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Brassinolide and Zinc Effect on Physio-Biochemistry of Garden Pea (*Pisum sativum* L.) under Water Deficit Condition

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ABSTRACT

Background: The experiment was carried out to examine the impact of different concentrations of exogenously applied of epi-brassinolide (e-BL) and zinc (Zn) on the physio-biochemical processes of garden pea (*Pisum sativum* L.) under subjected water-deficit stress environment.

Methods: A polyhouse experiment was performed using completely randomized design with three replications by e-BL pre-treating the seeds of garden pea genotype HUP-2 with concentrations of 0.01 and 0.05 mM and/or Zn before subjecting the plants to an induced water deficit stress at various stages of growth and development.

Results: Garden pea seeds that had been pre-treated with e-BL and Zn micronutrients showed a favorable reaction against a water shortage condition in terms of membrane stability and a variety of enzymatic activity. It is possible for e-BL and Zn to mitigate the impact of post-anthesis water shortage, which is more likely to result in yield reduction. Both seed pre-treatment with e-BL and basal administration of Zn have shown to be effective in protecting the HUP-2 genotype of Garden pea from the negative impact of water deficiency.

Key words: E-BL, Garden pea, Physio-biochemical traits, Water deficit, Zn.

INTRODUCTION

An Indian consume 15.4 kg of pulses annually. Pulses contain protein, fibre, minerals, nutrients and physiologically active compounds (Behera *et al.,* 2020). The UN and FAO named 2016 the “International Year of Pulses”, calling pulse seeds “nutritious seeds for a sustainable future” to boost pulse demand and supply. Biotic and abiotic stresses considerably affect pulse crop yields worldwide (Pavithra *et al.,* 2020). Water shortages affect pulse production most (Toker and Mutlu, 2011). Arhar, chickpea, gram and other pulses consumption are getting increased among the agricultural foods. Garden pea being an important pulse crop gained utmost attention by growers and consumers due to their quick growth habit, requiring low input costs, great yields, nitrogen fixation and versatility in fitting in any cropping system. This makes garden pea a formidable crop for agricultural diversification. Pea seeds are an excellent source to obtain daily intake of protein (23-25%), fibre (5%), soluble carbohydrates (5%), vitamins (B, K and E) and minerals (many of which are not found in other foods) (Cervenski *et al*., 2017; Zilani *et al*., 2017). Peas are laxative, anti-diabetic, antioxidant, antifungal, antibacterial and anticancer in nature (Mejri *et al.,* 2019). Irrigation water is scarce worldwide in agriculture (Cai *et al.,* 2011). Unreliable precipitation has caused drought in India. Closing stomata reduces transpiration, helping plants during drought (Copolovici *et al.,* 2014; Nabi *et al.,* 2019). Insufficient soil moisture, especially during flowering and pod-filling affects pea crop yields and may influence protein-to-starch ratio (Cervenski *et al.,* 2017).

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Brassinosteroids (BRs) regulate male sterility and other physiological processes (Cervenski *et al.,* 2017). Since rape plant pollen first identified BRs, various research has investigated their role in plant physiology *i.e*., flower buds,

pollen, fruit, seeds and leaves had phytohormone (Hussain *et al.,* 2020). These substances can also be free molecules, glucosides, or sulphates which are necessary for plant growth. Phytohormones are crucial in drought settings (Hussain *et al.,* 2020). This bio-regulator also interacts with other phytohormones to activate antioxidant enzymes and produce osmo-protectants (Zou *et al.,* 2018).

There are 28-homobrassinolide and 24-epibrassinolide (e-BL) are commercially available BR isomers with similar actions. Plants need mineral nutrients for metabolism and signal transduction particularly zinc (Zn). It promotes aldolase and carbonic anhydrase. Zn is needed to make lipids, proteins, auxin co-factors and nucleic acids in plants. Zn treatment improves crop yield and quality (Chattha *et al.,* 2017; Hassan *et al.,* 2019), but Zn deficiency lowers yield and quality (Chattha *et al.,* 2017; Hassan *et al.,* 2019). Zinc toxicity prevents cell division and elongation, reducing biomass production. According to research, zinc in small doses may benefit wheat, sunflowers, tomatoes and red cabbage (Sadoogh *et al.* 2014; Eslami *et al.,* 2014). As per studies, e-BL and zinc reduce drought negative effects. However, this raises questions about their modes of action and how they affect plant physiological and biochemical processes to make them drought-resistant. In light of this hypothesis, this article examines the effects of drought stress on plants and draws attention to the knowledge gaps in plant physiology, biochemistry and molecular biology regarding the effects of water deficit stress at different growth stages in garden pea and the effects of e-BL, Zn and their combination.

MATERIALS AND METHODS

The experiment was conducted in the poly house of Department of Horticulture at Institute of Agricultural Sciences, Banaras Hindu University during *rabi* seasons of 2016-17 and 2017-18. The growing season comprised of average temperature of 20°C and 40% relative humidity. The experimental setup under the poly house shielded it from the rain. The Department of Genetics and Plant Breeding supplied the garden pea var. HUP-2 seeds and the Department of Plant Physiology of the institute supplied e-BL and other biochemical analysis chemicals for the experiment. Uniform-sized seeds were treated in three surface-sterilized petri plates. One petri plate contained water, whereas the other two contained e-BL at 0.05 mM and 0.01 mM (Pradhan *et al.,* 2018). Pre-soaked seeds were planted in pots with 3 kg of soil and fertilizer and zinc. Only three seedlings from each container were transferred.

The seedlings were nurtured at the ideal moisture level. Plants were irrigated every alternate day. Stopping irrigation marked the beginning of water shortage therapy after 40 days assessment beginning with planting seeds till these are fully grown. Irrigation with tap water was performed every alternate day on the plants that were under control. Both water deficit patterns and two dosages of e-BL and/or Zn were employed in the treatment. The treatment comprised of Water deficiency before anthesis (WDBA), water deficiency after anthesis (WDAA), WDBA + 0.01 mM e-BL, WDBA + 0.05 mM e-BL, WDBA + Zn, T_0(control), T_1, T_2, T_3, T_4, T_5, T_6, T_7 and T_8.

Three completely randomized trials made up the experiment. The experiment repeated three times with each treatment and with calculated 5% significance of mean differences were calculated. Sairam (1994) method of membrane stability index (MSI) calculation was adopted. One sample with 0.2 mg of leaf was used in 10 ml of sterile water and tested for electrical conductivity (EC1) after 30 minutes at 40°C and EC2 was measured for other sample after 10 minutes at 100°C. The formula of MSI calculation as followed:

$$MSI\ (\%) = 1 - \frac{EC1}{EC2} \times 100$$

The relative water content (RWC) was estimated by using the protocol of Dionisio-Sese and Tobita (1998).

$$RWC\ (\%) = \frac{FW-DW}{TW-DW} \times 100$$

Where,
FW= Fresh mass.
TW= Turgid weight.
DW= Dry weight.

Proline was estimated by method described by Bates *et al*. (1973). A mortar and pestle homogenized 0.5 g of leaves in 5 ml of 3% sulphosalicylic acid for five minutes. The supernatant was preserved after 6000 g RPM for 10 minutes. 5 ml of a 3% sulphosalicylic acid aqueous solution extracted the residue three times. 15 ml of supernatant portions were mixed in a test tube contained 2 ml of extract, glacial acetic acid and ninhydrin reagent. After 30 minutes in a 100°C water bath, the reaction mixture turned brick red. After cooling, the reaction mixture was added 5 ml of toluene and passed to a separating funnel. The absorbance of mixture and toluene blank were compared by using spectrophotometer at 520 nm. Proline was measured proline in each plant sample using a standard curve.

The level of superoxide dismutase (SOD) activity was determined by following methods described by Dhindsa *et al.* (1982). 3 ml of the reaction mixture containing 0.1 ml of 1.5 M sodium carbonate, 0.2 ml of 200 mM methionine, 0.1 ml of 2.25 mM NBT, 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml of dimethyl sulfoxide and 0.1 ml of enzyme extract were placed in test tubes in duplicate. In order to serve as a control, two tubes were used, that were devoid of enzyme extract. After inserting the tubes for 15 minutes in the presence of two fluorescent lights rated at 15 W, the reaction was kicked off by adding 0.1 ml of riboflavin at a concentration of 60 M to each of the tubes. It was possible to put a stop to the reaction by turning off the light and covering the tubes with a dark cloth. The colour was obtained by the tubes that did not contain any enzyme extract. A non-irradiated, complete combination that

did not create any colour was utilized as a blank for the experiment. The absorbance was measured at 560 nm and one unit of enzyme was utilized as the amount of enzyme. This resulted in lowering of sample absorbance in comparison to the sample that did not contain enzyme. The EU was expressed as per g fresh weight basis, by using the following formula:

Enzyme Unit (EU) =

$$\frac{\text{Enzyme (-) light - (Enzyme (+) light - Enzyme (+) dark)}}{\frac{\text{Enzyme (-) light}}{2}}$$

(+) and (-) indicated with enzyme and without enzyme respectively. H_2O_2 content was measured in the first fully grown leaf of stressed and normal plants by Mukherjee-Choudhary method (1983). 10 mL of cold acetone was mixed with 0.1g of leaf sample. Whatman No. 1 filter paper filtered the homogenate. First 4 ml of titanium reagent and then 5 ml of concentrated ammonium solution were added to the extract to precipitate the peroxide-titanium complex. 10,000 rpm centrifugation lasted 5 minutes by dissolving the precipitate in 10 ml of 2% sulfuric acid after discarding the supernatant. After spinning to remove undissolved particles, absorbance was measured at 400 nm against a blank (2N H_2SO_4).

RESULTS AND DISCUSSION

The common indicator that is often noticed under stressful situations was the leaf proline content presented in Fig 1. During the first year, the treatment T_8 showed a larger proline content when it was seen at 48 days after sowing (DAS). Later, during the second year, the treatment T_{14} showed a larger proline content when it was noticed at 63 DAS. The results of the second year observations at 48 and 63 DAS revealed that the treatment T_5 contained significantly more proline than the other treatments. The lowest amount of proline found in either year samples with the control (T_0). Similar report also showing that the buildup of proline in response to drought stress may be caused by the promotion of its production, the inhibition of its breakdown, or the destruction of protein (Gomes *et al.,* 2010). When compared with the control group, the administration of 4M-EBL resulted in a considerable increase in the proline content (Mohammadi *et al.,* 2020). It has been observed that BRs increase the amount of proline in a substance by influencing the expression of genes involved in the process of proline biosynthesis (Talaat and Shawky, 2013). On the other hand, BRs encourage the production of nucleic acids via the process of biosynthesis in plant cells (Bajguz, 2000). Therefore, the rise in proline content that occurred after the application of EBL is most likely the result of an increase in the synthesis of nucleic acids in plant cells and their conversion into the necessary amino acids, such as proline. After the administration of EBL, it has been observed that the presence of BRs under stressful condition results in an increase in the amount of proline (Agami and Mohamed, 2013; Rady, 2011).

Garden pea was subjected to drought stress and the effect of 24-EBL and zinc on the membrane stability index (MSI) of the leaf as a percentage were recorded (Fig 2). The index of membrane stability in leaf tissue was shown to decrease as the amount of moisture stress increases. In the current experiment, this important sort of results in terms of membrane stability index was observed, which is consistent with the findings some researchers (Merwad *et al.,* 2018). However, brassinosteroid was able to lessen the effect that water stress had on the permeability of the plasma membrane (Siddique and Husen, 2021; Sahoo *et al.,* 2020). When compared with all the other treatments at 48 DAS, the post anthesis drought (T_5) exhibited the greatest impact. This was the case in the first year of the experiment. As the

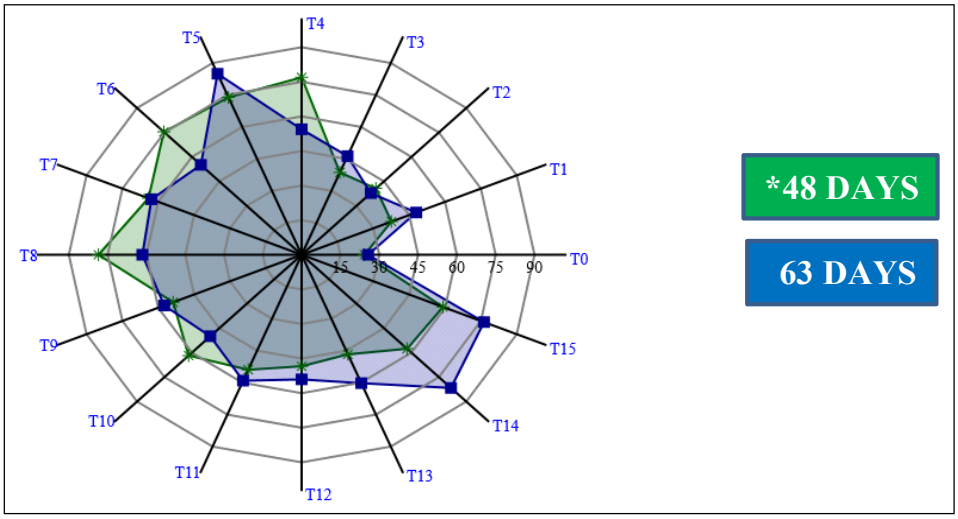

Fig 1: Effect of 24-EBL and zinc on leaf proline content (µg g^{-1} FW) in pea (*Pisum sativum* L.) genotype HUP-2 under induced drought.

days progress, observations were made at 63 DAS and 85 DAS, it was observed that the effects of the treatment were decreasing while the control was practically increasing. In a similar manner, in the second year, LDS + 0.05 mM 24-EBL + Zn (T_{15}) had the optimal values in comparison to all the other treatments at the 48 DAS, but diminished effects were reported at later stage. The control had the greatest recorded relative water content (RWC) of leaf in the first 48 days after emergence (DAS), whereas the treatment T_2 had the highest RWC of leaf during 63 DAS and 85 DAS during the first year presented in Fig 3. Similarly, the RWC % was found to be more in the control plants during the first few days (48 DAS) and it rose at a rate that was comparable across treatments T_2 and T_7. Some study showing under conditions of extreme water stress, the seedlings that were treated with 0.2 mg/L 24-EBL had a leaf water content that was much higher than it was when they were properly watered (Li *et al.,* 2012). The reason for this might be because there is a decrease in the amount of water that is lost from the leaf as a result of transpiration (Yavas and Unay,

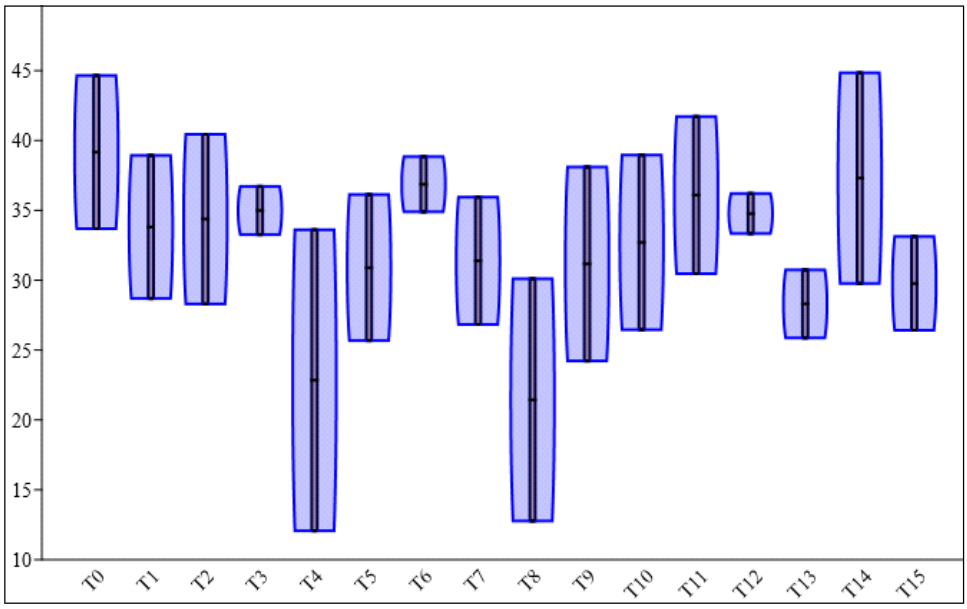

Fig 2: Effect of 24-EBL and zinc on membrane stability index of leaf (%) in *pea (Pisum sativum* L.) genotype HUP-2 under induced drought.

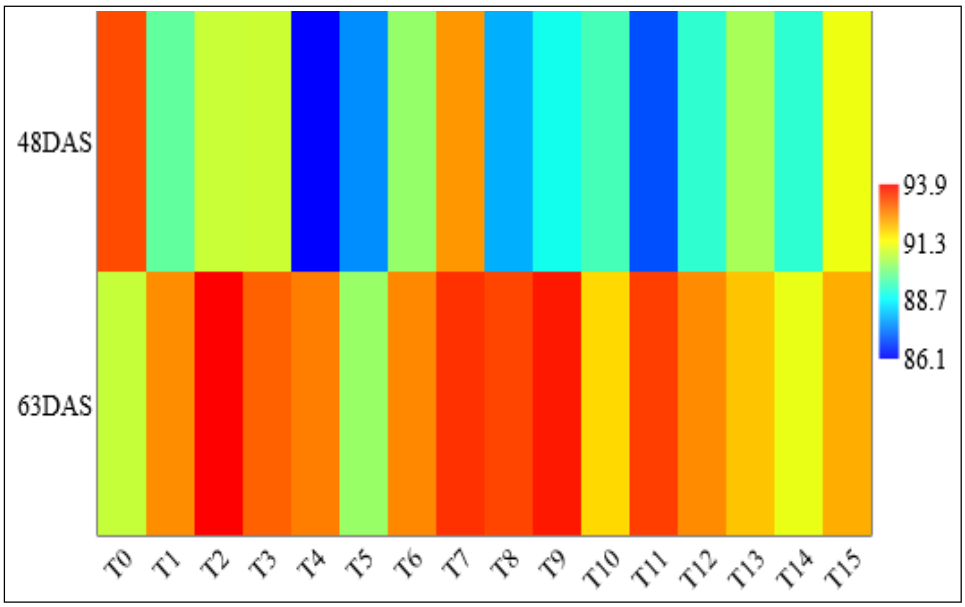

Fig 3: Effect of 24-EBL and zinc on relative water content (RWC) of leaf (%) in pea (*Pisum sativum* L.) genotype HUP-2 under induced drought.

2016). According to El-Khallal (2002), there is a possibility that brassinosteroids will regulate the expression of water stress-inducible proteins as well as induce the de-novo synthesis of specific polypeptides. This will encourage damaged plant cell membranes to recover and maintain homeostasis, which will result in higher RWC and a higher MSI.

Initially, it was observed that the activity of leaf superoxide dismutase (SOD) (Fig 4) was higher in control plants during the first 48 DAS. However, increased activity was found in treatment T_5 during the first 63 DAS and then the activity was once again higher in control during the first 85 DAS for the first year. However, results from the second year revealed that treatments T_5 and T_{12} produced more activity at both 48 and 63 DAS. At 48, 63 and 85 DAS after treatment, various treatments such as T_3, T_8 and T_{15} were shown to produce reduced activity. However, in the second year, treatments T_{13} and T_{10} had lower levels of activity at both 48 and 63 DAS. Reactive oxygen is produced and used by enzyme systems like catalase and SOD. Catalase and superoxide dismutase activities were stifled in pea plants that were put under the stress of drought (Li and

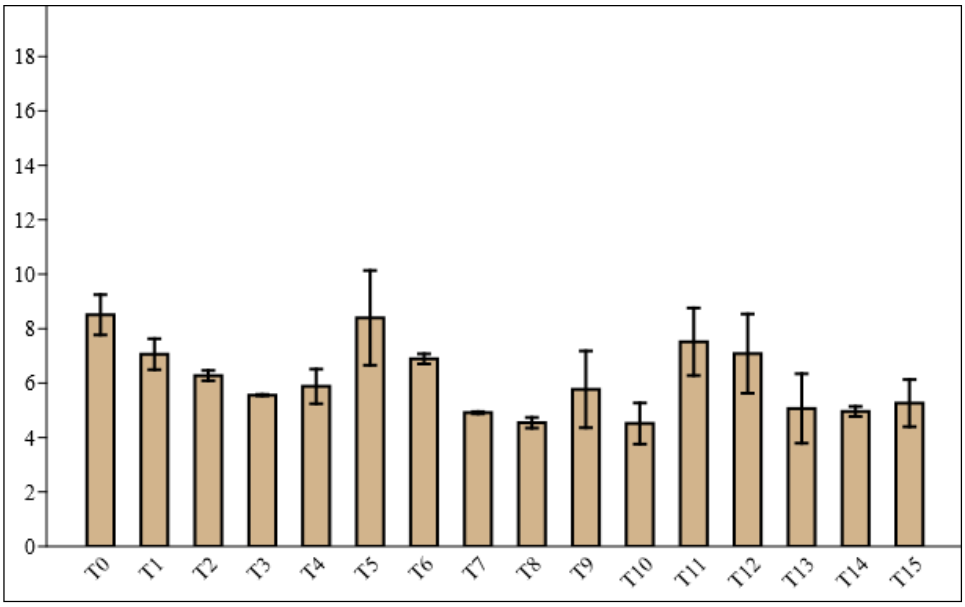

Fig 4: Effect of 24-EBL and zinc on leaf superoxide dismutase (SOD) activity (μM g^{-1} FW) in pea (*Pisum sativum* L.) genotype HUP-2 under induced drought.

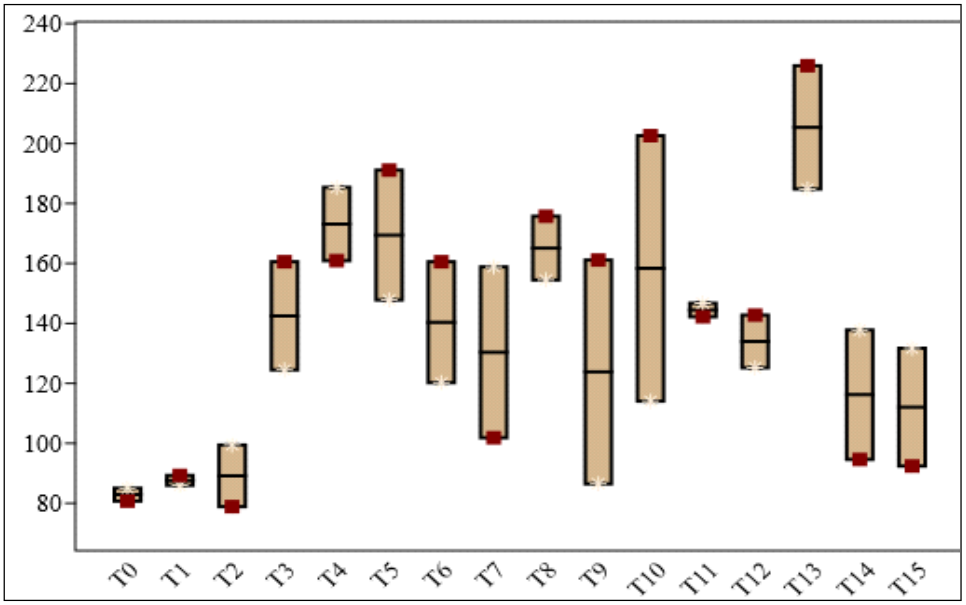

Fig 5: Effect of 24-EBL and zinc on leaf hydrogen peroxide (H_2O_2) content (μM g^{-1} FW) in pea (*Pisum sativum* L.) genotype HUP-2 under induced drought.

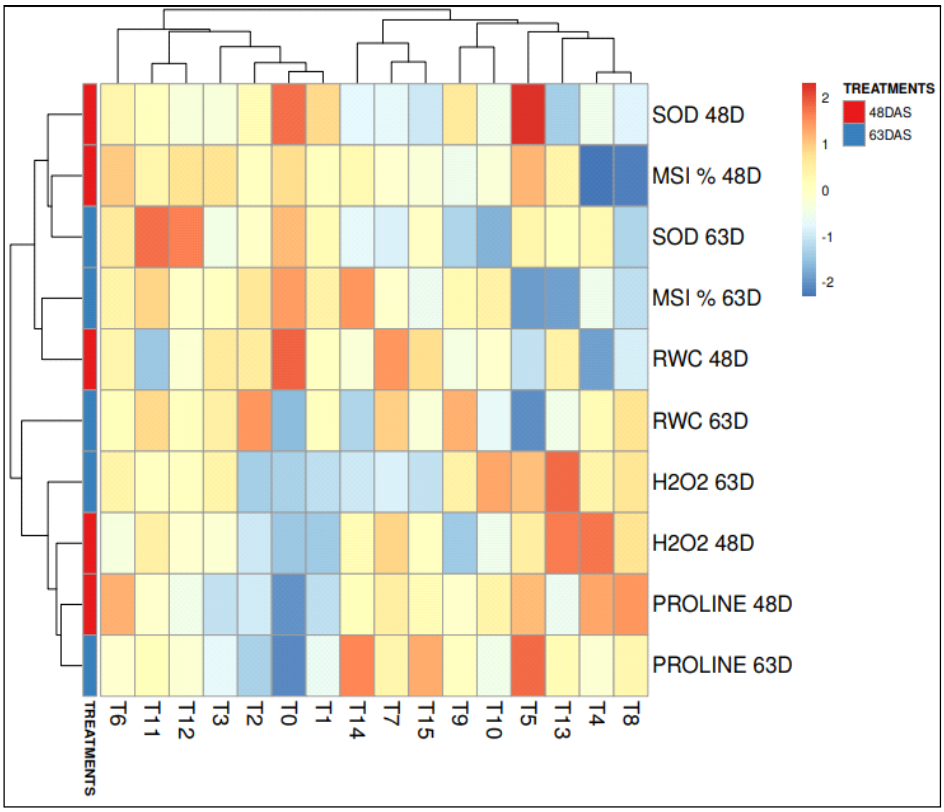

Fig 6: Effect of enzymatic activity.

Van Staden 1988 a, b). The use of BRs was shown in earlier studies to have a considerable impact on the antioxidant enzyme activity (Li and Van Staden, 1988 a, b). SOD activity was shown to be enhanced in rice after treatment with homo-brassinolide (Chen *et al*., 1997). Similar findings were seen in pea research conducted by (Alexieva *et al.,* 2001). The results of this experiment also demonstrated the same kind of repercussions. The activities of CAT, POD, APX and SOD all increased in response to drought stress (Bajguz and Hayat, 2009), which suggests that drought stress circumstances (Sahoo *et al.,* 2019) result in an inhibition of reactive oxygen species (Mohammadi *et al.,* 2020). The lowest level of catalase activity was likewise seen in conditions of water stress (Sairam and Srivastava, 2001; Sahoo *et al.,* 2021).

The amount of H_2O_2 found in leaf tissue was measured and documented in Fig 5 and Fig 6 for a number of different treatments under drought stress circumstances. In the beginning, the observations that were recorded demonstrated that treatment T_4, T_5, as well as T_{13} and T_8, had relatively greater levels of H_2O_2 in the leaf at 48 DAS, followed by T_3, T_5, T_8 and T_{13} on 63 DAS. The pre-anthesis drought treatment had the greatest H_2O_2 content at 48 DAS in the second year, but the greater quantity was found in treatment T_{13} at 63 DAS, which is comparable to the original first observation made in the first year. During the first year, the treatment with 0.05 mM 24-EBL (T_2) produced the results with the lowest content. In addition, the treatment T_9 had the least amount of an impact at 48 DAS and later at 63 DAS, despite the fact that the control plants had the least amount of leaf H_2O_2. Catalase is an enzyme that is present in virtually all living creatures that are exposed to oxygen. It is responsible for catalysing the breakdown of H_2O_2 into water and oxygen. Catalase may be found in bacteria, plants and mammals. BR treatment resulted in a considerable drop in H_2O_2 concentration (Damghan, 2009). It would appear that the application of brassinosteroids during times of stress improved the plant's ability to withstand drought by increasing the concentration of osmolites such as soluble sugars and proline. As a result, this aided in the maintenance of osmotic pressure within cells and led to an increase in the production of H_2O_2 (Zahedi *et al.,* 2019).

CONCLUSION

Anti-oxidant activity in cell increases when it adopt protection mechanism under stress conditions which ultimately influence the yield-related parameters. The combination treatments with 24-EBL and zinc maintained osmolyte buildup and ROS reduction, enhancing garden pea production. This study suggests that 24-EBL may reduce the impact of drought stress in plant. This influences cell signaling, which may regulate several plant traits. Its expansion helps anti-oxidants, compatible solutes and photosynthetic pigments aggregate. 24-EBL alleviated moderately dry conditions in the pea genotype HUP-2. There

is plenty of time to study this novel phytohormone and Its interaction with hormones and stress-related traits may help to generate drought-tolerant pea cultivars. However, Molecular and proteomic studies on mode of action of 24-EBL is crucial, as 24-EBL helps tissue-specific separation and plant component development.

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Author contribution

Jyostnarani Pradhan performed the experiment and wrote the first draft of the manuscript. Jyoti Prakash Sahoo, Laxmipreeya Behera, Kartik Pramanik, Siddhartha Shankar Sharma, and Jannila Praveena provided valuable information for preparation of the manuscript. Kailash Chandra Samal coordinated the process and gave valuable comments. All authors read and approved the final manuscript.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Conflict of interest

The authors declare no conflict of interest.

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