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Mycorrhiza-mediated potassium transport in *Medicago truncatula* can be evaluated by using rubidium as a proxy

Arjun Kafle [a], Danielle R. Cooney [a], Garud Shah [a,b], Kevin Garcia [a,*]

[a] Department of Crop and Soil Sciences, North Carolina State University, Raleigh, NC 27695, USA
[b] Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi considerably improve plant nutrient acquisition, particularly phosphorus and nitrogen. Despite the physiological importance of potassium (K^+) in plants, there is increasing interest in the mycorrhizal contribution to plant K^+ nutrition. Yet, methods to track K^+ transport are often costly and limiting evaluation opportunities. Rubidium (Rb^+) is known to be transported through same pathways as K^+. As such our research efforts attempt to evaluate if Rb^+ could serve as a viable proxy for evaluating K^+ transport in AM symbiosis. Therefore, we examined the transport of K^+ in *Medicago truncatula* colonized by the AM fungus *Rhizophagus irregularis* isolate 09 having access to various concentrations of Rb^+ in custom-made two-compartment systems. Plant biomass, fungal root colonization, and shoot nutrient concentrations were recorded under sufficient and limited K^+ regimes. We report that AM plants displayed higher shoot Rb^+ and K^+ concentrations and a greater K^+:Na^+ ratio relative to non-colonized plants in both sufficient and limited K^+ conditions. Consequently, our results indicate that Rb^+ can be used as a proxy to assess the movement of K^+ in AM symbiosis, and suggest the existence of a mycorrhizal uptake pathway for K^+ nutrition in *M. truncatula*.

1. Introduction

Arbuscular mycorrhizal (AM) fungi form a symbiotic association with the roots of most land plants, including major crops (Wang and Qiu, 2006). The hyphae of AM fungi develop a dense network that explores a larger volume of soil beyond the rhizosphere and provides water and nutrients for colonized plants (Smith and Read, 2008). In return, the plant provides AM fungi with carbohydrates in the form of lipids and sugars (Helber et al., 2011; Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). AM fungi have demonstrated a role in plant phosphorus (P) and nitrogen acquisition in natural and agro-ecosystems, resulting in many studies that explore the transport to these two elements in a symbiotic condition (Ezawa and Saito, 2018; Govindarajulu et al., 2005; Ohtomo and Saito, 2005; Tian et al., 2010; Kafle et al., 2019a; Plassard et al., 2019; Kafle et al., 2019b). Increasing efforts have been made to investigate the impact of AM symbiosis on the acquisition of other macro- and micro-nutrients, but our knowledge still remains very limited (Liu et al., 2000; Casieri et al., 2013; Garcia et al., 2016; Ruytinx et al., 2020).

In plants, potassium (K^+) is a key macro-nutrient that has a vital role in development and numerous vital processes, including membrane potential, enzyme activity, and biotic and abiotic stresses (Wang et al., 2013; Hasanuzzaman et al., 2018; Ragel et al., 2019). Although some studies reported that AM fungi have a positive impact on K^+ acquisition in colonized plants (Pallon et al., 2007; Scheloske et al., 2004; Estrada et al., 2013; Liu et al., 2019; Ye et al., 2017; Garcia et al., 2017), most current descriptions regarding the uptake, translocation, and regulation mechanisms of K^+ transport in plants occur under axenic conditions and/or on non-mycorrhizal plants (Alemán et al., 2011). Yet, more recent work identifies the fungal role in K^+ solubilization and transfer to plant roots, increasing interest and applicability for investigating these symbiotic associations in laboratory conditions and harnessing them in agricultural settings (Haro and Benito, 2019). Additionally, the first molecular mechanisms associated with the transport of K^+ from the soil to colonized roots have been identified in tomato (Liu et al., 2019), reinforcing the idea that AM fungi can actively transport K^+ towards host plants. Moreover, in ectomycorrhizal symbiosis, some fungal transporters and channels have also been recently identified, characterized, and described as active players for K^+ transfer from the soil to colonized roots (Garcia et al., 2014, 2020; Guerrero-Galán et al., 2018).

Tracking K^+ movement in AM symbiosis is crucial but also challenging due to cost and/or short half-life of K^+ isotopes. As such, a more

* Corresponding author.
E-mail address: kgarcia2@ncsu.edu (K. Garcia).

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desirable method is to use stable rubidium (Rb^+) as a proxy for K^+. Indeed, Rb^+ is transported through K^+ transport proteins, allowing the permeability of K^+ channels and transporters, and multiple studies have utilized Rb^+ to evaluate K^+ movements in yeasts and plants (Polley and Hopkins, 1979; Mulet and Serrano, 2002; Aiking and Tempest, 1977; Tyler, 1997; Rygiewicz and Bledsoe, 1984; Hawkes and Casper, 2002; Läuchli and Epstein, 1970a; Vallejo et al., 2005). However, possible K^+/Rb^+ discriminations by some plant species may make the use of Rb^+ tricky for absolute quantification (Marschner and Schimansky, 1971). Recently, we used Rb^+ as a proxy for K^+ transport in ectomycorrhizal symbiosis and observed that some fungi may also be able to discriminate between Rb^+ and K^+, allocating Rb^+ to the host plant in sufficient-K^+ condition and not in limited condition (Frank and Garcia, 2021).

In the model legume *Medicago truncatula* colonized by the AM fungus *Rhizophagus irregularis* DAOM 197198, we demonstrated previously a greater accumulation of K^+ compared to non-colonized plants, particularly under K^+-deficient conditions (Garcia et al., 2017). However, it was impossible to tell whether K^+ was directly taken up by roots or provided by the fungus since both plants and fungi were placed in single compartment pots. Here, in two-compartment systems, we decided to evaluate the role of Rb^+ as a proxy for K^+ transport in *M. truncatula* colonized by a different AM fungal isolate (*R. irregularis* isolate 09) under sufficient and limited K^+ regimes.

2. Material and methods

2.1. Plant and fungal materials

M. truncatula 'Jemalong A17′ seeds were scarified with concentrated sulfuric acid (18 M) for eight minutes, rinsed five times with autoclaved deionized water, surface sterilized with 8% commercial bleach for two minutes, and rinsed five times with water. Seeds were then placed on 1% (w/v) agar plates supplemented with 1 μM of gibberellic acid and kept at 4 °C for four days. Seeds were germinated after keeping them one night at room temperature. The germinated seedlings were axenically grown on modified Fahräeus medium (Catoira et al., 2000) for twelve days in a growth chamber at 23 °C/14 h and 18 °C/10 h day/night cycles. The AM fungus *R. irregularis* isolate 09 (Fellbaum et al., 2014); collected from southwest Spain by Mycovitro S.L. Biotechnología ecológica, Granada, Spain) used in this study was provided by Dr. Heike Bücking from the University of Missouri, U.S.A. The fungus was cultured in vitro utilizing axenic root organ cultures of Ri T-DNA-transformed carrot (*Daucus carota* clone DCI) grown on minimal medium (St-Arnaud et al., 1996) and fungal spores were extracted according to (Kafle et al., 2019a).

2.2. Design of two-compartment systems

Custom-made two-compartment systems were prepared using plastic boxes (12 cm × 8 cm x 8 cm; L x H x W; from Carno A/S) as described in (Frank and Garcia, 2021). Each box contained one compartment for roots (RC), paired together with a Magenta GA-7 Plant Culture Box (Bioworld) that was placed inside the box to form the fungal compartment (FC) where only fungal hyphae from the RC have access to. Ninety-nine 2 mm holes were drilled on one side of the Magenta box facing towards the RC and two 52-microns nylon mesh were placed on either side of the drilled holes to form an air gap (Fig. S1). The lowest perforations were made at 2 cm from the base to prevent any leakage of the nutrient solutions from one compartment to another. The mesh layers prevent the roots from entering the FC while still allowing the fungal hyphae from the RC to reach the FC. Six 5 mm holes were drilled at the bottom of the RC only for watering. Both compartments were filled with 300 ml of Turface® pre-washed with deionized water.

2.3. Mycorrhizal symbiosis and rubidium supply

Twelve-day-old *M. truncatula* seedlings were placed in the RC and were inoculated with 400 fungal spores and few colonized root segments of root organ cultures (AM), or kept non-inoculated (NM). After transplanting, six two-compartment systems were placed in 26 cm × 26 cm x 5 cm trays per treatment group. Plants were watered with 180 ml of sufficient K^+ (SK, 3.75 mM) or limited K^+ (LK, 0.05 mM) Long Ashton solutions at the base of each tray and protected with plastic covers for one week. Starting on day 5, 180 ml of the respective nutrient solution was provided to the RCs via filling the tray every three days until harvest. In order to fully control the concentration of available nutrients found within each FC, no drainage holes were added to the bottom of this compartment. Thus, each FC had a 20 ml 1/10 dilution of the corresponding solution (SK or LK) added to the top of the compartment. Diluted nutrient solution was provided to the FCs only once a week to prevent the over-accumulation of nutrients in these compartments as during the first few weeks of fungal growth and establishment, fungal access to the FC would be limited. Additionally, 30 ml of milli-Q water was periodically given in between weekly nutrient solution additions for each FC if needed to retain adequate moisture (Fig. S1).

Ten days prior to harvest, 20 ml of a Rb^+/K^+ solution was added every two days in the FCs of AM and NM conditions as follows: 3.25 mM, $RbNO_3$ and 0.5 mM, KNO_3 for the first experiment; 1.5 mM, $RbNO_3$ and 0.5 mM, KNO_3, or only 0.5 mM, KNO_3 for the second experiment (Fig. S1). The reasoning for these concentrations is the following: because we did not want to add too much K^+ in the LK condition, or too little in the SK conditions, we decided to give 0.5 mM of K^+ in the FCs when Rb^+ was added. Consequently, 3.25 mM of $RbNO_3$ was added to match a total Rb^+/K^+ concentration of 3.75 mM, similar to the K^+ concentration in the SK condition (see above). During the same period, the RCs were still watered as described above with SK or LK solutions. No watering solution was added on harvest day in any compartment, and plants were harvested seven weeks post-inoculation.

2.4. Biomass determination, ion content measurement, and mycorrhizal quantification

Upon harvest, fresh shoot weight was recorded, then dried for three days at 70 °C and dry weight was recorded. Dried shoot samples were sent to the Environmental and Agricultural Testing Service (EATS) at North Carolina State University to determine Rb^+, K^+, sodium (Na^+), and P concentrations by ICP-MS or ICP-OES. All these elements were expressed to mg or μg per g of dry weight before performing the statistical analyses.

Roots were washed with tap water, blotted with a paper towel, and fresh weights were recorded. After subsampling the roots for the quantification of AM colonization, the remaining roots were weighed again, dried for three days at 70 °C and dry weights were recorded. For mycorrhizal quantification, root samples were cleared with 10% KOH (w/v) at 95 °C for seven minutes, rinsed with water, and stained with 5% Sheaffer ink vinegar (v/v) at 95 °C for seven minutes. Root colonization was quantified using the grid line intersection method (McGonigle et al., 1990).

2.5. Statistical analyses

Due to a couple of plants that died during the experiment, the data analysis was based on five to six replicates for each treatment. All the figures were made with JMP software (JMP, SAS Institute Inc., NC, USA). Differences among means were analyzed with a two-way ANOVA or three-way ANOVA, depending on the experiment, followed by LSD post hoc tests. Pearson correlations were performed using Statistical Analytical software (Statistix 9, Tallahassee, Florida, USA).

3. Results

3.1. R. irregularis 09 improves the growth of M. truncatula

Our previous study demonstrated that *M. truncatula* plants colonized by the AM isolate DAOM 197198 displayed higher shoot K^+ concentrations than non-colonized plants, particularly under limited K^+ condition (Garcia et al., 2017). However, it was unclear whether the fungus played a direct or indirect role in obtaining supplementary nutrition beyond the direct access of plant roots. Indeed, these experiments were conducted in single pots in which both symbiotic partners had access to the same nutrient solution. Here, we utilized custom-made two--compartment systems in which *M. truncatula* was growing in one compartment uncolonized or in association with *R. irregularis* isolate 09 under sufficient and limited K^+ regimes (Fig. S1). These systems allowed the fungal symbiont exclusive access to the secondary compartment in which Rb^+ was added 10 days before the conclusion of experiment (Fig. S1).

Plant dry weight and AM root colonization were examined after seven weeks of co-culture (Fig. 1). We observed that the AM plants shoot and root biomass were significantly higher than NM plants under both SK and LK conditions (Fig. 1A, B). No significant difference was recorded for shoot and root biomass of NM plants between LK and SK conditions, that could be due to the plant tolerance to K^+ deprivation or a too short experimental period to observe growth differences. However, although shoot dry weight was similar under SK and LK conditions for AM plants (Fig. 1A), root biomass was significantly higher at LK than SK (Fig. 1B), indicating that the fungal colonization may have a role in host plant production of roots under K^+ deficiency. This improved root biomass at LK was not due to a difference in root colonization since around 45% of roots were colonized in both K^+ regimes (Fig. 1C).

3.2. Analysis of shoot nutrient concentration in M. truncatula colonized by R. irregularis isolate 09

After recording biomass, Rb^+, K^+, Na^+, and P concentrations were determined in shoots. Significantly higher shoot Rb^+ concentrations were detected in AM plants compared to NM plants in both SK and LK regimes (Fig. 2A). Yet, regardless of K^+ regime, no difference in shoot Rb^+ concentration was observed between all NM plants, as well as between AM plants (Fig. 2A). However, it should be noted that Rb^+ was also detected within the shoots of NM plants, indicating its presence in the growing medium, as reported previously (80 ppb of Rb^+, see (Frank and Garcia, 2021)). This Rb^+ quantity was much lower than what we added in the FCs, but it explains its detection in NM plants. Concerning shoot K^+ concentrations, NM plants growing at LK had significantly less shoot K^+ than those grown in SK, validating the sufficient and limited K^+ conditions used in this study (Fig. 2B). In both SK and LK conditions, AM plants had similar shoot K^+ concentrations, but significantly higher than NM plants (Fig. 2B). In contrast, shoot Na^+ concentration was significantly lower in AM plants compared to NM plants in both SK and LK (Fig. 2C). Interestingly, NM plants had higher Na^+ concentration in SK than LK conditions. Since K^+ and Na^+ are tightly connected ions in plant cells (Benito et al., 2014), we calculated the K^+:Na^+ ratio in NM and AM plants under SK and LK conditions (Fig. 3). We observed that the shoot K^+:Na^+ ratio of AM plants was significantly higher than NM plants in both SK and LK (Fig. 3). Additionally, the K^+:Na^+ ratio of AM plants in SK was greater than those in LK (Fig. 4). Finally, the shoot P concentration of AM plants was significantly higher than NM plants in both SK and LK conditions (Fig. 2D), indicating that the fungal P transport was not affected by the external K^+ availability.

In further considerations for evaluating nutrient relationships and the role of Rb^+ as a proxy for K^+ we tested the connections between shoot Rb^+ and shoot K^+, AM colonization, and plant biomass by determining Pearson correlations (Fig. 4). The concentrations of Rb^+ in the shoot demonstrated significant, strong positive correlation to shoot K^+

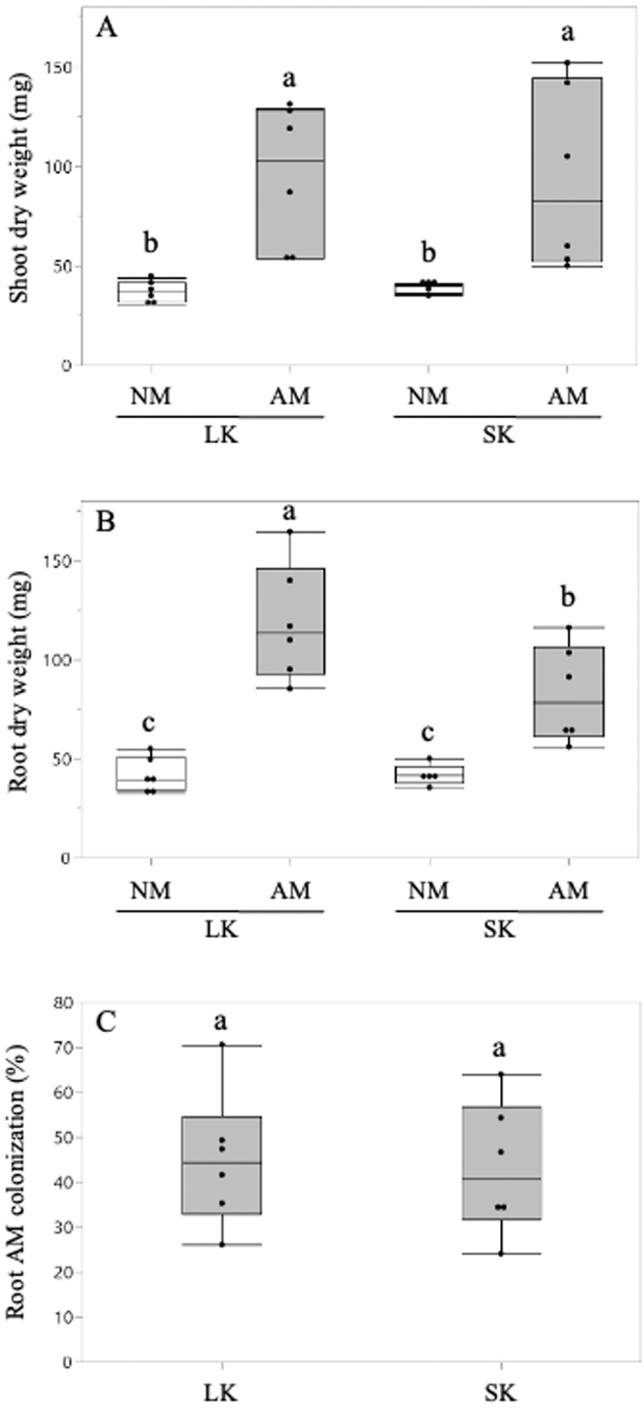

Fig. 1. Biomass and root colonization of *Medicago truncatula* plants inoculated by *Rhizophagus irregularis* isolate 09 under potassium-limited and -sufficient conditions. Shoot (A) and root (B) dry weights were determined in seven-week-old *M. truncatula* plants inoculated (AM) or not (NM) by the AM fungus *R. irregularis* isolate 09 in limited (LK, 0.05 mM) or sufficient K^+ (SK, 3.75 mM) conditions. Gray and white box plots display plants inoculated or not by the fungus, respectively. (C) The rate of fungal colonization was determined on *M. truncatula* roots grown under SK or LK conditions after seven weeks of co-culture using the grid line intersection method. Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). n = 5–6.

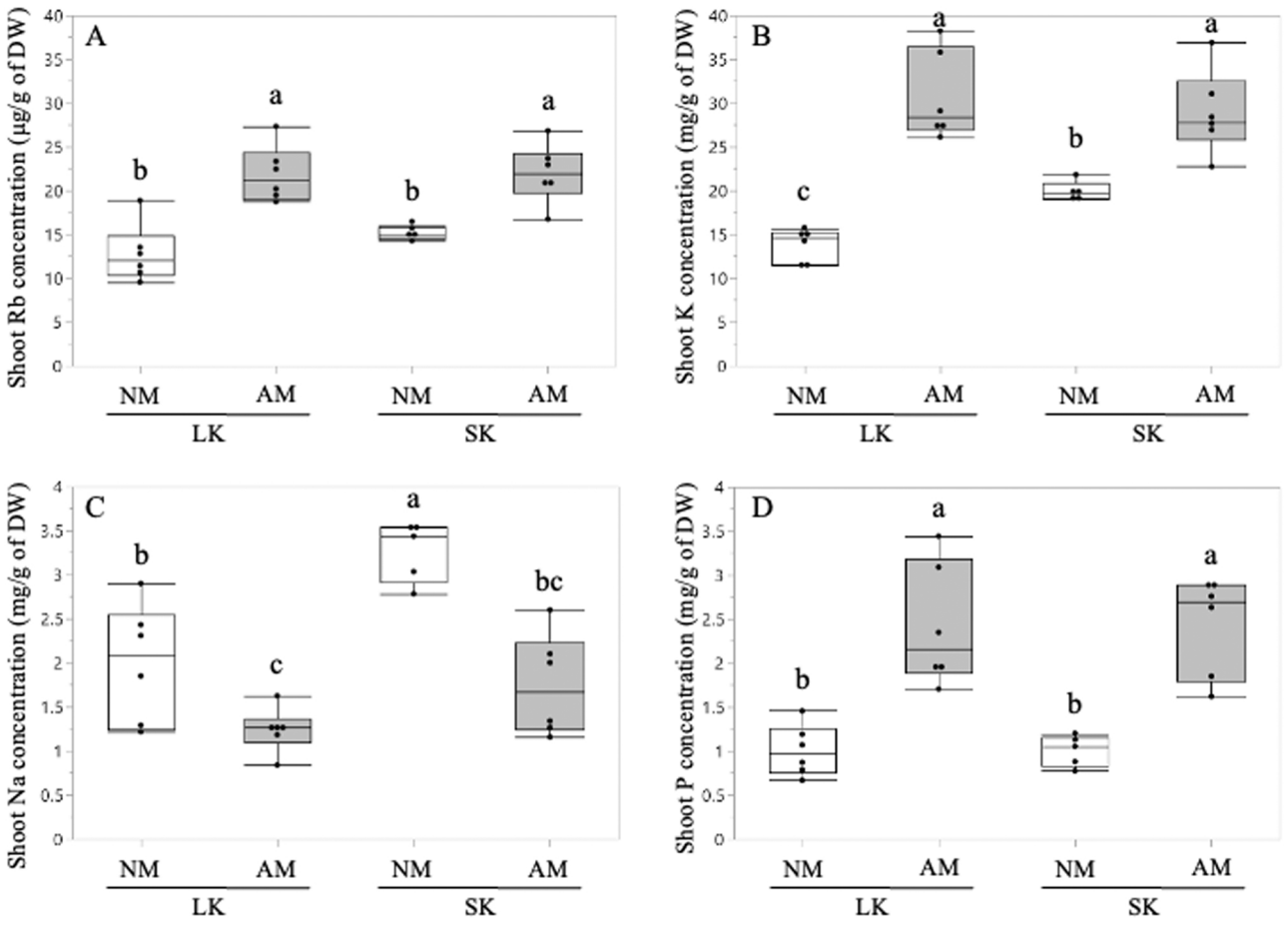

Fig. 2. Shoot rubidium, potassium, sodium, and phosphorus concentrations in *Medicago truncatula* inoculated by *Rhizophagus irregularis* isolate 09 under potassium-limited and -sufficient conditions. Rubidium (Rb, A), potassium (K, B), sodium (Na, C), and phosphorus (P, D) concentrations were determined by ICP-OES or ICP-MS in the shoots of seven-week-old *M. truncatula* plants inoculated (AM) or not (NM) by the AM fungus *R. irregularis* isolate 09 in limited (LK, 0.05 mM) or sufficient K^+ (SK, 3.75 mM) conditions. Gray and white box plots display plants inoculated or not by the fungus, respectively. Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). n = 5–6.

concentrations (Fig. 4A), indicating that Rb^+ could be used as a proxy for K^+ transport in our system. Furthermore, a significant, positive correlation occurred between shoot Rb^+ concentrations and the percentage of AM colonized roots (Fig. 4B), suggesting that the fungus is able to transport Rb^+ to the colonized plants. This latter observation was reinforced by the fact that no significant correlation was observed between shoot Rb^+ concentrations and root and shoot biomass in NM or AM plants (Fig. 4C; Fig. S2). Therefore, the bigger plants with a more developed root system are not the primary contributor to statistically significant Rb^+ accumulation, rather this is a clear indication on the role of the AM fungus in transporting Rb^+.

3.3. Lower Rb^+ supply can be used in two-compartment systems

To further confirm the role AM fungus had in the higher Rb^+ concentration observed in the shoots of colonized *M. truncatula* and to test whether a lower supply of Rb^+ can be used in the FC, we performed a supplemental experiment. We used the same conditions as the first experiment, but without Rb^+ added in the FC, or with only 1.5 mM of Rb^+ supplied (approximately half of the original concentration). Similar to the first experiment, we observed a trend that the shoot and/or roots of AM plants had increased biomass relative to NM plants, regardless of nutrient status SK or LK (Fig. 5A, B). Interestingly, AM plants from the systems containing 1.5 mM of $RbNO_3$ and 0.5 mM KNO_3 in the FC had significantly higher shoot biomass than AM plants where only 0.5 mM KNO_3 was added in the FC, for both SK and LK conditions (Fig. 5A). This effect could result from the difference in nitrogen source provided in the FCs between these two conditions. Biomass of AM plant roots was significantly greater at LK only when 1.5 mM of Rb^+ was added to the FCs compared to 0 mM of $RbNO_3$ (Fig. 5B). No difference was observed in root colonization between AM plants in SK, and those in LK (Fig. 5C). Additionally, all AM plants displayed more shoot P than NM plants (Fig. S3).

In both SK and LK conditions without Rb^+ addition, we did not observe any differences in shoot Rb^+ concentration between NM and AM plants (Fig. 6), confirming that AM plants were not able to acquire more Rb^+ from the substrate than non-colonized plants if not supplied to the FC. When the FCs were supplemented with 1.5 mM of Rb^+, we observed significantly higher Rb^+ concentration in AM plants in both SK and LK, compared to NM plants (Fig. 6A). This result indicates that Rb^+ was transported by the AM fungus from the FC, and thus validates its use as a proxy for evaluating K^+ transport in arbuscular mycorrhizal symbiosis.

4. Discussion

Maintaining an optimal K^+ concentration in plant cells is crucial for various processes, including photosynthesis, enzymatic and metabolic activity, or interaction with other nutrients (Wang et al., 2013; Ragel et al., 2019; Cui et al., 2019; Hernandez et al., 2012). The significant role of AM fungi in plant acquisition of K^+ has recently gained interest but still shows contrasting results (Haro and Benito, 2019; Garcia and Zimmermann, 2014; Domínguez-Núñez et al., 2016). Although recent

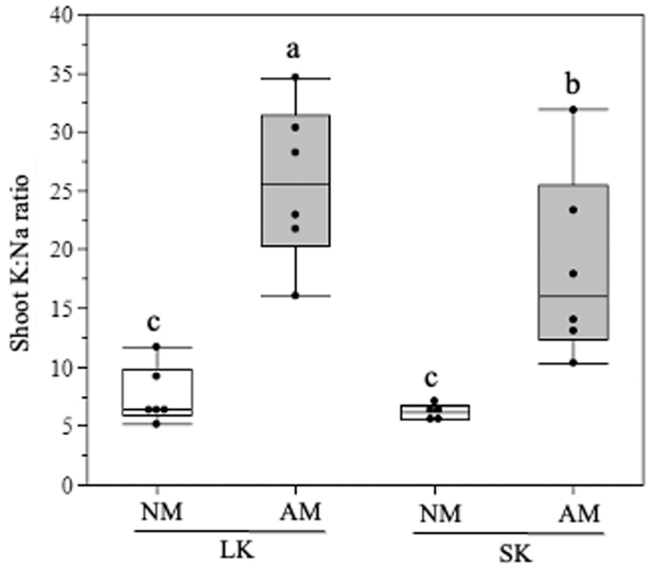

Fig. 3. Shoot K^+:Na^+ ratios in *Medicago truncatula* inoculated by *Rhizophagus irregularis* isolate 09 under potassium-limited and -sufficient conditions. Ratios between shoot K^+ and Na^+ concentrations were calculated for each *M. truncatula* seedlings that were colonized by *R. irregularis* isolate 09 (AM), or kept non-colonized (NM), and growing in limited (LK) and sufficient (SK) K^+ conditions. Gray and white box plots display plants inoculated or not by the fungus, respectively. Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). n = 5–6.

studies have revealed that the K^+ nutrition of some AM plants can be improved upon symbiosis, particularly under limited conditions (Liu et al., 2019; Garcia et al., 2017), an important remaining question is the direct vs. indirect involvement of AM fungi in K^+ movements from the soil to colonized roots. Since it is complicated to use K^+ isotopes to track these movements, we investigated the possible use of Rb^+ as a proxy for K^+ transport in *M. truncatula* as both cations share similar chemical properties and Rb^+ is transported through K^+ transporters in plants (Polley and Hopkins, 1979; Mulet and Serrano, 2002; Aiking and Tempest, 1977; Tyler, 1997; Rygiewicz and Bledsoe, 1984; Hawkes and Casper, 2002; Läuchli and Epstein, 1970a; Vallejo et al., 2005).

4.1. *R. irregularis isolate 09 improves K^+ nutrition, growth, and K^+:Na^+ ratio in M. truncatula*

Only the shoot nutrient concentrations were recorded due to the impossibility to discriminate between plant and fungal tissues in colonized roots. We observed that when plants were colonized by *R. irregularis* isolate 09, shoot K^+ concentrations increased under both SK and LK conditions and reached a similar level of K^+ concentration in plant tissue. This suggests that the AM fungus improved the plant K^+ nutrition under LK condition, but that this uptake may have been saturated at SK. In addition, it shows that this AM fungus can play an important role in improving *M. truncatula* K^+ nutrition, irrespective of the external K^+ availability. Interestingly, these observations contrast with our previous study in which more K^+ was recorded in AM plants growing under limited-K^+ conditions only, but not when sufficient amounts of K^+ was supplied (Garcia et al., 2017). In the present study, we used a different fungal isolate, *R. irregularis* isolate 09, which was previously described as a highly cooperative symbiont for P and nitrogen transport (Fellbaum et al., 2014; Kiers et al., 2011; Wang et al., 2016). Therefore, it is also possible that *R. irregularis* isolate 09 was able to provide more K^+ to the colonized plants relative to the one tested in our previous study (Garcia et al., 2017), suggesting that the mycorrhiza-mediated K^+ transport depends on the fungal ability to

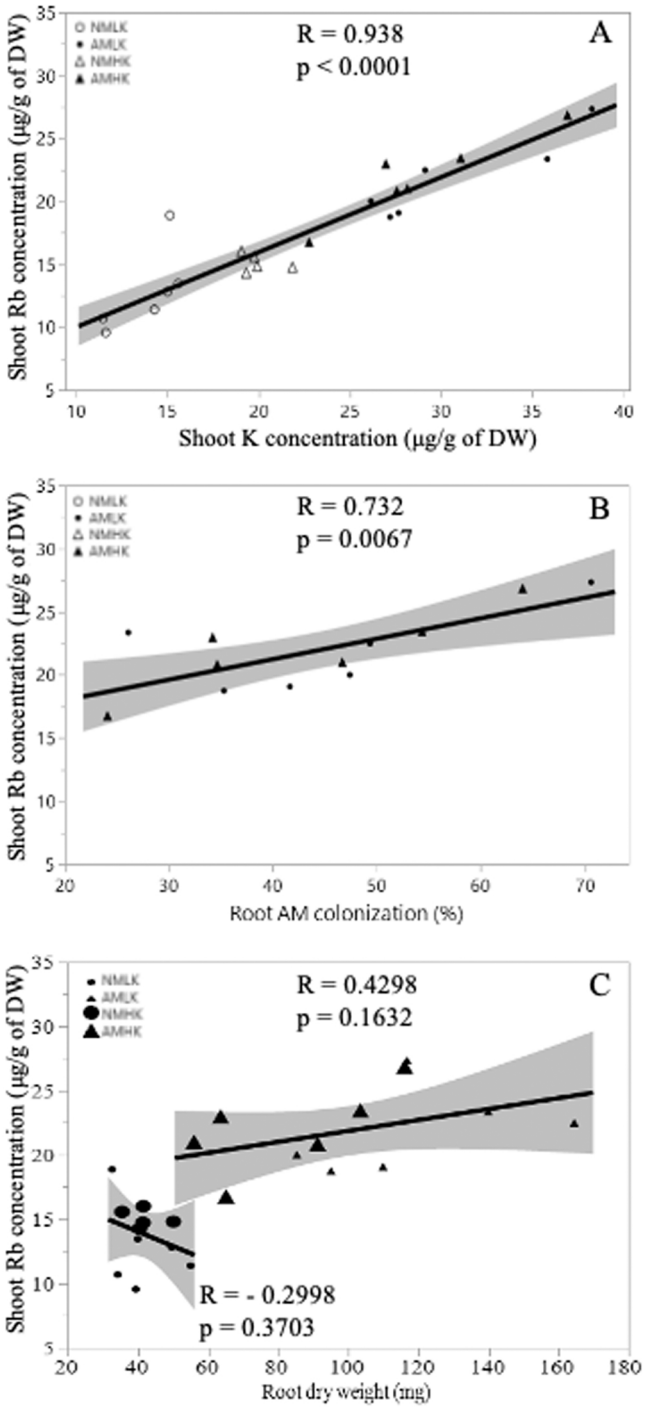

Fig. 4. Pearson correlations between shoot rubidium concentrations and shoot potassium concentrations, root biomass, or mycorrhizal colonization. Pearson correlations were calculated between shoot Rb^+ concentrations and K^+ concentrations (A), root dry weight (B), and root AM colonization (C) in *M. truncatula* seedlings colonized by *R. irregularis* isolate 09, or kept non-colonized.

acquire, translocate, and export K^+ to the host plant. Since it is hard to compare two independent experiments, a systematic comparative study between these two isolates, and other genera and species, should be performed to understand their respective ability to transport K^+.

Availability of K^+ in the soil generally plays a significant role in plant growth response and metabolism (White et al., 2021). Despite the difference in K^+ concentrations observed between LK and SK conditions of NM plants, the shoot and root growth responses of these plants remained

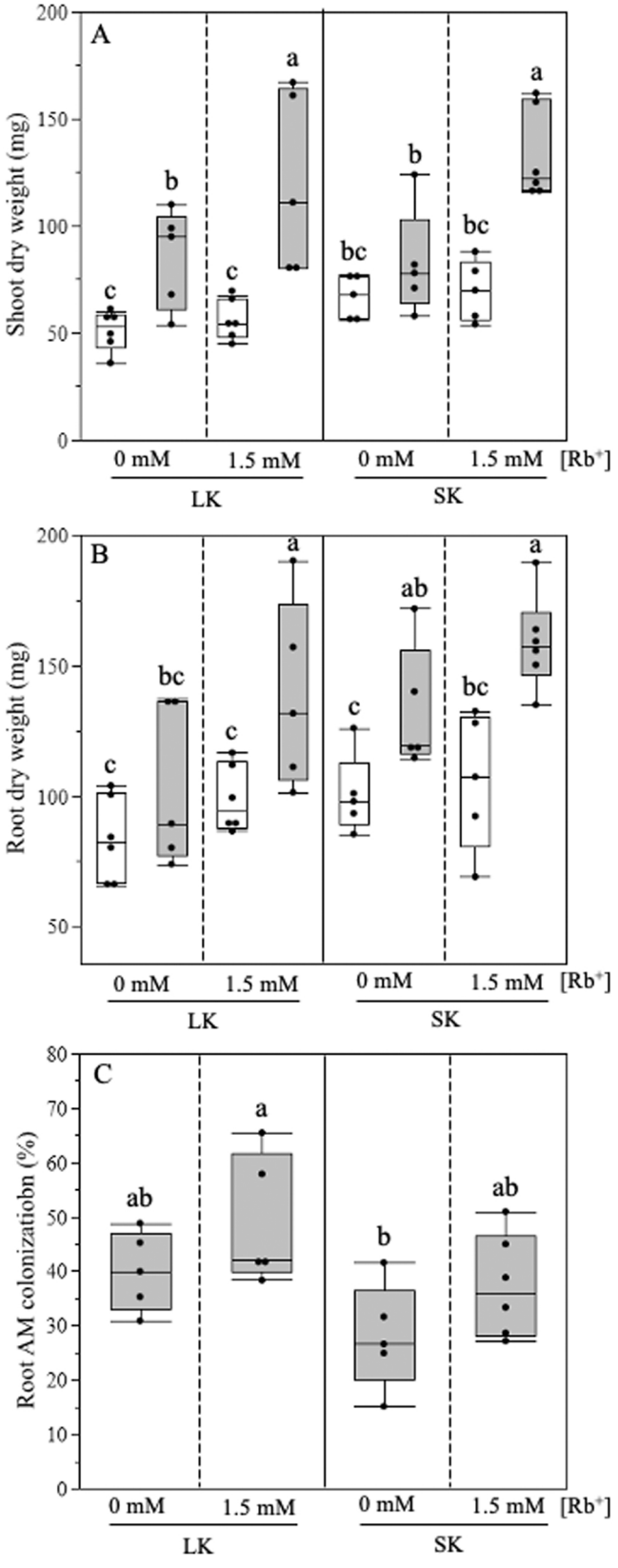

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Fig. 5. Biomass and root colonization of *Medicago truncatula* plants inoculated by *Rhizophagus irregularis* isolate 09 under potassium-limited and -sufficient conditions, and with 0 mM or 1.5 mM of rubidium nitrate added to the fungal compartment. Shoot (A) and root (B) dry weights were determined in seven-week-old *M. truncatula* plants inoculated (AM) or not (NM) by the AM fungus *R. irregularis* isolate 09 in limited (LK, 0.05 mM) or sufficient K^+ (SK, 3.75 mM) conditions. Gray and white box plots display plants inoculated or not by the fungus, respectively. (C) The rate of fungal colonization was determined on *M. truncatula* roots grown under SK or LK conditions after seven weeks of co-culture using the grid line intersection method. In FCs, 0 mM or 1.5 mM of $RbNO_3$ was added during each of the four watering sessions ([Rb^+]) ten days before harvest. Different letters indicate significant differences between treatments according to three-way ANOVA followed by LSD post hoc tests ($P < 0.05$). n = 5–6.

similar with no observable symptoms of K^+ deficiency recorded. This result suggests that this accession of *M. truncatula* (Jemalong A17) was rather tolerant to K^+ deprivation in our experimental conditions. We previously reported that this accession displayed limited response to low K^+ conditions compared to another accession (Garcia and Ané, 2017).

It has been reported that Na^+ accumulation in shoots leads to osmotic stress and further affects overall plant health (Hanin et al., 2016; Maathuis et al., 2014). In our study, higher accumulations of Na^+ was reported in NM plants relative to AM plants in both SK and LK conditions, suggesting that the fungus may have played a role in Na^+ ions allocation to the host plants. Some studies reported similar observations on compartmentalization of Na^+ and other toxic elements in AM hyphae (Garcia et al., 2017; Elhindi et al., 2018). Maintaining an optimal K^+:Na^+ ratio in plant cells is then crucial to tolerate K^+ deprivation and salt stress (Zhang et al., 2018; Assaha et al., 2017). Our study reveals that *M. truncatula* plants had a greater K^+:Na^+ ratio than NM plants, as already observed in other plant species such as in *Arundo donax*, *Zea mays*, or *Pinus taeda* (Estrada et al., 2013; Frank and Garcia, 2021; Pollastri et al., 2018). This result suggests that the AM fungus *R. irregularis* isolate 09 plays a significant role in the reduction of cytosolic Na^+ toxicity by maintaining an optimal K^+:Na^+ ratio.

4.2. Rb^+ can be used as a proxy for K^+ in AM symbiosis with some limitations

We recently assessed K^+ movements from four ectomycorrhizal fungi to the host plant *P. taeda* using Rb^+ as a proxy (Frank and Garcia, 2021). Yet, in AM symbiosis, there is a little evidence showing that Rb^+ could be transported in the soil-fungus-plant continuum. For example, when Rb^+ was injected in the soil more than 50 cm away from the roots of herbaceous species, its accumulation was observed in plant tissues, probably allocated by AM fungi (Hawkes and Casper, 2002). It has also been demonstrated that Rb^+ can be transferred between grasses and trees through AM fungal hyphae (Meding and Zasoski, 2008). Since it is believed that Rb^+ movement in plants takes the K^+ transport pathway (Vallejo et al., 2005; Läuchli and Epstein, 1970b), it would be possible to use Rb^+ for investigating the role of AM fungi in host plant K^+ nutrition. Although some plants and fungi may discriminate between these two elements (Marschner and Schimansky, 1971; Frank and Garcia, 2021), making the absolute quantification challenging, we compared the shoot Rb^+ status of non-colonized to AM plants.

In our experiments, when no Rb^+ was added to the FC, the shoot Rb^+ concentrations were similar between all plants but the biomass of AM plants was greater than the uninoculated ones. This result indicates that without supplemental Rb^+ provided to the FCs, AM plants were unable to acquire more Rb^+ from the substrate, validating a mycorrhiza-mediated Rb^+ transport. However, when Rb^+ was available to the fungus (1.5 mM or 3.25 mM, supplemented four times), significantly more Rb^+ was detected in the shoot of AM plants, under both SK and LK conditions. Even if it is hard to compare two independent experiments, it did not appear that more Rb^+ supplemented in the FC resulted in higher

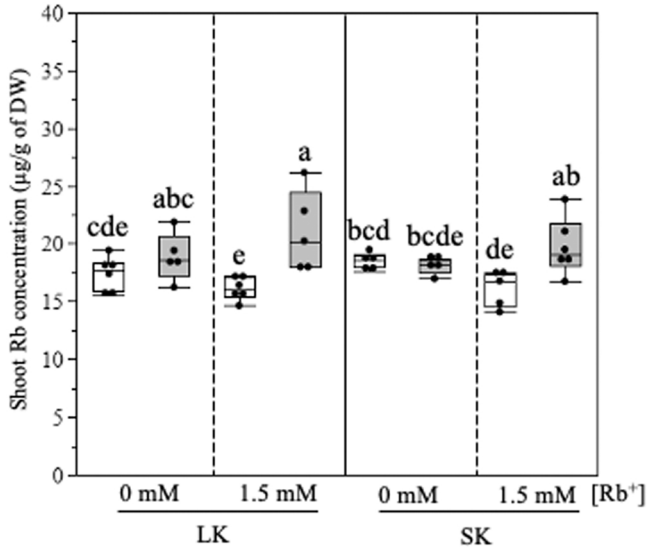

Fig. 6. Shoot rubidium concentrations in *Medicago truncatula* inoculated by *Rhizophagus irregularis* isolate 09 under potassium-limited and -sufficient conditions, and with 0 mM or 1.5 mM of rubidium nitrate added to the fungal compartment. Rubidium (Rb) concentrations were determined by ICP-MS in the shoots of seven-week-old *M. truncatula* plants inoculated (AM) or not (NM) by the AM fungus *R. irregularis* isolate 09 in limited (LK, 0.05 mM) or sufficient K^+ (SK, 3.75 mM) conditions. In FCs, 0 mM or 1.5 mM of $RbNO_3$ was added during each of the four watering sessions ([Rb^+]) ten days before harvest. Gray and white box plots display plants inoculated or not by the fungus, respectively. Different letters indicate significant differences between treatments according to three-way ANOVA followed by LSD post hoc tests ($P < 0.05$). n = 5–6.

Rb^+ accumulation in the corresponding plant shoots. These results align with a previous study showing that leaf Rb^+ levels of sugar beet plants remained similar even if a higher amount of Rb^+ was supplied in the growth medium (El-Sheikh et al., 1967). These authors also highlighted that Rb^+ can be toxic to the plant if used at too high concentration. It is also worth mentioning that the form of Rb^+ solution ($RbNO_3$) used in our experiments may not have been optimal. The anion type associated to Rb^+ cations can influence its absorption rate. Indeed, the Rb^+ uptake rate from barley roots was strongly reduced when Rb_2SO_4 was used, compared to RbCl (Emanuel et al., 1963). It is unknown whether the use of RbCl would provide a better Rb^+ transport than $RbNO_3$ upon symbiosis, but it deserves to be experimentally tested.

Thus, our experiments revealed that 1.5 mM of Rb^+ in the FC is sufficient to detect an accumulation of Rb^+ in the shoots of AM colonized plants. Finally, although AM plants were significantly bigger than NM plants, no correlation was detected between shoot Rb^+ concentration and plant biomass. The only positive correlations observed were between shoot Rb^+ concentration, shoot K^+ concentration, and colonization levels. Further reinforcing the idea that Rb^+ was transported by the fungus from the FC to the colonized plants, rather than the root ability to take up more Rb^+ as root mass increased. Since Rb^+ is speculated to be transported through K^+ transport proteins, its use in our system suggests that a symbiotic K^+ transport pathway exists in *M truncatula* even though the molecular players remain to be identified.

5. Conclusions

We investigated the possible use of Rb^+ as a proxy for K^+ in *M. truncatula - R. irregularis* isolate 09 symbiotic relationship. Altogether, our results indicate that (1) the accumulation of Rb^+ in the shoots of AM plants was related to the fungal colonization and K^+ movement only, but not to the increase of plant growth, (2) in our two-compartment growing system, Rb^+ can be used as a proxy for evaluating K^+ transport in AM symbiosis, and (3) K^+ availability was not a driver for Rb^+ transport from the FC to colonized roots. Altogether, our data indicate that the mycorrhizal pathway for K^+ transport exists in *M. truncatula* even if the molecular players remain to be identified.

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CRediT authorship contribution statement

AK and KG conceived and designed the research. AK and GS conducted the experiments. AK performed the statistical analysis. AK, DRC, and KG wrote the manuscript. All authors approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111364.

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