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Rapid formation of adventitious roots and partial ethylene sensitivity result in faster adaptation to flooding in the *aerial roots* (*aer*) mutant of tomato

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ABSTRACT

The frequency of extreme events such as droughts and floods has increased as a consequence of climate change. Many crops have not been improved to tolerate soil anoxia and, therefore, floods cause important economic losses. During submergence, *Solanum lycopersicum* L. exhibits three distinct responses which are adventitious root production, epinasty and aerenchyma formation. The development of a new adventitious root system is crucial as it can replace the original roots that succumb to the hypoxic environment. A*erial roots* (*aer*) is a tomato mutant characterized by the presence of numerous adventitious root primordia along the hypocotyl and older internodes. In this work, we have analyzed the *aer* mutant behavior to flooding to determine whether preformed adventitious roots represent an adaptive advantage with respect to biomass accumulation. We have also examined other morphological and anatomical responses of *aer* plants to detect differential adaptations under flooding. *Aer* plants form an abundant adventitious root system faster than Ailsa Craig cultivar, which results in flooded plants accumulating as much biomass as non-flooded *aer* plants. In addition, several ethylene-induced responses such as epinasty, hypertrophy, aerenchyma production, and apical hook formation are reduced in *aer*, suggesting a lower sensitivity of some tissues to ethylene. The *E4* expression level, an ethylene-induced gene, confirmed this observation since *E4* transcripts are less abundant in petioles and stems of ethylene-treated *aer* plants, coinciding with the tissues that present a lower degree of morphological and/or anatomical response. Evidence from the *Never ripe* mutant suggests a reduction in ethylene sensitivity could contribute to the attenuation of flooding effects. Therefore, our results indicate that the rapid formation of a new root system together with a reduction in ethylene sensitivity is responsible of a faster adaptation to flooding stress in the *aer* mutant.

1. Introduction

Climate change has brought about an increase in the frequency of climate extremes (Niu et al., 2014). Heat waves, frosts, droughts and heavy rainfall are some of the effects due to an increase in greenhouse gas emissions. In particular, intense precipitation that causes surface water flooding, river floods and snow melting have resulted in important losses in crop production all over the world (Bailey-Serres et al., 2012). This scenario is particularly alarming as most crops have not been selected for tolerance/resistance to flooding (Setter and Waters, 2003). Considering the increasing world population, one of the main challenges for the years to come is the development of high yielding varieties that are able to withstand extreme rainfall events – and therefore flooding – due to a continuously changing climate (Tester and Langridge, 2010). A better mechanistic understanding of how plants cope with the excess of water in the soil would provide a useful resource in breeding programmes aimed at maintaining or increasing agricultural productivity in flooded lands (Voesenek et al., 2014).

Tomato (*Solanum lycopersicum*) is the second most important vegetable in the world market after potatoes (FAO, 2015). It has been used as a model species to study different aspects of plant physiology and genetics, especially for those traits for which *Arabidopsis* is not a suitable option (Carvalho et al., 2011). Furthermore, tomato is more closely related to other horticultural crops such as lettuce than other widely studied species (Jiménez-Gómez and

Abbreviation: AR, adventitious root.

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Maloof, 2009), making it easier to transfer knowledge to those species.

Tomato has an additional advantage when used as model plant, which is the number of monogenic mutants that are available, most of them through the Tomato Genetics Resource Center (TGRC). Some of these mutants have been well characterized with respect to their physiology such as *Never ripe* (Lanahan et al., 1994), *diageotropica* (Kelly and Bradford, 1986), *epinastic* (Barry et al., 2001), *entire* (Mignolli et al., 2015), *ripening inhibitor* and *non ripening* (Osorio et al., 2011) whereas others, such as *aerial roots (aer)*, have never been studied. Phenotypically, the *aer* tomato mutant resembles a wild type plant except for the fact that numerous quiescent adventitious root (AR) primordia are formed along the stem.

After the onset of flooding, tomato plants exhibit some distinct responses, three of which are AR development (Jackson, 1955), leaf epinasty (Jackson and Campbell, 1976) and aerenchyma formation (Kawase, 1981). All three responses seem to act together to mitigate the impact of flooding. In particular, the contribution of a new AR system has long been associated with tomato plant recovery from flooding stress (Kramer, 1951; Jackson, 1955; Aloni and Rosenshtein, 1982; McNamara and Mitchell, 1990; Else et al., 2009). Indeed, tomato plant ability to produce a new root system allows the plant to adapt by replacing the original roots when damaged by the hypoxic/anoxic environment (Jackson and Drew, 1984; Colmer and Voesenek, 2009). Moreover, the production of ARs proves also beneficial when pathogens attack the original root system (Román-Avilés et al., 2004). However, the ability to respond to environmental stress depends on plant life cycle stage, as young plants are more plastic than older ones (Zhang et al., 2015).

The commercial use of submergence tolerant plants requires fundamental knowledge of basic aspects of tomato adaptive responses, as well as the identification of promising genotypes. In this paper, we characterized for the first time the *aer* mutant behaviour under flooding, considering morphological and anatomical changes (AR formation, stem porosity, stem hypertrophy, aerenchyma development and leaf epinasty) as well as the plant ability to resume growth while flooded. In particular, we tested the hypothesis whether preformed ARs present in *aer* stems result in faster adaptation to flooding during early life stages. Following, based on our morphological and anatomical observations, we addressed the question whether partial ethylene sensitivity in certain tissues contributes to mitigate the flooding stress effect on biomass accumulation.

2. Materials and methods

2.1. Plant materials and growing conditions

Tomato (*S. lycopersicum*) cv Ailsa Craig (AC, accession LA2838A) and *aerial roots* mutant seeds (*aer*, accession LA3205) were provided by the Tomato Genetics Resource Centre (TGRC, University of California, Davis). Since the genetic background of *aer* is unknown and nearly isogenic lines were not available, we selected the AC cultivar as a control line for our experiments. AC has been used as a control cultivar in physiological studies regarding AR formation (Negi et al., 2010; Vidoz et al., 2010) and in studies looking at anthocyanin production when near isogenic lines did not exist (Povero et al., 2011). The lack of spontaneous production of AR primordia along the stem, make AC a useful wild type to perform valid comparisons. To assess the effects of ethylene insensitivity, an experiment was performed using the ethylene perception mutant *Never ripe* (*Nr*, accession LA0162) and its wild type Pearson (accession LA0012) (Lanahan et al., 1994; Clark et al., 1999), provided by the TGRC.

One-week-old seedlings were transplanted on peat-based substrate (pH 5.5–6.5) amended with perlite (Dynamics 2 Q80, Buenos Aires, Argentina) in 220 mL plastic pots and irrigated every two days with 150 mL 1/4 strength Hoagland solution after the second week from sowing. Plants were maintained in a climatically controlled room with a temperature of $26 \pm 1\,^{\circ}C$ with 15 h photoperiod provided by high pressure sodium lamps (Vialox®, 400W, OSRAM GmbH, Germany). Average light intensity at the plant height was 254 $\mu mol\ m^{-2}\ s^{-1}$ and relative humidity fluctuated between 50 and 70%. Plants were grown for up to four weeks from sowing.

Unless specified otherwise, all flooding experiments were conducted with 4-week-old plants and lasted seven days. Groups of eight to fifteen plants were placed in $60 \times 40 \times 25$ cm ($h \times w \times d$) tanks and hypocotyls were submerged in tap water up to the cotyledons. Control plants were placed in similar containers and watered regularly, allowing soil to freely drain. When we assessed the effect of flooding duration on plant biomass, plants were flooded in 1/4 strength Hoagland solution so that the only factor affecting plants was flooding and not nutrient deficiency. In this case, control plants were regularly watered with 1/4 strength Hoagland solution as required.

2.2. Plant growth parameters

Biomass of roots, stems, leaves, and ARs of flooded and control plants was determined by oven-drying the material at 70 °C until constant weight.

Leaf area was determined by placing the leaves on a flat surface and covering them with a glass lamina to keep the material flat. Photos were taken and processed with the public-domain digital image processing software ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij) to determine the area.

2.3. Hypocotyl porosity determination

A modified pycnometer method (Jensen et al., 1969) was used in order to determine porosity. Hypocotyls from control and flooded plants approximately 1 cm long were excised and those from treated plants were carefully blotted with tissue paper. Hypocotyls were weighed (Z) and then infiltrated with distilled water under vacuum (−0.9 atm for 3 min) before being weighed again (Y). Their volume was determined by weighing a pycnometer full of water (X), and then the pycnometer with the infiltrated hypocotyl fully submerged (W). The volume of the water equivalent to the volume of the hypocotyl was determined with the following calculation: $[X - (W - Y)]$ / water density = $V_{hypocotyl}$. The volume occupied by air (V_{air}) was calculated as the difference between the weight of the hypocotyl infiltrated with water (Y) and the hypocotyl before this procedure (Z) divided by the density of water. Finally, the porosity was calculated as follows: $V_{air}/V_{hypocotyl} \times 100$.

2.4. Anatomical observations of flooded hypocotyls

Hypocotyl segments of control and 72 h flooded plants were first fixed in FAA (10 formaldehyde 40%: 5 acetic acid: 50 ethanol: 35 water v/v) applying a vacuum for 15 min. Samples were dehydrated using the tertiary butyl alcohol series and finally embedded in paraffin. Sections of 30 μm were stained with safranin for 2 h and counter-stained with Fast Green FCF for 5 min. Digital microphotographs were acquired with a Leica ICC50HD digital photographic camera coupled with a Leica DM LB2 (Leica Microsystems, Wetzlar, Germany) optical microscope.

2.5. Ethylene response assays

For the epinasty experiment, 4-week-old plants were placed for 16 h in an air-tight chamber enriched with 50 $\mu L\,L^{-1}$ ethylene. Ethylene gas was prepared from ethephon as described by Zhang and Wen (2010). Petiole angles of the first four basal leaves were measured at the beginning and at the end of the treatment by photographing leaf insertions perpendicularly and analyzing the photos with ImageJ. The experiment was performed twice with similar results.

The ethylene dose-response assay was performed on 5-day-old seedlings grown in darkness on water-agar medium. Seedlings were placed in sealed jars and exposed to 0, 1 and 10 $\mu L\,L^{-1}$ ethylene in darkness for 2 days. Finally, root and hypocotyl length, and apical hook curvature were measured. To determine the intensity of apical hook formation, 70 to 105 5-day-old seedlings of each genotype were treated with either air or 10 $\mu L\,L^{-1}$ ethylene for 2 days. Following, seedlings were classified into three categories according to the apical hook angle: <180°, = 180° and >180°.

2.6. Analysis of E4 gene expression

Samples of hypocotyls, leaves and petioles of AC and *aer* plants kept in presence of ethylene or air, as described before, were collected. Total RNA was extracted as described by Mignolli et al. (2012) with some modifications. Approximately 200 mg of frozen tissues were homogenized by the addition of 1 mL of TRI Reagent® (MRC, Cincinnati, OH, USA) and the supernatant was partitioned with chloroform. The aqueous phase was collected and 1:1 ratio of ice-cold isopropanol and high salt solution (0.8 M sodium citrate and 1.2 M sodium chloride, Sigma-Aldrich) was added. Finally, precipitated RNA was washed with 75% ethanol and resuspended in DEPC water. RNA was purified from contaminating DNA with the TURBO DNA free kit (Applied Biosystems/Ambion, Austin, TX, USA) and 5 μg of each sample were reverse transcribed into cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems).

Real Time RT-PCR was performed with an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). Transcript analysis of *E4*, an ethylene-induced gene (Lincoln et al., 1987), was performed using 50 ng of cDNA and SYBR® Green Master Mix (Bio-Rad, Hercules, CA, USA). Primer pair used for *E4* gene were: Fw 5′-GTGAAGACGGAGGTTGGGTA-3′ and Rv 5'-AGACATTCGGGTCAAACTGG-3'. Expression was normalized with the transcript levels of *LeEF1a* (Fw 5′-CATCAGACAAACCCCTCCGT-3′, Rv 5′-GGGGATTTTGTCAGGGTTGTAA-3′).

2.7. Statistical analyses

Two-way analyses of variance (ANOVA) were performed using Graphpad Prism 6.0 statistical software (www.graphpad.com). If main effects or interactions were significant, we compared differences between treatment means using Student's *t*-test ($P < 0.05$) or Tuckey's post test ($P < 0.05$).

3. Results

3.1. Aer and AC biomass accumulation after the onset of flooding

In order to determine whether the *aer* mutant phenotype improves plant adaptation to flooding, several growth parameters were studied. Plant height increased in plants placed under control and flooding conditions for 1, 2 or 3 weeks, in both AC cultivar and *aer* mutant (Fig. 1A). In AC, plants flooded for 1 or 2 weeks showed a decrease in height when compared to the control, whereas *aer* plants were higher or similar in height to control plants (Fig. 1A).

When AC plants were flooded for 2 or 3 weeks, leaf biomass was reduced by ~50% with respect to controls although it slightly increased from 1 to 3 weeks of flooding (Fig. 1B). On the contrary, a 3-fold increase in leaf biomass was observed in flooded *aer* plants after 3 weeks of treatment. At this time point, there was no difference between control and flooded *aer* plants (Fig. 1B). Total leaf area of flooded AC plants increased from 1 to 2 weeks of treatment and was always lower than control plant leaf area (Fig. 1C). Differently, *aer* plants increased total leaf area even after the longest exposure to flooding, reaching the same area as control plants (Fig. 1C).

Stem biomass of AC plants increased significantly with time in control and flooded conditions (Fig. 1D). However, the dry weight of stems from plants flooded for 2 and 3 weeks was not as high as that from plants growing under control conditions. In *aer* flooded plants, stem biomass not only increased but also reached control amounts after 3 weeks of flooding (Fig. 1D). A week of flooding caused a decrease in root biomass in both genotypes (Fig. 1E). These roots remained below the water level at all times and never resumed growth during the experiment. ARs were produced in both genotypes after a week of flooding and AR biomass increased 4-fold from week 1 to week 3 in AC and *aer* plants (Fig. 1F). In spite of having a similar behavior, *aer* AR biomass was 4 times greater than that of AC plants (Fig. 1F).

Total plant biomass revealed that, after 3 weeks of flooding, *aer* plants accumulated as much dry weight as plants placed under control conditions suggesting that they were able to overcome the flooding stress (Fig. 1G). Conversely, AC plants were unable to recover from flooding if the stress lasted longer than 1 week. After 2 and 3 weeks of flooding, AR/shoot ratio in flooded *aer* plants was similar to root/shoot ratio of control plants (Table 1). In AC, AR/shoot ratio in 3-week-flooded plants was still lower than root/shoot ratio of the control plants. Flooded plants of both genotypes exhibited a low root/shoot ratio, due to the prompt death of the original root system.

3.2. Comparison of AR production in aer and AC

The number and length of ARs in 2-, 3- and 4-week-old plants was assessed to determine whether plant age affects their response to flooding stress (Fig. 2A). In *aer*, 3-week-old plants exposed to flooding already produced the maximum number of ARs, which was maintained in 4-week-old plants (Fig. 2B). On the contrary, 3-week-old AC plants produced about half the number of ARs that arose in either older AC plants or *aer* plants of the same age.

Root length increased in 3-week-old AC plants respect to younger or older ones whereas it showed no difference between 3- and 4-week-old *aer* plants (Fig. 2C). In addition, ARs from 4-week-old AC plants were significantly shorter than those from *aer* plants (15 and 22 mm, respectively).

After 5 days, 4-week-old *aer* plants reached over 20% of the total number of ARs (Fig. 2D) whereas AC only produced about 5% of the total number. In spite of timing differences, the maximum number of ARs formed from submerged hypocotyls was the same for both genotypes (c.a. 100 ARs).

3.3. Hypertrophy, porosity and aerenchyma changes in aer

Stem hypertrophy was evident in all flooded plants and therefore we measured the diameter of control and flooded stems in both genotypes at the beginning and after a week of treatment. The increase in AC hypocotyls was larger (over 5-fold increase respect to control plants) than in *aer* (~3-fold increase respect to control plants) (Fig. 3A). The same trend was observed when stem porosity was measured using a modified pycnometer method, with a 3-fold increase in AC and only 1.4-fold increase in *aer* (Fig. 3A).

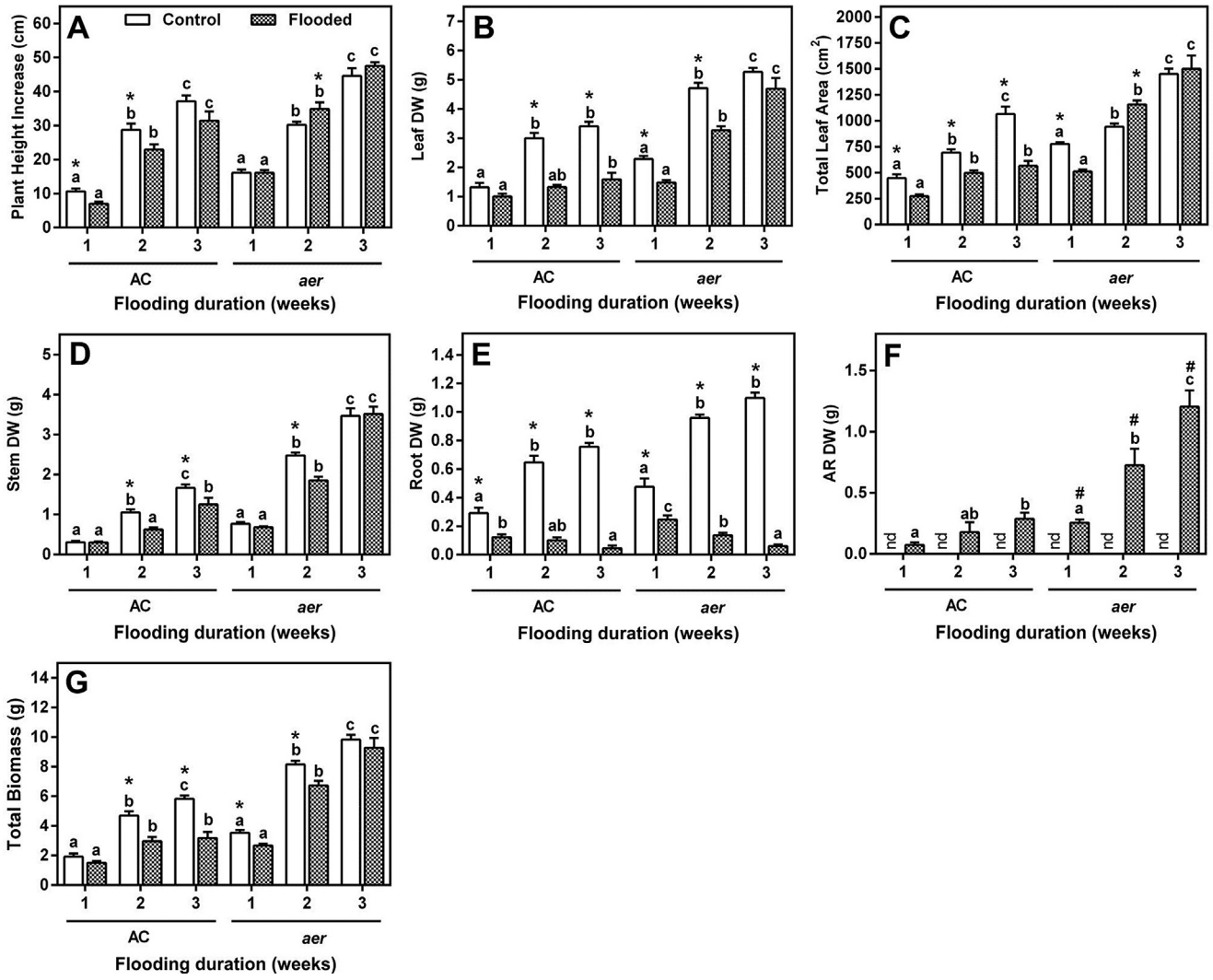

Fig. 1. Effect of flooding on plant height increase ($height_{final} - height_{initial}$, (A)), leaf biomass (B), leaf area (C), stem (D), root (E), adventitious root (F) and total (G) biomass accumulation of tomato cv AC and *aer* mutant plants after 1, 2 and 3 weeks of treatment. Plants were 4 weeks old at the beginning of treatments. Values are means ± SE. Except for diagram (F), all comparisons were performed within each genotype. Asterisks indicate significant differences between control and flooded plants by Student's *t* test within each flooding duration, whereas different letters indicate significant differences among flooding durations by Tukey's test within each treatment ($P < 0.05$; $n = 8$). Hash signs in (F) indicate significant differences between genotypes by Student's *t* test within each flooding duration. nd = not determined.

Table 1
Root to shoot ratio in tomato cv AC and *aer* mutant plants after 1, 2 and 3 weeks of flooding or under control conditions. Original roots/shoot ratio of control plants (control), original roots/shoot ratio of flooded plants ($flooded_{orig.\ roots}$), and adventitious roots/shoot ratio of flooded plants ($flooded_{adv.\ roots}$) are presented. Plants were 4 weeks old at the beginning of treatments. Values are means ± SE. Different letters indicate significant differences among the three ratios by Tukey's test within each flooding duration and genotype ($P < 0.05$; $n = 8$).

	AC			aer		
	Time of flooding treatment (weeks)			Time of flooding treatment (weeks)		
	1	2	3	1	2	3
Control	0.177 ± 0.009 (8) b	0.161 ± 0.008 (8) b	0.151 ± 0.008 (8) c	0.153 ± 0.015 (8) a	0.134 ± 0.004 (8) b	0.126 ± 0.004 (8) b
$Flooded_{orig.\ roots}$	0.089 ± 0.012 (8) a	0.050 ± 0.009 (8) a	0.018 ± 0.006 (8) a	0.117 ± 0.016 (8) a	0.027 ± 0.003 (8) a	0.007 ± 0.001 (8) a
$Flooded_{adv.roots}$	0.062 ± 0.022 (8) a	0.092 ± 0.035 (6) a	0.104 ± 0.012 (8) b	0.117 ± 0.008 (8) a	0.142 ± 0.023 (8) b	0.121 ± 0.015 (8) b

In order to determine whether flooding-induced hypertrophy and porosity were accompanied by aerenchyma formation, we examined stem anatomy in control and flooded plants. After 72 h of treatment, some cortical cells from flooded AC and *aer* hypocotyls underwent lysis causing the disorganization of the tissue but this process was less evident in *aer* (Fig. 3B). On the contrary, cortex sections from *aer* ARs presented more aerenchymatous tissue than those from AC (Fig. 3C).

3.4. Changes in ethylene responses in aer plants

Considering that flooded *aer* plants showed a lower degree of epinasty in previous experiments (Fig. 4A), 4-week-old plants of both genotypes were treated with ethylene in an air-tight chamber (Fig. 4B). After 16 h of treatment, AC plants were characterized by a pronounced increase in petiole curvature (~98°) whereas *aer* plants showed a milder increase in this parameter (63° in average, Fig. 4C).

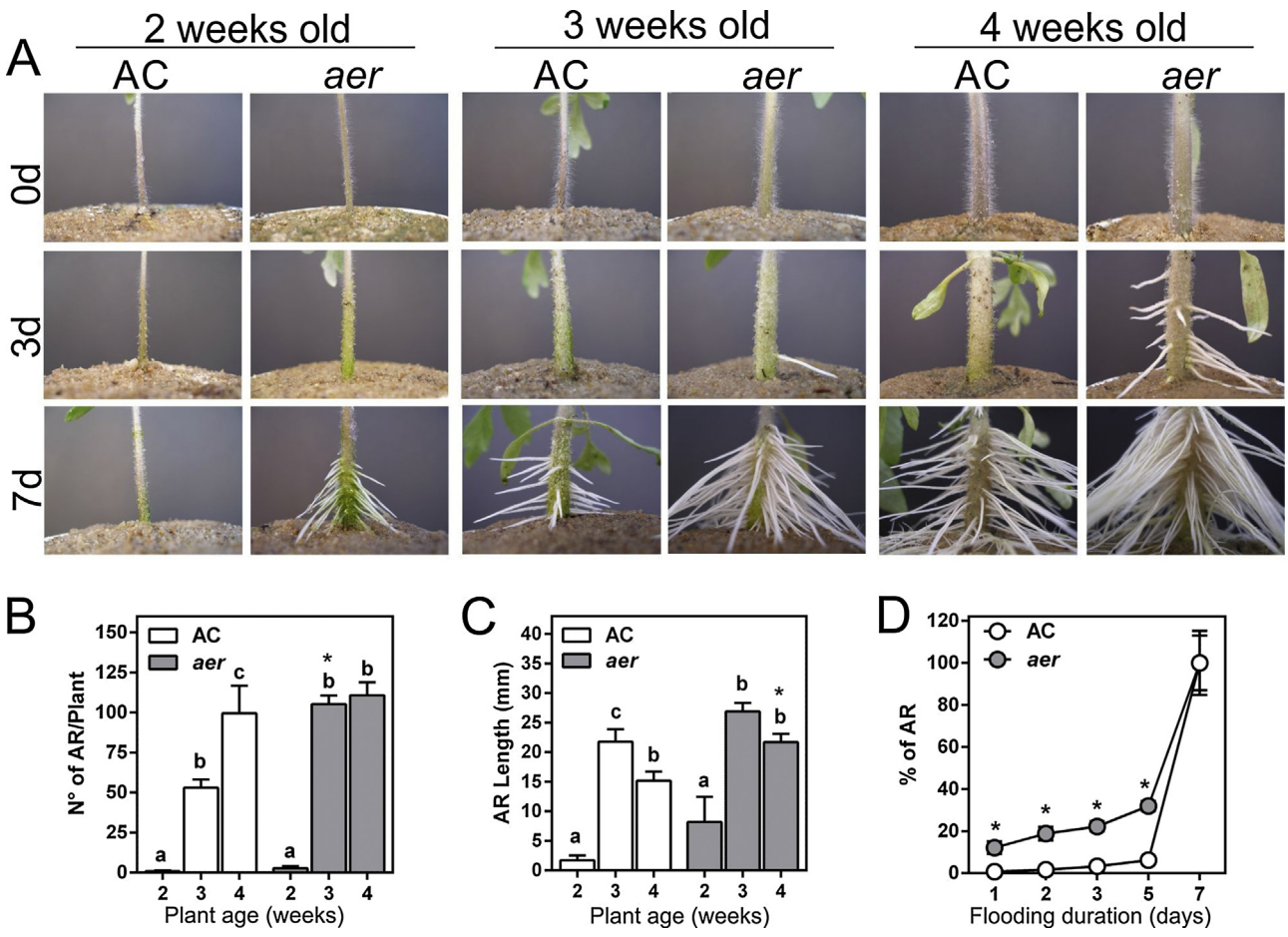

Fig. 2. Effect of plant age on adventitious root formation (A), number of adventitious roots (B) and adventitious root length (C) in flooded plants of tomato cv AC and *aer* mutant at 2, 3 and 4 weeks of age. Values are means ± SE. Asterisks indicate significant differences between AC and *aer* plants by Student's *t* test within each plant age, whereas different letters indicate significant differences among plants of different ages by Tukey's test within each genotype ($P < 0.05$; $n = 8$). Cumulative percentage of adventitious roots in 4-week-old plants of tomato cv AC and *aer* mutant after one week of flooding treatment (D). Values are means ± SE. Asterisks indicate significant differences between AC and *aer* plants by Student's *t* test within each point of time ($P < 0.05$; $n = 8$).

In order to confirm lower ethylene sensitivity in the *aer* mutant, seedlings of both genotypes were treated with ethylene in the absence of light. As ethylene concentration increased, hypocotyls became shorter following the same trend in both genotypes (Fig. 5A). When root length was measured, 1 $\mu L L^{-1}$ ethylene did not cause root shortening, but 10 $\mu L L^{-1}$ ethylene reduced root elongation in AC and *aer* mutant (Fig. 5B). Interestingly, increasing ethylene concentrations resulted in a higher degree of apical curvature in AC, whereas *aer* seedlings treated with 10 $\mu L L^{-1}$ ethylene showed no further increase in hook formation (Fig. 5C). Indeed, AC seedlings treated with 10 $\mu L L^{-1}$ ethylene pronounced an apical curvature of 180° or more while *aer* seedlings presented a similar proportion of apices over or below 180° of curvature (Fig. 5D).

In order to assess ethylene sensitivity, we analyzed the relative expression level of *E4* in leaves, petioles and hypocotyls of control and ethylene-treated plants. After treatment with 50 $\mu L L^{-1}$ ethylene, there was an increase in *E4* expression level in all three tissues of AC and *aer* plants. Although *E4* had similar expression levels in leaves of both genotypes (Fig. 5E) it had a lower expression in hypocotyls and petioles of the *aer* mutant.

The effect of partial ethylene insensitivity on biomass accumulation of flooded plants was studied using the *Nr* mutant. We observed that leaf biomass and total leaf area decreased in Pearson and *Nr* flooded plants, but this reduction was smaller in ethylene insensitive plants (Fig. 6A,B). Stem biomass was lower in flooded than in control Pearson plants but showed no significant variation between control and flooded *Nr* plants (Fig. 6C). The decrease in root biomass observed upon flooding was similar for Pearson and *Nr* plants (Fig. 6D). Regarding total biomass, there was a significant reduction in flooded plants of both genotypes, but *Nr* accumulated more biomass than Pearson (Fig. 6E). AR biomass and number were significantly lower in flooded *Nr* plants (Fig. 6F,G) but there was no variation between genotypes regarding AR length (Fig. 6H).

4. Discussion

Flooding is one of the abiotic stresses that negatively influence plant growth, reducing crop harvest in many parts of the world (Niu et al., 2014), and threatening global food production (Mickelbart et al., 2015). Tomato plants are known to be affected by the accumulation of water above the soil surface and grafting onto eggplant rootstocks has been suggested for plants cultivated in flood prone areas (Schwarz et al., 2010; Bhatt et al., 2015). The availability of a monogenic mutant stock in the TGRC has allowed us to identify a mutant, *aer*, which shows some degree of flooding adaptation. Indeed, flooded *aer* plants accumulate as much biomass as control plants after 3 weeks of treatment whereas, similarly to other tomato genotypes (Ezin et al., 2010), dry weight in flooded AC plants remains lower than in controls (Fig. 1G). In agreement with our results, Jackson (1955) observed that the initial decrease in growth of flooded tomato plants was reduced when ARs were removed as they developed but, after 9 days, the growth

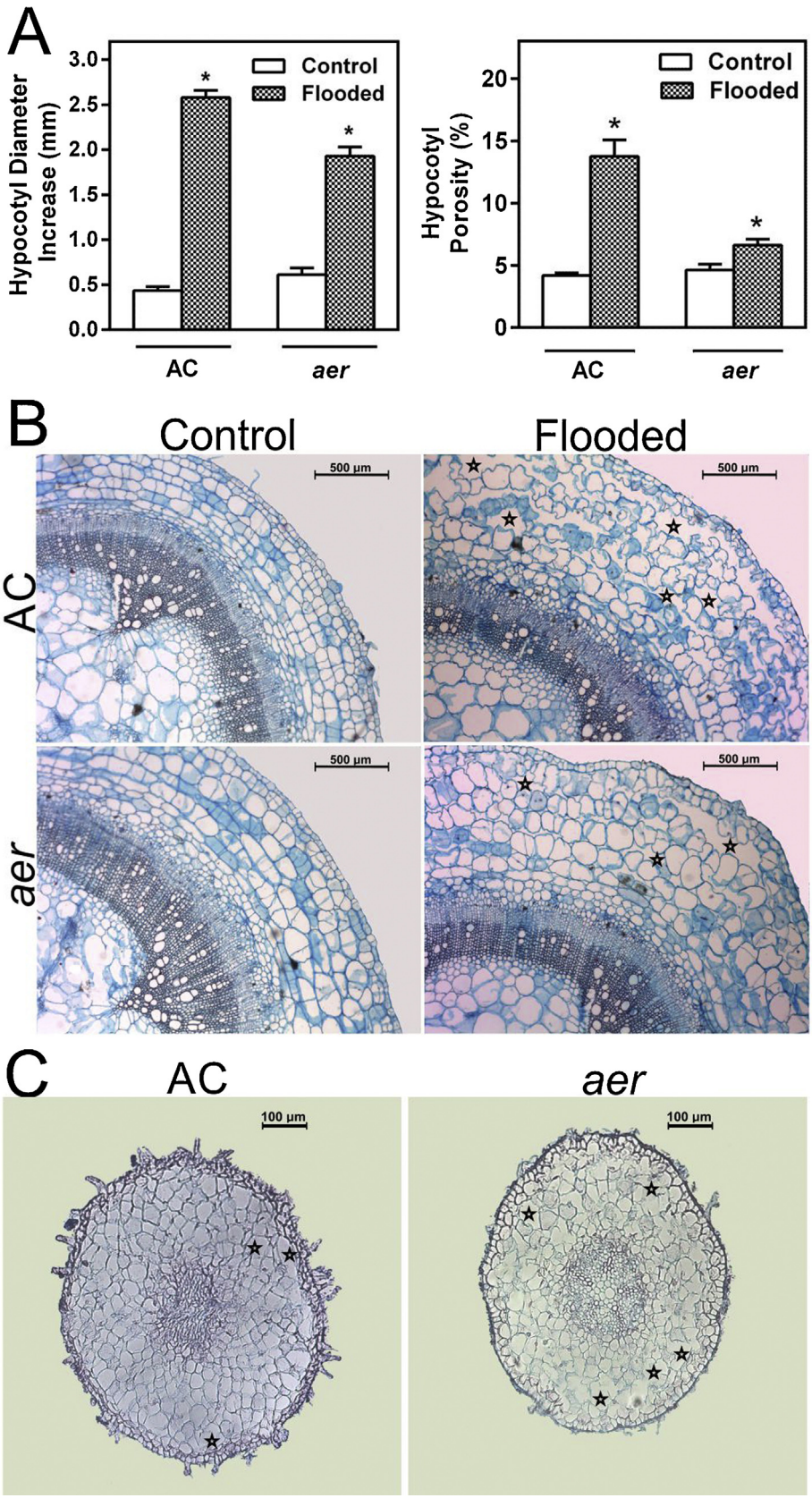

Fig. 3. Stem hypertrophy and porosity (A) in control and flooded plants of tomato cv AC and *aer* mutant after one week of treatment. Plants were 4 weeks old at the beginning of treatments. Values are means ± SE. Asterisks indicate significant differences between control and flooded plants by Student's *t* test within each genotype ($P < 0.05$; $n = 10$). Aerenchyma formation in AC and *aer* hypocotyls (B) and adventitious roots (C) of tomato after 72 h and one week of flooding, respectively. Stars indicate examples of cortical air spaces.

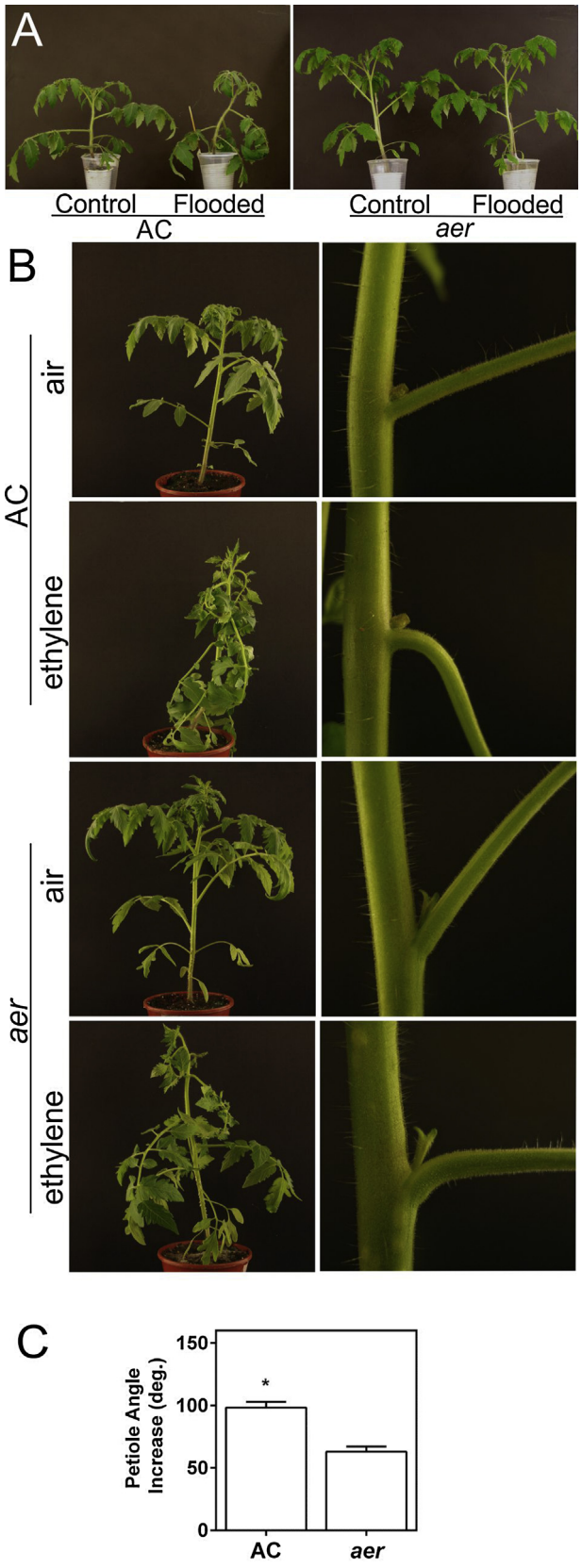

Fig. 4. Difference in epinastic response in 4-week-old flooded plants of tomato cv AC and *aer* mutant (A). Epinasty in 4-week-old AC and *aer* plants placed in an air-tight chamber in presence of air or 50 $\mu L\,L^{-1}$ ethylene (B) for 16 h. Increase in petiole insertion angle before and after ethylene treatment in AC and *aer* mutant (C). Asterisk indicates a significant difference between AC and aer by Student's *t* test ($P < 0.05$; $n = 12$).

rate was almost three times higher in plants with an intact AR system. Considering the reduced genetic base of the rootstocks used for commercial varieties of tomato (King et al., 2010), *aer* plant ability to resume biomass accumulation after flooding events, together with its vigour (Schwarz et al., 2010), constitute desirable traits if they have to undergo periods of abiotic stress such as waterlogging/submergence.

In agreement with previous reports involving different tomato genotypes (Kramer, 1951; Jackson, 1955; Aloni and Rosenshtein, 1982; McNamara and Mitchell, 1990; Else et al., 2009), *aer* plants ability to remain vigorous when exposed to flooding conditions corresponds with an abundant AR mass (Fig. 1F,G). Similarly, the tolerance of watermelon plants to flooding was observed to increase when grafted onto *Lagenaria siceraria* concomitantly with the rootstock ability to form ARs and aerenchyma (Yetisir et al., 2006). Indeed, the *aer* mutant ability to promptly form ARs (2B,D) could be a desired feature in rootstocks destined for use in flood-prone areas of the world, in which a fast response could be crucial to circumvent the effects of flooding (Dawood et al., 2014). As observed in another *Solanum* species (Zhang et al., 2015), the time required to produce ARs in tomato depends on plant age but is much shorter in *aer* than in AC (Fig. 2A,D).

The reduction in resistance to oxygen diffusion throughout stems and roots, and the decrease in the number of oxygen-consuming cells are some of the benefits that lysigenous aerenchyma formation brings to plants growing in stressful environments (Jackson and Armstrong, 1999; Drew et al., 2000; Shiono et al., 2008). In flooded tomato plants, the ability to form cortical lysigenous aerenchyma in submerged organs results in adequate provision of oxygen to the newly formed elongating roots (Fig. 3B,C). Although stem porosity, hypertrophy and aerenchyma formation are lower in *aer* plants when compared to AC (Fig. 3A), it does not hamper *aer* plant ability to recover biomass accumulation sooner than AC plants (Fig. 1G). Even though porosity in flooded *aer* hypocotyls is lower than in AC, it is similar to that found by McNamara and Mitchell (1990) in a tomato accession that was able to adapt to flooding. Both stem hypertrophy and aerenchyma formation have been attributed to ethylene (Wample and Reid, 1979; Evans, 2004) and would facilitate vertical oxygen movement within the plant.

Leaves from flooded plants can be re-oriented in order to adapt to new growing conditions. In some species such as tomato, they become epinastic within a few hours from the start of root submergence due to an increase in ethylene evolution (Jackson, 2002), thereby reducing leaf light interception, heat build-up and transpiration rate (Bradford and Hsiao, 1982).

At least three ethylene-induced responses to flooding are reduced in *aer* plants (epinasty, Fig. 4; aerenchyma formation, Fig. 3B and hypertrophy, Fig. 3A), which suggests that the *aer* mutation affects ethylene sensitivity in a tissue-specific manner so that some ethylene responses are reduced. Indeed, the partial response to ethylene observed in shoots is supported by the lower formation of the apical hook in ethylene-treated seedlings (Fig. 5C,D) which is part of the typical triple response to ethylene of dark-grown seedlings (Guzmán and Ecker, 1990).

The analysis of tissue-specific sensitivity to ethylene has been carried out by measuring the relative expression level of *E4*, an ethylene-induced gene (Lincoln et al., 1987), in control and ethylene-treated AC and *aer* plants. In agreement with the attenuation of some ethylene responses, expression of *E4*, an ethylene-induced gene, (Fig. 5E) is lower in ethylene-treated petioles and hypocotyls of the *aer* mutant, which are the tissues that have shown a reduced response to this hormone.

Although ethylene evolution is the trigger for many adaptive responses to stress, it has been reported that tomato plants inoculated with bacteria containing an ACC deaminase or expressing an

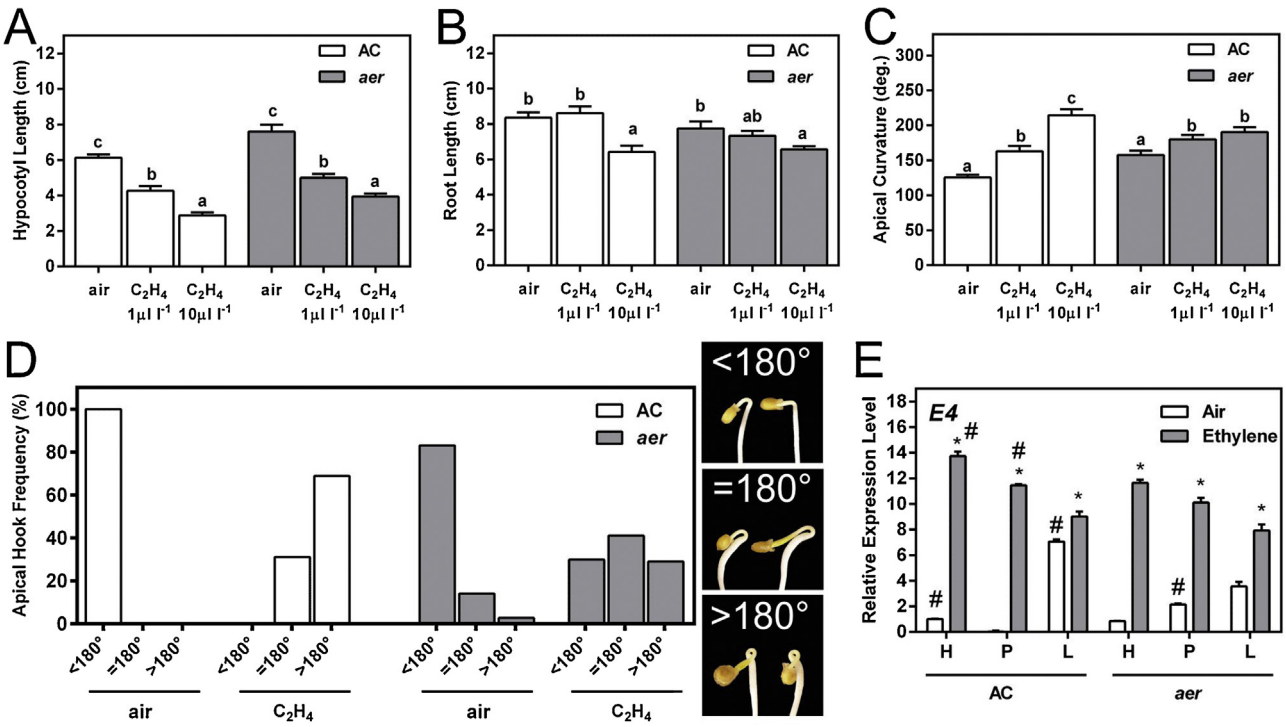

Fig. 5. Response of 5-day-old etiolated seedlings of AC and *aer* exposed to 0, 1 and 10 µL L^{-1} ethylene for 2 days. Hypocotyl (A) and root (B) length as well as apical hook curvature (C) were determined. Values are means ± SE. Different letters indicate significant differences among treatments by Tukey's test within each genotype ($P < 0.05$; $n = 20$). Apical hook frequency distribution (D) of 5-day-old etiolated seedlings of AC and *aer* exposed to 10 µL L^{-1} ethylene for 2 days. Relative expression level of *E4* gene in hypocotyls, H, petioles, P, and leaves, L, of 4-week-old AC and *aer* plants treated with 50 µL L^{-1} ethylene for 16 h (E). Transcript levels were normalized to *LeEF1A* expression and are indicated in relative units with AC hypocotyls in air assigned a value of 1. Asterisks indicate significant differences between control and flooded plants by Student's *t* test within each tissue and genotype, whereas hash signs indicate significant differences between AC and *aer* by Student's *t* test within each treatment and tissue ($P < 0.05$; $n = 3$).

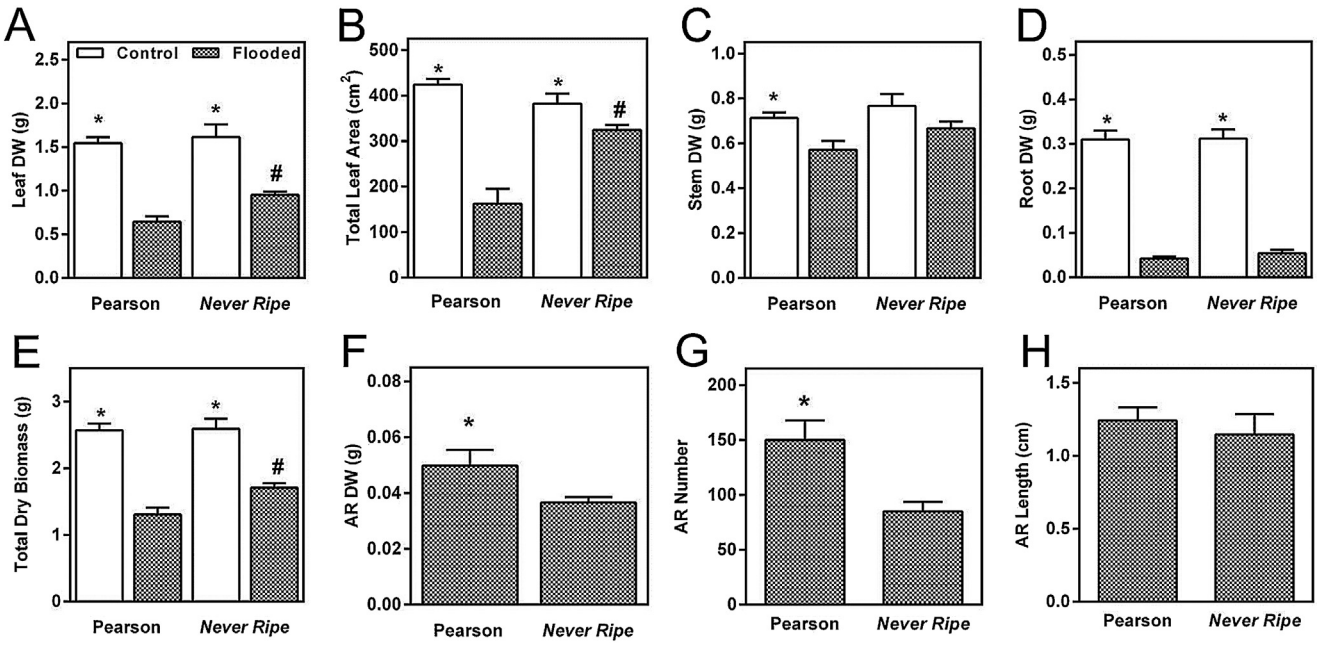

Fig. 6. Effect of flooding on leaf biomass (A), leaf area (B), stem (C), root (D) and total (E) biomass accumulation of tomato cv Pearson and *Nr* mutant plants after one week of treatment. Plants were 4 weeks old at the beginning of treatments. Values are means ± SE. Asterisks indicate significant differences between control and flooded plants by Student's *t* test within each genotype, whereas hash signs indicate significant differences between Pearson and *Nr* by Student's *t* test within each treatment ($P < 0.05$; $n = 8$). Adventitious root biomass (*F*), number of adventitious roots (*G*) and adventitious root length (*H*) in plants of Pearson and *Nr* after a week of flooding. Values are means ± SE. Asterisks indicate significant differences between Pearson and *Nr* by Student's *t* test ($P < 0.05$; $n = 8$).

ACC deaminase exhibited increased tolerance to flooding (Grichko and Glick, 2001a,b). Based on this and considering that *aer* plants regain biomass accumulation faster than AC plants, we analysed growth parameters in the *Nr* mutant and observed that flooded *Nr* plants are less affected than the wild type (Fig. 6A,B,C,E), though their ability to adapt seems to be somewhat counteracted by the lower production of ARs (Fig. 6F,G). Our results suggest that the partial ethylene insensitivity found in *aer*, together with the fast development of a new AR system, favour tomato plant adaptation to flooding. In fact, the proliferation of ARs is probably due to another effect of the mutation related to auxin as auxin-induced AR formation is independent of ethylene whereas ethylene promotion of ARs is dependent on auxin (Visser et al., 1996). Further experiments are being performed in order to identify the nature of the *aer* mutation.

5. Conclusions

Aer plants, characterized by the presence of AR primordia along the stem, rapidly regain biomass accumulation after the onset of flooding stress. Our studies revealed that the *aer* plant ability to promptly form a new adventitious root system and the reduction in ethylene sensitivity are two factors that contribute to faster adaptation to flooding in the mutant. In addition, the *aer* mutant proved a useful tool to study the adaptive importance of ARs in plants exposed to flooding stress as well as a potential rootstock for tomato plants grown in flood prone areas.

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