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Scientific Research

Thermo-Protective Role of 28-Homobrassinolide in *Brassica juncea* Plants

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Abstract

The present work was undertaken to study the effects of 28-homobrassinolide on growth, stress markers, antioxidative enzyme [superoxide dismutase (EC 1.15.1.1), guaiacol peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6)] activities and protein content in 10 days old seedlings of *Brassica juncea* L. treated with different degrees (4˚C, 44˚C) of temperature. 28-homobrassinolide at 10^{-9} M concentration lowered temperature stress. Different degrees of temperature treatment alone decreased the enzyme activities and protein concentration of seedlings. However, pre-sowing treatments of 28-homobrassinolide lowered the temperature stress and enhanced the contents of MDA and proline, activities of enzymes and protein concentration of seedlings.

Keywords

Antioxidative Enzymes, 28-Homobrassinolide, Brassinosteroids, Temperature Stress, Lipid Peroxidation, Guaiacol Peroxidase, Catalase

1. Introduction

Temperature is one of the most important environmental stresses that adversely affect plant growth and development thereby limiting plant productivity. It causes heat injury and chilling stress, which result in degradation and collapsing of proteins, lipids and bring changes in metabolism. These changes cause breakdown of cell products and membranes etc. Temperature stress accelerates generation of reactive oxygen species (ROS) that have the capacity to initiate lipid peroxidation and degrade proteins, lipids and nucleic acids [1]. Plants respond to temperature stress by changes in the levels of antioxidants and antioxidative enzymes [2] [3]. Several hor-

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mones are implicated in modulating the plant responses to oxidative stress, including ethylene [4], abscisic acid [5], salicylic acid (SA) [6] and brassinosteroids (BRs) [7].

Brassinosteroids are a class of plant polyhydroxy steroids that are ubiquitously distributed in the plant kingdom. These compounds, when applied to plants, improve their quality and yield. They have been further explored for stress-protective properties in plants against a number of stresses like chilling [8], salt [9], heat [10] and heavy metals [11] [12]. However, it is unclear whether BRs are involved in the modulation of plant responses to oxidative stresses. The influence of BRs on the response of the antioxidative enzymes of plants under stress conditions has been studied recently [7] [13]. The available data show that the changes induced in the activity of antioxidative enzymes by BRs differ with plant species and with stress conditions [9] [14] [15].

Brassica juncea L. is an important oilseed crop known for its oil content, edible and medicinal uses. The seed is a warming stimulant with antibiotic effects. The chemical constituents of *B. juncea* include glucosinolates, ascorbate, foliate, myrosinase, and sterols (brassicasterol, sitosterol and brassinosteroids). The present study was undertaken to observe the growth, contents of stress markers and antioxidants activities under the influence of 28-homobrassinolide (28-homoBR).

2. Materials and Methods

2.1. Chemicals

28-homoBL was obtained from Sigma-Aldrich Chemicals Pvt Ltd., INDIA. Chemicals and reagents used for various biochemical analyses were purchased from Merck. The experiments were performed under controlled conditions in plant growth chamber.

2.2. Collection of Seeds and Experimental Setup

Seeds of *Brassica juncea* CV-201 (certified) were procured from Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. Healthy seeds were manually selected and treated with 5% hypochlorite (v/v) for 5 minutes and then washed for 30 minutes in free flowing tap water followed by 4 - 5 times washing with deionised water. Seed priming was done to surface sterilized seeds with different concentrations of 28-homoBL (1 µM, 1 nM, and 1 pM) and DW as control, for 6 hours. 28-homoBL treated and untreated seeds were sowed in petriplates for seven days and then 7-day-old seedlings were exposed to 44˚C and 4˚C for 5 h daily for three days. After 44˚C and 4˚C temperature shock treatment, seedlings were transferred to normal lab conditions of plant growth chamber, observing 24˚C temperatures, 16/8 hours dark and light period and light intensity fall uniformly on each petriplate at 200 PAR while humidity was set at 80%. Present study was conducted to elucidate the effect of 28-homoBL on antioxidant system to mitigate toxic effect of temperature stress during seedling growth.

2.3. Treatment of 28-HomoBL and Temperature

Our experiment consisted of 12 treatments with 3 replication of each treatment. The treatment included 28-homoBL (1 µM, 1 nM, 1 pM) and temperature (24˚C, 4˚C, 44˚C) and combination of 28-homoBL and Temp (1 µM + 4˚C, 1 nM + 4˚C and 1 pM + 4˚C, 1 µM + 44˚C, 1 nM + 44˚C and 1 pM + 44˚C). The growth parameters in terms of root and shoot length were examined after 10 days after sowing (DAS).

2.4. Estimation of MDA and Proline Content

MDA and proline content was determined using colorimetric method. The level of MDA was measured by Thiobarbituric acid reaction method [15]. Proline estimation was done following method of Bates *et al*. [16]. The plant material was homogenized with 3% sulpho-salicylic acid. The homogenate was filtered and glacial ace-tic acid and acid ninhydrin was added to the supernatant. After shaking for 1 minute, the reaction mixture was incubated at 100˚C for 1 h. Reaction was stopped by adding toluene and absorbance was taken at 520 nm using spectrophotometer.

2.5. Preparation of Enzyme Extract

One gram of shoot tissue of *Brassica juncea* was homogenized in 3 ml of pre-chilled phosphate buffer, (pH 7.2)

in chilled pestle and mortar. The homogenates were centrifuged at 15,000 rpm for 15 minute at 4˚C and supernatant collected and used for enzyme activities of SOD and POD along with total proteins.

2.6. Superoxide Dismutase Activity (EC 1.15.1.1)

The assay of superoxide dismutase was carried out based on the reduction of nitro blue tetrazolium (NBT) [17]. To 0.5 ml of enzyme extract, 1.8 µl of 50 mM of Sodium Carbonate buffer (pH 10), 750 µl of 96 µM NBT and 150 µl Triton X-100 were added. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Absorbance was taken at 540 nm using spectrophotometer mentioned elsewhere, and activity of SOD was taken as an increase in absorbance for 2 min at 25˚C. The control was simultaneously run without enzyme extract. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of Units·mg^{-1} of protein.

2.7. Activity of Guaiacol Peroxidase (EC 1.11.1.7)

Guaiacol peroxidase was assayed by mixing 50 µl of Guaiacol, 30 µl of H_2O_2 and 3 ml of potassium phosphate buffer and enzyme extract. Blank was prepared by adding all the reagents except enzyme extract [18].

2.8. Activity of Catalase (EC 1.11.1.6)

CAT activity was measured according to Aebi [19] by taking 3 ml reaction mixture containing 1.5 ml of 100 mM phosphate buffer (pH 7.0), 0.05 ml of 75 mM H_2O_2 and 0.05 ml enzyme extract. The reaction was started by addition of H_2O_2 and CAT activity was measured as decrease in absorbance at 240 nm for 1 min.

2.9. Determination of Total Proteins

Total proteins were estimated by the method of Lowry *et al.* [20]. One ml of enzyme extract was kept in 1 ml of ice cold 20% TCA for 18 hours. Homogenate was centrifuged and pellet was dissolved in 0.1 N NaOH for protein estimation. The absorbance was measured at 750 nM.

3. Statistical Analysis

All analysis was done on a completely randomized de-sign. All data obtained was subjected to unpaired t-test. Each data was the mean of three replicates (n = 3) except for shoot and root length where n = 5 and comparisons of p-values < 0.05 were considered significant and different from control.

4. Results and Discussions

4.1. Effect of 28-homoBL and Temperature on Growth

28-homoBL treated seeds resulted in increased percent shoot length (**Table 1**) of *Brassica juncea* as compared to control. Maximum (39%) increase in shoot length was observed in seedlings treated with 10^{-9} M 28-homoBL concentrations. Seedlings grown in presence of 4˚C and 44˚C showed (13% and 27%) decrease respectively. Interestingly seeds grown in presence of 4˚C temperature after treatment with 10^{-9} M concentrations of 28-homoBL showed (16%) increase in shoot length rate as compared to control distilled water seeds.

Seedling growth in terms of root length showed synergistic mechanism of negative effect on growth particularly on root length. Root length affected negatively in all concentrations of 28-homoBL except 10^{-9} M where (1%) increase was found. Overall 28-homoBL showed stimulatory effect on shoot length and inhibitory effect on root length in presence or absence of temperature.

In case of fresh and dry weights, maximum decrease was found in seedlings treated with 44˚C temperature (14%) and (18%) respectively but when supplementation of different concentrations of 28-homoBL is given maximum increase (25%) fresh weight and (14%) dry weight was found in seedlings treated with 4˚C temperature stress.

4.2. Estimation of MDA and Proline Content

There are number of factors, plants showed at morphological and biochemical level which can be taken as stress

Table 1. Influence of pretreatment of 28-homoBL on shoot, root length and fresh, dry weights under temperature stress.

Treatment	Shoot Length (cm)	Root Length (cm)	Fresh Weight (mg)	Dry Weight (mg)
Control	3.99 ± 0.02*	4.7 ± 0.04*	1128 ± 0.05*	52 ± 0.04*
10^{-6} M 28-homoBL	4.55 ± 0.01*	4.59 ± 0.09	1439 ± 0.06	61.6 ± 0.01*
10^{-9} M 28-homoBL	5.55 ± 0.04*	4.76 ± 0.02*	1456 ± 0.05*	63.6 ± 0.03*
10^{-12} M 28-homoBL	5.16 ± 0.06	4.41 ± 0.01*	1424 ± 0.02*	55.3 ± 0.06
4˚C	3.49 ± 0.06	4.1 ± 0.07	1062 ± 0.02*	48.6 ± 0.05*
44˚C	2.93 ± 0.03*	2.83 ± 0.05*	971 ± 0.04*	43 ± 0.02*
10^{-6} M + 4˚C	3.82 ± 0.02*	4.33 ± 0.08	1266 ± 0.02*	54.3 ± 0.02*
10^{-6} M + 44˚C	3.6 ± 0.05*	3.22 ± 0.06	1164 ± 0.04*	47.3 ± 0.06
10^{-9} M + 4˚C	4.66 ± 0.07	4.38 ± 0.03*	1414 ± 0.03*	59.3 ± 0.02*
10^{-9} M + 44˚C	3.94 ± 0.04*	4.33 ± 0.02*	1202 ± 0.07	53 ± 0.01*
10^{-12} M + 4˚C	3.6 ± 0.01*	4.38 ± 0.05*	1336 ± 0.09	53.6 ± 0.01*
10^{-12} M + 44˚C	3.18 ± 0.03*	3.05 ± 0.05*	1119 ± 0.02*	46.6 ± 0.04*

Values are the mean of three replicates measurements. *Indicates significant difference compared to the control at $p < 0.05$.

indicators under inadequate environmental conditions. Lipid peroxidation (MDA) increases in presence of temperature (**Figure 1(A)**) stress indicated that extreme temperatures (4˚C, 44˚C) were lethal and responsible for disintegration of plasma membrane and thus caused decrease in growth of cell. In present study 20% and 23% of lipid peroxidation increase was observed in presence of 4˚C and 44˚C temperature. 28-homoBL decreases lipid peroxidation that was harmful to the cells [21]. 1 nM 28-homoBL treatment resulted in about 7% decrease in MDA content but in other 28-homoBL (1 μM and 1 pM) treatments 6% to 5% deccrease in MDA content was noticed. Enhancement of MDA content subjected to 1˚C and −10˚C temperatures was noticed in *Avena nuda*, a cold tolerant plant species [22]. It was noticed that instead of low temperature, high temperature also damages cellular membranes due to lipid peroxidation. The results showed that MDA level was significantly increased with rising temperature. There are reports that stresses disrupts membrane permeability is by peroxidation of the lipid membrane [23]. Membrane injury under High temperature is related to increased production of highly toxic reactive oxygen species [24]. Lipid peroxidation measured as the amount of produced MDA when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals. Lipid peroxidation is ascribed to oxidative damage and is often used as an indicator of increased damage [25]-[27]. 28-homoBL application protects membranes from damage by various stress factors by reducing MDA content [28]. From present study it was observed that this protection of cell membrane by 28-homoBL is very much dose-dependent along with absence or presence of any stress factor such as temperature in present case.

Proline is an amino acid which starts accumulating in higher amount in plants under inadequate environmental conditions which can be taken as stress marker. Presence of extreme temperatures such as 4˚C and 44˚C (**Figure 1(B)**) in seedlings growth environment enhanced proline content up to 34% and 13% as compared to control (untreated distilled water). On the other hand the promoting effect of temperature on proline content stimulation was higher in presence of 28-homoBL which increased with increase in 28-homoBL concentration. These results showed that in contrast to 28-homoBL, the temperature stimulatory effect on proline content was sustainable and that for the neutralization of its effect higher concentration of 28-homoBL was needed. 28-homoBL application can promote the biosynthesis of proline under heat stresses [29]. 1 nM 28-homoBL was most effective in proline accumulation in which about 126% of more proline accumulated as compared to untreated control distilled water seedlings. These results are in accordance with Rizhsky *et al.* [30] and Amirjani [31].

4.3. Superoxide Dismutase (SOD) Peroxidase (POD) and Catalase Activities

The presence of stress in the cell leads to the formation of ROS, which causes further severe oxidative damage

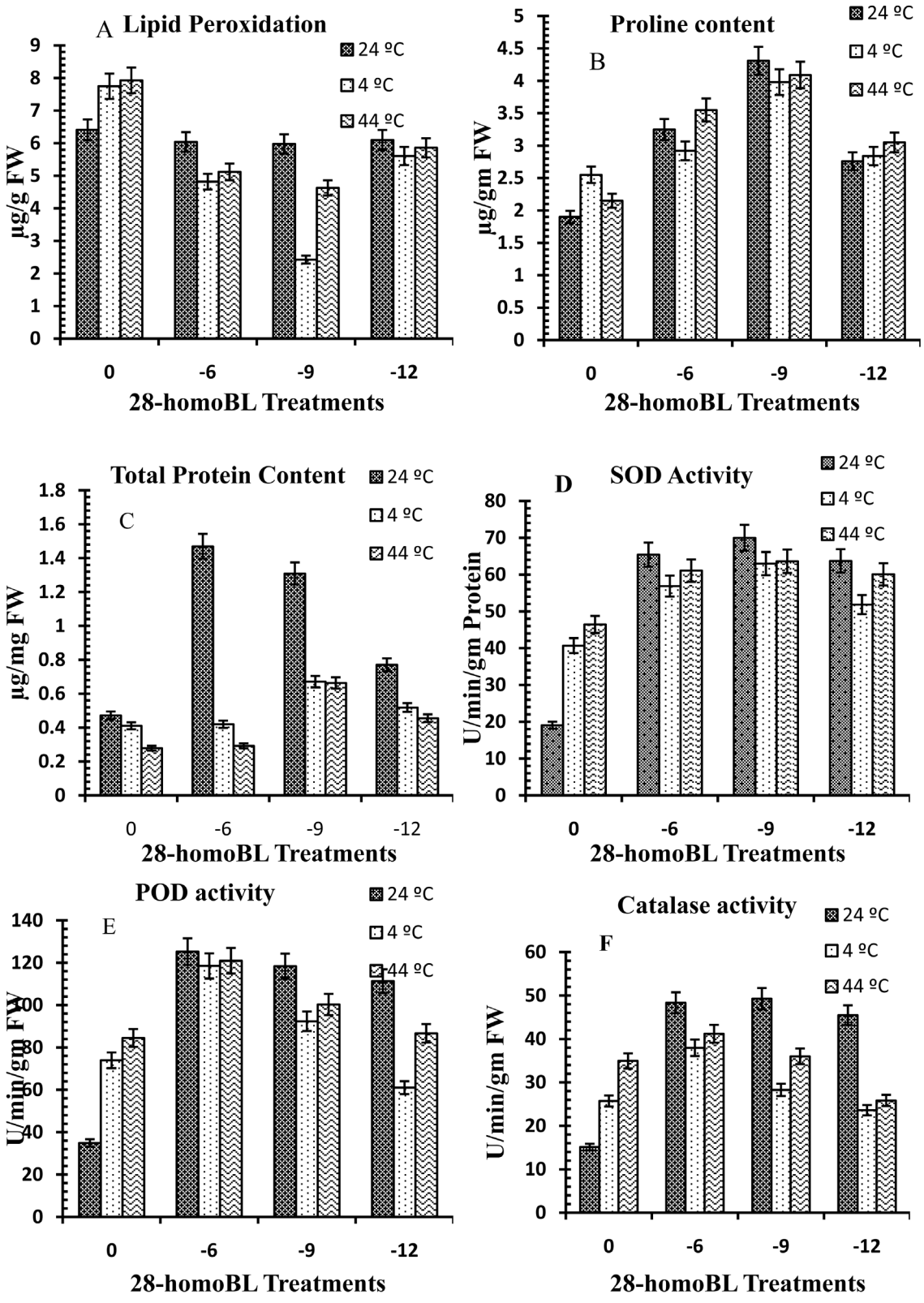

Figure 1. Levels of biochemical and antioxidant enzymes of *Brassica juncea* L. in presence and absence of 28-homoBL treatments and stress (A) Lipid peroxidaion (B) Proline content (C) Total Proteins (D) SOD activity (E) POD activity (F) CAT activity (values represent average of triplicates and expressed as mean ± SD).

to different cell organelles and biomolecules. To scavenge ROS, plants possess a well-organized antioxidative defense system comprising enzymatic and nonenzymatic antioxidants. The cooperative function of these antioxidants plays an important role in scavenging ROS and maintaining the physiological redox status of organisms [32]. The expressions of the antioxidant enzyme-related genes, *i.e.*, SOD, POD and CAT were up-regulated by extreme temperature treatments in comparison with the control. Up-regulation in the expression of genes, and hence the increased activities of the antioxidant enzymes suggest that temperature adaptation improved the antioxidant capacity, which may effectively lessen ROS injury during extreme temperature stresses.

In this study, a significant increase of SOD activity in seedlings was observed at extreme temperature (4˚C and 44˚C) and this increase was further enhanced in 28-homoBL treated seedlings (**Figure 1(C)**) of *Brassica juncea* L. and its involvement in plant tolerance to oxidative stress caused by abiotic stress. This may be attributed to the increased production of superoxide, resulting in the activation of existing enzyme pools or increased expression of genes encoding SOD [33]. The present study first time revealed that exogenous 28-homoBL application increases SOD activity to many folds which help the plant in upgrading its antioxidant capacity to scavenge more free radicles. Under temperature alone SOD activity increased to 2 folds as compared to control distilled water seedlings but his increase in SOD was 2 - 4 times more in seedlings but this increase in SOD was 2 - 4 times more in seedling shaving 28-homoBL treatment before exposing to extreme temperatures. Increased SOD activity caused by extreme temperatures has been previously observed in several plant species, and is routinely considered to be an adjustment response to stress [34].

Extreme temperatures decreased the CAT activity in *Brassica* seedlings and addition of BRs increased its activity (**Figure 1(D)**). CAT is an important oxidizing enzyme that helps in the removal of H_2O_2 and helps in detoxifying harmful metabolic products; its activity appears to be positively correlated with an increase in growth. A decrease in CAT activity, extreme temperatures can be attributed to inhibition of the CAT synthesis and other oxidase proteins [35]. A similar increase in CAT activity of sorghum seedlings under water stress caused by the application of brassinosteroids was reported previously [36]. In contrast to CAT activity, temperature stress increased the POD activity in *Brassica* seedlings (**Figure 1(E)**). An increase in POD activity is a common response to oxidative and abiotic stresses. In plants, POD protects cells against harmful concentrations of hydroperoxides and helps in a variety of cellular functions [37]. Increased total peroxidase activities in response to salinity were reported by [38]. A similar increase in POD was also observed after the application of Ni to the leaves of *Silene italic* [39]. The high POD activity in *Brassica* seedlings treated with extreme temperatures observed in the present study may indicate an initiation of disruption in the biochemical processes. However, we observed that 28-homobrassinolides applied to temperature stressed *Brassica* seedlings reduced the POD activity. Similarly, a reduction in POD activity in putrescine-alleviated salt stress in spinach leaves was reported [40].

4.4. Protein Content

The present finding revealed that exogenous application of 28-homoBL provokes accumulation of protein content (**Figure 1(F)**) as high as 177% more in 1 nM 28-homoBL treated seedlings as compared to untreated control seedlings. 28-homoBL seed pre-sowing treatments enhanced protein content in 10 days old *B. juncea* seedlings under temperature stress conditions as compared to control. Earlier reports indicated that 24-epibrassinolide-treated seedlings of *B. napus* showed maximum resistance to lethal heat treatments compared to control seedlings and this was found to correlate with higher levels of heat-shock proteins and corresponding mRNA during heat stress [8] [9] [41]. In this study 28-homoBL application provokes alterations in protein content in very dose-dependent and specificity manner in polypeptide profiles.

5. Conclusion

The present study shows that, although temperature is an essential factor for normal plant growth and physiological processes, extreme on both sides of mercury is toxic and may result in growth inhibition and altered metabolic processes. The observations of the present study clearly indicate temperature stress-protective properties of 28-homoBL in *Brassica juncea* plants. Stress ameliorative properties of BRs are clearly demonstrated by better growth, accumulation of proline content and antioxidative enzymes in seedlings to which different degrees of temperatures and concentration of 28-homobrassinolides are applied. It points to the possibility of 28-homoBL regulated stress-protection in plants but extensive studies are still needed on various aspects related to stress.

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