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MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL EFFECT OF SALICYLIC ACID ON ROOTS OF FABA BEAN (*VICIA FABA* L.) UNDER SALT STRESS

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Abstract

Legumes, such as faba beans, represent an important source of protein for human and animal nutrition. They also provide excellent natural soil fertilization through the symbiotic fixation of atmospheric nitrogen. Growth and development of *Vicia faba* L. can be limited by salinity, the major abiotic stress that affects crop yield, particularly in arid and semi-arid areas. Our study aims to demonstrate the long-term effect of the short-term salicylic acid seed treatment in two contrasting ecotypes of *Vicia faba* L. to mitigate the negative effect of salt stress.

Seed imbibition was applied with 0,25mM of SA at the early stage of germination, followed by100mM of NaCl until the flowering stage. Root length and some physiological and biochemical parameters are measured.

Salinity decreased root length, fresh and dry weight, and water content in roots of both faba bean ecotypes and increased non-enzymatic antioxidants (polyphenols and flavonoids) and proline accumulation. Interestingly, Lower levels of growth parameters under salinity were observed in "Nasmot" ecotype compared to "Tamelahet" ecotype.

The two ecotypes showed distinct responses to the exogenous SA Under salt conditions, the treatment enhanced the defense system of Nasmot by increasing proline content, polyphenols, and flavonoid concentration; it also increased FW, DW, and TWC. On the other hand, SA decreased all variables in Tamelahet, with the exception of increasing fresh and dry root weight.

Overall, salicylic acid can be recommended to develop tolerance in plants that show sensitivity to saline conditions.

Key words: Salinity, Salicylic acid, *Vicia faba* L., Defense system, Tolerant and Sensitive ecotypes.

Introduction

Vicia faba L. is one of the seed legumes that are distinguished by high levels of genotypic diversity related to their geographical origin and ecological habit (Wang *et al*., 2012; El-Esawi, 2017). Broad bean has a considerable agronomic and environmental role and is cultivated for its nutritional and medicinal value (Rabey *et al*., 1992; Cao *et al*., 2017; Dhull *et al*., 2022). Salinity is an ever-present constraint and a major threat responsible for significant losses in agricultural and legume crop production (Anon., 2008; Bimurzayev *et al*., 2021). It affects plant growth directly by increasing the osmotic pressure of the soil solution, accumulating certain ions at toxic concentrations in plant tissue, and altering the plant's mineral nutrition. It also induces oxidative stress (Allison, 1964; Kesawat *et al*., 2023).

Reclamation of the salt-affected plants can be achieved by several techniques, such as the exogenous application of plant growth regulators (Faghih *et al*., 2019). An optimal concentration of exogenous salicylic acid at different stages of plant growth, whether by seed priming, plant irrigation, or foliar spray, is known to improve the growth and survival of faba bean plants under salt stress conditions (Azooz, 2009; Orabi *et al*., 2013; Dawood *et al*., 2022).

The current study was conducted to assess the effect of the exogenous 0,25mM SA applied at the early stage of seed germination by imbibitions on the roots of two ecotypes of *Vicia faba* L., that have previously shown different responses (seeds vigor) under salt treatment. With three concentrations of NaCl (0, 100 and 200 mM) on differents ecotypes of faba bean. The results confirmed that Tamelahet had the best seeds vigor and more tolerant than other ecotypes like Nasmot (data not shown).

Material and Method

Two ecotypes of *Vicia faba* L. (Tamelahet and Nasmot) were chosen for the experiment from a collection produced during the year 2021 and taken from various geographic regions with a variety of environmental conditions (Table 1).

Table 1. Information about the ecotypes tested

	Tamelahet	**Nasmot**
Origin country	Tamelahet (East of the city of Tissemsilt, Algeria).	Nasmot (the southeast of the city of Mascara, Algeria)
Geographical coordinates	Latitude: 35.8126 Longitude: 1.62372 35° 48′ 45″ North, 1° 37′ 25″ East **Altitude: 684m** **(Köppen classification: Csa)**	Latitude: 35.25 Longitude: 0.383333 35° 15′ 0″ North, 0° 22′ 60″ East **Altitude: 935m** **(Köppen classification: BSk)**
Climate	Mediterranean climate with hot summer Average annual precipitation: 18.63mm Annual high temperatures: 22.47ºC (72.45ºF) Annual low temperatures: 12.2ºC (53.96ºF)	Dry and cold semi-arid climate Average annual precipitation: 15.41mm Annual high temperatures: 23.84ºC (74.91ºF) Annual low temperatures: 13.57ºC (56.43ºF)
Seeds description	Seeds have a round and oval shape and light brown color small seed size19×15mm	Seeds have a slightly flattened and oblong shape of beige and light brown color medium size 25×17mm

An unpublished study reveals that Tamelahet and Nasmot were two contrasting ecotypes (salinity tolerant and salinity sensitive, respectively) based on germination kinetics, radicular length, and final germination rate.

Seeds were surface sterilized with 10% sodium hypochlorite and rinsed with distilled water. The experimental arrangement applied to two ecotypes was composed of 4 treatments, including control (0 and 100mMNaCl): 4 replicates of 6 seeds per Petri dish for each treatment. Ten ml of hormonal solution (0.25 mM of salicylic acid) was added to petri dishes for imbibition for 24 hours, followed by salt solution (100mM NaCl) or distilled water for 10 days from the first day. Petri dishes were placed in the dark at 22°C. After the emergence of the radicals, seeds are transplanted into pots and kept in a greenhouse until the flowering stage. Plants were irrigated, followed with the corresponding solutions prepared with tap water.

At the end of the experiment, root length (RL) was measured using a meter scale, and it was weighed to determine their fresh weight (FW), and then oven-dried (80 °C for 48 h) to obtain their dry weight (DW).

Tissue water content (TWC) in roots was calculated with the following equation:

$$TWC\% = [(FW-DW)/FW] *100$$

where FW is fresh weight and DW is dry weight,

Proline was extracted according to the A.O.A.C. method (1955), modified by Nguyen & Paquin (1971). The determination was made according to the method of Bergman & Loxley (1970). The contents are expressed in µg/mg of dry vegetal material. Optic density reading at 515nm using a spectrophotometer was recorded. The results were obtained using a calibration curve with proline as the solution.

Extraction and quantification of polyphenols were performed according to the McDonald *et al*., (2001) method; the absorbance was measured with a spectrophotometer at 765nm using gallic acid and converted into µg/mg of dry material.

Flavonoids were determined according to Kim *et al*., (2003) method:400µl of methanolic extract was placed in a tube with 1200 µl of distilled water and 120 µl of 5% $Na^{+}NO_2$, followed by 120 µl of 10% $AlCl_3$ and 400 µl of 1 M NaOH. The absorbance was measured instantly at 510 nm. A calibration curve containing catechin was used to calculate the flavonoid content. The results are given in µg/mg of dry mass.

Statistical analyses

All data were analyzed statistically by factorial ANOVA using the STATISTICA program. Values are represented as the mean ± standard error and derived from four replicates (n=4). The bars show the standard errors. Different letters above the bars indicate significant differences, and bars with the same letter do not significantly differ at the five percent (p<0,05) significant level according to Duncan's tests.

Results

Morphological parameters

Roots length RL (cm): The effects of NaCl, SA, and their combined treatment on root length are presented in (Fig. 1). Statistical analyses showed a significant effect of each treatment (NaCl, SA, and NaCl*SA) on root length, while NaCl presented a significant negative correlation between salinity and RL (Tables 2 and 3). NaCl-only treatment reduced the root growth of both ecotypes by 10.34% and 33.71%; in contrast, it was maximized by SA treatment under non-saline conditions by 28,25cm and 30,25cm respectively, in Tamelahet and Nasmot. Salicylic acid accentuated an increase in root length in Nasmot by 1,72%, but it decreased in Tamelahet by 24,35% when compared with the stressed control.

Physiological parameters

Fresh and dry weight FW, DW (g): Table 3 presents a significant negative correlation between the treatments (NaCl and SA) and the FW and DW. It was also revealed in (Fig. 2) that NaCl treatment decreased FW and DW, respectively, by 90.79% and 90.94% in Tamelahet and 91.50% and 91.41% in Nasmot compared to the corresponding unstressed plants. It's also the effect of SA-only treatment, which decreased FW and DW by 22,07% and 1,9% in Tamelahet and 32,14% and 37,28% in Nasmot.

However, this pattern was reversed in the stressed plants treated with SA at their juvenile stage; the treatment increased FW and DW of roots in both ecotypes respectively by 37.74% and 54.77% in Tamelahet and 33.67% and 26.45 % in Nasmot when compared with the stressed control.

Table 2. Univariate tests of significance ANOVA for the effect of SA, Ecotype, NaCl, and their interactions in roots length (RL), fresh weight (FW), dry weight (DW), tissue water content (TWC), proline accumulation, polyphenols, and flavonoid concentration are given in Table 2.

	RL	FW	DW	TWC	Proline	Polyphenols	Flavonoids
Ecotyp	0,697187	0,028982	0,050016	0,671186	0,897370	0,144995	0,022538
NaCl	0,000000	0,000000	0,000000	0,195179	0,057989	0,000054	0,001983
SA	0,019617	0,000011	0,000735	0,196569	0,073286	0,271307	0,303721
Ecotyp*NaCl	0,146785	0,050670	0,070600	0,310605	0,775419	0,611416	0,951848
Ecotyp*SA	0,327824	0,824254	0,557978	0,332931	0,552486	0,182059	0,139536
NaCl*SA	0,004977	0,000038	0,002042	0,785697	0,828973	0,000099	0,077067
Ecotyp*NaCl*SA	0,448293	0,713346	0,541016	0,565052	0,385526	0,006698	0,484098

Table 3. The correlation matrices of morpho-physiological and biochemical attributes of two faba bean roots in response to 0,25mMsalicylic acid and salt stress were calculated using the STATISTICA program. Correlations were significant at *p*<0 ,05000.

	Ecotype	NaCl	SA	RL	FW	DW	TWC	Proline	polyphenols	Flavonoids
Ecotype	1,000000	-0,000000	-0,000000	-0,027404	0,129225	0,141649	-0,054191	-0,016153	-0,143460	0,263781
NaCl		1,000000	-0,000000	-0,797338	-0,799714	-0,750410	-0,166482	0,241281	0,424531	0,364872
SA			1,000000	0,168341	-0,277822	-0,252650	0,165957	-0,227552	-0,107835	0,116711
RL				1,000000	0,580366	0,551778	0,108165	-0,265214	-0,200284	-0,220934
FW					1,000000	0,955269	-0,080518	-0,127445	-0,409392	-0,289346
DW						1,000000	-0,285470	-0,110885	-0,388248	-0,252881
TWC							1,000000	-0,231709	0,138908	0,051228
Proline								1,000000	0,106644	0,108072
Polyphenols									1,000000	0,706050
flavonoids										1,000000

Tissue water content (TWC) (%): Results of the current study revealed that salinity caused a decrease in water content in Tamelahet (0.46%) and in Nasmot's roots (0.14%). Dehydration in the root of Tamelahet was caused by SA treatment under salt and normal conditions compared to the untreated control. Meanwhile, the same treatments increased water content in Nasmot only (Fig. 3). Statistically, the individual and interaction treatments had no significant effect on TWC.

Biochemical parameters

Proline accumulation (µg/mg DW): Results of (Fig. 4) showed that, NaCl increased root proline accumulation by 2,54% in Tamelahet and 16,71% in Nasmot, whereas SA treatment under salinity raised proline by 4,20% in Nasmot; meanwhile, it decreased in Tamelahet by 13,22 % as compared with the stressed control. Statistically, SA has no significant effect on proline accumulation (Tables 2 and 3).

Polyphenols were present in the roots of *Vicia faba* L. at normal conditions; NaCl improved a significant increase in the polyphenol concentrations by 681,78% in Tamelahet and 205,28% in Nasmot compared to the corresponding unstressed control (Fig. 5). On the other hand, NaCl had statistically significant effect on polyphenol synthesis (Table 2). SA treatment under saline conditions showed a significant decrease in polyphenol synthesis in Tamelahet by 61.02%. while a significant increase in Nasmot by 3.64% was observed to the stressed control.

Flavonoids (µg/mg DW): NaCl also improved a significant increase in flavonoids concentration by 109,672% in Tamelahet and 95,604% in Nasmot compared to the corresponding unstressed control (Fig. 6). In addition, NaCl had a statistically significant effect on flavonoid synthesis.

SA treatment under saline conditions showed a significant decrease in flavonoids synthesis in Tamelahet by 15.07%, while it was significantly increased in Nasmot by 42.05% compared to the stressed control.

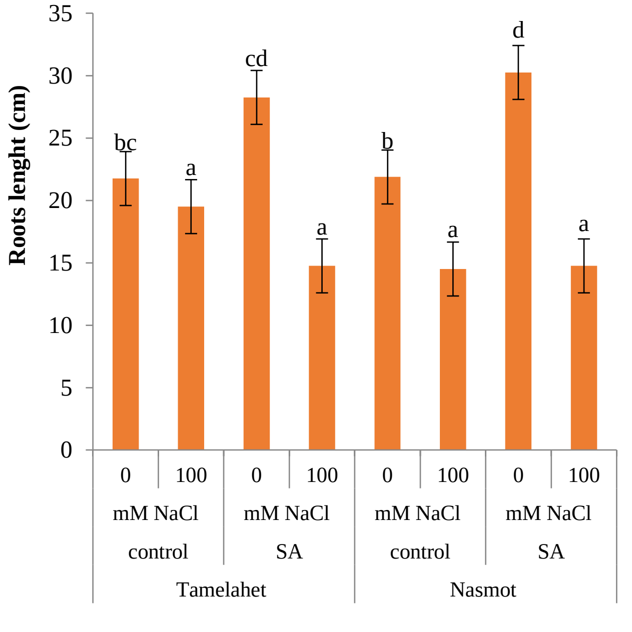

Fig. 1. Effect of SA treatment on roots length (RL) of two faba bean ecotypes (Tamelahet and Nasmot) under saline and non-saline condition.
Values are shown as the mean±standard and derived from four replicates (n=4). Bars indicate standard errors. Different letters above the bars indicate significant differences at the 5% significant level according to Duncan's test.

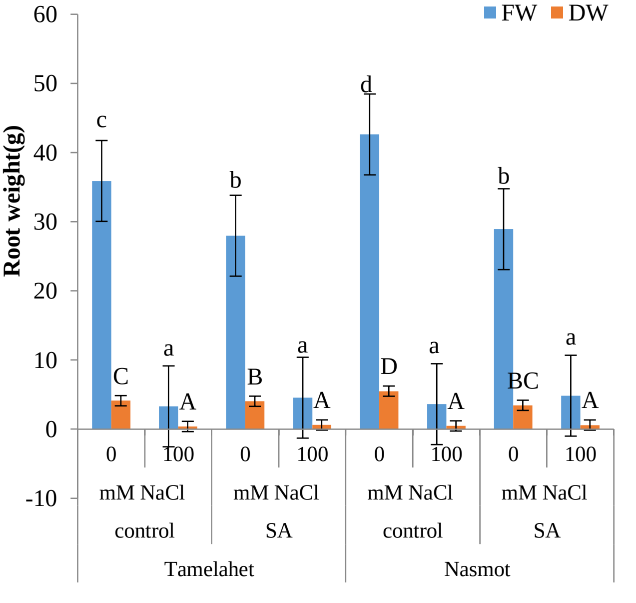

Fig. 2. Effect of SA on fresh and dry roots weight (FW, DW) of two faba bean ecotype (Tamelahet and Nasmot) under saline and non-saline condition.
Values are shown as the mean ± standard and derived from four replicates (n=4). Bars indicate standard errors. Different letters above the bars indicate significant differences at the five percent significant level according to Duncan's test. (lower case for FW, upper case for DW).

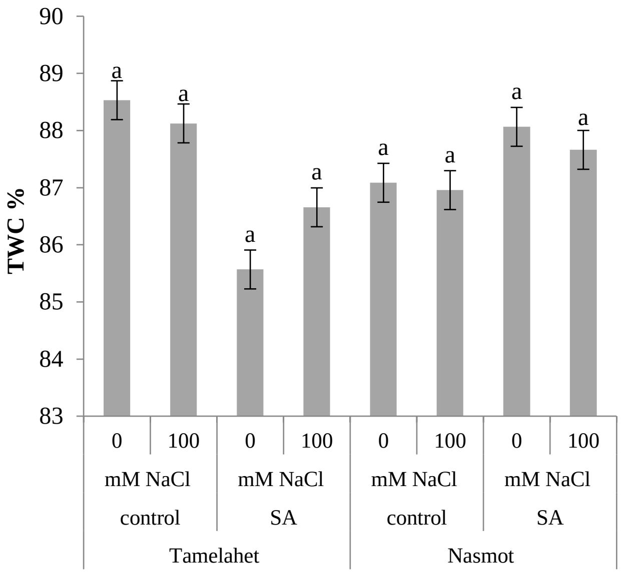

Fig. 3. The effect of SA, NaCl, and combined treatment on the variation of water content in two ecotypes of faba bean roots (Tamelahet and Nasmot).
Values are shown as the mean ± standard and derived from four replicates (n=4). Bars indicate standard errors. Different letters above the bars indicate significant differences at the five percent significant level according to Duncan's test.

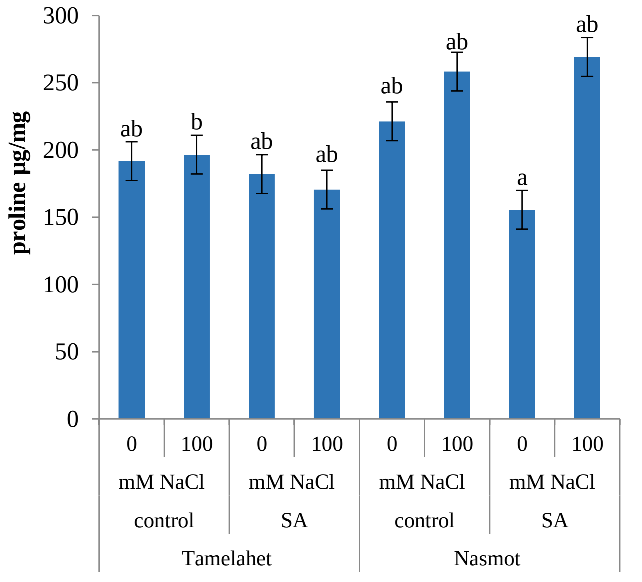

Fig. 4. The Effect of SA, NaCl and combined treatment on Proline accumulation in roots of two faba bean ecotype (Tamelahet and Nasmot).
Values are shown as the mean ± standard and derived from four replicates (n=4). Bars indicate standard errors. Different letters above the bars indicate significant differences at the five percent significant level according to Duncan's test.
Polyphenols (µg/mg DW)

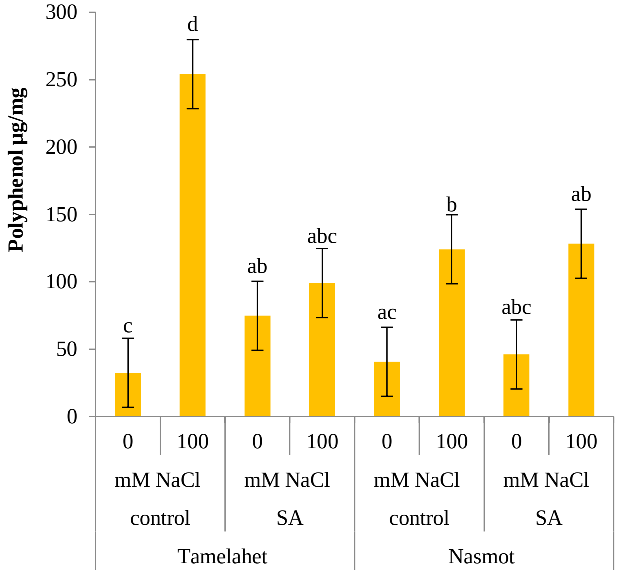

Fig. 5. The Effect of SA, NaCl and combined treatment on polyphenol content in roots of two faba bean ecotype (Tamelahet and Nasmot).
Values are shown as the mean ± standard and derived from four replicates (n=4). Bars indicate standard errors. Different letters above the bars indicate significant differences at the five percent significant level according to Duncan's test.

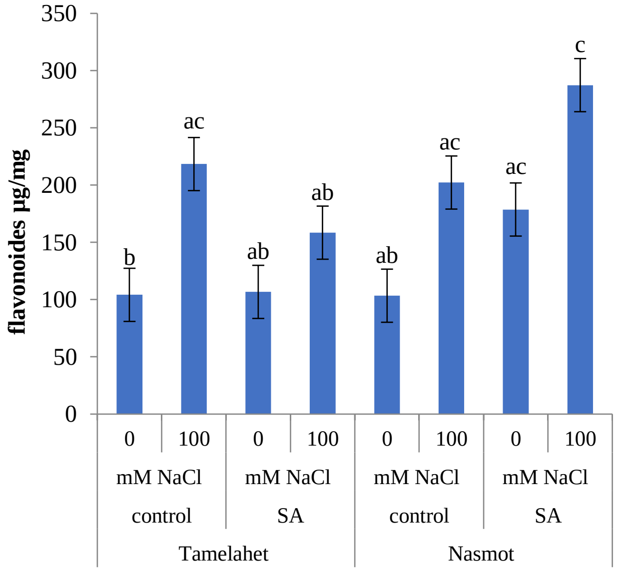

Fig. 6. The Effect of SA, NaCl and combined treatment on flavonoids content in roots of two faba bean ecotype (Tamelahet and Nasmot).
Values are shown as the mean ± standard and derived from four replicates (n=4). Bars indicate standard errors. Different letters above the bars indicate significant differences at the five percent significant level according to Duncan's test.

Discussion

The effect of salt stress on the roots of both ecotypes of *Vicia faba* L. presented in our results was similar to that reported in numerous studies. Salinity affects plant performance by reducing osmotic potential, which results in a decrease in plant water transmission. It also affects root growth because of the inhibition of cell division and the ionic toxicity caused by NaCl (Khodary, 2004; Hussain *et al*., 2011; Fahad&Bano, 2012; Ahmad *et al.*, 2018). Length of roots, fresh and dry weight, and other physiological parameters are usually used to determine plants sensitivity (Hniličková *et al*., 2019). In the present study based on the lower root growth parameters under salinity, the ecotype Tamelahet proved to be more tolerant than Nasmot. Because Tamelahet enable a good root protection with high antioxidants activity (polyphenols, and flavonoids) and osmoticum accumulation (proline) than the sensitive one under salinity.

Proline is an osmolyte protector; its accumulation is involved in osmoregulation, membrane protection, enzyme and protein stability, and free radical scavenging under salt conditions (Hayat *et al.*, 2012). In our results, proline accumulation in the roots of both faba bean ecotypes under salt stress varied according to plant salt sensitivity, which was in agreement with Ouiza *et al*., (2010) study.

Salt stress also induces oxidative stress, increasing the production of ROS (reactive oxygen species), which play a role in systematic signaling from roots to leaves, allowing plants to engage their defense systems for improved salt stress resistance (Abd Elgawad *et al*., 2016). In this way, both ecotypes of *Vicia faba* L., responded to salt conditions through the accumulation of antioxidants, polyphenols, and flavonoidsin their roots at various concentrations. The response to salinity in faba beans is genotype-specific, so that the tolerant and sensitive faba bean genotypes can develop different mechanisms of adaptation to salt stress (Azooz, 2009; Bimurzayev *et al*., 2021). An RNA sequence analysis was performed between two contrasting faba bean ecotypes to discover genes that may be correlated with salt tolerance, indicating that genes involved in ABA and ethylene hormone metabolism were found to be down-regulated in the salt-tolerant ecotype under salt conditions (Yang *et al*., 2020).Whereas, Souana *et al*., (2020) found that the difference between genotypes was rather quantitative than qualitative, even though one genotype showed better growth, physiological, and molecular responses under salt stress than the other.

Our results showed that the differences between the ecotypes of *Vicia faba* L., appeared in response to salicylic acid treatment. The exogenous application of SA ameliorated the inhibitory effect of salinity on root length, fresh and dry mass, and water content; it also effectively boosted proline accumulation and the non-enzymatic antioxidant biosynthesis (polyphenols and flavonoids) in Nasmot's roots. These findings are in agreement with those of Khodary (2004), Misra & Saxena (2009), Zahra *et al*., (2010), Li *et al*., (2014), Anaya *et al*., (2018), and Ahmad *et al*., (2020) in many plant species that value the role of exogenous SA in protecting plants from salinity.

Tamelahet had another response in this condition the root length and water content were decreased along with proline, polyphenols, and flavonoids following SA treatment under salinity. Our findings differed from those of previous studies. However, the fresh and dry weights were regulated in the same way as those of Nasmot.

According to studies (Knörzer *et al*., 1999; Gorni *et al*., 2023; Yu *et al*., 2023), an optimal exogenous SA at the optimal duration can create a non-serious oxidative stress and an activation of the cellular anti-oxidative system. It also plays an essential role in promoting root growth. The reactions of Tamelahet under the treatments applied were probably due to the fact that the tolerant ecotype had an intrinsic hormonal capacity such as salicylic acid biosynthesis, and was able to defend itself against salinity; however, an inappropriate exogenous concentration of these hormones caused a change in endogenous SA levels "an excess", resulting in extensive oxidative damage and consequently cell death (Rao *et al*., 1997; Kim *et al*., 2017; Hu *et al*., 2022).

Conclusion

Exogenous treatment with salicylic acid is regarded as an efficient method for improving plant tolerance in the sensitive faba bean plant. The tolerant ecotype « Tamelahet » enable better protection in the roots against the high NaCl level compared to the sensitive one by the increased of non enzymatic antioxidants (Polyphenols and flavonoids) and proline content, this data confirms the protective role of these molecules against oxidative stress. Moreover, it proves that NaCl treatment cause important modification in the root antioxidant mechanism and proline metabolism induced by the hormonal defense « salicylic acid » at root growth stage. The present work can be useful for understanding salt tolerance adaptation machinery and breeding salt-tolerant genotypes in faba bean legume for food security in Algeria and arid land.

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