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Molecular techniques (humeomics and metabolomics) to relate the structure of rhizosphere micro-bio-humeome to plants bio-stimulation

Tutor

Prof. Alessandro Piccolo

Candidate

Dr. Giovanni Vinci

Coordinator

Prof. Guido D'Urso

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Summary

The present study was aimed to evaluate the use of new sustainable and integrated agricultural practices as alternative to the conventional and unsustainable use of mineral fertilizers and pesticides, and understand how these interact with plants metabolism.

Specifically, bio-fertilizers based on recycled biomass like composts were tested, alone or in combination with plant bio-stimulants (bioeffectors), such as microorganisms and humic substances. The bio-effectors are frequently used in organic farming, to not only ensure food safety and increase soil biodiversity but as totally eco-sustainable instruments of plant growth.

The attempt to further understand the combined effect of microbes and humic compounds (the micro-bio-humeome) at the rhizospheric soil-plant interaction, was conducted by following the plants metabolome through the use of advanced analytical techniques, like gas-chromatography coupled with mass spectrometry (GC-MS) and Nuclear Magnetic Resonance spectroscopy(NMR).

Experimental results underlined a positive effect of the combined use of microbial and humic bio-stimulants on plants, thus suggesting the centrality of the microbio-humeome approach as valuable alternative to conventional agricultural practices in promoting the environmental sustainability and and food security of crop production.

All microorganisms tested, alone or in combination with AMF and humic acids from artichoke compost, have positively influenced the N and P content in plants. In particular, the best results on plants, were obtained by using a combination of *Bacillus amyloliquefaciens* AMF and humic acids as bio-stimulants and the compost from cow manure as fertilizer.

Additionally, in order to better understand which molecular class of compounds play a specific role in the micro-bio-humeome interactions, the Humeomics procedure applied directly on humic-rich materials like peats was developed to reach a comprehensive knowledge of the molecular structure of natural organic matter.

The results obtained have shown the Humeomics ability, when applied directly on peat, to simplify the complex organic mixture extract and precisely characterize the different molecular fraction obtained.

Different classes of compounds, such as phenols, dicarboxylic acids, fatty acids and alkanes, were characteristic of each peat studied, representing useful markers to identify the different origins of the OM accumulated during the periods of peat formation.

The employment of analytical techniques such as Metabolomics and Humeomics to enlarge our knowledge of the rhizospheric micro-bio-humeome interactions and mechanism, appears necessary to formulate future tailored preparations with specific bio-stimulants for different crops and soils characteristics.

CHAPTER 1

1.1 Introduction

Phosphorus (P), along with nitrogen (N), represents one of the most important macronutrients for plant growth. It is uptaken from soil solution in its inorganic form and becomes an integral component of membrane lipids and nucleic acids. Specifically, phosphorous plays a crucial role in the photosynthetic carbon cycle while it is present in many compounds involved in the primary metabolism of plants (Terry & Rao, 1991). In particular, the cellular inorganic orthophosphate (Pi) regulates enzyme activity and metabolic pathways as well as the transport processes. Many works indicate that P deficiency significantly depresses plant CO_2 assimilation, and thus alters the photosynthetic pathwyas (Rao & Terry, 1989; Jacob & Lawlor, 1991). Inhibition of photosynthesis by P limitation has often been explained with a reduction of the Calvin cycle activity, and specifically of the amount and activity of Rubisco and the regeneration of ribu-lose-1,5-bisphosphate (RuBP) (Brooks, 1986; Jacob & Lawlor, 1992; Pieters *et al*., 2001).

Limiting factors for plant growth and development in most soils are given by the bioavailability of macronutrients. It is well known that the plants uptake only a small percentage of P added with fertilizers, since this element is rapidly converted into insoluble complexes with Al and Fe oxides in acid soils, and calcium in alkaline soils (Rengel & Marschner, 2005; Lynch, 2011). This situation requires frequent applications of P fertilizers to soils, with the consequence that much of the P that has been applied to agricultural land over the years, is now stored in soils as a P capacity factor. It should be recalled that the "Green Revolution" allowed food production to satisfy population growth by paying the cost of intensive applications of supplementary nitrogen (N) and phosphate (P) nutrients in mineral fertilizers (Den Herder *et al*., 2010). However, at the present stage, the geochemical reserves of rock phosphate (RP), that represents the raw material for the production of inorganic fertilizers, including Triple Superphosphate (TSP), are expected to be drastically depleted within the end of this century (Schroder *et al*., 2011). Additionally, the

increasing dependence on mineral fertilizers is responsible for the degradation of environmental conditions. In fact, inorganic fertilizers are involved in severe alteration of soil micro-flora, structure and composition (Reddy *et al*., 2002; Koliaei *et al*., 2011), thus affecting soil fertility in the long term and leading to a substantial reduction of crops yields (Gyaneshwar *et al*., 2002). The development of alternative low-cost and eco-sustainable managements in crop production has therefore become mandatory to reduce the use of mineral fertilizers, preserve soil fertility and enhance high quality of foods (Cordell *et al*., 2009). A valuable alternative to the conventional agricultural practices is represented by organic farming, that, through amendments based on recycled organic matter, not only ensures food safety but also soil biodiversity. Organic farming provides the integrated soil management based on the sustainable use of natural resources, nutrient flows and soil biota, thus leading to a reduced dependence on agrochemical inputs and to an optimized agricultural production (Whipps, 2004; Avis *et al*., 2008). Among treatments based on organic matter, the use of compost from recycled iomasses, has been proved to be very efficient as both organic fertilizer and soil amendment (Barker, 2010; Hargreaves et al., 2008).

Within the objectives of organic farming, bio-effectors (BEs), including various plant growth promoting microorganisms (PGPMs) and/or active natural compounds, has been progressively addressed in the scientific literature. Commercial bio-preparations to support healthy growth and mineral nutrition of crop plants are available.

A third group of materials, referred to as “bio-stimulants”, enhance plant performance by direct growth stimulatory mechanisms, such as phytohormonal effects. In this group are included PGPMs and active natural compounds like Humic Substances (HS).

In contrast to conventional fertilizers and pesticides, the effectiveness of BEs is not based on their direct input per se. Conversely, their direct or indirect effect on plant performance is rather based on the functional implementation or activation of biological mechanisms, in particular those related to the with micro-bio-humeome interactions. However, the complexity of the multiple interactions in soil and, specifically, in the rhizosphere is poorly understood. In order to develop organic and

sustainable farming in the future, it is thus a scientific necessity to better understand the influences of biotic and abiotic factors on the activities of BEs in the natural environment and on their interaction.

1.1 Bio-fertilizers and Bio-amendments

Fertilizers and soil amendments are a wide array of materials added to soils to improve plant growth and soil propriety. The term Bio-fertilizer is referred to products that contain either micro/macro plant nutrients or specific organic components aimed to stimulate, directly or indirectly, plant growth through enhance microbial activity and nutrients availability from soil. Conversely, the term amendment is referred to any material that simply improves soil structure. However, soil amendments may also contain microbial populations (e.g. rhizobacteria) and other plant growth promotion agents (Perumal *et al.*, 2012). Many commercially available products have a dual purpose to serve as both bio-fertilizers and soil amendments. It is the case of compost made out of an aerobic recycling process of biomasses, or peat, that is a heterogeneous mixture of partially decomposed plant material (humus) that accumulates in water-saturated and oxygen-poor environments.

In particular, compost is frequently used in organic farming since it was proved to be very active as both organic fertilizer and soil amendment (Barker, 2010; Hargreaves *et al.*, 2008). Compost addition to agricultural soils increases soil porosity, structural stability, moisture and nutrient availability to plants, soil biological activity, root aeration and soil resistance to erosion (Garcia-Gil *et al.*, 2000; Kowaljow *et al.*, 2007; Weber *et al.*, 2007; Pane *et al.*, 2015). Compost benefits also include the ability to mediate soil-borne plant pathogen suppression with a significant impact on eco-friendly crop management (Pane *et al.*, 2011). Morevoer, composts from manure are good sources of bioavailable P for plants uptake. Therefore, the concomitant use of phosphate solubilizing bacteria (PSB) or other microorganisms with similar activity, is likely to lead to the

improvement of P bioavailability from soil, thereby representing a sustainable strategy to satisfy the P requirements of plants.

1.2 Bio-stimulants

A bio-stimulant is regarded as any substance or microorganism that enhances the plant nutrients uptake, tolerance to abiotic stresses and general crop quality traits. Commercial products used in agriculture as bio-stimulants contain mixtures of these substances and/or microorganisms (du Jardin, 2015), based on the current understanding of their functions to favour bio-inoculation in a range of soil types and cropping systems (Roesti *et al*., 2006; Ahmad *et al*., 2013). Despite recent efforts to clarify the regulatory status of bio-stimulants, there is no regulatory definition of plant bio-stimulants in the European Union or in the United States. Neertheless, some major categories are widely recognized by scientists, regulators and stakeholders, covering both substances and microorganisms (Calvo *et al*., 2014; du Jardin, 2015 ; Halpern *et al*., 2015). Microorganisms include beneficial plant growth promoting bacteria (PGPBs), Root Associated Fungi (RAF) and Arbuscular Mycorrhizas Fungi (AMF). Among these groups, microorganisms with specific capacity to mobilize P from RP and soil are termed phosphorus mobilizing microorganisms (PMMs). On the other hand, natural substances largely employed as Bio-stimulant are the mixtures of molecules which are referred to as Humic Substances (Piccolo, 2002).

1.3 Phosphorus Solubilizing Microroganisms (PSM)

Phosphate-solubilizing microorganisms (PSM) include a wide range of symbiotic and nonsymbiotic organisms, such as *Pseudomonas*, *Bacillus*, and *Rhizobium* species; *actinomycetes*; and various fungi like *Aspergillus* and *Penicillium* species (Richardson *et al*., 2009). PSM have the ability to

solubilize unavailable P form and make them available to plants by processes such as organic acid production, and consequent chelation and ion exchange reactions (Chung *et al.*, 2005). P-containing biofertilizers also bring along beneficial PSM to enhance P availability by increasing soil acidity or activity of phosphatases.

PSM have already been applied in the agricultural practices as bio-stimulants to increase crop productivity by solubilization of solid inorganic phosphates, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate. Among bacterial genera with this capacity, the Pseudomonas and Bacillus species result the most studied bacteria, and are progressively present in commercial formulations. Therefore, the use of PSM in agriculture would not only represent an alternative to the costly inorganic P fertilizers, also a concomitant tool to make P easily bioavailable to plants (Chang & Yang, 2009; Banerjee *et al*., 2010).

1.4 Root Associated Fungi (RAF)

As other microorganisms, Root Associated Fungi (RAF) reside in the rhizosphere and often within root tissues (endophyte). The beneficial effects conferred to plants by RAF include the induction of resistance to disease and tolerance to abiotic stresses (Rawat & Tewari, 2011). *Trichoderma spp.* represents the most studied fungi among RAF and it is of particular interest to sustainable agriculture since it establishes a mutualistic relationship with plants. In fact, while the fungus penetrates the external tissues of the root system and provides protection from pathogen, *Trichoderma spp.* benefits from the nutrient-rich ryzospheric environment. Moreover, *Trichoderma spp*. fungi are able to stimulate lateral plant root development trough the synthesis of auxins compounds (Benitez *et al*., 2004; Contreras-Cornejo *et al*., 2009) or modify host synthesis of nitric oxide under conditions of pathogen attack (Gupta *et al*., 2000).

Solubilization of P from soil or mineral fertilizers is a major beneficial role of *Trichoderma* in regards to the cited problem of depletion of phosphate sources and concomitant price increase of P fertilizers. Rudresch *et al*., (2005) reported that nine isolates of *Trichoderma spp*. were capable to successfully solubilize calcium phosphate, when compared with an efficient P-solubilizing bacterium, such as *Bacillus megaterium* subsp. *phosphaticum*. Additionally, *Trichoderma. harzianum* maintained its P-solubilizing capacity at varying concentrations of Cadmium, indicating that Trichoderma protect plants even in soils polluted with heavy metals (Rawat & Tewari, 2011). Plant stimulation and protection becomes even more efficient in combination of Trichoderma with Arbuscular mycorrhizal fungi (AMF) (Whipps, 2004; Avis *et al*., 2008).

1.5 Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) have gained a ever growing interest as bio-stimulants sice they representa key interface between plant hosts and soil nutrients. AMF is believed to reach more than 10% of the soil microbial biomass and to establish a mutual symbiosis with the majority of land plant species and agricultural crops (Smith & Read, 2008). They act as an extension of the plant's roots system that efficiently exchanges nutrients and sucrose to the fungus, while N/P is provided to the plant, through roots specialized structures named intracellular arbuscules. The basis of these mutualisms is the ability of fungi to form fine hyphae (with more favourable surface area to volume ratio for nutrient uptake) and to secrete enzymes/organic acids to mobilize nutrients. AMF belongs to the phylum Glomeromycota that are most widely active in agriculture, notably Rhizophagus (formerly Glomus) intraradices and Funneliformis (formerly Glomus) mosseae, both of which have been shown to increase P uptake in diverse crop plants (Cozzolino *et al*., 2013). Moreover, it was recently shown that AMF when in combination with a supply of humic matter to soil, are capable to significantly increase plant tolerance to Hg

contamination in polluted soils (Cozzolino *et al*., 2016). As an effect of the symbiosis, the AMF improve agriculture productivity by enhancing plant growth, seed production (Shumway & Koide, 1994) and protect plants from root pathogenic fungi and drought stress (Linderman, 2000; Augé, 2001). Moreover, AMF have a direct effect on the ecosystem, driving the structure of plant communities (van der Heijden *et al*., 1998 a,b) and ameliorating the quality of soil by improving its aggregation and organic carbon content.

1.6 Humic substances and their characterization by Humeomic

Humic substances (HS) are ubiquitous and relatively chemically stabilized materials, arising from degradation of plant and animal tissues through the natural occurring chemical and biological transformation. This natural organic matter is typically found in soils, sediments, coals, peat and waters (Stevenson,1994). HS have particular importance in controlling both the biogeochemistry of organic carbon and the environmental pollutants in the global ecosystem (Piccolo *et al*., 2003). HS are essential for the maintenance of soil fertility, acting on physical, physico-chemical, chemical and biological properties of the soil.

The bio-stimulant effects of HS regard the improvement of plant growth, through different mechanisms: 1) large contribution to the cation exchange capacity of soils, thereby increasing the plant nutrition capability of soils; 2) increase of phosphorus availability by strongly complexing with either calcium or iron in soil; 3) stimulation of plant physiology by increasing activity of plasma membrane H+-ATPases, which convert the free energy released by ATP hydrolysis into a transmembrane electrochemical potential used for the import of nitrate and other nutrients (Jindo *et al*., 2012). Additionally to HS, others humic-like substances, such as composts and vermicomposts, or geochemical deposits (lignites, peats) (du Jardin, 2012) have been shown to exert similar effects on the plant and soil systems.

The bioactivity of HS or other humic-like substances are related to their complex molecular composition, that originates from the random oxidation of plant biomolecules after the cell lysis and depends on different properties of each ecosystem, like climate and vegetation (Piccolo *et al*., 2003). HS are considered collections of heterogeneous compounds and originally categorized according to their molecular weights and solubility at different pH, into fulvic acids (FA) soluble in all pH conditions, humic acids (HA) soluble only under alkaline conditions, and humin (HU) insoluble at any pH. The traditional view of HS as macromolecular polymers has never been unequivocally demonstrated (Piccolo, 2001). A supramolecular theory more respectful of chemical thermodynamics and kinetics has been gaining consensus in recent years (Piccolo *et al*., 1996; Piccolo, 2001, 2002; Piccolo *et al*. 2001, 2002; Simpson, 2002; Piccolo *et al*., 2003). According to this new view, rather than being constituted by macromolecular polymers, as previously believed, HS are better described as supramolecular associations of heterogeneous molecules with average mass lower than 1000-1500 Da (Piccolo, 2001, 2002; Piccolo & Spiteller, 2003; Wershaw, 2004) and are held together by relatively weak forces (π-π, CH-π, and van der Waals interactions) in contiguous hydrophilic and hydrophobic domains of apparently high molecular sizes (Piccolo, 2002). The humic associations show only apparently large molecular dimensions and can be reversibly disrupted by treating humic solutions with small concentrations of mineral and mono- and di-carboxylic acids (Piccolo *et al*.,1999; 2002). Contrary to HS, the conformation of real macropolymers, where monomers are held together by covalent bonds, are not disrupted by the interactions with small amounts of organic acids (Piccolo *et al*., 2001, 2003).

This novel concept suggests that humic molecular complexity may be reduced by progressively breaking the inter- and intramolecular interactions that stabilize the complex suprastructures and single humic molecules be isolated. Their structural identification may then be achieved by combining advanced analytical techniques. This sequential chemical fractionation followed to an analytical identification of the singular subfractions is called Humeomics. Specifically, as reported by Nebbioso & Piccolo (2011, 2012), the Humeomic fractionation firstly use organic solvents in

order to extract the free unbound organic substance only by weak dispersive interactions. Two subsequent steps, which include the cleavage of covalent bonds in weakly bound esters by a mild transesterification using a solution of boron trifluoride-methanol (BF3-MeOH) and the cleavage of more strongly bound esters by an alkaline solvolysis using KOH-MeOH solutions. The Humic molecules released as a result of ester cleavage can be further fractionated by means of liquid/liquid or solid phase extraction (SPE). Finally, the strong ether and glycosidic bonds are cleaved by treatment with hydroiodic acid (HI) following a classic mechanism of protonation of the organic ether and subsequent nucleophilic substitution (SN) by iodide with an alcohol acting as a good leaving group. Consequently all sub-fraction deriving from the abovementioned process could be, quantitatively and qualitatively, identified through the use of advanced analytical technique, like the Nuclear magnetic resonance spectroscopy (NMR), gas- or liquid-chromatography coupled with mass spectrometry (LC- GC-MS). The Humeomics procedure, is able to characterize in a timely way Hs or parts of it, allowing to better understand the role performed by these in the micro-bio-humeome interaction.

1.7 Introducing the concept of the Micro-bio-humeome

It is growing the awareness that the intimate synergy occurring between soil micro-biota, humic substances and plants may produce mutually dependent effects (Cozzolino et al., 2016a), which permit to consider these three components as belonging to a simultaneously bioactive complex, that can be named the “micro-bio-humeome”. In fact, the beneficial effects resulting from the interactions between plants and soil microbiota, including rhizobia, are strongly affected by the soil HS to an extent that depends on their specific heterogeneous molecular composition. However, while the effects produced by the rhizospheric micro-bio-humeome system may be very complex, their detailed comprehension requires the application of advanced and integrated analytical

approaches such as plant Metabolomics and Humeomics, in combination with a deep evaluation of dynamics of the involved soil microorganisms.

1.8 Metabolomics

Metabolomics represent a relatively new "OMIC" science, that is able to determine the effects of different environmental stimuli on plant metabolism. The term metabolome was firstly described by Oliver *et al.*, (1998) as the range of low molecular weight compounds required for the maintenance, growth and normal function of a biological cell, in a particular physiological or developmental stage (Fiehn, 2002). The metabolome represents the final product of gene transcription and translation, that provides a precise picture of cells status at the moment of analysis. However, metabolites have a much greater variability in terms of atoms and subgroups variation, as compared to the linear 4-letter codes for genes or the linear 20 letter codes for proteins (Fiehn, 2002). Therefore, they cannot be sequenced like genes or proteins and the identification of all metabolites in one specific plant results much more complex. It estimated, for example, that the plant kingdom as a whole may develop more than 200,000 metabolites and phytochemicals (Fiehn, 2002). The specific physico-chemical properties of different groups of metabolites further adds to the complexity of metabolomics studies. However, the analytical procedure can be restricted to the identification and quantification of only a selected number of metabolites in a biological sample. The analytical process focused on the physico-chemical and molecular properties of specific compound is called *metabolite profiling* (Fiehn, 2002), while the approach to interpret the metabolic expression of different biological organism is called *metabolomics*. In this context several protocols of analysis and a wide range of analytical platforms have been developed, to help the identification of all metabolite extracted from biological samples (Dunn *et al.*, 2005). However, due to the complexity of the metabolome, one method does not exhaust the different

molecular variations of all metabolites, and the use of multiple protocols and analytical instruments is required (Zhang et *al.*, 2012).

The high precision of mass spectrometry (MS) and the reproducibility of nuclear magnetic resonance (NMR) spectroscopy combined with their ability to elucidate chemical structures (Kim *et al*., 2010) represent the most applied analytical technologies in metabolomics studies. In particular, gas chromatography coupled with mass spectrometry (GC-MS) facilitates the identification and quantification of a few hundred of metabolites in a single plant extract, thus resulting in fairly comprehensive coverage of the central pathways of the primary metabolism. The main advantage of this technology is that it has long been used for metabolite profiling and thus there are stable protocols for instrumental settings, and chromatographic evaluation , and mass spectra interpretation.

Conversely, NMR is a powerful method because it allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids and so on), besides abundantly identifying the primary metabolites (sugars, organic acids, amino acids and so on). Moreover, NMR signals are proportional to their molar concentration, thereby making possible the direct comparison of metbolites concentrations, without the need for calibration curves or derivatization procedure, as in the case of GC-MS analysis. Moreover, NMR is very useful for the elucidation of molecular structures. In fact, various two-dimensional NMR techniques can be used to identify structures in complex metabolic mixtures without the need of any further fractionation of extracts (Kim *et al*., 2010). Nevertheless, the application of NMR spectroscopy in the metabolomic analysis has several limitations, the major of which is perhaps its low sensitivity. In the light of the above, the best metabolomic approach is the one that can employ both GC-MS and NMR techniques with the aim to evaluate the influence exerted by different bio-stimulants or fertilizers on plants.

CHAPTER 2

2.1. Work objectives

The challenges associated with the development of sustainable alternative to the conventional agronomic practices are tackled from many different directions, including plant genetics, soil microbiology, formulation of new fertilizers, plant stimulation and protection from weeds and pests. The integration of various approaches will be necessary to deliver a truly effective solution to the challenge of attaining environmental sustainability, production of healthy foods, and maintainance of crop productivity.One of the aims of the present thesis work was to evaluate the effects of new sustainable technologies to increase plant growth, and concomitantly improve our understanding on how these technologies affect plants metabolism.

Chapters 3 and 4 report on the effects of different bio-effectors (BE) on the metabolome of *Zea mays* upon soil treatments with different organic or inorganic fertilizers.

The role of the micro-bio-humeome is studied in Chapter 5, by evaluating the possible synergistic effects of different bio-stimulants on maize plants. In particular, the combined bio-stimulants were the microorganisms and the organic fertilizers, which have been previously found to positively affect plant growth. The micro-bio-humeome effects on plant Metabolomics was evaluated here by gas-chromatography coupled with mass spectrometry (GC-MS) and Nuclear Magnetic Resonance spectroscopy (NMR) both in the solid and liquid state.

On the other hand, a comprehensive understanding of the role of the micro-bio-humeome requires also a full characterization of the molecular composition of the natural organic matter that becomes involved in the plant-microbe interactions. In fact, humic molecules not only represent a more or less bio-available metabolic carbon to microbes, but also respond with changes in conformation and bio-reactivity to the different composition of plant exudates.

In order to gain further insight in the complex molecular composition of a natural organic matter involved into the micro-bio-humeome, the Humeomics technique was successfully applied to

characterize the humic supramolecular associations from different sources. In Chapter 6, the Humeomics approach was applied on different peat materials with high HS content.

CHAPTER 3

Metabolomics reveal the effects of *Bacillus amyloliquefaciens* and different phosphorous-based fertilizers on *Zea Mays*

Abstract

The bioeffector *Bacillus amyloliquefaciens* was added here as a phosphate-solubilizing-bacterium (PSB) in a greenhouse study, whereby the colonized maize roots were grown in soils that received inorganic P fertilizers as triplesuperphosphate (TSP), rock phosphate (RP) and P-rich composts made of either cow- or horse-manure. The effects of both bioeffector addition and P amendments was investigated on the metabolic profiles of maize leaves. The determination of the plant leaves metabolome was achieved by both Gas chromatography–mass spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy**.** A broad range of primary polar metabolites, including sugars, organic acids, amino acids, phenolic acids, amino sugars, and sugar alcohols, were identified by both techniques. The results were elaborated by multivariate statistical analysis. The Principle Components Analysis (PCA) based on data matrix from both GC-MS and NMR showed the influence of *B. amyloliquefaciens* on the metabolomic profile of plants treated with different P-fertilizer. The findings indicated that the combination of P-rich organic composts with *B. amiloliquefaciens*, provide a valuable alternative to mineral fertilizers to satisfy plant P requirements.

Keywords: Metabolomics, *Bacillus amyloliquefaciens*, Compost, Rock phosphate, Triple Superphosphate, GC-MS, ^{1}H-NMR.

1. Introduction

Phosphorus (P), second only to nitrogen (N), represents an essential macronutrient for plant growth. Phosphorous is an integral component of plants membrane lipids and nucleic acids, plays a crucial role in the photosynthetic carbon cycle and it is associated to many compounds involved in the plants primary metabolism. However, the amount and bioavailability of P may represent limiting factors for plant growth in most soils.

Highly reactive P-based fertilizers, such as triple super phosphate (TSP) and di-ammonium phosphate (DAP), are applied to agricultural soils to maintain crop productivity. However, P is rapidly insolubilized in complexes with Al and Fe oxides in acid soils, fixed as calcium phosphates in alkaline conditions (Rengel & Marschner 2005; Lynch, 2011). Alternatively, rock phosphate (RP) represents a cheaper fertilizer largely used by farmers sue to a more convenient slow-release of P (Chien *et al*., 1995). The adoption of RP in combination with highly reactive P fertilizers leads to positive results, in terms of root development and P plant utilization (Chien *et al*. 1996; Zapata & Zaharah, 2002). The relentless population growth produces an ever larger demand for P fertilizers, despite the fact that the progressive consumption of natural RP reserves are expected to be drastically depleted within the end of this century (Schroder *et al*., 2011).

Moreover, the large overuse of these fertilizers not only is expansive, but also is environmentally unsustainable, being it responsible for the severe alteration of micro-flora, soil structure and composition (Reddy *et al*., 2002; Koliaei *et al*., 2011). Consequently, a long-term abuse of these fertilizers may affect the soil fertility and reduce the yield of crops (Gyaneshwar *et al*., 2002). The negative outlook of conventional agricultural practices calls for an alternative eco-sustainable management aimed to reduce the use of mineral P fertilizers while maintaining soil fertility (Cordell *et al*., 2009).

A valuable solution may be represented by combining the use of fertilizers based on stabilized organic matter with the inoculation of beneficial microorganisms. In particular, compost, that

results from a recycling process of biomasses, has been proved to be very efficient as both organic fertilizer and soil amendment (Barker, 2010; Hargreaves *et al.*, 2008). In fact, it has been demonstrated that the compost addition to agricultural soils may increase porosity, structural stability, moisture and nutrient availability, biological activity, root aeration and protects soil from erosion (Garcia-Gil *et al*., 2000; Kowaljow *et al*., 2007; Weber *et al*., 2007). Compost benefits also include the ability to mediate soil-borne plant pathogen suppression with a significant impact on eco-friendly crop management (Catello *et al*.,2013).

As composts are good sources of bioavailable P forms for plants, the concomitant use of phosphate solubilizing bacteria (PSB) can improve the bioavailability of phosphorous and provide an alternative strategy to satisfy plant P requirements. PSB are able to convert insoluble phosphate into plant available forms through processes of acidification, exchange reactions and production of organic acids (Liu *et al*., 2014; Rodríguez & Fraga, 1999; Sharma *et al*., 2013). Strains from bacterial genera of *Pseudomonas* and *Bacillus* represent the most efficient P-solubilizers in soil (Rodríguez & Fraga, 1999). Particularly, biofertilizer and biocontrol formulations prepared from endospore-forming *Bacillus* strains are increasingly applied due to their long shelf life (Qiao *et al*., 2014). For instance, *Bacillus amyloliquefaciens* strains belonging to subsp. *Plantarum* emerge as compared to other representatives of endospore-forming *B. amyloliquefaciens* because of their enhanced ability to colonize plant rhizosphere, to stimulate plant growth, and to suppress competing phytopathogenic bacteria and fungi (Qiao *et al*., 2014). Consequently, the use of this phosphate bio-fertilizers can decrease the detrimental effects of phosphate fertilizers on crop and soil health while maintaining good productive yields (Chang & Yang, 2009). However, a deep and exhaustive knowledge, in biochemical and molecular terms, on the effects exerted by this bio-fertilizers on the metabolic response of plants still lacks. On this basis, the purpose of the present work was to establish how different fertilizers, composed by P in inorganic (PR, TSP) or organic (compost) form, can influence maize plants, at a metabolic level, and their nutritional status. In addition, these fertilizers were tested either alone or in combination with inoculums of *Bacillus amiloliquefaciens*

aiming to clarify the possible synergistic role assumed by PSB in soil amendments. The metabolomics has emerged as a useful analytical approach to study the molecular responses induced in plants by different treatments or specific environmental conditions (Bundy *et al*., 2009). Therefore, the polar metabolites extracted from Zea mays plants grown with different P-based fertilizers were evaluated by a metabolomic analysis conducted on molecular data resulting from both gas chromatography coupled to mass spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR). Additionally the content of P and N was estimated in order to assess the general nutritional status of plant and support the interpretation of different metabolomes.

2. Materials and methods

2.1 Experimental design, plant growth, sampling and analyses

Maize plants (Zea mays cv. Colisee, KWS) were grown in pots (3 L) filled with 2.5 kg of substrate, prepared using an alkaline clay-loam soil mixed with quartz sand in the ratio of 2:1. The substrate was incubated in covered plastic boxes at 20 ± 2°C during 30 days prior to planting. Available soil P concentration (Olsen method) was 12 mg P/kg. The treatments consisted of factorial combinations of five types of P fertilizer: 1) P0, no P addition; 2) P1, triple superphosphate (TSP); 3) P2, rock phosphate (RP); 4) P3, composted buffalo manure; 5) P4, composted horse manure, and two microbial treatments : 1) B0, no inoculation; 2) B3, *Bacillus amyiloliquefaciens* (Rhizovital FZB42® ABiTEP GmbH, Berlin, Germany). All treatments were replicated five times, for a total of 50 pots. The P fertilizers were applied to pot soil at the rate of 50 mg P kg^{-1} dry substrate. Moreover, P3 and P4 were applied 15 days before sowing. A basal nutrients supply was performed adding nitrogen (N) as $Ca(NO_3)_2$ at the rate of 150 mg N kg^{-1} dry substrate and potassium (K) as Kalimagnesia (30% K_2O + 10% MgO) at the rate of 166 mg K kg^{-1} dry substrate. Maize seeds were

sown prior to microbial inoculation in the number of three seeds per pot. *Bacillus amyloliquefaciens* was applied as a suspension in demineralized water with 2.5 mM $CaSO_4$ by spraying on seeds surface at sowing, at the rate of $2.5x10^4$ spores, $2x10^6$ cfu and $2x10^6$ cfu g^{-1} dry substrate. The pot trial was conducted in a greenhouse. Watering with demineralized water was performed manually to maintain the water holding capacity between 40 and 70% throughout the experiment.

Eight weeks after sowing, the youngest fully expanded leaves for each plant, were collected between 08:00h and 12:00h. Harvested leaves were weighed, immediately frozen in liquid nitrogen, in order to quench the metabolism, and stored at -80°C.

Total P in maize shoots was obtained by first digesting 500 mg of dry powdered plant material by the ashing procedure of Gericke and Kurmies (1952), and then by colorimetrically determining P through the molybdenum blue assay method (Murphy & Riley 1962). Nitrogen was determined by an elemental analyzer Fisons EA 1108 (Fisons Instruments, Milano, Italy).

2.2 Extraction of plant leaves metabolites.

Maize leaves stored at -80°C were homogenized by using a mortar and pestle under liquid nitrogen. Then, 50±0.5mg of pooled homogenized plant material was weighed into pre-chilled 2 mL Eppendorf tube. The metabolites extraction was conducted by adding 1 mL of water/methanol/chloroform mixture (1:3:1 ratio) pre-cooled at -20°C, that also contained 20 μg ml^{-1} of Ribitol, as an internal standard. The samples were stirred for 30s and incubated for 15min at 70°C in order to inhibit the activity of possible enzymes present in the extract. The mixtures was then centrifuged for 10 min at 10000 rpm and 4°C, and the supernatants were recovered and transferred into 2 mL Eppendorf tubes. Pure water (400 μL) was added to allow the separation of polar and apolar phases corresponding to the methanol/water (upper) and chloroform (lower) phases, respectively. All extracts were finally stirred for 30 s and centrifuged for 10 min at 4°C and

10000 rpm. A volume of 400 μL was collected from the upper phase, transferred into 1.5 mL glass tubes for GC–MS analyses, dried under a flow of nitrogen and stored at -80 °C. Tree blank tubes were subjected to the same procedure.

2.3 Preparation of metabolites to analytical determination

Before derivatization for GC–MS analyses, the metabolic extracts were again dried under nitrogen flux in order to remove the humidity possibly acquired during storage at -80°C. Dried samples were redissolved in 50 uL of a solution of methoxyamine hydrochloride solubilized in pyridine (20mg ml^{-1}) and treated for 90 min at 30°C by applying a gentle shaking. After the methoximation step, the samples were silylated for 30 min at 37°C by using 50uL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) reagent. 30 minutes after the end of derivatisation, 2uL of each sample was injected into a GC column by using a split mode.

The same extraction was adopted to obtain NMR spectra, although both plant material and extraction volumes were doubled in order to overcome the lower sensitivity of NMR spectroscopy. In this case 800 μL of supernatant were dried under a flow of nitrogen, stored at -80 °C and resolved in the same volume of deuterated phosphate buffer (90 mM, pH 6.0) containing 0.05 mg mL^{-1} 3-(tri-methylsilyl) propionic-2,2,3,3-d4 acid (TMSPA, $\delta\ ^{1}H$ = 0 ppm; > 99%, Euriso-Top, France) which served as internal standard. Aiming to guarantee the complete dissolution of each metabolic extract, a 5 minutes long sonication was applied prior NMR analysis. Five replicates were prepared and examined for both NMR and GC-MS analyses.

2.4 GC-MS

Samples were analyzed using an Agilent 7683B Series Injector coupled to an Agilent HP6890 Series gas chromatograph system and a 5973 Mass Selective Detector, quadrupole type GC–MS system. The GC was performed with RTX-5MS WCOT capillary column (Restek, 30 m × 0.25 mm; film thickness, 0.25 mm) that was coupled, through a heated transfer line (250 °C), to a Turbomass-Gold quadrupole mass spectrometer. The gas chromatographic separation was performed by applying a 2 minutes long isothermal phase at 80 °C, followed by a temperature increase from 80 to 310°C (rate of 15°C min^{-1}) and culminating in a 10 minutes long isothermal phase at 310°C. Helium was used as carrier gas at 1 mL min^{-1}, as well as the injector temperature was set at 250 °C and the split flow applied for the split-injection mode was 25 mL min^{-1}. Mass spectra were obtained in EI mode (70 eV), scanning in the range included within 50 and 650 m/z, with a cycle time of 0.2 scan s^{-1}. The identification of mass spectra of eluted compounds was carried out by analyzing standard compounds as well as by evaluating the mass spectra reported in the library NIST 05 (http://www.nist.gov) and the library released by Max-Planck-Institute for Plant Physiology of Golm (Germany, http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html). In order to take into account possible coelutions and peak overlappings, the mass spectra for all eluted peaks were analyzed not only in their centre, but also at peak borders.

2.5 NMR

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm BBI Bruker probe and working at the ^{1}H frequency of 400.13 MHz, was used to conduct all NMR measurements at a temperature of 298 ± 1 K. Monodimensional ^{1}H spectra were acquired by setting 5 s of thermal equilibrium delay, a 90° pulse length ranging within 8 and 8.85 μs (−2 dB of attenuation), 128 transients, 32768 time

domain points, and 16 ppm (6410.3 Hz) as spectral width. The signal of residual water was suppressed by applying the on-resonance pre-saturation during thermal equilibrium delay. Assignment of NMR signals was performed by acquiring and interpreting 2D NMR spectra of both samples and standard compounds, and it was confirmed by the molecular characterizations of maize tissues reported in previous manuscripts (Broyart *et al.*, 2010; Castro *et al.*, 2008; Gavaghan *et al.*, 2011; Kuhnen *et al.*, 2010; Manetti *et al.*, 2006; Piccioni *et al.*, 2009). In particular, 2D NMR spectra consisted of ^{1}H–^{1}H homo-nuclear experiments, such as COSY (Correlation SpectroscopY), TOCSY (Total CorrelationSpectroscopY) and NOESY (Nuclear OverHauser SpectroscopY), and hetero-nuclear ^{1}H–^{13}C experiments, such as HSQC (Hetero-nuclear Single-Quantum Correlation) and HMBC (Hetero-nuclear Multiple Bond Correlation). All of 2D experiments were acquired with spectral widths of 16 (6410.3 Hz) and 300 (30186.8 Hz) ppm for ^{1}H and ^{13}C nuclei, respectively, and a time domain of 2048 points (F2) and 256 experiments (F1). Specifically, homo-nuclear 2D spectra consisted in 16 dummy scans and 64 total transients. Additionally, a mixing time of 80 ms and a trim pulse length of 2500 ms were set for TOCSY experiment. HSQC and HMBC hetero-nuclear experiments were acquired with 16 dummy scans, 80 total transients and 0.5 μs of trim pulse length. Respectively, these experiments were optimized by taking into account 145 and 6.5 Hz as the optimal ^{1}H –^{13}C short and long range J-couplings.

Spectra were processed by using both Bruker Topspin Software (v 2.1, BrukerBiospin, heinstetten, Germany) and MNOVA Software (v.9.0, Mestrelab Research, Santiago de Compostela, Spain). Phase- and baseline corrections were applied to all of mono- and bi-dimensional spectra. The free induction decays (FIDs) of 1D ^{1}H spectra were Fourier transformed with a function size of 32768 points and applying a 0.3 Hz apodization.

2.6 Statistical analysis of GC-MS and NMR results

The semiquantitative evaluation of GC-chromatograms was performed by normalizing the area of each peak by the area of the internal standard and further modulating it as a function of sample fresh weight (mg). Conversely, ^{1}H NMR spectra were divided in symmetrical *n*-intervals (buckets to 0.04 ppm) which were then integrated and normalized with respect to the internal standard TMSPA. Each ^{1}H NMR spectrum was integrated from 9.98 to 0.1 ppm, excluding the region involving the water signal suppression (4.86-4.74 ppm), and producing 244 variables. The total data set composed of GC-MS (49 variables) and NMR (244 variables) data was subjected to Principal Component Analysis (PCA) using a XLStat software v.9.0 (Addinsoft). PCA represents an unsupervised classification method requiring no *a priori* knowledge of the data set and reduces the dimensionality of multivariate data while preserving most of information, expressed in terms of variable variance. Significant differences in metabolome amounts as a function of the studied treatments were tested by one and two-way ANOVA, followed by Tukey's test ($p< 0.05$).

3. Results and Discussion

3.1 Phosphorus and nitrogen uptake

In order to evaluate the impact of *Bacillus amiloliquefaciens* inoculation and different P fertilization treatments on plant nutrition, at eight weeks after sowing, P and N levels were analyzed in maize shoots. P content was significantly affected by P fertilization in all treatments (Fig. 1A). Thus P content increased by 48, 44, 37 and 16% in P1, P2, P3 and P4, respectively, as compared to non-fertilized plants. These data suggest a greater plant P availability in inorganic P fertilizer treatments with respect to composted manures in non-inoculated plants. P content was also significantly affected by *Bacillus amiloliquefaciens* inoculation. In particular, the combination of *B.*

amiloliquefaciens inoculation and composted cow manure fertilization significantly increased P accumulation in shoot tissues to greater extent, as compared to other treatments. In fact, in this case P content increased by 42 % and 69% in P3 treatment as compared to B0P3 and B0P0 treatments , respectively. These results suggest that inoculation with *B. amiloliquefaciens* increased available-P levels as result of its potential ability to mineralize organic P forms.

In addition to the abovementioned effects on P nutrition, *B. amiloliquefaciens* inoculation in combination with composted manures also led to a significant increase in shoot-N content as shown by the comparison of B3P3 and B3P4 plants with the other treatments (Fig. 1B). P fertilization alone had no effect on shoot N content.

Overall these results indicated a considerable ability of *B. amyloliquefaciens* to solubilize organic fertilizers, thus increasing the availability of P and N in the soil.

The large content of N and P in the inoculated plants and fertilized with compost suggested a better nutritional status paving the way to a better growth and yield.

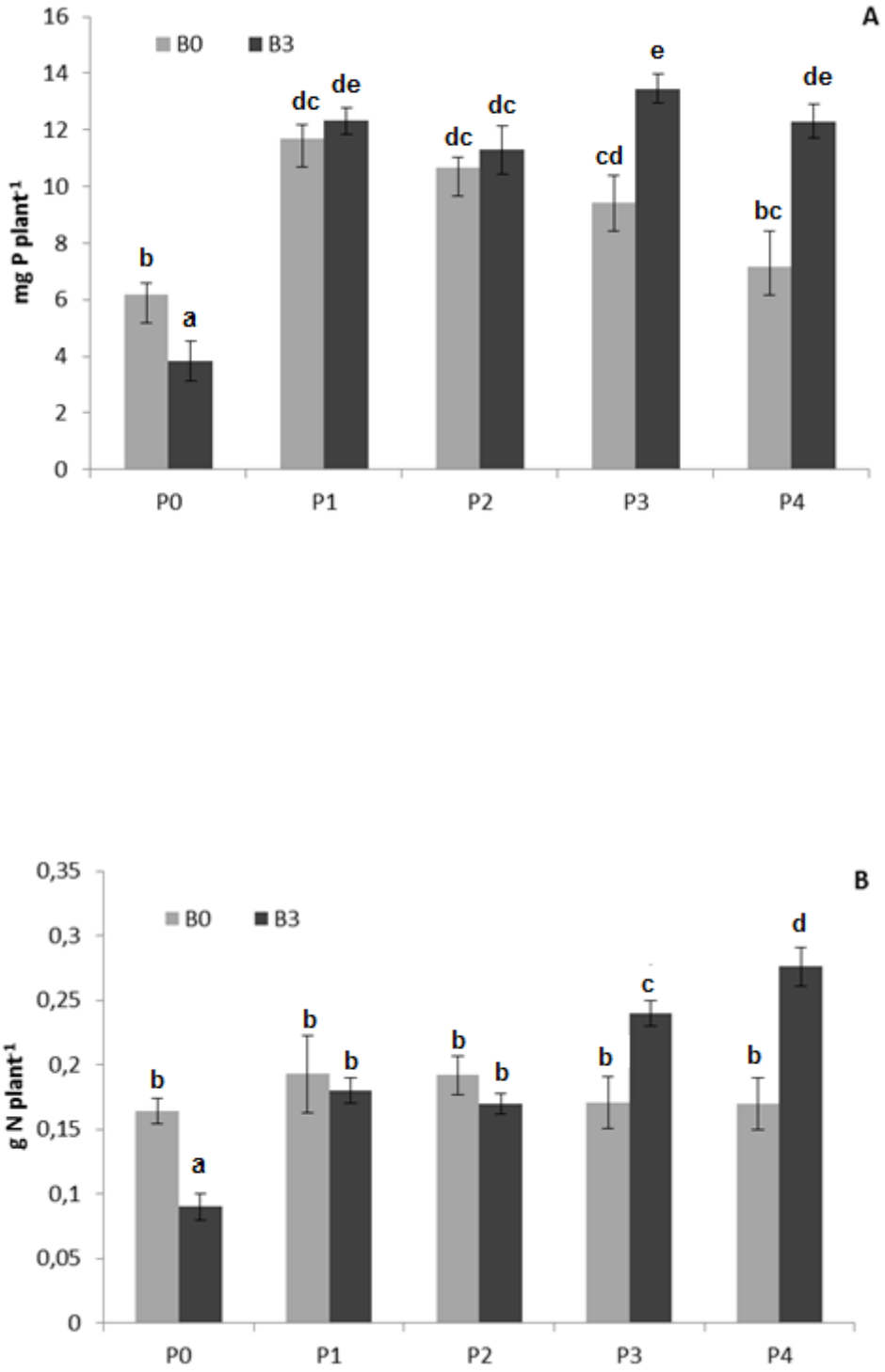


Figure 1. Phosphorous (A) and Nitrogen (B) content in the controls (B0) and inoculated plants (B3). Error bars indicate standard error (n=5) and different letters indicate significant differences by the Tukey's test at $P \leq 0.05$.

3.2 GC-MS and NMR metabolic profiling

In order to investigate differences in maize metabolites according to organic and inorganic P fertilization and in presence of plants inoculated with *Bacillus amyloliquefaciens,* the primary polar metabolites were analyzed by GC/MS and ^{1}H NMR. Figure 2 shows the GC chromatogram (Fig. 2A) and the NMR spectrum (Fig. 2B) of polar metabolites extracted from maize leaves of a representative sample (B0P0). The principal compounds identified were saccharides (mono- and di- saccharides), followed by organic acids, amino acids and amino-sugar Tabs (1a, 1b).

It is important to note that the combination of GC-MS and ^{1}H-NMR techniques enabled to attain a more detailed metabolic profile of maize leaves, thereby accounting for the advantages deriving from both these powerful and complementary analytical techniques.

The GC-MS showed an enhanced sensitivity in the identification of metabolites, such as organic acids, which were present in very small quantity into the metabolic extract. Conversely, the NMR results underlined an enhanced reproducibility in the identification of some amino acids in the replicates. Furthermore, the application of two-dimensional NMR techniques, that imply a better resolution in respect to one-dimensional ^{1}H NMR, has allowed to solve problems of signals overlapping. The visual inspection of both GC chromatograms and NMR spectra allowed to identify sucrose and its monosaccharide constituents, as the most abundant carbohydrates present in the leaves metabolome.

Hence, in order to understand how many and which metabolites were involved in the differentiation between the analyzed samples, the metabolic profiles of each treatment resulting from the use of both analytical techniques (GC-MS and NMR) was examined as unique data matrix by Principal Components Analysis (PCA). The PCA outputs consisted in score-plot and loading-plots, where the formers highlighted the differences existing among samples, while the latters described the involvement of variables in such differentiations.

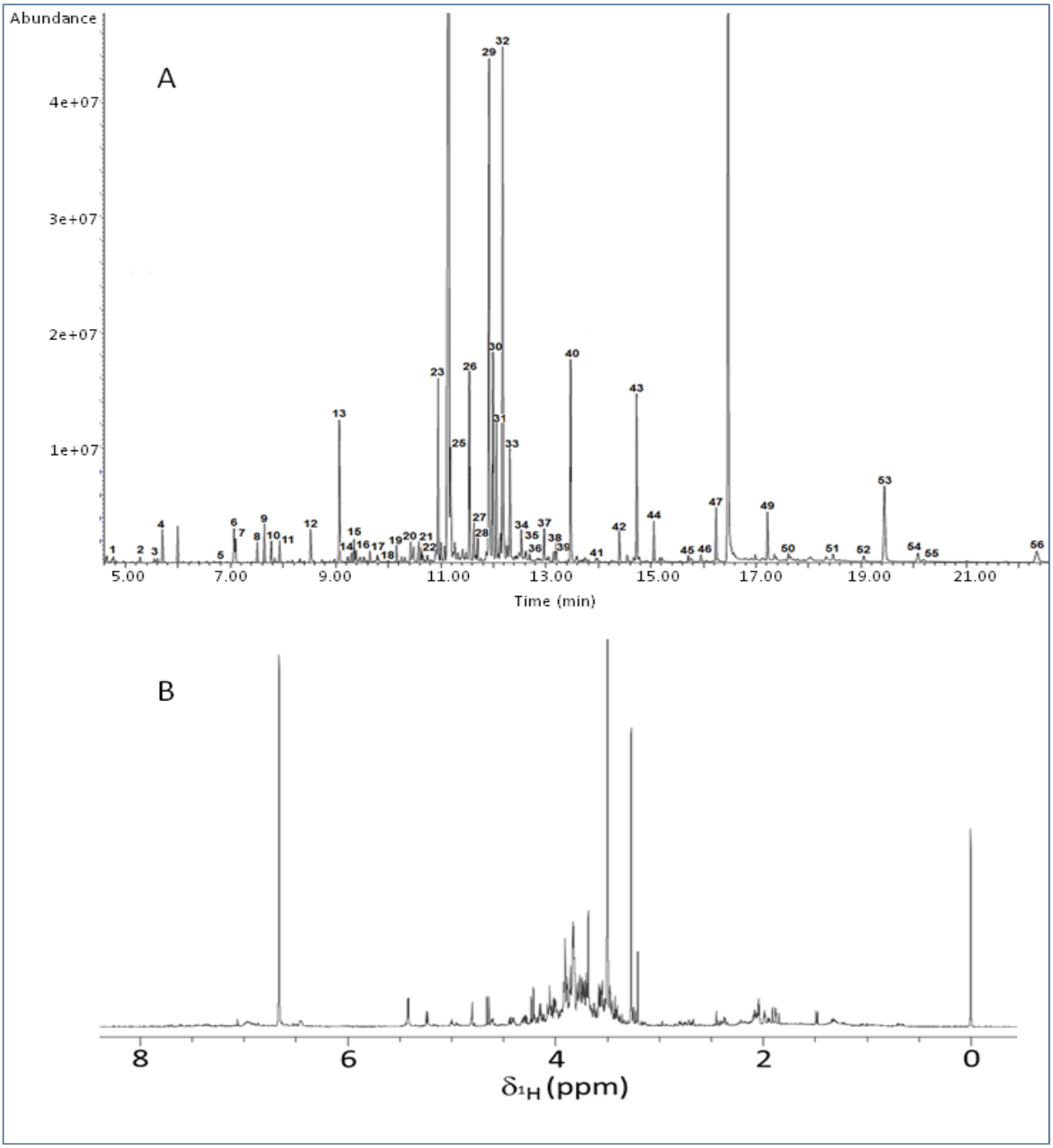


Figure 2. Total Ion Chromatogram (TIC) (A) and ^{1}H-NMR spectrum (B) of maize leaf extract (B0P0 sample).

Table 1a: List of primary metabolites from maize leaves identified by ^{1}H NMR

Amino acids	Assignment	δ (^{1}H)	Multiplicity
Alanine	β-CH_3	1.48	d
Alanine	β-CH_3	1.48	d
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.65	m
Arginine	β-CH_2	1.65	m
Arginine	β-CH_2	1.65	m
GABA	γ-CH_2	3.01	t
GABA	γ-CH_2	3.01	t
GABA	α-CH_2	2.28	t
Glutammic acid	γ-CH_2	2.34	t
Histidine	C4H ring	7.78	s
Histidine	C4H ring	7.78	s
Histidine	C2H ring	7.05	s
Isoleucine	γɜ-CH	1.45	m
Isoleucine	γ-CH	1.25	m
Isoleucine	γ-CH_3	1.02	d
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Phenylalanine	CH3,5	7.44	m
Phenylalanine	CH3,5	7.44	m
Phenylalanine	CH2,6	7.31	m
Phenylalanine	CH2,6	7.31	m
Proline	β-CH_2	2.32	m
Threonine	γ-CH_3	1.32	d
Tryptophan	C4H ring	7.72	d
Tryptophan	C4H ring	7.72	d
Tryptophan	C7H ring	7.54	d
Tryptophan	C7H ring	7.54	d
Tryptophan	C6H ring	7.28	t
Tryptophan	C6H ring	7.28	t
Tryptophan	C5H ring	7.20	t
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Valine	γ'-CH_3	1.04	d
Valine	γ'-CH_3	1.04	d
Valine	γ-CH_3	0.99	d

Carbohydrates	Assignment	δ (^{1}H)	Multiplicity
Fructose		4.08	m
Fructose		4.08	m
Fructose		3.99	ddd
Fructose		3.89	dd
Fructose		3.89	dd
Fructose		3.85	m
Fructose		3.80	m
Fructose		3.79	dd
Fructose		3.70	dd
Fructose		3.70	dd
Fructose		3.65	m
Fructose		3.57	d
Fructose		3.57	d
α-Galactose	C1H	5.27	d
β-Galactose	C1H	4.60	d
α-Glucose	C1H	5.24	d
β-Glucose	C1H	4.64	d
β-Glucose	C1H	4.64	d
Glucose		3.90	m
Glucose		3.90	m
Glucose		3.84	m
Glucose		3.83	m
Glucose		3.77	m
Glucose		3.73	dd
Glucose		3.72	dd
Glucose		3.54	dd
Glucose		3.54	dd
Glucose		3.54	dd
Glucose		3.51	t
Glucose		3.42	t
Glucose		3.40	t
Glucose		3.40	dd
Glucose		3.24	t
Glucose-6-phosphate		5.58	d
Glucose-6-phosphate		5.58	d
Inositol		4.05	t
Inositol		3.61	t
Inositol		3.61	t
Inositol		3.52	dd
Raffinose (Piccioni)	Gal1H	5.00	d
Raffinose (Piccioni)	Gal1H	5.00	d
Raffinose (Piccioni)	Fru3H	4.23	d
Sucrose	Glc1H	5.42	d
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose	$FruCH_2$-1'	3.68	s
Sucrose			
Sucrose			
Trehalose (Piccioni)	C1H	5.20	d
Trehalose (Piccioni)	C1H	5.20	d

Table 1a:continued

Nitrogenous compounds	Assignment	δ (^{1}H)	Multiplicity
Adenosine-like		8.34	s
Adenosine-like		8.34	s
Adenosine-like		8.34	s
Adenosine-like		8.27	s
Adenosine-like		8.27	s
Adenosine-like		8.27	s
Adenosine-like		8.11	d
Adenosine-like		5.99	d
Adenosine-like		5.99	d
Adenosine-like		5.99	d
UMP	C1'H ribose	8.11	d
UMP	C6 ring	5.99	d
Choline	N-CH_3	3.21	s
Choline	N-CH_3	3.21	s
Dimethylamine	CH_3	2.73	s
Glicine betaine (Piccioni)	N-CH_3	3.28	s
Phosphatidylcholine	CH_3	3.27	s
Trigonelline (HA)	HA	9.13	s
Trigonelline (HB, HC)	HB,HC	8.86	d
Trigonelline (HB, HC)	HB,HC	8.86	d
Trigonelline (HD)	HD	8.06	d
Trigonelline (HD)	HD	8.06	d
Trimethylamine	CH_3	2.91	s

Organic acids	Assignment	δ (^{1}H)	Multiplicity
Chlorogenic acid		7.6	d
Chlorogenic acid		7.13	d
Chlorogenic acid		6.87	d
Chlorogenic acid (Broyart)		6.35	d
Chlorogenic acid (Broyart)		5.316	td
Chlorogenic acid		5.316 1	td
Cis-aconitic acid	CH=C-	6.65	s
Cis-aconitic acid	CH=C-	6.65	s
Cis-aconitic acid	CH_2	3.49	s
Formic acid	HCOOH	8.46	s
Fumaric acid (α,β)	α,β CH=CH	6.53	s
Isobutirric acid	CH_3	1.14	s
Isobutirric acid	CH_3	1.14	s
Isocitric acid		2.96	q
Isocitric acid		2.96	q

Organic acids	Assignment	δ (^{1}H)	Multiplicity
Isocitric acid		2.46	dq
Isocitric acid		2.46	dq
Isocitric acid		2.46	dq
Maleic acid	α,β CH=CH	6	s
Malic acid	α-CH	4.32	dd
Malic acid	α-CH	4.32	dd
Malic acid	β-CH	2.68	dd
Malic acid	β-CH	2.68	dd
Malic acid	β-CH	2.68	dd
Malic acid	β–β'-CH_2	2.38	dd
Malic acid	β–β'-CH_2	2.38	dd
Quinic acid	H3	4.131	q
Quinic acid	H3	4.131	q
Quinic acid	H5	4.008	ddd
Quinic acid	H4	3.54	dd
Quinic acid	H6'	2.04	ddd
Quinic acid	H6'	2.04	ddd
Quinic acid	H6'	2.04	ddd
Quinic acid	H2	1.97	dd
Quinic acid	H2'	1.94	ddd
Quinic acid	H6	1.88	dd
Quinic acid	H6	1.88	dd
Quinic acid	H6	1.88	dd
Shikimic acid		6.42	m
Shikimic acid		6.42	m
Shikimic acid		4.38	t
Shikimic acid		4.38	t
Shikimic acid		2.77	dd
Shikimic acid		2.77	dd
Shikimic acid		2.77	dd
Shikimic acid		2.73	dd
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Succinic acid	α–β-CH_2	2.4	s

s= singlet d= doublet
dd= doubledoublet dd=doublet of a oubledoublet
ddq= doublet of a doublequartet q= quartet m= multiplet

Table1b : List of primary metabolites from maize leaves identified by GC-MS.

n°	Metabolites	RT (min)	MS fragmentation (m/z)	References
1	Lactic acid	4.77	52-73-75-117-147-149	NIST
2	Alanine	5.28	73-75-116-117-147-190	STD,MPI
3	Oxalic acid	5.7	73-74-75-147-148-149	NIST
4	Phosphoric acid	7.07	73-133-211-225-299-300	NIST
5	Glycerol tris TMS	7.09	73-117-147-205-299	NIST
6	Succinic acid	7.51	73-147-172-247	NIST
7	Glyceric acid	7.65	73-103-147-189-292	NIST
8	Itaconic acid	7.78	73-147-215-259	NIST
9	Serine	7.94	73-75-116-147-204-218	STD,MPI
10	Threonine	8.18	73-75-130-147-218	STD
11	Maleic acid	8.32	73-75-133-147-148-149	NIST
12	4-Ketaglucose	8.89	73-103-147-204	NIST
13	Arabino-hexose-2-ulose	8.98	73-103-147-205-234-262	NIST,MPI
14	Methylmaleic acid	9.07	73-147-189-233-265	NIST,MPI
15	Aspartic acid	9.35	73-75-100-147-232	STD
16	succinic anhydride	9.39	73-156-255	NIST
17	4-aminobutyric acid	9.47	73-130-175-205-231-260	NIST,STD
18	Tetronic acid	9.65	73-147-220-292	NIST
19	Glutaric acid	9.8	73-147-198-304	STD
20	Glutamine	10.16	73-75-128-147-156-246	NIST,MPI
21	Xylonic acid	10.43	73-117-147-175-217-244	NIST
22	Xylulose	10.66	73-103-147-217-307	NIST
23	**Ribitol IS**	10.95	73-103-147-217-319	STD
24	cis Aconitic acid	11.14	73-147-229-285-375	NIST,MPI
25	Cinnamic acid	11.42	73-147-204-245	STD,MPI
26	Shikimic acid	11.55	73-147-204-255	NIST,MPI
27	sugar	11.58	73-147-205-217	NIST
28	Isocitric acid	11.63	73-147-273-363	NIST
29	Quinic acid	11.92	73-147-191-255-345	NIST,MPI
30	Fructose	12	73-129-133-147-217-218	STD
31	Fructose	12.06	73-129-133-147-217-219	STD
32	Galactose	12.14	73-147-205-217-229	STD
33	Glucose	12.18	73-129-147-149-157-217	STD
34	Glucose	12.32	73-129-147-149-157-218	STD
35	UN	12.54	73-147-219-292	NIST
36	UN	12.97	73-147-221-449	NIST
37	Mucic acid	13.15	73-147-217-292-333	NIST
38	UN	13.35	73-103-147-221-449	NIST
39	Myo-Inositol	13.47	73-147-191-217-221-305	STD,MPI
40	Floridoside	13.73	73-103-147-204-337	NIST
41	Glucuronic acid	13.86	73-147-205-217-292-375	NIST
42	UN	14.11	73-147-204-321-361	NIST

Table 1b :continued

n°	Metabolites	RT (min)	MS fragmentation (m/z)	References
43	sugar	14.81	73-129-133-147-217-219	NIST
44	UN	15.1	73-147-204-321-361-406	NIST
45	oligosaccaride	15.5	73-147-217-361-450	NIST
46	oligosaccaride	15.53	73-147-217-361-450	NIST
47	oligosaccaride	16.2	73-147-217-361-450	NIST
48	Sucrose	16.47	73-169-217-243-361-362	NIST
49	UN	16.55	73-147-204-217-361	NIST
50	D-Turanose	17.35	73-147-217-361-450	NIST
51	Maltose	18.47	73-147-204-217-305-361	NIST
52	UN	19.05	73-147-219-255-345	NIST
53	Melbiose	19.45	73-103-129-204-217-361	NIST
54	UN	20.19	73-147-191-249-345-447	NIST
55	Chlorogenic acid	20.08	73-147-191-255-307-345	STD,MPI
56	Raffinose	22.35	73-147-129-205-217-361-437	NIST

NIST= mass spectra library NIST 05 (http://www.nist.gov); STD=standards; MPI=Max-Planck-Institute (Germany, http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html).

3.3 Metabolic analysis of plants treated with different P fertilization

The PCA score-plot (48.29% of the total variance) of samples from different P fertilization treatments is reported in Figure 3a, where a separation between treatments is noticeable. In particular, the PC1 allowed to distinguish the B0P1, B0P2 and B0P3 treatments from the B0P0 and B0P4 treatments. Such differentiation was statistically significant ($p < 0.05$) due to metabolites such as fructose, glucose, inositol, chlorogenic acid, shikimic acid, sucrose, quinic acid, galactose, trehalose, and raffinose, which were more and less abundant in the former and latter group of experiments, respectively. Remarkably, all these compounds correlated with the high concentration of P in plant from the B0P1, B0P2 and B0P3 treatments (Figure 1), thus suggesting an improved nutritional status due to the P2 inorganic and P3 compost fertilizers.

Conversely, the positive PC2 values (31% of the total variance) were associated with large concentrations of GABA, shikimic acid, isocitric acid, arginine, leucine, isoleucine, valine and phosphatidylcholine, which allowed to discriminate plant samples of the B0P4 treatments from those of other treatments. In particular, the GABA (γ-Aminobutyric acid) metabolite represents a significant component of the free aminoacidic pool, and the rapid increase of its content is frequently associated to abiotic stresses, althoughthe specific roles of GABA under these conditions is not yet clear (Kinnersley & Turano, 2000; Fait *et al*., 2008). Genetic studies showed that a change in GABA concentration had severe consequences on plant development (Bouché *et al*. 2003). However, a correlation of GABA content with a poor P and sugar metabolites concentrations suggests a state of stress on the plants, being a sign of insufficient plant growth.

The great content of phenylalanine, chlorogenic acid, quinic acid and three unassigned variables (UN6, UN8 and UN9) in plants from the B0P1 treatments allowed the discrimination of such plant metabolome along the negative values of PC2. The concomitant presence of aromatic amino acids, such as phenylalanine, chlorogenic acid and quinic acid, may indicate an enhanced production of secondary metabolites, which can be further attributed to plant stress conditions. The presence of phenylalanine may indicate the activation of phenylpropanoid pathway, that is a critical process to biosynthetize a plethora of secondary plant products such as, anthocyanins, lignin and phenols, while the chlorogenic acid formed between caffeic and quinic acids (Molgaardand, 1988) is commonly considered to be an intermediate in the lignin pathway (Schoch *et al*., 2001). These metabolite are reported to accumulate inside the vacuoles or in the apoplast during leaf ageing or stress condition (Takahama *et al*., 1998). Moreover, the mass fragmentation of the UN9 variable, shown in Supplementary Figure 1, indicates a metabolite structurally similar to chlorogenic acid, thereby supporting the hypothesis of an enhanced formation of secondary metabolites.

Conversely, the allocation of B0P0 plant extracts within quadrant III of the PCA was due to a lower concentration of most of the identified variables, such as sucrose, glucose, fructose and the amino acids arginine, leucine, isoleucine, valine and phosphatidylcholine. These results, correlated to the

lower content of P, suggest an altered photosynthesis in leaves and reduced carbon metabolisms, thereby negatively affecting plants growth (Foyer & Spencer, 1986; Fredeen *et al*., 1989; Rao & Terry,1995; Rao,1996).

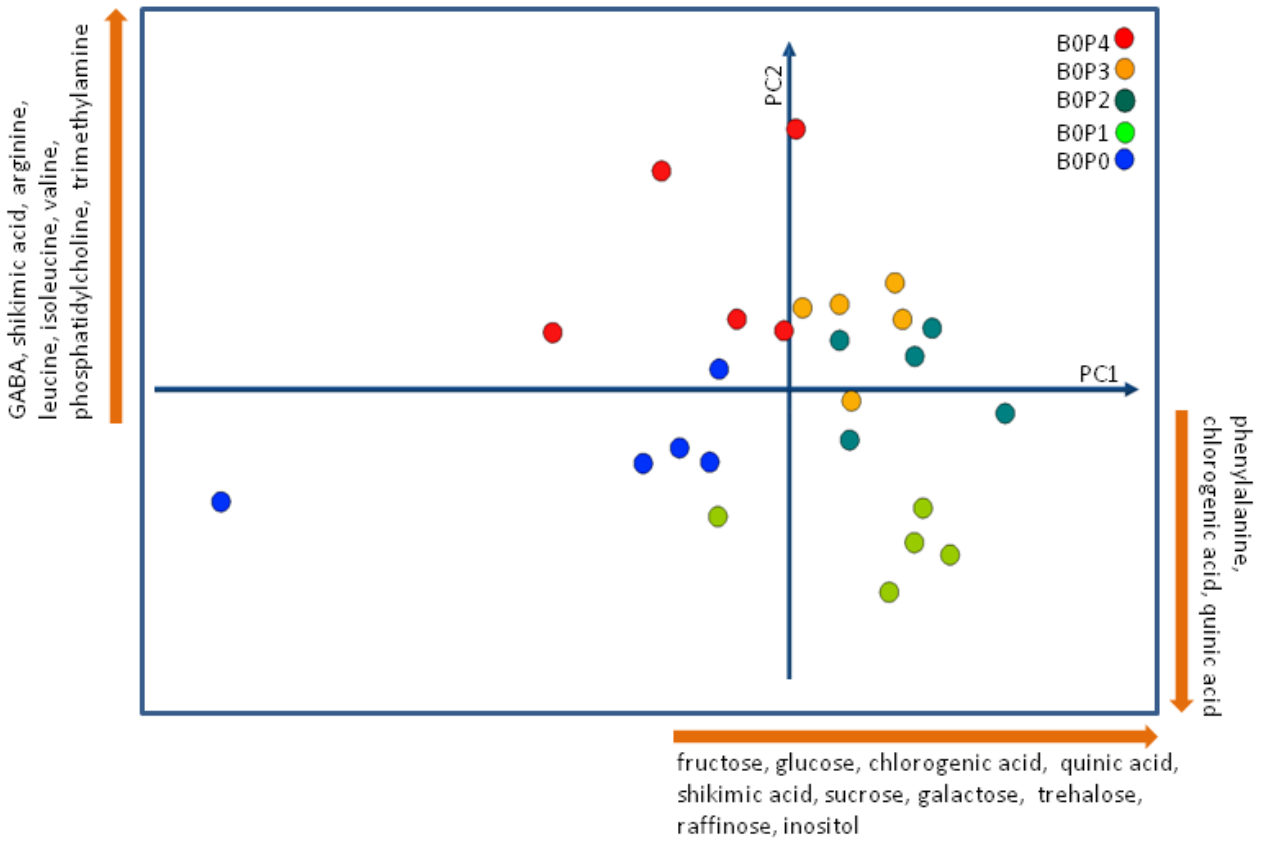


Fig. 3a. PCA score-plot obtained by processing GC-MS and NMR data of plants treated with different P-based fertilizers (P0-P4).

3.4 Effects of Bacillus amyloliquefaciens on the metabolite composition of plants with different P fertilization sources

The PCA model (Fig. 3b), with 68.44% of the total variance explained, revealed a good clustering of all plants that were inoculated with *Bacillus amyloliquefaciens* and subjected to the different phosphate fertilizations. Results showed a robust reproducibility among replicates. In particular, the first principal component (PC1) accounted for 45.49% of the total variance and well differentiated

the B3P0 and B3P1 treatments from the rest. Such differentiation was statistically significant ($p < 0.05$) for the variables (chlorogenic acid, shikimic acid, quinic acid, trehalose, raffinose, tryptophan, tyrosine and phenylalanine) which showed large concentrations in plant leaves and were placed along the positive values of PC1.

Furthermore the same variables, with the exception of chlorogenic acid, tryptophan and raffinose, enabled to separate the plants from the B3P3 and B3P4 experiments, which were treated with different compost. This discrimination between plant samples, suggests the that different molecular composition of the two composts may affect the plant metabolome (Spaccini and Piccolo 2009), thereby indicating the role of the micro-bio-humeome in these conditions.

Conversely, the metabolic profiles of plants treated with organic fertilizer (B3P3, B3P4) were differentiated from all other treatments along the PC2, that accounted for 22.94% of the toral variance. In particular, the plant extracts were discriminate along the positive values of PC2 on the basis of the large concentrations of glucose, fructose, galactose, GABA, alanine, phosphatidylcholine, coline, isoleucine, valine, shikimic acid, malic- and methylmaleic acid. These metabolites, were correlated with a large content of P and N in plant leaves. Specifically, the increased content of glucose and fructose, that represents the end product of photosynthesis was mainly affected by the high N concentration (Field & Mooney, 1986 ; Meir *et al*., 2002). These results appear to indicate an enhanced photosynthesis activity of plants, due to the combined use of bio-effectors. In fact, the high P and N uptake by plants affect also protein synthesis, thus explaining the great content of amino acids.

The large amount of GABA in samples from the B3P3 and B3P4 treatments, may represent an excellent indicator for the energy state of cell, particularly sensitive to the availability of C and N. In fact, this metabolite acts as signal to either stimulate the plant tissue to accumulate more energy, or reduce the excess energy (Michaeli & Fromm, 2015). Hence, these results suggest the the positive effects of *Bacillus amyloliquefaciens* on the nutritional state of plants under compost fertilization.

Plants under mineral fertilizers (B3P1, B3P2) showed a smaller content of sugars and amino acids than for plants treated with composts (B3P3, B3P4), and also revealed lower P and N content. In particular, the large content of tryptophan, tyrosine and phenylalanine in B3P1, suggests an enhanced production of secondary metabolites.

In particular, the relevant presence of phenylalanine and tryptophan serves as substrate for the phenylpropanoid pathway (Vogt, 2010), that, in turn, is a critical step for the biosynthesis of many secondary plant products, such as anthocyanins, lignin and phenolics (Leopold & Kriedman, 1975; Tzina & Galili, 2010). Moreover, also the presence of chlorogenic and shikimic acids confirms an enhanced synthesis of secondary metabolites.

Phenylalanine in the phenylpropanoid pathway represents a precursor for a variety of secondary plant products, such as anthocyanins, lignin, and phenolics. On the other hand, tryptophan is the precursor for the indolacetic acid that is involved in cell expansion and many other regulatory processes (Leopold & Kriedman,1975; Tzina &Galili, 2010). The synthesis occurs through the shikimate pathway, followed by the branched aromatic aminoacid metabolic pathway (Vogt, 2010). The great content of chlorogenic acid, shikimic acid, quinic acid in plant leaves indicates an enhanced synthesis of secondary metabolites, also partially involved in the biosynthesis of lignin (Schoch *et al*., 2001), which are probably due to various biotic and abiotic stress conditions, such as wounding, resistance to pathogen infection, metabolic stress or perturbations of cell wall structure (Caño-Delgado *et al*., 2003; Tronchet *et al*., 2010).

Despite the amount of P and N resulted identical for B3P2 and B3P1, the PCA reported in Figure 3b revealed a lower amount in B3P2 for all of variables mostly represented by PC2. This result suggested a similar nutritional status in these two plant groups, even though B3P1 was presumably subjected to a stronger condition of stress. Conversely, the lower content of P and N detected for B3P0 plants well reflected the scarce concentration of all metabolites represented by PC1 and PC2, thus inducing their PCA collocation along negative values of PC1 and PC2 (Figure 3). Such results concerning the control plants, may be attributed to a worse state of nutrition, a lower photosyntetic

activity and, probably, to a growth inhibition which, on the whole, confirm the positive and efficient action exerted by each of studied treatments.

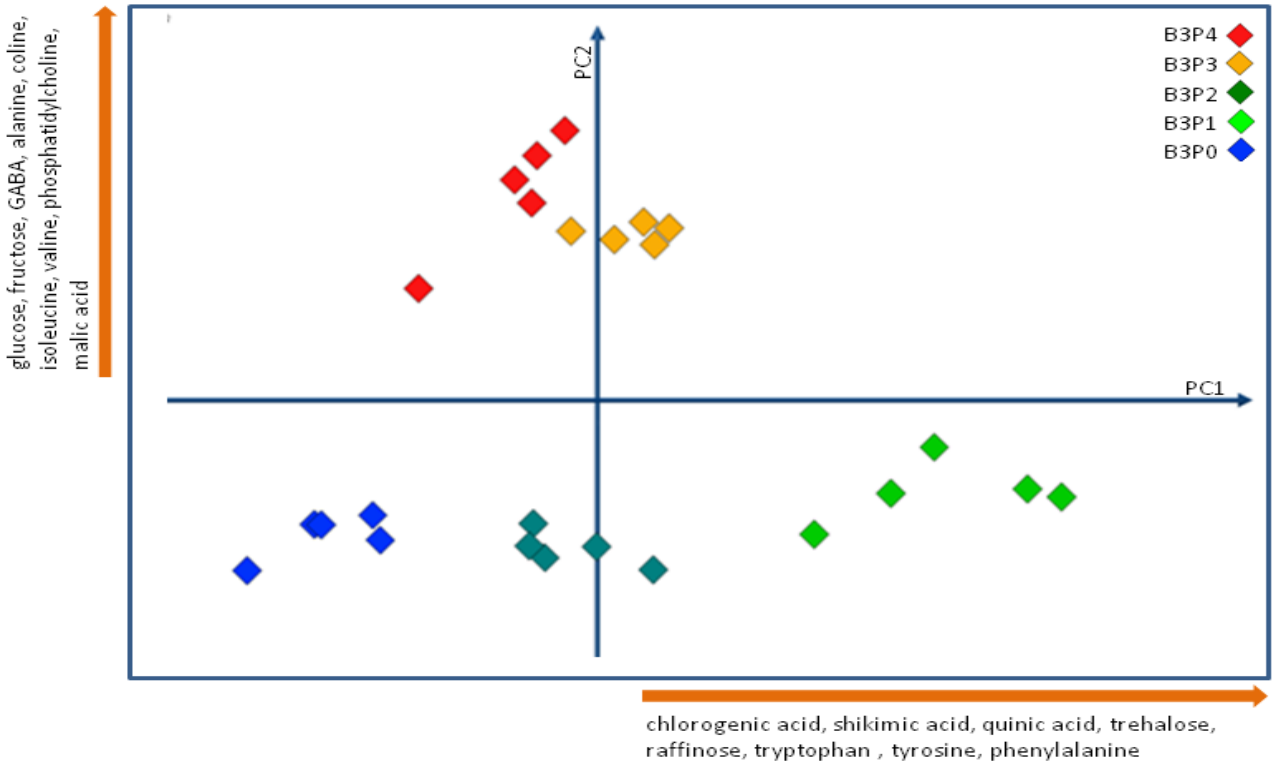


Fig. 3b. PCA score-plot obtained by processing GC-MS and NMR data of plants inoculated with *Bacillus amyloliquefaciens* and treated with different P-based fertilizers (P0-P4).

3.5 Comparison effects of different P fertilization with or without the inoculums of Bacillus amyloliquefaciens.

The PCA score-plot (Fig. 3c) obtained by collecting all variables from all treatments, with a total variance explanation of 57.4%, showed that the effect of *B. amyloliquefaciens* allowed a better separation and clustering of the inoculated plant samples from control samples. In particular, along the PC1 (accounted for the 38.8%) it was possible to distinguish the samples B3P1 from B3P0 that respectively reveled the larger and lower content of chlorogenic acid, shikimic acid, quinic acid, tirosine, threalose, raffinose and glutammate. The others samples showed intermediate values for these variables.

The PC2 was able to distinguish the samples B3P3 and B3P4 from the other for the higher concentration of glucose, fructose, galactose, GABA, alanine and valine. Remarkably, all of these compounds correlated with the high concentration of P and N found in the samples B3P3 and B3P4 (Figure 1) thus suggesting a better nutritional status in these plants and underlining the positive effect exerted by *Bacillus amyloliquefaciens* when associated to compost.

These results make it possible to better highlight the effects of *Bacillus amyloliquefaciens* on the different phosphate treatments. In fact, when inoculated, the plants shown an enhanced separation along the PC axis due to an increased weight of variables involved in such differentiation.

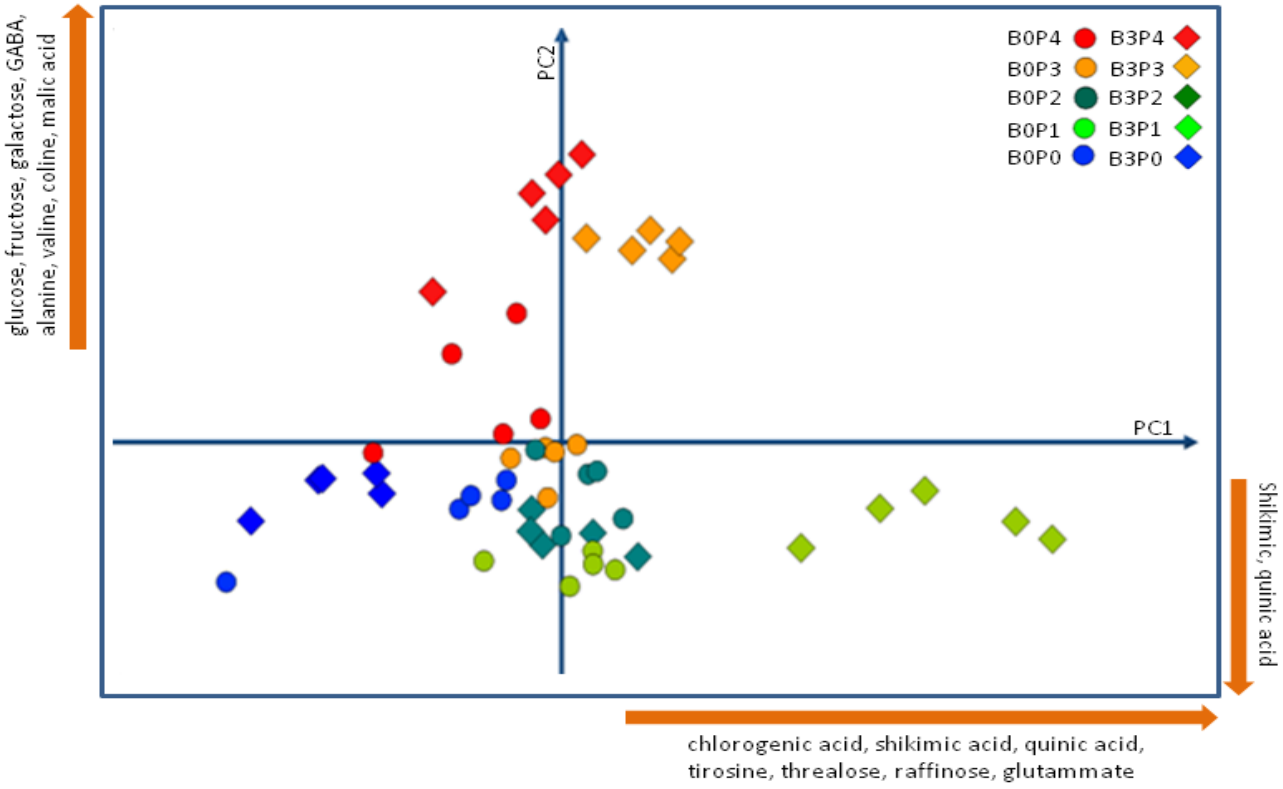


Fig. 3c. PCA score-plot obtained by processing GC-MS and NMR data of plants treated with different P-based fertilizers (P0-P4), either with (B3, rhombus) or without (B0, circle) the inoculation of *Bacillus amyloliquefaciens.*

3.6 Effects of different inorganic P fertilization with or without the inoculums of Bacillus amyloliquefaciens.

The effects on plants metabolites by *B. amyloliquefaciens* in the presence of different inorganic fertilizers are shown in Fig. 3d. According to the different metabolites concentrations, the PC1 accounted for a 53.6% of the total variance, and was able to discriminate samples from the B3P1 treatments from those of the B3P0 and B0P0 treatments. This discrimination was due to the large concentration of chlorogenic acid, shikimic acid, fructose, glucose, galactose, raffinose, inositol and glycin betaine found in samples of the B3P1 treatments.

Conversely, the variables glycerol, serine, threonine and melbiose associated to positive PC1 values and the variables valine, isocitric acid, GABA associated to negative PC1 values, were able to discriminate B3P2 samples from B0P2.

The PC3 axis, accounted for the 6.98% has allowed to separate the samples B0P1 by the others for the high concentration of phenylalanine and sucrose. Such samples exhibit also an low abundance of formic acid, adenosine metabolites, GABA, Alanine, glycerol, glyceric acid, and the floridoside.

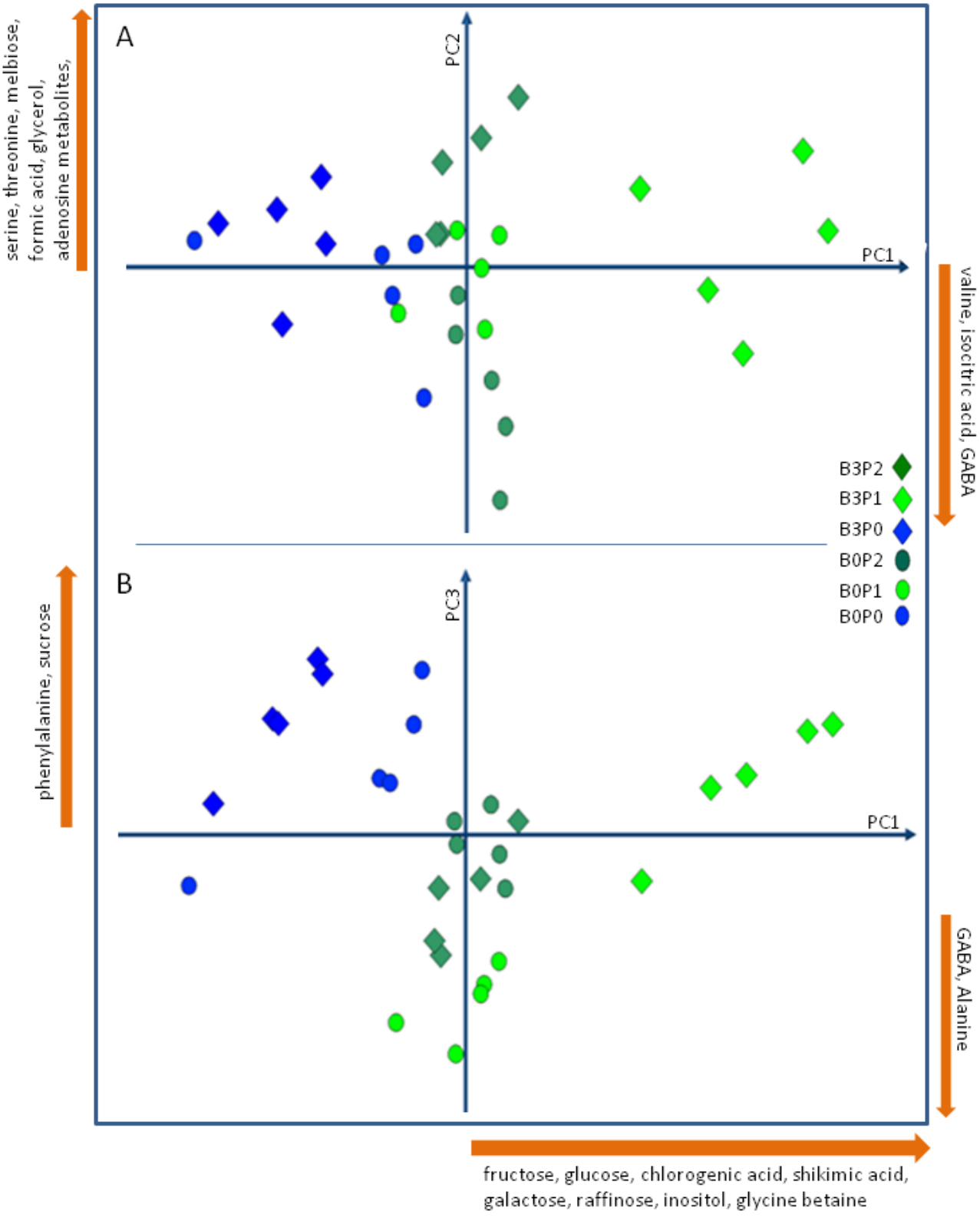


Fig. 3d : PCA score-plots (PC1/PC2, A) and (PC1/PC3, B) obtained by processing GC-MS and NMR data of plants treated either without (B0, circle) or with *Bacillus amyloliquefaciens* (B3, rhombus) and compared with plants amended with mineral fertilizers.

3.7 Effects of Bacillus amyloliquefaciens on the metabolite composition of plants treated with compost.

In Figure 4B is shown the PCA score-plot (48.56% total variance explained) of samples treated with organic fertilizers, either without or with the concomitant inoculum of *B. amyloliquefaciens*. The score-plot (Fig. 3e), with a total explanation variance of 53.66%, clearly shows a net and statistically significant discrimination between treatments. Particularly was evident a migration along the PC2 negative values of the samples inoculated with *B. amyloliquefaciens* when compared with controls. The results underlined also, that the two composts differently influence the plants metabolite profiles in all cases. To the positive values of PC1 (37.65% of variance) were associated high amounts of fructose, glucose, sucrose, tryptophan, phenylalanine, raffinose, inositol and shikimic acid. Such metabolites, in particular the high concentration of glucose, fructose and sucrose correlated with the high content of P and N in B3P3 samples, allow to speculate a more high photosynthetic activity of these plants. On the basis of these amount of metabolites were distinguished the groups of samples B3P3,B3P4 from B0P3,B0P4 and from B0P0,B3P0.

Conversely, the PC2 allow the separate the samples B3P4 from the other, as function of a higher concentration, of adenosine metabolites, alanine, serine, threonine, aspartic acid, glutamic acid and glycerol. Indeed, at positive values of PC2 were associated high levels of phenylalanine, shikimic acid, quinic acid, and tryptophan. These variables, characterize the groups of samples B0P0,B0P3 from the others, underlined an enhanced synthesis of protein or secondary metabolites probably induced upon various biotic and abiotic stress conditions (Caño-Delgado *et al*., 2003; Tronchet *et al*., 2010). Conversely the allocation of B3P0 along the negative values of PC1 was due to the low metabolite concentration as compared to the other samples. Such results, correlated to the lower content of P and N confirm a worst nutritional state and probably an inhibited growth.

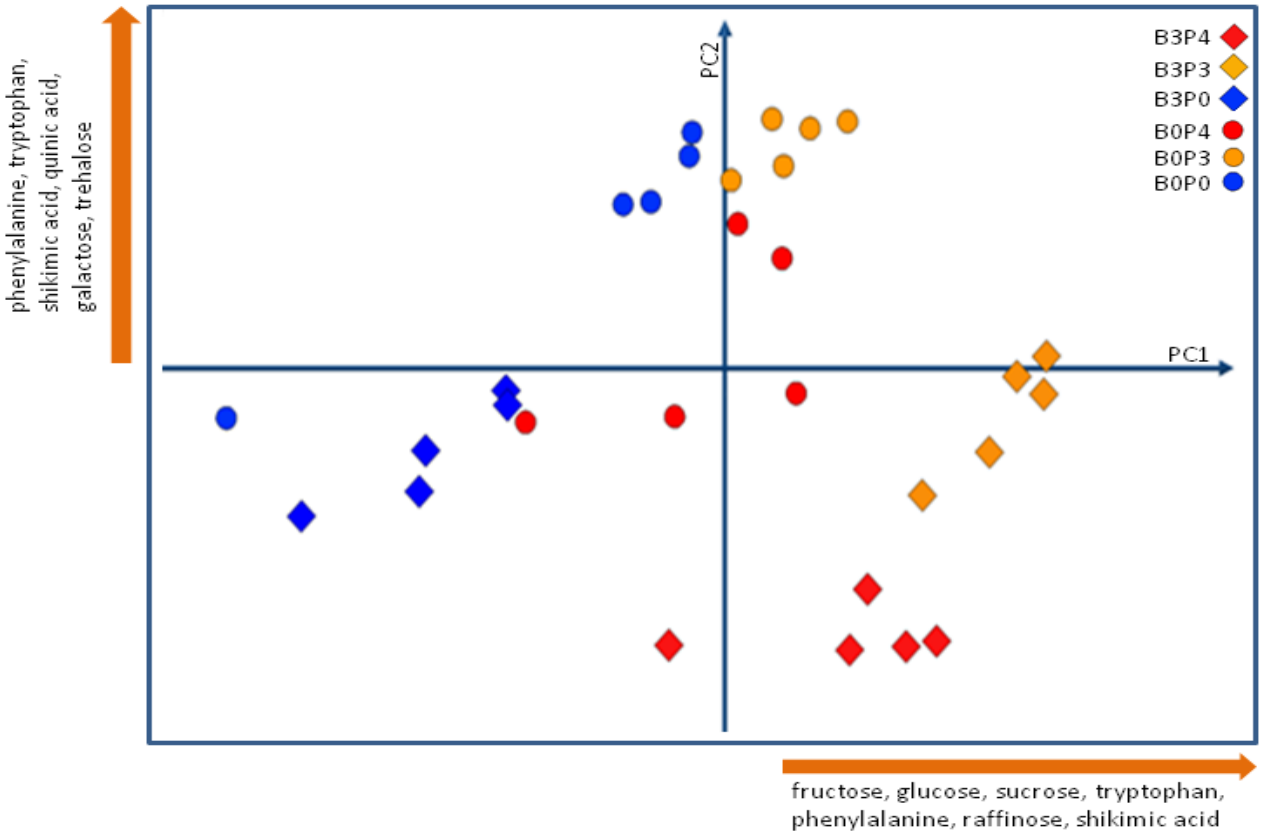
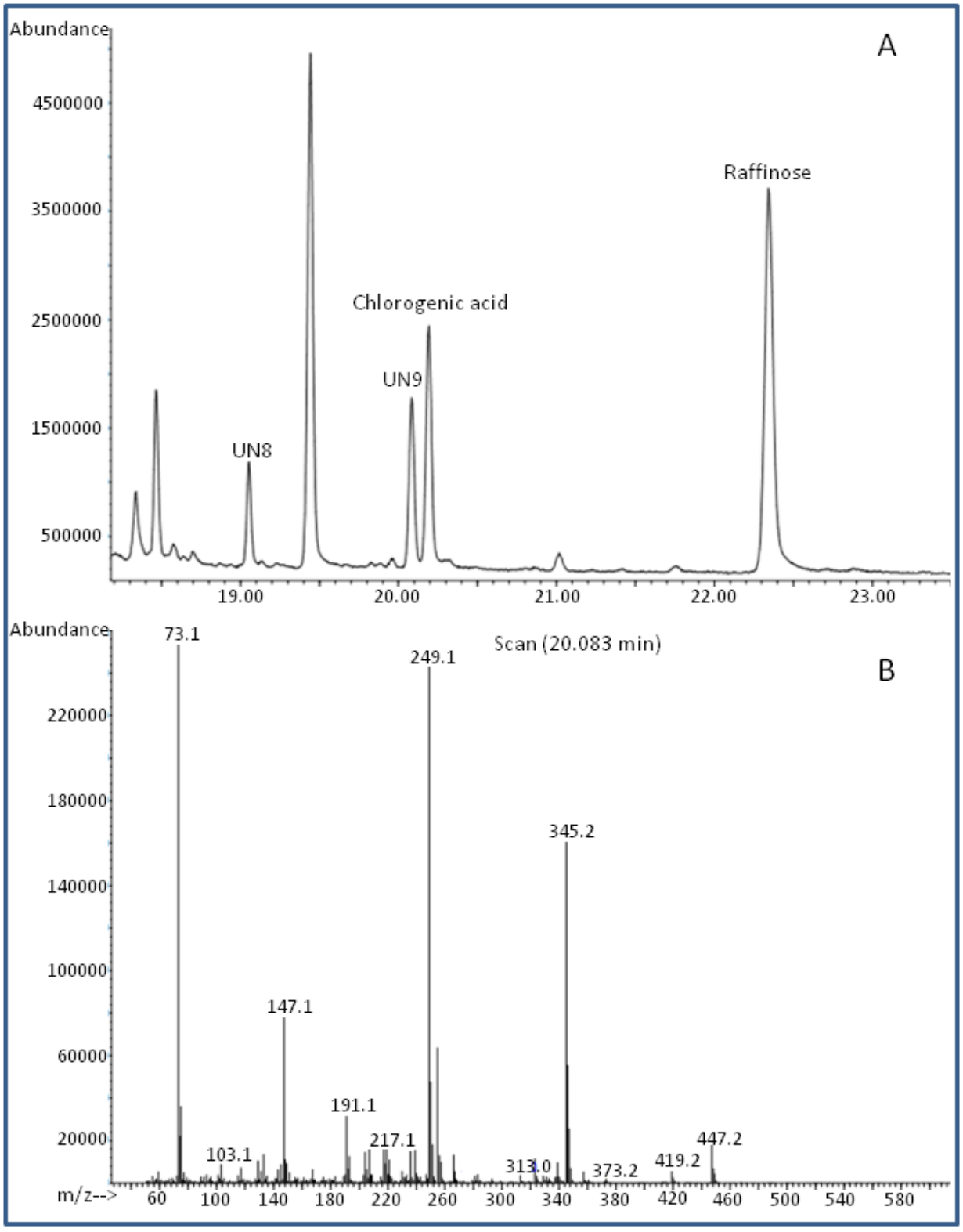


Fig. 3e. PCA score-plots obtained by processing GC-MS and NMR data of plants treated either without (B0, circle) or with *Bacillus amyloliquefaciens* (B3, rhombus) and compared with plants amended with different compost.

4. Conclusions

The purpose of the present work was to establish if different phosphatic fertilizer (RF, TSF) or different compost, may influence the metabolism of the maize plants, the content of P and N and consequently their growth. Furthermore, was evaluated the influence of *Bacillus amiloliquefaciens* as a promoter of plant growth according to its ability to increasing the P solubilization (Idriss *et al*., 2002; Richardson *et al*.,2009). To this end they were been performed metabolomic analyses of polar metabolites extracted from the youngest fully expanded leaves for each plant by GC-MS and NMR. The results underlined a different metabolic profile of the plants treated with different P source. The samples B0P1,B0P2 and B0P3 have been shown similar content of P and N correlated with high

values of glucose, fructose and sucrose metabolites underlined an higher photosynthetic activity and therefore, a better nutritional state to respect the other treatments. Conversely, B0P4 like B0P0 showed worst value of P and N uptake correlated to lower concentration of all metabolites involved into the photosynthetic activity. These condition do not allow thus an adequate plant growth. When the P treatments were associated with inoculums of *Bacillus amiloliquefaciens* , there has been an increased discrimination of all samples in the PCA. The enhanced discrimination was due to the highest content of P and N that influenced principally, the metabolic profiles of samples treated with composts (B3P3 and B3P4). These results allowed to highlighting a better nutritional state, showing the ability to sustain an higher growth of these plants. The *B. amiloliquefaciens* did not affect the content of P and N of samples treated with mineral fertilizers, thus showing an unchanged growth capacity. Conversely B3P0 samples were found to be adversely influenced to the *B. amiloliquefaciens* probably due to a state of competition between plants requests and microorganisms. Overall, these results confirm that the combination of organic fertilizer like composts, with phosphate solubilizing bacteria like *Bacillus amiloliquefaciens*, provide an valuable alternative to satisfy the plant P requirements, thus limiting the use of the more expansive and environmentally unsustainable mineral fertilizers.



Supplementary figure 1: TIC and mass fragmentation of the variable UN9 R.T. 20.08.

CHAPTER 4

^{1}H NMR and GC-MS Metabolomics reveals the influence exerted on *Zea Mays* by *Trichoderma harzianum* and different phosphorous based fertilizers

Abstract

During the past century, the worldwide agricultural productivity has been strongly improved by selecting efficient crop varieties and applying an intensive use of mineral fertilizers. However, the excessive use of such fertilizers is recognized to alter soil micro-flora, structure and composition. Conversely, organic farming may represent a valuable alternative since it consists of recycling organic matter to be used either alone or in combination with beneficial soil microorganisms. In this study, plants of *Zea mays* were grown in pots and amended with several P-based fertilizers whose composition was either mineral (RP and TSP) or organic (cow and horse compost). In addition, the set of treatments included the inoculation of *Trichoderma harzianum,* which represents one of the most efficient plant growth promoting fungi (PGPF). Hence, aiming to evaluate the influence exerted by such rizospheric interaction, called micro-bio-humeome, on plants metabolism, we used Gas Chromatography–Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) techniques to examine the leaves metabolome. The metabolomic profiles revealed a broad range of primary polar metabolites, including sugars, organic acids, amino acids, phenolic acids, amino sugars and sugar alcohols. GC-MS and NMR data were processed by PCA exercises which highlighted an enhanced influence exerted by studied fertilizers which was even enhanced when combined with *Trichoderma* inoculation. In particular, our results suggested that the amendment with organic compost, if combined with *Trichoderma*, may satisfy the plant P demand and promote the growth thus positively influencing maize plants and limiting the use of mineral fertilizers.

Keywords: Metabolomics, *Trichoderma harzianum*, Compost, RP, TSP, GC-MS, ^{1}H-NMR.

1. Introduction

Conventional farming represents the most common agricultural practice to guarantee a satisfying crop production and face an ever-increasing demand for human food. It has been estimated that in the next 50 years the total population will reach around 1.5 billion people thus making mandatory a further increase in food production (IFA, 2012). In the last decades, the so called "Green Revolution" has allowed food production to keep pace with worldwide population growth by paying the cost of intensive applications of supplementary nitrogen (N) and phosphate (P) nutrients through mineral fertilizers (Den Herder *et al*., 2010). This increasing dependence on mineral fertilizers, especially when accompanied by the use of chemical pesticides, is responsible to affect the optimal environmental conditions. In fact, inorganic fertilizers are involved in severe alteration of soil micro-flora, structure and composition (Reddy *et al*., 2002; Koliaei *et al*., 2011). Consequently, a long-term overuse of these fertilizers may affect the soil fertility and lead to a substantial reduction of crops yields (Gyaneshwar *et al*. 2002). Additionally, the reserves of rock phosphate (RP), which provide the raw material to produce inorganic fertilizers, including the Triple Superphosphate (TSP), are presumed to be drastically depleted within the end of this century (Schroder *et al*. 2011) thus implying inevitable repercussions on the costs of these fertilizers. Hence, the development of alternative low-cost and eco-sustainable managements in crop production has become mandatory to reduce the use of mineral fertilizers and preserve soil fertility (Cordell *et al*., 2009).

A valuable alternative to the conventional agricultural practices is represented by the organic farming, that, through amendments based on recycling organic matter, not only ensures food safety but also adds biodiversity to the soil. The organic farming also provides the integrated use of bio-stimulants composed mostly by autochthonous microorganisms that ensure beneficial effects on plants productivity and health (Whipps, 2004; Avis *et al*., 2008). Among the treatments based on organic matter, the compost represents one of the most utilized form of recycling product by land

farmers being it composed by stabilized organic matter serving as efficient soil amendment (Barker, 2010; Hargreaves *et al* 2008). In the last decades it has been demonstrated the ability of compost in increasing soil porosity, structural stability, moisture and nutrient availability, biological activity, root aeration and protects soil from erosion (Garcia-Gil *et al*., 2000; Kowaljow *et al*., 2007; Weber *et al*., 2007). The benefits also include the ability to mediate soil-borne plant pathogen suppression with a significant impact on crop management (Pane *et al*., 2013). Importantly, compost produced from animal manure represents an inexpensive source of bioavailable P for plants thereby it is frequently used in substitution or in combination with inorganic fertilizers.

The relationship between microorganisms and plants depends on sophisticated nutritional and chemical signaling as well as on soil and climate factors (Ortìz-Castro *et al*., 2009). At the same time, natural biostimulants suitable for a sustainable agriculture are represented by plant growth promoting fungi (PGPF). In particular, the fungal genus of *Trichoderma* is recognized as one of the most efficient and beneficial microorganism interacting with plants. In fact, it is well documented that the symbiosis between plants and *Trichoderma* strains may promote growth thus indirectly improving crop yields (Harman *et al*., 2004; Benítez *et al*., 2004). *Trichoderma* genus may also serve as biocontrol agent via mycoparasitism mechanisms (Harman *et al*., 2004), as well as it is responsible to enhance disease resistance in symbiotic plants (Reino *et al*., 2008; Vinale *et al*., 2008). These beneficial properties are accomplished via modulation of root architecture or through the exudation of substances capable to increase plant access to scarce or not available minerals in soil, including the sources of phosphorus (Hinsinger, 2001; Vance *et al*., 2003; Samolski et al., 2012). Such positive effects related to trichoderma-plant interactions are expected to reflect substantial changes in plant metabolism, thus influencing metabolome (Vinale *et al*.,2008; Yedidia *et al*., 2001), proteome and transcriptome (Marra *et al*., 2006; Alfano *et al*., 2007; Shoresh & Harman, 2008) of symbiotic plant.

Despite the beneficial potentialities ascribed to biostimulants such as compost organic fertilizers and *Trichoderma* inoculations, so far, a deep and exhaustive knowledge on biochemical and

molecular processes elicited in plants by these treatments still lacks. Therefore, at the best of our experience, we aimed to fill this gap by investigating the effects exerted on metabolome of *Zea mays* plants treated with *Trichoderma* fungi either alone or in combination with different fertilizers containing the same amount of available P but administrated in mineral (PR, TSP) or organic (composts) forms. This evaluation was carried out by using a metabolomic approach which is an emerging analytical methodology capable to provide precious insights into molecular responses induced by specific environmental and nutritional conditions (Bundy *et al*., 2009). We used Metabolomics to examine the polar metabolites which were extracted from *Zea mays* plants and detected by both gas chromatography coupled to mass spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR). Finally, the content of P and N was estimated in order to assess the general nutritional status of plant and support the metabolomic results.

2. Materials and methods

2.1 Experimental design, plant growth, sampling and analyses

Maize plants (*Zea mays cv. Colisee, KWS*) were grown in pots (3 L) filled with 2.5 kg of substrate, prepared using an alkaline clay-loam soil mixed with quartz sand in a ratio of 2:1. The substrate was incubated in covered plastic boxes at 20 ± 2°C during 30 days prior to seeding. Available soil P concentration (Olsen method) was 12 mg P/kg. The treatments consisted of factorial combinations of five types of P fertilizer: 1) P0, no P addition; 2) P1, triple superphosphate (TSP); 3) P2, rock phosphate (RP); 4) P3, buffalo manure composted; 5) P4, horse manure composted and two microbial treatments : 1) B0 (no inoculation); 2) B6 *Trichoderma harzianum* (OmG08).

All treatments were replicated five times, for a total of 50 pots. The P fertilizers were applied at the rate of 50 mg P kg^{-1} dry substrate. Moreover, P3 and P4 were applied 15 days before sowing. A

basal nutrients supply was performed adding nitrogen (N) as $Ca(NO_3)_2$ at the rate of 150 mg N kg^{-1} dry substrate and potassium (K) as Kalimagnesia (30% K_2O + 10% MgO) at the rate of 166 mg K kg^{-1} dry substrate. Maize seeds were sown prior microbial inoculation, three seeds per pot. *Trichoderma* was applied as a suspension of demineralized water with 2.5 mM CaSO4 spraying it on seeds surface at sowing, at the rate of 15 x 10^4 spores g^{-1} dry substrate. The pot trial was conducted in a greenhouse. Watering with demineralized water was performed manually to maintain the water holding capacity between 40 and 70% throughout the experiment.

Eight weeks after sowing, the youngest fully expanded leaves were collected for each plant, possibly in the time range included within 8:00 and 12:00 AM. Harvested leaves were weighed and immediately frozen in liquid nitrogen in order to quench the metabolism priot to be stored at -80°C.

Total P in maize shoots was obtained by first digesting 500 mg powdered dry plant material by the ashing procedure according to Gericke and Kurmies (1952) and then colorimetrically determining P by the molybdenum blue assay method (Murphy and Riley 1962). Nitrogen was determined by an elemental analyzer Fisons EA 1108 (Fisons Instruments, Milano, Italy). Then, total N and P have been calculated by proportionally modulating P and N values as a function of total dry biomass of shoots.

2.2 Extraction of plant leaves metabolites.

Maize leaves stored at -80°C were homogenized by using a mortar and pestle with liquid nitrogen. Then, 50±0.5mg of pooled homogenized plant material was weighed into pre-chilled 2 mL Eppendorf tube. The metabolites extraction was conducted by adding 1 mL of water/methanol/chloroform mixture (1:3:1 ratio) pre-cooled at -20°C, that also contained 20 μg ml^{-1} of Ribitol, as an internal standard. The samples were stirred for 30s and incubated for 15min at 70°C in order to inhibit the activity of possible enzymes present in the extract. The mixtures was then centrifuged for 10 min at 10000 rpm and 4°C, and the supernatants were recovered and

transferred into 2 mL Eppendorf tubes. Pure water (400 μL) was added to allow the separation of polar and apolar phases corresponding to the methanol/water (upper) and chloroform (lower) phases, respectively. All extracts were finally stirred for 30 s and centrifuged for 10 min at 4°C and 10000 rpm. A volume of 400 μL was collected from the upper phase, transferred into 1.5 mL glass tubes for GC–MS analyses, dried under a flow of nitrogen and stored at -80 °C. Tree blank tubes were subjected to the same procedure.

2.3 Preparation of metabolites to analytical detrmination

Before derivatization for GC–MS analyses, the metabolic extracts were again dried under nitrogen flux in order to remove the humidity possibly acquired during storage at -80°C. Dried samples were redissolved in 50 uL of a solution of methoxyamine hydrochloride solubilized in pyridine (20mg ml^{-1}) and treated for 90 min at 30°C by applying a gentle shaking. After the methoximation step, the samples were silylated for 30 min at 37°C by using 50uL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) reagent. 30 minutes after the end of derivatisation, 2uL of each sample was injected into a GC column by using a split mode.

In case of NMR analysis, it was used the same extraction procedure ruling out that both plant material to be extracted and the volumes of extraction solvents were doubled in order to take into account the lower sensitivity of NMR spectroscopy. In this case 800 μL of supernatant were dried under a flow of nitrogen, stored at -80 °C and resolved in the same volume of deuterated phosphate buffer (90 mM, pH 6.0) containing 0.05 mg mL^{-1} 3-(tri-methylsilyl) propionic-2,2,3,3-d4 acid (TMSPA, $\delta\ ^{1}H = 0$ ppm; > 99%, Euriso-Top, France) which served as internal standard. Aiming to guarantee the complete dissolution of each metabolic extract, a 5 minutes long sonication was applied prior NMR analysis. Five replicates were prepared and examined for both NMR and GC-MS analyses.

2.4 GC-MS

Samples were analyzed using an Agilent 7683B Series Injector coupled to an Agilent HP6890 Series gas chromatograph system and a 5973 Mass Selective Detector, quadrupole type GC–MS system. The GC was performed with RTX-5MS WCOT capillary column (Restek, 30 m × 0.25 mm; film thickness, 0.25 mm) that was coupled, through a heated transfer line (250 °C), to a Turbomass-Gold quadrupole mass spectrometer. The gas chromatographic separation was performed by applying a 2 minutes long isothermal phase at 80 °C, followed by a temperature increase from 80 to 310°C (rate of 15°C min^{-1}) and culminating in a 10 minutes long isothermal phase at 310°C. Helium was used as carrier gas at 1 mL min^{-1}, as well as the injector temperature was set at 250 °C and the split flow applied for the split-injection mode was 25 mL min^{-1}. Mass spectra were obtained in EI mode (70 eV), scanning in the range included within 50 and 650 m/z, with a cycle time of 0.2 scan s^{-1}. The identification of mass spectra of eluted compounds was carried out by analyzing standard compounds as well as by evaluating the mass spectra reported in the library NIST 05 (http://www.nist.gov) and the library released by Max-Planck-Institute for Plant Physiology of Golm (Germany, http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html). In order to take into account possible coelutions and peak overlappings, the mass spectra for all eluted peaks were analyzed not only in their centre, but also at peak borders.

2.5 NMR

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm BBI Bruker probe and working at the ^{1}H frequency of 400.13 MHz, was used to conduct all NMR measurements at a temperature of 298 ± 1 K. Monodimensional ^{1}H spectra were acquired by setting 5 s of thermal equilibrium delay, a 90° pulse length ranging within 8 and 8.85 μs (−2 dB of attenuation), 128 transients, 32768 time domain points, and 16 ppm (6410.3 Hz) as spectral width. The signal of residual water was

suppressed by applying the on-resonance pre-saturation during thermal equilibrium delay. Assignment of NMR signals was performed by acquiring and interpreting 2D NMR spectra of both samples and standard compounds, and it was confirmed by the molecular characterizations of maize tissues reported in previous manuscripts (Broyart *et al*., 2010; Castro *et al*., 2008; Gavaghan *et al*., 2011; Kuhnen *et al*., 2010; Manetti *et al*., 2006; Piccioni *et al*., 2009). In particular, 2D NMR spectra consisted of ^{1}H–^{1}H homo-nuclear experiments, such as COSY (Correlation SpectroscopY), TOCSY (Total CorrelationSpectroscopY) and NOESY (Nuclear OverHauser SpectroscopY), and hetero-nuclear ^{1}H–^{13}C experiments, such as HSQC (Hetero-nuclear Single-Quantum Correlation) and HMBC (Hetero-nuclear Multiple Bond Correlation). All of 2D experiments were acquired with spectral widths of 16 (6410.3 Hz) and 300 (30186.8 Hz) ppm for ^{1}H and ^{13}C nuclei, respectively, and a time domain of 2048 points (F2) and 256 experiments (F1). Specifically, homo-nuclear 2D spectra consisted in 16 dummy scans and 64 total transients. Additionally, a mixing time of 80 ms and a trim pulse length of 2500 ms were set for TOCSY experiment. HSQC and HMBC hetero-nuclear experiments were acquired with 16 dummy scans, 80 total transients and 0.5 μs of trim pulse length. Respectively, these experiments were optimized by taking into account 145 and 6.5 Hz as the optimal ^{1}H –^{13}C short and long range J-couplings.

Spectra were processed by using both Bruker Topspin Software (v 2.1, BrukerBiospin, heinstetten, Germany) and MNOVA Software (v.9.0, Mestrelab Research, Santiago de Compostela, Spain). Phase- and baseline corrections were applied to all of mono- and bi-dimensional spectra. The free induction decays (FIDs) of 1D ^{1}H spectra were Fourier transformed with a function size of 32768 points and applying a 0.3 Hz apodization.

2.6 Statistical analysis of GC-MS and NMR results

The semiquantitative evaluation of GC-chromatograms was performed by normalizing the area of each peak by the area of the internal standard and further modulating it as a function of sample fresh weight (mg). Conversely, ^{1}H NMR spectra were divided in symmetrical *n*-intervals (buckets to 0.04 ppm) which were then integrated and normalized with respect to the internal standard TMSPA. Each ^{1}H NMR spectrum was integrated from 9.98 to 0.1 ppm, excluding the region involving the water signal suppression (4.86-4.74 ppm), and producing 244 variables. The total data set composed of GC-MS (49 variables) and NMR (244 variables) data was subjected to Principal Component Analysis (PCA) using a XLStat software v.9.0 (Addinsoft). PCA represents an unsupervised classification method requiring no *a priori* knowledge of the data set and reduces the dimensionality of multivariate data while preserving most of information, expressed in terms of variable variance. Significant differences in metabolome amounts as a function of the studied treatments were tested by one and two-way ANOVA, followed by Tukey's test ($p< 0.05$).

3. Results and discussion

3.1 Phosphorus and nitrogen plant uptake

In order to evaluate the impact of *Trichoderma* inoculation and different P fertilization treatments on plant uptake and nutrition, P and N levels were analyzed in maize shoots eight weeks after sowing. As shown in Figure 1A, P content was significantly affected by P fertilization in all treatments. These data suggest a greater uptake of P in plants treated with both inorganic P-based fertilizers which can be attributed to a larger bioavailability of P in mineral fertilizers.

Despite the sole addition of *Trichoderma* (B6P0) reduced the amount of P in shoots, probably as a consequence of an adverse plant-fungi competition occurring in absence of fertilizers, a larger

amount of P was detected in shoots when *Trichoderma* was combined with all the studied fertilizers (Fig. 1A). In particular, the combination of *Trichoderma* inoculation with composted manure fertilizations significantly increased P accumulation in shoot tissues by revealing an extent which was significantly greater as compared to other treatments. In fact, B6P3 and B6P4 samples showed an abundance of P which was 29 and 56% larger than B0P3 and B0P4, respectively, as well as they presented a very larger amount of P (81 and 207%, respectively) as compared to the plants treated with the sole *Trichoderma* (B6P0). These results suggest that inoculation with *Trichoderma* brought significant benefits to plants which were accomplished through several mechanisms.

The combination of *Trichoderma* with composted manures not only increased the amount of P in plants, but also led to a relevant increase in shoot-N content as evidently shown by samples B6P3 and B6P4 in Figure 1B. Interestingly, since the sole application of composted manures did not influence the shoot N content (Figure 1A) such result suggests the synergistic role played by *Trichoderma* action. Summarily, the large content of N and P detected in plants inoculated with studied PGPF and fertilized with compost testified a better nutritional status induced in such plants which paved the way to a better growth and yield.

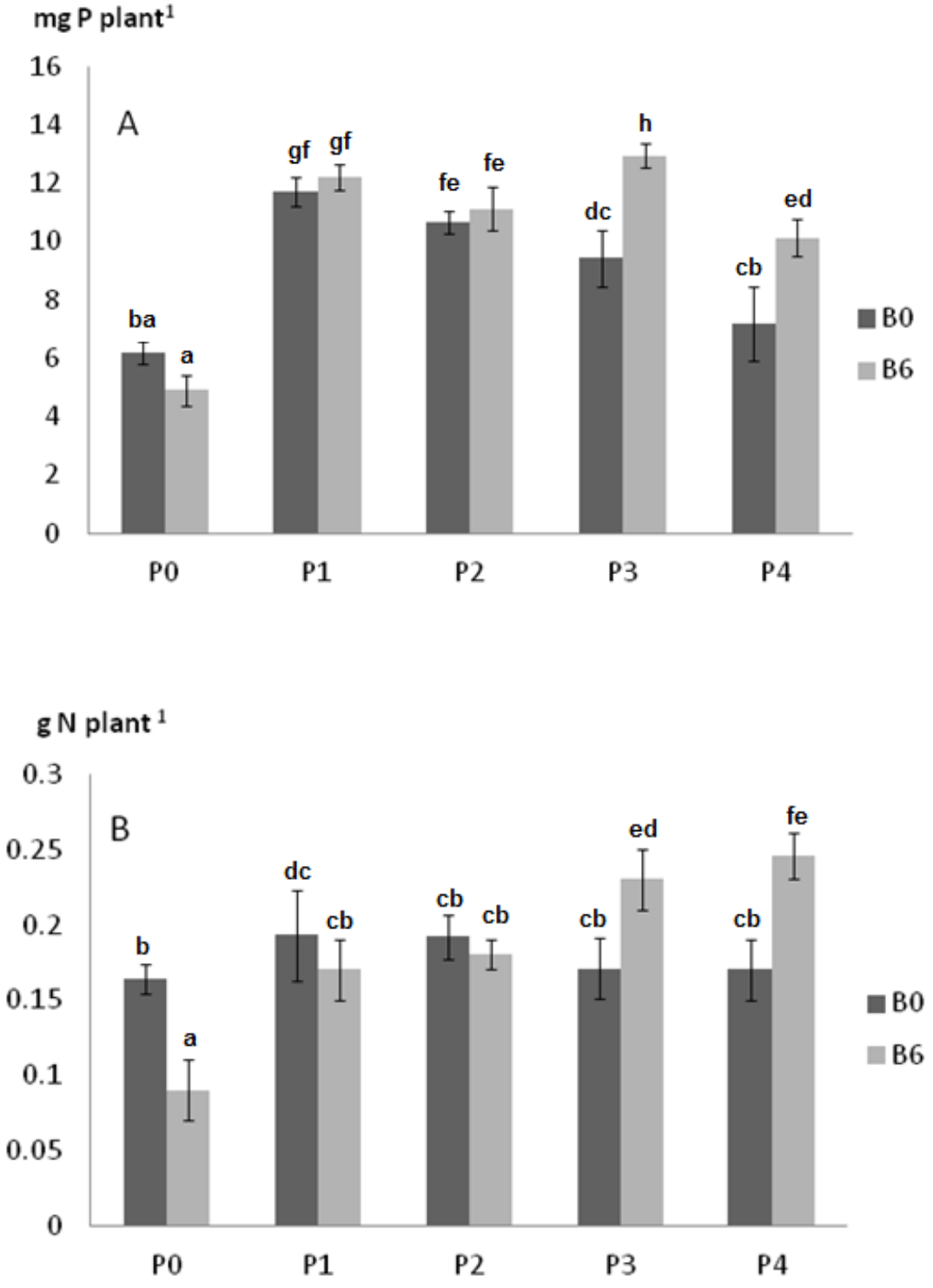


Figure 1. Phosphorous (A) and Nitrogen (B) content in the controls (B0) and inoculated plants (B6). Error bars indicate standard error (n=5) and different letters indicate significant differences by the Tukey's test at $P \leq 0.05$.

3.2 GC-MS and NMR metabolic profiling

Aiming to highlight the metabolic processes elicited in *Zea mays* plants by *Trichoderma,* the studied fertilizers, and their synergistic action, the primary polar metabolites extracted from leaves were detected by both GC-MS and ^{1}H NMR and evaluated by using a metabolomic approach. Figure 2 shows the GC chromatogram (Fig. 2A) and the NMR spectrum (Fig. 2B) of polar metabolites extracted from maize leaves of a representative sample (B0P0). The identified compounds included mostly saccharides (mono- and di- saccharides), organic acids, amino acids and amino-sugar (Table 1a, 1b). Importantly, the combination of GC-MS and ^{1}H-NMR techniques enabled the attainment of a more detailed metabolic profile of leaf accounting for the advantages deriving from both these powerful and complementary analytical techniques.

Specifically, GC-MS showed an enhanced sensitivity in identifying minor compounds, such as organic acids, which are typically scarce into the metabolic extract, whereas NMR allowed the revelation of several metabolites whose detection is typically difficult by GC-MS. As shown in Figure 2, the visual inspection of both GC chromatogram and NMR spectrum permits to assign cis-aconitic acid, quinic acid and shikimic acid as the most abundant organic acids detected into leaf metabolome. The predominant carbohydrates were sucrose and its monosaccharidic constituents. In addition, NMR enabled the neat detection of most of amino acids and nucleosides composing the leaf.

Hence, in order to identify the treatment-dependent responses induced in metabolome, the data resulting from both GC-MS and NMR analyses were merged into a unique matrix and semi-quantitatively examined by Principal Components Analysis (PCA). PCA is an unsupervised pattern-recognition method requiring no *a priori* knowledge of the data set and aiming to reduce the dimensionality of multivariate data while preserving most of total variance. The most informative outputs produced by PCA are the score-plot and the loading-plot. The former exhibits the distribution of samples in a plane defined by the principal components, while the latter describes

the relationship existing between examined variables and such components. Consequently, the loading-plot permits to easily correlate samples planar scoring with variables responses.

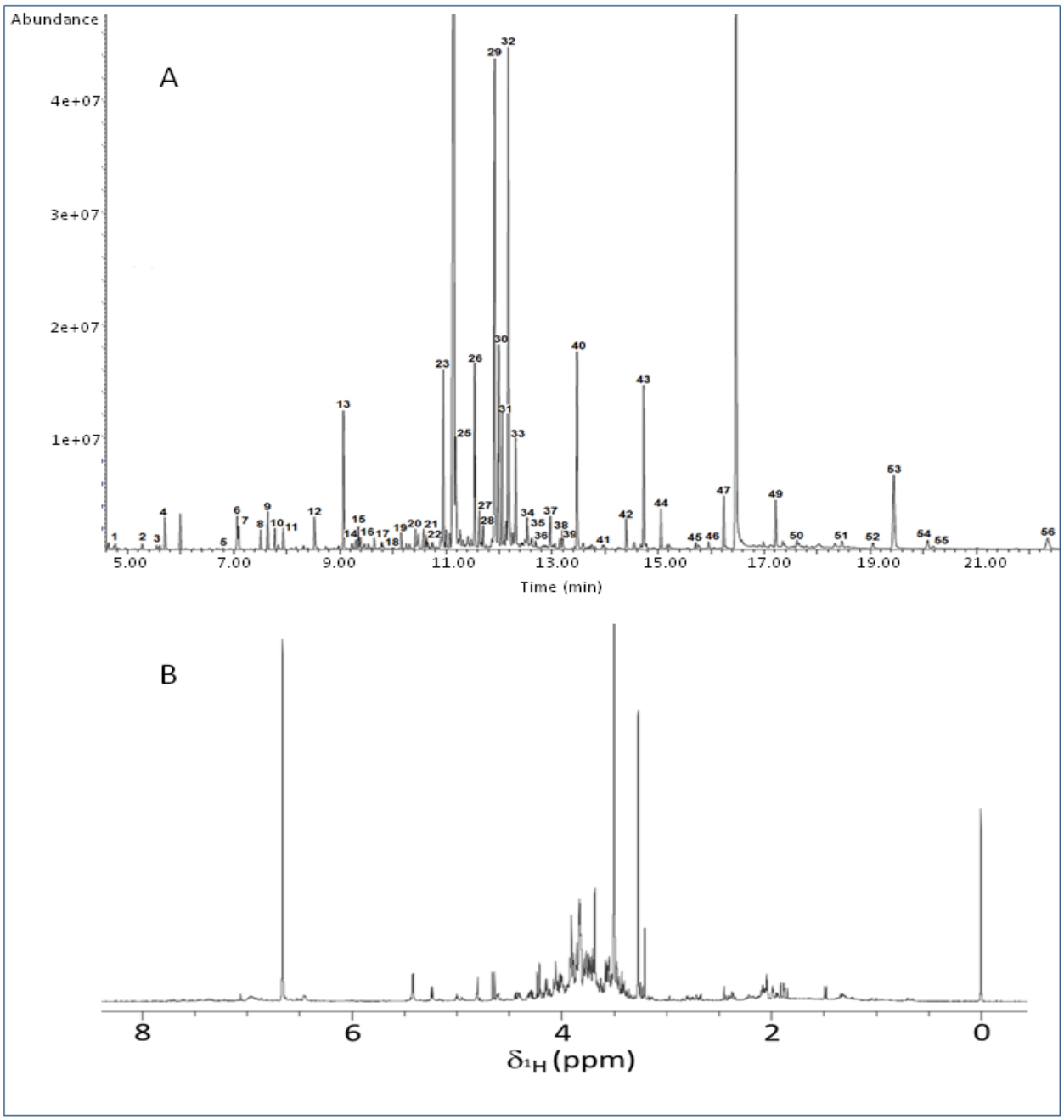


Figure 2. Total Ion Chromatogram (TIC) (A) and ^{1}H-NMR spectrum (B) of maize leaf extract (B0P0 sample).

Table 1a: List of primary metabolites from maize leaves identified by ^{1}H NMR

Amino acids	Assignment	δ (^{1}H)	Multiplicity
Alanine	β-CH_3	1.48	d
Alanine	β-CH_3	1.48	d
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.65	m
Arginine	β-CH_2	1.65	m
Arginine	β-CH_2	1.65	m
GABA	γ-CH_2	3.01	t
GABA	γ-CH_2	3.01	t
GABA	α-CH_2	2.28	t
Glutammic acid	γ-CH_2	2.34	t
Histidine	C4H ring	7.78	s
Histidine	C4H ring	7.78	s
Histidine	C2H ring	7.05	s
Isoleucine	γɜ-CH	1.45	m
Isoleucine	γ-CH	1.25	m
Isoleucine	γ-CH_3	1.02	d
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Phenylalanine	CH3,5	7.44	m
Phenylalanine	CH3,5	7.44	m
Phenylalanine	CH2,6	7.31	m
Phenylalanine	CH2,6	7.31	m
Proline	β-CH_2	2.32	m
Threonine	γ-CH_3	1.32	d
Tryptophan	C4H ring	7.72	d
Tryptophan	C4H ring	7.72	d
Tryptophan	C7H ring	7.54	d
Tryptophan	C7H ring	7.54	d
Tryptophan	C6H ring	7.28	t
Tryptophan	C6H ring	7.28	t
Tryptophan	C5H ring	7.20	t
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Valine	γ'-CH_3	1.04	d
Valine	γ'-CH_3	1.04	d
Valine	γ-CH_3	0.99	d

Carbohydrates	Assignment	δ (^{1}H)	Multiplicity
Fructose		4.08	m
Fructose		4.08	m
Fructose		3.99	ddd
Fructose		3.89	dd
Fructose		3.89	dd
Fructose		3.85	m
Fructose		3.80	m
Fructose		3.79	dd
Fructose		3.70	dd
Fructose		3.70	dd
Fructose		3.65	m
Fructose		3.57	d
Fructose		3.57	d
α-Galactose	C1H	5.27	d
β-Galactose	C1H	4.60	d
α-Glucose	C1H	5.24	d
β-Glucose	C1H	4.64	d
β-Glucose	C1H	4.64	d
Glucose		3.90	m
Glucose		3.90	m
Glucose		3.84	m
Glucose		3.83	m
Glucose		3.77	m
Glucose		3.73	dd
Glucose		3.72	dd
Glucose		3.54	dd
Glucose		3.54	dd
Glucose		3.54	dd
Glucose		3.51	t
Glucose		3.42	t
Glucose		3.40	t
Glucose		3.40	dd
Glucose		3.24	t
Glucose-6-phosphate		5.58	d
Glucose-6-phosphate		5.58	d
Inositol		4.05	t
Inositol		3.61	t
Inositol		3.61	t
Inositol		3.52	dd
Raffinose (Piccioni)	Gal1H	5.00	d
Raffinose (Piccioni)	Gal1H	5.00	d
Raffinose (Piccioni)	Fru3H	4.23	d
Sucrose	Glc1H	5.42	d
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose	$FruCH_2$-1'	3.68	s
Sucrose			
Sucrose			
Trehalose (Piccioni)	C1H	5.20	d
Trehalose (Piccioni)	C1H	5.20	d

Table 1a:continued

Nitrogenous compounds	Assignment	δ (^{1}H)	Multiplicity
Adenosine-like		8.34	s
Adenosine-like		8.34	s
Adenosine-like		8.34	s
Adenosine-like		8.27	s
Adenosine-like		8.27	s
Adenosine-like		8.27	s
Adenosine-like		8.11	d
Adenosine-like		5.99	d
Adenosine-like		5.99	d
Adenosine-like		5.99	d
UMP	C1'H ribose	8.11	d
UMP	C6 ring	5.99	d
Choline	N-CH_3	3.21	s
Choline	N-CH_3	3.21	s
Dimethylamine	CH_3	2.73	s
Glicine betaine (Piccioni)	N-CH_3	3.28	s
Phosphatidylcholine	CH_3	3.27	s
Trigonelline (HA)	HA	9.13	s
Trigonelline (HB, HC)	HB,HC	8.86	d
Trigonelline (HB, HC)	HB,HC	8.86	d
Trigonelline (HD)	HD	8.06	d
Trigonelline (HD)	HD	8.06	d
Trimethylamine	CH_3	2.91	s

Organic acids	Assignment	δ (^{1}H)	Multiplicity
Chlorogenic acid		7.6	d
Chlorogenic acid		7.13	d
Chlorogenic acid		6.87	d
Chlorogenic acid (Broyart)		6.35	d
Chlorogenic acid (Broyart)		5.316	td
Chlorogenic acid		5.316 1	td
Cis-aconitic acid	CH=C-	6.65	s
Cis-aconitic acid	CH=C-	6.65	s
Cis-aconitic acid	CH_2	3.49	s
Formic acid	HCOOH	8.46	s
Fumaric acid (α,β)	α,β CH=CH	6.53	s
Isobutirric acid	CH_3	1.14	s
Isobutirric acid	CH_3	1.14	s
Isocitric acid		2.96	q
Isocitric acid		2.96	q

Organic acids	Assignment	δ (^{1}H)	Multiplicity
Isocitric acid		2.46	dq
Isocitric acid		2.46	dq
Isocitric acid		2.46	dq
Maleic acid	α,β CH=CH	6	s
Malic acid	α-CH	4.32	dd
Malic acid	α-CH	4.32	dd
Malic acid	β-CH	2.68	dd
Malic acid	β-CH	2.68	dd
Malic acid	β-CH	2.68	dd
Malic acid	β–β'-CH_2	2.38	dd
Malic acid	β–β'-CH_2	2.38	dd
Quinic acid	H3	4.131	q
Quinic acid	H3	4.131	q
Quinic acid	H5	4.008	ddd
Quinic acid	H4	3.54	dd
Quinic acid	H6'	2.04	ddd
Quinic acid	H6'	2.04	ddd
Quinic acid	H6'	2.04	ddd
Quinic acid	H2	1.97	dd
Quinic acid	H2'	1.94	ddd
Quinic acid	H6	1.88	dd
Quinic acid	H6	1.88	dd
Quinic acid	H6	1.88	dd
Shikimic acid		6.42	m
Shikimic acid		6.42	m
Shikimic acid		4.38	t
Shikimic acid		4.38	t
Shikimic acid		2.77	dd
Shikimic acid		2.77	dd
Shikimic acid		2.77	dd
Shikimic acid		2.73	dd
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Succinic acid	α–β-CH_2	2.4	s

s= singlet d= doublet
dd=doublet of a doubledoublet
dd= doubledoublet
ddq= doublet of a doublequartet
q= quartet m= multiplet

Table1b : List of primary metabolites from maize leaves identified by GC-MS.

n°	Metabolites	RT (min)	MS fragmentation (m/z)	References
1	Lactic acid	4.77	52-73-75-117-147-149	NIST
2	Alanine	5.28	73-75-116-117-147-190	STD,MPI
3	Oxalic acid	5.7	73-74-75-147-148-149	NIST
4	Phosphoric acid	7.07	73-133-211-225-299-300	NIST
5	Glycerol tris TMS	7.09	73-117-147-205-299	NIST
6	Succinic acid	7.51	73-147-172-247	NIST
7	Glyceric acid	7.65	73-103-147-189-292	NIST
8	Itaconic acid	7.78	73-147-215-259	NIST
9	Serine	7.94	73-75-116-147-204-218	STD,MPI
10	Threonine	8.18	73-75-130-147-218	STD
11	Maleic acid	8.32	73-75-133-147-148-149	NIST
12	4-Ketaglucose	8.89	73-103-147-204	NIST
13	Arabino-hexose-2-ulose	8.98	73-103-147-205-234-262	NIST,MPI
14	Methylmaleic acid	9.07	73-147-189-233-265	NIST,MPI
15	Aspartic acid	9.35	73-75-100-147-232	STD
16	succinic anhydride	9.39	73-156-255	NIST
17	4-aminobutyric acid	9.47	73-130-175-205-231-260	NIST,STD
18	Tetronic acid	9.65	73-147-220-292	NIST
19	Glutaric acid	9.8	73-147-198-304	STD
20	Glutamine	10.16	73-75-128-147-156-246	NIST,MPI
21	Xylonic acid	10.43	73-117-147-175-217-244	NIST
22	Xylulose	10.66	73-103-147-217-307	NIST
23	**Ribitol IS**	10.95	73-103-147-217-319	STD
24	cis Aconitic acid	11.14	73-147-229-285-375	NIST,MPI
25	Cinnamic acid	11.42	73-147-204-245	STD,MPI
26	Shikimic acid	11.55	73-147-204-255	NIST,MPI
27	sugar	11.58	73-147-205-217	NIST
28	Isocitric acid	11.63	73-147-273-363	NIST
29	Quinic acid	11.92	73-147-191-255-345	NIST,MPI
30	Fructose	12	73-129-133-147-217-218	STD
31	Fructose	12.06	73-129-133-147-217-219	STD
32	Galactose	12.14	73-147-205-217-229	STD
33	Glucose	12.18	73-129-147-149-157-217	STD
34	Glucose	12.32	73-129-147-149-157-218	STD
35	UN	12.54	73-147-219-292	NIST
36	UN	12.97	73-147-221-449	NIST
37	Mucic acid	13.15	73-147-217-292-333	NIST
38	UN	13.35	73-103-147-221-449	NIST
39	Myo-Inositol	13.47	73-147-191-217-221-305	STD,MPI
40	Floridoside	13.73	73-103-147-204-337	NIST
41	Glucuronic acid	13.86	73-147-205-217-292-375	NIST
42	UN	14.11	73-147-204-321-361	NIST

Table 1b :continued

n°	Metabolites	RT (min)	MS fragmentation (m/z)	References
43	sugar	14.81	73-129-133-147-217-219	NIST
44	UN	15.1	73-147-204-321-361-406	NIST
45	oligosaccaride	15.5	73-147-217-361-450	NIST
46	oligosaccaride	15.53	73-147-217-361-450	NIST
47	oligosaccaride	16.2	73-147-217-361-450	NIST
48	Sucrose	16.47	73-169-217-243-361-362	NIST
49	UN	16.55	73-147-204-217-361	NIST
50	D-Turanose	17.35	73-147-217-361-450	NIST
51	Maltose	18.47	73-147-204-217-305-361	NIST
52	UN	19.05	73-147-219-255-345	NIST
53	Melbiose	19.45	73-103-129-204-217-361	NIST
54	UN	20.19	73-147-191-249-345-447	NIST
55	Chlorogenic acid	20.08	73-147-191-255-307-345	STD,MPI
56	Raffinose	22.35	73-147-129-205-217-361-437	NIST

NIST= mass spectra library NIST 05 (http://www.nist.gov); STD=standards; MPI=Max-Planck-Institute (Germany, http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html).

3.3 Influence exerted on leaves metabolome by Trichoderma inoculation

In Figure 3, it is shown the PCA score-plot including all of samples evaluated in this work and explaining 45.7% of total variance. Despite the relative overlapping of different samples, the holistic approach used in this PCA indicated evidently how *Trichoderma* was capable to enhance even more the separation among plants amended with different fertilizers. In particular, along the PC1 (26.2% of total variance) it was possible to distinguish the samples B6P3 and B6P4 from B6P0 samples as they revealed, respectively, the larger and the lower content of glucose, fructose, galactose, inositol, phosphoric acid, arginine, leucine and cis-aconitic acid. The remaining samples showed intermediate values for these variables. Remarkably, all of these compounds correlated with the high concentration of P and N found in the samples B6P3 and B6P4 (Figure 1) thus suggesting a

better nutritional status in these plants and underlining the positive effect exerted by *Trichoderma* when associated to compost. Conversely, B0P1, B0P2 and B6P1 were differentiated from samples B0P0, B0P3, B0P4 and B6P2 along the PC2 (19.5% of total variance) because they showed higher concentration of trigonelline, tryptophan, phenylalanine, threonine and valine. Specifically, the high concentration of trigonelline represents an index of stress condition for this group of plants (Tramontano & Jouve, 1997; Berglund *et al*., 1996). At the same time, also the abundant presence of aromatic amino acids, such as tryptophan and phenylalanine, may indicate the enhanced production of secondary metabolites which can be further attributed to plant stress conditions. In fact, the phenylalanine presence may indicate the activation of phenylpropanoid pathway which is a critical process to biosynthetize a plethora of secondary plant products such as, anthocyanins, lignin, growth effectors and phenols. On the other hand, tryptophan is the precursor for indolacetic acid which in turn is involved in the cell expansion and many others regulatory processes (Leopold & Kriedman, 1975; Tzina & Galili 2010). They are synthesized via the shikimate pathway followed by the branched aromatic amino acid metabolic pathway (Vogt, 2010). However, even though the holistic evaluation of all samples in a single PCA (Figure 3) put a determinant light on the impact brought by *trichoderma* on plant metabolome, the dense overlapping of observations resulting from different treatments prevented a clear and unambiguous identification of treatment-dependent influence. Therefore, several additional PCAs, reported as follows, were performed by restricting the number of theses in such a way to precisely focus on specific set of treatments.

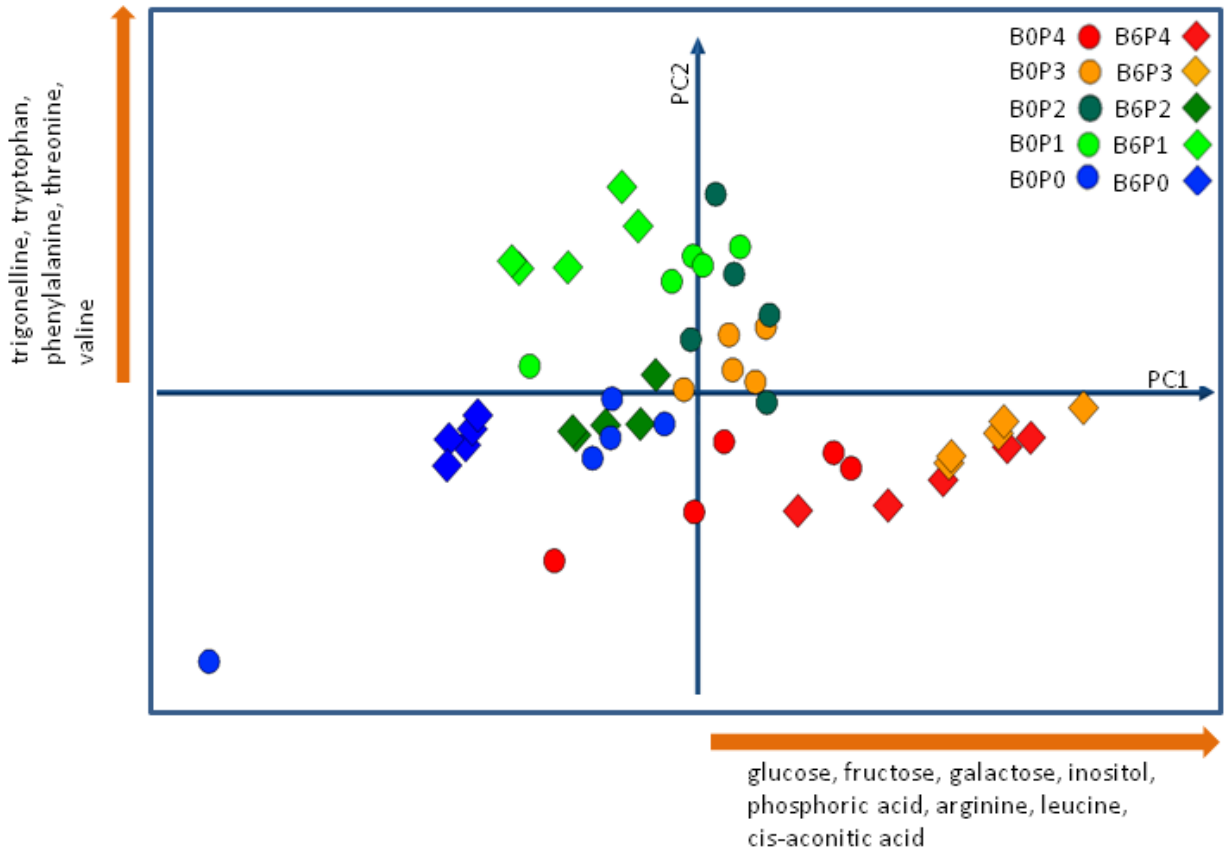


Figure 3: PCA score-plot obtained by processing GC-MS and NMR data of plants treated with different P-based fertilizers (P0-P4), either with (B6, rhombus) or without (B0, circle) the inoculation of *Trichoderma Harzianum.*

3.4 Metabolic analysis of plants inoculated with Trichoderma and treated with different fertilizers

Prior to evaluate the influence of *Trichoderma* on maize plants, we firstly considered the sole effect exerted by different fertilizers in absence of the fungal inoculum. Therefore, the score-plot of samples B0P0-P4, which has been already shown in our previous work (Chapter 3), is reported in Figure 4 for sake of completeness. Briefly, we observed a discrete separation among maize plants fertilized with different phosphorus source. Specifically, B0P1, B0P2 and B0P3 samples were collocated in positive PC1 values because of a higher amount of glucose, fructose, inositol and

sucrose. Such metabolites abundance was attributed to a higher photosynthetic activity and thus to a better nutritional state in plants. Conversely, B0P4 and B0P0 showed a lower amount of these compounds which was attributed to a less efficient biosynthesis of primary metabolites, such as carbohydrates, and was confirmed by the lower P and N uptake we measured in these plants (Chapter 3).

Analogously, we evaluated by PCA the metabolome of plants amended with the same fertilizers P0-P4 but inoculated with *Trichoderma.* The related score plot, which is shown in Figure 5 (54.79% of the total variance), confirmed the differentiability among plants treated with studied fertilizers as well as indicated that *Trichoderma* action strongly enhanced these differences. Specifically, the PC1 (36.78% of the total variance) differentiated samples treated with organic fertilizers (B6P3, B6P4) because of a higher amount of glucose, fructose, raffinose, cis-aconitic acid, choline, isocitric acid, alanine, arginine, proline and valine. Particularly, the increased content of glucose, which can be directly associated to photosynthesis, may be positively influenced by high N concentrations (Field & Mooney, 1986; Meir *et al*., 2002). Considering that B6P3 and B6P4 plants showed the highest concentration of P and N (Figure 1) these results could be thus correlated to an enhanced photosynthetic activity. However, the large uptake of N may also indicate the synthesis of proteins, as supported by the detection of abundant levels of amino acids such as alanine, arginine and valine. Summarily, these results suggest a positive effect of *Trichoderma* on the nutritional state of plants, especially when combined with compost.

Conversely, PC2 (18.01% of the total variance) permitted to neatly discriminate the plants treated with TSP (B6P1) (Figure 5). In fact, these samples have been associated to positive values of PC2 which, in turn, are correlated to high concentrations of trigonelline, fumaric acid, shikimic acid, chlorogenic acid, quinic acid, malic acid, trehalose, adenosine-like, threonine, leucine and isoleucine. Particularly, trigonelline, may serve as a cell cycle regulator as well as can accumulate in response to several stress conditions such as salt, oxidative and UV-stress (Tramontano & Jouve, 1997; Berglund *et al*., 1996). As proposed by Berglund, trigonelline may also work as potent

inducers of defensive metabolism in plants, including glutathione metabolism, and the accumulation of secondary defense compounds (Berglund *et al*., 1996). Such induction of defensive metabolism is also supported by the presence of high levels of chlorogenic acid, shikimic acid and quinic acid, which play important roles in the synthesis of secondary metabolites. However, the samples treated with compost (B6P3 and B6P4) were further differentiated by remaining ones also along the PC2, because they presented intermediate values for chlorogenic acid, shikimic acid and adenosine-like (Figure 5). Interestingly, a significant differentiation was also observed between B6P3 and B6P4, where the former exhibited a significantly larger amount of these three compounds as a probable consequence of different molecular composition of composts which influenced the plants metabolome at different extents (Spaccini & Piccolo 2009).

Despite the amount of P and N resulted identical for B6P2 and B6P1, the PCA reported in Figure 5 revealed a lower amount in B6P2 for all of variables mostly represented by PC2. This result suggested a similar nutritional status in these two plant groups, even though B6P1 was presumably subjected to a stronger condition of stress.

Conversely, the lower content of P and N detected for B6P0 plants well reflected the scarce concentration of all metabolites represented by PC1 and PC2, thus inducing their PCA collocation along negative values of PC1 and PC2 (Figure 5). Such results concerning the control plants, may be attributed to a worse state of nutrition, a lower photosyntetic activity and, probably, to a growth inhibition which, on the whole, confirm the positive and efficient action exerted by each of studied treatments.

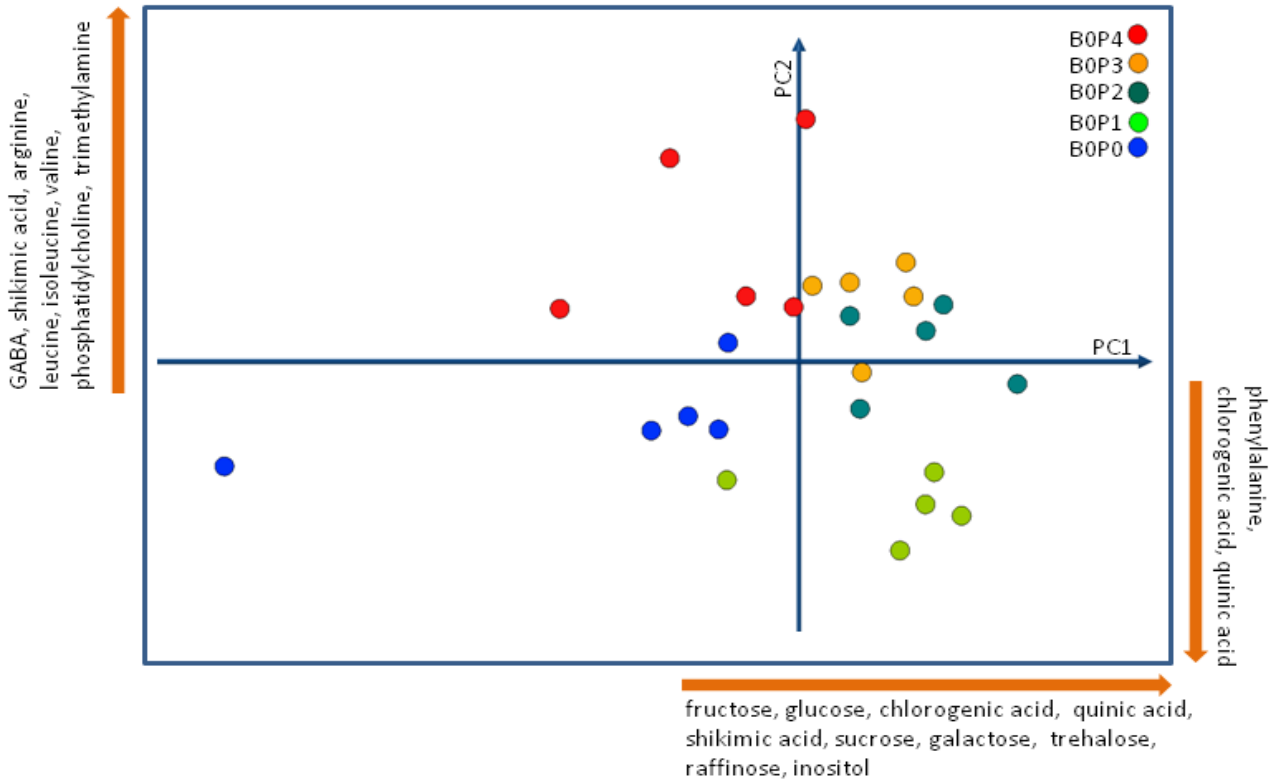


Figure 4: PCA score-plot obtained by processing GC-MS and NMR data of plants treated with different P-based fertilizers (P0-P4).

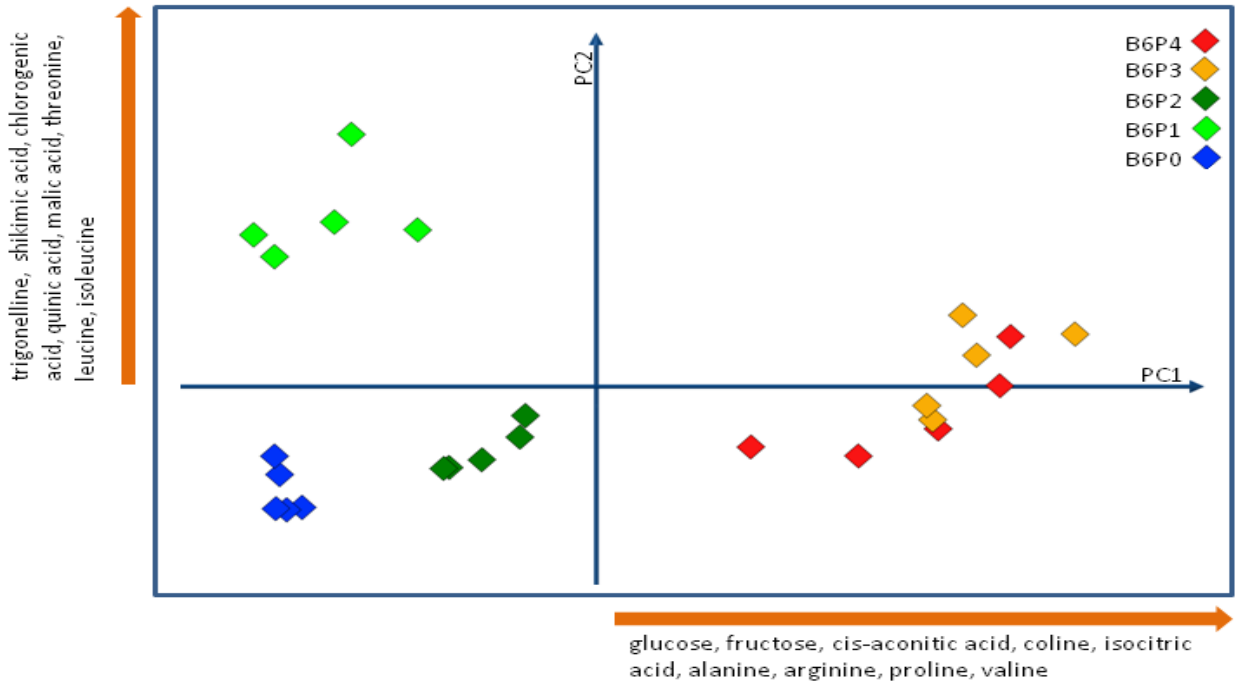


Figure 5: PCA score-plot obtained by processing GC-MS and NMR data of plants inoculated with *Trichoderma Harzianum* and treated with different P-based fertilizers (P0-P4).

3.5 Trichoderma effects on maize plants combined with mineral fertilizers

In Figure 6A is displayed the PCA score-plot of samples treated with mineral fertilizers RP and TSP, either without or with the concomitant inoculum of *Trichoderma Harzianum*. At positive values of PC1 (31% of the total variance) it was associated high concentration of trigonelline, glycine betaine, shikimic acid, chlorogenic acid, inositol, myo-inositol, raffinose , trehalose and galactose which permitted to discriminate the samples B0P1, B6P1 and B0P2 from all the remaining samples. Along PC4, the variables tryptophan and phenylalanine were more abundant in B0P1 as compared to B6P1 and B0P2 whereas samples B6P0 and B6P2 were collocated along positive values of PC4 because of their larger amount of histidine, adenosine-like, cis-aconitic acid and glutamic acid. The presence of trigonelline and glycine betaine in B0P1, B6P1 and B0P2 suggested a state of stress of these groups of plants (Tramontano & Jouve, 1997; Berglund *et al*., 1996). This supposition was supported also by the presence of the other abovementioned variables, probably involved in the formation of secondary metabolites, particularly during a condition of stress. Remarkably, the role of inositol and its isomer myo-inositol is particularly relevant in plants since they are involved in the phosphatidylinositol signaling pathway, auxin storage and transport, phytic acid biosynthesis, cell wall biosynthesis, and the production of stress related molecules (Loewus & Murthy, 2000; Loweus, 1990). In this context the concomitant presence of tryptophan, phenylalanine, chlorogenic acid and shikimic acid suggests an enhanced biosynthesis of secondary metabolites. The relevant presence of phenylalanine and tryptophan is very important since they serve as substrate for the phenylpropanoid pathway that, in turn, is a critical step for the biosynthesis of many secondary plant products such as, anthocyanins, lignin and phenolics (Leopold & Kriedman ,1975; Tzina & Galili , 2010). Indeed, also the presence of chlorogenic and shikimic acids confirms an enhanced synthesis of secondary metabolites. Since it is reported that stress conditions, such as wounding, resistance to pathogen infection, metabolic stress or perturbations in cell wall structure may enhance the biosynthesis of these compounds (Caño-

Delgado *et al*., 2003; Tronchet *et al*., 2010), their abundant presence associated to positive PC1 values may be correlated to a state of stress induced by mineral treatments.

Despite the restricted amount of variance explained by PC4 (8% of the total variance), this principal component enabled a more enhanced differentiation among studied samples and, in particular, that resulting from *Trichoderma* action. In fact, samples resulting from the same fertilizer treatment but combined with the fungal inoculum migrated systematically toward more positive PC4 values (Figure 6A).

3.6 Trichoderma effects on maize plants combined with organic fertilizers

In Figure 6B is shown the PCA score-plot (48.56% total variance explained) of samples treated with organic fertilizers, either without or with the concomitant inoculum of *Trichoderma Harzianum*. The figure clearly shows a neat discrimination between treatments and, in particular, indicates an evident dislocation toward PC1 positive values of those samples in which compost treatments (P3 and P4) were combined with *Trichoderma* inoculation. In line with the PCA reported in Figure 3, these results underlined that organic fertilizers, regardless the type of compost, significantly influenced the metabolic profile of studied plants by leading to a larger amount of glucose, fructose, inositol, raffinose, valine, alanine and threonine. Among these compounds, the highest concentration of carbohydrates, such as glucose, fructose and inositol, correlates with the high content of P and N in B6P3, B6P4 (Figure 1) thus suggesting a more enhanced photosynthetic activity induced in these plants. The samples B0P3 and B0P4 were collocated in the central area of PC1 because of the intermediate values detected for such compounds. Conversely the PC2, which accounted for a 13.98% of total variance, allowed the separation of B0P3 from all the other samples because of a higher content of chlorogenic acid, cis-aconitic acid, shikimic acid tryptophan, phenylalanine, leucine and isoleucine which are again potentially attributable to an enhanced biosynthesis of secondary metabolites.

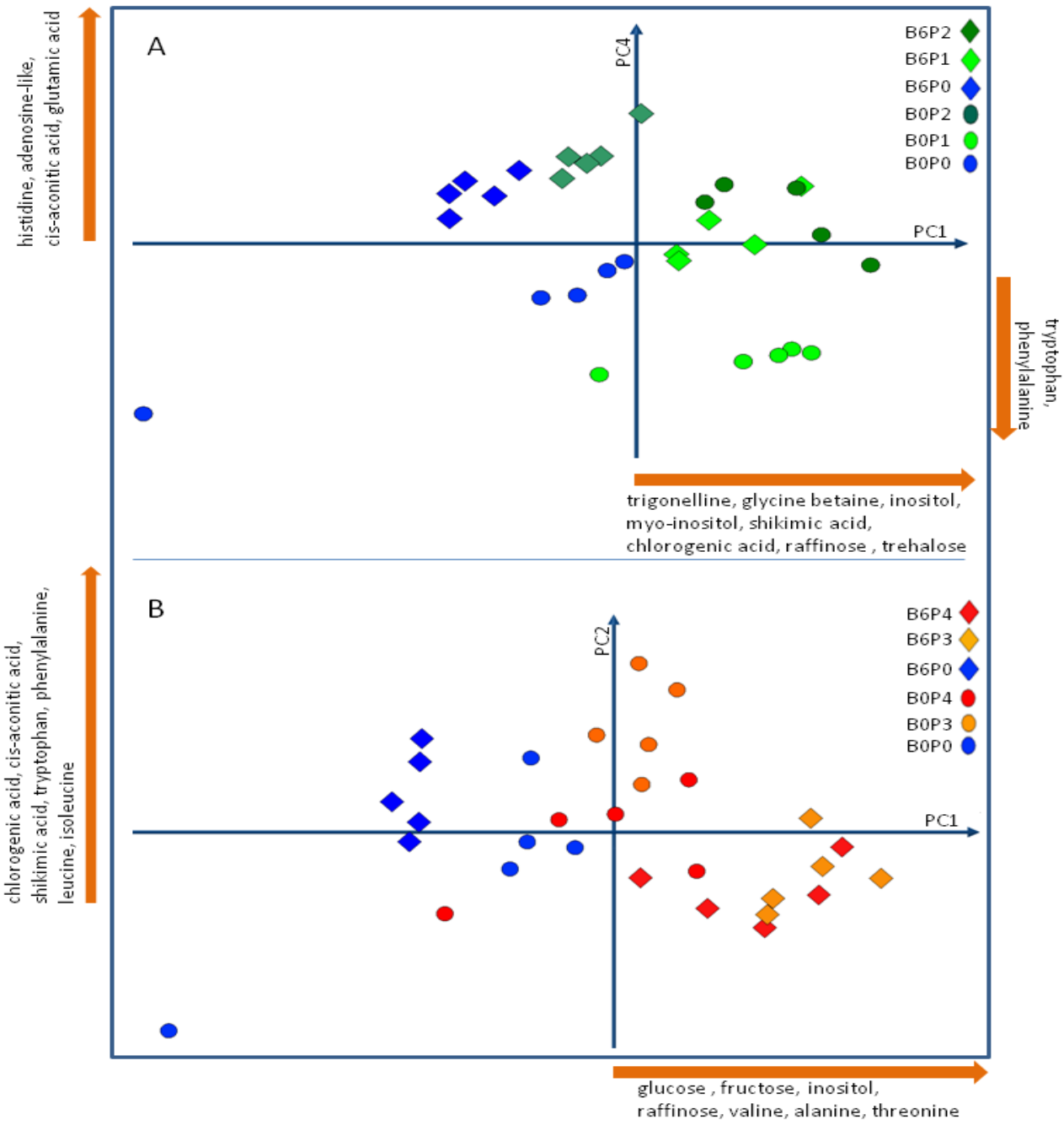


Figure 6: PCA score-plots obtained by processing GC-MS and NMR data of plants treated either without (B0, circle) or with *Trichoderma Harzianum* (B6, rhombus) and compared with plants amended with fertilizers based on mineral (A) or organic (B) forms of Phosphorous.

4. Conclusions

In this work we evaluated how the fungal inoculation of *Trichoderma* may influence plants of *Zea mays* treated either without or with different (mineral or organic) fertilizers, including compost. Behind the choice of treatments adopted in this work, there was the purpose to investigate on effects

induced on plants by agricultural strategies potentially capable to replace the conventional agriculture. To this end, the polar metabolites, extracted from treated plants subjected to studied treatments and detected by both GC-MS and ^{1}H NMR techniques, were grouped in a single data matrix and evaluated by Metabolomics. Importantly, the combination of GC-MS and NMR data enabled the identification of a more complete metabolic profile which, in a more reliable way, permitted to individuate compounds whose concentration varied in a treatment-dependent way. In particular, it was found a systematic response in several carbohydrates and in specific metabolites, typically occurring in stress conditions or involved in the biosynthethis of secondary metabolites.

The treatments based on different P sources resulted very effective since they specifically influenced both the metabolic profiles and the phosphorous-uptake of treated plants (Chapter3). However, when the P treatments were associated with *Trichoderma* inocula, such differentiations resulted significantly more enhanced, especially for the metabolome of samples treated with composts (B6P3 and B6P4). Such observation, along with the highest content of P and N detected in these plants suggested a better nutritional state in B6P3 and B6P4 plants which is expected to sustain a higher plant growth. A further evidence of beneficial properties of compost addition is represented by the fact that *Trichoderma* was not able to promote the content of P and N when combined with mineral fertilizers. Interestingly, B6P0 samples were found to be adversely influenced by *Trichoderma*, probably due to a state of plant-microorganism competition for the available nutrients. In conclusion, our results confirm that the combination of *Trichoderma* with organic fertilizers, such as composts, may provide a valuable alternative to satisfy the plant P demand, thus limiting the use of the more expansive and environmentally unsustainable mineral fertilizers.

CHAPTER 5

Metabolomic approach to study the influence exerted on *Zea Mays* by *different Biostimulants* in organic farming.

Abstract

The organic farming (OF) may represent a valuable alternative to the conventional agriculture practices, that not only ensures food safety and increases soil biodiversity but is considered totally eco-sustainable. Keys components of the OF are represented to the use of fertilizers based on recycling products, alone or in combination with plant bio-stimulants like microorganisms and humic substances. In order to evaluate the possible synergistic effects of such bio-stimulants, plants of *Zea mays* fertilized with compost, were treated with different microorganisms (*Pseudomonas spp, Bacillus amyloliquefaciens* , arbuscolar mycorrhizal fungi) alone or in combination with humic acids. The study of the influence on the plants metabolome of such rizospheric interaction, called micro-bio-humeome, was evaluated using analytical technique like Gas Chromatography–Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR). The metabolomic profiles revealed a broad range of primary polar metabolites, including sugars, organic acids, amino acids, phenolic acids, amino sugars and sugar alcohols. GC-MS and NMR data were processed by PCA exercises which highlighted an enhanced influence exerted by studied bio-stimulants. In particular, our results suggested that all treatment positively influence the nutritional status of plants. Furthermore, the results underlined that only the combination of *Bacillus amyloliquefaciens* with humic acids and AMF were able to create positive synergistic effects on plants.

Keywords: Metabolomics, compost, *Pseudomonas spp, Bacillus amyloliquefaciens*, Humic Acids, AMF, GC-MS, ^{1}H-NMR.

1.Introduction

Maintaining food production for a growing world population without compromising natural resources for future generations represents one of the greatest challenges for agricultural science. In this view, over the last few decades the use of organic farming has increased worldwide in order to ensure eco-sustainable crop productivity and quality together with low environmental impact and low cost of management (Lotter, 2003; Postma-Blaauw *et al*., 2010). One of the key components of the success of organic farming is represented to the use of fertilizers based on recycling products alone or in combination with plant biostimulants, like microorganisms and humic substances. Particularly, the compost represent an important form of recycling product based on stabilized organic matter and commonly utilized to the farmers as fertilizer and soil amendment (Barker, 2010; Hargreaves *et al* 2008). Several scientific research have been demonstrated the compost ability to increase soil porosity, structural stability, moisture and nutrient availability, biological activity and roots aeration (Garcia-Gil *et al*., 2000; Kowaljow *et al*., 2007; Weber *et al*., 2007). On the other hand, soil microorganisms represent key interface between plant and soil, within the rhizosphere, where they are stimulated by root exudates, and other organic substrates supplied by the plants. The beneficial activity of rhizosphere microorganisms include the increased availability and uptake of plant nutrients, protection against root pathogens and increased plant stress tolerance (Azcon Aguilar & Barea, 1992). Among the wide diversity of rhizosphere microorganisms, the arbuscular mycorrhizal fungi (AMF) and phosphorus solubilizing bacteria (PSB) represents two beneficial groups of microorganisms, frequently used in the organic farming as inoculants in order to improve the plant growth and health. The symbiosis between AMF and plants provide nutrients, stimulate plant growth and increase plant stress tolerance through several mechanisms (Miyasaka & Habte, 2001; Barea *et al*., 2005). Hyphae of plant associated AMF extend into soil from roots and greatly improve availability of insoluble P and others macro and micronutrients (Miyasaka & Habte, 2001; Govindrajulu *et al*., 2005; Cozzolino *et al*.,2013). In

sustainable agricultural systems, the inoculation of AMF is an important management practice when the native mycorrhizal potential of soil is inadequate (Koide & Mosse, 2004). Mycorrhizal colonized plants can also interact with several soil microorganisms including PSB improving the plant resistance to stress condition (Azcón *et al*., 2009, Barea *et al*., 2004; Dimkpa *et al*., 2009; Kasim *et al*., 2013). The use of PSB improve the bioavailability of phosphorous (P), they are able to convert insoluble phosphate into available forms for plants through processes of acidification, exchange reactions and production of organic acids (Liu *et al*., 2014; Rodríguez & Fraga, 1999; Sharma *et al*., 2013). Additionally, as been reported by Cozzolino and coworker, the simbiosis between AMF and plants not only enanche the plant nutrition but their interaction with humic matter play an important role in the plants tolerance to contaminants like mercury (Cozzolino *et al*., 2016). Despite, several studies confirm the beneficial effects of these biostimulants alone or combined between them, a deep knowledge in biochemical and molecular terms of the synergistic effects of micro-bio-humeome, understood as interaction between the organic matter with microorganism on the metabolic response of plants, still lacks. For these reasons, the present study was conduct with the aim to fill this gap by investigating the effects exerted on metabolome of *Zea mays* plants treated with *Bacillus amyloliquefaciens* and *Pseudomonas spp*., representing the two most efficient and studied P-solubilizers in the soil (Rodríguez & Fraga, 1999) either alone or in combination with different bio-stimulants, like AMF and Humic acid. This evaluation was carried out by using a metabolomic approach which is an emerging analytical methodology capable to provide precious insights into molecular responses induced by specific environmental and nutritional conditions (Bundy *et al*., 2009). We used Metabolomics to examine the polar metabolites which were extracted from *Zea mays* plants and detected by both gas chromatography coupled to mass spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR). Finally, the content of P and N was estimated in order to assess the general nutritional status of plant and support the metabolomic results.

2. Materials and methods

2.1 Experimental design, plant growth, sampling and analyses

Maize plants (*Zea mays*, cv Aphoteos, Limagrain), were grown in pots (5 kg), prepared using an alkaline clay-loam soil mixed with quartz sand in a ratio of 2:1. The compost from buffalo manures, used as organic phosphorus source (50 mg P/Kg^{-1}), was mixed and incorporated homogenously into the soil, and left to equilibrate for 3 weeks at room temperature before the experiment.

The treatments consisted of factorial combination of four microbial inoculums and one treatment with humic acids: 1) B0, (no inoculation); 2) B2, *Pseudomonas spp.*; 3) B3, *Bacillus amyloliquefaciens*; 4), M+, arbuscolar mycorrhizal fungi, 5) HA, humic acids extract from artichoke compost.

The commercial microbial products were: Proradix (*Pseudomonas* spp.), produced by Sourcon Padena GmbH & Co.; Rhizovital 42 (*Bacillus amyiloliquefaciens*) produced by ABiTEP GmbH and MYC Aegis based on a mixture of two arbuscolar mycorrhizal fungi strains (*Glomus mossae* and *Rhizophagus irregularis* produced by Italpollina S.p.a.). Humic acids were extracted from a green compost (composted from artichoke residues).

All treatments were replicated five times, for a total of 40 pots. Stock suspensions of each bacterial product were 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 (10 gr L^{-1} for Proradix and 26,8 gr L^{-1} for Rhizovital 42). MYC Aegis was applied directly as microgranule according to the product indications. Humic acids stock solution were prepared from freeze-dried powder dissolved in deionized water and diluted to the application rate (0,012 g Kg^{-1}). The pot trial was conducted in a greenhouse conditions (25–33°C, daily temperature range during maize growth). Watering with demineralized water was performed manually to maintain the water holding capacity between 40 and 70% throughout the experiment.

Eight weeks after sowing, the youngest fully expanded leaves were collected for each plant, possibly in the time range included within 8:00 and 12:00 AM. Harvested leaves were weighed and immediately frozen in liquid nitrogen in order to quench the metabolism prior to be stored at -80°C. Total P in maize shoots was obtained by first digesting 500 mg powdered dry plant material by the ashing procedure according to Gericke and Kurmies (1952) and then colorimetrically determining P by the molybdenum blue assay method (Murphy and Riley 1962). Nitrogen was determined by an elemental analyzer Fisons EA 1108 (Fisons Instruments, Milano, Italy).

2.2 Extraction of plant leaves metabolites.

Maize leaves stored at -80°C were homogenized by using a mortar and pestle with liquid nitrogen. Then, 50±0.5mg of pooled homogenized plant material was weighed into pre-chilled 2 mL Eppendorf tube. The metabolites extraction was conducted by adding 1 mL of water/methanol/chloroform mixture (1:3:1 ratio) pre-cooled at -20°C, that also contained 20 μg ml^{-1} of Ribitol, as an internal standard. The samples were stirred for 30s and incubated for 15min at 70°C in order to inhibit the activity of possible enzymes present in the extract. The mixtures was then centrifuged for 10 min at 10000 rpm and 4°C, and the supernatants were recovered and transferred into 2 mL Eppendorf tubes. Pure water (400 μL) was added to allow the separation of polar and apolar phases corresponding to the methanol/water (upper) and chloroform (lower) phases, respectively. All extracts were finally stirred for 30 s and centrifuged for 10 min at 4°C and 10000 rpm. A volume of 400 μL was collected from the upper phase, transferred into 1.5 mL glass tubes for GC–MS analyses, dried under a flow of nitrogen and stored at -80 °C. Tree blank tubes were subjected to the same procedure.

2.3 Preparation of metabolites to analytical detrmination

Before derivatization for GC–MS analyses, the metabolic extracts were again dried under nitrogen flux in order to remove the humidity possibly acquired during storage at -80°C. Dried samples were redissolved in 50 uL of a solution of methoxyamine hydrochloride solubilized in pyridine (20mg ml^{-1}) and treated for 90 min at 30°C by applying a gentle shaking. After the methoximation step, the samples were silylated for 30 min at 37°C by using 50uL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) reagent. 30 minutes after the end of derivatisation, 2uL of each sample was injected into a GC column by using a split mode.

In case of NMR analysis, it was used the same extraction procedure ruling out that both plant material to be extracted and the volumes of extraction solvents were doubled in order to take into account the lower sensitivity of NMR spectroscopy. In this case 800 μL of supernatant were dried under a flow of nitrogen, stored at -80 °C and resolved in the same volume of deuterated phosphate buffer (90 mM, pH 6.0) containing 0.05 mg mL^{-1} 3-(tri-methylsilyl) propionic-2,2,3,3-d4 acid (TMSPA, $\delta\ ^{1}H = 0$ ppm; > 99%, Euriso-Top, France) which served as internal standard. Aiming to guarantee the complete dissolution of each metabolic extract, a 5 minutes long sonication was applied prior NMR analysis. Five replicates were prepared and examined for both NMR and GC-MS analyses.

2.4 GC-MS

Samples were analyzed using an Agilent 7683B Series Injector coupled to an Agilent HP6890 Series gas chromatograph system and a 5973 Mass Selective Detector, quadrupole type GC–MS system. The GC was performed with RTX-5MS WCOT capillary column (Restek, 30 m × 0.25 mm; film thickness, 0.25 mm) that was coupled, through a heated transfer line (250 °C), to a Turbomass-Gold quadrupole mass spectrometer. The gas chromatographic separation was

performed by applying a 2 minutes long isothermal phase at 80 °C, followed by a temperature increase from 80 to 310°C (rate of 15°C min^{-1}) and culminating in a 10 minutes long isothermal phase at 310°C. Helium was used as carrier gas at 1 mL min^{-1}, as well as the injector temperature was set at 250 °C and the split flow applied for the split-injection mode was 25 mL min^{-1}. Mass spectra were obtained in EI mode (70 eV), scanning in the range included within 50 and 650 m/z, with a cycle time of 0.2 scan s^{-1}. The identification of mass spectra of eluted compounds was carried out by analyzing standard compounds as well as by evaluating the mass spectra reported in the library NIST 05 (http://www.nist.gov) and the library released by Max-Planck-Institute for Plant Physiology of Golm (Germany, http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html). In order to take into account possible coelutions and peak overlappings, the mass spectra for all eluted peaks were analyzed not only in their centre, but also at peak borders.

2.5 NMR

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm BBI Bruker probe and working at the ^{1}H frequency of 400.13 MHz, was used to conduct all NMR measurements at a temperature of 298 ± 1 K. Monodimensional ^{1}H spectra were acquired by setting 5 s of thermal equilibrium delay, a 90° pulse length ranging within 8 and 8.85 μs (−2 dB of attenuation), 128 transients, 32768 time domain points, and 16 ppm (6410.3 Hz) as spectral width. The signal of residual water was suppressed by applying the on-resonance pre-saturation during thermal equilibrium delay. Assignment of NMR signals was performed by acquiring and interpreting 2D NMR spectra of both samples and standard compounds, and it was confirmed by the molecular characterizations of maize tissues reported in previous manuscripts (Broyart *et al*., 2010; Castro *et al*., 2008; Gavaghan *et al*., 2011; Kuhnen *et al*., 2010; Manetti *et al*., 2006; Piccioni *et al*., 2009). In particular, 2D NMR spectra consisted of ^{1}H–^{1}H homo-nuclear experiments, such as COSY (Correlation SpectroscopY),

TOCSY (Total CorrelationSpectroscopY) and NOESY (Nuclear OverHauser SpectroscopY), and hetero-nuclear $^{1}H–^{13}C$ experiments, such as HSQC (Hetero-nuclear Single-Quantum Correlation) and HMBC (Hetero-nuclear Multiple Bond Correlation). All of 2D experiments were acquired with spectral widths of 16 (6410.3 Hz) and 300 (30186.8 Hz) ppm for ^{1}H and ^{13}C nuclei, respectively, and a time domain of 2048 points (F2) and 256 experiments (F1). Specifically, homo-nuclear 2D spectra consisted in 16 dummy scans and 64 total transients. Additionally, a mixing time of 80 ms and a trim pulse length of 2500 ms were set for TOCSY experiment. HSQC and HMBC hetero-nuclear experiments were acquired with 16 dummy scans, 80 total transients and 0.5 μs of trim pulse length. Respectively, these experiments were optimized by taking into account 145 and 6.5 Hz as the optimal $^{1}H –^{13}C$ short and long range J-couplings.

Spectra were processed by using both Bruker Topspin Software (v 2.1, BrukerBiospin, heinstetten, Germany) and MNOVA Software (v.9.0, Mestrelab Research, Santiago de Compostela, Spain). Phase- and baseline corrections were applied to all of mono- and bi-dimensional spectra. The free induction decays (FIDs) of 1D ^{1}H spectra were Fourier transformed with a function size of 32768 points and applying a 0.3 Hz apodization.

2.6 Statistical analysis of GC-MS and NMR results

The semiquantitative evaluation of GC-chromatograms was performed by normalizing the area of each peak by the area of the internal standard and further modulating it as a function of sample fresh weight (mg). Conversely, ^{1}H NMR spectra were divided in symmetrical *n*-intervals (buckets to 0.04 ppm) which were then integrated and normalized with respect to the internal standard TMSPA. Each ^{1}H NMR spectrum was integrated from 9.98 to 0.1 ppm, excluding the region involving the water signal suppression (4.86-4.74 ppm), and producing 244 variables. The total data set composed of GC-MS (49 variables) and NMR (244 variables) data was subjected to Principal Component Analysis (PCA) using a XLStat software v.9.0 (Addinsoft). PCA represents an unsupervised

classification method requiring no *a priori* knowledge of the data set and reduces the dimensionality of multivariate data while preserving most of information, expressed in terms of variable variance. Significant differences in metabolome amounts as a function of the studied treatments were tested by one and two-way ANOVA, followed by Tukey's test ($p < 0.05$).

3. Results and discussion

3.1 Phosphorus and nitrogen plant uptake

In order to evaluate the impact on plant nutrition, of different bio-stimulants used alone or in combination between them, at eight weeks after sowing, P and N levels were analyzed in maize shoots. As shown in Figure 1b, P content was significantly higher in all samples treated with bio-stimulants alone or in combination when compared to the control.

Additionally, the synergistic action deriving from the combined use of *Bacillus amyloliquefaciens*, AMF and HA have shown a greater positive effects on nutrients uptake when compared to the others bio-stimulants used alone or in combination.

Specifically, observing separately the groups of treatments composed by the two PSB bacteria (B2-B3), was possible to note that in the plants inoculated with *Pseudomonas spp*. (B2), the P content was slight higher than the others in the plants treated by combining the B2 with HA.

While, among the plants inoculated with B3, the combination of such bacteria with HA and AMF represent the best combination in terms of N and P contents.

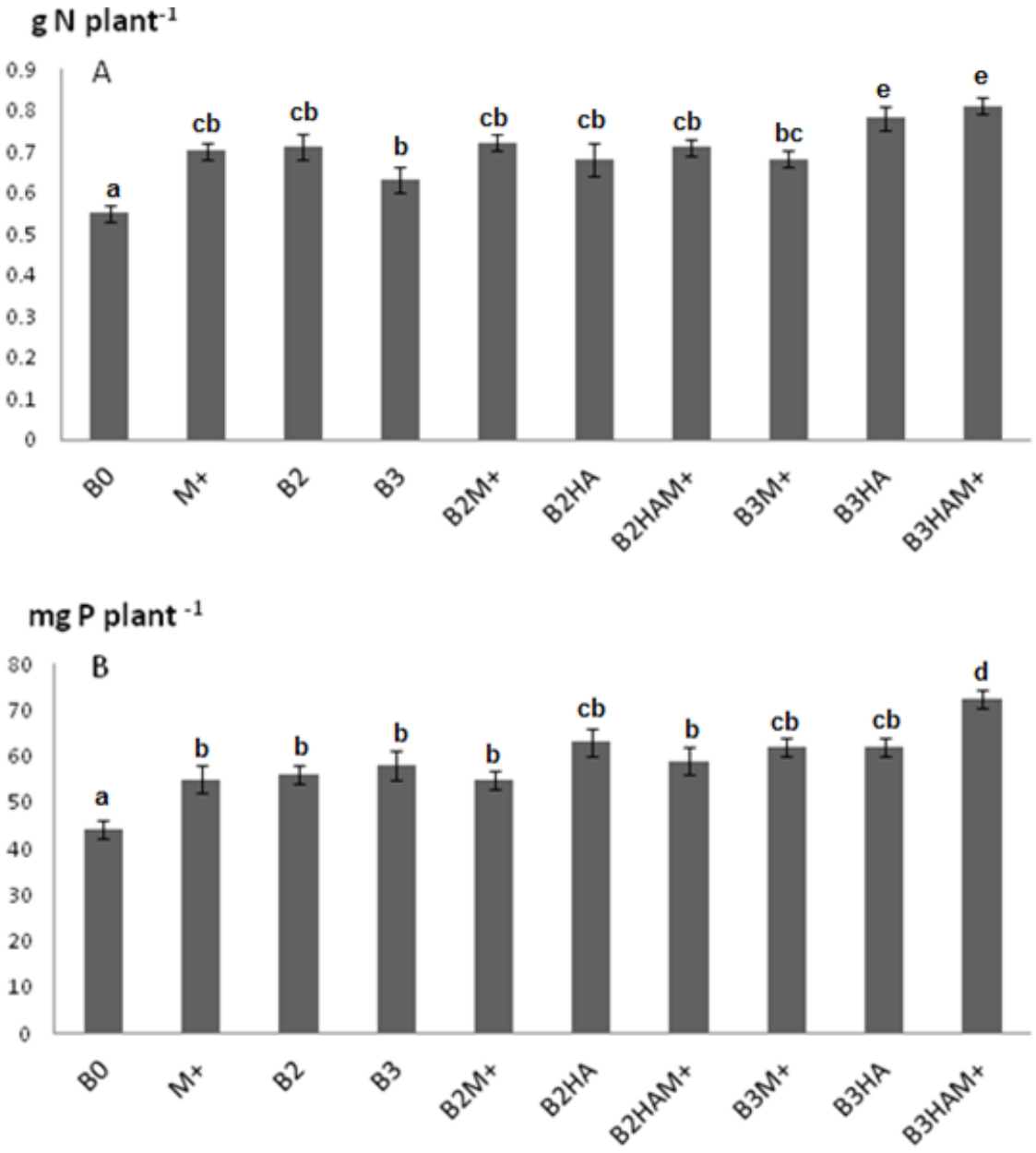


Figure 1. Nitrogen (A) and Phosphorous (B) content in the controls (B0) and inoculated plants. Error bars indicate standard error (n=5) and different letters indicate significant differences by the Tukey's test at $P \leq 0.05$.

3.2 GC-MS and NMR metabolic profiling

Aiming to highlight the metabolic processes elicited in *Zea mays* plants by different bio-stimulants and their synergistic action, the primary polar metabolites extracted from leaves were detected by

both GC-MS and ^{1}H NMR and evaluated by using a metabolomic approach. Figure 2 shows the GC chromatogram (Fig. 2A) and the NMR spectrum (Fig. 2B) of polar metabolites extracted from maize leaves of a representative sample (B0). The identified compounds included mostly saccharides (mono- and di- saccharides), organic acids, amino acids and amino-sugar (Table 1a, 1b). Importantly, the combination of GC-MS and ^{1}H-NMR techniques enabled the attainment of a more detailed metabolic profile of leaf accounting for the advantages deriving from both these powerful and complementary analytical techniques.

Hence, in order to identify the treatment-dependent responses induced in metabolome, the data resulting from both GC-MS and NMR analyses were merged into a unique matrix and semi-quantitatively examined by Principal Components Analysis (PCA). PCA is an unsupervised pattern-recognition method requiring no *a priori* knowledge of the data set and aiming to reduce the dimensionality of multivariate data while preserving most of total variance. The most informative outputs produced by PCA are the score-plot and the loading-plot. The former exhibits the distribution of samples in a plane defined by the principal components, while the latter describes the relationship existing between examined variables and such components. Consequently, the loading-plot permits to easily correlate samples planar scoring with variables responses.

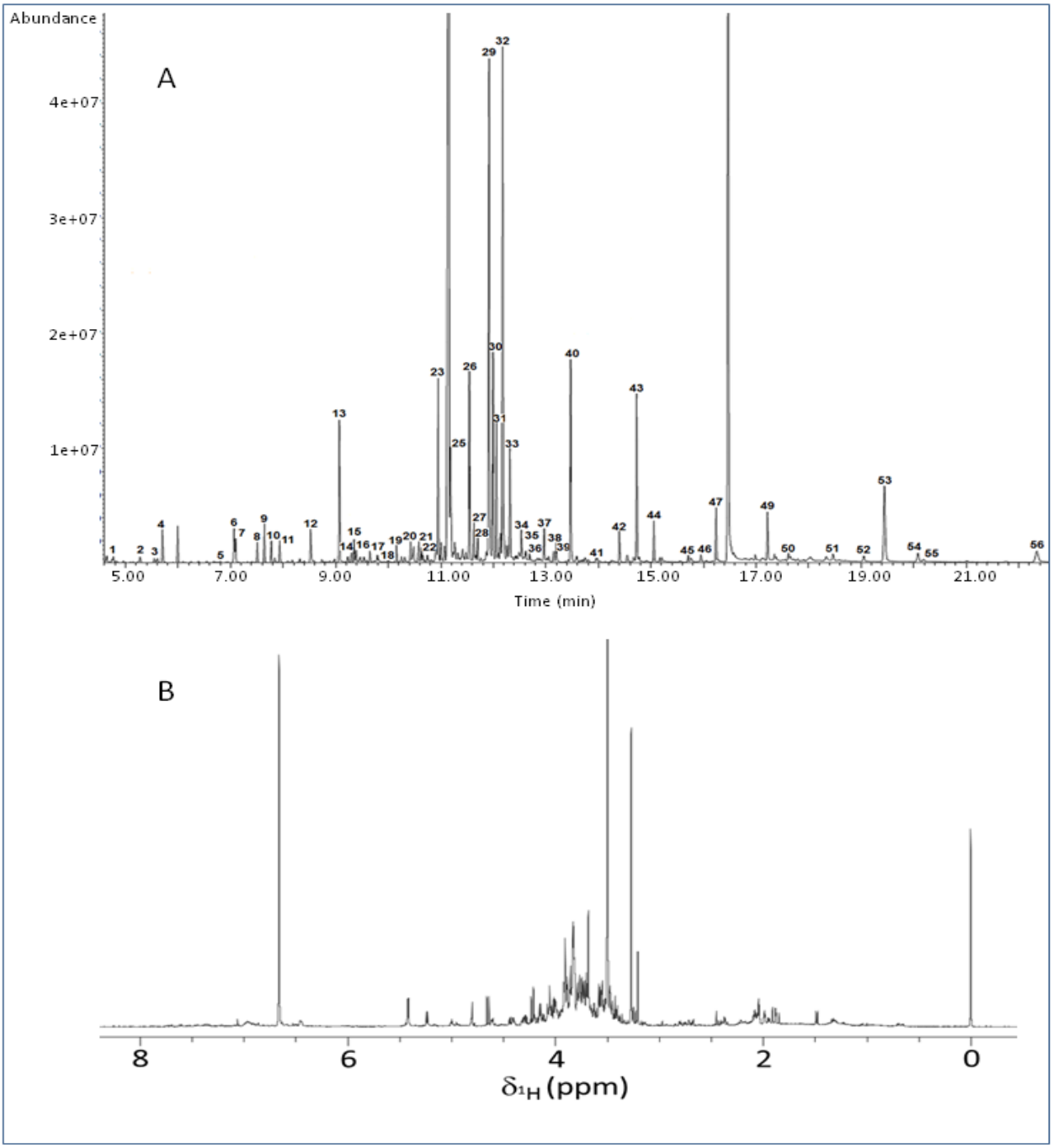


Figure 2. Total Ion Chromatogram (TIC) (A) and ^{1}H-NMR spectrum (B) of maize leaf extract (B0P0 sample).

Table 1a: List of primary metabolites from maize leaves identified by ^{1}H NMR

Amino acids	Assignment	δ (^{1}H)	Multiplicity
Alanine	β-CH_3	1.48	d
Alanine	β-CH_3	1.48	d
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.72	m
Arginine	β-CH_2	1.65	m
Arginine	β-CH_2	1.65	m
Arginine	β-CH_2	1.65	m
GABA	γ-CH_2	3.01	t
GABA	γ-CH_2	3.01	t
GABA	α-CH_2	2.28	t
Glutammic acid	γ-CH_2	2.34	t
Histidine	C4H ring	7.78	s
Histidine	C4H ring	7.78	s
Histidine	C2H ring	7.05	s
Isoleucine	γɜ-CH	1.45	m
Isoleucine	γ-CH	1.25	m
Isoleucine	γ-CH_3	1.02	d
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Isoleucine	δ-CH_3	0.93	t
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	γ-CH	1.69	m
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Leucine	δ'-CH_3	0.96	d
Phenylalanine	CH3,5	7.44	m
Phenylalanine	CH3,5	7.44	m
Phenylalanine	CH2,6	7.31	m
Phenylalanine	CH2,6	7.31	m
Proline	β-CH_2	2.32	m
Threonine	γ-CH_3	1.32	d
Tryptophan	C4H ring	7.72	d
Tryptophan	C4H ring	7.72	d
Tryptophan	C7H ring	7.54	d
Tryptophan	C7H ring	7.54	d
Tryptophan	C6H ring	7.28	t
Tryptophan	C6H ring	7.28	t
Tryptophan	C5H ring	7.20	t
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Tyrosine	C2,6H ring	7.18	d
Valine	γ'-CH_3	1.04	d
Valine	γ'-CH_3	1.04	d
Valine	γ-CH_3	0.99	d

Carbohydrates	Assignment	δ (^{1}H)	Multiplicity
Fructose		4.08	m
Fructose		4.08	m
Fructose		3.99	ddd
Fructose		3.89	dd
Fructose		3.89	dd
Fructose		3.85	m
Fructose		3.80	m
Fructose		3.79	dd
Fructose		3.70	dd
Fructose		3.70	dd
Fructose		3.65	m
Fructose		3.57	d
Fructose		3.57	d
α-Galactose	C1H	5.27	d
β-Galactose	C1H	4.60	d
α-Glucose	C1H	5.24	d
β-Glucose	C1H	4.64	d
β-Glucose	C1H	4.64	d
Glucose		3.90	m
Glucose		3.90	m
Glucose		3.84	m
Glucose		3.83	m
Glucose		3.77	m
Glucose		3.73	dd
Glucose		3.72	dd
Glucose		3.54	dd
Glucose		3.54	dd
Glucose		3.54	dd
Glucose		3.51	t
Glucose		3.42	t
Glucose		3.40	t
Glucose		3.40	dd
Glucose		3.24	t
Glucose-6-phosphate		5.58	d
Glucose-6-phosphate		5.58	d
Inositol		4.05	t
Inositol		3.61	t
Inositol		3.61	t
Inositol		3.52	dd
Raffinose (Piccioni)	Gal1H	5.00	d
Raffinose (Piccioni)	Gal1H	5.00	d
Raffinose (Piccioni)	Fru3H	4.23	d
Sucrose	Glc1H	5.42	d
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose			
Sucrose	$FruCH_2$-1'	3.68	s
Sucrose			
Sucrose			
Trehalose (Piccioni)	C1H	5.20	d
Trehalose (Piccioni)	C1H	5.20	d

Table 1a:continued

Nitrogenous compounds	Assignment	δ (^{1}H)	Multiplicity
Adenosine-like		8.34	s
Adenosine-like		8.34	s
Adenosine-like		8.34	s
Adenosine-like		8.27	s
Adenosine-like		8.27	s
Adenosine-like		8.27	s
Adenosine-like		8.11	d
Adenosine-like		5.99	d
Adenosine-like		5.99	d
Adenosine-like		5.99	d
UMP	C1'H ribose	8.11	d
UMP	C6 ring	5.99	d
Choline	N-CH_3	3.21	s
Choline	N-CH_3	3.21	s
Dimethylamine	CH_3	2.73	s
Glicine betaine (Piccioni)	N-CH_3	3.28	s
Phosphatidylcholine	CH_3	3.27	s
Trigonelline (HA)	HA	9.13	s
Trigonelline (HB, HC)	HB,HC	8.86	d
Trigonelline (HB, HC)	HB,HC	8.86	d
Trigonelline (HD)	HD	8.06	d
Trigonelline (HD)	HD	8.06	d
Trimethylamine	CH_3	2.91	s

Organic acids	Assignment	δ (^{1}H)	Multiplicity
Chlorogenic acid		7.6	d
Chlorogenic acid		7.13	d
Chlorogenic acid		6.87	d
Chlorogenic acid (Broyart)		6.35	d
Chlorogenic acid (Broyart)		5.316	td
Chlorogenic acid		5.316 1	td
Cis-aconitic acid	CH=C-	6.65	s
Cis-aconitic acid	CH=C-	6.65	s
Cis-aconitic acid	CH_2	3.49	s
Formic acid	HCOOH	8.46	s
Fumaric acid (α,β)	α,β CH=CH	6.53	s
Isobutirric acid	CH_3	1.14	s
Isobutirric acid	CH_3	1.14	s
Isocitric acid		2.96	q
Isocitric acid		2.96	q

Organic acids	Assignment	δ (^{1}H)	Multiplicity
Isocitric acid		2.46	dq
Isocitric acid		2.46	dq
Isocitric acid		2.46	dq
Maleic acid	α,β CH=CH	6	s
Malic acid	α-CH	4.32	dd
Malic acid	α-CH	4.32	dd
Malic acid	β-CH	2.68	dd
Malic acid	β-CH	2.68	dd
Malic acid	β-CH	2.68	dd
Malic acid	β–β'-CH_2	2.38	dd
Malic acid	β–β'-CH_2	2.38	dd
Quinic acid	H3	4.131	q
Quinic acid	H3	4.131	q
Quinic acid	H5	4.008	ddd
Quinic acid	H4	3.54	dd
Quinic acid	H6'	2.04	ddd
Quinic acid	H6'	2.04	ddd
Quinic acid	H6'	2.04	ddd
Quinic acid	H2	1.97	dd
Quinic acid	H2'	1.94	ddd
Quinic acid	H6	1.88	dd
Quinic acid	H6	1.88	dd
Quinic acid	H6	1.88	dd
Shikimic acid		6.42	m
Shikimic acid		6.42	m
Shikimic acid		4.38	t
Shikimic acid		4.38	t
Shikimic acid		2.77	dd
Shikimic acid		2.77	dd
Shikimic acid		2.77	dd
Shikimic acid		2.73	dd
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Shikimic acid		2.18	ddq
Succinic acid	α–β-CH_2	2.4	s

s= singlet d= doublet
dd=doublet of a doubledoublet
dd= doubledoublet
ddq= doublet of a doublequartet
q= quartet m= multiplet

Table1b : List of primary metabolites from maize leaves identified by GC-MS.

n°	Metabolites	RT (min)	MS fragmentation (m/z)	References
1	Lactic acid	4.77	52-73-75-117-147-149	NIST
2	Alanine	5.28	73-75-116-117-147-190	STD,MPI
3	Oxalic acid	5.7	73-74-75-147-148-149	NIST
4	Phosphoric acid	7.07	73-133-211-225-299-300	NIST
5	Glycerol tris TMS	7.09	73-117-147-205-299	NIST
6	Succinic acid	7.51	73-147-172-247	NIST
7	Glyceric acid	7.65	73-103-147-189-292	NIST
8	Itaconic acid	7.78	73-147-215-259	NIST
9	Serine	7.94	73-75-116-147-204-218	STD,MPI
10	Threonine	8.18	73-75-130-147-218	STD
11	Maleic acid	8.32	73-75-133-147-148-149	NIST
12	4-Ketaglucose	8.89	73-103-147-204	NIST
13	Arabino-hexose-2-ulose	8.98	73-103-147-205-234-262	NIST,MPI
14	Methylmaleic acid	9.07	73-147-189-233-265	NIST,MPI
15	Aspartic acid	9.35	73-75-100-147-232	STD
16	succinic anhydride	9.39	73-156-255	NIST
17	4-aminobutyric acid	9.47	73-130-175-205-231-260	NIST,STD
18	Tetronic acid	9.65	73-147-220-292	NIST
19	Glutaric acid	9.8	73-147-198-304	STD
20	Glutamine	10.16	73-75-128-147-156-246	NIST,MPI
21	Xylonic acid	10.43	73-117-147-175-217-244	NIST
22	Xylulose	10.66	73-103-147-217-307	NIST
23	**Ribitol IS**	10.95	73-103-147-217-319	STD
24	cis Aconitic acid	11.14	73-147-229-285-375	NIST,MPI
25	Cinnamic acid	11.42	73-147-204-245	STD,MPI
26	Shikimic acid	11.55	73-147-204-255	NIST,MPI
27	sugar	11.58	73-147-205-217	NIST
28	Isocitric acid	11.63	73-147-273-363	NIST
29	Quinic acid	11.92	73-147-191-255-345	NIST,MPI
30	Fructose	12	73-129-133-147-217-218	STD
31	Fructose	12.06	73-129-133-147-217-219	STD
32	Galactose	12.14	73-147-205-217-229	STD
33	Glucose	12.18	73-129-147-149-157-217	STD
34	Glucose	12.32	73-129-147-149-157-218	STD
35	UN	12.54	73-147-219-292	NIST
36	UN	12.97	73-147-221-449	NIST
37	Mucic acid	13.15	73-147-217-292-333	NIST
38	UN	13.35	73-103-147-221-449	NIST
39	Myo-Inositol	13.47	73-147-191-217-221-305	STD,MPI
40	Floridoside	13.73	73-103-147-204-337	NIST
41	Glucuronic acid	13.86	73-147-205-217-292-375	NIST
42	UN	14.11	73-147-204-321-361	NIST

Table 1b :continued

n°	Metabolites	RT (min)	MS fragmentation (m/z)	References
43	sugar	14.81	73-129-133-147-217-219	NIST
44	UN	15.1	73-147-204-321-361-406	NIST
45	oligosaccaride	15.5	73-147-217-361-450	NIST
46	oligosaccaride	15.53	73-147-217-361-450	NIST
47	oligosaccaride	16.2	73-147-217-361-450	NIST
48	Sucrose	16.47	73-169-217-243-361-362	NIST
49	UN	16.55	73-147-204-217-361	NIST
50	D-Turanose	17.35	73-147-217-361-450	NIST
51	Maltose	18.47	73-147-204-217-305-361	NIST
52	UN	19.05	73-147-219-255-345	NIST
53	Melbiose	19.45	73-103-129-204-217-361	NIST
54	UN	20.19	73-147-191-249-345-447	NIST
55	Chlorogenic acid	20.08	73-147-191-255-307-345	STD,MPI
56	Raffinose	22.35	73-147-129-205-217-361-437	NIST

NIST= mass spectra library NIST 05 (http://www.nist.gov); STD=standards; MPI=Max-Planck-Institute (Germany, http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html).

3.3 Influence exerted on leaves metabolome by different bio-stimulants

Prior to evaluate the possible synergistic effect exerted by PSB mixed with AMF and humic acids, we firstly considered the sole influence exerted by different microbial bio-stimulants. In figure 3 is displayed the PCA score-plot of samples treated with *Bacillus amyloliquefacuens, Pseudomonas spp*. and AMF respectively. The figure clearly shows a neat discrimination between treatments. Specifically, at positive values of PC1 (27.51% of the total variance) it was associated high concentration of glucose, fructose, alanine and valine which permitted to discriminate the M+ and B2 samples from the others. Particularly, among these groups of samples, M+ has shown a higher concentration of alanine and glucose. As reported by Weimer H. (1991), about a quarter of assimilated N is exported in the C4 plants as alanine. This is in line with our results, in fact the N content (fig1) in the samples M+ and B2 was significantly higher as compared to B3 and B0.

Conversely, along negative values of PC1, the high concentration of chlorogenic acid, tryptophan, phenylalanine, shikimic acid, isocitric acid and sucrose were associated to B0 and B3 samples. Such metabolites suggests an enhanced biosynthesis of secondary metabolites. The relevant presence of phenylalanine and tryptophan is very important since they serve as substrate for the phenylpropanoid pathway that, in turn, is a critical step for the biosynthesis of many secondary plant products such as, anthocyanins, lignin and phenolics (Leopold & Kriedman, 1975; Tzina & Galili, 2010). Indeed, also the presence of chlorogenic and shikimic acids confirms an enhanced synthesis of secondary metabolites. Since it is reported that stress conditions, such as wounding, resistance to pathogen infection, metabolic stress or perturbations in cell wall structure may enhance the biosynthesis of these compounds (Caño-Delgado *et al*., 2003; Tronchet *et al.*, 2010), their abundant presence associated to negative PC1 values may be correlated to a state of stress induced by treatments. Despite these observations are applicable to both B3 and B0 samples, the PC4, has permitted to distinguish the B3 and M+ samples from the others because of the highest amount of glycine, asparagine and malic acid. Additionally, albeit to a lesser extent than in M+ samples, the variables alanine and glucose allow to separate B3 from B0. All abovementioned variables, which are related to the photosynthetic activity, have been correlated with high contents of N and P in B3 than for as B0 samples thus underlining a better nutritional status induced by *Bacillus amyloliquefaciens.* These results, confirm the positive and efficient influence exerted by each of studied bio-stimulants. Conversely the variables involved in the discrimination of control plants, may be attributed to a worse state of nutrition, a lower photosynthetic activity and, probably, to a growth inhibition.

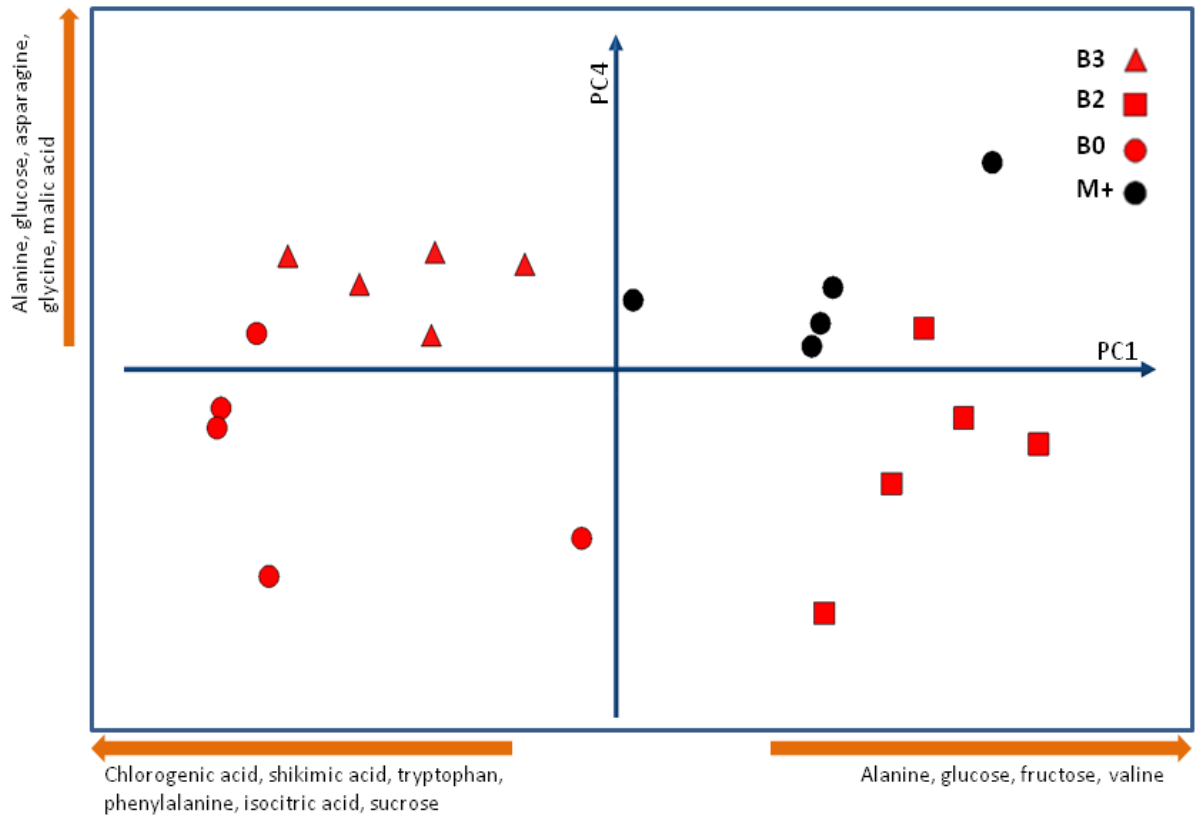


Figure 3. PCA score-plot obtained by processing GC-MS and NMR data of plants inoculated with different bio-stimulants (AMF (M+), *Bacillus amyloliquefaciens (B3), Pseudomonas spp.(B2),* control (B0)).

3.4 Metabolic analysis of plants inoculated with Pseudomonas spp., alone or in combination with AMF and humic acids

In the light of results obtained using the individual bio-stimulants, the next step was to study the possible synergistic effect exerted by PSB mixed with AMF and humic acids. In Figure 4 is displayed the PCA score-plot of samples treated with *Pseudomonas.spp* alone or in combination with AMF and humic acids. The PC1, accounted for 27.63% of the total variance, was able to discriminate the samples B2HAM+ and B2M+ from the others. Specifically, along positive values of PC1, the variables myo-inositol, tryptophan and phenylalanine feature the metabolic expression of maize plants inoculated with *Pseudomonas spp*. in presence of AMF. Remarkably, the role of

inositol and its isomer myo-inositol is particularly relevant in plants since they are involved in the phosphatidylinositol signaling pathway, auxin storage and transport, phytic acid biosynthesis, cell wall biosynthesis, and the production of stress related molecules (Loewus & Murthy, 2000; Loweus, 1990). In this context, the relevant presence of phenylalanine and tryptophan is very important since they serve as substrate for the phenylpropanoid pathway that, in turn, is a critical step for the biosynthesis of many secondary plant products such as, anthocyanins, lignin and phenolics (Leopold & Kriedman, 1975; Tzina & Galili, 2010). Conversely, the PC2 (24.64 of the total variance) clearly discriminate the samples B2HA and B2HAM+ from the others, due to a higher content of glucose. Such metabolite, which can be directly associated to photosynthesis, may be positively influenced by the presence humic acids.

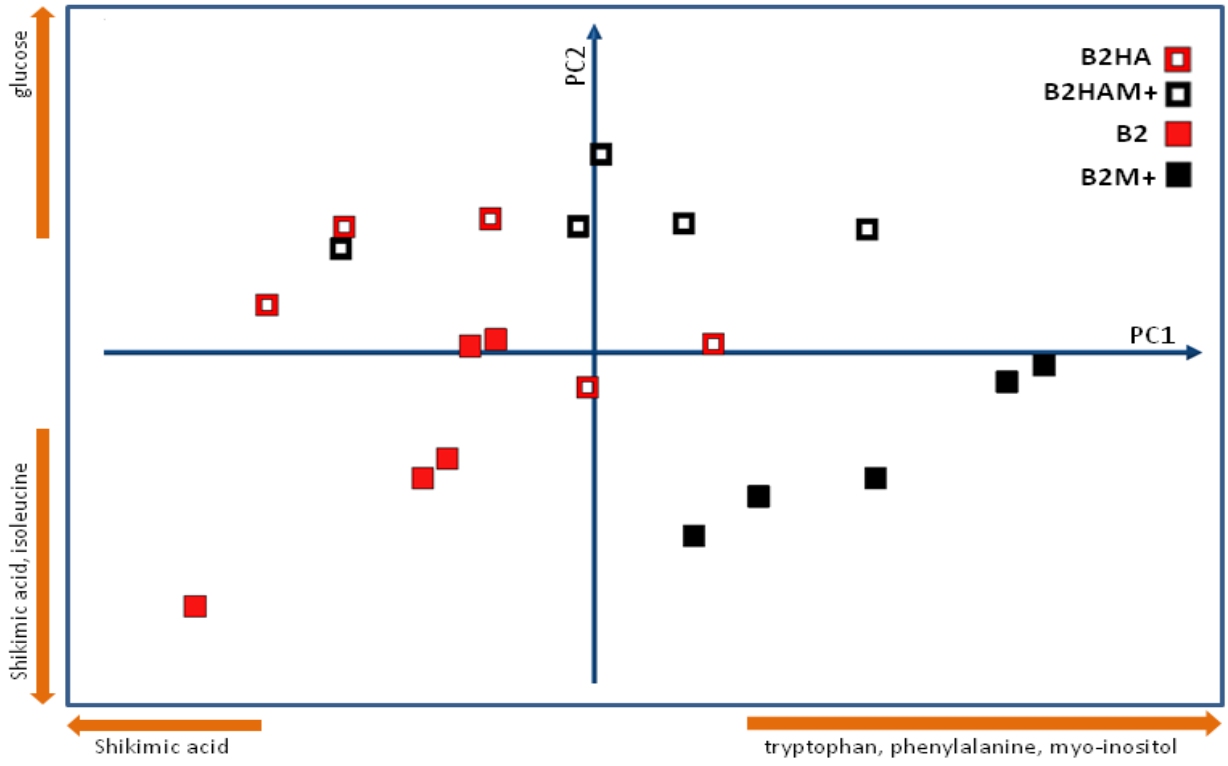


Figure 4. PCA score-plot obtained by processing GC-MS and NMR data of plants inoculated with *Pseudomonas spp.* (B2) an d different combination with AMF (M+) and humic acids(HA).

3.5 Metabolic analysis of plants inoculated with Bacillus amyloliquefaciens, alone or in combination with AMF and humic acids

In Figure 5 is shown the PCA score-plot (34.31% total variance explained) of samples inoculated with *Bacillus amyloliquefaciens*, either without or with the concomitant presence of humic acids and AMF. The figure shows a discrete discrimination between treatments and, in particular, indicates an evident separation toward PC2 values of those samples in which *B. amyloliquefaciens* (B3) were combined with AMF inoculation. In such case the samples B3M+ and B3HAM+ were discriminate along negative values of PC2 due to the highest concentration of isoleucine, valine and succinic acid. Such variables were correlated with highest content of P in B3HAM+ samples (Figure 1), thus suggesting a better nutritional status induced in these plants.

Instead, at positive values of PC2 it was associated high concentrations of chlorogenic acid, tryptophan, shikimic acid, quinic acid, tyrosine and alanine which permitted to discriminate the samples B3 from the others. Thus condition was probably attributable to an enhanced biosynthesis of secondary metabolites. Furthermore the samples B3 were associated with lower content of P and N, allowing therefore to explain the high levels of the abovementioned variables. In this context, B3HA samples were collocated in the central area of PC2 because of the intermediate values detected for all abovementioned variables.

Conversely, the PC4 which accounted for a 9.53% of total variance, was able to distinguish B3M+ samples from B3HAM+ samples because of their high content of sucrose and inositol respectively. Specifically, the increased expression of inisitol in B3HAM+ samples, was directly involved with the highest content of P , allowing to positively associate the combined effect of HA with AMF and *Bacillus amyloliquefaciens* .

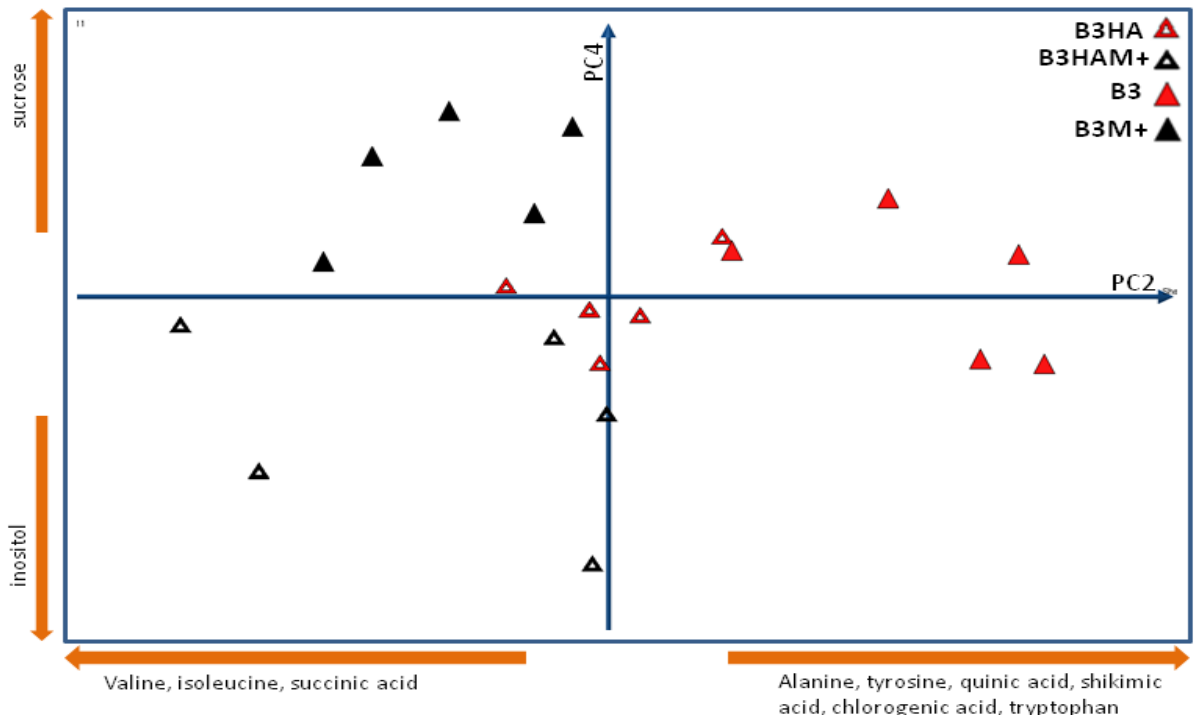


Figure 5. PCA score-plot obtained by processing GC-MS and NMR data of plants inoculated with *Bacillus amyloliquefaciens* (B3) an d different combination with AMF (M+) and humic acids(HA).

4. Conclusions

In this work, we evaluated the influence of micro-bio-humeome exerted by different bio-stimulants inoculated on plants of *Zea mays* fertilized with compost. Specifically, was evaluated the possible synergistic effect exerted by different PSB combined with AMF and humic acids. Behind the choice of treatments adopted in this work, there was the purpose to investigate on effects induced on plants by microorganism used in the organic farm in order to reduce the mineral fertilizers input.

To this end, the polar metabolites, extracted from treated plants subjected to studied treatments and detected by both GC-MS and ^{1}H NMR techniques, were grouped in a single data matrix and evaluated by Metabolomics. The combination of GC-MS and NMR data enabled the identification of a more complete metabolic profile which, in a more reliable way, permitted to individuate compounds whose concentration varied in a treatment-dependent way.

All microorganisms tested, alone or in combination with AMF and humic acids, have been positively influenced the N and P content in plants as compared with controls. Furthermore, all plants treated, have underlined a different metabolic expression linked to the different combination of bio-stimulant used.

Particularly, the highest content of P and N involved in the increased expression of isoleucine, valine, inositol and succinic acid shown in B3HAM+ samples, allow to highlight the greater synergistic effect of such combination of bio-stimulants, suggesting therefore, a better nutritional state, which is expected to sustain a higher plants growth. The combination of *Pseudomonas spp*. with HA and AMF, he has slightly improved the P content on plants, as compared to the other tested combinations. However, all others tested combinations of bio-stimulants, despite the different metabolic expression exerted on plants, he has not made marked improvements on the N and P content on plants.

Such results, allow to conclude that not all bio-stimulants combinations used create positive synergistic effects on plants in terms of N and P content.

CHAPTER 6

Molecular Characterization of Peats fractionated by Humeomics

Abstract

In order to enhance a deep knowledge on the molecular composition of peat, which is a very heterogeneous material relatively rich in organic matter (OM), we propose the Humeomic approach to characterize three ombrotrophic peats sampled in different geographical areas. The structural identification of the chemical fractionation products, subdivided according to their solubility in 4 residues (RES), 3 organ-soluble extracts (ORG) and 3 water-soluble extracts (AQU), was conducted by GC-MS, liquid state 1H NMR and solid state 13C CPMAS NMR.

The results obtained allow to well characterize three different peat materials on the basis of their molecular composition. GC-MS results provided a quantitative differentiation between the ORG fractions, in particular different classes of compounds, such as phenols, dicarboxylic acids, fatty acids and alkanes, characteristic for each peat samples may be used as markers to identify the different origin of OM accumulated during peat formation. At the same time, NMR results showed the molecular composition of the untreated peats and residue fractions, which permitted to follow the gradual depletion of OM resulting from the progressive humemoics extraction.

In this context, the Humeomic approach demonstrated to efficiently simplify the complex organic mixture extract from peats and improve the molecular identification of different compounds in the three ORG fractions.

Keywords: Peat, NOM, Humeomics, Fractionation, GC-MS, NMR

1.Introduction

Peat consists of a naturally occurring heterogeneous mixture composed by partially degraded organic matter (OM) prevalently deriving by plants residues which has accumulated in a water-saturated environment and in the absence of oxygen. The peats are characterized by anoxic and acidic conditions resulting from the fact that, especially for ombrothropic peats, the main inputs for water and nutrients are brought by the rain precipitations. Such relatively adverse conditions lead to the development of a selected microbiological community, mostly anaerobic, whose degrading action is relatively slow and may be further influenced by site-specific climate. In fact, the decomposer community, the depth, the water fluctuation and the oxygen availability may strongly control the degree of decomposition (Baber *et al*., 2003).Consequently, the accumulation of organic matter in peatlands is generally favoured since the rate of OM production is larger than the decomposition one. Generally, the most enhanced decomposition rate is reached in the upper layer of peats because of a relatively more aerated condition. In molecular terms, such a slow degradation offers the advantage to slow down the release in the atmosphere of greenhouse gases, such as CO_2 and CH_4 (Yu *et al*., 2010), as well as permits to go back, in a relatively easier way, to the botanical composition at the time of the deposition (Charman, 2002).

The most common chemical groups usually detected in peats include cellulose, hemicellulose, lignin, protein, cutin and suberin which are mostly ascribable to structural plant biopolymers. In particular, the peat OM contains plant biomarkers, such as lignin and lipids, which may provide information on plant origin. In fact, in anaerobic conditions, the lipids are almost resistant as well as the degradation of lignin is partial and proceeds at a relatively slow rate (Pancost *et al*., 2011). The content and the composition of lignin-derived compounds in peats is characteristic of the microhabitat developed at the moment of peat formation (Djurdjevic*et al*,. 2003; Tomson *et al*., 2010). In this context, such phenolic compounds may be present either as low-molecular-weight free compounds or as an aromatic components of peat lignin-humic complex (Tarnawski *et al*.,

2006). Concerning the lipids, the abundant presence of alkanes is attributable to leaf wax alkanes whose carbon chain length permits differentiate the type of plant from which they derived. Leaf wax of vascular plants (grasses, sedges, trees and shrubs) is dominated by long chain *n*-alkanes (C29–C31), whereas leaf wax of sphagnum, which is a moss genus efficiently adapted to peatlands, is characterized by medium chain length *n*-alkanes(C23–C25). Remarkably, this peculiarity has been also proved by compound-specific ^{13}C and ^{14}C studies of peat bogs (Nott *et al*., 2000; Pancost *et al*., 2002).

However, a deep and exhaustive knowledge on the molecular composition of peat, which is a very heterogeneous material relatively rich in OM, still lacks. So far, several analytical techniques have been developed and applied with the aim to improve the knowledge on this complex and heterogeneous material. Many of these, including chemolytic methods, are destructive techniques which produce artifacts preventing a reliable interpretation of results. For example, the analysis of pyrolysates may be very complex to be interpreted because of molecular rearrangements occurring during the induced pyrolysis. In light of this,^{13}C Cross Polarisation Magic-Angle-Spinning Nuclear Magnetic Resonance spectroscopy (CPMAS NMR), represents a valuable non-destructive method to examine peats since it provides meaningful information on carbon functional groups and indicate the most abundant molecular classes composing the studied OM in its solid form.

Recently, with the aim to examine very complex natural occurring materials, such as humic substances, it has been introduced a method, referred to as Humeomics (Nebbioso & Piccolo, 2011), which consists in a sequential chemical fractionation which simplify selectively the humic matter in several organo- and idrosoluble fractions whose molecular characterization results relatively easier and more complete. Since both the composition and conformation of peats are expected to be almost similar to those of humic substances, in this work the humeomics was applied for the first time to fractionate several peats deriving from different geographical area and different era of formation which were then characterized by advance techniques, such asGC-MS and ^{13}C CPMAS NMR.

2.Experimental

2.1. Samples

In this work we evaluated three ombrotrophic peats sampled in different geographical areas. In particular we examnined a sphagnum peat from Wales (WP), Canadian peat from Queen Charlotte Islands (QCI-27) and Swiss peat from Etang de la Gruere (2T-101). Additional informations on the samples dating, ash content and elemental analysis were reported in Table1. Each peat sample (500 mg) was placed in 300mL of 0.1M HCl and shaken overnight. After that, the samples were centrifuged (15min 4500rpm), washed by water until neutrality and air-dried prior to humeomic fractionation. All experiments were conducted in triplicate.

The determination of C, H and O was conducted directly on the pulverized samples by elemental analyzer (Fision EA1108), before the humeomic fractionation.

2.2. Fractionation

The sequential chemical called Humeomics fractionation was used to extract organic matter fractions by the peats (RES0). The unbound fraction (ORG1) was extracted by placing RES0 in a 2:1 v/v dichloromethane and methanol solution under magnetic stirring for 24h at room temperature. The supernatant was separated by centrifugation (15min, 7500rpm) and filtered through a Whatman 41 filter.This procedure was repeated two times and the residue (RES1) was air-dried.

For the extraction of weakly bound ester fractions (ORG2 and AQU2), the RES1 deriving from the previous step, was placed in a Teflon tube and treated two times using boron trifluoride in 12% methanol under N_2 atmosphere at 80°C for 12h. A ratio of 1ml of solution per gr of material was used. The derived supernatants were centrifuged (20 min, 3000 rpm) and combined. Then, the

residual BF3, was quenched with water and the MeOH removed by Rotavapor prior to subject the supernatant to liquid:liquid extraction using chloroform and water. The organo-soluble phase (ORG2) was separated, dried with anhydrous Na_2SO_4, filtered on a Whatman 41 filter and rotoevaporated. The aqueous phase (AQU2) was rotoevaporated to remove residual MeOH and Chloroform traces and dialyzed against distilled water using Amicon C membranes (1000 Da cutoff) until it was chloride-free and freeze-dried. The remaining solid residues (RES2), were water-washed and air-dried before the next step.

The strongly bound ester fraction (ORG3 and AQU3) were extracted from RES2 using a 1M KOH solution in methanol under N_2 atmosphere for 2h at 70°C (step repeated three times). After cooling, the supernatants were obtained by centrifugation (10 min, 3000 rpm).The residue was washed with 50mL of MeOH and centrifuged. The supernatants were combined, their pH adjusted to 2.0 with 37% HCl, and then liquid-liquid extracted three times with a mixture of DCM and water. The organo-soluble (ORG3) and hydro-soluble (AQU3) extracts were purified as for ORG2 and AQU2. The solid residue was water-washed and air-dried before the next step. Finally the last residue (RES4) and the strongly bound ether fraction (ORG4) and (AQU4), were obtained by placing RES3 in a 47% HI solution under N_2 atmosphere, stirring for 48h at 70°C. Finally, a liquid:liquid extraction of dichloromethane/water (50/50, v/v) was used to obtain ORG4 and AQU4.The last residue RES4 was dialyzed against water and freeze-dried.

2.3. Nuclear Magnetic Resonance (NMR) Spectroscopy

^{13}C-CPMAS NMR was applied to characterize the molecular composition of residue samples (RES0 to RES4) in the solid-state. ^{13}C-CPMAS NMR experiments were performed on a Bruker AV300. Samples were fitted into 4 mm Zirconia rotors with Kel-F caps and spun at a rate of 13000 Hz. Each carbon spectrum constisted of a recycle delay of 2s and an acquisition time of 3 ms.

Liquid-state NMR was applied to evaluate the water-soluble fractions (AQU2 to AQU4). The experiments were performed on a Bruker AV400 equipped with a 5mm inverse broadband (BBI) probe. The samples were placed into a 5.0 mm quartz tubes and dissolved with DMSO-d6. Monodimensional proton spectra were acquired by pre-saturating the signal of water. Data were processed with MestReC software.

2.4. GC-MS Spectrometry

Organosoluble fractions (ORG1 to ORG4) were derivatized before GC-MS analysis using acetyl chloride/methanol as methylating agent, followed by silylation using *N,Nbis[trimethylsilyl]trifluoracetamide / 1% trimethylchlorosilane*. The quantitative data were obtained adding tridecanoic acid as internal standard followed by an external calibration curve of specific standards for the different classes of compounds.

Samples were analyzed using an PerkinElmer chromatograph system coupled to an PerkinElmer Mass Selective Detector, quadrupole type GC–MS system. The GC was performed with RTX-5MS WCOT capillary column (Restek, 30 m × 0.25 mm; film thickness, 0.25 mm) that was coupled, through a heated transfer line (250 °C), to a Turbomass-Gold quadrupole mass spectrometer. The gas chromatographic separation was performed by applying a 2 minutes long isothermal phase at 100 °C, followed by a temperature increase from 100 to 300°C (rate of 4°C min^{-1}) and culminating in a 5 minutes long isothermal phase at 300°C. Helium was used as carrier gas at 1.6 mL min^{-1}, as well as the injector temperature was set at 250 °C and the split flowapplied for the split-injection mode was 25 mL min^{-1}. Mass spectra were obtained in EI mode (70 eV), scanning in the range included within 50 and 650 m/z, with a cycle time of 0.2 scan s^{-1}. The identification of mass spectra of eluted compounds was carried out by analyzing standard compounds as well as by evaluating the mass spectra reported in the library NIST 05 (http://www.nist.gov).

3.Results and discussion

3.1. Elemental composition and gravimetric analysis

The Table 1 shows a short description and the elemental composition of peat materials used in this work. Despite the different geographical origin and the different year of formation, the elemental composition indicated a very similar content of C,H and O.

The gravimetric evaluation of OM resulting from each step of humeomic fractionation has been listed in Table2 for all peats. The latter indicates that the most abundant yields have been achieved for ORG1, ORG2, AQU2 and RES4 fractions, and in particular for ORG2.

Remarkably, the ORG1 fraction, which is composed mostly by unbound molecular components of peat, totally and significantly differed according to the studied peat. In fact, peats QCI-27, WP and 2T-89, produced a ORG1 fraction corresponding to 6.2, 9.3 and 12.65%, respectively.

Relevant differences in yields were also observed for the ORG2 fraction (Table 2), which is composed by weakly bound peat components. In particular, the peat QCI-27 brought to the highest yield. However, the gravimetric results obtained for all other fractions did not show significant differences.

Our application of Humeomics led to a loss of material (U.M.) ranging within 30.4 and 41.1%, which is in line with results obtained by Nebbioso and Piccolo, (2011). This loss is probably due to the decarboxylation induced by the temperature, the presence of volatile substances and the release of occluded water. However, it has also to be taken into account that a the part of U.M. mostly composed by low molecular-size hydrophilic constituents may have been lost during dialysis. In fact, the application of humeomics fractionation is expected to change the Peat OM conformation through the opening of its supramolecular architecture which results in the release of relatively small molecular components (Piccolo, 2002).

Table1: Peats information and elemental composition.

Peats name	Peat classification	*Age (calendaryear)*	Ash (%)	C (%)	H (%)	O (%)
WP	*Welsh Sphagnum peat*	(recent)	3.39	51.44±0.35	5.78±0.01	41.32
QCI-27	*Canadian ombrotrophic peat (Queen Charlotte Islands)*	1904 A.D.	2.56	50.43±0.46	6.40±0.26	42.39
2T-101	*Swiss ombrotrophic peat (Etang de la Gruère)*	ca. 2100 B.P.	1.69	52.50±0.30	6.13±0.04	40.68

Table2: Gravimetric evaluation of organic matter after humeomic extraction. Results are expressed as the percentage related to the peat (RES0). U.M., Unaccounted material

Peats name	ORG1 (%)	ORG2 (%)	AQU2 (%)	ORG3 (%)	AQU3 (%)	ORG4 (%)	AQU4 (%)	RES4 (%)	U.M (%)
WP	9.3±1.1	13.3±1.5	11.4±2	0.7±0.1	2.2±0.2	0.1±0.2	2.1±0.5	32.4±7.2	30.4±5.2
QCI-27	6.2±1.3	16±1.2	10.8±2.1	0.45±0.1	0.16±0.2	1.0±0.9	3.7±0.2	19.3±4.5	41.1±7.2
2T-89	12.6±1.5	10.3±1.8	9.4±1.8	1.2±0.5	0.16±0.3	0.1±0.1	1.3±0.4	24.7±5.3	38.7±4.5

3.2. GC-MS Characterization

All of the organo-soluble fractions ORG1-4 extracted from peats samples were derivatized and analyzed by GC-MS. The results of ORG1-3 fractions are reported in Table 3A-C, respectively, and indicated a relevant abundance of aromatic compounds, such as phenolic acids, and saturated and unsaturated fatty acids characterized by medium-long chain (in particular C16:0 and C18:0). We characterized two main groups of phenolic acids, deriving principally from aromatic carboxylic acids: a) benzoic acid and b) cinnamic acid. Also several terpenoids, such as triterpenoids and sterols, were detected in all examined fractions. Concerning the carbohydrates, the most abundant amount was found principally in ORG1 and ORG2 fractions while in the ORG3 fraction they were

almost absent. However, from a quantitative point of view, GC-MS results revealed a different molecular compositions among ORG fractions. Despite the objective difficulties related to the interpretation of GC-MS chromatograms and spectra of such a heterogeneous and complex material, most of detected peaks were assigned. The peaks which were identified ranged within 10-20, 7-45 and 5-15% for ORG1,ORG2 and ORG3, respectively. Interestingly, such analytical yields, especially those obtained for ORG 2 fraction, may be considered relatively high as compared to the yield reported previously by Nebbioso and Piccolo (2011). However, in light of the intrinsic complex and heterogeneous nature of the studied peats, it is expected that a full detection of extracted molecules is prevented because of the technique which, despite the derivatization, may present several limitations. In fact, the compounds isolated in ORG 4 fractions were totally undetectable through the GC-MS. Additionally, it was not possible to include low intensity peaks in the quantitative calculations due to their poor reproducibility.

3.3 Unbound fraction ORG1

The GC-MS analysis of ORG1 fractions (Table 3A) revealed a greater percentage of identified compounds for QCI-27 than for the others peats. In fact, we identified the 20.5, 14,2 and 9.8% in QCI-27, 2T-89 and WP peats, respectively.

All extracts revealed that the most abundant class of compounds was represented by saturated fatty acids, then followed by aromatic compounds. These two compounds class represented more than the half of the totality of identified compounds.

The peat QCI-27 showed the highest concentration of fatty acids and aromatic compounds than the others, therefore indicating a greater extractability of such unbound material. Conversely, lowest values for the same compounds were observed in the 2T-89 peat, while for WP these were intermediate. Phenolic acids were the most representative compounds for the aromatic species since

their percentage ranged within 55.6% (QCI-27) and 100% (2T-89). Remarkably, the relevant differences detected in peats among phenolic abundance may be correlated to both dominant plants in the peatland as well as microhabitat features (Djurdjevic *et al*., 2003; Tarnawski *et al*., 2006; Tomson *et al*., 2010). Linear alcohols were only identified in QCI-27 and 2T-89 peats and showed similar content. Conversely, the dicarboxylic acids were found in QCI-27 and WP peats and accounted for 4.8 and 2.6 mg/g, respectively. Concerning the sugars, their total content was similar in QCI-87 and 2T-89 even if the ratio hexose/pentose was opposite. The Sphagnum peat WP showed slightly lower total content, but with similar ratio hexose/pentose of 2T-89.

Alkanes found in Welsh Sphagnum peat were circa 10 fold higher than 2T-89 and totally absent in QCI-27. Such peats were principally constituted by long chain *n*-alkanes (> C_{26}). These results, in line with those observed by Schellekens and Buurman(2011), where the long chain of *n*-alkanes (C_{29}+C_{31}), derived from woody plant species dominating in Sphagnum peat, while the C_{23} alkane were index of Sphagnum.

Table 3 A : GC-MS quantitative data of ORG1 fractions expressed as mg/g of extracted fractions.

Class of Compounds	QCI-27	2T-89	WP
	ORG1	ORG1	ORG1
Alkanes	-	1.5 ± 1.0	10.4 ± 0.1
Sugars tot.	5 ± 0.6	5.2 ± 0.3	3.8 ± 0.4
Hexose	1.4 ± 0.1	4.0 ± 0.2	2.7 ± 0.3
Pentose	3.6 ± 0.6	1.1 ± 0.1	1.2 ± 0.1
FattyAcids tot.	136.0 ± 13.4	83.3 ± 5.9	59.1 ± 7.8
Saturated	120.2 ± 12.3	80.9 ± 5.6	54.6 ± 7.3
Unsaturated	15.8 ± 1.1	2.5 ± 0.2	4.6 ± 0.5
DicarboxylicAcids	4.8 ± 0.2	-	2.7 ± 0.1
Linear Alcohols	3.6 ± 0.1	3.1 ± 0.1	-
Aromaticcompounds tot.	35.5 ± 0.6	18.2 ± 3.9	12.9 ± 1.7
PhenolicAcids	19.7 ± 0.1	18.2 ± 3.9	9.5 ± 1.5
Terpenoids	15.7 ± 0.1	25.7 ± 2.7	5.2 ± 0.3
TOT. (mg/g)	205.63 ± 15.8	142.17 ± 13.4	98.02 ± 11.8

3.4 Weakly bound ester fraction ORG2

In ORG2 fractions (Table 3B), we identified the 34.6, 46.9 and 7.1% for QCI-27, 2T-89 and WP, respectively. Differently than for ORG1, the most abundant class in ORG2 fraction was represented by the aromatic compounds.

As compared to the ORG1 fractions, a greater content of sugars was found in ORG2 which accounted for 0.2%, 2% and 2.3% in WP, QCI-27, 2T-89 peats, respectively and constituted principally by pentose monomers. Such a relative abundance is probably due to the transesterification that allowed the cleavege of ester bound moieties thus permitting the release of hydrophilic molecules possibly entrapped within the supramolecular structure of peat OM and protected from the degradative microbial activity. Obviously the most consistent part of sugars and others hydrophilic components were extracted by water and collected in the AQU fractions.

The second most abundant class of compounds was constituted by fatty acids. In particular, the QCI-27 and WP peats showed the highest and lowest content of saturated fatty acids, respectively, while the 2T-89 showed intermediate values, confirming therefore the same trend already observed for ORG1 fractions.

Dicarboxylic acids were found only in QCI-27 peat, whereas the linear alcohol were presents in a small only in WP. The terpenoid compounds were abundant in 2T-89, consistently with ORG1 fraction.

Table 3 B : GC-MS quantitative data of ORG2 fractions expressed as mg/g of extracted fractions.

Class of Compounds	QCI-27	2T-89	WP
	ORG2	ORG2	ORG2
Alkanes	-	-	-
Sugars tot.	20.6 ± 0.9	23.3 ± 1.2	2.3 ± 0.2
Hexose	1.7 ± 0.1	6.0 ± 0.5	0.3 ± 0.1
Pentose	18.9 ± 0.9	17.2 ± 0.7	1.9 ± 0.2
FattyAcids tot.	74.5 ± 7.4	48.2 ± 4.0	28.0 ± 3.0
Saturated	61.3 ± 5.3	39.2 ± 2.6	23.5 ± 4.1
Unsaturated	13.1 ± 1.3	8.9 ± 1.0	4.4 ± 0.1
DicarboxylicAcids	55.2 ± 4.4	-	-
Linear Alcohols	-	-	1.7 ± 0.2
Aromaticcompounds tot.	190.7 ± 15.2	377.5 ± 12.0	36.9 ± 4.6
PhenolicAcids	172.6 ± 14.0	366.7 ± 11.3	33.4 ± 4.3
Terpenoids	5.5 ± 0.7	19.8 ± 1.4	2.0 ± 0.2
TOT. (mg/g)	346.59 ± 28.7	469.02 ± 18.7	71.13 ± 8.4

3.5 Strongly bound ester fraction ORG3

The percentage yields of ORG3 fractions (Table 3C), revealed the 10.2% of identified compounds in QCI-27, 15.9% in 2T-89 and 4.7% in WP.

The organo-soluble fractions ORG3 (Table 3), obtained thought alkaline solvolysis, have been shown the presence of fatty acids as the main compounds, followed by aromatic compounds and terpenoids. Conversely to that observed in ORG1-2 fractions, the peat 2T-89 showed the highest content of fatty acids, while the other peats maintained the same difference.

Additionally, in such fraction, the phenols did not represent the main components of aromatics compounds as far as these were completely absents in peat QCI-27. The peat 2T-89 showed 6.2% of

aromatic compounds (2.8% were phenols), while WP showed 2.5% of aromatic (1% were phenols). The other compounds were totally absent in the extract.

Table 3 C : GC-MS quantitative data of ORG3 fractions expressed as mg/g of extracted fractions.

Class of Compounds	QCI-27	2T-89	WP
	ORG3	ORG3	ORG3
Alkanes	-	-	-
Sugars tot.	-	-	0.1 ± 0
Hexsose	-	-	0.1 ± 0
Pentose	-	-	-
FattyAcids tot.	71.82 ± 1.7	120.8 ± 0.6	26.2 ± 0.7
Saturated	63.1 ± 0.5	92.1 ± 1.3	24.9 ± 1.1
Unsaturated	8.8 ± 0.2	28.8 ± 1.7	1.3 ± 0.2
DicarboxylicAcids	0.1 ± 0	-	-
Linear Alcohols	-	-	1.5 ± 0.1
Aromaticcompounds tot.	15.9 ± 0.2	62.1 ± 4.5	25.4 ± 0.4
PhenolicAcids	-	28.5 ± 2.1	10.2 ± 0.2
Terpenoids	14.6 ± 0.8	5.1 ± 0.3	4.6 ± 0.4
TOT. (mg/g)	102.47 ± 2.5	159.57 ± 3.4	47.58 ± 1.5

3.6. NMR Characterization

^{13}C CPMAS NMR spectroscopy permits the identification of carbon functional groups and reveals the most abundant molecular classes composing peat OM. The ^{13}C NMR spectra of residues RES0-3 resulting from humeomic extraction have been reported in Figure 1A,B and C for peats 2T-89, QCI-27 and WP, respectively. The bulk materials (RES0), were characterized by a predominance of O-alkyl carbons (60-110 ppm), principally attributable to mono- and polysaccharides (Spaccini & Piccolo 2009). Specifically, 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 marker of anomeric carbon 1 of glucose chains in cellulose (De Marco et al., 2012). Interestingly, the most intense signals detected in all RES0 residues corresponded to carbohydratic moieties, where the highest and the lowest contents were detected in peats 2T-89 and QCI-27, respectively. However, since it is expected a relative abundance of lignin derived moieties in examined peats, part of signals rising in the region 55-60 ppm has to be attributed to lignin methoxyls.

The NMR peak resonating in the range 0-45 ppm indicated the presence of alkyl-carbons moieties ascribable to methylene and methyl groups in aliphatic chains and mainly deriving from lipidic substances such as plant waxes and polyesters (De Marco *et al*., 2012). Respectively, the peaks at 19 and 31 ppm may be assigned as , terminal methyls and methylenes (CH_2) segments of aliphatic chains present in various lipid molecules, such as wax, sterol, and cutin (Spaccini & Piccolo 2009; De Marco *et al*., 2012). As shown in Figures 1A-C these alkyl signals have been observed in RES0 samples and they were very intense for peat 2T-89.The wide peaks included within 110 and 145 ppm derive from unsubstituted and C-substituted aromatic carbons in either lignin monomers, lignans or flavonoids. The region (145–160 ppm) are attributable of quaternary carbons in phenolic compounds. Finally the region attributable to ester or acid carboxyles (160-190 ppm) did not show any peak for all the peats, thus suggesting a low abundance of these moieties.

As expected, the analysis of residues RES1-3 deriving from the humeomic fractionation showed a slow and progressive depletion of the organic substance contained in the bulk material RES0. Such depletion was in line with results obtained by analyzing the ORG fractions trough the GC-MS. In fact, the fact that ORG1 fraction was composed principally by fatty acids, terpenoids and phenolic acids well reflects the relevant decrease in intensity for RES1 fractions. At the same time, the progressive depletion of signals related to O-alkyl carbons (60-110 ppm) found in all RES3 samples was in agreement with the high presence of carbohydrates in the ORG2 fractions. Additionally, the signals in the last residue correlated with the low amount of compounds detected

in the ORG3 fractions thus confirming the difficulties faced to totally extract the remaining organic compounds presents in RES4 fractions.

The freeze-dried AQU water-soluble extracts from different peats were dissolved in dimethylsulfoxide (DMSO, ^{1}H signal at 2.5 ppm) prior to be analyzed by ^{1}H NMR.

Proton spectra (Figure 2) of AQU fractions evidenced significant similarities between 2T-89 and WP whereas a different composition was detected for . QCI-89 AQU2.

As previously reported (Simpson *et al.*, 2011), the proton spectra may be divided into three main regions: alkylic region (aliphatics components, 0-3 ppm), O-Alkyl region (prevalently oligo- and polysaccharides, 3-5 ppm) and aromatics region (mainly lignin components, 6-8 ppm). In particular, the quadrupolar triplet at 7 ppm has been ascribed to the presence of ammonium.

WP and 2T-89 samples revealed high concentration of O-Alkyl groups (3-5 ppm) thus indicating a relative abundance of alcohols and polysaccharides. Interestingly, the fact that in QCI-27 peat the peaks resonating in this region were almost sharp permitted to minimize the contribution of polysaccharides (typically associated to broadened signals) and indicated a larger presence of alcohols and oligosaccharides.

Concerning the alkylic spectral region, three intense peaks appearing in spectra, may be assigned as methyls (0.88 ppm) and methylene protons of alkylic chains (1,2 ppm) and found in all analyzed fractions. Aromatic peaks result in an overall broadened signal ranging within 6.5 and 8 ppm whose low resolution is typically due to the enhanced aggregation occurring in different and interacting aromatic moieties.

As shown in Figure 2, relevant differences were different according to among studied peats were found also in AQU3 fractions. This notwithstanding, in line with AQU2 spectra, the larger similarities were observed for 2T-89 and WP peats. In particular, AQU3 fractions appear depleted of O-alkyl compounds, as confirmed by low gravimetric results obtained for these fractions (Table 2). Intense signals were detected in the alkylic region (0-3ppm).Conversely, the QCI-27 AQU 3 fraction underlined a lower presence of the all compounds found in the other fractions.

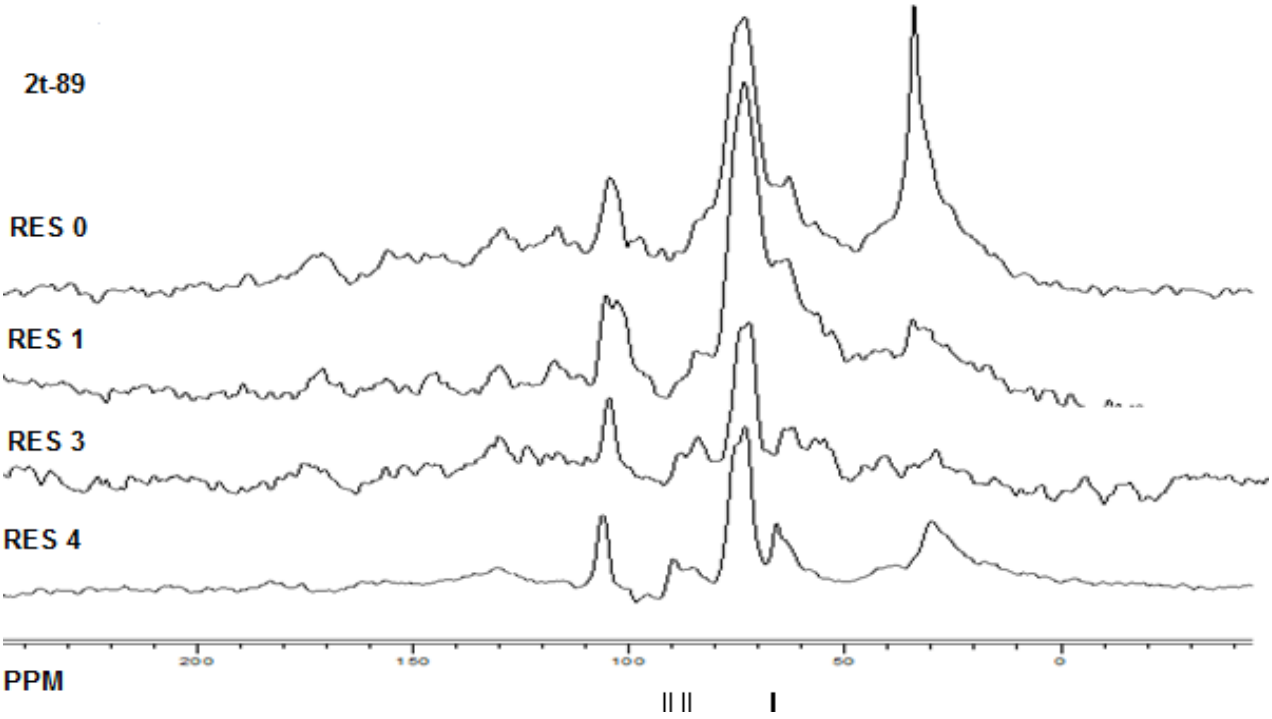


Figure 1a: ^{13}C NMR spectra of residues RES0-4 relative to the peat 2T-89.

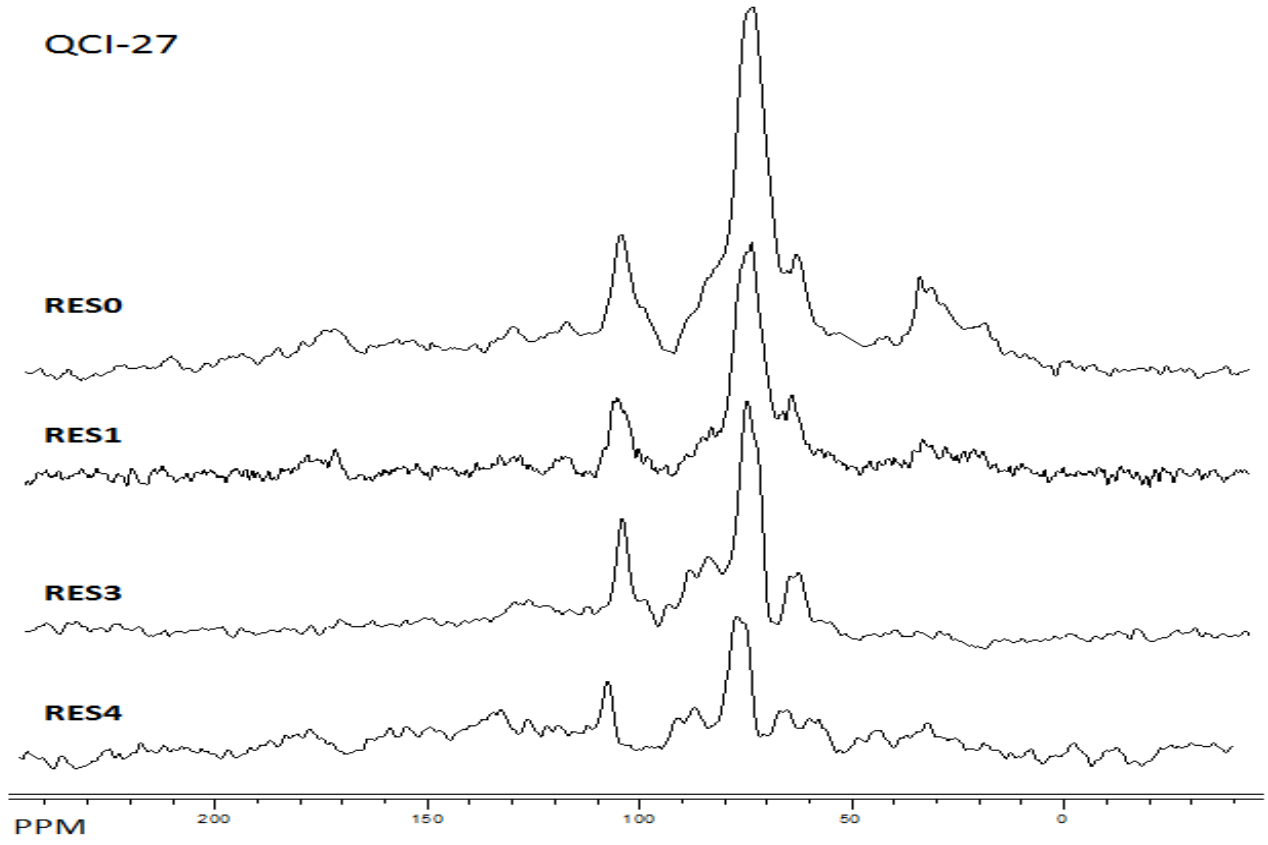


Figure 1b: ^{13}C NMR spectra of residues RES0-4 relative to the peat QCI-27.

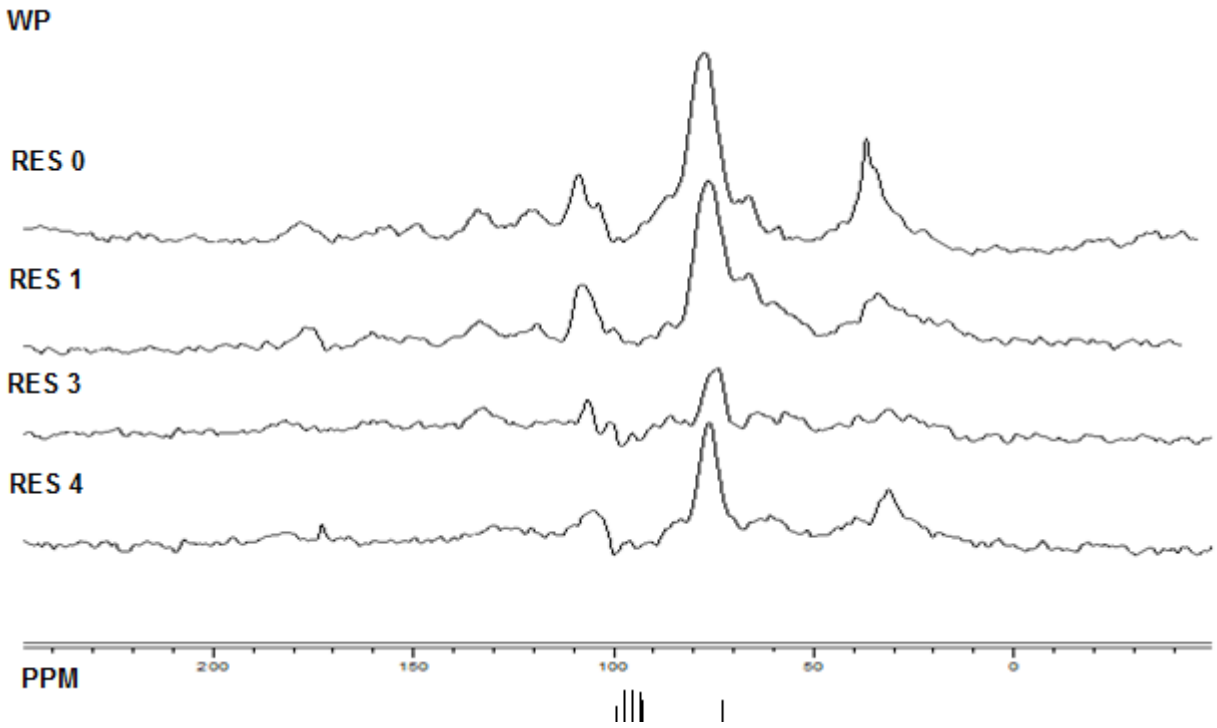


Figure 1c: ^{13}C NMR spectra of residues RES0- 4 relative to the peat WP.

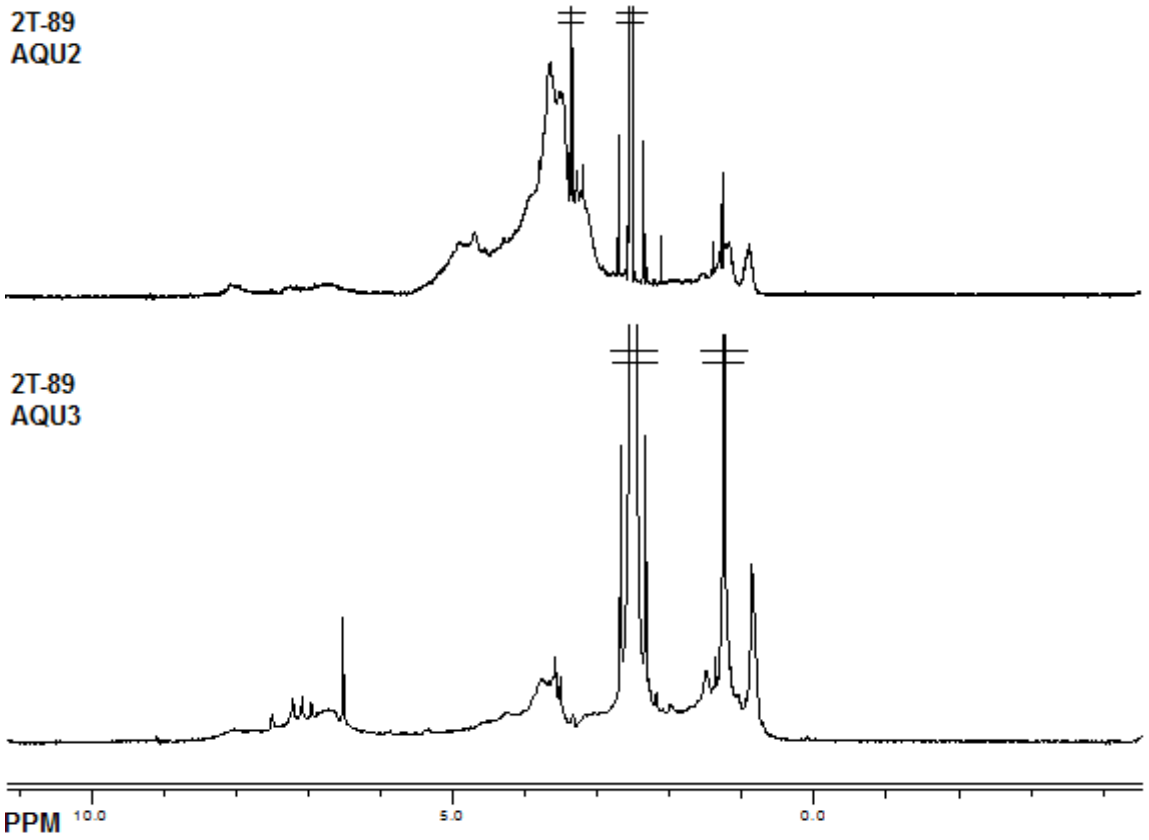


Figure 2a: ^{1}H NMR spectra of AQU2-AQU3 fractions relative to the peat 2T-89

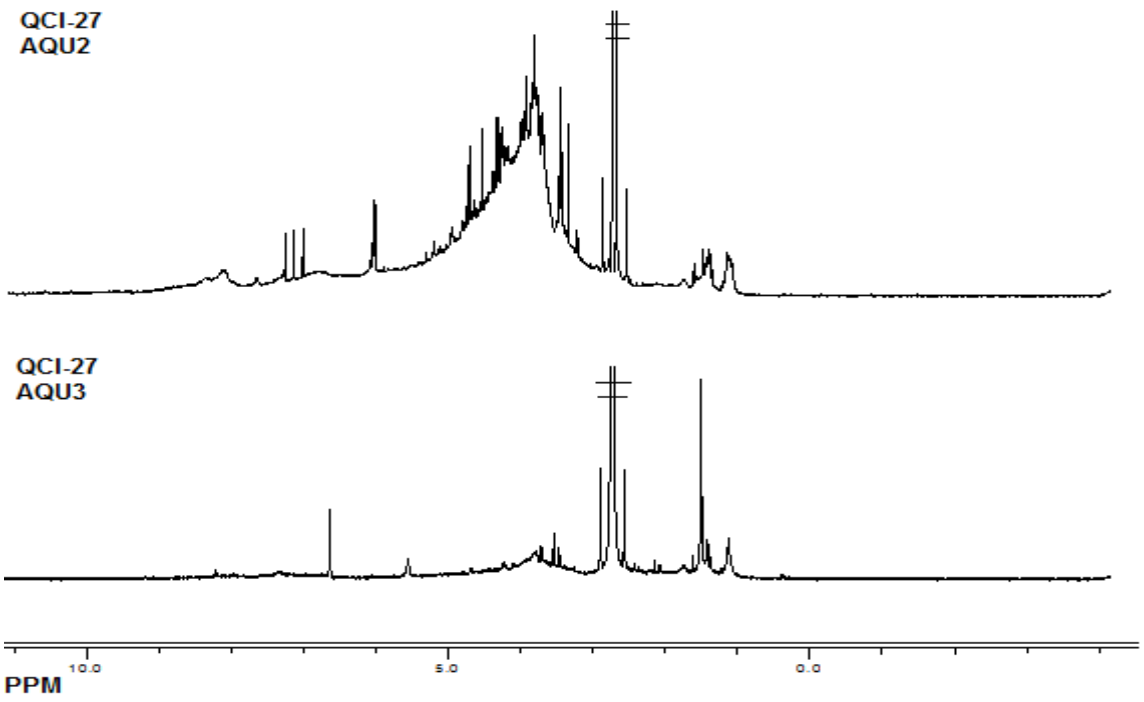


Figure 2b: ^{1}H NMR spectra of AQU2-AQU3 fractions relative to peat QCI-27.

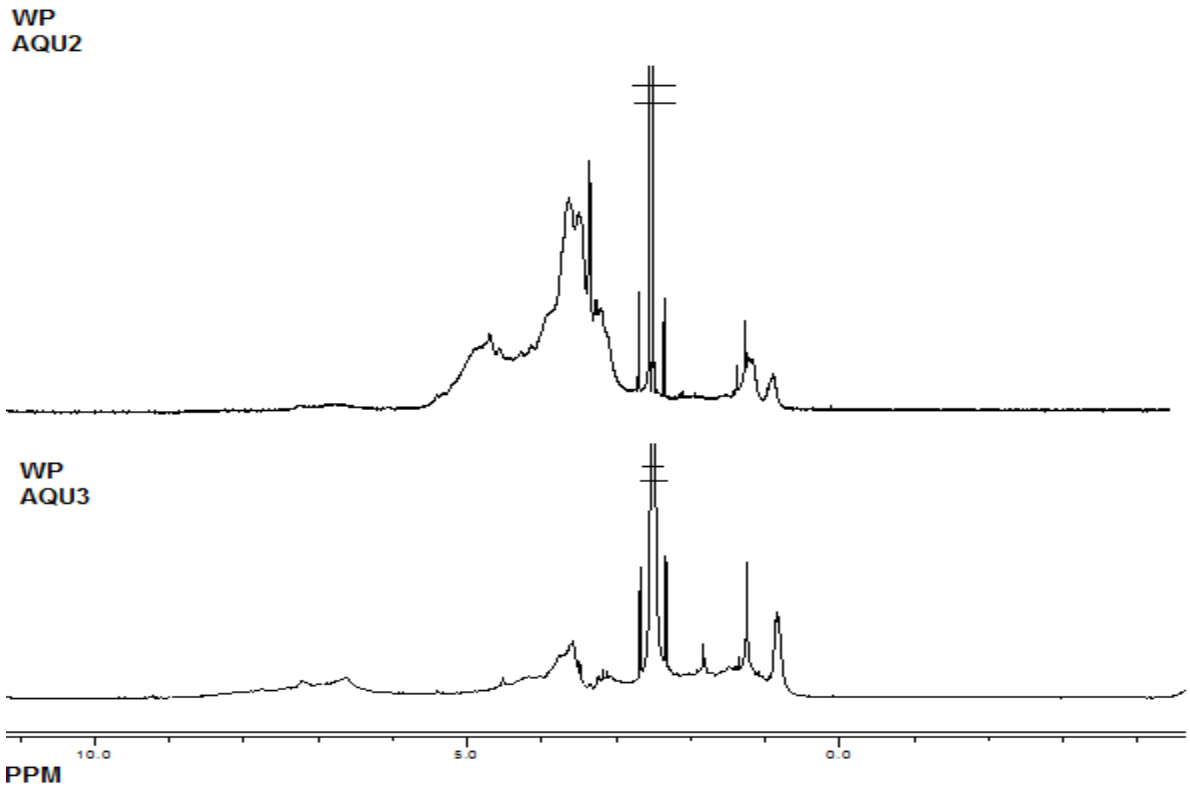


Figure 2c: ^{1}H NMR spectra of AQU2-AQU3 fractions relative to peat WP

4. Conclusions

In order to investigate the differences in the humification degree and identify possible markers to differentiate peats deriving from different geographical areas, we used the humeomics chemical fractionation. The original procedure of humeomics was designed to be applied on OM previously extracted from soils, in which the organic content is generally below 5%. In light of the high OM content typical for peat materials, we hereby described the efficient application of humeomics directly on peat materials, thus bypassing the preliminary OM extraction.

Despite, the similar elemental composition, the humeomic results, showed differences among the three peats used. Specifically, we have observed that the gravimetrical yields obtained for ORG1 and ORG2 fractions were characteristic of the different peats. GC-MS results, underlined different molecular compositions among ORG fractions analyzed under a quantitative point of view.

The different amount of compounds isolated and identified through humeomics, such as phenols, dicarboxylic acids, pentose sugars and alkanes, permitted to differentiate the studied peats.

In particular, long chain alkanes were very abundant in WP peat thus suggesting the dominant presence of woody plant species as compared to mosses in this Sphagnum peat. Of course, the different peat-specific classes of compounds may serve as marker to identify the different origins of the OM accumulated during the periods of peat formation. In this context, the humeomic approach demonstrated to efficiently simplify the complex organic mixture extract from peats and improve the molecular identification of different compounds in the three ORG fractions. At the same time, NMR results showed the molecular composition of the untreated peats and residue fractions, which permitted to follow the gradual depletion of OM resulting from the progressive humemoics extraction. Additionally the 1H NMR spectra showed the predominant presence of carbohydrates in AQU2 fractions. In light of these results we do believe that the Humeomic fractionation may represent a valuable tool to investigate on the molecular composition of peat.

CHAPTER 7

7.1 General Conclusions

The results obtained during the work of this thesis indicate that the combined use of GC-MS and NMR enabled a more reliable and exhaustive identification of the metabolic profile of plants under different treatments, than for each technique used alone.

The confidence in the analytical results allowed to reveal the positive effects on Zea mays plants of the combined soil treatments with manure composts and inocula of either bacteria or fungi. This combination promoted a better nutrient uptake and greater plant growth, as revealed by the larger concentration than other treatments of P and N in plant leaves, as well as by the larger presence of metabolites such as alanine, fructose and glucose. The beneficial effects of the compost/microorganisms combination were even more evident when compared to the lack of similar increase of N and P uptake when the same microorganisms were used in combination with mineral fertilizers. The results of this thesis also indicated that control treatments which received inoculation by bacteria or fungi without a fertilizer addition, did not show any improvement in plant nutrients and growth. This negative effect of the treatment with microbial bioeffectors was attributed to a state of wither plant-microorganism or microbe-microbe competition for available nutrients.

The best results on plants parameters were obtained under a treatment that combined the use of *Bacillus amyloliquefaciens* as bio-stimulant and cow-manure compost as P fertilizer. These preliminary results on the effectiveness of *B.amyloliquefaciens* added jointly with and cow-manure compost were used to study the impact of such treatment on the metabolic profile in plant leaves.

Moreover, in order to fulfill the purprose of unveiling the role of the micro-bio-humeome on the maize leaves metabolome, a further treatment (Chapter 5) was applied to a maize growing experiment based on the synergistic inoculation of microorganisms with AMF and in combination

with a HA isolated from a compost obtained from artichoke residues. The hypothesis of the bioactivity of the micro-bio-humeome was confirmed by finding that all microorganisms tested, alone or in combination with AMF and HA provided a larger N and P content in plants than for control. In particular, the greatest content of P and N in plants were found under the combined treatment with *B.amyloliquefaciens*, HA and AMF. These results indicate that the humeome represented by the soluble humic molecules in HA enabled a greater synergistic effect of the microbial bio-stimulants, thereby suggesting that a better nutritional state accompanies a better plants growth.

In conclusion, the results of this thesis confirm that the combination of microbial bio-stimulants with organic fertilizers, such as composts or readily soluble humic molecules, may provide a valuable alternative to the conventional agricultural practices, thus limiting the use of expensive and environmentally unsustainable mineral fertilizers.

This thesis thus showed the role played by the micro-bio-humeome in affecting the metabolome expression of plants, and, consequently, their growth. In Chapters 3 and 4, it was reveled that the different molecular composition of the composts used had different impact on plant N and P content and consequently on plant metabolome. It is therefore clear that the synergist effects represented by the micro-bio-humeome system must be addresses further by taking into consideration the different molecular composition of soil humus or exogenous humic or humic-like matters.

In order to complete the micro-bio-humeome concept, this thesis presented an application of Humomeomics, a procedure earlier developed in the thesis' laboratory, by which a detailed molecular characterization of some peat sample have been obtained (Chapter 6). Humeomics on peat samples simplified the complex organic mixture by selectively separating different molecular fractions on which a detailed spectrometric characterization was conducted. The findings on fractions weight and molecular composition highlighted that two organo-soluble fractions (ORG1 and ORG2) enabled a molecular differentiation between peat samples. Furthemore, GC-MS results provided a quantitative differentiation between the ORG fractions, thereby substantiating the

molecular differences among peat materials. In particular, different classes of compounds, such as phenols, dicarboxylic acids, fatty acids and alkanes, characteristic for each peat samples may be used as markers to identify the different origin of the organic matter OM accumulated during peat formation. These results therefore suggest that Humeomics can be profitably combined with metabolomics of plant leaves (and, possibly, roots) to reach a progressive deeper understanding of the micro-bio-humeome effects in the soil-plant rhizosphere system.

Based on the results of this thesis, it is believed that new tools are becoming available to enlarge our knowledge of the natural chemical and biological processes which take place in the rhizosphere, thereby contributing to the development of eco-compatible and sustainable agriculture.

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