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Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings

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Abstract

A humic acid (HA) isolated from a volcanic soil was separated in three fractions of decreasing molecular size (I, II and III) by preparative high performance size exclusion chromatography (HPSEC). The molecular content of the bulk soil HA and its size fractions was characterized by pyrolysis-GC-MS (thermochemolysis with tetramethylammonium hydroxide) and NMR spectroscopy. All soil humic materials were used to evaluate their effects on the enzymatic activities involved in glycolytic and respiratory processes of *Zea mays* (L.) seedlings. The elementary analyses and NMR spectra of the humic fractions indicated that the content of polar carbons (mainly carbohydrates) increased with decreasing molecular size of separated fractions. The products evolved by on-line thermochemolysis showed that the smallest size fraction (Fraction III) with the least rigid molecular conformation among the humic samples had the lowest content of lignin moieties and the largest amount of other non-lignin aromatic compounds. The bulk HA and the three humic fractions affected the enzyme activities related to glycolysis and tricarboxylic acid cycle (TCA) in different ways depending on molecular size, molecular characteristics and concentrations. The overall effectiveness of the four fractions in promoting the metabolic pathways was in the order: III > HA > II > I. The largest effect of Fraction III, either alone or incorporated into the bulk HA, was attributed to a flexible conformational structure that promoted a more efficient diffusion of bioactive humic components to maize cells. A better knowledge of the relationship between molecular structure of soil humic matter and plant activity may be of practical interest in increasing carbon fixation in plants and redirect atmospheric CO_2 into bio-fuel resources.

Keywords: Soil humic acids; Maize; Glycolytic pathway; Krebs cycle; High performance size exclusion chromatography; NMR spectroscopy; Thermochemolysis

1. Introduction

The past century has seen a marked increase in atmospheric carbon dioxide concentrations and a concomitant 'greenhouse warming' that has drawn scientific attention to the link between global carbon stocks and climate change (Cox et al., 2000). In particular, the decomposition and turnover of soil organic matter (SOM) due to intense agricultural production is recognized as an important determinant of carbon driven climate change (Briones et al., 2007). Moreover, SOM is recognized as a key factor in soil fertility since it controls the physical, chemical and biological properties of the rhizosphere (Nardi et al., 2002b; Gastal and Lemaire, 2002). In this respect, the unravelling of the biochemical and physiological events underlying the effect on plant growth of humic substances (HS), that are the major components of SOM, has become a primary goal to improve plant nutrition and, consequently increase photosynthate carbon (Nardi et al., 2002a).

The HS, heterogeneous organic compounds formed in soil as by-products of microbial metabolism on dead cell materials, were found up to now to exhibit a range of

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different effects on plant metabolism (Tan, 2003; Nardi et al., 1996, 2002a), depending on their origin, molecular size, chemical characteristics and concentration. According to a new view of their chemistry, HS are collections of heterogeneous, relatively low molecular-mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds (Piccolo et al., 1996; Piccolo and Conte, 1999; Piccolo, 2001; Sutton and Sposito, 2005). These associations are capable of organizing, in suitable aqueous environments, into supramolecular structures of only apparently large molecular sizes. This novel interpretation implies that root-exuded organic acids present in soil solution may affect the stability of humic conformation and hence their effect on permeability of root membranes (Nardi et al., 1996).

In the rhizosphere, an interaction between the root system and humic matter is possible when humic molecules, present in the soil solution, are able to flow into the apoplast and reach the plasma membrane. This event occurs in the vicinity of the root surface, where the simultaneous release of protons and organic acids by both roots and microbes enables the disruption of humic macrostructures and the subsequent release of the otherwise unavailable bioactive fractions (Piccolo et al., 1992). These substances may enter into the plant, translocate from roots to shoots (Vaughan and MacDonald, 1976; Nardi et al., 1996), and affect plant growth by different mechanisms: increasing respiration (Vaughan et al., 1985), enhancing mineral nutrition (Clapp et al., 2001; Varanini and Pinton, 1995, 2001), and/or stimulating hormonal activities (Vaughan et al., 1985; Visser, 1986; Nardi et al., 1988, 2000).

Visser (1987) showed that low molecular size (LMS) HS induced a more significant increase in respiration than high molecular size material in rat liver mitochondria. Moreover, in relation with concentration, they increased the efficiency of the oxidative phosphorylation process in vitro, particularly after contact time with the mitochondria for over 1 h. Khristeva et al. (1980) already showed an increase in ATP production due to HS. Following the hypothesis that an auxin-like activity may be exerted by HS on plant metabolism (Bottomley, 1914a, b), it has been elucidated that HS increase both the activity (Maggioni et al., 1987; Nardi et al., 1991) and amount (Canellas et al., 2002) of plasma membrane (PM) H^+ATPase, thereby allowing an apoplast acidification and an indirect cell elongation. Moreover, recent studies showed that LMS-HS stimulate nitrate uptake, possibly through the up-regulation of m-RNA synthesis of the major H^+ATPase form such as Mha2 (Quaggiotti et al., 2004). However, the effect of HS on the important glycolysis and respiration pathways are not yet well understood due to still insufficient experimental work that relates a detailed molecular description of humic matter to its biological activity (Vaughan et al., 1985; Chen and Aviad, 1990; Varanini and Pinton, 1995; Nardi et al., 2002a).

Glycolysis is of crucial importance in plants because it is the predominant pathway that "fuels" plant respiration. Moreover, a significant proportion of the carbon entering the plant glycolytic pathway and tricarboxylic acid (TCA) cycle is not oxidized to CO_2, but it is used in the biosynthesis of numerous compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids. The biosynthetic role of glycolysis and respiration is particularly important in actively growing autotrophic tissues (Plaxton, 1996).

The objectives of this work were: (i) to characterize by pyrolysis-GC-MS and NMR spectroscopy a soil humic acid (HA) and its three size fractions (I, II and III) separated by preparative high performance size exclusion chromatography (HPSEC), (ii) to test different concentrations of such soil humic materials on maize (*Zea mays* L.) seedlings, in order to evaluate their effects on metabolism through the measurement of enzymatic activities involved in glycolytic and respiratory processes. The enzymatic activities studied here and related to the glycolysis pathway were: glucokinase, phosphoglucose isomerase, PPi-dependent phosphofructokinase, pyruvate kinase, while those involved in respiration process were: cytrate synthase, malate dehydrogenase, and the cytosolic form of $NADP^+$-isocitrate dehydrogenase.

2. Materials and methods

2.1. Soil humic matter and separation of size fractions

A HA from a Fulvudand soil of the volcanic caldera of Vico, near Rome, Italy, was isolated by standard methods as reported elsewhere (Piccolo et al., 2002). The HA was titrated to pH 7.2 with a 0.5 m KOH solution in an automatic titrator (VIT 90 Videotitrator, Radiometer, Copenhagen) under N_2 atmosphere and stirring. After having reached the constant pH 7.2, the solution containing potassium humate was left under titration for 2 h, filtered through a glass microfibre filter (Whatman GF/C), and freeze-dried.

The mobile phase for HPSEC, a $NaCl/NaN_3$ ($2.89\,g\,l^{-1}$/$0.3\,g\,l^{-1}$) solution, was used to dissolve the HA to reach a concentration of $600\,mg\,l^{-1}$. Preparative separation of HA was conducted through a Biosep SEC-S-2000 (300 mm × 21.2 mm i.d.) column preceded by a Biosep SEC-S-2000 Guard Column (78.0 mm × 21.2 mm i.d.) by Phenomenex. A Gilson 305 pump (Gilson Inc., Middleton, WI, USA), a Gilson autosampler model 231, a Gilson FC205 fraction collector, and a Gilson 116 UV detector set at 280 nm were used to automatically isolate humic fractions in continuous. The nominal molecular-weight range of the preparative column was calibrated with polystyrene sulphonates of known molecular weights. The HA and standard solutions were injected with a Rheodyne rotary injector equipped with a 5 ml loop and the elution run at a $1.5\,ml\,min^{-1}$ flow rate. A Unipoint Gilson Software was used to automatically record all