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# 学位論文全文に代わる要約
# Extended Summary in Lieu of Dissertation


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学位論文題目：
Title of Dissertation　Tebuconazole and Trifloxystrobin-Mediated Abiotic and Biotic Stress Regulation in Plants: In Relation to Reactive Oxygen Species and Antioxidant Defense Systems (テブコナゾールとトリフロキシストロビンに媒介された植物における非生物的および生物的ストレス制御：活性酸素種と抗酸化防御システムについて)

学位論文要約：
Dissertation Summary

**Background of the study**

The world population increased many folds in the last century, by mid-2015 already crossed 7.3 billion and projected that world population will be reached 9.8 billion in 2050. For such large population world food demand also will be increased and projected that it will be 14.88 billion tons in 2050 (Islam and Karim 2019, Fig. 1). Therefore, in future, food security will be an important and priority issue for many nations especially for developing countries.

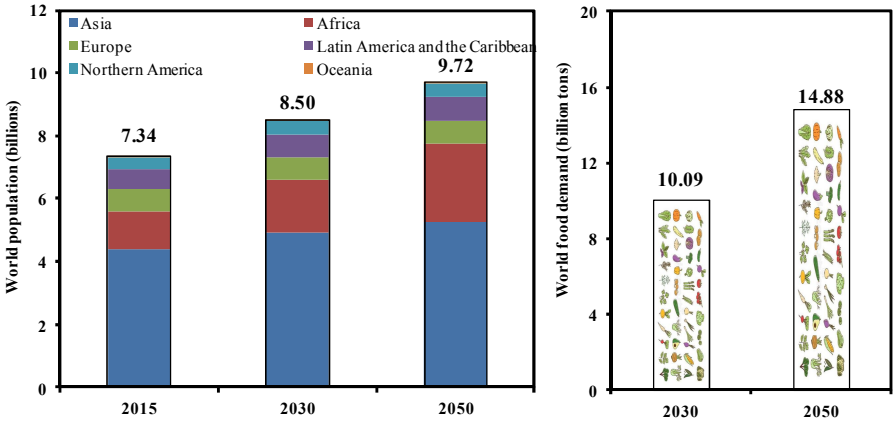


**Fig. 1** The projected world population and world food demand in the year 2050

Moreover, many abiotic and biotic factors are responsible to change the normal plant growing environment. As a sessile organism, plant cannot move to other places and faces various abiotic and biotic stresses, results in reduce crop productivity (Fraire-Velázquez and Balderas-Hernández 2013). Plants are always suffering by various abiotic stresses such as salinity, drought, metal toxicity, flooding, extreme temperature, nutrient deficiency, etc. and also affect by many biotic stresses such as fungi, bacteria, viruses, and insects thus cause the reduction of plant growth and yield significantly (Fig. 2). When plants attack by abiotic or/and biotic stresses, a series of physiological and biochemical mechanism changes occur, therefore, adversely affect the growth and productivity

of plants. Abiotic stresses, including salinity and cadmium (Cd) toxicity are serious threats to world agricultural productivity and future food sustainability.

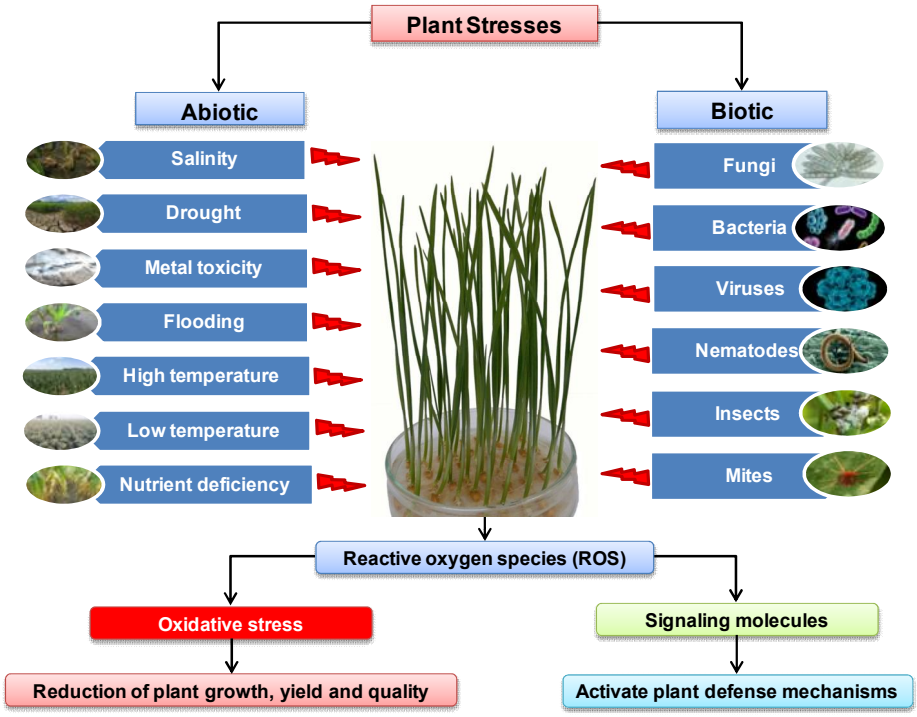


**Fig. 2** The common abiotic and biotic stressors in plants

Salinity significantly affects the agricultural crops, in the regions where crops are irrigated with saline water (Daliakopoulos et al. 2016; Mohsin et al. 2020a). About 20% of the world's irrigated land are currently affected by salinity, resulting in huge yield losses annually and restricting global food production (Liu et al. 2019; Ren et al. 2019). Plant growth and productivity are severely hampered by salinity because high salt levels create osmotic and ionic stresses that promote the generation of reactive oxygen species (ROS) and nutritional imbalances (Mohsin et al. 2020b). Higher salt concentration increases the amount of $Na^+$ in plant cells and inhibit the uptake of $K^+$ thus leading to ionic stress and disturbance of the intracellular ionic balance (Parvin et al. 2020). Therefore, hampering the $K^+$–mediated cellular mechanisms such as photosynthesis, enzymatic activity, and protein synthesis resulting generation of higher ROS such as singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\bullet-}$) hydroxyl radical ($^{\bullet}OH$), peroxide radical ($HO_2^{\bullet}$), peroxide ion ($HO_2^-$), etc. (Mohsin et al. 2019). Heavy metal, Cd toxicity triggers physiological changes in plants, including seed germination inhibition, growth retardation, and photosynthesis, transpiration, and respiration reduction (Khanna et al. 2019). At the cellular level, Cd inhibits function of electron transport and biosynthesis of chlorophyll (Chl) by interacting with different photosynthetic components (Guo et al. 2018). Cadmium alters cellular homeostasis by disrupting membrane integrity and also changes the function of hormone and antioxidant enzyme activity (Nahar et al. 2016; Mohsin et al. 2021). Usually, Cd is a redox inactive metal, which means it does not directly initiate the generation of ROS in plant cells (Rahman et al. 2016). However, it induces oxidative stress by disturbing the mineral balance, inhibiting the electron transport chain function, and reducing the antioxidant defense systems (Bhuyan et al.

2020). Cadmium-induced oxidative stress leads to the higher ROS generation resulting in damage to important cellular components such as proteins, DNA, and lipids (Hasanuzzaman et al. 2017; Raza et al. 2020). Pathogenic infection also responsible to hamper plant growth and productivity by increasing ROS-induced oxidative damage (Xu and Tian 2008).

In plants, ROS initially contribute to plant tolerance/disease resistance by showing antimicrobial activity, phytoalexin accumulation, cell wall strengthening, and acting as signalling molecules under stress conditions (Debona and Rodrigues 2016). However, the imbalance ROS production disrupts cell membrane integrity and normal cellular function by oxidizing carbohydrates, lipids, proteins, and nucleic acids (Fig. 3). Reactive oxygen species are considered as the main source of cell damage under both biotic and abiotic stresses (Kumar et al. 2015). Oxidative stress depends on the balance between production and scavenging of ROS, which in turn depends on growth conditions, stress duration, and the ability of the tissue to survive under imbalanced conditions (Costantini 2019).

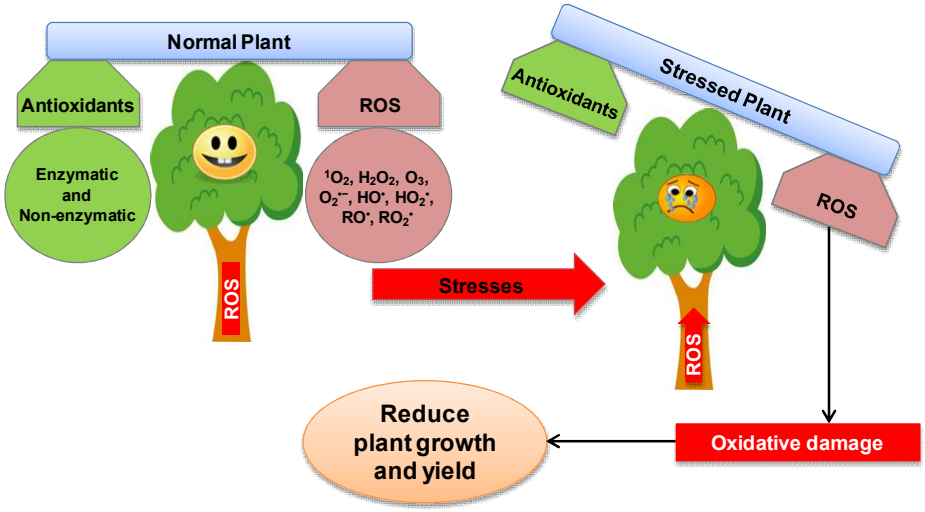


**Fig. 3** Oxidative damage in organisms by higher ROS generation under stresses

Plants can tolerate abiotic and biotic stresses by activating different molecular and biochemical responses at the cellular or whole-plant levels. The most common cellular strategies are ion absorption and exclusion, transfer of ions into the vacuole, organic solute synthesis in cytoplasm, and modification of membrane composition (Min et al. 2018). Plants have also developed several strategies to mitigate negative effect of abiotic and biotic stresses of at the whole-plant level, such as regulation of ion absorption by the roots, transportation to different plant parts, modification of enzymatic and non-enzymatic antioxidants, alteration of photosynthetic mechanisms, and induction of various plant hormone responses (Liu et al. 2018). The enzymatic (superoxide dismutase, SOD; catalase, CAT; peroxidase, POD; peroxidases, POX; polyphenol oxidase, PPO; peroxiredoxins, PRX; thioredoxin, TRX; ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR; glutathione peroxidase, GPX; and glutathione *S*-transferase, GST) and non-enzymatic (ascorbate, AsA; glutathione, phenolic compounds, alkaloids, non-protein amino acids,

α-tocopherols, etc.) components are involved in this system to mitigate ROS (Pompeu et al. 2017; Mohsin et al. 2021; Fig. 4). Plant stresses also initiate the generation of another cytotoxic compound, methylglyoxal (MG) that causes oxidative stress by damaging ultrastructural cellular components. However, glyoxalase–I (Gly–I) and glyoxalase–II (Gly–II) are two thiol-dependent enzymes, which can detoxify MG in the glyoxalase system (Mohsin et al. 2020b; Fig. 5).

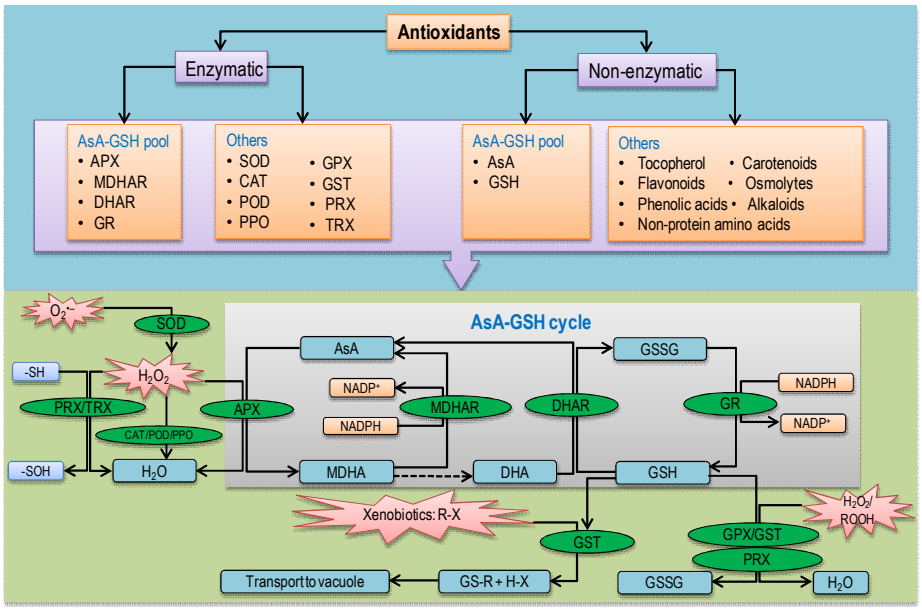


**Fig. 4** Antioxidant defense systems to detoxify overproduced ROS in living organisms

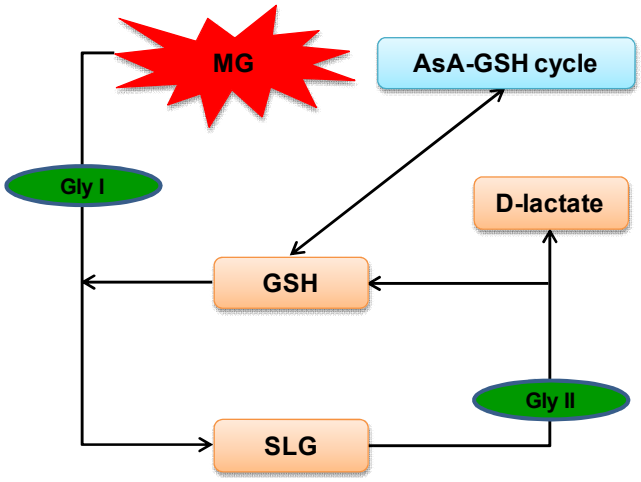


**Fig. 5** Mechanisms of MG detoxification by glyoxalase systems in living organisms

Moreover, sometimes plant antioxidant defense systems is not sufficient to minimize abiotic and biotic stress-induced oxidative damage due to higher cellular injury. Therefore, exogenous strategies are necessary to improve antioxidant defense systems for maintaining redox homeostasis in plant cells. Plant scientists are using various possible strategies such as plant breeding, transgenic approach, genome editing, and marker assisted

selection to develop tolerant/resistant varieties but these are costly and time consuming (Fig. 6). Due to low cost and easy application method, the use of phytoprotectants such as signaling molecules, osmolytes, plant hormones, trace elements, etc. becomes most popular technique to enhance plant stress tolerance (Hasanuzzaman et al. 2018). Stress tolerance of crop plants can also be aided by the use of synthetic chemicals that can function as phytoprotectants. Positive results of plant stress tolerance by exogenous phytoprotectants already reported by osmolites (Hasanuzzaman et al. 2014), polyamines (PAs) (Nahar et al. 2016), phenolic compounds (Parvin et al. 2019, 2020; Bhuyan et al. 2020), 2,4-D herbicides (Mohsin et al.2020b) etc.

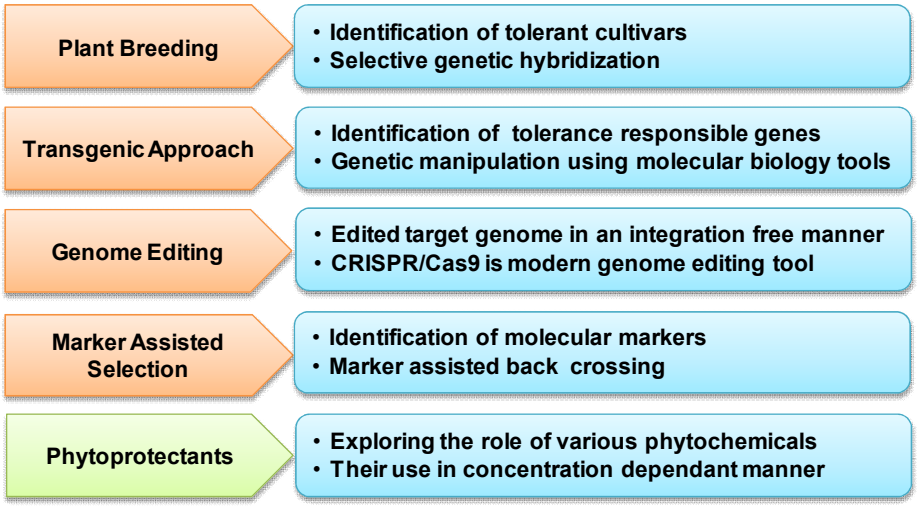


**Fig. 6** Possible strategies to make stress tolerance in plants

Fungicides are chemicals, which are normally used to control plant diseases by destroying disease-causing fungi. However, some fungicides such as triazole and strobilurin, are able to provide protection against both abiotic and biotic stresses. Strobilurin fungicides increase net photosynthesis in plants due to causes temporary reduction of respiration results in generating more energy thus improve plant physiology. This group of fungicides also helps to improve plant stress tolerance by increasing the activity of antioxidant enzymes and nitrate reductase. In addition, strobilurins increase the synthesis of abscisic acid (ABA), isopentenyl adenine (I6-ADE), and indoleacetic acid (IAA) in plants and indirectly improve the PAs synthesis by reducing the production of ethylene, therefore, helps to regulate the hormonal balance and leaf senescence in plants (Amaro et al. 2018). Another group of fungicides, triazoles also improves the plant growth by increasing ABA, PAs, and cytokinin synthesis, but inhibit the synthesis of gibbrellic acid (Fletcher et al. 2000; Fig. 7). The strobilurins also known as quinone outside inhibitors (QoIs), regulate the fungal growth by inhibiting mitochondrial respiration. These fungicides bind at the Qo site of cytochrome b that inhibit the transfer of electron between cytochrome b and c1, thus disrupts the energy cycle in fungal cells (Kunova et al. 2013). In fungal cell, triazole fungicides interfere with ergosterol biosynthesis which is the vital cellular component (Guirao-Abad et al. 2017). This fungicide increases the cytochrome-P450-dependant oxidase enzyme activity that causes demethylation of ergosterol through oxidative mechanisms. The inhibition of sterol synthesis decreases the membrane stability and initiate oxidative stress, thus cause cellular damage (Mohsin et al. 2019; Fig. 8).

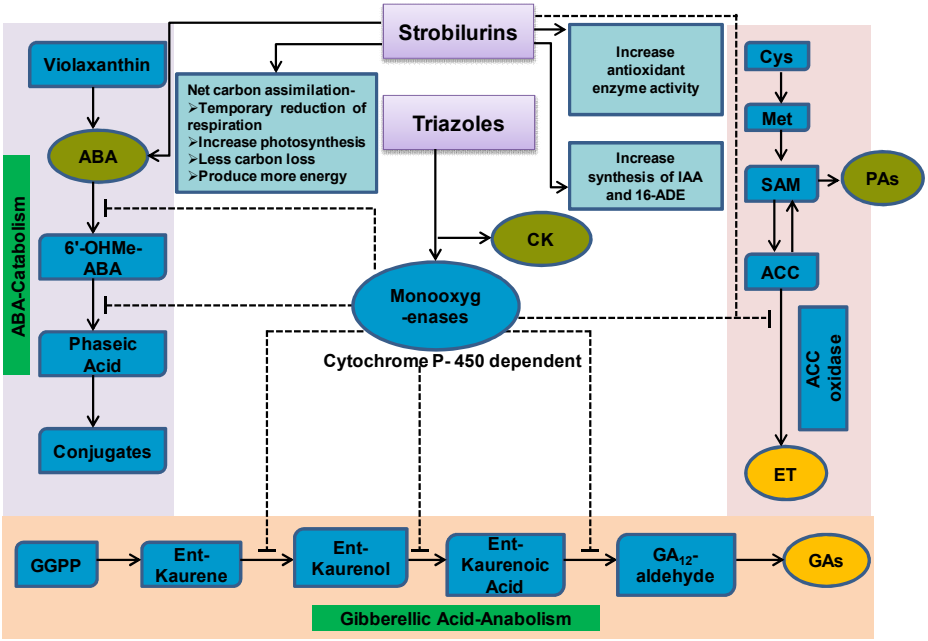


**Fig. 7** Mechanisms of strobilurins and triazoles fungicides to improve plant tolerance under abiotic stresses

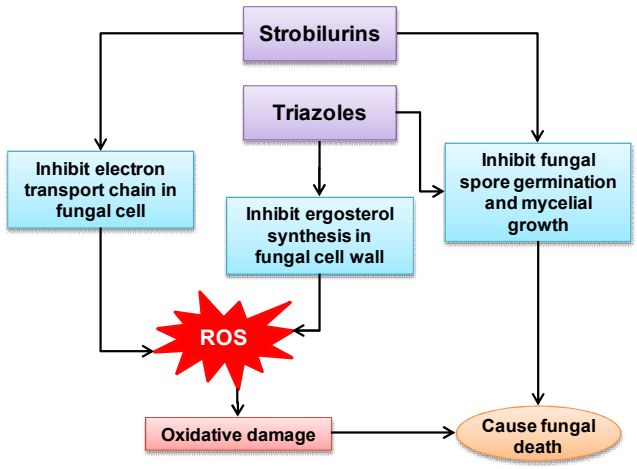


**Fig. 8** Mechanisms of strobilurins and triazoles fungicides to regulate fungal growth by causing cellular oxidative damage

Therefore, we hypothesized that strobilurin and triazole fungicides could regulate both abiotic and biotic stresses in plants by upregulating antioxidant defense systems. Thus, we have planed to study the effect of tebuconazole (TEB, triazole group) and trifloxystrobin (TRI, strobilurin group) on plant abiotic and biotic stresses in response of antioxidant defense mechanisms. Tebuconazole [(*R,S*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*1,2,4-triazol-1-ylmethyl)pentan-3-ol] is a systemic fungicide contain a chiral centre in the structure that is broadly used to control various plant diseases (Liu et al. 2015). Trifloxystrobin methyl (*E*)-methoxyimino-{(*E*)-α-[1-(α,α,α-trifluoro-m-tolyl) ethylideneaminooxy]-o-tolyl}acetate is a systemic fungicide and first strobilurin compound with an oximether side chain. The presence of the methyl in the oximether moiety and the trifluoromethyl group in the

phenyl moiety provides the bioefficacy against a variety of pathogenic microorganisms (Banerjee et al. 2006). Considering all these aspects, we investigated the potential role of TEB and TRI for abiotic and biotic stress regulation in wheat (for salinity and Cd stress), cucumber (for salinity stress), sweet potato (for black rot disease caused by *Ceratocystis fimbriata*), and citrus (for black rot disease caused by *Alternaria citri*) with the following objectives–

i. To investigate the physiological and biological responses under abiotic and biotic stresses in plants

ii. To identify the roles of TEB and TRI on antioxidant defense systems in plants under abiotic and biotic stress

iii. To observe the growth morphology, oxidative damage and antioxidant defense mechanisms in fungi after application of TEB and TRI

To accomplish these objectives, several experiments were conducted and the findings are described in five different titles as follows–

1. Tebuconazole and trifloxystrobin regulate the physiology, antioxidant defense and methylglyoxal detoxification systems in conferring salt stress tolerance in *Triticum aestivum* L.
2. Exogenous tebuconazole and trifloxystrobin regulates reactive oxygen species metabolism toward mitigating salt-induced damages in cucumber seedling
3. Protective role of tebuconazole and trifloxystrobin in wheat (*Triticum aestivum* L.) under cadmium stress via enhancement of antioxidant defense and glyoxalase systems
4. Effect of tebuconazole and trifloxystrobin on *Ceratocystis fimbriata* to control black rot of sweet potato: In processes of reactive oxygen species generation and antioxidant defense responses
5. Triazole and strobilurin fungicides-mediated citrus black rot disease management: Modulation of antioxidant defense systems in citrus and *Alternaria citri*

**Experiment 1: Tebuconazole and trifloxystrobin regulate the physiology, antioxidant defense and methylglyoxal detoxification systems in conferring salt stress tolerance in *Triticum aestivum* L.**

**Meterials and methods**

**Plant materials and test conditions**

Healthy and uniform seeds of the Japanese salt-sensitive wheat (*T. aestivum* L.) cultivar Norin 61 (Muranaka et al. 2002) were selected and sterilized in 70% ethanol for 10 min, then rinsed and soaked in distilled water ($dH_2O$) for a further 4 h. The seeds were germinated on moistened filter paper kept in darkness for 48 h, at 25 °C, and then transferred to a growth chamber maintained at 25 ± 2 °C, relative humidity of 65–70%, and 350 μmol photon $m^{-2}$ $s^{-1}$; 3300-fold diluted Hyponex was used as a source of nutrients. The nutrient solution contained N (8%), K (20.94%), P (6.43%), Ca (11.8%), Mg (3.08%), Mn (0.03%), Fe (0.24%), B (0.07%), Cu (0.003%), Zn (0.008%), and Mo (0.0014%). Prior to the main experiment, several trial experiments of different concentrations (from 0.01 to 100 μM) of TEB and TRI were conducted on wheat seedlings, and we selected 1.375 μM for TEB and 0.5 μM for TRI, which showed better performance under 250 mM NaCl as determined by phenotypic responses and

malondialdehyde (MDA) content. Three-day-old seedlings were pre-treated with fungicide (1.375 µM TEB+0.5 µM TRI) for 48 h, and then the resulting five-day-old seedlings were subjected to salt stress (250 mM NaCl) for the next five days. Control plants were grown in nutrient (Hyponex, Japan) solution only. The nutrient solution was renewed at every two days interval. The experiment was conducted following a completely randomized design (CRD) with three replications, and repeated thrice under the same conditions.

**Observation of physiological and biochemical parameters**

Ten-day-old seedlings were used to determine plant height, shoot fresh weight (FW) and dry weight (DW), root FW and DW, leaf relative water content (RWC), proline content, photosynthetic pigments content (Chl *a*, Chl *b* and carotenoid; Car), oxidative stress markers contents (MDA, $H_2O_2$, electrolyte leakage: EL, and MG), non-enzymatic antioxidant contents (AsA and reduced glutathione, GSH), enzymatic antioxidant activities (CAT, APX, MDHAR, DHAR, GR, GPX, and GST), glyoxalase enzymes activities (Gly-I and Gly-II), and minerals contents ($Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) by following established standard methods.

**Statistical analysis**

The measured data were statistically analyzed using XLSTAT 2018 software (AddinSoft 2018); three replications were used for analysis of variance (ANOVA). The mean differences were compared using Fisher's least significant difference (LSD) test at the 5% level of significance.

**Results**

Salt-induced ionic and osmotic stress a negatively affected the growth and physiology of the wheat seedlings, but TEB and TRI alleviated the salt toxicity (Fig. 9). Salt treatment alone resulted in oxidative damage and increased lipid peroxidation as evident by higher MDA and $H_2O_2$ content. Salt stress also decreased the Chl and RWC and increased the proline content. Furthermore, salt stress increased the dehydroascorbate (DHA) and glutathione disulfide (GSSG) content while AsA, AsA/DHA ratio, GSH and GSH/GSSG ratio decreased. However, a combined application of TEB and TRI significantly alleviated growth inhibition, photosynthetic pigments and leaf water status improved under salt stress. Application of TEB and TRI also decreased MDA, EL, and $H_2O_2$ content by modulating the contents of AsA and GSH, and enzymatic antioxidant activities. In addition, TEB and TRI regulated $K^+/Na^+$ homeostasis by improving the $K^+/Na^+$ ratio under salt stress. These results suggested that exogenous application of TEB and TRI rendered the wheat seedling more tolerant to salinity stress by controlling ROS and MG production through the regulationof the antioxidant defense and MG detoxification systems (Fig. 10).


**Fig. 9** Phenotypic appearance of ten-day-old wheat seedlings under different treatments. TT and S indicate 1.375 µM TEB+0.5 µM TRI and 250 mM NaCl, respectively

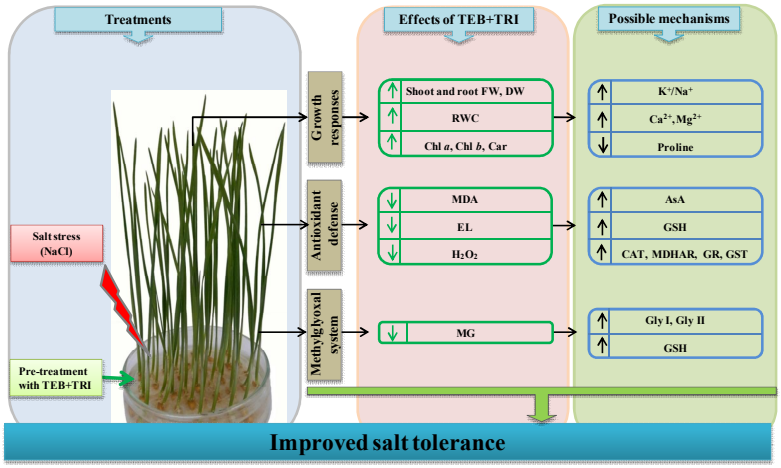


**Fig. 10** Possible mechanisms of TEB and TRI in improving salt tolerance in wheat seedlings based on the present investigation

**Experiment 2: Exogenous tebuconazole and trifloxystrobin regulates reactive oxygen species metabolism toward mitigating salt-induced damages in cucumber seedling**

**Materials and methods**

**Plant materials and test conditions**

Healthy and uniform cucumber (*Cucumis sativus* L. cv. Tokiwa) seeds were selected to perform this experiment. Cucumber plants were grown in a glasshouse at normal light conditions on 25–28 °C air temperature and relative humidity of 60–70%. Seedling plug trays were used for sowing the seeds, which filled with the mixture of vermiculite and peat at the ratio of 1:2 (v/v). After twenty-nine days of seed sowing, first true leaf was developed and then seedlings were transferred to plastic pots using grow rock and half-strength Hoagland solution was used as nutrients (Hoagland and Arnon 1950). After every 3 days nutrient solutions were changed during the growing period. Twenty-one days after transplanting the plants were exposed to salt (60 mM NaCl) and fungicides (1.375 µM TEB + 0.5 µM TRI; and 2.75 µM TEB + 1.0 µM TRI) solely and in combination for next 6 days. Un-treated plants were grown with nutrient solution only. The experiment was arranged in a CRD and each treatment was replicated three times.

**Observation of physiological and biochemical parameters**

Plants were used to determine plant height, number of leaves plant$^{-1}$, internodes length, shoot FW and DW, root FW and DW, RWC, proline content, photosynthetic pigments content (Chl *a*, Chl *b* and Car), oxidative stress markers contents (MDA, $H_2O_2$, and EL), non-enzymatic antioxidant contents (AsA and GSH), enzymatic antioxidant activities (CAT, APX, MDHAR, DHAR, GR, and GST), and minerals contents ($Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) by following established standard methods.

**Statistical analysis**

The measured data were statistically analyzed using XLSTAT 2018 software (AddinSoft 2018); three replications were used for analysis of variance (ANOVA). The mean differences were compared using Fisher's least significant difference (LSD) test at the 5% level of significance.

**Results**

The application of salt phenotypically deteriorated the cucumber plant growth that caused yellowing of the whole plant and significantly destructed the contents of Chl and Car (Fig. 11). The oxidative damage was created under salinity by increasing the contents of MDA, $H_2O_2$, and EL resulting in the disruption of the antioxidant defense system. Furthermore, in the leaves, stems, and roots of cucumber plants increased $Na^+$ content was observed under salt stress, whereas the $K^+/Na^+$ ratio and contents of $K^+$, $Ca^{2+}$, and $Mg^{2+}$ decreased. In contrast, the exogenous application of TEB and TRI reduced the contents of MDA, $H_2O_2$, and EL by improving the activities of enzymatic and non-enzymatic antioxidants. In addition, ion homeostasis was regulated by reducing $Na^+$ uptake and enhanced $K^+$ accumulation and the $K^+/Na^+$ ratio after application of TEB and TRI. Therefore, this study indicates that the exogenous application of TEB and TRI enhanced salt tolerance in cucumber plants by regulating reactive oxygen species production and antioxidant defense systems (Fig. 12).

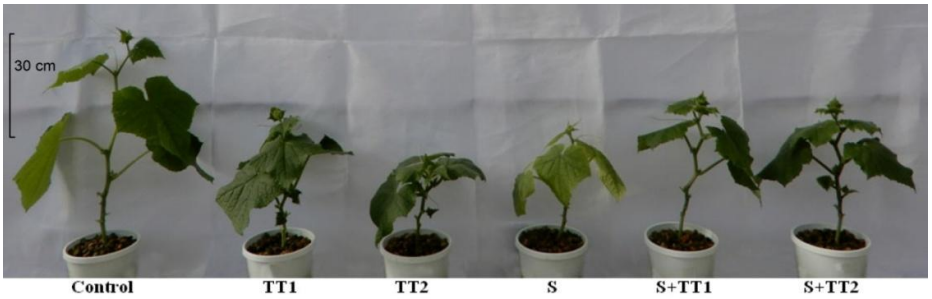


**Fig. 11** Phenotypic appearance of cucumber plants under different treatments. (TT1, 1.375 µM TEB+0.5 µM TRI; TT2, 2.75 µM TEB+1.0 µM TRI; S, 60 mM NaCl; respective treatments were applied on 50–d old plants for 6 days)

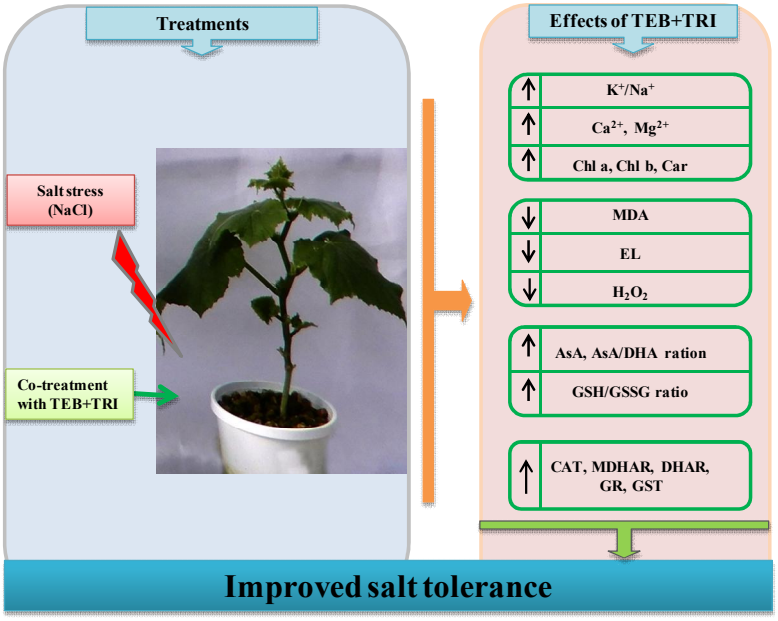


**Fig. 12** Possible mechanisms of TEB and TRI in improving salt tolerance in cucumber plants based on the present investigation

**Experiment 3: Protective role of tebuconazole and trifloxystrobin in wheat (*Triticum aestivum* L.) under cadmium stress via enhancement of antioxidant defense and glyoxalase systemsMaterials and methods**

**Materials and methods**

**Growth condition and stress treatments**

Wheat (*T. aestivum* L. cv. Norin 61) seeds were grown hydroponically. The seeds were sterilized (by 70% ethanol) and soaked in $dH_2O$ for 4 h. After soaking, the seeds were placed in Petri dishes (9 cm diameter) in an incubator at 25 °C for 48 h for germination. Following germination of the seeds, the seedlings were grown in a growth chamber maintaining proper relative humidity (65–70%), temperature (25±2°C), and light (350 µmol photon $m^{-2}$ $s^{-1}$). Hyponex solution (3300-fold diluted) was used as a nutrient solution. Prior to the experiment, several trials were conducted using different concentrations of TEB and TRI (from 0.01 to 100 µM) on wheat seedlings, and finally 2.75 µM for TEB and 1.0 µM for TRI were selected because they showed better phenotypic responses under 0.25 and 0.5 mM Cd stress. The seedlings (five-day-old) were exposed to Cd stress (0.25 and 0.5 mM $CdCl_2$ as mild and severe doses, respectively) alone and in combination with fungicides (2.75 µM TEB + 1.0 µM TRI) for four days. Only nutrient solution was used for untreated seedlings. The experiment was designed following a CRD with three repetitions.

**Observation of physiological and biochemical parameters**

Nine-day-old seedlings were used to determine shoot FW and DW, root FW and DW, RWC, proline content, photosynthetic pigments content (Chl *a*, Chl *b* and Car), oxidative stress markers contents (MDA, $H_2O_2$, EL, lipoxygenase: LOX activity, and MG), histocchemical detection of ROS, non-enzymatic antioxidant contents (AsA and GSH), enzymatic antioxidant activities (SOD, POD, CAT, APX, MDHAR, DHAR, GR, GPX, and GST), glyoxalase enzymes activities (Gly-I and Gly-II), and Cd and minerals contents ($Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) by following established standard methods.

**Statistical analysis**

The measured data were statistically analyzed using XLSTAT 2020 software (AddinSoft 2020); three replications were used for analysis of variance (ANOVA). The mean differences were compared using Fisher's least significant difference (LSD) test at the 5% level of significance.

**Results**

Cadmium toxicity hampered the physiological and biochemical functions of wheat seedlings (Fig. 13). Compared to control, the level of $H_2O_2$ in the seedlings exposed to mild and severe Cd stress alone increased by 81 and 112%, respectively. The accumulation of Cd also increased in the wheat seedlings along with declining mineral nutrients under Cd stress. The protective effect of TEB and TRI was observed with the enhancement of the antioxidant defense and methylglyoxalase systems and reduction in oxidative damage. Applying TEB and TRI reduced MDA (by 9 and 18%), EL (by 21 and 17%), MG (by 12 and 17%), and LOX activity (by 37 and 27%), respectively, relative to Cd stress alone. Cadmium uptake also decreased in the shoots (by 48 and 50%, respectively) and roots (by 23 and 25%, respectively) of the fungicide treated wheat seedlings under mild and severe Cd stress, relative to stress alone. These results indicate the exogenous application of TEB and TRI is a promising approach to improve Cd tolerance in wheat plants (Fig. 14).

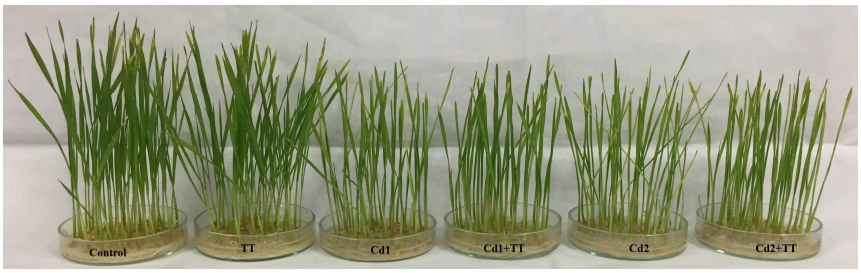


**Fig. 13** Effect of TEB (2.75 µM) and TRI (1.0 µM) on appearance of nine-day-old hydroponically grown wheat seedlings under Cd stress (0.25 and 0.5 mM $CdCl_2$, for four days). TT, Cd1, and Cd2 indicate 2.75 µM TEB+1.0 µM TRI, 0.25 mM $CdCl_2$, and 0.5 mM $CdCl_2$, respectively

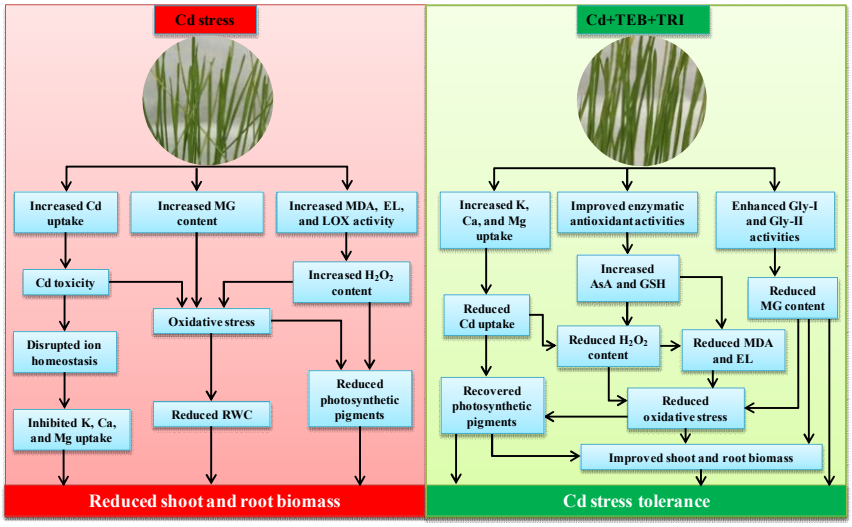


**Fig. 14** Possible mechanisms of TEB and TRI in improving cadmium tolerance in wheat seedlings based on the present investigation

**Experiment 4: Effect of tebuconazole and trifloxystrobin on *Ceratocystis fimbriata* to control black rot of sweet potato: In processes of reactive oxygen species generation and antioxidant defense responses**

**Materials and methods**

**Pathogen inoculation and incubation of sweet potato**

Sweet potato root parenchymatous tissues were cut into 3 cm diameter disks and surface sterilized with 1% sodium hypochlorite and rinsed several times in sterilized $dH_2O$, then placed on the net in a transparent plastic box and maintained high humidity by using two layers of wet paper towel. Prior to the experiment, several trials were conducted using different concentrations (from 0.01 to 100 µM) of TEB and TRI on *C. fimbriata* infected sweet potato disks, and some doses were selected based on their better phenotypic responses. The cut surface of the sweet potato disks was treated with selected different doses of TEB and TRI (0.1 µM TEB+0.04 µM TRI, 0.25 µM TEB+0.1 µM TRI, 0.5 µM TEB+0.2 µM TRI, and 1.0 µM TEB+0.4 µM TRI). After drying treated sweet potato disks were inoculated by 50 µl of *C. fimbriata* spore suspension ($1\times10^7$ spore $ml^{-1}$) counted by hemocytometer described by Mohsin et al. (2016). The control sweet potato disks were treated with sterilized $dH_2O$ only and then incubated in a growing room maintaining temperature 25-28 °C. Four days after incubation

biochemical and phytoalexins data were observed. The study was performed using CRD with three replications per treatments.

**Treatments and growing condition of *Ceratocystis fimbriata***

The potato sucrose agar (PSA) media was used as a growth medium for this study. The combination of TEB and TRI was applied by previously selected concentrations (0.1 µM TEB+0.04 µM TRI, 0.25 µM TEB+0.1 µM TRI, 0.5 µM TEB+0.2 µM TRI, 1.0 µM TEB+0.4 µM TRI) as food poisoning in the medium. Glass Petri dishes (9 cm) were filled with 20 ml of PSA, and a fifteen days old mycelial disk (6 mm in diameter) was placed in the centre of each plate, then incubated in an incubation chamber at 28±1 °C. Morphological and biochemical data were recorded after 11 days of incubation. The experiment was performed using CRD with three replications per treatments.

**Observation of physiological and biochemical parameters**

Sweet potato disks and fungal mycelia were used to determine oxidative stress markers contents (MDA, $H_2O_2$, EL, and LOX activity), histocchemical detection of ROS, non-enzymatic antioxidant contents (AsA and GSH), enzymatic antioxidant activities (SOD, POD, CAT, APX, MDHAR, DHAR, GR, GPX, and GST), phytoalexin detection, fungal growth, amylase activity, and melanin content by following established standard methods.

**Statistical analysis**

The measured data were statistically analyzed using XLSTAT 2020 software (AddinSoft 2020); three replications were used for analysis of variance (ANOVA). The mean differences were compared using Fisher's least significant difference (LSD) test at the 5% level of significance.

**Results**

Four days after inoculation of *C. fimbriata* in cut surface of sweet potato disks observed disease development reduced by different concentrations of TEB and TRI (Fig. 15). The infection of *C. fimbriata* increased the $H_2O_2$, MDA, EL, and LOX activity by 138, 152, 73, and 282%, respectively in sweet potato disks, relative to a control. In sweet potato disks, *C. fimbriata* reduced antioxidant enzyme activities as well as AsA and GSH contents by 82 and 91%, respectively, compared to control. However, TEB and TRI reduced the oxidative damage in *C. fimbriata*-inoculated sweet potato disks by enhancing antioxidant defense systems. On the other hand, 11 days after inoculation TEB and TRI application increased $H_2O_2$, MDA, EL, and LOX activity in *C. fimbriata*, where AsA and GSH content reduced, therefore, inhibited the growth of *C. fimbriata* (Fig. 16). These results suggest that TEB and TRI has significant impact to control black rot of sweet potato disease by inhibiting *C. fimbriata* growth (Fig. 17).

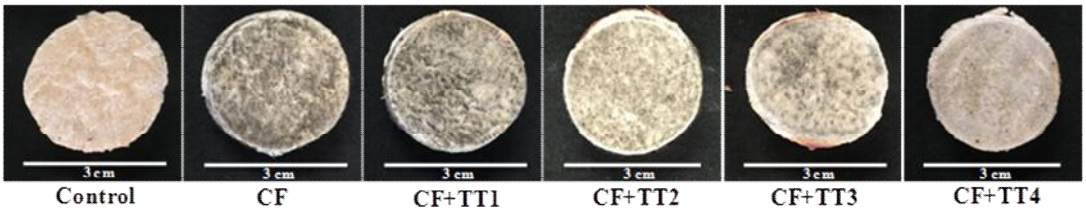


**Fig. 15** Effect of tebuconazole and trifloxystrobin on visual difference of *Ceratocystis fimbriata* growth in sweet potato disks at four days after inoculation. CF, TT1, TT2, TT3, and TT4 indicate *Ceratocystis fimbriata*, 0.1 µM tebuconazole+0.04 µM trifloxystrobin, 0.25 µM tebuconazole+0.1 µM trifloxystrobin, 0.5 µM tebuconazole+0.2 µM trifloxystrobin, and 1.0 µM tebuconazole+0.4 µM trifloxystrobin, respectively

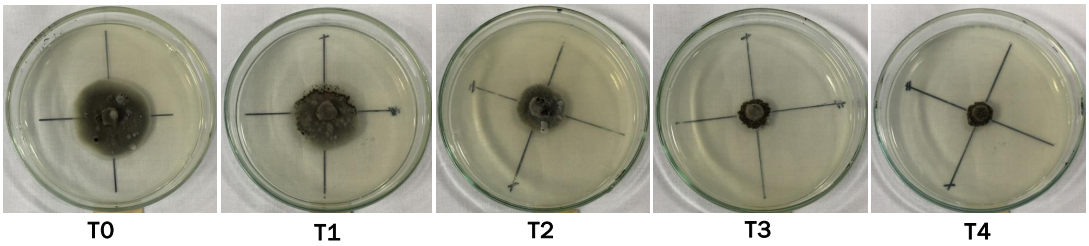


**Fig. 16** Effect of tebuconazole and trifloxystrobin on visual difference in diameter of mycelial growth of *Ceratocystis fimbriata* on potato sucrose agar amended culture plates at eleven days after inoculation. T0, T1, T2, T3, and T4 indicate control, 0.1 µM tebuconazole+0.04 µM trifloxystrobin, 0.25 µM tebuconazole+0.1 µM trifloxystrobin, 0.5 µM tebuconazole+0.2 µM trifloxystrobin, and 1.0 µM tebuconazole+0.4 µM trifloxystrobin, respectively

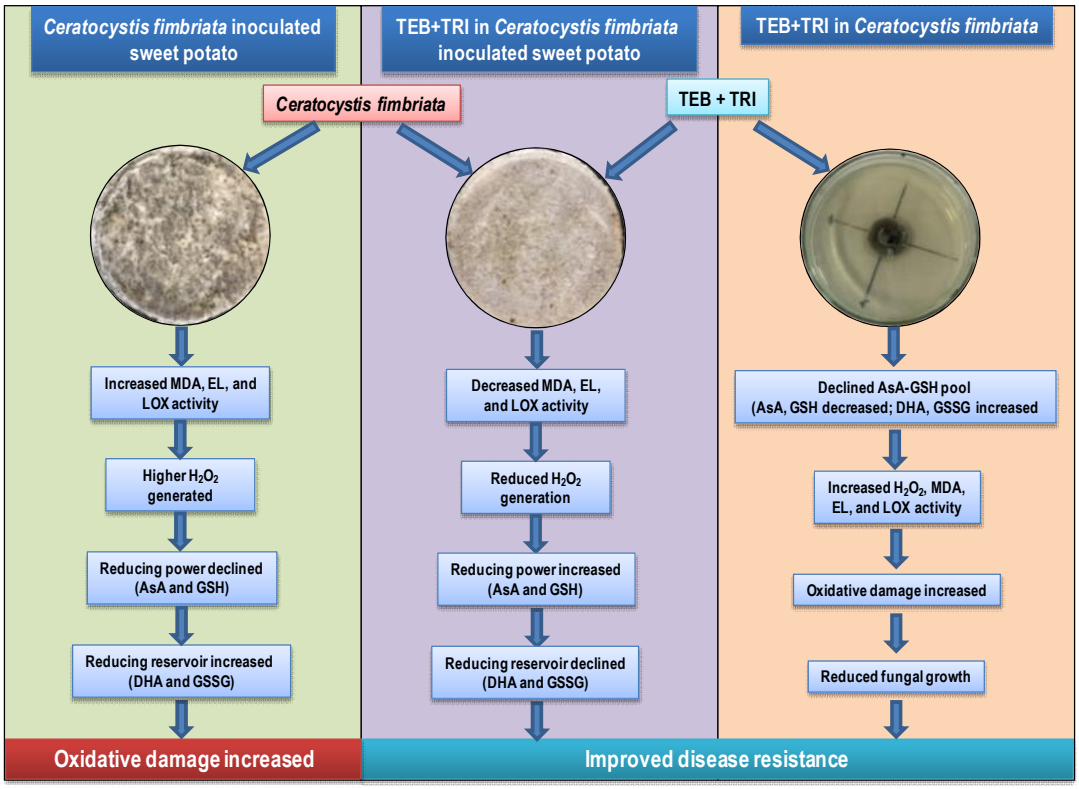


**Fig. 17** Possible mechanisms of tebuconazole and trifloxystrobin for improving disease resistance in sweet potato and growth inhibition of *Ceratocystis fimbriata* based on the present investigation. AsA, ascorbate; DHA, dehydroascorbate; EL, electrolyte leakage; GSH, reduced glutathione; GSSG, oxidized glutathione; H2O2, hydrogen peroxide; LOX, lipoxygenase; MDA, melondialdehyde; TEB, tebuconazole; TRI, trifloxystrobin

**Experiment 5: Triazole and strobilurin fungicides-mediated citrus black rot disease management: Modulation of antioxidant defense systems in citrus and *Alternaria citri***

**Materials and methods**

**Inoculation of fungus in citrus peels**

The peel segments (3×3.5 cm) were pre-sterilized and washed in several times and then placed on the net in a transparent plastic box by maintaining high humidity. The internal surface of peel segments was treated with different doses of triazole and strobilurin fungicides (0.1 µM TEB+0.04 µM TRI, 0.25 µM TEB+0.1 µM TRI, 0.1 µM PRO, and 0.25 µM PRO). After drying treated citrus peels were inoculated by 100 µl of *A. citri* spore suspension ($1\times10^6$ spore $ml^{-1}$). Sterilized $dH_2O$ were used to treat control citrus peels and then incubated at 25-28 °C. After five days, biochemical data were observed. The study was performed using CRD with three replications.

**Growing condition of *Alternaria citri* and treatments**

The fungicides at various concentrations (0.1 µM TEB+0.04 µM TRI, 0.25 µM TEB+0.1 µM TRI, 0.1 µM PRO, 0.25 µM PRO) were applied in the potato dextrose agar media. Then the media were poured in glass Petri dishes (9 cm diameter) and 20 ml for each. Mycelial disk (6 mm in diameter) was placed in the centre of each plate and incubated at 28±1°C. After ten days, morphological and biochemical data were determined. The experiment was performed using CRD with three replications.

**Observation of physiological and biochemical parameters**

Citrus peels and fungal mycelia were used to determine oxidative stress markers contents (MDA, $H_2O_2$, EL, and LOX activity), histocchemical detection of ROS, non-enzymatic antioxidant contents (AsA and GSH), enzymatic antioxidant activities (SOD, POD, CAT, APX, MDHAR, DHAR, GR, GPX, and GST), fungal growth, amylase activity, and melanin content by following established standard methods.

**Statistical analysis**

The measured data were statistically analyzed using XLSTAT 2020 software (AddinSoft 2020); three replications were used for analysis of variance (ANOVA). The mean differences were compared using Fisher's least significant difference (LSD) test at the 5% level of significance.

**Results**

Five days after inoculation of *A. citri* in citrus peels observed disease development reduced by different concentrations of triazole and strobilurin fungicides (Fig. 18). In citrus peels, *A. citri* increased $H_2O_2$ content with improving MDA , EL, and LOX activity. Fungal infection also reduced AsA and GSH contents as well as antioxidant enzyme activities. However, the application of fungicides in *A. citri* infected citrus peels reduced oxidative damage by improving antioxidant defense systems. On the other hand, 10 days after inoculation fungicide treatments increased oxidative damage in *A. citri* thus inhibited the fungal mycelial growth (Fig. 19). Both PRO and combination of TEB and TRI application increased $H_2O_2$ content in *A. citri* thus increased MDA, EL and LOX activity. Fungicides application reduced enzymatic and non-enzymatic antioxidants in *A. citri*, thus inhibited antioxidant defense systems. Therefore, the results indicated that triazole and strobilurin fungicide might be a promising strategy to manage citrus black rot disease (Fig. 20).

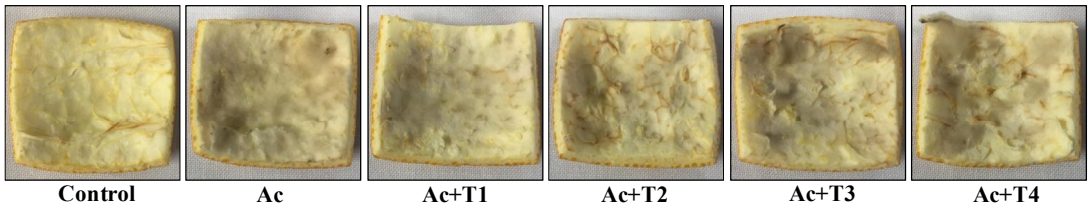


**Fig. 18** Effect of triazole and strobilurin fungicides on visual difference *Alternaria citri* growth in citrus peels at five days after inoculation. Ac, T1, T2, T3, and T4 indicate *Alternaria citri*, 0.1 μM TEB+0.04 μM TRI, 0.25 μM TEB+0.1 μM TRI, 0.1 μM PRO, and 0.25 μM PRO, respectively

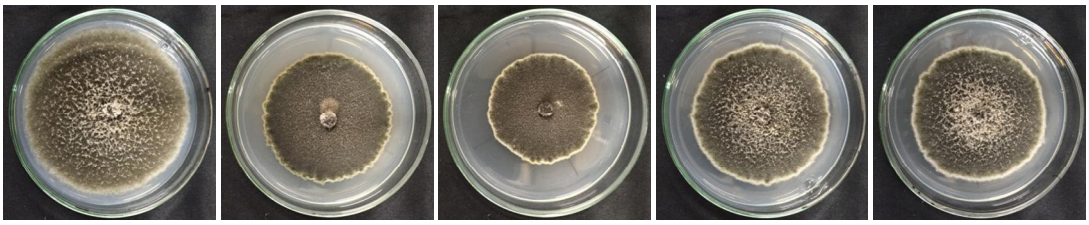


**Fig. 19** Effect of triazole and strobilurin fungicides on visual difference in diameter of radial mycelia growth of *Alternaria citri* on potato dextrose agar amended culture plates at ten days after inoculation. T0, T1, T2, T3, and T4 indicate control, 0.1 μM TEB+0.04 μM TRI, 0.25 μM TEB+0.1 μM TRI, 0.1 μM PRO, and 0.25 μM PRO, respectively

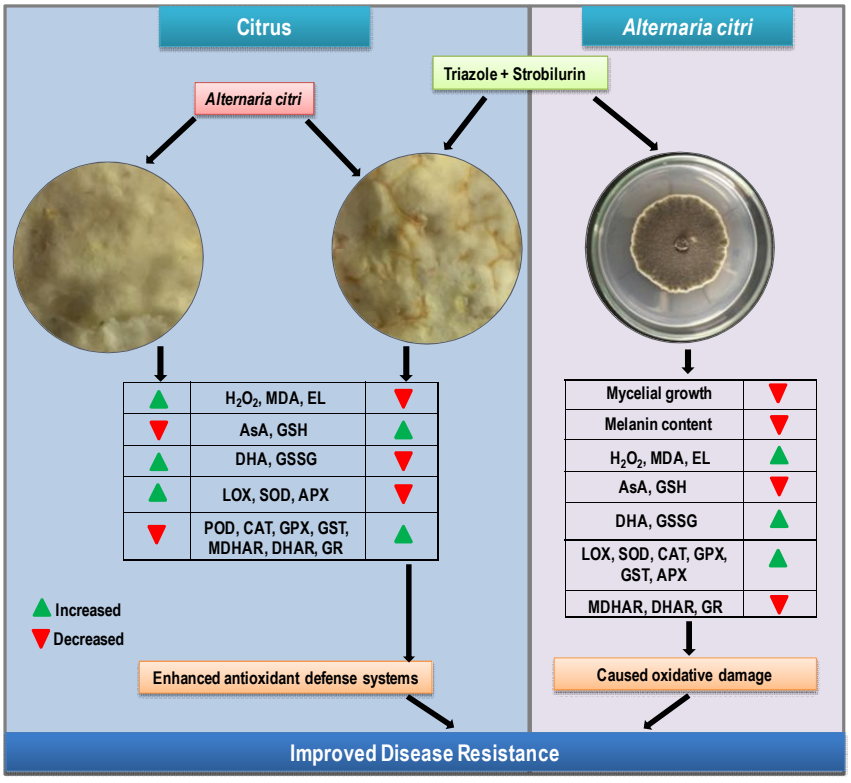


**Fig. 20** Possible mechanisms of triazole and strobilurin fungicides for improving disease resistance in citrus and growth inhibition of *Alternaria citri* based on the present investigation

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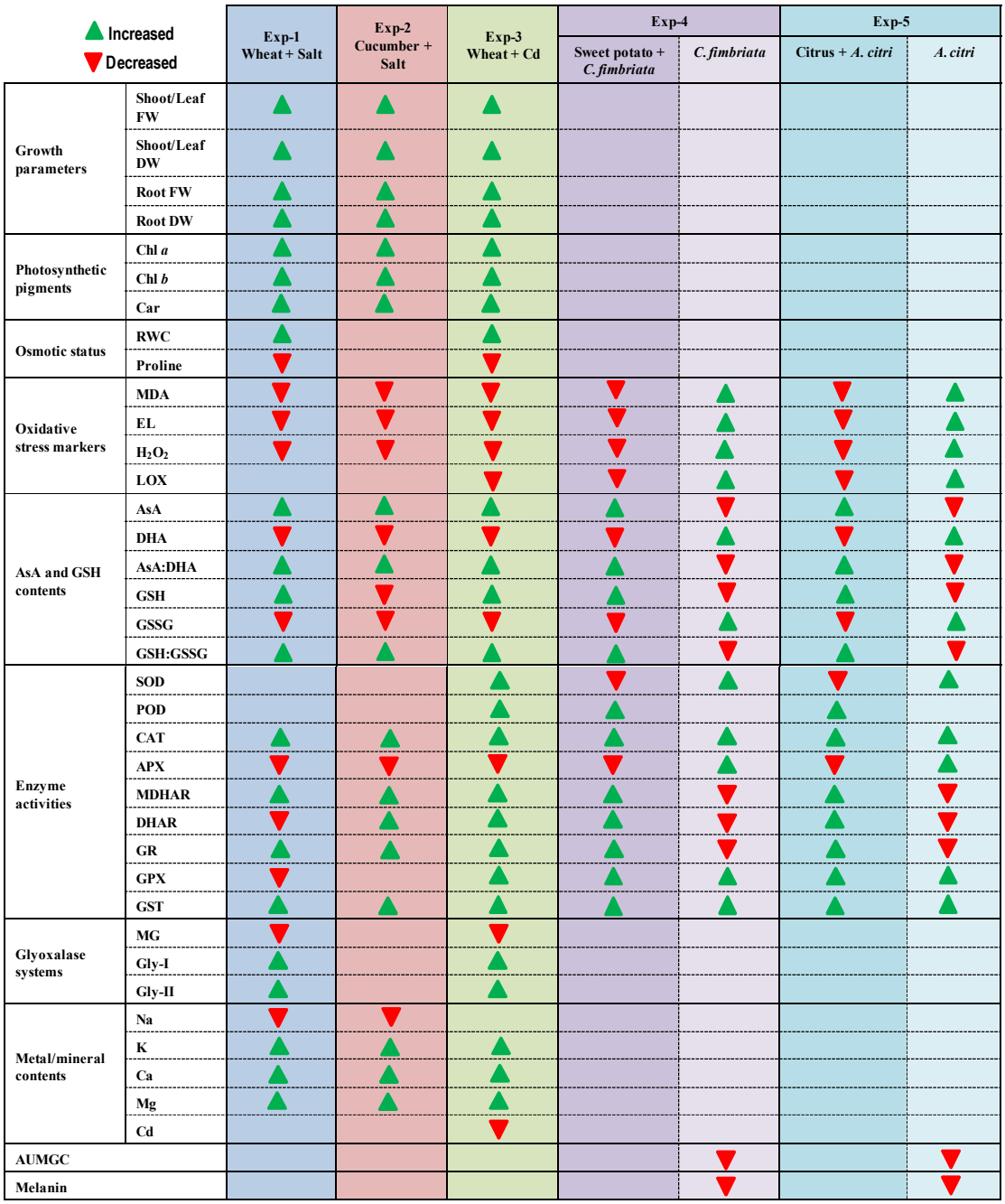


**Fig. 21** Overall summaries on possible mechanisms of TEB and TRI for improving plant tolerance under abiotic and biotic stress conditions based on the present investigation

**Overall summary**

In sum, the findings from all of the experiments provide information regarding the point of plant cellular damages under both abiotic and biotic stresses and the protective mechanisms of TEB and TRI in relation of antioxidant defense systems (Fig. 21). The application of TEB and TRI improved the activities of CAT, MDHAR, GR and GST in all five experiments under abiotic and biotic stress, that might be the possible reasons to improve plant stress tolerance. Thus, these findings might further assist in developing abiotic and biotic stress tolerant varieties.

Moreover, researchers might get information about TEB and TRI as phytoprotectants for integrated management of abiotic and biotic stresses. Therefore, further investigations are needed under field conditions in different crop species to justify the TEB and TRI-mediated plant tolerance mechanisms under abiotic and biotic stress conditions.

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