Olive mill wastewater spreading improves growth, physiological, and biochemical traits of _Phaseolus vulgaris_

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ABSTRACT

The present study aimed to valorize olive mill wastewater (OMW) as biofertilizers to improve plant growth and soil properties. We investigated purple beans (_Phaseolus vulgaris_ L.) subjected to OMW application at two moderate doses (50 and 100 $m^3 ha^{-1}$) once at the time of beans sowing under field conditions. Our results showed that both OMW doses greatly improved plant growth. Moreover, nutrient contents also increased using 50 $m^3 ha^{-1}$ dose of OMW. Physiological parameters as stomatal conductance increased significantly in plants treated with OMW, while electrolyte leakage (EL) was decreased in irrigated plants with OMW. Application of OMW at rate of 100 $m^3 ha^{-1}$ significantly increased peroxidase, polyphenoloxidase, endogenous peroxide hydrogen, and total phenols. Moreover, the catalase and lipid peroxidation activities were significantly increased at both doses of OMW. According to the canonical discriminant analysis (CDA), the three treatments were separated by the following growth, nutrient uptake, physiological, and biochemical traits: Shoot height, dry, and shoot weight, available P, gs, K^+, Ca^{2+}, total phenols, number of fruits, EL, and malonyldialdehyde activity.

Keywords: Purple beans; Bio-fertilizers; Plant growth; Antioxidant metabolism; Canonical discriminant analysis

1. Introduction

Agriculture in Morocco is a very important economic sector. The needs of a rapidly growing population have made intensive agricultural development necessary [1]. As a result, some farmers are moving towards the use of chemical fertilizers to increase crop growth and yield increases the sensitivity of plants and deteriorates soil quality [2,3]. Environmental risk is linked to massive pollution of soil and water, due to the irrational use of different agro-inputs,

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which reach watercourses and groundwater by infiltration [4]. One of the ways of reducing those environmental issues is the development of organic farming [5]. The olive oil processing industry produces 35 kg olive cake and 440 L of olive mill wastewater (OMW) per 100 kg of processed olives [6–8] and about 30 million $m^3 y^{-1}$ worldwide [1,9,10]. OMW is characterized by its dark liquid effluents, an acidic pH, high organic load, electrical conductivity, and phenolic compounds [11]. These effluents pose a major environmental problem, because of their overload of toxic substances such as phenolic compounds and a pollutant load of very high organic matter [12,13]. In addition, different physicochemical methods have been proposed to treat OMW, including simple evaporation [14], electro-coagulation [15], oxidation by ozone and Fenton reagent [16], membrane processing [17] as well as their agricultural spreading [1], which is an alternative among the suggested solutions. In Morocco, olive trees are the most cultivated plants with an area of 1,000,000 ha approximately and 1,500,000 tons of olives per year [18,19]. Nowadays, the improvement of agricultural production has become a challenge for Morocco, where OMW negatively affects soil properties, underground water reserves, and the surrounding environment. However, the agronomic application of OMW is limited by the doses to be applied and the risk of polyphenol accumulation in the soil after consecutive applications [20]. Recent studies reported the application of OMW to improve changes in physiology and nutritional quality of tomato [21], olive tree performance and oil quality [3]. Moreover, OMW enhances physiological and biochemical responses of plants of adult Chemlali olive trees [22], vegetative growth and nutrient uptake [23], and improves the main agro-physiological and biochemical responses of Sorghum bicolor [10].

In this context the present work, aims to (i) evaluate the response of irrigated plants with two doses of OMW (control: 0, 50, and 100 $m^3 ha^{-1}$) once at the time of beans sowing in the field, by measuring soil fertility and plant growth, physiological, and biochemical parameters, (ii) characterize the mechanisms and the tolerance of *Phaseolus vulgaris* cv. Extralon Purple Bean (EPL) to the stress caused by the application of OMW at different doses, (iii) and distinguish the differences in all measured parameters to separate the studied treatment.

2. Materials and methods

2.1. Study site

The experiment was conducted at a private farmer's field in Tamesloht commune (31°54′18″ N; 8°02′08′ W), Marrakesh, Morocco. The climate is semi-arid, with an average annual rainfall of 251 mm (from September to May) and temperature of 28.2°C in autumn, 18°C in winter, and 26°C in spring with 7°C and 32°C as a mean minimum and maximum annual temperatures, respectively. The farm is an agricultural land spread over 3 ha and equipped with a drip irrigation system with suitable internal drippers (sheath) which release 2 L/h. The distance between drip lines for the same board (block) is 0.4 m with 0.15 m as a distance between each internal dipper. The soil plots undergoing our experiment have never been benefited or treated by chemical fertilizers or other organic manures [24].

2.2. Experimental design

The experiment was carried out during 2016–2017 in the field in a complete randomized block design with three treatments and six blocks of 1.2 m^2 per each plot (1.5 m × 0.8 m). Each block has two rows of 10 plants and 0.5 m spacing between two blocks. Sampling seeds of purple beans were disinfected with 1/3 of diluted sodium hypochlorite (12°) and washed three times with sterile distilled water. Thereafter, the seeds were germinated for 72 h and watered with distilled water in the laboratory. Sprouted ones were sown directly on a loamy sand soil and irrigated once by OMW; 50 and 100 $m^3 ha^{-1}$. A similar number of nontreated plants were included as a control block irrigated by tap water (control: 0 $m^3 ha^{-1}$) and conducted under the same conditions to determine the effect of the native flora of agricultural soil on plant growth and productivity of purple bean crops. The main physicochemical characteristics of soil used are summarized in Table 1. The determination of the mineral elements was based on the methods of Olsen et al. [25] and Jackson [26].

2.3. Chemical characteristics of OMW

2.3.1. Volatile matter, dry matter, ash, and residual oil determinations

OMW were collected from a semi-modern three-phase olive mill located in Souihla, a region of Marrakesh (Morocco) and kept at 4°C until use. The volatile matter (VM) is determined by differentiating between the dry matter (DM) obtained by evaporation at 105°C, the ash residues resulting from the calcination at 550°C for 2 h and the percentage of residual oil was determined according to El

Table 1
Physicochemical characteristics of soil and OMW

	Soil analysis	OMW
Sand (%)	82.46	–
Loam (%)	10.77	–
Clay (%)	6.76	–
pH	8.14 ± 0.05	4.70 ± 0.08
$CaCO_3$ (%)	5.22 ± 0.62	–
Electrical conductivity (mS cm^{-1})	0.139.73 ± 19.65	23.5 ± 0.50
Organic matter (%)	0.74 ± 0.05	1.27 ± 0.05
Total organic carbon (%)	0.15 ± 0.01	0.26 ± 0.01
Available phosphorus (mg kg^{-1})	5.9 ± 0.0005	–
Potassium (mg kg^{-1})	16.63 ± 0.002	–
Calcium (mg kg^{-1})	62.6 ± 0.008	–
Sodium (mg kg^{-1})	28.93 ± 0.007	–
Magnesium (mg kg^{-1})	65.16 ± 0.004	94.86 ± 1.66
Dry matter (g L^{-1})	–	21.79 ± 0.50
Total suspended solid (g L^{-1})	–	11.35 ± 0.67
Ash (g L^{-1})	–	3.00 ± 0.08
Volatile matter (g L^{-1})	–	8.38 ± 0.14
Total phenol (g GAE L^{-1})	–	0.22 ± 0.01
Residual oil (%)	–	

Abbassi et al. [27]. The main characteristics of OMW are presented in Table 1.

2.3.2. Phenolic compounds determination

The total phenolic content of OMW was extracted using liquid-liquid extraction as described by El Abbassi et al. [27]. Firstly, the pH of OMW samples 5 mL was adjusted using HCl (2 M) to pH (2.0). OMW were defatting with n-hexane and extractions were performed thrice with ethyl acetate. All aqueous ethylic extracts were brought to dryness at 40°C under reduced pressure using rotary evaporator, and the extract was recuperated in methanol (5 mL). Total phenolic content was estimated following the Folin–Ciocalteu colorimetric method using gallic acid as standard. The total phenolic content was measured as gallic acid equivalent (GAE) and values are expressed as g of GAE L^{-1} of OMW.

HPLC analysis was performed in the center for analysis and characterization (Cadi Ayyad University of Marrakesh, Morocco) on Knauer apparatus; Smartline auto sampler 3950 series equipped with a Smartline manager 5000, Smartline pump 1000, Smart Mixmixer and Smartline PDA detector 2800 controlled by claritychrome software. Separation was performed using C_{18} column (Eurospher II 100–5,250 × 4.6 mm) with a gradient system run using eluting solution A = acetonitrile and eluting solution B = o-phosphoric acid/water (pH 2.6), (5/95 v/v). The gradient run starts with 5% A for 10 min, 13% until 12 min, 18% until 22 min, 25% until 39 min, 30% until 40 min, 85% until 49 min, 90% until 51 min, 95% until 53 min, 100% until 59 min and finally 5% until 60 min. The 10 μL was injected with a flow rate of 1 mL min^{-1} and pressure of 117 bar. The phenolic compounds were characterized according to their UV-Vis diode array detector at 280 nm spectrum, and they were identified by comparing their retention time (RT) with standards. The phenolic compounds were then quantified tentatively using a calibration curve of the corresponding standards. The results were expressed as gram per liter (g L^{-1}).

2.4. Assessment of growth parameters

After three months, the plant growth was evaluated by calculating the shoot height (SH), root length (RL), number of leaves, number of fruits and fruit weight (expressed per pod). The shoot height was measured at the level of the collar. Moreover, shoot and root parts were rinsed with tap water to remove dust and dried at 70°C for 48 h to measure dry shoot weight (DSW) and dry root weight (DRW).

2.5. Mineral nutrient contents

Determination of K^+, Na^+, Ca^{2+}, and P concentrations, was conducted by taking fresh leaves of purple beans, which were dried in the oven at 80°C for 48 h and incinerated at 600°C for 6 h in the furnace. The incinerated matter was digested in 5 ml of HCl (6 N) and K^+, Na^+, and Ca^{2+} concentrations were determined with flame spectrophotometer (AFP100) according to Brown and Lilleland [28], While, P concentration was measured according to the method described by Olsen et al. [25].

2.6. Assessment of physiological parameters

2.6.1. Chlorophyll fluorescence (F_v/F_m)

The chlorophyll fluorescence was estimated using a portable fluorometer (OS-30p + OPTI-SCIENCES) after 15–20 min of dark adaptation. The maximum quantum efficiency of Photosystem II (PSII) (F_v/F_m) was calculated as $F_v/F_m = (F_m−F_0)/F_m$, where F_0 is maximal and minimal fluorescence of dark-adapted leaves respectively, and F_v is variable fluorescence [29]. It measures the fluorescence signal (F_0) received from dark-adapted leaves using a low fluorescence intensity when all reaction centers are open, and the measure of maximum saturation (F_m) when all reaction centers are closed. The Photosystem II efficiency was also evaluated under stress by TFm (time to F_m), a parameter used to indicate the time at which the maximum fluorescence value was reached. Six plants per treatment per symbiotic combination were considered and grouped as three replicates.

2.6.2. Stomatal conductance (gs) (EL)

Using a portable porometer (Leaf Porometer LP1989, Decagon Device Inc., Washington, USA). The gs were measured at midday and the device was calibrated before each use with the supplied calibration plate. The measurements were carried out on the underside of the sheet. The gs was expressed in mmol H_2O m^2 s^{-1}.

2.6.3. Electrolyte leakage

Leaf membrane injury was calculated by the measure of electrolyte leakage (EL) following the method of Shanahan et al. [30] with slight modifications. Twenty leaf discs of 0.5 cm²were washed with tap-water to remove solutes released during cutting of the leaves, taken into a tube containing 10 mL of distilled water, and kept 24 h at 25°C under shaking. The initial conductivity (C1) was determined using a conductivity meter (Hanna HI 8733) and the final conductivity (C2) was recorded after taken the leaf samples were autoclaved at 120°C for 15 min EL was calculated as EL = (C1/C2) × 100.

2.7. Assessment of biochemical parameters

2.7.1. Extraction of enzymes

Samples of purple beans leaves from control and treated plants were grounded immediately in the presence of liquid nitrogen and 0.1 M potassium phosphate buffer (pH 7) containing 1% polyvinyl-poly-pyrrolidone (PVPP) and 1 mM Ethylene-diamine-tetra-acetic acid (EDTA). The homogenate was centrifuged at 15,000 g for 20 min at 4°C. The supernatant was used to determine the antioxidant enzymes (POD, PPO, and CAT) and protein [31].

2.7.2. Enzymes assays

Peroxidase (POD) activity was measured according to Tejera et al. [32]. The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 6), 20 mM guaiacol, 10 mM H_2O_2, and 0.1 mL of enzyme extract. The absorbance was determined at 470 nm and the reaction was allowed to

proceed for 3 min. POD activity was expressed in UE mg^{-1} of protein using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Polyphenoloxidase (PPO) activity was determined according to the modified method of Hori et al. [33]. The reaction mixture included 0.1 M potassium phosphate buffer (pH 6), 10 mM catechol and 0.1 mL of enzymatic extract. PPO activity was measured at 410 nm by monitoring the change of absorbance for 3 min. The PPO activity was expressed in UE mg^{-1} of protein using 71.3 mM^{-1} cm^{-1} as an extinction coefficient.

Catalase (CAT) activity was estimated according to the method of Aebi [34] with slight modifications. The reaction mixture contained 10 mM H_2O_2 and 0.2 mL enzymatic extract. CAT activity was determined at 240 nm using an extinction coefficient of 39.4 mM^{-1} cm^{-1} and expressed in nmol H_2O_2 decomposed per min per mg of protein. Total soluble protein was determined by the Bradford method [35] using Bovine Serum Albumin (BSA) as standard.

2.7.3. Total polyphenol content

The total phenol content was estimated using the colorimetric method of Folin–Ciocalteu [36]. Briefly, 500 mg of leaves were crushed in the presence of liquid nitrogen, homogenized with 10 mL of ethanol (80%) and centrifuged at 4,000 g for 20 min. 0.2 mL of each extract was added to 2.4 mL of distilled water and 0.4 mL of Folin's reagent. The mixture was homogenized and stirring. After 3 min, 1 mL of sodium carbonates 20% was added and the whole mixture was incubated for 1 h at ambient temperature in the dark. The concentration of total polyphenol was determined at 765 nm against a blank.

2.7.4. Hydrogen peroxide (H_2O_2)

H_2O_2 content was determined according to the modified method of Velikova et al. [37]. Briefly, aliquots of 100 mg were finely grounded with 3 mL of 20% (w/v) trichloroacetic acid (TCA) in a cold mortar. The homogenate was centrifuged at 12,000 g at 4°C for 10 min. The reaction mixture included 0.5 mL of supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of potassium iodide. The absorbance was measured after 1 h of incubation in the dark at 390 nm. The H_2O_2 concentration was determined using a standard curve and expressed in nmol H_2O_2 g^{-1} of fresh weight.

2.7.5. Malonyldialdehyde

Malonyldialdehyde (MDA) amount was estimated on leaves according to Heath and Packer [38] with some modifications. Fresh leaves (100 mg) were grounded in a cold mortar with 1 mL of 10% (w/v) TCA and 1 mL of cold acetone. The homogenate solution was centrifuged at 8,000 g for 15 min at 4°C. The assay mixture included 0.5 mL of 0.1% phosphoric acid, 0.5 mL of 0.6% thiobarbituric acid (TBA), and 0.25 mL of enzymatic extract. The mixture was boiled at 95°C for 30 min, and cooled quickly to stop reaction on an ice bath and 1.5 mL of 1-butanol was added the homogenate was centrifuged at 8,000 g for 15 min. The absorbance of the supernatant was determined at 532 nm and at 600 nm for the correction of nonspecific turbidity and MDA content was

determined using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed in nmol g^{-1} of fresh weight.

2.8. Statistical analysis

Data analysis was performed using SPSS (20.0), through one-way analysis of variance (ANOVA). Three to ten replicated per treatments were considered. The mean values and standard errors (SE) were calculated. Significant differences between means were determined by Tukey's Kramer test $p < 0.05$. A canonical discriminant analysis (CDA) was performed on plants of purple beans treated with two doses of OMW (50 and 100 m^3 ha^{-1}) and control plants (0 m^3 ha^{-1}) to identify the discriminating variables between them. CDA was carried out on growth, mineral content, physiological and biochemical parameters. Statistical tests were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Physicochemical characteristics of soil

The soil had a loamy sand texture, an alkaline character (pH 8.14) with a little amount of total limestone (5.22%), and content of organic matter was low (0.74%) according to the range (0.7%–2.4%) given by Loveland and Webb [39]. The electrical conductivity was low (0.139 mS cm^{-1}) as cited by Johnson et al. [40] which have been between 0.12 and 0.78 mS cm^{-1}. The available phosphorus and the other mineral element were either at an average or below the critical level (Table 1).

3.2. Physicochemical characteristics of OMW

Results in Table 1 showed that OMW used are characterized by an acidic pH (4.7) and high electrical conductivity (23.50 mS cm^{-1}) which reflects that OMW have a high average salinity has been in accordance to Ochando-Pulido et al. [41]. The acidic pH and salinity of OMW is mainly due to the presence of many ions such as K$^+$ and Ca^{2+} [42]. Moreover, OMW are characterized by a dark-color caused by lignin polymerized with phenolic compounds, increased acidity (pH = 5), and high electrical conductivity [43]. Their dry matter is 94.86 g L^{-1}, which found to be similar to that reported by El-Abbassi et al. [27] (90 g L^{-1}). OMW tested are rich in total polyphenols (8.38 g GAE L^{-1}); while their volatile matter is presented by 3 g L^{-1} and residual oil by 0.22%.

OMW showed high phenolic content. The qualification and quantification of major phenolic compounds that were determined using HPLC analysis were summarized in Fig. 1. Ten free compounds were tentatively identified and quantified in crude OMW by comparison of their RTs and UV spectra with standards that were analyzed under the same conditions (Fig. 1). HPLC chromatograms showed that hydroxytyrosol and tyrosol were the two major monomer phenolic compounds of OMW. The fast degradation of these monomers by the biologic activities of soil or their infiltration was shown in the deep layers of soil [44]. Piotrowska et al. [45] reported that OMW is characterized by slow biological mineralization, although its phenolic compound was gradually degraded with time and partially transformed in humic substances.

Table 2
Shoot height (SH), root length (RL), number of leaves (NL), number of fruits (NF), dry shoot (DSW) and dry root (DRW) weight and fruits weight (FW), Potassium (K^+), Sodium (Na^+), Calcium (Ca^{2+}), and available phosphorus (P) of bean seedlings irrigated with two doses of olive mill wastewater (Control: 0, 50 and 100 $m^3 ha^{-1}$)

	SH (cm)	RL (cm)	NL	NF	DSW (g)	DRW(g)	FW (g)	K^+ (mg/plant)	Na^+ (mg/plant)	Ca^{2+} (mg/plant)	P (mg/plant)
Control	35.17 ± 0.37[b]	23.69 ± 0.23[a]	12.90 ± 0.18[b]	12.60 ± 0.25[b]	7.74 ± 0.15[b]	0.93 ± 0.03[b]	30.37 ± 0.76[b]	47.89 ± 2.33[c]	14.06 ± 2.57[c]	93.64 ± 2.85[b]	12.47 ± 0.44[c]
50 $m^3 ha^{-1}$	44.78 ± 0.20[a]	26.48 ± 0.34[a]	15.60 ± 0.16[a]	16.70 ± 0.42[a]	13.82 ± 0.22[a]	1.41 ± 0.03[a]	37.19 ± 1.27[ab]	108.35 ± 3.62[a]	26.90 ± 1.38[a]	141.21 ± 3.67[a]	31.31 ± 0.96[a]
100 $m^3 ha^{-1}$	44.42 ± 0.36[a]	23.99 ± 0.43[a]	12.40 ± 0.19[b]	19.10 ± 0.24[a]	11.95 ± 0.21[a]	1.54 ± 0.04[a]	42.56 ± 0.39[a]	72.75 ± 3.88[b]	21.54 ± 1.40[b]	134.31 ± 3.20[a]	27.31 ± 1.20[b]
p-value	<0.0001	0.1547	0.0007	0.0003	<0.0001	0.0012	0.0163	<0.0001	0.0005	<0.0001	<0.0001
F	29.11****	2.00[ns]	9.55***	10.91***	25.52***	8.72**	4.81*	246.81***	35.50***	186.70***	342.61***
LSD (0.05)	2.92	3.14	1.61	2.88	1.78	0.31	8.08	6.69	3.74	6.50	1.85

Means values ± SE in the same column followed by different letters are significantly different at $p < 0.05$ by Tukey-Kramer test; ns: not significant at 0.05, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.3. Effects of OMW application on plant growth and biomass

Our results showed that the application of OMW has a positive response to purple beans culture (Table 2). Treated plants did not show any negative effect (morphological or physiological) on purple bean crops. When applying OMW at 50 or 100 $m^3 ha^{-1}$, a significant difference ($p < 0.0001$, $F = 29.11$) was observed in field growth in terms of shoot height compared to control. Hao et al. [46] suggested that OMW is a potential natural biofertilizers and can be taken to fertilize soils. Furthermore, the maximum shoot height of the treated plants was better than that of the control ones for *Vicia faba* and *Cicer arietinum* [47]. No significant difference ($p = 0.1547$, $F = 2.00$) was recorded in root length in plants amended and control. The number of leaves (NL) was greatly increased ($p < 0.001$, $F = 9.55$) by the application of OMW at 50 $m^3 ha^{-1}$. Ouzounidou et al. [21] reported that tomato root was more sensitive to the application of OMW at different doses than the upper parts of the plant grown either in the sand or in soil. This can be affected by the high organic matter and mineral nutrient brought by the higher doses of OMW, which mainly, in turn, exerts a high EC affecting RL and NL [10,48]. The dry biomass was significantly improved in terms of shoot weight ($p < 0.0001$, $F = 25.52$) and dry root weight ($p = 0.0012$, $F = 8.72$) with about 13.82 and 1.41 g, respectively. On the other hand, the application of OMW at a rate of 100 $m^3 ha^{-1}$ greatly increased the number of fruits ($p < 0.001$, $F = 10.91$) and dry root weight with values of 19.10 and 1.54 g, respectively compared to control plants. A significant difference was recorded in fruit weight with the application of OMW at 100 $m^3 ha^{-1}$ (42.56 g) compared to control plants with 30.37 g. Accordingly, the highest above ground biomass observed when applying OMW at a low rate (40 $m^3 ha^{-1}$) for spinach and beetroot was 98% and 93%, respectively [49]. Such increases in yielding traits with the application of OMW doses up to 100 $m^3 ha^{-1} y^{-1}$ during 8 y on olive trees have been reported by Magdich et al. [22].

3.4. Effects of OMW application on mineral nutrient accumulation

Analysis of the results summarized in Table 4 indicated that the application of OMW at 50 $m^3 ha^{-1}$ significantly increased ($p < 0.001$) the concentration of K^+, Na^+, Ca^{2+} and available P in leaves of purple beans in comparison with control plants, these augmentations were 108.35, 26.90, 141.21, and 31.31 mg per plant respectively. This is in line with other studies who suggested that pre-treatments of OMW at different rates improved significantly the accumulation of nutrient contents such as K^+, Na^+, Ca^{2+}, and P [49]. P, K^+, and Na^+ concentrations were significantly ($p < 0.0001$) high in purple beans amended with 50 $m^3 ha^{-1}$. While, the increment of Ca^{2+} was significantly higher in both treated plants than the control ones. These results corroborated with [22] who reported that olive trees treated with 50 and 100 $m^3 ha^{-1}$ significantly improved leaf nutrient concentrations such as K^+ Ca^{2+} and available phosphorus. Indeed, Piotrowska et al. [45] reported a sudden increase in some available phosphorus when applying OMW on soil. In addition, in line with these findings, other studies have also noted that the contribution of OMW to soil enrichment by K, P, Na remains significant
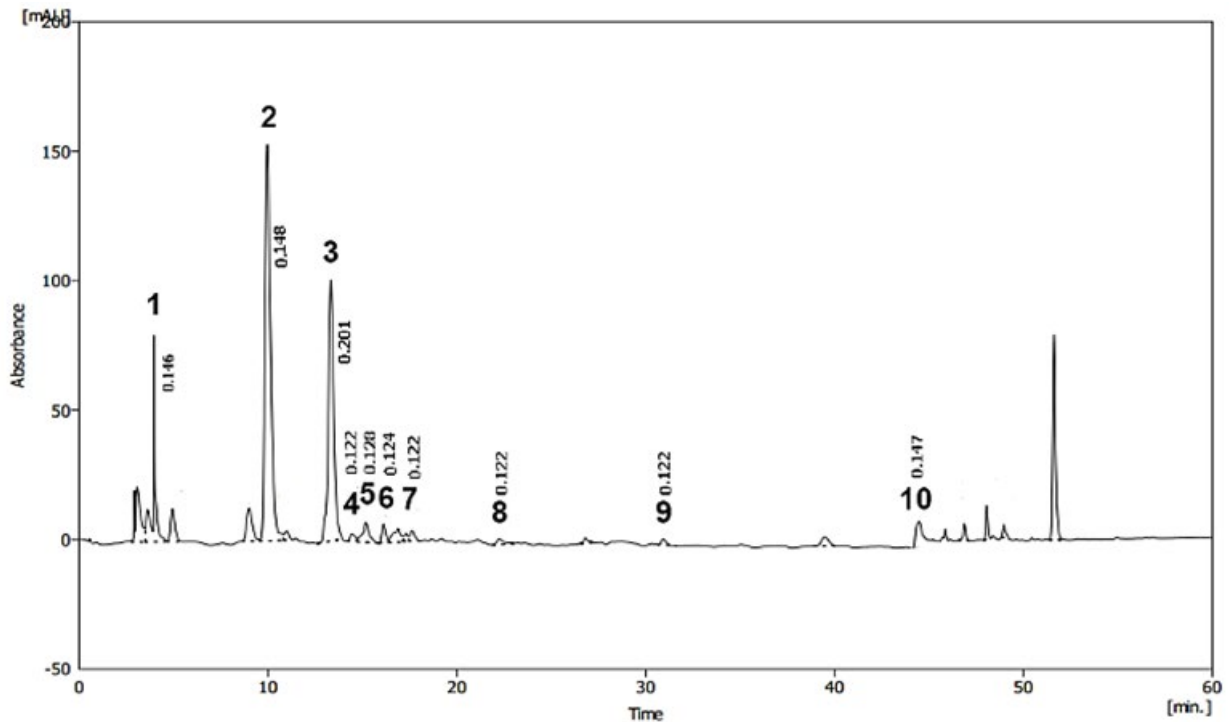
Fig. 1. HPLC chromatogram of the OMW phenolics. (1) Gallic acid, (2) Hydroxytyrosol, (3) Tyrocol, (4) Hydroxybenzoic acid, (5) 4-dihydroxybenzoic acid, (6) Vanillic acid, (7) Cafeic acid, (8) *p*-Coumaric acid, (9) Oleuropeinaglycone, (10) Quercetin (The amount of each phenolic compounds are given in front of its picture).

Table 3
Results of Lambda of Wilks, χ^2 and significance relative to growth, physiological, mineral contents, and biochemical traits studied in purple beans irrigated with two doses of OMW according to the canonical discriminant analysis (CDA)

	Function	Wilk's lambda	Chi-square (χ^2)	Df	*p*-Signification
Growth traits	1–2	0.095	61.07	6	<0.0001
	2	0.533	16.38	2	<0.0001
Physiological traits	1–2	0.000	58.47	8	<0.0001
	2	0.013	19.58	3	<0.0001
Mineral contents	1–2	0.000	41.86	4	<0.0001
	2	0.097	12.83	1	<0.0001
Biochemical traits	1–2	0.004	30.68	4	<0.0001
	2	0.446	4.44	1	<0.0001

Df: degree of freedom

[50,51]. Compared to the control plants, a significant increase in sodium, potassium calcium, and available phosphorus levels in response to OMW doses (Table 2).

3.5. Effects of OMW application on physiological parameters

The application of OMW at 50 and 100 m³ ha⁻¹ did not show a significant difference in chlorophyll fluorescence studied under field conditions (Fig. 2a), which is normal in plants grown under well-watered conditions and reflecting that there is no photo inhibitory to PSII complexes. Contrarily, the statistical analysis revealed that the stomatal conductance (gs) was improved significantly (*p* < 0.0001)

with OMW application, which are not significant between the doses applied (Fig. 2b). This agrees with the results of some works in which depend on the administered amounts of this effluent [22,52,53]. The high gs in irrigated plants with OMW showed that their stomatal are very open than control plants and leads to increase in the growth and development of plants [54]. The primary physiological effect of OMW is a reduction of EL. The cell membrane integrity was assessed by EL in leaves of purple beans (Fig. 2c). Our results showed that EL decreased significantly (*p* < 0.001) in leaves of plants treated in response to the application of increasing OMW doses. This damage was observed three months after the transplantation.
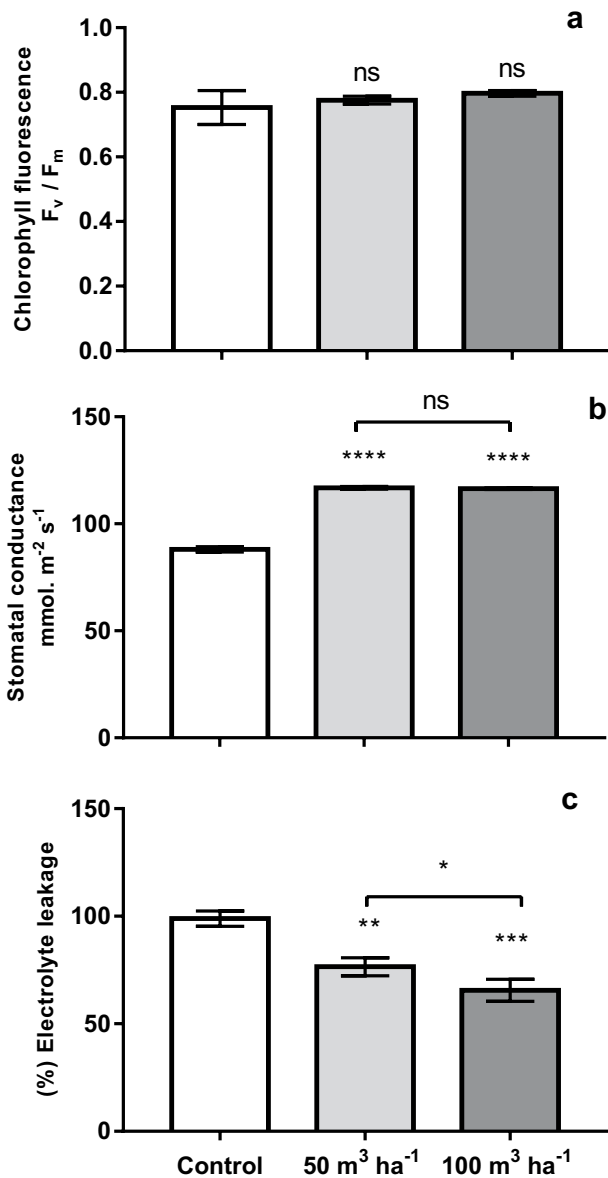
Fig. 2. (a) Chlorophyll fluorescence, (b) stomatal conductance, and (c) electrolyte leakage are determined in purple beans seedlings after three months of OMW agronomic application at different doses (control: 0, 50, and 100 m³ ha⁻¹). Mean values are compared to each other at $p < 0.05$ by Tukey-Kramer test. ns: not significantly, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.6. Effects of OMW application on biochemical parameters

Plant develops a complex of mechanism to alleviate damages induced by reactive oxygen species (ROS), which leads to changes in function and cellular structure causing eventual damage to proteins, nucleic acids, and lipids inhibiting their normal function [55–57]. ROS are well-known for playing a double role as both beneficial species and deleterious (signaling factors and protective). The production of antioxidant enzymes is considered as the most common mechanism to eliminate ROS and protects plants

against oxidative damages [58,59]. The results in Figs. 3a–c shows the variations in antioxidant enzymes (POD, PPO, and CAT) in purple beans leaves treated with OMW compared to plants treated with water (Control) under field conditions. The activities of POD and PPO were much more pronounced in irrigated plants with 100 m³ ha⁻¹ by about 989.05 and 101.80 UE mg⁻¹ of protein, respectively. This result reflects an efficient line of antioxidant defense against oxidative damage [60]. While CAT activity was not significant in irrigated plants with 50 and 100 m³ ha⁻¹ with values of 703.86 and 713.15 UE mg⁻¹ of protein, respectively. This enzyme is considered as an important component of protective systems [61]. Changes in total phenol significantly increased in parallel with the activity of the enzymes implicated in phenylpropanoid and flavonoid synthesis in response to the application of OMW, which presents a significant difference ($p < 0.0001$), compared to control plants (Fig. 3d). The total phenol content in purple beans leaves increased significantly ($p < 0.0001$, $F = 109$) in 100 m³ ha⁻¹. This is in line with Magdich et al. [22] who reported that treated olive trees with 200 m³ of OMW increased two-fold the phenolic compounds. Moreover, no phytotoxic effects on crops during their growth cycle were recorded, this remains related to the application modalities, the doses used and the phenological stage of the plant [13].We recorded a significant difference for POD, PPO, and total phenol contents in response to the increase of OMW doses (Fig. 3d). The induction and activation of antioxidant enzymes seem correlated with the applied OMW doses.

Table 4
Standardized canonical discriminant coefficients DF relative to growth, nutrient contents, physiological, and biochemical parameters according to CDA

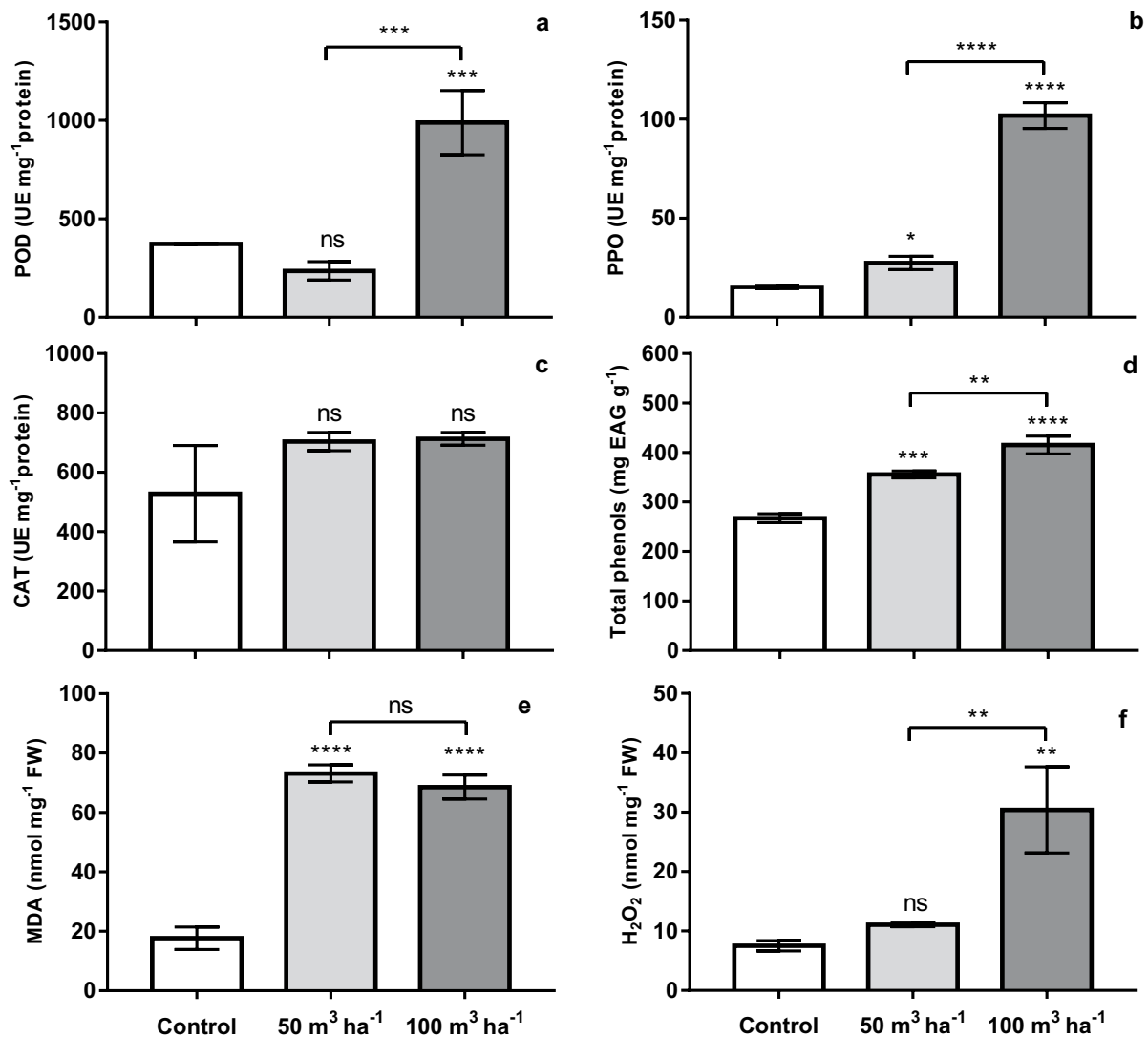| Parameters	Function	
	1	2
SH	0.65	0.44
RL	0.14	0.21
NL	0.20	−0.06
NF	−0.19	0.85
DSW	0.64	0.02
DRW	0.06	−0.13
FW	0.02	0.15
K	0.15	0.98
Na	0.04	−0.36
Ca	−0.42	−0.84
P	0.82	−0.56
gs	0.96	0.24
F_v/F_m	0.27	−0.36
EL	−0.35	0.93
PPO	0.14	−0.01
POD	0.05	−0.14
CAT	−0.15	−0.66
MDA	0.06	0.80
H_2O_2	0.03	0.00
TP	0.87	−0.16

Fig. 3. (a) Peroxidase, (b) polyphenyloxidase, (c) catalase, (d) total phenols, (e) malonyldialdehyde, and (f) hydrogen peroxide are determined in purple beans seedlings after three months of OMW agronomic application at different doses (control: 0, 50, and 100 $m^3 ha^{-1}$). Mean values are compared to each other at $p < 0.05$ by Tukey-Kramer test.
ns: not significantly, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

The H_2O_2 and MDA amount was evaluated as a metabolic indicator of purple beans leaves as an indicator of the status of their oxidative stress (Figs. 3e, f). The H_2O_2 accumulation was high ($p < 0.001$) in irrigated plants with 100 $m^3 ha^{-1}$ by about 30.39 nmol g^{-1} FW, whereas the lowest endogenous H_2O_2 content was shown in control plants (7.52 nmol g^{-1} FW). The MDA content was significantly high ($p < 0.0001$) in OMW treatments. This accumulation was not significant between plants treated with 50 and 100 $m^3 ha^{-1}$, which reflect that accumulation together with low EL observed in plant treated with OMW provides evidence of the high cell membrane damages due to the lipid peroxidation. Accumulation of MDA and H_2O_2 was abruptly increased by OMW application and accumulation of H_2O_2 was four times higher in treated plants with 100 $m^3 ha^{-1}$ as compared to control plants. This amount of H_2O_2 was generated in the process of acid degradation by peroxisomes as by-products [62]. Such

results corroborated with several previous studies, which have confirmed that OMW have serious phytotoxic effects when applied at large doses [1,10,22]. Up-regulation of antioxidant enzymes under stress was reported by several authors [63–65], taking into account the intensity and the period of drought stress.

3.7. Canonical discriminant analysis

CDA was carried out using growth parameters (7 variables) (CDAg), mineral nutrient contents (4 variables) (CDAn), physiological parameters (4 variables) (CDAp), and biochemical parameters (6 variables) (CDAb) as predictors of membership in a diagnostic group. This group corresponded to purple beans plants treated with control: 0 $m^3 ha^{-1}$, 50 $m^3 ha^{-1}$, and 100 $m^3 ha^{-1}$. According to CDA results, which showed that all measured parameters exhibit differences in

characteristics of studied biofertilizers (Fig. 4). High significant difference was obtained by Wilk's lambda of the model (0.533 for CDAg, 0.013 for CDAp, 0.097 for CDAn, and 0.446 for CDAb) and calculated F-value as well-presented significance ($p < 0.0001$) for these analyses (Table 3). Two discriminant functions (DF) were estimated, for 83.9% and 16.1% of the total variance for CDAg, 95.4% and 4.6% for CDAn, 98.9% and 1.1% for CADp and 98.7% and 1.3% for CDAb, respectively. The Khi-square (χ^2) test observed a significant

discriminatory power for the two functions ($p < 0.0001$). For CDAg, the eigenvalues of the first two functions (4.57 and 0.87, respectively) indicated them to explain most of the variance (83.9%) and their canonical discriminant correlations were $r_1 = 0.90$ and $r_2 = 0.68$. The CDAn present high eigenvalues (195.17 and 9.30, respectively) of the first two functions, showed them to illustrate the most of the variance (95.4%) and their canonical correlations were $r_1 = 0.99$ $r_2 = 0.95$. The eigenvalues for CDAp of the first two functions (116.94 and
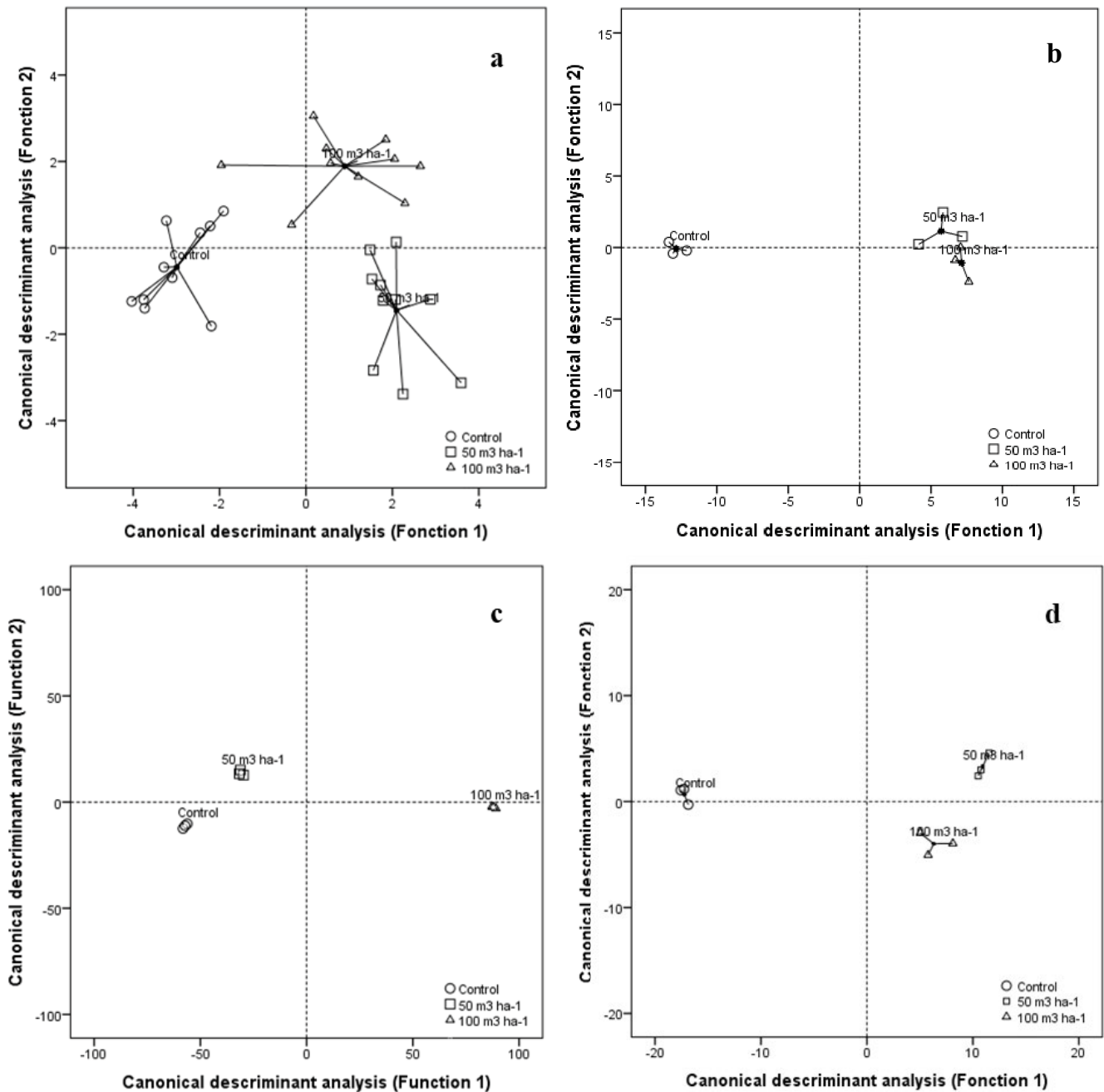

Fig. 4. 2D scatterplot showing the distribution of the study bio-fertilizers (control: 0, 50, and 100 m³ ha⁻¹) according to the gradient of the variance obtained by the two functions of the canonical discriminant analysis (CDA) for (a) growth parameters, (b) physiological parameters, (c) biochemical parameters, and (d) mineral contents.

1.24, respectively) indicated them to represent a total variance by about 98.9 % and their canonical correlations were $r_1 = 0.99$ and $r_2 = 0.74$. For CDAb, the eigenvalues of the first two functions (566.65 and 7.66, respectively) and their canonical analysis correlations were $r_1 = 0.97$ and $r_2 = 0.94$.

The bidimensional scatter plots of canonical discriminant space exhibit the distribution of the groups spanned by the first two functions (Fig. 4). The standardized coefficients of the canonical discriminant functions (DFs) of growth, nutrient, physiological, and biochemical parameters are summarized in Table 4 relative to CDAg, SH, and DSW were highly weighted in the positive part of first DF, while NF was highly weighted in the positive part of second DF. These scatter plots show a good separation of OMW doses applied to purple beans. For CDAg (Fig. 4a), the horizontal separation in the first DF was characterized. Thus, the first DF quantifies the level to which all treatment (control: 0, 50, and 100 $m^3\,ha^{-1}$) differ in growth parameters studied, which we argue to be the result of differences in their adaptation to the application of OMW. Both doses of OMW were mainly distinguished from control plants by high values of growth traits.

For CDAn, available P was highly weighted in the positive part of first DF and K^+ and Ca^{2+} were highly weighted in the positive and negative part of the second DF, respectively. gs indicated the highest standardized coefficient on first DF in the positive part, while EL was strongly weighted in the positive part of the second DF for CDAp. Concerning CDAn (Fig. 4d), the first DF suggested that irrigated plants with the two doses of OMW were mainly separated from control plants by high P, K^+, and Na^+ accumulation, confirming that application of OMW improves the nutrient uptake [50,51]. For CDAp (Fig. 4b), variability in physiological parameters studied were evident between treatment. The second DF indicated that control plants were mainly distinguished from OMW irrigated plants by its lower gs and high EL, reflecting that they have membrane intactness and well-watered. For CDAb, TP showed the highest standardized coefficient on the first DF in the positive part, whereas MDA and CAT were highly weighted in the positive and negative part of the second DF. Indeed, the first discriminant function contributed mainly to the separation between treatments (OMW and control). For CDAb (Fig. 4c), the first DF showed that treated plants with 100 $m^3\,ha^{-1}$ were mainly separated from other treatment (control and 50 $m^3\,ha^{-1}$) by high POD, PPO, TP, and H_2O_2 activities. Analysis of all parameters studied and their successive effects on the canonical discrimination between treatments studied, we suggested that H_2O_2, POD, PPO, EL could be indicated as selective parameters to select the toxic dose of OMW that we should avoid using for pre-treatment purple beans. The dose 100 $m^3\,ha^{-1}$ was clearly separated by the first DF in CDAb, while control plants were clearly distinguished from the two doses of OMW; 50 and 100 $m^3\,ha^{-1}$ by the second part of DF in CDAg, CDAn, and CDAp (Fig. 4).

4. Conclusions

From the present work, it is possible to conclude that irrigation of OMW at two doses once at the time of beans sowing improved significantly plant growth of purple beans. While, OMW at 50 $m^3\,ha^{-1}$ enhanced significantly the root length, the number of leaves, and nutrients uptake. Our results also reported that plants amended with 50 and 100 $m^3\,ha^{-1}$ increased significantly gs and decreased its EL. In addition, biochemical traits have indicated that at a dose-treatment 100 $m^3\,ha^{-1}$, OMW application strongly enhanced the production of PPO, POD, TP, and H_2O_2 activities. Whereas, plants amended with 50 $m^3\,ha^{-1}$ have been improved all measured parameters. Indeed, this dose has been the most acceptable to the purple beans vegetative development and productivity. A combination of growth, nutrient uptake, physiological, and biochemical parameters as an integrated approach has been conducted in our work to select the toxic dose of 100 $m^3\,ha^{-1}$ OMW that we should avoid when irrigating purple beans.

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