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Enzymatic Vegetable Organic Extracts as Soil Biochemical Biostimulants and Atrazine Extenders

Ana María García-Martínez,[†] Manuel Tejada,[‡] Ana Isabel Díaz,[§] Bruno Rodríguez-Morgado,[†] Juan Bautista,[†] and Juan Parrado*,[†]

[†]Dpto. Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Sevilla, C/Profesor García González 2, 41012, Sevilla, Spain, [‡]Dpto. Crystalografía, Mineralogía y Química Agrícola, E.U. ITA Universidad de Sevilla, Crta. Utrera, km 1, 41013 Sevilla, Spain, and [§]Gaiker-Centro Tecnológico, Parque Tecnológico, Edif. 202, 48170 Zamudio, Spain

The purpose of this study was to gather information on the potential effects of organic biostimulants on soil activity and atrazine biodegradation. Carob germ enzymatic extract (CGEE) and wheat condensed distiller solubles enzymatic extract (WCDS-EE) have been obtained using an enzymatic process; their main organic components are soluble carbohydrates and proteins in the form of peptides and free amino acids. Their application to soil results in high biostimulation, rapidly increased dehydrogenase, phosphatase and glucosidase activities, and an observed atrazine extender capacity due to inhibition of its mineralization. The extender capacity of both extracts is proportional to the protein/carbohydrate ratio content. As a result, these enzymatic extracts are highly microbially available, leading to two independent phenomena, fertility and an atrazine persistence that is linked to increased soil activity.

KEYWORDS: Biostimulants; atrazine extender; soil enzymatic activities

INTRODUCTION

Soil biostimulants (SB) are new agricultural organic products composed of peptides, amino acids, polysaccharides, humic acids, phytohormones, and other components (*1*). These are believed to work through a series of widely varying mechanisms, including activation of soil microbial activity and promotion or augmentation of the activities of critical soil enzymes, which would increase the microbial populations in the soil (*2*). The herbicide atrazine (2-chloro-4-ethylamine-6-isopropylamine-2,4,6-triazine) is widely used in corn (*Zea mays* L.), sorghum (*Sorghum bicolor* L. Moench), and sugar cane (*Saccharum officinarum* L.) production to control many broadleaf and certain grass weeds (*3*).

Atrazine is subject to both abiotic and biotic degradation, but repeated applications of a pesticide such as atrazine may selectively enrich the microbial community (*3*) with the capacity to use the herbicide as a growth substrate, thus microbial activity becomes the principal mechanism of atrazine dissipation from the environment (*4*). Many soil microorganisms are able to use atrazine as a C source and as an energy source (*5*−*7*), but it can also serve as a N source for a few microorganisms (*3*), thereby resulting in significantly enhanced rates of biodegradation (*8*−*10*). This accelerated process is characteristic of many control agents such as herbicides, insecticides, and fungicides in soils and leads to shorter periods of pesticide effectiveness and a failure to control the target pest due to the decrease in pesticide persistence. For these reasons, a loss of residual weed control has been reported during use of atrazine under these conditions (*11*, *12*).

Several studies of atrazine soil degradation have showed that this phenomenon is controlled by microbial adaptation and C and N availability (*13*). Atrazine biodegradation depends on the availability of the compound and other essential nutrients (*3*). Researchers have reported enhanced atrazine mineralization with the addition of organic amendments such as straw (*14*), starch, rice hulls, compost (*15*), and dairy manure (*16*) as a current environmental and agricultural practice for maintaining soil organic matter. These are used for reclaiming degraded soils and supplying plant nutrients, but inhibition of degradation has also been observed in soil amended with glucose, sodium citrate, and Sudan hay (*15*), suggesting that the source of organic material and not the C/N ratio may influence atrazine dissipation rate (*3*). On the other hand, it is well-known that the addition of an inorganic N source inhibits atrazine mineralization (*3*), suggesting that it is enhanced as a result of limited N availability.

We report in this work that the application of SB to soil leads to changes in atrazine mineralization potential, delaying the development of enhanced degradation linked to an increase in soil structure biological quality.

In this work, we evaluate the effects of two organic nitrogen sources, namely soil biostimulants, applied to a model soil contaminated with atrazine and the influence of relative protein content on some biochemical and biological soil properties and atrazine biodegradation.

MATERIALS AND METHODS

Experimental Design. The soil was collected from a University of Seville experimental research field located in Crta. Utrera, km.1 (Seville). The parcel selected had never been treated with agrochemicals. The associated soil characteristics are summarized in **Table 1**. Soil was homogenized and dried. It was spiked with atrazine powder at a final concentration of 100 mg/kg (Sigma Aldrich, 98% purity). Briefly, atrazine was solved in acetone and soil was dried and well mixed. Soil samples (250 g dry weight) were put into plastic containers (volume 250 mL) at 60% water-holding

*To whom correspondence should be addressed. Phone: +34 954556222. Fax: +34 954233765. E-mail: parrado@us.es.

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Table 1. Characteristics of the Experimental Soil (Data Represent the Mean of Three Samples)

pH (H_2O)	8.6 ± 0.2
CO_3^{2-} (g kg^{-1})	203 ± 12
fine sand (g kg^{-1})	142 ± 35
coarse sand (g kg^{-1})	387 ± 26
silt (g kg^{-1})	242 ± 19
clay (g kg^{-1})	229 ± 10
organic matter (g kg^{-1})	11.06 ± 0.8
total N (g kg^{-1})	0.4 ± 0.1
P (mg kg^{-1})	5.8 ± 1.1
K (mg kg^{-1})	212 ± 46

capacity (WHC). The soil−water content was maintained throughout the incubation period at 60% WHC. A biostimulating experiment was conducted. Soil biostimulants were added to the soil in a single dose of 1% of organic matter (w/w). The amount of biostimulants required to apply 1 g of organic matter were weighed, and they were dissolved in 100 mL of water and added to the contaminated soil. An abiotic control soil was performed using sodium azide (0.2%) to test inhibition of atrazine mineralization. All the samples were incubated in the dark in a cell culture chamber with temperature control (25 °C). All the treatments were performed in triplicate in a randomized way.

Soil Biostimulants (SB). The soil biostimulants used in this study were: (a) wheat condensed distillers solubles enzymatic extract (WCDS-EE), produced by enzymatic hydrolysis from wheat condensed distillers solubles (WCDS), a major byproduct of ethanol fermentation provided by Abengoa-Bioenergy (Bioethanol Galicia, Teixero, Spain), and (b) carob germ enzymatic extract (CGEE), produced also by enzymatic hydrolysis, but using carob germ as the raw material (*14*).

The proteins of the raw material were efficiently hydrolyzed, achieving a 37% degree of hydrolysis after 120 min of reaction using an endoprotease (obtained by liquid fermentation with *Bacillis lichiniformis* ATCC 21415 as a hydrolytic agent) in a bioreactor with controlled temperature (60 °C) and pH (pH = 8) (*15*).

Soil Biostimulants (SB) Analysis. *Physicochemical Properties Characterization*. Ash was analyzed according to standard AOAC methods (*17*). The total protein content was determined using the Kjeldahl procedure (*17*). Crude fat was determined gravimetrically after extraction with hexane for 12 h in a Soxhlet extractor (*17*). Total soluble carbohydrates were determined after extraction with a mixture of ethanol/water (2/3) for 2 h. After centrifugation at 4000*g*, the supernatant was filtered through no. 1 Whatman paper and total soluble sugars were estimated colorimetrically by the phenol-sulfuric acid method, using a standard curve of glucose (*17*).

Protein Characterization

Amino Acid Analysis. Amino acid composition was determined by reversed-phase HPLC analysis of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatives using γ-aminobutyric acid as internal standard. Samples were hydrolyzed using 6 M HCl/1% (w/v) phenol vapor at 110 °C for 24 h in vacuum. The amino acids were treated with AQC to form AQC derivatives, which were then analyzed using a Waters HPLC system (Millipore Ltd.) fitted with a reversed-phase C18 column (*1*).

Molecular Weight Analysis by Size-Exclusion Chromatography. Molecular-mass distribution of protein in the samples was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare), according to the procedure described by Bautista et al. (*18*), using a Superdex Peptide 10/300GL column (optimum separation range 0.1−7 kDa). Samples were centrifuged at 13300*g* for 15 min at 4 °C to remove insolubles, and the supernatant was passed through a 0.2 μm filter and loaded into a 0.1 mL loop connected to an Äkta purifier system. The column was equilibrated and eluted with 0.25 M Tris-HCl buffer (pH 7.00) in isocratic mode, at a flow-rate of 0.5 mL/min, and proteins/peptides were detected at 280 and 215 nm with a GE Healthcare UV900 module coupled to the column elution. A protein standard mixture (cytochrome C, 12500 Da; aprotinin, 6512 Da; vitamin B_{12}, 1255 Da; cytidine, 246 Da; glycine 75 Da) was used to cover the range of 100−7000 Da.

Biochemical Soil Assays: Soil Enzymes. *Dehydrogenase Activity* (*DHG*). Soil dehydrogenase activity was determined using 0.5 g of soil and was measured through the reduction of 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) to *p*-iodonitrotetrazolium formazan (INTF) by a modification of the INT assay (*19*). Samples were incubated for 30 min at room temperature. Products were measured at 485 nm using a spectrophotometer (GeneQuant 1300, GE Healthcare Bio-Sciences AB, USA).

Phosphatase Activity (*APHA*). Soil phosphatase activity was measured using *p*-nitrophenyl phosphate as a substrate (*19*). Soil (0.25 g) was incubated for 30 min with substrate buffer MUB (pH 6.5). Then 0.5 M $CaCl_2$ and 0.5 M NaOH were added to stop the reaction and extract the product, *p*-nitrophenol. The product concentrations were determined photometrically at 410 nm.

β-Glucosidase Activity (*BGA*). Soil β-glucosidase activity was measured using *p*-nitrophenyl-β-D-glucopyranoside as a substrate (*20*). Soil (0.25 g) was incubated for 30 min with substrate buffer MUB (pH 6). 0.5 M $CaCl_2$ and Tris buffer (pH 12) were added to stop the reaction and extract the product, *p*-nitrophenol. The product concentrations were determined photometrically at 410 nm.

Atrazine Soil Extraction and Analysis. Atrazine was extracted from soil samples using the literature procedure with some modifications (*21*). Soil samples (0.5 g) were extracted with 4.75 mL of distilled water and 10 mL of acetonitrile. The mixture was gently mixed for 1 h and then sonicated for 20 min. Finally, 10 mL of dichloromethane was added and the mixture was shaken and then centrifuged at 3000*g* for 5 min. The supernatant was filtered using a filter paper with a pore size of 0.45 μm, previously moistened with the solvent, and evaporated at 37 °C to dryness. The residue was dissolved in 1 mL of dichloromethane.

The solution was injected into an AUTOSPEC-Q spectrometer (GC/MS system, CE Instruments) to determine the atrazine concentration. Soil samples and a calibration standard (1,4-dibromobenzene; 100 mg/kg) were injected into a GC column. The conditions of the analysis were as follows: injection volume (1 μL) with splitless injection, injector temperature 280 °C and transfer line temperature 300 °C. The carrier gas was helium with flow rate of 1.5 mL/min at constant flow with vacuum compensation. A temperature program was used. The initial temperature was 80 °C and held for 0.5 min. The temperature was then increased to 180 at 30 °C/min and to 230 °C at the rate of 30 °C/min, then held for 5 min isothermal.

Microbial Analysis: Extraction of DNA and Denaturing Gradient Gel Electrophoresis (DGGE) Analysis. *DNA Extraction from Soil and PCR Amplifications*. Total DNA was extracted from all soil samples following a bead beating protocol using the UltraClean Soil DNA Isolation kit (MoBio Laboratories). The variable V3−V5 regions of 16S rDNA were amplified via PCR using primers F357-GC and R907 to conserve regions of the 16S rRNA genes. Primer F357-GC included a GC clamp at the 5′ end (F357: 5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3′ and R907: 5′-CCG TCA ATT CCT TTG AGT TT-3′). All PCR were performed with a Mastercycler (Eppendorf, Germany). Fifty μL of the PCR mixture contained 1.5 U of EcoTaq (Ecogen, BIOLINE), 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.5 μM, and 2 μL of DNA template. After 9 min of initial denaturation at 95 °C, a touchdown thermal profile protocol was used and the annealing temperature was decreased by 1 °C per cycle from 65 to 55 °C. Then, 20 thermal cycles at 55 °C of 1 min at 94 °C, 1 min of primer annealing, and 1.5 min at 72 °C were performed. Thermal cycling was completed with an extension step at 72 °C for 10 min. The PCR products were checked by electrophoresis in 2% (w/v) agarose-ethidium bromide gels.

Denaturing Gradient Gel Electrophoresis (*DGGE*). PCR products were dried and loaded onto a 6% (w/v) polyacrylamide gel (0.75 mm thickness) with a denaturing gradient of 40−60% (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1× TAE buffer using a DCode universal mutation detection system (BioRad) at 100 V and 60 °C for 16 h. Gels were stained for 5 min in 1× TAE buffer containing ethidium bromide and photographed. DGGE bands were excised from the polyacrylamide gels.

Analysis of DGGE Patterns. After incubating at 95 °C for 1 h to allow DNA to diffuse from the polyacrylamide gels, DNA extracts were used for reamplification as described previously. PCR products were purified and sequenced. All sequences were compared with the sequences in GenBank using BLAST.

Statistical Analysis. Analysis of variance (ANOVA) was performed for all variables and parameters considering all the data collected (with columns corresponding to incubation days and rows corresponding to soil

treatments) using the Statgraphics v. 5.0 software package (*22*). The means were separated by the Tukey's test, considering a significance level of $p < 0.05$ throughout the study. For the ANOVA analysis, triplicate data were used for each treatment and every incubation time.

RESULTS

Characterization of Soil Biostimulants. The final enzymatic vegetable extracts (CGEE and WCDS-EE) existed as brown syrups that were completely soluble in water. **Table 2** shows the main chemical characteristics. Proteins and carbohydrates are the main components, with protein content higher than in the original raw material (carob germ and wheat condensed distillers solubles) due to the use of proteases, which solubilize and hydrolyze the original proteins, increasing the protein concentration in the vegetable extracts (*1*, *23*).

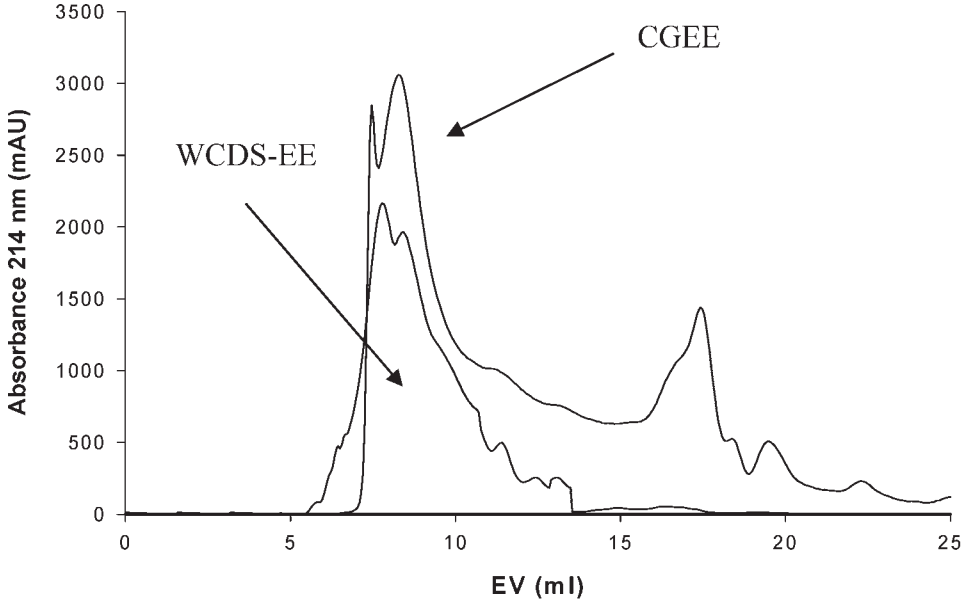
Table 2. Chemical Composition WCDS-EE and CGEE and Standard Error (Data Represent the Mean of Three Samples)

	WCDS-EE	CGEE
fat (g kg^{-1})	4.03 ± 0.8	3.0 ± 0.9
carbohydrates (g kg^{-1})	50.40 ± 5.6	26.0 ± 3.4
ashes (g kg^{-1})	6.45 ± 0.20	6.5 ± 1.9
protein (g kg^{-1})	39.11 ± 3.46	64.2 ± 3.8
ratio protein/carbohydrates	0.75	2.46

The main difference between the two enzymatic organic extracts is that the protein/carbohydrate ratio in CGEE (2.46) is superior than that in WCDS-EE (0.75); this ratio could have influence on soil stimulation and soil atrazine biodegradation.

Therefore, the molecular weight distribution of the protein content present in both extracts cover mainly peptides (< 10 kDa) and free amino acids, accounting for more than the 80% of the components under 1 kDa (**Figure 1**). The enzymatic process of protein hydrolysis makes the amino acids and peptides available for easy absorption by microorganisms.

Soil Enzymatic Activities. *Soil Dehydrogenase Activity*. **Figure 2** shows the activity of dehydrogenase during the incubation period. Soil dehydrogenase activity increased in all biostimulated samples during the incubation time; the highest values obtained were in soil treated with CGEE after 10 incubation days. After 10 days, DHG increased up to 20 and 48 times more than in controls in treated soils with WCDS-EE and CGEE, respectively. The enzymatic activity started to decline after the tenth day, but the biostimulated samples remained active. After 30 days of biostimulation, the soil dehydrogenase activity in soils biostimulated with WCDS-EE and CGEE remained 1.5 and 1.8 times higher,



Molecular weight (Daltons)	WCDS-EE	CGEE
> 10000	12.2 ± 1.0	6.1 ± 0.8
10000-5000	5.7 ± 0.8	4.8 ± 0.5
5000-1000	14.2 ± 1.4	9.1 ± 1.1
1000-300	27.1 ± 1.8	20.8 ± 1.6
< 300	40.8 ± 2.9	59.1 ± 2.2

Figure 1. Size exclusion chromatography of CGEE and WCDS-EE (Superdex peptide 10/300 GL high perfomace column, GE Healthcare) and molecular weight distribution of CGEE and WCDS-EE.

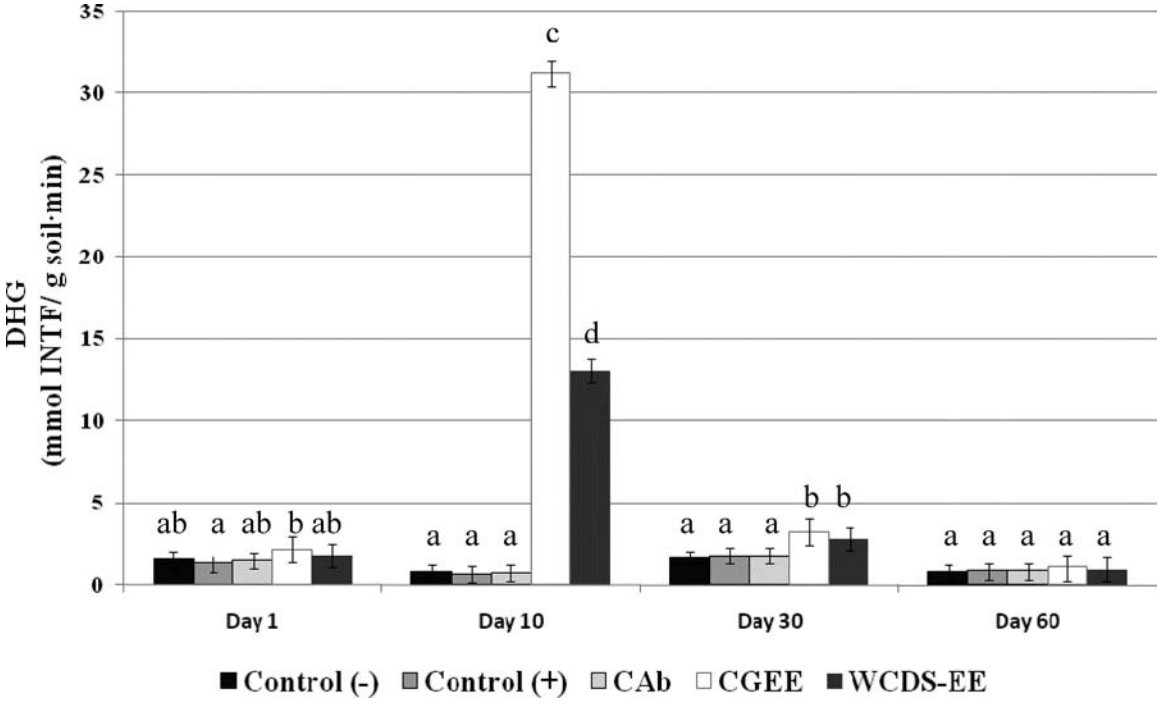


Figure 2. Dehydrogenase activity in atrazine-spiked soils after amendment with biostimulants (error bars represent the standard error of the means of three samples). Column (mean ± SE) followed by the same letter(s) are not significantly different ($p < 0.05$); INTF: 2-*p*-iodo-3-nitrophenyl formazan. Control (−), unpolluted soil; control (+), polluted soil; Cab, abiotic control. CGEE: carob germ enzymatic extract. WCDS-EE: wheat condensed distillers solubles enzymatic extract.

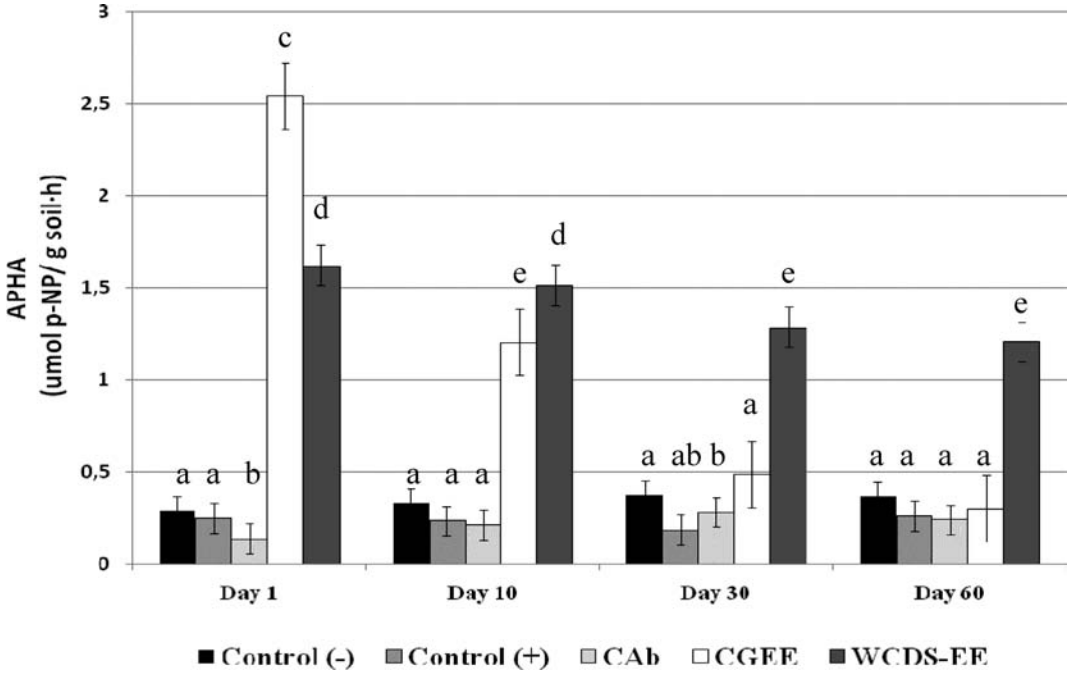


Figure 3. Phosphatase activity in atrazine-spiked soils after amendment with biostimulants (error bars represent the standard error of the means of three samples). Column (mean ± SE) followed by the same letter(s) are not significantly different ($p < 0.05$); *p*-NP: *p*-nitrophenol. Control (−), unpolluted soil; control (+), polluted soil; Cab, abiotic control. CGEE: Carob germ enzymatic extract. WCDS-EE: wheat condensed distillers solubles enzymatic extract.

respectively, than atrazine-contaminated control soils. After 60 days, the dehydrogenase activity values were similar in all treatments and similar to control soils.

In these results, there was a direct correlation between dehydrogenase activity induction and biostimulant protein/carbohydrate ratio (correlation coefficient, 0.95; slope, 21.236). We observed that CGEE induced a greater enzymatic increase than WCDS-EE (**Figure 2**).

Soil Phosphatase Activity. **Figure 3** shows the activity of phosphatase activity during the incubation period. The application of biostimulants had a strong stimulatory effect on phosphatase activity. Biostimulants showed differing effects 24 h after their addition. The highest enzymatic values were obtained with CGEE after 24 h, being nearly 10 times greater than those of contaminated control soil. After the same incubation time, treatments with WCDS-EE showed up to 6.75 times more phosphatase

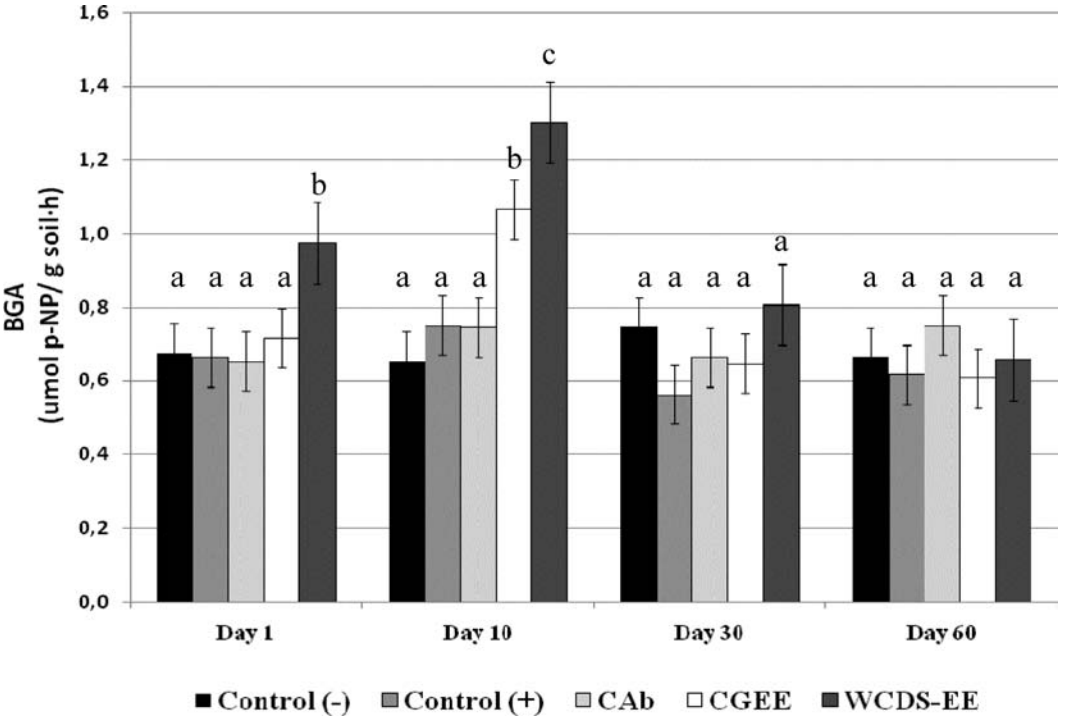


Figure 4. β-Glucosidase activity in atrazine-spiked soils after amendment with biostimulants (error bars represent the standard error of the means of three samples). Column (mean $\pm$ SE) followed by the same letter(s) are not significantly different ($p < 0.05$); *p*-NP: *p*-nitrophenol. Control (−), unpolluted soil; control (+), polluted soil; Cab, abiotic control. CGEE: carob germ enzymatic extract. WCDS-EE: wheat condensed distillers solubles enzymatic extract.

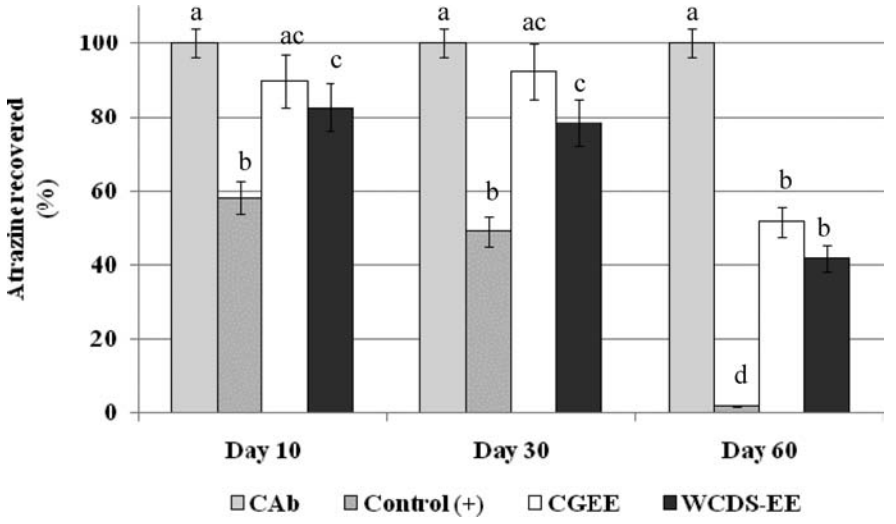


Figure 5. Atrazine recovered in soils amended with biostimulants (error bars represent the standard error of the means of three samples). Column (mean $\pm$ SE) followed by the same letter(s) are not significantly different ($p < 0.05$). Control (+), polluted soil; Cab, abiotic control. CGEE: carob germ enzymatic extract. WCDS-EE: wheat condensed distillers solubles enzymatic extract.

activity than atrazine control soil. At the same time, it was observed that the phosphatase activity decreased in soils amended with CGEE, whereas in WCDS-EE-biostimulated samples the phosphatase activity was maintained throughout the entire incubation time (60 days).

Soil β-Glucosidase Activity. **Figure 4** shows the evolution of soil β-glucosidase activity during the incubation period. β-Glucosidase catalyzes the hydrolysis of β-glucosides in soil and is one of the enzymes involved in the decomposition of plants (*24*). It reflects the state of the organic matter and the processes occurring therein (*25*). The biostimulants showed different effects 24 h after their addition and throughout the assay. In this case, it is found that treated soils with WCDS-EE, an organic biostimulant with the lesser protein/carbohydrate ratio (0.75), showed significantly higher enzymatic activity values than soils treated with a CGEE biostimulant, which has a 2.46 protein/carbohydrate ratio.

Atrazine Degradation. First, there was an insignificant decrease in atrazine concentration in abiotic control soil (**Figure 5**). To highlight the range of atrazine biodegradation in soils with different organic nitrogen sources, the atrazine concentration was examined throughout the incubation period (**Figure 5**).

The atrazine concentration decreased with incubation time. In contaminated soils, without organic nitrogen application, the decrease was certainly more pronounced than in biostimulated

Table 3. Microorganism Identification in Atrazine-Spiked Soils after Amendment with Biostimulants

DGGE band	accession no.	% similarity	closet organism in GenBank Database
B1	FJ006903.1	78	*Janthinobacterium* sp.
B2			not determined
B3	AB265150.1	98	*Sphingomonas* sp.
B4	FJ392837.1	97	*Lysobacter* sp. EM 0470
B5	AE005673.1	88	*Caulobacter crescentus* CB15
B6	DQ337559	98	*Pseudomonas* sp. BBCT
B7	FJ373039.1	98	*Bacillus* sp. 57-3
B8	AF368755.1	98	*Pseudomonas saccharaphila*
B9	AB264132.1	95	*Xantomonadaceae bacterium*

samples (**Figure 4**). After 10 incubation days, the atrazine levels approached nearly 58%, which decreased to 48% after 30 days. After 60 days, only 2% of the atrazine was recovered.

The effects of biostimulant application in contaminated soils were highly significant, and differences between the treatments were found. Compared with atrazine control soil, the treatments showed higher atrazine concentration in soil, with 89% and 82% of the atrazine added being recovered from CGEE and WCDS-EE treated soils, respectively, after 10 days. After one month, in CGEE biostimulated soil, biodegradation was inhibited and the atrazine levels were maintained. However, in WCDS-EE biostimulated samples, the biodegradation process occurred but the atrazine concentration in soil was higher than in control soil, with up to 1.5 times more atrazine. At the final measurement, after 60 days, only 2% of the atrazine remained in the control soil, in contrast to biostimulated samples, in which 42% (WCDS-EE) and 58% (CGEE) of the atrazine was still present.

Soil Microbial Diversity. Table 3 shows the microorganisms identified in soils treated with atrazine and biostimulants by denaturing gradient gel electrophoresis (DGGE) fingerprinting.

On the basis of visual inspection of the raw data, there were no differences in the DGGE profiles of the soil biostimulant treatments. These profiles were characterized by the presence of a limited number of defined bands. No well-defined predominant DGGE bands were detected in any sample. The main bands (in terms of intensity) were excised, reamplified, and sequenced. A BLAST analysis allowed for the identification of microorganisms with a high statistical significance. Identified microbial species are shown in **Table 3**.

We identified some *Pseudomonas* strains, such as *Pseudomonas sp.* BBCT and *Pseudomonas saccharaphila,* common atrazine-mineralizing microorganisms (*9*), which could be the bacteria responsible for the degrading process.

DISCUSSION

SB application induced a strong stimulant effect on the microbial populations in soil. The biodegradation activity of microorganisms results in the production of mineral compounds and essential elements that have a great significance on the physicochemical properties of soil, as these elements constitute the basis of plant nutrition. Therefore, in many cases, the soil microbial communities are a good indicator of soil fertility (*26*), and enzyme activities appear to be useful as a measure of the biological process. Their rapid response to environmental changes suggests that microbial activities are highly sensitive parameters in the assessment of environmental changes in soil. In the present work, DHG, APHA, and BGA were measured to evaluate the effects of SB on biochemical parameters in atrazine-contaminated soils (*27*). DHG represents a group of intracellular enzymes present in active microorganisms in the soil (*28*) that reflect the total oxidative activity of soil microflora, acting as an index of the total metabolic activity (*29*). As PHA and BGA are hydrolytic enzymes involved in the cycling of P and C, they are highly sensitive indicators of induced changes in soil properties due to their strong relationship with soil organic matter content and quality (*30*). APHA results correlated with DHG and BGA, thus enzymatic activities were sensitive indicators of the microbial activity response to SB application in an atrazine-contaminated soil.

Although atrazine could be subjected to abiotic degradation, our study agreed with previous research that the principal mechanism for atrazine degradation was biological based on the results of abiotic controls (with sodium azide 0.2% (w/w)), which did not show atrazine dissipation and where the atrazine concentration remained constant.

Although this herbicide has been termed as recalcitrant (*31*), a large variety of soil microorganisms are known to degrade atrazine, using it as a C source for growth (*5*, *9*, *10*). Also, the side chains and the N-rings can be used as a N source by some microorganisms to obtain energy and nutrients by N-dealkylation or dehalogenation reactions (*8*−*10*). Thus, in our study, atrazine biodegradation was carried out by the native microbial population, which was able to use the atrazine molecule. In fact, in our model, atrazine control soil (control (+)) showed an herbicide concentration decrease throughout incubation time. After one month, there was as much as 48% of the added atrazine remaining. After 60 days, only 2% was recovered. These results agree with previous studies: in semiarid soil (*29*) it was found that atrazine was eventually degraded in 45 days. Other studies showed that half-life of atrazine in soil ranged from 25 to 29 days (*32*, *33*).

Enhanced atrazine biodegradation in soils has been studied for years (*14*, *33*), and it is well-known that this process is affected by microbial adaptation and C and N availability. Addition of organic amendments may accelerate herbicide mineralization by stimulating microbial activity (*14*, *16*, *34*, *35*). From the data obtained, it can be deduced that atrazine did not interfere with enzymatic activity over time. Atrazine control soil showed similar DHG values to water control (control (−)) basal enzymatic values. However, the biodegradation process in atrazine control soil was considerably high (52% atrazine degraded in 10 days). Mineralization data suggest that this degradation is likely due to the native microbial population with the ability to mineralize the atrazine molecule as a sole C and N source. However, in biostimulated samples DHG was greatly increased (maximum nearly 50% more DHG in CGEE biostimulated soil). Although the microbial population activity was greatly stimulated in all treated soils, this increase was negatively correlated with the biodegradation process. These data suggest that under conditions where N was not limited, the microbial population changed the resource used to obtain energy and nutrients. Organic N in the form of peptides and free amino acids is a readily accessible N source due to the fact that enzymatic protein hydrolysis makes proteins available for easy absorption by microorganisms and may inhibit atrazine mineralization. In the present work, the SB used were organic N sources mainly in the form of peptides and free amino acids. From the results obtained, it is deduced that the organic nitrogen content had an important effect on the biostimulation and biodegradation processes.

In this study, we have shown that SB extend the life of the herbicide through the inhibition of metabolic biodegradation. The results of this study confirm those of previous studies showing an inhibitory effect of exogenous and organic N, in the form of peptides and free amino acids, on atrazine degradation.

Analyses like DGGE should be carried out to understand the effect of SB on the microbial community diversity of atrazine-contaminated soils. The DGGE analysis is a powerful and relatively easy way of comparing most of the species in different communities (*36*). The study of the bacterial populations by

PCR-DGGE reflected similar banding patterns between different biostimulant treatments during the period of study.

In agreement with this result, some authors found that at the same atrazine concentration the highest similarity was obtained and found that different atrazine concentrations reflected different DGGE binding patterns, thus a higher concentration than the one we used (100 mg/kg) significantly reduced total diversity compared with soils without added atrazine (*33*). In contrast to these results, we showed that there was no difference between water and atrazine control soils and biostimulated samples. Nevertheless, we have detected microorganisms such as *Pseudomonas* that have been implicated in atrazine biodegradation (*9*).

Biostimulants induce a rapid incremental increase of the microbial metabolic activity in treated soils with the consequent improvement of the soils' physical and chemical properties. Finally, WCDS-EE and CGEE showed activity as extenders which increase the persistence of atrazine in soil.

Our results show that the incorporation of organic matter in the form of peptides and free amino acids stimulated the soil enzymatic activity. Soils treated with WCDS-EE, an organic biostimulant with a lesser protein/carbohydrate ratio (0.75) show significantly higher enzymatic activity values than soils treated with a CGEE biostimulant, which has a 2.46 protein/carbohydrate ratio. These extenders increased the presence of atrazine in time that may increase the duration of its effectiveness, and due to the biodegradation depends on the SB relative protein content, CGEE, with a higher protein/carbohydrate ratio (2.46), has more extender capacity than WCDS-EE (0.75).

Also, in this study, we have shown that SB extend the life of the herbicide through the inhibition of metabolic biodegradation. The results of this study confirm those of previous studies showing an inhibitory effect of exogenous and organic N, in the form of peptides and free amino acids, on atrazine degradation.

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