Physiological and biochemical parameters controlling waterlogging stress tolerance in *Prunus* before and after drainage

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Abbreviations – APX, ascorbate peroxidase; CAT, catalase; POD, guaiacol peroxidase; PVP, polyvinylpyrrolidone; RH, relative humidity; ROS, reactive oxygen species; SOD, superoxide dismutase.

Abstract

Waterlogging is associated with poor soil drainage. As a consequence oxygen levels decrease in the root environment inducing root asphyxia and affecting plant growth. Some plants can survive under these conditions triggering complex anatomical and biochemical adaptations, mostly in the roots. Long- and short-term responses to waterlogging stress were compared in two trials using a set of two myrobalans (*Prunus cerasifera* Ehrh), 'P.2175' and 'P.2980', as tolerant rootstocks and two almond × peach [*Prunus amygdalus* Batsch × *Prunus persica* (L.) Batsch] interspecific hybrids, 'Garnem' and 'Felinem', as sensitive ones in two consecutive years. Stomatal conductance and chlorophyll content were measured in the long-term trials to assess survival performance, while the enzyme activities of superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (POD, EC 1.11.1.7), and catalase (CAT, EC 1.11.1.6) were measured in the short-term trials to study early antioxidant response. The incidence of the stress in the root environment was different as a result of the different plant development at the moment of the treatment, as a consequence of different environmental conditions both before and during the treatment between the 2 years. The activity of the different enzymes was higher in the sensitive genotype 'Felinem' than in the tolerant 'P.2175'. This result shows an activation of the antioxidant system and has been observed to depend of the different nature of the roots between the 2 years. As the antioxidant enzymes seem to work more efficiently when roots are more aerated, we cannot conclude that they are responsible for the higher tolerance observed in the myrobalan plums.

Introduction

Waterlogging is an abiotic stress inflicted by poor drainage in heavy soils caused either by heavy

rainfall in certain periods or by bad irrigation practices. Thus, water excess induces an oxygen concentration reduction in the environment around plant roots, making cellular oxygen levels drop, which is a phenomenon known as hypoxia (Drew 1997, Dennis et al. 2000). Crop plants require a free exchange of atmospheric gases for photosynthesis and respiration to keep their normal growth (Visser et al. 2003, Fukao and Bailey-Serres 2004, Suzuki and Mittler 2006); in flooding environment, the gas diffuses more slowly because of water saturation around the roots (Dennis et al. 2000), affecting directly to the nutrient absorption, plant grow and crop productivity (Visser et al. 2003, Fukao and Bailey-Serres 2004). Plants have developed different degrees of tolerance to flooding that are based on complex anatomical and biochemical adaptations (Drew 1997, Vartapetian et al. 2003, Liu et al. 2005). One of the early responses to waterlogging stress appears to involve the closing of stomata to avoid water loss, together with the regulation of the photosynthetic machinery (Domingo et al. 2002, Arbona et al. 2008). Previous studies on the physiology of stomata in higher plants suggest that the stomata influence the rate of CO_2 fixation in leaf mesophyll tissue (Wong et al. 1979), together with some degree of osmotic adjustment occurring when plants are submitted to stress periods (Munns 1988). On the other hand, antioxidant defense systems are a prominent element in plant response to environmental stresses especially in rootstocks. Under anaerobic conditions, an aerobic organism can be severely damaged by a partial oxygen reduction, induced by normal or aberrant metabolic processes. Reactive oxygen species (ROS) are known to cause oxidative damage to living tissues by oxidizing cellular components (Mittler 2002, Palma and Kermode 2003, Mittler et al. 2004). The toxic effect of ROS is circumvented by a combination of enzymatic and non-enzymatic mechanisms that can reduce the oxidative stress by converting ROS into harmless compounds. Superoxide dismutase (SOD) constitutes the first line of cellular defense followed by catalase (CAT) and guaiacol peroxidase (POD) (Blokhina et al. 2003, Mittler et al. 2004, Edreva 2005). Such aspects as the compartmentalization of ROS formation or antioxidants localization, synthesis and transport of antioxidants, the ability to induce antioxidant defenses and the cooperation between enzymes are the determinants for the activity (Mittler 2002, Blokhina et al. 2003, Mittler et al. 2004).

Prunus possess no physiological or anatomical adaptation to flooding, and *Prunophora* are more tolerant than *Amygdalus* subgenera. Traditionally, in *Prunus*, screening has been only done empirically, through the selection of rootstocks withstanding more time under flooding. Such observations allowed to consider 'Marianna-2624', 'myrobalan 29-C' and 'Marianna GF8-1' plums as the most tolerant ones, enduring 125 days of flooding and 'GF-557' and 'GF-677' almond × peach hybrids as the most sensitive ones (Durán 1976, Kozlowski 1997). Even though, few works

have been performed to find out the correlation between the tolerance and the physiological parameters controlling this stress in *Prunus* (Domingo et al. 2002, Dichio et al. 2003) and others fruit trees (Arbona et al. 2008). Interspecific rootstocks between myrobalan plum (*Prunus cerasifera* Erhr) and almond × peach hybrids [*Prunus amygdalus* Batsch × *Prunus persica* (L.) Batsch] have been created and are under evaluation in a current breeding program (Xiloyannis et al. 2007). The aim of this study was to compare the tolerance level of two myrobalans ('P.2175' and 'P.2980'), considered more tolerant and two almond × peach hybrids ('Felinem' and 'Garnem' used as rootstocks), considered more sensitive and the effects of recovery after drainage. Also, three antioxidant enzymes in one tolerant 'P.2175' and one sensitive 'Felinem' genotypes have been compared in flooding and recovery conditions to assess their early antioxidant responses.

The correlations between the physiological parameters, chlorophyll content, leaf conductance and antioxidant activity are discussed as possible indices enhancing the stress defense potential in myrobalans and in the hybrids, tolerant and sensitive rootstocks, respectively, which have been evaluated under waterlogging and recovery conditions during 2 years.

Materials and methods

Plant material and experimental conditions

Softwood cuttings of two myrobalan plums (*P. cerasifera* Erhr) 'P.2175' and 'P.2980' and two almond × peach hybrids ([*P. amygdalus* Batsch × *P. persica* (L.) Batsch] 'Felinem' and 'Garnem' were propagated during fall– winter 2005 and 2006. One-year-old cuttings from the four previously mentioned genotypes were transplanted in the two following winters during February (2007 and 2008) in 24-l (28 cm Ø) plastic pots and 47-l (37 cm Ø) pots in long- and short-term trials, respectively, filled with sand 100% as substrate. The two experiments were conducted outdoors under similar conditions, and during the treatments period, the climatic conditions were typically Mediterranean with a mean temperature ranging from 29.5 to 40°C in 2007 and from 30.4 to 36.1°C in 2008, while the percentage average relative humidity (RH) was 50.3% in 2007 and 55.7% in 2008. The monthly average of global solar radiation during the experiment in summer was of 27.1 MJ m^{-2} day^{-1} in 2007 and 26.0 MJ m^{-2} day^{-1} in 2008 mean- while, the total precipitation was 5.4 mm $month^{-1}$ in 2007 and 16.6 mm $month^{-1}$ in 2008. Data taken from Agro-climate Information System for Irrigation (SIAR).

Two trials with two experimental designs were set up, one to see the survival performance under waterlogging stress at long term and other to assess whether an early response of the antioxidants system is linked to waterlogging stress tolerance in two genetically distant genotypes in the short

term. Pots were drip-irrigated three times a day for 5 months, and a routine fertilization program was applied during the whole vegetative period, in which the plants were growing until the waterlogging treatments were imposed.

Long-term trial

This experiment was established with the four genotypes. At the end of June 2007 and 2008, 20 plants of each genotype 'P.2175', 'P.2980', 'Felinem' and 'Garnem' with similar size and appearance were submitted to two treatments: one control treatment, non-flooded, irrigated daily as indicated to maintain optimal soil–water conditions (C) and one waterlogging treatment where plants were flooded for 1 week (W). The experimental design was a random-block with 20 replicates of the four genotypes in 24-l pots, 10 control and 10 stressed. Waterlogging treatment was imposed, by placing each plant in plastic pots maintaining the water level 40 cm above the soil surface with a rubber tube individually enhanced to the button-hole drainage and the top of each pot. Flooding conditions started at 16.00 h and ended at the same time after 1 week, between June and July. Recovery was also studied over a period of 10 days by allowing the drainage individually of each pot. When needed, during the flooding week because of the transpiration effects, more water was added until to keep regularly the 40-cm water-level covering the whole root system. Independent preliminary experiments showed that after 2-weeks' flooding time in similar floodwater conditions, plants were rapidly and severely injured. Stomatal conductance and chlorophyll content were monitored in this trial.

Short-term trial

Two genotypes were used only in this trial, 'P.2175' and 'Felinem' and the plants were planted in 47-l pots with a set of two plants, one of each genotype. The whole plant was sampled to analyze the antioxidant activity, and a plastic layer sheet was used to divide the root system of each plant to facilitate individually the sampling. A total of five treatments were imposed: 0 h (control), 6 and 24 h (waterlogging treatments) and 6 and 24 h (recovery periods). Waterlogging treatment was imposed by placing the plants in plastic pots maintaining the water level 60 cm above the soil surface with a rubber tube individually enhanced to the button-hole drainage and to the top pot. After to separate the root system, three replicates of young roots were sampled for each treatment in both genotypes. For the analysis of roots, the sandy soil was removed and the fine roots were gently washed with tap water. Only those roots showing clear senescence symptoms were excluded. Roots were immediately frozen in liquid nitrogen and stored at −80°C until activity determination. Only stomatal conductance was monitored in this trial before the sampling for protein

128 determination.

129 **Physiological parameters**

130 During the waterlogging and recovery periods, the leaf chlorophyll concentration was estimated
131 using an SPAD 502 meter (Minolta Co. Osaka, Japan) on a set of four replicates in the long-
132 term trial on days 0, 2, 5 and 7 of the waterlogging treatment and on days 0, 2, 7 and 10 of the
133 recovery period. Midday leaf conductance (gl) was also measured with a Leaf Porometer
134 (Decagon Devices Inc. Pullman, WA), between 12:00 and 15:00 h on days 0, 2, 5 and 7 of the
135 waterlogging treatment and on days 0, 2, 7 and 10 of the recovery period on the whole set of
136 replicates in the long-term trial. The measurements were made in the 8 first top well-
137 developed leaves. Additionally, leaf conductance (gl) was also monitored in the two following
138 weeks after the treatment. Leaf conductance (gl) measurements were monitored in the same
139 leaves (first top) on the whole set of plants along the five treatments: 0, 6 and 24 h of
140 waterlogging and 6 and 24 h of recovery, in the short-term trial. Both trials' measurements were
141 carried out during the summers of 2007 and 2008.

142 **Antioxidant enzyme activity**

143 Three enzymes' activities were analyzed for the two genotypes 'P.2175' and 'Felinem'
144 submitted to water- logging in the short-term trial. The frozen roots (100 mg) were homogenized
145 in a mortar with liquid nitrogen and 40 mg polyvinylpyrrolidone (PVP). Proteins were
146 extracted with 250 µl buffer [MES–NaOH (pH 7.0) 100 m*M*, $MgCl_2$ 2.5 m*M*, ethylene
147 glycol tetraacetic acid (EGTA 2 m*M*, PVP 25.5 mg ml^{-1}, polyethylene glycol (PEG) 5.9
148 g l^{-1}, 1,4-dithiothreitol (DTT) 2 m*M*, glycerol 10% (v/v), Triton-100X 0.5% (v/v), (4-
149 amidinophenyl)-methanesulfonyl fluoride hydrochloride (APMSF) 20 µ*M*, leupeptin 1 µ*M* and
150 pepstatin A 1 µM]. The homogenate was kept at room temperature for 10 min and
151 centrifuged for 15 min at 21460 *g* and 2°C. The supernatants were collected for analysis.
152 CAT activity was assayed by adding 55 µl buffer [KH_2PO_4 pH 7, 0.05 *M*, hydrogen peroxide,
153 0.036%] to 2 µl extract. The hydrogen peroxide-dependent reduction catalyzed by CAT
154 activity was measured by the hydrogen peroxide absorbance depletion at 240 nm, in a
155 SmartSpect spectrophotometer at room temperature for 5 min at 10-s intervals. One CAT
156 activity unit was defined as the enzyme quantity needed to reduce 1 µl H_2O_2 min^{-1} at 25°C.
157 POD activity was analyzed by adding 100 µl buffer [KH_2PO_4 pH.7, 100 m*M*, guaiacol 10
158 m*M*, 5 µl hydrogen peroxide (0.3%)] to 40 µl extract, after the 5-min absorbance increase at
159 470 nm due to the formation of tetrahydroguaiacol after Guaiacol oxidation in a

SmartSpect 3000 spectrophotometer. One POD activity unit was defined as the quantity of enzyme required to reduce 1 µmol hydrogen peroxide min^{-1} at room temperature. SOD activity was determined following the O_2^- induced reduction of WST-1(2-(4-Iodophenyl)-3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)-2*H*-trezolium, monosodium salt) to WST-formazan using the xanthine–xanthine oxidase system, with an SOD assay kit (19160 Sigma). SOD activity (inhibition rate %) in the extracts was calculated with the following equation: [(Abs Blank 1 − Abs Blank 3) − (Abs sample − Abs Blank 2)]/(Abs Blank 1 − Abs Blank 3) × 100. One SOD activity unit was defined as the quantity of extract that caused a 50% absorbance inhibition at 450 nm and 37°C. Protein concentration in the extracts was determined with the protein-dye binding method using Coomassie Plus™ Protein Assay Reagent (Biorad-Germany). Enzyme activity was expressed as units per mg protein (U mg^{-1}).

Statistical analysis

Statistical analyses were performed by means of Statistical Analysis System software (SAS Institute 2004).

A t-distribution at $p < 0.05$ was applied for assessing significant differences in each genotype and each day between control and treated plants for stomatal conductance and chlorophyll content monitored in the long-term trial. The differences among treatments within each genotype were determined using analysis of one-way variance (ANOVA) for stomatal conductance and also for each enzyme and each year in the short-term trial. Means were separated by Duncan's test ($P < 0.05$).

Results

Chlorophyll concentration in the long-term trial

In both years, the colorimetric measurements did not show significant differences between the waterlogging and the recovery periods in either control or waterlogged plants of myrobalans 'P.2175' and 'P.2980' (Figs 1A, B and 2A, B). However, in 2007, the SPAD values in 'Felinem' and 'Garnem' were almost constant during the treatment period and higher in control plants than for the flooded plants, where a decrease was observed at the end of the recovery period (Fig. 1C, D). Moreover, in 2008, the control and waterlogged plants increased their values during both periods (Fig. 2C, D) but the differences were not statistically significant, and the values did not decrease as much as in the previous year, although there is a decrease in values at the end of recovery in 'Garnem' and 'Felinem'.

Leaf conductance in the long-term trial

Statistically significant decreases in leaf conductance (gl) occurred in the four genotypes during the 7 days of treatment, 10 days of recovery and the two following weeks in both years. In 2007 (Fig. 3), the leaf conductance reduction for 'P.2175' and 'P.2980' stressed plants was about 85 and 87%, respectively, compared with their control, meanwhile it was about 100% for the two hybrids 'Felinem' and 'Garnem'. In 2007, during the recovery period, both 'P.2175' and 'P.2980' myrobalan increased their leaf conductance through the recovery period, but at the end of the experimental period, significant differences were still found (Fig. 3A, B), although the plants were able to recuperate afterward without sign of death. However in the hybrids 'Felinem' and 'Garnem' after the 10- day recovery period, the readings were beyond the porometer range and all the stressed plants died, showing also significant differences (Fig. 3C, D). Meanwhile, in 2008 (Fig. 4), although a reduction of leaf functionality was observed for the four genotypes ('P.2175', 'P.2980', 'Felinem' and 'Garnem') during the flooding period, it increased during the recovery period, not only in the two myrobalans but also in the two hybrids. At the end of the 10-day recovery period, significant differences were found in the four genotypes. However, 1 week later, the differences were not statistically significant in the two myrobalans (Fig. 4A, B); and 2 weeks later, neither were in the two hybrids. (Fig. 4C, D). Moreover, the differences in temperature registered in the same days during the waterlogging and the recovery periods resulted statistically significant, up to 7°C between the 2 years (Table 1).

Leaf conductance in the short-term trial

Leaf functionality, measured by stomatal conductance in the short-term trial, showed similar pattern but with statistically differences in both years for the two cultivars evaluated 'P.2175' and 'Felinem'. Leaf conductance was lower in 2008 than in 2007 for all the treatments in both genotypes. Statistically significant difference was observed at 24 h W and 6 h recovery in both genotypes 'Felinem' and 'P.2175' (Fig. 5A, B), in both years. Those differences were not statistically significant between the 6 h W and 6 h recovery in either genotype in both years.

Antioxidant enzyme activity

Catalase

In response to flooding, CAT activity was higher in 'Felinem than in 'P.2175'. Flooded 'P.2175' plants showed CAT levels practically indistinguishable from those in the control treatment and recovery periods, and only statistically significant differences were found in 2007 (Fig. 6A). CAT activity in flooded 'P.2175' remained at basal levels for the 0- and 24-h recovery in both years.

While, in 2008, CAT activity increased 1.5-fold (compared with the control samples) in the recovery periods in 'Felinem' genotype, showing statistically significant differences at 24 h recovery (Fig. 7A), in the first year, CAT levels at 24 h recovery reached the control levels with no differences among the treatments (Fig. 6A). Overall, basal CAT levels in 'P.2175' were always lower than in 'Felinem'. In 'P.2175', values ranged between 0.2 and 0.5 U/mg protein; in 'Felinem', CAT activity varied between 1.5 and 4.0 U/mg protein.

Guaiacol peroxidase

This enzyme catalyzes the reduction of H_2O_2 using guaiacol as a co-factor. POD activity is higher in 'Felinem' than in 'P.2175' in both years, statistically significant differences were found only for 'Felinem' (Figs 6B and 7B) with an increase in 2008, but almost no visible change in the first year (2007). After 24 h of flooding treatment, POD activity in 'Felinem' roots initiated an increasing pattern that led, after 6 h of recovery period, to levels similar to those after 6 h flooding, but increased with statistically significant differences, and higher than in control plants. In 'P.2175', the pattern followed by POD was similar in both years, although higher activity was observed (Fig. 7A), in the first year, CAT levels at 24 h recovery reached the control levels with no differences among the treatments (Fig. 6A). Overall, basal CAT levels in 'P.2175' were always lower than in 'Felinem'. In 'P.2175', values ranged between 0.2 and 0.5 U/mg protein; in 'Felinem', CAT activity varied between 1.5 and 4.0 U/mg protein.

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Superoxide dismutase

Of the three antioxidant enzymes, SOD showed higher basal levels than CAT and POD. The pattern is similar for both genotypes in the 2 years, although higher activity was observed in 'Felinem'. In the first year (2007), both genotypes showed a minimum activity at the moment of maximum stress, at 24 h after flooding, lower than the control samples. 'P.2175' plants decreased SOD activity in response to flooding, showing levels 0.05 times lower than the control after 24 h of treatment. In 'Felinem', a rapid increase of SOD enzymatic activity was observed in 2007, at 6 h of flooding, with SOD levels higher than in the control samples. In both genotypes, SOD activity rose afterward, at the moment of recovery (Figs 6C and 7C), but significant differences were only found in 'P.2175' (Fig. 6C). In 2008, also both genotypes presented the same pattern, increasing at the moment of maximum stress and decreasing during the recovery period, and not showing any significant differences (Fig. 7C). Root SOD activity remained elevated in the recovery periods, with no statistically significant difference. Both 'Felinem' and 'P.2175' showed not marked differences between years.

Discussion

Physiological parameters

In the both long- and short-term trials in 2008, the plants of all genotypes were less developed at the beginning of the waterlogging period than in 2007.

As the experiments were carried out in the outdoors conditions mentioned, the less growth observed could be due to different environmental conditions in the month before the treatment: (17.2°C mean temperature, 62.4% RH, 22.9 MJ m^{-2} day^{-1} solar radiation and 31.4 mm $month^{-1}$ mean precipitation in 2007, and 16.5°C mean temperature, 64.7% RH, 19.3 MJ m^{-2} day^{-1} solar radiation and 162.4 mm $month^{-1}$ mean precipitation in 2008). So probably, this lower temperature and the higher precipitation in 2008 than in 2007 influenced the plant stage at the beginning of the treatment. Moreover, the temperatures registered during the waterlogging period were also significantly different. Although the chlorophyll content is not directly related to root asphyxia (Dichio et al. 2003), it could be considered as an index of plant development (data not shown), especially for the two red hybrid genotypes studied here. The chlorophyll meter quantifies green color in plants by immediately and non-destructively measuring relative quantity of chlorophyll (Turner and Jund 1994). The meter does not measure nitrogen or chlorophyll concentration; rather it provides immediate, on-site, quantitative measurements of leaf greenness. These hybrids have

286 'Nemared' as a male parent, with red leaves turning brownish-green to green as they mature in the
287 season when they continued growing (Gómez-Aparisi et al. 2001, Felipe 2009). Thus, even
288 though plant growth can be affected by waterlogging (Kozlowski 1997, Yu and Rengel 1999,
289 Visser et al. 2003), in 2008, the two hybrids' stressed plants continued growing (data not shown)
290 during the stress period (Fig. 2C, D), and the plant leaves were turning from red to green. Thus, in
291 2007, stressed plants' SPAD values were observed to decrease showing significant differences (Fig.
292 1C, D). Additionally, low root mass was observed in all the plant sampled for antioxidant analysis in
293 2008 compared with those in 2007 (data not shown). Although it is recognized that SPAD values are
294 influenced by plant growth stage (Turner and Jund 1994), a correlation exists between leaf color
295 and growth diagnosis through nutrient status (Furuya 1987). Therefore, we can readily assimilate
296 nutrient status as the responsible for plant development. We have observed that the same flooding
297 period, 1 week, had a different effect in plant tolerance in both hybrids in 2007 and 2008. So that,
298 SPAD values could be used in that case as an index of vegetative development, not only in the hybrids
299 that showed not significant differences in 2008 but also in the two myrobalans where SPAD values
300 of the stressed plants also were higher than in the control during the recovery period. As we assume
301 that plums are the tolerant genotypes (Domingo et al. 2002, Ruiz-Sanchez 2002), the release of
302 drainage also has a beneficial effect showing higher values, although not statistically different
303 between stressed and control plants (Fig. 2A, B).

304 Certainly, the most significant effect of waterlogging was manifested quickly observed on leaf
305 conductance values. No dieback or sign of death was appreciated in any of the two genotypes, only
306 after 24 h of root hypoxia due to water excess in either year in the short-term trial. In 2008, 2 weeks
307 after the recovery period, the differences were not statistically significant either in the myrobalans or
308 in the hybrids, while they were so in 2007 in the long-term trial. A higher capacity to recover after release
309 was observed in the myrobalan plants, where the conductance values 1 week after (Fig. 4A, B) the
310 recovery period between stressed and control plants were not statistically significant. In the hybrids,
311 the recovery was slower and it took one more week (Fig. 4C, D) to reach the value of the control plants.
312 Also, these results imply that a lower degree of stress was affecting the plants in 2008, most probably
313 due to a less biomass roots as a result of less growth, which implies more gas diffusion in a more
314 aerated root environment (Drew 1997, Dennis et al. 2000, Liu et al. 2005) that allowed plants of both
315 genotypes to recover faster. Even in 'Felinem' and 'Garnem', the control plants showed lower values
316 in 2008 (Fig. 4C, D) than in 2007 (Fig. 3C, D), but the 2008 decrease was not as steep as in 2007,
317 when they stopped growing and finally died. Moreover, the low 'Garnem' values at the moment of
318 maximum stress and high 'Felinem' values at the end of the experiment (Fig. 4C, D) could evidence

319 a higher tolerance of 'Felinem' than 'Garnem'. These leaf functionality values observed in the young
320 shoot of 'Felinem' together with a lower epinasty (data not shown) could indicate a slightly higher
321 tolerance to root asphyxia (Wong et al. 1979, Munns 1988, Kozlowski 1997) in moderate climatic
322 conditions. Previous work with the two hybrids did not establish differences (Dichio et al. 2003),
323 although both genotypes were flooded for a longer time and with different floodwater conditions.
324 Leaf damage was apparent (data not shown) in continuously flooded plants in 2007, and drainage
325 did not improve plant performance, but accelerated plant injury, similar results have been also
326 previously reported in citrus plants (Arbona et al. 2009, Hossain et al. 2009). Apparently, the lower
327 stress imposed in the root environment because of the less plant development observed in 2008 leads
328 the plant to survive better after drainage. As the same stress period imposed was not enough to injure
329 even the most sensitive genotypes, all of them (myrobalans and hybrids) recovered, reaching the level
330 established by the control plants. These results suggest that the plant vegetative stage influence their
331 behavior when overcoming a waterlogging period. Their eventual survival can be attributed to a
332 lesser vigor as pointed out by Xiloyannis et al. (2007). In our case, the root volume is limited by the
333 volume of pot, making the most vigorous plants more affected. As the temperature during the
334 flooding period (Table 1) in the 2 years was also significantly different, we can assume that the
335 temperature and other environmental conditions previous to the assay had also a remarkable influence
336 on the differences found in plant growth and hence in the tolerance response between the 2 years.
337 Those results indicate that waterlogging stress had a significant effect on leaf conductance in
338 continuously flooded plants, and similar results have been obtained in other crops (Visser et al. 2003,
339 Arbona et al. 2008, 2009, Sairam et al. 2008).

340 **Enzymatic activity**

341 Differences are especially noticeable in CAT activity, where the activation in the sensitive genotype
342 'Felinem' at the end of the aerated period becomes evident, with an increased free radical
343 scavenging activity in 2008. The lowest activity values were found for POD activity in both
344 genotypes, but similar low activity was also found in other peroxidases in *Citrus* (Hossain et al.
345 2009). Among the peroxidases, the one studied here is guaiacol peroxidase, acting the guaiacol as
346 electron donor (Blokhina et al. 2003). Few reports, among themselves in olive tree during rewatering
347 (Sofo et al. 2003), describe the role of this guaiacol peroxidase (POD), although most of them are
348 referred to ascorbate peroxidase (APX); both are involved in scavenging ROS from the different tissues
349 (Blokhina et al. 2003). Elevated APX activity has been involved in the oxidative damage observed in
350 many environmental stresses, such as salinity (Arbona et al. 2008) or waterlogging (Hossain et al.
351 2009). Contrarily, APX, have also been described as less affected by anoxia, have been stimulated

almost twofold during the aerated periods (Gogginn and Colmer 2005, Hossain et al. 2009). A decrease in guaiacol activity has been described as not affected by anoxia in tobacco (Blokhina et al. 2003). The two enzymes CAT and POD were activated under the stress level imposed in 2008, in a more aerated root environment especially at the end of the recovery period. The early CAT activation in 'Felinem', sensitive genotype, compared with 'P.2175', tolerant genotype, not only seems to be related to an active and efficient plant response to cope with waterlogging but also seems to be affected by the stress level suffered, as we observed low CAT in 2007 (also in the tolerant genotype) with a tiny increase in 2007, at the end of the waterlogging period. However, during 2008, less damage occurs in the more aerated soils, and induction occurred in the case of CAT and POD and both worked efficiently in the most sensitive genotype, 'Felinem'. Moreover, a higher accumulation of H_2O_2in 2007 may be partially responsible for causing membrane damage, which seems to be linked to oxidative damage and radical accumulation (Blokhina et al. 2003), not allowing plants with a strong level of stress to recuperate. CAT activity in the tolerant genotype 'P.2175' was lower than in the sensitive 'Felinem' during both years, which agrees with the hypothesis that CAT is described as an earlier response in the antioxidant system and also that acclimatizing has been shown to be dependent on the activation of such enzyme (Amor et al. 2000, Blokhina et al. 2003). Ultimately, SOD constitutes the first line of defense against ROS, and it seems directly modulate the amounts of O_2 and H_2O_2. This H_2O_2 is removed mainly by APX and CAT activities (Edreva 2005). The induction pattern of SOD is clearly the first response and may be independent of the cell membrane, as it is shown by its activity, which is higher than that of CAT and POD. Although induction occurs during both years in both genotypes, at the moment of maximum stress, SOD activity was higher in both genotypes in 2008 and lower in 2007, which could be explained also with the fact that in 2008 less injured roots were found at the moment of maximum stress. SOD activity decreased by 20% under continuous anoxia but ultimately returned with the aerated activity under intermittent anoxia, which are in concordance with results obtained in tissue roots (Gogginn and Colmer 2005).

It could be argued also in this short-term trial that high vegetative growth was related to higher old root abundance, so that roots in 2007 could respond later against oxidative damage than the younger roots in 2008 (Visser et al. 2003, Gogginn and Colmer 2005). A decline in SOD activity over the flooding period has been also described for citrus (Arbona et al. 2008, Hossain et al. 2009); however, the different tissue studied here could corroborate a different induction pattern depending of the tissues. The present results could be better explained by the different nature of the roots between the 2 years, and also, to the fact that active growth and continually renewing roots can respond faster

385 under an eventual defense mechanism to overcome stress. Moreover, a differential SOD response
386 to oxygen deprivation stress (anoxia and hypoxia) on different plants has been always contradictorily
387 described, depending of the experimental set-up or prolonged re-oxygenation (Blokhina et al. 2003).
388 In addition, although redox adjustments are central to most stress responses, the extent to which
389 intracellular ROS concentrations increase as a result of stress is highly variable (Foyer and Noctor
390 2005). Even though, the results shown here indicate an activation of the antioxidant system by
391 different degrees of stress, depending on the aerated environment. It has been known for a long
392 time that the main damage caused by anoxic stress occurs during re-admission of oxygen
393 (Blokhina et al. 2003), our results seem to be more in accordance with that situation, where post-
394 anoxia oxidative stress in the plant tissues would not include a hypoxic treatment before anoxia.

395 To our knowledge, for *Prunus* and other fruit trees, although differences in physiological
396 performance have been achieved (Arbona et al. 2009), no anatomical adaptations to flooding have been
397 reported as for wetland plants where anatomical adaptation has been observed (Vartapetian et al.
398 2003). Previous works describe differences in tolerance in several woody plant species (Vu and
399 Yelenosky 1991, Ruiz-Sánchez et al. 1996, Domingo et al. 2002, Parelle et al. 2006, Arbona et al.
400 2009), but, we report here the different response in antioxidant response between tolerant and
401 sensitive genotypes to waterlogging in *Prunus* species. We cannot conclude that antioxidant enzymes
402 are involved directly in the higher tolerance of 'P.2175' or in the scavenging of ROS compound. On
403 the contrary, tolerance could be more related to fermentative pathways that better modulate the toxic
404 accumulation of acetaldehyde and ethanol (Amador et al. 2009). Ultimately, the whole
405 photosynthetic machinery seems to play a more important role than the antioxidants system in the
406 tolerance response (Vu and Yelenosky 1991, Arbona et al. 2009). Flooding and submergence are
407 major abiotic stresses and rank alongside water shortage, salinity and extreme temperatures as major
408 determinants of species distribution worldwide (Sairam et al. 2008). Also, temperature stress can have
409 a devastating effect on plant metabolism, uncoupling major physiological processes (Suzuki and
410 Mittler 2006), and it seems to be the cause of the differences found in this work between the 2
411 years. Experimental conditions outdoors imposed not only waterlogging stress but apparently heat
412 stress, could influence the stress level response. Our results are more in accordance with the severity
413 of the stress imposed by the different growth and, ultimately, with the oxygen deprivation in the root
414 environment. Oxygen deprivation endurance depends upon plant cell and tissue type, developmental
415 stage and genotype, as well as the severity and duration of the stress. Light levels and ambient
416 temperature have also been pointed out (Fukao and Bailey-Serres 2004). Ultimately, we aim to
417 shed some light on the system and processes controlling the phenotypes used as rootstocks. The results

418 show that the release from stress has a beneficial effect of plant physiology, according to the stress level
419 either in the metabolism or in the activation of the antioxidant system, as it is indicated in the second year
420 by the plants' ability to recuperate.

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Tables and figures legends.

Table 1. Temperature (°C) in the long-term trial along the waterlogging and recovery periods in both years. For the same column, means values followed by the same letter are not different at $P < 0.05$ (according to t-test).

Fig. 1. Colorimetric measurements in the myrobalan 'P.2175' (A) and 'P.2980'(B), and almond × peach hybrid, 'Felinem'(C) and 'Garnem'(D) genotypes during the waterlogged (June 27 – July 4) and recovery (July 6 – July 13) periods in the year 2007. Ct, control; W, waterlogging. For the same genotype and date, mean values followed by an asterisk are different at $P < 0.05$.

Fig. 2. Colorimetric measurements in the myrobalan 'P.2175' (A) and 'P.2980' (B), and almond × peach hybrid, 'Felinem' (C) and 'Garnem'

(D) genotypes during the waterlogged (June 25 – July 2) and recovery (July 4 – July 11) periods in the year 2008. Ct, control; W, waterlogging. For the same genotype and date, mean values followed by an asterisk are different at $P < 0.05$.

Fig. 3. Stomatal conductance in the long-term trial along the waterlogging (June 27 – July 4) and recovery (July 6 – July 27) periods during 2007 in myrobalan 'P.2175' (A), myrobalan 'P.2980' (B), 'Felinem' (C) and 'Garnem' (D). T, treatment; C, control; W, waterlogging. For the same genotype and date, mean values followed by an asterisk are different at $P < 0.05$.

Fig. 4. Stomatal conductance in the long-term trial along the waterlogging (June 25 – July 2) and recovery (July 4 – July 25) periods during 2008 in myrobalan 'P.2175' (A), myrobalan 'P.2980' (B), 'Felinem' (C) and 'Garnem' (D). T, treatment; W, waterlogged; C, control. For the same genotype and date, mean values followed by an asterisk are different at $P < 0.05$ (according to t-test).

Fig. 5. Stomatal conductance in the short-term trial along the waterlogging and recovery hours for both genotypes in 2007 (A) and 2008 (B). W, waterlogging, REC, recovery. For the same genotype, mean values followed by the same letter are not significantly different at $P < 0.05$ on each year.

Fig. 6. Enzymatic activity in the myrobalan 'P.2175', and 'Felinem' genotypes of CAT (A), POD (B) and SOD (C) during the short-term trial periods in the year 2007. W, waterlogging; REC, recovery. For each genotype, mean values followed by the same letter are not significantly different at $P < 0.05$ within treatments

Fig. 7. Enzymatic activity in the myrobalan 'P.2175' and 'Felinem' genotypes of CAT (A), POD (B) and SOD (C) during the short-term trial periods in the year 2008. W, waterlogging; REC, recovery. For each genotype, mean values followed by the same letter are not significantly different at $P < 0.05$ within treatments.