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Mapping Single Nucleotide Polymorphism Markers Associated with the Pre-Flowering Morphological Performance of Fenugreek under Different Levels of Salt Stress

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Abstract: Salinity is a significant factor restricting plant growth and production. The effect of salinity stress on different growth parameters of 111 fenugreek genotypes was examined in an experiment with three salinity levels (0, 3000, 6000 mgL^{-1}). A completely randomized block design with two replicated pots per treatment was used. Non-significant treatment effects were observed on fresh weight (FW); however, all traits showed significant genotype-by-treatment (GxT) interactions. This GxT was reflected in substantial SNP x environment interactions. Of 492 significant SNPs associated with the measured traits, 212 SNPs were linked to the correlated traits using an arbitrary threshold of three. Several SNPs were associated with FW and dry weight, measured under the same salinity treatment. The correlation between both traits was 0.98 under the three salinity treatments. In addition, 280 SNPs with conditional neutrality effects were mapped. The identified SNPs can be used in future marker-assisted breeding programs to select salt-tolerant genotypes. The results of this research shed light on the salt-tolerant properties of fenugreek.

Keywords: *Trigonella foenum-graecum*; salinity tolerance; genetic variability; genotyping; single nucleotide polymorphisms

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1. Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is a dicotyledonous and annual diploid member of the *Fabaceae* family. Fenugreek leaves and seeds are consumed for their culinary and medicinal properties [1–3]. Its native range extends from Iran to northern India, and it is commonly grown in China, India, Egypt, Ethiopia, Morocco, Ukraine, Greece, and Turkey [4–6]. It has demonstrated health benefits such as improving insulin sensitivity and glucose tolerance, cardiovascular health, metabolic homeostasis, and anti-inflammatory effects. It has also been shown to strengthen muscle mass and exercise performance by increasing serum testosterone levels [7,8].

The growing human population and the limited land available for cultivation are two factors endangering agricultural sustainability [9,10]. Like many other leguminous crops, fenugreek production is affected by environmental stresses such as drought, salinity, and heat [11]. Salinity and sodicity affect more than 25% of all land and 33% of all irrigated land worldwide; therefore, they have been significant environmental hazards of the past century [10]. Soil salinization severely limits the amount of fenugreek biomass produced globally from agricultural farmlands [12–14].

Plants suffer from osmotic imbalances and toxic effects when salt concentrations exceed their maximum tolerance level. Salinity can potentially impede plant growth

in two ways: ionic, which accelerates the ageing of adult leaves, and osmotic, which restricts the development of young leaves. High ionic concentrations (primarily Na^+, but also other ions) within plant tissues are detrimental to plant metabolism, and high salt concentration in the growing medium prevents water from being absorbed by the roots (osmotic effect) [15–18]. Salinity stress generally limits seed germination, plant growth, fresh and dry mass production, chlorophyll content, root length, number of leaves, and yield [19–22]. Salt-tolerant plants use one of the three strategies to deal with salt stress. Ion exclusion: transporting Na^+/Cl^- in roots to reduce their accumulation within leaves. Tissue tolerance to accumulated ions: compartmentalizing high salt concentrations found in leaves at the cellular and intracellular levels. Osmotic tolerance: reducing shoot growth by long-distance signals triggered before shoot Na^+ accumulation [23,24].

Several genes quantitatively control morphological traits. The genomic regions that contain those genes are called quantitative trait loci (QTL) and are traditionally mapped using bi-parental populations, such as double haploid populations or recombinant inbred lines. Nevertheless, this method is hindered by the low mapping resolution and restricted variances in the mapping population. As a result, genome-wide association mapping (GWAM) was created, which relies on phenotyping many genotypes obtained from naturally evolved populations with more significant genetic variation. Due to past recombination processes spanning hundreds of generations, GWAM frequently identifies narrower intervals. Single nucleotide polymorphisms (SNPs), a codominant polymorphic marker, are used to identify such recombination events. These intervals and potential genes could offer essential features for marker-assisted selection, phenotypic introgression, or functional modification targets for crop development [25].

Environmental factors influence quantitative traits, and considering the effect of each significant SNP on the associated traits reveals several types: main effect or constitutive SNP effect, with positive or negative additive phenotypic effects, and SNP with substantial interaction with the environment. The latter includes those with effects stronger in one environment than another, SNPs with conditionally neutral effects detected only in one environment, and SNPs with antagonistic pleiotropic effects and opposite phenotypic effects in both environments [26].

Our recent study reported that fenugreek grows well in various habitats, including marginal areas, and is reasonably resistant to salinity [27]. Therefore, the current study aimed to investigate the genetic variability underlying the morphological traits in fenugreek genotypes grown under salinity stress and identify the SNP markers associated with the measured traits.

2. Results

The statistical analysis was performed on six morphological traits measured under control and two salinity levels. The correlation matrix (Figure 1) showed a positive correlation between all measured traits under three treatments. Comparing the same trait under different treatments revealed negative correlations between BN, FW, and DW measured under control and 6000 mgL^{-1} salinity levels. In contrast, BN, FW, and DW were positively correlated when comparing the control and 3000 mgL^{-1} salinity treatments. Positive correlations were observed between PHs measured under the three treatments.

The mean of DW across all genotypes was 0.56 g under control conditions, which increased to 0.63 g in 3000 mgL^{-1} and 0.95 g in 6000 mgL^{-1} treatments (Table 1). A similar response was observed for PH, LN, and BN.

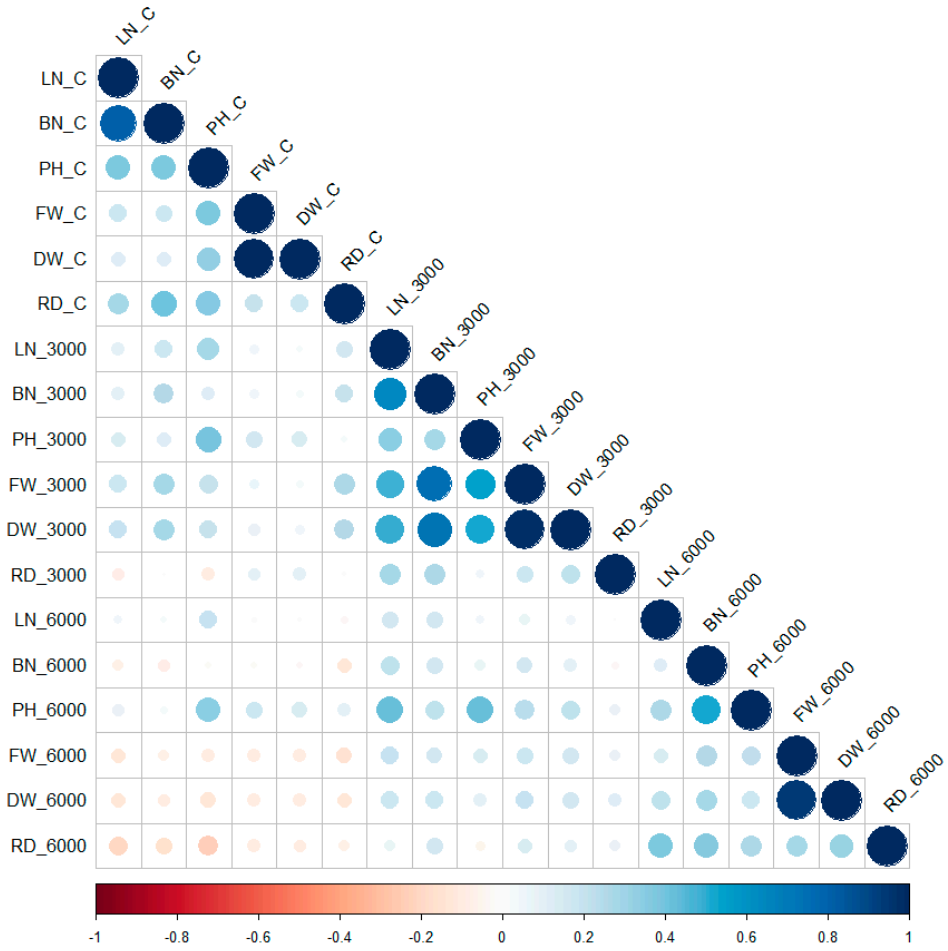


Figure 1. Pearson correlation analysis between measured traits; LN = number of leaves, BN = number of branches, PH = plant height, FW = fresh weight, DW = dry weight, and RD = root depth. The three treatments are expressed as C for control, 3000 for the salt stress at 3000 mgL^{-1}, and 6000 for the salt stress at 6000 gL^{-1}. The blue and red colors indicate positive and negative correlations, respectively.

Table 1. Population performance from the pot experiment expressed as the minimum (Min), maximum (Max), average (Avg), standard error (SE), and heritability (H^2) of 111 fenugreek genotypes under control and two levels of salinity, 3000 mgL^{-1} and 6000 mgL^{-1}.

Traits (Unit)	Control					3000 ppm					6000 ppm					MM		
	Min	Max	Avg	SE	H^2	Min	Max	Avg	SE	H^2	Min	Max	Avg	SE	H^2	T	G	GxT
LN	19.85	22.00	20.93	0.49	0.87	26.80	29.03	27.92	0.52	0.69	27.28	29.50	28.39	0.52	0.51	0.00	0.00	0.00
BN	1.78	2.27	2.03	0.09	0.43	1.95	2.43	2.19	0.10	0.44	2.56	3.04	2.80	0.10	0.44	0.00	0.00	0.00
PH (cm)	15.52	16.40	15.96	0.22	0.78	15.78	16.71	16.25	0.24	0.55	15.78	16.71	16.25	0.24	0.52	0.04	0.00	0.00
FW (g)	1.05	1.49	1.27	0.11	0.56	0.86	1.33	1.08	0.12	0.67	0.87	1.32	1.09	0.12	0.52	0.50	0.00	0.00
DW (g)	0.32	0.81	0.56	0.13	0.49	0.37	0.90	0.63	0.13	0.46	0.70	1.20	0.95	0.13	0.42	0.08	0.00	0.00
RD (cm)	12.53	14.30	13.41	0.33	0.68	11.76	13.51	12.64	0.35	0.55	12.87	14.63	13.75	0.34	0.43	0.00	0.00	0.00

Six traits, including the number of leaves (LN), number of branches (BN), plant height (PH), fresh weight (FW), dry weight (DW), and root depth (RD), were measured. Mixed-model (MM) analysis shows the level of significance for the treatments (T), genotype (G), and their interaction (GxT). Heritability ranged from 0.48 for BN to 0.87 for LN measured under the control treatment. All traits showed significant genotype effects and genotype-by-treatment (GxT) interactions. Significant treatment effects were observed for all traits except FW and DW. A significant genotypic variation and significant interaction were found for all measured traits.

Association Mapping

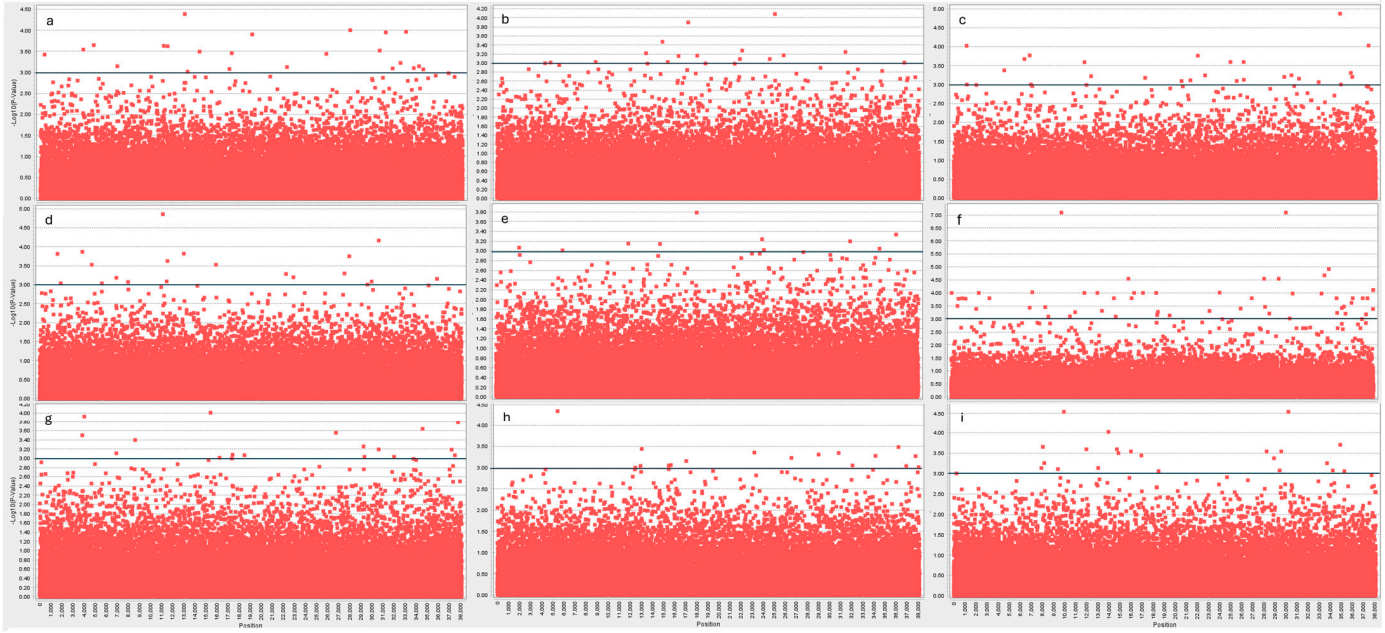
A total of 492 significant SNPs were associated with the measured traits, using an arbitrary threshold of three (Figure 2), of which 71 SNPs with $-\log10\ (p) \geq 4$ (Table 2 and Supplementary Table S1) and 212 SNPs were linked to correlated traits (Table 3 and Supplementary Table S1). The two most significant SNPs, dDocent_Contig_43225_248 and dDocent_Contig_43225_80, were mapped under 6000 mgL^{-1}, and each SNP was associated with BN and PH with $-\log10\ (p) = 7.1$ and 4.5, respectively.

Table 2. Significant SNPs with $-\log10\ (p) \geq 4$ were detected using a mixed linear model and associated with the measured traits in the control (C) and two salt treatments, 3000 mgL^{-1} (3000) and 6000 mgL^{-1} (6000).

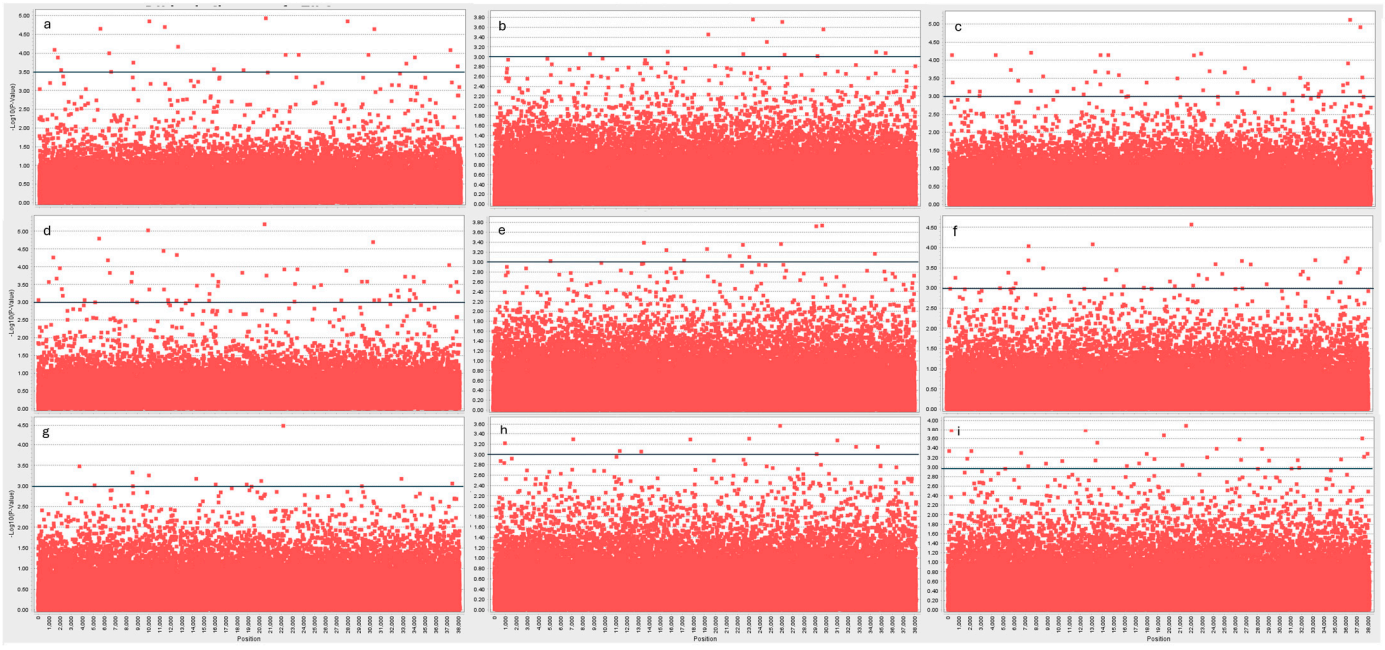
Trait	Marker	−Log10 (*p*)	R^2	Allele	Effect
LN_C	dDocent_Contig_30812_276	4.0	0.16	A(G)	−10.21
LN_C	dDocent_Contig_45644_257	4.0	0.16	A(C)	9.21
LN_3000	dDocent_Contig_9350_202	4.1	0.20	G(T)	−18.12
LN_6000	dDocent_Contig_31894_14	4.0	0.18	C(T)	16.03
LN_6000	dDocent_Contig_47529_298	4.9	0.22	A(G)	−34.51
BN_6000	dDocent_Contig_1933_583	4.1	0.18	A(G)	6.25
BN_6000	dDocent_Contig_1933_593	4.1	0.18	C(T)	−6.25
BN_6000	dDocent_Contig_29601_61	4.0	0.18	C(T)	6.73
BN_6000	dDocent_Contig_29746_105	4.0	0.23	G(T)	16.60
BN_6000	dDocent_Contig_29746_130	4.0	0.23	C(G)	16.60
BN_6000	dDocent_Contig_29746_136	4.0	0.23	C(G)	16.60
BN_6000	dDocent_Contig_29746_145	4.0	0.23	C(G)	16.60
BN_6000	dDocent_Contig_29746_24	4.0	0.23	C(T)	16.60
BN_6000	dDocent_Contig_29746_254	4.0	0.23	C(G)	16.60
BN_6000	dDocent_Contig_29746_281	4.0	0.23	A(G)	16.60
BN_6000	dDocent_Contig_29746_7	4.0	0.23	A(G)	16.60
BN_6000	dDocent_Contig_29746_96	4.0	0.23	C(G)	16.60
BN_6000	dDocent_Contig_6519_195	4.0	0.18	C(T)	−5.39
BN_6000	dDocent_Contig_797_289	4.9	0.23	A(G)	−8.46
PH_C	dDocent_Contig_373_122	4.0	0.17	C(T)	−3.69
PH_3000	dDocent_Contig_40266_46	4.4	0.21	A(G)	4.46
PH_6000	dDocent_Contig_6598_111	4.0	0.18	G(T)	4.61
FW_6000	dDocent_Contig_31699_109	4.1	0.18	A(G)	1.37
FW_6000	dDocent_Contig_31699_114	4.1	0.18	C(G)	1.37
FW_6000	dDocent_Contig_31699_37	4.1	0.18	C(T)	1.37
FW_6000	dDocent_Contig_31699_8	4.1	0.18	A(G)	−1.37
WC_C	dDocent_Contig_3179_151	4.8	0.21	C(G)	−0.07
RD_C	dDocent_Contig_53508_178	4.5	0.19	A(T)	−2.11

R^2 = explained phenotypic variance and the effect of each allele (alternate allele). Number of leaves (LN), number of branches (BN), plant height (PH), fresh weight (FW), dry weight (DW), and root depth (RD). The 50 nucleotide sequences flanking each SNP are presented in Table S1.

For example, 4 and 10 SNPs in the two contigs dDocent_Contig_57479 and dDocent_Contig_40671 were associated with BN measured under the control and 6000 mgL^{-1}, respectively. Five more SNPs were mapped in the dDocent_Contig_46795 and were associated with PH under 3000 mgL^{-1}. We also observed six more SNPs in dDocent_Contig_30005 associated with the two correlated traits, FW and DW, under the control treatment.



(**A**)



(**B**)

Figure 2. (**A**) Manhattan plots for the measured traits. Significant SNPs associated with the measured traits for the 111 fenugreek genotypes using 38,142 SNPs arranged randomly on the *x*-axis. The *y*-axis represents the −log10 (*p*) values. A black horizontal line defines an arbitrary threshold of 3. Manhattan plots represent the number of leaves (**a**–**c**), branch number (**d**–**f**), and plant height (**g**–**i**) under the control and two levels of salinity, 3000 mgL^{-1} and 6000 mgL^{-1}, respectively. (**B**) Manhattan plots for the measured traits. Significant SNPs associated with the measured traits for the 111 fenugreek genotypes using 38,142 SNPs arranged randomly on the *x*-axis. The *y*-axis represents the −log10 (*p*) values. A black horizontal line defines an arbitrary threshold of 3. Manhattan plots represent fresh weight (**a**–**c**), dry weight (**d**–**f**), and root depth (**g**–**i**) under the control and two levels of salinity, 3000 mgL^{-1} and 6000 mgL^{-1}, respectively.

Table 3. Overlapping significant SNPs between correlated traits under control, 3000 mgL^{-1}, and 6000 mgL^{-1} salt stress treatments.

Trait	Marker	−Log10 (*p*)	R^2	Allele	Effect
FW/DW_C	dDocent_Contig_30841_53	4.2	0.17	C(T)	2.01
		4	0.17	C(T)	4.22
	dDocent_Contig_31124_100	4.3	0.18	C(T)	−1.88
		4.2	0.18	C(T)	−3.98
	dDocent_Contig_31124_249	4.7	0.2	A(G)	−1.98
		4.6	0.2	A(G)	−4.25
	dDocent_Contig_31124_47	4.8	0.21	A(T)	2.19
		4.6	0.2	A(T)	4.64
	dDocent_Contig_35845_179	3.9	0.16	A(G)	−1.93
		4	0.16	A(G)	−4.19
	dDocent_Contig_35845_189	3.9	0.16	A(G)	−1.93
		4	0.16	A(G)	−4.19
	dDocent_Contig_36571_20	4	0.16	A(T)	−0.12
		3.5	0.14	A(T)	−0.37
	dDocent_Contig_43254_163	5.2	0.23	C(T)	−2.56
		4.9	0.22	C(T)	−5.36
	dDocent_Contig_43254_80	5	0.22	G(T)	−2.51
		4.8	0.21	G(T)	−5.3
	dDocent_Contig_49746_342	4	0.21	A(G)	−4.6
		4.1	0.22	A(G)	−10.2
	dDocent_Contig_5198_91	4.4	0.19	A(G)	−2.08
		4.7	0.2	A(G)	−4.66
	dDocent_Contig_57115_226	3.9	0.2	A(T)	−4.9
		4.9	0.26	A(T)	−13.6
	dDocent_Contig_6156_243	3.6	0.14	A(G)	−1.32
		4	0.16	A(G)	−3.04
	dDocent_Contig_63002_15	4.3	0.22	C(T)	−4.90
		4.1	0.22	C(T)	−10.30
FW/DW_3000	dDocent_Contig_35199_79	3	0.1323	G(T)	−0.20
		3	0.13144	G(T)	−0.34
	dDocent_Contig_64264_238	3.7	0.17492	A(G)	−0.23
		3	0.1342	A(G)	−0.33
	dDocent_Contig_64858_150	3.3	0.1699	C(G)	−0.24
		3.5	0.18123	C(G)	−0.43
	dDocent_Contig_85747_243	3.7	0.1758	C(T)	−0.24
		3.6	0.1653	C(T)	−0.38
	dDocent_Contig_9501_294	3.2	0.14269	A(C)	0.26
		3.1	0.13883	A(C)	0.43

Table 3. *Cont.*

Trait	Marker	−Log10 (*p*)	R^2	Allele	Effect
FW/DW_3000	dDocent_Contig_95794_210	3.4	0.15403	A(C)	0.46
		3.7	0.17421	A(C)	0.82
FW/DW_6000	dDocent_Contig_61819_183	3.3	0.16	C(T)	−0.35
		4.2	0.22	C(T)	−0.71
	dDocent_Contig_58866_61	4	0.18	C(T)	0.36
		4.2	0.19	C(T)	0.65
	dDocent_Contig_39980_178	4.6	0.21	A(G)	−0.52
		4.1	0.18	A(G)	−0.87
	dDocent_Contig_10975_317	3.7	0.16	G(T)	0.71
		5.1	0.24	G(T)	1.52
	dDocent_Contig_12419_342	3.4	0.14	A(T)	−0.69
		4.9	0.23	A(T)	−1.54
	dDocent_Contig_31895_105	4.1	0.18	C(T)	−0.73
		3.7	0.16	C(T)	−1.2

R^2 = explained phenotypic variance. The effect of each allele (alternate allele). Fresh weight (FW) and dry weight (DW). The 50 nucleotide sequences flanking each SNP are presented in Table S1.

3. Discussion

Growing the fenugreek population in pots enabled us to apply two levels of salinity stress. In addition, the sand-filled pots used in the experiment provide a greater imitation of the natural field environment to phenotype roots compared to other settings such as hydroponics, aeroponics, or agar plates. However, some root growth differences should still exist when comparing the adopted pot system with the natural field conditions [28]. As a result, we sought to compare the RD results from the current pot trial to the unpublished data from the field experiment [27], which demonstrated a positive association between RD measured across all treatments in both experiments. On the other hand, a negative correlation was found between BN recorded in the current pot experiment and an earlier field experiment [27], which could be attributed to the lower plant density in the pot experiment vs. the field condition.

Estimating broad-sense heritability revealed that most traits were moderately or highly heritable, indicating that the observed variation has a genetic foundation rather than being caused by environmental influences. Therefore, our study has enough power to map significant SNPs associated with the measured traits using the fenugreek population, previously genotyped with 38,142 polymorphic SNPs using ddRAD-seq technology [29].

The misleading positive association due to population structure is a common drawback of association studies [25,30], and to help solve this problem, two statistical correction procedures were evaluated to set a threshold value of 0.05 [31]. The first method, Bonferroni adjustment [32], calculates the significant *p*-value as the ratio of the significant threshold (α) to the number of markers (n). The false discovery rate (FDR) [33] is the other approach where *p*-values are organized in ascending order, and the rank of the *p*-value (r) is divided by the number of markers (n): ((r/n)). The criteria for both procedures revealed thresholds of 6.5 and 5.2, respectively, in the present fenugreek collection. Correction approaches, however, presuppose marker independence, which is not the case. Therefore, some previous research employed arbitrary thresholds between three and four [27,34–37]. As a result, we list candidate SNPs with thresholds greater than three if they have the same effect on correlated traits in two treatments.

We mapped 492 significant SNPs above the threshold −log10 (*p*) value of 3, of which 212 were linked to correlated traits, suggesting possible pleiotropic effects of those SNPs on

the associated traits. For example, 29, 5, and 21 SNPs were associated with FW and DW measured under the control, 3000 mgL^{-1}, and 6000 mgL^{-1} treatments, respectively, with a correlation of 0.98 between both traits in each of the three treatments. The effect of the same allele of each SNP on the trait was stronger in one environment than in the other. The same was observed for six SNPs associated with BN and LN measured under the control treatment, with a correlation of 0.85. In addition, we mapped 409 SNPs with conditional neutrality effects.

Fenugreek has been reported to tolerate drought [38], salinity [39], and heavy metals [40] and adapt to various climatic regions and marginal lands [27]. Stress-tolerant plants often exhibit stable growth patterns even in the presence of stressors. This stability can be observed in consistent development, biomass production, and overall plant architecture [39,41–44]. In agreement, stable DW was observed in the control and 3000 mgL^{-1} treatments; however, it increased in the 6000 mgL^{-1} treatments. Some salt-tolerant plants show adaptations by improving water use efficiency, allowing them to extract water from saline soils while minimizing water loss through transpiration [45–48].

Some plants have naturally evolved in saline environments and carry genetic alleles that confer salt tolerance. Other plants exposed to salt stress over long periods may undergo adaptive evolution, leading to the selection of individuals with traits that enhance salt tolerance [27,49–51]. One adaptive mechanism is developing deep or extensive root systems that explore larger soil volumes, allowing them to access water and nutrients from deeper soil layers [52,53]. This mechanism may explain the observed relative increase in RD regardless of increased salt levels, as most of the genotypes used here were collected from different places in Egypt with sandy soil of various degrees of salinity. As observed here, deep and extensive root systems promote overall growth and may increase plant yields under drought and salt stress, as reported in earlier studies [27,52].

4. Conclusions

Our screening for the fenugreek population identified several SNPs that showed either conditional neutrality or pleiotropic effects on the measured traits. Those SNPs can be used in future marker-assisted breeding programs to select the salt-tolerant genotypes. Our results shed light on the salt-tolerant properties of fenugreek. Additional genetic studies are required to dissect fenugreek's physiological, agronomical, and biochemical characteristics and unravel its ability to tolerate salinity and other abiotic and biotic stresses.

5. Materials and Methods

5.1. Plant Material and Experimental Design

The fenugreek population comprising 111 Fenugreek genotypes [29] were grown in pots under open field conditions at the Faculty of Agriculture, Cairo University, Giza, Egypt, in the 2022–2023 growing season. Temperature and humidity ranged between 18 and 30 °C and between 65 and 75%, respectively. Ten seeds were sown in pots 45 cm in height and 30 cm in diameter filled with 20 kg of washed sand. These were used as experimental units for our study. A total of 666 pots were divided into three treatments following a complete randomized block design, with two replicates per treatment. Pots were watered daily for two weeks to field capacity using tap water for the control treatment and 3000 mgL^{-1} and 6000 mgL^{-1} of NaCl solution for the two salinity treatments. A balanced fertilizer containing 18% NPK was applied twice to all pots at a 1 g/1 kg soil rate.

5.2. Phenotypic Measurements

After 30 days from seeding, three randomly selected plants were used to measure the following growth parameters: number of leaves (LN), number of branches (BN), plant height (PH), and whole plant fresh weight (FW). Dry weight (DW) was measured after drying plants at 65 °C for 72 h or after reaching a constant weight. Roots were collected and cleaned from sand, and their depths (RD) were measured.

5.3. Statistical Analysis

The Pearson correlation was performed in R for Windows 4.1.2, using the R package corrplot. SPSS v25 was used to perform a mixed model on the raw data from the three treatments to determine whether there were significant differences among genotypes (G), treatments (T), and their interaction (GxT). Broad-sense heritability (H^2) was calculated as the ratio of genetic variance (δ^2 g) to total phenotypic variance (δ^2 ph), as shown below:

$$H^2 = \delta^2 g / \delta^2 ph$$

$$\delta^2 ph = \delta^2 g + \delta^2 e$$

$$\delta^2 g = \left(MS_g - MS_e\right)/r$$

$$\delta^2 e = MS_e$$

where $\delta^2 e$ is the error variance, MS_g is the mean square of genotypes, MS_e is the error mean square, and r is the number of replications.

5.4. SNP Data and Mapping

The double-digest restriction site-associated DNA sequencing (ddRAD-seq) technique [54] was used for genotyping the 111 fenugreek genotypes. Briefly, 38,142 bi-allelic SNPs with a minimum quality score of 30, a maximum of 0.2% missing data per SNP, a minimum mean coverage depth of 20, and a minimum minor allele frequency (MAF) of 0.05 were used for our mapping analysis [29]. The mixed linear model (MLM), kinship matrix, and principal component implemented in the TASSEL software, version 5.0 [55], assisted in identifying SNP markers associated with the raw data of the measured traits. Two thresholds, 6.5 and 5.9, based on Bonferroni correction and a false discovery rate (FDR) of 0.05, respectively, were used to determine the significant association. A threshold of three was used to report potential SNPs, primarily if the SNP is linked to a correlated trait in different treatments.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/stresses4020017/s1. Table S1: Significant SNPs associated with the measured traits under three salinity levels with their explained variances and the 50 nucleotide sequences flanking each SNP.

Author Contributions: M.M.H.A.E.-W., M.S. and M.E.-S. contributed to the conception and design. A.M.D. and R.A.N. collected data. A.M.D. and M.E.-S. analyzed data. A.M.D., R.A.N. and M.E.-S. wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated and analyzed during the current study are available as Supplementary Materials or from the corresponding author on reasonable request.

Conflicts of Interest: The authors have no relevant financial or non-financial interests to disclose.

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