Scientia Horticulturae 215 (2017) 49–58

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Effects of phytotoxic extracts from peach root bark and benzoic acid on peach seedlings growth, photosynthesis, antioxidance and ultrastructure properties

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ARTICLE INFO

Article history:
Received 31 August 2016
Received in revised form
13 November 2016
Accepted 6 December 2016
Available online 15 December 2016

Keywords:
Antioxidant enzymes
Benzoic acid
Cyanide
Peach replant
Photosynthesis
Root bark extract
Ultrastructure

ABSTRACT

During growth cycle, the accumulation of autotoxins released from peach root into soil strongly restrains the perennial tree growth in the same soil plot. However, the toxication syndrome on plant developmental physiology and the identities of toxic substances from peach root are still elusive to date. In this study, we used pot trials to investigate the influence of peach root bark extracts (extracted either in water or ethanol) and benzoic acid (a major allelopathic agent in most plant species)on seedling performance. Subsequently, the chemical compounds from peach root extracts were characterized by Gas Chromatography-Mass. Interestingly, in our study, the major toxic cyanide was only detected in the water extracts of root bark not in the root wood part and more cyanide could be detected in the presence of β-glucosidase. After applying exogenous root extracts and benzoic acid, the seedlings physiological growth was inhibited as expected, and lipid peroxidation and the antioxidant enzymes were increased as well. The photosynthetic parameters, including net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration, were all significantly reduced by the exogenous root extracts application. At cellular level, transmission electron microscopy showed that most of the organelles were lost in the root tip cells treated by ethanol-based peach root extracts, while the irregular nuclei, amorphous mitochondria in root tip cells, dissolved middle lamella and elongated plastids were observed in benzoic acid treatment. Furthermore, for the first time we identified 49 chemicals from ethanol-based peach root extracts, among which, 2, 4-bis (1, 1-dimethylethyl) -phenol (10.40%), benzoic acid (8.65%) and palmitic acid (7.12%) occurred in the highest amounts. Altogether our comprehensive physiological profile by toxication analysis and the determined major toxins from the root bark extracts could provide critical clues for peach allelopathic studies.

1. Introduction

When peach seedlings are grown in the area where had been previously planted with the same or similar plant species, and consequently the trees will grow slowly, have low vigor and slowly switch to reproduction with small load and poor quality of fruits. This phenomenon is often termed as peach replant problem, sometimes also called peach soil sickness or peach replant disease. Two important players, microorganisms and phytotoxic metabolites have been reported to be involved in such peach replant problem (Bent et al., 2009; Yang et al., 2012), among them, the toxic substances from the peach root residues (autotoxins) left in the soil are frequently drawing the attentions of researchers. Cyanogenic glycosides, such as amygdalin and prunasin, are the leading and particularly important agents in peach replant problem (Mizutani, 1980; Sotomayor et al., 2006). Punasin is rich in peach roots, and originally it is not toxic, but could be decomposed by certain soil microorganisms into toxic hydrocyanic acid (HCN) (Benizri et al., 2005; Patrick, 1955). Additionally, in the case of cellular disintegration, the cyanogenic glycosides could contact with and finally be hydrolyzed by β-glucosidase into HCN and benzoic acid (BA) as final products, which are proved to be toxic to peach seedlings (Ballhorn, 2011; Benizri et al., 2005; Israel et al., 1973; Mizutani, 1980). Although growth inhibition by BA and ethanol extract of root bark (EERB) has been proved in peach (Israel et al., 1973;

Abbreviations: AC, activated charcoal; BA, benzoic acid; CAT, catalase; CN^-, cyanide; EERB, ethanol extracts of root bark; ML, middle lamella; MDA, malondialdehyde; POD, peroxidase; SOD, superoxide dismutase; SRL, specific root length; WERB, water extracts of root bark.

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http://dx.doi.org/10.1016/j.scienta.2016.12.004
0304-4238/

Mizutani, 1980), the toxication mechanism underlying is unknown, and whether allelopathic BA is accumulated in peach roots or not, as well as the main chemicals in EERB are never studied.

Alleolopathic compounds interfere in a broad range of plant growth and developmental programs at multiple layers, e.g., inhibition of enzymatic activity, damage of cellular membranes, disturbance in ion uptake and water balance (Weir et al., 2004). When plants are exposed to allelopathic agents, reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide are markedly produced and subsequently affect membrane permeability, and cause damage to DNA, proteins and generate lipid peroxide signaling molecules, thus inducing lipid peroxidation and causing a generalized cellular disruption that ultimately leads to cell death. (Li et al., 2012; Ye et al., 2006; Yu et al., 2003). Malondialdehyde (MDA), as the end product of lipid peroxidation, is also an important indicator of membrane deterioration. Plants have evolved a fine-tuned antioxidant system to cope with allelopathic stress. A series of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), are extremely induced to scavenge excess ROS and protect cells from damage. Briefly, SOD catalyze the dismutation of $O^{2-\cdot}$ to H_2O_2 which could then be converted into harmless O_2 and H_2O by CAT or POD (Li et al., 2012).

To our knowledge, the information about the role of antioxidant enzymes and MDA in roots of peach seedlings in response to peach root extracts is not available. Meanwhile, the response of antioxidant enzymes to exogenous BA treatment is not consistent in different plant species (Baziramakenga et al., 1995; Sunaina and Singh, 2015; Yadav and Singh, 2013; Zhang and Kirkham, 1996). In addition, very few information is available on photosynthesis and cellular changes poisoned by peach root extracts. The current study aims to study the plant biochemical and physiological response to root bark extracts and to identify the chemicals in peach root extracts by GC–MS for a better understanding of peach replant problem.

2. Materials and methods

2.1. Pot trials and experimental design

Seeds of peach [*Prunus persica* (L.) Batsch] rootstock were initially stratified at 4 °C for 12 weeks. After germination, the uniform seedlings were transferred into plastic pots (25 cm diameter × 18 cm depth) filled with sterilized perlite and quartz sand at the ratio of 1:1 (v/v) and grown in a plastic rain shelter with natural light condition at Huazhong Agricultural University (Wuhan, China) with semi-tropical monsoon climate and average temperature 16.8 °C in April and 28.2 °C in August, as well relative humidity 74% – 80%. Two weeks after transplanting, seedlings were drenched with 100 ml of EERB, water extracts of root bark (WERB), BA (2.5 mM or 5.0 mM) and distilled water (as control), respectively, at 4 days intervals for totally 16 weeks. Following cultivation under stress, all the seedlings were watered with 200 ml distilled water weekly (3 days gap after the drench) and 250 ml of full-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) every 10 days for each pot. Among the samples population, 75 seedlings were prepared for lipid peroxidation and antioxidant enzyme activity assays, and $n = 15$ for each treatment; 45 seedlings for growth parameter and gas exchange measurement analysis, and $n = 9$ for each intact analysis. Plant height was measured every four weeks, while stem diameter was measured both at beginning and end of the experiment. When harvesting, the shoot and root were separately collected and dried in an oven at 75 °C for 72 h for dry weight assay. Leaf area and root length was scanned by EpsonExpression10000xl scanner (Epson Inc., Japan) and analyzed with WinRhizo Pro (S) v. 2009c software (Regent Instrument Inc., Canada). The specific root length (SRL) was calculated as total root length per root dry weight.

2.2. Preparations of ethanol and water extracts of peach root bark

The EERB was obtained based on the previous study (Rutto and Mizutani, 2006a; Rutto and Mizutani, 2006b). The root bark was sampled from the roots of three six-year-old peach [*Prunus persica* (L.) Batsch] rootstock in peach orchard (Huazhong Agricultural University). Samples were dried in oven (101-2AB, Taisite Instrument, Tianjin, China) at 75 °C for 72 h and ground into powder using a disintegrator (FW100, Taisite Instrument). The powder was then passed through a 0.25 mm sieve. Root bark powder (10 g) was extracted with ethanol for three times, each time with 1000 ml of 80% ethanol at 2 °C for 24 h. The residual ethanol was removed by evaporating in a vacuum at 40 °C, the extracts were then filtered and pooled together. The final extraction was made up to 100 ml with distilled water and stored at 4 °C as stock solution, the working solution was diluted to 1:50 for later use.

Due to the national drug control policy, the highly toxic cyanide (CN^-) solution was not purchasable from the market, therefore we used water extracts of root bark instead, which contain large amounts of cyanide compound (Mizutani, 1980; Patrick, 1955). 10 g of root bark powder was extracted for three times, each time with 1000 ml of distilled water at 2 °C for 24 h, and then 10 g of non-sedimenting activated charcoal (Sigma, USA) was added to the extracts to eliminate organic compounds (Lee et al., 2006; Yu and Matsui, 1994), and finally the extracts were filtered, pooled together and stocked as EERB described.

2.3. Cyanide measurements

Cyanide concentration in root bark and root wood was assessed based on the work by Rumberger et al. (2007) but with slight modifications. Root bark and root wood were sampled and processed as abovementioned, and the root samples from each tree were evenly divided into two partitions. 1.5 g of root bark and root wood powder were extracted with 20 ml of distilled water, respectively. Another 1.5 g of abovementioned materials were extracted with the same volume of distilled water, and then stored in tightly sealed vessels. All the water extracts were sonicated at 2 °C for 5 h, then 1 ml of β-glucosidase (Sigma, USA) (it can hydrolyze cyanogenic glycoside and then yield cyanide) was added to the later water extracts. After incubating at 25 °C for 24 h, the water extract was then shaked for 2 h at 2 °C and diluted to a total volume of 25 ml. Centrifugation (Beckman, USA) at 3396 *g* for 15 min, all supernatants were filtered through a 0.22 μm micro sieve film and then sent to Spectroquant Cyanide Kit (Merck, Germany) for cyanide analysis.

2.4. Gas exchange measurement

A portable photosynthetic system (LI-6400XT OPEN 6.1, USA) was used to measure the net photosynthetic rate (*P*n), transpiration (E), stomatal conductance (*G*s), and intercellular CO_2 concentration (*C*i) of leaves on peach seedlings in pot trials. Measurements were conducted at 8:00-10:00 am at a saturating light flux 1000 $\mu mol\, s^{-1}\, m^{-2}$, relative humidity 75%, leaf temperature 30 ± 1 °C and ambient atmospheric CO_2 levels 391 ± 32 $\mu l\, l^{-1}$. Ten leaves were measured at the same node in the middle part of the seedlings.