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PHENOLICS METABOLISM IN BORON-DEFICIENT TEA [*CAMELLIA SINENSIS* (L.) O. KUNTZE] PLANTS

ROGHIEH HAJIBOLAND,[1,2]* SARA BAHRAMI-RAD[2] and SOODABEH BASTANI[2]

[1] Center of Excellence for Biodiversity, [2] Plant Science Department,
University of Tabriz, 51666 Tabriz, Iran

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Modification in the metabolism of phenolic compounds under boron (B) deficiency conditions was studied in tea plants. Plants were grown from seed, treated with low B in hydroponic medium under environmentally controlled conditions for six weeks. Dry matter production and B content of plants were significantly declined under B deficiency conditions. Boron starvation resulted in rising phenylalanine ammonia lyase activity in the young leaves and declining polyphenol oxidase activity in the roots. Soluble phenolics fraction was increased up to 3.4-fold in the young leaves while did not influence by B nutrition in the old leaves and roots. Cell wall (CW) bound phenolics and lignin content was lower in B-deficient plants compared with B-sufficient ones. Boron deficiency increased significantly activity of soluble peroxidase (POD) only in the leaves. Activity of ionically bound POD was decreased in the old leaf and roots while it increased in the young leaves upon B deprivation. Activity of covalently bound POD decreased in the roots and leaves of different age in low B plants. Our results suggested that tea plant is highly tolerant species to B deficiency and CW tightening and accumulation of oxidized phenolics are not mechanisms for growth inhibition under B deficiency conditions.

Keywords: B deficiency – peroxidases – phenylalanine ammonia lyase – polyphenol oxidase – tea plant

INTRODUCTION

Phenolic compounds are among the most widely distributed natural products in the plant kingdom and participate in significant ecophysiological phenomena. The phenolics content of plant tissues varies according to the environmental conditions [11, 32] and is also affected by the nutritional status of plants [4]. In general, when growth is restricted due to resource limitation, more carbon can be derived to defensive structures and to the production of carbon-based secondary metabolites such as phenolics [16]. Boron (B) is one of the nutrients which has been related to changes in the phenol content and metabolism [4]. The accumulation of phenols is characteristic for B-deficient tissues likely due to an increased synthesis and inhibited utilization in the CW synthesis [5, 6].

* Corresponding author; e-mail address: rhajiboland@yahoo.com

The phenylpropanoid pathway is responsible for the synthesis of a large variety of secondary metabolites including phenol esters, coumarins, flavonoids and lignin. All phenylpropanoids are derived from *trans*-cinnamic acid which is formed from L-phenylalanine by the action of phenylalanine ammonia-lyase (PAL, EC 4.3.5.1) the key enzyme in the biosynthesis of the phenolic compounds. This enzyme is activated in response to various stress conditions leading to the accumulation of phenolic compounds [11]. Effect of nutritional status on the activity of PAL was reported for nitrogen [18], phosphate [17, 23] and calcium [28]. Under B deficiency conditions, activity of PAL was reported to increase in tobacco leaves [7, 27].

Phenolics are oxidized by peroxidases (POD, EC 1.11.1.7) and particularly by polyphenoloxidase (PPO, EC 1.14.18.1). Apart from peroxidases which are specifically involved in the H_2O_2 detoxification, e.g. ascorbate peroxidase, different functions have been reported to be carried out by 'unspecific' PODs by catalyzing the oxidation of a wide range of phenolic substrates [30]. Individual POD isozymes are present in numerous cell compartments, such as the endoplasmic reticulum, Golgi apparatus, mitochondria, cytosol, the vacuole, and the CW [26, 35]. In the CW, PODs are involved in cross-linking between CW components and has been proposed to account for reduced plant cell expansion under some stress conditions such as drought [2]. Polyphenoloxidase oxidizes phenolics to quinones are known to be highly toxic and responsible for production of toxic oxygen species [4, 6]. It has been shown that quinones are more toxic than phenols from which they are derived [6, 25].

Accumulation of phenolics in B-deficient plants may activate enzymes that use phenolics as substrates. In response to high phenol accumulation, PPO activity rises in B-deficient tissues, accordingly, accumulation of substantial amounts of quinones has been considered as a major cause of damage and cessation of growth under B-deficient conditions [4, 6]. In leaf extracts of B-deficient plants activity of PPO progressively increased with the severity of B deficiency symptoms and it was higher in plant species with greater sensitivity to B deficiency than the plant species with less sensitivity [6]. However, other reports showed reduction of POD and PPO activity due to B starvation [27].

The esterification of phenols to the CW and cross-linking of such esters via ether bonding leads to the formation of lignin-like polymers by supplying lignin attachment sites to the matrix polysaccharides [19]. Therefore, identification of changes in the levels of soluble and CW-bound phenolics may elucidate the fate of phenolics accumulated in B-deficient tissues and indicate changes in the biochemical pathways and compartmentation of phenolics following B starvation. Forming complexes with B makes phenolics unavailable for oxidation, therefore, greater availability of phenols not bonded to B may results in higher oxidation of phenolics in B-deficient plants.

Tea [*Camellia sinensis* (L.) O. Kuntze] is a native species of south China and cultivated in humid and subhumid of tropical, subtropical and temperate regions of the world usually on acid soils. In Iran tea is cultivated on acid soils in the north of the country. In spite of a large amount of works on the phytochemistry of tea leaves, research works on mineral nutrition of this plant are rare. Boron deficiency is generally related to high rainfall areas and acid soil conditions common in soils of tea

plantations. Under acid soil conditions, B is more water-soluble and can therefore be leached below the root-zones of plants by rainfall or irrigations [29]. Boron requirements of dicots are generally higher than monocots [4] and because of the effect of B on the phenolic compounds and its importance in the quality of black tea [22], substantial metabolic and functional changes are expected in tea plants under B deprivation.

We observed previously that B deficiency influenced growth, nitrogen metabolism and antioxidant defense capacity [13] as well as tolerance to higher light intensities [14] in tea plants. To our knowledge, there is hardly any information on the effect of B nutritional status on the phenolics metabolism and fractionation in tea plants.

The objective of this work was to study the effect of low B supply on phenolic metabolism in tea plants. In addition of enzymes involve in the synthesis and degradation of phenolics, distribution of phenolics between cell fractions as affected by B deprivation was studied in young and old leaves as well as roots of intact tea plants.

MATERIALS AND METHODS

Plants culture and treatments

Seeds of tea [*Camellia sinensis* (L.) O. Kuntze] plants were collected from the garden of Tea Research Station in Fuman (Guilan Province, Iran). Hulled seeds were surface-sterilized with 1% active hypochlorite and germinated on perlite in dark and moistened by distilled water and saturated $CaSO_4$ every day. After the emergence of primary leaves, seedlings were transferred to the light. One-month-old seedlings were transferred to the nutrient solution [13] pH 4.2 without B (–B) or adequate (46 μM) B supply (+B). In order to minimize the B contamination of nutrient solution in –B treatment, 1 g L^{-1} of washed B specific resin (Amberlite IRA 743, Fluka) [1] packed in small textile bags were kept immersed in the nutrient solution throughout the plants cultivation. Nutrient solution and resin bags were replaced weekly. Plants were grown under controlled environmental conditions with a temperature regime of 25°/18 °C day/night, 14/10 h light/dark period, a relative humidity of 70–80% and at a photon flux density of about 400 μmol m^{-2} s^{-1}.

Plant harvest and analysis

Six weeks after treatment (10 weeks after sowing), plants were harvested. Plants were divided into shoot and root, washed with double-distilled water and after blotting dry, fresh weight was determined. Dry weight of plants was determined after drying at 70 °C for 2 days. For determination of B, samples were transferred to porcelain crucibles and dry-ashed with 10 mg $Ca(OH)_2$ at 550 °C for 5 h, resolved in 0.5 M HCl and made up to volume by double-distilled water. Boron was determined by azomethine-H method [21]. Determination of phenolics and activity of enzymes was per-

formed in three distinct organ including roots, second-youngest fully developed leaf (defined as young leaf) and third oldest leaf (defined as old leaf).

Extraction and assay of PAL and PPO

Phenylalanine ammonia lyase (PAL) activity was assayed after extraction in 50 mM sodium borate buffer (pH 7.0), containing 2 mM EDTA, 18 mM 2-mercaptoethanol and 2% (w/v) insoluble polyvinylpyrrolidone. After centrifugation, enzyme extract was incubated with 100 mM borate buffer (pH 8.8) containing 12 mM L-phenylalanine for 30 min at 30 °C. The absorbance was recorded at 290 nm and the amount of *trans*-cinnamic acid formed calculated using its extinction coefficient of 9630 M^{-1}. Enzyme activity was expressed as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid mg^{-1} protein min^{-1} [10]. Polyphenol oxidase (PPO) was extracted in 200 mM sodium phosphate buffer (pH 6.5). Assay solution consisted of 10 mM pyrogallol and 200 mM sodium phosphate buffer (pH 6.5) and reaction was started by adding enzyme extract at 30 °C. Change in the absorbance at 334 nm due to oxidation of pyrogallol was followed for 10 min and one unit of activity was calculated as ΔA at 334 nm mg^{-1} protein min^{-1} [31].

Extraction and assay of water soluble and CW bound phenolics

Leaf and root samples were used for extraction of water soluble and CW phenolics according to the method of Solecka et al. [33] and Ghanati et al. [12]. In brief, phenolics were extracted three times in 70% aqueous methanol at 4 °C in dark and after centrifugation, supernatant was used for determination of water soluble phenolics and the pellet for CW bound phenolics. The supernatant was dried and extracted with ethyl ether and after acidification and extraction with ethyl acetate, phenolics were determined after solubilizing in MeOH. The pellet was washed sequentially with water and Triton X-100, then the phenolics were liberated from the CW with 20 mM NH_4-oxalate (70 °C) and then with 100 mM NaOH for 24 h. After acidification and extraction with ethyl acetate, CW bound phenolics were determined after solubilizing in MeOH. Folin–Ciocalteu reagent was used for determination of phenolics using gallic acid as standard [34].

Extraction and assay of soluble and CW bound POD

Peroxidase (POD) was extracted and the activity was determined in soluble and CW bound fractions according to the method described by Ranieri et al. [26] and Ghanati et al. [12] with some modifications. The enzyme was extracted with 10 mM phosphate buffer (pH 7.0) and after centrifugation at 1000 *g* for 10 min at 4 °C, the supernatant was centrifuged again at 20,000 *g* for 20 min at 4 °C. The second supernatant

was used for assay of soluble POD. Pellets of the first and the second centrifugations were pooled, washed three times with 0.1% Triton X-100 and then incubated with 1M $CaCl_2$ for 2 h at room temperature and centrifuged at 18,000 *g* for 20 at 4 °C. The supernatant was used to determine the activity of ionically CW bound POD and the pellet was used directly for the assay of covalently CW bound POD [12, 26]. The POD activity was assayed with guaiacol test. Concentration of soluble protein was determined using a commercial reagent (Sigma) and BSA (Merck) as standard [3].

Experiment was undertaken in complete randomized block design with 4 replications. Statistical analyses were carried out using Sigma Stat (3.02) with Tukey test ($P<0.05$).

RESULTS

Plants dry matter production was significantly declined under B deficiency conditions. Reduction of dry weight was 38% and 41% for shoot and root, respectively. As expected, B content of plants was lower under low B supply compared with adequate B supply level (Table 1).

In B-sufficient plants activity of PAL was higher in the old leaves followed by the young leaves and roots. Activity of PAL was increased under low B supply only in the young leaves but not in the old leaves and roots (Table 2). In contrast to PAL, the highest activity of PPO was observed in roots followed by the old and young leaves. Boron nutritional status influenced PPO activity only in the roots. Roots of

Table 1

Shoot and root dry weight (g plant^{-1}) and B content (µg plant^{-1}) in tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (−B) boron supply

Treatments	Plant DW		Plant B content	
	Shoot	Root	Shoot	Root
+B	1.33±0.21^a	0.39±0.09^a	76±7^a	32±1^a
−B	0.82±0.12^b	0.23±0.08^b	48±3^b	23±1^b

Data in each column indicated by the same letter are not significantly different ($P<0.05$).

Table 2

Activity of phenylalanine ammonialyase (PAL, µg *trans*-cinnamic acid mg^{-1} protein 30 min^{-1}) and polyphenoloxidase (PPO, ΔA_{334} mg^{-1} protein min^{-1}) in different fractions in tea plants grown for six weeks at adequate (+B) or low (−B) boron supply

Plant organ	PAL		PPO	
	+B	−B	+B	−B
Young leaf	8.08±0.09^b	12.42±0.65^a	0.16±0.04^c	0.22±0.05^c
Old leaf	13.05±0.76^a	14.32±0.79^a	0.59±0.06^b	0.71±0.12^b
Root	5.12±1.01^c	4.09±1.46^c	1.53±0.29^a	0.74±0.02^b

Data of each enzyme indicated by the same letter are not significantly different ($P<0.05$).

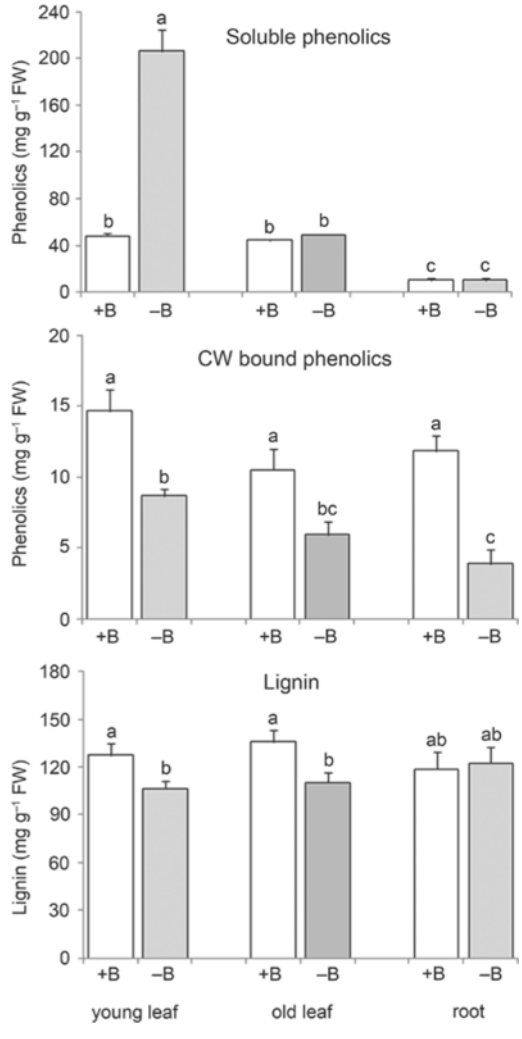

Fig. 1. Phenolics (mg g^{-1} FW) concentration in different fractions and concentration of lignin (mg g^{-1} FW) in tea plants grown for six weeks at adequate (+B) or low (−B) boron supply. Columns indicated by the same letter are not significantly different ($P<0.05$)

B-deficient plants showed lower PPO activity compared with that in the B-sufficient plants. Activity of PPO was only slightly influenced in the leaves due to B starvation (Table 2).

Soluble phenolics fraction was increased up to 3.4-fold in the young leaves due to B deficiency. In contrast, this fraction did not affect by B nutrition in the old leaves and roots (Fig. 1). Cell wall bound phenolics, however, was influenced by low B supply in all three examined organs. In B-deficient plants CW bound phenolics was

significantly lower compared with B-sufficient plants. Lignin concentration was significantly reduced by low B supply only in the leaves. In B-sufficient plants, CW bound phenolics and lignin content did not differ significantly among three examined organs while soluble fraction of phenolics was significantly lower in the roots compared with the shoot (Fig. 2).

Activity of POD in the soluble enzyme extract was higher in the roots followed by the old leaves and young leaves irrespective to the B nutritional status (Fig. 2). Boron deficiency increased significantly activity of soluble POD only in the leaves while it

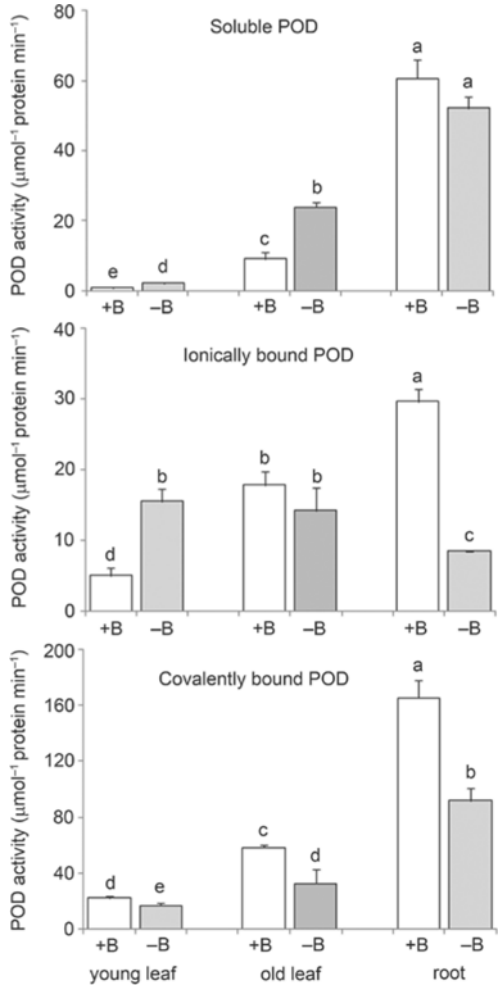

Fig. 2. Activity of peroxidase (POD, μmol mg^{-1} protein min^{-1}) in tissue extract and in different fractions including cytosolic, ionically and covalently CW bound POD (μmol mg^{-1} protein min^{-1}) in young and old leaves and roots of tea plants grown for six weeks at adequate (+B) or low (−B) boron supply. Columns indicated by the same letter are not significantly different ($P < 0.05$)

remained unchanged in the roots. Activity of ionically bound POD was decreased in the old leaf and roots slightly or significantly. In the young leaves, in contrast, significant rise in the activity of ionically bound POD was observed. Activity of covalently bound POD decreased in the roots and leaves of different age in low B plants. Irrespective to the B nutritional status and organ, the constitutive activity of covalently CW PODs was higher than two other PODs fractions (Fig. 2).

DISCUSSION

Growth of tea plants was declined under low B supply conditions. However, visual leaf symptom such as curling of leaf lamina and reduced apical dominance that are readily observable in susceptible species such as turnip [15] were not observed in tea plants. In addition, growth impairment was not observed visually in plants grown in the nutrient solution without B unless B traces were eliminated using specific resin in our work. These results indicated a high tolerance of tea plants to low B supply.

Although the constitutive activity of PAL was higher in the old leaves compared with the young leaves and roots, increase in the activity of PAL under B deficiency conditions was observed only in the young leaves. Increase in the activity of PAL was observed under deficiency of other nutrients such as nitrogen and phosphorus [17, 18, 23] as well as under some environmental stress conditions such as high light intensity and low temperature [11]. Increased PAL activity was also observed under Al treatment in the young and old leaves of tea plants (Hajiboland et al. unpublished results). Higher activity of PAL is responsible for phenolics accumulation in B-deficient plants observed in many plant species [6] and in this work (see below).

Polyphenol oxidase catalyses the oxidation of phenolic compounds into quinines, which can react with oxygen leading to the formation of toxic oxygen species [7]. The accumulation of these phenolics and toxic oxygen species in B-deficient tissues is a critical step in the initiation of impairment of several cellular functions [6]. Activity of PPO was strongly increased in the roots and leaves of turnip plants under B deficiency conditions [15]. In this work, however, activity of PPO was only slightly increased in the leaves of B-deficient plants, and in the roots even decreased upon B starvation. This may be another reason for higher tolerance of tea plants to B deficiency compared with susceptible species. Similar with our results, PPO activity was decreased under low B supply in tobacco plants [27] and increased only under excess B [12, 27]. It was also previously demonstrated that phenol oxidation is not involved in the loss of plasma-membrane integrity in the roots of squash plants under low B supply [9].

In the young leaves, in contrast to the old leaves and roots, concentration of water soluble phenolics was considerably increased in response to low B supply. In the young leaves, soluble phenolics may have some physiological and ecological functions such as protection against high light intensity [11] and chelation and detoxification of Al [24]. We have previously observed that photochemical events in the young leaves of tea plants were more protected against excess light compared with the old

leaves [13]. In addition, accumulation of phenolics in the young leaves that are used for production of black tea may influence the quality of tea beverage. The individual phenolic compounds may also be differentially changed by B treatment depending on leaf age as was observed for olive plants [20]. More detailed studies are needed to find out changes in qualitative and quantitative composition of phenolics under B deficiency conditions in tea plants.

In addition of the well-known role of B in CW synthesis and membrane integrity, B is necessary for the regulation of the biosynthesis of lignin and B deficiency causes an impaired xylem differentiation [4]. Among other PODs, CW-bound PODs are more efficient at catalyzing formation of cross-links between extension and feruloylated polysaccharids and for polymerization of phenolic monomers that causes CW stiffening and reduction of cell growth [2]. Under stress conditions, the enhanced PODs activity in the intercellular spaces, stimulates CW stiffening and reduces cell growth which might represent a mechanical adaptation to adverse conditions [26]. Several studies have presented evidence that PODs are involved in the normal cessation of cell elongation by CW stiffening as well as under stress conditions such as drought [36]. Activity of ionically and covalently bound PODs decreased in the old leaves and roots under B deficiency conditions, that was likely one of the reasons for reduction of CW bound phenolics and lignin in this organs. It is expected that, B-deficient tea plants would be susceptible to biotic stresses because of reduction in the rigidity of CW. Reduction in the activity of CW-bound PODs has also been reported for Fe-deficient plants [26].

Lower activity of CW-bound PODs in B-deficient compared with B-sufficient plants suggested that, in contrast to other stresses such as drought [2] and in contrast to susceptible species to B deficiency such as turnip [15], CW tightening is not a mechanism for growth inhibition under B deficiency conditions in tea plants. Down-regulation of various enzymes and proteins are involve in the CW growth and extension, e.g. xyloglucan endotransglycosylases and expansins under B deficiency conditions [8] may explain the B deficiency-induced cessation of cell elongation.

In the young leaves, the metabolism of phenolics as affected by low B supply was different than that in the old leaves. Unexpectedly, an increase in the activity of ionically-bound PODs was associated with lower CW bound phenolics and higher soluble phenolics in the young leaves. It implied that despite of an enhanced activity of POD fraction responsible for oxidative coupling of phenols, the majority of phenols have not been subjected to this enzymatic conversion and remained in the soluble fraction. Further studies are needed for quantitative analysis of various CW PODs using other substrates such as syringaldazine [26] or distinct phenolic compounds such as ferulic and caffeic acid in order to find relationship between phenolics oxidation in the CW and B nutrition in plants.

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