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Research Article

Wickerhamomyces anomalus WO2 inoculation protects *Suaeda fruticosa* from salt stress by the stimulation of the production of carotenoids and sugars and the augmentation of sodium translocation

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Abstract

The objectives of this study were to test the strain *Wickerhamomyces anomalus* WO2 as a plant growth-promoting yeast (PGPY), to evaluate the effect of its inoculation on the growth and physiological performance of *Suaeda fruticosa* subjected to salt stress, and to understand by which mechanism the yeast can protect the plant from high salinity. The results showed that the strain is halophilic, and grows at salt concentrations of up to 15%. Salinity caused decreases in chlorophyll a, b, and T in both inoculated and non-inoculated *S. fruticosa*. A significant increase in carotenoids was observed in *W. anomalus* WO2 inoculated plants. Inoculation enhanced the production of proteins, polyphenols and flavonoids at 1% of salinity, and sugars at all concentrations of NaCl. Although Na^+ and K^+ were higher in the leaves of non-inoculated plants compared to inoculated ones, the correlation of sodium translocation factor (TF) with salinity was very strong positive only in the inoculated plants. The production of carotenoids had a very strong positive relationship with salinity in inoculated plants, and a very strong negative correlation in non-inoculated plants. Soluble sugars were very strongly positively correlated with salinity in both inoculated and non-inoculated plants. However, a strong positive correlation of sugars with carotenoids was observed only in inoculated plants. This strain proved to be a promising candidate as a PGPY under salt stress. This work amends the PGPY bank with new strains having interesting abilities to resist high concentrations of NaCl and which can be used in the future as a biofertilizer.

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Keywords: carotenoids; growth; inoculation; PGPY; salt stress; sodium translocation; *Suaeda fruticose*; sugars; *Wickerhamomyces anomalus*

Introduction

Due to unsuitable agricultural practices and global warming, more and more lands around the world are suffering from degradation problems caused by the accumulation of salt. In fact, salinization is one of the most serious threats to agriculture worldwide. This problem is further aggravated in arid and semi-arid regions (Minhas *et al.,* 2020). According to FAO (2021), 424 million hectares of arable lands are affected by salinity. High salt concentrations disrupt plants survival by inhibiting various physiological and metabolic processes, which affects crop yields and threatens food security (Egamberdieva *et al*., 2019). In this worrying context, some solutions focus on limiting salt devastating effects. Thus, Plant Growth Promoting Microbes (PGPMs) are considered as a promising alternative to overcome this problem.

Plant Growth Promoting Microbes (PGPMs) are microorganisms present in the rhizosphere. Previous studies have already focused on the isolation of halotolerant PGPMs from the rhizosphere of halophyte plants (Fu *et al*., 2016; Etesami *et al*., 2018). These plants are not only a niche for the growth of PBPMs, but they also have the ability to sequester salts from salt contaminated soils (Etesami *et al*., 2018). *Suaeda fruticosa* (syn. *S. vera* Forssk. ex J.F. Gmel.) is a leaf succulent halophyte that grows in arid and semiarid saline habitats or salt marshes (Hameed *et al*., 2012). This plant species is widely distributed in halomorphic ecosystems in Algeria (Bahi *et al*., 2020). It has a strong ability to accumulate and sequester NaCl (Hameed *et al*. 2012), and shows the capacity to accumulate large amounts of heavy metals in its tissues without symptoms of toxicity. This ability makes it an excellent candidate for revegetation and remediation of metal-contaminated areas and salt-affected soils (Sidhoum and Fortas, 2019).

Plant Growth Promoting Microbes have a positive effect on plant physiology and can protect plants from biotic and abiotic stress (Chennappa *et al*., 2019). Among the best known and most widely used PGPMs, rhizobacteria and mycorrhizal fungi, are arousing enormous interest due to their ability to improve plant growth and health under saline conditions (Nadeem *et al*., 2014). Compared to rhizobacteria and mycorrhizal fungi, the use of yeast as growth promoters still in an early stage. Yeasts can be very useful, due to their production of molecules of interest and their ability to tolerate polluted and saline conditions (Shruthi *et al*., 2022). As plant growth promoters, some yeasts have demonstrated a great ability in terms of indole acetic acid production. Species belonging to *Hanseniaspora, Pichia, Candida, Sporidiobocus, Meyerozyma, Symmetrospora, Rhodotorula* were isolated from various sources (citrus peel, citrus leaves, citrus pulp and soil) (Shruthi *et al*., 2022). Other Plant Growth Promoting Yeasts (PGPYs) have been isolated from plant leaves (Fu *et al.,* 2016), grapes (Fernandez-San Millan *et al*., 2020) and water tanks (Gomes *et al*., 2015). This indicates that PGPYs can be isolated from unconventional sources. Saline habitats such as seas and salt lakes are an ideal place to isolate halotolerant microorganisms that could be used as PGPs. Recently, Krishnan *et al*. (2020) and Srinivasan *et al.* (2022) reported the isolation of the yeast *W. anomalus* from seaweed in Indian coast and its applications as PGP. In addition to a broad-spectrum antimicrobial activity against a variety of species, *W. anomalus* exhibits a multitude of applications with biotechnological importance (Padilla *et al*., 2018). This strain was applied in the production of fuel chemicals, in food and beverage production and in biocontrol for cereal grain preservation (Padilla *et al*., 2018). Although all the mentioned performances, little has been reported on *W. anomalus* environmental applications, except for its tolerance to some toxic metals such as chromium and cadmium (Breierová *et al*., 2002; Martorell *et al*., 2012), elimination of lead from water (Aibeche *et al*., 2022), and aromatic hydrocarbons degradation (Hesham *et al*., 2006), thus highlighting its potential role in environmental bioremediation.

Considering the lack of studies that relate the application of W. *anomalus* as a PGP, the objective of this study was to evaluate the effect of *W. anomalus* WO2 inoculation on the growth and physiological performance of the halophyte plant *Suaeda fruticosa* under saline stress and to understand by which mechanism the yeast can protect the plant from high salinity.

Materials and Methods

Strain and culture conditions

The yeast strain *W. anomalus* WO2 was used in the present study. It was isolated from water samples of Dayet Oum Ghellaz Lake (Province of Oran - North West of Algeria) and registered in GenBank database with the accession number MT331854. This strain is characterized by its ability to eliminate several heavy metals (copper, zinc, lead, cadmium, chromium) at high concentrations (Aibeche *et al*., 2022). The strain activation was carried out on YEPD broth for 72 h at 28 °C under continuous shaking at 150 rpm.

Effect of NaCl on yeast growth

The salinity tolerance of the studied yeast was evaluated by cultivating the strain in 200 mL flasks with 50 mL of Yeast Extract Peptone Dextrose medium (YEPD) supplemented with 0%, 3%, 5%, 10%, 15% and 20% of NaCl and incubated at 28 °C under continuous shaking at 150 rpm. Growth was monitored by optical density at 620 nm each 24 h for 96 hours. The experiment was performed in triplicate.

Plant material sampling and experimental design

The seeds of *S. fruticosa* were collected from the lakeside of Telamine Lake (35°42'50"N 0°23'30"W) located in the district of Gdyel (Province of Oran, Algeria). The proximity of Telamine lake to the industrial Zone II of Hassi Amer (7 km) makes it vulnerable to pollution hazards (Sidhoum and Fortas, 2019). The collected seeds were surface sterilized with a 12° sodium hypochlorite solution for 10 min and subsequently rinsed with distilled water. The seeds were sown in 250 mL capacity plastic pots containing a sterile cultivation substrate prepared by homogenously mixing peat and sand (w/w). The experiment was carried out in the experimental greenhouse of the University of Sciences and Technology of Oran, under natural light conditions. After 6 weeks of growth, uniformly developed seedlings were selected for inoculation.

Twenty germinated seedlings were inoculated by 20 ml of *W. anomalus* WO2 suspension grown in YEPD medium, prepared at a final concentration of 10^7 UFC mL^{-1}, after 24 h of growth at 28 °C under continuous shaking (150 rpm). Twenty non-inoculated controls received the same volume of medium in the absence of yeast.

After one week of cultivation, each group of plants (inoculated and non-inoculated) was divided into four sub-groups and were irrigated every three days for one month with 75 mL of water with separate solutions of NaCl at concentrations of 0%, 1%, 3%, and 5% (w/v).

Morphological, physiological and biochemical growth parameters

At the end of the experiment, shoot lengths, fresh and dry shoot weights were measured. The shoots water content was calculated as described by Turner (1981) using the following formula:

WC (%) = (F.W-D.W/F.W)100

Where F.W = Fresh weight and D.W = Dry weight.

For chlorophyll and carotenoid determination, fresh biomass (leaves) were homogenized in 80% ice-cold acetone in the dark. The mixture was then centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was used for the immediate determination of pigments. Absorbances of the solutions were determined spectrophotometrically at 663, 645 and 480 nm. Concentrations of total Chl (T-Chl), Chl a, Chl

b, and total carotenoids were calculated using the formula described by Arnon (1949). Chlorophyll and carotenoid concentrations were expressed as milligrams per gram of fresh weight.

For the evaluation of total protein content of fresh leaves, an amount of 1 g of different samples of *Suaeda fruticosa* leaves were ground in a mortar in 5 ml of 0.06 M phosphate buffer (pH 7). The mixture was then centrifuged at 10000 rpm for 10 min at 4 °C. Homogenates were used for protein quantification according to the method described by Bradford (1976), using bovine serum albumin (BSA) as a standard protein. The absorbance of the mixtures was measured spectrophotometrically at 595 nm.

The concentration of total polyphenol of plant leaves was determined according to the method of Folin-Ciocalteu (Singleton *et al*., 1999). 0.5 g of fresh leaves was mixed with 50 mL of 70% methanol; the mixture was then incubated at room temperature for 24 h under continuous stirring. After that, the solution was centrifuged at 3800 rpm for 20 min, the supernatant was collected and evaporated, then 5 mL of 70% methanol were added to the residues. 100 µL of each test solution was mixed with 500 µL of Folin-Ciocalteu (0.1 M). After 5 minutes, 400 µL of aqueous sodium carbonate Na_2CO_3 (7.5 %) was added to the mixture and incubated for 2 hours and the measurement was done at 760 nm. Total polyphenols values were expressed as milligram gallic acid equivalent per dry weight material (mg GAE g^{-1} DW).

Flavonoids were extracted by stirring 0.5 g of fresh leaves stirred in 5 ml of 80% methanol for 24 hours. The mixtures were filtered through a screen cloth, then centrifuged at 5000 rpm for 5 min. The total flavonoid content of each extract was measured at 510 nm using the aluminium chloride colorimetric method (Chang *et al*., 2002). The results are expressed in mg of catechin equivalent per 100 g of sample.

Total soluble sugar contents were determined using the anthrone reagent method (Yemm and Willis, 1954). Extraction of sugars was done by submersion of 100 mg of fresh leaves in 5.25 mL of 80% (v/v) ethanol at 25 °C for 20 h. Total soluble Sugars were analysed by reacting 2 mL of ethanolic extract, diluted 10 times previously in 80 % ethanol, with 4 ml freshly prepared anthrone (2 g anthrone + 1000 ml of H_2SO_4) in water bath at 92 °C for 8 minutes and the cooled samples were read at 585 nm.

For Na^+ and K^+ contents estimation, 100 mg of dried sample was digested by nitric acid (Lambert, 1975). The K^+ and Na^+ contents were determined by flame photometer (JENWAY PFP7). The translocation factor (TF), bioconcentration factor (BCF) and bioaccumulation factor (BAF) values were used to evaluate the potential of plants for phytoextraction and phytostabilization of sodium in soil according to Baker (1981). The factors were calculated by the following formula:

$$\mathrm{BCF} = \frac{\mathrm{Na_{root}}}{\mathrm{Na_{soil}}}$$

$$\mathrm{BAF} = \frac{\mathrm{Na_{leaf}}}{\mathrm{Na_{soil}}}$$

$$\mathrm{TF} = \frac{\mathrm{Na_{leaf}}}{\mathrm{Na_{root}}}$$

Where, Na_{leaf}, Na_{root}, and Na_{soil} are sodium concentrations in leaves, in roots, and in soil respectively.

Data analysis

Statistical analysis was performed using the SPSS software program (SPSS 23.0). Each treatment was replicated 3 times unless otherwise specified, and the data were presented as the mean value ± standard deviation (± SD). The relationship between the studied parameters was investigated by calculating the correlation coefficient R. Data were analysed by Two-way analysis of variance (ANOVA) and GLM (Generalized Linear Model). To determine the statistical significance of differences ($p< 0.05$) between means, the Tukey test was performed.

Results

Effect of NaCl on yeast growth

The strain *W. anomalus* WO2 exposed to increasing NaCl concentrations showed its best growth after 24 h of incubation at 0, 3 and 5% of NaCl (Figure 1). Yeast growth continued to progress to reach its maximum at 72 h. At 10% of NaCl, growth was slightly inferior compared to lower concentrations and it reached its maximum after 96 h. However, the growth of the yeast was very low at 15% of NaCl in the first 48 h, although it started to grow again to reach its maximum after 96 h of incubation. Finally, no growth was recorded for the yeast cultivated at 20% of NaCl.

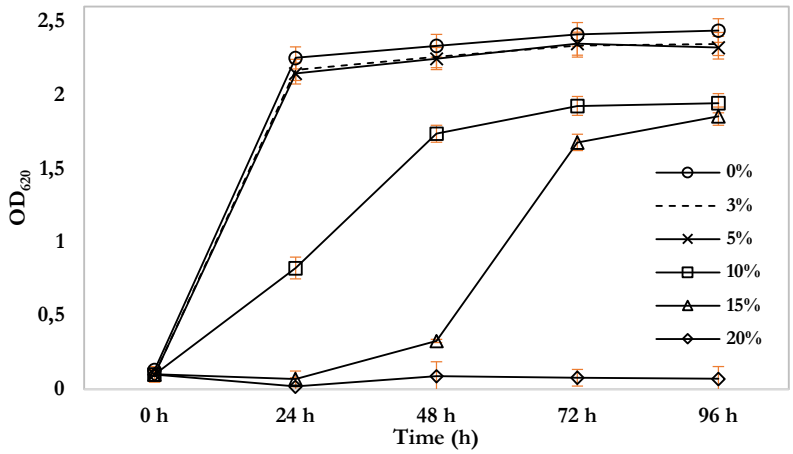

Figure 1. Growth of *Wickerhamomyces anomalus* WO2 in the presence of 0 %, 3 %, 5 %, 10 %, 15 % and 20 % NaCl concentrations at 28 °C

Effect of W. anomalus WO2 inoculation on plant growth parameters under NaCl stress

After 4 weeks of growth under different NaCl concentrations, the plants of *S. fruticosa* exhibited differences in growth parameters, but no mortality was detected at any concentration. Fresh and dry shoots weight (Figure 2 a, b) were at their maximum at 1% of NaCl, with a slight superiority in the inoculated plants. With increasing salinity, fresh and dry shoot weights progressively declined. Furthermore, shoot length (Figure 2 c) was around 20 cm for most concentrations, with a slight superiority for the inoculated plants. The maximum height was reached at 1% of NaCl in the inoculated plants (22.20 cm ± 1). As for shoots water content (Figure 2 d), an increase in values was noted as concentrations of salt increased until 3% of NaCl, with no differences registered between inoculated and non-inoculated plants. Shoots water content showed an important decrease at 5% of NaCl for non-inoculated plants (81.72% ± 4.5), while the yeast-inoculated plants maintained a high-water content (87.09% ± 5.8).

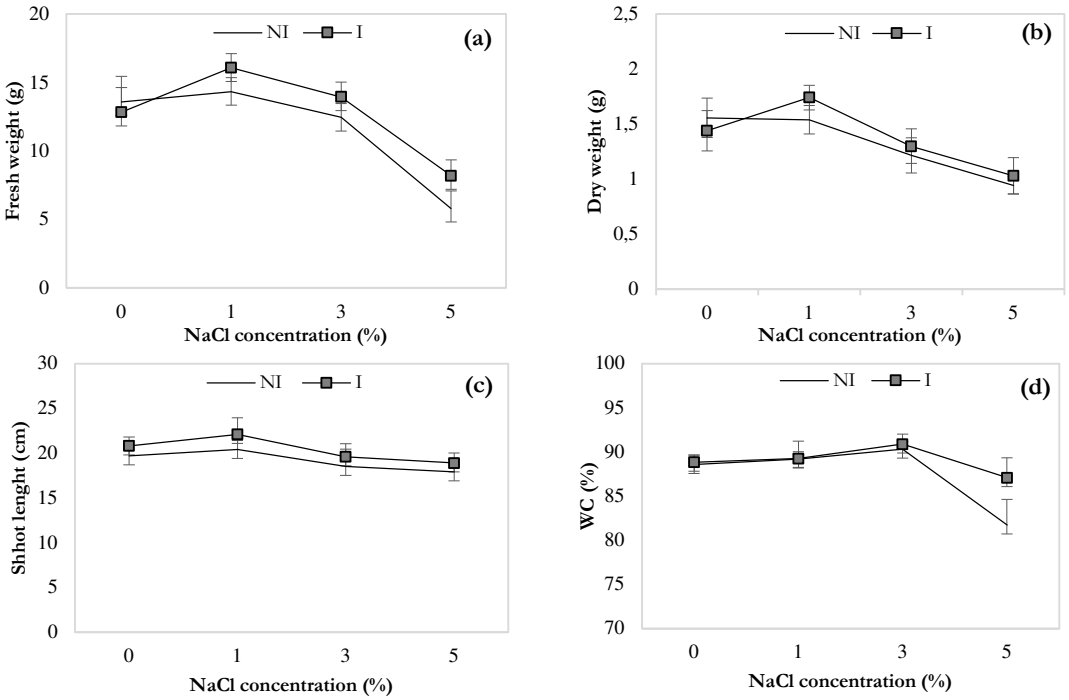

Figure 2. Effect of *Wickerhamomyces anomalus* WO2 inoculation on growth parameters of *Suaeda fruticosa* under salt stress; **(a)** Dry weight; **(b)** Fresh weight; **(c)** Shoot length; **(d)** Water content (WC)

Effect of W. anomalus WO2 inoculation on photosynthetic pigments under NaCl stress

The effect of salinity stress on photosynthetic pigments was studied (Figure 3). Under non-saline conditions, plants appeared green and healthy compared to the salt-stressed plants. Salt stress significantly decreased the concentrations of chlorophyll a, b and T in both inoculated and non-inoculated *S. fruticosa*. In the range from 0% to 5% of NaCl, the values varied from 17.63 mg g^{-1} ± 1 to 7.19 mg g^{-1} ± 0.65, from 7.82 mg g^{-1} ± 1.03 to 3.62 mg g^{-1} ± 0.58 and from 25.47 mg g^{-1} ± 1.87 to mg g^{-1} mg g^{-1} ± 0.56 for chlorophylls a, b and T respectively in non-inoculated plants. As for inoculated plants, the values of chlorophylls a, b and T went from 17.57 mg g^{-1} ± 1.16 to 8.18 mg g^{-1} ± 0.25, from 8.18 mg g^{-1} ± 1.06 to 2.75 mg g^{-1} ± 1.03 and from 25.74 mg g^{-1} ± 1.57 to 10.93 mg g^{-1} ± 0.77. Also, a decrease in carotenoid contents was observed in non-inoculated plants (Figure 3 d), with concentrations ranging from 2.95 mg g^{-1} ± 0.83 to 1.26 mg g^{-1} ± 0.24. However, a significant increase was noticed in inoculated plants where the concentrations of carotenoid ranged from 2.61 mg g^{-1} ± 0.6 to 7.89 mg g^{-1} ± 1.15 which represents an augmentation of 627% (Figure 3 d).

According to the GLM results, inoculation had no significant effect on the chlorophylls (total, a and b) contents, while it significantly increased the content of carotenoids at 1, 3 and 5% of NaCl (Table 1). A strong negative correlation was observed between salinity and Chl a, b and T in both non-inoculated and inoculated plants (Table 3). However, for carotenoids, a very strong negative correlation was observed in non-inoculated plants (r= -0.82), whereas a very strong positive correlation was obtained in inoculated plants (r= 0.94) (Tables 3 and 4).

Table 1. Significance of sources of variation for inoculation (I) and salt stress [NaCl] effects on morphological and physiological growth parameters using Generalized Linear Model (GLM)

	Fresh weight (g)	Dry weight (g)	Shoot length (cm)	WC (%)
I	3.26 ns	0.48 ns	1.46 ns	3.62 ns
[NaCl]	28.10 ***	9.79***	1.56 ns	10.60***
I*[NaCl]	1.04 ns	0.519 ns	0.025 ns	2.40 ns
	Chl a (mg g^{-1})	**Chl b (mg g^{-1})**	**Chl T (mg g^{-1})**	**Carotenoid (mg g^{-1})**
I	0.86 ns	0.045 ns	0.17 ns	33.87***
[NaCl]	104.19***	17.83***	85.48***	13.38***
I*[NaCl]	0.519 ns	0.42 ns	0.01 ns	39.80***
	Protein (mg g^{-1})	**Polyphenol (mg g^{-1})**	**Flavonoid (mg g^{-1})**	**Sugar (mg g^{-1})**
I	7.07*	311.57***	7651.64***	559.94***
[NaCl]	2.39 ns	138.06***	6388.37***	13.82***
I*[NaCl]	1.78 ns	181.57***	6109.88***	11.62***

WC: Water Content. Chl a: Chlorophyll a. Chl b: Chlorophyll b. Chl T: Chlorophyll T.
Significance levels: ns indicates no significant. * p< 0.05. ** p< 0.01. *** p<0.001.

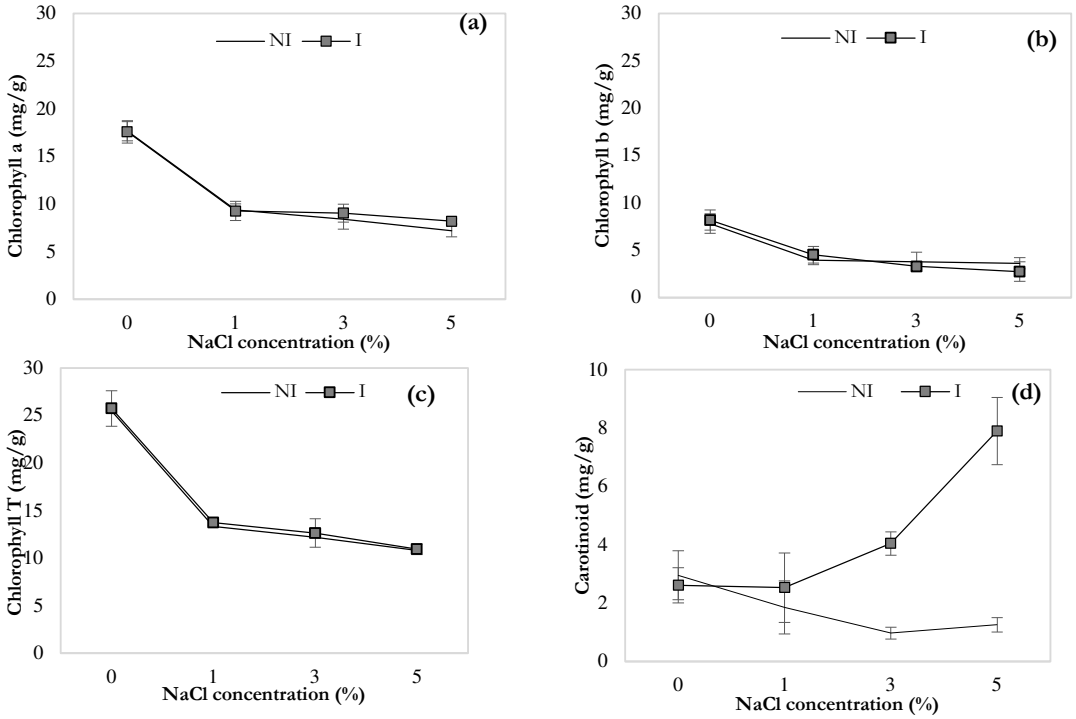

Figure 3. *Wickerhamomyces anomalus* WO2 inoculation effect on chlorophyll a **(a)**, chlorophyll b **(b)**, total chlorophyll **(c)** and carotenoid content **(d)** in leaves of *Suaeda fruticosa* under salts stress

Effect of W. anomalus WO2 inoculation on biochemical parameters under NaCl stress

The response of *S. fruticosa* seedlings to NaCl stress in the presence of of the halophilic yeast strain *W. anomalus* WO2 was investigated by determining the biochemical status of the plants, including protein, polyphenol, flavonoid and total sugars. The results are presented in Table 1 and Figure 4.

Both salinity and inoculation affected significantly polyphenols, flavonoids and sugar foliar contents according to the GLM results (Table 1). Hence, the assayed microbial inoculations affected significantly the foliar sugar concentrations of plants grown under non-stressing conditions.

The inoculation induced significant differences in protein content, and had a highly significant effect on polyphenols, flavonoids and sugar contents. This treatment increased highly the biochemical parameters at 1% NaCl concentration. Thus, the increasing of salt concentration (3% and 5%) in inoculated plants induced the production of sugar and the diminution of other parameters (Figure 4).

The correlation analysis revealed a weak between polyphenols and flavonoids with salinity, regardless of of the presence of yeast (Tables 3 and 4). As for protein content, a very strong positive relationship with salinity was found in non-inoculated plants (r= 0.99). The amount of soluble sugars strongly positively correlated with salinity in both inoculated and non-inoculated plants (r= 0.94 and 0.90 respectively) (Tables 3 and 4).

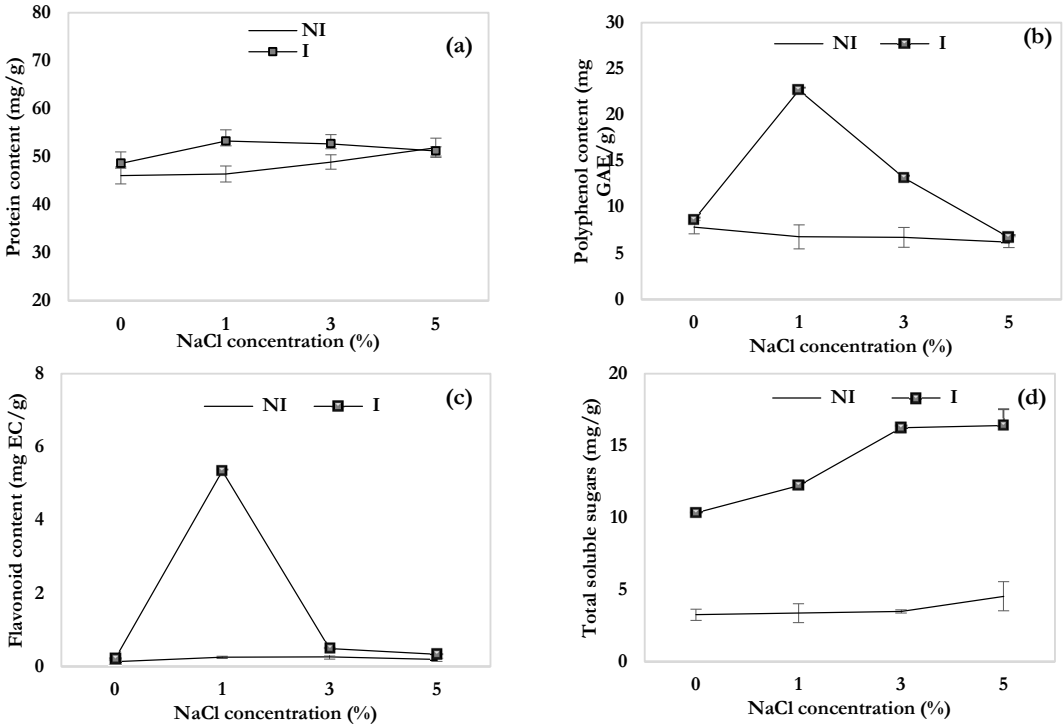

Figure 4. Effect of *Wickerhamomyces anomalus* WO2 inoculation on growth parameters of *Suaeda fruticosa* under salt stress; **(a)** Protein content; **(b)** polyphenol content; **(c)** Flavonoid content; **(d)** Carbohydrate content

Effect W. anomalus WO2 inoculation on bioconcentration (BCF), bioaccumulation (BAF) and translocation (TF) of NaCl

To investigate the mechanism underlying salt resistance of *S. fruticosa* seedlings, the amounts of Na^+ and K^+ accumulated in tissues and rhizospheric soil were measured. The K^+ and Na^+ concentrations in the rhizospheric soil were relatively similar with all external NaCl treatments. Nevertheless, in inoculated plants, an increase in Na^+ was observed with the increase of NaCl. Na^+ and K^+ tissue concentrations were higher in leaves compared to roots (Figure 5). In non-inoculated plants, Na^+ increased remarkably with NaCl with higher NaCl concentrations in both leaves and roots. However, in inoculated plants, Na^+ concentrations remained unchanged in leaves and slightly decreased in roots at higher NaCl levels. Regarding K^+ concentration in roots, a decrease was noticed in non-inoculated plants, while its concentration fluctuated in inoculated ones. In the leaves, K^+ presented changing concentrations for both inoculated and non-inoculated plants with the increasing of NaCl concentrations. Although, potassium accumulations in all tissues of *S. fruticosa* seedlings were reduced by external NaCl but increased in all leaves and roots of inoculated plants at higher NaCl concentrations (Table 2, Figure 5).

Similarly, a significant increase in the Na^+/K^+ ratio was noticed in the rhizospheric soil of inoculated seedlings as the NaCl concentrations increased, while the ratio was relatively unchanged in non-inoculated plants. Concerning roots tissue, the Na^+/K^+ ratio increased with increasing salt concentrations in inoculated seedlings, unlike the non-inoculated ones where the ratio increased until 3% NaCl and then declined afterward. In the leaves, the Na^+/K^+ ratio increased in both inoculated and non-inoculated plants, reaching its maximum at 3% NaCl, and then decreased at 5%.

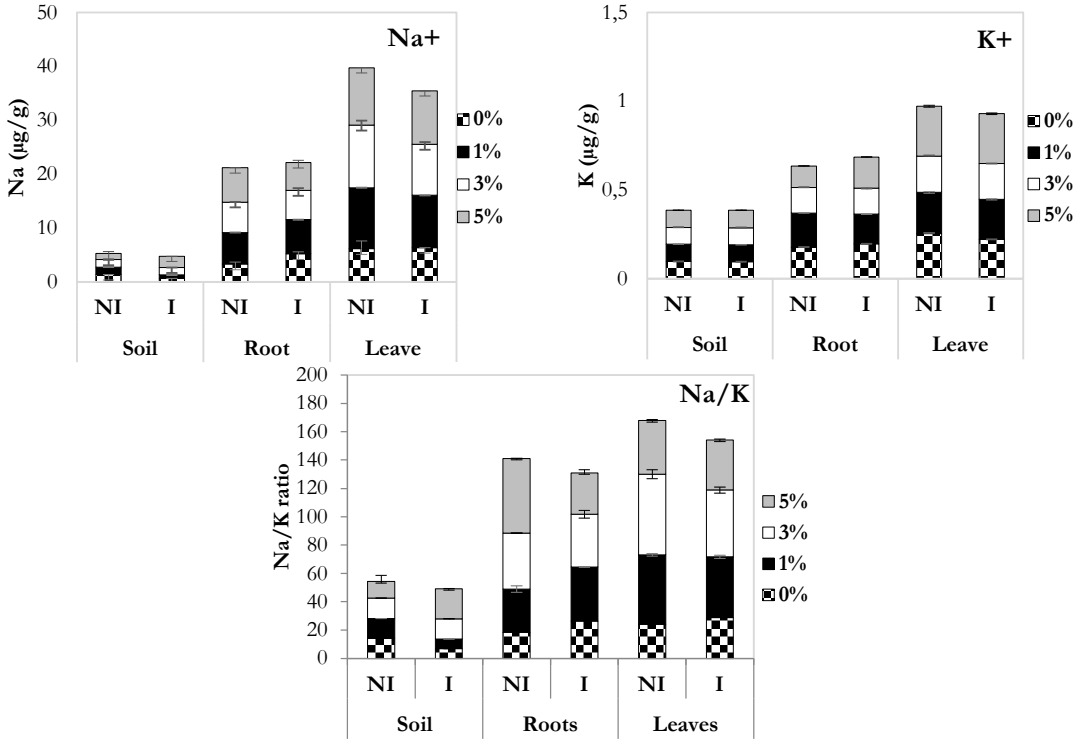

Figure 5. Effects of *Wickerhamomyces anomalus* WO2 inoculation on Na^+, K^+ and Na/K ratio in soil, root and leave of *Suaeda fruticosa* under different concentrations of NaCl solution (0%, 1%, 3% and 5%)

Table 2. Significance of sources of variation for organ (Org), inoculation (I) and salt stress [NaCl] effects on K, Na and Na/K, BCF, BAF, TF ratio using Generalized Linear Model (GLM)

	K mg g^{-1}	**Na** mg g^{-1}	**Na/K**	**BCF**	**BAF**	**TF**
Org	23076.57***	2252.64***	1197.30***	/	/	/
I	1.45 ns	10.24**	25.43***	15,39***	2,16 ns	13,76***
[NaCl]	589.87***	105.54***	185.03***	9,04***	7,76**	3,76*
Org * I	159.20***	15.52***	1.73 ns	/	/	/
Org * [NaCl]	595.84***	35.46***	54.11***	/	/	/
I * [NaCl]	155.05***	8.107***	11.15***	27,08***	15,63***	6,58**
Org*I*[NaCl]	131.21***	10.50***	52.68***	/	/	/

Significance levels: ns indicates no significant. * p< 0.05. ** p< 0.01. *** p<0.001.
BCF: bioconcentration factor; BAF: bioaccumulation factor; TF: The translocation factor.

The effect of *W. anomalus* WO2 inoculation on bioconcentration (BCF), bioaccumulation (BAF) and translocation (TF) factors of sodium in *S. fruticosa* plants under salt stress is represented in Figure 6. The highest BCF value was obtained at 1% NaCl (9.7% ± 1) in inoculated plants. A continuous increase of this factor was noticed for the non-inoculated plants across all salt concentrations, associated to a decrease in the

inoculated plants. For BAF, the highest value was noted at 1% NaCl (14.77% ± 1) in yeast inoculated plants. For the translocation factor (TF), it was superior in the non-inoculated plants at 0, 1, 3% NaCl (1.88% ± 0.12, 1.93% ± 0.13, 2.05% ± 0.14 respectively). However, at 5% NaCl the TF of yeast inoculated plants became higher (1.93% ± 0.14). A continuous increase proportional to salt concentration was noticed in inoculated plants (Table 2, Figure 6).

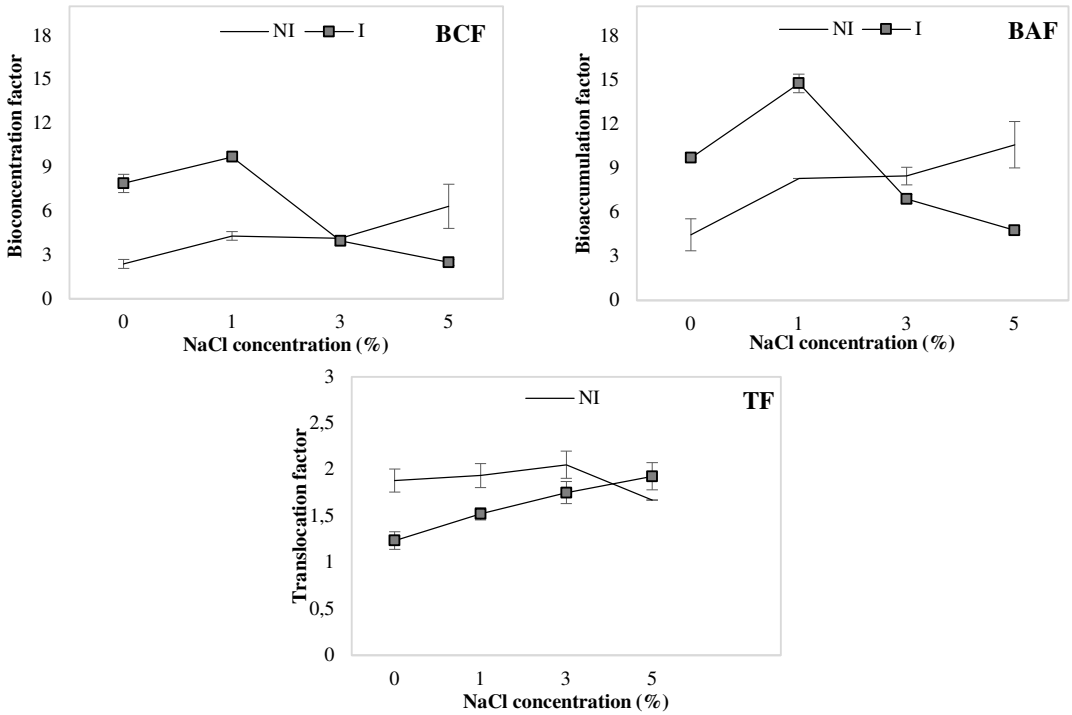

Figure 6. *Wickerhamomyces anomalus* WO2 inoculation effect on bioconcentration **(BCF)**, bioaccumulation **(BAF)** and translocation **(TF)** factors of sodium in *Suaeda fruticosa* plants under salt stress

The relationship between salinity and BCF, BAF and TF was studied. The results are presented in Tables 3 and 4. BCF and BAF had very strong positive correlation with salinity in non-inoculated plants (r= 0.91 and 0.89 respectively). Whereas the correlation was strong negative in inoculated plants (r= -0.90 and -0.76 respectively). As for translocation factor (TF), the correlation was very strong positive only in inoculated plants (r= 0.97).

Relationship between pigments and biochemical parameters

The relationship between pigments and biochemical parameters is represented in Tables 3 and 4. In non-inoculated plants, there was a very strong positive correlation between chlorophylls a, b, and T with carotenoids (r= 0.94, 0.92 and 0.94 respectively), while the correlation was weak negative in inoculated plants. The correlation between pigments (Chl a, b and T) and protein content was strong negative in inoculated plants and weak negative in non-inoculated plants. Also, protein content correlated negatively with carotenoid content in non-inoculated plants (r= -0.72).

There was a weak correlation between pigments and polyphenols in both inoculated and non-inoculated plants, contrary to flavonoids in non-inoculated plants, where a strong negative correlation with pigments was observed.

A strong negative correlation between total soluble sugars with Chl a, b and T (r= -0.80; -0.92 and -0.85 respectively) and a strong positive correlation with carotenoid were noticed in inoculated plants (r= 0.76). While, the relationship was moderate negative in non-inoculated plants (r= -0.52).

Relationship between bioconcentration (BCF), bioaccumulation (BAF) and translocation (TF) of NaCl with the studied parameters

The relationship between BCF, BAF with Chl a, Chl b and Chl T was very strong negative only in non-inoculated plants (Table 3). In inoculated plants (Table 4), the correlation was moderate positive with BCF and weak positive with BAF. The correlation was very strong negative between carotenoids and BCF and BAF regardless of yeast inoculation. While no correlation was noticed between pigments and TF in non-inoculated plants, a very strong negative correlation was registered after yeast inoculation with Chl a, b and T (r= -0.88, -0.96 and -0.91 respectively). In the opposite, TF was very strongly positively associated with carotenoid content (r= 0.84).

In non-inoculated plants (Table 3), the relationship was very strong positive between protein content and BCF and BAF (r=0.88 and 0.82), while it was moderate negative with TF (r= -0.59). The correlation was absent with BCF, weak positive with BAF and moderate positive with TF in inoculated plants (r= 0.53) (Table 4).

Polyphenols correlated only with BAF where a strong positive correlation was obtained for both plant groups (Tables 3 and 4). As for flavonoids, in non-inoculated plants, the relationship was weak positive with BCF and moderate positive with BAF and TF (Table 3). Whereas in inoculated plants (Table 4), it was strong positive with BCF, very strong positive with BAF and weak negative with TF. Finally, for soluble sugar content, the correlation was very strong to strong positive with BCF and BAF respectively and very strong negative with TF in non-inoculated plants (Table 3). While the opposite was noticed in inoculated plants where the correlation was very strong to strong negative with BCF and BAF respectively and very strong positive with TF (Table 4).

Table 3. Correlation coefficient between different parameters in non-inoculated plants

	Salt	Chl	Chl b	Chl T	Carot	Prot	Poly	Flav	Sug	BCF	BAF	TF
Salt	1.00											
Chl a	-0.80	1.00										
Chl b	-0.73	0.99	1.00									
Chl T	-0.78	1.00	1.00	1.00								
Carot	-0.82	0.94	0.92	0.94	1.00							
Prot.	0.99	-0.70	-0.61	-0.68	-0.72	1.00						
Polyph	-0.14	0.43	0.48	0.45	0.64	-0.02	1.00					
Flav	0.31	-0.78	-0.84	-0.80	-0.78	0.15	-0.81	1.00				
Sugar	0.90	0.60	-0.51	-0.58	-0.52	0.94	0.30	-0.03	1.00			
BCF	0.91	-0.87	-0.82	-0.86	-0.75	0.88	0.34	0.37	0.90	1.00		
BAF	0.89	-0.97	-0.93	-0.96	-0.87	0.82	0.97	0.59	0.78	0.97	1.00	
TF	-0.49	0.12	0.03	0.09	-0.06	-0.59	-0.37	0.51	-0.82	-0.59	-0.37	1.00

Chl a: Chlorophyll a. Chl b: Chlorophyll b. Chl T: Chlorophyll T. Carot: carotinoid content. Prot: protein content. Poly: polyphenols. Flav: flavonoids; Sug: Sugar; BCF: bioconcentration factor; BAF: bioaccumulation factor; TF: The translocation factor. P values were considered significantly ($P \leq 0.05$, *; $P \leq 0.01$, **).

Table 4. Title title title title title title title

	Salt	Chl a	Chl b	Chl T	Carot	Prot	Poly	Flav	Sug	BCF	BAF	TF
Salt	1.00											
Chl a	-0.74	1.00										
Chl b	-0.86	0.97	1.00									
Chl T	-0.79	1.00	0.99	1.00								
Carot	0.94	-0.53	-0.66	-0.58	1.00							
Prot.	0.30	-0.85	-0.74	-0.82	0.01	1.00						
Polyph	-0.48	-0.23	-0.02	-0.15	-0.68	0.68	1.00					
Flav	-0.35	-0.32	-0.08	-0.23	-0.45	0.63	0.91	1.00				
Sugar	0.94	-0.80	-0.92	-0.85	0.76	0.49	-0.30	-0.31	1.00			
BCF	-0.90	0.43	0.64	0.51	-0.86	0.00	-0.10	0.71	-0.87	1.00		
BAF	-0.76	0.17	0.40	0.25	-0.80	0.27	0.96	0.87	-0.71	0.96	1.00	
TF	0.97	-0.88	-0.96	-0.91	0.84	0.53	-0.62	-0.16	0.96	-0.81	-0.62	1.00

Chl a: Chlorophyll a. Chl b: Chlorophyll b. Chl T: Chlorophyll T. Carot: carotinoid content. Prot: protein content. Poly: polyphenols. Flav: flavonoids; Sug: Sugar; BCF: bioconcentration factor; BAF: bioaccumulation factor; TF: The translocation factor. P values were considered significantly ($P \leq 0.05$, *; $P \leq 0.01$, **).

Discussion

Soil salinization is becoming a major agricultural problem worldwide, mainly in arid and semi-arid regions. The increase in the level of salinity leads to negative impacts on soil properties and plant physiology. The objective of this study was to evaluate the effect of *W. anomalus* WO2 inoculation on *S. fruticosa* growth under salt stress.

The yeast W. anomalus WO2 is a halophilic strain

In this study, the yeast strain *W. anomalus* WO2 showed a great ability to grow at high salt concentrations. Being able to withstand salt concentrations of up to 15%, makes this strain a promising candidate to be applied in organic farming and the bioremediation of salt-affected soils. Similar studies have discussed the halophilic nature of *W. anomalus*. Bonatsou *et al*. (2015) reported that 16.32% was the minimum inhibitory concentration of salt for the strain *W. anomalus* Y18. Also, Praphailong and Fleet (1997) stated that *W. anomalus* did not grow at 20% of NaCl, while it grew at 15% which is in concordance with the current study. Therefore, based on this halophilic trait, this strain was selected for the study of the effect of its inoculation on *S. fruticosa* growth parameters under salt stress.

NaCl affects plant growth parameters regardless of yeast inoculation

The obtained results about the effect of *W. anomalus* WO2 inoculation on plant growth parameters under NaCl stress, revealed that, under 1% of NaCl, the fresh and dry shoot weights and shoot lengths of *S. fruticosa* were at their maximum. No significant difference was noticed between inoculated and non-inoculated plants. This indicates that the optimal concentration of salinity for the growth of *S. fruticosa* is 1%. However, this halophyte plant is able to survive and develop even at salinities up to 5% of NaCl (855.58 mM) for one month. The high tolerance of *S. fruticosa* to salinity up to 5.85% (1000 mM) was also observed in previous studies (Khan *et al*., 2000; Bankaji *et al*., 2016).

The water content increased until 3% of salinity concentration, which is superior to the results obtained by Khan *et al*. (2000), where they reported, maximum shoot tissue water content at 1.17% (200 mM) of NaCl. This parameter showed an important decrease at 5%, which is in concordance with the result of Khan *et al*. (2000) who observed a decreased in WC at higher salt concentrations. The present findings indicated that the yeast-inoculated plants had higher water content at 5% of NaCl, suggesting it may be one of the mechanisms

of the plant's defence system against high salt stress. Similarly, Chauhan *et al*. (2019) observed that the inculcation of rice with *Bacillus amyloliquefaciens* has improved water content under salt stress.

Inoculation enhances carotenoids content under salt stress

Regarding the effect of salinity on pigments, salt irrigation led to a decrease in chlorophyll a, b and T in inoculated and non-inoculated plants. Previous studies have reported a decrease in chlorophyll content in *S. prostrata*, *S. persica* and *S. europaea* with increasing soil salinity (Aghaleh *et al.*, 2009; Akcin *et al*., 2016). The decrease in chlorophyll content in salt-stressed plants is associated with an increase of chlorophyllase activity responsible for chlorophyl degradation (Santos, 2004). Also, high salinity induces ethylene production in plants, which inhibit chlorophyll biosynthetic pathway (Fahad *et al*., 2015).

Unlike non-inoculated plants, a significant increase in carotenoids was observed in plants inoculated with *W. anomalus* WO2. Inoculation of plants with PGPMs has been associated with an increase in carotenoids content (Katsenios *et al*., 2021). In a similar study, the yeast *Yarrowia lipolytica* FH1 increased carotenoids production in maize seedlings under salt stress (Gul jan *et al*., 2019).

Salinity at 1% boosts protein, polyphenols and flavonoids in inoculated plants

Despite the negative effects of salinity on plant growth parameters, the obtained results demonstrated that the inoculation of *S. fruticosa* plants with the *W. anomalus* WO2 improved protein, polyphenols, and flavonoids thus enhancing plant tolerance against salt stress.

Although the difference between inoculated and non-inoculated plants was not as great, the increase in total soluble proteins content could be attributed to the growth hormones produced by yeast and the direct stimulation of proteins synthesis (Gaballah and Gomaa, 2004; Stino *et al*., 2009; Khalil and Ismael, 2010; Agamy *et al*., 2013), providing plants with essential nutrient elements required for protein formation (Hayat, 2007).

The present investigation demonstrated that higher amounts of phenolic compounds and flavonoids have been observed in inoculated plants under 1% of salt stress. Thanks to the inoculation with the yeast, phenolic compounds and flavonoids production increased by 334% and 2136% respectively compared to non-inoculated plants at the same concentration of salt (1%). Recently, Muhammad *et al*. (2019), reported that increased levels of these compounds was observed in wheat inoculated by *Trichoderma reesei*. Indeed, improved flavonoids and phenols production may help the plants in various aspects, including growth, reproduction, resistance to pathogens and the protection against abiotic stress (Mona *et al*., 2017). In wheat and jointed goatgrass, polyphenols and flavonoids were observed to be involved against salt stress-induced oxidative stress (Kiani *et al*., 2021).

Inoculation of the plants enhance sugar production at all concentrations of salt

Compared with non-inoculated plants, the soluble sugar concentrations in inoculated plants were significantly higher in leaves at all NaCl concentrations. At higher concentrations of salt, total soluble sugars were increased by 3.6 times indicating a role of sugars in salt-stress tolerance. These results are in accordance with the study of Feng *et al*. (2002) which demonstrated that higher accumulation of soluble sugars in mycorrhizal plant tissue, especially in roots, could make mycorrhizal plants more resistant to osmotic stress induced by exposure to salt. Several studies have demonstrated that the accumulation of sugars and polyols was stimulated by salt stress in different plant species. A strong correlation has been established between the accumulation of sugars and the level of tolerance to salinity (Hanana *et al*., 2011).

Yeast inoculation protects the plant from salt stress at high concentrations

In the present study, the results showed that the presence of *W. anomalus* WO2 at higher concentrations of salt induced Na^+ accumulation in the soil, which caused a decrease of Na+ accumulation factors (BAF, BCF). Whereas its accumulation always took place in the leaves of non-inoculated plants. Also, the sodium translocation factor (TF) increased as the salt concentration increased in inoculated plants.

This sequestration of salt outside the plant is very important to avoid salt toxicity. It should be noted that the yeast species used in this study have certain properties that could have a direct positive effect on plant development and salt tolerance. Kasim *et al*. (2016) demonstrated that *W. anomalus* produces exopolymers in the extracellular space in stressful growth environment. As a response to the presence of nickel and cadmium stress, *W. anomalus* produces exoglycoproteins and phosphomannans as components of exopolymers (Breierová *et al*., 2002; Breierová *et al*., 2008). These exopolymers serve as an effective protective barrier against the penetration of heavy metal ions into the cells (Breierová *et al*., 2008). Under stressful conditions such as ion toxicity and osmotic stress caused by water limitation, some microorganisms develop biofilm as a strategy to increase the chances of survival (Enebe *et al*., 2018). The properties of this biofilm which functions as a barrier, depends on its composition and structure (Breierová *et al.,* 1996).

The decrease in Na^+ content in the roots of the inoculated plants under high salt stress could be explained by the ability of PGPMs to induce changes in the expression of the plant genes that control the Na^+ content or to actively participate in this ionic readjustment (Lanza *et al*., 2019). In this situation, PGPMs can accumulate Na^+ inside their cells, or actively remove it outside the roots of the plant (Lanza *et al*., 2019).

At higher NaCl levels, the Na^+ accumulation factors (BAF, BCF) decrease in inoculated plants was accompanied with an increase of K^+ concentration. Many studies on the mycorrhizal effect revealed that mycorrhizal plants can increase K^+ uptake while decreasing Na^+ accumulation in the cytoplasm under saline conditions compared to non-mycorrhizal plants (Giri *et al*., 2007; Hammer *et al*., 2011). To reduce cytosolic Na^+ concentration, some halophytes have developed a mechanism of ion compartmentation by sequestering excessive cytosolic Na^+ into the central vacuole, which alleviates the Na^+ toxicity, thus maintains ion homeostasis in saline conditions (Yamaguchi *et al*., 2013; Flowers et al., 2015).

The relationship between the studied parameters

According to the results of the correlation between the different parameters, yeast plays a central role in the protection of the plant against salt stress via the stimulation of the production of carotenoids. The translocation factor also seems to play an important role. In the presence of yeast, the more the salinity increased, the more this parameter increased and the more the BCF and BAF decreased. It seems also that there is a close relationship between flavonoids and BCF and BAF. In the absence of the yeast, the plant exhibited a different behaviour. The more salt was present, the more the plant produced protein and the more BCF and BAF increased.

Although sugar production correlated positively with salinity, regardless of whether the plant was inoculated or not. The obtained results proved that there was also a direct relationship between carotenoid content and sugar production and between sugar production and TF in inoculated plants. Whereas in the absence of yeast, there was an inverse relationship between sugar production and TF.

Carotenoids are known to be involved in protecting plants from salt stress (Li *et al.,* 2020). Gul jan *et al*. (2019) reported that the accumulation of carotenoids increased maize seedlings salt stress tolerance after plant inoculation with the yeast *Yarrowia lipolytica* FH1. Chlorophyll had a negative relationship with salinity in both inoculated and non-inoculated plants. Under these stressful conditions, carotenoids protected *S. fruticosa* by playing a role of accessory light-harvesting pigments, by extending effectively the range of light absorption which is a known role of carotenoids (Young, 1991). Another role of carotenoids is the protection of plants from the accumulation of Reactive Oxygen Species (ROS) at the cellular level (Young, 1991). When the plant is exposed to salt stress, ROS are generated at concentrations that may adversely affect the survival of the plant

(Latowski *et al*., 2011). To better adapt to these conditions, the plant produces carotenoids such as xanthophyll which is among the most important ROS scavenging mechanisms (Latowski *et al*., 2011).

Another adaptation strategy consists in synthesizing osmoprotectants, mainly sugars, and accumulating them in the cytoplasm and organelles. The key role of soluble sugars during stress includes carbon storage, osmo-protection, and hunting of the reactive oxygen species (Gupta and Huang, 2014, Hanana *et al*., 2011). These osmolytes, usually hydrophilic in nature, are poorly charged but polar and highly soluble molecules, suggesting that they can adhere to the surface of proteins and membranes to protect them from dehydration (Hanana *et al*., 2011). Moreover, in non-inoculated plants, it has been demonstrated through the correlation study, that proteins have close relationship with sugar content.

In this study translocation of sodium had a crucial role in yeast inoculated plants. It had a direct relationship with carotenoid and sugar productions. During salt stress, the energetic cost of Na^+ sequestration in the vacuoles increases. It was observed in this study how salt stress was accompanied by the decrease in chlorophyll levels. Indeed, the more the chlorophyll level decreased, the more the plant produced sugars. This is due to salt stress which reduces photosynthesis causing the plant to accumulate high content of sugars which could represent transient carbon storage (Sellami *et al*., 2019). Sugars also play an important role as a signalling molecule. At the genomic level, high glucose concentrations modify significantly the positive or negative expression of genes (Han *et al*., 2015). Solfanelli *et al.* (2006) discussed the role that sugars play as a signalling molecule in flavonoid biosynthetic pathways. This may explain why sugar production was closely correlated with carotenoid production. Indeed, sugar might act as signalling molecule in the biosynthesis of carotenoid in *S. fruticosa* in order to protect the plant from salinity. The expression of the genes involved in the carotenoid pathway has been observed previously in tomato and tobacco plants exposed to high concentrations of salts (Ann *et al*., 2011; Li *et al*., 2020).

Conclusions

W. anomalus WO2 is a salt-tolerant strain that has been successfully used as a Plant- Growth-Promoting Yeast (PGPY). Its wide range tolerance of salinity makes it a promising strain with potential applications in the field of soil remediation and sustainable agriculture. Inoculation of the halophyte plant *S. fruticosa* with the *W. anomalus* WO2 increased the tolerance of the plant to salt stress through the high increase of carotenoid production. This substance that compensates for the loss of chlorophyll caused by salinity, was not produced in the non-inoculated plants. *W. anomalus* WO2 also increased the tolerance of *S. fruticosa* by the excessive production of sugars which play an osmo-protective role and act as signalling molecules that trigger salt stress tolerance mechanisms. Increased sodium translocation is another mechanism by which inoculated plants resisted to high salt levels. These promising results indicate the possibility of using *W. anomalus* WO2 as PGPY to solve the problem of salinity affected soils, and to increase the tolerance to salt stress in high consumption plants in countries with arid and semi-arid climates.

Authors' Contributions

CA, NS and ADJ designed and supervised the study; CA, WS, FC, KE, AS and CM performed the laboratory experiments and bioassays, CA, WS, OK, SCH, SN and ADJ Writing - original draft, reviewed and edited the draft. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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