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Synergic effect of arbuscular mycorrhizal fungi and bradyrhizobia on biomass response, element partitioning and metallothionein gene expression of soybean-host under excess soil zinc

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ABSTRACT

The synergic effect of rhizobia (R) and arbuscular mycorrhizal (AM) fungi on the bioproduction, trace element partitioning and metallothionein gene expression of soybean-host was investigated under normal and elevated soil zinc conditions. In a completely randomized 3×4 factorial design, the experimental treatments – Zn addition (0, 200, and 400 mg Zn kg^{-1} soil) and inoculation (uninoculated control, R, AM, and RAM dual inoculation) were set up in the greenhouse for nine weeks. While the inoculants effectiveness was decreased in 400 mg Zn kg^{-1} soils, RAM induced significantly higher biomass production under all soil Zn treatments. The biomass response indicated that AM modulated stem and root bioproduction in favor of leaf/pod, while rhizobium favored root production and potentiated AM effect in dual inoculation. The partitioning of Zn and Mn in the hosts indicated synergic effects between AM and R, in RAM plants. Compared with control, AM lowered leaf Zn concentrations by reducing root Zn concentrations and modulating root-to-stem and stem-to-leaf Zn translocations. Compared to AM, RAM plants achieved lower leaf and pod Zn concentrations by mainly reducing root-to-stem Zn translocation. Zn treatment increased leaf and pod Mn in control plants, but symbionts countered this by regulating root-to-stem Mn translocation, especially in RAM. Type 1 metallothionein gene expression in roots was highest in RAM and lowest in control plants, but Zn effects were not dose-dependent. Synergisms in symbionts root colonization, number and greenness of leaves, element partitioning and metallothionein gene expression are indicated as important mechanisms underlying the effective partnership between AM and R, in the dual inoculation.

1. Introduction

Rhizobia (R) and arbuscular mycorrhizal (AM) fungi colonize legume roots and maintain symbiosis with the host. Utilized as biofertilizers and bioprotectants, these microorganisms support host growth and responses to biotic and abiotic conditions, while deriving shelter and photosynthates in return (Smith and Read, 2008; Polacco and Todd, 2011). Generally, improvement in host bioproduction is an important outcome expected of their utilization in plant production systems, including in sub-optimal conditions such as drought, deficient or excessive soil trace elements, salinity, etc. (Gamalero et al., 2009). One of the problems with the deployment of bioinoculants, however, is that the biomass response of the host may be positive, negative, or nil (Nogueira and Cardoso, 2003; Smith and Smith, 2011). It has been argued that aside insufficient time for the "maturity" of plant-AM symbiosis during short-term studies (Smith and Smith, 2011), evaluating inoculant effectiveness using only total biomass (as is commonly observed in literature) may mask biomass allocations between plant parts, and obscure a precise microsymbiont effect on host bioproduction (Jayne and Quigley, 2014). To evaluate the biomass partitioning effects of symbionts, the biomass response calculation for distinct parts of host-plant had been suggested (Poorter and Nagel, 2000; Veresoglou et al., 2012).

Many factors may contribute to a negative biomass response of a host to inoculants, as host response is a complex issue involving the plant-microbe-environment interaction (Smith and Smith, 2011). Understanding the factors underlying host response enhances the efficiency of the biological approach to optimizing crop productivity while minimizing the use of agrochemicals (Meena et al., 2017). Several reports indicate that due to synergic effects, dual inoculation with AM fungi (AMF) and R improved host performance more than single symbiont inoculations (Antunes et al., 2006; Chalk et al., 2006). But this is

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not always the case in a dual inoculation (Brown and Bethlenfalvay, 1987; Ray and Valsalakumar, 2010). The underlying synergistic responses during tripartite symbioses generally relate to microbe identity, physiology, and fitness under the prevailing soil conditions, plant nutrition, cultivar, etc. (Miransari, 2014). As these could be case-by-case (Gamalero et al., 2009), the elucidation of synergisms in AMF-rhizobial partnerships help clarify the mechanisms that underlie improved host bioproduction, in an efficient dual inoculation.

Excess soil zinc (Zn) conditions may be due to natural or anthropogenic factors and could affect plants as well as plant-microbe interaction outcomes (Chaney, 1993; Christie et al., 2004). For instance, some agricultural soils are known to contain moderately elevated Zn levels due to their volcanic origins (Okamoto et al., 2002; Ogiyama et al., 2005). Zn is also a prevalent contaminant in phosphate fertilizers and animal manures routinely applied on farms (Pinamonti et al., 1997; Lopez-Camelo et al., 1997). In Japan, the Zn regulatory level in the soil is 120 mg Zn kg^{-1}, while 200 mg Zn kg^{-1} was reported in the EU (Ogiyama et al., 2005, Tóth et al., 2016). However, elevated levels up to 400 mg Zn kg^{-1} has been reported in some farm soils, although the maximum allowable concentration (MAC) of Zn in agricultural soils varies from 100 to 300 mg kg^{-1} in most countries (ATSDR, 2005; Kabata-Pendias, 2011). As a high-value crop with many industrial applications in addition to its food/feed value, synergy between microbial inoculants for optimized soybean response under these range of soil Zn conditions, is valuable. Zn and manganese (Mn) nutrition in soybean has been implicated in the biomass response to symbionts (Nogueira and Cardoso, 2003; Ibiang et al., 2017). Both elements have similar ionic potential (charge/size ratio), may bioaccumulate together or antagonize one another, and share some metal transporters (Korshunova et al., 1999; Bravo et al., 2017). Metallothioneins (MTs) are a group (types 1–4) of cysteine-rich metal-binding proteins that are involved in metal homeostasis and ROS (reactive oxygen species) response, but their roles are not fully known in plants (Hassinen et al., 2011). While *MT1* is strongly expressed in roots (Guo et al., 2003), its role in host response to symbionts requires further elucidation within the context of AMF-rhizobia synergism. Our study examined the effect of single and dual AM fungal and rhizobial inoculants on bioproduction, organ-level partitioning of Zn and Mn, and type 1 metallothionein (*GmMT1*) gene expression in soybean-host, under normal and moderately elevated soil Zn conditions.

2. Materials and methods

2.1. Soil conditions

The soil utilized for the study was a mix of river sand and loam soil (sieved using a 2 mm mesh prior to mixing) in the ratio of 3:2 respectively. Characteristics of the unpolluted, unfertilized, unplanted soil-mix was determined to be: pH (6.27 ± 0.09), EC (3.99 ± 0.42 mS m^{-1}), available Zn (0.12 ± 0.04 μg g^{-1}), Fe (9.42 ± 0.44 μg g^{-1}), Mn (8.59 ± 0.23 μg g^{-1}) and Cu (0.18 ± 0.02 μg g^{-1}). Bulk soils in plastic bags were autoclaved (121 °C for 60 min) twice (at 24 h intervals), after which $ZnSO_4.7H_2O$ was dissolved in sterile distilled water and applied with mixing to the soils at 0 mg Zn kg^{-1} soil (Zn0), 200 mg Zn kg^{-1} soil (Zn200) and 400 mg Zn kg^{-1} (Zn400), and stored (25 °C) for one week before use. Zn treatments at Zn200 and Zn400 were chosen for their relevance to elevated Zn conditions in agricultural soils rather than to assuredly elicit metal toxicity (Pilon et al., 2009). On seeding day, soils were initially amended with dolomite (Ca: 278.7, Mg: 96.5 mg pot^{-1}), NPK fertilizer (N:30, P:44; K:83 mg pot^{-1}) and nutrient solution ($ZnSO_4.7H_2O$: 50, $MnSO_4.5H_2O$: 286, $CuSO_4.5H_2O$: 50, $CoCl_2.6H_2O$: 6; mg pot^{-1}, Fe-EDTA.$3H_2O$: 8.78 g pot^{-1}). Additional N fertilizer (35 mg pot^{-1}) was later applied at 4 and at 7 weeks after seeding.

2.2. Seeds

The soybean, *Glycine max* (L.) Merr. (cv. Enrei) was used in this study. Seeds were sterilized in 70% ethanol for 12 min, immersed in 10% H_2O_2 for 3 min, then rinsed in distilled water before sowing in pots containing soils (three seeds pot^{-1}, later thinned to one).

2.3. Experimental setup

The experiment was set up as a 3 × 4 factorial in a completely randomized design. Factor 1 was the Zn application (Zn0, Zn200, and Zn400), while factor 2 was the inoculation - rhizobium alone (R), AM fungus alone (AM), rhizobium + AM fungus (RAM), and uninoculated control (C). Each treatment was replicated 7 times giving a total of 84 experimental pots. All plants were routinely supplied with borehole water and pots were rotated weekly in the glasshouse for nine weeks.

2.4. Symbiont inoculations

The AMF used was *Claroideoglomus etunicatum* (supplied by Kyowa Hakko Kogyo Co. Ltd, now Kyowa Hakko Kirin Co. Ltd, Japan), while R was *Bradyrhizobium diazoefficiens* USDA 110 (USDA *Rhizobium* culture collection). AM fungal inoculum (soil bearing approx. 400 AM spores) was applied in the middle of soil in the pot, just prior to seeding. *Bradyrhizobium diazoefficiens* obtained from pure stock was maintained in yeast mannitol broth, and volume of broth was adjusted with sterile water as needed to obtain an average rhizobial cell concentration of 5.0×10^7 cells mL^{-1}. This diluted broth was used for rhizobial inoculation at the rate of 1 mL $seed^{-1}$ at time of seeding.

2.5. Plant harvest

At nine weeks after seeding, leaf greenness (SPAD 502 Plus Chlorophyll Meter, KONICA MINOLTA, JAPAN, INC.) were determined and five plants (n = 5) were randomly chosen for further analysis. Plants were wholly harvested by carefully emptying the soil from the plastic pots into labeled polythene bags, breaking apart soil mass loosely attached to roots in a bucket of water, washing in running tap water and rinsing in distilled water. The whole plant was then cut into the roots, stem, leaves, and pods. A portion of roots was subtracted for total RNA extraction (frozen in liquid nitrogen then stored at − 80 °C) and determination of mycorrhizal colonization before drying all fresh biomass in the oven at 80 °C for 48 h.

2.6. Plant bioproduction and response to inoculants

Total dry weight, as well as root, stem, leaf, and pod dry weights, were determined. Symbiont effectiveness was determined as total biomass response (Nogueira and Cardoso, 2003), while root biomass response (RBR), stem biomass response (SBR), leaf biomass response (LBR) and pod biomass response (PBR) were calculated separately (Watts-Williams and Cavagnaro, 2012) for each soil condition, using the dry weights of inoculated plants ($DW_{sym.}$) and uninoculated control ($DW_{control}$) as shown below:

$$Biomass response(\%) = (DW_{sym.} - meanDW_{control})/meanDW_{control}(\times 100) \quad (1)$$

2.7. Rhizobial nodule and mycorrhizal colonization

Symbiont colonization parameters were assessed in all the plants. Root nodules (R and RAM) were removed from roots, counted and weighed. Mycorrhizal colonization (AM and RAM) was determined in roots using the trypan blue staining technique previously described by Rajapakse and Miller (1994). Observation and scoring in a light microscope for mycorrhization indices was according to Trouvelot et al.