Long-term Aluminum Exposure Effects on Physiological and Biochemical Features of Highbush Blueberry Cultivars

Marjorie Reyes-Díaz

Center of Plant, Soil Interaction, and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Casilla 54-D, Temuco, Chile

Claudio Inostroza-Blancheteau, Rayen Millaleo, and Edgardo Cruces

Doctorado en Ciencias de Recursos Naturales, Facultad de Ingeniería, Ciencias y Administración, Universidad de La Frontera, Casilla 54-D, Temuco, Chile

Cristián Wulff-Zottele, Miren Alberdi, and María de la Luz Mora¹

Center of Plant, Soil Interaction, and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Casilla 54-D, Temuco, Chile

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ABSTRACT. We compared the aluminum tolerance of two highbush blueberry (*Vaccinium corymbosum*) cultivars, Legacy and Bluegold, grown in a greenhouse in Hoagland's solution with increasing concentrations of Al (0, 25, 50, 100, and 200 μM) for 7 to 20 days, using root lipid peroxidation (LP), radical scavenging activity (RSA), Al uptake by roots, and relative growth rate (RGR) as criteria. Leaf physiological [photochemical and non-photochemical parameters of photosystem II (PSII)] and biochemical (pigments, LP, RSA, and total soluble carbohydrates) responses to Al stress were also analyzed and then a principal component analysis (PCA) was performed. The results indicated that 'Bluegold' showed the highest Al uptake and LP in roots and a lower RGR in contrast to 'Legacy'. The photochemical parameters were more affected in 'Bluegold' than in 'Legacy', particularly at the beginning of the experiment. At this point, a sharp increase in RSA was found in 'Legacy'. According to these parameters, 'Legacy' was more Al tolerant than 'Bluegold'. PCA revealed that among the underlying processes affected by Al toxicity in the highbush blueberry, the photochemical efficiency of PSII followed by modifications of photosynthetic pigment contents are of greatest significance after long-term Al stress. Additionally, RSA plays an important role in the long-term acclimation response mechanisms to Al stress in highbush blueberry leaves.

It is well known that under soil acidification, aluminum (Al^{3+}) toxic ions are released into the soil solution, adversely affecting plant growth and crop yield and quality (Kochian, 1995; Meringa et al., 2004; Tang et al., 2002). The primary site of Al^{3+} accumulation in plants seems to be the distal elongation zone of roots, suggesting that Al^{3+} interacts with dividing and expanding cells, thus inhibiting cell root expansion (Rengel, 1996; Rout et al., 2001; Yamamoto et al., 2003). Although Al^{3+} cannot catalyze redox reactions, lipid peroxidation (LP) and the production of reactive oxygen species (ROS) are common and early symptoms of Al^{3+} toxicity in plants, followed by alterations in the integrity of the plasma membranes throughout LP (Jones et al., 2006; Tamás et al., 2006; Yamamoto et al., 2001). However, Babourina et al. (2006) reported that ROS production in *Triticum aestivum* seedling was activated primarily by low pH exposure rather than by Al stress. Reactive oxygen species and LP can result in nutritional and metabolic disorders

(Pavlovkin et al., 2009). Nonetheless, decreased levels of LP under Al stress were found in the roots of an Al-tolerant line of *Zea mays* (Giannakoula et al., 2008), but not in an Al-sensitive line (Hoshino et al., 2000). Therefore, the magnitude of Al stress is also dependent on the degree of Al tolerance in the species, lines, cultivars, or genotypes under investigation.

There is less knowledge about Al^{3+} effects in the aerial parts of plants (shoots and leaves) than in the roots. Rengel (1996) pointed out that the effects of Al^{3+} toxicity on shoots, such as restricted growth, become evident only after root growth is limited by exposure to toxic Al^{3+} levels in the rhizosphere, leading to mineral nutrition deficiencies in the aboveground tissues. Thus, the reduction in shoot growth and crop yield due to Al toxicity seems to be a long-term effect (Taylor, 1988). It has been reported that prolonged Al^{3+} stress also enhances LP associated with elevated ROS production in *Lemna minor* (Rout et al., 2001). Eventually, cell death or apoptosis may occur in plants subjected to long-term Al stress (Yamaguchi et al., 1999; Yamamoto et al., 1997). Other long-term effects are frequently related to chloroplast malformations, although high amounts of Al may not have been detected in this organelle, indicating indirect effects on chloroplast functioning (Moustakas et al., 1995). Decreased total chlorophyll content, disruption of photochemical capacity in photosystem II (PSII), and subsequent inhibition of the linear electron transport rate of photosynthesis in response to Al^{3+} have been found in some

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¹Corresponding author. E-mail: mariluz@ufro.cl.

plant species (Chen et al., 2005; Peixoto et al., 2002; Pereira et al., 2000). Al^{3+} -induced changes in the photosynthetic apparatus were accompanied by changes in the carbohydrate metabolism (Chen et al., 2005). Giannakoula et al. (2008) and Khan et al. (2000) reported an increased total soluble carbohydrate content in the leaves and roots of *Z. mays* lines due to Al stress, which was greater in Al-tolerant lines than in sensitive ones. The response of leaf antioxidant systems was also increased by Al toxicity in *Citrus reshni*, which coincided with the increased requirement for scavenging reactive species, whereas thermal energy dissipation of PSII decreased (Chen et al., 2005).

Currently, one of the major agronomic products in southern Chile is the highbush blueberry, which develops mainly in volcanic acid soils (Andisols) characterized by the presence of high concentrations of aluminum (Al^{3+}) (Borie and Rubio, 2003; Mora et al., 2004). Recently, Reyes-Díaz et al. (2009) studied the short-term (hours) physiological and biochemical responses in highbush blueberry cultivars subjected to acid Al toxicity. They found that the photochemical efficiency of PSII was least affected in the most Al-tolerant cultivar. Nevertheless, the long-term effect of Al toxicity has not been studied.

Our goal in this study was to examine the long-term effects (7–20 d) of Al toxicity on the physiological and biochemical performance and Al tolerance of highbush blueberry cultivars. We first ranked the Al tolerance of these cultivars according to root features such as LP and RSA in relation to Al uptake. Additionally, the cultivars with contrasting Al tolerance were compared with respect to their physiological and biochemical changes in leaves. To establish which physiological or/and biochemical profiles best characterized the leaf phenotypes of the cultivars with contrasting Al tolerance, we applied principal component analysis (PCA) (Roessner et al., 2001; Taylor et al., 2002). Principal component analysis is a method that reduces data dimensionality by performing a covariance analysis between factors. This method was successfully used for a rapid classification of a phenotypical mutant of *Arabidopsis thaliana* (Messerli et al., 2007), *Polaskia chichipe* (Carmona and Casas, 2005), and *Fucus spiralis* (Scott et al., 2001).

Materials and Methods

PLANT MATERIAL. ‘Legacy’ and ‘Bluegold’ highbush blueberry, frequently cultivated in southern Chile (Guerrero, 2006; Reyes-Díaz et al., 2009), were used for this study. One-year-old saplings of these genotypes grown in a substrate of oat shell:sawdust:pine needles at a 1:1:1 proportion were provided by the Maquehue Station of the Universidad de La Frontera, Temuco, Chile. Two groups of saplings were conditioned in plastic boxes filled with 18 L of Hoagland’s nutrient solution (Hoagland and Arnon, 1959) for 2 weeks. One group of saplings was used for physiological and biochemical analyses and the other group was used for growth determinations (see below). After conditioning, all saplings were subsequently transferred to a hydroponic solution containing Hoagland’s nutrient plus Al as $AlCl_3$ in concentrations of 0, 25, 50, 100, and 200 μM Al (treatment solution) for different times (0, 7, 14, and 20 d) and continuous aeration in a greenhouse. The solutions were prepared with sterile deionized water and were filter-sterilized through 0.2- μm pore diameter filters as per the method of Reyes-Díaz et al. (2009). The pH of control (without Al) and Al treatment solutions was monitored and adjusted daily to

a pH of 4.5 using 0.1 M HCl with a portable pH meter (model pH-0.13; Hi-Tech-Instruments, Shanghai, China). Greenhouse growth conditions were 25/20 °C (day/night), a 16/8-h (light/dark) photoperiod, 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux (PPF), and 70% relative air humidity. After aluminum treatment, roots were washed with 100 μM $CaCl_2$ and then rinsed three times with double distilled water as described in Yamamoto et al. (2001). After each, physiological and biochemical measurements were performed in roots and leaves from six individual plants per treatment as indicated below. For biochemical analyses, samples were harvested and stored at –80 °C until use.

PLANT GROWTH ANALYSES. After conditioning, the fresh weight of six whole plants from each cultivar was determined. From these plants, a group of three plants was separated for dry weight determinations (W_1), while the other three were subjected to the different Al treatments in the same conditions mentioned above. At the end of the experiment (20 d), the Al-treated plants were harvested for fresh and dry weight determinations (W_2). Growth was expressed as the relative growth rate (RGR) from the mean natural logarithm-transformed plant weights: $RGR = (\ln W_2) - (\ln W_1) / (t_2 - t_1)$ (Hoffmann and Poorter, 2002), where t_1 and t_2 are the time 0 and 20 d, respectively.

AL DETERMINATIONS. Leaves from the first to the fourth node of shoots and roots were dried separately at 70 °C in a forced-air oven for 48 h. Samples were ashed at 500 °C for 8 h and then treated with 2 M hydrochloric acid. Al was quantified using a simultaneous multielement atomic absorption spectrophotometer (model 969 atomic absorption spectrometer; Unicam, Cambridge, UK) as described in Sadzawka et al. (2004).

LP. In fresh material, the thiobarbituric acid-reactive substances (TBARS) were measured by Heath and Packer’s method (1968), modified by Du and Bramlage (1992). In this modified procedure, the absorbance was measured at 532, 600, and 440 nm to correct for the interference produced by TBARS-sugar complexes. Lipid peroxidation is a good criterion for determining Al tolerance in plants (Jones et al., 2006; Yamamoto et al., 2001); hence, it was used to establish tolerance to this metal in this study.

RADICAL SCAVENGING ACTIVITY (RSA). The RSA of roots and leaves was performed using the method of free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging as described by Chinnici et al. (2004), with some modifications. The absorbance was measured at 515 nm using Trolox as the standard.

PIGMENT DETERMINATIONS. Leaf pigments were extracted with 96% ethanol according to Lichtenthaler and Wellburn (1983). Total chlorophylls and total carotenoids were measured by a spectrophotometer (Genesys 5; Spectronic Unicam, Rochester, NY) at 663, 646, and 470 nm, and pigment concentrations were calculated according to Lichtenthaler and Wellburn (1983).

TOTAL SOLUBLE CARBOHYDRATES (TSC). Carbohydrates were extracted from fresh leaf tissues in 86% (v/v) ethanol:water with overnight agitation. Total soluble carbohydrate content was determined spectrophotometrically by the Resorcinol method (Roe, 1934) at a wavelength of 520 nm, using sucrose as the standard.

CHLOROPHYLL FLUORESCENCE PARAMETERS OF PSII. Leaf chlorophyll fluorescence from the second to fourth node of shoots was used to determine the photochemical efficiency of PSII using

a portable pulse-amplitude modulated fluorimeter (FMS 2; Hansatech Instruments, King's Lynn, UK). The Reyes-Díaz et al. (2009) protocol was used. Minimal fluorescence (F_0) was determined in dark-adapted (20 min) leaves by applying a weak-modulated light ($0.4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and maximal fluorescence (F_m) was induced by a short pulse (0.8 s) of saturating light ($9000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). After 10 s, actinic light ($120 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was turned on to obtain fluorescence parameters during steady-state photosynthesis. Saturating pulses were applied after steady-state photosynthesis had been reached to determine maximal fluorescence in light-adapted leaves (F_m') and steady-state fluorescence (F_s). Finally, the actinic light was turned off and a 5-s far-red (FR) pulse was immediately applied to obtain minimal fluorescence in light-adapted leaves (F_0'). In this article, the fluorescence parameters effective quantum yield (ΦPSII), electron transport rate (ETR), and non-photochemical quenching (NPQ) were estimated as described by Maxwell and Johnson (2000). $\Phi\text{PSII} = (F_m' - F_s)/F_m'$ is the indicator of the effective quantum yield of PSII. The ETR was calculated as: $\text{ETR} = \text{PPF} \times 0.5 \times \Phi\text{PSII} \times 0.84$ (Genty et al., 1989). Non-photochemical quenching was calculated as $\text{NPQ} = (F_m - F_m')/F_m'$ (Maxwell and Johnson, 2000).

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES. The experimental design is a split-plot design with two cultivars $\times$ five Al treatments $\times$ six replicates $\times$ three times for the physiological and biochemical determinations, and two cultivars $\times$ five Al treatments $\times$ three replicates $\times$ two times (0 and 20 d) for the RGR. Reported values correspond to the average of six individual replicates for each cultivar, Al treatments, and time for statistical analyses of physiological and biochemical measurements. RGR reported values correspond to three replicates for each cultivar. All data passed the normality and equal variance tests after the Kolmogorov-Smirnov test. Data were subjected to a two-way analysis of variance (where the factors were Al treatments and time), with repeated measurements for one factor (cultivars). A Tukey's honestly significant difference test was used to identify those values with significant differences. Both analyses were performed with Sigma Stat 2.0 software (SPSS, Chicago). Differences between the values were considered significant at $P \leq 0.05$. PCA, which is primarily concerned with the transformation of a large set of related variables to a new smaller set of uncorrelated variables (Jolliffe, 1986), was performed on leaves using the STATISTICA 6.0 (StatSoft, Tulsa, OK) software. Principal component analysis is appropriate when it is believed that a function of many attributes is involved in differences between the samples (Roessner et al., 2001; Taylor et al., 2002). Triplicate values of each physiological and biochemical measurement (described above) obtained from two cultivars selected according to their contrasting Al tolerance (Legacy and Bluegold are tolerant and sensitive, respectively) were compiled in a database designed in Excel (Microsoft, Redmond, WA). Subsequently, the data compiled were used to compute the average of the experiment results for each treatment. PCA was performed after $\log_{10}$ transformation of the average of the experiment results compiled in the data matrix previously described (Jolliffe, 1986). A $\log_{10}$ transformation of the 11 variables studied in each sample was carried out with the main objective of reducing the scattering of the data when the scores of each case, or variable, are plotted in the space generated by two principal components. A similar protocol is usually performed for metabolomic analysis in different plant species as described by Fiehn et al. (2000).

Results

RGR

No significant differences in the RGR for all the Al concentrations tested were found after 20 d in 'Legacy' compared with non-treated plants (Table 1). In contrast, a statistically significant decrease ($P < 0.05$) of RGR was registered in Al-treated 'Bluegold', the highest RGR decrease (5.8-fold) being at $200 \mu\text{M}$ Al. Statistically significant RGR differences between the two cultivars were found at 50, 100, and $200 \mu\text{M}$ Al concentrations ($P < 0.05$) (Table 1). When the RGR of roots and shoots were measured separately, the same tendency was observed (data not shown).

Root analyses

ALUMINUM CONTENT UNDER DIFFERENT AL TREATMENTS. The root Al content increased in parallel with the increase in the Al concentration of the treatment, up to $100 \mu\text{M}$ Al in 'Legacy' and 'Bluegold', and remained constant afterward (Fig. 1). 'Bluegold' showed 40% higher Al content in their roots than 'Legacy'. However, the root Al accumulation at almost all Al concentrations and all points in time was very similar in 'Legacy' and 'Bluegold', being 8.5-fold higher than the control at the highest Al treatments. Pearson correlations (r) between root Al amounts and Al concentration of treatments were statistically significant ($P \leq 0.001$) in the two cultivars as follows: Bluegold ($r = 0.85$) and Legacy ($r = 0.80$). Practically no differences in Al content were found at different times in the experiment (Fig. 1). Interactions between cultivars and Al treatment were statistically significant at 7, 14, and 20 d ($P < 0.001$, $P = 0.002$, and $P < 0.001$, respectively).

LP

Lipid peroxidation was more affected by Al toxicity in 'Bluegold' compared with 'Legacy' during the experiment, especially at 7 and 14 d of treatment, where the highest LP values were found in all the Al treatments in comparison with the control ($P < 0.05$). After 20 d of Al treatments, the LP values were significantly lower than those observed after 7 and 14 d

Table 1. Changes in the relative growth rates of highbush blueberry cultivars growing at increased Al treatments under acidic conditions for 20 d.

Cultivar	Al treatment (μM)	Relative growth rate [mean $\pm$ SE ($\text{g}\cdot\text{d}^{-1}$ DW)]
Legacy	0	0.025 ± 0.002 a ^z
	25	0.026 ± 0.002 a
	50	0.026 ± 0.004 a ^{*y}
	100	0.026 ± 0.001 a*
	200	0.025 ± 0.004 a*
Bluegold	0	0.023 ± 0.002 a
	25	0.022 ± 0.0002 a
	50	0.013 ± 0.002 b
	100	0.006 ± 0.0003 c
	200	0.004 ± 0.0003 c

^zDifferent lower case letters indicate statistically significant differences among the different Al treatments via Tukey's HSD at $P \leq 0.05$.

^yAsterisks indicates statistically significant differences between the cultivars at the same Al treatment via Tukey's HSD at $P \leq 0.05$.

Fig. 1. Aluminum uptake in roots of highbush blueberry cultivars in relation to the Al treatments at different times. Values are averages of six replicates $\pm$ SE. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al treatments for the same cultivar and exposure time. Different uppercase letters indicate differences (Tukey's HSD at $P \leq 0.05$) between exposure time for the same cultivar and similar Al treatment.

Fig. 2. Lipid peroxidation of roots of highbush blueberry cultivars grown under different Al treatments at different times measured as malondialdehyde (MDA). Values are an average of six replicates each $\pm$ SE. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al treatments for the same cultivar and exposure time. Different uppercase letters indicate differences (Tukey's HSD at $P \leq 0.05$) between exposure time for the same cultivar and similar Al treatment.

(Fig. 2). Lipid peroxidation under Al stress was 80% lower in 'Legacy' compared with 'Bluegold' (Fig. 2). Thus, in relation to LP, 'Legacy' was the most tolerant, while 'Bluegold' was the most sensitive to Al toxicity. Statistically significant correlations between root LP and Al treatment were detected in 'Bluegold' ($r = 0.76$; $P = 0.0009$) and 'Legacy' ($r = 0.59$; $P = 0.02$) (Fig. 2). A statistically significant interaction between time and treatment was found for LP in both cultivars ($P < 0.001$).

RSA. Higher RSA was found in 'Legacy' compared with 'Bluegold' (Fig. 3). A statistically significant increase ($P < 0.05$) at 7 d of treatment (around 94% with respect to initial values) was exhibited in 'Legacy' in all the Al treatments, whereas after 14 and 20 d of treatment, a decrease in RSA was found, reaching values near the control (Fig. 3).

Leaf analyses

AL CONTENTS. Leaves of Al-tolerant ('Legacy') and Al-sensitive ('Bluegold') cultivars exhibited similar patterns of Al accumulation up to 50 μ M of Al treatment at each time of the experiment, remaining constant at the highest Al treatments (Fig. 4). However, the largest Al leaf accumulation at the two highest Al treatments and times with respect to the controls was shown by the Al-sensitive cultivar (83% average of all times)

rather than in the Al-tolerant one (42% average of all times). There was no statistically significant interaction between the cultivar and the time with respect to the Al content of the leaves ($P = 0.279$).

Fluorescence parameters of the PSII

The maximum photochemical efficiency of PSII measured as Fv/Fm practically did not vary under any of the Al treatments (data not shown). It ranged from 0.73 to 0.79, which is normal for healthy leaves (Björkman and Demmig 1987; Maxwell and Johnson 2000). After 7 d of treatment, the Φ PSII and ETR of 'Legacy' were slightly lower (23%) than the control at each Al treatment ($P < 0.05$). Nonetheless, after 14 and 20 d, these parameters did not change under Al treatment, reaching values similar to the control (Table 1). 'Bluegold' exhibited a sharp statistically significant decrease in Φ PSII and ETR at 7 d (56%), 14 d (29%), and 20 d (28%) of Al treatments compared with controls (Table 2). In this cultivar, Φ PSII and ETR values were significantly lower than those of 'Legacy' under Al treatments ($P < 0.05$) (Table 2). A statistically significant increase in NPQ values, which are indicators of thermal energy dissipation, was found with respect to the control in 'Legacy' at 7 d of treatment in most Al treatments, whereas generally no changes in NPQ were found in 'Bluegold' (Table 2).

Fig. 3. Root-scavenging capacity of highbush blueberry cultivars grown under different Al treatments at different times measured as Trolox equivalents (TE). Values are an average of six replicates each $\pm$ SE. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al treatments for the same cultivar and exposure time. Different uppercase letters indicate differences (Tukey's HSD at $P \leq 0.05$) between exposure time for the same cultivar and similar Al treatment.

Pigment analyses

In 'Legacy,' carotenoid contents did not vary at the different times or Al treatments with the exception of 7 d (Fig. 5). At this time, carotenoid content increased compared with the control up to 50 μ M Al ($P \leq 0.05$) (Fig. 5). In 'Bluegold,' greater carotenoid contents were found after 14 d at higher Al treatments in relation to the other treatments ($P < 0.05$) (Fig. 5). The total chlorophyll content (Chl $a + b$) of 'Legacy' rose to 50 μ M Al at 7 d of treatment, and after 20 d, it remained constant despite the increase of Al concentrations, whereas at 14 d, Chl $a + b$ did not change at different Al treatments, remaining similar to the control (Fig. 5). While at 7 d of treatment, 'Bluegold' Chl $a + b$ contents decreased with respect to the control down to 25 μ M Al, remaining constant afterward. No changes were observed at the other time points (Fig. 5). Statistically significant interactions between cultivars and times were found in the Chl $a + b$ and Chl a/b ratios ($P \leq 0.001$).

RSA and TSC

Because TSC in leaves increase under Al stress in some plants concomitant with the RSA, we determined these parameters. Leaf RSA was significantly higher in 'Legacy' after 7 and 14 d of Al treatments, reaching values similar to the control

Fig. 4. Effect of different Al treatments on the Al contents of the leaves of two highbush blueberry cultivars with contrasting Al tolerance (Al-tolerant 'Legacy' and Al-sensitive 'Bluegold') at different times. Values are the average of six replicates each $\pm$ SE. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al treatments for the same cultivar and exposure time. Different uppercase letters indicate differences (Tukey's HSD at $P \leq 0.05$) between exposure time for the same cultivar and similar Al treatment.

after 20 d (Fig. 6). However, the RSA in 'Bluegold' did not show any changes after any of the Al treatments, with RSA values being significantly lower (62%) than in 'Legacy' ($P \leq 0.05$) (Fig. 6).

Total soluble carbohydrates of control leaves were higher in 'Legacy' than in 'Bluegold' ($P < 0.05$) (Fig. 6). In 'Legacy,' a statistically significant decrease in TSC in relation to the control ($P < 0.05$) was shown down to 50 μ M Al at each time point, remaining constant at higher Al concentrations. No significant changes in TSC were observed in 'Bluegold' at any time point or Al concentration (Fig. 6).

PCA of the physiological and biochemical features of leaves

Through PCA, we established the leaf physiological and biochemical phenotypes of two highbush blueberry cultivars with different Al tolerance under Al toxicity. Chlorophyll fluorescence parameters, the chemical analysis of Al uptake, and biochemical determinations in the photosynthetic pigment composition, TSC and RSA, were used to generate the data matrix employed for PCA. Moreover, PCA was performed on all the leaf samples collected from 'Legacy' and 'Bluegold' subjected to the complete set of experimental conditions (Fig.

Table 2. Photochemical and non-photochemical parameters of PSII in two highbush blueberry cultivars with contrasting Al tolerance grown under increasing Al concentrations for different times. Values are means of six replicates.

Duration of treatment (d)	Al treatment (μM)	Effective quantum yield [mean $\pm$ SE (relative unit)]	Electron transport rate [mean $\pm$ SE ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)]	Non-photochemical quenching [mean $\pm$ SE (relative unit)]
<i>'Legacy'</i>				
7	0	0.388 $\pm$ 0.024 a ^z A ^y	19.5 $\pm$ 1.2 aA	1.59 $\pm$ 0.03 aA
	25	0.297 $\pm$ 0.016 bA	15.0 $\pm$ 0.8 bA	2.02 $\pm$ 0.06 bA
	50	0.296 $\pm$ 0.022 bA	14.9 $\pm$ 1.1 bA	2.26 $\pm$ 0.23 bA
	100	0.286 $\pm$ 0.004 bA	14.4 $\pm$ 0.2 bA	2.40 $\pm$ 0.27 bA
	200	0.297 $\pm$ 0.009 bA	15.0 $\pm$ 0.5 bA	2.31 $\pm$ 0.20 bA
14	0	0.388 $\pm$ 0.024 aA	19.5 $\pm$ 1.1 aA	1.62 $\pm$ 0.03 aA
	25	0.371 $\pm$ 0.010 aA	18.7 $\pm$ 0.5 aB	1.65 $\pm$ 0.12 aB
	50	0.374 $\pm$ 0.045 aA	18.8 $\pm$ 2.3 aB	1.61 $\pm$ 0.15 aA
	100	0.369 $\pm$ 0.017 aA	18.6 $\pm$ 0.8 aB	1.62 $\pm$ 0.08 aA
	200	0.386 $\pm$ 0.014 aA	19.5 $\pm$ 0.7 aA	1.58 $\pm$ 0.17 aA
20	0	0.388 $\pm$ 0.024 aA	19.5 $\pm$ 1.2 aA	1.63 $\pm$ 0.03 aA
	25	0.376 $\pm$ 0.012 aA	18.9 $\pm$ 0.6 aB	1.63 $\pm$ 0.02 aB
	50	0.376 $\pm$ 0.013 aA	18.9 $\pm$ 0.7 bB	1.69 $\pm$ 0.02 aA
	100	0.373 $\pm$ 0.018 aA	18.8 $\pm$ 0.9 aB	1.70 $\pm$ 0.25 aA
	200	0.361 $\pm$ 0.023 aA	18.2 $\pm$ 1.1 aB	1.69 $\pm$ 0.11 aA
<i>'Bluegold'</i>				
7	0	0.399 $\pm$ 0.022 aA	20.1 $\pm$ 1.1 aA	1.82 $\pm$ 0.03 aA
	25	0.207 $\pm$ 0.023 bA	10.4 $\pm$ 1.1 bA	2.05 $\pm$ 0.01 aA
	50	0.185 $\pm$ 0.008 bA	9.3 $\pm$ 0.4 bA	1.98 $\pm$ 0.10 aA
	100	0.178 $\pm$ 0.012 bA	9.0 $\pm$ 0.6 bA	1.94 $\pm$ 0.25 aA
	200	0.181 $\pm$ 0.015 bA	9.1 $\pm$ 0.8 bA	1.92 $\pm$ 0.28 aA
14	0	0.399 $\pm$ 0.022 aA	20.1 $\pm$ 1.0 aA	1.83 $\pm$ 0.03 aA
	25	0.354 $\pm$ 0.014 aB	17.9 $\pm$ 0.7 aB	1.89 $\pm$ 0.01 aA
	50	0.280 $\pm$ 0.011 aB	14.1 $\pm$ 0.5 aB	1.87 $\pm$ 0.13 aA
	100	0.279 $\pm$ 0.025 aB	14.0 $\pm$ 1.3 aB	1.86 $\pm$ 0.07 aA
	200	0.281 $\pm$ 0.019 aB	14.1 $\pm$ 1.0 aB	1.80 $\pm$ 0.08 aA
20	0	0.399 $\pm$ 0.022 aA	20.1 $\pm$ 1.1 aA	1.82 $\pm$ 0.03 aA
	25	0.341 $\pm$ 0.023 aB	17.2 $\pm$ 1.2 aB	1.85 $\pm$ 0.13 aA
	50	0.290 $\pm$ 0.024 bB	14.6 $\pm$ 1.2 aB	1.83 $\pm$ 0.06 aA
	100	0.292 $\pm$ 0.007 bB	14.7 $\pm$ 0.3 bB	1.86 $\pm$ 0.07 aA
	200	0.288 $\pm$ 0.010 bB	14.5 $\pm$ 0.5 bB	1.86 $\pm$ 0.04 aA

^zDifferent lowercase letters indicate statistically significant differences between Al treatments for the same cultivar and exposure time via Tukey's HSD at $P \leq 0.05$.

^yDifferent uppercase letters indicate differences between exposure time for the same cultivar and similar Al treatment via Tukey's HSD at $P \leq 0.05$.

7A). This figure permitted an optimum visualization of physiological and biochemical phenotype separation between the Al-tolerant ('Legacy') and Al-sensitive ('Bluegold') cultivars. Note that in the non-Al-treated plants, the 'Bluegold' leaves displayed different leaf phenotypes compared with 'Legacy' and their clusters were separated into two independent groups (Fig. 7A, small circles). In addition, these results make it possible to conclude that the differences observed in Al-treated plants are caused by this stress and are unaffected by other biological factors, like senescence. The PCA also revealed that the first six highest-ranking components accounted for 97.9% of the total variance within the dataset (Fig. 8A), including the most influential physiological and biochemical measurements with the highest loading values on components 1, 2, and 3 (Fig. 8, B–D). Much of the variation in the dataset (82.2%) is explained by the first of the three principal components. The

assessment of the two first components allowed consistent classification of the samples in tolerant and sensitive cultivars under the different Al treatments and time during the experiment (Fig. 7, A–D).

COMPONENT 1. Component 1 (PCA 1) accounted for 41% of the variance, indicating a strong differential response in plants grown under Al and non-Al conditions (Fig. 7, A–C). PCA 1 was mostly influenced by modifications in the amounts of chlorophyll *b* (associated with the antenna complex of PSII), total chlorophyll, total carotenoids, the ratio of chlorophyll *a/b*, NPQ (heat dissipation of energy from PSII), and TSC amounts (products derived of the Calvin cycle and sugar metabolism) (Fig. 8B).

COMPONENT 2. Component 2 (PCA 2) comprised 25.5% of the variance within the dataset, indicating a strong differential response of plants grown in conditions of Al stress and

Fig. 5. Total chlorophyll content [Chl *a* + *b* (left figures)] and carotenoid content (right figures) in two highbush blueberry cultivars with contrasting Al tolerance (Al-tolerant 'Legacy' and Al-sensitive 'Bluegold') grown under increasing Al treatments at different times. Values are an average of six replicates each $\pm$ SE. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al treatments for the same cultivar and exposure time. Different uppercase letters indicate differences (Tukey's HSD at $P \leq 0.05$) between exposure time for the same cultivar and similar Al treatment.

non-Al-treated plants (Fig. 7, A and B). The scatter plot of the first principal component versus the second ones revealed that leaf samples from non-treated plants collected during the time course displayed different phenotypes in both cultivars, and that they cluster in two independent groups, thus identifying the phenotype of non-treated plants from each cultivar studied during the time period (Fig. 8A). Furthermore, PCA 2 made it possible to distinguish different response patterns to Al stress in the 'Legacy' and 'Bluegold' leaves during the experiment (Fig. 7). At day 7 of treatment, leaves from both highbush blueberry cultivars treated with different Al doses exhibited the most significant modifications to the leaf physiological and biochemical phenotypes compared with the control leaves and those from 14 and 20 d of Al stress (Fig. 7, B–D). Interestingly, PCA 2 demonstrated and confirmed that 'Bluegold' leaves were more affected by Al stress than 'Legacy' leaves (Fig. 7B). Among the physiological and biochemical parameters studied in the response to Al stress in leaves, alterations in the photochemical performance of PSII (Φ PSII and ETR) and the Al uptake were the most influential in the second component (Fig. 8C).

COMPONENT 3. Component 3 (PCA 3) made up 15.7% of the variance, and it corroborated differences in the physiological and biochemical phenotypes observed in the leaves of both cultivars after 7 d of Al treatment and detected in the second principal component (data not shown). Component 3 was affected by modifications in the RSA and the amount of chlorophyll *a*, and was also influenced by physiological and biochemical modifications observed in components 1 and 2, as shown in Fig. 8D. Some examples are the total amounts of

carotenoids and soluble sugars observed in component 1 and the Al uptake detected in component 2 (Fig. 8, B and C).

Discussion

Our results based on root LP and RSA demonstrated that 'Legacy' was the most Al-tolerant, while 'Bluegold' was the most Al-sensitive cultivar (Figs. 2 and 3). This assumption is also supported by the lower RGR under Al treatments of 'Bluegold' in comparison with 'Legacy' (Table 1). Standard growth parameters such as relative growth reduction in roots and shoots have been used as a marker of Al toxicity in rice (*Oryza sativa*) cultivars (Sivaguru and Paliwal, 1993). 'Legacy' accumulated a lower Al content in the roots than 'Bluegold', showing typical behavior for Al-tolerant and Al-sensitive cultivars, respectively. It is reported that after exposure to Al, an Al-sensitive *T. aestivum* genotype accumulates about 8-fold more Al in the root apex than an Al-tolerant genotype (Rincón and Gonzales, 1992). This higher Al accumulation in roots corresponded well with a higher LP in the most sensitive cultivar (Bluegold). Lipid peroxidation is a typical symptom of oxidative stress, and is considered a general index of oxidative membrane injury (Jones et al., 2006; Yamamoto et al., 2001). Similarly, it is accepted that membrane LP is an early event under Al toxicity (Giannakoula et al., 2008; Yamamoto et al., 2001). Furthermore, our results indicated that LP is also a manifestation of Al toxicity in days.

On the other hand, the higher root LP shown by 'Bluegold' was not accompanied with higher RSA. On the contrary, 'Legacy' showed very low LP compared with 'Bluegold' under

Fig. 6. Radical scavenging activity measured as Trolox equivalents [TE (left figures)] and total soluble carbohydrates (right figures) in the leaves of two highbush blueberry cultivars with contrasting Al tolerance (Al-tolerant 'Legacy' and Al-sensitive 'Bluegold') grown under increasing Al treatments for different times. Values are average of six replicates each $\pm$ SE. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al treatments for the same cultivar and exposure time. Different uppercase letters indicate differences (Tukey's HSD at $P \leq 0.05$) between exposure time for the same cultivar and similar Al treatment.

Al toxicity and a high RSA (Figs. 2 and 3). These results suggest an activation of the defense mechanism through RSA in 'Legacy' at 7 d, which afterward seems to have contributed to a decrease in LP. In Al-tolerant *T. aestivum* and *Z. mays*, low LP was also associated with high antioxidant capacity (Boscolo et al., 2003; Dong et al., 2002; Giannakoula et al., 2008). Thus, the high radical scavenging capacity of 'Legacy' seems to be an important feature related to its Al tolerance. Another important feature related to Al tolerance in 'Legacy' is the lower Al accumulation in roots than in 'Bluegold' under Al stress, as found in other plants (Rout et al., 2001). This suggests that an Al exclusion mechanism might be activated in 'Legacy'. Such a mechanism may involve the exudation of organic acids from the radical apices and subsequent chelation of the rhizosphere, as described for other plants (Kochian, 1995).

Our work showed that over the long term, Al-treatment effects on the photosynthetic apparatus were more pronounced in 'Bluegold' (Al sensitive) than in 'Legacy' (Al tolerant) (Table 1). Interestingly, a slight decrease in the photochemical parameters of PSII was only found in 'Legacy' at the beginning (7 d), but not in the long term, where a recovery was evident (Table 2). This may be explained by the high RSA of leaves in this cultivar (about 59% more than the sensitive ones). In addition, a high RSA may help to minimize membrane injury, protecting the thylakoid membranes and hence increasing its Al tolerance. On the other hand, 'Legacy' showed intrinsically higher TSC in leaves, decreasing with Al treatments at all times. In contrast, Giannakoula et al. (2008) reported an

increase in TSC in shoots/leaves of Al-tolerant *Z. mays* compared with sensitive ones, which could be related to osmoregulation. By contrast, seedlings of a non-tolerant *Z. mays* hybrid accumulated a greater quantity of carbohydrates in the apex of seminal roots in the presence of Al with respect to the tolerant genotype (Hoshino et al., 2000). This suggests that carbohydrate accumulation may not always be interpreted as a sign of Al tolerance in this species. It has also been taken into account that the causes of Al tolerance may not be attributed to only one factor but to various concomitant physiological processes (Hoshino et al., 2000).

It is important to mention that the photoprotective carotenoids increased in parallel with the higher NPQ after the 7 d of Al treatment in 'Legacy'. The role of carotenoids in the thermal energy dissipation of PSII in stressed plants is well established (Demmig-Adams and Adams, 1996).

Recently, Reyes-Díaz et al. (2009) studied the changes in the photochemical efficiency of PSII in highbush blueberry genotypes subjected to short-term (hours) Al stress. They found that the photochemical parameters decreased substantially due to Al treatments in 'Bluegold' followed by 'Legacy', while in 'Brigitta', a better PSII performance was found. According to this behavior, 'Brigitta' was considered an Al-tolerant cultivar and 'Bluegold' highly sensitive to Al stress in the short term. Interestingly, our results indicated that in long-term experiments, Al tolerance of these cultivars changed, with 'Legacy' being the most Al-tolerant followed by 'Brigitta' (not shown), whereas 'Bluegold' maintained its sensitivity to Al stress. This

Fig. 7. Principal component analysis (PCA) representing physiological and biochemical measurements from the leaves of two highbush blueberry cultivars with contrasting Al tolerance (Al-tolerant 'Legacy' and Al-sensitive 'Bluegold') growing under increasing Al treatments at different times. Principal component analysis was used to cluster leaf samples from the control and Al-treated plants of two blueberry cultivars at different times during the experiment. (A) A scatter plot of the first component against the second component; it shows the averages of the whole pool of data analyzed from the investigated samples. In A, a general overview and an introductory figure to the sequence of results in B through D are shown. Components 1 and 2 were chosen for the best visualization of leaf metabolic phenotype separation and include 66.5% of the information derived from physiological and biochemical variations. Leaves of 'Bluegold' (white squares enclosed in a black dotted-line cluster) displayed a metabolic phenotype distinct from leaves of 'Legacy' plants (black circles in a black long dash-line cluster) in A. (B–D) Visualization at days 7, 14, and 20 of each Al exposure for the two highbush blueberry cultivars, respectively.

suggests that 'Legacy' possesses a long-term dependent adjustment mechanism to Al stress. Therefore, the results obtained in the short-term experiments regarding the effect of aluminum stress in highbush blueberry cultivars may not be good predictors of the degree of tolerance to this stress given the possible existence of acclimation mechanisms that can be expressed in the longer term, as happens with other types of stresses (Taedo, 2000).

A PCA was conducted to visualize and classify the leaf physiological and biochemical phenotypes of the highbush blueberry cultivars studied at different times during the experiment. The PCA gave us information about which leaf physiological and biochemical features used during the experiment are the most determinant in response to Al toxicity. Considering the whole experimental data set, this analysis showed a clear separation and clustering between 'Legacy' and

'Bluegold' due to changes in many physiological and biochemical features, confirming Al tolerance and Al sensitivity, respectively. These leaf physiological and biochemical phenotype classifications were evident at 7 and 14 d, but not at 20 d. Another important consideration is that photosynthetic apparatus performance was the most affected by Al toxicity, followed by pigment biosynthesis and antioxidant activity in highbush blueberry, confirming the evidence mentioned above.

In summary, the results presented in this study allow us to conclude that among the underlying processes affected by Al toxicity in the highbush blueberry, growth, LP, and the photochemical performance of PSII followed by modifications of photosynthetic pigment contents have a preponderant importance after long-term Al stress. In addition, antioxidant activity also plays an important role in the long-term acclimation

Fig. 8. Variance of the highbush blueberry data explained using principal component analysis (A) and the most influential biophysical and biochemical measurements that contribute to components 1 (B), 2 (C), and 3 (D) derived from the analysis. TSC = total soluble carbohydrates, RSA = radical scavenging activity, ETR = electron transport rate, NPQ = non-photochemical quenching, phi PSII = effective quantum yield, Chl = chlorophyll.

response mechanisms against Al stress in leaves and roots of the highbush blueberry.

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