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Microbiological Features and Bioactivity of a Fermented Manure Product (Preparation 500) Used in Biodynamic Agriculture

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The fermented manure derivative known as Preparation 500 is traditionally used as a field spray in biodynamic agriculture for maintaining and increasing soil fertility. This work aimed at characterizing the product from a microbiological standpoint and at assaying its bioactive properties. The approach involved molecular taxonomical characterization of the culturable microbial community; ARISA fingerprints of the total bacteria and fungal communities; chemical elemental macronutrient analysis via a combustion analyzer; activity assays for six key enzymes; bioassays for bacterial quorum sensing and chitolipooligosaccharide production; and plant hormone-like activity. The material was found to harbor a bacterial community of 2.38×10^8 CFU/g dw dominated by Gram-positives with minor instances of Actinobacteria and Gammaproteobacteria. ARISA showed a coherence of bacterial assemblages in different preparation lots of the same year in spite of geographic origin. Enzymatic activities showed elevated values of β -glucosidase, alkaline phosphatase, chitinase, and esterase. The preparation had no quorum sensing-detectable signal, and no rhizobial *nod* gene-inducing properties, but displayed a strong auxin-like effect on plants. Enzymatic analyses indicated a bioactive potential in the fertility and nutrient cycling contexts. The IAA activity and microbial degradation products qualify for a possible activity as soil biostimulants. Quantitative details and possible modes of action are discussed.

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Key words: Preparation 500, hornmanure, biodynamic agriculture, biostimulants, auxin-like activity

Biodynamic (BD) agriculture is a form of organic farming that, in addition to ordinary organic farming practices, as soil building, composting, and crop rotations, uses two characteristic preparations as field sprays (referred to as 500 and 501) or additives for manure composting [15, 30]. Such products are included in the list of materials and techniques permitted in organic farming by an EC Regulation (834/2007).

The BD Preparation 500 is a fermented cow manure derivative used to improve the soil fertility and foster the formation of a strong root system, whose chemical features have been previously reported by our group [26]. Results of long-term field trials, such as those published in a Science paper by Mader *et al.* [17] proved that biodynamic practices, which primarily make use of Preparation 500, improve the overall soil quality; in particular, parameters such as organic matter, and microbial biomass and diversity were significantly higher in the biodynamic farming system in comparison with ordinary organic farming [16, 17]. As the difference between organic and BD farming lies essentially in the use of defined sprayed compounds such as 500 and 501, the basis for its effects can be sought in the features of these preparations.

Short-term field trials have shown that the use of both preparations is correlated with a higher yield of lentils per

unit plant biomass, lower grain carbon and crude protein content, greater NO_3^- content in soft white spring wheat, and greater NH_4^+ content in soil [6]. Their application was also found to be associated with higher levels of mineral carbon, which is considered an indicator of microbially available C [8], and differences in soil microbial fatty acid profiles in the first of two years of study [7].

We recently investigated the molecular composition of Preparation 500 by both solid-state nuclear magnetic resonance (NMR) spectroscopy and thermochemolysis, and found the product enriched in bio labile components compared with the starting manure, thus becoming potentially conducive to biostimulation of microorganisms and plants [26]. In comparison with initial manure, earlier reports suggested that Preparation 500 had lower values of pH, CO_2 respiration, and C:N ratio, higher nitrate content, and reduced losses of organic matter [3].

The aim of the present work was to perform a microbiological characterization of Preparation 500 and to seize some of its biological activities. The results, along with those of the recently assessed molecular properties, are envisaged to contribute to a clearer understanding of its ways of action in agricultural systems.

MATERIALS AND METHODS

Preparation 500

Different commercial samples of BD Preparation 500 from three leading Italian producers were studied. Samples were produced by “Società Agricola Biodinamica” (Labico, Rome), “La Farnia” (Rolo, Reggio Emilia), and “Biodynamic Agriculture Section” (Bolzano).

Briefly, the routine production comprises the following procedure: in early autumn, hollow cow horns are filled with cow manure from organic farming and buried underneath a biodynamically managed soil. The organic material is left to decompose during winter and cow horns are recovered in the following spring after almost 150–180 days of maturation. The material recovered from cow horns is moderately moist, dark, odorless, and humus-like.

The analyses included three preparations from 2010 (Rome, Bolzano, and Reggio Emilia), one preparation from 2011 (Rome) and its manure of origin (produced in 2010).

Elemental Content Analysis

The percent proportions of carbon, nitrogen, and sulfur of Preparation 500 or soils were determined on material dried for 2 days at 70°C using a CNS Macrovario combustion analyzer (Elementar Analysensysteme GmbH, Germany).

Culturable Microbial Population Analyses

Fresh aliquots of 3 g were resuspended by vigorous shaking for 1 h in 27 ml of physiological solution at room temperature and serial dilutions were plated on plate count agar (PCA, Difco, USA). Plates were incubated in aerobic conditions or within anaerobiosis jars. Colonies were sorted by morphology and confirmed by ARDRA using enzymes *HhaI* and *CfoI*.

DNA Extraction

Cells were lysed by resuspending a loopful of a plate-grown isolated colony in 50 μl of lysis buffer (0.25% sodium dodecyl sulphate, 0.05 M NaOH) in a 1.5 ml polypropylene tube, followed by stirring for 60 s on a vortex and heating at 95°C for 15 min. The lysate was centrifuged for 15 min and 10 μl of the supernatant was mixed with 90 μl of sterile water. Lysates were stored at 4°C prior to PCR.

PCR Amplification of the 16S rRNA Gene and ARDRA

One microliter of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad I-Cycler using the two 16S rRNA gene-targeted universal bacterial primers 63F (5'CAGGCCTAACACATGCAAGTC) [18] and 1389R (5'ACGGGC GGTGTGTACAAG) [22] at 1 μM each in a 25 μl reaction volume, using the following program: initial denaturation at 95°C for 2 min; 35 cycles at 95°C for 30 s, 55°C for 1 min, 72°C for 4 min, and a final extension at 72°C for 10 min. The PCR mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 1 μM of each primer, and 2.5 U Taq DNA Polymerase, recombinant (Invitrogen Life Technologies, Italy). Amplicons were digested overnight at 37°C upon mixing 5 μl from the 25 μl reaction volume with 1 μl of *CfoI* enzyme (Pharmacia, Sweden) and 2 μl of 10 \times reaction buffer. Digested DNA was loaded on a 1.5% agarose gel, run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera. Upon ARDRA, the isolates were sorted and selected for sequencing.

DNA Sequencing and Bacterial Molecular Taxonomy Analysis

One microliter of the amplicon resulting from the above-described PCR amplification was mixed with 1 μl containing 6.4 picomoles of the above-described forward primer 63F in a 0.2 ml polypropylene tube and then dried by incubating the tube open for 15 min at 65°C in an I-Cycler thermal cycler. The template and primer mix was directly used for dideoxy cycle DNA sequencing with fluorescent terminators (Big Dye; Perkin-Elmer/Applied Biosystems, USA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Australia). BLAST analysis against nucleotide databases was performed *via* the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

Estimation of Overall Bacterial and Fungal Species Richness by ARISA

Genomic DNA extraction from Preparation 500 and soils used for comparison was performed upon drying the material and starting from 250–550 mg amounts using a Genomic DNA from Soil kit from Macherey Nagel (Macherey-Nagel Inc., USA) as recommended by the manufacturer. The protocol involves a mechanical lysis with beads and a lysis based on SDS. The lysate was purified by passage through a Nucleospin Inhibitor Removal Column, and eluted in 100 μl . Quality was assessed spectrophotometrically upon absorbance ratios 260/280. The PCR amplifiability was verified using three primer pairs on conserved bacterial and fungal targets (16S rRNA, 18S rRNA, and ITS).

Samples were amplified using two primer pairs (forward 6-FAM, labeled blue; reverse VIC, labeled green) and run along with a marker (LIZ1200) on a capillary sequencer AB3730. The first pair amplifies the region between genes of the bacterial 16S rRNA and

23S rRNA subunits, and the second allows to amplify between the fungal 18S rRNA and 28S rRNA. Data analyses using Gene Mapper ver. 4.0. on the ARISA data were performed by converting peak heights into values normalized upon the percent fluorescence of each run, and running community comparisons by cluster analyses and non-metric multidimensional scaling (NMDS) using the PAST software ver. 2.12 (<http://folk.uio.no/ohammer/past>).

Enzymatic Assays

The activities of the enzymes arylsulfatase, β -glucosidase, acetate-esterase, leucine-aminopeptidase, alkaline phosphatase, and chitinase, present in Preparation 500, soil, or other material, were determined upon applying an extraction/desorption procedure [14] and using fluorescent analogs of each enzyme's substrate on microplates. Extracts were obtained as follows: 0.5 g of material was put in a 2 ml eppendorf tube together with 1 ml of a 50 mM THAM-HCl buffer containing 4% bovine serum albumin and 1% Triton X-100 and glass beads. Tubes were subjected to bead-beating at 30 strokes/s for 3 min, and then centrifuged at $20,000 \times g$ for 5 min. Supernatants containing desorbed enzymes were dispensed in 96-well microplates with the appropriate buffer to determine enzymatic activities using fluorescent 4-methyl-umbelliferyl-based substrates.

Analysis of the Presence of Quorum Sensing Signals

The presence of *N*-acyl homoserine lactone molecules able to induce the quorum sensing circuitry of cell-to-cell communication in Gram-negative bacteria was assessed on serial dilutions of a resuspension of 1 g of Preparation 500 in 10 ml of water, using the *Agrobacterium tumefaciens* reporter strain NTL4 as previously described [25].

Analysis of *nod* Gene-Inducing Compounds

The presence of compounds of possible plant origin or derived from microbial processing, able to induce the rhizobial nodulation genes, was assessed by the β -galactosidase assay using a *nodC-lacZ* gene fusion in a *nodD*-carrying derivative of *Rhizobium leguminosarum* bv. *viciae* as described previously [23].

Bioassays to Test the Biological Activity of Preparation 500 on Plants

The biological activity of Preparation 500 (a resuspension of 200 mg in 60 ml of water, equal to the dilution proportions used for its field applications) was assessed by checking the growth reduction of watercress (*Lepidium sativum* L.) roots (auxin activity) and the increase in the length of lettuce (*Lactuca sativa* L.) shoots (gibberellic acid activity). The Audus method [1] with its further modifications [21, 24] was used.

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After rinsing 5 times with sterile distilled water, 10 seeds were aseptically placed on filter paper contained in a Petri dish. For watercress, the filter paper was wetted with 1.2 ml of 1 mM CaSO_4 (control); or 1.2 ml of 0.1, 1, 10, and 20 mg/l indoleacetic acid (IAA) (Sigma, USA) to obtain the calibration curve; or 1.2 ml of a serial dilution of the products into 1 mM CaSO_4 . For lettuce, the experimental design was the same as for watercress, except that the sterile filter paper was wetted with 1.4 ml of the above solutions and the calibration curve was a progression of 0, 0.01, 0.1, and 10 mg/l gibberellic acid (GA) (Sigma, USA).

Table 1. C, N, S percent content and C/N ratio of different batches of Preparation 500.

Sample	% N	% C	% S	C/N
Starting manure (Rome)	2.57	32.02	0.46	12.46
Preparation 500 (Rome)	2.74	24.80	0.58	9.06
Preparation 500 (Bolzano)	2.39	27.06	0.52	11.33
Preparation 500 (Reggio Emilia)	2.21	26.30	0.56	11.89

The seeds were germinated in the dark at 25°C. After 48 h for watercress and 72 h for lettuce, the seedlings were removed and the root or shoot lengths were measured.

RESULTS

Elemental Content Analysis

Table 1 shows the percent content of the three macroelements carbon, nitrogen, and sulfur and the C/N ratio. The values of the three Preparation 500 samples were rather constant and individuated a C/N ratio around the level of 10. The starting manure, before being packed and buried, showed a higher carbon content. This is indicative of a fermentative process leading to a C volatilization without nitrogen loss, suggesting an assimilation of the mineralized N by the microbial biomass.

Culturable Microbial Population Analyses

The PCA culturable aerobic fraction of the Preparation 500 population yielded a value of 2.38×10^8 CFU/g dw ($\pm 1.2 \times 10^7$ SD). Incubation in anaerobiosis allowed to count 7.85×10^7 CFU/g dw ($\pm 4.3 \times 10^6$ SD). Fungal colonies amounted to 1.1×10^6 /g dw ($\pm 6.1 \times 10^5$ SD). The ARDRA sorting allowed to select members of each morphotype and to sequence their 16S RNA, which gave rise to the composition shown in Table 2. A dominance by Gram-positives with minor occurrences of Actinobacteria and Gammaproteobacteria characterized the counts. No significant differences among the different batches and sources were observed. Such community structure is coherent with active transformative potential in conditions of low redox values conducive to slow proteolysis and fermentation of organic matter. The picture is in line with the underground incubation conditions at which the maturation of Preparation 500 is carried out.

Estimation of Overall Bacterial and Fungal Species Richness by ARISA

ARISA is a method suitable for the analysis of complex microbial communities. It is based on fluorescent primers and targets the amplification of the variable length region of the internally transcribed spacer (ITS) starting from the genomic DNA of the microbial communities. Products are separated through capillary electrophoresis, and a chromatogram is obtained in which each peak corresponds

Table 2. Identities and proportions of the bacterial members of the culturable community from Preparation 500.

Species	Taxonomical group	Percent in CFU
<i>Bacillus megaterium</i>	Firmicutes	46.83
<i>Bacillus safensis</i>	Firmicutes	44.99
<i>Rhodococcus coprophilus</i>	Actinobacteria	3.65
<i>Pseudoxanthomonas dajeonensis</i>	Gammaproteobacteria	1.42
<i>Microbacterium</i> sp.	Actinobacteria	1.06
<i>Aeromonas rivuli</i>	Gammaproteobacteria	0.74
<i>Bacillus pumilus</i>	Firmicutes	0.46
<i>Nocardia globerula</i>	Actinobacteria	0.39
<i>Agromyces fucosus</i>	Actinobacteria	0.32
<i>Sphingopyxis macrogoltabida</i>	Alphaproteobacteria	0.11
<i>Pseudomonas fulva</i>	Gammaproteobacteria	0.04

to a distinct operational taxonomical unit. The system is highly sensitive and has a resolution level of differences of as little as a single nucleotide.

On average, 1.2 µg of total DNA was obtained, with a mean A260/280 ratio of 1.6, testifying a fair sample purity. The ARISA for Preparation 500 showed a minimum of 58 and a maximum of 65 peaks for bacterial species and values ranging from 32 to 60 for fungal species (Fig. 1). The bacteria to fungi ratio for Preparation 500 was on average 1.6. The prevalence of bacteria over fungi is in line with data stemming from its chemical characterization [26], in that its main changes during maturation showed an increase in lignin compounds, whose breakdown would have been mostly due to fungal activity.

Upon converting the ARISA peak heights into normalized values (percent of the total sample fluorescence), a matrix was obtained suitable for community comparisons. The phenons generated by a neighbor-joining tree (Fig. 2) showed a clustering of the three preparations from 2010, which are set apart from the product of 2011, which in turn is in the same phenon of the 2010 manure, which was its own starting material.

The NMDS analysis (Fig. 3) also showed a clear separation by year and a coherence of the three preparations of 2010

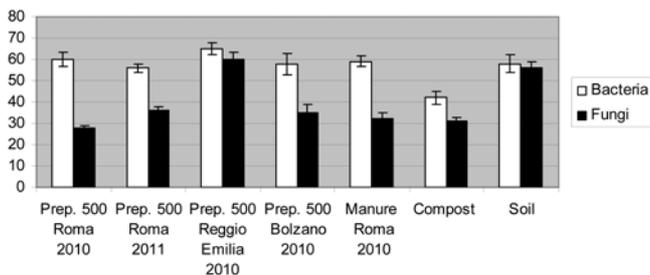


Fig. 1. ARISA number of peaks comparison between Preparation 500 and other substrates or soils.

notwithstanding their geographic distances. The outgroups, represented by an unrelated compost preparation and of a soil community, were well separated along the two coordinate axes. The same analyses on the fungal assemblages gave a less-defined grouping (data not shown). Bacteria seem to be the community that mostly exerts characteristic differences to the material, whereas fungi appear less specific.

Enzymatic Assays

The six activities examined (Fig. 4) were chosen as representative indicators of the four major nutrient cycles (carbon, nitrogen, phosphorus, and sulfur), of the catabolic potential towards both fungal/arthropods polymers (chitinase), and of global enzymatic activity (esterase). Esterase activity can be carried out by a variety of hydrolytic enzymes,

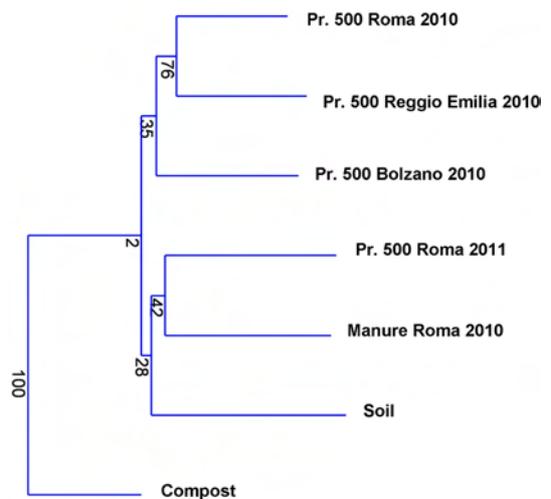


Fig. 2. Cluster analysis of normalized ARISA data on bacterial communities. Neighbor-Joining tree with the Bray–Curtis distance method. Soil and compost were used as outgroups.

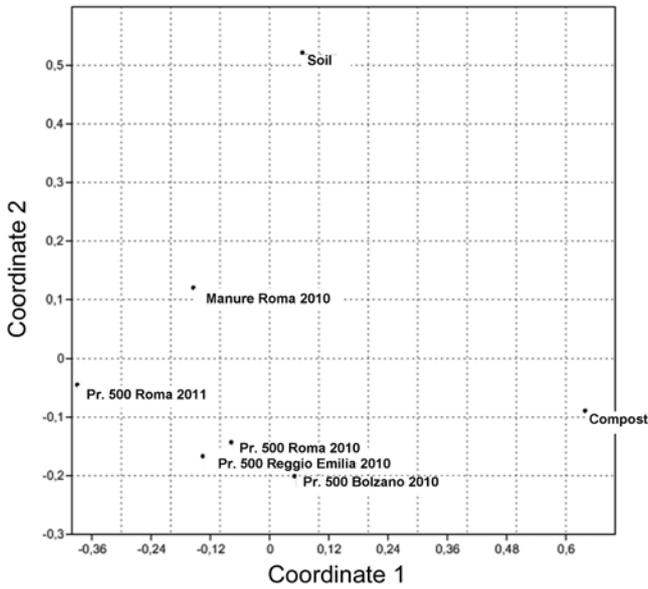


Fig. 3. Non-metric multidimensional scaling (NMDS) analysis on normalized ARISA data on bacterial communities. Euclidean method, scatter diagram.

including aspecific esterases, proteases, and lipases [20]. Preparation 500 displays high specific levels of activity, particularly in the degradative attitudes such as β -glucosidase, including those for complex polymers (chitinase). A high alkaline phosphatase activity indicates its potential to hydrolyze organic P esters. Preparation 500 is relatively low in leucine aminopeptidase activity, which is an enzyme involved in nitrogen cycling. The activity of this enzyme, in contrast, is highest in silkworm feces, from which a stinging odor of ammonia is normally released, consistent with high rates of amino acid breakdown.

In this enzymatic test, the two animal cast products (Preparation 500 and silkworm feces) were compared with a series of soils including a biodynamic farm, a number of conventional soils, and soils of natural environments. The non-cropped soils showed good levels of activity in comparison with agricultural ones. Preparation 500 displayed high levels of activity, which were in the same range (arylsulfatase and alkaline phosphomonoesterase) or much higher than those of uncropped soils (Fig. 4). In general, it could be observed that conventionally cropped systems respond with higher activities when supplied with organic inputs, as shown by the manure-fertilized corn. In this respect, the organic farming practices, coupled with bioactive stimulants such as Preparation 500, could possibly contribute in restoring the balance to values closer to those of natural contexts. The latter have usually a more intense carbon cycle, warranting an efficient biological turnover, and a nitrogen cycling conducive to sustainable fertility.

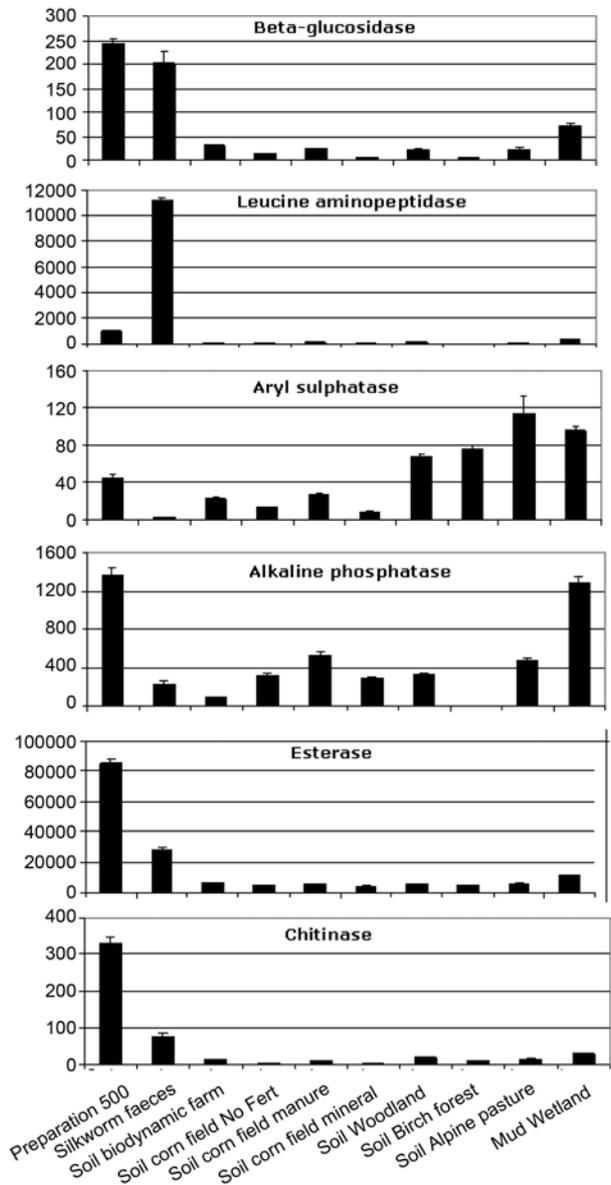


Fig. 4. Enzymatic assay results.

Activities are expressed as nM per gram of material dry weight and per hour. Silkworm feces are freshly collected scats from fifth instar larvae of *Bombyx mori* fed on mulberry leaves. The biodynamic soil is from a farm in which Preparation 500 has been regularly applied for over a decade. The three corn field soils are from a conventionally cropped site comparing zero fertilization (No Fert) with manure (230 t ha⁻¹ per year) and with mineral fertilization (1245 N + 3240 P₂O₅ + 2160 K₂O kg ha⁻¹ per year). The two forest soils include a Mediterranean ash-hornbeam woodland and a birch stand. The alpine pasture is a relatively overgrazed mountain meadow and the wetland is a man-managed lowland mire receiving agricultural runoff.

Analysis of the Presence of Quorum Sensing Signals

The assay scored negative, indicating the absence of *N*-acyl homoserine lactones (AHLs) of medium and short chain size. The datum is coherent with the fact that the prevailing populations in Preparation 500 appear to be Gram-positives,

and their signaling strategies would not make use of AHLs but rather of peptides.

Analysis of *nod* Gene-Inducing Compounds

No flavonoid-like activity was observed in such assay (data not shown). There were no apparent plant-derived inducing compounds such as legume flavons, *etc.* that could have accumulated in the intestinal tract of the animal, nor inducers of microbial origin. This rules out the possibility that Preparation 500 might act as a source of inducers for the *nod* genes of rhizobia, whose pre-exposure to flavonoids prior to encounters with the plant would improve nodulation and crop productivity.

Plant Hormone-Like Activity

A preparation is defined as endowed with biostimulation activity when it produces effects comparable to those of indol-3-acetic acid (IAA) and gibberellic acid (GA₃), even if the actual molecules are absent. Upon treating 500 seeds of watercress (*Lepidium sativum* L.) with dilutions of Preparation 500, positive auxin-like activity was observed corresponding to 1 mg/ml = 0.03 ppm of indol-acetic acid. Instead, as regards GA-like activity, upon examining the growth of 500 hypocotyls of cichory (*Cychorium intybus* L.), no effects were observed.

As for the auxin-like activity, the concentration occurring in the suspension that is actually used in the field (200 g in 60 L to be dispensed in 1 hectare) was in the same range of the IAA activity observed in some known commercially used biostimulants, such as the alfalfa hydrolysate [12] and the lignosulfonate-humates [13]. Data support the possibility that an active hormone-like level of activity is delivered to crops when Preparation 500 is sprayed.

DISCUSSION

Aiming at overall considerations on the possible action in soil, one can point out the following. Preparation 500 is substantially endowed with enzymatic-specific activities, rich in microbial cells, whose culturable fraction is dominated by the *Bacillus* genus, and exhibits an auxin-like activity. The presence of a rich microbial population is in agreement with our parallel detailed chemical characterization [26] that highlighted the dominant presence of ramified alkanes, whose origin is acknowledged as microbial. The auxin-like activity is also consistent with the low fungal presence, in that the abundance in lignin compounds, which are good candidates for IAA-like activity, is likely linked to a limited action of the lignin degrading fungi. Similarly to humic-like materials obtained from green compost or vermicompost, the large amount of undegraded lignin residues found in Preparation 500 by Spaccini *et al.* [26]

may account for the biostimulations towards microbes and plants. In fact, several studies have shown that humic extracts from compost have physiological effects on plants similar to auxin hormones [4, 11], and literature therein. Moreover, it is not unlikely that the large content of carbohydrates and peptides in Preparation 500 [26] may turn on microbial proliferation, and, thus, greater rhizospheric activity [5].

Preparation 500 is reported to increase soil biological activity and stimulate root formation [15, 30], notwithstanding the fact that its application is carried out at very low quantities. The BD agricultural protocol prescribes the dispersion of about 200 g of Preparation 500 in 60–70 L of water and the distribution over an hectare of soil. An example of dilution ratios over such scale is useful to determine the final concentration that can be attained. Assuming, as an example, a MW of about 250 for a putative active molecule, when applying 200 g of such compound over a whole hectare, one should first consider over which solution volume it will end up. The weight of a hectare of land, considering a depth from zero to 20 cm as useful to roots, is about 2,000 tons. The water in soil on average represents ¼ of its weight and is therefore 500 tons; i.e., 500,000 L. If 200 g of that compound were dissolved in a final volume of 500,000 L, a concentration of 0.0004 grams per liter is achieved. A molecule with a low molecular weight (*e.g.*, 250) whose 1 M solution is 250 g/l will be thereby diluted to 2.5 µM. This concentration, in the micromolar range, is definitely to be regarded as very high in terms of biological activities. There are many evidences on the range of activities of compounds of microbial origin that are effective in triggering plant physiological changes and morphogenesis reorganization. For example, the chitolipooligosaccharides that induce legume nodulation start their activity at concentrations as low as 0.1 nanomolar (10⁻¹⁰ M) [10]. Furthermore, there are several examples in biology of molecules active even at femtomolar concentrations (10⁻¹⁵ M). Hence, assuming that the 200 g of Preparation 500 is obviously not made of a pure active substance, even if the active principles in it were lower than 1/10,000th of its weight, they will already be delivered at a 10⁻¹⁰ M concentration. It is therefore not surprising that a distribution of the prescribed doses is fully capable of bringing in soil ranges of molecular signals that are well within their expected windows of biological activity.

Therefore, it is unlikely that, at the dose utilized, this product is effective as a structural organic fertilizer or as microbial inoculant of biofertilizer species [31], whereas it cannot be excluded that it might act through soil bacterial regulation. Bacteria detect and react to extremely low levels of signal molecules in their environment, as shown in work on quorum sensing [19]. Many higher plants have been shown to produce signal-mimicking compounds, thereby

affecting bacterial density relationships [2]. Potentially bioactive compounds could be abundant in Preparation 500 (e.g., low-molecular-weight peptides), due to its microbially mediated slow maturation under low oxygen conditions, and the consequent proteolytic activity. This is reminiscent of the situation observed in the build-up of biostimulating potential reported by other studies, whereby starting from very different substrates, once these are transformed *via* subtilisin digestion, a remarkable plant growth-enhancing capability arises [28].

Another possible mode of action of Preparation 500, which does not exclude the former, may be through hormonal effects on crop growth and development. Deffune and Scolfield [9] found that humic acids extracted from this and other BD preparations (505 and 507) caused a positive growth response in wheat seedlings relative to the control, with BD preparations and IAA effective at concentrations of 0.2×10^{-11} and lower. Preparation 500 was also reported to exhibit high cytokinin activity [27] and, as we showed in the present paper, an effective auxin activity. Thus, the mode of action of the product could be supposedly by stimulating root formation and consequently activating plants to access soil stocks of nutritional compounds that would be otherwise overlooked and unexploited. This assumption is consistent with the recommended practice in biodynamic agriculture that prescribes, in order for the Preparation 500 to be effective, the existence of an organic matter stock in the soil. It is also worth considering that auxin has been reported to stimulate bacterial growth [29], and therefore its effects could be in part mediated by microorganisms rather than acting directly on plants.

Experiments are in progress to assess further aspects of Preparation 500, such as the successional maturation of its whole bacterial community, which will be determined upon metagenomic approaches run on samples at increasing levels of maturation. These tests will cast further light on the biological peculiarities of this biodynamic product.

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