HORMONAL REGULATION OF TOMATO RESISTANCE TO COMBINED SALT AND POWDERY MILDEW STRESS

MASTER THESIS

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Thesis Report

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ABSTRACT

Three tomato Ol lines,; Ol-1, ol-2 & Ol-4, which carry resistance genes to powdery mildew, and their crosses with hormone mutants, epinastic (ethylene overproducer), notabilis (ABA deficient) and def (jasmonic acid deficient) were evaluated for combined resistance against powdery mildew and tolerance to salt stress (50 mM). The growth habit and senescence of those tomato genotypes was monitored in both treatments. The Ol-gene x mutant combinations were segregating F_2 and F_3 plants and homozygous lines of those genotypes were selected using PCR marker assays. The disease index measurement after 10 and 15 days post inoculation showed that Ol-1 was highly affected by salt stress while differential response was observed in its mutants. Ol-1 x notabilis showed reduced susceptibility and senescence, while Ol-1x epinastic showed higher susceptibility. Ol-2 resistance was slightly broken in Ol-2x notabilis and Ol-2x epinastic combinations. Ol-4 resistance was not affected by any treatment and mutant combinations. Fifteen genes which have important role in biotic & abiotic signalling pathways related to ROS signalling pathways, ABA synthesis and the plant defense hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways were used for gene expression analysis in this study in the Ol-1 hormone mutant combinations. The PAL and ICS genes were downregulated during combined PM and salt treatment which probably leads to SA reduction and triggers more disease susceptibility and senescence. Expression of the JA marker gene was higher under combines stress and as SA and JA interact antagonistically this may contribute to senescence. Lower RBOH expression found in Ol-1 genotypes potentially affected HR resulting plant susceptibility under stress conditions. Increased expression of CKX1 was found in the Ol-1 line indicating less cytokinin produced, which ultimately may trigger increased senescence. Further research on these genotypes will help to find specific genes that are responsible for the different responses under combined powdery mildew and salt stress.

Key words: Powdery mildew, Gene expression, Hormones, salt stress and Ol lines

1. INTRODUCTION

Tomato (*Solanum lycopersicum*) is considered both a vegetable and a fruit, and grown all over the world. It is popular due to its natural abundant source of vitamin C with other vitamins and a special carotenoid pigment lycopene, that has anti-oxidant ability helping against cancers and heart disease. China is the highest tomato producing country in the world followed by India. On the basis of its public demand, tomato is ranked the fourth largest vegetable crop in the world (http://faostat.fao.org). Besides its economic and nutritional importance, the diversity of natural genetic resources and easy genetic manipulation make the tomato as an excellent species for plant genetic analysis (Gupta et al. 2009). It is diploid species and member of the *Solanaceae* family, has a small genome (950 Mb) with a short period of life. Tomato is cross-pollinated, can tolerate inbreeding and maintaining related to breeding is easy. For the above attributes of tomato, it opens the doors of intensive breeding by marker assisted based genetic research and analysis of specific gene function.

1.1 Salinity stress

Soil salinity is a remarkable issue to crop production all over the world. Due to increase in saline area all over the world and occupation of cultivated land every year, soil salinity is considered as a very important abiotic stress for crop production. The saline soil occupies more than 6% of the total arable land which is more than 800 million hectare land area of the world (FAO. 2005). Every year about 2 million ha (1%) of arable land is affected by salt (FAO. 2005). Sodium chloride (NaCl) is the most abundant salt which is also highly soluble. Normally, soil is considered as saline when Electrical Conductivity (EC) of the soil is minimum 4 dS/m, which is equivalent to 40 mM NaCl that creates 0.2 Mpa osmotic pressure. Salinity stress in plant affect plant growth in two ways: the osmotic stress due to increase in internal osmotic pressure and other is ionic stress. The osmotic stress first appears when salt accumulate around the root zone and reduce water availability for plant that reduce the growth of shoot, leaf and bud. The ion toxicity affect at later stage when salt accumulate in leaves and inhibit the essential enzymatic activity by displacing potassium by sodium. The Na^+ cation strives with K^+ due to their similar ionic pattern and hamper essential cellular processes in plant cell. Salt stress impairs ion homeostasis, low water content and imbalance redox status in plant cell resulting in excess Na^+ uptake and also interfere with essential K^+ uptake by root which leads to lower production and plant cell death (Sun et al. 2010). So, maintaining Na^+ and K^+ homeostasis is very important to keep the toxic ion concentration low and to accumulate

essential ions in plant. Plant store excess Na^+ in the vacuole of cell and try to keep normal growth and prevent death. Plant cells use H^+-ATPase and H^+ - pyrophosphatases enzyme to drive the antiporters of H^+, K^+ and Na^+ by creating a proton motive force that enhances transport of ions and metabolites. Osmotic stress activates ABA synthesis and results in upregulated AtNHX1 gene expression. This gene encodes a vacuolar Na^+/K^+ exchanger (Shi and Zhu 2002). The Na^+ inside the cell is sensed by Ca^+ dependent SOS1 (Salt Overlay Sensitive), a membranous protein. SOS1, acts as both a transporter and a sensor of Na^+, is also maintaining Na^+/ K^+ transport activity which is essential for Na^+ efflux from Arabidopsis cells (Chinnusamy et al. 2006). Na^+ accumulates in the plant cell by K^+ transporter HKT1 gene and also some non-selective cation channels. A functional interlink of SOS1 and HKT1 system may achieve ion homeostasis in the plant cell during salinity stress (Olías et al. 2009). In tomato, SOS1 acts in partitioning the Na^+ on plant organs and retaining Na^+ in the stem and finally prevent the Na^+ to reach the leaf (Olías et al. 2009). Salinity also increases cytosolic Ca^+ level which leads to the production of reactive oxygen species (ROS) by activation of NADPH oxidase (Olías et al. 2009).

1.2 Disease resistance

Plant have evolved defence strategies that are controlled by a complex signalling structure depending on the different types of defence:- PTI (PAMP triggered immunity) and ETI (Effector trigger immunity). PAMPs are directly recognized by pattern recognition receptors (PRRs) which leads to PTI and the infection is halted. Some effector can interfere with PTI leading to ETS (Effector –trigger susceptibility) and introduce secondary immune response. Plant resistance proteins (R proteins) are found in the plasma membrane or intercellular space of cell and these can recognise those effectors resulting in effector-trigger immunity. R proteins have a series of leucine-rich repeats (LRRs), a nucleotide-binding site (NBS) and a signalling domain. They are collectively known as NBS-LRR proteins. Pathogen again in order to overcome ETS produce modified or new effectors to take advantage against the host leading to ETS. The plant again produce resistance gene (R-gene) to recognize the modified effector and this ongoing arm race continues. Plant hormones, SA, JA, ET, Auxin, ABA, and GA, Cytokinins and even Brassinosteroids are shown to be involved in both ETI and PTI (Bari and Jones 2009). SA pathway acts as an essential constituents in effector-triggered immunity (ETI), PAMP-triggered immunity (PTI) and systemic acquired resistance (SAR) against biotrophs (Corina Vlot et al. 2009). JA along with low levels of ET is able to trigger response to herbivores and wounding, while high ET levels and JA trigger responses to necrotrophic

pathogens (Grant and Jones 2009). Jasmonic acid (JA) also leads to Proteinase inhibitors (PIs) induction (Turner et al. 2002). During stress situation, proteinase inhibitor I and II were noticed to induce JA and its volatile ester methyl Jasmonate (MEJA) in tomato leaves (Turner et al. 2002).

1.3 Abiotic and Biotic Interaction in Plant

According to (Solomon S 2007) the average temperature of the globe will rise up to 3-5^0 C within hundred years that will affect detrimentally on world agricultural production. As a consequence, the sea levels will rise leading to extensive salinization of agricultural land with severe effect on yield and quality of crops (Morison et al. 2008). Due to increasing temperature, the intensity of pests and pathogens will increase which will threaten world crop production (Pautasso 2012). Plants are always fighting against both abiotic and biotic stress and evolved certain molecular signalling pathways to keep themselves resistant against pathogen and tolerant to abiotic stresses such as transcription factor (TF) mediated signalling and resistance protein (R) vs pathogen effector interactions (Dubos et al. 2010). Different types of stress such as salt, drought, water, injury and pathogen attack involve JA, ethylene, ABA and SA production. These hormonal signals are transduced by a number of genes that help to protect plants. The interactions among these hormonal pathways show antagonistic and synergistic effects (Atkinson and Urwin 2012). The components of stress interactions are summarized in the following parts.

1.3.1 ABA

It is very common that when plants are facing stress, different plant hormones accumulate to protect the plant. For example, when wounding occurs, it can induce the production of auxin, ABA and ethylene. Among all hormones, ABA is considered an abiotic stress hormone whereas salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling pathways are playing as main phytohormone networks in plant immune system (Pieterse et al. 2009). In many plant tissues, NCED (9-*cis*-epoxycarotenoid dioxygenase) is a key enzyme in ABA biosynthesis (Destefano-Beltrán et al. 2006). ABA is involved in physiological processes of plant, including stomatal closure, embryo morphogenesis, development of seeds, synthesis of storage proteins & lipids, seed germination, leaf senescence and defence against pathogens (Zhang et al. 2006). ABA is an essential facilitator in triggering plant responses in many crops during adverse environmental stress including tomato. Plant with ABA overproduction

increased transpiration rate and hydraulic conductivity of root and leaf size (Thompson et al. 2007). ABA enhanced salt tolerance by also altering root growth and exogenous ABA application increased root water content more in salt tolerant variety than salt sensitive variety (Etehadnia et al. 2008). Lowest root dry weight, lower root water content, lower shoot dry weight and less growth rate were found in ABA-deficient mutant plant (Etehadnia et al. 2008). During drought stress, an ABA-deficient tomato mutant (*flacca*), was not able to close stomata. Rice seedlings are known as chilling-sensitive and unable to grow in cold temperatures and rice suspension cells were seriously injured by exposure to 4°C. But exogenous application of ABA can induce to some extent even freezing tolerance (up to -4^0 C) in rice seedlings (Shinkawa et al. 2013).

As a whole, ABA is considered as a negative controller of plant immunity (Mauch-Mani and Mauch 2005), because of its antagonistic interaction with SA-ET-JA signaling pathways (Anderson et al., 2004). Insect injured plants induce proteinase inhibitor which is influenced by ABA hormone, and plays an important role in preventing insect damage. After local wounding of tomato plants, proteinase inhibitors (PI-I and PI-II) were accumulated in the aerial parts of this plants (Swamy and Smith 1999). On the other hand, another ABA-deficient mutant of tomato (*sitiens*) showed significant reduction in these genes expression after artificial wounding. Abscisic acid (ABA) was shown to upregulate expression of the proteinase inhibitor II in ABA-deficient mutant tomato (Pena-Cortes et al. 1995). Under salt stress, ABA deficient mutants show poor growth. ABA deficient plant can show enhanced resistance against biotic stress. For example, Tomato *sitiens* mutant with reduced ABA showed high accumulation of SA dependent defence gene PR1 which enhanced tolerance to *Botrytis cinerea* fungus (Asselbergh et al. 2007).

1.3.2 Production of ROS

Plants have a complex controlling network to facilitate both biotic and abiotic stress responses which depend on production of ROS, ROS scavenging and signalling. Reactive Oxygen Species (ROS) play an important role in abiotic and biotic stress response. Plants experiencing salt stress induce stomatal closure thus reducing CO_2 availability of leaves that inhibit carbon fixation leading to production of ROS in plant cell. The ROS products are mainly two types: free radicals and non-radical molecules. Superoxide anion (O_2^-), hydroxyl radicals (OH^-) are free radicals and singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) are the example of non-radical molecular product which are highly toxic and cause damage to proteins, lipids,

carbohydrates and even DNA and leading to cell death (Gill and Tuteja 2010). The various environmental stresses like salinity, drought, chilling, pathogen attack etc promote ROS generations in plants as result of cellular disruption. ROS play two opposing roles in plants; they act as signalling molecules in low concentration, which is detected in early events of pathogen attack and plays an important role in pathogenesis signal transduction regulators and causes cellular damage in high concentration. After wounding of tomato, ROS acted as messengers for the induction of defence related genes and generated adjacent to the cell wall of vascular bundle producing H_2O_2 from wound inducible polygalacturonase for defence gene activation in mesophyll cells (Orozco-Cárdenas et al. 2001). High concentration of ROS causes cellular damage of lipid, protein, DNA, it hampers enzyme activity, causes inhibition of protein synthesis resulting plant cell death. Plants increase endogenous antioxidant defence level to avoid oxidative damage of cell, by producing ROS scavenging enzymes. Plants upregulated large number of antioxidant defence related genes and these played an important role to protect cells from oxidative damage in response to salt stress in *Physcomitrella patens* (Wang et al. 2008). During salt stress, *Plantago maritima,* a salt tolerant species, produced low level of MDA (Malondialdehyde) and showed protection against oxidative damage by increasing SOD, CAT and APX genes activity (Hediye Sekmen et al. 2007).

Both ROS production and scavenging enzymes are found in different plant organelles like chloroplasts, mitochondria and peroxisomes (Sharma et al. 2012) . In normal plant growth condition, the production of ROS and ROS scavenging enzymes remain in equilibrium but during salt stress, excess ROS production leading to oxidative damage resulting in plant cell death (Miller et al. 2010). The ROS scavenging enzymes include Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), Guaicol peroxidase (GPX), Monodehydro ascorbate reductase (MDHAR), Dehydro ascorbate reductase (DHAR) and Glutathione reductase (GR) (Gill and Tuteja 2010). ROS can also directly or indirectly interact with SA, JA and ET defence signalling pathways.

1.4 Leaf senescence

Leaf senescence means death of leaf which occurs as a result of declining chlorophyll content and photosynthetic activity of leaf (van Doorn and Woltering 2004). However, leaf senescence is not only a degradation process, but also has vital role in nutrient mobilization especially nitrogen (Himelblau and Amasino 2001). Leaf senescence is associated with cellular death of leaf by changing cell structure, metabolism, and gene expression, enhanced by biotic and

abiotic stress (Sedigheh et al. 2011). There are several factors involved in the initiation of leaf senescence. These are pigment degradation, proteolysis, nucleic acid breakdown, respiration of carbohydrates and lipids, nitrogen and phosphate remobilization to other parts of plant. Excess salt concentration results in premature senescence and reduces the photosynthetic area of the plant (Munns 2002). Oxidative stress caused by biotic stress is also responsible for senescence. Rubisco is responsible for carbon fixation and is the most abundant protein in plants and is also involved in senescence. ROS influence Rubisco specific protease to deactivate and degrade Rubisco enzyme which results in leaf senescence (Sedigheh et al. 2011).

Phytohormones are signalling molecules produced within the plant and are essential for the growth and development of plants. The role of each hormone is difficult to assess as they are affecting in different developmental and environmental events in a complex way (Pyung et al. 2007). The level of ET, JA and ABA are increased at senescence time which induces the expression of stress-related defence genes. Ethylene is involved different types of plant cell death and senescence and also has an important role in responses to biotic and abiotic stress. In *Arabidopsis,* the level of ethylene increases during leaf senescence. Accordingly, the ethylene ACC oxidase is upregulated in senescing leaves. The two mutants of *Arabidopsis ethylene-resistant 1* (*etr1*) and *ethylene-insensitive 2* (*ein2*) which were ethylene deficient exhibiting significant delayed in leaf senescence (Chen et al. 2011). ABA is considered as plant stress hormone and usually responds to adverse environmental conditions. Exogenous application of ABA promotes leaf abscission and senescence. During drought, saline condition and chilling stress, ABA content increases in leaves that potentially leads to senescence.

Cytokinin (CK) is leaf senescence-inhibiting hormone leading to delayed senescence and leaf senescence occurs if CK level is reduced below a threshold level and exogenous application cytokinin promotes delayed leaf senescence (Zhang and Gan 2012). The cytokinin-mediated delaying of leaf senescence is positive correlated with the function of extracellular invertase. Extracellular invertase (LIN6) is the key enzyme of an apoplasmic phloem unloading pathway which is involved in a source-sink transition in plants promoting a high hexose/sucrose ratio.

Many transcription factors exhibit a senescence-associated pattern, including NAC (Zhang and Gan 2012), WRKY (Zentgraf et al. 2010) and MYB (Zhang et al. 2011) domain transcription factors.. Cysteine proteinase (CYP) play an essential role in senescence and programmed cell death in tomato leaves. CYP is also involved in different hormonal signalling pathways in response to biotic and abiotic stresses (Grudkowska and Zagdańska 2004). Sugars are

important signal molecule for plant development and metabolism. Pathogens attack may influence leaf senescence by modification of sugar status in cell affecting carbon metabolism or by regulating plant hormones level of plant (Wingler and Roitsch 2008). During drought stress, induction of senescence occurs due to interactions between sugar and abscisic acid (ABA) signalling whereas in chilling stress, this same interaction may lead to delayed senescence (Wingler et al. 2006).

1.5 Importance of salt stress and powdery mildew disease in tomato

Tomato is an important culinary and popular vegetable all over the world. Tomato cultivation is threatened by many pests and diseases during their growth and development. The cultivated tomato is moderately salt-sensitive plant with 50% production loss when grown in saline media with 7.5 ds/m (Katerji et al. 2003). Different studies proved that when growing different tomato cultivars in high salt solutions, the Na^+ ions were transported and accumulated in leaf and inhibit photosynthetic activities (Zhou et al. 2009). Several genetic mechanism is responsible during tomato plant grown in saline stress condition (Foolad 2004). Plants rapidly alter their gene expression to recognize and respond to salt stress and to adapt in this situation with some modification of their physiological and biochemical state (Amini et al. 2007). The seed germination, stem height, fresh and dry weight of stem, leaves, and root of tomato plant were decreased by salt stress (Zhou et al. 2009). Maintaining the redox reaction balance in plant cells is an important criterion for normal growth and production activities. Rubisco activase is suppressed during salt stress resulting lower CO_2 assimilation in tomato leaves, less photosynthesis and hampered glucose synthesis (Zhou et al. 2009). Salt stress interferes with carbohydrate metabolism and partitioning in various organs in tomato leaves (Khelil et al. 2007). Tomato powdery mildew (*O. neolycopersici*) is a very important biotrophic fungal disease of tomato all over the world. In temperate region like, Europe, this disease causes severe damage in tomato production, mainly in greenhouse production. After infection of tomato leaves, it turns in brown color and causing rapid death of infected leaves. This fungus has a wide host range including pepper, eggplant, etc. which can survive in weed hosts as mycelium before infection The cultivated tomato varieties are susceptible. The resistance gene of *O. neolycopersici* was identified in wild tomato *Lycopersicon* species. The hypersensitive reaction (HR) is a form of apoptosis-like Programme Cell Death (PCD) which is the major mechanism of resistance to *O. neolycopersici* (Hofius et al. 2009).

1.6 Powdery mildew resistant lines

Most of the popular cultivated tomato species are susceptible to powdery mildew. The powdery mildew resistant lines are found from some wild species of tomato. Scientists identified nine loci that can confer resistance against PM. The introgressed locus from those resistance lines into cultivated species is the best way to get PM resistance tomato varieties. The isogenic lines Ol-1, ol-2 and Ol-4 carry segments from *S. habrochaites* G.1.1560, *S. lycopersicum* var cerasiforme LA 1230 and *S. peruvianum* LA 2172 respectively, which confer resistance to powdery mildew. The dominant resistance genes Ol-1 and Ol-4 were mapped on the long and short arm of chromosome 6 respectively (Bai et al., 2004)., whereas the recessive resistance gene ol-2 gene was mapped on chromosome 4 (Bai et al. 2008). Ol-1 gene confers delay cell death when cell is invaded by PM fungus and allows restricted fungal growth where as Ol-4 triggers hypersensitive resistance leading to complete resistant to O. neolycopersici. The ol-2, recessive resistance gene induce callose deposition and reinforcement of cell wall to prevent extent of PM at the time of fungus invasion (Bai et al. 2008).

1.7 Previous research on tomato resistance to combined salt stress and PM

Assessing the resistance Ol-1, ol-2 and Ol-4 under different levels of salt stress (0, 50, 100 and 150 mM NaCl), it was found that mild salt stress (50, 100 mM) compromises the resistance of line Ol-1, inducing both increased pathogen sporulation and extended senescence (Sunarti 2012). On the other hand lines ol-2 and Ol-4 were unaffected for both these aspects. Expression analysis of candidate genes in line Ol-1, showed increased expression of ET/JA biosynthesis and signaling genes.

1.8 Objectives

Crosses of the isogenic lines Ol-1, ol-2 and Ol-4 with lines carrying mutant genes, in different hormonal pathways, namely ET/JA/ABA will be used to study tomato resistance to combined salt and powdery mildew (PM) stress and its hormonal regulation. The objective of present study is:

- To select lines homozygous for Ol-genes and the hormonal pathways genes.

- To investigate the involvement of different hormonal pathways (SA, JA, ET and ABA) in resistance against powdery mildew (PM) and combination of PM and salt stress.
- To assess how senescence is affected in those lines under combined stress.
- To measure the gene expression at different levels of salt stress and severity of PM .
- How do hormonal pathways interact with disease resistance of Ol-1, ol-2 and Ol-4 mutants.

2. MATERIALS AND METHODS

2.1 Plant materials

Crosses between three tomato mutants not (ABA-deficient), def (JA-deficient) and epi (ET over-producer) and the isogenic lines Ol-1, ol-2 and Ol-4 (Bai et al., 2008, Bai et al., 2004) were assessed for powdery mildew resistance line in combination with salt stress. Further details on the plant material used in this thesis is listed in Table 1. Seeds for all crossing combinations with isogenic lines except epi were sown in pots in the greenhouse of Unifarm,Wageningen University. Seeds of Ol-1 x epi, ol-2 x epi and Ol-4 x epi crossing combinations were germinated in vitro, to be selected after germination in the dark. Seeds were disinfected before placing in media for germination. The following procedures were followed during this task: i) Seeds were washed in sterile water in ethanol for one minute, ii) The seeds were then sterilized in 1.5% NaOCl solution for 15 minutes followed by three washes in sterile water. iii) The disinfected seeds were then sown in 1/2 MS medium (pH 5.8, 0.8% agar and antibiotic cefotaxime 30 mg/l).

Table 1. Crossing combination of three isogenic lines with epi, def and not mutants in F_2 and F_3 generation

Isogenic lines	Generation of isogenic lines	Crossing combination with mutant	No of planted seed	No of selected plant per treatment
Ol-1	F_3	Ol-1 X epi	25-30	6-7
	F_2	Ol-1 X def	*200-220	6-7
	F_2	Ol-1 X not	*200-220	6-7
ol-2	F_3	Ol-2 X epi	50-55	6-7
	F_3	Ol-2 X def	50-55	6-7
	F_3	Ol-2 X not	50-55	6-7
Ol-4	F_2	Ol-4 X epi	*200-220	6-7
	F_2	Ol-4 X def	*200-220	6-7

* In F_2 generation more seeds are needed due 1:15 segregation ratio (selection for two genes compared to 1:3 in F_3 lines (Ol-genes fixed).

2.2 Ol-lines selection

We collected leaf sample from different Ol- lines and their mutants for DNA extraction. We used Ol-gene markers for screening and selection of homozygous parental materials. For PCR we isolated plant DNA by the following method. The table 2 showed a short description about the marker of isogenic Ol lines.

Table 2 . List of marker of three Ol lines

Isogenic lines	**Markers**	**Sequence**
Ol-1	H9A211 lower band	Forward: TGCTCTAACAAAATCACCAAAATC Reverse: AAATGGTCAAACAAAGTCTATTGAG
ol-2	Deletion marker-ol-2 lower band	Forward: ACCCTTAAGAAATAGGGCAAA Reverse: ACCATCATGAACCCATGTCT
Ol-4	Deletion marker-Ol-4 higher band	Forward: GAACCGGATGTGTCCTTGAC Reverse: TTCTCCGAGACTTTGAACAAGA

2.2.1 DNA extraction

We did quick DNA isolation by using NaOH protocol. For this, we sampled approximately 10mg of plant tissue in tubes in a 96-well plate format .

After adding 20 µl NaOH (0.5 M) we crashed the samples. 20 µl of 100mM Tris-HCl was added to the slurry and mixed appropriately. Then 5 µl of the slurry was diluted in 95 µl of 100mM Tris-HCl in a 96-well plate

2.2.2 PCR protocol

We used 2 µl 5 x phire buffer- 0.4 µl dNTPs-, 2 µl F-primer-, 2 µl R-primer-, 0.12 µl Phire enjyme (10 µ/ml)-, 2.5 µl MQ- and 1 µl PVP-to a final volume 10 µl master mix. Then add 1 µl of the sample above containing the extracted DNA. The temperature profile of PCR was 98^0 –30 sec, (98^0 –5 sec, 54^0 –5 sec, 72^0 –10 sec) for 30 cycles,72^0 –100 sec, 94^0 –30 sec, 25^0 –30 sec.

2.3 Hormone mutants selection

Mutants were selected according to the MSc thesis of (Lara 2013). The tomato epi mutant (VFN8 background), is an ethylene over producer mutant and ethylene is a growth inhibiting hormone. The segregating population contianing 'epi' mutants was germinated *in vitro* in the dark. During dark condition, 'epi' mutants were stunted as ethylene suppressed the plant growth. The plants which had normal growth in dark condition were selected as 'EPI' (WT plants). In case of 'def' mutant (Castlemart background), plants segregating for the mutation were grown in the greenhouse in normal conditions. Leaflets of 3 week-old plants were wounded with scissors to activate JA signalling, and 24 hrs afterwards were sampled and the RNA that was isolated was used for qPCR. The LAP marker gene, a marker of JA signalling pathway evaluation, was used to evaluate the activation of downstream responses after wounding. Plants homozygous for the mutation (def) showed no upregulation of the LAP gene, in contrast with heterozygous and WT plants (DEF). In case of 'not' mutant (Ailsa Craig background), plant were germinated in the greenhouse in normal conditions. After DNA isolation and PCR amplification of the NCED gene where the mutation is located, the PCR amplicons were sequenced and examined for the presence of the mutation. Homozygous plants were selected, that were either carrying (not), or not the mutation (NOT).

2.4 Experimental set up

The experiment was arranged in a completely randomized split plot design with 4 replications for each genotypic combination per treatment except for the Ol-1x epi and Ol-2x epi mutants for which 7 replicates per treatment were used. The main plots were 2 levels of salt treatment (S_0- normal nutrient solution and S_1- solution with 50 mM NaCl added). NaCl was applied on 4-week old plants by watering the plants with 50 mM NaCl solution. Irrigation was repeated every day. Eight days after the salt treatment start, plants were inoculated with powdery mildew.

2.5 Fungal materials and sporulation

For disease tests, *Oidium neolycopersici* isolate was used. Powdery mildew fungus (*Oidium neolycopersici*) is maintained in growth chambers at 20±3 °C with 70±15 % relative humidity (RH) upon Money maker (MM) tomato variety and was used as source of the inoculum.

Fungal spores were washed off from heavily infected tomato leaves and then diluted to the concentration of 2.5×10^4 spores per ml. The inoculum was then sprayed on the 5 weeks old plants.

2.6 Disease data collection

The severity of disease was expressed by disease index (DI) which was assessed at 10 and 15 days post inoculation (dpi). The disease index was evaluated by comparison with MM which is considered very susceptible.

The values where corresponding to macroscopic observations of PM growth and sporulation where 0 = healthy plant, no visible sporulation, 1 = < 0.1-10 % of foliar area affected, slight sporulation, 2 = 10-20 % area affected, 3 = 20 – 30 % area affected, 4 = 30-50 % area affected and 5 = > 50 % area with abundant sporulation.

2.7 Leaf Senescence data collection

The senescence of different Ol lines and their mutants in both treatments (powdery meldew and combined powdery mildew & salt stress) were measured after 10 and 15 days post inoculation (dpi) of disease in both. The senescence score was 0-5 according to the leaf damage and plant fitness

The following measurement was done during leaf senescence data collection:

0 = healthy plant, no visible sporulation

1 = < 5 % of foliar area affected, very few spot surrounded by chlorotic or necrotic flecks

2 = 5-10 % foliar area affected, chlorotic or necrotic flecks

3 = 11 – 20 % foliar area affected, chlorotic or necrotic flecks

4 = 21-50 % foliar area affected, chlorotic or necrotic flecks

5 = > 50 % foliar area affected chlorotic or necrotic flecks

2.8 Chlorophyll content

Chlorophyll content was measured using SPAD meter 502 (Minolta) on the second and third leaf counting from bottom, immediately before pathogen inoculation.

2.9 Gene expression analysis

Leaf sample (middle leaflets from the 3rd and 4th leaf) was collected at 10 dpi and preserved in -80^{o}C for further gene expression study. Fifteen genes responsible for different physiological and molecular activities were used to monitor different signalling pathways.

2.9.1 Candidate genes

Genes involved in biotic and abiotic stress signaling pathways were assessed. In tomato, NCED (9-cis-epoxycarotenoid dioxygenase) gene is involved in ABA metabolism, AOS (Allene oxide synthase) gene was used as marker for JA pathway, ICS (Isochorismate synthase) and PAL (phenylalanine ammonia lyase) genes are part of the SA pathway and ACCase (1-aminocyclopropane-1carboxylic acid synthase), ACO3 and ERF2 genes were used as marker of ethylene pathway. RBOHD, RBOHF, SOD and APX genes are involved in ROS production and detoxification. CKX1 and LIN6 genes are involved in senescence and CYP3 and MCA1 genes in autophagy (Table 3).

Table 3. Candidate genes for gene expression analysis and function.

Genes	Name	Function
SOD	Superoxide dismutase	Defence against ROS (Alscher et al. 2002)
APX	Ascorbate peroxidase	ROS detoxification system enzymes (Caverzan et al. 2012)
RBOHD	Respiratory burst oxidase D	Promotes ROS production (Peer et al. 2011)
RBOHF	Respiratory burst oxidase F	Generation of ROS production (Zhu et al. 2013)
NCED	9-*cis*-epoxycarotenoid dioxygenase	Responsible for ABA biosynthesis (Behnam et al. 2013)
PAL	Phenylalanine ammonia-lyase	Flavonoid biosynthesis (Chang et al. 2008)
ICS	Isochorismate synthase	Salicylic acid synthesis. (Garcion et al. 2008)
AOS	Allene oxide synthase	Jasmonic acid biosynthesis (Wasternack et al. 2006)
ACCase	ACC synthase	Ethylene biosynthesis (Schulte et al. 1997)
ERF2	Ethylene Responsive Factor	Activate the transcription of basic type defense-related genes, pathogenesis-related (PR) genes (Pirrello et al. 2006)
ACO3	Acc oxidase 3	Ethylene biosynthesis (Argueso et al. 2007)
CYP3	Cysteine protease	Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence (Grudkowska and Zagdańska 2004)
MCA	Metacaspase	Key roles in programmed cell death (PCD) (Choi and Berges 2013)
CKX1	CK dehydrogenase	Cytokinins regulating enzymes (Saravanakumar et al. 2011)
LIN6	Invertase 6	Key enzyme of an apoplasmic phloem unloading pathway (Proels and Roitsch 2009)

2.9.2 Primer design and testing

Primers were designed by using the NCBI primer design tool. Primers were designed manually using the alignment in regions where discrimination between different genes will possible. Some important criteria like the length of the primer, the size of amplicon, GC content, melting temperature, annealing temperature were considered during primer design. Finally, the following primer pairs were selected for the genes of interest.

Table 4. List of primer of candidate genes for salinity tolerance of tomato

Primer	Sequence (in 5'----> 3' order)
NCED1_F1	TCGAAAACCCGGATGAACAAGTGA
NCED1_R1	AACCAGAAACTTTTGGCCATGGTTC
AOS_F1	CCGGCGGGAAGATCACGATG
AOS_R1	TCGAAAACGGCGTCGTGTGA
ICS_F1	GGCAATAGATGCACTTCAGGCCA
ICS_R1	CGCATGGTCCCAAGACGCTTT
PAL_F1	GCTGTCAAGAACACAGTGAGCCA
PAL_R1	GGTAGGTGGAGCTGCAGGGA
ACCase_F2	CGCGATGAGGTTAGGTAAAAGGCA
ACCase_R2	GTCGATTCCCTTAAAAGTGGACGCA
ACO3_F1	ATCCAGGAAATGACGCGGTG
ACO3_R1	TCTTGGCTCTTTTGGCTGAAAC
ERF2_F1	GGAGGCGGCTAGAGCTTATG
ERF2_R1	CGGACTCGATGACTCCACAG
RBOHD_F1	TCAGGTCAAGCATCAAAGCCGTT
RBOHD_R1	TGGTGAAACCGCAGCACAGT
RBOHF_F1	GGAGTGGAGGGTGTGACTGGA
RBOHF_R1	GGTGCGAGTACCAGAACGCA
SOD_F1	CCTCTCACTGGTCCACAGTCCA
SOD_R1	AGCAGTTAACCCTGGAGGCCA
APX_F	CCATTTGGAACAATCAGGCACCCG
APX_R	CGGGGCCTCCCGTAACTTCA
CKX1_F1	GAAGCGGCTAGAGGTAGTGG
CKX1_R1	GCTACGCAGGAGAGGATAGGT
LIN_F1	TTGGTTCAATGGCCTGTTCAAG
LIN_R1	TTCAACGTCAGCCTGTGCAA
CYP3_F1	ACACCGGCAAGAATGGCATA
CYP3_R1	GCCTAACCAATGCAACTGCG
MCA_F1	CACTCTTTGACGTCTTTGGCG
MCA_R1	AACCATACCCATGAACCCGC
EF-la_ F1	GAA CCA TCC AGG CCA AAT AA
EF-la_ R1	CCG TTC TTC CAC CAC TGA TT

2.9.3 qPCR

The RNA was extracted from the 3rd and 4th leaves of tomato by using RNeasy™ mini kit (Qiagen). The concentration of the RNA has been measured spectrophotometrically using the Nanodrop equipment. The quality of the RNA was checked by running it on 1.5% agarose gel. One (1) µg of RNA was treated with deoxyribonuclease I (DNase) and inactivated according to the manufacturer's guidelines. DNase treated RNA was then reverse-transcribed in cDNA: the Reverse transcription reaction mix contains 5x iScript reaction mix, iScript reverse transcriptase, nuclease-free water and RNA template. Real time PCR was performed in a BioRad ($CFX96^{TM}$) with IQ SYBR green super mix. Two technical replicates were used. The reaction mix contained iQ SYBR green supermix- 5 µl, 1 µl forward primer (0.3mM)-, 1 µl reverse primer (0.3mM)-, cDNA template- 3.0 µl (5ng/Ul) to a final volume 10 µl. Thermocycling condition were: 95^0C for 3 min followed by 42 cycle of 95^0C for 10 sec and 60^0C for 30 sec. The target genes were normalized by using Elongation factor 1-alpha as a housekeeping gene. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.10 Statistical analysis

The data was arranged and prepared for analysis by using Genestat 14th edition. Two way ANOVA was used to determine the significance ($P<0.05$) differences together with Fisher's Least Significant Difference (LSD), among the plant height, chlorophyl content and gene expression analysis in order to investigate if the difference in genotypes is dependent on the treatment received.

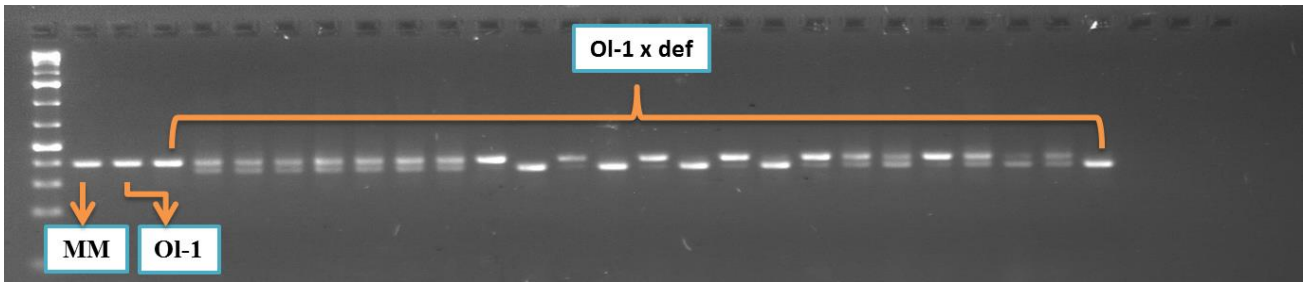
3. RESULTS

Plants were segregating for either both the Ol-genes and the hormonal mutations (F_2 populations), or only for the hormonal mutations (F_3 populations). After germination a sample from the first leaf was collected and checked from all crossing combination of Ol- gene by doing PCR and gel electrophoresis analysis. Specific Ol-gene markers (Table 2) was used for screening the parental materials

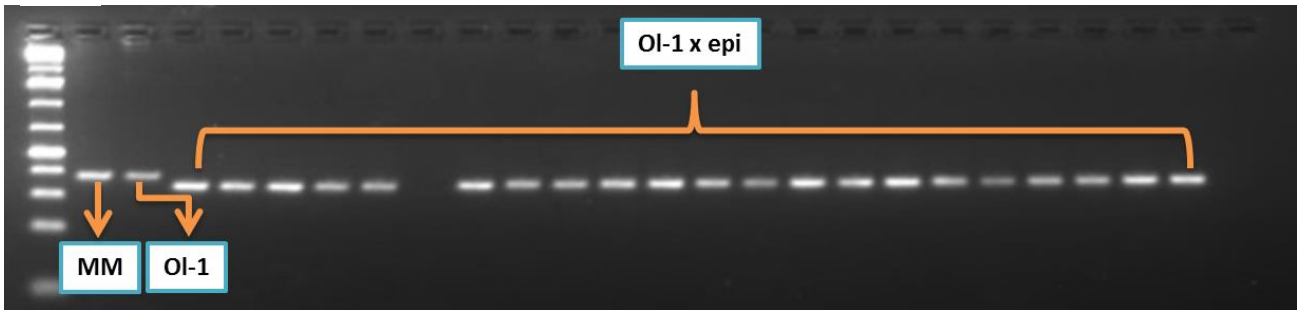
3.1 Selection and confirmation of Ol lines and their mutants

3.1.1 Selection and confirmation of Ol-1 line and its mutants

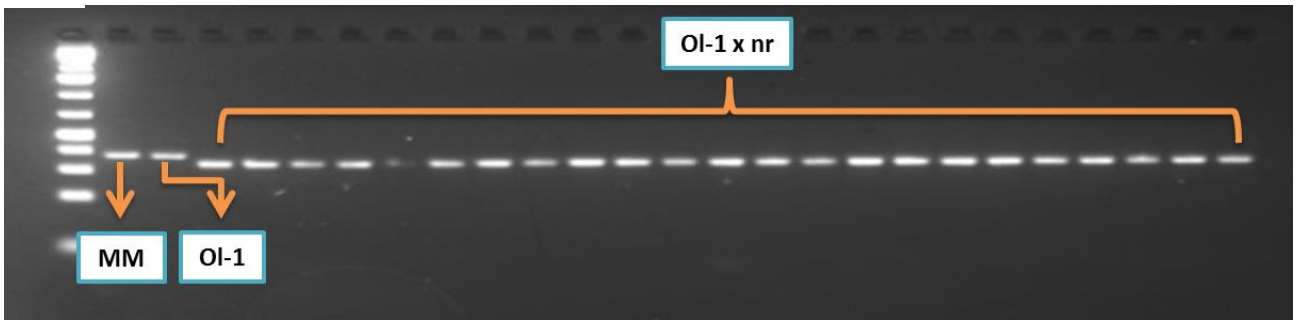
The crossing combination of Ol-1 with different hormonal mutants were either from F_2 generation or F_3 generation and plants with homozygous Ol-1 alleles were selected therefore Ol-1 gene was fixed (Table 1) . The Ol-1 marker `H9A11' is co-dominant, so it can discriminate between homozygous and heterozygous plants. The heterozygous plants and plants not carrying the Ol-1 gene were discarded. Surprisingly the Ol-1 genotype showed the upper band similar to MM. Probably, the reason is that the Ol-1 genotype used has the region fine mapped and the marker `H9A11' lies outside this region and therefore corresponds to MM allele. Both homozygous and heterozygous individuals were found from the PCR product of Ol-1 x def crossing combination as this mutant came from F_2 generation but we selected homozygous line having the lower band (Fig. 1 a). The crossing combination of Ol-1 x epi and Ol-1 x nr was F_3 generation and as a result their PCR product gave homozygous lines as well (Fig. 1 b,c). We did not use plants from Ol-1 x nr crossing combination due to lack of number of plants for further study.



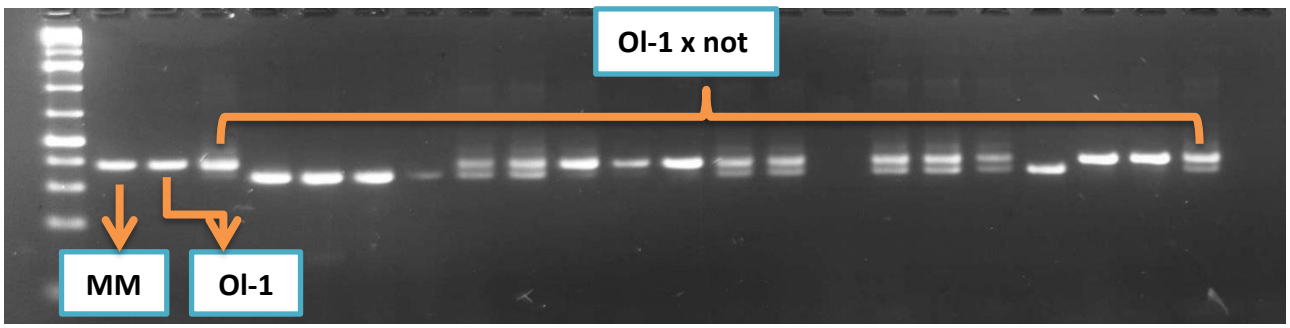
(a)



(b)



(c)

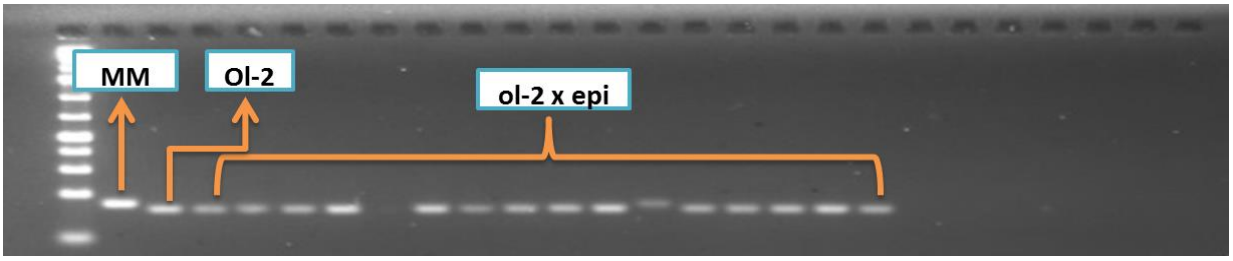


(d)

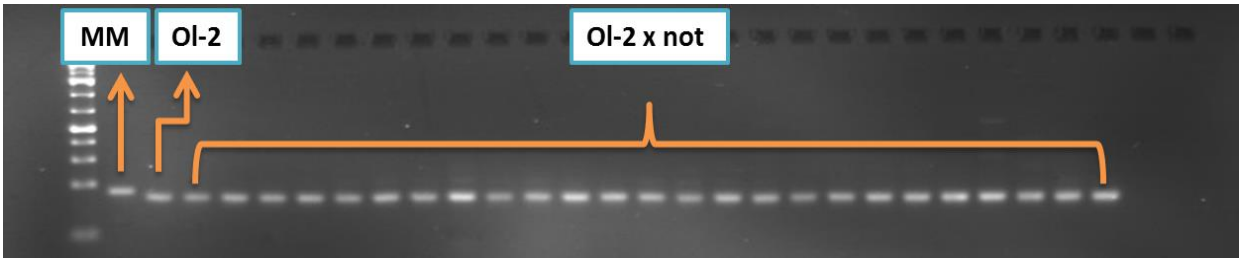
Figure 1. *PCR products of Ol-1 x mutants crossing combination screened with deletion marker H9A11, (a) Ol-1x def (b) Ol-1 x epi (c) Ol-1 x nr and (d) Ol-1 x not. The lower band corresponds to the Ol-1 resistance gene.*

3.1.2 Selection and confirmation of Ol-2 line and its mutants

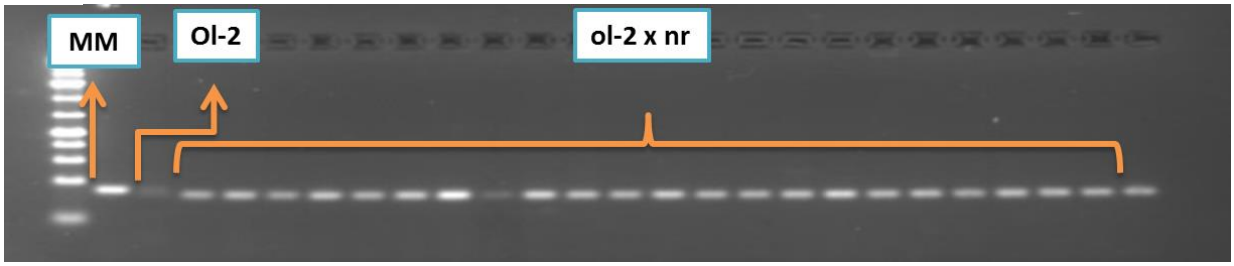
Deletion marker of ol-2 was used to detect ol-2 mutant in a PCR product with the lower band corresponding to the mutant allele. The ol-2 deletion marker is codominant, so It was expected homozygous line from PCR product of ol-2 mutants, as all ol-2 mutants derived from F_3 generation and were selected previously in the F_2 for the presence of the gene. The PCR product after gel electrophoresis, all ol-2 mutants populations (ol-2 x epi, ol-2 x not, ol-2 x nr and ol-2 x def) showed homozygous lines (Fig. 2 a,b,c and d). We did not use plants from Ol-2 x nr crossing combination due to lack of number of plants for further study.



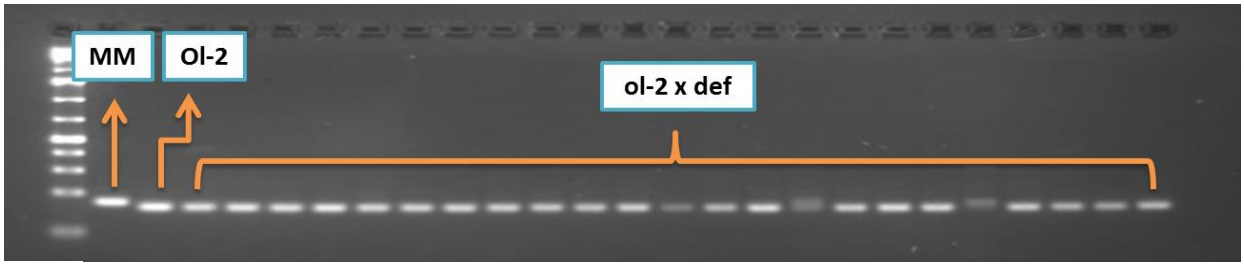
(e)



(f)



(g)

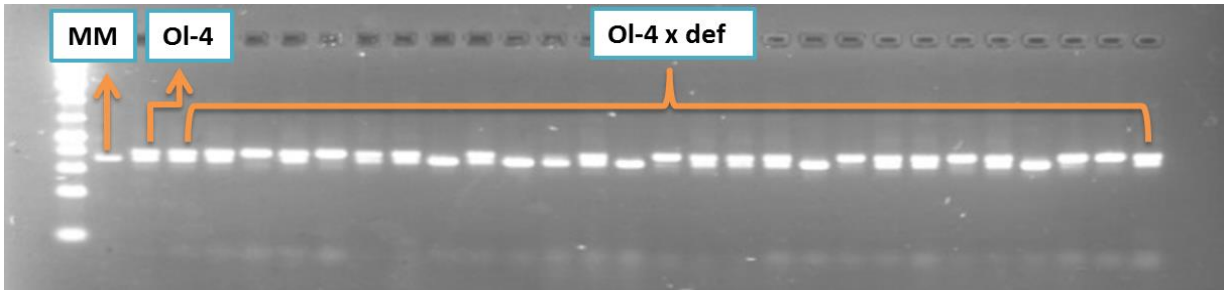


(h)

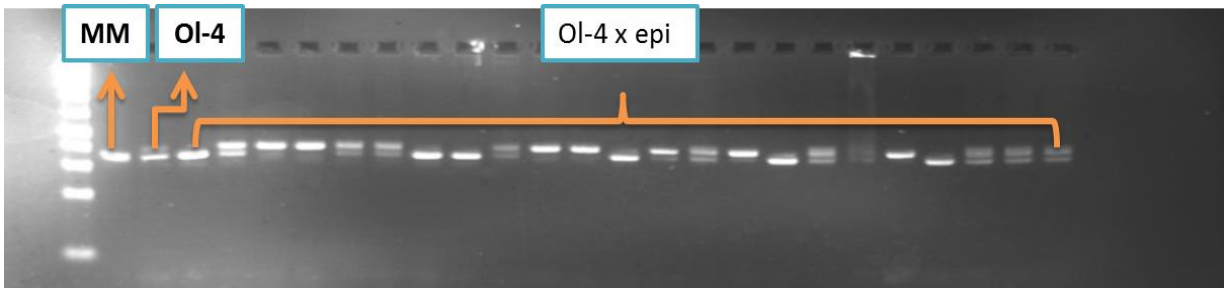
Figure 2. *PCR product of ol-2 x mutants crossing combination screened with deletion marker (a) ol-2 x epi, (b) ol-2 x not, (c) ol-2 x nr and (d) ol-2 x def. The lower band corresponds to the ol-2 resistance gene.*

3.1. 3 Selection and confirmation of Ol-4 line and its mutants

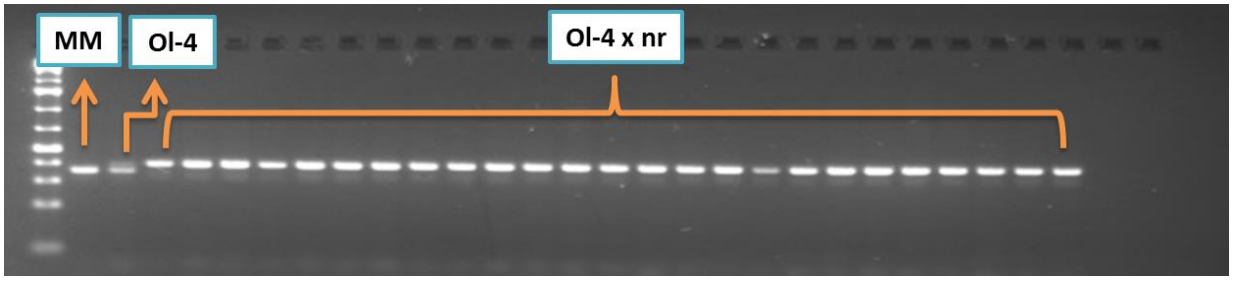
Deletion marker of Ol-4 was used to detect Ol-4 mutant from PCR product and appeared in higher band. The Ol-4 deletion marker is codominant, so it can also discriminate between homozygous and heterozygous plants. The heterozygous plants and plants not carrying the Ol-4 gene were discarded. Segregating population was found from the PCR product of Ol-4 x def and Ol-4 x epi crossing combination as this population came from F_2 generation and then, we selected homozygous line for experiment (Fig. 3 a and b). On other way around, the Ol-4 x nr products showed homozygous line because this crossing combination was from F_3 generation (Fig. 3. c). We did not use plants from Ol-4 x nr crossing combination due to lack of number of plants for further study.



(i)



(j)



(k)

Figure 3. *PCR product of Ol-4 x mutants crossing combination screened with deletion marker (a) Ol-4 x def , (b) Ol-4 x epi and (c) Ol-4 x nr. The upper band corresponds to the Ol-4 resistance gene.*

3.2 Phenotypic analysis of mutants during salt and powdery mildew combined stress

3.2.1 Plant height

After selection for the homozygosity in Ol-genes when possible (in F_2 population) plants carrying (lowercase naming) or not the hormonal mutation (uppercase naming) were selected to observe effects due to different parental backgrounds.

Plant height was measured 7 days after the application of salt stress and before the inoculation of powdery mildew. Growth was reduced in all crossing combination regardless of the presence of the hormone mutant gene, showing the significant effect of the genetic background of the mutants on growth. The epi mutants were the shortest plants. Epinastic mutation is characteristic by its short plant growth, excessive leaf curling (Barry et al. 2001). No difference was found between the treatments.

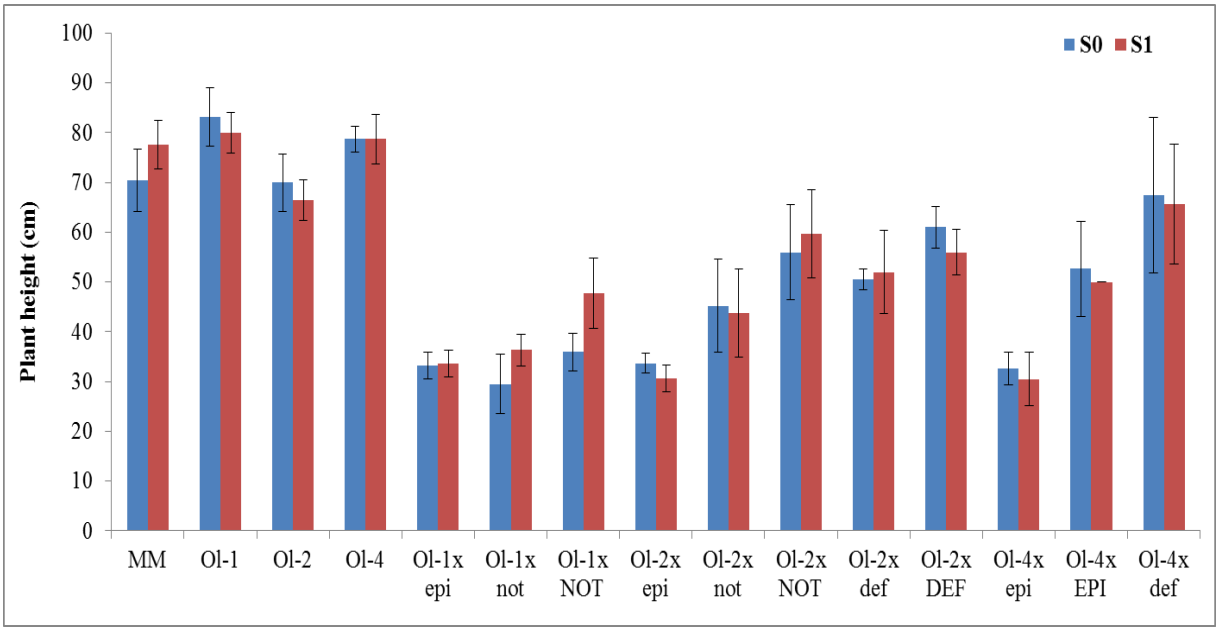


Figure 4. *Plant height of MM, Ol lines and crossing combination of Ol lines with mutants under PM infection (S0) and combined PM and NaCl 50mM solution (S1). Error bars represent standard deviation.*

3.2.2 Chlorophyll content

Chlorophyll content was measured before the application of powdery mildew in the plants. There was no significant difference among the genotypes in both treatments.

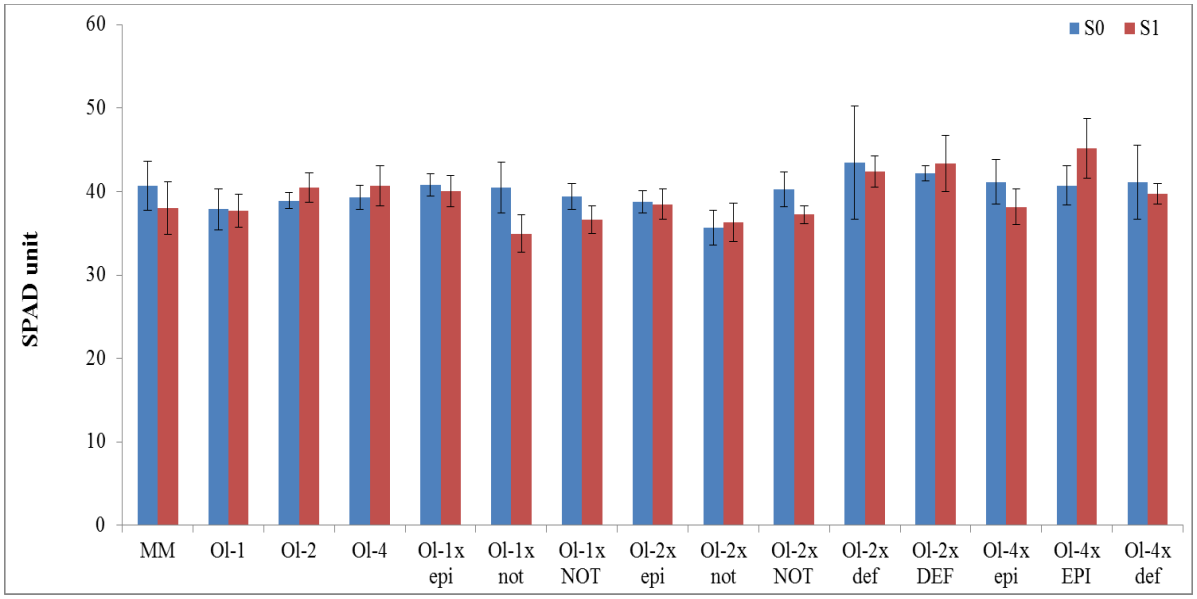
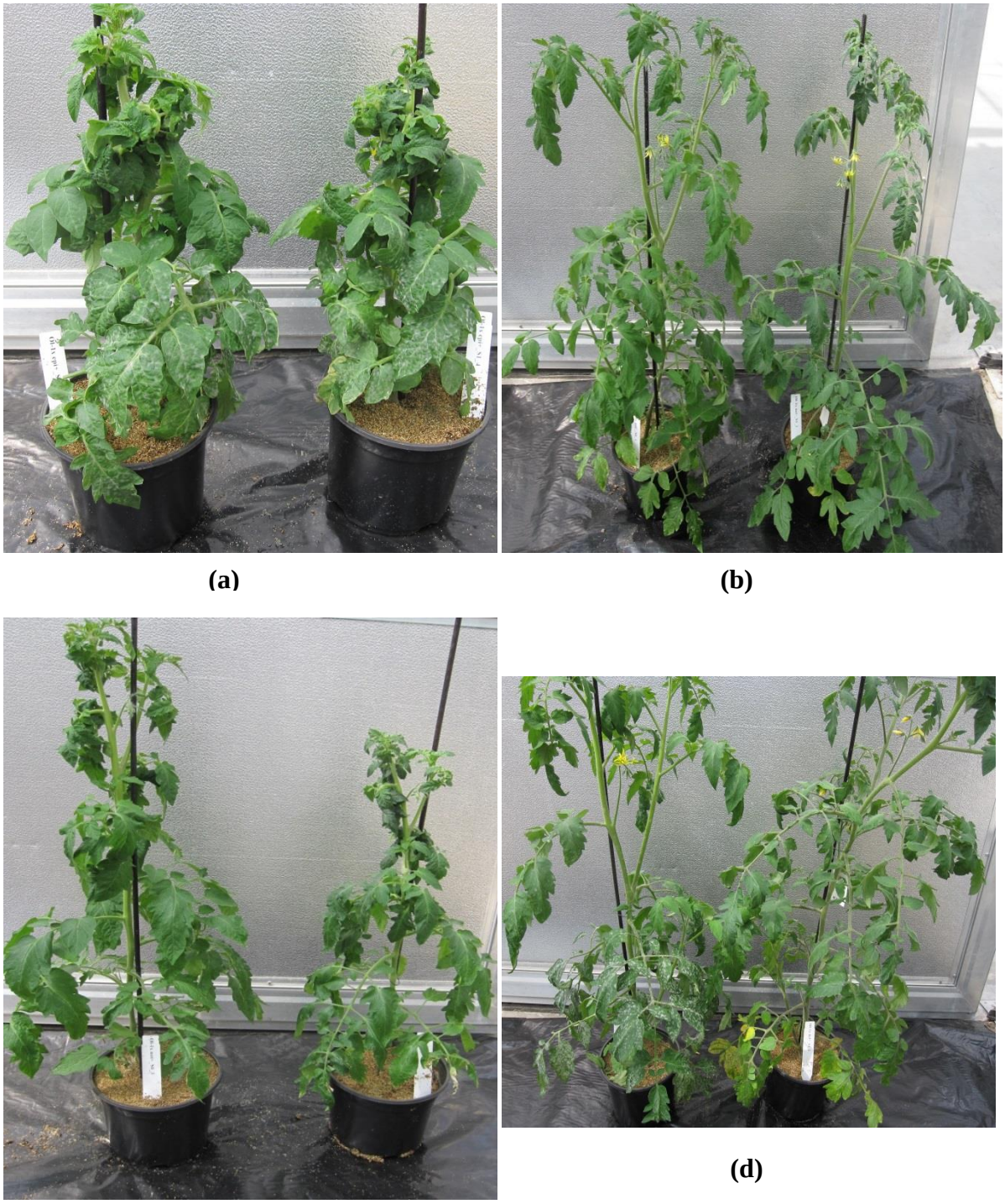


Figure 5. Chlorophyll content of MM, Ol lines and crossing combination of Ol lines with mutants under PM infection (S0) and combined PM and NaCl 50mM solution (S1). Chlorophyll content is represented in SPAD units. Error bars represent standard deviation.

3.3 Phenotypic performance

The phenotype of Ol-1 was showing more senescence among all genotypes (Figure 6.b). The Epinastic mutant combination with Ol-1, ol-2 and O-4 were known by short plant growth and excessive leaf curling (Figure 7.a, 9.b and 9.c). Less senescence was found in all ol-2 and Ol-4 mutants (Figure 8, 9)

Figure 6. *Phenotypes of (a) MM, (b) Ol-1, (c) ol-2 and (d) Ol-4 isogenic line, under PM infection (left) and combined PM and NaCl 50mM solution (right) at 15 dpi.*



(a) **(b)**

(c) **(d)**

Figure 7. *Phenotypes of (a) Ol-1 x epi, (b) Ol-1 x NOT, (c) Ol-1x not and (d) Ol-1 x def mutants under PM infection (left) and combined PM and NaCl 50mM solution (right) at 15 dpi.*

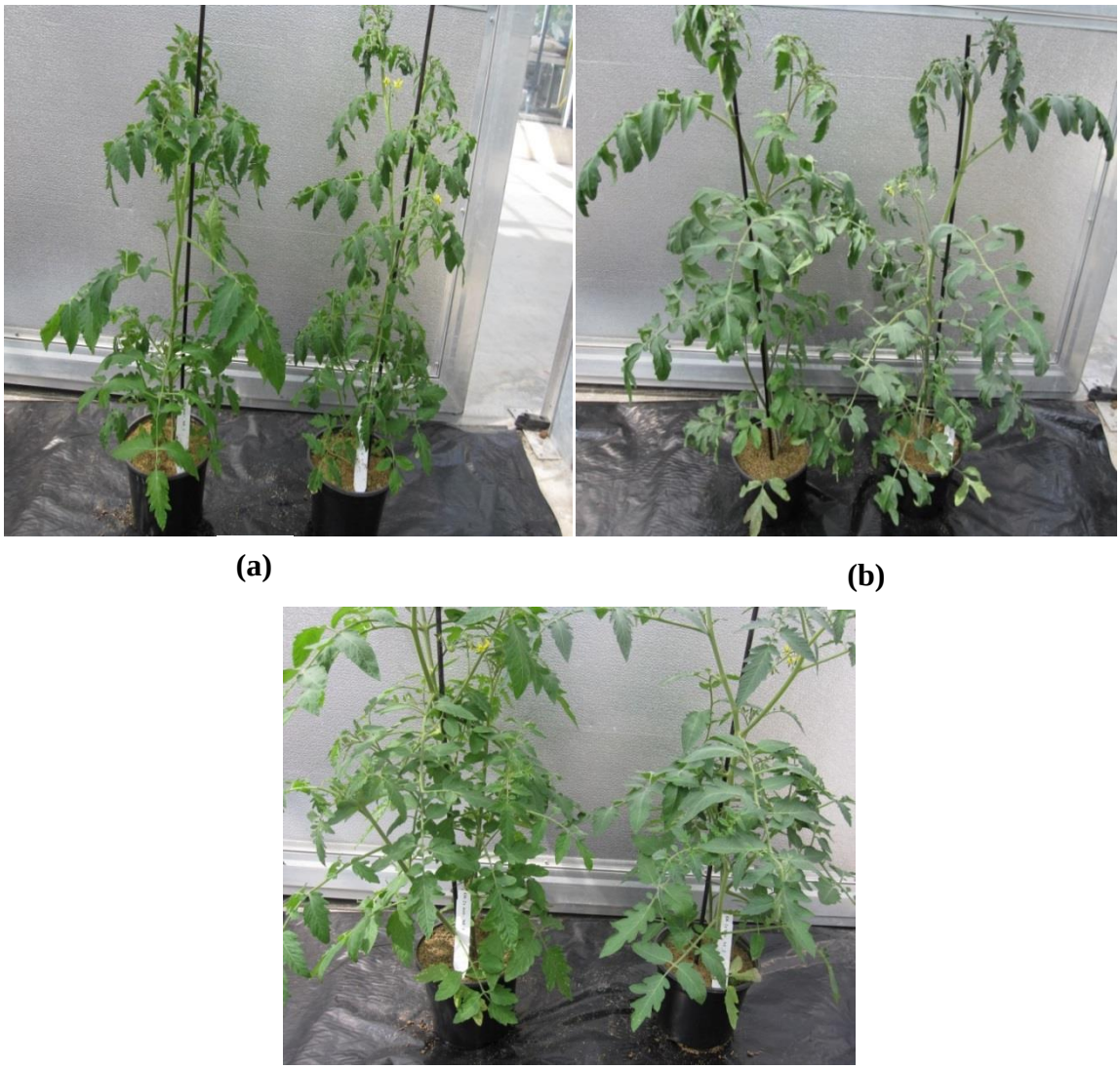


Figure 8. *Phenotypes of (a) ol-2x not, (b) ol-2 x def and (c) ol-2x NOT under PM infection (left) and combined PM and NaCl 50mM solution (right) at 15 dpi.*

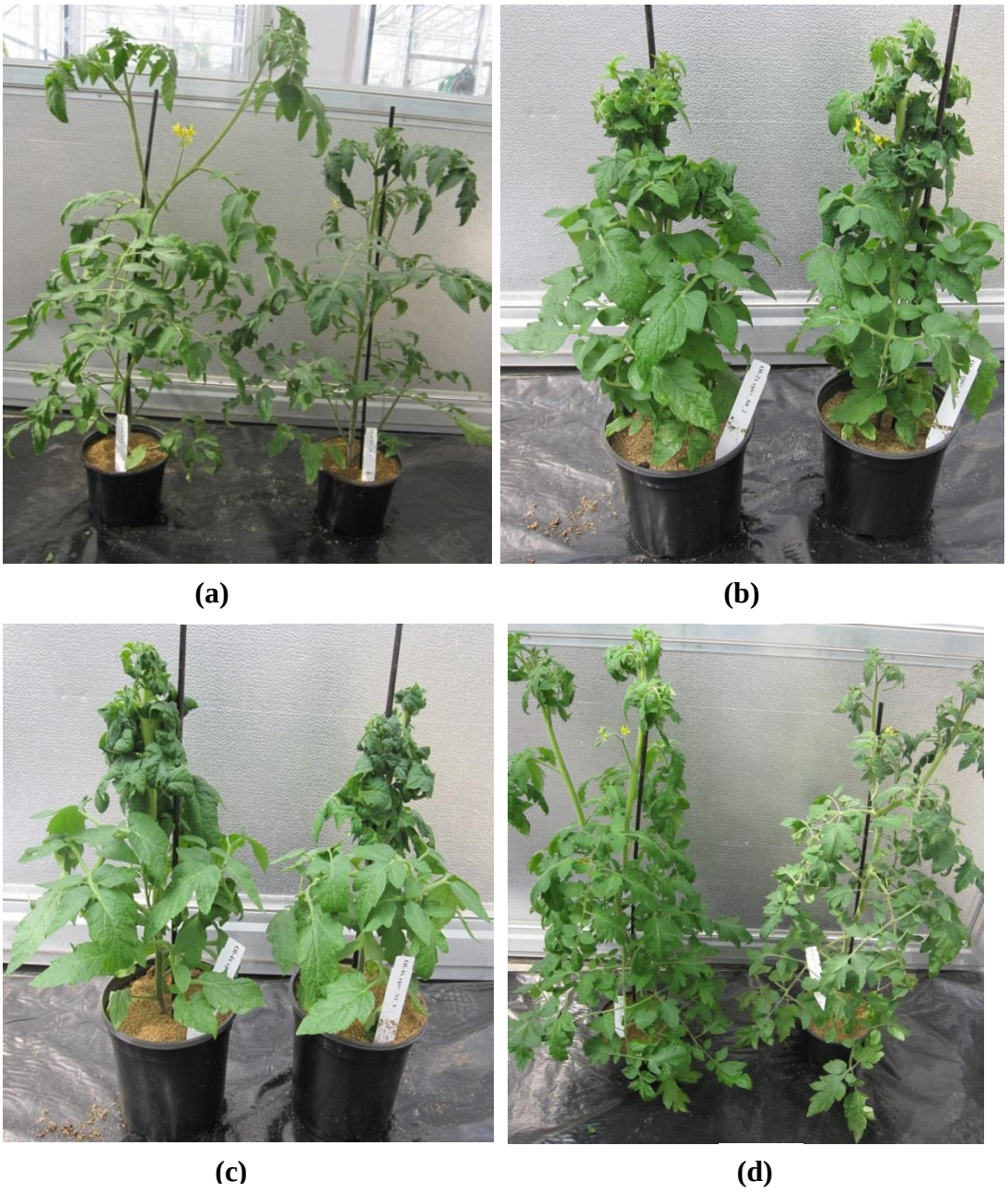


Figure 9. *Phenotypes of (a) ol-2x DEF, (b) ol-2 x epi, (c) Ol-4 x epi and (d) Ol-4-def mutant under PM infection (left) and combined PM and NaCl 50mM solution (right) at 15 dpi.*

3.4 Disease index

Disease index was measured both 10 and 15 days post inoculation (dpi) in the greenhouse. After 10 dpi, Ol-1 and its mutants became susceptible to disease but ol-2 and Ol-4 showed resistant to disease in both PM and combined PM & salt treatment (Figure 10).

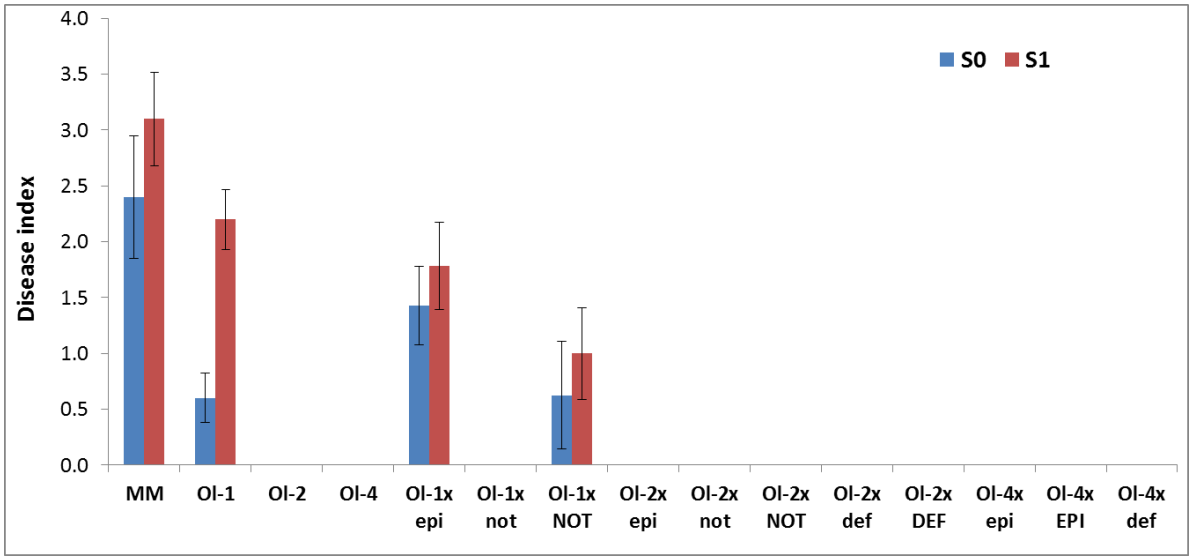


Figure 10. *Disease index under PM and combined PM with salt solution among different OL mutants along with Money maker (MM) after 10 dpi. S0= PM, S1= PM with 50 mM salt solution. DI is expressed in a scale from 0 (no conidiospore developed) to 5 .*

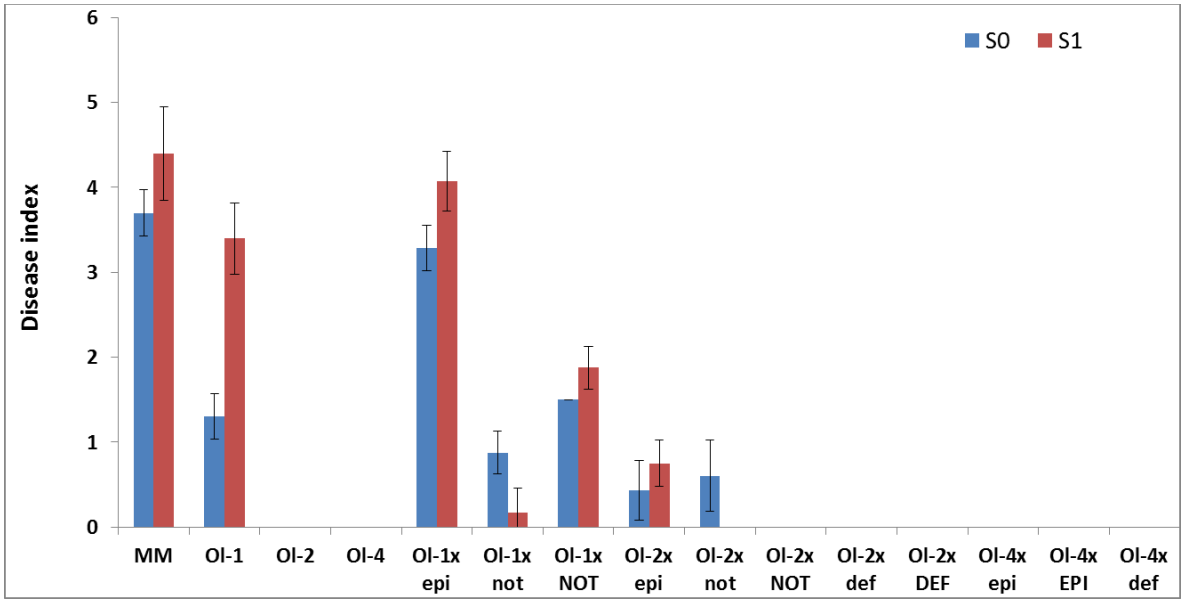


Figure 11. *Disease index under PM and combined PM with salt solution among different Ol mutants along with Money Maker (MM) after 15 dpi. S0= PM, S1= PM with 50 mM salt solution. DI is expressed in a scale from 0 (no conidiospore developed) to 5 .*

At 15 days post inoculation (dpi), disease index was increased more significantly in Ol-1, Ol-1 x epi, and Ol-1 x NOT mutants than at 10 dpi during combined PM and salt stress (Figure 11). The newly affected mutant at 15 dpi were Ol-1 x not, ol-2 x epi and ol-2 x not. So, epi mutant was found more susceptible in case of crossing with Ol-1 and ol-2 lines in both treatments. In case of Ol-1 x not mutant, disease index was significantly decreased in combined PM and salt treatment. There was no disease symptom in ol-2, Ol-4, Ol-2 x NOT, ol-2 x def, ol-2 x DEF, Ol-4 x epi, Ol-4 x EPI and Ol-4 x def crossing combination. Comparing all genotypes, the Money maker (MM) showed the highest disease susceptibility in both PM and combined PM & salt treatment (Figure 11).

The pictures of all leaflets were taken after 15 dpi and it was found that the Ol-1 x not and Ol-1 x NOT crossing combination showed moderate resistance in salt stress. The Ol line leaflets and their rachis turned blackish and soft resulting in more senescence. More fungal growth was found in Ol-1 x epi mutant. The leaflet of Ol-1 x not had far less fungal growth and was greener than other mutants whereas the leaflet of Ol-1 x NOT mutant was more yellowish , an indication of higher senescence (Figure 12). In case of ol-2 lines, ol-2- x epi and ol-2 x not mutants were infested but other ol-2 mutants remained without disease infestation (Figure 13). All Ol-4 mutants were remained without disease infestation during both treatments (Figure 14).

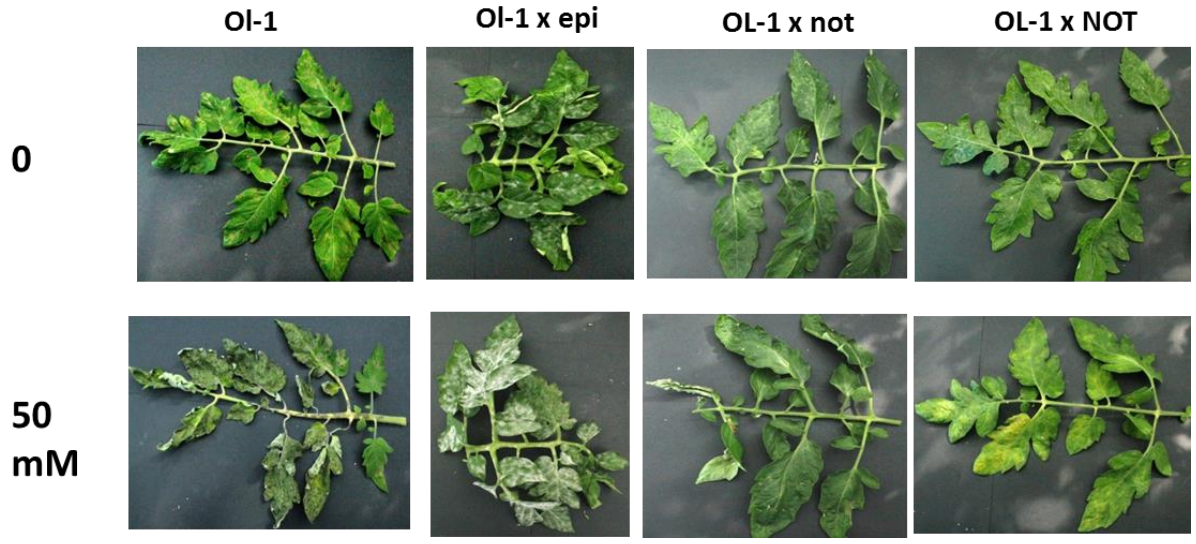


Figure 12. *Leaflets of Ol-1 line and its mutants, showing disease susceptibility in both treatments S0= PM, S1= PM with 50 mM salt solution at 15 dpi.*

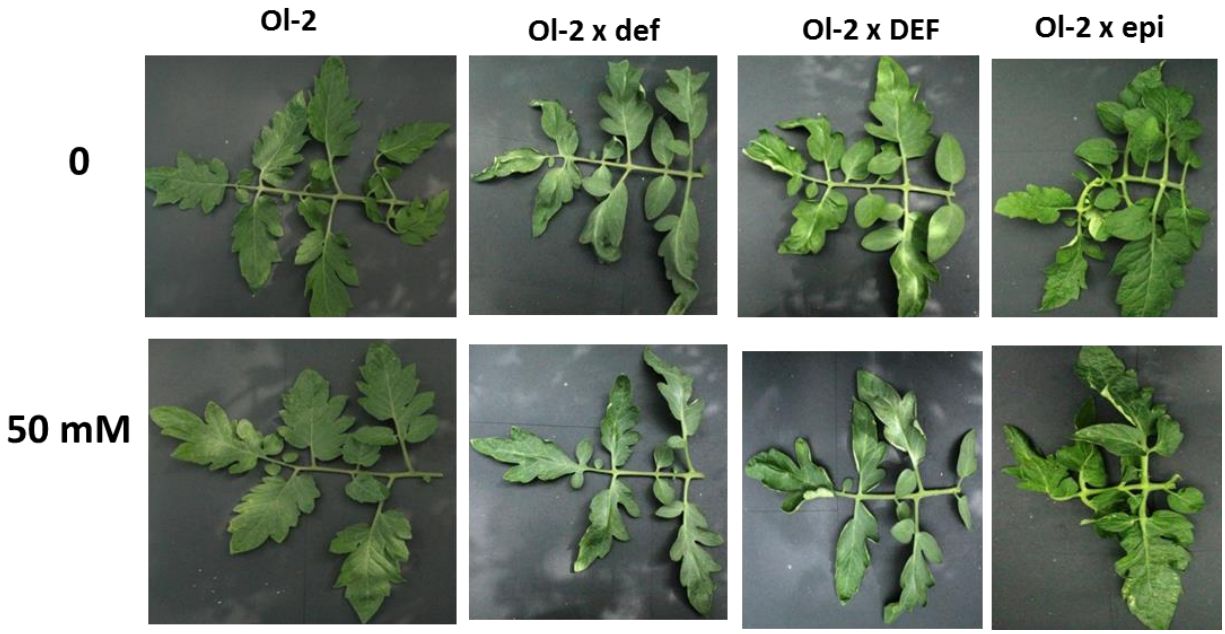


Figure 13. *Leaflets of ol-2 line and its mutants, showing disease resistance except ol-2 x epi crossing combination in both treatments S0= PM, S1= PM with 50 mM salt solution at 15 dpi.*

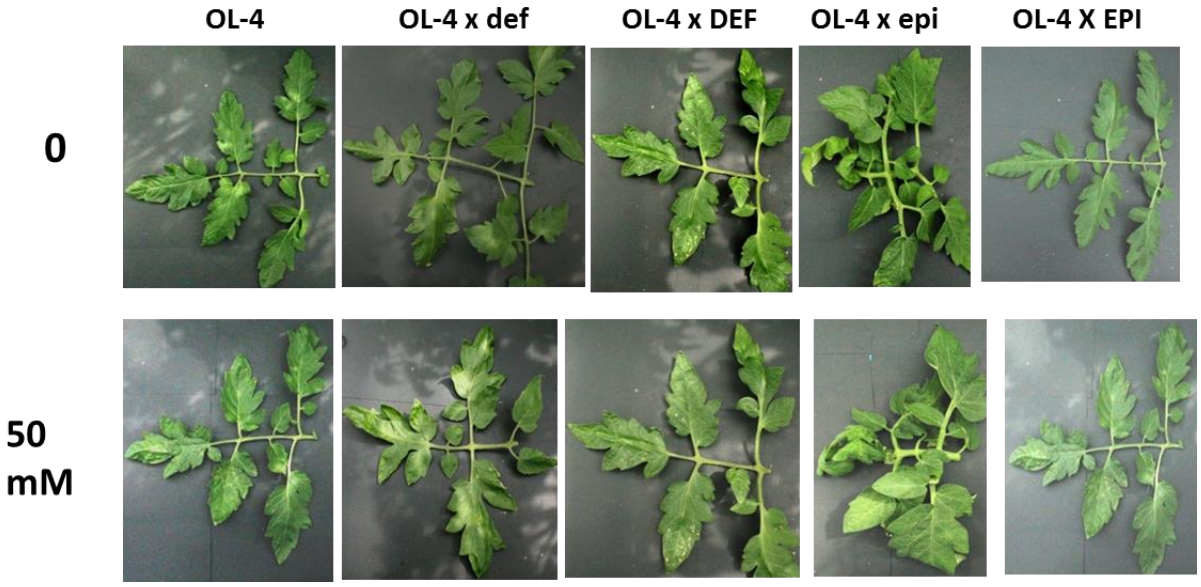


Figure 14. *Leaflets of Ol-4 line and its mutants, showing disease resistance in both treatments S0= PM, S1= PM with 50 mM salt solution at 15 dpi.*

3.5 Senescence

In general, senescence is increased during PM in combination with salt stress. After 10 dpi under stress combination, senescence was especially increased in MM and Ol-1 and slightly increased in, Ol-1 x epi, Ol-1 x NOT, Ol-4 x EPI and Ol-4 x def.

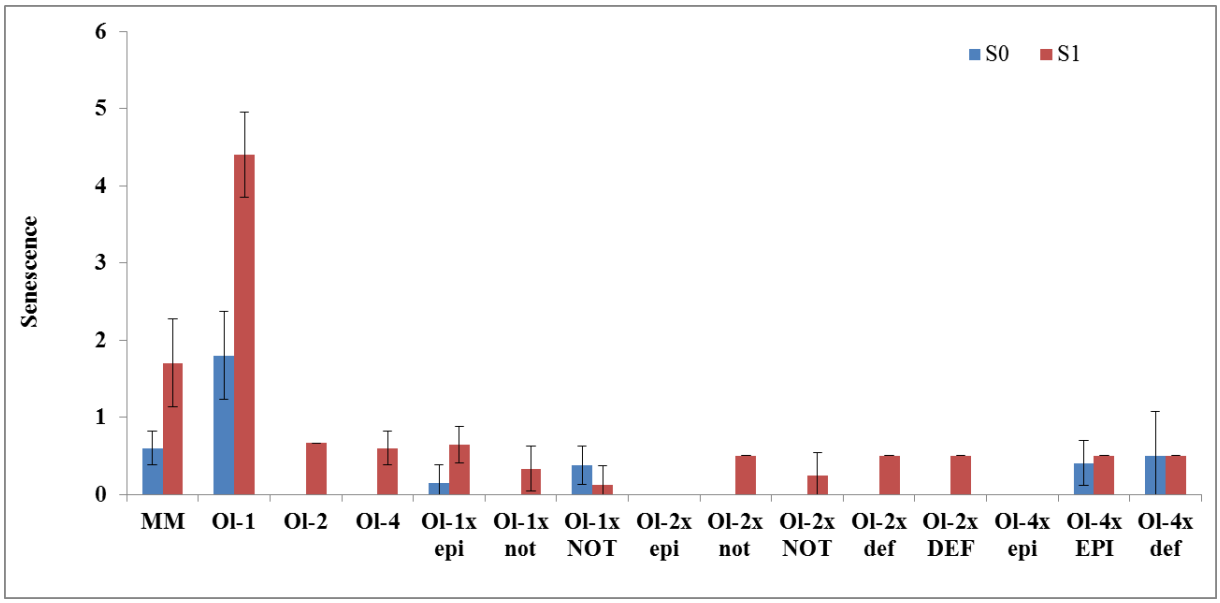


Figure 15. *Senescence under PM and combined PM with salt solution among different Ol mutants with Money maker (MM). S0= PM, S1= PM with 50 mM salt solution after 10 dpi . Error bars represent standard deviation.*

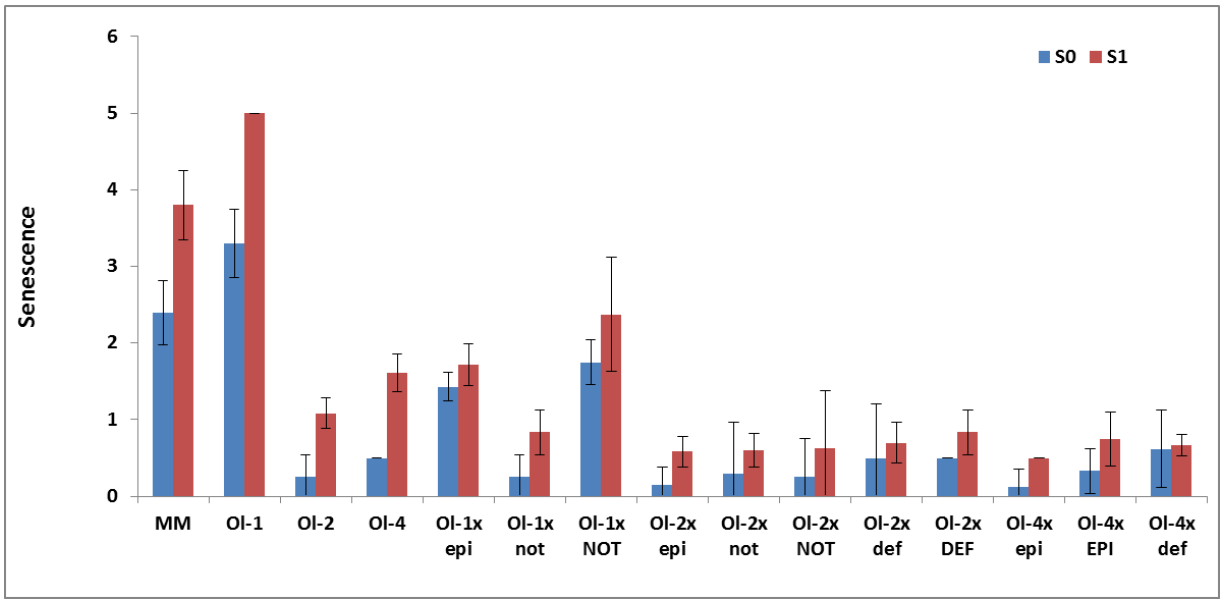


Figure 16. *Senescence under PM and combined PM with salt solution among different Ol mutants with Money maker (MM). S0= PM, S1= PM with 50 mM salt solution after 15 dpi . Error bars represent standard deviation.*

After 15 dpi, the senescence was increased in combined PM & salt treatment compared to only PM treatment in all genotypes. Senescence was significantly increased in all Ol-lines but the highest was found in Ol-1 line under both PM with salt stress Among the Ol-1 mutants Ol-1 x epi and Ol-1 x not showed reduced senescence and Ol-1 x NOT showed higher senescence than the Ol-1x not. In all ol-2 and Ol-4 hormone mutant combinations that senescence was not increased.

3.6 Gene Expression

The expression of 15 genes related to ABA metabolism, ROS signalling pathways, and the plant defense-related hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways was measured. Ol-1 line and the mutants Ol-1 x epi and Ol-1 x not (but not Ol-1 x def) showed treatment and genotype effects, they were used for further characterization by gene expression. ol-2 and Ol-4 genes and their mutants showed resistance to *O. neolycopersici* that wasn't highly affected in our experiments.

To calculate the relative gene expression of target gene, it is necessary to use appropriate 'housekeeping gene' to normalize the target gene expression data. In this experiment, EF1-a (Elongation factor 1-alpha) was used as a housekeeping gene.

3.6.1 ROS scavenging pathway

Both SOD and APX are ROS scavenging enzymes responsible for ROS detoxification. These two genes showed different expression levels in response to both treatments only PM and combined PM & salt stress. The expression level of SOD was increased during combined PM and salt treatment compared to PM treatment in all genotypes, among them significantly to MM, Ol-1, Ol-1 x not, Ol-1 x epi mutants. The highest level of gene expression was found in Ol-1 in both treatments (PM and combined PM & salt treatment) (Figure 17).

In case of APX gene, gene expression was unaltered in combined PM and salt stress, with the exception of in MM for which high variation between replicates was observed and therefore no conclusions can be made.

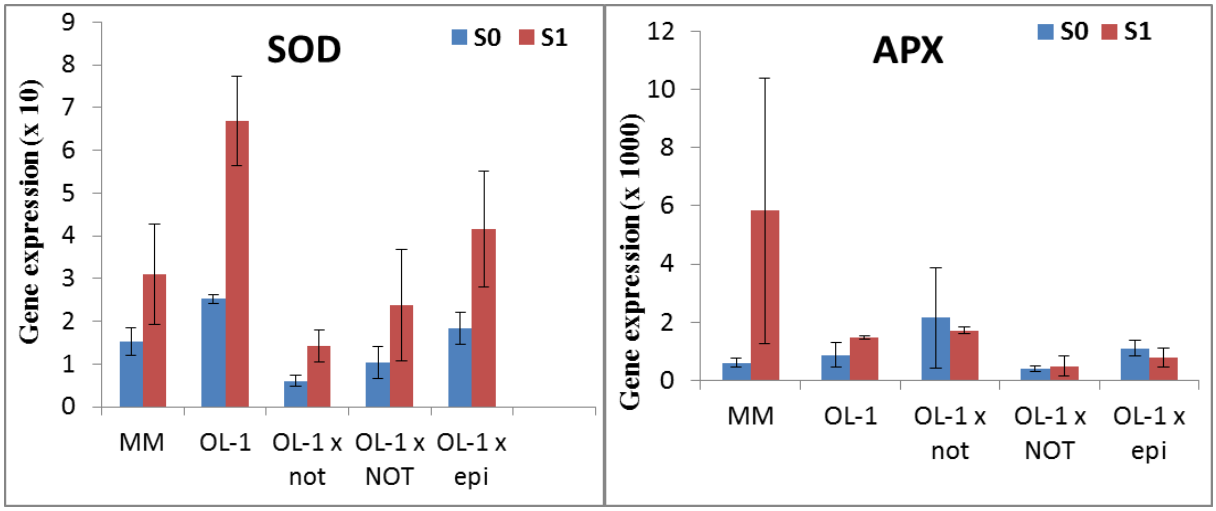


Figure 17. *Expression level of SOD and APX genes in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.2 NADPH oxidases pathway

NADPH oxidase creates superoxide by releasing electrons from NADPH inside the cell. This superoxide can spontaneously form hydrogen peroxide that can produce reactive oxygen species (ROS). The genes RBOHD and RBOHF are homologues known as respiratory burst oxidase and also responsible for promoting ROS production (Mersmann 2010).

In general, the RBOHD gene expression level was decreased in all the genotypes under combined PM & salt treatment. The level of expression was significantly decreased in MM, Ol-1 x not and Ol-1 x NOT mutants during combined PM and salt treatment. The highest gene expression level in both treatments was found in Ol-1 line, while in comparison Ol-1 mutants had lower expression in both treatments.

In case of RBOHF gene, the expression level was slightly increased under combined PM and salt stress and showed the highest expression level in MM genotype. Ol-1 x not mutant, had significantly lower expression in comparison to Ol-1 in both treatments.

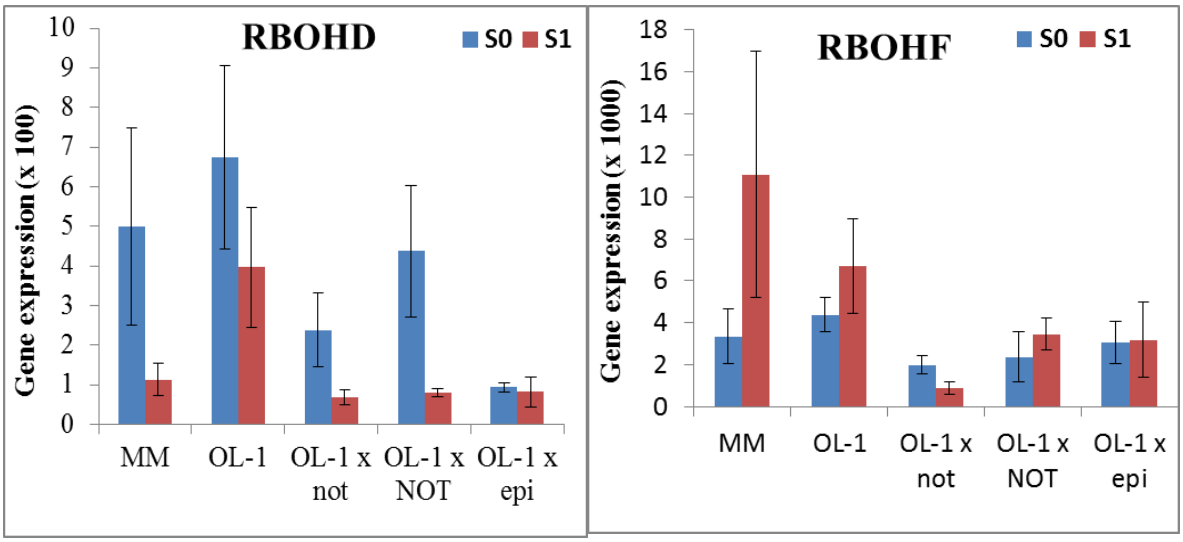


Figure 18. *Expression level of RBOHD and RBOHF genes in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.3 Abscisic acid pathway

NCED (9-*cis*-epoxycarotenoid dioxygenase) gene is involved in ABA and is the gene that is mutated in the notabilis mutant (frameshift mutation). In case of NCED gene, the level of expression was significantly increased in Ol-1 under combined PM and salt treatment, in comparison to the other genotypes where no significant differences were observed. Gene expression level was significantly decreased in Ol-1 x not mutant during combined PM and salt treatment.

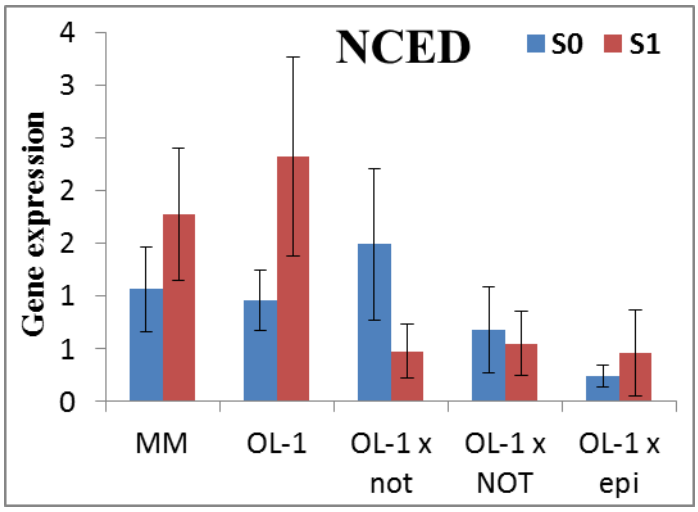


Figure 19. *Expression level of NCED gene in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.4 Salicylic acid pathway

Salicylic acid (SA) is an important signal molecule in plants. PAL is the rate limiting biosynthetic enzyme of the phenylpropanoid pathway and is induced during abiotic and biotic stress condition (Chen et al. 2009).

The PAL gene expression was significantly decreased in case of MM, Ol-1 and Ol-1 x not mutant during combined PM and salt treatment. In case of Ol-1 x NOT mutant, the PAL gene expression level was slightly increased in combined PM and salt treatment. The highest gene expression level was found in Ol-1 genotype during PM treatment among the all genotypes.

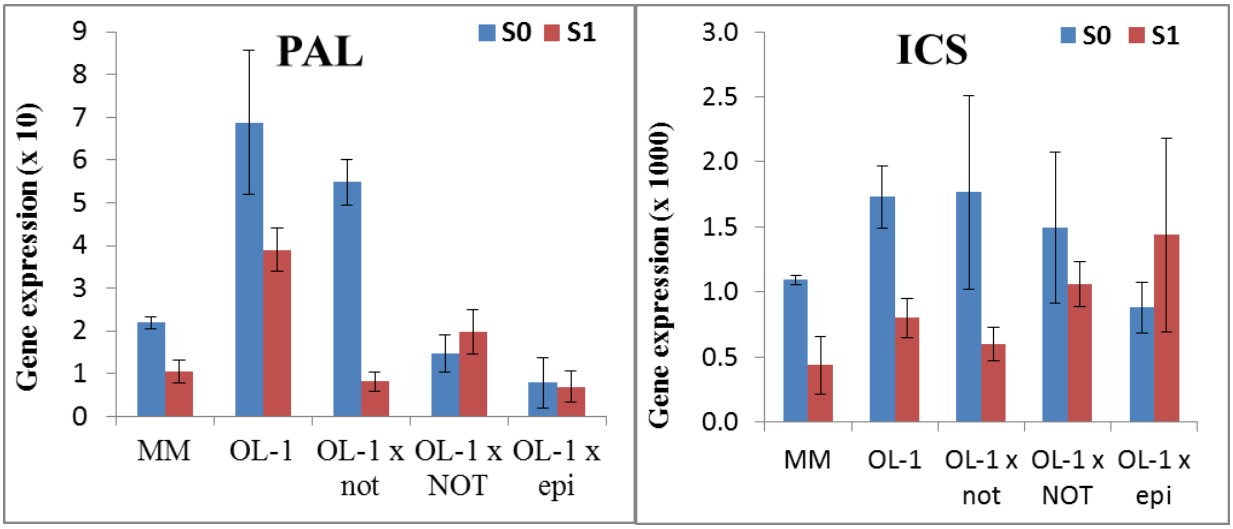


Figure 20. *Expression level of PAL and ICS genes in PM and combined PM & salt treatment. Error bars represent standard deviation.*

ICS gene expression pattern was similar and decreased significantly to that of PAL in case of MM, Ol-1 and Ol-1 x not mutant during combined PM and salt treatment. The Ol-1 line had the highest expression of both PAL and ICS genes under PM treatment.

3.6.5 Jasmonic acid pathway

JA is lipid-derived signals and synthesized by the octadecanoid pathway. In tomato like other plant species, JA is the specific signal in wound response to necrotrophic pathogens. The defenceless (def) mutant of tomato has limited ability to raise JA signalling level after wounding and produce little protease inhibitor and more susceptible to herbivore attack (Howe et al. 1996). Allene oxide synthase (AOS) is a key enzyme for the biosynthesis of jasmonic acid (JA).

The level of AOS gene expression was increased significantly in case of MM, Ol-1, Ol-1 x NOT during combined PM and salt treatment compare to PM treatment. The highest gene expression was found in Ol-1 line among the all genotypes during combined PM and salt treatment. The level of gene did not change in Ol-1 x not and Ol-1x epi crossing combinations.

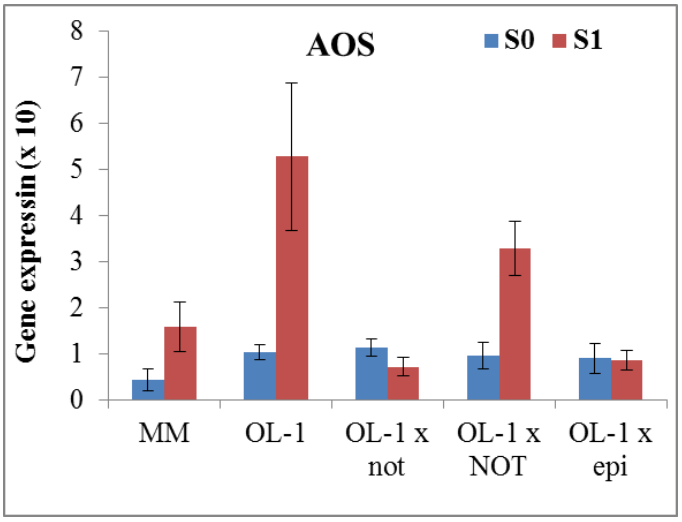


Figure 21. *Expression level of AOS gene in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.6 Ethylene pathway

Beside the function of ripening, ethylene is involved in abiotic and biotic stress responses (Gazzarrini 2001). Ethylene is formed from methionine via S-adenosyl-l-methionine (AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC).

In case of ACCase gene, the expression was significantly increased in MM and Ol-1 line during combined PM and salt treatment and strong up regulated expression found in Ol-1 line. Gene expression was decreased significantly in Ol-1 x not mutant during combined PM and salt treatment.

On the contrary, the level of ACO3 gene expression was markedly increased in Ol-1 x not in combined PM and salt stress compare to PM. In case of MM and Ol-1 line, the expression level was significantly decreased during combined PM and salt treatment.

The expression level of ERF2 (Ethylene Response Factor 2) gene was decreased significantly in all genotypes during combined PM & salt treatment. Among the all genotypes, the gene expression was remarkably decreased in Ol-1 x not mutant during combined PM & salt treatment among the all genotypes.

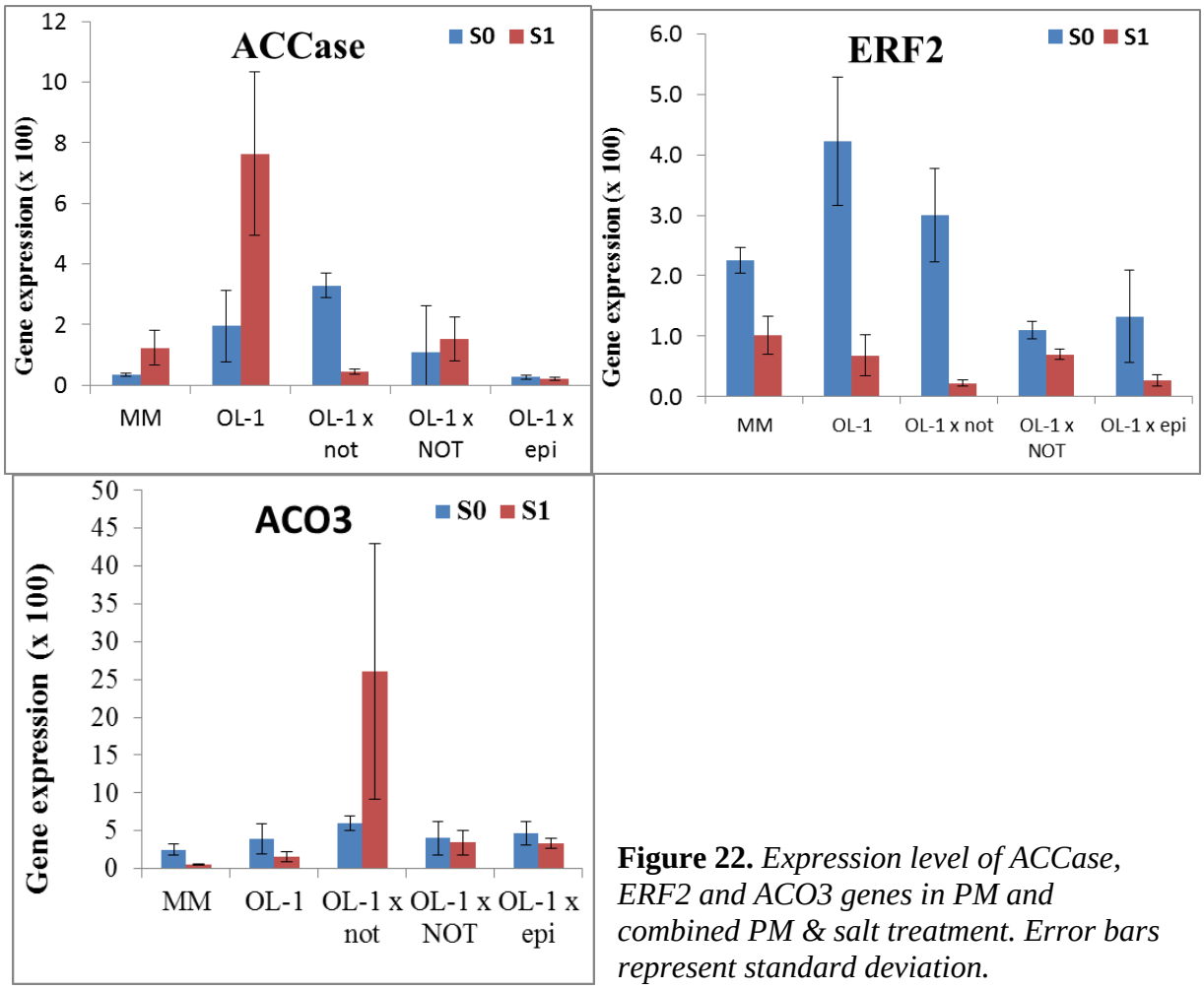


Figure 22. *Expression level of ACCase, ERF2 and ACO3 genes in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.7 Apoptosis cell death pathway

CYP3 and MCA are involved in apoptotic cell death in plants. Cysteine proteases (CYPs) is the key enzyme for programmed cell death (PCD) or apoptosis in plant and PCD is initiated by generating reactive oxygen species (ROS) (Jabs et al., 1996). The level of CYP3 gene expression was slightly increased in MM, Ol-1 and Ol-1x NOT during combined PM and salt treatment than only PM treatment. The highest gene expression found in Ol-1 in both treatments (PM and combined PM & salt treatment). In Ol-1 x not and Ol-1 x epi mutants, the CYP3 gene expression level was slightly decreased in combined PM and salt treatment. The lowest gene expression was observed in Ol-1 x epi among all of the genotypes during both treatments (PM and combined PM and salt).

In general, plant metacaspases gene is responsible for programmed cell death (PCD) and this PCD occurs in response to various biotic and abiotic stresses (Frank et al. 2003). Two types of metacaspase, type I and type II are present in tomato in which type II has already been characterized (MCA). The level of MCA gene expression was decreased in all genotypes at

combined PM & salt treatment compare to PM treatment. The MCA gene expression was significantly higher in Ol-1 during PM treatment among all genotypes. The gene expression was the lowest in Ol-1 x epi among all of the genotypes during both treatments (PM and combined PM and salt).

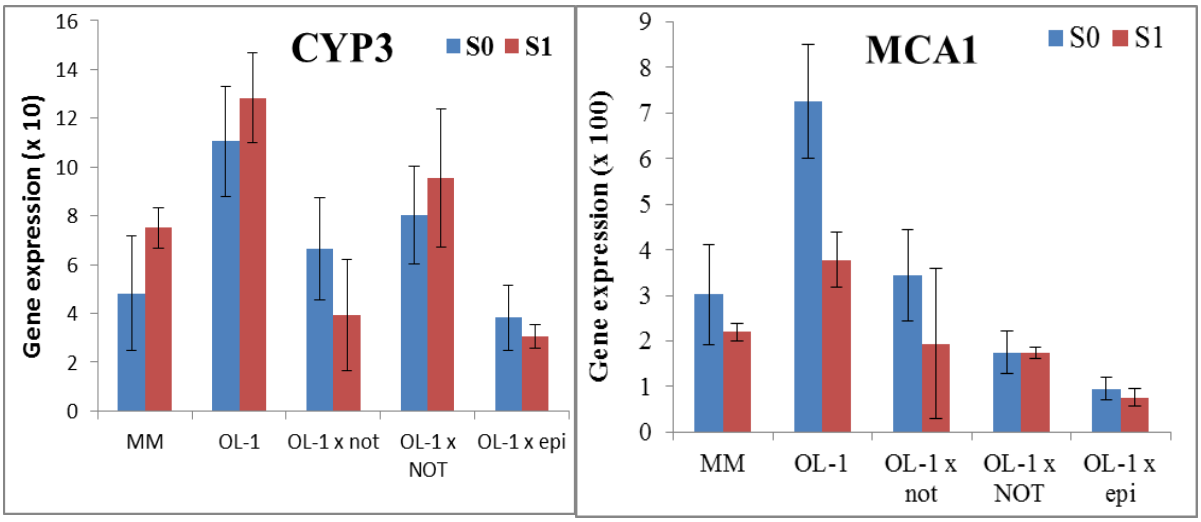


Figure 23. *Expression level of CYP3 and MCA1 genes in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.8 Cytokinin regulating enzymes

CKX (cytokinin oxidase/dehydrogenase) plays a vital role and maintains the homeostasis of endogenous CK levels required for plant growth and development in plant (Kamínek et al. 1997). The level of CKX gene expression was slightly increased during combined PM and salt treatment. The highest gene expression was found in Ol-1 x epi mutant among the all genotypes in both treatments (PM and combined PM & salt).

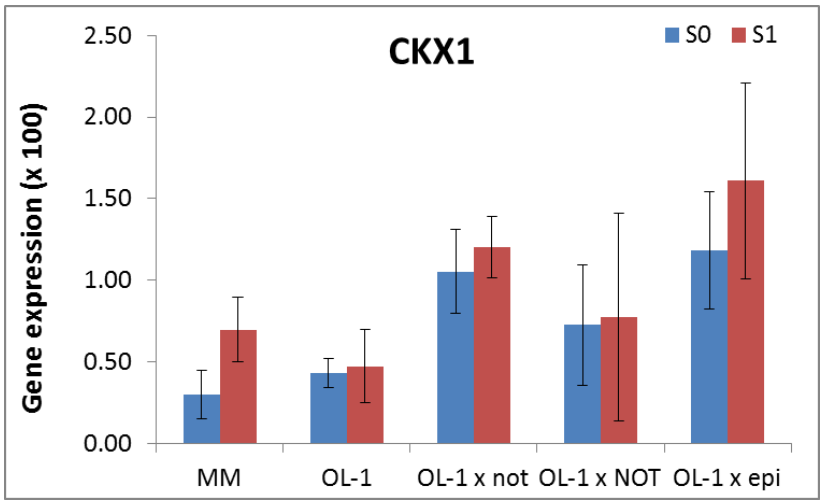


Figure 24. *Expression level of CKX1 gene in PM and combined PM & salt treatment. Error bars represent standard deviation.*

3.6.9 Phloem unloading pathway

LIN6 (Extracellular invertase) is the key enzyme of an apoplastic phloem unloading pathway and catalyses the hydrolytic cleavage of the transport sucrose. In young plant, LIN6 is expressed in the veins of leaves. In case of LIN6 gene, the level of gene expression was significantly decreased in MM, Ol-1 and Ol-1 x not crossing combination during combined salt and PM treatment. The gene expression level had the highest in case of Ol-1 x not mutant among all genotypes at PM treatment. The gene expression was the lowest in Ol-1 x epi among all of the genotypes during both treatments (PM and combined PM and salt).

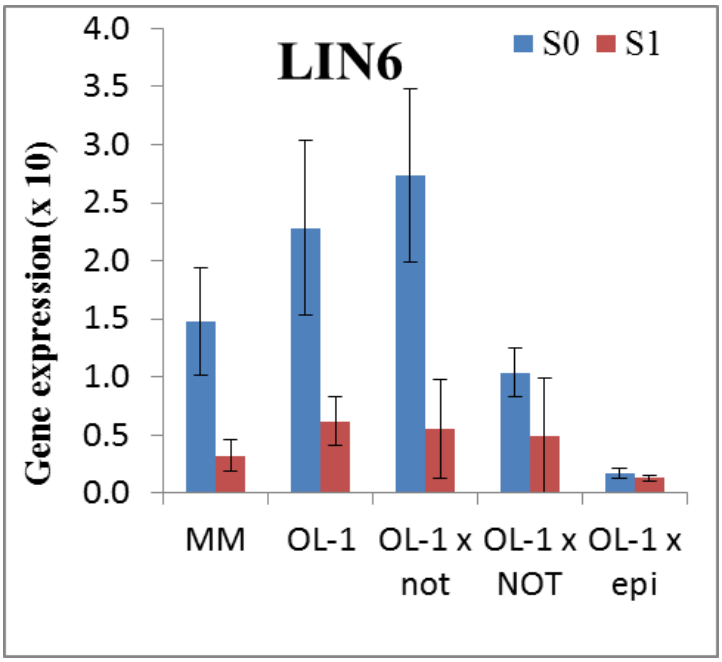


Figure 25. *Expression level of LIN6 gene in PM and combined PM & salt treatment. Error bars represent standard deviation.*

4. DISCUSSION

4.1 Selection and confirmation of Ol mutants

SCAR (Sequence Characterized Amplified Region) is PCR based co-dominant marker. This marker is designed from nucleotide sequence which is cloned from DNA fragment that is linked with trait of interest (Kiran et al. 2010). The developed SCAR marker is used to identify the target allele from related species by giving single, distinct band of the desired allele. The maker can differentiate homozygous and heterozygous characteristics of same species. So, we used three SCAR deletion markers to detect the polymorphism between allele of a gene in a particular Ol-gene in this study. This SCAR deletion markers can distinguish between homozygous and heterozygous line. However in Ol-1 genotype the alleles could not be distinguished. Probably, the reason is that the Ol-1 genotype used has the region fine mapped and the marker `H9A11' lies outside this region and therefore corresponds to MM allele. The marker co-segregated perfectly with resistance in all Ol-1 segregating populations.

4.2 Plant height and chlorophyll content

In this study, we measured plant height 7 days after salt stress (50 mM) application and before the powdery mildew inoculation, with no significant differences observed between the stressed and control plants. The seed germination, stem height, fresh and dry weight of stem, leaves, and root of tomato plant were decreased by salt stress (Zhou et al. 2009). The low salt stress concentration and short interval after its application perhaps resulted in no significant differences. The plant height decreased with increasing salinity in all tomato plants (Sunarti 2012), but significant differences were observed at higher salt concentrations (100, 150mM NaCl).

During combined PM and salt stress, the chlorophyll content was increased as the level of salt stress increased (Sunarti 2012). On the other hand adverse effects on membrane stability was found after salt stress resulting decreased chlorophyll content of leaves of different tomato cultivars (Sholi 2012). In this study, chlorophyll content was also not affected significantly due to low concentration of salt (50 mM). These differences may also be a result of different experimental conditions like, the developmental stage of the plants or other environmental factors such as temperature and irradiance.

4.3 Phenotypic performance

4.3.1 Disease index

The Ol-1, ol-2 and Ol-4 lines crossed with the hormone mutants were assessed for their response to combined powdery mildew infection and salt stress to investigate the involvement of different hormonal pathways (ABA, JA and ET) to resistance against combined PM and salt stress. ABA is well known as a stress hormone, it is especially important for abiotic stress. Salicylic acid (SA) and jasmonic acid /ethylene signalling pathways are defence hormones that are important in the response to biotic stress.

The Ol-1 gene was mapped as a cluster together with the Ol-5 gene from wild tomato species *S. habrochaites* (Bai et al. 2005). The Ol-1 gene confers non-race specific resistance and showed slow HR (delayed cell death) during fungal attack. In salt stress condition, Ol-1 was affected by *O. neolycopersici* and its crosses with mutants showed a differential response. The most significant changes were observed with Ol-1 x epi mutant which showed high susceptibility under PM infection only and was further increased in salt stress combination. Fusarium wilt of tomato (*Fusarium oxysporum* f. sp. *Lycopersici*) and inhibition of vegetative growth was induced by ethylene (Primrose 1979). So, in our study, ethylene overproducer mutant may be influenced disease during crossing with Ol-1. On the other hand Ol-1x not showed decreased susceptibility and senescence especially under combined stress. The tomato mutant *sitiens* (ABA-deficient) was shown more resistant than money maker to *Oidium neolycopersici* (Achuo et al. 2006) and according to (Curvers et al. 2010) an ABA deficient mutant increased the permeability of cuticle which leaded to disease resistance. These two studies may be linked with our study.

The recessive gene ol-2 confers race-non-specific resistance via papilla formation, a non-HR-based mechanism (Bai et al. 2005) and is homologous to the recessive barley gene *mlo* that confers durable resistance to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) through the apposition of papillae at the cell wall (Piffanelli et al. 2002). During combined PM and salt stress, ethylene overproducer gene (epi) and ABA deficient mutant (not) compromised disease when combined with the ol-2 resistance gene. It may be concluded that excess ET makes the plant susceptible to PM. Similarly, an , epi mutant (ethylene overproducer) of tomato showed susceptibility *to Botrytis cinerea* compared with its wild-type progenitor (Díaz et al. 2002).

ABA may be important for resistance conferred by the ol-2 gene. ABA helps the ol-2 gene to confer resistance to PM probably by inducing callose deposition (Bai et al. 2008). Here, ol-2 without ABA (ol-2 x not) was not able to confer resistance probably because the low ABA levels negatively affect callose deposition, making the cell wall vulnerable to extention of the PM fungal spore. According to (Flors et al. 2005) ABA has an important role in helping the plant to deposite callose during pathogen attack.

The ROS production, MAP kinase signaling and different plant hormone (SA, ET and JA) signaling are triggered by PTI and ETI. (Tsuda and Katagiri 2010). Ol-4 is an R-gene that confers resistance through fast HR which triggers apoptosis-like PCD during PM infection. (Mang et al. 2012) found that both high ABA level and high temperature suppressed the plant defense responses by inhibiting specific R gene function. In addition, Systematic acquired resistance was suppressed and ABA level was increased by up to 3.6-fold during salt stress (Yasuda et al. 2008) which reduced nuclear accumulation of R proteins SNC1 and RPS4 in Arabidopsis resulting in compromised disease resistance. However both SNC1 and RPS4 are TIR types of NB-LRR R proteins that have nuclear localization, while CC types of R proteins, such as the Mi-type R-gene that Ol-4 encodes, do not localize to nucleus. Also the abiotic stress used is different (salt vs heat) so there may be different responses induced. However, we observed that Ol-4 resistance was not affected in any of the treatments or hormone mutant combinations.

4.3.2 Senescence

Senescence is a common phenomenon of the plant developmental process that finally leads to plant cell death through a genetically regulated process. Accumulation of toxic ions during salt stress (e.g. Na^+) in the leaf may result in induced leaf senescence. Plant hormones are involved in regulating the senescence process. In addition, oxidative burst leads to leaf senescence (Sedigheh et al. 2011) which occur by rapid release of large amount of reactive oxygen species (ROS) like superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen etc. from different plant cell specially in chloroplast in response to various abiotic and biotic stresses. As oxidative burst is an outcome of both salt stress and pathogen infection, the combination of those different stresses may differentially influence leaf senescence and thus it was increased during combined PM and salt stress differently in the three Ol- lines and their mutants on the basis of their resistance ability against pathogen. Senescence was not significantly affected in ol-2 and Ol-4 and their mutants. Probably because senescence under combined stress is

amplified because of higher pathogen susceptibility, as these line were resistant also senescence was not affected.

4.4 Gene expression analysis

The crosstalk between biotic and abiotic interaction and the molecular mechanisms that are involved can be discovered by gene expression analysis. The expression of genes that are important in hormone synthesis pathways were found to be affected during abiotic and biotic stress, and give insight in the importance of these hormonal pathways for PM resistance. The Ol-1 lines and the mutants (Ol-1 x epi and Ol-1 x not) showed treatment and genotype effects, so they were used for further characterization by gene expression. The gene expression results for different hormonal pathways are discussed below.

4.4.1 Abscisic acid pathway

NCED is involved in ABA biosynthesis and ABA has an antagonistic interaction with other hormones SA/JA/ET. Balance between ABA and SA/JA/ET hormone signalling is an important determinant for successful defence against migratory nematode *Hirschmanniella oryzae* in rice (Nahar et al. 2012). ABA signalling has a complex function in Arabidopsis basal resistance, negatively regulating SA/JA/ET- mediated resistance to biotrophic and necrotrophic fungi. Ol-1 x not crossing combination is ABA deficient mutant and shows reduced susceptibility and senescence under combined stress, potentially linking ABA production with these responses under combined stress (Figure 19). The level of expression of this NCED gene was the highest in Ol-l line under combined stress means more ABA biosynthesis occurs in this line, which probably results in disease susceptibility and also increased senescence.

4.4.2 Salicylic acid pathway

PAL is the rate limiting enzyme for secondary metabolites and indirectly influences SA biosynthesis, whereas ICS is the key enzyme for SA synthesis (D'Maris Amick Dempsey et al. 2011). The main precursor of SA synthesis in plant is Phenylalanine and PAL acts as catalysing agent for the conversion of phenylalanine to trans-cinnamic acid (D'Maris Amick Dempsey et al. 2011). In case of Ol-1 line, during PM treatment, higher ICS expression is observed in comparison to the other genotypes which correlates with its higher resistance to PM. Both PAL and ICS genes were down regulated during combined PM and salt treatment

means that probably down regulation of secondary metabolism and decreased SA biosynthesis triggered more disease susceptibility (Figure 20). However the same trend was also observed in Ol-1x not, which showed no increase in susceptibility under combined stress, therefore other mechanisms are probably involved in its resistance.

4.4.3 Jasmonic acid pathway

JA plays a regulatory role in response to necrotrophic pathogen attack and senescence (Breeze et al. 2011). SA and JA interact antagonistically. AOS is the key enzyme for JA biosynthesis (Wasternack et al. 2006). Higher levels of AOS gene expression in all genotypes except Ol-1x not under combined stress may be linked to higher levels of JA produced and lower SA biosynthesis, which would make the plant more susceptible to disease with increased senescence (Figure 21).

4.4.4 Ethylene pathway

The plant hormone ethylene is involved in many physiological processes, including plant growth, development and senescence. Ethylene also plays a pivotal role in plant response or adaptation under biotic and abiotic stress conditions. Both ACCase and ACO3 (ACC oxidase 3) are responsible for biosynthesis of ethylene. The level of ACCase gene expression was the highest in OL-1 line during combined stress whereas in case of ACO3 (ACC oxidase 3) gene was down regulated in all genotypes except Ol-1 x not mutant during combined stress (Figure 22). Ethylene also triggers the biosynthesis of abscisic acid (ABA), and mainly through the stimulated ABA it mediates stomatal closure which limits photosynthetic activity, biomass production, and induces growth inhibition and senescence accompanied by an overproduction of reactive oxygen species (Grossmann 2003). Ethylene responsive factors (ERF2) are also important plant-specific transcription factors. Expression of the ERF2 gene in tomato was induced by ethylene, MeJA, abscisic acid (ABA) and salt treatment indicating that ERF2 might act as a connector among different signal transduction pathways. However, in this experiment, the ERF2 expression was highly decreased under combined stress compared to PM aloneprobably the PM defense interacts with the ERF2 response to salt stress..

4.4.5 ROS scavenging pathway

When plants are exposed to stressful environmental conditions like salt stress, the production of Reactive Oxygen Species (ROS) increases and can cause significant damage to the cells. Antioxidant defences, which can detoxify ROS, are present in plants. Both SOD and APX are ROS scavenging enzymes responsible for ROS detoxification and they are the first line defence against reactive oxygen species (ROS) under biotic and abiotic stress (Sharma et al. 2012). APX plays a key role catalysing the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor (Apel and Hirt 2004). During combined PM and salt stress, SOD gene transcriptional level was found up-regulated in all genotypes resulting huge amount of ROS scavenging was produced to combat with ROS in cell and tried to balancing between them (Figure 17). The expression of APX genes is regulated in response to biotic and abiotic stresses as well as during plant development (Caverzan et al. 2012). The differential regulation of SOD and APX may indicate unbalanced ROS detoxification, as SOD detoxifies superoxide to H_2O_2 which needs to further be reduced to H_2O by APX, however APX expression was down regulated. This imbalance may lead to the symptoms of increased senescence observed under combined stress.

4.4.6 NADPH oxidases pathway

NADPH oxidases generate reactive oxygen species during ROS mediated signalling in plants. The level of RBOHD gene expression was found to be down regulated in all genotypes during combined stress (Figure 18). A lack of RBOHD expression may lead to very low levels of ROS production, resulting in low HR that may lead to plant susceptibility in stress condition. RBOHD-dependent ROS generation is associated with pathogen recognition during the oxidative burst linked with PTI, ETI and hypersensitive response (HR). In case of RBOHF gene, the expression analysis was difficult to explain due to show opposite behaviour of RBOHD. Functional RBOHD triggers cell death during pathogen attack but simultaneously inhibits death in neighboring cells through the suppression of free salicylic acid and ethylene levels (Pogány et al. 2009). Therefore its reduced expression may lead to the spread of cell death, and the increased senescence symptoms observed in Ol-1.

4.4.7 Apoptosis cell death pathway

Cyp3 (Cysteine proteases) and MCA1 (metacaspases) are responsible for apoptosis (programmed cell death; PCD) cell death in plants. This PCD is initiated by generating

reactive oxygen species (ROS). In Arabidopsis thaliana, the type I metacaspases gene expression was up regulated by bacterial infection and type II metacaspases was involved in PCD which was caused by fungal attack (Watanabe and Lam 2011)). In pepper (*Capsicum annuum)* the metacaspases gene (Camc9) was also involved in PCD and silencing of this gene enhanced disease resistance whereas overexpression of this gene made the plant susceptible to disease (Kim et al. 2013). The level of expression of both CYP3 and MCA1 genes were down regulated in Ol-1 x not mutant, as this mutant is ABA deficient. And this may explain its increased resistance, however similarly reduced expression was observed in Ol-1x epi mutant so no solid conclusions can be made (Figure 23).

4.4.8 Cytokinin regulating enzymes

Cytokinins are hormones that play an essential role in plant growth and development. Cytokinin oxidase open reading frame called CKX1 also induced by abscisic acid, which may control cytokinin oxidase expression. CKX1 is the catabolic enzyme of Cytokinin (CK). More expression of CKX1, results in lower cytokinin levels. CKX1 gene was not significantly changed during combined PM and salt stress, so its involvement in symptom development cannot be concluded (Figure 24).

4.4.9 LIN6

Extracellular invertase Lin6, is the key enzyme of the apoplastic phloem unloading pathway. The expression of LIN6 gene, is an inducible component of the defence/stress responses. The expression of LIN6 gene was up-regulated by various abiotic and biotic stresses. (Roitsch et al. 2003). Expression of LIN6 was reduced under combined stress. Down regulation of cell wall invertases results in increased susceptibility to pathogens, probably due to the decreased availability of carbohydrates for the defence responses at the site of infection (Essmann et al. 2008). Lin6 is possibly a target for differental hormonal regulatory pathways, and may also be linked to senescence. Lin6 expression in the Ol-1 x epi mutant was remarkably low, which may be linked to the retarded growth of the epi mutants under all conditions (Figure 25).

5. CONCLUSIONS

1. Under combined PM and salt stress, Ol-1 resistance was negatively affected, while ol-2 and Ol-4 were still completely resistant to PM under salt stress

2. The rate of senescence under combined PM and salt stress was high in Ol-1 gene whereas less senescence was found in the resistant, under these conditions, ol-2 and Ol-4 genes .

3. The crosses of Ol-1, ol-2 and Ol-4 with the mutants ‘epi’ and ‘not’ had pleiotropic effects in growth.

4. The susceptibility of Ol-1 was increased in the epi (ethylene overproducing) mutant and reduced in not (ABA deficient mutant) in both powdery mildew and salt stress.

6. Gene expression analysis in Ol-1 line and its crossing combination with epi and not mutants showed that reduced expression of SA biosynthesis may trigger disease susceptibility and high expression of JA biosynthesis result in greater senescence under combined stress. RBOHD and LIN6 had lower expression in combined PM and salt stress and they may be related to response to combined stress.

6. RECOMMENDATIONS

1. Experiment should be repeated with materials that have the gene fixed and is not initially segregating because the duration of selection and the handling genotypes may have caused unintended effects.

2. Content of hormones such as ABA, JA, ET in the Ol- lines and its mutant combinations should be measured under both treatment to verify the effect of the mutations and directly link hormone content with the phenotype.

3. Whole genome expression analysis in genotypes with different response (Ol-1 and Ol-1 x not) to measure the precise effect of treatments and the mutants on on expression of defense responses.

4. For PM resistance breeding, Ol-4 will be the best line for combined stress condition because Ol-4 is based on resistance gene (R-gene) and was resistant in all treatment and hormone mutant crossing combinations

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