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Agronomic Impact and Phytotoxicity of Olive Mill Wastewater as a Biofertilizer on *Vicia faba* L.

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ABSTRACT

The Moroccan olive oil industries generate a substantial amount of olive mill wastewater (OMW), causing a significant environmental issue. Consequently, its valorization represents a sustainable agroecological solution to the environmental problems caused by this uncontrolled discharge. In this context, the use of OMW as an organic biofertilizer in agriculture has been suggested as an alternative to restore soil fertility and improve agricultural production. To comprehensively understand its impact, the conducted study focused on the local cultivar *Vicia faba* L., investigating the consequences of daily applications of OMW at varying doses (5%, 10%, 20%, 30%). In the experimental design, NaCl solutions, mimicking the electrical conductivity of OMW, were used to pinpoint the potential phytotoxic agents within the wastewater. The results revealed that a high dose of OMW had significant effects on morphological, physiological, and biochemical parameters. Additionally, phytotoxicity depended on both OMW concentration and the growth stage of *Vicia faba* L., causing growth delays, germination inhibition, as well as the accumulation of proline and soluble sugars. These findings underscore the complexity of using OMW in agriculture and highlight the critical importance of precise control over application rates for the success of this approach. While reusing OMW presents a viable and practical solution, a nuanced understanding of its impact on specific crops and a meticulous approach to dosage management are imperative to realize its potential benefits and mitigate any potential risks to crop productivity.

Keywords: olive mill wastewater, *Vicia faba* L., biofertilizer, phytotoxicity, morphological parameters, physiological parameters, biochemical parameters.

INTRODUCTION

In Morocco, the olive sector is one of the most important crops that allow the supply of oil mills. Morocco is the world's second-highest producer of preserved olives and the sixth-most producer of olive oil, following the European Union, Tunisia, and Turkey (El Qarnifa et al., 2019). About 30 million tonnes of olive mill wastewater (OMW) is produced per olive growing season by the main olive oil-producing countries in the Mediterranean region. OMW constitutes the residual by-product resulting from the olive crushing process and stands as a principal waste product in the olive oil industry (Cedola et al., 2020). Its uncontrolled discharge leads to significant social, economic, and environmental challenges in the Mediterranean region associated with olive oil production, such as surface and groundwater pollution (Bounadi et al., 2023), and soil degradation (Zahra El Hassani et al., 2023).

OMW constitutes complex effluents with a high variation related to different characteristics, recognizable by their reddish-brown color and cloudiness (Zenjari et al., 2006), composed of the vegetation water of the olive tree fruits, water used for washing and processing is easily fermentable (Goula and Gerasopoulos 2017), It is characterized by an acidic pH value ranging from 3 to 6 (Azzam and Hazaimeh 2021; Tsigkou et al., 2022), and high salinity depending mainly on the natural richness of dissolved mineral salts in vegetable waters and the traditional practice of salting olives before crushing for preservation (Es Saouini et al., 2023), which is primarily water-based (83–96%), mineral salts (0.5–2%), and organic constituents (3.5 to 15%) (Kıpçak and Akgün 2018a). Moreover, the organic content is reflected in the high concentrations of BOD_5 (over 100 g O_2/L), COD (over 220 g O_2/L), and unpleasant odor (Alami and Fattah 2020). This effluent contains phenolic compounds (2 to 5%), which constitute the most recalcitrant fraction contributing to the phytotoxic effects of OMW (Babić et al., 2019). The composition of olive mill wastewater varies depending on different factors, such as cultivars, geographical origin, agronomic practices, harvest time, and extraction methods (Stillitano et al., 2019).

Olive mill wastewater has been widely utilized on soils as fertilizer due to its high organic matter and nutrient levels. However, its use, when implemented without proper environmental management, may be detrimental (Alrowais et al., 2023; Khalil et al., 2024). In the Moroccan eastern region, Bouknana et al. (2021) concluded that farmers used olive mill wastewater for irrigation= of cultivated fields because of their richness in water (more than 82%) and mineral elements like potassium, sodium, and phosphate (N, P, K). This practice is scarcely used (1%) as a result of its adverse effects on receiving waters, and plant species. Therefore, it is an indispensable plant nutrient source (Khdair and Abu-Rumman 2020). In this context, Caruso et al. (2018) as well as Chatzistathis and Koutsos (2017) published two valuable studies on the impacts of OMW spreading on soil and crops, their findings revealed that utilizing OMW induced beneficial effects on soil. Moreover, these effluents have been reported to exert adverse effects on plant growth, leading to delays in germination and impeded plant development, primarily attributed to elevated concentrations of phenolic compounds or heavy metals, particularly in non-diluted solutions (Khalil et al., 2021). Despite these documented negative implications, it is crucial to acknowledge that these effluents contain significant nutrient content. The fertility status of these effluents can be further explored for potential applications in agriculture (Tzortzakis and Chrysargyris 2024).

Dilution of olive mill wastewater emerges as a recommended and effective strategy to ameliorate its inherent toxicity. However, the success of this operation should be controlled by adhering to appropriate OMW doses (Sierra et al., 2007). In Greece, applying OMW as fertilizer is allowed (OGGHR, 2016). In Spain, Portugal, and Italy, OMW is permitted as a biofertilizer up to 80 m^3 ha^{-1} $year^{-1}$ (Koutsos et al., 2018).

Salinity is a significant constraint for plant production (Gohari et al., 2023). Excess Na^+ and Cl^{-1} affect plant growth and development (Kamran et al., 2020). Plants are generally subjected to two types of stress: biotic and abiotic stress. Viruses, insects, fungi, etc. mainly cause the first type of stress (Parween et al., 2020). However, abiotic stress results from environmental factors like salinity, drought, and cold, leading to a decrease in leaf area, leaf thickness and succulence, leaf abscission, root and shoot necrosis, and internode length (Rahneshan et al., 2018). Furthermore, both biotic and abiotic stress can induce multiple adverse impacts on plant growth across physiological, molecular, and biochemical, resulting in a significant loss of agricultural productivity (Evelin et al., 2019). In addition, the sensitivity to salt stress depends on the growth stage at which the stress is applied (Bensidhoum and Nabti 2021).

Faba bean (*Vicia faba* L.) belongs to the Fabaceae family and stands as the globe's ancient crop cultivated plants, it is native to Mediterranean countries. It is used to prepare different dishes due to its edible seeds (Çilesiz et al., 2023), it provides an essential source of protein, starch, fiber, vitamins, and minerals (Badjona et al., 2023). Faba bean has also a high potential to promote human health as well as prevent obesity and diseases such as cholesterol, glycemic, coronary heart diseases, and colon cancer (Steen et al., 2010). Additionally, this species exhibits high antioxidant and anti-inflammatory effects (Badjona et al., 2023).

The main objective of this study was to conduct a comprehensive exploration into the implications of dispersing olive mill wastewater and applying saline solutions on the growth and developmental stages of *Vicia faba* L. Throughout the entire experimental process, the broad bean plants received exclusive daily inputs of olive mill wastewater and saline solution, beginning from the initial germination phase and extending through the entirety of the growth cycle. This systematic approach sought to comprehensively evaluate the impact of these inputs on both growth and biochemical parameters, providing valuable insights into comprehending how these conditions may influence the overall performance of *Vicia faba* L. cultivation.

MATERIALS AND METHODS

Experimental site

The experimental research took place within the open field located at the research station of the Faculty of Sciences in Oujda, Morocco, over a three-month duration. This experimental station is situated at an altitude of 661 meters, with coordinates of 34° 39' 06.71'' North and 01° 53' 58.80'' (Mzabri et al., 2021). The local climate is characterized as arid with mild winters.

Plant materials

Faba bean seeds were selected as the plant material due to their high germination capacity and growth rate. Furthermore, these seeds were used as a plant model to investigate the phytotoxicity and ecotoxicity of various pollutants. To align with the local context and conditions, a local variety of seeds commonly cultivated in eastern Morocco was chosen for this research.

Olive mill wastewater source and characteristics

The olive mill wastewater was sourced from an olive oil mill utilizing a semi-traditional system located in the city of Aklim Berkane, Morocco. The samples were collected from the storage bays, transferred to the experimental station, and subsequently stored in cool, humid environments shielded from direct sunlight to maintain consistent physicochemical characteristics.

Preparation of different concentrations, cultivations, and stress applications

The seeds of *Vicia faba* L. were carefully chosen based on their morphology, color, and health condition. To ensure sterility, they underwent a 5-minute disinfection process with a 12% sodium hypochlorite solution at a 5% concentration, followed by thorough rinsing with distilled water to eliminate any residual chlorine. Subsequently, the seeds were soaked in water at room temperature for 24 hours to expedite germination and then planted in plastic pots filled with peat.

For optimal and rapid growth, these pots were placed in a controlled glass greenhouse for two weeks, with adequate watering provided as needed. The seedlings were transplanted into black plastic pots when they reached the four to five-leaf stage to encourage root system development, using topsoil as the substrate. Later, the seedlings were transferred to the field and irrigated for a week with well water located at the Faculty of Sciences in Oujda, with an electrical conductivity (EC0) of 0.56±1 μS/cm, this phase was intended to adapt the plants to their new environment to avoid thermal shock before the onset of stress.

The application of olive mill wastewater and saline solution commenced on the 20th day after sowing, with daily irrigation adjustments based on daily evapotranspiration levels. The broad bean plants were subjected to four different concentrations of olive mill wastewater (5%, 10%, 20%, 30%). To achieve equivalent electrical conductivity levels as in olive mill wastewater, solutions containing NaCl (S1, S2, S3, S4) were meticulously prepared as indicated in the Table 1.

Germination test

The seeds used were selected for their uniformity. Subsequently, they were immersed in a 10% sodium hypochlorite solution with a concentration of 12% for 10 minutes; this was done to prevent any fungal development and to ensure the sterility of their surface. Following this treatment, the seeds underwent multiple rinses with distilled water. The phytotoxicity test involved the placement of 10 seeds, evenly spaced, within a Petri dish containing filter paper. Before this, the seeds underwent a 24-hour soaking process in distilled water. Each Petri dish was then irrigated with 15 ml of the respective treatment solutions, while distilled water was used for the control test. Subsequently, the seeds were

Table 1. The different treatments used in this experiment

Treatment	OMW concentration	OMW volume (L)	Water volume (L)	Saline solution	Saline solution concentration (mM)
T0	Control	0	30	-	-
M1	5%	1.5	28.5	-	-
M2	10%	3	27	-	-
M3	20%	6	24	-	-
M4	30%	9	21	-	-
S1	-	-	-	0.9 g/l	15.37 mM
S2	-	-	-	1.8 g/l	30.74 mM
S3	-	-	-	3.6 g/l	61.48 mM
S4	-	-	-	5.4 g/l	92.22 mM

incubated under controlled conditions in a growth chamber, which provided complete darkness to eliminate the influence of photosynthesis, maintaining a day/night temperature of 22±1 °C. The assessment of seed germination percentage and radicular elongation was carried out. Each experiment was repeated in triplicate. The assessment of the influence of OWM on the germination of *Vicia faba* L. seeds involved examining the following parameters: germination rate (GR) and germination index (GI). The calculation of the relative germination percentage and germination index was performed following the Phytotoxicity test method described by Zucconi et al. (1981). The germination percentage is determined by dividing the number of seeds that have germinated at a specific time “t” by the total number of seeds, and it is then presented as a percentage.

$$(\%)\ germination = \frac{Nt}{Ni} \times 100 \quad (1)$$

where: *Nt* represents the count of germinated seeds at the particular time “t.” and *Ni* is the total number of germinated seeds under consideration.

Germination index

The calculation of the relative germination percentage was carried out using the formula:

$$RG \text{ in } (\%) = \frac{Gs}{Gc} \times 100 \quad (2)$$

In the equation, “Gs” and “Gc” represent the counts of germinated seeds in the Petri dishes that were subjected to the treatments and the control, respectively.

$$RL \text{ in } (\%) = \frac{Ls}{Lc} \times 100 \quad (3)$$

With “Ls” and “Lc” denoting the average lengths of seed roots exposed to the treatments and water, a value of zero was assigned when no observable growth occurred. Consequently, the germination index was computed using the following formula:

$$IG \text{ in } (\%) = \frac{Gs}{Gc} \times \frac{Ls}{Lc} \times 100 \quad (4)$$

Physicochemical and mineral analysis of olive mill wastewater

The hydrogen potential (pH) and electrical conductivity (EC) measurements were carried out by using a pH meter type pH-2006 and a conductivity meter type Thermo Scientific Orion. The total suspended solids (TSS) were acquired through the centrifugation of a 50 ml aliquot of raw olive mill wastewater samples at 4000 rpm for 20 minutes. The TSS value was established by assessing the weight differential before and after centrifugation, followed by drying in an oven at 105 °C for two hours (AFNOR T 90-105). The sample weight loss was employed to ascertain the total solids content. This determination was carried out by exposing the sample to an oven till a stable weight was attained.

The chemical oxygen demand (COD) quantifies the quantity of oxygen consumed by organic matter, this measurement is established through the oxidation of organic compounds with potassium dichromate under high-temperature and acidic conditions (APHA 1992). The measurement of BOD_5 was conducted according to the (T 90-103) standards; this parameter represents the amount of oxygen necessary for the decomposition of biodegradable organic substances.

Ammonium (NH^{4+}), nitrite (NO^{2-}), and total phosphorus (P_T) were quantified following (AFNOR, 1983). The assessment of nitrate (NO^{3-}) levels was performed through the utilization of molecular absorption spectrometry, specifically the sodium salicylate method (ISO 7890-3 December 1998). The determination of chlorides involved the application of the Mohr titration method, utilizing silver nitrate and potassium chromate for precise measurement (AFNOR NF ISO 9297).

The evaluation of both major and trace metallic elements is achieved through the use of atomic absorption spectrometry with a flame (NF T90-020). Specifically, the quantification of sodium and potassium (AFNOR NF T 90-019) for assessing water quality, is performed using the same atomic absorption spectrometry method. Furthermore, the analysis of metallic elements, including Fe (iron), Cd (cadmium), Cu (copper), Zn (zinc), and Pb (lead), was conducted through the utilization of atomic absorption flame techniques. These analytical processes encompass both direct assays and methodologies involving complexation and extraction. The total polyphenols were quantified using the Folin Ciocalteu colorimetric technique following the procedure outlined by Kähkönen et al. (1999) with modifications. Specifically, 50 µl of the phenolic extract was mixed with 1.35 ml of distilled water and 200 µl of the Folin Ciocalteu solution. After a 3-minute incubation, 400 µl of 20% sodium carbonate (Na_2CO_3) solution was added, resulting in the development of a blue color. The mixture was then incubated in a water bath at 40 °C for 20 minutes after the absorbance was measured at 760 nm. The results were determined using a standard gallic acid curve for calibration. Total flavonoids were determined according to the method described by Ayoola et al. (2008), and total sugars were determined according to the technique of Dubois et al. (2002).

Growth parameters, physiological and biochemical analysis

Morphometric measurements were conducted on all cultivated plants throughout the entire growth period. These measurements were taken biweekly over three months to monitor the evolution of growth parameters. The measurements encompassed determination of both the height of aerial parts and stem diameter, utilizing a caliper for precise measurements. Additionally, the count of branches was recorded. Leaf area was determined using Mesurim software, where three leaves were sampled from the base, middle, and apical sections of each plant. Root length was measured at the beginning of the experiment and its end. Careful collection of pods was undertaken to prevent damage to the plants, and these pods were individually weighed to ascertain their fresh weight (FW). The chlorophyll pigment content was assessed using the procedure detailed by Tran et al. (1995), the leaf proline content was determined following the method outlined by Monneveux and Nemmar (1986). The quantification of soluble sugars in leaves was conducted by referencing the procedure of Yemn and Willis, as reported and modified by Sidari et al. (2008). The quantum yield of PSII is measured using a portable Fluorometer model FMS (FMS2 Pulse Modulated Chlorophyll Fluorescence Monitoring System, Hansatech, England).

Experimental device and statistical analysis

In this experiment, the conditions were heterogeneous, as the work was conducted in a field setting. Consequently, the authors opted for a complete randomized block (CRB) design (Figure 1), consisting of three blocks, each containing 81 bean plants (3 plants per treatment, with 9 treatments per block). The blocks served as a means of replication, while the treatments were represented within sub-blocks. The outcomes are displayed as the mean ± standard deviation. To ascertain the significance of variations among different tests, an Analysis of Variance (ANOVA) was employed. Statistical data analysis was conducted using IBM SPSS Statistics software (version 20), and the Tukey's test was applied at a 5% significance level.

RESULTS AND DISCUSSION

Physicochemical characteristics of olive mill wastewater

Olive mill wastewater constitutes a highly contaminated effluent with a composition that varies significantly. This variability depends on several factors, such as the type of olives used (the Moroccan picholine species being the predominant variety in Morocco), the degree of ripeness (which varies depending on the harvesting period), the cultivation methods employed, the salting practices applied to preserve the olives, prevailing climatic conditions, and the specific olive oil extraction processes utilized (Stillitano et al., 2019). Olive mill wastewater is known for

Figure 1. Complete randomized block design

its distinctive and increasingly potent odor as it is stored. Initial observations revealed that OMW exhibits a brown to reddish-brown hue, which darkens during storage, resulting in a cloudy appearance and a pronounced acidic odor (Paraskeva and Diamadopoulos 2006). The conducted investigations have confirmed the acidic nature of OMW, with a pH of 5.48, attributed to the existence of organic acids, including phenolic acids and fatty acids (Bounadi et al., 2023). This pH value falls within the range reported in the existing literature (Azzam and Hazaimeh 2021; Tsigkou et al., 2022). Similarly, Gueboudji and Kadi (2023) observed that OMW is acidic and rich in organic and mineral constituents.

Electrical conductivity varied between 18 and 50 mS/cm (Di Serio et al., 2008). It is worth noting that this parameter alone may not provide an immediate indication of the mineral content (Paredes et al., 2005). The obtained results align with findings in the literature (Gueboudji et al., 2021; Benaddi et al., 2022). Specifically, a value of 23.07 mS/cm was measured, denoting a substantial salt content in these effluents, caused by the practices of salting during olives preservation. Regarding water content, the conducted analysis recorded a value of 96.3%, these values follow those reported by Kıpçak and Akgün (2018b). Therefore, the valorization of these effluents is of significant importance, particularly as a potential source of water.

The values for total suspended solids and total solids are 6 g/L and 37 g/L, respectively. Similar findings were reported by Es Saouini et al. (2023) indicating that OMW is notably laden with total suspended solids and dry matter. The considered samples, drawn from the storage bays during the peak oil season, exhibited elevated levels of suspended matter and dry matter. This could be attributed to the origin of the OMW sampling, the high concentrations of total suspended solids and total solids may result from incomplete settling in the storage bays or due to the effects of wind and/or agitation during the unloading of OMW. Regarding organic matter (23.12 g/L) and ash content (13.85 g/L), the obtained results align with those found in the existing literature (El Yamani et al., 2020; Zaier et al., 2017; Gueboudji and Kadi, 2023).

Olive mill wastewater is notably rich in organic matter expressed in BOD_5 (biological oxygen demand) and COD (chemical oxygen demand). According to Table 2, values of 4.7 g/L were obtained for BOD_5 and 6.65 g/L for COD. These results are relatively lower when compared to those reported by a majority of authors (El Hajjouji et al., 2007; Bouknana et al., 2021; Rais et al., 2017). The BOD_5 value obtained is 4.7 g O_2/L, indicating that the BOD_5 levels below 8 g/L do not present any phytotoxicity, as reported by El-Rhaouat et al. (2014). It is worth noting that the daily direct discharge of olive mill wastewater (OMW) can lead to the asphyxiation of the receiving environment.

The content of phenolic compounds exhibited a range between 2 g/L and 12 g/L (Zbakh and El Abbassi, 2012; Tosti et al., 2013). The total polyphenols and flavonoid content were measured at 0.53 g/L and 0.19 g/L, respectively. These values are lower compared to the majority of authors (Bargougui et al., 2019; Meftah et al., 2019). This variation in phenolic content could be attributed to the nature of the olive mill wastewater (OMW). The recorded total sugar content is 1.86 g/L, which falls at the lower end of the range documented by Bouknana et al. (2014). The obtained mineral content analysis results demonstrate that olive mill wastewater is a complex effluent with a wide variation in macro, micro, and oligo-elements, the concentrations of

Table 2. Physicochemical characteristics of raw olive mill wastewater

Parameters	Unite	Value
pH (25°C)	-	5.48
Electrical conductivity (25°C)	(mS/cm)	23.07
Chemical oxygen demand	(g/L)	6.65
Biochemical oxygen demand	(g/L)	4.7
Water content	(%)	96.3
Total solids	(g/L)	37
Total suspended solids	(g/L)	6
Organic matter	(g/L)	23.12
Ash content	(g/L)	13.85
Phenolic compounds	(g/L)	0.53
Total flavonoid contents	(g/L)	0.19
Total sugars	(g/L)	1.86
Ammonium (NH4+)	(mg/L)	0.382
Nitrite (NO^{2-})	(mg/L)	0.317
Nitrate (NO^{3-})	(mg/L)	8.72
Phosphorus (P)	(g/L)	0.19
Sodium (Na)	(g/L)	2.08
Chlorides (Cl)	(g/L)	0.37
Potassium (K)	(g/L)	4.7
Calcium Ca^{2+}	(mg/L)	65.94
Zinc (Zn)	(mg/L)	12.32
Iron (Fe)	(mg/L)	45.36
Copper (Cu)	(mg/L)	0.36
Cadmium (Cd)	(mg/L)	0.025
Lead (pb)	(mg/L)	0.91

total ammonium (NH^{4+}), nitrate (NO^{3-}), and nitrite (NO^{2-}) were measured at 0.382 mg/L, 8.72 mg/L, and 0.317 mg/L, respectively. these values are comparatively lower than those reported by the majority of authors (Yalcuk et al., 2010; Khattabi Rifi et al., 2021). However, it is essential to note that a previous study by Kıpçak and Akgün (2018b) found OMW to have a very high content of mineral salts. The obtained results reveal values of 0.19 g/L for phosphorus (P), 2.08 g/L for sodium (Na^+), 0.37 g/L for chlorides (Cl^-), 4.7 g/L for potassium (K^+), and 65.94 mg/L for calcium (Ca^{2+}).

The presence of heavy metals in olive mill wastewater (OMW) is significant, particularly in the case of iron and zinc, whereas copper, cadmium, and lead are only found in trace amounts. Specifically, the values of 12.32 mg/L were measured for zinc (Zn), 45.36 mg/L for iron (Fe), 0.36 mg/L for copper (Cu), 0.025 mg/L for cadmium (Cd), and 0.91 mg/L for lead (Pb). Consequently, the agronomic valorization of these effluents is of great value as a source of essential fertilizing elements. Several studies have concluded that the controlled spreading of OMW onto agricultural soils can be a successful strategy for its valorization, provided that it is carried out under controlled conditions and with appropriate dosages (Chatzistathis and Koutsos 2017; Meftah et al., 2019; Mekki et al., 2018).

Growth parameter of *Vicia faba* L.

The obtained findings demonstrate that the application of olive mill wastewater and salt stress leads to a decrease in stem height, foliar surface, the number of branches, and root biomass diameter in the Faba bean. This application has a detrimental effect on these growth parameters, as it hampers the development of both aerial and underground plant parts. These results are consistent with the findings reported by Al-Mefleh et al. (2020). A previous study conducted by Dakhli and Maalej (2017) also observed a substantial reduction in plant growth and barley productivity as a result of OMW application.

During the vegetation stage, all morphological parameters exhibited gradual increases across all treatments. Notably, it was observed that the different concentrations did not have a significant impact on these parameters during this stage, and this observation was substantiated by statistical analyses. The data revealed that there were no significant differences in various concentrations compared to the control. The effects of applying olive mill wastewater and salt solutions became noticeable starting from the eighth week and remained consistent during both the flowering and fruiting stages (10 weeks). The highest values for length and diameter, measuring 42.33 cm and 1.08 cm, respectively, were observed in control plants. However, these values gradually decreased with increasing salt and olive mill wastewater (OMW) stress, reaching 33.28 cm in length and 0.75 cm in diameter. This reduction is highly statistically significant ($p \leq 0.01$).

These results align with those obtained by Rusan et al. (2015) where untreated OMW was found to be phytotoxic to maize plants, resulting in reduced plant weight. The reduction in growth may be attributed to an increase in abscisic acid concentration in the aerial part or a decrease in cytokinins (Itai, 2018). Sdiri Ghidaoui et al.

Table 3. Impact of stress on the morphological parameters of *Vicia faba* L. plants. Measured every two weeks for three months

Time of treatment	Before treatment								
	T0	[M1]	[M2]	[M3]	[M4]	[S1]	[S2]	[S3]	[S4]
Height (cm)	11.56^{ns}	11.22^{ns}	12.78^{ns}	10.56^{ns}	11.78^{ns}	11.72^{ns}	12.22^{ns}	11.44^{ns}	11.06^{ns}
Collar diameter (cm)	0.54^{ab}	0.56^{ab}	0.61^{b}	0.48^{a}	0.57^{ab}	0.57^{ab}	0.57^{ab}	0.54^{ab}	0.56^{ab}
Numbers of branches	4.11^{ns}	3.67^{ns}	4.33^{ns}	3.56^{ns}	3.67^{ns}	3.89^{ns}	4.00^{ns}	4.33^{as}	3.78^{ns}
	2 weeks after the treatment								
Height (cm)	18.00^{ns}	17.56^{ns}	18.78^{ns}	17.22^{ns}	18.17^{ns}	18.89^{ns}	20.06^{ns}	16.83^{ns}	16.94^{ns}
Collar diameter (cm)	0.73^{ns}	0.7^{ns}	0.71^{ns}	0.66^{ns}	0.74^{ns}	0.73^{ns}	0.77^{ns}	0.77^{ns}	0.74^{ns}
Numbers of branches	7.56^{ns}	7.11^{ns}	7.44^{ns}	6.78^{ns}	6.67^{ns}	6.89^{ns}	7.67^{ns}	7.78^{ns}	7.33^{ns}
	4 weeks after the treatment								
Height (cm)	24.56^{ns}	26.28^{ns}	24.56^{ns}	23.72^{ns}	25.50^{ns}	25.56^{ns}	27.22^{ns}	24.17^{ns}	23.00^{ns}
Collar diameter (cm)	0.84^{ns}	0.78^{ns}	0.75^{ns}	0.79^{ns}	0.87^{ns}	0.85^{ns}	0.88^{ns}	0.89^{ns}	0.77^{ns}
Numbers of branches	10.56^{ns}	10.33^{ns}	10.33^{ns}	11.33^{ns}	10.78^{ns}	10.33^{ns}	11.22^{ns}	11.22^{ns}	10.89^{ns}
	6 weeks after the treatment								
Height (cm)	32.78^{ns}	32.56^{ns}	29.67^{ns}	28.11^{ns}	31.11^{ns}	30.89^{ns}	33.11^{ns}	28.67^{ns}	28.33^{ns}
Collar diameter (cm)	0.97^{ns}	0.97^{ns}	0.89^{ns}	0.8^{ns}	0.89^{ns}	0.98^{ns}	0.87^{ns}	0.93^{ns}	0.89^{ns}
Numbers of branches	14.89^{ns}	13.89^{ns}	13.78^{ns}	13.33^{ns}	14.56^{ns}	14.22^{ns}	16.11^{ns}	14.56^{ns}	14.89^{ns}
Leaf area (cm^2)	34.36^{ns}	42.59^{ns}	39.39^{ns}	37.09^{ns}	37.00^{ns}	38.80^{ns}	35.46^{ns}	33.14^{ns}	32.09^{ns}
	8 weeks after the treatment								
Height (cm)	42.11^{a}	40.56^{bc}	34.44^{bc}	34.33^{bc}	37.7^{abc}	37.78^{abc}	37.44^{abc}	32.67^{c}	32.33^{c}
Collar diameter (cm)	1.02^{ns}	1.06^{ns}	0.98^{ns}	0.89^{ns}	0.94^{ns}	1.07^{ns}	0.89^{ns}	1.01^{ns}	0.96^{ns}
Numbers of branches	17.78^{ns}	16.44^{ns}	15.89^{ns}	15.44^{ns}	17.00^{ns}	17.33^{ns}	15.56^{ns}	15.89^{ns}	14.78^{ns}
Time	10 weeks after the treatment								
Height (cm)	42.33^{a}	40.33^{ab}	35.50^{bc}	33.28^{c}	37.8^{abc}	38.67^{abc}	37.11^{abc}	33.33^{c}	34.33^{bc}
Collar diameter (cm)	1.04^{a}	0.94^{ab}	0.94^{ab}	0.76^{b}	0.79^{ab}	1.04^{a}	0.95^{ab}	0.93^{ab}	0.87^{ab}
Numbers of branches	15.78^{ns}	14.83^{ns}	14.44^{ns}	14.67^{ns}	16.11^{ns}	15.50^{ns}	15.78^{ns}	14.89^{ns}	14.78^{ns}
	12 weeks after the treatment								
Height (cm)	42.33^{a}	40.33^{ab}	35.50^{bc}	33.28^{c}	37.8^{abc}	38.67^{abc}	37.11^{abc}	33.33^{c}	34.33^{bc}
Collar diameter (cm)	1.08^{a}	0.95^{abc}	0.94^{abc}	0.75^{c}	0.78^{bc}	1.04^{ab}	0.95^{abc}	0.93^{abc}	0.85^{abc}
Numbers of branches	15.78^{ns}	14.39^{ns}	14.00^{ns}	14.11^{ns}	15.78^{ns}	15.28^{ns}	15.61^{ns}	14.61^{ns}	14.28^{ns}
Leaf area (cm^2)	18.56^{ns}	21.55^{ns}	22.22^{ns}	20.29^{ns}	20.96^{ns}	21.66^{ns}	19.45^{ns}	19.21^{ns}	20.55^{ns}

Note: Each value is expressed as mean ± standard deviation (n=3) (ns: $p>0.05$, $^{a, b}$ and ^{c} $p\leq 0.0001$). Groups consisting of two or three letters indicate interaction between them.

(2019) discovered that the application of olive mill wastewater at high rates had an impact on morphological, physiological, and biochemical parameters in *Vicia faba* L. Similarly, Bibi et al. (2012) demonstrated that salt stress reduced the stem height of *Vicia faba* L., even at lower concentrations. These studies corroborate the drawn observations regarding the effects of OMW and salt stress on plant parameters.

The observed reduction in seedling vegetative growth can be attributed to the increase in osmotic pressure due to NaCl. Furthermore, it may result from the inhibition of central axis elongation (Maaouia-Houimli et al., 2011), and a decrease in the number of cell divisions (Benmahioul et al., 2009). The obtained findings are consistent with those of Sdiri Ghidaoui et al. (2019) who observed increased shoot elongation in the plants grown in the soil treated with OMW.

The roots serve as the initial point of contact among the various constituents of the soil. It is through these specialized plant structures that plants establish their first interaction with the surrounding environment. When elements such as salts and olive mill wastewater are present in the soil, the roots are the first to come into contact with these compounds. In this context, the obtained findings revealed a notably significant

Table 4. Impact of stress on root length, pod weight (g), and number of pods per plant of *Vicia faba* L. at the end of the experiment

Treatment	Number of pods per plant	Pod weight (g)	Root length (cm)
T0	4.67 ^{ns}	518.35 ^{a}	38.30 ^{a}
[M1]	4.33 ^{ns}	400.23 ^{b}	35.10 ^{d}
[M2]	3.67 ^{ns}	328.71 ^{f}	35.00 ^{d}
[M3]	3.56 ^{ns}	348.59 ^{e}	30.20 f
[M4]	2.89 ^{ns}	308.68 ^{g}	28.60 ^{g}
[S1]	4.11 ^{ns}	385.82 ^{c}	36.20 ^{b}
[S2]	3.44 ^{ns}	365.5 ^{d}	35.80 ^{c}
[S3]	4.44 ^{ns}	266.41 ^{H}	33.00 ^{e}
[S4]	3.00 ^{ns}	239.06 ^{i}	30.20 ^{f}

Note: Each value is expressed as mean ± standard deviation (n=3) (ns: p>0.05, $^{a\text{-}i}$ p≤0.0001).

adverse impact on root growth, stemming from both salt stress and the application of olive mill wastewater solution (p≤0.01), with a more pronounced reduction at higher concentrations. It is evident that the plants irrigated with olive mill wastewater experience more marked stress compared to the plants subjected to saline water. Indeed, the control group exhibited the longest growth (38.3 cm), while the lowest length (28.6 cm) was recorded in the M4 concentration group.

Rekik et al. (2017) reported similar outcomes, showing that the use of untreated OMW led to a reduction in root and stem length in seedlings. The presence of salt can limit the plant's access to calcium, consequently inhibiting root emergence and growth, including the development of absorption hairs (Saadallah et al., 2001). Various authors have confirmed that polyphenols can impede root growth (Sampedro et al., 2004; Massoudinejad et al., 2014; Babić et al., 2019).

The phytotoxicity of OMW is primarily attributed to its high concentration of aromatic compounds, like tannins and phenolic compounds (Babić et al., 2019). Similarly, Enaime et al. (2020) noted that the phytotoxicity of OMW is contingent on elevated salinity and an acidic pH.

The results indicate a statistically significant reduction in the fresh weight of pods under stress conditions (p ≤ 0.01), the control plants displayed a weight of 518 g, whereas the plant exposed to the highest level of treatment with olive mill wastewater (M4) exhibited a weight of 308 g. This reduction may be attributed to the hindrance in the absorption of essential ions like Ca^{2+}, K^{+}, and NO^{3-}, which are critical for plant growth and development. Additionally, the decline in fresh weight can be attributed to the fact that NaCl elevates the osmotic pressure in the cultivation environment, thereby impeding water absorption by the root system (Munns 2002). The decline in growth is frequently accompanied by noticeable symptoms such as wilting, chlorosis (yellowing of leaves), and leaf drop. These manifestations collectively contribute to a reduction in crown diameter and a decrease in the number of branches.

Physiological and biochemical analysis of *Vicia faba* L. leaves

Chlorophyll pigment content and the quantum yield of PSII

The analysis of variance indicated that the presence of both NaCl and olive mill wastewater results in a decrease in total chlorophyll levels, although this reduction is statistically insignificant. In contrast, the photosynthesis yield, particularly PSII, is significantly affected (p≤0.05), with a consistent reduction observed in all stressed plants compared to the control plants. These findings align with those of Rusan et al. (2015), who demonstrated that OMW application inhibits photosynthesis. The observed reductions in chlorophyll content in the considered salt- and OMW-stressed variety can be attributed to the inhibitory influence of Na^{+} and Cl^{-} ions on chloroplast structure, subsequently impacting the biosynthesis of various pigments (Mane et al., 2010).

Parida and Das (2005) took into account that the initial reaction to salt stress entails a decrease in the expansion of the leaf area. Furthermore, the impact of NaCl on photosynthesis is manifested through reduced chlorophyll content, leaf area, leaf number, transpiration rate, and stomatal conductance (Aldesuquy et

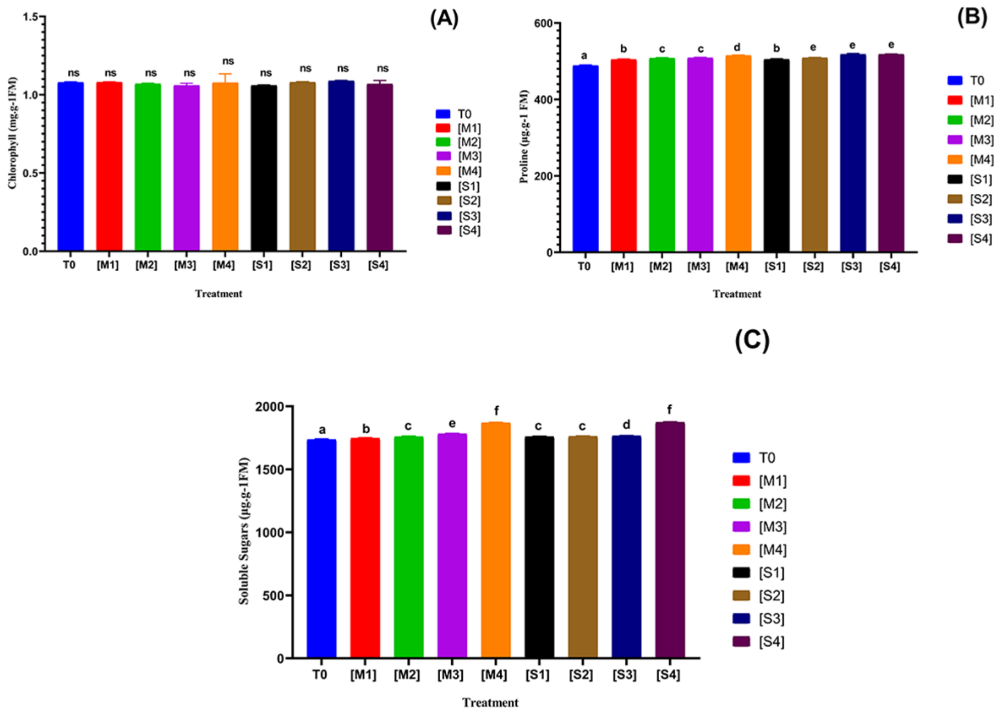

Figure 2. The influence of different salt stress levels and the application of olive mill wastewater after six weeks on various biochemical and physiological characteristics assessed in a *Vicia faba* leaf. Each value is expressed as mean ± standard deviation (n=3) (ns: p>0.05, a–f: p ≤ 0.0001) and A – chlorophyll pigment content after six weeks; B – proline content after six weeks; C – soluble sugars content after six weeks

al., 2014; Talaat 2019). Mechri et al. (2011) and Asfi et al. (2012) reported similar findings, illustrating a significant reduction in the quantum yield of photosystem II in the olive and spinach plants treated with OMW. High salt concentrations led to observable symptoms, including leaf yellowing, chlorosis, and even necrosis, ultimately resulting in the death of older leaves. This yellowing is a consequence of the damage inflicted upon chlorophyll pigments, leading to a reduction in pigment content (DAJIC, 2006). Furthermore, there is a decrease in chlorophyll synthesis, photosynthesis, and photochemical efficiency (Ahanger et al., 2019).

Proline content

The imposition of stress generally leads to an increase in proline content in stressed plants in comparison to control plants, and this elevation is typically directly related to the applied concentration. The treatment (M4) yielded the highest proline values, reaching 976.03 $\mu g \cdot g^{-1}$ FM, during the last week of the experiment. The proline content for all treatments saw a significant reduction, with this decrease being of high statistical significance ($p \leq 0.01$).

Similar observations have been made by Mansour and Ali (2017), when dealing with elevated levels of NaCl and OMW, they induced varying degrees of proline accumulation in bean leaves. Proline plays a crucial role in plant defense against various abiotic stresses, including salinity, as it helps protect membranes from damage (Slama et al., 2015; Shafi et al., 2019). The increase in proline content has also been documented by El Sayed and El Sayed (2011) and Hassanein et al. (2012) in *Vicia faba* L., with a more significant increase observed under severe stress conditions.

Some researchers suggest that changes in proline content may be linked to the loss of turgor and/or stress-induced stimulation of its synthesis (El-Iklil et al., 2002), others claim that proline accumulation is a result of the inhibitory impact of stress on its oxidation and integration into proteins. Additionally, proline accumulation might be attributed to the induction of the expression of proline biosynthesis genes (Yoshiba et al., 1995; Sdouga et al., 2019).

Soluble sugars content

The increase in soluble sugar content in plants has been extensively documented as a reaction to

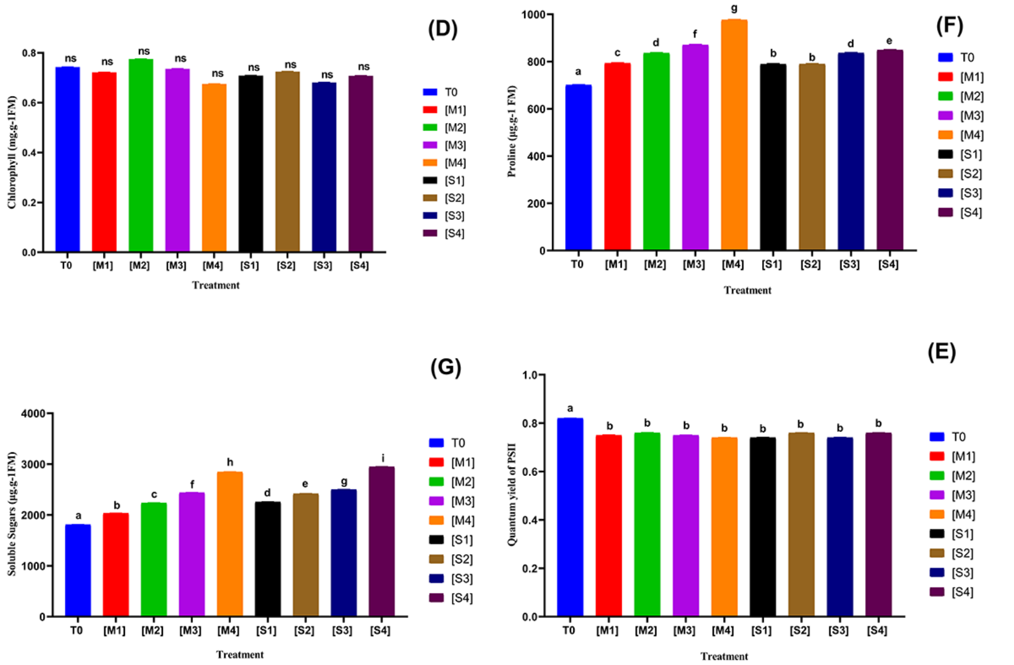

Figure 3. The influence of different salt stress levels and the application of olive mill wastewater after twelve weeks on various biochemical and physiological characteristics assessed in a *Vicia faba* leaf. Each value is expressed as mean ± standard deviation (n=3) (ns: $p>0.05$, a-i: $p\leq0.0001$) and D – chlorophyll pigment content after twelve weeks; F – proline content after twelve weeks; G – soluble sugars content after twelve weeks; E – the quantum yield of PSII after twelve weeks.

abiotic stress (Sami et al., 2016; Jogawat 2019), including instances of salt stress in *Pisum sativum* (Ahmad et al., 2018). In line with these findings, the obtained results reveal that the plants irrigated with high concentrations of salt and olive mill wastewater accumulate more sugars than those irrigated with lower concentrations. This increase is particularly significant at lower doses and exhibits an exponential rise with elevated salt and olive mill wastewater concentrations. Specifically, the presence of soluble sugars in plants exhibited an increase, going from 1,808.48 ($\mu g \cdot g^{-1}$ FM) for the control plant to 2,844.80 ($\mu g \cdot g^{-1}$ FM) for the plant that received the highest amount of olive mill wastewater, and this is statistically highly significant ($p\leq0.01$).These outcomes align with those reported by Magdich et al. (2016).

The ability of plants to resist various types of stress, including drought, salinity, and oxidative stress, is attributed to the accumulation of soluble sugars (Martínez-Noël and Tognetti 2018; Peng et al., 2016). This accumulation plays a role in down-regulating photosynthesis to maintain homeostasis and has been demonstrated to serve as an Osmo protective mechanism (Banerjee and Roychoudhury 2018; Slama et al., 2015) The elevation in soluble sugar content could stem from the degradation of starch reserves, swiftly transforming them into sucrose (Munns et al., 2006). Alternatively, it may arise from modifications in the enzymatic activities linked to carbohydrate metabolism (Dubey and Singh 1999).

Germination test

Rate of germination

The results of the study point towards a clear correlation between the dosage of olive mill wastewater and the germination rates of the seeds. As the concentration of olive mill wastewater rises, a discernible reduction in the ability of seeds to germinate becomes evident. However, this reduction in germination rates is statistically insignificant. The highest germination rate was observed in the control group and the medium enriched with 5% OMW, with rates approaching 97%. Consequently, this 5% concentration appears to be conducive to seed germination. This can be attributed to the dilution of OMW, which reduces the concentration of elements and promotes germination. Enaime et al. (2020) have reported that a significant dilution is required to

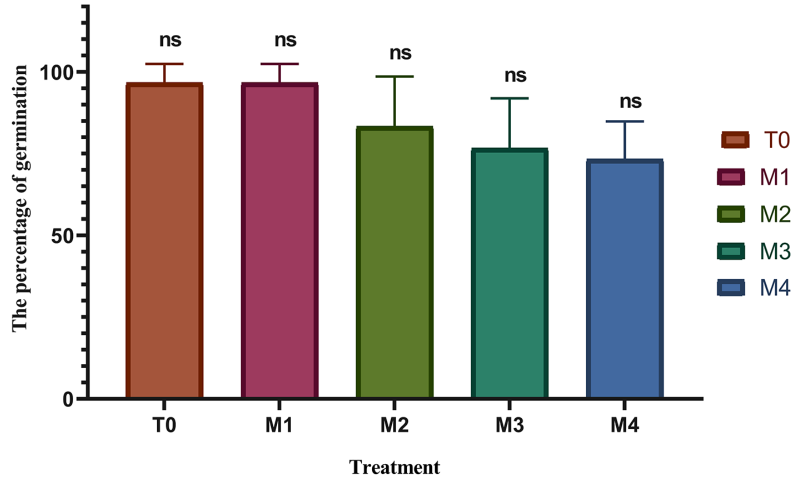

Figure 4. The germination rate of *Vicia faba* L. seeds following treatment with olive mill wastewater. Each value is expressed as mean ± standard deviation (n=3) (ns: p>0.05)

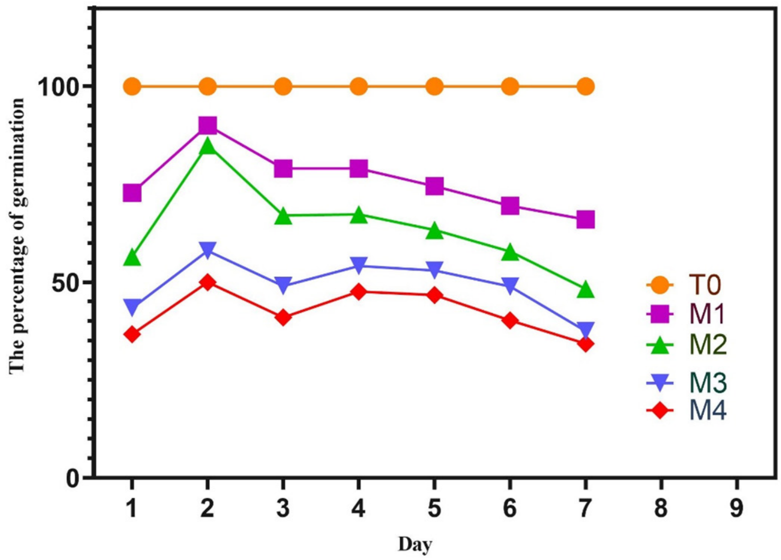

Figure 5. Germination index within seven days

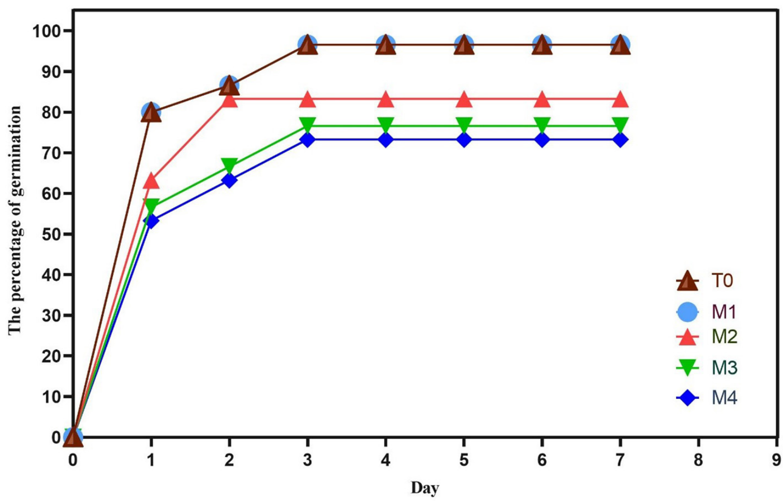

Figure 6. Germination kinetics within seven days

decrease the phytotoxic impact of undiluted olive mill wastewater on tomato plant.

On the other hand, at a concentration of 30%, the germination rate exhibits a significant decline to 73%. Many authors have also noted that high doses of OMW are phytotoxic and effectively prohibit seed germination (Enaime et al., 2020; Popolizio et al., 2022). This inhibition at elevated concentrations can be attributed to the toxic impact of salinity and the presence of polyphenols in OMW, the suppressive impacts of these phenolic compounds are more evident in terms of inhibiting root elongation as compared to seed germination (Hanafi et al., 2011; Bouknana et al., 2019).

The reduced germination rates at concentrated OMW levels can be attributed to the highly phytotoxic properties of concentrated OMW, primarily due to its phenolic content and its salt content (Barbera et al., 2014; Benidire et al., 2015). Rusan et al. (2015) and Komilis et al. (2005) have demonstrated that phytotoxicity decreases as OMW is diluted with water. Similarly, recent studies by Zahra El Hassani et al. (2023) and Saf et al. (2023) have found that OMW application either partially or completely inhibits the germination of seeds from various plant species.

Kinetics of germination

Through the analysis of different concentrations and time periods, it was possible to identify three distinct phases in the germination process:

- an imbibition phase is essential for the initial appearance of germination. The duration of this phase varies depending on the concentration of OMW and is relatively short;
- an exponential phase is characterized by a rapid increase in the germination rate, which progresses proportionally with time;
- the final phase represents the ultimate percentage of germination achieved.

CONCLUSIONS

The phytotoxicity study reveals that the daily application of fresh olive mill wastewater on broad beans throughout their entire growth cycle has adverse agronomic consequences. It was observed that phytotoxicity is contingent on the concentration of OMW and the phenological stage. Notably, the most significant phytotoxicity was detected with high doses of OMW, resulting in a sequence of morphological, physiological, and biochemical changes, this included growth delays, germination inhibition, increased levels of sugar and proline, degradation of chlorophyll pigments, and a reduction in the quantum yield of photosystem II. Three key factors contribute to this phytotoxicity: salinity, acidity, and phenolic compounds, serving as the primary determinants of the phytotoxic effect. Nevertheless, the use of appropriate doses is crucial for the success of this practice, especially in the regions where water resources are limited. The presence of valuable fertilizing elements and water in OMW can be particularly advantageous in such areas. It could be considered a useful and cost-effective amendment and fertilizer.

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