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RELATIONSHIP BETWEEN BORON AND PHENOLIC METABOLISM IN TOBACCO LEAVES

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Abstract—Tobacco plants (*Nicotiana tabacum* var. Sevilla) were treated with boron (B) as H_3BO_3 in the following concentrations B1: 0.5 μM, B2: 5 μM, B3: 10 μM and B4: 20 μM, and we analysed: total B, total phenols, orthodiphenols, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD) and dry weight. The effect of the B treatments were differentiated by: (1) foliar accumulation of phenols induced by the minimum B treatment (B1) and maximum (B4); and (2) oxidation of the phenols induced by the intermediate treatments (B2 and B3). The two predominant forms in which B is found in leaves (forming complexes with pectins and phenols, or in the free form) appears to determine and explain the effect of this element on the metabolism of the phenols.

INTRODUCTION

Phenolics are among the most influential and widely distributed natural products in the plant kingdom, and carry strong physiological and ecological implications [1–5]. The phenolics are also known to interact with growth-regulating compounds, peroxidases (POD, EC 1.11.1.7) and IAA oxidase [6]. The phenolics which have been most thoroughly studied are concerned with pest and disease resistance [7] and to discoloration of leaves and fruit caused by their oxidation [8, 9].

Boron (B) is one of the nutrients which has been related to changes in the phenol content and metabolism. The accumulation of phenols is characteristic of B-deficient tissues due to increased synthesis and inhibited utilization of phenols in cell-wall synthesis. In response to high phenol accumulation, polyphenol oxidase (PPO, EC 1.14.18.1) activity rises in B-deficient tissues [10, 11].

The reaction catalysed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is commonly regarded as a key step in the biosynthesis of phenols [12–14], and is affected by a number of factors [12]. Phenolics are degraded by POD and principally by PPO [15, 16]. Many studies have demonstrated that both enzymes increase in response to biotic and abiotic stress [17, 18].

Many studies have investigated the relationship between B deficiency and phenols. Our aim was to determine the effect that increasing dosages of B exert on the content and metabolism of the phenolics in the leaves of tobacco plants, where discoloration (browning), for example, diminishes the quality of tobacco.

RESULTS AND DISCUSSION

There are significant differences in B concentrations in leaves between treatments (Table 1). B concentration increased with the amounts of B supplied, so that in B4 the concentration of B was 7-fold higher than B1. A similar increase in B induces a parallel increase of B concentrations in broccoli leaves [19], where it was demonstrated that when excessive doses of B are applied, the leaf concentration of B remains practically constant. Taking into account these findings, the foliar concentrations of B in plants treated with B1 could be considered as deficient, those treated with B2 and B3 would qualify as normal, and those treated with B4 would be considered high.

The different B treatments also induced significant differences in the activity of PAL ($p < 0.001$). Maximum activities were recorded for B1 and B4 (Table 1), with increases over the minimum activity found for B2 of 53 and 65%, respectively. The foliar levels of orthodiphenols and total phenols ($p < 0.001$) followed the same pattern as did the PAL activity, since B1 and B4 presented the maximum con-

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Table 1. Effect of the B treatments (B1: 0.5 μM H_3BO_3; B2: 5 μM H_3BO_3; B3: 10 μM H_3BO_3; B4: 20 μM H_3BO_3) on the foliar B concentration, content and metabolism of phenolics and leaf dry weight. Data are means ± s.e. ($n = 6$)

Treatment	Total B	PAL	OrthodPhOH.	Total PhOH.	PPO	POD	Dry wt.
B1	9.5 ± 1.2	81.6 ± 3.7	2.56 ± 0.02	5.15 ± 0.03	3.37 ± 0.33	3.71 ± 0.19	0.43 ± 0.03
B2	36.9 ± 3.1	53.3 ± 2.6	1.56 ± 0.02	3.30 ± 0.02	5.98 ± 0.20	5.87 ± 0.27	0.74 ± 0.05
B3	59.4 ± 3.9	57.5 ± 2.5	1.35 ± 0.02	3.11 ± 0.02	8.12 ± 0.38	8.61 ± 0.36	0.89 ± 0.05
B4	83.9 ± 4.2	87.7 ± 3.9	2.71 ± 0.03	5.33 ± 0.02	2.25 ± 0.14	3.46 ± 0.20	1.01 ± 0.07

[Total B: $\mu g\ g^{-1}$ dry wt.; PAL: μmol cinnamic acid mg^{-1} protein h^{-1}; Orthodiphenols (OrthodiPhOH.) and Total Phenolics (Total PhOH.): μg of caffeic acid g^{-1} fr. wt; PPO: μmol caffeic acid oxidised mg^{-1} protein min^{-1}; POD: μmol guaiacol oxidised mg^{-1} protein min^{-1}; Leaf dry weight: mg dry wt $leaf^{-1}$].

centrations and the B2 and B3 minima (Table 1). These results, with a significant correlation between PAL activity and phenolic compounds ($r^2 = 0.974$***), are consistent with a role for PAL in the synthesis of phenols [12–14], and suggest that B activity could be reflected directly in PAL activity and less directly in the synthesis of foliar phenols.

PPO may also participate in the accumulation of phenol compounds. In contrast to the enzymatic activity of PAL, the treatments B2 and B3 increase PPO activity ($p < 0.001$), presenting the maximum at B3, with an increase of 140% with respect to the minima at B1 and B4 (Table 1). POD activity ($p < 0.001$) shows a trend similar to that of PPO (Table 1). The relationship in our experiment between the PPO and the concentration of phenols was negative and significant (PPO-total phenols, $r^2 = -0.939$***). All the observed correlations are consistent with the suggested role of PAL in synthesizing phenols, and of PPO and POD in their degradation.

Other work [20, 21], indicates that B applied at deficient or excessive dosages forms complexes (>90% of the foliar content), mainly with pectins and phenols in the cell wall and plasma membrane, respectively, increasing the stability of these structures under these conditions. In contrast, when B is applied at low but adequate levels, more than 60% of its foliar content remains in the free form, thus exercising other metabolic functions.

In our experiment, taking into account the foliar B concentrations, the B1 and B4 treatments appear to reflect conditions of deficient and high B levels, respectively, where the proportion of free B is minimal, and the proportion of bonded B or B forming complexes predominate. In this way, we can explain the increased PAL activity at the B1 and B4 levels, since the conditions in both treatments would be similar to those of tissues deficient in B [8], and also the accumulation of phenolics because they are complexed with B and so unavailable for oxidation.

On the other hand, the treatments B2 and B3 appear to produce adequate levels of free (or metabolic) B. The high levels of free B possibly affect the depressed PAL activity (Table 1) and increased phenolic oxidation, due principally to two causes: (1) a greater availability of phenols not bonded to B [8], and (2) the effect of B increasing the activity of such oxidative enzymes as PPO and POD [22]. In our experiment the plants treated with B3 developed purple-brown pigmentation in the leaves. Finally, the increase in dry weight with B treatments, and especially in B4 (Table 1), indicates the positive effect of this micronutrient on plant growth [19].

EXPERIMENTAL

Crop design

Seeds of *Nicotiana tabacum* var. Sevilla were sown in September. The seedlings were grown in individual pots of peat in an experimental greenhouse in southern Spain (Granada) for 45 days and then transferred to a cultivation chamber under controlled environmental conditions with relative humidity of 60–80%, temp. 30/20° (day/night), and 16 h photoperiod at a PPFD of 350 μmol $m^{-2}\ s^{-1}$ (measured at the top of the plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, U.S.A.). The plants grew in individual pots (25 cm upper diam., 17 cm lower diam., 25 cm in ht), filled with vermiculite. For one month (from day 45 until day 75 after sowing), before the experimental treatments, the plants received a nutrient soln of: 6 mM KNO_3, 2 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$ and 0.5 μM H_3BO_3. The nutrient soln (pH 5.5 to 6.0) was renewed every 3 days.

At 75 days after sowing, we applied the different levels of B as H_3BO_3 considering the initial level 0.5 μM as (B1), 5 μM (B2), 10 μM (B3) and 20 μM (B4). The experimental design was a randomized complete block with 4 treatments, arranged in individual pots with 4 plants per treatment, each one replicated 3 times.

Plant sampling

The plants were sampled beginning at the 14-leaf stage, just before the onset of flowering. From the same plants, leaves were subjected to 2 sampling dates: at the first one, day 105 after sowing, leaves were

picked from the nodes 10 and 11. The second sampling, 2 weeks later, leaves from nodes 12 and 13 were picked. All the sampled leaves were in the mature state with lengths of more than 10 cm. The material was rinsed three times in H_2O after disinfecting with 1% non-ionic detergent [23], then blotted on filter paper. The leaves from the nodes 10 and 12, were used fresh for the analysis of PAL, PPO and POD, total phenolics and orthodiphenols, performing triplicate assays for each extracting. Those picked from nodes 11 and 13 were dried in a forced air oven at 70° for 24 h. Dry wt was recorded and expressed as mg dry wt leaf^{-1}. Protein was estimated by the method of Ref. [24] using BSA as a standard.

Plant analysis

Extraction and assay of PAL. The extraction was by the method of Ref. [25]. Fresh plant material was ground in cold (4°) with 50 mM buffer (Na_2HPO_4/KH_2PO_4) pH 7.0 containing 5% PVP (M_r 44,000), 50 mM Na ascorbate, 18 mM 2-mercaptoethanol and 0.1% Triton X-100. The homogenate was filtered and centrifuged at 20,000 *g* for 10 min. $(NH_4)_2SO_4$ was added to the supernatant (to 35% satn), which was then centrifuged for 20 min at 20,000 *g*. More $(NH_4)_2SO_4$ was added to this supernatant to reach a final satn of 80%. This fraction was centrifuged at 20,000 *g* for 20 min and the pellet resuspended in extraction buffer. This soln was used for PAL assays.

PAL activity was assayed by a modification of the method of Refs [26, 27]. The assay mixture consisted of 0.06 M Na borate buffer, pH 8.8, and crude enzyme. The reaction was initiated by the addition of 11 mM L-phenylalanine. Tubes were incubated at 30° for 1 h and the reaction stopped by the addition of 35% (w/v) TFA. Tubes were then centrifuged for 5 min at 5000 *g* to pellet the denatured protein. PAL activity was determined from the yield of cinnamic acid, estimated from *A* 290 nm in presence and absence of phenylalanine.

Extraction and assay of PPO. Fresh plant material was ground with 0.1 M buffer (Na_2HPO_4/KH_2PO_4) pH 7 containing 1.5% PVP. The homogenate was filtered and centrifuged at 15,000 *g* for 15 min [28]. The supernatant was used for PPO assays. All these procedures were carried out at 4°. The assay mixture consisted of 100 mM buffer (Na_2HPO_4/KH_2PO_4), pH 7, 0.58% Triton X-100 and enzyme extracts. The reaction was initiated by the addition of 30 μM of caffeic acid. PPO activity was measured by the change in *A* 370 nm of the assay mixture (30°) based on the disappearance of caffeic acid.

Extraction and assay of POD. The method used was that of Ref. [29]. Fresh plant material was ground with 50 mM Tris-acetate buffer, pH 7.5, containing 2 mM EDTA and 0.5% PVP. The homogenate was filtered and centrifuged at 40,000 *g* for 10 min. The pellet was discarded and the supernatant used for POD assays. All procedures were carried out at 4°.

POD activity was determined following the change of *A* at 485 nm due to guaiacol oxidation [30]. The reaction mixture contained in 3 ml: 100 μM Tris-acetate buffer pH 5.0, 1 μM EDTA, 0.1 μM guaiacol and 0.003 μM H_2O_2.

Extraction and quantification of orthodiphenols and total phenols. The phenols of the plant material were extracted with MeOH. Total phenolic content was assayed quantitatively by *A* at 765 nm with Folin-Ciocalteau reagent [31]. The orthodiphenolics were determined by *A* at 360 nm, as in Ref. [32]. The results obtained were expressed as mg of caffeic acid g^{-1} fr. wt.

Estimation of total boron. Total B was analyzed after digestion of dry and milled leaf material with 12N H_2SO_4 and H_2O_2. To measure B in leaves, the azomethine-H$^+$ method was followed, and *A* was read at 410 nm [33]. The concn of B was expressed as μg g^{-1} dry wt.

Statistical analysis

The data shown are mean values ± s.e. Differences between treatment means were compared using the LSD at the 0.05 probability level. Levels of significance are represented by * at $p < 0.05$, ** at $p < 0.01$, *** at $p < 0.001$ and ns: not significant.

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