Effects of an Alfalfa Protein Hydrolysate on the Gene Expression and Activity of Enzymes of the Tricarboxylic Acid (TCA) Cycle and Nitrogen Metabolism in *Zea mays* L.

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The effects through which an alfalfa protein hydrolysate (EM) possessing gibberellin- and auxin-like activity may promote plant nitrogen (N) nutrition have been investigated in *Zea mays* L. Treatment with 0.01 or 0.1 mg L^{-1} EM for 48 h resulted in enhanced plant growth and leaf sugar accumulation. Concomitantly, the level of nitrates decreased, whereas total N percentage was unchanged. The activity of a number of enzymes involved in carbon (C) metabolism (malate dehydrogenase, MDH; isocitrate dehydrogenase, IDH; citrate synthase, CS) and N reduction and assimilation (nitrate reductase, NR; nitrite reductase, NiR; glutamine synthetase, GS; glutamate synthase, GOGAT; aspartate aminotransferase, AspAT) was significantly induced by EM supply to plants, and the transcription pattern of MDH, IDH, CS, and NR strongly correlated with data of enzyme activity. The transcript accumulation of asparagine synthetase (AS) was also induced by EM in the roots. The results suggest that EM might promote nitrogen assimilation in plants through a coordinate regulation of C and N metabolic pathways and open the way for further research on protein hydrolysates as a valid tool to improve N use efficiency and, as a consequence, to reduce the intensive use of inorganic N fertilizers in agriculture.

KEYWORDS: Protein hydrolysate; *Zea mays*; TCA cycle; nitrogen metabolism; gene expression; enzyme activity

INTRODUCTION

Agricultural water pollution is regarded as a major area of concern worldwide. Indeed, the intensification of agricultural practices that make widespread use of fertilizers and pesticides has determined an increasing impact on the quality of ground and surface waters (*1*). Pesticides, phosphorus, and nitrate represent the main agricultural water pollutants. The use of pesticides leads to the indirect emission of toxic substances, whereas phosphorus has been associated with environmental pollution through the eutrophication of lakes, bays, and non-flowing water bodies. High nitrates (NO_3^-) come from nitrogen (N) fertilization; around 50% of nitrogen fertilizers applied to crops is left behind as residue, which can move throughout the soil profile into groundwater (leaching) (*2*). Therefore, rinsing nitrates threatens the quality of drinking water and, in surface waters, results in decreased ability of aquifers to support plant and animal life and makes them less attractive for recreation. A further hazard associated with excessive use of nitrogen fertilizers is the gaseous loss of nitrogen into the atmosphere. High doses of carbon dioxide and ammonia that escape into the atmosphere both from fertilizer manufacturing plants and from soils may affect human health (*3*).

In recents years, innovations in production have been evolving toward low-cost, organic, sustainable, and environmentally friendly systems that must contemporarily ensure the yield and high quality of crops. Some authors have proposed the use of biostimulants in plant nutrition instead of inorganic fertilizers to improve nutrient uptake or their utilization by plants (*4*).

Biostimulants are organic molecules that are often referred to as positive plant growth regulators or as metabolic enhancers and are presently recognized and included in the legislation controlling the marketing of fertilizers (*5*). When applied in small amounts, biostimulants can promote plant development, increase yields, and support plants to overcome stress situations by acting directly or indirectly on plant physiology (*6*). Organic biostimulants can also reduce the need for high levels of nitrogen fertilization by increasing the efficiency of nutrients and water uptake (*7*).

Biostimulants include three groups of molecules: plant hormones, humic substances, and free amino acids/peptides. Research on plant hormones and their role in the regulation of plant growth and development has been extensively documented (*8*). Humic substances (humic acids, fulvic acids, and humin) are known to improve shoot and root growth, induce seed germination, and facilitate seedling establishment (*9, 10*). The

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mechanisms through which humic substances produce such responses remain unclear, although they have been shown to improve soil structure, cation exchange capacity (CEC), and microbial activity (*11*). The third group of biostimulants is also known as protein hydrolysates (PHs). They are natural bio-stimulants, comprising oligo- and polypeptides, and free amino acids, which can be obtained through chemical and/or enzymatic hydrolysis of organic matrix from plant or animal sources (*12*). Foliar application of PHs usually leads to the improvement of plant nutrition and metabolism and increases the tolerance of plants to abiotic stress (*13*). To evaluate the quality of a protein hydrolysate based biostimulant, the content of free amino acids and the characterization of the peptide fraction are required. Indeed, evidence suggests that the low molecular weight compounds are more responsible for the biostimulant properties of the PHs, as plants easily absorb low molecular size peptides and free amino acids, which can significantly influence plant metabolism (*14*). Free amino acids in PHs may positively affect plant physiology by acting on photosynthesis, protein synthesis, lignification rate, and mechanisms involved in abiotic stress resistance (*14*). Additionally, they possess hormone-like activity (*15*).

Because the application of PHs may reduce the use of inorganic N fertilizers in agriculture by improving N plant nutrition, in the present work we want to verify the biostimulant activity of an alfalfa hydrolysate based fertilizer through bioassays and to test its effects on the activity and gene expression of enzymes involved in the tricarboxylic acid cycle (TCA) and nitrogen metabolism, which are both strictly related to plant productivity. This study could also help to obtain insight in the complex cross-signaling between N and carbon (C) metabolism and in the still unknown mechanisms of actions of PHs in plants.

MATERIALS AND METHODS

Characterization of the Protein Hydrolysate. The protein hydrolysate used in this study (EM) was produced by fully controlled enzymatic hydrolysis (FCEH) by ILSA S.p.A (Arzignano, VI, Italy) using vegetal material from alfalfa plants. Moisture was determined by weight loss at 105 °C; ash by residue on ignition at 550 °C; organic matter (OM) by loss on ignition (OM = dry matter − ash); pH in water (3/50, w/v); electrical conductivity (EC) in water (1/10, w/v); total organic carbon (TOC) by wet oxidation method with potassium dichromate; total nitrogen (TKN) via Kjeldahl method; ammonium nitrogen (NH_4^+-N) by extraction with diluted HCl and steam distillation with magnesium oxide; and total organic nitrogen (TON) by difference (TON = TKN − NH_4^+-N). Total phosphorus, sulfur, and metals were determined by acid digestion with ultrapure nitric acid (Merck, Darmstadt, Germany) and determination by induced coupled plasma atomic emission spectroscopy (Spectro Ciros[CCD], Kleve, Germany). Free amino acids (FAA) were extracted using 0.1 M HCl for 1 h and determined by RP-HPLC after derivatization with FMOC. Total tryptophan was determined by RP-HPLC as reported by Cavani et al. (*16*). The degree of hydrolysis (DH) was calculated on the basis of free α-amino nitrogen/total organic nitrogen ratio (*17*). The free α-amino nitrogen was determined using the *o*-phthaldialdehyde (OPA)−N-acetylcysteine (NAC) spectrophotometric assay. The apparent molecular weights of PH were determined by size exclusion chromatography (HP-SEC) using a Bio-Sil SEC 125-5 (Bio-Rad, Hercules, CA) column (300 mm × 7.8 mm) and 0.05 M phosphate buffer 0.15 M NaCl at pH 6.8 as a mobile phase. The protein hydrolysate was diluted in the mobile phase at the concentration of 1 g of TOC L^{-1} and filtered on 0.2 μm filters (Millipore, Bedford, MA) before injection into the column. The corresponding chromatogram
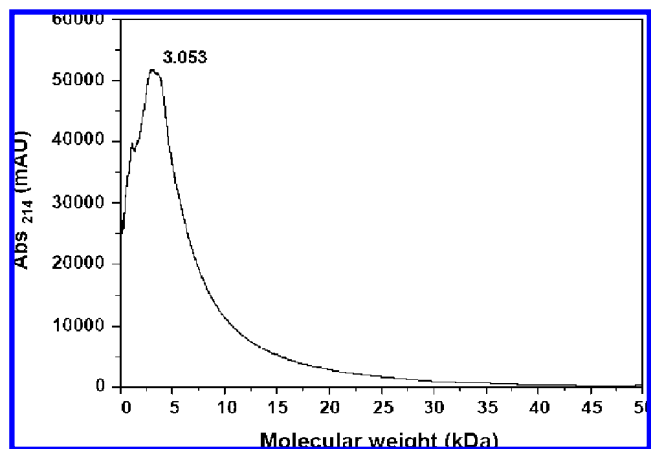

Figure 1. Chromatogram obtained after elution of the protein hydrolysate EM on the Bio-Sil Sec 125-5 column. The polypeptide standards molecular mass ranged within 1.13 (vitamin B$_{12}$) and 670 (thyroglobulin) kDa.

was recorded at $\lambda = 214$ nm using a UV detector (**Figure 1**). The calibration line was built up using polypeptide standards with molecular masses ranging within 1.13 (vitamin B$_{12}$) and 670 (thyroglobulin) kDa.

Audus test. The biological activity of the protein hydrolysate (EM) was assessed by measuring the root growth reduction of watercress (*Lepidium sativum* L.) and the increase in the shoot length of lettuce (*Lactuca sativa* L.) (*18*). Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After five rinsings with sterile distilled water, 10 seeds were placed on sterile filter paper in a sterile Petri dish. For watercress, the filter paper was wetted with 1.2 mL of 1 mM CaSO$_4$ (control) or with 1.2 mL of 0.01, 0.1, 1, or 10 mg L^{-1} indoleacetic acid (IAA) (Sigma, St. Louis, MO) to obtain the calibration curve or with 1.2 mL of a serial dilution of EM into 1 mM CaSO$_4$. For lettuce, the experimental design was the same as for watercress except that the sterile filter paper was wetted with 1.4 mL of the above solutions and the calibration curve was a progression of 0.1, 1, 10, and 100 mg L^{-1} gibberellic acid (GA) (Sigma). The seeds were allowed to germinate in the dark at 25 °C. The seedlings were removed, and the root or shoot lengths were measured after 48 h for watercress and 72 h for lettuce. The values obtained were the means of 20 samples and 5 replications.

Because the hormone-like activity of the protein hydrolysate could be influenced by the presence of potential cross-reacting compounds (e.g., arabinoxylans via 8-8-coupled diferulates, complex feruloylated heteroxylan sidechains), EM was purified by chromatography using a Sephadex G-25 embebbed column or Amberlite IR 120 in H$^+$ form. The hormone-like activity measured in the purified products via Audus bioassay was further compared with the one recovered in the nonpurified EM and served as control.

Plant Material. Maize seeds (*Zea mays* L. cv. DK 585) were soaked in distilled water for one night in running water and germinated for 60 h in the dark at 25 °C on a filter paper wetted with 1 mM CaSO$_4$ (*25*). Seedlings were then raised in a hydroponic setup using a Hoagland no. 2 modified solution (*19*), with the following composition: KH$_2$PO$_4$ (40), Ca(NO$_3$)$_2$ (200), KNO$_3$ (200), MgSO$_4$ (200), FeNaEDTA (10), H$_3$BO$_3$ (4.6), CuCl$_2$·2H$_2$O (0.036), MnCl$_2$·4H$_2$O (0.9), ZnCl$_2$ (0.09), NaMoO$_4$·2H$_2$O (0.01). Plantlets were grown inside a climate chamber with a 14 h light/10 h dark cycle, air temperature of 27/21 °C, and relative humidity of 70/85% and at a photon flux density (PFD) of 280 mol m^{-2} s^{-1}. Twelve-day-old plants were treated for 48 h with different concentrations of an alfalfa protein hydrolysate: 0 (control), 0.01, or 0.1 mg L^{-1}. Fresh samples of roots and leaves were harvested and analyzed for physiological determinations. Part of the plant material was frozen in liquid nitrogen and kept at −80 °C for further molecular analyses.

Soluble Sugars, Nitrate, Total N (TKN) Content, and Proteins. Foliar tissues (100 mg) of five independent plants were dried for 48 h at 80 °C, ground in liquid nitrogen, and then extracted with 2.5 mL of 0.1 N H$_2$SO$_4$. Samples were incubated in a heat block for 40 min at 60

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Table 1. Oligonucleotide Primer Name and Sequence for Primers of *Z. mays* Used in RT-PCR Reactions[a]

gene	forward primer (5′−3′)	reverse primer (5′−3′)
NR	GACGCCATCCACTACGACATG	ACCTTGACGAGGAGGTCCAAGT
MDH	GCCAGATTTCTGAGAGACTTAATGTCCA	TCGAGGCATGAGTAAGCAAGCGTCTT
IDH	AAACTCGAGGCTGCTTGCGTTGAGA	ATAATTAGCTTGCATCGAAACTGCGG
CS	GTTTGGTCATGGAGTTCTGCGTAA	GGAGGTACAACTTCATACAACTTGGACAC
AS	CATCATTGAGCTCTCGCGCAGGTTAC	GGGGGAAATGTTATGAAGCGTTCACAA
Zm actin	TGTTTCGCCTGAAGATCACCCTGTG	TGAACCTTTCTGACCCAATGGTGATGA

[a] NR, nitrate reductase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; CS, citrate synthase; AS, asparagine synthethase; *Zm* actin, actin.

°C and then centrifuged at 6000*g* for 10 min at 4 °C. The supernatants were analyzed by HPLC (Perkin-Elmer 410) after being filtered. The soluble sugars were separated using a Bio-Rad Aminex 87 C column (300 × 7.8 mm) using H_2O as eluent at a flow of 0.6 mL min^{-1}. Sugar concentration was expressed in grams per kilogram of dry weight (g kg^{-1} of dwt).

For nitrate content determination, root and leaf tissues (1 g) of five individual plants were frozen in liquid N_2 and homogenized (1:5 w/v) in 10 mM HCl. The extract was filtered through two layers of muslin and clarified by centrifugation at 35000*g* for 15 min. All steps were performed at 4 °C. The supernatant was filtered through 0.22 μm, and the quantification of NO_3 was performed via HPLC on an AS 4S-SC anionic exchange column (Dionex, Sunnyvale, CA), equipped with a Dionex suppressor and a 431 conductivity detector (Waters-Millipore, Milford, MA). A solution of sodium bicarbonate and sodium carbonate (1.7 mM NaHCO$_3$/1.8 mM Na$_2$CO$_3$) was used as eluent at a 2 mL min^{-1} flow rate. As a reference standard, sodium nitrate was used (Fluka, Buchs, Switzerland). Nitrate content was expressed as NO_3^- micromoles per gram of fresh weight (μmol g^{-1} of fwt).

Total nitrogen was estimated by Kjeldahl methods, and the values obtained were the average of five replicates and expressed in percent (w/w). Three independent experiments were performed for each determination.

The total content of protein was estimated via the Bradford method (*20*) and was expressed in milligrams per gram of fresh weight (mg g^{-1} of fwt).

Expression Analysis via Semiquantitative RT-PCR. Total RNA was extracted from roots and leaves of *Z. mays* plantlets. After harvest, the plant samples were immediately frozen in liquid N and kept in a freezer at −80 °C until further analysis. RNA isolation was kept in a freezer at −80 °C until further analysis. RNA isolation was performed using the Nucleon Phytopure kit (Amersham-Pharmacia, U.K.) following the protocol provided by the manufacturer. The RNA amount and quality were analyzed spectrophotometrically. Subsequently, an electrophoresis analysis was carried out in a 1% (w/v) agarose gel to verify the absence of nucleic acid degradation. Fifty micrograms of total RNA was treated with 10 units of DNase RQ1 (Promega, Milano, Italy) in a heat block at 37 °C for 30 min. At the end of the DNase reaction, the samples were extracted with phenol/chloroform (3:1) and the RNA was precipitated. The pellets obtained were washed in 1 mL of 75% (v/v) ethanol and resuspended in 20 μL of sterile RNase-free water, and the RNA amount was estimated as described previously. After DNase treatment, 5 μg of RNA of each sample was used to synthesize the first-strand cDNAs, by means of 200 units of ImProm-II reverse transcriptase (Promega) and oligodT as primers, in 20 μL reactions. The reaction conditions were 37 °C for 60 min and 70 °C for 5 min. Cooling to 4 °C for 5 min stopped the RT reaction.

RT-PCR experiments with specific primers were carried out to evaluate the expression level of the *Z. mays* genes reported in **Table 1** (*21*). For all PCR reactions 1 μL of the cDNA obtained was used in 20 μL reactions, using 0.025 unit/μL of Taq-polymerase (Amersham-Pharmacia-Biotech, Piscataway, NJ), and a set of different numbers of cycles ranging from 14 to 30 was tested to determine the optimal number of cycles, corresponding to the exponential phase in the amplification for each gene. In this phase, increasing numbers of PCR cycles resulted in a higher amount of PCR product, indicating that the reactions were not in the stationary phase and the reaction components were not limiting. Each PCR cycle consisted of 3 min of initial denaturation at 95 °C, 30 s of denaturation at 95 °C, 30 s of annealing,

30 s of extension at 72 °C, and 7 min of final extension at 72 °C. The annealing phase temperature was modified depending on the gene transcript.

The constitutively expressed *Z. mays* actin gene (J0128) was used as internal control to normalize the obtained gene expression results. RT-PCR analysis was performed employing the Gen Amp PCR system 9700 (PE Biosystems, Branchburg, NJ), and PCR products were separated by electrophoresis in a 1−1.5% agarose gel stained with ethidium bromide. The DNA fragments were visualized under UV light and quantified through the ImageJ program (ImageJ 1.23J, Wayne Rasband, U.S. National Institute of Health). Furthermore, to confirm the expression analysis results, PCR reactions were carried out on cDNAs obtained from two different RNA extractions performed on roots of seedlings of two independent experiments and were repeated at least four times for each cDNA. PCR products obtained from the gene expression analysis were further sequenced to verify the specificity of amplification of each gene. DNAs were extracted from the agarose gel and eluted by the "QIAquick Gel Extraction-Kit Protocol" (QIAGEN) kit. The eluted DNAs were then electrophoresed in agarose gel together with a molecular marker to be quantified. Gene sequencing was carried out at the CRIBI at Padua University, using the ABIPRISM original Rhodamine Terminator kit (PE Biosystems) and specific and universal primers. Blastx and Blastn (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) programs were used to compare the gene sequences.

Sequence data from this paper can be found in the GenBank/EMBL data libraries under accession numbers NR (M27821), CS (W49861), IDH (W21690), MDH (T27564), and AS (T27564).

Enzyme Extraction and Assay Conditions. For the extraction of N reduction and assimilation enzymes, root and leaf tissues (1 g) were ground in a mortar with added 100 mM Hepes−NaOH solution at pH 7.5, 5 mM $MgCl_2$ solution, and 1 mM dithiothreitol (DTT) solution. The ratio of plant material to mixture solution was 1:3. The extract was filtered through two layers of muslin and clarified by centrifugation at 20000*g* for 15 min. The supernatant was used for enzymatic analysis. All steps were carefully performed at 4 °C.

The activity of nitrate reductase (NR) was assayed according to the method of Lewis et al. (*22*), whereas the activity of nitrite reductase (NiR) was determined on the basis of the drop in NO_2^- concentration in the reaction medium. After incubation at 30 °C for 30 min, the NO_2^- content was determined colorimetrically at $\lambda = 540$ nm.

Glutamate synthase (GOGAT, EC 1.4.7.1) assay contained 25 mM Hepes−NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na$_2$EDTA, and 100 μL of enzyme extract in a 1.1 mL final volume. GOGAT activity was measured spectrophotometrically by monitoring NADH oxidation at $\lambda = 340$ nm.

For glutamine synthetase (GS, EC 6.3.1.2) assay the mixture contained 90 mM imidazole−HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM KAsO$_4$, 3 mM MnCl$_2$, 0.4 mM ADP, 120 mM glutamine, and the appropriate amount of enzyme extract. The assay was performed in a final volume of 750 μL. The enzymatic reaction was developed for 15 min at 37 °C. The γ-glutamyl hydroxamate was colorimetrically determined by the addition of 250 μL of a mixture (1:1:1) of 10% (w/v) FeCl$_3$·6H$_2$O in 0.2 M HCl, 24% (w/v) trichloroacetic acid, and 50% (w/v) HCl. The optical density was recorded at $\lambda = 540$ nm.

The activity of aspartate aminotransferase (AspAT, EC 2.6.1.1) was measured spectrophotometrically by monitoring NADH oxidation at $\lambda = 340$ nm at 30 °C. The assay medium (final volume of the reaction

Table 2. Selected Chemical Properties of the Alfalfa Protein Hydrolysate (EM)[a]

property	unit	
moisture	%	43.5
ash	%	18.9
organic matter	%	37.6
pH		5.9
ECw	dS m^{-1}	16
TOC	% w/w	18.8
TKN	% (w/w)	2.29
NH$_4^+$-N	% (w/w)	0.38
NO$_3^-$-N	% (w/w)	0.03
C/N ratio		8.10
total Na	% (w/w)	0.19
total S	% (w/w)	0.13
total K	% (w/w)	1.65
total Ca	% (w/w)	4.16
total Mg	mg kg^{1-}	0.08
total P	mg kg^{1-}	0.01
total Fe	mg kg^{1-}	38
total Zn	mg kg^{1-}	103
total Cu	mg kg^{1-}	18
total Mn	mg kg^{1-}	65
total Ni	mg kg^{1-}	1.3
total Pb	mg kg^{1-}	0.3
total Cr	mg kg^{1-}	24
α-NH$_2$-N	mg of N g^{-1}	5.59
DH	%	29.3
MW	%	6.3

[a] ECw, electrical conductivity; TOC, total organic carbon; TKN, total nitrogen; DH, degree of hydrolysis; MW, weight-average molecular weight. Values are the means of five replicates, with standard errors always ≤5% of the mean.

Table 3. Free Amino Acid Contents of the Alfalfa Protein Hydrolysate (EM)[a]

amino acid	% (w/w)
Asp	0.077
Glu	0.094
Ala	0.517
Arg	<LQ
Phe	0.129
Gly	0.295
Hyp	<LQ
Ile	0.071
His	0.082
Leu	0.156
Lys	0.13
Met	<LQ
Pro	0.104
Ser	<LQ
Trp	0.001
Tyr	0.081
Thr	<LQ
Val	0.18
total	1.916

[a] Values are the means of five replicates.

mixture = 2.4 mL) contained 100 mM Tris−HCl, pH 7.8, 240 mM L-aspartate, 0.11 mM pyridoxal phosphate, 0.16 mM NADH, 0.93 kU malate dehydrogenase (MDH), 0.42 kU lactate dehydrogenase (LDH), 12 mM 2-oxoglutarate, and 200 μL of enzyme extract.

For the extraction of TCA cycle enzymes, leaves and roots (1 g) were ground in a mortar using 100 mM Tris−HCl buffer, pH 8.2, containing 5 mM β-mercaptoethanol (Sigma), 1 mM Na$_2$EDTA, and 10% glycerol. Extracts were then filtered and centrifuged. All steps were performed at 4 °C.

For cytrate synthase (CS, EC 1.11.1.6), the activity was assayed by adding 50 μL of 0.17 mM oxalacetic acid, 50 μL of 0.2 mM acetyl-coenzyme A (acetyl-CoA), and 10 μL of extract to 3 mL of 0.1 M Tris−HCl, pH 8.0, and measurements were carried out spectrophotometrically at 25 °C by monitoring the reduction of acetyl-CoA to CoA, at λ = 232 nm.

For NADP$^+$−isocitrate dehydrogenase assay (NADP$^+$-IDH, EC 1.1.1.42), 50 μL of crude extract was added to a 2.85 mL final volume of a reaction mixture containing 88 mM imidazole buffer, pH 8.0, 3.5 mM MgCl$_2$, 0.41 mM β-NADP sodium salt, and 0.55 mM isocitrate sodium salt. The assay was performed at 25 °C following the formation of NADPH at λ = 340 nm.

For the assay of malate dehydrogenase (MDH, EC 1.1.1.37), 94.6 mM phosphate buffer, pH 6.7, 0.2 mM β-NADH sodium salt, 0.5 mM oxalacetic acid, and 1.67 mM MgCl$_2$ were used. MDH activity was assayed at 25 °C, following the formation of NAD$^+$ at λ = 340 nm.

Measurement of enzyme activity was performed in five replicates, and the absorbance in the samples was measured using a JASCO V-530 UV−vis spectrophotometer.

Statistical Analysis. The results obtained were processed statistically with the Student−Newman−Keuls test, and the standard deviations are shown in the tables and figures.

RESULTS

Characterization of EM. The main chemical properties of EM are shown in **Table 2**. Compared to two biostimulants tested by Cavani et al. (*16*), the amount of organic matter in EM was low 37.6% (w/v), whereas the content of ash and the electrical

conductivity (EC$_w$) were both high, with values of 18.9% (w/v) and 16 dS m^{-1}, respectively. The percentage of inorganic nitrogen in the form of ammonia and nitrates was low (0.38% w/w and 0.03 w/w, respectively) (**Table 2**), whereas the total amount of free amino acids was high, up to 1.916% (w/w) (**Table 3**) and correlated with the free α-amino nitrogen (α-NH$_2$-N) (**Table 2**). The hydrolytic process employed for EM production was effective in the weight-average molecular weight (MW) reduction, as confirmed by data on EM hydrolysis degree (DH) and MW (**Table 2**).

Audus Test and Effect of EM on Plant Growth, Sugars, Nitrate, and Total Nitrogen Content. The Audus test was used to examine the biostimulant properties of the alfalfa protein hydrolysate, and this revealed the auxin- and gibberellin-like activity of EM (**Figure 2**). The response induced in terms of gibberellin-like activity, that is, the increase in epicotyl length, was dose-dependent and similar to that elicited by exogenous gibberellic acid. Compared to the IAA-like activity, while increasing concentration of pure indoleacetic acid strongly reduced the root length of watercress, the application of EM determined a dose-dependent slight reduction of root length. Because the existence of cross-reacting compounds that can interfere with the analysis could not be excluded, the bioassays were also performed using the protein hydrolysate after its elution on a column embebbed with Sephadex-G25 gel or Amberlite in H$^+$ form. The purification of EM resulted in no GA-like activity and in a sharp non-dose-dependent auxin-like response (data not shown).

Following these findings, EM was administered to maize plants for 48 h at concentrations of 0.01 and 0.1 mg L^{-1}, and its effect on a number of physiological parameters related to plant productivity such as plant growth, sugars, and nitrate content was evaluated.

Results indicated that both concentrations of EM supplied to plants could promote leaf growth, whereas the root dry weight was only slightly, although significantly, enhanced by EM 0.01 mg L^{-1} (**Figure 3**). Treating maize plants with EM 0.1 mg L^{-1} resulted in a significant increase of the sugar content in foliar tissues (**Table 4**). In particular, the amounts of fructose and sucrose in EM-treated plants were about 2.5 and 1.8 higher, respectively, than those recorded in the controls. Conversely, the level of glucose only weakly increased.
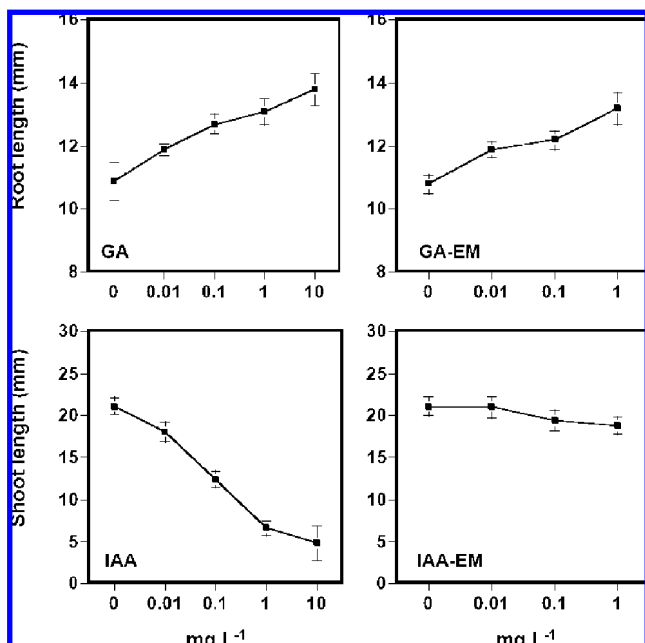
Figure 2. Auxin- and gibberellin-like activities of the EM hydrolysate evaluated via Audus test, that is, by measuring the reduction of root length (mm) of watercress and the increase in shoot length (mm) of lettuce, respectively. The values obtained were the means of 20 samples and 5 replications. GA, gibberellin acid; IAA, indoleacetic acid; GA-EM, gibberellin-like activity of the alfalfa protein hydrolysate; IAA-EM, auxin-like activity of the alfalfa protein hydrolysate.
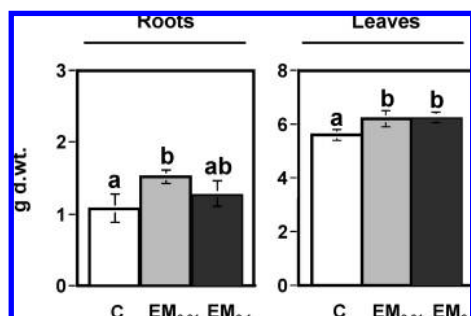

Figure 3. Effect of EM treatment on root and leaf dry weight (dwt) of *Z. mays* plants grown for 12 days in a Hoagland modified complete nutrient solution and treated for 2 days with EM at 0.01 or 0.1 mg L^{-1}. Data are the means of 10 values each from 3 independent experiments ($\pm$SD). Different letters on bars indicate significant differences between treatments ($P < 0.05$). C, control; EM 0.01, EM 0.01 mg L^{-1}; EM 0.1, EM 0.1 mg L^{-1}.

Table 4. Contents of Sucrose, Glucose, and Fructose in Leaves of *Z. mays* Plants Treated for 48 h with the EM Protein Hydrolysate at 0.01 or 0.1 mg L^{-1a}

	sucrose (mg g^{-1} of dwt)	glucose (mg g^{-1} of dwt)	fructose (mg g^{-1} of dwt)	proteins (mg g^{-1} of fwt)
C	116.73 a	26.964 a	7.127 a	284 a
EM 0.01	133.22 b	28.563 a	4.962 a	312 a
EM 0.1	213.03 c	30.152 a	17.529 b	451 b

^{a} Values are the means of five replicates. Means within each column followed by the same letter do not differ at $P = 0.05$ based on Student−Newman−Keuls test. dwt, dry weight; fwt, dry weight.

As expected, nitrate content was higher in roots than in leaves because nitrate reduction mainly occurs in leaves. While total nitrogen (TKN) percentage remained unchanged, a remarkable decrease of nitrate was observed in EM-treated maize plants
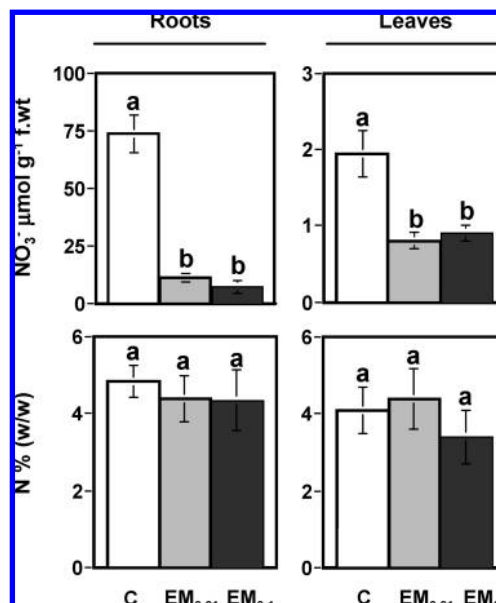

Figure 4. Effect of EM treatment on nitrate and nitrogen contents in roots and leaves of maize plants grown for 12 days in Hoagland modified complete nutrient solution and treated for 2 days with EM at 0.01 or 0.1 mg L^{-1}. N content was estimated in percent on the basis of the fresh weight. Data are the means of five values each from three independent experiments ($\pm$SD). Different letters on bars indicate significant differences between treatments ($P < 0.05$). C, control; EM 0.01, EM 0.01 mg L^{-1}; EM 0.1, EM 0.1 mg L^{-1}.

(**Figure 4**). Indeed, under EM conditions the amounts of nitrate in roots and leaves accounted for 10−15 and 41−46% of those measured in the controls, respectively. The content of total protein was significantly increased by 0.1 mg L^{-1} EM supply to maize plants compared to the control plants (**Table 4**).

Effect of EM on the Activity of N-Assimilation and Carbon Metabolism Related Enzymes. Nitrogen and carbon metabolisms as influenced by EM application to plants were investigated in *Z. mays* by measuring the activities of enzymes that catalyze key steps in the nitrogen organication and in the TCA cycle.

The protein hydrolysate increased the activities of the enzymes involved in the nitrogen assimilation pathway compared to the control. Compared to the cytoplasmic nitrate reductase (NR) and the chloroplastic nitrite reductase (NiR), which catalyze the two-step reduction of nitrate to ammonia, the treatment of maize plants with EM influenced the activity of NR and NiR in a similar way (**Figures 5** and **6**). Specifically, the application of EM 0.1 mg L^{-1} increased their activity by 27% (NR) and 11% (NiR) in roots (**Figure 5**) and by 78% (NR) and 24% (NiR) in leaves, over the control (**Figure 6**). The ammonia produced by NR and NiR activities is assimilated into glutamine and glutamate through the glutamine synthetase (GS)/glutamate synthase (GOGAT) system, which allows the further incorporation of N in organic compounds. At the concentration of 0.1 mg L^{-1}, the activities of GS and NAD(P)H$^+$−GOGAT were enhanced over the control by 39 and 19% in roots (**Figure 5**), respectively, and by 24 and 43% in leaves (**Figure 6**). Glutamate coupled with the oxalacetate produced in TCA is a substrate of the enzyme aspartate aminotransferase (AspAT) for the biosynthesis of the amino acid aspartate (ASP), which serves as a N donor in several amino transferase reactions. The activity AspAT was increased in roots (**Figure 5**) and leaves (**Figure 6**) by both concentrations of EM (0.01 and 0.1 mg L^{-1}).

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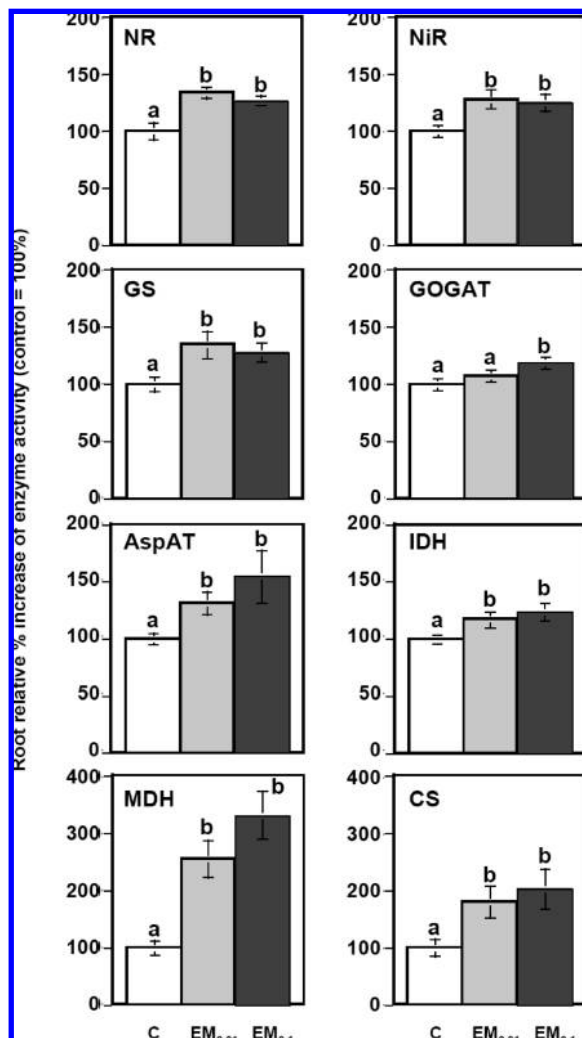

Figure 5. Root enzyme activity of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), citrate synthase (CS), and aspartate aminotransferase (AspAT) in *Z. mays* plants grown for 12 days in Hoagland modified complete nutrient solution and treated for 2 days with EM at 0.01 or 0.1 mg L^{-1}. Data are the means of five values each from three independent experiments ($\pm$SD). Different letters on bars indicate significant differences between treatments ($P < 0.05$). C, control; EM 0.01, EM 0.01 mg L^{-1}; EM 0.1, EM 0.1 mg L^{-1}.
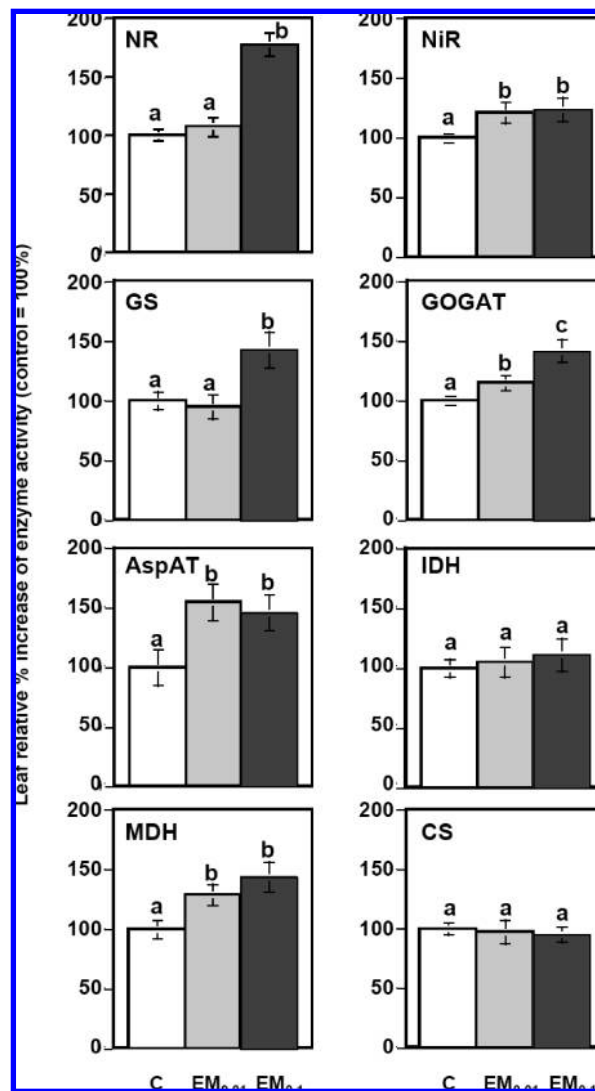

Figure 6. Leaf enzyme activity of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), citrate synthase (CS), and aspartate aminotransferase (AspAT) in *Z. mays* plants grown for 12 days in Hoagland modified complete nutrient solution and treated for 2 days with EM at 0.01 or 0.1 mg L^{-1}. Data are the means of five values each from three independent experiments ($\pm$SD). Different letters on bars indicate significant differences between treatments ($P < 0.05$). C, control; EM 0.01, EM 0.01 mg L^{-1}; EM 0.1, EM 0.1 mg L^{-1}.

With regard to the activity of enzymes functioning in the Krebs cycle (TCA), the supply of EM to maize plants led to a significant increase of malate dehydrogenase (MDH) activity relative to the control, more remarkable at root level (plus 156 and 232% at EM 0.01 and 0.1 mg L^{-1}, respectively) (**Figure 5**). In roots, the activity of isocitrate dehydrogenase (IDH) was weakly increased by the protein hydrolysate (plus 17 and 24%, at EM 0.01 and 0.1 mg L^{-1}, respectively) (**Figure 5**), whereas citrate synthase (CS) activity was greatly enhanced (plus 79 and 103% at EM 0.01 and 0.1 mg L^{-1}, respectively). Conversely, following EM treatment, the activity of IDH and CS in leaves did not vary (**Figure 6**).

Effect of EM on Gene Expression. To confirm the effect of stimulation exerted by the alfalfa protein hydrolysate on the nitrogen and/or carbon metabolisms in maize plants, the transcriptional pattern of genes coding for enzymes functioning in the TCA cycle (malate dehydrogenase, MDH; isocitrate dehydrogenase, IDH; citrate synthase, CS) and nitrogen assimilation (nitrate reductase, NR; asparagine synthetase, AS) was analyzed via RT-PCR (**Figures 7** and **8**). The gene expression of the enzyme asparagine synthetase was also evaluated because the enzyme catalyzes the synthesis of the amino acid asparagine (Asn), which fulfills a fundamental role in N mobilization and in the photorespiratory process (*23*).

The molecular results indicate a significant increase of the mRNA abundance in roots of plants treated with the EM protein hydrolysate compared to the controls (**Figure 7**). In particular, the induction of such genes was strongly dependent on the concentration of the protein hydrolysate supplied to plants because the transcript accumulation was about 1.5–2.5-fold higher at 0.1 mg L^{-1} than at 0.01 mg L^{-1}. The supply of maize plants with EM did not influence the transcript level of genes coding for IDH, CS, and AS in leaves, whereas the genes encoding NR and MDH were up-regulated by the protein hydrolysate, their transcript levels accounting for 1.4- and 2-fold the amount of those recorded in control plants, respectively (**Figure 8**). However, differently from the gene expression pattern observed in roots, no variation in leaf transcript

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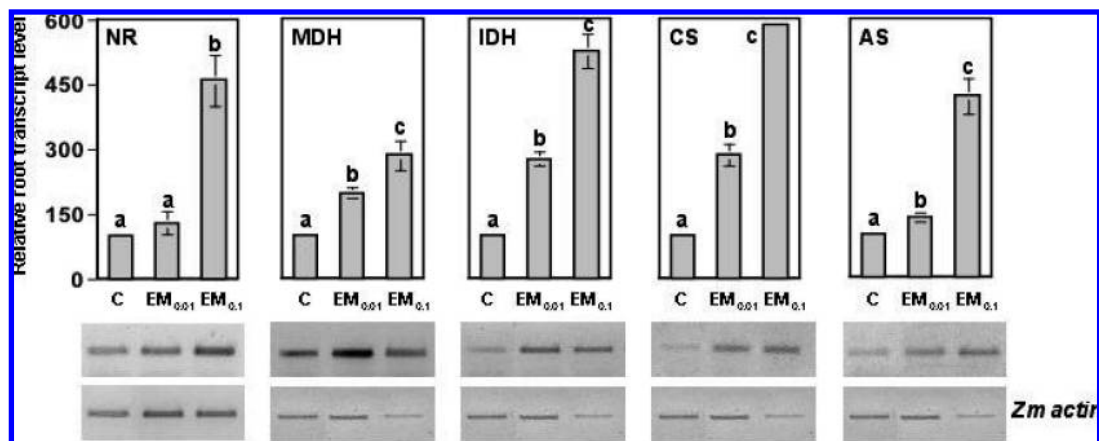
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Figure 7. Gene expression (mRNA level) and relative transcript accumulation of nitrate reductase (NR), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), citrate synthase (CS), and asparagine synthetase (AS) in roots of *Z. mays* plants grown for 12 days in Hoagland modified complete nutrient solution and treated for 2 days with EM at 0.01 or 0.1 mg L^{-1}. Quantification of transcript amounts was performed using the semiquantitative PCR method in the linear range, and *Zm* actin was used as internal control. The accumulation of the gene transcript was first normalized relative to *Zm* actin transcript and then expressed relative to the control (=100%). Analyses were repeated at least three times for each cDNA obtained from two different RNA extractions. Different letters on bars indicate significant differences between treatments ($P < 0.05$, ± SD). C, control; EM 0.01, EM 0.01 mg L^{-1}; EM 0.1, EM 0.1 mg L^{-1}.
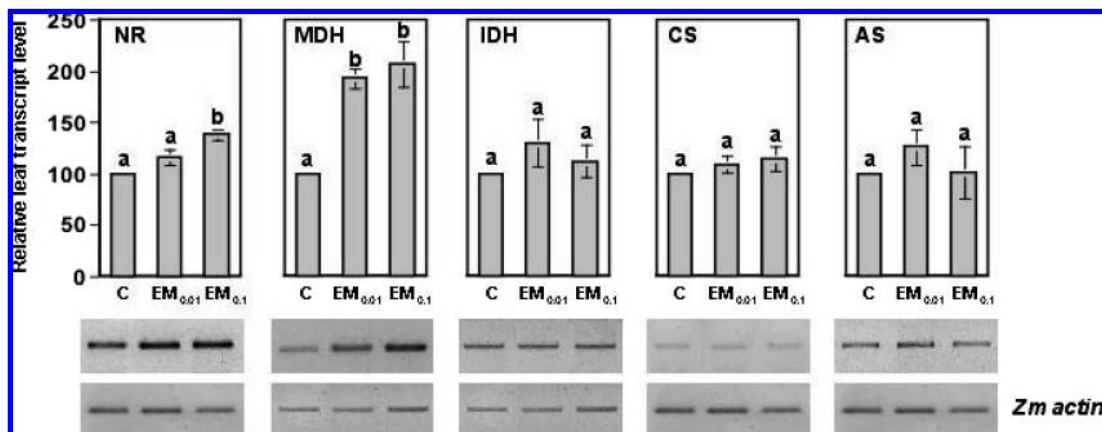

Figure 8. Gene expression (mRNA level) and relative transcript accumulation of nitrate reductase (NR), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), citrate synthase (CS), and asparagine synthetase (AS) transcript accumulation in leaves of *Z. mays* plants grown for 12 days in Hoagland modified complete nutrient solution and treated for 2 days with EM at 0.01 or 0.1 mg L^{-1}. Quantification of transcript amounts was performed using the semiquantitative PCR method in the linear range, and *Zm* actin was used as internal control. The accumulation of the gene transcript was first normalized relative to *Zm* actin transcript and then expressed relative to the control (=100%). Analyses were repeated at least three times for each cDNA obtained from two different RNA extractions. Different letters on bars indicate significant differences between treatments ($P < 0.05$, ± SD). C, control; EM 0.01, EM 0.01 mg L^{-1}; EM 0.1, EM 0.1 mg L^{-1}.

accumulation of MDH occurred among plants treated with different concentrations of EM.

DISCUSSION

Nitrogen being the major limiting factor in plant growth and productivity, a prerequisite of plant adaptation to environmental conditions (soil quality, climate, pH) is the nitrogen use efficiency (NUE) and the capacity of specific enzymes to catalyze nitrogen reduction and assimilation (*24*). The enzymes nitrate reductase (NR) and nitrite reductase (NiR) catalyze the two-step reduction of nitrate (NO_3^-) to ammonium (NH_4^+), which is rapidly incorporated into organic compounds through the activity of the enzyme glutamine synthetase (GS) (*25, 26*). GS works in association with the enzyme glutamate synthase (GOGAT) to produce glutamate (Glu) from glutamine and α-ketoglutarate in the GS/GOGAT cycle (*26*). Glu is the amino nitrogen donor for the enzyme AspAT, which uses oxalacetate and glutamate as substrates to form aspartate (Asp). The latter

is the precursor for several essential amino acids that are involved in osmotic regulation and represent the link between the nitrogen and carbon metabolic pathways (*27*). Indeed, the oxalacetate required to synthesize Asp is produced from malate in the TCA cycle by the activity of malate dehydrogenase (MDH), whereas α-ketoglutarate can be formed by isocitrate dehydrogenase (IDH) in the TCA cycle and by AspAT during Asp production (*28*).

The interactions between N and C pathways and their dynamics are hugely important for crop production, and it has been speculated that the increased photosynthetic production of carbon skeletons might stimulate the nitrogen assimilation in plants (*29*). As a consequence, certain metabolites such as sucrose and amino acids play an important role in the regulation of the enzymes involved in nitrogen assimilation.

Because of this and in view of evaluating the use of protein hydrolysates as a tool to improve N fertilizers' use efficiency, the effectiveness of an alfalfa protein hydrolysate (EM) to

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promote plant growth and productivity has been investigated in *Z. mays* by analyzing the activity and gene expression of several enzymes involved in nitrogen and carbon metabolisms, as well as a number of physiological parameters.

Before the supply to plants, the protein hydrolysate was characterized to evaluate its quality. The chemical analysis of EM revealed a high content of ash, which could be ascribed to the high quantities of total calcium, iron, manganese, and zinc. However, most of the cations in the protein hydrolysate were at such low concentrations to exclude a direct mineral nutrient fertilizing effect by EM. In support of this was the low concentration of EM used during plant treatments and the relatively low content of inorganic nitrogen.

To test and quantify the biostimulant activity of organic molecules on plants, bioassays that compare the physiological responses induced by such molecules with the responses elicited by hormones are commonly used. Among bioassays, the Audus test is the most reliable in terms of reproducibility and repeatability and makes it possible to determine whether the molecules possess auxin- and/or giberellin-like activity. The EM protein hydrolysate was shown to possess biostimulant properties because it displayed both auxin- and giberellin-like activities, as confirmed by the Audus test. Such activities may be in part due to the biological action of small peptides and amino acids, which are considered to be hormone precursors. Indeed, several studies proved that peptide signaling is important in various aspects of plant development and growth regulation including meristem organization, leaf morphogenesis, and defense responses to biotic and abiotic stress (*29*). The loss of giberellin-like activity of EM after elution in Amberlite may be due to acidification of the protein hydrolysate during the purification procedure. This hypothesis arises from previous studies on humic substances, where the GA-like activity could be shown only at neutral pH values (*30*). Conversely, the auxin-like activity of the purified EM was confirmed, in agreement with the release of the IAA-like activity from humic substances at acidic pH values as previously observed by Nardi et al. (*31*).

The tested protein hydrolysate was effective in promoting carbon and nitrogen metabolisms in terms of transcript accumulation and enzyme activity. Indeed, EM strongly induced the root mRNA accumulation and the activity of the main enzymes working in the TCA cycle, such as malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), and citrate synthase (CS). The latter is considered to be the most important enzyme of TCA cycle because it catalyzes the reaction that controls the rate of the respiratory pathway (*28*). We investigated the cytosolic form of NAD^+ isocitrate dehydrogenase because it is assumed to be the enzyme that is primarily responsible for isocitrate to α-ketoglutarate conversion in the Krebs cycle (*28*). In fact, the NAD^+IDH enzyme is considered a key step in the generation of 2-oxoglutarate for ammonium assimilation and amino acid biosynthesis in higher plants (*28*).

The root gene expression of two enzymes that take part in the nitrogen assimilation pathway (nitrate reductase, NR; and asparagine synthetase, AS) and the activity of five enzymes of N metabolism (nitrate reductase, NR; nitrite reductase, NiR; glutamine synthetase, GS; glutamate synthase, GOGAT; and aspartate aminotransferase, AspAT) were also increased by EM treatment. These findings indicated EM-enhanced N assimilation, which was confirmed by the reduction of nitrate content in leaves of plants supplied with the protein hydrolysate and could be ascribed to the faster consumption of nitrate to produce amino acids under EM treatment as the total nitrogen percentage was unchanged. In support of this, the content of proteins in

plants supplied with 0.1 μg L^{-1} protein hydrolysate was significantly higher (451 μg g^{-1} of fwt) compared to the controls (284 μg g^{-1} of fwt). Induced production of N assimilates by humic substances in maize plants was also shown in a previous work (*30*).

The strong correlation between gene expression and enzyme activity observed in this study suggested that coordinate mechanisms of regulation of gene transcription and signal transduction for C and N metabolic pathways may occur under EM condition, which are important to maintain the N/C ratio balance. This hypothesis is supported by data on sugars, as the content of sucrose was significantly higher in plants treated with EM compared to the controls. Indeed, sucrose, which is synthesized from carbohydrates produced by the fixation of atmospheric CO_2 via the reductive pentose phosphate (Calvin) pathway, represents the starting compound for the respiratory pathway, and its increase may justify the improved activity of the TCA cycle and nitrogen assimilation. Recently, sucrose has been found to be involved in the regulation of asparagine synthetase (AS) gene expression in *Phaseolus vulgaris* roots and nodules (*31*) and to increase the content of the amino acid asparagine (Asn). In other reports sucrose has been shown to directly induce the activity and gene expression of NR (*32*) and GS (*33*) following exogenous application. Coruzzi et al. (*25*) proposed that the metabolic regulation of GS expression in plants might be controlled by the relative abundance of carbon skeletons versus amino acids. This would allow nitrogen assimilation into glutamine to proceed (or not) depending on the metabolic status and biosynthetic requirements of the plant.

In conclusion, our results encourage further research on PHs by suggesting that the protein hydrolysate EM may improve the N use efficiency of maize roots, thereby contributing to the reduction of inorganic N fertilizer applications in agriculture. The root gene expression as well as the leaf and root enzyme activity of TCA and N assimilation enzymes in some respects relied on EM concentration supplied to plants. In particular, at the higher EM concentration (0.1 mg L^{-1}) the maximum transcript accumulation and enzyme activity were often observed. Therefore, an EM concentration of 0.1 mg L^{-1} could promote plant growth, likewise 0.01 mg L^{-1}, but was more effective in promoting plant N assimilation.

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