Activation of fulvic acid-like in paper mill effluents using H_2O_2/TiO_2 catalytic oxidation: Characterization and salt stress bioassays

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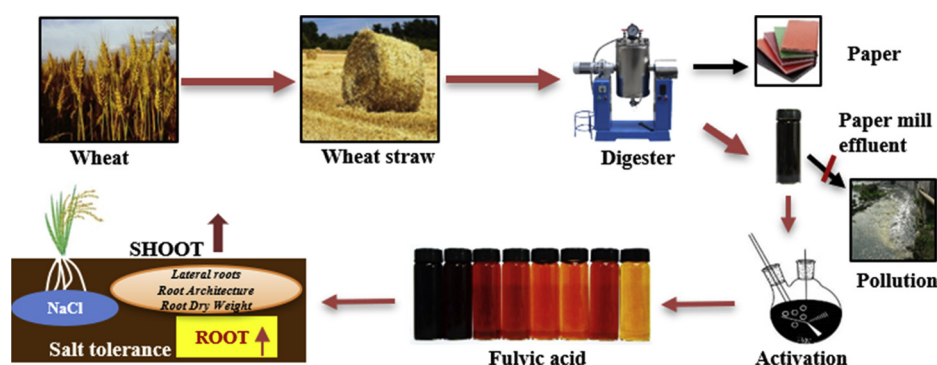[d] Key Laboratory of Humic Acid Fertilizer of Ministry of Agriculture, Shandong Agricultural University Fertilizer Technology Co. Ltd, Feicheng, Shandong, 271600, China
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GRAPHICAL ABSTRACT

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ABSTRACT

Increasing environmental concerns about organic waste in paper mill effluents demand alternative wastewater management technology. We reported novel activation of fulvic acid-like in paper mill effluents using hydrogen peroxide (H_2O_2) as oxidizer and titanium oxide (TiO_2) as catalyst. Spectroscopic characteristics of fulvic acid-like in paper mill effluents before and after activation (PFA and PFA-Os, respectively) were compared with a benchmark fulvic acid extracted from leonardite (LFA). Results indicated that PFA-Os exhibited less lignin structures, more functional groups and lower molecular weight than PFA, sharing much similarity with LFA. Among PFA-Os with varying degrees of oxidation, PFA-O-3 activated with 1:2 vol ratio of paper mill effluent and 30% H_2O_2 for 20 min digestion at 90 °C stands out to be the optimal for further examination of its biological activity. Bioassays with rice seed/seedling indicated that applications of LFA at 2–5 mg-C/L and PFA-O-3 at

60–100 mg-C/L significantly increased rice seed germination rate and seedling growth under salt stress imposed with 100 mM NaCl. The mechanism was mainly through reduced oxidative damage via activation of anti-oxidative enzymes and lipid peroxidation. This study provides the needed technical basis of safer and cleaner technologies for innovative management of paper mill effluents.

1. Introduction

The pulp and paper industry, one of the most important industry sectors, ranks the world's sixth largest industrial polluter, posing a serious threat to the environment and human health [1–4]. Conventional chemical and/or biological treatment methods aiming to reduce organic waste loads are often challenged by the recalcitrant nature and high toxicity of the effluents [5–8]. The high cost involved with treatment of paper mill effluents inserts further economic burden to the industry [9,10]. Thus, a cost-effective, green technology is critically needed for treatment of paper mill effluents. In this paper, we studied an alternative technology to utilize the organic waste in paper mill effluents for sustainable agricultural.

Humic substances, which are complex, heterogeneous mixtures of polyelectrolytic macromolecules, are widely used in sustainable agriculture to enhance plant growth and physiology [11–13]. Sources of humic substances are primarily non-renewable resources such as peats and leonardite [14]. The rising market of using humic substances as plant stimulator has generated a focal interest in using organic wastes as a new sustainable resource for commercial humic substances products. Previous work verified that the recalcitrant organic matters in paper mill effluents, mainly degradation products of starch, carbohydrates, lignin derivatives, and proteins, shared similar spectroscopic characteristics with humic substances [15–18]. Particularly, the organic matters in paper mill effluents resemble fulvic acid (FA), an essential component of humic substances, which accounted for 54–68% of the total organic carbon (C) [19].

The benefits of FA in regulating plant growth has been well recognized [20]. In general, FA can induce changes in the primary and secondary metabolic pathways of plants associated with abiotic stress tolerance. For example, FA can increase root growth and decrease membrane damage, thereby promoting the growth of trees under salt stress [21]. The biological activity of FA as plant growth promoters depends largely on its rich functional groups and low molecular weight (MW), whereby FA molecules can easily penetrate cell membranes [22]. The organic compounds in paper mill effluents, however, feature high MW, thereby lacking the needed biological activity for agricultural applications [6,23,24].

The hypothesis of this study is that the fulvic acid-like in paper mill effluents can be chemically activated to enhance its biological activity as a plant growth promoter. Oxidation of organic compounds with H_2O_2 into low MW derivatives has been reported by several workers [25,26], and TiO_2 may serve as a catalyst to enhance the reaction [27–29]. However, no reports about appropriate use of H_2O_2/TiO_2 for activation of fulvic acid-like in paper mill effluents are available in the literature. Therefore, the first objective of this study is to identify the optimal reaction condition for activation of fulvic acid-like in paper mill effluents using H_2O_2 as oxidizer and TiO_2 as catalyst. To achieve this goal, we varied H_2O_2 dosage, reaction temperature, and reaction time in our experimental design. The second objective of the study is to compare the elemental composition and spectroscopic characteristics of fulvic acid-like in paper mill effluents before and after activation (PFA and PFA-Os, respectively) with a benchmark FA extracted from leonardite (LFA). Spectroscopic techniques including Fourier transformed infrared spectrophotometry (FTIR), size exclusion chromatography (SEC), ^{13}C nuclear magnetic resonance with cross-polarization/magic angle spinning (CP/ MAS ^{13}C NMR) were employed to determine the molecular weight and structures as well as functional groups of these fulvic acid-likes.

The PFA-O with a relatively high yield, low MW and abundant functional groups is further studied to evaluate its biological activity. Thus, the third objective of this study is to identify a functional application range of the selected PFA-O with rice germination and seedling growth bioassays under salt stress. The antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) as well as lipid peroxidation are measured to evaluate the biological activity of PFA-O in relation to oxidative damages induced by salt stress at the cellular level. Our results can provide a technical basis for safer and cleaner technologies to manage the paper mill effluents. Sustainable utilization of organic matter in paper mill effluents can help address the environmental impacts associated with the paper-making process.

2. Materials and methods

2.1. Materials

The leonardite was from Shanxi Province, China. All chemicals were purchased at the analytical grade. Solutions were prepared with distilled water.

2.2. Paper effluents manufacturing process

The pulp and paper mill effluents were produced with the technology kindly provided by Shandong Tranlin group (Shandong, China). Briefly, wheat straws harvested from the Agricultural Experimental Station of Shandong Agricultural University were air dried and grounded into pieces. The raw material was weighed and added into a digester. Solid $(NH_4)_2SO_3$ and ammonia water was added to reach a concentration of 20% (w/w) and 0.1% (v/v), respectively, based on the dry weight of the raw material. Digestion was facilitated with steams of 240 °C under 0.2 mPa for 400 min. After digestion, the mixture was passed through a 200-mesh screen. The effluents were kept in a container and stored at 4 °C.

2.3. Activation of fulvic acid-likes in effluent

An aliquot of 100 mL effluent, after equilibrated with the room temperature, was mixed with 100 mL 0.1 M NaOH, 0.1 g TiO_2 and a fixed dosage of 30% H_2O_2 in a digester under N_2 atmosphere. The system conditions examined in this study consisted of three H_2O_2 dosages (effluent to H_2O_2 volume ratio of 1, 2, 3), three reaction temperatures (70, 80, 90 °C), and three reaction time (20, 40, 60 min) for a total of seven treatments (PFA-O-1~7, Table 1).

Table 1
Treatments of the paper mill effluent activation.

Treatments	Paper mill effluent/H_2O_2 volume ratio	Temperature (°C)	Time (min)
PFA-O-1	1:1	70	20
PFA-O-2	1:1	90	40
PFA-O-3	1:2	90	20
PFA-O-4	1:2	80	40
PFA-O-5	1:3	80	20
PFA-O-6	1:3	70	40
PFA-O-7	1:3	90	60

2.4. Fulvic acid-likes isolation

Extraction of FA and fulvic acid-likes from leonardite and paper mill effluents before and after activation followed the International Humic Substance Society (IHSS) procedure (http://humic-substances.org). The final solution containing fulvic acid-like was freeze-dried and the obtained fulvic acid-like was kept in a desiccator over silica gel for further analysis. Fulvic acid-like yields of PFA and PFA-Os was calculated as the percentage of organic carbon recovered after extraction from the original effluent. Organic C was determined by the potassium dichromate oxidation method [30].

2.5. Characterization of fulvic acid-likes

Elemental analysis (C, H, O, N and S) was determined by a Vario-EL Elemental Analyzer (Germany). Each treatment was performed in triplicate. Infrared spectra (FTIR) were recorded with a Nicolet Magna-IR 550 spectrometer over the 4000–400 cm^{-1} range, with a resolution of 2 cm^{-1} [31]. Size exclusion chromatography (SEC) was performed on solutions of the sample using a Sephadex G-100 medium gel (Code No. 17-0060-02 Pharmacia Biotech AB) [32]. Solid-State CP-MAS ^{13}C NMR Spectroscopy was acquired with an Avance 600 MHz (Bruker, Karlsruhe, Germany) spectrometer [31].

2.6. Rice seed germination bioassay

The rice seeds (*Oryza sativa* L, Jinghua 208) were surface sterilized with 5% NaClO for 10 min, rinsed five times with distilled water, and air dried. These seeds were then presoaked for 24 h in different concentrations of LFA (1, 2, 5, 10, 20 mg-C/L) and the selected PFA-O (40, 60, 80, 100, 120 mg-C/L) under dark condition. Seeds were washed five times, air dried, and transferred into petri dishes with filter paper wetted by 10 mL of 100 mM NaCl solution to introduce salt stress. Germination tests were carried out on four replicates of 200 seeds with 50 seeds in each dish. All seeds were kept in a growth incubator set at 28 °C. Germination rate was recorded every 24 h for 120 h. A seed was considered germinated when the emerging shoot reached on half of the seed length. Preliminary germination tests following the above procedure with NaCl concentrations of 0, 50, 100, 150, and 200 mM, respectively, established that 100 mM NaCl introduced appropriate salt stress.

2.7. Rice seedling growth bioassay

To determine whether the selected PFA-O can alleviate salt stress on rice seedling growth, five-day-old seedlings germinated in water were transferred to 1/4 international rice nutrient solution in a growth incubator. The nutrient solution was renewed daily for 5 days. The seedlings were then treated with the following for 2 days: (1) nutrient solution; (2) nutrient solution containing LFA (1, 2, 5, 10, 20 mg-C/L); (3) nutrient solution containing the selected PFA-O (40, 60, 80, 100, 120 mg-C/L). Then, seedlings were grown in nutrient solution containing 0 or 100 mM NaCl for 3 days. The seedlings were harvested, measured, weighed, and frozen in liquid nitrogen for further analysis. Seedling tests were carried out with four replicates with 32 seedlings for each treatment and 8 seeding in each pot.

2.8. Antioxidant enzymes and lipid peroxidation

The superoxide dismutase (SOD) activity was assayed based on its ability to reduce nitroblue tetrazolium by super anion generated by riboflavin system under illustration [33]. Peroxidase (POD) activity was measured by the ability with catalytic H_2O_2 to oxidize guaiacol to produce a dark brown product [34]. Catalase (CAT) activity was spectrophotometrically measured by monitoring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for at least

3 min [35]. The level of lipid peroxidation was assessed by determining the activity of malondialdehyde (MDA) [36].

2.9. Quality control and quality assurance

The quality control and quality assurance program were conducted to assure the repeatability and reproducibility of collected data. The activation experiments, determination of organic C as to calculate the fulvic acid-like yield and elemental analysis were performed in triplicate as to lower its error. For the seed germination and seedling growth experiment under salt stress, each sample were carried out with four replicates. Control samples was conducted with the same concentration of NaCl but without fulvic acid or fulvic acid-like. It helped to confirm the results of fulvic acid and fulvic acid-like on the salt tolerance of rice. The determination of salt tolerance indicators was also performed in four replicates.

2.10. Statistical analyses

All statistical analyses were conducted using Statistical Analysis System (SAS Version 9.2). Significant differences among treatments were determined using mean separation tests (Duncan's multiple range test) at $p < 0.05$.

3. Results and discussion

3.1. Elemental compostion and fulvic acid-like yields

The elemental analy sis results showed that PFA contained more C, H, N and S but less O than LFA and the purpling process with $(NH_4)_2SO_3$ resulted in the incorporation of S in PFA (Table 2). The lower O/C ratio of PFA than LFA indicated that PFA contained less carboxyl functional group than LFA, and this can influence its bioactivity in some extent. After the oxidation with H_2O_2, the enriched N, S and O in PFA-Os indicated that the contents of N, O or S functional groups increased (Table 2). The higher H/C and O/C ratios of PFA-Os than PFA suggest that activation resulted in lower MW and more abundant functional groups in PFA-Os [37,38]. Visual observation indicated that the black color of PFA was changed to varying degrees of orange after oxidation by H_2O_2 (Fig. S1).

For a given quantity of organic C in the effluent, fulvic acid-like yields, which is calculated as the percentage of organic C retained in fulvic acid-like from the original effluent, ranged from about 33 to 59% (Fig. 1). The yield of PFA-O-1 (58.2%) was similar to that of PFA (56%), indicating minimum degree of oxidation. The yield decreased with further increase in H_2O_2 dosage, reaction temperature, and reaction time. For example, PFA-O-7 has the lowest yield (33.3%), PFA-O-3 had a yield of 48.5%. The depleted C, lower MW and higher content of functional groups in PFA-Os showed the depolymerization of

Table 2
Elemental composition of LFA, PFA, and PFA-Os.

Treatments	Mass (%)					Atomic ratio	
	C	H	N	O	S	H/C	O/C
LFA	28.17 c	1.91 g	0.39 h	68.74 a	0.79 e	0.81 g	1.83 a
PFA	38.66 a	4.26 b	2.27 g	48.08 f	6.73 d	1.32 f	0.93 f
PFA-O-1	28.46 c	3.78 d	5.34 d	50.29 e	11.49 a	1.60 d	1.33 e
PFA-O-2	26.54 e	4.44 a	7.93 a	50.11 e	10.47 c	2.01 a	1.42 d
PFA-O-3	24.81 f	4.05 c	7.60 b	52.94 d	10.43 c	1.96 ab	1.60 b
PFA-O-4	29.4 b	3.21 f	2.55 f	53.19 cd	11.05 b	1.31 f	1.36 e
PFA-O-5	26.97 d	3.32 ef	3.85 e	53.69 c	11.63 a	1.48 e	1.49 c
PFA-O-6	24.28 g	3.95 c	7.54 b	52.97 d	10.93 c	1.95 b	1.64 b
PFA-O-7	22.92 h	3.38 e	6.25 c	55.14 b	11.62 a	1.77 c	1.80 a

Mean values with the same letter did not differ significantly at $p < 0.05$. (n = 3).

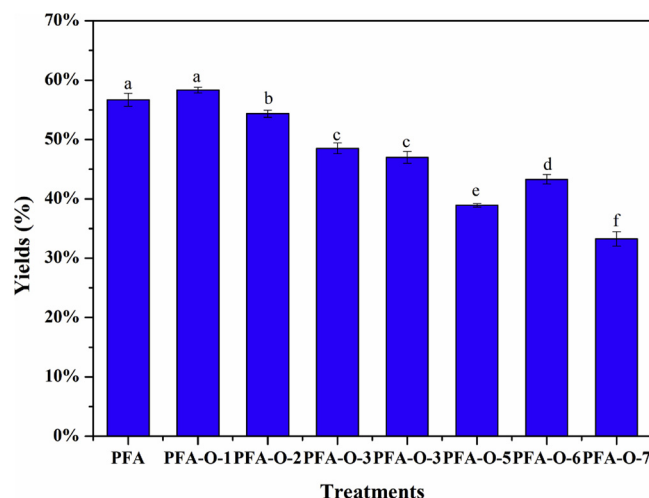**Fig. 1.** Fulvic acid-like yields from paper mill effluents. Mean values with the same letter did not differ significantly at $p <$ 0.05. Error bars represent the stand error (n = 3).
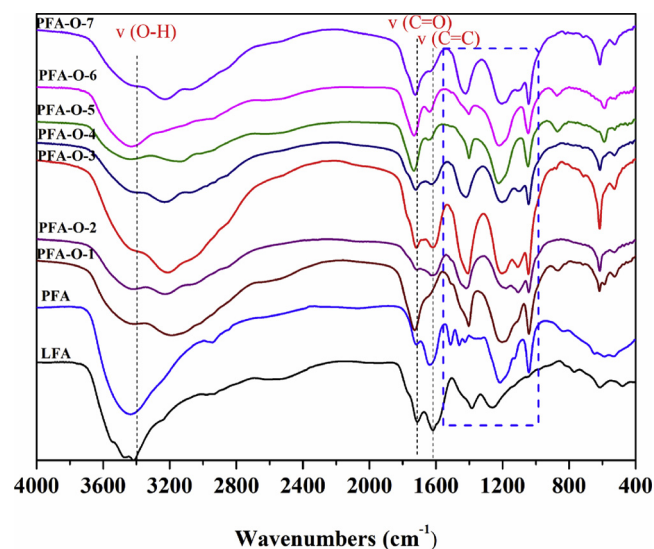

Fig. 4. Solid CP-MAS ^{13}C NMR spectra of LFA, PFA, and PFA-O-3.

Table 3
Integration Area of ^{13}C CPMAS-NMR Spectra from fulvic acid-likes.

Treatments	chemical shift (ppm)						
	0–45	45–65	65–95	95–110	110–140	140–160	160–190
LFA	16.65	11.38	7.75	3.35	34.97	8.98	16.92
PFA	11.16	21.18	12.96	6.92	23.95	16.45	7.38
PFA-O-3	16.76	18.29	24.44	4.91	9.92	6.54	19.14

macromolecules is accompanied with the release of CO_2.

3.2. FTIR spectroscopy

The FTIR spectra (Fig. 2) exhibit typical absorption bands of FA [35,36,40,41]. All spectra showed a large broad band at approximately 3400-3200 cm^{-1}, which are normally attributed to stretching of O−H bonds in phenol, hydroxyl and carboxyl groups [42]. The band at 1720-1700 cm^{-1} is related to C−O vibration, indicative of carboxyl groups. The bands at 1660–1600 cm^{-1} is attributed to C═C stretching of aromatic rings, and to C═O stretching of conjugated carbonyl groups. Effects of oxidation are obvious at the 1515, 1465–1450, 1426, 1420–1384, 1200 and 1125–1080 cm^{-1} bands (Fig. 2). The band at 1515 cm^{-1} in PFA represents lignin [23], which is also assigned to C═C stretching of aromatic rings. This band does not exist in PFA-Os,

Fig. 2. FTIR spectra (4000-400 cm^{-1}) of LFA, PFA, and PFA-Os.

macromolecules with H_2O_2 mediated oxidation, consistent with the results that H_2O_2 can mediated the oxidation of organic matter in water [39]. The lower yield after activation indicates that oxidation of
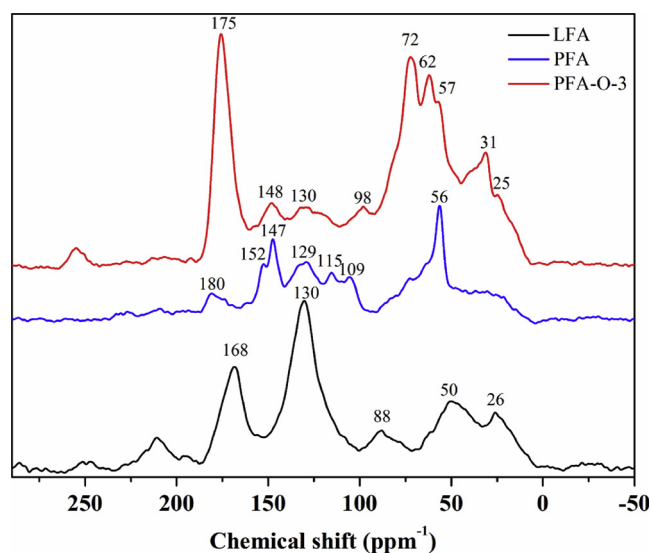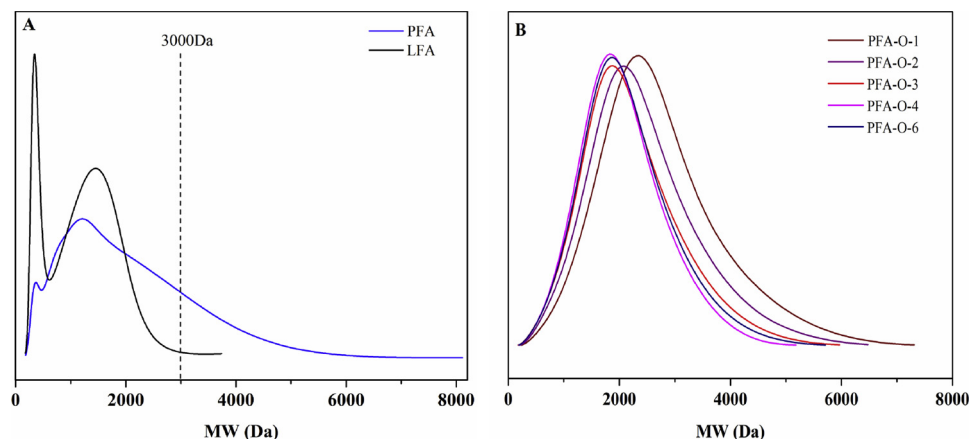

Fig. 3. Size exclusion chromatograms monitored by refractive index detector for (A) LFA and PFA; (B) PFA-Os.
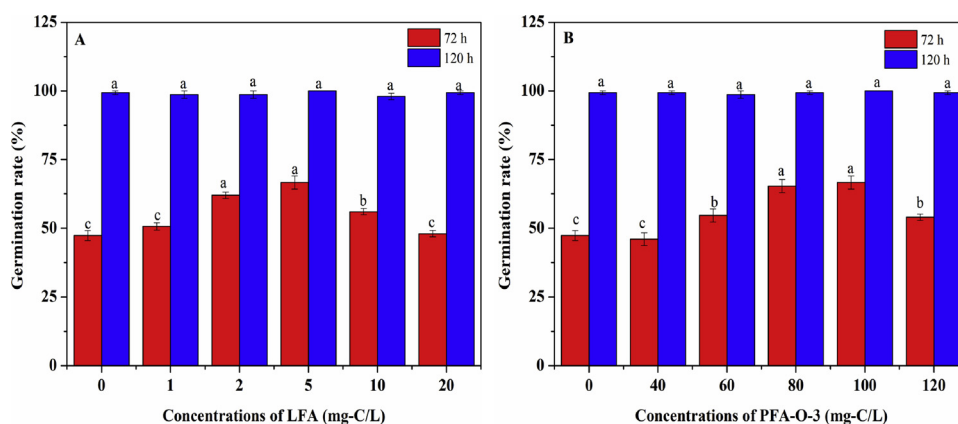
Fig. 5. Effects of fulvic acid-like on rice seed germination under salt stress condition measured at 72 h and 120 h: (A) LFA (B) PFA-O-3. Mean values with the same letter did not differ significantly at $p < 0.05$. Error bars represent the stand error (n = 4).
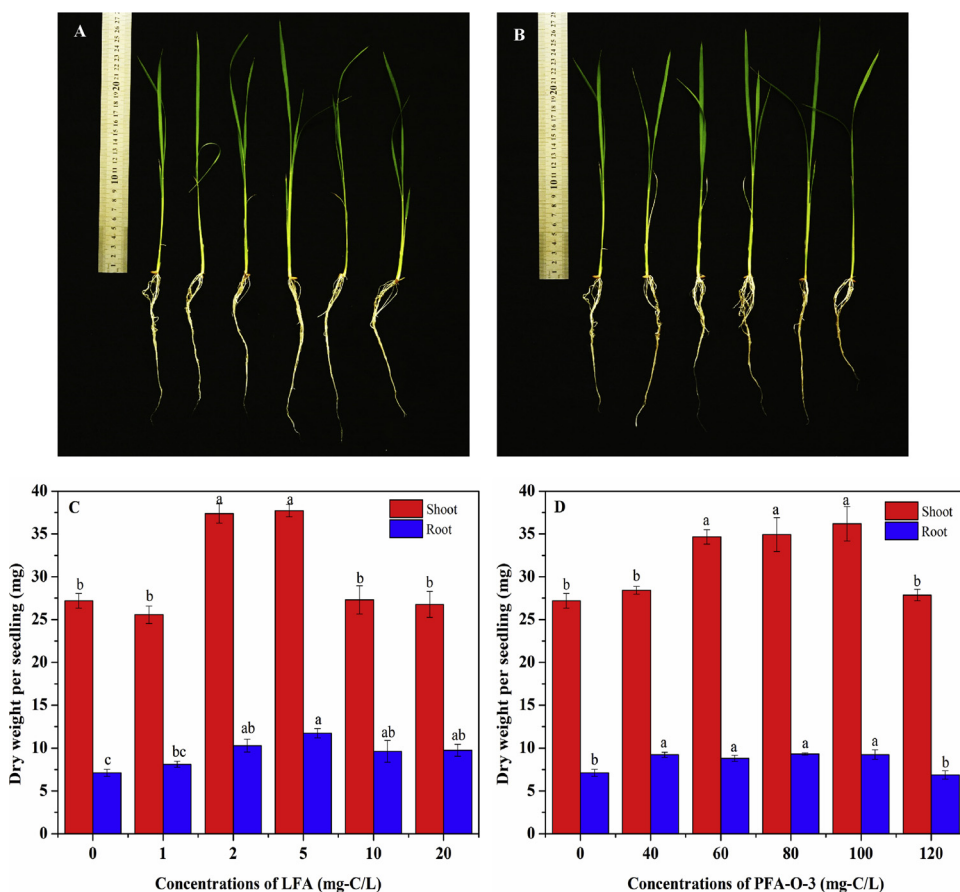

Fig. 6. Effects of fulvic acid-like on rice seedling growth under salt stress condition. (A) picture of seedings treated by LFA, (B) biomass of seedling treated by LFA, (C) picture of seedings treated by PFA-O-3, and (D) biomass of seedling treated by PFA-O-3. Mean values with the same letter did not differ significantly at $p < 0.05$. Error bars represent the stand error (n = 4).

indicating that the lignin structures had been oxidized, which is in consist with the results that H_2O_2 can mediate the oxidation of kraft lignin [43]. Similarly, the band at 1465 cm^{-1} in PFA, attributed to C−H stretching of CH_2 and CH_3 groups of aliphatic chains, and the band at 1426 cm^{-1} in PFA, attributed to stretching of O−H bonds in carboxyl, are not shown in PFA-Os. There are also bands that are intense in PFA-Os but do not exist in PFA. For example, the band at 1420-1384 cm^{-1} in PFA-Os, assigned to antisymmetric COO− stretching, aliphatic C−H deformation and the C−O stretching in phenols [44,45], and the band at approximately 1200 cm^{-1} in PFA-Os, attributed to stretching of O−H and C−O deformation of COOH, are not present in PFA. Those results reveal that H_2O_2 oxidized the lignin structure in paper mill effluent accompanied with the increase of functional groups. The results are in line with the report that the polyfunctional monomeric compounds,

such as aromatic aldehydes and carboxylic acids, are the mainly oxidation products of lignin [46]. Furthermore, compared with LFA and PFA, PFA-Os have a distinct strong band at 615 cm^{-1}, which is attributed to the S−O stretching of sulfonic groups. This unique band in PFA-Os indicates that oxidation helped formation of sulfur-containing functional groups. Among all PFA-Os, the main differences are the intensity of signals. For PFA-O-5, PFA-O-6 and PFA-O-7, the signal bands and their intensities had no significant difference, and the yield of fulvic acid-like decreased, suggesting that H_2O_2 is overdosed and the overdose of H_2O_2 resulted in the overoxidation of functional groups and loss of fulvic acid-like. For PFA-O-1, PFA-O-2, PFA-O-3 and PFA-O-4, the signal intensities are obviously different. The stronger signals of PFA-O-1 and PFA-O-3 means that the control of reaction time can regulate the content and oxidization degree of functional groups in fulvic acid-like.

Table 4
Superoxide dismutase (SOD), peroxide (POD), and catalase (CAT) activities in rice seedlings under salt stress in response to fulvic acid-likes.

FA	Concentrations (mg-C/L)	SOD (Ug^{-1} FW)	POD ($Ug^{-1} min^{-1}$FW)	CAT ($Ug^{-1} min^{-1}$FW)
	0	313.65 b	379.99 d	14.76 b
	1	517.95 a	436.09 c	14.07 bc
LFA	2	505.52 a	441.19 c	16.55 a
	5	514.17 a	481.02 c	11.17 d
	10	517.13 b	606.67 a	13.64 bc
	20	505.47 a	549.87 b	13.00 c
	0	313.65 c	379.99 d	14.76 b
	40	385.97 b	461.77 b	12.06 d
PFA-O-3	60	472.33 a	397.00 cd	13.43 c
	80	515.44 a	430.19 bc	13.12 c
	100	396.10 b	434.53 bc	16.33 a
	120	485.03 a	519.93 a	13.63 c

Mean values with the same letter did not differ significantly at $p < 0.05$. (n = 4).

In all, PFA-O-3 has the most diversified and strongest absorption bands.

In conclusion, the FTIR of PFA-Os shows more similarities in spectroscopic characteristics with LFA than PFA after the acticetion of H_2O_2 with TiO_2 as catalyst. Besides, PFA-Os showed higher contents of functional groups and less lignin structure than PFA. The best treatment is PFA-O-3.

3.3. SEC analysis

SEC analysis provides a qualitative assessment of MW distribution of fulvic acid-likes. Consient with the literature, our results also showed that PFA had a higher MW than LFA [23,24] (Fig. 3A). The low MW fraction (MW < 3 kDa) was 98.9% for LFA and 75.9% for PFA. The MW distribution of PFA-O-1, 2, 3, 4 and 6 showed a much lower MW than PFA with only one well-separated peak centered around 1.8–2.8 kDa (Fig. 3B). The ratio of low MW fraction (MW < 3 kDa) was 80.1%, 86.1%, 90.8%, 93.54% and 92.4%, respectively, for PFA-O-1, 2, 3, 4 and 6. The results showed that H_2O_2 lowered the molecular weight of fulvic acid-like under the control of reaction conditions.

When compare the MW distribution, functional groups, and FA yield with different treatments, we found that the control of H_2O_2 dosage have strongly influenced on oxidation products and the control of reaction time within suitable H_2O_2 dosage can influence the content of functional groups. By controlling the reaction conditions (H_2O_2 dosage, reaction temperature, and reaction time), PFA-Os with lower molecular weight, more functional groups and higher yield can be produced, and PFA-O-3 with 1:2 vol ratio of paper mill effluent and 30% H_2O_2 for 20 min digestion at 90 °C stands out as the optimal option for further

analysis.

3.4. Solide-^{13}C NMR analysis

Detailed C functionality distributions and structural information of LFA, PFA and PFA-O-3 were obtained by solid-state CP-MAS ^{13}C NMR (Fig. 4). Typical peaks of FA in chemical shifts existed in all fulvic acid-like fractions, indicating that they share similar structural features [21]. The relative distribution of signal areas normalized by integrating the ^{13}C chemical shift intervals is summarized in Table 3. The sharp peaks of LFA, PFA-O-3 and broad signals of PFA in the alkyl C (0–45 ppm) range reveal the presence of alkyl chains (−CH_2− and −CH_3 groups). Both LFA and PFA-O-3 have a higher alkyl-C content than PFA (Table 3). The observed relative lower intensity of methoxy substituent (45–65 ppm) in the spectra of LFA is indicative of mineralization of bioavailable compounds. The main signal centered on 56 ppm of PFA represents lignin derivatives [21,47]. For PFA-O-3, the strong signal within the 45–60 ppm chemical shift can be attributed to the C−N bond in the amino acid moiety, consistent with the elemental analysis results (Table 1). The intense signal at 72 ppm indicates a higher content of O-alkyl-C, which are assigned to the polysaccharide chains of wheat tissue [48]. The signal at 109 ppm in the spectra of PFA indicates a higher content of anomeric carbons of carbohydrates. The increase of carbohydrates after oxidization was also observed in kraft pulp mill effluent when treated by sun irradiation [18]. Our results indicated that the lignin fraction in paper mill effluent had been oxidized since the release of carbohydrate generally happens with the conversion of lignin.

The most distinctive features of LFA, PLA and PFA-Os were in the aromatic/olefinic-C region (110–140 ppm). The larger relative intensity around 130 ppm of LFA showed a higher aromatic C, consistent with elemental analysis. For PFA and PFA-O-3, the different resonances around 110–140 ppm are related to the unsubstituted C of lignin monomers. PFA-O-3 has much lower ratio in this region, confirming the effects of oxidation. The signal intensity at 147 ppm and 152 ppm shown by the specific O-aromatic region and phenols (140–160 ppm) in PFA confirmed the structures of lignin by the incorporation of O-substituted ring C, but the signals are relatively lower in PFA-O-3 and LFA. Finally, the strong signal in the carbonyl region (160–190 ppm) at 168 ppm of LFA and 175 ppm of PFA-O-3 suggests the contribution of carbonyl groups to those two FAs, but PFA has much lower absorbance at 180 ppm (carboxyl groups).

While, PFA-O-3 shares much similarity with LFA, and LFA has a higher content of aromatic C and phenol. Meanwhile, compared with PFA, PFA-O-3 has less lignin derived aromatics structure, and more carboxyl C, alkyl-C and carbohydrates. The decrease of aromatic structure is contrary to the results that sun irradiation increased the aromatic structure [18]. The reason may be that the strong oxidization
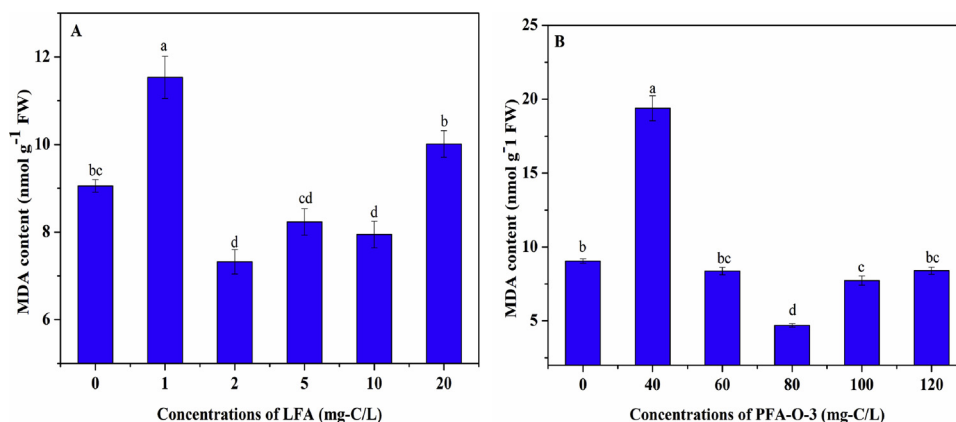

Fig. 7. Malondialdehyde (MDA) contents in rice seedlings under salt stress pretreated with (A) LFA (B) PFA-O-3. Mean values with the same letter did not differ significantly at $p < 0.05$. Error bars represent the stand error (n = 4).

of H_2O_2 triggered the oxidative ring-opening cleavage reactions of fulvic acid-like. Furthermore, the addition of NaOH can cause hydrolysis of lignin structure in fulvic acid-like under mild condition. This may lead to the cleavage of ether linkages [49,50], and the strong oxidation of H_2O_2 may lead to the production of carboxyl groups.

3.5. Rice seed germination bioassay

Seed germination is critical for crop production in saline environment as it determines whether the crop can be successfully established [51]. While salt stress inducted by 100 mM NaCl inhibated rice seed germination in this experiment, LFA and PFA-O-3 both alleviated this effect (Fig. 5). Compared with the control, LFA at 2, 5, 10 mg-C/L increased germination rate by 15.2–29.7% after 72 h of observation. Germination rate decreased with further increase in LFA concentration (Fig. 5A). PFA-O-3 at 60–120 mg-C/L improved seed germination rate by 12.5–28.9% at 72 h. The other concentrations had no significant effects (Fig. 5B). After 120 h, germination rate of all treatment reached close to 100%, indicating that LFA and PFA-O-3 were safe for use with rice seeds. Our results showed that both LFA and PFA-O-3 can improve the rice germination under salt stress, which are in consistent with the results that the application of FA improved seed germination of barley under salt stress and wheat under drought and heat stress [52,53].

3.6. Rice seedlings growth

Both LFA and PFA-O-3 showed significant effects on the growth of rice seedlings under salt stress condition (Fig. 6). Application of LFA at 2 and 5 mg-C/L significantly increased the length and biomass of seedlings including both shoot and root (Figs. 6A&B; S2A). However, when LFA were applied at 10 and 20 mg-C/L, the effects were only significant for root biomass. LFA at 1 mg-C/L had no effects. For PFA-O-3, application at 60, 80 and 100 mg-C/L significantly increased the length and biomass of shoot and root (Figs. 6C&D; S2B). By contrast, application at 40 and 120 mg-C/L had no significant effects on seedling growth. Visual observation of seeding root also indicated that LFA and PFA-O-3 increased the number of lateral root (Fig. S3). Our results support prior observations that humic substances improved plant growth and physiology with increased root length and density, thus favoring nutrient uptake in plants via increase in absorptive surface area [54–57]. Several workers verified that the ability of FA in promoting the growth of plant is associated with their structural features and only the fractions with less content of aromatic structure and high content of carboxyl groups can interact with cell membranes and improve the growth of root [58,59]. The chemical structure features of PFA-O-3 with less aromatic structure and high carboxyl groups may help understand the mechanism in promoting the growth of rice root.

3.7. Antioxidant enzymes activity and lipid peroxidation

To further determine fulvic acid-like's relieving effects on oxidative damages induced by salt stress at the cellular level, changes of antioxidant enzymes activities (SOD, POD and CAT) in rice seedlings were compared. With salt stress imposed by 100 mM NaCl, LFA and PFA-O-3 significantly increased the activity of SOD and POD at all tested concentrations. The activity of CAT was increased by LFA only at 2 mg-C/L and by PFA-O-3 at 100 mg-C/L (Table 4). Anjum et al. [34] also indicated that exogenous FA enhanced levels of antioxidant enzymes (SOD, POD, and CAT) under drought and well-watered condition. Production of these antioxidant enzymes helps reduce oxidative damages. Functional groups, especially the phenolic hydroxyl group, have hydrogen donation and electron transfer capacities to scavenge reactive oxygen species such as OH˙ [60–62]. The rich phenolic hydroxyl group in LFA and PFA-O-3 can thus explain the alleviating effects with respect to salt stress in this study.

Lipid peroxidation, reflected by the reduced content of MDA, is

another process associated with increased stress resistance [63]. LFA at 2, 5, 10 mg-C/L reduced MDA content by 9–19%. Further increase in LFA concentrations induced accumulation of MDA (Fig. 7A). In contrast, PFA-O-3 at 80, 100 mg-C/L reduced MDA content by 14.5–48%; lower concentrations at 40 mg-C/L increased MDA content (Fig. 7B). The application of FA has also been shown to reduce MDA content in soybean under heat and salt stress and in maize in response to water stress [33,64].

4. Conclusions

A novel method to activate and extract fulvic acid-like from paper mill effluents using H_2O_2 as oxidizer (effluent to 30% H_2O_2 volume ratio = ½) and TiO_2 as catalyst is now developed and proven effective. With a higher H/C and O/C ratios and less lignin structure than the original fulvic acid-like in paper mill effluents, the activated fluvic acid-like features lower molecular weight and richer functional groups, resembling much of the structure characteristics of benchmark fulvic acid extracted from leonardite. Furthermore, the activated fluvic acid-like functions are similarly with that of the benchmark fulvic acid in alleviating salt-induced inhibition of seed germination and early stage seedling growth. These biological activities are evidenced by reduced oxidative damage by salt stress via activation of antioxidative enzymes and lipid peroxidation. The functional concentrations for improving rice seed germination rate and seedling growth under salt stress condition are 2–5 mg-C/L for the benchmark fulvic acid and 60–100 mg-C/L for the activated fulvic acid extracted from paper mill effluents. This study provides the needed technical basis for resourceful utilization of paper mill effluents to produce fulvic acid as a plant growth promoter for sustainable agriculture production.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.05.095.

References

[1] M. Ali, T.R. Sreekrishnan, Aquatic toxicity from pulp and paper mill effluents: a review, Adv. Environ. Res. 5 (2001) 175–196.

[2] C. Yu, L. Shi, Y.T. Wang, Y. Chan, B.D. Cheng, The eco-efficiency of pulp and paper industry in China: an assessment based on slacks-based measure and Malmquist-Luenberger index, J. Clean. Prod. 127 (2016) 511–521.

[3] D.T. Sponza, Application of toxicity tests into discharges of the pulp-paper industry in Turkey, Ecotoxicol. Environ. Saf. 54 (2003) 74–86.

[4] E. Lee, C.M. Glover, F.L. Rosario-Ortiz, Photochemical formation of hydroxyl radical from effluent organic matter: role of composition, Environ. Sci. Technol. 47 (2013) 12073–12080.

[5] X.Q. Chen, C.L. Si, P. Fatehi, Pretreatment and in situ fly ash systems for improving the performance of sequencing batch reactor (SRB) in treating thermomechanical pulping effluent, ACS Sustain. Chem. Eng. 5 (2017) 6932–6939.

[6] T. Leiviskä, H. Nurmesniemi, R. Pöykiö, J. Rämö, T. Kuokkanen, J. Pellinen, Effect of biological wastewater treatment on the molecular weight distribution of soluble organic compounds and on the reduction of BOD, COD and P in pulp and paper mill effluent, Water Res. 42 (2008) 3952–3960.

[7] G. Thompson, J. Swain, M. Kay, C.F. Forster, The treatment of pulp and paper mill effluent: a review, Bioresour. Technol. 77 (2001) 275–286.

[8] J. Hynynen, A. Palomäki, J.J. Meriläinen, A. Witick, K. Mäntykoski, Pollution

history and recovery of a boreal lake exposed to a heavy bleached pulping effluent load, J. Palolimnol. 32 (2004) 351–374.

[9] K. Niu, J. Wu, F. Yu, J.L. Guo, Construction and operation costs of wastewater treatment and implications for the paper industry in China, Environ. Sci. Technol. 50 (2016) 12339–12347.

[10] N. Merayo, D. Hermosilla, L. Blanco, L. Cortijo, Á Blanco, Assessing the application of advanced oxidation processes, and their combination with biological treatment, to effluents from pulp and paper industry, J. Hazard. Mater. 262 (2013) 420–427.

[11] J.E. Tomaszewski, R.P. Schwarzenbach, M. Sander, Protein encapsulation by humic substances, Environ. Sci. Technol. 45 (2011) 6003–6010.

[12] S. Trevisan, O. Francioso, S. Quaggiotti, S. Nardi, Humic substances biological activity at the plant-soil interface: from environmental aspects to molecular factors, Plant Signal. Behav. 5 (2010) 635–643.

[13] L.P. Canellas, F.L. Olivares, Physiological responses to humic substances as plant growth promoter, Chem. Biol. Technol. Agric. 1 (2014) 1–11.

[14] N.A. Kulikova, I.V. Perminova, Binding of atrazine to humic substances from soil, peat, and coal related to their structure, Environ. Sci. Technol. 36 (2002) 3720–3724.

[15] A.M. Amat, A. Arques, M.A. Miranda, F. López, Use of ozone and/or UV in the treatment of effluents from board paper industry, Chemosphere 60 (2005) 1111–1117.

[16] D. Pokhrel, T. Viraraghavan, Treatment of pulp and paper mill wastewater-a review, Sci. Total Environ. 333 (2005) 37–58.

[17] K.P. Kringstad, K. Lindstroem, Spent liquors from pulp bleaching, Environ. Sci. Technol. 18 (1984) 236A–248A.

[18] S.I. Carvalho, M. Otero, A.C. Duarte, E.H.B. Santos, Spectroscopic changes on fulvic acids from a kraft pulp mill effluent caused by sun irradiation, Chemosphere 73 (2008) 1845–1852.

[19] H. Ma, H.E. Allen, Y. Yin, Characterization of isolated fractions of dissolved organic matter from natural waters and a wastewater effluent, Water Res. 35 (2001) 985–996.

[20] L.P. Canellas, F.L. Olivares, Physiological responses to humic substances as plant growth promoter, Chem. Biol. Technol. Agric. 1 (2014) 1–11.

[21] D. Martinezbalmori, R. Spaccini, N.O. Aguiar, E.H. Novotny, F.L. Olivares, L.P. Canellas, Molecular characteristics of humic acids isolated from vermicomposts and their relationship to bioactivity, J. Agric. Food Chem. 62 (2014) 11412–11419.

[22] Y. Qin, H. Zhu, M. Zhang, H.F. Zhang, C. Xiang, B.C. Li, GC-MS analysis of membrane-graded fulvic acid and its activity on promoting wheat seed germination, Molecules 21 (2016) 1–11.

[23] R.M. Duarte, E.B. Santos, A.C. Duarte, Spectroscopic characteristics of ultrafiltration fractions of fulvic and humic acids isolated from an eucalyptus bleached Kraft pulp mill effluent, Water Res. 37 (2003) 4073–4080.

[24] S. Ciputra, A. Antony, R. Phillips, D. Richardson, G. Leslie, Comparison of treatment options for removal of recalcitrant dissolved organic matter from paper mill effluent, Chemosphere 81 (2010) 86–91.

[25] F.G. Chen, S.L. Xie, X.L. Huang, X.H. Qiu, Ionothermal synthesis of Fe_3O_4 magnetic nanoparticles as efficient heterogeneous Fenton-like catalysts for degradation of organic pollutants with H_2O_2, J. Hazard. Mater. 322 (2017) 152–162.

[26] O. Rozas, C. Vidal, C. Baeza, W.F. Jardim, A. Rossner, H.D. Mansilla, Organic micropollutants (OMPs) in natural waters: oxidation by UV/H_2O_2 treatment and toxicity assessment, Water Res. 98 (2016) 109–118.

[27] Z. Liu, T. Wang, X. Yu, Z.X. Geng, Y.H. Sang, H. Liu, In situ alternative switching between Ti^{4+} and Ti^{3+} driven by H_2O_2 in TiO_2 nanostructures: mechanism of pseudo-Fenton reaction, Mater. Chem. Front. 1 (2017) 1989–1994.

[28] J.C. Wang, H.H. Lou, Z.H. Xu, C.X. Cui, Z.J. Li, K. Jiang, Y.P. Zhang, L.B. Qu, W. Shi, Natural sunlight driven highly efficient photocatalysis for simultaneous degradation of rhodamine B and methyl orange using I/C codoped TiO_2 photocatalyst, J. Hazard. Mater. 360 (2018) 356–363.

[29] Y. He, G. Huang, C. An, J. Huang, P. Zhang, X. Chen, X. Xin, Reduction of *Escherichia coli* using ceramic disk filter decorated by nano-TiO_2: a low-cost solution for household water purification, Sci. Total Environ. 616 (2018) 1628–1637.

[30] M.M. Alguacil, E. Torrecillas, F. García-Orenes, A. Roldán, Changes in the composition and diversity of AMF communities mediated by management practices in a Mediterranean soil are related with increases in soil biological activity, Soil Biol. Biochem. 76 (2014) 34–44.

[31] R. Baigorri, M. Fuentes, G. González-gaitano, G. Almendros, F.J. Gonzalez-Vila, Complementary multianalytical approach to study the distinctive structural features of the main humic fractions in solution: gray humic acid, brown humic acid, and fulvic acid, J. Agric. Food Chem. 57 (2009) 3266–3272.

[32] H.G. Müller, T. Gasser, Changes of chemical properties of humic acids from crude and fungal transformed lignite, Fuel 85 (2006) 2402–2407.

[33] S. Xu, S.S. Zhu, Y.L. Jiang, N. Wang, R. Wang, W.B. Shen, J. Yang, Hydrogen-rich water alleviates salt stress in rice during seed germination, Plant Soil 370 (2013) 47–57.

[34] S.A. Anjum, L. Wang, M. Farooq, L. Xue, S. Ali, Fulvic acid application improves the maize performance under well-watered and drought conditions, J. Agron. Crop Sci. 197 (2011) 409–417.

[35] B.K. Huang, S. Xu, M. Xuan, M. Li, Z.Y. Cao, K.L. Liu, T.F. Ling, W.B. Shen, Carbon

[36] R.S. Dhindsa, W. Matowe, Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation, J. Exp. Bot. 32 (1981) 79–91.

[37] A.A. Ivanov, E.V. Maltseva, N.V. Yudina, Composition of fulvic acids after the mechanical activation of peats, Solid Fuel. Chem. 50 (2016) 7–11.

[38] E.B.H. Santos, R.M.B.O. Duarte, O.S. Filipe, A.C. Duarte, E. Rosenberg, Structural characterisation of the coloured organic matter from an eucalyptus bleached Kraft pulp mill effluent, Int. J. Environ. Anal. Chem. 78 (2000) 333–342.

[39] S.K. Ling, S. Wang, Y. Peng, Oxidative degradation of dyes in water using Co^{2+}/H_2O_2 and $Co^{2+}/peroxymonosulfate$, J. Hazard. Mater. 178 (2010) 385–389.

[40] J. Dai, W. Ran, B. Xing, M. Gu, L. Wang, Characterization of fulvic acid fractions obtained by sequential extractions with pH buffers, water, and ethanol from paddy soils, Geoderma 135 (2006) 284–295.

[41] C. Landry, L. Tremblay, Compositional differences between size classes of dissolved organic matter from freshwater and seawater revealed by an HPLC-FTIR system, Environ. Sci. Technol. 46 (2012) 1700–1707.

[42] J.Z. Xie, Y.Y. Yang, B. Gao, Y.S. Wan, Y.C. Li, J. Xu, Q.H. Zhao, Biomimetic superhydrophobic biobased polyurethane-coated fertilizer with atmosphere "Outerwear", ACS. Appl. Mater. Interfaces 9 (2017) 15868–15879.

[43] F. Napoly, N. Kardos, L. Jean-Gérard, G.H. Catherine, B. Andrioletti, M. Draye, H_2O_2-mediated kraft lignin oxidation with readily available metal salts: what about the effect of ultrasound, Ind. Eng. Chem. Res. 54 (2016) 6046–6051.

[44] J. Yoo, T. Shim, J. Hur, J. Jung, Role of polarity fractions of effluent organic matter in binding and toxicity of silver and copper, J. Hazard. Mater. 317 (2016) 344–351.

[45] Y. Chao, L. Peng, Water dispersibility and temperature dependence of electrical conductivity of conductive polypyrrole nanoparticles doped with fulvic acids, J. Chem. Eng. Data 56 (2011) 899–904.

[46] C. Li, X. Zhao, A. Wang, G.W. Huber, T. Zhang, Catalytic transformation of lignin for the production of chemicals and fuels, Chem. Rev. 115 (2015) 11559–11624.

[47] E.B.H. Santos, A.C. Duarte, The influence of pulp and paper mill effluents on the composition of the humic fraction of aquatic organic matter, Water Res. 32 (1998) 597–608.

[48] N.O. Aguiar, F.L. Olivares, E.H. Novotny, L.B. Dobbss, D.M. Balmori, L.G. Santos-Júnior, J.G. Chagas, A.R. Façanha, L.P. Canellas, Bioactivity of humic acids isolated from vermicomposts at different maturation stages, Plant Soil 362 (2013) 161–174.

[49] Z.S. Yuan, S.N. Cheng, M. Leitch, C.B. Xu, Hydrolytic degradation of alkaline lignin in hot-compressed water and ethanol, Bioresour. Technol. 101 (2010) 9308–9313.

[50] J.M. Lavoie, W. Baré, M. Bilodeau, Depolymerization of steam-treated lignin for the production of green chemicals, Bioresour. Technol. 102 (2011) 4917–4920.

[51] J. Song, G.U. Feng, C. Tian, F. Zhang, Strategies for adaptation of *Suaeda physophora*, *Haloxylon ammodendron* and *Haloxylon persicum* to a saline environment during seed-germination stage, Ann. Bot. 96 (2005) 399–405.

[52] K. Çavuşoğlu, H.G. Ergin, Effects of humic acid pretreatment on some physilolgical and anatomical parameters of barley under salt stress, Bangladesh J. Bot. 44 (2015) 591–598.

[53] A. Blum, A. Ebercon, Cell membrane stability as measure of drought and heat tolerance in wheat, Crop Sci. 21 (1981) 43–47.

[54] W. Schmidt, S. Santi, R. Pinton, Z. Varanini, Water-extractable humic substances alter root development and epidermal cell pattern in Arabidopsis, Plant Soil 300 (2007) 259–267.

[55] S. Nardi, D. Pizzeghello, A. Muscolo, A. Vianello, Physiological effects of humic substances on higher plants, Soil Biol. Biochem. 34 (2002) 1527–1536.

[56] S. Nardi, D. Pizzeghello, M. Schiavon, A. Ertani, Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism, Sci. Agric. 73 (2016) 18–23.

[57] R. Grene, Oxidative stress and acclimation mechanisms in plants, The Arabidopsis Book 1 (2002), p. e0036.

[58] M.T. Rose, A.F. Patti, K.R. Little, A.L. Brown, W.R. Jackson, T.R. Cavagnaro, A meta-analysis and review of plant-growth response to humic substances: practical implications for agriculture, Adv. Agron. 124 (2014) 37–89.

[59] A. Muscolo, M. Sidari, O. Francioso, V. Tugnoli, S. Nardi, The auxin-like activity of humic substances is related to membrane interactions in carrot cell cultures, J. Chem. Ecol. 33 (2007) (2007) 115–129.

[60] P. Cos, L. Ying, M. Calomme, J.P. Hu, M. Cimanga, B. Van Poel, L. Pieters, A.J. Vlietinck, D. Vanden Berghe, Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers, J. Nat. Prod. 61 (1998) 71–76.

[61] J.W. Chen, Z.Q. Zhu, T.X. Hu, D.Y. Zhu, Structure-activity relationship of natural flavonoids in hydroxyl radical-scavenging effects, Chin. J. Pharmacol. 23 (2002) 667–672.

[62] V. Butković, L. Klasinc, W. Bors, Kinetic study of flavonoid reactions with stable radicals, J. Agric. Food Chem. 52 (2004) 2816–2820.

[63] A. Blum, A. Ebercon, Cell membrane stability as measure of drought and heat tolerance in wheat, Crop Sci. 21 (1981) 43–47.

[64] B.S. Dinler, E. Gunduzer, T. Tekinay, Pre-treatment of fulvic acid plays a stimulant role in protection of soybean (*Glycine max* L.) leaves against heat and salt stress, Acta Biol. Crac. Ser. Bot. 58 (2016) 29–41.