Environmental and Experimental Botany 135 (2017) 126–135

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Culturable endophytic fungal diversity in the cadmium hyperaccumulator *Solanum nigrum* L. and their role in enhancing phytoremediation

Abdur Rahim Khan[a], Muhammad Waqas[a,b], Ihsan Ullah[c], Abdul Latif Khan[d], Muhammad Aaqil Khan[a], In-Jung Lee[a], Jae-Ho Shin[a,*]

[a] School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] Department of Agriculture, Abdul Wali Khan University Mardan, Mardan, Pakistan
[c] Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan
[d] UoN Chair of Oman's Medicinal Plants & Marine Natural Products, University of Nizwa, 616 Nizwa, Oman

ARTICLE INFO

Article history:
Received 9 December 2015
Received in revised form 10 March 2016
Accepted 11 March 2016
Available online 14 March 2016

Keywords:
Fungal community
Solanum nigrum
Mutualism
Heavy metal tolerance
Enhanced phytoextraction

ABSTRACT

In this study, the endophytic fungal community associated with *Solanum nigrum* was isolated and characterized for cadmium (Cd) tolerance and host plant growth modulation under Cd contamination for the first time. In total, 42 culturable endophytic fungal isolates representing various morphotypes were isolated, among which 14 different genera of the fungal phylum Ascomycota were identified based on the analysis of the internal transcribed spacer (ITS) region of the rRNA gene. Among the identified genera, *Colletotrichum* (18%) represented the dominant genus followed by *Alternaria* (14%), and *Fusarium* (14%). The majority of the endophytic isolates were sensitive to a lower concentration (0.5 mM) of Cd. However, *Glomerella truncata* PDL-1, and *Phomopsis fukushii* PDL-10 showed the highest tolerance to a concentration gradient of Cd (0.5–2 mM). Owing to the levels of Cd tolerance detected, in order to simulate a tripartite plant-microbe-metal interaction, *S. nigrum* plants were inoculated with PDL-1 and PDL-10 under Cd spiking of 0, 5, 15, and 25 mg kg^{-1}. The results indicated that PDL-10 inoculated plants had significantly higher Cd content in shoots as well as in roots than observed in the PDL-1 inoculated plants. Additionally, irrespective of Cd stress, PDL-1 and PDL-10 inoculation significantly improved plant growth attributes such as shoot and root length, chlorophyll content, and fresh and dry shoot/root weight as compared to those of non-inoculated control plants. The results of this study highlight the possible role of fungal endophytes harbored inside *S. nigrum*, which has the potential to improve the efficiency of phytoremediation or phytostabilization in the cleanup of Cd-contaminated soil.

1. Introduction

Cadmium (Cd) is one of the most toxic heavy metals and has recently been considered an environmental pollutant due to the excessive release of this element from various industrial and agricultural activities into soil and water (Gallego et al., 2012). In living organisms, Cd tends to accumulate in different tissues and interfere with numerous biochemical and physiological processes, thus adversely affecting the organism and environment (Solis-Dominguez et al., 2007). Presently, different research studies are moving towards the application of eco-friendly *in situ* strategies, characterized by a low cost and greater remediation potential for contaminated soil. In this regard, biological remediation methods for the detoxification of contaminated sites has received much attention worldwide.

Phytoremediation, unlike many conventional technologies, is an efficient, environment-friendly, and cost-effective alternative for the decontamination of heavy-metal-laden soils (Garbisu and Alkorta 2001). Certain plant species, known as hyperaccumulators, have the capacity to accumulate a higher concentration of toxic metals in their aboveground parts without showing any visible symptoms of toxicity (Buendia-Gonzalez et al., 2010). Specifically, *Solanum nigrum* L., an annual or perennial herb, which is a wild species with remarkable ecological adaptability throughout the world, has been characterized as a Cd-hyperaccumulator (Wei et al., 2004; Khan et al., 2014). However, some major drawbacks that limit the efficient application of phytoremediation approaches include its low Cd tolerance, slow growth, low biomass production

* Corresponding author.
E-mail address: jhshin@knu.ac.kr (J.-H. Shin).

http://dx.doi.org/10.1016/j.envexpbot.2016.03.005
0098-8472/

due to the toxic effects of higher concentrations of metals, and finally variation in the bio-availability of heavy metals for the plants used (Gerhardt et al., 2009; Glick 2010). These factors considerably reduce the efficiency of phytoremediation of contaminated soils. Therefore, it is necessary to update this potential eco-friendly process by increasing the growth of hyper-accumulator plants to yield higher biomass and simultaneously to nullify or lessen the level of phytotoxicity of metals.

Fungal endophytes are symbiotic organisms, and colonize the inner tissues of healthy plants to establish a harmonious relationship with their host without causing any visible disease symptoms (Saikkonen et al., 1998; Schulz and Boyle 2005). Fungal endophytes have been isolated from various plant species, ranging from herbaceous crop plants to higher giant woody forest trees, suggesting their universal presence in nearly all higher plants (Luo et al., 2011a,b,c).

Previous studies have indicated that the association of fungal endophytes can significantly enhance plant growth, including biomass as well as production yield (Ahemad and Kibret 2014; Hoffman et al., 2013). The beneficial effects of associated fungi (both endophytic fungi and ecto-mycorrhizae) on host plants mitigate biotic and abiotic stresses by increasing nutrient availability, enhancing tolerance to contaminants, and competing with and essentially inhibiting pathogenic organisms (Luo et al., 2009; Singh et al., 2011). During this plant-fungal symbiotic relationship, the associated fungi operate multiplex mechanisms to wipe out the repercussions of heavy metal stress using different detoxification strategies. For example, the fungi can induce the immobilization of heavy metals on the surface of living tissues using chemical bonding groups in the cell wall or by complexation of the heavy metal with small molecular organic compounds exuded in the rhizosphere (Luo et al., 2014). Fungal endophytes colonizing the host plant growing in heavy metal contaminated soil have marked tolerance for heavy metals, which suggests their potential application in phytoremediation strategies for the enhanced extraction of pollutants (Firmin et al., 2015; Soleimani et al., 2010b). For instance, *Festuca arundinacea* and *Festuca pratensis* inoculated with *Neotyphodium* spp. fungal endophytes showed higher production of biomass and Cd accumulation than plants not inoculated with endophytes in Cd-contaminated soils (Soleimani et al., 2010a,b). Such heavy metal resistant endophytic fungi are capable of promoting host plant growth, biomass production, and enhanced metal extraction. Furthermore, they alleviate the toxic effect of heavy metals by regulating various biochemical processes inside the plant through the production of different metabolites and phytohormones that help the host plant avoid metal stress toxicity (Hoffman et al., 2013; Redman et al., 2011). Fungal isolates belonging to different genera such as *Trichoderma*,*Aspergillus*, and arbuscular mycorrhizal fungi (AMF) have been studied for their potential role in the phytoremediation of contaminated soil (Firmin et al., 2015).

In the last decade, extensive research has focused on *S. nigrum* associated bacterial endophytes and their interactions in a Cd-contaminated environment (Chen et al., 2014, 2010; Khan et al., 2015b). However, the interactions of the endophytic fungi associated with *S. nigrum* in the presence of Cd have not yet carefully investigated. For instance, only one study by Xiao et al. (2010) confirmed that *Microsphaeropsis* sp. LSE10, an endophytic fungus isolated from the stem of *S. nigrum*, exhibited biosorption of Cd *in vitro* from an aqueous solution of growth media. The isolation and characterization of *S. nigrum* associated fungal endophytes is essential for enhancing phytoremediation technology employed at Cd contaminated sites. Up until now, there has not been sufficient information available concerning the potential role that interactions among endophytic fungi isolated from *S. nigrum* might play in Cd phytoremediation. In this study, we aimed to investigate the interactions among isolates representing the fungal diversity associated with the host plant, *S. nigrum*, in a Cd contaminated environment, in order to understand the role of these symbionts in phytoremediation.

2. Materials and methods

2.1. Experiment 1. Fungal diversity in S. nigrum and their tolerance against Cd

2.1.1. Isolation of endophytic fungi

Samples of naturally occurring healthy plants were randomly collected from fields in Daegu, South Korea. Whole plants along with the rhizospheric soil were carefully uprooted, packed in plastic bags, and immediately transferred to the laboratory. Plant samples were separated into leaves, stems and roots, and then washed with sterilized distilled water three times to completely remove the surface dust. The tissues were cut into 1-cm-long and 0.5-cm-wide fragments. Subsequently, the fragments were sterilized by immersion in 70% ethanol for 3 min and a sodium hypochlorite solution (2.5%) for 30 min, and finally washed with sterile distilled water three times. To confirm protocol efficiency, drops of sterile distilled water from the final wash step were spread on potato dextrose agar (PDA) plates. The three different media used for endophytic fungi isolation were: (1) Hagem agar media (0.5% glucose, 0.05% KH_2PO_4, 0.05% $MgSO_4{\cdot}_7H_2O$, 0.05% NH_4Cl, 0.1% $FeCl_3$, and 1.5% agar, pH 5.6 ± 0.2), (2) Czapek agar media (1% glucose, 1% peptone, 0.05% KCl, 0.05% $MgSO_4{\cdot}_7H_2O$, 1.5% agar, and 0.001% $FeSO_4{\cdot}_7H_2O$; pH 7.3 ± 0.2), and (3) potato dextrose agar media (PDA) (20.0 g/L dextrose, 4.0 g/L potato extract and 15 g/L agar, pH 5.6 ± 0.2). Fragments of plant tissues were randomly selected and transferred to different media containing 50 mg/L of chloramphenicol. The plates were incubated at 25 °C for 7 days, and after daily observation, newly emerged fungal spots were transferred to fresh PDA plates and repeatedly transferred until purified. The purified fungal isolates were maintained at 4 °C prior to freezing in glycerol (50%) and finally stored at −80 °C. Initially, the fungal isolates were grouped based on their morphological characteristics, such as mycelium color, growth rate, exudate production and color, spore length, and morphology.

2.1.2. Identification of fungal endophytes

The fungal isolates were identified by first extracting the genomic DNA, then completing a polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region, and finally sequencing the fragment obtained. The gDNA was isolated using the CTAB method. The ITS1-ITS4 region was amplified using the primer set ITS-1F (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4R (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR was performed with a thermal cycler using the following steps of amplification: pre-heating for 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C with a final extension of 3 min at 72 °C. The size of the amplified PCR products was verified with electrophoresis using a 0.8% agarose gel. The PCR products were purified using PCR purification kits and sequenced. The raw sequences were aligned using MEGA6, edited manually, and BLAST searched for the best match using the ntBLAST option in NCBI GenBank (http://www.ncbi.nih.gov/index.html). Homologous fungal ITS sequences were retrieved from NCBI and a phylogenetic tree was constructed using the neighbor-joining option with 1000 bootstrap replicates.

2.1.3. Screening of endophytic fungi for Cd tolerance

Fourteen different representative isolates of each genus were selected for preliminary screening of Cd tolerance using PDA plates