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RESEARCH ARTICLE

Effect of salinity on DNA methylation and antioxidant phenolic compounds of wild watercress (*Rorippa nasturtium-aquaticum* L.)

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Abstract

Epigenetic changes are involved in plant responses to stress. Cytosine methylation is one of the most important epigenetic changes, regulating gene expression. In this paper, the MSAP (methylation-sensitive amplification polymorphism) method was used to find out how the watercress (*Rorippa nasturtium-aquaticum* L.) genome changed in response to 0, 60, 80, and 100 mM NaCl and how that affected phenylalanine ammonium lyase (PAL) activity, phenolic content, and antioxidant capacity. The results showed an inverse correlation between methylation levels and PAL activity and the contents of total phenolics and flavonoids, indicating salt stress-induced reprogramming of the methylation pattern of watercress, which has a negative effect on the synthesis of phenolics. The results revealed a significant decrease in phenolic contents and antioxidant activity under low and moderate salinity compared to control and an increase under strong salinity compared to moderate salinity. The findings of this study contribute to our understanding of the reprogramming of DNA methylation under salinity and its effect on watercress phenolic metabolism.

Keywords

watercress; methylation; metabolites; stress; phenolics; antioxidant

Introduction

Abiotic stress has a big effect on the growth and yield of plants (1). It does this by causing plant cells to make too many reactive oxygen species (ROS), which are highly toxic to proteins, lipids, and nucleic acids and damage or kill cells (2). Plants counteract the ROS overproduction by diverse endogenous defence systems, which include a variety of antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and glutathione peroxidase, among others) and specialized metabolites (phenolic acids, flavonoids, carotenoids, alkaloids, glutathione, among others) (3, 4).

Abiotic stresses can change the natural DNA methylation patterns in plants, modulating gene expression, which has revealed that DNA methylation is essential in mediating plant interactions with the environment (5, 6). Thus, DNA methylation is an important epigenetic regulator that affects a wide range of biological processes, such as morphological development, including agronomic features (7), and the biosynthesis and accumulation of specialized metabolites (8).

DNA methylation is a chemical change that happens when methyltransferases (DNMT) help add methyl groups to the CpG sequence to make 5-methylcytosine (9). Cytosine methylation is thought to be essential for genomic imprinting, transcriptional regulation of genes and transposable elements, and gene silencing (10). There are several methods to test DNA methylation. The methylation-sensitive amplified polymorphism (MSAP), which is a change to the AFLP method, gives information about cytosine methylation patterns without knowing the genome sequences beforehand (11).

Watercress (*R. nasturtium-aquaticum*) is an edible aquatic plant member of the Brassicaceae family. Its phytochemical composition confers on it multiple biological properties, such as antioxidant, antibacterial, anticancer, and anti-inflammatory (12). Watercress naturally occurs in several springs in Durango, Mexico, where it is consumed as a vegetable and used as a medicinal plant to treat kidney and lung disorders, stimulate metabolism, and promote bile secretion (13). However, the salinization of inland waters is a current widespread phenomenon, increased by global warming and anthropogenic activities (14, 15). Salinity affects the accumulation of phenolic compounds (16, 17) through epigenetic regulation of genes involved in the plant phenylpropanoid pathway (18). To the best of our knowledge, the variation of the methylation level and the phenolic compound accumulation of watercress exposed to different concentrations of NaCl have not been explored. The register of this variation would contribute to revealing the potential of adaptation to saline conditions in this plant, as well as the potential of salt stress as a tool for the manipulation of phenolic accumulation. The current study aimed to determine the variation in the methylation level and the phenolic compound accumulation of watercress exposed to different salinity conditions.

Materials and Methods

Plant material, salt treatments, and growth conditions

R. nasturtium-aquaticum plants were collected from the springs of Berros, Nombre de Dios, Durango, Mexico (between 23° 93'19'' N, 104° 27' 23'' W, and 23° 91' N, 104° 26' W) in July 2016. A voucher specimen was deposited at Herbarium CIIDIR (curatorial number 16895). The experiments were carried out in a greenhouse located in Durango, Mexico (24° 03' 06'' N, 104° 36' 25'' W). The temperature varied from 18°C to 37°C, the relative humidity was 87%, and the daily photoperiod was 13 h throughout the study.

After being collected, plants were tap-water washed, transferred to individual plastic plots, and grown in hydroponic irrigation systems with a nutrient solution (Hydro Environment®) for 7 days. After this adaptation period, experimental lots of 15 plants were formed. Each saline treatment (0, 60, 80, and 100 mM NaCl, corresponding to 1.40, 7.81, 9.91, and 11.87 dS/m, respectively, for 15 days) was formed by three experimental lots (replicas). The electrical conductivity was monitored daily with a conductivity meter to maintain the salt level of the nutrient solution.

Preparation of phenolic extracts

Aerial parts from the 15 plants in each experimental lot of each treatment were collected, combined, dried at 40 °C for 48 h, ground, and independently analyzed. Phenolic extracts were prepared according to Gutiérrez-Velázquez et al. (19).

Total phenolics (TP) and flavonoids (TF)

The concentrations of TP were estimated using the Folin-Ciocalteu reagent according to Skotti et al. (20) from a standard curve constructed with seven concentrations (0.0039-0.25 mg/mL) of gallic acid (GA) (Abs_{725} = 0.013[GA] + 0.11, r = 0.99). Contents were expressed as milligrams equivalents of gallic acid per gram of dry extract (mg GAE/g DE).

The estimations of TF were carried out according to Ordoñez et al. (21), from a standard curve constructed with six concentrations (0.005-0.03 mg/mL) of quercetin (Q) (Abs_{420} = 0.326[Q] + 0.027, r = 0.99). Contents were expressed as milligrams equivalents of quercetin per gram of dry extract (mg QE/g DE).

Phenylalanine ammonium lyase (PAL) activity

PAL activity was determined according to Anand et al. (22). The enzymatic activity was calculated using the calibration curve constructed with five concentrations (0.9-31.25 µg/mL) of trans-cinnamic acid (CA) (A_{290}= 0.079 [CA] + 0.029, r = 0.99). One unit of PAL activity was defined as the amount of PAL that produces 1 µmol of CA in 1 h. The results were expressed as micrograms of cinnamic acid per millilitre per hour (µg CA/mL/h).

Antioxidant activity

The $DPPH^{\cdot}$ (2,2-diphenyl-1-picrilhidrazil) scavenging activity, determined according to Medina-Medrano et al. (23), was used to assess the antioxidant capacity of samples. The antiradical activity was expressed as the efficient concentration at 50% (EC_{50}), defined as the antioxidant concentration (mg/mL) needed to decrease by 50% the initial $DPPH^{\cdot}$ concentration.

The ABTS (2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonic acid) assay was carried out according to Re et al. (24). The $ABTS^{\cdot}$ inhibition capacity was expressed as the efficient concentration at 50% (EC_{50}), defined as the antioxidant concentration (mg/mL) needed to provide 50% inhibition of $ABTS^{\cdot}$.

The antioxidant capability of samples was also evaluated by their iron-reducing power (IRP) according to Chavan et al. (25). Results are expressed as extract concentration, giving an absorbance of 0.5 (EC_{50}).

Methylation-sensitive amplification polymorphism (MSAP) analysis

DNA extraction was carried out according to Bhau et al. (26). The epigenetic variation was performed by the methylation-sensitive amplification polymorphism (MSAP)

Table 1. Sequences of primers and adapters used for MSAP analysis.

	***EcoRI* Secuences**	***HpaII/MspI* Secuences**
Pre-amplification Primers	5'GACTGCGTACCAATTC-3'	5'-ATCATGAGTCCTGCTCGG-3'
	5'GACTGCGTACCAATTC**AC**-3'	5'-ATCATGAGTCCTGCTCGG**TCAA**-3'
Selective amplification Primers	5'GACTGCGTACCAATTC**AG**-3'	
	5'GACTGCGTACCAATTC**AAC**-3'	5'-ATCATGAGTCCTGCTCGG**AAT**-3'
	5'GACTGCGTACCAATTC**AT**-3'	
Adapter pair	5'-CTCGTAGACTGCGTACC-3'/	5'-GATCATGAGTCCTGCT-3'/
	3'-CATCTGACGCATGGTTAA-5'	3'-AGTACTCAGGACGAGC-5'

assay (11), using separately the combination of restriction enzymes *EcoRI/MspI* and *EcoRI/HpaII* (Table 1).

Digestion

An aliquot (3.5 µL) of DNA was digested with 0.5 µL (6 U) of *EcoRI* enzyme, 0.5 µL (5 U) of *HpaII* enzyme, 0.1 µL of BSA (0.05 mg/mL), and 5 µL of multicore buffer (1X), in a final volume of 20 µL. The mixtures were incubated at 37 °C for 3 h. Other digestions, using *MspI* instead of *HpaII,* were carried out under the same conditions.

Ligation

The ligation reaction contained 20 µL of DNA combined with 1 µL (5 pmol) of *EcoRI* adapter, 1 µL (50 pmol) of *MspI/HpaII* adapter, 0.3 µL (0.9 U) of T4 DNA ligase, and 3 µL (1X) T4 DNA ligase buffer, in a final volume of 30 µL. Adaptors were ligated for one day at room temperature.

Preamplification

The preamplification reaction contained 2 µL of ligated DNA, combined with 0.63 µL of 10 µM *EcoRI* primer and 0.52 µL of 10 µM *MspI/HpaII* primer, 0.4 µL Taq polymerase (2 U), 0.8 µL of 0.4 mM dNTPs, 4 µL of 1X PCR buffer, and 1 µL of 25 mM $MgCl_2$, in a final volume of 20 µL. The amplification conditions were as follows: 25 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. The preamplifier products were diluted with 200 µL of ultrapure water and stored at -20 °C before being used in the selective amplification reaction.

Selective amplifications

Each of the eight combinations formed by mixing each of four *EcoRI* selective primers with each of two *MspI/HpaII* selective primers was used independently. Table 2 displays the various combinations and volumes employed.

Each combination of selective primers was mixed with 5 µL of preamplified product according to Table 3, 0.4 µL of 0.2 mM dNTPs, 1 µL of 25 mM MgCl2, 4 µL of 1X PCR buffer, and 0.4 µL of Taq polymerase (2 U), in a final volume of 20 µL. The selective amplification conditions were 12 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, decreasing the annealing temperature by 0.7 °C per cycle, and then 24 cycles at 94 °C for 30 s, 56 °C for 1 min, and 72 ° C for 2 min, with a final period of 5 min at 72 °C. The amplified samples were separated by electrophoresis on polyacrylamide gels (5%).

Table 2. Selective primer combinations used in the MSAP analysis.

Combination	* *EcoRI* Selective primer	Volume (µL)	** *MspI/HpaII* Selective primer	Volume (µL)
1	AG	0.53	TCAA	0.44
2	AG	0.53	AAT	0.45
3	AC	0.53	TCAA	0.44
4	AC	0.53	AAT	0.45
5	AAC	0.52	TCAA	0.44
6	AAC	0.52	AAT	0.45
7	AT	0.55	TCAA	0.44
8	AT	0.55	AAT	0.45

***** Refers to selective amplification primers of *EcoRI* sequences, which are in bold in Table 1. ****** Refers to selective amplification primers of *HpaII/MspI* secuences, which are in bold in Table 1.

Table 3. Effect on total phenolic (TP), flavonoids (TF), and activity of phenylalanine ammonium lyase (PAL) in watercress (*R. nasturtium-aquaticum*) grown under different NaCl concentrations.

NaCl (mM)	TP (mg GAE/g DE)	TF (mg QE/g DE)	PAL (µg CAE/mL/h)
0 (Control)	37.45 ± 0.80^{a}	15.45 ± 0.39^{a}	1.69±0.17^{a}
60	36.14 ± 0.68^{b}	10.27 ± 0.37^{b}	0.51±0.09^{b}
80	27.47 ± 0.69^{d}	7.21 ± 0.22^{c}	0.18±0.07^{d}
100	30.23 ± 0.72^{c}	7.49 ± 0.23^{c}	0.30±0.09^{c}

*__GAE__, Gallic Acid equivalents; **QE**, Quercetin equivalents; **CAE**, Cinnamic acid equivalents; **DE**, dry extract. Different letters on the same column indicate significant differences (Tukey test, p<0.05).

Data analysis

All assays were performed in triplicate for five individuals per treatment. The results were expressed as mean ± standard deviation (SD). Data were subjected to ANOVA, and the means were compared by the Tukey test ($p<0.05$). Data were also submitted to principal component analysis (PCA), cluster analysis, and correlation analysis (Spearman method, $p<0.05$). R v4.1.1 programming language (27), operated by R Studio, was used to carry out all statistical analyses.

For the MSAP analysis, only the clearly resolved bands were considered. The methylated loci (5'-C5mCGG-3') were estimated by the bands amplified in the *EcoRI/MspI* combination and absent in the *EcoRI/HpaII* combination for the same locus. Fragments amplified by both combinations (*EcoRI/MspI* and *EcoRI/HpaII*) indicated non-methylated loci (5'-CCGG3'), whereas fragments present in the *EcoRI/HpaII* combination but absent in the *EcoRI/MspI* combination were considered as hemimethylated loci, which represent methylation in external cytosines in the context of 5'-CCGG-3'. The absence of loci in both isoschizomers (*MspI* and *HpaII*) can be the result of hypermethylation or the absence of restriction sites (28). Binary matrixes of the presence (1) or absence (0) of the individual amplified loci per selective primer combination were constructed, from which the number of methylated, non-methylated, hemimethylated, total, and % methylation loci were calculated for the plants of the different saline treatments. Matrixes were used to calculate the epigenetic intra-treatment variability through Nei's index, diversity index, and polymorphism (P, 95%) using InfoGen/E software. The epigenetic inter-treatment variability was estimated with the polymorphism (95%), average heterozygosis, and Nei's unbiased heterozygosity, using the Popgene 1.32 program.

Results and discussion

Total phenolics and flavonoids

Significant differences were found in both the TP and TF contents among treatments (Table 3). Both TP and TF were significantly higher in control plants than in plants from any saline treatment. At 100 mM NaCl, an increase of 1.10 times in TP was observed compared to the concentration of 80 mM NaCl. The response trend to salinity here found for watercress was similar to that reported for radish (*Raphanus sativus* L.) (29), in which the highest value of TP was observed in the control, whereas at 100 mM NaCl, the authors observed an increment compared to the concentrations of 10 and 50 mM NaCl. This is interesting since both species (watercress and radish) belong to the same family (Brassicaceae). However, different responses have been reported for other plant species, for instance, for *Salvia mirzayanii* Rech. f. & Esfand, in which an increase in the total phenolic content with the increase in salt concentration was observed (30), for *Shizonepeta tenuifolia* Briq., in which phenolics increased at low (25 mM) and moderate (50 mM) salt concentrations but decreased at severe concentrations (75 and 100 mM) (31), and for common bean (*Phaseolus vulgaris* L.), in which a constant decline in the content of phenolics with an increase in NaCl was revealed. The diversity of responses suggests that different plant species may have evolved particular and genetically controlled mechanisms of salt tolerance, although some similarities may exist between species of the same group of plants.

It has been reported that an increase in the accumulation of phenolic compounds contribute to avoiding oxidative stress (33, 34). But for some plant species, like *Limonium delicatulum* Kuntze (36), the main defence against salinity is not the accumulation of phenolics but the activity of antioxidant enzymes (35), or the accumulation of osmolites like proline, like in *Physalis ixocarpa* Brot. ex Hornem. (37). The fact that salt-stressed watercress didn't make more phenolic compounds suggests that, for this species, phenolic compounds don't help protect against salinity, especially at low and moderate levels of salinity.

PAL activity

The results of PAL activity are shown in Table 3. The highest activity was recorded in the control plants. The concentrations of 60 and 80 mM NaCl caused a decrease in enzyme activity. Nevertheless, at 100 mM a significant increase compared to 80 mM NaCl was observed, which agrees with what was here found for total phenolics and flavonoids. The PAL activity here found for salt-stressed watercress followed the same trend found for TP and TF (Table 3), revealing a high relationship between them (Figure 1). PAL is a crucial enzyme of the phenylpropanoid pathway, which leads to the biosynthesis of phenolic compounds, such as flavonoids, from the amino acid phenylalanine. Plants synthesize phenolics at the same time as the PAL activity rises (38). The current findings are in disagreement with what was reported for *Jatropha curcas* L., in which NaCl-dependent increases in PAL activity were observed, except in the highest concentration evaluated (200 mM) (39). Our results also differ from what was reported for *Zea mays* L., in which an increase in PAL activity was observed for corn stressed by salt (40). The PAL activity here registered confirmed that the phenylpropanoid biosynthesis pathway was not enhanced in watercress under low and moderate saline stress, showing a slight improvement under the high salinity condition. These results account for the levels of TP and TF found in

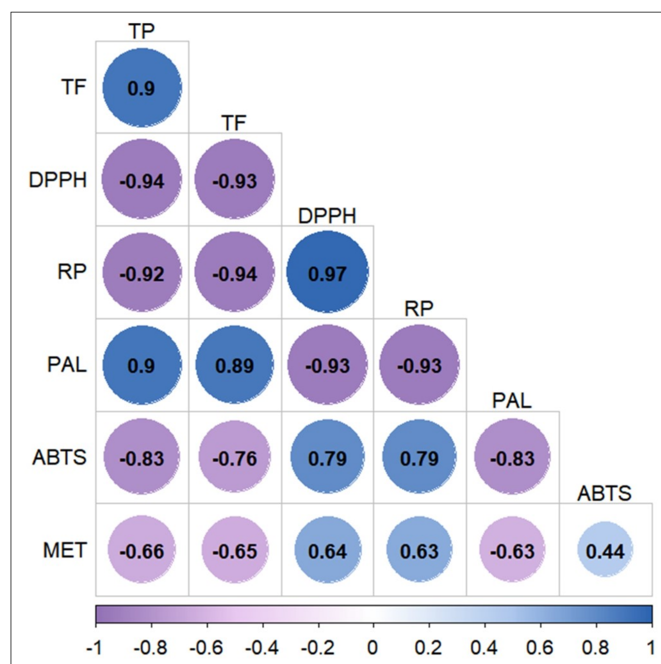

Fig. 1. Heat map representing the correlations between total phenolics content (**TP**), flavonoid content (**TF**), DPPH scavenging activity (**DPPH**), ABTS scavenging activity (**ABTS**), iron reducing power (**RP**), phenylalanine ammonia lyase activity (**PAL**), and methylation percentage (**MET**) of watercress (*R. nasturtium-aquaticum*) grown under different concentrations of NaCl. Values correspond to correlation coefficients (Spearman method, $p<0.05$).

watercress under the different salt conditions evaluated and suggest some regulation of the genes involved in the pathway by saline conditions.

Antioxidant activity

The antioxidant properties of watercress extracts are summarized in Table 4. The control treatment showed stronger scavenging activities as well as the highest potential to reduce Fe (III) to Fe (II). The lowest antioxidant capacity was observed for plants from the 80 mM NaCl treatment, in which the lowest values of TP, TF, and PAL activity were also found (Table 4). The antioxidant activities displayed a similar trend to those of TP, TF, and PAL activities of watercress under increased NaCl concentrations (Table 3).

Table 4. Effect of different NaCl concentrations on the antioxidant activity of watercress (*R. nasturtium-aquaticum*).

NaCl (mM)	$EC_{50/DPPH}$ (mg/mL)	$EC_{50/ABTS}$ (mg/mL)	$EC_{50/RP}$ (mg/mL)
0 (Control)	0.13±0.00^{c}	0.056±0.00^{b}	0.229±.007^{c}
60	0.19±0.00^{b}	0.055±0.00^{b}	0.292±.005^{b}
80	0.23±0.00^{a}	0.082±0.00^{a}	0.408±.041^{a}
100	0.22±0.00^{a}	0.069±0.00^{c}	0.306±.012^{b}

The DPPH scavenging capacity here found for control watercress (Table 4) was similar to that reported for *Nasturtium officinale* R. Br. (watercress) from Iran (EC_{50} = 114.7 µg/mL) (41). The ABTS^{+} scavenging capacities were partially similar to those reported for *S. tenuifolia*, for which the low level of salinity (25 mM) stimulated the highest antioxidant capacity, and the levels of 50, 75, and 100 mM of NaCl caused a decrease. Nevertheless, unlike what was observed for watercress, for *S. Tenuifolia*, an increase in the sequestering capacity of ABTS$^{•+}$ was not observed at the highest salt concentration evaluated (200 mM) (31). The iron-reducing power showed a similar trend to that of the scavenging capacity of both DPPH and ABTS^{+}, and the three antioxidant capacity evaluations were related to TP, TF, and PAL activity (Figure 1). The current results were different from those reported for *Cuminum cyminum* L., in which an increase in the iron-reducing power exposed to saline stress was observed (42). Salinized and non-salinized watercress displayed better iron-reducing power than other Brassicaceae species, such as cabbage and cauliflower (9.07 and 16.75 mg/mL, respectively) (43), highlighting the important antioxidant capacity of *R. nasturtium-aquaticum*.

Methylation profiles

Table 5 shows that each of the eight EcoRI-MspI/HpaII primer combinations showed different levels of methylated, unmethylated, and hemimethylated loci in watercress plants grown in different salty conditions. The most informative combination of selective primers (the one that produced the highest number of amplified loci) under any saline treatment was *EcoRI* (5'-GACTGCGTACCAATTC**AG**-3') and *MspI/HpaII* (5'-ATCATGAGTCCTGCTCGG**TCAA**-3'), with a total of between 75 loci for plants of the 60 mM NaCl treatment and 79 loci for plants of the 100 mM NaCl treatment. The less informative selective primer combination in all cases was *EcoRI* (5'-GACTGCGTACCAATTC**AAC**-3') and *MspI/HpaII* (5'-ATCATGAGTCCTGCTCGG**AAT**-3'), with a total of between 26 loci for plants of the 60 mM NaCl treatment, and 45 loci, for plants of the 80 mM NaCl treatment.

Table 5. Methylation patterns of watercress (*R. nasturtium-aquaticum*) grown under different NaCl (mM) concentrations, were determined by eight combinations of selective initiators (*EcoRI* and *MspI/HpaII*).

Selective initiator *EcoRI*	Selective initiator *MspI/HpaII*	Methylated loci	Unmethylated loci	Hemimethylated loci	Amplified loci/primer combination	Methylation (%)
			0 mM (Control)			
AG	TCAA	2	72	2	76	2.63
AG	AAT	6	43	14	63	9.52
AC	TCAA	3	42	5	50	6.00
AC	AAT	12	15	29	56	21.43
AAC	TCAA	3	46	2	51	5.88
AAC	AAT	0	25	12	37	0.00
AT	TCAA	10	33	2	45	22.22
AT	AAT	16	32	6	54	29.63
TOTAL		52	308	72	432	12.16
			60 mM			
AG	TCAA	7	67	1	75	9.33
AG	AAT	8	56	8	72	11.11
AC	TCAA	10	41	8	59	16.95
AC	AAT	6	36	23	65	9.85
AAC	TCAA	9	40	13	62	14.52
AAC	AAT	7	17	2	26	17.50
AT	TCAA	8	26	10	44	17.50
AT	AAT	12	49	12	73	16.44
TOTAL		67	332	77	476	14.15

			80 mM			
AG	TCAA	8	65	3	76	10.53
AG	AAT	8	49	14	71	11.27
AC	TCAA	9	44	12	65	13.85
AC	AAT	19	27	29	75	25.33
AAC	TCAA	7	46	8	61	11.48
AAC	AAT	10	30	5	45	22.22
AT	TCAA	13	35	8	56	23.21
AT	AAT	12	41	1	54	22.22
TOTAL		86	337	80	503	17.51
			100 mM			
AG	TCAA	16	53	10	79	20.25
AG	AAT	7	50	16	73	9.59
AC	TCAA	17	35	10	62	27.42
AC	AAT	21	32	22	75	28.00
AAC	TCAA	14	37	6	57	24.56
AAC	AAT	3	27	5	35	8.57
AT	TCAA	16	26	7	49	32.65
AT	AAT	13	40	2	55	23.64
TOTAL		107	300	78	485	21.84

The total of methylated loci was highly variable, with a range of 52 for the control plants and 107 for the plants grown under the salt concentration of 100 mM. The number of unmethylated loci varied between 300 for the plants from the 100 mM NaCl treatment and 337 for the plants from the 80 mM NaCl treatment. The number of hemimethylated loci values ranged from 72 for control plants to 80 for plants from the 80 mM NaCl treatment. These differences by themselves indicate epigenetic variations between the watercress plants from the different salt treatments and suggest a high inner cytosine polymorphism between plants from each treatment.

The number of methylated, unmethylated, and hemimethylated loci changed between watercress that was not under stress and watercress that was under stress. Increases in the percentage of methylation according to increases in NaCl were found (between 12.16% for control plants and 21.84% for 100 mM salinized plants). However, there wasn't a dose-dependent change in the number of unmethylated or hemimethylated loci, which went down when the strongest salt treatment was used (Table 5). There was an inverse relationship between the percentage of methylation and PAL activity (-0.63), TP (-0.66), and TF (-0.65), indicating that salt stress-induced epigenetic changes had a significant impact on PAL, TP, and TF.

The levels of methylation found for the aerial parts of non-stressed watercress were lower than those reported for the mature leaves of 30 genotypes of *Brassica oleracea* (between 30% and 41%) (28), and also lower than those found for the expanded leaves of two *Capsicum* sp. genotypes (64.36–67.00%) (44), but higher than those found for the leaves of in vitro grown potato (1.4%) (45). All these different values suggest that in the plant kingdom, there is a high natural variation in methylation profiles. The different methylation levels found in the leaves of watercress may be related to the gene-specific expression of the phenylpropanoid biosynthesis pathway as needed by plants to adapt under each particular salt condition.

Some of the selective primer combinations here were also used for *Laguncularia racemosa* L. to estimate the epigenetic variability (11). For *L. recemosa*, the authors reported that the AAC/AAT combination was the most informative (with 70 out of a total of 209) (11). On the contrary, the combination AAC/AAT was the least informative for the watercress analyzed in the current study, which supports the proposal mentioned above about the high variation of the natural methylation profiles in the plant kingdom.

Several studies suggest that DNA methylation may be associated with transcriptional regulation of genes involved in plant stress responses, suggesting that variation in DNA methylation is important in mediating plant interaction with environmental factors (46, 47). The variations here registered for *R. nasturtium-aquaticum* could have prepared the plant to cope with the salinity levels evaluated. Some modifications may be stable and inherited by future generations to enable them to deal with extreme environments as an evolutionary process involved in adaptive responses and stress tolerance (48).

Epigenetic variability

The epigenetic variation among plants from the same saline treatment is shown in Table 6. The values of Nei's index, Shannon's index, and polymorphism revealed that the plants from the 60 mM NaCl treatment were the most epigenetically variable, while the 80 mM treatment was the most epigenetically homogeneous.

Table 6. Intraepigenetic variability of watercress (*R. nasturtium-aquaticum*) grown in different concentrations of NaCl.

	Control	60 mM	80 mM	100 mM
Nei 's diversity index	0.1127	0.1509	0.0855	0.1064
Shannon's index	0.1701	0.2263	0.1292	0.1597
Polymorphism (%)	31.25	40.91	23.86	28.98

Plants under saline stress can exhibit greater levels of epigenetic variability (49). Our results revealed that rearrangement of DNA methylation took place in salt-stressed watercress, generating more epigenetic variability in watercress from the treatment of 60 mM NaCl (Table 6). This indicates that a higher inner cytosine polymorphism can exist among the plants treated with 60 mM NaCl than among the plants treated with any of the other treatments.

Table 7 displays Nei's measures of epigenetic identity and epigenetic distances among watercress plants from the different saline treatments. The highest epigenetic identity (0.9458) and the lowest epigenetic distance (0.0558) were found between the plants from the 80 and 100 mM treatments, while the lowest epigenetic identity (0.9140) and the highest epigenetic distance (0.0900) were found between the plants from the 60 and 100 mM treatments. Genetic distance is the degree of genetic differences between populations or species. There is an inverse relationship between genetic distance and genetic identity, so the greater the genetic distance between two groups, the lower the genetic identity between them. Analogously, epigenetic distance can represent epigenetic differences between groups of organisms and can provide an estimation of how close, in epigenetic terms, different groups are. In the present study, the greater the epigenetic distance between the plants from two different saline treatments, the greater the differentiation in the levels of methylation. Thus, the greatest epigenetic closeness was found between plants from the 80 mM NaCl and 100 mM NaCl treatments. The plants from the 60 mM NaCl and 100 mM NaCl treatments were the most epigenetically dissimilar.

Table 7. Epigenetic identity (above the diagonal) and epigenetic distance (below the diagonal) between watercress (*R. nasturtium-aquaticum*) grown in different concentrations of NaCl.

Treatment	Treatment			
	Control	60 mM	80 mM	100 mM
Control	****	0.9377	0.9411	0.9158
60 mM	0.0644	****	0.9442	0.9140
80 mM	0.0607	0.0574	****	0.9458
100 mM	0.0880	0.0900	0.0558	****

Cluster analysis was done on the different methylation profiles to find out how the watercress grown in different salt conditions is related to each other epigenetically. The results (Figure 2) revealed a clear separation between control plants and plants from the 60 mM NaCl treatment on the one hand, and plants from the 80 mM NaCl and 100 mM NaCl treatments on the other hand. However, the cluster analysis did not distinguish plants from each saline treatment. These results showed that epigenetic changes occurred when salt levels increased from 60 mM to 80 and 100 mM.

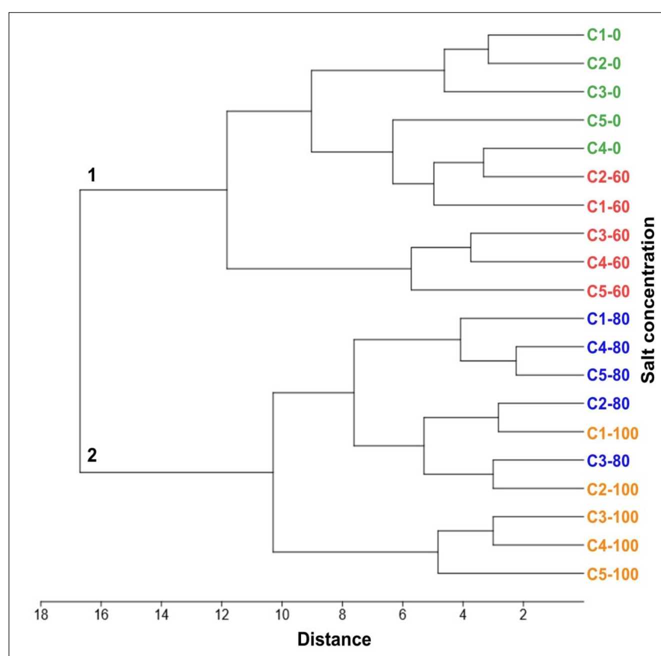

Fig. 2. Results of the cluster analysis comparing the methylation profiles of five individuals of watercress (*R. nasturtium-aquaticum*) grown under different concentrations of NaCl (C1-0 – C5-0: control; C1-60 – C5-60: 60 mM NaCl; C1-80 – C5-80: 80 mM NaCl; C1-100 – C5-100: 100 mM NaCl).

The results of the present study showed that salt stress produced increases in methylation levels associated with increased stress conditions. As indicated by the correlation analysis (Figure 1), when methylation levels increase, PAL activity decreases, and consequently, phenolic and flavonoid contents and antioxidant capacity also decreases. According to previous reports, increases in genome methylation inhibit the expression of particular genes (49), which could explain the phytochemical changes in watercress under saline stress. The variation of methylation level and polymorphism can be related to genomic plasticity, which is revealed in the phenotypic variation (50). Thus, the variations in TP, TF, antioxidant activities, and PAL here found for watercress could be determined by the epigenetic polymorphism estimated.

When all methylation levels, TP, TF, PAL, and antioxidant activity values here found for salt-stressed and non-stressed watercress were subjected to a PCA (Figure 3A), plants from each treatment were clearly discriminated. Plants of the treatments 80 mM NaCl and 100 mM NaCl were separated from plants of the control treatment and 60 mM NaCl by their high methylation level and high antioxidant capacity. The same data subjected to a cluster analysis revealed that the chemical, metabolic, and epigenetic alterations allowed the differentiation among plants of watercress from the different saline treatments (Figure 3B). The current results revealed that TP, TF, PAL activity, antioxidant capacity, and methylation level of non-stressed and salt-stressed watercress, taken together, represent important markers with agronomic and food quality implications.

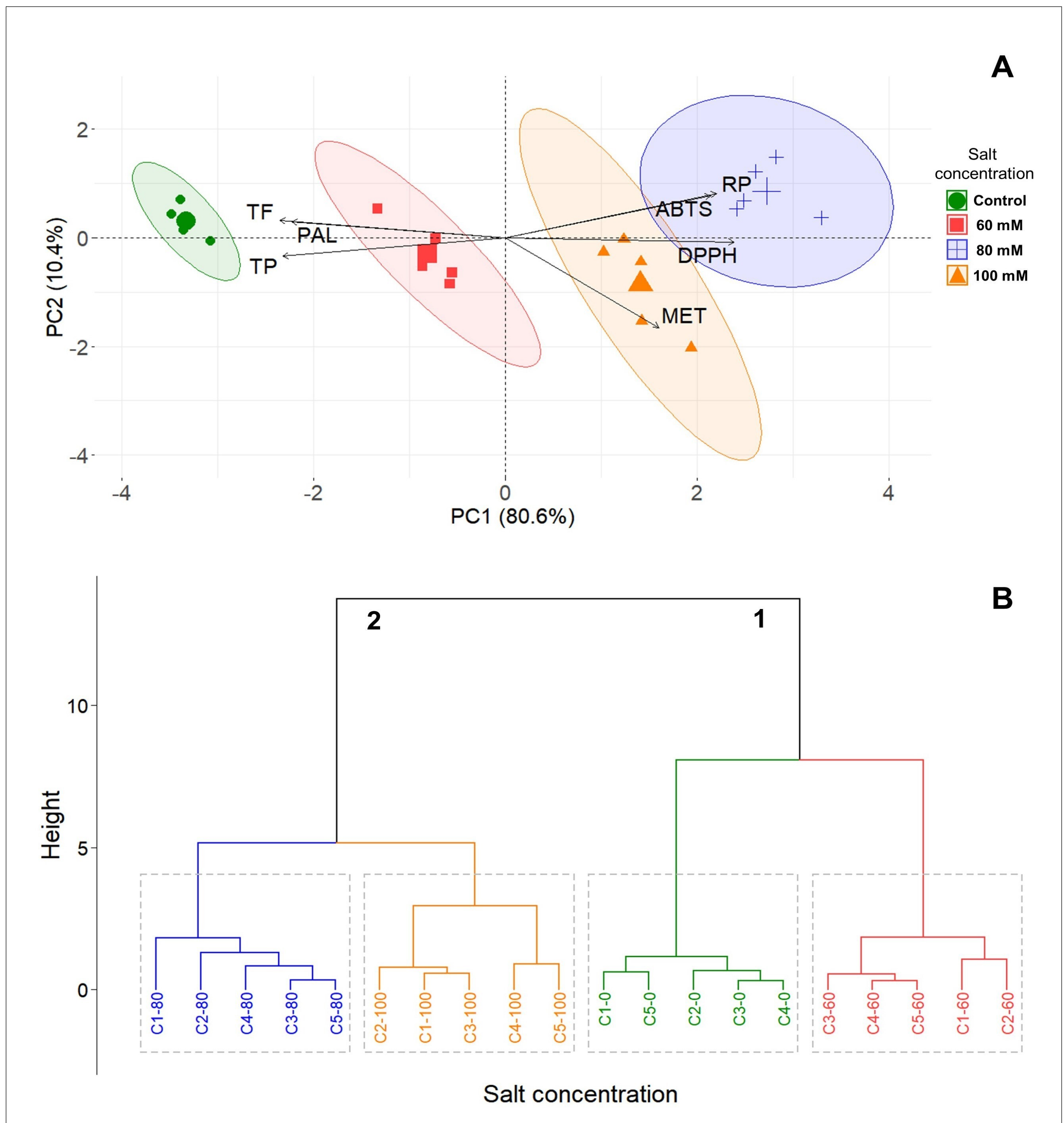

Fig. 3. Results of the Principal Component Analysis (**A**) and cluster analysis (**B**), comparing the contents of total phenolics (**TP**) and flavonoids (**TF**), DPPH scavenging activity (**DPPH**), ABTS scavenging activity (**ABTS**), iron reducing power (**RP**), phenylalanine ammonia lyase activity (**PAL**), and methylation percentage (**MET**) of watercress (*R. nasturtium-aquaticum*) grown under different concentrations of NaCl (C1-0 – C5-0: control; C1-60 – C5-60: 60 mM NaCl; C1-80 – C5-80: 80 mM NaCl; C1-100 – C5-100: 100 mM NaCl).

Conclusion

Salt stress induces reprogramming of the methylation pattern, which affects PAL activity, the contents of total phenolics and flavonoids, and the antioxidant properties of *R. nasturtium-aquaticum*. The reprogramming was modulated by the intensity of salinity. Since epigenetic and chemical responses can vary according to NaCl concentration, it is necessary to accurately register these types of responses to determine the adaptation potential of this economically important plant. The findings of this study contribute to our understanding of DNA methylation and its effect on watercress phenolic metabolism, which could aid in elucidating its mechanisms of expression regulation and stress adaptation. The knowledge of the phenolic contents and methylation levels of watercress, and the changes occurring under different saline concentrations may support genetic improvement programs focused on developing new salt-tolerant varieties. Taken together, the chemical, biochemical, and epigenetic attributes here evaluated represent markers for the growth conditions of the species, which could support the development of fingerprinting with implications in agronomy and food quality.

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Authors contributions

MVGV and NAA conceived and designed the study, participated in the collections of plant material, performed the statistical analysis, and analyzed and interpreted data. MVGV and HMMG carried out the epigenetic analysis. JAAR and EADA participated in the collections of plant material and carried out the total phenolics determinations. DYRB, LSGV and RTR carried out the antioxidant analysis. AVS carried out the enzymatic determinations. All authors made a critical revision, providing intellectual content. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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