Benzoate, Gallate, And Salicylate Regulate Redox-Enzymes, Ultrastructure, And Accumulation of Boron To Counteract Boron Toxicity On Tomato Callus Cells

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Abstract

The participation of benzoate (BA), gallate (GA), and salicylate (SA) in various biochemical and physiological processes in plants under conditions of excessive boron (EB) is largely unknown to date. Here, the relationships between phenolic acids (PAs) and the regulation of redox-enzymes, the ultrastructure of cells, and boron forms in the mitigation of EB-induced oxidative stress within tomato calli were studied. Tomato calli were exposed to 2 mM boron (B) in the presence or absence of three concentrations of benzoate, gallate, and salicylate. The data showed that different concentrations of PA counteracted the inhibition of growth and oxidative stress of EB stress by reducing hydrogen peroxide ($H_2O_2$) production, lipoxygenase (LOX) activity, boron accumulation forms, cell wall thickening, and moderate concentrations were the most effective. Applications of PAs reduced the catalytic impacts of EB on superoxide dismutase (SOD) and catalase (CAT) activity. Likewise, benzoate and gallate increased the influences of EB stimulation on peroxidase (POD) and ascorbate peroxidase (APX) activities; whereas, SA reduced these effects on both enzymes. PA treatments enhanced the insignificant catalytic effect of EB on the activity of phenylalanine ammonia-lyase (PAL), as well as the stimulation of the negative influence of EB on polyphenol oxidase (PPO) activity. The findings highlight that PAs play an important role in alleviating EB stress in tomato plants by regulating redox enzymes, B-accumulation forms, and cell wall thickening. This study provides new perspectives for strategies related to excess boron tolerance in tomato plants and thus can be used as plant growth promoters.

Introduction

Boron is known as a micronutrient and is essential in relation to vascular plants, which have important physiological and representational roles and thus regulate crop productivity (Landi et al. 2019). Climate change leads to a decrease in precipitation and a rise in sea levels in many parts of the world, resulting in increased levels of boron (B) in irrigation water, which has an impact on plant growth (Cervilla et al. 2012; Princi et al. 2016). B is toxic for plants when present in excessive amounts. After exposure to (a)biotic stress such as excess boron (EB), certain ion channels and kinase cascades are activated (Fraire-Velázquez et al. 2011), reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) (Laloi et al. 2004), reprogramming of genes that perform to appropriate defensive actions and increase plant resistance to reduce damage due to stressor (Fujita et al. 2006). Among the compounds participate in the development of adaptive actions, there are important roles played by components of the lipoxygenase (LOX) pathway that possess the key signaling system properties (Rejeb et al. 2014). Recently, in tomatoes, accumulation of B, $H_2O_2$, lipid peroxidation in tissues resulting from EB has been stated (Kaya et al. 2020; Farghaly et al. 2021).

Plants have a complex defensive response strategy, which can appear either primarily or after a stress challenge. The plant's constitutive defense mechanism, after stress recognition, triggers a complex defense signaling chain that varies from one stress to another (Rejeb et al. 2014). Plants constitutively cope with oxidative stress through internal defense strategies consisting of various enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX),
peroxidase (POD), and non-enzymatic antioxidants (Hasanuzzaman et al. 2020). Under EB, Kaya et al. (2020) reported an increase in CAT, SOD, and POD activity within tomato plants. However, other studies have demonstrated that APX activity in lettuce (Eraslan et al. 2007) and CAT in citrus leaves (Han et al. 2009) decreased or was insensitive in tomatoes to some EB doses (Cervilla et al. 2007). In plants, phenolics are also important secondary metabolites that serve as effective antioxidants (Hossain et al. 2009). Phenylalanine ammonia-lyase (PAL) is the main enzyme in phenolate synthesis, and EB has been shown to enhance PAL activity in grape cultivars (Sarabandi et al. 2019). Furthermore, Sofo et al. (2005) concluded that under abiotic stress, a decrease in polyphenol oxidase (PPO, having catechol oxidase activity) was related to the enhancement of the antioxidant ability and phenolics accumulation.

Phenolics have been harnessed in many applications such as plant growth promotion, antioxidant, allelochemical and bioremediation (Bujor et al. 2015). Benzoate (BA), gallate (GA), and salicylate (SA) are phenolic compounds involved in the regulation of growth and development of plants, and their responses to (a)biotic stress factors (Khan et al. 2015; Farghaly et al. 2021). Senaratna et al. (2003) stated that BA is known to provide abiotic stressor tolerance similar to that reported for SA. BA has been proven to provide resistance against extreme-temperature (Senaratna et al. 2003), drought (Anjum et al. 2013), heavy metals (Pan-pan et al., 2020), and excess boron (Farghaly et al. 2021). In plants, gallate's participation in defense against abiotic stress is not yet largely known, and it is only known to reduce the effect of ozone stress (Rudolphi-Skórska and Sieprawska 2016), alleviate cold stress (Ozdan-Konakci et al. 2019), increase tolerance against Cd and Cu stress (Ozdan-Konakci and Kabakci 2020; Yetişsina and Kurtb 2020), and relief of EB stress (Farghaly et al. 2021). SA plays a critical role in controlling many physiological activities and plant tolerance to various environmental stresses (Khan et al. 2019). Additionally, SA regulates ROS production by enhancing antioxidant systems (Radi et al. 2014; Farghaly et al. 2021).

Tomatoes are among the most cultivated vegetable crops worldwide (Srividya et al. 2014) and a rich source of vitamins, minerals (USDA 2016) and antioxidants (Di Masico et al. 1989). They are considered one of the most prominent vegetable crops in Egypt, they are cultivated throughout the year. The incidence of B toxicity in tomato cultivation fields has been reported in many countries from dry areas of the world, including Egypt (Landi et al. 2012). The effects of benzoate and gallate applications on redox enzymes, B-accumulation forms, and cell wall thickening were not reported in tomatoes under excess boron conditions, while SA is an active hormone for mitigating boron excess stress on antioxidant enzymes (Radi et al. 2014). This investigation provides new insights into the coordinating effects of redox enzymes induced by BA, GA, or SA and reducing forms of B accumulation, cell wall thickening to improve resistance against oxidative stress induced by EB in tomato calli.

**Materials And Methods**

**Plant tissue culture**
The *in vitro* experiment was performed with the cultivar "Castle Rock" of tomato (*Solanum lycopersicum* L.). Seeds were disinfected with 5% NaClO prior to germination with half strength of MS (Murashige and Skoog 1962). Seedlings were grown (10-12 days) under growth chamber conditions (16/8 light period, temperature 25 ± 1 °C). The explants (1.0 cm of the hypocotyl) were grown in a pre-prepared MS medium and incubated in a growth chamber for a month (Farghaly et al. 2021).

The medium included MS (4.4 g L\(^{-1}\)), sucrose (30 g L\(^{-1}\)), a-naphthalene-acetic acid (0.1 mg L\(^{-1}\)), and 6-benzyl-amino-purine (1 mg L\(^{-1}\)), 4 various treatments were as follows:

1. The control set was MS-nutrient medium alone (control) and 2 mM boric acid on the MS-nutrient medium
2. The BA group was: 0.1 μM BA + 0 mM B, 1 μM BA + 0 mM B, 10 μM BA + 0 mM B, and 0.1 μM BA + 2 mM B, 1 μM BA + 2 mM B, 10 μM BA + 2 mM B
3. The GA group was: 1 μM GA + 0 mM B, 10 μM GA + 0 mM B, 20 μM GA + 0 mM B, and 1 μM GA + 2 mM B, 10 μM GA + 2 mM B, 20 μM GA + 2 mM B
4. The SA group was: 5 μM SA + 0 mM B, 50 μM SA + 0 mM B, 100 μM SA + 0 mM B, and 5 μM SA + 2 mM B, 50 μM SA + 2 mM B, 100 μM SA + 2 mM B

Calli were harvested after a month, rinsed with sterile distilled water (dw), sucked with filter paper, for fresh weight (FW) assessment (FW), oven-dried for dry weight (DW) assessment, and the other calli were frozen at -80°C.

**Hydrogen peroxide**

The \( \text{H}_2\text{O}_2 \) content in the callus was assessed by the Velikova et al. (2000) method. After the callus sample was homogenized in 0.1% (w/v) trichloroacetic acid (TCA), the mixture was properly centrifuged, the filtrate (0.5 mL) was treated with 0.5 mL of 0.1 M K phosphate buffer (PPB; pH 7.0) and 1 mL of 1 M KI then after 20 min, the absorbance was assessed at 390 nm and expressed in mg g\(^{-1}\) fresh weight (FW).

**Enzyme extraction**

Frozen samples (0.5 g of callus tissue) were ground in 5 mL of 100 mM PPB (pH 7.8) comprising 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 g polyvinylpyrrolidone and then properly centrifuged. Lowry et al. (1951) technique was used to quantify the soluble protein in the supernatants. The activities of the examined enzymes were calculated based on the difference in absorption wavelength (nm) per mg protein per min.

**Lipoxygenase (EC 1.13.11.12)**

The Minguez-Mosquera et al. (1993) technique was followed to assess LOX activity. To dw (5 mL), Tween-20 (5 μL), and linoleic acid (35 μL) were mixed and NaOH (0.2 M) was added until the mixture was
clear and the pH was adjusted to 9.0, then the mixture was completed to 100 mL with PPB (0.1 M) and
the pH was adjusted to 6.5. To the previous mixture (2.95 mL) enzyme aliquots (50 μL) were added and
then the change in absorbance was measured at 234 nm.

Superoxide dismutase (EC 1.15.1.1)

The Misra and Fridovich (1972) technique was followed to assess the SOD activity. The substrate mixture
contained Na₂CO₃ buffer (0.05 M; pH 10.2), EDTA (0.1 mL), enzyme aliquots (50 μL), and epinephrine
(100 μL), then the change in absorbance was assessed at 480 nm.

Catalase (EC 1.11.1.6)

The Aebi (1984) technique was followed to assess the CAT activity. The substrate mixture contained PPB
(0.05 M; pH 7), H₂O₂ (0.01 M), and enzyme aliquots (100 μL), then the change in absorbance was
measured at 240 nm.

Peroxidase (EC 1.11.1.7)

The Zaharieva et al. (1999) technique was followed to assess the POD activity. The substrate mixture
contained PPB (0.03 M; pH 7), H₂O₂ (0.0065 M), guaiacol (0.0015 M), and enzyme aliquots (100 μL), then
the change in absorbance was measured at 470 nm.

Ascorbate peroxidase (EC 1.11.1.11)

The Nakano and Asada (1981) technique was followed to assess the APX activity. The substrate mixture
contained PPB (0.05 M; pH 7), EDTA (0.0001 M), H₂O₂ (0.0012 M), AsA (0.0005 M), and enzyme aliquots
(50 μL), then the change in absorbance was assessed at 290 nm.

Phenylalanine ammonia-lyase (EC 4.3.1.5)

The Havir and Hanson (1968) technique was followed to evaluate the PAL activity. The substrate mixture
contained borate buffer (0.05 M; pH 8.7), phenylalanine (1 mg L⁻¹), and enzyme aliquots (500 μL), then
the change in absorbance was assessed at 290 nm.

Polyphenol oxidase (EC 1.14.18.1)

The Kumar and Khan (1982) technique was followed to assess the PPO activity. The substrate mixture
contained PPB (0.1 M; pH 6), catechol (0.1 M), and enzyme aliquots (200 μL), and the change in
absorbance was then assessed at 495 nM.

Boron forms

The different B forms were extracted as reported by Du et al. (2002) and Li et al. (2017). To dry callus
powder, dw was added, left on a shaker at 25°C for 24 h, filtered, and free B was measured in the filtration,
and NaCl (1 M) was put on the residue and left on a shake at 25°C for 24 h, filtered. Semi-bound B was measured in the filtrate, then HCl (1 M) was put on the residue and left on a shaker at 25°C for 24 h, filtered, and the bound B was measured in the filtrate. The curcumin-acetate method was applied to measure the B forms concentration (Mohan and Jones 2018).

Transmission electron microscope imaging

The plant samples were prepared using an adaptation of the method used by Bozzola and Russell (1991). Samples were viewed using field-emission TEM (JEOL transmission electron microscope JEM-100CX II).

Statistical analysis

The results obtained were a mean (± standard deviation) of 4 replicates, with all three technical measurements, in most cases, and statistical tests were calculated by SPSS software. ANOVA (One-way analysis of variance) was employed and followed by Multiple Range Tukey Test for all PA treatment without or with EB. Pearson correlation test was performed to perceive the relationship between the mean rate of different parameters of tomato under BA, GA, or SA without or with EB and asterisks show significant correlation (* and ** at 5 and 1%, respectively). Correspondence analysis was performed to investigate the relationships between redox enzymes and different levels of each treatment with PA with or without EB. Excel's t-test was used to investigate the significant difference between treatment with PA with or without EB (ns = non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Results

Callus growth

EB stress has been declared to inhibit cell and plant growth factors. ANOVA analysis of FW and DW data of tomato calli showed that EB stress resulted in a significant decrease in the mass gain of the studied tomato calli (Fig. 1A-B, Tables S1-6). However, the three PAs applied to EB-stressed calli alleviated this growth deficiency. EB in the nutrient medium caused a significant decrease in callus biomass in terms of FW and DW compared to B-unstressed calli, and the reduction was 46.97% and 42.76%, respectively. PA treatments increased callus biomass compared to EB-stressed calli and recorded high increases in moderate concentrations of benzoate, gallate, and salicylate, respectively. Compared to EB-stressed calli, the use of moderate concentrations of benzoate, gallate, and salicylate increased the FW and DW of EB-stressed calli by approximately 158.75%, 66.21%, 48.70% for FW, 83.53%, 32.53%, 43.86% for DW, respectively. Under MS-B conditions, our results indicated that the use of BA (0.676*, 0.392, 0.809**), GA (0.897**, 0.811**, 0.559), and SA (0.461, -0.788**, 0.586*) resulted, in most cases, a strong relation between free, semi-bound, and bound boron content and DW of callus, respectively. As expected, under excess boron stress, BA (-0.805**, -0.658*, -0.722**), GA (-0.544, -0.729**, -0.586*), and SA (-0.614*, -0.883**, -0.872**) showed strong negative correlations between free, semi-bound and bound boron content and DW of callus, respectively.
Hydrogen peroxide

The $\text{H}_2\text{O}_2$ contents, a product of redox metabolites, were evaluated within tomato calli that underwent various treatments to assess the degree of oxidative regulation resulting from EB stress and the influences of benzoate, gallate, and salicylate in reducing this negative damage (Fig. 2A, Tables S1-6). As revealed in Fig. 2A, EB stress increased hydrogen peroxide content in tomato calli by 60.22% compared to EB-unstressed calli. PA treatments of boron-stressed calli reduced the effect of stimulating EB stress on $\text{H}_2\text{O}_2$ content. Compared to EB-stressed calli, calli treated with moderate concentrations of benzoate, gallate, and salicylate showed reductions in $\text{H}_2\text{O}_2$ content of 23.00%, 47.94%, and 25.77%, respectively. The higher concentration of BA and SA caused an insignificant decrease in $\text{H}_2\text{O}_2$ content; whereas, GA significantly reduced its content.

Under MS-B-conditions, the low and moderate levels of the BA and GA treatments did not alter the $\text{H}_2\text{O}_2$ content; whereas, its content increased with the higher levels in the MS medium. Moreover, the $\text{H}_2\text{O}_2$ content gradually increased as the SA level increased in the MS medium. Interestingly, the application of BA (0.848**, 0.607*, 0.770**), GA (0.900**, 0.931**, 0.967**), and SA (0.552, 0.916**, 0.893**) to EB-stressed calli showed strong positive correlations between $\text{H}_2\text{O}_2$ and free, semi-bound and bound B content.

Lipoxygenase activity

To test whether the positive effects of PA treatments on EB-stressed calli were related to their ability to reduce membrane damage, LOX activity was assessed in calli exposed to EB stress with or without PAs application (Fig. 2B, Tables S1-6). LOX activity in calli was increased with an increase of B in the MS nutrient medium and the increase was 65.96% compared to the control calli. Treating EB-stressed calli with the three tested PAs attenuated the negative impact of EB stress on LOX activity. LOX activity decreased significantly, in most cases, and recorded the highest drops in moderate levels of benzoate, gallate, and salicylate; whereas, compared to EB-stressed calli the decrease was 36.43%, 25.64%, and 15.65%, respectively.

Under MS-boron conditions, significant increments in LOX activity were noticed with the application of PAs, and only BA recorded non-significant changes. Moreover, the application of benzoate, gallate, and salicylate induced strong positive relations between LOX activity and $\text{H}_2\text{O}_2$ content in calli-treated with or without EB.

Superoxide dismutase activity

SOD activity was estimated as a remarkable scavenger for ROS (Fig. 3A, Tables S1-6). SOD activity in callus cells significantly increased when the callus was exposed to EB, compared to the control calli, and the recorded increase was 23.63%. The use of gallate and salicylate did not considerably alter SOD activity in EB-stressed calli; whereas benzoate statistically reduced its activity.
Under MS-boron conditions, benzoate and gallate treatments did not alter SOD activity, however, its activity gradually increased with increasing salicylate level in the nutrient medium. Furthermore, the relations between SOD activity and \( \text{H}_2\text{O}_2 \) content were minimal in calli-treated with PAs and stressed or unstressed with EB, only the correlations in calli-treated with SA (0.895**) without EB and BA (0.579*) with EB were significant.

**Catalase activity**

CAT activity was estimated in tomato calli subjected to different levels of PAs with or without EB because it aids in the decomposition of \( \text{H}_2\text{O}_2 \) into \( \text{H}_2\text{O} \) and \( \text{O}_2 \) (Fig. 3B, Tables S1-6). As appeared in Fig. 3B, it can be observed that the EB had a considerable catalytic effect on CAT activity in the callus (about 2-fold above the control calli). However, applications of the three concentrations of benzoate, gallate, or salicylate to EB-stressed calli reduced the stimulating effect of EB on CAT activity.

Treatment of EB-stressed calli with a moderate concentration of GA showed a higher decrease in CAT activity (60.51%) compared to moderate concentrations of benzoate and SA, which recorded a decrease of 39.46% and 34.44%, respectively, of B-stressed calli.

Under MS-B cases, the three phenolic acids applied, in the most cases, significantly increased CAT activity in callus cells compared to MS-B-unstressed calli. Interestingly, PA applications caused strong positive relations between CAT activity and \( \text{H}_2\text{O}_2 \) content in calli stressed or unstressed with EB, only GA without EB stress caused a non-significant relationship.

**Peroxidase activity**

POD activity was determined because it induces oxidation by \( \text{H}_2\text{O}_2 \) for a wide range of organic materials (Fig. 3A, Tables S1-6). POD activity was stimulated with increased B in the MS-nutrient medium which showed a 35.11% increase in POD activity compared to MS-B-unstressed calli. Supplementation of benzoate and gallate at different concentrations with excess boron increased POD activity, while salicylate treatments decreased its activity significantly. Compared to EB-stressed calli, moderate levels of BA and GA showed increases in POD activity of 26.16% and 83.47%, respectively, while SA at moderate concentration reduced its activity by 43.63%.

Under MS-B conditions, benzoate and gallate treatments progressively improved POD activity in the corresponding absolute controls. In contrast, POD activity decreased significantly with the application of SA levels in the MS medium. Furthermore, in the callus treated with excess boron and benzoate, gallate, and salicylate the correlation between POD activity and \( \text{H}_2\text{O}_2 \) content was significant (-0.603*, -0.789**, and +0.865**, respectively), while it was insignificant in the PAs-treated-callus only.

**Ascorbate peroxidase activity**
APX activity was determined because it induces the hydrogen peroxide dependent oxidation of AsA in plants (Fig. 3B, Tables S1-6). Treating tomato callus with 2 mM B positively affected APX activity. Under EB states, the increase in APX activity, compared to MS controls, was 34.99%. Treatment with different levels of benzoate and gallate plus excess boron enhanced APX activity (10.46%, 22.34%, 16.23% for benzoate and 10.25%, 9.16%, 20.32 for gallate, respectively, over B-stressed calli), while salicylate treatments did not significantly alter its activity.

Under MS-B states, BA treatments reduced APX activity in calli, only the higher level did not alter its activity. GA treatments catalyzed APX activity, in most cases, under MS conditions. However, salicylate applications did not alter APX activity, only the higher level enhanced its activity, under MS conditions. Additionally, our results showed that APX activity in calli treated with only SA (0.655*) and BA with EB (-0.744**) showed a strong association with H$_2$O$_2$ content; whereas, in other PA treatments with or without excess boron, the correlations were insignificant.

**Phenylalanine ammonia-lyase activity**

PAL activity was examined to mark whether co-applications of PAs with or without EB treatments affected phenolic biosynthesis in tomato calli (Fig. 4A, Tables S1-6). The data showed that EB significantly failed to enhance PAL activity in tomato calli. Nonetheless, the moderate and high concentrations of the tested PAs significantly enhanced PAL activity compared to EB-stressed calli. Combined application of BA, GA, SA, and EB at the highest level resulted in increased PAL activity, which showed a higher increase, in most cases, of 16.83%, 42.03%, and 18.95%, respectively, than that found in EB-stressed calli. Also, our results manifested that correlations between PAL activity and H$_2$O$_2$ content were not significant in the existence of PAs with EB, only there was a significant relationship in the BA case with EB (-0.684*).

Correspondingly, under MS-B conditions, PAL activity decreased by 21.74% and 29.82%, respectively, at moderate and high concentrations of BA compared to MS- calli. However, gallate and salicylate treatments did not affect PAL activity in calli exposed to MS-B conditions; only the highest level of SA boosted its activity (46.39%). Moreover, under BA without EB treatments, PAL activity showed a negatively strong association with H$_2$O$_2$ content (-0.673*); whereas, the same relationship was positively strong in cases of GA (0.714**) and SA (0.820**) without EB.

**Polyphenol oxidase activity**

PPO activity in calli was examined to look at the degree of phenolic oxidation induced by PAs with or without EB treatments (Fig. 4B, Tables S1-6). EB negatively affected PPO activity in calli, with PPO activity reduced by 31.86%, compared to EB-unstressed calli. The PA treatments had, in most cases, significant catalytic effects on PPO activity in calli under EB conditions. GA in high concentration was the most active PA in stimulating PPO activity in callus tissues; the stimulation rate was 179.19%, compared to EB-stressed calli. Moreover, the results manifested that the PAs (BA, GA, and SA) together with the EB
treatments caused significant relationships between PPO activity and H₂O₂ content in calli (-0.777**, -0.918**, and -0.763**, respectively).

PA treatments without EB led to altered effects on PPO activity in callus tissues. The applications of different levels of benzoate significantly reduced PPO activity; however, gallate greatly enhanced its activity. In contrast, SA did not considerably alter PPO activity under MS-B states. Additionally, the results revealed that the PAs without EB treatments induced non-significant relationships between PPO activity and H₂O₂ content in calli.

**Boron forms**

Under MS-B cases, B was found mainly in free form in the tomato callus, with free B present at 68.4%, semi-bound B at 23.4%, and bound B at 8.2% of total B. However, in cases of EB, free and semi-bound B decreased by 6.90% and 4.38% to increase bound B by 11.28%. It can also be seen that EB caused significant increases in free, semi-bound, and bound B contents in B-stressed calli higher than in non-stressed calli (Fig. 5A-C, Tables S1-6). EB stress increased the free, semi-bound, and bound B content in calli by 74.55%, 57.74%, and 362.39%, respectively compared with the control callus. Benzoate, gallate, and salicylate applications reduced free, semi-bound, and bound B accumulation in EB-stressed calli as indicated by 40.35%, 28.67%, 14.93% for free B, 14.43%, 25.02%, 42.28% for semi-bound B, 41.46%, 35.06%, and 30.83% for bound B at moderate concentrations compared to EB-stressed calli, respectively. Under MS-B cases, benzoate and gallate treatments resulted in significant or non-significant increases in different concentrations of B form in calli compared to the corresponding absolute control. However, the different SA treatments did not alter the different forms of B in calli.

**Transmission electron microscopy**

TEM enables an estimate of the cell's microstructure, particularly in the internal features of cells and organelles. Hence, this technique was harnessed to look at the differences in ultrastructural resulting from excessive B stress and whether applications of phenolic acid enhanced callus cell resistance (Fig. 6 and 7). Imaging obtained from TEM showed that EB-untreated (control) cells have a normal shape, large nuclei, large vacuoles, and thin cell walls (ranging from 333 to 559 nm). The EB caused the cell walls to be thicker between 1167 and 1347 nm, which means 2.4 to 3.5 times more than the control cell wall. However, the moderate concentration of benzoate, gallate, and salicylate treatments reduced cell wall thickness in EB-stressed calli. Compared to B-stressed calli, the highest decrease in cell wall thickness was found at 78.17-85.00%, 42.54-64.52%, 83.59-83.72%, respectively after exposure to benzoate, gallate, and salicylate. Moreover, the addition of benzoate, gallate, and salicylate alone without increasing B to the nutrient medium reduced the thickness of the cell walls.

**Discussion**

In plants, PAs are a subclass of phenols that have a single COOH group with or without one or more OH groups, causing the H atom to be donated to produce antioxidant properties. However, the performance of
the action of PAs in mitigating the inhibition of EB stress on plant growth, cell ultrastructure, accumulation of B forms, and antioxidant enzymes have not been quite proven. Therefore, this research was conducted to test the mechanisms underlying PAs stimulating plant growth, to find out the response of antioxidant enzymes, the association of boron forms, and modification of the cell ultrastructure in alleviating EB stress.

Under our investigational conditions, we found that the use of PAs mitigated the negative effect of EB stress on tomato calli, including their growth. Consistent with our previous studies (Metwally et al. 2018; Farghaly et al. 2021), the increase in boron in the present study remarkably impeded the growth of callus and the gain of fresh and dry biomass. In this research, EB stress (2 mM) induced oxidative stress (Fig. 2) that could be linked to a defect in antioxidant enzymes. Moreover, inhibition of callus growth could be due to the accumulation of boron in various forms in the callus cells that were in direct contact with EB and the thickening of the cell walls. Inhibition of callus growth due to excessive boron stress may result from inhibition of cell division and rate of cell expansion, which is mainly caused by cell wall thickening. Accordingly, the increase in cell wall-bound B (Fig. 6) and increased cell wall thickening (Fig. 8) in this study may refer to decreased tomato callus growth. Podgórnska et al. (2017) reported that the thickness of cell walls depends on B ion availability.

However, when PAs were co-applied with EB, a significant increase in FW and DW of calli was observed in boron-stressed calli (Fig. 1). Furthermore, our results indicated that in the presence or absence of EB, BA was the most active PAs in improving calli growth, and for all PAs used, moderate concentration was most active in increasing growth. The strong negative relations between callus growth, \( \text{H}_2\text{O}_2 \) content, and the concentration of B forms under the treatments of PAs and EB confirmed that the higher the callus growth, the less B accumulation and the decreased oxidative stress in the plants. These results indicate that phenolic acids play significant roles in mitigating the damage to tomato calli that occurs under conditions of excess boron stress, as they regulate antioxidant enzymes involved in detoxifying ROS, and reduce the accumulation of boron forms in cell walls and cytoplasm. Likewise, the increase in callus growth was recorded in tomatoes using PAs under EB (Farghaly et al. 2021).

As with other ions, excessive boron stress leads to ROS formation (Kaya et al. 2020). Hence, EB supply may facilitate \( \text{H}_3\text{BO}_3 \) transport to cells that can be partially converted into borate due to the high internal pH of the cellular sap (Wimmer et al. 2002), thus releasing ROS, like \( \text{H}_2\text{O}_2 \) (Dat et al. 2000). In this investigation, the \( \text{H}_2\text{O}_2 \) content in calli was assessed to screen for oxidative stress triggered by EB stress. The results (Fig. 1A) revealed that EB in MS-medium increased \( \text{H}_2\text{O}_2 \) content in callus tissues. This result indicates that EB in tomato calli, predominantly, stimulates the NADPH oxidase component, the gp\(^{91}\)phox protein (Agrawal et al. 2003), which reduces \( \text{O}_2 \) to the \( \cdot\text{O}_2^- \) that dismutates into \( \text{H}_2\text{O}_2 \). Moreover, we speculate that EB stimulates \( \text{H}_2\text{O}_2 \) production by affecting electron transport in pro-plastids and mitochondria. Accordingly, Shah et al. (2017) reported that EB toxicity leads to oxidative stress predominantly by destroying electron transport during photosynthesis. This result confirmed our previous results with wheat varieties (Metwally et al. 2012; El-Shazoly et al. 2019).
However, PAs applied at most concentrations significantly controlled H$_2$O$_2$ content in calli stressed with EB. This result indicated that the applied PAs contributed to controlling ROS generation under EB stress by regulating boron uptake (Farghaly et al. 2021). Accordingly, Gawlik-Dziki et al. (2012) and Chen et al. (2019) reported that phenolics are effective in neutralizing or scavenging ROS and reducing oxidative stress of DNA, proteins, and lipids thus reducing cellular oxidative damage. Among the PAs tested, gallic acid was the most active in reducing the H$_2$O$_2$ content in the EB-treated calli, this indicates that it has the largest number of hydroxyl groups of the PAs tested. Recently, Chen et al. (2020) stated that the presence and location of the OH group in PAs play important roles in the reaction of antioxidant activity. Moreover, Mensor et al. (2001) and Sekher Pannala et al. (2001) referred that the more OH groups of PA that an antioxidant molecule has, the greater the capability to eliminate hydroxyl radicals. These reports confirmed the lowest effect of BA in eliminating H$_2$O$_2$ accumulation under EB stress. The high H$_2$O$_2$ content in calli that underwent SA treatments and high levels of BA and GA without EB stress indicated that H$_2$O$_2$ acted in the onset of SA in establishing acquired systemic resistance (Rao et al. 1997).

Since H$_2$O$_2$, one species of ROS has a significant membrane permeability (Fischer et al. 2005), exogenous application of H$_2$O$_2$ can cause deleterious effects on cells (Lee et al. 2005) such as the oxidative stimulation that will propagate, resulting in damage into cellular molecules. Under different stress conditions, lipid degradation and membrane-bound fatty acid peroxidation are bound with increased LOX activity (Babenko et al. 2017). Our results declared that LOX activity considerably increased in EB-stressed-calli; whereas, the PA treatments, in most cases, considerably reduced its activity under EB-stress. This indicates that the PAs speeded the removal of ROS from tomato calli under EB-stress. Therefore, PAs can reduce the oxidative stressor in tomatoes caused by EB-stress. The strong positive correlation between LOX activity and H$_2$O$_2$ content in calli confirmed these results, which indicated that PAs with or without EB reduced the oxidative stress that increased LOX and H$_2$O$_2$. In general, benzoate, gallate, and salicylate treatments at moderate concentrations can reduce H$_2$O$_2$ content and LOX activity, thus increasing tomato calli resistance to EB stress. In this context, the emergence of ROS species, parameters for assessing the stress point of EB, ionic imbalance, have been stated in tomato seedlings (Cervilla et al. 2012). Accordingly, Siquet et al. (2006) reported that the antioxidant action of PAs should be regarded as a beneficial method to delay membrane phospholipids peroxidation. Likewise, the roles of benzoate, gallate, and salicylate in improving the impacts of stress on free radical removal activities of other plants were investigated by other researchers, which could support our results (Franck et al. 2013; Singh et al. 2017; Shaki et al. 2019).

In contrast, considerable increases in LOX activity in GA and SA treated calli without EB indicate that the oxidative metabolites of fatty acids resulting from LOX activity are predominantly involved in growth rather than aging. Consistent with our results, Siedow (1991) stated that oxygenated fatty acid products of LOX activity have a clear role in growth, aging, and reaction to external stress.

Most of the enzymatic antioxidants were affected under EB stress with or without PAs, indicating that those enzymes enable calli to withstand the EB stress.
The SOD enzyme is metallic in nature, which forms the primary frontier of the defense against ROS (Jackson et al. 1978). Our data exhibited that SOD activity considerably increased in calli stressed with EB; indicating that EB stimulates the antioxidant enzymatic defense that helped the calli resisted this stress. Similarly, Kaya et al. (2020) found an increase in SOD activity within tomato seedlings under EB stress. Statistics showed that GA and SA did not alter SOD activities in calli stressed with EB, indicating that calli had sufficient endogenous antioxidants to remove low ROS content corresponding to low H$_2$O$_2$ content and LOX activity, compared to calli stressed with EB. These results confirmed the non-significant relationship between SOD activity and H$_2$O$_2$ content in GA and SA treated-calli and the excess of boron. Nonetheless, the strong positive correlation between SOD activity and H$_2$O$_2$ content in calli-treated with BA with EB also confirmed the least effect of BA in eliminating H$_2$O$_2$ accumulation under EB stress. Consistent with our data, Chandrakar et al. (2016) found that SA, PA hormone, treatment did not alter SOD activity in arsenic stressed soybean seedlings. In contrast, an increase within SOD activity with SA treatments in MS-B conditions is bound with an increase in H$_2$O$_2$ content, and these results are in agreement with earlier reports, which indicated that SA enhances H$_2$O$_2$ content by disrupting H$_2$O$_2$-removal enzymes and increases H$_2$O$_2$-generation of enzymes as SOD (Rao et al. 1997). Moreover, the strong positive correlation between SOD activity and H$_2$O$_2$ content in SA-treated calli without boron increase confirmed this data.

CAT plays a critical function in withstanding plant stress, and increasing CAT activity under stressor conditions is a signal for oxidative stress tolerance, as it degrades H$_2$O$_2$ the SOD product (Mittler 2002). According to our data, CAT activity in calli-stressed with EB significantly increased; indicating that overexposure to EB increased the content of ROS in tissues; however, it also improved defense systems. CAT activity has likewise been reported with EB use in tomato seedlings (Kaya et al. 2020). However, B-induced that CAT activity in calli considerably reduced by PA supplementation, which confirmed the remarkable roles of these hormonal molecules in reducing EB-caused oxidative damage. Moreover, these results suggested that other enzymes may be sufficient to suppress low levels of H$_2$O$_2$. The strong positive correlation between CAT activity and H$_2$O$_2$ content in calli-treated with EB and PAs confirmed the low H$_2$O$_2$ content in calli as CAT has a low affinity for H$_2$O$_2$ and requires a high amount of H$_2$O$_2$. This indicates that H$_2$O$_2$ did not increase and therefore, CAT activity also did not increase in calli treated with EB and PAs. Accordingly, Foyer et al. (2009) concluded that CAT has a substantially low affinity for H$_2$O$_2$, and thus removes only the high H$_2$O$_2$ content. In line with our results, Ozfıd an-Konakci and Kabakci (2020) found that GA alone and GA + Cd did not induce CAT activity in wheat seedlings. In addition, Amist and Singh (2018) observed a decrease in CAT activity of wheat seedlings-treated with hydrotic stress and BA. Parallel to our finding, decreased CAT activity was detected during EB-toxicity and SA application in canola plants (Radi et al. 2014).

Both CAT and POD, in concert with SOD, play a remarkable defensive role in removing ROS (Jaleel et al. 2009). The current results demonstrated that with increased B in the MS-nutrient medium, POD activity in calli increased; indicating that tomato calli adapt to EB through effective defense systems. Moreover, this
increase in activity was bound with high phenolic content, as observed in the current study. Likewise, Kaya et al. (2020) found that excess boron increased POD activity in SC 2121 tomato cultivars. Under MS or EB conditions, treatments with benzoate and gallate were further active in stimulating POD activity in calli; whereas, SA treatments reduced this activity. Our results also revealed that the association between POD activity and H$_2$O$_2$ content was significantly negative in calli-treated with EB + BA or EB + GA, while it was positively significant in calli-treated with EB + SA. Nonetheless, the same association was minimal in the callus treated with PAs without increasing boron. These results suggest that, in the existence of EB, this enzyme was responsible for H$_2$O$_2$ elimination in calli-treated with benzoic or gallic acid; however, it was not responsible for this elimination in calli-treated with salicylate. Yadav and Singh (2013) concluded that the elevated activity of POD by application of BA to Cd-stressed-wheat seedling is dose-dependent. Recently, Yetişsin and Kurt (2020) found that GA stimulated POD activity in Cu-stressed maize seedlings. The decrease in POD activity in EB-stressed calli confirmed by a previous study on canola plants treated with EB and SA (Radi et al. 2014).

APX is one of the main redox-enzymes responsible for suppressing toxic levels of H$_2$O$_2$ (Apel and Hirt 2004), and it may have a pivotal role in cleaning up ROS because even very low concentrations are sufficient for H$_2$O$_2$ degradation (Anjum et al. 2014; Sofo et al. 2015). The results clarified that EB stimulated APX activity in the calli, indicating that EB stimulates redox-enzymes that help calli withstand this stress. Moreover, this increase in APX activity may be maintained by a high ascorbate content, as was noticed in our previous study in EB-treated tomato callus (Farghaly et al. 2021). Similarly, the increment in APX activity in tomato plants during boron toxicity was previously detected by Cervilla et al. (2007).

Also, our results revealed that there was an additional improvement in APX activity in EB-stressed calli through benzoate and gallate applications due to their active participation in ROS detoxification, in addition to intracellular oxidative stress management. The strong negative correlation between APX activity and H$_2$O$_2$ content in BA-treated plants under EB confirmed earlier reports by Amist and Singh (2018) who reported that POD and APX were the common peroxidases that converted H$_2$O$_2$ into H$_2$O using guaiacol and ascorbate, and increased in BA-treated-drought-stressed wheat seedlings. Our results, additionally, are consistent with those of Ozfidan-Konakci and Kabakci (2020) who reported GA-induced APX activity in wheat exposed to Cd stress. However, treatments of SA with or without EB, in most cases, did not alter APX activity indicating that this enzyme was not responsible for H$_2$O$_2$ clearance in these calli. Likewise, in canola, APX was not significantly altered by applying SA to boron stressed or unstressed plants (Radi et al. 2014).

PAL is an important enzyme in plants, linking secondary and primary metabolism, and moreover, it is key to the biosynthesis of stress-resistant phenolic compounds and powerful antioxidants (Naoumkina et al. 2010; Singh et al. 2010). Statistics manifested that the EB did not stimulate PAL activity in the tomato callus, indicating that the phenolics were synthesized through other pathways. This impact on PAL activity was not as strong as reported in previous studies (Sarabandi et al. 2019), due to the different
plants or the low B concentration that was used in our treatment. Nonetheless, the moderate and high levels of tested PAs significantly enhanced PAL activity compared to calli-stressed with EB, indicating that the PAs boosted the phenylpropanoid pathway, to support antioxidant systems and callus cell resistance against EB stress. Accordingly, Kang et al. (2003) concluded that SA treatments can significantly induce tolerance to various (a)biotic stresses. Further, Wen et al. (2008) reported that the application of SA can catalyze the accumulation of PAL-mRNA, biosynthesis of novel PAL protein, and enhance PAL activity under high-temperature stress. Strong correlations between PAL activity and $H_2O_2$ content in calli treated with BA (negative), GA (positive), and SA (positive) without EB confirms Kováčik et al. (2009) who stated that an increase in PAL activity could indicate SA induced stress and may shed-light on the significance of PAL in modifying these conditions. This finding could indicate the decrease in PAL activity under BA treatments, which had low concentrations to influence PAL activity; however, PAL activity increased under high concentrations of GA and SA.

In plants, PPO is able to perform different tasks in response to different (a) biotic stresses (Thipyapong et al. 2007). The present results illustrated that, with EB in the MS-nutrient medium, PPO activity decreased within tomato callus cells, and these results predominantly indicated that the decrease was connected with increased other redox-enzymes as is the case here with CAT, APX, and POD. These findings are similar to previous research that found the negative effect of EB on PPO activity in gram plants (Chatterjee et al. 2005). Moreover, Sofo et al. (2005) concluded that under abiotic stress, reduced PPO activity was related to the enhancement of antioxidant capacity and phenolics accumulation. However, PPO activity in callus cells significantly increased by PA treatments, confirming the important role of this enzyme in reducing ROS overproduction and oxidative stresses caused by B toxicity. The negative strong relations between PPO activity and $H_2O_2$ content in calli confirms a previous report by Taşgın et al. (2006) who found that protein accumulation, decreased CAT activity, and increased POD, and PPO activities in the apoplastic region could be the best strategy to tolerate cold temperature in wheat leaves. In calli treated with BA without EB, the decrease in PPO activity was mostly related to the increase in cysteine content (Farghaly et al. 2021), as Mishra and Gautam (2016) reported that cysteine inhibited PPO activity. Under excess boron, an increase in PPO activity and decreased cysteine content in calli could confirm the previous suggestion (Farghaly et al. 2021). These results require additional investigation.

Boron possesses a pKa of 9.2 and is thus present in the MS-nutrient medium in an uncharged form that can be absorbed by passive diffusion across the plasma membranes. Boron is normally present in the cell in three major forms, free, semi-bound, and bound, the free is soluble B that is directly available for potential physiological roles, semi-bound is mainly involved in the synthesis of the cell wall, and bound is a component of cell walls (Du et al. 2002). The results illustrated that the increased supply of B into the MS-nutrient medium led to increased uptake of B forms into calli (Fig. 6). The results illustrated that free B (61.5%) was most prevalent in tomato calli, which may be related to symptoms of EB stress.

Interestingly, the bound B was increased by about 11% under EB stress than the control calli, this may be due to the increased binding sites on cell walls by the rhamnogalacturonan RG-II. Thickening of callus cell walls under the EB confirmed the increase of bound B thus decreasing callus expansion and growth.
Accordingly, Reid (2007) stated that additional boron binding on the cell wall may further disrupt the cell expansion and growth processes.

However, when PAs were co-applied with EB, a significant decrease in the accumulated B forms was observed in B-stressed tomato calli (Fig. 6), resulting in a considerable increase in the growth criteria of the stressed calli (Fig. 1). Our results clearly demonstrated that the applications of PAs reduced free, semi-bound, and bound B accumulation (Fig. 6), which resulted in the overall enhancement in the callus growth under excess boron stress (Fig. 1). Free boron was likely related to plant chlorosis (Wang et al. 2014), and decreased amount could be related to plant tolerance of EB stress (Landi et al. 2015). Decreased amount of bound B in calli could be related to callus growth promotion under co-application of PAs with excess boron stress as indicated reduction of cell wall thickening by TEM micrographs. Our results are consistent with previous findings by Metwally et al. (2018) and Farghaly et al. (2021) who found that phenolic acids reduced the boron content in barley tissues and tomato plants under excess boron stress.

**Conclusion**

The participation of benzoate, gallate, and salicylate in various biochemical and physiological processes in plants under conditions of EB is largely unknown to date. This study disclosed that tomatoes-treated with benzoate, gallate, and salicylates under excessive boron stress altered the accumulation of H$_2$O$_2$ and boron forms (free, semi-bound, and bound), and the activity of antioxidant enzymes to withstand EB stress. This study suggests that phenolic acid treatments alleviated excess boron stress in the tomato plant by modulating antioxidant enzymes and reducing H$_2$O$_2$ accumulations and boron forms (Fig. 9A-B). The strategy of the three PAs applied was to promote plant growth under EB by reducing H$_2$O$_2$ production through the activation of CAT activity and reducing cell wall thicknesses by reducing the accumulation of various B forms, especially the bound form. These results suggested that the most effective phenolic acids were BA > GA > SA. Moreover, benzoate was more active at the moderate concentration used (1 µM), which was the lowest concentration of the total concentrations of the three phenolic acids used. These results could contribute to improving the management strategy to alleviate excess boron stress in tomatoes, but more studies are needed.

**Abbreviations**

ANOVA: One-way analysis of variance

AsA: Ascorbic acid

APX: Ascorbate peroxidase

B: Boron

BA: Benzoic acid
Declarations

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Ethics declarations

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Declaration of Competing Interest

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Author contributions

Abeer Radi, Fatma Farghaly, and Afaf Hamada conceived and designed the research. Abeer Radi, Fatma Farghaly, and Hussein Salam conducted experiments. Abeer Radi, Fatma Farghaly, Hussein Salam, and Afaf Hamada analyzed the data and wrote the manuscript.

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**Figures**

**Figure 1**

Fresh (A) and dry (B) weight of Solanum lycopersicum calli under the influence of different concentrations (low, L; moderate, M; high, H) of benzoic acid (BA), gallic acid (GA), or salicylic acid (SA) and excess boron for 30 days. The data are means ± SD (n = 4). The different letters, capital for phenolic acid without boron treatments and small for phenolic acid with boron treatments, indicate significance (one-way ANOVA; Tukey HSD-post-hoc), and asterisks indicate significant differences between treatments with or without boron (t-test; * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant).
Figure 2

The H2O2 content (A) and lipoxygenase (LOX; B) activity of Solanum lycopersicum calli under the influence of different concentrations (low, L; moderate, M; high, H) of benzoic acid (BA), gallic acid (GA), or salicylic acid (SA) and excess boron for 30 days. The data are means ± SD (n = 4). The different letters, capital for phenolic acid without boron treatments and small for phenolic acid with boron treatments, indicate significance (one-way ANOVA; Tukey HSD-post-hoc), and asterisks indicate significant differences between treatments with or without boron (t-test; * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant).

Figure 3

The superoxide dismutase (SOD; A) and catalase (CAT; B) activity of Solanum lycopersicum calli under the influence of different concentrations (low, L; moderate, M; high, H) of benzoic acid (BA), gallic acid (GA), or salicylic acid (SA) and excess boron for 30 days. The data are means ± SD (n = 4). The different letters, capital for phenolic acid without boron treatments and small for phenolic acid with boron treatments, indicate significance (one-way ANOVA; Tukey HSD-post-hoc), and asterisks indicate significant differences between treatments with or without boron (t-test; * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant).
Figure 5

The phenylalanine ammonia-lyase (PAL; A) and polyphenol oxidase (PPO; B) activity of Solanum lycopersicum calli under the influence of different concentrations (low, L; moderate, M; high, H) of benzoic acid (BA), gallic acid (GA), or salicylic acid (SA) and excess boron for 30 days. The data are means + SD (n = 4). The different letters, capital for phenolic acid without boron treatments and small for phenolic acid with boron treatments, indicate significance (one-way ANOVA; Tukey HSD-post-hoc), and asterisks indicate significant differences between treatments with or without boron (t-test; * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant).

Figure 6

Free (A), semi-bound (B), and bound (C) boron of Solanum lycopersicum calli under the influence of different concentrations (low, L; moderate, M; high, H) of benzoic acid (BA), gallic acid (GA), or salicylic acid (SA) and excess boron for 30 days. The data are means + SD (n = 6). The different letters, capital for phenolic acid without boron treatments and small for phenolic acid with boron treatments, indicate significance (one-way ANOVA; Tukey HSD-post-hoc), and asterisks indicate significant differences between treatments with or without boron (t-test; * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant).
Figure 7

Optical micrographs showing tomato (Solanum lycopersicum L.) callus cells under moderate concentrations of benzoic acid, gallic acid, or salicylic acid and excess boron for 30 days.
| Control | Boron (2 mM) |
|---------|-------------|
| ![Control](image1) | ![Boron (2 mM)](image2) |

| Benzoic acid | Benzoic acid + Boron |
|--------------|----------------------|
| ![Benzoic acid](image3) | ![Benzoic acid + Boron](image4) |

| Gallic acid | Gallic acid + Boron |
|-------------|---------------------|
| ![Gallic acid](image5) | ![Gallic acid + Boron](image6) |

| Salicylic acid | Salicylic acid + Boron |
|----------------|------------------------|
| ![Salicylic acid](image7) | ![Salicylic acid + Boron](image8) |

**Figure 8**

Transmission electron micrograph showing tomato (Solanum lycopersicum L.) callus cells under moderate concentrations of benzoic acid, gallic acid, or salicylic acid and excess boron for 30 days. Chl.: chloroplast, CW: cell wall, Nu: nucleus, Ni: nucleolus, Vs: vacuoles, Mt: mitochondria, S: starch.
Figure 9

Analysis of growth, antioxidant enzymes, and boron forms of tomato (Solanum lycopersicum L.) calli under the influence of different concentrations of benzoic acid, gallic acid, or salicylic acid without excess boron (A) or with excess boron (B) for 30 days. BAL = benzoic acid at low level; BAM = benzoic acid at moderate level; BAH = benzoic acid at high level; GAL = gallic acid at low level; GAM = gallic acid at moderate level; GAH = gallic acid at high level; SAL = salicylic acid at low level; SAM = salicylic acid at moderate level; SAH = salicylic acid at high level. LOX = lipoxygenase; SOD = superoxide dismutase; CAT = catalase; POD = peroxidase; APX = ascorbate peroxidase; PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase; B-f = free boron; B-s-b = semi-bound boron; B-b = bound boron.

Supplementary Files

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