Chemical and Biological
Technologies in Agriculture

New insights into municipal biowaste derived products as promoters of seed germination and potential antifungal compounds for sustainable agriculture

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Abstract

Background: Municipal biowaste management may generate a negative impact on the environment; therefore, their biomasses could be valorised as an alternative feedstock to fossils to produce high performance compounds useful for agricultural applications. The main objective of this study was to evaluate the potential agricultural applications of bioproducts (BPs) obtained by alkaline hydrolysis of the solid anaerobic digestate of municipal biowastes (ADMBW) and of one oxidized (ozonized) product (ADMBW BP OX). Both products were chemically characterized and used for agricultural in vitro assays.

Results: BP preparations were tested for their potential effect as enhancers of seed germination process using five concentrations (1, 10, 100, 1000, and 5000 mg L^{-1}) and three different species: cress (*Lepidium sativum*), tomato (*Solanum lycopersicum*), and lettuce (*Lactuca sativa* L.). At this aim several germination indices were calculated to establish the priming effect of these substances on the selected seeds. Moreover, the potential in vitro antifungal effects of BPs at three concentrations (100, 1000, and 5000 mg L^{-1}) on many dangerous fungal phytopathogens of economically important cultivated crops were evaluated and compared to Benzothiadiazole, one of the most-used plant disease suppressants. Results show that these ADMBW derived BPs exert a seed specie-specific positive effect on germination process, inducing better performances in the several calculated indices at all the concentration tested, except for the ADMBW BP OX 5000 mg L^{-1}, which showed at the highest concentration a strong phytotoxic effect on tomato seeds.

Conclusions: The fungicidal potency of ADMBW BP OX was for the first time clearly demonstrated on multiple targets as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Monilia* sp., *Sclerotium rolfsii*, and *Phytophthora nicotianae*, by calculating their relative EC_{50} and, when it was possible, also EC_{95} and MIC values. These results are of great impact in the actual historical moment, as from a biowaste as DMBW, which is worldwide constantly produced, may be possible to obtain agrochemicals and fertilizers without the usual feedstocks, which are more and more expensive.

Keywords: Digestate, Biostimulant, Fungicidal potentiality, Germination indices, Biopolymers, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Monilia sp.*, *Sclerotium rolfsii*

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Graphical Abstract
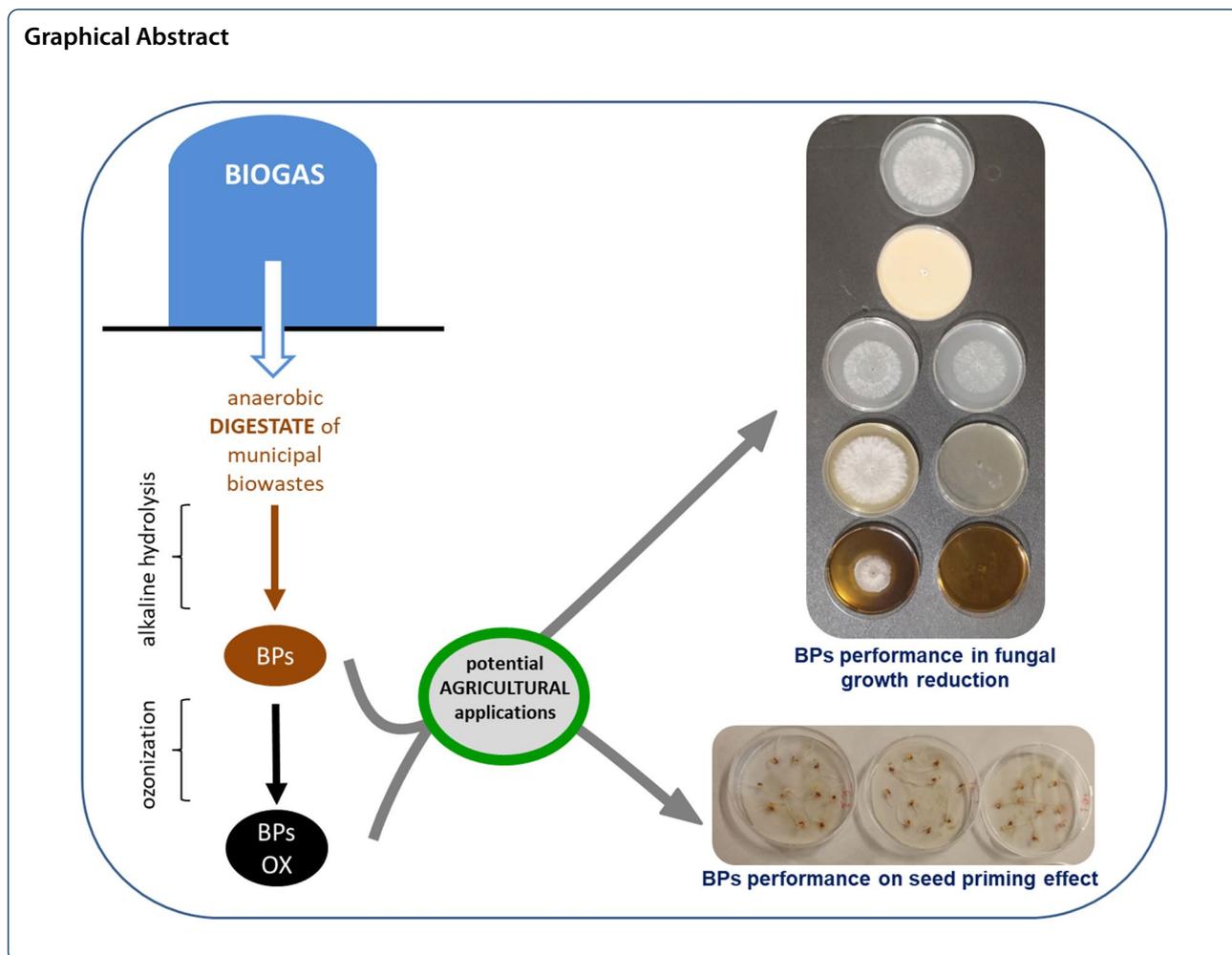
Introduction

Currently, much research is being carried out to valorise biomass from different sources as alternative to fossil feedstock for the production of fuel, chemicals, and materials. Cultivation of dedicated plants is pursued to provide a relatively low entropy source of bioproducts to use in different sectors, where synthetic products are currently used. This practice raises social moral concern, due to the subtraction of soil to food production in a world, where 690 million people suffer starvation [1]. From this point of view, valorisation of biowastes from municipal, agriculture and agro-industrial source is unanimously accepted. Indeed, using biowaste as feedstock to produce valued added chemicals and materials will also decrease the amount of landfilled biowastes. In this context, municipal biowaste (MBW) is potentially the most sustainable renewable feedstock. It is readily available in every urban settlement in the world and is a negative cost feedstock, since its collection costs are already paid off by citizens' taxes [2–4]. Under these circumstances, turning

a MBW treatment plant into a biorefinery producing biofuel and biobased value added chemical specialities is an attracting perspective. A typical MBW treatment plant is the *ACEA Pinerolese Industriale S.p.A.*, located in Pinerolo (Turin, Italy). It processes MBW by fermentation and produces biogas and compost. Over the past 20 years, the University of Turin group has developed chemical hydrolysis and oxidation processes to obtain new value-added soluble bioproducts (BPs), either from the ACEA anaerobic digestate and compost [5], and from exhausted food plants. The BPs have been demonstrated competitive with fossil sourced commercial chemicals for use in several sectors of agriculture and the chemical industry [6].

The present work reports the isolation process and the outstanding antifungal properties of a new BP. The product contains a wide range of organic molecules belonging to the class of oximes. Oximes are starting reagents for the synthesis of several functionalised organic N compounds [7]. They are also biologically active as

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antimicrobial agents, insecticides and antifungals [8, 9]. Moreover, new herbicides, such as cyclohexanedione oximes have been developed as they are effective at low doses and easily decomposable, to reduce herbicide impact in the environment [10].

Plant fungi lower crop production and quality [11]. The current population is expected to grow by 30% in 2050. Consequently, higher food production will be necessary. At the same time, the concern for more consumption of current commercial pesticides is increasing, due to their negative effects on human health and environment. Moreover, they induced arising of pathogen resistance phenomena to synthetic fungicides. The development of efficient eco-friendly antifungal compounds is a critical urgent objective to achieve to protect agricultural crops from disease.

The depicted scenario gives the feeling of the level of the economic, environmental, and social issues addressed by the present work. Comparing the performances of the new BPs and Benzothiadiazole (BTH) allows assessing the potential market value of the BP, considering that the global pesticide market is estimated 4 M tons/yr [9]. BTH is one of the most-used plant disease suppressants [11], and its market price is up to 820 USD kg^{-1} [12].

The general aim of the present study was to increase the added value of biobased products obtained from the digestate of municipal waste, by applying these substances in the agricultural field. Based on the above considerations, the specific objectives of this paper were i) the production of high performance biobased products (BPs) from municipal biowaste; ii) testing the effect of BPs on seed germination process of three different species cress (*Lepidium sativum*), Tomato (*Solanum lycopersicum*) and Lettuce (*Lactuca sativa* L.); iii) to detect the multiple fungicidal potentiality of BPs derived from the anaerobic digestate of municipal biowaste (ADMBW) versus many threatening fungal pathogens of cultivated crops and eventually to compare their in vitro performances with a standard product as BTH.

Materials and methods
Materials
The BP was obtained from the solid ADMBW sampled from the Acea plant. The digestate was hydrolysed in water at pH 13 and 60 °C according to a previously reported process [13]. In essence, the liquid hydrolysate was separated from the insoluble residue by sedimentation, followed by centrifugation and ultrafiltration though 5 kDa cutoff polysulphone membrane. The membrane retentate was dried at 60 °C to yield the solid ADMBW BP. This product was dissolved in pH 10 water and oxidised by bubbling a 4% ozone–oxygen stream through the aqueous solution for 64 h [14]. During this time, the

solution pH 10 was maintained by KOH addition. The final alkaline solution was filtered through a 0.2 kDa polysulphone membrane to separate the membrane permeate. This was dried at 60 °C to obtain the solid ADMBW BP OX. All the analytical methods applied for the products compositional characterization have been previously reported [13, 14].

Experimental conditions for BPs performance on seed germination
Different species of seeds were evaluated: (i) cress, which represents the most sensitive species to many toxic compounds, and have been commonly used in germination test [15], (ii) tomato and (iii) lettuce, as they represent host plant species for some of the isolated pathogens used in this study.

In detail, the plant material used in these trials were cress (*Lepidium sativum* cultivar Dadas), Tomato (*Solanum lycopersicum* cultivar Missouri) and Lettuce (*Lactuca sativa* L. cultivar Romana) seeds, provided by a local nursery located in Catania, Sicily (Italy).

The seeds were rinsed with sterilized water and maintained moistened in distilled water for 1 h. BP solutions (ADMBW BP and ADMBW BP OX) were prepared by dissolving the powder in distilled water at the final concentration of 5000 mg L^{-1}. Different amounts of 5000 mg L^{-1} BP solutions were diluted in distilled water to yield solutions at different concentrations. These were used to moisten the filter papers (Whatman 3 MM) in the trials. For both BPs, hydrolized and ozonized, five different concentrations were tested (1, 10, 100, 1000, and 5000 mg L^{-1}) yielding 10 treatments. Tests were conducted using 90 mm plastic Petri dishes and three layers of filter papers. Then, seeds (20 seeds per Petri dish) were placed on the filter papers previously moistened with the BP solutions (5 mL). Controls in distilled water moistened paper were routinely performed.

Germination was carried out in a growth chamber at 25 ± 1 °C in the dark. Seeds were considered germinated when a radicle of at least 2 mm emerged. The germinated seeds were counted and monitored daily until no further seed germinated (48 h for cress, 96 h for lettuce, 144 h for tomato). Radicle lengths were measured using a digital caliper to the nearest 0.01 mm. The experimental trial was repeated twice in a complete randomized block design, and for each treatment three replicates were tested, according to methods of the Association of Official Seed Analysts [16].

Seed germination indices
To evaluate the positive or phytotoxic effects of BP treatments on seeds, several germination indices were calculated as detailed below [17]:

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i) The Germination Index (GI) was calculated to assess the overall response variability between treatments [18]:

$$GI = (Nt \times Lt/Nc \times Lc) \times 100$$

where Nt and Nc are the medium number of germinated seeds in treated (t) and control (c) conditions, respectively, and Lt and Lc are the average radicle lengths of germinated seeds in treated (t) and control (c) conditions, respectively.

ii) The Germination Percentage (GP) was calculated for each treatment and for each seed species as percentage of total germinated seeds from sowing:
GP = (number of germinated seeds/number of total seeds for bioassay) × 100

iii) The Mean Daily Germination (MDG), representing the mean number of germinated seeds per day, was calculated at 24 and 48 h for cress, at 48, 72, and 96 h for lettuce, and at 48, 72, 96, and 144 h for tomato [19]:
MDG = GP/t
where GP is the germination percentage at time t, which is the number of days after the sowing.

iv) The Seedling Vigor Index (SVI) was calculated at 24 and 48 h for cress, at 48, 72, and 96 h for lettuce, and at 48, 72, 96, and 144 h for tomato [19]:
SVI = GP × L
where GP is the germination percentage and L is the seedling length of germinated seeds at each monitoring time.

v) The Mean Germination Rate (MGR), also known as rate of Maguire [20]:
MGR = [number of germinated seeds/days of first count] + …………. + [number of germinated seeds/days of final count]

vi) The Mean Germination Time (MGT) was calculated at the last day of germination process for each species, according to Ruan et al. [21] as follows:
MGT = $\sum(n,t)/\sum n$
where n is the number of newly germinated seeds at time t.

vii) The Germination Energy (GE) was calculated according to Ruan et al. [21] as follows:
GE = (Percentage of germinated seeds at the starting day of germination/Total number of seeds sets for bioassay) × 100

viii) The Speed of Emergence (SE), was calculated according to Soltani et al. [22] as follows:
SE = (Number of germinated seeds at the starting day of germination/Number of germinated seeds at the final days of measurement) × 100

ix) The Coefficient of the Rate of Germination (CRG) was calculated as reported in Chiapusio et al. [23] as follows:
CRG = [(N1 + N2 + …..   Nn)/(N1 × T1) + (N2 × T2) + ….. + (Nn × Tn)] × 100
where N1, N2, … Nn are the number of germinated seeds on time in days T1, T2, …. Tn.

x) The Time required for 50% germination (T50), was calculated according to Coolbear et al. [24] as follows:
T50 = ti + [((N/2) − ni) × (ti − tj)]/(ni − nj)
where N is the final number of germinated seeds, ni and nj the cumulative numbers of seeds germinated by adjacent counts at times ti and tj.

In vitro BP performances against phytopathogenic fungi

The effects of ADMBW BP and ADMBW BP OX in reducing/inhibiting mycelial growth of 9 fungal pathogens affecting vegetables, fruit and ornamental crops (Table 1) were assessed according to amended media technique and using potato dextrose agar (PDA, Lickson, Vicari, Italy) as basic substrate. Acibenzolar-S-methyl [syn. benzothiadazole, (BTH), Bion® 50% active ingredient, water granules, Syngenta Italia S.p.A. Milano MI, Italy] was also included and served as standard active ingredient of reference [25]. The molecule was chosen, since it has been reported both as fungicide and plant resistance activator on a several commercial crops including lettuce and tomato, and as having multi-target effects against several classes of plant pathogens. In detail, BPs were tested at 0, 100, 1000 and 5000 mg L^{-1}, whereas BTH was tested at a single concentration, 3000 mg L^{-1}, to compare its performance with that achieved by the highest rate of BPs (Table 1). The BTH concentration of 3000 mg L^{-1} was chosen, since it is reported in literature as the discriminatory and most effective one for in vitro mycelial reduction of phytopathogenic fungi and in vivo for disease control [26, 27].

For the BPs–BTH comparison, stock solutions of ADMBW BPs and BTH were preliminarily dissolved in sterilised distilled water (SDW), vortexed and sterilised by passing through by 0.45 µm pore size membrane filter (Sigma-Aldrich, St. Louis, MO, USA). Afterward, the solutions were added to media cooled at about 45–50 °C. Autoclaved media were amended with appropriate volumes of stock solutions containing the active ingredients to reach the above fixed concentrations. No fungicide was added to the control dishes.

Following the media preparation, the mycelial plugs of each fungal pathogen were cut from the edge of an actively growing culture on agar media and placed upside down on the centre of each fungicide-amended or control dish. Dishes were incubated at 21 ± 2 °C in darkness

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Table 1 Target fungal pathogens including relative hosts and induced symptoms employed in in vitro assays performed to evaluate BP, BP OX and BTH effects in reducing mycelial growth

Target pathogens[*]	Host	Symptom	mg L^{-1} of active ingredient	
			BP and BP OX	BTH
Botrytis cinerea Di3A-E2	Eggplant	Fruit rot	100, 1000 and 5000	3000
Monilia spp. Di3A-A1	Apricot	Fruit rot	»　　» 　　»	»
Sclerotinia sclerotiorum Di3A-L3	Lettuce	Lettuce head rot	»　　» 　　»	»
Fusarium spp. Di3A-C1	Cucurbit	Vascular infection	»　　» 　　»	»
F. oxysporum f.sp. *radicis-lycopersici* Di3A-T1	Tomato	Vascular infection	»　　» 　　»	»
Sclerotium rolfsii Di3A-TP3A	Tomato	Basal stem rot	»　　» 　　»	»
Alternaria alternata Di3A-CT7	Carob	Leaf spot	»　　» 　　»	»
Colletotrichum gloeosporioides Di3A-O4	Orange	Fruit anthracnose	»　　» 　　»	»
Phytophthora nicotianae Di3A-B1	Buxus	Root and crown rot	»　　» 　　»	»

[*] All tested fungal isolates obtained for relative hosts are monosporic colonies

for about 5 days. For each concentration, three dishes were used, and colony diameter was measured in two perpendicular directions for the calculated value. Triplicate assays were performed. Radial growth on each dish was measured as average and the raw data from three replicates used to calculate growth reduction $(GR) = [1-(\text{radius in amended plates/radius of control dishes})] \times 100$.

Determination of EC$_{50}$, EC$_{95}$ and MIC versus target fungi to evaluate BP potentiality

Dose–response curves of single isolates for each fungicide were generated by plotting all raw growth reduction data against compound concentration and the effective BP's concentration to inhibit 50% and 95% of mycelial growth (EC$_{50}$ and EC$_{95}$) was calculated for each isolate by linear regressions (by determining coefficient of determination R^2) of the mycelial growth reductions versus the active ingredient concentrations [28, 29]. Minimum inhibitory concentration (MIC i.e., lowest concentration of compound that inhibit the mycelial growth) was also calculated when possible (i.e., when falling within the concentration range).

Statistical analyses

Statistical treatment of data collected from each experiment was performed using the Statistics package software (version 10; Statsoft Inc., Tulsa, OK, USA). The analysis of the variance was performed on raw data considering all the single replicates. Since the laboratory assays were performed three times, *F* and *P* values were calculated to evaluate whether the effects of single factor as treatment, concentration and assay were significant. In post-hoc analyses, the means were always separated by the Fisher's least significance difference test ($\alpha = 0.05$).

Results and discussion

BP production processes

The BP production process was designed and realised in a pilot production facility to represent a model chemical plant to be integrated in a conventional anaerobic MBW treatment plant [13]. To this purpose, the Acea Pinerolese MBW treatment plant was taken as case study. This plant is a typical example of the most advanced European plants performing anaerobic fermentation of unsorted food wastes from separate collection source to produce biogas and digestate, followed by aerobic composting of the digestate mixed with green residues. The pilot facility applied conventional chemical hydrolysis and oxidation reactions to the anaerobic digestate and composts of different mixes with the intent to produce water soluble biobased chemical specialities for use in different sectors of agriculture and the chemical industry.

The pilot facility comprised a 500 L reactor, a product separation unit, and a drying oven. The product separation section included a sedimentation vessel to separate the insoluble residue from the liquid hydrolysate, a centrifuge to separate residual fine solid particles from the liquid hydrolysate and a tangential flow filtration unit. This unit was suitable to accommodate a series of membrane with different cutoffs from 750 to 0.2 kDa to be operated in sequential flow mode, where the permeate through the higher cut off membrane was the feed to the lower cut off membrane. In this fashion, several fractions were separated according to their molecular weight ranges: i.e., ≥ 750 kDa, 750–150, 150–100, 100–50, 50–30, 30–20, 20–5, 5–0.2 and ≤ 0.2 kDa. The alkali reagent was recovered in the final permeate through the 0.2 kDa cut off membrane and recirculated as such to the hydrolysis reactor. As the insoluble hydrolysate residue and the soluble molecular fractions were proven valued

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added products in different sectors of agriculture and the chemical industry, the process did not produce secondary products and excess unreacted alkali needing further disposal treatment [5, 13, 14].

The same pilot facility was suitable to perform both the hydrolysis and oxidation process. MBW hydrolysis at pH 13 and 60 °C allowed producing the above BP fractions. These are complex mixes of different molecules with molecular weight ranging from 0.2 to above 750 kDa, which contain different aliphatic and aromatic C types substituted by a variety of acid and basic functional groups bonding mineral ions inherited from the pristine native biopolymers. Thanks to their water solubility and chemical composition features, the BPs exhibited, in field tests, useful properties as soil fertilisers, plant growth biostimulants, animal feed supplements, sequestering agents for the remediation of polluted soil and industrial waters containing trace metals and organics, emulsifiers, photo sensitizers and reagents for the production of composite plastic articles, biosurfactants, bioplastics.

The ozonisation of the crude BP hydrolysate obtained from the Acea ADMBW was found to change the pristine molecular weight and functional groups distribution [14]. The oxidized product (BP OX) had lower molecular weight and higher content of carboxylic functional groups. The ozonisation reaction yielded 30% biopolymers with molecular weight from 100 to over 750 kDa exhibiting remarkable surfactant properties, and 70% of small molecules with molecular weight ≤ 0.2 kDa. On the contrary, ADMBW contained 95% of the high molecular weight biopolymers and less than 5% molecules with molecular weight ≤ 0.2 kDa. The remarkable surfactant properties of the oxidised biopolymers prospect high market value for these products, which can compensate largely their production cost. The high yield of the molecules with molecular weight ≤ 0.2 kDa was contrary to the design thinking of the authors and expectations from the room temperature oxidation of ADMBW BP. Surprisingly, the here in after reported results of the present work demonstrate that the oxidised ADMBW BP OX fraction with low molecular weight has far higher potential market value, compared to the high performance polymeric biosurfactants.

Characterization of ADMBW BP and ADMBW BP OX

According to LC–MS/MS studies, ADMBW BP OX contained a mix of compounds with 2–7 C atoms, such as mono, di and tri carboxylic acids, hydroxy- and keto-carboxylic acids, aldehydes, ketones, and oxymes [14]. The ^{13}C spectra of ADMBW BP OX showed the presence of aliphatic, oxime and carboxylic acid C atoms resonating at δ 17–53, 162 and 173–176 ppm in the relative ratios 2.5/1/4, respectively. The content of aromatic C
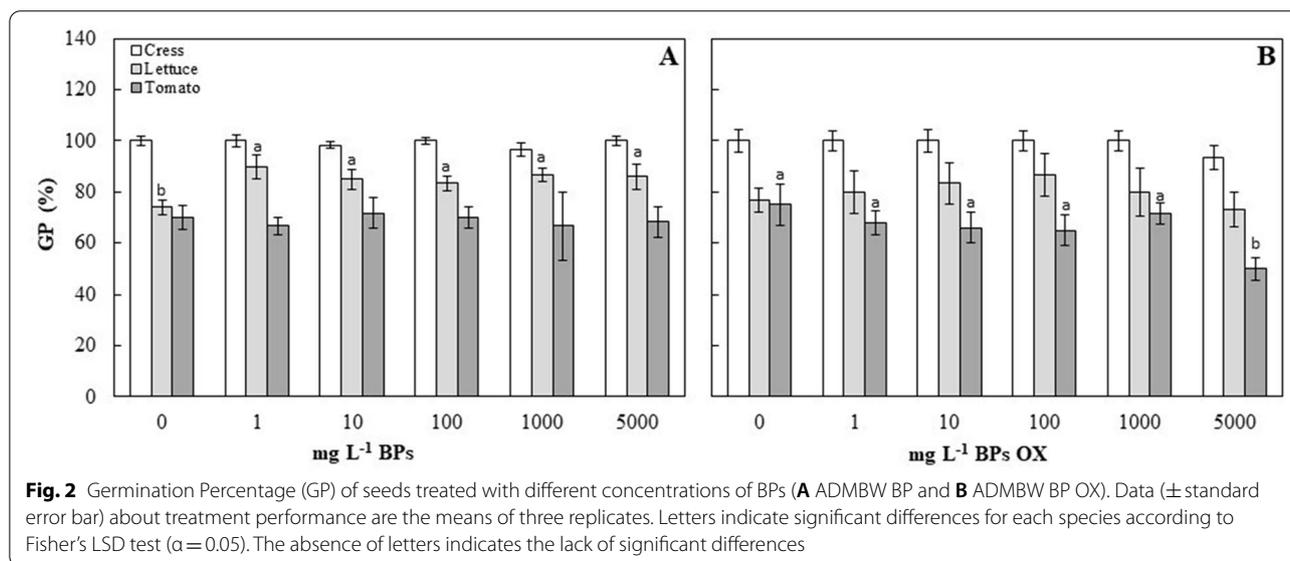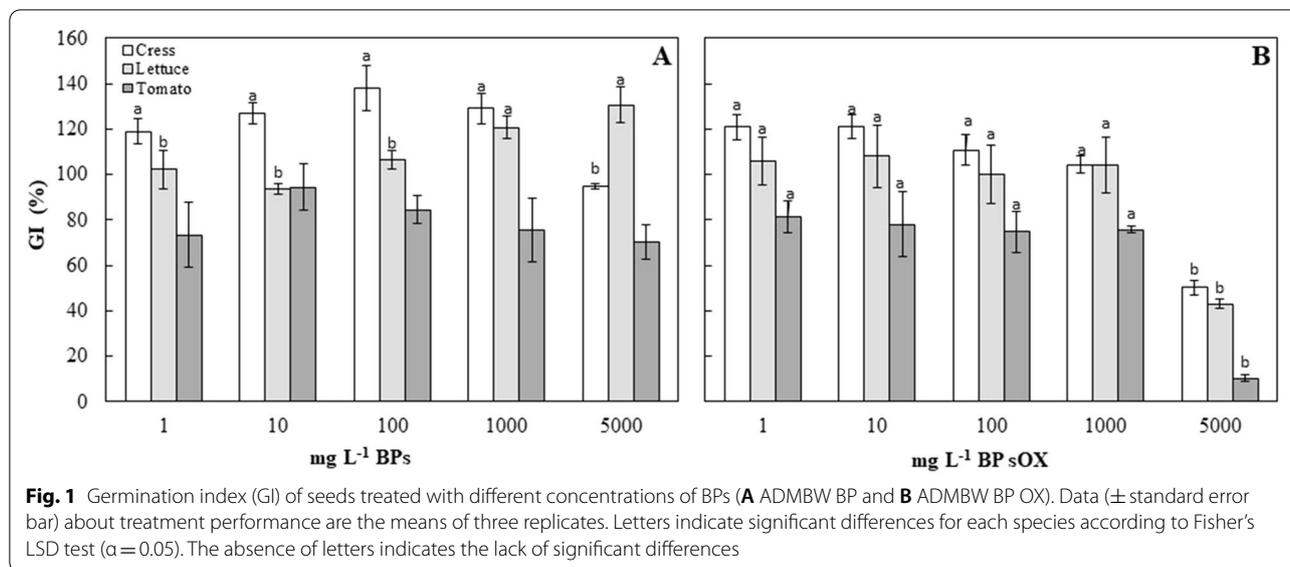
was negligible. For ADMBW BP OX, the virtual structure $HC(COOH)_2–[CH(COOH)]_2–C(H)=N–OH$ with empirical formula $C_8H_9NO_9$ and 245.31 g mass was consistent with the ^{13}C spectra data, the measured C/N 7 ratio and the 0.2 kDa cutoff of membrane through which the product permeated during the separation from the mix of higher molecular weight ozonized products. These results suggest that ADMBW BP OX is mainly a mix of aliphatic polycarboxylic acids and oxymes. By comparison, ADMBW BP had C/N 5.7 and molecular weight in the 5–750 kDa range. The ^{13}C spectra of ADMBW BP showed that the organic C was distributed over 43% aliphatic, 10% ammine, 4% methoxy, 10% alkoxy, 3% anomeric, 13% aromatic, 7% carboxylic C, 9% amide C, 1% ketone C [30]. For the higher number of functional groups and molecular weight, the ADMBW BP composition is much more complex than that for ADMBW BP OX.

BP performance on seed germination

Germination indices of seeds subjected to the experimental trials were calculated to evaluate the performance of the treatments and their concentration effect, using five different amounts of BPs and BPs OX.

The germination indices (GI) of seeds are reported in Fig. 1. The ADMBW BP treatment on cress and lettuce at all the tested concentrations did not negatively affect the germination process. Interestingly, data in Fig. 1A show also a beneficial effect on germination GI (GI > 100%) at concentrations ranging from 1 to 1000 mg L^{-1} on the cress, and at concentrations of 1000 and 5000 mg L^{-1} on lettuce (Fig. 1A). These results are validated by Alvarenga et al. [31] findings for other biodegradable organic residues, such as sewage sludge from municipal wastewater treatment, compost from the organic fraction of unsorted municipal solid waste, and garden waste compost which confirmed that cress was a very sensitive indicator of compost toxicity. For tomato, Fig. 1A shows GI quite lower than 100%, with values being constant at all concentrations. The ADMBW BP OX data (Fig. 1B) show no significant differences (p < 0.05) between concentration ranging from 1 mg L^{-1} to 1000 mg L^{-1} for each species. However, at the highest BP OX applied concentration (5000 mg L^{-1}), a phytotoxic effect was observed for all species. Tomato, exhibiting the lowest GI values, appear as the most sensitive species.
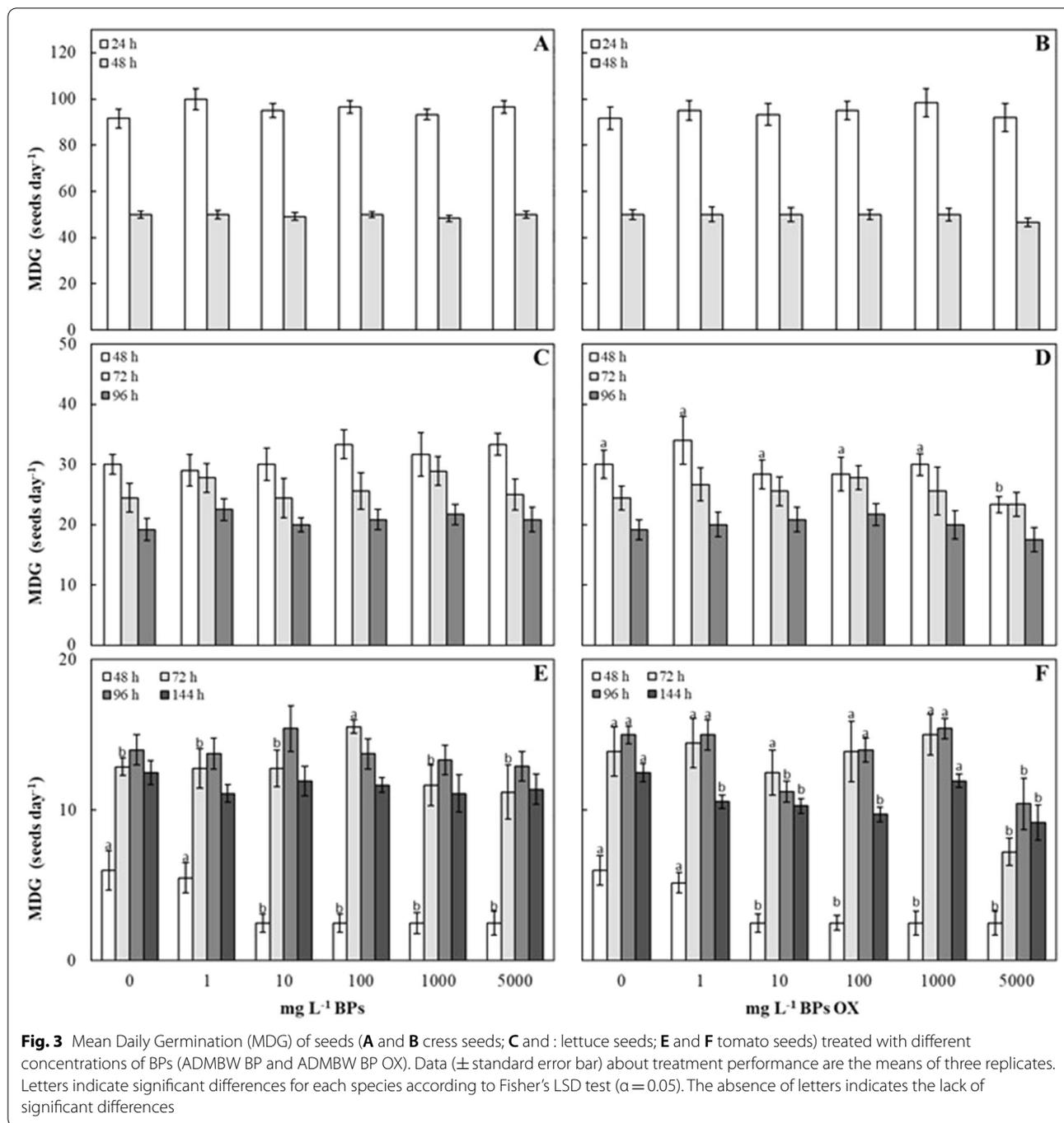
The presence of both BPs during cress seed germination did not affect the GP values at any concentration (Fig. 2). Moreover, in lettuce seeds treated with ADMBW BPs, an increase in GP values with respect to the control was observed at all concentrations, suggesting a promoting effect on seed germination process (Fig. 2A). By comparison, ADMBW BP OX, at all concentrations, did

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Fig. 1 Germination index (GI) of seeds treated with different concentrations of BPs (**A** ADMBW BP and **B** ADMBW BP OX). Data (± standard error bar) about treatment performance are the means of three replicates. Letters indicate significant differences for each species according to Fisher's LSD test (α = 0.05). The absence of letters indicates the lack of significant differences

Fig. 2 Germination Percentage (GP) of seeds treated with different concentrations of BPs (**A** ADMBW BP and **B** ADMBW BP OX). Data (± standard error bar) about treatment performance are the means of three replicates. Letters indicate significant differences for each species according to Fisher's LSD test (α = 0.05). The absence of letters indicates the lack of significant differences

not exhibit significant effects on lettuce seeds (Fig. 2B). As regard tomato, a slight reduction of GP value was recorded only using 5000 mg L^{-1} of ADMBW BP OX (Fig. 2B). These results suggest that as regard cress and lettuce, BP OX 5000 did not show any phytotoxic effect, as the reduction in GI values was mostly related to the length of the radicle produced during the germination process than related to the number of germinated seeds.
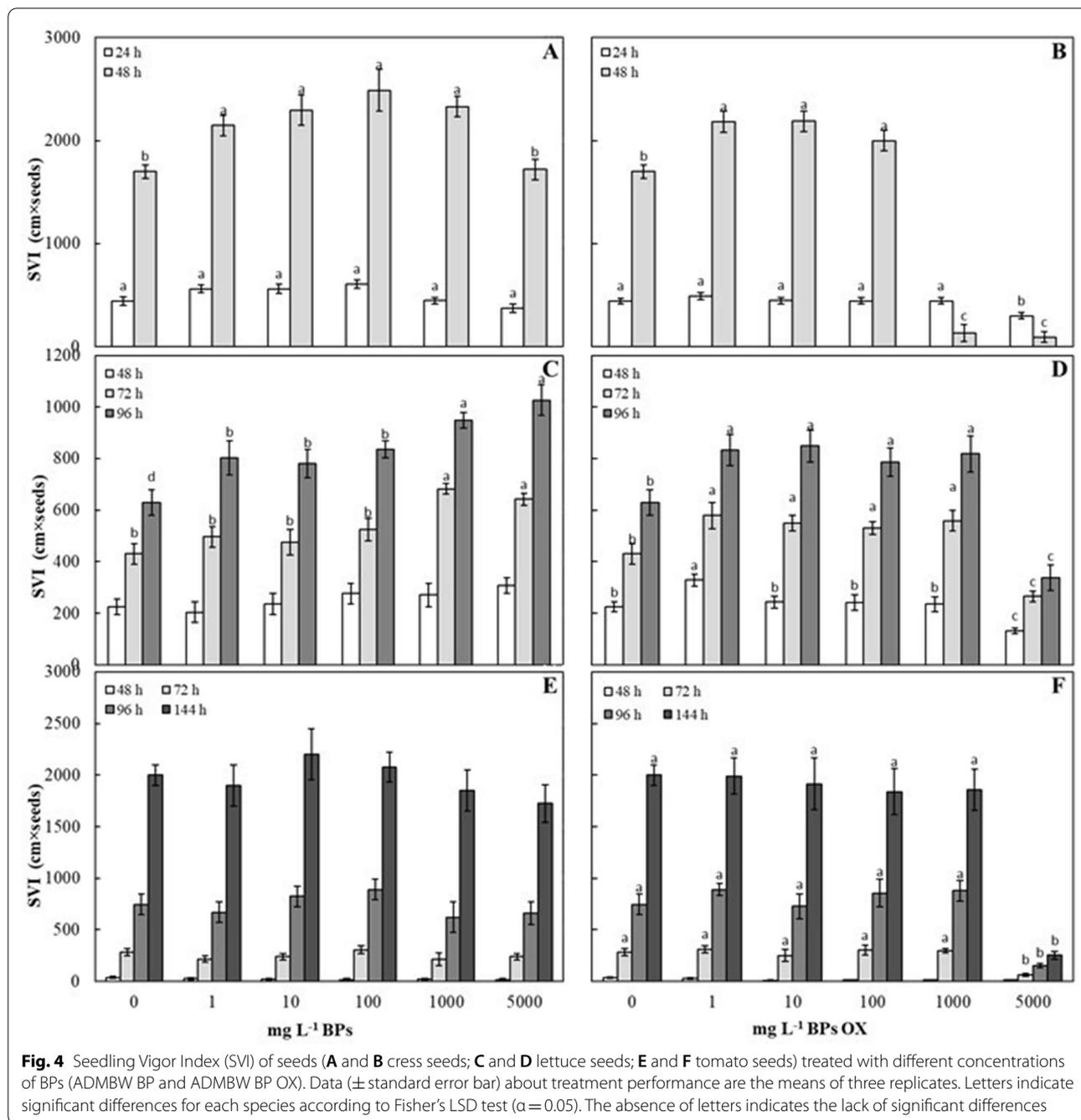
As regard to the number of germinated treated seeds per day (MDG), in cress no significant effect was proven at all monitoring times, and with all the tested BP concentrations (Fig. 3A, B). For lettuce no

significant effect at all concentrations and monitoring times was detected, except using 5000 mg L^{-1} ADMBW OX BP, for which a slightly reduced value of MDG was detected after 48 h, although at the end of the germination process the MDG value was similar to that calculated for the control (Fig. 3C, D). The most sensitive species resulted to be once again tomato, as already observed for previous indices. In particular, ADMBW BP did not affect MDG values after 72 h until the end of the germination process (Fig. 3E), whereas ADMBW OX BP reduced the MDG values with respect to the control at the highest concentration of 5000 mg L^{-1} at all the monitoring times (Fig. 3F).

Fig. 3 Mean Daily Germination (MDG) of seeds (**A** and **B** cress seeds; **C** and : lettuce seeds; **E** and **F** tomato seeds) treated with different concentrations of BPs (ADMBW BP and ADMBW BP OX). Data (± standard error bar) about treatment performance are the means of three replicates. Letters indicate significant differences for each species according to Fisher's LSD test (α = 0.05). The absence of letters indicates the lack of significant differences

The Seedling Vigor Index (SVI) (Fig. 4) showed that a different effect was exerted by each BP concentration on each seed species. For cress seeds, after 48 h, all concentrations except 5000 mg L^{-1} showed an improved effect on SVI values. The highest value was attained from BP 100 mg L^{-1} (1.4-fold increase with respect to the control) (Fig. 4A). The ADMBW BP OX (Fig. 4B) exerted, after 48 h, a positive effect with respect to the control

at the concentrations 1, 10 and 100 mg L^{-1}, whereas BP OX 1000 mg L^{-1} and 5000 mg L^{-1} drastically decreased SVI values (13 and 19 times lower than the control, respectively). Lettuce seeds, at the beginning of the germination process (48 h), treated with ADMBW BP at all applied concentrations, did not show significant differences (Fig. 4C), whereas after 72 h and at the end of the germination process, BP1000 and BP5000 significantly

Fig. 4 Seedling Vigor Index (SVI) of seeds (**A** and **B** cress seeds; **C** and **D** lettuce seeds; **E** and **F** tomato seeds) treated with different concentrations of BPs (ADMBW BP and ADMBW BP OX). Data (± standard error bar) about treatment performance are the means of three replicates. Letters indicate significant differences for each species according to Fisher's LSD test (α = 0.05). The absence of letters indicates the lack of significant differences

increased the SVI values. ADMBW BP OX after 48 h increased SVI values at 1 mg L^{-1} (1.5-fold higher than the control), whereas concentrations up to 1000 mg L^{-1} did not affect the indices. Interestingly, after 72 and 96 h, an increase of SVI values at all concentrations was observed up to 1000 mg L^{-1}, while the highest concentration provoked a decrease in SVI values at all times (Fig. 4D).

On tomato seeds, ADMBW BP did not determine a significant effect at all concentrations and at all monitoring

times (Fig. 4E). Conversely, ADMBW OX BP determined at each time a strong negative effect at 5000 mg L^{-1}, showing at the end of the germination process a value of SVI around eightfold lower than the control. However, after 48 h from sowing no effect was detected (Fig. 4F).

Islam et al. [32] reported that in different species, among which cress and lettuce, higher SVI values than in the control corresponds to positive biostimulant effects on seed germination. Accordingly, the ADMBW BP

results would prove this product as germination inducers for cress and lettuce. For tomato seeds, ADMBW BP did not affect the germination process. Its effect was not evident at the early stage of germination. The positive effect was relevant at the end of the process (in cress after 48 h and in lettuce after 96 h). A different behaviour was proven for ADMBW BP OX. In cress, which is an annual weed, concentrations ranging from 1 to 100 mg L^{-1} showed a promoting effect, whereas 1000 and 5000 mg L^{-1} resulted phytotoxic. These results suggest a potential use of BP OX at these concentrations as a possible herbicide, according to Sevilla-Moran et al. [10]. Putatively, the presence of oxime in BP OX may be associated to the phytotoxic effect at high concentrations. Sandin-Espana et al. [33] found that oxime-based herbicides, such as cyclohexanedione oxime, determine a rapid reduction of growth followed by the destruction of shoot meristems in susceptible species. This could explain the negative effect of BP OX at high concentrations, which occurred mostly on the length of the radicle rather than on seed germination process. These herbicides seem to inhibit the fatty acid biosynthesis in isolated chloroplasts, cell cultures, or leaves showing a greater susceptibility for some species, such as corn, wheat, and wild oats [34, 35]. However, other plants, such as soybean, spinach, and sugar beet, have been reported to be more tolerant to oxime [33].

Table 2 report the Mean Germination Rate (MGR), the Germination Energy (GE), the Speed of Emergence (SE), the Coefficient of the Rate of Germination (CRG), the Mean Germination Time (MGT), and the Time required for 50% germination (T50) of seeds treated with different concentrations of BPs. Islam et al. [32] showed that in cress (*Lepidium sativum*), lettuce (*Lactuca sativa*), and other different species MGR, GE, SE and CRG values are positively correlated with the stimulant effect on seed germination. On the contrary, MGT and T50 values are negatively correlated. In the present work, MGR and GE values showed that OX BP at 5000 mg L^{-1} was phytotoxic for all the tested seeds. However, tomato seeds showed GE values lower than the control at all BP concentrations, except at 10 mg L^{-1} (Table 2).

SE values appeared to depend on the species and the BP concentration. Tomato seeds were the most sensitive species to both tested BP and BP OX, especially at the highest concentrations. The results showed also that cress and tomato SE values were similar or higher than the control using BP. The BP OX treatment did not exert any significant effect on cress at all concentrations while showing reduced SE values on lettuce at the highest concentration (Table 2). The MGT, CRG and T50 were not significantly modified by all the tested BPs (Table 2).

The overall analyses of these germination indices suggest that only BP OX 5000 may induce a phytotoxic effect

on germination, in particular in tomato seeds, which results the most sensitive species. This treatment negatively affected the radicle emerging rather than the seed germination process *sensu strictu*. By comparison, the analysis proves BP as inducer of the germination process at the tested concentrations, in particular on lettuce and cress.

Effects of BP, BP OX and BTH in reducing mycelial growth of phytopathogenic fungi

In laboratory assays, no significant effect was found for the 4 tested pathogens (*A. alternata*, *Fusarium* sp., *F. oxysporum* f.sp. *radicis-lycopersici*, *C. gloeosporioides*) for each factor (*data not reported*), whereas a significant effect of ozone (oxidation) treatment and BP concentration was detected on fungal growth reduction of *B. cinerea*, *Monilia* sp., *S. sclerotiorum*, *S. rolfsii* and *P. nicotianae* (Table 3). The significance of the ozonization treatment may be probably related to a greater accumulation of oximes in OX BPs than BPs. Although the putative effects of these compounds against these pathogens should be definitively demonstrated, it can be assumed that higher amounts of oximes have played an important role for the expressed anti-fungal activity as well as it was widely reported in literature [8, 9]. As regards to BP rate effects, our findings are in perfect agreement to the expected ones, with an increasing effect with the concentration. Diversely, for the same targets, no significant effect of the assay was detected on mycelial reduction (Table 3), underlining the data reproducibility. Consequently, data of the three assays were combined and analyzed to calculate parameters of BP fungicidal potency based on mycelial growth reductions (Table 4).

Values of EC$_{50}$, EC$_{95}$ and MIC of BPs not ozonized were not found for almost all of tested targets except for *Monilia* sp., of which only EC$_{50}$ was determined (Table 4). Otherwise, in BPs OX amended media EC$_{50}$ values were determined for 5 pathogens (*B. cinerea*, *S. sclerotiorum*, *Monilia* sp., *S. rolfsii*, and *P. nicotianae*), EC$_{95}$ for 3 pathogens (*S. sclerotiorum*, *Monilia* sp., *S. rolfsii*) and, only for *S. rolfsii* MIC value was also intercepted. For *Fusaria*, *A. alternata* and *C. gloeosporioides*, BP OX, although supplemented at the highest concentration, was not effective and no fungal growth parameter was detected. Comprehensively, noteworthy were the antifungal potentialities of BP OX against basidiomycete *S. rolfsii*, followed by those found for ascomycetes *S. sclerotiorum* and *Monilia* sp. Although to a lesser extent, also BP OX performances *versus* *B. cinerea* and the oomycete *P. nicotianae* are encouraging. Based on our preliminary data, we may hypothesize for these sustainable compounds a wide spectrum of activity versus key fungal pathogens of

Table 2 Mean Germination Rate (MGR), Mean Germination Time (MGT), Germination Energy (GE), Speed of Emergence (SE), Coefficient of the Rate of Germination (CRG), and Time required for 50% germination (T50) of seeds treated with different concentrations of ADMBW BP (BP) and ADMBW BP OX (BP OX)

mg L^{-1}	Species	MGR (seeds day^{-1}) BP	MGR BP OX	MGT (days) BP	MGT BP OX	GE (%) BP	GE BP OX	SE (%) BP	SE BP OX	CRG (%) BP	CRG BP OX	T50 (days) BP	T50 BP OX
0	Cress	19.17±0.36	19.17±0.36a	1,08±0,04	1,08±0,04	91.67±3.60c	91.67±3.60	91.67±3.60b	91.67±3.60	92.62±3.15	92.62±3.15	1.00±0.00	1.00±0.00
	Lettuce	7.06±0.32	7.06±0.32a	2.26±0.06	2.26±0.06	60.00±4.71b	60.00±4.71b	77.98±3.98a	77.98±3.98a	44.30±1.19	44.30±1.19	2.57±0.06	2.57±0.06
	Tomato	4.64±0.22	4.64±0.22a	3.63±0.20	3.63±0.20	66.67±18.00a	66.67±18.00a	17.07±3.92a	17.07±3.92a	27.80±1.61	27.80±1.61	3.44±0.17	3.44±0.17
1	Cress	20.00±0.00	19.50±0.24a	1.00±0.00	1.05±0.02	100.00±0.00a	95.00±2.36	100.00±0.00a	95.00±2.36	100.00±0.00	95.38±2.14	1.00±0.00	1.00±0.00
	Lettuce	7.67±0.54	7.78±0.74a	2.48±0.12	2.07±0.06	53.33±7.20c	73.33±7.20	58.98±6.19b	92.59±6.05a	40.53±1.88	48.33±1.36	2.64±0.12	2.50±0.00
	Tomato	4.06±0.29	4.11±0.12a	3.65±0.18	3.29±0.08	41.67±13.61b	41.67±6.80b	12.69±4.26b	13.49±2.59b	27.58±1.44	30.50±0.80	3.61±0.18	3.46±0.10
10	Cress	19.33±0.36	19.33±0.14a	1.05±0.01	1.07±0.01	95.00±2.36a	93.33±1.36	96.58±1.40a	93.33±1.36	96.75±1.33	93.80±1.18	1.00±0.00	1.00±0.00
	Lettuce	7.00±0.57	7.33±0.48a	2.43±0.12	2.40±0.03	53.33±5.44c	56.67±2.72b	66.27±4.21b	68.25±1.30c	41.38±1.89	41.65±0.50	2.70±0.16	2.65±0.06
	Tomato	4.22±0.39	3.47±0.59a	3.70±0.09	4.02±0.17	66.67±18.00a	25.00±0.00c	7.12±0.58c	8.81±1.54c	27.11±1.08	25.01±1.08	3.85±0.01	3.65±0.06
100	Cress	19.67±0.27	19.50±0.24a	1.03±0.03	1.05±0.02	96.67±2.72b	95.00±2.36	96.67±2.72a	95.00±2.36	96.97±2.47	95.38±2.14	1.00±0.00	1.00±0.00
	Lettuce	7.67±0.24	7.61±0.75a	2.28±0.06	2.39±0.04	66.67±2.72a	56.67±9.81b	80.09±3.09a	64.23±6.45c	44.01±1.26	41.85±0.67	2.63±0.06	2.56±0.05
	Tomato	4.19±0.06	3.69±0.40a	3.69±0.11	3.38±0.09	25.00±0.00c	25.00±0.00	7.14±0.00c	8.86±0.96c	27.17±0.82	29.67±0.77	3.85±0.00	3.65±0.04
1000	Cress	19.00±0.41	19.83±0.14a	1.03±0.01	1.02±0.01	93.33±1.36a	98.33±1.36	96.67±1.36a	98.33±1.36	96.83±1.30	98.41±1.30	1.00±0.00	1.00±0.00
	Lettuce	7.89±0.33	7.28±0.92a	2.27±0.08	2.31±0.09	63.33±7.20a	60.00±8.16b	73.15±7.89b	74.72±5.28b	44.24±1.52	43.51±1.66	2.50±0.00	2.60±0.08
	Tomato	3.86±0.72	4.33±0.20a	3.76±0.08	3.59±0.04	25.00±0.00c	25.00±0.00	8.84±2.24c	7.01±0.28c	26.65±0.55	27.90±0.33	3.69±0.11	3.72±0.01
5000	Cress	19.67±0.27	18.00±0.41b	1.03±0.03	1.07±0.04	96.67±2.72b	86.67±1.36	96.67±2.72a	93.25±3.59	96.97±2.47	93.99±3.10	1.00±0.00	1.00±0.00
	Lettuce	7.56±0.51	6.22±0.09b	2.35±0.06	2.33±0.04	66.67±2.72a	46.67±2.72c	81.07±4.54a	66.67±3.89c	42.73±1.13	42.89±0.70	2.78±0.04	2.50±0.00
	Tomato	3.83±0.34	3.06±0.33b	3.96±0.03	3.99±0.08	25.00±0.00c	25.00±0.00	7.49±0.68c	9.58±1.28c	25.27±0.22	25.11±0.49	3.70±0.03	3.62±0.05

Data (± standard error bar) about treatment performance are the means of three replicates. Letters indicate significant differences for each species according to Fisher's LSD test ($\alpha = 0.05$). The absence of letters indicates the lack of significant differences

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important commercial crops that should be verified in future in in vivo experiments.

Figure 5 visually shows the in vitro potentiality of BP, BP OX and BTH against fungal targets. Relating to the fungal pathogens previously found sensitive to the BP OX treatments (Table 3, Fig. 5), Fig. 6 shows the compared effects of BP OX at 5000 mg L^{-1} and BTH at 3000 mg L^{-1} in reducing fungal growth of each target. In detail, it may

be observed that the performances in reducing fungal growth of BTH at 3000 mg L^{-1} are significantly higher than those achieved with BP OX at 5000 mg L^{-1}, for *B. cinerea*, *Monilia* sp. and *P. nicotianae* but significantly lower for *S. rolfsii* and similar (data not significant) in the case of *S. sclerotiorum*.

Comprehensively, our data from repeated bioassays represent very remarkable and promising findings, since

Table 3 ANOVA effects of single factors in laboratory on mycelial reductions of some targeted pathogen

Factor	*B. cinerea* Di3A-E2		*S. rolfsii* Di3A-TP3A		*S. sclerotiorum* Di3A-L3		*Monilia* sp. Di3A-A1		*P. nicotianae* Di3A-B1	
	F	P value	F	P value	F	P value	F	P value	F	P value
BP Ox treatment	7712.9	< 0.0001	8244.6	< 0.0001	11197.1	< 0.0001	1784.02	< 0.0001	212.07	< 0.0001
BP concentration	5560.8	< 0.0001	4646.2	< 0.0001	9339.80	< 0.0001	2862.2	< 0.0001	1825.45	< 0.0001
Assay	0.234	0.7924 ns	1.95	0.1577 ns	1.20	0.3135 ns	3.11	0.0565 ns	0.09	0.9148 ns

P value of fixed effects associated to *F* test

Table 4 EC$_{50}$, EC$_{95}$ and MIC (mg L^{-1}) values of BP and BP OX *versus* sensitive pathogens

Fungal target	BPs			BPs OX		
	EC$_{50}$	EC$_{95}$	MIC	EC$_{50}$	EC$_{95}$	MIC
Botrytis cinerea Di3A-E2	–	–	–	4678.7	–	–
Monilia spp. Di3A-A1	5134.4	–	–	2469.7	5652.7	–
Sclerotinia sclerotiorum Di3A-L3	–	–	–	2885.9	5315.0	–
Sclerotium rolfsii Di3A-TP3A	–	–	–	798.5	4446.0	5000.0
Phytophthora nicotianae Di3A-B1	–	–	–	5608.3	–	–

– not detected

Fig. 5 Exemplificative overview of in vitro performances of ADMBW BP and ADMBW BP OX compared with those of BTH in reducing/inhibiting mycelial growth of *Botrytis cinerea*, *Monilia* sp., *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Phytopthora nicotianae* on PDA amended at different concentrations 108 h after fungal inoculation

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Fig. 6 Compared mycelial reductions detected at the highest rates of BP OX (5000 mg L^{-1}) and BTH (3000 mg L^{-1})
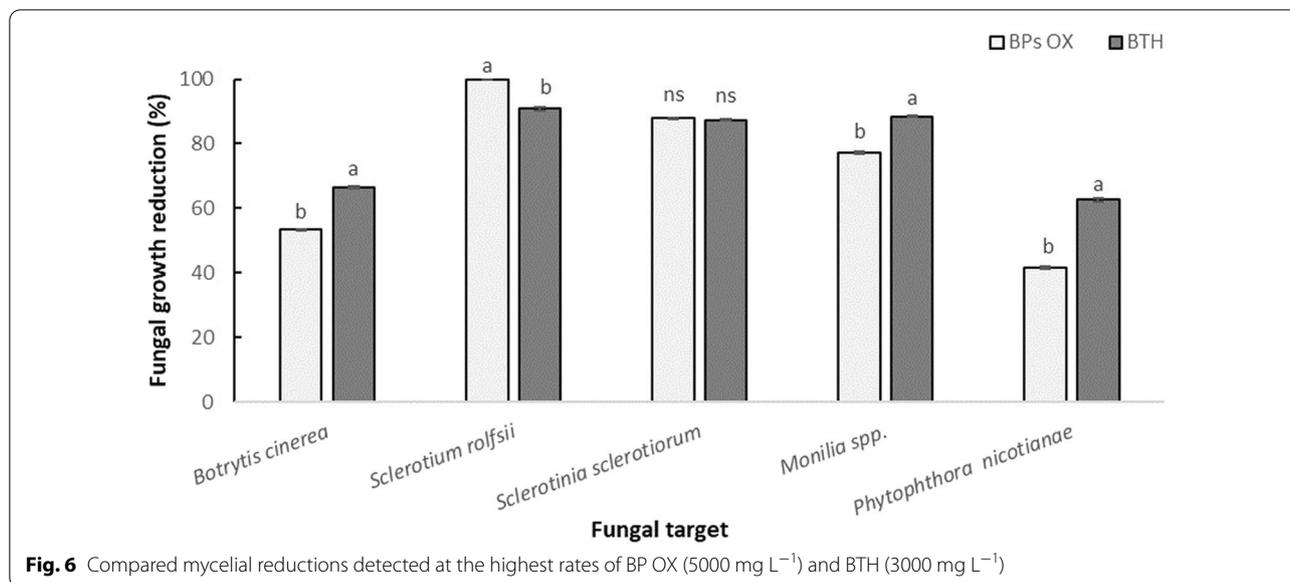
EC_{50}, EC_{95} and MIC are considered as the most important basic parameters to express antifungal properties and compare preliminary potencies among antifungal compounds [36]. The BP antifungal properties were previously reported only for *Leptosphaeria maculans*, causal agent of blackleg of oilseed rape (*Brassica napus* L.) [25]. For this past report, ADMBW BP revealed good performances, although to a lesser extent than BTH, in reducing leaf necrosis. Our findings, although should be confirmed by *in planta* assays, clearly showed as BPs OX could presumably exert a fungicide activity *versus* a wide spectrum of key phytopathogenic fungi including ascomycetes, basidiomycetes and oomycetes representing heavy threats for vegetable, fruit and ornamental crops worldwide. Unlike to previous data [25], further remarkable aspects of this research are that the antifungal properties of BP OX depend on the target and, surprisingly, this compound could exhibit in some cases similar or superior performances than BTH. The collected data obtained in the present work with the BP and BPox encourage to implement further R&D work to assess whether the use of BPs from other biowastes of urban and agriculture sources will produce similar or better results.

Conclusions

The present work provides further insights into the BP biostimulant properties reported in previous studies performed on the cultivation of several food and ornamental plants. Specifically, it points out that, depending on the cultivated plant species and the applied doses, BPs may induce increased plant growth or phytotoxic effects. The detailed analyses of germination indices suggest that the effect of BPs depends on the species to which is applied and BP concentration. The ADMBW BP proves to act as inducer of the germination process, at all the concentrations tested on cress and lettuce, exerting no effect on tomato seeds. ADMBW BP OX showed a different behaviour, as it resulted a positive effector on cress and lettuce at low concentrations, but phytotoxic at the highest one, in particular in tomato seeds, which was the most sensitive species. Moreover, to the best of authors' knowledge, this paper shows for the first time the multiple high potentiality of oxidized biowaste compound to be used simultaneously against several key phytopathogenic fungi. Likewise, the oxidation of BPs obtained from other biowaste materials may yield products inhibiting the mycelial growth of other specific pathogens that threaten different vegetable and ornamental crops. Although these results need an in vivo confirmation, this paper opens the way to a further sustainable exploitation of biowaste materials through which it might contribute for a more circular economy, aiming to a multiuse purpose of BPs as biostimulant, herbicide, and antifungal compounds, depending on their concentration and species. Comprehensively, the results of the herein reported work prospect a feasible development of new BPs-based farming practices for a more sustainable agriculture. These findings constitute a further case demonstrating how the application of low temperature chemical reactions to biowaste might yield high value products. On the other hand, they allow estimating the economic contribution of the new BP to support the implementation of the rising biobased chemical industry, with specific reference to new MBW fed biorefineries integrating chemical and

biochemical processes. Moreover, in the actual historical moment, these results are very interesting, because from a biowaste as ADMBW, which is worldwide constantly produced, may be possible to obtain agrochemicals and fertilizers without the usual feedstocks, which are more and more expensive.

Abbreviations
BPs: Bioproducts; ADMBW: Anaerobic digestate of municipal biowastes; ADMBW OX: Oxidized anaerobic digestate of municipal biowastes; EC: Effective concentration; MIC: Minimum inhibitory concentration; MBW: Municipal biowaste; LG: Lignin; BTH: Benzothiadazole; GI: Germination Index; GP: Germination Percentage; MDG: Mean Daily Germination; SVI: Seedling Vigor Index; MGR: Mean Germination Rate; MGT: Mean Germination Time; GE: Germination Energy; SE: Speed of Emergence; CRG: Coefficient of the Rate of Germination; T50: Time required for 50% germination.

Author contributions
EM: conceptualization: AB, AV, IP, and EP: formal analysis: FF, IC and EP: investigation; FF and IC: methodology; EM. IP, AV, AB: validation; AB and AV: supervision; AB: project administration; EM, IP, FF, IC, AV, AB: writing manuscript draft; IP, AV, AB and EM: providing final draft. All authors read and approved the final manuscript.

Availability of data and materials
The data used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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