



Salicylic acid increases tolerance of *Vigna mungo* cv. T9 to short-term drought stress

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Abstract

Drought stress severely affects plant growth and productivity. Black gram is an extensively cultivated legume crop worldwide. Its production has not improved much in the last decade as it is adversely affected by biotic and abiotic stresses among which drought is a major factor. Salicylic acid (SA) pre-treatment to a *Vigna mungo* variety significantly increases chlorophyll, proline, carbohydrate, and total phenolic content. APX, GPX and SOD activities also increase and CAT activity decreased. At molecular level, induced expression of various stress-related genes, i.e. heat shock protein (Hsp), calmodulin (CAM), malate dehydrogenase (MD), metallothionein (MT), mitogen-activated protein kinase (MAPK), tryptophan synthase (TSN), zinc finger (ZF), phenylalanine ammonia lyase (PAL) and WRKY proteins are analysed by quantitative RT PCR after 1 mM SA treatment under short-term drought stress. It is observed that 1 mM SA pre-treatment is optimum to increase tolerance against short-term drought stress.

Keywords *Vigna mungo* · Salicylic acid · Drought stress

Introduction

Black gram, originated in India, is a short duration (90–120 days) legume crop, accounting for about 20% of world pulse production (Pandey 2019). Globally, higher production and consumption of black gram is reported in India, and here, it is the third important pulse (Pandey and Chakraborty 2016). It has a high nutritive value and is also used in sustainable cropping systems, medicinal preparations, nutraceuticals and cosmetics. The production of black gram has not improved in last decade mainly due to various stresses (Pandey and Chakraborty 2015). Drought stress has some typical features; it is a slow event, difficult to find out the starting and end points, no single indicator, difficult to quantify and is estimated to decrease crop productivity by half globally (Feng et al. 2020).

Plant response to drought may be instantaneous by change in protein phosphorylation and may extend to prolonged

period by altered gene expression. The response intensity depends on different features, i.e. species and genotype of plants, length and severity of drought, age and stage of development of plants, organ and cell type and the sub cellular compartment of plants. Several biochemical and molecular parameters are modulated in response to drought.

Chlorophyll content is reduced under drought stress by chlorophyll degrading enzymes in sunflower (Kiani et al. 2008), *Vaccinium myrtillus* (Tahkokorpi et al. 2007) and also in cotton (Massacci et al. 2008). Reduced chlorophyll content is linked to reduced Rubisco activity, decreased gas exchange, instable protein complexes and damaged chlorophyll (Bota et al. 2004).

Reduced pigment content and closure of stomata under drought leads to reduction in photosynthesis and ultimately results in reduced carbohydrate content (Yazdanpanah et al. 2011).

In addition, increased lipid peroxidation and membrane injury index under drought disrupts leaf integrity and further reduces photosynthetic capacity (Zlatev et al. 2006).

Proline (a metal chelator) accumulation is a very early response of plants against water deficit. It inhibits lipid peroxidation, scavenges singlet molecular oxygen and stabilises protein structures (Ashraf and Foolad 2007). It is a

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key antioxidant and able to prevent programmed cell death (Chen and Dickman 2005).

Drought stress in plants increases reactive oxygen species (ROS) which can be disruptive if not brought under tolerable limits. H_2O_2 produced under stress is a signal molecule at lower concentrations but highly destructive at higher concentrations and may cause programmed cell death (Breusegem et al. 2001; Quan et al. 2008). H_2O_2 prevents CO_2 fixation (up to 50%) in plants by oxidising the SH groups of the enzymes in Calvin cycle (Foyer and Shigeoka 2011).

To bring down the levels of ROS, plants have non enzymatic-enzymatic clean-up mechanism (Halliwell and Gutteridge 2006) where antioxidants play major role in the detoxification of ROS. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) are key enzymatic antioxidants.

Polyphenolic compounds (secondary metabolites) protect plants against biotic and abiotic stress and phenylalanine ammonia lyase (PAL) play central role in their synthesis (Yang et al. 2016). Increase in phenolic content is reported under drought, high and low temperature, low soil fertility, high light intensity, water logging and UV radiations (Mole et al. 1988; Delalonde et al. 1996; Close and McArthur 2002; Ali and Abbas 2003; Gholizadeh 2011; Kabiri et al. 2014). Phenyl alanine ammonia lyase (PAL) is a component of antioxidative defence system and facilitates the protection against different stresses such as drought. Superoxide dismutase (SOD) works as a first line of defence against oxidative stress (Wang et al. 2020). Increased SOD activity is studied by Sharma and Dubey (2005) in rice, Zlatev et al. (2006) in common bean and also by Wang et al. (2008) in white clover and alligator weed against drought stress.

Catalase is a light sensitive enzyme that converts H_2O_2 into H_2O and O_2 . Sharma and Dubey (2005) reported the decrease in CAT activity in rice seedlings under drought stress. Scavenging efficiency of CAT for H_2O_2 is lower when compared with peroxidase (Erdal and Dumlupinar 2010).

Ascorbate peroxidase (APX) uses ascorbate as a hydrogen donor to reduce the H_2O_2 in water-water and AA-GSH cycles (Asada 2000). APX has higher affinity (μM range) for H_2O_2 when compared with CAT and POD (mM range). Increased APX activity is reported by Yang et al. (2008) in *Picea asperata* and by Zlatev et al. (2006) in *Phaseolus vulgaris* against drought stress.

Guaiacol and pyrogallol work as electron donor for Guaiacol peroxidase (GPX) activity (Jebara et al. 2005). Increased GPX activity was reported by Zhang et al. (2006) and by Pan et al. (2006) in liquorice under stress conditions.

At molecular level, plants turn on or turn off a series of genes under different stresses. Heat shock protein (Hsp), calmodulin (CAM), malate dehydrogenase (MD), metallothionein (MT), mitogen-activated protein kinase (MAPK), tryptophan synthase (TSN), zinc finger (ZF), phenylalanine

ammonia lyase (PAL) and WRKY directly or indirectly protect plants against stress conditions.

Pre-treatment with nitric oxide, ethylene and salicylic acid (SA) that are involved in plant signalling process have been used to increase tolerance to environmental stresses. SA is a phenolic compound and acts as a plant growth regulator; is able to affect various physiological, biochemical and molecular processes. Under stress condition, it protects photosynthetic pigments and enhances the activity of antioxidative enzymes (Gill et al. 2016; Wang et al. 2017). Involvement of SA against environmental stresses, particularly against biotic stresses is well established in black gram (Kundu et al. 2012).

In the present study, *V. mungo* plants are given SA pre-treatment and put under short-term drought stress to evaluate its effectiveness in increasing tolerance.

Materials and methods

Seeds of T9 plants were obtained from Division of Plant Biology, Bose Institute, Kolkata, India. Germinating seeds were transferred to soil filled plastic pots (capacity: 1L). The pots were kept in the growth chamber at $(30 \pm 2^\circ C)$. Three-week-old test plants (21 DAS) were grouped into 5 sets, each set consisted of 12–15 replicate plants as follows: set 1—healthy control plants; set 2—untreated plants; set 3, 4 and 5—leaves of T9 plants were pre-treated with either 0.5 mM, 1 mM or 3 mM of SA, respectively, by spraying until run-off and subsequently subjected to drought stress by withholding water after three days (72 h). Physiological, biochemical and molecular studies were done at the time of SA treatment, after 72 h of SA treatment, after 3 days of drought stress and after 24 h of re-watering (recovery).

Physiological study

The leaves of T9 plants were noted at the time of SA treatment, after SA treatment, after short drought stress and on recovery for any necrotic symptoms.

Biochemical study

Chlorophyll content (Arnon 1949), carbohydrate content (Hedge and Hofreiter 1962), H_2O_2 content (Alexieva et al. 2001) total phenolic content (Singleton et al. (1999) method with modifications as mentioned by Chakraborty et al. (2008), proline content (Bates et al. 1973), and lipid peroxidation (De Vos et al. 1989) were determined.

To determine the activities of antioxidant enzymes, fresh leaves (0.5 g) were homogenised in a mortar and pestle under ice-cold condition with 5 ml extraction buffer (50 mM phosphate buffer pH 7.0, 1 mM EDTA, 1 mM ascorbate, 1 mM

PVP and 0.05% tritonX100). It was centrifuged at 5000 g for 20 min at 4 °C. Supernatant was collected and stored at – 20 °C for the assay. Activity of superoxide dismutase (Beauchamp and Fridovich 1971) as modified by Madamanchi et al. (1994); catalase (Miyagawa et al. 2000), ascorbate peroxidase (Chen and Asada 1989), guaiacol peroxidase (Kar and Mishra 1976) and PAL activity (Chakraborty et al. 2008) were determined.

Statistical analyses

Data are analysed by two-way analysis of variance to detect the overall significant ($p < 0.05$) differences between all means using SYSTAT (Ver. no. 13.00.05 SYSTAT software Inc. 2009).

Quantitative RT PCR

Quantitative RT PCR was performed and the activity of the following genes was observed: heat shock protein (Hsp), calmodulin (CAM), mitogen-activated protein kinase (MAPK), zinc finger (ZF), malate dehydrogenase (MD), tryptophan synthase (TSN), metallothionein (MT), phenylalanine ammonia lyase (PAL), rubisco activase (RA) and WRKY proteins, at the time of 1 mM SA treatment, after 72 h of SA treatment, after short-term drought stress and on recovery. Prior to RT PCR, RNA isolation and cDNA synthesis were done using Spectrum Plant Total RNA Kit (Sigma Aldrich, catalog no. STRN50) and high-capacity cDNA reverse transcription kit (Applied Biosystems, catalog no. 4368814), respectively. RT PCR was done using DyNamo Color Flash SYBR Green qPCR kit (Thermo scientific, catalog no. F-416L) in a Qiagen Rotor Gene Q system. Quantitative study of the candidate genes was done according to Livak and Schmittgen (2001).

Results and discussion

Drought stress limits the plant growth and yields. Exogenous application of salicylic acid, an endogenous plant growth regulator, protects plants against stress conditions.

In the present study, wilting of leaves was observed in *V. mungo* plants under short-term drought stress. SA treatment at 1 mM concentration lowered wilting after 3 days of drought stress and on recovery while 3 mM SA treatment damaged the leaves and necrotic regions were observed. It is reported earlier the lower concentration of SA works as a defence strategy against drought stress, while higher concentration of SA generally damages the plants (Joseph et al. 2010).

Reduction in chlorophyll content was observed under drought stress (Fig. 1a, b, c). When the plants were re-watered, increased chlorophyll content was observed in all plants except untreated plants when compared with drought-stressed plants of the same set. Higher chlorophyll content was observed in 1 mM SA-treated plants when compared to other concentrations. Alam et al. (2013) reported reduced chlorophyll content under drought stress.

In the present study, highest H_2O_2 content was observed in 3 mM SA-treated plants (Fig. 2a) and this may be the cause of necrotic regions observed in the leaves. SA treatment is reported to increase H_2O_2 and may lead to injury to plant tissue (Habibi 2012). H_2O_2 at lower concentration plays important role in signal transduction against stress conditions (Gong et al. 2005) while at higher concentration it is harmful to plants.

Total phenolic content was enhanced under drought stress as well as after SA treatment in the present study (Fig. 2b). Previously, Ali et al. (2007) reported enhanced level of total phenolics on SA treatment. Due to higher antioxidant properties that decrease ROS level under stress condition, phenolic compounds may facilitate plant defence against environmental stresses (Chakraborty et al. 2008).

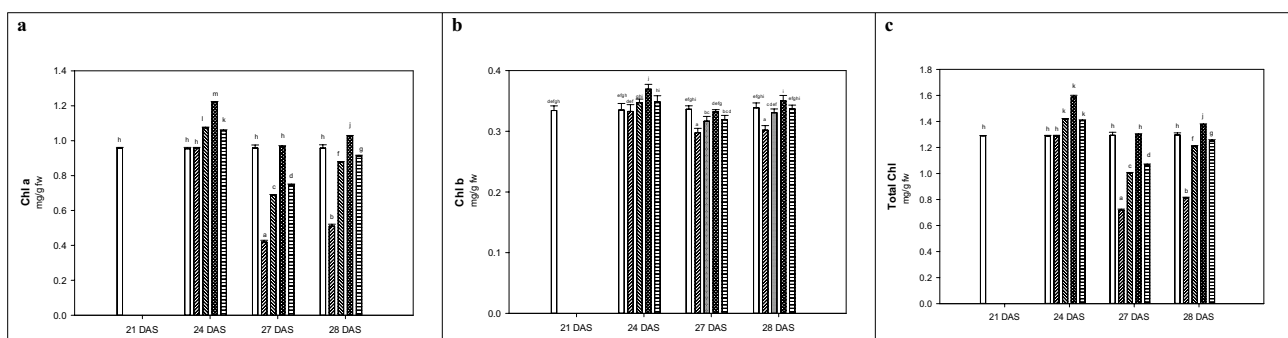


Fig. 1 Effect of drought stress and SA treatment on chlorophyll content (mg/g fw) of *V. mungo* leaves; **a** chlorophyll a, **b** chlorophyll b, **c** total chlorophyll c (bars with same alphabets are not significantly different at $p = 0.05$)

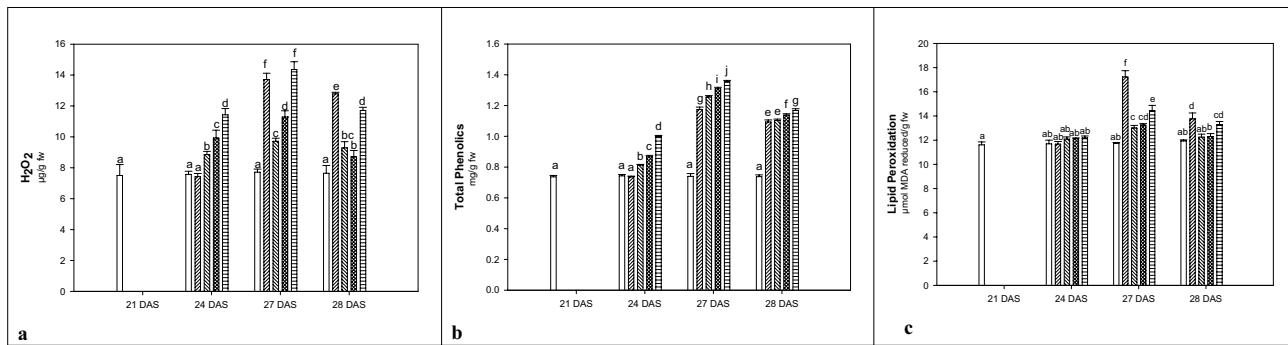


Fig. 2 Effect of drought stress and SA treatment on **a** H₂O₂ content, **b** total phenolic content, and **c** lipid peroxidation in *V. mungo* (bars with same alphabets are not significantly different at $p=0.05$)

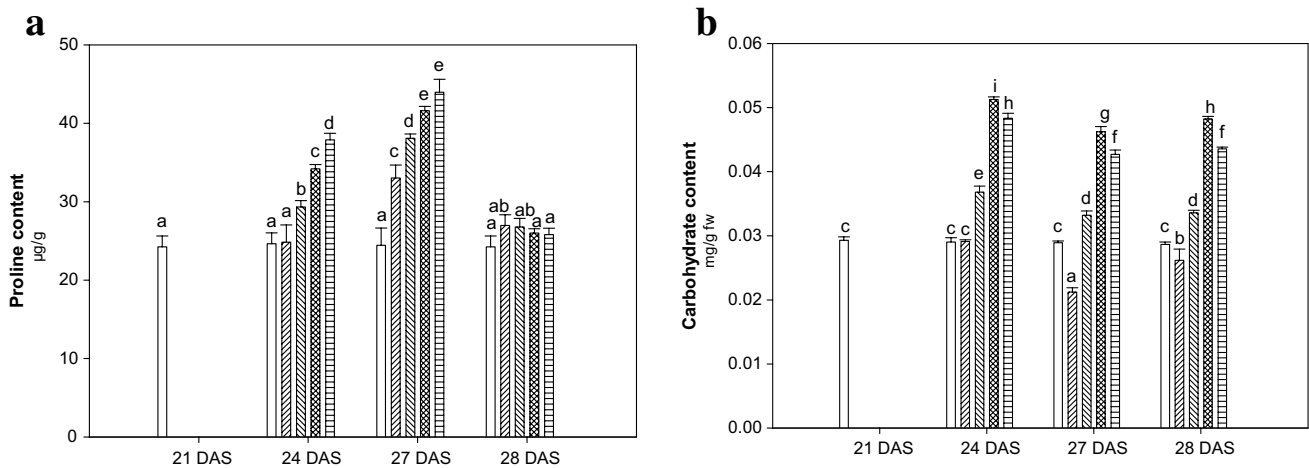


Fig. 3 Effect of drought stress and SA treatment on **a** proline content, **b** carbohydrate content in *V. mungo* (bars with same alphabets are not significantly different at $p=0.05$)

Lipid peroxidation increased in drought-stressed plants after 3 days of short-term drought stress while lower level of lipid peroxidation was observed in 0.5 mM SA-treated plants and 1 mM SA-treated plants (Fig. 2c). Decrease in lipid peroxidation has been reported on SA treatment under drought stress (Alam et al. 2013; Kabiri et al. 2014).

Higher proline content was observed in 3 mM SA-treated plants, while it was lower in drought-stressed plants under short-term drought stress (Fig. 3a). Enhanced level of proline under drought stress is reported by Yazdanpanah et al. (2011) and Patel and Hemantaranjan (2012) on SA treatment and may act as a protective mechanism.

Drought stress adversely affected the ability of the plant metabolism and decreased carbohydrate content was observed after 3 days of drought stress (Fig. 3b). Treatment with SA could prevent the adverse effects of drought and an increase in carbohydrate concentration was observed. Increased carbohydrate content after SA treatment is previously reported by Yazdanpanah et al. (2011).

PAL activity increased on SA treatment in a concentration-dependent manner and also after 3 days of short-term drought stress (Fig. 4). The increase in PAL activity explains

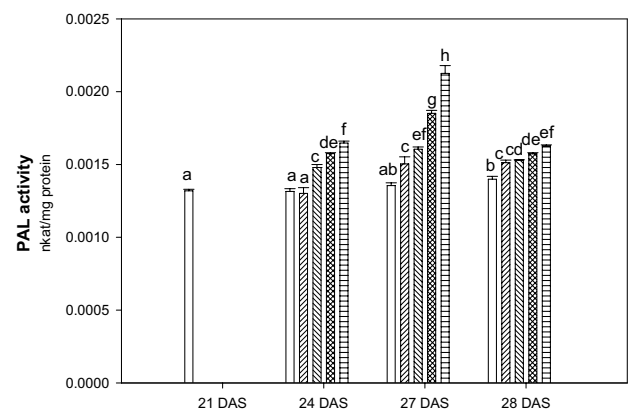


Fig. 4 Effect of drought stress and SA treatment on PAL activity in *V. mungo* (bars with same alphabets are not significantly different at $p=0.05$)

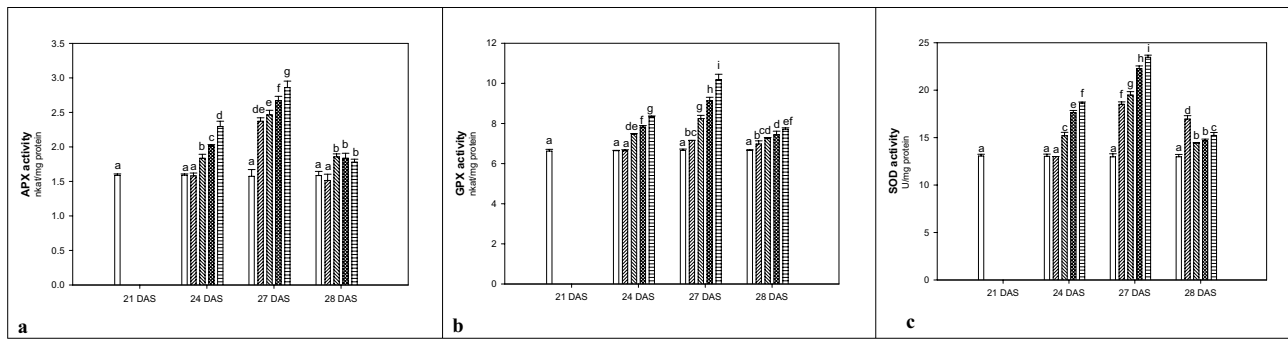


Fig. 5 Effect of drought stress and SA treatment on **a** APX, **b** GPX and **c** SOD activity in *V. mungo* (bars with same alphabets are not significantly different at $p=0.05$)

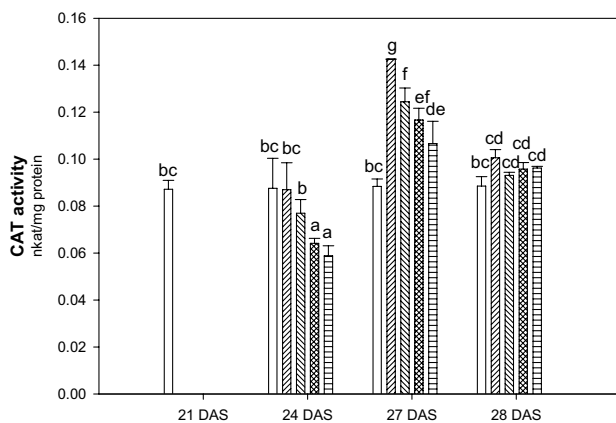


Fig. 6 Effect of drought stress and SA treatment on CAT activity in *V. mungo* (bars with same alphabets are not significantly different at $p=0.05$)

the increased phenolic concentration in the leaves and is previously reported by Ali et al. (2007).

The activity of the antioxidant enzymes, APX, GPX and SOD activity (Fig. 5a–c) increased, while CAT activity (Fig. 6) decreased on SA treatment. Increased activity of the antioxidant enzymes APX (Saruhan et al. 2012), GPX

(Horvath et al. 2007) and SOD (Saruhan et al. 2012), and decreased activity of CAT (Shakirova, (2007) on SA treatment is due to the activation of antioxidant defence mechanism of plants to increase tolerance under stress conditions.

Induced Hsp (heat shock protein) expression is reported in biotic as well as abiotic stress conditions (Reddy et al. 2014; Pavlova et al. 2009), while there are no reports of their induction under normal conditions (Zhang et al. 2008). In the present study, increased Hsp expression was observed after SA treatment as well as under short-term drought stress and among all treatments higher fold change was observed for 1 mM SA-treated plants after short-term drought stress (Fig. 7a). HSPs work as molecular chaperones, highly conserved polypeptides and play pivotal role under biotic and abiotic stress (Kotak et al. 2007). HSPs also increase the membrane stability and scavenge ROS by regulating antioxidant enzymes. They help in protein folding and restrict irreversible mis fold of proteins to maintain cellular homeostasis against biotic and abiotic stress (Ahuja et al. 2010).

Intracellular plant Ca^{2+} level is influenced by different environmental stress conditions including salinity and drought stress, during plant growth and development (Xu et al. 2011). Intracellular Ca^{2+} plays important role in plant defence mechanisms as it acts as a signal molecule against

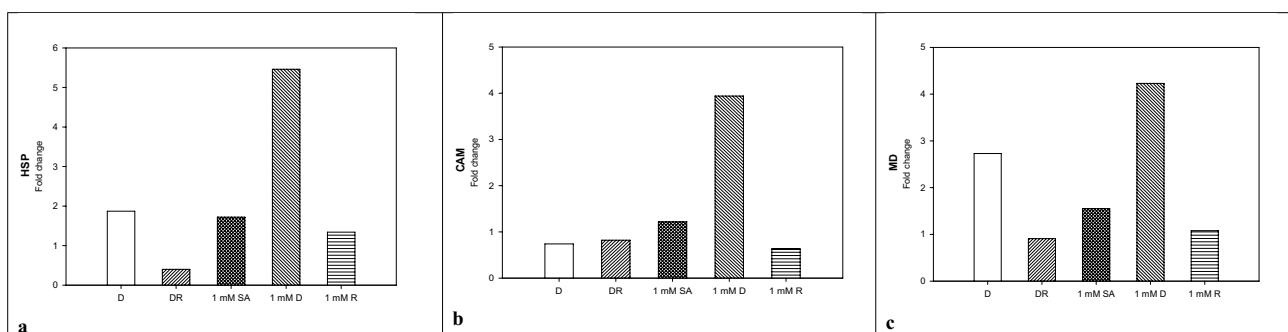


Fig. 7 Effect of drought stress and 1 mM SA treatment on **a** Hsp, **b** CAM, **c** MD gene expression in *V. mungo*

stress conditions (Yang et al. 2010). Calmodulin (CAM) is a Ca^{2+} sensor, important in different calcium-dependent signalling pathways (Sun et al. 2001). AtCML9 (Magnan et al. 2008), AtCML24 (Delk et al. 2005) and AtCML42/43 (Chiasson et al. 2005) are modulated under different stress conditions.

In the present study, expression of CAM was induced after SA treatment and among all treatments higher expression was studied in 1 mM SA-treated plants under short-term drought stress (Fig. 7b).

Malate dehydrogenase (MD) expression was induced after SA treatment as well as under short-term drought stress in the present study (Fig. 7c). MD is a key enzyme of malate/aspartate shuttle and TCA (tri-carboxylic acid cycle). Different isoforms of MD differ in their localization and specificity for the NAD or NADP. Higher accumulation of MD is reported in soyabean leaf against drought and heat stress (Das et al. 2016). Increased expression of malate dehydrogenase like protein is reported in common bean against water-deficit conditions (Recchia et al. 2013).

A higher expression of Metallothionein (MT) was observed on 1 mM SA treatment under 3 days of short-term drought stress. Induced expression of MT was also studied after SA treatment but it was less when compared with 1 mM SA treatment under drought stress (Fig. 8a). MTs keep intracellular metal homeostasis and work as a ROS scavenger (Zhou et al. 2006). Yang et al. (2009) found the over expression of OsMT1a that increased the level of APX, CAT and POD in transgenic rice plants when compared to wild-type rice plants under drought stress. Accumulation of OsMT1a (Yang et al. 2009) and MT3 (cotton metallothionein protein) (Xue et al. 2009) are reported under water-deficit condition that protect plants from harmful effects of ROS. On the other hand, MT2 (metallothionein protein) in *Arabidopsis* is not induced by SA treatment (Murphy and Taiz 1995).

Mitogen-activated protein kinase (MAPK) cascades play pivotal role in various signalling pathways and respond to drought, wounding, salt stress, cold and oxidative stress (Ichimura et al. 2000; Yuasa et al. 2001). In the present work, MAPK expression was induced after SA treatment but not under short-term drought stress (Fig. 8b). Among all treatments, higher fold change was observed in 1 mM SA-treated plants on 3 days of drought stress. MAPK genes as AtMPK3, AtMPK4, AtMPK6, OsMPK1, OsMPK5, OsMPK12, GhMPK7 and p48 SIP kinase, etc. are reported to be induced by SA treatment and are essential for signal transduction (Li et al. 2012). Expression of OsMAPK44 is induced in rice against water deficit and salt stress (Jeong et al. 2006).

Tryptophan synthase is an important enzyme in tryptophan and indole synthesis. Higher expression of TSN gene was observed at 1 mM SA treatment after drought stress (fold change: 3.81) and lower expression in recovered drought-stressed plants (fold change: 0.33) (Fig. 9a). Here, expression of this gene was induced after drought stress (D) and SA treatment (1 mM SA and 1 mM D). There is no literature available on the effect of SA treatment on TSN expression. Camalexin (3-thiazol-2'-yl-indole) which is originated from tryptophan is a phytoalexin of *Arabidopsis thaliana*. Induced expression of Camalexin is studied by plant pathogens and salicylic acid plays important role in it (Glawischig 2007).

Zinc finger motifs are involved in RNA binding, transcriptional regulations, apoptosis, protein-protein interactions and growth and development of plants. These motifs are able to protect plants against salt and drought stress and suitable for engineering crop plants with enhanced resistance (Xu et al. 2008). Expression of various ZF proteins, i.e. ZFP252, ThZF1, GsZFP1, OsZnI, AtZAT6, AtTZF2, AtTZF3, GhTZF1 and OsTZF1 are reported under drought stress (Jan et al. 2013; Shi et al. 2014; Zhou et al. 2014).

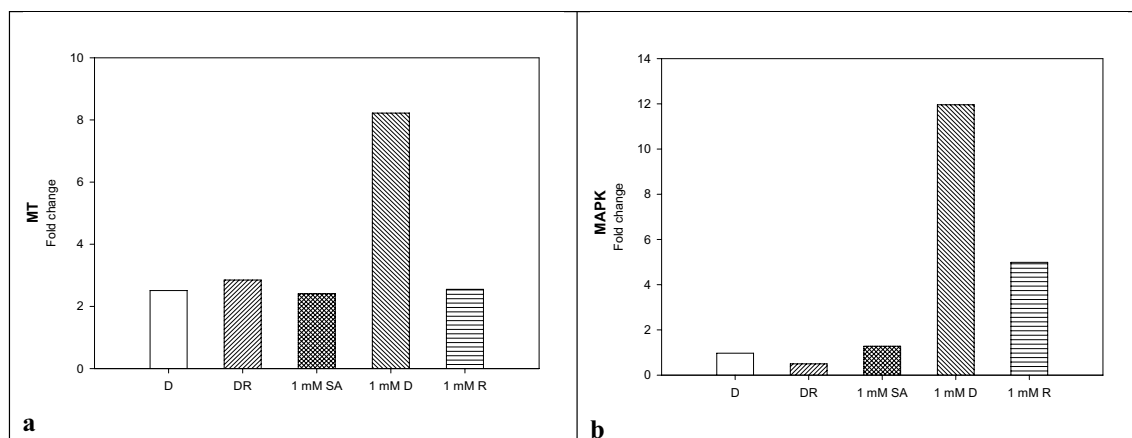


Fig. 8 Effect of drought stress and 1 mM SA treatment on **a** MT and **b** MAPK gene expression in *V. mungo*

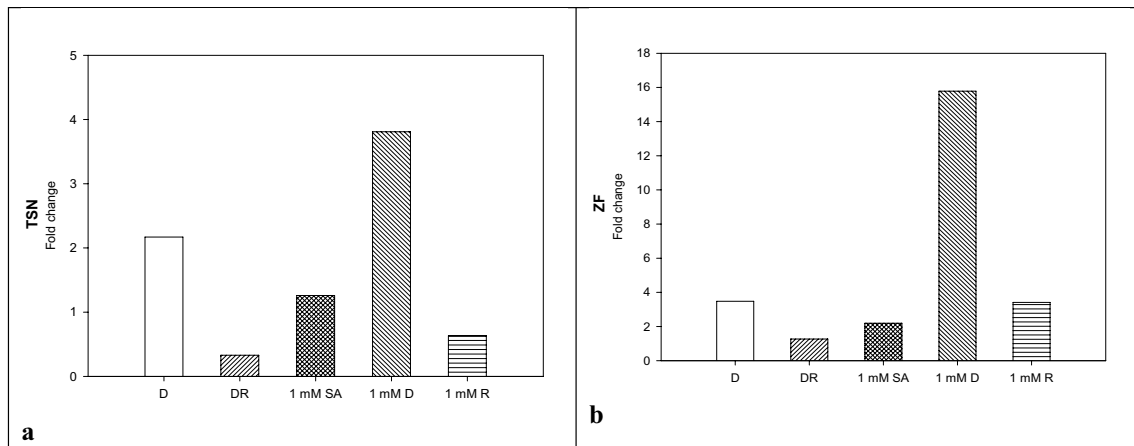


Fig. 9 Effect of drought stress and 1 mM SA treatment on **a** TSN and **b** ZF gene expression in *V. mungo*

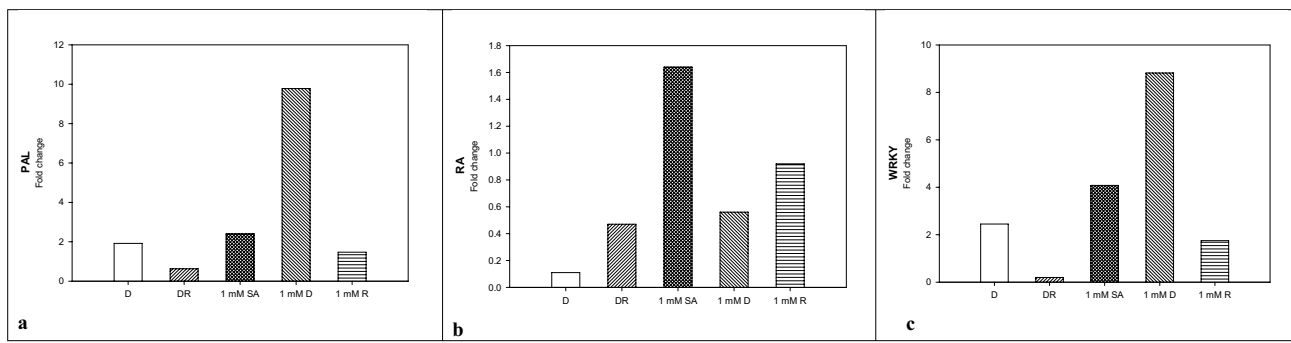


Fig. 10 Effect of drought stress and 1 mM SA treatment on **a** PAL, **b** Rubisco, **c** WRKY gene expression in *V. mungo*

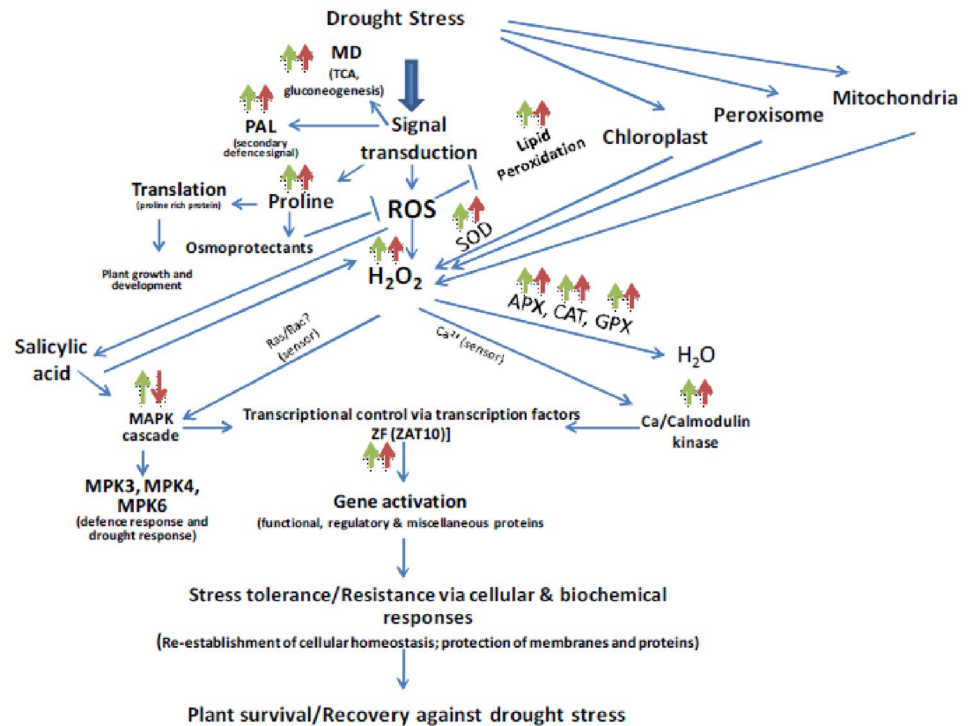
ZF activity was induced after SA treatment and also after short-term drought stress (Fig. 9c). Different ZF proteins as CaKR1, GhTZF1 and OsTZF1 are regulated by SA (Jan et al. 2013).

Collaborating the biochemical study, PAL gene was also induced on SA treatment and under short-term drought stress (Fig. 10a). Higher fold change was studied for 1 mM SA-treated plants after 3 days of drought stress. These findings are in accordance to the results obtained by Phimchan et al. (2014). Regulation of PAL activity by SA has been reported by Xu et al. 2012.

Rubisco gene expression was increased after SA treatment but after short-term drought stress, it decreased (Fig. 10b). Drought stress damages the Rubisco protein and Rubisco activase (RA) works as a chaperone and protects synthesis of chloroplast protein from drought-induced damage. Aranjuelo et al. (2011) and Zhou et al. (2007) reported reduced RA activity in plants under drought stress while some workers reported minor or even no hindrance in Rubisco activity (Pelloux et al. 2001; Flexas et al. 2006).

WRKY genes (group of transcription factors) are involved in plant development, dormancy, drought tolerance, embryogenesis and thermal hysteresis (Xie et al. 2005) and some of them are reported to be regulated by SA (Zhang et al. 2012). WRKY transcription factors play pivotal role in stress response or tolerance and also regulate different plant processes (Phukan et al. 2016). Ding et al. (2016) in wheat, Wang et al. (2015) in soybean and Wu et al. (2017) in common bean studied that WRKY proteins facilitate tolerance against abiotic stresses. Niu et al. (2012) studied the role of TaWRKY2 (wheat WRKY gene) in growth, development and also against stress condition. Increased level of WRKY21 is reported in *Arabidopsis* that facilitates the drought tolerance (Jiang et al. 2012). In the present study, expression of WRKY was induced under short term of drought stress and also after SA treatment (Fig. 10c). Higher fold change was observed for 1 mM D (1 mM SA-treated plants after 3 days of drought stress).

Fig. 11 Modulation of biochemical and molecular processes in *V. mungo* plants treated with SA and then put under drought stress



Conclusion

A summary of modulation of biochemical and molecular processes in *V. mungo* plants treated with SA and then put under drought stress is represented in Fig. 11. The role of SA against biotic and abiotic stresses as a plant defence molecule is well established but there is no literature available for the effect of SA pre-treatment in pulses under short-term drought stress. 1 mM SA treatment was found to suitable for T9 plants to tolerate short term of drought stress while higher concentrations, i.e. 3 mM damaged the plants.

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Author contribution Sonali Pandey conducted the study, compiled the results. Dipjyoti Chakraborty conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors disclose no conflict of interest.

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