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Effects of Seed Priming on Mitigating the Negative Effects of Increased Salinity in Two Varieties of Sweet Pepper (*Capsicum annuum* L.)

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Abstract: The increase in soil salinity has a negative effect on the growth and yield of plants. Mitigating the negative effects of soil salinity is therefore a difficult task and different methods are being used to overcome the negative effects of salt stress on crop plants. One of the often-used approaches is seed priming that can increase plants' vigor and resilience. In this paper, we tested the effects of hydropriming, proline priming, and salicylic acid priming on the mitigation of the negative effects of salt stress on two bell pepper varieties (*Capsicum annuum* L.): Herkules and Kurtovska kapija. Sweet bell pepper seeds were primed following desiccation to achieve the original water content, and subsequently cultivated in salt-supplemented medium. The positive effects on vigor (in the form of increased germination and seedling establishment) as well as on level of tolerance for salt stress were recorded for both cultivars. The positive effects varied between the priming treatments and pepper cultivar used. The results of germination, seedling performance, photosynthetic pigments, and osmolytes were measured for seedlings grown from unprimed and primed seeds with under 0, 25, and 50 mM of NaCl. Both cultivars demonstrated greater germination when primed with proline and salicylic acid, while the Herkules cultivar demonstrated a higher tolerance to salt when proline was used as the priming agent. Priming with salicylic acid and proline in the seed improved germination and seedling performance, which could be related to the increase in proline content in the seedlings.

Keywords: seed priming; sweet pepper; soil salinity; proline; salicylic acid

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1. Introduction

The reduction in water flow and the lengthening of dry periods is increasing the salinity of soil, especially in irrigated agricultural areas. In some parts of the world, 50% of irrigated land is affected by increased soil salinity [1]. Globally, a total of 33% of all agricultural land is salinized and there is a trend of a 10% annual increase due to increased surface evaporation (due to increased temperatures), decreased precipitation, and other climate change-related factors [2]. Soil salinity refers to increased concentrations of cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) and anions (HCO^{3-}, Cl^-, NO_3, ${SO_4}^{2-}$, and ${CO_3}^{2-}$) that influence plant metabolism [3] and negatively affect plant growth and yield, often leading to crop failure [4]. Mitigating the effects of soil salinity on plants is a challenging task for scientists to accomplish. It is recognized worldwide that it is useful to use sustainable methods. Recent advances in understanding the mechanisms of salt stress have enabled the development of approaches to induce plant defense mechanisms using beneficial molecules or microorganisms at different growth stages of crops to increase the salt tolerance of crops and mitigate the negative effects of soil salinity. One of these approaches is seed priming [5]. Salt stress is one of the limiting factors in crop food yield production, and prolonged exposure to salt stress can result in crop loss [6]. Salt induces direct and indirect physiological disturbances in plant tissues, including osmotic stress, reduced plant growth, and nutrient uptake, as well as the generation of reactive oxygen molecules, which further induce damage to proteins, carbohydrates, lipid membranes, and DNA [7,8]. One of the commonly used methods is the application of different molecules at the seed level, called seed priming, which relies on the controlled hydration of the seed to take up water and trigger metabolism before germination, which involves the activation of enzymes, metabolite build-up, and other processes that contribute to shortening the lag time during germination [9–11]. Seed priming includes different types, depending on the method/compound used: hydropriming (water), halopriming (inorganic salt), osmopriming (osmotic solution), hormonal priming (phytohormones), biopriming (microorganisms), and priming with nanoparticles [5].

Sweet pepper (*Capsicum annuum* L.) is the third most important crop from the Solonaceae family (after tomato and potato) with differently colored fruits ranging from yellow, orange, red, purple, and brown [12]. Peppers are usually grown in greenhouses where constant irrigation is required to ensure optimum water availability. Often, the fertilizer requirement is high, and mineral fertilizers have been used to meet the nutrient requirements that can lead to increased soil salinity [13]. According to Abdellal et al. [14], salt stress in sweet peppers leads to a decrease in the chlorophyll content, relative water content, and fruit yields, whereas electrolyte leakage, malondialdehyde (MDA), proline concentration, reactive oxygen species (ROS), and the activities of antioxidant enzymes increase under salt stress. Also, salt stress in sweet peppers can cause a decrease in the sugar content, the total and individual organic acid contents, but also an increase in the content of capsaicinoids [13]. Foliar applications of ascorbic acid [15], salicylic acid, proline [14], silicone [16], and glycine betaine [17] to sweet peppers can help mitigate the negative effects of soil salinity. However, the reaction to salt stress within the *Capsicum* genus is genotype-, fruit part-, and salinity level-dependent [13].

The aim of the present study is to evaluate how seed priming can effectively be utilized in the alleviation of salt stress effects on two bell pepper varieties and how seed priming can influence salt tolerance levels. In this study, we investigated the possible effects of seed treatments with water (hydropriming), 1 mM of salicylic acid, and 1 mM of proline on mitigating the negative effects of soil salinity on the early developmental stage of the plants of two bell pepper varieties—Kurtovska kapija and Herkules. Both varieties are important to produce peppers for industrial processing, for the production of the popular vegetable spread in the Balkans, *ajvar*. We measured the possible effects of priming on seed germination, seedling growth parameters, photosynthetic pigments, proline content, and total soluble carbohydrate content under salt stress, and compared the results with control plants grown without salt in a medium.

2. Materials and Methods

2.1. Plant Material, Seed Priming, and Experimental Design

Seeds of two sweet pepper varieties, Herkules Red 023 (ECN: 09H3100161) and Kurtovska kapija (ECN: 800134), were obtained from Gene bank, Research Institute of Crop Production (VURV) Praha–Ruzyně. Healthy seeds of both cultivars were primed by immersing the seeds in a suitable priming medium (water—hydropriming, 1 mM of salicylic acid, and 1 mM of proline) for 24 h at 8 °C. After priming, the seeds were briefly washed with distilled water, dried to the original moisture content, and grown under control or salt conditions. Non-primed seeds were used as a control grown on salt-free and salt-supplemented media. For each variety and priming medium, 150 seeds were primed.

To control all other environmental factors and only investigate the influence of soil salinity, we grew the plants in vitro in a growth chamber under controlled conditions. All plants were cultivated in vitro using aseptic techniques on a nutrient medium.

The experiment consisted of testing three priming agents (hydropriming—H_2O; proline priming—1 mM of proline, and salicylic acid priming—1 mM of salicylic acid) and two levels of salinity (25 mM and 50 mM). Non-primed seeds were used as control seeds, while medium without the supplementation of NaCl served as the control medium.

2.2. Growing Condition and Stress Treatment

Media for plant cultivation contained vitamins, iron, and minerals [18]; sucrose was used as a carbon source and 1% (*w*/*v*) agar puris was added as a gelling agent after the pH adjustment of the media to 5.8. Salt (NaCl) was added to the medium prior to pH adjustment, where appropriate.

For each variety, 5 petri dishes per salt level (0, 25, and 50 mM of NaCl) and per priming treatment (non-primed, hydroprimed, proline-primed, and salicylic acid-primed seeds) were used. The experiment was repeated in triplicates. All cultures were kept in a growth chamber with regulated temperature (23 ± 2 °C), light (16 h photoperiod provided by FLORA Osram lamps with Luminous flux at 25 °C 1000 lm), and humidity (70%).

2.3. Determination of Germination Percentage and Seedling Growth Parameters

To evaluate the germination percentage (%), the number of germinated seeds in relation to the number of cultivated seeds per treatment was evaluated after two weeks for all experiments. Seedling fresh weight (FW) and dry weight (DW), biomass production (BP), and total water content (TWC) were evaluated after 4 weeks of culture. Biomass production was calculated in correlation to the dry weight of non-primed seedlings grown on control or salt stress media, according to the formula [11]:

$$\%BP = \frac{DW_p - DW_c}{DW_c} \times 100 \quad (1)$$

where %BP is the biomass production percentage; DW_p is the dry weight (g) of primed plants grown on corresponding media; and DW_c is the dry weight (g) of non-primed plants grown on control medium.

Total water content (%) was calculated using the fresh and dry weights of plants (after drying at 50 °C until a constant mass was achieved) [11]:

$$\%TWC = \frac{FW - DW}{FW} \times 100 \quad (2)$$

where %TWC is the percentage of total water content; FW is fresh weight (g), and DW is dry weight (g).

2.4. Measurements of Photosynthetic Pigment Content

Photosynthetic pigments were determined spectrophotometrically in 100% acetone extracts of obtained shoots, against blank (100% acetone), and quantified according to Lichtenthaler [19]. The results are expressed as mg of pigment per gram of shoot fresh weight (mg/g FW).

2.5. Measurements of Osmolyte Adjustment Potential

The potential of plants for osmolyte adjustment was evaluated according to the production of osmolytes (proline and sugars). Proline content was analyzed according to the method of Carillo [20] using 70% ethanol extract from dry plant material from which chlorophylls were previously removed to avoid interference with the readings (using absolute acetone). Prior to proline extraction, pellets were dried removing the remaining acetone. Quantification of proline was performed using a calibration curve of L-proline and expressed as mg of proline per g of DW.

Total soluble carbohydrate content was evaluated according to the method of Teslova et al. [21] using anthrone reagent and quantified using a calibration curve of glucose. The results are expressed as mg/g DW. Content of starch was determined in a hydrolyzed pellet following the extraction of soluble sugars. Quantification of sugars was performed according to the calibration curve of glucose, where the obtained results were multiplied by 0.9 to convert molecules of glucose to molecules of starch and expressed as mg of soluble sugar/starch per g DW, respectively.

2.6. Statistical Analysis

All the data were analyzed using STATISTICA 10.0 software (Statsoft Inc., Hamburg, Geramny, StatSoft GmbH). Experimental results are presented as the mean ± standard deviation of three independent replications. The obtained data were subjected to a variance analysis (ANOVA), and the Newman–Keuls post hoc test was carried out to identify significant differences between the extract types. Mean values with $p < 0.01$ were considered statistically significant. Principal component analysis (PCA) was performed on the whole dataset.

3. Results and Discussion

3.1. Seed Priming Effects on Germination and Seedling Growth Performance under Salt Stress

Seed priming can enhance plants' tolerance toward stress, stimulating a quicker response and higher seedling and plant vigor under salt stress conditions [22]. Sweet bell peppers have varying degrees of tolerance toward salt stress [13,23], with the investigated variety Kurtovska kapija being more susceptible to salt stress than Herkules. The establishment of seedlings from primed and non-primed seeds varies among the used priming agents and salt concentrations (Table 1). Kurtovska kapija, in general, had a low germination rate and low seedling establishment, especially under high salt stress, where even germinated seedlings presented altered root morphologies (shortening of the root and a lack of root hairs).

Table 1. The performance of two sweet pepper cultivars under different concentrations of salt stress (0, 25, and 50 mM) grown from unprimed and primed (hydroprimed, 1 mM of proline, and 1 of mM salicylic acid) seeds.

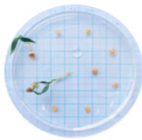
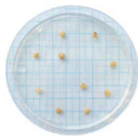
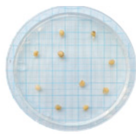
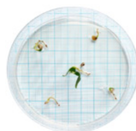
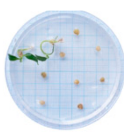
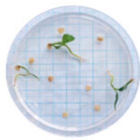
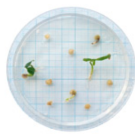
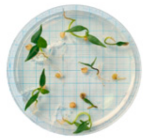
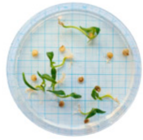
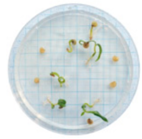
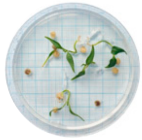
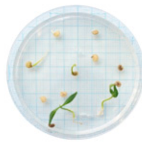
		NaCl Concentration (mM)		
	Variety	**0**	**25**	**50**
Non-primed	Kurtovska kapija			
	Herkules			
Hydroprymed	Kurtovska kapija			
	Herkules			

Table 1. *Cont.*

		NaCl Concentration (mM)		
	Variety	**0**	**25**	**50**
1 mM proline-primed	Kurtovska kapija			
	Herkules			
1 mM salycilic acid-primed	Kurtovska kapija			
	Herkules			

The germination percentages of Kurtovska kapija and Herkules non-primed seeds under control conditions were below 50% (45% and 22%, respectively) (Table 2). Low germination rates can be improved using different priming agents, which was demonstrated in our study; but, the priming agent used can have a significant influence on the plants' germination and vigor. Vigor and plant response to stress are often determined by the plant's fast germination and fast establishment of seedlings, and seed priming can enhance both (germination speed and seedling vigor) [24]. The use of different priming agents can induce different responses and increase stress tolerance in plants growing from primed seeds. In our study, proline priming appeared to be the best priming treatment, significantly increasing the germination rates of both varieties (Kurtovska kapija and Herkules), reaching 66% and 100%, respectively. The significance of the increase in the germination rate is the most evident under moderate salt stress, where proline-primed seeds show an increase in germination rate compared to the control despite the stress with a germination rate of 100% for the Herkules variety and over 50% for Kurtovska kapija.

The Kurtovska kapija variety also showed an increase in dry matter, while Herkules showed a significant increase in fresh matter in proline-treated plants. The positive effects of proline priming are also reflected in the salt tolerance of the plants. The increased tolerance of the plants to salt stress after proline- and salicylic acid-priming treatments was reflected in the higher germination rates and a higher survival rate of the primed plants (both varieties) compared to non-primed and hydroprimed plants (Tables 1 and 2). According to our results, hydropriming is not beneficial for the plants and these results are in accordance with the paper published by [2], which tested the effect of hydropriming on two sweet pepper varieties and also found that hydropriming reduced germination in both tested cultivars. Also, hydropriming did not have an effect on seedling performance under salt stress, which was evident from fresh and dry weights and total water content and biomass production parameters. On the other hand, proline- and salicylic acid-priming measures increased the germination and seedling performance of non-primed seeds and induced a higher tolerance of salt stress (Table 1), which is evident from the increase in biomass production (Table 2). But this effect was variety-specific and, for example, proline tended to be more beneficial for Herkules for Kurtovska kapija; these effects were mild but still beneficial. Proline-priming of Herkules seeds increased the fresh weight of control plants 2fold(grown on 0 mM of NaCl) and improved the performances of seedlings grown under 25 mM and 50 mM, concentrations which were lethal for non-primed seeds of Herkules. Hossinifarahi et al. [25] also reported that seed priming using salicylic acid may be beneficial for germination. However, similar to our study, different effects of priming on different varieties were noticed in the study where the authors evaluated the effects of hydropriming, osmopriming, and thermopriming on the mitigation of salt stress effects on sweet pepper cultivars [26]. Also, Yadev et al. [27] found that primed pepper seeds with warm water, $CuSO_4$, potassium nitrate, and polyethylene glycol demonstrated increased rates as well as percentages of seed germination and showed improved tolerance to stress resulting in an overall improved seedling performance. Salicylic acid can regulate ion uptake and enhance defense mechanisms under salinity stress by influencing the biosynthesis of different molecules involved in the alleviation of salt stress [28].

3.2. Seed Priming Effects on Photosynthetic Pigments

Fully formed seedlings of two pepper varieties were further tested to evaluate the effects of salt stress on photosynthetic activity through the evaluation of changes in photosynthetic pigments. The photosynthetic pigments of sweet pepper varieties Kurtovska kapija and Herkules grown from non-primed and primed seeds are shown in Table 3.

In general, the content of photosynthetic pigments was low in our samples, which was not surprising since we analyzed young shoots that did not yet have fully developed organs. Similarly, shoots grown in vitro can demonstrate lower chlorophyll contents, as recorded by Karalija et al. [8] for lettuce seedlings grown in vitro. As can be seen in Table 3, there are no major differences and no clear trend between these parameters within the analyzed samples. Similar results were also reported for *Brassica* seedlings under salt stress [29]. On the other hand, it has been reported in the literature that salt stress can reduce photosynthetic parameters in sweet peppers [14,30] and that measuring the photosynthetic parameters can be useful to determine the measures that may be beneficial for mitigating salinity in more developed plants [14]. The reduction in chlorophyl content after priming with salicylic acid has been previously recorded when a higher concentration of salicylic acid was used (1 mM), which is in concordance with the results obtained in this study, while lower concentrations of salicylic acid can have enhancing effects on chlorophyll content [28].

3.3. Seed Priming Effects on Osmolyte Adjasment and Total Protein Content

Plants' adaptation to salt stress involves, among other mechanisms, the synthesis of osmolytes in a process of osmolyte adjustment to new osmotic conditions experienced by the root. Osmolyte adjustments under stress in primed and non-primed sweet pepper seedlings were assessed according to the concentrations of proline, soluble sugars, and starch in the shoots and roots of fully developed seedlings in plants under osmotic stress in relation to control plants (Table 4).

Table 2. Effects of seed priming on germination percentage and shoot biomass of two sweet bell pepper varieties grown from hydroprimed (HP) seeds and seeds primed with proline (PP) and salicylic acid (SAP) and non-primed (NP) seeds under stress.

		Kurtovska kapija				*Herkules*			
		NP	**HP**	**PP**	**SAP**	**NP**	**HP**	**PP**	**SAP**
Control	G (%)	45.00 ^{e} ± 2.00	15.00 ^{g} ± 1.00	66.00 ^{b} ± 1.00	50.00 ^{d} ± 1.00	22.00 ^{f} ± 1.00	22.00 ^{f} ± 2.00	100 ^{a} ± 0.00	61.00 ^{c} ± 1.00
	FW (mg/plant)	77.06 ^{c} ± 0.21	16.33 ^{e} ± 0.35	47.33 ^{d} ± 0.19	42.86 ^{d} ± 0.43	77.07 ^{c} ± 2.3	90.37 ^{b} ± 2.4	147.53 ^{a} ± 9.81	77.60 ^{c} ± 1.12
	DW (mg/plant)	3.03 ^{e} ± 0.01	1.33 ^{f} ± 0.03	4.53 ^{d} ± 0.09	4.03 ^{d} ± 0.02	11.67 ^{a} ± 0.02	8.60 ^{b} ± 0.13	8.70 ^{b} ± 0.06	6.40 ^{c} ± 0.14
	TWC (%)	96.06 ^{a} ± 1.09	91.83 ^{b} ± 2.1	90.42 ^{b} ± 3.10	90.59 ^{b} ± 2.2	84.86 ^{c} ± 1.23	90.48 ^{b} ± 1.14	94.10 ^{ab} ± 1.19	91.75 ^{b} ± 4.1
	BP (%)	-	−56.04 ^{a} ± 2.2	49.45 ^{b} ± 0.98	32.96 ^{d} ± 1.09	-	−26.29 ^{e} ± 0.24	−25.43 ^{e} ± 0.98	−45.14 ^{c} ± 1.1
25 mM NaCl	G (%)	15.00 ^{h} ± 1.00	17.25 ^{g} ± 1.45	52.50 ^{b} ± 1.25	47.50 ^{c} ± 1.25	30.00 ^{e} ± 1.00	25.00 ^{f} ± 2.00	100 ^{a} ± 0.00	40.00 ^{d} ± 1.00
	FW (mg/plant)	35.10 ^{b} ± 0.19	31.43 ^{d} ± 0.13	51.43 ^{a} ± 1.01	50.00 ^{a} ± 1.10	NM	34.47 ^{bc} ± 1.02	34.37 ^{bc} ± 0.27	33.25 ^{cd} ± 0.23
	DW (mg/plant)	4.30 ^{b} ± 0.09	3.03 ^{c} ± 0.02	4.60 ^{b} ± 0.21	4.50 ^{b} ± 0.20	NM	6.97 ^{a} ± 0.03	6.87 ^{a} ± 0.77	3.60 ^{c} ± 0.04
	TWC (%)	87.74 ^{b} ± 2.1	90.34 ^{ab} ± 2.1	91.05 ^{a} ± 1.95	91.00 ^{a} ± 2.0	NM	77.69 ^{c} ± 2.23	79.79 ^{c} ± 2.34	89.17 ^{b} ± 2.14
	BP (%)	-	−26.29 ^{a} ± 1.22	6.97 ^{b} ± 0.11	4.65 ^{b} ± 1.13	-	*	*	*
50 mM NaCl	G (%)	LD	LD	25.00 ^{c} ± 1.00	32.50 ^{b} ± 1.50	21.00 ^{d} ± 1.00	LD	81.00 ^{a} ± 2.00	22.00 ^{d} ± 2.00
	FW (mg/plant)	LD	LD	30.35 ^{d} ± 0.99	35.45 ^{c} ± 0.02	NM	LD	69.55 ^{a} ± 2.13	54.60 ^{b} ± 1.3
	DW (mg/plant)	LD	LD	3.85 ^{c} ± 0.09	3.25 ^{c} ± 1.72	NM	LD	9.45 ^{a} ± 0.15	4.57 ^{c} ± 0.04
	TWC (%)	LD	LD	87.31 ^{b} ± 3.1	90.83 ^{a} ± 0.08	NM	LD	86.41 ^{b} ± 3.12	91.64 ^{a} ± 4.1
	BP (%)	-	LD	*	*	NM	LD	*	*

G—gemination percentage; FW—fresh weight; DW—dry weight; TWC—total water content; BP—biomass production; NM—not measured due to bad shape of the plants—dried out plants or dying plants. LD—lethal dose. *—biomass production could not be calculated since no control plants survived the stress. Parameters within one row sharing the same letter do not differ significantly according to the ANOVA post hoc analysis of variance using the Newman–Keuls test at the level of significance of $p < 0.01$.

Table 3. Effect of seed priming of two sweet bell pepper varieties grown from hydroprimed (HP) seeds and seeds primed with proline (PP) and salicylic acid (SAP) and non-primed (NP) seeds under stress.

	NaCl (mM)	*Kurtovska kapija*				*Herkules*			
		Chl *a* (mg/gFW)	Chl *b* (mg/gFW)	Total Chls (mg/gFW)	Car (mg/gFW)	Chl *a* (mg/gFW)	Chl *b* (mg/gFW)	Total Chls (mg/gFW)	Car (mg/gFW)
NP	0	0.69 ^{b} ± 0.01	0.34 ^{a} ± 0.01	1.04 ^{b} ± 0.03	0.38 ^{a} ± 0.01	0.14 ^{bcd} ± 0.01	0.06 ^{bc} ± 0.00	0.19 ^{d} ± 0.01	0.06 ^{c} ± 0.00
	25	0.84 ^{a} ± 0.01	0.35 ^{a} ± 0.00	1.19 ^{a} ± 0.01	0.40 ^{a} ± 0.00	NM	NM	NM	NM
	50	LD	LD	LD	LD	NM	NM	NM	NM
HP	0	0.59 ^{c} ± 0.01	0.26 ^{b} ± 0.00	0.85 ^{c} ± 0.01	0.28 ^{b} ± 0.00	0.16 ^{bc} ± 0.01	0.07 ^{ab} ± 0.01	0.23 ^{b} ± 0.02	0.07 ^{bc} ± 0.00
	25	0.54 ^{e} ± 0.01	0.22 ^{cd} ± 0.01	0.76 ^{d} ± 0.02	0.24 ^{cd} ± 0.00	0.20 ^{ac} ± 0.01	0.08 ^{a} ± 0.00	0.28 ^{a} ± 0.01	0.08 ^{ab} ± 0.00
	50	LD	LD	LD	LD	LD	LD	LD	LD
PP	0	0.34 ^{i} ± 0.01	0.17 ^{ef} ± 0.00	0.51 ^{fg} ± 0.01	0.22 ^{de} ± 0.00	0.14 ^{bcd} ± 0.06	0.06 ^{bc} ± 0.01	0.20 ^{cd} ± 0.02	0.06 ^{cd} ± 0.01
	25	0.38 ^{g} ± 0.01	0.19 ^{de} ± 0.00	0.56 ^{f} ± 0.01	0.20 ^{e} ± 0.00	0.12 ^{bd} ± 0.01	0.05 ^{cd} ± 0.00	0.18 ^{d} ± 0.02	0.05 ^{de} ± 0.01
	50	0.36 ^{h} ± 0.00	0.15 ^{fg} ± 0.00	0.51 ^{fg} ± 0.01	0.16 ^{f} ± 0.00	0.10 ^{d} ± 0.01	0.04 ^{d} ± 0.01	0.14 ^{e} ± 0.01	0.04 ^{e} ± 0.01
SAP	0	0.56 ^{d} ± 0.05	0.24 ^{bc} ± 0.02	0.80 ^{cd} ± 0.08	0.26 ^{bc} ± 0.02	0.15 ^{cd} ± 0.01	0.06 ^{bc} ± 0.00	0.20 ^{cd} ± 0.01	0.06 ^{cd} ± 0.00
	25	0.45 ^{f} ± 0.00	0.19 ^{de} ± 0.00	0.64 ^{e} ± 0.01	0.20 ^{e} ± 0.00	0.21 ^{a} ± 0.01	0.08 ^{a} ± 0.01	0.29 ^{a} ± 0.02	0.08 ^{ab} ± 0.01
	50	0.33 ^{j} ± 0.00	0.14 ^{g} ± 0.00	0.47 ^{g} ± 0.01	0.15 ^{f} ± 0.00	0.12 ^{bd} ± 0.02	0.05 ^{cd} ± 0.01	0.17 ^{de} ± 0.03	0.05 ^{de} ± 0.01

NM—not measured due to bad shape of the plants—dried out plants or dying plants. LD—lethal dose. Parameters within one column sharing the same letter do not differ significantly according to the ANOVA post hoc analysis of variance using the Newman–Keuls test at the level of significance of $p < 0.01$.

Table 4. Osmolyte adjustments in two sweet bell pepper varieties grown from hydroprimed (HP) seeds and seeds primed with proline (PP) and salicylic acid (SAP) and non-primed (NP) seeds under stress.

		NaCl mM	*Kurtovska kapija*				*Herkules*			
			NP	HP	PP	SAP	NP	HP	PP	SAP
Soluble sugars (mg/gDW)	**Shoot**	0	8.89 [b] ± 5.39	ND	33.43 [a] ± 5.55	5.01 [b] ± 4.53	1.42 [c] ± 0.09	0.76 [c] ± 0.15	1.55 [c] ± 0.94	0.78 [c] ± 0.41
		25	ND	4.29 [b] ± 0.89	3.58 [bc] ± 0.59	7.49 [a] ± 1.55	NM	ND	2.00 [c] ± 0.05	ND
		50	LD	LD	ND	ND	LD	LD	ND	ND
	Root	0	ND	ND	13.77 [a] ± 0.55	1.09 [b] ± 0.75	NM	ND	1.41 [b] ± 0.15	ND
		25	ND	ND	7.11 [b] ± 0.35	ND	NM	ND	11.68 [a] ± 5.86	ND
		50	LD	LD	ND	ND	NM	LD	ND	ND
Starch (mg/gDW)	**Shoot**	0	19.97 [d] ± 1.52	ND	30.36 [b] ± 3.99	27.38 [c] ± 4.32	6.51 [e] ± 1.81	9.20 [e] ± 2.50	21.56 [d] ± 3.92	49.44 [a] ± 3.94
		25	23.23 [b] ± 1.67	11.15 [d] ± 0.95	33.78 [a] ± 2.14	25.79 [b] ± 3.22	NM	ND	14.37 [cd] ± 5.99	20.22 [bc] ± 2.60
		50	LD	LD	11.18 [c] ± 5.05	21.03 [a] ± 0.89	NM	LD	11.67 [c] ± 2.58	17.31 [b] ± 1.76
	Root	0	ND	ND	14.57 [bc] ± 4.87	13.10 [c] ± 5.04	20.62 [b] ± 3.96	9.04 [c] ± 0.73	20.59 [b] ± 3.52	32.16 [a] ± 7.04
		25	ND	ND	24.46 [a] ± 1.13	11.93 [c] ± 1.29	NM	ND	17.77 [b] ± 3.68	22.61 [a] ± 2.48
		50	LD	LD	14.11 [b] ± 4.55	10.22 [b] ± 0.99	NM	LD	22.56 [a] ± 1.81	16.81 [b] ± 2.16
Proline (mg/gDW)	**Shoot**	0	8.89 [e] ± 0.39	ND	33.43 [a] ± 0.59	5.00 [f] ± 0.05	19.97 [d] ± 0.51	ND	30.36 [b] ± 0.99	27.38 [c] ± 0.32
		25	LD	4.29 [e] ± 0.09	3.58 [e] ± 0.54	7.49 [d] ± 0.07	23.23 [b] ± 1.67	11.15 [c] ± 0.98	33.77 [a] ± 1.14	25.79 [b] ± 0.22
		50	LD	LD	ND	ND	NM	LD	11.18 [b] ± 0.57	21.04 [a] ± 0.89
	Root	0	ND	ND	13.77 [a] ± 0.54	ND	NM	ND	14.57 [a] ± 0.87	13.11 [a] ± 0.41
		25	ND	ND	ND	ND	NM	ND	24.46 [a] ± 1.13	11.93 [b] ± 0.29
		50	LD	LD	ND	ND	NM	LD	14.12 [a] ± 0.52	ND

NM—not measured due to bad shape of the plants—dried out plants or dying plants. LD—lethal dose. Parameters within one row sharing the same letter do not differ significantly according to the ANOVA post hoc analysis of variance using the Newman–Keuls test at the level of significance of $p < 0.01$.

Salt stress limits the ability of plants to absorb water due to osmotic pressure created by an increased concentration of ions outside the root. Therefore, the accumulation of osmolytes in plant tissues, especially in the roots, can increase their tolerance to salt stress [31] through an increase in the osmotic potential in root cells, ensuring water retention in root cells. One of the main osmolytes that is often accumulated as a response to salt stress is proline; a variation in salinity stress resistance among species is attributed to differences in the proline content [16,29,32]. In our investigation, it was demonstrated that seedlings developed from primed seeds could accumulate higher amounts of proline than non-primed plants. It was found that unprimed shoots of the Herkules variety contained a 2.25-fold-higher amount of endogenous proline than control plants when exposed to salt stress. Interestingly, the proline content in the shoot and root of the Herkules variety increased when the seed was treated with proline and salicylic acid, while this trend was not observed in the salt-sensitive Kurtovska kapija variety. An increase in proline content correlating to higher abiotic stress tolerance has been confirmed in other studies in different plant species [7–9].

On the other hand, unprimed shoots and roots of the Kurtovska kapija variety grown without salt showed a higher content of soluble sugars and starch than Herkules. Priming with proline significantly increased the contents of soluble sugars and starch in Kurtovska kapija, while treatment with salicylic acid increased the starch content in both varieties. Different mechanisms of osmolyte adjustments induced by seed priming (accumulation of more proline in Herkules, a more tolerant plant, and the accumulation of more soluble sugars in Kurtovska kapija, a more sensitive plant) as well as a mutual response (accumulation of starch) seem to be related to the use of proline and salicylic acid as priming agents. Compared to other varieties, we noted that salicylic acid priming induced a higher increase in osmolytes in Herkules varieties, while for Kurtovska kapija, proline seemed to be more effective as a priming agent. According to our results, both the endogenous proline content and the increase in proline content by priming seeds are important for salt stress tolerance; so, the choosing a priming agent that is capable of increasing the proline level can be a target for the mitigation of the negative effects of salt stress.

3.4. Comparative Analysis of Priming Effects on Salt Stress Tolerance in Two Pepper Varieties

The results were summarized and a PCA analysis was performed to evaluate the correlation between the type of priming agent and the level of salt tolerance in the analyzed pepper varieties. We performed two PCAs: in one, we analyzed photosynthetic pigment content variation and in second the osmolyte variation in relation to priming agent selection and the level of salt stress (Figure 1).

The PCA analysis of photosynthetic pigment variation in relation to priming agent and salt stress level revealed a clear distinction between the two varieties of tested peppers, with the Kurtovska kapija variety showing a more robust response with little variation in the chlorophyte content in relation to salt stress, while the Herkules variety showed a scattered response in relation to salt concentration (Figure 1). This is to be expected considering that these varieties are differently susceptible to salt stress and their photosynthetic apparatus is probably differently adapted to salt stress.

The responses of both cultivars in terms of osmolyte adaptation are more strongly related to the priming agent used, showing that sugar synthesis is strongly influenced by priming with proline, and this priming agent clearly contributes to a higher tolerance of plants to salt stress (Figure 2). It is also evident that osmolyte synthesis (primarily of sugars) is more strongly affected in Kurtovska kapija, while Herkules regulates both proline and sugar synthesis to increase tolerance levels to salt stress. The role of osmolytes in the tolerance of abiotic stressors induced by seed priming has been previously confirmed in several plants [8–11].

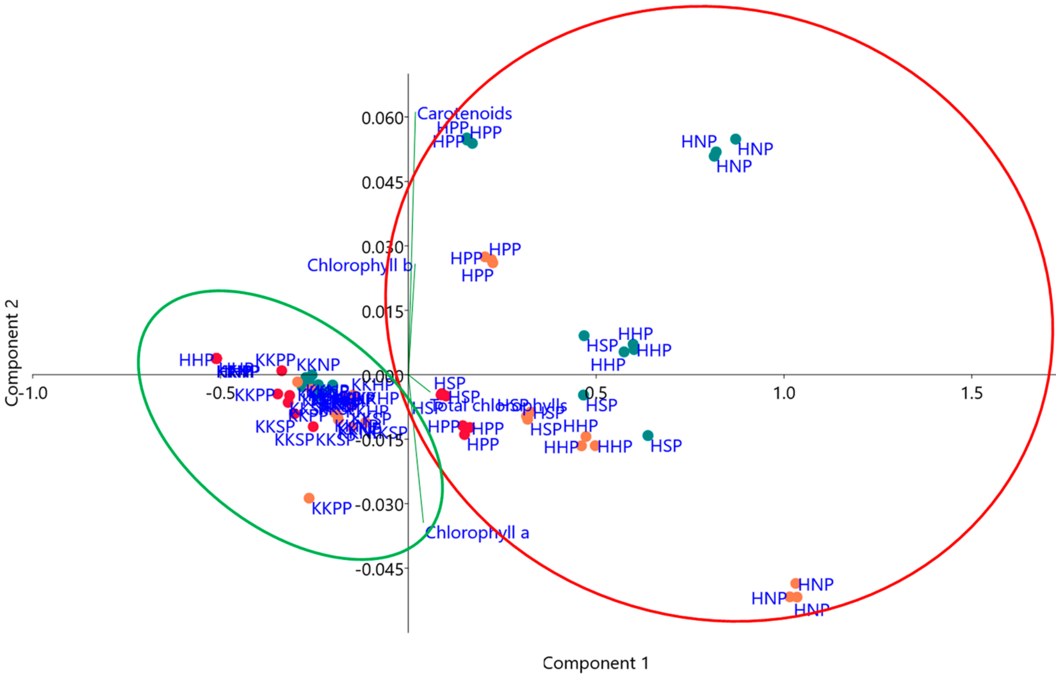


Figure 1. PCA analysis of chlorophyll content variation in shoots in relation to priming agent, level of salt stress, and variety. **KK**—Kurtovska kapija, **H**—Hercules, **NP**—non-primed; **HP**—hydroprimed; **PP**—proline-primed; **SP**—salicylic acid-primed. **Blue color** –0 mM NaCl; **orange**—25 mM NaCl; **red**—50 mM NaCl.

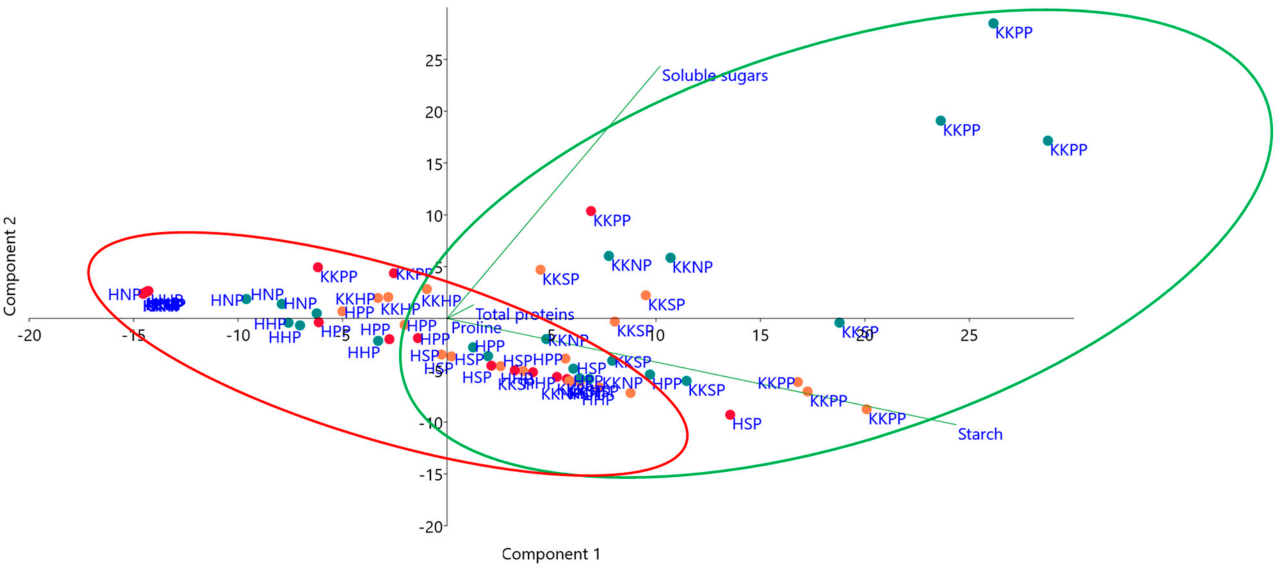


Figure 2. PCA analysis of chlorophyll content variation in shoots in relation to priming agent, level of salt stress, and variety. KK—Kurtovska kapija, H—Hercules, NP—non-primed; HP—hydroprimed; PP—proline-primed; SP—salicylic acid-primed. Blue color—0 mM NaCl; orange—25 mM NaCl; red—50 mM NaCl.

4. Conclusions

Plants' tolerance to salt and methods to enhance this tolerance or alleviate the stress effects are essential for rising issues of soil salinity in arid areas due to climate change. Peppers are generally sensitive to salt stress and increasing their tolerance can be crucial for crop success, but also for fruit quality. Utilizing seed priming, it is possible to increase peppers' tolerance to salt stress as well as enhance some already existing tolerance mechanisms.

Mechanisms of tolerance to salt stress can be triggered using different priming agents and the presented research suggests that the increase in tolerance in more salt-tolerant bell pepper varieties occurs due to the increased activation of existing mechanisms, while in more susceptible varieties, an additional increase in sugar synthesis leads to an increased tolerance to salt stress, suggesting an activation of novel mechanisms. Simple protocols for seed priming and simple priming-agent formulations can be used in crop production, and efforts should be made for the commercialization of such products.

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