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## Stress combination: When two negatives may become antagonistic, synergistic or additive for plants?

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### ABSTRACT

Plants in their natural environment are constantly subjected to various abiotic and biotic stressors and, therefore, have developed several defense mechanisms to maintain fitness. Stress responses are intricate and require various physiological, biochemical, and cellular changes in plants. The reaction mechanisms in plants subjected to drought, salinity, or heat stress alone have been explained in numerous studies. However, the field conditions are significantly different from the controlled laboratory conditions. In the field, crops or plants are simultaneously exposed to two or more abiotic and/or biotic stress conditions, such as a combination of salinity and heat, drought and cold, or any of the abiotic stresses combined with pathogen infection. Studies have shown that plants' reactions to combinations of more than two stress factors are distinct and cannot be explicitly deduced from their responses to different stresses when applied separately. Therefore, additional research is needed to understand the complete mechanism of plant responses to stress by analyzing data between single stress and multiple stress responses. This review aims to provide an overview of current research on plant responses to a combination of various stress conditions and their influence on the metabolic, transcriptional, and physiological characteristics of plants.

**Key Words:** abiotic stress, abscisic acid, biotic stress, defence mechanism, multifactorial stress, reactive oxygen species, stress response

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### INTRODUCTION

It is a great challenge for farmers to continuously supply food to the increasing world population, while natural resources remain the same. According to the Food and Agriculture Organization (2017), approximately one third of the total land area is classified as potentially suitable for arable agriculture, and only 3.5% of the total land area remains ecologically unaltered. Multifactorial stresses affecting plants are prominent in most agricultural fields worldwide and are identified as one of the most persistent threats in the field. According to IPCC (2014), abiotic stress situations, mainly drought, salinity, and high temperature, are the center of attention of intense agricultural research as the losses they inflict each year are projected to be US\$14–19 million worldwide. A wide variety of biotic components (fungi, bacteria, nematodes, phytoplasmas, viruses, viroids, *etc.*) and abiotic stress components (drought, heat, salinity, heavy metals, metalloids, *etc.*) regularly and simultaneously challenge plants in their natural habitats (Mantri *et al.*, 2014; Stork, 2018). Consistent exposure to biotic and abiotic stresses

alters morpho-physiological, biochemical, and molecular processes in plant cells, which work orderly to control reactive oxygen species (ROS) levels. These affect growth and productivity (Hasanuzzaman *et al.*, 2020).

Based on the number of interacting factors, stresses are broadly classified into three groups: single, multiple individual, and combined. In single stress, only one stress factor affects plant growth and development. In the case of multiple individual stresses, two or more stresses exist at different times without any overlap, whereas a combined stress is characterized by at least some overlap between the stresses. The concurrence of drought and high temperatures in hot seasons is an example of combined abiotic stress, whereas the simultaneous invasion of plants by bacterial and fungal pathogens is an example of combined biotic stress. Plant biologists have conventionally studied plant responses to single abiotic or biotic stresses in order to develop cultivars that are more tolerant to the particular stressor (Matsui *et al.*, 2008; Shaik and Ramakrishna, 2013). Such studies have greatly aided our knowledge of plant responses to environmental stressors. This kind of investigation, on the

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other hand, may not mimic the actual field conditions where plants face multiple stresses. In this regard, recent studies have revealed that the reaction of plants to a combination of stresses is unique and cannot be inferred from the reactions to individual stresses (Atkinson and Urwin, 2012; Prasch and Sonnewald, 2013; Pandey *et al.*, 2015; Zhang and Sonnewald, 2017).

Whether coexisting stresses are additive, antagonistic, or synergistic, enhancing or weakening the effects of a particular stress, remains equivocal (Maher *et al.*, 2019). Sinha *et al.* (2017), who studied the interaction between drought and pathogens and their effect on chickpea, noticed an increased occurrence of fungal infections such as dry root rot (causal agent, *Rhizoctonia bataticola*) and black root rot (causal agent, *Fusarium solani*) under extreme drought when compared to irrigated field conditions. Studies on the simultaneous pathogen and high temperature suggest that elevated temperatures enhance the vulnerability of plants to diseases. Sharma *et al.* (2007) observed intensified symptoms of spot blotch, a fungal leaf disease of wheat caused by *Cochliobolus sativus*, for six consecutive years, which they attributed to the rise in average night temperatures. However, one beneficial consequence of plants' exposure to multiple stresses is that while defending against one stress it may become resistant to other stresses. This phenomenon is called cross-tolerance, and it demonstrates that plants develop a complex regulatory mechanism to adapt effectively to unstable environments (Rejeb, 2018). For example, high-temperature, adult-plant resistance, which is expressed only in adult plants at high temperatures, strengthens disease resistance in *Triticum aestivum* to stripe rust (causal agent, *Puccinia striiformis* f. sp. *tritici*) (Carter *et al.*, 2009).

Furthermore, in a concentration-dependent manner, salinity has been reported to improve resistance to powdery mildew (causal agent, *Blumeria graminis*) in *Hordeum vulgare* (Wiese *et al.*, 2004). The ion and osmotic toxicity influenced by salinity stress can restrain the intensification of pathogens. The combined influence of two stresses on plants may have a detrimental or beneficial effect; the subsequent stress is typically the one that triggers considerable distress (Atkinson and Urwin, 2012). Studies on multiple stresses have attempted to simulate the natural environment; however, the environmental conditions cannot be controlled in the field. The primary stress protection response of plants can be strongly regulated by one stressor (Fujita *et al.*, 2006). It is usually challenging to design combined stress experiments that truthfully replicate natural conditions, and the results of combinatorial stresses are highly reliant on the experimental setting. The outcomes of the experiments will be determined by plant growth stage, duration and frequency of stress applications (consecutive or parallel stresses), and magnitude and nature of individual stresses. Regardless

of these differences, some common reactions have been reported among different treatments. Subsequent exposure to different stresses activates specific ion channels and kinase cascades (Fraire-Velázquez *et al.*, 2011). This leads to the accumulation of ROS, plant hormones, and reorganization of genetic mechanisms, which assists in many defensive responses, increases plant resistance, and ultimately reduces crop yield losses (Rejeb *et al.*, 2014).

This review aims to enhance and clarify the existing perspective of stress combinations by outlining certain basic principles relevant to combined stresses, point out their worldwide prevalence, and evaluate their impact on metabolic, transcriptional, and physiological characteristics. We also include a general update on various stress combinations and address their impact on the most relevant crops.

## STRESS COMBINATION RESEARCH: A REALITY CHECK

In previous years, a significant number of studies have focused on the responses of plants to environmental factors. Most of them were conducted in a restricted environment, especially under laboratory conditions, which is the optimal approach to create reproducible research. These studies were mostly focused solely on the responsiveness of plants to a single stress. In contrast, aside from prominent research on the impact of concurrent drought and heat stresses (Rizhsky *et al.*, 2004; Mittler, 2006), the effect of stress combinations has been little studied. Until now, the characterization and investigation of physiological, biochemical, and molecular pathways associated with plant reactions have been primarily concentrated on individual stresses, whereas studies on the effects of multiple stresses have been inadequately represented (Suzuki *et al.*, 2014; Pandey *et al.*, 2017; Zhang and Sonnewald, 2017). In recent decades, several review articles have discussed the potential effects of climate variation on plant pathogens and diseases (Juroszek *et al.*, 2020). Surprisingly, the number of studies on the impact of climate change on plant pathogens and agricultural diseases has been declining since 2014, indicating a waning interest in this subject. In the past few decades, there has been a substantial gap in data obtained from these studies and the information that explains the evolution of plants and crops with improved resistance to field conditions. While the available data illustrate the processes leading to improved resistance in several transgenic plants to specific biotic or abiotic stress situations under laboratory conditions, they cannot be applied to field conditions. Hence, to bridge this gap in knowledge and assist the cultivation of crops and plants with enhanced tolerance under field stress conditions, research should focus on the molecular, physiological, and metabolic aspects of stress combinations.

Some researchers successfully developed the technically difficult experimental setups that allow testing of combina-

torial stress factors under controlled laboratory conditions. Hence, in addition to the two revolutionary investigations on the combined influence of heat and drought stresses on the genomic profiles of tobacco and *Arabidopsis* (Rizhsky *et al.*, 2002, 2004), many recent genomic studies have analyzed plant responses to synchronized stresses (Shaar-Moshe *et al.*, 2017; Sinha *et al.*, 2017; Osthoff *et al.*, 2019; Sewelam *et al.*, 2020; Lopez-Delacalle *et al.*, 2021).

#### NECESSITY OF STUDYING STRESS COMBINATION

According to IPCC (2014), the temperature is predicted to increase between 1.8 and 4.0 °C by the year 2100. Increased frequency of droughts and/or extreme temperatures, strong winds, and precipitation events will alter the global agricultural system. The dwelling territories of pests and pathogens will also be affected by climate change, as elevated temperatures facilitate pathogen transmission (El-Sayed and Kamel, 2020); for instance, elevated temperatures are known to promote pathogen spread (Luck *et al.*, 2011; Madgwick *et al.*, 2011). Furthermore, numerous abiotic stress situations have been proven to decrease plant defense mechanisms, rendering them more susceptible to pathogen infection (Mittler and Blumwald, 2010; Atkinson and Urwin, 2012).

Consequently, primary crops in future fields will be exposed to a broader range of abiotic and biotic factors, as well as their combinations. Longer growing seasons instigated by climate change will lengthen pathogen reproduction and spread. For example, higher temperatures hasten spore germination and stimulate spore production of powdery mildew (*Podosphaera plantaginis*), a natural pathogen of *Plantago lanceolata*; contrary to sexual traits, all asexual traits perform better under increased temperatures (Vaumourin and Laine, 2018).

Lopez-Delacalle *et al.* (2021) demonstrated that an exclusive redesign of metabolic pathways occurs when salinity and heat stresses are combined, including alterations in the expression of 1 388 genes and the accumulation of 568 molecular characteristics. Using transcriptomics and metabolic results, these authors showed that the proline and ascorbate pathways perform concurrently to maintain cellular redox homeostasis, and they identified important transcription factors from the basic leucine zipper domain (bZIP), Cys2His2 zinc finger, and trihelix families as possible controllers of the up-regulated genes under combined salinity and heat stresses (Lopez-Delacalle *et al.*, 2021). Such research explains how plants adapt to multifactorial environmental challenges and highlights the synergy between critical cell metabolic pathways for efficient ROS detoxification.

In a large-scale microarray experiment, Rasmussen *et al.* (2013) studied both abiotic and biotic stresses alone and in combination by correlating transcriptomic changes in 10 *Arabidopsis thaliana* ecotypes using cold, heat, high-light,

salt, and flagellin treatments as single stress factors and their double combinations. Approximately 61% of the alterations under combination stresses could not be determined from the single stress treatment, and plants favored only 5%–10% of the responding transcripts between potentially antagonistic responses (Rasmussen *et al.*, 2013). Analysis of the transcriptomic profile of plants exposed to different abiotic stresses revealed that different stresses elicit a substantially unique reaction, and only minor similarities in transcript expression could be established between plant responses to abiotic stresses such as heat, water, cold, salt, or mechanical stress (Fracasso *et al.*, 2016; Baillo *et al.*, 2019; Demirel *et al.*, 2020; Kang *et al.*, 2020; Liu *et al.*, 2020). Sun *et al.* (2015) studied the effects of combined salt and drought stresses on maize plants by treating them either singly or simultaneously with soil water deficiency (60% soil water content) and high salinity (5 mL of 250 mmol L<sup>-1</sup> NaCl) for seven days. They observed that the effect of combined stresses differed at both metabolic and physiological levels compared to those under drought or salt stress alone. Maize plants exhibited a completely different paradigm of metabolic reactions whereby a few metabolites responded significantly and their reactions differed between combined stress and individual stress treatments (Sun *et al.*, 2015).

Sewelam *et al.* (2020), who studied the responses of *Arabidopsis* to salt, osmotic, and heat stresses individually and in combination, reported that, based on the metabolite profile, the type of employed stress determines the generation of explicit compatible solutes. Furthermore, they reported that treatments that included heat stress downregulated the transcription of genes that code for abundant photosynthetic proteins and proteins regulating the cell life cycle while upregulating the genes involved in protein degradation. Upon exposure to combined stresses, the plants switch their metabolism to the survival mode, characterized by a minimum yield. Studies on multifactorial stresses provide molecular basis for the threats to plant yield and the anticipated world food security due to heatwaves arising from global warming. These findings, along with those from other studies on this topic, contemplate abiotic stress combinations for engineering or breeding plant tolerance to actual field scenarios.

The information about stress combinations from the fields, laboratories, and molecular studies is increasing daily. These results suggest that plants counter the stress combination in a non-additive way, generating outcomes that cannot be extrapolated from the effects of individual stresses (Kissoudis *et al.*, 2014). Multifactorial stress combinations could have unforeseeable consequences for agricultural areas or ecosystems. For instance, because of a small level of individual stressors, it might not be possible to detect a distinct

reduction in crop productivity. However, despite the small level, the introduction of new stressors might initiate a negative interaction between the stressors, prompting a striking reduction in crop productivity and shifting the ecosystem towards a rapid decline.

#### CROSS-TOLERANCE BETWEEN ABIOTIC AND BIOTIC STRESSES

Plants acclimatize to biotic and abiotic stresses by inducing a cascade or a network of series that start with stress recognition and finish with the transcription of various target genes. These include stress stimuli, signal transduction, transcription regulators, target genes, and stress responses such as changes in morphology, biochemistry, and physiology. Plant responses to different stresses are synchronized by complicated and generally interrelated signaling pathways that regulate several metabolic systems (Rejeb *et al.*, 2014). This process, known as cross-tolerance, helps plants to adjust or acclimatize to various stresses following experience with a particular stress. Such interactions may emerge as a mechanism that allows plants with minimum suitable biological approaches to counter stress. Signaling cascades stimulate ion channels, kinase cascades, and ROS and hormone accumulation, arresting abiotic and biotic stresses. Recent studies have shown that defense genes that are usually associated with the combination of abiotic or biotic stresses show a substantial overlap either shared or as overall stress-reaction genes (Massa *et al.*, 2013; Narsai *et al.*, 2013; Shaik and Ramakrishna, 2013; Sham *et al.*, 2014, 2015; Zhang *et al.*, 2016). Plants, however, direct considerable transcriptional reprogramming in response to a combination of stresses and demonstrate a unique transcription response that is different from either of their responses to individual stresses, despite the significant overlap in responsiveness to the combination of ecological stresses (Atkinson, 2011; Prash and Sonnewald, 2013; Rasmussen *et al.*, 2013; Sham *et al.*, 2015).

In general, environmental pressure through abiotic and biotic stresses can induce resistance in plants. In addition, specific atmospheric stresses can predispose plants by permitting them to react swiftly and in a resistant way to further threats. Cross-tolerance between abiotic and biotic stresses will thus have a beneficial impact by improving tolerance in plants and allowing for substantial agriculture ideas. Interestingly, abiotic stress regulates the defense mechanism at the pathogen infection site and other components, thus amplifying plant resistance mechanisms (Venegas-Molina *et al.*, 2020). Prior studies on the interaction of drought with various pathogens in trees found that drought and pathogen infection had a synergistic effect (Desprez-Loustau *et al.*, 2006). However, few plants were more vulnerable to a single

stress than to a simultaneous treatment of different stresses (Suzuki *et al.*, 2014).

Mota *et al.* (2021) studied the transcriptional dynamics elicited by root-knot nematode infection combined with drought stress through a meta-analysis of wild *Arachis* RNA-sequencing data. Overexpression of the cross-stress-tolerant endochitinase-encoding gene (*AsECHI*) from *Arachis stenosperma* led to the reduction of *Meloidogyne incognita* infection by approximately 30% and stimulated post-drought improvement in *Arabidopsis* plants exposed to both stresses. Biotic and abiotic factors individually promote hormone-responsive genes in the jasmonic acid (JA) and abscisic acid (ABA) pathways, whereas multiple stressors appear to stimulate ethylene hormone pathways. The detection of a network of cross-stress regulated genes in *Arachis* better our understanding of the complicated process of regulation of biotic and abiotic stresses in plants, thereby promoting a more effective crop breeding for combined stress tolerance. Davis *et al.* (2015) reported that cereal virus (barley yellow dwarf virus) infection increases the level of phytohormones and salicylic acid (SA) in *Triticum aestivum* in a time-dependent manner, indicating a possible biochemical basis for virus-induced hormonal responses that modify plant reactions to environmental stress. In another study, the introduction of bacteria (*Bacillus pumilus*) increased the morphological parameters and elevated drought tolerance in *Glycyrrhiza uralensis* by protecting the chloroplast submicroscopic structure, thus increasing the chlorophyll content, photosynthetic rate, and water status (Zhang *et al.*, 2019). Various plant growth-promoting bacteria have been reported to enhance drought resistance in maize (Vardharajula *et al.*, 2011), wheat (El-Afry, 2012), bean (Sarma and Saikia, 2014), and *Brassica* species (Saeed *et al.*, 2016). Similarly, numerous mycorrhizal fungi and rhizobacteria have been found to induce stress tolerance in many crops through various defense mechanisms, including the production of antioxidants, restriction of ethylene generation, and improvement of ABA regulation (Grover *et al.*, 2011). Application of these microorganisms will offer an innovative approach for gene modification and plant breeding to deliver rapid, but successful, enhancement of crop stress resistance (Grover *et al.*, 2011).

#### EFFECT OF STRESS COMBINATION ON PLANT PHYSIOLOGY

##### *Examples of beneficial interactions between stresses*

Certain stress combinations may have positive effects on plants compared to individual stresses when applied separately. Some recent studies that reported positive interactions between stresses are listed in Table I.

Iyer *et al.* (2013) studied the reaction of *Medicago trun-*

TABLE I

Recent experimental studies showing positive effects of combinatorial stresses on plants

Stress combination	Plant	Effect	Reference
Salinity + heat	Tomato <i>Solanum lycopersicum</i>	Reduced accumulation of reactive oxygen species Improved photosynthetic activity and less oxidative damage than in plants exposed to salinity only	Rivero <i>et al.</i> (2014) Lopez-Delacalle <i>et al.</i> (2021)
High CO <sub>2</sub> + high light Drought + barley yellow dwarf virus	Lettuce <i>Triticum aestivum</i>	Increased biomass production and antioxidant capacity Infected plants recovered from severe stress events more readily than non-infected plants	Pérez-López <i>et al.</i> (2013) Davis <i>et al.</i> (2015)
Salinity + <i>Oidium neolycopersici</i>	<i>S. lycopersicum</i>	Decreased pathogen symptoms under severe stress due to Na <sup>+</sup> and Cl <sup>-</sup> accumulation	Kissoudis <i>et al.</i> (2016)
Soil moisture stress + <i>Sclerotium rolfisii</i> Drought + waterlogging	Chickpea <i>Zea mays</i>	Highly upregulated defense response genes Enhanced growth parameters (leaf area, plant height, and stem diameter) and improved stress tolerance	Tarafdar <i>et al.</i> (2018) Rafique <i>et al.</i> (2019)

*catula* to ozone, drought, and their combination. The authors noticed that short-term ozone stress (70 nmol mol<sup>-1</sup>) for 6 h per day for six consecutive days caused chlorosis and small necrotic lesions, while drought (withholding water for a period of 10 d) caused wilting and breaking down of entire leaves along with chlorosis. Surprisingly, when these stresses were combined, the impact caused by ozone or drought alone was dramatically eliminated. It is believed that the important factor that might be linked to the reduction in mutual effects of ozone and drought stress is minimal stomatal conductance (Iyer *et al.*, 2013). Individual exposure to drought or ozone caused an increase in ROS levels. In contrast, ROS levels were reduced to a value equivalent to that of the control state when exposed to combinatorial stress. Similarly, in tomato plants, the combined exposure to salinity and heat stress reduces ROS accumulation to a considerable level (Colmenero-Flores and Rosales, 2014), which protects plants from damage caused by salt stress (Rivero *et al.*, 2014).

Abiotic stresses, primarily drought and salinity, decrease pathogen resistance (Suzuki *et al.*, 2014). However, in some of the experiments conducted by researchers, it has also been observed that specific abiotic stresses often promote resistance in plants to pathogens. For example, to investigate the effects of NaCl on the existing relationship amongst tomatoes and powdery mildew, Kissoudis *et al.* (2016) analyzed effects of three different levels of salt stress, mild, moderate, and severe (50, 100, and 150 mmol L<sup>-1</sup> NaCl, respectively), on powdery mildew resistance and general performance of tomato introgression lines with contrasting levels of partial resistance, as well as near-isogenic lines (NILs) having the resistance gene *Ol-1* (associated with a slow hypersensitivity response (HR)), *Ol-2* (a *mlo* mutant associated with papilla formation), and *Ol-4* (an *R* gene associated with a fast HR). Increased susceptibility was observed in susceptible and partially resistant lines during mild salt stress (50 mmol L<sup>-1</sup>), followed by increased senescence. In contrast, severe salt stress (150 mmol L<sup>-1</sup>) lowered disease symptoms. Under severe stress, sodium (Na<sup>+</sup>) and chloride (Cl<sup>-</sup>) ion

concentrations in the leaves were linearly associated with reduced pathogen symptoms. The effect of simultaneous salinity and powdery mildew on tomato plants is determined by the intensity of salt stress and the disease-resistance mechanism. Under mild salt stress, negative interactions, such as greater powdery mildew susceptibility, leaf senescence, and reduced biomass, were observed in most agricultural scenarios. Extreme salt stress partially reversed these effects while significantly influencing plant biomass. The expression of ethylene and JA pathway genes and the cell wall invertase gene *LIN6* were attributed to greater sensitivity and senescence in NIL-*Ol-1* to the combination of stresses. The build-up of Na<sup>+</sup> and Cl<sup>-</sup> distinguishes salt stress from other abiotic conditions, such as drought or heat (Kissoudis *et al.*, 2016). This accumulation generally has harmful consequences on the plant and the fungus as well, as NaCl is known to be an antifungal agent (Blomberg and Adler, 1993).

A weak negative correlation was found between sulfate (SO<sub>4</sub><sup>2-</sup>) and calcium (Ca<sup>2+</sup>) ion concentrations and enhanced disease resistance, in conjunction with Na<sup>+</sup> and Cl<sup>-</sup>. Because both SO<sub>4</sub><sup>2-</sup> and Ca<sup>2+</sup> promote disease resistance (Kruse *et al.*, 2007; Jiang *et al.*, 2013), disrupting their equilibrium under combined stress may lead to defense failure in plants. These results highlight the significance of stress severity and resistance type on plant performance under a combination of abiotic and biotic stresses. Drought stress has also been found to augment resistance in tomatoes against *Botrytis cinerea*, which may stimulate overlapping pathogen-defense pathways that might not necessarily include ABA (Achuo *et al.*, 2006). Similar positive interactions have also been reported for other abiotic stresses, such as heavy metal stress. Some non-toxic metal ions induce defense responses to non-hyperaccumulator plants, which are not competent enough to flourish in heavy metal-stressed soils. Introduction of healthy *Triticum aestivum* ‘Sonalika’ seeds with a mild dose of cadmium (Cd), provided as 50 μmol L<sup>-1</sup> CdCl<sub>2</sub> for 48 h and then washed off, offered resistance to the following infection by *Fusarium oxysporum* inoculum. This resistance is correlated with Cd-binding protein expression—an increase in ROS levels prompted by metal may also activate defense responses (Mitra *et al.*, 2004).

### Examples of negative interactions between stresses

In general, the ability of plants to recognize and adapt to different stress combinations is crucial when these stresses have a detrimental influence on the growth and reproduction of plants. Studies showing negative impact of combined exposure to drought and heat stresses on plant development and productivity are listed in Table II. One such example is the damage caused by multiple stresses on spring wheat, where drought stress hampers grain filling, flower growth, and ovule activity, whereas heat stress affects pollen fertility and grain quantity (Prasad *et al.*, 2011). Reproductive tissues are more sensitive to the combined stress than to drought or heat stress alone, which will have a more significant harmful impact on crop yield. The combination of drought and heat stresses in *Populus yunnanensis* resulted in a significant decline in photosynthetic activity and higher ROS generation; however, in *Festuca arundinacea* and *Lolium perenne*, the same stress combination caused a significant decline in photosystem II (PSII) photochemical efficiency (Jiang and Huang, 2001; Li *et al.*, 2014). It has also been reported that drought situations in England and Wales are associated with increased occurrence of common scab (causal agent, *Streptomyces scabiei*) in potatoes (Potato Council News, 2011).

In contrast to individual stresses, synchronized treatment of *Arabidopsis* with drought and *Turnip mosaic* virus contributed to a greater decline in plant weight and leaf number (Prasch and Sonnewald, 2013). Silva *et al.* (2013) assessed the contrasting primary physiological reactions of *Jatropha curcas* with salt stress alone and in combination with heat stress. Plants were treated with salt stress (100 mmol L<sup>-1</sup> NaCl) before and after exposure to 43 °C for 6 h. The outcomes of salt stress treatment were much more

severe than those of heat stress, and when the stress factors were combined, salinity was augmented. Substantial reductions in carbon dioxide (CO<sub>2</sub>) assimilation rate and stomatal conductance contributed to the adverse effects of the combined treatments. Stress combination increased Na<sup>+</sup> and Cl<sup>-</sup> build-up in the leaves because of membrane destruction and lipid peroxidation. In another study, Mahalingam and Bregitzer (2019) studied the influence of short-term heat, drought, and their combination on barley varieties. They explained that susceptibility to the stress combination was generally higher than that to heat or drought stress alone. In addition, severe yield loss (over 95%) was reported when a stress combination was imposed during the heading stage.

In another study conducted by Zhou *et al.* (2019), the activity of the photosynthetic apparatus was reduced in tomato plants when treated with a combination of drought and heat stress. Regarding control set-ups, tomato plants lowered their electron transport rate (ETR) and quantum yield of PSII ( $\varphi$ PSII) when subjected to drought (combined or not with heat stress). Interestingly, plants under drought conditions could repair ETR and  $\varphi$ PSII following a recovery period, but plants under stress combinations could not. Hence, the photosynthetic apparatus was irreversibly damaged by the combined stress, and tomato plants could not recover PSII performance following the combined-stress period. In a similar study, two citrus genotypes, ‘Carrizo’ citrange and ‘Cleopatra’ mandarin, demonstrated different capabilities to handle elevated temperatures alone or when combined with drought stress. The leaf relative water content (RWC) in both genotypes showed a similar reduction under water scarcity, suggesting that the effect of drought was identical in both genotypes. In contrast, heat stress increased transpiration in both citrus genotypes. However, there was an extreme decline in RWC when both stresses were introduced in combination.

TABLE II

Recent experimental studies showing negative effects of combinatorial stress on plants

Stress combination	Plant	Effect	Reference
Drought + heat	Lentil	Decreased concentrations of sucrose and starch in leaves and seeds caused by inhibited sucrose synthase and starch phosphorylase activities	Sehgal <i>et al.</i> (2017)
	Canola	Significantly lowered oil production due to a decrease in photosynthetic carbon absorption	Elferjani and Soolanayakanahally (2018)
	<i>Solanum lycopersicum</i>	Reduced electron transport rate and photosystem II	Zhou <i>et al.</i> (2019)
	Barley <i>T. aestivum</i>	Severe yield loss (over 95%) Hypo-additive response, water relation characteristics affected	Mahalingam and Bregitzer (2019) Sattar <i>et al.</i> (2020)
Heat + high light	<i>T. aestivum</i>	Quick stomatal closure led to decreased photosynthetic rate as CO <sub>2</sub> assimilation and rubisco activity decreased	Chen <i>et al.</i> (2017)
Salt stress + <i>Pseudomonas syringae</i> pv. <i>lachrymans</i>	Cucumber	Negative impact of NaCl on plant growth intensified	Chojak-Koźniewska <i>et al.</i> (2017)
Salinity + heat	<i>Carrizo citrange</i>	Enhanced Cl absorption in leaves due to high transpiration rate generated by high temperatures, which counteracted physiological responses of plants to salt stress	Balfagón <i>et al.</i> (2018)
High temperature + drought	<i>T. aestivum</i>	Pollen susceptibility and impairment to female reproductive organs resulted in nearly 55% yield reduction	Fábián <i>et al.</i> (2019)

This might be due to the additive effect of the individual stresses (drought-induced water loss and heat-increased transpiration). Likewise, the compatible osmolyte proline accumulation was also at maximum levels in drought + heat stress treatments (Zandalinas *et al.*, 2016b). The osmotically active molecule proline is also amassed in response to other stresses (García-Sánchez *et al.*, 2007; Moustakas *et al.*, 2011).

Consequently, in addition to its well-known function as a compatible osmolyte, proline has a number of other defense properties, such as redox balance and radical scavenging, protein structure preservation, functioning as a chaperonin, improving the activities of various enzymes, and helping to reduce cell membrane damage (Shao *et al.*, 2008; Szabados and Saviouré, 2010). An increase in proline concentration was linked to water loss caused by drought conditions, whereas the increase in transpiration rates was connected to high temperatures or both (Zandalinas *et al.*, 2016b). As a result, large regulatory amounts of proline inhibit further osmolyte production unless more extreme stress conditions are encountered, enabling the linear relationship between the RWC and the biochemical response to be disrupted.

Sattar *et al.* (2020) investigated the combined and individual effects of drought and heat stress on water relations, photosynthetic pigments, osmolyte accumulation, and antioxidant defense mechanisms in the flag leaf of bread wheat. The authors found that the collaborative outcomes of both stresses were hypo-additive. Similarly, water stress and fungal infection (*Erysiphe cruciferarum*) had an additive effect on *Alliaria petiolata* growth (Enright and Cipollini, 2007). Although water stress lessened disease expansion (percentage of infected leaf area dropped under water stress conditions), plants under drought stress were significantly smaller than those watered regularly and powdery mildew spread over the entire leaf area by the end of the experiment (Enright and Cipollini, 2007).

#### UNIQUE TRANSCRIPTOMIC FEEDBACK OF PLANTS TO STRESS COMBINATION

Despite the plethora of studies on single-stress conditions, few have focused on understanding the molecular mechanisms adopted by plants to resist the combination of two or more different stresses. Fortunately, at present, there has been a rise in the number of transcriptomic studies that aimed to interpret the reaction of plants to stress combinations (biotic and abiotic). These studies included the cold + drought (Zheng *et al.*, 2016), salinity + heat (Suzuki *et al.*, 2016), drought/flooding + herbivore attack (Nguyen *et al.*, 2016), drought + pathogen (Sinha *et al.*, 2017), drought + heat (Ashoub *et al.*, 2018; Liu *et al.*, 2018; Wang *et al.*, 2018), salinity + ozone (Natali *et al.*,

2018), and light + heat (Balfagón *et al.*, 2019) stress combinations. In the response of plants to high temperatures and other stresses, the heat shock factor (HSF) family of transcriptional regulators plays a pivotal role by influencing both immediate responses and epigenetic control of heat stress memory (Jacob *et al.*, 2017; Ohama *et al.*, 2017). Shaar-Moshe *et al.* (2017) studied the transcriptional patterns and morpho-physiological acclimatization of *Brachypodium distachion* to single salinity, drought, and heat stresses and to their double and triple combinations. Hierarchical clustering analysis of morpho-physiological acclimatization in plants exposed to stress combinations revealed deterioration in various attributes. Only 37% of differentially expressed genes conserved their responses under single and combined stresses, demonstrating restricted expression constancy among partly overlapping stresses. When common stress genes were compared to those exclusively expressed in response to a combination of stresses, a substantial switch from augmented intensity to antagonistic responses was discovered. The varied transcriptional fingerprints reflect a change in the mechanism of action when multiple stresses are combined and the inadequate potential to anticipate plants' responses as different stresses are combined.

Osthoff *et al.* (2019) revealed that intricate transcriptomic responses regulate the recognition and signaling of stress combinations in barley. They studied the impact of drought and salt stresses individually and in combination on barley seminal root development and transcriptomic plasticity. Drought and combined stress notably decreased the total root length when the plants were exposed for more than four days. Furthermore, the transcriptome sequence illustrated that 60%–80% of stress type-specific gene expression responses detected 6 h following exposure were also present after 24 h of stress treatment. After 24 h of stress treatment, hundreds of extra genes were stress-regulated compared to 6 h of application. The combination of salinity and drought resulted in a distinct transcriptomic reaction.

Moreover, transcription factors promote global reprogramming with the persistent preponderance of basic helix-loop-helix transcription factors, HSF, and ethylene response factors. Rizhsky *et al.* (2002) employed cDNA arrays, together with physiological parameters, to analyze the consequences of drought and heat shock on tobacco plants. Drought + heat shock application triggered closing of the stomata along with a reduction in photosynthesis and increased respiration. During their combinatorial treatment, the transcripts triggered at the time of drought exposure (such as those encoding catalase and dehydrin) and heat shock (thioredoxin peroxidase and ascorbate peroxidase) were eliminated. However, under combined drought and heat shock exposure, additional transcript expressions such as glutathione peroxidase, pathogen-related proteins, a WRKY

transcription factor, and an ethylene response transcriptional co-activator were explicitly activated. Photosynthesis genes were silenced, while transcripts expressing certain enzymes of the glycolysis and pentose phosphate pathways were triggered, indicating the activation of these pathways under stress conditions.

Balfagón *et al.* (2019) studied the responses of *Arabidopsis thaliana* plants to high light and heat stress combinations. High light and heat stress combination caused permanent destruction of PSII, decreased D1 (encoded by *PsbA* gene) protein levels, and elevated transcriptional response suggestive of PSII repair activation. Transcripts specific to high light and heat stress combination are engaged in several other processes, such as redox mechanisms, transportation of proteins, protein catabolic processes, or photosynthesis, and are associated with Cd responses, salinity, or involvement in the citric acid cycle. The *HSF* transcription factors exhibited an additive expression, with *HSFA2*, *HSFA7A*, *HSFB1*, *HSFB2A*, and *HSFB2B* displaying the maximum expression values under high light and heat stress conditions. A mutant deficient in JA biosynthesis (allene oxide synthase) showed increased susceptibility to high light and heat stress conditions, indicating that JA is necessary for altering numerous transcriptional responses exclusive to stress combination. Johnson *et al.* (2014) studied drought and heat single stress conditions or their combinations to understand the transcriptional response of *Sorghum bicolor* using microarrays. Drought and heat stress caused expression alterations in 4% and 18% of the genes investigated, respectively. Approximately 20% of the probes were differentially expressed in response to drought and heat stress combinations. In *Sorghum* combined stress response, ontological investigation of these ‘unique’ transcripts revealed a possible involvement of particular transcription factors such as *MYB78* and *ATAF1*, chaperones such as *HSP*, and biochemical functions such as polyamine synthesis.

#### SIGNALING PATHWAYS INDUCED BY RESPONSES TO MULTIPLE STRESSES

Earlier studies on biotic and/or abiotic stress combinations concerning two or three different stress factors (Rasmussen *et al.*, 2013; Zhou *et al.*, 2019; Zandalinas *et al.*, 2020) confirmed that plants respond to stress combinations in an exclusive manner and that their responses do not correspond to the sum of the plants’ responses to each of the individual stresses that contribute in combination. However, in some cases of stress combinations, the reaction to one of the involved stressors could be more prevalent compared with the reaction to the other (Zhou *et al.*, 2019; Sewelam *et al.*, 2020), or the different stresses might have an overall additive effect (Vile *et al.*, 2012; Bansal *et al.*, 2013; Shaar-Moshe *et al.*, 2017). Nearly all research on plants’ responsiveness

to stress combinations include transcripts, proteins, and metabolites that are unique to the combination (Prasch and Sonnewald, 2013; Kissoudis *et al.*, 2014; Sewelam *et al.*, 2020).

#### Abscisic acid signaling

As a chemical stimulus, ABA intensifies alternations in plant physiological and developmental approaches in response to atmospheric factors, thereby contributing to the acclimatization of plants to different abiotic and biotic stresses in combination and alone (Ramegowda and Senthil-Kumar, 2015; Berens *et al.*, 2019; Gull *et al.*, 2019). Stress-combination studies demonstrated that ABA might play a role in the modified response of plants to drought and heat stress exposure, either in combination or alone. For instance, upon exposure to either drought or heat stress alone, the accumulation of the 9-cis-epoxy-carotenoid dioxygenase (NCED) protein essential for ABA synthesis was significantly upregulated in poplar (Li *et al.*, 2014). In contrast, in drought + heat stress, NCED protein accumulation first increased and then decreased. These findings show differential ABA regulatory activities under single and combinatorial stressors. In addition, ABA serves as a pivotal regulator of water status and stomatal activity. During water stress, plants elicit and accumulate ABA, which causes stomatal closure, leading to water conservation. The cellular and molecular pathways involved in ABA-induced stomatal closure have been comprehensively studied and reviewed (Lim *et al.*, 2015; Zhao *et al.*, 2017; Niu *et al.*, 2018; Bharath *et al.*, 2021). In addition to contributing to water management under drought conditions, stomatal closure acts as a protective mechanism to stop pathogen incursions. In addition to involved in stomatal closure that restricts pathogen entry, ABA also influences pathogen interactions by intervening with other hormones involved in plant defense mechanisms (Oide *et al.*, 2013; Lim *et al.*, 2015; Boba *et al.*, 2020).

Plants identify pathogen-associated molecular patterns (PAMPs), which activate innate immunity. It was reported that PAMPs participate in closing the stomata and curbing the entry of pathogens (Choi and Klessig, 2016). However, some studies using *ost1* mutants that do not respond to ABA (Mustilli *et al.*, 2002) and ABA-deficient *aba3-1* mutants (Léon-Kloosterziel *et al.*, 1996) have shown that stomatal closure is not caused by PAMPs. Therefore, it has been suggested that in guard cells, PAMP-regulated stomatal closure involves activation of the ABA signaling pathway. Consequently, by activating stomatal closure, ABA does indeed have a beneficial impact on disease resistance. Tossi *et al.* (2014) reported that UV-B irradiation in *Arabidopsis thaliana* enhanced the production of ABA and the synthesis of nitric oxide (NO) and ROS, thus contributing to stomatal closure. Abscisic acid-induced stomatal closing might



be more significant for acclimating plants to the drought + UV-B stress combination than for modifying plant responses to the drought + heat stress conditions.

Several plant hormones, such as SA, JA, and ethylene, play a significant role in pathogen resistance. Generally, SA is associated with systemic acquired resistance and biotrophic pathogen resistance, whereas JA and ethylene are associated with induced systemic resistance and necrotrophic pathogen resistance (Glazebrook, 2005; Pieterse *et al.*, 2009). Abscisic acid is associated with the SA, JA, and ethylene signaling pathways and functions by working either synergistically or antagonistically with these hormones (Anderson *et al.*, 2004; Mosher *et al.*, 2010). At the gene transcription level, ABA has been shown to have a deleterious effect on JA and ethylene-dependent pathogen resistance (Anderson *et al.*, 2004). Abscisic acid-deficient mutants triggered the usual JA and ethylene marker genes, including *PDF1.2* and *CHI*, more intensely than the wild-type *Arabidopsis*; however, exogenous ABA application downregulated these genes (Anderson *et al.*, 2004). A transcriptomic study of salinity and heat individual stresses and their combination in *Arabidopsis thaliana* found that the expression of 699 transcripts was explicitly stimulated in response to stress combination (Suzuki *et al.*, 2016). Strangely, among these 699 transcripts, those correlated with the ABA signaling pathway were extensively characterized. In comparison, transcripts associated with SA and gibberellic acid, which might antagonize the signaling mechanism of ABA, were the least influential (Ishibashi

*et al.*, 2012). Recent studies have discovered a complicated coordination mode between various hormone signals in *Arabidopsis* and other plants in response to drought, heat, and their combination. For instance, ABA accumulates swiftly in citrus plants in response to drought treatment alone, whereas a combination of drought and heat stresses causes a surge in ABA accumulation, although to a considerably lesser amount compared with that under drought alone (Zandalinas *et al.*, 2016a). Unlike ABA, a higher level of SA, accumulated under the drought + heat stress combination compared to its levels under individual stress application, may be implicated in the suppression of the ABA signaling pathway (Moeder *et al.*, 2010). Under these single and combined stresses, citrus plants showed a different ABA and SA accumulation profile to that of *Arabidopsis*, which suggested a maximum or minimum amount of ABA or SA accumulation, respectively, under identical scenarios (Zandalinas *et al.*, 2016a).

#### Reactive oxygen species

The main variables that limit crop productivity and yield are abiotic stresses, such as heavy metals, water stress, salinity, and high temperature. These stresses are linked to the generation of specified harmful chemical entities known as ROS (Fig. 1), hydrogen peroxide ( $H_2O_2$ ), superoxide radical ( $O_2^{\cdot-}$ ), hydroxyl radical, and others. Reactive oxygen species can induce cellular damage through protein degradation, enzyme inactivation, gene alterations, and interference with

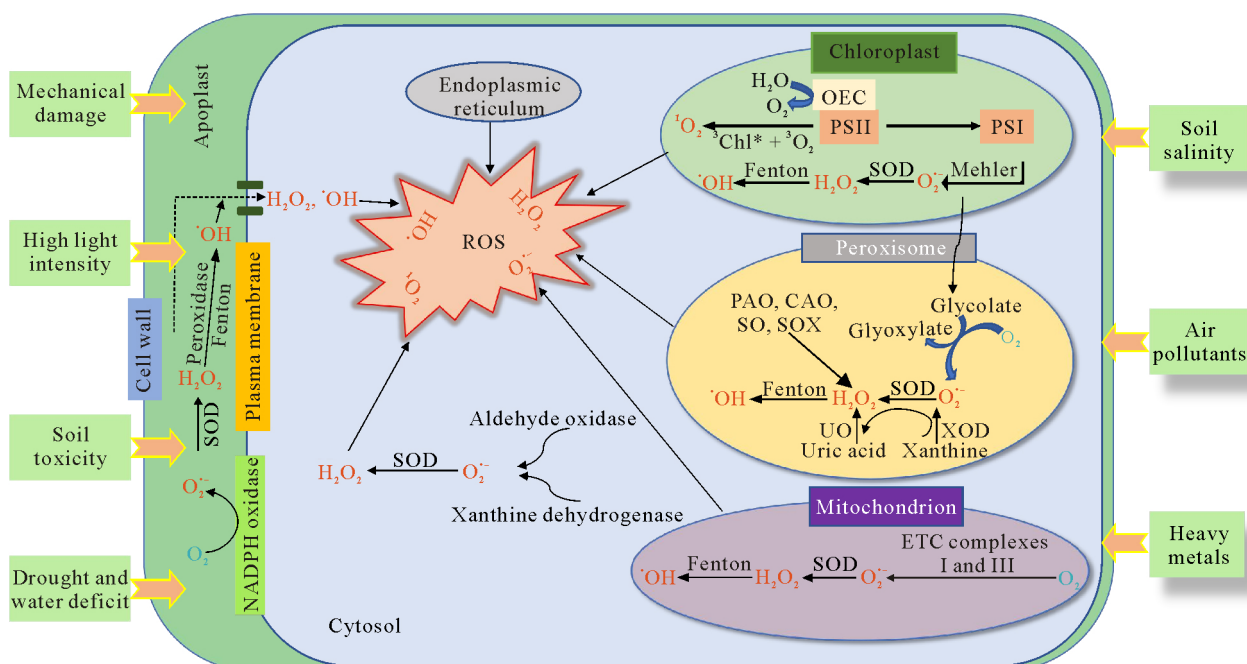


Fig. 1 Production of reactive oxygen species (ROS) in plants in relation to different abiotic stress factors (modified from Hasanuzzaman *et al.* (2020)). SOD = superoxide dismutase; NADPH = nicotinamide adenine dinucleotide phosphate; OEC = oxygen-evolving complex; PSI = photosystem I; PSII = photosystem II;  $^1O_2$  = singlet oxygen;  $^3O_2$  = triplet oxygen;  $^3Chl^*$  = triplet excited chlorophyll; UO = urate oxidase; XOD = xanthine oxidase; PAO = polyamine oxidase; CAO = copper amine oxidase; SO = sulfite oxidase; SOX = sarcosine oxidase; ETC = electron transport chain.

various metabolically essential pathways. It has been concluded from multiple studies that during stress combination, the specific sequence of ROS accumulation, synthesis of antioxidants, and expression of different scavenging enzymes is comparative to the sequence obtained by introducing these stresses individually. These modifications have been displayed in the levels of  $O_2^-$  and  $H_2O_2$ , expression of enzymes such as peroxidases, glutathione-S-transferase, and glutathione reductase, and concentrations of antioxidants and osmoprotectants such as proline, glycine betaine, trehalose, and sucrose (Rivero *et al.*, 2014; Suzuki *et al.*, 2014; Pandey *et al.*, 2015; Vuleta *et al.*, 2015; Carvalho *et al.*, 2016; Jin *et al.*, 2016; Martinez *et al.*, 2016).

It has been reported that transgenic plants overexpressing ROS scavengers or their mutants with greater ROS-scavenging capacity exhibit improved resistance to environmental stresses, suggesting that ROS leads to stress damage (Hasegawa *et al.*, 2000; Kocsy *et al.*, 2001). Upon penetration of the vascular pathogen *Verticillium* spp., ROS is implicated in stress-induced tolerance in *Arabidopsis thaliana* by escalating drought tolerance because of the development of *de novo* xylem and the consequent increase in water flow (Xia *et al.*, 2009). Brassinosteroid-treated cucumber plants caused an increase in  $H_2O_2$  levels and prepared the plant for biotic and abiotic stress tolerance (Xia *et al.*, 2009). The salt resistance activation of  $H_2O_2$  in citrus significantly increased the concentrations of oxidized and S-nitrosylated proteins, which persisted after stress treatment, whereas non-treated plants were more stress-sensitive (Tanou *et al.*, 2009). Hypoxic or anoxic conditions caused by flooding or waterlogging (WL) produce toxic compounds that impede plant metabolism, resulting in ROS overproduction and oxidative damage (Loreti *et al.*, 2016). Anee *et al.* (2019) studied the WL-sensitive *Sesamum indicum* for different time periods (2, 4, 6, and 8 d) of WL stress and found that malondialdehyde (MDA) and  $H_2O_2$  increased in a time-dependent manner. *Solanum lycopersicum* showed an equivalent increase in these indicators of oxidative stress (Rasheed *et al.*, 2018). After exposure to WL conditions for 7 d, an Antarctic plant, *Deschampsia antarctica*, generated increased MDA and  $H_2O_2$  (Park and Lee, 2019). Furthermore, mutants lacking the function of the ABA- and ROS-regulated protein PP2Cs (*abi1*) have been shown to be particularly susceptible to drought and heat stress combinations, along with salinity and heat, indicating the involvement of ROS-ABA interactions in plant tolerance to stress combinations (Suzuki *et al.*, 2016; Zandalinas *et al.*, 2016a).

A vast number of studies have shown increased ROS response transcripts as a significant constituent of the stress combination-acclimatization response pathway, highlighting the importance of ROS in plant acclimation to stress combinations (Suzuki *et al.*, 2014). In this expanding and essential

line of plant stress research, novel roles for ROS in plant adaptation to stress combinations will likely be highlighted in future studies. Moreover, ROS and ABA appear to be two of the major influencers that facilitate the acclimatization of plants to stress combinations for the time being.

## CONCLUSIONS

Plant sciences have achieved major advances in elucidating multiple abiotic and biotic stress effects. We have described the versatile role of abiotic stress and biotic stress conditions when plants are exposed to stress combinations, such as the simultaneous impact of abiotic stress conditions (high temperature, high salt, drought, *etc.*) along with biotic stress conditions. Transcriptomics, metagenomics, and metabolomics data have been used to address the bottleneck of plant-stress interaction research. Interestingly, transcriptome data of plants exposed to a combination of stress stimuli indicate that the number of genes that are differentially expressed might be predictable from the combination of single stress. However, key knowledge gaps remain, especially regarding the biological relevance of the changes in rhizosphere microbiome under different stress conditions. Understanding how plant responses to stress conditions are initiated will allow us to answer fundamental questions on how plants induce biochemical, physiological, and molecular modifications regulated by abiotic, biotic, and both stress types. It will also help us to understand what is required for a plant to tolerate stressful conditions. Such an increased understanding of plant-stress connections in natural systems is strongly needed, and, as this review shows, molecular research efforts in crops have to be strengthened considerably.

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