

## Thiamine-induced nitric oxide improves tolerance to boron toxicity in pepper plants by enhancing antioxidants

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**Abstract:** The role of thiamine (THI)-induced nitric oxide (NO) synthesis in the improvement of tolerance to boron (B) toxicity in pepper (*Capsicum annuum* L.) was studied. A solution of THI (50 or 100 mg L<sup>-1</sup>) was sprayed to the foliage of pepper plants exposed to high B regime (2.0 mM H<sub>3</sub>BO<sub>3</sub>) once a week for 4 weeks. A scavenger of NO (0.1 mM), cPTIO, along with THI was sprayed once a week to ascertain whether or not endogenous NO played a role in the alleviation of B stress in pepper plants by THI. There were significant reductions in plant dry weight, PSII quantum efficiency ( $F_v/F_m$ ), total chlorophyll, leaf calcium (Ca<sup>2+</sup>) and potassium (K<sup>+</sup>) contents as well as leaf water potential by 47.57%, 28.78%, 34.42%, 45.45%, 55.53%, and 471.4%, respectively, but there was a significant increase in the leaf proline, ascorbate (AsA), glutathione (GSH), hydrogen peroxide, malondialdehyde, electron leakage, B levels and NO by 2.6-, 2.3-, 1.9, 3.7-, 5.6-, 2.2-, 3.6-, and 3.0-fold, respectively in B-stressed plants. Both treatments of THI mitigated B-induced oxidative damage and led to a further increase in NO synthesis. The positive effect of THI on plants grown under B stress was fully eliminated by the cPTIO application by lowering leaf NO content. These findings exhibit that NO may function as a downstream signal in THI-induced tolerance to B toxicity in pepper plants by reversing oxidative stress, enhancing the antioxidant defense mechanism and sustaining mineral nutrient homeostasis. Thus, NO and THI both contributed to improved B toxicity tolerance in pepper plants.

**Key words:** Antioxidant system, boron toxicity, nitric oxide, pepper, thiamine

### 1. Introduction

Sweet pepper is commonly grown in dry areas where the soils are enriched with boron (B), so it was chosen as a test crop for the present investigation. It is moderately sensitive to B toxicity (Ayers and Westcot, 1985), so B toxicity can damage it when the concentration of B in soil solution exceeds 1–2 mg L<sup>-1</sup> (Yermiyahu et al., 2008). Since B is needed at very low quantities for plants, it is regarded as a micronutrient (Fang et al., 2016). Conversely, its levels are reasonably high in arid and semiarid areas wherein precipitation and irrigation practices are not adequate (Pardossi et al., 2015). Furthermore, use of water containing high B for irrigation purpose is another possible cause of B toxicity in plants (Wakeel et al., 2018). Boron deficiency is often easy to counteract by using fertilizers enriched with B, but boron toxicity seems to be a relatively more difficult issue because the harmful effects of high B levels on plants, especially in areas where annual rainfall is less than 550 mm, cannot be reversed (McDonald et al., 2010; Wang et al., 2014). Boron toxicity causes some of the characteristic symptoms on plants, such as restriction of

plant growth and induction of chlorosis due to inhibition of chlorophyll synthesis (Nali et al., 2015). It also leads to deprivation of lipids, increased membrane leakage, reduced photosynthesis, and impaired regulation of oxidative defense mechanisms (Mesquita et al., 2016; Shah et al., 2017; Kaya et al., 2018a).

Boron toxicity could be relieved by using bio-promoters such as thiamine (THI) (Yusof et al., 2015). Thiamine is involved in all living beings since it participates in a number of vital events including the synthesis of carbohydrates, ATP, NADPH, and nucleic acids (Nosaka 2006). Thiamine is believed to activate defense systems in plants (Bettendorf and Wins, 2013; Yusof et al., 2015) and it improves tolerance to DNA damage induced by stress factors (Tunc-Ozdemir et al., 2009). Furthermore, thiamine was reported to efficiently allay the harmful effects of water stress and saline stress in maize (Rapala-Kozik et al., 2008), reduce oxidative stress induced by low temperature, high light intensity, and osmotic and salt stresses in *Arabidopsis* (Tunc-Ozdemir et al., 2009). In the former investigations, thiamine has been proved to be a

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potential biochemical for improving tolerance to stress in plants, so it is proposed that the continuous synthesis of thiamine may improve severe stress tolerance in plants (Ribeiro et al., 2005; Abidin et al., 2016). Although thiamine has a crucial function in alleviating deleterious effects on plants under a stress as mentioned earlier, there appears to be a rare investigation concerning its effects against boron toxicity, so it logically justifies that thiamine should be examined in boron-stressed plants.

Nitric oxide (NO) is reported to control different metabolic processes such as stomatal regulation, photomorphogenesis, leaf senescence, plant resistance, pollination, blossoming, etc. (Misra et al., 2011; Simontacchi et al., 2015; Fancy et al., 2017). NO also contributes to tolerance mechanisms in plants exposed to exceptionally unfavorable environments (Ahmad et al., 2016; Tripathi et al., 2017). As an example, it maintains reactive oxygen species (ROS) at low levels through changing the activities of key antioxidant enzymes (Niu and Guo, 2012; Groß et al., 2013; Ahmad et al., 2016). Various investigations have shown that NO is implicated in several hormonal and biological responses in plants (Hasanuzzaman et al., 2012; Corpas and Palma, 2018), but the likely contribution of NO to thiamine-induced antioxidant defense needs to be elucidated. Thus, this study aimed to assess if thiamine-induced synthesis of NO was implicated in alleviation of B stress in pepper plants.

## 2. Material and methods

### 2.1. Plant growth and treatments

Greenhouse experimentation was set up with pepper (*Capsicum annuum* L.) cv. "Semerkand". Before designing the study, the seeds of pepper were sterilized with NaOCl (1%) solution. For germination purpose, 5 seeds were sown in a pot consisting of perlite (5 L in each pot), and then three plants were retained to grow in each pot alone for assessing various traits mentioned below. The plants were irrigated using a nutrient solution (0.5 strength). The composition of NS was outlined in detail by Kaya and Ashraf (2015) and the pH of NS was kept at 5.5. The plants were grown at 11 h light period per day during the entire experimentation period.

The experiment comprised the following treatments: control treatment of nutrient solution only with 0.05 mM B or 2.0 mM (toxic) B. One week after germination, the seedlings were subjected to two B levels. There were two levels of thiamine (THI) (50 or 100 mg L<sup>-1</sup>) in 0.01% Tween-20; the treatment solutions were sprayed to plant leaves once a week for 4 weeks. To gain insight into if NO participated in mitigation of B stress by THI, an NO scavenger (0.1 mM), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (cPTIO) was sprayed once a week for 4 weeks along with

THI application to plants subjected to nonstressful and stressful regimes. The control plants were supplied with an equal volume of solution with no THI. Depending on plant size, 100–1000 mL of 1/2-strength NS was supplied every other day to each pot. The trial consisted of 3 replications, each having 9 plants.

Four weeks after the start of B supplementation, three plants from each replication were divided into shoot and roots and their fresh weights were noted. The leaves (fully expanded) of the remaining six plants of each replicate (e.g., 18 plants per treatment) were used to appraise different traits mentioned below. For appraising dry weights of the plant samples, they were subjected to 72 °C for 3 days.

After quantifying the fresh mass, the roots and shoots of plants were divided and dried at 75 °C for 24 h to quantify dry mass. The plant growth and physio-biochemical traits described below were determined.

### 2.2. Chlorophyll determination

A leaf sample (1.0 g) was homogenized in a 90% acetone solution. The mixture was then filtered and absorbance was measured using a spectrophotometer to quantify chlorophyll levels following the method of Strain and Svec (1966).

### 2.3. Chlorophyll fluorescence measurements

Prior to determining this parameter, the leaf samples were adapted in dark for 30 min and then used to measure the PSII photochemical efficiency ( $F_v/F_m$ ) using Mini-PAM Photosynthesis Yield Analyzer (Walz, Germany).

### 2.4. Leaf water potential

Leaf water potential was quantified by using a recently extended leaf of plant by subjecting it to a water potential measurement apparatus (PMS model 600, USA).

### 2.5. Free proline

Free proline was quantified by employing the ninhydrin protocol outlined by Bates et al. (1973). A fresh leaf tissue (each 500 mg) was macerated in aqueous sulfosalicylic acid (10 mL, 3%) and then the macerated solution was filtrated. An aliquot (2 mL) was treated with 2 mL each of glacial acetic acid and acid ninhydrin. All samples were kept at 80 °C in a water bath for 1 h. Next, to last the reaction, the samples were kept in an ice bath. By adding toluene (4 mL) to the mixture, a mixer was used to mix the solution for 15–20 s. The absorbance readings of all the treated samples were noted at 520 nm.

### 2.6. Determination of ascorbate (AsA)

The method outlined by Mukherjee and Choudhuri (1983) was employed to appraise AsA. Leaves were homogenized in liquid nitrogen followed by in a cocktail containing dinitrophenyl-hydrazine (2%) and trichloroacetic acid (6%) prepared in ½-strength H<sub>2</sub>SO<sub>4</sub> and 10% thiourea dissolved in 70% ethanol. The sample solution was boiled in a water bath for 15 min and then cooled down to 25

°C. After centrifugation at  $1000 \times g$  for 10 min at 4 °C, the resultant pellet was liquefied in  $H_2SO_4$  (80%). The absorbance values were noted at 530 nm.

### 2.7. Glutathione (GSH) assay

The total GSH was quantified employing the method of Ellman (1959). A supernatant of sulfosalicylic acid (3 mL of 4%) was added to 0.5 mL of the extract in phosphate buffer. Following centrifugation of the mixture at  $3000 \times g$ , a 0.5 mL of sample supernatant was reacted with Ellman's reagent. The absorbance values were recorded at 412 nm after an interval of 10 min.

### 2.8. Chemical analysis

Determination of the leaf B was performed following the Azomethine-H method outlined by Wolf (1971). Further details are given in Kaya et al. (2019). For the quantification of leaf  $Ca^{2+}$  and  $K^+$ , ground leaf samples were heated in a muffle furnace at 500 °C for 6 h. A 5 mL of hot HCl (2 M) were added to the resultant ash, and then the mixture volume was completed to 50 mL by adding deionized water. An ICP was used to read the samples for the quantification of  $Ca^{2+}$  and  $K^+$  (Chapman and Pratt, 1982).

### 2.9. Analysis of nitric oxide (NO)

Leaf NO content was quantified following the protocol outlined by Zhou et al. (2005) with slightly modified. Following homogenising fresh leaf material (0.6 g) in 50 mM cold acetic acid buffer (3 mL) at pH 3.6 containing 4% zinc diacetate, the mixture was centrifuged at  $10,000 \times g$  at 4 °C for 15 min. The collected aliquots were mixed followed by adding 100 mg of charcoal to each sample. After the samples were filtered and vortexed, the mixture (1 mL) and the Greiss reagent (1 mL) at room temperature were added. The absorbance readings were noted at 540 nm.

### 2.10. Hydrogen peroxide ( $H_2O_2$ )

The method outlined by Velikova et al. (2000) was followed to appraise leaf  $H_2O_2$  content. A fresh leaf material (each 500 mg) was extracted in 3 mL of TCA (1% w/v). After centrifuging each sample, an aliquot of 0.75 mL of the sample was added to 0.75 mL of K buffer (10 mM) and 1.5 mL of KI (1 M). The absorbance readings were recorded at 390 nm.

### 2.11. Leaf malondialdehyde (MDA)

The method depicted by Weisany et al. (2012) was employed to quantify leaf MDA, a lipid peroxidation product.

### 2.12. Electrolyte leakage (EL)

The EL was appraised by employing the detailed procedure outlined by Dionisio-Sese and Tobita (1998). Detailed information on the procedure used can be found in Kaya and Ashraf (2015).

### 2.13. Leaf soluble proteins

Leaf soluble protein was appraised adopting the procedure reported by Bradford (1976). Fresh leaf material (each 500 mg) was homogenised in the solution of phosphate buffer (0.2 mM) at pH 6.2. The homogenized samples were then centrifugated at  $2000 \times g$  for 10 min. The volume of the aliquot was brought up to 10 mL by adding the phosphate buffer solution. A supernatant of the Coomassie Brilliant blue reagent (5 mL) was added to the solution of sample (1 mL). It was then shaken in a vortex mixer for 30 s. The absorbance values were noted at 595 nm.

### 2.14. Activities of antioxidant enzymes

A proportion of fresh leaf (500 mg) was homogenized in Na-P buffer (50 mM) containing soluble polyvinyl pyrrolidone (1%). The solution so extracted was centrifuged at 4 °C at  $20,000 \times g$  for 15 min. The CAT activity was appraised following Kraus and Fletcher (1994), the SOD activity by Van Rossum et al. (1997), and that of POD by Chance and Maehly (1955).

### 2.15. Statistical analysis

The data for various traits were subjected to a two-way analysis of variance using the statistical package, CoStat program (Version v6.303). The Duncan's Multiple Range test at 5% probability was adopted to assess if the means varied significantly from one another.

## 3. Results

When pepper plants were exposed to B toxicity (BT), substantial decreases were found in dry mass, chlorophyll contents,  $Fv/Fm$  and  $\Psi l$  of the plants ( $P \leq 0.05$ ) by 47.6%, 34.4%, 28.8%, and 471.4%, respectively in comparison with those in the nonstressed plants (Table; Figures 1A–1D). Treatments of THI, 50 and 100 mg  $L^{-1}$ , considerably ( $P \leq 0.05$ ) improved total dry weight by 50.1% and 57.0%, chlorophyll content by 52.5% and 56.3%,  $Fv/Fm$  by 21.4% and 33.9%,  $\Psi l$  by 21.7% and 33.0%, respectively in comparison with those in BT plants.

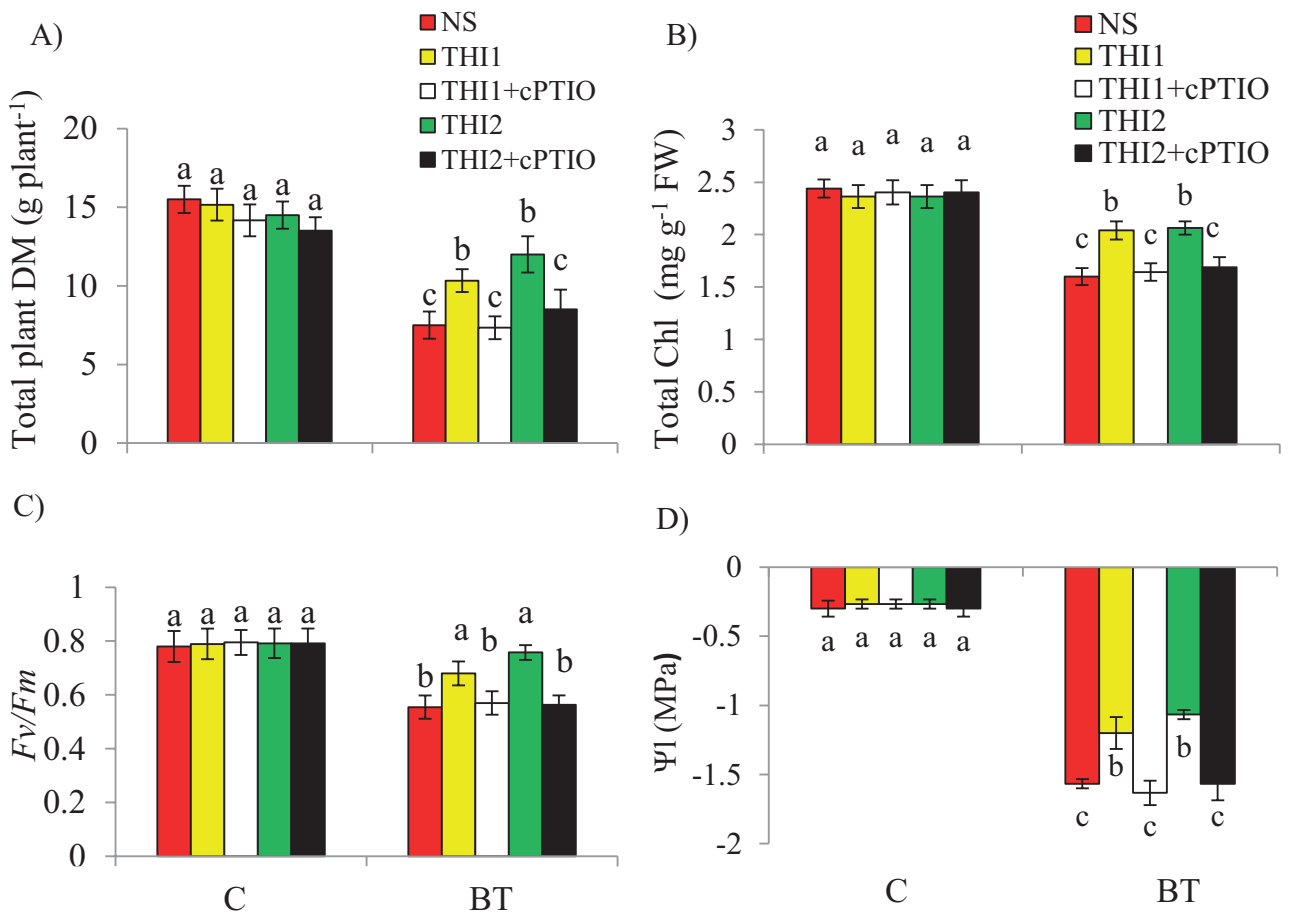
To understand the possible involvement of endogenous NO in THI-improved B toxicity stress tolerance in pepper plants, cPTIO, a scavenger of NO, was sprayed together with THI to pepper plants under BT stress. The use of cPTIO plus THI completely overturned the benefit effects of THI on the traits mentioned above. These findings obviously provide a strong evidence that NO was participated in THI-improved BT tolerance in pepper plants.

Leaf potassium ( $K^+$ ) and calcium ( $Ca^{2+}$ ) levels considerably ( $P \leq 0.05$ ) decreased (Figures 2A and 2B), but boron (B) content (Table; Figure 2C) increased in the boron-stressed plants. However, THI supply significantly ( $P \leq 0.05$ ) increased leaf  $K^+$  and  $Ca^{2+}$  contents, but reduced leaf B in the boron-stressed plants. When cPTIO along with THI was applied to the BT-stressed plants, the positive effects of THI applications on these parameters were reversed, and this suggests that lowering NO in the

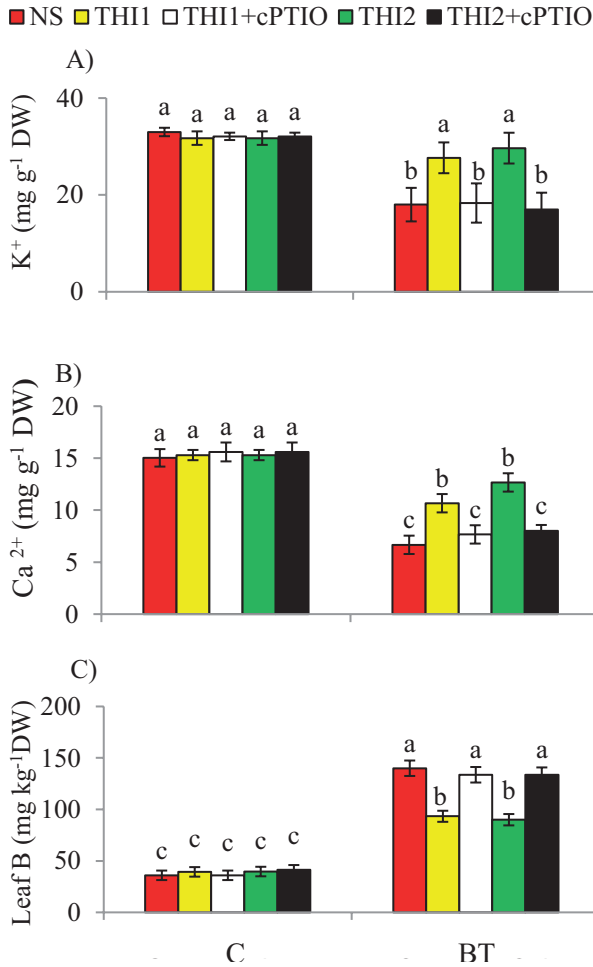
**Table.** Two-way completely randomized analyses of variance (ANOVA) of the data (mean squares) for growth and different biochemical attributes of pepper plants.

Source of variations	df	Plant dry weight	Total Chl.	$F_v/F_m$	Leaf water potential	Leaf $K^+$	Leaf Ca
BS	1	221***	2.54***	0.203***	9.52***	750***	295***
T	4	7.23ns	0.57ns	0.013ns	0.106**	48.4ns	9.12**
BS x T	4	6.63ns	0.98*	0.012ns	0.090*	3.07*	9.31**
Error	20	2.708	0.28	0.007	0.014	7.947	1.89
		<b>Leaf B</b>	<b>Proline</b>	<b>AsA</b>	<b>GSH</b>	<b>H<sub>2</sub>O<sub>2</sub></b>	<b>MDA</b>
BS	1	47600***	395***	64.9***	4.3***	6077***	3141***
T	4	818***	7.0ns	9.7**	0.28ns	198.2**	172***
BS x T	4	973***	6.8ns	14.3***	0.30ns	165.4**	110**
Error	20	99.0	4.2	1.57	0.12	34.0	20.0

BS: Boron stress; T: Treatments; ns = nonsignificant; \*, \*\* and \*\*\* = significant at 0.05, 0.01, and 0.001 levels, respectively.



**Figure 1.** Total plant dry matter [DM; A)], total chlorophyll on fresh weigh (FW) basis (B), maximum fluorescence yield [ $F_v/F_m$ ; C)] and leaf water potential [ $\Psi_l$ ; D)] in the leaves of pepper plants grown under control (C) and boron toxicity (BT) sprayed with thiamine (THI1: 50 and THI2: 100 mg L<sup>-1</sup>) combined with 0.1 mM scavenger of NO, cPTIO, or nonsprayed (NS). (Mean  $\pm$  SE). Mean values carrying different letters within each parameter differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.

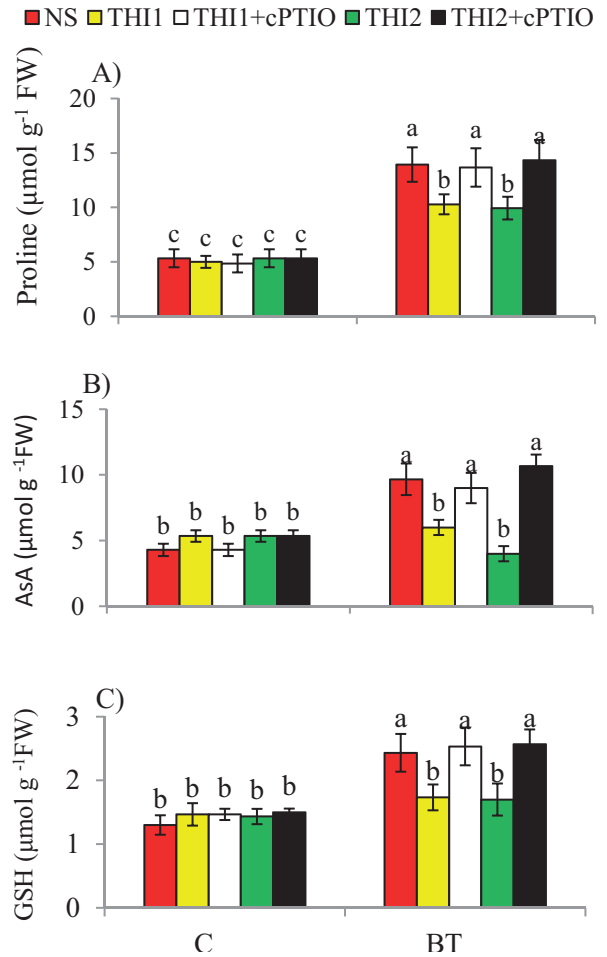


**Figure 2.** Leaf potassium [K<sup>+</sup>; A), calcium [Ca<sup>2+</sup>; B) and boron [B; C) contents on dry weight (DW) basis in the leaves of pepper plants grown under control (C) and boron toxicity (BT) sprayed with thiamine (THI1: 50 and THI2: 100 mg L<sup>-1</sup>) combined with 0.1 mM scavenger of NO, cPTIO, or nonsprayed (NS). (Mean ± SE). Mean values carrying different letters within each parameter differ significantly (P ≤ 0.05) based on Duncan's multiple range test.

leaves of plants possibly caused THI applications being not effective in increasing leaf K<sup>+</sup> and Ca<sup>2+</sup> contents and decreasing leaf B in the plants subjected BT stress.

Leaf proline content, ascorbate (AsA), and glutathione (GSH) increased significantly (P ≤ 0.05) under BT stress, but they all were reduced due to THI supply (Table; Figures 3A–3C). When BT-stressed plants were sprayed along with cPTIO, the effects of THI applications on these traits were reversed, suggesting that when NO was possibly blocked, THI applications were not effective in reducing these traits in the plants under BT stress.

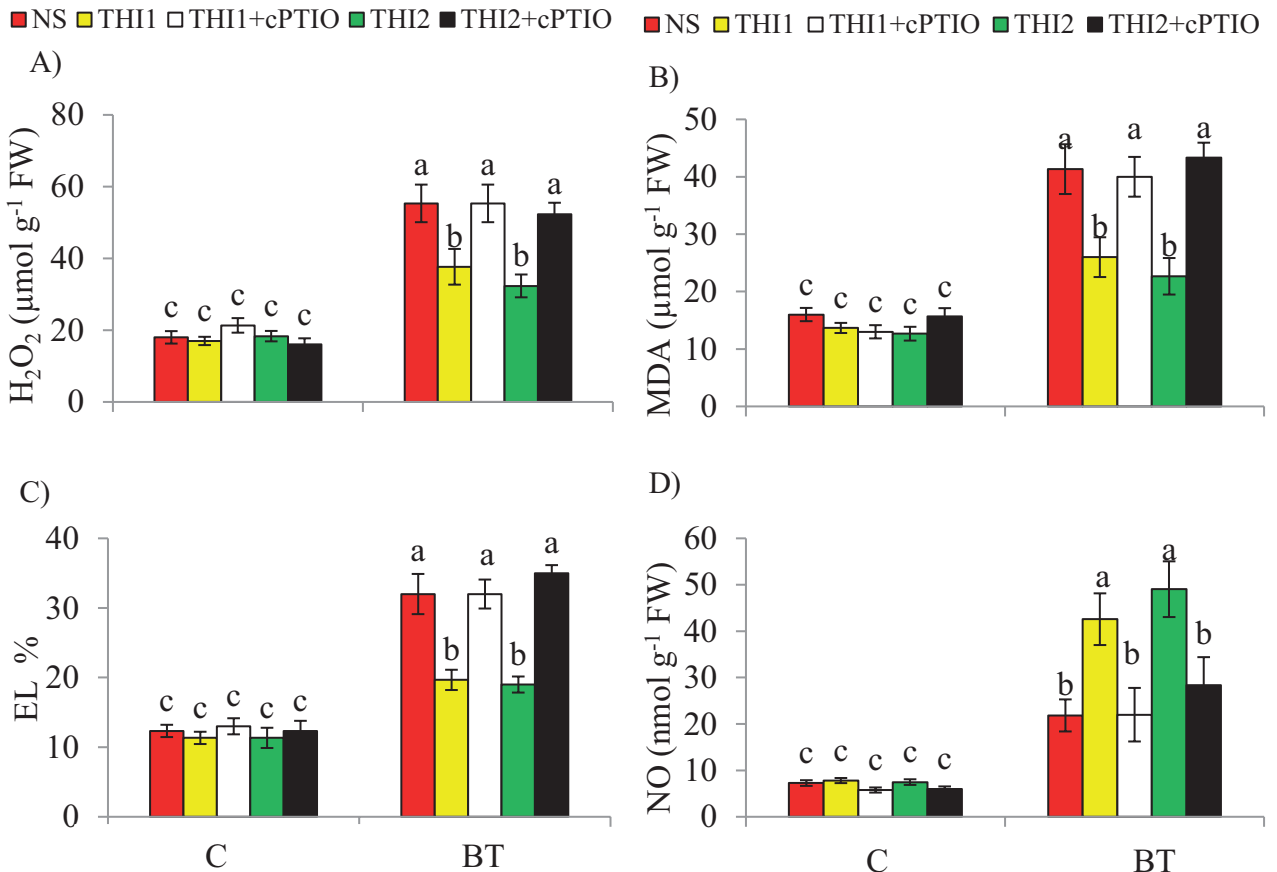
The overgeneration of hydrogen peroxide and malondialdehyde as well as electrolyte leakage were



**Figure 3.** Leaf proline (A), ascorbate [AsA; (B) and glutathione content [GSH; (C) on fresh weight (FW) basis in the leaves of pepper plants grown under control (C) and boron toxicity (BT) sprayed with thiamine (THI1: 50 and THI2: 100 mg L<sup>-1</sup>) combined with 0.1 mM scavenger of NO, cPTIO, or nonsprayed (NS). (Mean ± SE). Mean values carrying different letters within each parameter differ significantly (P ≤ 0.05) based on Duncan's multiple range test.

significantly augmented in pepper plants under BT stress (Figures 4A–4C). Both THI treatments relieved BT-induced the oxidative stress by significantly reducing oxidative stress parameters, but supplementation of cPTIO completely reversed the alleviation effects of the THI treatments. When endogenous NO was blocked by using cPTIO, THI alone was not effective in reducing the oxidative stress. Thus, these findings further reveal that NO and THI might jointly play a vital role in improving BT stress tolerance of pepper plants due to THI application.

Boron stress considerably (P ≤ 0.05) elevated the endogenous NO in the leaves of pepper plants (Figure 4D). The treatments of THI, 50 and 100 mg L<sup>-1</sup>, led to further



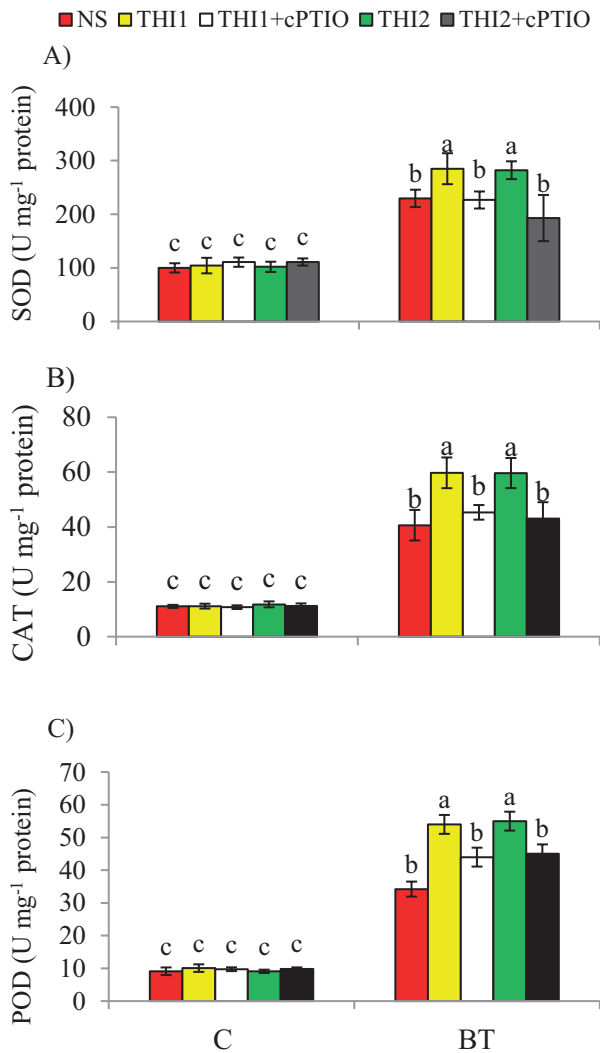
**Figure 4.** Hydrogen peroxide [ $H_2O_2$ ; A)], malondialdehyde [MDA; B)], electrolyte leakage [EL; C)] and nitric oxide [NO; D)] on fresh weight (FW) basis in the leaves of pepper grown under control (C) and boron toxicity (BT) sprayed with thiamine (THI1: 50 and THI2: 100 mg L<sup>-1</sup>) combined with 0.1 mM scavenger of NO, cPTIO, or nonsprayed (NS). (Mean  $\pm$  SE). Mean values carrying different letters within each parameter differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.

increases significantly ( $P \leq 0.05$ ) in this metabolite in the leaves of pepper plants under BT condition relative to those in BT-stressed plants alone. The significant increases in endogenous NO induced by THI treatments were reversed totally with the supply of cPTIO. These results indicate that THI triggers NO in the leaves and so NO may be a downstream signal produced by THI.

Antioxidant defense system which might be induced by THI in the BT-stressed plants was investigated and it was also proposed to get an insight into whether THI was involved in endogenous NO-triggered antioxidant system under BT stress. The results clearly indicate that BT stress considerably ( $P \leq 0.05$ ) augmented the activities of antioxidant enzyme (SOD, POD, and CAT) (Figures 5A–5C). The treatments of THI led to additional elevations in the activities of all these enzymes. However, combining cPTIO with THI completely reduced these enzyme activities. These results reveal that both endogenous NO and THI are jointly responsible in regulating the antioxidant defense system in pepper plants under BT stress.

#### 4. Discussion

The results of many investigations show that although B is needed in a small amount for higher plants, it can be toxic at high concentration in a growing medium (Kot 2008; Kayihan et al., 2017). An obvious decline in plant biomass under BT could be due to the harmful effects of B toxicity on physiological processes and mineral acquisition (Metwally et al., 2018). The possible reason of increased plant growth by the THI in the present experimentation might have been that THI is needed for division of meristematic stem cells and organ primary cells (Martinis et al., 2016). Furthermore, the accumulation of some osmo-regulators, e.g., free soluble sugars and amino acids in the THI-treated plants can enhance the water status of plants thereby raising the turgor pressure, required for cell expansion and plant growth (Sayed and Gadallah, 2002). Thiamine has been tested in various stress situations, e.g., under salinity and water stress in *Arabidopsis* (Rapala-Kozik et al., 2012), under salt stress in maize (Kaya et al., 2015), under drought stress in white clover (Ghaffar et al.,



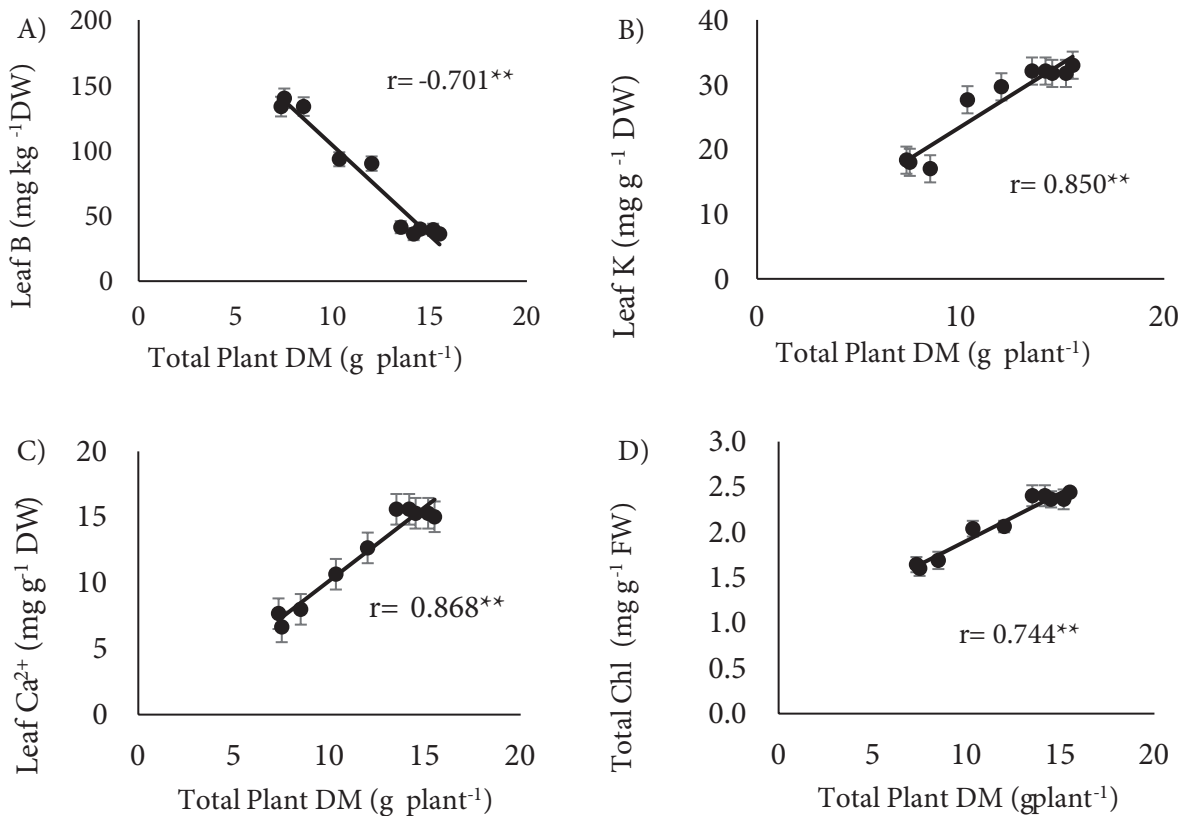
**Figure 5.** Activities of superoxide dismutase [SOD; A)], catalase [CAT; B)], and peroxidase [POD; C)] in the leaves of pepper plants grown under control (C) and boron toxicity (BT) sprayed with thiamine (THI1: 50 and THI2: 100 mg L<sup>-1</sup>) combined with 0.1 mM scavenger of NO, cPTIO, or nonsprayed (NS). (Mean ± SE). Mean values carrying different letters within each parameter differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.

2019), and under osmotic, oxidative and salt stresses in oil palm (Abidin et al., 2016). However, a rare report seems to be available in the literature on the effect of thiamine tested on the B-stressed plants. Thus, one of the priorities of our investigation was to assess its possible positive effects on plants under B toxicity stress. The levels of THI (50 or 100 mg L<sup>-1</sup>) chosen for foliar spray to maize plants were already used for the same crop in a previous study (Kaya et al., 2015). The allaying effect of THI on BT-induced suppression in plant growth is likely due to reduced leaf B content, and enhanced K<sup>+</sup> and Ca<sup>2+</sup> contents, chlorophyll

content and *Fv/Fm*, which were otherwise suppressed by BT stress. In the present investigation, plant dry matter was nonlinearly correlated ( $P \leq 0.001$ ) with leaf B content ( $r = -0.701$ ) (Figure 6A), but linearly correlated ( $P \leq 0.001$ ) with leaf K<sup>+</sup> and Ca<sup>2+</sup> content, and chlorophyll content ( $r = 0.850$ ,  $r = 0.868$ , and  $r = 0.744$ , respectively) (Figures 6B–6D). These results showed that THI might have participated in the response of the pepper plants to BT, as already proposed by Kaya et al. (2015) in salinity-stressed maize plants.

Although NO acts as a signaling molecule in the response of plants to a variety of stressful environments (Singh et al., 2008; Panda et al., 2011), there is no sufficient information in the literature on the interaction between NO metabolism and BT-induced stress. An elevated NO content in pepper plants under BT stress was recorded, as previously shown for other plants subjected to growth conditions supplemented with various heavy metals (Yuan and Huang, 2016; Farag et al., 2017). Therefore, these findings propose that NO could effectively participate in some key metabolic phenomena in plants subjected to boron toxicity. Moreover, THI treatments caused a further elevation in endogenous NO synthesis in the plants under BT stress. Thus, it is soundly promising that the treatment of THI may induce endogenous NO synthesis, which may be involved in improving stress tolerance of plants as an antioxidant. However, it is believed that oversynthesis of NO can cause damaging effects in plants (da-Silva et al., 2018). Therefore, an optimum level of cellular NO is needed for mitigating the deleterious effects of a stress. In the present experiment, the levels of endogenous NO induced by THI treatments were not higher than the threshold level for pepper plants; thus, its destructive effects on physiological processes could be expected. There seems to be no report relating to the interaction of THI with NO in the response of plants to a stress in the available literature. Thus, this study provides a novel insight into our understanding in this respect. Nitric oxide could be a downstream signal molecule induced by THI in enhancing tolerance to boron toxicity in pepper plants. A similar suggestion was put forth by Liu et al. (2015) who proposed that NO might be a downstream signal of melatonin in the improvement of tomato tolerance to alkaline conditions. On the other hand, when NO synthesis was scavenged by treating plants with cPTIO, the alleviation effects of THI were reverted thereby resulting in reduced leaf NO content.

Environmental stress is widely believed to deleteriously affect chlorophyll synthesis (Kalaji et al. 2016), but during the mechanism of photosynthesis, the chlorophylls are required in a large amount to absorb a considerable amount of light to expedite the shifting of electrons to the reaction center. Similarly, *Fv/Fm* is known as an important indicator of assessing photo-oxidative outcome



**Figure 6.** Correlation of total plant dry mater (DM) with leaf B (A), potassium [K<sup>+</sup>; B]) and calcium [Ca<sup>2+</sup>; C]) contents on dry weight (DW) basis, and total chlorophyll (D) content on fresh weight (FW) basis in the leaves of pepper plants under boron toxicity, supplied with thiamine and sprayed with or without 0.1 mM scavenger of NO, cPTIO, \*\*: Correlation is significant ( $P \leq 0.001$ )

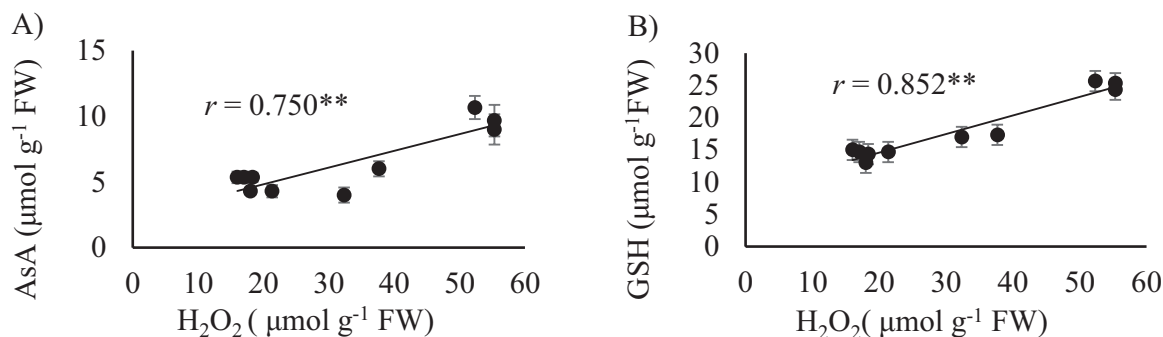
of photosystem II (Wang et al., 2013). In our study, the treatments of THI enhanced chlorophyll levels and *Fv/Fm* of pepper plants under BT stress (Figures 1B and 1C). In an earlier study, Soltani et al. (2014) noted that exogenously supplied THI improved the chlorophyll contents in marigold plant. Thiamine is known to scavenge reactive oxygen species, thereby improving the chlorophyll content (Mady, 2009; Kaya et al., 2018b). The present results additionally show that the overproduction of H<sub>2</sub>O<sub>2</sub> within the leaves of pepper plants subjected to BT could be connected to decreased chlorophyll level. Thus, it can be inferred that THI is efficiently involved in alleviating the injurious effect of BT on chlorophyll synthesis, likely by reducing the production of H<sub>2</sub>O<sub>2</sub>. An alternative reason for improvement of tolerance of pepper plants to BT could be because of the role of THI in improving the antioxidant defense mechanism to eliminate H<sub>2</sub>O<sub>2</sub>, thereby elevating the levels of chlorophyll. This statement was similarly verified in our study where THI applications further improved SOD, CAT, and POD, and chlorophyll levels. In view of the available literature, it is likely that thiamine has a defensive effect on the D1 protein of PSII; therefore, it

might have resulted in increased PSII activity in pepper plants under BT as previously reported in wheat treated with ascorbic acid (Athar et al., 2008).

It has been reported that BT can disturb water uptake by affecting the water flow (Wimmer and Eichert, 2013). Severe deterioration of the xylem vessels can decrease the flow of water to plant shoots (Wimmer and Eichert, 2013). An explanation of greater leaf water potential ( $\Psi_l$ ) by THI treatment might have been that application of thiamine lowered the osmotic stress by accumulating proline, thereby enhancing leaf water potential of the pepper plants under BT stress, as shown in the present experiment. Similar ameliorating effects of THI treatment on leaf water and proline contents have already been reported in sunflower (Sayed and Gadallah, 2002), and maize (Tuna et al., 2013) under saline stress.

It has been well documented that ascorbate (AsA) and glutathione (GSH) are the nonenzymatic substances which scavenge the ROS including H<sub>2</sub>O<sub>2</sub> to mitigate oxidative stress induced by boron stress (Metwally et al., 2018). Similarly, the results of the present study clearly show that the leaf AsA and GSH levels were increased in the plants





**Figure 7.** Correlation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) with ascorbate [AsA; (A)] and glutathione [GSH; (B)] on fresh weight (FW) basis of pepper plants under boron toxicity supplied with thiamine and sprayed with or without 0.1 mM scavenger of NO, cPTIO, \*\* and \*: Correlation is significant at ( $P \leq 0.001$ ) and ( $P \leq 0.05$ ), respectively.

under BT stress, wherein H<sub>2</sub>O<sub>2</sub> content was also increased. The correlation coefficients indicate that leaf H<sub>2</sub>O<sub>2</sub> content is significantly and linearly correlated with ASA and GSH (Figures 7A and 7B). When pepper plants were exposed to BT stress, they also produced ASA and GSH to ameliorate B-induced oxidative stress.

Boron stress has been reported to decrease Ca<sup>2+</sup> contents in apple (Mouhtaridou et al., 2004) and K<sup>+</sup> and Ca<sup>2+</sup> contents in sweet pepper (Piñero et al., 2017) as similarly shown in our experiment. This could have been due to the decrease in the uptake of nutrients mediated by reduced transpiration (Piñero et al., 2017). THI-induced improvement in tolerance to B toxicity of the pepper plants could be related to decreased B levels and increased leaf K<sup>+</sup> and Ca<sup>2+</sup>. In the present study, it could be inferred that THI treatments enhanced the nutrient concentrations by improving plant growth, thereby improving nutrients' uptake from the growing medium.

Boron toxicity caused significant increases in H<sub>2</sub>O<sub>2</sub>, MDA, and EL in the pepper plants. The present investigation proposes that B toxicity led to an injurious effect on membrane integrity; thus, membrane damage was increased. Oxidative impairment and membrane peroxidation were the most usual symptoms appearing in plants under B stress (Wang et al., 2010; Pandey, 2013). Earlier studies have shown that B stress triggers H<sub>2</sub>O<sub>2</sub> and superoxide radicals in tomato plants (Cervilla et al., 2009). These alterations might disturb consistent ion exchange capability of plasma membrane and the whole metabolic events linked with cell membrane functions (Wang et al., 2016; Moussa and Algamal, 2017). The redox potential of the cell can be impaired by over-accumulation of H<sub>2</sub>O<sub>2</sub> and this leads to the elevations of antioxidants and the reinforcement of antioxidant defense system (Hassanein et al., 2015). However, THI treatments significantly mitigated BT-induced oxidative impairments by lowering H<sub>2</sub>O<sub>2</sub> accumulation, MDA levels, and EL.

Plants evolve a strategy to improve their ability to protect plant cells against the damage and dysfunctions caused by heavy metal contaminated soil by triggering antioxidant enzyme activities including SOD, CAT, and POD to scavenge H<sub>2</sub>O<sub>2</sub> (Sharma and Dubey, 2005). Furthermore, in the present experiment, THI enhanced the antioxidant defense system in the pepper plants under B toxicity which was evident by lowered H<sub>2</sub>O<sub>2</sub> generation and lipid peroxidation, and improved chlorophyll content and antioxidant defense system. Blocking the NO synthesis induced by THI caused THI to be ineffective in plants grown under B toxicity. This further proves that the alleviating effect of THI on oxidative damage caused by BT stress is NO-dependent.

## 5. Conclusion

Overall, thiamine-triggered nitric oxide was found to participate in improving tolerance to B toxicity in pepper plants. The results suggest that NO could be a downstream signaling molecule induced by THI in pepper plants under B toxicity. When NO was scavenged using cPTIO, THI alone was not effective in improving tolerance to B toxicity. Thus, externally applied THI and endogenous NO are both jointly responsible for improved tolerance to B toxicity in pepper plants. Furthermore, endogenous NO induced by THI might be a key metabolite which improves antioxidant defense system to reduce oxidative damage in pepper plants under B toxicity. On the other hand, we need to further investigate how far the molecular network works in the case of THI, which increases tolerance of plants to B toxicity. Additional studies are still needed to investigate the role of other signaling molecules in response of plants to BT stress induced by THI.

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