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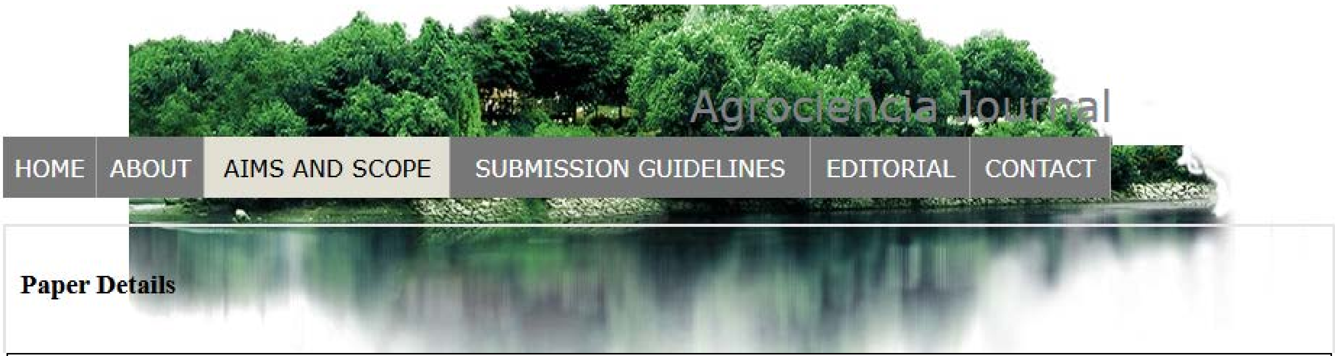

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Keywords: alfalfa, legumes, bio stimulant, abiotic stress, seed germination

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Farina di Basalto® application ameliorates the adverse effects of salinity on seed germination and early seedlings growth of *Medicago sativa* L.

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Abstract

Salinity is one of the major constraints prevailing in the environment, affecting plant growth, agricultural productivity, and soil fertility. The application of beneficial silicon-rich bio-stimulants presents an alternative strategy to ensure agricultural sustainability, as their use and expansion can help mitigate the adverse effects of salinity and reduce the excessive use of synthetic chemicals. The objective of this study was to evaluate the interaction between salinity levels and the use of Farina di Basalto® Type XF (FdBXF) on the germination and early seedling growth of two alfalfa (*Medicago sativa* L.) varieties (Gabes and Azzura). Five levels of sodium chloride (0, 50, 100, 150, and 200 mM) and five levels of Farina di Basalto® Type XF (0, 1, 3, 5, and 10%) were tested in 30 treatments under a completely random design. Germination percentage, mean germination time, radicle length, hypocotyl and cotyledon length, and biomass were measured at the end of the experiments. Sodium chloride significantly reduced all germination and seedling growth parameters. The reduction in the measured parameters was inversely proportional to increasing concentrations of NaCl. The application of

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Keywords: alfalfa, legumes, bio stimulant, abiotic stress, seed germination

1. Introduction

In several areas around the globe, salinization is recognized as the major process of land degradation (Chen et al. 2022). On average, the world loses 10 hectares of cultivable land per minute, with 3 hectares attributed to salinization. Furthermore, 10 to 15% of irrigated areas (20 to 30 million hectares) are affected to varying degrees by salinization problems (Mermoud 2006). The phenomenon remains the greatest constraint, affecting both agricultural soils and rangelands. It significantly reduces soil fertility, often rendering it sterile and unsuitable for cultivation or the growth of diverse vegetation, except for halophytes. This problem leads to a reduction in cultivable areas and, combined with other factors, poses a threat to the food balance in arid and semi-arid regions (Hachicha 2007; Ltaeif et al. 2021).

The germination stage is considered the most critical and crucial stage in the life cycle of higher plants and for seedling development and establishment (Wolny et al. 2018). This stage may be negatively affected by salinity, causing both water stress and ion toxicity, which can interfere with the enzymatic activity of higher plants (Parmoon et al. 2018). Under salinity, seed germination decreases with increasing salinity, leading to a delay in seed establishment (Taghvaei et al. 2022). Furthermore, salinity stress causes damage to enzymes and other biological functions, resulting in malfunction and ionic leakage from cell membranes (Munns & Tester 2008; Malik et al. 2022). All these factors influence seed germination traits, as well as the length of the root, hypocotyl, and cotyledons, and affect the dry biomass.

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The response of plants to salinity depends on several variables, including the species, the variety, stress severity, growth conditions, and the stage of development (Ben Naceur et al. 2001). To address salinity, selecting appropriate crops presents a valuable alternative. In this context, alfalfa (*Medicago sativa* L.), known as the queen of forage crops, is highly regarded compared to other forage legumes due to its high dry matter yield, nutritional value, and tolerance to drought and salinity (Midoun & Kadri 2015). However, the effectiveness of crop choice may be limited in the face of the alarming growth rate of salinization. In response to salinity, the application of bio-stimulants beneficial to the growth and development of plants can serve as a complementary and practical strategy.

Various management strategies have been tested to mitigate the adverse effects of challenging environmental conditions (Soliman et al. 2019; Alharbi et al. 2018; Shedeed et al. 2022).

The aim of this study was to determine whether the application of different concentrations of Farina di Basalto® type XF could mitigate the effect of increasing doses of NaCl on the germination and early seedling growth of two *M. sativa* varieties, Gabes and Azzura. We hypothesised that Farina di Basalto® type XF could alleviate the adverse effects of salinity on seed germination and early seedling growth of *M. sativa* and that the response to salinity would depend on both the variety and the concentration. Various management strategies have been tested to mitigate the adverse effects of challenging environmental conditions (Soliman et al. 2019; Alharbi et al. 2018; Shedeed et al. 2022).

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2. Material and methods

"Farina di Basalto®": Farina di Basalto® type XF consists of micronized particles (⌀ < 20 µm) obtained through an industrial process. The raw material utilized to produce Farina di Basalto® is sourced from the Castel Viscardo deposit (TR) - Italy. Basalt is an effusive volcanic rock belonging to a predominantly mafic chemical composition. It is a basic rock devoid of free crystalline silica, amentiferous minerals, or other substances harmful to the environment or animal health. Farina di Basalto® is derived by the mechanical grinding of the pure mineral, using ceramic tools, without the addition of other minerals or substances. Furthermore, no materials containing washing water with flocculants, or undesirable and harmful products for agriculture, are used in its production. There are various types of basalt, each with unique characteristics related to their composition, structure, and origin. Based on its chemical composition, mineralogy, and physical characteristics, the basalt used for the production of Farina di Basalto® (FdB) can be classified as phonolitic tephritic basalt and has unique characteristics. The basalt used for the production of Farina di Basalto® is particularly prized for its unique composition of micro and trace elements that are useful for plants.

Farina di Basalto® is known for its fertilizing effects, due to its richness in Silicon, Alumina, Potassium, and Calcium (Elimem et al. 2022). Silica (silicon dioxide) is the main component of Farina di Basalto® type XF, with a percentage of 47% (Table 1).

Table 1. Physicochemical characteristics of Basalt powder Farina di Basalto® type XF

Physicochemical characteristics	
Solid physical state	Powdery
Water solubility	Not soluble in water
Colour	Slightly gray
Odour	Not noticeable
pH	9±0.5 (Log[H+])
Electrical conductivity (2:1 extract)	1.14 (dS /m)
Cation exchange capacity	9 (meq/100g)
Assimilable iron (As. Fe)	377 (mg/kg)
Density	2.70 (kg/dm^3)
SiO_2	47%
K_2O	9%
Fe_2O_3	6.85%
CaO	8%
MgO	2.25%
Na_2O	3.55%
P_2O_5	0.65%
TiO_2	0.6%
Mn	636 mg/Kg
S	536 mg/Kg
B	81 mg/Kg
Cu	51 mg/Kg
Zn	68 mg/Kg

2.2. General germination procedure

All the experiments were conducted at the Agricultural Production Systems and Sustainable Development Laboratory (Higher School of Agriculture of Mograne, Tunisia). Two varieties of *M. sativa* were used: Azzura, supplied by Basalti Orvieto®, and Gabes, from the HSA of Mograne seed bank. Seeds were randomly selected from a bulk sample and manually cleaned to remove debris and damaged seeds and were stored in paper bags in a seed storage room (temperature: 15–5°C; humidity: 40%–60%) until their use. The surface of the seeds was sterilized with 5% commercial bleach for 15 seconds and then carefully washed for 2 minutes with sterile deionized water. For each condition, 10 replicates of 10 seeds were used. Seeds were placed on Petri dishes containing 2 layers of Whatman #1 filter paper, initially moistened with 5 ml of distilled water or the assigned test solution, and incubated under each condition in an incubator with fluorescent cold white lights producing a PPFD (photosynthetic photon flux) of 40 µmol.m^{-2}.s^{-1} (measured with the SB quantum 190 sensor, Li-Cor Inc., Lincoln, NE, USA). The Petri dishes were closed and sealed with adhesive tape (Parafilm™) to prevent desiccation. The photoperiod in the incubator was set at 12 h to coincide with the highest temperature range (Baskin & Baskin 2014). Seed germination was evaluated 14 days after the start of the experiment. Seeds were considered germinated when a radicle reached 2 mm in length. Seed viability was checked by physically pricking the seed to see if the embryo was fleshy rather than hollow inside. To determine the 1000 seed weight (TSW), 4 replicates of 100 seeds were randomly selected, counted by hand, and weighed using an analytical balance with an accuracy of 0.0001 g (Kaya et al. 2012; Tonguç & Erbaş 2012). The levels of the different treatments were determined based on the results of the preliminary tests that established the tolerance interval.

2.3. Treatments application

NaCl application: The impact of salt stress on seed germination in both varieties was assessed under five levels of salt stress. This was accomplished by incubating the seeds with solutions of 0, 50, 100, 150, and 200 mM sodium chloride (NaCl). Distilled water was used as a control.

Farina di Basalto® type XF application: The different concentrations were prepared by dissolving four different weighed quantities of Farina di Basalto®: 0, 1, 3, 5, and 10 g per 100 ml of distilled water at room temperature.

Combined effect of NaCl and Farina di Basalto® type XF: The combined effect of NaCl and Farina di Basalto® type XF was applied by combining the five levels of NaCl (0, 50, 100, 150, and 200 mM) and the five levels of Farina di Basalto® type XF (0, 1, 3, 5, and 10%).

2.4. Measured parameters

Quantifying effects on germination: The germination percentage (GP) was calculated according to Hamdi et al. (2022): GP (%) = (Nt × 100) / N, where Nt is the number of seeds germinated in the respective treatments, and N is the total number of seeds used in the bioassay.

Quantifying effects on seedling growth parameters: At the end of the experiment, a destructive harvesting approach was applied by separating the seedlings into parts (cotyledon, hypocotyl, and radicle), immediately freezing them, and storing them in a freezer at -20°C for further analysis. The dry weight of seedlings, root, hypocotyl, and shoot lengths were then recorded.

Quantifying effects on seedling water relations: The water content (WC) was measured according to Ltaeif et al. (2021): WC (%) = [(FW - DW) / FW] × 100. The samples were placed in a forced air oven at 70°C for 24 hours, and the dry weight (DW) was recorded in mg per plant.

2.5. Data analysis

Each trial was carried out according to a completely randomized design (CRD) with four repetitions. Statistical analyses were carried out using SPSS v. 25 (SPSS Inc., Chicago, IL), applying a significance level (α) of 0.05. Deviations from the arithmetic mean were calculated as the standard error (SE). Prior to statistical analysis, data series were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. Cotyledon and radicle lengths were compared between varieties, [NaCl], [Basalt], and their concentrations and interactions using general linear models (GLM). Since normality or homogeneity of variance was not achieved, the final germination percentage, radicle length, and dry weight were analysed using generalized linear models (GLZ) with the Wald Chi-square test (χ^2) (Ng and Cribbie, 2017). The effect of each NaCl and Basalt concentration on the germination and seedling traits of each species was analysed using three-way ANOVA in PLABSTAT v. 3A (Utz, 2011). We used the Mann-Whitney U test as a post hoc test to identify significant differences between treatment means with honest significant difference (HSD) at $p < 0.05$.

3. Results and discussion

Effect of Farina di Basalto® type XF on germination under salt stress: Under control conditions (0 mM NaCl), the application of Farina di Basalto® had no significant effect on the germination percentage of the two varieties of *M. sativa* ($p > 0.05$) (Figure 1). In comparison to the control, salt stress significantly reduced the germination percentage (Figure 1A, Table 1). In *M. sativa* varieties Gabes and Azzura, the lowest NaCl concentrations (50, 100, and 150 mM for Gabes, and 50, 100 mM for Azzura) did not cause a significant difference compared to the control ($p > 0.05$). Above these concentrations, the plants began to show signs of toxicity, attributed to the accumulation of chlorides within plant tissues. The most notable reduction was recorded at a concentration of 200 mM for both varieties: Gabes (ca. -82%) and Azzura (-74%). However, the addition of Farina di Basalto® type XF mitigated the reduction in germination percentage caused by salt stress ($p < 0.01$). The most significant mitigation was observed with 1% Farina di Basalto® in *M. sativa* var. Gabes, showing a 122% increase in germination percentage under 200 mM NaCl. Additionally, using 3% Farina di Basalto® under the same conditions for treated seeds of *M. sativa* var. Azzura resulted in a greater mitigation, with a ca. 127% increase in germination percentage.

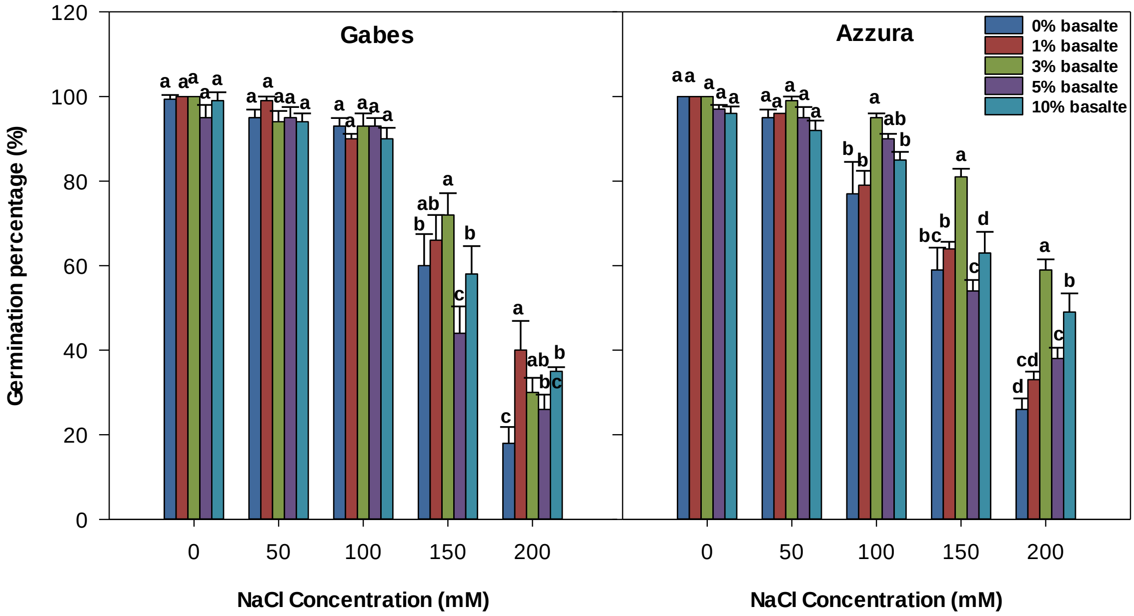

Figure 1. Effect of Farina di Basalto® type XF on the germination percentage of *M. sativa* var Gabes and var Azzura under NaCl. Data are shown as the means ± SE (n=4). Different letters indicate significant differences (one-way ANOVA according to Mann-Whitney U multiple comparison test, $p<0.05$).

These beneficial effects of Farina di Basalto® type XF could be attributed to silicon (Si), a non-essential element for plants (Meng et al. 2020). Our findings align with previous reports on the positive effects of silicon in mitigating the adverse effects of salinity on biochemical and physiological parameters in bean (*Phaseolus vulgaris* L.) (Oral et al. 2019), as well as on germination and juvenile growth in barley (*Hordeum vulgare* L.) (Ellouzi et al. 2023). Recently, several studies have summarized that Si plays an essential role in regulating various physiological and biochemical mechanisms to enhance plant growth under harsh conditions (Mir et al. 2022). The beneficial effects of Si can be attributed to several processes related to water balance, ion homeostasis, osmotic adjustment, and most importantly, the attenuation of toxic chemicals (Debona et al. 2017). Moreover, the positive impact of Si in terms of exogenous application was strongly associated with reactive oxygen species (ROS) homeostasis and improved antioxidant defence under salt stress (Zhu et al. 2019).

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Effects of Farina di Basalto® on seedling growth under salt stress: Under salinity, a significant decrease in the length of cotyledons, hypocotyls, and radicles ($p < 0.05$) was observed in all treatments when compared to the control (Figure 2A, B). A progressive decrease was recorded for these parameters, reaching their minimum values at a concentration of 200 mM NaCl. For a concentration of 3% Farina di Basalto® under salt stress, the lengths of the cotyledons and hypocotyls increased significantly compared to the control ($p < 0.01$) (Figure 2).

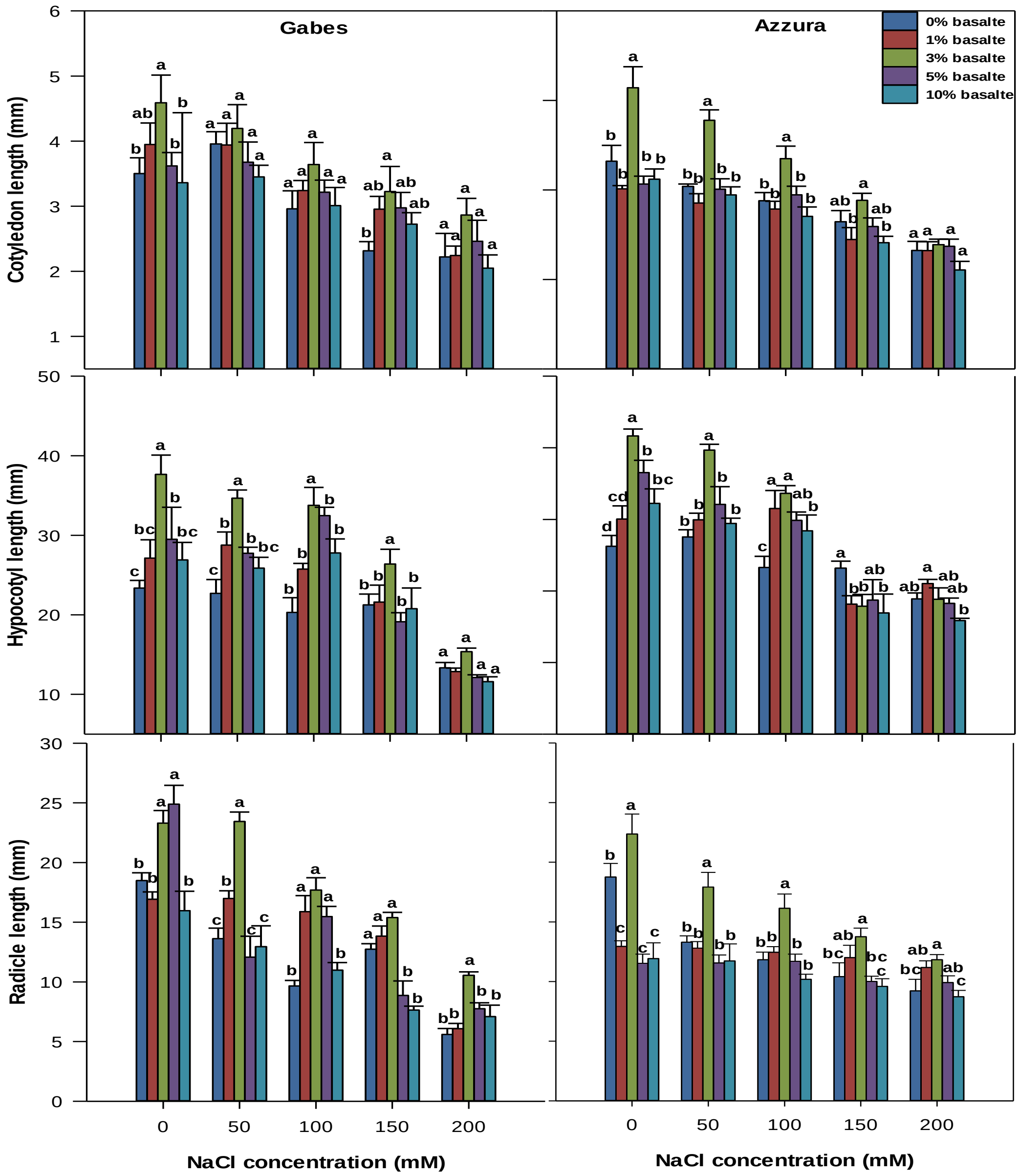

FIGURE 2. Effect of Farina di Basalto® type XF on the length of cotyledons, hypocotyl and radicle of *M. sativa* var Gabes and var Azzura under NaCl. Data are shown as the means ± SE (n=4). Different letters indicate significant differences (one-way ANOVA according to Mann-Whitney U multiple comparison test, $p<0.05$).

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For cotyledon length, under control conditions, the addition of Farina di Basalto® significantly increased cotyledon length, with the highest values recorded at 3% basalt for both varieties, Gabes and Azzura, resulting in an increase of 102.4% and 57%, respectively. For *M. sativa* var. Gabes, salt stress significantly reduced cotyledon length. The most significant reduction in cotyledon length was recorded at 200 mM, with a reduction of 26.55%. The addition of basalt significantly increased this parameter under 150 mM, particularly at 3% basalt, resulting in a substantial increase of 22.4% in cotyledon length. The highest cotyledon length in both control and treated seedlings was recorded with 3% Farina di Basalto®. Similarly, for *M. sativa* var. Azzura, salt stress significantly reduced cotyledon length, with the highest reduction recorded at 200 mM, resulting in a 37.55% decrease. The addition of basalt significantly increased cotyledon length at all NaCl concentrations, except at 200 mM. The highest cotyledon length was recorded with the addition of 3% Farina di Basalto® to treated seedlings.

Regarding hypocotyl length, the addition of basalt significantly increased the hypocotyl length in seedlings of both varieties, Gabes and Azzura, under control conditions. The highest hypocotyl length was recorded with 3% Farina di Basalto®, resulting in increases of 82.56% and 58.66% for *M. sativa* var. Gabes and var. Azzura, respectively, compared to control seedlings. For *M. sativa* var. Gabes, salt stress led to a reduction in hypocotyl length, with the highest reduction recorded at 200 mM (43.66%) compared to the control. The addition of Farina di Basalto® type XF significantly mitigated the effect of salt stress, with the highest mitigation recorded when 3% basalt was added to seedlings treated with NaCl. For *M. sativa* var. Azzura, under control conditions, the highest hypocotyl length was recorded in seedlings treated with 3% Farina di Basalto®, compared to those without basalt. Salt stress reduced hypocotyl length in seedlings, with the most significant reduction recorded at 200 mM, showing a 28% reduction compared to the control. The addition of 3% Farina di Basalto® type XF proved to be the most effective concentration for mitigating the effects of salt stress compared to the other concentrations of basalt studied.

For radicle length, under control conditions, the addition of Farina di Basalto® at 3% and 5% for *M. sativa* var. Gabes and at 3% for var. Azzura seedlings has significantly increased this parameter by 61.64% and 52.21%, respectively. Similar to cotyledon and hypocotyl length, the radicle length of treated seedlings from both varieties significantly decreased with the increase in NaCl concentration compared to non-treated seedlings. The most substantial reduction in radicle length was recorded at 200 mM, with a reduction of 69.74% and 25.56% for *M. sativa* var. Gabes and var. Azzura, respectively. However, the addition of Farina di Basalto® significantly mitigated the impact of salt stress. At 200 mM of NaCl, the highest mitigation was recorded with the addition of 3% Farina di Basalto®, resulting in an increase in radicle length of 88.52% and 6.23% for *M. sativa* var. Gabes and var. Azzura, respectively, compared to treated seedlings without basalt (Figure 2).

Si, the major constituent of Farina di Basalto® type XF, has garnered much interest in recent years as a defensive bio-stimulant when applied exogenously in cultivated plants against environmental stresses, including salinity (Ellouzi et al. 2022). The positive impact of Si through exogenous application can be strongly associated with the regulation of ROS homeostasis and improved antioxidant defence (Zhu et al. 2019). Similar results were obtained in cultivated sainfoin (*Onobrychis viciifolia* Scop.) (Oral et al. 2019; Wu et al. 2017).

The concentrations of 50 and 100 mM NaCl had no significant effect on the total biomass of *M. sativa* var. Gabes. However, above these concentrations, salt significantly decreased this parameter. For *M. sativa* var. Azzura, only the concentration of 50 mM NaCl had no effect on the total biomass of treated seedlings. The most substantial reduction in this parameter was recorded at 200 mM for both varieties, and the highest mitigation was observed with the addition of 3% Farina di Basalto® type XF (Figure 3). This result can be explained by the

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accumulation of chlorides in the tissues of the plant. However, following the application of Farina di Basalto®, type XF, the seedlings exhibited behaviour almost identical to that of the control seedlings. Consequently, it appears that Farina di Basalto® type XF intervenes in the process leading to the accumulation of salts in the plant's organs. Similar results were observed in rice (*Oryza sativa* L.) (Isa et al. 2010) and wheat (*Triticum durum* Desf.) (Bijanzadeh et al. 2019).

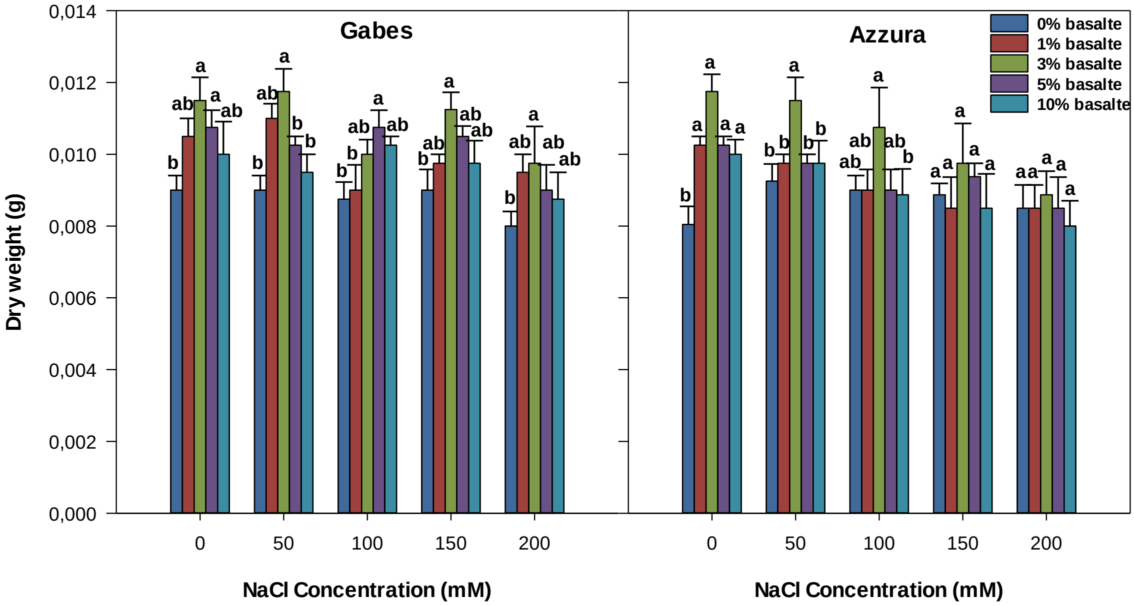

Figure 3. Effect of Farina di Basalto® type XF on seedling biomass of *M. sativa* var Gabes and var Azzura under NaCl. Data are shown as the means ± SE (n=4). Different letters indicate significant differences (one-way ANOVA according to Mann-Whitney U multiple comparison test, $p<0.05$).

Conclusion

In conclusion, *M. sativa* var. Gabes exhibited a higher tolerance to NaCl compared to *M. sativa* var. Azzura. Furthermore, seed priming with Farina di Basalto® Type XF mitigated the negative impacts of salinity by enhancing germination and seedling growth, offering a promising alternative for sustainable agriculture. Given the challenges of food insecurity driven by climate change, such applications could prove beneficial in the future. Field trials are recommended to explore the potential of seed priming in additional crops.

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Supplemental material

Table S1. General linear models (LM) for cotyledon and hypocotyl lengths for *Medicago sativa* var. Gabes and var. Azzura comparing between varieties, NaCl and basalt concentrations. F, Fisher statistic; WS, Wald statistic; df, degree of freedom; p, probability.

Tests of Model Effects	Cotyledon length			Hypocotyl length		
Source	Type III			Type III		
	WS	df	Sig.	WS	df	Sig.
Intercept	34963.680	1	0.000	11827.866	1	0.000
Variety	4.688	1	0.030	22.921	1	0.000
NaCl	3492.663	4	0.000	586.483	4	0.000
Basalt	79.322	4	0.000	222.431	4	0.000
Variety * NaCl	51.801	4	0.000	131.296	4	0.000
Variety * Basalt	33.327	4	0.000	108.557	4	0.000
NaCl * Basalt	116.392	16	0.000	158.819	16	0.000
Variety * NaCl * Basalt	29.554	16	0.020	250.959	16	0.000