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Effect of exogenous H_2O_2 on antioxidant enzymes of *Brassica juncea* L. seedlings in relation to 24-epibrassinolide under chilling stress

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Hydrogen peroxide is most stable molecule among reactive oxygen species, which play a vital role in growth and development of plant as signaling molecule at low concentration in response to various abiotic and biotic stresses. Exogenous application of H_2O_2 is known to induce chilling tolerance in plants. Brassinosteroids are plant steroid hormones known for their anti-stress properties. In this study, effect of exogenous H_2O_2 on antioxidant defense system of *Brassica juncea* L. seedlings was investigated in 24-epibrassinolide (24-EBL) treated and untreated seedlings under chilling stress. The surface sterilized seeds of *B. juncea* L. were germinated in petriplates containing different concentrations of H_2O_2 alone and in combination with 10^{-8} M 24-EBL. Chilling treatment (4 ºC) was given to 10-days old seedlings grown in different treatments for 6 h daily up to 3 days. 24 h recovery period was given to chilling treated seedlings by placing at 25ºC ± 2ºC and harvested for antioxidant enzymes on 14th day after sowing (DAS). Treatment of 24-EBL in combination with H_2O_2 (15 and 20 mM) helped in reducing the toxicity of seed and seedlings due to H_2O_2 exposure on their germination rate, shoot and root length respectively. 24-EBL treatment at seed and seedling stage helped in alleviating the toxic effect of H_2O_2 through antioxidant defense system by increasing the activities of various enzymes involved in antioxidant defense system such as catalase (CAT, E.C. 1.11.1.6), ascorbate peroxidase (APOX, E.C. 1.11.1.11), and superoxide dismutase (SOD, E.C. 1.15.1.1). In conclusion, exogenous pretreatment of H_2O_2 to seeds of *B. juncea* L. adapted the seedlings to tolerate chilling stress, which was further ameliorated in combination of H_2O_2 with 24-EBL.

Keywords: Antioxidants, ROS, H_2O_2, Brassinosteroids, 24-Epibrassinolide, *Brassica juncea* L., Chilling stress

The plants due to their sessile nature are exposed to various environmental stresses, such as temperature, light intensity etc. which affect their growth. The most typical kinds of ecologically important environmental factors affecting plant growth, development and productivity are temperature, light and water. Fluctuations in temperature are a significant factor because both low and high temperatures limit plant productivity. Low temperature causes production of H_2O_2 as a result of photochemical reaction during fog, which may be the cause of death of seedlings due to lipid peroxidation at cellular level, leading to production of reactive oxygen species (ROS). ROS production to high level can damage membrane lipids, proteins and nucleic acids, resulting in cell death[1]. To mitigate high production of ROS, plants have well developed antioxidant defense system operating at cellular level.

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ROS, such as H_2O_2 at low concentration play a pivotal role as signaling molecule for proper growth and development. The ability to adjust their antioxidant system to changing ROS concentrations may be vital to all species under stress conditions[2,3]. Low temperature-induced photoinhibiton also has an important role in the chilling damage to young maize plants[4]. Chilling stress reduces the capacity of photosynthetic system to utilize incident photon and leads to photoinhibiton[5]. Internally in plants, H_2O_2 is produced during the normal course of metabolism and is one of the stable ROS. It is produced as result of enzymatic activity of superoxide dismutase (SOD) on free radicals produced by the electron transport machinery of the plant. For normal growth and development of the plant, the ROS production must be in equilibrium with their scavenging rate. This equilibrium between the production and scavenging of ROS may be disturbed by number of adverse abiotic stress factors such as high light intensity, drought, low and high temperature[6-9].

A stressful environment leads to rapid synthesis of H_2O_2 in chloroplast and other cell organelles and in apoplast[10]. The generation of ROS and especially H_2O_2 is acknowledged as a signal for activation of plant defense mechanism under biotic and abiotic stress[8,10,11]. Application of H_2O_2 at low concentration has been shown to induce stress tolerance in plants. The preliminary H_2O_2 treatment of *Arabidopsis* or tobacco protects the plant from oxidative damage due to high light intensity[12,13]. Tolerance to low temperature is demonstrated after treatment with low concentration of H_2O_2 in maize seedlings, *Phalaenopsis* and *Vigna radiata*[8,14,15] and similarly treated potato nodal explants are found to be resistant to high temperature[10,16]. It has been proved that pretreatment with H_2O_2 causes alteration in the activity of several antioxidant enzymes and/or the level of antioxidants such as glutathione. Studies with exogenously applied H_2O_2 have confirmed the role of H_2O_2 as a cell death trigger and show that high concentration can cause necrosis[17].

Brassinosteroids are the plant steroids which have been implicated in protecting plants from various types of stresses like drought, salt, heat[18-22]. In an earlier study, it has been reported that 24-epibrassinolide (24-EBL) protects the cultured cells of *Chorispora bungeana* by enhancing antioxidant defense system under chilling stress[40]. *Brassica juncea* is a cold seasoned crop which frequently faces cold temperature shocks in natural field environment. Under these circumstances of low temperature, production of H_2O_2 in the environment is a natural phenomenon, to which this crop met without any excuse. In the present study, effect of exogenous H_2O_2 application has been studied with or without supplementation of 24-EBL on various antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APOX) and SOD in modulating the tolerance of *B. juncea* L. seedlings to chilling stress.

Materials and Methods

Plant material and treatment

The seeds of *Brassica juncea* L. cv. PBR 210 were procured from Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. The seeds were sterilized with 0.01 % $HgCl_2$ and rinsed 5-6 times with double-distilled water. The surface sterilized seeds were kept in different concentrations of H_2O_2 (15 and 20 mM) alone and in combination overnight. Next day, the treated seeds were grown in petriplates in laboratory conditions. After 10 days of with 10^{-8} M 24-EBL for pre-sowing soaking treatment sowing, the seedlings were subjected to chilling treatment (4°C) for 6 h daily upto 3 days. Seedlings were placed at 25°C ± 2°C for 24 h after chilling treatment for recovery and harvested for various biochemical assays on 14th day after sowing. Morphological data in terms of percent seed germination, shoot length and root length were prepared.

In vitro antioxidant activity

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was estimated by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are generated by the auto-oxidation of hydroxyl amine hydrochloride[23]. The reduction of NBT was followed by increase in absorbance at 540 nm in reaction mixture containing 50 mM Na-carbonate buffer (pH 10.0), 96 µM NBT, 0.6% Triton X-100. The reaction was initiated by addition of 20 mM hydoxylamine-HCl (pH 6.0) and 2 min later, enzyme sample was added. The enzyme activity was calculated as the SOD concentration inhibiting reduction of NBT by 50%.

Catalase (CAT, E.C. 1.11.1.6) activity was assayed by measuring the rate of H_2O_2 decomposition followed by decrease in absorbance at 240 nm in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 150 mM H_2O_2, and enzyme extract[24]. Enzyme activity was determined using the extinction coefficient of 6.93×10^{-3} $mM^{-1}cm^{-1}$.

Ascorbate peroxidase (APOX, E.C. 1.11.1.11) activity was estimated according to the method of Nakano and Asada[25], following the decrease in absorbance at 290 nm of the reaction mixture containing 100 mM potassium-phosphate buffer (pH 7.0), 5 mM ascorbate, 0.5 mM H_2O_2 and enzyme extract. Enzyme activity was determined using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$, and calculated as the amount of enzyme required to oxidize 1 µM ascorbate $min^{-1}g^{-1}$ tissue.

Total protein content was estimated by the method of Lowry *et al.*[26]. The amount of protein was expressed as mg g^{-1} FW.

Results

Treatments of different sub-lethal concentrations of H_2O_2 modulated the metabolism of *B. juncea* seedlings exposed to low temperature. When supplemented with 24-EBL, survival and growth of seedlings were improved by reducing the toxic effect

of H_2O_2. This effect was observed in terms of altered morphological and antioxidant enzyme activities.

Morphological parameters

Effect to different concentrations of H_2O_2 alone and in combination with 24-EBL pre-sowing soaking treatments on percentage germination, root length and shoot length was observed on 10th day after sowing (DAS) and presented in Table 1. Germination rate, root length and shoot length of 10 DAS seedlings decreased with increasing concentration of H_2O_2 alone as compared to control seedlings (distilled water). However, when 24-EBL pre-sowing soaking treatment (10^{-8} M) was given, all the morphological attributes as percentage germination on 3rd day, root length and shoot length on 10th DAS were improved as compared to H_2O_2 treatments. The 20 mM H_2O_2 concentration showed maximum stress promoting effect on percentage germination (45.33 ± 2.02), root length (4.1 ± 0.15 cm) and shoot length (4.57 ± 0.11 cm) as compared to control distilled water and when supplemented with 24-EBL on all the morphological attributes.

Biochemical analysis of 10 DAS seedlings indicated that H_2O_2 (15 mM and 20 mM) caused stress which was further ameliorated by chilling treatment (4ºC) to 14 DAS seedlings.

Total protein content

Restrained stress of H_2O_2 (15 and 20 mM) and chilling (4°C) resulted in degrading total protein content in 10 and 14 DAS seedlings as compared to control distilled water (Fig. 1A & B). 24-EBL treatment helped in upgrading total protein content, which increased in combined treatment of 20 mM H_2O_2 with 24-EBL (18.04 ± 0.61) as compared to control distilled water (17.80 ± 0.52). Chilling treatment deteriorated the protein content to lowest level in control distilled water seedlings (13.59 ± 0.39). Supplementation of 24-EBL in 20 mM H_2O_2 treated seedlings proved to be better in making the seedling more tolerant to chilling stress (15.04 ± 0.36). 24-EBL treatment helped in ameliorating stress injuries caused due to H_2O_2 and chilling by increasing total protein content.

Antioxidant enzyme activity

Seedlings exposed to chilling temperature showed increase in activities of antioxidant enzymes as a self invulnerability phenomenon, which further increased to upper level, when supplemented with different concentrations of H_2O_2 alone and in combination with 24-EBL. Maximum increase (10.61 ± 0.89) in SOD activity (Fig. 2A) was observed in seedlings treated with 24-EBL + 20 mM H_2O_2 under chilling stress as compared to chilling treated only (7.79 ± 0.53). SOD activity was significantly enhanced in 24-EBL + 20 mM H_2O_2 treated seedlings (6.8 ± 0.76) as compared to control seedlings (3.9 ± 0.34) on 10th DAS.

Activity of CAT was increased under chilling stress to comparable level when supplemented with 24-EBL + H_2O_2. Maximum CAT activity (32.18 ± 0.45) was observed in 24-EBL + 15 mM H_2O_2 under chilling stress (Fig. 2B) as compared to chilling treated only (17.00 ± 0.89).

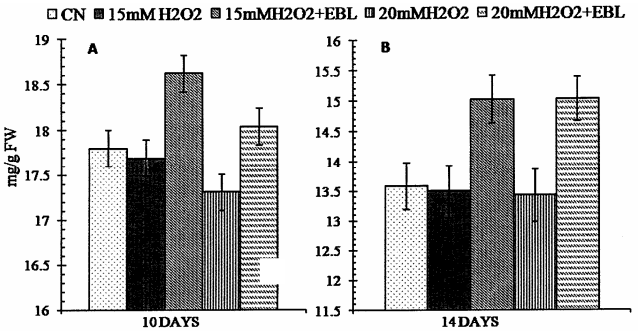

Fig. 1—Total protein content on 10th day (A) before chilling and 14th day (B) after chilling treatment in H_2O_2 alone and in combination with 24-EBL treated seedlings of *B. juncea* L.

Table 1—Effect of different concentrations of H_2O_2 alone and in combination with 24-EBL on percentage germination, root length and shoot length on the 10th day after treatment

[Values expressed as mean ± S.E.]

Treatments	Germination (%)		Root length (cm)		Shoot length (cm)	
	Mean ± S. E.	'*t*' value	Mean ± S. E.	'*t*' value	Mean ± S. E.	'*t*' value
Control	65.66 ± 2.02	-	8.7 ± 0.29	-	6.89 ± 0.46	-
15 mM H_2O_2	52.33 ± 1.45	5.36*	5.0 ± 0.03	9.02*	5.54 ± 0.22	2.63
15 mM H_2O_2 + EBL	63.00 ± 2.64	0.80	5.4 ± 0.12	10.62*	6.66 ± 0.19	0.44
20 mM H_2O_2	45.33 ± 2.02	7.11*	4.1 ± 0.15	14.29*	4.57 ± 0.11*	4.90*
20 mM H_2O_2 + EBL	57.00 ± 1.73	3.25*	4.6 ± 0.20	11.82*	5.34 ± 0.20*	3.08*

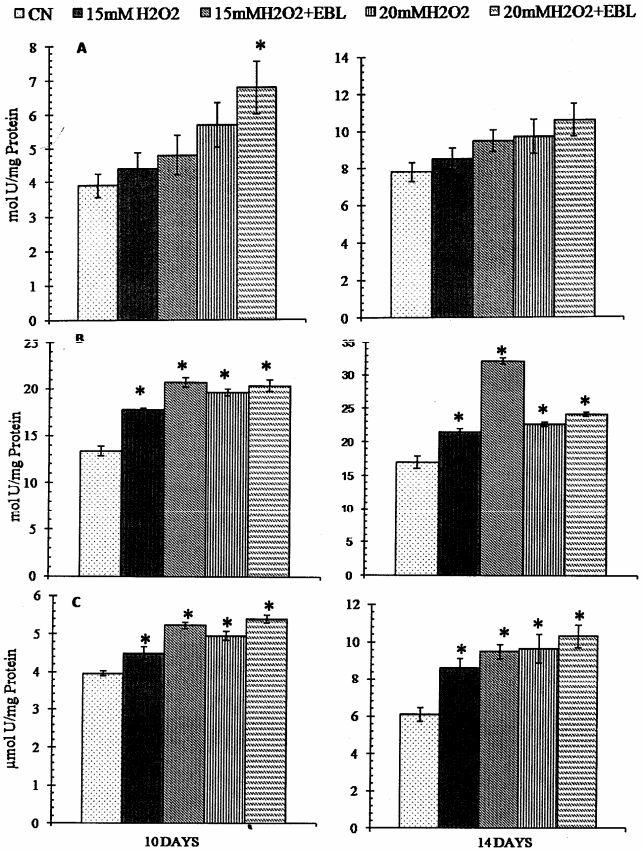

Fig. 2—Activity of SOD (A), CAT (B) and APOX (C) on 10th day before chilling and 14th day after chilling treatment in H_2O_2 alone and in combination with 24-EBL treated seedlings of *B. juncea* L.

For APOX activity, seedlings treated with 24-EBL + 20 mM H_2O_2 (10.33 ± 0.60) showed highest increase under chilling stress (Fig. 2C) as compared to chilling treated only (6.10 ± 0.36). CAT and APOX activities increased considerably in 24-EBL treated seedlings supplemented with H_2O_2 under normal and chilling temperature. Increased H_2O_2 toxicity ameliorated the antioxidant system of plant which ameliorated further to significant level, when supplemented with 24-EBL, making the seedlings to tolerate any toxic effect of H_2O_2 and/or chilling stress.

Discussion

Acclimatization process during stress conditions, particularly temperature stress involved responses which are operating at morphological, physiological, biochemical and/or molecular levels make the plant resistant towards adverse conditions. H_2O_2 is one of the toxic ROS which is available to plants during its normal course of growth and development and enhanced its production level, whenever the plant is under stress. *Brassica* is cold seasoned crop which is exposed to exogenous H_2O_2 during low temperature conditions as a result of photochemical reaction occurring in presence of fog and cloudy environment. Hariyadi and Parkin[30] have observed that chilling treatments increase the production of ROS in cucumber plants. Brassinosteroids play an important role in modulating the toxic effect of H_2O_2 and chilling stress and also play an important role in plant growth and development[21,29]. Anti-stress properties of brassinosteroids have been studied by various workers on different crops like rice, tomato, maize, and brassica[27-29].

Present study revealed the effect of 24-EBL in reducing the toxic effect of H_2O_2 and chilling stress, improving the germination rate, shoot and root length by modulating enzymatic activities at cellular level. Our results indicated that pre-sowing soaking treatment of seeds with H_2O_2 make the seedlings more tolerant to chilling stress by increasing antioxidant activities to higher level as compared to untreated control seedlings. Hung *et. al.*[39] reported that H_2O_2 functions as stress signal at low concentration, but when alleviated to permissible level can interact with cysteine residues of various proteins that could potentially alter protein conformation and affecting protein activity. In present experiment, it was observed that protein content was degraded under H_2O_2 and chilling treatment. However, H_2O_2 treated seedling supplemented with 24-EBL enhanced protein content, but not to the level which was observed in control untreated plants.

In an earlier study on H_2O_2 in relation to plant stress, it is reported that H_2O_2 is produced indirectly by spontaneous or SOD-mediated dismutation of superoxides[31]. SOD directly acts on superoxide radicals to form H_2O_2. In present study, 1.5-fold increase in SOD activity was observed after chilling treatment in comparison to normal seedlings. Dismutation of superoxide anion by SOD might be the primary step in defense mechanism against low temperature conditions. Minor increase in activity of SOD has been detected in H_2O_2-treated pea plants[33]. APOX activity in *B. juncea* seedlings continued to increase during chilling treatment. This result was in accordance with Asada and Takashi[37] who reported that after dismutation of superoxide radicals into H_2O_2 by SOD, APOX leads to their breakdown into water. H_2O_2 treatment increased CAT activity as compared to control seedlings. Supplementation of exogenous H_2O_2 has been shown to stimulate the expression of

CAT[8]. Earlier study[38] has clearly demonstrated that CAT effectively modulates H_2O_2 to O_2 and H_2O. In our results, it was observed that addition of 24-EBL increased all the antioxidant enzyme activities in modulating the stress caused due to high production and accumulation of H_2O_2 supplemented with chilling stress.

Currently, research data show that H_2O_2 can play a dual role in cells which at present concentration proved to be toxic for *B. juncea* L. The present results also showed that exogenous application of H_2O_2 makes the plant more tolerant towards chilling stress by well rehearsing at biochemical and cellular levels. Earlier studies have reported that H_2O_2 treatment provides protection to plants subjected to various stresses and particularly in plants exposed to chilling stress[13,15]. The exogenous application of H_2O_2 prior to chilling treatment influenced the activities of antioxidant defense enzymes, especially APOX, which was further augmented when 24-EBL was added to pretreatment solutions of H_2O_2. However, more investigations are needed to understand the mechanism of H_2O_2 and brassinosteroids protection, singly or in combination in cold tolerated crops like *B. juncea*.

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