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EXOGENOUSLY APPLIED COUMARIN-INDUCED SALT TOLERANCE IN A MULTIPURPOSE CROP *SORGHUM BICOLOR* UNDER SALINE CONDITIONS

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ABSTRACT

Secondary metabolites mainly the phenolic compounds play an essential role in scavenging the reactive oxygen species in plants which occur due to salt stress. This study primarily focused on pre-treatment of *Sorghum bicolor* var. SS77 seeds with a potent phenolic compound (Coumarin) to induce salt tolerance in seedlings. The seeds were hydroprimed with distilled water and with two different concentrations (50 ppm and 100 ppm) of coumarin. They were germinated under different salinity regimes of 0 mM, 100 mM and 200 mM NaCl by incubating in growth chamber at 28 ± 1°C in dark for 12 h and seedlings were harvested after 12 days. Coumarin (100 ppm) effectively enhanced vegetative growth and antioxidant enzyme activities (catalase, ascorbate and guaiacol peroxidase) of sorghum seedlings under different salinity regimes. However, salt stress has considerably reduced vegetative growth and antioxidant enzyme activities of sorghum seeds under different salinity regimes. Increase in vegetative growth parameters (shoot and root lengths, fresh and dry weights) and antioxidant enzyme activities (catalase, ascorbate peroxidase and Guaiacol peroxidase) were recorded at different salinity regimes. Coumarin 100 ppm dosage was found to be more effective as compared to 50 ppm coumarin and also give better results at higher salt concentration.

Key words: Seed priming, coumarin, sorghum, salt tolerance, antioxidant enzymes.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench; Family Poaceae) is characterized as moderately salt tolerant C_4 crop (Greenway and Munns, 1980). It is fifth most important food crop known to cover approximately 44 million hectares of land in more than 100 countries with an average annual production of 60 million tons (Iqbal *et al.*, 2010). Sorghum is a multipurpose crop extensively utilized as food, fodder and feed stock production (Ngara *et al.*, 2012). Due to greater resemblance to maize, sorghum can be utilized as a substitute of corn by domestic animals feed. However, growth and biomass potential of sorghum is hampered by salinity stress around the world.

Salinity has affected about 800 million hectares of land throughout the world which incapacitate more than 7% of the total land area (FAO, 2008). Salt may ascend naturally in the subsoil or acquainted by brackish water irrigation. Improper management of irrigation due to water scarcity and farming has exaggerated this most damaging abiotic stress around the world, particularly in arid and semi-arid areas (Abraha and Yohannes, 2013*;* Munns and Gilliham, 2015). Salinity induced hyper-ionic and osmotic stress generates alteration in major metabolic processes in plants (Tavakkoli *et al.*, 2010), including membrane damage, nutrient imbalances, altering concentrations of growth regulators and enzymatic imbalances, which reduces the photosynthetic activities (Hasanuzzaman *et al.*, 2012).

Phenolic compounds are most abundant group of secondary metabolites in higher plants that help in stress resistance. More commonly found phenolic compounds in plants are simple phenyl-propanidids, coumarin, benzoic acid derivatives, lignin and lignin precursors, tannins and flavonoids (Vermerris and Nicholson, 2006). Coumarin and its derivatives belong to efficient class of phenols synthesized by all higher plants (Razavi, 2011). Coumarin actively promotes antioxidants, cytotoxic and antimicrobial properties of plants resulting enhanced growth and development (Lupini *et al*., 2010).

Crops at the initial phase of seed germination are hypersensitive to saline environment (Shitole and Dhumal, 2012). To resolve this issue priming of seeds with different stress ameliorating compounds were suggested in previous reports (Maiti, *et al.*, 2011*;* Tavili *et al.*, 2011). Seed priming is a controlled hydration strategy to enhance seed germination, imbibition to break seed dormancy, activate utilization of stored food material and activates several enzymes (Khalil *et al.*, 2010*;* Ghassemi-Golezani *et al.*, 2011). Increased in salt tolerance of cereals by seed priming techniques has reported in various studies (Jafar *et al.*, 2012.,Tabassum *et al.*, 2017). Seed priming promoted growth by increasing the antioxidant enzymatic activities, synthesis of osmolytes and osmo-protectants which reduced free radical stress in plants generated due to salt stress (Chen and Arora, 2013). Therefore, current

study was designed to determine the potential of coumarin priming under saline conditions in *Sorghum bicolor* var. SS77

MATERIALS AND METHODS

Seed germination and Seedling growth conditions

A study was designed to assess the effects of coumarin seed priming on seedling growth of *Sorghum bicolor* var. SS77 under saline stress *in vitro*. Seeds were primed with distilled water (hydropriming), 0 (non-primed), 50 and 100 ppm of coumarin (dissolved in 0.1% ethanol) for 4 h at 25 °C, air dried and were transferred to Petri plates containing different salinities (0, 100 and 200 mM NaCl). The plates were incubated at 28±1 °C in dark for 12 h for seed germination and then exposed to light/dark (14 h/10 h) periods at 28±1 °C for 7 days. Seedling growth and antioxidant enzyme activities were recorded at the end of 7th day.

Extract Preparation for Proteins and Enzymes

Fresh leaf material (0.2 g) were grinded with liquid nitrogen, homogenized in 6 mL of potassium phosphate buffer (100 mM, pH 7.5) and centrifuged at 12,000 x *g* at 4 °C for 20 minutes. The supernatant (protein extract) was transferred quickly into Eppendorf tubes and stored at -20 °C in a refrigerator.

Proteins Estimation Methodology

Proteins were estimated by following method of Bradford (1976) using Comassie blue dye. Fifty milligram of Comassie blue G-25 dye was dissolved in 25 mL of ethyl alcohol in a beaker and shake virgously, then 50 mL of 85% phosphoric acid was added into this solution and made up to 100 mL with distilled water. The solution color was dark red. The assay reagent was prepared by diluting 1 volume of the dye stock with 4 volumes of the distilled water. The solution was dark-brown in color and had pH of 1.1. Five mL of the reagent (prepared diluted dye stock) was placed in the test tubes with an addition of 100 µL enzyme extract and absorbance was recorded at 590 nm and values of protein was calculated by standard curve of bovine serum albumin, the formula for the protein estimation is as follows:

Protein mg/g = (Value from standard curve * total volume of the extract * dilution factor) / Weight of material

Antioxidant enzymes estimation

Different antioxidant enzyme activities like catalase (Aebi, 1984), Ascorbate peroxidase (Nakano and Asada, 1981) and Guaiacol peroxidase (Polle *et al.*, 1994) were determined in protein extracts of sorghum seedlings.

Catalase Enzyme assay

Protein extract was employed for estimation of catalytic activity in sorghum seedlings. Potassium phosphate buffer (100 mM), pH = 7.0 was prepared and 15 mM H_2O_2 and 100 µL of enzyme extract was taken for estimation of catalytic enzymatic activity. Three mL reaction mixture was taken in a quartz cuvette containing, potassium phosphate buffer, H_2O_2 for blank and for test in this 3 mL reaction mixture, 100 uL of enzyme extract was added and immediately noted absorbance at 240 nm for 0, 30 and 60 s carefully. The assay was performed at 25 °C. After determining the linear decrease in absorbance of the given extract, the catalytic activity was calculated by the given formula:

Catalytic activity (Units mL^{-1} enzyme) = Δ 240nm/min * 3 * dilution factor/ EnC * volume of extract used for test.

Where, EnC = 39.4 $M^{-1}cm^{-1}$.

Ascorbate Peroxidase Enzyme Assay

Ascorbate peroxidase was estimated by taken 3 mL of the reaction mixture containing potassium phosphate buffer (100 mM, pH 7.0) having 0.5 mM ascorbic acid and 0.1 mM H_2O_2 as blank and for enzymatic activity 100 uL of the prepared protein extract was added into it and immediately noted the linear decrease in absorbance for 0, 20 and 60 s (Nankano and Asada, 1981). Ascorbate enzymatic activity was further calculated by the given formula:

Ascorbate peroxidase enzymatic activity (Units mL^{-1}) = Δ 290 * 3 * dilution factor / EnC* volume of extract

Where, EnC= 2.8 $mM^{-1}Cm^{-1}$. APX

Guaiacol Peroxidase Enzyme Assay

Guaiacol peroxidase was estimated by taken 3 mL of the reaction mixture containing potassium phosphate buffer (100 mM, pH 7.0) having 20 mM guaiacol, 10 mM H_2O_2 as blank and for enzymatic activity 50 uL of enzyme

extract was added into this mixture and immediately noted the increase in absorbance at 470 nm for 0 and 60 seconds. The guaiacol peroxidase enzymatic activity was calculated by the formula:
Guaiacol peroxidase enzymatic activity (Units ml^{-1} Enzyme) = Δ 470/min * 3 * dilution factor / EnC * volume of Extract.
Where, EnC= 26.6 $mM^{-1}Cm^{-1}$.

Further all enzymes specific activities were determined by dividing enzyme activity (Units mL^{-1} enzyme) to the protein concentration ($mg.mL^{-1}$) of the respective samples.

Statistical Analysis of Data

The data are presented as mean ± S.E. of three replicates (n = 3). Statistical analysis was carried out using SPSS Ver. 14.0 for Windows (SPSS Inc., Chicago, IL, USA; SPSS 2012). Two-way Analysis of variance (ANOVA) was performed to test for difference ($P < 0.05$) among treatments and the follow up of ANVOA was done by Least Significant Difference (LSD) to compare the significance of priming and salinity treatments.

RESULTS

Germination

Increasing salt treatment reduced seed germination in non-primed and primed seeds. Seed priming with coumarin under salt stress condition slightly increased the germination percentage but the difference was non-signification between different treatments (Fig. 1).

Table 1. Salinity induced percent reduction / promotion over non-saline control of different growth parameters of seedling under the influence of different seed priming treatments.

		Root				Shoot			
		NP	HP	COP 50	COP 100	NP	HP	COP 50	COP 100
Length	100 mM NaCl	-29.2	-17.8	-12.7	-9.4	-29.9	-12.3	-18.1	-11.4
	200 mM NaCl	-41.3	-51.5	-21.8	-16.5	-58.4	-28.0	-40.0	-14.3
Fresh Weight	100 mM NaCl	-47.9	-23.4	-17.2	-33.7	-26.1	-23.6	-21.0	-5.7
	200 mM NaCl	-60.1	-35.3	-31.3	-33.9	-42.5	-24.0	-28.8	-32.3
Dry Weight	100 mM NaCl	-30.9	-23.7	-22.2	-9.6	-50.9	-36.8	-34.1	-18.4
	200 mM NaCl	-43.2	-26.3	-6.3	-22.9	-52.9	-41.7	-35.0	-26.6

NP = Non-priming; HP = Hydropriming; COP 50 = Priming with 50 ppm Coumarin and COP 100 = Priming with 100 ppm Coumarin

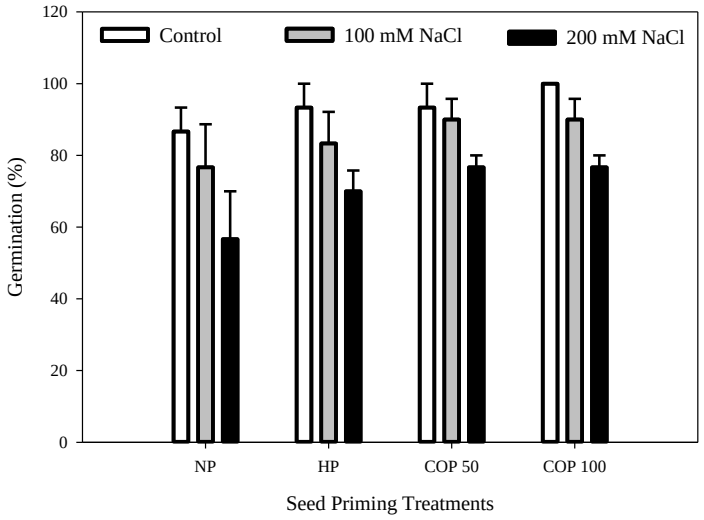

Fig. 1. Effect of seed priming on germination percentage of *Sorghum bicolor* under salt stress. NP = Non-Priming; HP = Hydropriming, COP 50 = Priming with 50 ppm Coumarin; COP 100 = Priming with 100 ppm Coumarin.

Seedling growth

Sorghum seedlings displayed higher vegetative growth when treated with coumarin (50 and 100 ppm) compared to non-prime and hydro-prime controls. Sorghum seedlings showed decrease in vegetative growth at higher salinity treatments. Root length, shoot length, root fresh weight, shoot fresh weight and dry weights of shoot and root were enhanced significantly ($P< 0.01$) in all salinity regimes (0, 100, 200 mM NaCl) with coumarin (50, 100 ppm) treatments compared to non-primed and hydro-primed plants (Fig.2, A-F). Highest root and shoot fresh weights were recorded in plants treated with 100 ppm of coumarin at 100 mM NaCl concentration. Non-primed plants revealed lowest root-fresh weights in all tested salinities. It was observed that dry weights of shoot and root decrease in non-prime seedlings in all salinity regimes as compared to coumarin treated seedlings. Salinity induced percent reduction over non-saline control of different growth parameters of seedlings under the influence of seed priming treatments are presented in Table 1.

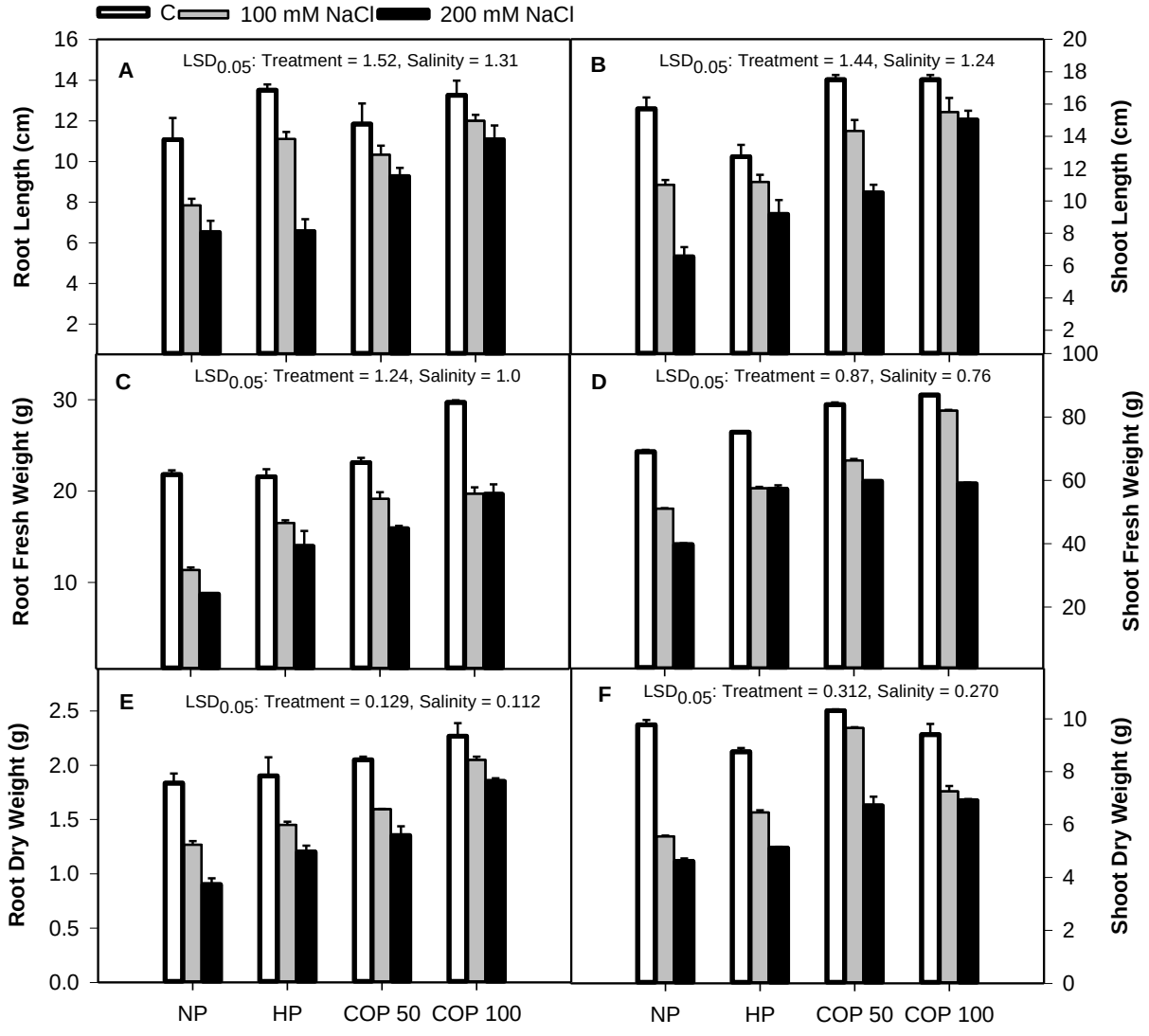

Fig. 2. Effect of seed priming on growth of *Sorghum bicolor* seedlings under salt stress conditions (100 mM and 200 mM NaCl). A) Root length, B) Shoot length, C) Root fresh Weight, D) Shoot Fresh weight, E) Root Dry weight and F) Shoot Dry weight. NP = Non-Priming; HP = Hydropriming, COP 50 = Priming with 50 ppm Coumarin; COP 100 = Priming with 100 ppm Coumarin

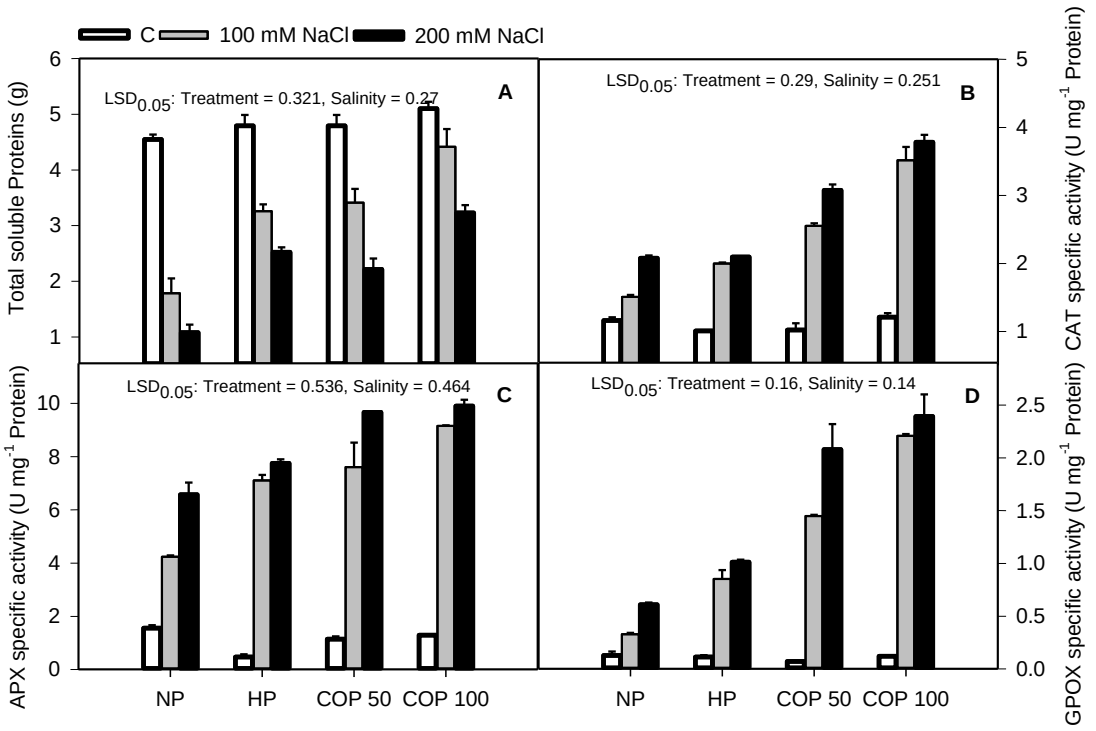

Fig. 3. Effect of seed priming on Protein and Antioxidant enzymes of *Sorghum bicolor* seedlings under salt stress conditions (100 mM and 200 mM NaCl). A) Total soluble Proteins, B) Catalase activity (CAT), C) Ascorbate peroxidase activity (APX) D) Guaiacol peroxidase activity (GPOX). NP = Non-Priming; HP = Hydropriming, COP 50 = Priming with 50 ppm Coumarin; COP 100 = Priming with 100 ppm Coumarin

Protein and Antioxidant enzymes

It was evaluated that antioxidant enzymatic activities (Catalase, Ascorbate and Guaiacol peroxidase) were decreased in non-prime plants at all salinity treatments (0, 100, 200 mM NaCl) as compared to coumarin (50 and 100 ppm) treated seedlings (Fig. 3). Highest antioxidant enzymatic activities (Catalase, Ascorbate and Guaiacol-peroxidase) were observed in coumarin (100 ppm) treated seedlings at highest salinity regimes. Results of protein content indicated that amount of protein gradually decreased with the increase in salinity regime in non-prime, coumarin treated and hydro-primed seedlings at different salinity regimes. At 200 mM salinity regime, greater decrease in protein content was observed in non-prime seedlings compared to both coumarin prime and hydropriming treatments.

DISCUSSION

A two-way ANOVA interaction between salt and coumarin implied significant effect of coumarin in ameliorating vegetative growth (root length, shoot length, fresh and dry weights of root and shoot) of sorghum under different salinity regimes. Salt hazardous effects reduced seedling growth in non-prime seedlings and this effect was drastically occurred at 200 mM salinity. Ali *et al*. (2013a, b) have investigated salt tolerance of three Australian sorghum cultivars at germination and early seedling growth and found that it is more tolerant at germination phase, whereas in growth phase it is more susceptible to salinity. They found that salinity reduced germination velocity relatively in higher magnitude in Cv. Honey Graze. Fifty percent reduction corresponded to 421.57, 335.57 and 327.46 mM of NaCl in Cv. Mr. Buster, Cv. Extra Sweet and Cv. Honey Graze, respectively. The parameters like seedling phytomass, number of leaves per seedling and total leaf area per seedling declined with salinity. Fifty percent reduction in dry phytomass corresponded with 82.9, 82.1 and 72.7 mM of NaCl in Cv. Extra Sweet, Cv. Honey Graze and Cv. Mr. Buster, respectively.

An increase in root length of seedlings in all salinities (0, 100 and 200 mM NaCl) was recorded when primed with 100 ppm coumarin, compared to non-treated plants. This increase in root length of coumarin treated plants grown under salt stress was occurred may be due to similar action of coumarin like growth promoter (e.g. auxin)

which has also previously been reported in a study on root growth of *Petunia hybrida* (Abenavoli *et al.,* 2001). However, reduced root length of non-prime in current investigation is due to salt stress which alters phytohormones level in plants which has been reported (Davies, 1995). Furthermore, at higher salinity (200 mM NaCl) increase in root length at coumarin (100 ppm) treatment may have involved coumarin promoting activity of essential phytohormones (auxin, gibberellin and ABA) which has already been reported in some studies (Gurmani *et al.,* 2011). However, differential growth responses in root and shoot lengths in coumarin treated seedlings as compared to non-prime are may be due to differential accumulation of coumarin. Likewise, increase in root growth of plant by coumarin application under saline condition has also reported in a study on wheat (Saleh and Madany, 2015). In view of improved shoot growth, foliar application of coumarin will be suggested along with seed priming which could adequately improve shoot growth in *Sorghum* under saline condition. The reduced biomass (fresh and dry weights) of non-prime in treatments of salinity may be due to salt stress as recorded in various studies (Meloni *et al.,* 2001). Many studies utilized coumarin in ameliorating stress and increasing growth of plants including, in root tips of onion (Podbielkaowska *et al.,* 1996), sunflower hypocotyl and wheat coleoptile segments (Alexieva *et al.,* 1995). Similar findings regarding growth enhancing function of coumarin under saline stress have reported in studies on *Arabidopsis*, pea, wheat, cucumber, soybean and faba bean (Lupini *et al.,* 2014; Stanchey *et al.,* 2010). Coumarin 100 ppm dosage found more productive in enhancing seed germination and seedling growth. The coumarin application may enhance synthesis of plant essential growth regulators including auxin and gibberellin and inhibit abscisic acid in plant tissues of *Sorghum* plants by pre-soaking seeds in coumarin solutions. Another study recommended that coumarin perform function similar to gibberellin due to stimulatory effect observed by the elongation of pea stem and second leaf sheath of wheat seedlings (Saleh and El-Soud., 2015).

Protein has a pivotal role in increasing salt tolerance and maintaining cellular activities of plant under salt stress. Increase or decrease in protein contents due to salt stress has been studied in various proteomic studies (Beltagi *et al.,* 2006*;* Kapoor and Shrivastava, 2010). In present investigation decrease in protein content with the increasing salinity regime might be due to salt harmful effects caused by increase in osmotic stress due to which protease activity may have increase resulted in reduced protein content of seedling (Parida *et al.,* 2002). Similar findings like present results of decreased in protein content in non-prime with the increased in salt concentration was investigated in a study on sorghum (Ali *et al.,* 2013b) and Kodo millet (*Paspalum scrobiculatum*) germplasm under saline condition (Kumari and Vishnuvardhan, 2013). Likewise, present investigation reduction in protein content of plant with the increasing salt concentration has studied in study on *Catharanthus roseus* (L.) and barley (Jaleel *et al.,* 2008*;* Khosravinejad *et al.,* 2009). Reduction in protein content was observed to be lesser in coumarin treated seedlings at 100 mM salinity. It may be probably due to coumarin-priming might have inhibited protease activity.

Oxidative stress triggers cellular and metabolic damages in plant oxidizing protein, lipid and nucleic acids and inhibiting enzymatic activity (Laluk *et al.,* 2011). Plants activate antioxidant enzymes system for protection against the destruction of ROS (Malik *et al.,* 2010). The activities of antioxidant enzymes are reported to increase under stressed environment (Apel and Hurt, 2004*;* Abbasi *et al.,* 2014). However, plants acquire other metabolic mechanisms against oxidative stress like accumulation of osmo-protectants and increase production of phenolic compounds (Gupta and Huang, 2014). In present investigation coumarin priming effectively enhanced antioxidant enzymatic activities of catalase, ascorbate and peroxidase enzymes in order to reduce oxidative stress generated by salt stress in seedlings under saline conditions. Likewise, present investigation seed priming with coumarin in improvement of antioxidant enzyme activities has previously been studied in a study on wheat (Saleh and Madany, 2015). Activities of catalase and peroxidases were increase in plants treated with phenolic compounds under normal and stressful environment as also reported in various studies (Li *et al.,* 2013, Singh *et al.,* 2013). The present results indicated that coumarin priming alleviates growth of sorghum seedlings under saline condition.

Conclusion: It is concluded that coumarin-priming was beneficial in alleviating salt stress in *Sorghum bicolor* seedlings under different saline conditions. It improved vegetative growth of the seedling through modulating antioxidant enzymatic activities and scavenging ROS.

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