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Low temperature induced metabolic regulation in maize crop is coupled to nitrification and denitrification processes

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Abstract

Maize is an important cereal crops however, its growth is retarded in response to environmental stimuli although the mechanism is not clarified. In this respect, plants (*Zea mays* L.) were grown in plastic pots and were given low temperature (4~8 ^{o}C) for 24h, 48h and 72h in cold chamber. In response to low temperature for 24h, the amount of protein was increased (34.72%) compared to control plants kept in ambient temperature. Similarly, the amount of protein was increased by 64.68% and 16.28% for 48h and 72h respectively however, the effects were pronounced at 48h of treatment. Low temperature was shown to enhance PPO activity by 291.52% and 79.79% respectively after 24h and 48h while the effects were assumed to be reduced after 72h. H_2O_2 plays the potential role on signaling of diverse molecules and is regulated by low temperature stress. In separate studies, plants were exposed to cold for 24h, 48h and 72h periods and the accumulation of H_2O_2 in the extract was determined. H_2O_2 was exaggerated by 19.59%, 15.54% and 62.16% respectively compared to respective controls. It is assumed that the above findings are coupled to nitrification and denitrification process. Therefore, we also examined the effect of low temperature on nitrate content in maize tissue extract. Nitrate accumulation in response to low temperature was increased by 23.41% and 25.30% respectively after 24h and 48h. Similarly, accumulation of nitrate after 72h was recorded as 21.97% when compared to respective controls. Nitrate accumulation and degradation are consecutive processes. The results show clearly that H_2O_2 induced biological processes in seedlings are coupled to internal nitrification and denitrification processes.

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Introduction

Maize is an important cereal crops and grown in different countries. It is also grown in different parts of Bangladesh. However, its cultivation and uses in Bangladesh should be enhanced. To understand the uses and production of maize, its biochemical investigation is necessary.

Although maize cultivation is observed in some areas of Bangladesh, however, maize production is retarded than the expected yield. Due to change of climate and other environmental factors, insufficient growth and production of maize are observed. Therefore, it is substantial to take the measure and make a plan to develop the strategy for identifying the causes and remedy for the development of higher maize production and its cultivation. Although few studies involved in maize research were done in Bangladesh, the current research project might be a significant aspect in higher yielding of maize crop.

Nitrogen is the most limiting element in plant nutrition. Efficient recycling of reduced nitrogen present in the form of urea is important for plant growth since urea contains a significant amount of this element. In addition to internally generated urea, externally applied urea can also be utilized by plants. Moreover, recent investigation suggests that deficiency of amino nitrogen causes pathogenic syndromes, pale appearance associated with other physiological disorders in plants. Nitrogen deficiency affects crop growth and development, with symptoms, including leaf loss, plant dwarfing, thickening of root cell walls, and reduced yield (Kusano *et al.*, 2011). The disappearance of amino nitrogen from urea is an important aspect in nitrogen metabolism and an essential step for utilization to promote growth.

Nitrate accumulation in plants is affected greatly by different ways. It is assumed that environmental factors might be involved greatly for this impairment in nitrate accumulation in plants. Moreover, low temperature is considered to be involved in causing higher oxidative stress (Baek and Skinner, 2012), therefore, formation of hydrogen peroxide (H_2O_2) in response to this environmental stimuli in maize crop is an important aspect of this study although not clarified well. In adverse environment, plants survive in the environment by expression of new proteins and other anti-oxidative enzymes particularly polyphenol oxidase (PPO) enzyme. The formation of these molecules might be linked to the signaling of H_2O_2 and is not well understood.

Due to global climate change, temperature stress is becoming the major area of concern for the researchers worldwide. The reactions of plants to these stresses are complex and have devastating effects on plant metabolism, disrupting cellular homeostasis and uncoupling major physiological and biochemical processes. As the biological process in plants is disrupted by the oxidative stress, the uptake of nitrate in maize crop might be influenced by the oxidative stress induced by low temperature. It is assumed that nitrification and denitrification processes are coupled to the oxidative stress caused by low temperature although not clarified well. Therefore, the present study has been undertaken regarding this phenomenon in this crop to examine the influence of low temperature on the biosynthesis of protein and polyphenol oxidase (PPO) (an anti-oxidative enzyme); accumulation of H_2O_2 and nitrate in maize seedlings.

Materials and methods

Plant materials and low temperature treatment

For this experiment, two plastic pots were used; each pot size was 30 cm in diameter and 11.3 cm in height. An adequate amount of soil was taken in each plastic pot and the plastic pots were seeded with *Zea mays* L. The soil was brought from the university campus. For the germination of seeds, the strong seeds were collected from the nearby source and the efficiency of seed germination was 60%–70%. After 8~10 days of germination, the two different pots were described as control and low temperature induced plant. Control pot was used for 24h, 48h and 72h treatments in the room temperature without cold acclimation. The second pot was used for 24h, 48h and 72h duration in

the temperature controlled cooling chamber and given cold exposure (4~8 °C) with full aeration. After the treatments, maize seedlings (without roots) were collected consecutively from each pot with scissors for 24h, 48h and 72h duration and kept in –20 °C. Plants collected from the above treatments were used for the assay of protein, polyphenol oxidase activity (PPO) according to the conventional procedure as described below. In separate examinations, plants were similarly grown in pots and after 8~10 days of germination, plants were exposed to low temperature for 24h, 48h and 72h periods and the control plants were kept in the ambient room temperature for the above mentioned periods. After the treatment, plants (without roots) were collected from the pots with scissors and kept in –20 °C. The plants were used for the assay of hydrogen peroxide (H_2O_2) and nitrate content.

Assay of tissue protein content

Maize tissues were homogenized with pre-cooled water and were centrifuged at 8000 rpm for 15 min. The supernatants from each tissue homogenate were used as crude extract for assay of protein by using 50 µL extract. The protein content in tissue was determined by the procedure of Lowry *et al*. (1951). Briefly, alkaline solution was prepared by mixing 50 mL of alkaline Na_2CO_3 solution (2% Na_2CO_3 in 0.1N NaOH) and 1.0 mL of copper-sodium potassium tartarate solution (1 g sodium potassium tartarate and 0.5 g $CuSO_4$. $5H_2O$ were dissolved in 100 mL distilled water). Fifty micro liters of tissue extract was taken to the test tube and made up to 1 mL with distilled water. For blank, 1 ml water was used in place of tissue extract. Five milliliters of alkaline solution was added to each tube and mixed well. The tubes were allowed to stand for 10 min at room temperature and 0.5 mL of diluted FCR (Commercial FCR was diluted with equal volume of water) was added and mixed well. After 30 min, the absorbance was taken at 650 nm against the blank. The protein content in each plant tissue was calculated from the standard graph of bovine albumin (1 mg mL^{-1}) and is expressed as mg/g of tissue weight.

Assay of polyphenol oxidase (PPO) activity

The plants of the different treatments (24h and 48h and 72h) and their respective controls were homogenized with 26 mL of distilled water in a mortar kept on ice. Approximately, 2.80 g of low temperature induced and their respective control plants were used for homogenization. The homogenates were centrifuged at 8000 rpm for 15 min and the supernatants were used as crude extract for assay of PPO activity spectrophotometrically as described by Mahadevan and Sridhar (1982) based on an initial rate of increase in absorbance at 495 nm where, catechol was used as substrate. One unit of enzyme activity is defined as a change in absorbance of 0.001 min^{-1} mL^{-1} of enzyme extract. For determination of PPO activity in maize tissue, 4 mL of 0.1 M phosphate buffer (pH 6.0) and 1 mL of crude enzyme extract were taken in the test tube and kept on ice.

The contents were mixed, placed in a spectrophotometer using a cuvette and the absorbance was adjusted to zero at 495 nm. The cuvette was removed, 1 mL of catechol (0.1 M) was added, quickly mixed by inversion and the changes in absorbance at 495 nm were recorded for up to 3 min (1, 2, 3 min). In all experiments, three replicates were performed for each sample. The following calculation was used to assay PPO activity in a sample: change in $A_{495} = A_f - A_i$, where, A_i = initial absorbance reading and A_f = final absorbance reading. Change in A_{495} for each sample was used to calculate the units of PPO activity and the activity is expressed as Unit g^{-1} of tissue weight.

Determination of hydrogen peroxide content (H_2O_2)

The plants of the different treatments (24h and 48h and 72h) and their respective controls were homogenized with 26 mL of 0.1% (w/v) TCA (0.1g in 100 ml distilled water) in a mortar kept on ice. Approximately, 2.84 g of low temperature induced and their respective control plants were used for homogenization. The homogenates were centrifuged at 8000 rpm for 15 min and the supernatants were used as crude extract for the assay of hydrogen

peroxide content (H_2O_2). The assay of H_2O_2 in maize seedlings was followed by the procedure of Borriboon *et al.* (2018). Briefly, the supernatant (0.5 mL) was transferred to a 25-mL test tube and 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (Germany, Merk) were added to the test tube and mixed thoroughly. Absorbance of the mixture was measured spectrophotometrically at 390 nm (U-1800 spectrophotometer, Hitachi, Japan). The amount of hydrogen peroxide (H_2O_2) was expressed as µmole g^{-1} of tissue weight. For preparation of the standard curve, 20 vol. of H_2O_2 (Jolly Chemicals, Dhaka, Bangladesh) was used. To prevent the decomposition of H_2O_2, the assay tubes were kept in dark.

For H_2O_2 standard curve, 0.1 mL of 20% H_2O_2 was taken and diluted with distilled water and made up to 100 mL (1000X dilution). Then further 10 times (10X) dilution was made. (Taken 1 mL from above and diluted in 99 mL of distilled water).

Test tube	1	2	3	4	5	6	7
H_2O_2	0	0.1	0.2	0.4	0.6	0.8	1
PBS (pH 7.0)	1	0.9	0.8	0.6	0.4	0.2	0
1 M KI	1	1	1	1	1	1	1

Determination of nitrate content

The maize seedlings of different treatments (24h and 48h and 72h) and their respective controls were homogenized with 26 mL of distilled water in a mortar kept on ice. Approximately, 2.9 g of maize seedlings were used for homogenization. The homogenates were centrifuged at 8000 rpm for 15 min and the supernatants were used as crude extract for the assay of nitrate as described by Cataldo *et al.* (1975). Briefly, 0.2 mL extract of each treatment was mixed with 0.4 mL reagent (5% salicylic acid in conc. H_2SO_4: 2.5 g salicylic acid was taken in beaker. 30 mL conc. H_2SO_4 was added, shaked and dissolved completely. It was made up to 50 mL with conc.

H_2SO_4 and kept in brown glass bottle). After 20 min at room temperature, 9.4 mL of 2M NaOH was added slowly to the tubes. The tubes were cooled at room temperature and absorbance at 410 nm was determined spectrophotometrically. For blank, 0.2 mL H_2O was taken in tube, 0.4 mL reagent and 9.4 mL 2M NaOH were used. The tubes were cooled at room temperature and absorbance at 410 nm was determined similarly. Sodium nitrate ($NaNO_3$) (20 mM in distilled water) was used as standard for determination of nitrate content in maize tissue. The results were expressed as µmole of nitrate per gram of tissue weight.

Statistical analysis

Results of the experiments were expressed as mean and standard error of different groups. The reported data represent the mean of three replications (n=3). The differences between the mean values were evaluated by ANOVA followed by paired t-test using SPSS software for comparison of changes at $p = 0.05$ level of significance.

Results

Effects of low temperature on protein content in extract of maize seedling

To examine the role of low temperature on the regulation of protein synthesis in tissues of *Zea mays* L., plants in the pot were exposed to 4~8 ˚C in the temperature controlled cooling chamber for 24h period and the respective control was kept in ambient room temperature. As demonstrated in Fig. 1A, the amount of protein accumulated in maize seedlings in response to low temperature was 18.57 ± 0.02 mg g^{-1} of tissue whereas for control plants, the protein content was 13.79 ± 0.04 mg. The results demonstrated that protein contents had been significantly enhanced and stimulated (34.72%, $p < 0.01$) by low temperature compared to the respective control. The results appeared to indicate that the protein contents were affected by cold acclimation. Therefore, it is reasonable that an adaptive response by the species was created and some stress proteins were synthesized.

As illustrated in Fig. 1B, the amount of protein in tissues of plant in response to low temperature has been recorded to determine the effect of low

temperature on protein synthesis for extension of time. After 48h of treatment, the tissue protein was estimated as 15.51 ± 0.13 mg for control and for low temperature induced plant, the value was 25.54 ± 0.01 mg g^{-1} of tissue weight. Low temperature causes a significant and more pronounced increase in protein accumulation in tissue by 64.68% ($p < 0.01$) when compared to the respective control. The increase of protein in maize seedling was found to be higher than the previous 24h of exposure as demonstrated in Fig. 1A and Fig. 1B. Therefore, the protein content in tissue is assumed to be regulated by the variation of temperature and be strictly followed by the extension of time. The accumulation of protein in seedlings was found to be augmented in response to low temperature time dependently. The results suggest that the increased protein in maize seedling might be due to the higher sensitivity of temperature. Because, the abiotic stress like low temperature causes higher oxidative stress.

To survive in the adverse environment, plant needs the formation of stress proteins thereby the higher synthesis of protein might be considered as the survival factor as well as index for characterization of physiology of tissue of this species.

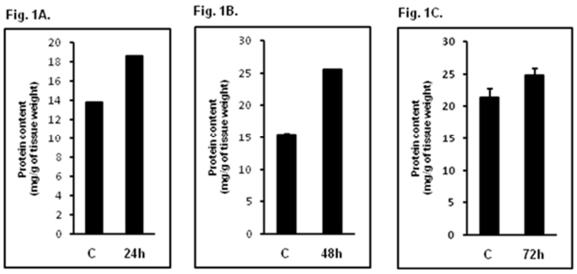

Fig. 1. Alteration of protein contents in seedlings of *Zea mays* L. during cold acclimation. The plants were exposed to 4~8 °C for 24h (1A), 48h (1B) and 72h (1C) in cold chamber, however the respective controls were used without any cold acclimation and kept in ambient temperature. After the treatment, the plants and its respective controls were used for determination of protein. The results are means of ± SE for three values in each group

We further extend the duration to get optimum effect of low temperature on the regulation of protein synthesis in maize seedling of *Zea mays* L., plants in the pot were exposed to 4~8 °C in the temperature controlled cooling chamber for 72h period and the respective control was kept in ambient room temperature. As demonstrated in Fig. 1C, the amount of protein accumulated in maize seedlings in response to low temperature was 24.81 ± 1.06 mg g^{-1} of tissue whereas for control plants, the protein content was 21.34 ± 1.34 mg. The results demonstrated that protein contents had been significantly enhanced and stimulated (16.28%, $p < 0.01$) by low temperature compared to the respective control however, the results were assumed to be lower than 48h of treatment. Therefore, low temperature causes the accumulation of protein in tissues of maize and the synthesis is dependent on the exposure of duration. It is assumed that prolonged exposure may cause the synthesis of new genes in maize to survive in the adverse environment. The results appeared to indicate that the protein contents were affected by cold acclimation. Therefore, it is reasonable that an adaptive response by the species was created and some stress proteins were synthesized.

Effect of low temperature on polyphenol oxidase (PPO) activity in maize seedling

To properly identify physiological responses to environmental stress such as low temperature, plants were exposed to 4~8 °C in the temperature controlled cooling chamber for 24h periods and the respective controls were kept in ambient room temperature. Polyphenol oxidase activities in maize tissue exposed to the cold temperature for the above mentioned periods were examined at 100 mM catechol, substrate for the enzyme. As shown in Fig. 2A, the average PPO activity in maize seedling in response to low temperature for 24h period was 578.79 ± 45.39 Unit g^{-1} of tissue whereas for control plant kept in ambient temperature, the PPO activity was 147.83 ± 18.82 Unit. A significant 291.52% ($p < 0.01$) increased PPO activity was observed after 24h when compared to the control plant. The results appeared to indicate that

the PPO activities were affected by cold acclimation. Therefore, it is reasonable that an adaptive response by the species of plant was created and the higher synthesis of PPO was observed to serve as the factor in adverse environmental situation and might be sensitive to the temperature variation.

Tissues of *Zea mays* L. were exposed to low temperature for 48h period and the average PPO activity was 690.17 ± 34.38 Unit while for the respective control plant, the enzyme activity was 383.87 ± 39.75 Unit g^{-1} of tissue. The results indicated that 79.79% ($p < 0.01$) increased PPO had been found after 48h in response to low temperature compared to the control plant as illustrated in Fig. 2B. The increased synthesis of PPO in tissue in response to low temperature might be involved in the regulation of metabolic functions of this species of plant. The alteration of PPO level in maize tissue is an index for characterization of the sensitivity to the environmental temperature. The results suggest that the increased PPO induced by low temperature might be caused by such abiotic stress and could be considered as the survival factor for this species of plant in critical environment. The higher activity of PPO might be involved to prevent the oxidative stress caused by low temperature.

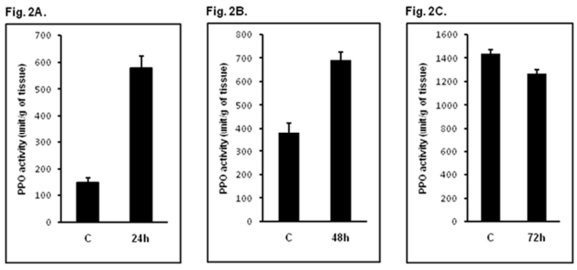

Fig. 2. Polyphenol oxidase (PPO) activity in response to 100 mM catechol in leaves of *Zea mays* L. during cold acclimation. The plants were exposed to 4~8 °C for 24h (2A), 48h (2B) and 72h (2C) in cold chamber, however, the respective control was used without any cold acclimation and kept in ambient temperature. After the treatment, the plants and its respective control were used for the assay of PPO activity. The results are means of ± SE for three values in each group

To find the optimum physiological responses, plants were further exposed to 4~8 ˚C in the temperature controlled cooling chamber for 72h periods and the respective controls were kept in ambient room temperature. After the exposure, plants were homogenized and tissue extract was prepared to assay of PPO activity where 100 mM catechol was used for the substrate of the enzyme. As shown in Fig. 2C, the average PPO activity in maize seedling in response to low temperature for 72h period was recorded as 1267.40 ± 20.90 Unit g^{-1} of tissue whereas for control plant kept in ambient temperature, the PPO activity was 1434.34 ± 14.56 Unit. A significant 11.63% ($p < 0.01$) reduced PPO activity was observed after 72h when compared to the control plant. Although enhanced PPO activity was observed after 24h and 48h of treatment, however 72h of exposure causes much lower responses on PPO synthesis. The results indicate that the prolonged exposure may reduce the effect on PPO synthesis however synthesis of PPO is dependent on the time of exposure.

Effect of low temperature on hydrogen peroxide (H_2O_2) content in maize seedling

H_2O_2 has been proposed to be involved in the signal transduction pathways leading to acclimation and protection from abiotic stresses. Hydrogen peroxide (H_2O_2) is a signal molecule which mediates a wide range of physiological and biochemical reactions during the whole period of plant growth. Moreover, the formation of the above molecules in response to low temperature might be guided by H_2O_2 concentrations. Therefore, we examined the effect of low temperature on the amount of hydrogen peroxide content in maize seedlings for 24h, 48h and 72h periods.

Plants were exposed to 4~8 ˚C in the temperature controlled cooling chamber for 24h periods and the respective controls were kept in ambient room temperature. As shown in Table 1, the average H_2O_2 content in maize seedling in response to low temperature for 24h period was 0.39 ± 0.02 µmole g^{-1} of tissue whereas for control plant kept in ambient temperature, the H_2O_2 level was 0.32 ± 0.01 µmole.

A significant 19.59% ($p < 0.06$) increased H_2O_2 level was observed after 24h when compared to the control plant. The higher synthesis of H_2O_2 in response to low temperature might be a contributory factor to survive the plants in adverse environment and may serve as a signaling molecule to drive the biological responses.

Tissues of *Zea mays* L. were exposed to low temperature for 48h period and the average H_2O_2 concentrations were 1.64 ± 0.05 µmole g^{-1} of tissue while for the respective control plant, the accumulation of H_2O_2 was 1.42 ± 0.08 µmole g^{-1} of tissue weight. The results indicated that 15.54% ($p < 0.05$) increased H_2O_2 had been found after 48h in response to low temperature compared to the control plant as illustrated in Table 1. The increased synthesis of H_2O_2 in tissue in response to low temperature might be involved in the regulation of metabolic functions of this species of plant.

Table 1. Hydrogen peroxide (H_2O_2) contents in maize seedling in response to low temperature after 24h, 48h and 72h of exposure. After the treatment, the plants were immediately removed from the respective pots and sampling was performed. Control plants were similarly used except giving low temperature exposure

Treatments	Hydrogen peroxide (H_2O_2) content (µmole/g of tissue weight)
Control	0.32 ± 0.01
24h	0.39 ± 0.02 [A]
Control	1.42 ± 0.08
48h	1.64 ± 0.05 [B]
Control	0.59 ± 0.02
72h	0.95 ± 0.04 [C]

The results are means of ± SE for three values in each group. [A] $p < 0.06$ versus respective control. [B] $p < 0.05$ versus respective control and [C] $p < 0.01$ versus respective control.

In separate experiment, plants were grown in the pots and were exposed to low temperature for further extended time. As shown in Table 1, the average H_2O_2 content in maize seedling in response to low temperature for 72h period was 0.95 ± 0.04 µmole g^{-1} of tissue weight whereas for control plant kept in ambient temperature, the H_2O_2 level was 0.58 ± 0.02 µmole. A significant 62.16% ($p < 0.01$) increased H_2O_2 level was observed after 72h when compared to the control plant. The results appeared to indicate that H_2O_2 concentrations were affected by cold acclimation. The increase of H_2O_2 may induce the cellular effects so that the maize seedlings may survive in the environment.

Effects of low temperature on nitrate accumulation in extract of maize seedling

Nitrate is the potent stimulator for plant growth. The formation and degradation of nitrate is a consecutive process. In adverse environment, severe oxidative stress causes the formation of ROS in plant seedlings. Simultaneously, to survive in that environment, PPO changes, up regulation of genes and stress proteins were synthesized. Moreover, accumulation of H_2O_2 in different areas of seedling was demonstrated. Plant needs much food during critical environment and energy deficiency might be possible. It is assumed that plant uses nitrate as a source of energy which will be enhanced in response to low temperature. To examine whether low temperature induced phenomenon is linked to the synthesis of nitrate in maize, we examined the effect of low temperature on nitrate content in seedlings. In this respect, plants in the pot were exposed to 4~8 ˚C in the temperature controlled cooling chamber for 24h period and the respective control was kept in ambient room temperature. As demonstrated in Fig. 3A, the amount of nitrate accumulated in maize seedlings in response to low temperature was 56.42 ± 1.34 µmole g^{-1} of tissue whereas for control plants, the nitrate content was 45.71 ± 2.64 µmole.

The results demonstrated that nitrate contents had been significantly enhanced and stimulated (23.41%, $p < 0.05$) by low temperature compared to the respective control. The results appeared to indicate that the enhanced nitrate contents were affected by cold acclimation and might be due to the energy deficiency during critical environment. It is assumed

that enhanced nitrate in seedlings in response to low temperature is because of the up regulation of cellular nitrate transporter. Therefore, it is reasonable that an adaptive response by the species was created and nitrate in plant were synthesized.

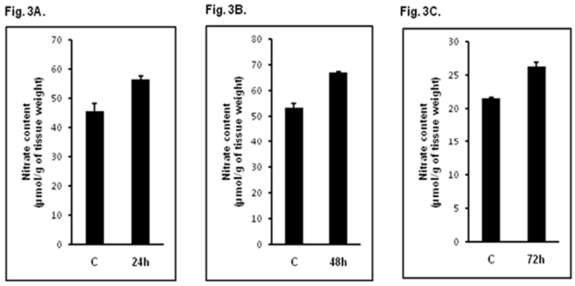

Fig. 3. Accumulation of nitrate in maize seedling (*Zea mays*) during cold acclimation. The plants were exposed to 4~8 °C for 24h (3A), 48h (3B) and 72h (3C) in cold chamber. After the treatment, the plants were immediately removed and sampling of tissue was performed. Control plants were similarly used except giving low temperature exposure. The results are means of ± SE for three values in each group

As illustrated in Fig. 3B, the amount of nitrate in tissues of maize plant after 48h of treatment was estimated as 53.49 ± 1.60 µmole for control and for low temperature induced plant; the value was 67.03 ± 0.14 µmole g^{-1} of tissue weight. Low temperature causes a significant and more pronounced increase in nitrate accumulation in tissue by 25.30% ($p < 0.05$) when compared to the respective control. The increase of nitrate in maize seedling was found to be higher than the previous 24h of exposure as demonstrated in Fig. 3A and Fig. 3B. Therefore, the nitrate content in tissue is assumed to be regulated by the variation of temperature and be strictly followed by the extension of time. The accumulation of nitrate was found to be augmented in response to low temperature time dependently. The results suggest that the increased nitrate in maize seedling might be due to the prolonged exposure of time and caused by temperature stress in the environment where they survive. For the plant growth during adverse environment, nitrate is the major energy sources and foods.

To find the optimum effect, plants were exposed to 4~8 ˚C in the temperature controlled cooling chamber for 72h periods and the respective controls were kept in ambient room temperature. As demonstrated in Fig. 3C, the average nitrate content in maize seedling in response to low temperature for 72h period was 26.25 ± 0.67 µmole g^{-1} of tissue whereas for control plant kept in ambient temperature, the nitrate level was 21.52 ± 0.10 µmole. A significant 21.97% ($p < 0.05$) increased nitrate level was observed after 72h when compared to the control plant however the values were lower than that of 24h and 48h of periods (Fig. 3A and Fig. 3B). The results shows that nitrate accumulation in plants are affected by cold acclimation however with the extension of time depletion of nitrate might be possible because nitrate accumulation and degradation are consecutive process in the biological system. Our investigation concludes that nitrification and denitrification process are influenced by environmental stimuli particularly low temperature plays the critical role in this respect. The metabolic processes including the biosynthesis of protein, enhancement of PPO activity and the formation of H_2O_2 are coupled to nitrification and denitrification process.

Discussion

To understand the mechanism of plant species responses to low temperature regarding the physiological and adaptive responses, assay of protein, PPO activity, H_2O_2 and nitrate accumulation in tissue of *Zea mays* L. was performed. In this respect, plants were grown in pot and exposed to 4~8 ˚C for 24h, 48h and 72h periods. In the present study, low temperature has been found to be involved in causing higher protein contents and PPO activities in maize leaves however the effects were more pronounced after 48h of the exposures. The mechanism of formation of these proteins and enzymes in response to the temperature stress is not known in this species of plant, however, several lines of evidences might be involved to clarify and recognize the formation of these molecules in such adverse situation. It has been shown that low temperature causes the higher oxidative stress

inducing the synthesis of active oxygen species (AOS) (Baek and Skinner, 2012) and increases tolerance to AOS in cereals and with an increase in anti-oxidative enzymes (Soengas *et al*., 2018). Anti-oxidative enzymes can neutralize AOS (Odaira *et al*., 2000) and thereby prevents the cellular membranes and organelles from the damaging effects of AOS. It is reasonable that fluctuation of temperature can cause stress to the normal physiological functions of plants, and hence alteration of metabolic activities in tissue of the maize plant might be observed. Previous studies revealed that low temperature had been associated with pronounced modifications in the ultrastructure of leaf cells, disorganization of cellular compartments (Stefanowska *et al*., 2002) and therefore, may induce the synthesis of new enzymes and proteins. *Zea mays* L. is a common crop grown in Bangladesh and other countries during winter and summer seasons. Therefore, the plants have the higher sensitivity to these temperatures; however, the plants survive in very cold environment although the mechanism is not clarified. Since low temperature causes the significant alteration in metabolic functions of plant and has been revealed to cause reactive oxygen species (ROS), therefore might be involved in causing the synthesis of diverse metabolites essential for various purposes. The reactive oxygen species (ROS) have been shown to cause the injury in plants during the critical circumstance and therefore, to survive in this environment, plants generate different mechanisms and synthesize the compounds.

Abiotic stress leads to a series of molecular, biochemical, physiological and morphological changes that adversely affect plant growth and productivity. Low temperature is a major factor limiting the productivity and geographical distribution of many species, including important agricultural crops. Higher plants manifest a unique capability of the synthesis of a large amount of diverse molecules so-called secondary metabolites, such as phenolic compounds and the synthesis and release of phenolics are induced by various biotic and abiotic factors (Makoi and Ndakidemi, 2007). It has been demonstrated in the previous study that these enzyme activities are increased in response to different types of stress, both biotic and abiotic (Yadegari *et al*., 2007). More specifically, both enzymes have been related to the appearance of physiological injuries caused by thermal stress. During the experiment, it was observed that both low temperature induced and the respective controls caused the color pigmentation quickly, however low temperature induced seedlings had higher pigmentation than the control, therefore, it is reasonable that cold acclimation causes the higher oxidation of phenolic compounds and might be an effective approach for producing the colored pigment essential for the several purposes.

Enzymatic browning is a significant problem in a number of fruits and vegetables such as strawberry (Chisari *et al*., 2007), potato (Lee and Park, 2007) and lettuce (Gawlik-Dziki *et al*., 2008). The discoloration in fruits and vegetables by enzymatic browning, resulting from conversion of phenolic compounds to o-quinones which subsequently polymerize to be a brown or dark pigment and the enzymes involved these processes are PPO and POD (Jiang *et al*., 2004).

The previous studies reveal that cold acclimation adversely affects physiological and morphological structures of plants (Zhang *et al*., 2023) and the nutritional deficiency has been observed in response to cold. Therefore, it is reasonable that adverse oxidative effects caused by cold acclimation might be correlated to the alteration of physiology of leaf of *Zea mays* L. and also to the nutritional deficiencies particularly the uptake of essential nutrients from the soil and also from the environment. Measurement of protein and PPO in tissue of *Zea mays* L. might be an essential approach and will give a new insight to clarify the mechanism of diverse metabolic functions of plant as well as help in analysis of physiology of *Zea mays* L. Moreover, regulation of these enzymes and proteins is not only mediated by cold environment but also might be by other chemical mediators in the environment.

It is well known that low temperature causes higher oxidative stress. To clarify whether low temperature induced enhancement of protein and PPO activity is linked to the oxidative stress, we also examined the H_2O_2 concentration in maize seedlings. Since this molecule is recognized to be a major signaling molecule triggering intracellular synthesis of diverse molecules. In this study, H_2O_2 has been found to be enhanced in response to the oxidative stress caused by low temperature. Hydrogen peroxide (H_2O_2), as a reactive oxygen species, is widely generated in many biological systems. It has been considered as an important signaling molecule that mediates various physiological and biochemical processes in plants. Normal metabolism in plant cells results in H_2O_2 generation from a variety of sources. To date, it has become accepted that H_2O_2 plays important roles in plant developmental and physiological processes including seed germination, programmed cell death (PCD); senescence, flowering, root system development, stomatal aperture regulation and many others. A number of discussions showed that H_2O_2 could respond to abiotic stresses such as cold (Orabi *et al.*, 2015) and high temperatures (Wang *et al.*, 2014; Wu *et al.*, 2015). It is clear from these studies that H_2O_2 could enhance abiotic stress resistance through protecting organelle structure under abiotic stress conditions. For instance, H_2O_2 may protect chloroplast ultrastructure to preserve photosynthesis under abiotic stress. Similarly, to improve plant abiotic stress tolerance, H_2O_2 may modulate the expression of resistance genes and antioxidant enzyme activities during abiotic stress response. During adverse environment caused by low temperature, H_2O_2 in maize seedling was increased up to 48h of treatment however after 72h of period; the formation of this molecule was prevented. As it is well known that hydrogen peroxide is the signaling molecule and triggers the intracellular regulation of genes and other biomolecules. In response to severe oxidative stress caused by low temperature, hydrogen peroxide is synthesized. The previous observations revealed that low temperature causes the enhancement of H_2O_2 (Lee *et al.*, 2004). Therefore, our findings are compatible to their findings.

It is assumed that the increase of protein and PPO level in maize seedlings in response to low temperature might be coupled to nitrification and denitrification processes. As the growth was enhanced after germination of maize seeds, thereby it is reasonable that nitrate content in seedling might be altered. Low temperature may induce the synthesis of nitrate in seedlings although not understood well. Nitrogen is a key element for the biosynthesis of nucleic acids, protein and chlorophyll in plants. Among the various nitrogen sources, nitrate is largely taken up from the soil by plant roots. In plants, nitrate accumulation depends on its absorption and metabolism. Some of the root-absorbed nitrates are assimilated in the roots but most are transported to the shoots and assimilated by nitrate reductase (NR) and other nitrogen metabolic enzymes in plant leaves. The uptake, assimilation and translocation of nitrates in plants are regulated by multiple internal cues (expression of related genes and enzyme activities) and also by external environmental factors. It has been shown that environmental low temperature affects nitrate uptake in plants (Silalahi *et al.*, 2016). Their findings suggest that nitrate accumulation is regulated by the environmental stimuli.

In adverse environment, plant needs nitrogenous sources to synthesize different anti oxidative enzymes and proteins and cellular DNA synthesis. In our findings, higher nitrate accumulation was observed after 24h and 48h of treatment of low temperature and after 72h of period, the accumulation was assumed to be reduced. Therefore, it is expected that nitrate accumulation has been shown up to 48h of period and then degradation of nitrate might be possible. The previous investigation revealed that nitrate accumulation was enhanced in spinach leaves in response to low temperature (Aydin and Nalbantoglu, 2011). Their findings are correlated to the current investigation. Nitrate uptake and degradation is a consecutive process and is affected by environmental stress particularly low temperature. Further studies are needed to clarify the mechanism. Collectively, our overall findings suggest that low temperature causes diverse metabolic alteration in

maize seedlings and hydrogen peroxide may play the critical role in this respect. It is assumed that the biological process enhanced by low temperature is coupled to the internal nitrification and denitrification process in maize seedlings.

Conclusion

The adverse effect of low temperature on plant growth is an important aspect in the current investigation. Low temperature is a major abiotic environmental stimuli causing higher oxidative stress. However, to survive in the adverse environment, plant needs adaptive response.

In this study, low temperature triggers the biosynthesis of protein and up regulation of PPO activity after 24h, 48h and 72h of treatment however, the effects were assumed to be reduced after 72h of treatment. It is assumed that up regulation of genes and accumulation of PPO might be involved to survive in the adverse atmosphere. Since H_2O_2 plays the potent role in signaling of diverse molecules, higher accumulation of H_2O_2 in maize seedling may induce the up regulation of the above molecules and is linked to these biological processes. In this study, nitrate accumulation in response to low temperature for 24h, 48h and 72h periods was investigated. Nitrate is the potent and major stimulator for plant growth. It is observed that nitrate accumulation was potentially enhanced in response to low temperature after 24h and 48h of periods, however after 72h of treatment, the accumulation was assumed to be reduced.

Therefore, it is expected that nitrate accumulation has been shown up to 48h of period and then degradation of nitrate might be possible. Nitrate uptake, assimilation and translocation in plants are regulated by expression of related genes and enzyme activities and also by external environmental factors. Nitrate accumulation and degradation are consecutive process and is affected by environmental stress particularly low temperature plays the critical role in this respect. Our findings suggest that low temperature causes diverse metabolic alteration in maize seedlings and H_2O_2 may play the critical role in this respect. It is assumed that the biological processes enhanced by low temperature are coupled to the internal nitrification and denitrification process in maize seedlings.

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