Black Sea Journal of Agriculture
doi: 10.47115/bsagriculture.1110338

Open Access Journal
e-ISSN: 2618 – 6578

Research Article
Volume 5 - Issue 3: 329-335 / July 2022

COMPARATIVE EVALUATION OF SALICYLIC ACID (SA) AND 2,4-DICHLORO-6-{(E)-[(3METHOXYPHENYL)IMINO]METHYL} PHENOL (DPMP) ON GROWTH AND SALT STRESS TOLERANCE IN FORAGE PEA (PISUM SATIVUM SSP. ARVENSE L.)

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Abstract: Alleviation of salt stress is becoming one of the urgent needs of agricultural production. Even though enhancement of tolerance levels with genetic variation is a common approach, exogenous applications of various compounds are a newly emerging field. Here, the effects of two different plant elicitors, salicylic acid (SA) and 2,4-dichloro-6-{(E)-[(3methoxyphenyl)imino]methyl} phenol (DPMP) on growth and stress tolerance levels of forage pea (*Pisum sativum* ssp. *arvense* L.) were evaluated. Plants were exposed to salt stress (100 mM) in addition to DPMP, SA, or DMSO (Solvent) foliar spraying. The results revealed contrasting effects for each elicitor. Under non-stressed conditions, DPMP applied plants had higher values in plant height, shoot dry weight (SDW), and taproot length, while SA applied plants had significantly higher shoot fresh weight (SFW), and DMSO applied plants had higher values in root fresh (RFW) and dry (RDW) weights, and root/shoot ratios. When we evaluated stress tolerance index (STI) levels, DPMP applied plants had higher STI values in SFW, SDW, RFW, and RDW. DPMP improved STI and biomass allocation better than SA and DMSO. These elicitors may have significant potential in abiotic stress tolerance, in addition to their well-known biotic stress eliciting roles. There is a need for further research to define appropriate doses and application times.

Keywords: Elicitor, Forage pea, Root development, Salicylic acid, Salt stress

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Received: April 28, 2022
Accepted: June 25, 2022
Published: July 01, 2022

Cite as: Özkurt N, Bektaş Y. 2022. Comparative evaluation of salicylic acid (SA) and 2,4-dichloro-6-{(E)-[(3methoxyphenyl)imino]methyl} phenol (DPMP) on growth and salt stress tolerance in forage pea (*Pisum sativum* ssp. *arvense* L.). BSJ Agri, 5(3):329-335.

1. Introduction

Salt stress affects approximately 6% of the world's agricultural areas and it is becoming one of the most urgent limitations in agriculture (Yang and Guo, 2018; Acosta-Motos et al., 2020). When plants face salinity, they show common osmotic stress symptoms, and productivity gets declined or is completely prevented, based on salt accumulation, duration, and the tolerance level of the plant (Yang and Guo, 2018; Liang et al., 2018; Grozeva et al., 2019). The source of the salt stress could be irrigation water or accumulation of salt in the soil may lead to excessive salt stress for plants. Plant roots are exposed to salinity in the first place and the induction of stress signals from roots warn stomata to open less frequently. The stress signals minimize photosynthetic activities eventually. In addition to these initial salt stress responses, ion toxicity, as well as osmotic stress, occurs as secondary stresses (Yang and Guo, 2018; Liang et al., 2018). There have been a significant number of publications to understand plants' responses to salt stress (Singh et al., 2021). The effects of genotype on stress tolerance (Li et al., 2020; Acosta-Motos et al., 2020; Zhang et al., 2021), and its molecular mechanism (Mullan and Barrett-Lennard, 2010; Cornacchione and Suarez, 2017; Amoah et al., 2020; Li et al., 2020) were evaluated with in-depth observations.

Forage pea (*Pisum sativum* ssp. *arvense* L.) is a member of the Legume family. It is mostly used for fresh or dry herbage production and it has a significant role in soil nitrogen sustainability (Ateş and Tekeli, 2017; Çaçan et al., 2019). Legume forage crops have a soil nitrogen recovery advantage compared to forage crops from the Poaceae family due to nitrogen fixation ability (Den Herder et al., 2010). Genetic diversity (Demirkol and Yılmaz, 2019), common root trait diversity (Acikbas et al., 2022), yield and yield components (Uzun et al., 2012; Ateş and Tekeli, 2017; Tan and Kadıoğlu, 2018), and PEG induced osmotic stress (Bektas, 2022) responses of forage pea were previously reported. In a study conducted by (Demirkol et al., 2019) germination characteristics of forage pea were also evaluated. According to their results, 90 mM was the tolerance threshold level for the genotypes tested. A similar study (Grozeva et al., 2019) reported significantly reduced shoot and root biomass compared to less affected plant height in three different pea cultivars.

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Plant elicitors are synthetic or organic compounds that aim to induce plants' response to abiotic or biotic stress factors (Bektas and Eulgem, 2015). Even though relatively new, they are mostly tested against biotic stress factors (Bektas and Eulgem, 2015; Tripathi et al., 2019), and some for abiotic stresses (Tripathi et al., 2019; Palmer et al., 2019; Koo et al., 2020; Ahmad et al., 2021). Salicylic acid (SA) is a phytohormone and one of the most important regulatory compounds of the plant immune systems. Research showed that the exogenous application of SA is also important for abiotic stress response, in addition to biotic stress, and has been used extensively in various applications (Larqué-Saavedra and Martin-Mex 2007; Koo et al., 2020). Also, some research provides information about the promising role of Salicylic acid (SA) as a plant growth regulator and the enhancement of plant adaptation to different stress conditions (Li et al., 2013; Samota et al., 2017; Wani et al., 2016; Zhao et al., 2017). Increasing evidence has shown that exogenous application of SA can improve plant tolerance to salinity. Some research showed its promising role in salt stress including; reduced salt stress by improving photosynthesis and growth in mustard (Nazar et al., 2015) alleviation of salt stress by enhancing antioxidant systems (Zhang et al., 2014), increasing enzymatic and non-enzymatic pathways (Shamili et al., 2021). In addition to SA, some studies also demonstrated the activity of other compounds to increase plant adaptation to abiotic stresses. For example; pretreatment with β-aminobutyric acid (BABA) increases salt stress tolerance in rapeseed (Mahmud et al., 2020) and barley (Mostek et al., 2016). Chitosan application increased the salt-adaptive factors in stevia (Gerami et al., 2020), and coating seeds with chitosan improved growth performance under salinity stress (Peykani and Sepehr, 2018). All of the above reports provide strong insight that plant defense elicitors may have the potential to increase abiotic stress tolerance and reduce the severity of stress factors including salt stress. Recently 2, 4-dichloro-6-{(E)-[(3-methoxyphenyl) imino] methyl} phenol (DPMP) is described as an analog of SA and promising synthetic elicitor. Its activity against some pathogens including oomycetes *Hyaloperonospora arabidopsidis* (*Hpa*) (Bektas et al., 2016) and bacterial pathogens*; Pseudomonas syringae* pv. *tomato* (*Pst*) and *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) were revealed (Bektas et al., 2016; Bektas, 2021). In our previous study, we also showed its activity against PEG-induced osmotic stress (Bektas, 2022). However, there were no studies evaluating DPMPs role against salt stress and comparison of its activity with a well know defense-related phytohormone, SA. The effects of SA and DPMP, as well as their mode of action under salt stress, have not been revealed. Therefore, we aimed to comparatively evaluate the effects of SA and DPMP on seedling above and below-ground growth and development as well as their effects on stress tolerance index values under controlled conditions.

2. Material and Methods

2.1. Plant Material and Growth Conditions

Forage pea (*Pisum sativum* ssp. *arvense* L.) was selected as a model organism to investigate the possible roles of two plant elicitors, salicylic acid (SA) and 2,4-dichloro-6-{(E)-[(3methoxyphenyl)imino]methyl} phenol (DPMP), under salt-stressed and non-stressed conditions on the plant above- and below-ground growth and stress tolerance indexes. DMSO (Sigma Aldrich GMBH) was used as the solvent for DPMP and considered the control. Experiments were conducted under controlled conditions in the Department of Agricultural Biotechnology, Siirt University, Siirt, Türkiye (37°58'13.20"N - 41°50'43.80"E). The study was conducted following a modified cigar-roll method (Hohn and Bektas, 2020; Acikbas et al., 2021) according to randomized complete blocks design (RCBD) with three replications and ten plants per replication. During experiments, mean temperature and relative humidity ranged between 25-27°C and 60-70%, respectively, with 12/12 h day and night periods. Three different subsets (DPMP, SA, and DMSO) were prepared following Bektas and Eulgem (2015 and Bektas et al. (2016). There were a total of six different treatment groups, DPMP_control (10 µM DPMP), SA_control (100 µM SA), DMSO_control (0.2% DMSO), and DPMP_NaCl (10 µM DPMP+100 mM NaCl), SA_NaCl (100 µM SA++100 mM NaCl), and DMSO_ NaCl (0.2% DMSO+100 mM NaCl). SA was ordered from Sigma-Aldrich Chemie GmbH, Germany, and DPMP was kindly obtained from Prof. Dr. Thomas Eulgem, University of California, Riverside, USA.

An adequate number of seeds for the "Gap pembesi" cultivar were surface sterilized with 70% ethyl alcohol (C_2H_5OH) and 5% sodium hypochlorite (NACIO) for 5 minutes each and rinsed under running water. It is followed by placing seeds of similar size on germination papers (60 x 40 cm) as 10 seeds per paper and covered with a second paper layer (Hohn and Bektas, 2020). Each set is rolled and placed in large beakers filled with distilled water or saline solution (100 mM). After seedlings emerged, plant elicitors were applied as a foliar spray on the 7th and 10th days after the initial establishment of the experiments on May 20th, 2020.

2.2. Data Collection and Image Analysis

The experiments were completed on the 15th day when the roots of the %50 plants reached 40 cm depth. Germination papers were taken out of beakers and placed on the bench, and root images were collected using a portable hand-held scanner (Iscan Color Mini Portable Scanner) at 300 DPI resolution. Above and below-ground fresh and dry weights were measured using a precision scale (Weight lab instruments). Image analysis was performed to collect root length using ImageJ software (Rueden et al., 2017). Stress tolerance indices were calculated according to Moursi et al. (2020). The effects of SA and DPMP were evaluated by comparing their effects on above and below-ground traits listed in

Table 1. Analysis of variance (ANOVA) and variance groupings (TUKEY's Honest Significant Difference (HSD) test) was calculated using the Statistix software package (Analytical Software; Tallahassee, FL, USA).

Table 1. Seedling above- and below-ground growth-related traits and stress tolerance index traits evaluated under controlled conditions on forage pea

Trait name	Abbreviation-Calculation
Plant height	PH
Taproot length	TapRL
Shoot fresh weight	SFW
Root fresh weight	RFW
Shoot dry weight	SDW
Root dry weight	RDW
Plant height/Taproot length ratio	PH/RL
Shoot fresh weight/root fresh weight ratio	RFW/SFW
Shoot dry weight/root dry weight ratio	RDW/SDW
Stress tolerance index	
Stress tolerance index	STI
Reduction of PH	PH_Control–PH_NaCl
Reduction of SFW	SFW_Control–SFW_NaCl
Reduction of SDW	SDW_Control–SDW_NaCl
Reduction of RFW	RFW_Control–RFW_NaCl
Reduction of RDW	RDW_Control–RDW_NaCl
TapRL_STI	(TapRL_NaCl/TapRL_Control)*100
PH_STI	(PH_NaCl/PH_Control)*100
RFW_STI	(RFW_NaCl/RFW_Control)*100
SFW_STI	(SFW_NaCl/SFW_Control)*100
RDW_STI	(RDW_NaCl/RDW_Control)*100
SDW_STI	SDW_NaCl/SDW_Control)*100

3. Results

3.1. Root-Shoot Growth and Seedling Vigor

The effects and comparative performances of DPMP and SA were evaluated under salt-stressed and non-stressed conditions. Plant growth indicators including PH, SFW, RFW, SDW, RDW, RFW/SFW, RDW/SDW, and TapRL were evaluated. According to the results, there were significant differences between treatments for all the above traits except TapRL. At the non-stressed conditions, DPMP had significantly ($p<0.05$) higher values in PH, SDW and TapRL compared to SA and DMSO (Figure 1a, d, h). On the other hand, SFW was significantly higher in SA compared to DPMP and DMSO (Figure 1b). Finally, DMSO caused significantly higher values in RFW, RDW, RFW/SFW ratio, and RDW/SDW ratio (Figure 1c, e, f, and g). These results suggest different levels of effects in each chemical compared to one another.

Plant height (PH) was the highest (24.93 cm) in DPMP application, followed by SA (23.76 cm) and the least was in DMSO (21.80 cm). Under salt stress, all PH values were reduced to about one-third of the non-stressed conditions between 6.90 cm (DPMP) and 6.64 cm (SA) (Figure 1a). Shoot fresh weight (SFW) was the highest in SA with 0.73 g, and lowest in DPMP with 0.54 g in the non-stressed, while it was the opposite in the salt-stressed conditions with DPMP having the highest and SA having the lowest SFW values (Figure 1b). Root fresh weight (RFW) was the highest (0.68 g) in DMSO (no elicitor applied, only solvent) and lowest (0.50 g) in DPMP under non-stressed, while the highest in DPMP (0.16 g) and lowest in SA (0.11 g) under salt-stressed conditions (Figure 1c). Shoot dry weight (SDW) was the highest in DPMP application (0.10 g) and lowest in DMSO (0.08 g) under non-stressed conditions and the same ranking was observed under salt-stressed conditions (Figure 1d). RDW was found to be not significant within each treatment (non-stressed and salt-stressed), the ranking under non-stress was DMSO, DPMP, and SA, while it was ordered as DPMP, SA, and DMSO, respectively, under salt-stressed conditions (Figure 1e). Plant carbon allocation patterns can be estimated by the ratios of root and shoot biomass using fresh or dry weights. The highest RFW/SFW ratio was obtained in DMSO, followed by DPMP and SA under non-stressed and salt-stressed conditions (Figure 1f). When we evaluated RDW/SDW ratios, DMSO_NaCl was followed by DMSO_control, DPMP_NaCl, DPMP_control, SA_control, and SA_NaCl, respectively (Figure 1g). Our last growth-related trait was Taproot length (TapRL). Even though the longest TapRL was obtained in DPMP under non-stressed conditions it was not significantly different from DMSO and SA under non-stressed conditions (Figure 2). Similar outcomes were obtained in salt-stressed conditions (Figure 1h).

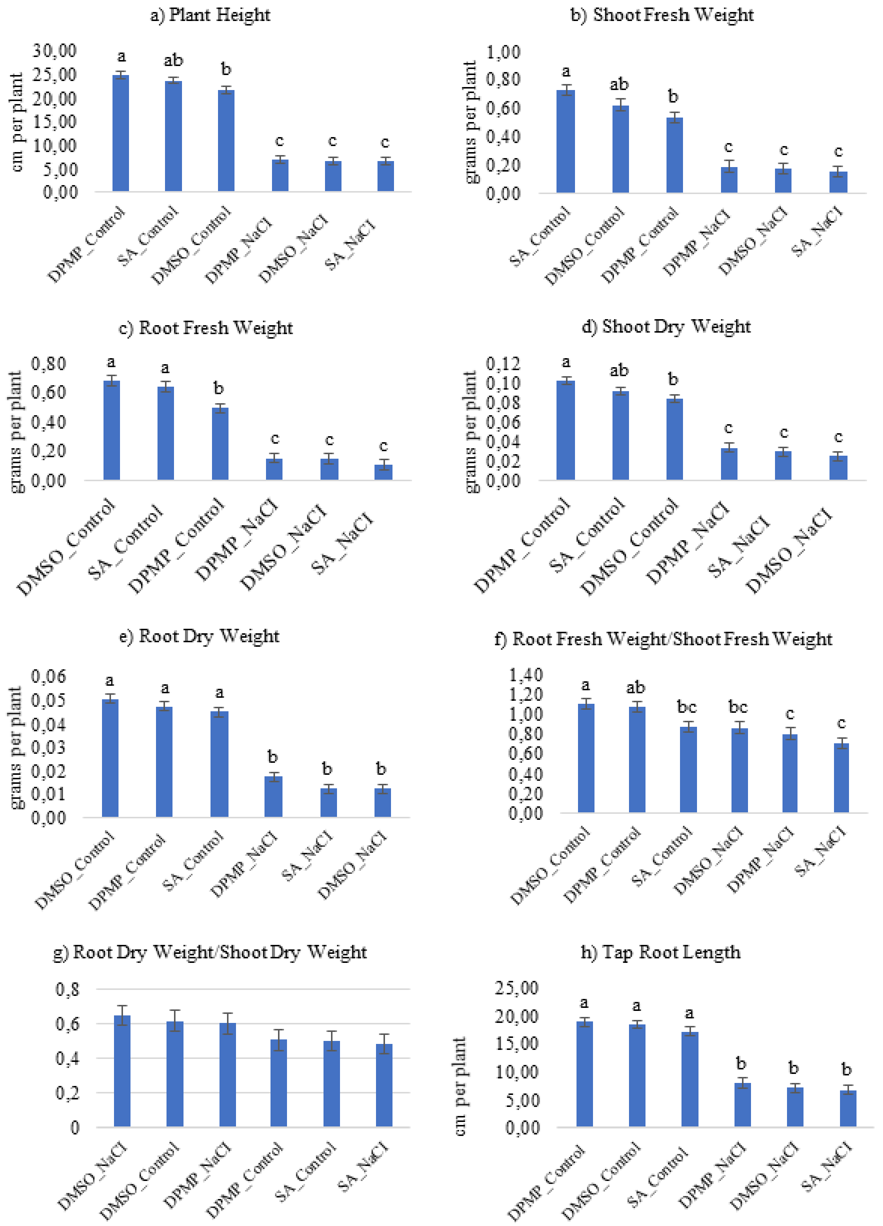

Figure 1. Mean values for plant height, shoot fresh and dry weight, root fresh and dry weight, root/shoot fresh and dry ratios, and taproot length. For each trait, means followed by different letters are significantly different at a $p < 0.05$ level according to the TUKEYs honest significant difference (HSD) test.

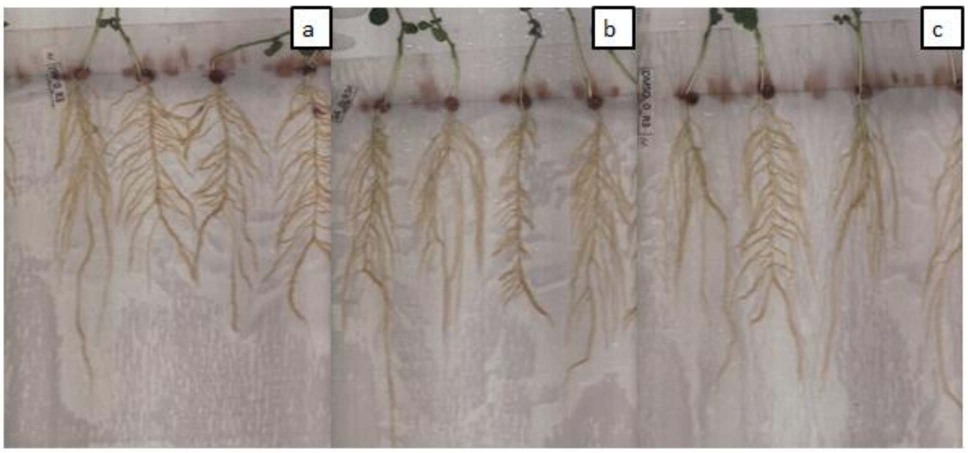

Figure 2. The TapRL was obtained under a) DPMP non-stressed conditions, b) SA non-stressed conditions, and c) DMSO non-stressed conditions.

3.2. Relative Efficiency of DPMP and SA Against Salt Stress

To evaluate the effectiveness of DPMP, we tested it against a well-known plant elicitor, SA, under non-stressed and salt-stressed conditions. This provided a better comparison efficiency. The values for each morphological trait under non-stressed conditions were compared with its values under salt-stressed conditions. The % decrease values and STIs for PH, SFW, SDW, RFW, RDW, and TapRL were calculated. According to the results, the lowest decrease in the values of SFW, SDW, RFW, and RDW were obtained in DPMP with 63.99, 65.56, 66.88, and 62.79% decrease compared to non-stressed trials of the same traits. For the PH, DMSO had the lowest decrease ratio with 68.91% and for the TapRL, SA had the lowest decrease ratio with 46.62%. These values were used to compute stress tolerance index (STI) values. In line with the percent decrease ratios, DPMP had the highest STI on SFW (36.00), SDW (34.44), RFW (33.12), and RDW (37.21). While DMSO had the highest STI in PH (31.09) and SA had the highest STI value in TapRL (53.38) (Table 2).

4. Discussion

Salt stress is one of the most destructive abiotic stress factors, following drought stress (Liang et al., 2018; Yang and Guo, 2018). It is affecting more than %6 of the global agricultural land and this rate is increasing with wrong or excessive irrigation as well as fertilizer applications (Yang and Guo, 2018). One of the ways to cope with salt and other osmotic stresses is to select/breed new varieties. There is a significant genetic diversity available in the wild or domesticated gene pools (Shabala et al., 2016; Liang et al., 2018; Kumar et al., 2021) for the improvement of abiotic stress tolerance in crops. However, replacing all cultivars with the tolerant/resistant ones in the world is almost impossible. There is a need for enhancing the growth potential and stress tolerance efficiencies of the currently grown cultivars. A relatively new approach, plant elicitors, or enhancers, started to gain interest due to their practicality and reduced or minimal effects on the environment. Salicylic acid (SA), γ- aminobutyric acid (GABA), β-aminobutyric acid (BABA), and Acibenzolar-S-methyl (ASM) are the well-known plant elicitors, while newly identified organic or synthetic substances are introduced as possible elicitors against biotic or abiotic stress factors or just to enhance plant growth and development (Bektas and Eulgem, 2015). DPMP is one of those new chemicals that has proven effective against several pathogens (Bektas et al., 2016; Bektas, 2021). DPMP is reported to be effective against *Pst,* while its activity on plant growth and stress tolerance levels is not well defined. Here, we evaluated DPMP by comparing its role with phytohormone SA in crop growth under non-stressed and salt-stressed conditions.

Table 2. Stress tolerance index (STI) values for plant height (PH), shoot fresh weight (SFW), shoot dry weight (SFW), root fresh weight (SFW), root dry weight (SFW), and taproot length (TapRL) evaluated salt-stressed compared non-stress conditions. The results are presented as % decrease (non-stress -salt stress) and STI values

Trait	Control vs. NaCl	Value Decrease	% Decrease	STI
	DPMP	18.06	72.46	27.54
PH	DMSO	15	68.91	31.09
	SA	17.15	72.19	27.81
	DPMP	0.35	63.99	36
SFW	DMSO	0.45	72	28
	SA	0.57	78.02	21.98
	DPMP	0.07	65.56	34.44
SDW	DMSO	0.06	69.41	30.59
	SA	0.061	66.45	33.55
	DPMP	0.33	66.88	33.12
RFW	DMSO	0.53	77.34	22.66
	SA	0.53	81.73	18.27
	DPMP	0.03	62.79	37.21
RDW	DMSO	0.04	75.39	24.61
	SA	0.03	71.78	28.22
	DPMP	9.5	47.41	52.59
TapRL	DMSO	11.32	158.59	38.68
	SA	9.21	46.62	53.38

4.1. Effects of DPMP and SA on Plant Growth and Development

According to the results on above and below-ground morphological traits, DPMP, SA, and DMSO (only solvent) had differing effects on seedling growth. DPMP applied plants had higher values in SDW, PH, and TapRL (Not significant). These results suggest that DPMP has a comparable, even higher effect on plant growth than SA. The role of SA on plant growth, development, and stress tolerance (abiotic or biotic) enhancement is well known and documented by multiple reports (Filgueiras et al., 2019; Tripathi et al., 2019; Koo et al., 2020). So, its role in growth is no surprise, while there were no reports on the effect of DPMP on plant growth, except in our previous report (Bektas, 2022). According to (Koo et al., 2020) SA acts as a plant hormone and regulates, plant immunity, growth, and development. It has crosstalk with absisic acid, ethylene, jasmonic acid, and auxin. With these roles, SA can be considered a key element in plants. Even though there is not much knowledge on the role of DPMP on plant hormones and regulation, it may be listed as analogous to SA (Bektas et al., 2016).

4.2. Comparative Evaluation of DPMP and SA on Salt Stress Tolerance Index (STI)

Stress tolerance index (STI) calculation is a way to evaluate the effects of genotypic differences or applied substances on plant growth under stressed conditions. Here, the STI was calculated according to Moursi et al. (2020) with slight modifications. Accordingly, DPMP applied plants had higher biomass allocation and STI values compared to SA or DMSO applied plants under salt-stressed conditions. As previously reported (Filgueiras et al., 2019; Koo et al., 2020; Ahmad et al., 2021) SA has well-known effects as a plant growth enhancer. Here, SA applied plants had longer TapRL under salt-stressed conditions, compared to DPMP and DMSO. On the other hand, DMSO caused taller plant stature (PH) compared to other chemicals. DPMP applied plants had higher biomass and STI values under salt-stressed conditions, compared to SA and DMSO. According to the results of the current experiment, DPMP helps plants to cope with the negative effects of salt stress. Its mode of action and specific hormonal effects are yet to be identified. DPMPs' role against *Pst* is confirmed by morphological and molecular observations (Bektas et al., 2016). It induced SA-related defense genes and reduced the disease severity of *Hyaloperonospora arabidopsidis, Pst,* and *Cmm*. The results of the current study provided preliminary evidence for the positive effects of DPMP on salt stress tolerance. It seems to enhance biomass production potential under the currently applied salt dose. However, there is a need for defining optimal doses, application frequencies, and effective application procedures. We are currently working on identifying its role on other abiotic and biotic stress factors as well as on other crops. Synthetic or organic compounds may provide new insights into plant stress tolerance improvement and growth enhancement.

Author Contributions

N.Ö. organized (100%), analyzed, and interpreted the data (100%) and wrote the original manuscript (70%). Y.B. initiated the research idea (100%), supervised the research (100%), suggested the research methods (100%), structured the paper (100%), and edited the manuscript (30%). All authors reviewed and approved final version of the manuscript.

Conflict of Interest

The authors declared that there is no conflict of interest.

Acknowledgments

The authors are grateful to Assoc. Prof. Dr. Mehmet Arif Özyazıcı for providing seeds and Prof. Dr. Thomas Eulgem for providing DPMP.

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