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Low-temperature Stress Influences Organic Osmolytes, Antioxidants and Physiological Traits at Reproductive Stage of Rice (*Oryza sativa* L.) Varieties in Late-*Kharif* Planting

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Rice (*Oryza sativa* L.) is a staple food for most countries, originated from tropical areas and sensitive to low-temperature or temperate regions. A field experiment was conducted in at ARS, Ganagavathi, UAS, Raichur, Karnataka for two consecutive years (2020&2021). The experiment was laid out in two factorial randomized block design (RBD) in two dates of transplanting that is *Kharif* (K-15th September) and late-*Kharif* season (LK-30th September) with four varieties *i.e*., GNV-10-89 and GNV-1108 (short duration) and GNV-1801 and BPT-5204 (long duration) in three replications. The low-temperature at reproductive stage of late-*Kharif* season was 14±1°C., the results obtained at reproductive stage, proline content was higher in LK than K. Long duration varieties showed higher than short duration varieties. The total soluble sugar was higher in K and lower in LK, short duration varieties possessed higher than long duration varieties. The antioxidants activity like catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), ascorbate peroxidase (EC 1.11.1.11) and super oxide dismutase (EC 1.15.1.1) was higher in the low-temperature stress condition that is LK than K and among varieties, long duration varieties showed higher antioxidants activity than short duration varieties. Photosynthetic rate and transpiration rate was higher in K and lower in LK, short duration varieties obtained higher than long duration varieties. Grain yield per hill was higher in K than LK and short duration varieties recorded higher than long duration varieties. This study concludes that the low-temperature encounter the reproductive stage of long duration varieties transplanted in LK.

Keywords: Rice crop; low-temperature stress; late-Kharif transplanting; antioxidant enzyme activity; organic osmolytes accumulation and grain yield.

ABBREVIATIONS

ARS : (Agriculture Research Station);
UAS : (University of Agricultural Sciences);
K : (Kharif season-15th September);
LK : (late-Kharif season-30thSetember);
GNV : (Gangavati);
BPT : (Sambha masuri variety);
D_1 : (date of transplanting-1 (Kharif);
D_2 : (date of transplanting-2 (late-Kharif);
CAT : (catalase);
POD : (peroxidase);
APX : (Ascorbate peroxidase);
SOD : (superoxide dismutase);
F.W. : (fresh weight).

1. INTRODUCTION

"Rice (*Oryza sativa* L.) is one of the three major food crops of the world and forms the staple diet of about half of the world's population. Rice belongs to the genus *Oryza* which contains 25 recognized species, of which 23 are wild species and two are *O. sativa* and *O. glaberrima* are cultivated" [1-3]. "*O. sativa* is the most widely grown of the two cultivated species. Rice is originated from tropical or subtropical zones due to which rice growth responds differently to low-temperature stress during different developmental stages. The optimum temperature required for rice cultivation is about 25°C to 35°C" [4] "moreover in temperate regions rice growth is impressed by limited period that favours growth and development. In general, low-temperature of 0-15 °C can reduce the crop survival rate, inhibit photosynthesis, retard growth and block proteins, lipids and carbohydrates synthesis" [5-9] reported that "rice was more sensitive to low temperature at seedling stage, since it retard seedling growth, resulting in leaf curving, shoot shortening and reduced number of tillers". "Accumulation of proline has been reported during low-temperature stress which serves as osmoprotectants and hence their determination during stress can be used as indicators to evaluate the potential low- temperature stress tolerance at reproductive stage of rice crop. Rice possesses strategies to adapt to cold stress. For example, cold-treated rice plants accumulate proline that stabilizes protein synthesis and thereby maintains the optimal function of rice cells" [10]. "Low-temperature stress damage to rice is an extremely complicated biophysical and biochemical process. When exposed to low-temperature, rice experiences changes in stability and functioning of the cell membrane, the efficiency of photosynthesis, amount of antioxidants and osmoprotectants" [11,12].

It is now well recognized that low-temperature stress at the reproductive stage is serious constraint to rice productivity. The effects of low-temperature on vegetative and reproductive

stages are difficult to evaluate the growing conditions as the plant reaches vegetative growth but sets lesser grain filling because of low-temperature can affect the amount and rate of uptake of water and nutrients under cold conditions the cell liquids can freeze, causing plant death because of desiccation and starvation.

"The major adverse effect of cold stress in plants has been seen in terms of plasma membrane damage which affects all physiological activities of plants. In cereals, the effects of low temperature stress at the reproductive stage delay heading and result in pollen sterility, which is thought to be one of the key factors responsible for the reduction in grain yield" [13]. "Rice is more likely to suffer from low temperature stress especially during floral development" [14,15]. "Mean daily temperature of less than 20 °C can cause anther dehiscence, pollen load on stigma leads to flower abortion thus, low temperature stress, with non-inductive photoperiod, results in subsequent flowering and delay in panicle initiations" [16]. "At booting, low temperature stress inhibits the growth of pollen, which affects spikelet fertility in rice. During the grain development, low temperature stress results in partial and late maturation of grain and grain development is regulated through source-sink relationship, which is adversely affected by temperature. Under low temperature stress, grain filling period and rate are declined, which lead to small grain size" [17,18]. According to [19] "low temperature stress resulted in delayed heading or maturation and yield reduction due to spikelet sterility in rice crop". "Low-temperatures that occur at critical reproductive stages can adversely affect grain quality or cause yield reductions in high-latitude and high-altitude regions of China, Japan, Korea, and other parts of the world" [20]. "The antioxidant enzymes, POD, SOD and CAT, act as a protective enzyme system to limit the levels of free radicals and prevent their damage, maintaining a balance between the antioxidants and free radicals" [21].

This investigation was conducted under Tunga Bhadra Command Area (TBCA) where, the water availability to the farmers of Gangavathi region was reaching late as compared to other regions of TBCA therefore the transplanting of paddy in the main field will also delay for about 15-20 days which is called late-*Kharif*. In this experiment we took two short duration varieties and two long duration varieties to know the effect of low-temperature in late-*Kharif* season. The varieties used were BPT-5204 and GNV-1801 (long duration varieties) and GNV-10-89 and GNV-1108 (short duration varieties).

2. MATERIALS AND METHODS

2.1 Organic Osmolytes Estimation

Organic osmolytes such as proline (L-proline) and total soluble sugars in the leaf tissue was estimated by the method adopted by [22] and [23] respectively, total soluble sugars was estimated by DNS method.

2.1.1 Proline (L-proline) estimation

Fresh leaf tissue was crushed with 10 mL of 3% aqueous sulphosalicylic acid, centrifuged at 10,000 rpm for 20 minutes, and the supernatant was utilized to determine the amount of L-proline. A test tube was filled with 2.0 mL leaf extract, 2.0 mL glacial acetic acid, and 2.0 mL freshly made acid ninhydrin solution. After one hour in a boiling water bath, the mixture was transferred to an ice bath to stop the reaction. Toluene was then added and vigorously mixed for 30 seconds. The toluene layer that had formed was then separated, and the absorbance of the light pink colour intensity was measured at 520 nm against a toluene blank. Equation (A.1) was used to calculate the amount of proline present in the source using a standard curve made from pure L-proline and expressed on a fresh weight basis.

2.1.2 Proline (L-proline) standard curve

From the stock proline solution, different concentrations of proline (0, 20µg, 40µg, 60µg, 80µgand 100 µg) were put into a series of test tubes. The total volume was made up to 2.0 mL with distilled water. A reagent was added, and the process was carried out as it was prepared for the leaf extract. Fig. (1.A) show the standard curve between L-proline (µg) and absorbance at 520 nm.

2.1.3 Total soluble sugar estimation

The sugars were extracted from one gram of fresh leaf material using 80% ethanol three times, each time adding 15 mL by homogenization. The sample extract was then collected and used for the estimation after being filtered through muslin cloth. Because alcohol affects the estimation of sugar, it was eliminated from the extract before the estimation of sugar. A test tube containing 1.0 mL of alcohol extract

was placed in a water bath at a temperature of 50 °C for 20 minutes after being held in a boiling water bath for 10 minutes. The extract was then diluted to a volume of 5 mL with distilled water. After cooling, the phenolphthalein indicator was added, and it was thoroughly mixed. Drops of 1.0 N NaOH are added till the solution turns pink. Added 0.1 N HCl drop by drop until the solution becomes colourless to reneutralise the excess alkali. The volume was made to 10 mL with distilled water. The total soluble sugars were determined using 1.0 mL of alcohol evaporated extract. Where test tubes containing 1.0 mL of the extract and 0.5 mL of the DNS reagent were used, the test tubes were placed in a boiling water bath at 50 °C for 10 minutes. By adding distilled water, a volume of 10 mL was made and the reddish brown or red colour that resulted was measured at 510 nm. The concentration of total soluble sugars was estimated from the glucose (D-glucose) standard curve (Fig. 1.B) and expressed in mg per gram fresh weight.

2.1.4 Total soluble sugars standard curve

The stock solution of glucose (D-glucose) was prepared by mixing 100 mg of glucose with 100 mL of distilled water to get the desired volume. Different concentrations from the stock solution, including 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL, were drawn into a series of test tubes and diluted with distilled water to make the volume 1.0 mL. The addition of 0.5 mL of DNS solution, which was then heated in a water bath for 10 minutes, cooled, and finally diluted to a final volume of 10 mL using distilled water. The colour created, which is reddish brown or red, was measured at a wavelength of 510 nm. A standard curve between 510 nm absorbance and D-glucose concentration was created.

2.2 Antioxidative Enzymes Catalase (CAT), Peroxidase (POD), L-Ascorbate Peroxidase (APX) and Superoxide Dismutase (SOD) Activity of Rice Crop under Low-Temperature Stress

The antioxidant enzymes such as CAT (EC 1.11.1.6), POD (EC 1.11.1.7), APX (EC 1.11.1.11) and SOD (EC 1.15.1.1) play a major role of protecting plants under stress conditions. In this investigation the above mentioned four antioxidative enzymes activity was estimated where in One gram of fresh leaf tissue grounded in pre cooled pestle and mortar with 20 mL sodium phosphate buffer (0.1 M) under ice-cold condition (1- 4 °C) and centrifuged at 10000 rpm for 15 minutes. The supernatant collected for enzyme assay and stored at 1- 4 °C temperature till the assay carried out and used for the estimation of enzymes *viz.,* CAT (EC 1.11.1.6) according to the method described by [24], POD (EC 1.11.1.7) procedure elucidated by [25], APX (EC 1.11.1.11) according to [26] and SOD (EC 1.15.1.1) determined as stated by [27]. The details of calculation mentioned in the Appendix 1.

2.2.1 Catalase (CAT) enzyme activity

In a cuvette 1.5 mL sodium phosphate buffer (0.1 M), 1.2 mL hydrogen peroxide and 0.3 mL enzyme extract was taken, the final volume of the reaction mixture was 3.0 mL and a blank containing enzyme solution but having H_2O_2 free phosphate buffer, the time required for a decrease in absorbance was recorded, if the time taken was greater than 60 seconds then repeat the measurements with a more concentrated enzyme source. The absorbance recorded at 240 nm in ultra violet spectrophotometer against a blank. The catalase (EC 1.11.1.6) enzyme activity calculated by using the equation Eq. (A.2) and expressed in the unit activity in m mole min^{-1} g^{-1} F.W.

2.2.2 Peroxidase (POD) enzyme activity

The total volume of the reaction mixture was 2.0 mL which consists, 1.0 mL sodium phosphate buffer (0.1 M),0.2 mL guaiacol solution (20 Mm), 0.1 mL enzyme extract followed by 0.5 mL distilled water. The reaction was initiated by adding 0.2 mL H_2O_2, the mixture shaken thoroughly then brown colour formed after mixing it well. The change in absorbance from 0 minute to three minutes was recorded at 470 nm. The activity of POD (EC 1.11.1.7) enzyme calculated using the equation Eq. (A.3) and expressed in the unit m mole min^{-1} g^{-1} F.W.

2.2.3 L-Ascorbate peroxidase (APX) enzyme activity

The reaction mixture in the cuvette consists of 1.5 mL of 0.1 M (pH 7) sodium phosphate buffer solution, 0.3 mL ascorbate, 0.6 mL H_2O_2 and 0.6 mL enzyme extract the total volume made up to 3.0 mL, the mixture was shaken thoroughly then the decrease in absorbance recorded at 290 nm in UV-spectrophotometer. One unit of the APX (EC 1.11.1.11) activity was calculated as the amount of enzyme required oxidizing 1.0 µM of ascorbate $min.^{-1}$ g^{-1}F.W. (fresh weight) by the equation Eq. (A.4) and expressed in m mole min^{-1} g^{-1} F.W.

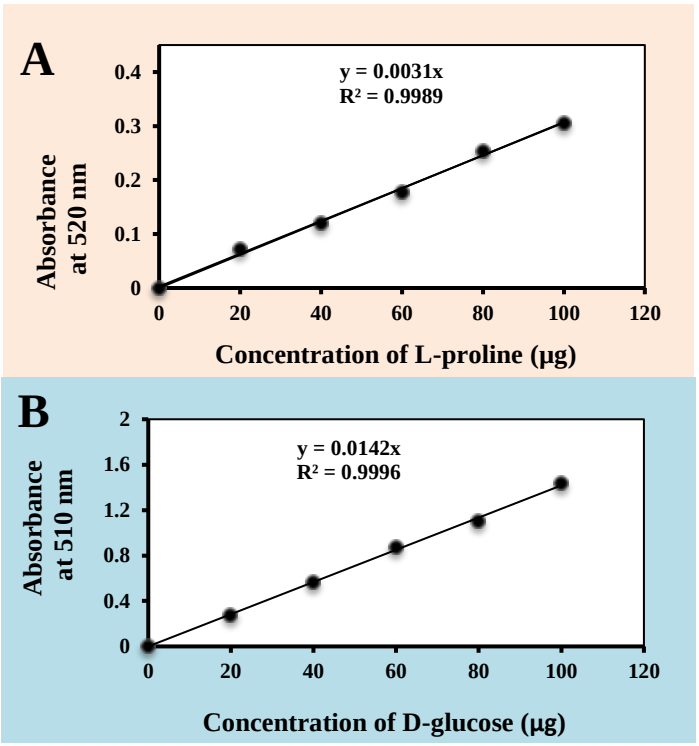


Fig. 1. (A) The standard curve between the L-proline (µg) and absorbent at 520 nm a linear regression was observed between the absorbance values at 520 nm and L-proline contents at 0-120 µg (R^2 = 0.996). (B) The standard curve between the D-glucose (µg) and absorbent at 510 nm a linear regression was observed between the absorbance values at 510 nm and D-glucose contents at 0-120 µg (R^2 = 0.998)

2.2.4 Superoxide dismutase (SOD) enzyme activity

In the test cuvette, the reaction mixture containing 1.3 mL of 50 mM sodium carbonate buffer (pH 10), 0.5 mL of 0.096 mM NBT (Nitroblue Tetrazolium) and 0.1mL Triton X-100 was taken. The reaction was initiated by the addition of0.1 mL hydroxylamine hydrochloride. After 2 minutes, 0.7 mL enzyme extract was added. The percentage inhibition in the rate of NBT reduction was recorded as an increase in the absorbance at 540 nm. The activity of SOD (EC 1.15.1.1) enzyme was calculated using the equation Eq. (A.5). The unit activity of enzyme expressed in unit min^{-1} g^{-1}F.W. or µL mL^{-1} min^{-1} g^{-1} F.W.

2.3 Physiological traits

2.3.1 Photosynthetic rate (µ moles CO_2 m^{-2} s^{-1})

Photosynthetic rate was measured by using Infra Red Gas Analyzer (IRGA) TPS-2 portable photosynthesis system version 2.01. The TPS-2 passes a measured flow of air over a leaf sealed into a chamber called the leaf cuvette using a valve, the TPR-2 first samples the CO_2 and H_2O in the air going to the cuvette and then in the air leaving the flow rate and changing in the CO_2 and H_2O concentrations. The assimilation rate of CO_2 and the transpiration rate of water were determined. This is commonly referred as the "open system method of measurement". Hence the measurements were made on the portion of leaves exposed directly to sunlight on five plants and it expressed in µ mol. CO_2 m^{-2} s^{-1}.

2.3.2 Transpiration rate (m moles H_2O m^{-2} s^{-1})

Transpiration rate was recorded between 10:00 am to 12:00 noon on the abaxial surface of the top fully expanded leaf by using IRGA (TPS-2 portable photosynthesis system version 2.01) and expressed in the unit m mole H_2O m^{-2} s^{-1}.

2.4 Yield Traits

In this investigation the yield traits such as spikelet fertility and grain yield per hill was observed the values and expressed in the particular unit.

2.4.1 Spikelet fertility (%)

The spikelet fertility of four varieties at different date of transplanting was calculated by separating the number of filled spikelet per panicle manually from total number of spikelets of a panicle and calculated by using the below formula and expressed in percentage.

2.4.2 Grain yield (g hill^{-1})

Five hills from each plot were selected randomly all grains were separated from panicle and sun dried. The average of five hills computed and expressed in grams per hill.

2.5 Statistical Analyses

All data collected from the research investigation was subjected to statistical analyses in Web Agri Stat Package 2.0 (WASP) which is developed by Ashok Kumar Jangam and Pranjali Ninad Wadekar (2015). The levels of significance used in 'F' and 't' tests was at the probability level of 0.05 ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 Proline (L-proline) Accumulation under Low-Temperature Stress at Reproductive Stage of Rice Crop

In order to enhance the stress tolerance level of plants, proline act as a mediator of osmotic adjustment, proteins and membrane stabilizer, an osmotic stress-related genes inducer and ROS scavenger, so that plants can perform better under stress [28-30]. In this investigation proline (L-proline) accumulation was determined in both *Kharif* (K) and late-*Kharif* (LK) in four rice varieties at reproductive growth stage the results presented in the Table 1. Higher accumulation of proline was observed in late-*Kharif*. Between four varieties BPT-5204 showed more proline accumulation followed by GNV-1801 and less accumulation was observed in GNV-1108 followed by GNV-10-89.The differences in proline content of leaf were non-significant in the interactions between two seasons and varieties. Under cold stress proline accumulation enhanced and protects cellular enzymes from denaturation [31]. In this investigation proline accumulation increased in late-*Kharif* season it might be due to the influence of low-temperature (13^{o}C - 15 ^{o}C in December month) stress at reproductive stage comparatively in normal *Kharif* season. As stated by [7] who studied that the effect of low temperature stress on oats seedlings under low temperature stress proline content increased as compared to control. Similarly sensitive varieties (BPT-5204 & GNV-1801) possessed higher proline content than tolerant varieties (GNV-10-89 and GNV-1108). Increased proline content has been widely observed in rice varieties under low temperatures. The significant correlations between proline contents and cold tolerance confirmed the function of proline during cold response in rice [32].

3.2 Total Soluble Sugar Content of Rice Crop under Low-Temperature Stress at Reproductive Stage of Rice Crop

The results with respect to total soluble sugars in the leaf tissue showed significant difference at reproductive stage, presented in the Table 1. The higher total soluble sugar content was observed in *Kharif* season as compared to late- *Kharif* season. The low-temperature stress tolerant varieties (GNV-10-89 & GNV-1108) recorded the higher total soluble sugar as compared to the sensitive varieties (BPT-5204 & GNV-1801). It was non-significant among the interactions. Soluble sugars are highly sensitive to environmental stresses, which act on the supply of carbohydrates from source to sink. The total sugar content increased as the plant growth advanced that is, from vegetative to reproductive stage. These results were similar with the investigation of [33] they stated that, the biochemical parameters such as total sugar contents of the leaf tissue were higher at flowering stage compared to those contained at tillering and panicle initiation stages. [34] reported that sugars possess a positive correlation with cold stress tolerance. Sugars under low temperature stress contribute to preventing the water within the plant cells to freeze because of its typical compatible osmolyte property, hence reducing the availability of water for the ice nucleation process in the apoplast. Sugars play a role in scavenging reactive oxygen species and contribute to enhanced stabilization of membranes [35,36].

3.3 Catalase(CAT) Enzyme Activity

In this experiment the CAT activity at reproductive stage was higher in late-*Kharif* season (512.7m mole min^{-1} g^{-1} F.W.) than *Kharif* season (247.2m mole min^{-1} g^{-1} F.W.) among varieties, GNV-10-89 (518.5 m mole min^{-1} g^{-1} F.W.) and GNV-1108 (453.6 m mole min^{-1} g^{-1}

F.W.) showed higher CAT activity compared to BPT-5204 (246.8m mole min^{-1} g^{-1} F.W.) and GNV-1801 (299.3m mole min^{-1} g^{-1} F.W.). Among interactions CAT activity was high in late-*Kharif* and GNV-1089 (719.2m mole min^{-1} g^{-1} F.W.) combination and less CAT activity (179.1m mole min^{-1} g^{-1} F.W.) was found in *Kharif* and BPT-5204 (Fig. 2A).CAT is an important enzyme that acts to dissociate hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water (H_2O). According to [37] growth of rice seedling reduced in cold-stress with different extents among eight rice cultivars. Under cold treatment, the tested cultivar with more growth rate had a higher level of hydrogen peroxide in the shoot but lower level in the root. Similarly, [38] also reported that, the activity of CAT was studied in attached and detached rice leaves where CAT activity in tolerant rice varieties increased. The sensitivity of rice to low temperature stress especially at the reproductive stage which was a primary factor of rice yield fluctuation. According to [39] the activity of CAT was significantly increased in resistant (LJ25) but decreased in susceptible variety (LJ11) under low temperature stress.

3.4 Peroxidase (POD) Enzyme Activity

Low-temperature stress (14±^{o}C) treated plants at reproductive stage showed the significantly higher POD activity (Fig. 2B) in late-*Kharif* season (521.6m mole min^{-1} g^{-1} F.W.) as compared to *Kharif* season (375.6 m mole min^{-1} g^{-1} F.W.). [40] found that alteration of POD activity observed with cold stress. Hence, it induced an increase in the amount of quantitative and qualitative POD at the beginning of stress in both types, tolerant and less tolerant plants in all four species. GNV-10-89 (517.7 m mole min^{-1} g^{-1} F.W.) and GNV-1108 (459.9 m mole min^{-1} g^{-1} F.W.) possessed high POD activity in comparison to BPT-5204 (346.7 m mole min^{-1} g^{-1} F.W.) and GNV-1801 (359.0 m mole min^{-1} g^{-1} F.W.). Among interactions POD activity was high in late-*Kharif* and GNV-10-89 (549.0m mole min^{-1} g^{-1} F.W.) combination and less enzyme activity was found in *Kharif* and BPT-5204 (201.3m mole min^{-1} g^{-1} F.W.). As stated by [41] in two contrasting cold tolerance cultivars of barley, tolerant cultivar (M0103) had significantly higher POD activity than the sensible cultivar (Chumai) after 72 hours recovery in cold treated plants.POD activity in oats was higher under low-temperature stress than normal temperature, slowly increased on the first three days, rapidly increased on the third to fifth day of stress and reached the max on the fifth day more than 4 times of control this increased activity improved cold tolerance [7].

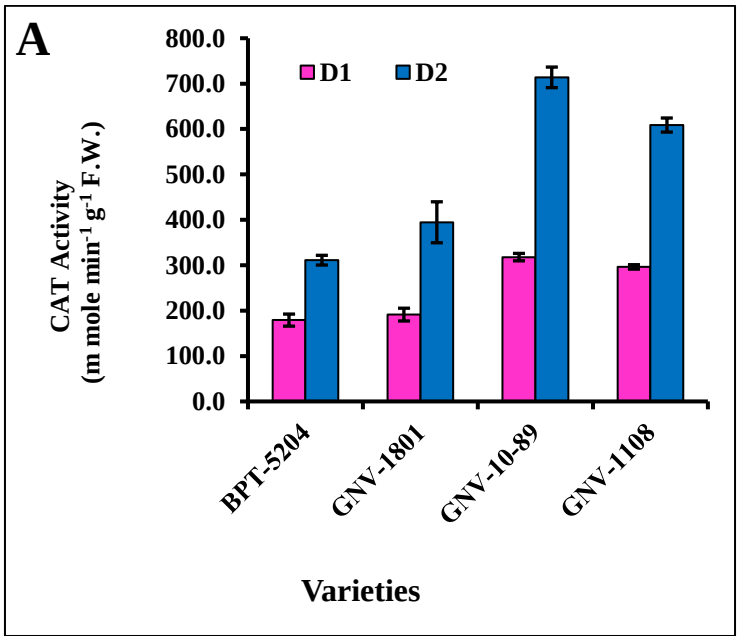
3.5 L-Ascorbate Peroxidase (APX) Enzyme Activity

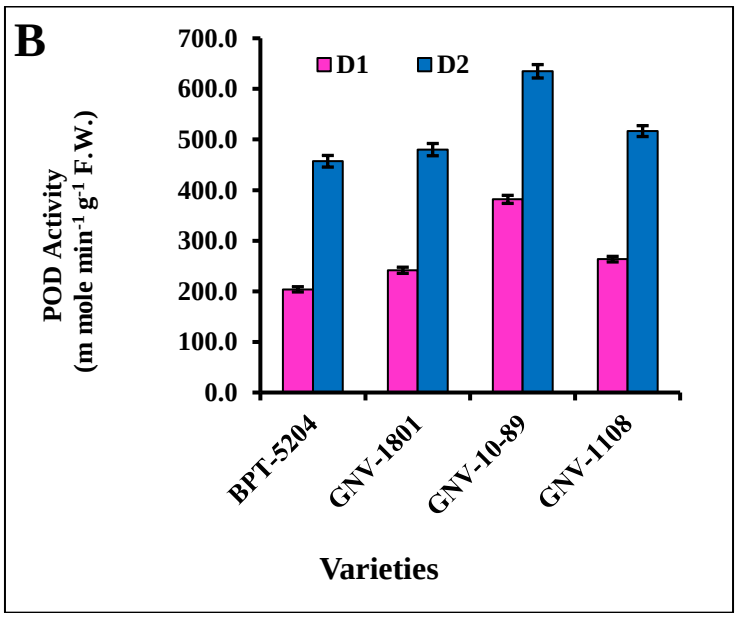
The enzyme APX activity (Fig. 2C) recorded in the late-*Kharif* season (7.28m mole min^{-1} g^{-1} F.W.) was significantly higher than *Kharif* season (4.84m mole min^{-1} g^{-1} F.W.). Among the varieties GNV-10-89 evidenced higher (7.29m mole min^{-1} g^{-1} F.W.) followed by GNV-1108 (6.39 m mole min^{-1} g^{-1} F.W.) and less APX activity was found in BPT-5204 (5.96 m mole min^{-1} g^{-1} F.W.) which was accompanied by GNV-1801 (4.61m mole min^{-1} g^{-1} F.W.). Between interactions the higher APX was reported in late-*Kharif* and GNV-10-89 (9.31 m mole min^{-1} g^{-1} F.W.), significantly less APX activity was noticed in BPT-5204 (4.40 m mole min^{-1} g^{-1} F.W.). Exposure of rice seedlings to low-temperature during germination and vegetative growth was limiting factor to the establishment and development of rice seedlings hence the activity of APX was higher in the tolerant genotypes than in the sensitive genotype [42]. The APX responses are directly involved in the protection of plant cells against adverse environmental conditions [43]. According to [44] reported that in maize seedlings, cold acclimation enhanced the activity of antioxidant enzyme ascorbate peroxidase and concluded that, enzymatic antioxidants accumulate under low-temperature stress and are actively involved in the detoxification of ROS thus enhancing the resistance of the plants. [45] reported an increase in free ascorbate content in 15 days old seedlings of two wheat varieties after cold treatment.

3.6 Superoxide Dismutase (SOD) Enzyme Activity

Among two season of rice transplanting the significantly higher SOD activity (Fig. 2D) was found in the lat-*Kharif* season (166.6 UA min^{-1} g^{-1} F.W.) and less SOD activity was recorded in *Kharif* season (76.6 UA min^{-1} g^{-1} F.W.) Between the four varieties GNV-10-89 (139.2 UA min^{-1} g^{-1} F.W.) showed the more SOD activity which was accompanied by GNV-1108 (99.6 UA min^{-1} g^{-1} F.W.) whereas the low temperature sensitive varieties possessed decreased SOD activity BPT-5204 (70.9 UA min^{-1} g^{-1} F.W.) followed by GNV-1801 (91.2 UA min^{-1} g^{-1} F.W.). The SOD activity among the interactions of transplanting seasons and varieties was higher in the late-*Kharif* and GNV-10-89 (242.5 UA min^{-1} g^{-1} F.W.)

and reduced SOD activity was found in *Kharif* and BPT-5204 (34.4 UA min^{-1} g^{-1} F.W.). [37] reported the among eight rice cultivars of Taiwan growth of rice seedling diminished under cold stress. The tested cultivars with higher growth rate had a higher level of H_2O_2 in the shoots but lower level in the roots. In contrast, the tested cultivates with low growth rate had higher levels of H_2O_2 in the roots but a lower level in the shoots, suggested that cold stress might induce oxidative stress in the roots, not in the shoots therefore cold stress increased SOD activity in the roots. [7] studied the effect of low temperature stress on *Avena nuda* L. Seedlings, which leads to changes in the antioxidant enzymes such as superoxide dismutase (SOD) activity. Maize is another widely studied crop in the area of oxidative stress induced by chilling temperature. The activity of superoxide dismutase was found to be higher in cold tolerant maize genotype than the cold sensitive genotype [46].





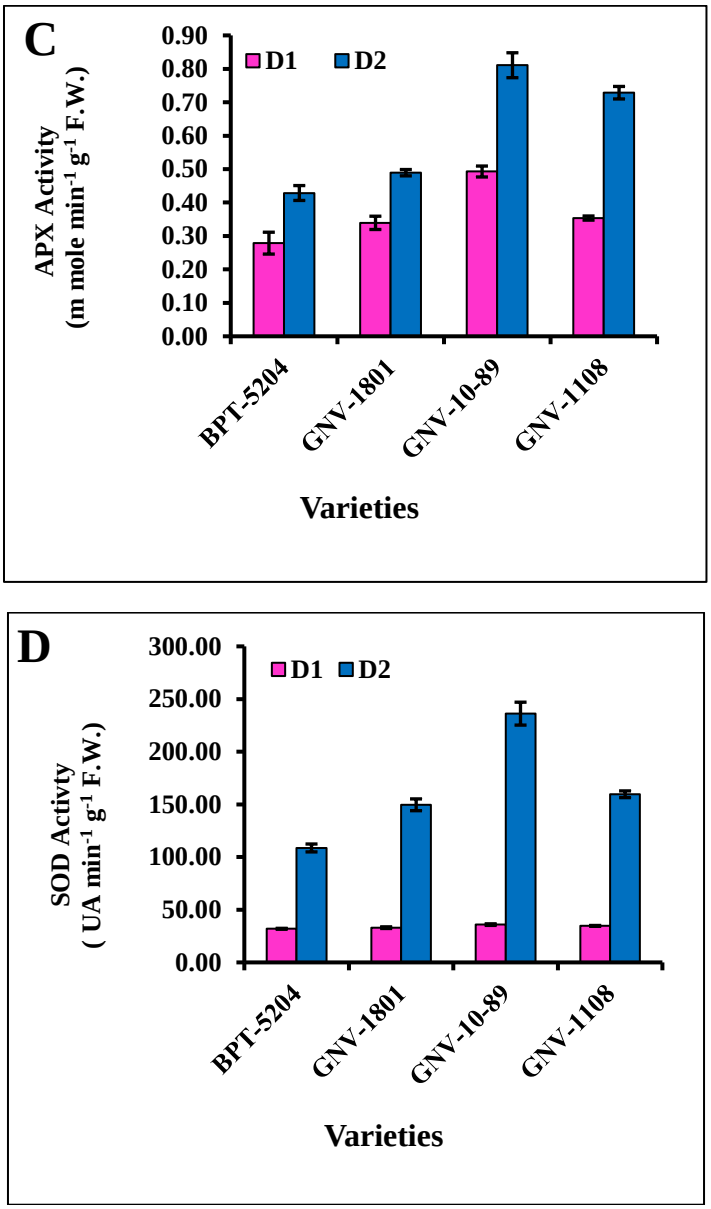


Fig. 2. (A) Catalase(CAT) activity, (B) Peroxidase (POD) activity, (C) L-Ascorbate peroxidase (APX) activity and (D) Superoxide dismutase (SOD) activity in four rice varieties (BPT-5204, GNV-1801, GNV10-89 and GNV-1108) andtwo dates of transplanting that is D_1 (*Kharif*) and D_2 late-*Kharif*). The statistical analyses have been done by ICAR-WASP-2.0 software with the mean values of results obtained. The standard deviation was computed also included the error bars on the graph. The level of significance used in 'F' and 't' tests was at the probability level of 0.05 (P ≤ 0.05)

4. PHYSIOLOGICAL TRAITS

4.1 Photosynthetic Rate (µ moles CO_2 m^{-2} s^{-1})

In this research experiment the results obtained on photosynthetic rate recorded at reproductive stage (Table 1.) The significantly higher photosynthetic rate was noticed in *Kharif* season as compared to late-*Kharif* season. Among the varieties GNV-10-89 recoded more photosynthetic rate followed by GNV-1108 and less photosynthetic rate was observed in BPT-5204 which was accompanied by GNV-1801. The interactions between transplanting seasons and varieties showed non-significant difference. At early growth stages, cold stress reduces the cytochrome path of electron transport and

enhances alternative respiratory pathways [47 and 48]. Cold stress in rice reduces the photosynthesis activity by reducing the chlorophyll content. Cold stress inhibits the chlorophyll synthesis, increases the membrane permeability [49] damages the chloroplast [50] and finally decreases the photosynthetic activity. In chloroplast stroma cold stress lowered the activity of dark reaction [51] reduced the light dependent reaction activity in thylakoid membrane [52] and decreased the carbon dioxide (CO_2) assimilation activity. Cold stress also reduces the Hill reaction activity and inhibited the electron transmission activity [53].

4.2 Transpiration Rate (m moles H_2O m^{-2} s^{-1})

The significantly higher transpiration rate was observed in *Kharif* season recorded at reproductive growth stage (Table 1.) and lower transpiration rate was detected in late-*Kharif* season where the temperature was 14±1 ^oC in the month of December. The present study revealed that higher transpiration rate led to water circulation and optimum photosynthetic efficiency which consequently increased yield in normal *Kharif*. Likewise [54] revealed that, the low temperature deteriorated plant growth, chlorophyll content, net photosynthetic rate stomatal conductance, inter cellular CO_2 and transpiration rate in zoysia grass. The variety GNV-10-89 and GNV-1108 showed adaptability measures to cope up stress and environmental constraints as compared to BPT-5204 and GNV-1801. Higher transpiration rate reflected in high dry matter and grain yield in tolerant varieties (GNV-10-89 and GNV-1108).Temperature greatly influences the magnitude of the driving force for water movement out of a plant rather than having a direct effect on stomata. As temperature increases, the water holding capacity of that air increases sharply. Warmer air can hold more water, its relative humidity is less than the same air sample at a lower temperature, or it is 'drier air'. Because cooler air holds less water, its relative humidity increases or it is 'moister air'. Therefore, warmer air will increase the driving force for transpiration and cooler air will decrease the driving force for transpiration [55].

5. YIELD TRAITS

5.1 Spikelet Fertility (%)

The data on spikelet fertility recorded (Table 1.) was significantly higher in the *Kharif* season than late-*Kharif* season might be due to the effect of low-temperature stress at reproductive stage wherein more sterile spikelets or unfilled spikelets per panicle were found which lead to reduced spikelet fertility in the late-*Kharif* season. Among the varieties GNV-10-89 showed the higher spikelet fertility followed by GNV-1108 and less was observed in BPT-5204 which was pursued by GNV-1801. Between interactions it was found non-significant difference. Rice is more likely to suffer from low-temperature stress especially during floral development [14]. Mean daily temperature of less than 20 °C can cause anther dehiscence, pollen load on stigma leads to flower abortion thus, low temperature stress, with non-inductive photoperiod, results in subsequent flowering and delay in panicle initiations [16]. At booting, low temperature stress inhibits the growth of pollen, which affects spikelet fertility in rice. During the grain development, low-temperature stress resulted in partial and late maturation of grain [17] and grain development regulated through source-sink relationship, which was adversely affected by temperature. Under low-temperature stress, grain filling period and rate are declined, which lead to small grain size [18]. [56] reported that the low-temperature at reproductive stage of *Kharif* sown rice crop recorded reduced spikelet fertility. The negative effects of low temperature during flowering, grain filling percentage and grain yield were mainly attributed to the decreased spikelet fertility, which might be the results of short anther dehiscence, poor pollen grains and low pollen germination on stigma [57 and 58]. In addition the reduced panicle exertion was also reported to be responsible for the decreased spikelet fertility under chilling stress [59].

5.2 Grain Yield (g $hill^{-1}$)

The significantly higher grain yield was obtained in the *Kharif* season as compared to late-*Kharif* season which was exposed to low-temperature stress at reproductive stage (Table 1.). Among the varieties the low-temperature stress tolerant varieties that is GNV-10-89 and GNV-1108 showed more grain yield per hill whereas, the sensitive varieties possessed lower grain yield per hill. Likewise between interactions of transplanting seasons and varieties, *Kharif* and GNV-10-89 combination recorded the higher grain yield per hill and lower grain yield per hill was observed in late-*Kharif* and BPT-5204 combination. [60] reported that the poor spikelet fertility under low-temperature (LT) stress limits

Table 1. Influence of low-temperature stress on osmotic adjustment substances (proline and total soluble sugar) at reproductive stages of rice varieties transplanted in *Kharif* and late-*Kharif* seasons

Treatments	Proline content (m moles g^{-1} F.W.)	Total soluble sugar content (mg g^{-1} F.W.)	Photosynthetic rate (µ mole CO_2 m^{-2} s^{-1})	Transpiration rate (m mole H_2O m^{-2} s^{-1})	Spikelet fertility (%)	Grain yield (g $hill^{-1}$)
Transplanting seasons						
K	20.8	24.0	14.5	12.9	84.3	29.3
LK	22.8	21.0	13.4	11.0	75.9	20.9
S.Em.±	**0.40**	**0.28**	**0.13**	**0.06**	**0.61**	**0.59**
C.D. at 5%	**1.24**	**0.88**	**0.39**	**0.19**	**1.86**	**1.83**
Varieties						
BPT-5204	26.9	16.0	13.5	10.0	75.2	16.8
GNV-1801	22.8	19.0	13.2	11.5	76.0	21.3
GNV-10-89	17.5	31.0	15.2	13.6	85.7	34.8
GNV-1108	9.9	24.0	14.1	12.5	83.4	27.3
S.Em.±	**0.57**	**0.41**	**0.18**	**0.06**	**0.86**	**0.84**
C.D. at 5%	**1.75**	**1.24**	**0.55**	**0.20**	**2.63**	**2.58**
Interactions						
K×BPT-5204	25.1	17.0	14.3	10.7	81.2	21.3
K× GNV-1801	21.7	19.0	13.6	12.5	78.7	23.9
K× GNV-10-89	17.0	32.0	15.6	14.6	90.5	42.1
K× GNV-1108	19.2	25.0	14.7	13.6	87.0	29.7
LK×BPT-5204	28.8	15.0	12.7	9.4	69.3	12.3
LK× GNV-1801	23.9	18.0	12.8	10.5	73.4	18.7
LK× GNV-10-89	18.0	30.0	14.8	12.6	81.1	27.6
LK× GNV-1108	20.7	23.0	13.5	11.5	79.8	24.9
S.Em.±	**0.81**	**0.57**	**0.26**	**0.12**	**1.21**	**1.19**
C.D. at 5%	**NS**	**NS**	**NS**	**NS**	**NS**	**3.65**
C.V.	**7.92**	**5.79**	**3.02**	**2.40**	**2.77**	**11.65**

K(Kharif - 15th September), LK(Late-Kharif - 30th September); Varieties: BPT-5204, GNV-1801, GNV-1089 &GNV-1108; NS: Non-significant F.W.: Fresh Weight. The statistical analyses done by using ICAR-WASP-2.0 software with the mean values of results obtained. The level of significance used in 'F' and 't' tests was at the probability level of 0.05 (P ≤ 0.05)

the possibility of high yield potential in indica-japonica hybrid rice, leading to reduced stability of grain yield. Low temperature stress at reproductive stage is the main constraint to temperate Japonica rice production and affects rice cultivars by delaying vegetative growth and heading, reducing spikelet fertility and affecting grain quality, reduced tillering, pollen sterility a key factor responsible for the reduction in grain yield of rice [61]. [62] studied the effect of low temperature stress influenced by date of transplanting on yield attributes and yields of two rice varieties. They reported that there was a significant reduction in yield due to delayed transplanting. Spikelet sterility was increased by late transplanting due to low temperature at panicle emergence stage. It was also reported that cold stress delays phenological development and increases spikelet sterility, resulting in low yield [63, 64 and 65].

6. CONCLUSION

This investigation conclude with the following evidence, under low-temperature (14±1^{o}C) stress that is in late-*Kharif* planting or delayed planting the organic osmolyte *i.e*., proline accumulation and antioxidants activity increased to protect cell damage due to oxidative stress. In this research area quite often the temperature drops below 20^{o}C during the month of December and January when the reproductive stage of late planting starts which resulted in poor growth of rice seedlings. The low-temperature (14±1^{o}C) encounters the reproductive stage especially at the fertilization stage which leads to less spikelet fertility found in late-*Kharif* planting. The crucial factor that is grain yield was reduced in late-*Kharif* planting. Low temperature stress resulted in prolonged vegetative growth stage and more chaffy grains in the BPT-5204. Based on the results obtained and grain yield affected in late-*Kharif* planting the four rice varieties used in this study were categorized into sensitive and tolerant to low-temperature stress such as GNV-10-89 and GNV-1108 were moderately tolerant, GNV-1801 was moderately sensitive and BPT-5204 was found sensitive to low-temperature stress. The reproductive stage of *Kharif* season planting was escaped from low-temperature stress at reproductive stage. Thus this investigation revealed that the late-*Kharif* planting of the above said rice varieties were was not suitable in this region or any temperate regions.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX 1.

$$Prolinecontent\ (mmoles/g/F.W.) = \frac{\text{Proline (µg/mL)}\times \text{Toluene (mL)}\times 5}{115.5 \times \text{Sample (g)}} \quad \text{Eq. (A.1)}$$

Where: F.W. = fresh weight (g); 115.5 is molecular weight of proline (L-proline)

$$Catalase\ activity\ (mmoles/min/g/F.W.) = \frac{\text{Absorbance/min}\times \text{Total vol (mL)}}{\text{Ext.Coefficient}\times\text{Sample vol.(mL)}} \quad \text{Eq. (A.2)}$$

Where: Extinction coefficient = 6.93 $\times 10^{-3}$ mM^{-1} cm^{-1}

The unit activity of enzyme expressed in m mole min^{-1} g^{-1} F.W.

$$Peroxidase\ activity\ (mmoles/min/g/F.W.) = \frac{\Delta OD\times \text{Rm V (mL)}}{\varepsilon 470\times \text{EV (mL)}} \quad \text{Eq. (A.3)}$$

Where: ΔOD = Change in absorbance; RmV = Reaction mixture volume (mL); Ɛ 470 = Molar extinction coefficient of H_2O_2 at 470 nm (6.375 M^{-1} cm^{-1}); EV = Enzyme extract volume (mL); The unit activity of enzyme expressed in m mole min^{-1} g^{-1} F.W. (Fresh Weight).

$$Ascorbate\ peroxidase\ (mmoles/min/g/F.W.) = \frac{\text{Absorbance/min.}\times \text{Total Vol.(mL)}}{\varepsilon 290\times \text{EV (mL)}} \quad \text{Eq. (A.4)}$$

Where: Ɛ 290 = Molar extinction coefficient of substrate at 290 nm (2.8 $mM^{-1}cm^{-1}$); EV = Enzyme extract volume (mL); The unit activity of enzyme expressed in m mole min^{-1} g^{-1} F.W.

Hydroxylamine hydrochloride was auto-oxidized by superoxide radicals to nitrite. The addition of NBT induces an increase in absorbance at 540 nm due to the accumulation of blue formazon. With the addition of superoxide dismutase (EC 1.15.1.1), superoxide radicals get trapped and there was reduction of NBT to blue formazon. The per cent inhibition of NBT reduction was calculated as below,

$$y\ (\%) = \frac{\text{Change in abs./min.(blank) – change in abs./min.(test)}}{\text{change in abs./min.(blank)}} \times 100 \quad \text{Eq. (A.5)}$$

y (%) = inhibition is produced by 70 µl of sample

$$\frac{50 \times 70}{y} = z\ \mu\text{L of sample}$$

Where: Therefore, z µL of enzyme is required to inhibit 50 % NBT; The unit activity of enzyme expressed in unit min^{-1} g^{-1} F.W. or µL mL^{-1} min^{-1} g^{-1} F.W.

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