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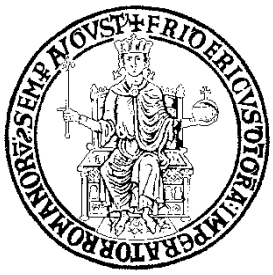

DOTTORATO IN

SCIENZE AGRARIE ED AGROALIMENTARI

XXXI° CICLO

Omeprazole, a proton-pump inhibitor on humans, acts as a growth enhancer and stress protectant in plants

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CHAPTER 1.

LITERATURE REVIEW

1.1 Introduction: benzimidazoles

Benzimidazole is the benzo derivative of imidazole (Figure 1), synthesized for the first time by Hoebrecker in 1872 (Bansal, 2002). Imidazole is a five member-ring with formula $C_3N_2H_4$. It is an organic compound contained in many natural products like alkaloids in plants.

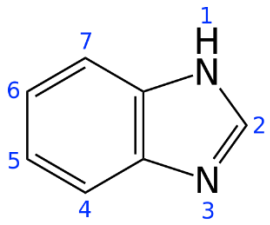

Figure 1. Skeletal formula of benzimidazole, showing the numbering convention for substituent groups

The initial research on benzimidazoles can be traced back to 1944, when Woolley hypothesized that, because of their similarity with purines, benzimidazoles could act as this heterocyclic compound to induce some biological responses (Woolley, 1944). In nature, the benzimidazole moiety is a part of vitamin B12 complex, and some of the first artificial benzimidazoles had a vitamin B12-like activity (Brink and Flokers, 1949).

All seven positions on the benzimidazole nucleus can be substituted with a variety of atoms. However, most of the biologically active benzimidazole based compounds have the functional groups at 1, 2 and/or 5, 6 positions (Bansal and Silakari, 2012). Many benzimidazoles derivatives are bioactive molecules widely used for their pharmacological and pharmacokinetic activities. Benzimidazole bioactivity in humans has been well documented (Salahuddin *et al*., 2017).

Substituents around the benzimidazole nucleus give rise to many drugs with diametrically opposed activities, including antiparasitic, anticonvulsants, analgesic,

antiulcer, anticancer, proton pump inhibitors, and many others (Bansal and Silakari, 2012). For this reason, the research on benzimidazoles has gathered broad interested in the last years, due to the need of new therapeutic molecules.

The benzimidazole moiety is also the nucleus of many fungicides (Gupta and Pathak, 2011; Dias, 2012; García *et al*., 2003). These derivatives control a broad range of fungi at relatively low application rates thanks to their ability to inhibit β-tubulin polymerization, consequently blocking cytoskeleton formation. The most used benzimidazole fungicides are benomyl (a), carbendazim (b), thiabendazole (c) and fuberidazole (d) (Figure 2).

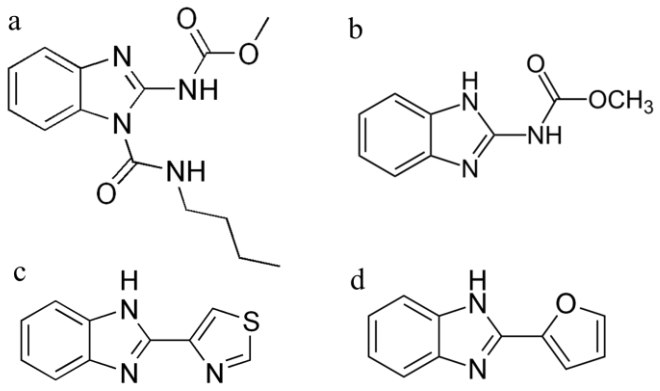

Figure 2 Skeletal formula of the most used benzimidazole fungicides

However, due to benzimidazole resistance by several pathogens their use as fungicide has declined and it is currently prohibited for some of them (Ma and Michailides, 2005; Lucas *et al*., 2015; https://ec.europa.eu/food/plant_en).

Interestingly, many authors observed both beneficial and detrimental effects of different benzimidazole based fungicides on plants, which represent their off target activity (García *et al*., 2003). For this reason, verification of the direct and/or indirect effects of benzimidazoles on plants metabolism and physiology deserves further attention, considering that the target of these molecules is still unknown. While many reviews on the therapeutic potential of benzimidazoles for humans have been published (Bansal and

Silakari, 2012; Salahuddin, 2017), a comprehensive review on use, potential use and likely target of benzimidazoles in plants is missing.

1.2 Review on benzimidazole effect on plants

The first paper in which a benzimidazole has been used on plants was published in 1955, with a further 35 papers since then. The majority of the literature involved testing the effects of different benzimidazole derivatives on various plants, reporting a plethora of effects induced by these molecules on various aspects of plant growth and physiology. A comprehensive list of experiments involving benzimidazoles and their effects is enumerated in Table 1. However, considering that the concentrations and the nature of the benzimidazole derivatives used in each experiment was different, it is not easy to find a common trait involved in the bioactivity of these molecules. Indeed, what the authors found in these experiments is contrasting: some papers reported that benzimidazoles had a positive effect on the overall growth of treated plants (***IPO-1***, Kołodziej *et al*., 2006; ***Benzimidazole***, Dyar and Shade, 1974; ***Carbendazim***, García *et al*., 2002; ***Carbendazim***, Debergh, 1993; ***Benomyl***, Skene, 1972; ***Benomyl/carbendazim,*** Magnucka *et al*., 2007; ***Benzimidazole,*** Dyar, 1967; ***Benzimidazole***, Hillman, 1955), while others showed reductions in shoot and root biomass and in root length as well as phytotoxicity (***Benzimidazole***, Hillman, 1955; ***Benomyl***, Boyle, 1973; ***Benzimidazole***, Klingensmith, 1961; ***Flubendazole***, Stuchlíková; 2018; ***Benomyl***, Siddiqui and Zaman, 2004; ***Mercaptobenzimidazole***, Rebstock *et al*., 1955; ***Benomyl***, Mihuta-Grimm *et al*., 1990; ***Carbendazim***, Siddiqui and Bano, 2018; ***Benzimidazole***, McCorquodale and Duncan, 1957; ***Carbendazim***, García *et al*., 2002). In addition to direct effect on plant growth and/or health, benzimidazole have been reported to delay excised leaves senescence (***Benzimidazole***, Harris-Shultz *et al*., 2015; ***Benzimidazole*** Nowierski *et al*., 1995; Tripathi *et al*., 1982; ***Benzimidazole***, Sayeed *et al*., 1985), the production of specific compounds like phenols (***Benomyl***, Siddiqui and Zaman, 2004), phytocystatin (***Omeprazole***, Braga *et al*., 2018) and ginsenoids (***IPO-1***, Kołodziej *et al*., 2006), and in seed germination (***Benomyl/carbendazim***, Thomas, 1973). It should be noted that the

positive effect on plants growth were found only when benzimidazole derivatives were used at low concentrations (less then 1 mM), while with higher doses detrimental effects were observed. A list of the reviewed papers is shown in Table 1.

Year	Molecule	Dose	Specie	Reference
1955	Benzimidazole	2.5 mM	*Lemna minor*	Hillman, 1955
1955	Mercaptobenzimidazoles	5 mM	*Cucumis sativa*	Rebstock *et al*., 1955
		6 mM	*Phaseolus vulgaris*	
1957	Benzimidazole	1.9 mM	*Vicia faba*	McCorquodale and Duncan, 1957
1961	Benzimidazole	1 mM	*Cucumis sativa*	Klingensmith, 1961
		10 mM	*Solanum lycopersicum*	
		10 mM	*Bushbean*	
1967	Benzimidazole	0.5-1.5 mM	*Nicotiana tabacum*	Dyar, 1967
1972	Benomyl	80-160 µM	*Raphanus raphanistrum subsp. sativus*	Skene, 1972
		80-160 µM	*Glicine max*	
1973	Benomyl	2-4 mM	*Allium cepa*	Boyle, 1973
1973	Benomyl	0-3.4 mM	*Apium graveolens*	Thomas, 1973
	Thiabendazole	0-5 mM	*Apium graveolens*	
	Carbendazim	0-5.2 mM	*Apium graveolens*	
1973	Benzimidazole	10-100 µM	*Thelypteris felix-mas*	Dyar and Shade, 1973
1982	Carbendazim	26-520 µM	*Triticum vulgare*	Tripathi *et al*., 1982
1985	Benzimidazole	0.5 mM	*Triticum vulgare*	Sayeed *et al*., 1985
1990	Benomyl	0-479 µM	*Solanum lycopersicum*	Mihuta-Grimm and Rowe, 1990
1993	Carbendazim	0-800 µM	*Cordyline terminalis*	Debergh, 1993
		0-800 µM	*Prunus avium*	
1995	Benzimidazole	1 mM	*Hordeum vulgare*	Nowierski *et al*., 1995
2002	Carbendazim	1.3 mM	*Nicotiana tabacum*	García *et al*., 2002
		2.5 mM	*Nicotiana tabacum*	
		5.2 mM	*Nicotiana tabacum*	

2004	Benomyl	3.4-6.8 mM	*Zea mays*	Siddiqui and Zaman, 2004
2006	IPO-1	*unknown MW*	*Panax quinquefolium*	Kołodziej *et al*., 2006
2007	Benomyl	34 µM	*Secale cereale*	Magnucka *et al*., 2007
		52 µM	*Secale cereale*	
2015	Benzimidazole	1 mM	*Sorghum bicolor*	Harris-Shultz *et al*., 2015
		1 mM	*Zea mays*	
2018	Omeprazole	30-115 µM	*Allium cepa*	Braga *et al*., 2018
2018	Flubendazole	10 µM	*Plantago lanceolata*	Stuchlíková; 2018
		10 µM	*Plantago lanceolata*	
2018	Carbendazim	10-100 µM	*Allium sativum*	Siddiqui and Bano, 2018

Table 1 List of the paper reporting the effect of various benzimidazole derivatives on plants from 1955 to 2018

Most of the above experiments were performed to test the phytotoxicity of the most commonly used fungicides on crops. When the literature is examined more closely, it becomes evident that some of these molecules, when used at lower concentrations than optimal as fungicides, can induce an increase in plants biomass production (García *et al*., 2003). Strikingly, none of these experiments demonstrates the biochemical or molecular targets for growth enhancement benzimidazole activity in plants. This is likely due to the scarce tools available to researchers in the period in which these experiments were performed. For this reason, the aim of this thesis is to understand the mechanisms involved in benzimidazole function on plants, and specifically the family of substituted benzimidazoles known to inhibit proton-pumps activity in humans. Molecules that are able to perturb ion homeostasis in plants can be useful to understand the mechanisms responsible for plant tolerance to osmotic and ionic stresses, with direct agricultural applications and for the study of basic biology.

1.3 Proton pump inhibitors

The inhibition of proton pumps is the most effective strategy for the treatment of gastrointestinal diseases, such as gastroesophageal reflux or peptic ulcer. The first proton pump inhibitor (PPI), omeprazole (OP) was approved in humans in 1989, and subsequently followed by other six molecules of the same class, namely pantoprazole, esomeprazole, rabeprazole, lansoprazole, and dexlansoprazole. All of the PPI's have a benzimidazole nucleus and a pyridine with substituents on both of the rings, which are principally linked with bioavailability and catabolism of the drug. Specifically, omeprazole possesses a methoxy chain attached to its benzimidazole ring. The efficacy of this class of drugs is due to their ability to block the gastric acid secretion through the inactivation of the hydrogen-potassium adenosine triphosphatase enzyme system (H^+/K^+-ATPase). In the acidic environment of the parietal cells in the stomach lumen, these molecules undergo to a protonation in both the pyridine and benzimidazole ring that leads to the formation of the active sulfonamide derivative, which then binds covalently to cysteine moieties of the H^+/K^+-ATPase. This inactivation is irreversible and long lasting (Strand *et al*., 2017). After inhibition, the acid production will requires new H^+/K^+-ATPase protein complexes to replace the inactivated proteins. These molecules are able to maintain stomach pH higher than 4 from 15 to 21 hours (Wolfe and Sachs, 2000). Moreover, their effectiveness is not reduced with long-term treatment. These two qualities of PPI's represent the revolutionary aspect of these molecules compared to the ones used previously for the treatments of gastric disease, the histamine H2-receptor antagonists. Indeed, the latter had an efficacy of maximum 8 hours, with a high chance to incur in tachyphylaxis as soon as within 3 to 5 days (Wilder-Smith *et al*., 1990).

1.4 Small bioactive molecules

While a number of ATPase inhibitor have been demonstrated to inhibit activity of plant ATPases, no studies have tested the effects of benzimidazole based PPIs on plant growth

and tolerance to stress (Pedersen and Palmgren, 2017). The use of small bioactive molecules represents a promising approach, not only to study the molecular target divergence between different biological systems, but also to find mechanisms involved in different aspects of plant biology (Kaschani and der Hoorn, 2007). The use of human-to-plant and plant-to-human application of bioactive molecules is well established (Dillworth, 2009). The general trend is to use molecules well-characterized in one system and evaluate their effect on other model systems. This approach has led to a number of molecules now widely used cross-kingdom. In plant biology, many have been applied to study the protein activity, enzymes, aquaporins, and ATPases (Table 2).

Compound	MW (Da)	Function/target	References
Modulators of phosphorylation			
PD98059	372.41	MAP kinase kinase inhibitor	Zhang *et al*., 2006
K-252a	467.47	Kinase inhibitor	Dai et al., 2006
Wortmannin	428.43	Kinase inhibitor	Robatzek *et al*., 2006
Cantharidin	168.15	Phosphatase inhibitor	Luan, 2003
Modulators of proteolysis			
E-64	357.41	Inhibitor of cysteine proteases	Van der Hoorn, 2004
AEBSF	203.23	Inhibitor of serine proteases	Pak and Van Doorn, 2005
MG132	475.3	Proteasome inhibitor	Robatzek *et al*., 2006
Bestatin	308.17	Inhibitor of aminopeptidases	Zheng *et al*., 2006

Modulators of membrane trafficking			
Latrunculin B	395.51	Inhibitor of actin polymerization	Robatzek *et al*., 2006
N-ethylmaleimide (NEM)	125.13	Inhibitor of exocytosis/actinomyosin complex	Davies *et al*., 2006
Brefeldin A	280.36	Inhibitor of vesicle trafficking	Nomura *et al*., 2006
Colchicine	399.44	Inhibitor of microtubule assembly	Fiorani and Beemster, 2006
Oryzalin	346.36	Inhibitor of tubulin polymerization	Robatzek *et al*., 2006
Other modulators			
U73122	464.64	Inhibitor of phosphatidyl inositol phospholipase C	Hetherington and Brownlee, 2004
Quinidine	326.43	Inhibitor of root exudation	Hoat *et al*., 2006
Paclobutrazol	293.79	Inhibitor of gibberellin biosynthesis	Saito *et al*., 2006
Naphthylphthalamic acid	291.3	Inhibitor of auxin transport	Dai *et al*., 2006
Cerulenin	223.27	Inhibitor of fatty acid synthase	Koo *et al*., 2005
Cycloheximide	281.35	Inhibitor of protein synthesis	Dreher *et al*., 2006
W7	340.87	Inhibitor of calmodulin-dependent proteins	Davies *et al*., 2006
Forskolin	410.5	Activator of adenyl cyclase	Davies *et al*., 2006
BTH	210.28	Induces systemic acquired resistance	Van Hulten *et al*., 2006
Mevinolin, lovastatin	404.54	Inhibitor of the MVA pathway	Rodriguez-Concepcion *et al*., 2004

Isoxaben	332.39	Inhibitor of cellulose biosynthesis	Paredez *et al*., 2006

Table 2 List of small bioactive molecules (<500 Da) active on different aspects of plant biology adapted from Kaschani and der Hoorn (2007)

1.5 Aim of the study

We chose to test OP, the ancestor molecule of benzimidazole based PPI. OP can be considered a small bioactive molecule, being smaller than 500 Da. Using a chemical genetics approach, we hypothesized that substituted benzimidazoles that act as PPIs in humans may have an effect in plants exposed to environmental stresses such as salinization of the root zone. To test this hypothesis, we chose to subject different plant species (tomato and basil) to osmotic and ionic stress. In plants, proton pumps are essential for establishing the proton motive force used to exclude toxic ion and maintain homeostasis (Ji et al 2013; Oh et al., 2010). In addition, we used the model species *Arabidopsis thaliana* to assess changes in root architecture under the effect of omeprazole. Our results can be valuable as an innovative approach for the study of unknown mechanisms involved with the tolerance to salt stress, that we know is one of the main constrains of plant productivity worldwide. For this reason, considering the high impact that this specific stress has on food safety, this study could give us important insights on this topic from both a biological as well as an applicative point of view, which are pivotal for the comprehensiveness and thoroughness of a scientific work.

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CHAPTER 2.

A BENZIMIDAZOLE PROTON PUMP INHIBITOR INCREASES GROWTH AND TOLERANCE TO SALT STRESS IN TOMATO

2.1 Abstract

Pre-treatment of tomato plants with micromolar concentrations of omeprazole (OP), a benzimidazole proton pump inhibitor in mammalian systems, improves plant growth in terms of fresh weight of shoot and roots by 49 and 55% and dry weight by 54 and 105% under salt stress conditions (200 mM NaCl), respectively. Assessment of gas exchange, ion distribution, and gene expression profile in different organs strongly indicates that OP interferes with key components of the stress adaptation machinery, including hormonal control of root development (improving length and branching), protection of the photosynthetic system (improving quantum yield of photosystem II) and regulation of ion homeostasis (improving the K^+:Na^+ ratio in leaves and roots). To our knowledge OP is one of the few known molecules that at micromolar concentrations manifests a dual function as growth enhancer and salt stress protectant. Therefore, OP can be used as new inducer of stress tolerance to better understand molecular and physiological stress adaptation paths in plants and to design new products to improve crop performance under suboptimal growth conditions.

2.2 Introduction

Soil salinization is a major problem for agriculture. It is estimated that by 2050 salinization will lead to up to 30% degradation of cultivated land (Rengasamy, 2006; FAO, 2011; Aragüés *et al*., 2015; Lal, 2015). The effects of soil and water salinity on plant growth and development have been well documented. Excess of Na^+ and Cl^- ions in proximity of the roots generate osmotic and ionic stress and activate signals inhibiting cell division and plant growth (Deinlein *et al*., 2014). Metabolic dysfunction and

nutritional disorders associated with Na^+ and Cl^- loading in plant tissues and organs translate in further growth reduction and eventually irreversible cell damage. Upon exposure to salt stress, the control of growth, ion and water homeostasis becomes an essential part of an adaptation program that helps resuming growth, albeit at a reduced rate (Maggio *et al*., 2006; Park *et al*., 2016; Van Oosten *et al*., 2016; Annunziata *et al*., 2017). During adaptation, ion movement through cellular compartments is essential to detoxify the cytoplasm and re-establish osmotic balance (Hasegawa, 2013; Ji *et al*., 2013; Mancarella *et al*., 2016). Plasma membrane and vacuolar H^+-ATPases play a fundamental role in this physiological process since by generating active transport of proton H^+ across the membranes they create pH gradients and electrical potentials that drive transport of ions and molecules (including NO_3^-, PO_4^-, K^+, Na^+, sucrose, hexoses, and amino acids) across membranes (Pardo *et al*., 2006; Batelli *et al*., 2007; Deinlein *et al*., 2014). H^+-ATPases can be activated/deactivated in response to many environmental cues such as abiotic stresses (Hasegawa, 2013). It occurs that salinization stimulates vacuolar H^+-ATPase and H^+-PPase activities which in turn facilitate tonoplast Na^+/H^+ antiporter function and cytoplasm detoxification (Pardo *et al*., 2006; Ji *et al*., 2013). It has been shown that increasing cellular proton pump activity via co-overexpression of the vacuolar H^+-pyrophosphatase gene AVP1 and the vacuolar Na^+/H^+ antiporter gene AtNHX1, enhanced salt stress tolerance, most likely by potentiating ion compartmentalization functions (Shen *et al*., 2015). In contrast, cell treatment with vanadate (an inhibitor of the plasma membrane H^+-ATPase) increases the Na^+/K^+ ratio in plant tissues and enhances sensitivity to salinity (Li *et al*., 2014).

In animals, homologs of plant proton pumps operate through the H^+/K^+ ATPase mechanism (Axelsen and Palmgren, 1998). Similar to plants, animal proton pumps working across membranes generate acidification of organismal compartments. For decades proton pump inhibitors (PPIs) have been successfully used to inhibit gastric acid secretion (McTavish *et al*., 1991). As matter of fact, benzimidazole based PPIs are common treatments used with gastro-esophageal reflux disease (GERD) and peptic ulcers (Baumann and Baxendale, 2013). In animals, omeprazole, the most common

benzimidazole PPI, affects P-Type IIC ATPases. These P-Type IIC ATPases represent a large family of ATP driven transporters, which are responsible for moving ions across membranes. These include membrane Ca^{2+} pumps, Na^+/K^+ transporters, and H^+/K^+ transporters. Omeprazole is largely used as PPI that suppresses stomach acid secretion in the gastric mucosa (Wallmark, 1986; Shin *et al*., 2009). The specific inhibition of the P-Type IIC H^+/K^+ ATPase located in the parietal cells is irreversible and specific. Plants are not known to possess P-Type IIC ATPases that transport Na^+ or K^+, instead relying on the family of NHX-type Na^+ and K^+/H^+ antiporters for plasma membrane extrusion and compartmentation into the vacuoles and endosomes. Plants do possess P-Type IIA and III ATPases, primarily SERCA-like, which are not known to transport Na^+ or K^+. SERCA-like ATPases show very low homology (approximately 25%) to P-Type IIC ATPases that transport Na^+ or K^+ (Sweadner and Donnet, 2001). The SERCA-like ATPases are primarily endoplasmic reticulum transporters of calcium (Altshuler *et al*., 2012) and manganese (Mills *et al*., 2008). Plants also have P-Type III ATPases typically found in the plasma membrane, but none have been functionally characterized as Na^+ or K^+ transporters. While plants are not known to possess P-Type IIC ATPases that transport Na^+ or K^+ which are the target of omeprazole, we wanted to verify whether plant treatment with omeprazole might actually alter the transmembrane control of ion fluxes and disrupt plant tolerance to saline stress. In contrast to what we may have expected based on our current understanding of plant ATPases and plant responses to salt stress, here we demonstrate that tomato treatment with micromolar concentrations of omeprazole greatly enhanced plant growth and improved its tolerance to saline stress.

2.3 Materials and methods

2.3.1 Plant Growth Conditions

Tomato seeds (cultivar M82, accession LA3475) obtained from the Tomato Genetics Resource Center (TGRC, University of California, Davis, USA) were germinated in plates containing MS media and transplanted to a hydroponic system after 1 week when

cotyledons were fully expanded. Air temperature (Ta, °C), humidity (RH, %), and solar radiation (Rs, W m^{-2}) were acquired by a data logger (Spectrum Technologies, Plainfield, IL, United States). The average air humidity and temperature were 58% and 24 °C during the day and 86% and 18 °C during night-time, under short day conditions. Plants were grown in hydroponic solution containing: 1.5 mM $Mg(NO_3)_2 \cdot 6H_2O$, 3.4 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM KNO_3, 1.8 mM K_2SO_4, 1.5 mM KH_2PO_4, and 14 mg/L Hidromix (Valagro, Atessa (Chieti) Italy). Eight plants per treatment were grown in 4 L tanks with constant aeration. At 26 Days After Sowing (DAS), the first treatment of OP was added to the nutrient solution (1, 10, and 45 µM). Salt stress was initiated 36 DAS by adding 75 mM NaCl to salt treatments. At 42 DAS the NaCl concentration was increased to 150 mM and on 46 DAS the NaCl concentration was raised to 200 mM NaCl. Destructive harvest and biometrics were taken at 50 DAS (14 days of salt stress). Roots were separated from shoots in order to obtain their individual FW. To measure relative water content (RWC), shoots were transferred in deionized water for 24 h to induce maximum turgidity and weighed. Shoots and roots were dried for 3 days at 64°C and weighed individually. Root length was measured with a ruler; root area was measured using ImageJ (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA) as per Yoo *et al*. (2010).

2.3.2 RNA Extraction and Quantitative RT-PCR

Leaves of 7-week-old hydroponically grown plants (50 DAS, 14 DAST), treated with 1, 10, and 45 µM OP, with 0 and 200 mM NaCl, were harvested and immediately frozen at −80°C. Leaves from the same treatment were mixed and three replicates per bulk were analyzed. 100 mg of fresh leaf tissue per sample was homogenized with liquid nitrogen and extracted with 1 ml of TRIzol (Life Technologies). First-strand synthesis was performed with a QuantiTect Reverse Transcription Kit (QIAGEN) using 1 µg of total RNA. Real-time qPCR reactions, using 10 ng of cDNA per reaction, two experiments, four replicates per experiment, were carried out on an ABI 7900HT qPCR detection system using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Life

Technologies). Each qRT-PCR experiment was repeated at least twice to confirm results. Primers were designed based on gene models and EST sequences available in GenBank. All qRT-PCR primers were determined to be within 3% efficiency of each other. Relative expression levels were calculated using EF1α as an internal standard and the ΔΔCt method for relative quantification. The list of the primers used and the gene name is reported in Table 3.

Primer Name	Sequence	Accession #
Sl707A1-F	CTGAACAGAAAGTTATTTGGCAGTC	Solyc04g078900.2
Sl707A1-R	ATGATACTAGCCATTCTCAGTGTCTC	
Sl707A3-F	GCTCCCAAACCCAATACCTAC	Solyc04g071150.2
Sl707A3-R	CAGTTTGGCGAGTTCATTTCC	
SlHKT1.1-F	TCTAGCCCAAGAAACTCAAAT	Solyc07g014680.2
SlHKT1.1-R	CTAATGTTACAACTCCAAGGAATT	
SlNCED1-F	CATAATCGAAAACCCGGATG	Solyc07g056570.1
SlNCED1-R	AACTTTTGGCCATGGTTCAG	
SlNHX1-F	CACGATATGGTGGGCTGGTT	Solyc06g008820.2
SlNHX1-F	GGGTGTGGCCAAATCTCGTA	
SlNHX2-F	ATTGGAGGATCGGCAGGAAC	Solyc04g056600.2
SlNHX2-R	CCATGGAGCCAGATTGACCA	
SlNRT1.1-F	TAGCGCCGCGATGATATTAGGG	Solyc06g074990
SlNRT1.1-R	TGTAACGTTGTTGGCTGAACTTGC	
SlP5CD-F	CACAGGTAGCTCAAGGGTGG	Solyc02g089620
SlP5CD-R	GGCCCAAGGATCTTCCAGTC	
SlP5CS-F	AACTGAGCTTGATGGCAAGG	Solyc08g043170
SlP5CS-R	ACCAGAGGCTGAGCTGATGT	
SlSOS1-F	TCGAGTGATGATTCTGGTGG	Solyc01g005020
SlSOS1-R	ATCACAGTGTGGAAAGGCT	
SlLEA-F	CGAAGGAGAAGGCTAGTGGA	Solyc03g116390.2
SlLEA-R	AGCGATGCTCCTCACTTGTT	
SlAPX2-F	ATGGTAGCTGGAGGAGACCT	Solyc06g005150
SlAPX2-R	TTGAGGGAGCATGGACCAAC	
SlCAT1-F	GGACAATAATGGCAGGGCAA	Solyc12g094620
SlCAT1-R	TTACAGCCAGTTGGTCGCTT	
SlCHLBP-F	GAGCATTCTAGCAGTATTGG	Solyc07g063600
SlCHLBP-R	TGTTGCCTTCACCAACTCCA	
SlPSII-F	CGAAGAAGGGGTTCTAACGT	Solyc09g064500

SlPSII-R	GTCTCGTGGAGTCCGCATAA	
SlFTSH-F	AAGGACGTGGATCTGTCAGC	Solyc03g112590
SlFTSH-R	TGGCATATTTGCATGCTCGC	

Table 1 List of primers and gene names used in this study.

2.3.3 Ion Measurements

Ions measurements were performed according to a procedure described by Carillo *et al*. (2011), with following modifications. 100 mg of powdered dried material was suspended in 10 mL of MilliQ grade water (Milli-Q PLUS, Millipore, United States), and subjected to four freeze-thaw cycles by freezing in liquid nitrogen and thawing at 40°C. Samples were centrifuged at 34000 × g for 10 min and the clear supernatants were analyzed by ionexchange chromatography using a DX500 apparatus (Dionex, Olten, Switzerland) with an IONPACATC1 anion trap column (Dionex), an IONPAC-AG11 guard column (Dionex) and an analytical IONPAC-AS11 4-mm column (Dionex), fitted with an ASRSII 4-mm suppressor for anions (Dionex), and an IONPACCTC cation trap column (Dionex), an IONPAC-CG12A guard column (Dionex) and an analytical IONPAC-CS12A 4-mm column (Dionex), fitted with a CSRS 4-mm suppressor for cations (Dionex), with detection by a CD20 conductivity detector (Dionex), according to the manufacturer's instructions.

2.3.4 Chl a Fluorescence Emission and Gas Exchange

To determine chlorophyll a (*Chl a*) fluorescence, a LED light source with emission peaks centered at 465 (blue) and 635 nm (red) generated a PPFD equal to 1500 µmol (photons) m^{-2} s^{-1} (90% red, 10% blue). A modulated fluorometer analyzer, Li-6400XT (Li-Cor Biosciences, Lincoln, NE, United States), was used to assess the fluorescence parameters. The measuring beam was set at intensity 5 (according to the instrument manual) with a modulation of 20 kHz. After the measurement of *Chl a* fluorescence emission at steady-state under light conditions, F*'*, the maximum fluorescence emission, F_m, was assessed upon induction by a 0.8 s saturating light pulse at 6000 µmol (photons)

m^{-2} s^{-1} at 20 kHz. After that, actinic light was briefly switched off while a far-red light of 8 µmol (photons) m^{-2} s^{-1} for 6s was used to discharge the PSII to allow measurement of the minimum fluorescence emission under light conditions, F_0'. Photochemical quenching (qP) was calculated from the previously mentioned parameters. Net photosynthetic CO2 assimilation rate (A, µmol m^{-2} s^{-1}) and stomatal conductance of water vapor (gs, mol m^{-2} s^{-1}) were measured using a portable open-system gas exchange (Li-6400XT).

Measurements of photosynthetic rates (A) and stomatal conductance (gs) were taken at saturating light on well-exposed and fully expanded top leaves of six plants per treatment. Leaf chamber CO_2 was set to 400 µmol CO_2 mol^{-1} air. Measurements were taken for each time point between 10:00 and 13:00 for the duration of the experiment (November 10^{th}– 23^{rd}, 2016). Gas-exchange parameters using the von Caemmerer and von Farquhar (1981) model were calculated by instrument software (Li-Cor, 2011). The effective quantum yield of PSII photochemistry in light-adapted leaves was calculated using: $\Phi_{PSII} = (F_m' - F')/F_m'$ (Genty *et al*., 1990). Anatomical analysis on a leaf area of 0.069 mm^2 were performed as described in Yoo *et al*. (2010).

2.3.5 Statistical Analysis

Biometric measurements were statistically analyzed using the Student's t-test. Photosynthesis and gas exchange results were statistically analyzed using two-way ANOVA procedure with Sidak multiple comparisons test. Ion quantification was analyzed using a two-way ANOVA and Duncan's multiple range to determine differences between means ($P \leq 0.05$). Ions for roots and shoots were statically analyzed in two separate analyses.

2.4 Results

2.4.1 Plant Growth

In order to assess the potential for omeprazole to affect growth and salt tolerance, we conducted a hydroponic experiment at increasing OP concentrations. Measurements were taken to separate phenotypes of roots and shoots. Our results indicate that OP induces two specific phenotypes: (1) increased growth of roots and shoots and (2) increased tolerance and growth under high salt stress. Specifically, for growth we observed that OP works in a dose dependent manner (Figure 1). Low doses, 1 µM, stimulated significant increases in growth while a higher dosage either had no stimulatory effect (10 µM) or, in the case of 45 µM, were inhibitory to growth. Application of OP at 1 µM resulted in 49 and 48% increases in shoot FW and DW, respectively (Figure 1). The highest dose (45 µM) inhibited growth of shoots and reduced FW and DW by 37 and 32%, respectively (Figure 1). The stimulatory effect of OP was not limited to shoots; we also observed that 1 µM stimulated root growth and biomass accumulation by increasing FW and DW of roots by 55 and 56%, respectively. Higher concentrations, 10 and 45 µM, did not significantly affect root growth (Figure 1). In control growth conditions, low concentrations of OP did not increase root length significantly. However, at 45 µM root length was severely inhibited, with a reduction of 53% (Figure 2). While OP did not significantly increase the maximal lengths of roots, low doses greatly increased root mass. OP also had effect on later root branching resulting in changes to the overall root area.

Low concentrations of 1 µM increased root area by 19%. Again, 45 µM had an inhibitory effect, decreasing root area by 44% (Figure 2).

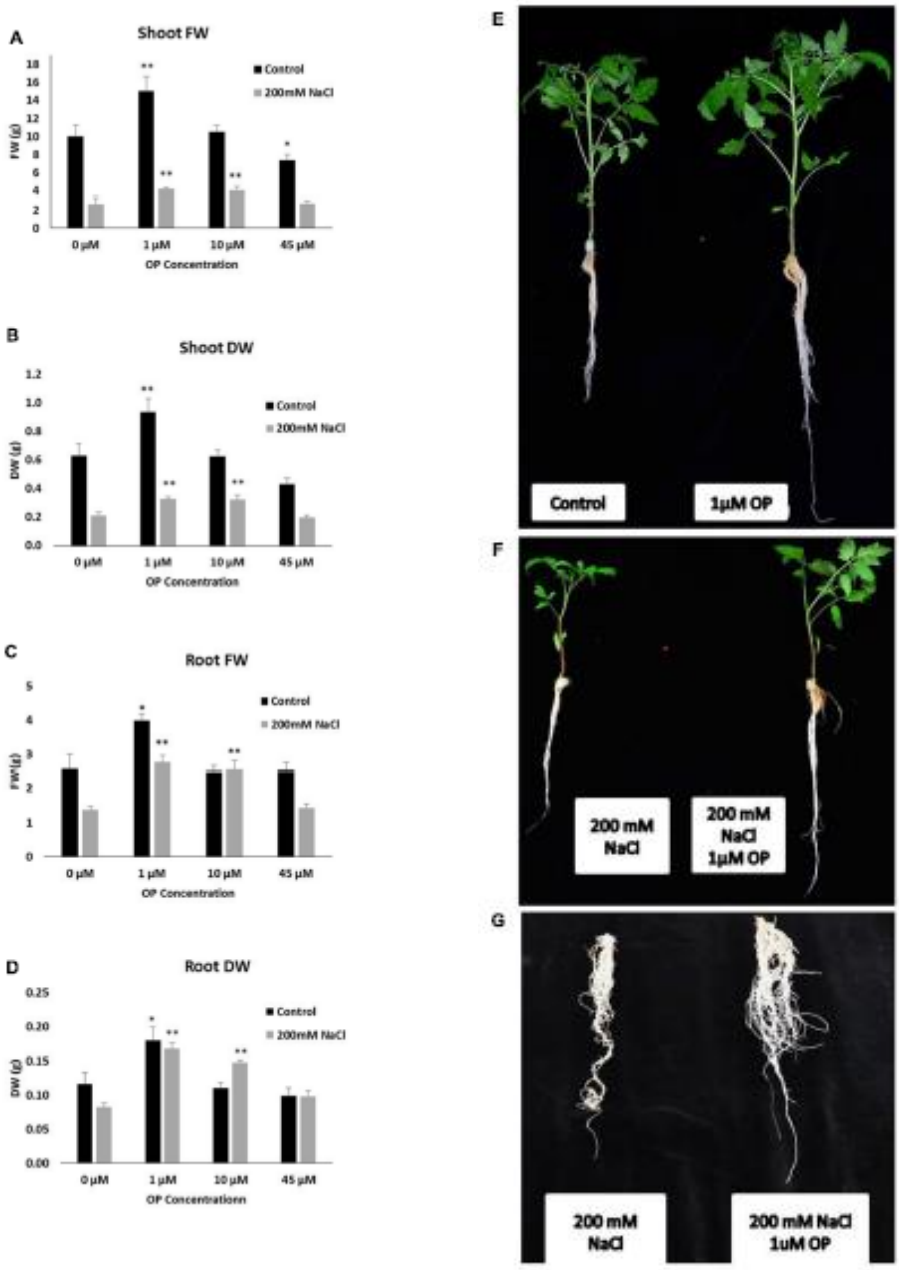


Figure 1 Omeprazole (OP) enhances shoot and root growth of Solanum lycopersicum var. M82 plants under control and salt stress conditions. Photos (right panel series) show representative plants (E,F) or roots (G) treated with 1 µM OP and controls, with and without 200 mM NaCl. The left panel series indicates Shoot fresh weight (FW) and dry weight (DW) (A,B) and Root FW and DW (C,D). Plants were grown in a hydroponic solution containing 0, 1, 10, and 45 µM OP, with and without 200 mM NaCl. Plants were harvested after 2 weeks of salt treatment (50 DAS, 14 DSS) and average shoot and root FW and DW was calculated. Values indicate average SE (n = 7). Single asterisks denote significant differences according to student (P < 0.1) between untreated controls and OP treated plants, double asterisks denote (P < 0.01) between untreated controls and OP treated plants.

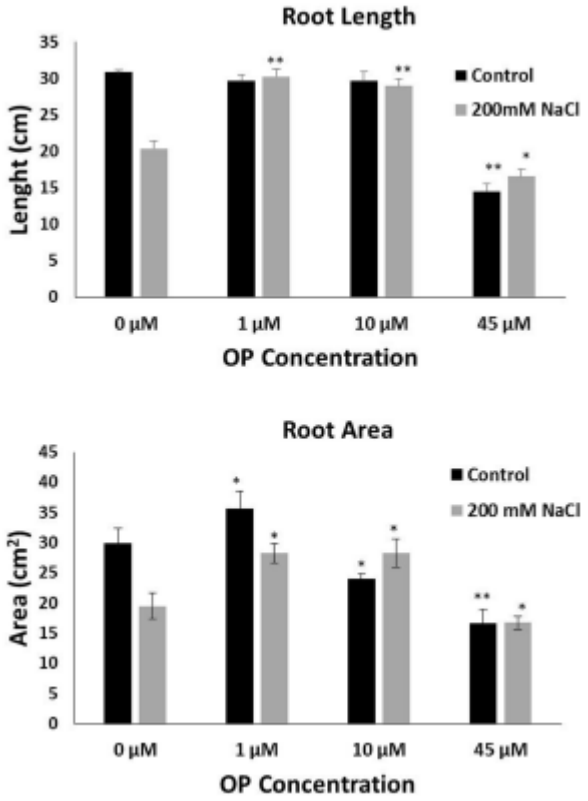


Figure 2 Omeprazole enhances root growth of Solanum lycopersicum var. M82 plants under control and salt stress conditions. Average Root Length (top) and Average Root Area (bottom). Plants were grown in a hydroponic solution containing 0, 1, 10, and 45 µM OP, with and without 200 mM NaCl. Plants were harvested after 2 weeks of salt treatment (50 DAS, 14 DSS) and root length and area were measured. Values indicate average SE (n = 7). Single asterisks denote significant differences according to Student (P < 0.1) between untreated controls and OP treated plants, double asterisks denote (P < 0.01) between untreated controls and OP treated plants.

2.4.2 Salt stress tolerance

The role of OP on salt stress tolerance was assessed in hydroponic culture. OP had significant effects on shoot growth and remarkable effects on root growth in the presence of NaCl stress. Plants grown under severe salt stress, 200 mM NaCl, and low concentrations of OP were able to maintain growth. Treatment with 1 µM under 200 mM NaCl increased shoot FW and DW over untreated controls by 56 and 54%, respectively (Figure 1). Similar results in shoots were observed with 10 µM treatments and salt stress. The application of 45 µM did not increase either of these shoot growth parameters under severe salt stress. Growth promotion under severe salt stress was more

pronounced in roots. Both root FW and DW of 1 µM OP treated plants was double (103 and 105%, respectively) that of untreated controls under severe salt stress (Figure 1). While 10 µM had no significant effect in control conditions, we observed increased tolerance in salt stress conditions. Salt stressed plants treated with 10 µM demonstrated increases of 52% of FW and DW over untreated plants subjected to 200 mM NaCl (Figure 1). High concentrations of OP (45 µM) did not induce significant gains under salt stress. Average root length for 1 and 10 µM treated plants under salt stress was similar to controls and unstressed plants, showing no reduction in root length, while salt stress reduced average root length in untreated controls by one third (Figure 2). OP treated plants also showed increased root area under severe salt stress, with 1 and 10 µM treated plants having an average of 45% more root area that untreated controls (Figure 2). Treatment with OP did not significantly alter RWC of leaves in either control or salt stressed plants (90 ± 0.04% for controls and 88 ± 0.04% for OP treated plants and 68 ± 0.03% for salt stressed plants and 71 ± 0.01% for salt stressed plants treated with OP).

2.4.3 Gas Exchange and Chl a Fluorescence Emission

Gas exchange and *Chl a* fluorescence emission were measured to assess direct effects of OP treatment on these physiological parameters. For this purpose, we conducted a second experiment with plants grown in soil. The second soil experiment was conducted using what was deemed to optimal concentration for OP (1 µM) and a NaCl concentration of 150 mM. Before stress imposition, A, Net photosynthetic CO_2 assimilation rate (A, µmol m−2 s−1) and gs stomatal conductance of water vapor (gs, mol m^{-2} s^{-1}) did not show any significant difference with respect to OP treatments, and they averaged 25.8 and 0.397 µmol m^{-2} s^{-1}, respectively. In non-stress conditions, we did not observe any effect by OP on gas exchange or photosynthesis. Before salt stress imposition there was no statistical difference between untreated controls and OP treated plants where the average, Φ_{PSII} and qP were 0.255 and 0.456 respectively.

We did observed a protective effect on photosystem integrity in 1 µM OP treated plants subjected to salt stress. Both Φ_{PSII} (quantum yield of photosystem II) and qP (photochemical quenching) were significantly affected by salt stress and OP (Figure 3). After 14 days of salt stress Φ_{PSII} was 0.124 and qP 0.279 in OP treated plants, 37 and 43%, respectively, higher than untreated controls (Figure 3). Stomatal Index and Stomatal Density were found to be similar between OP treated plants and controls (data not shown).

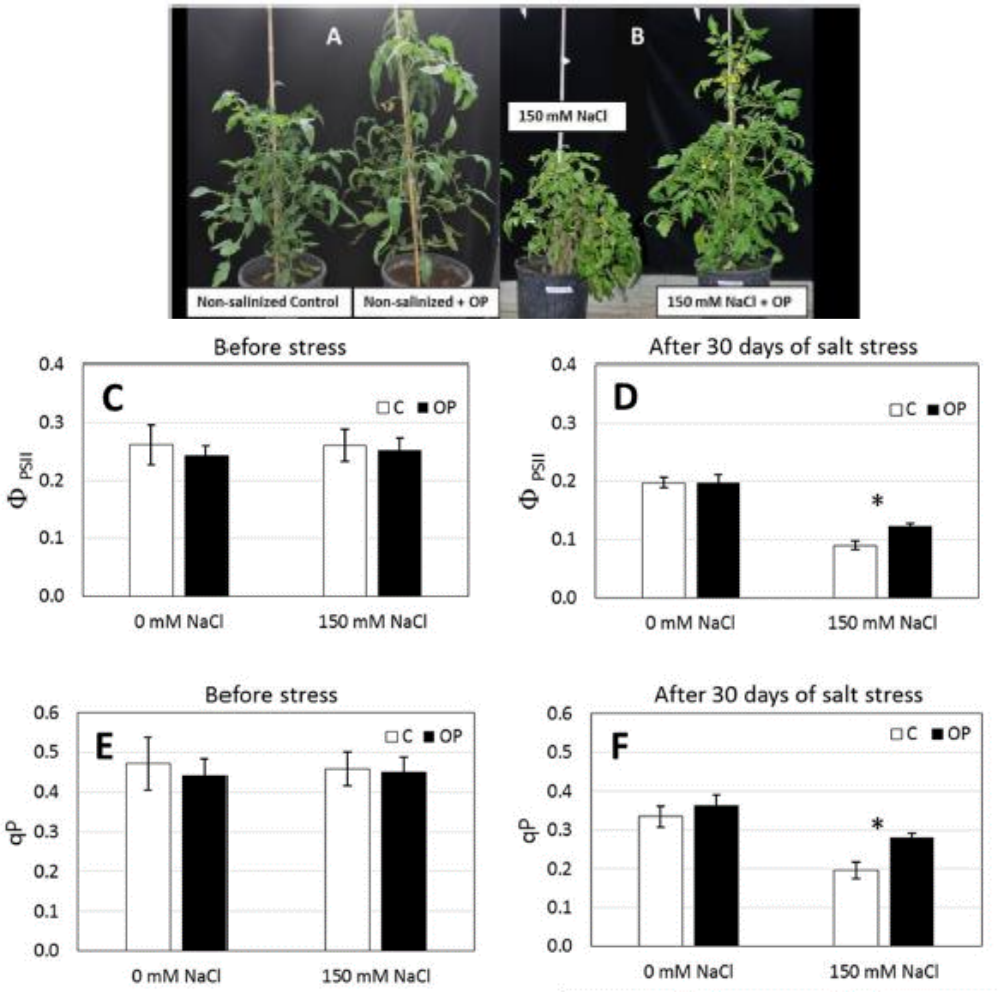


Figure 3 Omeprazole enhances growth and tolerance to salt stress of Solanum lycopersicum var. Red Setter plants. Plants were grown in soil, unsalinized or salinized with 150 mM NaCl and irrigated with 0 and 1 µM OP. Photos (A,B) of representative plants were taken after 2 weeks of salt treatment (72 DAS, 14 DSS) and efficiency of Photosystem II (8PSII, C,D) and photochemical quenching (qP, E,F) was measured. Values indicate average ± SE (n = 6). Single asterisks denote significant differences according to Student (P < 0.01) between untreated controls and OP treated plants.

2.4.4 Ion Profile of Omeprazole Treated and Salt Stressed Plants

To better understand the mechanisms that OP affects to increase growth and tolerance to salt stress, tissue ion concentrations were profiled in all hydroponic treatments. OP altered the ion accumulation of tomato plants in control conditions and under severe salt stress. In unstressed conditions, OP increased K^+ accumulation in roots treated with 1 µM (Figure 4); however, it did not increase Na^+ accumulation. OP did affect the Na^+/K^+ ratio of salt stressed leaves and roots. The root Na^+/K^+ ratio of roots under salt stress was reduced by 12, 23, and 35% in 1, 10, and 45 µM OP treated plants, respectively (Figure 4). Calcium accumulation was also affected by OP treatment. In shoot, lower OP concentrations, 1 and 10 µM, decreased shoot calcium concentration significantly. Interestingly, 45 µM OP increased calcium concentration in roots and shoots. Root chloride accumulation was observed to be elevated in roots of plants treated with OP when compared to controls only under stress condition. Treatment with OP was observed to increase nitrate content of roots at 1 and 45 µM. OP treatment with salt stress did not result in any significant increase in nitrate accumulation (Figure 4).

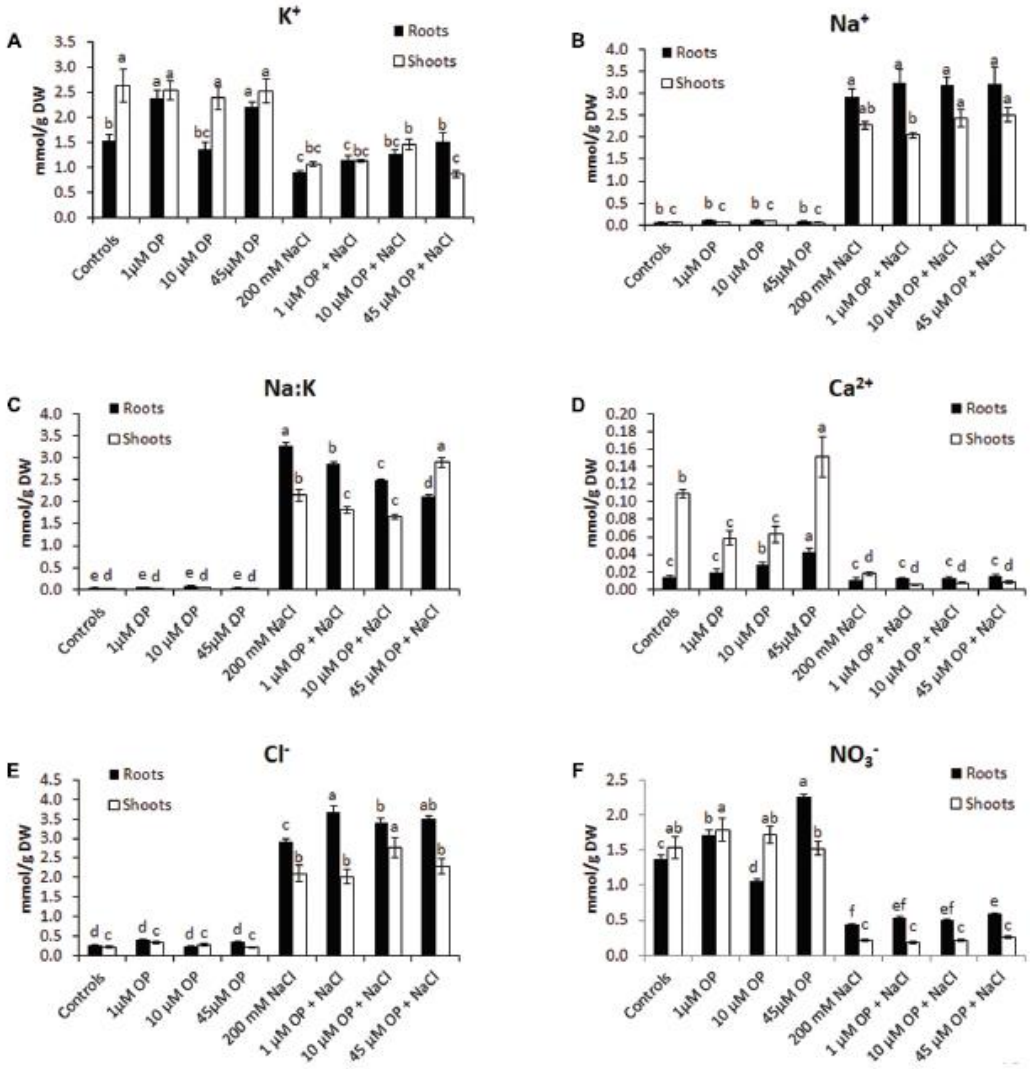


Figure 4 Ion profiles of Solanum lycopersicum var. M82 plants treated with omeprazole. Plants were grown in a hydroponic solution containing 0, 1, 10, or 45 µM OP, with and without 200 mM NaCl. Plants were harvested after 2 weeks of salt treatment (50 DAS, 14 DSS) and used for ion analysis. Values for K^+ (A), Na^+ (B), Na:K ratio (C), Ca^{2+} (D), Cl^- (E), and NO_3^- (F) are shown. Values indicate average ± SE (n = 6). Different letters indicate significant differences at P < 0.05 between an OP treated sample and the corresponding untreated control.

2.4.5 Gene Expression

Gene expression analysis was used to characterize the downstream mechanisms affected by OP that resulted in improved growth and salt tolerance in hydroponically grown tomatoes (Figures 5–7). We looked at three general categories of genes: ion transporters, stress signal transduction and osmotic response components, genes involved in

antioxidant and photosynthetic systems. Gene expression was evaluated in roots and shoots. We found that OP treatment affected a number of genes in non-stress conditions and augmented responses of numerous key genes involved in salinity stress responses and adaptation.

For ion accumulation, exclusions and transport, we selected a few ion transporters known to play key roles in responses to salinity: the plasma membrane Na^+ antiporter SlSOS1 (Salt Overly Sensitive 1), two tonoplast located K^+ antiporters, SlNHX1 and SlNHX2 (Sodium/hydrogen exchanger 1 and 2) and the Na^+ transporter SlHKT1.1 (High affinity K^+ transporter) (Figure 5). SlSOS1 was significantly upregulated in all OP treatments, with augmented expression over untreated controls under salt stress in roots and shoots (Figure 5). This may have likely contributed to the lower Na^+/K^+ ratio seen in the ion analysis. For the two tonoplast located potassium antiporters, SlNHX1 and SlNHX2, which also mediate critical functions, including turgor maintenance, stomatal function and ion homeostasis under hyperosmotic stress (Zhang and Blumwald, 2001; Pardo *et al*., 2006), both genes were significantly upregulated under 1 µM OP treatment with increased expression over controls under salt stress (Figure 5). We also analyzed the expression pattern of HKT transporters, which play an important role in limiting the influx and subsequent accumulation of sodium into the shoot as well as sodium loading into root xylem (Asins *et al*., 2013; Ali *et al*., 2016). HKT1.1 expression in shoots was higher in OP treated plants compared to untreated controls in non-stress and salinity stress conditions (Figure5). In roots, we found that OP treatment decreased SlHKT1.1 expression slightly in non-stress conditions and remarkably under salt stress. Decreased expression of HKT1 transporters may have significantly reduced sodium entry into roots, protecting them from ionic stress. Furthermore, HKT1 expression increased in salt stressed shoots. This may have favored sodium recirculation into the xylem, a function that coupled with decreased uptake and loading in roots could be a key role OP plays in salt tolerance. This result is consistent with a reduced Na^+/K^+ ratio found upon OP treatment. In non-stress conditions, treatment with OP increased the nitrate content of roots and moderately in shoots (Figure 4). In order to link the observed nitrate

accumulation profile with gene functions, we examined the gene expression of the bidirectional transporter SlNRT1.1 (High affinity nitrate transporter 2) responsible for uptake and transport of nitrate (Jossier *et al*., 2010). Expression of SlNRT1.1 was upregulated in OP treated plants, in roots and shoots, under non-stress conditions. We also observed higher SlNRT1.1 expression in salt stressed plants with OP application although no significant increases in nitrate content were detected under stress conditions.

With respect to the stress signal transduction components, we analyzed the expression of an ABA biosynthesis gene, SlNCED (9-cis-epoxycarotenoid dioxygenase), and an ABA catabolism gene SlCYP707A3 (ABA 8'-hydroxylase). We found that OP treatment decreased SlNCED expression in roots and shoots in non-stress conditions. Interestingly, SlNCED was induced upon salt stress, yet OP treatment caused an opposite response compared to what was observed in control plants (Figure 6). Specifically, in contrast to control plants, shoot expression of SlNCED was elevated over untreated controls in shoots under salt stress while root expression was significantly reduced. SlCYP707A3 expression was highly dysregulated under 1 µM OP treatment. While shoot expression was less than a third of controls, root expression was nearly three times that of untreated roots. Under salinity stress and OP treatment, expression of SlCYP707A3 was not downregulated. To better explain the expression pattern of ABA related genes, we examined the expression of SlLEA (Late embryogenesis abundant protein) a highly inducible marker in response to abiotic stress (Iovieno *et al*., 2016). While SlLEA was induced in salt stress conditions, it was less highly upregulated in the roots of OP treated plants under salt stress. This seems to correlate with decreased ABA signal transduction in the roots. In salt stressed shoots treated with OP, SlLEA demonstrated drastic upregulation, 10-fold higher than in untreated salt stress controls.

We also examined the antioxidant machinery and osmotic adaptation expression profiles of genes associated to ascorbate and proline biosynthesis. The cytosolic ascorbate peroxidase, SlAPX2 (Ascorbate peroxidase), was highly upregulated in shoots and roots of salt stressed plants. In OP treated plants under salt stress, root SlAPX2 expression was highly induced, compared to salt stress controls, indicating a more robust ROS

scavenging response induced by OP. With respect to proline and osmotic stress response, while expression of pyrroline-5-carboxylate synthetase, SlP5CS was not significantly altered in OP treated controls, it showed increased expression in salt stressed roots and shoots. Expression of the genes encoding for the catabolic pyrroline-5carboxylate dehydrogenase, SlP5CD (Proline dehydrogenase), showed a similar pattern in shoots of OP treated plants under salt stress. This may have contributed to a differential accumulation of proline in the roots and shoots.

The last set of genes we examined were those involved in the protection of the photosynthetic system. Expression levels of the tomato photosystem II reaction center psb28-like protein (PSII) were significantly upregulated under low concentrations of OP. These increases were also observed in OP treated plants under severe salt stress (Figure 7). In the leaves of OP treated plants under salt stress we found significant upregulation of the tomato catalase gene, SlCAT1 (Catalase 1) (Figure 7). High SlCAT1 expression levels have been found to enhance salt stress tolerance by reducing photoinhibition from damage to the photosystem by H_2O_2 (AlTaweel *et al*., 2007). We also examined the tomato homolog of FtsH, an ATP-dependent protease that plays a key role in degradation and repair of photosystem II (Kato *et al*., 2009; Sun *et al*., 2010). Expression of SlFTSH was below detectable thresholds in controls and OP treated plants. However, salt stress induced SlFTSH expression with an even greater upregulation under salt stress and OP treatment.

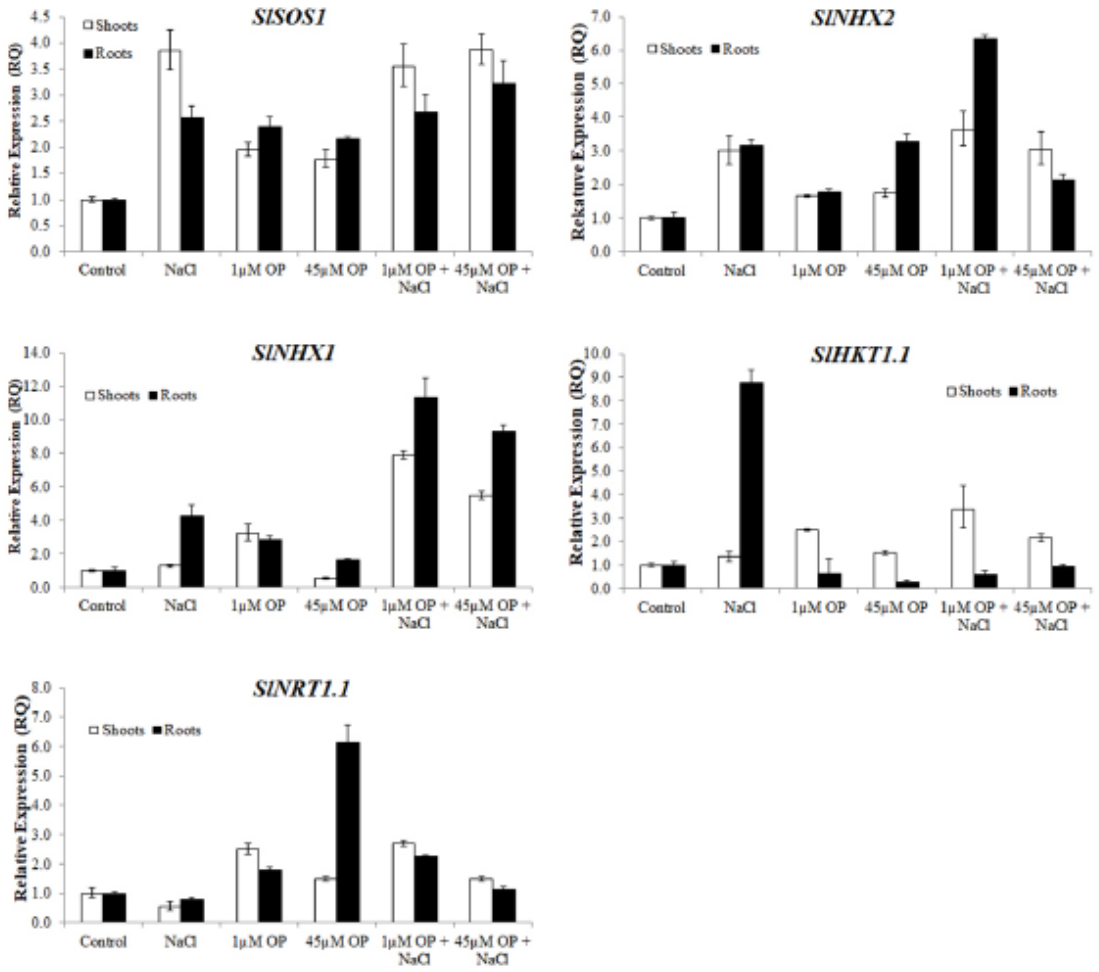


Figure 5 Ion transporter gene expression in plants treated with omeprazole. Plants were grown in a hydroponic solution containing 0, 1, and 45 µM OP, with and without 200 mM NaCl. Samples for qRT-PCR were harvested after 2 weeks of salt treatment (50 DAS, 14 DSS) and harvested for ion analysis. Values indicate average ± SD (n = 3).

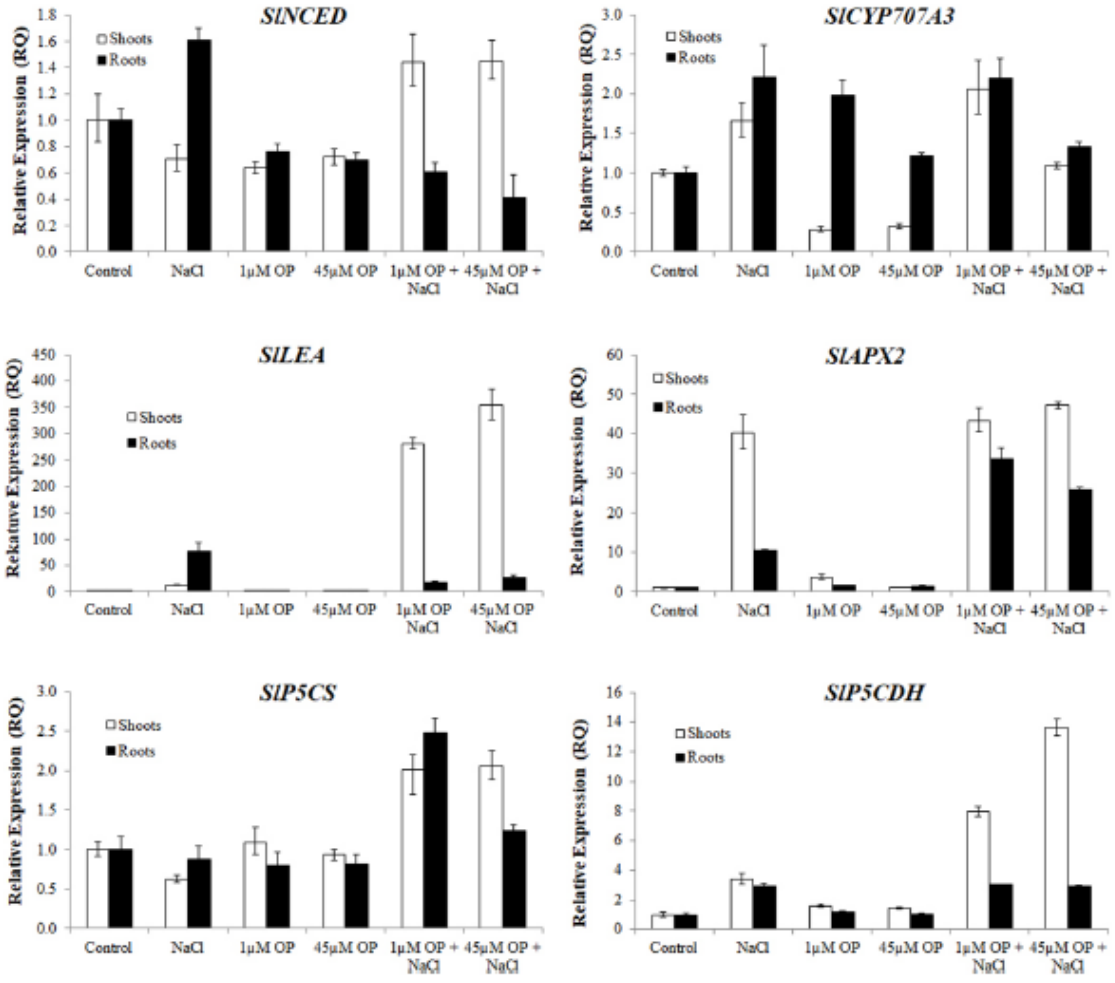


Figure 6 Secondary metabolism and stress signaling gene expression in plants treated with omeprazole. Plants were grown in a hydroponic solution containing 0, 1, and 45 µM OP, with and without 200 mM NaCl. Samples for qRT-PCR were harvested after 2 weeks of salt treatment (50 DAS, 14 DSS) and harvested for ion analysis. Values indicate average ± SD (n = 3).

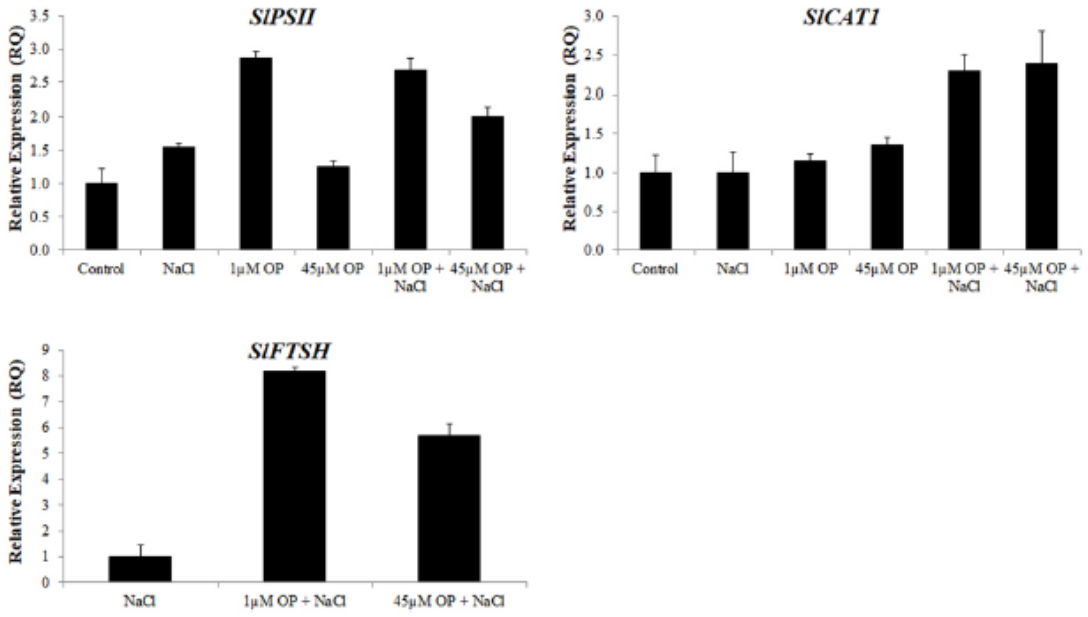


Figure 7 Photosynthetic gene expression in plants treated with omeprazole. Plants were grown in a hydroponic solution containing 0, 1, and 45 µM OP, with and without 200 mM NaCl. Samples from leaves for qRT-PCR were harvested after 2 weeks of salt treatment (50 DAS, 14 DSS) and harvested for ion analysis. Values indicate average ± SD (n = 3).

2.5 Discussion

2.5.1 OP Improves Plant Growth and Salt Stress Tolerance

In this work we demonstrated that by feeding tomato roots with hormonal concentrations of omeprazole, a benzimidazole PPI in animal systems, we can significantly improve plant growth and ability to tolerate saline stress. OP treatment with 1 µM increased shoot FW by 49% and DW by 48%. FW of roots was increased by 55% and DW by 56% in the absence of stress. Under saline stress, shoot growth was maintained, with a 56% increase in shoot FW and 54% increase in DW. Roots showed the most dramatic phenotype under salt stress, with a doubling of FW and DW over untreated controls (Figures 1, 2). Although this morphological change was not the only component that may have enhanced salt tolerance of OP treated plants, this response may have important implications with respect to growth and adaptation in saline environments (Julkowska *et al.*, 2014; Feng *et al.*, 2016). Longer, more extensive roots may help to escape

salinization by exploring non salinized areas of the soil profile (De Pascale *et al*., 2012; Lynch *et al*., 2014; Feng *et al*., 2016; Annunziata *et al*., 2017). OP seems also to interfere with ABA responses. Lateral root formation is highly sensitive to ABA concentrations, with inhibition of lateral root primordia being an order of magnitude more sensitive than seed germination (De Smet *et al*., 2003). While ABA deficient mutants have impaired growth, endogenous ABA levels have been clearly shown to be inhibitory to root growth at low osmotic potentials (Sharp *et al*., 2004; Duan *et al*., 2013; Zhao *et al*., 2014). The effects of OP on lateral root formation are likely due to changes in ABA biosynthesis, catabolism, and/or perception. By decreasing biosynthesis of ABA in roots under salt stress and increasing catabolism, OP may overcome its inhibitory effects on root growth under low osmotic potential. These results also shed some light on the role of root systems in plant salt stress adaptation.

2.5.2 OP has multiple effects on cellular mechanisms that enhance salt stress tolerance

Hyperaccumulation of Na^+ in the cytoplasm during salinity stress results in toxicity and disturbs essential cellular metabolisms such as protein synthesis, enzyme activity, and photosynthesis (Maggio *et al*., 2006; Hasegawa, 2013). Glycophytes cope with salinity stress by maintaining low cytosolic Na^+ levels and by acquisition and maintenance of K^+ (Flowers and Colmer, 2015). Sodium exclusion and potassium uptake are essential adaptations in response to high salinity in the environment that improve salt tolerance. OP appears to augment these adaptive mechanisms by affecting the regulation of a number of ion transporters. Under OP, increased expression of SlSOS1, SlNHX1, and SlNHX2 (Figure 5) establishes a pattern of sodium exclusion and increased potassium uptake, a result that was confirmed by the ion analysis (Figure 3). The plasma membrane sodium antiporter SOS1 is essential for excluding sodium from the cytoplasm and a key component in maintaining ion homeostasis (Ji *et al*., 2013). Similarly, NHX1 and NHX2 have been shown to enable maintenance of turgor, ion homeostasis, stomatal movements, growth regulation, cell expansion, and potassium uptake (Bassil *et al*., 2011; Barragán *et al*., 2012). In Arabidopsis, NXH1 selectivity has been associated to

vacuolar calcium concentrations (Yamaguchi *et al*., 2005). The low concentrations of calcium found in shoot of OP treated plants at 1 and 10 µM may have likely been correlated to a reduced calcium entry into the roots and consequently to effects on the selectivity of NHX1, as confirmed by the low Na^+/K^+ ratio of root and shoot of OP treated plants (Figure 3). The expression pattern of SlHKT1.1 we found in root and shoot of OP treated plants is also of particular relevance. The dysregulation of SlHKT1.1 under salt stress and OP, with 1) increased expression in shoots to facilitate sodium recirculation to the roots and 2) decreased expression in roots to reduce sodium loading into the xylem and subsequent transport to sensitive photosynthetic tissues indicates that OP treatment augments the plant's ability to control sodium accumulation in sensitive tissues (Figure 5). HKT transporters play an important role in limiting the influx of sodium into the shoot and subsequent accumulation as well as sodium loading into root xylem (Hauser and Horie, 2010; Ali *et al*., 2016). High sodium shoot accumulation has been linked to low AtHKT1.1 expression in roots in a number of Arabidopsis ecotypes (Rus *et al*., 2006). HKT1 transporters and non-selective cation channels (NSCCs) are the major contributors to sodium uptake in cells (Hasegawa, 2013). Decreasing the expression of HKT1 transporters in roots, while increasing HKT1 expression in shoots, could therefore be a key consequence of OP activity in plants under salt stress. Based on the ion profiles, it is clear that the phenotype of salt stress tolerance seen under OP treatment is due in part to a re-partitioning of ions under stress conditions. The increased expression of SlNRT1.1 (Figure 5) and the increased nitrate content in roots (Figure 3) indicate that OP may contribute to nitrogen uptake efficiency and resultant improvement in plant nutritional status. OP treated plants have a growth phenotype (Figures 1, 2) and increased nitrogen uptake would certainly contribute to increased growth in ideal conditions.

2.5.3 OP protects the photosynthetic system

The reduction of A, gs, Φ_{PSII} and qP in non OP and OP treated plants exposed to 150 mM of NaCl compared to the 0 NaCl treatments indicated that salt stress reduced the efficiency of PSII reaction centers and impaired electron transport in the photosynthetic apparatus (Maxwell and Johnson, 2000; Baker, 2008). OP treatment seemed to improve the actual quantum yield of PSII (Φ_{PSII}) and the photochemical quenching (qP) in the salt stressed leaves. We found that a number of key photosynthesis genes involved in photosystem II repair and ROS scavenging were upregulated under OP treatment (Figures 6, 7). Expression of catalase is a clear indicator of increased ROS scavenging and removal of potentially harmful accumulation of H_2O_2 (Mittova *et al*., 2000; Das and Roychoudhury, 2014). The upregulation under OP treatment of two key components of photosystem II repair, SlPSII (Photosystem II reaction center psb28 protein) and SlFTSH, seems to indicate that while salt stress does damage the photosystem, repair mechanisms required to maintain a nominal level of photosynthesis are less impeded. Transcript and protein accumulation of low molecular mass proteins (PSII like) have been observed in response to ROS and abiotic stress (Hihara *et al*., 2003; Kosmala *et al*., 2009; Shi *et al*., 2012). Arabidopsis mutants of SlPSII and SlFTSH genes show a decreased capacity for photosynthesis under abiotic stress (Sun *et al*., 2007) and in tomato, SlFTSH content is decreased after drought stress (Tamburino *et al*., 2017). The decreased expression of a key rate-limiting step of ABA biosynthesis in OP roots and shoots indicates that ABA levels are likely altered under OP treatment. More importantly, roots and shoots respond very differently under OP treatment, in the presence of salt stress. ABA responses under OP and salt treatment appear to be upregulated in shoots while at the same time, downregulated in roots. This is observed in the expression of SlNCED, SlCYP707A3, and the ABA responsive SlLEA gene. One possible explanation for this gene expression profile and observed growth phenotype under salt stress is that OP inhibited root ABA biosynthesis and activated shoot ABA biosynthesis which would allow root growth and branching under stress conditions (normally inhibited by ABA) (Sharp and LeNoble, 2002) and control ethylene

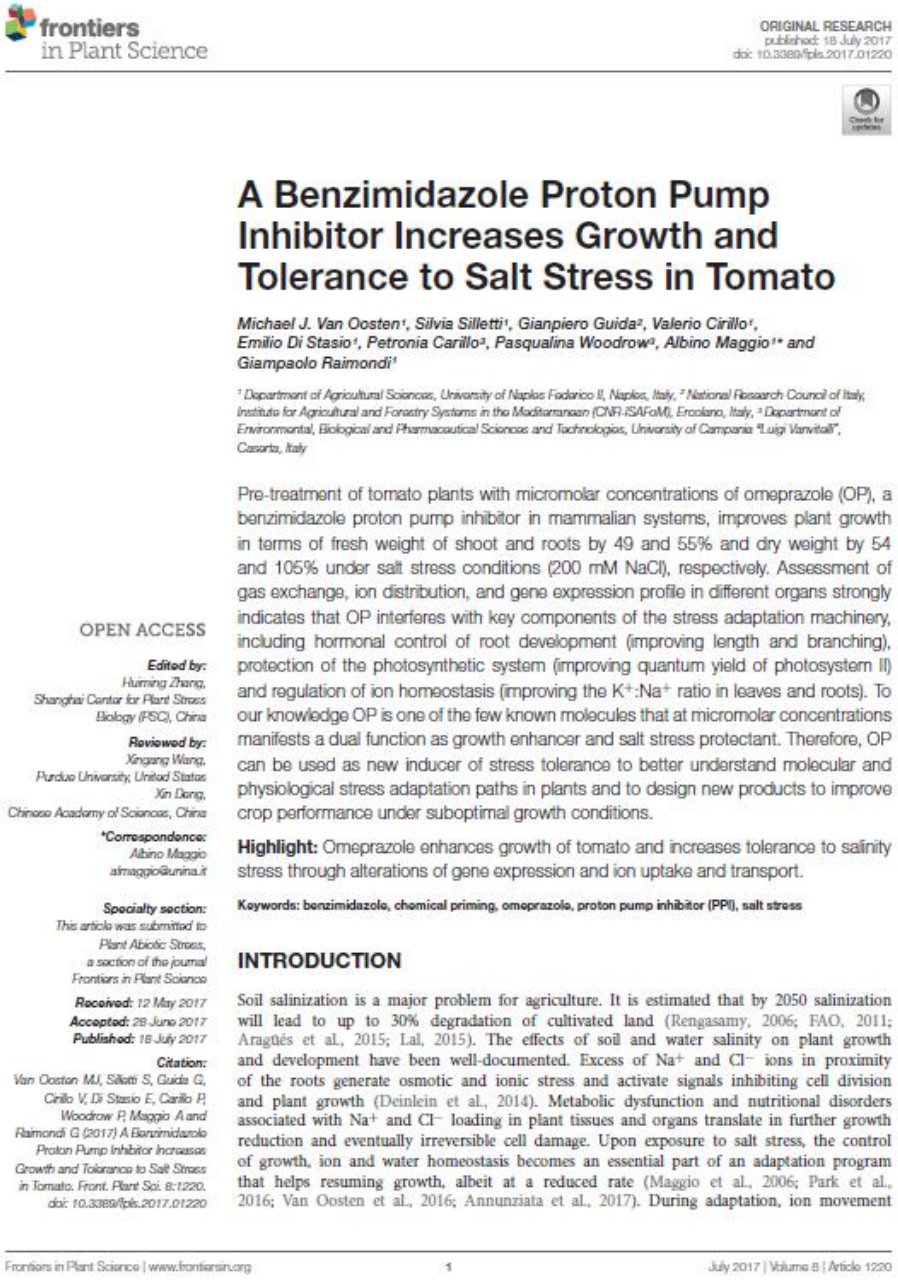
production in the shoot which would otherwise inhibit growth (Sharp, 2002). This hypothesis could also be aligned with the expression levels of SlP5CS and SlP5CD genes that may have contributed to an increased accumulation of proline in the roots, a response that typically, but not always, follows high ABA levels (Mattioli *et al*., 2009; Barbieri *et al*., 2012).

2.5.4 Possible targets of OP in plants

At the moment we do not have yet conclusive evidence for the molecular target(s) of OP. The OP concentrations we used and dose responses indicate that OP acts with a hormone-like behavior with growth stimulation between 1 and 10 µM and inhibitory effects at higher concentrations (Figures 1, 2). Similar responses have been reported for other molecules including phytohormones (Bartoli *et al*., 2013; Huot *et al*., 2014; Lozano Durán and Zipfel, 2015). However, only for a few of these single-molecule effectors a function on growth enhancement and stress tolerance has been demonstrated. Plants lack TypeIIC ATPases that transport K^+ and Na^+, the known target of OP, and the closest related classes of ATPases in plants share very low homology with animal H^+/K^+-ATPase (Sweadner and Donnet, 2001). However, based on the well-characterized function as H^+/K^+-ATPase inhibitor in animal systems, we can also hypothesize that OP is inhibiting an ATPase present in plants. This hypothesis is difficult to come to terms with, since very little room exists in our current paradigm of ATPase driven proton gradients and ion transport, where inhibition of one or more of these components would actually increase growth or tolerance to salinity. Ion homeostasis is key to growth and adaptation to osmotic stress, a clear mechanism for the role of OP does not readily present itself. The possibility that OP is exerting its effect through a mechanism of action, which is unrelated to an ATPase inhibitory function in plants should also be considered. OP appears to be one of a few molecules with a dual function of growth enhancer and stress protectant and it represents an excellent candidate to explore key mechanisms that could shed some light on how plant growth inhibition and adaptation in response to salt stress can be uncoupled (Ruggiero *et al*.,

2004). While the exact target of omeprazole remains unclear, the physiological effects open new avenues for understanding the mechanisms that allow plants to grow under adverse conditions.

2.6 Published paper front-page



frontiers in Plant Science

ORIGINAL RESEARCH
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A Benzimidazole Proton Pump Inhibitor Increases Growth and Tolerance to Salt Stress in Tomato

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Pre-treatment of tomato plants with micromolar concentrations of omeprazole (OP), a benzimidazole proton pump inhibitor in mammalian systems, improves plant growth in terms of fresh weight of shoot and roots by 49 and 55% and dry weight by 54 and 105% under salt stress conditions (200 mM NaCl), respectively. Assessment of gas exchange, ion distribution, and gene expression profile in different organs strongly indicates that OP interferes with key components of the stress adaptation machinery, including hormonal control of root development (improving length and branching), protection of the photosynthetic system (improving quantum yield of photosystem II) and regulation of ion homeostasis (improving the K^+:Na^+ ratio in leaves and roots). To our knowledge OP is one of the few known molecules that at micromolar concentrations manifests a dual function as growth enhancer and salt stress protectant. Therefore, OP can be used as new inducer of stress tolerance to better understand molecular and physiological stress adaptation paths in plants and to design new products to improve crop performance under suboptimal growth conditions.

Highlight: Omeprazole enhances growth of tomato and increases tolerance to salinity stress through alterations of gene expression and ion uptake and transport.

Keywords: benzimidazole, chemical priming, omeprazole, proton pump inhibitor (PPI), salt stress

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INTRODUCTION

Soil salinization is a major problem for agriculture. It is estimated that by 2050 salinization will lead to up to 30% degradation of cultivated land (Rengasamy, 2006; FAO, 2011; Aragüés et al., 2015; Lal, 2015). The effects of soil and water salinity on plant growth and development have been well-documented. Excess of Na^+ and Cl^- ions in proximity of the roots generate osmotic and ionic stress and activate signals inhibiting cell division and plant growth (Deinlein et al., 2014). Metabolic dysfunction and nutritional disorders associated with Na^+ and Cl^- loading in plant tissues and organs translate in further growth reduction and eventually irreversible cell damage. Upon exposure to salt stress, the control of growth, ion and water homeostasis becomes an essential part of an adaptation program that helps resuming growth, albeit at a reduced rate (Maggio et al., 2006; Park et al., 2016; Van Oosten et al., 2016; Annunziata et al., 2017). During adaptation, ion movement

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CHAPTER 3.

PHYSIOLOGICAL AND METABOLIC RESPONSES TRIGGERED BY OMEPRAZOLE IMPROVE TOMATO PLANT TOLERANCE TO NaCl STRESS

3.1 Abstract

Interest in the role of small bioactive molecules (< 500 Da) in plants is on the rise, compelled by plant scientists' attempt to unravel their mode of action implicated in stimulating growth and enhancing tolerance to environmental stressors. The current study aimed at elucidating the morphological, physiological and metabolomics changes occurring in greenhouse tomato (cv. Seny) treated with omeprazole (OP), a benzimidazole inhibitor of animal proton pumps. The OP was applied at three rates (0, 10, or 100μM) as substrate drench for tomato plants grown under nonsaline (control) or saline conditions sustained by nutrient solutions of 1 or 75mM NaCl, respectively. Increasing NaCl concentration from 1 to 75mM decreased the tomato shoot dry weight by 49% in the 0μM OP treatment, whereas the reduction was not significant at 10 or 100μM of OP. Treatment of salinized (75mM NaCl) tomato plants with 10 and especially 100μM OP decreased Na^+ and Cl^- while it increased Ca^{+2} concentration in the leaves. However, OP was not strictly involved in ion homeostasis since the K^+ to Na^+ ratio did not increase under combined salinity and OP treatment. OP increased root dry weight, root morphological characteristics (total length and surface), transpiration, and net photosynthetic rate independently of salinity. Metabolic profiling of leaves through UHPLC liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry facilitated identification of the reprogramming of a wide range of metabolites in response to OP treatment. Hormonal changes involved an increase in ABA, decrease in auxins and cytokinin, and a tendency for GA down accumulation. Cutin biosynthesis, alteration of membrane lipids and heightened radical scavenging ability related to the accumulation of phenolics and carotenoids were observed. Several other stress-related compounds, such as polyamine conjugates, alkaloids and sesquiterpene lactones, were altered in response to OP. Although a specific and well-

defined mechanism could not be posited, the metabolic processes involved in OP action suggest that this small bioactive molecule might have a hormone-like activity that ultimately elicits an improved tolerance to NaCl salinity stress.

3.2 Introduction

Salinity affects more than 45 million hectares (20%) of irrigated soils accounting for one-third of worldwide food production (Machado and Serralheiro, 2017). In Europe, about 4 million hectares have been impoverished by human activities, in particular along the Mediterranean coast (Daliakopoulos *et al*., 2016). Climate change, rise in evapotranspiration, intensive farming, excessive over-pumping of groundwater for irrigation (especially in coastal areas with consequent sea-water infiltration into fresh aquifers) and use of low quality water (brackish water or treated wastewater) in irrigation contribute synergically to soil salinization (Rana and Katerji, 2000; Costantini and Lorenzetti, 2013; Daliakopoulos *et al*., 2016). Under these circumstances, continuous exposure to hyperosmotic stress and seasonal effects linked to salt accumulation in the roots highly affect crop yield (Rana and Katerji, 2000).

Osmotic stress and ion toxicity are the main problems that affect salt stressed plants (Munns and Tester, 2008; Gorham *et al*., 2010). Under high salinity, roots are unable to uptake water from the soil and toxic concentrations of sodium and chloride build up in the cytosol and organelles, resulting in plant nutritional disorders and oxidative stress (Hasegawa *et al*., 2000; Munns, 2002; Tavakkoli *et al*., 2010). Sodium interferes with potassium and calcium uptake, negatively affecting stomatal control; moreover, it can replace potassium in key enzymatic reactions. Therefore, the salt stress status of a crop depends mainly on the potassium-to-sodium ratio than on the absolute amount of sodium in the cytosol (Shabala and Cuin, 2008; Asins *et al*., 2013; Annunziata *et al*., 2017). Instead, chloride competes with nitrate for uptake and translocation within the plants by nitrate transporter proteins, exerting direct and indirect effects mediated by nitrate decrease on chlorophyll degradation as well as on the PSII quantum yield and photochemical quenching (Carillo *et al*., 2005; Tavakkoli *et al*., 2011). This double

effect reduces plant growth and causes irreversible cell damage. However, plants try to adapt to salinity by osmo-regulating cellular compartments and controlling ion and water homeostasis to reduce stress damage and resume growth (Hasegawa *et al*., 2000; Woodrow *et al*., 2017). In particular, a ubiquitous mechanism of plant cells involves compartmentalization of toxic ions in the vacuoles as inexpensive osmotica and synthesis and/or accumulation of organic osmolytes in the cytosol for osmotic adjustment and protection against oxidative stress (Carillo *et al*., 2008; Hasegawa, 2013; Shabala, 2013). In this important process, plasma membrane and vacuolar H^+-ATPases have a key role in cytosol detoxification by creating an electrochemical H^+ gradient across the membranes used to drive a secondary active transport for Na^+ compartmentalization within the vacuole or its extrusion from the cell (Blumwald *et al*., 2000; Pardo *et al*., 2006; Ji *et al*., 2013). In fact, it is generally accepted that salt stress induces H^+-pumping capacity in plant tissues, mainly to energize Na^+/H^+ exchanger activity (Cuin *et al*., 2011; Bose *et al*., 2015). Moreover, the electrochemical gradient built up can be channeled for driving the active co-transport with H^+ of nitrate, phosphate, sulfate, sucrose, hexoses, and amino acids against their gradient (Batelli *et al*., 2007; Silva and Gerós, 2009; Conde *et al*., 2011).

Proton pump activity is continuously modulated by all the important factors controlling the plant physiology, subject to activation/deactivation foremost in response to abiotic stresses (Chelysheva *et al*., 1999; Hasegawa, 2013). Salt tolerance in Arabidopsis is enhanced as a result of increased ion compartmentalization facilitated by overexpressing vacuolar H^+-PPase AVP1 (Fuglsang *et al*., 2011) and, furthermore, by co-overexpressing vacuolar H^+-PPaseAVP1 and Na^+/H^+ antiporter AtNHX1 genes simultaneously (Shen *et al*., 2015). Conversely, inhibition of plasma membrane H^+-ATPase by vanadate decreases the K^+/Na^+ ratio rendering the plant more susceptible to salinity (Li *et al*., 2014).

Homologues of plant proton pumps are the gastric H^+/K^+ATPases, members of the P2-type ATPase family, responsible for gastric acid secretion, which include also membrane Ca^{2+} pumps and Na^+/K^+-transporters (Shin *et al*., 2009). The introduction and use of

substituted benzimidazoles as proton pump inhibitors (PPI) targeted to the gastric H^+/K^+-ATPases has been essential for the treatment of peptic ulcers and gastroesophageal reflux disease (Fellenius *et al*., 1981). Omeprazole (OP) has been the first PPI pharmaceutical introduced in the market, which specifically and irreversibly inhibits the P2-type ATPases (Shin and Kim, 2013). It is thus used for the treatment of dyspepsia, peptic ulcer, gastroesophageal reflux disease or Helicobacter pylori infection (Seoane *et al*., 2017).

Over the past few decades, plant scientists have started to identify the targets and mode of action in plants of signaling small molecules (< 500 Da) derived from human/animal research (Kaschani and van der Hoorn, 2007; Lace and Prandi, 2016). These small bioactive molecules created on the basis of natural or synthetic low-molecular weight compounds could be considered an efficient and safe approach to stimulate plant growth and elicit tolerance to environmental stressors (Kaschani and van der Hoorn, 2007; Lace and Prandi, 2016; Tsygankova *et al*., 2016).

Notwithstanding P2-type ATPases are not present in plants, Van Oosten *et al*. (2017) have demonstrated OP (345.4 Da) as being effective at micromolar (µM) concentrations in stimulating tomato plant growth and enhancing tolerance to salinity. However, the experiments discussed by Van Oosten *et al*. (2017) pertained to a short term trial, while in horticultural context plants growing on saline soils usually encounter long-term sodium chloride salinization. Moreover, though the effectiveness of OP in inducing stress tolerance has been partially clarified, conclusive evidences regarding its molecular targets have not become available yet. Nonetheless, it is important to unravel the molecular basis of the improved stress tolerance imparted by OP treatments, in order to elucidate the physiological and biochemical mechanisms involved, thus supporting a rationale for their application in agriculture. In this context, an untargeted approach facilitated by metabolomics has proved a powerful strategy for shedding light onto the role of secondary metabolites in mediating plant response to abiotic stressors (Nakabayashi and Saito, 2015).

Indisputably, the elucidation of fundamental plant molecular responses to OP can be instrumental to unraveling adaptive strategies against salinity stress; hence the aim of this study was to investigate morphological, physiological, and metabolic changes in response to OP application onto greenhouse tomato subjected to salt stress conditions. Untreated and treated tomato plants were characterized and compared in terms of growth, root morphology, ion content, gas exchange parameters, water relations and metabolic profiling.

3.3 Materials and methods

3.3.1 Plant Material, Greenhouse Conditions, and Crop Management

The experimental trial was carried out in the 2016 summer season in an unheated glasshouse at the experimental station of the University of Naples Federico II, located in Bellizzi, Salerno province (43° 31′ N, 14° 58′ E; 60 m asl), Italy. The tested vegetable species for the current experiment was tomato (*Solanum lycopersicum* L.) cv. Seny (Seminis Monsanto, Milano, Italy). Tomato plants were grown under natural light conditions and the daily air temperature inside the glasshouse was maintained between 18 and 30°C.

Cultivar Seny is a round-fruited, indeterminate tomato vine widely cultivated under greenhouse conditions in Italy due to its high productivity and resistance to cracking. Tomato seedlings were transplanted on May 2, at the three-true-leaf phenological stage into plastic pots (h 20 cm; D 20 cm) containing 5.3 L of a peat/perlite mixture in 2:1 volume ratio. The Lithuanian peat containing sphagnum peat moss (Agraria Di Vita, Pistoia, Italy) had the following physicochemical properties: 80% water holding capacity, pH 4.0, electrical conductivity 0.1 dS m^{-1}, 11 g kg^{-1} N, 0.1 g kg^{-1} P, 0.1 g kg^{-1} K, 1.8 g kg^{-1} Ca, 2.0 g kg^{-1} Mg, 70 mg kg^{-1} Fe, 15 mg kg^{-1} Mn, and 4 mg kg^{-1} Zn. Plastic pots were arranged in double rows. Plant rows were 0.9 m apart, and the space between plants within a row was 0.3 m. The distance between the centers of double rows was 2.22 m, resulting in a plant density of 3 plants m^{-2}, as normally practiced among fresh

tomato greenhouse growers. Pathogens and pests were controlled based on standard phytoprotective practices used by commercial tomato growers in Italy.

3.3.2 Experimental Design, Omeprazole Application, and Nutrient Solution Management

The experiment was designed as a two-way factorial design encompassing combinations of two sodium chloride (NaCl) concentrations (1 mM nonsaline control and 75 mM NaCl) in the nutrient solution and three omeprazole (OP) application levels (0 control, 10 and 100 µM OP). The treatments were arranged in a randomized complete-block design with four replicates, amounting to a total of 24 experimental units with four plants each (n = 96 plants). The OP was applied as substrate drench treatment five times during the growing cycle at weekly intervals starting on 10 May (9 days after transplanting; DAT). All OP applications were delivered at a uniform rate of 100 mL per plant.

The basic (nonsaline) nutrient solution had the following composition: 13.6 mM N-NO_3, 2.0 mM S, 1.4 mM P, 6.0 mM K, 4.5 mM Ca, 2.0 mM Mg, 1 mM Na, 1 mM Cl, 20 µM Fe, 9 µM Mn, 1.5 µM Cu, 3 µM Zn, 20 µM B, and 0.3 µM Mo with an electrical conductivity (EC) of 2.0 dS m^{-1}. The saline nutrient solution treatment consisted of the same basic composition plus an additional 75 mM NaCl, yielding an EC value of 9.2 dS m^{-1}. The pH of the two nutrient solutions was 6.2 ± 0.3. The nonsaline and saline nutrient solutions were prepared using deionized water. Saline treatment was initiated on May 18 (17 DAT).

The nutrient solution was pumped from independent tanks and delivered through a drip irrigation system with one emitter per plant at a flow rate of 2 L h^{-1}. All plants received the same amount of solution with a leaching fraction of 20% to avoid build up of salinity into the substrate. A leaching fraction of 20% is needed to maintain the EC in the substrate to a similar level to the nutrient solution EC (Colla *et al*., 2012, 2013).

3.3.3 Yield, Growth Measurements, and Root Characteristics

The number of fully ripe fruits as well as the fresh weight of marketable fruit of the first two trusses were recorded on all plants. At the end of the experiment (July 5, 65 DAT), plants were separated into leaves, stems and roots. All plant tissues were dried at 80°C for 72 h until they reached a constant weight, which corresponded to their dry biomasses. Shoot dry weight was equal to the sum of the aerial vegetative parts (leaves + stems), and the root-to-shoot ratio was also calculated. Dried plant tissues were sampled for ion analyses. The total leaf area per plant was measured using an electronic area meter (Li-Cor3000, Li-Cor, Lincoln, NE, USA).

The plant height as well as the number of leaves per plant were counted. Also, the root system architecture components were determined. Root system collection and sample preparation were performed following the protocol described previously by Lucini *et al*. (2015) and Rouphael *et al*. (2017a). The determination of root morphology characteristics was performed using WinRHIZO Pro (Regent Instruments Inc., Canada), connected to an image analysis scanner (STD 4800 Regent Instruments Inc., Canada). Three-dimensional images were captured and the following root characteristics were determined: root diameter, total root length and surface.

3.3.4 Leaf Water Potential, Relative Water Content, and Leaf Gas Exchange Measurements

On June 6 (36 DAT), leaf water potential (Ψl) measurements were performed on three replicates per treatment, using a dewpoint psychrometer (WP4; Decagon Devices, Pullman, WA). The Relative Water Content (RWC) of basal and apical tomato leaves was calculated following the formula described by Jones and Turner (1978) (RWC = [FW-DW]/[TW-DW] × 100); where FW, DW and TW corresponded to fresh, dry and turgid weight, respectively.

At 58 DAT, the net CO_2 assimilation rate (A_{CO2}), stomatal resistance (rs) and transpiration rate (E) were measured with a portable gas exchange analyzer (LCA-4;

ADC BioScientific Ltd., Hoddesdon, UK) equipped with a broadleaf chamber (cuvette window area, 6.25 cm^2). This measurement was carried out within 2 h across solar noon (i.e., between 11.00 and 13.00) on the youngest fully expanded leaves, using six replicates for each treatment. Photosynthetically active radiation (PAR), Relative humidity (RH) and CO_2 concentration (593 ± 8 µmol m^{-2} s^{-1}, RH 50 ± 0.6% and 377 ± 0.6 mg kg^{-1}, respectively) were set at ambient value and the flow rate of air was 400 mL s^{-1}. The Water Use Efficiency (WUE) was calculated as A_{CO2}/E.

3.3.5 Ion Analyses

Dried plant tissues (leaf, fruit, and root) were ground separately in a Wiley mill (IKA, MF10.1, Staufen, Germany) to pass through 0.5 mm sieve, and then were used for ion analyses.

For the cations (K^+, Ca^{2+}, Mg^{2+}, and Na^+) and anions, PO_3^{4-}, and Cl^-) analysis, 250 mg of dried material was extracted in 50 mL of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) using a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80°C for 10 min as described previously by Rouphael *et al*. (2017b, d). The mixture was centrifuged at 6000 rpm for 10 min (R10 M, Remi Elektrotechnik Limited, India), then filtered through a 0.20 µm filter paper (Whatman International Ltd., Maidstone, U.K.). The monovalent and bivalent cations were separated by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) and quantified through an electrical conductivity detector. An IonPac CG12A (4 × 50 mm, Dionex, Corporation) guard column and IonPac CS12A (4 × 250 mm, Dionex, Corporation) analytical column were used for the separation of the four cations, whereas for anions an IonPac AG11-HC guard (4 × 50 mm) column and IonPac AS11-HC analytical column (4 × 250 mm) were used.

3.3.6 Collection of Samples and Metabolomic Analysis

Two terminal leaflets were sampled from the first fully expanded leaves of two plants per experimental plot at the end of the experiment, and immediately frozen in liquid nitrogen before stored at -80°C for metabolomic analysis. Tissue samples (1.0 g) of four replicates per treatment were extracted in 10 mL of 0.1% HCOOH in 80% methanol, using an Ultra-Turrax (Ika T-25, Staufen, Germany), then filtered through a 0.22 µm cellulose membrane disposable filter and finally transferred to an amber vial for analysis. The untargeted metabolite screening was carried out using a 1290 UHPLC liquid chromatography system coupled to a G6550 quadrupoletime-of-flight mass spectrometer, equipped with a JetStream dual Electrospray ionization source (UHPLC-ESI/QTOF-MS) (Agilent Technologies Santa Clara, CA, USA).

The parameters for metabolomic investigations in plant tissues were set out in previous experiments (Pretali *et al*., 2016). Briefly, chromatographic separation was achieved on an Agilent Zorbax Eclipse-plus column (75 × 2.1 mm i.d., 1.8 µm) using a mobile phase consisting of water (A) and methanol (B), flowing at 220 µL min-1 and 35°C. The gradient was initiated with 5% B and increased to 90% B within 35 min, whereas the mass spectrometer was run in positive scan mode (range of 100– 1200 m/z) using a nominal mass resolution of 30,000 FWHM. Concerning electrospray conditions, nebulizer pressure was 60 psig, capillary voltage was 4 kV, sheath gas was nitrogen at 10 L min^{-1} (350 °C), and drying gas was nitrogen at 10 L min-1 (280°C).

Raw data were processed using Profinder B.05 (from Agilent Technologies) for feature initial deconvolution. Compounds identification was carried out using the whole isotopic pattern (i.e., accurate mass, isotope accurate spacing and isotope ratio). Compounds were aligned for both mass and retention time, then annotated using the database PlantCyc 9.5 (Plant Metabolic Network, http://www.plantcyc.org; released November 2014). A filter-by-frequency post-processing was applied retaining only those compounds that were present in 100% of replications within at least one treatment. Therefore, identification was carried out as Level 2 (putatively annotated compounds), according to COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi).

3.3.7 Statistical Analysis of Experimental Data

Experimental data were subjected to two-way analysis of variance (ANOVA) using the SPSS 10 software package. Treatment means within each measured parameter were separated by Duncan's multiple range test performed at a significance level of $P \leq 0.05$. Principal component analysis (PCA) was also performed using Minitab 16.2.1 statistical software, aimed to extract trends by formulating new variables correlated to the original ones (Lawless and Heymann, 2010; Rouphael *et al*., 2017d). The PCA outputs included variable loading to each selected component and treatment component scores (Ciarmiello *et al*., 2015; Rouphael *et al*., 2017c).

Metabolomics data were formerly elaborated using Agilent Mass Profiler Professional B.12.06 (from Agilent Technologies). Compounds were filtered by abundance (area > 10,000 counts), normalized at the 75th percentile and baselined to the median of control. Unsupervised hierarchical cluster analysis was carried out setting similarity measure as "Euclidean" and "Wards" linkage rule. Fold-change analysis was also carried out, using a cut-off value of 2. Thereafter, the dataset was exported onto SIMCA 13 (Umetrics, Malmo, Sweden), UV-scaled and elaborated for partial least square discriminant analysis (PLS-DA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modeling together with unsupervised methods (Worley and Powers, 2013). Hierarchical cluster analysis can be applied in order to reveal differences between classes without supervision, whilst the utilization of class membership in OPLS-DA allows a better separation between classes in score plot hyperspace while effectively separating Y-predictive variation from Y-uncorrelated variation in X. In particular, OPLS-DA allowed separating variation between the groups into predictive and orthogonal (i.e., ascribable to technical and biological variation) components. Outliers were excluded using the distance from the origin in the OPLS-DA model, according to Hotelling's T2 and adopting 95 and 99% confidence limits for suspect and strong outliers respectively. Model overfitting was excluded through cross validation CVANOVA ($p < 0.01$) and permutation testing. Model parameters (goodness-of-fit R2Y and goodness-of-prediction Q2Y) were also produced. Regarding Q2Y prediction

ability, a value >0.5 was adopted as a threshold to identify acceptable models, according to software recommendation and as set out in literature (Rombouts *et al*., 2017). Variable importance in projection (VIP analysis) was used to evaluate the importance of metabolites and to select those having the highest discrimination potential (VIP score >1.3). To achieve information on the regulation of biochemical processes related to OP treatment either under salinity or nonsaline control, a following fold-change analysis was performed for those metabolites highlighted by VIP analysis.

3.4 Results

3.4.1 Morphological Parameters, Yield, and Root Characteristics

Plant height, number of leaves per plant, total leaf area as well as shoot biomass were influenced by salinity and omeprazole (OP) treatments with significant salinity × OP interaction. In treated and untreated tomato plants, the plant height number of leaves, leaf area and dry biomass decreased as the salinity level increased, with a more detrimental effect recorded in untreated plants (Figures 1, 2). In fact, increasing NaCl concentration in the nutrient solution from 1 to 75 mM decreased the tomato shoot biomass by 49% in the control treatment, whereas the dry shoot reduction was not significant when 10 µM (-10%) and 100 µM (-7%) of OP were used, with no significant difference between the two OP concentrations.

Except from the root diameter, which was not affected by either salinity or OP, root dry weight, total root length and surface as well as the root-to-shoot ratio (R/S) incurred significant salinity × OP interaction (Figure 1). The root dry weight, total length and surface area were negatively influenced by salt stress treatment (Figure 3). Under nonsaline conditions, the drench application of OP elicited dose-dependent increases in root dry weight, total length and surface, whereas under saline conditions significant differentiation was observed with respect to the 0 µM control but not between the 10 and 100 µM treatments (Figures 1, 3).

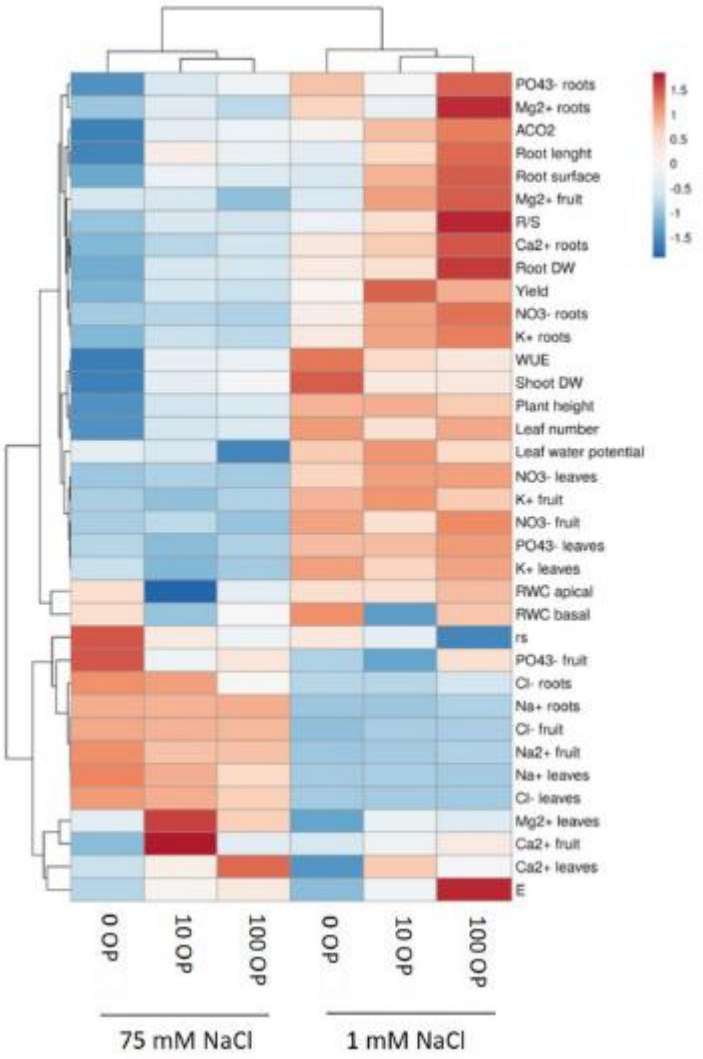

Figure 1 Heat map analysis summarizing the plant responses to NaCl concentration in the nutrient solution and OP treatments. Results were calculated as Logarithm base 2 (Log2) of untreated and OMP-treated plants under to salinity levels (1 or 75mM NaCl) and were visualized using a false color scale with red indicating an increase and blue a decrease of plants values compared to values relative to those in control condition. No differences were visualized by white squares.

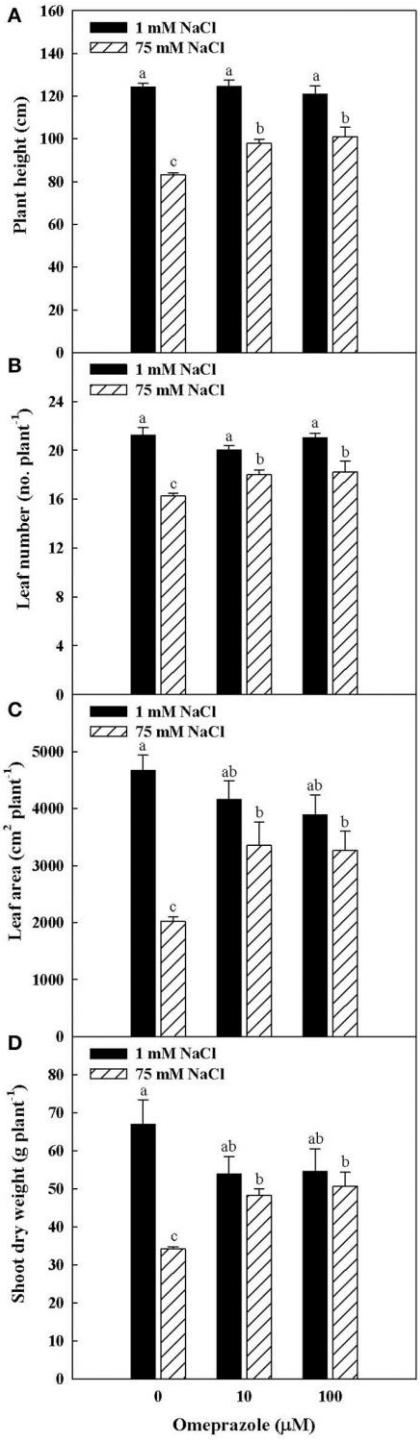

Figure 2 Effects of NaCl concentration in the nutrient solution and omeprazole application on plant height (A), number of leaves per plant (B), total leaf area (C), and shoot dry biomass (D) of greenhouse tomato plants. Different letters indicate significant differences according to Duncan's test (P = 0.05). The values are the means of four replicate samples. Vertical bars indicate ± SE of means.

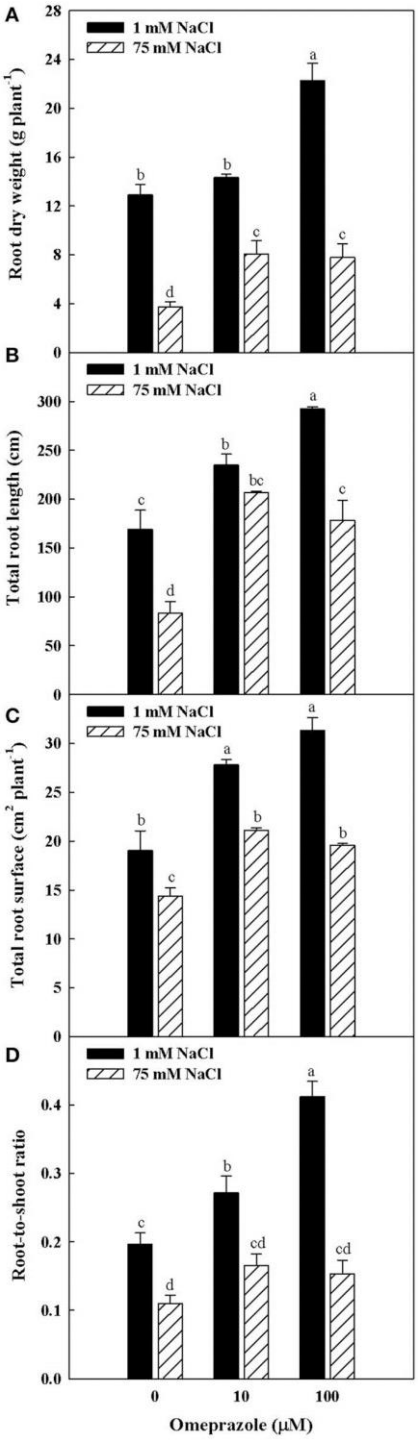

Figure 3 Effects of NaCl concentration in the nutrient solution and omeprazole application on root dry weight (A), total root length (B), total root surface (C), and root-to-shoot ratio (D) of greenhouse tomato plants. Different letters indicate significant differences according to Duncan's test (P = 0.05). Vertical bars indicate ± SE of means.

Tomato yield and the mean fruit weight were significantly affected by salinity and OP treatments with no salinity × OP interaction. Neither salinity nor OP treatment had a significant effect on tomato fruit number (data not shown). Irrespective of OP treatment, fresh tomato yield decreased with increasing salinity in the nutrient solution (Figure 4). Moreover, when averaged over salt-treatment levels, the yield of OP treated plants was higher than those of untreated plants by 44.5% (Figure 4).

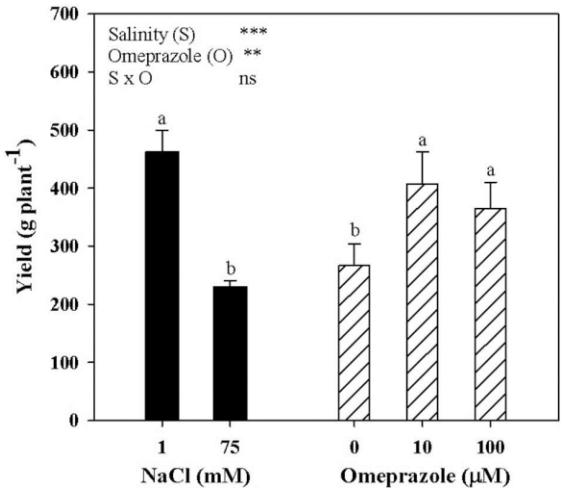

*Figure 4 Mean effects of NaCl concentration in the nutrient solution and omeprazole application on yield of greenhouse tomato plants. Different letters indicate significant differences according to Duncan's test (P = 0.05). The values are the means of four replicate samples. Vertical bars indicate ± SE of means. ns, **, *** Non significant or significant at P ≤ 0.01, and 0.001.*

3.4.2 Physiological Parameters

The net CO_2 assimilation rate (A_{CO2}) and stomatal resistance (rs) of tomato plants were significantly affected by salinity and OP treatments, with no salinity × OP application interaction, whereas the leaf water potential (Ψ_l) and WUE were only affected by the salinity treatment (Table 1). Increasing the sodium chloride concentration in the nutrient solution from 1 to 75 mM reduced Ψ_l, A_{CO2}, and WUE by 27, 37, and 34%, respectively, while it increased rs values by 23% (Table 1). Substrate drench application of OP induced significant increase of A_{CO2} (+48%), with no significant difference between the two OP concentrations (Table 1). The higher A_{CO2} in OP-treated tomato plants was accompanied by an increase in E values. Averaged over salinity treatments, OP

application induced lower values of rs in comparison to untreated plants (Table 1). Conversely to the leaf gas exchange parameters, no significant differences between treatments were recorded in RWC of basal leaves (Table 1).

Source of variance	Ψ_l MPa	RWC apical (%)	RWC basal (%)	A_{CO2} (μmol CO_2 m^{-2} s^{-1})	r_s (m^2 s^1 mol^{-1})	E (mol H_2O m^{-2} s^{-1})	WUE (μmol CO_2 mol^{-1} H_2O)
Salinity (S)	***	NS	NS	***	*	NS	*
Omeprazole (OP)	NS	*	NS	*	**	*	NS
S × O	NS	NS	NS	NS	NS	NS	NS
Salinity (mM NaCl)							
1	-1.39 a	83.88	91.51	6.22 a	16.84 b	1.78	3.58 a
75	-1.90 b	77.33	86.94	3.95 b	20.64 a	1.69	2.35 b
Omeprazole (μM)							
0	-1.58	83.37 a	95.57	3.87 b	22.06 a	1.41 b	3.02
10	-1.54	76.36 b	81.36	5.46 a	18.65 ab	1.70 ab	2.95
100	-1.81	82.10 a	91.81	5.92 a	15.52 b	2.03 a	2.93
S ×OMP							
1 mM NaCl × 0 μM OP	-1.42	83.29	96.66	5.22	19.86	1.38	4.13
1 mM NaCl × 10 μM OP	-1.28	83.08	80.14	6.36	17.73	1.66	3.41
1 mM NaCl × 100 μM OP	-1.46	85.28	93.93	7.09	12.91	2.27	3.16
75 mM NaCl × 0 μM OP	-1.74	83.44	92.31	2.53	24.25	1.46	1.37
75 mM NaCl × 10 μM OP	-1.80	69.64	82.57	4.56	19.56	1.73	2.64
75 mM NaCl × 100 μM OP	-2.15	78.92	88.63	4.75	18.12	1.79	2.70

ns,*,**, *** Nonsignificant or significant at P ≤ 0.05, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05).

Table 1 Analysis of variance and mean comparisons for leaf water potential (Ψl), relative water content (RWC) of apical and basal leaves, net CO2 assimilation rate (A_{CO2}), stomatal resistance (rs), transpiration rate (E), and water use efficiency (WUE) of tomato plants grown under two salinity levels and treated with omeprazole (OMP) at three rates of application.

3.4.3 Ion Content and Partitioning

Except for the bivalent cations (Ca^{2+} and Mg^{2+}) in leaf tissue, the nitrate, phosphate and K^+ in both leaves and roots as well as Ca^{2+} and Mg^{2+} in roots, were negatively affected by 75 mM NaCl in the nutrient solution (Table 2). Moreover, the concentrations of both toxic elements (Na^+ and Cl^-), which accumulated mainly in leaves and to a lesser extent in roots, were significantly influenced by salt stress treatment (Table 2). In OP untreated plants, the concentrations of Na^+ and Cl^- were 50- and 23-fold higher as the salinity level in the nutrient solution increased (Table 2). The K^+/Na^+ ratio, initially equal to 21.4 in leaves and 11.9 in roots, was drastically reduced at 75 mM of NaCl to a value of 0.3 and 0.4, respectively. The OP treatment averaged over salt stress levels, affected

nitrate and Ca^{2+} concentrations in leaf tissue which were higher by about 23% than in OP untreated tomato plants (Table 2).

Source of variance	NO_3^- (mg g^{-1}dw)		PO_4^{3-} (mg g^{-1}dw)		K^+ (mg g^{-1}dw)		Ca^{2+} (mg g^{-1}dw)		Mg^{2+} (mg g^{-1}dw)		Na^+ (mg g^{-1}dw)		Cl^- (mg g^{-1}dw)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Salinity (S)	***	***	***	*	***	***	NS	***	NS	***	***	***	***	***
Omeprazole (O P)	**	**	NS	NS	NS	*	*	NS	NS	*	NS	NS	*	NS
S × O	NS	*	NS	NS	NS	NS	NS	NS	NS	**	*	NS	*	NS
Salinity (mM NaCl)														
1	24.33 a	26.29 a	23.1 a	8.81 a	39.46 a	26.45 a	18.99	4.10 a	4.80	1.83 a	2.82 b	1.94 b	10.16 b	4.19 b
75	3.32 b	3.24 b	16.0 b	6.37 b	26.42 b	9.83 b	20.34	2.28 b	5.15	1.24 b	81.12 a	17.89 a	173.94 a	21.11 a
Omeprazole (µM)														
0	11.96 b	9.89 b	11.3	6.99	34.23	14.04 b	16.95 b	2.77	4.75	1.47 b	48.63	9.84	99.81 a	12.83
10	14.90 a	16.09 a	19.0	7.10	31.27	19.75 a	20.73 a	3.12	5.16	1.40 b	43.04	9.63	94.28 ab	12.32
100	14.61 a	18.31 a	20.0	8.68	33.32	20.63 a	21.31 a	3.68	5.01	1.73 a	34.25	10.27	82.06 b	12.81
S x OMP														
1 mM NaCl × 0 µM O P	21.19	17.33 b	22.8	8.92	40.61	20.76	16.00	3.53	4.62	1.8 b	1.9 c	1.75	8.29 c	3.35
1 mM NaCl × 10 µM O P	25.84	28.43 a	22.7	7.49	37.51	28.11	21.47	3.95	4.91	1.43 c	3.67 c	1.52	10.89 c	3.49
1 mM NaCl × 100 µM O P	25.96	33.12 a	23.9	10.02	40.26	30.48	19.49	4.83	4.86	2.25 a	2.9 c	2.53	11.29 c	5.74
75 mM NaCl × 0 µM O P	2.73	2.46 c	16.4	5.06	27.85	7.32	17.90	2.01	4.88	1.14 c	95.35 a	17.92	191.33 a	22.30
75 mM NaCl × 10 µM O P	3.96	3.74 c	15.4	6.71	25.04	11.39	19.99	2.30	5.41	1.37 c	82.41 ab	17.73	177.66 a	21.15
75 mM NaCl × 100 µM O P	3.27	3.5 c	16.3	7.34	26.37	10.78	23.14	2.53	5.16	1.21 c	65.6 b	18.01	152.84 b	19.88

ns,*,**, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05).

Table 2 Analysis of variance and mean comparisons for nitrate, phosphate, potassium, calcium, magnesium, sodium and chloride ions in leaves and roots of tomato plants grown under two salinity levels and treated with omeprazole (OMP) at three rates of application.

Interestingly, under nonsaline conditions the application of 10 and 100 µM of OP as substrate drench induced a significant increase of nitrate in root tissue (Table 2). A significant OP × salinity interaction was observed as the OP treatment effectively reduced the Na^+ and Cl^- accumulation in leaf tissue under saline (75 mM NaCl) but not under nonsaline (1 mM NaCl) conditions. Under saline conditions the OP application significantly reduced Na^+ and Cl^- accumulation in leaf tissue in a dose-dependent manner: -14 and -31% Na^+, and -7 and -20% Cl^- in response to 10 and 100 µM OP, respectively. However, significant reductions in leaf Na^+ and Cl^- concentrations were attained in response to the 100 µM OP level. Root Na^+ and Cl^- concentrations were lower by 31 and 20%, respectively, when 100 µM OP was delivered to tomato plants (Table 2, Figure 1).

3.4.4 Metabolic Profiling of Leaves

The salinity × OP application interaction was also analyzed using an untargeted metabolomics approach based on UHPLC-ESI/QTOF-MS. Overall, this analytical approach allowed annotating 2,019 compounds.

Both the non-averaged unsupervised hierarchical cluster analysis and the supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) multivariate statistical approaches (see Materials and Methods section) allowed differentiating between treatments (Figures 5 and 6), suggesting that the metabolic profiles were affected by the treatments. In particular, when looking at the heat-map based on the fold-change analysis (Figure 5), two main clusters could be identified, representing 1 and 75 mM NaCl respectively. These findings indicated that salinity was the main clustering factor. Nonetheless, OP treated plants could be discriminated from those without OP, and a dose-dependent response was also observed under salt stress conditions (i.e., 75 mM NaCl). Indeed, three separated sub-clusters could be defined under salinity, whereas OP treated plants clustered together under 1 mM NaCl conditions. The following OPLS-DA supervised multivariate analysis provided an output that was consistent with hierarchical clustering, suggesting that salinity was the principal factor followed by OP concentration (Figure 6). To better point out the response related to OP itself rather than its specific biochemical role in promoting salt stress tolerance, two different OPLS-DA models were built, under 1 and 75 mM NaCl, respectively. Both models fitting parameters were more than adequate, being goodness-of-fit R2Y = 0.99 under both salinity conditions and goodness-of-prediction Q2Y 0.59 and 0.73 for 1 and 75 mM NaCl, respectively. Both models provided 100% accuracy in class prediction (Fischer's probability: 0.0002), whereas cross validation CV-ANOVA ($p < 0.01$) and permutation testing excluded model overfitting. No outlier replicates could be identified using Hotelling's T2 under 95 and 99% confidence limits for suspect and strong outliers, respectively.

Given the adequate fitting of OPLS-DA models, a subsequent investigation was done aiming to identify the compounds differences could be attributed to. With this purpose,

the investigation of the most discriminant compounds in the OPLS-DA model (i.e., variables of importance in projection— VIP analysis) was carried out. Table 3 reports the metabolites identified (i.e., >1.3) by VIP analysis, together with individual scores and their standard error, as well as Log fold-change values and regulation. Overall, 84 compounds were identified as those variables mostly contributing to class discrimination in OPLS-DA. Discriminating compounds were grouped in functional classes; hormones, membrane lipids, terpenes, and alkaloids were the most represented classes. Among hormones, compounds related to almost all classes could be discerned. In more detail, the brassinosteroid brassinolide, auxin inactivation compounds (oxindole-3-acetyl-aspartateN-beta-glucosyl-beta-1,4-glucose; 2-oxindole-3-acetyl-hexose; indole-3-acetyl-tryptophan), inactive forms of gibberellins (A34, A98, A51-catabolite), a precursor and a catabolite of abscisic acid (abscisic aldehyde and dihydroxyphaseic acid respectively), methyl jasmonate and a cytokinin (trans-zeatin riboside triphosphate) were identified. Among lipids, several membrane lipids (glyco- and phospholipids) were identified in VIP analysis, together with cutin biosynthetic intermediates [9,10 epoxystearate and (9R,10S)-dihydroxystearate]. Sesquiterpene lactones were also among discriminating compounds, including lubimin-related sesquiterpenoid phytoalexins (3 hydroxylubimin and 2-dehydrolubimin), parthenolide, two costunolide-related compounds [3-beta-hydroxycostunolide and germacra-1(10), 4, 11(13)-trien-12-oate] as well as zealexins A1 and A3. Furthermore, several alkaloids and phenolics were outlined as OPLS-DA discriminants; among the seconds, tetramethylquercetagetin, conjugated cyanidins, and hydroxycinnamics were the most represented. However, ajmaline and sarpagine, lupanine, and cinchona alkaloids were the most common. Carotenoids (mainly ascribable to xanthins), polyamines and their conjugates, pteridine as well as porphyrin biosynthetic precursors were also selected among discriminating compounds. Among amino acids, asparagine, lysine, saccharopine (a lysine degradant), and cystathionine (involved in cysteine/homocysteine interconversion) were pointed out. Finally, some other compounds could be recognized as differential, including L-dopachrome (intermediate in eumelanin biosynthesis), 6, 7-dimethyl-8-(1-D-ribityl) lumazine (flavin biosynthesis), a plastoquinone, an acetyl-hexosamine and two glucosinolaterelated

compounds (9-methylthiononylhydroximoyl-glutathione and 7-methylthioheptyldesulfoglucosinolate).

Compound	OPLS-DA VIP		[OP 100µM] vs. [OP control], 1 mM NaCl		[OP 100µM] vs. [OP control], 75 mM NaCl	
	Score	SE	LogFC	Regulation	LogFC	Regulation
AMINO ACIDS						
L-asparagine	1.33	0.89	−4.13	Down	−3.18	Down
L-cystathionine	1.33	1.31	0	Down	−1.50	Down
L-lysine	1.39	0.73	0.52	Up		
L-saccharopine	1.31	0.45	0	Down		
HORMONES						
brassinolide	1.38	0.36	−0.37	Down	−2.98	Down
oxindole-3-acetyl-aspartate-N-beta-glucosyl-beta-1,4-glucose	1.3	0.39	−6.66	Down	−1.75	Down
a 2-oxindole-3-acetyl-hexose	1.64	0.54	−4.40	Down		
indole-3-acetyl-tryptophan	1.56	0.43	6.72	Up		
gibberellin Asub34/sub	1.47	0.17	20.34	Up		
gibberellin Asub98/sub	1.39	0.85	−5.29	Down	−1.35	Down
gibberellin Asub51/sub-catabolite	1.32	0.67	−9.51	Down		
trans-zeatin ribosidetriphosphate	1.38	0.96	0	Down	−3.92	Down
(+)-cis-abscisic aldehyde	1.38	1	9.3	Up		
dihydroxyphaseic acid	1.36	0.86	5.48	Up	−0.03	Down
methyl jasmonate	1.36	1.08	0.59	Up	−0.01	Down
LIPIDS						
9,10-epoxystearate	1.35	0.65	0.37	Up		
(9R,10S)-dihydroxystearate	1.34	0.66	0.45	Up		
1-18:0-2-18:1-phosphatidylethanolamine	1.43	0.79	1.4	Up		
1-16:0-2-18:3-diacylglycerol-trimethylhomoserine	1.39	0.93	4.94	Up	−1.56	Down
1-18:3-2-16:3-monogalactosyldiacylglycerol	1.31	0.96	0.01	Up	−0.23	Down

1-18:1-2-16:1-monogalactosyldiacylglycerol	1.39	0.57	10.83	Up	−6.62	Down
1-18:1-2-16:0-monogalactosyldiacylglycerol	1.36	1.23	9.91	Up	−0.14	Down
1-18:2-2-18:2-monogalactosyldiacylglycerol	1.31	0.9	−0.21	Down	−0.38	Down
1-18:1-2-16:0-phosphatidylglycerol	1.38	0.91	5.2	Up	−2.57	Down
1,2-dipalmitoyl-phosphatidylcholine	1.37	0.4	−5.29	Down	−3.94	Down
1-18:1-2-18:3-phosphatidylcholine	1.43	0.45	−0.15	Down	−2.57	Down
1-18:3-2-18:2-phosphatidylcholine	1.38	0.58	0.11	Up	−0.19	Down
1-18:1-2-18:1-sn-glycerol-3-phosphocholine	1.37	1.06	0.09	Up	−0.60	
1-18:3-2-18:3-phosphatidylcholine	1.35	0.65	−0.13	Down	−2.41	Down
1-18:3-2-18:1-phosphatidylcholine	1.32	0.68	−0.15	Down	−0.60	Down
linolenate	1.34	0.74	6.84	Up		
(9S,10S)-9,10-dihydroxyoctadecanoate	1.34	0.66	0.26	Up		
4-alpha-carboxy-5-alpha-cholesta-8,24-dien-3-beta-ol	1.31	0.93	18.25	Up	−4.50	Down
SESQUITERPENE LACTONES						
zealexin A1	1.42	0.71	0.05	Up	−1.65	Down
zealexin A3	1.34	0.87	0.44	Up		
parthenolide	1.38	1	9.3	Up		
germacra-1(10),4,11(13)-trien-12-oate	1.42	0.71	−5.10	Down	−1.65	Down
3-beta-hydroxycostunolide	1.38	1	9.3	Up		
3-hydroxylubimin	1.5	0.55	4.21	Up	Down	
2-dehydrolubimin	1.33	0.78	−4.83	Down		
ALKALOIDS						
10-deoxysarpagine	1.38	0.8	−5.41	Down	−1.44	Down
vellosimine	1.36	0.37	−0.20	Down		
17-O-acetylajmaline	1.35	1.03	0.08	Up		

1,3,7,9-tetramethylurate	1.39	1.15	−4.22	Down	−0.08	Down
(S)-n-methylcanadine	1.4	1.05	5.16	Up	−0.08	Down
quinidinone	1.4	0.62	0.42	Up	−5.61	Down
cinchoninone	1.36	0.37	−4.84	Down		
lupanine	1.34	0.6	−0.02	Down		
17-oxosparteine	1.34	0.6	−0.02	Down		
PHENOLICS						
glyceollin I/II	1.38	1.06	0	Down		
maysin	1.35	0.56	−3.19	Down		
1-naphthol glucoside	1.38	0.87	0.54	Up		
tetramethylmyricetin/tetramethylquercetagetin	1.32	0.67	0.14	Up		
2′-hydroxy 3,6,7,4′-tetramethylquercetagetin	1.32	0.58	0.53	Up	−0.49	Down
cyanidin 3-O-glucoside-7-O-(6-O-(p-hydroxybenzoyl)-glucoside)	1.31	0.65	4.88	Up	−0.07	Down
cyanidin 3-O-glucoside-7-O-(6-O-(4-O(6-O-(p-hydroxybenzoyl)-glucosyl)-oxybenzoyl)-glucoside)	1.49	0.65	−2.81	Down		
cinnamaldehyde	1.43	0.63	14.91	Up		
beta-D-glucosyl-2-hydroxycinnamate	1.4	0.68	0.48	Up	−0.62	Down
PORPHYRIN						
protoporphyrin IX	1.38	0.35	−8.53	Down		
uroporphyrinogen-III	1.41	0.94	−0.11	Down		
CAROTENOIDS						
3,4,3′,4′-tetradehydroisozeaxanthin	1.41	0.55	6.19	Up		
capsanthin	1.34	0.86	0.12	Up	−0.14	Down
sulcatone	1.31	0.58	0	Down		
caloxanthin	1.34	0.86	0.12	up	−0.14	Down
5,6-epoxy-3-hydroxy-9-apo-beta;-caroten-9-one	1.36	1.08	0.42	Up	−0.01	Down
zeinoxanthin	1.43	1.05	0.24	Up	−0.08	Down
POLYAMINES AND CONJUGATES						
dihydroxyferuloyl-sinapoyl spermidine	1.38	0.54	0	Down	−3.72	Down

acetylspermidine	1.39	0.46				
tyramine	1.69	0.45	0	Up	−7.96	Down
cinnamoyltyramine	1.31	0.88	−4.92	Down		
PTERIDINE						
a 5,6,7,8-tetrahydropteridine	1.35	0.35	5.53	Up		
6-hydroxymethyl-7,8-dihydropterin	1.34	1.25	0.21	Up	−2.88	Down
tetrahydropteroyl-α-glutamylglutamate	1.33	0.8	0.03	Up	−5.91	Down
OTHERS						
L-dopachrome	1.33	0.66	−0.08	Down	−1.06	Down
6,7-dimethyl-8-(1-D-ribityl)lumazine	1.33	0.97	0	Down	−1.73	Down
(R)-pantoate	1.43	0.47	0.54	Up		
dehydroascorbate (bicyclicform)	1.38	0.49	−0.31	Down		
a plastoquinone	1.44	0.82	0.09	Up		
an N-acetyl-D-hexosamine	1.4	0.55	−0.09	Down	−0.82	Down
9-methylthiononylhydroximoyl-glutathione	1.32	0.7	15.32	Up	−4.79	Down
7-methylthioheptyldesulfoglucosinolate	1.31	0.88	5.87	Up		

Compounds were gained through UHPLC-ESI/QTOF-MS metabolomics and selected by OPLS-DA discriminant analysis followed by VIP (Variables of Importance in Projection) analysis. Compounds are grouped in functional classes and provided together with VIP score, VIP score standard error, as well as fold-change analysis.

Table 3 Leaf metabolites discriminating tomato plants under two salinity levels and treated with omeprazole (OMP) at three rates of application.

3.4.5 Principal Component Analysis

To obtain a broad overview on the morphological and physiological changes of greenhouse tomato plants in response to OP application under both saline and nonsaline conditions, the PCA was carried out. The first two principal components (PCs) were related with Eigen values > 1 and explained 85.2% of the total variance with PC1 and PC2 accounting for 70.5 and 14.7%, respectively (Figure 7). PC1 was positively

correlated to nitrate and potassium concentration in leaf tissues, plant height, net photosynthetic rate, yield as well as leaf number and area. PC1 was also negatively correlated to both toxic ions as well as to rs. Moreover, PC2 was positively correlated to Ca^{2+} and Mg^{2+} in leaves, transpiration and root length and root surface, and negatively correlated to RWC basal and apical, rs and K^{+} in leaves and fruit. Furthermore, the score plot of the PCA clearly divided the two nutrient solutions (1 and 75 mM NaCl) along PC1 with nonsaline treatment concentrating most of the plant growth parameters, yield and phosphate, Ca^{2+}, Mg^{2+} in roots and physiological parameters, whereas the saline treatment stands out for toxic ions (Na^{+} and Cl^{-}) (Figure 7). The OP applications were clustered in respect to PC2, with both 10 and 100 µM OP on the positive side of the PC2 that is characterized by improved physiological status, root characteristics, photosynthetic performance, R/S and yield (at 1 mM NaCl) and higher leaf Mg^{2+} as well as lower Na^{+} and Cl^{-} concentrations in both leaves and roots (at 75 mM NaCl; Figure 7).

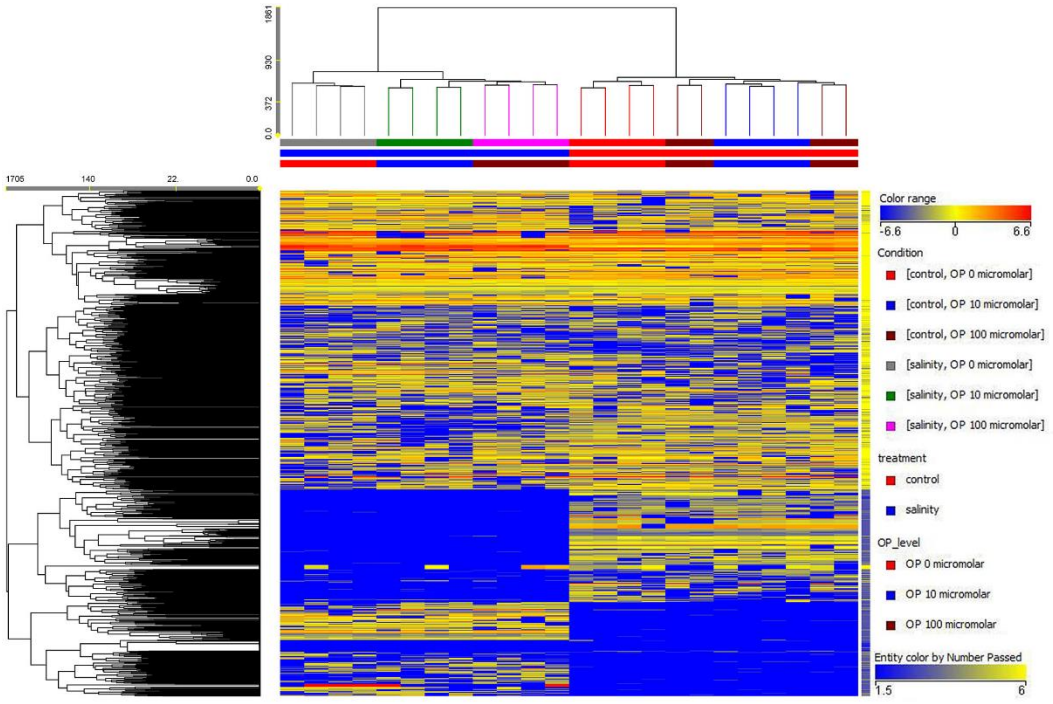

Figure 5 Unsupervised hierarchical cluster analysis of leaf samples from metabolomic profile of tomato plants grown under nonsaline (1mM NaCl) or saline nutrient solution (75mM NaCl), following OP application at three rates (0, 10, or 100µM). Clustering was carried out on both conditions (treatments, vertical dendrogram) and compounds (metabolites, horizontal dendrogram). Dendrograms were built on the basis of fold-change based heat map (similarity: Euclidean, linkage rule: Ward).

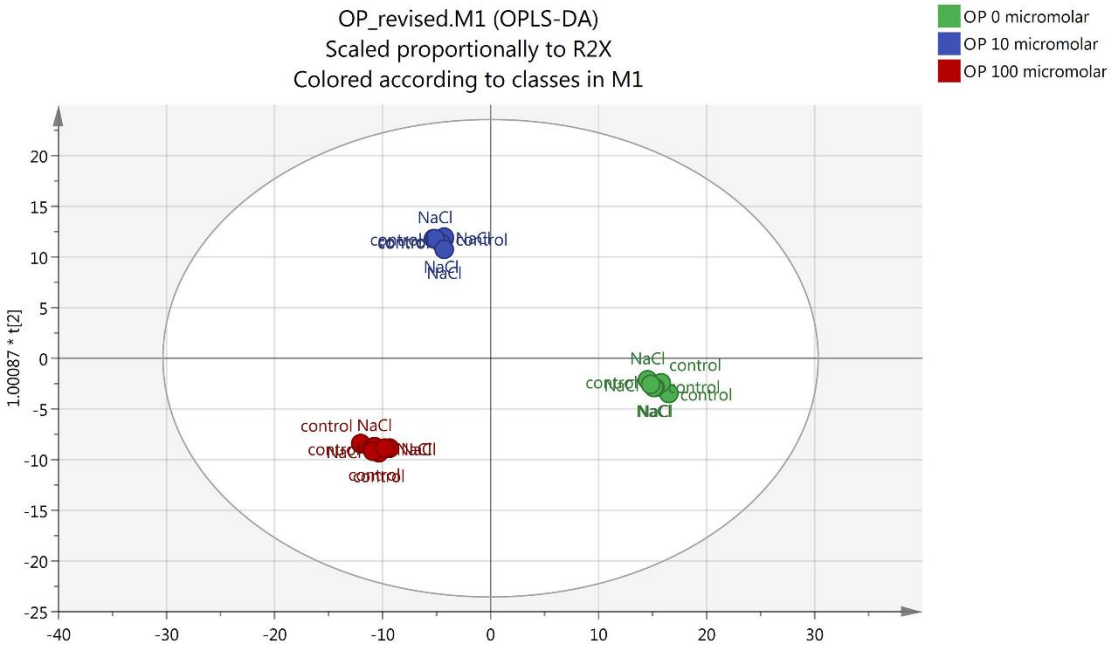

Figure 6 Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) on tomato leaves metabolome from plants grown under nonsaline (1mM NaCl) or saline nutrient solution (75mM NaCl), following OP application at three rates (0, 10, or 100µM). Individual replications are given in the class prediction model score plot.

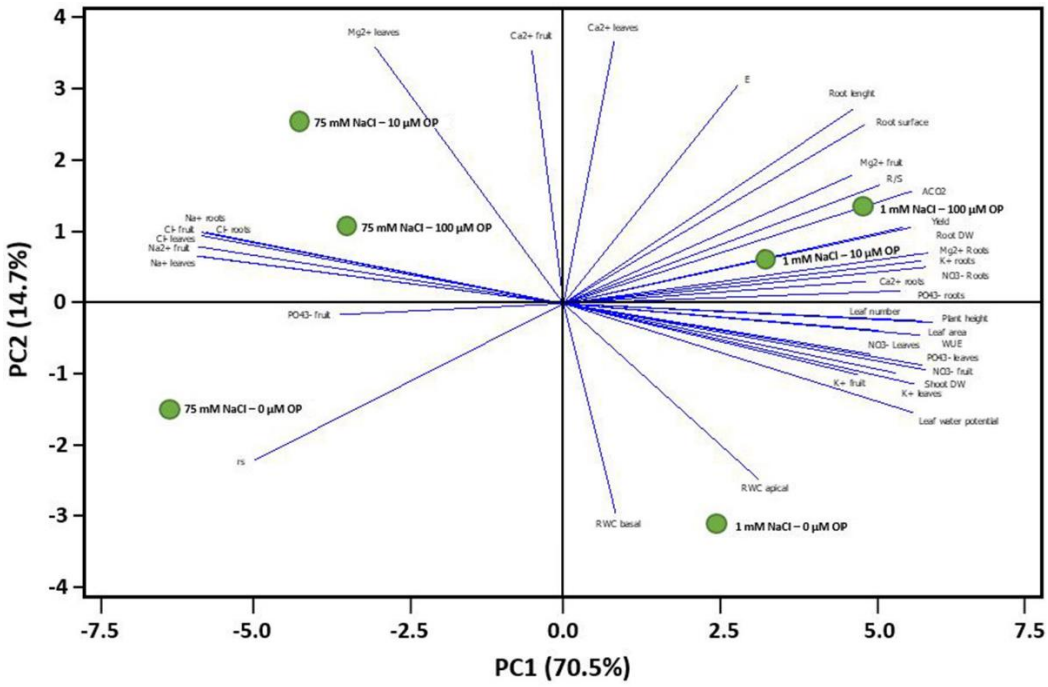

Figure 7 Principal component loading plot and scores of principal component analysis (PCA) of morphological, physiological traits and ion contents of greenhouse tomato grown under nonsaline (1mM NaCl) or saline nutrient solution (75mM NaCl), following OP application at three rates (0, 10, or 100µM).

3.5 Discussion

3.5.1 Implications of Omeprazole for Morphological and Physiological Parameters

Vegetable crops respond to excessive sodium chloride concentration in soil or irrigation water with growth inhibition and yield reduction (Tester and Davenport, 2003), and the severity of crop production losses may fluctuate in relation to several interconnected variables such as cultural environment, genetic material (species and/or cultivars) as well as the concentration, time of exposure, and type of salts (Colla *et al*., 2010). The significant depression of plant growth parameters (plant height, leaf number, leaf area, biomass production) as well as yield reduction with increasing NaCl in the nutrient solution has been reported previously in several greenhouse experiments on potted leafy and fruit vegetables, including tomato (Colla *et al*., 2006; Savvas *et al*., 2011; Rouphael *et al*., 2012, 2016, 2017b; Lucini *et al*., 2016). Furthermore, high concentration of NaCl in the nutrient solution will induce a broad range of biochemical, physiological, anatomical, and metabolic changes such as impairment of root activity, nutrient imbalance, chlorophyll degradation and decrease of the net photosynthetic rate (Munns, 2002; Munns and Tester, 2008), as observed in the present experiment on tomato plants supplied with 75 mM NaCl. Significant decrease in morphological and physiological traits in NaCl-treated tomato plants occurred; and that effect varied in relation to the OP application. However, the positive effects of OP, for example on WUE, were found at the end of the salinity treatment, suggesting a time-dependent action of OP. It is probable that the WUE improved under salinity thanks to another mechanism adopted by plants to minimize water loss even at low rs, that is leaf area restriction, a feature shown by both halophytes and non–halophytes under high salinity (Maas and Nieman, 1978). The expected stimulator effect of OP, a benzimidazole inhibitor of animal proton pumps, on plant growth parameters (i.e., plant height, leaf number and area, shoot dry biomass), previously reported in Van Oosten *et al*. (2017), was not evidenced in the absence of salt stress. However, other morphological and physiological parameters were increased by OP application independently of salinity, in particular root dry weight, root length and surface, R/S, transpiration and photosynthetic net rate. An explanation for

this different response, observed against the former study which was performed in the absence of stress, could be attributed to the different growing conditions, variation between the tomato cultivars employed (determinate type "M82" vs. indeterminate type "Seny") and not least the length of the growth cycle (14 vs. 65 days) as the former study was terminated before the plants reached their reproductive stage. On the other hand, the detrimental impact of NaCl was clearly mitigated when 10 and 100 µM of OP were applied to tomato plants by substrate drench. The improvement of plant growth parameters induced by OP application could be associated with the stimulation of the root system architecture (increased root dry biomass, total root surface, and length), which may improve nutrient use efficiency and total biomass production. The application of OP may have also triggered a signal transduction pathway mediated by endogenous phytohormone (i.e., elicitation of root auxin synthesis), which resulted in significant increase of root length and density, thus inducing a "nutrient acquisition response" that favored nutrient uptake and translocation.

Omeprazole application could be also responsible for the inhibition of swelling-dependent chloride channels (Schmarda *et al*., 2000). The presence of non-isosmotic conditions can alter intracellular and extracellular osmolality, generating a passive flow of water thus causing cell swelling or shrinkage (Sardini *et al*., 2003). In plants, shrinkage is much more dangerous than swelling that is counteracted by vacuolar and cell wall action. When a cell swells, it attempts to restore its original volume by activating channels or transporters in order to release appropriate osmolytes, typically K^+, Cl^- and organic osmolytes. If the osmolyte and/or ion efflux is blocked (OP effect), it is possible that root cells undergo an enlargement of cell volume thus having a positive effect on root characteristics (total root length and surface area).

Another putative mechanism supporting the inductive role of OP in stress tolerance is the higher CO_2 assimilation rate, through: (i) better osmotic adjustment, (ii) improved balance between the uptake and loss of water, (iii) higher efficiency in absorbing macro and microelements from the substrate, thus boosting tomato performance. In addition,

the application of OP may improve photosynthesis by reducing the stomatal resistance, as observed in the current experiment as well as by Van Oosten *et al*. (2017).

3.5.2 Implications of Omeprazole for Ion Homeostasis

The maintenance of ion homeostasis, in which salt overly sensitive (SOS) pathway plays a key role, is a major adaptation strategy against salinity, highly implicated in plant salt tolerance (Soni *et al*., 2013). In the present study, the high concentration of Na^+ and Cl^- in the nutrient solution depressed cation and anion uptake, translocation and accumulation based on the strong decrease of nitrate, phosphate (in both leaf and root tissues) and Ca^{2+} and Mg^{2+} (in roots) as previously reported by Grattan and Grieve (1999) on a wide range of horticultural commodities. It is well established that Cl^- competition for nitrate transporter proteins affects nitrate uptake and transport and reduces the loading of nitrate into the root xylem (Carillo *et al*., 2005, and references therein). Moreover, since nitrate is necessary to induce nitrate reductase (NR), the first key enzyme of the nitrogen assimilation process, decrease of nitrate flux from roots under salinity stress severely affects NR and nitrogen assimilation in leaves (Campbell, 1999). High concentration of Na^+, in turn, impairs not only K^+ translocation from root to shoot but also its uptake by plasma membrane transport (Gao *et al*., 2016). Moreover, Na^+ can depolarize and damage the plasma membrane favoring K^+ leakage, further decreasing the cytosolic K^+ content (Wang *et al*., 2013). When Na^+ concentration is much higher than that of K^+, it can substitute K^+ in key enzymatic reactions and damage metabolic pathways in cytosolic compartments. Therefore, plants are less sensitive to the absolute amount of Na^+ than to K^+/Na^+ ratio (Shabala and Cuin, 2008; Cuin *et al*., 2009). However, salt tolerant tomato plants were able to retain higher Na^+ and Cl- levels in leaves than in roots, suggesting the presence of an active inclusion mechanism in plants as a trait of salt tolerance (Läuchli and Epstein, 1990; Rodriguez *et al*., 2005). In fact, taking into account the high genotypic diversity of tomato plants with respect to ion homeostasis, the more tolerant species/accessions are able to accumulate higher amounts of salts in shoots (leaves and stems), while those more sensitive accumulate salts

principally in roots (Cuartero and Fernández-Muñoz, 1998). It has been ascertained that tomato roots can sense and control the Na^+ concentration reaching aerial parts depending on the intensity of the stress, probably thanks to the SOS pathway (Olías *et al*., 2009).

Accordingly, an intriguing current result was that OP treatment decreased Na^+ and Cl^- concentration in leaves, especially the 100 µM application, and increased leaf Ca^{2+} concentration. However, K^+/Na^+ ratio did not significantly increase in leaves but also in the roots of OP treated plants where K^+ slightly increased. These findings suggest the induction of a salt-tolerance mechanism other than vacuolar sequestration of Na^+, which is a common means for decreasing Na^+ concentration in the cytoplasm, thereby contributing to the osmotic adjustment while maintaining water absorption under salt stress conditions (Silva and Gerós, 2009). Mitochondria and plastids can also sequester some Na^+ contributing to its compartmentalization (Conde *et al*., 2011). At the same time, cytosolic K^+ concentration can be maintained at a constant level or, thanks to the K^+ stored in the vacuole, even increased, osmoregulating the cell and avoiding the impairment of plant metabolism under salinity.

3.5.3 Implications of Omeprazole for the Metabolomic Profile of Tomato Leaves

The metabolomic profile of tomato leaves was clearly affected by the OP treatment as highlighted by both unsupervised and supervised multivariate statistics. Hierarchical cluster analysis, i.e., the unsupervised chemometric approach, evidenced two distinct clusters, comprising NaCl saline stress and nonsaline control. Looking at sub-clusters within main clusters, the effect of OP was still evident, resulting in a mixed cluster in control (comprising both 10 and 100 µM OP) separate from 0 µM OP. However, three distinct sub-clusters could be evidenced in the main salinity cluster representing 0, 10, and 100 µM OP. The former clustering, therefore, evidenced that OP treatment had an impact on tomato leaf metabolomic profile even under nonsaline conditions. This effect became dose related when 75 mM NaCl was applied. On these premises, it can be

postulated that the effect of OP far exceeded the osmoregulation of ions. This is coherent with the fact that OP is known to interfere with P-Type IIC ATPases, a large family of ATP-driven transporters (Shin *et al*., 2009) that have not been reported in planta (Van Oosten *et al*., 2017). As a further confirmation, Na concentration in salinized roots was unrelated to the OP treatment. Interestingly, looking at plants grown in 75 mM NaCl, Na^+ concentration in leaves was reduced by OP in a dose-dependent manner but the K^+/Na^+ ratio was almost unaffected. These findings support the fact that a complex metabolic response might be involved, potentially including hormonal network balance and compounds trafficking.

To investigate further the effect of OP on tomato leaf metabolome, a supervised tool was carried out. It is reported that OPLS-DA is a powerful supervised approach in metabolomics (Worley and Powers, 2013). Indeed, the utilization of class membership in OPLS-DA allows a better separation between classes in score plot hyperspace, while effectively separating Y-predictive variation from Y-uncorrelated variation in X. The VIP score, being calculated as a weighted sum of the squared correlations between the OPLS-DA components and the original variables, is next able to summarize the contribution a variable provides to the model. The excellent OPLS-DA model parameters achieved starting from UHPLC-ESI/QTOF-MS profiles, suggest that differences were actually represented within our dataset.

Hormone compounds were among the most represented in VIP analysis. A complex fine-tuning of plant hormone profiles is occurring under salt stress conditions, as recently reviewed (Ryu and Cho, 2015). Abscisic acid (ABA) is a key enzyme in regulating the response to saline stress since its increase induces stomatal closure, accumulation of osmolytes and growth defects thus ensuring plant survival under salinity. Indeed, ABA precursors accumulated in OP-treated tomato under 1 mM NaCl, suggesting that the treatment might trigger an improved tolerance to salinity. Coherently, a decrease was observed for both auxins (catabolites decreased and an inactive conjugate form increased under 1 mM NaCl) and a cytokinin (a zeatinriboside derivative in 75 mM NaCl). Auxins are known to cause hypersensitivity to salt stress, likely because they

interfere with the salt-mediated remodeling of root architecture (Ryu and Cho, 2015 and references therein). Analogously, cytokinins have a negative role in response to salinity, as their receptors modulate environmental signals and because of their ABA-antagonistic activity (Ryu and Cho, 2015 and references therein). Finally, also a decrease in gibberellins (GA) can trigger salt tolerance, as GA-deficient mutants exhibited enhanced salt stress response (Ryu and Cho, 2015). Notably, GA catabolites were found among discriminant metabolites in our experiments. It must be also pointed out that a complex network of cross-talking enzymes might be considered. As an example, it is known that auxin regulation of GA biosynthesis has a key role in regulating growth between different organs/tissues, and that this aspect relates to survival under salinity conditions (Yamaguchi, 2008). Ethylene is a further hormone known to play a central role in abiotic stress response. Our analytical approach could not detect ethylene, as this is a volatile small metabolite. The brassinosteoid brassinolide was also involved in OP-related response. Brassinosteroids are involved in plant stress response through cross-talking with ABA, and are supposed to have a positive role in stress tolerance via modulation of stomatal conductance (Ryu and Cho, 2015 and references therein). Unexpectedly, the current trend was not consistent with previous findings, as brassinolide down accumulated in 75 mM NaCl treated tomato plants. Nonetheless, the decrease in L-cystathionine (involved in de novo synthesis of ethylene precursor methionine) was found down accumulated in OP-treated tomato leaves under 75 mM NaCl. Although the changes in phytohormonal profile induced by OP deserve further investigation, the above-reported information clearly suggests that OP treatment significantly altered hormonal balance in tomato. Looking at the changes in growth and physiological parameters, these OP-induced alterations might have contributed toward the increase in salt stress tolerance we observed.

Besides hormonal imbalance, lipids were also involved in the response to OP treatment. Interestingly, two cutin-related compounds were up accumulated in leaves treated with OP. Cuticular lipids are reported to be induced by NaCl and drought, with the aim of limiting water losses thanks to their ability to postpone the onset of cellular dehydration

(Kosma *et al*., 2009). Several membrane lipids, mainly glycosylated lipids or phospholipids, were also involved in stress response. The plasma membrane H^+ ATPase pumps play essential roles in signal transduction, cell expansion, stomatal opening, and salt stress response (Sun *et al*., 2010a). These pumps counteract salt activated K^+ efflux, which is mediated by depolarization activated channels and accelerate H_2O_2 production via NADPH oxidases (Zhang *et al*., 2017), thus triggering Ca^{2+} influx (Sun *et al*., 2010b). Subsequently, elevated Ca^{2+} levels activate the SOS signaling pathway (Zhang *et al*., 2017). SOS pathway alters, among others, the cytoskeleton, root architecture and mineral partitioning (Ji *et al*., 2013). The changes observed in membrane lipids might be the result of these membrane related processes occurring under salinity. Analogously, the accumulation in phenolics and carotenoids could be related to the increased H_2O_2 production, with the aim of strengthening the antioxidant capacity of leaves under salt stress. Coherently, the oxidized form of ascorbic acid was found to be down accumulated in OP treated plants under 75 mM NaCl.

Also terpenes and alkaloids are compounds that can be triggered by environmental stress, and by NaCl salinity in particular (Chadwick *et al*., 2013; Lucini *et al*., 2015); among terpenes, sesquiterpenoids are reported to possess antioxidant capacity (Chadwick *et al*., 2013), and therefore might be also implicated in the aforementioned response to oxidative stress at the membrane level.

Spermidine and tyramine polyamine conjugates were also altered by OP treatment. These compounds can be acylated for regulatory purposes likely altering their biological functionality; they are reported to be involved in a wide range of plant developmental processes such as cell division, flowering, and responses to environmental stress (Luo *et al*., 2009). Although their specific role in OP response is still unclear, their recruitment was highlighted in the present results, consistently with previous findings on abiotic stress response (Shi and Chan, 2014; Rouphael *et al*., 2016). The involvement of pteridine, needed for folate biosynthesis, is also not surprising. Indeed, plant metabolism involves a wide range of interconversion and donation of one-carbon (C1) units, through

reactions where folates are essential cofactors. Folates participate moreover in the THF-mediated glycine-serine conversion in photorespiration.

Overall, a very articulated and complex metabolic response was observed in response to OP treatment, involving hormonal balance and cross-talking, membrane processes and oxidative stress there occurring under salinity, as well as a range of other stress elicited chemical compounds. These processes differ from the classical mechanisms through which plants increase tolerance to salinity. Typically, salt-resistant plants possess an improved capacity for H^+ pumping activity, which enables salinized cells to retain K^+/Na^+ homeostasis and avoid ionic toxicity. According to our results, the processes related to OP treatment transcend typical ion homeostasis. Although a single well-defined specific mechanism could not be outlined, a hormone-like activity has been postulated. On this basis, the mechanism(s) through which OP affects tolerance to NaCl salt stress involve different biochemical/metabolic responses. Taken together, these OP-related changes finally end in an improved capacity of counteracting the detrimental processes triggered by salinity.

3.6 Conclusions

Under climate change scenario, the pressure of abiotic stressors and in particular salinity on vegetable productivity is expected to further challenge food security in the coming decades.

Thus, it is important to explore the potential role of small bioactive molecules resourced from human/animal research in increasing vegetable plant tolerance to conditions of salinity. The metabolic profile of plants was found significantly affected by OP treatment, and dose-dependent changes in key metabolites were identified under 75 mM NaCl salt stress conditions. OP was not strictly involved in homeostasis of ions, even though it was able to decrease leaf Na^+ and Cl- concentration under salinity stress. This is in agreement with the fact that plants do not possess P-Type IIC ATPases, i.e., the known target of OP. However, this small bioactive molecule appeared to be involved in

a signal transduction pathway regulating endogenous hormones responsible for the increase of morphological root parameters, and consequently for the "nutrient acquisition response." Hormonal network was significantly affected by OP, eliciting increase in ABA, decrease in auxins and cytokinin, as well as a tendency in GA down accumulation. Furthermore, membrane processes were affected by the OP treatment, involving stimulation of cutin biosynthesis, alteration of membrane lipids and an improved capacity for counteracting radical-mediated oxidative processes via the accumulation of phenolics and carotenoids. Several other stress-related compounds were affected by the OP treatment, including polyamine conjugates, alkaloids and sesquiterpene lactones. Taken all together, OP heightens this essential adaptation mechanism and increases tomato nutrient uptake and allocation, photosynthesis and plant performance under salt stress conditions thus improving resource use efficiency and tolerance to salinity. Although large-scale commercial application of OP to mitigate plant salinity stress might currently not be economically viable, the present findings further corroborate the potential for development of a new class of formulations based on OP analog molecules.

3.7 Published paper front-page

frontiers in Plant Science

ORIGINAL RESEARCH
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Physiological and Metabolic Responses Triggered by Omeprazole Improve Tomato Plant Tolerance to NaCl Stress

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Interest in the role of small bioactive molecules (< 500 Da) in plants is on the rise, compelled by plant scientists' attempt to unravel their mode of action implicated in stimulating growth and enhancing tolerance to environmental stressors. The current study aimed at elucidating the morphological, physiological and metabolomic changes occurring in greenhouse tomato (cv. Seny) treated with omeprazole (OMP), a benzimidazole inhibitor of animal proton pumps. The OMP was applied at three rates (0, 10, or 100 μM) as substrate drench for tomato plants grown under nonsaline (control) or saline conditions sustained by nutrient solutions of 1 or 75 mM NaCl, respectively. Increasing NaCl concentration from 1 to 75 mM decreased the tomato shoot dry weight by 49% in the 0 μM OMP treatment, whereas the reduction was not significant at 10 or 100 μM of OMP. Treatment of salinized (75 mM NaCl) tomato plants with 10 and especially 100 μM OMP decreased Na^+ and Cl^- while it increased Ca^{2+} concentration in the leaves. However, OMP was not strictly involved in ion homeostasis since the K^+ to Na^+ ratio did not increase under combined salinity and OMP treatment. OMP increased root dry weight, root morphological characteristics (total length and surface), transpiration, and net photosynthetic rate independently of salinity. Metabolic profiling of leaves through UHPLC liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry facilitated identification of the reprogramming of a wide range of metabolites in response to OMP treatment. Hormonal changes involved an increase in ABA, decrease in auxins and cytokinin, and a tendency for GA down accumulation. Cutin biosynthesis, alteration of membrane lipids and heightened radical scavenging ability related to the accumulation of phenolics and carotenoids were observed. Several other stress-related compounds, such as polyamine conjugates, alkaloids and sesquiterpene

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CHAPTER 4.

OMEPRAZOLE TREATMENT ELICITS CONTRASTING RESPONSES TO SALT STRESS IN TWO BASIL GENOTYPES

4.1 Abstract

Omeprazole has been shown to act as a plant growth regulator and enhances tolerance to salt stress. In this study, two *Ocimum basilicum* genotypes were tested for their responses to omeprazole (OP) under salt stress. The two genotypes, Napoletano a salt sensitive genotype, and Genovese a salt tolerant genotype, had contrasting responses to omeprazole treatment. Napoletano demonstrated increases in terms of growth (+36%) and salt tolerance (+19%) upon treatment while Genovese had a growth increase (+35%) and OP enhanced sensitivity to salt stress (-13%). OP treatment also had an effect on the post-harvest behavior of these two genotypes by increasing Napoletano shelf life while decreasing Genovese shelf life. The contrasting responses to OP in these two genotypes has provided insight into the role of this molecule in mediating growth and adaptation to stress and, more importantly, into the complexity of the mechanisms mediating these processes.

4.2 Introduction

Improving crop performances in saline environments is becoming increasingly important since climate change and population growth will put unprecedented pressure on agricultural systems and, consequently, will exacerbate salinization of soils and irrigation water (Rengasamy, 2006; Godfray *et al*., 2010; De Pascale *et al*., 2011). Current estimates indicate that 33% of irrigated agricultural land faces salinization problems, with a predicted 10% annual increase in salinization (Shrivastava and Kumar, 2015). Although over the last thirty years we have gained a solid understanding of the key mechanisms that plants activate in response to salt stress (Deinlein *et al*., 2014) and

of those that, if perturbed, make plants more salt sensitive (Flowers and Colmer, 2015; Ali *et al*., 2016). However, we do not have high performance plants that are salt stress tolerant and have passed the field test (Roy *et al*., 2015; Munns and Gilliham, 2015). It is therefore concluded that those tolerance determinants that have been potentiated via traditional breeding programs and/or genetic engineering are not sufficient to confer tolerance, most likely because they are partial components of a more complex stress tolerance system that have not been completely elucidated. In addition to most recent discoveries on epigenetic control of hormonal regulation and abiotic stress responses (Yamamuro *et al*., 2016; Pandey *et al*., 2017) there are additional fundamental mechanisms that are still poorly understood and hinder our ability to enhance salt stress tolerance in plants, including what regulates the dichotomy between stress adaptation and growth and/or the level of growth inhibition under stress (Golldack *et al*., 2014; Mickelbart *et al*., 2015). Although there is a clear and well documented correlation between overexpression of adaptation traits and growth reduction (Maggio *et al*., 2002; Munns and Gilliham, 2015), there are also a few examples in the literature indicating that best performers under stress are those that have a reduced perception of the stress and, therefore, grow relatively well in the presence of moderate root zone salinity (Barbieri *et al*., 2012; Mancarella *et al*., 2016; Orsini *et al* 2012). *Ocimum basilicum* cultivars are grown globally with a large number that vary in morphology, secondary metabolites and flavor (Hiltunen and Holm, 2003). The genetic diversity of basil has only been marginally addressed. It has been demonstrated that there is great genetic diversity within the *Ocimum* family while cultivated *O. basilicum* has less genetic diversity (Vieira *et al*., 2003; Carović-Stanko *et al*., 2010). Two genotypes of basil, predominantly used in Italy (Barbieri *et al*., 2012), Napoletano (NAP) and Genovese (GEN) have different morphology, flavor and, most importantly, tolerance to salinity. Previously, these two genotypes were assessed for their salinity tolerance and NAP was found to be much more sensitive to salinity than GEN (Barbieri *et al*., 2012; Mancarella *et al*., 2016).

Omeprazole (OP) is a member of a family of benzimidazoles that act as proton pump inhibitors (PPI) and affects P-Type IIC ATPases by irreversibly binding and inactivating the P-Type IIC H^+/K^+ ATPase found in the parietal cells of the gut lumen. OP is the most common of the benzimidazole PPI drugs and is on the WHO Model List of Essential Medicines (EML). OP is used to suppress excess acid secretion in the stomach (WHO Expert Committee, 2009). The P-Type IIC ATPases are responsible for moving ions across membranes. Meanwhile plants lack this type of ATPase and rely on the family of NHX-type Na^+ and K^+/H^+ antiporters for plasma membrane extrusion and transport into the vacuole (Hasegawa, 2013). These antiporters are coupled with membrane-bound ATPases that establish proton gradients. While the target of OP is well understood in animals its role in plants is largely unknown. It has been shown that in tomato OP enhances growth and tolerance to salt stress (Van Oosten *et al*., 2017; Rouphael *et al*., 2018). The mechanism of action is the subject of scrutiny and will provide a valuable point of control for modifying growth and osmotic tolerance in plants. Here we report the first evidence that OP functions in a second species, basil, with contrasting responses to salt stress between two genotypes, one sensitive and one tolerant. We also observed alteration of post-harvest shelf life affected by OP treatment. These finding contribute to the understanding of the role of OP in growth and stress tolerance while also elucidating the key mechanisms that basil utilizes to adapt to salinity.

4.3 Materials and Methods

4.3.1 Plant material

Two commercial basil genotypes, Genovese (GEN) and Napoletano (NAP), were sown on two layers of wet paper filter in dark conditions. Germination occurred after 4 days and plantlets were transferred to cellpacks. At 28 Days After Sowing (DAS) plants were transferred to 10 cm diameter pots and regularly watered with tap water. Plants were treated with 1µM OP at 35, 41 and 55 DAS. An initial salt treatment with 100 ml of 100

mM NaCl was given at 41 DAS, 0 Days After Stress Treatment (DAST). A second salt treatment was given at 48 DAS (7 DAST) increasing the NaCl concentration to 200mM. Plants were randomly arranged on the greenhouse bench.

4.3.2 Biometric measurements

At 62 DAS (21 DAST) plants were harvested and separated into leaves, stems, and roots for fresh biomass determination and their tissues were dried to constant weight in a forced-air oven at 80°C for 5 days for the dry biomass determination. The final plant heights, leaf number, plant leaf area, stem diameters (basal and apical) and first and second to last internode length were also measured. The average leaf area was measured with ImageJ (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA) leaf area analysis using ten leaves from each genotype and treatment (Abramoff *et al*., 2004).

4.3.3 Relative Water Content and ion leakage assays

Relative Water Content (RWC) of leaves was performed on 10 mature leaves for each genotype and treatment as per Mancarella *et al*. (2016). Ion leakage was performed on 10 mature leaves in a total volume of 50 ml for each genotype and treatment as per Ishatani *et al*., (1998).

4.3.4 qRT-PCR and gene expression

Gene expression was performed as detailed in Mancarella *et al*. (2016) and analyzed using the Comparative CT method. Primers used in this study are reported in Mancarella *et al*., 2016.

4.3.5 Mineral analysis of plant tissue

Ion measurements were performed according to a procedure described by Van Oosten *et al*. (2017). Plant material was dried and pulverized using a cutting-grinded head (IKA, MF10.1, Staufen, Germany). The powder was extracted in Milli-Q water (Merck Millipore, Darmstadt, Germany) for 10 minutes at 80° C in a thermostatic bath (ShakeTemp SW22, Julabo, Seelbach, Germany) and centrifuged at 6000 rpm for 10 minutes. The samples were filtered at 0.20 µm and stored at -20° C. A Dionex ICS-3000 system (Sunnyvale, CA, USA) equipped with suppressed conductivity detection was used to determine the ion content of the samples. The ion separation was carried out with two different ion-exchange columns: for cations separation an IonPac CS12A column (250 x 4 mm) was eluted with 20mM methanesulfonic acid (flow rate 1 ml/min); anions were separated on an IonPac AS11-HC column (250 x 4 mm) with a potassium hydroxide gradient eluent (flow rate 1.5 ml/min).

4.3.6 Post-harvest experiment

Fourteen basil plants per treatment were harvested and placed in low density polyethylene plastic bags and stored in dark room at controlled temperature (10°C). Cuttings were weighed every three days to quantify water loss and rated visually for the presence of necrosis and/or surface molds. Excess moisture was removed from the bag was removed at weighing. Necrotic leaves have been removed to assess fresh weight loss from each bag. Shelf life was defined as the time until the first obvious sign of deterioration, based on visual analysis only (Lange and Cameron, 1994).

4.3.7 Statistical analysis

Data were analyzed by ANOVA and Least Significant Different (LSD) multiple range comparison tests were used to determine differences between means ($P \leq 0.05$).

4.4 Results

4.4.1 Growth responses induced by OP

GEN plants were more salt tolerant than NAP plants as also reported in our previous findings (Barbieri *et al*., 2012; Mancarella *et al*., 2016). Unlike our previous work, in this study salt stress was induced incrementally and over a longer period in order to study the role of adaptation to salt stress and the influence of OP on these mechanisms. We found that GEN plants subjected to 200 mM NaCl stress showed a reduction of 16% and 1.1 % in leaf area and shoot fresh weight compared to unstressed controls (Figure 1, lower panel; Figure 2), while for NAP we observed a 36% reduction in leaf area and a 36% reduction in shoot fresh weight (Figure 1,upper panel; Figure 3). GEN plants under salt stress did not show any significant reductions in shoot dry weight (Figure 2) while NAP showed a 27 % reduction (Figure 3).

Figure 1 Phenotype of two basil genotypes in salt stress conditions with and without OP treatment. Napoletano (upper panel) and Genovese (lower panel). Controls, 200 mM NaCl, 1 µM Omeprazole, and 200 mM NaCl & 1 µM Omeprazole treated plants (from left to right).

Treatment with OP on unstressed GEN and NAP plants resulted in a growth increase in both genotypes. OP treatment increased GEN leaf area by 31%, shoot fresh weight by 35%, shoot dry weight by 43% (Figure 2). The NAP genotype responded similarly with leaf area increasing by 36%, shoot fresh weight increasing by 26%, and shoot dry weight by 43% (Figure 3).

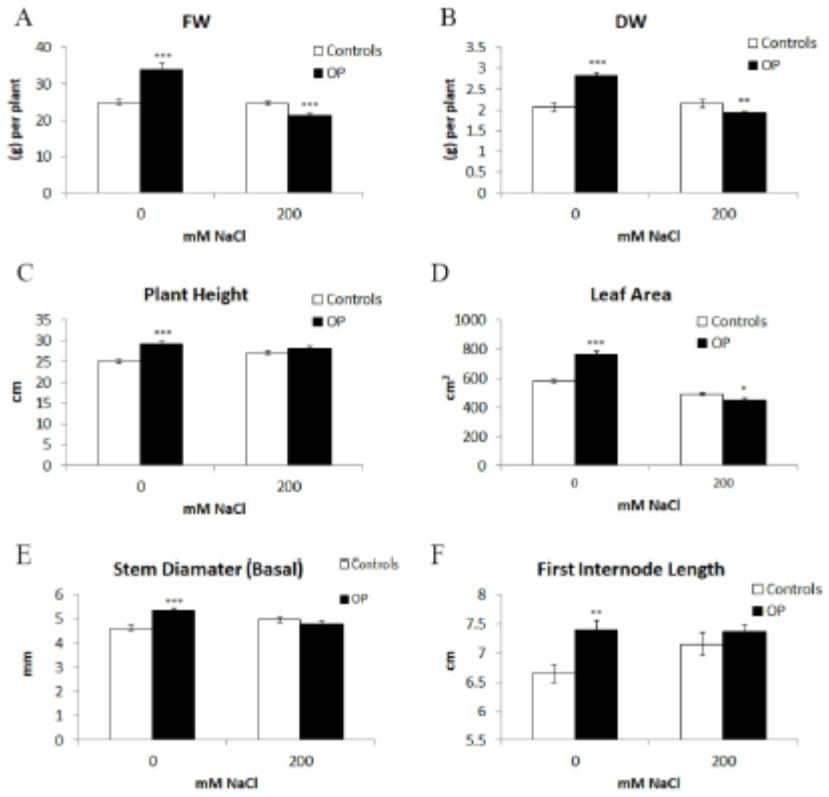

Figure 2 Biometrics of salt stressed Genovese basil plants with OP. (A) Fresh Weight, (B) Dry Weight of shoots, (C) Plant Height, (D) Total Leaf Area, (E) Basal Stem Diameter, (F) Fourth Internode Length. Values indicate average ± SE (n=10). Single asterisks denote significant differences according to Student (P<0.05) between untreated controls and OP treated plants, double asterisks denote (P<0.05) and triple asterisks denote (P<0.01) between untreated controls and OP treated plants.

The two basil genotypes responses to salt stress in conjunction with OP treatment diverged greatly, however. When GEN plants were treated with 200 mM NaCl and 1 µM OP shoot fresh biomass decreased by 13% (Figure 2). The NAP genotype showed a significant increase in salt tolerance upon OP treatment with increases of 30% for leaf area, 19% for shoot fresh weight, and 22% for shoot dry weight over untreated plants

under salt stress. Overall, under salt stress, OP improved plant growth of the salt sensitive NAP genotype, whereas it had a minor detrimental effect on the salt tolerant GEN genotype (Figure 3).

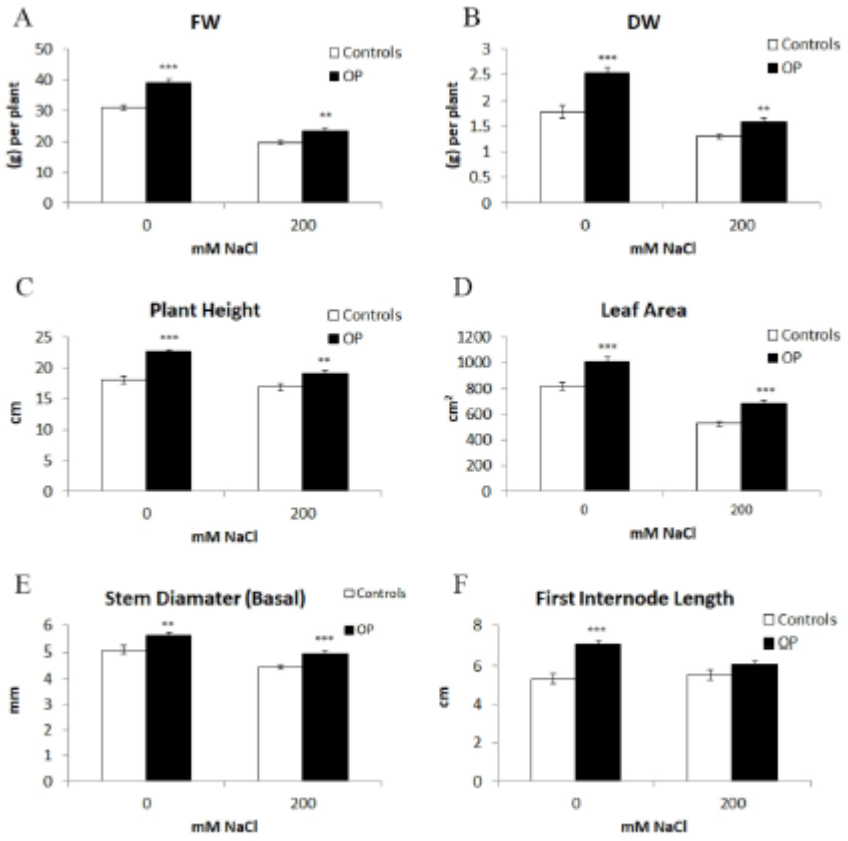

Figure 3 Biometrics of salt stressed Napoletano basil plants with OP. (A) Fresh Weight, (B) Dry Weight of shoots, (C) Plant Height, (D) Total Leaf Area, (E) Basal Stem Diameter, (F) Fourth Internode Length. Values indicate average ± SE (n=10). Single asterisks denote significant differences according to Student (P<0.05) between untreated controls and OP treated plants, double asterisks denote (P<0.05) and triple asterisks denote (P<0.01) between untreated controls and OP treated plants.

4.4.2 Water relations and ion leakage

Genovese plants showed a higher relative water content (RWC) in unstressed conditions, while no difference was observed between GEN and NAP plants under salt stress. However, RWC was affected by OP treatment. When treated with OP, GEN showed no change in unstressed conditions, while the RWC of NAP increased by 8% (Figure 4).

When subjected to salt stress in the presence of OP, both GEN and NAP plants showed a significant increase of 14% and 15%, respectively (Figure 4). This result indicates that OP treatment increases RWC under severe osmotic stress.

Under stress condition, OP induced a strong reduction of the ion leakage level in NAP

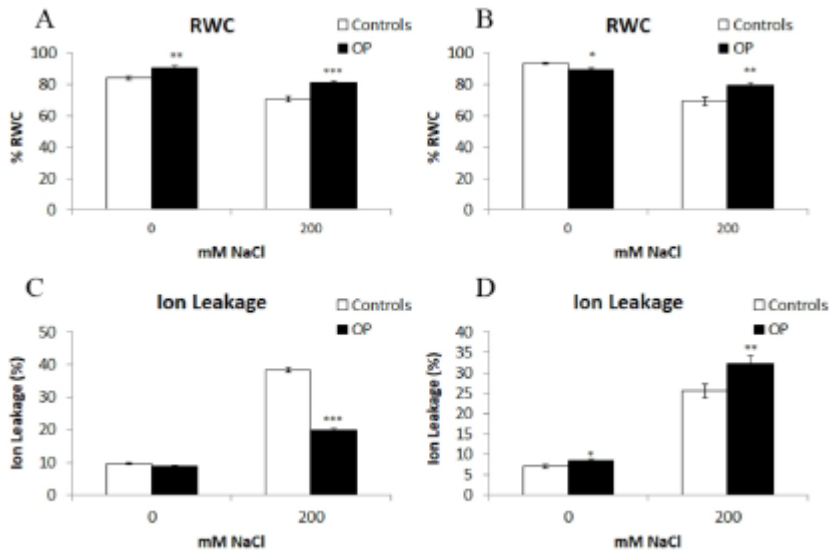

Figure 4 Ion leakage and Relative Water content of salt stressed Napoletano and Genovese basil plants (A) NAP Relative Water content, (B) GEN Relative Water content, (C) NAP Ion leakage and (D) GEN Ion leakage. Values indicate average ± SE (n=10 for RWC and n=8 for Ion Leakage). Single asterisks denote significant differences according to Student (P<0.1) between untreated controls and OP treated plants, double asterisks denote (P<0.05) and triple asterisks denote (P<0.01) between untreated controls and OP treated plants.

4.4.3 Ion content of OP treated plants

OP led to interesting changes on ion content of the shoot of basil plants for both the genotypes used in this experiment. OP reduced sodium concentration under salt stress by 9% in GEN and 20% in NAP (Figure 5A). It is interesting to highlight that the sodium concentration levels in the two genotypes subjected to salt stress without OP was significantly higher in NAP compared to GEN. This result suggests a mechanism missing in the NAP that does not allow this genotype to cope with salt stress as well as GEN does. In line with other results provided in this study, also for other ions concentration OP had a different effect on the two genotypes. Under salt stress, chloride

concentration showed an opposite trend between the two genotypes, with a 23% increase in GEN and a 36% reduction in NAP (Figure 5B). No significant differences were observed in unstressed conditions.

Under salt stress the Na/K ratio also showed a similar contrasting trend, where GEN and NAP showed a 26% reduction and a 15% increase, respectively (Figure 5C). This result is due to the changes in tissue sodium concentration, as well as the changes in potassium concentration. OP led to an increase of potassium in GEN for both unstressed and salt stressed conditions. Potassium increased in unstressed plants by +61% and by +24% in salt stressed plants. Conversely, OP treatment in NAP led to a 30% reduction of potassium concentration under salt stress and no change in unstressed conditions (Figure 5D). While sodium accumulation shows the same trend in both of the genotypes, the differences in potassium accumulation between GEN and NAP explain the different Na/K ratio under OP treatment.

Major changes were observed in nitrate concentration, but again with a contrasting trend between the two genotypes. Strikingly, GEN plants accumulated 2.5 fold more nitrate when treated with OP under unstressed conditions, while no significant difference was found under salt stress (Figure 5E). Similar results have been previously reported on tomato plants treated with OP (Van Oosten *et al*., 2017; Rouphael *et al*., 2018). On the contrary, no significant changes were observed in unstressed conditions in NAP plants, while the nitrate accumulation was reduced of 65% by OP treatment under salt stress (Figure 5E).

Sulfate accumulated in GEN plants when treated with OP with a 116% increase both under unstressed and salt stressed conditions (Figure 5F). As observed for other parameters, NAP plants treated with OP showed an opposite trend in sulfate accumulation, with no differences observed in unstressed conditions and a 31% reduction under salt stress (Figure 5F).

Citrate followed a similar pattern; with OP treated GEN plants accumulating high concentrations in both unstressed (+204%) and stressed conditions (+61%). Conversely

OP treated NAP plants showed a 65% increase in unstressed plants and a 50% decrease in salt stressed (Figure 5G).

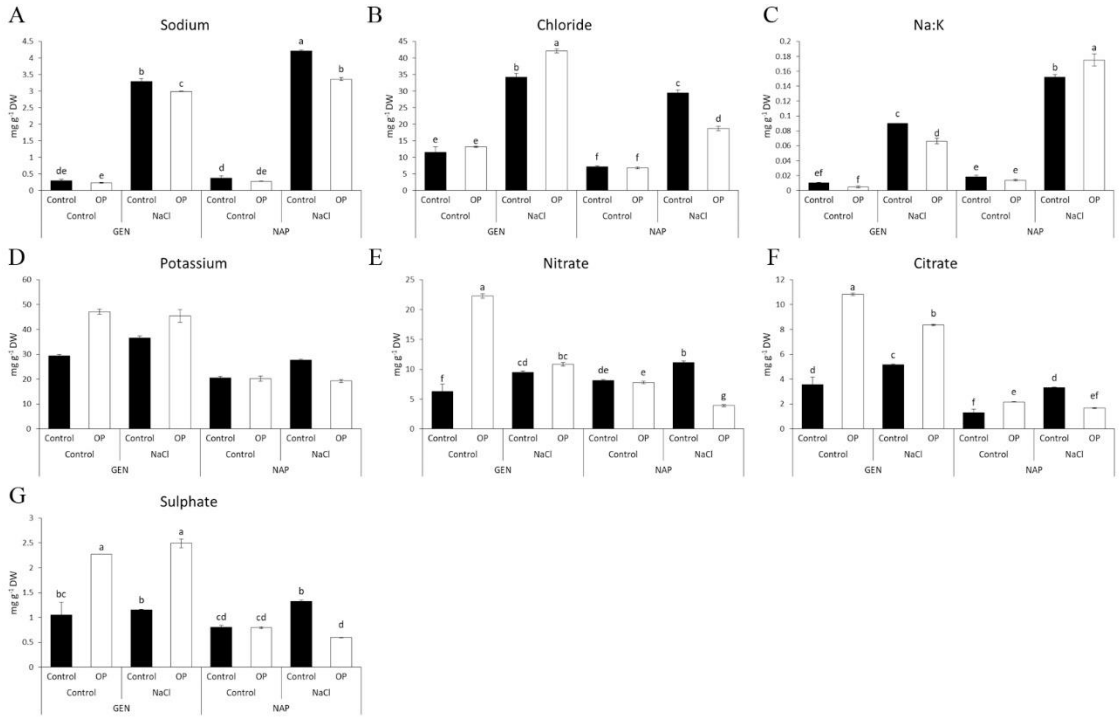

Figure 5 Ion Content of salt stressed Napoletano (NAP) and Genovese (GEN) basil plants. Sodium (A), Chloride (B), Sodium:Potassium Ratio (C), Potassium (D), Nitrate (E), Citrate (F), and Sulphate (G). Values indicate average ± SD (n=3). Different letters indicate significant differences at P ≤ 0.05.

4.4.4 OP induced changes in gene expression

Treatment with OP induced a number of changes in gene expression relating to key stress regulatory genes, stress signaling, and response to reactive oxygen species. Two key markers responsible for the control signal cascades activated in response to abiotic stress (LEA5) and adaptation to salt (STO1) were selected to evaluate responses under OP treatment. The expression of LEA5 was strongly induced in NAP under salt treatment, but similar to unstressed plants and the salt tolerant genotype GEN when treated with OP (Figure 6A). Expression of STO1 in GEN under salt treatment was elevated compared to unstressed plants, as previously observed (Mancarella *et al*., 2016). OP treatment reduced the expression of STO1 in GEN plants under salt stress.

Interestingly, NAP showed a small increase in STO1 expression only under salt stress and OP treatment did induce major changes in expression (Figure 6B). The photosystem reaction center protein B (CP47) involved in stabilization of the D1 protein of reaction center of PSII under stress was highly upregulated in NAP under salt stress with a weaker induction under OP treatment. Expression of CP47 was not induced in GEN plants under salt stress and OP treatment decreased expression in unstressed and salt stressed conditions. (Figure 6 C)

Abscisic acid metabolism was significantly altered under OP treatment in GEN plants. In both stressed and unstressed conditions, OP treatment significantly increased ABA Aldehyde Oxidase (AAO3) gene expression while in NAP was upregulated under salt stress with little or no induction in salt stress and OP treatment (Figure 6 D).

With respect to ROS scavenging, expression of Ascorbate Oxidase (AO) was not induced by OP in either genotype but was strongly induced under salt stress in NAP plants with attenuation in salt stress under OP treatment (Figure 6 E). Expression was decreased in salt stressed GEN with and in the absence of OP.

To evaluate the responses of each genotype to salt stress under OP treatment the expression of three key genes involved in ROS signaling were evaluated. Salt stress drastically increased CAT1 and GST1 expression in NAP however in the presence of OP, the expression of these genes were similar to that of unstressed controls (Figure 6 F and 5G). Interestingly, OP treatment increased GST1 expression in GEN plants in the absence of stress, but to a far lesser degree in salt stressed plants. Ascorbate peroxidase (APX1) was upregulated upon OP treatment in NAP plants in both unstressed and salt stress conditions. Under salt stress GEN drastically downregulated APX1 and to a lesser extent in conjunction with OP treatment (Figure 6H).

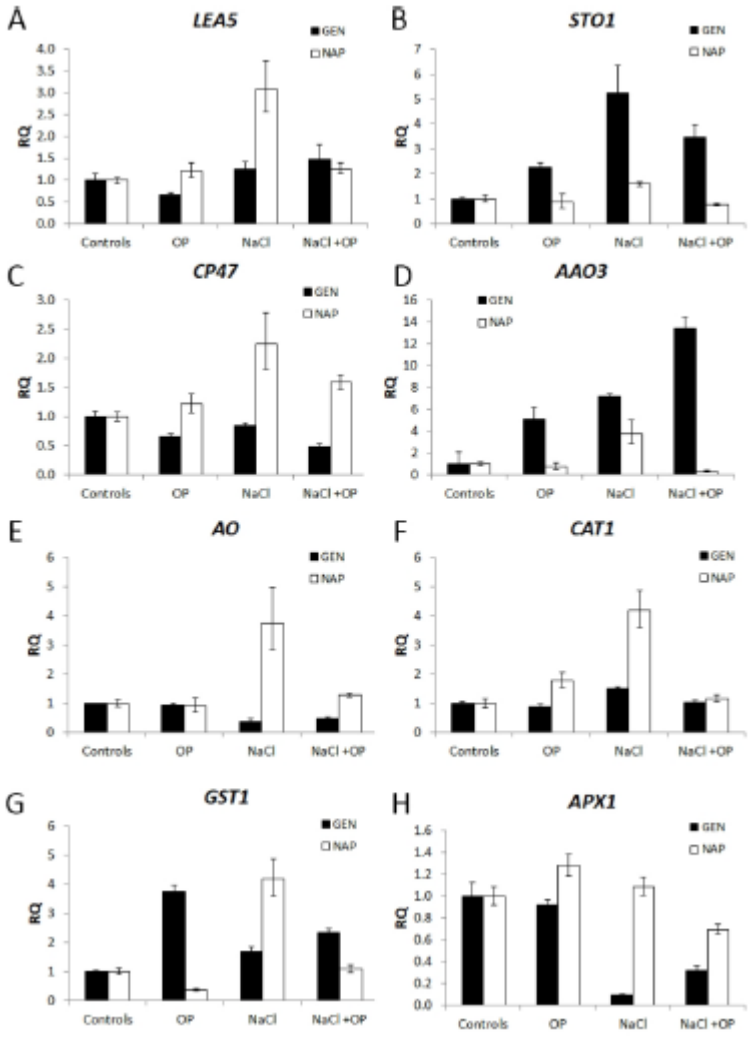

Figure 6 Gene expression of salt stressed Napoletano and Genovese basil plants. LEA (A), STO1 (B), CP47 (C), AAO3 (D), AO (E), CAT1 (F), GST1 (G), and APX1 (H). Relative expression is based on the ΔΔCt Method.

4.4.5 OP effects on post-harvest shelf-life

Basil plants of both genotypes were harvested and shoots were bagged and subsequently stored in the dark at 6 °C. Water loss and number of necrotic leaves were measured over a period of 20 days. Water loss did not vary between treatments and genotypes (data not shown). Overall, NAP showed a shorter shelf-life than GEN with a greater number of necrotic leaves starting at 13 days (Figure 7). OP had a significant effect on the shelf life of both genotypes. OP treated NAP plants had fewer, 55% less, necrotic leaves than

untreated controls at the end of 20 days. GEN treated with OP had an adverse effect, increasing the number of necrotic leaves by 3.3 fold at the end of 20 days (Figure 7). OP treatment had contrasting effects on the two genotypes similar to what was observed in the salt stress experiments (Figure 2 and 3).

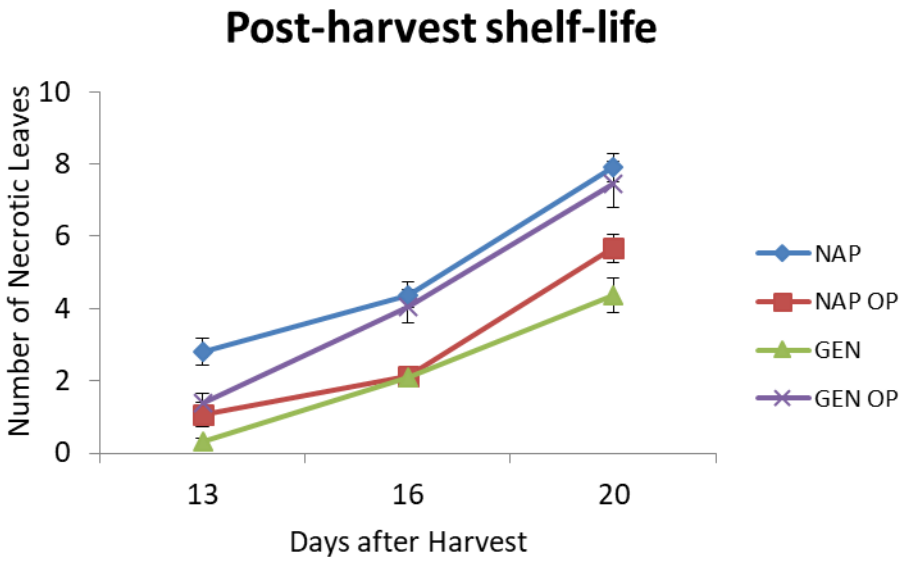

Figure 7 Post-harvest shelf life of salt stressed Napoletano and Genovese basil plants. Number of necrotic leaves. Values indicate average ± SE (n=16). All data points had a P<0.01 between untreated controls and OP treated plants.

4.5 Discussion

The debate on plant abiotic stress tolerance of the past decades has largely focused on four fundamental physiological processes that are critical for determining plant performance under salinity and drought stress conditions: 1) osmotic and ionic adjustments (Seki *et al*., 2007), 2) control of radical oxygen induced injury (Zhu, 2016), 3) general metabolic homeostasis (Weng *et al*., 2016), and 4) growth control (Wang *et al*., 2018). These mechanisms, along with the signal networks controlling them have been the target of most efforts in genetic engineering of crop stress tolerance (Umezawa *et al*., 2006). However, experimental evidence is building up demonstrating that remarkable tolerance to moderate abiotic stresses can be achieved by targeting

mechanisms , and or controlling systems, which turned out to be mostly hormonal based (Wang *et al*., 2018) that somehow uncouple growth and adaptation strategies (Castiglioni, 2008; Miao *et al*., 2018). It has also been demonstrated that successful tolerance mechanisms in plants may rely on a reduced perception of the stress environment and lack of activation of alarm reactions that trigger many stress related responses, including reduced growth (Barbieri *et al*., 2012; Orsini *et al*., 2012; Mancarella *et al*., 2016; Jakab *et al*., 2005; Yang and Guo, 2017) Based on these findings, it seems critical to understand how growth and stress adaptation are functionally connected. We have recently demonstrated that feeding omeprazole (OP) via root systems enhances plant growth (in the absence of salinity) but also improves salinity (Van Oosten *et al*., 2017, Rouphael *et al*., 2018) stress tolerance. Considering that we observed these responses under micromolar treatments, we attributed to this molecule a signaling and/or hormonal action that could be of interest in exploring the relationship between plant growth and stress adaptation (Savvides *et al*., 2018). While our previous work focused on the shock of salt stress, in this study we used plants that were adapted to salt stress over a long period. OP is a benzimidazole used in humans as a potassium ATPase inhibitor (Wallmark, 1968). In plants, it has been proposed that OP plays a role in partitioning and sequestration of toxic ions accumulated during salt stress. The two basil genotypes, GEN and NAP, use differing strategies to respond and adapt to salt stress with GEN being better able to accumulate and partition sodium in saline conditions (Figure 7) (Barbieri *et al*., 2008; Mancarella *et al*., 2016). NAP appears to be more sensitive due to less efficient mechanisms involved in salt stress adaptation. Based on previous results (Van Oosten *et al*., 2017), we hypothesized that OP treatment would increase the salt tolerance in basil by augmenting salt stress adaptation mechanisms present in both basil genotypes. Interestingly, while OP treatment increased growth, only the salt sensitive genotype, NAP, responded positively to OP treatment under salt stress. The highly tolerant GEN genotype became more sensitive to salt stress when treated with OP. This was reflected in lowered FW and DW (Figures 1, 2, and 3) and increased ion leakage of OP treated GEN plants (Figure 4). In contrast, NAP became more tolerant with lowered ion leakage and more extensive growth under salt stress. NAP

demonstrated a greater ability to exclude chloride and sodium ions under OP treatment while GEN accumulated less sodium but more chloride (Figure 7). Previous experiments with these two genotypes and rapidly induced salt stress showed high levels of sodium accumulation with NAP accumulating more than GEN in early stages of salt stress while GEN accumulating an overall higher quantity of sodium after prolonged stress. In this study, incremental salinization and a longer period of adaption to salinity resulted in lower levels of accumulated sodium, with NAP accumulating more sodium than GEN (Mancarella *et al*., 2016). Interestingly, OP treatment augmented GEN's ability to uptake potassium, but this increased accumulation did not correlate with increased salt tolerance. The ability of the GEN genotype to tolerate salt stress seems to be due to compartmentalization of toxic ions rather than balancing with potassium or exclusion from the cell (Hasegawa, 2013; Flowers *et al*., 2015). This strategy of adaptation to salinity has been observed in a number of halophytic species (Munns and Tester, 2008). While OP treatment increased salt tolerance in NAP, it did not enhance potassium uptake which indicates that different mechanisms of adaptation may exist in this genotype (Figure 7). The NAP genotype's sensitivity to salt could be due to a different perception of salt stress compared to GEN (Shabala *et al*., 2015). Alternatively, NAP may be impaired in the regulator mechanisms that muster salt stress responses. Transcription factors from the DREB/CBF, AP2/ERF, and bZIP families may be differentially regulated between the two genotypes and resulting metabolic adjustment in response to salt stress (Golldack *et al*., 2014). However, as the vast majority these genes remain functionally uncharacterized in basil, further study is warranted.

The phenotype observed under OP treatment indicates that the NAP genotype's sensitivity may be due to its inability to exclude chloride from the cytoplasm. Induction of salt tolerance in NAP by OP treatment was not the result of higher exclusion of sodium or increased uptake of potassium, but instead the greater capacity to exclude chloride. The "Siam Queen" genotype of sweet basil demonstrated reductions in growth from $CaCl_2$ similar to NaCl, indicating that Cl^- alone can induce salt stress (Scagel *et al*., 2017). Exclusion of chloride from the cytosol is essential for avoiding adverse effect on

the metabolism (Geilfus, 2018). The exclusion is maintained by sequestration in the vacuole or extrusion across the plasma membrane (Flowers *et al*., 2015). Critically, loading of chloride into the root xylem is a key point of control in salt stress. Transport and loading of chloride is tightly regulated and driven by proton ATPases establishing proton gradients used by Cl^- symporters to transport Cl^- out of the cytosol (Li *et al*,. 2017). The NAP genotype appears to retain the mechanisms present in GEN that support adaptation to high levels of sodium but appears to lack regulator mechanisms that compensate for increased chloride in the environment. While the regulation of Cl^- transport is not nearly as well as understood as Na^+, the ability to transport and accumulate Cl^- in basil merits further study.

Omeprazole treatment also affected the post-harvest shelf-life of both genotypes. The GEN genotype typically has a longer shelf life than NAP and this has contributed to its commercial success (Figure 5). OP treatment decreased the number of necrotic leaves in NAP while increasing the decay in GEN, shortening the shelf-life. This change in phenotype is likely due to the level of reactive oxygen species and their scavengers. OP treatment in GEN increased ion leakage over untreated controls (Figure 4) which is indicative of ROS accumulate induced by stress (Savvides et a., 2018). Glutathione-S-transferase1 (GST1) was upregulated four-fold in OP treatment GEN plants while expression was down regulated in NAP (Figure 6). GST1 in carnation has been shown to be linked with ethylene biosynthesis and plays a role in senescence (Maxson and Woodson, 1996; Tripathi and Tuteja, 2007). While increased GST1 expression in GEN was not sufficient to protect against ROS generated during senescence, it may indicate elevated ethylene levels as GST1 is induced by ethylene during senescence (Marrs, 1996; Keunen *et al*., 2016). The increase in necrotic leaves observed during the shelf-life assessment may be due more to dysregulation of senescence rather than excess ROS generation as APX1 and CAT1 were not significantly dysregulated in GEN under OP treatment

While the targets of OP in plants are still unknown, the beneficial effect on plants is becoming more evident. The contrasting responses of two genotypes, one tolerant and

one sensitive, indicate that the cellular and physiological responses activated are determined at the genetic level. A few potential candidates have been previously identified, but further characterization of these two genotypes will offer a better understanding of the mechanisms at work. It remains to be seen if the observed results in basil between contrasting genotypes are also present in other species. *Arabidopsis thaliana*, a glycophyte, and its close euhalophyte relatives Eutrema salsugineum and Thellungiella parvula are the ideal model species that can be used to answer this question (Dassanayake *et al*., 2011; Wu *et al*., 2012).

Here we have reported a second species, basil, which demonstrates growth enhancement and increased salt tolerance in response to OP treatment. Interestingly, while this confirms the elicited phenotype we previously observed in tomato, we have determined that highly salt tolerant genotypes may not benefit from OP treatment under salt stress (Van Oosten *et al*., 2017). Our results also seem to indicate that the salt sensitive genotype NAP has the capacity to tolerate sodium, but not chloride. These findings will help in the identification of the cellular and molecular targets of OP and assist in characterizing the mechanisms involved in salt tolerance.

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CHAPTER 5.

OMEPRAZOLE INDUCES CHANGES IN ARABIDOPSIS ROOT SYSTEM ARCHITECTURE

5.1 Abstract

A major topic in plant science is understanding the signaling network that induces changes in root morphogenesis, since this leads to modifications of various aspects of plant development and growth. Roots act as plant sensors for the external environment,, and can mediate specific developmental changes Therefore, knowing those drivers that can change root morphology is pivotal. Results from previous experiments have shown that Omeprazole (OP) induced a remarkable increase of the volume of the tomato root apparatus. However, these results did not unravel specific morphological root traits (e.g. lateral/adventitious root induction, primary root elongation) involved in the observed OP effect, which would be important to define the functional link between root morphology and stress tolerance phenotypes. For this reason, we performed a series of experiments at the Purdue University (Indiana, USA) with the aim to quantify the effect of OP on root morphology.

5.2 Introduction

Uncovering root anatomy and architecture regulation is one of the most exciting challenges in plant biology. Roots are the first sensors for soil nutrients in plants, as well as biotic and abiotic stresses. For this reason, it is expected that the first modifications arising in plants in a continuously changing environment from where they cannot escape will occur in roots (Deak and Malamy, 2005).

Variations in root architectures can lead to different plant performance, both under favorable and unfavorable growth conditions. Indeed, the most plastic trait in plants are roots, that driven by environmental cues may regulate growth speed and direction as

well as to the position of later roots initiation. The main goal of root branching is to improve water and nutrient uptake while allowing soil root anchoring (Tian *et al*., 2014).

Being able to shape root architecture in order to improve plant performance is an essential aim for the future, because of the never-ending increase of inhospitable environments for plants all over the world (Rengasamy, 2006). However, few research groups tried to uncover the way it can be achieved using small bioactive molecules as specific triggers of root branching.

The use of small bioactive molecules is a promising approach to induce changes in plant growth. These molecules can exert various effects on plants, both beneficial (growth regulators, hormone-like activity) and detrimental (herbicides). The search of the biological targets of a molecule can be an important step to increase our knowledge of many biological events occurring in plants (Kaschani and der Hoorn, 2007).

Previous experiments on tomato showed an effect of Omeprazole (OP) on root biomass (Van Oosten *et al*., 2017; Rouphael *et al*., 2018). The inhibitor effect of OP on primary root elongation has been recently shown in *Arabidopsis thaliana* (Okamoto *et al*., 2018) and *Allium cepa* (Braga *et al*., 2018). However, no mention has been done on the specific changes induced by OP on root system architecture (RSA). For this reason, the main goal of the experiments performed has been to test the ability of OP to induce the formation of lateral and adventitious roots, as well as to confirm recent findings on primary root shortening.

5.3 Materials and methods

5.3.1 Plants growth conditions

Arabidopsis thaliana seeds were surface sterilized with 30% bleach plus Tween-20 for 7 minutes, and then rinsed 5 times with distilled sterile water. The seeds were stratified for 2 days at 4°C. For root traits evaluation, plantlets were grown on solid media made of 4.33g/L of Murashige & Skoog Basal Salt Mixture and 1% sucrose. The pH of the media

was finally adjusted to 5.7 using KOH. Seedlings were grown vertically under continuous light in a chamber set at 23°C. To evaluate the effect of Omeprazole (OP) on root traits, seven plantlets per plate were transferred to MS and MS+OP plates at three days after the germination (DAG). OP was added after autoclaving at a concentration of 1 and 10 μM.

5.3.2 Determination of root traits

Root traits were evaluated at 4 (D1) and 6 (D2) days after transfer (DAT) with SmartRoot, an ImageJ plugin (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA). Total lateral root length represents the sum of the length of all the lateral roots per plants. The total root length apparatus is the sum of the primary and the lateral roots length per plant.

5.3.3 Measurement of shoot biomass

At 6 DAT shoot fresh weight was evaluated according to Roycewicz and Malamy (2012), using chlorophyll shoot content to estimate shoot biomass ($r^2 = 0.904$, $y = 21.38x$). The result is expressed as absorbance at 430nm, the wavelength at which the samples were analyzed on the plate reader.

5.3.4 Statistical analysis

Data were subjected to a two-way analysis of variance (ANOVA) using the SPSS 10 software package. Treatment means within each measured parameter were separated by Duncan's multiple range test performed at a significance level of $P \leq 0.05$.

5.4 Results

The effect of OP on various RSA's traits has been evaluated. On primary root, 10 μM OP induced a reduction of length, while 1 μM did not exert any effect, both at D1 and D2. This is in agreement with Okamoto *et al*. (2018) that showed a similar reduction in primary root length starting at 10 μM.

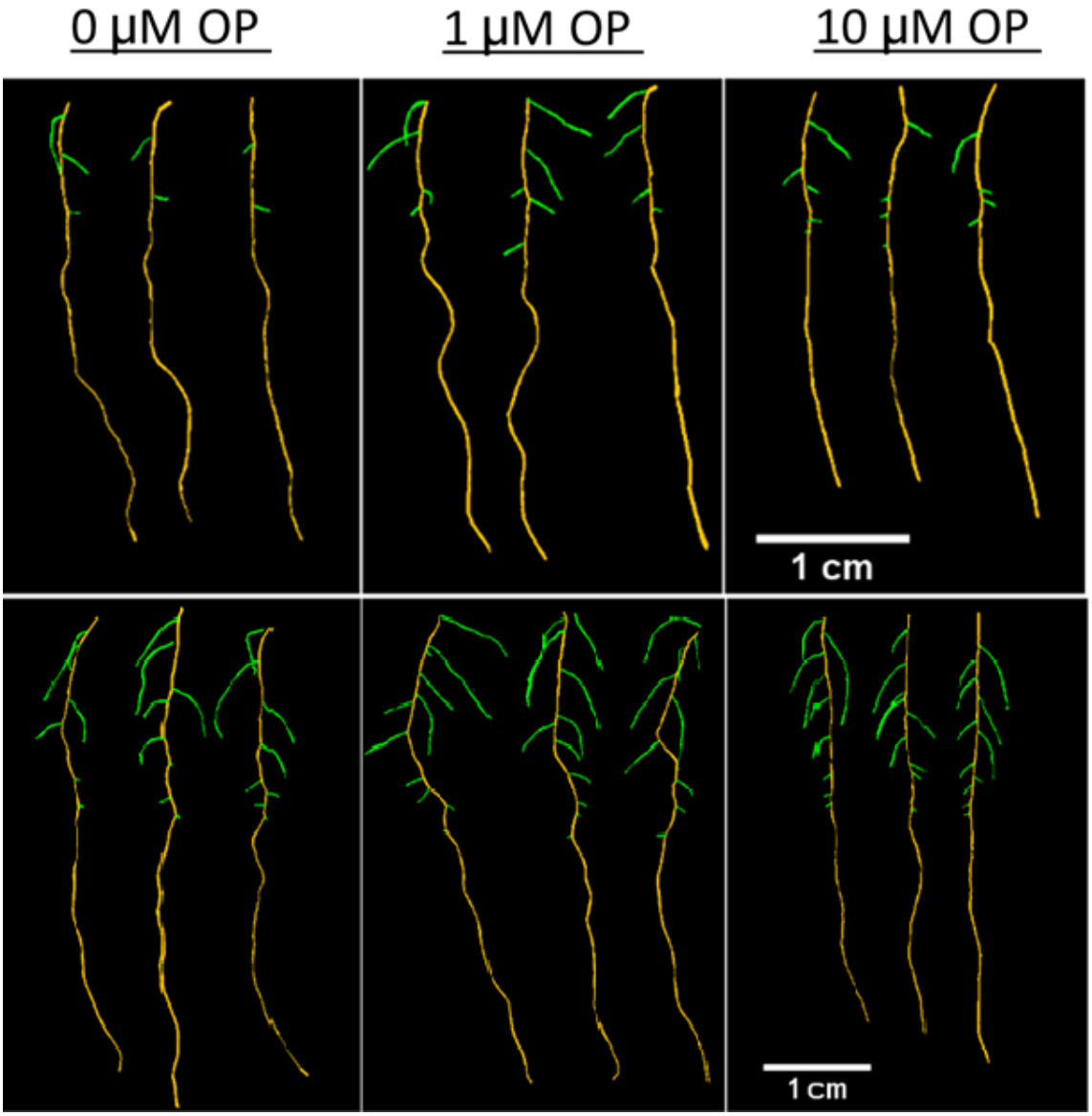

Figure 1 Output of SmartRoot plugin of ImageJ at D1 (top) and D2 (bottom) showing three representative plantlets per treatment. OP clearly induces the emergence of lateral roots. At 10 μM, OP inhibits primary root elongation.

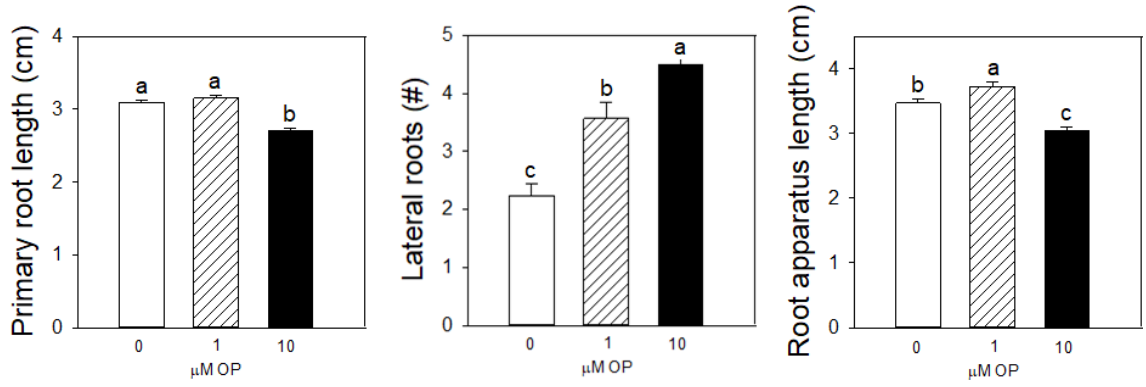

Figure 2 Quantification of the main RSA traits at D1. Different letters indicate significant differences according to Duncan's test (P < 0.05). The values are the means of 35 replicates. Vertical bars indicate ± SE of means

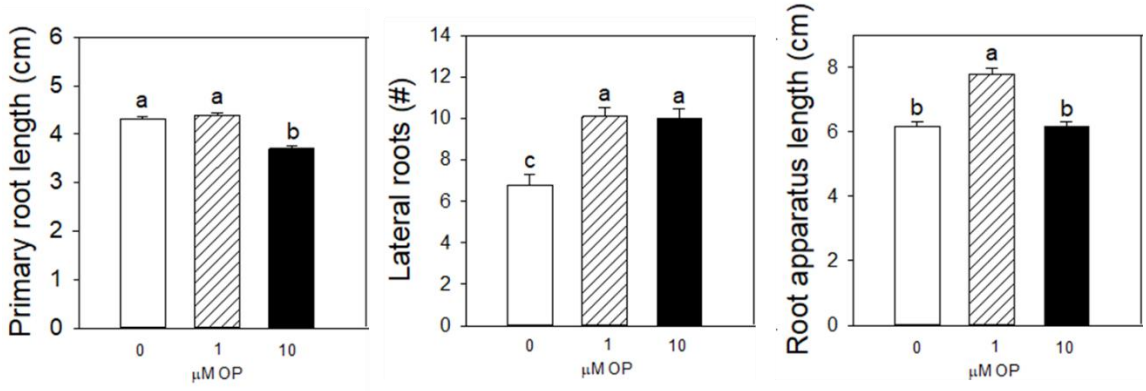

Figure 3 Quantification of the main RSA traits at D2. Different letters indicate significant differences according to Duncan's test (P < 0.05). The values are the means of 35 replicates. Vertical bars indicate ± SE of means.

The main result of this experiment is the effect of OP on lateral root formation. OP strongly induced lateral root formation both at D1 and at D2. For this reason, total root length was longer in plants treated with 1 µM OP. In contrast, at 10 µM OP there was no difference in this trait when compared to the control. This was caused by a trade-off between primary root length and lateral roots formation.

Finally, shoot biomass was higher in plants treated with 1 µM, while at 10 µM, no difference has been found when compared to the control.

5.5 Discussion

In this experiment, OP had a significant effect on RSA of Arabidopsis plants grown in vitro. Previous experiments reported a reduction of the primary root length when plants were treated with OP concentrations higher than 10 μM. However, the authors did not evaluate the differences that OP treatment exerted on the RSA. The most affected trait by OP was the number of lateral roots which increased significantly in OP treated plants. OP seemed to anticipate lateral roots emergence when compared to the control, resulting in a bigger root apparatus.

Shaping plants root architecture and understanding how root systems develop is critical to improve plant resource acquisition and eventually plant yield (Lynch, 2013). In fact, different RSA can lead to different use of water and nutrients in the soil, resulting in completely different outputs concerning growth and tolerance to stress (Uga *et al*., 2013). Considering that the fluctuating environment in which plant have to survive and develop causes changes in RSA, it is fundamental to understand the physiological and molecular mechanisms involved in lateral root formation (Giehl *et al*., 2013).

OP effects on RSA in Arabidopsis can be functionally used to unravel the biology of lateral root emergence and the relationship with environmental adaptation. The next planned experiments on this topic will be to investigate a functional correlation between different RSA and different levels/types of stress, including osmotic, ionic and nutritional.

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General conclusions

An increasing global population puts a greater emphasis on food security in the modern era. Food security is affected by environmental fluctuations that can drastically decrease crop yields. For this reason, the scientific community is employing all the tools currently available to develop plants and methods that can be utilized in a robust and sustainable system, less sensitive to severe yield losses in response to environmental changes. However, even if many strategies have proven to be effective in achieving this goal, agriculture still lacks crops that have stable yields under fluctuating environmental conditions. Synthetic molecules and complex mixtures like "biostimulants" have been shown to prime stress tolerance or increase tolerance to abiotic stresses. The mechanisms responsible for these increases in stress tolerance remain largely unknown, either due to the complexity of signal perception for small synthetic molecules or a lack of interest in their specific chemical composition in the case of biostimulants.

For this reason, the small bioactive molecule approach can shed light on previously obscure aspects of plant stress tolerance. Medicine and agriculture offer a plethora of small bioactive molecules that have the potential to affect plant responses to abiotic stress. This approach can further give information on the evolution of mechanisms in common between different taxa.

We choose to test an ATPase inhibitor in humans on plants, seeking to determine if a shared mechanism existed in plants. Omeprazole is one of the most used and studied drugs in human science, able to inhibit gastric acid production and giving relief against different gastric illnesses. Its ability to affect plants, would be of high interest since plants does not possess the same sub-class of P-Type ATPases. Our work has demonstrated that omeprazole has a bioactive effect in plants. Furthermore, in plants omeprazole enhances tolerance to environmental hyperosmotic stress. Our results show that this molecule is bioactive at very low concentrations (two order of magnitude lower than in humans) and acts as an inhibitor of growth a high concentrations (greater than 50µ). Omeprazole enhances tolerance to osmotic stress through alterations of ion composition and transport, secondary metabolism, and gene expression. Omeprazole

also can alter developmental patters at a macro level through changes root morphology. Treatment can also increase yield in solanaceae plants. We have demonstrated that omeprazole is bioactive in more than one species, with similar results observed across taxonomic orders. The biochemical target of omeprazole represent a key point of control that could be used to enhance growth and yields as well as make plants more tolerate salt stress. The target also represents a potential subject for gene editing and chemical genetic screening for natural homologues. Considering the high efficacy of this molecule that promotes growth and stress tolerance, all of the above applications have great potential for agriculture and enhancing our understanding of plant biology.

The novelty of this PhD thesis, in conclusion, is using molecules with known biological activity in non-plant species and evaluating their ability to alter plant metabolism. This "small bioactive molecule" approach has been quite successful in yielding a new molecule for use in plants that acts at hormonal concentrations and has lasting effect on plant metabolism. However, more experiments have to be performed to identify and characterize the target of omeprazole in plants.

Appendix

Salinity is one of the most critical constrain of crop yield, due to the osmotic and ionic stress induced by sodium and chloride on plants. The 20% of cultivated lands worldwide are affected by salinity, and this phenomenon is expanding at an annual rate of 10% (Shrivastava and Kumar, 2015). Salinity induces, at the soil level, a reduction in soil hydraulic conductivity, surface crusting, soil structure degradation, leading to losses of arable lands (Warrence et al., 2002). The classification of soil salinity classes based on the electrical conductivity (EC) of the soil extract is reported in Table 1.

Salinity Class	EC (electrical conductivity)
	dS m^{-1} or mmhos cm^{-1}
Nonsaline	0-2
Very slightly saline	2-4
Slightly saline	4-8
Moderately saline	8-16
Strongly saline	>16

Table 1 Classification of soil sainity according to USDA (Scianna, 2002).

However, each plant specie has a specific tolerance to salt stress, summarized by Maas and Hoffman relationship in 1977 (Figure 1).

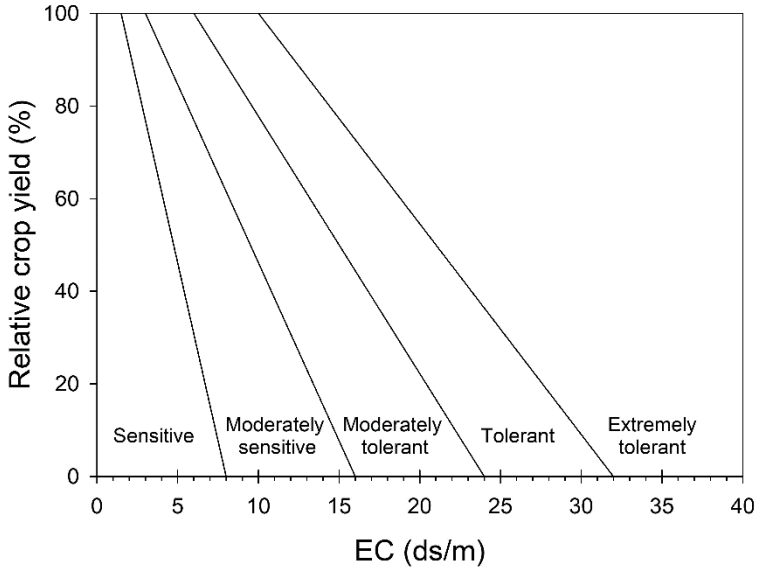

Figure 1. Boundaries of salinity tolerance categories, from sensitive to extremely tolerant, obtained based on the Maas and Hoffman relationship (1977) (Tanji and Kielen, 2002).

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