JOURNAL OF ENVIRONMENTAL SCIENCES 37 (2015) 184–191

Available online at www.sciencedirect.com

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JOURNAL OF ENVIRONMENTAL SCIENCES

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Piriformospora indica confers cadmium tolerance in *Nicotiana tabacum*

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ARTICLE INFO

Article history:
Received 10 October 2014
Revised 12 May 2015
Accepted 3 June 2015
Available online 17 July 2015

Keywords:
Plant endophytic fungus
Malondialdehyde
Proline
Chlorophyll
Stress-related genes
Tobacco plants

ABSTRACT

Piriformospora indica, a root-colonizing endophytic fungus of Sebacinales, promotes plant growth and confers resistance against biotic and abiotic stresses. In order to confirm the influence of *P. indica* on growth, proline, malondialdehyde (MDA), chlorophyll, and cadmium (Cd) amounts in *Nicotiana tabacum* under Cd stress, hydroponics, pot and field trials were conducted. The results showed that *P. indica* can store Cd in plant roots and reduce leaf Cd content, reduce the concentration of MDA, and increase the proline and chlorophyll content and the activities of catalase, peroxidase, and superoxide dismutase under hydroponic Cd stress. RT-PCR analysis showed that the relative expression level of genes *Gsh2*, *TaPCS1*, *oas1*, *GPX*, and *Hsp70* in colonized plants was 4.3, 1.4, 2.9, 1.7, and 6.9 fold higher than in un-colonized plants respectively. Cd exposure significantly reduced un-colonized plants' agronomic traits compared to *P. indica*-colonized ones. Our results suggested that *P. indica* can sequester Cd in roots, so that much less cadmium was transported to leaves, and the increased concentrations of antioxidant enzymes, pigments and proline contents, as well as the higher expression of stress-related phytochelatin biosynthesis genes in *P. indica*-inoculated plants, may also serve to protect *N. tabacum* plants against oxidative damage, enhancing Cd tolerance.

Introduction

Cadmium (Cd) is a ubiquitous toxicant which has been recognized as one of the most deleterious heavy metals that are present in the environment. In plants, Cd disturbs various physiological and biochemical processes, leading to inhibition or reduction of growth (Sandalio et al., 2001; Xu et al., 2009), and plants' stressed tissues develop an array of protective and repair systems that minimize the oxidative damage resulting from heavy metals (Khalvati et al., 2010). In addition to the intrinsic protective systems of plants against stresses, plants grow in association with rhizospheric microorganisms like arbuscular mycorrhizal fungi (AMF) that can further alleviate stress symptoms (Talaat and Shawky, 2011; Moradi and Salimi, 2013).

Arbuscular mycorrhizal fungi are widely believed to protect host plants from the detrimental effects of abiotic stresses (Al-Karaki et al., 2004; Ruiz-Lozano, 2003). Like the AMFs, the root endophytic fungus *Piriformospora indica* has been shown to colonize the roots of many plant species, promote plant growth and confer resistance against biotic and abiotic stresses, resulting in a large increase in the biomass (Varma et al., 1998, 1999; Sherameti et al., 2008; Stein et al., 2008; Oelmuller et al., 2009; Franken et al., 2010; Sun et al.,

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http://dx.doi.org/10.1016/j.jes.2015.06.005
1001-0742/

2010; Unnikumar et al., 2013; Varma, 2013). Although *P. indica* interacts with various plant species and the mechanism shares similarities with mycorrhizal fungi and several plant growth rhizobacteria (Harrison, 2005; Oldroyd et al., 2005; Stein et al., 2008; Lugtenberg and Kamilova, 2009), there are not many reports concerning the molecular mechanism by which *P. indica* promotes plant properties. Here, we report that *P. indica* can promote Cd accumulation and segregation in roots so that much less cadmium is transported to leaves, and improve the Cd tolerance of *Nicotiana tabacum*. *P. indica* can adopt various metabolic regulatory functions and adapt to maintain cell biomembrane system stability and reduce the level of membrane lipid peroxidation of plants, and upregulate the expression of phytochelatin biosynthesis and stress-related genes.

1. Materials and methods

1.1. Endophytic fungus, plant materials and co-culture

P. indica was grown on modified Kaefer medium (Hill and Kaefer, 2001) at 25 °C for 7 days in the dark. A glass petri dish (9 cm × 9 cm) containing 30 mL MS medium was inoculated with two mycelium plugs (5 mm each) and incubated at 25 °C for 7 days under continuous illumination of 6000 lx.

N. tabacum seeds were soaked in sterile tap water for 36 hr, then surface-sterilized in 70% ethanol for 1 min, in 1.0% sodium hypochlorite solution (5% active chlorine) for 10 min, rinsed repeatedly using sterile water, and planted in Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) for 14 days. The seedlings were then transferred to the MS medium and placed at 1 cm distance from the mycelium plugs. The plants inoculated with aseptic agar plugs were mock-inoculated as a control. The plants were kept at 25 °C with a 16-hr-light (6000 lx)/8-hr-dark photoperiod for 30 days.

1.2. Microscopy and detection of *P. indica* in colonized roots

After 30 days of co-cultivation, the plant roots were washed thoroughly and cut into small pieces (1 cm) for microscopy. The fungal structures were observed under an Olympus fluorescence microscope BX51 (Nikon Corporation, Tokyo, Japan).

1.3. Hydroponics, pot and field experiments

After *N. tabacum* co-cultivation with *P. indica* for 30 days, one part was transferred to pots (7 cm × 9 cm) filled with vermiculite, watered with Hoagland nutrient solution, while the other part was transferred to pots (7 cm × 9 cm) filled with a 4:2:1 mixture of sphagnum:vermiculite:perlite, and watered with ordinary water. Plants were allowed to grow in a greenhouse maintained at 25:16 °C (day: night), and a photoperiod of 16 hr:8 hr (light: dark). Control plants were also transferred to similar conditions. After 30 days, the first portion of plants was transferred to the nutrient solution to culture for one week, then various concentrations of Cd were added, to give Cd concentrations of 0.5, 1.0, and 1.5 mg/L. Plant roots, stems and leaf samples were retrieved after two weeks to determine Cd content and other physiological and biochemical indexes. After 45 days, the second portion of plants was transferred to pots (20 cm × 27 cm), containing 10 kg field soil treated with cadmium nitrate one week previous, containing 5 mg cadmium nitrate per kilogram of soil. Plant root, stem and leaf samples were retrieved after treatment at regular intervals of 14, 30 and 50 days to determine Cd content and other indexes. In addition, some of the second group of plants were transferred to the field, and designated in 5 districts respectively, with each district having 30 tobacco plants. 70 days later, *P. indica* and mock-treated *N. tabacum* plants were treated with 500 mL of 0.1 g/L cadmium nitrate solution, while water was used for the control plants. The control and each treatment were repeated 10 times. Leaf samples were retrieved after treatment for 14, 30 and 50 days to determine Cd content (Appendix A Fig. S2 shows the process of the experiment).

1.4. Cd determination

The dried samples of finely ground (0.5 g) plant tissue were digested with a mixture (2:1, V/V) of HNO_3 and H_2O_2. Cd concentrations in digested solutions were determined by inductively coupled plasma-MS spectrometry (Dai et al., 2009).

1.5. Determination of the malondialdehyde, proline, and chlorophyll and the enzyme activity

The malondialdehyde (MDA) content was determined according to Zhang and Fan (2002). The free proline content in leaves was determined according to Dobrá et al. (2011) with minor modifications.

The leaf samples (0.2 g) were homogenized in 10 mL of 80% acetone (v/v). Each homogenate was transferred to a 25 mL brown flask. The supernatant was used to measure absorbance at 661.6, 644.8, and 470 nm to calculate chlorophyll levels (Sims and Gamon, 2002).

For activities of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) extractions were carried out according to the methods introduced in previous publications (Giannopolitis and Ries, 1977; Zhang and Kirkham, 1994; Cakmak and Marschner, 1992), respectively.

1.6. RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted from the leaves of mock- and *P. indica*-inoculated plants after 1 mg/L Cd treatment for 7 days using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA). For real-time Polymerase Chain Reaction (PCR), a total volume of 25 mL mixture containing 12.5 mL of 2 × SYBR Premix Ex Taq TM (Takara Bio Inc., Shiga, Japan), 2 μL of diluted cDNA and 0.25 mL of 25 mmol/L gene-specific forward/reverse primers (Table 1) on a Mastercycler ep realplex Thermal Cycler (Eppendorf, Hauppauge, NY, USA) was performed using the following PCR program: 2 min at 95 °C, and 40 cycles of 5 s at 95 °C and 31 s at 60 °C. A melting curve analysis was performed to ensure that only a single product was obtained. Ct values were