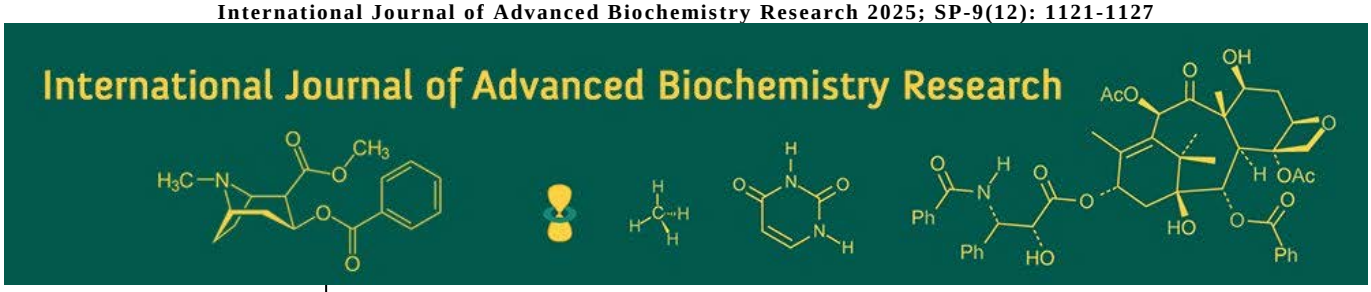

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Amelioriative effect of salicylic acid on rabi sorghum under drought stress

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Abstract
A field experiment was conducted during the Rabi season of 2024-25 at Mahatma Phule Krishi Vidyapeeth, Rahuri, to standardize the optimum concentration of salicylic acid under drought stress in sorghum (*Sorghum bicolor* (L.) Moench). Two contrasting rabi sorghum varieties, Phule Yashoda (drought tolerant) and Phule Revati (drought susceptible), were grown under normal and drought conditions imposed using a rainout shelter. Foliar application of SA at 0, 0.25, 0.50, 0.75, and 1.0 mM was carried out at 20, 40, and 60 days after sowing. Drought stress significantly reduced relative leaf water content (RLWC) and chlorophyll content while increasing lipid peroxidation, with greater damage observed in Phule Revati. Application of SA, particularly at 0.50-0.75 mM, effectively mitigated drought effects by improving RLWC, maintaining chlorophyll content, enhancing proline accumulation, and stimulating antioxidant enzymes (SOD and CAT), thereby reducing oxidative damage. Phule Yashoda exhibited higher inherent tolerance, whereas Phule Revati showed greater relative improvement in response to SA treatment. Overall, foliar application of salicylic acid at 0.50-0.75 mM proved effective in enhancing physiological and biochemical tolerance of rabi sorghum under drought stress.

Keywords: Salicylic acid, drought, sorghum

1. Introduction
Sorghum (*Sorghum bicolor* (L.) Moench) is a C_4 cereal crop belonging to the family Poaceae and ranks as the fifth most important cereal globally after wheat, maize, rice, and barley. It is widely cultivated in regions characterized by high temperatures, low rainfall, and marginal soils, including saline and drought-prone areas. Sorghum is a multipurpose crop used for food, feed, fodder, fuel, fiber, fermentation, and organic fertilizer production. Its grains are nutritionally rich, containing carbohydrates, proteins, vitamins, and minerals, making it a vital food and feed resource, particularly in water-limited environments (Carvalho *et al*., 2002) [9]. In India, sorghum is grown during both kharif and rabi seasons, with rabi sorghum primarily consumed as food and kharif sorghum used for fodder and industrial purposes. Nearly 95% of sorghum cultivation in India is rainfed, with major production concentrated in Maharashtra and Karnataka. Rabi sorghum plays a crucial role in the semi-arid Deccan Plateau; however, its productivity is declining due to erratic rainfall and terminal drought stress during critical growth stages (Kumar *et al*., 2022) [20]. Sorghum exhibits high tolerance to drought and moderate tolerance to salinity, making it a model crop for studying stress resilience in cereals. Abiotic stresses such as drought and salinity disrupt cellular homeostasis, induce osmotic stress, and enhance the generation of reactive oxygen species (ROS), leading to oxidative damage. Physiological traits such as relative water content, membrane stability, lipid peroxidation, and antioxidant enzyme activities are widely used indicators of stress tolerance and yield performance under drought conditions (Shahbazi *et al*., 2012) [25]. Salicylic acid (SA) is an endogenous phenolic plant growth regulator that plays a pivotal role in regulating plant responses to abiotic and biotic stresses. SA influences physiological and biochemical processes including photosynthesis, transpiration, ion uptake, ethylene inhibition, osmolyte accumulation, and antioxidant defense, thereby enhancing stress tolerance (Arfan *et al*., 2007; Hashempour *et al*., 2014) [3, 15]. Exogenous application of SA has been shown to increase abscisic acid levels and proline accumulation,

contributing to improved drought adaptation (Shakirova *et al*., 2003) [26]. Additionally, SA acts as a key signaling molecule in systemic acquired resistance and modulates interactions with other phytohormones, thereby coordinating plant growth and defense mechanisms under stress conditions (Bosch *et al*., 2007; Ghosh and Roychoudhury, 2024) [7, 12].

2. Materials and Methods

The seeds of Phule Yashoda (drought tolerant) and Phule Revati (drought susceptible) were collected from All India Co-ordinated Research Project on Sorghum, MPKV, Rahuri and used for experiment. Seeds of these varieties were grown in *Rabi* season of the year 2024-25 in the field and rainout shelter with 30. 10. 2024 sowing date. Salicylic acid was sprayed @ 0, 0.25, 0.50, 0.75, 1mM at 20, 40, 60 DAS. The leaf samples were collected and evaluated for RLWC, proline, chlorophyll, membrane stability index, antioxidative enzymes (SOD, CAT etc.) at 30, 50 and 70 DAS. The data was analysed by statistically for ANOVA using Split split plot design by using OPSTAT programme. Treatments were compared with CD values at 5% level of significance.

2.1 Proline

Proline content in leaf tissue of both stressed and control leaf sample of sorghum was determined based on the method described by Bates *et al*. (1973) [6] using nihydrine reagent. The proline content in the leaves was expressed in micromoles per gram of fresh weight (µmoles g^{-1} F. W.). Leaf sample (250 mg) was homogenized with 2 ml of sulphosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. The filtrate was reacted with 2 ml of acid ninhydrin and 2 ml glacial acetic acid in a test tube then boiled in hot water bath for 1 hr at 100ºC and the reaction was terminated by placing the test tube in ice bath. After the reaction terminated the reaction mixture was extracted by adding 4ml toluene and mixed vigorously for 15 to 20 sec. The toluene containing chromophore was separated from the aqueous phase and the absorbance was measured at 520nm using toluene as blank. The concentration of proline was determined from the standard curve and calculated on a fresh weight basis (µmoles g^{-1} F. W.).

2.2 Lipid peroxidation

Lipid peroxidation is the oxidative degradation of lipid / fatty acid by reactive oxygen species (ROS). The level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS content) described by Heath and packer (1968) [16]. Leaf sample (0.25 g) was homogenized in 5 ml of 0.1% TCA, then homogenate was centrifuge at 15,000 x g for 15 min and the supernatant was used to estimate the thiobarbituric acid reactive substance (TARS) content. TBA 0.5% in 20% TCA reagent (4 ml) was added in l ml aliquot of the supernatant. The reaction mixture was heated to 95ºC for 30 min in the hot water bath and then cooled down in an ice bath to stop reaction. The aliquot was centrifuge at 10,000xg for 10 min. The absorbance of supernatant was recorded at 532 nm. The value of nonspecific absorption was recorded at 600nm and was subtracted by value recorded at 532 nm. The TBARS content was calculated by using existing coefficient E=115 $mM^{-1}cm^{-1}$. Lipid peroxidation was expressed in µmoles MDA g^{-1} F.W.

2.3 Total Chlorophyll content

The total leaf chlorophyll content of sorghum was determined by the method described by Arnon (1949) [4] using 80% acetone solution. The sorghum leaf samples were weighed (0.2 g) and cut into small pieces. The fresh leaf sample was macerated with 20 ml of 80% acetone in the motor and pestle. The homogenate was centrifuge at 5000xg for 10 min then, the supernatant was collected. The pellet was obtained. The absorbance of supernatant was recorded at 645 nm and 663 nm with 80% acetone as blank.

The amount of total chlorophyll was calculated by using the following formula and expressed in mg g^{-1} F.W.

$$\text{Total chlorophyll} = \frac{20.2\,(A_{645}) + 8.02\,(A_{663}) \times V}{1000 \times W}$$

Where, A= Absorbance at specific wave length V= Final volume of chlorophyll extract in 80% acetone (ml), W= Fresh weight of leaf sample in grams (g).

2.4 Relative Leaf Water Content

Relative leaf water content was estimated by the procedure described by Molaei *et al*. (2012). For the determination of relative leaf water content (RLWC), five leaf samples from each treatment were taken, then weighed for fresh weight (FW), then placed in distilled water for 4 hr at 25 ºC and their turgid weights (TW) were measured. The leaf samples were then oven dried for 24 hr at 75 ºC and weighed fir determining the dry weight (DW) of the leaf samples.

$$\text{RLWC}\,(\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Where, RLWC= Relative leaf water content, DW = Dry weight (g), FW = Fresh weight (g), and

2.5 Supeoxide dismutase

Superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method described by Dhindsa *et al*. (1981) [10]. The reaction mixture contained: 0.2 ml methionine, 0.1 ml NBT, 0.1 ml EDTA, 1.5 ml phosphate buffer, o.1 ml sodium carbonate, o.1 ml enzyme extract, 0.9 ml distilled water and o.1 ml riboflavin. The reaction was started by adding 0.1 ml riboflavin and placing the test tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gives the maximal colour, served as control. Switching off the light and putting the tubes into dark stopped the reaction. The non-irradiated complete reaction mixture served as blank. The absorbance was measured at 560 nm against blank reaction mixture without enzyme extract. Calculation one unit of SOD activity was defined by the amount of SOD enzyme required to inhibit photo reduction of 50 % nitroblue tetrazolium dye.

2.6 Catalase

Catalase activity was measured immediately in fresh extract as described by Aebi (1984) [1]. The hydrogen peroxide dependent oxidation was estimated by the decrease in absorbance at 240 nm. Three ml enzyme reaction mixture contained: 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 200 µl enzyme extract, 800 µl of distilled water and 12.5 mM hydrogen peroxide (0.5 ml of 75 mM).

The reaction was initiated with addition of 0.5 ml of 75 mM H_2O_2. For measurement of catalase enzyme activity, the

decline in absorbance was recorded at 240 nm for three min at an interval of 30 sec. The amount of hydrogen peroxide decomposed was determined from molar extinction co-efficient (ε 36 M^{-1} cm^{-1}). The enzyme activity was expressed as µmoles of H_2O_2 decomposed mg^{-1} protein min^{-1}.

3. Results and Discussion

The present investigation was undertaken to evaluate the ameliorative effect of salicylic acid on biochemical responses in *Rabi* sorghum variety subjected to drought stress. Two variety, Phule Revati (susceptible) and Phule Yashoda (tolerant), were grown under rainout shelter (stress) and open field (control) conditions. Salicylic acid was applied the foliar spray at different concentrations (0, 0.25, 0.5, 0.75, and 1 mM) at 20, 40, and 60 days after sowing (DAS). Biochemical analysis was recorded at 30, 50, and 70 DAS. The data collected were subjected to appropriate statistical analysis.

3.1 Relative Leaf Water Content

Foliar application of salicylic acid (SA) and drought stresssignificantly influenced relative leaf water content (RLWC) in sorghum (Fig. 1). Drought stress caused a marked reduction in RLWC in both varieties, with a greater decline observed in the drought-susceptible variety Phule Revati compared to the tolerant Phule Yashoda. Under stress conditions, Phule Revati showed reductions in RLWC of 24%, 18%, and 14% at 30, 50, and 70 DAS, respectively, while corresponding reductions in Phule Yashoda were 15%, 18%, and 13% over untreated control. Foliar application of SA, particularly at 0.75 mM, effectively mitigated drought-induced reductions in RLWC under both control and stress conditions. Under drought stress, 0.75 mM SA increased RLWC by 27%, 20%, and 21% in Phule Yashoda and by 26%, 37%, and 32% in Phule Revati at 30, 50, and 70 DAS, respectively, showing the highest recovery among all treatments. Similar improvements were also observed under control conditions. RLWC is a reliable indicator of plant water status and drought tolerance (Kadioglu *et al*., 2011) [17]. The improvement in RLWC following SA application suggests enhanced cellular water retention under moisture stress. These findings are consistent with earlier reports in wheat, where SA application improved RLWC under drought conditions.

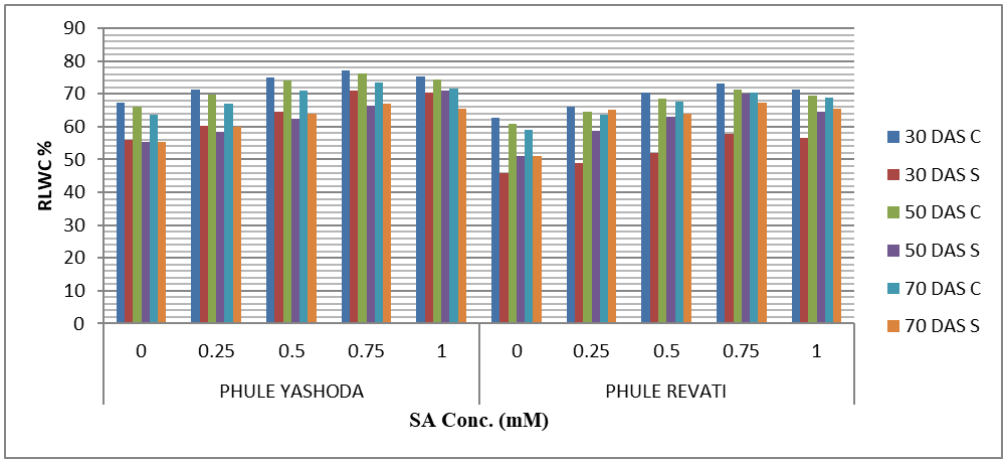

Fig 1: Effect of salicylic acid on relative leaf water content of sorghum at different growth stages under drought stress.

3.2 Lipid peroxidation

Lipid peroxidation, measured as malondialdehyde (MDA) content, increased significantly under drought stress in both sorghum varieties (Fig. 2), with higher accumulation observed in the drought-susceptible variety Phule Revati compared to the tolerant Phule Yashoda. In untreated stressed plants, MDA levels in Phule Yashoda increased by 6%, 6%, and 6% at 30, 50, and 70 DAS, respectively, indicating enhanced oxidative damage under moisture deficit. Foliar application of salicylic acid (SA) significantly reduced lipid peroxidation under stress conditions, with 0.50 mM SA being the most effective concentration. Under drought stress, Phule Yashoda recorded the lowest MDA values (3.62, 4.00, and 4.12 µmol g^{-1} FW at 30, 50, and 70 DAS), corresponding to reductions of 39%, 37%, and 40% over untreated stressed plants. Similarly, in Phule Revati, 0.50 mM SA reduced MDA content by 39%, 37%, and 28% at 30, 50, and 70 DAS, respectively, reflecting the greatest mitigation of oxidative damage among all treatments. Under control conditions, SA application also significantly lowered MDA content in both varieties. At 0.50 mM SA, reductions of 36-39% were observed in Phule Yashoda, while Phule Revati showed 34-36% lower MDA levels compared to untreated control plants. The reduction in lipid peroxidation following SA application indicates improved membrane stability through effective scavenging of reactive oxygen species (ROS).

Similar protective effects of SA against oxidative damage have been reported in wheat under salinity stress (Mahatma *et al*.), where SA reduced lipid peroxidation by 22%. Increased ROS accumulation under abiotic stress is known to disrupt membrane integrity and cellular metabolism, leading to oxidative stress (Gill, 2010) [13].

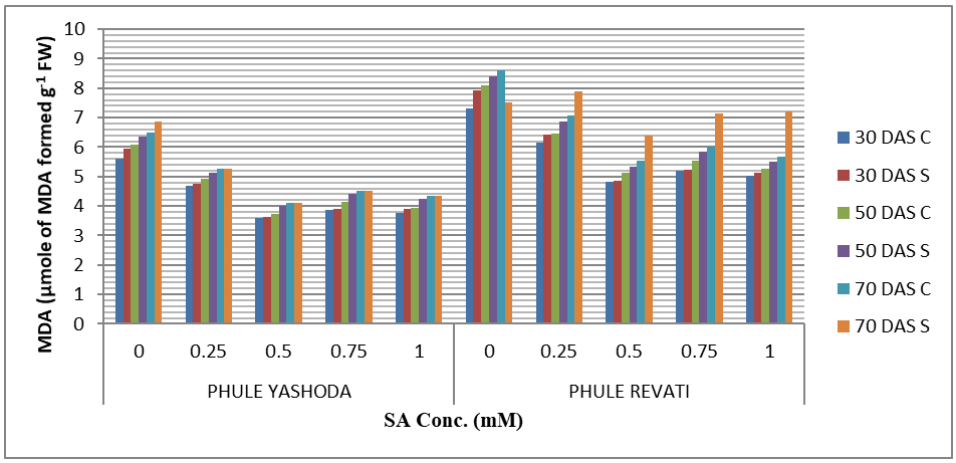

Fig 2: Effect of salicylic acid on Lipid peroxidation rate of sorghum at different growth stages under drought stress.

3.3 Total Chlorophyll content

Drought stress significantly reduced total chlorophyll content in both rabi sorghum varieties (Fig. 3), with a greater decline in the susceptible Phule Revati than in the tolerant Phule Yashoda. In untreated stressed plants, chlorophyll content decreased by 25-27% in Phule Yashoda and 48-52% in Phule Revati across growth stages. Foliar application of salicylic acid (SA) significantly mitigated this reduction, with 0.50 mM SA being most effective under control conditions and 0.50-0.75 mM under drought stress. Under stress, SA increased chlorophyll content by 31-36% in Phule Yashoda and by 68-91% in Phule Revati compared to untreated stressed plants, indicating substantial improvement in chlorophyll retention, particularly in the susceptible variety. Under extreme drought, photosynthetic efficiency is often impaired due to reduced Rubisco enzyme activity and limitations in gas exchange (Bota *et al*., 2004) [8]. In addition, (Sinha *et al*., 1993) [27] reported that maize plants exposed to lead stress exhibited increased chlorophyll and carotenoid content following SA treatment.

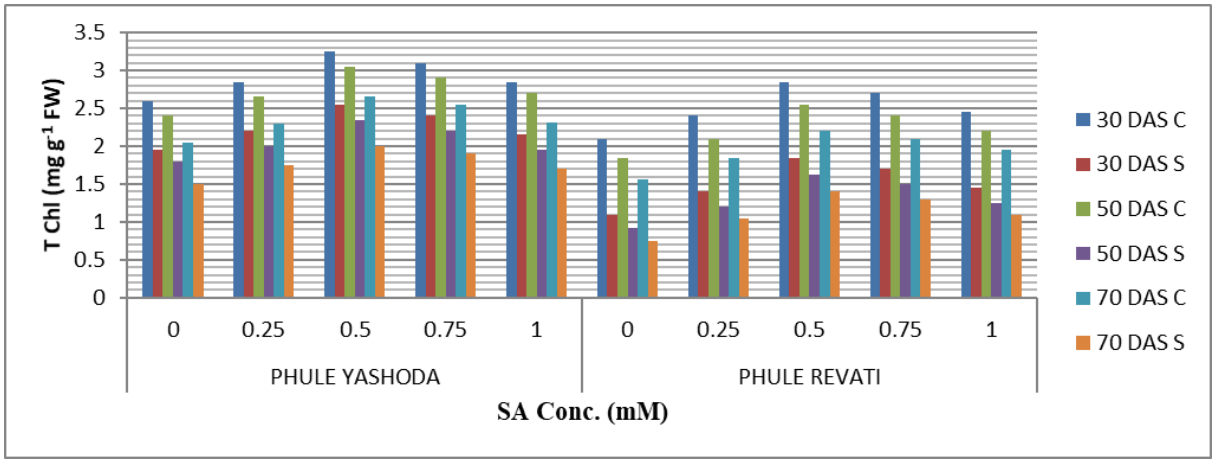

Fig 3: Effect of salicylic acid on Total chlorophyll content of sorghum at different growth stages under drought stress.

3.4 Proline

Proline content increased significantly in both sorghum varieties under drought stress (Fig. 4), with higher accumulation observed in the tolerant variety Phule Yashoda. In untreated stressed plants, proline levels increased by 86-113% in Phule Yashoda across growth stages. Foliar application of salicylic acid (SA) further enhanced proline accumulation under both control and stress conditions. Under drought stress, 0.50-0.75 mM SA resulted in maximum proline accumulation, increasing levels by 59-124% in Phule Yashoda and by 100-108% in Phule Revati over untreated plants. Under control conditions, SA also significantly increased proline content, with 0.50 mM SA producing the highest increases in both varieties. These results indicate that SA enhances osmotic adjustment through increased proline accumulation, particularly under drought stress. The application of salicylic acid (SA) externally has been shown to enhance proline accumulation in several plant species subjected to various biotic stresses (Yusuf *et al*., 2008) [29]. The proline content increased with the increased salt stress in all the sorghum vareity. Similar observations were reported in maize (Turan *et al*., 2009), sugarcane (Gomathi *et al*., 2010) and thus, the results obtained in the present investigation are in conformity with the earlier reports.

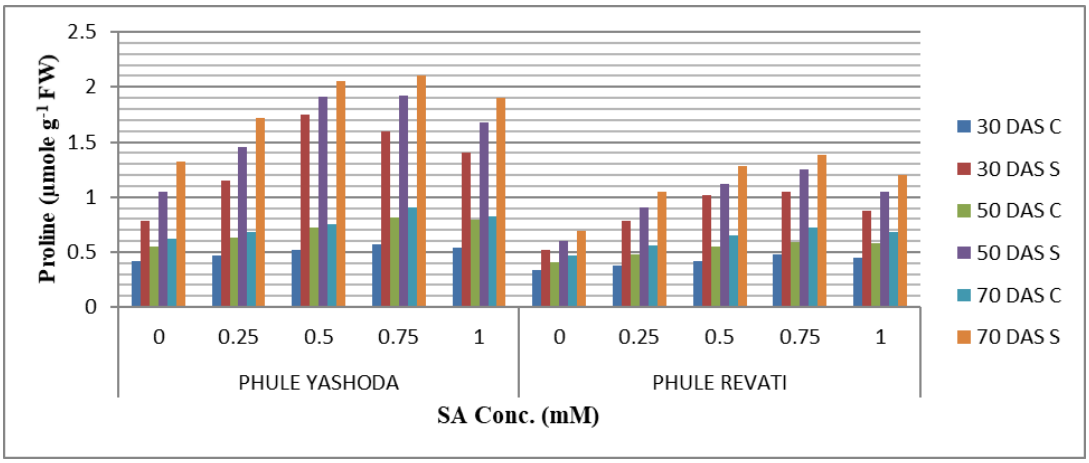

Fig 4: Effect of salicylic acid on proline of sorghum at different growth stages under drought stress.

3.5 Superoxide dismutase

Superoxide dismutase (SOD) activity increased significantly in both sorghum varieties under drought stress (Fig. 5), with higher activity observed in the tolerant variety Phule Yashoda than in the susceptible Phule Revati. In untreated stressed plants, SOD activity increased by 32-36% in Phule Yashoda and by 12-31% in Phule Revati across growth stages. Foliar application of salicylic acid (SA) further enhanced SOD activity, with 0.50 mM SA showing the maximum effect under both control and stress conditions. Under drought stress, 0.50 mM SA increased SOD activity by 75-84% in Phule Yashoda and by 88-94% in Phule Revati over untreated stressed plants, indicating a strong activation of antioxidant defense mechanisms.

These findings are in agreement with earlier research, which demonstrated that exogenous salicylic acid enhances the activity of antioxidative enzymes, thereby protecting cellular membranes from ROS-induced damage during drought stress (Saruhan *et al*., 2012; Shakirova *et al*., 2003) [23, 26]. (Tanou *et al.* 2009) [28] observed that antioxidant activities resulted in elimination of reactive oxygen species and change it to low-risk material, that this the process is a kind of mechanism of stress tolerance in plants. Antioxidant activity includes enzyme activity such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) and other enzymes, including glutathione cycle and ascorbate.

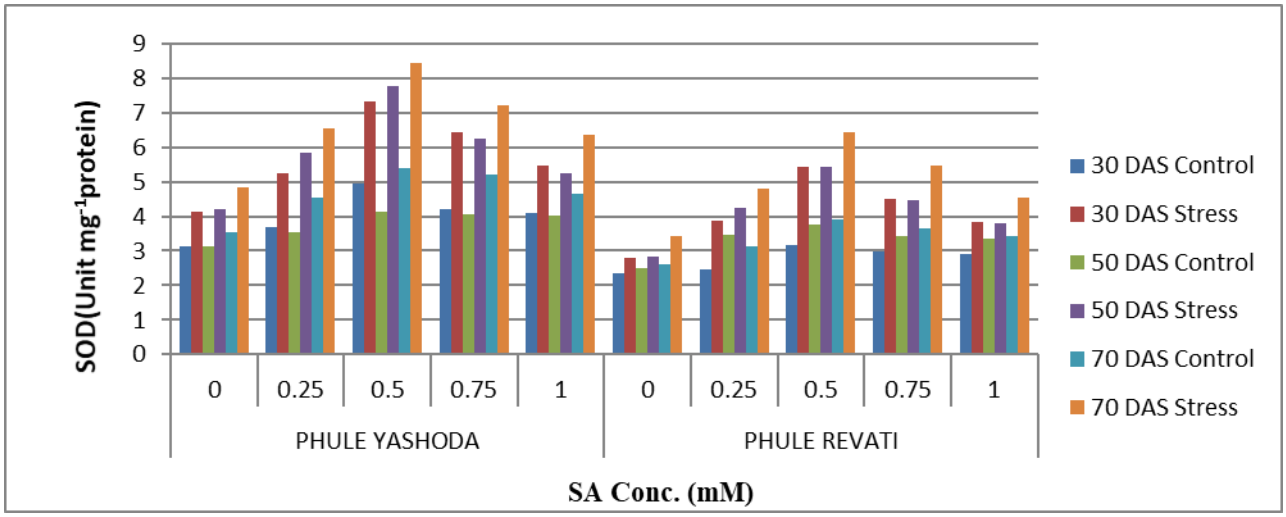

Fig 5: Effect of salicylic acid on Superoxide dismutase of sorghum at different growth stages under drought stress.

3.6 Catalase

Catalase (CAT) activity increased significantly in both sorghum varieties under drought stress (Fig. 6), with higher activity observed in the tolerant variety Phule Yashoda compared to the susceptible Phule Revati. In untreated stressed plants, CAT activity increased by 6-7% in Phule Yashoda and by 8-10% in Phule Revati across growth stages. Foliar application of salicylic acid (SA) further enhanced CAT activity, with 0.50 mM SA showing the maximum effect under both stress and control conditions. Under drought stress, 0.50 mM SA increased CAT activity by 19-23% in Phule Yashoda and by 21-30% in Phule Revati over untreated stressed plants, indicating enhanced antioxidant capacity. Khalvandi *et al*., (2021) [18] reported that, SA treatment under drought conditions enhanced CAT activity, leading to reduced membrane permeability and improved photosynthetic performance. Positive responses in maize (Khodary, 2004) [19] and barley (El-Tayeb, 2005) treated with salicylic acid under salt stress have been attributed to elevated levels of antioxidant enzymes such as CAT, SOD, and APX.

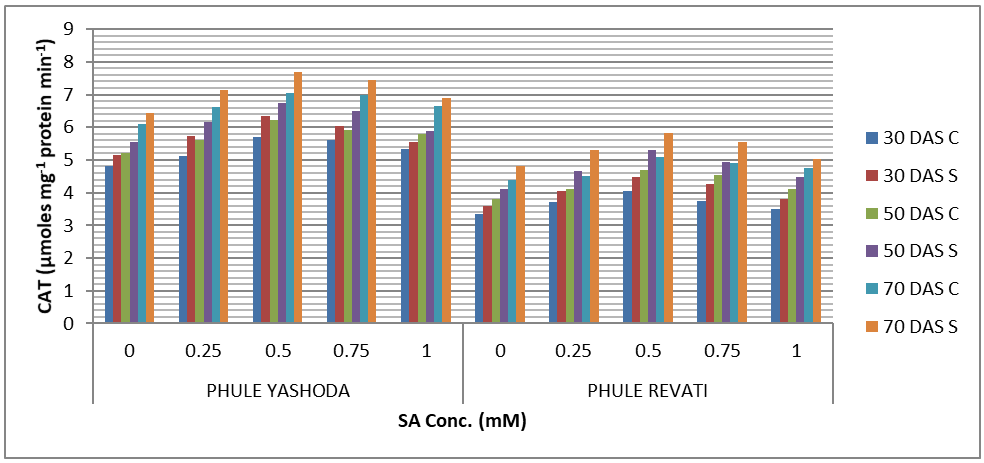

Fig 6: Effect of salicylic acid on Catalase of sorghum at different growth stages under drought stress.

4. Conclusion

Drought stress significantly reduced RLWC and chlorophyll content, especially in the susceptible variety Phule Revati, indicating severe impairment of water retention and photosynthetic capacity. Foliar application of salicylic acid (0.50-0.75 mM) effectively mitigated drought effects by improving RLWC, stabilizing total chlorophyll content, and enhancing membrane stability across all growth stages. SA treatment 0.50 mM enhanced proline accumulation and P_5CS activity, contributing to improved osmotic adjustment under drought, particularly in the tolerant variety Phule Yashoda across all stages but in 70 DAS more effective.

Foliar application of salicylic acid (0.50mM) effectively enhanced antioxidant enzyme activities (SOD, CAT) were significantly upregulated under drought and further amplified by SA, reducing oxidative damage and lipid peroxidation (MDA). Overall, salicylic acid improved biochemical resilience of sorghum under drought stress and control, with more pronounced benefits in the drought-sensitive variety, suggesting its potential as a drought-mitigation strategy.

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