BABA in priming tomato for enhanced tolerance to drought, salinity and fungal stress and combinations thereof

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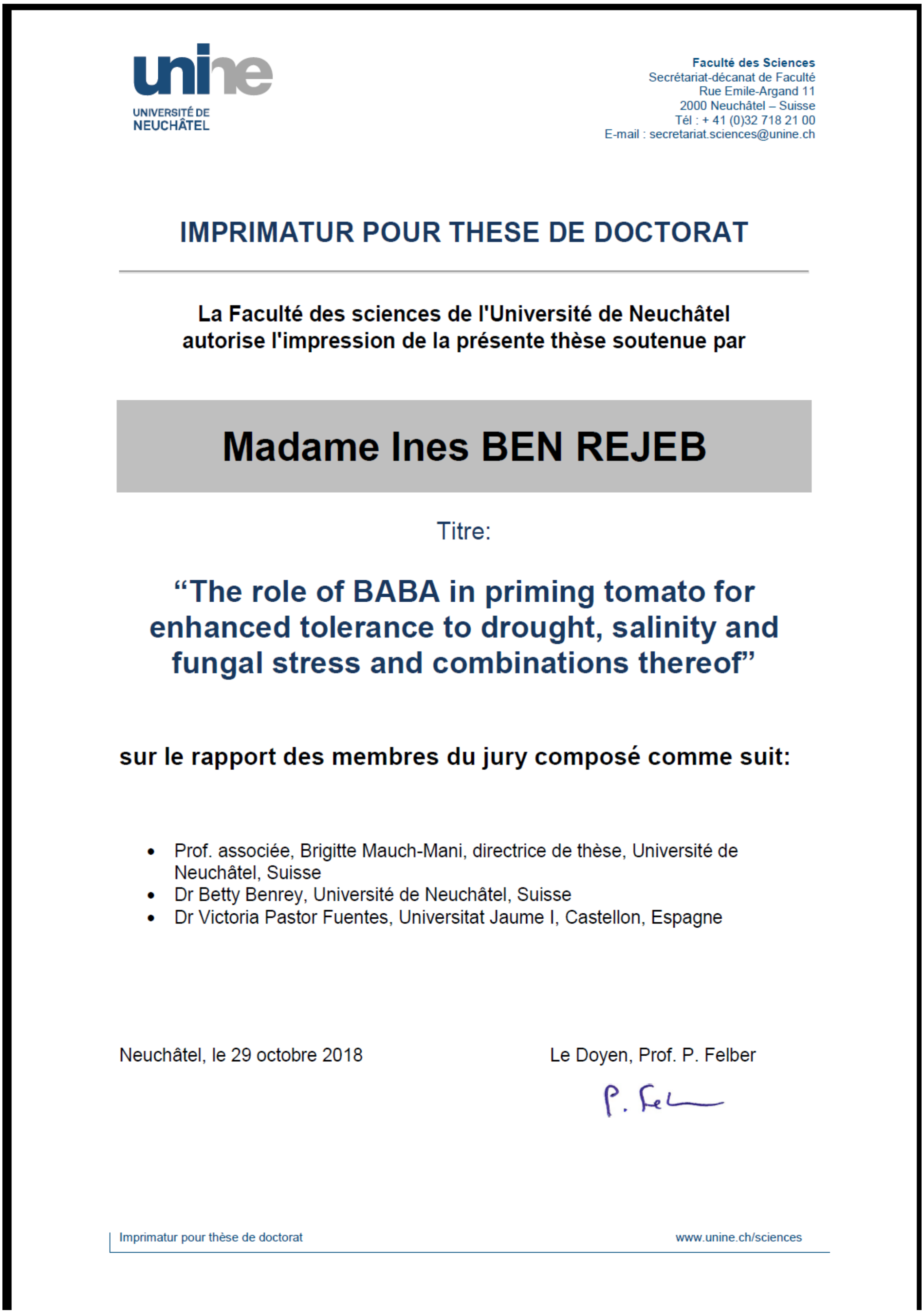

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Abstract

Tomatoes are among the most produced and consumed vegetable-fruit in many countries and is considered an economically important crop. However, drought, salinity and pathogen infection are the main causes of crop yield reduction and variability. Although plants have developed defensive capacities against these stresses, but plant tolerance and resistance depend on the degree and intensity of stress and on the species. In this thesis, we investigated the role of β-aminobutyric acid (BABA) in the induction of tolerance and resistance against drought stress, salt stress and combination of salinity and infection with *Botrytis cinerea* in two tomato cultivars (cv Marmande: a stress-resistant cultivar and cv Coeur de Boeuf: a stress-sensitive cultivar). Our results showed that BABA significantly reduces the harmful effects of a single and combined stress. Treated tomato plants with BABA in response to drought or salt stress accumulate more chlorophyll a and b, anthocyanin, ABA and genes related to abiotic stress compared to non-treated plants. We found a reduction in reactive oxygen species that are related to greater increase of antioxidant in treated-plants with BABA compared to non-treated ones. What surprised us is that BABA induces more tolerance to Marmande than to Coeur de Boeuf. In addition, plants treated with BABA show better resistance against the combination of both stresses compared to non-treated plants by induction of callose accumulation, H_2O_2, ABA, SA and the expression of *PR1* and *PR5*. This opens many perspectives on the effect of BABA on metabolic study.

Keywords: BABA, tomato plants, Induction of resistance, drought stress, salt stress, *Botrytis cinereal*, combined stresses.

Résumé

La tomate compte parmi les légumes-fruits les plus importants sur le plan économique dans plusieurs pays. Cependant la sécheresse, la salinité et le les pathogènes constituent les principales causes de réduction et de variabilité des rendements des cultures. Bien que les plantes aient développé des capacités défensives contre ces stress, leur tolérance et résistance dépend du degré et de l’intensité du stress et aussi de l’espèce. Dans cette thèse, nous avons étudié le rôle de l’acide β-aminobutyrique (BABA) dans l’induction de la résistance contre le stress hydrique, le stress salin et la combinaison de salinité et infection avec *Botrytis cinerea* chez deux cultivars de tomate (cv Marmande : un cultivar résistant au stress et cv Cœur de Bœuf : un cultivar sensible au stress). Nos résultats ont montré que BABA atténue considérablement les effets nocifs d’un seul stress et aussi des stress combinés. Les plants de tomate sous contrainte hydrique ou saline, traités avec BABA accumulent plus de chlorophylle a et b, anthocyanine, ABA et montrent aussi une induction des gènes reliés au stress abiotique comparé aux plants non-traités. Nous avons trouvé une réduction des espèces réactives d’oxygène qui sont liées à une augmentation des antioxydants plus importante chez les plants traités avec BABA par rapport aux plants non-traités. Ce qui nous a surpris c’est que BABA induit plus de tolérance au cv Marmande qu’au cv Cœur de Bœuf. De plus, les plants traités avec BABA présentent une meilleure résistance contre la combinaison des deux stress par rapport aux plants non-traités suite à l’induction de l’accumulation de callose, d’H_2O_2, d’ABA, de SA et aussi l’expression de *PR1* et *PR5*. Ceci ouvre de nombreuses perspectives quant à l’effet de BABA sur les métabolites.

Mots-clés : BABA, plant de tomate, induction de la résistance, stress hydrique, stress salin, Botrytis cinerea, combinaison de deux stress.

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Glossary

ABA	Abscisic acid
AOX	Alternative oxidase
APX	Acorbate peroxidase
AREB	ABA-responsive element-binding proteins
BABA	β aminobutyric acid
BIT	1,2- benzisothiazole-1,1-dioxide
BTH	Benzothiadiazole
bZIP	Basic leucine zipper
CAT	Catalase
CBF	C-repeat binding factors
CBL	Calcineurin B-like protein
CDPKs	Calmodulin-dependent protein kinases
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CIPK	CBL-interacting protein kinase
DAMPs	Damage-associated molecular patterns
ETI	Effector-triggered immunity
GABA	γ-aminobutyric acid
GPX	Guaiacol peroxidase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
INA	2,6-dichloroisonicotinic acid
IR	Induced resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
JA-Ile	Jasmonic isoleucine
LEA	Late-embryogenesis-abundant proteins
MAMPs	Microbe-associated molecular patterns
MAPKs	Mitogen-activated protein kinases
MAPKK	MAPK kinases
MAPKKK	MAPK kinase kinases

MDHAR	Monodehydroascorbate reductase
MeSA	Methyl salicylic acid
MYB	Myeloblastosis
NAC	No Apical Meristem, ATAF and Cup-Shaped Cotyledon
NB-LRR	Nucleotide-binding leucine-rich repeat
NCI	N-cyanomethyl-2- chloroisonicotinamide
NO	Nitric oxide
NPR1	Nonexpressor of PR genes
$O^{\bullet -}_2$	Superoxide radical
$^1O_2$	Singlet oxygen
$OH^{\bullet}$	Hydroxyl radical
PAMPs	Pathogen-associated molecular patterns
PGPF	Endophytic fungi
PGPR	Rhizobacteria
R6K	Ribosomal protein kinases
POX	Peroxidase
PR	Pathogenesis related protein
PRKs	Receptor protein kinases
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
SOS	Superoxide scavenging
TDL	3’- chloro-4,4’-dimethyl-1,2,3- thiadiazole-5-carboxanilide (tiadinil)
TFs	Transcription factors

Summary

Tomato (*Solanum lycopersicum*), a member of the family *Solanaceae*, is one of the widely produced and consumed vegetables and is considered an economically important crop. Tomato plants can be cultivated around the world and are also one of the main generators of employment in rural regions. However, this crop is confronted with a multitude of abiotic and biotic stresses that are responsible to affect plant growth, development, and crop productivity. To protect themselves against these constraints, plants have evolved a broad range of defense mechanisms that are present or that can be enhanced by treatment with various synthetic and natural compounds. β aminobutyric acid (BABA) is an inducer of resistance against a large variety of attackers such as pathogens and herbivores as well as against abiotic stress like salinity and drought stress. BABA is capable to improve the immune system of the stressed plants and make it more resistant, this is called priming. Primed plants express faster and stronger enhanced defense upon encountering either abiotic or biotic stress. The vast majority of induced resistance studies were conducted on a single type of stress (abiotic or biotic stress). However, there are no studies about induced tolerance in plants under a combination of abiotic and biotic stress. Thus, the aim of this thesis was to investigate the effect of BABA treatment under salinity, drought stress, and under a combination of salinity and *Botrytis cinerea* in tomato plants. We were interested to identify some of the mechanisms involved in resistance induced by BABA.

First, due to water deficit and soil salinity problems in field-grown tomato cultures, two tomato cultivars (a highly stress-susceptible (Coeur de Boeuf) and a tolerant one (Marmande) were chosen to determine the effect of BABA treatment in the induction of resistance against drought and salinity. BABA-treated plants induced defense-related genes faster than nontreated plants. Microscopic analysis of leaves treated with BABA at different levels of water and salt stress revealed induction of lignin accumulation and reduction of hydrogen peroxide (H_2O_2) production. In addition, chemical analysis showed an increase in total antioxidant activity, as well as of Chlorophyll ’a’ and ‘b’ comparing to untreated plants. These results showed an effective priming of defense responses in treated tomato plants under abiotic stress, thus increasing their tolerance.

Second, a combination of salinity and infection with *Botrytis cinerea* was studied in order to identify the beneficial effect of BABA to induce resistance in tomato. BABA-treated plants showed earlier and higher expression of genes related to stress following the combination of salt stress and *B. cinerea* infection compared to the nontreated plants. Interestingly, salt stress improved the protective effect of BABA by increasing callose accumulation, thus BABA and salt stress together might play an important role in the antifungal defense.

Third, the phytohormone induction in treated tomato plants with BABA during the combination of salinity and *B. cinerea* infection was examined. BABA-treated plants exhibited higher ABA and SA levels upon combined stress, which could serve as a chemical arsenal during salinity tolerance and antifungal immunity in tomato.

Chapter I

General introduction

In their environment, plants are constantly confronted with the climatic variations or aggressions caused by other organisms that may prevent their proper development. Abiotic stress such as drought, high salinity, extreme temperatures and all kinds of unfavorable environmental conditions is a worldwide phenomenon. The most common manifestations following these stresses are not only limits in plant growth and development but also disruption of the cellular structures and impairment of key physiological functions. Hence, environmental stresses result in numerous physiological changes in plants (Wilkinson and Davis, 2010; Roy et al., 2011) and lead to a series of molecular and biochemical reactions that adversely affect plant growth and productivity (Ait Barka and Andran, 1997). This is a problematic situation which causes food insecurity for large parts of the population, particularly in rural areas. Abiotic stresses are the major factors of poverty for millions of people. Many regions in the world suffer from an insufficient water availability and salinization of the land. Only 14.51% of agricultural areas are equipped for irrigation (http://www.faostat.fao.org) and 23% of all cultivated land is affected by salt (Keren, 2000). Drought and salinity affect more than 10% of arable land, which results in more than 50% decline in the average yields of important crops worldwide (Wang et al., 2003).

As sessile organisms, plants cannot move like animals to escape the danger. It is crucial for them to adapt and to develop tolerance to maintain growth, complete their life cycle and reach their full genetic potential. As mentioned above, abiotic stresses may affect multiple stages of plant development (Chinnusamy et al., 2004). Therefore, in the course of evolution, plants have developed specific mechanisms and strategies to defend themselves against these challenges (Alkinson and Urwin, 2012). The difference in plant reactivity subsequently leads to alterations in morphology, physiology, gene expression and to metabolic changes aimed at restoring cellular homeostasis.

In addition to abiotic stress, plants in their environment are exposed to biotic stress such as fungi, bacteria, virus, nematodes and herbivorous insects. Some studies report that the exposure to one type of stress induces resistance to the second one (Abou Qamar et al., 2009). This phenomenon of cross-tolerance confers enormous regulation and often allows plants to adapt to severe conditions (Bartoli et al., 2013). In this project we used salt stress (50mM) and infection with *Botrytis cinerea*. The use of this low concentration of NaCl is based on the small morphological and physiological effect compared to the control (Shavrukov, 2013). Fifty Mm of NaCl is responsible to decrease plant growth, which is the result of the disturbed osmotic processes and the toxic effect of Na^+ and Cl^- but the plants can acclimate for a definite period. We combined salt stress with *Botrytis cinerea* which is known as grey mold that can infect more than 200 plant species (Asselbergh et al., 2007). This fungus induces cell death in the vegetative tissues (Dean et al., 2012). *Botrytis* penetrates through wounds or stomata and starts to kill plant tissues by secreting toxic compounds or lytic enzymes (Van Kan, 2006). This infection leads to necrosis of the host. As demonstrated in the *sitiens* tomato mutant, ABA deficiency increases the resistance to *Botrytis* (Audenaert et al., 2002; Asselbergh et al., 2007). In addition, the application of exogenous ABA increases the susceptibility of plants to *Botrytis* attacks (Audenaert et al., 2002).

Despite the effectiveness of defenses induced following abiotic and biotic stress, symptoms and damage of plants are sometimes observed in the field leading to both economic and humanitarian problems. To minimize the losses caused by these stresses, farmers have various solutions, for example, the intensive use of phytosanitary products. However, these treatments have a consequence on the environment and also on human health. In parallel, other alternatives are now considered providing effective resistance of plants under adverse conditions. One of these alternatives is to produce transgenic plants but the very low public acceptance of such technology limits its use. Combating pathogens through the use of antagonistic microorganisms is also one of the methods (James Cook, 1993). Finally, the stimulation of natural defense in plants by inducing resistance is considered one of the best strategies. Such a priming treatment allows the plant to respond faster and stronger to abiotic or biotic stress than their non-primed counterparts (Prime-A-Plant Group, 2006). Priming can also be achieved via treatment with chemicals. There are various synthetic and natural compounds like salicylic acid (SA), benzothiadiazole (BTH), β-aminobutyric acid (BABA) (Lawton et al., 1996; Jakab et al., 2001) which are capable to control various stresses in plants by improving the immune system of the stressed plant and make the plant more resistant. These substances have the ability to induce resistance in the treated plants. Numerous studies have shown that the nonprotein amino acid β-aminobutyric acid induces protection in many plant species

against various stresses (Cohen et al., 2016).
The tomato plant has been chosen for our study. Tomato has been used as a convenient model system before and is also one of the most important economical vegetable plants in the *Solanaceae* family (Gong et al., 2010). We use tomato as a model to study its physiological, biochemical and molecular responses under different kinds of stress. For this thesis we chose two tomato cultivars based on their difference of the defensive capacity of each one under stress and also for their agronomic and economic importance in Tunisia.

- Cv Marmande

This variety is originally from France from the city of Marmande from which it owes its name. This tomato is characterized by its high productivity and resistance to Fusarium (*Fusarium oxysporum* f.sp.) and to other stressors (El-Saka et al., 2016; Ghanem et al., 2016). Its growth is indeterminate, and the medium-sized fruits grow as a cluster of 4 or 5 fruits.

- Cv Cœur de bœuf

This tomato variety is native from Italy and characterized by an indeterminate growth. Its fruit is big (can reach 200 to 300g and sometimes 500 to 700g depending of the culture conditions), fleshy and has a carmine red color. This cultivar is known to be snensitive to stress (Dasgan et al., 2002).

Chapter I - General Introduction

IA: Induced resistance in tomato plants against abiotic and biotic stress

A. Morphological, physiological and molecular effect of biotic stress on plants

Plants are confronted to a multitude of abiotic stress such as drought, salinity, cold, heat and heavy metals that are responsible to limit their growth, development and productivity. Drought and salinity are two important abiotic stresses and plant responses to these stressors are often similar.

Sensing the stress

In order to be able to react, a plant has first to be aware of a stress. Following drought or salt stress, receptors present on the plant cells membrane perceive the danger. The transduction of the generated signal is the result of a rapid rise in cytosolic Ca^{2+} levels which plays the role of second messenger and signal transmitter to differentiate such kind of stress (Xiong et al., 2002; Capiati et al., 2006). Following each stress, there is a rapid peak of Ca^{2+}. According to the quantitative difference of such Ca^{2+} peaks, plants recognize which stress is developed. Kiegle et al. (2000) showed that different cell types in Arabidopsis roots could differentiate between cold, osmotic and salt stress through calcium. Ca^{2+} activates the signaling cascade amplification in response to multiple adverse environmental conditions through interacting protein phosphatases and protein kinases (Lecourieux-Ouaked et al., 2000). Among the protein kinases involved in the signaling cascade, there are calmodulin-dependent protein kinases (CDPKs), receptor protein kinases (RPKs), ribosomal protein kinases (S6K) and mitogen-activated protein kinases (MAPKs) (Capiati et al., 2006). Indeed, MAPK participates in a cascade of phosphorylation that generally involves three types of kinase proteins: MAPK kinase kinases (MAPKKK), MAPK kinases (MAPKK) and MAPK. In response to a stimulus, MAPKKK activates by phosphorylation a MAPKK, which in turn activates and phosphorylates MAPK leading to the transmition of the signal (Rodriguez et al., 2010). This cascade launches the defense responses in plants including expression of genes encoding defense proteins. MAPKs for example, have been shown to be involved in rice during multiple abiotic stresses (Agrawal et al., 2003).

Reactive oxygen species (ROS) appear early during MAPK activation and are responsible for a direct toxicity of H_2O_2 (Mittler et al., 2011). An excessive amount of ROS is very toxic to the plant and can cause peroxidation and de-esterification of membrane lipids and also protein denaturation (Bowler et al., 1992). To avoid damaging effects and cell death during such oxidative stress, plants activate scavenging enzymes or directly repress the generation of ROS (Vinocur and Altman, 2005). In addition to their toxicity, ROS are also important signal transduction molecules. The signal perception of an abiotic stress by the plasma membrane is followed by the generation of not only calcium as a second messenger but also ROS and inositol phosphates. The role of ROS is to modulate the calcium level in the cells. This recognition of the stress leads to the expression of major stress-responsive genes ensuring plant adaptation.

Effects of abiotic stress on plants

Plants are continuously confronted to a multiple environmental factor. Abiotic stress is considered as the primary reason of reducing yields for the majority of crop loss. Abiotic environmental factors are responsible to cause more than 50% of crop loss worldwide (Bray et al., 2000; Wang et al., 2003). Drought stress and high salinity are the two major abiotic stress factors causing a deficit in water leading to a dramatic disruption of plant functioning. Both stresses are the primary cause of the reduction and limitation of plant yield (Cushman and Bohnert 2000; Witt et al., 2012). The impact on plants under drought and/or salinity has a high degree of similarity with respect to physiological, biochemical, molecular and genetic effects (Sairam and Tyagi, 2004). During drought, the amount of water in the soil available for the plant is insufficient for the growth causing a decrease in the soil water potential. However, in the most saline soil, the total amount available of water in the soil is constant but under low water potential. This physiological drought happens when soluble salt levels in the soil solution are high enough to limit water uptake due to low water potential, thereby inducing drought stress (Carrow and Duncan, 1998). The responses of the plant to water deficit differ significantly at various organizational levels and it is related to the intensity and the duration of stress as well as to the plant species and its stage of development (Shao et al., 2008). Both stresses lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm into the intracellular space resulting in a reduction of the cytosolic and vacuolar volumes. Early responses to water and salt stress are largely identical except for the ionic component in the cells of plants under salt stress.

Effects of drought stress on plants

Plant growth is achieved by cell division, cell enlargement, and differentiation. It also involves morphological, physiological, ecological and genetic events and their complex interactions.

-Water relations: Under severe scarcity of water, cell growth and elongation can be inhibited by interrupting the flow of water from the xylem to the surrounding elongating (Nonami, 1998). Moreover, water deficit is responsible to decrease plant biomass through growth inhibition which depends on the plant species, the stage of development and the degree and duration of stress (Cramer et al., 2007; Tavakkoli et al., 2010). Indeed, this stress causes a decrease of fresh material production as well as dry material production of leaves, roots, and stem (Wang and Nil, 2000; Zeid and Shedeed, 2006). During water stress, relative water content, turgor potential, transpiration, stomatal conductance and water-use efficiency are decreased (Egilla et al., 2005; Nerd and Nobel, 1991). Impaired mitosis and cell elongation result in reduced plant height, leaf area and crop growth under water deficit (Nonami, 1998; Kaya et al., 2006; Hussain et al., 2008) (Figure 1).

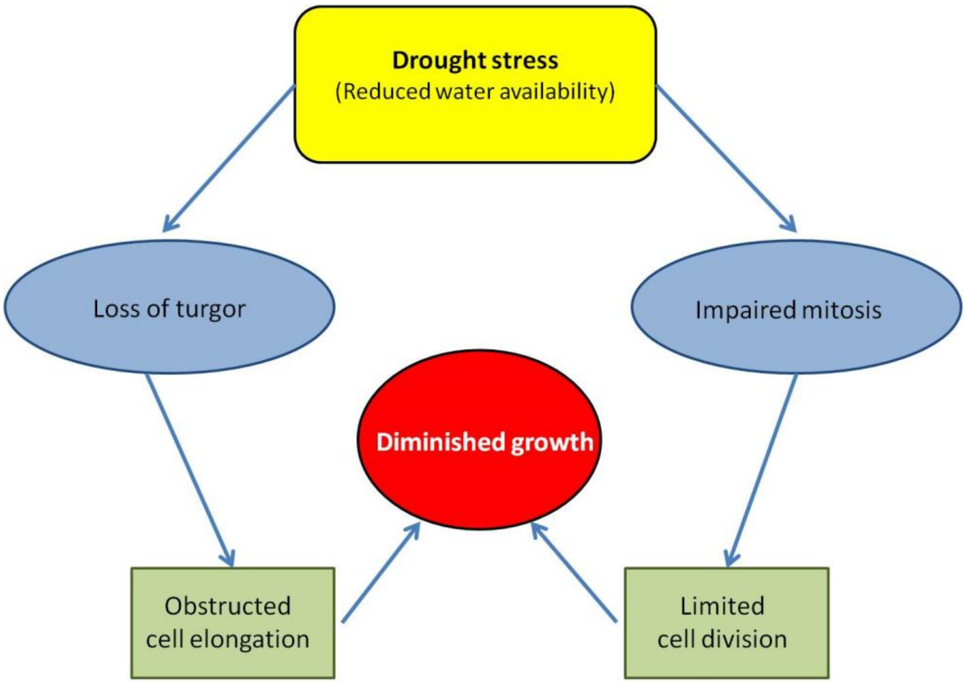

Fig. 1. Description of plant growth reduction under drought stress. Under drought stress conditions, cell elongation is inhibited by reduced turgor pressure. Cell division is also affected by the reduction of the plant water uptake. As a result, impaired mitosis, cell elongation and expansion lead to reduced growth (According to Farooq et al., 2009).

-Nutrient relations: Drought stress usually results in decreased total nutrient uptake by the root and their transport to shoots and their reduced tissue concentrations in crop plants. The cause of lower absorption of the inorganic nutrients may be the result of interference in nutrient uptake and unloading mechanism, and reduction of transpiratory flow (Garg, 2003). Drought induces an increase in nitrogen (N) and a definitive decline in phosphorus (P) (Garg, 2003). Drought stress influences plant nutrition resulting in a perturbation of plant growth and development, which can be related to decreasing the available energy for assimilation of $NO^{-}{}_{3}$ /$NH^{+}{}_{4}$, $PO^{3-}{}_{4}$ and $SO^{2-}{}_{4}$. This energy might be transformed in energy-dependent processes (Grossman and Takahashi, 2001). In addition, P and $PO^{3-}{}_{4}$ contents in the plant tissues decrease under drought, which is the result of low moisture availability that affects $PO^{3-}{}_{4}$ mobility (Peuke and Rennenberg, 2004).

Effects of salt stress on plants

-Osmotic effects: NaCl accumulation at high concentrations decreases the osmotic potential of the soil solution at the root zones of the plants which hinder the absorption of water, the effects of which are reflected in a reduction of growth, then wilting and sometimes complete desiccation (Lugan et al., 2010). In fact, growth arrest occurs when turgor is reduced below a critical threshold and dehydration of cells begins when they cannot compensate for the loss of turgor (Cramer, 2002). In this case, an osmotic adjustment is necessary so that the cellular hydric potential remains lower than that of the extracellular medium and that of the soil (Chinnusamy et al., 2005). This phenomenon ensures, on the one hand, the continuation of the water absorption from the soil, and on the other hand, the retention of the intracellular water and the maintenance of the turgor (Lugan et al., 2010).

-Nutritional effects: Salinity induces a disturbance in the nutritional balance thus limiting the absorption and transfer of major ions essential for growth. Na^+ competes with K^+ and Ca^{2+} in addition, Cl^- competes with NO_3^- and SO_4^{2-} (Tavakkoli et al., 2010). Na^+ can substitute for K+ in its osmotic functions but cannot replace it in its physiological functions (Mian et al., 2011). The similarity of the physico-chemical structure of Na^+ and K^+ is responsible for a competition at the level of K^+ transport sites, which leads to a potassium deficiency and an increase in the Na^+/K^+ ratio (Tavakkoli et al., 2010).

-Toxic effects: Na^+ is necessary for the growth of some plants, but high concentrations of NaCl affect the glycophytes growth (Ellouzi et al., 2011). Munns (2002) attributes the inhibition of growth to toxicity of salt accumulated in the leaves, accelerating senescence and foliar necrosis. The latter is due to an accumulation of Na^+ in the apoplasm, in the absence of an effective vascular compartmentalization (Pett and Moller, 2010).

However, the action of the accumulated ion in large amounts can be direct resulting in changes in lipid structure or protein denaturation at the membrane level, or indirectly related to changes in metabolic reactions (Lugan et al., 2010). The direct and indirect toxic effects caused by sodium and chlorine are related to two extremely important problems: on the one hand, the regulation of the Na^+ or Cl^- absorption at the roots and the cells, on the other hand, the distribution of these ions (Pett and Moller, 2010).

Oxidative stress: generation of ROS under abiotic stress

Under severe environmental conditions like drought and salt stress, the closure of the stomata allows the plant to limit its losses in water but reduces the entry of CO_2 into the leaf thus leading to insufficient intracellular CO_2 concentrations. This again leads to a decrease in photosynthesis following the decrease of photosynthetic processes that allow the passage of electrons from a donor (H_2O) to an ultimate acceptor (NADPH) (Miller et al., 2009). Photosynthesis provides the energy needed to provide ATP from ADP and PI via the creation of a photon gradient. When the stomata are closed, the light energy becomes more than the photon utilization capacities by the metabolism with a risk of photo-inhibition. Therefore, this phenomenon favors the production of ROS which can cause severe damage to both the photosystems PSI and PSII (Jaspers and Kangasjavi, 2010; Nishiyama et al., 2011). The first molecular species directly derived from the reduction of oxygen in chloroplasts and formed directly from the chain of electron transporters is superoxide radical ($O^{\bullet -}{}_2$). However, these ions are the precursors of other oxidative molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^\bullet$) (Asada, 2006). These oxidative molecules are generated from only 1–2% of the total O_2 consumed by plants (Bhattacharjee, 2005).

- H_2O_2 is toxic for the cell because it inactivates certain enzymes of the Calvin cycle (a cycle that intervenes in the fixation of the carbon of the air) (Halliwell, 2006).
- $OH^{\bullet}$ is the most reactive and toxic ROS. It is generated at neutral pH by the Fenton reaction between H_2O_2 and $O^{\bullet -}_2$ catalyzed by transition metals like iron and copper (Das and Roychoudhury, 2014).
- $^1O_2$ is an atypical ROS which is generated by the photoexcitation of chlorophyll (Chl) and its reaction with the oxygen (Gill and Tuteja, 2010).

The production of ROS in plants is mainly localized in different cellular compartments such as chloroplast, mitochondria, and peroxisomes. In addition, there are other sites of ROS production like the endoplasmic reticulum, cell membrane, cell wall and the apoplast (del Rio et al., 2006; Navrot et al., 2007). Under several environmental stress conditions like drought and salt stress, ROS levels increase dramatically inducing oxidative stress by the imbalance of the equilibrium between ROS generation and antioxidants (Gill and Tuteja, 2010) (Figure 2). Thus, oxidative stress is the consequence of an increase of ROS production and a decrease of antioxidant levels in plants under abiotic stress.

ROS affect many cellular functions by causing damage on proteins, pigments, nucleic acids, carbohydrate, lipid peroxidation (LPO) and loss of PSII activity which eventually amalgamate in the cell death of plants (Wagner et al., 2004; Foyer and Noctor, 2005; Krieger-Liszkay et al., 2008).

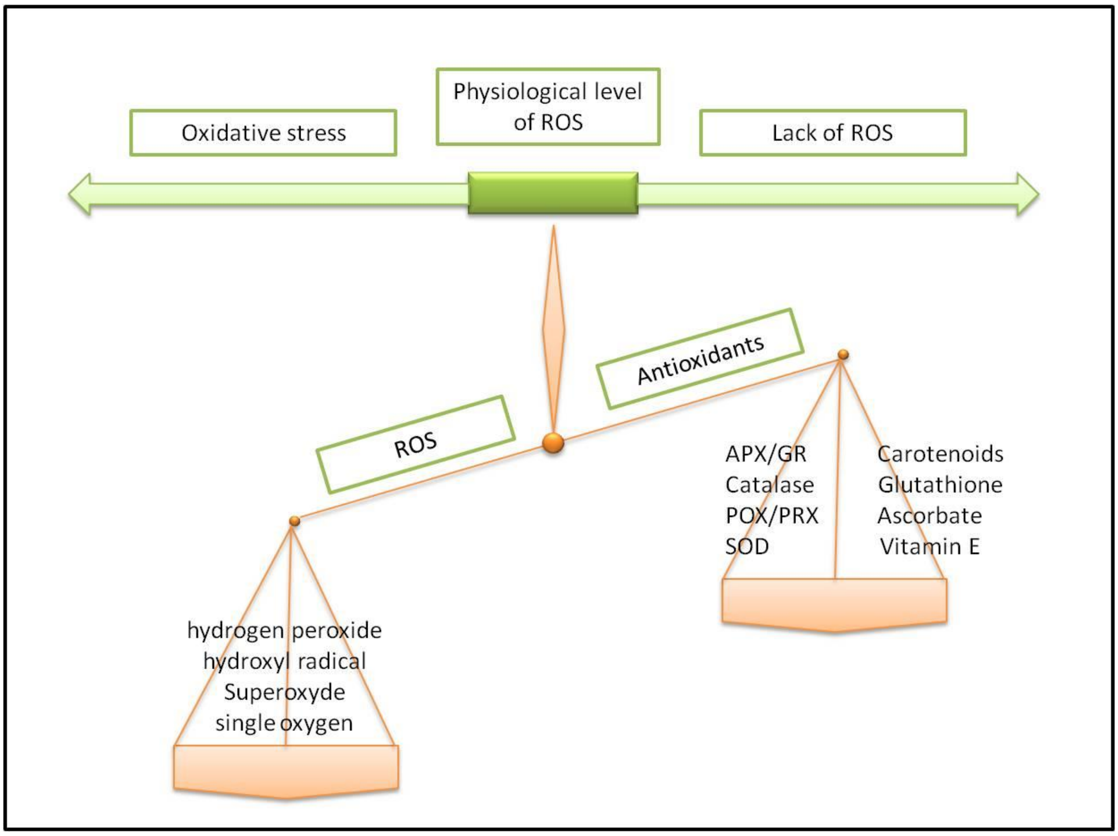

Fig. 2. Oxidative stress description. The imbalance between ROS and enzymatic and nonenzymatic antioxidants is detected in plants in response to abiotic stress.

Adaptative plant response to drought and salt stress

To survive in a hostile environment, plants adopt various defense strategies to adjust their osmotic potential to prevent loss of turgor allowing the plant to escape unfavorable conditions of growth (Choi et al., 2011). These stress-tolerance strategies include a large number of morphological (reduction of growth, chlorosis, leaf abscission, wilting, formation of adventitious roots, localized necrosis), physiological (stomatal closure, reduction of photosynthesis), molecular and biochemical processes (accumulation of organic solutes and proteins, modification of the activity and the function of the enzymes, induction of the gene expression) which are altered to help the plant to overcome stress (Bhargava and Sawant, 2013).

Morphological responses

Drought stress and salinity are the most important environmental factors that affect the growth and productivity of several plants (Munns, 2002; Munns et al., 2010; Fathi and Tari, 2016). The unavailability of water due to drought or salinity (physiological drought) (Strogonov, 1964) is the cause of these changes.

To sustain themselves, complete their life cycle and survive under severe environmental conditions, plants avoid stress by reducing the aerial parts due to the diminution of the activity of cyclin-dependent kinases responsible for cell division slowdown (Munns and Tesler, 2008; Farooq et al., 2009). A decrease of cell elongation and expansion leads to a reduction of plant height and leaf area (Hussain et al., 2008). The reduction in leaf area leads to a lowered water uptake from the soil and results in a decrease in transpiration which can be considered as a positive response to prevent the negative impact of water stress (Álvarez and Sánchez-Blanco, 2015; Fathi and Tari, 2016). Under salt stress, the leaf area of *Myrtus communis* decreases resulting in a beneficial change in water relations (Acosta-Motos et al., 2014).

The roots are the first line of defense under drought stress and present plasticity in response to this constraint (Bengough et al., 2006; Siopongco et al., 2009). For example, a reduction in the length of the root system was detected in soybeans, rice, and maize under drought stress condition and allows the maintenance of stable water status (Nouri et al., 2012; Zhang et al., 2008; Hammer et al., 2009). Other studies, however, have shown that root system proliferation allows them to penetrate the deeper layer of soil for extracting water and nutrient under abiotic stress (Kavar et al., 2007; Franco et al., 2011). Leaf senescence is a strategy adopted by plants in order to escape these conditions. Mahajan and Tuteja (2005) showed that leaf senescence is accelerated in cotton to escape the stress.

Physiological responses

- Stomatal closure

Drought and salt stress enhance both the transpiration rate and the pH of leaf sap, which can directly affect stomatal conductance through the increase of abscisic acid (ABA) accumulation (Wilkinson and Davies, 2002). This indicates that the first plant response to a decline in leaf turgor and/or water potential is the closure of their stomata to prevent water loss through transpiration (Bhattacharjee and Saha, 2014).

Therefore, leaf water status and stomatal conductance interact positively under stressed conditions. Stomatal closure decreases the rate of transpiration which reduces the inflow of CO_2 into the leaves and produces more electrons for the formation of ROS (Farooq et al., 2009). In addition, stomatal closure is followed by reduced photosynthesis under water deficit conditions (Cornic, 2000). The role of ABA (ABA production in dehydrating roots and ABA circulation in the plant) is well documented in closing the stomata under drying conditions (Wilkinson and Davies, 2002). In stressed *Vicia faba* roots, ABA concentrations increased at the guard cell apoplast which correlated with the stomata closure (Zhang and Outlaw, 2001). Stoll and colleagues (2000) demonstrated that the increase of ABA in xylem sap and leaf in grapevine upon drought stress leads to stomatal closure. Stomatal conductance decreases under salt stress in tomato plants (Orsini et al., 2010) in order to minimize water loss.

- Photosynthetic pigments

Photosynthesis is one of the most important processes in plants. The concept of photosynthesis is to convert the sun's energy to chemical energy (sugar) through photosynthetic pigments such as chlorophyll. These photosynthetic pigments are found in the chloroplasts of the plant cells. In plants Chlorophyll a (Chl a) and chlorophyll b (Chl b) are the main photosynthetic pigments and plays a crucial role in converting light energy. However, drought and salt stress conditions are responsible for damaging of the photosynthetic pigments and ensure the deterioration of thylakoid membrane (Singh and Dubey, 1995; Arivalagan and Somasundaram, 2015). Both the chl a and b decreased with increasing drought and/or salt stress (Jaleel et al., 2009; Heidari et al., 2014). In addition, the activity of the chlorophyll degrading enzymes chlorophyllase and peroxidase increased under severe conditions accordingly to the decrease of the chlorophyll content (Rao and Rao, 1981). The limitation of photosynthesis under drought and/or salt stress has been reported in sunflower (Akram et al., 2009; Kiani et al., 2008), in tomato (Ciobanu and Sumalan, 2009), cotton (Massacci et al., 2008), sunflower (Heidari et al., 2014), and radish (Jamil et al., 2007). However, contradictory results indicated that, under abiotic stress, chlorophyll content increased in soybean (Wang et al., 2001) and cotton (Higlie et al., 2010). The reason for reduced photosynthesis in plants during drought and/or salinity is generally due to the stomatal closure, metabolic impairment and reduction of photosynthetic pigment contents (Reddy et al., 2004; Farooq et al., 2009). On the contrary, other studies demonstrated that the increase of photosynthesis under salt stress may be due to an increase in leaf thickness and in the number of chloroplasts (Papp et al., 1983; Misra et al., 1997).

Biochemical responses

- Accumulation of antioxidant

When the level of ROS increases in the plants and exceeds the normal levels under stressful conditions like drought or salt stress, it induces oxidative stress which can cause damage to some biomolecules (Sharma et al., 2010). Therefore, the equilibrium between ROS production and ROS scavenging is perturbed. In order to detoxify and limit the production of ROS under drought and/or salt stress, the intervention of the antioxidant defense system in the plant cell is necessary. The antioxidant system is constituted of enzymatic components like superoxide dismutase (SOD), alternative oxidase (AOX), catalase (CAT), peroxidase (POX), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR) as well as non-enzymatic components including carotenoids, flavonoids, glutathione and ascorbate (Noctor and Foyer, 1998; Gill and Tuteja, 2010) (Table 1). The degree of detoxification depends on the species, stage of development of the plant and the duration and intensity of the stress. These antioxidants play a crucial role in the plant's responses to drought and salinity. Antioxidant activities increased in sesame upon drought stress (Hussein et al., 2016). In wheat, it has been reported that NaCl causes an increase in ROS and that seedlings of this species are capable of inducing antioxidant defenses (Sairam et al., 2002). Research in two maize genotypes has shown that NaCl increases the activity of SOD, ascorbate peroxidase (APX), Guaiacol peroxidase (GPX) and GR at the leaf level. This increase is more pronounced in the tolerant genotype than in the susceptible genotype (De Azevedo Neto et al., 2006). Transgenic tomato plants expressing *CaKR1* showed low levels of $O^{\bullet -}_2$ and H_2O_2 as well as an increase in resistance to salinity and oxidative stress (Seong et al., 2007). It was demonstrated that these transgenic tomato plants produced higher levels of transcripts coding for pathogenesis-related proteins (PR) as well as transcripts coding for antioxidant proteins such as Superoxide dismutase (*LeSOD2*) and ascorbate peroxidase (*LeAPX2* and *LeAPX3*), which indicates that *CaKR1*is responsible to regulate the antioxidant metabolism and the response to stress.

Table 1. Important enzymatic and non-enzymatic antioxidants that detoxify plants from ROS

	Name		Localization	Affinity to	References
Enzymatic antioxidant	Alternative oxidase (AOX)		Thylakoids Mitochondria	$O^{\bullet -}_2$	Yoshida et al., 2006
	Superoxide dismutase (SOD)	MnSOD	Mitochondria Glyoxysome Peroxisome membrane	$O^{\bullet -}_2$ $OH^{\bullet}$	Rodriguez-Serrano et al., 2007
		FeSOD	Stroma of chloroplasts Peroxisome Mitochondria		Asada, 2000 Moran et al., 2003
		CuZnSOD	Cytosol Peroxisome Apoplast Thylakoids membrane		Bowler et al., 1994 Arora et al., 2002
	Catalase (CAT)		Peroxisome Chloroplasts	H_2O_2	Smirnoff, 1998
	Peroxidase (POX)		Apoplast	H_2O_2	Mika et al., 2004
	Ascorbate peroxidase (APX)		Peroxisome Mitochondria Chloroplasts Cytosol	H_2O_2	Wang et al., 2006
Non-enzymatic antioxidant	Ascorbate (ASC)		All cell and extracellular compartments	$O^{\bullet -}_2$ $^1O_2$ $OH^{\bullet}$	Foyer and Noctor, 2005
	Glutathione		Cytosol Mitochondria	H_2O_2	Noctor and Foyer, 1998
	Carotenoids		Associate with PSII	$^1O_2$	Miller et al., 1996

- Accumulation of stress metabolites

Osmotic adjustment is also one of the mechanisms that protect membranes from abiotic stress. It is provided by osmoprotectants such as proline, sugar, glutamate, glycine betaine, sorbitol, mannitol, polyols, polysaccharides and inorganic ions (Krasensky and Jonak, 2011). These compounds help the plant to maintain a state of osmotic balance and turgor in the cell (Vinocur and Altman, 2005) and are important for membrane stability (Clémentine et al., 2010). For example, the increase of proline content in stressed tobacco plants by osmotic stress reduces free radical levels (Hong et al., 2000). Overexpression of LEA (Late Embryogenesis Abundant) proteins has been correlated with osmotic stress, for example in transgenic rice this molecule confers dehydration tolerance (Chandra Babu et al., 2004). This protein contributes to membrane stabilization (Wang et al., 2003).

Hormonal responses

- Phytohormones involved under drought and salt stress

Plant hormones are among the most important compounds which intervene in the development and growth of plants and play a key role in stress signal transmission. It is known that phytohormones play an important role in abiotic stress signaling; especially abscisic acid (ABA) (Figure 3). ABA is generated as a signal controlling seed germination and developmental processes. The biosynthesis of ABA is related to the changing of the water status in plants normally caused by abiotic stress. Thus, this endogenous messenger function is considered to be the regulation of plant water content and osmotic stress tolerance (Orellana et al., 2010). In response to an environmental condition, plants adjust ABA levels constantly to induce plant resistance. The increase of ABA level in vegetative tissues during abiotic stress is responsible for regulating stomatal closure, stimulating the accumulation of osmo-compatible solutes and triggering the activation of many stress-related genes (Christmann et al., 2006; Nakashima et al., 2012). Stomatal closure minimizes intracellular water loss under drought stress; therefore, ABA is named a stress hormone. Mittler and Blumwald (2015) demonstrated that during environmental stress, there is a positive interaction between ABA and ROS to control stomatal function and gene expression (Mittler and Blumwald, 2015). ABA-deficient mutants in tomato, tobacco, and maize show growth problems under drought stress and through a long period of stress, these mutants are not able to survive (Huang et al., 2012). ABA-deficient mutants in Arabidopsis, namely *aba1, aba2,* and *aba3* have smaller size even in optimal conditions, but they can easily die in case of the persistence of abiotic stress (Xiong et al., 2001). Compared to wild-type, transgenic plants that overexpressed the key regulator gene in ABA biosynthesis can improved the drought stress tolerance by increasing ABA levels and maintained low stomatal conductance (Iuchi et al., 2000; Qin and Zeevaart, 2002). Exogenous application of ABA can increase abiotic stress tolerance in maize and turfgrass plants stressed by water deficit (Lu et al., 2009; Zhang et al., 2011). These results illustrate the protective role of ABA in the defense mechanisms against abiotic stresses. In addition, application of ABA can induce tolerance against salt and drought stress in *Arabidopsis thaliana* (Jakab et al., 2005). In a recent study in creeping bentgrass (*Agrostis stolonifera*) Li and colleagues (2016) report that exogenous ABA, γ-aminobutyric acid (GABA) and salicylic acid (SA) are responsible to improve the drought-induced damages by accumulating special metabolites and maintaining membrane stability and leaf water status. Hyperactivation of ABA metabolism is the result of osmotic stress to increase plant resistance (Nambara and Marion-Poll, 2005). Maize plants treated with brassinolides enhance drought stress tolerance by increasing ABA biosynthesis (Zhang et al., 2011).

In addition to ABA, salicylic acid can also increase plant abiotic stress tolerance (Khan et al., 2015). SA-deficient *NahG* transgenic of Arabidopsis lines are sensitive against salinity, the reason is related to the decreased activity of antioxidant enzymes (Cao et al., 2009). SA-accumulating mutants (*cpr5* and *acd6*) in *A. thaliana* enhance drought tolerance through stomatal closure (Okuma et al., 2014). Pal et al. (2014) demonstrated that SA biosynthesis is responsible to increase drought tolerance in rice. SA-primed wheat plants increase antioxidant activity which confers a tolerance to salt stress (Afzal et al., 2011). Interestingly, SA treatment decreases H_2O_2 accumulation in wheat under salt stress (Erdal et al., 2011), whereas, H_2O_2 level increased by the same treatment in maize under drought stress and resulted in an enhanced tolerance (Saruhan et al., 2012). Furthermore, salicylic acid (SA) and jasmonic acid (JA) are known to respond to ROS accumulation (Kiffin et al., 2006). These results show the positive role of SA in the defense mechanisms against drought and salt stress. Moreover, expression patterns of various plant genes are regulated by drought and high salinity and also by ABA. Gene expression patterns induced by these stressors can be differentiated from each other by their dependence on ABA. Some of the genes are fully or even partially ABA-dependent (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2002).

ABA and various stress signals cross-talk and interact antagonistically or synergistically to maintain cellular homeostasis and to induce plant defensive capacity against abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 2000).

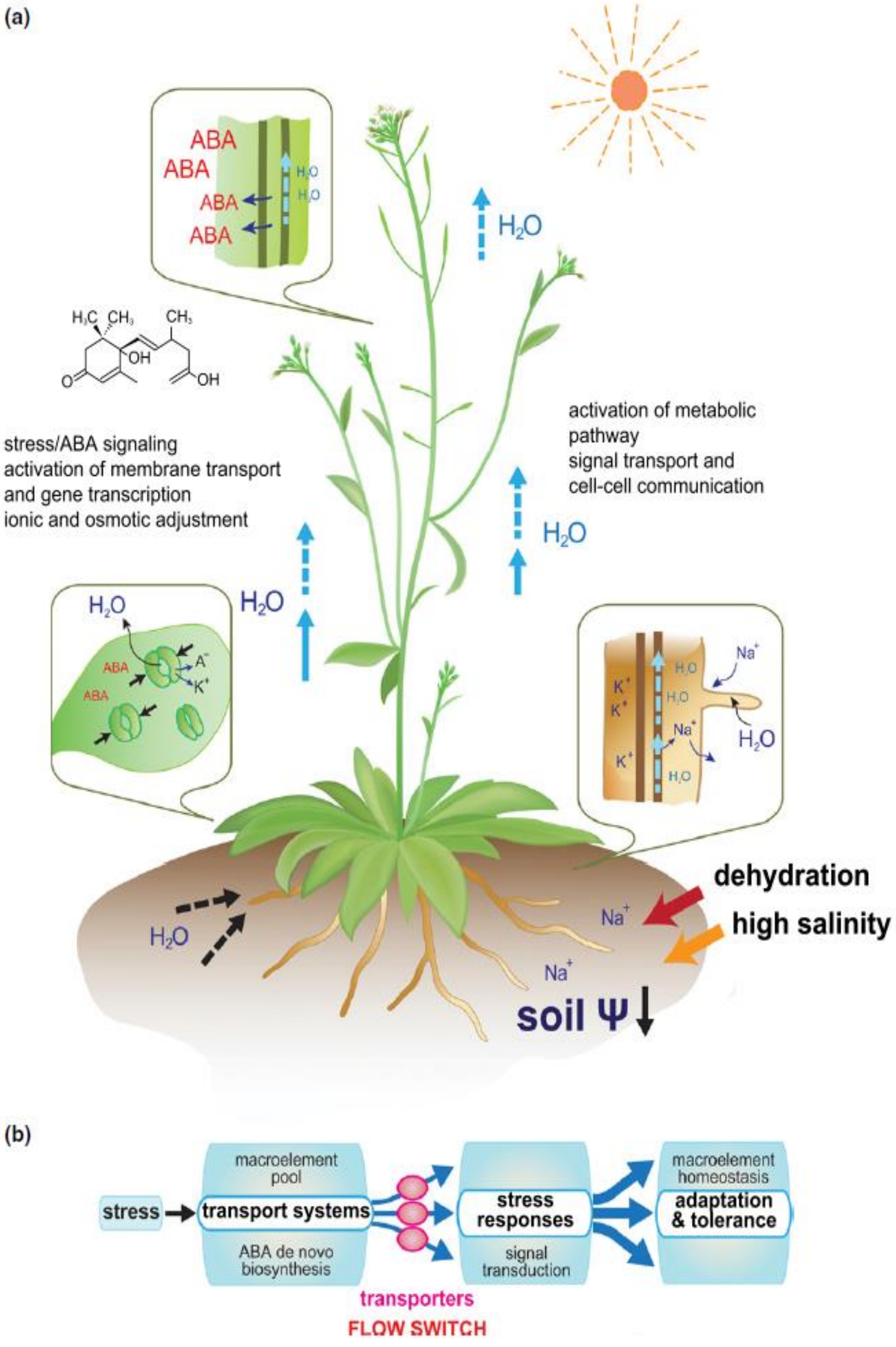

Fig. 3. Plant transport system and responses to drought and high salinity stresses. (a) Abiotic stressors stimulate the biosynthesis and transport of plant hormones, like abscisic acid (ABA), triggering of signaling networks, activation of membrane transport systems and transcriptional activation of a number of stress-responsive genes. All these processes control the plant cellular homeostasis and ability to survive under stress conditions. (b) The transport systems enhance and accelerate the plant responses to stress (According to Osakabe et al., 2013).

Molecular responses

- Genes and transcription factors involved in ABA-dependent and ABA-independent

Abiotic stress, especially drought and salt stress are responsible for serious problems resulting in environmental deterioration. Salinization for example increases from year to year (Wang et al., 2003). Zhu (2001) shows that drought and salt stress are often interconnected because they induce similar cellular damage. However, the tolerance mechanisms at the physiological and genetic levels are characterized by their complexity. Abiotic stress is controlled by molecular mechanisms which are responsible for the activation and regulation of specific genes related to this kind of stress. These genes are involved in the signal transmission, protection of the membranes and proteins, and code for molecular chaperones and enzymes that contribute to cellular detoxification (Umezawa et al., 2006b). Many abiotic stress responsive genes have been identified in plants, including rice, Arabidopsis (Fower and Thomashow, 2002) and tomato (Gong et al., 2010) by using molecular techniques such as microarray analysis. There are two major categories of genes that are involved in the responses to environmental stress: the first are functional proteins which include the osmoprotectants, free radical scavengers, and late-embryogenesis-abundant proteins (LEA) (Zhu, 2001; Wang et al., 2003) that are responsible for the protection of the membrane and proteins. The second is regulatory proteins involved in signal perception and signal transduction like MAP kinases, superoxide scavenging (SOS), phospholipase and transcription factors (Tripathi et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 1997).

Transcription factors (TFs) such as ABA-responsive element-binding proteins (*AREB*), C-repeat binding factors (*CBF*), No Apical Meristem, ATAF and Cup-Shaped Cotyledon (*NAC*), basic leucine zipper (*bZIP*) and Myeloblastosis (*MYB*) regulate many genes involved in plant water stress tolerance. Their binding targets are present in the promoter region of different stress-responsive genes. Their function is to ensure the activation of genes responsible for tolerance enhancers (Akhtar et al., 2012) and they regulate many biochemical and physiological processes.

The response pathways under abiotic stress can be classified into two categories, ABA-dependent and ABA-independent (Umezawa et al., 2006a; Nakashima et al., 2012; Tripathi et al., 2013). The transcriptional regulatory networks (including these pathways) of abiotic stress signals and gene expression has been described in detail (Figure 4). *AREB* for example is one of the main *cis*-acting elements in ABA-depending signaling of water stress responses (Shinozaki and Yamaguchi-Shinozaki, 2007). AREBs are activated via phosphorylation of a specific site (Furihata et al., 2006). It has been shown that *AREB* controlling ABA-dependent signaling of the Arabidopsis *RD29B* gene via two (*bZIP*) transcription factors (Uno et al., 2000). The overexpression of *AREB* is responsible for an increase in drought tolerance in Arabidopsis (Uno et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2007; Kang et al., 2002). This transcription factor has been reported in different species like rice (Lu et al., 2009), tomato (Orellana et al., 2010) and Arabidopsis (Choi et al., 2000; Uno et al., 2000).. *AREB* induces antioxidative processes to protect membranes in tomato plants (Orellana et al., 2010). *AREB* is responsible to activate two dehydrin genes (Hsieh et al., 2010).

Another important TF is *CBF3,* (C-repeat binding factors, also known as dehydration-responsive element binding proteins, DREB*s*) promote higher tolerance to drought and salinity (Kasuga et al., 1999). *CBF3* is related to ABA-dependent pathways and regulates the LEA-like genes. In addition, the overexpression of *CBF3* in tobacco and in rice (Kasuga et al., 2004) increases tolerance to low temperature and drought stress (Oh et al., 2005). The abiotic stress signaling components of *CBFs* might be regulated by specific ubiquitin-mediated degradation (Chinnusamy et al., 2004). *CBF* genes are considered as “master switches” (Hsieh et al., 2002). Their expression increases freezing tolerance in transgenic Arabidopsis plants in the absence of cold stimulation by activating the expression of *COR* genes (Lee and Seo, 2015). In addition, the overexpression of *CBF3* increases not only freezing tolerance but also salt and drought tolerance in transgenic Arabidopsis via the *RD29A* promoter (Kasuga et al., 1999), in transgenic tomato plants (Hsieh et al., 2002) and in rice (Oh et al., 2005). The expression of *CBF3* is responsible to reduce the accumulation of ROS in transgenic tomato (Rai et al., 2013).

NAC is one of the largest families of TFs in plants (Ma et al., 2013) that is localized in the nucleus (Ma et al., 2013). Their role is to regulate a number of biochemical processes that protect plants under different stress conditions (Tran et al., 2010). Proteins of this family have been identified in Arabidopsis (Nakashima et al., 2009).

However, overexpression of *ZmSNAC1* at the germination stage in Arabidopsis after osmotic stress enhances tolerance to dehydration compared with wild-type seedling (Lu et al., 2012). In rice, *SNAC1* strongly enhances plant resistance under drought and salt stress with no effect on yield (Hu et al., 2006; Song et al., 2011a). *NAC3* is a regulator of ROS metabolism in tobacco plants (Liu et al., 2013). NAC proteins share a highly conserved N-terminal domain which is responsible for DNA-binding activity (Ooka et al., 2003). *NAC* had earlier been shown to enhance tolerance to drought and salt stress in transgenic rice (Hu et al., 2006), was also significantly activated by salt and water-deficit stresses, but relatively weakly by ABA in seedling leaves in wheat (Mao et al., 2012).

RABC2a encodes a hydrophilic, glycine-rich protein (18.5KDa). *RAB* is characterized by its similarity in the other plant species (Lang and Palva, 1992). *RAB* genes are expressed during late embryogenesis, the beginning of seed dehydration and also in the vegetative tissues stressed with drought, salt or exogenous ABA. They are expressed in both mono- and dicotyledonous plant species. *RAB* is related to ABA-responsive secretion (Jose et al., 2013). The expression of *RAB18* in *Arabidopsis thaliana* increases when the plants are subjected to drought stress, which is due to the increase of the endogenous ABA level (Lang et al., 1994).

Genetic analysis demonstrates that the components induced by ABA-dependent and ABA-independent pathways can converge or cross-talk in the signaling pathways (Tuteja, 2007). For example, the expression of *RD29* gene in *A. thaliana* is regulated according to both ABA-dependent and ABA-independent (Yamaguchi-Shinozaki and Shinozaki, 1993). In addition, under drought and cold stress, proline accumulation in plants can be induced by both ABA-dependent and ABA-independent signaling pathways (Mahajan and Tuteja, 2005). The upregulation of pea DNA helicase 45 (PDH45), under salt stress, is mediated by ABA-dependent pathway (Sanan-Mishra et al., 2005), while, under salt stress, ABA-independent pathways induced calcineurin B-like protein (CBL) and CBL-interacting protein kinase (CIPK) from pea that plays an important role in response to calcium and stress (Mahajan et al., 2006 a,b). In general, gene expressions induced upon osmotic stress are mediated essentially by the ABA-dependant pathways.

Recently, the expression of *PR* genes was demonstrated to have a key role in abiotic stress tolerance. In *A. thaliana*, the increase of *PR* genes (*PR1*, *PR2*, and *PR5*) expression mediated by SA-pathway enhanced stomatal closure under drought condition (Liu et al., 2013). Exogenous SA controls the expression of PR proteins in order to increase *Triticum aestvum* resistance to drought and freezing (Horváth et al., 2007).

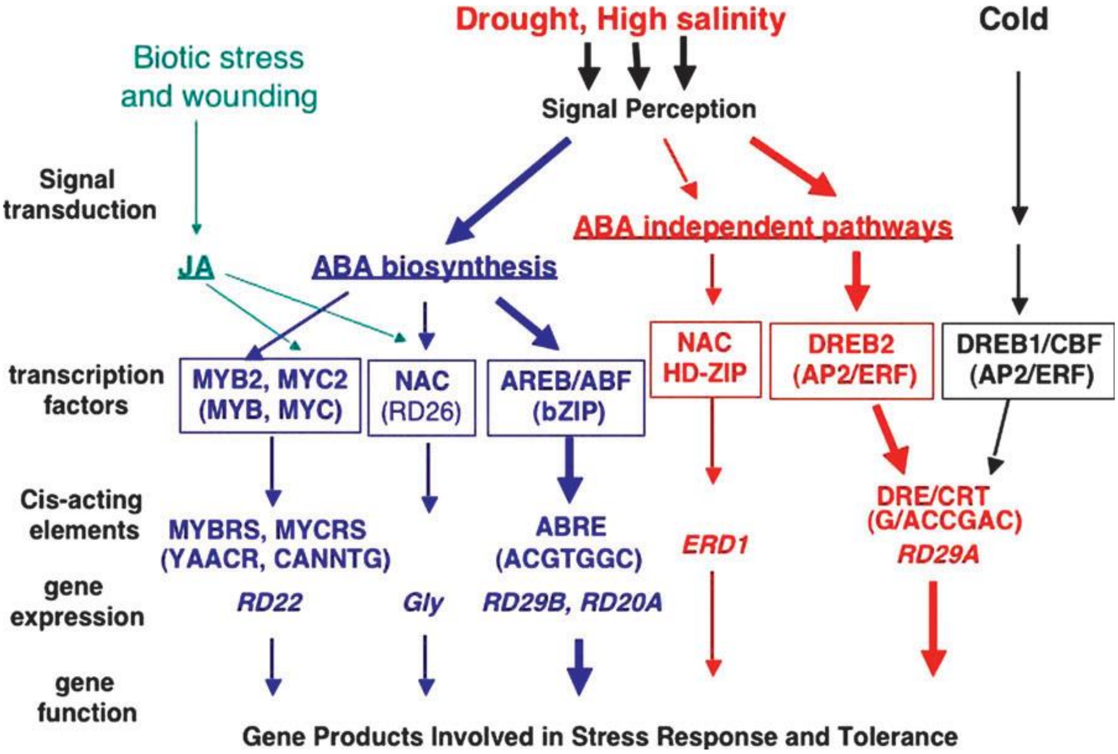

Fig. 4. Transcriptional regulatory networks of abiotic stress signals and gene expression. At least six signal transduction pathways exist in drought, high salinity, and cold-stress responses: three are ABA-dependent and three are ABA-independent (According to Shinozaki and Yamaguchi-Shinozaki, 2007).

Inducing abiotic stress tolerance

Induction of resistance is a way to help the plants under severe environmental conditions. This induction of tolerance can be achieved by different factors. Acclimation is one of the classical strategies which can be installed when there is gradual exposure of plants to an increased level of stress. The tolerance of these plants becomes higher than in not acclimated ones (Thomashow, 2001). The combination of two abiotic stresses, applied successively, shows unexpectedly an increasing tolerance in plants (Mittler, 2006). The combination of abiotic and biotic stress induced resistance against biotic stress in plants. For example, drought stress enhanced the capacity of tomato plants to defend themselves against pathogen attacks (Pye et al., 2013). In addition to their beneficial role in plant defense against pathogen attacks, beneficial microorganisms induce also tolerance against abiotic stress. Banana plants are able to grow normally in saline soil and this is due to the presence of mycorrhiza (Yano-Melo et al., 2003). Plant growth-promoting rhizobacteria (PGPR) are able to increase tolerance in plants by inducing phytohormones and antioxidants and also increase the expression of stress-response genes. Nadeem et al., (2010) report that PGPR induce salt tolerance in maize and wheat. In addition to these strategies, it has been shown that the addition of chemical compounds like inositol protects onion plants against salt stress (Chatterjee and Majumder, 2010), Pyraclostrobin and strobilurin help maize plants to cope with drought stress (Beckers and Conrath, 2007).

B. Plant response to biotic stress

Stress recognition

In their environment, plants are confronted with different pathogens and pests that are responsible to disrupt the development of the plants. Thus, plants have developed, through evolution, an efficient and complex immune system to defend themselves against pathogen attack. Plants possess pre-existing defense machinery to induce specific defense mechanisms against attackers that have the power to overcome the barriers (Spoel and Dong, 2012). During the first step of the plant defense response that acts at the plasma membrane, attackers are recognized by a pathogen or microbe-associated molecular patterns (PAMPs/MAMPs) like Flagellin, chitin, glycoproteins, and lipopolysaccharides and by trans-membrane pattern recognition receptors (PRRs) (Zipfel, 2009; Bittel and Robatzek, 2007). After perception of a MAMP by the plant cell, diverse responses such as calcium influx and accumulation of ROS and nitric oxide (NO) occur inducing a resistance-mediating process commonly named “PAMP-triggered immunity” (PTI) (Jones and Dangl, 2006). In addition to PAMPs and MAMPs, plants are also able to recognize pathogen attack by damage-associated molecular patterns (DAMPs). DAMPS are known as polysaccharides released from cell wall or as endogenous peptides. Hufflaker and colleagues (2011) identified an orthologue of AtPep1 from maize which is an endogenous elicitor against attackers.

The second step of the plant innate immune system takes place in the cytoplasm when the pathogens and pests succeed to face the first lines of defense and became able to suppress or mitigate plant defense mechanisms. Attackers deliver several effectors proteins into plant cells. These effectors also called avirulence (Avr) proteins manipulate the plant defense mechanisms through suppressing PTI (Espinosa and Alfano, 2004; Grant et al., 2006). Ellis et al. (2007) demonstrated that AVRK and AVRA10 proteins are employed by Blumeria graminis f. sp. hordei in barley cells to suppress PTI. During the second step, the Avr protein is recognized and attenuated by nucleotide-binding leucine-rich repeat (NB-LRR) proteins, which are encoded by plant resistance (R)-genes (Elmore et al., 2011). The recognition of Avr proteins by plant R-proteins results in amplification in the plant response mechanisms; this phenomenon is called effector-triggered immunity (ETI) or R gene-mediated resistance (Jones and Dangl, 2006). This plant defense response often results in cell death in order to inhibit pathogen spread, this is known as a hypersensitive response (HR) (Greenberg and Yao, 2004).

PTI and ETI can suppress attackers by inducing downstream responses that can lead in a local and systemic induced resistance through callose deposition, lignin accumulation, accumulation of PR proteins, and production of secondary compounds.

In addition to the basal defenses there is a possibility to induce resistance against a broad spectrum of pathogens and insects by ways of systemic acquired resistance and induced systemic resistance.

Systemically induced resistance

Many plants are able to develop and enhance their biological defensive response against further pathogen attack through the perception of specific stimuli. This kind of effective plant protection against attackers is named induced resistance (IR) (Van Loon, 2000). Systemic acquired resistance (SAR) is a classical form of IR, which is observed in distal plant parts upon pathogen infection (Durrant and Dong, 2004; Vlot et al., 2008). Also, root colonization by beneficial micro-organisms, is able to induce resistance against necrotrophic pathogens and pests, this resistance is called induced systemic resistance (ISR) (Van Loon, 2007; Van Wees et al., 2008).

- Systemic acquired resistance (SAR)

After pathogen attack, which is responsible to cause a hypersensitive response (HR; Fu et al., 2012), distal leaves receive sophisticated defense mechanisms to induce resistance responses (Vlot et al., 2008). This phenomenon is known as systemic acquired resistance (SAR; Shah, 2009) and it is effective against a broad range of biotrophic and hemibiotrophic pathogens (Grant and Lamb, 2006). SAR is regulated by the accumulation of SA which triggers the activation of the transcription factor activator NPR1 (nonexpressor of *PR* genes). NPR1 induces important changes in the defense gene transcriptome through specific interaction with transcription factors (Maleck et al., 2001; Mou et al., 2003). Moreover, SAR increases *PR* gene expressions like *PR1* and *PR5*. This up-regulation in *PR* genes leads to induce resistance in maize and barley (Morris et al., 1998; Walters et al., 2011). In addition, SAR induced PR proteins like chitinase, glucanase, and thaumatin which have antimicrobial power against attackers (Mauch et al., 1988; Van Loon et al., 2006). Methyl salicylic acid (MeSA) is a one among the mobile distant signals involved in SAR in Arabidopsis and tobacco which can to be converted back to SA (Park et al., 2007). Some studies presented the positive effects of increasing SA on Arabidopsis resistance against attackers like *Staphylococcus aureus* (Prithiviraj et al., 2005a), *Pseudomonas aeruginosa* (Prithiviraj et al., 2005b) and *Agrobacterium tumefaciens* (Yuan et al., 2007). Exogenous application of SA or other synthetic chemicals that can activate SAR such as 2,6-dichloroisonicotinic acid (INA; Métraux et al., 1990), S-methyl benzo1,2,3-thiadiazole-7-carbothioate (BTH; Ryals et al., 1996; Wang et al., 2005), 3-allyloxy-1,2-benziso-thiazole-1,1- dioxide (probenazol; Nakashita et al., 2002 a), 1,2- benzisothiazole-1,1-dioxide (BIT; Yoshioka et al., 2001), N-cyanomethyl-2- chloroisonicotinamide (NCI; Nakashita et al. 2002b) and 3’-chloro-4,4’-dimethyl-1,2,3- thiadiazole-5-carboxanilide (tiadinil (TDL); Yasuda et al., 2004, Pye et al., 2013) increase plants resistance to certain pathogens by inducing SAR. For example, Radhakrishnan et al. (2011) demonstrated that BTH induces SAR in cucumber against *Pythium aphanidermatum*. TDL induced resistance in tomato plants mediated by SA against *P. syringae* (Pye et al., 2013). Exogenous SA attenuates crown gall disease caused by *Agrobacterium tumefaciens* in *Nicotiana benthamiana* plants whereas transgenic plants silenced for genes involved in SA biosynthesis and signaling (NahG) were sensitive to *Agrobacterium* infection (Anand et al., 2008).

- Induced systemic resistance (ISR)

Plant resistance can be induced systemically following root colonization by some beneficial soil microbes like plant growth promoting rhizobacteria (PGPR) or endophytic fungi (PGPF) (De Vleesschauwer and Höfte, 2009; Trillas and Segarra, 2009; Pieterse et al., 2014). ISR increases plant resistance against a large spectrum of necrotrophic pathogens and pests (Van Loon, 2007; Pineda et al., 2010; Berendsen et al., 2012). To better understand rhizobacteria-mediated ISR, *Pseudomonas fluorescens* WCS 417r has been used as a protective agent in Arabidopsis against several leaves and root pathogens such as *Pseudomonas syringae* pv. *tomato* (bacterial leaf pathogen), *Xanthomonas campestris* pv. *armoraciae* (bacterial leaf pathogen), *Alternaria brassicicola* (fungal leaf pathogen), *Fusarium oxysporum* (fungal root pathogen), and *Peronospora parasitica* (oomycete leaf pathogen) (Ton et al., 2002). Moreover, Van Oosten and colleagues (2008) identified in Arabidopsis that ISR is effective against the herbivorous insects *Spodoptera exigua*. However, MYC2-impaired *jin1* mutants are not able to benefit from ISR mediated by WCS 417 (Pozo et al., 2008). Several studies on ISR demonstrated that, at the site of pathogen infection, an enhanced accumulation of plant defense proteins like chitinase and peroxidase was detected following infection by *Xanthomonas oryzae* pv. *oryzae* or *Gaeumannomyces graminis* var. *tritici* (Sari et al., 2008; Chithrashree et al., 2011). ISR involves signaling pathways that are JA and ET-dependent but requires SA independent (Pieterse et al., 1998). Research on rice demonstrated that ISR induced by *P. fluorescens* WCS374r was dependent on JA/ET-signaling pathways but was independent of SA-signaling (De Vleesschauwer et al., 2008). In barley, ISR mediated by *Pseudomonas fluorescens* involved JA-signaling pathway to increase resistance against *Fusarium* (Petti et al., 2010). NPR1 protein is required in ISR and acts through JA and ET-dependent signaling and is responsible to increase resistance against *P. syringae* (Van Wees et al., 2000).

Surprisingly, beneficial soil microbes can, in a specific case, act similarly to SAR by increasing resistance against biotrophic pathogens through SA-dependent signaling leading to PR protein accumulation (Molitor et al., 2011). Interestingly, Van Wees and colleagues (2000) presented the additive effect of combined SAR and ISR to enhance plant resistance against *P. syringae,* despite the known antagonism between SA and JA (Koornneef and Pieterse, 2008).

C. Combination of abiotic and biotic stress

The field is very different from the controlled conditions used in laboratory studies, and often involves the combined exposure of plants to more than one stress condition.

There are several kinds of stress combinations such as a combination of drought and salt stress, drought and heat, cold and salinity, or any of the major abiotic stresses like salinity combined with pathogen infection such as *B. cinerea*. According to the results of recent studies, the plant response to combined stressors is different from the plant response to a single stress (Suzuki et al., 2014). In addition, the plant response to simultaneous abiotic and biotic stress cannot be predicted. There is a cross-talk between plant the hormones ABA and SA in plant defense and it was shown that ABA negatively regulates SA-dependent defense signaling (Jiang et al., 2010). Sorghum and bean stressed by drought stress present a higher susceptibility to *Macrophomina phaseolina* (Diourte et al., 1995; Mayek-Petez et al., 2002). The same result was obtained from Arabidopsis exposure to drought stress and *P. syringae* (Mohr and Cahill, 2003). In contrast, it has been shown that, in some case, the exposure of plants to abiotic stress enhances resistance to pathogen attacks (Bowler and Fluh, 2000; Mittler and Blumwald, 2010) via the positive effect of ABA on callose deposition (Mauch-Mani and Mauch, 2005; Ton et al., 2009) and the induction of gene expression in response to both kinds of stress (Abou Qamar et al., 2006). Achuo et al., (2006) demonstrate the positive effect of drought stress to enhance resistance against *B. cinerea* in tomato. This positive interaction can be due to stomatal closure reducing water loss from infected tissues, or the high levels of defense compound accumulated after a period of abiotic stress.

D. Priming for inducing resistance

To survive and to be able to complete their life cycle in unfavorable conditions, plants must respond quickly and appropriately to stress. The resistance of the plant depends on the speed and intensity of the establishment of defensive mechanisms. Although plants can acclimate (not heritable modifications) or adapt (heritable modifications) to stressful conditions, the protection remains insufficient if the intensity and the period of stress are too strong. Over the past years, it has been shown that plants can be primed for more efficient activation of defense responses by reacting rapidly and efficiently to a stress. This “primed state” is often associated with an enhanced and induced resistance to biotic and abiotic stresses (Conrath et al., 2002, Mauch-Mani et al., 2017). Usually, this state appears after pre-treatment with biological stimuli such as rhizobacteria, mycorrhizal fungi, and virulent or avirulent pathogens, or can also be triggered by chemicals like SA, BTH, and BABA etc (Figure 5) (Conrath et al., 2006).

The mode of action of these compounds remains mostly unclear, but it is known that such compounds play an important role to strengthen the behavior of stressed plants (Muthukumarasamy et al., 2000; Flors et al., 2007; Vicedo et al., 2009). To respond faster and stronger, primed plants accelerate and increase their ability to activate the defense against pathogen attack (Prime-A-Plant Group, 2006). Priming is considered as a critical process responsible to cope with a certain stress situation and the response becomes faster and more intense (Mauch-Mani et al., 2017). Priming has been described in various plant defenses to be a common feature of a plant's immune system that protects against attackers (SAR and ISR) and against abiotic stress (Zimmerli et al., 2001; Conrath, 2011; Ton et al., 2009) (Figure 5).

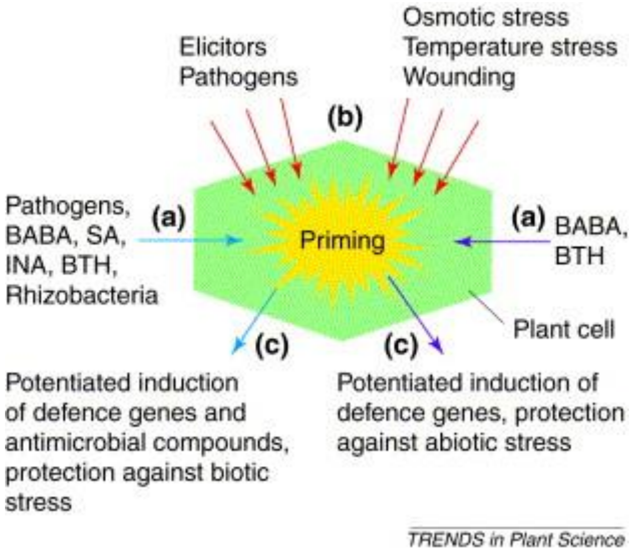

Fig. 5. A pretreatment with Salicylic acid (SA), β-aminobutyric acid (BABA), Dichloroisonicotinic acid (INA) or Benzothiadiazole (BTH) primed the plants to react faster and stronger under stress. (a) Priming step. (b) Challenge with biotic or abiotic stress. (c) Potential response (According to Conrath et al., 2002).

BABA-IR

β-aminobutyric acid (BABA) is a non-protein amino acid and known as an excellent priming inducer (Baccelli and Mauch-Mani, 2016; Mauch-Mani et al., 2017). BABA has been known for more than 50 years for its effective resistance in stressed plants against a high number of biotic stress like fungi, virus, bacteria and pests as well as abiotic stress such as drought, salinity, cold or heat (Jakab et al., 2001; Ton et al., 2005; Balmer et al., 2015; Cohen et al., 2016). The high efficacy of BABA to induce resistance can be related to its ability to potentiate several defense signaling pathways

(Baccelli and Mauch-Mani, 2016) (Figure 6). BABA-induced resistance (BABA-IR) depends on SA and JA/ET signaling pathways against pathogens (Jakab et al., 2001) and depends also on ABA signaling for callose accumulation (Ton and Mauch-Mani, 2004); or against abiotic stress via ABA and SA defense signaling (Jakab et al., 2005; Khan et al., 2015) (Figure 5). Interestingly, it has been shown that the primed state of a plant by BABA can be transferred to the next generation through the seeds. The progeny of such primed plants reacts naturally faster to a given stress and become better protected (Slaughter et al., 2012). This transgenerational priming is probably based on epigenetic mechanisms (Luna et al., 2012; Slaughter et al., 2012). For more than 50 years, BABA has been known to be xenobiotic, but recently, it has been demonstrated that various plants do synthetize BABA and can be implicated in the induction of plant resistance against such stress (MauchMani et al., 2017; Thevenet et al., 2017; Baccelli et al., 2017). Accordingly, endogenous BABA levels increase in Arabidopsis and other crops after salt stress and infection with virulent pathogens (Thevenet et al., 2017). Baccelli et al. (2017) demonstrated that the accumulation of BABA increased rapidly after infection with avirulent *Pst AvrRpt2* leading to enhance the defense reaction (Baccelli et al., 2017). Interestingly, being present naturally in plants, BABA presents a great deal for enhancing the plants resistance against various abiotic and biotic stresses.

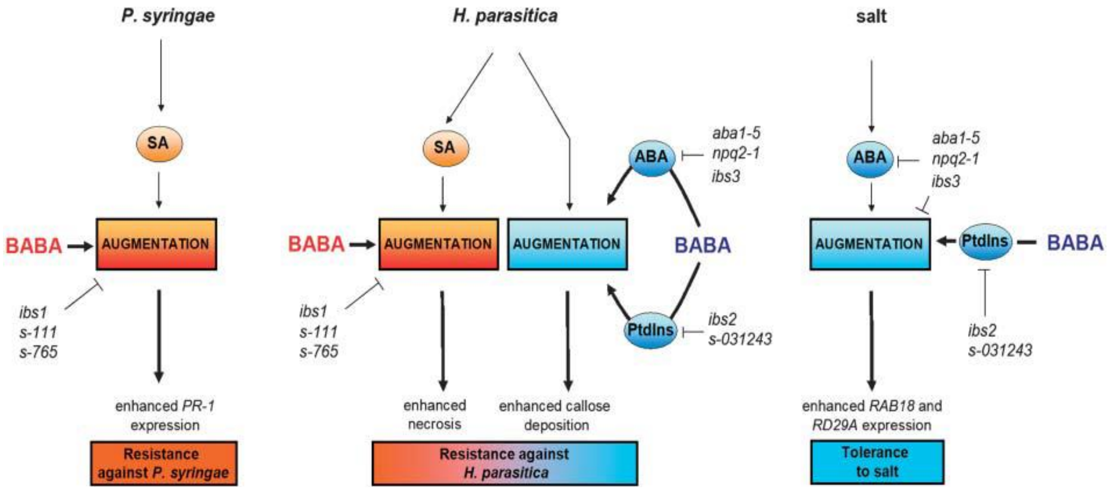

Fig. 6. Model for the priming mechanisms by BABA against abiotic (salt stress) and biotic (*P. syringae* and *H. parasitica*) stresses. Salt stress toleramce is mediated by ABA-dependent pathway, *P. syringae* resistance is mediated by SA and *H. parasitica* is mediated by both SA and ABA (According to Ton et al., 2005).

Effect of BABA on abiotic stress

BABA is a potent inducer of resistance against abiotic stress (Ton et al., 2005; Jakab et al., 2005). The signaling pathways are generally controlled by ABA and/or SA under different kind of abiotic stress (Jakab et al., 2005). It was shown that BABA induces drought resistance in crabapple (Macarisin et al., 2009). In contrast, mutants in the ABA signaling pathway, *aba1* and *aba4* lose tolerance induced by BABA to salt stress and drought (Jakab et al., 2005). Zimmerli et al. (2008) showed that the thermotolerance induced by BABA is associated with accumulation of genes encoding transcription factors related to the ABA pathway. The PR proteins were previously shown to be induced only after pathogen attack, while, other studies have proved that these PR family members are also induced under abiotic stress conditions and play a crucial role in BABA-inducing resistance. Following BABA treatment, salinity and drought tolerance increased in Arabidopsis. BABA primes the expression of ABA-dependent *RAB18* and *RD29* to enhance the response of plants to drought stress (Ton et al., 2005). Similarly, the expression SA-dependent *PR-1* and *PR-5* after salt and drought stress are higher in plants treated with BABA, although induced resistance is still observed in the transgenic plant *nahG* and the mutant *npr1* (Jakab et al., 2005). It was reported that endo-1,3-b-glucosidase (chitinase), which belongs to the PR protein family participated in the plant defense against salinity (Song et al., 2011b) and drought (Macarisin et al., 2009; Faghani et al., 2015). Mosteck et al. (2016) indicated that BABA-induced the up-regulation of antioxidant enzymes, PR proteins and chaperones in barley enhancing the plant defense and detoxification processes against drought stress. ABA accumulation induced a partial stomatal closure leading to reduce water use under drought conditions in treated wheat with BABA (Du et al., 2012). Similarly, an acceleration of stomatal closure was detected in BABA-treated Arabidopsis through an enhanced ABA upon salt and drought stress (Jakab et al., 2005). Under severe drought stress, ROS accumulation was lower in treated wheat with BABA compared to untreated plants, which is due to the enhancement of antioxidant enzymes activities (SOD, CAT, and GR) responsible to mitigate the negative effect of ROS and reducing the oxidative (Du et al., 2012). A significant increase was detected in several antioxidants like APX and SOD responsible to induce resistance in BABA-treated plants under acid rain (Liu et al., 2011). Upon BABA treatment, the majority proteomic differences detected under salt stress result in enhanced detoxification in barley (Mostek et al., 2016). Furthermore, BABA induced photosynthesis inhibition under salt stress through changes in carbohydrate metabolism. After BABA priming, a significant up-regulation of the proteins involved in cell respiration (glyceraldehyde-3-phosphate dehydrogenase, enolase, and 6-phosphogluconate dehydrogenase) has been shown in barley, which ensures the increase in required energy (Mostek et al., 2016). The accumulation of these proteins might be related to plant adaptation under oxidative stress (Rasoulnia et al., 2011). In addition, BABA has a positive effect against abiotic stress by inducing lignin deposition, but there are only a few works studying this subject.

Lignin biosynthesis can be regulated by transcription factors like MYB (v-myb avian myeloblastosis viral oncogene homolog). The overexpression of MYB58 and MYB63 produced the cell lignifications through the expression of lignin biosynthesis-genes (Guo et al., 2017; Liu et al., 2018). On the opposite, a decrease in lignin biosynthesis occurred in BABA-treated crabapple seedling under drought stress through the removal of COMT (enzyme implicated in the lignin biosynthesis) (Macarisin et al., 2009).

Effect of BABA on biotic stress

The signaling pathway controlling BABA-induced resistance (BABA-IR) depends on SA and ABA against both biotrophic and necrotrophic pathogens (Jakab et al., 2001; Ton and Mauch-Mani, 2004; Mauch-Mani and Mauch, 2005). Moreover, BABA-IR has been demonstrated to be effective against nematodes and insects (Oka and Cohen, 2001; Hodge et al., 2006). Treatment with BABA primes *Arabidopsis* infected with *Plectosphaerella cucumerina* for an enhanced formation of callose-rich papillae (Pastor et al., 2013). The increase of callose deposition at the site of attempted penetration in BABA-treated plants is regulated by ABA, which allows the plants to resist pathogens (Ton and Mauch-Mani, 2004). Furthermore, BABA is capable to increase resistance through SA-dependent *PR1* gene expression (Po-Wen et al., 2013; Zhong et al., 2014). In the Arabidopsis-*P. syringae* pv. *tomato* pathosystem, SA is required to induce resistance by BABA (Zimmerli et al., 2000). BABA enhanced the resistance in various species and against different attackers like in *A. thaliana* against *B. cinerea* or against *Pectobacterium* ssp. *carotovorum* (Pcc) (Zimmerli et al., 2001; Po-Wen et al., 2013), in grapevine against *P. viticola* (Cohen et al., 1999; Hamiduzzaman et al., 2005), in tomato against *P. infestans* or against early blight

(Cohen et al., 1994; Roylawar et al., 2015), *brassicicola* and *P. cucumerina* (Ton and Mauch-Mani, 2004), in harvested peaches against *Rhizopus stolonifier* (Wang et al., 2018) or in potato against *P. infestans* (Floryszak-Wieczorek et al., 2012). The Arabidopsis mutant *pmr4*, deficient in callose synthase, has lost the resistance induced by BABA to *A. brassicicola* but maintains a basal resistance to *P. syringae* (Flors et al., 2008). Under biotic stress, the expression *LOX-9* and *PR-4* (JA-regulated genes) were enhanced by BABA responding to *P. viticola* (Hamiduzzaman et al., 2005). *PR-1* was upregulated in treated plants with BABA in response to *B. cinerea* (Zimmerli et al., 2000). BABA-IR acts like the typical SAR by inducing the accumulation of PR proteins through SA, JA or ET signaling pathways in infected plants (Van Loon et al., 2006). BABA-primed plants were reported to increase PAL activity (lipophilic substance that inhibit the development of the pathogens at the site of infection) and induction of lignin accumulation in grapevine against *P. viticola* (Cohen et al., 1999) and downy mildew (Hamiduzzaman et al., 2005), in potato against Phytophthora infestans (Bengtsson et al., 2014) and in rice against nematodes (Ji et al., 2015). The increase of lignin deposition in BABA-treated plants leads to increase resistance against attackers.

It has been reported that ROS has a crucial role in the plant signaling network (Mittler et al., 2011). In lettuce, BABA enhanced ROS accumulation upon infection with *Bremia lactucae* (Cohen et al., 2001). The increase of H_2O_2 in BABA-treated grape was detected after *P. viticola* infection (Dubreuil-Maurizi et al., 2010). Moreover, Pastor and colleagues (2013) found that BABA induces H_2O_2 and callose accumulation in Arabidopsis against *P. cucumerina* or treated with chitosan, suggesting that ROS homeostasis is regulated by BABA.

Depending on the plant species and the intensity of the stress, ROS homeostasis can change in BABA-treated plants.

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Chapter I - General Introduction

IB: Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms

Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms

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Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms

Abstract

Plants are constantly confronted by both abiotic and biotic stresses that seriously reduce their productivity. Plant responses to these stresses are complex and involve numerous physiological, molecular, and cellular adaptations. Recent evidence shows that a combination of abiotic and biotic stress can have a positive effect on plant performance by reducing the susceptibility to biotic stress. Such an interaction between both types of stress points to a crosstalk between their respective signaling pathways. This crosstalk may be synergistic and/or antagonistic and include among others the involvement of phytohormones, transcription factors, kinase cascades, and reactive oxygen species (ROS). In certain cases, such crosstalk can lead to a cross-tolerance and enhancement of a plant's resistance against pathogens. This review aims at giving an insight into cross-tolerance between abiotic and biotic stress, focusing on the molecular level and regulatory pathways.

Keywords: cross-tolerance; biotic stress; abiotic stress; plant hormones

1. Introduction

Plants have to deal with various and complex types of interactions involving numerous environmental factors. In the course of evolution, they have evolved specific mechanisms allowing them to adapt and survive stressful events. Exposure of plants to biotic and abiotic stress induces a disruption in plant metabolism implying physiological costs [1–4], and thus leading to a reduction in fitness and ultimately in productivity [5]. Abiotic stress is one of the most important features of and has a huge impact on growth and, consequently, it is responsible for severe losses in the field. The resulting growth reductions can reach >50% in most plant species [6]. Moreover, biotic stress is an additional challenge inducing a strong pressure on plants and adding to the damage through pathogen or herbivore attack [7–11].

A crucial step in plant defense is the timely perception of the stress in order to respond in a rapid and efficient manner. After recognition, the plants' constitutive basal defense mechanisms [12] lead to an activation of complex signaling cascades of defense varying from one stress to another [13,14]. Following exposure to abiotic and/or biotic stress, specific ion channels and kinase cascades [15] are activated, reactive oxygen species (ROS) [16], phytohormones like abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) [17] accumulate, and a reprogramming of the genetic machinery results in adequate defense reactions and an increase in plant tolerance in order to minimize the biological damage caused by the stress [18].

In recent years, research has mainly concentrated on understanding plant responses to individual abiotic or biotic stresses [19–22], although the response to simultaneous stresses is bound to lead to a much more complex scenario [18]. From the perception of the stimulus (stress) to the final response in cells, plants use various signaling pathways depending on the challenge(s). It seems that plants respond in a specific manner when they have to face more than one stress simultaneously, and the response cannot be predicted based on the plant’s response to the individual single stresses [23]. Research on multiple stresses has been trying to simulate natural conditions, but in the field, conditions are not controlled, and one stress can strongly influence the primary stress defense response of the plants [18]. Moreover, plants can show different degrees of sensitivity depending on the field condition and the developmental stage of the plant [24]. Additional factors that can influence an interaction are the intensity of the stress and the plant species. Various interactions can take place between the defenses induced after perception of the stresses. They depend on the specific combination of stresses and even on the degree of simultaneity [15,25,26]. It is not clear whether simultaneous stresses are rather antagonistic, synergistic or additive, inducing more or less susceptibility to a specific kind of stress [27,28]. Combination of two stressors can have a negative and additive effect on plants, the second stress being the one that leads to a greater damage [29]. On the other hand, the combination of stresses can also lead to antagonistic responses in the plants [30,31]. Common beans exposed to drought stress display more symptoms when infected by *Macrophomina phaseolina* [29] and treatment of detached tomato leaves with exogenously applied ABA increases the susceptibility of wild type plants to *Botrytis cinerea* [32].

Interestingly, one possible outcome of multiple stress exposure is that plants that are able to defend themselves facing one stress can become more resistant to other stresses [33]. This phenomenon is called cross-tolerance, showing that plants possess a powerful regulatory system that allows them to adapt quickly to a changing environment [33–35]. Wounding, for instance, increases salt tolerance in tomato plants [34]. Furthermore, in tomato plants again, localized infection by *Pseudomonas syringae* pv. tomato (*Pst*) induces systemic resistance to the herbivore insect *Helicoverpa zea* [36]. The association between abiotic and biotic stress is also possible [13], as demonstrated by the reduced infection of tomato by *Botrytis cinerea* and *Oidium neolycopersici* following the application of drought stress [37].

Ozone exposure can induce resistance to virulent *Pseudomonas syringae* strains in *Arabidopsis* [38]. Conversely, biotic stress can also interfere to increase the resistance to abiotic stress. This effect is visible when plants are under pathogen attack. Infection may cause stomatal closure to hinder pathogen entry and as a consequence water loss is reduced and leads to an enhanced plant resistance under abiotic stress [39]. Xu and colleagues [40] show that viral infection protects plants against drought stress. Verticillium infection in Arabidopsis plants induced the expression of the Vascular-Related No Apical meristem ATAF and Cup-Shaped Cotyledon (NAC) domain (VND) transcription factor *VND7*. VND7 induced *de novo* xylem formation ensuring the water storage capacity and as a consequence, increased plant drought tolerance [41]. Stress combination induces different signaling pathways, which share some components and common outputs [14–25]. This could help plants to minimize energy costs and create a flexible signaling network [42].

Resistance to both biotic and abiotic stress has been well documented in a variety of crops through priming of defenses. This component of induced resistance can be achieved through specific chemical stimuli like the resistance inducers BABA (beta-aminobutyric acid) or BTH (benzothiadiazole) [43,44], genetic manipulation of genes and proteins [45] or by previous contact with a pathogen [46]. Due to the complexity of interactions in defense, in the present review, we aim to focus on the cross-tolerance between abiotic and biotic stress as a part of induced resistance for defense.

2. Cross-Tolerance between Abiotic and Biotic Stress

Plants are able to manage simultaneous exposure to abiotic and biotic stress, and there is evidence for a link between the responses to these two stressful situations [23,47–49]. Usually, environmental pressure by abiotic and biotic stress can induce plant resistance. However, some plants confronted with each stress individually have also been reported to be more susceptible compared to a simultaneous exposure to two different stresses [50,51]. In addition, certain environmental stresses have the possibility to predispose the plant in order to allow it to respond faster and in a resistant manner to additional challenges. Therefore, cross-tolerance between environmental and biotic stress may induce a positive effect and enhanced resistance in plants and have significant agricultural implications. Interestingly, abiotic stress regulates the defense mechanisms at the site of pathogen infection as well as in systemic parts, thus ensuring an enhancement of the plant's innate immunity system [31]. Likewise, osmotic and proton stress are inducers of resistance in barley against powdery mildew. This induced resistance depends on the formation of callose-containing papillae capable of blocking fungal growth [48]. This kind of resistance is similar to the chemically induced resistance by BTH and INA (isonicotinic acid) [52]. Achuo *et al.* [37] demonstrated that drought stress increased the ABA content of tomato leaves, concomitantly with increasing the resistance against the necrotophic fungus *Botrytis cinerea* and that salt stress reduced susceptibility towards the biotrophic fungus *Odium neolycopersici* but not against *Botrytis cinerea*. This difference between drought and salt stress is in accordance with the observation that they both induce different gene expression patterns [53]. Additionally, the acclimation of *Nicotiana benthamiana* to moderate drought stress (60% of field capacity) reduced the growth of *P. syringae* pv. *tabaci* [26]. Recently, Atkinson and Urwin [23] reviewed the interaction of abiotic and biotic stress where they showed the common threads in pathways leading to a regulation of plant responses. Therefore, in order to prepare the plant for the battle, the activation of various detoxifying enzymes, control hormones, signaling pathways, and gene expression are indispensable [4,42,54].

The defense response of plants exposed to different stressors is expected to be complex including the interconnection of various signaling pathways regulating numerous metabolic networks [55].

3. Signaling Pathways Induced by Multiple Stress Responses

The interaction between abiotic and biotic stress induces complex responses to the different stressors. Under stress, the accumulation of certain metabolites positively affects a plant's response to both stresses and therefore protects it from multiple aggressors [25,47]. Callose accumulation, changes in ions fluxes, ROS, and phytohormones are the first responses induced to combat the stress and the resulting signal transduction triggers metabolic reprogramming towards defense [31,56].

3.1. Reactive Oxygen Species

A rapid generation of ROS is observed after stress sensing [57,58]. One of the major roles of ROS is to serve as signaling molecules in the cells [58–62]. The production of ROS is fine-modulated by the plant to avoid tissue damage [58,63–71]. ROS have long been known to be destructive and harmful compounds in stressed organisms. However, it has been shown that while high levels of ROS lead to cell death, lower levels are mostly responsible to regulate the plant's stress responses [67–69]. In biotic stress, ROS are mainly involved in signaling. This again might attenuate the oxidative stress caused by abiotic stress [70]. Furthermore, ROS could interfere in cross-tolerance [33]. ROS are involved in stress-induced tolerance in *Arabidopsis thaliana* after infection with the vascular pathogen *Verticillium* spp. by increasing drought tolerance due to *de novo* xylem formation and the resulting enhanced water flow [68]. Additionally, the production of ROS can help in cell-to-cell communication by amplifying the signal through the *Respiratory Burst Oxidase Homologue D* (*RBOHD*; [72]) and can act as a secondary messenger by modifying protein structures and activating defense genes [61,73]. ROS respond to abiotic and biotic stress, but differently from one stress to another [47]. Davletova *et al.* [74] showed that the transcription factor *Zat12* was involved in both abiotic and biotic stress and that *Zat12* could be a regulator in ROS scavenging. ROS may possibly be the central process mediating cross-tolerance between abiotic and biotic stress responsive networks [23]. In Arabidopsis, ROS production can be sensed by ROS-sensitive transcription factors [75,76] leading to the induction of genes participating in the stress responses. Gechev *et al.* [77] proposed that ROS were inducers of tolerance by activating stress response-related factors like mitogen-activated protein kinases (MAPKs), transcription factors, antioxidant enzymes, dehydrins, and low-temperature-induced-, heat shock-, and pathogenesis-related proteins.

Priming for stress tolerance induced after application of specific chemicals is responsible for certain modifications in ROS signaling [70–78]. Treatment of cucumber plants with brassinosteroids lead to a rise in H_2O_2 levels and primed the plants for both biotic and abiotic stress tolerance [68]. H_2O_2 priming for salt tolerance in citrus moderately increased the abundance of oxidized and *S*-nitrosylated proteins, and the level remained the same after stress application, however, non-treated plants were more sensitive to the stress [78].

3.2. Mitogen-Activated Protein Kinase (MAPK) Cascades

Following perception and recognition of stress stimuli, Mitogen-Activated Protein Kinase (MAPK) cascades are activated. They control the stress response pathways [79,80]. MAPKs are highly conserved in all eukaryotes and are responsible for the signal transduction of diverse cellular processes under various abiotic and biotic stress responses, and certain kinases are involved in both kind of stress [18,81,82]. Since MAPKs are involved in different stress responses, they could have a role in the combination of abiotic and biotic stress [83,84]. For instance, in cotton the kinase *GhMPK6a* negatively regulates both biotic and abiotic stress [85]. MAPK pathways activated by pathogen attack are mediated by SA, and the resulting expression of *PR* genes induces defense reactions [86]. The Arabidopsis protein VIP1 is translocated into the nucleus after phosphorylation by MPK3 and acts as an indirect inducer of *PR1* [87]. Chinchilla *et al.* [88] showed that pathogen associated molecular patterns (PAMPs) like flagellin trigger MAPK cascades in order to establish pathogen response signaling. In addition, MAPK such as MPK3, MPK4, and MPK6 also responded to various abiotic stresses [89,90]. MAPK cascades are important in controlling cross-tolerance between stress responses [12]. MPK3 and MPK6 are essential to show full primed defense responses [91], therefore, these two kinases could be important for mediating tolerance to further stresses. Over-expression of the *OsMPK5* gene and also kinase activity of OsMPK5 induced by ABA contributes to increased abiotic and biotic stress tolerance. *OsMPK5* seems to play a double role in the rice stress response, one as a positive regulator of resistance to the necrotrophic brown spot pathogen *Cochliobolus miyabeanus* and the second as a mediator of abiotic stress tolerance [81,92]. Tomato plants activate MPK1 and MPK2 against UV-B, wounding, and pathogens in order to enhance their defense reactions [93]. MAPK signaling also interacts with ROS and ABA signaling pathways leading to enhanced plant defense and induction of cross-acclimation to both abiotic and biotic stress [94–96].

3.3. Relevance of Hormone Signaling under Stress Interaction

The control of every kind of stress by specific hormones allows defense responses against defined environmental conditions. ABA is considered the primary hormone involved in the perception of many abiotic stresses [97]. Increases in ABA concentration modulate the abiotic stress-regulation network [98] while biotic stress responses are preferentially mediated by antagonism between other stress hormones such as SA and acid JA/ET [99]. In certain cases, ABA has been shown to accumulate after infection [18,27,100,101]. For instance, higher levels of ABA were observed after *Pst* DC 3000 infection [102], and this provoked a suppression of other defense responses [103]. However, recent findings show a positive effect of ABA on biotic stress resistance [30,104,105]. This dual effect makes ABA a controversial molecule that can switch from “good to bad” depending on the environmental conditions (type and timing of the stress; [105]). Moreover, under combination of abiotic and biotic stress, ABA mostly acts antagonistically with SA/JA/ethylene inducing a susceptibility of the plant against disease and herbivore attack [28,31,32,106,107]. However, since an increase of ABA under the effect of abiotic stress induces stomatal closure, as a “secondary effect”, the entry of biotic assailants through these passive ports of the plant is prevented. Hence, under such circumstances, the plant is protected from abiotic as well as from biotic stress [108]. There are three different phases showing the influence of ABA on pathogen infection [23,30]. The first effect of ABA on the combination of both, abiotic and biotic stress is related only to abiotic stress because an infection takes more time to establish itself and the plants react therefore later to it [30–109]. At this moment, ABA induces stomatal closure [110], which allows a reduction in water loss and, as a consequence, the maintenance of a beneficial water potential. In this first phase, SA, JA and ethylene might not yet be activated and ABA can antagonize their induction. In this situation, future responses against potential pathogens are modified. The second phase concerns the post-infection reactions. Callose is an important inducible defense that can prevent pathogen invasion [111]. After infection, an intact ABA signaling pathway is required to increase callose accumulation in attacked plants [44,112], and the presence of ABA can induce or repress additional callose accumulation [98] depending on the environmental conditions. Therefore, ABA variation by a previous stress can influence the final output following biotic stress, such as strengthening the resistance phenotype through accumulation of callose or by inducing other defense pathways [96,108]. The third phase finally starts when PAMPs stimulate the accumulation of specific hormones that are SA, JA, and ethylene in order to regulate the defense reaction [27,96,113]. In summary, the exact role of ABA as a regulator of the dialogue between abiotic and biotic stress strongly depends on the timing of the stress perception: does the infection hit a plant that had already been exposed previously to abiotic stress or does an infected plant become additionally exposed to abiotic stress [30,97,114]?

The beneficial role of SA in the relationship between plants and pathogens has been extensively studied. What is known is that ABA and SA have an antagonistic role in plant defense against stressors [31]. However, Miura and Tada [88] have shown that in addition to ABA, SA seems to also be important in plant responses to drought stress. Furthermore, SA increased barley resistance against water deficit [115].

3.4. Transcription Factors and Molecular Responses in Cross-Tolerance

Changes in gene expression occur after detection of a given stress, and the reprogramming of the molecular machinery is regulated by the action of transcription factors. The altered expression of certain genes is a key event in helping plants to set up an effective defensive state, and there is convincing evidence that many genes are multifunctional and able induce tolerance in plants towards more than one stress [49–116]. The activity of such genes involved in defense is mediated by specific phytohormones like ABA, SA, JA, and Ethylene. For example, the activity of the *BOTRYTIS SUSCEPTIBLE1* (*BOS1*) gene is mediated by both ABA and JA and induces resistance against osmotic stress and necrotrophic pathogens [117], and *bos1* mutant plants are more susceptible to both stresses [117]. In Arabidopsis, the transcription factor *MYB96* plays an important role in plant protection under pathogen infection by mediating the molecular link between both ABA induced by drought stress and SA expressed following pathogen infection [118]. *SlAIM1* in tomato responds positively to the combination of abiotic stress and infection with *Botrytis* *cinerea* [13] and *OsMAPK5*, which has kinase activity, is a positive regulator of the rice response to drought, salt, and cold tolerance and disease resistance [86].

Interestingly, many *PR* genes are also induced upon exposure of a plant to abiotic stress ensuring disease resistance [118]. PR proteins are crucial for plant resistance against pathogens, and their expression is strongly up-regulated when plants are attacked [118]. Over-expression of certain transcription factors in plants confronted with cold stress and infection activates cold-responsive *PR* genes, thereby conferring protection against both stressors [119]. The up-regulation of some transcription factors after exposure to abiotic stress leads to an accumulation of PR proteins. The transcription factors C-repeat Binding Factors (*CBF*), Dehydration-Responsive Element-Binding proteins (*DREB*) and No Apical meristem ATAF and Cup-Shaped Cotyledon (*NAC*) have been extensively studied as players of the primary abiotic stress signaling pathways ensuring tolerance under stress [120–122]. *CBF* is induced under cold stress together with a group of PR proteins [123]. Transgenic Arabidopsis overproducing the *NAC* transcription factor *NTL6*, which is induced by cold stress, enhance their defense response against pathogen attack by promoting an up-regulation of the *PR1* gene [118–124]. Tsutsui *et al.* [125] showed that the transcription factor *DREB* could regulate the response of cross-tolerance between abiotic and biotic stress insuring the resistance of Arabidopsis response to cold and pathogen (Figure 1).

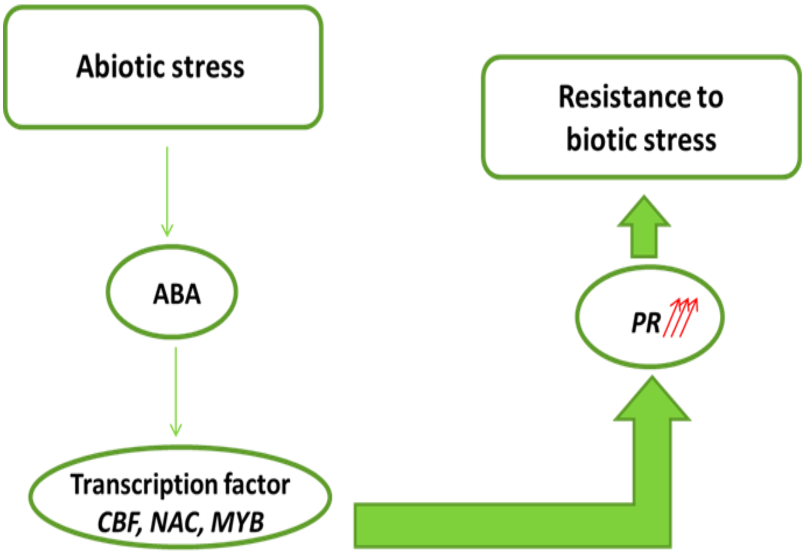

Figure 1. Abiotic stress can enhance the expression of specific transcription factors (TFs) like C-repeat Binding Factors (*CBF*), No Apical meristem ATAF and Cup-Shaped Cotyledon (*NAC*), *MYB* mediated by abscisic acid (ABA). Although the exact role of ABA in plant pathogen interactions is still a matter of debate, in some specific cases it has been shown to promote resistance against biotic stress following abiotic stress. This is attributed to the over-expression of TFs inducing the up-regulation of *PR* genes

Recently, it has been proposed that the WHIRLY1 protein and *REDOX-RESPONSIVE TRANSCRIPTION FACTOR1* (*RRTF1*) could participate in the traffic of communication between plastids and the nucleus [126]. WHIRLY1 perceives the redox changes in the plastid and carries the information to the nucleus in an NPR1-independent manner. The authors propose this protein as an ideal component in retrograde signaling that will lead to acclimation and adaption to new stresses. In the same way, *RRTF1,* which is induced by biotic and abiotic stresses, could be priming distant leaves to defend themselves against further stresses.

4. Conclusions and Outlook

A plant's response following exposure to abiotic/biotic stress strongly depends on its developmental stage [127] and the environmental conditions to which it is subjected [99]. Many stress combinations lead to phenotypic damage and, as mentioned above, the expression of defense is affected according to the type of abiotic stress and the pathogens involved. Overall, the complex response of the plant stems from the interplay of specific signaling pathways involved in abiotic and biotic stress. The combination of both stress types leads to an increased accumulation of a large number of signaling compounds that, in an ideal case, will be expressed as cross-tolerance (Figure 2).

Plants perceive the information signal of each stress and consequently activate specific molecules. Only some of them, which are common to both stressors, will participate in the defense response to the specific stress combination and thus contribute to protect the plant and enhance its resistance.

Various novel approaches can help plants to resist under combinatorial stress. The "Omics" technology is one of these approaches. Transcriptomics, proteomics, and metabolomics have revealed plant responses under stress and their underlying mechanisms and point to potential target genes, proteins or metabolites for inducing tolerance and improve plant responses. Little is known about the "Omics" characterization of abiotic and biotic stress combinations, but recently, several reports have addressed this question [16,51,70,128,129]. Although complete genome sequences are available for an increasing number of crop and model plants, in comparison, protein and metabolite databases are still rather incomplete, hence complicating the task of integrating all observations. Additionally, different plant species or even cultivars may behave differently, plant responses are also often organ-dependent, and results obtained with whole plants may be misleading.

Another approach might consist of molecular engineering of specific genes and their introduction into crop plants. By modifying a gene coding for a small antimicrobial peptide and introducing it into potato, the resistance of potato to biotic and abiotic stress was increased [130].

The manipulation of common regulators is also a promising approach. Boosting the accumulation of flavonoid biosynthesis mitigates the negative effects of abiotic and biotic stress [131,132]. Polyamines are another example. These substances have long been known to mediate resistance to pathogens [133] but they are also involved in abiotic stress resistance [134]. Genetic manipulation of polyamine accumulation could lead to multi stress tolerance [135].

A further possibility to promote cross-tolerance is the exploitation of priming. Some chemicals have been shown to prime plants for both biotic and abiotic stresses under laboratory conditions [136], and their application might allow a better management of multiple stresses under field conditions. The ultimate goal in every case is to maintain or even enhance plant performance, yield, and productivity under adverse conditions

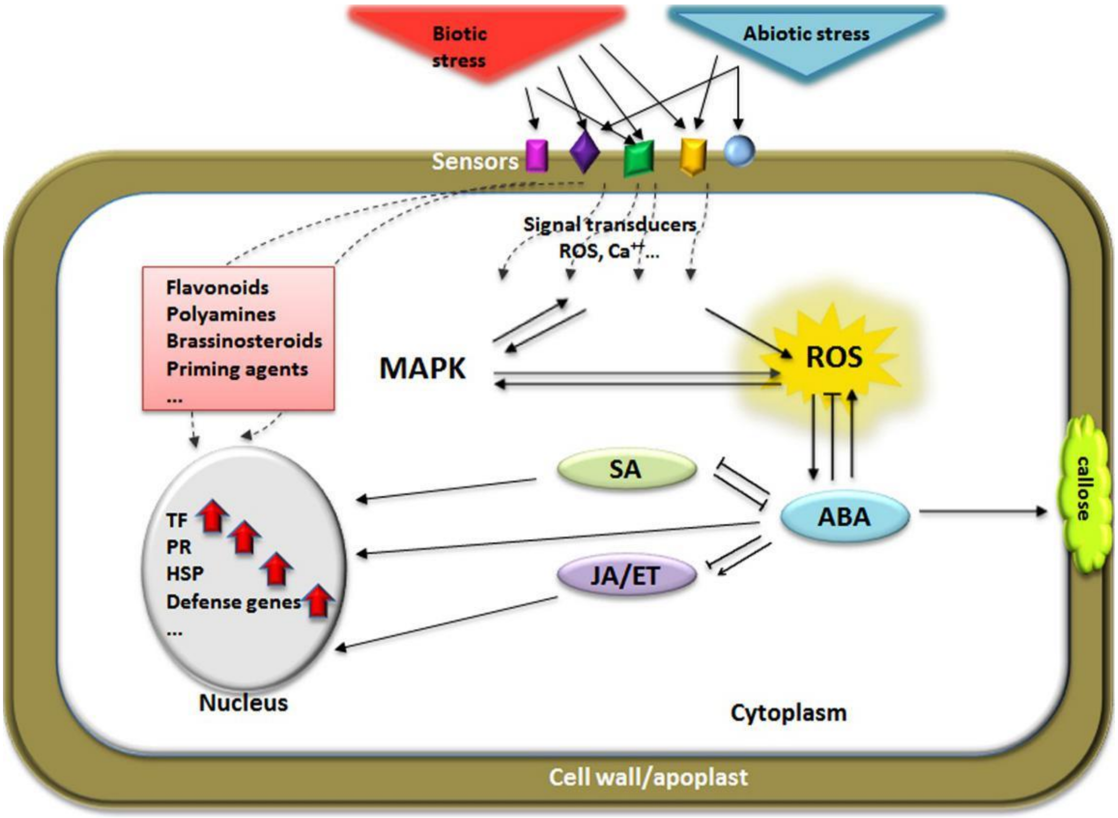

Figure 2. Elements possibly involved in cross-tolerance between biotic and abiotic stress. Both biotic and abiotic stress have to be first sensed by the plant cell, and then the information is transduced to appropriate downstream-located pathway(s). Sensors as well as signal transducers might be shared by both types of stressors. Reactive oxygen species (ROS) and Ca^{++} are known among others to play a prominent role as transducers (messengers) and mitogen-activated protein kinases (MAPK) cascades have been shown to be used by both types of stresses. MAPKs are centrally positioned in Ca^{2+}-ROS crosstalk as well as in the signal output after exposure to a specific stress. The importance of ROS has repeatedly been described for both types of stresses too, and, therefore, ROS might represent crucial elements in the integration of both stresses during cross-tolerance. Plant hormone signaling is of utter importance for stress adaptation. While abscisic acid (ABA) is predominantly involved in abiotic stress adaptation, salicylic acid (SA) and jasmonate/ethylene (JA/ET) are more responsible for the plant's reaction to biotic stress. However, there is a tremendous amount of crosstalk taking place between the various hormonal pathways, and the exact nature of this crosstalk during simultaneous biotic and abiotic stress remains to be investigated. ABA signaling contributes positively to pre-invasion defense and is responsible for enhancing callose deposition. ABA presents a positive interaction with JA/ET signaling. The activation of SA signaling by pathogen challenge can attenuate ABA responses. ABA signaling negatively affects signals that trigger systemic acquired resistance, enhancing pathogen spread from the initial site of infection. The interaction of SA, JA, and ET signaling results in increased resistance to pathogens. Hormones, secondary metabolites, priming agents, and further chemicals located in the cytoplasm finally up-regulate transcription factors (TF), pathogenesis related (PR) and defense genes, heat shock protein (HSP) genes, and further genes involved in protection against stress and thus lead to the phenotypic expression known as cross-tolerance. Arrows: induction; flat-ended lines: repression.

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Author Contributions

All authors contributed equally to this review article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Chapter II

β-aminobutyric acid induces tolerance and is involved in defense responses of two tomato cultivars against drought and salt stress

β-aminobutyric acid induces tolerance and is involved in defense responses of two tomato cultivars against drought and salt stress

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β-aminobutyric acid induces tolerance and is involved in defense responses of two tomato cultivars against drought and salt stress

Abstract

Plants are sessile organisms and cannot escape from biotic and abiotic stresses. Thus, a plant’s defensive capacity can be enhanced by specific stimuli. A well-known inducer of biotic stress tolerance in tomato is the non-protein amino acid β-aminobutyric acid (BABA). Since BABA has been shown to also induce tolerance to abiotic stress in Arabidopsis, in this study, we investigated the effect of the BABA-induced priming for drought and salt stress tolerance in two tomato cultivars (a highly stress-susceptible (cv Coeur de Boeuf) and a tolerant one (cv Marmande)). Due to water deficit and soil salinity problems in field-grown tomato cultures, a better adaptation of these plants to such stresses is highly desirable.

BABA increased stress-induced ABA production, leading to a decrease of stomatal conductance. In addition, microscopic analysis of leaves treated with BABA at different levels of water and salt stress revealed induction of lignin accumulation and reduction of hydrogen peroxide (H_2O_2) production. Moreover, chemical analysis showed an increase of total antioxidant activity, as well as of Chlorophyll ’a’ and ‘b’ comparing to untreated plants. These results show an effective defense priming response in treated tomato plants under abiotic stress, thus increasing the tolerance.

Introduction

During their growth, plants are continuously exposed to a multitude of environmental stress such as drought, salinity, cold, heat, high light. These stressors have negative impacts on plant growth, development, and restriction on crop production (Buchanan et al., 2000). Drought stress and salinity together are considered the most limiting plant productivity. Under these severe conditions, all plant processes are affected by the reduction of water uptake from the soil. As a consequence, crop plants have evolved wide changes such to activate specific defenses as evoking defensive mechanisms and adjusting their cellular metabolism to cope with abiotic stress (Wang et al., 2008). Plant responses to these abiotic stressors vary considerably from plant to plant depending on their tolerance capacity (Krasensky and Jonac, 2011). Drought and salt stress inhibit plants growth in order to reduce absorbing water and induce stomatal closure limiting leaf transpiration. In addition, the homeostasis of reactive oxygen species (ROS) must be well controlled under stress by the activity of antioxidant compounds, which increase generally during stress and are able to eliminate the excess of ROS, thereby preventing oxidative stress (Khanna-Chopra and Selote, 2007). However, under severe and continuous abiotic stress, the balance between the generation of ROS and the detoxification with enzymatic and non-enzymatic antioxidant could be disturbed and may induce a subsequent oxidative damage (Asada, 1999; Wang et al., 2008). The increase of plant tolerance under severe abiotic stress including drought and salinity is insured by an efficient antioxidative system in order to mitigate the oxidative damage (Arbona et al., 2003). The equilibrium between ROS and antioxidant defense systems is modulated by the increase endogenous abscisic acid (ABA) upon abiotic stress (Jiang and Zhang, 2002a, b; Fan et al., 2009). However, face of future food supply insecurities due to the increase of the global population and global climate change, researchers are trying to develop scientific techniques in order to increase plant tolerance upon abiotic stress (Roeckner, 1992; Schmidhuber and Tubiello, 2007; Munner and Tester, 2008; Godfray et al., 2010). An interesting approach was found to increase plants protection against harmful environmental conditions, which is the induction of tolerance by specific chemical elicitors (Conrath et al., 2006). These chemicals induced resistance to future stress factors exposure including abiotic and biotic stress and leads to faster and effective plant responses, this phenomenon is called priming (Goellner and Conrath, 2008; Macarisin et al., 2009). β-aminobutyric acid (BABA) is one among various chemicals which enhanced plant stress protection. Prior studies have demonstrated that BABA confers plant protection against a wide range of stresses such as biotic stress like attacks by fungi, bacteria, virus and nematodes, as well as abiotic stress like drought, salinity, cold and heat (Jakab et al., 2001; Jakab et al., 2005; Ton et al., 2005; Huang et al., 2011; Balmer et al., 2015). It is well known that BABA enhanced resistance in Arabidopsis and many other plants against various necrotrophic and biotrophic pathogens was achieved via the activation of both salicylic acid (SA) and abscisic acid (ABA)-dependent defense mechanisms (Zimmerli et al., 2000, 2001; Ton and Mauch-Mani, 2004). In addition, it has been shown that BABA mediated protection in grape against downy mildew by potentiating jasmonic acid (JA)-dependent mechanisms (Hamiduzzaman et al., 2005). Depending on the biotic stress to be encountered, BABA-treated plants present several changes at the molecular level like boosting hormonal signaling pathways, increasing the expression of many defense regulatory genes and transcription factors (TFs) and accumulation of proteins, and at physiological responses like stomatal closure, the ROS and callose deposition (Jakab et al., 2005; Flors et al., 2008; Van der Ent et al., 2009; Macarisin et al., 2009; Pastor et al., 2013).

Both drought stress and high salinity induce osmotic stress in plants and are among the most aggressive stressors by disrupting membrane homeostasis and activating secondary effects leading to cell death (Hasegava et al., 2000; Ozturk et al., 2002). In order to characterize the effects of BABA-induced plant tolerance to drought and salt stress, research has been focusing on several putative mechanisms to demonstrate the reduction of plant sensitivity to stress upon BABA treatment. An early response to drought stress and salinity is the decrease of stomatal conductance through the action of abscisic acid (ABA) induced in BABA-treated Arabidopsis (Jakab et al., 2005, Du et al., 2012). Thus, the decrease in stomatal conductance leads to improve water use efficiency ensuring plant tolerance under stress. It was found that primed plant-induced abiotic stress tolerance was primarily mediated through ABA signaling cascades (Jakab et al., 2005). Moreover, BABA can induce the activation of the abscisic acid (ABA), salicylic acid (SA) and jasmonic acid signaling pathways (Liu et al., 2011; Du et al., 2012). In wheat, BABA decreased ROS accumulation and increased antioxidant enzyme activities in response to drought stress (Du et al., 2012). These results concluded that BABA treatment can boost antioxidant defense system and then minimize oxidative damage in plant cells upon stress (Du et al., 2012; Hossain et al., 2012). These studies suggested that BABA leads to a faster and stronger activation of stress-specific defense mechanisms in response to abiotic stress.

There is a limited number of studies describing the potential of BABA priming of defense against abiotic stresses. Therefore, the present study was conducted to analyze the ability of BABA to induce tolerance in tomato plants. The leaves fresh and dry weight differences were determined, stomatal conductance as well as phytohormones alterations between BABA-treated and non-treated tomato plants under drought or salt stress conditions. In addition, some transcription factors (TFs) were analyzed in response to drought and salinity. In order to precisely elucidate the defense responses activated by BABA, two tomato cultivars with contrasting drought/salinity tolerance were used.

Material and methods

Culture conditions

Seeds of both tomato cv Marmande and cv Coeur de Boeuf were obtained from Quedlinburger (Aschersleben, Germany) and Catros-Gerand (France). For sterilization, tomato seeds were rinsed in 70% ethanol, incubated for 15 minutes in 2% bleach and washed 4 to 5 times with sterile distilled water. After 24 hours at 4°C, the sterilized seeds were pre-germinated for 6 days in a Petri dish with a humid sterile Whatman filter paper in the dark in a plant growth chamber (Percival AR-95L, CLF Plant Climatics GmbH, Wertingen, Germany or Canada). Tomato seedlings were then transferred to multi-cell growing trays filled with commercial soil (Compost (25%), sand (12%) and peat (63%); Ricoter Erdaufbereitung AG, 3270 Aarberg, Switzerland or Fafard Ltd., St Bonaventure, Canada) watered with Solbac (Andermatt Biocontrol, Switzerland) solution to prevent fungus gnat problems. Ten days later, seedlings were individually transferred to small plastic pots (Volume=100 mL) filled with the same soil. All the plants were well watered and kept in thes same plant growth chamber under with the following conditions: 16 hours day at 26°C, 8 hours night at 18°C, 60% relative humidity and an irradiance of 245 μmol m^{-2} s^{-1} until they reached the stage of four fully expanded true leaves (from the tip, excluding petiole). At this stage, plants of uniform size were used for the experiments.

β-Aminobutyric acid (BABA) treatment

β-Aminobutyric acid (Sigma-Aldrich, Switzerland) was dissolved in water and applied as a soil drench (5 mM BABA soil concentration) (Oka et al., 1999) to 4-week-old plants, 2 days prior to the imposition of the drought or salt stress. Control plants were watered with tap water. The time of BABA treatment was considered to be day 0.

Drought and salt stress application

Tomato plants were well watered (Control) or exposed to drought stress 60% (moderate stress) or 20% (severe stress) of water and control plants were well watred, 48 hours following BABA treatment. The stress was applied to all the plants treated or non-treated with BABA to compare later the effect of priming in the plants under different levels of stress. For drought stress every day the water quantity in the pots was assessed using a TDR 100, FIELDSCOUT from Illinois (USA) and was adjusted to the desired percentage of water. With this instrument, it is possible to keep the same degree of stress in each pot.

Tomato plants were well watered with tap water (control) or exposed to salt stress 50, 100 or 200 mM NaCl. The NaCl concentrations were added to the pots every 3 days (Capiati et al., 2006).

Plant growth, physiological parameters

A week after the beginning of the salt and drought stress, the stomatal conductance was measured at noon on the abaxial surface of the third and the fourth fully expanded leaves on six plants per treatment with a diffusion porometer (AP-4, Delta-T Devices, Cambridge, UK). After two weeks of stress, this measurement was repeated on the same leaves. At the end of the experiments (two weeks after drought/salt stress), fresh and dry weights were recorded. These measurements were performed using 6 independent biological replicates per sample. These experiments were repeated two times with similar results.

Gene expression

Plant material was harvested after 24 hours (day 1) and 48 hours (day 2) following BABA treatment also after the 3rd, 5th and the 7th day of drought or salt stress, flash frozen in liquid nitrogen and kept at -80°C until use. Three biological replicates were collected per time point.

RNA extraction and Real-Time qRT-PCR

Total RNA was extracted from the frozen tomato leaf tissues. RNA isolation was performed according to manufacturer’s instructions using the RNeasy Plant Mini kit (Qiagen, http://www.qiagen.com). RNA was treated with DNase (Qiagen) and reverse transcribed into cDNA using SuperScript III RT (Invitrogen, http://www.invitrogen.com). Primers for qRT-PCR were designed using the universal probe library assay design tool from Roche (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). Primer efficiency was determined by performing a qRT-PCR with serial diluted cDNA; the minimal accepted efficiency for the primers was set to 0.8. The qRT-PCR was performed using the SensiMix SYBR kit (Bioline, http://www.bioline.com) on a Rotor-Gene 6000 cycler (Qiagen). The reaction volume was 10 μL, consisting of 2.5 μL nuclease-free water, 5 μL SensiMix SYBR mastermix, 0.25 μL forward and reverse primer (each 10 μM) and 2 μL cDNA. PCRs were performed using 3 independent biological replicates per sample, each replicate consisting of a pool of 3 plants. PCR reaction were performed in technical duplicates as a three-step reaction (initial hold step, 95°C for 10 min; 40 cycles of amplification, 95°C for 15 s, 60°C for 20 s, 72°C for 20 s) with a final melting curve analysis (68°C-95°C). Melting curve and cycle threshold (Ct) analysis were performed on the Rotor-Gene 6000 software 1.7. Relative gene expression of stressed tissue was calculated relative to control treated plants in regard to the two housekeeping genes *Actin* and *Tubulin* and the specific primer efficiencies with the help of REST 2009 (Qiagen). The gene expression data were further visualized using the software MeV viewer (http://www.tm4.org).

Table 1. Primers for abiotic stress genes.

Stress	**Gene**	**Accession**	**Fw primer**	**Rev primer**
Drought	*CBF3*	AY497899	CTGTTTTCCATGCCAGGATT	GGGGAGGAGGTAGCATGAG
Drought	*AREB*	NM_001247667	TGGTGAAACTGTTATTCAGTCTGC	GTGTGGATCTGACCCCATTC
Salt	*NAC3*	XM_004244154	TTCAAAAAGGCCACTAGCAAA	CCTTTTTGTTGATTGAGAATTATCGT
Salt	*RABC2a*	XM_004249983	TGGAGATTCTGGTGTTGGAA	GAAAGATCTTGAAAAAGATGATGTGA
Reference gene	*Actin-7-like*	XM_004249818	GGTTGGAATGGGTCAGAAAG	GATACCCCTCTTGGATTGAGC
Reference gene	*Tubulin*	NM_001247878.2	TACTGAAGGCGCAGAGTTGA	TTCTCCGCTTCTTTACGAACA

Pigment Analysis

The third and the fourth leaves of two tomato cv Marmande and cv Coeur de Boeuf was harvested after the 3rd, 5th and 7th day of drought or salt stress and was frozen immediately in liquid nitrogen. Leaf tissue was freeze-dried and was finely ground for pigment analysis. Three independent biological replicates were used.

- **Chlorophyll (chl) contents**

The chl pigment was extracted from 10 mg of finely ground freeze-dried samples. Leaf tissue was taken into 1.5 mL falcon centrifuge tube, 4 mL of 96% methanol (v/v) was added and gently vortexed. The samples were incubated in the dark at 4° C for overnight (Lichtenthaler and Wellburn, 1983; Thompson et al., 1989; Kleinhenz et al., 2003). The samples were then centrifuged at 6000 rpm for 10 minutes. The supernatant was collected for analysis, the absorbance of chl a and chl b in 96% methanol (v/v) was measured in a spectrophotometer (Power Wave XS, BioTek, USA), at respective wavelengths of 663 and 653. Standard curved were developed using solutions containing chl a chl b (Sigma Aldrich, USA). chl a (C_a) and chl b (C_b) was calculated from the following equation:

$C_a = 15.65A666 – 7.34A653$

$C_b = 27.05A653 – 11.21A666$

- **Total Anthocyanins**

The total anthocyanin was extracted from 10 mg of finely ground leaf tissue. Each sample was added to the falcon tubes. Four ml of 2% HCl in methanol was added to the tube and incubated for 24 h at 4° C (Revila et al., 1998; Kleinhenz et al., 2003). The mixture was centrifuged at 6000 rpm for 10 minutes. Each sample replication of 200 µL was transferred to assay plates (microtitre plates) and absorbance was measured at 535 nm using spectrophotometer (Power Wave XS, BioTek, USA).

Total antioxidant

Leaf extracts for antioxidant analysis were obtained by homogenized 10mg of ground leaf tissue in 750µL of 100% methanol using a vortex for 3 seconds and then incubated at 4°C in the dark for 24 hours. The homogenates were centrifuged at 15,000rpm for 15min at 4°C. The supernatant was recovered in a small Eppendorf of 1.5mL. The pellet was re-dissolved with 750µL methanol and homogenized. The homogenates were centrifuged at 15,000rpm for 15min. the supernatant was recovered and added to the first supernatant and adjusted to 1.5mL with methanol. Extracts were stored at -20°C until used in antioxidant assay procedures.

Total antioxidant was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, Canada) assay according to the method described by Brand-Willians et al. (1995) with some modifications. Stock solutions were prepared as following:

-Butylated hydroxyanisole (BHA; Sigma-Aldrich, Canada) was dissolved in the absolute methanol at the concentration of 100ppm. BHA solution was covered and kept in dark bottle at 4°C until use.

-DPPH was dissolved in absolute methanol at a concentration of 1M. The flask was placed in the ultrasonic bath for 4min at 40°C and the volume was adjusted using a volumetric flask. The working solution was obtained by diluting DPPH solution methanol and then kept in dark for 20min to obtain an absorbance of 0.97±0.02 units at 517nm using the spectrophotometer (Power Wave XS, BioTek, USA) (Hussain et al., 2008; Saeed et al., 2012). The standard curve was linear between 0 and 30ppm BHA.

270µL of tomato leaf extract were mixed with 1620µL DPPH. The mixture was shaken vigorously and allowed to stand for 20 min at room temperature in the dark. The absorbance was read at 517 nm. BHA was used as the positive control for comparison and DPPH solution diluting with methanol was taken as the blank. A lower absorbance value of the reaction mixture indicates a higher free radical scavenging activity. All tests were carried out in triplicate. The capability to scavenge the free radical DPPH in percentage of sample was calculated according to the following formula; Scavenging (%) = $100 \times (A_{blank} \_ A_{sample} / A_{blank})$

Where A_{blank} is the absorbance of the DPPH solution and A_{sample} is the absorbance of the extract solution. Extract concentration providing 50% scavenging (IC_{50}) was calculated from the graph-plotted inhibition percentage against extract concentration.

Three independent biological replicates were used.

DAB staining, microscopy analysis, and H_2O_2 quantification.

In situ H_2O_2 production was revealed by brown precipitates after 3,3’-diaminobenzidine (DAB) staining. The 3rd and the 4th fully expanded leaves after 7 days of drought or salt stress were harvested. Harvested leaf discs were stained in 1 mg of DAB (Sigma Aldrich-Canada) per milliliter at pH < 3 (a low pH is necessary in order to solubilize DAB) for 24 h in the dark and were subsequently detained in chloral-hydrate, as described previously (Thordal-Christensen et al., 1997; Luna et al., 2011). H_2O_2 is visualized as a reddish-brown deposit in DAB-treated leaves. DAB staining intensities were quantified from digital photographs (Zeiss stereo discovery v20 microscope (Carl Zeiss Canada ltd., Toronto, Ontario, Canada)) by the number of dark-brown DAB pixels relative to total pixels corresponding to plant material, using Image J. Six biological replicates were collected for analysis and average of ROS measurements were based on at least 20 photographs from 6 different tomato plants.

Histochemical analysis of lignin

Histochemical determination of lignin was performed using Wiesner's test (general staining for lignin). The 3rd and the 4th fully expanded leaves after 7 days of drought or salt stress were cut into discs. Sections were treated with a saturated solution of phloroglucinol (Sigma Aldrich, Canada) in 85% (v/v) ethanol, for 3 min at room temperature and washed in 25% (v/v) HCl (Roth et al., 1997; Sanchez-Aguayo et al., 2004). Lignin was visualized as a pink color at the ribs of plants. Lignin was quantified from digital photographs (Zeiss stereo discovery v20 microscope (Carl Zeiss Canada ltd., Toronto, Ontario, Canada)) using Image J. Six independent biological replicates were used.

Hormone quantification

The third and the fourth leaves of two tomato cv Marmande and cv Coeur de Boeuf was harvested after the 3rd, 5th and 7th day of drought or salt stress and was frozen immediately in liquid nitrogen. Leaf tissue was freeze-dried and was finely ground for hormone quantification. ABA, SA, JA, and JA-Ile were quantified simultaneously in single samples using an optimized ultrahigh pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method (Glauser et al., 2012) with some modifications. In brief, hormones from 4 mg dry weight were extracted in EtOAc: formic acid, 99.5:0.5 (v/v). Before extraction, an internal standard solution containing isotopically labeled ABA, SA, JA and JA-Ile (10 ng/mL) was added to the samples. The extracts were evaporated to dryness and resuspended in 100 μL of aqueous methanol (70%). After centrifugation, 5 μL of that solution was injected in UHPLC-MS/MS. The hormones were quantified by calculating a calibration equation obtained by linear regression from 5 calibration points for each analyze. Peak areas of the hormones measured in the samples were normalized to the internal standard before applying the calibration equation. Three independent biological replicates were used.

Statistical analysis

The significance of differences between control and treated plants of the phenotypic part was analyzed with a one-way ANOVA; control and stressed plants ($P<0.05$) were then compared using the Tukey's range and *t*-test. This comparison allows seeing the significant differences between two sample groups. All statistical analyses were accomplished using Sigma Plot 11.0 (http://www.sigmaplot.com).

Results

Morphological and physiological responses of treated tomato plants with BABA

To determine the effect of BABA treatment on the two cultivars of tomato plants (cv Marmande and cv Coeur de Boeuf) under different stress regimes, we measured fresh and dry weight. Four-week-old tomato plants were treated with BABA (5mM) or with water. Two days later, some plants were stressed with drought (moderate drought stress, where the soil water content was equal to 60%; or severe water stress, where the soil water content was equal to 20%) or with salt (50, 100 or 200mM NaCl) and the rest kept under normal conditions which were watered daily (control plants). These conditions were maintained for 2 weeks. The fresh and the dry weight in treated and non-treated plants with BABA decreased in both cultivars depending on the degree of stress (Figure 1). Treated plants with BABA tended to have higher fresh and dry weight in both tomato cultivars compared to non-treated plants, even under higher levels of drought (20%) and concentrations of salt (100 or 200mM). In cv Marmande and upon 100 or 200 mM of salt there was a significant difference in fresh and dry weight between treated and non-treated plants with BABA. These levels of stress decreased the biomass of the plants, but BABA has a positive effect on the plant's growth. However, there was no significant difference between cv Marmande plants treated and non-treated with BABA under a different level of drought stress. While BABA was not effective in cv Coeur de Boeuf, there was no significant difference in fresh and dry weight between treated and non-treated plants with BABA under drought and salt stress. These results showed that BABA enhanced the biomass in cv Marmande under salt stress but has no effect on the plant's growth under drought stress.

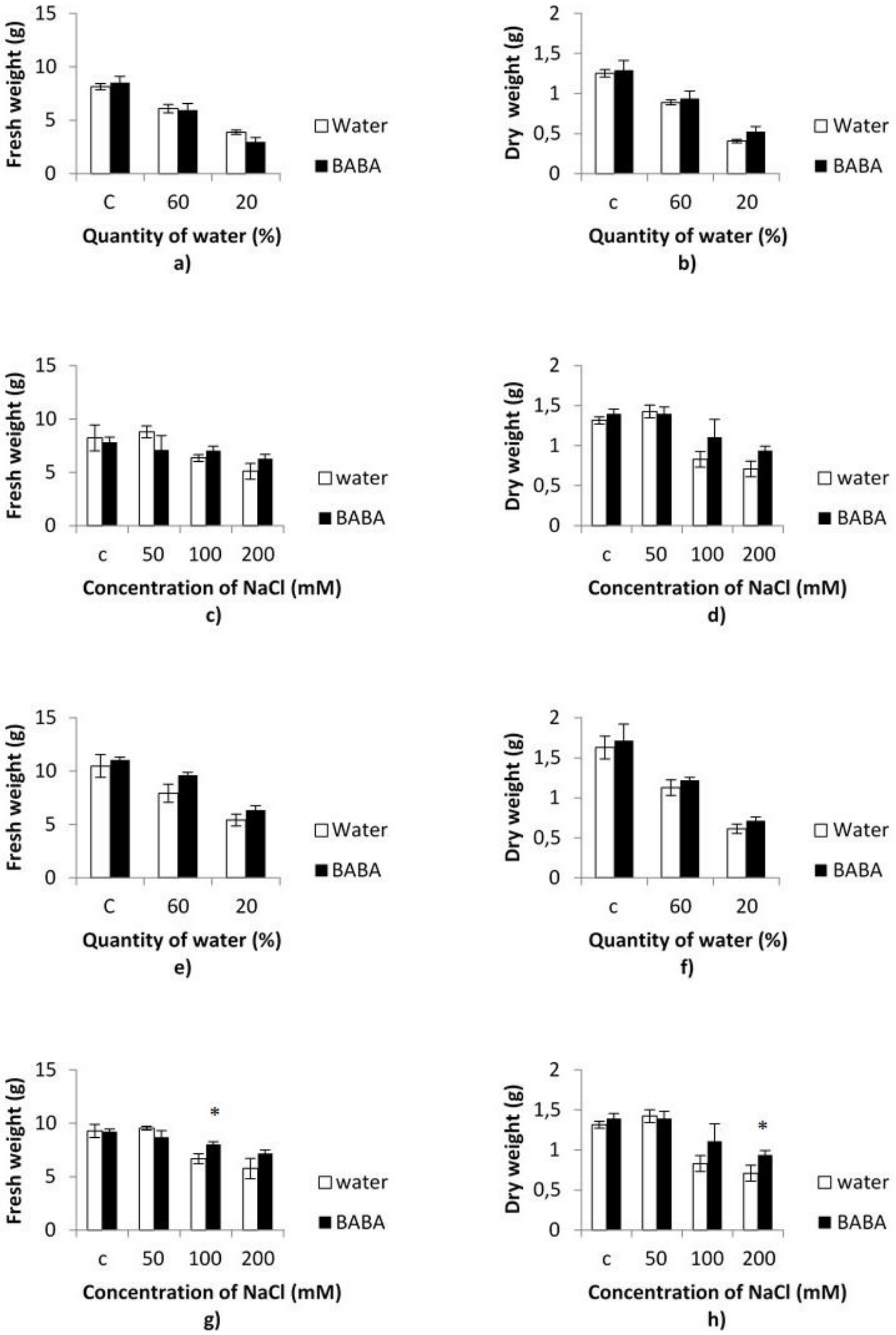


Fig. 1. Fresh and dry weight of tomato leaves after 2 weeks of drought or salt stress in two tomato cultivars (Coeur de Boeuf and Marmande). a) and b) Fresh and dry weight of cv Coeur de Boeuf tomato leaves under drought stress. c) and d) Fresh and dry weight of cv Coeur de Boeuf tomato leaves under salt stress). e) and f) Fresh and dry weight of cv Marmande tomato leaves under drought stress. g) and h) Fresh and dry weight of cv Marmande tomato leaves under salt stress. Error bars indicate the standard errors for the average values of 6 replicates. Asterisks indicate a significant difference in a Student *t*-test (* = $p<0.05$).

Stomatal conductance (Physiological characteristic)

We measured the relative leaf stomatal conductance in salt or drought-affected plants treated and non-treated with BABA (Supplemental material figure S1). The addition of BABA decreased the stomatal conductance in any stress condition. After one week of stress, the stomatal conductance decreased similarly in both cultivars, Marmande and Coeur de Boeuf, at different concentrations of salt and also at the different levels of drought stress compared to control. However, there was no significant difference between plants treated with BABA or not except in plants subjected to severe stress. There was a significant difference between plants treated and non-treated with BABA with 20% of water and also with 100 and 200 mM of NaCl. This suggests that BABA had no effect on stomatal conductance after 1 week of stress unless the stress was severe (Supplemental material figure S1) and BABA did not influence the sensitivity of the stomata to the leaf water status.

When extending the period of stress to 2 weeks, stomatal conductance in plants treated with BABA became higher than that for plants not treated in both experiments (salt and drought stress). The stomatal conductance continued to decrease as the soil dried in treated/non-treated plants with BABA (Figure 2). The decrease of the stomatal conductance in both tomato cultivars is responsible to induce a slowdown of respiration and minimize transpiration, and the result is a reduction of water loss in the plants. As a hypothesis, BABA changed the stomatal pore opening compared to non-treated plants. The narrowing of the stomatal pores induced stomatal closure in non-treated tomato with BABA under salt and drought stress. Interestingly, in plants treated with BABA the stomatal conductance was significantly higher compared to the non-treated plants. As a hypothesis, after a longer period of stress, BABA-treated plants were less susceptible to osmotic stress compared to control plants; therefore, BABA may enhance the tolerance of tomato plants.

The difference between the stomatal conductance in control plants and stressed plants with 200mM NaCl or 20% of water was higher in cv Coeur de Boeuf than in cv Marmande.

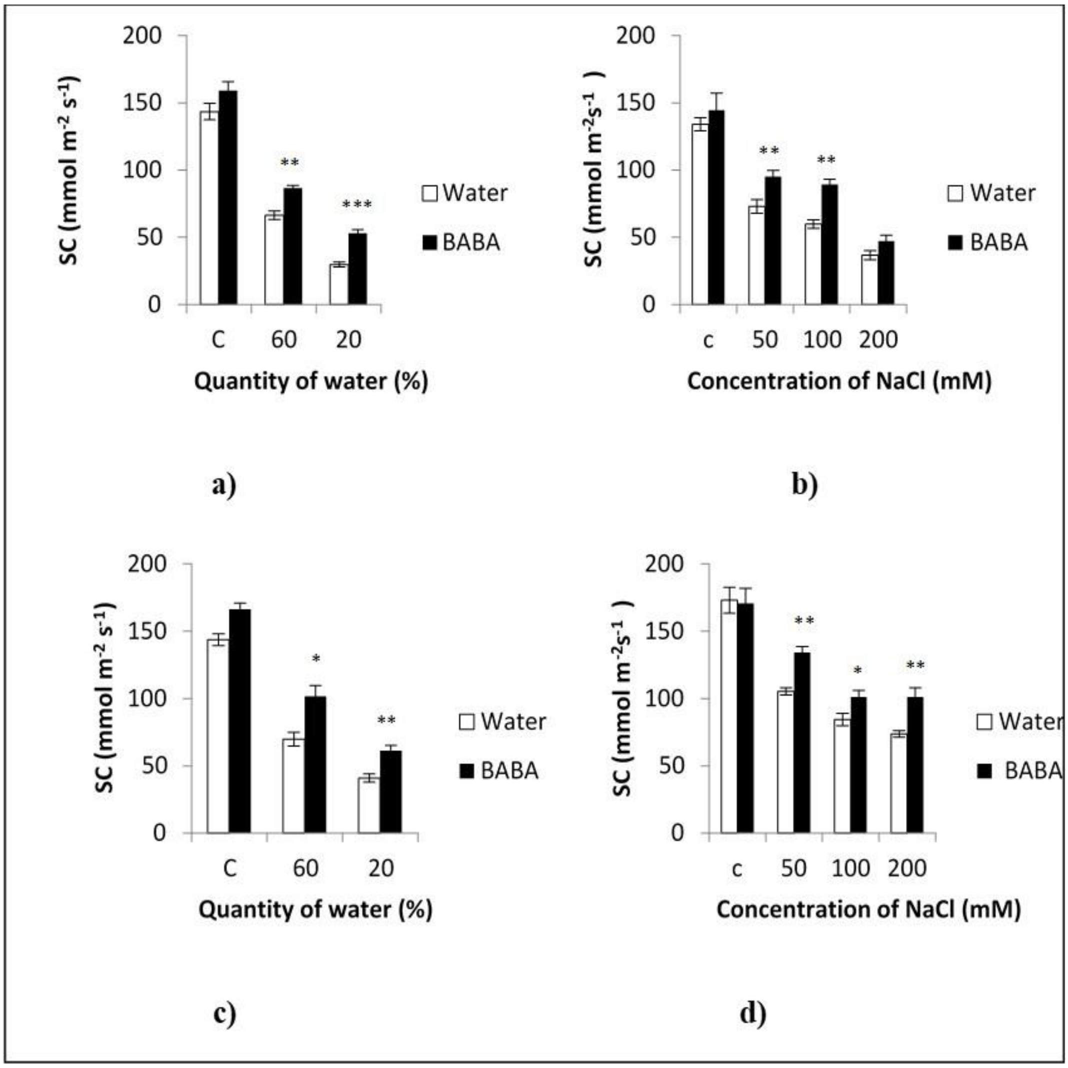


Fig. 2. Effect of BABA treatment on the stomatal conductance in two tomato cultivars after 2 weeks of salt or drought stress a)-b) Stomatal conductance in cv Coeur de Boeuf leaves under drought or salt stress c)-d) Stomatal conductance in cv Marmande leaves under drought or salt stress. Error bars indicate the standard errors for the average values of 6 replicates. Asterisks indicate a significant difference in a Student's t-test (* = $p<0.05$, ** = $p<0.01$, ***=$p<0,001$).

Chlorophyll content

The chlorophyll a (Chl a) and chlorophyll b (Chl b) contents were very much affected by drought and salt stress in cv Marmande as well as in cv Coeur de Boeuf and in treated and untreated plants with BABA (Figure 3 and 4). The level of Chl a and b decreased depending on the degree of drought or salt stress in treated and untreated plants with BABA compared to control. However, plants treated with BABA showed the highest level of Chl a and b compared to the non-treated plants with BABA in control and stressed plants with drought or salt. There were significant differences in Chl a and b between treated and untreated cv Coeur de Boeuf plants with BABA after 5 and 7 days of severe drought stress (20%) and under 50 and 100mM od salt stress. While, in cv Marmande, the significant differences between treated and non-treated plants with BABA were detected at 5 and 7 days of stress in control and at all drought stress levels. The same was the case for salt stress, where BABA-treated cv Marmande plants showed a significant increased chl a and b content compared to untreated plants except for 200mM after 5 days of stress where the difference was not significant between treatments. This result indicated that the syntheses of pigments are significantly inhibited by drought or salt stress while promoted by BABA treatment.

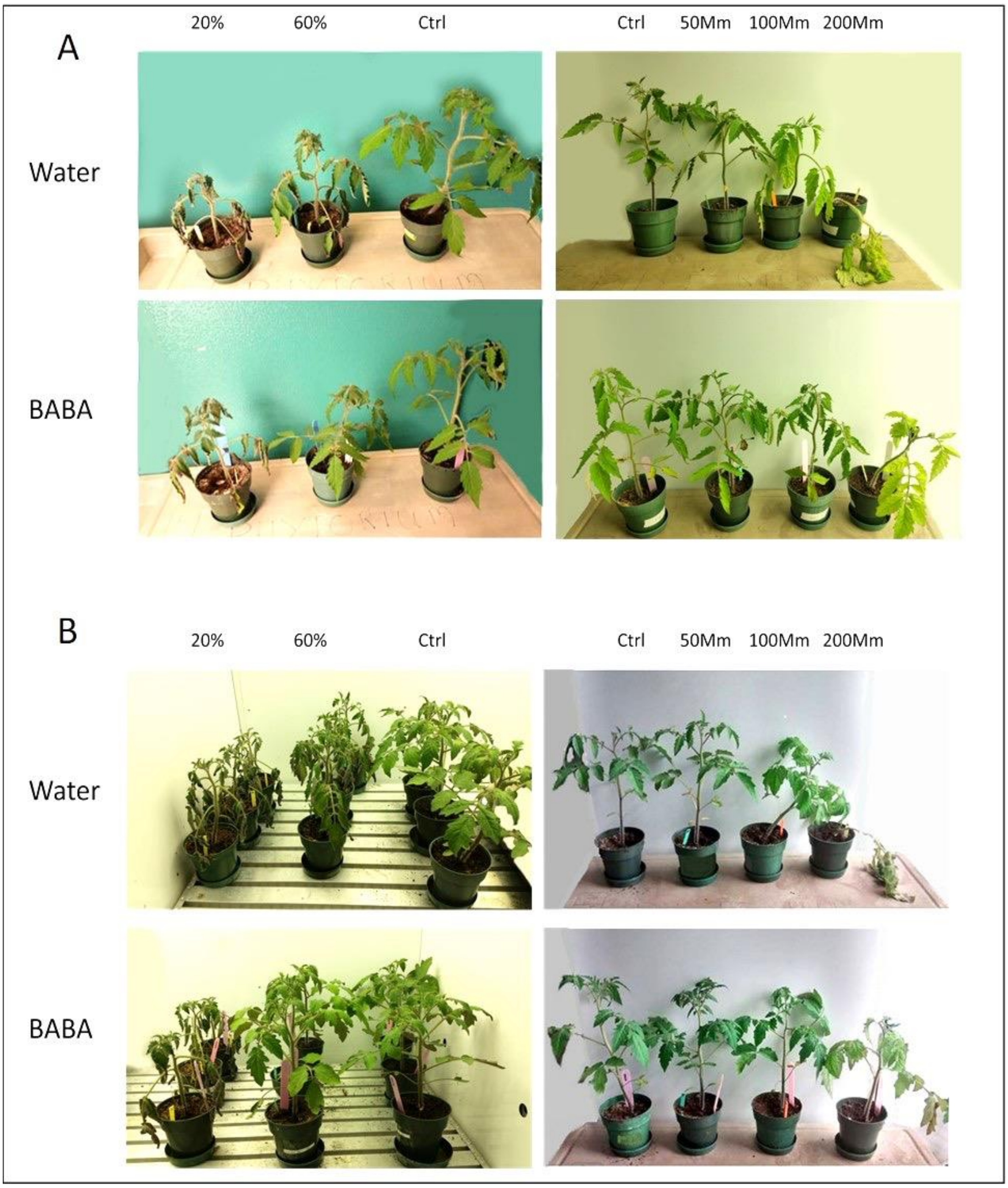
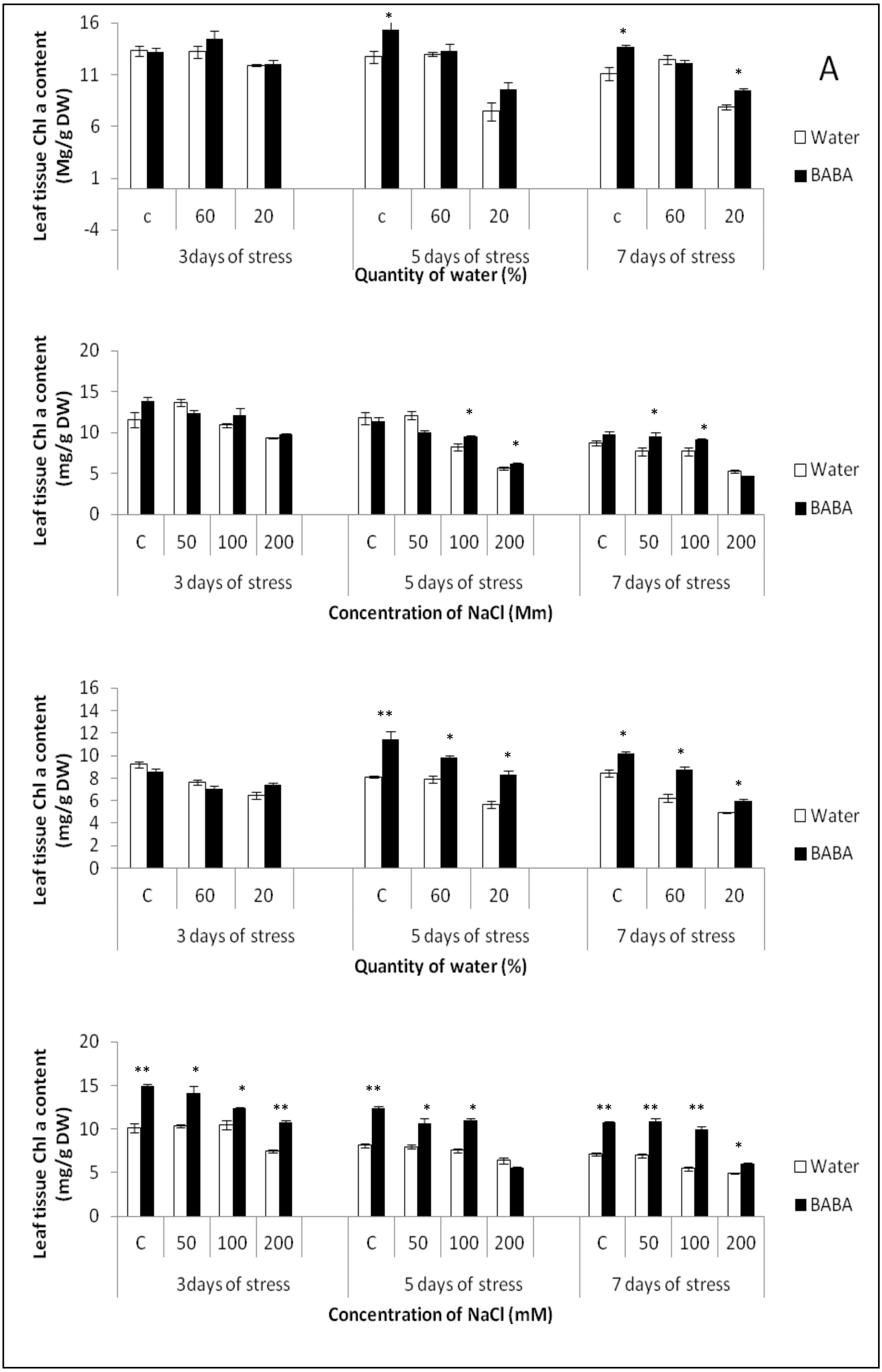


Fig. 3. Picture of water-or BABA-treated tomato plants after 7 days of drought or salt stress. A) Coeur de Boeuf B) Marmande.


A
Leaf tissue Chl a content (Mg/g DW)
16
11
6
1
-4
c
60
20
3days of stress
5 days of stress
7 days of stress
Quantity of water (%)
□ Water
■ BABA
Leaf tissue Chl a content (mg/g DW)
20
15
10
5
0
C
50
100
200
3 days of stress
5 days of stress
7 days of stress
Concentration of NaCl (Mm)
□ Water
■ BABA
Leaf tissue Chl a content (mg/g DW)
16
14
12
10
8
6
4
2
0
C
60
20
3 days of stress
5 days of stress
7 days of stress
Quantity of water (%)
□ Water
■ BABA
Leaf tissue Chl a content (mg/g DW)
20
15
10
5
0
C
50
100
200
3days of stress
5 days of stress
7 days of stress
Concentration of NaCl (mM)
□ Water
■ BABA

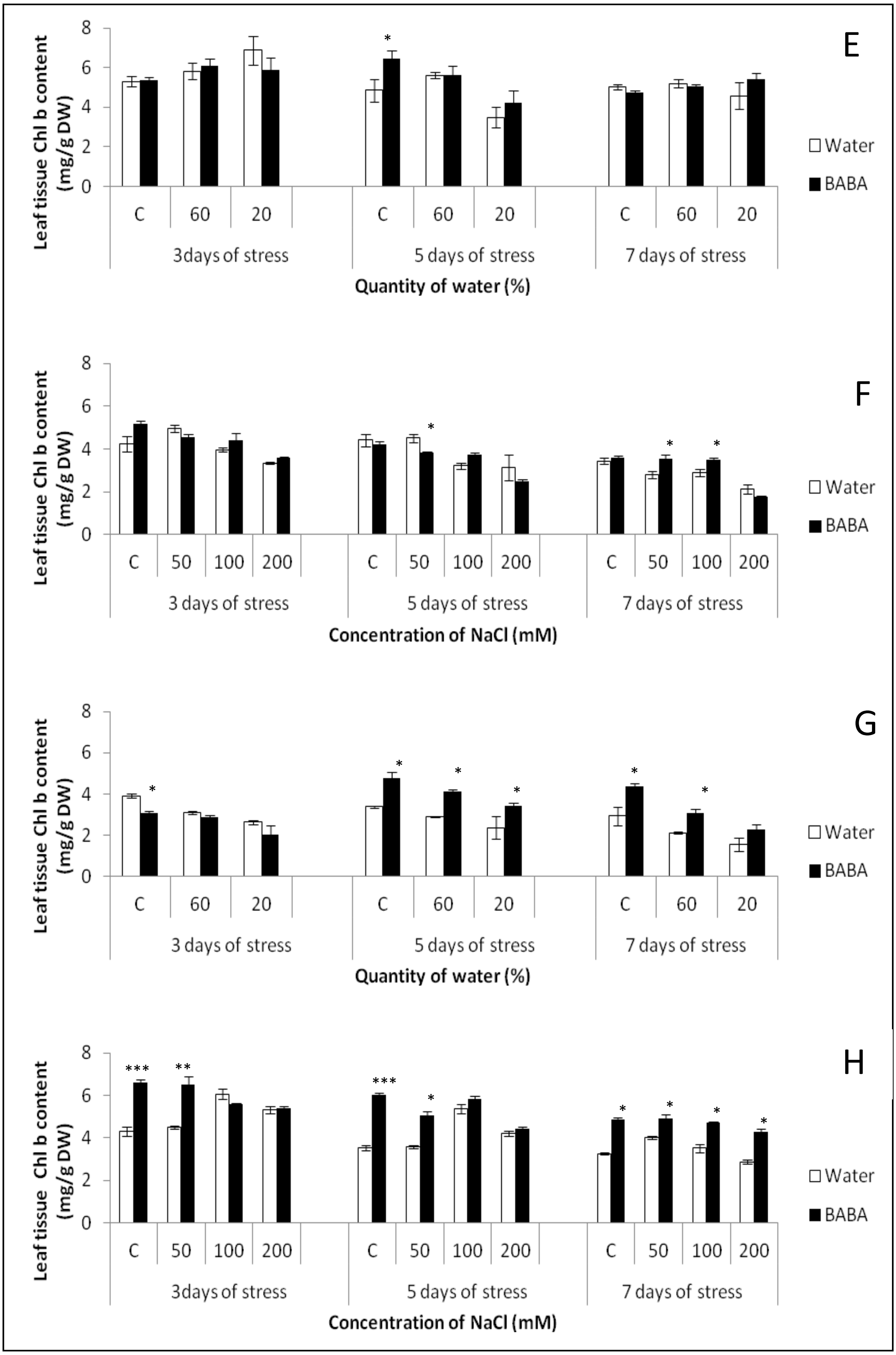


Fig. 4. Chlorophyll content of two tomato cultivars (Marmande and Coeur de Boeuf) of 30-day-old plants treated and untreated with BABA (5 mM) at 48h before establishing different levels of drought and salt stress. Third and fourth leaves of each plant were detached at 3-time points (3rd, 5th and 7th day of stress (lack of water (60%

and 20% relative to the total pot capacity of water) or with salt (50,100 and 200mM)). (A) Chl a content in cv Coeur de Boeuf under drought stress (B) Chl a content in cv Coeur de Boeuf under salt stress (C) Chl a content in cv Marmande under drought stress (D) Chl a content in cv Marmande under salt stress (E) Chl b content in cv Coeur de Boeuf under drought stress (F) Chl b content in cv Coeur de Boeuf under salt stress (G) Chl b content in cv Marmande under drought stress (H) Chl b content in cv Marmande under salt stress. Asterisks indicate statistically significant differences between treated and untreated plants with BABA under different levels of drought or salt stress, according to Student's *t*-test; $\alpha = 0.05$. The bars represent the standard deviation of the mean; $n = 3$). The experiment was repeated twice with similar results. (* = $p<0.05$, ** = $p<0.01$, ***=$p<0,001$).

Anthocyanin contents

Drought and salt stress were thought to impact anthocyanin synthesis (Parida and Das, 2004; Nakabayashi et al., 2014; Kovinich et al., 2014; Kovinich et al., 2015) induced by higher accumulations of anthocyanins in the epidermal cells, which often contributes to enhanced stress tolerance in plants. In this study, we examined the degree of this protection related to anthocyanin contents in BABA-treated plants in response to abiotic stress. Anthocyanins were induced in response to drought stress in cv Coeur de Boeuf treated and non-treated with BABA (Figure 5). In addition, both plants treated and non-treated with BABA exhibited a gradual accumulation of anthocyanin by increasing the level of drought stress in Coeur de Boeuf. BABA-treated cv Coeur de Boeuf plants exhibited higher anthocyanin levels than non-treated ones. However, anthocyanin content decreased in response to salt stress in untreated cv Coeur de Boeuf Plants. Contrariwise to untreated cv Coeur de Boeuf plants with BABA, in response to salt stress, BABA-treated plants showed higher and more stable levels of anthocyanin. In non-treated cv Marmande plants with BABA, there was no significant difference between drought stress intensities. The same results were found under salt stress, except for 200 mM of salt stress, where anthocyanin content was higher than the control. Anthocyanin content exhibited a significantly higher level in treated cv Marmande plants with BABA than non-treated plants, especially after 7 days of stress. This result indicated that BABA contributed to stress tolerance in plants. Cv Marmande treated and untreated with BABA exhibited lower anthocyanin accumulation than cv Coeur de Boeuf in response to salt or drought stress. This result demonstrated that cv Marmande accumulated less anthocyanin in their epidermal cells than Coeur de Boeuf.

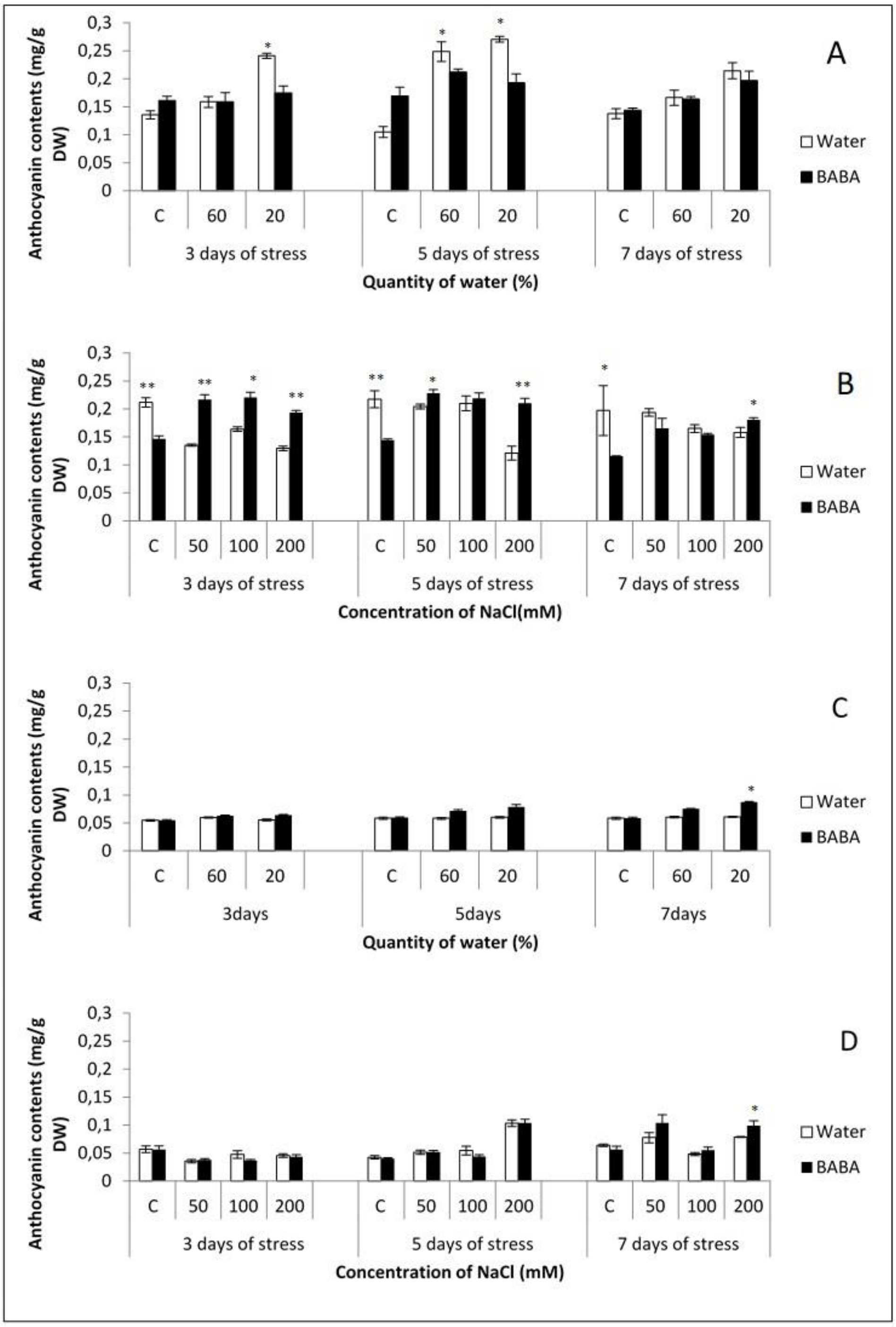


Fig. 5. Anthocyanin contents of two tomato cultivars (Marmande and Coeur de Boeuf) to 30-day-old plants treated and untreated with BABA (5 mM) at 48h before establishing different levels of drought and salt stress. Third and fourth leaves of each plant were detached at 3-time points (3rd, 5th and 7th day of stress (lack of water (60% and 20% relative to the total pot capacity of water) or with salt (50,100 and 200mM)). (A) Anthocyanin content in cv Coeur de Boeuf under drought stress (B) Anthocyanin content in cv Coeur de Boeuf under salt stress (C) Anthocyanin content in cv Marmande under drought stress (D) Anthocyanin content in cv Marmande under salt. Asterisks indicate statistically significant differences between treated and untreated plants with BABA under different levels of drought or salt stress, according to

Student's *t*-test; α = 0.05. The bars represent the standard deviation of the mean; *n* = 3. The experiment was repeated twice with similar results. (* = $p<0.05$, ** = $p<0.01$).

Impact of stress on H_2O_2 accumulation and the positive effect of BABA treatment

Because BABA is known as an inducer of tolerance in plants subjected to unfavorable growth conditions, we examined to what extent this protection related to endogenous H_2O_2 levels. To this end, tomato plants growth under the same controlled conditions and treated with BABA at the stage of 4 true leaves and then subjected to lack of water or salt stress 48h after BABA treatment. Leaves were fixed in acidic 3,3-diaminobenzidine (DAB) staining solution (pH<3) after 7 days of stress. H_2O_2 levels were quantified digitally by the relative number of dark-brown pixels after 24 h of staining. Control and stressed leaves from cv Marmande and cv Coeur de Boeuf plants treated with BABA exhibited lower staining than non-treated leaves with BABA (Figure 6). Further, this result was confirmed by the quantification of H_2O_2 accumulation (Figure 7) where the accumulation of H_2O_2 was found to be significantly lower in leaves of treated plants with BABA compared to non-treated plants in both cultivars of tomato and at different stress levels. This result confirmed that BABA helps to minimize drought and NaCl-induced oxidative stress *in situ*. A heavy staining was observed in the presence of salt stress or lack of water, where H_2O_2 levels were higher in plants treated and non-treated with BABA compared to control in both cultivars. In addition, plants treated and non-treated with BABA both exhibited a gradual accumulation of H_2O_2 by increasing the levels of drought stress and NaCl concentration in both cultivars. Cv Marmande leaves treated and untreated with BABA exhibited lower H_2O_2 accumulation than cv Coeur de Boeuf in the presence of salt or drought stress with the exception of 60% drought stress (Figure 6). This result demonstrated that BABA reduces the level of ROS in cv Marmande and also in cv Coeur de Boeuf under drought and salt stress. In addition, the protection by BABA from ROS accumulation is better in cv Marmande than in cv Coeur de Boeu

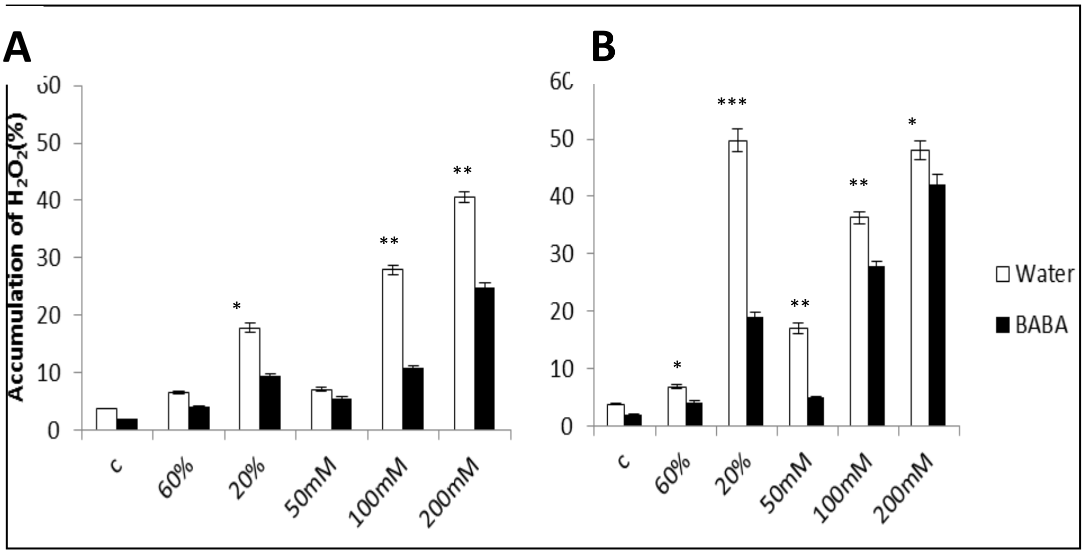


Fig. 6. Impact of stressed conditions on H_2O_2 accumulation in leaves to 30-day-old plants for 2 tomato cultivars ((M) cv Marmande and (CB) cv Coeur de Boeuf) treated and untreated with BABA (5 mM) at 48h before the establishment of drought and salt stress. Third and fourth leaves of each plant were detached at 7th days of stress (lack of water (60% and 20% relative to the total pot capacity of water) or with salt (50,100 and 200mM)) and staining with DAB. (A) Accumulation of H_2O_2 on cv Marmande leaves stressed and treated with BABA compared to stressed and non-treated plants. (B) Accumulation of H_2O_2 in cv Coeur de Boeuf leaves stressed and treated with BABA compared to non-treated plants. What is shown is the percentage value of relative 3,3-diaminobenzidine (DAB) staining intensities (± standard error of the mean; $n > 20$) at 7 days. DAB accumulations were quantified as the number of dark-brown spots- corresponding pixels relative to the total number of pixels covering plant material. Asterisks indicate statistically significant differences between stressed and treated or untreated plants with BABA (Student's *t*-test; $\alpha = 0.05$). The experiment was repeated twice with similar results. (* = $p<0.05$, ** = $p<0.01$, ***=$p<0,001$).

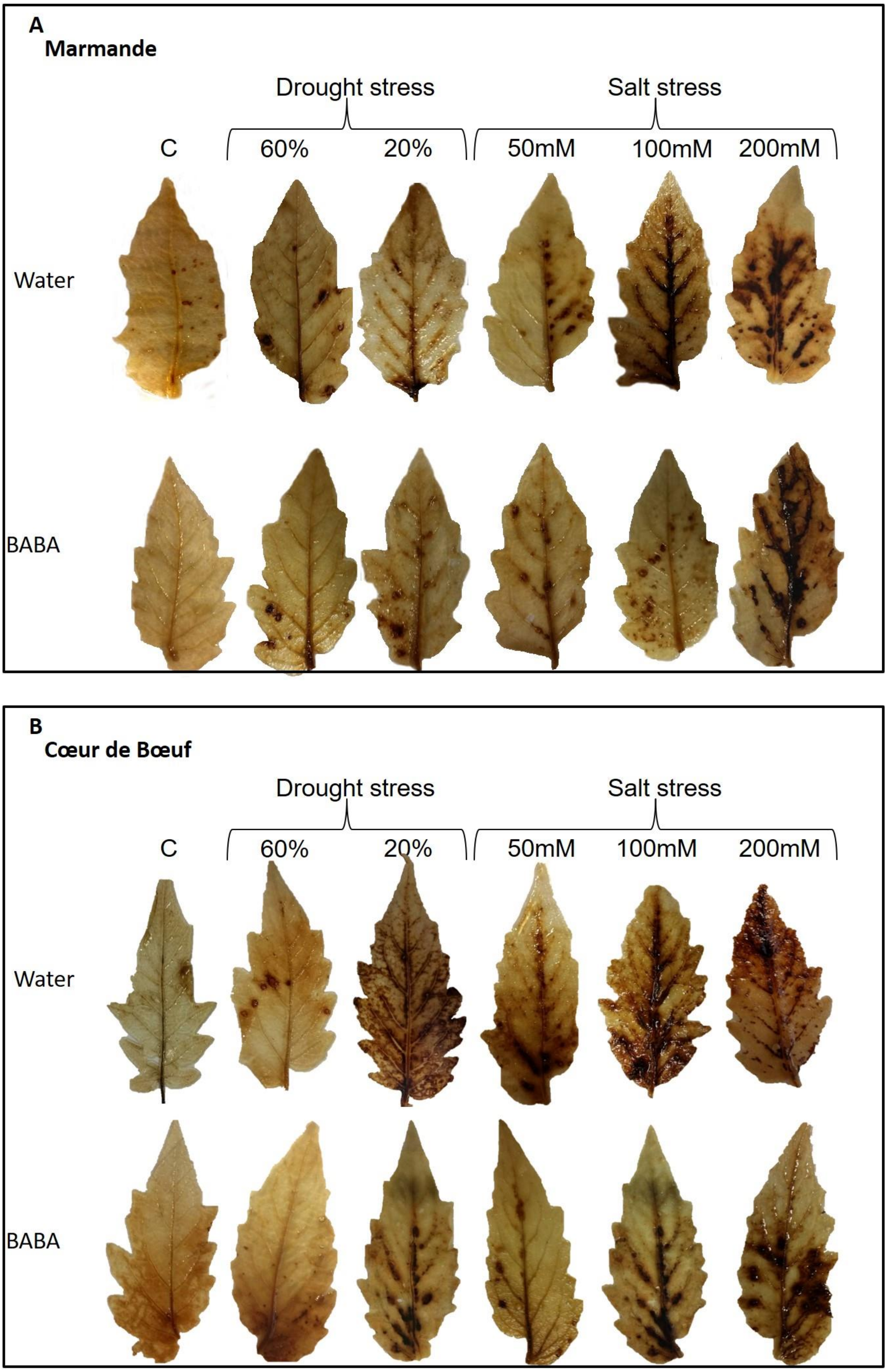


Fig. 7. *In situ* detection of hydrogen peroxide using DAB staining on tomato leaves treated or non-treated with BABA at 7 days after stress with lack of water (60% and 20% relative to the total pot capacity of water) or with salt (50,100 and 200mM). (A)

Photographs of H_2O_2 localization on cv Marmande leaves. (B) Photographs of H_2O_2 localization on cv Coeur de Boeuf leaves. Photographs show stained leaves with 3,3-diaminobenzidine (DAB). Thirty-day-old plants were treated or not with BABA then, after 48 hours, plants were stressed with lack of water or salt.

Impact of stress on total antioxidant accumulation in plants treated with BABA

Decreases in soil water potential induced by drought or salt stress must be followed by an adaptation in order to increase tolerance in plants. However, after long periods of stress inducing strong reductions in plants water potential and oxidative damage, stressed plants become not able to survive. The total antioxidant held an important role in the detoxification of the high accumulation of ROS in plants under abiotic stress. To evaluate the effect of BABA-treated plants compared to non-treated plants in the ability of antioxidant capacity, total antioxidant was extracted by using DPPH methods from 30-day-old tomato plants treated or not with BABA and subjected to different levels of drought or salt stress. Total antioxidant capacity was measured at 3 time-points (3, 5 and 7 days after stress). Control and stressed leaves from cv Marmande and cv Coeur de Boeuf plants treated with BABA exhibited a higher level of total antioxidant than non-treated leaves with BABA (Figure 10). This result confirmed that BABA boosts minimize drought and NaCl-induced oxidative stress. Plants treated and non-treated with BABA both exhibited a gradual accumulation of total antioxidant following the increase of the level of drought stress or NaCl concentration in both cultivars at different time points. In addition, upon 7 days of drought or salt stress, cv Coeur de Boeuf showed a significant difference between treated and non-treated plants with BABA and the level of antioxidant was higher compared to cv Marmande which indicated that oxidative damage was higher in cv Coeur de Boeuf than in cv Marmande.

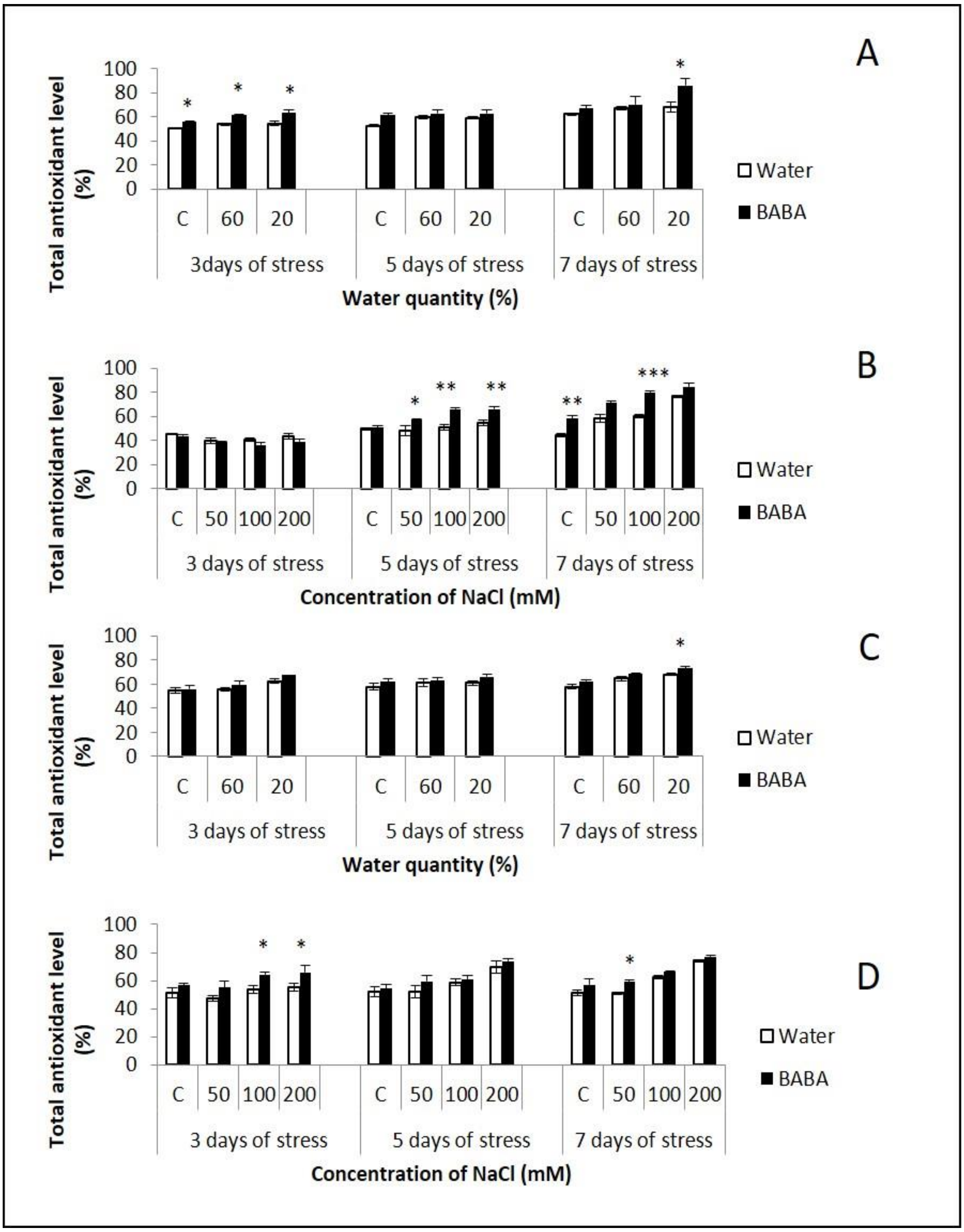


Fig. 10. Total antioxidant capacity to 30-day-old plants of 2 tomato cultivars ((M) Marmande and (CB) Coeur de Boeuf) treated and non-treated with BABA (5 mM) at 48h before the establishment of drought or salt stress. Third and fourth leaves of each plant were detached at 3 different time points (3, 5 and 7 days of stress (lack of water (60% and 20% relative to the total pot capacity of water) or with salt (50,100 and 200mM)). (A) Total antioxidant capacity in cv Coeur de Boeuf leaves treated and non-treated with BABA under drought stress (B) Total antioxidant capacity in cv Coeur de Boeuf leaves treated and non-treated with BABA under salt stress. (C) Total antioxidant capacity in cv Marmande leaves treated and non-treated with BABA under drought stress (D) Total antioxidant capacity in cv Marmande leaves treated and non-treated with BABA under salt. Total antioxidant capacity was analyzed by

the DPPH methods. (± Standard error of the mean; *n* > 3). Asterisks indicate statistically significant differences between stressed and treated or non-treated plants with BABA (Student's *t*-test; α = 0.05). The experiment was repeated twice with similar results. (* = p<0.05, ** = p<0.01, ***=p<0,001).

Lignin deposition in BABA treated plants in response to drought and salt stress

It is known that lignin is a major constituent of xylem cell walls and attributes hydrophobicity and mechanical strength to plant vessel conductivity and lignin deposition helps plants to withstand the negative effect of abiotic stress (Sanchez-Aguayo et al., 2004). To assess the effect of lignin in BABA-treated tomato plants stressed with drought and salt, lignin depositions in leaf vessels were stained with phloroglucinol-HCl and were quantified numerically by the relative pink staining intensity of the vessels. Control and stressed leaves from cv Marmande and cv Coeur de Boeuf plants treated with BABA exhibited higher staining levels than non-treated leaves with BABA (Figure 8). Furthermore, this result was confirmed by quantification of lignin deposition (Figure 9) where lignin was found to be significantly higher in leaf discs of treated plants with BABA compared to non-treated plants in both cultivars of tomato and at different stress levels. This result confirmed that BABA helps to increase lignifications and therefore, induces tolerance to abiotic stress. In cv Marmande treated with water, there was no significant difference between stressed plants with different level of drought or with salt stress, except for treated plants with water and stressed with 200mM of NaCl where lignin deposition was lower than the rest. However, lignifications in cv Coeur de Boeuf in treated plants with water and stressed with severe drought stress (20%) or with severe salt stress (100 or 200mM) were lower than control plants. Furthermore, plants treated with BABA exhibited a gradual decrease of lignin deposition by increasing the level of drought stress or NaCl concentration in both cultivars. This result shows that lignin deposition was suppressed in response to severe dehydrative stressors as well as severe salt stress in both cultivars. Cv Marmande leaves treated with BABA exhibited higher lignin deposition than cv Coeur de Boeuf in the presence of salt or drought stress. This result demonstrates that BABA-treatment leads to an increase in lignin accumulation in both cultivars in response to drought and salinity. In addition, BABA leads to a better protection in cv Marmande by accumulating more lignin than cv Coeur de Boeuf.

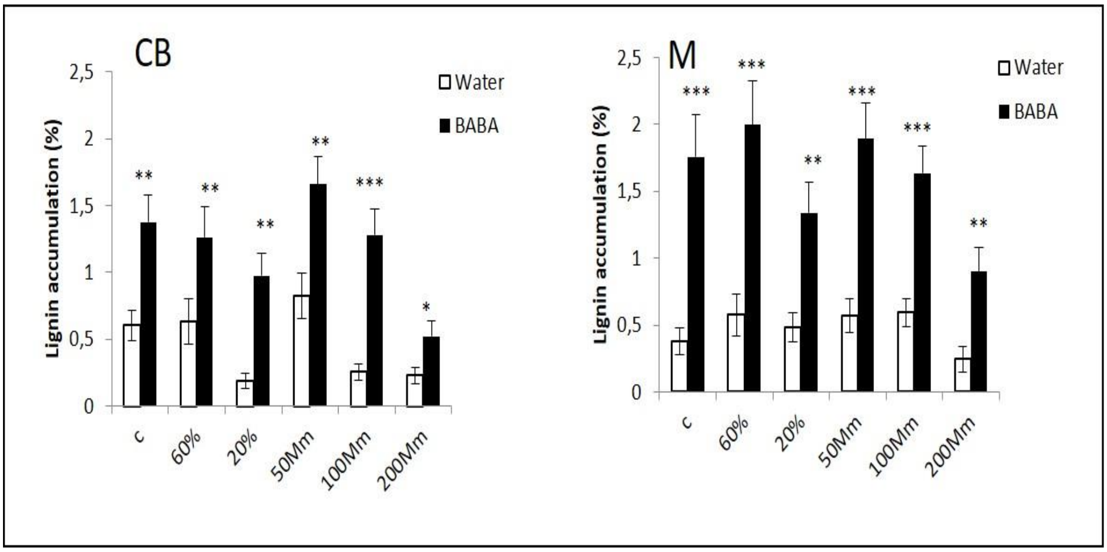


Fig. 8. Lignin deposition in the vascular tissues to 30-day-old plants of 2 tomato cultivars ((M) cv Marmande and (CB) cv Coeur de Boeuf) treated and untreated with BABA (5 mM) at 48h before the establishment of drought and salt stress. Third and fourth leaves of each plant were detached at 7th days of stress (lack of water (60% and 20% relative to the total pot capacity of water) or with salt (50,100 and 200mM)) and cut in leaf discs (r=0.25cm). (A) Lignifying tissues in cv Coeur de Boeuf leaves stressed and treated with BABA compared to stressed and non-treated plants. (B) Lignifying tissues in cv Marmande leaves stressed and treated with BABA compared to non-treated plants. Lignified cells were stained pink with phloroglucinol-HCl. What is shown is the percentage value of relative lignin intensities (± standard error of the mean; *n* > 20) which were quantified as the intensity of pink coloration in leaf veins. Asterisks indicate statistically significant differences between stressed and treated or non-treated plants with BABA (Student's *t*-test; α = 0.05). The experiment was repeated twice with similar results. (* = p<0.05, ** = p<0.01, ***=p<0,001).

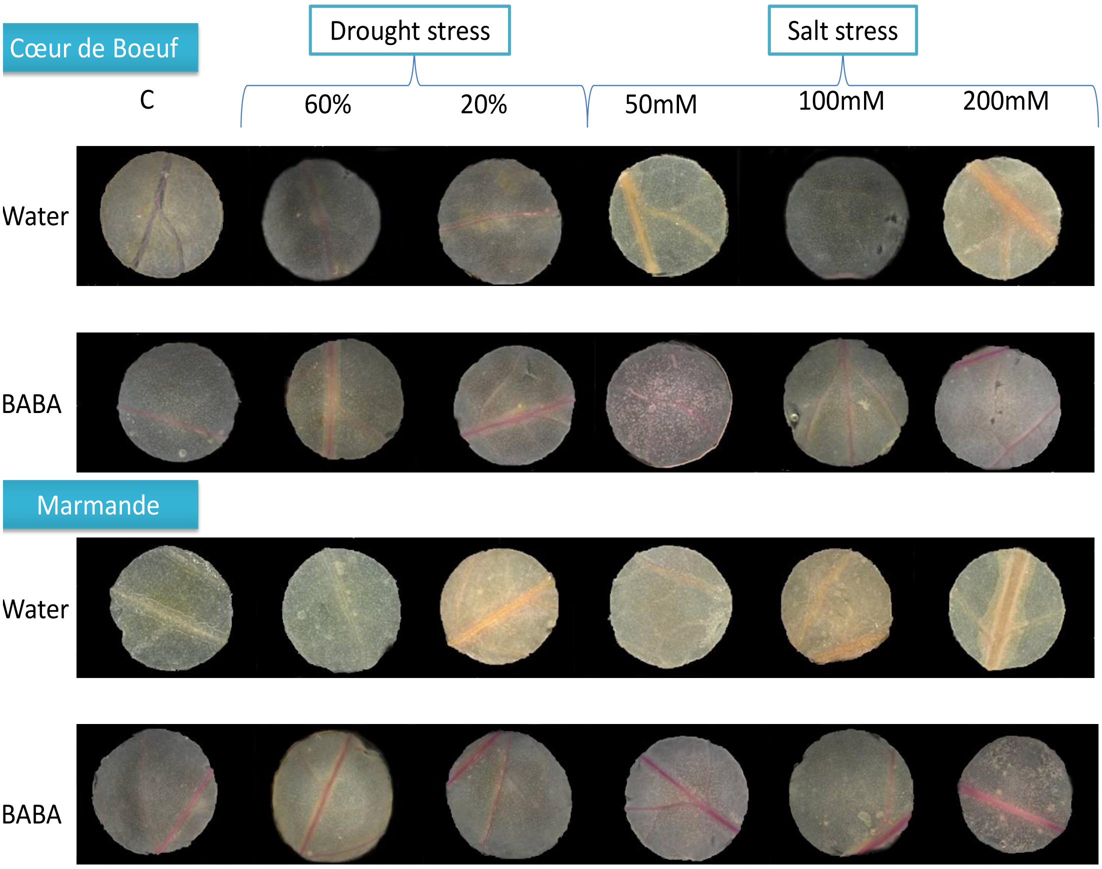
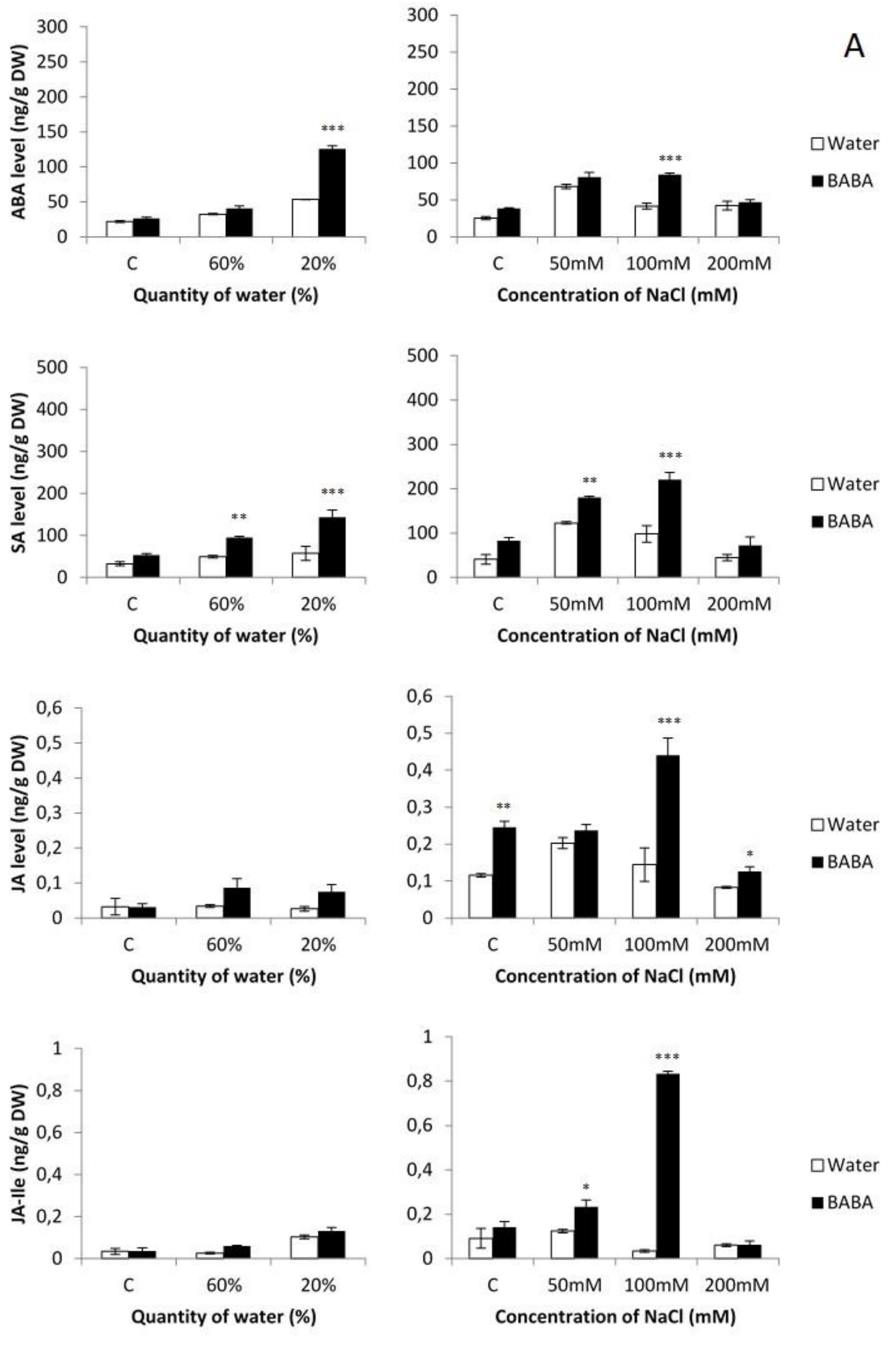


Fig. 9. Photographs of lignin deposition on cv Marmande and cv Coeur de Boeuf leaf discs. *In situ* detection of lignin accumulation on tomato leaf discs where plants were treated or non-treated with BABA and then subjected to 7 days of stress with lack of water (60% and 20% relative to the total pot capacity of water) or with salt (50, 100 and 200mM). Thirty day-old plants of were treated or not with BABA then, after 48 hours, plants were stressed with lack of water or salt. Lignification is visibleas pinkisch coloration.

Effect of BABA treatment on phytohormones in stressed plants with drought and salt stress

The importance of phytohormones (ABA, SA, JA, JA-Ile) as a regulator in various abiotic stress tolerance levels has been studied extensively (Yamaguchi-Shinozaki and Shinozaki, 2006; Fujita et al., 2011). Nevertheless, the role of this plant hormone in the regulation of primed tomato plants in response to drought or salt stress remains unclear. To examine whether BABA-treated plants tolerance is associated with a similar induction of various phytohormones-dependent defense, we investigated the response of ABA, SA, JA and JA-Ile in primed plants under drought or salt stress conditions using ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS). Only results after 7 days of drought/salt stress have been used for this study. At 7 days after abiotic stress, leaf ABA content increased with drought or salt stress for treated and non-treated cv Coeur de Boeuf and cv Marmande plants compared to control. BABA-treated plants showed a higher ABA accumulation compared to non-treated plants under drought or salt stress (Figure 11). A significant difference was observed under 60 and 20% lack of water and under 50 and 100 mM of salt stress in treated plants with BABA compared to the non-treated plants in both cultivars. This result demonstrated BABA-induced tolerance in plants by increasing ABA under stressed conditions. However, under 200mM of salt stress, ABA levels decreased in treated and non-treated plants with BABA in both cultivars and there were no significant differences between treatments. This result might be due to the toxic effect of 200mM of salt on tomato plants and it is highly probable that BABA treatment had no effect on plant protection. In addition, leaf SA content increased with drought or salt stress for treated and non-treated plants in both cultivars. BABA-treated plants showed a higher SA level compared to non-treated plants under drought or salt stress. A significant difference between treated and non-treated tomato plants was detected in control, 60 and 20% lack of water in both cultivars and in 50, 100 mM of salt stress in cv Coeur de Boeuf. Whereas, no significant difference was detected between treated and non-treated cv Marmande plants under salt stress. Morover, JA-dependent tolerancewas higher upon drought or salt stress in BABA-treated plants compared to non-treated-BABA- plants in both cultivars, while JA-dependent tolerance in non-treated plants revealed no significant differences under different levels of stress. Similarly, this effect was observed in JA-Ile upon drought and salt stress. ABA and SA levels were higher in cv Marmande than in cv Coeur de Boeuf. While JA and JA-Ile level were lower in cv Marmande than in cv Coeur de Boeuf. This result demonstrated that BABA increased the level of ABA, SA, JA and JA-Ile in both cultivars and under abiotic and biotic stress. In addition, the level of these phytohormones is higher in cv Marmande than in cv Coeur de Boeuf. Therefore, BABA is responsible to increase tolerance in tomato plants against drought and salinity.


A
ABA level (ng/g DW)
300
250
200
150
100
50
0
C
60%
20%
Quantity of water (%)
***
C
50mM
100mM
200mM
Concentration of NaCl (mM)
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□Water
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SA level (ng/g DW)
500
400
300
200
100
0
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JA level (ng/g DW)
0,6
0,5
0,4
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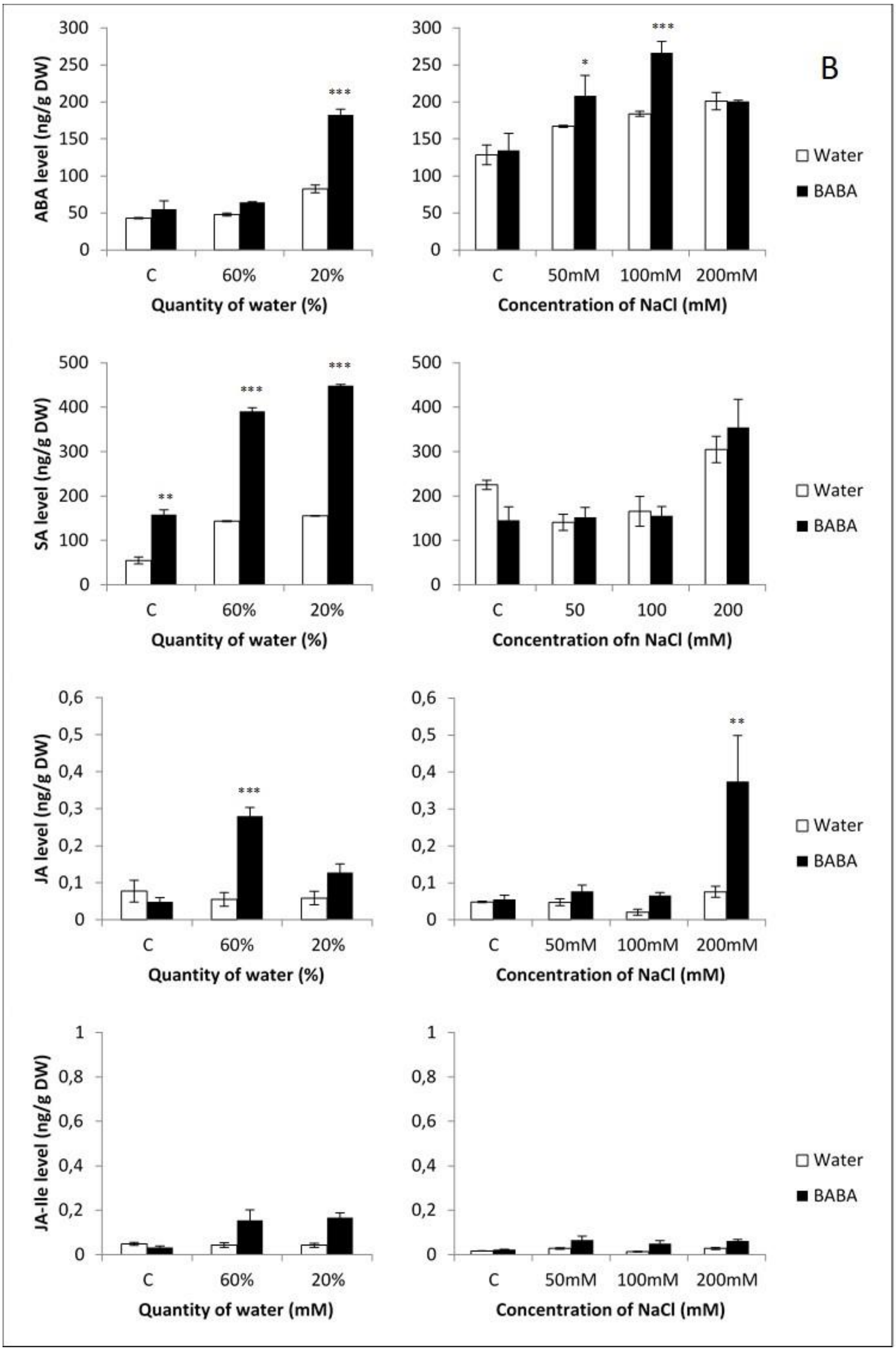

Fig. 11. Effect of BABA on phytohormones concentrations (Abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and jasmonoyl-L-isoleucine (JA-Ile)) to 30-day-old plants of cv Coeur de Boeuf (A) and cv Marmande (B) after 7 days of stress (lack of water (60% and 20% relative to the total pot capacity of water) or with NaCl (50,100 and 200mM)). Phytohormones were measured in freeze-dried leaves by

HPLC-MS/MS. Asterisks indicate statistically significant differences between treated and non-treated plants with BABA (Student's *t*-test; $\alpha = 0.05$). Each bar is the average of three independent measurements ± standard error. The experiment was repeated twice with similar results. (* = $p<0.05$, ** = $p<0.01$, ***=$p<0,001$).

Effect of BABA on the transcription factor genes involved in drought and salt stress

The specific defense responses at the molecular level were investigated by quantitative real-time PCR (qRT-PCR). The effect of the tolerance induced by BABA in the response of tomato leaves at distinct time points of drought and salt stress including lifestyle transition points was compared as shown in Figure 12.

The transcriptional state of cv Marmande leaves stressed by salt

All the genes tested in this experiment were up-regulated after the second day of treatment with BABA, with the only difference being in the level of the gene expression which became higher on the second day (Figure 12 A). The explanation for this could be that BABA enhanced the tolerance of tomato plants and prepared the plants for any type of stress. Similarly, the gene expression in control plants, in the third day of BABA treatment, was higher than the days before. This suggests that all genes reach a peak after 3 days of BABA treatment even though there was no stress and it started to decrease on the fourth day. Moreover, with 50mM of salt, all genes were up-regulated after 1 day of salt stress and the level was higher compared to the second day of stress. However, with 100 and 200mM of salt during the first and the second day of stress, the general gene expression was less up-regulated with few exceptions. Interestingly, the induction of these TFs in leaves showed an earlier and higher defense response in the plants on the first day compared to the second day of stress. *AREB, CBF3, NAC3,* and *RABC2a* which are ABA-dependent were up-regulated; hence, ABA played an important role in the interaction of BABA to enhance the tolerance of tomato plants under abiotic stress.

The transcriptional state of cv Coeur de Boeuf leaves stressed by salt

Under normal conditions, treated cv Coeur de Boeuf plants with BABA showed a difference on the level of gene expression (Figure 12 B). The expression data demonstrated an increase in the transcription level under salt stress conditions which became more pronounced on the third day of BABA treatment. This level was kept for the fourth day of BABA treatment. Even though there were no stressors, the level of all gene expressions remained up-regulated on the third and the fourth day of stress. Notably, the expression of *CBF3* and *NAC3* were up-regulated during the period of salt stress. With the different concentrations of salt stress, BABA induced a high level of gene expression on mainly the second day of stress. This indicates that the induction of these TFs promoted by salt stress was delayed in cv Coeur de Boeuf compared to cv Marmande. As a hypothesis, the perception of the stress signaling occurred later than the other cultivar and ABA reached the maximum value after 2 days of salt stress.

The transcriptional state of cv Marmande leaves stressed by drought

The characterization of the molecular behavior of cv Marmande treated with BABA under drought stress was made by analyzing the expression of *AREB*, *CBF3*, *NAC3*, and *RABC2a*. In primed plants without stress, the expression patterns of all genes analyzed were up-regulated after 1 day of treatment. The level decreased the second day of BABA treatment. This result indicated that the level of the TFs expression had reached the maximum on the first day after BABA treatment (a peak) and then decreased on the second day (Figure 12 C). All genes were down-regulated the third day of BABA treatment. Under the stressed conditions, there was a difference in the gene expression between the degree of stress and also at different time points. In the moderate dehydration stress (60%), only *CBF3* became up-regulated after 3 days of stress. However, under severe drought stress (20%), transcript levels of all genes were up-regulated and rapidly induced in leaves after 1day of water deficit shock followed by a second and third day of stress, but with a few exceptions. This suggests that severe stress-induced a higher gene expression related to ABA rather than a moderate one.

The transcriptional state of cv Coeur de Boeuf leaves stressed by drought

The expression of these TFs was up-regulated during the first and the second day after BABA treatment (Figure 12 D). The response was faster and stronger, but the levels of the gene expression decreased the second day. There is a similarity between this result and the result with cv Marmande. In a severely stressful condition (20%), *CBF3* was up-regulated at different time points except for the second day. However, under moderate drought stress (60%), the expression level of all genes was low during a specific period of stress, except the second day where *CBF3* and *NAC3* were up-regulated.

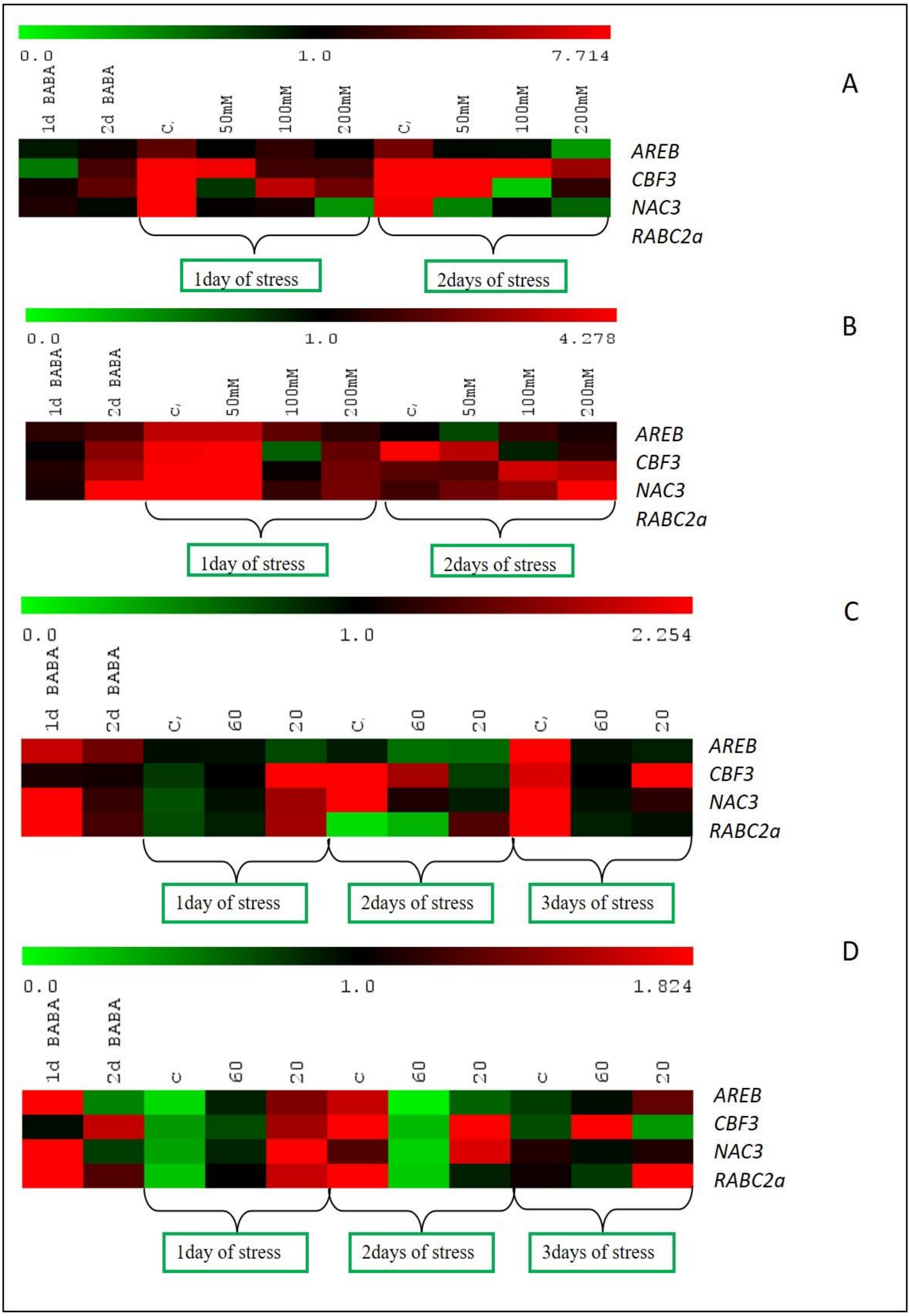

Fig. 12. Comparative analysis of TFs expression to 30-day-old plants of 2 tomato cultivars ((M) cv Marmande and (CB) cv Coeur de Boeuf) treated and non-treated with BABA (5 mM) at 48h before the establishment of drought or salt stress. (A) Comparative analysis of TFs expression in cv Coeur de Boeuf plants treated with BABA under salt stress (0, 50, 100 and 200mM). (B) Comparative analysis of TFs expression in cv Marmande plants treated with BABA under salt stress (0, 50, 100 and 200mM). (C) Comparative analysis of TFs expression in cv Coeur de Boeuf plants treated with BABA under drought stress (0, 60 and 20%). (D) Comparative analysis of TFs expression in cv Marmande plants treated with BABA under drought stress (0, 60 and 20%). Gene expression was assessed by qRT-PCR using total RNA from the leaves of two tomato cultivars after 1 and 2 days of BABA treatment, and after 1 and 2 days of salt stress or 1, 2 and 3 days after application of drought stress. The TFs expression is indicated by non-treated fold induction compared to BABA-treated plants. Green = down-regulated genes, red = up-regulated genes. This is a comparison between treated and non-treated plants

Discussion

Priming is known as a sensitization to stress response and plants primed by chemical stimuli are stronger and more resistant to different kinds of stress (Conrath et al., 2002; Conrath et al., 2006). In this study, BABA was proven to enhance better tolerance in cv Marmande than in cv Coeur de Boeuf under salt or drought stress. Plants treated with BABA demonstrated higher fresh and dry weight than control plants under salt stress. However, under moderate and severe drought stress, plants treated with BABA did not show any differences compared to non-treated ones. In addition, all plants decreased in growth under different levels of stress. Osmotic stress provoked similar responses in all plants. In addition, plants treated with BABA grew better under osmotic stress. The positive relationship between the dry and fresh weights of plant growth with and without osmotic stress (Figure 1) suggested that the higher biomass production promoted by BABA is responsible for the enhancement of growth under osmotic stress. Such enhancement of growth under both stress and non-stress conditions has also been observed in soybean where plant treatment with BABA had a small influence on plant growth (Zhong et al., 2014). Many studies show the aggressive effect of high concentration of BABA inducing biomass inhibition and damage in the leaves (Zhong et al., 2014), nonetheless, the best concentration of BABA is responsible to enhance the resistance of treated plants under abiotic and biotic stress (Zimmerli et al., 2000; Wu et al., 2010). Jakab et al. (2005) showed that BABA was responsible for inducing drought tolerance in Arabidopsis by delaying the onset of wilting (35% of water loss) compared to the control (70% of water loss).

Other studies demonstrated that BABA leads to a significant rise of dry and fresh weight content inducing an increase of stress tolerance upon salt stress (Mostek et al., 2015; Mostek et al., 2016).

The stomatal closure reduced the evapotranspiration and, as a consequence, kept maximum levels of water in the leaves. This phenomenon is generated directly or indirectly after a transmission of the signal by the roots of the stressed plant in case of drought or salt stress (Jackson, 2002). There is another possibility showing the earlier closure of stomata as a response to the increase of ABA levels in the plant (Ton et al., 2009). The accumulation of ABA increases the tolerance of plants under abiotic stress (Pérez-Alfocea et al., 2011). Further studies showed that in Arabidopsis, BABA induced abiotic stress tolerance through ABA-dependent responses (Jakab et al., 2005). In our study, the stomatal conductance was higher, after 2 weeks of stress, in plants treated with BABA, than in the control treatment in both tomato cultivars under salt/drought stress, which indicated that stomata remain partially closed after BABA-priming following ABA accumulation compared to the control plants that have a tendency towards total closure (Figure 2). Previous research has shown that stomatal closure was related to the increase of ABA in two cultivars of wheat (Du et al., 2012). It was reported that BABA increases the sensitivity of stomata to ABA in wheat (Du et al., 2012), but the results showed BABA-treated plants are less susceptible to the stress than control plants. It could be that BABA acts differently in monocotyledonous than in dicotyledonous plants. These results could switch tomato plants to be in a more alarmed state in response to abiotic stress.

From previous studies examining the induction of plant rtolerance it is known that BABA is capable of activating many defense mechanisms depending on the type of stress and also the species. It is possible to induce more callose deposition as a physiological barrier in the case of biotic stress (Ton and Mauch-Mani, 2004), reactive oxygen species, biosynthesis of secondary metabolites and increase the activity of enzymes (Du et al., 2012). In addition to all these defensive mechanisms, there is activation of defense genes which were involved in the response of environmental stressors. In this study, BABA showed an enhanced transcription of some TFs in tomato plants. The primed state may enhance the accumulation of *AREB, CBF3, NAC3* and *RABC2a* which are responsible for inducing tolerance under abiotic stress. The interesting result in cv Marmande and cv Coeur de Boeuf showed that all these TFs tested in our experiment, involved in ABA signaling, were up-regulated after 1day of BABA treatment then were increased and maintained at higher levels until the fourth day without any kind of stress (Figure 12). BABA stimulated the expression of these TFs before any stressor to prepare the plant's immunity system. The plants are ready to battle (Conrath et al., 2006). Plants become primed to activate a faster and stronger defense in the case of stress (Juing et al., 2009). Under salt stress (50mM), cv Marmande showed an up-regulation of all these genes after one day of stress. The increased level of TFs under stress in primed plants is the result of priming (Ton et al., 2009). That is visible as a faster and stronger effect of primed plants under salt stress, like in Arabidopsis under salt stress (Jakab et al., 2005). But the level was lower with 100 and 200mM after the first and also the second day of stress which could be due to the toxicity effect of these concentrations in plants. In cv Coeur de Boeuf, *CBF3* and *NAC3* were more expressed in control and stressed plants. *CBF3* and *NAC3* reduced the accumulation of ROS and enhanced the antioxidative capacity, which had a detoxification role in transgenic tomato and tobacco under drought stress (Rai et al., 2013; Liu et al., 2013). The intense induction of these genes in primed plants induced the accumulation of proline and LEA proteins which reduced the level of ROS and lead to membrane stabilization, thus providing better defense for plants under osmotic stress (Chkarabortee et al., 2007). During salt stress, it was noticed, that the expression levels of genes were much higher in cv Coeur de Boeuf compared to cv Marmande and this could be related to the sensitivity of cv Coeur de Boeuf to abiotic stress. Surprisingly, under drought, stress levels of the TFs were lower in both tomato cultivars compared to salt stress (Figure 12 C, 12 D). The expression of these TFs reached the maximum under a severe drought stress (20%) in both cultivars with some exceptions in cv Marmande. This result indicated that tomato plants were suffering at 20% drought stress. In Arabidopsis and tobacco *AREB* transactivation of *AtRD29A* was induced at a high degree of desiccation to improve tolerance (Yamaguchi-Shinozaki and Shinozaki, 1993; Hseih et al., 2010). Under moderate drought stress (60%), TF expression was up-regulated the third day of stress in cv Marmande. However, the expression of TFs in cv Coeur de Boeuf started earlier after 2 days of stress and then the level decreased the third day. This difference between cv Marmande and cv Coeur de Boeuf could be due to the sensitivity of the second cultivar to drought stress. *CBF3* showed a higher level in both cultivars under water deficit. Over-expression of *CBF3* enhanced tolerance in drought exposed plants. This

TF possessed a higher capacity to defend the plant and to eliminate ROS. These results were confirmed in transgenic tomato exposed to water deficit (Rai et al., 2013).

Drought and salt stress have a negative effect on plant growth and development and in large part, they are the cause of all metabolic and cellular disturbances. One of these major perturbations is associated with the decrease of chlorophyll pigment levels (Tuba et al., 1996). It is at the chloroplasts where a part of the carboxylation phenomena is located by using the enzymatic process which depended upon the degree of the light photons used as well as the concentration of chlorophylls, or, more precisely, the active photosynthetic pigments. Photosynthetic activity depended on genotype (Juan et al., 2005) and environmental stress (El-Sharkavy, 2006). In our study, Drought and salinity have a considerable effect on the synthesis of chlorophyll pigments (a, b and anthocyanin) in cv Marmande as well as in cv Coeur de Boeuf. The chl a and chl b content of the drought or salt-stressed tomato plant was decreased in treated and non-treated plants with BABA when compared to control plants (Figure 4). A similar report was obtained in *Paulownia imperialis* and *Sorghum bicolor* (Astorga and Melendez, 2010; Oraki et al., 2012; Ebrahimia et al., 2014; Arivalagan and Somasundaram, 2015) under drought stress. BABA treatment increased, in some cases, the chl a and b content in stressed plants when compared to stressed and non-treated plants with BABA. Similar results were reported in sorghum treated with propiconazole (PCZ) and salicylic acid (SA), a well-known inducer of tolerance in plants to water and salt stress, (Arivalagan and Somasundaram, 2015) in *Solenostemon rotundifoliu* (Kishorekumar et al., 2008) and in wheat (Arfan et al., 2007). Similar results were also observed in groundnut plants treated with ABA, which has been known to be a messenger in stress-perception response pathways and plays a direct role in mediating the photosynthesis to respiration in leaves (Zhang et al., 2001; Zhou et al., 1998) under drought stress (Sankar et al., 2013) in *Kentucky bluegrass* (Wang et al., 2003) and in tomato (Thompson et al., 2000). BABA is responsible for increasing chlorophylls which may be required to increase the leaf’s thickness and force a direct impact on the photosynthesis intensity.

In response to drought stress, cv Coeur de Boeuf showed an increase of anthocyanin content in plants treated and non-treated with BABA. Similar results were found in Arabidopsis under osmotic stress (Kovinish et al., 2015). However, under salt-stressed, cv Coeur de Boeuf plants showed a decrease of anthocyanin content in non-treated plants when compared to control (Figure 5). Similar results were observed in tomato (Berova et al., 2000). This difference between cv Coeur de Boeuf-defense responses to stress may be due to sensitivity to salt stress. In contrast, cv Marmande plants have no significant effect upon drought or salt stress in non-treated plants except for 200mM of salt where anthocyanin content was significantly higher than the control. BABA-treated plants increased anthocyanin content in response to drought or salt stress in both cultivars. Similar results were observed in *Arachis hypogaea* plants treated with paclobutrazol (PBZ) which is a plant growth regulator (Sankar et al., 2013). Therefore, BABA might be the cause for the increased anthocyanin content upon drought or salt stress.

H_2O_2 functioned as stress signals in plants, mediating a range of defense responses to environmental stress (Neill et al., 2002; Zaninotto et al., 2006; Zhang et al., 2009; Hossain et al., 2015). However, higher production of ROS like H_2O_2 in plants subjected to various severe abiotic stresses such as drought and salt stress may have broken a balance between ROS and antioxidative systems and; therefore, abolished the signal and leads to lignin in plant tissues (McAinsh et al., 1996; Hosain et al., 2010; Mostafa and Fujita, 2013; Nahar et al., 2014).

Analysis of the *in vivo* localization of ROS via DAB-staining demonstrated an increase of H_2O_2 accumulation upon 7 days of drought or salt stress in treated and non-treated plants with BABA in two tomato cultivars (Figure 7). A similar result was shown in Arabidopsis where the exogenous application of ABA was responsible for triggering H_2O_2 accumulation (Xing et al., 2008; Luna et al., 2011). In response to salt stress, transgenic tobacco plants accumulated more ROS when compared to the control (Yadav et al., 2012). Our results revealed that BABA-treated plants in both cv Marmande and cv Coeur de Boeuf maintained less ROS in epidermal cells after response to drought and salt stress compared to non-treated plants (Figure 6). This result was similar to that observed previously in two wheat cultivars treated with BABA under soil drying conditions (Du et al., 2012). We here provide evidence that BABA reduced the accumulation of H_2O_2 in tomato under drought and salt stress, suggesting that lower H_2O_2 levels might be responsible for reducing oxidative damage resulting from abiotic stress. A similar result was found in Arabidopsis where inhibition of H_2O_2 production compromised stomatal closure induced by the increase of ABA and then conferred to stress tolerance (Shintaro et al., 2007). Cv Marmande accumulated the lower level of H_2O_2 in response to drought or salt stress than cv Coeur de Boeuf. This difference between cv Marmande and cv Coeur de Boeuf may be related to the plant genotype.

Increased lignification in plants under abiotic stress would help xylem vessels to reduce transpiration by increasing the stiffness to reduce water permeability and; therefore, withstand or cope with low water potential (Whetten and Sederoff, 1995; Huang et al., 2010; Le Roy et al., 2017). In our study, lignin accumulation decreased in treated and non-treated tomato plants with BABA under drought or salt stress (Figure 8 and 9). Similarly, in another study, it was indicated that lignin biosynthesis is suppressed upon drought stress in Maize (Alvarez et al., 2008). Lignifications were strongly suppressed in BABA-primed crabapple seedling under drought stress (Macarisin et al., 2009). Thus, lignin deposition was decreased upon severe abiotic stress in BABA-primed plants which may be due to the absence of growth according to a restriction of cell elongation under drought or salt stress. A similar result was found in seedling of crabapples treated with BABA (Macarisin et al., 2009). Interestingly, BABA-treated cv Marmande and cv Coeur de Boeuf presented a higher level of lignin deposition compared to the non-treated plants. This result was similar to Cohen et al. (2000) study, where BABA was shown to enhance lignin deposition in grapevines. The increase in lignifications in treated plants with BABA was one of the reactions included in a general adaptation strategy of plants faced with dehydration and may result in an increase of mechanical strength and/ or water impermeability.

The increase of ROS production in plants can be the consequence of unfavorable environmental conditions like drought or salt stress. To protect themselves against these toxic compounds, plants activated their antioxidant defense systems (Apel and Hirt, 2004; Tuteja, 2007; Khan and Singh, 2008; Gill and Tuteja, 2010). It is known that BABA treatment augmented the antioxidant defenses increasing the total antioxidant activity and then enhanced tolerance to oxidative stress (Hossain et al., 2012). In our study, we found that total antioxidants increased upon drought or salt stress in treated and non-treated plants with BABA in both cultivars (Figure 10). Furthermore, BABA-treated plants showed higher total antioxidant levels than non-treated plants in both cultivars. After BABA treatment, a reduction of H_2O_2 accumulation and an increase in total antioxidants are activated, together this helped to avoid high levels of oxidative cellular damage. This suggests that BABA can induce the capacity to cope with oxidative stress in tomato plants. BABA-induced tolerance could be mediated by a faster osmoregulation process. Similar results have been described in pea seedling after SA treatment (Srivastava and Dwivedi, 1998) and in tomato plants treated with DAAME (Flors et al., 2007). Other research indicated that BABA-primed plants presented a greater accumulation of active antioxidant defense mechanisms compared to non-treated plants under cadmium-induced oxidative stress (Hossain et al., 2012) and salt stress (Hossain et al., 2014), which might act to remove the free radical induced by stress which is correlated with enhancing plant protection and minimizing oxidative stress damage (Mostek et al., 2016). A similar study was shown in wheat lines where BABA decreased ROS accumulation and enhanced antioxidant enzyme activity, which lead to reduced oxidative damage to lipid membranes (Du et al., 2012). Under acid rain, BABA-treated plants presented significantly higher levels of ascorbate peroxidase and superoxide dismutase, as well as other low-molecular-weight antioxidants and anti-oxidative enzymes, which lead to enhanced tolerance in treated plants (Liu et al., 2011).

It is well known that the hormonal system plays a crucial role in the regulation of plant growth and abiotic or biotic stress tolerance (Robert-Seilaniantz et al., 2007; Shimizu et al., 2013; Miura and Tada, 2014). Although, drought and salt stress both created osmotic stress in plant organisms, which eventually caused desiccation and tolerance to water uptake in plants; fortunately, plants can enhance stress-induced phytohormones' accumulation to escape these unfavorable conditions. In this study, plants treated and non-treated with BABA showed a higher accumulation of ABA, which is the primary hormonal regulator of abiotic stress, upon drought/salt stress in both cultivars (Figure 11 A and B). The increase of ABA levels in response to drought/salt stress lead to the induction of stress tolerance by regulation of the water balance in the cells and controlling stress response in plants (Yamaguchi-Shinozaki and Shinozaki, 2006; Nakashima and Yamaguchi-Shinozaki, 2013; Zhang et al., 2006, 2013; Riccardi et al., 2016). ABA-induced in response to abiotic stress was involved in the expression of genes' encoded dehydrins associated proteins and antioxidants and repressed growth and chlorophyll contents (Wang et al., 2013). ABA-induced stomatal closure was involved to limit leaf transpiration (Trejo et al., 1993; Pham and

Desikan, 2012). In addition, ABA-induced tolerance affected ROS accumulation which could be connected to the increase of antioxidant activities upon abiotic stress (Kao, 2014; Karuppanapandian et al., 2011). BABA was shown to enhance drought/salt-induced ABA accumulation in the leaves of cv Marmande and cv Coeur de Boeuf more than non-treated plants. Thus, BABA treatment in stressed plants leads to hypothesize that ABA would be mediated by the BABA-induced tolerance. Similar results were demonstrated in tomato treated with DAAME in response to salt stress (Flors et al., 2007). Significant accumulation of endogenous SA under drought/salt stress in treated and non-treated tomato plants was shown in our results in both cultivars (Figure 11 A and B). The similar result was found in Okuma et al., (2014) study where a significantly higher SA level was detected in Arabidopsis under drought stress. Other studies demonstrated that SA treatment contributed to the induction of tolerance to salt stress in Arabidopsis, wheat and tomato (Shakirova et al., 2003; Singh and Gautam, 2013). This result suggested that accumulation of endogenous SA could confer an increase of plant tolerance in response to stress. Contrariwise, the study of Borsani et al., (2001) presented the negative effect of SA in arabidopsis seedlings under osmotic stress. Tomato plants treated with BABA presented a higher level of SA compared to non-treated plants under drought/salt stress in both cultivars. This result indicated that BABA treatment helped the plants to become more tolerant to abiotic stress. Similar results were shown under exogenous SA conditions where the activities of antioxidants were enhanced with the decrease of H_2O_2, which indicated that SA induced tolerance to oxidative damage under drought stress in stressed mustard seedlings (Alam et al., 2013) and maize (Saruhan et al., 2012). However, contradictory results were found in Németh et al. (2002) study, which suggested that exogenous SA could be responsible for decreasing tolerance in Maize and wheat under drought stress. In our results SA levels decreased in response to 200 mM of salt in cv Coeur de Boeuf. This result suggested that 200 mM may be toxic to the plants. Finally, accumulation of SA suggested that this hormone can elicit plant-adaptive responses to drought (MunoEspinoza et al., 2015).

JA has a similar function as ABA and a significant role in plant responses to abiotic stress by the activation of specific stress responses. Notably, this hormone can be suppressed by SA or they can be involved in the same signaling pathways (Wasternack, 2007). Our results showed that JA in treated plants with BABA increased during drought/salt stress compared to non-treated plants (Figure 11 A and B). However, non-treated plants presented lower and no significant difference to JA levels under different degrees of stress. These results suggested that BABA induced JA in response to drought/salt stress. The increase of JA levels might be connected to enhanced ABA levels under drought stress in apples and barley (Shan and Liang, 2010). It was reported in other study that the gene expression of SA and JA-stress responses-dependent was mediated by H_2O_2 (Mhamdi et al., 2010). It was noteworthy that the high level of JA content in treated and stressed tomato plants may be due to the coordination action between JA and SA. Previous studies have raised the possibility that JA-dependent processes may confer enhanced plant tolerance to salt-mediated effects (Tsonev et al., 1998).

JA-Ile, which is a major active form of Jasmonates, had a prominent role in plant defense responses (Shimizu et al., 2013). In our study, JA-Ile increased in treated and non-treated cv Coeur de Boeuf plants under drought stress conditions but there was no significant difference between treatments (Figure 11 A and B). The same results were found in non-treated cv Marmande plants in response to drought stress, while treated cv Marmande plants with BABA presented a higher level of JA-Ile compared to non-treated plants. Similar results were shown in cv Marmande under salt stress. These results suggested that BABA was effective in increasing JA-Ile in cv Marmande upon drought stress. In addition, JA-Ile was higher in treated and non-treated cv Coeur de Boeuf plants under salt stress. However, the level of JA-Ile decreased in response to 200 mM where there was no significant difference between treated and non-treated plants. Thus, 200 mM may be toxic for cv Coeur de Boeuf. These differences of JA-Ile levels in response to different stresses could be related to plant sensitivity. For example, the accumulation of JA and JA-Ile in two grapevine cell lines was more prevalent in the sensitive *Vitis riparia* than in the *Vitis rupestris*, which is a salt-tolerant line (Ismail et al., 2012, 2014). The accumulation of JA-Ile was lower compared to the rest of the phytohormones. It is conceivable that BABA treatment leads to an increase of the bioactivity of JA, but further studies are still not clear.

Taking together the results presented in this study, the induced tolerance based on BABA treatment has been shown to be effective in tomato plants under drought or salt stress. Thus, the use of this compound in agriculture could help in the future to fight against abiotic stress (especially drought and salinity) in a natural and unaggressive way towards nature.

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Supporting information

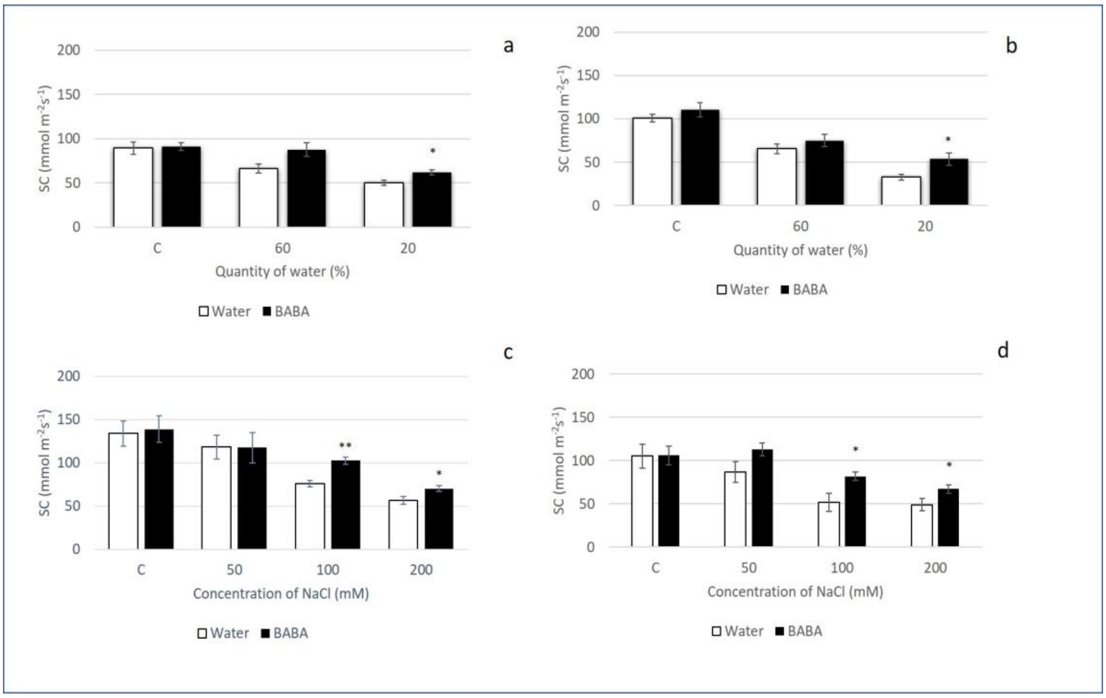

Fig S1. Measurement of stomatal conductance after 1 week of drought and salt stress a) and b) cv Marmande and cv Coeur de Boeuf under different levels of water deficit c) and d) cv Marmande and cv Coeur de Boeuf stressed by different concentrations of salt. Error bars indicate the standard errors for the average values of 6 replicates. Asterisks indicate a significant difference in a Student t-test (* = $p<0.5$).

Chapter III

Impact of β-aminobutyric acid on induced resistance in tomato plants exposed to a combination of abiotic and biotic stress

Impact of β-aminobutyric acid on induced resistance in tomato plants exposed to a combination of abiotic and biotic stress

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Abstract

A plant's defensive capacity can be enhanced by treatment with various synthetic and natural compounds capable of improving its immune system and make it more resistant. This is called priming. Primed plants express faster and stronger enhanced defense upon encountering either abiotic or biotic stress. Traditionally, plant stress has been studied by applying a single type of stress such as drought, salinity or infection and analyzing phenotypic and molecular aspects of the resulting plant phenotype. However, this type of analysis is in sharp contrast to natural conditions where plants are simultaneously subjected to a combination of different abiotic and biotic stresses that limit crop yields. Recent evidence shows that a combination of abiotic and biotic stress can have a positive effect on plant performance by reducing the susceptibility to biotic stress. Such an interaction between both types of stress points to crosstalk between their respective signaling pathways. Using the non-protein amino acid β-aminobutyric acid (BABA) to prime tomato plants, we found that BABA-treated plants showed earlier and higher expression of *PR1* and *PR5* genes following combination of salt stress and infection with *Botrytis cinerea* compared to unstressed plants exposed to salt. Histochemical analysis revealed that in BABA-treated plants, induced levels of callose deposition and lignin accumulation were higher than in non-treated controls, while the spread of *B. cinerea* was strongly reduced. A rapid H_2O_2 accumulation detected in BABA-treated plants under combined stress, may have contributed to the observed decrease in the pathogen's proliferation.

Keywords: β-aminobutyric acid, tomato, salt stress, *Botrytis cinerea*, combined stresses, induction of resistance.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important crops and widely cultivated around the world. In 2013, it was seventh in the ranking of food crops worldwide, while production and consumption are constantly increasing [1,2]. Tomato provides valuable compounds like lycopene, known for its anti-oxidative and anticancer properties [3]. However, the yield potential of tomato is strongly affected by biotic stress factors including *Botrytis cinerea* [4,5]. This necrotrophic fungus induces cell death in the vegetative tissues [6,7]. *B. cinerea* penetrates through wounds or stomata and kills plant tissues by secreting toxic compounds or lytic enzymes [8]. This infection leads to necrosis of the host. Constitutive barriers, such as a stronger cell wall or stored metabolites, and inducible defenses, such as the synthesis of phytoalexins, help plants cope with such fungal attacks [9]. However, in their natural habitats, plants are often challenged not only by biotic stress but at the same time with abiotic stresses. The simultaneous action of both abiotic and biotic stressors can activate a multitude of compounds in the plant that act with different modes of convergence ensuring the regulation and the modification of the plant response pattern which could be completely different from the predicted plant responses to a single constraint [10-13].

The interaction between abiotic and biotic stresses in plant responses is controlled by different hormonal signaling pathways and by a variety of molecular mechanisms that work together in a very complex way [10,14-16]. Narusaka et al. [17] indicated that plant responses to a simultaneous abiotic and biotic stress are associated with changes in complex gene networks in order to withstand the stress. However, studies in this field suggested that abiotic stress can have additive (increasing the stress impact) or reductive (increasing stress tolerance) effects on plants upon pathogen attack and vice versa [10,18-20]. The rapid accumulation of abscisic acid (ABA) in the plants as an adaptive response to abiotic stress for example increases the susceptibility of plants to pathogen attacks [21]. Sorghum and bean stressed by drought stress present a higher susceptibility to *Macrophomina phaseolina* [22,23]. The same holds true for Arabidopsis exposed to drought stress and *P. syringae* [24]. Moreover, under salt stress, increased plant tolerance through Na^+ compartmentalization in the vacuoles may have adverse effects on pathogen feeding and development [12]. In contrast, in some cases the exposure of plants to abiotic stress enhances resistance to pathogen attacks [25,26] via the positive effect of ABA on callose deposition [27,28] and the induction of gene expression in response to both kinds of stress [29]. Drought stress enhances resistance against *B. cinerea* in tomato [30]. This positive interaction can be due to stomatal closure reducing water loss from infected tissues or the high levels of defense compound accumulated after a period of abiotic stress as shown in the *sitiens* tomato mutant where the abscisic acid deficiency increases the resistance to *B. cinerea* [31]. It is still not clear why some studies on to the combination of abiotic and biotic stresses report an increase of plant resistance against pathogen attacks while others an increased susceptibility to infection [32].

Generation of reactive oxygen species (ROS) as inducible defense response following abiotic and biotic stress depends on the strength and duration of the stress [33-35]. Low levels of ROS occur primarily as stress signal transduction molecules that insure plant acclimation against many stresses. They can, however, become toxic for plants if levels continue to increase leading to oxidative stress [36]. To minimize damage under abiotic stress conditions, plants produce antioxidant and ROS-scavenging enzymes [37,38]. In contrast, plants actively generate ROS and act positively on plant resistance against pathogen infection by mediating the hypersensitive response (HR)-like cell death, a process known as the oxidative burst. In this case, the accumulation of ROS limits pathogen spread [39-41]. Furthermore, ROS accumulation in response to a combination of abiotic and biotic stress may create a stress-specific signal to both stressors in order to induce the acclimation response to the plants. Laluk et al. [42] demonstrated that Pentatricopeptide Repeat Protein for Germination on NaCl (*PGN*) controls the role of ROS in the combination of abiotic and biotic stress conditions, which indicates that PGN is responsible to regulate ROS homeostasis in the mitochondria.

Priming plants puts them into a stage where they show an increased and accelerated ability to activate various defense mechanisms upon encountering biotic and abiotic stresses [43,44]. Among the many inducers of resistance, β-aminobutyric acid (BABA) has proved to be an effective agent for the induction of resistance to both biotic and abiotic stressors [45,46]. Depending on the challenging stressor, BABA-primed plants will mount their defense via the appropriate defense signaling pathway. BABA-induced resistance against downy mildew and *Pseudomonas* for example depends on salicylic acid(SA) signaling leading to the accumulation of pathogenesis-related (PR) proteins while defense against necrotrophic fungi depends on ABA [47,45]. PR proteins are induced not only in response to pathogen attack but also under abiotic stress conditions [45,48-50]. Unfortunately, there is a lack of information related to BABA-induced tolerance in plants subjected to a combination of abiotic and biotic stress. The objective of this study was to determine the effect of pre-treatment of tomato plants with BABA on salt-induced predisposition to the foliar pathogen *B. cinerea.* We also evaluated the impact of BABA on the expression pattern of *PR* genes and some transcription factors involved in the defense responses to a combination of salinity and infection with *B. cinerea*.

Material and methods

Culture conditions

Controlled conditions

Seeds of tomato cv Marmande were obtained from Quedlinburger (Aschersleben, Germany). For sterilization, tomato seeds were rinsed in 70% ethanol, incubated for 15 minutes in 2% bleach and washed 4 to 5 times with sterile distilled water. After 24 hours at 4°C, sterilized seeds were pre-germinated for 6 days in a Petri dish with a humid sterile Whatman filter paper in the dark in a plant growth chamber (Percival AR-95L, CLF Plant Climatics GmbH, Wertingen, Germany). Tomato seedlings were then transferred to multi-cell growing trays filled with commercial soil (Compost (25%), sand (12%) and peat (63%); Ricoter Erdaufbereitung AG, Aarberg, Switzerland) watered with Solbac (Andermatt Biocontrol, Switzerland) solution to prevent fungus gnat problems. Ten days later, seedlings were individually transferred to small 100-mL plastic pots filled with the same soil. All plants were well watered and kept in the same plant growth chamber with the following conditions: 16-hour day at 26°C, 8-hour night at 18°C, 60% relative humidity and an irradiance of 245 μmol m^{-2} s^{1} until they reached the stage of four fully expanded true leaves (from the tip, excluding petiole). At this stage, plants of uniform size were used for the experiments.

Greenhouse conditions

Seeds of tomato cv Marmande were sterilized and pre-germinated as described above. Tomato seedlings were then transferred to multi-cell growing trays filled with soil mixture (Compost (25%), sand (12%) and peat (63%), Fafard, Canada) and further processed in a greenhouse (Plant Research Facility Greenhouse, McGill University, Canada) under the same conditions as described above for growth chamber grown plants.

Priming and stress application

β-Aminobutyric acid (BABA) (Sigma-Aldrich, Switzerland and Canada) was dissolved in water and applied as a soil drench (5 mM BABA final soil concentration) [51] to 4-week-old plants 2 days prior to applying salt stress (0 and 50mM NaCl). Control plants were watered with tap water. The time point of BABA treatment is considered to be day 0 (Figure 1). Tomato plants (cv Marmande) were subjected to pre-treatments with various concentrations of BABA, followed by the salt stress 48 hours later. The inoculation with *B. cinerea* was performed after 24 hours of the salt stress.

Inoculations

B. cinerea was isolated from an infected tomato fruit and was routinely cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Switzerland and Canada) at 24°C. Conidia were harvested from sporulating colonies (15-day-old cultures) with 5 mL of sterile water. Conidia were filtered through gauze, washed by centrifugation (10 min, 1533 *g*, 20°C) and centrifuged another time (4 min, 13552 *g*). The pellet was re-suspended in 1 mL of sterile water and the final conidia suspension was made with 1 mL of 1/2 strength PDB (Potato Dextrose Broth) (Difco Laboratories, Detroit) and 10 µL of sucrose (10mM as a final concentration) at a density of 10^6 conidia mL^{-1}. The suspension was pre-incubated without shaking for 2 hours. Six µL droplets of the suspension were placed on the third and the fourth true leaves. All plants were transferred to a container with a transparent lid (growth chamber) or covered with transparent plastic bags (greenhouse) to keep 100% of relative humidity. These experiments were repeated two times with similar results.

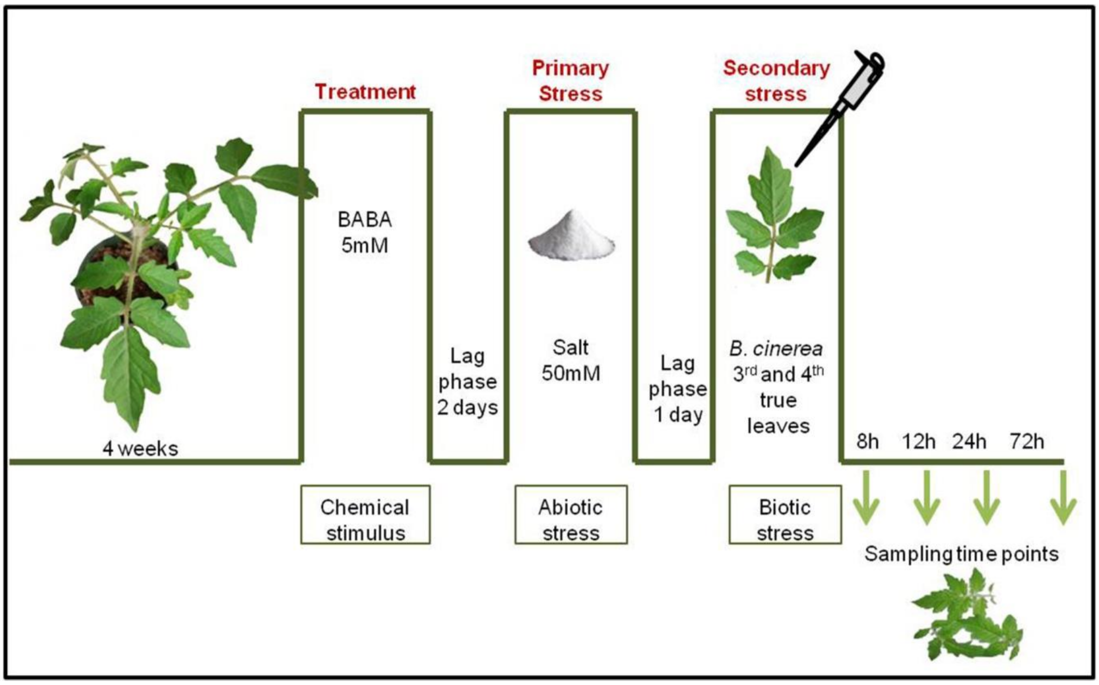

Fig. 1. Time course of the combined stress experiments in BABA-treated tomato plants. Four-week-old tomato plants were exposed to a chemical stimulus, BABA, by soil drench. After two days, the primary stress 'salt stress' was applied as a soil drench. After one day, a secondary stress '*B. cinerea'* was applied by placing 6µ droplets of the fungal suspension on the third and the fourth true leaves. Leaf tissues were harvested at four-time points: (1) after 8 hours post inoculation (hpi); (2) after 12 hpi; (3) after 24 hpi; (4) after 72 hpi.

Size of infected area

Seventy-two hours after inoculation, the area of infection was calculated by measuring with a caliper square (two diameter values for each infection site) and the infected leaves were directly put into 100% ethanol for later callose staining. Average sizes of infection were based on at least 20 measurements from six tomato plants. Six biological replicates were collected.

Callose staining

Seventy-two hours after inoculation, tomato leaves were collected, discolored in 95% EtOH and stained with aniline-blue (Sigma Aldrich, Switzerland) as described previously by Ton et al. [45], with some modifications. Briefly, leaves were incubated for at least 24 h in 100% ethanol until all tissues were transparent, washed in 0.07 M phosphate buffer (pH = 9), and incubated for 1 to 2 h in 0.07 M phosphate buffer containing 0.01% aniline-blue (Sigma Aldrich, Switzerland), prior to microscopic analysis. Observations were performed with an epifluorescence microscope with UV filter (BP 340 to 380 nm, LP 425 nm). Callose was quantified from digital photographs by the number of white pixels (callose intensity) or the number of depositions relative to the total number of pixels covering plant material, using Image J software. Callose was selected automatically, using the “Color Range” tool. The accuracy of resulting callose selection was visually verified before proceeding. Average callose measurements were based on at least 20 photographs from different tomato plants. Six biological replicates were collected.

Gene expression

Plant material was harvested at 0h, 8h, 12h, and 24h after inoculation with *B. cinerea*, flash frozen in liquid nitrogen and kept at -80°C until use. Three biological replicates were collected per time point.

RNA extraction and gene expression analysis

Total RNA was extracted from the frozen tomato leaf tissues. RNA isolation was performed according to manufacturer’s instructions using the RNeasy Plant Mini kit (Qiagen, http://www.qiagen.com). RNA was treated with DNase (Qiagen) and reverse transcribed into cDNA using SuperScript III RT (Invitrogen, http://www.invitrogen.com). Primers for qRT-PCR were designed using the universal probe library assay design tool from Roche (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). The genes and their corresponding primers used in this study are listed in Table 1. Primer efficiency was determined by performing a qRT-PCR with serial diluted cDNA. Minimal accepted efficiency for the primers was set to 0.8. The qRT-PCR was performed using the SensiMix SYBR kit (Bioline, http://www.bioline.com) on a Rotor-Gene 6000 cycler (Qiagen). The reaction volume was 10 µL, consisting of 2.5 µL nuclease-free water, 5 µL SensiMix SYBR mastermix, 0.25 µL forward and reverse primer (each 10 µM) and 2 µL cDNA. PCRs were performed using 3 independent biological replicates per sample, each replicate consisting of a pool of 3 plants. PCR reactions were performed in technical duplicates as a three-step reaction (initial hold step, 95°C for 10 min; 40 cycles of amplification, 95°C for 15 s, 60°C for 20 s, 72°C for 20 s) with a final melting curve analysis (68°C-95°C). Melting curve and cycle threshold (Ct) analysis were performed on the Rotor-Gene 6000 software 1.7. Relative gene expression of stressed tissue was calculated relative to control treated plants in regard to the two housekeeping genes *Actin* and *Tubulin* and the specific primer efficiencies with the help of REST 2009 (Qiagen).

Table 1. Primers used to assess transcription factor and gene expression in tomato leaves under combination of abiotic and biotic stress.

Stress	Gene	Accession	Fw primer	Rev primer
Salt	*CBF3*	AY497899	CTGTTTTCCATGCCAGGATT	GGGGAGGAGGTAGCATGAG
Salt	*AREB*	NM_001247667	TGGTGAAACTGTTATTCAGTCTGC	GTGTGGATCTGACCCCATTC
Biotic stress	*PR1*	EU_589238	AAACCTAGCTGCCGCTTTC	TTGCTTCTCATCAACCCACA
Biotic stress	*PR5*	NM-001247422	GTGAATGCCCTGGTTCACTT	TCCGAATGTAGTACAAGGGTTG
Reference gene	*Actin-7-like*	XM_004249818	GGTTGGAATGGGTCAGAAAG	GATACCCCTCTTGGATTGAGC
Reference gene	*Tubulin*	NM_001247878.2	TACTGAAGGCGCAGAGTTGA	TTCTCCGCTTCTTTACGAACA

DAB staining, microscopy analysis, and H_2O_2 quantification

In situ hydrogen peroxide (H_2O_2) production was revealed by brown precipitates after 3,3'-diaminobenzidine (DAB) staining. The 3rd and the 4th fully expanded leaves at 72hpi were cut into discs (0.5 cm in diameter). Harvested leaf discs were stained in 1 mg mL^{-1} of DAB (Sigma Aldrich, Canada) at $pH < 3$, put for 24 h in the dark and subsequently destained in saturated chloral-hydrate (Sigma, Canada), as described previously [52,53]. H_2O_2 is visualized as a reddish-brown deposit in DAB-treated leaves. DAB staining intensities were quantified from digital photographs (Zeiss stereo discovery v20 microscope (Carl Zeiss Canada ltd., Toronto, Ontario, Canada)) by the number of dark-brown DAB pixels relative to total pixels corresponding to plant material, using Image J. Six biological replicates were collected for analysis and average of ROS measurements were based on at least 20 photographs from different tomato plants.

Histochemical analysis of lignin

Histochemical determination of lignin was performed using Wiesner's test. The 3rd and the 4th fully expanded leaves were cut into discs at 72hpi. Discs were treated with a saturated solution of phloroglucinol (Sigma Aldrich, Canada) in 85% (v/v) ethanol, for 3 min at room temperature and washed in 25% (v/v) HCl [54,55]. Lignin was visualized as a pink color around the infection site. Pictures of lignin were taken under a Zeiss stereo discovery v20 microscope (Carl Zeiss Canada ltd., Toronto, Ontario, Canada).

Statistical analysis

Significance of differences between control and treated plants was analyzed with a two-way ANOVA; control and stressed plants ($P<0.05$) were then compared using the Tukey's range and *t*-test. All statistical analyses were accomplished using Sigma Plot 11.0 (http://www.sigmaplot.com).

Results

Disease progress in leaves

The objective of this experiment was to evaluate the effect of BABA on the induction of resistance in plants subjected to a combined stress in two different, namely controlled (growth chamber) and semi-controlled (greenhouse) conditions. Progress of the disease differed between cv Marmande plants treated and non-treated with BABA and stressed or not with salt (Figure 2 and 3). Compared with non-treated control plants, BABA-treated cv Marmande plants showed a statistically significant reduction in lesion size at 72 h after combined stress in both conditions (Figure 2). The effect of BABA on the proliferation of *B. cinerea* in the abscisic acid (ABA)-deficient *sitiens* tomato mutant (Figure S2 and S3) was better than the results obtained with cv Marmande, under semi-controlled conditions which *sitiens* exhibited a higher level of resistance against *B. cinerea* (Figure 2). The size of infections in the *sitiens* mutant was smaller compared to both cv Rheinlands Ruhm and cv Marmande. In addition, concomitant treatment with BABA and 50mM of salt lead to a higher protection against *B. cinerea* compared to non-treated and stressed cv Marmande plants. This induction of resistance was also reflected by a statistically significant reduction of the infection size, but there was no significant difference between control plants treated with BABA and plants treated with BABA + 50mM of salt which still looked healthy under controlled and semi-controlled conditions (Figure 3).

However, plants subjected to 100 and 200mM of salt, respectively, were severely infected by the fungus, independently if they had been treated with BABA or not under controlled conditions (Figure S1). This suggests that high salt concentrations (100 and 200mM) were toxic to the plants and BABA was not able to protect them under these severe conditions.

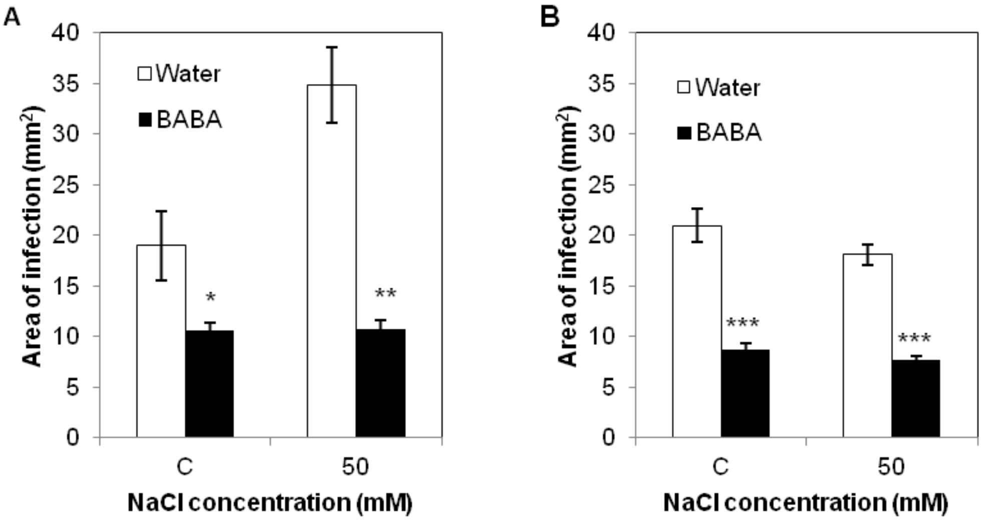

Fig. 2. Size of lesions caused by *B. cinerea* infection 72h post-inoculation of tomato leaves (cv Marmande) treated with water or BABA and then challenged with abiotic stress (salt stress: water control and 50mM NaCl). A) Size of infection area in tomato grown under controlled conditions. B) Size of infection in tomato from greenhouse conditions. Data shown are average values (±standard error of the mean; n > 6 independent replicates) of relative area. Asterisks indicate significant differences (Student's *t*-test, $p<0.001$) in the area of infection between plants treated with water and plants treated with BABA. The experiment was repeated 2 times with similar results. (* = $p<0.05$, ** = $p<0.01$, ***=$p<0,001$).

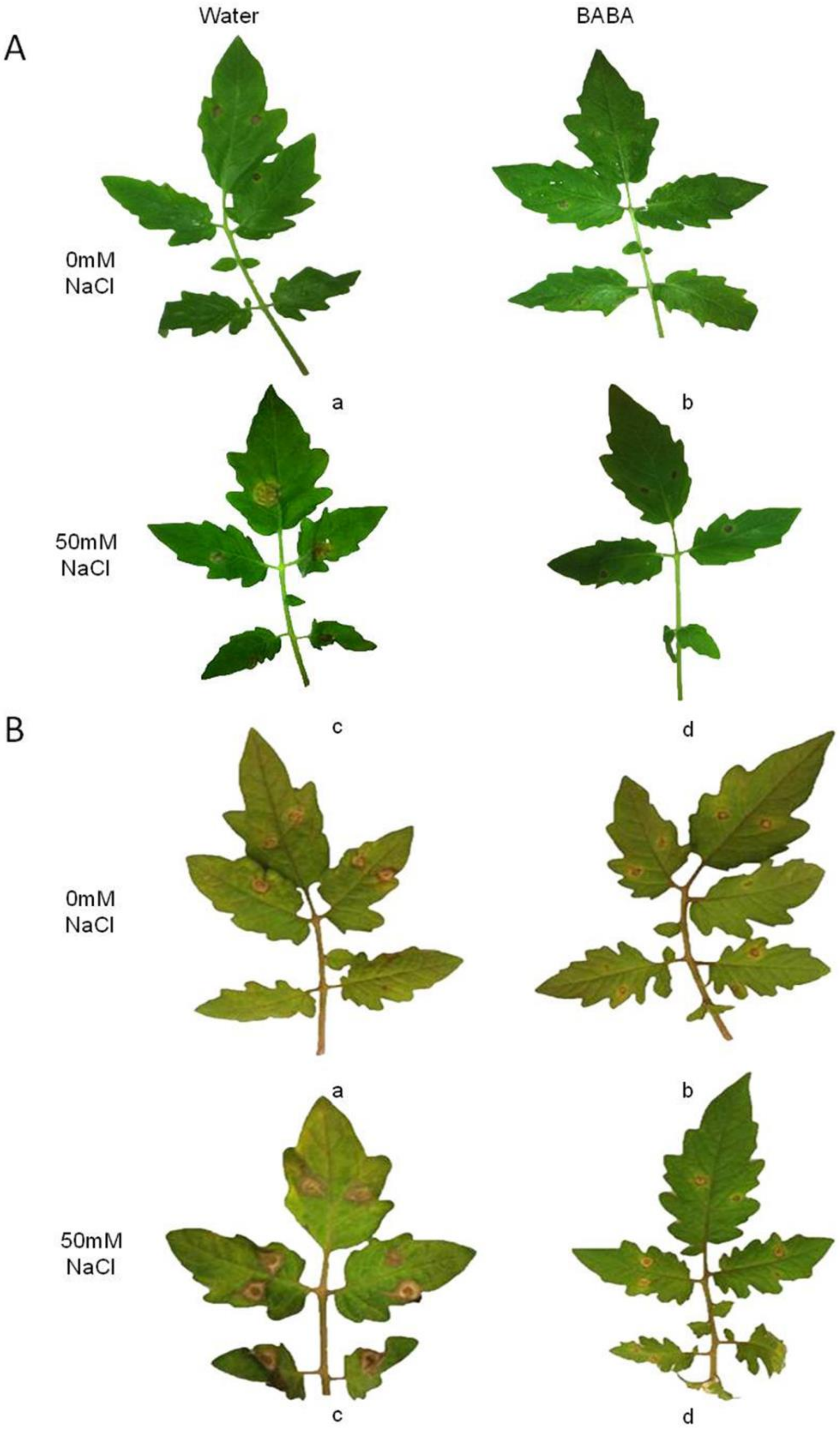

Fig. 3. Responses of tomato plants (cv Marmande) treated with water or BABA to the combination of salt stress (50mM) and *B. cinerea.* A) Leaves from tomato plants subjected to combined stresses, experiment was conducted under controlled conditions: Leaves treated with water or BABA (representative of two replicate treatments) from control (a and b) and stressed with 50 mM of salt (c and d). B)

Leaves from tomato plants subjected to combined stresses, experiment was conducted under greenhouse conditions: Leaves treated with water or BABA (representative of two replicate treatments) from control (a and b) and stressed with 50 mM of salt (c and d). Symptoms photographed 72h post-inoculation.

Localization and intensity of callose deposits induced by *B. cinerea*

To investigate the role of stress combination (salt stress and inoculation with *B. cinerea)* on BABA-induced callose, we examined the dynamics of callose accumulation in BABA-primed and non-primed plants in response to a stress combination (Figure 4 and S4). We did find a statistically significant increase in callose deposition upon concomitant treatment with BABA and the combination of two stresses compared to plants treated with BABA and just a single stress (inoculation with *B. cinerea*; Figure 4). These results suggest that callose induction in BABA-treated plants was improved by the simultaneous application of two stresses. However, in the case of water-treated control plants, there was a significant difference between plants subjected to a single or double stress with a lower level of callose detected upon stress combination.

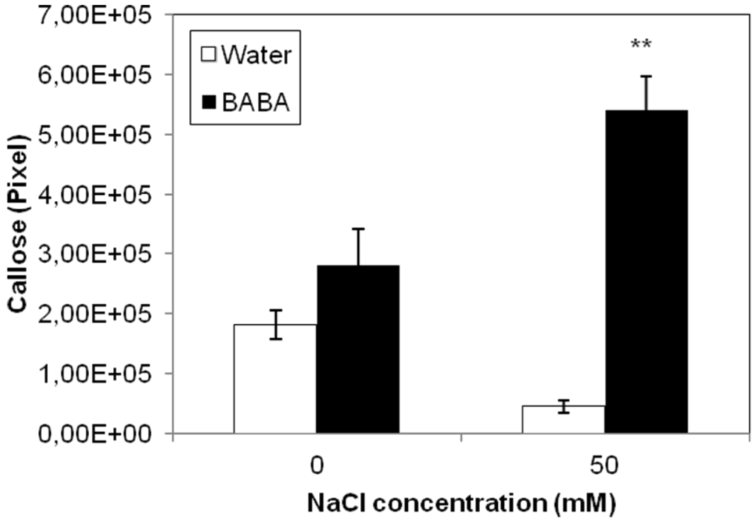

Fig. 4. Callose deposition in tomato leaves in response to *B. cinerea* infection in water or BABA-treated plants additionally exposed or not to salt stress (50mM). Data shown are average values (± standard error of the mean; $n > 20$) of relative callose intensities at 72 hpi with *B. cinerea*. Asterisks indicate statistically significant changes in response to BABA treatment and combined stress (Student's *t*-test; $\alpha = 0.05$). ($p = 0.0272$).

Involved defense signaling pathways

In order to assess the effect of BABA treatment and saline stress on *B. cinerea* resistance at the transcriptomic level, we evaluated the expression of a chosen set of genes and transcription factors (TFs) known to be involved in the induction of salinity tolerance and/or *B. cinerea* resistance (Table 1; [56-59]. The expression of tomato defense genes was studied to determine the cross-talk between abiotic (low salinity (50 mM)) and biotic stress (*B. cinerea*) and also the defense pathways affecting both kinds of stressors (Figure 5). Control infected tomato plants at 8 hpi presented no significant differences between plants treated or not with BABA for all genes tested except *PR1*, which was slightly down-regulated. Interestingly, at the same time, BABA induces ABA-responsive element-binding proteins (*AREB*) and C-repeat binding factors 3 (*CBF3*) expressions after combination of salt stress (50mM) *B. cinerea*, contrary to *PR1* and *PR5* that were down-regulated. This suggests that at this initial phase of colonization, only TFs related to abiotic stress (*AREB* and *CBF3*) were activated. Twelve hours post infection the comparison between plants treated or not with BABA under single stress (infection with *B. cinerea*) showed an up-regulation in the expression of *CBF3* and *PR1*. This shows that BABA has a positive effect on the expression of *CBF3* and *PR1* in plants infected with *B. cinerea*. Surprisingly, *CBF3* was down-regulated after 12 hpi in plants treated with BABA and subjected to double stress. In addition, *PR1* and *PR5* were up-regulated at 24hpi in BABA-treated plants under combined stress. *PR1* and *PR5* expressions were induced in plants treated with BABA under combined stress and were expressed higher than under single.

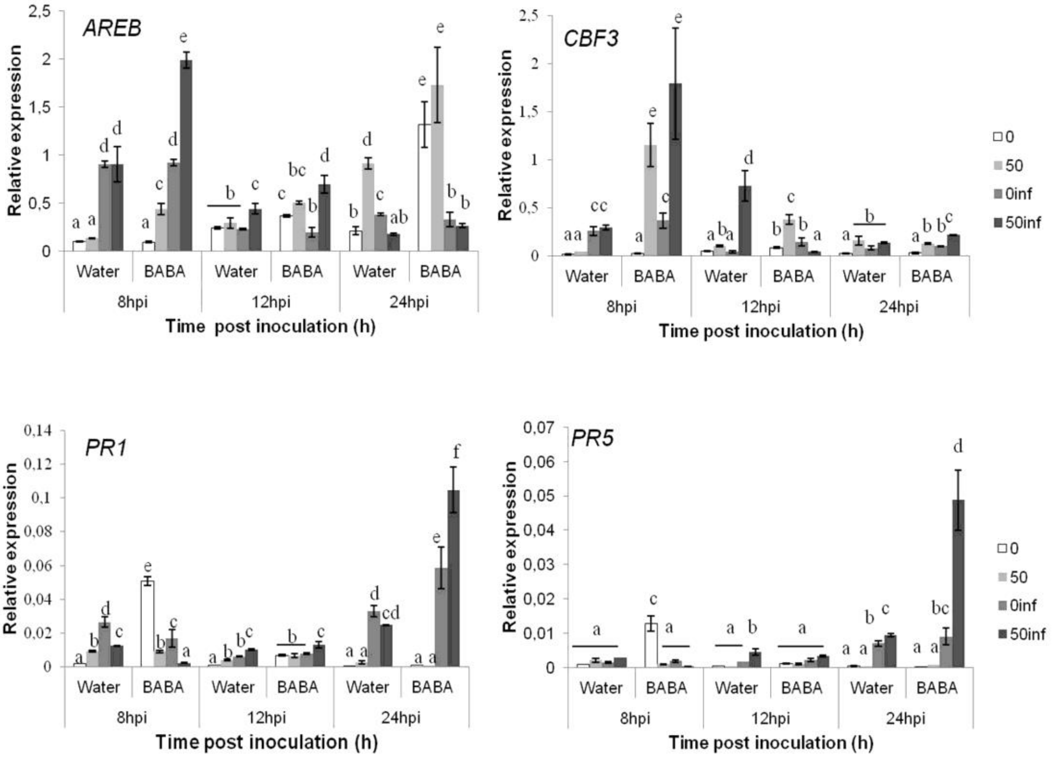

Fig. 5. Comparative analysis of defense gene and TF expression in tomato leaves (cv Marmande) stressed with or without 50mM of NaCl and infected with *B. cinerea* at 8, 12 and 24 hpi. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of AREB, CBF3, PR1, and PR5 in 4-week-old tomato plants pretreated with β-aminobutyric acid (BABA) 1 day before salt stress and 2 days before infection with Botrytis cinerea. RNA was isolated from leaves at 0, 8, 12 and 24 h after inoculation, converted to cDNA and the analyzed by qRT-PCR. Bars represent mean ± standard deviation (SD), *n*=3 independent replicates (Fisher's least significant differences test; α=0.05).

Impact of stress on H_2O_2 accumulation and positive effect of BABA treatment

A possible involvement of ROS in the response of plants subjected to combined abiotic and biotic stress was addressed. To this end, leaves of treated plants were subjected to DAB staining and H_2O_2 accumulation at the inoculation site was quantified digitally after 24 h of staining. Control and stressed leaves from tomato plants (cv Marmande) treated with BABA exhibited higher staining at the inoculation site than leaves from water controls (Figure 6). Further, this result was confirmed by quantification of H_2O_2 accumulation (Figure 6) where the accumulation of H_2O_2 was significantly higher in leaves of plants treated with BABA compared with non-treated plants. This confirms that BABA positively influences ROS accumulation *in situ*. In non-treated plants, a heavy staining was observed in the presence of salt stress. The

H_2O_2 levels were higher in stressed plants compared to the control. A combination of salt stress and inoculation with *B. cinerea* increased H_2O_2 accumulation compared to plants under single stress (*B. cinerea).* Surprisingly, there was no significant difference of H_2O_2 accumulation in BABA-treated plants under double or single stress ($p=0.0966$). In addition, H_2O_2 accumulation was significantly higher under combined stress in BABA-treated *sitiens* and its wild-type compared to non-treated plants Moreover, H_2O_2 levels were higher under combined stress in BABA-treated plants than under single stress and it was observed that this level was higher in the *sitiens* mutants compared to its wild-type and cv Marmande (Figure S5). These results may be connected to the effect of ABA in all defense responses in plants under single or double stress.

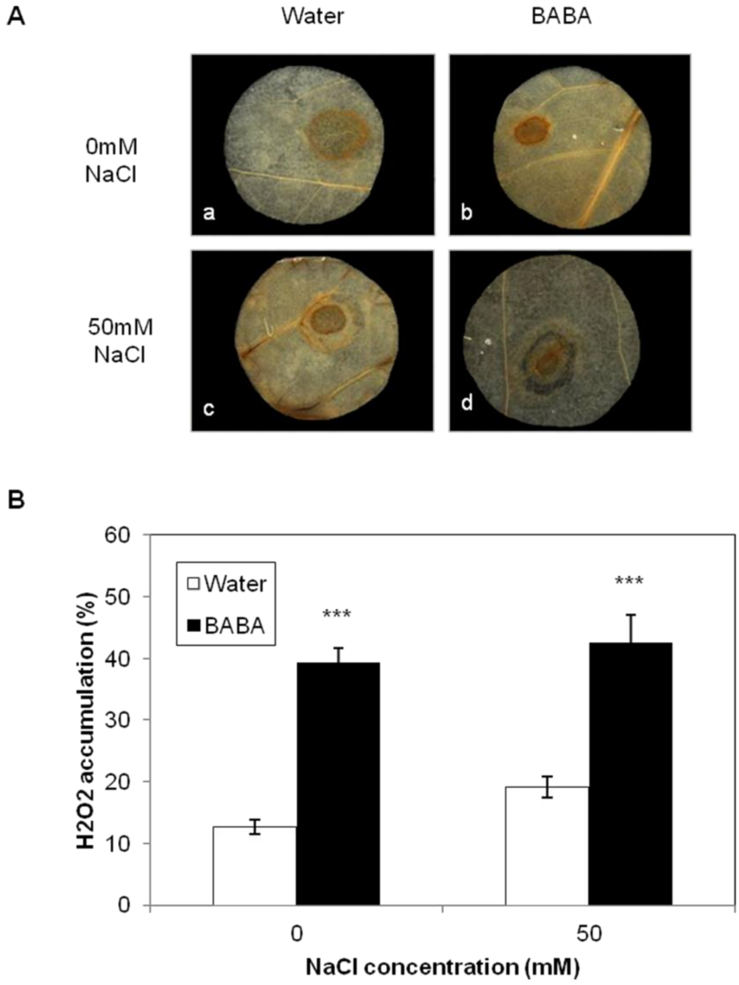

Fig. 6. Impact of stressed conditions on H_2O_2 accumulation in leaves to 30 day-old tomato plants (cv Marmande) treated with water or BABA (5 mM) 48h before exposing them to salt stress. The results are expressed as percentage of dark-brown spots coloration relative to the total leaf disc surface (± standard error of the mean; *n*

> 20) at 72 hours after infection. A) Photographs of stained leaf discs. Photographs show stained leaf discs with 3,3-diaminobenzidine (DAB) at 72 hours post inoculation (hpi) and analyzed by light microscopy. a) Nontreated control inoculated with *B. cinerea*; b) BABA-treated tomato plants and inoculated with *B. cinerea*; c) Nontreated stressed plants and inoculated with *B. cinerea*; d) BABA-treated tomato plants and subjected to a combination of salt and inoculated with *B. cinerea*. B) H_2O_2 accumulation in treated or non-treated tomato plants under combination of abiotic and biotic stress. Asterisks indicate statistically significant differences between salt stressed and water or BABA-treated plants (Student's *t*-test; $\alpha = 0.05$). The experiment was repeated twice with similar results. (***=p<0,001).

Lignin deposition around the infection sites

BABA-treated tomato plants stressed by salt and infected with *B. cinerea* were stained with phloroglucinol-HCl to visualize lignification in the tissues. A strong accumulation of lignin was observed in BABA-treated tomato plants in both control and stressed plants compared to non-treated plants (Figure 7). However, there was no difference in lignin accumulation in BABA-treated plants under single or double stress. This confirms that BABA helps to increase lignification and therefore induce resistance to biotic stress and combination of abiotic and biotic stress. Similar results were obtained with cv Rheinlands Ruhm tomato plants and the *sitiens* mutant where plants treated with BABA presented a more pronounced staining under single and combined stress compared to water-treated plants (Figure S6).

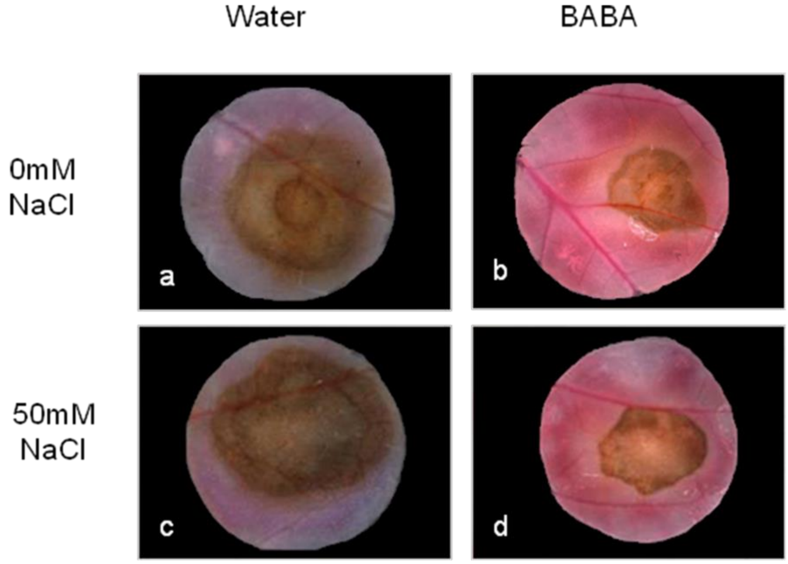

Fig. 7. Photographs of lignin deposition in leaf discs of tomato (cv Marmande) from the third and fourth true leaves treated with water or BABA followed by a combination of salt stress (50mM) and infection with *B. cinerea*. a) through d), leaf disks (0.5 cm in diameter) of tomato. a) Water controls inoculated with *B. cinerea*; b)

Water-treated, salt-stressed plants, inoculated with *B. cinerea*; c) BABA-treated plants inoculated with *B. cinerea*; d) BABA-treated plants subjected to salt stress and inoculated with *B. cinerea.*

Discussion

The goal of the study was to evaluate the effect of BABA treatment on the defensive capacity of tomato plants exposed to a combination of biotic and abiotic stress. For the biotic stress we used the grey mold pathogen *B. cinerea*, a necrotrophic fungus, and mild abiotic stress was achieved through soil drench with 50 mM NaCl solution. Our results shown that exposure to salt does not negatively interfere with BABA-induced induction of resistance against grey mold. Similar results have been reported in tomato plants treated with the resistance inducers Benzothiadiazole-S-methyl ester BTH and N-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide TDL, [60] to a combination of salt stress and infection with *Pseudomonas syringae* pv *tomato* [61]. These results contradict earlier findings of Yasuda et al. [62] that showed that exogenous ABA (simulating the effect of abiotic stress on plants) suppressed the induction of systemic acquired resistance (SAR) induced by 1,2-benzisothiazol-3 (2H)-one-1,1-dioxide BIT or BTH [63,60] in Arabidopsis plants infected with *Pseudomonas syringae* pv *tomato*. Interestingly, under mild salt stress (50mM) the average size of necrotic lesions was significantly larger than in water-treated control plants. These results are in agreement with some studies reporting a negative impact of abiotic stress on disease resistance [62,12,64]. Previous reports have shown that ABA, which is induced during abiotic stress, increases plant susceptibility to many fungi. Treatment with ABA reduced the resistance of potato tuber slices against *Phytophthora infestans* and *Cladosporium cucumerinum* [65]. Similarly, the increase of susceptibility was demonstrated in rice treated with ABA and infected with *Magnaporthe grisea* [66].

Contradictory to our observations, relatively mild salt-stress on tomato plants was shown to have no effect on *B. cinerea* [30]. Thaler and Bostock [67] likewise demonstrated that salt stress did not affect tomato plant resistance to *P. syringae*. In contrast to the above report, it was found that drought stress was responsible to delay powdery mildew disease development in *Alliaria petiolata* (garlic mustard), which could be due to stomatal closure that reduces the ability of the pathogen to penetrate into the leaf [67,68]. The high resistance of the BABA-treated ABA deficient *sitiens* mutant of tomato against *B. cinerea* compared to its wild-type and cv Marmande suggests that the lower concentration of ABA in *sitiens* is correlated to the increased resistance against the necrotrophic fungus.

The β-1,3-glucan polymer callose is considered a strong physical barrier against pathogen penetration [59,69]. It is deposited in the form of papillae at the sites of attempted fungal penetration. This defense mechanism of the plants is enhanced after priming [12]. Lignin is an important phenolic polymer that participates in the formation and rigidity of cell walls. The induction of lignin-like deposits by BABA has been shown to be correlated to enhanced resistance against fungi [70,71]. Our results show that the increase of callose deposition and lignin accumulation at the cellular level could contribute to preventing the infection of *B. cinerea* in BABA-treated plants. Both callose deposition and lignin accumulation were more pronounced in BABA-treated than in water-treated tomato plants. Moreover, lignin accumulation was strongly induced in ABA-deficient BABA-treated *sitiens* mutants. Callose deposition and lignin accumulation might restrict penetration and development of *B. cinerea* in BABA-treated plants, correlating with the increase of resistance in tomato plants against *B. cinerea*. In support of our data, Pastor et al. [69] demonstrated that treatment with BABA induced priming of callose and decreased disease symptoms caused by *Plectosphaerella cucumerina*. An increase of callose deposition in Arabidopsis plants treated with BABA before infection with *Alternaria brassicicola* or *P. cucumerina* has also been shown [47,72]. Our results are in line with the observation of Hamiduzzaman et al. [71] where callose deposition and lignification were more pronounced in BABA-treated than in water-treated grapevine. In addition, concomitant treatment with BABA and 50mM of salt lead to a higher accumulation of callose compared to water-treated plants and also compared to BABA-treated plants. This shows that 50mM of salt increased resistance against *B. cinerea* in BABA-treated plants, suggesting that plants exposed to the combination of abiotic and biotic stress reacts more strongly to BABA treatment than plants exposed to single stress. As reported in previous studies, the application of ABA has a similar effect as BABA on the increase of callose deposition and consequently can be implicated in the induction of plant resistance upon pathogen attack [47,73,27].

Callose deposition was however significantly affected by a combination of salt and infection with *B. cinerea* in water-treated tomato plants, suggesting that salt stress may have an additive effect on a plant's response to *B. cinerea*. In support of our data, Wiese et al. [74] found a positive role for ABA in plant-pathogen resistance, where ABA-treated barley primed for papillae-mediated resistance against powdery mildew. Similar results were found in tomato plants under a combination of salt stress and powdery mildew [64], where abiotic stress increased plant susceptibility to biotic stress. Contrary to our results, previous studies reported that treatment with ABA, a regulator of plant responses to abiotic stress, triggers callose-mediated resistance against necrotrophic pathogens [75,76,47].

Plants respond differently to multiple stresses compared to individual stress, and in this context, we studied the effect of gene expression levels in primed plants under a stress combination. Transcription factors like *AREB* and *CBF3* are major transcriptional activators that regulate ABA-dependent gene expression and increased abiotic stress tolerance [77-80]. Our transcriptional analysis of some marker genes involved in abiotic stress has shown an up-regulation of *AREB* and *CBF3* expression in BABA-primed plants at 8hpi and subjected to combined stress compared to non-primed plants. On the opposite, the expression of the genes involved in biotic stress (*PR1* and *PR5*) was down-regulated. Salt-stressed and infected plants did not favor biotic stress resistance which may have increased the defense response against abiotic stress, at this stage. At this early time point (8hpi), BABA-induced *AREB* and *CBF3* expression may contribute to enhanced resistance in coordination with reduced expression of *PR1* and *PR5* in plants under combined stress. In this context, it is worth mentioning that *AREB* and *CBF3* were both up-regulated, contributing to the plant induced-resistance against salt stress. Overexpression of *AREB1* was shown to induce drought tolerance in Arabidopsis, rice and soybean [57,81-83]. Plants including tomato, tobacco, and wheat overexpressing *DREB1*/*CBF*, under abiotic stress, showed an increase of stress-responsive gene expression in order to induce stress tolerance [84,85]. A similar result was found in transgenic plants where the overexpression of *DREB1*/*CBF3* led to increased tolerance to abiotic stress such as salinity [86,87]. Over-expression of abscisic acid-responsive element binding protein 1 identified in tomato (*SlAREB1*) transcripts in tomato and *CBF3* in transgenic oats conferred resistance to salt stress and is responsible to activate defense-related genes [88,89].

This is in line with *AREB1* and *CBF3* expression which showed up-regulation in response to drought stress in leaf and root tissues of five grape varieties, which are responsible to increase stress tolerance [90]. In BABA-treated plants, the pathogenesis-related genes *PR1* and *PR5* were up-regulated at 24hpi, especially under stress combination (Figure 5). This could be related to the onset of plant protection against *B. cinerea*. Similar results were found in tomato plants treated with BTH, where *PR1* was enhanced and contributed to plant protection against *B. cinerea* [31]. Surprisingly, *AREB* and *CBF3* were suppressed in BABA-treated plants under combined stress at 24hpi. This is probably due to BABA being responsible for a higher accumulation of ROS, and, as a consequence, better protection against *B. cinerea* [69]. Previous studies demonstrated that the PR proteins are induced not only following pathogen attack but also in response to abiotic stress conditions [91]. BABA-primed plants show an induction of the expression of salicylic acid-dependent (*PR1*, *PR5*) and ABA-dependent (rab-related gene 18 (*RAB18*), responsive to dessication 29 A (*RD29A*)) genes upon salt stress [48,45]. Several studies reported that endo-1,3-ß glucanase, which belongs to the PR protein family, contributed to plant defense against salinity [92] and drought [49,50]. The highest increased expression of *PR1* and *PR5* in 50 mM NaCl + *B. cinerea* in BABA-treated plants at 24hpi compared to single stress (infection with *B. cinerea*) could be related to salt stress. Recently, Chojak-Kozniewska et al. [93] showed that the up-regulation of *PR1* in cucumber plants exposed to stress combination resulted from SA-independent induction by salt stress. Other studies have demonstrated that the induction of *PR* genes upon abiotic stress could probably be the consequence of an establishment of a complex signaling crosstalk between abiotic and biotic stress responses [94].

Interestingly, BABA is able to induce stress combination resistance in tomato plants and the observed priming for the induction of *PR* expression is in agreement with the significantly smaller average area of necrotic lesions in leaves of plants pre-treated with BABA under stress combination compared to non-treated stressed plants. Recently, Thevenet et al. [95] found that BABA is a natural product of plants and endogenous levels of BABA increase directly after pathogen attack or abiotic stress. Moreover, the increase of endogenous BABA levels after infection depends on the plant’s immune system [96]. These findings can explain the importance of the effectiveness of BABA against a wide range of stresses including the combination of abiotic and biotic stress.

Increased ROS production is a common characteristic of cells exposed to a wide range of stresses. Plants have evolved the ability to take advantage of these molecules to defend themselves against abiotic and biotic stress. ROS have a dual beneficial role depending on the type of pathogen that attacks the plants. On the one hand, against biotrophic pathogens, they act as a key defense compound but on the other hand, ROS serve as the molecules helping the necrotrophic pathogen to exploit these responses [4]. In both cases, the accumulation of ROS can be effective in hindering pathogen proliferation. Here, we show that the high level of resistance to the necrotroph *B. cinerea* in tomato plants treated with BABA was partially due to an increase of H_2O_2 accumulation at the site of infection. ROS increasing directly after the pathogen attack acts as second messengers in order to induce cellular defense responses [97]. In addition, a reduced capacity to scavenge pathogen-inducible ROS contributes to reinforce and modify the cell wall, which is known to form the physical barriers against the progress of *B. cinerea* [4,98,99]. However, decreased resistance of salt-stressed tomato plants to *B. cinerea* may suggest that abiotic stresses increase susceptibility to necrotrophic pathogens in non-treated tomato plants. A contradictory result shows that there is a positive link between ABA and ROS to increase resistance against *B. cinerea* [100]. Others have shown that ABA treatment could induce the resistance of Arabidopsis against *B. cinerea* [29]. It can be concluded that the interaction of abiotic and biotic stress is complex, but BABA has the power to induce resistance against these stressors. Pastor et al. [69] demonstrated that BABA-IR against *P. cucumerina* in *Arabidopsis thaliana* was dependent on H_2O_2 accumulation. Mutants NADPH/respiratory burst oxidase protein D (*rbohD*) and phytoalexin-deficient 2 (*pad*2) (mutant in ROS generation or scavenging) are impaired in BABA-induced priming of H_2O_2 accumulation, and consequently decreased *P. cucumerina* susceptibility [69]. We demonstrate that in BABA-treated and stressed plants the accumulation of H_2O_2-dependent-defenses was tend to be higher compared to control treated plants, despite that there is no significant difference. We hypothesize that salt, which is known to increase ABA, and ROS accumulation are interconnected components to increase the capacity of BABA-induced resistance against *B. cinerea*. A similar result was presented by Ton and Mauch-Mani [47] where the induction of resistance by BABA in Arabidopsis against *A. brassicicola* and *P. cucumerina* was mediated by ABA. Thus, we can conclude that there is an establishment of a link between BABA, ABA and ROS to increase resistance against *B. cinerea*. In contrast, in BABA-treated tomato, the ABA-deficient *sitiens* mutant presented a higher level of resistance than the wild-type to *B. cinerea*, which coincided with a rapid accumulation of H_2O_2 under single or double stress (Figure S5). Furthermore, *sitiens* presented also a higher H_2O_2 concentration comparing to cv Marmande. Despite the number of studies examining the influence of ABA on the response of plants to pathogen attack, more research is needed to clarify these controversial results.

H_2O_2 accumulation was higher in water-treated *sitiens* mutants than its wild-type and cv Marmande. Similar results were found in the study of Asselbergh et al. [4] where H_2O_2 accumulation was higher in *sitiens* mutants than in wild-type plants ensuring a direct fungitoxic effect on the pathogen. In this case, H_2O_2 induced an oxidative burst and HR-like response in *sitiens* that may play an important role in defense signaling. Taken together, it is possible that ABA deficiency in the *sitiens* mutant represents an adequate defense against *B. cinerea*. Strikingly, H_2O_2 accumulation increased in *sitiens* mutant plants treated with BABA and under combined stress and H_2O_2 levels were higher than under single stress. Based on these results, we hypothesize that BABA and salt stress proceed together to increase H_2O_2 accumulation in the *sitiens* mutant.

The increase of H_2O_2 levels in plants under stress conditions may be harmful to the plants and affect the redox status and turn to oxidative stress [101]. However, in this case, the earlier accumulation of ROS always remained within adequate concentration and under what would be a toxic level for plant cell [102,69].

Conclusion

BABA is well known as a priming inducer boosting the defensive response capacity of a plant against stress, but as mentioned previously, there are limited studies about the role and the mode of action of BABA to induce combined stress tolerance in plants. Here, we conclude that BABA has the capacity to induce resistance in tomato plants under a combination of salt stress and infection with *B. cinerea*. However, a negative interaction was observed in water-treated tomato plants under combined stress. BABA primed for an increase in callose deposition, lignification, and H_2O_2

accumulation in order to improve plant performance under combined stress and especially to increase pathogen resistance. Up-regulation of *PR* gene expression in BABA-treated plants showed also the effectiveness of BABA to induce resistance not only against abiotic or biotic stress but also against a combination of both. There is a transition from early to later time points in the induction of genes. ABA-related genes seem to be more induced at the beginning (8hpi) and followed by a down-regulation while the SA-dependent *PR* genes behave in an opposite manner. Plant defenses are dynamic and change along the time of infection. The results reported in this study could be reproduced on other crops, at different growth stages and other combined challenges to better understand the impact of BABA on plant defensive response.

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Supporting informations

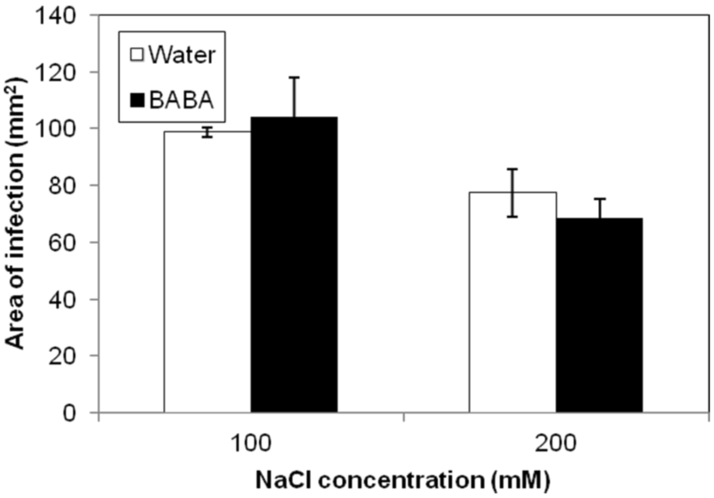


Fig. S1. Size of infection in tomato leaves (cv Marmande), treated or not with BABA and challenged with both abiotic (salt stress: 100 and 200mM) and biotic stress (*B. cinerea*) after 72h post-inoculation and grown under controlled conditions. Data shown are average values (±standard error of the mean; n > 6) of relative area. The experiment was repeated 2 times with similar results.

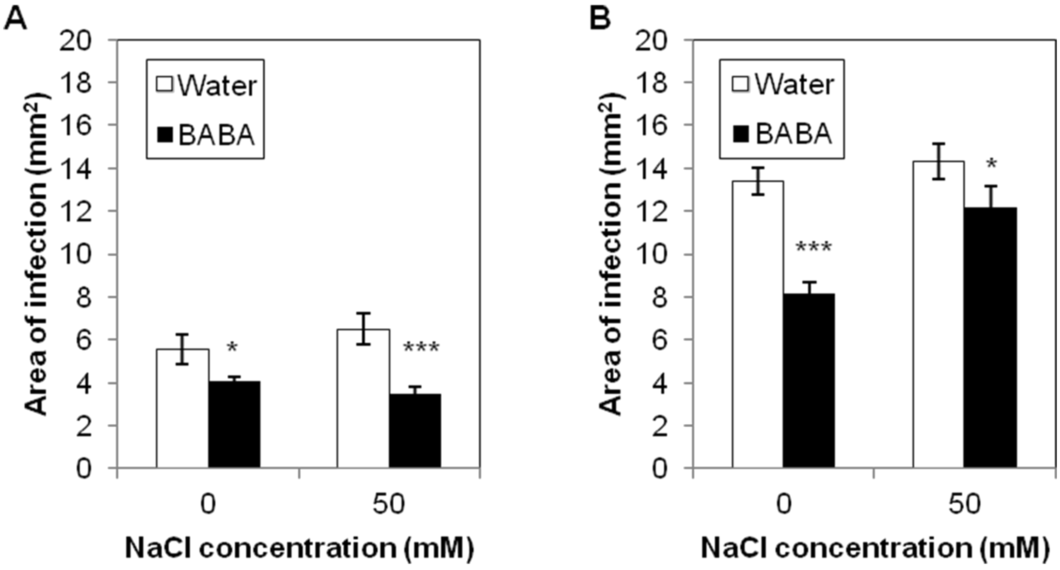


Fig. S2. Size of infection in tomato leaves, treated or not with BABA and challenged with both abiotic (salt stress: control and 50mM) and biotic stress (*B. cinerea*) after 72h post-inoculation under greenhouse conditions. A) Size of infection in *Sitiens* (ABA mutant) leaves. B) Size of infection in Rheinlands Rhum (Wild-type of *Sitiens*) leaves. Data shown are average values (±standard error of the mean; n > 6) of relative area. The asterisk indicates significant differences (Student's *t*-test, p<0.001) in the area of infection between plants treated with water and plants treated with BABA. The experiment was repeated 2 times with similar results. (* = p<0.05, ***=p<0,001).

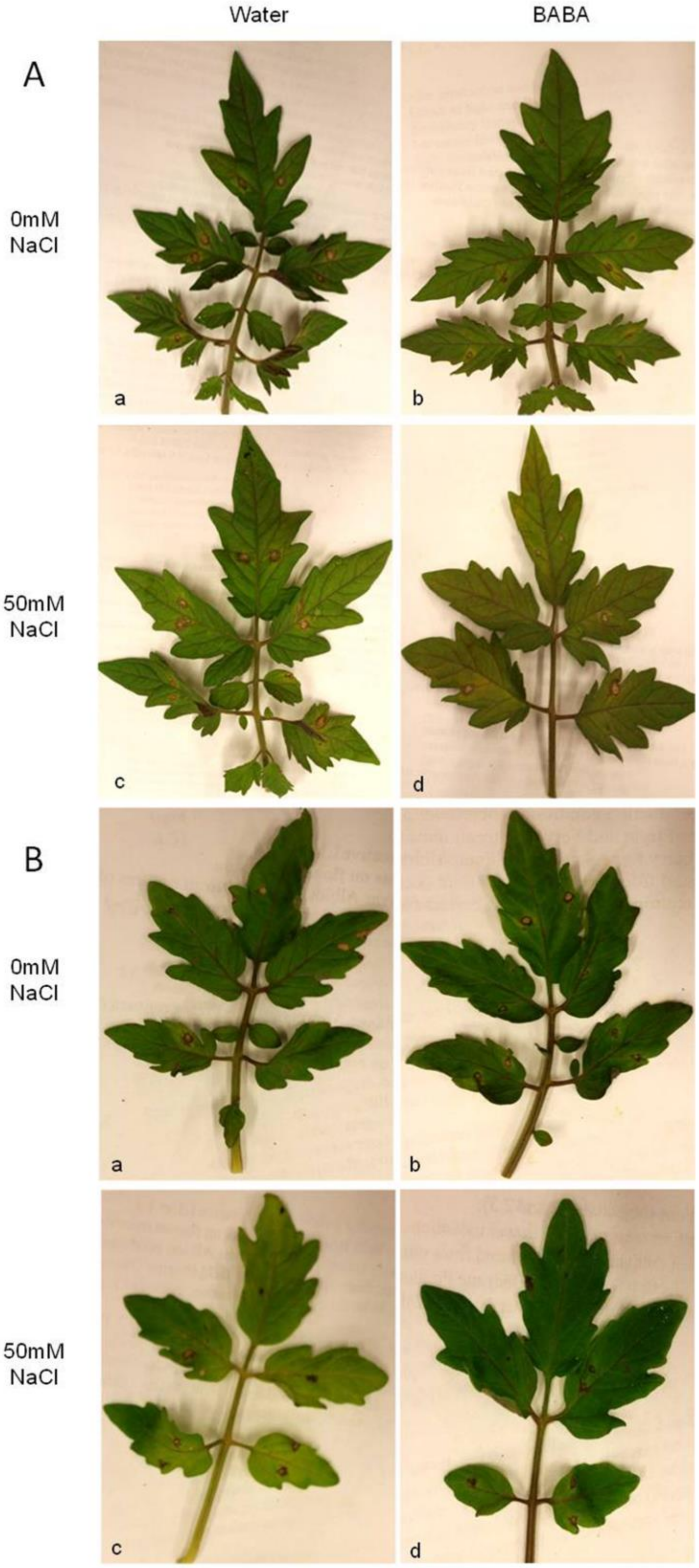


Fig. S3. Responses of tomato plant treated or nontreated with BABA to the combination of salt stress (50mM) and *B. cinerea* under greenhouse conditions. A) Leaves of Rheinlands Ruhm (Wild-type of *Sitiens*): Leaves treated or nontreated with BABA (representative of two replicate treatments) from control (a and b) and stressed with 50 mM of salt (c and d). B) Leaves of *sitiens*: Leaves treated or nontreated with

BABA (representative of two replicate treatments) from control (a and b) and stressed with 50 mM of salt (c and d). Symptoms photographed at 72h post-inoculation.

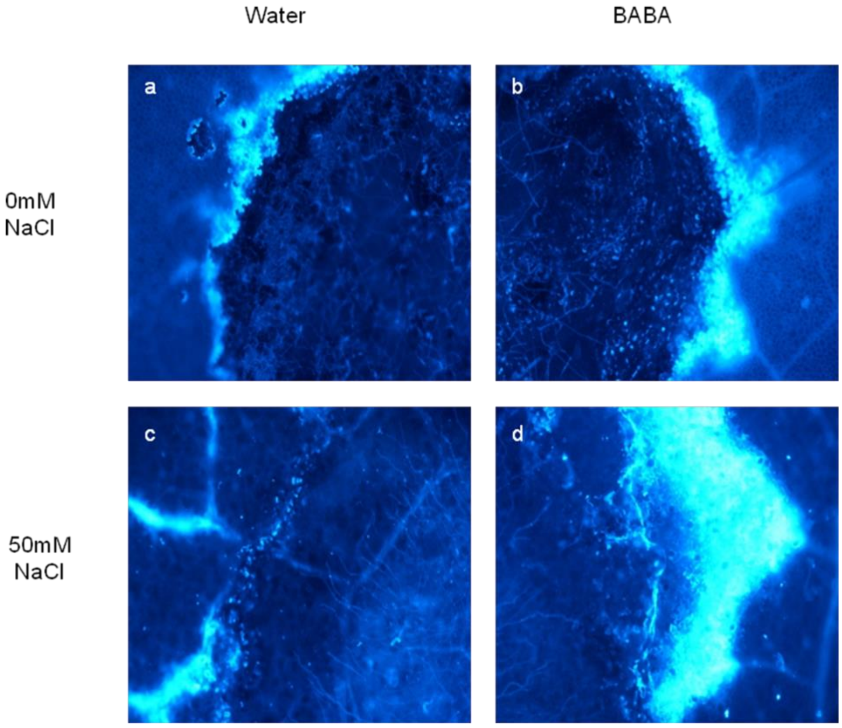
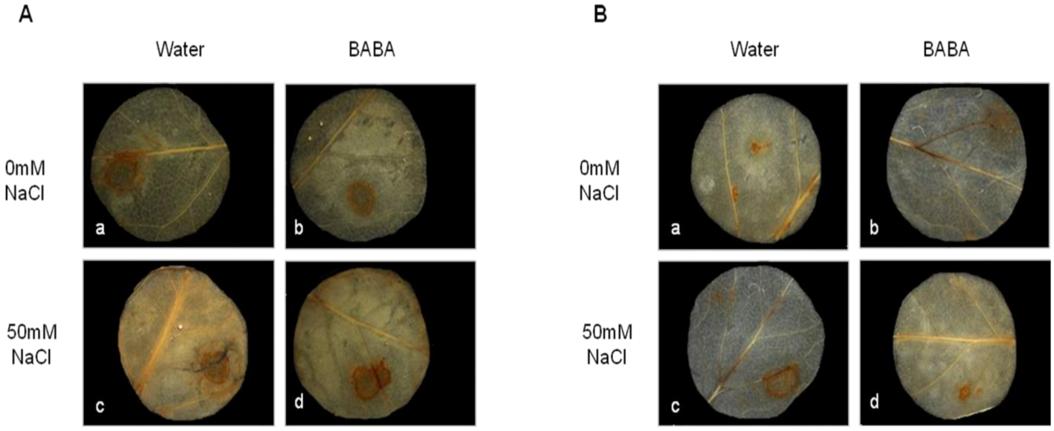


Fig. S4. Localization of callose at 72 hpi by *Botrytis cinerea* in tomato leaves treated with BABA and stressed with salt (50mM). Photographs show stained leaves (Aniline-blue) exposed to UV light. a) Control tomato plants and inoculated with *B. cinerea* b) BABA-treated tomato plants and inoculated with *B. cinerea*. c) stressed tomato plants with 50mM of salt and inoculated with *B. cinerea*. d) BABA-treated tomato plants and subjected to a combination of salt and inoculation with *B. cinerea*.



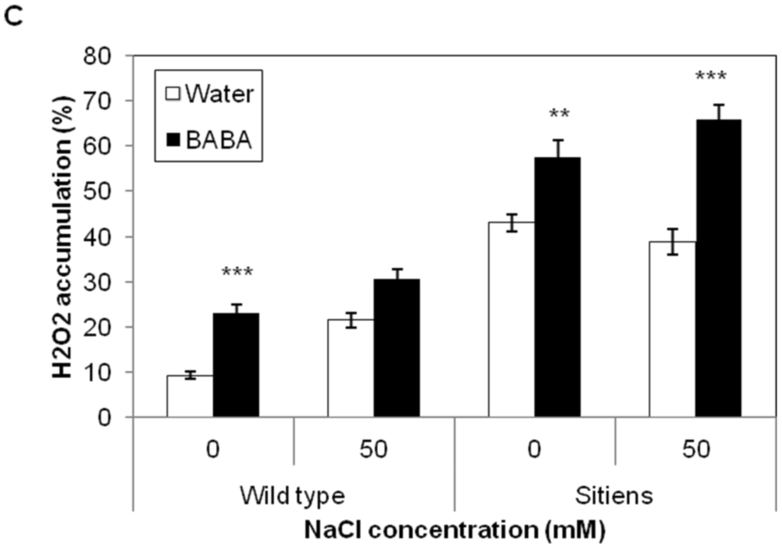


Fig. S5. Impact of stressed conditions on H_2O_2 accumulation in leaves to 30-day-old tomato plants (*Sitiens* and Rheinlands Ruhm (Wild type of *sitiens*)) treated and nontreated with BABA (5 mM) at 48h before establishing of salt stress. Third and fourth leaves of each plant were detached after 72 hours of infection by *B. cinerea* and staining with DAB. The result indicated the percentage value of dark-brown spots coloration relative to the total leaf disc surface (± standard error of the mean; $n > 20$) at 72 hours after infection. DAB accumulations were quantified as the number of dark-brown spots-corresponding pixels relative to the total number of pixels covering plant material. A) Photographs of stained wild type leaves. B) Photographs of stained *sitiens* leaves. C) H_2O_2 accumulation in *sitiens* and Rheinlands Ruhm leaves under combination of abiotic and biotic stress. Photographs show stained leaf discs with 3,3-diaminobenzidine (DAB) at 72 hours post inoculation (hpi) and analyzed by light microscopy. a) Nontreated control inoculated with *B. cinerea*; b) BABA-treated tomato plants and inoculated with *B. cinerea*; c) Nontreated stressed plants and inoculated with *B. cinerea*; d) BABA-treated tomato plants and subjected to a combination of salt and inoculated with *B. cinerea*. Asterisks indicate statistically significant differences between stressed and treated or nontreated plants with BABA (Student's *t*-test; $\alpha = 0.05$). The experiment was repeated twice with similar results. (** = $p<0.01$, ***=$p<0,001$).

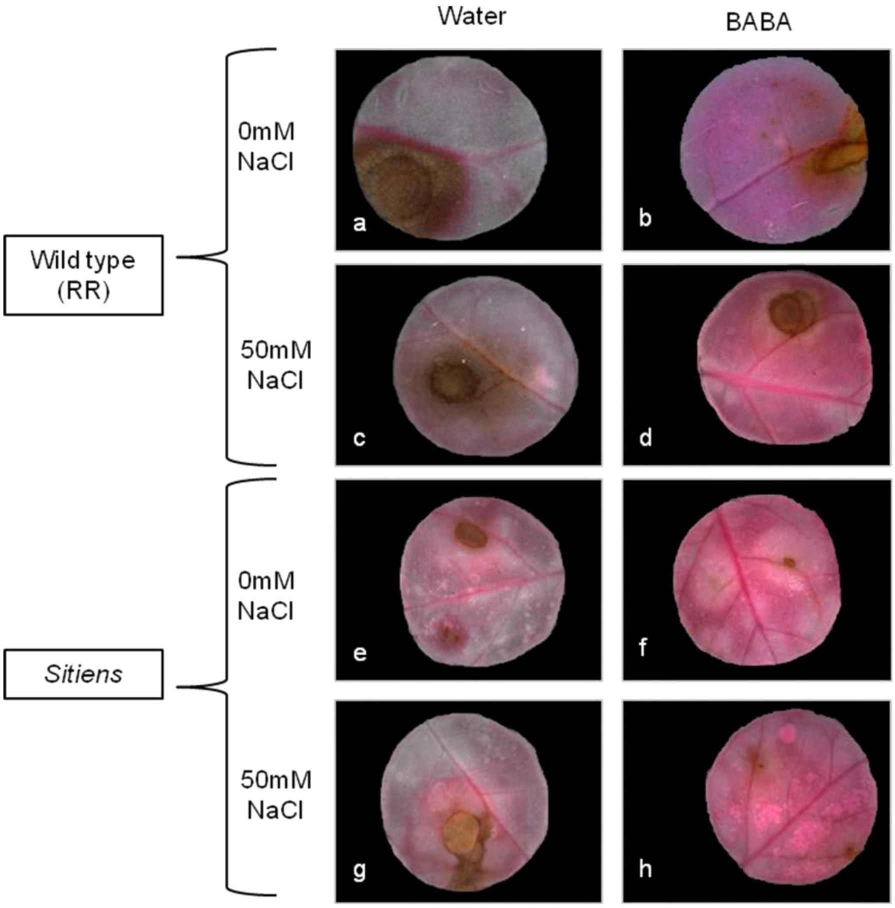


Fig. S6. Photographs of lignin deposition on *Sitiens* and Rheinlands Rhum (wild-type of *Sitiens*) leaf discs from the third and fourth true leaves treated or nontreated with BABA following combination of salt stress (50mM) and inoculation with *B. cinerea*. a) through h) Leaf disks (0.5 cm in diameter) of tomato. Plants of 30-days-old were treated or not with BABA then, after 48 hours, plants were stressed with salt and 24 hours later inoculated with *B. cinerea*. Lignified cells were stained pink with phloroglucinol-HCl and analyzed by light microscopy at 72 hours post inoculation (hpi). a) and e) Nontreated control inoculated with *B. cinerea*; c) and g) Nontreated stressed plants and inoculated with *B. cinerea*; b) and f) BABA-treated tomato plants and inoculated with *B. cinerea*; d) and h) BABA-treated tomato plants and subjected to a combination of salt and inoculated with *B. cinerea.*

Chapter IV

Effect of stress combination on hormone balance in primed tomato plants

Effect of stress combination on hormone balance in Primed tomato plants

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Abstract

In their environment, plants are constantly exposed to a combination of abiotic and biotic stress which limits growth and crop yields. Only recently researchers have begun to focus their research in this field and have started understanding the physiological and molecular responses of plants to different stress interactions. Plant responses to the combination of abiotic and biotic stress are suggested to be completely different than individual stress and have provoked complex responses controlled by different signaling pathways. The interaction of plant signaling pathways in primed plants under combined stresses is still poorly understood. To assess this, we studied the concomitant effect of BABA and salt on inducing resistance in tomato plants to *Botrytis cinerea* and their effects on hormonal profiles. The results showed that treated tomato plants with BABA presented higher levels of ABA, SA, and JA-Ile in response to combined salt and *B. cinerea* infection. The interaction between ABA, SA, and JA-Ile signaling may be important in optimizing the response in treated and non-treated plants with BABA under combined stress, making plants more resistant to such stress.

Key words: Tomato, stress combination, BABA, abscisic acid, salicylic acid, jasmonic acid-isoleucine, salt stress, *Botrytis cinerea*.

Introduction

Plants are often confronted with a multitude of environmental constraints that limit their growth, development, and productivity. These sessile organisms are not only confronted with individual abiotic or biotic stress but; unfortunately, plants are often exposed to a simultaneous combination of stresses. Many studies have suggested that under combined stress plants exhibit adequate and shared physiological and molecular responses to increase stress tolerance which could be completely different from individual stress (Atkinson and Urwin, 2012; Rasmussen et al., 2013). As the frequency and extent of salt and pathogen spread are projected to increase due to climate change (Garrett et al., 2006; Munns and Tester, 2008; Millar and Bennett, 2016), it is essential to understand how plants respond and improve resistance to multiple stresses. Therefore, the risk of plants encountering both abiotic and biotic stress at the same time in the future will definitely increase and will become frequent in the environment. Under stressed conditions, plants have developed various physiological and biochemical responses in order to acquire stress resistance and thus have developed adaptive defense responses to multiple stresses. Some molecular functions act in an antagonistic manner and some responses prevail in time over others under multiple stresses (Glazebrook, 2005; Yasuda et al., 2008). Plant physiological, biochemical and molecular responses to abiotic and biotic stress are mediated by phytohormone networks, which are generally defined as "chemical regulators". Furthermore, hormonal interactions have been studied, under combined stress conditions, to assist in the explanation of direct and indirect plant defense changes in plants (Kissoudis et al., 2014). Among the plant hormone groups, abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) are classified as hormone-like regulators of plant development and defense against abiotic and biotic stress (Bari and Jones, 2009).

ABA is an isoprenoid phytohormone which is the primary regulator of several abiotic stress responses (Zhu, 2002; Wasilewska et al., 2008). In addition, ABA plays a crucial role in the plant's defense against biotic stress, but it is a highly multifaceted defense response which depends on the type of attacker and the specific stage of defense (Mauch-Mani and Mauch, 2005; Ton et al., 2009; Ramegowda and Senthil-Kumar, 2015). Generally, ABA acts as either a negative or positive chemical regulator of plant defense responses to different pathogens. The consensus is that ABA is responsible for negatively regulating the plant's defense responses against both biotrophic and necrotrophic pathogens through the suppression of SA and JA/ET biosynthesis and signaling (Achuo et al., 2006; Yasuda et al., 2008; Sanchez-Vallet et al., 2012). ABA-deficient mutations show the sensitization of these signaling pathways and increased resistance to different pathogens (Cao et al., 2011). Conversely, exogenous ABA applications increase disease development (de Torres-Zabala et al., 2007). However, ABA can also positively contribute to regulating defense responses against some necrotrophic pathogens (Adie et al., 2007). ABA positively mediates resistance through the closure of stomata, which is the entry point for attackers, such as *Pseudomonas syringae* (Melotto et al., 2006). Moreover, ABA-induced resistance is based upon callose accumulation ensuring cell wall reinforcement against pathogens penetration (Ton and Mauch-Mani, 2004; Adie et al., 2007; Garcia-Andrade et al., 2011). It is known that ABA acts antagonistically with SA and JA signaling pathways under pathogen attacks. However, recently, researchers suggest that ABA acts both synergistically and antagonistically with pathogen signaling, which can create a complex network of interaction with special cross-talk at different levels (Fujita et al., 2006; Yasuda et al., 2008).

SA is a phenolic compound synthesized via the phenylpropanoid pathway in the plant (Metraux, 2002). SA levels increased upon pathogen attacks and are essential in both local and systemic resistance reactions (Murphy and Carr, 2002). In addition, SA acts as a mediator of systemic acquired resistance (SAR) (Rasmussen et al., 1991). The increase of SA is a greater inducer of defense against pathogens by minimizing the spread and the severity of infections (Vlot et al., 2009; Pye et al., 2013). The expression of pathogenesis-related (PR) genes is induced upon the increase of SA levels in infected plants, which is responsible for protecting these plants against the spread of the pathogen (Cao et al., 1997; Barriuso et al., 2008). However, SA and ABA act antagonistically in plants response to biotic stress (Mohr and Cahill, 2007; Jiang et al., 2010). Interestingly, the *ahg2-1* mutant in Arabidopsis accumulates high endogenous ABA and SA, which indicates that there is a complex cross-talk between ABA and SA in this double mutant; thus, ABA and SA do not always act antagonistically (Nishimura et al., 2009). In addition, SA signaling, induced by biotic stress, can mitigate ABA signaling, which is responsible for plant adaptation to abiotic stress (Kim et al., 2011). In addition, SA was demonstrated to induce tolerance to abiotic stress (Khan et al., 2015; Yuan and Lin, 2008). SA was shown to induce salinity tolerance of *Torreya grandis* through the increase of chlorophyll content and the activity of antioxidant enzymes that eventually lightened oxidative stress (Li et al., 2014). Furthermore, it has been demonstrated that SA improves plant tolerance to major abiotic stresses such as drought (Fayez and Bazaid, 2014), osmotic (Alavi et al., 2014) and heat stress (Khan et al., 2013). Exogenous application of SA makes many crop plants more tolerant to abiotic stresses (Horváth et al., 2002).

Moreover, JA and its methyl ester, methyl jasmonate (MeJA), are natural regulators of plant development and responses to external constraints (Creelman and Mullet, 1995).

JA signaling is activated in different plant species in response to herbivore attack and it has played an important role in regulating the plant's defense responses against necrotrophic pathogens (Pieterse et al., 2009). In addition, JA plays a crucial role during induced systemic resistance (ISR) (Pieterse et al., 2009). Intriguingly, JA is known to be a negative regulator of SA-dependent defenses (Bari and Jones, 2009). Importantly, JA-Ile has been identified as an activator of the majority of JA-induced responses (Staswick et al., 2002; Wang et al., 2008).

To ensure the success of plant resistance to abiotic and biotic stress, it is necessary to induce plant resistance by the non-protein amino acid β-aminobutyric acid (BABA), which is known to be a potent inducer of resistance against pathogen infection and abiotic stress (Jakab et al., 2001; Jakab et al., 2005). BABA exerted its function, in some cases, by potentiating SA-dependent defense mechanisms (Zimmerli et al., 2000). In addition, there have been other cases wherein BABA acts through priming of ABA-dependent signaling pathways (Ton and Mauch-Mani, 2004). As mentioned earlier, both pathways could contribute to abiotic stress tolerance. In this study, it was determined that BABA is able to increase ABA, SA and JA-Ile levels to protect tomato against a combination of salt stress and *B. cinerea* infection.

Material and methods

1. Culture conditions

Seeds of tomato cv Marmande were obtained from Quedlinburger (Aschersleben, Germany), were rinsed in 70% ethanol, incubated for 15 minutes in 2% bleach for sterilization and washed 4 to 5 times with sterile distilled water. After 24 hours at 4°C, the sterilized seeds were pre-germinated for 6 days in a Petri dish with a humid sterile Whatman filter paper and placed in the dark. Tomato seedlings were then transferred to multi-cell growing trays filled with a soil mixture (Compost (25%), sand (12%) and peat (63%), Fafard, Canada) and were kept in the greenhouse (Plant Research Facility Greenhouse, McGill University, Canada) under the following conditions: 16 hours a day at 26°C, 8 hours a night at 18°C, with 60% relative humidity and the light intensity of 245 μmol m^{-2} s^{-1}. Ten days later, seedlings were individually transferred to small plastic pots (Volume=100 mL) filled with the same soil mixture. All the plants were well watered and kept under the same conditions until they reached the stage of four fully expanded true leaves (from the tip, excluding petiole). At this stage, plants of uniform size were used for the experiments.

2. BABA treatment

BABA (Sigma-Aldrich, Canada) (5mM final concentration in the soil) (Oka et al., 1999) was applied as a soil drench to 4-week-old plants. Control plants were watered with tap water.

3. Abiotic and biotic stress application

The abiotic stress used in this experiment was salt stress (50mM of NaCl) and the necrotrophic fungus was *B. cinerea* and was isolated from an infected tomato leaf (Laval University, Quebec, Canada), control plants were well watered with tap water. Tomato plants were subjected to different pretreatments with BABA and then salt stress after 48 hours. The inoculation with *B. cinerea* was performed after 24 hours of salt stress.

4. Inoculations

Botrytis cinerea was routinely cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, USA) at 24°C. Conidia were harvested from sporulating colonies (15-day-old cultures) with 5 mL of sterile water. The conidia were filtered through gauze, washed by centrifugation (10 min, 3700 rpm, 20°C) and centrifuged another time (4 min, 11000 rpm).

The pellet was re-suspended in 1 ml of sterile water. The final conidia suspension was in 1 mL of PDB1/2 strength (Potato Dextrose Broth) (Difco Laboratories, Detroit, USA) and 10 µL of sucrose (10mM as a final concentration) at a density of 10^6 conidia mL^{-1}. The suspension was pre-incubated without shaking for 2 hours. Six µL droplets of the suspension were placed on the third and the fourth true leaves. All plants were covered with transparent plastic bags to keep 100% of relative humidity.

4. Hormone quantification

The third and the fourth leaves of tomato plants were harvested after 0, 8, 12 and 24 hours post inoculation (hpi) and were frozen immediately in liquid nitrogen. For each biological sample, leaves from three plants were pooled. Leaf tissue was finely ground and was freeze-dried for hormone quantification. ABA, SA, JA, and JA-Ile were quantified simultaneously in single samples using an optimized ultrahigh pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) modified method (Glauser et al., 2012). Essentially, hormones from 4 mg dry weight were extracted in EtOAc: Ethyl acetate: formic acid, 99.5:0.5 (v/v). Before extraction, an internal standard solution containing isotopically labeled ABA, SA, and JA-Ile (20 ng/mL) were added to the samples. The extracts were evaporated to dryness and re-suspended in 200 μL of aqueous methanol (70%). After centrifugation, 5 μL of this solution was injected in UHPLC-MS/MS. The hormones were quantified by calculating a calibration equation obtained by linear regression from 5 calibration points for each analysis. Peak areas of the hormones measured in the samples were normalized to the internal standard before applying the calibration equation. Three biological replicates were collected per time point

5. Statistical analysis

The significance of differences between control and treated plants of the phenotypic part was analyzed with a two-ways ANOVA; control and stressed plants ($P<0.05$) were then compared using the Tukey's range and *t*-test. This comparison allowed us to see the significant differences between two sample groups. All statistical analyses were accomplished using JMP 11.0.

Results

Study of the Hormonal Pattern in Tomato Plants

To understand the effect of BABA to increase the resistance level in tomato plants under a combination of abiotic and biotic stresses; we analyzed the accumulation of stress-related hormones (ABA, SA, and JA-Ile) in tomato plants under a combination of salt stress and *B. cinerea*. Phytohormones were quantified by UHPLC-MS/MS (ultra-high-pressure liquid chromatography-tandem mass spectrometry) at 0, 8, 12 and 24 hpi (Figure 1). Our analyses showed that BABA-treated plants have significant effects on hormone concentrations compared to non-treated plants. Significant increases of ABA levels were shown in BABA-treated plants compared to non-treated plants at 0, 12 and 24 hpi in stressed and non-stressed plants. However, no significant difference was detected at 8hpi between treated and non-treated plants under combined stress. In addition, ABA levels were higher in treated and non-treated plants at 0 hpi compared to the ABA levels at 24 hpi (Figure 1 a). This might be due to the beginning of an osmotic adjustment after 48 hours of the experiment. Plants subjected to a combined stress showed a statistical increase of SA levels in treated plants compared to those non-treated with BABA at 0, 8 and 24 hpi. However, no significant difference was detected at 12hpi. In addition, SA levels were higher in treated and non-infected plants compared to the control plants (Figure 1b). Interestingly, SA levels increased with time which could be due to the effect of combined stress. JA-Ile levels were higher in treated plants under combined stress at 24hpi compared to non-treated plants. However, no significant difference of JA-Ile level was detected in treated tomato plants under single stress (noninfected plants) compared to non-treated plants except for stressed plants at 0 and 12 hpi (Figure 1c). Accordingly, BABA strongly induced an increase in the accumulation of ABA, SA and JA-Ile compared to the non-treated plants under a combination of abiotic and biotic stress and also under single stress, suggesting an involvement of these hormones in the induction of plant resistance. At 24 hpi, our analyses showed that *Botrytis* had significant effects on the accumulation of ABA, SA, and JA-Ile. Whereas, concomitant treatment with BABA and 50mM of NaCl lead to a higher accumulation of SA and JA-Ile under combined stress conditions, which may have induced a higher protection against *B. cinerea*.

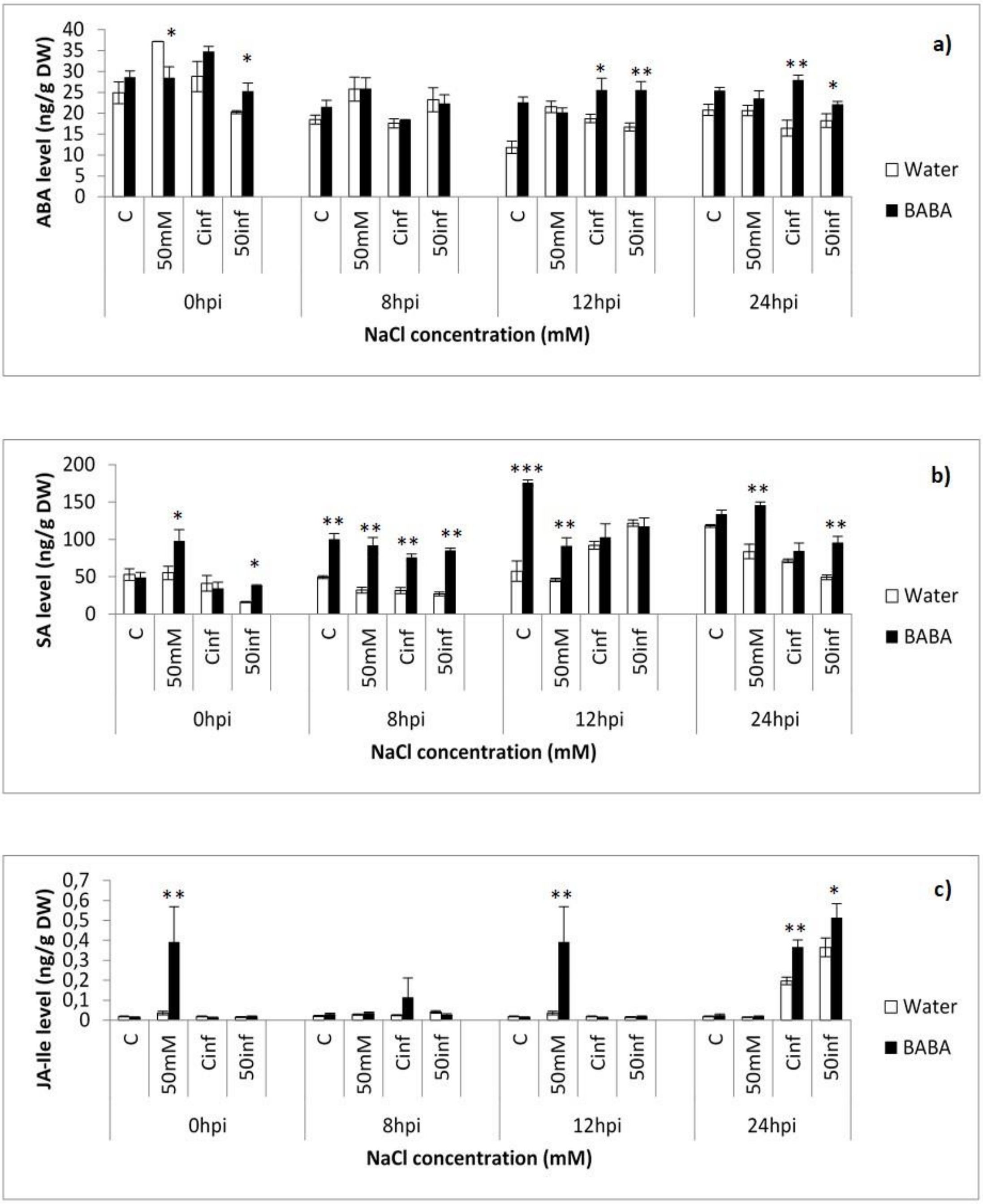

Fig. 1. Quantification of hormone levels in treated and non-treated tomato plants with BABA under a combination of salt stress and inoculation with *Botrytis cinerea*. Leaves were collected for hormone analysis at 0, 8, 12 and 24. Abscisic acid (ABA), salicylic acid (SA) and jasmonoyl-L-isoleucine (JA-Ile) levels were determined in the lyophilized material by high-performance liquid chromatography-mass spectrometry. (a) Quantification of ABA in treated and non-treated plants with BABA upon salt stress and inoculation with *Botrytis cinerea*. (b) Quantification of SA in treated and non-treated plants with BABA upon salt stress and inoculation with *Botrytis cinerea.* (c) Quantification of JA-Ile in treated and non-treated plants with BABA upon salt stress and inoculation with *Botrytis cinerea.* Plants were compared using Tukey's

range test; asterisks indicate statistically significant differences (n= 3, $P < 0.05$). The experiment was performed one time. (* = $p<0.05$, ** = $p<0.01$, ***=$p<0,001$).

Discussion

The interaction of multiple signaling cascades is of utmost importance in regulating the response of plants during stressed conditions such as the combination of abiotic and biotic stress. However, the success of a plant's response to resist simultaneous salt stress and pathogen infection largely depends upon the speed and intensity by which they can be activated and control their inducible defenses. It is well known that BABA presents a beneficial resistance strategy in plants under abiotic and biotic stress (Jakab et al., 2005; Ton et al., 2005, Cohen et al., 2016). The goal of this study was to evaluate the role of BABA treatment on the pattern of phytohormonal adaptation during the combination of salt stress and *B. cinerea* infection. We have reported that Marmande cv. exhibits significant increases in ABA, SA and JA-Ile levels following treatment with BABA compared to non-treated plants. In addition, at 24 hpi, the hormones were all accumulating at higher levels in treated plants under combined stress. This fits the attenuated and symptomless disease progress observed for *B. cinerea* (previous study), proving that BABA really contributes to a more effective defense under combined stress. Similar results showed that BABA exerted its function by inducing resistance via priming of ABA- and/or SA-dependent response mechanisms against abiotic and/or biotic stress (Zimmerli et al., 2000; Ton and Mauch-Mani, 2004; Jakab et al., 2005; Ton et al., 2009).

Early exposure of plants to salt stress results in increased ABA levels in BABA-treated and non-treated plants which also played a crucial role in reducing and suppressing the *B. cinerea* effect. Similar results were found in maize, Arabidopsis and tomato (Jia et al., 2002; Mauch-Mani and Mauch, 2005; Achuo et al., 2006; Zorb et al., 2013). The increase of resistance observed in this study may be due to reduced pathogen spread achieved by ABA-mediated callose biosynthesis. Similar results were observed when the inhibition and the degradation of pathogens were the results of callose accumulation (Anderson et al., 2004; Jacobs et al., 2003). A previous study suggested that the increase of callose accumulation was mediated by ABA in BABA-treated plants (Ton and Mauch-Mani, 2004). Flors et al. (2008) demonstrated that ABA levels increased the colonization by *Alternaria brassicicola* in treated plants with BABA and priming for callose deposition. Contradictory to our observations, Vargas et al. (2012) showed an increased susceptibility of plants treated with ABA upon pathogen infection. ABA deficiency in the *sitiens* mutant of tomato was more resistant to *B. cinerea* than wild-type, while exogenous ABA increased susceptibility (Asselbergh et al., 2007). The explanation for the difference between our results and the results of Vargas and colleagues and Asselbergh and colleagues may be due to the changing role of ABA during disease progression depending on the challenging pathogen (Ton et al., 2009).

In response to a necrotrophic pathogen, at 24hpi, the plant hormones SA and JA-Ile increased in treated and non-treated plants contributing positively to the plant's resistance. Similar results were found in plant response to *B. cinerea* infection through the increase of SA, JA and ethylene (ET) (Zimmerli et al., 2001; Ferrari et al., 2003; Vicedo et al., 2009). JA-Ile was repressed at all-time points and under single (salt stress) and double stresses, except under combined stress at 24hpi. This result can be explained by the positive effect of JA-Ile on preventing disease progression. Contradictory to our results, Scalschi et al. (2013) demonstrated that in primed tomato plants by hexanoic acid, SA increased upon being challenged with *Pseudomonas syringae*; however, JA-Ile was repressed.

In order to understand the complex responses of plants against two simultaneous stresses, we made a model reflecting three phases of primed plants under combined stresses. In the first phase, the increase of ABA levels at 0 hpi was the result of plant defense response to abiotic stress which also stimulated host resistance. The second phase, or intermediate phase (which was the post-invasion defense response) showed a deposition of callose to strengthen cell walls against *B. cinerea*. In the third phase, when the pathogen had penetrated the host tissue, the induction of SA and JA-Ile levels were responsible for regulating a broad spectrum of defensive compounds acting against the spread of *B. cinerea* and ABA might have interfered with reactive oxygen species (ROS) production resulting in increased pathogen susceptibility. Interestingly, ABA was connected to the SA and JA-Ile signaling pathways and, in this case, acted synergistically with these phytohormones at 24hpi and under combined stresses. A similar result was found in ABA hypersensitive germination2-1 (ahg2-1) mutant in Arabidopsis where the increase of gene-related defense was related to both ABA and SA accumulation, which indicated that ABA and SA acted synergistically (Nishimura et al., 2009). Therefore, ABA accumulation may have an important role in plant response to multiple stresses. According to Ton et al. (2009), in addition to the positive effect of ABA in BABA-treated plants upon salt stress, ABA was also responsible also for increasing the plant's defensive capacity against fungus without SA and JA-dependent defense activations.

SA levels were high at all-time points especially in plants treated with BABA, which may have resulted in the positive role of this hormone in plants resistance not only against biotic stress but also against abiotic stress. It has been demonstrated that SA is responsible for improving plant tolerance against abiotic stress through the regulation of several plant physiological processes like antioxidant defense systems (Besseau et al., 2012; Khan et al., 2015; Nazar et al., 2015). Moreover, SA has been shown to induce salinity stress tolerance-mechanisms (Palma et al., 2013; Nazar et al., 2015; Szepesi et al., 2009). Surprisingly, JA-Ile and SA levels were higher in treated plants with BABA under combined stress compared to plants under single stress (infection with *B. cinerea*), suggesting that salt stress increased plants' protection against *B. cinerea* in treated plants with BABA. It is known that ABA can suppress SA and JA signaling involved in enhanced resistance to a necrotrophic pathogen (Anderson et al., 2004; Lorenzo et al., 2004, Mauch-Mani and Mauch, 2005; Mohr and Cahill, 2007). However, Anderson and colleagues (2004) demonstrated that the exogenous application of methyl-JA and ET cannot restore the defense gene mediated by exogenous ABA. These results have suggested that under simultaneous salt and necrotrophic pathogen attacks, the interaction between the ABA, SA and JA-Ile signaling pathways play crucial roles in the crosstalk between biotic and abiotic stress signaling, thereby allowing plants the ability to regulate both stresses. In addition; our data suggested that ABA was a dominant process in treated plants with BABA for the avoidance of combined stress effects on plants.

Unfortunately, we were unable to analyze the repetition of this experiment to confirm our results. The reason for this was that we had a lot of problems with the UHPLC-MS/MS instrument. Our samples gradually contaminated the machine and the risk that major damage could happen to it coupled with our observations of the peak distortion that occurred during the batches forced us to dilute our samples, but we continued to have our samples contaminated.

Dr. Gaëtan Glauser (Senior Research Scientist, Department of Chemistry, Neuchâtel) hypothesized that there was a high concentration of metabolites in these samples due to salt stress that may responsible for the gradual contamination of the machine.

Dr. Victor Flors (Professor, Department of Agricultural and Environmental Sciences at Universitat Jaume I, Spain) hypothesized that the reason for this contamination may have been due to missing a step in our protocol, whereby we were meant to clean our samples using organic partitioning, which meant that only hormones and few more organic compounds were collected and; therefore, the salts should be removed from the samples. In his experience, the solution is to dilute the samples and then the background (the interference) will enormously reduced and the ratio signal to noise will increase. As I mentioned before, we diluted our samples, but we continued to experience contamination.

Dr. Victoria Pastor (Researcher, Department of Agricultural and Environmental Ssciences at Universitat Jaume I, Spain) suggested that the problem may have come from the amount of organic solvent used for extraction. According to her scientific experience, high amounts of organic solvent does not produce the best conditions for the equipment and can affect the sensibility of the instrument because all the substances that are extracted with these solvents may interfere in the analysis and the detector gets dirty quickly.

It is our duty to prevent damage whenever possible, so the instrument does not become unavailable, generates excessive costs for repair, or decommissioned entirely. For this reason, we decided to halt the experiment and not to do the hormonal extraction of the rest of the samples.

Conclusion

The cross-talk between ABA, SA, and JA-Ile is complicated and still unclear, especially under a combination of abiotic and biotic stress. Furthermore, there is a lack of information about signaling pathways interaction in treated plants with BABA under various stress conditions. Here, we conclude that BABA has the capacity to induce resistance in tomato plants by increasing ABA and SA levels compared to non-treated plants upon a combination of salt stress and infection with *B. cinerea*. It has become increasingly clear that ABA and SA are interconnected and contribute to increased resistance in BABA treated and non-treated plants under combined stress.

We can conclude that ABA had a positive role with BABA in a plant’s response to a combination of abiotic and biotic stresses.

To further progress our understanding of the complex interactions between phytohormone signaling, induction of resistance and combination of abiotic and biotic stress, it will be necessary to conduct further research with different plant species and various simultaneous stresses.

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Chapter V

General conclusions and perspectives

General conclusions and perspectives

Conclusions

Plants are well equipped to defend themselves against abiotic stress as well as biotic stress through constitutive or inducible defenses. In addition to these natural defenses, plants have the ability to acquire an induced defensive capacity by the application of various stimuli such as BABA. Recently, it has been demonstrated that BABA is a natural compound which various plants do synthetize it. This endogenous BABA increased following stress and can be implicated in the induction of plant resistance which makes it even more important (MauchMani et al., 2017; Thevenet et al., 2017; Baccelli et al., 2017). The induction of resistance by exogenous BABA has been associated with a faster and stronger plant response against abiotic stress and attackers (Conrath et al., 2002). The main objective of this thesis was to elucidate the physiological and molecular mechanisms of priming in BABA-treated tomato under single stress or double stress scenarios.

The following section summarizes our findings on the induction of resistance by BABA on tomato plants under stressed conditions and showcases our perspectives of future research in the field with the goal of enhancing resistance under abiotic and biotic stress.

Role of BABA to induce resistance in tomato plants upon drought stress and salinity

The effect of BABA to induce resistance against abiotic stress was demonstrated in several studies. In case of drought and salt stress, Arabidopsis pre-treated with BABA showed a positive effect comparing to the control after 1 week of stress (Jakab et al., 2005; Ton et al., 2005). The results presented in this thesis showed that BABA enhanced the defense response of tomato plants during drought and salt stress. BABA had no effect on tomato growth; and the fresh and the dry weight showed a decrease in growth following the increase of stress levels. An effect on stomatal conductance was detected, wherein the plants that were treated with BABA presented a high stomatal conductance compared to the control plants. BABA increased the total antioxidant in stressed tomato plants inducing a reduction of H_2O_2 accumulation. We hypothesized that BABA activated defense systems in order to prevent oxidative stress (Du et al., 2012). Interestingly, lignin accumulation was higher in BABA-treated plants. The increase in lignifications may have been due to one of the adaptation strategies of plants faced with dehydration and could have resulted in enhanced mechanical strength and/or water impermeability. This difference in resistance levels between treated and untreated plants induced by BABA treatment could explain the priming state of this compound under different abiotic stressors. Comparing the changes that occur at the molecular and biochemical level between treated and non-treated plants with BABA has shown that control plants react rapidly after BABA treatment and an up-regulation of ABA-dependent transcription factors was detected on the second day of BABA treatment and concordingly ABA levels increased. Primed plants responded faster than non-treated ones in the up-regulation of defense-related genes after salt/drought stress. Altogether the results we observed demonstrated that cv Marmande is more resistant than cv Coeur de Boeuf.

Role of BABA to induce resistance in tomato plants under a combination of salt stress and infection with *B. cinerea*.

The combination of abiotic and biotic stress is an interesting field of research wherein information about priming effects is lacking. An important result was found in primed plants treated with salt and infected with *B. cinerea* which demonstrated that BABA treatment enhances the plant’s response against both stressors. The area of infection in treated plants with BABA is smaller than in non-treated plants. The same result was found in plants treated with BABA and 50Mm of salt. In addition, callose accumulation, lignifications, and H_2O_2 accumulation were higher in primed plants which indicated the positive effects of BABA on enhancing plant resistance upon combined stresses. Interestingly, *AREB* and *CBF3* were up-regulated at the beginning of the experiment (8hpi), while the pathogenesis-related genes *PR1* and *PR5* were repressed. This result indicated that the plants react to defend against salt stress. However, *PR1* and *PR5* were up-regulated at a higher level, after 24hpi, to defend the plants against this fungus. Surprisingly, the expression of *PR1* and *PR5* were significantly higher in BABA-treated plants under combined stress than under single stresses. So, it can be hypothesized that there is cross-talk between abiotic and biotic stress, thus confirming that salt stress enhanced the resistance of primed plants against *B. cinerea.*

Importance of phytohormones signaling in the plant’s defensive responses

To unravel the role of BABA to induce resistance against salinity and infection with *B. cinerea,* we studied the phytohormones signaling pathways in order to obtain an idea about the crosstalk between them. SA, JA, and ET are the major hormones that induced defense activation (Glazebrook *et al.*, 2003). Other research demonstrated that ABA (abiotic stress signaling) is also a regulator of defense signaling under biotic stress (Mauch-Mani and Mauch, 2005). In this thesis, we demonstrated that ABA and SA levels were higher in treated plants with BABA than non-treated plants under combined stresses. Thus, a positive crosstalk was observed between ABA and SA responsible for increasing resistance to both abiotic and biotic stress. However, only JA-Ile increased at 24hpi in plants treated with BABA under double stress. We therefore hypothesize that JA-Ile-depending signaling may have strongly primed the defense response against *B. cinerea.* These results demonstrate a positive relationship between the ABA, SA and JA-Ile pathways in BABA-primed plants. A further understanding of defense mechanisms involved in the induction of resistance in treated-tomato with BABA under combination of abiotic and biotic stresses could be of a great importance for future crop protection methods.

Perspectives

For future research, from this thesis the following perspectives arose and are mentioned below:

- Does BABA treatment influence the leaf area and the number of stomata upon abiotic stress?

Determination of leaf area would help to have a better idea about the effect of BABA on tomato growth under abiotic stress. In addition, the number of stomata would be interesting to obtain further explanation concerning stomatal conductance.

- Could a metabolite study give us an idea about priming and abiotic stress?

It would be interesting to detect compounds interfering in the resistance of BABA-treated tomato plants under drought/salt stress (essentially membrane proteins) and those involved in the rigidity of the cell wall.

- Could the study of gene expressions related to ROS and antioxidant synthesis in BABA-treated plants and stressed by abiotic stress and the combination of abiotic and biotic stress help to better understand the priming under these conditions?

Although this thesis demonstrates that the induction of total antioxidants in primed plants is in concordance with the increase of ROS under drought and salinity, the mechanism of action of antioxidants (enzymatic and non-enzymatic antioxidants) and ROS remains elusive. It would be interesting to study the genes involved in ROS and detoxification in primed plants under single and double stresses to obtain a clearer understanding of this defense response.

What are the mechanisms of BABA-IR in tomato under a combination of abiotic and biotic stress?

There are several studies which have demonstrated the positive effects of BABA on enhancing resistance against individual stresses. However, it seems that plants respond in a specific manner when they have to face combined stressors and the role of BABA in inducing resistance to this remains unclear. In addition to the further understanding of BABA-IR under a combination of stresses in this thesis, it would be interesting to focus on the intricate molecular crosstalk of plants treated with BABA in order to develop clearer information about the plant's defensive responses upon simultaneous stresses.

- **What is the role of BABA against other kinds of combined stressors?**

This thesis is the first study on the role of BABA in inducing resistance in plants against a combination of salinity and infection with *B. cinerea*. It would be interesting to test whether BABA is effective against other combined stresses and other plant species.

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Appendix

Appendix

A preliminary experiment involving abiotic stress have been conducted during this project. This assay is of potential interest for further investigations on the relationship between abiotic stress and tomato plants. For this reason, the related results are presented here.

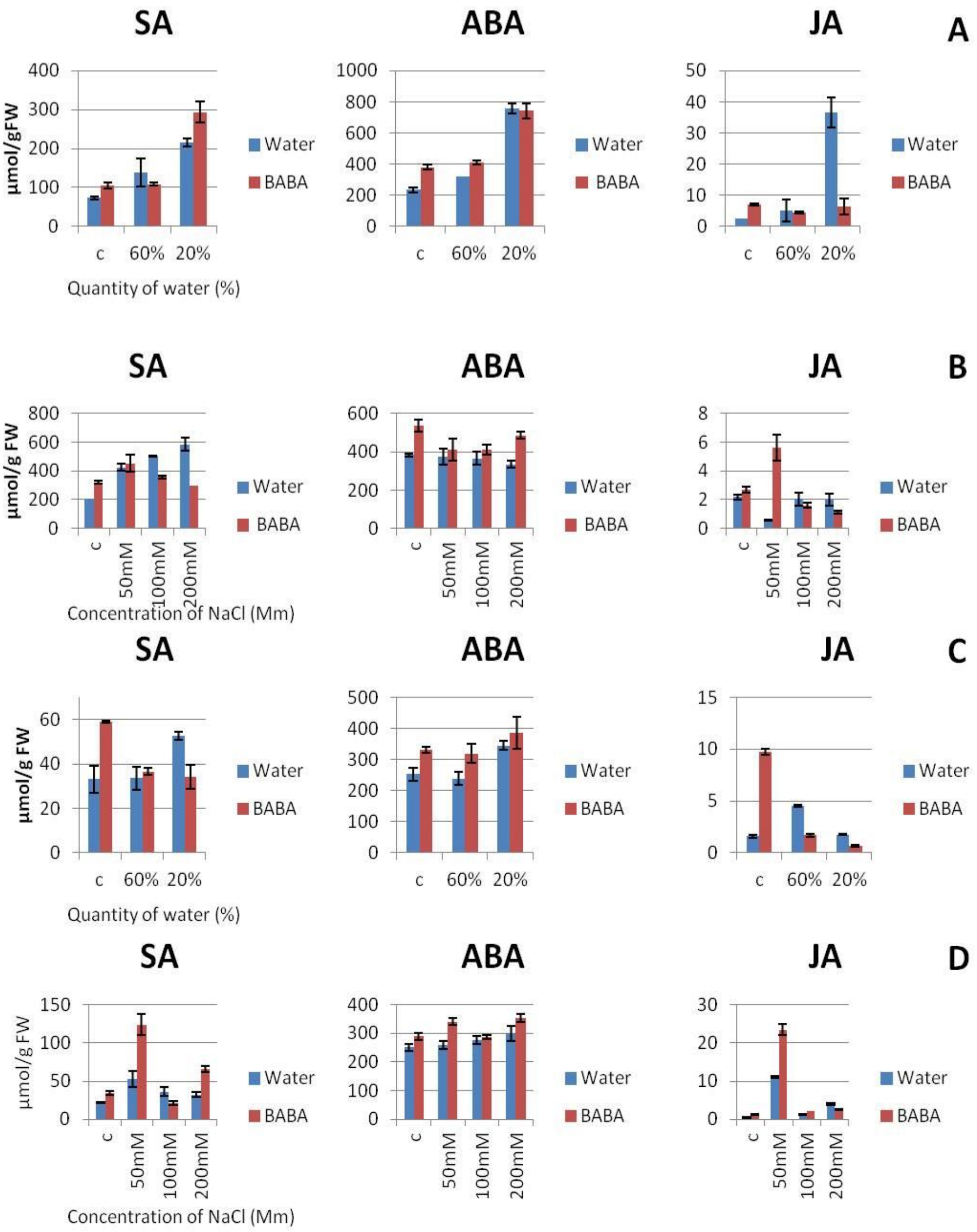

Figure. Quantification of hormone levels in treated and non-treated tomato plants with BABA under drought or salt stress. Leaves were collected for hormone analysis after 2 weeks os stress. Abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) levels were determined in the fresh material by high-performance liquid chromatography-mass spectrometry. (A) Hormonal level in Coeur de Boeuf after 2 weeks of drought stress. (B) Hormonal level in Coeur de Boeuf after 2 weeks of salt stress. (C) Hormonal level in Marmande after 2 weeks of drought stress. (D) Hormonal level in Marmande after 2 weeks of salt stress.

List of publications

Ben Rejeb I, Spicher L, Cordier M, Mauch-Mani B (2013) Induced tolerance and priming for abiotic stress in plants. In: Gaur RK, Sharma P, 1st edition, (eds): Molecular approaches in plants abiotic stress, CRC Press, Boca Raton pp 33-43.

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Awards

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    - Practicals in biology (Medicine 1st year). Introduction to Biology (use of technical equipment), test on lipids, proteins and carbohydrate and protein analysis methods.
    - Practicals in molecular biology and phytopathology (Bachelor). Introduction to research: a complete project planning experience in the laboratory, inducing infections with different pathogens (*Hyaloperonospora arabidopsidis*, *Colletotrichum graminicola*, *Pseudomonas syringae* and *Botrytis cinerea*) on different plants (Arabidopsis, maize and tomato).
    - Practicals and Workshops for Master students in laboratories. Creation of a complete project with different learning methods such as qRT-PCR for gene expression, hormonal analysis, detection of infection by different colors (trypan blue) and callose staining.

CommunicationsParticipation in meetings and conducting conferences:

SCOPES Meeting 2012: Gödöllő, Hungary.
Prime-Time Workshop 2013: Leukerbad, Switzerland.
PhD meeting 2014, Neuchâtel, Switzerland.
SPPQ conference 2015, Château Mont-Ste-Anne, Beaupré, Québec, Canada.
Canadian Society of Agronomy conference 2016, Montréal, Québec, Canada.

Scientific publications

Ben Rejeb I., Atauri Miranda L., Cordier M. and Mauch-Mani B. (2013). Induced tolerance and priming for abiotic stress in plants. Molecular Approaches in Plant Abiotic Stress. Editors: Rajarshi Kumar Gaur and Pradeep Sharma.

Ben Rejeb I., Pastor V. and Mauch-Mani B. (2014). Biotic and abiotic stress can work together to protect plants. *Plants*, 3(4):458-75. doi:10.3390/plants30x000x.

Ben Rejeb I., Pastor V., Gravel V. and Mauch-Mani B. (2018). Impact of β-aminobutyric acid on induced resistance in tomato plants exposed to a combination of abiotic and biotic stress. *Journal of Agricultural Science and Botany*, In press.

Course interuniversity doctoral program in organismal Biology

-Public speaking.
-Planning a career strategy: Networking and job finding methodology.
-Writing reviews.
-Introduction to statistical software R.
-Advances in Swiss research on the chemical ecology of plant defense.
-Microscopy SEM (Scanning Electron Microscopy).

Language skills: French and English, Arabic **(mother tongue)**.