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Growth, Yield, and Fruit Quality of Pepper Plants Amended with Two Sanitized Sewage Sludges

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Organic wastes such as sewage sludge have been successfully used to increase crop productivity of horticultural soils. Nevertheless, considerations of the impact of sludges on vegetable and fruit quality have received little attention. Therefore, the objective of the present work was to investigate the impact of two sanitized sewage sludges, autothermal thermophilic aerobic digestion (ATAD) and compost sludge, on the growth, yield, and fruit quality of pepper plants (*Capsicum annuum* L. cv. Piquillo) grown in the greenhouse. Two doses of ATAD (15 and 30% v/v) and three of composted sludge (15, 30, and 45%) were applied to a peat-based potting mix. Unamended substrate was included as control. ATAD and composted sludge increased leaf, shoot, and root dry matter, as well as fruit yield, mainly due to a higher number of fruits per plant. There was no effect of sludge on fruit size (dry matter per fruit and diameter). The concentrations of Zn and Cu in fruit increased with the addition of sewage sludges. Nevertheless, the levels of these elements remained below toxic thresholds. Pepper fruits from sludge-amended plants maintained low concentrations of capsaicin and dihydrocapsaicin, thus indicating low pungency level, in accordance with the regulations prescribed by the Control Board of “Lodosa Piquillo peppers” Origin Denomination. The application of sludges did not modify the concentration of vitamin C (ASC) in fruit, whereas the highest doses of composted sludge tended to increase the content of reduced (GSH) and oxidized (GSSG) glutathione, without change in the GSH/GSSG ratio. There were no effects of sludge on the transcript levels of enzymes involved in the synthesis of vitamin C, L-galactono-1,4-lactone dehydrogenase (GLDH) or in the ascorbate−glutathione cycle, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), and glutathione reductase (GR). Results suggest that the synthesis and degradation of ASC and GSH were compensated for in most of the treatments assayed. The application of sanitized sludges to pepper plants can improve pepper yield without loss of food nutritional quality, in terms of fruit size and vitamin C, glutathione, and capsaicinoid contents.

KEYWORDS: Capsaicinoids; fruit yield; gene expression; pepper (*Capsicum annuum* L. cv. Piquillo); sewage sludge; vitamin C

INTRODUCTION

Peppers (*Capsicum* spp.) contain significant levels of biologically active compounds that provide health benefits beyond basic nutrition. Peppers have exceptionally high ascorbate (ASC) or vitamin C content, as well as significant amounts of glutathione, two antioxidant compounds which play important roles in protecting cells against free radicals and oxidative damage (*1*). Pepper fruits can have also large quantities of capsaicinoids, a group of pungent phenolics derived from the phenylpropanoid pathway (*2*), with strong physiological and pharmacological properties (*3*), of which capsaicin and dihydrocapsaicin occur in quantities $>80\%$.

As a consequence of the growing global demand for food, the need to increase crop production through specific agricultural fertilization practices arises (*4*). In such context, sewage sludges have been used to increase crop productivity (*5*−*7*) due to their beneficial effects on the physical, chemical, and biological properties of soils (*5*−*9*). However, the agricultural use of sewage sludges has some potential risks associated with the presence of heavy

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metals, toxic organic compounds, and pathogenic microorganisms, aspects that must be taken into account to achieve a safe use of this byproduct as a soil conditioner (*10*). To prevent the spread of pathogenic microorganisms, the U.S. Environmental Protection Agency (U.S. EPA), through Part 503 Regulation (*11*), asserts that sludge should be appropriately treated to satisfy specific microbial standards before its application to land. In this respect, Part 503 Regulation establishes two classifications of biosolids based on the level of pathogen and vector attraction reduction: class A biosolids, treated with processes that further reduce pathogens (PFRPs) to the point at which they are not longer detectable, and class B biosolids, treated with technologies that significantly reduce pathogens but that do not meet the degree of sanitation established in the regulation. Part 503 also establishes site restrictions for class B biosolids to prevent disease transmission. As a result, there is considerable interest in sludge treatment processes that further reduce pathogens. Among these, composting and autothermal thermophilic aerobic digestion (ATAD) are recognized by the U.S. EPA as technologies capable of fulfilling class A pathogen requirements and, consequently, allow the production of a sludge that could be used for soil amendment with few restrictions. Composting of raw organic wastes has a number of agronomic benefits including reduction in material mass and water content, pathogen suppression, decreased weed viability, and the production of a stabilized organic material that is easier to handle and spread (*12*). This process requires relatively simple mechanical equipment, is simple to operate, and is more appropriate for urban areas, where agricultural lands often are not available for disposal of digested sludges. Nevertheless, it requires careful control of process parameters to ensure complete pathogen destruction (*7*), relatively large areas, and odor control (*13*). Innovative methods of thermophilic stabilization include ATAD technology, commonly referred to as "liquid composting". The ATAD process achieves a high level of disinfection and a high sludge treatment rate and stabilization (*14*). Among the environmental benefits of the ATAD process are a high volatile solids reduction capability (between 38 and 50%), as well as reduced emissions of methane and odors. The small volume, low energy, and low instrumentation requirements make the process cost-competitive with composting (*15*). Such a process is more appropriate for small- and medium-sized treatment plants. Nevertheless, one of the main disadvantages of ATAD technology is that the product may need to be dewatered before land application (*15*).

Previous studies have shown that organic wastes, including sewage sludge, can enhance pepper growth and yield through the increase of soil fertility (*6*, *7*, *16*). Differences in the management of soil fertility affect soil dynamics and plant metabolism, which may result in differences in plant composition and nutritional quality (*17*). However, there are few reports about the effect of sewage sludges as soil amendments on the content of compound indicators of the nutritional value of fruits and vegetables, such as bioactive substances and antioxidant compounds. Furthermore, metal accumulation in fruits and vegetables destined for human consumption, as a result of sewage sludge additions, remains a public concern. Therefore, the objective of the present study was to evaluate the effect of different doses of two sanitized sludges, ATAD and composted sludge, on the content of functional compounds such as vitamin C, glutathione, and capsaicionids, as well as on the accumulation of heavy metals on pepper fruits.

MATERIALS AND METHODS

Organic Amendments. Two sanitized sewage sludges treated with processes that reduce pathogens according to Part 503 Regulation (*11*) were used in the experiment: ATAD and composting. ATAD is an exothermic process in which sludge is subjected to temperatures above 55 °C with a hydraulic retention time of 6−15 days. The process of composting was driven after the mixing of raw sewage sludge with pine bark and gardening waste. Temperatures above 55 °C were reached during more than one month, and the complete process of stabilization lasted for >3 months. ATAD and composted sludges were obtained from Tudela and Pamplona (Navarra, Spain) wastewater plants, respectively. The main properties of the sludges are shown in **Table 1**.

Table 1. Sewage Sludge and Hoagland Nutrient Solution Properties

	ATAD	compost
dry matter (%)	7.34	47.44
pH	6.4	5.1
electric conductivity (dS m^{-1})	6.3	4.5
total organic carbon (%)	37.6	27.6
C/N	28	10

	ATAD	compost		Hoagland
$N_{Kjeldahl}$ (%)	1.18	2.65	N (g L^{-1})	0.105
$N\text{-}NO_3$ (%)	0.43	0.01	$N\text{-}NO_3$ (g L^{-1})	0.105
$N\text{-}NH_4$ (%)	0.13	0.50		
P (%)	1.74	2.43	P (g L^{-1})	0.035
K (%)	0.51	1.19	K (g L^{-1})	0.137
Ca (%)	8.15	10.36	Ca (g L^{-1})	0.100
Mg (%)	0.73	1.30	Mg (g L^{-1})	0.024
S (%)	1.39	1.04	S (g L^{-1})	0.032
Na (%)	0.42	0.15	Na (g L^{-1})	0.001
Fe (%)	0.99	1.19	Fe (g L^{-1})	0.003
Mn (mg kg^{-1})	200.0	230.0	Mn (mg L^{-1})	0.253
B (mg kg^{-1})	60	70	B (mg L^{-1})	0.250
Cd (mg kg^{-1})	0.70	0.60	Cd (mg L^{-1})	<0.001
Cu (mg kg^{-1})	103.0	117.5	Cu (mg L^{-1})	0.012
Ni (mg kg^{-1})	7.0	10.8	Ni (mg L^{-1})	<0.001
Pb (mg kg^{-1})	42.3	48.1	Pb (mg L^{-1})	<0.001
Zn (mg kg^{-1})	523.0	498.9	Zn (mg L^{-1})	0.078
Hg (mg kg^{-1})	0.47	0.61	Hg (mg L^{-1})	<0.001
Cr (mg kg^{-1})	17.1	28.0	Cr (mg L^{-1})	<0.001

Experimental Design and Growth Conditions. A peat-based commercial container medium mixed with perlite and sand (4:1:1 v/v/v) was packed into pots with a capacity of 2 L. Two doses of ATAD sludge, 15 and 30%, v/v (A1 and A2, respectively), and three of composted sludge, 15, 30 and 45% (v/v) (CP1, CP2, and CP3, respectively), were assayed. Pots containing substrate without the addition of sludge were included as a control (C) group. The sludges were added to the substrate 1 month before transplanting. This period of time allows the level of phytotoxic substances (e.g., excess ammonium) to decrease, as well as the mixture to homogenize microbiologically. Soil samples were taken before transplanting for use in microbiological assays. One pepper seedling (*Capsicum annuum* L. cv. Piquillo) (two- or three-leaf stage) was transplanted into each pot. Plants were grown until maturity stage in a greenhouse maintained at 25/15 °C day/night, supplemented with irradiation from high-pressure sodium lamps Son-T-Agro (Philips Nederland B.V., Eindhoven, The Netherlands) during a 14 h photoperiod and were irrigated with half-strength Hoagland's nutrient solution (*18*). At the end of the experiment (plants with at least three red fruits), two whole red fruits per plant were oven-dried at 55 °C for 4−5 days and ground in a Polytron grinder for heavy metals and capsaicinoid analysis. Longitudinal strips from red fruits (including exocarp, mesocarp, and endocarp without seeds) were cut, immediately plunged into liquid N_2, and stored at −80 °C for ascorbate, glutathione, and gene expression analysis. The experiment was repeated twice with similar results. Data from both repetitions were pooled (total of 10 plants per treatment) and analyzed.

Growth and Fruit Yield Parameters. Fruit fresh weight and diameter were determined for each plant. Leaf, shoot, and root dry matter (DM) were determined after drying at 80 °C for 2 days. Fruit DM was calculated after drying at 60 °C for 45 days.

Substrate Microbiological Properties. Global microbial activity of the substrates was assessed by measuring basal respiration, total microbial biomass carbon (MBC), and the metabolic quotient (qCO_2). Respiration rates were measured in hermetically sealed flasks, in which a 30 g substrate sample was kept in darkness at 28 °C and 60% of its water-holding capacity (WHC) for 33 days. The CO_2 emitted was measured with an infrared gas analyzer (IRGA) as described by Johnson et al. (*19*). MBC was determined according to the technique of substrate-induced respiration (SIR) (*20*). Fifteen grams of substrate was placed in bottles of 135 mL at 50% of its WHC with a solution of glucose (2.5%). Substrates were incubated for 3 h at 22 °C in darkness, and the percentage of CO_2 in each bottle was measured by an IRGA as described above. The qCO_2 was calculated as the ratio of the basal respiration, expressed as micrograms of C-CO_2 per gram of substrate per hour, and the MBC, expressed as micrograms of C per gram of substrate.

Heavy Metal Content. To determine the concentrations of Cr, Mn, Fe, Ni, Cu, Zn, Cd, and Pb in pepper fruits, 0.25 g of dried sample was digested with nitric acid (HNO_3, 65% v/v) in a microwave unit Xpress model CEM MARS 5 (CEM Corp., Matthews, NC), according to the EPA-3051-A method (*21*). Samples were centrifuged, and the supernatants were analyzed by inductively coupled plasma−mass spectrophotometry (ICP-MS) with an Agilent 7500 series spectrophotometer.

Determination of Ascorbate (ASC), Reduced Glutathione (GSH), Oxidized Glutathione (GSSG), and Capsaicinoids (Capsaicin and Dihydrocapsaicin). ASC, GSH, and GSSG were extracted according to the method of Rellán-Álvarez et al. (*22*). Plant tissue (150 mg of pericarp) was ground with mortar and pestle in liquid N_2. Isotopically labeled standards of reduced glutathione (GSH*) and ascorbic acid (ASA*) were added at the moment of sample grinding. The dry powder was homogenized with 1 mL of cold (4 °C) extraction solution (5% w/v metaphosphoric acid and 1 mM EDTA in 0.1% formic acid), supplemented with 1% (w/v) polyvinylpolypyrrolidone (PVPP) just before use. Homogenates were centrifuged at 15000*g* for 20 min at 4 °C. Supernatants were collected, and the pellets were resuspended with 0.5 mL of the same extraction solution and centrifuged again under the same conditions. The second supernatants obtained were combined with the first one and taken to a final volume of 2 mL with extraction solution. Then, they were filtered through 0.22 μm polyvinylidene fluoride filters (PVDF) (Millipore, Bedford, MA), immediately frozen in liquid N_2, and stored at −80 °C until analysis. All steps were done in a cold chamber at 4 °C under green safelight to avoid ASC degradation.

Capsaicinoid extraction was based on the procedure of Garcés-Claver et al. (*23*). Dried tissue (100 mg) was extracted with 1 mL of pure acetonitrile, containing a small amount of the internal standard 4,5-dimethoxybenzyl-4-methyloctamide (DMBMO), 5 μM. The suspension was constantly shaken for 1 h at room temperature and, then, heated in a water bath at 65 °C for 1 h. The mixture was shaken again for 1 h in the conditions indicated above. Later, the suspension was centrifuged for 15 min at 16000*g*. The supernatants were collected, brought to a volume of 1 mL with acetonitrile, filtered successively through 0.45 and 0.22 μm PVDF membrane filters (Millipore), and stored at −20 °C until analysis.

Quantification of ASC, GSH, GSSG, and capsaicinoids was carried out according to the methods of Rellán-Álvarez et al. (*22*) and Garcés-Claver et al. (*23*), respectively, with some modifications. Chromatographic separation was performed on an Alliance 2795 HPLC system (Waters, Milford, MA) coupled to a micrOTOF II ESI-TOFMS high-resolution mass spectrometer apparatus (Bruker Daltoniks GmbH, Bremen, Germany). Analyses were done in the negative mode for antioxidants and in the positive mode for capsaicinoids. Capillary, end plate, hexapole RF, drying gas temperature, nebulizer pressure, and drying gas flow were set at 3000 V, −500 V, 128.6 V, 180 °C, 1.6 bar, and 8 L min^{-1} and 3000 V, −500 V, 80 V, 180 °C, 1.4 bar, and 6 L min^{-1} for antioxidants and capsaicinoids, respectively. The system was controlled with the software packages MicrOTOF Control v.2.2. and HyStar v.3.2. (Bruker Daltonics). Data were processed with Data Analysis v.3.4. software (Bruker Daltonics).

Gene Expression Analysis. The expression of L-galactono-1,4-lactone dehydrogenase (GLDH, EC 1.3.2.3), ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDAR, EC 1.6.5.4), and glutathione reductase (GR, EC 1.8.1.7) was measured as follows. Total RNA was isolated from pepper fruits with the Trizol reagent (Gibco BRL) and quantified as described by Mateos et al. (*24*). Two micrograms of total RNA was used as a template for the reverse transcriptase (RT) reaction. It was added to a mixture containing 1 mM dNTPs, 1.6 μg of polydT_{23} primer, 1× RT-buffer (25 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, and 2 mM DTT), 0.9 U RNasin ribonuclease inhibitor, and 10 U AMV RT (FINNZYMES). The reaction was carried out at 42 °C for 40 min, followed by a 15 min step at 70 °C and then by cooling to 4 °C for 10 min.

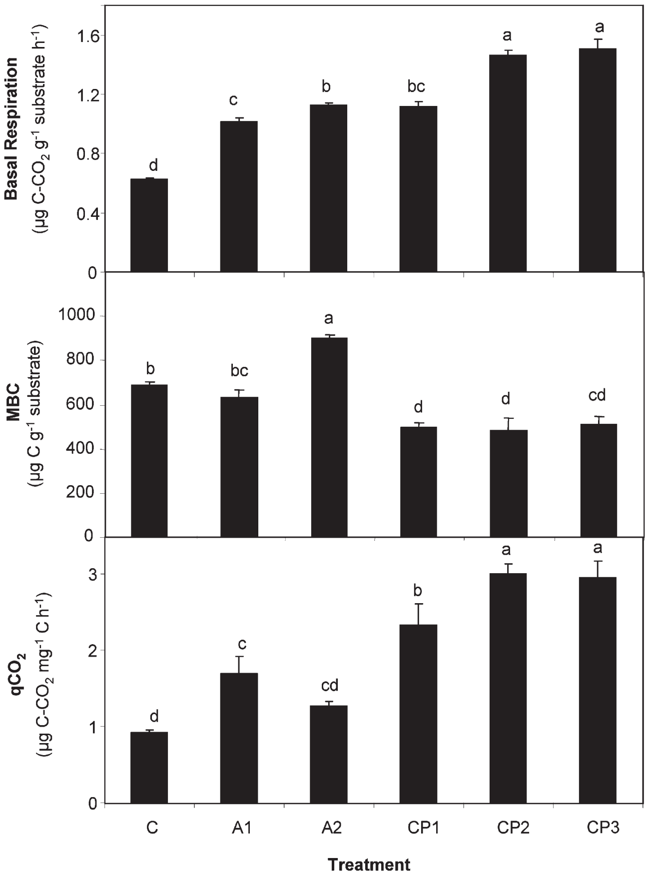

Figure 1. Basal respiration, microbial biomass carbon (MBC), and metabolic quotient (qCO_2) of unamended substrate (C) and substrate amended with various doses of ATAD and composted sewage sludge: A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Each bar represents the mean ± standard error (SE) of five determinations. The different letters indicate significant differences ($p < 0.05$) between treatments based on LSD test.

The sequences of gene-specific primer pairs used in this work are presented in Table S1 (Supporting Information). cDNAs were amplified by PCR, according to the method of Mateos et al. (*24*), as follows: 1 μL of each cDNA (30 ng) was added to 200 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 1 U of Hot Master Taq DNA polymerase (Eppendorf), and 1 μM of each primer, in a final volume of 20 μL. Reactions were carried out in a Hybaid thermocycler (Ashford, U.K.). A first step of 2 min at 94 °C was followed by 28−33 cycles (depending on the gene) of 20 s at 94 °C, 20 s at 55 °C, and 30 s at 65 °C, with a final extension of 10 min at 65 °C. Then, amplified PCR products were detected after electrophoresis in 1% (w/v) agarose gels and staining with ethidium bromide. To standardize the results, the relative abundance of *Actin* was also determined and used as the internal standard. Quantification of the bands was performed using a Gel Doc system (Bio-Rad Laboratories) coupled with a highly sensitive CCD camera. Band intensity was expressed as relative absorbance units. The ratio between each specific gene and *Actin* amplification was calculated to normalize for initial variations in sample concentration. Then, the relative increase or decrease in gene expression in ATAD and compost treatments was calculated by dividing the normalized band density of the gene from ATAD and compost treatments by that of the same gene from the control treatment (C), with a value of 1.

Statistical Analysis. Analysis of variance was performed for each parameter. Means ± standard errors were calculated, and, when the *F* ratio

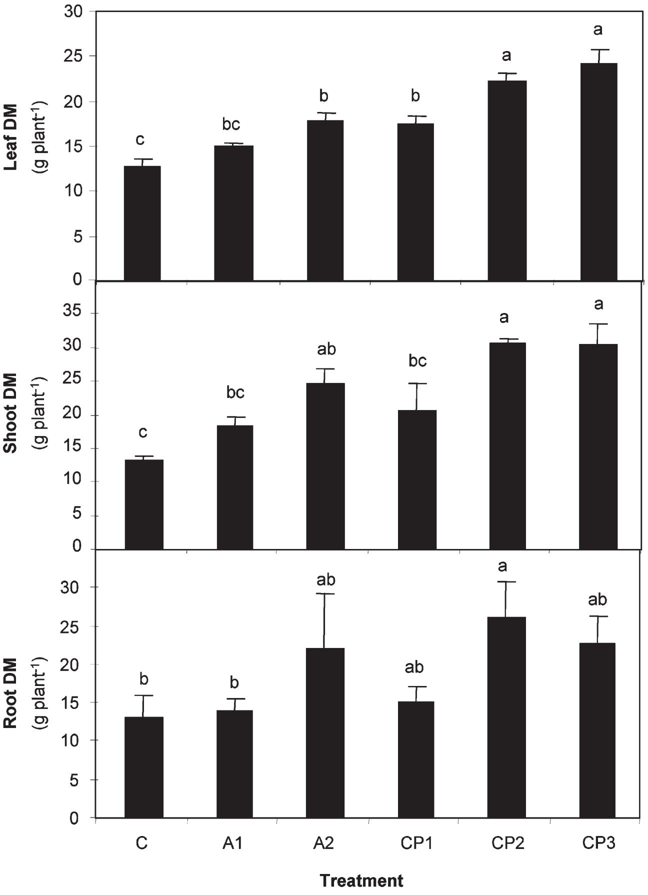

Figure 2. Leaf, shoot, and root dry matter (DM) of plants grown in unamended substrate (C) and substrate amended with various doses of ATAD and composted sewage sludge: A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Values are means ± SE of 10 plants. The different letters indicate significant differences ($p < 0.05$) between treatments based on LSD test.

was significant ($p < 0.05$), a least significant difference (LSD) test was applied as available in the SPSS statistical package version 12.0 programs for Windows XP.

RESULTS

The application of both ATAD and composted sludge stimulated significantly the basal respiration of the soil, especially in those substrates amended with compost (**Figure 1**). Soil respiration increased as the dose of sludge increased. The microbial biomass carbon (MBC) increased only with the highest dose of ATAD (A2), whereas it decreased with the application of compost, independently of the dose applied. With regard to the metabolic quotient (qCO_2), there was a very significant increase in the treatments with compost and with the lowest dose of ATAD (A1), whereas it does not change in the A2 compared with control.

The application of the highest doses of ATAD (A2) and all of the doses of composted sewage sludge (CP1, CP2, and CP3) increased significantly leaf DM (36−89%) and shoot DM (86−132%) per plant, compared to control (**Figure 2**). Root DM increased significantly only in the treatment CP2. Pepper yield and the number of fruits per plant increased significantly (28−43 and 30−98%, respectively) as the dose of ATAD and composted sludge increased, although the differences between CP2 and CP3 were not statistically significant (**Figure 3**). The application of both sludges modified neither fruit dry matter nor fruit diameter compared to control (**Figure 3**). There were no remarkable differences in DM production between both types of sludge when compared at similar doses on a volume basis. However, compost exhibited slightly higher leaf and fruit DM production, as well as higher pepper yield at dose 30% v/v.

Figure 4 represents the correlation between pepper yield and the plant available nitrogen in the treatments assayed ($N_{available}$). Available nitrogen per plant represents the amount of nitrogen present in mineral forms (N-NO_3 and N-NH_4) that can be readily taken up by the plant during the growth period. $N_{available}$ was calculated by taking into account the N supplied with the Hoagland's nutrient solution (**Table 1**) and the mineral N applied with the sludge (**Table 1**), as well as the N mineralized during the growth period, which was estimated according to the U.S. EPA (EPA/625/R-95/001) (*25*). The increase in the available N per plant led to increases in pepper yield until a maximum in the treatment CP2, where a plateau phase was reached.

The application of ATAD sludge increased significantly the concentration of Cu in fruit, whereas the compost led to an increase in Cu and Zn as the dose of compost increased (**Table 2**). By contrast, the levels of Cr, Mn, Fe, Ni, Cd, and Pb did not increase in the plants amended with sludge, even decreased, as in the case of Cr, Fe, Ni, Cd, and Pb in the treatment ATAD and Mn, Fe, and Cd in compost.

Figure 5 shows the concentrations of ASC, GSH, and GSSG and the ratio of GSH/GSSG in pepper fruits. The application of ATAD and composted sewage sludge did not modify the concentration of ASC in fruits. By contrast, the concentrations of GSH and GSSG increased significantly in CP2 and tended to increase in CP3 compared to control. In general, the addition of sludge did not modify significantly the GSH/GSSG ratio, except in treatment A1, which exhibited a great increase in the ratio of GSH/GSSG. No differences in the level of *GLDH*, *APX*, *MDAR*, and *GR* expression were observed in pepper fruits under the treatments assayed (**Figure 6**).

The addition of sewage sludge to soil did not affect the concentration of capsaicin and dihydrocapsaicin, regardless of the type of sludge applied (Supporting Information, Table S2).

DISCUSSION

The application of ATAD and composted sewage sludge increased plant growth (21−103%) and fruit yield (28−75%), in line with previous results (*6*, *7*, *16*). Such increase in fruit yield was mainly due to an increase in the number of fruits per plant, instead of an increase in fruit size (dry matter per fruit or diameter). Similarly, Arancon et al. (*16*) reported that the effect of vermicompost on fruit weight (grams per fruit) was not as pronounced as the effect on pepper yield. The increase in growth and yield of plants amended with organic wastes has been previously attributed directly to nutrient availability (*26*−*28*). Sewage sludge is an excellent source of macro- and micronutrients, especially nitrogen. In the present work, the estimated available N in the amended substrates was higher compared with control (from 17% in A1 to 192% in CP3), which may partially explain the increase in plant growth and fruit yield of these treatments. In addition, the treatments with ATAD and composted sludge were also fertilized with Hoagland's nutrient solution. It is very likely that the sludge not only slowly released nutrients but, also, prevented nutrient losses of fertilizer (denitrification, volatilization, and leaching) by binding such elements (*29*). It must be noted that pepper yield increased as the dose of ATAD and composted sludge increased until a maximum in the treatment CP2. Then, a plateau was reached, which can signal the beginning of the so-called luxury uptake by the plant.

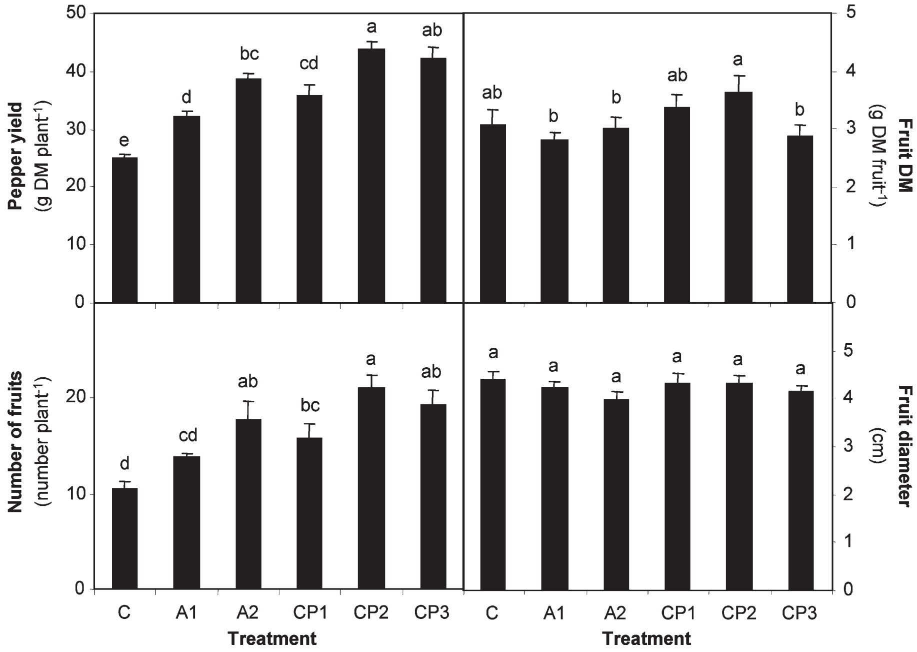

Figure 3. Pepper yield (red and green fruits), number of fruits per plant (red and green fruits), fruit dry matter (DM) (red fruits), and fruit diameter (red fruits) of plants grown in unamended substrate (C) and substrate amended with various doses of ATAD and composted sewage sludge: A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Values are means ± SE of 10 plants. The different letters indicate significant differences ($p < 0.05$) between treatments based on LSD test.

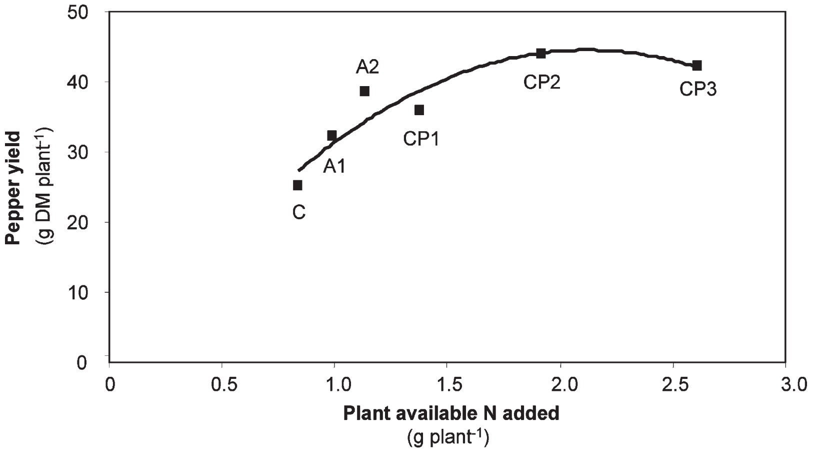

Figure 4. Relationship between pepper yield and plant available nitrogen added in the treatments assayed, unamended substrate (C) and substrate amended with various doses of ATAD and composted sewage sludge: A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Plant available nitrogen was calculated as the sum of N added with Hoagland's nutrient solution and the N available in the sludge, estimated according to the U.S. EPA (EPA/625/R-95/001 (*25*)). It was assumed that the mineralization rates of organic nitrogen were 30 and 10% for aerobically digested and composted sludges, respectively (*25*).

Table 2. Heavy Metals Concentration (mg Kg DM^{-1}) in Red Fruits of Plants Grown in Unamended Substrate (C) and Substrate Amended with Various Rates of ATAD and Composed Sewage Sludge[a]

treatment	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
C	0.24 ± 0.03 a	8.58 ± 0.22 a	43.68 ± 3.48 a	0.14 ± 0.04 a	3.13 ± 0.24 e	25.24 ± 0.84 c	0.14 ± 0.04 a	0.13 ± 0.01 a
A1	0.22 ± 0.03 ab	8.38 ± 0.13 a	27.42 ± 2.80 b	0.13 ± 0.09 ab	4.14 ± 0.19 d	25.44 ± 0.80 bc	0.06 ± 0.01 b	0.12 ± 0.01 a
A2	0.17 ± 0.01 b	9.13 ± 0.32 a	27.02 ± 2.33 b	0.03 ± 0.01 c	4.64 ± 0.25 cd	26.04 ± 1.00 bc	0.02 ± 0.01 c	0.04 ± 0.01 b
CP1	0.21 ± 0.01 ab	8.89 ± 0.43 a	28.60 ± 4.13 b	0.08 ± 0.03 bc	5.09 ± 0.43 cd	26.94 ± 1.52 bc	0.03 ± 0.01 c	0.04 ± 0.01 b
CP2	0.21 ± 0.02 ab	7.52 ± 0.21 b	30.78 ± 1.84 b	0.08 ± 0.03 bc	6.87 ± 0.27 b	28.01 ± 0.86 ab	0.02 ± 0.01 c	0.06 ± 0.01 ab
CP3	0.19 ± 0.01 ab	5.91 ± 0.22 c	25.72 ± 1.88 b	0.11 ± 0.01 ab	8.04 ± 0.21 a	30.45 ± 0.25 a	0.02 ± 0.04 c	0.10 ± 0.04 ab

[a] A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Values are means ± SE of five determinations (one pepper per plant from five different plants per treatment). Within each column, values followed by different letters are significantly different ($p < 0.05$) based on LSD test.

Besides nutrient supply, other factors such as increased rhizosphere microorganisms activity and/or the presence of humic substances in the sludge may have indirectly influenced plant growth in the treatments with sludge. Arancon et al. (*16*) and

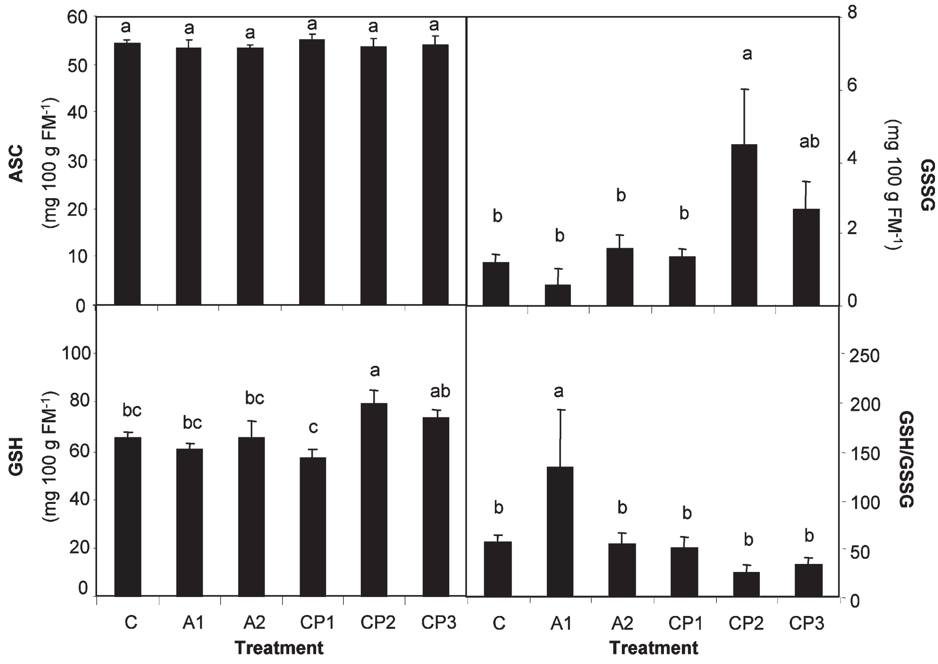

Figure 5. Ascorbate (ASC), reduced glutathione (GSH), and oxidized glutathione (GSSG) concentrations and GSH/GSSG ratio in red fruits of plants grown in unamended substrate (C) and substrate amended with various doses of ATAD and composted sewage sludge: A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Each bar represents the mean ± SE of five determinations (one sample per plant from five different plants per treatment). The different letters indicate significant differences ($p < 0.05$) between treatments based on LSD test.

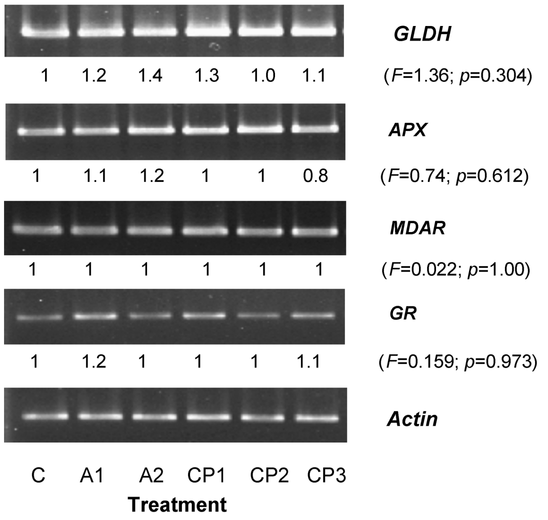

Figure 6. Analysis of the mRNA expression of *GLDH* (L-galactono-1,4-lactone dehydrogenase), *APX* (ascorbate peroxidase), *MDAR* (monodehydroascorbate reductase), and *GR* (glutathione reductase) in red fruits of plants grown in unamended substrate (C) and substrate amended with various doses of ATAD and composted sewage sludge: A1 (15% v/v), A2 (30% v/v), CP1 (15% v/v), CP2 (30% v/v), and CP3 (45% v/v). Semiquantitative reverse transcription-PCR was performed on total RNA isolated from fruit samples from at least four different plants per treatment. The relative abundance of *Actin* was used as the internal standard. Images shown are representative agarose electrophoresis gels of the amplification products visualized by ethidium bromide staining under UV light. Numbers are means of three different experiments and express the change in the gene band intensity relative to the control (as detailed under Materials and Methods).

Pascual et al. (*7*) reported that the positive effects of sewage sludge on pepper yield could be due to the stimulation of soil microbiota, which may have an indirect influence on plant growth. In the present work, microbial biomass of the sludge-amended soils (measured as MBC) did not increase, in general, when compared to control. Nevertheless, microbial activity increased significantly in all of the substrates amended with sludge with respect to the control, as shown by the basal respiration and metabolic quotient (qCO_2). Microbial activity in soils can contribute to the improvement of soil structure (*30*). Because plants in the present experiment were not cultivated in soil, but in a peat/perlite-based growing medium, such an effect on soil structure is not expected to have a significant impact on plant growth in our case. However, the increased microbial activity after sludge addition can also influence the root environment and plant growth through the production of plant growth hormones, including indoleacetic acid, gibberellins, and cytokinins, by a wide variety of rhizosphere microorganisms (*31*). Additionally, sewage sludges, especially those composted, contain significant amounts of humic substances, which is expected to have an important and positive impact on soil fertility (*32*−*34*). The presence of humic substances in the ATAD and composted sludge may also explain the improvement in growth and fruit yield of amended peppers in the present work.

It must be pointed out that there were not remarkable differences between the effects of ATAD and compost on plant growth and yield parameters, when compared at similar doses on a volume basis. Only the composted sludge seemed to have a greater effect on leaf DM, pepper yield, and fruit DM (**Figures 2** and **3**), which may be related with the higher nutrient content, N especially, of this sludge (**Table 1**). In addition, the composted sludge had lower water content than ATAD; therefore, for the same dose on a volume basis, the amount of dry material added with the compost was greater than in the case of ATAD. This implies higher amounts of nutrients and organic matter added with the compost, which may also explain the stronger effect of such treatment on the substrate microbial activity (**Figure 1**) as well as the differences observed in some growth and yield parameters.

One of the main problems of the agricultural use of sewage sludge is related to the presence of heavy metals in this waste, which can bioaccumulate in vegetable and crop tissues, thus involving a serious health hazard problem (*35*). Among the heavy metals present in the sludge, Zn and Cu have been reported to be the most mobile (*5*, *36*−*38*). Previous works have reported an increase in the bioavailability of Cu and Zn in the soil after sludge addition, associated with increases in the concentrations of these elements in the root and shoot of ryegrass and subterranean clover (*36*, *38*), as well as in the grain of barley plants (*5*). In the present study, the concentrations of Cu and Zn in pepper fruit increased significantly as the dose of sludge rose, which indicates the existence of a notable translocation of these metals from the soil to the reproductive organs of the plant. Compost-amended plants exhibited higher concentrations of Cu with respect to ATAD plants, which may be related to the greater Cu content of compost (**Table 1**). In addition, as indicated above, the low water content of composted sludge led to higher applications of such material on a dry weight basis and, thus, of heavy metals. We cannot discount differences in the bioavailability of heavy metals present in the sludges due the different natures of the material assayed, as well as the different technologies employed to treat them. Although Zn and Cu seemed to be the most mobile elements, in our work, their values remained within the normal ranges reported for pepper fruits (*39*) and far below the toxicity thresholds established by Kabata-Pendias and Pendias (*40*) for plants. With regard to human health risks, Miller and Miller (*41*) reported that Zn and Cu are toxic to plants before reaching the plant tissue concentrations that affect human health. Therefore, because in the present work there was no evidence of phytotoxicity, we can rule out a risk for human health due to these heavy metals. Nevertheless, we cannot discount higher accumulations of Zn and Cu in the fruit after repeated applications of sewage sludge, as reported by Antolín et al. (*5*). Contrary to Zn and Cu, the levels of other metals decreased in fruits from the amended treatments. Such result may be explained by a lower bioavailability of these elements in the sludge-treated substrates. In addition, it is possible that their translocation from the vegetative to reproductive organs was lower with respect to Zn and Cu, thus being mainly accumulated in the root. One of the most common strategies of plants for storage and inactivation of toxic elements is their accumulation in the root (*42*). Cadmium and lead are the heavy metals of greatest concern to human health, and they are the only two metals for which concentration in vegetables is regulated by the European Union (EU) (*43*, *44*) and the Codex Alimentarius (Food and Agriculture Organization of the United Nations-World Health Organization, FAO-WHO) (*45*). In the present work, the levels of both trace metals in the fruit remained below the threshold values established by such regulations.

Peppers have exceptionally high vitamin C content, the major water-soluble antioxidant in plant cells, which plays a major role in protecting cells against free radicals and oxidative damage (*1*). Plants and most animal species synthesize their own vitamin C, but humans cannot, although they require 60−100 mg a day of this vitamin and, therefore, it must be obtained from the diet (*46*). The role of ASC in the human diet is thought to be significant in preventing common degenerative conditions (*47*). Because the bioavailability of ASC in fruits and vegetables is equal to the availability of synthetic L-ascorbic acid (*48*), plant sources of vitamin C are considered to be more beneficial to human health, because they provide other essential nutrients and phytochemicals (*49*). Previous studies dealing with the effect of organic amendments on ASC concentration in plant tissues report variable and somewhat contradictory results. Several authors have observed an increase in the concentration of vitamin C of different crops amended with organic wastes (*1*, *50*, *51*), suggesting that compost causes changes that favor the accumulation of antioxidants (*1*). By contrast, other studies show no significant differences in the ASC concentration of fruits and vegetables amended with organic wastes compared to conventional fertilization (*52*, *53*). In the present study, the application of ATAD and composted sludge did not modify the concentration of vitamin C in fruits, remaining in the range of previous papers (*3*, *55*). In accordance with these results, the expression of *GLDH*, an enzyme involved in the synthesis of ASC (*56*, *57*), did not change in the treatments with sludge. Similarly, transcript levels of *APX* and *MDAR*, enzymes that catalyze the oxidation of ascorbate to monodehydroascorbate and the reduction of this to ascorbate, respectively, were not modified in the sludge-treated plants. These results suggest that the synthesis and removal of ascorbate through the ascorbate−glutathione cycle were compensated for in all of the treatments assayed. Vitamin C can be considered as a biomarker for nutritional quality of fruits and vegetables (*58*). Interestingly, the agronomic use of sewage sludge as soil amendment increased pepper yield without affecting the vitamin C content, thus indicating that fruit quality was maintained in the treatments with sludge.

Glutathione is another important antioxidant compound present in peppers, which has been called the master antioxidant because of its critical roles in metabolic mechanisms and pathways (*59*). This nonproteinogenic tripeptide is found in two interconvertible forms inside the cell, that is, oxidized (GSSG) and reduced (GSH). High ratios of GSH to GSSG are mandatory for normal functioning of a cell in various environmental conditions, because, for example, the regeneration of ascorbate requires GSH as electron donor in the ascorbate−glutathione cycle. In the present experiment, the concentration of reduced glutathione (GSH) tended to increase with the addition of the highest doses of compost (CP2 and CP3). Our results agree with those of Wang et al. (*1*) and Planquart et al. (*60*), who reported that the application of compost, from sewage sludge in the last case, induced the formation of GSH in strawberry fruits and colza, respectively. The accumulation of GSH in several plant and bryophyte species has been associated with an intrinsic response of plants upon heavy metal exposure (*61*−*63*). Besides its role as an antioxidant molecule in the ascorbate−glutathione cycle, GSH is also the substrate for the biosynthesis of phytochelatins, which are involved in heavy metal detoxification (*64*). Although the levels of heavy metals in the compost used in this experiment were below the permissible limits for its agronomic use (*11*) and their concentrations, at least in fruit, were far below toxic thresholds, the higher availability of trace metals in the substrates with the highest doses of compost may have slightly induced the synthesis of glutathione by the plants. The parallel increase in both GSH and GSSG contents observed in CP2 and CP3 suggests that the oxidation of GSH to GSSG and its reduction to GSH were compensated for in these treatments, as shown by the maintained GSH/GSSG ratios. In line with this, we found no change in the expression of GR, an enzyme that maintains the reduced glutathione pool by catalyzing the reduction of GSSG to GSH, in the sludge-treated plants. The high GSH/GSSG ratio in treatment A1 was not related to significant changes in the expression of GR.

The concentration of capsaicinoids in pepper fruits varies depending on the different cultivars. As far as we know, the data for capsaicin and dihydrocapsaicin for the cultivar Piquillo have not been reported before. The levels of the capsaicinoids in Piquillo peppers were in the range of pungent *Capsicum* genotypes with low levels of capsaicinoids, such as Sincap and Agridulce, and far below more pungent cultivars, such as Tabasco and Orange Habanero (*23*). Besides genetic components, the pungency level of peppers is determined by environmental components, and the concentration of capsaicinoids increases with increased environmental stress (*65*). Among other factors, fertilization has been reported to affect the pungency level of fruits. Johnson and Decoteau (*66*) and Estrada et al. (*67*) reported that mineral supplementation, especially with N, affected significantly the capsaicin content in Jalapeño and Padrón pepper fruits, respectively. Bajaj et al. (*68*) observed higher levels of capsaicin in sweet peppers fertilized with high doses of N and P compared with those not fertilized. In this regard, the addition of sludge, which contains significant amounts of plant nutrients, may be expected to affect the concentration of capsaicinoids in peppers. Nevertheless, in the present work, there were no changes in the levels of capsaicin and dihydrocapsaicin with respect to the control, nonamended plants. To explain such a result, it must be noted that in our work, control plants received nutritive solution with optimum nutrient rates for pepper growth, whereas in the case of Bajaj et al. (*68*) and Johnson and Decoteau (*66*) the control treatments received suboptimum nutrient doses or they did not receive nutrient supply, leading probably to some nutrient deficiencies, with the consequent decrease in capsaicinoid content. Another possible explanation for the absence of the variations in the capsaicinoid content may be that the pungency level of the cultivar Piquillo is strongly influenced by the genetics of the plant rather than other environmental factors as suggested by previous research (Macua, http://www.lukor.com/not-neg/sectores/0411/20161540.htm). The Control Board of "Lodosa Piquillo peppers" Origin Denomination specifies that Piquillo pepper should be just slightly pungent (*69*). In this way, it must be noted that peppers amended with ATAD and composted sludge maintained a low pungency level, which is in accordance with such regulation.

In summary, the application of both ATAD and composted sewage sludges to a peat-based potting mixture increased plant biomass and fruit yield of pepper plants, which were partially attributed to a higher nutrient availability as well as to the improvement of the biological properties of the substrate mixture. Although the heavy metal content in fruit remained below toxic thresholds, it is important to monitor Zn and Cu contents if long-term applications of sludge are proposed, to ensure that they will not exceed the tolerance levels for animal feed or human food. The concentrations of some functional compounds, such as vitamin C and capsaicinoids, were not markedly affected by sludge addition. The agricultural application of sanitized sludges treated with ATAD and composting processes may be an interesting practice, which increases pepper yield without loss of the food nutritional value. Nevertheless, further research involving different types of growth media and soils is needed before sewage sludge amendments can be adapted to practical field applications. In addition, sewage sludge is a potential source of organic pollutants, which should be taken into consideration in future research in this field to avoid potential risks for human health.

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Supporting Information Available: Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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