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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

Physiological basis of phenolics-induced salt stress recovery in tomato: Relevance of antioxidant defense associated systems
(トマトにおけるフェノール類により誘導される塩ストレスからの回復の生理学的基礎：抗酸化防御に係るシステムの関連性)

学位論文要約：
Dissertation Summary

Background of the study

World population entirely depend on plants for food, shelter, clothes, medicine, etc. and such increasing demand also putting pressure on the environment due to its rapid growth. Therefore, plants suffer from growth reduction and disturbance in developmental processes which together contribute to yield loss. It will be worst with the imminent global climatic changes by generating extreme environmental changes along with ever-increasing world population, degradation of arable land and fresh water scarcity in the near future (Batth et al. 2017). As sessile organism plant can not escape these stresses. In addition, the severity of any abiotic stress effects depends on stress duration, age of the plant, species and cultivar of plant. Among abiotic stresses, salinity is one of the most devastating abiotic stresses because most of the crop plants are sensitive to this stressor (Hasanuzzaman et al. 2013). Around 20% of the irrigated land has already been affected by salinity (Liu et al. 2019). Due to its worst impacts and inundation rate, it has been predicted that around half of world arable land will be salinized as well as lost by 2050 (Frukh et al. 2020). Plants exposed to higher levels of salinity are affected by both hyperosmotic and hyperionic stress through accumulating Na^+ and Cl^- which causes membrane damage (Figure 1), nutrient imbalance, enzymatic inhibition, metabolic dysfunction, photosynthesis inhibition, and hampers other major physiological and biochemical processes those ultimately lead to growth inhibition or death of the plant (Rahman et al. 2016a).

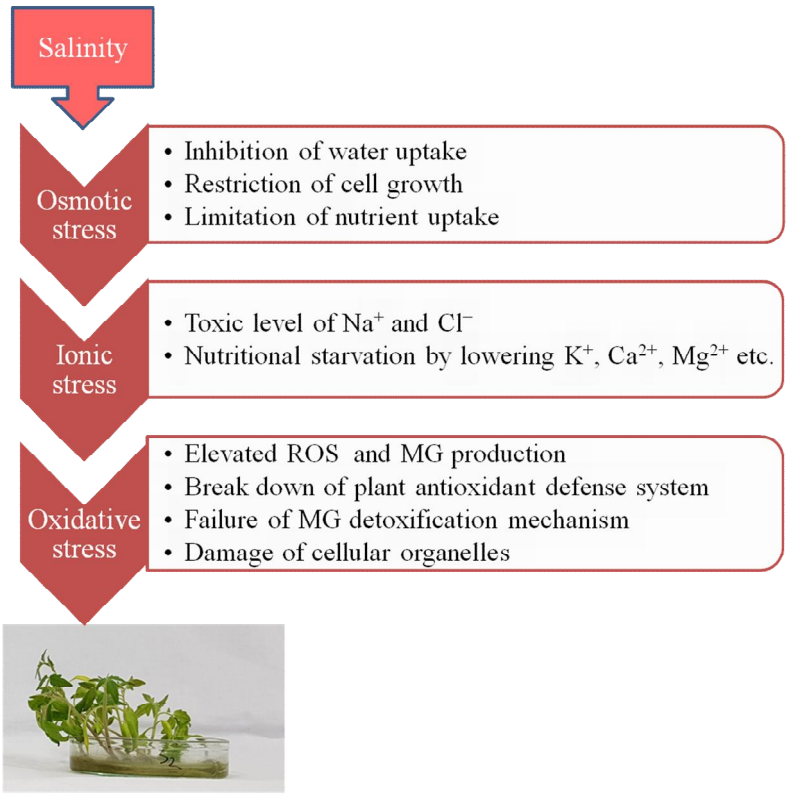

Figure 1. Salinity-induced primary and secondary stresses in plants

Higher levels of salt in plant growth medium increase Na^+ uptake and decrease K^+ content as Na^+ causes K^+ efflux and triggers K^+ leakage from plant cells. As a result, under salt-stress conditions, Na^+ content exceeds that of K^+, resulting in a higher Na^+/K^+ ratio as well as nutrient imbalance (Mohsin et al. 2020). Production of reactive oxygen species (ROS) in the plant is unavoidable, where plant regulates its balance for ensuring its signaling role through antioxidant defense system. But, salinity causes the overproduction of ROS including singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$), along with disruption of antioxidant defense system which consequently results in oxidative stress (Figure 2) (Hasanuzzaman et al. 2013; Rahman et al. 2016a,b; Mohsin et al. 2020). This salinity mediated secondary stress thus causes oxidative damage and cell membrane injury. Moreover, salinity accelerates the generation of cytotoxic methylglyoxal (MG) through the glycolysis pathway and causes oxidative damage as well as disruption of protein synthesis (Rahman et al. 2016a).

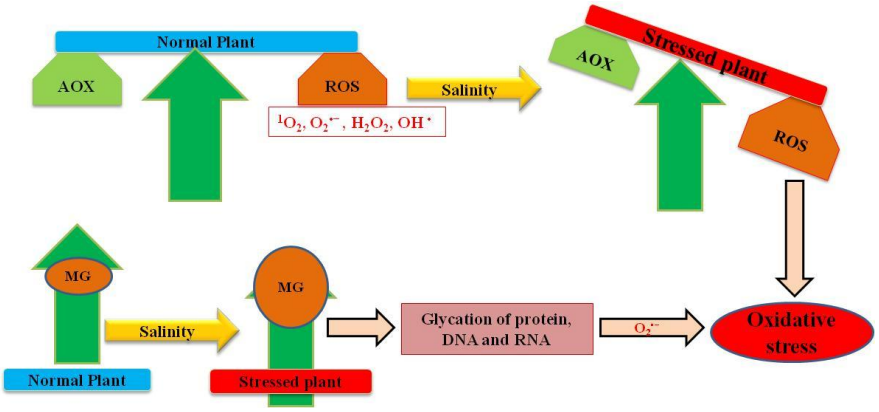

Figure 2. Reactive oxygen species (ROS) and methylglyoxal (MG)-mediated oxidative stress. (AOX: antioxidants)

Naturally plants activate their protective mechanisms to alleviate salinity-induced toxicity. Tolerant plant species are able to cope with salt toxicity by their in-build defense mechanisms where they regulate proper osmotic balance, inhibition of toxic ions accumulations and free radical scavenging (Saleh and Madany 2015). According to, osmoregulation is one of the vital mechanisms for stimulating osmolytes synthesis including proline (Pro), glycine betaine (GB), trehalose (Tre) or others (Rahman et al. 2016a; Parvin et al. 2019a). These osmolytes not only regulate the osmotic pressure but also able to protect cellular organelles from oxidative injury through upregulating the antioxidant defense mechanism. Ion exclusion, along with transferring the absorbed ions to the vacuole is another strategy to counter ionic stress of salt tolerant plants (Min et al. 2018). To cope with acute ROS and MG toxicity, the plant possesses antioxidant defense and glyoxalase system (Figure 3), respectively where a very systematic correlation exists between these mechanisms (Rahman et al. 2016a; Hasanuzzaman et al. 2018a; Mohsin et al. 2020).

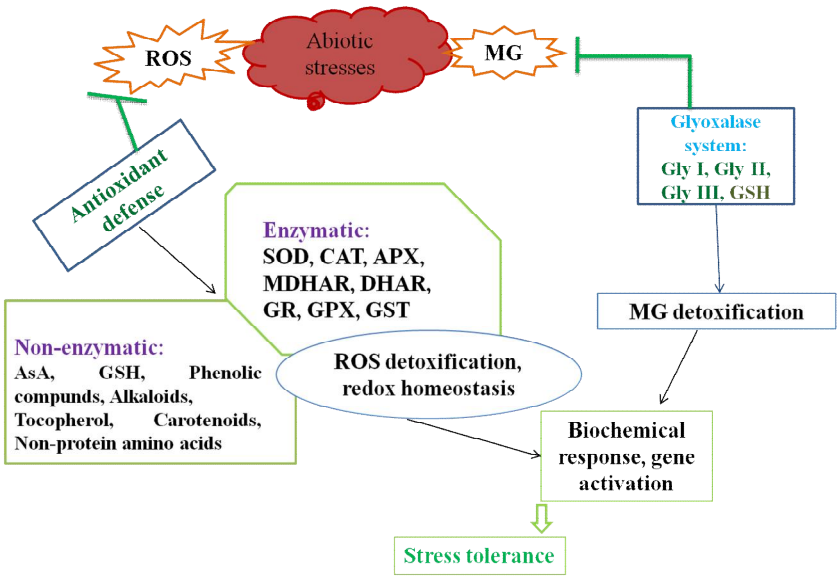

Figure 3. Defense mechanisms in plant for salt tolerance to combat ROS and MG

The antioxidant defense system includes non-enzymatic antioxidants (ascorbate, AsA; glutathione, GSH; tocopherol, phenolic compounds (PCs), alkaloids, carotenoids, non-protein amino acids etc.) and enzymatic antioxidants (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR; glutathione peroxidase, GPX; and GST, glutathione *S*-transferase) (Figure 4) (Hasanuzzaman et al. 2019). Here, APX, MDHAR, DHAR and GR are four enzymes, and AsA and GSH non-enzymatic antioxidants composing the AsA-GSH cycle. The SOD catalyzes the $O_2^{\bullet-}$ in to H_2O_2 which is then detoxified by the activity of CAT and through the AsA-GSH cycle involving their enzymatic components for further recycling back (Hasanuzzaman et al. 2018a). Besides, GSH also possesses an important role in activating the GPX and GST enzymes for direct scavenging of H_2O_2, while GST also has the properties of xenobiotics detoxification with the incorporation of GSH. Moreover, among the non-enzymatic antioxidants PCs are secondary metabolites which can chelate the diverse range of ions and scavenge ROS consequently hinder the oxidation of lipid and DNA damage (Rani et al. 2018).

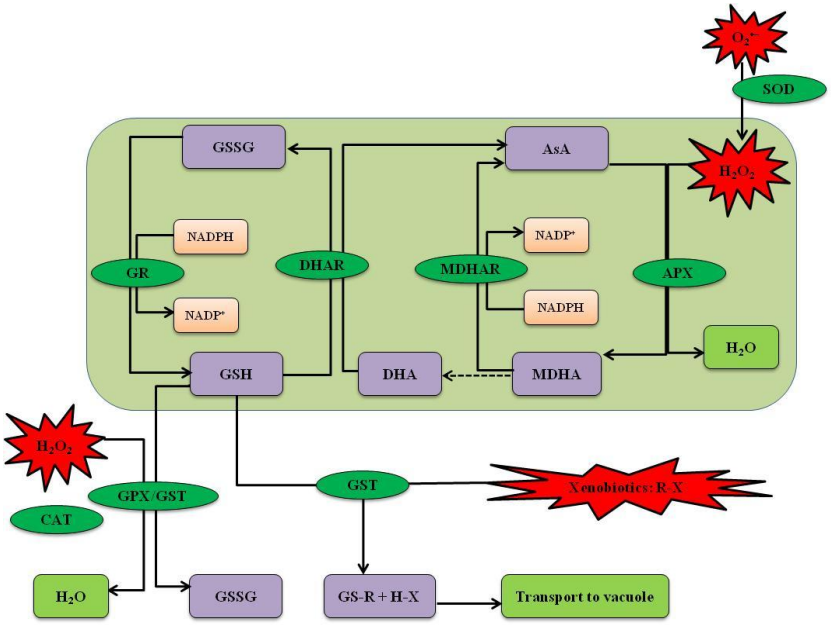

Figure 4. Antioxidant defense system– the pathways for ROS detoxification in plants

For lessening the elevated level of MG from the cell under stress condition, the plant has a unique system called glyoxalase system (Figure 5). This system includes glyoxalase I (Gly I) and glyoxalase II (Gly II), glyoxalase III (Gly III) (Mohsin et al. 2020). These enzymes play a key role in the detoxification of toxic MG with the help of GSH from plant cell and confer stress tolerance against MG-induced oxidative stress. The enzymes of the glyoxalase system can detoxify MG effectively through two-step reactions. In the first step, MG is converted to S-D-lactoyl glutathione (SLG) by using the Gly I enzyme, where GSH acts as co-factor, and in the second step, SLG is converted to D-lactate in cooperation with the Gly II enzyme, where GSH is recycled back. Both antioxidant defense and glyoxalase system detoxify ROS and MG, respectively and maintain redox balance which generate biochemical response, cellular signaling and activate different genes (Hasanuzzaman et al. 2018a).

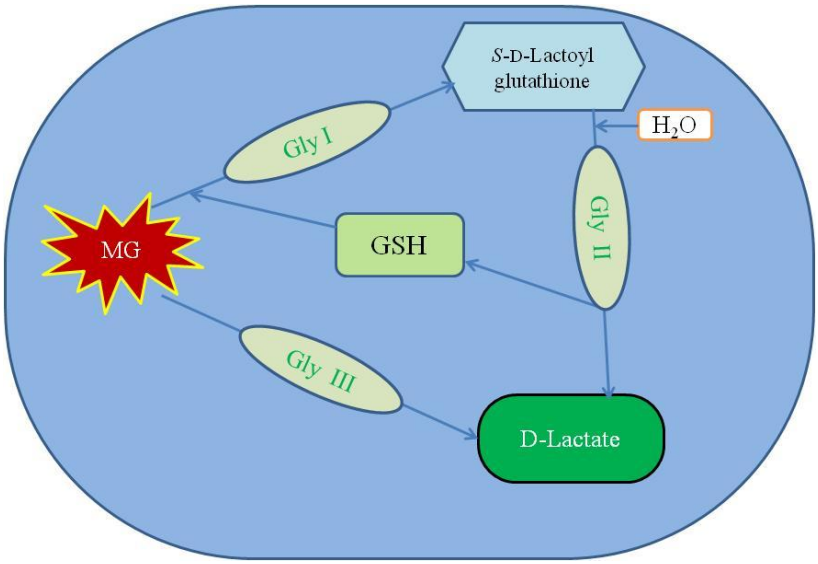

Figure 5. Glyoxalase system for MG detoxification

Improving ion homeostasis and regulating both the antioxidant and glyoxalase systems are necessary to develop salt-stress tolerance. Researchers are currently testing diverse groups of chemicals including phytohormone, organic acid, essential nutrient molecules, antioxidants, and other plant-derived secondary metabolites as phytoprotectants to enhance salinity tolerance. Phenolic compounds are plant secondary metabolites and non-enzymatic antioxidants, increase of which are considered as stress response markers (Quan et al. 2016). They are able to directly scavenge ROS by their structural hydrogen atom and/donating an electron and thus increases the stress tolerance (Waśkiewicz et al. 2013).

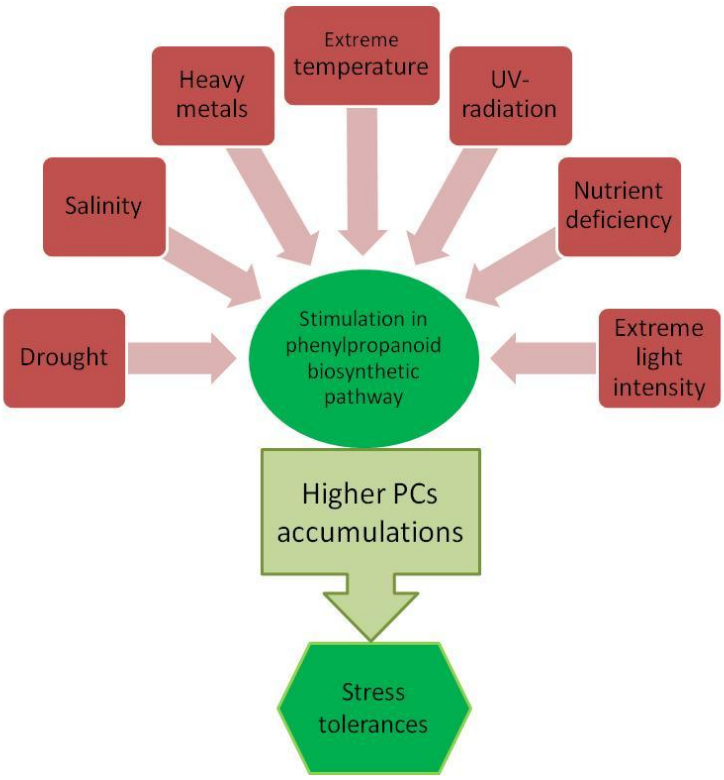

Figure 6. Role of phenolic compounds (PCs) in plant to attain stress tolerance

Although synthesis of PCs via phenylpropanoid metabolism by involving the shikimic acid metabolic and malonic acid pathway in plants is endogenously controlled process but is also regulated by external environmental factors — light, temperature and wounding (Sharma et al. 2019). These compounds act as signaling molecules and protect plants from environmental stressed-induced ROS generation and thus stimulating stress tolerance. Higher biosynthesis of secondary metabolites including polyphenols is one of the plants respond towards abiotic stresses and thus enhances the evolutionary fitness. Indeed, these accumulated PCs give plant higher tolerance against abiotic stresses including salinity, drought, heavy metal, temperature, pesticides and UV radiations (Figure 6) (Naikoo et al. 2019). Thereafter, plants have the ability to enhance PCs biosynthesis upon adverse environmental conditions (Selmar 2008). As PCs are non-enzymatic antioxidants and that's why capable of reducing cell membrane peroxidation by detoxifying ROS as well as protects plants from oxidative stress (Sharma et al. 2019). The structural variability is the main reason behind the diversification of PCs in nature (Waśkiewicz et al. 2013) which consisting of a single aromatic ring to complex polymeric substances, thus PCs are classified into further various subgroups (Figure 7). Activities of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and other enzymes are responsible to PCs accumulation (Naikoo et al. 2019).

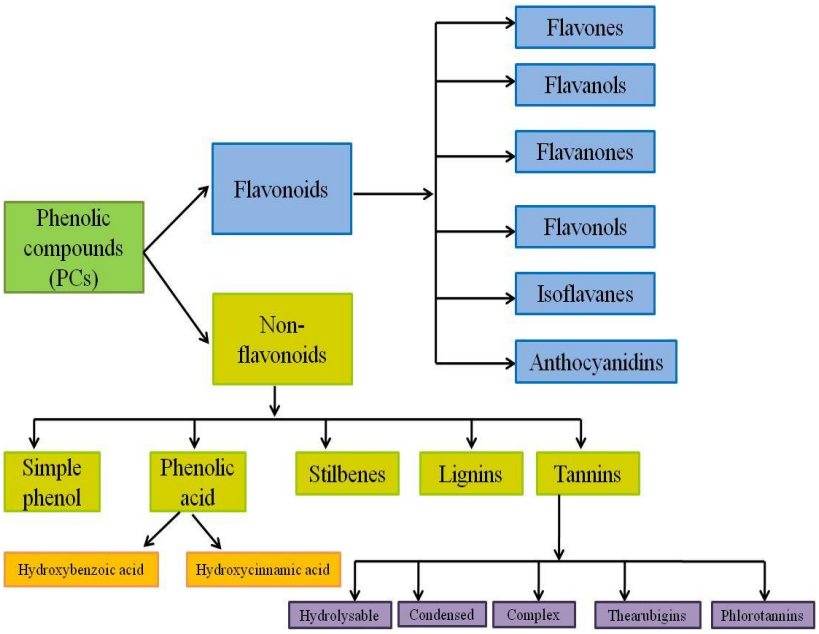

Figure 7. Classification of phenolic compounds

Upon salt stress, plants showed higher accumulation of PCs along with stimulation in phenylpropanoid biosynthetic pathway as a part of antioxidant defense and thus scavenged stress-induced toxic ROS (Bistgani et al. 2019). Salt-induced excessive ROS in plants need the active participation of antioxidants to be scavenged for gaining higher tolerance. Consequently, the total phenolic and flavonoid contents reduced in salt exposed *Oryza sativa* because of elevated ROS and showed their antioxidants properties (Rahman et al. 2016b). Some of the reports are available about PCs mediated plant tolerance against different kinds of abiotic stresses by using vanillic acid (Xuan and Khang 2018), quercetin (Kurepa et al. 2016), cinnamic acid (Li et al. 2011), ferulic acid (Li et al. 2013), ellagic acid (Abu El-Soud et al. 2013), coumarin (Saleh and Madany 2015). But PCs -induced coordinated action of antioxidant defense and glyoxalase systems of plants under stress is not investigated yet. Moreover, this group of chemicals can be used as very potential source to scavenge ROS as well boost up the plant inherent defense system for attuning higher tolerance.

Tomato (*Solanum lycopersicon* L.) is one of the most widely cultivated fruit vegetable due to its high nutritional value and diversified growth responses upon different climatic conditions. In keeping it in mind along with its moderate tolerance nature to salinity, here it has been taken as test material.

Therefore, we investigated quercetin, vanillic acid and coumarin–mediated response of tomato seedlings under salinity by involving plant antioxidant defense, glyoxalase systems and mineral homeostasis for attuning tolerance. Present studies are the first evidence on PCs -induced salt stress tolerance in tomato, where the coordinated actions of antioxidant defense, glyoxalase system, and mineral homeostasis were addressed together. Considering the above-mentioned strategies, there were some objectives for carrying out several studies -

i. To identify the tolerance and recovery strategies through physiological and biochemical responses of *S. lycopersicum* L. to salinity
ii. To investigate the regulatory roles of different PCs as phytoprotectants under salt stress for enhancing tolerance of *S. lycopersicon*
iii. To investigate the coordinated actions between the antioxidant defense and glyoxalase systems in conferring salt tolerance in *S. lycopersicum* L. with or without exogenous PCs application

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

1. Comparative physiological and biochemical changes in tomato (*Solanum lycopersicum* L.) under salt stress and recovery: Role of antioxidant defense and glyoxalase systems
2. Quercetin mediated salt tolerance in tomato through the enhancement of plant antioxidant defense and glyoxalase systems
3. Exogenous vanillic acid enhances salt tolerance of tomato: Insight into plant antioxidant defense and glyoxalase systems
4. Coumarin alleviates salt toxicity in tomato by promoting ion homeostasis, antioxidant defense and glyoxalase systems

Experiment I: Comparative physiological and biochemical changes in tomato (*Solanum lycopersicum* L.) under salt stress and recovery: role of antioxidant defense and glyoxalase systems

Meterials and methods

Plant materials and stress treatments

Uniform and healthy tomato (*S. lycopersicum* L. cv. Pusa Ruby) seeds were surface sterilized with 70% ethanol (5 min). Fifteen seeds were placed on two layers of moistened filter paper in Petri plates and incubated in a germination chamber. After 5 d, the number of plants per plate was reduced to 10 healthy plants and the plates were transferred to a growth chamber. The plants were supplied with full strength Hoagland nutrient solution (Hoagland and Arnon 1950) and grown under controlled conditions (light: 350 µmol photon $m^{-1} s^{-2}$, photoperiod: 16/8 h of light/dark, temperature: 25 ± 2 °C and relative humidity: 65–70%) for the next 10 d. Several trials were conducted prior to the actual experiment to determine the highest salt level exposure with the shortest recovery period. We found that tomato plants recovered within 48 h from the damage induced by 250 mM NaCl. Therefore, we selected 150 and 250 mM NaCl and 96 h as the stress conditions and a subsequent 48 h in normal nutrient solution for the recovery condition to investigate the recovery mechanism. After 96 h of salt stress, the plants were moved to the recovery solution by removing the salt solution, washing the plants with distilled water, and then supplying the nutrient solution. The third and fourth leaves of the tomato plants were analyzed after both the stress and recovery phases. The whole experiment was conducted three times and included three replications per treatment, with 10 plants per replication. Morphological data were obtained as the averaged values from 10 randomly selected plants.

Determination of physiological and biochemical parameters

After definite treatment duration, plant height, root length, stem girth and fresh weight (FW) were measured while dry weight (DW) collected after dried at 80°C for 48 h. Different physiological and biological parameters. leaf relative water content (RWC), photosynthetic pigments content (chlorophyll; Chl *a*, Chl *b* and carotenoid; Car), proline (Pro) content, lipid peroxidation (malondialdehyde, MDA content), H_2O_2 content, histchemical detection of ROS, lipoxygenase (LOX) activity, MG content, antioxidant defense system including content of non-enzymatic component (AsA and GSH content), activity of enzymatic components (SOD, CAT, GPX, GST, APX, MDHAR, DHAR, GR) and glyoxalase enzymes activities (Gly I and Gly II), contents of 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 and summary

Under saline conditions, plants showed osmotic stress responses that included low LRWC and high Pro content. Salinity induced oxidative stress by the over-accumulation of ROS (H_2O_2 and $O_2^{\bullet-}$) and MG. Salinity also impaired the non-enzymatic and enzymatic components of the antioxidant defense system. On the other hand, excessive Na^+ uptake induced ionic stress which resulted in a lower content of other minerals (K^+, Ca^{2+}, and Mg^{2+}), and a reduction in photosynthetic pigment synthesis and plant growth. After 2 days in the normal nutrient solution, the plants showed improvements in antioxidant and glyoxalase system activities, followed by improvements in plant growth, water balance, chlorophyll synthesis and higher mineral contents (Figure 8). The antioxidant and glyoxalase systems worked in concert to scavenge toxic ROS, thereby reducing lipid peroxidation and membrane damage. Taken together, these findings indicate that tomato plants can tolerate salinity and show rapid post-stress recovery by enhancement of their antioxidant defense and glyoxalase systems.

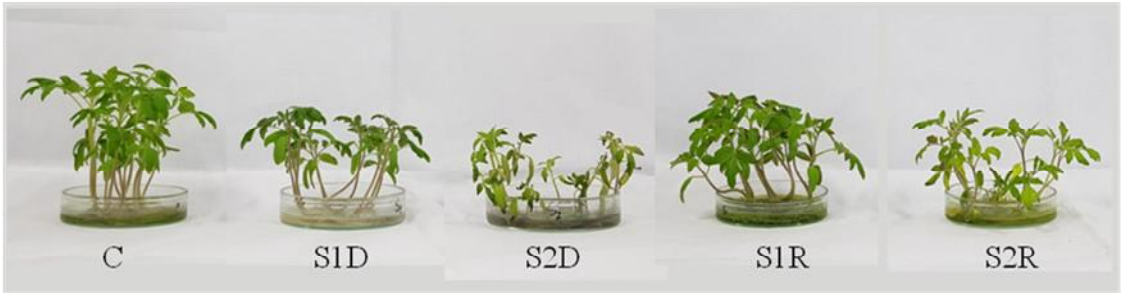

Figure 8. Visual differences of tomato plants under salinity (150 and 250 mM NaCl) and after a 48 h recovery period. (C: control, S1D: 150 mM NaCl, S2D: 250 mM NaCl, S1R: recovered 150 mM NaCl and S2R: recovered 250 mM NaCl).

Experiment II: Quercetin mediated salt tolerance in tomato through the enhancement of plant antioxidant defense and glyoxalase systems

Materials and methods

Growth of seedling and stress treatment

Seedling were grown as described in Experiment-I. Then 10-d old seedlings were treated with salt (NaCl, 150 mM) and Qu (15 and 25 μM) in solely and in combination as a co-treatment. The respective salt treatment was incorporated with nutrient solution and renewed every day with and/or without Qu during the whole period of study. Quercetin was dissolved in alcoholic solution and prepared just before of application. Control treated seedlings received neither salt not Qu; only nutrient solution. Data were collected from 3rd and 4th leaves of tomato seedlings after 5 days of treatment. For clarification and validation, the experiment was executed three times. Each time there were three replications for each treatment. For data collection in each replication we kept two Petri plates where in each Perti plates there were 10 seedlings. Moreover, morphological data was measured from 10 randomly selected seedlings from each treatment and expressed from its average value.

Determination of physiological and biochemical parameters

All the studied physiological and biochemical parameters were measured by following Experiment-I

Statistical analysis

Statistical analysis was same as Experiment-I

Results and summary

Quercetin (Qu) is a strong antioxidant among the phenolic compounds having physiological and biochemical roles in plants. Hence, we have studied the Qu evolved protection against salinity in tomato. Salinity caused ionic toxicity by increasing Na^+ content in seedlings along with nutritional starvation of K^+, Ca^{2+}, and Mg^{2+}. While osmotic stress was detected by higher free Pro content and lower LRWC in salt-stressed seedlings. Salt toxicity also induced higher H_2O_2 generation, MDA content and LOX activity as a sign of oxidative stress. Tomato seedlings suffered from MG toxicity, degradation of chlorophyll, along with lower biomass accumulation and growth due to salt exposure. However, Qu application under salinity resulted in lower Na^+/K^+ due to reduced Na+ content, higher LRWC, increased Pro, and reduction of H_2O_2 and MDA content, and LOX activity, which indicated alleviation of ionic, osmotic, and oxidative stress respectively. Quercetin caused oxidative stress, lessening through the strengthening of both enzymatic and non-enzymatic antioxidants. In addition, Qu increased GST activity in salt-invaded seedlings, which might be stimulated ROS scavenging along with higher GSH content. As a result, toxic MG was detoxified in Qu supplemented salt-stressed seedlings by increasing both Gly I and Gly II activities. Moreover, Qu insisted on better plant growth and photosynthetic pigments synthesis in saline or without saline media (Figure 9). Therefore, exogenous applied Qu may become an important actor to minimize salt-induced toxicity in crops.

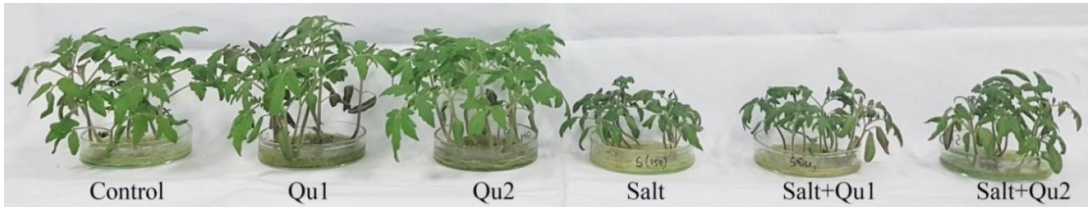

Figure 9. Visual differences of tomato seedlings treated with salt (150 mM NaCl) with and/or without quercetin (Qu1; 15 µM and Qu2; 25 µM) for 5 days.

Experiment III: Exogenous vanillic acid enhances salt tolerance of tomato: Insight into plant antioxidant defense and glyoxalase systems

Materials and methods

Seedling growth and treatments

Seedling were grown as described in Experiment-I. Prior to main experiment, we have selected the best doses (40 and 50 µM) of VA (4-hydroxy-3-methoxy benzoic acid) from several trial experiments consisting of 10, 20, 30, 40, 50 and 60 µM concentration those confer better performance under 150 mM NaCl in tomato seedlings by the phenotypic responses. Then, seedlings were exposed to 150 mM NaCl alone and in combination with 40 and 50 µM of VA as co-treatment for 5 days with daily change of solutions. 25 ml of Hoagland nutrient solutions were used for preparing respective treatment with NaCl and VA for each Patri plate where only Hoagland nutrient solution was used for control treatment. Afterward, required data were collected from 3rd and 4th leaves. Growth and biomass related data were collected from 10 seedlings per treatment. This experiment was conducted in three repeated times consisting of three replications per repetition by following similar procedures.

Determination of physiological and biochemical parameters

All the studied physiological and biochemical parameters were measured by following Experiment-I

Statistical analysis

Statistical analysis for studied all data was similar withxperiment-I

Results and summary

Ten-d-old tomato seedlings were treated with salt (NaCl; 150 mM) and vanillic acid (VA; 40 and 50 µM) separately and in combination with salt. Salinity restricted seedlings growth, biomass accumulation, chlorophyll and carotenoid contents. Salt-induced osmotic stress was indicated by lower LRWC and elevated Pro content, where higher Na^+/K^+ ratio indicated the ionic toxicity. Tomato seedlings went through oxidative damage due to acute ROS production and LOX activity with confirmation by higher lipid peroxidation and membrane damage under salinity. Conversely, exogenous VA reduced osmotic and ionic toxicity in stressed-seedlings by enhancing the RWC and Pro level, and lowering Na^+/K^+ ratio, respectively. Exogenous VA up-regulated the components of antioxidant defense system in salt-treated seedlings resulted in the reduction of ROS production, LOX activity and membrane damage in stressed-seedlings. Additionally, VA application caused the reduction of toxic MG accumulation under salt stress through the enhancement of glyoxalase system. Thus, VA-induced alleviation of

osmotic, ionic and oxidative stresses leading to improve plant growth and chlorophyll synthesis in stressed-seedlings (Figure 10 and 11). So, VA significantly improves salinity tolerance and plant growth performance by involving the actions of plant antioxidant defense and glyoxalase systems.

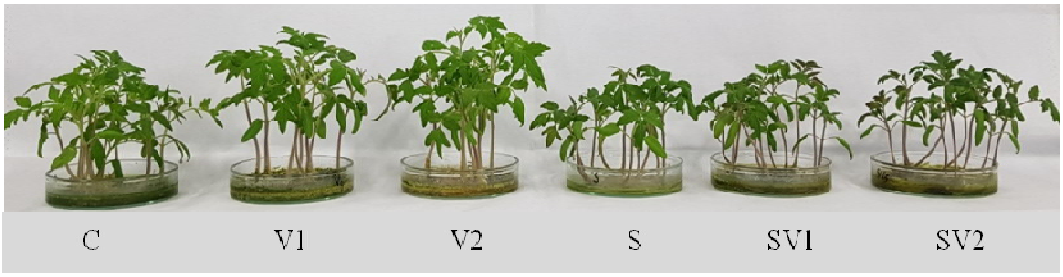

Figure 10. Phenotypic appearance of tomato seedlings treated with salt (S: 150 mM NaCl) with and/or without vanillic acid (V1: 40 µM and V2: 50 µM) for 5 days.

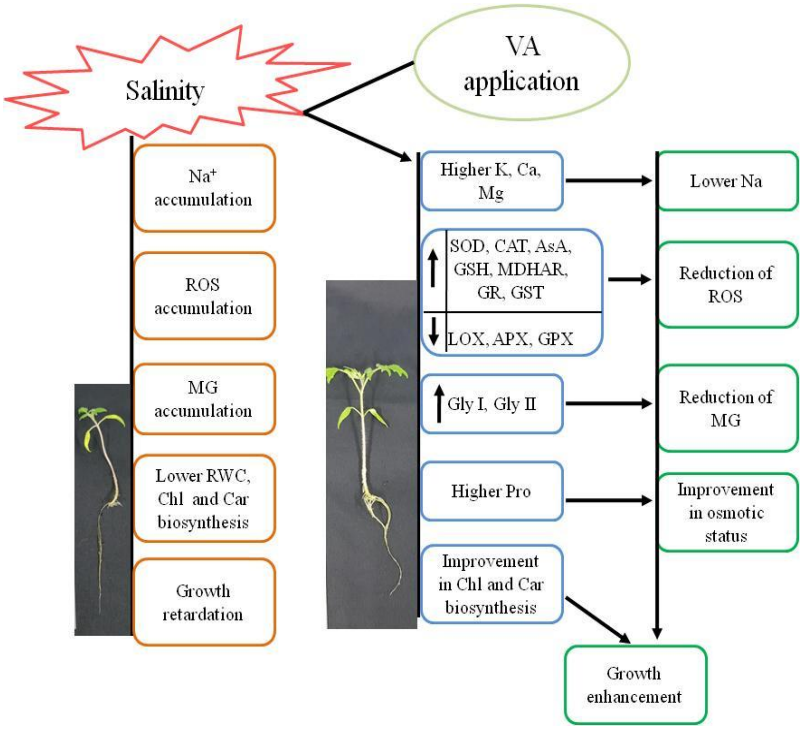

Figure 11. Diagrammatic presentation of VA-induced improvement in salt tolerance of tomato

Experiment IV: Coumarin alleviates salt toxicity in tomato by promoting ion homeostasis, antioxidant defense and glyoxalase systems

Materials and methods

Plant material and treatments details

Seedling were grown as described in Experiment-I. At 8 days of old, seedlings were treated with coumarin (COU, 20 and 30 µM) for next 2 days. Then 10-d old seedlings were exposed to salt stress (NaCl, 100 and 160 mM)

made with full strength Hoagland nutrient solution for next 5 days. Control treated seedlings grown by only nutrient solution and for every respective treatment, nutrient solution were renewed every day accordingly. Morphological and biochemical data were collected from 3rd and 4th leaves of seedlings while, whole seedling including root was used for observing the growth and biomass accumulations at 15-d old. The whole experiment was carried out following a completely randomized design (CRD) with three replications.

Determination of physiological and biochemical parameters

All the studied physiological and biochemical parameters were measured by following Experiment-I

Statistical analysis

The collected data were subjected to statistical analysis using XLSTAT v.2020 (Addinsoft 2020) where one way analysis of variance (ANOVA) was used. The mean differences were compared using Fisher's least significant difference (LSD) test at the 5% level of significance.

Results and summary

The protective role of COU has been noticeable through the improvement in seedling growth, biomass content, Chl and Car contents (Figure 12). But only salt stressed seedlings suffered from osmotic, ionic and oxidative stress with suffering from water shortage, ionic toxicity with mineral deficiency and cellular damage from lipid peroxidation, membrane instability. As a consequence tomato growth, biomass accumulation and photosynthetic pigmentation were hampered due to salinity. In addition, COU pretreated seedlings showed higher water status with elevated Pro content while toxic Na^+ accumulation restricted with higher content of K^+, Ca^{2+} and Mg^{2+} under stressed condition. Pretreatment with COU caused the stimulation in non-enzymatic antioxidant status with higher activities of enzymatic antioxidants and resulted in inhibition of ROS, MDA content, EL of stressed seedlings. Its first time where COU also showed the significant detoxification of MG in salt stressed tomato seedling through higher responses of glyoxalase enzymes through the support of GSH. These results can be explained by COU-pretreatment induced up regulation in antioxidants response, glyoxalase enzymes activity with a correlated action of glutathione. In addition, pretreated seedling showed the higher growth under salinity along with better mineral homeostasis which were because of COU-mediated relief from oxidative stress.

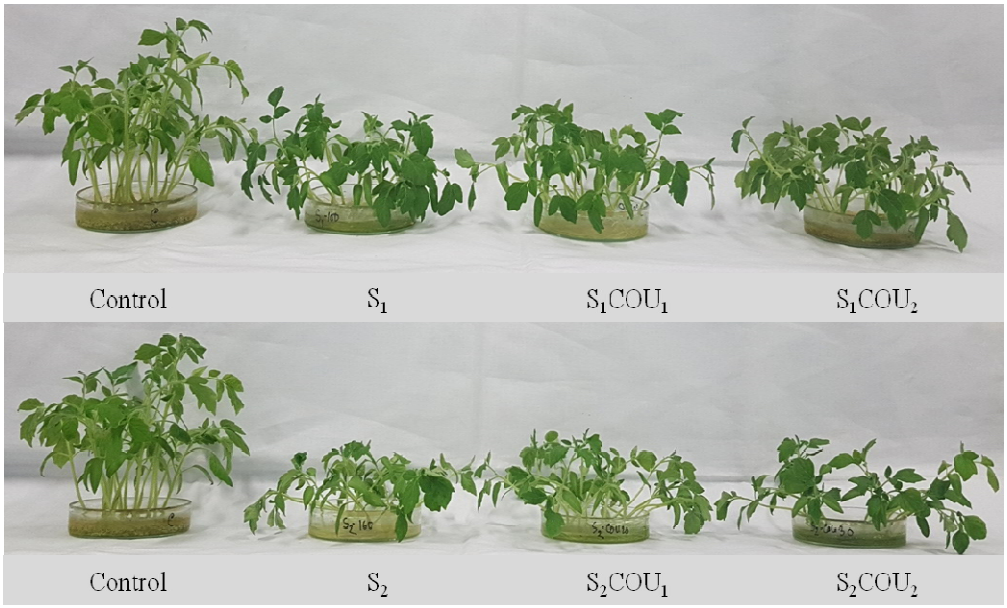

Figure 12. Visual appearance of tomato seedlings by pretreatment with and/or without coumarin (COU1, 20 µm and COU2, 30 µm; 2 days) under salt stress (100 and 160 mM NaCl) for 5 days

In sum, phenolics (Qu, VA and COU) up-regulated the plant antioxidants defense including non-enzymatic and enzymatic components and glyoxalase enzymes activities which corroborated with suppression of ROS and MG contents, respectively under salt stress. So, phenolics-mediated higher growth, biomass contents and photosynthetic pigmentation of tomato seedlings demonstrated the mitigation of salt-induced ionic, osmotic and oxidative stress. Therefore, the findings of these studies provide the protective roles of phenolics as a potent source of phytoprotectants to be used in chemical biology for abiotic stress tolerance through enhancing plant defense mechanisms of existing crops cultivars. Further more studies are demanded about phenolics-induced crops tolerance upon abiotic stresses along with other signaling molecules including hormones for higher osmoregulation with antioxidant defense and glyoxalase systems.

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