

Production, Characterization, and Effects on Tomato of Humic Acid-like Polymerin Metal Derivatives from Olive Oil Mill Waste Waters

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The dark polymeric organic fraction rich in potassium recovered from olive oil mill waste waters (OMWW) and named polymerin and the potassium salified deglycosylated polymerin derivative (K-SDpolymerin) were easily transformed into their metal derivatives by saturation with various metals, including Na, Cu, Zn, Mn, Fe, and Al. Saturated metal polymerins were characterized by diffuse reflectance infrared Fourier transform spectroscopy and atomic absorption spectrometry. Tests on tomato plants of the various polymerins showed that only the soluble polymerin, K-SDpolymerin, and the insoluble Mn-SDpolymerin were significantly toxic. The toxic effects of OMWW on tomato at the original concentration and diluted 1:10 were much stronger than those of any polymerin. The possible exploitation of polymerins as bioamendments and/or metal biointegrators as a function of their phytotoxic effects, their humic acid-like nature, and their richness in macro- and micronutrient metals is also discussed.

KEYWORDS: *Olea europea*; olive oil mill waste waters; polymers; polysaccharides; proteins; melanin; metals; humic acid-like substances; diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS); atomic absorption spectrometry (AAS); *Lycopersicon esculentum*; phytotoxicity test; bioamendments

INTRODUCTION

Recycling of olive oil mill waste waters (OMWW) as an amendment in agriculture, either in raw form or after various treatment (1–5), and recovery of their organic components for use in agriculture and in industry (6–8) are the approaches commonly proposed for their disposal. The high polluting properties of OMWW, as documented by the high chemical oxygen demand (COD) and biological oxygen demand (BOD) (9), render inadequate this material to be discharged into sewage plants, but it can be spread on the soil according to the laws of various producing countries.

In the framework of studies of ecological and useful disposal processes, we recently found (9) that metal cations naturally occurring in OMWW were mainly bound to the organic polymeric fraction and that K was the most abundant metal, followed, in decreasing order, by Ca, Mg, Na, Zn, Fe, and Cu. In addition, the COD and BOD of this biomaterial markedly decreased in comparison with those of the raw OMWW (9).

These findings prompted the recovery of the metal polymeric organic fraction, which was named polymerin, with the aim of studying its possible recycling in agriculture and use in environmental technology processes (10). The potential employ-

ment of this biomaterial in agriculture as a bioamendment and/or metal biointegrator is motivated by its humic acid-like nature and its richness in macro- and micronutrients such as K and, to a lesser extent, Ca, Mg, Fe, and Zn.

The present paper describes (a) the production of metal-polymerins obtained separately by saturation of polymerin with various micronutrients (Cu, Zn, Mn, and Fe) and other metals of interest (Na and Al) and (b) their characterization by diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and atomic absorption spectrometry (AAS).

Another objective of this paper is to illustrate the production and characterization of the metal Cu, Zn, Mn, Fe, and Al salts of deglycosylated polymerin (Me-SDpolymerins), obtained separately by saturation of a potassium salt of deglycosylated polymerin (K-SDpolymerin) (10).

In view of the possible application of these biomaterials, their effects on tomato (*Lycopersicon esculentum* L.) cuttings were studied compared with the effect of raw OMWW. Finally, the possible exploitation of these materials is discussed in relation to their humic acid-type nature and natural presence of nutrient metals.

MATERIALS AND METHODS

Materials. Samples of OMWW were supplied by a pressure processing plant located in Monteroduni (Isernia, Italy) and were kept refrigerated at $-20\text{ }^{\circ}\text{C}$ in tightly closed PVC vessels until use. Solvents were of HPLC grade and reagents of analytical grade. High-quality

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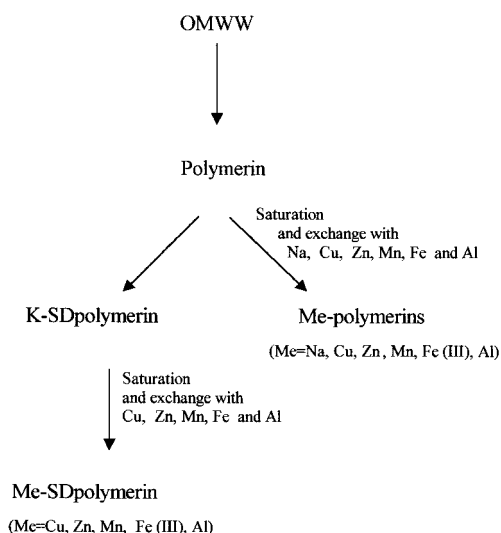


Figure 1. Scheme of preparation of Me-polymerins and Me-SDpolymerins from polymerin and K-SDpolymerin, respectively.

water (hqw), obtained using a Milli-Q system (Millipore), was used throughout the experiments.

Production of Metal-Polymerins (Me-polymerins) from Polymerin and of Metal-Deglycosylated Polymerins (Me-SDpolymerins) from K-SDpolymerin. Polymerin and K-SDpolymerin were first prepared according to the procedure previously described (10). Aliquots of 100 mg of either polymerin or K-SDpolymerin were mixed separately with each saturated solution (10 mL) of NaCl, CuCl₂, ZnCl₂, MnCl₂, FeCl₃, and AlCl₃ and left overnight under magnetic stirring, at room temperature. The mixture was then transferred to an ultrafiltration cell (200 mL), equipped with a magnetic stirrer and a membrane having a cutoff of 1000 Da. The cell was placed under a nitrogen flow and connected to an hqw reservoir (1000 mL). The operating pressure inside the cell was regulated at 3.5 bar.

The ultrafiltration process was performed to eliminate the excess metal cations, which were not adsorbed on the polymerins, and was stopped when no metal was detected by AAS in the permeated fraction. The nonpermeated fraction, which mainly consisted of a brown precipitate, except for the very soluble Na-polymerin, was removed from the cell and lyophilized. A residual brown material was obtained with a yield of 95% for Me-polymerins and 93% for Me-SDpolymerins (Figure 1).

Metal Determination by AAS. Metal cations were determined by a Perkin-Elmer model 3030 B atomic absorption spectrometer equipped with deuterium-arc background correction. Either air-acetylene or nitrous oxide-acetylene flames were used as the atomization source. Measurements were performed using the manufacturer's recommended operating parameters. The spectrometer was controlled by AA Winlab software (Perkin-Elmer, Norwalk, CT). Background correction (AA-BG) was used for lower wavelength elements (Zn, Mn, Fe, and Cu), which could be more susceptible to interferences from molecular absorbance. The average and standard deviation of three absorption measurements were recorded for each sample.

All glassware, plasticware, and storage bottles used were previously immersed for several hours in a solution containing 10% w/v concentrated HCl and 20% w/v concentrated HNO₃ (Carlo Erba, Milan, Italy), to avoid any kind of contamination, and air-dried before use. Stock standard solutions of each metal cation (1 g L⁻¹) were obtained from BDH Reagents (Poole, U.K.).

Aliquots of 100 mg of polymerin and its derivatives Na-polymerin, Cu-polymerin, Zn-polymerin, Mn-polymerin, Fe-polymerin, and Al-polymerin and of K-SDpolymerin and its metal derivatives Cu-SDpolymerin, Zn-SDpolymerin, Mn-SDpolymerin, Fe-SDpolymerin, and Al-SDpolymerin were digested with 8 mL of HNO₃ (65% w/v) and 2 mL of HClO₄ (72% w/v) on a heating sand bath at 80 °C. Once dried, samples were added with 5% HCl solution, then filtered through a Whatman No. 40 paper filter, Ø 12.5 cm, and finally raised to a volume of 50 mL with 5% HCl.

DRIFT Spectroscopy. For DRIFT analysis 0.2 mg of each sample was mixed with 200 mg of KBr (FTIR grade, Aldrich Chemical Co., Milwaukee, WI), ground in an agate mortar, pressed surface smoothed with a microscope glass slide, and finally transferred to a sample holder. The DRIFT spectra were obtained using a Perkin-Elmer 1720 X FT-IR spectrophotometer and a diffuse-reflectance device (Perkin-Elmer). The diffuse-reflectance cell containing the samples was flushed with N₂ gas for 10 min before scanning to remove atmospheric H₂O vapor and CO₂. The resolution of 1 cm⁻¹ was used in recording all spectra.

Phytotoxicity Test of OMWW. The phytotoxicity of OMWW at the original concentration and diluted 1:10 was tested on cv. Marmande tomato plants using the following procedure: 10 young tomato plantlet cuttings were dipped with each cut end in 1 mL of the test solution and stored at 25 °C; necrosis symptoms were evaluated in a range of 5–96 h and rated on a 0–4 necrosis index scale, where 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100% of plant necrotized.

Phytotoxicity Test of Polymerins. The phytotoxicity of polymerin and Me-polymerins (Me = Na, Cu, Zn, Mn, Fe, and Al) was tested on cv. Marmande tomato plants at a concentration of 1 (6 μM) and 5 mg/mL (30 μM), considering an average molecular weight of 150 kDa (10), whereas a concentration of 0.2 (30 μM) or 1 mg/mL (150 μM), considering an average molecular weight of 6.3 kDa (10), was used for K-SDpolymerin and Me-SDpolymerins (Me = Cu, Zn, Mn, Fe, and Al).

The following procedure was used: 10 young plantlet cuttings were dipped with each cut end in 1 mL of the test solution and stored at 25 °C; the phytotoxicity was thus estimated in a range of 5–96 h as the percentage of wilted plantlets.

RESULTS AND DISCUSSION

Metal-Polymerins. Metal-polymerins were prepared from polymerin (10) according to the scheme reported in Figure 1.

Their composition is reported in Table 1, and their DRIFT spectra are shown in Figure 2 and Table 3.

With respect to polymerin in Na-polymerin the concentration of K decreased ~5-fold while the concentration of Na increased and the concentration of Ca and Mg decreased much more than in the other polymerins. The concentration of Cu and Zn collapsed to 0.00, indicating that the two metals were completely removed by Na. The concentration of Fe(III) presented only a small change. Mn and Al were detected only in Mn- and Al-polymerins, but not in polymerin, Na-polymerin, or all the other Me-polymerins.

In Cu-polymerin, K decreased ~10-fold with respect to polymerin, while Cu increased. The concentration of Ca, Mg, and Fe(III) decreased, due to the exchange with Cu, with an extent lower than that observed for K because of the higher stability of Ca, Mg, and Fe(III), chelated with the carboxylate anions and OH, SH, and NH₂ groups occurring in polymerin (10). The concentration of Zn was higher than in polymerin, possibly because of small Zn impurities in the Cu salt utilized for the exchange. The concentration of Na remained surprisingly constant, despite the single charge of this ion. This peculiar behavior, which was also observed in other polymerins, may be related to the small size of Na, which remained entrapped in small sterically hindered sites.

In Zn-polymerin the concentration of K was close to that of Cu-polymerin and the concentration of Zn correspondingly increased, indicating the similar physicochemical behaviors of the Zn and Cu cations with respect to K. Similar to Cu-polymerin, the concentration of Ca, Mg, Fe(III), and Cu, decreased only slightly, compared with that of polymerin, suggesting the same explanation reported above for Cu-polymerin.

In Mn-polymerin, the concentration of K showed a value similar to that of Zn-polymerin and the concentration of Mn

Table 1. Metal Composition of Polymerin and Its Metal Derivatives Obtained by Saturation of Polymerin with Na, Cu, Zn, Mn, Fe, and Al

	K		Na		Ca		Mg		Cu	
	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹
polymerin	8.26 ± 0.66	0.21 ± 0.017	0.82 ± 0.041	0.036 ± 0.0018	0.85 ± 0.094	0.042 ± 0.0046	0.46 ± 0.03	0.038 ± 0.0025	0.020 ± 0.0008	6.3 × 10 ⁻⁴ ± 2.5 × 10 ⁻⁵
Na-polymerin	1.79 ± 0.13	0.046 ± 0.0033	7.42 ± 0.28	0.32 ± 0.012	0.42 ± 0.04	0.021 ± 0.002	0.11 ± 0.006	0.010 ± 0.0005	0.00	0.00
Cu-polymerin	1.07 ± 0.1	0.027 ± 0.0025	0.83 ± 0.044	0.036 ± 0.0019	0.71 ± 0.081	0.035 ± 0.004	0.17 ± 0.01	0.014 ± 0.0008	6.38 ± 0.28	0.20 ± 0.0088
Zn-polymerin	0.82 ± 0.05	0.021 ± 0.0012	0.70 ± 0.04	0.030 ± 0.0018	0.74 ± 0.065	0.037 ± 0.0032	0.30 ± 0.026	0.025 ± 0.0022	0.012 ± 0.0007	0.00037 ± (2.2 × 10 ⁻⁵)
Mn-polymerin	0.73 ± 0.08	0.019 ± 0.002	0.82 ± 0.036	0.036 ± 0.0016	0.74 ± 0.092	0.037 ± 0.0046	0.21 ± 0.015	0.017 ± 0.0012	0.012 ± 0.0005	0.00037 ± (1.5 × 10 ⁻⁵)
Fe-polymerin	0.39 ± 0.03	0.010 ± (8 × 10 ⁻⁴)	0.79 ± 0.028	0.034 ± 0.0012	0.37 ± 0.051	0.033 ± 0.0026	0.26 ± 0.017	0.021 ± 0.0014	0.020 ± 0.0012	0.0063 ± (3.7 × 10 ⁻⁵)
Al-polymerin	0.27 ± 0.015	0.007 ± (4 × 10 ⁻⁴)	0.68 ± 0.03	0.030 ± 0.0013	0.31 ± 0.025	0.016 ± 0.0013	0.064 ± 0.004	0.0054 ± 0.00033	0.010 ± 0.0005	0.0030 ± (1.5 × 10 ⁻⁵)
	Zn		Fe		Al		Mn		Σ	
	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹
polymerin	0.11 ± 0.008	0.0030 ± 0.00022	0.54 ± 0.055	0.019 ± 0.0011	0.00	0.00	0.00	0.00	11.06	0.35
Na-polymerin	0.00	0.00	0.31 ± 0.039	0.017 ± 0.0014	0.00	0.00	0.00	0.00	10.05	0.41
Cu-polymerin	0.11 ± 0.008	0.0035 ± 0.00026	0.37 ± 0.036	0.013 ± 0.0013	0.00	0.00	0.00	0.00	9.63	0.35
Zn-polymerin	6.29 ± 0.41	0.19 ± 0.013	0.37 ± 0.044	0.013 ± 0.0016	0.00	0.00	0.00	0.00	9.23	0.31
Mn-polymerin	0.046 ± 0.005	0.0014 ± 0.00015	0.36 ± 0.028	0.013 ± 0.001	0.00	0.00	5.94 ± 0.4	0.23 ± 0.015	8.86	0.34
Fe-polymerin	0.00	0.00	5.03 ± 0.47	0.27 ± 0.017	0.00	0.00	0.00	0.00	7.16	0.37
Al-polymerin	0.00	0.00	0.09 ± 0.013	0.003 ± 0.00047	2.9 ± 0.2	0.32 ± 0.023	0.00	0.00	4.96	0.38

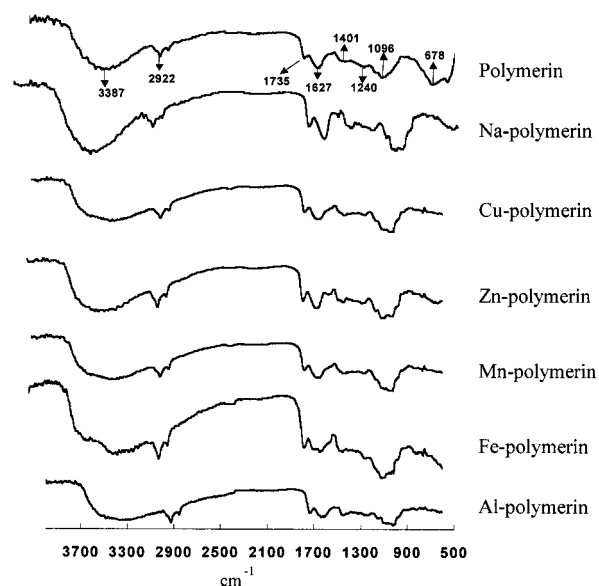


Figure 2. DRIFT spectra of polymerin and Me-polymerins (Me = Na, Cu, Zn, Mn, Fe, or Al).

correspondingly increased. The concentrations of Ca, Mg, Fe(III), Zn, Cu, and Na were similar to those in Zn-polymerin and Cu-polymerin.

In Fe-polymerin, the concentration of K showed a more marked decrease and the concentration of Fe correspondingly increased more than in the other metal-polymerins considered above. This behavior is consistent with the trivalent charge of Fe(III). The concentrations of Na, Ca, and Mg showed the same trend observed in the above-considered metal-polymerins. The concentration of Cu remained constant, whereas Zn was completely replaced by Fe(III).

Finally, in Al-polymerin the concentration of K showed the greatest decrease in comparison with that of the above-considered metal-polymerins and the concentration of Al increased much more than the other metals, such as for Na in Na-polymerin. The concentration of Na remained constant, and that of all the other metals Ca, Mg, Cu, Zn, and Fe showed a marked decrease compared with those observed in the other metal-polymerins. The peculiar behavior of Al can be attributed to two factors: the high charge and the small size of this ion, which allow an easy site occupation and the possibility to form chelates.

Generally, the metal content in metal-polymerins, in terms of weight percentage, decreased from 11.06 in polymerin to 4.96 in Al-polymerin, whereas the sum in milliequivalents percentage showed very similar values except for Na-polymerin, as shown in the last two columns of Table 1.

In brief, the production of Na-, Cu-, Zn-, Mn-, Fe-, and Al-polymerins by saturation of polymerin with the corresponding metals occurred mainly through replacement of K, which was not only the most highly concentrated but also the most removable metal, due to its single charge and large size. Correspondingly, there was a large increase of the metals added and substitution of those native in polymerin, that is, Cu, Ca, Mg, Zn, and Fe(III). The total replacement of Cu occurred only by Na in Na-polymerin, whereas that of Zn by Na, Fe, and Al occurred in Na-polymerin, Fe-polymerin, and Al-polymerin, respectively.

All of the considered Me-polymerins were essentially solid precipitates except for the very soluble polymerin and Na-polymerin, and their DRIFT spectra showed that their chemical

Table 2. Metal Composition of K-SDpolymerin and Its Metal Derivatives Obtained by Saturation of K-SDpolymerin with Cu, Zn, Mn, Fe(III), and Al

	K		Na		Ca		Mg		Cu	
	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹
K-SDpolymerin	2.88 ± 0.25	0.075 ± 0.0064	1.21 ± 0.05	0.053 ± 0.0022	1.69 ± 0.17	0.085 ± 0.0085	0.10 ± 0.006	0.0083 ± 0.0005	0.00	0.00
Cu-SDpolymerin	0.29 ± 0.026	0.0074 ± 0.00067	1.28 ± 0.05	0.057 ± 0.0022	1.48 ± 0.14	0.074 ± 0.007	0.080 ± 0.005	0.0066 ± 0.00042	2.7 ± 0.12	0.087 ± 0.0035
Zn-SDpolymerin	0.25 ± 0.020	0.0069 ± 0.0005	1.22 ± 0.084	0.053 ± 0.0036	1.48 ± 0.2	0.074 ± 0.01	0.090 ± 0.006	0.0067 ± 0.0005	0.00	0.00
Mn-SDpolymerin	0.25 ± 0.018	0.0069 ± 0.00046	1.28 ± 0.076	0.057 ± 0.0033	1.65 ± 0.12	0.083 ± 0.006	0.060 ± 0.004	0.0049 ± 0.0032	0.00	0.00
Fe-SDpolymerin	0.23 ± 0.023	0.0059 ± 0.0006	1.27 ± 0.06	0.055 ± 0.0026	0.6 ± 0.05	0.030 ± 0.0025	0.050 ± 0.003	0.0041 ± 0.00025	0.00	0.00
Al-SDpolymerin	0.27 ± 0.015	0.0069 ± 0.00039	1.25 ± 0.054	0.054 ± 0.0023	0.07 ± 0.0045	0.0035 ± 0.00023	0.00	0.00	0.00	0.00
										Σ
	Zn		Fe		Al		Mn			
	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹	mg 100 mg ⁻¹	mequiv 100 mg ⁻¹
K-SDpolymerin	0.08 ± 0.005	0.0024 ± 0.0015	0.15 ± 0.012	0.0054 ± 0.0004	0.00	0.00	0.00	0.00	6.11	0.23
Cu-SDpolymerin	0.108 ± 0.008	0.0033 ± 0.0024	0.19 ± 0.02	0.0068 ± 0.00072	0.00	0.00	0.00	0.00	6.21	0.25
Zn-SDpolymerin	2.80 ± 0.18	0.085 ± 0.0055	0.15 ± 0.016	0.0054 ± 0.00057	0.00	0.00	0.00	0.00	5.99	0.23
Mn-SDpolymerin	0.11 ± 0.007	0.0034 ± 0.0021	0.14 ± 0.01	0.0050 ± 0.00036	0.00	0.00	2.20 ± 0.18	0.09 ± 0.0065	5.69	0.25
Fe-SDpolymerin	0.00	0.00	2.70 ± 0.22	0.15 ± 0.0079	0.00	0.00	0.00	0.00	4.85	0.25
Al-SDpolymerin	0.00	0.00	0.07 ± 0.0060	0.0025 ± 0.00022	1.7 ± 0.15	0.19 ± 0.017	0.00	0.00	3.36	0.26

nature was similar to that of polymerin (**Figure 2** and **Table 3**), the DRIFTS characterization of which was previously reported (10).

In particular, the main features of DRIFT spectra are as follows: (a) strong absorptions observed in the range between 3273 and 3413 cm⁻¹ due to alcoholic and phenolic OH and peptide NH; (b) medium absorption bands in the 2922 and 2932 cm⁻¹ region attributed to saturated CH stretching; (c) medium-intensity bands in the range between 1720 and 1741 cm⁻¹ attributed to the stretching of a C=O methyl ester bond and C—O—C linkage stretching weak bands in the range between 1240 and 1257 cm⁻¹; the latter overlap those due to the stretching of the diphenyl ether bond occurring in the melanin system of polymerin (10). Other strong, characteristic bands are observed in the range between 1601 and 1627 cm⁻¹, due to the stretching of C=O of peptide nature, C=C of aromatic nucleus, and symmetric stretching of COO⁻. Other characteristic weak bands are observed in the range between 1398 and 1456 cm⁻¹, which can be attributed to the symmetric stretching of COO⁻. The very strong, important bands occurring in the range between 1024 and 1096 cm⁻¹ are attributed to the stretching of C—OH groups of the sugars, which are contained in high amount in polymerin (10).

A significant DRIFTS difference of the Me-polymerins compared with Na-polymerin and polymerin is the greater bandwidth in the region between 3000 and 4000 cm⁻¹ (**Figure 2**), which is particularly evident in Mn-, Fe-, and Al-polymerin, due to macroaggregation of Me-polymerins, except for the soluble Na-polymerin.

In a previous paper on the characterization of polymerin (10), a high chemical similarity to humic acids was shown. Hence, the latter can also be considered humic acid-like substances.

Metal—SDpolymerins. Metal-SDpolymerins were prepared from K-SDpolymerin (10) following the scheme reported in **Figure 1**.

Their metal composition is reported in **Table 2**, and their chemical nature was identified by DRIFT spectroscopy (**Figure 3** and **Table 3**).

With respect to K-SDpolymerin, in Cu-SDpolymerin the concentration of K decreased almost 10-fold, while the concentration of Cu increased. The concentration of Ca and Mg decreased, to a much lesser extent, than that observed for K because of their chelation with the carboxylate anions and other functional groups such as OH, SH, and NH₂ occurring in K-SDpolymerin (10). The concentrations of Zn and Fe(III) were higher due to small impurities of Zn and Fe(III) in the Cu salt utilized for the exchange. The concentration of Na remained almost constant as was observed in Me-polymerins and will be observed also in the other Me-SDpolymerins.

The metals Mn, Al, and Cu were detected only in Mn-, Al-, and Cu-polymerins respectively, as they were originally absent in K-SD-polymerin and all the other Me-SDpolymerins.

Also in Zn-SDpolymerin the concentration of K showed an almost 10-fold decrease, compared with that of K-SDpolymerin, and the concentration of Zn correspondingly increased, indicating that the latter replaced not only K but also other native metals, that is, Ca and Mg.

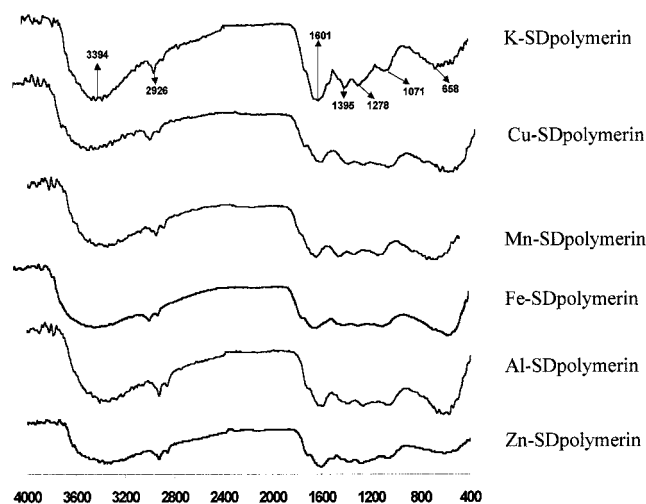
In Mn-SDpolymerin the concentration of K also decreased and the concentration of Mn increased with replacement of also Mg and partly Ca and Fe(III). The concentration of Zn increased slightly due to the presence of small impurities in the salt.

In Fe-SDpolymerin the concentration of K showed a marked decrease in comparison with that of the other Me-SDpolymerins, and the concentration of Fe markedly increased, also as a

Table 3. DRIFT Spectral Data of Polymerin and K-SDpolymerin and Their Corresponding Metal Derivatives

	DRIFT ν^a (cm ⁻¹)							
	stretching							bending
	OH, NH	saturated CH	C=O ester	C=O amidic, COO-symmetric, and C=C aromatic nucleus	COO-asymmetric	C-O-C ester and ether and C-OH phenol	C-OH alcohol (sugar and amino acid) and C-NH ₂ amino acid	melanin nucleus
polymerin	3387 (s)	2922 (m)	1735 (m)	1627 (s)	1401 (w)	1240 (w)	1096 (s)	678 (s)
Na-polymerin	3401 (s)	2929 (m)	1736 (m)	1617 (s)	1416 (w)	1256 (w)	1075 (s)	
Cu-polymerin	3339 (s)	2927 (m)	1738 (m)	1627 (s)	1398 (w)	1241 (w)	1083 (s)	
Zn-polymerin	3413 (s)	2925 (m)	1741 (m)	1627 (s)	1413 (w)	1257 (w)	1075 (s)	
Mn-polymerin	3274 (s)	2932 (m)	1720 (m)	1601 (s)	1417 (w)	1262 (w)	1074 (s)	
Fe-polymerin	3352 (s)	2925 (m)	1736 (m)	1606 (s)	1418 (w)	1226 (w)	1097 (s)	
Al-polymerin	3365 (s)	2928 (m)	1736 (m)	1635 (s)	1456 (w)	1243 (w)	1024 (s)	
K-SDpolymerin	3394 (s)	2926 (m)	1719 (w)	1601 (s)	1395 (m)	1279 (m)	1071 (w)	658 (w)
Cu-SDpolymerin	3397 (s)	2927 (m)	1725 (w)	1604 (s)	1386 (m)	1262 (m)	1076 (w)	670 (s)
Zn-SDpolymerin	3345 (s)	2926 (m)	1720 (w)	1604 (s)	1383 (m)	1283 (m)	1058 (w)	604 (m)
Mn-SDpolymerin	3381 (s)	2922 (m)	1718 (w)	1589 (s)	1407 (m)	1281 (m)	1077 (w)	670 (s)
Fe-SDpolymerin	3357 (s)	2924 (m)	1711 (w)	1605 (s)	1384 (m)	1270 (m)	1084 (w)	570 (s)
Al-SDpolymerin	3381 (s)	2924 (m)	1728 (w)	1604 (s)	1406 (m)	1265 (m)	1071 (w)	670 (s)

^a s = strong, m = medium, w = weak.

**Figure 3.** DRIFT spectra of K-SDpolymerin and Me-SDpolymerins (Me = Cu, Zn, Mn, Fe, or Al).

consequence of the replacement of Ca, Mg, and Zn, the concentrations of which decreased.

Finally, in Al-SDpolymerin the concentration of K decreased and that of Al increased markedly as a consequence of the more extended replacement not only of K but also of Ca, Mg, Zn, and, surprisingly, Fe(III).

Interestingly, Al and Na confirm in the Me-SDpolymerins the same peculiar behavior observed in Me-polymerins with respect to the other metals.

Generally, the content of metal in the Me-SDpolymerins, expressed in terms of weight percentage, decreased from 6.11 in K-SDpolymerin to 3.36 in Al-SDpolymerin, whereas the sum in terms of milliequivalents percentage shows very similar values, as shown in the last two columns of **Table 2**. This means that the number of negative sites which linked the metals in the considered Me-SDpolymerins remained substantially constant with respect to K-SDpolymerins after the metal exchange.

In brief, the production of Me-SDpolymerins occurred mainly by replacement of K and, to lesser extent, Ca, Mg, Zn, and Fe, naturally occurring in the Me-SDpolymerins. The total replacement of Zn and Mg was performed by Al in the corresponding Al-SDpolymerin, whereas the total replacement of Zn was performed only by Fe in Fe-SDpolymerin. In Me-SDpolymerins

Na remained almost constant; in addition, Fe and, in particular, Al strongly replaced the other metals, as has been also observed in Me-polymerins, due to the small size of the first metal ion, the high charge of the second, and both the small size and high charge of the third metal ion, respectively.

Similar to Me-polymerins, Me-SDpolymerins were substantially obtained as solid precipitates, except for K-SDpolymerin, and their chemical nature also appeared similar from DRIFT spectra analysis (**Figure 3** and **Table 3**).

The DRIFT spectra of K-DS- and Me-SDpolymerins (**Figure 3** and **Table 3**) showed some significant differences from those of polymerin and Me-polymerin (**Figure 2** and **Table 3**). In particular, a strong decrease of the bands in the 1071–1084 cm⁻¹ region is observed. This result can be attributed to the stretching of C–OH groups of residual sugars that remained in K-SDpolymerins, and consequently in the Me-SDpolymerins, after the hydrolysis of polymerin for the preparation of K-SDpolymerin (10). A further significant difference was the appearance of Me-SDpolymerins of absorptions between 570 and 670 cm⁻¹, due to the bending of aromatic rings of the melanin system, which was previously identified in K-SDpolymerin (10).

The only significant DRIFT difference of the Me-SDpolymerin aggregates compared with K-SDpolymerin is the enlargement of the band in the 3000–4000 cm⁻¹ region (**Figure 3**), which can be attributed to their macroaggregation as a consequence of the chelating effect of the metals.

Effects on Tomato Plants. As previously reported (10) the DRIFT spectra of K-SDpolymerin and, consequently, those of the Me-SDpolymerins are very similar to the DRIFT spectra of Na- and Cu-humate reported previously by Piccolo and Conte (11). The humic acid-like nature of polymerin (10), Me-polymerins, K-SDpolymerin, and Me-SDpolymerins suggests these materials can be exploited as potential bioamendments and biointegrators in agriculture. In this perspective their effects on tomato plants with cut stems were tested at laboratory scale, and the data obtained are summarized in **Figure 4**.

In particular, the completely soluble polymerin at concentrations of both 30 and 6 μ M caused the wilting of all plants (100%) after 5 h (**Figure 4a**). However, at the concentration of 6 μ M, the plants recovered progressively until total recovery after 96 h. In contrast, plants treated with a concentration of 30 μ M remained wilted until 48 h; but after 96 h,

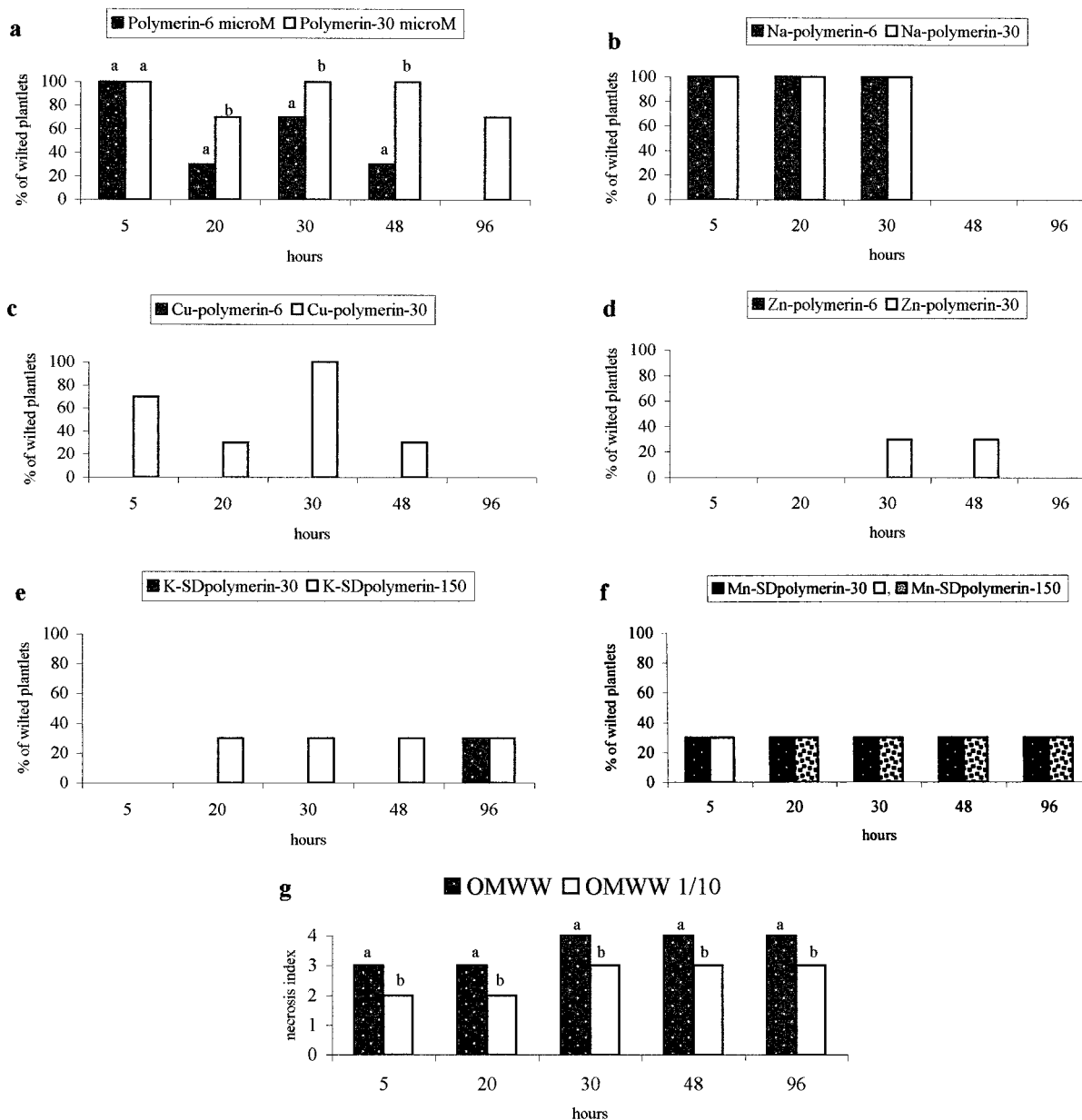


Figure 4. Effects on tomato cuttings in a 5–96 h period at 25 °C of polymerin (a), Na-polymerin (b), Cu-polymerin (c), Zn-polymerin (d), K-SDpolymerin (e), Mn-SDpolymerin (f), and OMWW (g). The dotted rectangles in panel f indicate that the leaves have been also affected by necrosis in a 20–96 h period. The necrosis index of OMWW (g) was measured on all 10 plantlet cuttings. Different letters distinguish different means for $p < 0.05$.

three recovered (30%) and the remaining seven wilted (70%). These data suggested that polymerin obstructed mechanically the water flow through the xylem mainly at the level of the leaf petiole, because the wilting essentially affected the leaves and this obstruction may have been caused by the high molecular sizes of polymerin. However, the plants were capable of metabolizing the polysaccharide and/or the protein components of the polymerin, causing the disaggregation of the polymeric mixture and, consequently, removing the obstruction more easily at a lower concentration (6 μM), but with more difficulty at a higher concentration (30 μM).

The completely soluble Na-polymerin caused the wilting of all the plants after 5 h at both concentrations, but after 48 h they all recovered (Figure 4b). Possibly, the small size of Na caused a marked volume contraction of Na-polymerin with respect to polymerin, with a consequently easier removal of the xylem pathway obstruction at both concentrations.

Cu- and Zn-polymerin caused symptoms of wilting only at 30 μM and until 48 h, whereas all the plants recovered after 96

h (Figure 4c,d). The temporary wilting of the plants could depend on the residual solubility of these Me-polymerins, which were absorbed from the plants at very low concentrations and were consequently more easily disaggregated. Finally, Mn-, Fe-, and Al-polymerin were shown to be inactive, possibly due to their total insolubility and, consequently, they were not able to be absorbed from the plant.

The very soluble K-SDpolymerin caused the wilting of 30% of the plants after only 20 h at a concentration of 150 μM (Figure 4e). This result suggested the obstruction of the leaf petiole xylem pathways, which occurred at a concentration 5 times higher than the concentration (30 μM) that caused the wilting of 30% of the plants after 96 h. Possibly, at this latter concentration the accumulation of the K-SDpolymerin occurred at leaf petiole after 96 h.

None of the other Me-SDpolymerins showed any activity at either concentration except for Mn-SDpolymerin (Figure 4f), which, at a concentration of 30 μM , caused symptoms

of wilting in 30% of tomato plants starting from 20 h after the beginning of the experiment, whereas at a concentration of 150 μ M showed, in addition to wilting, leaf necrosis, possibly caused by interference of Mn with the plant cell redox system.

In general, K-SDpolymerin showed weaker wilting effects at 150 μ M than polymerin at 30 μ M (Figure 4a,e). This behavior may be explained by the 25 times smaller (6.3 kDa) molecular size with respect to polymerin (150 kDa). Hence, the latter obstructs the xylem pathways of leaf petioles more efficiently.

In conclusion, the soluble polymerin and its derivative K-SDpolymerin and the insoluble Mn-SDpolymerin were only significantly toxic among the considered polymerins. However, the wilting effect of the soluble polymerins can be attributed to a mechanical obstruction of xylem pathways of tomato, whereas that of Mn-SDpolymerin may be related to the interference with the plant metabolism.

Possible Exploitation of Polymerins. On the basis of these results, the strong phytotoxicity of OMWW on tomato observed at both original and diluted 1:10 concentrations (Figure 4g) may be ascribed mainly to a synergistic effect of polyphenols, which act on plant metabolism (12, 13), and, to a much more lesser extent, to the polymeric fraction (polymerin), which acts through a mechanical obstruction apparent only at relatively high concentrations.

In a previous study (9) the COD and BOD of the OMWW polymeric fraction appeared to be strongly reduced with respect to the whole waste waters. In addition, the results of the present study show that polymerins are only in part phytotoxic. These findings, their humic acid characteristics, and the presence of macronutrient (K, Mg, and Ca) and micronutrient (Cu, Zn, Fe, and Mn) metals suggest their promising exploitation as bio-amendments and/or metal-biointegrators. However, the possible employment of Me-polymerins with respect to Me-SDpolymerins is likely of greater interest, as their simpler and less expensive production process appears to be much more convenient (Figure 1).

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