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Benefits provided by four ectomycorrhizal fungi to *Pinus taeda* under different external potassium availabilities

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Abstract

Ectomycorrhizal fungi contribute to the nutrition of many woody plants, including those in the Pinaceae family. Loblolly pine (*Pinus taeda* L.), a native species of the Southeastern USA, can be colonized by multiple species of ectomycorrhizal fungi. The role of these symbionts in *P. taeda* potassium (K^+) nutrition has not been previously investigated. Here, we assessed the contribution of four ectomycorrhizal fungi, *Hebeloma cylindrosporum*, *Paxillus ammoniavirescens*, *Laccaria bicolor*, and *Suillus cothurnatus*, in *P. taeda* K^+ acquisition under different external K^+ availabilities. Using a custom-made two-compartment system, *P. taeda* seedlings were inoculated with one of the four fungi, or kept non-colonized, and grown under K^+-limited or -sufficient conditions for 8 weeks. Only the fungi had access to separate compartments in which rubidium, an analog tracer for K^+, was supplied before harvest. Resulting effects of the fungi were recorded, including root colonization, biomass, and nutrient concentrations. We also analyzed the fungal performance in axenic conditions under varying supply of K^+ and sodium. Our study revealed that these four ectomycorrhizal fungi are differentially affected by external K^+ and sodium variations, that they are not able to provide similar benefits to the host *P. taeda* in our growing conditions, and that rubidium may be used with some limitations to estimate K^+ transport from ectomycorrhizal fungi to colonized plants.

Keywords Ectomycorrhizal symbiosis · *Pinus taeda* · Plant nutrition · Potassium · Rubidium · Sodium

Introduction

Ectomycorrhizal (ECM) fungi are an integral part of forest ecosystems, particularly in the northern hemisphere. Approximately 95% of short roots in boreal and temperate forests are colonized by ECM fungi (Martin et al. 2001). While the mycelium can comprise 32% of microbial biomass in the soil of a temperate forest, only 3–5% of land plants globally form these associations (Becquer et al. 2019). ECM fungi play an important role in nutrient cycling and soil composition, and provide a wealth of services necessary for the growth of woody plants, including nutritional benefits and tolerance to various environmental stresses (Smith and Read 2008). They provide essential hydromineral resources to the plant in exchange for carbohydrates produced by the host's photosynthesis (Garcia et al. 2016). When colonizing plant roots, ECM fungi form three distinct pseudo-tissues. These include extraradical hyphae scavenging the soil for water and nutrient acquisition, a mantle surrounding the roots and isolating them from the soil, and a network of hyphae around epidermal and cortical cells, the Hartig net, where nutrient exchanges take place. A wide variety of fungal and plant transport proteins are expressed at the soil-fungus and fungus-plant interfaces, allowing a bidirectional transfer of nutrients between the partners (Casieri et al. 2013; Guerrero-Galán et al. 2018b).

Although research on ECM symbiosis has thus far focused mainly on nitrogen and phosphorus (Nehls and Plassard 2018; Plassard et al. 2019), recent evidence showed that potassium (K^+) is also transported through ECM fungi towards woody host plants (Garcia et al. 2014; Guerrero-Galán et al. 2018a, b, c; Peng et al. 2020). In trees, K^+ is a vital nutrient that, if lacking in surrounding soils, can cause growth limitations (Fromm 2010). K^+ is an essential element for plant cell metabolic functions, such as plasma membrane polarization, stomatal aperture regulation, and turgor pressure (Wang and Wu 2013; Ragel et al. 2019; Wang et al. 2021). Cellular concentrations of K^+ in plants are typically 100 to 200 mM, whereas soil concentrations of plant

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available K^+ range from 0.1 to 1 mM depending on soil type (Asher and Ozanne 1967). Indeed, K^+ is often not sufficiently present in plant-available forms, which represents approximately 1–2% of total soil K^+ (Zörb et al. 2014). This very low availability combined with the constitutive K^+ demand of plants often leads to the development of K^+ depletion zones around the roots. In addition, under K^+ deficiency, other salts such as sodium (Na^+) can substitute K^+ for some of these metabolic functions. However, this might represent a problem for a host plant due to the toxicity of high Na^+ concentrations (Benito et al. 2014).

Loblolly pine (*Pinus taeda* L.) is an ecologically and economically important tree species of the Southeastern USA that has been a major source of timber for more than a century (Ashe 1915; Li et al. 1999; Schultz 1999; McKeand et al. 2006). It accounts for nearly 45% of the timber supply in the USA and is known for its short growing time resulting in a higher planting turnover (Li et al. 1999). Fertilizer demands to optimize inputs and maximize yields have been extensively investigated in *P. taeda* plantations (Albaugh et al. 2009; Jokela et al. 2010; Carlson et al. 2014). In particular, it has been described that K^+ fertilizers affect growth and foliar responses in *P. taeda* (Bengtson 1976; Shoulders and Tiarks 1990). However, the role of ECM fungi on K^+ acquisition and tolerance to varying external K^+ availabilities in loblolly pine remains unexplored (Plassard and Dell 2010). Tracking the transport of K^+ in ECM associations is challenging because most isotopes are not stable enough or are too costly to reasonably work with. An easier and safer method is to use stable rubidium (Rb^+) as an analog tracer for K^+. Indeed, Rb^+ is transported through K^+ transport proteins—no Rb^+-specific transporter has been identified in any organism so far—and multiple studies have utilized Rb^+ to track K^+ movements in plants (Polley and Hopkins 1979; Rygiewicz and Bledsoe 1984; Tyler 1997; Hawkes and Casper 2002). However, Rb^+ should be used cautiously to evaluate K^+ fluxes due to possible K^+/Rb^+ discriminations by some plant species (Marschner and Schimansky 1971).

In this study, we investigated the impact of four species of ECM fungi, *Hebeloma cylindrosporum*, *Paxillus ammoniavirescens*, *Laccaria bicolor*, and *Suillus cothurnatus*, on the K^+ acquisition of *P. taeda* growing under K^+-sufficient and -limited conditions. We designed custom two-compartment systems that allowed the fungi to access a plant-free compartment in which stable Rb^+ was added. Fungal colonization, biomass, and Rb^+, K^+, Na^+, and calcium (Ca^{2+}) concentrations were determined in ECM and non-colonized plants. Additionally, we analyzed the growth performance of these four fungal symbionts using liquid media containing contrasted amounts of K^+ and Na^+. We demonstrated that these symbionts are differentially affected by variations in K^+ and Na^+ availability, that they are not able to provide similar benefits to the host *P. taeda* in our growing conditions, and that Rb^+ may be used to estimate K^+ transport from ECM fungi to colonized plants.

Materials and methods

Plant and fungal materials

P. taeda seeds (Sheffield's Seed Co. Inc, collected in Arkansas, USA) were surface sterilized with 30% H_2O_2 for 15 min, rinsed five times, 10 min each, with autoclaved milli-Q water, and kept in the final rinse at 4 °C for 72 h. Sterilized seeds were placed in Petri dishes containing agar (15 g l^{-1}) and glucose (2 g l^{-1}), and germinated in a growth chamber (16 h day, 8 h night, 23 °C, relative moisture 60%, luminosity 210 µmol m^{-2} s^{-1}) for 3 weeks.

Four fungal species of ECM Basidiomycota fungi were used; all growing at 26 °C. *H. cylindrosporum* Romagnesi h7 (Debaud and Gay 1987), *L. bicolor* strain S238N (Orton 1960), and *S. cothurnatus* VC 1858 (Singer 1945) were maintained on solid YMG medium (Rao and Niederpruem 1969). *P. ammoniavirescens* Pou09.2 (Dessi and Contu 1999) was maintained on solid modified Melin-Norkans (MMN) medium.

Design of two-compartment systems

Seedlings were transplanted into custom-made two-compartment systems that were prepared using plastic boxes (12 cm × 8 cm × 8 cm; L × H × W; from Carmo A/S) (Fig. 1a). In each box, a Magenta GA-7 Plant Culture Box (Bioworld) was placed to form a compartment for the development of fungal hyphae (FC). The remaining of the plastic box formed the root compartment (RC). A total of 99 holes (each having a diameter of 2 mm) were drilled on the side of the Magenta box facing the RC. A 52-µm nylon mesh was glued over the perforation to the outside of the box. Another 52-µm nylon mesh was held against the perforation on the inside of the box, forming air gaps (Fig. 1b). Due to their size, the mesh layers allowed fungal hyphae to reach the FC but restricted the roots from doing so. The placement of holes in the plastic compartment divider and the water level in the pots were both carefully managed to ensure that water was not moving between the compartments and transferring nutrient solutes. FCs were watered from the top at the opposite side of the meshes. Six holes (5 mm each) were drilled in the bottom of only the RCs for watering.

Ectomycorrhiza production and rubidium supply

RC and FC compartments were filled with 300 ml of Turface®, pre-washed with deionized water, and one 3-week-old *P. taeda* seedling was placed in each RC. One-month-old

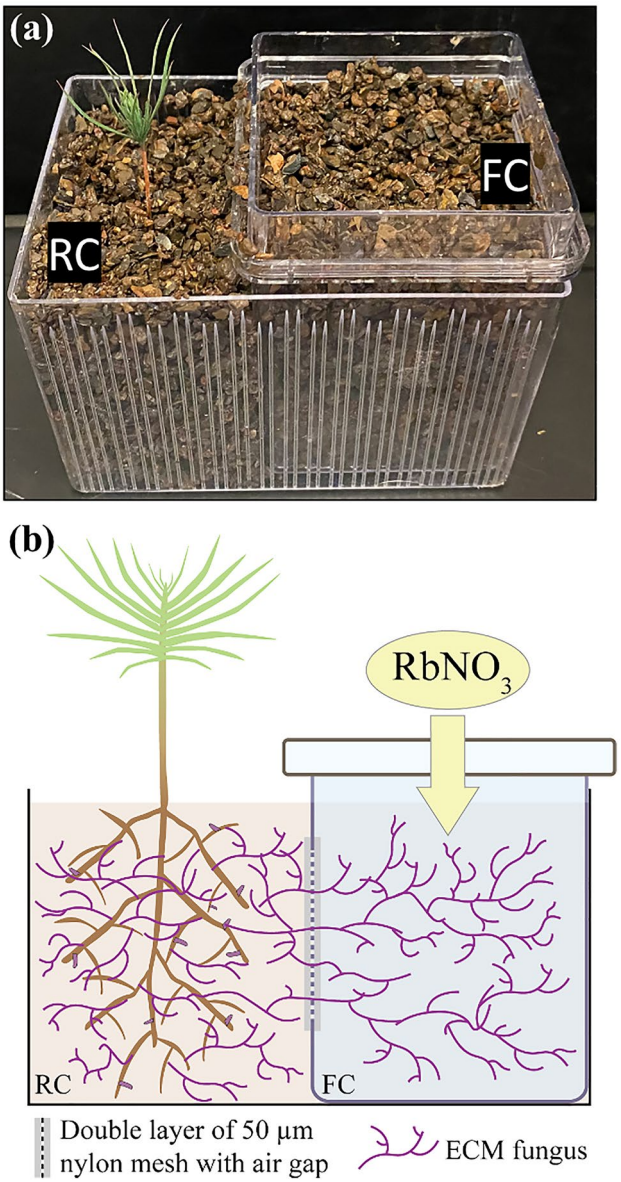


Fig. 1 Design of two-compartment systems to study ectomycorrhizal interactions in *Pinus taeda*. (**a**) Photo of the custom two-compartment systems used in this manuscript. Plants were transplanted and inoculated or not in one side of the system that is referred to as the root compartment (RC). A Magenta GA-7 Plant Culture Box (Bioworld) with 99 holes on one side was placed in the larger box and constitutes the fungal compartment (FC). (**b**) Two meshes were placed on each side of the Magenta box, forming an air gap that prevented the roots, but not the fungus, from reaching the FC. Both compartments were filled with Turface® and watered with the corresponding solutions. In the last 10 days of experiments, $RbNO_3$ was provided to the FC only

fungal cultures growing in jars containing 50 ml of liquid YMG or MMN media, depending on the fungus, were rinsed in 40 ml of limited K^+ (LK, 0.05 mM K^+) or sufficient K^+ (SK, 1 mM K^+) liquid N1 media that were described in Garcia et al. (2014). Rinsed thalli were then ground with a homogenizer (Fisher) in 40 ml of LK or SK solutions and poured in the RCs containing the pine seedlings. For non-mycorrhizal plants, seedlings were placed in the RC and 40 ml of LK or SK media were poured on top. Two-compartment systems were placed in trays by treatment groups, and 30 ml per pot of nutrient solution (LK or SK) was provided to the base of each tray. Trays were transferred to the growth chamber with clear plastic covers (16 h day, 8 h night, 23 °C, relative moisture 60%, luminosity 210 µmol m^{-2} s^{-1}). After 1 week, the covers were removed and 30 ml of a 1/10 dilution of LK or SK media was given to the FCs only. Plants were watered regularly from the bottom with 180 ml of the corresponding (LK or SK) nutrient solution given to each tray. To prevent the over-accumulation of nutrients in the FCs, they were given 30 ml 1/10 dilution of the corresponding medium once a week only for the duration of the experiment, with plain milli-Q water supplied from the top between these days if needed. Trays were rotated every 10 days to eliminate possible biases within the growth chamber. Starting 11 days before harvest, 20 ml of a Rb^+/K^+ solution (0.6 mM, $RbNO_3$; 0.05 mM, KNO_3) was given to each FC every other day, for a total of 100 ml per FC (Fig. 1b). Two independent experiments with different fungal strains (1: non-mycorrhizal/*H. cylindrosporum*/*P. ammoniavirescens*; 2: non-mycorrhizal/*L. bicolor*/*S. cothurnatus*) were set up due to space limitation in the growth chamber. Six 2-month-old plants per condition were collected in each experiment.

To validate that the systems really prevent the transfer of nutrient from one compartment to another and to ensure that adding K^+ solution increase the amount of K^+ available in the Turface®, we performed a control check experiment. There were three different treatment groups. The first group was given 30 ml of milli-Q water to the RC and the FC. The second group was given 30 ml of the SK solution to the RC and 30 ml of milli-Q water to the FC. The third group was given 30 ml of the LK solution to the RC and 30 ml of milli-Q water to the FC. Each treatment group had three replications. The boxes were placed in a growth chamber overnight, and the next day the Turface® was harvested for analysis. Three grams of each sample were submitted to the Environmental and Agricultural Testing Services (EATS) at North Carolina State University for K^+ concentration determination by ICP-OES.

Dry weight determination, ion content measurement, and mycorrhizal quantification

Eight weeks after inoculation, seedlings were harvested. The roots were dipped in milli-Q water and shaken to remove as much Turface® as possible. Roots and shoots were separated, and fresh weights were recorded. The shoots were dried at 70 °C for 1 week, and the roots were examined for colonization. For inoculated seedlings, all non-colonized

short roots and ECM root tips were counted with a binocular scope, and the percentage of ectomycorrhiza per plant was calculated. For non-mycorrhizal plants, a visual inspection confirmed the absence of mycorrhizal colonization. Roots were then dried at 70 °C for 1 week. Shoot and root dry weights were recorded, and shoots were ground and submitted to the EATS at North Carolina State University for K^+, Rb^+, Na^+, and Ca^{2+} concentration determination by ICP-OES and ICP-MS.

Various potassium and sodium regimes in fungal pure cultures

To assess the growth of *H. cylindrosporum*, *P. ammoniavirescens*, *L. bicolor*, and *S. cothurnatus* in axenic condition under various high and low K^+ and Na^+ regimes, four media were prepared as described in Garcia et al. (2020). Two of them were the exact same ones used for the ECM assays: the LK and SK solutions that contained 0.2 mM of NaCl. The other two were LK and SK media supplemented with 1 mM of NaCl instead (LK + Na^+ and SK + Na^+, respectively). After 4 weeks of culture at 26 °C on an agar-supplemented (10 g/l) version of these media, the four ECM fungal species were subcultured into 50 ml of their corresponding liquid media and incubated at 26 °C. Thalli were collected 28 days later, and fresh weights were determined.

Statistical analyses

Data are presented as the mean of five to six replicates. Differences between averages were analyzed by two-way ANOVA followed by LSD post hoc tests, or by Student's *t*-test, depending on the experiment. Box plots were performed with R software (ggplot2 package). Pearson correlations were also performed with R software (ggpubr package) at the 5% level of statistical significance.

Results

Low potassium availability does not impact the root colonization of *P. taeda* seedlings by ectomycorrhizal fungi

To evaluate the ability of each fungal species to colonize pine roots, and assess the impact of external K^+ availability on fungal colonization, pine seedlings were harvested after 8 weeks of co-culture. We counted all of the short roots on each plant, as well as the number of short roots that had formed ectomycorrhizae, by looking for the presence of fungal hyphae and the absence of root hairs (Fig. S1). Inspection of root systems showed successful colonization of the seedlings by all four fungi (Fig. 2). *H. cylindrosporum*, *P. ammoniavirescens*, and *L. bicolor* all colonized over 90% of the total short roots of their host plants, while *S. cothurnatus* had significantly lower rates of colonization, around 50%. In addition, no difference was observed between plants growing at LK and those at SK (Fig. 2). All control plants not inoculated with any fungi appeared not to be colonized upon inspection, confirming them as non-mycorrhizal controls.

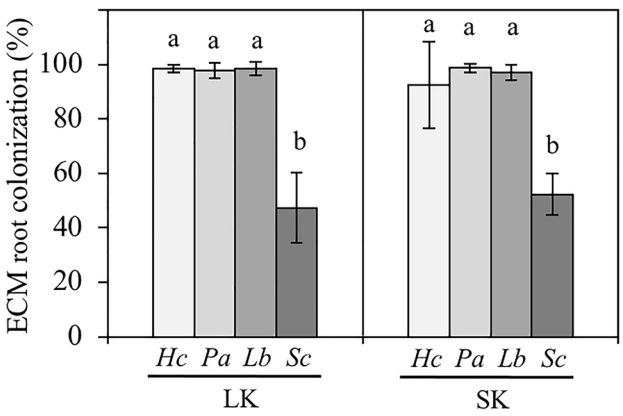


Fig. 2 Ectomycorrhizal colonization rates of *Pinus taeda* seedlings grown under potassium-limited and -sufficient conditions. The colonization rate for each treatment group represents the percentage of short roots exhibiting ECM morphology relative to the total number of short roots in 8-week-old plants colonized by *H. cylindrosporum* (*Hc*), *P. ammoniavirescens* (*Pa*), *L. bicolor* (*Lb*), or *S. cothurnatus* (*Sc*), and growing in limited (LK) and sufficient (SK) K^+ conditions. Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). $n = 5–6$

The impact of fungal colonization on *P. taeda* seedling biomass depends on external potassium availability

Control check experiments validated our two-compartment systems since no transfer of K^+ from one compartment to another was detected without the presence of any plant or fungi (Fig. S2). Additionally, around 80 ppb of Rb^+ was detected in the Turface®, which is far less that what was added in the FCs and detected in plant tissues (see below). We examined the impact of the four fungal symbionts on the growth of *P. taeda* seedlings under LK and SK conditions by recording fresh and dry biomass after 8 weeks of co-culture. Shoots and roots were harvested, weighed separately, dried, and weighed again. Significantly higher shoot dry biomass were observed in seedlings colonized by *P. ammoniavirescens* in LK condition only, but not in SK condition, in comparison to the control plants (Figs. 3a and S3a). Plants colonized by *H. cylindrosporum* displayed similar shoot biomass in both LK and SK compared to control plants (Figs. 3a and S3a). The shoots from plants inoculated with *L. bicolor* grown in both LK and SK conditions, as well

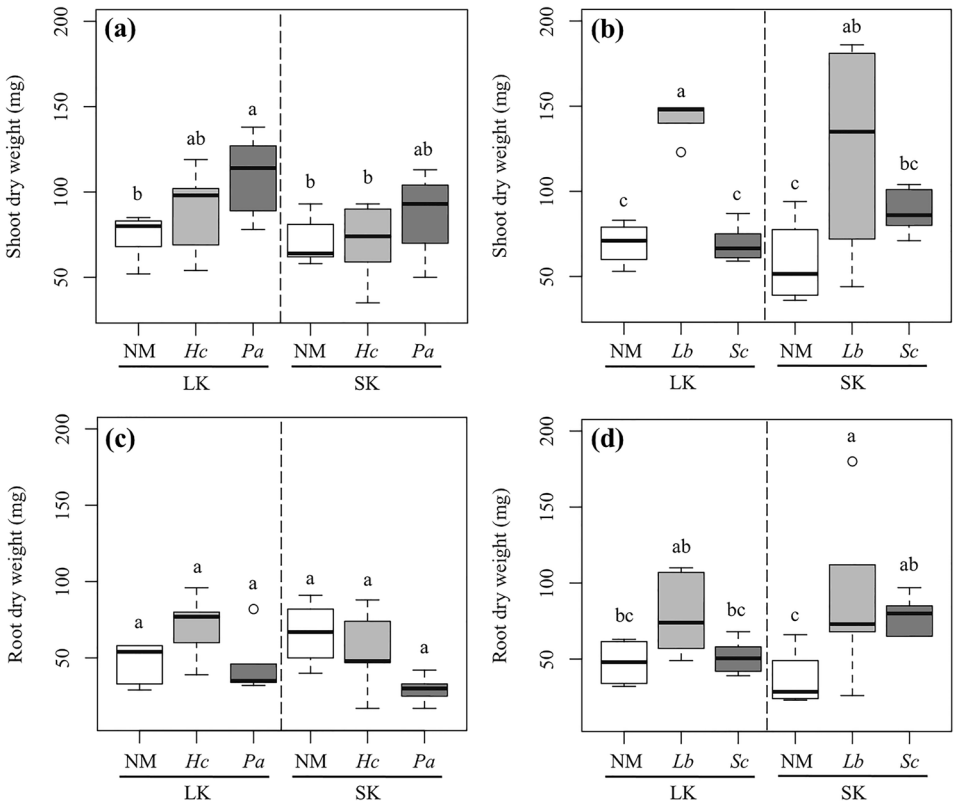


Fig. 3 Dry weight of ectomycorrhizal and non-colonized *Pinus taeda* seedlings grown under potassium-limited and -sufficient conditions. Shoot (**a**, **b**) and root (**c**, **d**) dry weights were determined in seedlings colonized by *H. cylindrosporum* (*Hc*) (**a**, **c**), *P. ammoniavirescens* (*Pa*) (**a**, **c**), *L. bicolor* (*Lb*) (**b**, **d**), or *S. cothurnatus* (*Sc*) (**b**, **d**), or kept non-colonized (NM), and growing in limited (LK) and sufficient (SK) K^+ conditions. Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). $n = 5–6$

as the plants grown in SK with *S. cothurnatus*, displayed higher biomass than the non-colonized plants (Figs. 3b and S3b). Concerning the roots, no difference in fresh and dry weights was observed for plants colonized by *P. ammoniavirescens*, whatever the external K^+ availability (Figs. 3c and S3c). Seedlings inoculated with *H. cylindrosporum* displayed higher root fresh, but not dry, biomass than the control plants in both LK and SK conditions (Figs. 3c and S3c). Plants colonized by *L. bicolor* and *S. cothurnatus* displayed higher root fresh and dry biomass in SK condition only in comparison to the control plants (Fig. S3d). Between LK and SK conditions, significant differences were spotted only for *H. cylindrosporum*, *L. bicolor*, and *S. cothurnatus* (fresh weights only), indicating an impact on external K^+ availability on growth benefits provided by some ECM fungi (Fig. S3d).

Altogether, these results indicate that in our experimental conditions, the external K^+ availability influenced the impact of each ECM fungus on the host biomass.

Analysis of shoot nutrient concentrations in ectomycorrhizal *P. taeda* seedlings under standard and low potassium conditions

After collecting dry biomass data, shoots of colonized and control plants were ground and the concentrations of Rb^+, K^+, Na^+, and Ca^{2+} were obtained by ICP-OES and ICP-MS. Root nutrient concentrations were not recorded due to the impossibility to discriminate between plant and fungal tissues. Our analyses showed that seedlings grown in co-culture with *H. cylindrosporum* or *P. ammoniavirescens* displayed significantly greater shoot Rb^+ concentrations compared to the non-mycorrhizal control plants, in SK condition only (Fig. 4a). Plants colonized by *L. bicolor* or *S. cothurnatus* did not yield any significant differences in Rb^+ levels, whatever the external K^+ level (Fig. 4b). Seedlings colonized by *H. cylindrosporum* or *P. ammoniavirescens* grown in either LK or SK conditions had significantly higher shoot concentrations of K^+ than control plants (Fig. 4c). Plants inoculated with *L. bicolor* had greater K^+ concentration than non-mycorrhizal plants in SK only (Fig. 4d). Once again, there was no observed difference with non-colonized plants in K^+ concentrations in the seedling shoots colonized by *S. cothurnatus* (Fig. 4d). This similar pattern of shoot K^+ and Rb^+ concentrations in *P. taeda* seedlings was confirmed by the strong positive correlation result (Fig. 5). Alternatively, K^+ and Rb^+ concentrations were not correlated to the plant shoot and root biomass (Fig. S4). Plants inoculated with *P. ammoniavirescens* or *L. bicolor* displayed lower Na^+ concentration than non-colonized plants, but in LK condition only (Fig. 4e, f). In contrast, there was no significant difference in shoot Na^+ concentration in plants inoculated

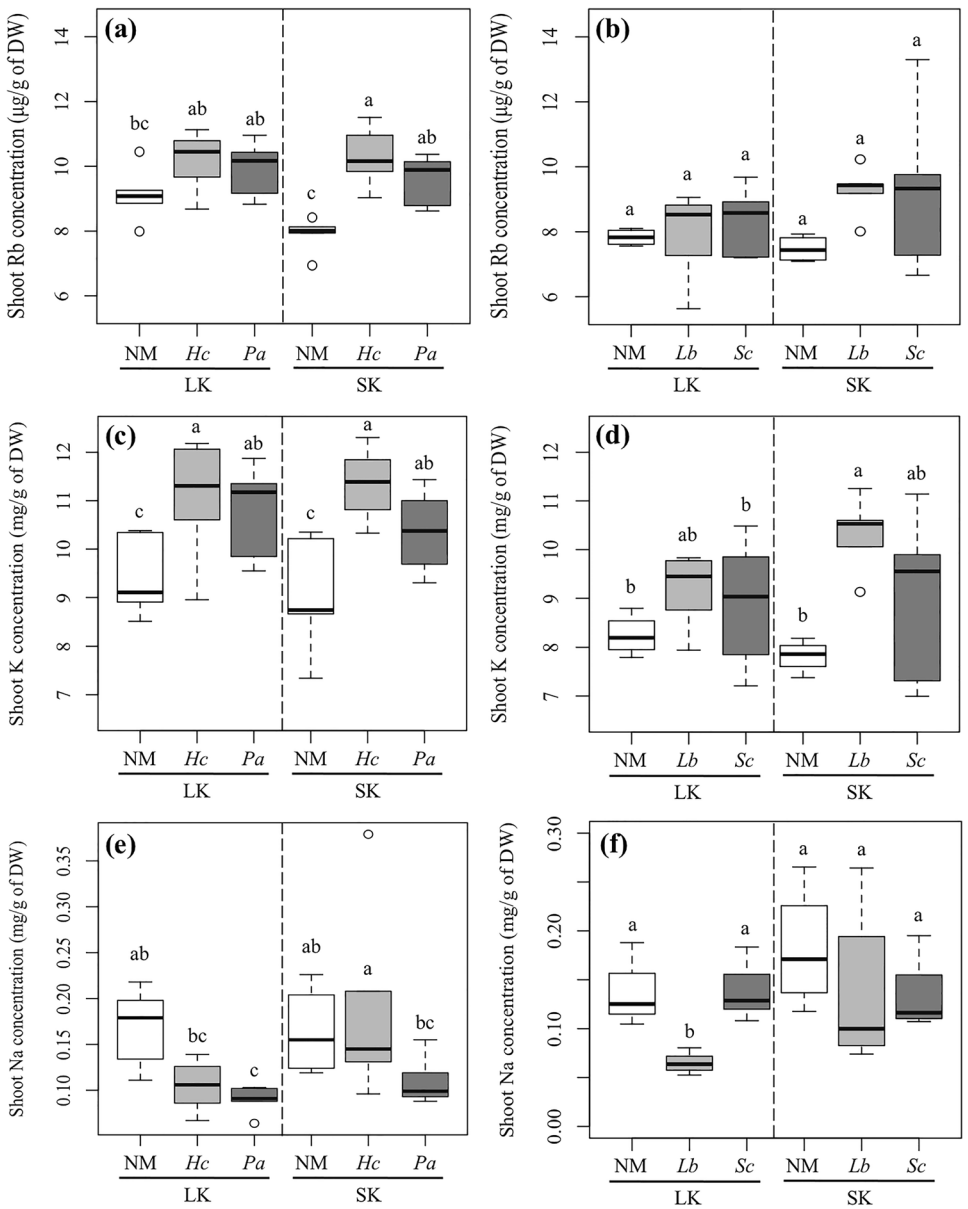


Fig. 4 Shoot rubidium, potassium, and sodium concentrations in ectomycorrhizal and non-colonized *Pinus taeda* seedlings grown under potassium-limited and -sufficient conditions. Rubidium (Rb; **a**, **b**), potassium (K; **c**, **d**), and sodium (Na; **e**, **f**) concentrations were measured by ICP-OES and ICP-MS in the shoots of eight-week-old *P. taeda* seedlings that were colonized by the ectomycorrhizal fungi *H. cylindrosporum* (*Hc*) (**a**, **c**, **e**), *P. ammoniavirescens* (*Pa*) (**a**, **c**, **e**), *L. bicolor* (*Lb*) (**b**, **d**, **f**), or *S. cothurnatus* (*Sc*) (**b**, **d**, **f**), or kept non-colonized (NM), and growing in limited (LK) and sufficient (SK) K^+ conditions. Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). $n = 5–6$

with *H. cylindrosporum* or *S. cothurnatus* (Fig. 4e, f). Comparing mycorrhizal plants under LK and SK conditions, we also observed that those colonized by *H. cylindrosporum* and *L. bicolor* displayed significantly higher concentrations of Na^+ in SK compared to LK (Fig. 4e, f). This indicates that changes in external K^+ availability could affect the ability of some ECM fungi to prevent Na^+ accumulation in host tissues.

Because K^+ and Na^+ are closely connected ions in plant processes, one being replaced by the other, particularly under K^+ deficiency (Benito et al. 2014), we calculated the shoot K^+:Na^+ ratio for each seedling (Fig. 6). A significantly greater K^+:Na^+ ratio in plants colonized by *H. cylindrosporum*, *P. ammoniavirescens*, or *L. bicolor* was observed in LK condition only, compared to control plants (Fig. 6a, b). No significant difference was observed in the K^+:Na^+ ratio of seedlings colonized by *S. cothurnatus*, compared to non-mycorrhizal plants (Fig. 6b).

Significantly lower Ca^{2+} concentrations were observed in plants inoculated with *H. cylindrosporum* or *P. ammoniavirescens* in K^+-limited condition only (Fig. S5a). Plants colonized by *L. bicolor* or *S. cothurnatus* did not display any differences in Ca^{2+} concentration in either LK or SK conditions, compared to control plants (Fig. S5b). Additionally, pine seedlings colonized by *H. cylindrosporum* displayed significantly higher Ca^{2+} concentration in SK compared to the LK condition (Fig. S5a).

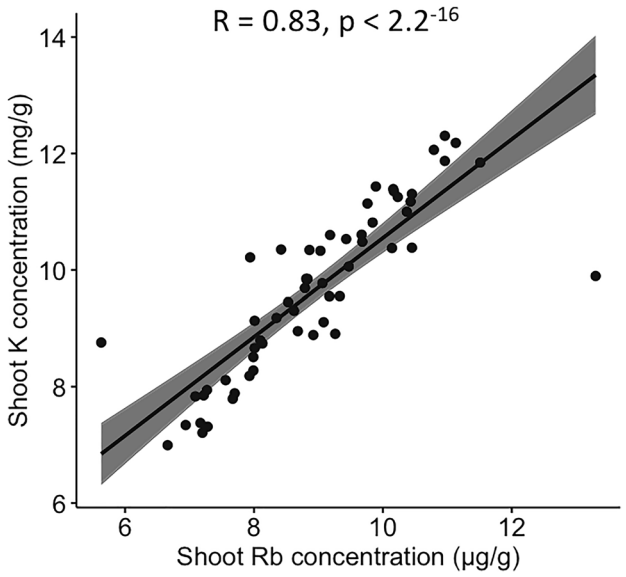


Fig. 5 Pearson correlations between shoot potassium and shoot rubidium concentrations. Pearson correlations in *P. taeda* seedlings colonized by *H. cylindrosporum*, *P. ammoniavirescens*, *L. bicolor*, *S. cothunartus*, or kept non-colonized. Strong positive correlation was observed

H. cylindrosporum, P. ammoniavirescens, L. bicolor, and *S. cothurnatus* are differentially affected by high and low potassium and sodium regimes

To assess the ability of *H. cylindrosporum*, *P. ammoniavirescens*, *L. bicolor*, and *S. cothurnatus* to grow in K^+-limited and -sufficient conditions, we placed the fungi in liquid LK and SK solutions, respectively, and recorded the fresh weights of 28-day-old thalli (Fig. 7). In addition, plants colonized by these fungi showed contrasted accumulation of Na^+ in their tissue (Fig. 4e, f). Consequently, we grew the fungi in LK and SK liquid media used for ECM assays that contained 0.2 mM of Na^+, and in LK + Na^+ and SK + Na^+ media containing 1 mM of Na^+ (Fig. 7). Our analysis showed that no significant difference can be spotted for the growth of *H. cylindrosporum* and *P. ammoniavirescens* between LK and SK solutions. However, biomass of *P. ammoniavirescens* thalli was significantly higher in both LK and SK solutions than in any other fungi growing in any media (Fig. 7). The only significant difference observed for *H. cylindrosporum* was between fungi growing in LK and SK solutions supplemented with Na^+: thalli were bigger in SK + Na^+ than in LK + Na^+ solution, showing that the tolerance to high Na^+ concentrations for this fungus depends on the external K^+ availability (Fig. 7). Concerning *P. ammoniavirescens*, a significantly greater biomass was recorded for the thalli growing in LK + Na^+ than in LK and SK + Na^+ solutions, indicating that the fungus can better tolerate the external Na^+ accumulation under K^+ deficiency than the other fungi

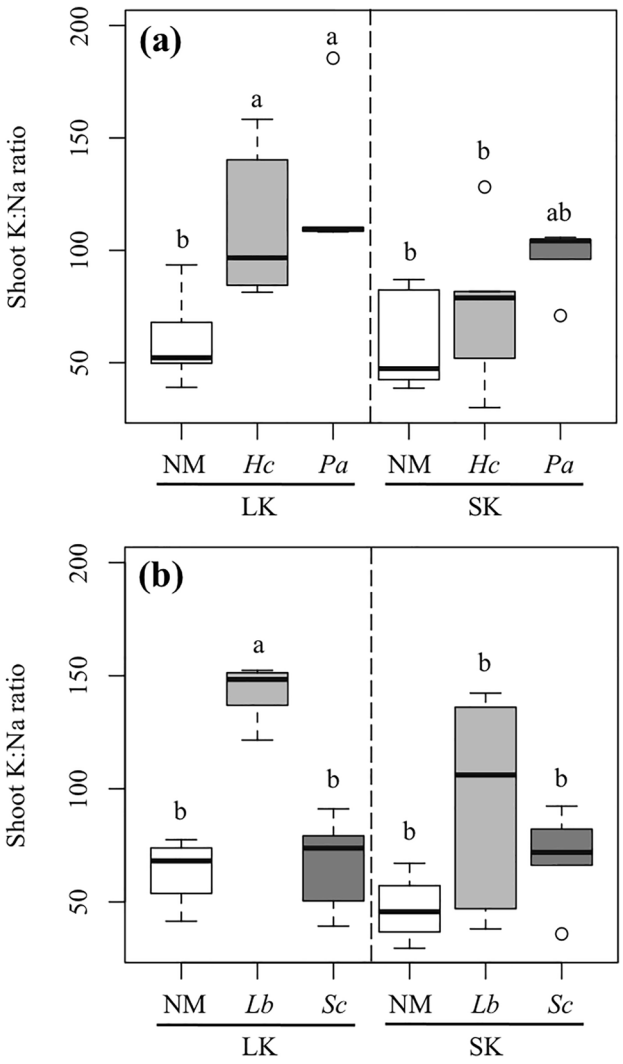


Fig. 6 Shoot K^+:Na^+ ratios in ectomycorrhizal and non-colonized *Pinus taeda* seedlings grown under potassium-limited and -sufficient conditions. Ratios between shoot K^+ and Na^+ concentrations were calculated for each *P. taeda* seedlings that were colonized by the ectomycorrhizal fungi *H. cylindrosporum* (*Hc*) (**a**), *P. ammoniavirescens* (*Pa*) (**a**), *L. bicolor* (*Lb*) (**b**), or *S. cothurnatus* (*Sc*) (**b**), or kept non-colonized (NM), and growing in limited (LK) and sufficient (SK) K^+ conditions. 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. 7). Interestingly, very limited growth was observed for *L. bicolor*, and no growth at all for *S. cothurnatus*, in any condition.

Discussion

The ability of ECM fungi to improve the K^+ nutrition of their host plants, and the direct transport of K^+ ions from the soil to colonized roots is still largely debated (Garcia and Zimmermann 2014; Becquer et al. 2019). Indeed,

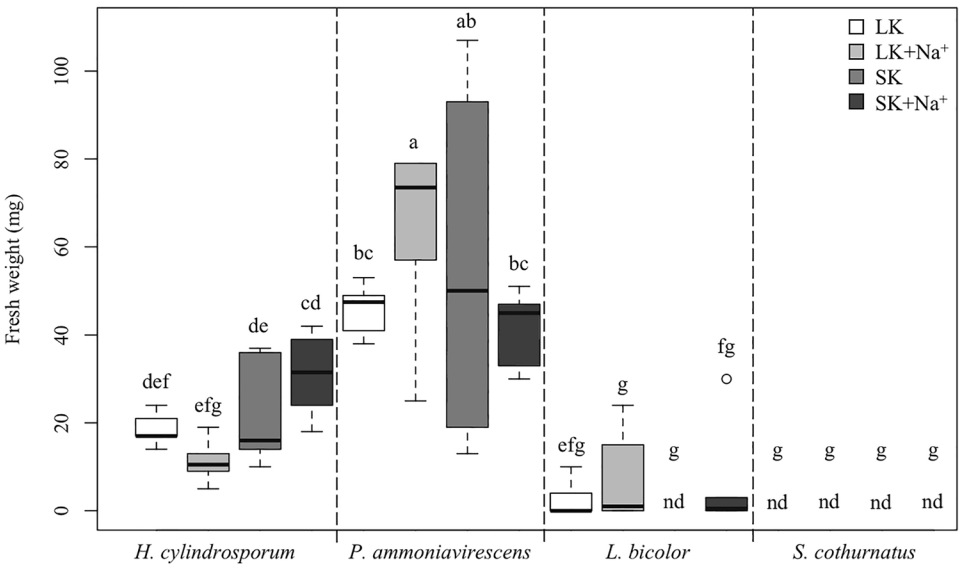


Fig. 7 Biomass of *Hebeloma cylindrosporum*, *Paxillus ammoniavirescens*, *Laccaria bicolor*, and *Suillus cothurnatus* under high and low potassium and sodium regimes. Fresh weights of the ECM fungi *H. cylindrosporum*, *P. ammoniavirescens*, *L. bicolor*, and *S. cothurnatus* were determined after 28 days of culture in limited (LK) and sufficient (SK) K^+ conditions supplemented (1 mM) or not (0.2 mM) of sodium (Na^+). Different letters indicate significant differences between treatments according to two-way ANOVA followed by LSD post hoc tests ($P < 0.05$). $n = 5–6$

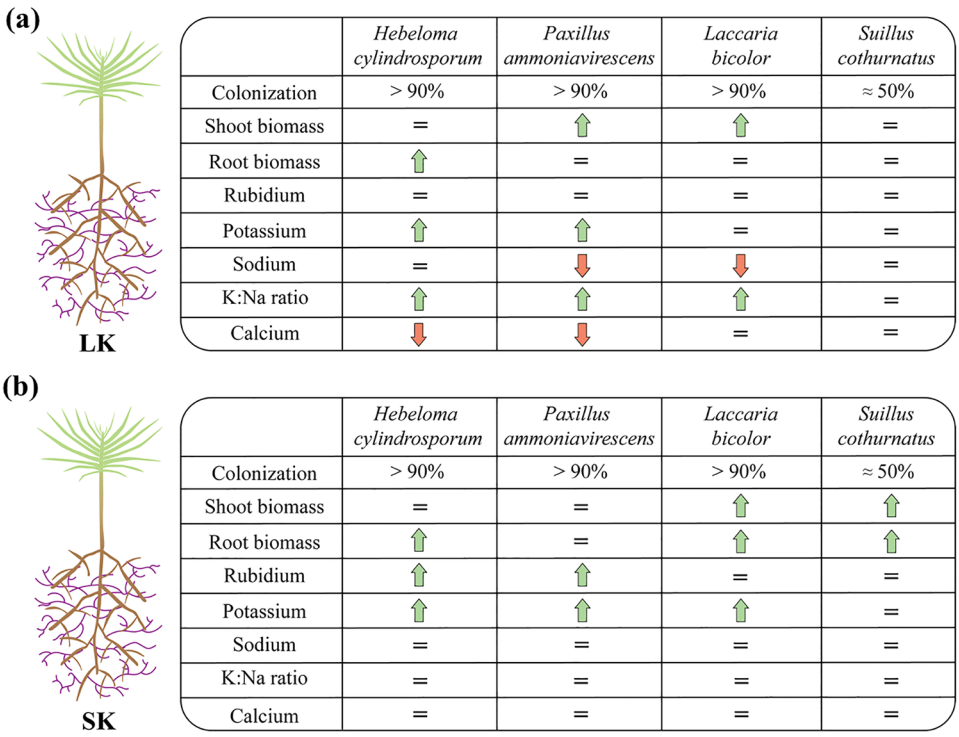
contradictory results revealed that the ectomycorrhiza-mediated movements of K^+ are context dependent. For example, oak and pine trees colonized by the ECM fungus *Tuber melanosporum* displayed similar, or even reduced, K^+ concentrations compared to non-mycorrhizal plants (Domínguez Núñez et al. 2006, 2007). On the other hand, recent studies reported that various ECM fungi can significantly improve soil K^+ availability and/or its transfer towards their host plant (Garcia and Zimmermann 2014; Guerrero-Galán et al. 2018a, b, c; Sun et al. 2019; Peng et al. 2020). Here, we investigated the role of four ECM fungi on K^+ acquisition of *P. taeda* seedlings under K^+-limited and -sufficient conditions.

P. taeda seedlings colonized by *H. cylindrosporum*, *P. ammoniavirescens*, and *L. bicolor* tolerate better low potassium conditions than non-mycorrhizal plants

We decided to focus on nutrient concentrations in shoots only due to the impossibility of discriminating between plant and fungal tissues in the roots, and because it reflects the translocation of nutrients from belowground to aerial parts. Pine seedlings colonized by two of the investigated fungi, *H. cylindrosporum* and *P. ammoniavirescens*, showed an increase of shoot K^+ concentrations, whatever the external K^+ availability, while plants interacting with *L. bicolor* displayed greater K^+ concentrations only in SK (Fig. 8). Since K^+ concentrations in non-colonized plants were not affected by the external K^+ availability, it reveals that *P. taeda* can efficiently tolerate K^+ deficiency, at least for a short period of time, but is also dependent on some ECM fungi to take up K^+. Concerning *H. cylindrosporum*, it contrasts with the results observed on the maritime pine (*Pinus pinaster*). Indeed, *P. pinaster* seedlings colonized by *H. cylindrosporum* displayed greater K^+ contents only under K^+ deficiency (Garcia et al. 2014), indicating that the benefits provided by this ECM fungus depend on the host plant and growing conditions.

In addition, plants colonized by *H. cylindrosporum*, *P. ammoniavirescens*, and *L. bicolor* showed a higher K^+:Na^+ ratio than non-colonized seedlings (Fig. 8a). In plants, maintaining a balanced cytosolic K^+:Na^+ ratio is crucial to tolerate salt stress and fluctuations in K^+ availability (Assaha et al. 2017; Zhang et al. 2018). Therefore, our results indicate that these three fungi are able to prevent the accumulation of Na^+ into loblolly pine tissues upon colonization, as already seen in plants colonized by arbuscular mycorrhizal fungi (Estrada et al. 2013; Garcia et al. 2017; Pollastri et al. 2018). Interestingly, *P. ammoniavirescens* is the only fungus, out of the four investigated, that was not affected by the addition of Na^+ in pure culture, and that even showed a biomass increase when Na^+ was supplemented in the K^+-deficient medium (Fig. 7). Although additional experiments are still needed, particularly on the expression of fungal transport genes and possible Na^+ exclusion mechanisms, *P. ammoniavirescens* could become a promising model symbiont to elucidate the tolerance of ECM trees to salinity (Guerrero-Galán et al. 2019).

In plants, K^+ deficiency triggers a wide variety of physiological, biochemical, and molecular responses (Ashley et al. 2006; Wang and Wu 2010; Hafsi et al. 2014). Among these responses, Ca^{2+} is central: Ca^{2+} channels open at the root epidermis (Véry and Davies 2000), Ca^{2+} concentration increases upon K^+ deficiency (Allen et al. 2001), and Ca^{2+} acts as a signal transduced through the CBL-CIPK pathways (Wang et al. 2018; Tang et al. 2020). Loblolly pine seedlings colonized by *H. cylindrosporum* or *P. ammoniavirescens* displayed lower shoot Ca^{2+} concentrations than non-colonized



	Hebeloma cylindrosporum	*Paxillus ammoniavirescens*	*Laccaria bicolor*	*Suillus cothurnatus*
Colonization	> 90%	> 90%	> 90%	≈ 50%
Shoot biomass	=	⇧	⇧	=
Root biomass	⇧	=	=	=
Rubidium	=	=	=	=
Potassium	⇧	⇧	=	=
Sodium	=	⇩	⇩	=
K:Na ratio	⇧	⇧	⇧	=
Calcium	⇩	⇩	=	=

	Hebeloma cylindrosporum	*Paxillus ammoniavirescens*	*Laccaria bicolor*	*Suillus cothurnatus*
Colonization	> 90%	> 90%	> 90%	≈ 50%
Shoot biomass	=	=	⇧	⇧
Root biomass	⇧	=	⇧	⇧
Rubidium	⇧	⇧	=	=
Potassium	⇧	⇧	⇧	=
Sodium	=	=	=	=
K:Na ratio	=	=	=	=
Calcium	=	=	=	=

Fig. 8 Summary of benefits provided by *Hebeloma cylindrosporum*, *Paxillus ammoniavirescens*, *Laccaria bicolor*, and *Suillus cothurnatus* to *Pinus taeda* seedlings grown under potassium-limited and -sufficient conditions. Variations in shoot and root biomass, nutrient concentrations, and shoot K^+:Na^+ ratio in *P. taeda* seedlings in co-culture with *H. cylindrosporum*, *P. ammoniavirescens*, *L. bicolor*, or *S. cothurnatus* and growing in limited (LK; **a**) and sufficient (SK; **b**) K^+ conditions were displayed as follow: green arrow, significant increase; red arrow, significant decrease; equal sign, no change. These variations reflect data described in the other figures

plants, under low external K^+ condition only (Fig. 8). These results suggest that the Ca^{2+}-mediated responses to low K^+ were attenuated in *P. taeda* because of colonization under K^+-limited condition.

Altogether, our results indicate that *H. cylindrosporum*, *P. ammoniavirescens*, and to some extent *L. bicolor* are able to play a determinant role in host K^+ nutrition and/or to prevent the accumulation of Na^+ and Ca^{2+} in plant tissues, but show varying benefits depending on K^+ availability and on the fungal species.

S. cothurnatus has limited effects on *P. taeda* tolerance to low potassium condition

As mentioned above, *P. taeda* seedlings were also colonized by another ECM fungus, *S. cothurnatus*, which displayed limited benefits to the plant under low K^+ condition compared to the other symbionts, in our experimental setups. Indeed, K^+ nutrition was not improved in seedlings colonized by *S. cothurnatus* under both K^+-limited and -sufficient conditions (Fig. 8). In addition, no reduction of Na^+ accumulation in shoots was observed, compared to non-colonized plants. *S. cothurnatus* can symbiotically interact with *P. taeda* in the forest, which makes incompatibility an unlikely reason for such differences (Liao et al. 2016). However, in our experimental conditions, this was the only species with a significantly lower colonization rate compared to the other fungi (around 50% vs > 90%, respectively), which might explain the reduction of benefits. It is also possible that this low colonization rate was due to poor fungal growth in the nutrient media used for the ECM assays. Indeed, *S. cothurnatus* pure cultures did not grow at all in LK and SK media supplemented or not with Na^+ (Fig. 7). Although hyphae of all fungal species were visually observed in their respective FCs, it is possible that due to its lower growth rate, *S. cothurnatus* hyphae were not invading enough the FCs, limiting the benefits provided to the host plant. Interestingly, *L. bicolor* performed poorly in the liquid LK and SK media as well, but was able to highly colonize *P. taeda* and to provide some benefits to the host plant in ECM assays. Consequently, it is unclear why both *L. bicolor* and *S. cothurnatus* did not grow well in the LK and SK liquid media, and why they have different abilities to colonize *P. taeda* roots.

Interestingly, *S. cothurnatus* colonized *P. taeda* less than the other tested fungi, but was associated with greater shoot and root biomass under K^+-sufficient condition (Fig. 8b), compared to non-colonized plants. It is unclear why *S. cothurnatus* did not perform well in axenic condition, and multiple hypotheses can be raised, including the absence of plant roots and/or solid substrate, difference in temperature, or difference in nutrient availability. Further work could require complementary investigation into *S. cothurnatus* growth preferences, using different media, to increase colonization rate and optimize benefits during symbiosis.

Limitations on the use of stable rubidium to track the transport of potassium in ectomycorrhizal symbiosis

Tracking K^+ transport in mycorrhizal symbiosis has been challenging for a long time due to the difficulty of using radioactive isotopes (Garcia and Zimmermann 2014). Hawkes and Casper (2002) used Rb^+ as a proxy to track K^+ movement in arbuscular mycorrhizal symbiosis. Indeed, no Rb^+ transport proteins have been identified so far, and it appears that Rb^+ ions enter in plants through the K^+ transport pathway (Vallejo et al. 2005). However, it has been shown that some plant species can discriminate between K^+ and Rb^+, making it difficult to evaluate K^+ fluxes (Marschner and Schimansky 1971). In our two-compartment systems, Rb^+ was provided to the FC, making it accessible to the plant only via the fungal hyphae. While successful tracing would not require a 1:1 K^+:Rb^+ ratio, it was reasonable to expect similar trends in response to fungal colonization, confirming the use of Rb^+ as an analog tracer for K^+. Additionally, one hypothesis was that *P. taeda* seedlings would be more reliant on K^+ from the fungus under limited K^+ conditions. In our experiments, although we observed an increase in K^+ concentrations in plants colonized by *H. cylindrosporum* and *P. ammoniavirescens* under K^+-limited and/or -sufficient conditions, as well as a strong positive correlation between shoot K^+ and Rb^+ concentrations, higher amounts of Rb^+ were recorded only in plants watered with the SK solution (Fig. 8b). This suggests that these fungi may have been able to discriminate between K^+ and Rb^+, and in doing so, preferentially allocated K^+ when it was in higher demand by the plant. Another limitation of the use of stable Rb^+ in our experimental design was the fact that Rb^+ was also detected in non-mycorrhizal plants. Based on various quality checks, we are confident that no leaking was possible from the FCs to the RCs (Fig. S2), indicating that Rb^+ ions were present in the synthetic soil used in our system. Nevertheless, observing an increase of Rb^+ concentration in ECM plants under K^+ sufficient conditions, except for those colonized by *L. bicolor* and *S. cothurnatus*, implies an active transport from the other fungi towards colonized roots and confirms that $RbNO_3$ can be used to some extent to track K^+ movement in mycorrhizal symbioses. Further research into the connection between these two nutrients in ECM symbiosis involving a concentration gradient of Rb^+ in the FCs, or the possible use of radioactive Rb^+ isotopes or stable cesium chloride ($^{133}CsCl$), would deepen our understanding of the ectomycorrhiza-mediated transport of K^+.

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Declarations

Conflict of interest The authors declare no competing interests.

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