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Exogenous hydrogen peroxide positively influences root growth and exogenous hydrogen peroxide positively influences root growth and metabolism in leaves of sweet potato seedlings

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Abstract

Hydrogen peroxide (H_2O_2) is a signal molecule which mediates a wide range of physiological and biochemical reactions during the whole period of plant growth. We studied the effects of various concentrations of exogenous hydrogen peroxide as well as a hydrogen peroxide scavenger, ascorbic acid (AsA) on the growth and development of adventitious roots and leaves of sweet potato seedlings. In culture solutions with lower concentrations of H_2O_2 (less than or equal to 2.5 mM), the formation of adventitious root and the growth of sweet potato seedlings were induced, while the physiological properties of roots were also significantly increased, especially in 0.5 mM H_2O_2 treatment. However, when the concentration of H_2O_2 came up to5 mM, it played an opposite role to inhibit the growth of adventitious roots and seriously damaged them. Moreover, the growth of adventitious root could also be significantly inhibited by the mono-addition of AsA. Treatment with 4mM AsA followed by 2.5 mM H_2O_2 for three days inhibited the elongation of adventitious roots to some degrees. In conclusion, the growth of adventitious roots in sweet potato could be induced by exogenous H_2O_2 below the injury level (0.5 mM) and could be reversed by AsA treatment. Some levels of endogenous H_2O_2 are indispensable for sweet potato in the course of adventitious roots formation. The effect of H_2O_2 on leaf physiology resulted from its effects on root growth. The best concentration for inducing adventitious roots and leaf growth was 0.5 mM H_2O_2.

Keywords: adventitious root; ascorbic acid; hydrogen peroxide; sweet potato; root growth.
Abbreviations: ABA: abscisic acid, AsA: ascorbic acid, CAT: catalase, H_2O_2: hydrogen peroxide, MDA: malondialdehyde, SOD: superoxide dismutase.

Introduction

Hydrogen peroxide (H_2O_2) is continually generated from various sources during normal metabolism in plant cells. The electron transport processes during photosynthesis and respiration generate basal levels of H_2O_2, which increase in response to environmental stress (Desikan et al., 2004). In addition, there are enzymatic sources of H_2O_2, including NADPH oxidase, cell wall peroxidases, amine oxidases, as well as other flavin containing enzymes (Neil et al., 2002). Although the precise intracellular concentrations of H_2O_2 that are likely to be toxic do vary, high rates of H_2O_2 production are normally balanced by very efficient antioxidant systems (Corpas et al., 2001; Gechev et al., 2006), such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT); antioxidants such as ascorbic acid and glutathione are also crucial for plant defence against oxidative stress (Møller et al., 2007). An oxidative burst, with rapid H_2O_2 synthesis is a common response to pathogens, elicitors, wounding, heat, ultra-violet light and ozone (Apel et al., 2004). Recent biochemical and genetic studies confirm that H_2O_2 is a signaling molecule in plants which mediates various physiological and biochemical processes (Mittler et al., 2004), including systemic acquired resistance (SAR) and hypersensitive resistance (HR) (Torres et al., 2006), senescence and programmed cell death (PCD) (Delledonne et al., 2001; Ren et al., 2002; Gechev et al., 2006; Van Breusegem et al., 2006), stomata closure (Mustilli et al., 2002; Suhita et al., 2004; Bright et al., 2006), root gravitropism and lateral root development, formation of adventitious roots (Su et al. 2006; Dunand et al. 2007), cell wall development (Carol et al., 2006), pollen-stigma interactions and development, etc (McInnis et al., 2006; Potocký et al., 2007). The formation of adventitious roots involves redifferentiation, in which predetermined cells switch from their morphologenetic path to act as mother cells for the development of a meristematic tissue and then root primordial. After removal of the primary root system, higher auxin concentrations are required during the root induction phase (de Klerk et al., 1999). Su et al., (2006) found the effects of these treatments on the development of soybean lateral roots were consistent with the changes in endogenous H_2O_2 levels. The development of soybean lateral roots was associated with the oxidative degradation of polyamines, and their products, especially H_2O_2, are likely to play an important role in the growth of soybean lateral roots. Li et al., (2007) demonstrated that H_2O_2 could promote the formation and growth of adventitious roots of seedling explants in cucumber. The effects of H_2O_2 on the formation and growth of adventitious roots could be removed or inhibited partly or completely by H_2O_2 scavengers or inhibitors of its synthesis. However, Zhang et al., (2009) found that exogenous H_2O_2 had the same inhibition effect to root growth in Arabidopsis Columbia as did ABA, but

the inhibition effect can be reversed by ascorbic acid. Thus, it can be deduced that H_2O_2 mediate the inhibition of root growth by ABA. Sweet potato is an easy-to-high-yield crop and multi-functional health food whose harvest is also easy; yields of sweet potato crop ranked second in the world after potatoes. China accounts for more than 80% accounted for in the world of the total output of sweet potato (Islam et al., 2006). Sweet potato is considered as the main food, feed and industrial raw material in China (Farmer et al., 2007; Liu et al., 2011). The propagation of sweet potato is mainly based by vine cuttings; therefore, the faster and more intense the root formation and growth, the greater the survival rate of seedlings and expected output (Alem et al., 2010). The formation of a large root system in the early stage will promote the robust growth of seedlings in early development, and flourishing stems and leaves will be conducive to overall yield. Thus, the study of whether H_2O_2 could induce adventitious root formation and growth of sweet potato seedlings presents practical significance. Effects of H_2O_2 signals on root growth have been extensively reported and exogenous H_2O_2 mediated both the growth of lateral roots and root hairs (Carol et al., 2006; Zolla et al., 2010) and primary root (Šimonovičová et al., 2004; Shin et al., 2004; Dunand et al., 2007; Jiang et al., 2012). Studies on the influence of exogenous H_2O_2 signal impact on growth and development of adventitious roots in sweet potato has barely been reported to date. In this study, various concentrations of culture solutions of exogenous H_2O_2 and its scavenger AsA were used to determine the effect of exogenous H_2O_2 on adventitious roots and leaf physiology of sweet potato. The study provided theoretical and practical foundations for the study of H_2O_2 signals and its practical application to sweet potato production.

Results

Effect of H_2O_2 on adventitious root growth

Observations and measurements are shown for the whole adventitious root system (Fig. 1 A) and parameters of root weight (Fig. 1 B), average root length (Fig. 1 C), total root surface area (Fig. 1 D) and root number (Fig. 1 E) per plant which indicate root growth. The growth of the whole adventitious roots (Fig. 1 A) differed significantly across treatments. Compared with treatment No.1 (control), the adventitious root growth in treatments No.2 (0.5 mM H_2O_2) and No.3 (2.5 mM H_2O_2) were significantly better, especially that of treatment No.2. As the concentration of H_2O_2 increased to 5 mM in treatments No.4 and No.5, the growth of roots were almost completely inhibited; even some damage was observed. For the No.5 treatment, as the concentration of H_2O_2 increased gradually from 0.5 mM to 5 mM, the inhibition was slightly lighter than treatment No.4. For treatments No.7 and No.8 in which AsA, a scavenger of H_2O_2 was added (at AsA 4 and 10mM, respectively), the growth of all the adventitious roots was almost completely inhibited, and the inhibition in No.7 was a little lighter. For treatment No.6, in which sweet potato was cultured in 4 mM AsA for 3 d after prior treatment with 2.5 mM H_2O_2 for 3 d, the elongation of root was somewhat inhibited compared with treatment No.1, but the number of roots was higher. Results of the adventitious root weight (Fig. 1 B) showed that the dry or fresh weight of roots under treatments Nos. 2 and 3 were greater than that under treatment No.1 by 3- and 2-fold. The root weight of treatment No.6 was almost the same as treatment No.1, but those of treatments Nos.4- 8 were not measured. The total root surface area among treatments (Fig. 1 C) varied similarly as root weight. Treatments No.2 and No.3 also produced larger surface areas than No.1, with No.2 having the largest area. Treatment No.6 produced a slightly less surface area than treatment No.1, but treatment No.5 produced substantially less surface area than No.1. The root surface area of treatments Nos.4, 7 and 8 could not be measured. To understand the effect of H_2O_2 on the formation of adventitious root, the visible root numbers were counted (Fig. 1 D). The root numbers under treatments Nos.2, 3 and 6 were more than those of treatment No.1, and treatment No.6 had the most roots. Treatment No.7 had the same root number as No.1. However, the root numbers under treatments Nos.4 and 5 were less than those of treatment No. 1, no visible roots were counted for treatment No.8. To determine the effect of H_2O_2 on the elongation of adventitious roots, the average root length was measured (Fig. 1 E). The average roots of treatments Nos.2 and 3 were longer than those of treatment No.1, with treatment No.2 having the longest roots. Compared with treatment No.1, treatment No.6 produced shorter roots. Roots in other treatments could not be measured, because they had no measurable adventitious roots.

Effect of H_2O_2 on activity and microstructures of root

An examination, with the aid of a microscope, showed marked deformation of roots in treatments Nos.4 and 5 cultured in 5.0 mM H_2O_2 (Fig. 2 A). The adventitious root was damaged, but the roots in other treatments were normal. For treatments Nos.6 and 7, in which AsA was added, root hairs were observed. There was no photo of treatment No.8 because no root formation occurred in the treatment. α-naphthylamine oxidation method was used to stain the activity of peroxidase enyzme in roots, which is also a way to measure root activity. The results of the staining (Fig. 2 B) showed that, except for treatment No.6, roots in other treatments such as Nos. 1, 2, and 3 were stained to dark red; by comparison, the colour of treatments Nos.2 and No.3 were heavier than that of treatment No.1, especially for treatment No.2. Thus, the root activities in these treatments were stronger than those in treatment No.1, while the root activity in treatment No.6 was weaker, because the colour was lighter.

Effect of H_2O_2 on the repair of damaged stem section of seedlings

The accumulation of materials for repairing damage to the cut section is helpful to prevent outflow of organics and block the intrusion of pathogens. The 5 treatments in which H_2O_2 was added to culture solution during the whole observation period accumulated lots of solid materials, used for damage repair on the section of seedling stem such as lignin. The other 3 treatments, especially treatments Nos.7 and 8 (that contained AsA) showed almost no accumulation of solid materials for damage repair. Treatments Nos.7 and 8, which contained AsA throughout the period of study, induced lots of mucus flow from stem into solution via section, in contrast to other treatments with H_2O_2.

Effect of root treatment with H_2O_2 or AsA on leaf growth

Leaves in treatments Nos. 7 and 8 containing AsA exhibited wilting and less vigorous while those in other treatments were quite normal (Fig. 3 A). When analysing the relative water content of leaves in each treatment, resultsshowed that the relative water content of leaves in treatment No.2, which had abundant root system, was the only one higher than that of leaves in treatment No.1; treatments Nos.3 and 6 had the same level as treatment No.1 (Fig. 3 B). The other treatments, especially treatments Nos.7 and 8, with almost no roots, were lower.

Table 1. The description of treatment groups. Different concentrations of H_2O_2 and AsA were added into the Hoagland solution respectively. The whole experiment lasted 6 days (from DAY 0 to DAY 5).

Treatment groups	The detail protocol of each treatments
NO.1	Control group with no H_2O_2 and no AsA
NO.2	From DAY 0 to DAY 5: 0.5 mM H_2O_2
NO.3	From DAY 0 to DAY 5: 2.5 mM H_2O_2
NO.4	From DAY 0 to DAY 5: 5 mM H_2O_2
NO.5	DAY 0: 5.0 mM H_2O_2 ; DAY 1 to DAY 2: 2.5 mM H_2O_2 ; DAY 3 to DAY 5: 5.0 mM H_2O_2
NO.6	DAY 0 to DAY 2 : 2.5 mM H_2O_2; DAY 3 to DAY 5: 4.0 mM AsA
NO.7	From DAY 0 to DAY 5: 4.0 mM AsA
NO.8	From DAY 0 to DAY 5: 10.0 mM AsA

*The concentration of H_2O_2 or AsA in each treatment depended on its concentration in Hoagland nutrient solution.

Effect of root treatment with H_2O_2 or AsA on leaf physiology

To determine changes in leaf physiology, the contents of MDA (Fig. 4 B) and leaf pigments (Fig. 4 C) in leaves were measured. The content of endogenous H_2O_2 in leaves (Fig. 4 A) had also been determined to study the effect of exogenous H_2O_2 on endogenous H_2O_2 in leaves, and the reasons for the effect of exogenous H_2O_2 on leaf metabolic processes. The MDA is a metabolite of membrane peroxidation, and the amount of which reflected the damage level of membrane. The levels of MDA (Fig. 4 B) in all the treatments except treatments No.2 and No.3 were much higher than that in treatment No.1. The leaf MDA contents in treatments No.2 and No.3 were slightly higher than treatment No.1, but not to a significant level. This indicated that leaves in all the treatments, except treatments No.2 and No.3, were damaged to some extent. The two chlorophyll levels showed the same trend as MDA among treatments (Fig. 4 B); contents of chlorophyll A and B were higher in treatments Nos.2, 3 and 6 than in treatment No.1. The other treatments had no remarkable difference than control (No.1). There was a consistency between root growth and leaf chlorophyll level. And the lutein remained almost at the same level among treatments except treatment No.4 which had a lower level than treatment No.1. The endogenous H_2O_2 concentrations in all the treatments showed almost the same level; there was no consistency between concentrations of endogenous H_2O_2 in leaves and the effect of different concentrations of H_2O_2 or AsA on roots. Thus, it may be considered that there was negligible or no transportation of exogenous H_2O_2 or Vc from roots to leaves.

Discussion

Data showed that treatment of sweet potato seedlings with exogenous H_2O_2 at lower concentrations, especially at 0.5mM (treatment No.2) substantially enhanced root growth as measured by root weight, root number, average root length and total surface area per plant or root activity. When seedlings were treated with medium (2.5mM) concentration of H_2O_2 (as in treatment No.3), the enhancement of growth of adventitious roots was still noticeable, but slightly weaker than that observed with the 0.5mM treatment. However, at higher concentrations (5mM) H_2O_2 treatment (as in treatments No.4 and No.5), root growth was seriously inhibited or even completely damaged; thus, this concentration could be considered as the threshold concentration for adventitious roots. Under treatments No.7 and No.8 which contained AsA during the whole experimental period, the root growth was also seriously inhibited, especially as the AsA level increased as in treatment No.8. Not only the growth of adventitious root, but the recovery from damage to cut seedling sections, was prevented. For treatment No.6, cultured in 4 mM AsA for 3 d after treatment with 2.5 mM H_2O_2 for 3 d, only root elongation

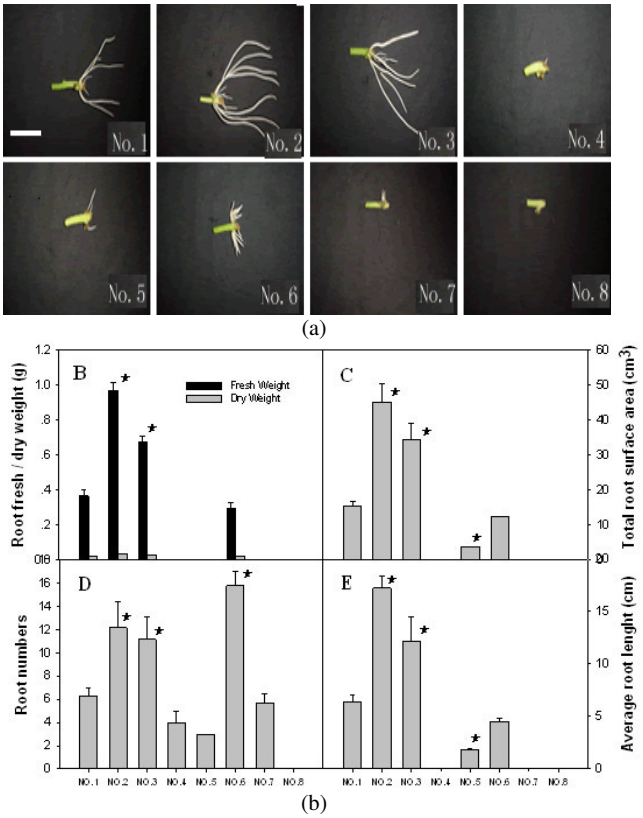

Fig 1 The adventitious root growth (A), root weight (B), total root surface area (C), root number (D) and average root length (E) of sweet potato seedlings in treatment groups with different concentrations of H_2O_2 and AsA. Data represents the means and SD of three independent measurements with 9 explants for each treatment. Bar = 5 cm. Error bars are SD for three replicate reactions. * Indicates significant difference between the means ($P < 0.05$).

was inhibited to some extent, and the root activity was much lower, but root number increased. Therefore we suggest that the growth of adventitious root of sweet potato could be induced by exogenous H_2O_2 at concentrations lower than the injury level. The AsA is a scavenger of H_2O_2 (Miller et al., 2008); it can scavenge the endogenous H_2O_2 in root or stem, thereby inhibiting the adventitious root formation and elongation induced by exogenous H_2O_2. This proves that there was a link between root growth and H_2O_2 , not only that a certain concentration of exogenous H_2O_2 can induce the growth of adventitious roots, but also that some level of endogenous H_2O_2 is indispensible for root growth as reported previously (Li et

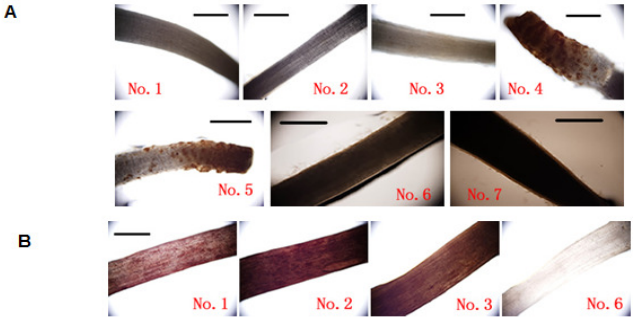

Fig 2. The microstructure of adventitious roots (A) and root activities (B) of sweet potato seedlings in root treatment groups with different concentrations of H_2O_2 and AsA. The root activity was represented by the activity of peroxidase enyzme in roots. Bar = 1mm.

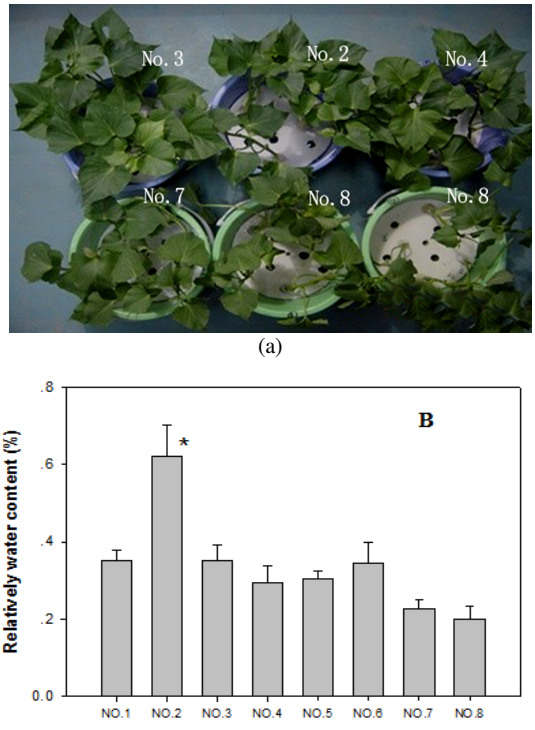

Fig 3. Effect of root treatments with different concentrations of H_2O_2 and AsA on leaf growth (A) and leaf relative water content (B). In Fig. 3A, the leaf status of NO.3 is better than that of NO.8. Data represents the means and SD of three independent measurements with 9 explants for each treatment. Error bars are SD for three replicate reactions. * Indicates significant difference between the means ($P < 0.05$).

al., 2009; Liao et al., 2009). We also found that the inhibition of adventitious root elongation by AsA was more sensitive than the formation of root, as 4mM AsA had significant inhibition of root elongation, but only slight inhibition of the formation of adventitious roots. This suggests that the root elongation stage needs higher concentrations of endogenous H_2O_2 than the adventitious root formation stage. The staining activity following the addition of H_2O_2 was more intense than control and treatments that received AsA, so it could be deduced that the exogenous H_2O_2 could induce the activity of peroxidase enzyme in roots. The peroxidase enzyme mediates the formation of cell wall (Liszkay et al., 2003) and contributes to cell expansion. We found that the elongation zone of root was darkly stained and this may suggested that H_2O_2 could promote the elongation of root by stimulating the elongation of cells in this area. However, how doses of peroxidase enzyme mediate the induction of root growth needs to be further studied. NADPH oxidases control development by making ROS, that regulate plant cell expansion through the activation of Ca^{2+} channels; then activates MAPK cascades， as calcium and MAPKs regulate the activity of other signaling proteins to induce the adventitious root formation as well as root elongation (Foreman et al., 2003; Liu et al., 2009; Zhang et al., 2009). Therefore, in our experiment, addition of low concentration of H_2O_2 stimulated the response of downstream signals, subsequently promoting formation and growth of adventitious roots. At higher concentrations of exogenous H_2O_2, the roots suffered oxidative damage and some structures of the cells were destroyed (del Carmen Cordoba-Pedregosa et al., 2003), particularly a number of new cells, for example, the root primordial cells. However, the addition of AsA, a scavenger of H_2O_2, removed the endogenous H_2O_2 in plants, hence the downstream signals could not be stimulated and root growth was inhibited. For treatments Nos. 6 and 7, in which 4mM AsA was added during the last 3 days, root hairs were generated, but not in treatments with added H_2O_2. This result was in contrast to what has been reported by Foreman (Foreman et al., 2003). We consider that in the root elongation process, the added AsA might greatly reduced the content of endogenous H_2O_2 in the cell elongation zone, thereby lowering the activated Ca^{2+} concentration in the cytoplasm and inhibiting cell elongation. However, to elevate their capacity for water absorption , epidermal cells concentrated the limited Ca^{2+} concentration on the site where root hairs grow, to increase the absorption area of the roots in order to compensate the water deficit caused by the inhibition of root growth (Pitzschke et al., 2006; Potters et al., 2007). From what showed in Fig. 5, it can be inferred that the mucus synthesized by the plant for repairing damage to stem cutting sections can be oxidised into solid matter and accumulated on the stem section to prevent outflow of organic matter and block the intrusion of pathogens, only when some levels of H_2O_2 were converged on this area. Kawasaki (Kawasaki et al., 2006) proved that reactive oxygen species were necessary for the biosynthesis of lignin used for damage repair. In the presence of H_2O_2, the catalytic dehydrogenation of peroxidase enyzme, coniferyl, alcohol was turned into phenoxy radical and resulted to the synthesis of lignin. Therefore, H_2O_2 was confirmed to play a significant role in the repair of tissue damage during our experiment. In treatments Nos. 7 and 8 which contained AsA, the mucus used for repairing tissue damage could not be oxidized to solid matter due to failure to concentrate enough H_2O_2 on the cut surface. As a result, the outflow of organic matter into the culture solution could not be prevented. The parameters determined during in this study, including the relative water content, MDA and leaf pigments showed reasonable consistency with adventitious root growth. However, there was no consistency between concentrations of H_2O_2 or AsA around roots and the endogenous H_2O_2 concentrations in leaves. Thus, it may be considered that there was no substantial exogenous H_2O_2 or AsA transportation from roots to leaves. As aforementioned, peroxidase is an enzyme that scavenges H_2O_2 (Mittler, 2002), and our data showed that H_2O_2 could induce its activity (Fig. 2 B). A peroxidase activity in the root could more efficiently reduce H_2O_2 concentration in roots and prevent its leakage into other parts of the plant, thereby blocking the long transportation of H_2O_2 from root to leaf and limiting its effects to the site of application (Basta et al., 2005; Sagi et al., 2006).

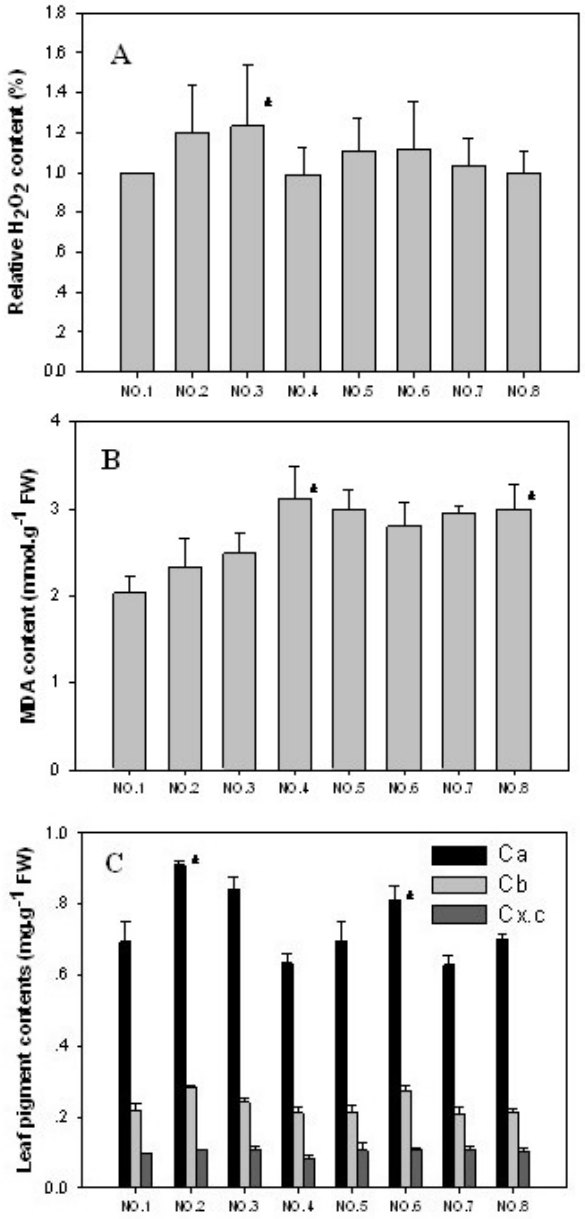

Fig 4. Contents of H_2O_2 (A), MDA (B) and leaf pigments (C) in the leaves of sweet potato seedlings in root treatment groups with different concentrations of H_2O_2 and AsA. Data represents the means and SD of three independent measurements with 9 explants for each treatment. Ca induced chlorophyll A; Cb induced chlorophyll B; Cx.c induced lutein. Error bars are SD for three replicate reactions. * Indicates significant difference between the means ($P < 0.05$)

Besides, during root elongation, along with the thickening and longitudinal elongation of cell walls, a large number of reactive oxygen species were needed (Kawasaki et al., 2006). Thus, the peroxidase enzyme in the cell wall and NADPH oxidase on the cell membrane produced a large amount of H_2O_2 to oxidise substances used for the synthesis of cell wall (Foreman et al., 2003), as well as the formation of new cell walls and thickening of cell wall. Also, the damage repair on the cut section needs massive H_2O_2 reactive oxygen species. In treatments with AsA, the AsA would scavenge lots of H_2O_2 reactive oxygen species, but the AsA was in turn consumed by the H_2O_2 reactive oxygen species, thereby reducing the concentration of AsA entering the gap in the cell wall. Consequently, the transportation of a large amount of AsA to leaves was also blocked. The exogenous H_2O_2 and AsA affected leaf growth via the root accepting signals directly and then producing different growth-stimulating signals to mediate root physiological processes, such as root activity or root growth, changing water absorption capacity and altering leaf water condition, finally affecting leaf metabolic processes. That is why the adventitious root growth in treatments Nos.2, 3 and 6 were better, and could promote the water absorption capacity of these treatments, and transport of water to leaves, hence the relative water content was higher. With no water deficit in leaves, their metabolism was much normal, as the leaf pigment levels were higher and MDA content much lower than other treatments (Slesak et al., 2007).

Materials and methods

Cultivation and sampling

Clippings of sweet potato [Ipomoea batatas (L.) Lam. cv. Yulm] seedlings with 5 leaves (explants) were cultured in Hoagland nutrient solution in a growth chamber. The nutrient solution was prepared into plastic containers with a diameter of 25cm and carried 4 seedlings each. The nutrient solution was changed every 3 days. Different concentrations of H_2O_2 or AsA were added in the culture solution in a total of 8 treatments (Table 1). Each treatment was replicated three times. The seedlings were cultured under a light intensity of 600 u mol m^{-2} s^{-1}, 12 h photoperiod, routine ventilation and temperature of about 25 °C /18 °C.

Measurement of root growth

After six days of treatment, observations were made on the whole adventitious root system, including root weight, average root length, total root surface area and root number per plant. Nine plants were sampled from each treatment, with three samples per replicate. We also photographed the whole root system and determined the fresh and dry weights, average root length as well as total root surface area using the root system scanner. Healthy leaves sampled from the main vein were chopped into small pieces and quickly frozen in liquid nitrogen, and then preserved at -80 °C for use in determining a variety of physiological parameters.

Lipid peroxidation

Lipid peroxidation was determined as the amount of malondialdehyde (MDA, e=155 mM±1 cm±1), a product of lipid peroxidation followed the method described by Hodges et al (Hodges et al., 1999). 1 ml of saved supernatant (which has also been used to determinate antioxidant enzyme) was mixed with 3 ml reaction buffer including 5% Trichloroacetic acid (TCA) and 0.5% Thiobarbituric acid (TBA) was heated in 100 °C water for 15 min, then cooled immediately and centrifuged at 10,000 rpm for 10 min. The absorbance was monitored at 450, 532 and 600 nm.

Leaf pigments content

Mature and well-exposed leaves from plant (1 g fresh weight) were homogenised in a mortar and pestle using 5 ml of chilled 80% acetone. The homogenate was centrifuged at 10 000 rpm at 4°C for 10 min. The absorbance of the supernatant was

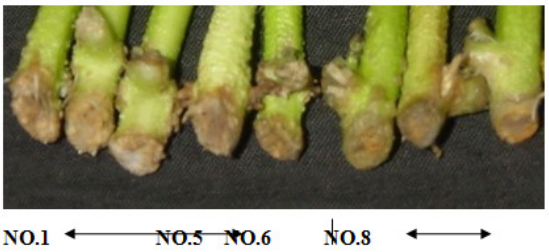

Fig 5. The reparation of seedling stem sections in root treatment groups with different concentrations of H_2O_2 and AsA. Mucus synthesized from the stem section of sweet potato seedlings in treatment groups of N0.1 to NO.5, but no in treatment groups of NO.6 to NO.8.

measured at 646, 663 and 750 nm, respectively, and leaf pigments content was calculated as per the method of Arnon et al. (1974).

H_2O_2 content

H2O2 was determined by measuring luminol-dependent chemiluminescence following a modification of the method described by Warm et al., (1982) and Chen et al., (1993). Plant tissue (approximately 0.5 g), consisting of the apical region of the shoot including the cotyledons, was ground in liquid N2 and extracted in 3 mL of ice-cold 5% TCA. The crude extracts were centrifuged for 20 min at 1400*g*. A sample of supernatant (0.5 mL) was passed through a column containing 0.5 g of Dowex resin previously equilibrated with 5% TCA. The column was washed with a further 3.5 mL of 5% TCA; all eluates were collected together. H_2O_2 content was measured by adding 0.5 mL of eluate to 0.5 mL of 0.5 mm luminal and the volume was made to 5.5 mL with 0.2 m NH_4OH (pH 9.0). This mixture (0.5 mL) in a borosilicate glass tube was analyzed using a chemiluminescence meter. Chemiluminescence was initiated by injecting 50 mL of 0.5 mm potassium ferricyanide in 0.2 m NH_4OH. The emitted photons were counted for 5 s. Recovery estimates (which consisted of adding a known concentration of H_2O_2 to aliquots of the initial extracts that were then processed in parallel) indicated that an average 93% (SD 5.38) of the H_2O_2 was recovered, and were used as correction factors for each sample.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Three replications were performed and recorded as the mean ± SD. Significant differences (Pvalues<0.05) between treated group and control group were determined using one way *ANOVA* followed by the Tukey test.

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