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Plant growth stimulation in *Prunus* species plantlets by BTH or OTC treatments under *in vitro* conditions

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ABSTRACT

The effects of benzothiadiazole (BTH) and L-2-oxothiazolidine-4-carboxylic acid (OTC) on the growth and viral content of micropropagated, *Plum pox virus* (PPV)-infected peach [(*Prunus persica* (L.) Batsch] 'GF305' plantlets were analyzed. Low BTH and OTC concentrations resulted in a significant increase in the growth of GF305 peach and plum plants, with greater effects in PPV-infected than in healthy GF305 peach plantlets. Neither BTH nor OTC reduced the virus content. In fact, the highest growth and viral contents coincided, especially with the 10 μM BTH treatment. Differing effects on the antioxidative metabolism of PPV-infected GF305 peach plantlets were observed, depending on the compound and the concentration used: BTH decreased GSH, whereas OTC increased it. In PPV-infected plants, the 50 μM OTC treatment produced a decrease in ascorbate peroxidase, catalase, and glutathione peroxidase, but an increase in superoxide dismutase. However, BTH produced a rise in peroxidase activity. Both 10 μM BTH and 50 μM OTC produced H_2O_2 accumulation that was correlated with the histochemical detection of H_2O_2 by 3,3′-diaminobenzidine staining. PPV infection induced *NPR1* expression and a synergistic effect occurred in the presence of 50 μM OTC, since this compound produced an up-regulation of *NPR1* in both healthy and PPV-infected GF305 peach plantlets. The results showed that GSH, as previously suggested, and/or H_2O_2 could be involved in the regulation of *NPR1* expression. Globally, the results show that both OTC and BTH improved the vigor of *Prunus* species, including peach and plum, under *in vitro* conditions, producing positive effects on growth, antioxidative metabolism and *NPR1* expression. All of these improvements could be critical for more successful *ex vitro* acclimatization as well as for improved responses to different stresses.

Introduction

Studies on the effects of biotic stress at the physiological and biochemical levels in fruit trees are hampered by the long growth cycles involved. Different factors, including the use of woody plants, the inoculation method and the growth cycle with periods of dormancy, have made it difficult to study short-term responses to virus infection. Thus, previous results were obtained for long-term infection: for example, the response of *Prunus* to viruses such as *Plum pox virus* (PPV) or *Apple chlorotic leaf spot virus* (ACLSV) (Hernández et al., 2001, 2004, 2006; Díaz-Vivancos et al., 2006; García-Ibarra et al., 2011). *Plum pox virus* (PPV), a member of the genus *Potyvirus*, is the most damaging pathogen of stone-fruit trees in the Mediterranean zone, being responsible for important losses in apricot, plum, peach and possibly cherry orchards and representing a severely limiting factor for fruit production in sharka-affected areas (Rubio et al., 2009). The use of a susceptible herbaceous plant allows the study of the short-term biochemical and physiological responses to virus infection. This information can also help to develop defense strategies against virus infection, which could be effective in *Prunus* species. This is the case for the PPV-*Pisum sativum* (cv. Alaska) interaction, in which we studied both the initiation (early response) and the disease development phases (Díaz-Vivancos et al., 2008; Clemente-Moreno et al., 2010). However, these models can show differences in their response to infection when compared with the natural host of the virus (*Prunus* spp.). An approach to resolve this problem could be the use of *in vitro* culture techniques. Different authors support the idea of developing stress-tolerant plants through *in vitro* selection

Abbreviations: ASC–GSH cycle, ascorbate–glutathione cycle; APX, ascorbate peroxidase; BTH, benzothiadiazole; CAT, catalase; DHAR, dehydroascorbate reductase; G6PDH, glucose-6-P-dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; MDHAR, monodehydroascorbate reductase; OTC, L-2-oxothiazolidine-4-carboxylic acid; $^1O_2$, singlet oxygen; •OH, hydroxyl radical; O_2, superoxide radical; POX, peroxidase; PPV, plum pox virus; SAR, systemic acquired resistance; SOD, superoxide dismutase; TBARS, hiobarbituric acid-reactive substances.

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(Rai et al., 2011). Recently, micropropagated *Prunus* explants have been used to study Fe deficiency, a common problem that affects *Prunus* species (Cellini et al., 2011). Moreover, in previous work, we described the micropropagation of healthy and PPV-infected GF305 peach plantlets for their use as a continuous source of PPV inoculums and as an interesting tool to study physiological stress over a timescale shorter than in *ex vitro* conditions, especially for woody plants (Clemente-Moreno et al., 2011).

Several lines of evidence support the regulatory role that cellular antioxidants – especially reduced glutathione (GSH) and GSH-related enzymes – play in the biochemical and physiological responses of plants to biotic stress (Gullner et al., 1999; Fodor et al., 1997). Exposure of tobacco leaf discs to the cysteine precursor L-2-oxo-4-thiazolidine-carboxylic acid (OTC) led to a massive accumulation of GSH as well as to reduced contents of TMV coat protein and suppression of disease symptoms in TMV-inoculated tobacco plants (Gullner et al., 1999). Similarly, OTC treatment increased GSH at the subcellular level and resulted in the suppression of ZYMV-induced symptom development over a period of five weeks in pumpkin plants (Zechmann et al., 2007). In recent work, we observed that OTC and (2,1,3)-benzothiadiazole (BTH) treatments of pea plants *in vivo* reduced the percentage of leaves showing sharka symptoms (Clemente-Moreno et al., 2010). BTH is a functional analog of salicylic acid (SA) and has been shown to protect wheat plants systemically against powdery mildew infection (Görlach et al., 1996), tobacco against TMV (Friedrich et al., 1996), *Arabidopsis* against *Pseudomonas syringae* (Lawton et al., 1996) and tomato plants against *Cucumber mosaic virus* (Anfoka, 2000). In addition, it is known that SA, or BTH, is involved in the activation of systemic acquired resistance (SAR) (Goellner and Conrath, 2008). SAR is characterized by a broad range of disease resistance and the inducible expression of pathogenesis-related (PR) genes (Ryals et al., 1996). Non-Expressor of Pathogenesis-Related Genes 1 (*NPR1*) is a key regulator in the signal transduction pathway that leads to SAR. Mutations in the *NPR1* gene result in a failure to induce PR genes in systemic tissues and a heightened susceptibility to pathogen infection, whereas over-expression of the NPR1 protein leads to increased induction of the PR genes and enhanced disease resistance (Kinkema et al., 2000).

Only a few reports have described the effects of BTH or OTC in different plant–virus interactions, but none of them involved woody plants. In the present work, we analyzed the effects of BTH and OTC with respect to improving the defense capacity of micropropagated peach plantlets against PPV infection and on the virus content. We also tested the effects of different concentrations of these compounds on the growth of peach plantlets, the antioxidative metabolism, the H_2O_2 contents and the expression of the *NPR1* gene. To check the positive effect of BTH or OTC on *Prunus* sp. development, the effects of these compounds on plant growth were also tested using plum (*Prunus dulcis* cv. Stanley) plantlets.

Materials and methods

Plant material

Healthy and PPV-infected GF305 peach [(*Prunus persica* (L.) Batsch] seedlings, inoculated by grafting a chip from an herbaceous GF305 plant showing strong sharka symptoms were used for the establishment of micropropagated culture, as described by Clemente-Moreno et al. (2011). The micropropagated GF305 plantlets obtained were sub-cultured at 4 week intervals, as described previously (Clemente-Moreno et al., 2011), for their micropropagation. Plum explants were obtained by regeneration from plum seed hypocotyls (cv. Stanley), as described by Petri et al. (2008), and cultured in the same micropropagation medium used for GF305 peach explants (Clemente-Moreno et al., 2011).

BTH and OTC treatments

Once the micropropagation of GF305 plants was established, we added different concentrations of BTH or OTC to the multiplication medium used for the micropropagation. In previous experiments, we tested the effects of different BTH (37, 75, 150, 250 and 500 μM) and OTC (0.1, 0.25, 0.5, 1 and 2 mM) concentrations on the growth of peach plantlets under *in vitro* conditions. The BTH or OTC was added from a 50-mM aqueous stock solution, sterilized by filtration, after the medium had been autoclaved and poured into sterile glass flasks (100 ml of medium in each one). In the assays with lower BTH (5, 10 μM) or OTC (10, 20, 50 μM) concentrations, a 10-mM stock solution of either BTH or OTC was used. In the case of micropropagated plum explants, we assayed 5, 10 and 20 μM BTH and 10, 20, 50 and 100 μM OTC. All treatments were applied for up to 4 weeks. The use of both compounds in the micropropagation of fruit trees has been submitted for a Spanish patent application ("Treatments to increase vigour in fruit trees under *in vitro* conditions," patent number P201130888).

Determination of antioxidative enzymes

To analyze enzymatic activities, GF305 explants (1 g) were homogenized with a mortar and pestle in 2 ml of ice-cold 50 mM Tris-acetate buffer pH 6.0, containing 0.1 mM EDTA, 2% (w/v) PVP, 2% (w/v) PVPP and 0.2% (v/v) Triton X-100. For APX activity, 20 mM ascorbate was added. The homogenate was centrifuged at 14,000 × *g* for 20 min and the supernatant fraction was filtered through Sephadex G-25 NAP columns equilibrated with 50 mM Tris-acetate buffer pH 6.0, containing 2 mM ascorbate for APX activity.

The enzymatic activities from the ASC–GSH cycle [ascorbate peroxidase (APX, EC 1.11.1.1, monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2)], catalase (CAT, EC 1.11.1.6), peroxidase (EC 1.11.1.7), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione-S-transferase (GST, EC 2.5.1.18), glucose-6-P-dehydrogenase (G6PDH, 1.1.1.49) and superoxide dismutase (SOD, EC 1.15.1.1) were analyzed according to Hernández et al. (2006).

Glutathione analysis

Samples were prepared by homogenizing GF305 peach explants in cold 5% meta-phosphoric acid. The homogenate was centrifuged at 13,000 × *g* for 15 min at 4 °C, and the supernatant was collected for analysis of glutathione. Aliquots (500 μl) were neutralized with 750 μl of 0.5 M potassium phosphate buffer (pH 7.5) and then 25 μl of MilliQ water were added. This sample was used to assay the total glutathione (GSH + GSSG). Another 500-μl aliquot was neutralized with 750 μl of 0.5 M potassium phosphate buffer (pH 7.5) and then 25 μl of 2-vinylpyridine were added to mask the GSH. This second sample was incubated for 60 min at room temperature and used to assay the oxidized glutathione (GSSG).

The total and oxidized glutathione were assayed by the NADPH-driven, glutathione-dependent reduction of dithio-bis-2-nitrobenzoic acid in the presence of NADPH and GR, according to the method of Zhang and Kirkham (1996). The GSH was calculated as the difference between total glutathione and GSSG. The glutathione content was measured in 1 ml of reaction mixture containing 0.2 mM NADPH, 50 mM potassium phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM DTNB and 3 units of GR. The reaction rate was monitored by measuring changes in absorbance at 412 nm for