Plant Environment Development 8(2): 19-24, 2022 (December)
©Department of Botany, University of Rajshahi
e-mail: plant.environ.dev@ru.ac.bd

Print ISSN 1994-1501
Online ISSN 2311-3529
http://plantenvdev.com

Research Paper

Iron biofortification potentialities of arbuscular mycorrhizal symbiosis in alfalfa and sorghum

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Abstract

Arbuscular mycorrhizal fungi (AMF) are a group of beneficial microorganisms for plants. This study deals with the mechanisms of AMF-mediated Fe enrichment in alfalfa (Strategy I) and sorghum (Strategy II) plants., AMF supplementation did not improve plant biomass, chlorophyll synthesis and photosynthesis efficiency in either alfalfa or sorghum. However, both of the plant species showed a significant accumulation of Fe in root and shoot due to AMF surplus. Further, ferric chelate reductase activity was the key to boost Fe mobilization in roots of alfalfa while sorghum plants showed no specific mechanism but constitutively regulates the Fe uptake and its related genes in roots. The qPCR analysis also showed the upregulation of Fe-related genes (*MsIRT1*, *MsNramp1*, and *MsFRO1*) in roots of alfalfa, while sorghum plants showed the constitutive expression of *SbDMAS2*, *SbNAS3*, and *SBYS1* in roots following AMF supplementation. It suggests that AMF is useful for Fe enrichment purposes to boost Fe both Strategy I and II plants.

Keywords: Arbuscular mycorrhizal fungi , Fe uptake, biofortification, FCR, PS release.

INTRODUCTION

Iron (Fe) is an essential element for plants. Fe participates in various processes in plants, including co-factor in cellular processes, mitochondrial respiration, photosynthesis, regulation of protein structure (Zhang et al. 2019; Jiang et al. 2007). Biofortification is mainly considered as a sustainable approach to eliminate nutrient deficiency in plants; perhaps, it can also be used to increase nutrients in the edible or useful parts of the plants. Enrichment of nutrient is not only significant for plants but also the human and animals dependent on them Fe uptake in plants involved a wide range of mechanisms, mainly divided them into Strategy I and Strategy II plants (Brumbarovaet al. 2015, Banakaret al. 2017). Sunflower (*Helianthus annuus*), a strategy-I plant show reduction-based Fe uptake system through ferric reductase activity (*FRO*) and iron-regulated transporter (*IRT1*, *NRAMP1*, etc.) mainly in roots (M'sehli et al. 2011; Vert et al. 2002). Strategy-II plant-like sorghum (*Sorghum bicolor*) posses chelation-based mechanisms by which plants release phytosiderophores (PS), an organic compound, in the rhizosphere to mobilize inorganic Fe-III compounds by forming Fe-PS complex (Vert et al. 2002). The 2′-deoxymugineic acid (DMA) is derived from S-adenosyl-l-methionine (SAM) catalyzed by nicotianamine synthases (NAS) in Strategy II plants (Kobayashi et al. 2010; Bashir et al. 2006). The chelation of insoluble Fe due to DMA enhances the solubility and uptake of Fe(III)-DMA complex into roots. In addition, the *YS1* gene (phytosiderophore transporter yellow stripe 1) is known to acts as a proton-coupled symporter for PS and NA-chelated metals (Curie et al. 2001).

Arbuscular mycorrhizal fungi (AMF), especially the fungi of the phylum Glomeromycota establish a mutualistic relationship with plants (Sieh et al. 2013). Most of the studies showed that AMF improved the nutritional status of plants mainly through the connection of fungal hyphae under different mineral deficiencies (Kabir et al. 2020). Metabolic pathways linked to S

metabolites are induced due to AMF, which in turn increases nutrient availability for plants (Chu et al. 2016). AMF symbiosis has also been reported to enhance Fe uptake in plants under Fe deficiency (Kabir et al. 2020; Rahman et al. 2020). In addition, plants often face different biotic and abiotic stresses that lead to the accumulation of reactive oxygen species (ROS). However, a number of ROS-scavenging enzymes regulate the elevated oxidative burst for protecting plants (Mittler 2002). AMF symbiosis is also associated with the protection of host plants from stress from oxidative damages (Borde et al. 2001; Hajiboland et al. 2009).

Despite the enormous potentialities of AMF on agriculture, the knowledge on the increment of bioavailable Fe to the aerial parts of Strategy I and II plants is still vague. Therefore, this study was performed in order to investigate whether and how AMF supplementation affects morphological features, photosynthesis parameters, and Fe accumulation in alfalfa and sorghum.

MATERIALS AND METHODS

Used Inoculant

Commercial AMF inoculum consisted of the mixture of endomycorrhizal spore (*Glomusintraradices, Glomusmosseae, Glomusaggregatum, Glomusetunicatum*) were used (Myco Supreme, USA).

Plant cultivation

In this study, we have used alfalfa (Strategy I) and sorghum(Strategy II) seeds on top of wet tissue paper placed in a plastic tray at room temperature for germinations. Once germinated within 3 days, the plants were separately transferred to the 500 g of the sterile mixed substrate [1:1 (v/v), perlite: sand] supplemented with micro and macro-elements (Kabir et al. 2020; Hoagland and Arnon 1950). For AMF treatment, 250 mg AMF /pot was added. The mixed cultivation substrate was irrigated with the solution every three days. The plants were kept in the growth chamber at 25°C having 60% relative humidity, 200 $\mu molm^{-2}\ s^{-1}$ light intensity, and long-day light conditions (14 h light/10 h dark) for four weeks.

Iron analysis in root and shoot

After harvesting, root specimens were rinsed one-time with 0.1 mM $CaSO_4$ and multiple times with Milli-Q water to expel exterior constituent. The roots and shoots were separately dried in an oven for 72h at 70º C. Afterwards, from each treated group dried samples were weighed and dissolved with a solution of $HClO_4/HNO_3$, 1:3 v/v, The Fe concentration in digested solution was then determined from the standard known solution of these elements by ICP-MS (inductively coupled plasma mass spectrometry) (Agilent 7700, ICP-MS).

Characterization of photosynthetic parameters

The longest root and shoot were taken for the measurement of length using a digital caliper. The dry weight of root and shoot was taken after drying for 3d at 80°C in an electric oven. The chlorophyll score was measured on young leaves by SPAD meter (Minolta, Japan) as instructed by Yuan et al. (2016). Furthermore, photosynthesis biophysics through chlorophyll fluorescence kinetic (OJIP), such as Fv/Fm (quantum efficiency of photosystem II), Pi_ABS (photosynthesis performance index), and Mo (approximated initial slope in ms^{-1} of the fluorescent transient) of young leaves kept for 1 h at dark using FP 100 (Photon Systems Instruments, Czech Republic).

Iron chelate reductase activity

The Fe (III) chelate reductase (FCR) activity was determined, as described by Kabir et al. (2020). Initially, roots were rinsed with 0.2 mM$CaSO_4$ and deionized water. Afterward, root and shoot tissue was transferred to 1.5 mL Eppendorf tubes filled with 100 mMFe(III) EDTA, 0.10 mM MES-NaOH (pH 5.5), 300 mMferrozine. The samples were placed in the shaker in the dark for 20 min. Finally, the absorbance of aliquots was measured 562 nm in a spectrophotometer. The FCR activity was determined using the molar extinction coefficient for ferrozine ($M^{-1}cm^{-1}$).

PS release in roots

The PS release from roots was determined as previously described (Kobra and Singh 2014). Briefly, harvested plants were submerged in a beaker containing working solution (1N NaOH, 4mM $FeCl_3$) aerated by a pump for 3 h. Afterward, the solution was passed through Whatman paper to eliminate microbial degradation. The Fe(III) was then reduced due to the addition of 8% hydroxylaminochloride at 55^0C for 20 min. The PS release as ferric ion was then determined at 562 nm after adding 10 mMferrozine and 0.5 M Na-acetate buffer (pH 4.6) in a spectrophotometer.

Real-time PCR analysis in roots

We have selected a few candidate genes, which are commonly responsible for Fe uptake Strategy I and II

plants. The total RNA was isolated from the root tissues of tomato plants as instructed (SV total RNA isolation system kit, Promega, USA). In brief, from each condition, 80 mg of root samples were used to homogenize in a mortar pestle with RNA extraction buffer, and 1% (v/v) β-mercaptoethanol (β-ME) was added before to centrifuge for 2 min at 12000 rpm. After subsequent washing steps, the RNA was restored from the RNA spin columns additional RNase-free water, and the final yield of the RNA was checked by a Nanodrop Spectrophotometer. The first-strand cDNAwas synthesized from RNA using the PrimeScript™ RT kit (Takara, United States). The real-time PCR was performed in CFX-96™ real-time PCR (BIORAD, United States) for the expression of a candidate gene using gene-specific primers (Supplementary Table S1) designed based on the sorghum gene database. The PCR program was as follows: 95 °C for 30 sec, followed by 40 cycles at 95 °C for 5 sec, 60 °C for 30 sec.The expression level of target genes was assessed following the $2^{-\Delta\Delta Ct}$method (Livak and Schmittgen, 2001), where the *Actin* house keeping gene considered as an internal control. For each condition, three separate replications were performed.

Statistical analysis

All experiments were done on complete randomized design having at least three replications for each sample. Statistical analyses and graphs were (two-tailed paired t-test) were performed using Microsoft Excel and GraphpadPrism 5 software, respectively. Statistical significance was set at 5% level.

RESULTS

Given the well-known efficacy of commercial AMF inoculum in plant growth promotion, we directly used it in our experiments. Morphological analysis showed that AMF supplementation did not demonstrate any significant improvement in root and shoot features in either alfalfa or sorghum compared to non-treated controls (Fig. 1, Fig. 2). In addition, parameters related to photosynthesis, such as SPAD score, Fv/Fm, Pi_ABS, did not show any changes in leaves of alfalfa and sorghum due to AMF compared to control (Fig. 3).

Interestingly, although FCR activity showed a significant increase in alfalfa, the PS release did not significantly change in roots of sorghum under AMF supplementation compared to controls (Fig. 3). However, the addition of AMF showed a significant increase in Fe concentration in both root and shoot of alfalfa and sorghum (Table 1).

Table 1. ICP-MS analysis of Fe (mg g^{-1} DW) in root and shoot of alfalfa and sorghum cultivated with or without AMF.

Alfalfa			
root		shoot	
Control	+AMF	Control	+AMF
207±31.1[a]	341±24.8[b]	76±5.2[a]	114±4.8[b]
Sorghum			
root		shoot	
Control	+AMF	Control	+AMF
178±22.4[a]	269±23.4[b]	68±12.4[b]	99±4.7[b]

[Different letters indicate significant differences between means ± SD of treatments (n=3) at a $P < 0.05$ significance level.]

Fig.1. Photographs showing the phenotype of 7-day old alfalfa and sorghum cultivated with or without AMF.

Supplementary Table S1. Primers sequences used in qPCR experiments.

Gene	Accession Number	Primer	Sequence
MsActin	XM_024774779.1	Forward	ACCGGTGTGATGGTTGGTAT
		Reverse	GCCACACGAAGCTCATTGTA
MsIRT1	KX641478.1	Forward	TTTACCCTTGGCGACACGTT
		Reverse	CATGAACCCGGTCCCAAGAA
MsNramp1	XM_024781241.1	Forward	GCATTGCTAGCCTCAGGACA
		Reverse	TCCATGCGTATGCAGGTGAT
MsFRO1	AY439088.1	Forward	GGTGACACGTGGATCATCTG
		Reverse	TTGCAATCCACAGGAACAAA
SbActin	XM_021463392.1	Forward	TCTTTGCGCCCAAGAAATGC
		Reverse	GAAGAAAGCGGTGGCCGATA
SbDMAS2	XM_002442161.2	Forward	TGGTTGCCTTGTTCTCCCAC
		Reverse	GATCACGAGCAACAAGATAAAAACT
SbNAS3	XM_002463385.2	Forward	CCTCTGCTGCGAGATGGAAG
		Reverse	TCGAACAAGAAAGGCAAGCAC
SbYS1	XM_002452787.2	Forward	GTAGCAAATGGTGGGGAAGA
		Reverse	GCCGGAATATATGTGGGATG

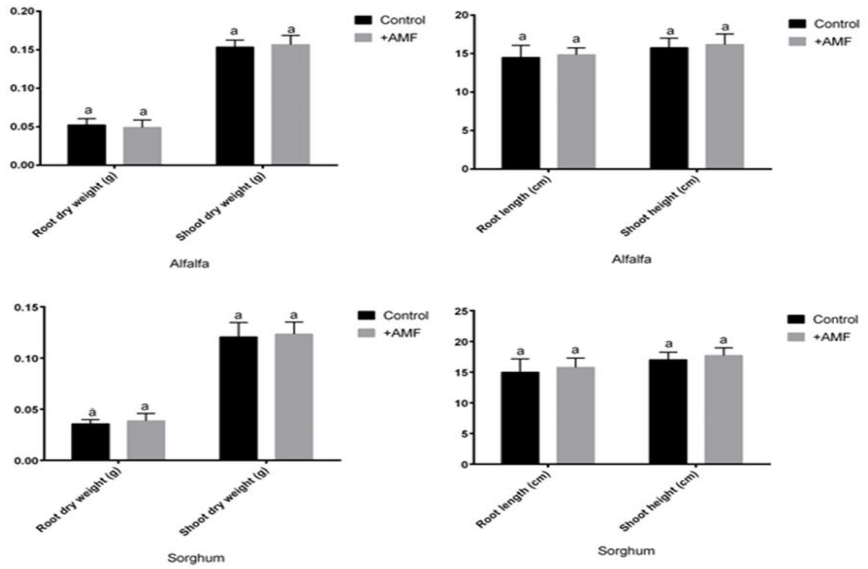

Fig. 2. Morphological features of alfalfa and sorghum cultivated with or without AMF. Different letters indicate significant differences between means ± SD of treatments (n=3) at a $P < 0.05$ significance level.

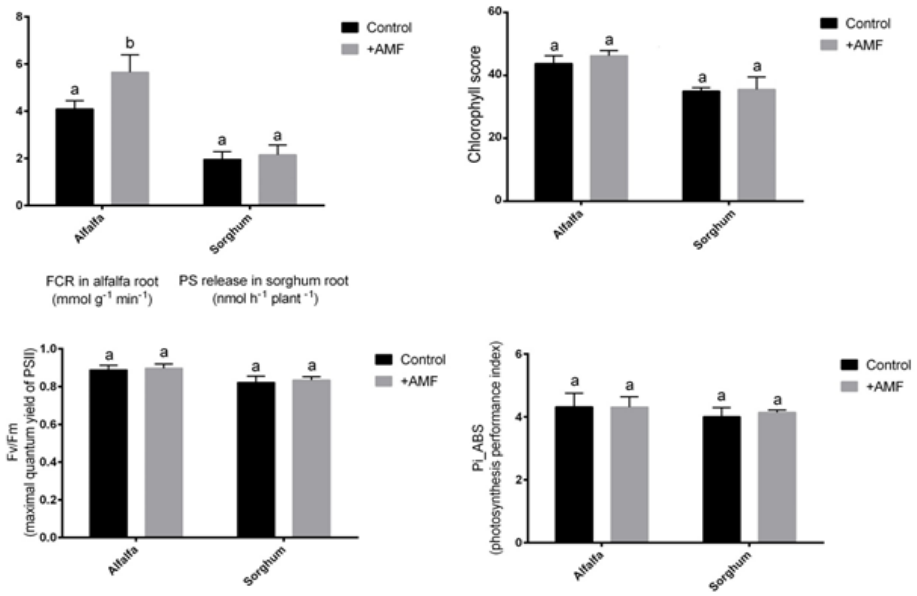

Fig. 3. FCR activity,PS release and photosynthesis parameters in alfalfa and sorghum cultivated with or without AMF. [Different letters indicate significant differences between means ± SD of treatments (n=3) at a $P < 0.05$ significance leve]l.

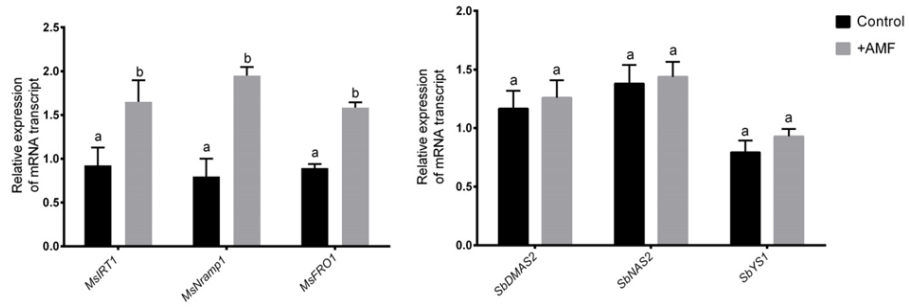

Fig. 4. Quantitative expression of candidate genes in roots of alfalfa and sorghum cultivated with or without AMF. [Different letters indicate significant differences between means ± SD of treatments (n=3) at a $P < 0.05$ significance level.]

Interestingly, genes responsible for Fe uptake in Strategy I and II plants showed distinct expression patterns in response to AMF surplus in roots. In alfalfa, *MsIRT*, *MsNramp1*, and *MsFRO1* genes showed a significant increase in expression in roots following AMF supplementation compared to controls (Fig. 4). In contrast, the expression of *SbDMAS2*, *SbNAS3*, and *SbYS1* genes showed no significant changes in the roots of sorghum due to AMF compared to non-treated controls (Fig. 4).

DISCUSSION

The approach of this study was to investigate the iron biofortification efficiency of AMF in alfalfa and sorghum in normal growth conditions. Results suggest that AMF is not able to play a positive role in boosting morphological features in either root and shoot in tested Strategy I and II plants. In contradictory to these findings, the improvement of biomass was also reported in other plants belonging to Strategy I and II categories (Li et al. 2015; Ingraffia et al. 2019). Photosynthesis is an integral part of plant growth and development in which chlorophyll synthesis is an indicator. Neither of the tested plant species was found to be influenced by AMF supplement in chlorophyll synthesis. However, AMF supplementation improved Fe level in both root and shoot of alfalfa and sorghum, although photosynthesis efficiency was not notably improved. It may be possible that the elevation of Fe is not directly linked to photosynthesis in these tested plants and may dependant on the co-factors involved with photosynthesis. In a similar study, AMF positively increased the growth and uptake of Fe in wheat but not in faba bean (Ingraffia et al. 2019). Therefore, it implies that AMF efficiency in plants is a genotypic dependant regardless of the Fe-acquisition strategy.

At the biochemical level, two main Fe acquisition mechanisms, FCR and PS release in roots, respectively associated with Strategy I and II plants, were notably varied due to AMF supplementation. This evidence is consistent with the expression of pattern of candidate genes responsible for Fe uptake in alfalfa and sorghum. It suggests that alfalfa possess upregulation Strategy I mechanism, while sorghum follows constitutive regulation of Strategy II mechanisms, to enable increased Fe accumulation to the aerial plants under AMF supplementation. In conclusion, AMF supplementation can be a useful eco-friendly strategy to initiate the Fe biofortification program in significant crop and vegetable plants.

Acknowledgment
We are grateful to the University of Rajshahi for providing research facilities.

Conflict of interest
We do not have any conflict of interest.

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