p-Coumaric acid increases lignin content and reduces hyperhydricity in in vitro-grown *Arabidopsis* seedlings

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Abstract

Hyperhydricity (HH) occurs when plants are faced with accumulation of water and reduction of air in the apoplast of the leaves. One of the characteristics of hyperhydric plants is the reduction of cell wall lignification (hypolignification), but how this is related to the abnormalities is still unclear. Lignin is hydrophobic and based on this it can be speculated that a reduction in lignin levels leads to more capillary action of the cell wall and, consequently, to more water in the intercellular spaces. p-coumaric acid is a hydroxy derivative of cinnamic acid and a precursor for lignin and flavonoids in higher plants. In the present work, the relative amount of apoplast water and air to the total apoplast volume of *Arabidopsis thaliana* wild-type (Col-0) leaves was evaluated. Exogenously applied *p*-coumaric acid can be channelled into the phenylpropanoid pathway through action of the enzyme 4-hydroxycinnamoyl-CoA ligase (4CL), ultimately resulting in an increase in the total lignin content. Exogenously applied p-coumaric acid led to increases in apoplastic air and lowering of apoplastic water in seedlings grown on medium solidified with gelrite. The symptoms of HH are also greatly diminished. These findings corroborate our hypothesis that lignin plays a role in the development of the HH and that an increase of lignin production by exogenously applying *p*-coumaric acid can lead to a decrease of water in the apoplast and thus to a reduction in the occurrence of HH.

Keywords: *Arabidopsis*, apoplast hyperhydricity, lignin, *p*-coumaric acid

INTRODUCTION

Vegetative propagation by tissue culture is the most efficient way to produce a large number of uniform individuals displaying a genotype of interest. However, during tissue culture, plants are undergoing unnatural and extreme conditions, which may result in physiological disorders, such as HH (Debergh et al., 1981; Rojas-Martinez et al., 2010; van den Dries et al., 2013). The altered morphology and anatomy of hyperhydric plants were found to influence various physiological parameters and biochemical processes. HH is defined as physiological, morphological and anatomical disorder which is associated with a reduction in of propagation and survival after transplanting (Badr-Elden et al., 2012). This occurs when plants are grown on gelrite-gelled and liquid media, more than on agar-gelled media. Pereira Machado et al. (2014) observed that hyperhydric plants displayed abnormal phenotypes such as curled, water-soaked, large, thick, crispy and translucent leaves. Changes in anatomical features of hyperhydric leaves were also found, including a poorly developed epicuticular wax layer (Olmos and Hellín, 1998), a reduced number of palisade cells (Picoli et al., 2001) and large intercellular spaces in the mesophyll (Jausoro et al., 2010). Further, abnormalities such as low lignification (Kevers et al., 1987), chlorophyll deficiency (Franck et al., 1998) and malformed stomata (Apóstolo and Llorente, 2000) have been reported.

van den Dries et al. (2013) reported that flooding of the apoplast is a key factor in the development of HH. They revealed that not only the entry and removal of water determine flooding of apoplast (the cell wall continuum and the intercellular spaces) are crucial, but also the characteristics of the cell walls bordering the intercellular spaces. The most likely mechanism to keep the intercellular spaces from being flooded is by maintaining a

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hydrophobic monolayer on the surfaces of the adjacent cells, but this is still debated (Raven, 1996). The polysaccharide components of plant cell walls are highly hydrophilic and thus permeable to water, whereas lignin is more hydrophobic. The crosslinking of polysaccharides by lignin is an obstacle for water absorption into the cell wall. Thus, lignin makes it possible for the plant's vascular tissue to conduct water efficiently (Sarkanen and Ludwig, 1971). In higher plants, the cell wall is one of the first tissues affected by stress signals, which are then transmitted to the cell interior and influence several process (Komatsu et al., 2010).

Lignification, which is the metabolic process of sealing plant cell walls by lignin deposition, occurs during the course of normal tissue development and it plays a crucial role in conducting water in plant stems. Lignin is a polymer formed by oxidative coupling of *p*-hydroycinnamyl alcohol monomers (monolignols), which are products of the phenylpropanoid pathway (Vanholme et al., 2012). Recently, Salvador et al. (2013) reported that exogenously applied cinnamic acid inhibited root growth, increased 3-indoleacetic acid (IAA) oxidase and cinnamate 4-hydroxylase (C4H) activities. In addition to this, the allelochemical increased the total lignin content, thus altering the sums rations of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin monomers. The allelochemicals can be channelled into the phenylpropanoid pathway, which in turn may increase the lignin monomer amount, thus solidifying the cell wall and inhibiting the root growth of soybean plants (dos Santos et al., 2008; Zanardo et al., 2009; Bubna et al., 2011). Kevers et al. (1987) suggested that hyperhydric plants have reduced cell wall synthesis and hence cell wall expansion through turgor pressure due to the deficiency in both cellulose and lignin. Based on these reports, we hypothesized that the deficiency of lignin makes the cell wall more hydrophilic thereby more water is taken up by the apoplast and this causes HH. The objective of this study was to analyse the effects of *p*-coumaric acid on the relative amount of apoplast water and air to the total apoplast volume and total lignin content of *Arabidopsis thaliana* wild-type (*Col-0*).

MATERIALS AND METHODS

Plant material and treatments

Arabidopsis thaliana wild-type (*Col-0*) seeds are firstly agitated in ethanol 70% (v/v) for 1 min, then immersed in 2% (w/v) sodium hypochlorite for 20 min. Later, seeds are rinsed three times for 10 min with distilled water. Then, the sterile seeds were transferred to Petri dishes with half-strength Murashige and Skoog (MS) basal salt mixture, including vitamins (Murashige and Skoog, 1962) supplemented with 1.5% (w/v) sucrose and solidified with 0.7% (w/v) Micro-agar (Duchefa Biochemie). The seeds were stratified at 4°C in the dark for 3 days and then germinated at 21°C in a growth chamber with 16 h light/8 h dark (30 µmol m^{-2} s^{-1}, Philips TL33). To study the role of lignin in HH, 7-day-old seedlings (9 seedlings $dish^{-1}$) were transferred to the same nutrient medium solidified with 0.7% microagar (control), 0.2% (w/v) gelrite (Duchefa Biochemie) and 0.4% (w/v) gelrite supplemented with 0, 10, 100 and 500 µM of *p*-coumaric acid. Each treatment was replicated 3 times.

Evaluation of apoplastic water and air volumes in leaves

The apoplastic water was extracted from 14-day-old leaves by mild centrifugation (Terry and Bonner, 1980; van den Dries et al., 2013). First, the leaves were excised from plants, weighed and then subsequently put into a spin mini filter microcentrifuge tube (Starlab, Ahrensburg, Germany). Leaves were then centrifuged at 3000 g for 20 min at 4°C. The leaves were reweighed immediately after centrifugation. The apoplastic water volume (V_{water}) in µL g^{-1} fresh weight (FW) was calculated by using the formula: $V_{water} = [(FW-W_{ac})\times\rho H_2O]/FW$; where FW = fresh weight of leaves in mg, W_{ac} = weight of leaves after centrifugation, and ρH_2O = water density (the water density was taken as equal to 1 g mL^{-1}).

The apoplastic air volumes in leaves were assessed using a pycnometer with a stopper (van den Dries et al., 2013). Leaves were excised, weighed and subsequently put into the pycnometer, which was then filled with distilled water and stoppered. Filter paper was used to remove any excess water on the exterior of the pycnometer. The combined weight of the

full pycnometer and leaves was measured. The pycnometer, with both water and leaves, underwent a vacuum (about 500 mmHg) for 5 min, in order to remove air from the apoplast and replaced it with water. If needed, this vacuum treatment was repeated until all of the air was removed off from the apoplast and the leaves had sunk to the bottom of the pycnometer. After vacuum infiltration, the pycnometer was refilled, dried and then weighted again. The apoplastic air volume (V_{air}) in µL g^{-1} fresh weight (FW) was calculated by using the formula: $V_{air} = [(W_{bv}–W_{av})×\rho H_2O]/FW$; where W_{bv} = weight in mg of the pycnometer including leaves and water before vacuum infiltration, W_{av} = weight of the pycnometer including leaves and water after vacuum infiltration, FW = fresh weight of leaves, and ρH_2O = water density.

The relative amount of apoplast water and air was calculated to the total apoplast volume (TAV) in percentage (%) by using the formula: % $V_{water} = V_{water}/(TVA)×100$ and % $V_{air} = V_{air}/(TVA)×100$; where $TAV = V_{water} + V_{air}$.

Extraction of cell walls and lignin determination

Cell walls were extracted from the powdered samples according to Estrada et al. (2000) with modifications. A 100 mg portion of powder was incubated in 10 mL of methanol (MeOH) at 20°C for 15 min. The suspension was centrifuged at 2750 rpm for 5 min, then the supernatant was discarded. Fresh 10 mL MeOH was added to the pellet, and a second 30 min at 60°C incubation was performed; the mixture was centrifuged at 2750 rpm for 5 min and then the supernatant was removed. The extraction with MeOH was repeated and centrifuged until the supernatant was colourless. The pellet was resuspended in 10 mL of milli-Q water for three times. The last pellet was resuspended once in 10 mL of 0.5 M phosphate buffer (pH 7.0) containing 5% ethanol (EtOH) and 0.02% protease (Pronase E, Sigma Aldrich). This suspension was incubated for 18 h at 37°C and centrifuged at 2750 rpm for 5 min, after which the resuspension of the pellet in 10 mL of solvent and centrifugation under the same conditions was performed consecutively: 3× with distilled water as the solvent, 3× with 95% EtOH, and 2× with absolute EtOH. The final pellet (cell wall material) was dried at room temperature for one day and weighed. The lignin content was determined using the acetyl bromide method (Fukushima and Kerley, 2011).

Statistical analysis

The experiment design was completely randomized and each plot was represented by one Petri dish with 9 seedlings. The data were expressed as the mean of 3 independent experiments ± SE. An analysis of variance to determine significant differences was performed with Genstat 18.1 and the differences between the parameters were evaluated by Fisher's protected least significance test. P values≤0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The effect of *p*-coumaric acid on apoplastic water and air volume

The development of HH symptoms on *Arabidopsis* wild-type (*Col-0*) is shown in Figure 1. Seedlings cultured on medium solidified with 0.2% (w/v) gelrite showed the most severe hyperhydric phenotype with elongated petioles, wrinkle, brittle and thick leaves (Figure 1B) compared to the other treatments. It is known that the use of gelrite instead of agar consistently resulted in HH, but generally gelrite also has a great effect on shoot growth and is remarkably transparent in comparison to agar as solidifying agent. The effect of gelrite on HH is due to increased availability of water, thereby allowing a higher uptake rate of water. Chelators excreted by plants may liquefy the gelrite medium and in this way, it resembles culture on liquid medium (van den Dries et al., 2013). Kevers et al. (1988) found that the lignin levels were increased in both stem and leaves of carnation in the solid cultures, while decreased in the corresponding organs from explants in liquid cultures. In order to investigate the role of the cell wall component lignin, we checked the effect of adding exogenously a precursor in lignin biosynthesis, i.e., *p*-coumaric acid. It was added to 0.4% (w/v) gelrite media instead of 0.2% gelrite, because we felt that the effect could be better monitored and measured there. Medium solidified with 0.4% (w/v) gelrite already showed less pronounced

HH symptoms compared to 0.2% (w/v) gelrite (Figure 1B, C). These findings are in agreement with Quiala et al. (2014) and Ivanova and van Staden (2010) who reported that increasing the gelrite concentration, reduced significantly the occurrence of HH on *Tectona grandis* L. and *Aloe polyphylla* due to decreased water availability in the media. Exogenously applied *p*-coumaric decreased the symptoms of HH (Figure 1D) when compared to 0.4% gelrite alone (Figure 1C).

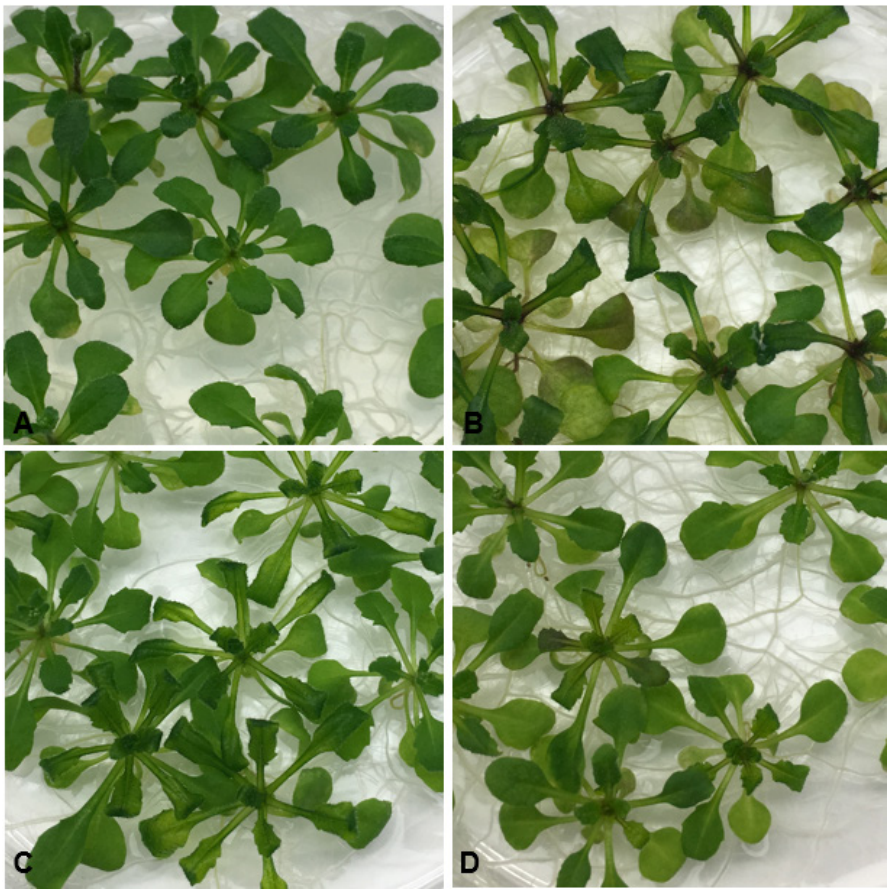

Figure 1. *Arabidopsis* wild-type (*Col-0*) seedlings cultured for 14 days on medium (A) agar control, (B) 0.2% gelrite, (C) 0.4% gelrite, and (D) 0.4% gelrite + 500 µM *p*-coumaric acid.

In addition to the visual evaluation on phenotypical abnormalities of HH, the important parameter to monitor the extent of HH was by measuring the amount of water accumulating in the apoplast together with the amount of apoplast air. In Figure 2, the results of these measurements are given showing the amount of apoplast water and air relative to the total apoplast volume of *Arabidopsis thaliana* wild-type (*Col-0*) seedlings. The data show that the seedlings on 0.2% (w/v) gelrite accumulated remarkably more water (84%) and less air (16%). in the apoplast. Conversely, on the agar control, the percentages of water and air in volume were 14 and 86%, respectively. When the concentration of gelrite was increased to 0.4% (w/v) gelrite, the percentage of the water volume declined and the percentage of the air volume increased compared to 0.2% (w/v) gelrite. Still, this was not enough to prevent HH symptom development. In comparison with 0.4% (w/v) gelrite, the percentage of the water volume sharply declined from 56 to 38% and the percentage of the air volume increased from 43 to 62% by addition of *p*-coumaric acid from 10 to 500 µM. The difference between 100 and 500 µM *p*-coumaric acid on the percentages of water and air volumes was not significant.

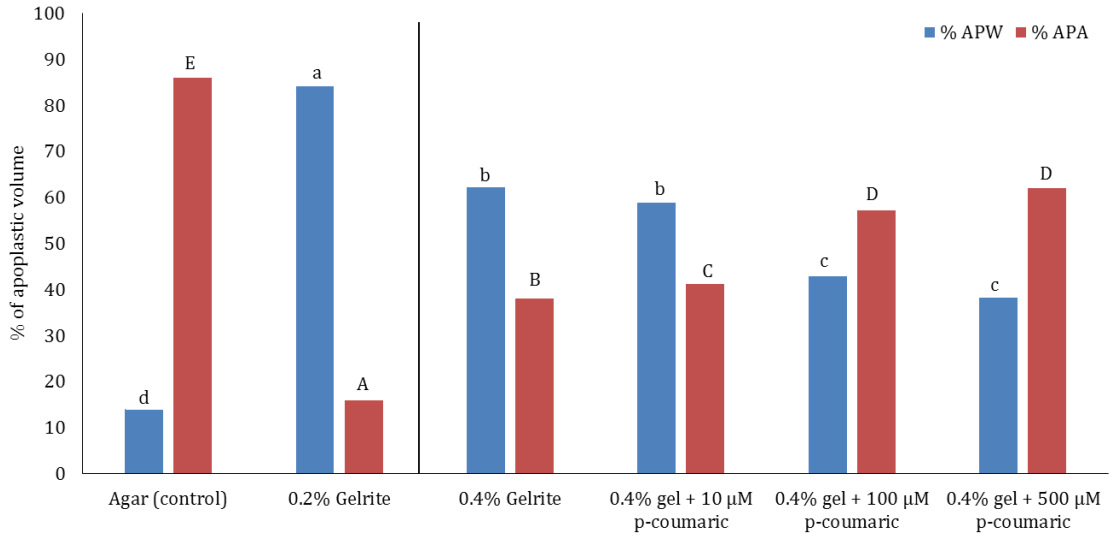

Figure 2. Effect of *p*-coumaric acid on *Arabidopsis* wild-type (*Col-0*) seedlings after 14 days of culture. Values (n=3) that are significantly different marked with different letter (P≤0.05) by Fisher's protected least significant test. APW, apoplasic water; APA, apoplastic air.

The effect of *p*-coumaric acid on total lignin content

In agreement with the effect of *p*-coumaric acid observed on the relative amount of apoplast water and air to the total apoplast volume, the total lignin content was investigated. *p*-coumaric acid treatments (10, 100 and 500 µM) increased the total lignin content in comparison with 0.2% and 0.4% (w/v) gelrite (Table 1). These results on lignin content in HH, in vitro plants confirmed earlier results by Kevers et al. (1988) and extended them adding the effect of gelrite percentage and *p*-coumaric acid addition. The positive effect of precursor application on lignin production was found earlier by Schilmiller et al. (2009) and Salvador et al. (2013) in *Arabidopsis* and soybean seedlings, respectively.

Table 1. Lignin content of *Arabidopsis Col-0* (wild type) seedlings as determined by acetyl bromide derivatization.

Line	Lignin content (A_{280} mg^{-1} cell walls ± SE)[a]
Col-0 Agar (control)	0.0222±0.0004
Col-0 0.2% Gelrite	0.0102±0.0003
Col-0 0.4% Gelrite	0.0119±0.0002
Col-0 10 µM *p*-coumaric acid	0.0134±0.0004
Col-0 100 µM *p*-coumaric acid	0.0146±0.0007
Col-0 0.4% Celrite + 500 µM *p*-coumaric acid	0.0181±0.0002

[a]A_{280}, absorbance at 280 nm; n=3.

CONCLUSIONS

Exogenously applied *p*-coumaric acid revealed that lignin plays an important role in reducing the occurrence of HH. *p*-coumaric acid was chosen as allelochemical because in future research we aim to use an *Arabidopsis* lignin mutant (*ref 3*) which has a mis-sense mutation in the gene encoding cinnamate 4-hydroxylase (*C4H*), the enzyme producing *p*-coumaric acid.

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