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Original article

Contribution of glomalin to Pb sequestration by arbuscular mycorrhizal fungus in a sand culture system with clover plant

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ABSTRACT

Glomalin (a glycoprotein) is produced by arbuscular mycorrhizal fungi and has the ability to sequester toxic heavy metals. We hypothesized that an increase in the concentration of Pb would lead to increased glomalin production and consequently higher Pb-sequestration. In a two compartment pot culture experiment, clover plants *(Trifolium repens* L.) were inoculated with arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, in sterile washed sand and then treated with Pb concentrations of 0, 150, 300 and 450 μM as $Pb(NO_3)_2$. The root compartment (RC) was isolated from the hyphal compartment (HC) by nylon mesh (37 μm). The glomalin was extracted from both RC and HC and assayed by using Bradford method and monoclonal antibody MAb32B11. The amount of sequestrated Pb was determined after wet digestion of glomalin. The results showed that the amount of Bradford reactive and immunoreactive glomalin is not correlated with hyphal biomass at different Pb levels. There was a positive and significant relationship between the percentage of root colonization and both immunoreactive and Bradford reactive glomalin. The content of Bradford and immunoreactive glomalin in both RC and HC was significantly increased by rising Pb levels, compared to the unleaded control. The maximum immunoreactive glomalin in HC (10.04 μg/mg hyphae) and in RC (7.79 μg/mg root) was recorded at 450 μM of Pb, which was markedly different from other levels of Pb. Total Pb sequestrated by HC glomalin enhanced as the level of Pb increased and it was 665.72 μg Pb/mg HC glomalin at 450 μM of Pb, while the total Pb sequestrated by RC glomalin was enhanced as the levels of Pb increased up to 300 μM (228.91 μg Pb/mg RC glomalin). Also, there were positive and significant correlations between both immunoreactive and Bradford reactive glomalin in HC with the amount of Pb sequestration. Consequently, this study provides evidence on the role of glomalin in Pb sequestration which should be considered for biostabilization of toxic elements in polluted sites.

1. Introduction

Glomalin is a component of spore and hyphal cell wall of arbuscular mycorrhizal (AM) fungi discovered in 1996 by Wright and co-workers [43]. It was thought to be exuded by the living fungus [41], but Driver and co-workers [8] found that glomalin is tightly bound in AM fungi hyphal and spore walls and small amounts (<20%) of glomalin are released by an AM fungi into the soil environment. Glomalin, though still not biochemically defined, is an N-linked glycoprotein composed of 3–5% N, 36–59% C, 4–6% hydrogen, 33–49% oxygen, and 0.03–0.1% P [33]. Glomalin also contains 0.8–8.8% Fe [29], which may be responsible for the reddish color of glomalin [42]. Glomalin is a stable compound, insoluble in water and resistant to heat degradation [39]. Apart from the Glomeromycota, no other fungal group produces this glycoprotein in significant amounts [39,41].

A number of studies have reported the contributions of glomalin to phytoremediation [6,12]. While examining the roles of glomalin in heavy metals sequestration of two polluted soils, González-

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Chávez et al. [12] stated the potential of glomalin in reducing availability and toxicity of "potentially toxic elements" such as Cu, Cd and Pb. Furthermore, Cornejo et al. [6] reported that glomalin related soil proteins (GRSP) bind to about 28% Cu and 6% of Zn in a soil highly polluted with these heavy metals. From their study, it appears that the higher concentration of the pollutants, the higher ability of GRSP to bind to them and make the pollutants unavailable. These studies are proof of the significant contributions of AM fungi to phytoremediation through glomalin production. Cornejo et al. [6] found correlations between GRSP production and heavy metal concentrations in the soil. They explained that toxicity induced stress by heavy metals may enhance glomalin production by AM fungi [24].

Glomalin is usually determined by two methods, the Bradford dye-binding protein assay and enzyme-linked immunosorbent assay (ELISA). Since many laboratories are not equipped to carry out the ELISA assay, many studies measure GRSP using the colorimetric Bradford method which measures the total protein [3,31]. Critical assessments of using the Bradford assay to quantify GRSP have shown cross-reactivity with non-AM fungi proteins added before autoclaving, and with other non-proteinaceous materials added to the GRSP extract [30,37]. This can result in an overestimation of GRSP stocks. Wright et al. [39] employed an ELISA assay with monoclonal antibody MAb32B11 developed against crushed spores of *Glomus intraradices* for the measurement of glomalin in soil and plant roots. Despite the reported correlations between ELISA and Bradford values [39,41,43], Bradford method is for total protein assay; hence, it is less specific for glomalin [40]. The Bradford total protein assay involves the use of Coomassie dye that binds to almost all protein [31,37]. Both microorganisms and plant roots secrete some proteinaceous substances, such as amino acids [7,14], which may be co-extracted with glomalin and detected by the Bradford assay [31,32,33]. These findings imply that Bradford-reactive soil protein (BRSP) is not completely related to AM fungi and needs thorough investigation [37]. Also, several studies confirmed that the ELISA technique is more sensitive and specific for GRSP quantification [27,43]. Based on the other findings, the Bradford method and ELISA assay may be useful in measuring glomalin pools when organic matter concentrations are low such as washed, autoclaved soil or in other controlled experimental conditions [18,31,43].

The objectives of this study were to determine whether increased Pb concentration would increase glomalin production. We also examined the amount of Pb sequestrated by the glomalin extracted from root and hyphal compartments at different levels of the applied Pb. By using glomalin-free sand culture system, we could avoid cross-reaction of the Bradford and ELISA assays with other proteins that could interfere with glomalin measurements.

2. Materials and methods

2.1. Pot experiment

Coarse sand was passed through 2 mm sieve and rinsed with water until all the silt and clay-sized particles were removed. The sand was acid-washed with 3 N HCl, and then GRSP was pre-extracted with 50 mM sodium citrate (pH 8.0 for 1 h at 121 °C). The extract was decanted, and the sand was thoroughly washed with water. After that, the sand was air dried and autoclaved at 121 °C for 1 h. Sterile glomalin-free sand was poured in 15 cm diameter (1500 g) pots. Fungal inoculum and clover seeds were placed together in the center of the pot in 8 cm diameter nylon mesh (37 μm, NBC Meshtec Inc.) bags to form a root compartment (RC) and a root-free area for hyphae compartment (HC) outside the bag. The seeds were surface sterilized by immersion in 70% (v/v) ethanol for 2 min, followed by transferring them to 0.5% (v/v) sodium hypochlorite for 10 min, and then rinsing 10 times with sterile tap water. Thirty uniform seeds of *Trifolium repens* L. (belonging to the Gene Bank, Research Institute of Forests and Rangelands, Iran) were aseptically sowed inside the bag containing autoclaved sand (800 g) and were inoculated with fungal inoculum. For preparing the inoculum, contents of six plates of *in vitro* culture of *Rhizophagus irregularis* (formerly, *Glomus intraradices*) on T-DNA transformed roots of *Daucus carota* L. were thoroughly mixed with sterile, glomalin-free sand (<0.5 mm diameter) and then a thin layer of inoculum (10 g) was spread just below the seeds. After plant establishment, they were thinned to 20 plants per pot. The pots were placed in the growth room with 16 h photoperiod (~300 μmol m^{-2} s^{-1}) at 25/20 °C day/night temperatures. They were watered every other day with the Hoagland's nutrient solution [20] having reduced phosphorus (40 μM) and containing Pb levels of 0, 150, 300 and 450 μM as $Pb(NO_3)_2$, and 0.5 mM MES buffer to maintain the pH at 6.1. Sodium nitrate was added to the treatments at concentrations that equalize the nitrate effects of $Pb(NO_3)_2$ levels [13]. After four weeks, P concentration in the Hoagland's solution was diminished to 20 μM for better mycorrhizal establishment. The plants were grown for a total of 16 weeks (14 weeks of exposure to the different levels of Pb). The controls received the basic Hoagland' nutrient solution without Pb. At harvest, the nylon bag containing roots (RC) was removed, and the roots were rinsed several times with sterilized and distilled water; then they were air-dried and kept frozen at −20 °C for glomalin extraction. The hyphal compartment (outside the nylon bag) was also removed and kept frozen at −20 °C until glomalin assessment. Extraradical hyphae in HC were collected by rinsing the sand with water and washing the hyphae on a series of stacked sieves (250,150, and 53 μm).

2.2. Mycorrhizal root colonization

A subsample of roots (0.1 g fresh weight) were cut into 1 cm length and fixed in 50% ethanol. The roots were placed in individual test tubes and cleared with 10% KOH (w/v), stained with 0.05% trypan blue in lactic acid:glycerol:water (1:1:1) solution, and finally destained with lactic acid:glycerol:water solution [4]. The percent of root colonization was determined by the gridline intersect method under a stereo microscope [10]. Briefly, all root fragments that crossed the grid lines were counted. The presence of hyphae, arbuscule, spore or vesicle scored the roots as colonized ones. The percentage of AM root colonization was expressed as the percentage of total number of colonized roots over the total number of roots counted.

2.3. Glomalin assays

The extraradical hyphae or root samples were separately macerated with five glass beads in tubes containing 50 mM sodium citrate extraction buffer at pH 8. For glomalin extraction, the samples were autoclaved at 121 °C for 60 min in three cycles, and the supernatants from each 1-h extraction cycle were combined after centrifugation at 10,000 × g for 10 min [31]. After the extraction process, the hyphal and root samples were transferred into weigh boats, dried at 70 °C and weighed. The glomalin content in a 1-mL subsample of the crude extract was determined by both Bradford and ELISA techniques as described below.

In the crude extracts, Bradford reactive glomalin was determined using a modified Bradford protein assay [39] which utilizes an acidic solution of Coomassie Brilliant Blue G-250 dye which binds to protein amino acid residues [8]. 200 μl sample extract reacted with 5 ml Coomassie Brilliant Blue G-250 dye (Merck