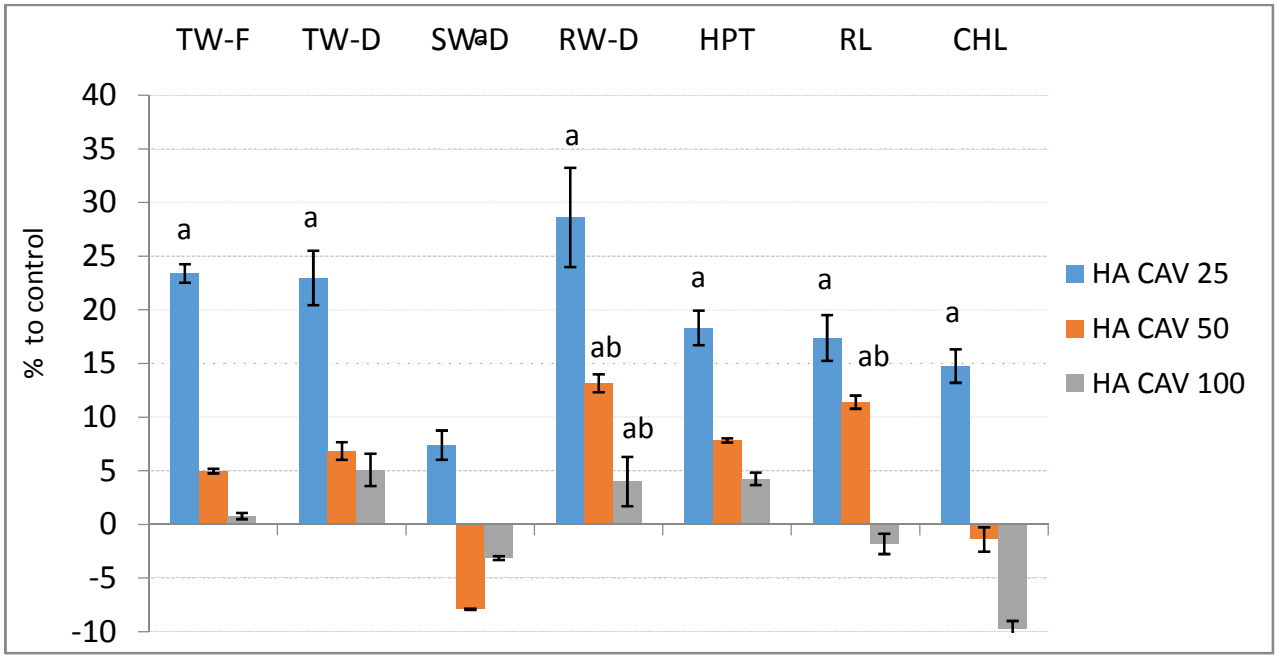

Figure 6. Effects of humic acids (cauliflower) at different concentration on plantlets growth parameters. TW-F=Total weight fresh, TW-D=Total weight dry, SW-D=Shoot weight dry, RW-D=Root weight dry, HPT=Plant height, RL=Root length, CHL=Chlorophyll. Values expressed in % respect to control. Columns (mean ± S.D.) followed by different letters indicate significant difference at $P<0.05$ (Tukey's test).

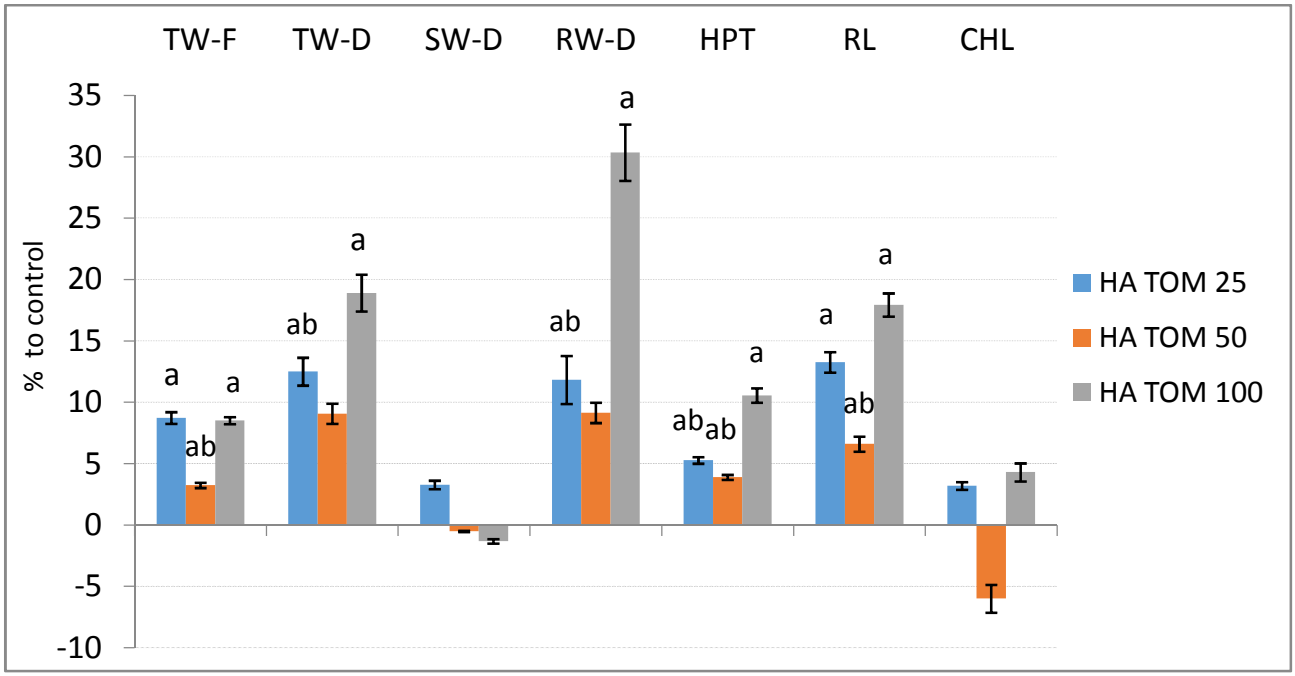

Figure 7. Effects of humic acids (tomato) at different concentration on plantlets growth parameters. TW-F=Total weight fresh, TW-D=Total weight dry, SW-D=Shoot weight dry, RW-D=Root weight dry, HPT=Plant height, RL=Root length, CHL=Chlorophyll. Values expressed in % respect to control. Columns (mean ± S.D.) followed by different letters indicate significant difference at P<0.05 (Tukey's test).

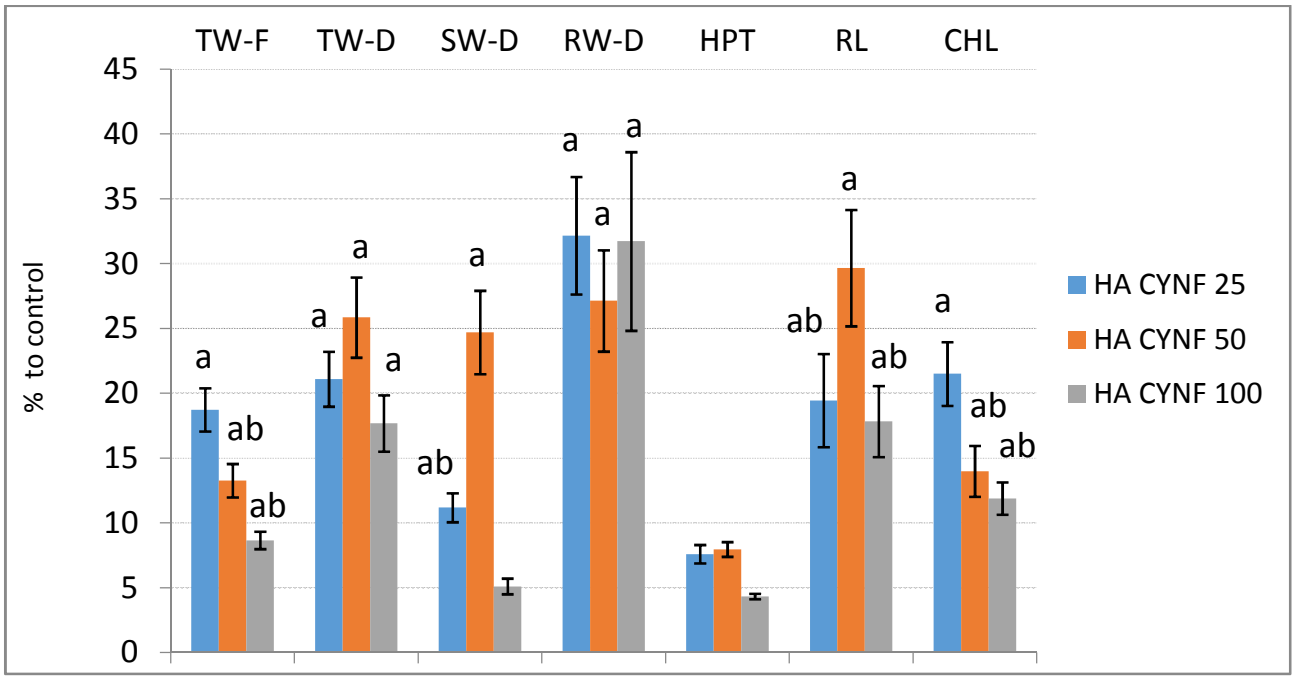

Figure 8. Effects of humic acids (artichoke+fennel) at different concentration on plantlets growth parameters. TW-F=Total weight fresh, TW-D=Total weight dry, SW-D=Shoot weight dry, RW-D=Root weight dry, HPT=Plant height, RL=Root length, CHL=Chlorophyll. Values expressed in % respect to control. Columns (mean ± S.D.) followed by different letters indicate significant difference at P<0.05 (Tukey's test).

3.7 Multivariate analysis

Principal component analysis explained 60.59% of total variance showing a clear separation of plant treatments with different humic acid (Fig. 9). The first PC, explaining 46.25% of variance, separated HA-CYN and HA-CYNF treatments from HA-TOM and HA-CAV, these last shifted on the positive axis. The second PC, explaining 14.34% of variance, mainly separated HA-CAV and HA-TOM on positive and negative axis, respectively. The distribution of variables on bi-plot suggests that ALKYL-H, H/C ratio and LigR ratio are the major contributors to the increase of plant growth and were mainly associated with HA-CYN and HA-CYNF. However, HA-CAV and HA-TOM were respectively associated with the hydrophobic and aromatic components, and still brought positive results. An apparent discrepancy were due to the spread of aromatic indicators along the first PC, disclosed by the bi-plot of PC2-PC3 (Fig. 10) in which all aromatic indicators clustered together. Finally, it is interesting to note that the highest concentration treatments are generally shifted towards the aromatic properties of humic substances, indicating that dose-effect observed in all treatment is correlated with the structure stability degree of humic acids.

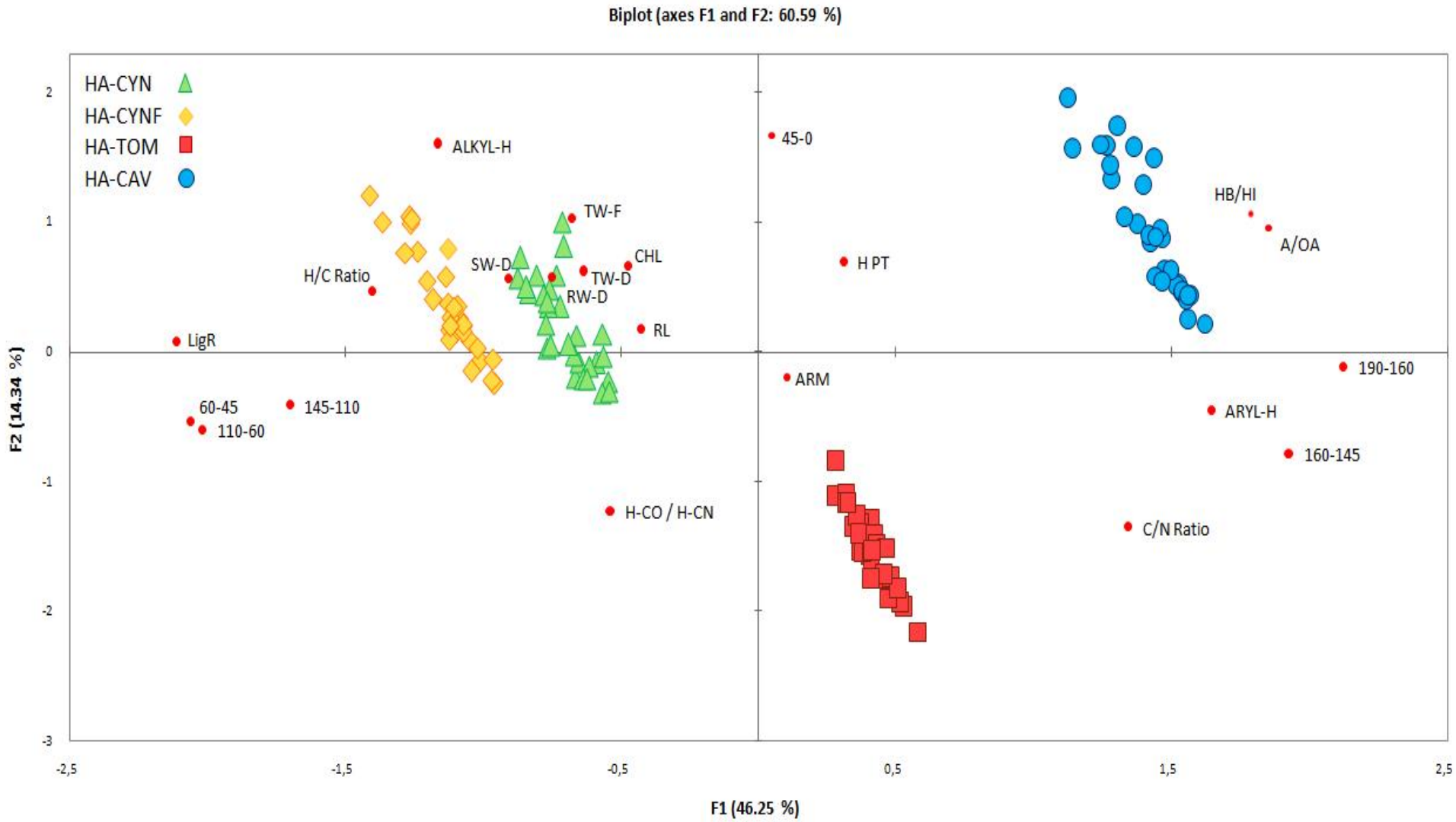

Figure 9. PCA bi-plot (PC1-PC2), indicating good separation of treatments in different groups according to the humic acids used. Position of the variables along the PCs indicates their importance for that PC.

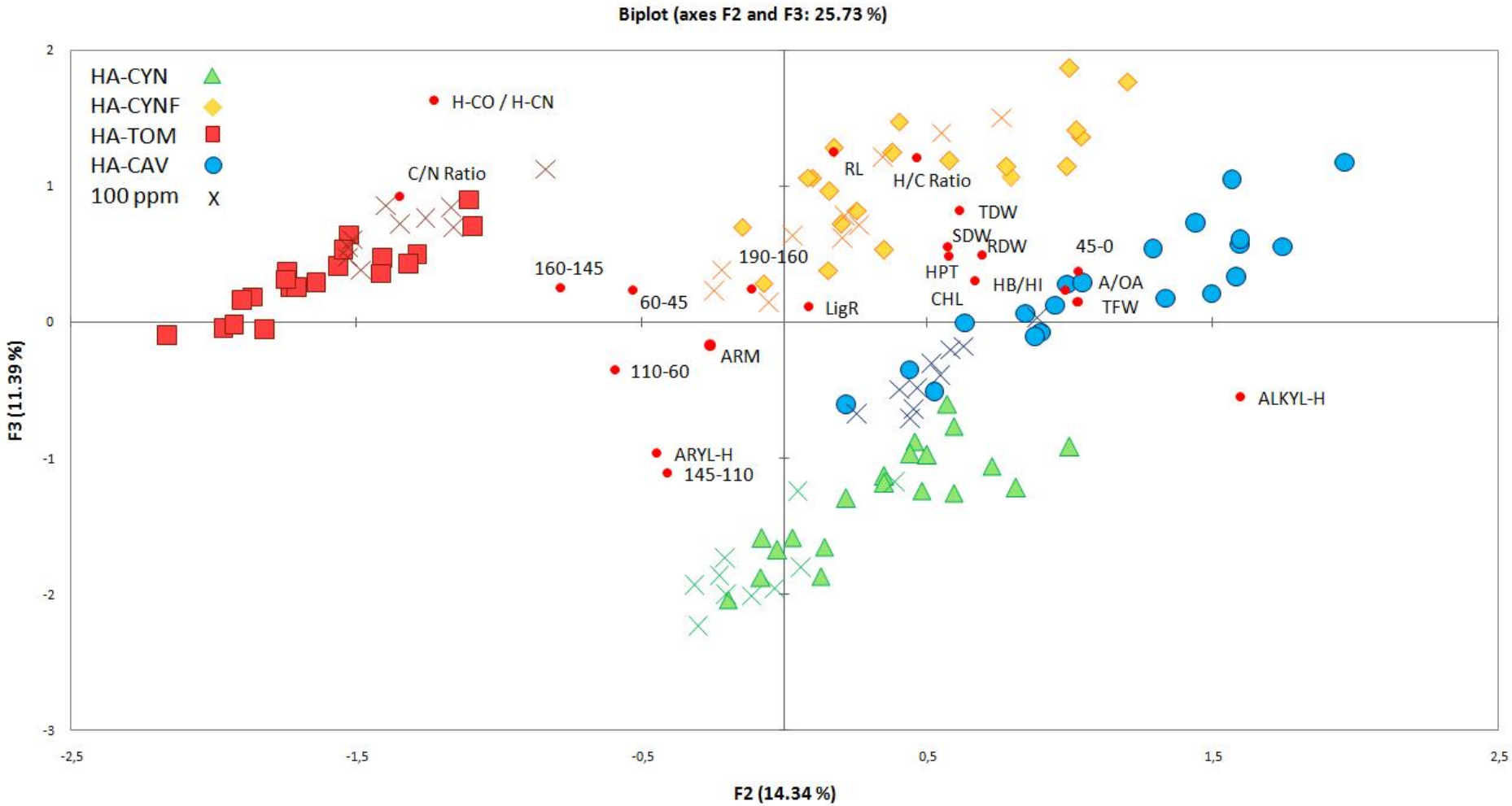

Figure 10. PCA bi-plot (PC2-PC3), indicating that 100ppm treatments grouped towards the aromatic indicators (ARM, ARYL-H, 145-110). Position of the variables along the PCs indicates their importance for that PC.

4. Discussion

In this study we correlate the structural composition of humic acids from recycled biomasses with the biological effects on plant growth. Despite the differences observed between the humic substances by NMR and infrared spectroscopy as well as elemental and thermal analysis, all the humic substances assayed favored plant growth, in a very similar pattern with relatively large responses at low application rates.

HA-CYNF structural indices indicated the significative content of aromatic components, associated with the presence of peptidic compounds as revealed by the nitrogen content (Table 1) as well as by the large LigR ratio (Table 2). In the plant growth bioassay, all parameters were positively influenced by the application of HA-CYNF, in particular with HA-CYNF 50 ppm dose that yielded the largest dry biomass and root length, but also the best development of the aerial part.

In response to treatments of the plants with 25 and 50 ppm of both HA-CYN and HA-CAV, general performances increased significantly ($P \leq 0.05$), but decreased when the concentration of humic acids was higher. In particular, the root development was favored over the aerial-part growth, except for HA-100 that showed a decrement in root length. A similar behavior was observed by Vaughan and Malcolm (1985) who described the tendency of humic substances to stimulate root growth at lower concentration then those necessary for the shoot development. From the chemical point of view, HA-CAV showed the greatest hydrophobic character highlighted by the value of HB and A/OA index (Table 2). The low LigR value showed the minor incorporation of lignin-derived structures, therefore suggesting the major contribution of lipidic materials to the hydrophobicity. On the other hand, the termogravimetric profile of HA-CYN was characterized by one step thermal degradation (Fig. 4, B), suggesting the prevalent aromacity of this material as suggested by the lowering of H/C ratio compared to the original biomasses. The presence of aromatic and hydrophobic

components has been closely related to the ability of humic substances to stimulate root development (Balmori et al., 2014; Canellas et al., 2014), although a positive metabolic effect of soluble carbohydrates, as those found in the liquid NMR analyses of HA-CYN, may not be excluded (Nardi et al., 2007; Xu et al., 2012).

A dose-response response was also observed with the application of HA-TOM. The biological assay reported the best performances when seedlings were treated with HA-TOM at highest concentration. However, this material resulted the least effective on plant growth. HA-TOM has a larger content of aromatic components, but their contribution arise mainly from phenolic carbons, as confirmed by NMR analysis (Table 2) and by the thermal degradation of phenols at 350-400°C visible in Figure 4. According to literature, phenolic compounds are potential inhibitors of nitrogen uptake and may have interfered, at lowest concentrations, with the correct development of plantlets (Muscolo et al., 2005; Muscolo and Sidari, 2006). However, the integration of ^{1}H NMR spectra highlight the most abundant content of proteinaceous material, in accordance with nitrogen content (Table 1) and IR spectra results (Fig. 1). Mechanisms involved in the stimulatory effect of humic substances are not clearly defined and could be ascribable to their chemical and physical properties.

Humic substances are described as a supramolecular aggregation of relatively small heterogeneous molecules, whose structural domains creates an hydrophobic microenvironment that favors molecular interactions with organic molecules. Piccolo (2002) stated that the occurrence of hydrophobic humic clusters are assumed to incorporate and protect polar molecules randomly, which may be released by conformational changes of humic associations in solution and become available to the plant root system.

As consequence of this concept, the effective bioactivity of humic matter may be mainly related to variable conformation and molecular strength association by which humic components are mutually combined and aggregated to each other in natural environments. In this scenario, hydrophobic to hydrophilic ratio could play a key role for the bioactivity of

humic extracts. In our experiments all humic acids brought positive results and retained an overall balanced distribution between hydrophobic and hydrophilic components.

As suggested by Nardi et al. (2007), it´s plausible that the biological activity of HS result from a particular arrangement of humic molecules in solution, where the distribution of hydrophilic bioactive molecules within the hydrophobic environment generates different degree of conformational flexibility, relied on concentration, that allow the interaction of active humic molecules with root cells. The flexibility of the structure would therefore explain the dose-response effect observed.

5. Conclusion

One of the major goal of the modern agriculture is to increasingly rely upon sustainable practices such as the use of environmental-friendly biostimulants. The present results confirm that compost is a viable way to extract bioactive humic acids, which represent an alternative biological product for a wide range of agricultural practices. The relationship between humic composition and the biological effect on plant performances need to be futher investigated, but a detailed molecular characterization is of remarkable importance for a more a comprehensive understanding of the chemical and biological interaction underlying bioactivity mechanisms and for a more aware use of humic substances as plant growth promoters.

CHAPTER V

Synergistic effects of phosphate solubilizing bacteria, AMF and humic acids enhance plant growth in a compost-based organic farming

Keywords: PSB; Arbuscular mycorrhizal fungi; *Pseudomonas* proradix; *Bacillus amyloliquefaciens*; Humic acids; Bioactivity; Microbial ecology; PGPR; PLFA; DGGE

Abstract: This study evaluated the interactions between two phosphate solubilizing bacteria (PSB), *Pseudomonas* spp. and *Bacillus amyloliquefaciens*, a mixture of two arbuscular mycorrhizal fungi (AMF), *Glomus mosseae* and *Rhizophagus irregularis*, and a humic acid (HA) extracted from artichoke compost, with respect to their effects on growth of maize plants and on the modifications of the soil microbial community structure. All plants inoculated showed a significant increase of biomass, phosphorus and nitrogen content when compared to plants grown without inoculation. The best effects on plant growth were obtained with the combination of *B. amyloliquefaciens*, AMF and HA. PLFA and DGGE fingerprinting analysis were used to detect changes in the soil microbial composition and dynamics. Inoculation with *Pseudomonas* spp. did not affect the composition of microbial community, except for the AMF and HA co-inoculated treatment, in which a decrease of AMF markers were observed, thus suggesting a negative interference and a plausible re-allocation of carbon sources. DGGE data confirmed a moderate effect of *Pseudomonas* inoculation, although a distinct variability were observed, in particular when comparing plants with and without inoculation. On the contrary, combination of *B. amyloliquefaciens*, AMF and HA increased

AMF markers and reduced the presence of saprophytic fungi, showing a synergistic effects that benefited general plant health status. Permutation test based on DGGE data confirmed a significant variation in both bacterial and fungal community structure induced by co-inoculation.

1. Introduction

The intensive use of mineral fertilizers and agrochemicals have been driving the agricultural productivity of the past century. Primary challenges for future agriculture require innovative cropping technologies for a more efficient management of the limited natural resources to preserve soil fertility and minimize the adverse environmental impact of current agricultural production (Timothy et al., 2016).

Organic fertilizers, such as compost, have the advantage of recycling nutrients that are already available in the agro-ecosystem, enriching soil with organic matter that converts nutrients to a stable form less susceptible to leaching, but, at the same time, less accessible due to their low solubility, thus requiring more input of energy to be easily processed by plant roots (Chen, 2006; King and Torbert, 2007).

Increasing efficiency of plant nutrient uptake represent the final goal to contribute to the sustainable intensification of agriculture. In this context, the utilization of bio-effectors (BEs), including various plant growth promoting microorganisms (PGPMs) and/or active natural compounds could represent an important determinant of soil fertility serving as brokers between plants and the soil in which they exert their activity (Watt et al., 2006; Marschner, 2012; Neumann and Römheld, 2012).

Bacillus and *Pseudomonas* species belong to the most common and abundant bacteria in the rhizosphere and phyllosphere of various crops, suggesting a high competence to colonize plant surfaces and tissues. They provide numerous beneficial traits, such as root growth

promotion, solubilisation of sparingly soluble nutrients and stimulation of root colonization by mycorrhizal fungi (Kloepper et al., 2004; Frey-Klett, 2007; Weller, 2007; Borriss, 2011). This last play a key functional role by mediating the transfer of carbon from roots to the soil as a source of energy for microbial life and contribute to plant uptake of mineral nutrients and water (Römheld and Neumann, 2006).

However, none of the known PGPM per se has the potential to fulfill the requested requirements of providing a viable alternative to mineral fertilizers. In recent literature, it has been recognized that the purposeful combination of plant growth promoting microorganisms can result in interactive and synergistic effects that are not achievable with single applications. Symbiosis of mycorrhizal fungi with associated microorganisms is considered to be of crucial importance for the development of more complex tri- or multi-partite interplay that could benefit plant fitness (Bonfante et al., 2009). Garbaye (1994) introduced the term mycorrhization helper bacteria for bacteria associated with mycorrhizal fungi, which consistently promote mycorrhizal development, hyphal branching (fungal architecture), growth, survival, reproduction, exudates composition and production of antibacterial metabolites (Frey-Klett and Garbaye, 2005; Riedlinger et al., 2006; Frey-Klett et al.,2007). Cooperation between beneficial microorganisms has been also confirmed for different species of phosphate-solubilising bacteria (e.g. *Bacillus* spp., *Pseudomonas* spp.) in diverse plant species (Kim et al., 1998; Singh and Kapoor, 1998; Widada et al., 2007).

Besides synergistic interactions between different microbial bio-effectors, also combinations including natural compounds can provide additional benefits for plant growth. Plant growth-promoting effects have been reported for a wide range of natural extracted materials. Humic substances have earned a rising attention from farmers and scientific community owing to their contribute to the regulation of many crucial ecological and environmental processes. Humic substances sustain plant growth and microbial life regulating both soil carbon and nitrogen cycling, the growth of plants and microorganisms, the fate and transport of

anthropogenic-derived compounds and heavy metals, and the stabilization of soil structure (Piccolo, 1992; Nardi et al., 2000; Delgado et al., 2002; Gryndler et al., 2005; Young et al., 2006; Hrselová et al., 2007; Canellas et al., 2008; Chen and Wang, 2008; Dobbss et al., 2010). However, because synergistic effects are often the result of complex interactions between many factors, the required conditions for the best performance of BE combinations need to be further investigated. The objectives of our research were to study the effects of the combination of microorganisms (*Bacillus amyloliquefaciens*, *Pseudomonas* spp., arbuscular mycorrhizal fungi mixture) and humic acids, on maize plants , with the final goal to verify the overcoming nutrient limitations and to evaluate the effect on microbial population of the rhizosphere.

2. Materials and methods

2.1 Soil and compost material

To set up the pot experiment, we used the surface layer (0–15 cm) of soil collected at the Long Term Experimental field site of the University Farm of Agricultural Dept. (University of Naples "Federico II"), located in Castel-Volturno (CE). Soil and compost pH and electrical conductivity (EC) were measured in a 1:5 soil/water suspension (w/v) after 1 h end-over-end shaking at 25 °C. Soil organic carbon (C) was determined by the Walkley–Black procedure (Nelson and Sommers, 1982) and total nitrogen (N) by the Kjeldahl digestion method (Bremner and Mulvaney, 1982). Available soil phosphorus (P) was extracted with sodium bicarbonate (Olsen method) and then determined by the molybdenum-blue method (Murphy and Riley, 1962). The soil was a clay loam (44.6%, 28% and 27.4% sand, silt and clay), alkaline (pH 8.6) and classified as a Vertic Xerofluvent, containing 1.11 g kg^{-1} total N, 10.5 g kg^{-1} organic carbon, 11 mg kg^{-1} of $NaHCO_3$-extractable P.

The composting process was conducted using the on-farm composting facility at the LTE experimental station. The composting method is based on a static pile with air insufflation system, formed by a rotative pump connected to a frame of perforated rubber tubes. The tubes were placed on a bed of dry corn residues (4x8 meters). The composting pile were made up by a mixture (base matrix) of cow and buffalo manure (70% w/w) and maize straw and poplar trimming as structuring woody material (30% w/w); the mixed material was uniformly spread by a power shovel to cover the insufflation system and forming the final pile height of approximately 1.5 m.

The composting process lasted 100 days, with a periodic monitoring of external and internal temperature level (5 min interval) and oxygen percentage (60 min interval). During the first 50 days the minimum percentage of oxygen was set at 10%, then subsequently at 5%.

Before chemical analysis, the compost samples were oven-dried at 40°C until constant weight and sieved at 500 μm. Elemental content (C, H, N) of compost samples (Table 1) were determined by elemental analyzer Fisons EA 1108 (Fisons Instruments, Milano, Italy).

Table 1. Elemental composition of composts. Total C, N and H are given as % dry matter.

	Unit	**COMPOST**
Total C	%	27,99±0,01
Total N	%	2,13±0,02
Total H	%	4,06±0,03
C/N Ratio		13,14

2.2 Bioeffectors

Bioeffectors addressed in the experiment consisted of three commercial microbial inocula and one humic acid. The microbial products were: Proradix® (*Pseudomonas* spp.) produced by Sourcon Padena GmbH & Co., Rhizovital 42® (*Bacillus amyiloliquefaciens*) produced by ABiTEP GmbH, and Aegis® based on a mixture of two arbuscular mycorrhizal fungi strains *Glomus mosseae* and *Rhizophagus irregularis,* provided by Italpollina S.p.a.

Stock suspension of each bacterial product was prepared under sterile conditions using demineralized water with 2.5 mM CaSO4. The volume required to inoculate each pot was calculated in 30 ml of diluted product. MYC Aegis was applied directly as microgranule.

Humic acids were extracted from a green compost (composted artichoke residues) through the following extraction procedure: an aliquot of 100 g of each air-dried compost (2 mm sieved) was suspended in 500 ml 0.1 mol L^{-1} NaOH and 500 ml 0.1 mol L^{-1} $Na_4P_2O_7$ and mechanically shaken for 24 h. The suspension was then centrifuged at 7000 rpm for 20 min and glass wool filtered. The extraction was repeated 2 times (1h agitation step). The suspension was acidified to pH 1.5 with 6 mol L^{-1} HCL to allow the precipitation of humic acids. After 24h, the samples were centrifuged at 4000 rpm for 20 min and humic acids collected and dialysed against deionized water using 1-kD cutoff spectrapore membrane until the electrical conductivity resulted lower than 0.5 dS m^{-1}. Stock solution were prepared from freeze-dried powder dissolved in deionized water and diluted to the application rate (0.012 g Kg^{-1}).

2.3 Pot preparation and experimental design

The pot experiment was conducted from May to July 2014, under greenhouse conditions (25–33 °C, daily temperature range during maize growth). The soil was sieved to 5 mm, mixed with quartz sand at the ratio of 2:1 (w/w) and thoroughly homogenized. The pot

experiment was conducted using this soil/sand substrate. The substrate was incubated in covered plastic boxes at 20 ± 2°C during 30 days prior to planting. Maize (*Zea mays*, cv Aphoteos, Limagrain S.p.a.) plants were grown in pots filled with 5 kg of substrate. The composted cow manure, used as recycled organic P source (50 mg P/Kg^{-1}), was mixed and incorporated homogenously into the soil, and the mixture was left to equilibrate for 3 weeks at room temperature before the experiment. The pot trial was designed as follows:

B0	Control (no inoculation)
B0M	Control + Mycorrhizal inoculum
B2	*Pseudomonas* spp.
B2M	*Pseudomonas* spp. + Mycorrhizal inoculum
B2HA	*Pseudomonas* spp. + Humic acids
B2HAM	*Pseudomonas* spp. + Humic acids + Mycorrhizal inoculum
B3	*Bacillus amyiloliquefaciens*
B3M	*Bacillus amyiloliquefaciens* + Mycorrhizal inoculum
B3HA	*Bacillus amyiloliquefaciens* + Humic acids
B3HAM	*Bacillus amyiloliquefaciens* + Humic acids + Mycorrhizal inoculum

Each treatment was replicated five times for a total of 50 pots. Three maize seeds were sown 3 cm below the soil surface in each pot. The different microbial inocula were applied as a suspension of demineralized water with 2.5 mM $CaSO_4$ spraying it on seeds surface at sowing, at the rate of $2x10^6$CFU g^{-1} dry substrate of B2 and B3 respectively, using an automatic pipette with sterile pipette tips, into each planting hole, whereas for the application of mycorrhizal inoculum 2.5 g of product were placed under the seeds. Holes were closed and the top surface of the soil were covered with 150 g (~ 0.5 cm thick layer) of quartz sand (0.6-1.2 mm) to avoid the formation of surface crusts after watering. After emergence (5 days after

sowing), only one maize seedling per pot was then left to grow and additional 15 ml of BE solution was applied to the top layer. During crop growth, soil water content was maintained at 60% of field capacity by periodically adding water to compensate evapotranspiration losses. The plant performances were monitored recording heights and symptoms of N deficiencies were corrected, if needed, by supplying the nutrient in solution at rate of 0.50 g pot^{-1} N (as $CaNO_3$). The plants were harvested 8 weeks after sowing. Plant height and leaf numbers were measured, shoot and root separated and fresh and dried biomass measured, after drying in a forced-air oven at 70 °C for 2 days. The soil strictly adhering to roots was considered as rhizospheric soil and separated by brushing after a gentle shake. The soil falling from roots as well as the rest of soil was considered bulk soil. Rhizospheric soil was sieved at 2 mm and stored at −20 °C for PLFA and molecular fingerprinting analysis, while the bulk soil was air-dried and sieved at 2 mm at room temperature and stored for physical–chemical analysis. Total P in maize shoots was obtained by digesting the dried plant samples with diluted HNO_3 and HCL (1:3 v/v) (Gericke and Kurmies, 1952) and then colorimetrically determined by molybdenum blue assay method (Murphy and Riley, 1962). Total N was determined by Kjeldahl digestion method (Bremner and Mulvaney, 1982).

Characterisation of microbial communities

2.4 PLFA analyses

Selected fatty acids pertaining to the soil phospholipid (PLFA), and used as biomarkers for specific soil microbial communities, were extracted using the modified Bligh and Dyer (1959) technique, as described by Bardgett et al. (1996). Total soil lipids were extracted from 2 g of soil by a chloroform/methanol (MeOH)/citrate buffer (1:2:0.8 v/v). Separation of lipid classes was conducted in silica gel columns. Neutral, glyco-, and phospholipid fractions were

obtained by sequentially eluting silica gel columns with chloroform, acetone, and methanol, respectively. Phospholipid fractions were dried under a N2 flow at 37 °C and stored at −20 °C. Fatty acid methyl esters were formed by a mild alkaline methanolysis. Thirty microliters of methyl nonadecanoate fatty acid (19:0; Sigma-Aldrich) were added as internal standard, and the methylated samples were dried under N2 flow. Samples were dissolved in 200 µl of hexane for analysis by a Perkin-Elmer Autosystem XL (GC) equipped with a PE Turbomass-Gold quadrupole mass spectrometer. Chromatographic separation was achieved through a 60m Supelco Capillary column (SLB-5 ms) using helium as carrier gas (1 ml min−1). Samples (2.5 µl) were injected in split less mode with the injector held at 250 °C. The initial oven temperature, 100 °C, was held for 5 min, raised to 210 °C at a rate of 2 °C min−1, then raised from 210 to 250 °C at a rate of 5 °C min−1, and held for 12 min. Mass spectra were obtained in EI mode (70 eV), scanning in the range of m/z 50–600, with a cycle time of 1s. The abundance of individual PLFA was derived from the relative area under each chromatographic peak, as compared to that of internal standard (19:0) and related to the calibration curve of the 19:0 standard fatty acid dissolved in hexane. Each PLFA content was expressed as nmol of PLFA per gram of dry soil. Fatty acids were named according to the ω-designation described as follows: total number of carbons followed by a colon; the number of double bonds; the symbol ω; and the position of the first double bond from the methyl end of the molecule. Cis- and trans-configurations are indicated by c and t, respectively; iso and anteiso forms of methyl-branched fatty acids are indicated by i- and a-, respectively. 10Me indicates a methyl group placed on the tenth C atom from the carboxyl end of the molecule; cy refers to cyclopropane fatty acids. The C18:2ω6c and C18:1ω9c PLFA were used as biomarkers for fungal biomass (Frostegard and Baath, 1996). The aC15:0, iC15:0, iC16:0, iC17:0, and aC17:0 PLFA were chosen to represent Gram(+) bacteria (Sundh et al., 1997), while the C16:1ω7c, C18:1ω7c, C18:1ω5c, cyC17:0, and cyC19:0 PLFA were related to Gram(−) bacteria (Sundh et al., 1997), and the 10MeC16:0, 10MeC17:0, and 10MeC18:0

PLFA to actinomycetes. Total PLFA concentration was the sum of single identified PLFA. The PLFA ratios relative to fungal/bacterial, Gram(+)/Gram(−) bacteria, and AMF/ saprotrophic fungi were calculated by using the sum of the respective fatty acid biomarkers and were assumed to represent the relative abundance of these groups. Data obtained were subjected to normality test (Shapiro-Wilk test) to evaluate the distribution of the samples, means values were compared using one-way analysis of variance (ANOVA) and tested for significance with Tukey´s HSD test in SPSS software (IBM SPSS Statistics). Statistical significance was defined for $p<0.05$. Multivariate Principal component analysis were used to analyze PLFA biomarkers by means of XLSTAT software (Version 2014.4.08, Addinsoft, Inc., Brooklyn, NY, USA).

2.5 Total microbial community DNA (TC-DNA) extraction

Total community microbial DNA (TC-DNA) was extracted using four out of five replicates subjected to TC-DNA extraction (each 0.5 g wet weight) after a harsh lysis step, using FastPrep FP24 bead-beating Instrument (twice repeated, 30 s, 5.5 m s-1) (MP Biomedicals), by means of the FastDNA spin kit for soil (MP Biomedicals). Purification of extracted TC-DNA was performed using GeneClean spin kit (MP Biomedicals) according to the manufacturer protocol. DNA yield was checked by agarose gel electrophoresis (0.8% w/v agarose; 100 V, 40 min) using ethidium bromide staining and estimated using the 1-Kb Plus DNA ladder. Purified DNA was stored at -20°C.

2.6 Amplification of bacterial 16S rRNA

The purified TC-DNA was diluted 1:10 with deionized water before PCR amplification. F984GC/R1378 universal eubacterial primer pair was used to amplify TC-DNA as described by Heuer et al. (1997). All primers used in this study are provided in Table 2. PCR master

buffer consisted of: 1µl of template DNA (1 to 5 ng), 5 µl GoTaq Flexi Buffer, 3.5 µl MgCl (25 mM), 2 µl acetamide (50%), 2.5 µl deoxynucleotide triphosphates (dNTPs, 2 mM), 0.5 µl primers (10 µM), 0.125 µl GoTaq polymerase (5 u/µl) 9.625 µl deionized water. PCR program included an initial denaturation step at 94°C for 5 min, followed by 35 thermal cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 10 min.

A nested PCR approach was applied for the amplification of 16S rRNA genes of *Pseudomonas* and *Bacillus* as previously described (Heuer et al., 1997; Gomes et al., 2001; Costa et al., 2007; Weinert et al., 2009). Amplification of *Pseudomonas* specific group was conduted using F311Ps/R1459Ps primer set and the following master buffer: 1µl of template DNA, 2.5 µl TrueStart buffer, 2.5 µl dNTPs (2 mM), 2.5 µl MgCl (25 mM), 2 µl acetamide (50%), 0.5 µl primers (10 µM), 1.25 µl bovine serum albumin (BSA), 0.125 µl TrueStart Taq polymerase (10u/µl), 12.125 µl of deionized water. PCR thermal cycler was programmed as follows: 94° denaturing step for 7 min was followed by 30 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were diluted 1:10 and used as templates for the nested PCR with primer pair F984GC/R1378.

Amplification of *Bacillus* specific group was achieved using BacF/R1378 specific primers and the master buffer was prepared as follows: 1µl of template DNA, 5 µl GoTaq Flexi Buffer, 2.5 µl dNTPs (2 mM), 3.75 µl MgCl (25 mM), 0.5 µl primers (10 µM), 0.025 µl BSA, 0.25 µl GoTaq polymerase (10u/µl), 8.975 µl of deionized water. The following setting program was used: 94° denaturing step for 5 min was followed by 30 cycles of 94°C for 1 min, 65°C for 1,5 min, and 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were diluted 1:10 and used as templates for the nested PCR with primer pair F984GC/R1378. PCR products were analyzed on conventional agarose gel and diluted 1:10 before DGGE analysis.

Table 2. Primer set used to amplify bacterial and fungal community.

Primer pair	Primer	Sequence (5'–3')[a]	Taxonomic group	Annealing temp (°C)
F984GC/R1378	F984 R1378	GC clamp-AACGCGAAGAACCTTAC CGGTGTGTACAAGGCCCGGGAACG	*Bacteria*	53
F311Ps/R1459Ps	F311Ps R1459Ps	CTGGTCTGAGAGGATGATCAGT AATCACTCCGTGGTAACCGT	*Pseudomonas*	63
BacF/R1378	BacF	GGGAAACCGGGGCTAATACCGGAT	*Bacillus*	65
ITS1F/ITS4	ITS1F ITS4	CTTGGTCATTTAGAGGAAGTAA TCCTCCGCTTATTGATATGC	*Fungi*	55
ITS1FGC/ITS2	ITS2	GCTGCGTTCTTCATCGATGC	*Fungi*	55

[a] The GC clamp sequence was CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.

2.7 Amplification of fungal ITS fragments

The fungal community was studied based on the fungal internal transcribed spacer (ITS) fragment and was amplified using a nested PCR approach as described by Weinert et al. (2009) with primer set ITS1F/ITS4 (Gardes and Bruns, 1993; White et al., 1990). The reaction mixture for the first PCR was composed of: 1µl of template DNA, 2.5 µl TrueStart Buffer, 2.5 µl dNTPs (2 mM), 3.75 µl MgCl (25 mM), 0.5 µl primers (10 µM), 0.5 µl DMSO, 0.125 µl TrueStart Taq polymerase (5u/µl), 13.625 µl of deionized water. Thermal cycle started with denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. Samples were used as templates for the second PCR. The buffer for the second PCR was the same as the one used for the first PCR, except for the use of 0.2 µl TrueStart Taq polymerase and different primer set (ITS1F-GC/ITS2). The PCR conditions were the same as those described for the first PCR except for the number of cycles, which were reduced to 25. Final amplified samples were checked by means of electrophoresis agarose gel prior to further DGGE analysis.

2.8 Analysis of 16S rRNA and ITS gene fragments by denaturing gradient gel electrophoresis (DGGE)

DGGE analysis were performed using a PhorU2 apparatus (Ingeny, Goes, The Netherlands), according to Weinert et al. (2009). Two different double gradient gel were used to separate gene fragments: a gel composed of 46.5–65% denaturants (100% denaturant was defined as 7 M urea and 40% formamide) and acrylamide (6.2% to 9%) was used for both community and group-specific 16S rRNA, meanwhile the gradient consisted of 23% to 58% denaturant and 8% acrylamide for fungal gene fragments. The gels were ran in Tris-acetate- EDTA buffer and voltage was kept constant at 140 V for 17 h at 58°C for bacterial community and 100 V for 18 h at 60°C for fungal community. After electrophoresis the gels were silver stained as described by Heuer et al. (2001), air dried and scanned for acquiring digital pictures. DGGE profiles were processed by using GelCompar II software ver. 6.5 (Applied Maths) as described by Rademaker et al. (1999) and modified by Smalla et al. (2001). Cluster analysis of DGGE fingerprint patterns were carried out using pairwise Pearson correlation coefficients calculated by means of unweighted pair group method using arithmetic averages (UPGMA). Pearson similarity matrices were used to test significant differences between treatments by the application of permutation test with PERMTEST software (10000 simulations), calculating the d-value (dissimilarity value) from the average overall correlation coefficients within the groups minus the average overall correlation coefficients between groups from treatments compared, in accordance with Kropf et al. (2004).

3. Results

3.1 Plant growth and nutrient content

Application of bioeffectors significantly increased total dry plant biomass (Fig. 1). The best effects were shown by B2M and B3HA accounting for +60% and +73% increase as compared

to control. However, among inoculated treatments, no marked differences were observed, except for B3HA whose biomass resulted significantly enhanced. Both N and P content resulted increased in all inoculated plants, in particular after B3HAM application (Fig. 2, Fig. 3). In fact, B3HAM treatment showed N and P increase ranging from 30 to 36% respectively when compared to B0 (Fig.2, Fig.3).

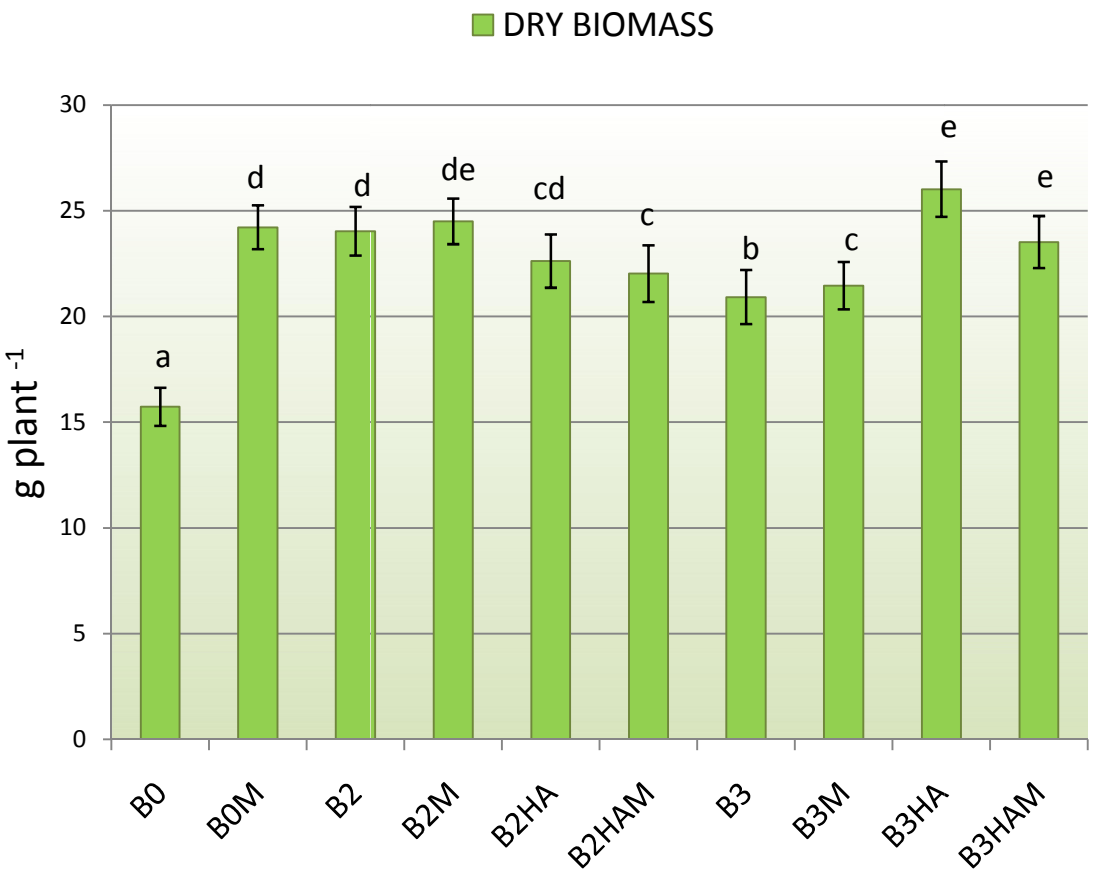

Figure 1. Dry biomass content as affected by different treatments**.** Columns (mean ± S.D.) followed by different letters indicate significant difference at P<0.05 (Tukey's test).

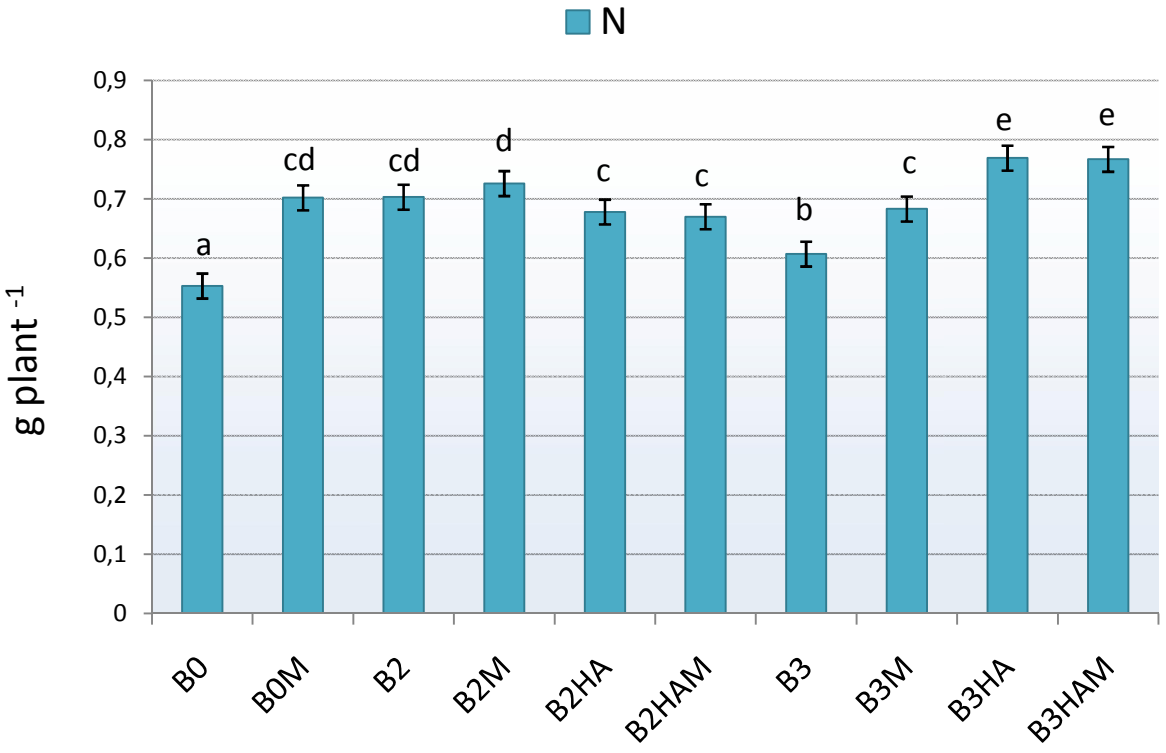

Figure 2. Nitrogen content under different treatments**.** Columns (mean ± S.D.) followed by different letters indicate significant difference at P<0.05 (Tukey's test).

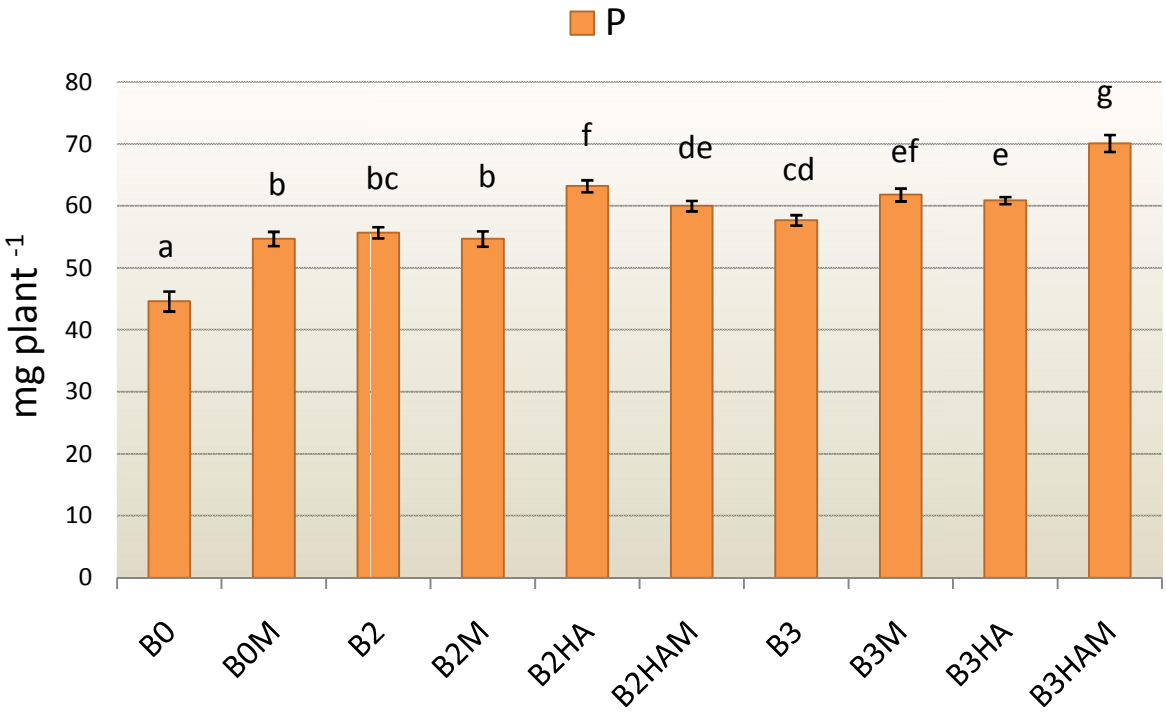

Figure 3. Phosphorus content under different treatments**.** Columns (mean ± S.D.) followed by different letters indicate significant difference at P<0.05 (Tukey's test).

3.2 PLFA analysis

The total amount of PLFA, a measure of total microbial biomass, did not changed significantly under all bioeffectors treatments, except for B3M, B3HA and B3HAM (Table 3). However, significant differences were highlighted when considering specific microbial groups. Gram(-) and fungal PLFA were larger in control (B0) than under all bioeffectors treatments. Conversely, the marker of arbuscular mycorrhizal fungi (16:1ω5c) increased with mycorrhizal and/or bacterial inoculation (B2 and B3), in particular, it was the greatest for B3HAM treatment. The ratios of specific fatty acids rather than single fatty acids were capable to reveal differences in the composition of soil microbial communities due to bioeffectors inoculation. The Gram(+)/Gram(-) and Fungal/Bacterial ratios in B0 were the smallest and the largest, respectively, as compared to all treatments (Table 3). Under mycorrhizal inoculation treatments, fungi to bacteria ratios were the smallest (Table 3, Fig. 4). Principal components analysis (PCA) of the selected soil biomarker PLFAs showed the changes in microbial composition in soils under bioeffectors treatments (Fig. 5). The first two principal components (PC1 and PC2) accounted for 65.4 % of total variation and separated the variables in terms of bioeffectors treatments. All PLFA markers were positively aligned along the PC1 with loading values for PC1 larger than 1, except for 16: 1ω5c. The PC1 explained 50.34 % of variance. The placement of B2 and B3 in the positive region suggested an increase of all PLFA markers in these treatments. Since C16:1ω5 PLFA was negatively spread along the PC1, the placement of B2 in the positive PC1 region suggested that this marker decreased in soil samples under B2 treatment. The most significant variables responsible for the differentiation along PC2 were represented by the i14:0, a14:0, a15:0, i15:0 that had a positive spread over this principal component (Fig. 5) while 16:1ω5c and 10Me18:0 showed a negative correlation to PC2. The placement of all treatments with *B.*

amyloliquefaciens (B3, B3M, B3HA, B3HAM) in the negative region indicated an increase of mycorrhizal marker in these treatments.

Nutrients content plotted together with microbial markers provided additional information shown in Figure 6. The radar chart revealed that P content varied principally in accordance with the content of AM fungi and saprophytic fungi, resulting larger when AMF/fungi ratio was higher. This tendency was slightly visible for Gram(-) too. Also nitrogen showed a similar trend but appeared less affected by each treatment.

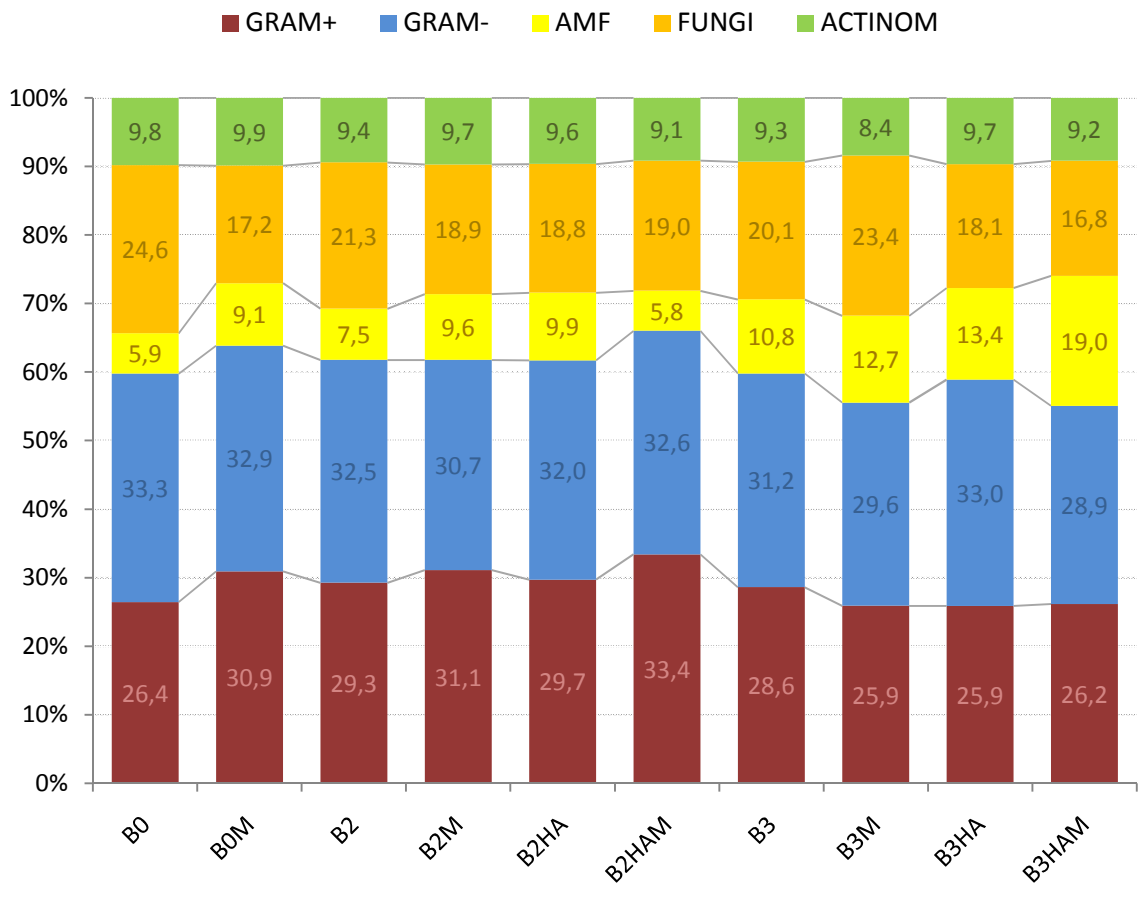

Figure 4. Abundance (%) of bacterial Gram(+),Gram(−), arbuscular mycorrhizal fungi, saprophytic fungi and actinomycetes, estimated by PLFA biomarkers by means of five replicates.

Table 3. Abundance of actinomycetes, bacterial, fungal, Gram(+), Gram(-), total PLFA as well as PLFA ratios Fungi/Bacteria and Gram(+)/Gram(-).

Treatments	Gram(+)	Gram(-)	Saprotrophic Fungi	Actinomycetes	Fungi/Bacteria	Gram(+)/Gram(-)	Total PLFA
B0	63,78±2.44	102,91±5.7	75,85±1.14	30,24±3.53	0,46±0.01	0,62±0.03	272,79±8.75
B0M	73,83±1.11	97,61±3.27	50,82±3.23	29,26±1.8	0,30±0.01	0,76±0.02	251,53±7.85
B2	84,51±3.78	115,17±7.23	75,61±1.55	33,13±3.31	0,38±0.01	0,73±0.02	308,41±14.41
B2M	81,71±5.15	97,91±9.73	59,58±5.36	30,52±3.31	0,33±0.04	0,84±0.07	269,72±16.35
B2HA	75,32±4.62	100,61±2.87	59,06±2.57	30,27±1.52	0,34±0.03	0,75±0.03	265,27±7.35
B2HAM	73,79±3.36	88,20±3.65	51,27±1.24	24,85±2.10	0,32±0.01	0,84±0.06	238,11±3.38
B3	78,70±7.45	102,83±5.73	66,49±5.36	30,67±2.17	0,37±0.01	0,77±0.07	278,69±17.30
B3M	67,87±5.04	88,49±6.98	52,80±2.52	19,15±2.54	0.35±0.04	0,77±0.04	228,30±15.81
B3HA	68,95±3.10	89,38±6.0	53,31±1.96	23,16±2.06	0,34±0.02	0,77±0.08	234,80±5.59
B3HAM	67,99±1.84	93,28±6.13	54,19±4.25	29,58±2.95	0,34±0.01	0,73±0.03	245,04±14.53

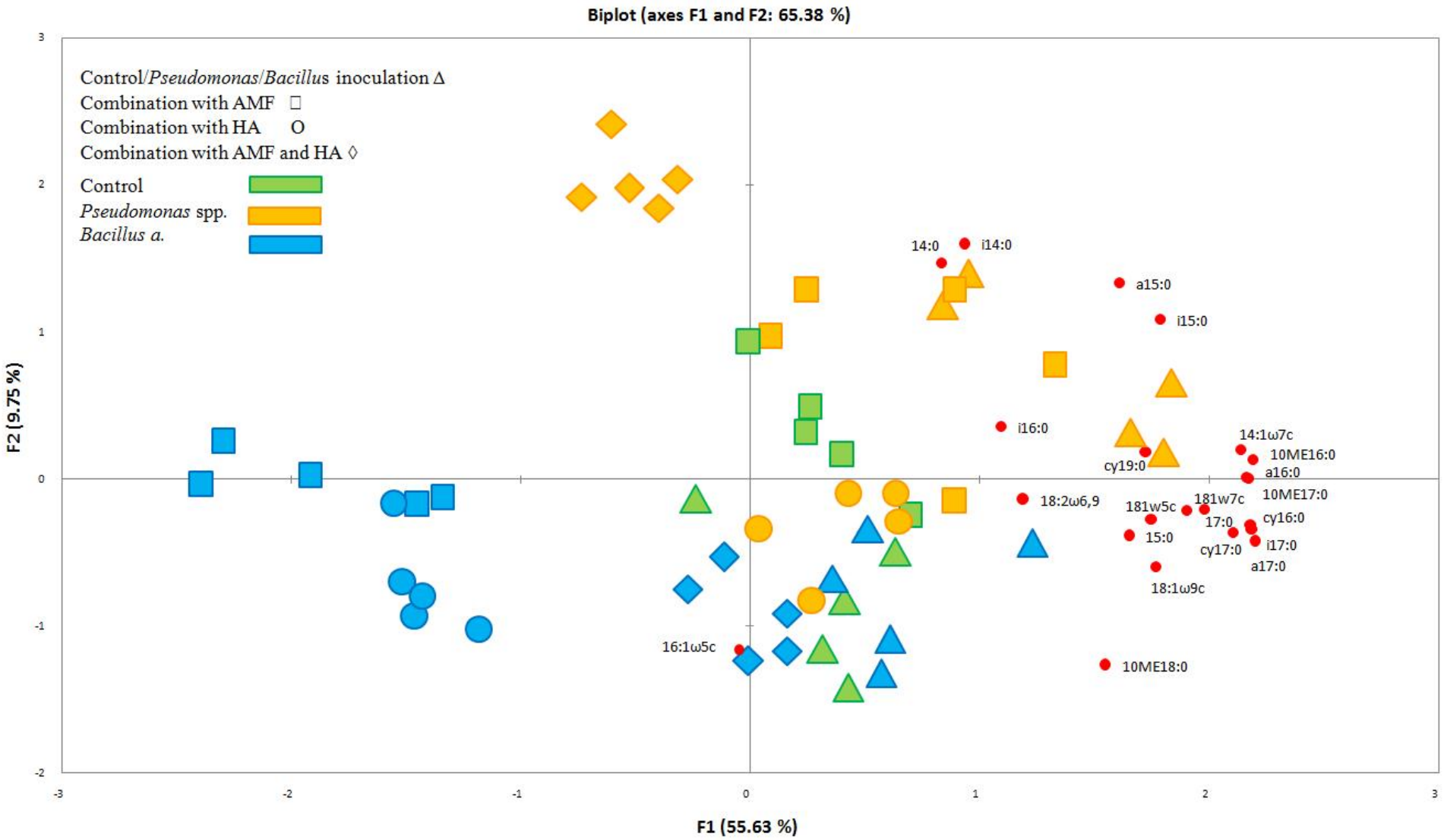

Figure 5. PCA score-plot of microbial PLFA markers.

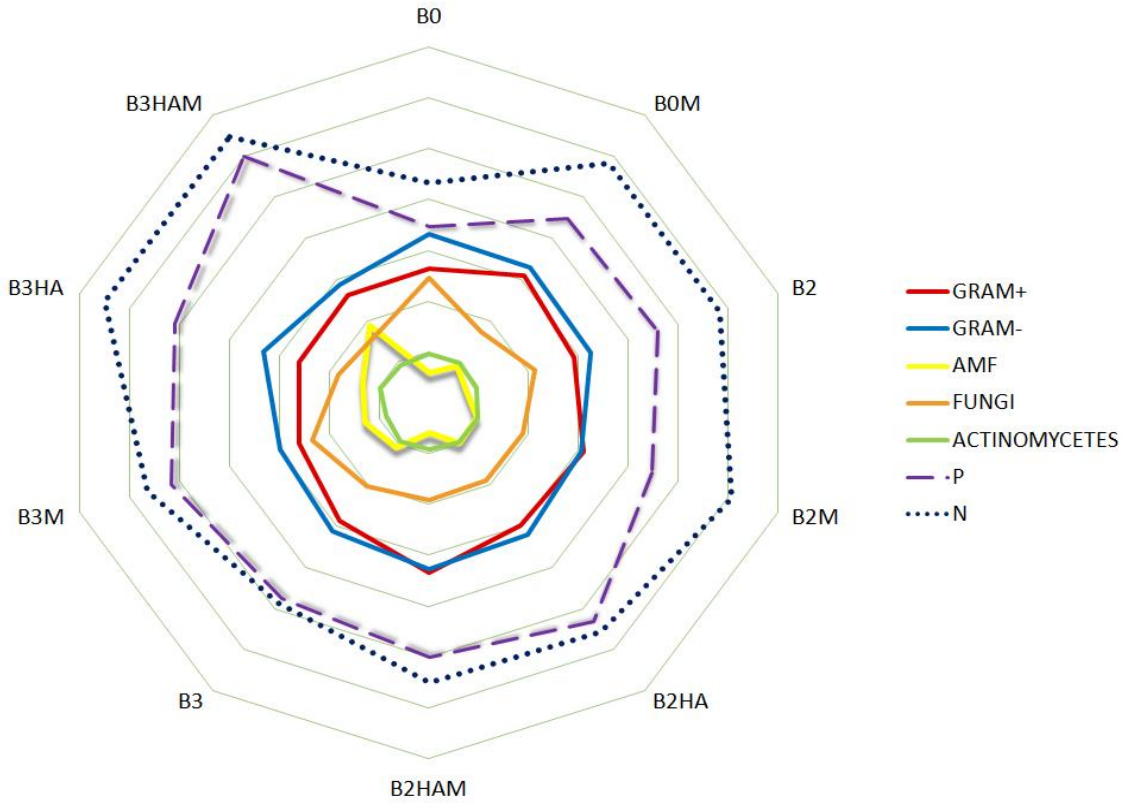

Figure 6. Nitrogen and phosphorus content and PLFA biomarkers variation across treatments. Data were transformed in order to show relationship between parameters.

3.3 PCR-DGGE fingerprint analysis

The analysis of DGGE fingerprints by UPGMA revealed that the bacterial community composition of the different treatments shared approximately 40% similarity (Fig. 7). All replicates clustered according to the own treatment delineating consistent differences between each other. According to pairwise comparisons (Table 4), B3HAM was the treatment with the major shift in the bacterial community composition (d=82.95, p<0.05), as well as B3HA (d=67.51 p<0.05), although similar differences were observed for all treatments inoculated with B2.

Profiles generated by group specific fingerprints of *Pseudomonas* and *Bacillus* communities are shown

in Figure 8 and Figure 9, respectively. Clustering of each treatment in *Pseudomonas* fingerprint showed a large similarity in the range between 70% and 98%, except for B2HA replicates that showed a distinctive cluster. Differences carried out by statistical analysis suggested an overall moderate inoculation effect of *Pseudomonas*, however, *d*-values calculated on pairwise comparisons (Table 4) suggested a discrete shift of bacterial community when B2 was combined with mycorrhizal fungi and humic acids (B2HAM d=14.16, p<0.05).

Bacillus microbial population did not allowed a clear separation of clusters (Fig. 9), but this result was partly supported by pairwise group comparisons, since leastwise B2M and B3HA resulted statistically different from control, showing a quite large dissimilarity values (d=22.28, d=14.48 p<0.05, respectively). However, except for B2M, all of B3 related treatments were found to generally display the largest distance, in terms of microbial shift, when compared to control (Table 4).

Fungal communities gel showed a remarkable abundance of ITS fragments, suggesting a high level of internal variability. Cluster profile of B3 group treatments was clearly visible, evidencing how this group strongly affected fungal structural composition in soil (Fig. 10). The inoculation effect was confirmed by the permutation test that showed a large dissimilarity values when treatments inoculated with B3 were compared to control (Table 4). However, it is noteworthy that both inoculated treatments showed major differences when combination of bioeffectors occurred, in particular with B2HA and B3HAM (Table 4).

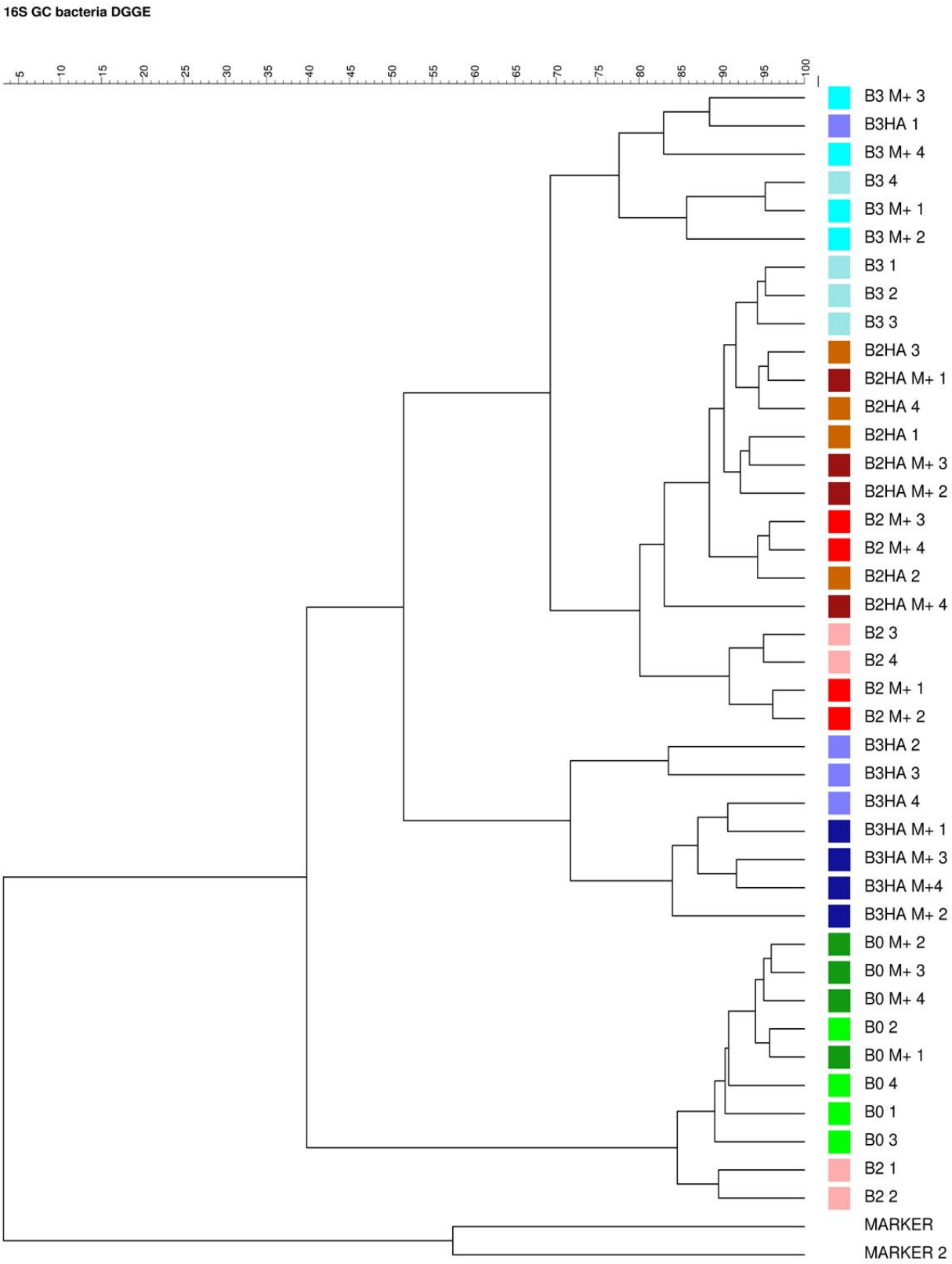

Figure 7. UPGMA Cluster analysis based on Pearson similarity matrix of total bacterial community (16S rRNA).

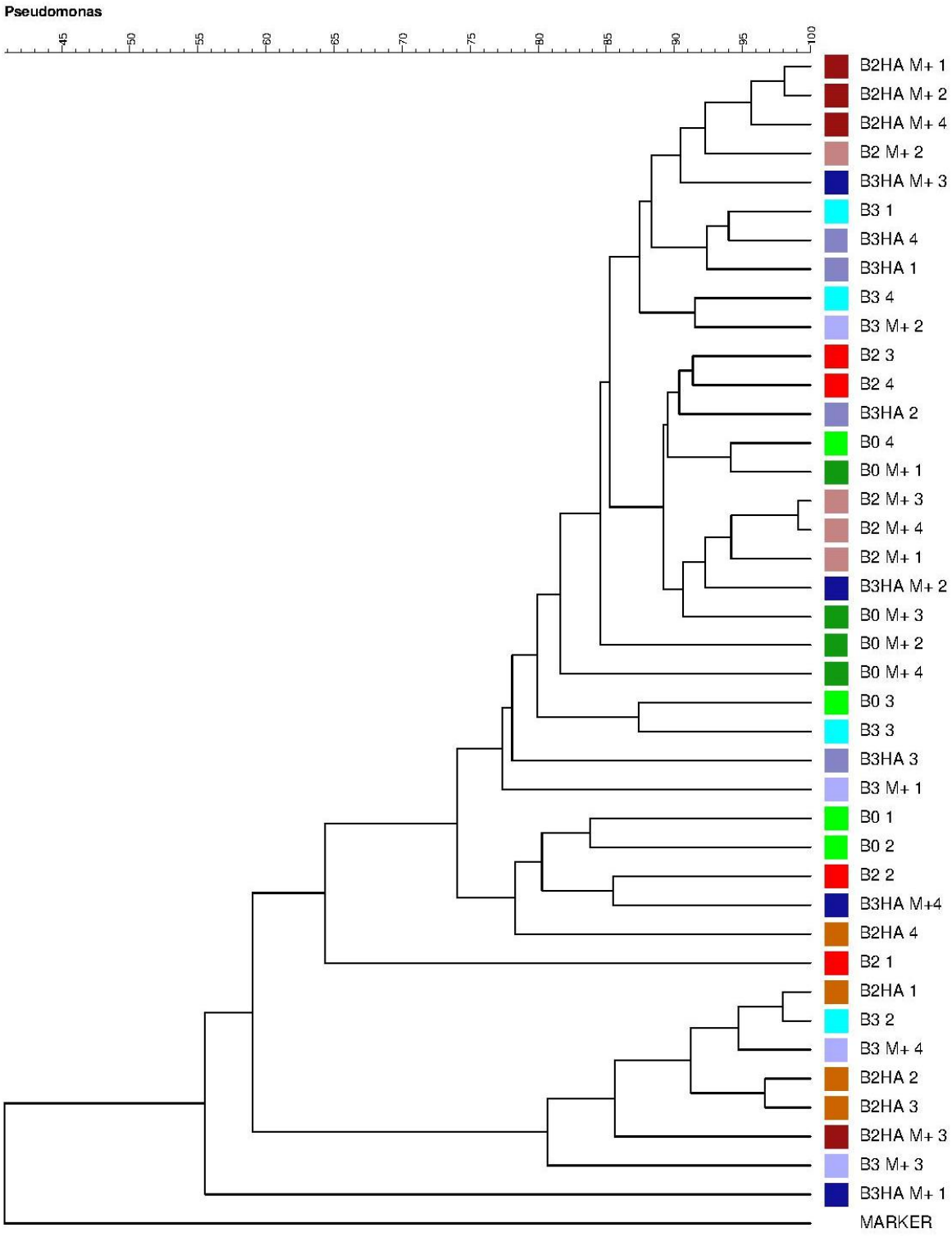

Figure 8. UPGMA Cluster analysis based on Pearson similarity matrix of *Pseudomonas* group-specific community.

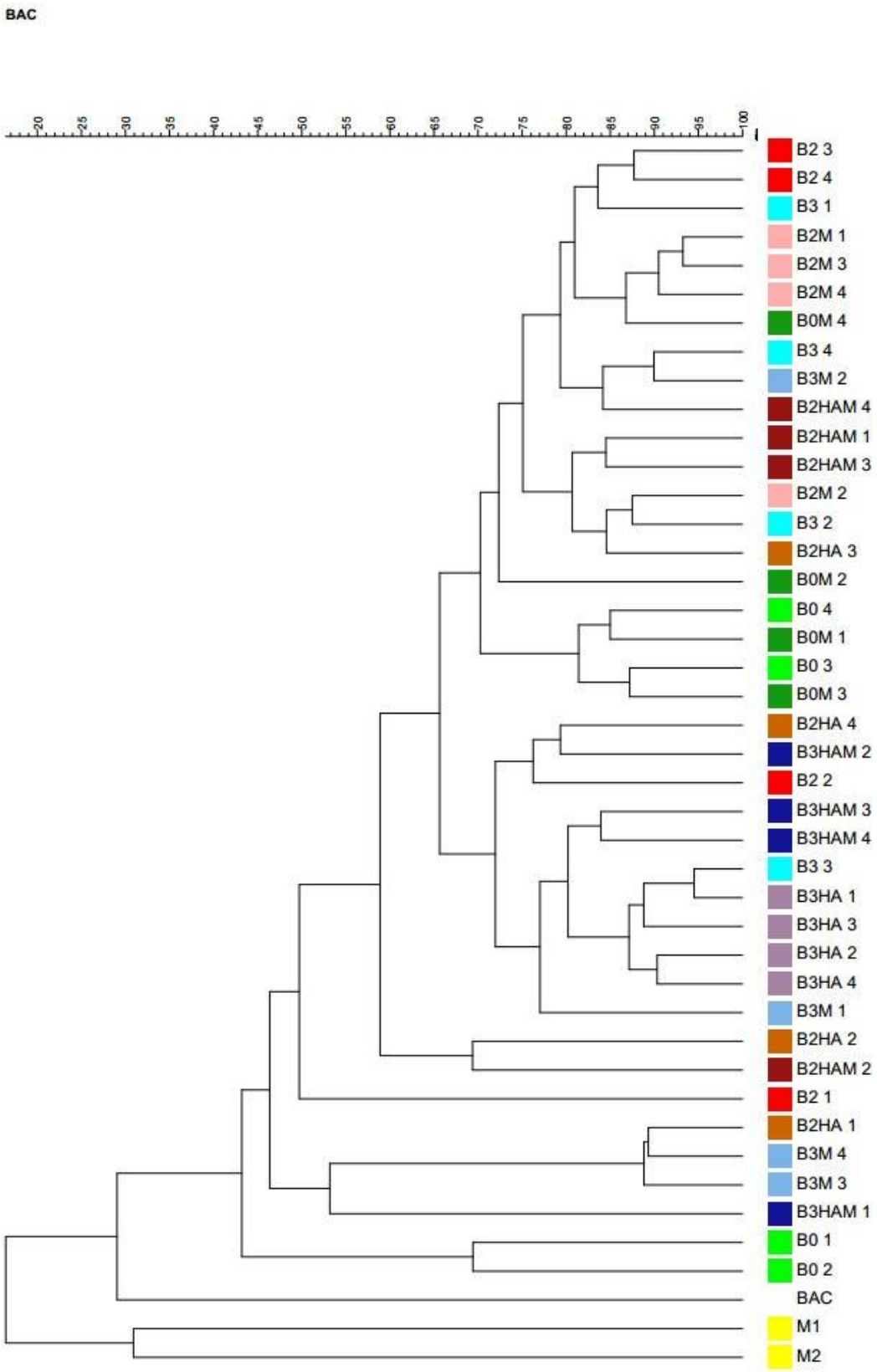

Figure 9. UPGMA Cluster analysis based on Pearson similarity matrix of *Bacillus* group-specific community.

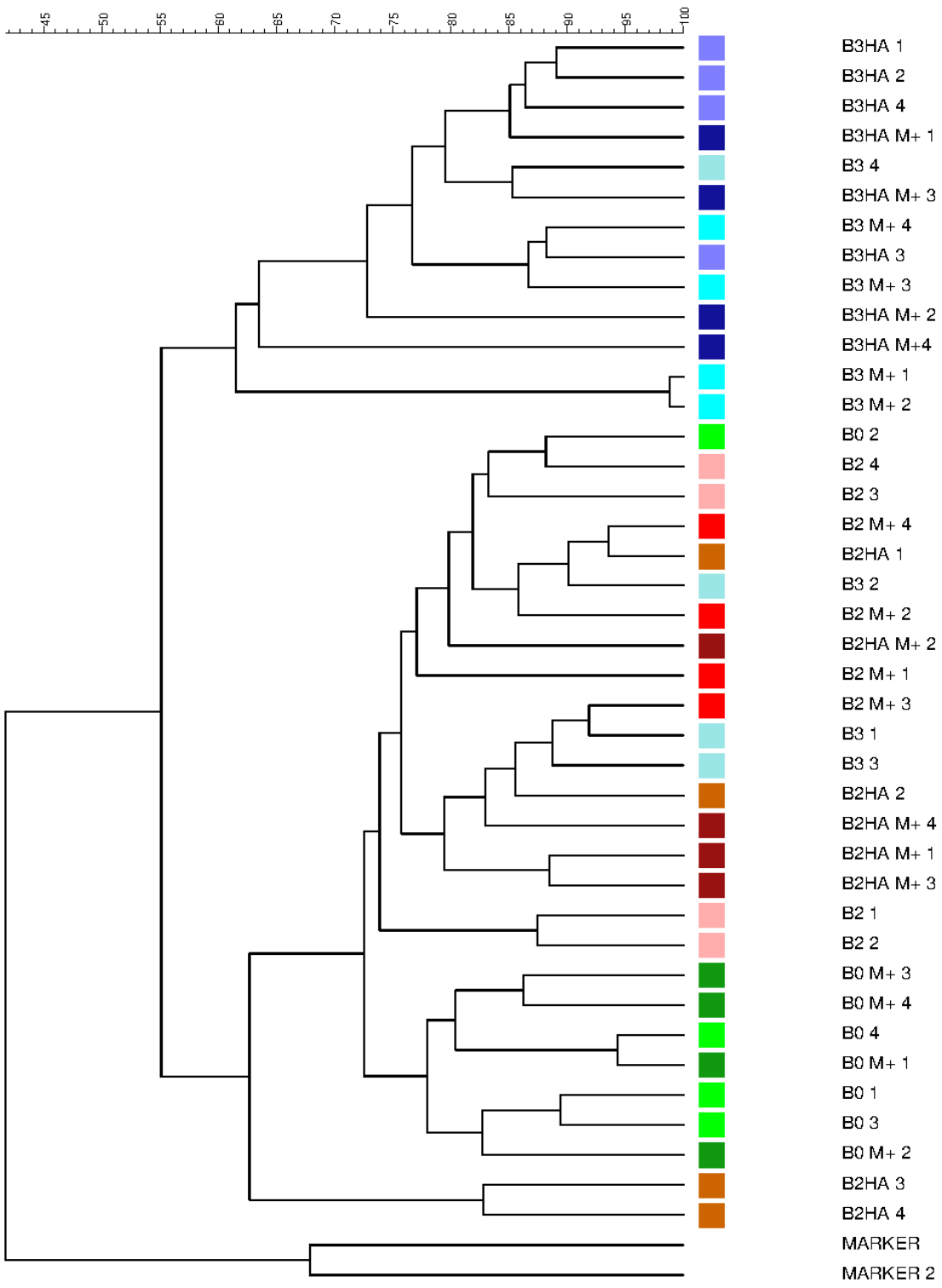

Figure 10. UPGMA Cluster analysis based on Pearson similarity matrix of fungal community.

Table 4. Treatment-dependent differences (d-values) of total bacterial, fungal and group specific communities in the rhizosphere of maize. Dissimilarity values = average within-group pairwise Pearson´s correlation – average between-group pairwise Pearson´s correlation.

Inoculated Treatments	Bacteria	Pseudomonas	Bacillus	Fungi
	dissimilarity values			
B2	9.34*	0.89	4.88	8.56*
B2M	46.19*	5.98*	22.28*	8.56*
B2HA	58.71*	6.41	3.07	18.73*
B2HAM	54.21*	14.16*	7.94	10.14*
B3	50.36*	6.97*	7.66	7.81*
B3M	47.125*	9.94	15.05	22.65*
B3HA	67.51*	4.8*	14.48*	18.72*
B3HAM	82.95*	2.27	9.98	22.88*
B0M	0.74	1.43	7.94	3.23

** Significant ($p \leq 0.05$) difference between inoculated treatment and control (not inoculated).*

4. Discussion

This study screened two different microorganisms in combination with humic acids and mycorrhizal inoculum, evaluating plants response such as growth and N and P uptake. Our results suggested an overall capacity of each bio-effector, when inoculated individually, to positively influence plant fitness, even though the best performances were brought by their combination. In fact, compared to control, the treatments that positively influenced plant performances were B2M, B2HA, B3HA, B3HAM.

Within plants inoculated with *Pseudomonas* spp., B2M yielded the greatest biomass and N content, whereas B2HA showed the largest P uptake. As far as microbial community is concerned, no major shift occurred. Similarity shown by cluster analysis (Fig. 7) was assessed around 75%, data confirmed by PLFA analysis, in which no major differences between microbial taxa in all B2 group treatments

were observed (Fig. 4, Fig.5), except for a decrease of fungal and actinomycetal PLFA markers in combination with mycorrhizal inoculation (Table 3, Fig. 4).

Several studies indicated that *Pseudomonas* spp. generally increase plant growth and favor root colonization by co-inoculation with AMF (Zaidi and Khan, 2005; Vyas and Gulati, 2009). Roesti et al. (2006), found that *Pseduomonas* spp. did not affect the root colonization by AMF and its establishment, whereas the co-inoculation improved plant yield and grain quality of wheat. Provided that general plant health still benefitted from co-inoculation, in B2HAM treatment we found a decrease of the 16:1ω5c PLFA biomarker of AMF, thus suggesting a possible competitive effect. Interestingly, as P content in plants treated with humic acids enhanced, this findings suggest that antagonistic activity could be attributable to a changed pattern in the availability of carbon source that favored co-metabolism processes in *Pseudomonas* population, in accordance with Haan (1974). This finally could explain both the moderate inoculation effect and the major solubilization of phosphorus observed in *Pseudomonas* treated plants.

Compared to *Pseudomonas* treatments, *B. amyloliquefaciens* alone or in combination with AMF resulted in a less effective stimulation of plant growth and N uptake, but raised significantly P content. However, B3HA and B3HAM showed the best performances even when compared to *Pseudomonas* treatments (B2).

Several studies pointed out that combination of *Bacillus* microbial strains, HA and AMF benefits plants health status. Adesemoye et al. (2009) found that mixture of *B. amyloliquefaciens* and AMF at 70% of fertilization rate produced the same yield as the full fertilization rate without inoculants. Furthermore, in a field trial was showed that interaction of *Bacillus* and AMF promoted maize growth, yield and nitrogen content (Adesemoye et al., 2008). Humic fertilizer in addition to *Bacillus* spp. has also been addressed as a powerful combination since increase in nutrients uptake and chlorophyll contents as well

as enhanced plant development has been evidenced (Bohme, 1997; Rekha et al., 2007; Pishchik et al., 2016).

In our study, the combination of *Bacillus*, AMF and HA shifted microbial community in favor of AMF with a decrease in the content of saprophytic fungi (Fig. 6). Whether AMF influenced bacteria or vice versa is still difficult to carry out. Singh and Kapoor (1998) recorded an increase in *Bacillus* population after AMF inoculation, while Yusran et al. (2010) reported that co-inoculation of *B. amyloliquefaciens* and AMF promoted mycorrhizal root colonization. Cell wall-degrading enzymes such as B-1,3 glucanase and chitinase are known to be involved in the activity of some P-solubilising microorganisms (Vassilev et al., 2006). Wang et al. (2002) isolated two extracellular chitinases from *B. amyloliquefaciens* that resulted active against fungi. This could explain the decrease in the population of saprophytic fungi observed in B3HAM. An enhanced plant growth and nutrient uptake could be also favored by the growth inhibition of a competitive component of soil microflora such as saprotrophic fungi (Cozzolino et al., 2016). Furthermore, Vassilev et al. (2006) stated that the production of lytic enzymes can be stimulated by the presence of organic substances rich in lignocellulose and the presence of compost as well as humic substances could have enhanced the microbial biological control that benefited plant health. On the other hand, the addition of humic acids can influence processes such as sporulation, production of external mycelium and mycorrhizal colonization (Gryndler et al. 2005; Yan, 2009; Nobre et al., 2013; Sensoy et al., 2013). As suggested by Canellas et al. (2013), humic substances could increase the surface area available for attachment and establishment of endophytic microorganisms by stimulating plasma membrane H+ -ATPase that, in turns, generates a proton force that initiates cell- wall loosening and extension growth (Hager, 2003). Furthermore, the increased efflux of organic acids exudates into the rhizosphere (Nardi et al., 2009) could attract microbial population to the roots, as they provide carbon source, and, at the same time, trigger the association and colonization of lateral roots (Canellas et al., 2008; Puglisi et al., 2008).

5. Conclusion

This study revealed that the mixed combination of PGPR, AMF and humic acids improved plant growth and nutrient uptake in comparison to not inoculated or single inoculated plants. As a result of co-inoculated bioeffectors, modifications of the microbial communities composition were found. The key role of AM colonization could be successful increased by the association with selected rhizospheric bacteria and by the addition of organic matter such as humic substances. However, it is difficult to predict the outcome of interactions, therefore their successful application need further to be validated under different condition.

RESEARCH IN PROGRESS I

Effects on P availability to plants and soil microbial community composition in a greenhouse treatments with organic and inorganic P sources and bioinoculants.

Abstract

The need for efficient use of phosphorus (P) in agriculture has been recently questioned because of concerns about the finite amount of P fertilizer resources. Microbes play a key role in soil P dynamics, uptake, solubilization and mineralization. Therefore a better understanding of the relationship between type of P amendment, microbial activity and changes in soil P availability is important to better manage soil P. The effects of the application of two bio-effectors strains (*Trichoderma harzianum* T-22 , B1and *Bacillus amiloliquefaciens*, B3) alone (B1P0, B3P0) and in combination with inorganic (triple superphosphate, P1 and rock phosphate, P2) or organic fertilizer (composted cow manure, P3, and composted horse manure, P4) on growth, N and P uptake of maize (*Zea mays* L.), were investigated in a greenhouse experiment. Available P and indigenous microbial community structure in soil and the arbuscular mycorrhizal colonization of maize were examined. Organic and inorganic fertilization increased plant growth, P and N uptake. The application of B1and B3 increased plant growth, P and N uptake. The magnitude of this effect varied among different fertilizing treatments and between the two bio-effectors strains. In comparison to P0 treatment, application of P fertilizers increased the total relative abundance of microorganisms as indicated by PLFA profiles, but not that of treatments inoculated with microbial strains. In all treatments, the application of *B. amiloliquefaciens* resulted in

the increase of the arbuscular mycorrhizal colonization of maize, the arbuscular mycorrhizal NLFA marker 18:1ω5c, and the AMF/saprotrophic fungi ratio. We conclude that application of PGPR can promote P supply of crops in moderate soil P level, in combination with organic and inorganic P fertilization. Interactive effects of applied bacterial strains and organic fertilization depend on the sort of organic fertilizer and microbial strain used.

Introduction

Phosphorus (P) deficiency is a constraint to plant growth worldwide (Cordell et al., 2009). Phosphorus availability to crops can be increased by application of organic or inorganic amendments such as manures, composts, crop residues, rock phosphates or various manufacture inorganic P fertilizers (Ayaga et al., 2006). However, about 85% of added inorganic P become unavailable to plants in the year of application due to absorption and precipitation with Fe, Al and Ca in the soil (Brady and Weil, 2008). On the other hand, the effect of the organic amendments on soil P availability depends on their decomposability and P concentrations (Reddy et al., 2005). Soil microorganisms play a fundamental role in the biogeochemical cycling of inorganic P and organic P in the rhizosphere (Marschner et al. 2003; Richardson and Simpson, 2011), in transformation of P by uptake into and release from the microbial biomass, and also the solubilization of inorganic P and formation and mineralization of organic P (Malik et al., 2012). Plants can also interact with other soil microorganisms in the rhizosphere, e.g., arbuscular mycorrhizal fungi (AMF) (Toljander et al., 2007). Arbuscular mycorrhizal fungi contribute essentially to the mobilization and transport of P in soils (Jeffries et al., 2003; Read and Perez-Moreno, 2003). Their hyphae increase the absorptive surface area of the host-plant root systems (Johansson et al., 2004; Cozzolino et al., 2013). Additive effects between AMF and plant growth–promoting rhizobacteria (PGPR) were observed, e.g., after application of *Pseudomonas* spp.

(Gamalero et al., 2004) and *Bacillus circulans* (Singh and Kapoor, 1999). Several reports have demonstrated that the interaction of AMF and *Trichoderma* spp. may be beneficial for both plant growth and plant disease control (Barea et al., 1997; Saldajeno et al., 2008; Martínez-Medina et al., 2009). For example, it has been reported that some *Trichoderma* strains may influence AMF activity (Calvet et al., 1993; Brimner and Boland, 2003; Martinez et al., 2004; Martínez-Medina et al., 2009). A synergistic effect of some saprophytic fungi on AMF spore germination and colonization has been confirmed (Calvet et al., 1993; McAllister et al., 1996; Fracchia et al., 1998).

Understanding the plant-microbial interactions is considered an opportunity for manipulating specific microorganisms and therefore enhancing P availability in soil (Richardson and Simpson, 2011). Agricultural management practices can have large impacts on the size and activity of soil microbial communities (Bolton et al., 1985; Kirchner et al., 1993). Furthermore, the effects of a PGPR application on P mobilization and plant P supply are scarcely predictable, evoked by the great importance of environmental factors. The nutrient status of soil, fertilizer type, are among these factors (Richardson et al., 2009; Sørensen et al., 2001). Zheng et al. (2009) found that both the bacterial and fungal populations were significantly higher under balanced fertilization than under nutrient-deficiency fertilization. Generally, organic fertilization can increase the soil microbial activity (Zaller and Köpke, 2004; Bünemann et al., 2006). On the other hand, as organic fertilization usually implies an improved soil nutritional status and higher competition by the autochthonous microflora, a smaller effect of applied bioeffectors could be expected. Phospholipid fatty acid (PLFA) analysis is a biochemical method being considered to be highly sensitive indicators of the most active microbial community, and has been used to detect changes in the soil microbial community resulting from various disturbances (Vestal and White, 1989; Liu et al., 2012; He et al., 2013; Cozzolino et al., 2016). Several previous studies have reported that chemical fertilizer applications increased microbial biomass and activity without significantly changing bacterial community structure (Chu et al., 2007; Islam et al., 2011),

while others showed that chemical fertilizers decreased arbuscular mycorrhizal fungal (AMF) diversity (Islam et al., 2011; Lin et al., 2012).

The aim of the present work was to evaluate the effects of organic and inorganic P fertilizations and of two bioeffectors inoculation 1. to promote growth, N and P uptake of maize plants; 2. to influence mycorrhiza formation of maize and soil microbial community composition by examining the PLFA and NLFA profiles; 3. to support soil P mobilization.

Materials and methods

2.1 Experimental design

A pot experiment was conducted in a completely randomized design with 15 treatments in 5 replicates. Maize plants (Zea mays, cv Colisee, KWS) were grown under 5 different P-fertilization treatments. 1. P0, untreated control, 2. P1, triple superphosphate, 3. P2, rock phosphate, 4. P3, composted cow manure, 5. P4, composted horse-cow manure. Moreover, three microbial bioeffectors treatments were used. 1. B0, control without bioeffector application; 2. B1, *Trichoderma harzianum* strain T-22 (Trianum® Koppert B.V. The Netherlands), 3. B3, *Bacillus amyiloliquefaciens* (Rhizovital FZB42® ABiTEP GmbH, Berlin, Germany). The microbial strain have been previously selected on the basis of their P-mobilization efficacy in vitro (EU-project "Biofectors").

2.2 Soil preparation and plant growth

The surface layer (5-20 cm) of a clay-loam soil (Vertic Xerofluvent) was collected at Castel Volturno (CE) Experimental Station, Department of Agriculture, University of Naples. The main soil physical

and chemical characteristics are given in Table 1, indicating a suboptimal content of double lactate–soluble P, that is assumed to represent the amount of plant-available P. The soil was sieved to 5 mm, mixed with quartz sand at the ratio of 2:1 (w/w) and thoroughly homogenized. The pot experiment was conducted using this soil/sand substrate. The substrate was incubated in covered plastic boxes at 20 ± 2°C during 30 days prior to planting. Maize plants were grown in pots (3 L) filled with 2.5 kg of substrate. The P fertilizers were applied at the rate of 50 mg Pkg^{-1} dry substrate. Moreover, P3 and P4 were applied 15 days before sowing. A basal nutrients supply was performed adding nitrogen (N) as Ca(NO3)2 at the rate of 100 mg N kg^{-1} dry substrate and potassium (K) as Kalimagnesia (30% K2O + 10% MgO) at the rate of 166 mg K kg^{-1} dry substrate. The fertilizers were added in powder form to the substrate (soil/sand mix) of each individual pot and mixed thoroughly. Maize seeds were sown prior microbial inoculation, three seeds per pot. The different microbial inocula were applied as a suspension of demineralized water with 2.5 mM CaSO4 spraying it on seed surface at sowing, at the rate of 2.5x104 spores and 2x106CFU g^{-1} dry substrate of B1 and B3 respectively. The trial was carried out from May to July 2013 under open greenhouse conditions and after emergence (5 days after sowing), only one maize plant per pot was then left to grow. During crop growth, soil water content was maintained at 60 % of field capacity by periodically adding water to compensate for evapotranspiration losses. All plants were harvested after 8 weeks of growth. At harvest shoots were cut, oven-dried at 65°C for 48 h, weighed, and milled. The soil adhering to root segments was considered as the rhizosphere soil and separated by brushing after a gentle shake. The soil falling from roots as well as the rest of soil was regarded as the bulk soil. Soil samples were divided in two subsamples: one subsample of rhizosphere soil was sieved at 2 mm and stored at −20 °C for PLFA–NLFA analyses, while the other subsample of bulk soil was air-dried and sieved at 2 mm at room temperature for physical–chemical analyses.

Table 1. Characteristics of the soil used for the pot experiment.

Soil type	Texture			pH	Organic C	Available P	N total	Carbonate
	Sand	Silt	Clay	($CaCl_2$)				
	(%)	(%)	(%)		(%)	(mg P kg^{-1})	%	%
	19,0	44,5	36,5	7.3	1.34	12	0.114	17

2.3 Plant and soil analyses

Total P in maize shoots was measured after dry ashing using the molybdenum blue assay method (Murphy and Riley, 1962). Nitrogen was determined by an elemental analyzer Fisons EA 1108 (Fisons Instruments, Milano, Italy). Shoot-biomass P and N uptake per pot was calculated by multiplying shoot biomass (g) with shoot P and N concentration (mg g^{-1}), respectively. Available soil P was extracted with sodium bicarbonate (Olsen method) and then determined by the molybdenum-blue method (Murphy and Riley, 1962).

2.4 Mycorrhizal colonization

Estimation of root mycorrhizal colonization was assessed by a modification of the Phillips and Hayman method (1970). Maize roots were washed with tap water, cleared in 10 % KOH for 10 min at 90 °C in a water bath, rinsed in water, and then soaked in 2 M hydrochloric acid for few minutes. After soaking, the roots were stained in a lactoglycerol solution containing 0.01 % acid fuchsin for 5 min at 90 °C in a water bath. The roots were then rinsed in water and stored in a solution of lactic acid until evaluation of root colonization. Mycorrhizal colonization was calculated by the gridline intersect method (Giovannetti and Mosse, 1980).

2.5 PLFA and NLFA analyses

Selected fatty acids pertaining to the soil phospholipid (PLFA) and neutral lipid (NLFA) fractions, and used as biomarkers for specific soil microbial communities, were extracted using the modified Bligh and Dyer technique (1959), as described by Bardgett et al. (1996). Total soil lipids were extracted from 2 g of soil by a chloroform/methanol (MeOH)/citrate buffer (1:2:0.8 v/v). Separation of lipid classes was conducted in silica gel columns. Neutral, glyco-, and phospholipid fractions were obtained by sequentially eluting silica gel columns with chloroform, acetone, and methanol, respectively. While the glycolipid fraction was discarded, neutral and phospholipid fractions were dried under a N_2 flow at 37 °C and stored at −20 °C. Fatty acid methyl esters were formed by a mild alkaline methanolysis. Thirty microliters of methyl nonadecanoate fatty acid (19:0; Sigma-Aldrich) were added as an internal standard, and the methylated samples were dried under a N_2 flow. Samples were dissolved in 200 µl of hexane for analysis by a Perkin-Elmer Autosystem XL (GC) equipped with a PE Turbomass-Gold quadrupole mass spectrometer. Chromatographic separation was achieved through a 60-m Supelco Capillary column (SLB-5 ms) under helium as carrier gas (1 ml min−1). Samples (2.5 µl) were injected in splitless mode with the injector held at 250 °C. The initial oven temperature, 100 °C, was held for 5 min, raised to 210 °C at a rate of 2 °Cmin−1, then raised from 210 to 250 °C at a rate of 5 °C min−1, and held for 12 min. Mass spectra were obtained in EI mode (70 eV), scanning in the range of m/z 50–600, with a cycle time of 1 s. The abundance of individual PLFA was derived from the relative area under each chromatographic peak, as compared to that of internal standard (19:0) and related to the calibration curve of the 19:0 standard fatty acid dissolved in hexane. Each PLFA content was expressed as nmol of PLFA per gram of dry soil.

Fatty acids were named according to the ω-designation described as follows: total number of carbons followed by a colon; the number of double bonds; the symbol ω; the position of the first double bond

from the methyl end of the molecule. Cis- and trans-configurations are indicated by c and t, respectively; iso and anteiso forms of methyl-branched fatty acids are indicated by i- and a-, respectively. 10 Me indicates a methyl group placed on the tenth C atom from the carboxyl end of the molecule; cy refers to cyclopropane fatty acids.

The C18:2ω6c and C18:1ω9c PLFA were used as biomarkers for fungal biomass (Frostegard and Baath, 1996). The aC15:0, iC15:0, iC16:0, iC17:0, and aC17:0 PLFA were chosen to represent Gram(+) bacteria (Sundh et al., 1997), while the C16:1ω7c, C18:1ω7c, C18:1ω5c, cyC17:0, and cyC19:0 PLFA were related to Gram(−) bacteria (Sundh et al., 1997), and the 10MeC16:0, 10MeC17:0, and 10MeC18:0PLFA to actinomycetes. As C16:1ω5 PLFA is entirely specific not only for AMF but also for some Gram(−) bacteria, both C16:1ω5 NLFA and PLFA were used as indexes for AMF. Although the C16:1ω5 NLFA is specific, it indicates storage material rather than the biomass characteristics suggested by the PLFA biomarkers. Thus, the use of C16:1ω5 for the NLFA/PLFA ratio is a suitable measurement of different communities, with its large level (>1) reflecting the prevalence of AMF contribution (Olsson, 1999). Total PLFA concentration was the sum of single identified PLFA. The PLFA ratios relative to fungal to bacterial, Gram(+) to Gram(−) bacteria, and AMF to saprotrophic fungi were calculated by using the sum of the respective fatty acid biomarkers and were assumed to represent the relative abundance of these groups.

2.6 Statistical analyses

The significant difference between mean values was determined by the one-way analysis of variance, while application of Tukey's test to differentiate among results was given at the $P<0.05$ probability level by using the Statgrafics Centurion software version XV. Data were checked for normality and

homogeneity of variance and transformed when necessary. XLSTAT software (Addinsoft, v. 2014) was adopted to elaborate multivariate principal component analysis (PCA).

Results

3.1 Plant growth and nutrients uptake

P fertilization enhanced the growth, P and N uptake of maize (Fig. 1). In B0 treatment, the triple superphosphate application (P1) increased growth of maize up to 45.8% and P and N uptake up to 49% and 15%, respectively, compared to the unfertilized control. Likewise, P2 application increased maize yield (37%) and maize P uptake (44%) when compared to control. The yield increase upon organic fertilization was small (19%) after P3 application, and insignificant after P4 application. The increase in P uptake was 37% and 19% for P3 and P4 treatments respectively, whereas N uptake was significantly smaller.

The effect of bioeffectors application on maize growth without P fertilization was smaller than for the P fertilization (Fig. 1-A). The application of B1 and B3 strains had either no significant effects or even negative (B3) on plant growth in P0 and P2 treatments, but increased plant growth in P1 and compost (P3 and P4) fertilization (Fig. 1-A). An increased P and N uptake were observed after B1 application, and, in particular, after B3 treatment under compost fertilization (Fig. 1-B,C). In fact, B3 inoculation, increased P and N uptake by about 40 and 63%, respectively. However, there was no significant response to the application of both B1 and B3 strains in P uptake for either P1 or P2 treatments. The P and N uptake of maize was even decreased after B1 application in combination with P1 and P2 fertilizers, and after B3 application in the P unfertilized experiment (Fig. 1-B,C).

3.2 Mycorrhizal colonization

Both P fertilization and bioeffector applications slightly but significantly affected the mycorrhizal colonization of maize (Fig. 1-D). For B0 control and B3 treatments, a greater mycorrhizal colonization was found in combination with P1 and P2 fertilization. The largest effect was found for the P2 treatment, whereby B3 application increased mycorrhizal colonization by 16% more than B0 (Figure 1-D). The differences were significant between control and composted cow manure under B3 application (Fig. 1-D). Compared to control without bioeffector, B1 application in combination with P1 caused a smaller mycorrhizal colonization, that was not observed for the P0, P2 and P3 treatments (Fig. 1-D).

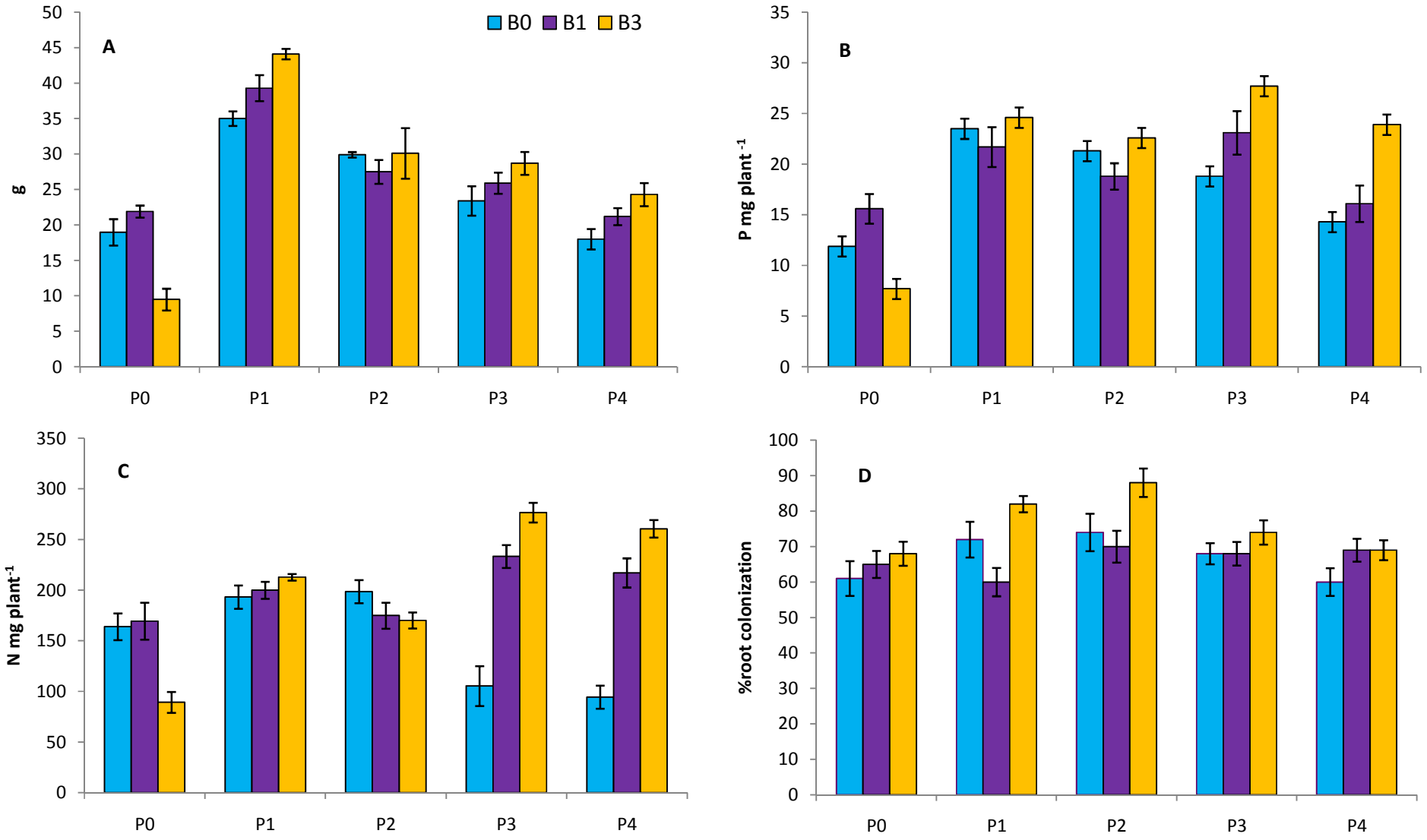

Figure 1. Total maize biomass (A), content of P in leaves (B), content of N in leaves (C), and percentage of root colonization by AMF (D) under different bioeffectors inoculation and P fertilizer treatments.

3.3 Microbial community composition

A total of 25 PLFA were identified in the different soil treatments. PLFA profiles were dominated by cy19:0, 18:1ω9, 18:1 ω7, 18:2ω6,9 and a15:0 fatty acids, which together accounted for approximately 40% of total PLFA for all treatments. The total PLFA biomass is an indicator of the total microbial biomass, thereby assuming that changes in PLFA profiles indicate shifts in soil microbial communities. Different P fertilizers and bioeffectors treatments significantly changed the total relative abundance of selected PLFA (Table 2, Fig. 2). Application of composted cow manure (P3) caused the relative greatest total microbial biomass for each inoculation treatment, except for B3 (Table 2; Fig.2). Compared to control (P0), application of P fertilizers generally increased soil microbial communities, although the total relative abundance of PLFA showed no significant difference after P4 application, and decreased notably in B1P1, B1P2 experiments (Table 2, Fig. 2).

However, the amounts of total PLFA, bacterial, Gram(+), Gram (-) and fungal PLFA were found to be greatest in the B0P3 treatment, followed by the B0P2 and B3P1 treatments, whereas it was the least in the B1P1 treatment (Table 2, Fig. 2). The amount of actinomycetal PLFA was greatest in the B1P3 (Table 2). On the other hand, inoculation with B3 increased AMF fungal growth in terms of spores and propagules, as indicated by the general increase of the C16:1ω5 NLFA in all fertilizer treatments (Table 2). However, the largest values for this NLFA indicator of AMF were found with P1 and P2 fertilizer.

Principal components analysis (PCA) of the selected soil biomarker PLFAs and NLFA showed the changes in microbial composition in soils under different P fertilizer and bioeffectors treatments (Fig. 3). The first two principal components (PC1 and PC2) accounted for 81.3 % of total variation and separated the variables both in terms of P fertilizers and bioeffectors treatments. All PLFA markers were positively aligned along the PC1 with loading values for PC1 larger than 1. The PC1 explained 74.9 % of variance and well differentiated the B0 and B3 treatments under P1, P2, P3 fertilization from

all the B1 treatments, except for B1P3, while their placement in the positive region suggested an increase of all PLFA markers in the B0 and B3 treatments.

The positive loadings for PC2 was associated with the monounsaturated fatty acids (16:1ω7c, 18:1ω9c, 18:1ω7c, 18:1ω5c), the cyclopropane fatty acids (cy17:0), as well as the fatty acids with methyl groups (10Me17:0, 10Me18:0) and contrasted with the iso- and anteiso-branched saturated fatty acids (i14:0, a14:0, i 15:0, i16:0) placed on the negative coordinate axis (Fig. 3). Furthermore, PC2 permitted to separate B3 treatment under P1, P2, P3 fertilizers from that of the B0 treatment, based on the relatively larger amount of NLFA 16:1ω5c and Gram(+) markers for the B3 treatments.

The ratios of specific fatty acids rather than single fatty acids were capable to reveal differences in the composition of soil microbial communities due to bioeffectors inoculation and fertilizer application . The Gram(+)/Gram(−) PLFA ratio was the largest for B1 treatments, whilst it was the smallest for B0 (Fig. 4, A). This ratio increased with B3 inoculation under P1 and P3 fertilizers, where fungal/bacteria ratio decreased (Fig. 4, A,B). This trend is also highlighted in Figure 5, showing the increase of bacterial PLFA, in particular of Gram(+), passing from 23% in P0 treatment to 30 and 32% in P1 and P3 treatments, respectively, and the concomitant decrease of fungal and actinomycetal PLFAs, passing from 26 and 11% in P0 to 16 and 6% in P3, respectively**.** Furthermore, B3 inoculation determined the greatest values of AMF over saprotrophic fungal biomass, as shown by the C16:1ω5/(C18:2ω6,9+C18:1ω9) ratio (Fig. 4C).

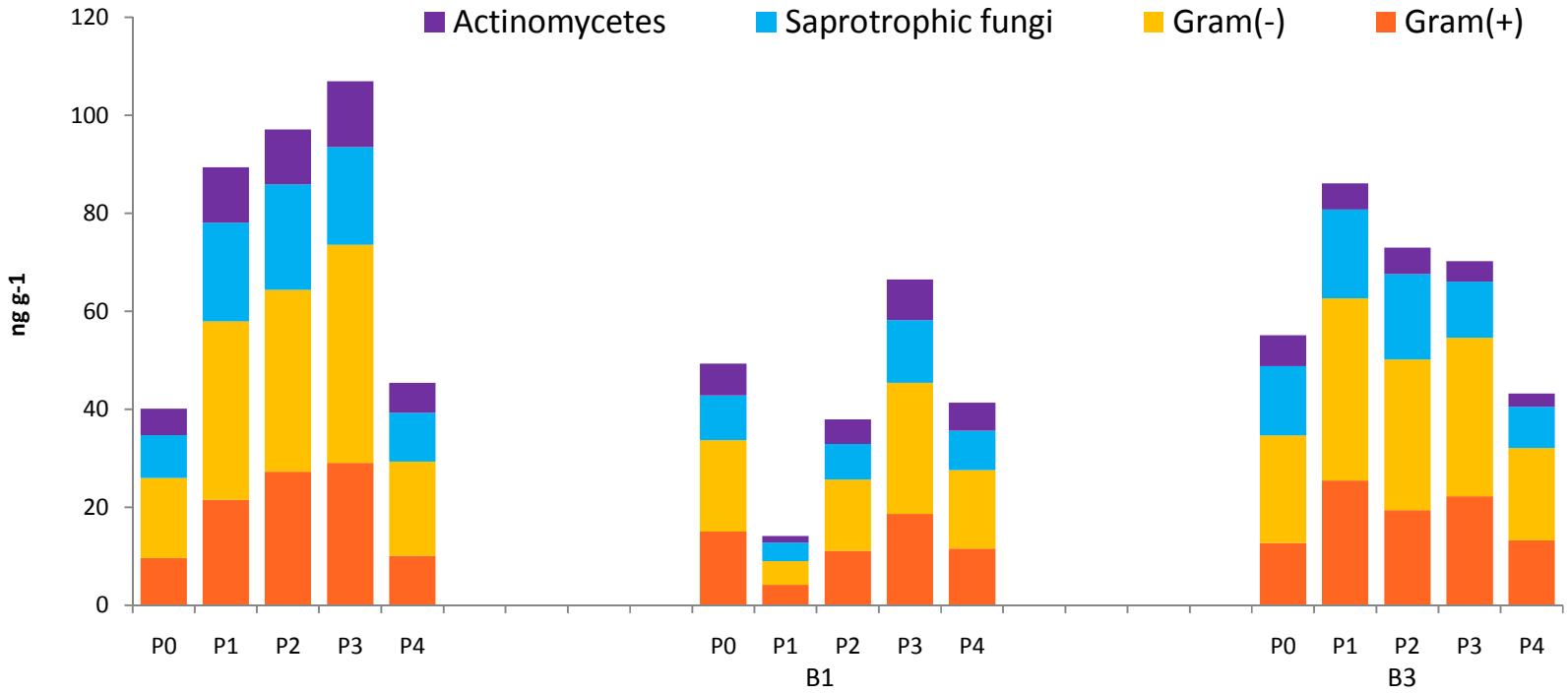

Figure 2. The sum of selected phospholipid fatty acids (PLFAs) in soils under three inoculation and five phosphate fertilization treatments.

Table 2. Abundance of actinomycetes, bacterial, fungal, Gram(+), Gram(-), total PLFA as well as NLFA 18:1ω5c.

	Fungal PLFA	Bacterial PLFA	Bacterial Gram(+)	Bacterial Gram(-)	Actinomycetes	Total PLFA	NLFA 18:1ω5c
Bioeffectors				ng g^{-1}			
B0							
P0	8.7±0.25	26.0±1.15	9.7±0.5	21.5±1.1	5.4±0.4	42.3±2.2	6,93±1.9
P1	20.15±1.7	56.9±2.7	21.5±1.1	36.4±1.8	11.4±1.1	93.4±5.4	29,9±5.6
P2	21.5±1.4	64.3±3.1	27.2±1.9	37.1±1.4	11.2±0.8	103.7±4.8	28,8±2.3
P3	19.96±1.4	73.5±6	29.1±1.2	44.5±5.4	13.4±1.4	113.5±1.4	17,6±3.8
P4	9.1±0.7	33.7±2.6	15.03±1.9	18.7±1.1	6.5±0.3	47.5±3.7	4,83±0.5
B1							
P0	9.6±0.7	33.7±2.6	15.03±1.9	18.7±1.1	6.5±0.3	51.9±3.5	13,56±2.5
P1	3.7±0.2	9.04±0.9	4.2±0.3	4.8±0.6	4.3±0.3	14.8±1.13	5,03±0.2
P2	7.3±0.8	25.7±2.3	11.2±1.0	14.5±1.4	14.5±1.4	40.6±3.3	17,91±2.8
P3	12.8±1.1	45.4±4	18.7±1.2	26.7±2.0	26.7±2.0	71.3±4.5	18,34±2.8
P4	8.02±0.5	31.9±8.3	11.6±1.0	20.3±2.9	20.3±2.9	48±8.7	7,07±1.5
B3							
P0	14.2±1.4	34.7±3.8	12.7±1.7	22.0±2.2	6.2±0.8	57.9±5.7	14,30±3.2
P1	18.1±1.7	62.6±7.7	25.5±2.7	37.2±5.1	5.3±0.6	91.2±1.34	52,40±6.0
P2	17.5±2.1	50.2±4.1	19.4±2.9	30.8±2.2	5.3±0.8	77.8±4.2	56,10±5.5
P3	11.5±1.25	54.6±3.4	22.2±1.	32.4±1.9	4.2±0.4	76.1±5.4	38,54±5.3
P4	8.4±0.8	32.1±3.2	13.2±1.7	18.9±1.6	2.7±0.12	45.8±4.2	18,28±3.6

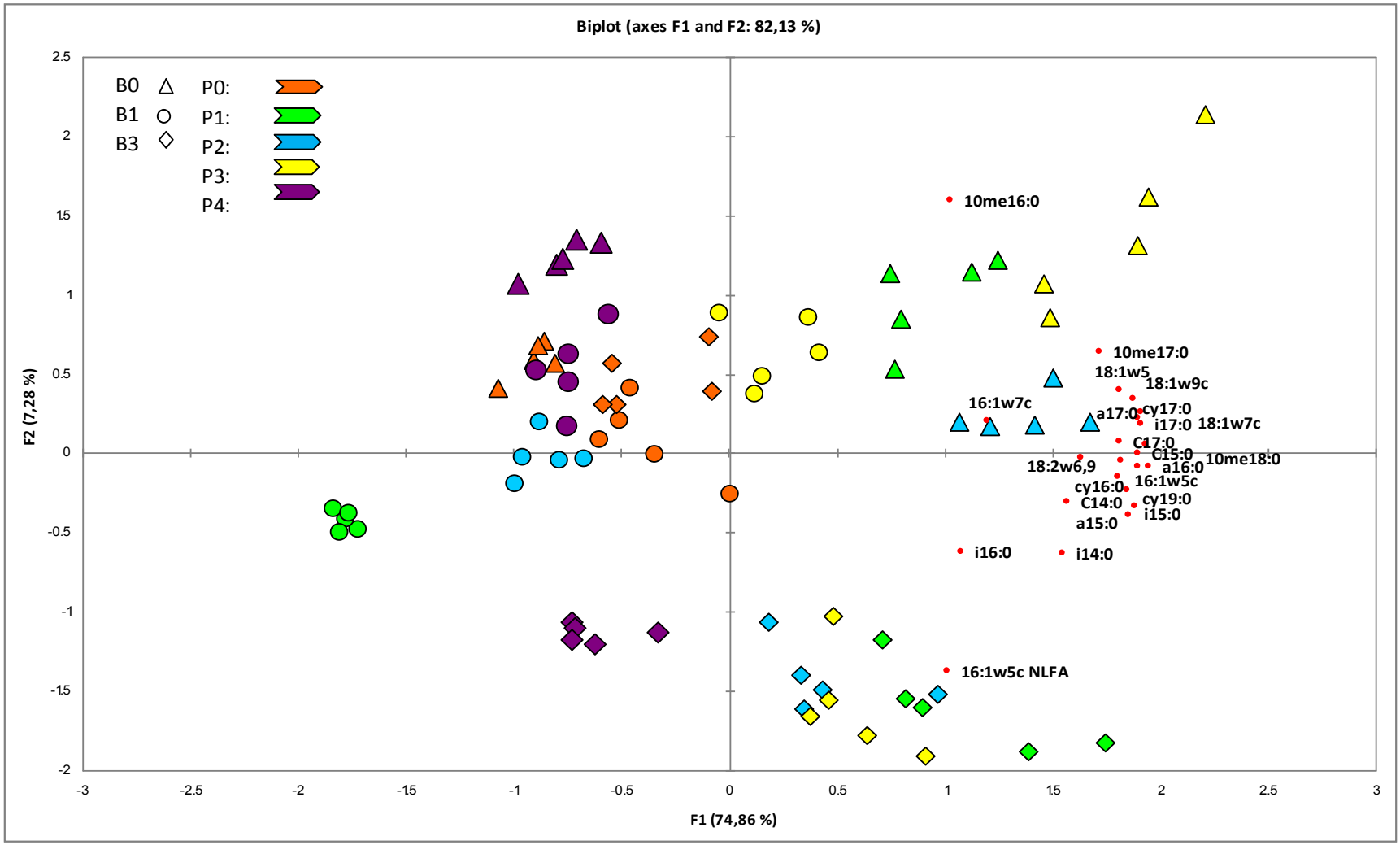

Figure 3. Principal component analysis of the PLFA patterns from soils under different bioeffectors inoculation (B0, B1, B3) and P fertilizers (P0, P1, P2, P3, P4). Factors 1 and 2 accounted for 74.86 % and 7.28 %, respectively, of the variance.

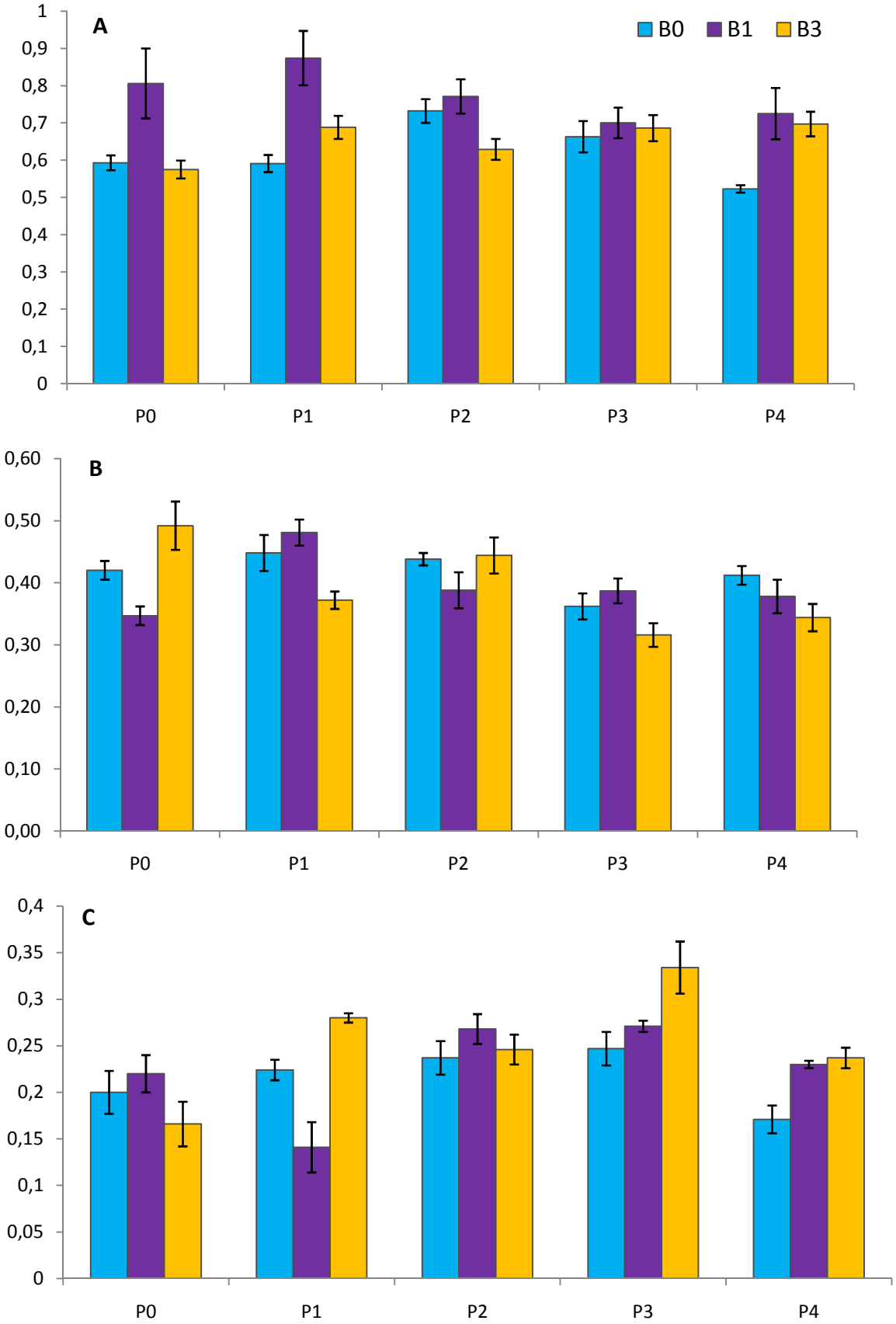

Figure 4. PLFA ratios in soils under different treatments : (A) Gram-positive (Gram+)/Gram-negative (Gram−); (B) fungal/bacteria; (C) AMF/saprotrophic fungi.

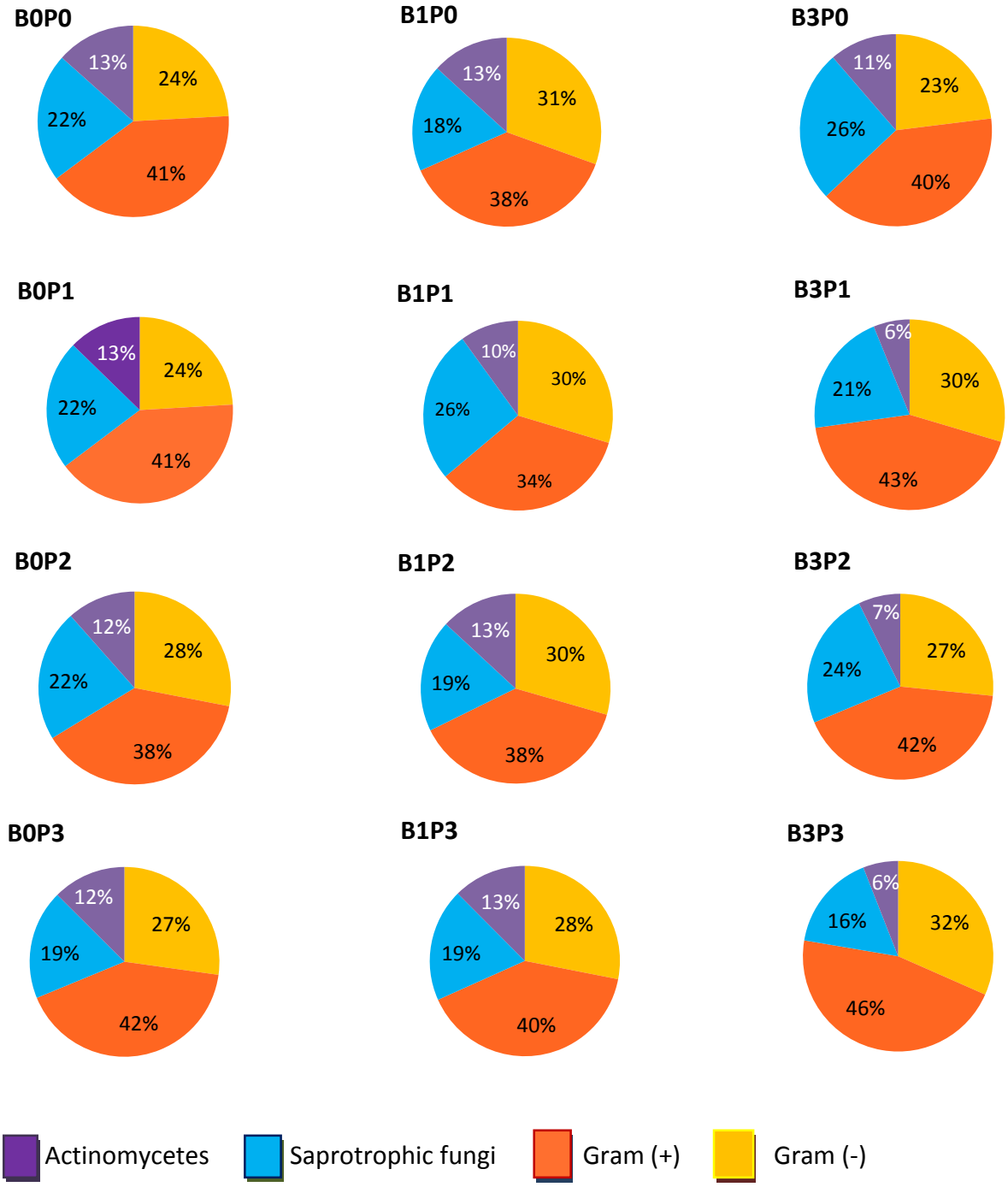

Figure 5. Percentage of main soil microbial groups, under different bioeffectors inoculation and P fertilizers treatments.

RESEARCH IN PROGRESS II

The effect of *Pseudomonas* spp. treatment on maize (*Zea mays L.*) growth, yield and phosphorus uptake in field conditions under mineral and low-input organic fertilizers.

Introduction

Although strong evidence supports the functional significance in plant growth promotion of phosphate solubilising microorganisms in laboratory experiments and knowledge on specific mechanisms of various BEs has accumulated, unknown factors often limit the reproducibility of effects under variable field conditions. This is of particular importance when crops are subjected to several different environmental conditions, with constraints from poor soils, water deficits, nutrient deficiencies, non-favourable temperatures and other stresses (Neumann and Römheld, 2002; Drinkwater and Snapp, 2007).

Development of innovative plant nutrition strategies to improve plant growth and nutrient acquisition in organic and low-input systems, must be considered in order to decrease the mineral input and enhance the nutrient use efficiency of organic and sparingly available mineral fertilizers.

To ensure strong practical relevance in the field, a major emphasis lies on prevailing issues in alternative fertilization strategies. By combining recycling mineral nutrients contained in organic and industrial recycling products such as compost and phosphate solubilizing microorganisms, insufficient availability of mineral nutrients in low input and organic farming could be overcome. However, field experiments represent the final step in order to

verify the application of new technologies. Here, we briefly exanimate preliminary results obtained in a field experiment to assess the potential efficacy of a phosphate solubilizing bacteria, *Pseudomonas* Proradix® spp. in combination with organic amendments based on cow/buffalo manure and evaluate the differences compared to mineral fertilizers (triple super phosphate, TSP). The main objective of this study is to verify the effectiveness and sustainability of innovative BE-based plant nutrition strategies for the supply of mineral nutrients to crop plants in comparison to current standards of agricultural practice and with respect to their agronomic viability and sustainability.

Materials and methods

2.1 Soil and compost

The soil used in the experiment was a clay loam (446, 280 and 274 g kg^{-1} respectively sand, silt and clay), alkaline (pH 8.6, 1:2.5 soil:water) and classified as a Vertic Xerofluvent, containing 1.11 g kg^{-1} total N, 10.5 g kg^{-1} organic carbon, 11 mg kg^{-1} of $NaHCO_3$-extractable P. The composting process was conducted using a static pile with air insufflation system, formed by a rotative pump connected to a frame of perforated rubber tubes. The tubes were placed on a bed of dry corn residues (4x8 meters). The composting pile were made up by a mixture (base matrix) of cow and buffalo manure (70% w/w) and maize straw and poplar trimming as structuring woody material (30% w/w); the mixed material was uniformly spread by a power shovel to cover the insufflation system and forming the final pile height of approximately 1.5 m. The composting process lasted 100 days, with a periodic monitoring of external and internal temperature level (5 min interval) and oxygen percentage (60 min interval). During the first 50 days the minimum percentage of oxygen was set at 10%, then subsequently at 5%.

2.2 Bioeffector

The bio-effector used here consisted of microbial mixture of *Pseudomonas* spp. (Proradix®, produced by Sourcon Padena GmbH & Co). This bio-effector was used at concentration of 10^9 cfu kg^{-1} . Before the application to soil, a suspension was prepared freshly in non-chlorinated tap water diluting 1g of product in 2 liters water m^{-2}. Band application was achieved by supplying the product on top of plant rows (0.23 g m^{-1} row). The bio-effector was supplied at sowing, while application was repeated at the 2-3 leaves stage on plants.

2.3 Experimental setup

The field trial was conducted at the Experimental Station of the Department of Agriculture of the University of Naples Federico II, located at Castel Volturno (CE), in an agricultural area 60 km north of Naples, Campania, Italy. The experimental area was divided into 40 m^2 plots. Maize (*Zea may*s L. cv Aphoteoz, Limagrain) plants were seeded at a row distance of about 10 cm and 75 cm between rows, with a plant density of 7 plants m^{-2}. Six treatments with 4 replicates in completely randomized blocks were established, with a total of 24 plots. The treatments consisted of:

B0P0 = Control with no phosphorus addition

B0P1 = Control with TSP (triple super phosphate) (50 kg P/ha)

B0P2 = Control with composted cow/buffalo manure (125 q/ha)

B1P0 = *Pseudomonas proradix* spp. with no phosphorus addition

B1P1 = *Pseudomonas proradix* spp. with TSP (triple super phosphate) (50 kg P/ha)

B1P2 = *Pseudomonas proradix* spp. with composted cow/buffalo manure (125 q/ha)

Nitrogen was supplied as urea (135 kg/ha total N). Nutrient concentrations in the compost were determined prior to application and P and N were generally supplemented to equal the doses of TSP treatments. The experiment was conducted from June to October and plant performances were monitored recording all symptoms of nutrient deficiencies. Sampling was performed at four phenological stages (30, 60, 90 and 120 days after sowing). At harvest, all cobs were collected at the end of October. Plant height and leaf numbers were measured, and the fresh and dry weights of shoots and leaves were recorded (after 48 h in oven at 70 °C). Root samples were removed, washed, weighed and stored at 4°C in ethanol, for later determination of mycorrhizal colonization. At 60 and 90das, rhizospheric and bulk soil samples were collected, the first sieved at 2 mm and stored at −20 °C for further PLFA analysis, while the bulk soil was air-dried and sieved at 2 mm at room temperature and stored for physical–chemical analysis. At final harvest, grain yield was evaluated. Total P in maize grain was obtained by digesting the pulverized grain samples with diluted HNO_3 and HCl (1:3 v/v) (Gericke and Kurmies, 1952) and then colorimetrically determined by molybdenum blue assay method (Murphy and Riley, 1962). Total grain nitrogen was determined by Kjeldahl digestion method (Bremner and Mulvaney, 1982).

Preliminary results

At 2-3 weeks after sowing, plants in P0 treatments showed severe P deficiency symptoms. At one month after sowing some plants have undergone a great stress due to an attack of larvae of *Agriotes* spp., but then they recovered completely.

At first sampling the best performances in all treatments, in terms of plant growth, were obtained with Proradix inoculation. The largest biomass was shown in TSP treatments. However, during the second and third sampling no significant differences were noted between TSP and compost treatments. The final yield production showed a slight decrease in

treatments amended with compost (Fig. 1). Similar trend were shown by nitrogen and phosphorus content (Fig. 2, Fig. 3). However, when compost based treatments were compared, inoculation with *Pseudomonas* brought higher grain nutrient content. Further analysis are required to evaluate the impact on microbial community, with particular attention to arbuscular mychorrizae. However, considering that 125 q/ha of compost used in this study was far less the recommended dosage usually applied for maize crops (between 30 and 35 ton/ha^{-1}), we consider our results excellent in view of sustainable crop production and the inoculation of *Pseudomonas* promising due to the better use efficiency of available resources.

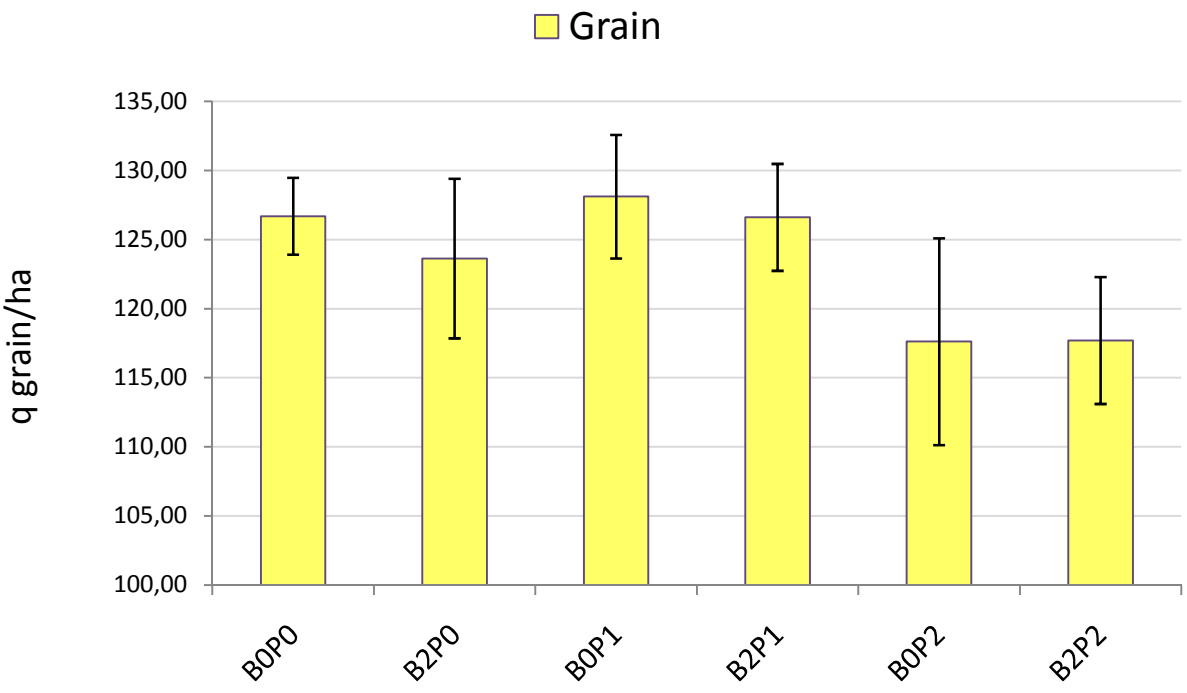

Figure 1. Dry grain content as affected by different treatments (mean ± S.D.).

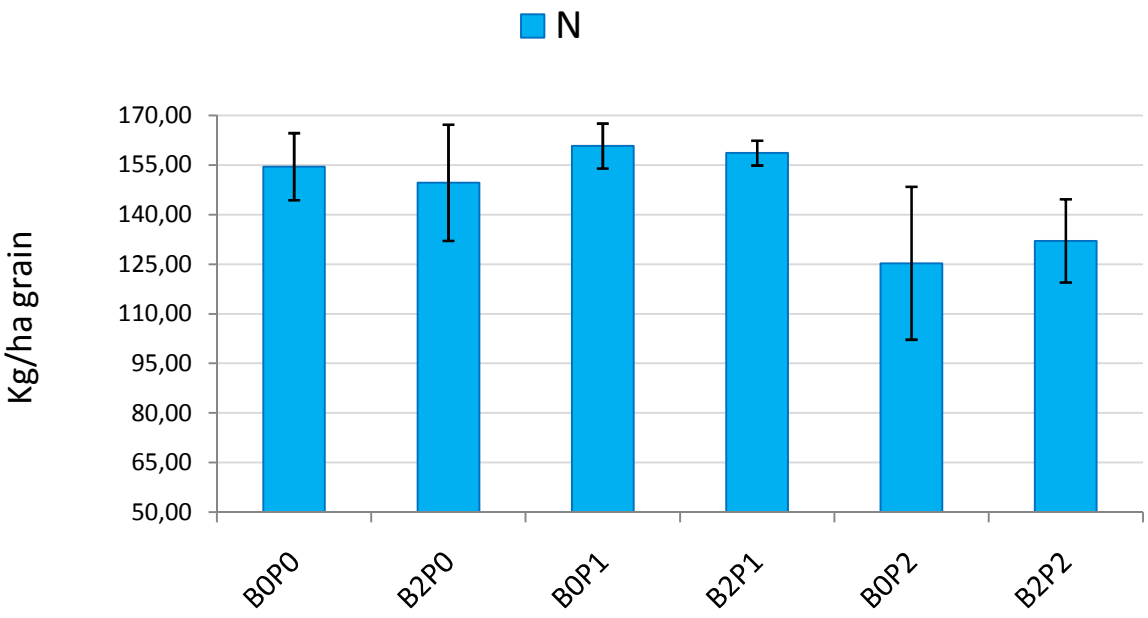

Figure 2. Grain nitrogen content as affected by different treatments (mean ± S.D.).

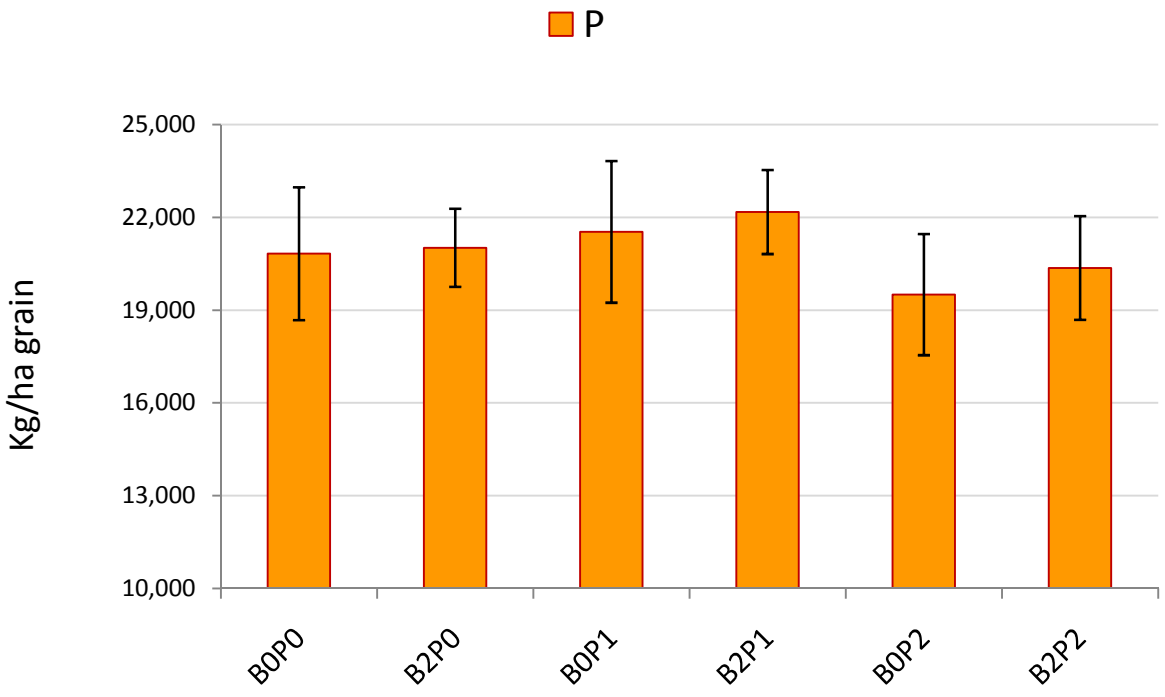

Figure 3. Grain phosphorus content as affected by different treatments (mean ± S.D.).

GENERAL DISCUSSION

This thesis followed two research line: the first regarded the production and characterization of natural extraction products (water-extract and humic acids) followed by the evaluation of plant growth promotion effects, while the second research line consisted in the application of nutrient mobilizing microorganisms in greenhouse experiments and the identification of functional combinations showing synergistic interactions.

It was found that water-extracts and humic acids extracted from composts exerted an overall positive effect on plant growth and root development, depending on their physical-chemical properties and molecular composition. Bioactivity appeared to be related to the changes of conformational structure of humic substances in solution, thereby allowing a more efficient release and diffusion of bioactive molecules towards the root cellular membranes that directly or indirectly affected plants development. This mechanism of action was a function of the ratio of hydrophobic to hydrophilic components in each humic material, that was ultimately responsible for the mutual association strength among humic molecules. Furthermore, the results presented in this thesis confirm that compost raw source, from which bioactive humic substances can be obtained at low cost, thereby represents an alternative to other humified products for a wide range of agricultural applications.

The application to a low phosphorus soil of phosphate solubilising microorganisms, such as *Trichoderma harzianum* and *Bacillus amyloliquefaciens,* was shown to increase nitrogen, phosphorus, and micronutrients content in plants. In particular, when combined with organic amendments, *Bacillus amyiloliquefaciens* enhanced plant height and shoot biomass by 37% and 33% under treatments with composted cow/buffalo and horse manure, respectively. These outcomes suggest that a modification in the structure and composition of soil microbial

communities were induced by the microbial inoculation, especially when organic amendments were used. The thesis showed that the total amount of PLFA, a measure of total microbial biomass, was significantly enhanced in all treatments amended with composted cow/buffalo manure. Moreover, measures of the quantity of c16:1ω5 NLFA, as an indicator of AMF spores and propargules, suggested an enhanced AM fungal growth following the inoculation with *Bacillus amyiloliquefaciens*, also confirmed by the increase of root colonization percentage. These results, in accordance with literature, indicated a synergistic interaction between *B. amyloliquefaciens* and AMF for the enhancement of plant growth.

To support this synergistic hypothesis, this thesis also investigated the effects of different combinations of *Bacillus amyiloliquefaciens* and *Pseudomonas* spp. with AMF and HA extracted from green compost. The results indicated not only a general positive effect on plant growth of each microbial bio-effector inoculated alone, but even greater effect when added in combination with HA and AMF. Plants inoculated with *Pseudomonas* spp. and AMF yielded the greatest biomass and nitrogen content, while co-inoculation with HA showed the largest P accumulation. No major shift of soil microbial communities were concomitantly observed. However, when HA were added in combination with *Pseudomonas* spp. and AMF, a negative shift in AMF population was noticed. This may be explained by the arising of co-metabolism processes with *pseudomonas* or of competitive microbial populations, thereby resulting detrimental for the development of the AMF infection. Nevertheless, the combination of *B. amyiloliquefaciens,* AMF and HA was found to be a very effective in the increase of plant nutrients uptake and growth. Furthermore, the inoculation with *B. amyiloliquefaciens* shifted the soil microbial community in favor of AMF with a simultaneous decrease in the content of saprophytic fungi. These results confirmed the synergism between both groups of microorganisms which are capable to mutually interact for the improvement of their bioactity

in the rhizosphere, whose extent is also increased by the presence of avialble humic molecules with their capacity to stimulate plasma membrane H^+-ATPase and induce root structural changes and elongation. A consequence may be the increase of root surface area available for the infection by AM fungi and the production of root organic acids exudates favoring the establishment of *B. amyiloliquefaciens.*

The results of this thesis not only validated most of the hypothesis on the synergistic cooperation between microbial bioeffectors and natural organic matter in the plant rhizoshere but also contributed to cast the basis of new fertilization technologies in the framework of a sustainable agriculture.

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