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

氏名：　Hossain Md Shahadat
Name

学位論文題目：
Title of Dissertation

Study on physiological and biochemical mechanisms of acetate-induced abiotic stress tolerance in lentil (*Lens culinaris* Medik)
(レンズ豆（*Lens culinaris* Medik）における酢酸塩誘導ー非生物的ストレス耐性の生理・生化学的メカニズムに関する研究)

学位論文要約：
Dissertation Summary

The world population is increasing day by day and is expected to be 11 billion by 2100 (FAO, 2017). To feed the 11 billion people, we need to improve the crop productivity. Unfortunately, due to climate change, sustaining crop production is becoming more challenging (FAO, 2017; Mantri et al. 2012). A number of abiotic stresses as shown in Fig. 1 may cause severe yield loss depending on the extent and duration of the stresses. For example, soil salinity is one of the major constraints that reduce crop yield (Hanin et al. 2016). When plants expose to salt stress, plants experience osmotic shock immediately. This is because the presence of salt in the growing media lowers the osmotic potential (Munns and Tester, 2008). As a result, seedlings cannot absorb water. Furthermore, with time, plants uptake excessive amount toxic ion from salts mostly, sodium (Na^+). Higher amount Na^+ disturbs physiological and biochemical processes in the plants (Munns and Tester, 2008; Ismail and Horie, 2017). Excess Na^+ replaces potassium from the active sites of some enzymes results in inactivation of these enzymes and also excludes potassium from the cell. Severe potassium leakage from the cell might activate cell death processes (Shabala and Pottison, 2014). In short, plants expose to salt stress experience osmotic stress and ion toxicity in the cell. Later on, both of these accelerate ROS generation and successive oxidative stress and cell death (Abogadallah, 2010; Shabala and Pottison, 2014). Reactive oxygen species (ROS) such as singlet oxygen, super oxide radical, hydroxyl radical and hydrogen peroxide are produced as byproduct in the cell (Gill and Tuteja, 2010; Choudhury et al., 2017). Under non-stressed conditions, plants detoxify ROS continuously so that the level of ROS cannot do much harm for the plants (Fig. 2). However, under abiotic stress conditions such as salt, Cu, As and Cd, ROS generation increases. Plant may not increase the detoxification rate to detoxify excess ROS (Fig. 2). As a result, the amount of ROS increases in the cell and react with important cellular components such as lipid, protein and DNA to oxidize them. Finally, plants experience oxidative stress and cell death (Gill and Tuteja, 2010; Choudhury et al., 2017). Being in stressed condition for long time, the adverse changes at cellular level become conspicuous at physiological and morphological level of plants showing stunted growth, chlorosis, leaf senescence, lower photosynthesis and CO_2 assimilation and at the end yield loss depending on the severity of the salt, Cu, As and Cd stress (Ismail and Horie 2017). Extensive research on salt tolerance mechanism reveals that inhibition of Na^+ uptake, lower Na^+/K^+, lower translocation of Na^+ from root to shoot, better osmolyte synthesis ability and higher ROS detoxification are related to salt tolerance in plants (Ashraf, 2009; Hasanuzzaman et al. 2013; Shabala and Pottison, 2014; Savvides et al. 2016).

Release of heavy metals/metalloids such as copper (Cu), cadmium (Cd) and arsenic (As) from the industry contaminates agricultural land which in turn affect crop production and human health when heavy metals enter to our body through the food (Yang et al. 2005; Singh et al. 2016; Abbas et al. 2018).

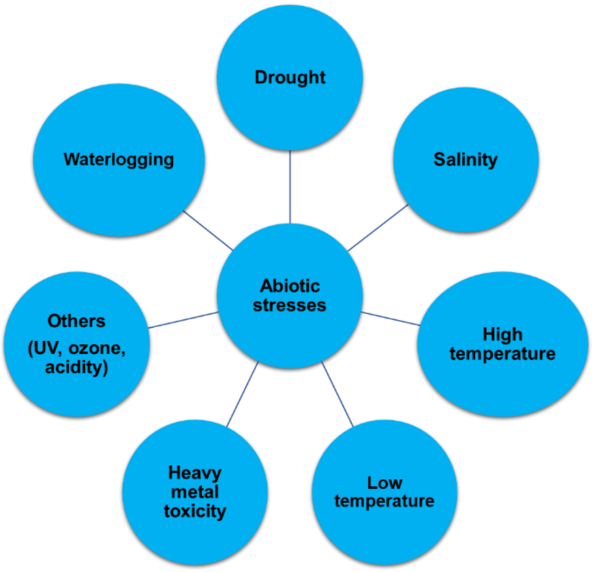


Fig. 1 Type of abiotic stresses

Due to rapid progress in industrialization and pesticide industry, use of Cu and Cd increased dramatically (Adrees et al. 2015; Huybrechts et al. 2019). Absence of proper regulations on pesticide use and ignorance of the danger of excess Cu in living beings including animals and plants lead to the contamination of soil and water (Wuana and Okieimen, 2011). Unlike Cd, Cu is required for proper growth and development for plants, more than optimal level, Cu is detrimental for plants. Being a metal element, Cu and Cd cannot be destroyed, as a result Cu and Cd persists in nature and found in different living organisms including human and plants (Adrees et al. 2015; Shanmugaraj et al. 2019). Ground water contamination with arsenic (As) is another major problem in south-east Asia including Bangladesh and India (Panaullah et al. 2009; Alam et al. 2019). People in these are exposed to As either directly by drinking As-contaminated water or by taking food that grown in As-contaminated soil. In addition, irrigation with As-contaminated ground water has dramatic negative impact on crops (Abbas et al. 2018). Therefore, exposure to Cu, Cd and As is not good for the living organisms. When crop plants is grown in these heavy metals/metalloids contaminated agricultural land, heavy metals/metalloids (Cu, Cd, and As) concentration build up in plant cell and different adverse effect become prominent at cellular, morphological and physiological level such as disturbance in nutrient uptake, chlorosis, stunted growth, cell death, biomass reduction, impaired photosynthesis, and at the end yield decreases (Wojas et al. 2010; Adrees et al. 2015; Abbas et al. 2018). Heavy metals/metalloids can disrupt the antioxidant defense system which results in severe oxidative damage in plants through overproduction of ROS (Wojas et al. 2010; Adrees et al. 2015; Abbas et al. 2018).

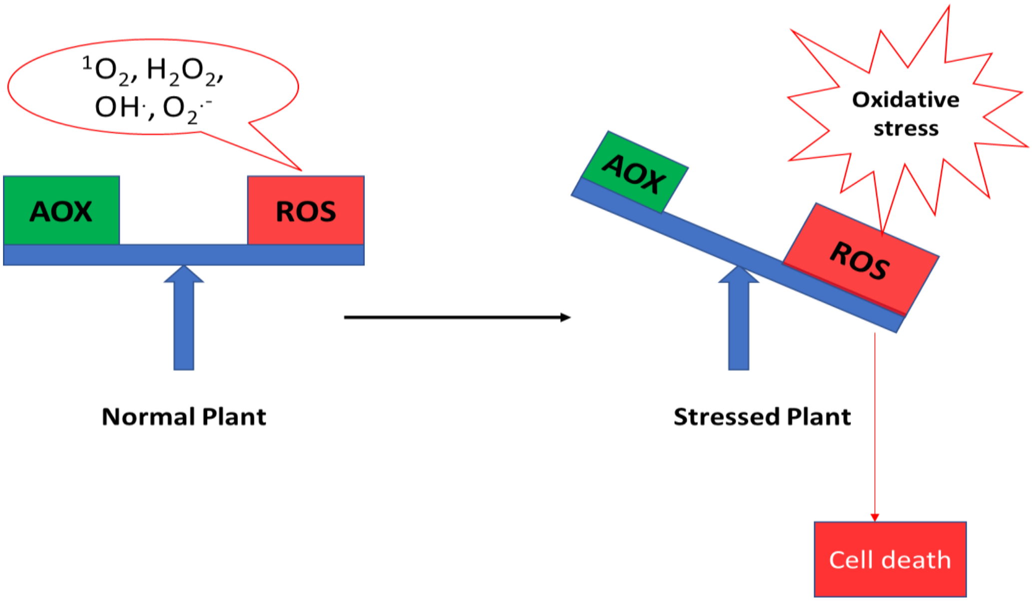


Fig. 2 ROS-mediated damage in stressed plants. AOX, antioxidant defense system; ROS, reactive oxygen species; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, super oxide radical, $OH^{\cdot}$, hydroxyl radical and H_2O_2, hydrogen peroxide

To detoxify ROS, plants are equipped with antioxidant defense system (Fig. 3). This system includes enzymatic antioxidant and non-enzymatic antioxidants (Gill and Tuteja, 2010; Hasanuzzaman et al. 2017). The enzymatic antioxidants are catalase; CAT, ascorbate peroxidase; APX, monodehydro ascorbate reductase; MDHAR, dehydroascorbate reductase, DHAR and glutathione reductase, GR. The non-enzymatic antioxidants are ascorbate, AsA and glutathione, GSH. At normal growing condition, the rate of ROS generation and the rate of ROS detoxification by antioxidant defense pathway are tightly controlled as shown in Fig. 2 and 3. However, under unfavorable growing conditions, antioxidant defense system enhanced to detoxify extra ROS (Gill and Tuteja, 2010; Choudhury et al. 2017). After certain time, antioxidant defense system decreased or cannot keep pace with ROS generation, as a result, oxidative stress occurs in plants (Choudhury et al. 2017). However, by any means, stimulation of this pathway may further increase the effectiveness of this system. As oxidative stress is a common phenomenon under abiotic stress, enhancement of antioxidant defense system might be an effective approach for stress management.

Along with the enhancement of antioxidant system to detoxify for ROS, to sustain under metal/metalloid toxicity, plants possess other mechanisms which includes synthesis of thiol metabolite like GSH, production of phytochelatin to make complex with metals/metalloids and storage it metals/metalloids vacuole, reduction in the metals/metalloids uptake and translocation from root to shoot (Adrees et al. 2015; Wang et al. 2016; Tiwary and Sarangi, 2017; Abbas et al. 2018).

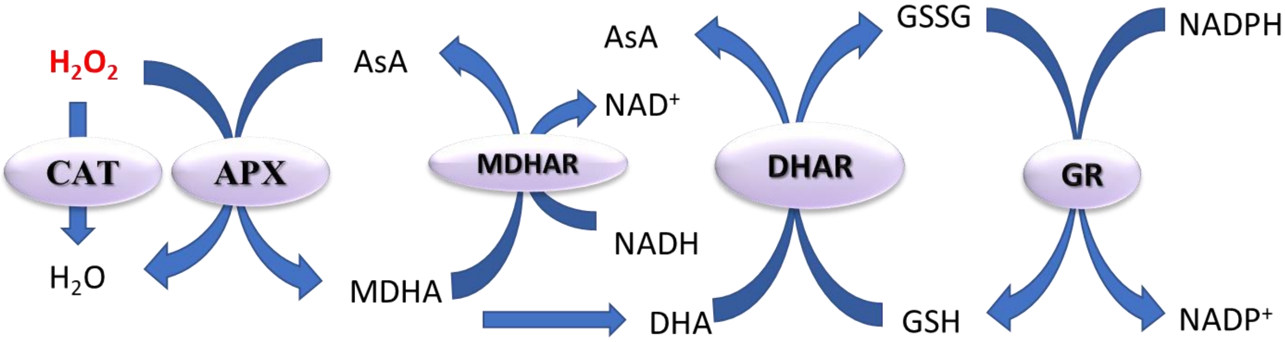


Fig. 3 Antioxidant defense system in plants. Catalase; CAT, ascorbate peroxidase; APX, mondehydro ascorbate reductase; MDHAR, dehydroascorbate reductase, DHAR and glutathione reductase, GR; ascorbate, AsA; glutathione, GSH and oxidized glutathione, GSSG.

Use of chemicals to enhance abiotic stress tolerance is an effective way thus gaining attention recently for a number of reasons. Treating plants with a chemical bring changes at molecular and cellular level and triggers a number of biochemical responses sometimes may not be manifested at morphological level (Savvides et al. 2016; Mauch-Mani et al. 2017). Due to these changes at molecular level, when the plants exposed to environmentally challenged condition, they responded more quickly and effectively compared to non-treated plants (Fig. 4) (Savvides et al. 2016; Mauch-Mani et al. 2017). This chemical biology approach has some other advantages, for example, chemicals can be applied to any plants at any time whenever necessary. A number of chemicals such as silicon (Si), salicylic acid (SA), indole acetic acid (IAA), jasmonic acid (JA), hydrogen sulfide (H_2S), nitrogen oxide (NO), polyamines, ascorbate (AsA), glutathione (GSH), gamma amino butyric acid (GABA) and beta amino butyric acid (BABA) have been reported for the effectiveness to induce stress tolerance (Savvides et al. 2016; Mauch-Mani et al. 2017; Nguyen et al. 2018). Still, chemicals are being screened for more effectiveness and cost effective. Kim et al. 2017 reported the acetate-mediated drought tolerance in *Arabidopsis*. They showed that acetate activate JA signaling pathway and modify histone protein which in turn contribute to stress tolerance in plants. Considering the potential of acetate, I hypothesized that acetate could enhance other abiotic stress tolerance.

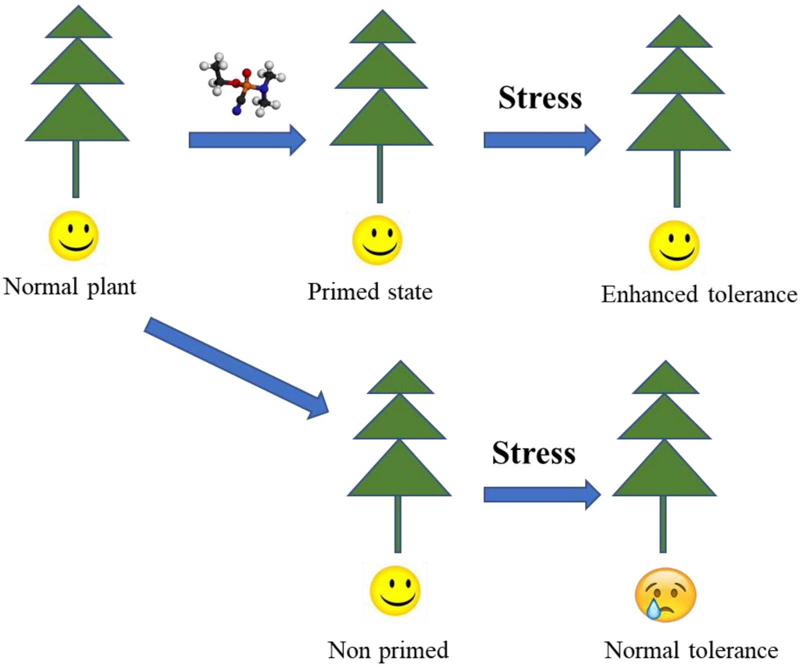


Fig. 4 Chemical priming enhances stress tolerance in plants

Lentil is a beneficial crop in many ways such as it adds up atmospheric nitrogen thus reduces the production cost and improves soil health; it is rich in protein like other beans (Rahman et al. 2012; Samaranayaka, 2017). Even though, lentil is very important crop due to its socio-economic and environmental viewpoints, very little research has been conducted on lentil compared with rice, wheat, maize and Brassica (Manners and van Etten, 2018). Like other crops, lentil production will also be affected due to adverse environmental conditions. To develop the lentil production, we need to develop proper technology that helps lentil to survive under ever changing environment.

Considering the importance of lentil, I investigated the tolerance mechanisms in lentil under different abiotic stresses after application of acetate.

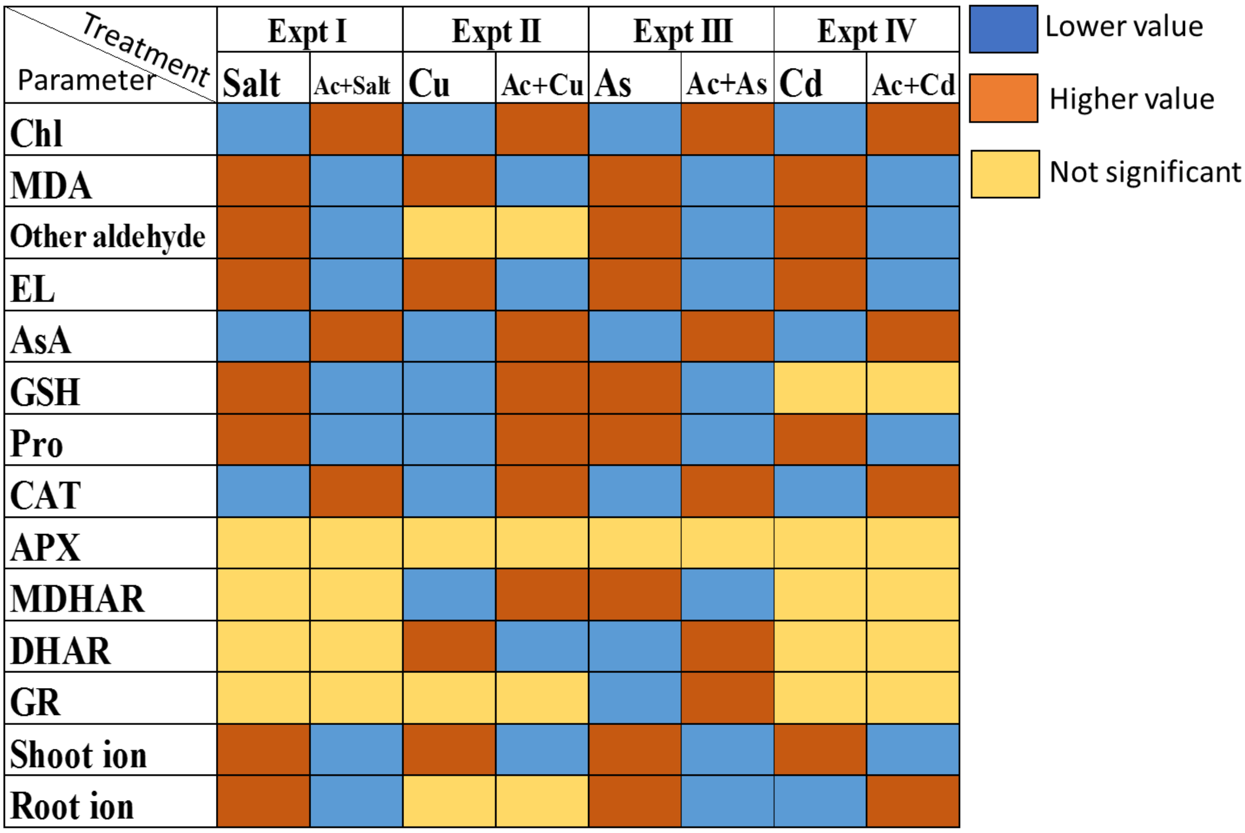
I studied the response of lentil seedling under salt (NaCl), copper (Cu), arsenic (As) and cadmium (Cd) stress conditions. All these stresses severely affected plant growth by reducing chlorophyll and carotenoids contents, and disturbing water balance. After trying different chemicals, I observed that acetate could enhance salt stress tolerance in lentil indicated by higher survival percentage and better growth. I then investigated the role of exogenous acetate under Cu, As and Cd stress conditions. Surprisingly, I found that acetate application improved the phenotypic appearance under these stress conditions. In most cases acetate application improved growth parameters, photosynthetic pigments (chlorophyll and carotenoids) and water status compared to stressed seedlings. It is hypothesized that to adjust water balance in the cell, proline content would increase under these stress conditions. As expected, I observed increased proline content compared to control seedlings under these stress conditions. And acetate treated seedlings had lower proline content in all cases except for Cu stress (Fig. 5). Mostofa et al. (2015) observed significant proline accumulation in rice under Cu stress. In this study, I observed higher proline content in lentil seedlings Cu stress after acetate pretreatment compared with Cu stresses seedlings alone, indicating that pro accumulation is not related with water content of plants. This is because acetate treated seedlings had higher water content in Cu stressed seedlings compared with Cu stressed seedlings alone. Kastori et al. (1992) reported higher accumulation of proline in Cu stressed seedling compared with Cd stressed seedling, even though both Cu and Cd stressed seedling had similar water content. Their findings strongly support our result that pro accumulation is not an indicator of osmotic stress under heavy metal stress. I assume that acetate-induced pro accumulation might play role as an antioxidant to scavenge ROS. However, proline synthesis is a costly process often results in growth penalty (Munns and Tester 2008). In this study, to synthesize more proline, seedlings spent more energy that is why I found no change in DW in acetate pretreated seedlings under

Cu stress. To reveal the exact function of proline in lentil under different stress conditions with or without acetate, further investigation is needed.

Under these stress conditions, malondialdehyde (MDA), other aldehydes, hydrogen peroxide (H_2O_2) and electrolyte leakage (EL) increased compared to control. This means that higher oxidative damage happened to seedlings under these stress conditions. Interestingly, under all kinds of stresses I tested, acetate application reduced the MDA, other aldehydes, H_2O_2 and EL contents, suggesting that acetate application reduced oxidative damage in plants (Fig. 5).

I then investigated the antioxidant defense system under these stress conditions. Acetate treated and non-treated seedlings had different responses under different stress conditions (Fig. 5). I found a common pattern in the response of antioxidant pathway under these stress conditions. Ascorbate (AsA) content and catalase (CAT) activity decreased under salt, Cu, As and Cd stress condition, indicating that antioxidant defense pathway could not function properly due to unfavorable conditions (Fig. 5). Actually, plants try to metabolize the excessive ROS through the antioxidant pathway which is composed of non-enzymatic components such as AsA and glutathione (GSH), and enzymatic components such as CAT, APX, DHAR, MDHAR and GR. However, antioxidant defense pathway cannot cope up with the ROS production level when the severity and duration of stress prolong. However, acetate application improved the AsA content and CAT activity which in turn results in lower oxidative damage under these stress conditions. Glutathione is an important antioxidant which also act as chelator under metals/metalloids stress. Under Cu stress condition, I observed acetate treated seedling had higher GSH content than only Cu stressed seedlings while acetate treatment had no effect in GSH content under Cd stress condition. From these results, it is clear that GSH accumulation and functions may vary depending on type of stresses. To clarify the role of GSH under Cd stress, I investigated the acetate-mediated tolerance mechanism in lentil seedling using GSH inhibitor (buthionine sulphoximine, BSO). BSO treatment made the seedling more susceptible in terms of phenotypic appearance and oxidative stress under Cd stress condition both acetate treated and non-treated seedlings. However, acetate treated seedling were still better than acetate non treated seedlings after BSO treatment, suggesting that acetate might not require GSH to enhance tolerance (to some extent) under Cd stress condition.

Under these stress conditions, it is observed that higher amount sodium (Na) ion, Cu, As and Cd accumulated in shoots and roots of lentil seedlings which might results in higher oxidative damage. Surprisingly, in all cases, acetate application reduced the toxic metal accumulation in above ground plant part (shoots) which is another reason for better survival of lentil seedlings under salt, Cu, As and Cd stress condition (Fig. 5). However, acetate application could not reduce the accumulation of Cu and Cd in roots of lentil seedlings.



Treatment / Parameter	Expt I Salt	Expt I Ac+Salt	Expt II Cu	Expt II Ac+Cu	Expt III As	Expt III Ac+As	Expt IV Cd	Expt IV Ac+Cd
Chl	🟦	🟧	🟦	🟧	🟦	🟧	🟦	🟧
MDA	🟧	🟦	🟧	🟦	🟧	🟦	🟧	🟦
Other aldehyde	🟧	🟦	🟨	🟨	🟧	🟦	🟧	🟦
EL	🟧	🟦	🟧	🟦	🟧	🟦	🟧	🟦
AsA	🟦	🟧	🟦	🟧	🟦	🟧	🟦	🟧
GSH	🟧	🟦	🟦	🟧	🟧	🟦	🟨	🟨
Pro	🟧	🟦	🟦	🟧	🟧	🟦	🟧	🟦
CAT	🟦	🟧	🟦	🟧	🟦	🟧	🟦	🟧
APX	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
MDHAR	🟨	🟨	🟦	🟧	🟧	🟦	🟨	🟨
DHAR	🟨	🟨	🟧	🟦	🟦	🟧	🟨	🟨
GR	🟨	🟨	🟨	🟨	🟦	🟧	🟨	🟨
Shoot ion	🟧	🟦	🟧	🟦	🟧	🟦	🟧	🟦
Root ion	🟧	🟦	🟨	🟨	🟧	🟦	🟦	🟧

🟦 Lower value
🟧 Higher value
🟨 Not significant

Fig. 5 Responses of lentil seedlings under salt, copper (Cu), arsenic (As) and cadmium (Cd) with or without acetate treatment. For salt stress, hydroponically grown six-day-old seedlings were exposed to 100 mM NaCl with or without 10 mM Na-acetate. After 2-day stress treatments, seedlings were allowed to recover for 2-day in the nutrient solution only, then, shoots were harvested for biochemical and physiological study. The treatments are 100 mM NaCl+2 day recovery (salt) and 100 mM NaCl+10 mM Na-acetate +2 day recovery (Ac+salt). For Cu stress, two sets hydroponically grown six-day-old seedlings, one set treated with 10 mM Na-acetate and another set non-treated with 10 mM Na-acetate, were exposed to 3.0 mM copper sulfate ($CuSO_4$. $5H_2O$) for four days. After four days, shoots were harvested for biochemical and physiological study. The treatments are 3.0 mM $CuSO_4$ (Cu), 10 mM Na-acetate + 3.0 mM $CuSO_4$ (Ac + Cu). For As stress, two sets hydroponically grown six-day-old seedlings, one set treated with 10 mM Na-acetate and another set non-treated with 10 mM Na-acetate, were exposed to 320 µM Na arsenate for four days. After four days, shoots were harvested for biochemical and physiological study. The treatments are 320 µM Na arsenate (As), 10 mM Na-acetate + 320 µM Na arsenate (Ac + As). For Cd stress, two sets hydroponically grown six-day-old seedlings, one set treated with 10 mM Na-acetate and another set non-treated with 10 mM Na-acetate, were exposed to 3.0 mM cadmium chloride ($CdCl_2$) for four days. After four days, shoots were harvested for biochemical and physiological study. The treatments are 3.0 mM

$CdCl_2$ (Cd), 10 mM Na-acetate + 3.0 mM $CdCl_2$ (Ac + Cd). Orange colored box represents significantly higher value than blue colored box. Two adjacent gold colored boxes represent no significant difference between the values of the boxes. Expt I, Expt II, Expt III and Expt IV indicates four individual experiments. Chlorophyll (a + b) content (Chl), malondialdehyde content (MDA), other aldehyde content (Other aldehyde), percentage of electrolyte leakage (EL), ascorbate content (AsA), reduced glutathione content (GSH), proline content (Pro), catalase activity (CAT), ascorbate peroxidase activity (APX), dehydroascorbate reductase activity (DHAR), monodehydroascorbate reductase activity (MDHAR), glutathione reductase activity (GR), metals/metalloids (Na, Cu, As and Cd) content in shoots (shoot ion) and metals/metalloids (Na, Cu, As and Cd) content in roots (root ion).

It is surprising how a single chemical, acetate provides tolerance against multiple stresses. Still, we need to conduct further research to know the effect of acetate on long term stress on different crops, to know the effect of acetate on yield of different crops under stress condition and to find the effective mode of application so that farmers can easily adopt this technology.

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