J Plant Growth Regul (2012) 31:406–415
DOI 10.1007/s00344-011-9250-8

Tolerance of *Arabidopsis thaliana* to the Allelochemical Protocatechualdehyde

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Received: 14 September 2011 / Accepted: 22 November 2011 / Published online: 23 December 2011

Abstract We investigated the effects of the secondary metabolite protocatechualdehyde (PCA, 3,4-dihydroxybenzaldehyde) on stress markers, including fluorescence parameters and the concentrations of pigments, free radicals, protein, and lipid peroxides, in adult plants of *Arabidopsis thaliana*. The content of proline, carotenoids, and chlorophylls *a* and *b* peaked 9 h after administration of 3 mM PCA (the highest concentration used in this study), although malonyldialdehyde and dry mass contents peaked 24 h following PCA treatment. Leaf staining revealed peak production of O_2^- between 30 and 90 min post-treatment and peak production of H_2O_2 between 1 and 9 h post-treatment. Several effects, including the observed furling of leaf margins (leaf rolling), the increases in proline content and dry to fresh weight ratio, and the oxidative burst, are reminiscent of the plant response to drought. Early dehydration in PCA-treated plants was confirmed by decreases in leaf water potential, relative water content, and stomatal opening in the first hours of treatment. Thus, PCA seems to be either inducing water deficiency stress (probably through action in the roots) or directly triggering antidrought defenses. In either case, plants showed tolerance to the concentrations employed in this study, with most of the parameters observed having recovered control values within a week of treatment.

Keywords Plant tolerance · Early response · Mode of action · Phytotoxicity · Secondary metabolite

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Introduction

Over 100,000 plant secondary metabolites have been identified (Wink 2010). These low-molecular-weight compounds include mediators of communication with other plants or insects and substances with essential roles in defense against pathogens and herbivores (Gershenzon 2002; Unsicker and others 2009). Each is produced by only a limited number of species and often only in appropriate circumstances. Organisms exposed to secondary metabolites following their release into the environment (and possible transformation by microorganisms) may or may not respond in an ecologically significant fashion; if they do, the ecological function fulfilled may depend on the receiving organism (Hadaçek 2002; Kliebenstein 2004). The characterization of the metabolic response of receptor species is essential for a full understanding of interspecies interactions. In the context of plant-plant interactions, secondary metabolites have attracted much attention in recent years due to their ability to regulate growth and development of receptor plants. Therefore, some of these compounds have been proposed in weed control as potential bioherbicides (Macías 1995; Duke and others 2002). Several natural compounds previously screened for phytotoxic activity have been found to strongly inhibit growth of adult plants, increasing their potential in the field (Macías and others 1999).

3,4-Dihydroxybenzaldehyde, also known as protocatechualdehyde (PCA), is a secondary metabolite found in seeds and young plants of wheat, the stem of *Ilex litseaefolia*, the roots of *Salvia miltiorrhiza*, the leaves of *Vitis vinifera*, and the bacterium *Streptomyces lincolnensis* M-20 (Weber and others 1995; Zhang and others 2005; Kim and others 2008). In Chinese folk medicine it is used to treat cardiovascular complaints (Yu and others 2006) and has

been reported to have anticarcinogenic, antifibrotic, and anticoagulant activities; to inhibit the replication of the hepatitis B virus; and to protect hepatocytes and hepatic microsomes against oxidative stress (Hsu and others 2005; Zhou and others 2006; Kim and others 2008; Wang and others 2008). The few studies of its interactions with other plants have observed it to inhibit germination and root growth at relatively low concentrations (Reigosa and Pazos-Malvido 2007), but little is known of its role in plant receptor and plant emitting species.

Based on the previously demonstrated phytotoxic potential of this compound on seedling metabolism, we investigated the effects of PCA on markers of stress, including fluorescence parameters and the concentrations of pigments, free radicals, protein, and lipid peroxides, in adult plants of *Arabidopsis thaliana* in both the short term (1–24 h) and the medium term (4–8 days) to establish the phytotoxic PCA action on adult plant development.

Material and Methods

Seeds of *Arabidopsis thaliana* L. (Heyn.) ecotype Columbia (Col-0) were sterilized by successive 3-min immersions in 0.01% solutions of Triton X-100 in aqueous EtOH (50%) and NaOCl (0.5%), and then were washed thrice in autoclaved water, vernalized for 48 h at 4°C in 0.1% agar to favor synchronized germination, and transferred to Petri dishes containing agar with Murashige-Skoog nutrients (Sigma-Aldrich) and 1% sucrose. The Petri dishes were kept for 15 days under 60 μmol m^{-2} s^{-1} of light in a growth chamber at 22 ± 2°C. The plantlets produced were transferred to individual pots 5 cm in diameter and 6 cm high containing inert Perlite moistened with 50% Hoagland nutrient solution (Hoagland and Arnon 1950).The pots were placed in a growth chamber at 22 ± 2°C, a photocycle of 8 h of light (120 μmol m^{-2} s^{-1}) and 16 h of darkness, and a relative humidity of 55%. The plantlets were watered with 50% Hoagland nutrient solution twice a week during the first 2 weeks, and then every other day until the age of 5 weeks, when they had an average of nine fully developed leaves. At this point (day 0 of the experiment) each plant was watered with 15 ml of a 0, 1, or 3 mM solution of PCA in 50% Hoagland nutrient solution, with each treatment being applied by root feeding to 60 plants. During the remainder of the experiment, the plants were watered with these solutions every other day.

After applying the treatments, different measurements were taken both before and after harvest. At the end of the experiment the plants were harvested. Part of the fresh plant material was weighed and frozen at −80°C for measurement of photosynthetic pigments, free proline, lipid peroxidation, and total protein content, whereas the remaining plants were used for measurements of fresh and dry biomass, water status-related parameters, and in vivo measurements. Fresh aerial parts were weighed, then the plant material was dried in an oven (70°C, 48 h) after which dry weight was recorded.

Stomata density and stomata opening (Meister and Bolhar-Nordenkampf 2001), relative water content (RWC) (Weatherley 1950; Slatyer 1967; González and Reigosa 2001), and water potential (Ψ_W) with the pressure chamber technique (Scholander Chamber, Soil Moisture Equipment, Santa Barbara, CA, USA)(Scholander and others 1965; González 2001) were measured 1, 12, 48, and 192 h after treatment.

All the measurements were done at least in triplicate, and the experiment was duplicated in time. Replicates were randomly distributed in the chamber.

Fluorescence Measurements

Immediately before PCA treatment and 1, 2, 3, 6, 9, 12, 24, 96, 144, and 192 h later, fluorescence measurements were performed on three whole plants (the same three on each occasion) using a Maxi Imaging PAM Chlorophyll Fluorescence System from Walz (Effeltrich, Germany). On each occasion, after measurement of red and near-infrared emission (R, NIR) for calculation of absorptivity (Abs) as 1 − R/NIR, the plants were kept in darkness for 5 min. Then the leaves were successively illuminated with an intensity of 0.5 μmol m^{-2} s^{-1} for measurement of F_o, the minimum fluorescence of dark-adapted leaves, with a saturating pulse of intensity of 2,700 μmol m^{-2} s^{-1} for measurement of F_m, the maximum fluorescence of dark-adapted leaves; and for 5 min during which actinic illumination at 110 μmol m^{-2} s^{-1} (with measurement of the corresponding fluorescence level F_s) was interrupted every 20 s with 800 ms of saturating pulses of 2,700 μmol m^{-2} s^{-1} for measurement of F'_m, the maximum fluorescence of light-adapted leaves. These measurements were employed by the instrument software for calculation of F'_o (the minimum fluorescence of light-adapted leaves) using the formula of Oxborough and Baker (1997), which is valid for the lake model of PSII (Klughammer and Schreiber 2008). The lake model, based on a Sterm-Volmer approach and opposite to what happens in the puddle model, considers that reaction centers are connected by shared antenna and is a more appropriate indicator of Q_A redox state for terrestrial plants (Kramer and others 2004). $F_v = F_m - F_o$ and $F'_v = F'_m - F'_o$ are the variable fluorescence of dark- and light-adapted leaves, respectively; F_v/F_m and F'_v/F'_m are the maximum quantum efficiencies of dark- and light-adapted PSII; $\varphi_{II} = (F'_m - F_s)/F'_m$ is the effective photochemical quantum yield, or operating efficiency, at PSII; φ_{NO} is the quantum yield of chlorophyll fluorescence and unregulated nonfluorescent nonphotochemical de-excitation, using the

formula of Kramer and others (2004); $\varphi_{NPQ} = 1 - \varphi_{II} - \varphi_{NO}$ is the quantum yield of regulated nonfluorescent nonphotochemical de-excitation; q_N is the nonphotochemical quenching coefficient, calculated as $(F_m - F'_m)/(F_m - F'_o)$; $q_P = (F'_m - F_s)/F'_v$ is the photochemical quenching coefficient; $q_L = q_P(F'_o/F_s)$ is the fraction of PSII centers that are open (Kramer and others 2004); and ETR is the apparent electron transport rate. Note that when the formula of Oxborough and Baker (1997) for F'_o is used in the formula of Kramer and others (2004) for φ_{NO}, the latter becomes identical to the much simpler and more widely applicable formula $\varphi_{NO} = F_s/F_m$ (Klughammer and Schreiber 2008). All parameters were averaged over the whole area of the whole plant image, the 15 values so obtained for each replicate were plotted against time, and the area under the graph was calculated and divided by the time between the first and last measurement to obtain a mean value for that period. Values reported herein are means-over-replicates of the mean values so obtained.

Chemistry

Semiquantitative Determination of H_2O_2 In Situ

Hydrogen peroxide was determined using a modified version of the method of Van Acker and others (2000). Leaves were cut 1, 3, 6, 9, 12, and 24 h after PCA treatment and were vacuum infiltrated for 5 min with a 1-mg ml^{-1} solution of 3,3′-diaminobenzidine (DAB) in water brought to pH 3.8 with 0.1% HCl. After 8 h in the dark at room temperature in the same solution, the leaves were illuminated for 15 min (by which time brown stains marked areas with H_2O_2) and were then washed twice in 96% EtOH to remove chlorophyll and fixed in 70% glycerol. H_2O_2 was determined semiquantitatively by image analysis.

Semiquantitative Determination of O_2^- In situ

Leaves were cut 30, 60, 90, and 120 min after PCA treatment and were immediately immersed in distilled water, after which the base of each leaf was cut to prevent embolisms. The leaves were then vacuum infiltrated for 5 min with a 0.65-mg ml^{-1} solution of NaN_3 in K-P buffer (pH 7.8) containing 0.1% of nitroblue tetrazolium (NBT) (Halliwell and Gutteridge 1985), after which they were left in darkness in the same buffer for 20 min and then illuminated until the appearance of stains. Thereafter they were treated in the same way as for determination of H_2O_2.

Photosynthetic Pigment Concentrations

Chlorophyll *a*, chlorophyll *b*, and carotenoids were determined in 100-mg samples collected in triplicate 3, 9, and 24 h after PCA treatment. The samples were homogenized in 1.5 ml of methanol and centrifuged for 5 min at 170×*g*; the absorbance of the supernatant at 470, 653, 666, and 750 nm was recorded; and pigment concentrations were calculated as per Wellburn (1994) and expressed as percentage of dry weight.

Total Protein Content

Total protein content 3, 9, 24, 48, 96, and 192 h after PCA treatment was determined in triplicate by Bradford's method as described by Pedrol and Ramos (2001). For each replicate, 100 mg of leaf material was homogenized in 0.8 ml of 0.05 M Tris buffer (pH 8.0) containing 0.05 g of the insoluble antioxidant polyvinyl polypyrrolidone, and the mixture was centrifuged at 2,860×*g* for 20 min. A 0.1-ml sample of the supernatant was then mixed with Bradford's reagent, and absorbance at 595 nm was measured and translated into protein content using a calibration line constructed with bovine serum albumin standards. Protein content was calculated per gram of dry weight.

Lipid Peroxidation

Lipid peroxidation 3, 9, 24, 48, 96, and 192 h after PCA treatment was determined by measurement of malonyldialdehyde (MDA) content as per Hodges and others (1999). Prefrozen plant material was homogenized in 80% ethanol and centrifuged at 3,000×*g* for 10 min at 4°C; the supernatant was incubated at 95°C with 20% TCA containing 0.01% hydroxytoluenebutylate, with and without 0.5% thiobarbituric acid (TBA); and absorbance was measured at 440, 532, and 600 nm. MDA (nmol/ml) was calculated as $10^3 \times [(A - B)/157]$, where $A = (Abs_{532} - Abs_{600})_{TBA+} - (Abs_{532} - Abs_{600})_{TBA-}$ and $B = 0.0571 \times (Abs_{440} - Abs_{600})_{TBA+}$. MDA content was calculated per gram of dry weight.

Free Proline

Free proline was determined by the method of Bates and others (1973), with modifications described by Pedrol and Ramos (2001), 9, 24, and 48 h after treatment with 1 or 3 mM PCA. On each occasion, triplicate 100-mg samples were homogenized in 5 ml of 3% (w/v) sulfosalicylic acid. Then the homogenate was filtered through Whatman No. 2 paper, mixed with acid ninhydrin and glacial acetic acid in 1:1:1 ratio, and heated at 100°C for 1 h. After cooling in ice, 2.5 ml of toluene was added, the organic and inorganic phases were separated, and absorbance was measured at 520 nm. Proline concentration was read from a calibration line constructed with pure proline standards and was expressed as percentage of dry weight.

Statistical Analyses

Following testing for non-normality by the Kolmogorov–Smirnov test and for heteroscedasticity by Levene's test, the statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests in the case of homoscedastic data and by Tamhane's T2 test in the case of heteroscedastic data.

Results and Discussion

Eight days after PCA treatment, treated plants exhibited quantitatively similar growth to that of controls, with the same number of leaves and no statistically significant differences in fresh weight. However, the leaves of treated plants, especially those administered 3 mM PCA, were darker and narrower and had furled margins (Fig. 1b), all of which are typical signs of water deficit (Begg 1980; Kadioglu and Terzi 2007). In keeping with this, plants treated with 3 mM PCA showed a slight but statistically significant increase in the ratio between dry weight and fresh weight throughout the first 48 h after treatment (Fig. 1a). It has been suggested that the furling of margins (ultimately leaf rolling) constitutes a common strategy for accommodation to drought, because decreased effective leaf area can reduce transpiration without necessarily being accompanied by the prevention of photosynthesis that is caused by stomatal closure (O'Toole and others 1979; Clarke 1986; Kadioglu and Terzi 2007).

DAB-stainable areas indicative of H_2O_2 production appeared transiently at the leaf margins of PCA-treated plants in a dose-dependent fashion, with the higher PCA dose being associated with earlier appearance than the lower dose (<1 h vs. 1–3 h), greater size and stain intensity, and later disappearance (>12 h vs. ~9 h) (Fig. 2a). In keeping with the visual appearance of the leaves (Fig. 1b), the expansion of affected areas at the leaf margin is reminiscent of the effects of drought. A somewhat similar pattern of O_2^- production was shown by NBT staining (Fig. 2b), albeit on a shorter time scale (O_2^- production is known to be an early response; Gill and Tuteja 2010). Its disappearance within 24 h is in keeping with its triggering processes of defense and recovery, analogous to those mediated by H_2O_2 in response to ABA and other stimuli or stressors (McAinsh and others 1996; Pei and others 2000;

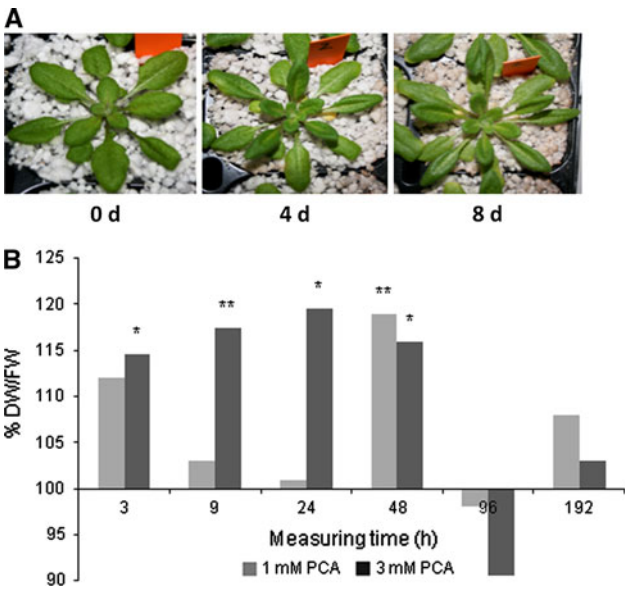

Fig. 1 **a** *Arabidopsis* plants before and 4 and 8 days after treatment with 3 mM PCA, showing progressive furling of leaf margins. **b** Ratio between dry weight and fresh weight of adult *Arabidopsis* leaves treated with 1 or 3 mM PCA. Values are expressed as percentage at various times post-treatment compared to the value obtained for untreated control plants. Statistical significance with respect to controls: $*P < 0.05$; $**P < 0.01$

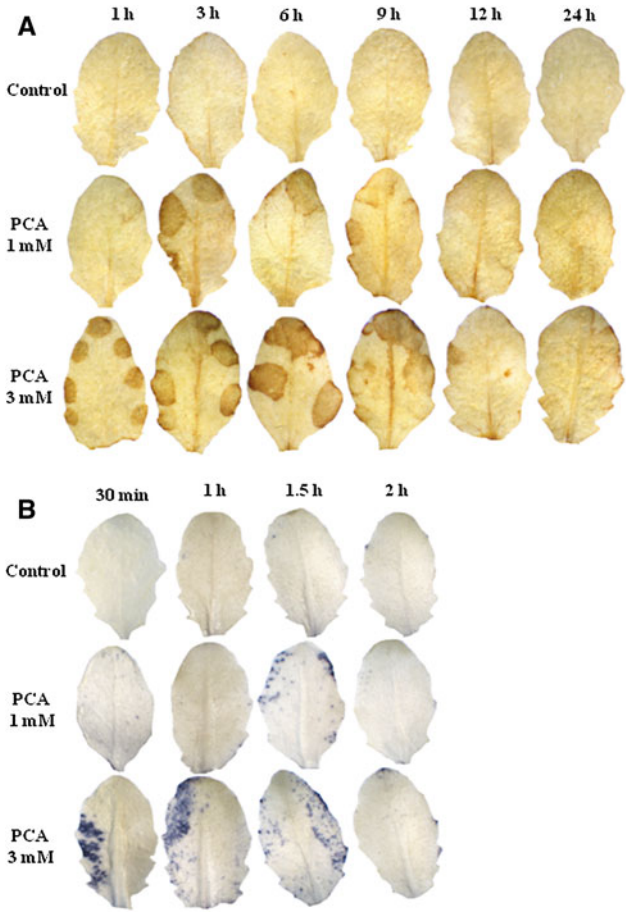

Fig. 2 *Arabidopsis* leaves stained with DAB (**a**) or NBT (**b**) at various times after treatment with 0, 1, or 3 mM PCA. Brown areas show H_2O_2 production and blue areas show O_2^- production

Neill and others 2002; Bright and others 2006; Huang and others 2009). In view of the ability of H_2O_2 to induce stomatal closure by activating calcium channels in the plasma membrane of guard cells (Pei and others 2000; Zhang and others 2001), it seems that stomatal closure may have preceded the leaf rolling observed subsequently (Fig. 1b).

The effects of the observed oxidative burst are reflected by the measurements of lipid peroxidation. Relative to those of control plants, MDA levels in PCA-treated plants increased significantly during the first 24 h and then fell back to those of control levels, the increase being greater and the subsequent fall more rapid with the 3 mM PCA dose than the 1 mM dose (Fig. 3). A rise in MDA levels is typical in plants subjected to drought or other stressors and generally indicates an increase in membrane permeability (Chempakam and others 1993; Zhang and Kirkham 1996).

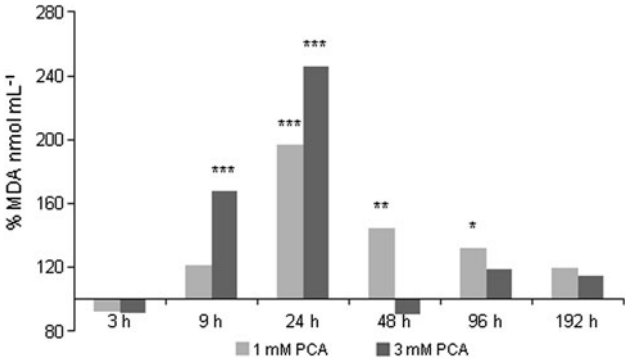

Fig. 3 Malonyldialdehyde content of adult *Arabidopsis* leaves treated with 1 or 3 mM PCA expressed as a percentage of the value for untreated control plants, at various times post-treatment. Statistical significance with respect to controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

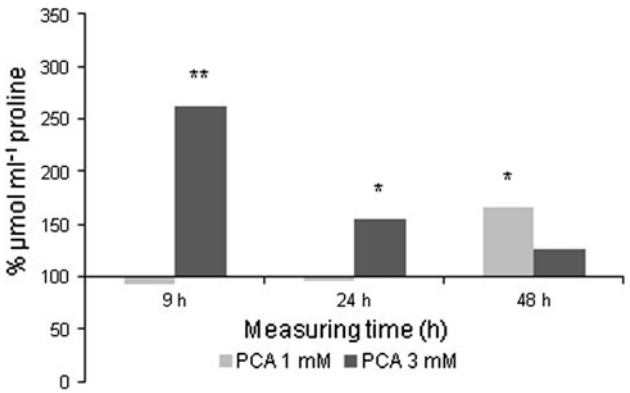

Fig. 4 Proline content of adult *Arabidopsis* leaves treated with 1 or 3 mM PCA, expressed as a percentage of the value for untreated control plants, at various times post-treatment. Statistical significance with respect to controls: $*P < 0.05$; $**P < 0.01$

An alteration that is characteristic of drought in drought-resistant sorghum and other drought-resistant plants (Singh and others 1973; Sivaramakrishnan and others 1988; Gzik 1996), and that was also observed in this study, was a rise in proline levels (Fig. 4). With 3 mM PCA, proline had already peaked by the time of the first measurement, 9 h after treatment, and by 48 h had fallen to control levels, whereas with 1 mM PCA, proline levels appeared to be still increasing after 48 h. Together with other solutes, proline helps adjust osmolality during drought-related leaf rolling (Hsiao and others 1984; Kadioglu and Turgut 1999; Kadioglu and Terzi 2007).

Like the concentrations of the analytes reported on above, those of chlorophyll *a* and *b* and carotenoids rose

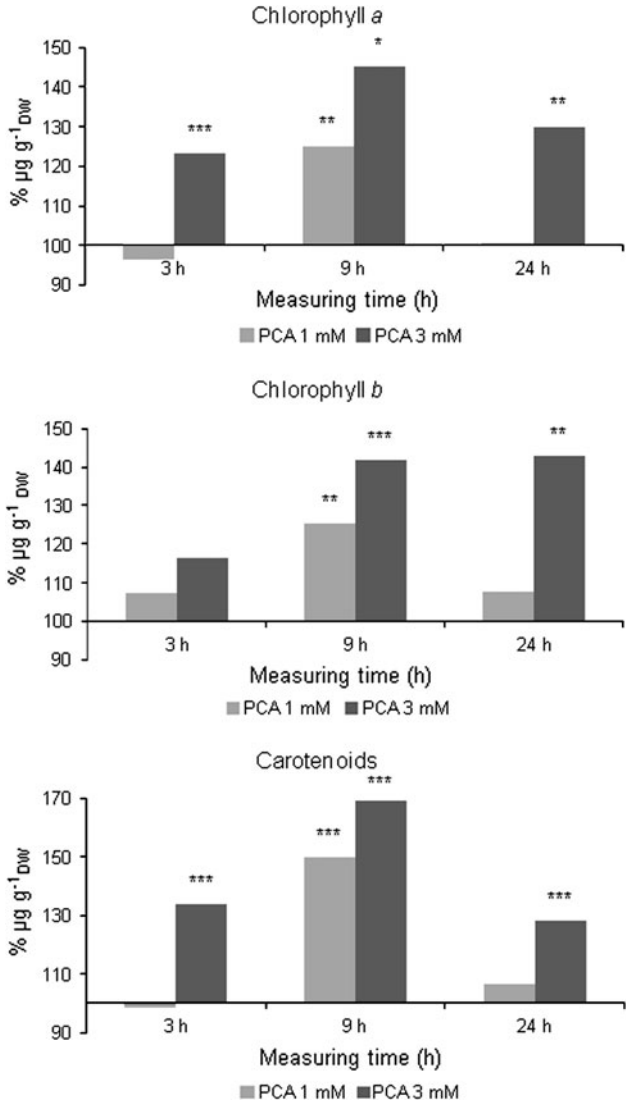

Fig. 5 Chlorophyll *a*, chlorophyll *b*, and total carotenoid contents of adult *Arabidopsis* leaves treated with 1 or 3 mM PCA, expressed as a percentage of the value for untreated control plants, at various times post-treatment. Statistical significance with respect to controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

and then fell relative to controls, generally peaking around 9 h after treatment (Fig. 5). With 1 mM PCA the rise was smaller than with 3 mM treatment, and was only significant at the peak. With 3 mM PCA, chlorophyll *a* and carotenoids were already significantly higher than in controls 3 h after treatment and were still so after 24 h, whereas chlorophyll *b* was slower to rise but still remained at peak values 24 h after treatment. As reported by Kraus and others (1995), pigment levels are usually higher in drought-tolerant plants than in others, and the particularly pronounced rise in carotenoid levels may constitute a response to the PCA-induced oxidative burst, given the capacity of carotenoids to sequester free radicals (Lu and others 2003; Yin and others 2010). In fact, the rise in chlorophyll levels may have been at least partly due, not to increased *de novo* synthesis, but to increased protection by carotenoids; this

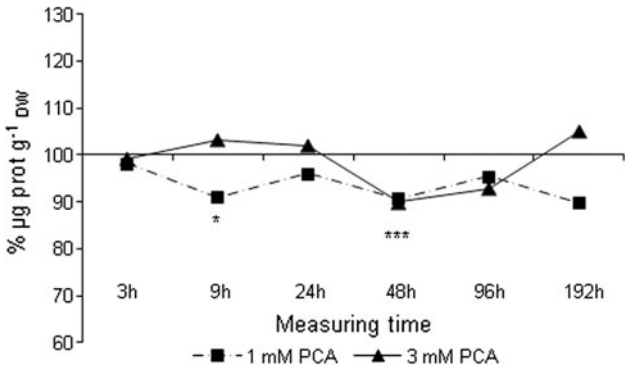

Fig. 6 Total protein content of adult *Arabidopsis* leaves treated with 1 or 3 mM PCA, expressed as a percentage of the value for untreated control plants, at various times post-treatment. Statistical significance with respect to controls: $*P < 0.05$; $***P < 0.001$

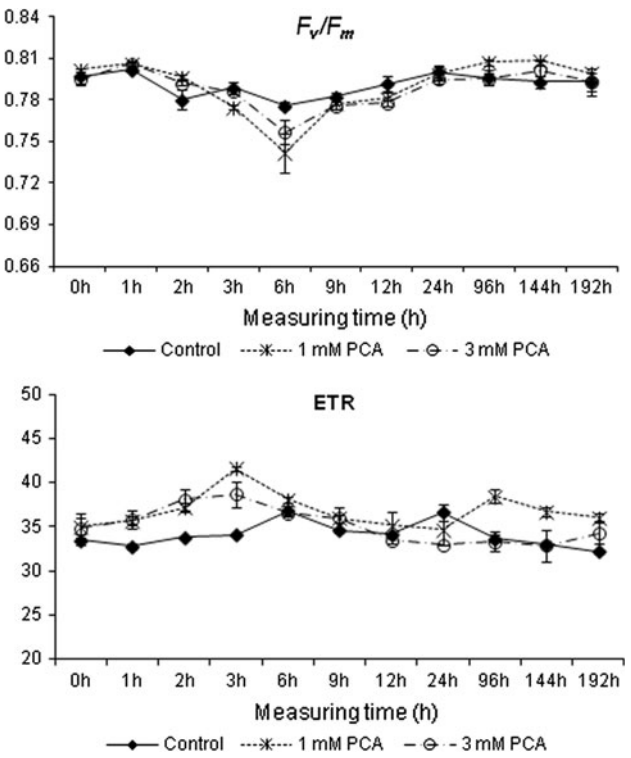

Fig. 7 Values of the maximum efficiency of PSII in dark-adapted plants (F_v/F_m) and of apparent electron transport rate in light-adapted plants (ETR), at various times after treatment of adult *Arabidopsis* plants with 0, 1, or 3 mM PCA (see Table 1 for the statistical significance of differences between treated plants and controls)

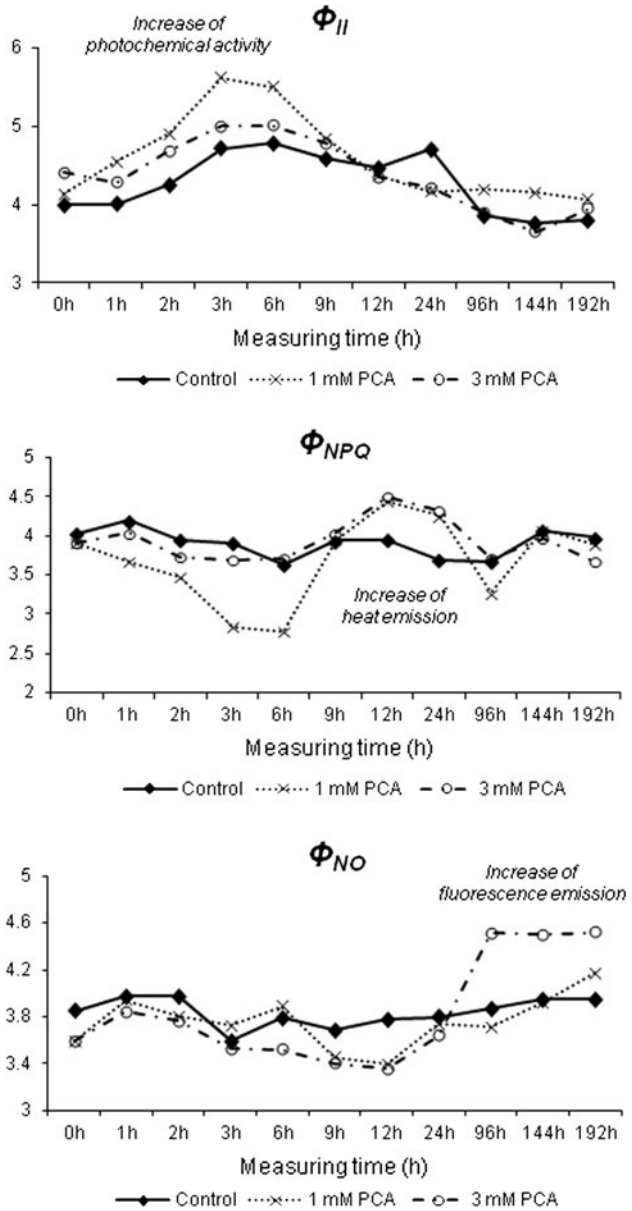

Fig. 8 Values of the operating efficiency of PSII (φ_{II}), the quantum yield of regulated nonfluorescent nonphotochemical de-excitation (φ_{NPQ}), and the quantum yield of chlorophyll fluorescence and unregulated nonfluorescent nonphotochemical de-excitation (φ_{NO}) at various times after treatment of adult *Arabidopsis* plants with 0, 1, or 3 mM PCA (see Table 1 for the statistical significance of differences between treated plants and controls)

interpretation would seem to be in keeping with the finding that the only alteration of total protein concentration was a slight reduction of less than 15% (Fig. 6).

Like the chemical parameters, photochemical parameters were altered during the first 24 h after PCA treatment, but within a week had in most cases acquired values very similar to those of control plants (Figs. 7, 8; Table 1). Curiously, it was initially plants treated with the lower PCA concentration that differed most from controls. Thus, 1–6 h after 1 mM PCA treatment, φ_{II} and apparent ETR were significantly greater than control levels and φ_{NPQ} and q_N were significantly less, but after 3 mM PCA treatment, neither φ_{II} nor φ_{NPQ} exhibited significant alteration throughout this period. This apparently inverse dose dependence may possibly be related to the observed increase in chlorophyll concentrations. Assuming that this increase was a response driven by increased demand for photosynthesis (for synthesis of proline, antioxidants, and so on; Gonzáles and others 2008), then the plants in which chlorophyll concentration increased most rapidly (those receiving the greater PCA dose) may have been able to satisfy the demand for photochemistry mainly through an increase in photochemical capacity, without needing to achieve more than a relatively small increase in operating efficiency (φ_{II}) by reducing regulated dissipation of light energy (φ_{NPQ}). In this respect it may be noted that apparent ETR was significantly greater in plants treated with 3 mM PCA than in controls, which in the absence of a correspondingly large increase in φ_{II} indicates increased absorptivity.

Because the decrease in F_v/F_m values 3–9 h after treatment coincides with increasing chlorophyll content (which would increase F_m), it must be attributed to an increase in F_o, possibly due to an increased antenna/PSII ratio. The marked increase in q_L relative to controls suggests the maintenance of an elevated demand for photosynthesis.

Between 9 and 24 h after treatment, an increase in downregulation of PSII (φ_{NPQ}) resulted in decreased operating efficiency and/or φ_{NO}. It was during this period, coinciding with the greatest rise in MDA and a slight reduction in pigment levels, that whole-plant images of F_v/F_m showed the greatest number of small damaged areas, all of them at peripheral sites. The highest F_v/F_m values were shown by the young leaves at the center of the rosette (Martínez-Peñalver and others 2011).

At post-treatment times longer than 24 h, plants treated with 3 mM PCA showed an increase in φ_{NO}. Increased fluorescence emission has been previously found for plants treated with other secondary metabolites (such as BOA; Sánchez-Moreiras and others 2011), and may perhaps reflect increased fluorescence associated with a continuing fall in pigment levels.

Based on the above results, a new experiment was conducted to analyze the water relationships in PCA-treated plants. Stomatal opening, water potential, relative water

Table 1 Statistically significant levels of differences in chlorophyll fluorescence parameters between adult *Arabidopsis* plants treated with 1 or 3 mM PCA and untreated controls, at various times post-treatment

PCA 1 mM / PCA 3 mM	F_v/F_m	ETR	ϕ_{II}	ϕ_{NPQ}	ϕ_{NO}	q_N	q_L
1 h	+ / +	++ / ++	++	−−		−−	+
2 h	++ / +	++ / +++	+++ / ++	−	−	−−−	
3 h	−−−	+++ / +++	+++	−−−	+	−−− / −	+++ / +++
6 h	−− / −	+	++	−			+++ / +++
9 h	− / −		+		−−	+ / ++	++
12 h	− / −			+++ / +++	−− / −−	+++ / +++	++
24 h			−− / −−	+++ / +++		+++ / +++	
96 h		++	+++	−	++	−	+++
144 h		+	+		+		+
192 h		+		−	+ / ++		+++ / +

Plus signs indicate positive differences with respect to controls and *minus signs* negative differences. The number of *plus or minus signs* indicates statistical significance: one, $P < 0.05$; two, $P < 0.01$; three, $P < 0.001$

Table 2 Number of open and closed stomata 1, 12, 48, and 192 h after treatment of adult *Arabidopsis* plants with 0 or 3 mM PCA

		Open stomata	Closed stomata
1 h	Control	$34^{a} \pm 11.3$	$26^{a} \pm 11.2$
	PCA	$18^{b} \pm 7.82$	$33^{a} \pm 10.6$
12 h	Control	$35^{a} \pm 5.9$	$36^{a} \pm 8.4$
	PCA	$18^{b} \pm 9.4$	$63^{c} \pm 17.5$
48 h	Control	$38^{a} \pm 13.8$	$33^{a} \pm 16.3$
	PCA	$43^{a} \pm 19.8$	$63^{c} \pm 17.9$
192 h	Control	$48^{ac} \pm 16.8$	$67^{bc} \pm 15.6$
	PCA	$54^{c} \pm 16.9$	$44^{b} \pm 13.2$

Letters represent statistically significant differences between treatments at $P < 0.05$

content, dry and fresh weights, and specific leaf area were monitored to confirm the previously suggested dehydration process. As shown in Table 2, significant differences in the number of open stomata after 1 and 12 h and closed stomata after 12 and 48 h were detected between control and treated plants. Although the stomatal density did not change, there was a clear induction of stomatal closure after PCA treatment, which supports other findings presented before. As previously suggested, stomatal closure has been observed to precede leaf rolling (Zhang and others 2001; Kadioglu and Terzi 2007). Plants also showed a positive relationship between stomatal closure and formation and accumulation of H_2O_2 (see Fig. 2a), optimizing water use efficiency as previously demonstrated by other authors (Zhang and others 2001; Deuner and others 2011). In addition, the values of RWC and Ψ_W confirm the effect on water status in PCA-treated plants (Fig. 9). RWC and Ψ_W values significantly decreased very quickly, already 1 h after the treatment, when compared to the control. This effect is in accordance with the oxidative burst detected in the margins of the leaves, with increased contents of H_2O_2 during the first 48 h of PCA treatment (see Fig. 2a). The effect on water relationships was also consistent with the stomatal behavior previously discussed. However, 8 days after the treatment the values of these parameters were similar to control values, which supports the suggested recovery of these plants within a week of treatment. The same was found for the ratio dry weight to fresh weight, as the values 8 days after treatment were not statistically different from those of control plants. The furling of leaf margins, which decreases effective leaf area (see SLA values in Fig. 9), seems to be induced as a common strategy for accommodation to drought, avoiding permanent stomatal closure and lower values of water potential. Although harvesting was not directly related to watering

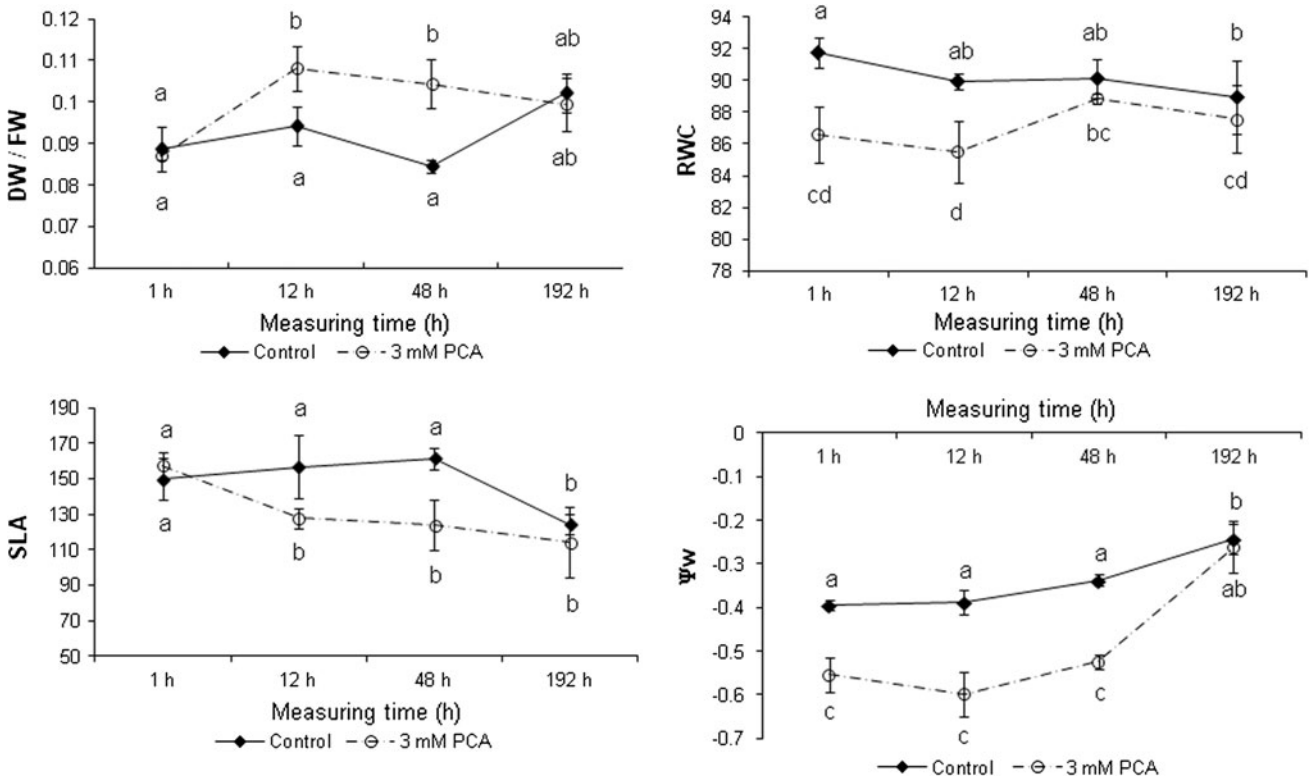

Fig. 9 Values of the ratio of dry weight to fresh weight (DW/FW), specific leaf area (SLA), relative water content (RWC), and water potential (Ψ_W) at various times (1, 12, 48, and 192 h) after treatment of adult *Arabidopsis* plants with 0 or 3 mM PCA. Letters represent statistically significant differences between treatments at $P < 0.05$

(which was done every 48 h), the measurement of chlorophyll *a* fluorescence suggests that there is an acclimation process to PCA action in treated *Arabidopsis* plants, probably through the increase of regulated energy dissipation (φ_{NPQ}) and antidrought responses.

It should be considered at this point that PCA was imposed by root feeding every 2 days, which could be masking the effects on transient parameters. Further studies on root morphology and physiology would be necessary to fully understand the effect of PCA on *Arabidopsis* metabolism.

Conclusions

Although PCA has been found to have relatively small IC_{50} and IC_{80} values for inhibition of *Arabidopsis* germination and root growth (Reigosa and Pazos-Malvido 2007), its phytotoxic action on adult plants appears to be quite different. Several of its effects, including the furling of leaf margins, the increase in proline content, the increase in the ratio of dry weight to fresh weight, the decrease of RWC, Ψ_W, and stomatal opening, and the oxidative burst, are reminiscent of the response to drought. Thus, PCA seems to be either inducing water deficiency stress (probably through action in the roots) or directly triggering anti-drought defenses. In either case, the plant seems to have little difficulty in responding adequately to the concentrations employed in this study, with most of the parameters observed having recovered to the level of control values within a week of treatment. Phytotoxic activity previously found for PCA in *Arabidopsis* seedlings (Reigosa and Pazos-Malvido 2007), is therefore not relevant in adult plants.

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