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RESEARCH ARTICLE

# Improved yield, fruit quality, and salt resistance in tomato co-overexpressing *LeNHX2* and *SlSOS2* genes

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**Abstract** The $K^+$, $Na^+/H^+$ antiporter LeNHX2 and the regulatory kinase SlSOS2 are important determinants of salt tolerance in tomato plants and their fruit production ability. In this work, we have analyzed the effects of *LeNHX2* and *SlSOS2* co-overexpression on fruit production, quality in tomato plants (*Solanum lycopersicum* L. cv. MicroTom), and analyzed physiological parameters related to salt tolerance. Plants overexpressing *LeNHX2*, *SlSOS2* or both were grown in greenhouse. They were treated with 125 mM NaCl or left untreated and their salt tolerance was analyzed in terms of plant biomass and fruit yield. Under NaCl cultivation conditions, transgenic tomato plants overexpressing either *SlSOS2* or *LeNHX2* or both grew better and showed a higher biomass compared to their wild-type plants. Proline, glucose and protein content in leaves as well as pH and total soluble solid (TSS) in fruits were analyzed. Our results indicate that salinity tolerance of transgenic lines is associated with an increased proline, glucose and protein content in leaves of plants grown either with or without NaCl. Salt treatment significantly reduced yield, pH and TSS in fruits of WT plants but increased yield, pH and TSS in fruits of transgenic plants, especially those overexpressing both *LeNHX2* and *SlSOS2*. All these results indicate that the co-overexpression of *LeNHX2* and *SlSOS2* improve yield and fruit quality of tomato grown under saline conditions.

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## Introduction

Salt stress is one of the major factors that limit plant growth and crop productivity, especially in the arid and semi-arid regions where soil salinity poses a severe threat to food security. Plants respond to salt stress by osmotic adjustment, generally by increasing the concentrations of solutes such as proline and soluble sugars in order to adjust osmotic potential for a better uptake of water (Li et al. 2014; Wu et al. 2016). Proline is accumulated in leaves in order to maintain chlorophyll level and cell turgor to protect photosynthesis activity under salt stress (Silva-Ortega et al. 2008). The soluble sugars are highly accumulated in vacuoles and produce high turgor pressure that are involved in response to abiotic stresses such as salt stress by affecting osmotic potentials (Rasheed et al. 2011). Veeranagamallaiah et al. (2007) reported that plants respond and adapt to salt stress through the synthesis of specific proteins, and the synthesis of stress-induced proteins is part of the stress tolerance mechanism.

The overexpression of $Na^+$, $K^+/H^+$ antiporters and their regulatory proteins has been evidenced as an important method to overcome the adverse effects of salt stress on tomato plants and improve plant growth as well as crop productivity (Rodríguez-Rosales et al. 2008, 2009; Gálvez et al. 2012; Huertas et al. 2012, 2013; Cagnac et al. 2020; Maach et al. 2020). The intracellular NHX transporters constitute the first Cation/Proton exchanger family studied in plants. It was shown that overexpression of this protein in various plants improves salt tolerance, indicating a role of the protein in vacuolar $Na^+$ accumulation (Apse et al.

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1999; Zhang and Blumwald 2001; Venema et al. 2002). In *Arabidopsis*, the SOS signal transduction pathway is responsible for $Na^+$ homeostasis and salinity tolerance by maintaining favourable $K^+/Na^+$ ratios in the cytoplasm through the action of the plasma membrane $Na^+/H^+$ antiporter SOS1, which mediates $Na^+$ extrusion out of the root cell and long-distance $Na^+$ transport from roots to shoots (Shi et al. 2002; Zhu 2002). In this pathway, a calcium-binding protein, SOS3, senses cytosolic calcium changes elicited by salt stress (Ishitani et al. 2000). SOS3 physically interacts with and activates the serine/threonine protein kinase, SOS2 (Liu et al. 2000). The SOS3/SOS2 kinase complex phosphorylates and activates the plasma membrane $Na^+/H^+$ exchanger encoded by the SOS1 gene (Qiu et al. 2002; Quintero et al. 2002; Shi et al. 2002; Olías et al. 2009). Coordination of the activity between the $Na^+/H^+$ exchangers of the tonoplast and plasma membrane were suggested by Qiu et al. (2004) who observed that the mRNA levels of *NHX* genes were up-regulated in the *sos1* mutant. Moreover, an increased fruit production under NaCl conditions was reported in tomato plants co-overexpressing the *Pennisetum glaucum* vacuolar $Na^+/H^+$ antiporter and *Arabidopsis* $H^+$-pyrophosphatase (Bhaskaran and Savithramma 2011). Also, in *Arabidopsis*, the co-overexpression of *NHX1* and *SOS3* was demonstrated to further improve salt tolerance relative to plants overexpressing only *NHX1* (Yang et al. 2009) and an increased silique production under salt stress was detected in double transgenic plants overexpressing *AtNHX1* and *SOS1* (Pehlivan et al. 2016). Recently, we have also reported that in tomato that the co-overexpression of *LeNHX2* and *SlSOS2* increases salt tolerance and fruit production (Baghour et al. 2019).

Besides conferring salt tolerance, plant antiporters play an important role in improving the organoleptic characteristics of fruits as well as those related to fruit ripening (Hanana et al. 2009; Huertas et al. 2012). Total soluble solid (TSS) and pH are important fruit quality characteristics for suitability of tomato in industrial processing (Teka 2013). In this context, the aim of the present study is to investigate the effects of *LeNHX2* and *SlSOS2* co-overexpression on tomato fruit production and quality as well as determining the role of sugar, proline and protein accumulation during salt stress tolerance of tomato transgenic plants overexpressing *LeNHX2*, *SlSOS2* or both genes.

## Materials and methods

### Plant Material and growth conditions

Tomato plants (*Solanum lycopersicum* L. cv. Microtom) overexpressing *LeNHX2*, *SlSOS2* or both genes were used in this work. T3 tomato plants homozygous and mono-locus for the transgenes *LeNHX2* (Huertas et al. 2013) and *SlSOS2* (Huertas et al. 2012) were cross-pollinated. After crossing, plants were grown and seeds were removed from mature fruits derived from the cross-pollinated flowers.

The flower of the *SlSOS2* overexpressing plants from line L-82 were used as female parents and those from the *LeNHX2* overexpressing from L-932 were used as male parents. Analysis by real time PCR of F1 plants harbouring the constructs for *SlSOS2* and *LeNHX2* co-overexpression showed a higher expression of both genes compared to wild-type and single transgenic lines (Baghour et al. 2019).

Seeds from WT and single transgenic plants overexpressing *LeNHX2* or *SlSOS2* as well as seeds derived from the crosses between the T3 homozygous *LeNHX2* and *SlSOS2* transgenic plants were germinated and the resulting plants were used for experiments. Tomato seeds were sown in seedbeds containing peat-moss. The seedbeds were kept in a greenhouse from Mohamed I University in Nador (Morocco) and irrigated with tap water for 7 weeks, and then the plants were transferred to 1.2 L pot (1 plant per pot) containing peat-moss and kept in a greenhouse. These plants were irrigated with tap water for 1 week and then with either 100 mL tap water or 100 mL of 125 mM NaCl three times every week for 16 weeks.

### Plant analysis

Plant materials were gently removed from their substrate at 17 weeks after transplanting. The roots, leaflets, stems, and fruit were rinsed three times in distilled water after decontamination with nonionic detergent and then blotted on filter paper (Wolf 1982). Then, samples were dried in a forced air oven at 70 °C for 48 h.

### Glucose and proline determination

Samples of 0.5 g of plant tissues were crushed in 5 mL 95% (v/v) ethanol. The insoluble fraction of the extract was washed twice with 5 mL of 70% ethanol. All soluble fractions were centrifuged at 3500 × *g* for 10 min. The supernatants were collected and stored at 4 °C for glucose and proline determinations (Irigoyen and Emerich 1992). Glucose concentration was determined spectrophotometrically at 650 nm using the colorimetric assay with the anthrone reagent (Irigoyen and Emerich 1992). The free proline content was measured spectrophotometrically at 515 nm by the method of Paquin and Lechasseur (1979).

### Soluble-protein determination

Fresh plant samples (0.5 g) were crushed with cold phosphate buffer (50 mM $KH2PO_4$, pH 7.0) and centrifuged at

12 000 × *g* for 15 min. The resulting supernatant was used for the determination of soluble proteins using Bradford G-250 reagent (Bradford 1976). The results are expressed as mg bovine serum albumin/g of fresh weight.

### Fruit yield and quality

At the corresponding ripening stage, 16 weeks after transplanting (at full maturity stage with an orange-red to red color), all fruits were collected and weighed to determine the yield and part of samples was immediately used to determine Total soluble solid (TSS) and pH (Papadaki et al., 2017; Baghour et al., 2019; Maach et al., 2020). For these determinations, we used 5 repetitions with two plants for each treatment (10 plants for each treatment).

TSS was measured in fruits at full maturity stage with an organe-red to red color as $^{o}$Brix in a few drops of the juice of mature fruit using a digital refractometers (ATAGO Co., Ltd., Tokoyo, Japan). Tests for pH were performed on homogenate of fully mature fruit using a portable pH meter.

### Statistics

All data in this report were obtained from an experiment with three to five repetition for each parameter. Statistical analyses were performed with Statgraphics Plus (Statistical Graphics Corp, StatPoint Inc., Herndon, VA). Analysis of variance (ANOVA) was used to assess difference between treatments and significance level was determined at $P \leq 0.05$. Significant differences according to the Duncan's multiple range test (DMRT) are indicated with different letters in the figures and tables.

## Results

To study the effects of the co-overexpression of *SlSOS2* and *LeNHX2* on the plant tolerance towards NaCl, we measured the fresh weights of roots, stems and leaves of wild type and transgenic plants grown under control conditions (0 mM NaCl) and in the presence of 125 mM NaCl (Fig. 1a, b). Under control conditions (no NaCl added to the culture), our data demonstrate that plants co-overexpressing *SlSOS2* and *LeNHX2* had significantly higher fresh weights of roots, stems and leaves ($P < 0.01$). Treatments with 125 mM NaCl reduced roots, stems and leaves fresh weight of wild-type, while transgenic plants expressing either *LeNHX2* or *SlSOS2* or both significantly showed significant increase in roots, stems and leaves fresh weight ($P < 0.001$; Fig. 1a, b). Phenotypic evaluation of transgenic lines co-expressing either *LeNHX2* or *SlSOS2* was also performed in plants grown in the absence (Fig. 1b) or the presence (Fig. 1c) of 125 mM NaCl. Under saline conditions, all transgenic plants, especially those co-expressing *LeNHX2* and *SlSOS2*, showed a better growth compared to untransformed plants.

The glucose contents were measured in leaves of WT and transgenic lines overexpressing either *LeNHX2* or *SlSOS2* or both grown in the presence or in the absence of NaCl (Fig. 2a). Our results showed that under the normal conditions, plants co-overexpressing *LeNHX2* and *SlOS2* accumulated 80% more glucose in leaves compared to the WT and single transgenic lines ($P < 0.001$). However, under saline condition, all transgenic plants showed an increased level of this carbohydrate. Interestingly, plants simultaneously overexpressing *LeNHX2* and *SlOS2* accumulated almost three times more glucose compared to wild type plants under NaCl treatment.

In relation to the proline accumulation, our data demonstrate that NaCl treatments significantly increased proline levels in wild-type and transgenic lines ($P < 0.001$; Fig. 2b). Furthermore, all transgenic plants, especially those overexpressing *LeNHX2*, accumulated higher contents of proline in either normal or saline conditions.

Regarding the effect of *LeNHX2* and *SlSOS2* co-overexpression on protein content in tomato leaves, our results showed that under normal conditions single transgenic plants that overexpress *LeNHX2* present the highest protein levels ($P < 0.01$; Fig. 2c). Nonetheless, under NaCl stress, increase in protein content was detected in plants overexpressing *LeNHX2* alone or in double transgenic lines, compared with wild-type and single transgenic plants overexpressing *SlSOS2*.

We have measured fruit production (Fig. 3a), total soluble solids (Fig. 3b) and pH (Fig. 3c) in NaCl-treated and untreated wild-type and transgenic plants. Interestingly, the effect of *LeNHX2* and *SlSOS2* co-overexpression on fruit yield (g/plant) is particularly evident under salt treatment ($P < 0.001$).

Under control conditions, (no NaCl added), the highest TSS levels were observed in wild-type plants (Fig. 3b). Treatment with NaCl stimulated an increase of TSS values in all transgenic plants ($P < 0.001$), reaching the highest value in fruits of double transgenic plants with an increase of 39% relative to WT fruits.

In relation to fruit pH, our results showed no significant differences between NaCl-untreated wild-type and transgenic plants (Fig. 3c). Relative to the fruit pH of untreated tomato plants, NaCl application significantly increased the pH of fruits from *LeNHX2*, *SlSOS2* and *LeNXH2SlSOS* transgenic plants while decreased the pH of fruits of wild-type plants ($P < 0.001$).

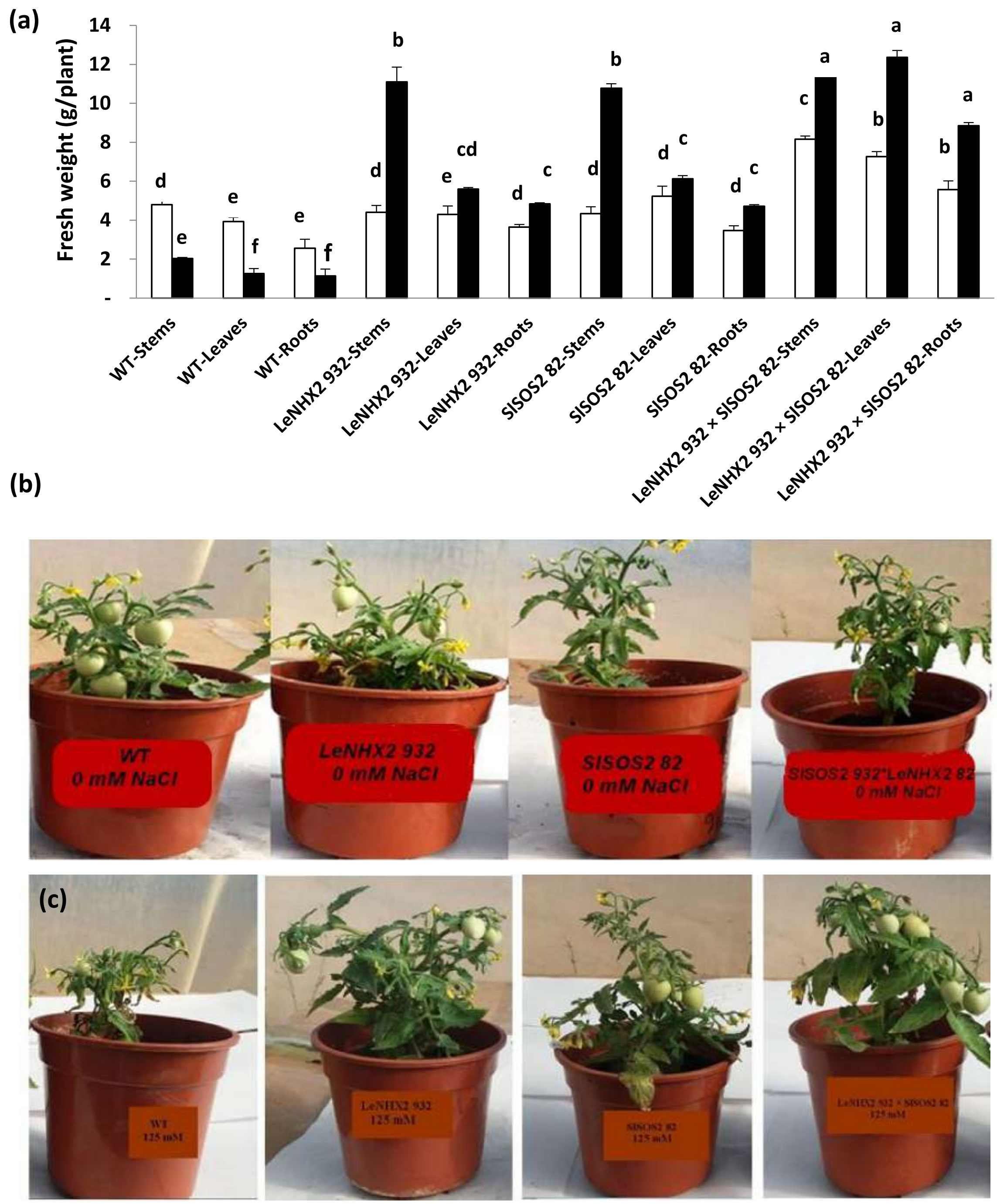


**Fig. 1** **a** Fresh Weight of wild-type, single and double transgenic plants overexpressing *LeNHX2* and *SlSOS2* grown either under the absence (empty bars) or the presence of NaCl (black bars) of 125 mM NaCl. Representative images of WT, single and double transgenic plants grown in the absence (**b**) or the presence (**c**) of under 125 mM NaCl. Means that have different letters at the top of each bar are significantly different at $P < 0.05$ according to Duncan's multiple range test

## Discussion

The effect of overexpression of ion transport genes on plant's salt tolerance could be further enhanced when more than one of these genes was overexpressed. It has been reported in *Arabidopsis* that overexpression of the vacuolar $Na^+/H^+$ antiporter AtNHX1 or the plasma membrane $Na^+/H^+$ antiporter *SOS1* improve salt tolerance in transgenic plants, but the improved salt tolerance is limited to NaCl concentrations lower than 200 mM (Pehlivan et al. 2016). LeNHX2 is a $K^+$, $Na^+/H^+$ antiporter and therefore participates in $Na^+$ accumulation in cell compartments when

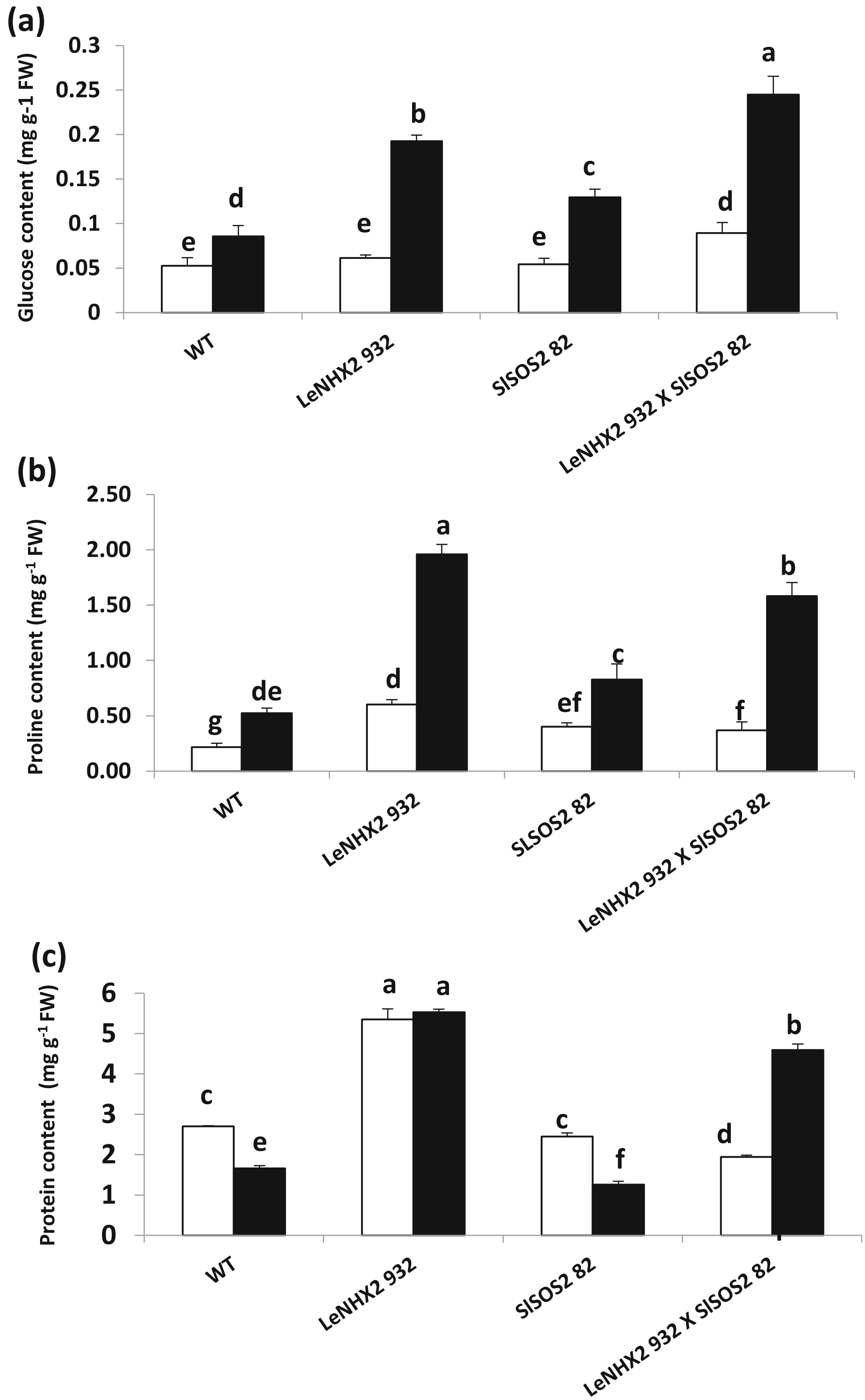


**Fig. 2** Effect of *LeNHX2* and *SlSOS2* co-overexpression on glucose (**a**), proline (**b**) and protein content (**c**) of tomato plants. WT and *LeNXH2, SlSOS2* and *LeNXH2 x SlSOS2* plants were cultivated in peat-moss for 17 weeks in the absence (empty bars) or the presence (black bars) of 125 mM NaCl. Means that have different letters at the top of each bar are significantly different at $P < 0.05$ according to Duncan's multiple range test

plants are grown under saline conditions. Intracellular $Na^+$ and $K^+$ accumulation could promote water influx to cell compartments and thus contribute to the increased fresh weight of transgenic plants grown in the presence of NaCl. Plants co-overexpressing the *LeNHX2* and *SlSOS2* genes showed an improved growth performance with higher root, stem, and leaf fresh weights under salt stress compared to WT and single transgenic plants (Fig. 1a–c). These results

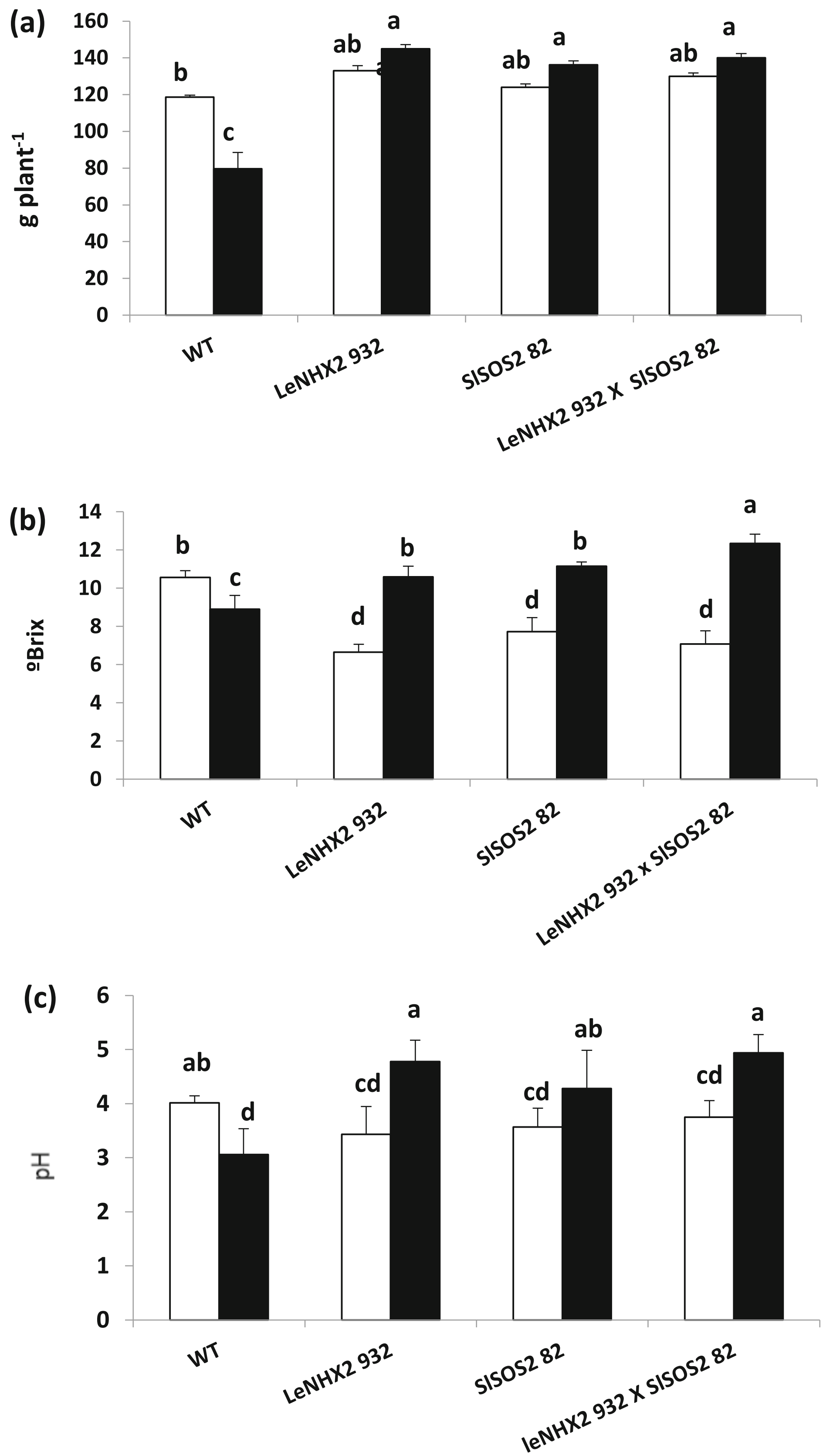


**Fig. 3** Effect of LeNHX2 and SlSOS2 co-overexpression on yield (**a**), TSS (**b**) and pH (**c**) of tomato plants. WT and *LeNXH2, SlSOS2* and *LeNXH2 x SlSOS2* plants were cultivated in peat-moss for 17 weeks in the absence (empty bars) or the presence (black bars) of 125 mM NaCl. Means that have different letters at the top of each bar are significantly different at $P < 0.05$ according to Duncan's multiple range test

are in accordance with previous studies on the effects of co-overexpression of several ion transport genes on salt tolerance and biomass production in *Arabidopsis*, cotton and tomato (Zhao et al. 2006; Shen et al. 2015). Shen et al. (2015) have reported that transgenic cotton plants expressing the Arabidopsis vacuolar $Na^+/H^+$ antiporter gene *AtNHX1* and *AVP1* produced significantly higher biomass compared to WT and single transgenic plants under salt stress conditions. Gouiaa et al. (2012) showed that tobacco plants co-expressing the wheat $Na^+/H^+$ antiporter *TNHXS1* and $H^+$-pyrophosphatase *TVP1* produced significantly higher number of leaves compared to mono-transgenic lines and the WT plants. Moreover, a possible explanation of the increased fresh weight of double transgenic plants under control (no NaCl added) conditions could be related to a better $K^+$ nutrition of double transgenic plants allowing for a better plant growth. All these reports indicate that expression of multiple salt-related genes could further improve salt tolerance and enhance biomass production.

Soluble sugars have been reported to increase in leaves under salinity conditions in order to maintain the osmotic adjustment in the plant (Yu et al. 2015; Pérez-Jiménez and Pérez-Tornero 2020; Shafiq et al. 2020). Our data indicate that salinity stress enhances leaf glucose accumulation both in WT and transgenic plants (Fig. 2a), and this increase is more pronounced in the transgenic lines. These results are in agreement with those reported in sugarcane by Gandonou et al. (2011) and in *Populus* by Watanabe et al. (2000) showing an increased level of soluble sugars as a result of salt stress enhances the plant tolerance to salt stress.

Proline is an osmoprotective agent and an important compatible solute that accumulates in plants under different types of stresses such as drought, cold, heat and salinity (Maach et al. 2020). The accumulation of this amino acid under salinity stress is an important mechanism in maintenance of the osmotic adjustment and salt tolerance (Kahlaoui et al. 2018; Ahanger et al. 2020). The higher accumulation of proline in all transgenic tomato plants analyzed in this work (Fig. 2b), especially in plants overexpressing *LeNHX2* together with *SlSOS2* could represent an adaptive mechanism to salt stress. Our results shows agreement with Bhaskaran and Savithramma (2011) who observed a significant increase in proline content in transgenic tomato plants co-expressing *Pennisetum glaucum PgNHX1* and *Arabidopsis AVP1* compared to WT and single transgenic lines expressing either *PgNHX1* or *AVP1*. Contrary to what was expected, proline and protein content in the co-overexpressing plants are lower compared to those overexpressing *LeNHX2*, under both normal and saline conditions. These results could be explained by the interaction between SlSOS2 and LeNHX2 proteins. More recently, Dong et al (2021) have reported that PgNHX proteins shared the same putatively interactive protein AVP1, HKT1, SOS2 and SOS3. Similarly, Pehlivan et al. (2016) have reported that although the SOS2 can regulate AtNHX1's activity, there does not appear to be a synergy between overexpression of *AtNHX1* and *SOS3*.

Protein accumulation has been reported to play an important role for cell survival and membrane stabilization under saline conditions (Goudarzi and Pakniyat 2009). The increase in the soluble protein content can be the result of the enhanced *de novo* synthesis of proteins for cell protection (Teixeira et al. 2005). In our study, the soluble proteins significantly increased in single and double transgenic plants overexpressing *LeNHX2* alone or together with *SlSOS2* (Fig. 2c). Previously, Gandonou et al. (2011) and Kahlaoui et al. (2018) showed that the content of soluble proteins increases in leaves and roots of salt tolerant cultivars whereas it decreases in the sensitive ones, suggesting that these components could play a key role in sugarcane and tomato salt tolerance. All these results are consistent with the previous findings that salt resistant plants are able to respond and adapt to salt stress through the synthesis of stress-induced proteins as a tolerance mechanism (Veeranagamallaiah et al. 2007; Maach et al. 2020).

The high expression level of some *NHX* isoforms in known sinks for potassium like fruits or flowers, where growth is dependent on cell expansion, point to a role of these isoforms in vacuolar $K^+$ accumulation (Rodríguez-Rosales et al. 2009). Silencing of the gene in tomato has a severe effect on growth and fruit and seed Production (Rodríguez-Rosales et al. 2008). It has been shown that potassium plays a vital role in photoassimilate transport from source to sink (Schobert and Tschesche 1978; Lalonde et al. 2003) and thus improved potassium homeostasis could enhance fruit production. In this study, tomato plants overexpressing both *LeNHX2* and *SlSOS2* showed a better yield compared to plants overexpressing only one gene (Fig. 3a). Our results have been supported by the observations of Pehlivan et al. (2016) who reported that co-overexpression of AtNHX1 and SOS1 could significantly reduce yield loss in *Arabidopsis* plants grown under 250 mM NaCl. Moreover, Shen et al. (2015) observed an increase in yield of transgenic cotton plants co-expressing the *Arabidopsis* vacuolar $Na^+/H^+$ antiporter gene AtNHX1 and $H^+$-pyrophosphatase gene AVP1 when cultivated under salt stress. Our results clearly show that plants ovexpressing *LeNHX2*, *SlSOS2* or both genes grow better and produce more fruits than WT plants both under control and NaCl irrigation conditions (Fig. 1a, 3a). In addition, results in Figs. 1a and 3a show that compared to plants irrigated with water, adding 125 mM NaCl to the irrigation water improves growth of single and double transgenic plants without affecting fruit yield. Furthermore, the co-overexpression of *LeNHX2* and *SlSOS2* improves fruit quality of tomato plants grown under saline conditions. All these results support the potential biotechnological interest of overexpressing *LeNHX2*, *SlSOS2* or both to enhance tomato fruit yield by plants cultivated either under saline or not saline irrigation conditions. In addition, TSS is an important quality criterion reflecting concentration of sugars in fruits (Flores et al. 2010). Shabani et al. (2013) have reported in tomato that salt treatment decreased fruit juice pH and increased TSS concentrations. We have observed that high level of NaCl treatment reduced TSS and pH value in WT plants (Fig. 3b, c), while transgenic plants, especially those co-overexpressing *LeNHX2* and *SlSOS2* reached higher levels of these two parameters suggesting that the co-expression of these two genes have improved fruit quality. The high levels of TSS observed in fruits of transgenic lines co-overexpressing *LeNHX2* and *SlSOS2* is probably due to a high transport of sugars from leaves to fruits. In this respect, Yin et al. (2010) reported that salinity stress enhances carbohydrates accumulation from the source leaves to the fruit of tomato plants. In relation to the pH of fruit juice, as reported by Mitchell et al. (1991) and Coban et al. (2020), we have observed that salinity significantly reduced the pH of fruits from wild-type plants. However, transgenic plants, especially those co-overexpressing *LeNHX2* and *SlSOS2*, showed an increase in pH values of fruits in plants cultivated under NaCl stress. Similarly, Maach et al. (2020) have reported that the overexpression of LeNHX4 antiporter improved the fruit quality in tomato by TSS and pH under salt stress.

## Conclusions

In this work, we have demonstrated that co-overexpression of the antiporter *LeNHX2* and the regulatory kinase *SlSOS2* confer salt tolerance and improves fruit yield and quality by increasing fruit pH and total soluble solid. Proline, glucose and protein accumulation in leaves of transgenic plants under either normal or stress conditions could be related to the better salt tolerance of these plants relative to WT tomato. Finally, our results suggest that multiple-gene co-expression results in higher salt tolerance.

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**Declarartions**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This manuscript does not imply human participants or studies on animals.

**Informed consent** Not required.

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ORIGINAL ARTICLE

# Overexpression of *LeNHX4* improved yield, fruit quality and salt tolerance in tomato plants (*Solanum lycopersicum* L.)

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**Abstract**

The function of the tomato $K^+$, $Na^+/H^+$ antiporter LeNHX4 has been analyzed using 35S-driven gene construct for over-expressing a histagged LeNHX4 protein in *Solanum lycopersicum* L. Compared to wild-type plants, the expression of *LeNHX4* was enhanced in most of plants transformed with a gene construct for *LeNHX4* overexpression although some plants showed a decreased *LeNHX4* expression. Overexpression of *LeNHX4* was associated to an increased fruit size while silencing of this gene was related to a decreased fruit size. We have investigated the effect of *LeNHX4* overexpression on fruit production and quality and we have also evaluated salt tolerance in two different overexpression lines by measuring proline, protein and glucose concentrations in tomato leaves grown either under control (0 mM NaCl) or saline (125 mM NaCl) conditions. Plants overexpressing *LeNHX4* showed a higher amount of fruits than WT plants and accumulated higher contents of sugars and cations ($Na^+$ and $K^+$). The application of 125 mM NaCl, affected negatively fruit production and quality of WT plants. However the transgenic lines overexpressing *LeNXH4* increased fruit quality and yield. In relation to salt tolerance, overexpression lines showed higher levels of leaf proline, glucose and proteins under NaCl treatment. The overexpression of *LeNHX4* in tomato plants, improved salinity tolerance and increased fruit yield and quality under both normal and salinity stress conditions.

**Keywords** *Solanum lycopersicum* · Proline · Soluble sugars · Protein · Salinity tolerance · LeNHX4 antiporter

## Introduction

Soil salinity is the most severe factor limiting plant growth and crop productivity. This problem increases in arid and semi-arid regions. According to the report of FAO, over 6% of the world's land is affected by either salinity or sodicity which accounts for more than 800 million ha of land throughout the world [1].

High levels of salinity may cause both ionic and osmotic stress effects, which lead to a decline of turgor, membrane damage, the inhibition of water and essential ions uptake, disordered metabolism, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction, including photosynthesis which ultimately leads to plant death [2–6]. On the other hand, potassium (K) is an essential element required for various physiological and biochemical processes that influence plant growth and development. In plants $K^+$ is also a key player in the maintenance of osmotic adjustment and cell turgor [7] and plays an important role in enzyme activation, photosynthesis and respiration, assimilate transport, protein metabolism and stomatal regulation [8]. Due to similar physicochemical properties, $Na^+$ and $K^+$ ions have the potential to compete each other for uptake by plant-root cells. Under salt stress, the uptake of $Na^+$ is increased drastically, causing a decrease in the absorption and accumulation of $K^+$ and subsequent deficiency of this element [9–11].

Plant cell adaptation to salinity involves avoiding sodium toxicity and potassium loss (CITAS Adecuadas [12, 13]).

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Therefore, keeping a high $K^+/Na^+$ ratio in the cytosol is considered a fundamental process for salinity tolerance [14, 15]. For this purpose, plant cells have developed mechanisms of restriction of $Na^+$ influx, active $Na^+$ efflux, and compartmentalization of $Na^+$ in the vacuole or other intracellular compartments [16]. Both $Na^+$ efflux and $Na^+$ compartmentalization need the operation of $Na^+/H^+$ antiporters located at the plasma membrane or intracellular membranes [17].

In previous works we have identified in tomato several $Na^+$, $K^+/H^+$ antiporters belonging to the NHX family of proteins and determined their expression level in the salt sensitive cultivated species *Solanum lycopersicum* L. cv. Volgogradskij and the salt tolerant wild species *Solanum pimpinelifolium* L. in response to salt stress [18]. This study showed a higher expression of *LeNHX4* in the wild tomato and an accumulation of $Na^+$ in aerial parts of these plants. Interestingly, results from these studies showed that unlike other NHXs isoforms, tomato *LeNHX4* is highly expressed in reproductive tissues and thus may have a role in fruit production and quality.

Regulation of osmotic adjustment under salt stress is also an important determinant of salt tolerance. In many plant species, the accumulation of compatible osmolytes (i.e., proline and sugars) is an important strategy leading to stress tolerance [19]. These molecules regulate the osmotic adjustment in plants grown under salt stress and enables plants to reestablish the water and osmotic homeostasis [20]. Proline is accumulated preferentially in leaves in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress [21]. In addition, soluble sugars are highly accumulated in vacuoles and produce high turgor pressure affecting osmotic potentials [22–25]. Interestingly, a relationship between *NHX* gene expression and sugar and proline accumulation has been reported [26–30].

Based on all the above, in this work we have studied whether the overexpression of the vacuolar $Na^+$, $K^+/H^+$ antiporter LeNHX4 affect tomato fruit yield and quality under unstressed and NaCl stress cultivation conditions.

## Materials and methods

### Transformation and molecular characterization of transgenic plants

For stable expression of the LeNHX4 protein in tomato (*S. lycopersicum* L. cv. MicroTom), the LeNHX4 coding sequence to which the sequence for a C-terminal RGS(H)10 tag was added, was cloned under control of the 35S promoter in the pCAMBIA1303 plant expression vector as described. The plasmid pCAMBIA 1303 carrying the LeNHX4-RGS(H)10 fragment was transferred in the LB4404 *Agrobacterium tumefaciens* strain [31] and used for tomato cotyledon transformation as has been described by Ellul et al. [32].

The presence of the LeNHX4-RGS(H)10 construction in selected tomato transgenic lines was assessed by polymerase chain reaction (PCR) analysis using specific primers to amplify a 468 bp fragment of the hygromicin resistance gene harboured in the plant expression cassette (Forward: 5′-GATGTTGGCGACCTCGTATT-3′, Reverse: 5′-GTGCTTGACATTGGGGAGTT-3′) and DNA obtained from tomato leaves following a method by Edwards et al. [33].

The level of expression of *LeNHX4* in leaves of untransformed and transgenic plants was assessed by real time PCR using gene-specific primers as previously described [18].

### Plant material and growth conditions

Plants used for molecular characterization and fruit yield and quality determinations were cultivated in hydroponics for 4 weeks in 1/4 Hoagland nutrient solution [34]. For this purpose, seed were surface sterilized in ethanol 70% for 1 min followed by 50% commercial bleach for 5 min and 3 washes with sterile distilled water. Seeds were then cultivated in polystyrene boxes containing quartz sand, watered for 1 week with one-tenth strength Hoagland nutrient solution [34] and another 2 weeks with one-fourth strength of the same solution. Seedlings with four leaves were then transferred to pots for hydroponic cultivation. Typically, plants were grown for 4 weeks in a 2.5 L pot containing an aerated one-fourth strength Hoagland nutrient solution. Hydroponic cultivation was performed in a growth chamber at 24/20 °C day/night, under an illumination of 140 $\mu mol/m^2/s$ (photoperiod of 16 h light and 8 h darkness) and 40–50% relative humidity.

Alternatively, and in order to extend the growing period, tomato seeds were sown in seedbeds containing peat-moss. The seedbeds were kept in a greenhouse and irrigated with tap water for 7 weeks. 50-day-old tomato plants were transferred to 1.2 L pot (1 plant per pot) containing peat-moss and kept in a greenhouse. These plants were irrigated with tap water for 1 week and then with either tap water or 125 mM NaCl three times ever week for 16 weeks.

### Determination of fruit quality parameterss

Ion content was measured in untransformed and transgenic tomato plants grown in hydroponics for 4 weeks in growth chamber under the conditions described above. Fruits were dried for 48 h at 80 °C, milled to powder and digested in a concentrated $HNO_3:HClO_4$ (2:1, v/v) solution. $K^+$ and $Na^+$ concentrations were determined in the digested material by inductively coupled plasma spectrometry (Varian ICP 720-ES).

Plant water content was calculated as $[(FW - DW)/FW)] \times 100$.

Sugar analysis by GC–MS was carried out by a method modified from Schauer et al. [35]. Five tomato fruits were taken at postharvest stage to perform the GC–MS determinations.

Fruits were harvested and the fleshy, edible tissue was frozen in liquid nitrogen and crushed to powder with a grindomixer (Retsch, Haan, Germany). Samples were stored at − 80 °C until further analysis. For each individual fruit, frozen tomato tissue (150 mg) was extracted with 700 µL MeOH solution containing ribitol (9 µg ribitol/mL MeOH) as an internal standard. The mixture was extracted for 15 min at 70 °C and mixed vigorously with 700 µL of distilled water. To separate polar and non-polar metabolites, 325 µL chloroform was added to the mixtures. After centrifugation at 14,000 rpm for 5 min, the upper methanol/water phase (500 µL) was taken and dried overnight under vacuum.

The residue was re-dissolved in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine and derivatized at 37 °C for 90 min followed by a 30 min treatment with 60 µL MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamine) at 37 °C. Sample volumes of 1 µL were then injected into the GC column.

Total soluble solid (TSS) was measured as °Brix in a few drops of the juice using a digital refractometer (ATAGO Co., Ltd., Tokyo, Japan).

Tests for pH were performed on fruit homogenate using a portable pH meter.

### Glucose, proline and protein determination in leaves

For glucose and proline determination samples of 0.5 g of plant materials were used by following the methods described by Irigoyen et al. [36] and Paquin and Lechasseur [37], respectively.

Fresh leaves were used for the determination of soluble proteins using Bradford G-250 reagent [38], and expressed as mg/g of fresh weight.

### Statistics

All data in this report were obtained from at least three independent experiments with three or four replicates each. Analysis of variance was used to assess difference between treatments and plant genotypes were considered as significant when $P \leq 0.05$. Significant differences according to the Duncan's multiple range test (DMRT) are indicated with different letters in the figures and tables.

## Results

### Molecular characterization of transgenic plants

The cDNA corresponding to *LeNHX4* gene was expressed under the cauliflower mosaic virus 35S gene promoter in tomato (*S. lycopersicum* L. cv MicroTom). T3 homozygous plants with a single insertion of the transgene were selected on the basis of PCR amplification of hygromicin resistance gene and the analysis of hygromicin resistance segregation after seed germination in selective media containing the antibiotic (Supporting Information Fig. S1). From these plants, three independent lines, L-2, L-3 and L-5, were further characterized. Real-time RT-PCR analysis showed a higher expression level of *LeNHX4* in leaves of L-3 and L-5 transgenic lines than in those of untransformed controls (Fig. 1a). Surprisingly, the expression level of *LeNHX4* in plants from line L-2 was half of that in WT plants, which indicate the silencing of *LeNHX4* in this line (Fig. 1a).

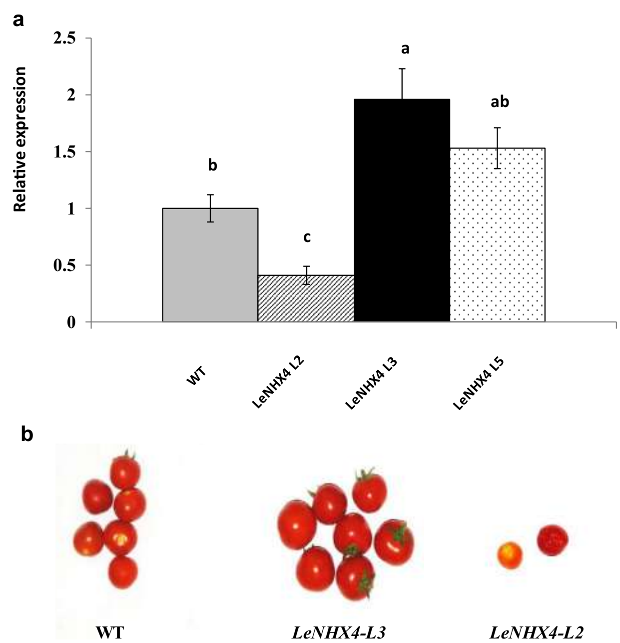


**Fig. 1** **a** Transcript levels of *LeNHX4* in leaves of WT and transgenic plants. Plants were cultivated in hydroponics for 4 weeks. The results show the expression of *LeNHX4*, quantified by real-time qPCR and expressed as increase in transcript levels relative to the level in leaves of WT plants, to which value 1 is assigned. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$. **b** Image of the fruits obtained from WT and transgenic plants (lines L-3 and L-2). It can be observed that relative to WT plants, the low expression of *LeNHX4* in L2 causes a lower fruit size while, while the high expression of the gene in L3 line provokes an increase of fruit size

## Characterisation of fruits

Since *LeNHX4* is highly expressed in fruits [18] we tested whether overexpression of *LeNHX4* have an effect on fruit yield. Fruit size was related to the level of expression of *LeNHX4* in the transgenic lines (Fig. 1b). Plants ovexpressing *LeNHX4* (lines 3 and 5) showed the highest fruit size, while plants in which *LeNHX4* (line 2) was silenced showed the lowest fruit size. Fruit production was measured in untransformed and transgenic tomato plants overexpressing *LeNHX4* grown in hydroponics for 4 weeks in Hoagland nutrient solution (Fig. 2a). Our data demonstrate that overexpression of *LeNHX4* lead to a significant increase in fruit production, while the lowest production was reached in WT plants. Fruit fresh weight (Fig. 2b) showed trends similar to those of fruit production, reaching high values in transgenic lines L-3 and L-5, exceeding WT by 61% and 88% respectively.

The overexpression of *LeNHX4* increased the fruit water content respect to the value in WT plants (Fig. 2c). The concentrations of glucose, fructose and sucrose were determined in fruits of WT plants and plants overexpressing *LeNHX4* in order to study the effect of the overexpression of *LeNHX4* on the quality of tomato fruits (Fig. 2d). Fructose and glucose levels were strongly increased in transgenic plants in comparison to WT plants. However, the concentration of sucrose was not significantly different between WT and transgenic plants.

Figure 3 shows the contents of $Na^+$ and $K^+$ in fruits of WT plants and plants overexpressing *LeNHX4*. The highest $Na^+$ contents were found in the *LeNHX4* overexpressing lines, with values of 0.10 and 0.06% of dry weight, respectively, while the WT fruits reached the lowest value. Similar results were obtained for fruit $K^+$ content, with transgenic lines L-3 and L-5 showing 14 and 18% higher $K^+$ content than WT fruits.

The effect of *LeNHX4* overexpression in fruit yield and quality was also studied in plants grown in the presence or the absence of NaCl for longer time periods. Our results demonstrate that plants overexpressing *LeNHX4* showed higher fruit production that WT plants when both were irrigated either with or without NaCl (Fig. 4a). Compared to untreated plants salt-treated wild type plants reduced fruit production by 33%, while fruit production by *LeNHX4* overexpressing plants was slightly increased as a result of salt treatment (Fig. 4a).

Fruit quality characteristics such as total soluble solids (TSS% or °Brix), and pH are shown in Table 1. Under normal conditions (without 125 mM NaCl), transgenic lines overexpressing *LeNHX4* showed a significant reduction in TSS. In relation to pH, we have not found significant differences between WT and both transgenic lines. However,

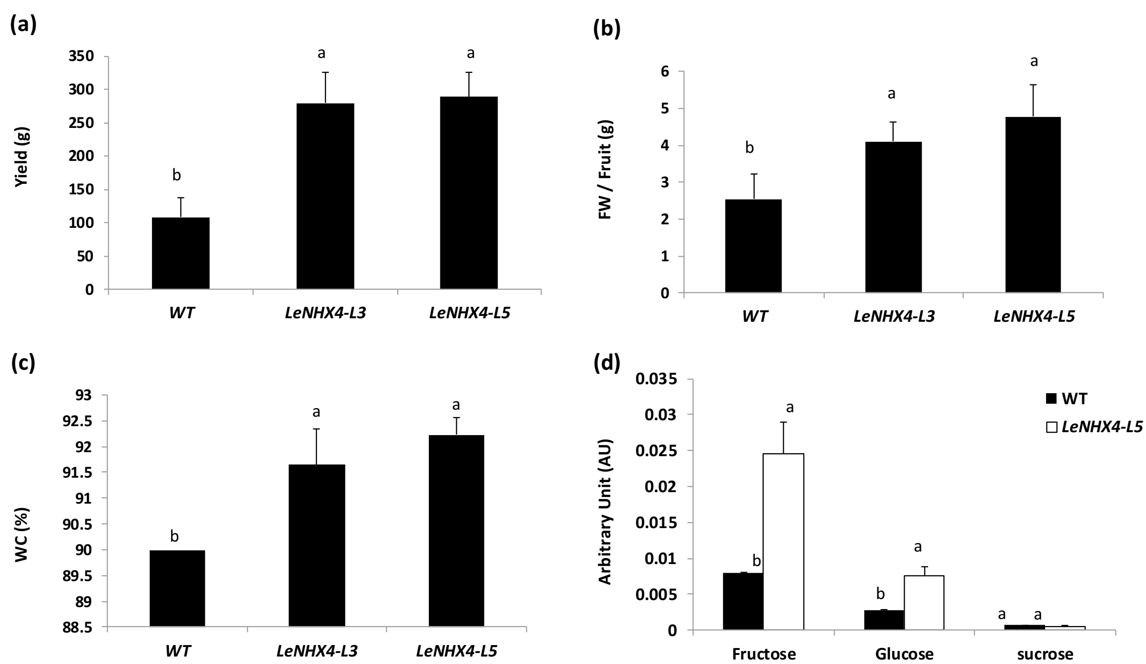


**Fig. 2** Effect of *LeNHX4* overexpression on fruit production (**a**), fruit weight (**b**), water content (**c**) and soluble sugars (**d**) of tomato plants. WT and *LeNHX4* plants were cultivated in hydroponics for 4 weeks. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$

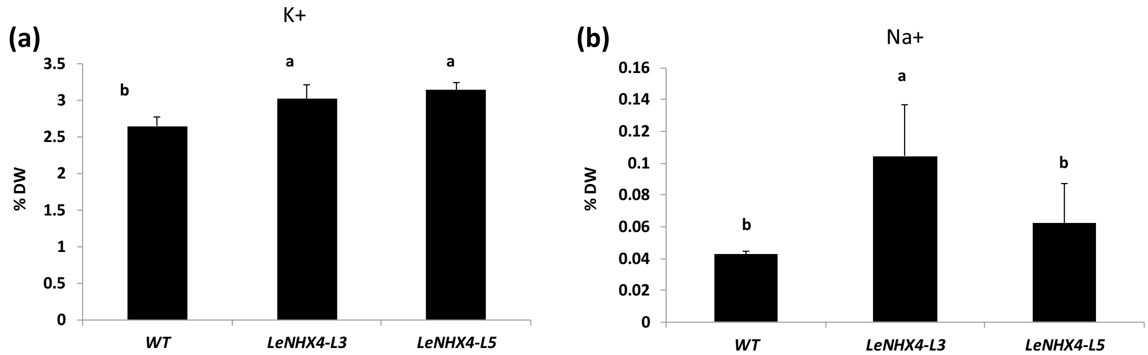


**Fig. 3** $K^+$ (**a**) and $Na^+$ (**b**) contents in fruit of WT and transgenic tomato plants overexpressing *LeNHX4*. Plants were cultivated in hydroponics for 4 weeks. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$

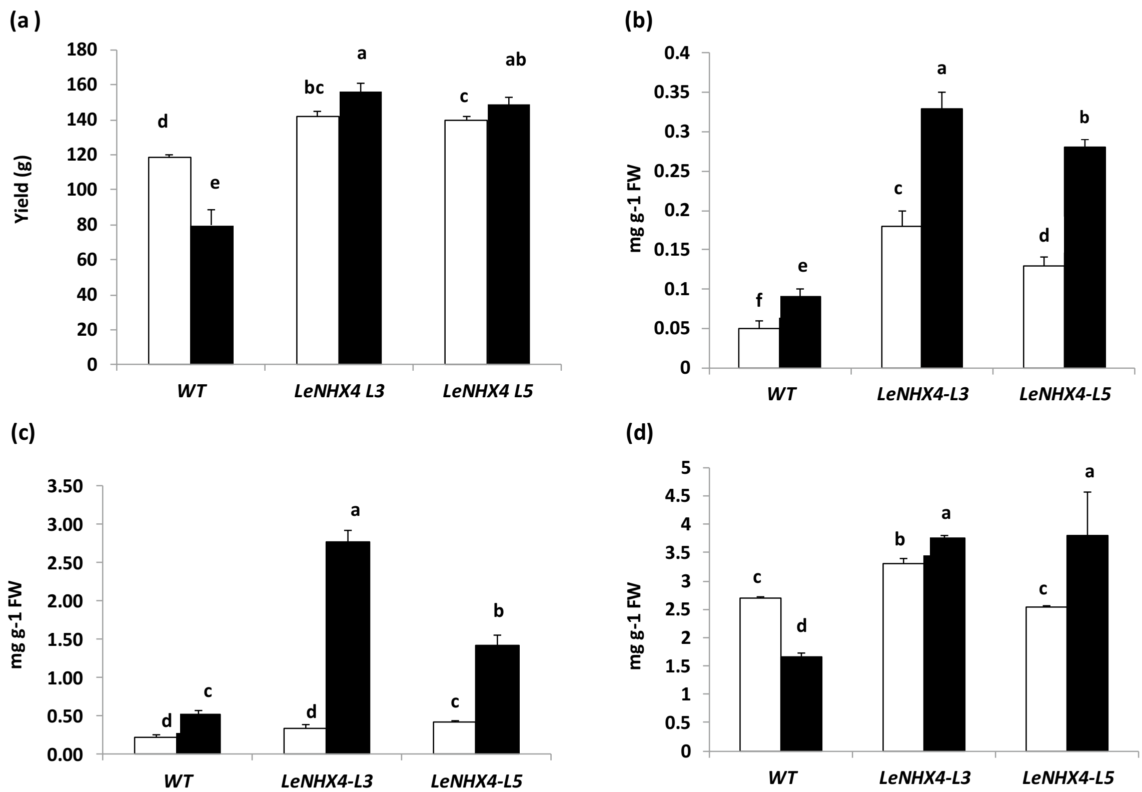


**Fig. 4** Effect of *LeNHX4* overexpression on fruit production (**a**) and glucose (**b**), proline (**c**) and protein concentrations (**d**) of tomato plants. WT and *LeNHX4* plants were cultivated in peat-moss for 16 weeks in the absence (empty bars) or the presence (black bars) of 125 mM NaCl. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$

the addition of 125 mM NaCl to the irrigation solution significantly increased total soluble solids in *LeNHX4* overexpressing lines and decreases it in WT plants. Similarly, the pH decreased in WT and increased in transgenic lines under salinity conditions.

## Glucose, proline and proteins in leaves of WT and transgenic plants overexpressing *LeNHX4*

Figure 4b shows the glucose concentration in leaves of WT and the transgenic lines overexpressing *LeNHX4* (lines L-3 and L-5). The NaCl treatment increases the glucose level in all plants, above all in *LeNHX4* overexpressing plants.

**Table 1** pH and solid solute content (°Brix) in fruit of wild-type and transgenic plants overexpressing *LeNHX4* grown under control conditions (0 mM NaCl) and 125 mM NaCl

| | TSS (°Brix) | | pH | |
|---|---|---|---|---|
| | 0 mM NaCl | 125 mM NaCl | 0 mM NaCl | 125 mM NaCl |
| WT | 10.56 ± 0.36 a | 9.58 ± 0.33 b | 4.01 ± 0.13 a | 3.43 ± 0.42 b |
| *LeNHX4-L3* | 6.36 ± 0.74 b | 11.3 ± 0.36 a | 3.79 ± 0.41 a | 4.92 ± 0.28 a |
| *LeNHX4-L5* | 6.08 ± 0.28 b | 10.84 ± 0.56 a | 3.83 ± 0.41 a | 4.53 ± 0.33 a |

Plants were cultivated in peat-moss for 16 weeks in the absence or the presence of 125 mM NaCl. Values are means ± standard deviation of three independent experiments with three replicates each. Means followed by different letters are significantly different at $P \leq 0.05$

Our results show that NaCl treatment significantly increased the proline content in WT and *LeNHX4* overexpressing plants, being the increase more pronounced in the transgenic plants (Fig. 4c).

Figure 4d shows the effect of NaCl treatment on protein contents. Significant differences were found in the protein content between untreated and treated plants. Under NaCl stress, transgenic plants showed higher protein content than untreated plants, while the protein level in WT plants was negatively affected.

## Discussion

Salt stress is a major abiotic factor limiting plant growth and productivity. Plants exhibit various physiological and biochemical responses to salt stress. An important mechanism to overcome salt stress is the exclusion of $Na^+$ from the cytoplasm, by the operation of $Na^+/H^+$ antiporters at the plasma membrane or tonoplast. Plant NHX antiporters play a key role in NaCl tolerance by the extrusion of $Na^+$ out of cytosol [18, 39].

In tomato, four LeNHX proteins were reported by Gálvez et al. [18]. From these proteins, LeNHX2 was studied in relation to its role in salt tolerance. Indeed, Huertas et al. [40] showed that tomato plants overexpressing *LeNHX2* grew better in the presence of 120 mM NaCl than untransformed controls and Baghour et al. [14] demonstrated that the joint overexpression of *LeNHX2* and *SlSOS2* increased even more the plant salt tolerance. In addition to the increased salt tolerance, an increased fruit production was found in plants overexpressing *LeNHX2*, *SlSOS2* or both [14, 41]. In relation to this, Bassil et al. [42] reported that *AtNHX1* and *AtNHX2* are required for growth and floral development in *Arabidopsis*, while *AtNHX3* and *AtNHX4* play role in seed development [43, 44]. More recently, Sharma et al. [45], showed that in *Triticum aestivum* the *TaNHX4* group genes were highly expressed in later development stages of leaf, spike and grain.

In previous work, we have demonstrated that *LeNHX4* is highly expressed in flowers and fruits of tomato plants, indicating specific roles of this isoform in the reproductive tissues [18]. In this work, data of fruit production of tomato plants cultivated in media lacking NaCl indicate an improvement of fruit yield in transgenic lines overexpressing the *LeNHX4* antiporter (Fig. 2a, b), suggesting a role of *LeNHX4* in fruit production. Fruit of tomato plants overexpressing *LeNHX4* showed a higher water content than those of WT plants (Fig. 2c), which together with the higher accumulation of sugars (Fig. 2d), $K^+$ and $Na^+$ (Fig. 3) in fruits of these plants under NaCl cultivation conditions could suggest that plants overexpressing *LeNHX4* withstand better a shortage of water under salinity conditions. In agreement with our results, Li et al. [46] showed that *Arabidopsis* transgenic plants overexpressing *RtNHX1* increased their capacity for osmotic adjustment and improved salinity tolerance due to a high relative water content and accumulation of solutes allowing osmoregulation.

The total soluble solids (TSS) and pH are important quality parameters, which plays a significant role in fruit selection. Reina-Sánchez et al. [47] recorded that an increase in fruit quality represented an increase of total soluble solids and titratable acidity. Salinity positively affect the levels of TSS, including sugars, organic acids, and amino acids in fruits [48–54]. Salinity stress enhances gluconeogenesis, as well as metabolic flow in ripe fruit, resulting in high-Brix fruit [54]. In relation to the role of NHX ion transporters on fruit quality Hanana et al. [55] reported that the NHX antiporters play a critical role in plant growth and development, and improve organoleptic characteristics of fruits as well as those related to fruit ripening [41]. Contrary to what was expected, TSS levels are lower in overexpressing lines than in wild type plants cultivate in the absence of NaCl. However the overexpression of *LeNHX4* gene improved the fruit quality in tomato by increasing the solid solute content and pH under salt stress (Table 1). As opposed to our results, Zhang and Blumwald [56] reported that overexpression in tomato of the *Arabidopsis* antiporter *AtNHX1* did not affect the total soluble solids content in plants grown under 200 mM NaCl.

The increase in the concentration of soluble sugars such as glucose enhances plant tolerance to several abiotic stresses, such as drought, salinity and cold [57] because of their involvement in osmotic adjustment. Our results in tomato showed an increase of glucose and fructose content in fruits of transgenic plants under normal conditions (Fig. 2d) and suggest that the increased expression of *LeNHX4* could have a positive effect on glucose synthesis under non stressful conditions. Similar results were reported by Leidi et al. [26], who reported that transgenic tomato plants overexpressing *AtNHX1* accumulated higher level of free sugars even if they were not transferred to salt. NaCl treatment significantly increased the concentration of glucose in plants overexpressing *LeNHX4* in relation to WT plants (Fig. 4b). Lieu et al. [58] did not find any significant differences on the sugar contents of WT and transgenic *Beta vulgaris* plants overexpressing *AtNHX3*. However, with the application of high NaCl treatment (300 or 500 mM), a greater sugar accumulation was observed in transgenic plants, suggesting that the expression of *AtNHX3* in sugar beet improved sugar synthesis in transgenic plants and increased the plants salt tolerance by influencing the transcription of genes involved in the sugar synthesis pathways.

A large number of studies show a positive correlation between proline accumulation and plant stress and proline concentration has been shown to be generally higher in salt tolerant than in salt sensitive plants. The accumulation of osmotic compounds such as proline is a common response of plants to high salinity to combat osmotic stress [59, 60]. Proline is accumulated preferentially in leaves in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress [21]. Plants overexpressing *NHXs* show an enhanced capacity for osmotic adjustment and improved salinity tolerance due to a better relative water content and accumulation of proline [26–30]. Our data showed that under control conditions, the proline content in leaves was slightly increased in the transgenic lines (Fig. 4c). However, when exposed to saline conditions, the level of this aminoacid dramatically increased in plants overexpressing *LeNHX4*. Recently, Wang et al. [61] reported that the overexpression of *Ipomoea batatas IbNHX2* significantly increased proline content, superoxide dismutase (SOD) activity, and photosynthetic rate in sweet potato. Similarly, transgenic *Arabidopsis* plants overexpressing the $Na^+/H^+$ antiporter *RtNHX1* from *Reaumuria trigyna* accumulated more proline than WT and *nhx1* mutant plants under 200 mM NaCl treatment [46]. The increase of proline during salt stress has been associated to the up-regulation of proline biosynthesis genes (AtP5CS1 and AtP5CS2) in transgenic plants [46, 61].

There are many evidences that proteins could have a role in osmotic adjustment [62–64]. Plants are also able to respond and adapt to salt stress through the synthesis of specific proteins, and the synthesis of stress-induced proteins is part of that stress tolerance mechanism [65]. Our results show that the NaCl treatment affected negatively the protein content in WT plants (Fig. 4d). However, the overexpression of *LeNHX4* increased the protein content in transgenic lines L-3 and L-5. These results are consistent with those reported by Tester and Davenport [66], who suggested that the disruption in protein synthesis appears to be an important cause of damage caused by $Na^+$. Comparing the total soluble protein content between the salt-tolerant cultivar (Rio Grande) and the salt-sensitive one (Heinz-2274), Kahlaoui et al. [67] found that the protein levels significantly increased more in the salt-tolerant tomato cultivar when irrigated with saline water. The accumulation of these proteins under saline conditions may provide a storage of nitrogen that is re-utilized when stress is over [68] and may play a role in osmotic adjustment [67]. However, there are also studies reporting no differences in protein content as a result of NaCl tolerance. Ashraf and Fatima [69] found that salt-tolerant and salt-sensitive safflower accessions did not differ significantly in soluble leaf proteins.

In conclusion, the results reported in this study indicate that overexpression of *LeNHX4* in tomato increases size, sugar (glucose and fructose) and ion ($Na^+$ and $K^+$) contents of fruits. Moreover, relative to WT plants, plants overexpressing *LeNHX4* show an enhanced fruit yield under both non-stress and salinity stress conditions as well as higher levels of osmoregulatory compounds (proline and proteins) in leaves.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This manuscript does not imply human participants or studies on animals.

**Informed consent** Not required.

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# Application of biostimulants improves yield and fruit quality in tomato

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# Application of biostimulants improves yield and fruit quality in tomato

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**ABSTRACT**

Tomato (*Solanum lycopersicum* L.), grown in saline soils, exhibit reduced yield quantity and quality which may be alleviated with the use of biostimulants. These materials can be applied to roots, foliage or seed and are designed for a specific action and target a particular stage of plant development. Tecamin Flower® (TF) and Tecamin Brix® (TB) are foliar biostimulants formulated to improve flowering and fruit quality. The biostimulants were used separately, or in combination, to determine their effects on tomato yield quantity and quality. Tecamin Brix® applied alone, or in combination with Tecamin Flower®, resulted in higher yield. Both biostimulants increased fruit quality, with the highest levels of firmness, total soluble solids, pigment and pH obtained with joint application of Tecamin Brix® and Tecamin Flower®. The combined application of Tecamin Brix® and Tecamin Flower® might be effective in improving tomato fruit yield and quality, especially in soils with high salt concentrations.

**ARTICLE HISTORY**

**KEYWORDS**
*Solanum lycopersicum*; biostimulants; quality; salinity; soil

Tomato (*Solanum lycopersicum* L.) fruit are an important source of bioactive compounds with known beneficial effects including vitamins, minerals and natural antioxidants (Frusciante et al., 2007). In the coastal areas of the Mediterranean countries, especially in Morocco and Spain, tomato is an economically important crop (Baghour et al., 2019). However, the Nador Region of Morocco is characterized by saltwater intrusion, with many coastal agricultural areas suffering loss of productivity.

Tomato organoleptic quality is an important parameter in addition to yield. Fruit quality and market value are determined by size, shape, firmness, color, taste and solid content which vary with season (Ho and Hewitt, 1986). Yield is determined mainly by flower and fruit set and fruit development. Water-soluble chemical fertilizers can be applied to foliage to increase nutrient availability and promote growth (Splittstoesser, 1990).

Biostimulants are used to improve nutrient uptake and increase yield and crop quality. They stimulate natural processes that benefit growth and

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response to high or low temperatures, lack of water or excess salt (Du Jardin, 2015). Tecamin Flower® (TF) and Tecamin Brix® (TB) are biostimulants specifically formulated for stimulating fruit set, development and ripening. Tecamin Flower® contains amino acids from plant origin, which stimulates flowering and contains phosphate and boron both of which are involved in fertility and fruit setting, and boron positively affects tomato production and quality (Nonnecke, 1989; Stanley et al., 1995). Foliar application provides a source of boron which is directly relocated to meristems, young leaves, fruit and flowers when needed. Tecamin Flower® contains potassium and seaweed extract. Tomatoes have a high potassium requirement. Deficiency in K can affect yield quantity and quality (Marschner, 1995; Mengel and Kirkby, 1987; Usherwood, 1985). Potassium participates in the regulation of translocation of assimilates, activation of a metabolic enzyme and regulation of opening and closing stomata (Hawkesford et al., 2012). The objective of the study was to determine the effects of foliar application of Tecamin Flower® and Tecamin Brix® on tomato fruit yield and quality.

## Materials and methods

The experiment was conducted under field conditions. Seed of the commercial elongated tomato, cv. Nador F1, were surface sterilized in 70% ethanol for 1 min, followed by 50% commercial bleach for 5 min and 3 washes with sterile-distilled water. After that, on 15 February 2019, seed were germinated in 77-hole seed trays (50 × 33 × 4.70 cm), filled with peat and kept in a growth chamber under fluorescent light (140 $mmol{\cdot}m^{-2}{\cdot}s^{-1}$), with a photoperiod of 16/8 h (light/dark period) and temperature of 24/18°C (day/night), and irrigated every 2 days with tap water.

After 1 month, at the 3-leaf stage, plants were placed manually in the field at an intra- and inter-row spacing of 45 and 90 cm, respectively, and treated with a systemic fungicide Difenoconazole (1 mL/10 L) by backpack sprayer. Plants were irrigated with water from the Moulouya impoundment (EC of 0.2 $ms{\cdot}cm^{-1}$) for 1 h per day with drip irrigation tube placed 5 cm from each plant. Emitters were 20 cm apart and flow rate was 1 $L{\cdot}h^{-1}$. Treatments were Control (no biostimulants), and TB or TF alone, and TB+TF with 10 plants for each. Treatments were replicated 3 times (120 plants in total). The clayey soil, formed into raised beds, was composed of: CaO (7109 $mg{\cdot}kg^{-1}$), $P_2O_5$ (104 $mg{\cdot}kg^{-1}$), $K_2O$ (1736 $mg{\cdot}kg^{-1}$), MgO (5.41 $mg{\cdot}kg^{-1}$), $Na_2O$ (1150 $mg{\cdot}kg^{-1}$), EC (1.07 $ms{\cdot}cm^{-1}$); with the trace elements: Cu (2.51 $mg{\cdot}kg^{-1}$), Mn (5.11 $mg{\cdot}kg^{-1}$), Fe (11.60 $mg{\cdot}kg^{-1}$), Zn (2.06 $mg{\cdot}kg^{-1}$) and organic matter (OM) (2.24%). No additional fertilizer was applied. Tecamin Flower® contains Boron (1%), seaweed extract (4%), L-amino acids (3%), nitrogen (3%), phosphorus ($P_2O_5$, 10%) and molybdenum (0.5%);

Tecamin Brix® is composed of potassium (18%), boron (0.2%) and seaweed extract (10%).

Treatments consisted of foliar application, with a hand sprayer, of 3 mL·L$^{-1}$ of each material when applied alone and 3 mL·L$^{-1}$ of each material when combined, in distilled water, by hand sprayer, between 8 and 10 AM every 15 days. The Tecamin Flower® was applied at the appearance of the first flower and 15 days after that Tecamin Brix® was applied. The experiment lasted 12 weeks (10 March to 1 June 2019).

Fruit were harvested once weekly, beginning at 5 weeks after transplanting, when ripe (at full maturity stage with an orange-red to red color), and continued weekly for 5 weeks. All fruit were collected and weighed to determine the yield. Fruit were classified based on weight as: small (<60 g), medium (60 to 120 g) and large (>120 g). Tomato firmness was measured with a portable penetrometer according to established standards (Anonymous, 1998), and is based on the pressure necessary to push a plunger of specified size into the pulp of the fruit up to a specific depth (Islam et al., 2012). A portable refractometer (PAL-1, Atago, Tokyo, Japan) was used to measure total soluble solids (TSS) in a few drops of tomato juice and expressed as °Brix.

Lycopene was estimated following Suwanaruang (2016). Fresh fruit samples (0.001 g) were placed in 1 mL of distilled water and vortexed in a water bath at 30°C for 1 h; then, 8.0 mL of hexane:ethanol:acetone (2:1:1) (v/v) was added. Samples were capped and vortexed immediately, and incubated out of bright light. After at least 10 min, 1 mL of distilled water was added to each sample and they were again vortexed. Samples were set aside for 10 min to allow phases to separate and air bubbles to disappear. Absorbance was determined at 503 nm by spectrophotometry. The lycopene level in hexane extracts was calculated according to Anthon and Barrett (2007) and Godwin et al. (2015). The pH was determined on fruit homogenate using a portable pH meter. Data were subjected to analysis of variance in Statgraphics Plus (Statistical Graphics Corp, StatPoint Inc., Herndon, VA). Means were separated with Duncan's multiple range test.

## Results and discussion

Biostimulants are used to increase yield and improve crop quality. Treatment with Tecamin Flower® and Tecamin Brix® affected small, medium and large size fruit production (Table 1). Control plants produced fewer small, medium and large size fruit than plants treated with Tecamin Flower® and Tecamin Brix® (Table 1). More fruit of all sizes were produced when the biostimulants were applied jointly. Tecamin Flower® and Tecamin Brix® contain potassium, nitrogen and boron, which are essential for plant growth and yield production (Al-Shammari et al., 2018; Hartz et al., 2005; Islam et al., 2013; Prema et al., 2011; Taber et al., 2008). Combined application of TC and TF is important to

**Table 1.** Effects of Tecamin flower® (TF), Tecamin brix® (TB) and their combined (TF±TB) application on total fruit yield (kg/10 plants).

| Fruit size | | | |
|---|---|---|---|
| Treatment | SmallMedium | Medium | Large |
| Control (water only) | 6.7c[a] | 23.5c | 20.9c |
| Tecamin flower® (TF) | 9.5b | 32.6b | 26.3b |
| Tecamin brix® (TB) | 11.4ab | 42.0a | 34.3a |
| TF±TB | 13.2a | 44.7a | 36.5a |

[a]Values in a column followed by the same letter are not significantly different, $P < 0.05$, Duncan's multiple range test.

increase tomato yield by providing fruit tissues with essential elements and hormones necessary for growth and development.

Firmness is an important parameter of tomato fruit quality and an indicator of the state of maturity. Firmness is affected by cell wall structure, cell size, turgidity and membrane properties (Chapman et al., 2012; Huang et al., 2018). There was an increase in fruit firmness (Table 2), especially when Tecamin Flower® and Tecamin Brix® were used in combination. The action of biostimulants can be explained by their effects on ethylene synthesis and their potassium content (Sonntag et al., 2019); also, algae extract has a beneficial effect on turgidity and cell wall components, which could benefit flexibility of cell membranes and on fruit firmness (Hawkesford et al., 2012).

Brix is a parameter that determines the quality of tomato concentrate; decreased Brix is interpreted as a loss of product quality. The brix value of dark-red tomatoes, due to high levels of lycopene, treated with Tecamin Flower® and Tecamin Brix® was higher than controls (Table 2). Brix degree and lycopene play an important role in tomato selection (Dube et al., 2020; Maach et al., 2020). Application of these biostimulants improved tomato fruit quality, especially when applied jointly. Lycopene is a carotenoid compound giving red color to tomato fruit (Nguyen and Schwartz, 1999). Lycopene has strong antioxidant activity (Di Mascio et al., 1989), which plays a major role in determining tomato fruit quality (Fogliano et al., 1999; Miller and Rice-Evans, 1997). Tecamin Flower® and Tecamin Brix® did not have a remarkable effect on pH (Table 2). The values were within the optimal range for a good and acceptable quality of tomato fruit (Benton Jones (2007)).

**Table 2.** Effect of Tecamin flower® (TF) or Tecamin brix® (TB) application alone, or in combination, on tomato fruit quality.

| Treatment | Firmness ($kg \cdot cm^{-2}$) | TSS[a] (°Brix) | Pigment ($mg \cdot g^{-1}$ fresh weight) | pH |
|---|---|---|---|---|
| Control | 2.00d[b] | 4.38 c | 17.22d | 4.08c |
| TF | 3.17c | 5.08b | 37.10c | 4.24b |
| TB | 4.17b | 4.54 c | 42.65b | 4.28b |
| TF+TB | 4.50a | 5.96a | 46.01a | 4.35a |

[a]TSS = total soluble solids.
[b]Values in columns followed by the same letter are not significantly different, $P < 0.05$, Duncan's multiple range test.

In the present study, we investigated the effects of the application of Tecamin Flower® and Tecamin Brix® on tomato yield and fruit quality. Application of Tecamin Brix®, alone or in combination with Tecamin Flower® increased production of small, medium and large fruit. The combined Tecamin Brix®+Tecamin Flower® improved fruit quality by increasing the organoleptic parameter firmness, pH and TSS. Treatment with biostimulants can reduce the amount of fertilizers applied and were efficient in promoting yield and fruit quality of tomato plants grown on saline land.

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ORIGINAL ARTICLE / ORIGINALBEITRAG

# Gibberellic Acid and Indole Acetic Acid Improves Salt Tolerance in Transgenic Tomato Plants Overexpressing *LeNHX4* Antiporter

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**Abstract**

Salinity is the major environmental factor that limits plant growth and productivity. High concentrations of $Na^+$ and $Cl^-$ ions in soil solution negatively affect $K^+$ uptake and may cause physiological and metabolic disorders, which adversely affect the plant growth and crop yield. In plants, NHX antiporters play an important role in salt tolerance by catalyzing $Na^+$ accumulation in vacuoles. In this work, we studied the effect of exogenous plants hormones auxin (IAA) and gibberellic acid ($GA_3$), on salinity tolerance, fruit production and quality in transgenic tomato plants overexpressing LeNHX4 antiporter grown under 125 mM NaCl. Here, we have found that IAA and $GA_3$ positively affected leaf proline, glucose, soluble proteins and *ortho*-diphenol. In addition, both hormones increased tomato production and fruit quality parameters. These results suggest that $GA_3$ and IAA improved salinity tolerance and increased fruit yield and quality, probably by increasing the expression or the activity of LeNHX4 antiporter.

**Keywords** Solanum lycopersicum · LeNHX4 antiporter · Salinity tolerance · Yield · Phytohormones

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| ABRE | ABA-Responsive Element |
| ARF2 | Auxin response factor |
| AuxRE-core | Core of the auxin response region |
| CHX | Cation/$H^+$ exchanger |
| CPA | Cation-proton antiporter |
| DELLA | Aspartic acid-glutamic acid-leucine-leucine-alanine |
| GA | Gibberellins/gibberellic acid |
| GARE | Gibberellin-responsive element |
| HAK5 | High-affinity $K^+$ transporter 5 |
| IAA | Indole-3-aceticacid |
| NHX | $Na^+$/$H^+$ exchanger |
| KEA | $K^+$-efflux antiporter |
| TGA element | TGA sequence |

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## Introduction

Several studies have shown that crop yields must double in the next three decades to meet projected global food demand (Nuccio et al. 2015). However, various biotic and abiotic stresses substantially contribute to the reduced production. Salinity stress is one of the most important abiotic stress factors that limit crop production around the world, especially in the arid and semi-arid regions and this problem is intensified by climate change. In saline soils, the high concentrations of $Na^+$ and $Cl^-$ may cause hyperionic and hyperosmotic stress, which can lead to the reduced absorption of water and essential nutrients such as $K^+$ (Baghour et al. 2019; Maach et al. 2020; 2021).

In plants, an important mechanism to overcome salt stress is by the exclusion of $Na^+$ from the cytoplasm, by the operation of $N^+$/$H^+$ antiporters at the plasma membrane or tonoplast (Rodríguez-Rosales et al. 2009; Baghour et al. 2019; Cagnac et al. 2020; Malakar and Chattopadhyay 2021). These antiporters are involved in the exchange of cations and protons across cell membranes and contribute to the regulation of cell pH and they play an essential role in cellular homeostasis (Hanana et al. 2009). The identification and characterization of genes involved in $Na^+$ and

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$K^+$ homeostasis is an essential and indispensable task for improving the salt tolerance of tomato plants.

Among these transporters, NHX proteins have been suggested to be important for salt tolerance by promoting accumulation of $Na^+$ or $K^+$ inside vacuoles (Rodríguez-Rosales et al. 2009; Gálvez et al. 2012). In plants, *NHX* genes encode intracellular cation/$H^+$ antiporters regulating $Na^+$, $K^+$ and $H^+$ levels in cytoplasm and intracellular compartments, providing osmotic and ionic protection. In tomato, we have previously characterized different $Na^+$, $K^+/H^+$ antiporters belonging to the NHX protein family (Gálvez et al. 2012).

On the other hand, plant hormones are involved in the response of plants to a wide range of abiotic environmental stresses. These hormones mediates gene expression involved in plant physiological responses to stress such as drought and salinity, and regulate many aspects of plant growth, development and in several cases have also been linked to salt tolerance (Bhattacharyya et al. 2015). Phytohormones play a central role in the integration of different upstream signals into different adaptive outputs such as changes in the activity of ion-channels, protein modifications, protein degradation, and gene expression (Riemann et al. 2015). A plant hormone auxin (indole-3-acetic acid, IAA), in addition to their role in the regulation of plant growth and development, are also involved in plant tolerance to various biotic and abiotic stresses (Lu et al. 2019; Salem et al. 2020). During salinity stress, the loss of phospholipase D function negatively affects auxin redistribution, which suppresses root meristem activity and, therefore, causes a significant reduction in the growth of the primary roots (Wang et al. 2019). Other reports suggest that the final target of auxin action is the plasma membrane $H^+$-ATPase, which excretes $H^+$ ions into the cell wall compartment. $H^+$ extrusion out of the cells facilitates $K^+$ ions uptake through the activity of $K^+$ transporters (Hager 2003). More recently, Malakar and Chattopadhyay (2021) reported that auxin response factor (ARF2) has been shown to suppress the high affinity $K^+$ transporter, HAK5 expression by binding to its promoter. Moreover, gibberellic acid (GA), also known as gibberellin, is one of the major plant hormones, which regulate many developmental processes from seed germination to plant senescence (Gupta and Chakrabarty 2013; Luo et al. 2019). GAs induce DELLA repressor protein degradation and thereby control a number of critical developmental processes as well as responses to stresses (Achard et al. 2007; Lantzouni et al. 2020). In plants, various stress signals and phytohormones share common elements in the signaling pathway and these common elements cross-talk with each other, to maintain cellular homeostasis (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). Gibberellin-responsive elements (the GARE motif) and auxin-responsive elements (TGA-element and AuxRR-core) were also found in the CPA (Cation Transport Antiporter) genes of moso bamboo (Wu et al. 2021). In this study, we examine how the exogenous application of GA and AIA affects the production and quality of transgenic tomato plants, overexpressing LeNHX4 antiporter, grown under saline conditions, as well as assessing the response of certain physiological and biochemical indicators under such conditions.

## Materials and Methods

### Plant Material and Growth Conditions

Seeds from wild type and transgenic tomato plants (*Solanum lycopersicum* L. cv. MicroTom) overexpressing *LeNHX4* (lines 3–3 and 3–4) generated as described by Maach et al. (2020) were sown in seedbeds containing peat-moss. The seedbeds were kept in a greenhouse and irrigated with tap water for 6 weeks. 40-days-old tomato plants were transferred to 1.2 L pot (1 plant per pot) containing peat-moss and kept in a greenhouse. These plants were irrigated with tap water for 10 days and then with 125 mM NaCl three times every week and were treated with $GA_3$ (T1) or IAA (T0) by spraying with 10 ppm of these hormones twice a week for two months.

### Plant Sampling

70 days (Ten weeks) after transplanting, the plants were gently removed from their substrate. Next, the roots, leaflets, stems, and fruits were rinsed in abundant tap, and distilled water, blotted on filter paper, and dried in a forced-air oven at 70 °C for 48 h.

### Plant Analysis

***Glucose and Proline Determination*** Samples of 0.5 g of plant materials were used to determine glucose and proline levels by using the methods described by Irigoyen et al. (1992) and Paquin and Lechasseur (1979), respectively.

***Soluble-protein Determination*** Fresh leaves were used for the determination of soluble proteins using Bradford G-250 reagent (Bradford 1976), and expressed as mg/g of fresh weight.

***Extraction and Quantification Ortho-diphenol*** The phenols of the plant material were extracted with MeOH. Total phenolic content was assayed quantitatively at 765 nm with Folin-Ciocalteau reagent (Singleton and Rossi 1965). The results obtained were expressed as mg of caffeic acid per g. fresh weight.

***Yield and Fruit Quality Parameters*** Fruits were harvested at the mature stage at 70 days after transplanting. All fruits were collected and weighed to determine the yield and part of samples was immediately used to determine total soluble solid and pH. For these determinations, we used 5 repetitions with two plants (10 plants per treatment).

***Total soluble solid (TSS)*** was measured as °Brix in a few drops of the fruit juice using a digital refractometer (ATAGO Co, Ltd, Tokyo, Japan).

***Tests for pH*** were performed on fruit homogenate using a portable pH meter.

## Statistics

In this report, all data were obtained from experiments with three to five repetitions for each parameter. Analysis of variance (ANOVA) was used to assess difference between treatments and plant genotypes were considered as significant when $P \leq 0.05$. Significant differences according to the Duncan's multiple range test (DMRT) are indicated with different letters in the figures and tables.

## Results

To gain insight into the role of phytohormones, in salinity resistance of plants overexpressing LeNHX4 antiporter, we examined the effect of exogenous IAA and $GA_3$ on the content of osmoprotectants such as glucose, proline, proteins (Fig. 1) and *ortho*-diphenol (Fig. 2) in wild-type (WT) and two tomato transgenic lines (L-33 and L-34) overexpressing the *LeNHX4* gene. We analyzed these osmoprotectants in leaves of wild-type and transgenic lines grown under NaCl stress and treated with either 10 ppm IAA or 10 ppm $GA_3$ (Fig. 1a–c). Application of exogenous IAA or $GA_3$ significantly enhanced the glucose content in wild-type and transgenic plants overexpressing *LeNHX4* gene (Fig. 1a; $P < 0.001$). Similarly to glucose, significant proline accumulation was noted in gibberellin and auxin treated plants and it was considerably high for both transgenic lines ($P < 0.01$; Fig. 1b). In relation to soluble proteins (Fig. 1c), statistical analysis showed that there is no significant difference between different treatments ($P > 0.05$), although under normal conditions or under IAA treatment, L-34 transgenic plants accumulated slightly higher levels of proteins. *Ortho*-diphenol content was higher in transgenic than WT plants treated or not with IAA or $GA_3$ (Fig. 2). IAA treatment significantly increased the *ortho*-diphenol concentrations in the wild type and L-33 plants.

To investigate the effects of plant hormones on fruit production under salt stress, the wild-type and transgenic plants overexpressing *LeNHX4* were grown under 125 mM NaCl and treated with either gibberellins or auxins (Fig. 3). Our results show a higher fruit production in WT than *LeNHX4* transgenic plants. However, while fruit production in WT plants was not affected by IAA or $GA_3$ treatments, transgenic plants showed an increased production of fruits after being treated with IAA (lines 3.3 and 3.4) or $GA_3$ (line 3.4).

Fruit quality parameters such as total soluble solids (TSS% or °Brix) and pH are presented in Table 1. Exogenous gibberellin and auxin treatments increased the TSS values in wild-type and transgenic plants oveexpressing *LeNHX4* ($P < 0.001$), the increase being more pronounced in lines L-34. Treatment with IAA or $GA_3$ decreased pH value in fruits of WT plants but did not affect pH value in fruits of the transgenic lines L3.3 and L3.4 ($P > 0.05$).

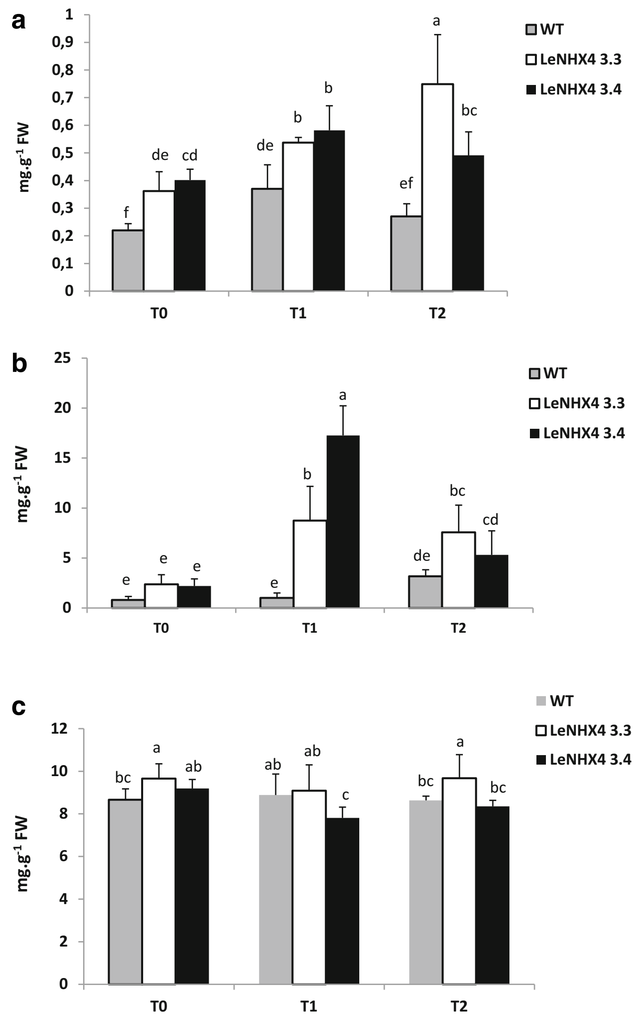


**Fig. 1** Effect of exogenous $GA_3$ and IAA application on glucose (**a**) proline (**b**) and protein content (**c**) in WT, *LeNHX4-3.3* and *LeNHX4-3.4* lines. Plants were cultivated in peat-moss for 6 weeks and treated with 125 mM NaCl mM for 60 days. T1 (10 ppm gibberellic acid) and T2 (10 ppm IAA) were sprayed twice a week 40 days after germination. Means that have different letters at the top of each bar are significantly different at $P < 0.05$ according to Duncan's multiple range test

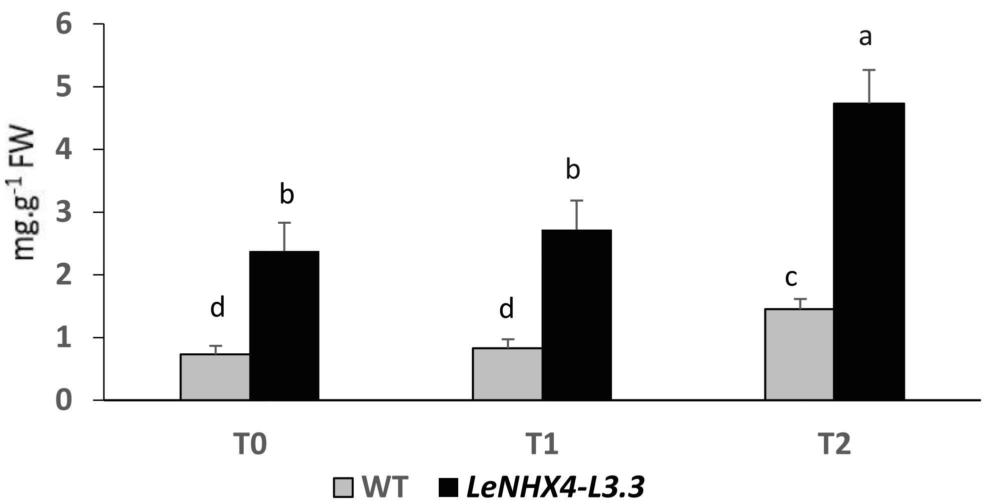


**Fig. 2** Effect of exogenous $GA_3$ and IAA application on *ortho*-diphenol concentrations. Plants were cultivated in peat-moss for 6 weeks and treated with 125 mM NaCl mM for 60 days. T1 and T2 treatments were sprayed twice a week with 10 ppm gibberellic acid and AIA, respectively. Means that have different letters at the top of each bar are significantly different at $P < 0.05$ according to Duncan's multiple range test

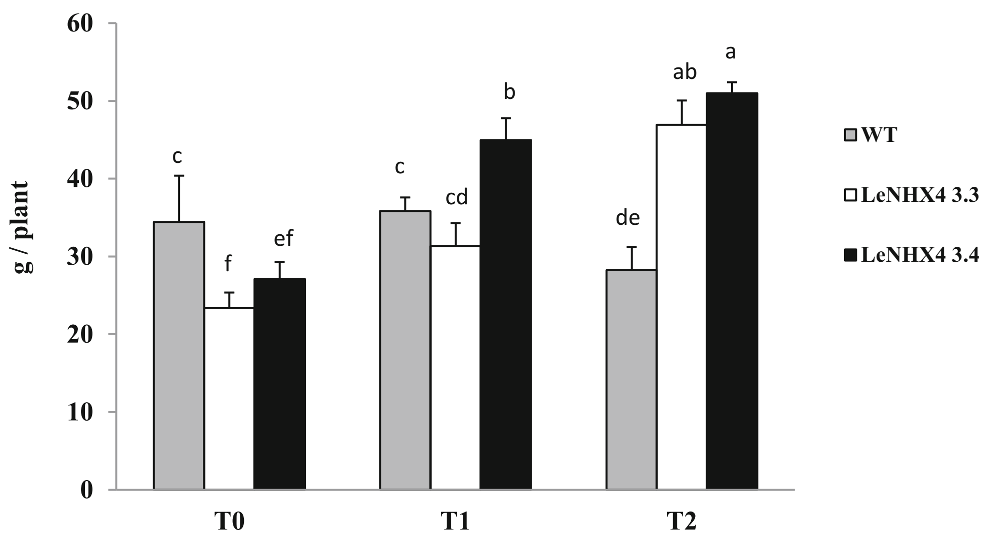


**Fig. 3** Effect of exogenous $GA_3$ and IAA application on fruit production. Plants were cultivated in peat-moss for 6 weeks and treated with 125 mM NaCl mM for 60 days. T1 and T2 treatments were sprayed twice a week with 10 ppm gibberellic acid and AIA, respectively. Means that have different letters at the top of each bar are significantly different at $P < 0.05$ according to Duncan's multiple range test

## Discussion

Plant NHX proteins have been suggested to be important for salt tolerance by promoting accumulation of $Na^+$ or $K^+$ inside vacuoles. *LeNHX4* shows a high expression level especially in stems and fruits, indicating to specific roles for this isoform in the reproductive tissues (Rodríguez-Rosales et al. 2009; Baghour et al. 2019; Kong et al. 2021). More recently, we have demonstrated that the overexpression of *LeNHX4* in tomato plants, improved salinity tolerance and increased fruit yield and quality under both normal and salinity stress conditions (Maach et al. 2020). To test whether salinity tolerance in transgenic lines could be enhanced by exogenous IAA and GA treatments, we have assessed the effect of these two hormones on some biochemical parameters such as glucose, proline, soluble proteins (Fig. 1a–c) and *ortho*-diphenol contents (Fig. 2). NHX antiporters have been suggested to be regulated by many phytohormones (Fu et al. 2020). These plant hormones play an important role in regulating plant responses to salinity and $N^+/K^+$ homeostasis (Bhattacharyya et al. 2015; Iqbal et al. 2014). Exogenous gibberellin increased $K^+$ ion concentration, while reduced the oxidative stress and $Na^+$ accumulation under salinity (Shahzad et al. 2021). In *Arabidopsis*, the expression of *AtNHX1, AtNHX2, AtNHX4* and *AtCHX17* is induced by ABA (Jia et al. 2018). Furthermore, Kong et al. (2021) have shown that the 26 *ZmCPA* genes harboring ABA-Responsive Element (ABRE), especially the 10 *ZmCPAs* (*ZmCHX1*, *-7*, *-10*, *-12*, *-14, ZmKEA1*, *-5, ZmNHX1*, *-4*, and *-9*), were probably regulated by abscissic acid (ABA). More recently, Malakar and Chattopadhyay (2021) reported that auxin response factor (ARF2) has been shown to suppress HAK5 expression by binding to its promoter. In *Phyllostachys heterocycla*, gibberellin-responsive elements (the GARE motif) and auxin-responsive

**Table 1** pH and solid solute content (ºBrix) in fruit of wild-type and transgenic plants overexpressing *LeNHX4* grown under saline conditions and treated with GA3 and IAA

| | TSS (⁰Brix) | | | pH | | |
|---|---|---|---|---|---|---|
| | T0 | T1 | T2 | T0 | T1 | T2 |
| **WT** | 7.08 ± 1.04 e | 9.72 ± 0.92 bc | 7.98 ± 0.93 de | 4.30 ± 0.08 b | 3.92 ± 0.0.18 c | 3.90 ± 0.04 c |
| *LeNHX4-L3.3* | 9.57 ± 0.57 bc | 9.02 ± 0.85 cd | 10.0 ± 0.91 bc | 4.40 ± 0.04 ab | 4.41 ± 0.07 ab | 4.46 ± 0.08 a |
| *LeNHX4-L3.4* | 9.50 ± 0.99 bc | 10.6 ± 0.93 b | 12.1 ± 0.97 a | 4.31 ± 0.10 b | 4.41 ± 0.03 ab | 4.46 ± 0.08 a |

Plants were cultivated in peat-moss for 6 weeks and treated with 125 mM NaCl mM for 60 days. T1 and T2 treatments consist of spraying leaves with 10 ppm gibberellic acid or AIA twice a week. Values with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test

elements (TGA-element and AuxRR-core) were also found in the cation/proton antiport genes (Wu et al. 2021).

Previously, Gaxiola et al. (2007) have monitored the effect of exogenous abscisic acid (ABA), auxin and gibberellin (GA) on $Na^+/H^+$ antiporter (HvNHX1), and found that ABA and auxin induced the expression of the *HvNHX1* genes, while GA had no effect. Iqbal et al. (2011) have shown that the beneficial effects of $GA_3$ can be attributed to its effect on ionic uptake and partitioning (within shoots and roots) in the salt stressed wheat plants. More recently, Geng et al. (2020) have shown that *NsNHX1* can be induced by gibberellins and abiotic stresses such as salt and drought. These findings are consistent with the present results showing that the exogenous IAA and GA treatment, positively affected the glucose, proteins, glucose (Fig. 1a–c) and *ortho*-diphenol (Fig. 2) contents in transgenic tomato plants. In agreement with our results, Shahzad et al. (2021) have found a significant increase in total soluble protein and phenolic compounds in GA-treated plants, indicating that higher accumulation of phenolic compounds could be pivotal for plant tolerance against oxidative stress, contributing to osmotic adjustment and stabilization of membranes in plants (Saeidi-Sar et al. 2013). Phenolic compounds is a physiological response and adaptive mechanism of higher plants to cope with biotic and abiotic stresses such as drought and salinity (Ruiz et al. 1998; Caliskan et al. 2017).

GA is highly likely to be connected with the vacuolar membrane function for plant growth (Konishi et al. 2005). This plant hormone was shown to play an important role in plant salt stress tolerance through regulating DELLA transcription factors (Achard et al. 2007). GAs play a key role in these adaptive responses by stimulating the degradation of growth-repressing DELLA proteins (Conti et al. 2014; Blanco-Touriñán et al. 2020). Moreover, auxin also plays important roles in salt stress response, since endogenous IAA contents in leaves and roots were significantly decreased in plants under salinity condition (Du et al. 2013; Liu et al. 2015). Treatment with auxin increases the expression of a plasma membrane $H^+$-ATPase gene in *Zea mays* (Frias et al. 1996), and the expression of *HvNHX1* in barely roots (Fukuda and Tanaka 2006).

More recently, we have shown that the overexpression of *LeNHX4* in tomato plants, increased fruit yield and quality under salinity stress conditions (Maach et al. 2020). Here, we have tried to extend our understanding of the effects of plant hormones on yield and fruit quality of transgenic tomato plants overexpressing LeNHX4 antiporter. $K^+$ plays a vital role in photoassimilate transport from source to sink (Lalonde et al. 2003; Schobert et al. 1998) and thus improved potassium homeostasis by overexpressing NHX antiporters could enhance fruit production and quality (Baghour et al. 2019; Maach et al. 2021). Here, the increase in yield (Fig. 3) and fruit quality (Table 1) observed in transgenic tomato plants treated with $GA_3$ and IAA could be attributed to the positive effects of these two hormones on LeNHX4 antiporter, which mostly expressed in fruits and flowers (Gálvez et al., 2012; Rodríguez-Rosales et al. 2009). In this respect, Riemann et al. (2015) reported that plant hormones play an important role in the integration of different upstream signals into adaptive outputs such as changes in the activity of ion channels, which could explain the higher production of *LeNHX4* transgenic tomato plants treated either with IAA or with GA.

## Conclusion

Based on the present findings, it can be inferred that both $GA_3$ and IAA positively affected fruit production and quality of tomato plants overexpressing of *LeNHX4* gene, enhancing fruit yield and TSS values. In addition, both hormones alleviated salinity stress in transgenic plants by enhancing the production of total phenols and osmoprotectants such as proline, glucose and proteins. Our results suggest that hormones gibberellins and indole acetic acid could improve salt tolerance by regulating the activity of LeNHX4 antiporter.

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**Conflict of interest** M. Baghour, M. Akodad, A. Dariouche, M. Maach, H. El Haddaji, A. Moumen, A. Skalli, K. Venema and M.P. Rodríguez-Rosales declare that they have no competing interests.

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# Valorization of Moroccan Bentonite Deposits: "Purification and Treatment of Margin by the Adsorption Process"

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**Abstract:** The main objective of this work was to contribute to the reduction in the contamination of phenolic compounds contained in margin by an adsorption process on two types of raw bentonite. The margin used in the studies was collected from a semi-modern oil mill located in the Nador–Morocco region. The results of the physico-chemical analyses showed that the effluents of the oil mills showed that they are highly polluted, particularly in terms of the total suspended solids (TSS), chemical oxygen demand (COD), and iron content of around 154.82 (mg/L), and copper content of 31.72 (mg/L). The mineralogy of bentonites studied by X-ray diffraction (XRD) reveals the existence of two types of montmorillonite; theoretically, the diffraction peak (001) of the montmorillonite appears at 15 Å, with a basal spacing that corresponds to a calcium pole, and the diffraction peak (001) appears at 12Å, with a basal spacing that corresponds to a sodium pole. The specific surface area of the bentonite used is characterized by a large specific surface area, varying between 127.62 $m^2 \cdot g^{-1}$ and 693.04 $m^2 \cdot g^{-1}$, which is due to the presence of hydrated interleaved cations. This surface is likely to increase in aqueous solution depending on the solid/liquid ratio that modulates the degree of hydration. With a high cation exchange capacity (CEC) (146.54 meq/100 g), samples of margin mixed with raw bentonites at different percentages vary between 5% and 100%. The potential of Moroccan bentonite for the phenol adsorption of 9.17 (g/L) from aqueous solutions was investigated. Adsorption tests have confirmed the effectiveness of these natural minerals in reducing phenolic compounds ranging from 8.72% to 76.23% contained in the margin and the efficiency of heavy metal retention through microelements on raw bentonites. The very encouraging results obtained in this work could aid in the application of adsorption for the treatment of margin.

**Keywords:** raw bentonites; retention; margin; heavy metal; adsorption; phenolic compounds

## 1. Introduction

Oil mills are among the industries with significant pollution emissions following the extraction of olive oil [1]. Morocco, characterized by a wealth of natural resources, is among the Mediterranean countries with the highest production of olive oil [2,3]. This industry, so beneficial for the national economy, leaves polluting and toxic liquid discharges (margin) [4]. Margin is also characterized by a high concentration of organic matter, which is poorly biodegradable and highly toxic [5]. They consist of water (83% to 92%), organic compounds (4% to 16%), and inorganic compounds (1% to 2%) [6]. These liquid discharges from oil mills must not be discharged directly into the sewage system. In this context, various studies have been carried out to eliminate phenolic compounds before they are discharged to the receiving environment [2,3,7–9]. Adsorption is one of the techniques that are widely used for the removal of pollutants which can occur between a solid and a gas or between a solid and a liquid [10,11]. It can be defined as the phenomenon of fixation of atoms or molecules on the surface of the solid by weak interaction forces of van der Waals type [12]. It is thus the passage from the dissolved to the adsorbed state. The opposite process is desorption [13]. Adsorption is caused by charged sites on the surface of the adsorbent, and the adsorption capacity is directly related to the number of these sites per unit area [14]. Bentonites can be used as adsorbents to respond to environmental problems [14–16].

This use has become one of the most important applications of bentonite in Europe and America, although in Africa, due to its abundance in nature and its low cost compared to other adsorbent materials, bentonite is considered to be a crucial element, especially in the adsorption process [2]. This study proposes the physico-chemical characterization of the liquid wastes from oil mills and to study the feasibility and efficiency of the treatment of phenolic compounds by adsorption with respect to two Moroccan crude bentonites, Trebia and Iboughrdayn. Additionally, we reflect upon the characterization of raw bentonites (without modification).

## 2. Results and Discussion

### *2.1. Characterization of Adsorbent*

#### 2.1.1. Physicochemical Properties

Table 1 provides the results of the physicochemical properties of the bentonites used, such as the adsorbent. Bentonite of Trebia is characterized by the clay fraction (<2 μm) in the order of 9.09%, the silt fraction from 58.69% and the sand fraction from 32.20% [17]. Moreover, Bentonite of Iboughrdayn presents a clay fraction from 17.3, the silt fraction from 38.7 and the silt fraction 43.9%. All samples indicate mild alkalinity with pH values between 8.7 and 9.12 due to the presence of alkali carbonates and bicarbonates or silicates. These soluble and basic salts are generally associated with the composition of clay. The surface area of the bentonites is very important; we deduce that it is characterized by a significant amount of Smectite [17,18]. The free swelling index of bentonites varies between 36.53% and 59.18%, suggesting that the bentonites used have significant swelling properties.

**Table 1.** Physicochemical properties of bentonite samples used in the study.

| Sample | % Sand (>60 μm) | % Silts (2–60 μm) | % Clay (<2 μm) | IG (%) | C (%) | $S_{ST}$ ($m^2$/g) | CEC (meq/100 g) | pH |
|---|---|---|---|---|---|---|---|---|
| TrB | 32.20 | 58.69 | 9.09 | 59.18 | 36 | 518.92 | 42.42 | 9.12 |
| IB | 43.9 | 38.7 | 17.3 | 36.53 | 45 | 630 | 127.12 | 8.7 |

Note: TrB: bentonite of Trebia; IB: bentonite of Iboughrdayn; $S_{ST}$: specific surface area; CEC: cation exchange capacity; IG: swelling index; C: colloidality.

#### 2.1.2. XRD Characterization

The results of the XRD on the disoriented powder of the bentonite samples are shown in Figure 1. XRD analysis of raw Iboughrdayn bentonite shows the presence of montmorillonite, with the first peak located at a spacing of 12Å at $d_{001}$ reflection; this shows that the natural bentonite is in the form of calcium, and the other three peaks are located at 4.45Å ($d_{110}$), 2.567 Å ($d_{200}$), and 1.49 Å ($d_{060}$). The $d_{060}$ reflection indicates the dioctahedral character of the smectite [19,20]. The plagioclase feldspar group with a calcium pole is manifested by anorthite peaks and orthoclase (K-feldspar). It is characterized by a low amount of hematite, quartz, ekmanite, dolomite, zeolite, and cristobalite. In the Trebia deposit, bulk mineralogy is characterized by the association of two types of montmorillonite;

the first diffraction peaks ($d_{001}$) of montmorillonite occur at a basal spacing of 15Å and 12Å. Theoretically, the peak at 12Å ($d_{001}$) corresponds to a calcium pole (M-Ca) and the peak at 15Å ($d_{001}$) corresponds to a sodium pole (M-Na). The other three peaks are located at 4.48 Å ($d_{110}$), 2.16Å ($d_{200}$), and 1.49 Å ($d_{060}$). A moderate amount of K-feldspar, hematite, anorthite, and cristobalite; and secondary minerals quartz, anatase, dolomite was found, with traces of pyrite, zeolite, and xenotime.

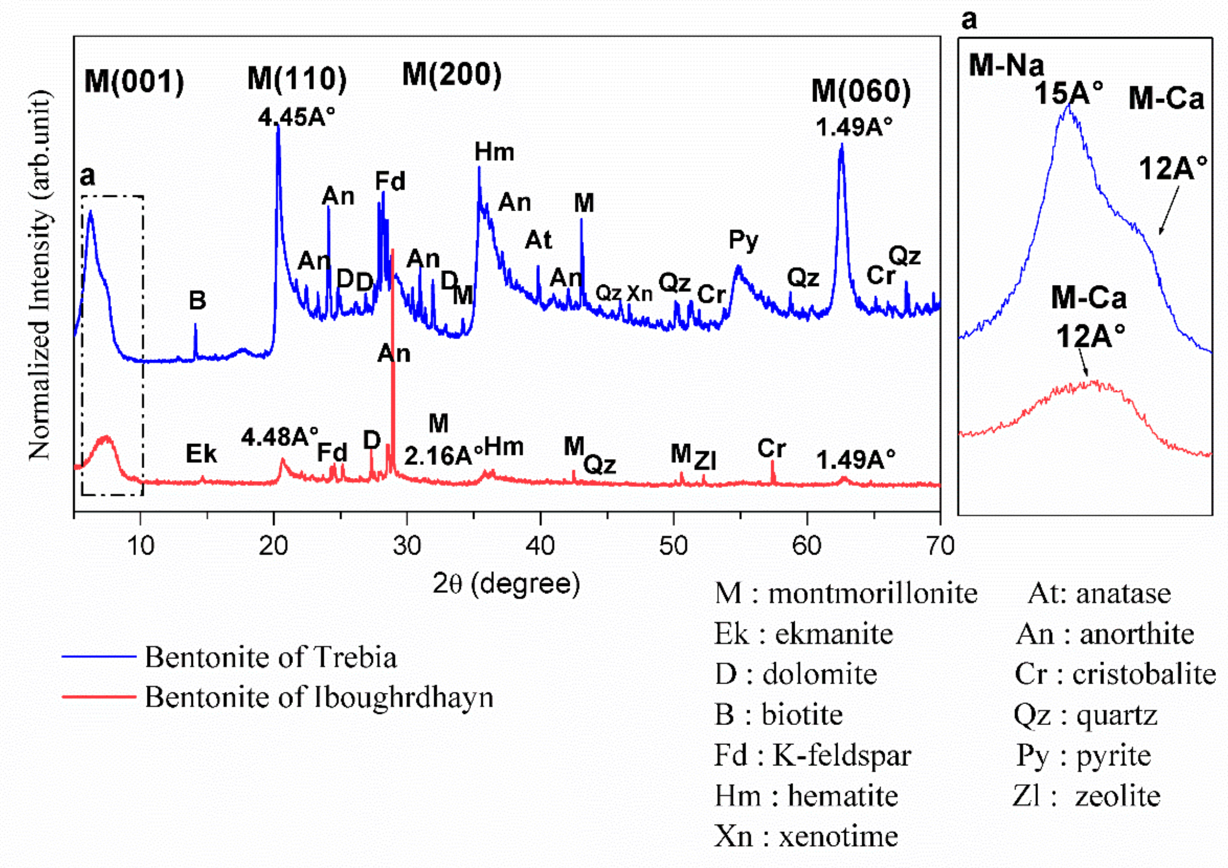


**Figure 1.** X-ray diffractograms of the raw bentonite samples.

### 2.1.3. Spectrum Analysis

The FTIR analyses of the raw bentonite samples were taken in the range of 400–4000 $cm^{-1}$ (Figure 2). The sample of Iboughrdayn is characterized by very strong multiple absorption of montmorillonite [21], observed in the range of 3626.10 to 3423.65 $cm^{-1}$; these bands are attributed to the elongation vibrations of the binding of internal O-H groups in the structure of montmorillonite [ν OH] at the octahedral layer. As well as the peaks produced at around 3626 $cm^{-1}$, this probably shows the presence of magnesium in the structure of the analyzed bentonites [22–25]. The spectrum of Terbia's bentonite exhibits an absorption band at 3699.47 $cm^{-1}$, assigned to the stretching and bending vibrations of the OH groups for the water molecule of hydration, which adsorbed on the bentonite surface [26]. This was followed by a large band at 3251.98 $cm^{-1}$ in the mineral, attributed to OH stretching ($\nu_3$) the structural hydroxyl groups and water present in the mineral. This indicates the possibility of a hydroxyl bond between the octahedral and tetrahedral layers [27]. The intense band present in the range between 1635 and 1637 $cm^{-1}$ corresponds to the asymmetric ($\nu_2$) OH (deformation mode) of the interlayer water and a structural part of the mineral [27–29]. Moreover, Iboughrdayn bentonite's very strong absorption band observed at 1035.77 $cm^{-1}$ is recognized for its stretching vibration Si-O bands, which are characteristic of stratified montmorillonite silicate mineral and attributed to the triple degenerate extension Si-O $\nu_3$ (in the plane) [30]. The presence of a band at 844.82 $cm^{-1}$ in all samples is attributed to Al-Mg-OH deformations [31,32]. The bands located at 700 $cm^{-1}$ and 723 $cm^{-1}$ indicate

Si-O stretching due to the presence of quartz [33,34]. The bands are at 520 for Si-O-Al (octahedral) and 462 $cm^{-1}$ for Si-O-Si bending vibrations.

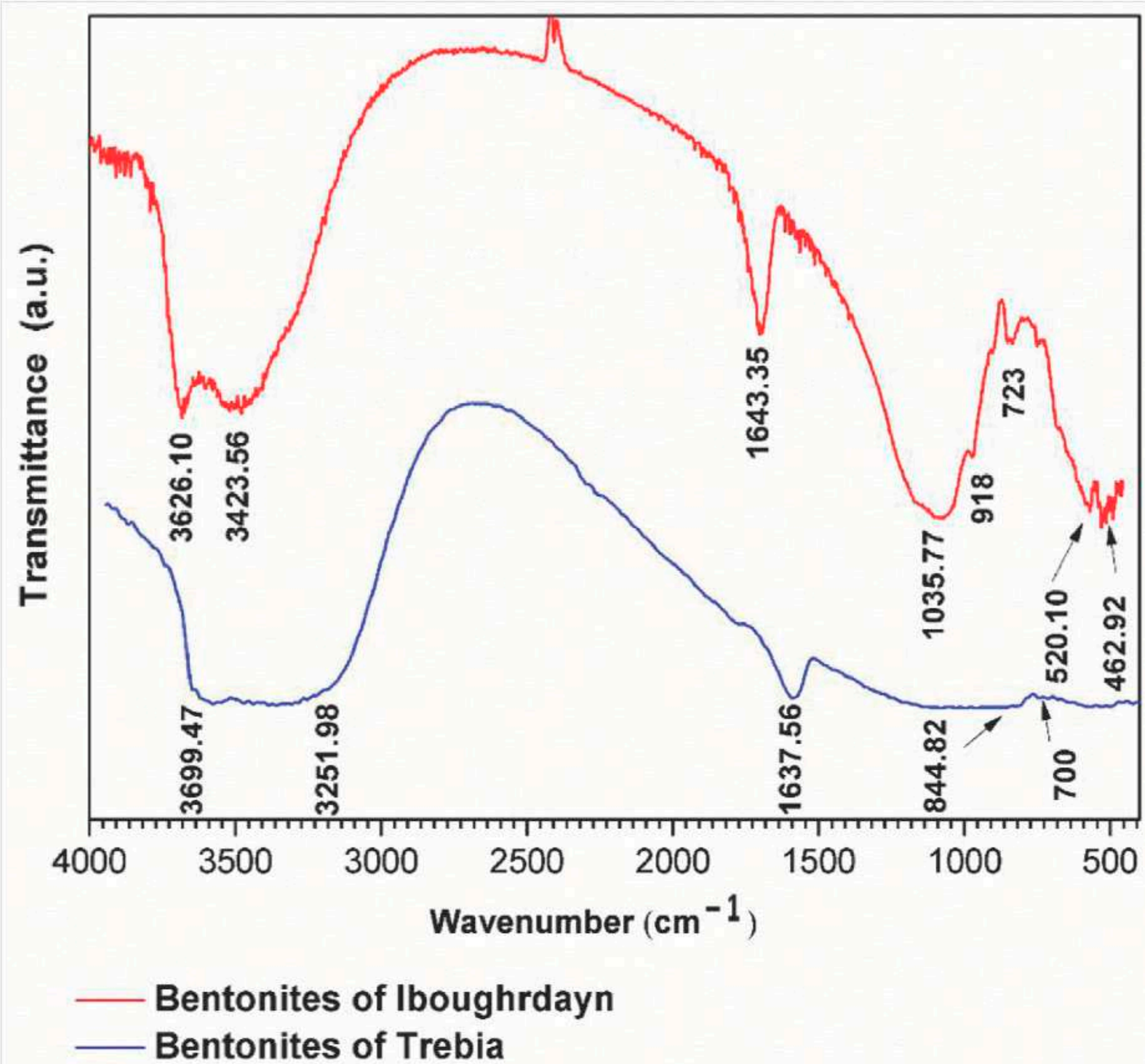


**Figure 2.** FTIR spectra of raw bentonite studied.

### 2.1.4. XRF Characterization

The chemical constitution of the adsorbents is represented in Table 2. The bentonite samples are characterized by a large amount of Fe, between 28.6% and 30.93%, related to iron ore such as hematite ($Fe_2O_3$), identified by both XRD analysis and FTIR spectra. The silice content, from 0.1% to 7.94%, is related to quartz, cristobalite and feldspar. Samples are characterized by a medium content of calcium, ranging from 11.1% to 14.01%, and the aluminum content, between 4.81% to 7.3%, which are part of the mineralogical composition of montmorillonite. Magnesium was identified in the samples, although $Al^{3+}$ ions can be partially substituted by $Mg^{2+}$ ions in the octahedral layers, which explains the decrease in aluminum content in the bentonite of Iboughrdayn offset by magnesium [35].

**Table 2.** Chemical composition of bentonite.

| Weight (%) | IB | TrB |
|---|---|---|
| Ca | 14.01 | 11.1 |
| Fe | 30.93 | 28.6 |
| Al | 4.81 | 7.3 |
| Si | 7.94 | 0.1 |
| Mn | 0.18 | 0.8 |
| K | 4.06 | 4.4 |
| Sr | 0.68 | 0.4 |
| P | 2.12 | 3.1 |
| S | 1.04 | 1.6 |
| Th | 0.033 | 0.1 |
| Rb | ND | 0.05 |
| Y | 0.033 | 0.02 |
| Mg | 32.1 | 29.3 |
| Zn | 0.55 | 0.08 |
| Sn | ND | 0.07 |
| Ti | 1.25 | 1.2 |
| Pb | 0.05 | ND |
| Zr | 0.18 | 0.3 |
| As | ND | 0.02 |

Note: ND; not detected.

### 2.2. *Characteristics of Raw Margin*

The margin sample was analyzed prior to treatment for different physico-chemical properties, and the results of the analysis are presented in Table 3. These values represent the averages of three determinations. According to the compositions of these raw margins, it appears that it is a highly polluting and harmful organic effluent of an organic nature. It is worth noting that the parameter values are in good agreement with those reported in the literature. It can be seen that the pH of margin is relatively low (pH = 4.03). This is mainly due to the presence of compounds such as organic acids and phenols. Indeed, the recorded electrical conductivity is relatively average, in the order of 16.93 ms·cm$^{-1}$. The margin used contained fewer suspended solids of 5.93 g·L$^{-1}$. Organic matter expressed in COD has a very high value of the order 172, 72 g·L$^{-1}$. A high content of compounds such as phenolic acids and fatty acids was found, with total polyphenols present at a rate of almost 9.17 g·L$^{-1}$. The concentrations of nitrogen elements (TKN: 2.39 g·L$^{-1}$), and phosphate elements (total phosphorus: 1.16 mg·L$^{-1}$) are relatively high.

**Table 3.** Physico-chemical characteristics of the raw margin.

| Parameter | Unit | Mean ± Standard Deviation (*n* = 3) |
|---|---|---|
| pH | (Unit) | 4.03 |
| Electrical conductivity | (mS/cm) | 16.93 |
| Chemical oxygen demand | (g·L$^{-1}$) | 172.72 |
| Total suspended solids | (g·L$^{-1}$) | 5.93 |
| Total Kjeldahl nitrogen | (g·L$^{-1}$) | 2.39 |
| Total polyphenols | (g·L$^{-1}$) | 9.17 |
| Total phosphorus | (mg·L$^{-1}$) | 1.16 |
| Micro-element concentrations (g·L$^{-1}$) | | |
| Ca | (g·L$^{-1}$) | 0.62 |
| K | (g·L$^{-1}$) | 6.17 |
| Na | (g·L$^{-1}$) | 1.04 |
| Cl | (g·L$^{-1}$) | 1.72 |
| Heavy metal concentrations (mg·L$^{-1}$) | | |
| Fe | (mg·L$^{-1}$) | 154.82 |
| Cu | (mg·L$^{-1}$) | 31.72 |
| Zn | (mg·L$^{-1}$) | 36.14 |
| Pb | (mg·L$^{-1}$) | 5.28 |

*2.3. Adsorption Experiments*

2.3.1. Evolution of Physico-Chemical Parameters

The results in Figure 3 show the variation in the pH of the bentonite/margin mixture as a function of the increase in the percentage of bentonites.

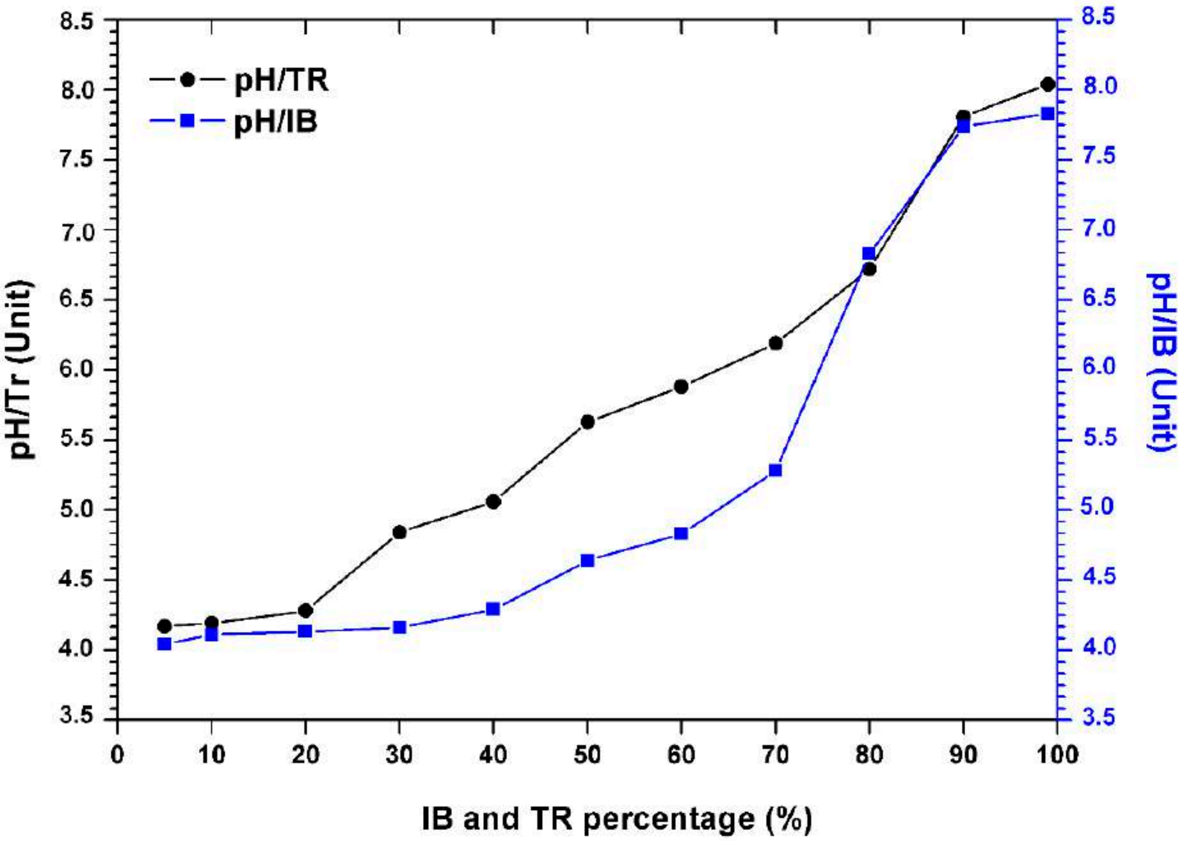


**Figure 3.** Evolution of the pH of the margin–bentonite mixtures.

There is an increase in pH (Table 4). However, the acid pH varies between 4.04 and 4.17, at the alkaline pH from 7.83 to 8.04 between 5% and 80% of the concentration of bentonite.

**Table 4.** Evolution of the pH and the electrical conductivity of the margin-bentonite mixtures.

| **Sample** | **$M_r$** | **$Tr_{5\%}$** | **$Tr_{10\%}$** | **$Tr_{20\%}$** | **$Tr_{30\%}$** | **$Tr_{40\%}$** | **$Tr_{50\%}$** | **$Tr_{60\%}$** | **$Tr_{70\%}$** | **$Tr_{80\%}$** | **$Tr_{90\%}$** | **$Tr_{99\%}$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH (Unit) | 4.03 | 4.17 | 4.19 | 4.28 | 4.84 | 5.06 | 5.63 | 5.88 | 6.19 | 6.72 | 7.81 | 8.04 |
| EC (mS/cm) | 16.93 | 16.88 | 15.37 | 14.82 | 12.76 | 10.04 | 8.82 | 7.45 | 5.92 | 3.28 | 0.93 | 0.17 |
| **Sample** | **$M_r$** | **$Ib_{5\%}$** | **$Ib_{10\%}$** | **$Ib_{20\%}$** | **$Ib_{30\%}$** | **$Ib_{40\%}$** | **$Ib_{50\%}$** | **$Ib_{60\%}$** | **$Ib_{70\%}$** | **$Ib_{80\%}$** | **$Ib_{90\%}$** | **$Ib_{99\%}$** |
| pH (Unit) | 4.03 | 4.04 | 4.11 | 4.13 | 4.16 | 4.29 | 4.64 | 4.83 | 5.28 | 6.83 | 7.74 | 7.83 |
| EC (mS/cm) | 16.93 | 16.72 | 16.02 | 15.83 | 14.94 | 11.73 | 9.24 | 6.38 | 5.29 | 3.82 | 0.93 | 0.12 |

$M_r$: Raw margin; $Tr_{x\%}$: percentage of Trebia's bentonite; $Ib_{x\%}$: percentage of Iboughrdayn bentonite.

Still, a sudden increase in pH was noted when the percentage of bentonite was over 80%. This increase is probably related to the composition of the bentonite which is rich in soluble and basic salts such as alkali carbonates and bicarbonates or silicates, which have a strong capacity to neutralize the acidic pH of the mixture.

At a percentage of 90%, Trebia bentonite tends to neutralize the pH value of the 7.81 mixture. In addition, for Iboughrdayn bentonite, the average pH value is around 7.74.

Figure 4 shows the electrical conductivity, showing that this parameter decreases steadily throughout the experimental period. The passage of the raw margin through the mixed margin shows a significant and successive decrease in electrical conductivity in the Trebia and Iboughrdayn mixtures, from 16.88–16.72 to 0.17–0.12 µS/cm.

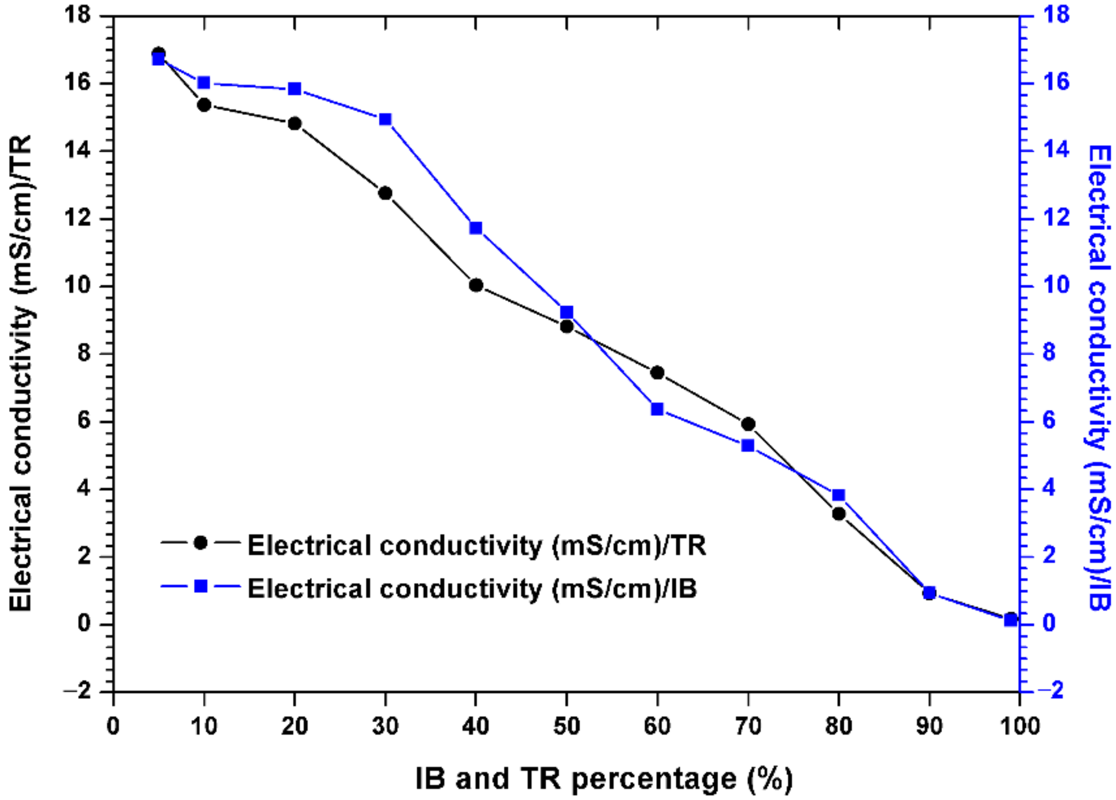


**Figure 4.** Evolution of the electrical conductivity of the margin–bentonite mixtures.

### 2.3.2. Total Suspended Solid Removal

The influence of adsorbent dose on the adsorption of total suspended solids from the margin is shown in Figure 5. An increase in suspended solid removal with an increase in adsorbent dose was recorded, starting at a concentration of 50%. The TSS concentration for the Iboughrdayn bentonite mixture ranges from 3.19 to 5.59 $g \cdot L^{-1}$. In combination, the TSS concentration for Trebia bentonite decreased from 5.88 $g \cdot L^{-1}$ to 3.82 $g \cdot L^{-1}$. Trebia's bentonite corresponds to a reduction of 35.58%. Iboughrdayn bentonite has a reduction rate of 46.21%. The variation in the rate of reduction of TSS can be explained by the particle size composition of the bentonites (Table 1). Therefore, the bentonite mixture plays the role of the primary barrier, which led to a reduction in TSS.

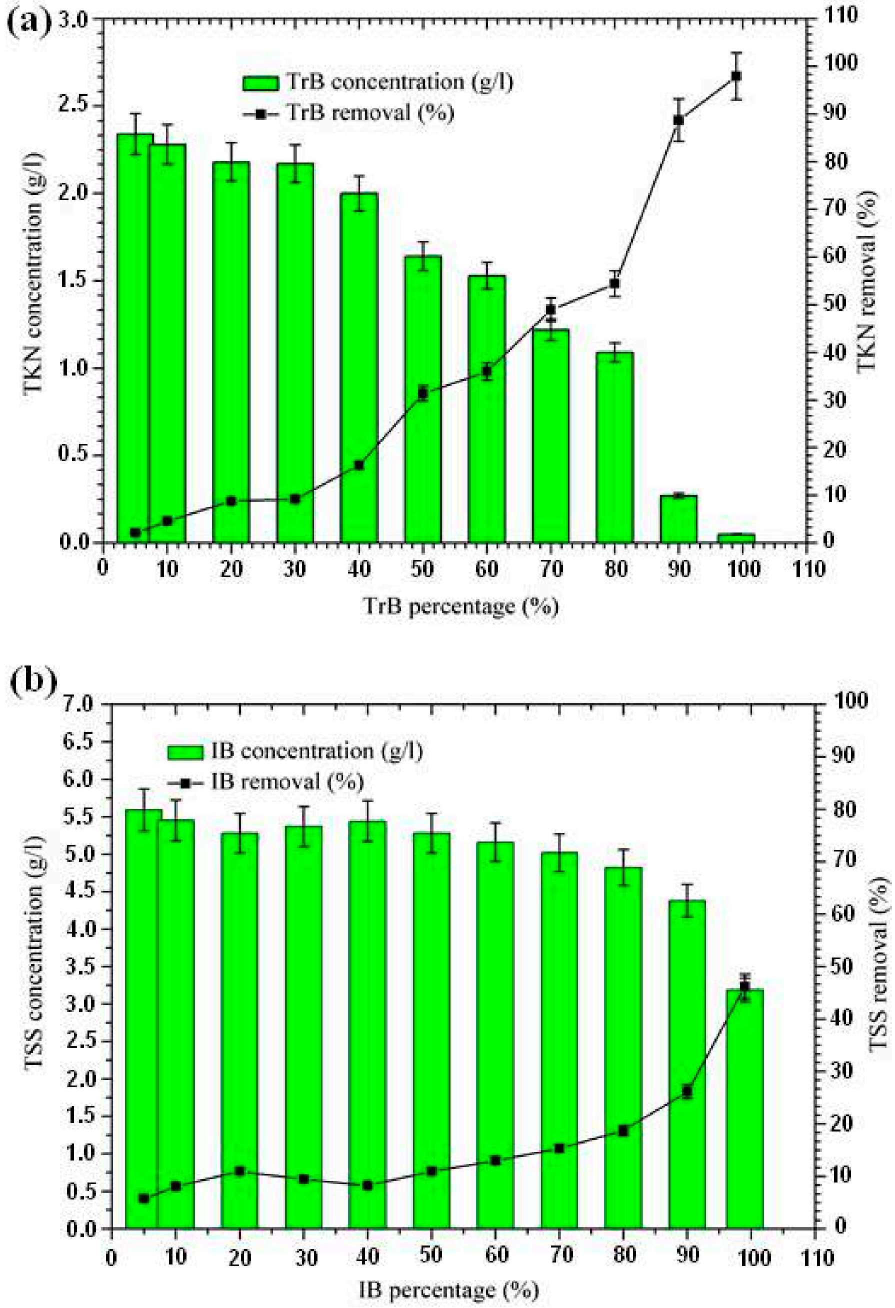


**Figure 5.** (**a**) Concentration and removal percentage of TSS by TrB; (**b**) concentration and removal percentage of TSS by IB.

### 2.3.3. Organic Matter Reduction

Figure 6 shows a significant reduction in COD, in contrast to Bentonite of Terbia and Iboughrdayn, which varies between 82.80 and 93.73%. This reduction is due to the degradation of organic matter by bentonite. Figure 6 shows the variation in total COD concentration and the rate of reduction as a function of the percentage of bentonite. The evolution of this parameter was divided into three distinct phases:

- The first phase (5–50%): where the COD concentrations for the margin mixture with Iboughrdayn bentonite were reduced from 161.03 to 77.83 g·L$^{-1}$. At the same time, we noticed an increase in the COD removal rate from 6.77% to 54.94%. In addition, Trebia margin/bentonite, characterized by reduced concentrations from 165.81 to 105.70 g·L$^{-1}$, corresponds to elimination rates from 4.00% to 38.80%;

- The second phase (50–70%): the COD concentrations for the almost constant margin/bentonite mixture vary successively for the Iboughrdain and Trebia bentonite in the interval between 73 and 96.5 g·L$^{-1}$. In addition, COD removal rates vary between 57% and 44%;
- The third phase beyond 70%: we distinguished a remarkable increase at the end of the treatment of the rate of elimination, with Iboughrdayn and Trebia bentonite reaching up to 93.73% and 82.80%, respectively.

Then, the reduction in the COD rate is probably due to the reduced decomposition of organic matter affected by the formation of the adsorption conditions [34].

In fact, the decrease in COD is related to the reduction in organic matter decomposition, which is now adsorbed on the bentonite, forming the organoclay complex. The bentonite in this complex plays a protective role against the decomposition of organic matter.

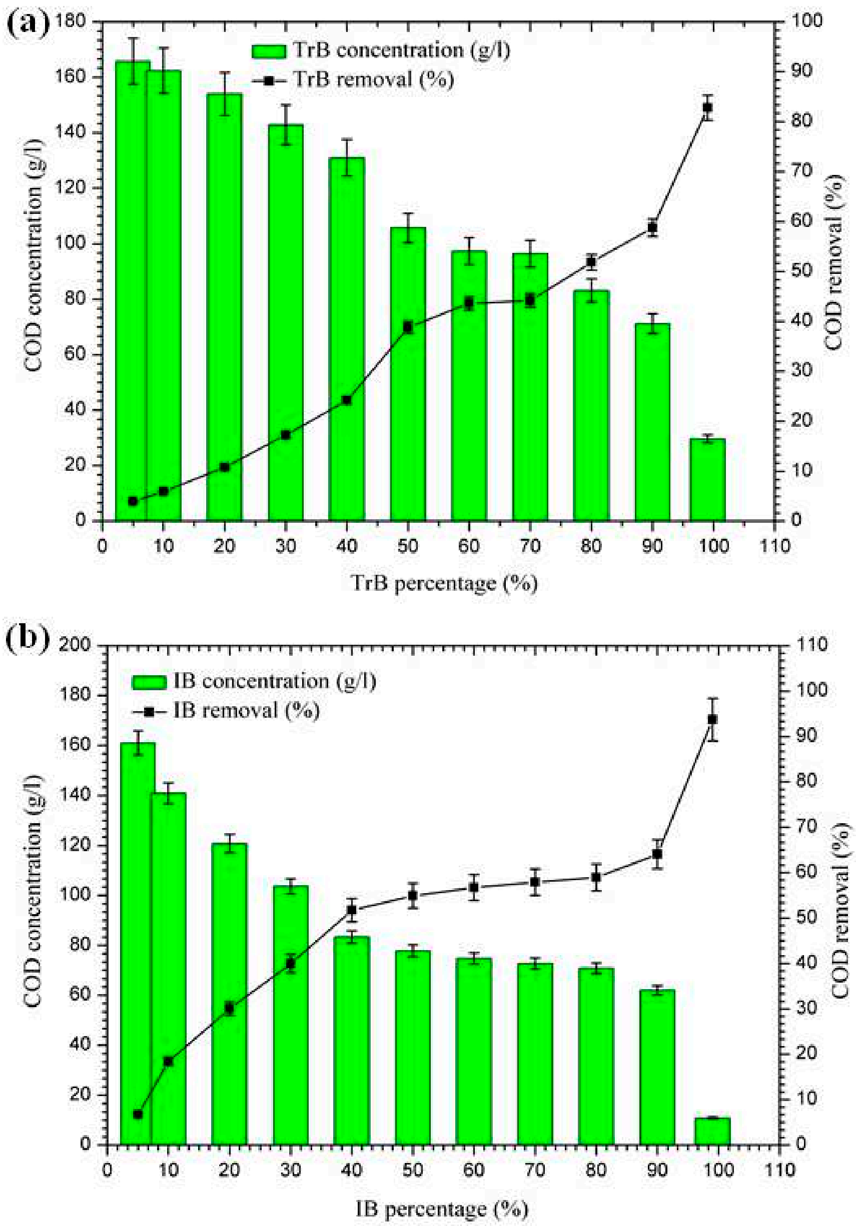


**Figure 6.** (**a**) Concentration and removal percentage of COD by TrB; (**b**) concentration and removal percentage of COD by IB.

2.3.4. Nitrogen Removal

The concentration of TKN nitrogen in Iboughrdayn's bentonite-treated margin is of the order of 2.27 g·L$^{-1}$, and decreases to 0.11 g·L$^{-1}$, effectively reduced at a rate of 95.40%. However, Trebia bentonite can remove TKN nitrogen concentrations of from 2.34 to 0.05 g·L$^{-1}$. At the beginning of the experiment, we recorded a percentage reduction of 2.09% in NTK nitrogen, which gradually increased to reach a maximum value of 97.91% (Figure 7). In fact, the increase in removal efficiency for the concentration of TKN nitrogen in the bentonite can be explained by the texture of the bentonites used for the development of aerobic conditions and the denitrification process, as well as the adsorption on the surface of the bentonite, due to their large specific surface area (Table 1).

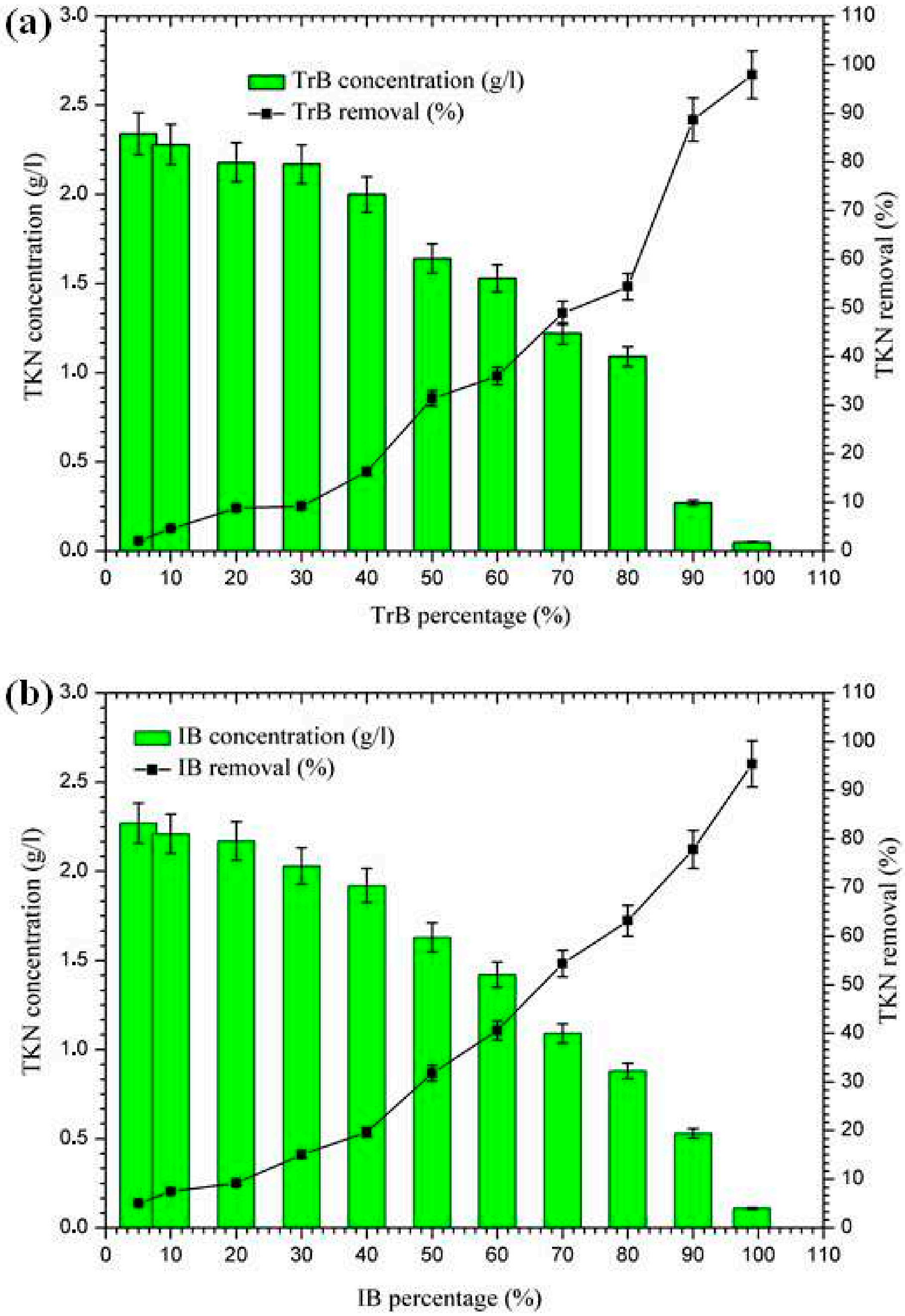


**Figure 7.** (**a**) Concentration and removal percentage of TKN by TrB; (**b**) concentration and removal percentage of TKN by IB.

2.3.5. Phosphorus Removal

According to the results presented in Figure 8, phosphorus removal occurs in two phases:

- The first Phase (5–20%): where the maximum phosphorus retention rate by Trebia bentonite, on the one hand, reached 34.48%, and on the other hand, Iboughrdayn bentonite at abatement reached 32.76%;
- The second phase (20–99%): where the maximum total phosphorus removal reaches values between 75% and 98.14%. Phosphorus can be removed by adsorption on iron and aluminum hydroxides ($Al^{3+}$), by the precipitation process [36–39], which is indeed part of the chemical composition of bentonite (Table 2).

Therefore, the difference in the rate of phosphorus removal observed during treatment with Trebia bentonites and Iboughrdayn bentonite could be related to variations in the mineralogical composition and micromorphological and textural characteristics of these bentonites.

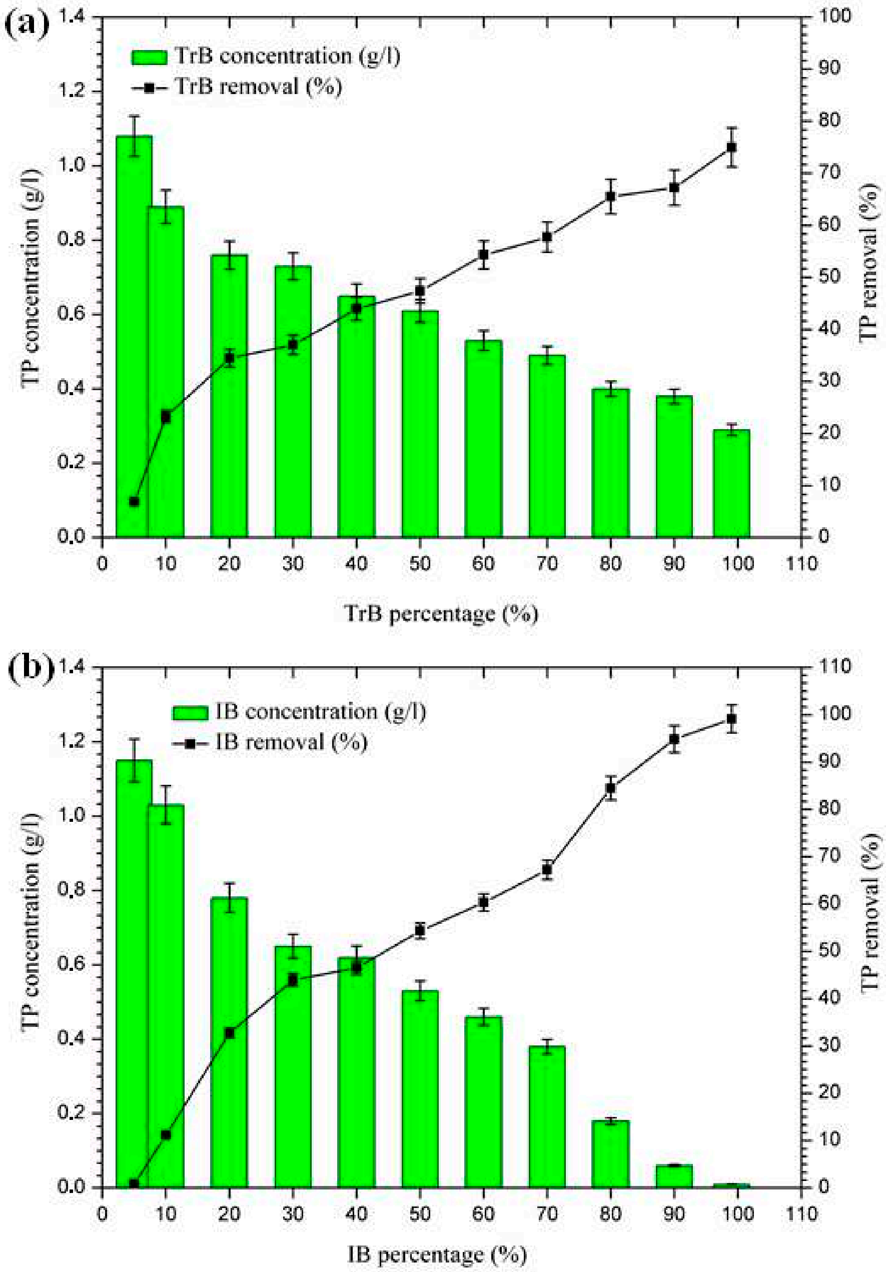


**Figure 8.** (**a**) Concentration and percentage of TP removed by TrB; (**b**) concentration and percentage of TP removed by IB.

2.3.6. Total Phenolic Compound Removal

The mixing of margin with Iboughrdayn bentonite at high rates of elimination of phenolic compounds (59.32–76.23%) was recorded for percentages between 70% and 99%, with a maximum observed when the contents of phenolic compounds were very low at the end of treatment. On the other hand, Trebia bentonite, with fairly average rates of elimination of phenolic compounds (30.53–58.23%), corresponds to percentages between 60% and 99%, with a maximum observed when the levels of phenolic compounds are very low, obtained at the end of treatment. However, the maximum removal rate was recorded at 60% bentonite (Figure 9).

The decrease in phenolic compounds would be related to the higher and/or increased pH [40,41]. Then, the phenolic compounds could be removed by adsorption on the surface of the bentonite, perhaps due to the higher dose of adsorbent providing more active adsorption sites or surfaces [9]. These data may explain the high removal rate of phenolic compounds found in the second phase (60% to 99%), where the percentages of bentonites and pH were significant.

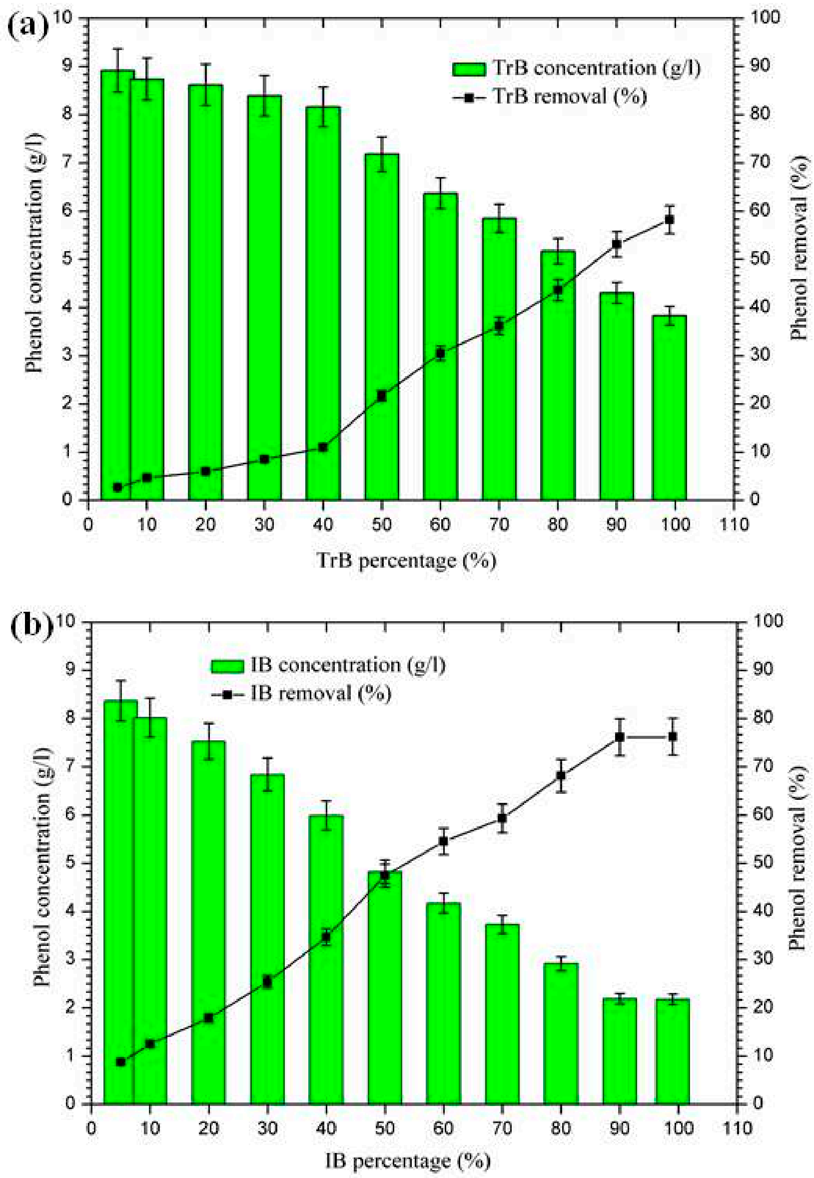


**Figure 9.** (**a**) Concentration and removal percentage of the phenol by TrB; (**b**) concentration and removal percentage of the phenol by IB.

2.3.7. Variation of Microelements

Figure 10 shows the variation in microelements in the margin/bentonite mixture. The potassium concentration varies from 6.14 g·L$^{-1}$ to 4.62 g·L$^{-1}$ in the margin/bentonite mixture of Iboughrdayn. In comparison, the concentration of potassium decreases from 6.11 g·L$^{-1}$ to 5.08 g·L$^{-1}$ in the mixture of margin with Trebia bentonite, with removal rates varying in both the Trebia margin/bentonite mixture and the Iboughrdayn margin/bentonite mixture from 17.67% to 25.12%.

The mixture of margin with Trebia bentonite has sodium (Na) concentrations ranging from 1.04 g·L$^{-1}$ to 0.60 g·L$^{-1}$, corresponding to an elimination rate of the order of 42.31%. However, the mixture the margin with Iboughrdayn bentonite has sodium (Na) concentrations which decreased from 1.02 g·L$^{-1}$ to 0.32 g·L$^{-1}$. As well as this, we observed that the elimination rate of Na increased from 1.92% to 69.23%. Jointly, the concentrations of calcium (Ca) and chloride (Cl) increased with the increase in the percentage of bentonite. Indeed, the increase in these two elements is denoted from 20% onwards.

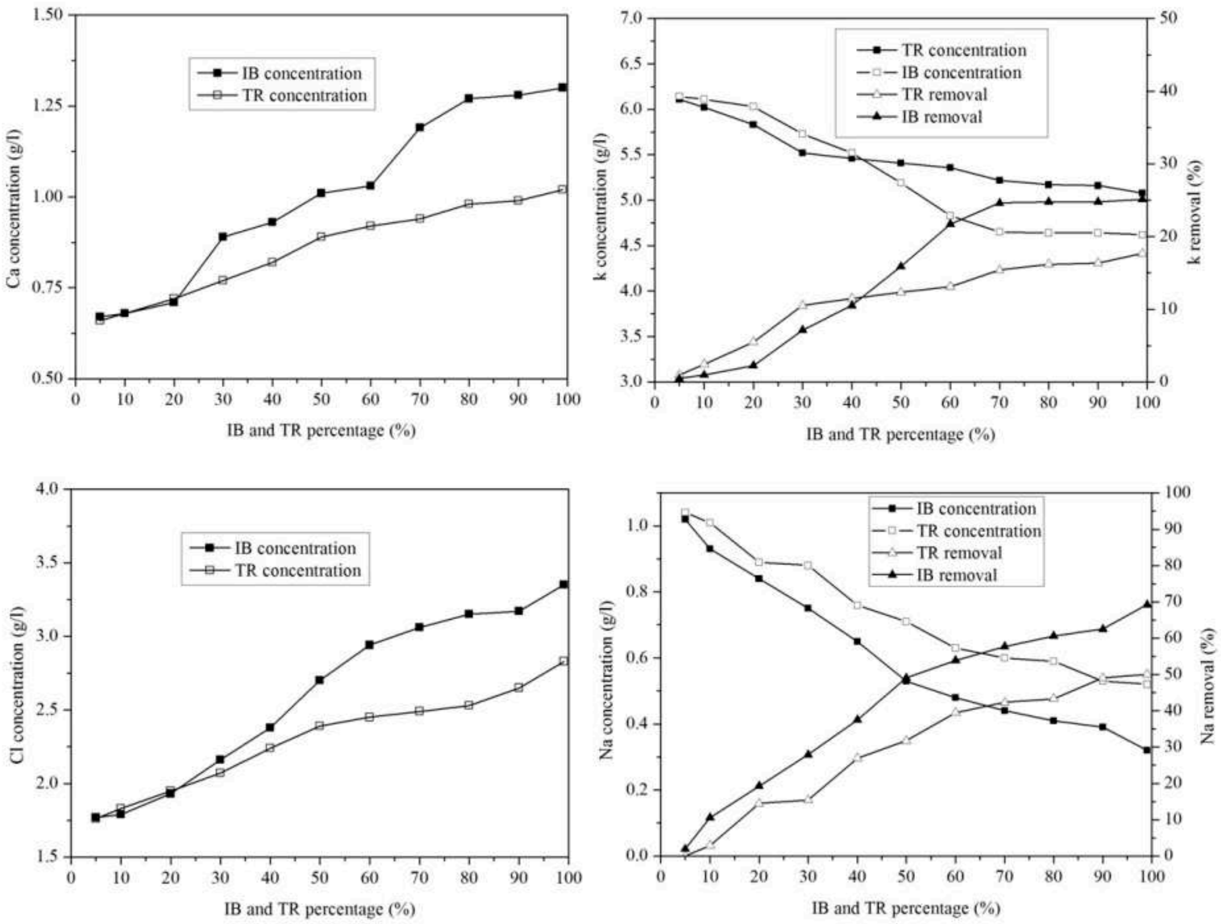


**Figure 10.** Concentration of and variation in micro-elements as a function of bentonite content.

2.3.8. Heavy Metal Ion Removal

In order to compare the adsorption potentials, raw bentonite from Trebia and Iboughrdayn was chosen and used for the optimal adsorption of metal ions from margin.

The concentration of Zn in the mixture of margin with Iboughrdayn bentonite varies between 31.72 g·L$^{-1}$ and 0.01 g·L$^{-1}$. The evolution of this element was split into two phases: in the first phase, percentages of the 90% lower bentonite correspond to rates between 11.93 and 48.12% (Figure 11). In the second phase, a sudden increase in the rate of elimination of Zn was observed at a maximum value of around 94.94%. In addition, the mixture of margin with Trebia bentonite was characterized by an ideal absorption power, where the concentrations recorded at the end of the experiment maintain that traces of Zn do not exceed 0.02 g·L$^{-1}$.

This corresponds to a maximum rate of 99.97%. It was deduced that the adsorption of Pb occurs in a total way, where the elimination rate is denoted successively for Trebia and Iboughrdain bentonite from the percentages of 70% and 10%. Knowing this, the same Pb elimination rates were recorded in the order of 99.81%. The variation in the Cu adsorption in the two adsorbents obtains the same results, with an elimination rate of 99.97% Cu at percentage 99%, corresponding to concentrations of 0.01 g·L$^{-1}$.

The decrease in the concentration of Fe in the mixture of margin with Iboughrdayn bentonite from 151.03 to 0.17 g·L$^{-1}$ corresponds to a maximum rate of 99.89%. Moreover, the mixture of the curbstone with Trebia bentonite is characterized by an Fe removal rate of 87.19%.

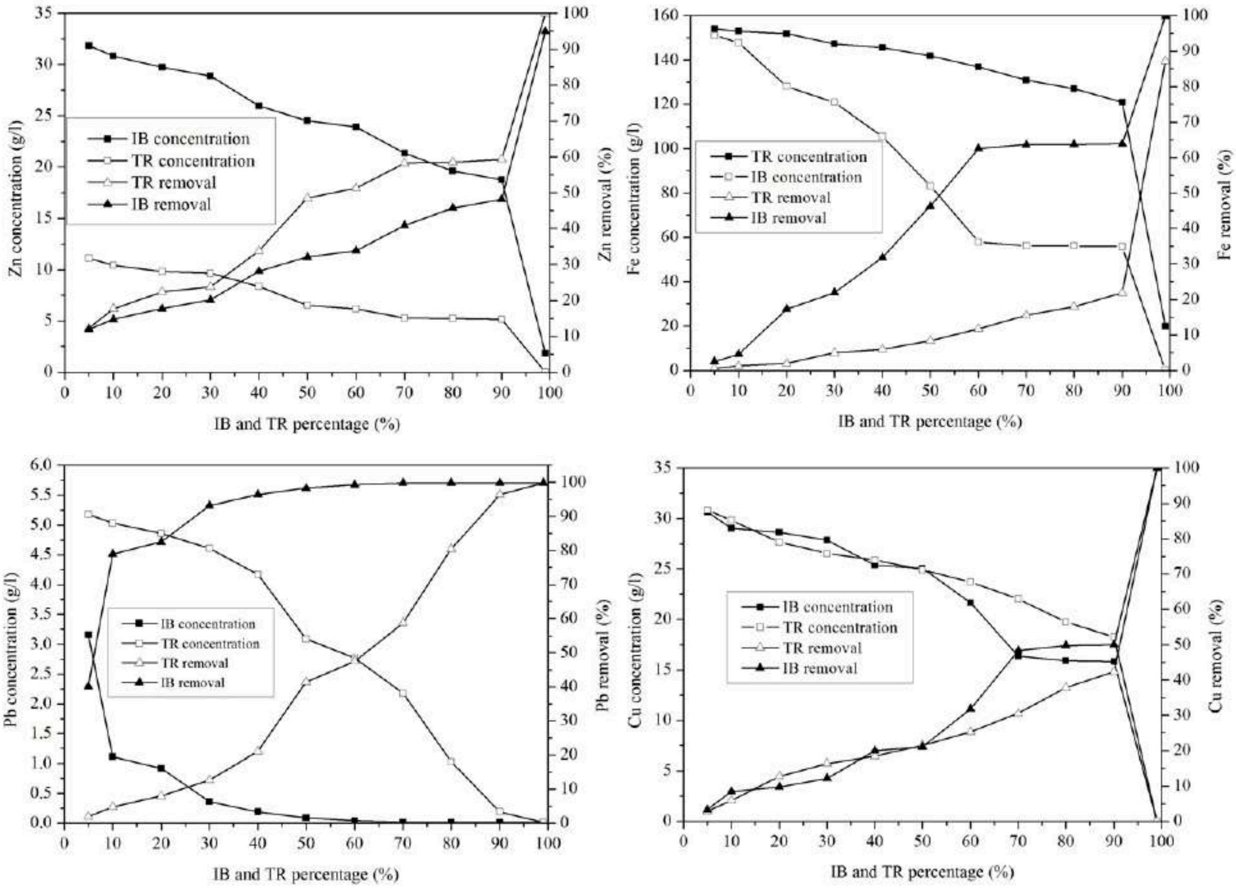


**Figure 11.** Concentration of and variation in heavy metals.

### *2.4. Phenol Adsorption Mechanism on Bentonite*

The adsorption process results from interactions between the surface of the adsorbent (bentonite) and the adsorbate (phenol, heavy metal, etc.).

The mechanisms responsible for the fixation of organic compounds on the bentonite material (formation of the organoclay complex) are multiple and are controlled by the properties of the adsorbent, particularly its specific surface, the value and location (tetrahedral or octahedral) of the electric charge, and the different characteristics of the organic adsorbate, of which the nature, size, structure, and basicity of the organic molecules are the dominant parameters.

Indeed, bentonite treatment of margin increases the pH, which promotes the adsorption of phenols by attractive forces of chemical nature, causing a transfer or pooling of electrons and, consequently, the destruction of the individuality of molecules and formation of a chemical compound on the surface of bentonite (Figure 12a).

The bentonites studied have basic pH values (Table 1). The neutrality of the medium is probably related to various properties of bentonite material, including its high CEC; its adsorptive power; its composition, which is rich in basic salts such as carbonates, bicarbonates, oxides, hydroxides, etc.; and its ability to form an organo–mineral complex with certain organic compounds of the margin. This latter property plays a primordial role in the ionic fixation and increases the property of adsorption, bentonites' excellent material of the depollution, etc., and so the heavy metals are easily introduced to the interfoliar space of the bentonite (Figure 12b).

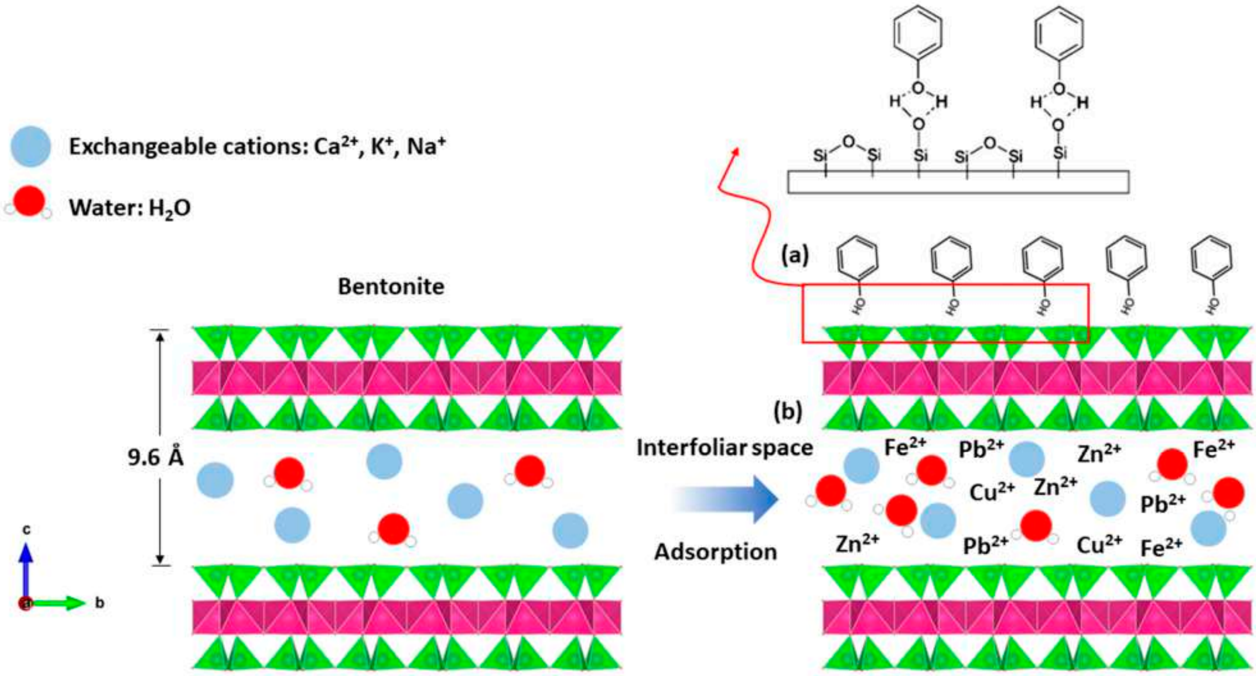


**Figure 12.** Adsorption mechanism between; (**a**) phenol and bentonite; (**b**) heavy metal and bentonite.

## 3. Materials and Methods

### *3.1. Materials*

The bentonite used in this work is distributed in the external domain of the Rifaine chain on the western flank of Nador city in the Kert neogenous basin (Figure 13). Bentonite comes from the Trebia and Iboughrdayn deposit in Nador (North-East Morocco). The elemental composition of the raw bentonite is illustrated in Table 1. The margin used in this study was collected from a semi-modern oil mill located in the province of Driouache, about 60 km from the city of Nador. The samples were taken during the olive oil extraction seasons (December–March) and during the olive-growing season 2018–2019.

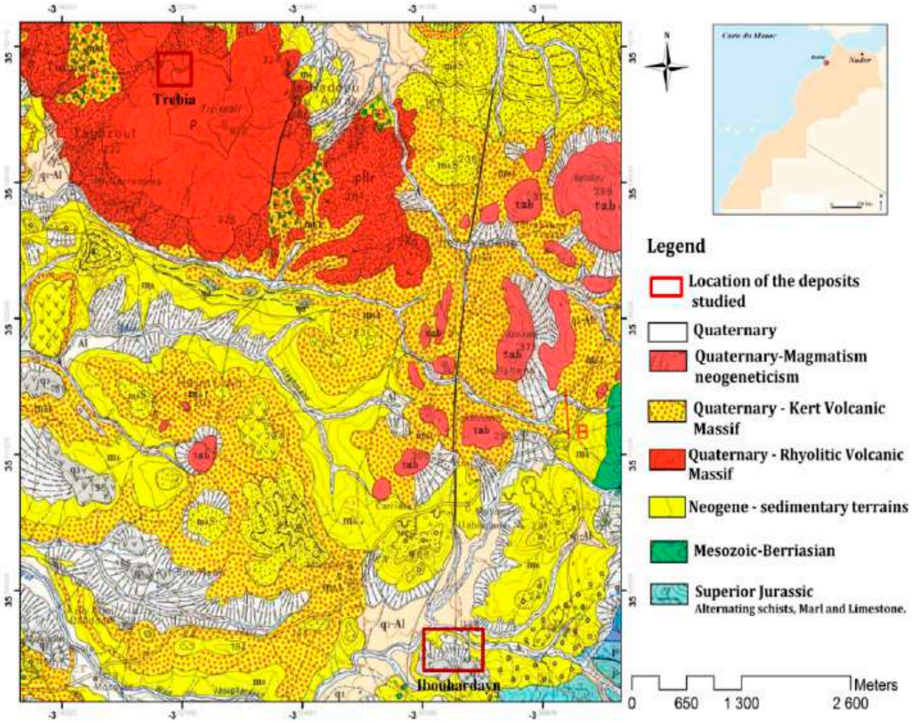


**Figure 13.** Geological map of the deposits studied.

### 3.2. Preparation of the Adsorbent/Adsorbate

The experiments were performed in beakers with a capacity of 100 mL. For each test, the solution was mixed with the margin and a mass of the adsorbent (bentonites). The mixture was stirred permanently. To achieve the objectives of this study, we progressively substituted the bentonite contents with increased margin volumes. The percentages of applied margin were calculated in relation to the following mass loads: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 99%.

### 3.3. Characterization Methods

The adsorbant samples were characterized by X-ray diffraction (XRD) (San Giovanni Valdarno (AR), Italy), Fourier transform infrared spectroscopy (FTIR) (Nador, Morocco), particle size distribution (Marrakech, Morocco), and chemical analyses (XRF) (Nador, Morocco). The crystal phases were determined by X-ray diffraction analysis, an XRD patterns were collected using an XPERT-PRO diffractometer (San Giovanni Valdarno (AR), Italy), operating at a voltage of 40 kV and an intensity of 30 mA with radiation (Cu), on the powdered sediments following the normal procedure for clay analysis [42]. Chemical analysis of the samples was determined using an X-ray fluorescence analyzer (Nador, Morocco). IR spectra were made using a Shimadzu FTIR-8400S spectrometer (Nador, Morocco) over a range of 400–4000 $cm^{-1}$. The particle size distribution of the samples was analyzed on bulk sediment using a laser diffraction particle analyzer Horiba 300. Characterization of the physico-chemical parameters was performed by measuring acidity (pH), swelling index, and swelling rate.

The specific surface areas and the cation exchange capacity of the samples were found by the methylene blue test [43,44]. The values of the cation exchange capacity (CEC) were determined according to (1) [44]

$$\text{CEC (meq/100g)} = V_{MB} \times N_{MB} \times (100/m_0) \quad (1)$$

where $V_{MB}$ = the volume of MB added to the sample; $N_{MB}$ = the normality of methylene blue; $m_0$ = the weight of the dry powdered sample.

*3.4. Adsorption Methodology*

The efficiency the margin treatment with bentonite was monitored by measuring the evolution of the physico-chemical parameters. The hydrogen potential (pH) of the mixture was determined by a pH meter according to the protocol described by Alkama et al. (2009) [45]. The electrical conductivity was measured using a Consort C933 conductivity meter. Suspended solids (SS) were determined by filtration on membranes with a pore diameter of 45 µm. The TSS content was determined by the difference in weight of the filter before and after filtration and drying in an oven at 105 °C for 4 h [46]. The determination of nitrogen (TKN) was carried out according to the Kjeldahl method described by Bargaz et al. (2012) [47]. The chemical oxygen demand (COD) was determined according to the standard method [48], with oxidation of the organic matter contained in the sample at 150 °C by an excess of potassium dichromate in an acidic medium and in the presence of silver sulphate. The excess potassium dichromate was determined by colorimetry at $\lambda$= 620 nm. Total phosphorus (TP) was determined according to standard methods by AFNOR (1997), APHA (2005), and Rodier (2009) [48]. This was measured after mineralization in acidic solution with the presence of sodium persulfate at 200 °C for two hours, followed by an analysis of the orthophosphates [49].

Total polyphenols were obtained according to the method recommended by Macheix et al. (1990) [50]. Total polyphenols were quantified from the 200 µL Folin–Cioccalteu reagent. They were then oxidized by blue oxides of tungsten and molybdenum, followed by 400 µL of a sodium carbonate solution (20%) [51,52]. The mixture was placed in an incubator at 40 °C for 30 min. Finally the absorbance reading was taken at 760 nm. The contents of phenolic compounds are expressed in $g \cdot L^{-1}$.

The contents of iron, copper, zinc, potassium, sodium, calcium, chloride, and lead were analyzed by atomic absorption spectrometry (model AFP100, Biotech Management Engineering Co. Ltd., Nicosia, Cyprus) [53,54].

Mass percent of the removal metal ion was calculated using the following Equation (2) [55].

$$\%\ \text{Removal} = (C_0 - C_f/C_0) \times 100 \quad (2)$$

where $C_0$ and $C_f$ (mg/L) are the initial and final equilibrium concentrations of solution.

## 4. Conclusions

The treatment and adsorption of margin on raw bentonite from Morocco was studied in an aqueous medium. This adsorption depends on the physicochemical parameters involved (pH, electrical conductivity, and mass of adsorbent). From all the results, obtained at the end of the application for adsorption by the raw bentonite, it could be concluded that the studied margins present a major risk for the environment. Therefore, the study of adsorption has allowed us to note that the efficiency of phenolic compounds' removal from the copings by natural bentonite is important. This is explained by the presence of free sites on the surface of the particles of the adsorbent (bentonite), which correspond to the progressive decrease in concentrations. Given the very encouraging results which were obtained, we aim to study the application of adsorption for the treatment of margins. This method is simple, economical, ecological, non-toxic and fast, which is why we recommend its use for the treatment of domestic wastewater in developing countries.

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# Simultaneous adsorption behaviour of heavy metals from Oil Mill Wastewater onto natural clay

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**Abstract.** The present work reports the synergistic and inhibitory adsorption effects involved in the multicomponent adsorption of heavy metal ions (Fe (II), Pb (II)), and major elements from oil mill liquid waste (OMW) using natural bentonite as adsorbent cames from Nador (North-East Morocco). Morocco is one of the most olive oil producing Mediterranean countries. This industry, which is so beneficial to the national economy, leaves two toxic and non-biodegradable residues (liquid/solid). OMW or margin is a current liquid pollutant that has been listed by the United States Environmental Protection Agency (EPA). The classical methods used for phenol removal are expensive or limited to large-scale applications such as biological and thermal decomposition methods. The margins used in the studies were collected from a semi-modern oil mill (Nador-Morocco). The results of the physicochemical analyses showed that the effluents of the oil mills showed that they are highly polluted, in particular the suspended solids, COD, and iron contents of around 154.82 (mg/l) and copper 31.72 (mg/l). Samples of OMW mixed with raw bentonites at different percentages vary between 10 % and 80 %. Different interactions between bentonite and metal ions dealing with the decrease of the concentrations. This study proves that this bentonite is an effective adsorbent for the elimination of heavy metals from OMW.

## 1. Introduction

Agricultural production is one of the primary sources of waste [1, 2]. In several countries where olive oil is produced, the olive oil production of these countries represents 94% of world production [3-5]. Thus, the olive oil extraction process has large amounts of waste [6]. These residues can be in liquid or solid form and contain important phytochemicals with high added value. The liquid part of these wastes is named "margin" or "Oil Mill Wastewater" (OMW). By the way, the margins are strongly concentrated in organic matter , which is not very biodegradable and highly toxic [5]. They are made of water, organic compounds, and inorganic compounds.
The phenolics are identified as priority pollutants because they are harmful to organisms at low concentrations [7, 8]. As well as, hazardous pollutants because of their potential danger to human health [9].
On the other hand, inorganic compounds are manifested by heavy metals and major elements.

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Heavy metal contamination is one of the major concerns in today's world [1, 10].
Heavy metals are defined as metallic elements that have a relatively high density compared to water and is toxic or poisonous even at low concentration [6, 3]. Generally, heavy metals are toxic [11, 12]. So in our study, we used bentonites clay as an adsorbent and the margin as adsorbate. Bentonite is a clay mineral particle with two tetrahedral silica sheets and one aluminium octahedral sheet, and the main mineral is montmorillonite which has a double diffusion layer [13, 14]. The margin used in this study it is a highly polluting and harmful acidic effluent of an organic nature, with a pH of around 4, total polyphenols of 9.17 $g.L^{-1}$, total phosphorus of 1.16 $mg.L^{-1}$ and high COD was 172.72 $g.L^{-1}$ [15].
Concerning the bentonite clay used in this work characterized by a very high water retention capacity and therefore a high swelling capacity [16], $CaCO_3$ was very low [17], it has high porosity and permeability [18] is also characterized by a significant specific surface area [19]. This study therefore, aims at developing a

low-cost novel adsorbent of removing Fe (II), Pb (II) and major elements.

## 2. Materials and methods

### 2.1. Materials

Raw bentonite acquired from North-East Morocco was employed as the adsorbent material. Oil mill liquid waste used in this research was collected from the city of Nador, North-East Morocco (Fig. 1). The different properties of the studied bentonite are presented in table 1. Firstly, bentonite sample was dried at 40°C for 48 h, and then characterized by particle size distribution, X-ray diffraction (XRD), Infrared Spectroscopy (FTIR), X-ray fluorescence (XRF), and Atterberg limit. The particle size distribution of the raw bentonite has been determined by was determined by sedigraphy with the SediGraph III device, following the standards ASTM D422-C958-E1617 (Laboratory Prove, Geotechnical Centre of the University of Siena) [20]. The mineralogical composition was identified by X-ray diffraction using X'pert HighScore, was used in the range of 0-80° at the scan speed of 4°/min with copper anticathode CuKα radiation (λ = 0.1918 Ǻ, 40kV, 40mA). The quantitative mineralogical analysis was obtained from the powder XRD data using an internal standard for each mineral [21]. Chemical analysis of the major elements was carried out by X-ray fluorescence spectroscopy (XRF). Plasticity was obtained by determining Atterberg limits: liquid limit (IL), plastic limit (PL) plasticity and index (PI) with stand ASTM D3418 1983 [22]. Test Method for Shrinkage Factors of bentonite by the Mercury Method [23].

**Table 1.** Granulometric and geotechnical characteristics of Nador bentonite.

| Sample | % Gravel | % Sand | % Limon | % Clay | Cu | % PL | % LL | % PI | % $W_S$ |
|---|---|---|---|---|---|---|---|---|---|
| $B_M$ | 0.1 | 43.9 | 38.7 | 17.3 | 96.2 | 44.8 | 90.7 | 45.9 | 15.5 |

* **$B_M$:** Moroccan bentonite; **Cu**: Uniformity coefficient; **PL**: plasticity limit; **LL**: Liquidity limit; **PI**: plasticity index; **$W_S$:** shrinkage limit.


**Fig.1.** Olive oil extraction site (Nador - Morocco); **(a)** Storage of margins in basins; **(b)** Harmful effects of margins on soil quality.

### 2.2. Measured parameters

In this study, we have taken oil mill liquid waste as an adsorbate. In order to ensure a satisfactory including the preparation of the mixes adsorbent / adsorbate follows loads: 5%, 10%, 30%, 50%, 70%, and 90%. The contents of iron, lead, potassium and sodium, were analyzed by atomic absorption spectrometry (model AFP100, Biotech Management Engineering Co. Ltd., UK) [24]. Nitrogen (NTK) dosing, were performed according to the Kjeldahl method described by Bargaz et al (2012) [25]. It was determined by the mineralization of the samples in sulfuric acid of organic nitrogen in the presence of selenium ($K_2SO_4$).

Chemical oxygen demand (COD) was determined using the Standard Method for the Examination of Water and Wastewater [26]. Total phosphorus was measured after mineralization in acid conditions with sodium persulfate at 200°C/2h, followed by an analysis of orthophosphates according to the methods standardized by AFNOR (1997) [27]. The determination of phenols was quantified with 200 µl of FolinCioccalteu according to the process recommended by Macheix et al. 1990 [28]. The absorbance reading of the solution was read at the wavelength of maximum dye using a UV-Vis spectrophotometer. The mass per cent of the removal metal ion was calculated using the following equation (1) [29]:

$$\% \ Removal = (Ci - Ce / Ci) \times 100 \qquad (1)$$

Where Ci and Ce are the initial and final equilibrium concentrations (mg.$L^{-1}$).

## 3. Results and discussion

### 3.1. Characterization of bentonite

#### *3.1.1. Particle distribution*

Table 1 presents the percentages of particle size fractions of the bentonite used in this work. The Nador bentonite analyzed show a wide variation in particle size distribution; four particle size fractions were identified: clay (17.3%), silt (38.7%), sand (43.9%) and gravel (0.1%). The grain sizes of clay characterize by the uniformity coefficient or Hazen coefficient allows to express the spread of the curve granulometric, and the curvature coefficient is used to describe the shape of the particle size curve [30]. The particle size curves of the bentonite investigated is well spread out (Fig. 2), although the value has a uniformity coefficient greater than 2 (Cu = 96.2) [31].

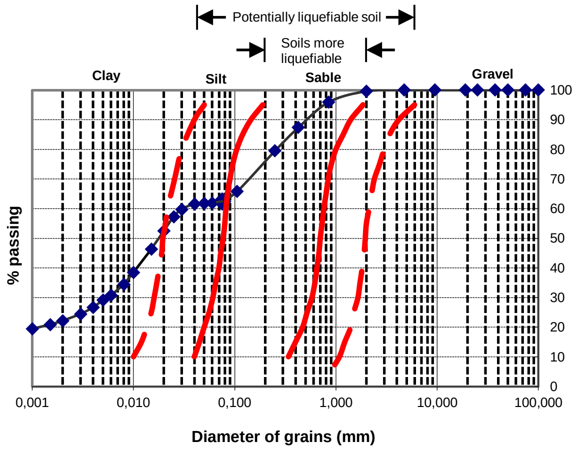


**Fig. 2.** Particle size distribution curve and limit of liquefiability potential according to Tsuchida (1970) [32].

### *3.1.2. Mineralogical composition*

The results obtained from the analysis of the diffractograms of the total rock show that the mineralogical composition is diversified (fig. 3 a). The samples consist mainly of anorthite (56.04%), feldspar (20.34%), and montmorillonite (15.34%) (fig. 3 b), with moderate cristobalite contents (4.85 %). Zeolite (1.43%), quartz (1.34%) and muscovite (0.62 %) are presents as traces in the bentonite samples.

These results are complemented by infrared spectroscopy (FTIR) (fig. 4). A simple analysis of the spectra indicated the presence of a strong absorption peak located at 3632 $cm^{-1}$, characteristics of O-H stretching vibration in the bentonite structure [32-34]. Other bands were assigned to the vibration H-O-H around 3626 and 1635 $cm^{-1}$ [35]. An intense band between 1035 and 465 $cm^{-1}$ indicates the stretching vibration of Si-O corresponding to quartz and cristobalite [36].

The one located at 520 $cm^{-1}$ is related to the elongation vibrations of the Si-O-Al bonds [37].

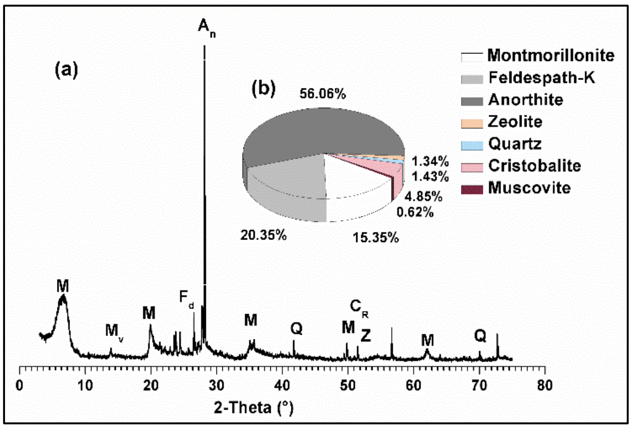


**Fig. 3. (a)** Total mineralogical composition obtained by X-ray diffraction; **(b)** Semi-quantitative estimation of the total mineralogy of Nador bentonite.

***Legend:** **Q**= Quartz; **M** = Montmorillonite; $\mathbf{F_d}$ = Feldspar; $\mathbf{C_R}$ = Cristobalite; **Z** = Zeolite; $\mathbf{M_v}$ = Muscovite; **An** = Anorthite.

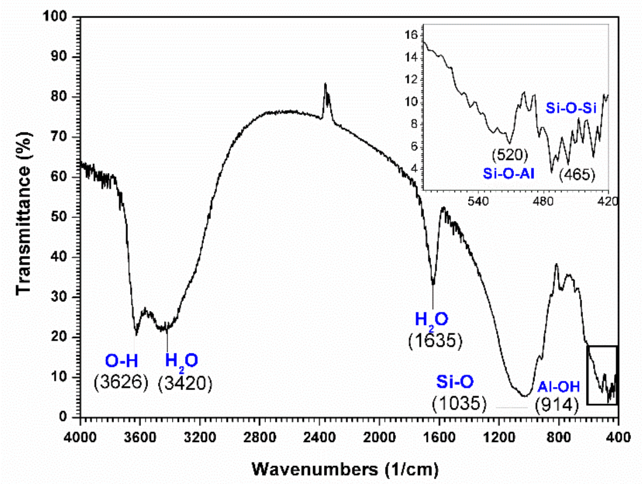


**Fig. 4.** Typical bentonite spectrum obtained by the infrared spectroscopy.

### *3.1.3. Geotechnical proprieties*

Atterberg limits are represented in the plasticity map of Casagrande. Atterberg limit test was performed through determination of Plasticity index (PI) value from the difference of liquid limit (LL) and plastic limit (PL) [14]. Natural bentonite was reported the value of LL, PL and PI were 90.7, 44.8 and 45.9, respectively (Table 1). The plasticity map shows that the bentonites used are classified as very plastic (fig. 5). The shrinkage limit ($W_S$) of the bentonite is 15.5% (fig. 6), has a maximum volumetric weight of about 1.87 $g.cm^{-3}$ (fig. 7).

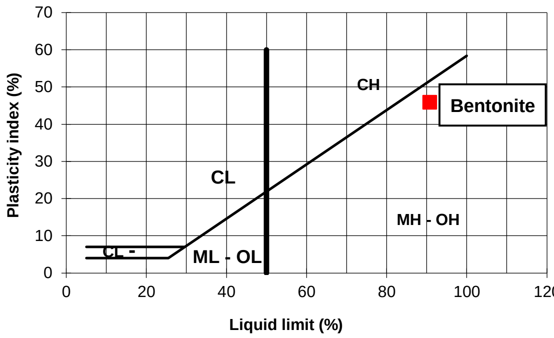


**Fig. 5.** Bentonite projection in the Casagrande plasticity map.

***Legend: CL** : mineral clays with low to medium plasticity; **ML-OL** : inorganic silts and veryfine sands with very low plasticityand organic silts and silt–clay mixtures of low plasticity; **OH-MH** : organic silts and clays of medium plasticity; **CH**, Inorganic clays of high plasticity.

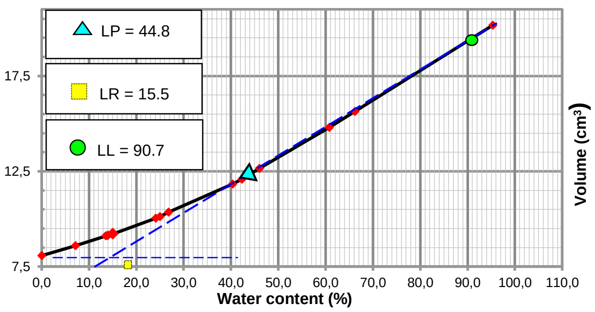


**Fig. 6.** Volumetric Shrinkage Curve.

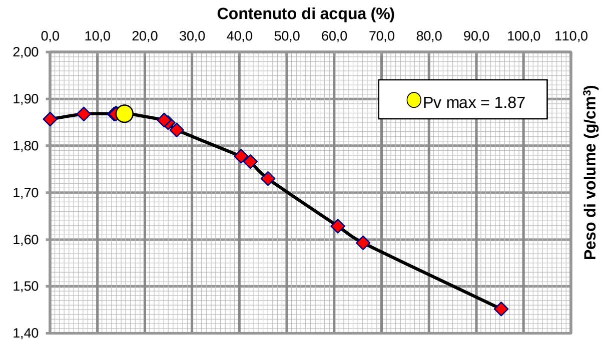


**Fig. 7.** Variation in volumetric weight as a function of water content

### *3.1.4. Chemical composition*

Table 2 shows the XRF results of all elements presents in the natural bentonite sample. Results of the XRF show that bentonite is mainly composed of Mg, Fe and Ca. High levels of Mg and Fe indicate the presence of iron oxides or substitution of Si and Al by Fe and Mg, also revealed that there are meager proportions of P, S, Ti, Mn…etc.

**Table 2.** Elements in natural clay as determined using XRF analysis.

| Element | * Mg | * Fe | K | * Ca | Si | Al | P | S | Ti | Mn | Sr | Zr | Th | Zn | Y | Pb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weight % | 32.1 | 30.93 | 4.06 | 14.01 | 7.94 | 4.81 | 2.12 | 1.04 | 1.25 | 0.18 | 0.68 | 0.18 | 0.033 | 0.55 | 0.033 | 0.05 |

(*) : maine composition.

## 3.2. Purifying efficiency bentonite

### *3.2.1. Abatement of COD*

Figure 8 represents the variation of the total COD concentration and the abatement rate as a function of the percentage of bentonite. COD concentrations in this study ranged from 161.03 and 62.04 g.L$^{-1}$. In turn, the COD removal rates varied between 6.77 and 64.08%. So the reduction in the COD rate is most likely due to the reduced decomposition of organic matter [38, 13].

### *3.2.2. Abatement of nitrogen*

NTK presents the main form of nitrogen in wastewater. The reduction of NTK concentration during the treatment of OMW is shown in Fig. 9.
The concentration of NTK fluctuates from 2.27 to 0.53 g.L$^{-1}$ with an optimal removal of 77.82%. The elimination of the concentration of NTK nitrogen in OMW can be explained by the texture of the bentonites that serve for the development of aerobic conditions and the denitrification process [39].

https://doi.org/10.1051/e3sconf/202131408002

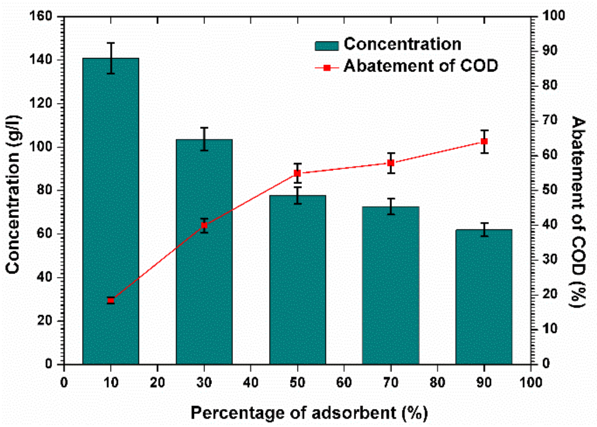


**Fig. 8.** Concentration and abatement of COD.

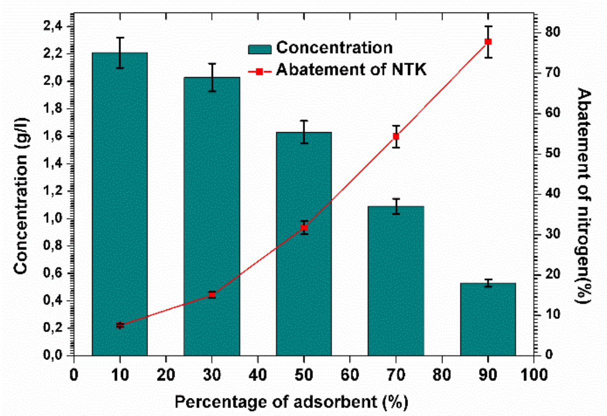


**Fig. 9.** Concentration and abatement of NTK.

### *3.2.3. Abatement of phenol*

The analysis of the phenolic fraction as a complex of the margins allowed us to evaluate the variation and the evolution of these polyphenols according to the bentonite (fig. 10).

In this study, the maximum recorded removal rate of 76.12%. In fact, the decrease of phenolic compounds would be linked to the more significant and/or higher pH [15, 40].

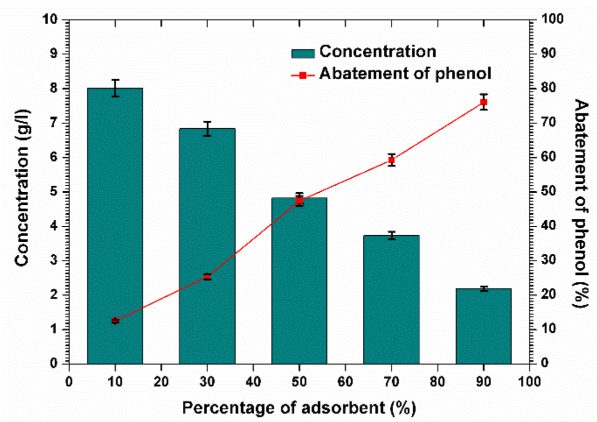


**Fig. 10.** Concentration and abatement of phenol.

### *3.2.4. Abatement of phosphorus*

In OMW, phosphorus is present in both dissolved and particulate forms. Phosphorus can usually be removed from municipal OMW by the enhanced adsorption phosphorus removal method [41]. The maximum phosphorus retention rate of the analyzed OMW bentonite reached 94.83%. The concentration decreases from 1.15 to 0.06 $g.L^{-1}$ (fig. 11).

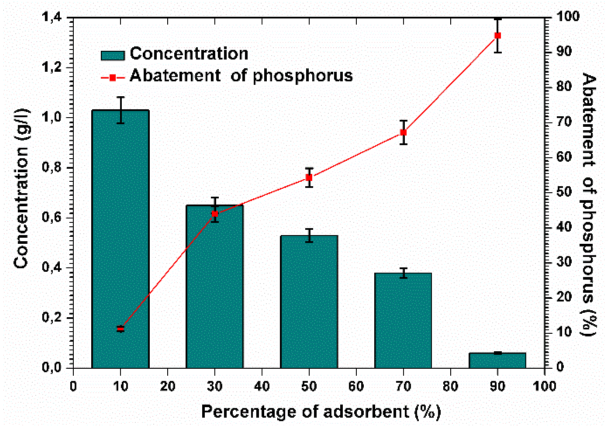


**Fig. 11.** Concentration and abatement of phosphorus.

### *3.2.5. Abatement of major elements*

The evolution of the reduction of the element major K and Na are presented successively in figure 12 and 13. The concentration of potassium (K) diminishes from 6.14 to 4.64 $g.L^{-1}$ (fig. 12), including abatement rates ranging from 1 to 26%. Concerning the concentrations of sodium (Na) ranging from 0.93 $g.L^{-1}$ to 0.39 $g.L^{-1}$, corresponds to the removal rate of Na increased from 10.58 to 62.5% (fig. 13). Our bentonite is characterized by the strong plasticity, whose mineralogical composition indicates significant contents of montmorillonite, although the low content of K and Na (Table 2). These data may explain the considerable rate of K and Na removal.

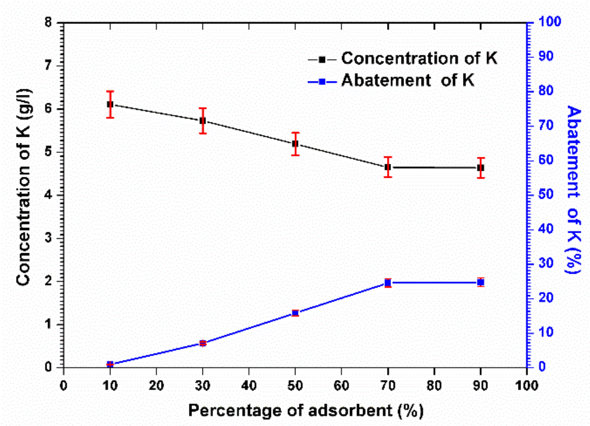


**Fig. 12.** Concentration and reduction of potassium.

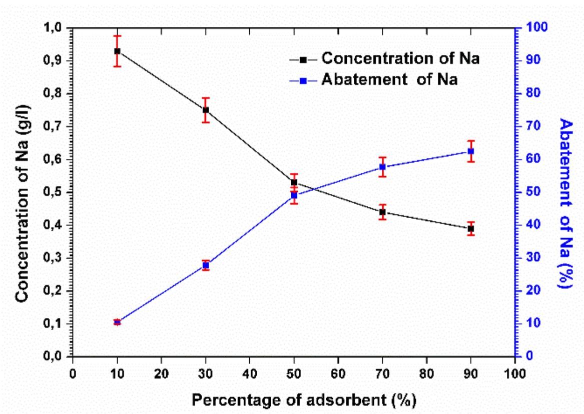


**Fig. 13.** Concentration and reduction of sodium.

### *3.2.6. Abatement of heavy metals*

Concentration and reduction of heavy metals in margin by bentonite are shown in the following figures 14 and 15. The iron concentrations were lowered from 147.86 to 55.93 $g.L^{-1}$. Of which the optimal reduction of iron was of the order of 63.87% (fig.14). Nonetheless, the concentration of lead recorded has significantly decreased from 1.11 to 0.01 $g.L^{-1}$, corresponds to elimination rates ranging from 78.98 to 99.81% (fig.15). So, can be explained by the decreases of the iron, which is due to the precipitation of metal ions in the form of hydroxide compounds [42].

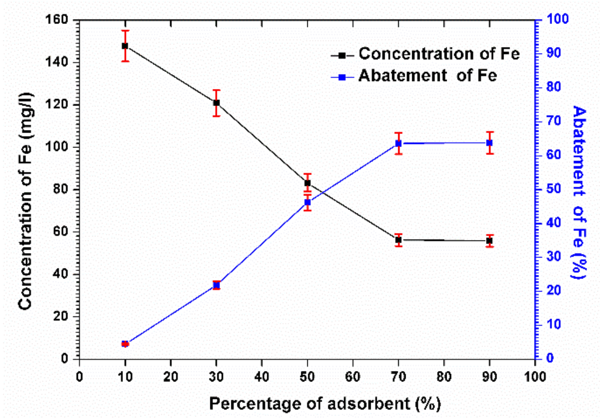


**Fig. 14.** Concentration and reduction of iron.

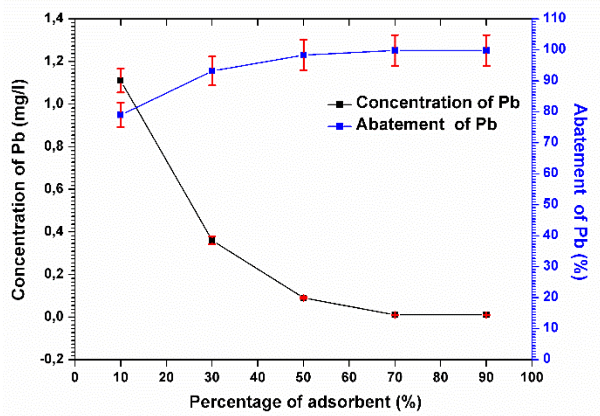


**Fig. 15.** Concentration and reduction of lead.

## 4. Conclusion

In this study, we studied the effectiveness of the treatment of heavy metals and microelements by bentonite. The results obtained show that the natural clay was a potential and promising adsorbent for the removing heavy metals and microelements by bentonite from an aqueous solution. Yet, natural clay is an inexpensive adsorbent that can remove up to 99.81% of lead and 63.87% of iron for bentonite mass loadings of about 80%. This research allows to optimize the adsorption conditions but requires understanding the adsorption kinetics is necessary for the adsorption performance of bentonites.

### Nomenclature

**CH :** Inorganic clays of high plasticity.
**CL :** Mineral clays with low to medium plasticity.
**COD :** Chemical Oxygen Demand.
**Cu :** Uniformity coefficient.
**LL :** Liquidity limit.
**ML :** Inorganic silts and very fine sands with very low plasticity.

**OH/MH :** Organic silts and clays of medium plasticity.
**OL :** Organic silts and silt–clay mixtures of low plasticity.
**OMW :** Olive Mill Wastewater (margin).
**PI :** Plasticity index.
**PL :** Plasticity limit.
**TKN :** Total Nitrogen Kjeldahl.
**Ws :** Shrinkage limit.

**Compliance with ethical standards**

**Conflicts of Interest**

The authors declares that there are no conflicts of interest regarding the publication of this manuscript.

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# Bio-regulators: Silicon, Salicylic Acid, Ascorbic Acid Improve Salt Tolerance in Cucumber (*Cucumis sativus* L.)

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**Abstract:** Soil salinity is currently considered as a major environmental problem. This issue is accentuated by climate change, especially in arid and semi-arid regions. In these areas, drought and high level of salt in soils and irrigated waters are the main abiotic factors limiting plant growth and productivity. On the other hand, rapid population growth and reduction of arable land are major factors that could affect food security. Regarding salt tolerance, most crop plants show sensitivity to high salt levels, but it differs strongly between species and slightly between cultivars within the same species. Cucumber (*Cucumis sativus* L.) is an important economic crop and is sensitive to salinity. To enhance cucumber performance and resistance to high levels of NaCl, different strategies can be employed, In recent decades, an increased research interest has been devoted to develop new strategies to overcome the deleterious effects of salinity on cucumber plants by using molecular markers and genetic transformation as tools to generate salinity-tolerant genotypes together with the implementation of some cultural techniques. Among the other strategies currently being used is the exogenous application of biostimulants and bioregulators. In this regard, and in order to improve salt tolerance of cucumber plants, we have discussed here the effect of foliar application of bio-regulators such as silicon, salicylic acid, and ascorbic acid on plant metabolism and yield.

**Keywords:** Salinity, Cucumber, Silicone, Salicylic Acid, Ascorbic Acid

## 1. Introduction

Salinity is one of the main abiotic stresses in plant agriculture worldwide. It affects a large part of the world's land surface and is a major threat to agricultural production. Most plants are constantly subjected to a range of environmental stresses, such as salt, drought, and cold [1]. In this regard, almost 20 percent of the cultivated area and about half of the irrigated land in the world are affected by salinity. High soil salinity reduces plant growth and thus global food production [2]. It is also one of the most serious constraints affecting agricultural production [3]. Under conditions of salt stress, excessive concentrations of $Na^+$ decrease essential nutrients such as $K^+$ and $Ca^{2+}$ in plants [4, 5]. In addition, certain high levels of salinity can cause some ionic and osmotic disorders which will lead to a decrease in turgidity, membrane damage, and inhibition of water and essential ion absorption, metabolic disorders are manifested by altered levels of growth regulators, enzymatic inhibition, and also metabolic dysfunction, including photosynthesis which will eventually result in plant death [6, 7].

Salt stress is characterized, in most cases, by two stresses mainly at the plant tissue level: one is osmotic stress caused by relatively high concentrations of soil solutes that disrupt the homeostasis of water potential, and the other is ion- specific stress resulting from excessive accumulation of $Na^+$ and $Cl^-$ and altered ratios $Na^+/Na^+$ [8, 9].

One of the most effective ways to overcome salinity problems is the introduction of salt-tolerant crops. At present, selection for salinity tolerance of crops has generally been limited by the lack of reliable breeding characteristics. Multiple genes appear to act in concert to increase salinity tolerance, and some proteins involved in protection against salinity stress have also been recognized. However, the development of methods and strategies to improve the deleterious effects of salt stress on plants has received considerable attention in recent years. In this respect, and in this chapter we will mention some products (bio-regulators) with applications commonly used in agriculture to improve salt tolerance in cucumbers (*Cucumis sativus* L), namely silicon, salicylic acid, and ascorbic acid.

### *1.1. Origin and Classification*

The cucumber (*Cucumis sativus* L.), belongs to the family Cucurbitaceae, and comprises 30 species divided into two groups according to the number of chromosomes [10]. It's considered to be native to Asia, as the wild cucumber exists in India and a closely related species is found in the eastern Himalayas [11]. Cucumber (*Cucumis sativus* L.) is one of the oldest cultivated vegetables that has been known historically for more than five thousand years and probably originated in India. From India, the plant seems to have spread eastwards into China and westwards into Asia Minor, North Africa, and Southern Europe long before written history. Cucumber is a thermophilic and frost-sensitive plant species, which grows best at temperatures above 20°C; as a result, it is cultivated in almost all countries with temperate climates.

### *1.2. Nutritional Value of the Cucumber*

The table below 1 shows main energy and mineral values in the cucumber fruit. In 100g of edible portion of cucumber you will find a significant amount of the following elements, Vitamin A (45 IU), Vitamin C (12mg), Magnesium (15mg), and Phosphorus (24mg).

***Table 1.*** *Nutritional value in cucumber [12].*

| Edible portion (per 100g) | |
|---|---|
| Water | 96 g |
| Protein | 0,6 g |
| Fat | 0,1 g |
| Carbohydrates | 2,2 g |
| Vitamin A | 45 UI |
| Vitamin Bl | 0,03 mg |
| Vitamin B2 | 0,02 mg |
| Vitamin C | 12 mg |
| Niacin | 0,3 mg |
| Valcium | 12 mg |
| Iron | 0,3 mg |
| Magnesium | 15 mg |
| Phosphorus | 24 mg |

# 2. Improvement Salt Stress Tolerance in Cucumber (*Cucumis sativis* L) by Using Bio-regulators Described Below

### *2.1. Definition*

Silicon (Si) is a quantitatively important inorganic constituent of higher plants, but it is almost absent from the main scientific publications on higher plants. (Si) is the second most common element in the soil after oxygen and is a beneficial element for plants, especially under adverse environmental conditions [13]. The earth's crust is made up of 27.61170 of the Silicon, which comes in a wide variety of forms and stabilities. In a simplistic model, Si is found at each intermediate stage in a gradual transition between molecular solubility (silicic acid), homogeneously dispersed colloids (hydrosols), non-rigid gels (hydrogels), and rigid gels (xerogels) [14]. The boundaries of this continuum are not clear-cut, and the entities are only arbitrarily definable. The application of silicon has been shown to be a promising method for reducing the adverse effects of salt stress on plants [4].

### *2.2. Silicon Improves Salt Stress in Cucumber (Cucumis sativis L)*

Silicon considers being one of the most important and also most possible mechanisms that reduce the activity of toxic metal ions in the environment. The concentration of Si in soil solutions varies in ~~the~~ range of 0.1-0.6 mM, and changes little over the whole range of physiological pH values. When culture solutions with much higher concentrations are used, polymerization and precipitation are of concern [15].

### *2.3. Improving Cucumber (Cucumis sativus L) Under Salt Stress Using Silicon*

To face this problem mentioned in the introduction, there will be some strategies to improve and adapt the salt stress of plants, among them, the application of exogenous substances can be a promising solution that could save on the one hand time and on the other hand the necessary labor force.

So, the key to this problem will be presented in the application of silicon, the second most common element in the soil [13]. It can be accumulating in different ways, also in different varieties of plant species. Plants can be classified as high, intermediate and non-accumulators according to their modes of silicon uptake (active, passive, and rejector [3].

Silicon has been shown and also considered beneficial in lowering some of the risks of environmental stresses, including biotic stresses such as plant diseases and pest damage [16, 17], also abiotic stresses such as salinity, drought, frost, and finally heavy metal toxicity [18, 19].

Silicon plays an important role in mitigating the damage caused by salt stress, which has been widely published in various crops, namely, wheat, sorghum, barley and soybean. However, his application can be an alternative approach to increase agricultural production in saline soils.

In this chapter, we have focused our study on cucumber (*Cucumis sativus* L.), which belongs to the most important vegetable group and also the most sensitive to salt stress. Furthermore, cucumbers are characterized by low silicon accumulation due to a lower transport density [20]. According to [21], cucumber is among the horticultural crops important that may be more sensitive to salinity As a result, [21], showed that bio-regulator silicon has a considerable role to play in mitigating the rate of salt toxicity in cucumber plants by increasing the activity of key antioxidant enzymes and thereby decreasing oxidative membrane damage. In addition, earlier work by [21] also suggested that the silicon-mediated salt tolerance of cucumbers has been attributed to reduced oxidative stress. Nevertheless, has not yet cleared whether this is an action of silicon or simply a consequence of stress reduction. In this respect [22], suggest that silicon improves the salt tolerance of cucumber plants (*Cucumis sativus* L.), mainly by improving water absorption, especially in the root path, and the positive regulation of aquaporin gene expression may partly contribute to increased water absorption. Silicon application reduced both $Na^+$ and $Cl^-$ accumulations in the roots under the effect of salt stress.

In addition, silicon will regulate the osmotic adjustment in which plants respond to saline stress by osmotic adjustment, typically by increasing concentrations of solutes such as proline and soluble sugars to adjust the osmotic potential for better water absorption [23]. As a result of the osmotic adjustment, cells can recover their volume and cell turgidity in a few hours. However, the rate of cell elongation is very low, resulting in a change in cell size [24-27]. This whole procedure necessarily depends on a phenomenon that depends on the genotype's mechanism of absorption of water by plants thanks to silicon under salt stress. [28], reported that the application of Silicon has an important effect on the decrease of transport of ions from $Na^+$ and in parallel improves the ratio $K^+/Na^+$ in salt-stressed wheat. However, the decrease in accumulation of $Na^+$ due to silicon is not always the case [29, 3], suggesting the involvement of other mechanisms. Recently, a number of studies have been carried out on the attenuating effect of silicon on salinity induced osmotic stress and found that the addition of silicon improved the water status of the plant, as in tomatoes [29], maize, sorghum, grass, and cucumber.

# 3. Salicylic Acid Improves Salt Stress in Cucumbers (*Cucumis sativis* L)

### *3.1. Definition*

Salicylic acid belongs to an extraordinarily diverse group of plant phenols generally defined as substances with an aromatic ring bearing a hydroxyl or its functional derivative. The first author who introduced and gave a general presentation for salicylic acid was Raffaele Piria in 1838 [30], who said that the name salicylic acid (SA), coming from the Latin word Salix, a willow tree, was given to this active ingredient. During the 19th century SA and other salicylates, mainly methyl esters and glucosides which are easily convertible to SA, were isolated from various plants, including spirea and wintergreen. The first commercial production of synthetic SA started in Germany in 1874. Aspirin, the trade name for acetylsalicylic acid, was introduced by the Bayer company in 1898 and quickly became one of the world's best-selling drugs. Aspirin was as effective as SA and caused much less irritation to the human digestive system.

Salicylic acid free and very soluble in polar organic solvents and moderately soluble in water, it is a crystalline powder that melts at 157-l59°C. The pH of a saturated aqueous solution of SA is 2.4. SA fluoresces at 412 nm when excited at 301 nm, and this property can be used to detect this compound in a number of facility systems [31]. The presence of SA in plants, long suggested, has been confirmed by several investigators using modern analytical techniques [31-34]. An exhaustive study of SA in the leaves and reproductive structures of 34 agronomically important species confirmed the ubiquity of this compound in plants. Rice, crabgrass, green foxtail, barley, and soybean had SA levels above 1 µg/g fresh weight.

### *3.2. Pathway and Biosynthesis of Salicylic Acid (SA) in Plant*

Salicylic acid (SA) is an important signal molecule in plants, two routes of SA biosynthesis have been proposed in plants. The most important mechanism for the formation of benzoic acids in plants is the degradation of the side chain of cinnamic acids [35], which are important intermediates in the shikimic acid pathway. Therefore, a priori, SA (ortho-hydroxybenzoic acid) could be considered as a derivative of cinnamic acid. The conversion of cinnamic acid to sulphuric acid will probably be done by one of the two routes described in the figure below (Figure 1).

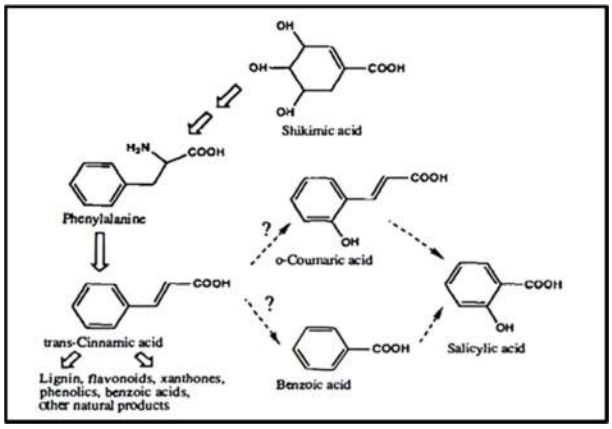


***Figure 1.*** *Proposed pathway for the biosynthesis of salicylic acid in plants [36].*

### *3.3. Salicylic Acid Improves the Quality of Cucumbers Under salt Stress*

The cucumber (*Cucumis sativus* L.) is one of the most important horticultural crops in the world. In addition, it is one of the main commercial agricultural crops in many countries, but it is very sensitive to salt, water, and temperature stresses. It is considered to be a market garden crop that is cultivated worldwide. Cucumber growth and yield are reduced by salinity stress [37]. The demand for food is a major global problem worldwide. Soil salinity, drought, soil erosion and other climate changes are reducing food production in arid and semi-arid areas.

Salinity is one of the main obstacles to declining agricultural productivity, and the production of salt-tolerant crops has been given high priority in sustainable agricultural research [38]. It reduces plant growth and yield and interferes with many biochemical processes such as photosynthesis, respiration, synthesis of phytohormones, homeostasis of reactive oxygen species (ROS), and antioxidant systems. In addition, the water deficit is becoming a serious and significant problem in many countries of the world. Cucumber is one of the most important horticultural crops and cucumber and other cucurbits have a root system and high sensitivity to water deficit stress. Cucumber and other Cucurbits have a high transpiration rate and sensitivity to water deficit stress [39]. It is well known that drought stress increases the production of oxygen species (ROS) in different cell organelles. However, salinity stress produces reactive oxygen species (ROS) in plant cells, which can be damaged if the ROS are not rapidly produced. SOD, POD, and CAT in the detoxification of ROS by catalyzing the exchange of free $O_2^-$ to $O_2$ and $H_2O^2$. Reactive oxygen species are more numerous in plants exposed to saline stress than controls. ROS includes both radical (superoxide radical, hydroxyl radical and alkoxy radical), and non-radical (hydrogen peroxide and singlet oxygen) forms, which damage chlorophyll, proteins, DNA, lipids, and other macromolecules. Several reports have suggested that changes in unsaturated fatty acid content can improve plant tolerance to salinity [40]. Thus, these ROS negatively affect different biological and physiological processes and death will occur if stress is prolonged [41, 42]. Ascorbic acid (AsA) is ~~a~~ low molecular weight but powerful antioxidant. In this respect, various studies have shown that salinity increases the ROS trapping enzymes in cucumber. [43], were able to prove that AsA can be considered appropriate and affordable plant bio-regulators to overcome the negative and destructive effects of water stress on the cucumber plant. Exogenously applied SA and Kin induced TOD and CAT activity in saline soil conditions has been reported previously for wheat, mung bean [4] and tomato. POD is one of the main antioxidant enzymes that contribute to protection against salt-induced oxidation and stress in cucumber plants.

In addition, AsA participates in a wide range of plant processes, namely photosynthesis and cell expansion, and also increases plant resistance to various abiotic stresses. Among the different bio-regulators already tested on various plant species, gamma aminobutyric acid (GABA) has emerged as a promising multi-protective agent with the capacity to rapidly increase plant tolerance to various abiotic stresses such as water accumulation, high temperatures, heat, shocks, salt and water deficit stresses. In plants, GABA is involved in a multitude of physiological functions such as growth regulation and tolerance to various abiotics stresses [44].

Research by these groups of researchers [4, 45] shows that the stimulation of salt tolerance through seed pre-treatment and foliar application of salicylic acid and kinetin have been well reported for many crop species. However, the mechanism of improvement due to seed pre-treatment with salicylic acid and kinetin is not yet fully understood. In addition, salinity stress can alter the hormonal balance in plants, so hormonal homeostasis is a possible mechanism for the salinisation of cytokinin and salicylic acid [4]. The site of salicylic acid application could contribute to a mechanism for the regulation of physiological processes, such as stoma closure, ion transport and absorption, membrane permeability and photosynthesis [4]. Concerning cytokinin is involved in some growth strategies and thus physiological processes such as cell division, apical dominance, lateral root initiation, stomatics and vascular behavior and development under abiotic stress.

Another study carried out by a few researchers showed that the experimental pre-treatment of seeds with salicylic acid improved the salinity tolerance of cucumber genotypes. The beneficial effects of seed pre-treatment with SA on plant biomass and physiological responses have been reported in a number of species grown under abiotic stresses [39]. SA has enhanced the defense mechanism against various in plant species [39]. SA also plays an important role in growth and development [4, 5].

# 4. Improvement Salt Tolerance in Cucumbers (*Cucumis sativis* L) by Ascorbic acid Foliar Application

### *4.1. Definition*

Ascorbic acid (AsA), as an antioxidant, is defined as a small water-soluble molecule that acts as a primary substrate in the cyclic detoxification and neutralization of superoxide radicals and singlet oxygen. In addition, it is very effective in improving plant biomass [46].

### *4.2. Ascorbic Acid Improves Salt Stress in Cucumber (Cucumis sativis L)*

Water deficit is becoming a serious and significant problem in many countries of the world [42]. Cucumber is one of the most important horticultural crops, cucumbers and other cucurbits have a fibrous root system and are very sensitive to water stress [39].

Salt stress can affect plant growth and development directly through its potential toxic effects, and indirectly through its osmotic effects [47]. Recently, several studies have reported that salinity also causes increased production of reactive oxygen species, which may themselves lead to secondary signals. ROS are highly reactive in the absence of any protective mechanism. They can severely disrupt normal metabolism by oxidative damage to essential membrane lipids, proteins and pigments [48]. To recover ROS, [49] showed that plants synthesize different types of defense systems composed of non-enzymatic antioxidants such as ascorbic acid and enzymatic antioxidants such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (AP) and glutathione reductase (GR). Scavenging system has the potential to extinguish ROS in stress-tolerant plants. Osmotic adjustment is the cellular response to reduced turgidity. The cytosolic and organellar machinery of glycophytes and halophytes is equally sensitive to $Na^+$ and $Cl^-$; osmotic adjustment is therefore achieved in these compartments by the accumulation of compatible osmolytes and osmo-protectors [50].

However, $Na^+$ and $Cl^-$ are energy efficient osmolytes for osmotic adjustment and are compartmentalized in the vacuole to minimize cytotoxicity. Many oxidants, such as the hydroxyl radical, contain an unpaired electron and are therefore highly reactive and harmful to plants at the molecular level. Ascorbate plays a central role in photosynthesis, as is implied by its high concentration in chloroplasts. To deal with this problem, ascorbic acid (AsA) is involved in the regulation of many critical biological processes such as photo-inhibition and cell elongation ([51]. Ascorbic acid is also involved in the cell cycle and many other important enzymatic reactions (e.g. ethylene biosynthesis). In this chapter we have been able to prove that AsA can be considered as suitable and affordable plant bio-regulators to overcome the negative and destructive effects of salt and water stress on the cucumber plant.

According to the data and studies provided by Liang et al. [52], growing cucumbers under water stress conditions had a significant negative effect on the physiological condition of the plant and also resulted in a decrease in the quality and quantity characteristics of the cucumber plant. Secondly, water stress can be an equally serious trade problem for cucumber growers. Therefore, the use of cheap, available and affordable plant-based bio-regulators can be considered as a better and appropriate solution to this problem. But different crops may have different responses to the use of different plant bio-regulators in their cultivation process [52]. Thus, another study conducted by [43], demonstrated that foliar application of AsA can be considered among the main bio-regulators to mitigate the effects of and physiological responses to drought stress damage and effects on morphological, physiological, and biochemical properties.

# 5. Conclusion

Salinity is becoming a major constraint to crop production, and it is becoming severe with climate change; which could have an impact on food security. Currently, bio-regulators such as silicon, salicylic acid, and ascorbic acid play essential roles in abiotic stresses tolerance such as drought and salinity. These three bio-regulators are among relevant and affordable plant bio-regulators to overcome the negative and destructive effects of salt and drought stress in cucumber plant by enhancing the osmolytes the osmolytes accumulation and improving osmotic adjustment. Besides, they are extremely effective at low concentrations; on the other hand, they are very beneficial for the health of the environment through their low use in terms of quantity applied in the field.

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# Assessment of potential contamination of groundwater in abandoned mining region of Ben Taieb, Northeastern Morocco using statistical studies

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***Abstract---***The objective of the study is to raise awareness of the effect of anthropogenic activity on groundwater in the urban area, water samples were collected from 12 wells in the area in April and September 2019. From these samples, pH and conductivity were determined using a pH meter/conductivity meter. From these samples, pH and conductivity were determined using a pH meter/conductivity meter, while heavy metal concentrations (Mn, Fe, Pb, Cu and AL) were determined by atomic analysis and ICP-MS. The study showed that groundwater in the study area is ($0.01mg/l<Mn^{2+}<0.06$ mg/l) and contains ($0.14mg/l<Cu^{2+}<0.03$ mg/l), ($0.02mg/l<Fe^{2+}<0.4$ mg/l), ($0.02mg/l<Zn^{2+}<1.8mg/l$) and ($0.01mg/l<AL^{3+}<0.05mg/l$). These concentrations are above the permissible limits recommended by the World Health Organization (0.05, 0.2, 0.3, 5 and 0.2 mg/l, respectively; $p > 0.05$). Bacteriological analyses show that the vast majority of the wells studied in the Ben Taib area harbour high densities of Faeceal coliform (0 to more than 420 CFU/100ml) and Fecal Streptococci (0 to over 25 CFU/100ml). The presence of these high quantities of pathogens, which are indicators of pollution and bacteriological contamination by wastewater, could be due to anthropic activities.

***Keywords---***groundwater, ICP-MS, landfill, heavy metal contamination.

## Introduction

Groundwater, which is located in permeable geological formations named aquifers, is a valuable renewable resource. In India, groundwater is a precious resource for agriculture and mining, and it is mostly utilized as drinking water (Pande et al. 2020; Sharma, Tripathi, and Chandra 2020). The study of environmental contamination by discharges containing heavy minerals (Baize 1997) have shown that the increase in concentrations of heavy metal contamination is mainly generated by anthropogenic activity. It is true that mining activity(Su 2014), through the processes of ore extraction, milling usually in the open air (dust) and discharge of liquid and solid tailings, necessarily leads to the contamination of the surrounding environment by its tailings, dust and untreated water that is discharged into nature.(El-Fadeli et al. 2015; Falck 2015; Edraki et al. 2014; Candeias et al. 2018; Gueddari Hicham et al. 2021).

This article is the first to investigate the deterioration of water resources caused by the region's lithology, mining tailings, and, in particular, the discharge of wastewater caused mostly by the town of Ben Taib's autonomous sanitation. Microbiological testing was used to determine the influence of wastewater on groundwater quality and public health. Precipitation, which is a transit factor for pollutants from mining waste, is irregular in the study region(Van Lam, Van Hoan, and Duc Nhan 2019). The influx of wastewater from village activities, on the other hand, is constant and might be the primary source of contamination from the abandoned M'hajer mine.

## Material and Methods

### Study area

The Ben Taieb commune is located in the Driouch province's northwest region, at an altitude of 250 to 400 m. Several rivers flow through the area, such as the Oued Aghbal and the Oued Ouchane (Fig.1).The areas are located at the level of quaternary alluvium, within which at least three in which at least three levels of alluvial terraces can be identified: Late Quaternary, Amirian, Tensiftian-Presosoltanian, and Rhabian-Soltanian (Barathon, Delibrias, and Weisrock 1988). In the northern and north-western parts of the areas, there are lenses of calcareous Cretaceous calcareous shales. The north-eastern part of the area is marked by the presence of lenses of red silts dating from the Villafranchian period(Carlier 1973). Finally, to the west and north of the areas, there are scree and slope deposits.The slopes are low overall and moderate to the north of the areas, in contact with the gentle landforms. There does not appear to be a particularly dominant slope process.

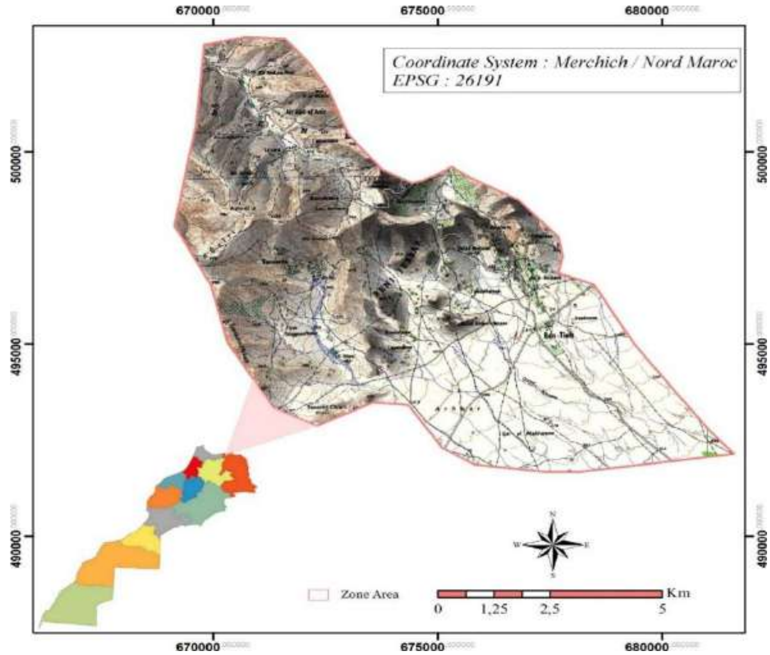


Figure 1. Location of the study area.

**Bacteriological analyses**

Total coliforms, faecal coliforms, and faecal streptococcus were the germs we were looking for in the waters of the eight sites studied. These measures are used to evaluate the quality of drinking water. The membrane filter method was used to search for bacteria in the water examined, and it conformed to "AFNOR" criteria.(Rodier, J, Bernard LEGUBE 2009). All water samples were promptly chilled to 4°C in a portable cooler and transported to the laboratory. The protocols used to conduct the chemical analysis of the main ions are as follows:(Yahya et al. 2017; Zouhri et al. 2019):

- Bacteriological analysis was carried out in the laboratory immediately in the following sample. The results are presented as the number of colonies per unit of volume. The investigation includes the following elementsTotal Coliforms (TC), Fecal Coliforms (FC), and Fecal Streptococci (FS) according to ISO 9308-1, ISO 9308-1 and ISO 7899-2 respectively:
- Enumeration of total coliform bacteria per 100 $cm^3$, using membrane filtration (0.45 µm) and culture on Tergitol TTC 7 Agar 24 h at 37°C ;
- Faecal coliform bacteria count per 100 $cm^3$, using membrane filtration (0.45 µm) and culture on Tergitol Agar TTC 7 Agar 24 h at 44°C;
- Enumeration of faecal streptococcal bacteria per 100 $cm^3$, using membrane filtration (0.45 µm) and culture on Slanetz Bartley Agar 24-48 h at 37°C.

**Physical and chemical analyses**

For each sample, certain measurements were taken in situ to be able to quantitatively determine the parameters that change after sampling; such as temperature (T (°C)), hydrogen potential (pH), electrical conductivity (Cond (µS/cm)), and dissolved oxygen (O2d). We performed these measurements using a MULTI 350I portable multi-parameter meter. -The other parameters were measured in the laboratory according to the analysis methods recommended by(Rodier, J, Bernard LEGUBE 2009):

- The determinations are carried out by Flame Atomic Absorption Spectrometry (Varian Model 475-AA) is a method that essentially allows the determination of metals in solution, we used it as a method of identification and quantification of 4 metals (Zn, Mn, Fe, Cu).
- The determinations are carried out by mass spectrometry with inductively coupled plasma ICP-MS according to the ISO17294-2:2016 standard, used for the determination of trace elements (Hg, AL, Cd, Cr, Br, Ni, Pb).

**Discussion**

**Microbiological quality of analysed waters**

Ben Taieb's aquifers are more contaminated with faecal coliforms and faecal streptococci (Fig.1). The existence of extremely high levels of pathogenic germs, which are indicators of wastewater pollution and microbiological contamination, clearly indicates the negative impact of human activities and insufficient waste management on the quality of groundwater utilized in the region. The measurement of total coliforms provides information on a well's potential sensitivity to surface contamination. Thus, it is possible to hypothesize that the contamination rate is particularly high during wet seasons, but the total coliform rate is high during dry periods. Observations show that a high EC/SF ratio (>4) can be considered as a good indication of man-made pollution(Yahya et al. 2017; Singh 2017; G Hicham et al. 2021).

- Coliforms in total (TC): TC was detected in almost all of the tested wells and springs. Their amounts varied between wells and water springs and were affected by the sample time, whether wet or dry. During the dry period, we found that the rate of TC rose. TC values vary from 475 to 2000 CFU/100 mL in June during the monsoon season of 2019, and from 420 to 1150 CFU/100 mL in November during the dry period of the same year (Fig.A;C-2).
  Faecal coliforms (FC):FC is an indication of faecal pollution and can be found in virtually all wells and springs that have been sampled. Their rates were higher during the dry period, and their amounts were often lower when compared to the TC. During the rainy season, FC levels varied from 0 to 420 CFU/100 mL in June, whereas during the dry season, FC values ranged from 0 to 310 CFU/100 mL in November (Fig.B;C-2).
- Faecal streptococci (FS): FS are indicators of faecal contamination and are present in almost all wells and water springs, but generally with smaller rates than FC. In the wet period, FS values ranged between zero to 25 CFU/100 mL. In the dry period, the FS values varied between zero to 15 CFU/100 mL in November (Fig.D;C-2).

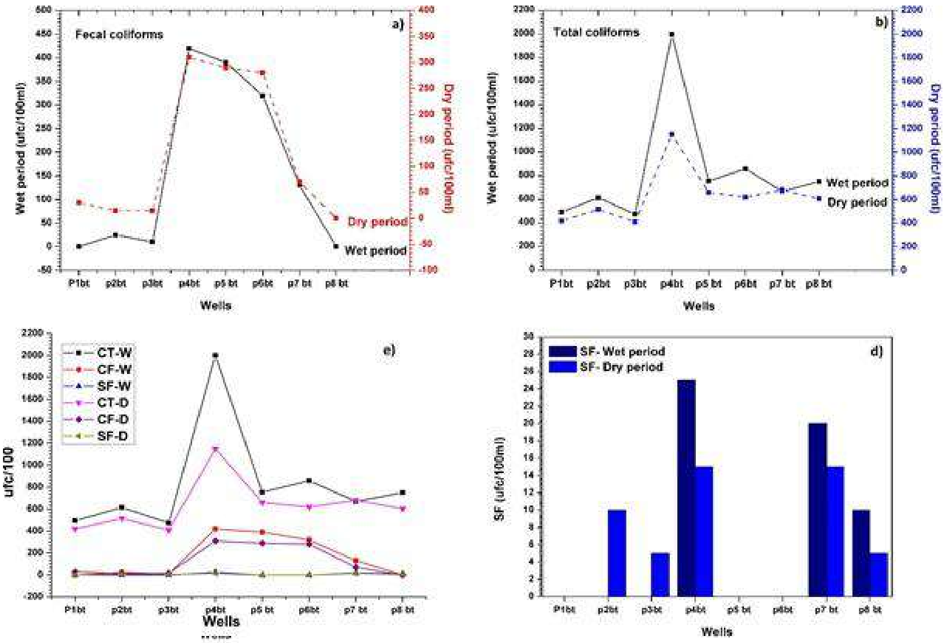


Figure 2.Graphic variation of Bacteria in the Ben Taieb water table (Wet period): A-fecal Coliforms, B-Total Coliforms, C-Comparison of all bacteria, D-Fecal Streptococci

**Physicochemical quality of analysed waters**

The results of the studies of the twelve water samples are given in graphs that illustrate the spatial distribution of the various elements investigated. While cadmium and mercury are nearly absent in all samples, a similar result was found for the Nekor basin (Ghalit et al. 2017), the other elements are present in various amounts (Table.1).

Table 1
Descriptive statistics of metal elements Variable

| Variable (mg/l) | Mean | Minimum | Maximum | WHO Standard (Edition 2011) |
|---|---|---|---|---|
| Lead | 0.0015 | 0.001 | 0.0025 | 0.01 |
| Magnesium | 0.034 | 0.01 | 0.06 | 0.05 |
| Nickel | 0.018 | 0.002 | 0.05 | 0.02 |
| Copper | 0.072 | 0.03 | 0.14 | 0.2 |
| Zinc | 0.4 | 0.02 | 1.8 | 5 |
| Chromium | 0.02 | 0.0005 | 0.054 | 0.05 |
| Iron | 0.146 | 0.02 | 0.4 | 0.3 |
| Aluminum | 0.026 | 0.01 | 0.05 | 0.2 |
| Brome | 0.035 | 0.02 | 0.05 | - |

The research revealed that the various metal concentrations of Zn, Cu, Cd, Pb, Ni, Br, and Al in the abandoned mine wells are within the WHO recommended limits, excluding iron, which has a concentration range of 0.110 - 0.4 mg/l, which exceeds the WHO permissible value of 0.3 mg/l, indicating that all three wells are contaminated (Fig.3). The majority of the samples examined had manganese concentrations below 0.05 mg/L (Fig.3), which corresponds to the WHO standard (Edition 2011). Except for one sampling point, the manganese levels in the wells were around 0.05 mg/L. This is considered above the recommended standards.

Groundwater, which is considered the purest form of drinking water as compared to surface water (Offodile 2014), has a higher concentration of iron than water from the abandoned mine, which would be expected to have higher values due to the mining activity that has occurred in the area(Luzati et al. 2016). This high concentration of iron in groundwater can be attributed to the leaching of common fractures generated by geology and lead-zinc mineralization (Leach et al. 2001; Velasco et al. 2003), with which iron was commonly associated. The leaked metal slowly percolates through the earth's strata, reaching and polluting the groundwater(Zhang et al. 2020).

In addition, iron, which occurs naturally in the earth's crust, is adsorbed. In the earth's crust it is adsorbed to oxide minerals; adsorbed to clay mineral surfaces(Truche et al. 2018); associated with sulphide minerals or organic carbon and these clay minerals are ubiquitous in the study area(D. Liu et al. 2019; Zhang et al. 2020).The mineralization was determined to be an iron oxide (Fe2O3) containing 35% iron in M'hajer (Table.2), a deposit that had been recognized since the Spanish colonization's inception. The tests conducted by the Spanish organization (Usit Color) have resulted in the following Mineralization conclusions: iron oxyde (Hematite and Oligiste)(Knouz et al. 2016). Natural dissolution or desorption of iron from these source materials can introduce iron into groundwaterthe iron and manganese content of groundwater in Ben taib is affected by several major factors, particularly Fe- and Mn-based chemicals and organic matter deposited all through the Holocene(Lenstra et al. 2020; Zawierucha, Kozlowski, and Malina 2016; W. Liu et al. 2021). During the field study, it was discovered that once colourless groundwater is collected from the wells, it will become red or yellow after a brief period of contact with air. This implies that the groundwater is depleted (Carretero and Kruse 2015).A microbially driven redox mechanism mediated by organic matter can result in Fe, Mn mobilisation in groundwater (Neidhardt et al. 2014;Hem 1985). Under these conditions, iron and manganese from soiland aquifers will be discharged into groundwater(Palmucci, Rusi, and Di Curzio 2016).

Table 2
Levels of chemical components of Hematite mineral in M'hajer Mine(Knouz et al. 2016)

| Chemical components | Levels % |
|---|---|
| Fe | 36.46 |
| Zr | 0.0178 |
| Sr | 0.0061 |
| Pb | 0.0393 |

| | |
|---|---|
| As | 0.0086 |
| Zn | 0.0143 |
| W | 0.0072 |
| Co | 0.2838 |
| Mn | 0.0758 |
| Mo | 0.0011 |

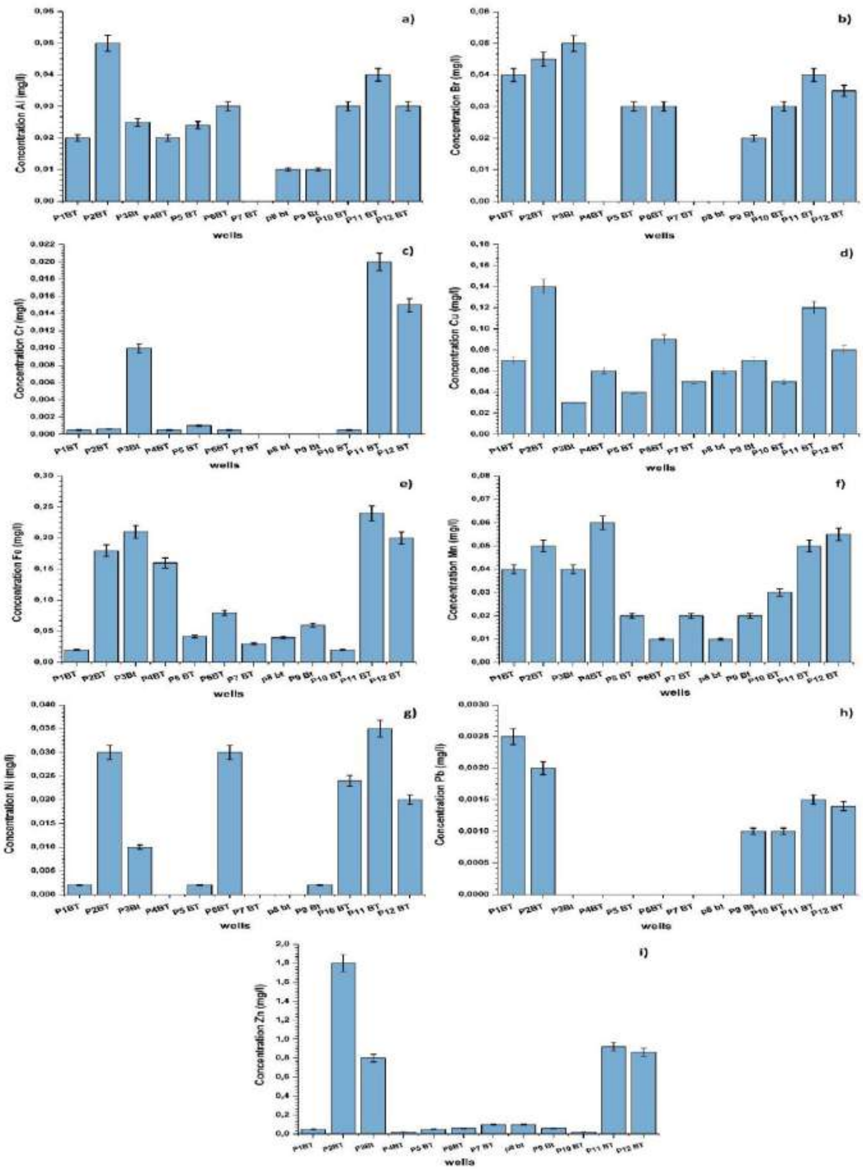


Figure 3.Graphic variation of the distribution of chemical parameters in Ben Taieb aquifer during the high-water period: A-Aluminum, B-Brome, C-Chromium, D-Copper, E-Iron, F-Manganese, G-Nickel, H-Lead, I-Zinc

**Statistical studies**

The physicochemical data are analyzed statistically employing Principal Component Analysis (PCA) on a table of 13 variables (Pb, Cu, Al, Fe, Zn, Cr, Cd, and Ni Electrical conductivity (Cond), PH, O2d, and T) and 42 people (wells and sources). The correlation matrix (table 1) of the various parameters investigated indicated a tight link between TDS and electrical conductivity on one side, and zinc with chromium, copper, and aluminium on the other. The correlation coefficients show this link. The major linkages are most likely due to the elements' shared origins. In our research, we used a statistical technique known as multivariate Principal Component Analysis (PCA). This is a quantitative and impartial approach for classifying groundwatersamples based on their geochemical characteristics.

Table 3
Correlation between variables and factors

| | ZN | CU | CR | PB | AL | FE | Mn | Br | NI |
|---|---|---|---|---|---|---|---|---|---|
| ZN | 1 | 0.68 | 0.48 | -0.2 | 0.3 | 0.74 | 0.56 | 0.59 | 0.59 |
| CU | 0.68 | 1 | 0.27 | [illegible] | 0.42 | 0.45 | 0.35 | 0.33 | 0.7 |
| CR | 0.48 | 0.27 | 1 | -0.17 | 0.63 | 0.77 | 0.51 | 0.45 | 0.48 |
| PB | -0.2 | [illegible] | -0.17 | 1 | [illegible] | -0.33 | 0.11 | 0.23 | -0.25 |
| AL | 0.3 | 0.42 | 0.63 | [illegible] | 1 | 0.51 | 0.43 | 0.59 | 0.8 |
| FE | 0.74 | 0.45 | 0.77 | -0.33 | 0.51 | 1 | 0.73 | 0.44 | 0.5 |
| Mn | 0.56 | 0.35 | 0.51 | 0.11 | 0.43 | 0.73 | 1 | 0.34 | 0.26 |
| Br | 0.59 | 0.33 | 0.45 | 0.23 | 0.59 | 0.44 | 0.34 | 1 | 0.59 |
| NI | 0.59 | 0.7 | 0.48 | -0.25 | 0.8 | 0.5 | 0.26 | 0.59 | 1 |

The factorial plane (1x2) projection of the variables reveals that 51.86 per cent of the total variance is represented (Fig.4). T, Electrical conductivity Pb, Cu, Al, Fe, Zn, Cr, Cd, and Ni are grouped in the positive pole while PH is grouped in the negative pole on the factor 1 axis, which accounts for 51.86 per cent of the total variation (Table 3, Fig.4). As a result, the factor 1 axis may be thought of as an axis that characterizes groundwater mineralization in the research region. The analysis of individual projections on the factorial plane F1 reveals that the measurement points may be split into two groups:

- Group 1: Factor I accounts for 12.8 per cent of the variation and is governed by Mn, Fe, Cr, Zn. linked to minerals ($FeCr_2O_4$,$PbCrO_4$, $FeS_2$ and $Fe_2O_3$). These locations are on the positive side of axis 1.
- Group 2 comprises of the three individuals: - Factor II is responsible for 51.86 per cent of the variance; it has been shown that the most mineralized sites are AL, Cu, Br, and Ni linked to minerals ($Al_2MgO_4$ and $Al_2O_3$). Besides that, correlation explains pollution indices for agricultural and domestic activities. These locations are on the axis's negative side.

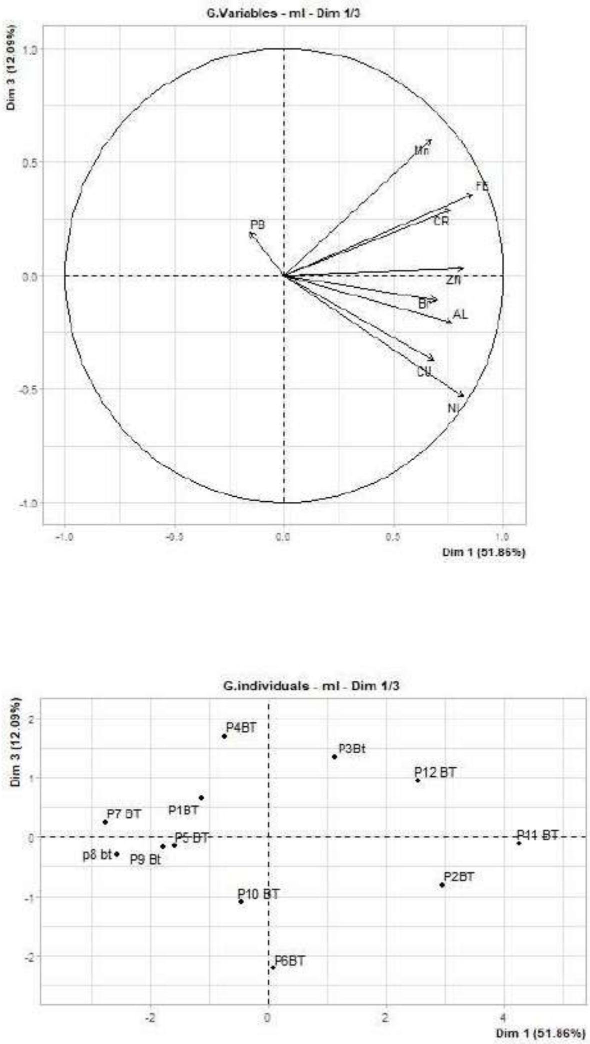


Figure 4. Main components analysis circle

## Conclusion

The results of the physicochemical and bacteriological tests led us to the assumption that the contamination of the well water analyzed is widespread. In reality, the water under investigation contains characteristics that make it unfit for human consumption. This is mostly due to the following factors: This is mostly related to electrical conductivity, hardness, nitrates, nitrites, ammonium, trace metals (Fe, Mn, and Al), total germs, and total coliforms. Animal faeces and livestock effluents are the sources of pollution at the microbiological level. In terms of faecal coliforms and faecal streptococci, it appears that contamination is consistent.It is thus strongly advised that polluted wells be cleaned as soon as possible to protect groundwater, particularly at depth, from probable contamination spread. When compared to the literature measuring groundwater quality on a national and continental scale, it was discovered that the water from the wells in the Ben Taib region did not significantly surpass Moroccan or worldwide criteria. Certain precautions must, however, be taken to restrict the sources of contamination. In the future, not only will it be required to analyze the health concerns connected with the amount of pollution in these waters.

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# Support Vector Machine: A Case Study in the Kert Aquifer for Predicting the Water Quality Index in Mediterranean Zone, Drouich Province, Oriental Region, Morocco

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## ABSTRACT

The expansion of urbanization and the amplification of anthropic activities in the Rif region require the establishment of wells. However, the irrational exploitation of water and natural conditions have generated the rise of the water table and the increase in pollution. Thus, the assessment of water quality has emerged as a significant concern. This study's goal is to assess the adequacy of groundwater quality in two aquifers in the vicinity of the Mediterranean Zone - Drouich Province and Oriental Region, Morocco, for drinking water needs by taking 62 water samples of the Kert aquifer for 2019. The Water Quality Index (WQI) classifies water quality: as excellent, good, poor, very poor, etc. That is essential for conveying information about water quality to people and decision-makers in the affected area. The WQI in the Kert aquifer varies from 62.3 to 392.3. The calculation of the water quality index (WQI) of the Kert aquifer view is based that 45.16% of groundwater samples are of poor quality, making them acceptable for drinking. The study's analysis is established with a geographic information system (GIS) setting. The index map provides decision-makers with a complete and interpretable picture for better water resource planning and management. SVM models are shown to account for 87.71% of the varying water quality score. Different statistical and intelligence models may make the index more predictable. These forecasts assist us in better managing the aquifer's water quality.

## INTRODUCTION

Public health issues involving the chemical contamination of groundwater must be addressed immediately. The availability of water for human use is a critical global and regional concern (Azizullah et al. 2011, El Yousfi et al. 2022, Schweitzer & Noblet 2018, Vesali Naseh et al. 2018). Water Quality Indices (WQIs) are an easy-to-understand tool that managers and decision-makers can use to evaluate a specific water body's quality and potential applications (Kumari & Sharma 2019, Rawat & Singh 2018). WQI is a mathematical technique for integrating detailed water quality data into a numerical score describing the general view of the water's quality (Gueddari et al. 2022, Mukate et al. 2019, Ponsadailakshmi et al. 2018, Singh et al. 2019) as a whole. In essence, the WQI aims to offer a system for presenting a cumulative score and an expression numerically describing a certain level of water quality (Deshpande et al. 2014, Tziritis et al. 2014). Horton's work led to the development of the first WQI in the US, which has been used in Europe since the 1970s, initially in the UK. Later, the United States National Sanitation Foundation refined this concept (Banda & Kumarasamy 2020, de Andrade Costa et al. 2020,

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Kachroud et al. 2019). As a result, the WQI principle has been the subject of extensive effort since then, using concepts that have been slightly modified (Brilli et al. 2013, Eden & Ackermann 2001, Von Zur Gathen & Gerhard 2013).

Prior research in this field has concentrated on groundwater quality (the source of salinity, the aquifer's geochemistry, and investigations of heavy metals). (Elgettafi et al. 2013, Hicham et al. 2021a, 2021b). This publication predicts the Water Quality Index in the unconfined Kert aquifer using a Support Vector Machine.

Water is required for the life of all organisms. As such, it is an irreplaceable resource (Butler 2017, Malekzadeh et al. 2019, Toolabi et al. 2021) We found that the WQI can be predicted using physical values and no sub-index computation of the input for physical and chemical parameters in this study.

## MATERIALS AND METHODS

### Study Area

North-eastern Morocco is home to the Kert Basin, which covers a space of around 250 square kilometers are shown in Fig. 1. The western Gareb chain forms its eastern boundary. The Kert basin's most important river, the seasonal Temsamane, flows around the massive Temsamane metamorphic mountain, which surrounds the plain to the north and northwest. The river's overall length is 90 km and its catchment area is 2710 sq. km (Zielhofer et al. 2008). According to some studies on the current regular fault system, Kert Basin was formed during the Messinian Paleocene. (Azdimousa 2007). Pump testing and electro-geophysical investigations were used to develop this model. The Kert plain is encircled by an open aquifer that flows through the Miocene Bluish Marls (bedrock aquifer). Numerous hydrogeological formations exist, and their stratification is visible in Fig. 2. Transgressed Miocene marls are overlain by an identical layer of limestone and conglomerate that serves as the aquifer's bedrock. The only other formation in the region, we think, is the Miocene, which contains gypsum. This sequence is completed with villafranchian gravel, silt, and clay. Two Miocene vertical faults created the Kert basin. As a result, the northern and southern bounds of the plain were extended. These faults may connect the aquifer to the Jurassic unit below

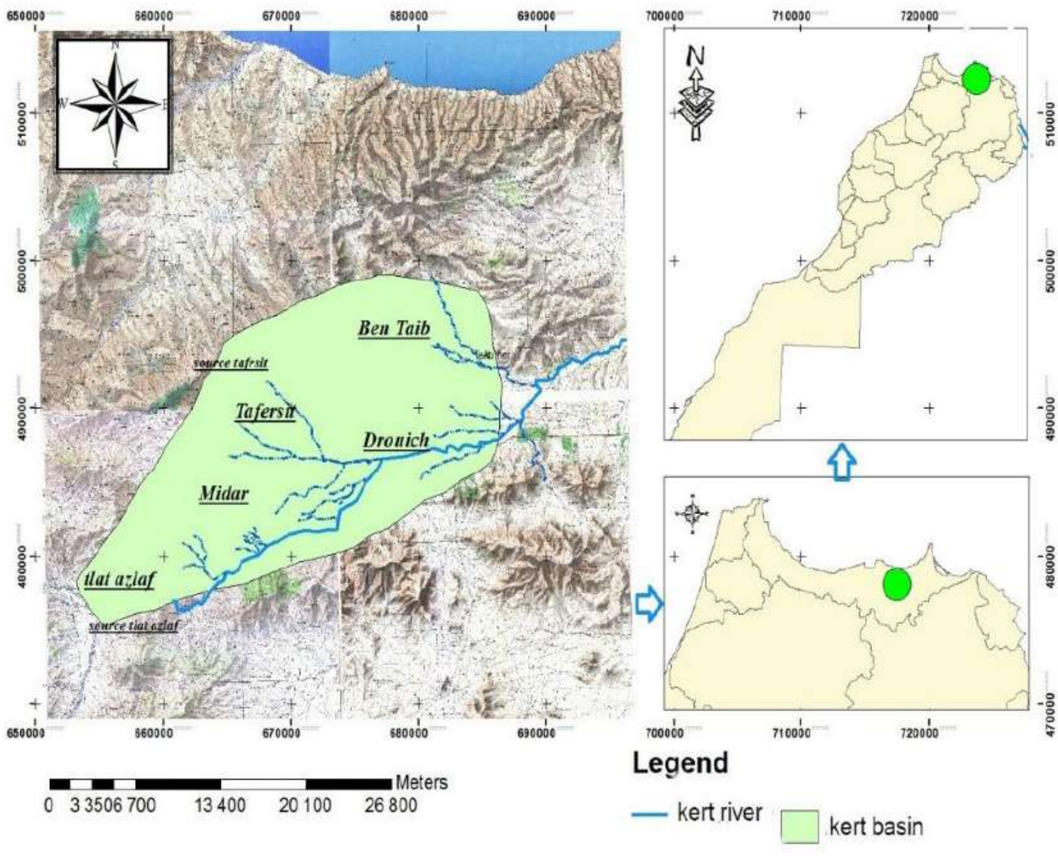


Fig. 1: The Kert basin's placement.

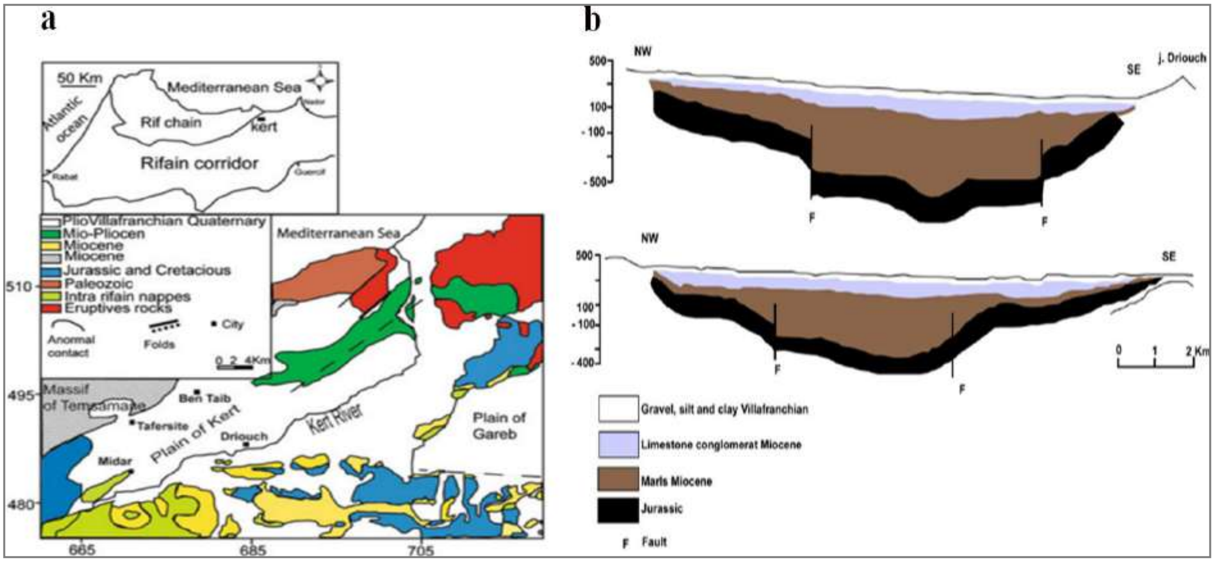


Fig. 2. a - The Kert Plain's base map and b - Hydrogeological section of the kert aquifer system (Carlier 1973).

(Carlier 1973). With an annual rainfall of just 276.9 mm, the area is semi-arid. Temperatures range from 10°C to 25°C annually. The Moulouya Hydraulic Basin Agency pegs the average annual evapotranspiration as 291.6 mm.year$^{-1}$.

## Field Sampling

During the low water season of 2019, sixty wells and two springs are tested from the Kert groundwater. The groundwater samples are subjected to a physicochemical evaluation (Rodier et al. 2009), and a physico-chemical analytical procedure is used. Coolers were used to retain the models at 4$^{o}$Celsius right away. The ISO 5665 standard is used to collect the samples. The pieces were kept cool (2 to 4°C) in labeled plastic bottles to examine chemical parameters. EC, pH, main elements ($Cl^-$, $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Na^+$, $SO_4^{2-}$, $HCO_3^-$, $NO_3^-$, and $PO_4^{3-}$) were investigated.

## Data Processing

Physicochemical data is evaluated using Principal Component Analysis-based multivariate statistical methodologies (PCA). The statistical method is often used in water research to investigate water mineralization and pooling and determine the relationship between WQI and salinity phenomena. The statistical analysis was carried out using the R program. A comparison is made between the measured factors and the quality of drinking water requirements set by the WHO. Thematic maps employing Geographic Information Systems are used to display the results (GIS).

The water quality index (WQI) was developed to quantify the influence of human and natural activities on groundwater chemistry. While assigning weights for the WQI computation, the relative relevance of the physicochemical parameters in determining the overall quality of water for drinking water applications is evaluated. Between one and five, the weight is given. TDS, pH, EC, $SO_4$, nitrate, and

Table 1: Physicochemical parameters relative weight Parameters (WHO Edition 2011).

| Chemical variables | WHO standards (Edition 2011) | Weight (wi) | Relative weight $Wi = wi / \Sigma^n_{i=1} wi$ |
|---|---|---|---|
| pH (on the scale) | 6.5-8.5 | 4 | 0.114 |
| EC [mS.cm$^{-1}$] | 250 | 4 | 0.114 |
| TDS [mg.L$^{-1}$] | 500 | 5 | 0.142 |
| $HCO_3^-$ [mg.L$^{-1}$] | 500 | 3 | 0.086 |
| $Cl^-$ [mg.L$^{--1}$] | 600 | 3 | 0.086 |
| $SO_4^{2-}$ [mg.L$^{-1}$] | 400 | 4 | 0.114 |
| $NO_3^-$ [mg.L$^{-1}$] | 50 | 5 | 0.142 |
| $Ca^{++}$ [mg.L$^{-1}$] | 200 | 2 | 0.057 |
| $Mg^{++}$ [mg.L$^{-1}$] | 150 | 1 | 0.029 |
| $Na^+$ [mg.L$^{-1}$] | 200 | 2 | 0.057 |
| $K^+$ [mg.L$^{-1}$] | 10 | 2 | 0.057 |

Table 2: WQI-based water quality rating ranges and kinds range water type (Bhargava 1983).

| Range | Type of waters |
|---|---|
| ≥50 | Excellent water |
| 50-100 | Good water |
| 100-200 | Poor water |
| 200-300 | Very poor water |
| ≤300 | Water that is unfit for consumption |

$HCO_3$ have been assigned a maximum weight of 5, 4 for pH, EC, $SO_4^{2-}$, 3 for $HCO_3^-$, and two for $Ca^{++}$, $Na^+$, and $K^+$ (Table 1). (Akhtar et al. 2021, Bhimanagouda et al. 2020, Gueddari et al. 2022, Karunanidhi et al. 2021, Selvam et al. 2014)). The comparative weight is calculated using the subsequent equation (eq.1).

$$\text{Wi} = wi / \sum_{i=1}^{ni} wi \quad \ldots(1)$$

Where:

Wi denotes the weight relative to another.

wi denotes the parameter's weight.

The argument count is n.

The level for evaluating the quality of each parameter is determined by dividing each water sample's concentration by the applicable standard (WHO Edition 2011) and multiplying the results by 100 (eq.2).

$$Qi = \left(\frac{ci}{si}\right) \times 100 \quad \ldots(2)$$

Where,

Qi denotes the standard of quality.

ci denotes every chemical parameter's concentration in

The weight of each sample is determined, and the concentration is given in milligrams per liter. si is the recommended limit for every chemical parameter in milligrams per liter by the World Health Organization (WHO Edition 2011). Begin the last phase of WQI calculation by computing the SI for each parameter (Table.2). The water quality index is calculated by adding the SI values for each sample. (Abbasnia et al. 2019, Mukate et al. 2019) (eq.3 and 4).

$$Sli = Wi \times qi \quad \ldots(3)$$

$$WQI = \Sigma Sli \quad \ldots(4)$$

Where

Sli is the $i^{th}$ parameter's sub-index.

qi is the score assigned to the ith parameter depending on its concentration.

The number n denotes the number of parameters.

## Physical and Chemical Analysis

Because the parameters vary after sampling, and cannot be identified qualitatively, we took measurements of T (°C) and dissolved oxygen in situ, as well as pH, Cond (S.cm-1), and hydrogen potential (pH) (O2d). For these tests, we utilized a MULTI 350I multi-parameter meter that is easily portable.

Methods for analyzing various other variables were used in a laboratory, as per recommendations from Rodier et al. (2009):

- To identify and quantify ($K^+$, $Na^+$), we employed Flame Atomic Absorption Spectrophotometry (Varian Model 475-AA).
- According to the ISO17294-2:2016 standard, the measurements are made using spectrophotometry and colorimetric dose ($SO_4^{2-}$, $NO_3^-$, $PO_4^{3-}$).
- Complexometric measurements are used for calcium and magnesium. To measure the concentration of main anions, we used them as a technique.
- Chloride precipitation and bicarbonate titrimetry with 0.01 N HCl are two methods to determine precipitation. It is common practice to measure chemical levels in $mg.L^{-1}$ units.

## Support Vector Machine (SVM)

Identifying the root causes of data is well-suited to the use of SVM-based supervised machine learning models. The statistical learning theory and the notion of structural risk reduction underlie these strategies (Bennett & Demiriz 1999, Evgeniou et al. 2002). The kernel approach in regression transforms non-linear correlations between inputs and outputs into a higher-dimensional regression analysis. (Üstün et al. 2007, Xu et al. 2006). D = x I,y I i = 1: n, where x is the contribution vector, y is the output variable, and n is the number of observations.

The SVR model aims to find the best function f defined (Kavousi-Fard & Kavousi-Fard 2013, Mohammadi & Mehdizadeh 2020) (eq.5):

$$f(x) = w^T \varphi(x) + b \quad \ldots(5)$$

Where *a* is a nonlinear function that translates the data received into high dimensional space, w and b represent the weights and the bias that are determined by minimizing the regularized risk function (eq.6):

$$R = \frac{1}{2} w^T w + C \frac{1}{n} \sum_i^n L_\varepsilon(g(x_i), y_i) \quad \ldots(6)$$

Where $L_\varepsilon$ is the ε – I insensitive loss function; $C \frac{1}{n} \sum_i^n L_\varepsilon(g(x_i), y_i)$ is the empirical error and C is a positive Trade-off parameter between the observed error's magnitude and the model's flatness.

When attempting to forecast river water quality using an ungauged catchment in a dual scenario, this study turned to RBF (Model-Based Support Vector Machine Model for Predicting Water Quality) (eq.7).

$$K(x_i, x_j) = \exp\left(-\frac{\| x_i - x_j \|^2}{2\sigma^2}\right) \quad \ldots(7)$$

Where $\|x_i - x_j\|^2$ is the squared Euclidean distance between the two input vectors and, and is the band-

Table 3: Individual water quality index (WQI) categorization.

| Wells | WQI | Water quality classification type | Wells | WQI | Water quality classification type |
|---|---|---|---|---|---|
| w1 | 219.4 | Very poor water | w34 | 84.1 | Good water |
| w2 | 334.98 | Water that is unfit for consumption | w35 | 139.6 | Poor water |
| w3 | 311.02 | Water that is unfit for consumption | w36 | 190.7 | Poor water |
| w4 | 264.48 | Very poor water | w37 | 201.5 | Very poor water |
| w5 | 138.29 | Poor water | w38 | 175.1 | Poor water |
| w6 | 202.1 | Very poor water | w39 | 206.3 | Very poor water |
| w7 | 197.5 | Poor water | w40 | 216.2 | Very poor water |
| w8 | 96 | Good water | w41 | 224.1 | Very poor water |
| w9 | 138.4 | Poor water | w42 | 322.5 | Water that is unfit for consumption |
| w10 | 282.8 | Very poor water | w43 | 392.3 | Water that is unfit for consumption |
| w11 | 179.7 | Poor water | w44 | 301.1 | Water that is unfit for consumption |
| w12 | 296.5 | Very poor water | w45 | 320.1 | Water that is unfit for consumption |
| w13 | 234.7 | Very poor water | w46 | 186.7 | Poor water |
| w14 | 228.6 | Very poor water | w47 | 126.1 | Poor water |
| w15 | 128.4 | Poor water | w48 | 169.3 | Poor water |
| w16 | 273.5 | Very poor water | w49 | 74.6 | Good water |
| w17 | 227.6 | Very poor water | w50 | 138 | Poor water |
| w18 | 197.7 | Poor water | w51 | 89.6 | Good water |
| w19 | 223.6 | Very poor water | w52 | 74.9 | Good water |
| w20 | 260.1 | Very poor water | w53 | 99.5 | Good water |
| w21 | 318.1 | Water that is unfit for consumption | w54 | 110.2 | Poor water |
| w22 | 245.2 | Very poor water | w55 | 98.2 | Good water |
| w23 | 222.6 | Very poor water | w56 | 92 | Good water |
| w24 | 179.3 | Poor water | w57 | 96 | Good water |
| w25 | 213.5 | Very poor water | w58 | 152.5 | Poor water |
| w26 | 116.6 | Poor water | w59 | 158.8 | Poor water |
| w27 | 109.3 | Poor water | w60 | 185.3 | Poor water |
| w28 | 139.3 | Poor water | S1 | 95 | Good water |
| w29 | 183.4 | Poor water | S2 | 69.8 | Good water |
| w30 | 153.2 | Poor water | | | |
| w31 | 128.3 | Poor water | | | |
| w32 | 163.2 | Poor water | | | |
| w33 | 62.3 | Good water | | | |

width parameter of the RBF function (Sun & Fox 2014).

## RESULTS AND DISCUSSIONS

### Water Quality Index (WQI)

An assessment of water quality and the sustainability of drinking water can only be made using the Water Quality Index (WQI) (Bhimanagouda et al. 2020, Nong et al. 2020, Ponsadailakshmi et al. 2018). The Water Quality Index (WQI) is described as a technique of assessment that delivers the final impact of each water quality indicator (Adimalla 2019, Tripathi & Singal 2019).With an average of 110.16 28.55, 60 wells and two springs are evaluated for the Water Quality Index (WQI). Sixty percent of the water points have a WQI of more than 100, which indicates poor water quality. Because 88.89 percent of these boreholes had Nitrate levels over 50 $mg.L^{-1}$, it seems that high levels of nitrates influence

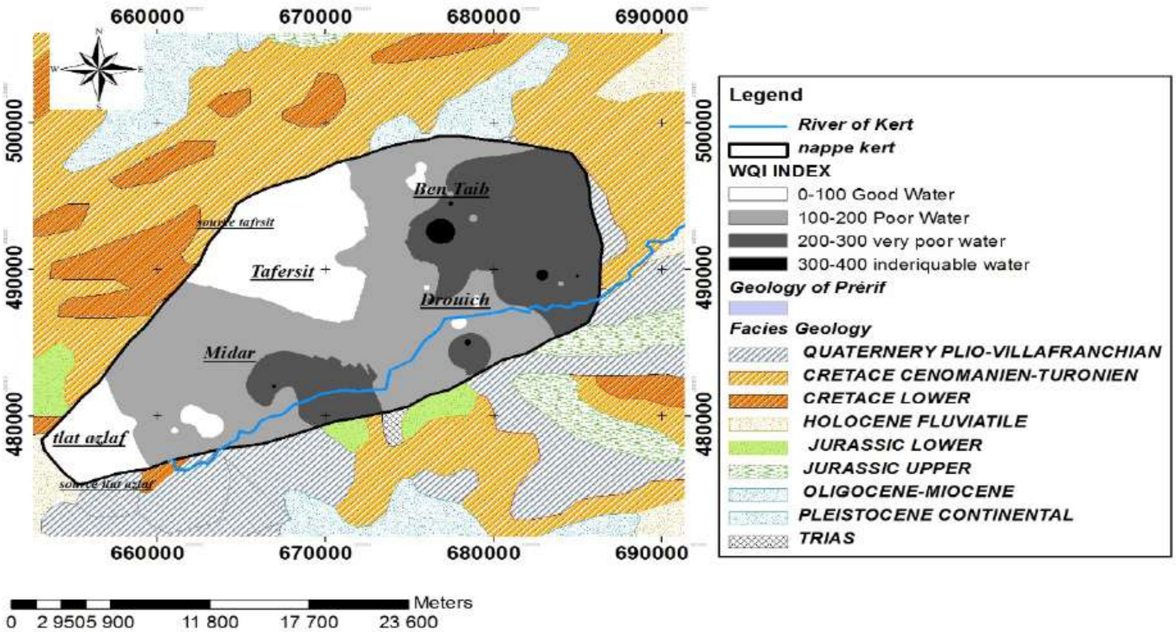


Fig. 3: Water quality classification ranges.

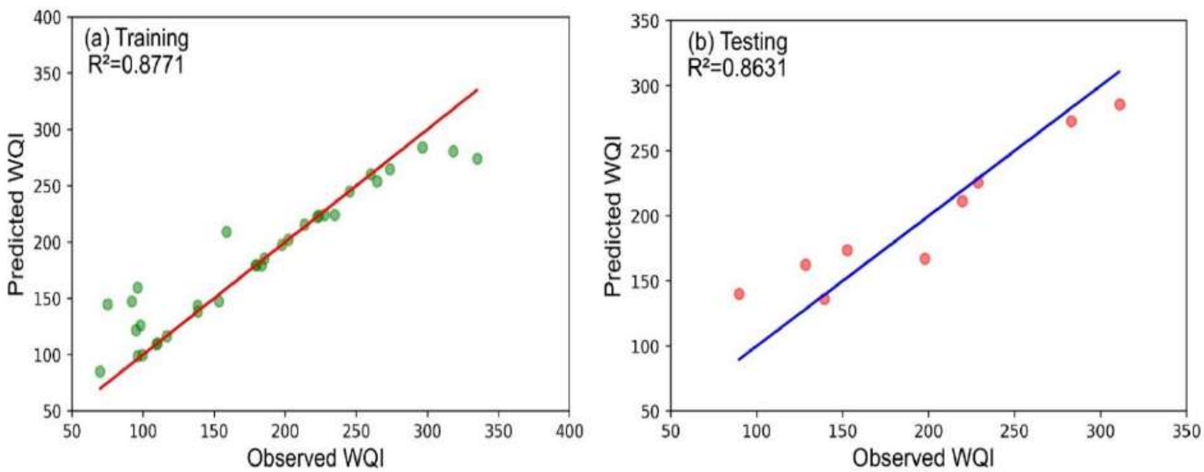


Fig. 4: Plot of prediction versus SVM-Model test data.

the WQI and the deterioration of water quality is shown in (Table.3). Fig. 3 illustrates how an examination of WQI and other metrics demonstrates that natural processes are crucial to the deteriorating water quality of the Kert Aquifer. Gypsum in rock formations may cause this phenomenon since rock salt dissolves readily in water (Gueddari et al. 2022, Liu et al. 2020, Lu et al. 2015, Zaier et al. 2021)). The elevated concentrations of EC, chloride, salt, and calcium demonstrate unequivocally that rock-water interactions are the dominant driver of water quality degradation in the studied region (Kawo & Karuppannan 2018, Mukate et al. 2019, Vaiphei et al. 2020).

All simulations were written with Python 3.8 using the sci-kit-learn library. Table 4 depicts the achieved results in

Table 4: Table predictive performances of the SVM model.

| | $R^2$ | RMSE |
|---|---|---|
| Training | 0.8771 | 25.6819 |
| Testing | 0.8631 | 25.7497 |

Table 5: Correlation between variables and factors.

| | pH | CE | TDS | $Cl^-$ | $Mg^{2+}$ | $Ca^{2+}$ | $K^+$ | $NO_3^-$ | $SO_4^{2-}$ | $HCO_3^-$ | $Na^+$ | WQI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | 1.00 | | | | | | | | | | | |
| CE | -0.61 | 1.00 | | | | | | | | | | |
| TDS | -0.55 | 0.89 | 1.00 | | | | | | | | | |
| $Cl^-$ | -0.56 | 0.95 | 0.82 | 1.00 | | | | | | | | |
| $Mg^{2+}$ | -0.28 | 0.58 | 0.64 | 0.61 | 1.00 | | | | | | | |
| $Ca^{2+}$ | -0.37 | 0.51 | 0.72 | 0.37 | 0.45 | 1.00 | | | | | | |
| $K^+$ | -0.19 | 0.14 | 0.24 | 0.05 | 0.17 | 0.25 | 1.00 | | | | | |
| $NO_3^-$ | 0.11 | -0.20 | -0.15 | -0.19 | 0.03 | -0.14 | -0.12 | 1.00 | | | | |
| $SO_4^{2-}$ | -0.31 | 0.44 | 0.78 | 0.29 | 0.45 | 0.82 | 0.38 | -0.12 | 1.00 | | | |
| $HCO_3^-$ | 0.00 | 0.23 | 0.28 | 0.11 | 0.03 | 0.07 | -0.14 | 0.14 | 0.18 | 1.00 | | |
| $Na^+$ | -0.58 | 0.89 | 0.92 | 0.86 | 0.40 | 0.47 | 0.13 | -0.18 | 0.57 | 0.31 | 1.00 | |
| WQI | -0.58 | 0.86 | 0.93 | 0.80 | 0.57 | 0.56 | 0.19 | -0.14 | 0.68 | 0.31 | 0.89 | 1.00 |

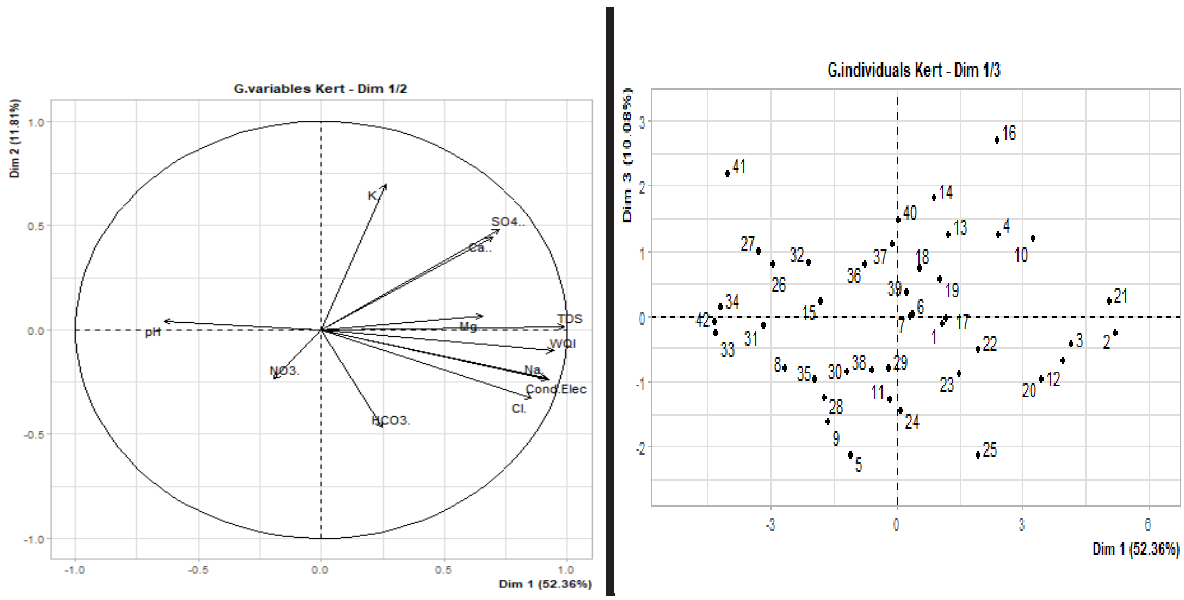


Fig. 5: Main components analysis circle.

both the training and testing stages. As this table indicates, the root means square error rose only by 0.26%, while $R^2$ decreased slightly by 1.6% during the testing phase. These results showed that the SVM model is stable (Table 4). Fig. 4 illustrates the SVM model's regression plots between the measured and predicted WQI values. As demonstrated by this figure, the SVM model showed a reasonable correlation between observed and estimated values with values of $R^2 = 0.8771$ and $R^2 = 0.8631$ in both the training and testing stages, correspondingly. These results reveal the SVM approach's resilience in predicting WQI.

## Statistical Studies

Principal Component Analysis (PCA) is used to analyze the physicochemical data. A table of 15 variables ($Ca^{2+}$, $Mg^{2+}$, $Na^+$, $K^+$, $HCO_3^-$, $SO_4^{2-}$, $Cl^-$, $NO_3^-$, $PO_4^{2-}$ Electrical conductivity (Cond), PH, O2d, and T) and 62 persons is used (wells and springs) (Table 5). The correlation matrix (Table 5) for the various parameters investigated revealed a strong link between Na+, $SO_4^{2-}$, $Cl^-$, and electrical conductivity. The correlation matrix, a square matrix characterized by a correlation coefficient, may determine the relationship between two variables. All water samples examined were put into a correlation matrix (Table 5).

After examining the correlation matrix, we discovered the following intriguing correlations between the variables:

- TDS (0.93) has the strongest correlation with the WQI, whereas Electric conductivity (0.86), $Cl^-$ (0.8), $Na^+$ (0.89), $SO_4^{2-}$(0.68), and $Mg^{2+}$ have modest correlations (0.57). CE and $Cl^-$(0.95) and CE and $Na^+$ have strong relationships (0.89). Only a little correlation exists between Calcium and WQI (0.56) and $SO_4^{2-}$(0.86), showing that the two are intertwined and affect the Kert alluvial aquifer's groundwater quality. The number of essential factors was calculated using the Kaiser criteria ( 1958). Based on these criteria, aquifer hydrogeochemistry variation can only be explained by components with eigenvalues larger than or equal to one.

A total of 64.83 percent of the variation may be attributed to the four primary kept components as shown in (Table 5). $Na^+$, $Cl^-$, WQI, CE and TDS positively influence Principal Component 1, accounting for 52.36 percent of the overall variance. Evaporitic rocks are responsible for this problem, as are residential and agricultural pollutants are shown in Fig. 5. $Ca^{2+}$, $SO_4^{2-}$, and $K^+$ contribute to Principal Component 2, accounting for 11.81 percent of the variation. The salinity of the water is consequently the determining element here. It results from surface salts being dissolved in water and then remineralized. Each of the four variables (components) represents one of the primary processes that explain how to understand how the Kert aquifer acquired and evolved its chemist; it is necessary to look at four variables (components): water-rock interaction, agricultural pollution, and the aquifer itself. As a result of these interactions between water and rock and pollution from home and industrial agriculture, the four variables (or components) reflect the primary processes that explain how the chemist of the Kert aquifer came to be. There are three families of water quality founded on the projection of persons shown in Fig. 5, the first and second of which are situated in the south and eastern regions of the research area, respectively. The third family characterizes Salinity-polluted environments.

## CONCLUSIONS

Aquifer lithology and recharge areas, which favor geological dissolution, are connected to groundwater chemistry, associated with the Triassic marl-sulfate formations and the Quaternary evaporate formations. On the other hand, the Kert aquifer's groundwater is of low quality. According to a principal component analysis (PCA) chemical factors, such as sodium, calcium, magnesium, sulfates, and chlorides, have the most significant effect on the quality of Kert's quaternary water table. The Water Quality Index (WQI) reveals that 79% of the drillings had WQI values over 100. As salt levels rise, the Oust of Kert is particularly susceptible since it contains more than the WHO's recommended amount of salinity. Soil treatment is needed in light of this condition and conservative water management measures, such as establishing a strict residential discharge monitoring system.

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