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RESEARCH ARTICLE

Protective Effect of Methyl Jasmonate on Photosynthetic Performance and Its Association with Antioxidants in Contrasting Aluminum-Resistant Blueberry Cultivars Exposed to Aluminum

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Abstract

Methyl jasmonate (MeJA) protective effect on photosynthetic performance and its association with antioxidants in two highbush blueberry (*Vaccinium corymbosum* L.) cultivars with contrasting aluminum (Al) resistance under Al toxicity was determined. Legacy (Al-resistant) and Bluegold (Al-sensitive) cultivars were subjected to control, MeJA, Al, and their combination (Al+MeJA) for 0, 24, and 48 h under greenhouse conditions. Al concentration, oxidative damage (malondialdehyde (MDA) and H_2O_2 concentrations), antioxidant activity (AA), superoxide dismutase (SOD) and catalase (CAT) activities, total polyphenols (TPP), chlorogenic acid, and in vivo photosynthetic performance were determined. The exposure to Al toxicity increased the Al concentration (up to 15-fold) and oxidative damage (up to 5.5-fold) compared to the control at 48 h, despite the antioxidant responses (SOD and CAT activities) were increased (up to 4-fold), mainly in the Al-sensitive cultivar at 48 h. Concomitantly, the photosynthetic performance was strongly reduced in the Al-sensitive cultivar (1.6-fold), while the Al-resistant cultivar was more stable during the experiment. However, when cultivars were exposed to Al+MeJA, the Al accumulation and oxidative damage strongly decreased (7-fold and 1.6-fold, respectively), increasing AA, SOD and CAT activities, and TPP in both cultivars during the first hours of Al exposure. The MeJA application decreased Al uptake and stimulated antioxidant pathways, which may counteract the toxic Al effects, protecting the photosynthetic apparatus in both cultivars, being more evident in the Al-sensitive cultivar.

Keywords Chlorophyll fluorescence · Methyl jasmonate · Photosynthesis · Pigments · Oxidative stress

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1 Introduction

The acidity of soils (pH < 5.5) allows the solubilization of aluminum (Al) complexes to phytotoxic aluminum (Al^{3+}) (Meriño-Gergichevich et al. 2010; Ryan and Delhaize 2010), which limits physiological and metabolic plant functions (Li et al. 2012). The toxic effect of Al^{3+} appears early in roots, inhibiting their cell division and elongation. This prevents water and nutrient absorption, essential for the cellular and plant metabolism, which lead to decrease plant productivity and quality (Kochian et al. 2015). At the cellular level, the primary target of Al^{3+} is the plasma membrane and organelle, where Al^{3+} binds to the negative-charged phospholipids leading to a reduction of membrane fluidity (Meriño-Gergichevich et al. 2010), disrupting the membrane functions, which may lead to an increase of reactive oxygen species (ROS) and thereby oxidative stress and lipid peroxidation (LP) (Inostroza-Blancheteau et al. 2008). To counteract the Al toxic

damage induced by oxidative stress, plants can activate antioxidant systems (Inostroza-Blancheteau et al. 2008; Meriño-Gergichevich et al. 2015). Enzymatic activities as catalase (CAT), peroxidase (APX), and superoxide dismutase (SOD) and non-enzymatic compounds have been reported as ROS scavengers in the Al detoxification in many plants (Inostroza-Blancheteau et al. 2008; Meriño-Gergichevich et al. 2015). However, when the antioxidant systems are not sufficiently effective to counteract the toxic Al effects, main processes such as photosynthetic performance, including the photochemical activities of photosystem II (PSII) (Jiang et al. 2008; Hasni et al. 2015a) and CO_2 assimilation, are strongly affected (Chen et al. 2005a; Yang et al. 2015; Banhos et al. 2016). Although stomatal and non-stomatal limitations have been suggested as important factors for the Al-dependent depression of photosynthesis (Quinteiro Ribeiro et al. 2013), the increased closure of PSII reaction centers, reduced capacity for PSII-dependent electron transport (Chen et al. 2005a; Moustaka et al. 2016), and/or increased photorespiration (Chen et al. 2005a) have been recognized as critical causes for the limited CO_2 assimilation in plants exposed to Al toxicity.

Indeed, a number of studies in various herbaceous and woody plants have shown that Al toxicity decreases photochemical efficiency of PSII, measured as the effective quantum yield of PSII (Φ_{PSII}) in wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), and *Citrus grandis* plants (Jiang et al. 2008; Moustaka et al. 2016). It has been reported that the maximal photochemical efficiency of PSII, measured as the maximal quantum yield of PSII (F_v/F_m), and the Φ_{PSII} were also reduced in tangerine (*Citrus reshni*) (Chen et al. 2005b) and in tobacco (*Nicotiana tabacum*) seedlings (Li et al. 2012) exposed to toxic doses of Al (up to 2 mM). Furthermore, our previous studies also found a strong decrease of Φ_{PSII} in the sensitive highbush blueberry cultivar when plants were subjected to Al toxicity in a nutrient solution at short and long terms (Reyes-Díaz et al. 2009, 2010). Likewise, a significant inhibition of the photosynthetic CO_2 assimilation (up to 77%) was reported in Al-treated barley (*Hordeum vulgare* L.) (Ali et al. 2011), highbush blueberry (Reyes-Díaz et al. 2011), and eucalyptus (*E. grandis* × *E. urophylla*, *E. urophylla* × *E. camaldulensis*, *and E. urophylla*) (Yang et al. 2015). It is also reported that stomatal conductance and water-use efficiency (WUE) were strongly inhibited in soybean (*Glycine max*), reaching up to 67% (Zhang et al. 2007), while in cacao (*Theobroma cacao*) plants reached up to 40% (Quinteiro Ribeiro et al. 2013) as a result of Al toxicity. Some studies strongly have suggested that the decline of photosynthesis at high Al levels might be due to a reduced photosynthetic electron transport at PSI level (Lidon et al. 1999; Hasni et al. 2015b). Photosynthetic pigments (chlorophylls and carotenoids) are strongly reduced by the effect of Al toxicity in various plants such as cacao (Quinteiro Ribeiro et al. 2013) and rye (Silva et al. 2012). In contrast, a significant increase of both chlorophyll *a* (Chl*a*) and chlorophyll *b* (Chl*b*) contents was reported in Al-treated maize (*Zea maize*) plants (Lidon et al. 1999). These conflicting results may reflect a differential-, species-, and/or genotype-specific responses to Al toxicity.

Several studies have reported that jasmonates (JAS) such as jasmonic acid (JA) or methyl jasmonate (MeJA) have a protective effect on plants against various environmental stresses such as salt stress (Ismail et al. 2012), UV stress (Larronde et al. 2003), and toxic metals (TM) (Keramat et al. 2009; Ulloa-Inostroza et al. 2017). However, studies about the protective effects of JAS on plants under Al toxicity are scarce, with the exception of a few reports like Xue et al. (2008) in tora (*Cassia tora*), Roselló et al. (2015) in rice (*Oryza sativa*), and Ulloa-Inostroza et al. (2017) in highbush blueberry. The reports on the interaction between TM and MeJA on plants have been controversial. Thus, favorable plant responses under TM have been observed with the MeJA application on herbaceous and woody plant species, showing an increase of antioxidant responses (enzymatic and non-enzymatic) and a decrease in the oxidative stress (LP and ROS) (Piotrowska et al. 2009; Keramat et al. 2009; Chen et al. 2014; Ulloa-Inostroza et al. 2017). In contrast, negative effects by higher MeJA dose application with arsenic (As) in peppers (*Capsicum frutescens*) and cadmium (Cd) in *Kandelia obovata* has been reported, where increased LP and content of hydrogen peroxide (H_2O_2) and decreased antioxidant responses were found (Yan et al. 2013; Chen et al. 2014). Important responses to JAS application are the changes in the photosynthetic apparatus functionality in *K. obovata* and pepper seedlings under cadmium stress (Yan et al. 2013; Chen et al. 2014), runner bean (*Phaseolus coccineus*) with copper (Cu) exposition (Hanaka et al. 2016), and canola (*Brassica napus*) and soybean with As and nickel (Ni) stress, respectively (Farooq et al. 2016; Sirhindi et al. 2016). These reports showed protective effect of MeJA, controlling the decrease of photosynthetic performance provoked by TM stress. Despite the intensive research described above, the role of plant hormones, especially MeJA in mitigating toxic metal effects on plant physiology, is poorly understood. Thus, the aim of this study was to determine the protective effect of MeJA on photosynthetic performance and its association with antioxidants in two highbush blueberry cultivars with contrasting Al resistance exposed to Al toxicity.

2 Materials and Methods

2.1 Plant Material

Two-year-old blueberry (*Vaccinium corymbosum*) cultivars with contrasting Al resistance (Legacy, Al-resistant, and

Bluegold, Al-sensitive), according to Reyes-Díaz et al. (2009, 2010), were used in this study. They were provided by Berries San Luis, Quillém, Lautaro, Chile (38° 29′ S, 72° 23′ W). The cultivars were produced in vitro and grown in a substrate of oat shell:sawdust:pine needles at a 1:1:1 proportion. Uniform size and healthy plants were selected for the experiment.

2.2 Growth Conditions in Nutrient Solution

The experiment was carried out in a greenhouse of the Instituto de Agroindustria, Universidad de La Frontera, Temuco, Chile (38° 45′ S, 72° 40′ W). Blueberry cultivar shrubs were grown in 18-L pots with Hoagland's nutrient solution under greenhouse conditions: temperature 25 ± 0.2 °C, 400 μmol photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD), and 70% relative humidity. The plants were pre-conditioned during 7 days in nutrient solution aerated continuously with an aquarium pump.

2.3 Treatments and Experimental Design

The experimental design was completely randomized with three replicates per treatment giving a total of 54 plants of both cultivars. At the start of the experiment, Al was applied as $AlCl_3$ (100 μM) with Al^{3+} 26.8% as free metal (Geochem speciation, Shaff et al. 2010) to the Hoagland nutrient solution (Hoagland and Arnon 1959), maintaining a pH of 4.5 and at 20 °C under continuous aeration. The MeJA was applied by spraying on leaves according to Ulloa-Inostroza et al. (2017). Plants were treated with and without toxic Al and MeJA application as follows: (a) without Al and MeJA (control), (b) 5 μM MeJA (MeJA), (c) 100 μM Al (Al), and (d) 100 μM Al + 5 μM MeJA (Al+MeJA) for 0, 24, and 48 h. After each time, in vivo photosynthesis and fluorescence measurements were performed. Leaves were harvested and separated in two groups, (1) fresh material for MDA and (2) frozen material at − 80 °C for further chemical and biochemical analyses.

2.4 Chemical Analysis

The Al concentration in leaves was determined after drying the plant material in a forced air oven (70 °C) until obtaining dry weight, which was weighed and incinerated at 500 °C for 8 h and digested with 2 M hydrochloric acid. The Al concentrations were determined in a spectrophotometer simultaneous multi-element atomic absorption (model 969; UNICAM, Cambridge, UK) according to Sadzawka et al. (2004).

2.5 Biochemical Determinations

The malondialdehyde (MDA) concentration was determined in fresh leaves as an indicator of oxidative stress. Thiobarbituric acid reacting substance (TBARS) assay was used according to the modified method by Du and Bramlage (1992). The quantification of the MDA was measured at 532, 600, and 440 nm in a UV–VIS spectrophotometer. MDA equivalent (nmol/mL) was calculated as reported by Du and Bramlage (1992) as follows: $[[(A_{532} - A_{600}) - [(A_{440} - A_{600})$ (MA of sucrose at 532 nm/MA of sucrose at 440 nm)]]/ 157,000]10^6 and expressed as MDA nmol g^{-1} FW.

The H_2O_2 concentration was measured at 390 nm in leaf samples in a UV–VIS spectrophotometer according to Ulloa-Inostroza et al. (2017). It was expressed as H_2O_2 μmol g^{-1} FW.

The total AA in leaves was determined by using the 2.1-diphenyl1-1-picrylhydrazyl (DPPH) method according to Chinnici et al. (2004) and Reyes-Díaz et al. (2010). The leaf extracts were measured at 515 nm in a UV–VIS spectrophotometer and were expressed by Trolox equivalents (TE).

The SOD was assayed according to Giannopolitis and Ries (1977) by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm in a UV–VIS spectrophotometer. Enzymatic activity was expressed as protein content determined by Bradford's method (Bradford 1976).

The CAT activity was measured by monitoring the conversion of H_2O_2 to H_2O and O_2 (Pinhero et al. 1997). The quantification enzyme activity was estimated by H_2O_2 consumption for 60 s at 240 nm in a UV–VIS spectrophotometer. Enzymatic activity values were standardized by the total protein content by the Bradford method (Bradford 1976).

Total polyphenols (TPP) were determined with the Folin-Ciocalteu reagent using the method described by Slinkard and Singleton (1977). The absorbance of all samples was measured at 765 nm using a UV–VIS spectrophotometer and the results were expressed as milligrams of chlorogenic acid equivalent per gram of fresh weight.

Chlorogenic acid was measured by HPLC analysis as described by Ribera et al. (2010). The signals were detected at 320 nm. The mobile phase was (A) acidified water (phosphoric acid 10%) and (B) 100% acetonitrile, and the gradient was as follows: 0–9 min of 100% A, 9.1–19.9 min of 81% A and 19% B, and 20–25 min of 100% B.

2.6 Photosynthetic Performance

The in vivo net photosynthesis and stomatal conductance were measured with a portable infrared gas analyzer (Licor-6400, LI-COR Bioscience, Inc., Lincoln, NE, USA) according to Reyes-Díaz et al. (2011). Intrinsic water-use efficiency of photosynthesis

(WUE_{PH}) was calculated through the photosynthesis and stomatal conductance according to Locke and Ort (2014), where:

WUE_{PH} = Photosynthesis (μmol CO_2 m^{-2} s^{-1})/Stomatal conductance (mol H_2O m^{-2} s^{-1}).

The Chl*a* fluorescence parameters of PSII as the maximal quantum yield of PSII [$F_v/F_m = (F_m - F_o)/F_m$], effective quantum yield of PSII (Φ_{PSII}), electron transport rate (ETR), and non-photochemical quenching (NPQ) were measured according to Reyes-Díaz et al. (2009, 2010) with a fluorometer (FMS 2; Hansatech Instruments, King's Lynn, UK) and calculated as described in Maxwell and Johnson (2000).

2.7 Pigment Analysis

Photosynthetic pigments (chl*a*, chl*b*, beta carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin, and neoxanthin) were extracted from leaves with 100% HPLC grade acetone at 4 °C under a green dim light and the extracts were centrifuged at 4 °C and quantified by HPLC-DAD according to Garcia-Plazaola and Becerril (1999) with minor modifications, where the signals were detected at 295, 410, and 445 nm and the data were expressed as micro-grams per gram of fresh weight (μg g^{-1} FW). The mobile phase was (A) acetonitrile:methanol:Tris-buffer 0.1 M pH 8 (84:2:14) and (B) methanol:ethyl acetate (68:32). The pigments were eluted using a linear gradient from 100% A to 100% B for the first 12 min, followed by an isocratic elution with 100% B for the next 6 min. This was followed by a 1-min linear gradient from 100% B to 100% A and an isocratic elution with 100% A for a further 6 min to allow the column to re-equilibrate with solvent A prior to the next injection.

2.8 Statistical Analysis

The results are based on three replicates. All data passed the normality and equal variance tests according to Kolmogorov-Smirnov normality test. Statistical data analyses were carried out by three-way ANOVA (where factors were treatment, time, and cultivar) using Sigma Stat 2.0 software. Significantly, different means were determined using Tukey's multiple comparison test (statistical significance $P \leq 0.05$).

3 Results

The statistically significant interaction among cultivars, times, and treatments was observed for Al concentration ($P \leq 0.001$). In both cultivars, the Al concentrations in leaves were increased under Al^{3+} treatment during the first hours of exposure to Al toxicity compared to the control ($P \leq 0.05$; Fig. 1), being the differences higher and statistically significant in the Al-sensitive cultivar (Bluegold) ($P \leq 0.05$; Fig. 1b). The Al-resistant cultivar (Legacy) showed an increase up to 1.7-fold Al concentration at 48 h ($P \leq 0.05$; Fig. 1a), while the Al-sensitive cultivar exhibited the highest Al concentration (14-fold), when exposed to the Al treatment in comparison to control ($P \leq 0.05$; Fig. 1b). In the resistant cultivar, Al concentration was significantly decreased (0.8-fold) as a result of MeJA application under Al toxicity at 48 h compared to the Al treatment ($P \leq 0.05$; Fig. 1a), while in Bluegold the Al concentration was also reduced by MeJA application, reaching similar values to the control ($P \leq 0.05$; Fig. 1b).

For MDA concentration, there was a statistically significant interaction among cultivars, times, and treatments ($P \leq 0.001$). The MDA was increased as a result of Al^{3+} exposure

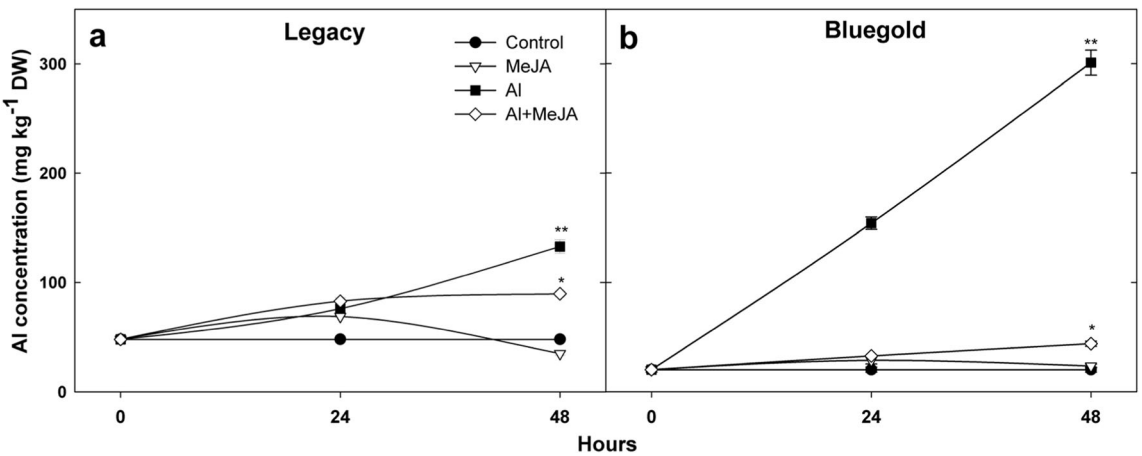

Fig. 1 Aluminum accumulation in leaves (mg kg^{-1} DW) of blueberry cultivars with contrasting Al resistance exposed separately to Al (100 μM Al) and MeJA (5 μM MeJA) and to a combination of both (Al+MeJA) at different times. A Hoagland nutrient solution was used as control. **a** Al-resistant and **b** Al-sensitive cultivars. Mean values ± S.E. were calculated from 3 replicates. Asterisks indicate statistically significant differences at $P \leq 0.05$ (*) and $P \leq 0.001$ (**)

in a time-dependent manner in both cultivars, and the increase was much higher in the Al-sensitive cultivar ($P \leq 0.05$; Fig. 2a and b). In both cultivars, the Al+MeJA application reduced the MDA to values similar to that observed in the control at 24 h of Al exposure ($P \leq 0.05$; Fig. 2a and b). However, the MDA decreased sharply at 48 h for about 21% and 37% in the Al-resistant and Al-sensitive cultivars respectively, compared to the Al treatment ($P \leq 0.05$; Fig. 2a and b). The H_2O_2 concentration was significantly higher in the Al-resistant cultivar in all treatments than in the Al-sensitive cultivar ($P \leq 0.05$; Fig. 2c and d). A small increase (~ 8%) in H_2O_2 concentration was observed in the Al-resistant cultivar after 48-h treatment with MeJA and Al^{3+} treatments in comparison to control ($P \leq 0.05$; Fig. 2c). The H_2O_2 concentration in the Al-sensitive cultivar did not change through the experiment (Fig. 2d).

In both cultivars, the total AA in leaves was enhanced under Al^{3+} treatment during the first hours of Al exposure compared to the control, being higher in the Al-resistant cultivar in each treatment during the experiment ($P \leq 0.05$; Fig. 3a and b). In the Al-resistant cultivar, a strong increase (31%) of AA was observed in samples treated with Al^{3+} treatment at both time points of measurements compared to the control ($P \leq 0.05$; Fig. 3a). In the Al-sensitive cultivar, the Al treatment increased AA from 33 to 40% at 24 and 48 h, respectively compared to the controls ($P \leq 0.05$; Fig. 3b). This increase was around 14% lower as a result of combined Al+MeJA application in both cultivars at 24 h compared to Al treatment. Nevertheless, in both cultivars, the values for AA were statistically similar at 48 h to their respective values observed in plants treated with Al^{3+} treatment ($P \leq 0.05$; Fig. 3a and b).

A higher SOD activity was observed in the Al-sensitive than in the Al-resistant cultivar under control conditions ($P \leq 0.05$; Fig. 3c and d). Similar trend in the SOD activity was observed in both cultivars subjected to MeJA treatment ($P \leq 0.05$; Fig. 3c and d). The highest increase (1.6-fold) of SOD activity was found in the Al-resistant cultivar under the combined Al+MeJA treatment at 24 h, whereas at 48 h the SOD activity was reduced (12%) in relation to the control ($P \leq 0.05$; Fig. 3c). In the Al-sensitive cultivar, the SOD activity was gradually increased during the treatment, showing a strong increase of 33% under the $Al^{3+}$and 1.4-fold increase under the combined Al+

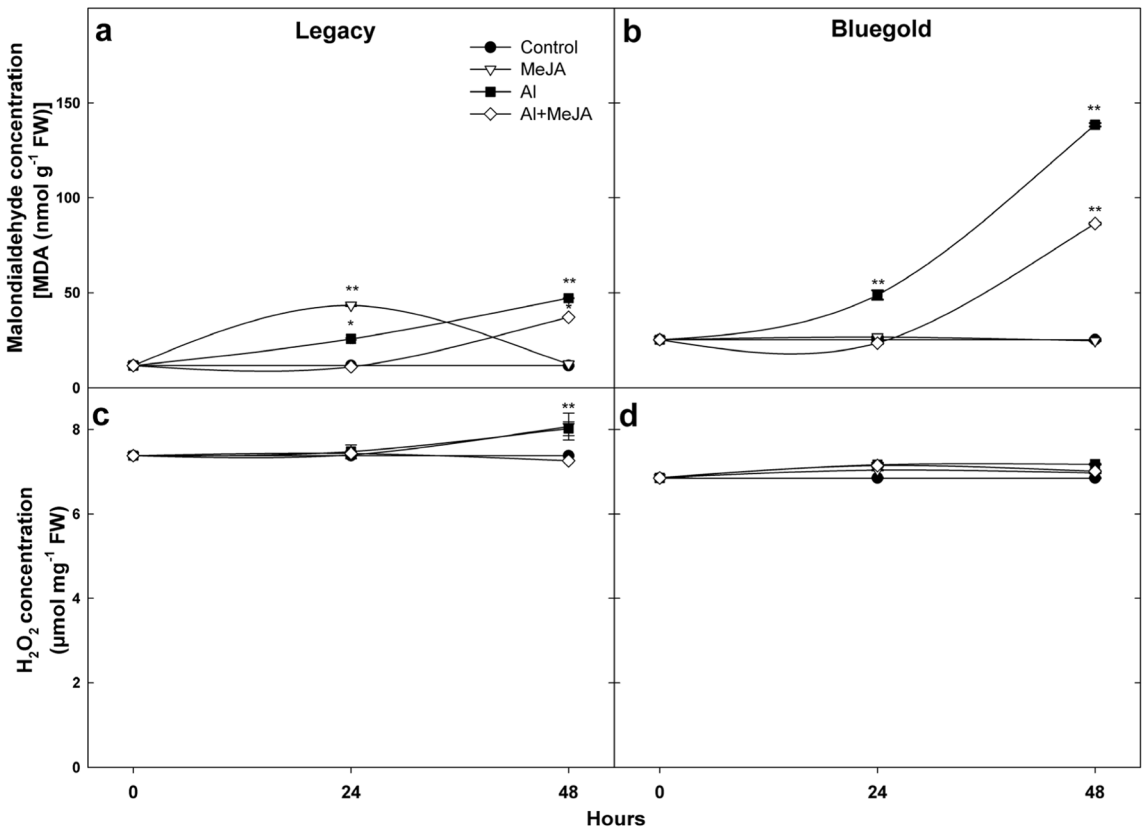

Fig. 2 The malondialdehyde (MDA) concentration (nmol g^{-1} FW) (**a**, **b**) and H_2O_2 concentration (µmol mg^{-1} FW) (**c**, **d**) of blueberry cultivars with contrasting Al resistance exposed separately to Al (100 µM Al) and MeJA (5 µM MeJA) and to a combination of both (Al+MeJA) at different times. A Hoagland nutrient solution was used as control. **a**, **c** Al-resistant and **b**, **d** Al-sensitive cultivars. Mean values ± S.E. were calculated from 3 replicates. Statistically significant levels were estimated at $P \leq 0.05$ (*) and $P \leq 0.001$ (**)

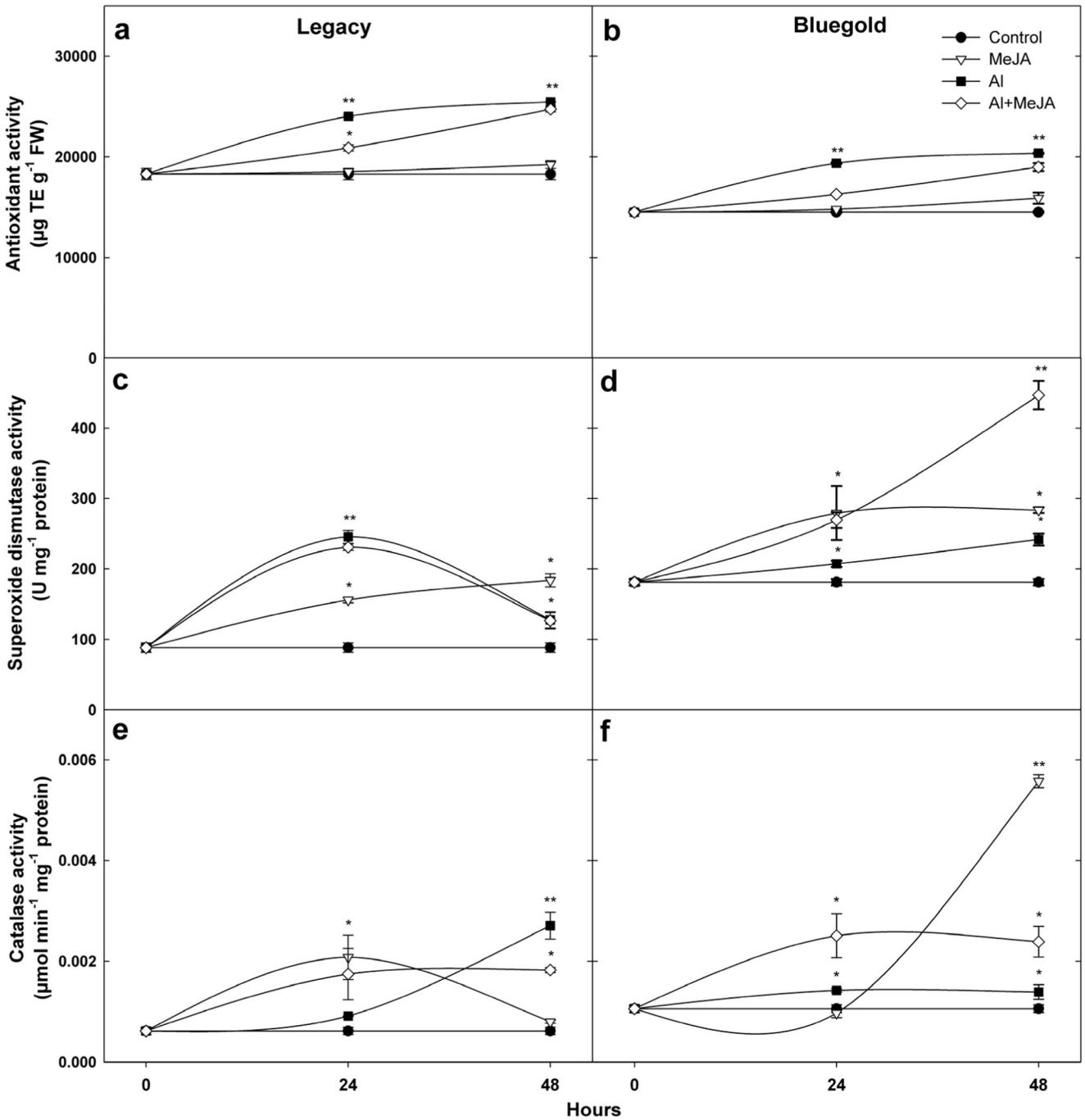

Fig. 3 The antioxidant activity (µg TE g^{-1} FW) (**a**, **b**), superoxide dismutase activity (U mg^{-1} prot) (**c**, **d**), and catalase activity (µmol min^{-1} mg^{-1} of prot) (**e**, **f**) of blueberry cultivars with contrasting Al resistance exposed separately to Al (100 µM Al) and MeJA (5 µM MeJA) and to a combination of both (Al+MeJA) at different times. A Hoagland nutrient solution was used as control. **a**, **c**, and **e** Al-resistant and **b**, **d**, and **f** Al-sensitive cultivars. Mean values ± S.E. were calculated from 3 replicates. Statistically significant levels were estimated at $P \leq 0.05$ (*) and $P \leq 0.001$ (**)

MeJA treatment at 48 h respectively, in comparison to the control ($P \leq 0.05$; Fig. 3d).

The CAT activity was generally higher in the Al-sensitive compared to the Al-resistant cultivar ($P \leq 0.05$; Fig. 3e and f). The application of MeJA treatment strongly stimulated (2.3-fold) the CAT activity after 24 h of treatment in the Al-resistant cultivar, while the highest increase of CAT activity (4.5-fold) in the Al-sensitive plants was observed after 48 h compared to their respective controls ($P \leq 0.05$; Fig. 3e and f). Exposure of both cultivars to toxic Al^{3+}resulted in a small

increase of CAT activity after 24 h (Fig. 3e and f), while after 48 h the increase of CAT activity was observed only in the Al-resistant cultivar (3.5-fold) ($P \leq 0.05$; Fig. 3e). The combined treatment (Al+MeJA) of both cultivars for 24 h resulted in similar enhancement (around 1.6-fold) of the CAT activity and the higher activity was maintained after 48 h compared to the controls ($P \leq 0.05$; Fig. 3e and f).

The concentrations of TPP were higher in the Al-resistant cultivar than in the sensitive one ($P \leq 0.05$; Fig. 4a and b). Treatment with MeJA resulted in strongly enhanced amounts of TPP in the Al-resistant cultivar after 24 h and 48 h compared to control, being lower in the Al-sensitive cultivar at the same time points ($P \leq 0.05$; Fig. 4a and b). In both cultivars, only a small and comparable increase of TPP concentration was observed under toxic Al^{3+} treatment (Fig. 4a and b). The combined (Al+MeJA) treatment caused an increased TPP of 20% after 48 h compared to the control Legacy cultivar ($P \leq 0.05$; Fig. 4a). In contrast, the increase of TPP in the Bluegold cultivar was higher (27%) after 24 h and even more at 48 h (39%) compared to the control plants ($P \leq 0.05$; Fig. 4b). Chlorogenic acid concentrations in the Al-sensitive cultivar were significantly reduced in plants treated for 24 h and 48 h with Al^{3+} and MeJA treatments, with the exception of MeJA treatment for 48 h when a sharp increase of the chlorogenic acid concentration was observed ($P \leq 0.05$; Fig. 4d). In contrast, the Al-resistant cultivar showed a transient increase in chlorogenic acid concentration after 24 h of exposure to Al+MeJA and Al^{3+}, which decreased after 48 h ($P \leq 0.05$; Fig. 4c).

The effects of toxic Al^{3+}, MeJA, and the combined Al+MeJA treatments on the photosynthetic performance of the contrasting Al-sensitive (Bluegold) and Al-resistant (Legacy) blueberry cultivars were assessed following the changes in CO_2 assimilation rates, stomatal conductance, and WUE_{PH}, as well as parameters derived from modulated chlorophyll fluorescence measurements. Gas exchange measurements revealed only a small decline in the rates of CO_2 assimilation and stomatal conductance in the Al-resistant cultivar exposed to toxic Al^{3+} (Fig. 5a and c), while the WUE_{PH} remained unaffected during the time course of treatment (Fig. 5e). The application of MeJA and the combined (Al+MeJA) treatments did not show any statistically significant effects on the same parameters measured in Al-resistant plants ($P \leq 0.05$; Fig. 5a, c, and e). Control plants of the Al-sensitive Bluegold cultivar exhibited lower CO_2

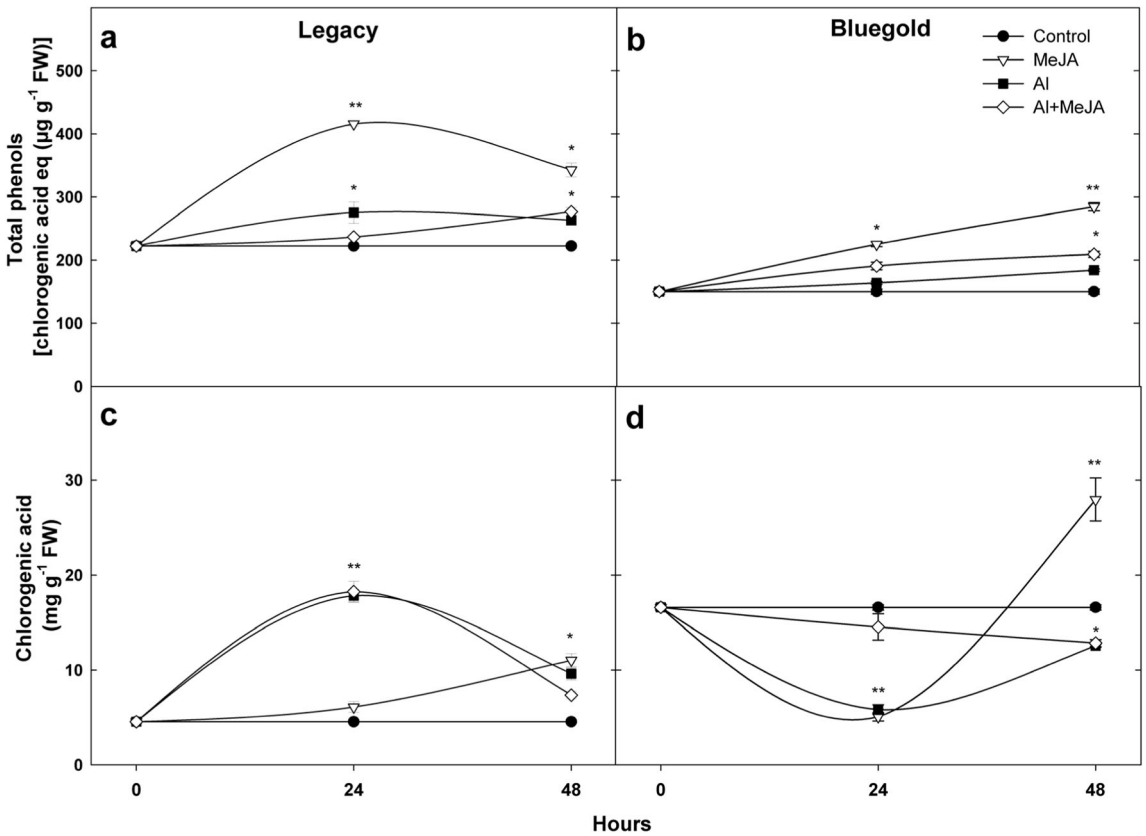

Fig. 4 Total polyphenols (chlorogenic acid (µg g^{-1} FW)) (**a**, **b**) and chlorogenic acid (mg g^{-1} FW) (**c**, **d**) of blueberry cultivars with contrasting Al resistance exposed separately to Al (100 µM Al) and MeJA (5 µM MeJA) and to a combination of both (Al+MeJA) at different times. A Hoagland nutrient solution was used as control. **a**, **c** Al-resistant and **b**, **d** Al-sensitive cultivars. Mean values ± S.E. were calculated from 3 replicates. Statistically significant levels were estimated at $P \leq 0.05$ (*) and $P \leq 0.001$ (**)

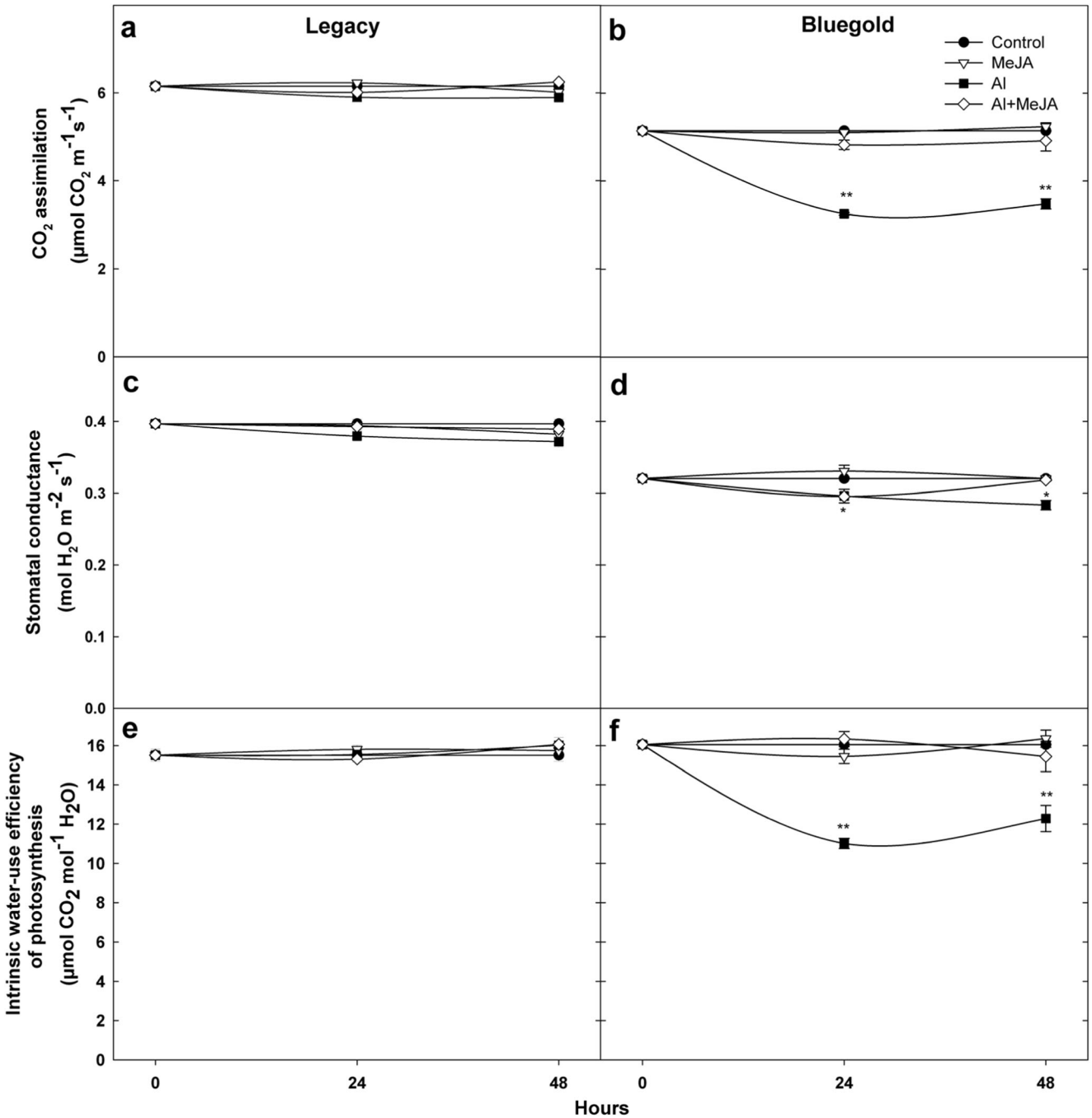

Fig. 5 The CO_2 assimilation (μmol CO_2 m^{-2} s^{-1}) (**a**, **b**), stomatal conductance (mol H_2O m^{-2} s^{-1}) (**c**, **d**), and water utilization efficiency (μmol CO_2 $mmol^{-1}$ H_2O) (**e**, **f**) of blueberry cultivars with contrasting Al resistance exposed separately to Al (100 μM Al) and MeJA (5 μM MeJA) and to a combination of both (Al+MeJA) at different times. A Hoagland nutrient solution was used as control. **a**, **c**, and **e** Al-resistant and **b**, **d**, and **f** Al-sensitive cultivars. Mean values ± S.E. were calculated from 3 replicates. Statistically significant levels were estimated at $P \leq 0.05$ (*) and $P \leq 0.001$ (**)

assimilation (17%) and stomatal conductance (20%) compared to Legacy plants (Fig. 5a and b). In contrast, to the Legacy cultivar, the Al^{3+} treatment of Bluegold plants resulted in a strong decrease of CO_2 assimilation (39%) and WUE_{PH} (31%) during the first 24 h of treatment, while stomatal conductance decreased gradually after 24 (10%) and 48 h (13%) in comparison to the control ($P \leq 0.05$; Fig. 5b, d, and f). Application of MeJA during the toxic Al^{3+} treatment (Al+MeJA) restored the CO_2 assimilation rates, stomatal conductance, and WUE_{PH} to similar levels of values observed in control plants ($P \leq 0.05$; Fig. 5b, d and f).

The values of F_v/F_m did not exhibit statistically significant variations in both cultivars and all treatments (0.83 ± 0.02 Legacy and 0.82 ± 0.01 Bluegold, data not shown) and were within the typical range for healthy plants. However, the values of the Φ_{PSII}, the rates of linear photosynthetic ETR, and the NPQ measured in the Al-sensitive Bluegold cultivar under control conditions were significantly lower by 20%, 21%, and 31% respectively, compared to the control plants of Al-resistant Legacy plants (Fig. 6). Moreover, in contrast to the Al-resistant cultivar, exposure of Bluegold plants to toxic Al^{3+} caused a drastic decrease of Φ_{PSII} (77%) and ETR (60%) values compared to non-treated control plants (Fig. 6b and d). Interestingly, the levels of NPQ remained unchanged under the same conditions (Fig. 6f). It should be also noted that the reduced Φ_{PSII} and ETR levels were substantially, but not completely recovered by the presence of MeJA during the Al^{3+} treatment (Al+MeJA) of Al-sensitive plants.

The contents of all pigments were significantly higher in the Al-sensitive than in the Al-resistant cultivar under control growth conditions ($P \leq 0.05$; Table 1). Treatment

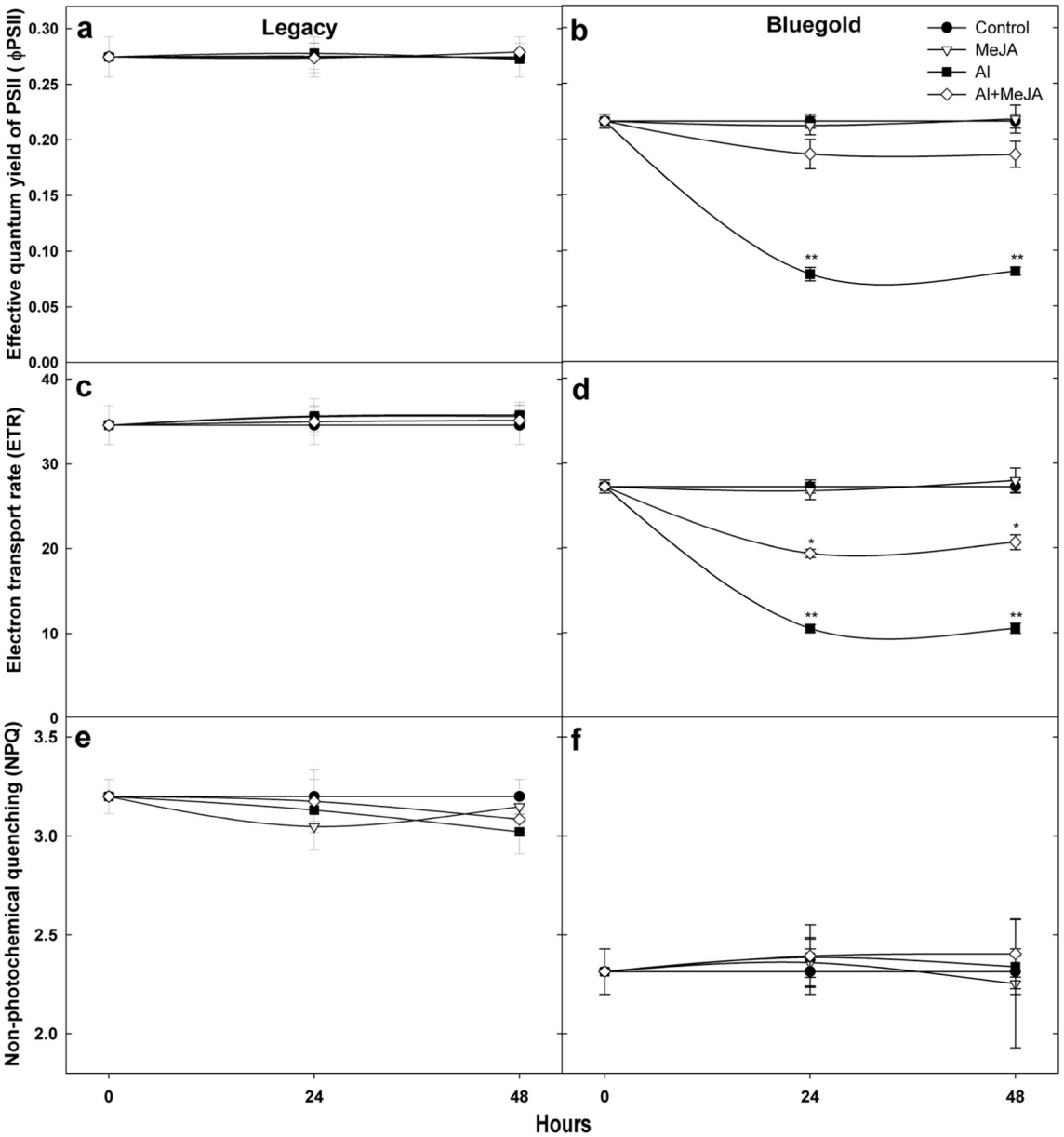

Fig. 6 The effective quantum yield of PSII (Φ_{PSII}) (**a**, **b**), electron transport rate (ETR) (**c**, **d**), and non-photochemical quenching (NPQ) (**e**, **f**), of blueberry cultivars with contrasting Al resistance exposed separately to Al (100 μM Al) and MeJA (5 μM MeJA) and to a combination of both (Al+MeJA) at different times. A Hoagland nutrient solution was used as control. **a**, **c**, and **e** Al-resistant and **b**, **d**, and **f** Al-sensitive cultivars. Mean values ± S.E. were calculated from 3 replicates. Statistically significant levels were estimated at $P \leq 0.05$ (*) and $P \leq 0.001$ (**)

Table 1 Effects of MeJA application on pigment concentration (μg g^{-1} FW) and composition in two blueberry cultivars with contrasting Al resistance exposed separately to Al (100 μM Al) and MeJA (5 μM MeJA) and to a combination of both (Al+MeJA) at different times.

Cultivar	Hour	Treatment	Chlorophyll a+b	Chlorophyll a/b	Beta carotene	Lutein	Neoxanthin	Violaxanthin	Antheraxanthin
Legacy	24	Control	598.1 ± 31.3 bA	1.5 ± 0.0 bA	110.8 ± 6.1 bA	240.9 ± 17.7 aA	64.6 ± 2.4 bA	78.3 ± 3.5 aA	3.8 ± 0.2 bcA
		MeJA	494.4 ± 25.1 bA	1.6 ± 0.0 bA	98.4 ± 5.1 bA	229.1 ± 5.4 aA	52.6 ± 3.2 cB	60.0 ± 3.5 bB	5.1 ± 0.3 aB
		Al	578.1 ± 27.9 bA	1.4 ± 0.1 bB	101.7 ± 6.4 bA	225.9 ± 7.0 aAB	53.8 ± 1.5 cB	62.7 ± 3.4 bB	3.1 ± 0.1 cA
		Al+MeJA	730.4 ± 32.1 aA	2.0 ± 0.1 aA*	148.9 ± 5.2 aA	279.7 ± 14.7 aA	80.1 ± 4.8 aA	87.9 ± 3.5 aA	4.5 ± 0.2 abB
	48	Control	598.1 ± 31.3 bA	1.5 ± 0.0 bA	110.8 ± 6.1 abA	240.9 ± 17.7 aA	64.6 ± 2.4 aA	78.3 ± 3.5 aA	3.8 ± 0.2 cA
		MeJA	509.0 ± 30.1 bA	1.5 ± 0.0 bA	106.0 ± 0.5 bA	225.2 ± 12.2 abA	50.8 ± 3.1 bB	57.2 ± 3.5 bB	6.7 ± 0.1 aA
		Al	520.4 ± 19.7 bA	2.0 ± 0.0 aA	102.5 ± 2.0 bA	176.0 ± 6.4 bB	46.0 ± 2.7 bB	55.3 ± 1.7 bB	3.4 ± 0.1 cA
		Al+MeJA	700.6 ± 7.7 aA	1.7 ± 0.0 bB*	128.0 ± 3.3 aB	269.3 ± 4.5 aA	71.4 ± 0.2 aAB	82.9 ± 1.5 aA	5.5 ± 0.0 bA
Bluegold	24	Control	888.7 ± 24.2 aA*	1.6 ± 0.0 aA	187.3 ± 2.9 aA*	332.3 ± 11.5 aA*	87.2 ± 2.5 aA*	89.9 ± 1.7 bA*	13.2 ± 0.7 bA*
		MeJA	853.6 ± 13.5 aA*	1.6 ± 0.0 aB	177.4 ± 9.5 aA*	330.0 ± 12.4 aA*	80.3 ± 0.8 aA*	82.4 ± 4.0 bA*	20.1 ± 0.1 aB*
		Al	807.2 ± 9.4 aA*	1.5 ± 0.0 aB	146.8 ± 3.7 bB*	348.7 ± 7.3 aA*	81.6 ± 3.6 aA*	106.2 ± 4.8 aA*	19.1 ± 1.1 aA*
		Al+MeJA	798.6 ± 25.2 aA*	1.5 ± 0.0 aA	145.0 ± 6.2 bB	351.6 ± 21.3 aA*	78.6 ± 2.5 aA	92.4 ± 4.0 bA	20.0 ± 0.9 aA*
	48	Control	888.7 ± 24.2 aA*	1.6 ± 0.0 aA	187.3 ± 2.9 aA*	332.3 ± 11.5 aA*	88.0 ± 4.1 aA*	91.2 ± 3.4 aA	12.2 ± 1.1 bA*
		MeJA	573.1 ± 23.4 cB	1.3 ± 0.0 cbA	108.7 ± 6.4 dB	270.7 ± 10.3 bB	60.3 ± 4.9 bB	59.1 ± 3.2 bB	24.6 ± 1.5 aA*
		Al	696.6 ± 23.7 bB*	1.8 ± 0.0 aA	143.1 ± 3.2 cB*	271.2 ± 10.3 bB*	60.1 ± 3.8 bB*	84.4 ± 3.9 aB*	12.5 ± 0.5 bB*
		Al+MeJA	792.2 ± 37.0 aA	1.5 ± 0.0 abA	155.3 ± 12.2 aB	324.5 ± 18.9 aA*	82.5 ± 2.1 aA*	89.3 ± 3.0 aA	13.1 ± 0.8 bB*

Mean values ± S.E. were calculated from 3 replicates. Different lowercase letters show statistically significant differences among the treatments at each time point of MeJA application (0, 24, or 48 h) in the same cultivar. Different capital letters show significant differences between time points of pigment analyses for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time points of analyses ($P \leq 0.05$). The start of experiment (0 time) for each parameter did not show statistically significant differences with the control at each time (24 and 48 h) and therefore they were not indicated in the table

of the Al-resistant cultivar with Al^{3+} for 24 h resulted in a decreased amounts of the xanthophyll pigments as follows: neoxanthin (16%), violaxanthin (20%), and antheraxanthin (18%), whereas after 48 h of treatment the amounts of lutein, neoxanthin, and violaxanthin decreased by approximately 28% in relation to the control ($P \leq 0.05$; Table 1). In the Al+MeJA treatment of the Legacy cultivar, all the pigments were increased with the exception of lutein after 24 h compared to Al^{3+} treatment ($P \leq 0.05$; Table 1). In addition, the total chlorophyll (Chl*a*+*b*) was also significantly higher after 24 h compared to the all treatments ($P \leq 0.05$; Table 1). In the Al-sensitive cultivar, beta carotene decreased at 24 and 48 h by approximately 23%, while the amount of antheraxanthin was increased at 24 h (around 40%) under the toxic Al^{3+} treatment and the combined Al+MeJA treatment relative to the control. Similarly, violaxanthin showed an increase of 18% in plants exposed to Al^{3+} treatment for 24 h in comparison to the control ($P \leq 0.05$; Table 1). On the contrary, a significant reduction in the amounts of chlorophyll rate (Chl*a*+*b*) (22%), beta carotene (24%), lutein (18%), and neoxanthin (32%) was observed after the Al^{3+} treatment for 48 h, while in the combined Al+MeJA treatment the amounts of these pigments were similar to the control ($P \leq 0.05$; Table 1).

4 Discussion

In agreement with a number of previous studies (Chen et al. 2005a; Yang et al. 2015; Banhos et al. 2016), the results presented in this study also demonstrated a significant reduction of CO_2 assimilation in the Al-sensitive cultivar (Bluegold) of highbush blueberry exposed to toxic Al^{3+} (Fig. 5b) and this response is cultivar specific (Reyes-Díaz et al. 2009, 2010; Ulloa-Inostroza et al. 2017). It has been suggested that the increased closure of PSII reaction centers might be the key limiting factor causing the reduced CO_2 assimilation in plants exposed to toxic Al^{3+} (Chen et al. 2005a; Li et al. 2012). However, in contrast to a number of previous studies examining the effects of toxic Al^{3+} in various higher plants reporting a significant decrease of the maximal quantum yield of PSII, measured as F_v/F_m (Lidon et al. 1999; Jiang et al. 2008; Li et al. 2012; Hasni et al. 2015a), our results fail to demonstrate any significant Al^{3+}-induced changes of the F_v/F_m values in both Al-resistant and Al-sensitive blueberry cultivars, although the Φ_{PSII} and the ETR were markedly reduced in the Al-sensitive Bluegold plants (Fig. 6b and d). These results clearly imply that the limiting step(s) of the photosynthetic electron transport in plants exposed to toxic Al^{3+} conditions might be located downstream of the PSII reaction center. Indeed, the studies of Lidon et al. (1999) and Hasni et al. (2015b) have suggested that the reduction of CO_2 assimilation at toxic levels of Al may well be due to a reduced photosynthetic electron transport at PSI level.

To prevent possible photoinhibition and photooxidative damage of the photosynthetic apparatus in plants exposed to toxic Al^{3+} conditions, the excess energy not used for CO_2 assimilation should be dissipated before the accumulation of toxic ROS (Nishiyama et al. 2006). Non-radiative (thermal) dissipation of excess light energy by ΔpH- and/or xanthophyll cycle-dependent NPQ occurring within the pigment bed of light-harvesting chlorophyll a/b-protein complex of PSII (LHCII) chlorophyll-protein complexes has been considered the major protective mechanisms against photoinhibitory damage of the photosynthetic apparatus (Demmig-Adams and Adams 2006). Thus, a strong enhancement of NPQ should be expected in Al^{3+}-treated Bluegold plants. Surprisingly, and regardless of the higher amount of all xanthophylls observed in Al-sensitive compared to the Al-resistant cultivar (Table 1), the values of NPQ remained unchanged during the exposure of both cultivars under toxic Al^{3+} (Fig. 6e and f). Moreover, the values of NPQ in the Al-resistant cultivar even under control conditions were 30% higher compared to the Al-sensitive plants (Fig. 6e and f). These results suggest that zeaxanthin-dependent NPQ could not be the dominant photoprotective mechanism under Al stress conditions. Alternatively, the lack of significant differences in NPQ values in Al-resistant plants exposed to toxic Al conditions suggests that other quenching mechanisms/processes not related to ΔpH- and zeaxanthin-dependent NPQ, but rather to excitation energy quenching occurring within the reaction center PSII (Ivanov et al. 2008) may be more directly involved and play a significant role in protecting the photosynthetic apparatus of the Al-resistant cultivar under toxic Al^{3+} conditions, since Φ_{PSII} and ETR did not vary (Fig. 6a and c).

On the other hand, simultaneous application of MeJA and toxic Al^{3+} reduced gradually the accumulation of Al (Fig. 1) as well as the oxidative damage in both cultivars (Fig. 2), and recovered the photosynthetic performance after 48 h of combined exposure to Al+MeJA, the effects being more evident in the Al-sensitive cultivar (Figs. 5 and 6). In this cultivar, Al toxicity disturbed the photosynthetic performance, showing the highest reductions of CO_2 assimilation, stomatal conductance, WUE_{PH}, Φ_{PSII}, and ETR compared with Al untreated plants (Figs. 5b, d, f and 6b and d). One possible explanation of the reduced photosynthetic capacity under toxic Al^{3+} was provided by the model proposed by Li et al. (2012), suggesting that the toxic Al can react and/or replace the non-heme iron (Fe^{2+}) between quinones (Q_A and Q_B) in the reaction center of PSII. Replacing the non-heme iron by Al within

the $Q_AFe^{2+}Q_B$ complex would impede the electron transfer between Q_A and Q_B leading to over-reduction of the PSII reaction center and strong decrease of the photosynthetic ETR and the proton (H^+) transfer from stroma to the lumen. This would reduce the generation of ATP and NADPH needed for the Calvin cycle, and would explain the reduced CO_2 assimilation in plants subjected to toxic Al^{3+} (Li et al. 2012). Bearing in mind that the functional photosynthetic apparatus requires 22–23 iron atoms (Briat et al. 2015), of which PSI reaction center complex is the most Fe-abundant (14 Fe atoms) component (Balk and Schaedler 2014), may be accepted that the chemical signature of Al and Fe ions is quite similar based on several reports about that Al can replace Fe in Fe-containing proteins (Fleming and Joshi 1987) or directly interacting with Fe-S groups of complexes I and III in the mitochondrial electron transport chain (Li and Xing 2011). These possibilities make the iron-rich PSI reaction center could be a primary target of Al toxicity (Li et al. 2012), supporting by earlier findings of Al-induced reduction of PSI-dependent electron transport (Lidon et al. 1999; Hasni et al. 2015b).

Alternatively, the increment of ROS ($O_2^{\bullet-}$ and H_2O_2) induced by Al^{3+} in the chloroplasts of stomatal guard cells would depolarize the plasma membrane and activate the calcium influx to the cytoplasm of the guard cells (Zhang et al. 2001). This calcium enhancement would induce the output of chloride and potassium from stomatal guard cells to subsidiary cells, modulating its closure (McAinsh et al. 1996). Moreover, our findings showed that MeJA application improved these responses (Figs. 5b–f and 6b and d). Interestingly, the MeJA application significantly reduced the Al concentration in leaves, especially in the sensitive cultivars (Fig. 1). It has been reported that the xylem loading by heavy metals is mainly influenced by plant transpiration (Yan et al. 2015), suggesting that the reduction in Al concentration in blueberry cultivars might be a result of stomatal closure and a decreased transpiration. On the other hand, it is reported that blueberry plants exhibited a lower root Al uptake and limited Al mobilization to leaves by MeJA application under Al toxicity (Ulloa-Inostroza et al. 2017), decreasing the direct damage in the redox balance of PSII (Li et al. 2012). In addition, the reduction of the LP by MeJA application decreased the direct oxidative damage in the photosynthetic apparatus by Al toxicity (Figs. 2, 5, and 6).

Our experimental results clearly demonstrate that MeJA and toxic Al stimulated differential antioxidant responses in the studied cultivars, depending on their degree of Al resistance. In general, in the Al-resistant cultivar strongly depends on the increased amount and capacity of the phenolic compounds to bind Al^{3+} (by hydroxyl and carboxyl groups) and thus decreasing its availability (Fig. 4a and c), while the Al-sensitive cultivar is more dependent on the increased activity/amount of the ROS scavenging enzymes to reduce the oxidative damage caused by Al stress (Fig. 3d and f). It is important to emphasize that both antioxidant responses were stimulated by MeJA application in both blueberry cultivars under Al stress, thus limiting the Al mobilization and its toxic effects (Figs. 3 and 4). Expectedly, the higher amounts of phenolic compounds would result in higher Al binding at cellular level, which would limit the exposure of chloroplasts to toxic Al. In this sense, phenolic compounds, due to their redox properties, also play important roles in absorbing and neutralizing ROS (Emamverdian et al. 2015; Kulbat 2016; Tighe-Neira et al. 2018). Recently, detailed analyses of phenolic compounds in Legacy and Bluegold cultivars indicated that the elevated amounts of chlorogenic acid, caffeic acid, ferulic acid, and myricetin observed under combined Al+ MeJA treatment positively correlated with the higher Al resistance of the Legacy cultivar, while in the Bluegold (Al-sensitive) cultivar, these compounds were decreased or even not present under the same conditions (Ulloa-Inostroza et al. 2017). Despite of the reduced Al mobilization by MeJA application mentioned above, the possibility that some Al^{3+} could reach the chloroplasts could not be rejected. To minimize the photosynthetic damage imposed by the presence of toxic Al^{3+} within the chloroplasts, alternative mechanisms for Al detoxification may be activated. The increased amounts of xanthophylls (Table 1) and the higher enzymatic activities of SOD and CAT (Fig. 3) observed in the presence of MeJA clearly indicate that both mechanisms could be highly effective in neutralizing the ROS induced by toxic Al^{3+}. Probably, in our study, the anteraxanthin and lutein from the xanthophyll cycle participated in the dissipation of the excess of energy absorbed by chlorophylls, protecting the light-harvesting complexes and reaction center. Products derived from β-carotene (neoxanthin, violaxanthin, anteraxanthin) were increased earlier (24 h) than those derived from α-carotene (lutein), which increased later (48 h) (Table 1). This time-depending behavior seems to be also associated with the Al resistance of blueberry cultivars. It is remarkable that reports about the carotenoid concentration with toxic metals and JAS application are scarce. In this sense, Piotrowska et al. (2009) found that in the aquatic plant *Wolffia arrhizal* exposed to Pb and JAS the total carotenoid concentrations were increased.

Furthermore, in the present work, the Al toxic and MeJA application decreased the oxidative damage by the action of the SOD activity. Later, the CAT (Fig. 3e and f) and probably ascorbate peroxidase (thylakoid-APX) convert the H_2O_2 back into water, decreasing the damage induced by ROS. Thus, possibly the ROS did not depolarize the plasma membrane of the stomatal guard cells without changes of the cytoplasmic calcium, chloride, and potassium concentration. Therefore, the stomatal guard cells were maintained open allowing the exchange of CO_2, resulting in a recovery of photosynthetic performance of both cultivars, being more evident in the Al-sensitive cultivar.

5 Conclusion

The MeJA application under toxic Al^{3+} conditions has a significant protective effect on photosynthetic performance in the Al-sensitive (Bluegold) and to a much lesser extends in Al-tolerant (Legacy) blueberry cultivars. The experimental results presented in this study imply that the possible protective role of MeJA could be provided by (1) decreasing the accumulation of toxic Al^{3+} in the leaves; (2) stimulating the antioxidant response through non-enzymatic (phenolic compounds) and enzymatic (SOD and CAT activities) mechanisms, thus providing more effective detoxification of the ROS induced by toxic Al^{3+}; and (3) protecting the PSII photochemistry through the increased pool of xanthophyll pigments. Combining all of these MeJA-stimulated mechanisms could provide sufficient protection of the photosynthetic apparatus and maintain its effective functioning under the unfavorable toxic Al condition, particularly in the Al-sensitive cultivar.

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