The ectomycorrhizal fungus *Paxillus ammoniavirescens* influences the effects of salinity on loblolly pine in response to potassium availability

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**Abstract**

Salinity is an increasing problem in coastal areas affected by saltwater intrusion, with deleterious effects on tree health and forest growth. Ectomycorrhizal (ECM) fungi may improve the salinity tolerance of host trees, but the impact of external potassium (K\(^+\)) availability on these effects is still unclear. Here, we performed several experiments with the ECM fungus *Paxillus ammoniavirescens* and loblolly pine (*Pinus taeda* L.) in axenic and symbiotic conditions at limited or sufficient K\(^+\) and increasing sodium (Na\(^+\)) concentrations. Growth rate, biomass, nutrient content, and K\(^+\) transporter expression levels were recorded for the fungus, and the colonization rate, root development parameters, biomass, and shoot nutrient accumulation were determined for mycorrhizal and non-mycorrhizal plants. *P. ammoniavirescens* was tolerant to high salinity, although growth and nutrient concentrations varied with K\(^+\) availability and increasing Na\(^+\) exposure. While loblolly pine root growth and development decreased with increasing salinity, ECM colonization was unaffected by pine response to salinity. The mycorrhizal influence on loblolly pine salinity response was strongly dependent on external K\(^+\) availability. This study reveals that *P. ammoniavirescens* can reduce Na\(^+\) accumulation of salt-exposed loblolly pine, but this effect depends on external K\(^+\) availability.

**INTRODUCTION**

Soil salinity is an increasing problem for agriculture and forest management worldwide (Kearney et al., 2019; Munns & Tester, 2008). Due to elevated storm surge intensity and sea level rise associated with climate change, saltwater intrusion is impacting coastal groundwater and soils globally (Cao et al., 2021), with deleterious effects on plant health in coastal cropland and forest systems previously unadapted to high salinity (Antonellini & Mollena, 2010; Martinez & Ardón, 2021; Poulter et al., 2009; Ross et al., 2020; Tully et al., 2019). Saltwater intrusion is responsible for an increasing occurrence of forest decline in many areas of the lower Coastal Plain of the Southeastern United States, where soil salinity is gradually surpassing the tolerance threshold of many established forest species (Ury et al., 2020).

Among the affected species, loblolly pine (*Pinus taeda* L.) is economically important for timber production due to its high productivity and short maturation time, and occupies most of the 12 million hectares of managed plantation area within its native range (Matallana-Ramirez et al., 2021). Negative responses to soil salinity have been observed in loblolly pine when exposed to salinity levels greater than ~5 ppt (ca. 90 mM of NaCl; Poulter et al., 2008) in normal field
conditions. In plants, soil salinity causes osmotic stress, inhibits root water uptake, and creates ion imbalances due to increased toxic accumulations of sodium (Na\(^+\)) in competition with the potassium (K\(^+\)) necessary for vital plant functions, ultimately inducing oxidative stress and negatively impacting plant photosynthesis and metabolism (Azevedo et al., 2009; Kumari et al., 2021). Due to the chemical similarity of the monovalent cations K\(^+\) and Na\(^+\), K\(^+\) availability and transport mechanisms can play an important role in plant mitigation of Na\(^+\) toxicity and maintenance of intracellular ion homeostasis in vital plant tissues during salt exposure (Benito et al., 2014; Hauser & Horie, 2010). Plants can employ a variety of strategies to cope with salt exposure, including selective ion sequestration, exclusion, or sequestration in vacuoles (Maathuis et al., 2014; Wu et al., 2018). Trees also interact with beneficial root-associated microbes that could influence salinity responses, such as ectomycorrhizal (ECM) fungi (Bai et al., 2021; Guerrero-Galán et al., 2019).

ECM fungi form symbiotic associations with the roots of most tree species in temperate and boreal forests (Becquer et al., 2019), and may play a crucial role in influencing loblolly pine nutrition in natural forests and managed plantations (Hackman et al., 2022). Through the expression of various transport proteins, the extraradical fungal hyphae transport essential nutrients and water from the soil toward host roots (Garcia et al., 2016). Inside colonized roots, they are exchanged for plant photosynthates within a network of hyphae known as the Hartig net developed between cortical cells. ECM fungi have been reported to improve the salinity tolerance of their host trees, but benefits may vary depending on species partnership and soil conditions (Guerrero-Galán et al., 2019; Usman et al., 2021). In each case, different strategies may be involved in the ECM enhancement of host salt tolerance, such as fungal mitigation of root Na\(^+\) uptake (Li et al., 2012), or improvement of plant K\(^+\) nutrition (Luo et al., 2011), as observed in Populus × canescens inoculated with Paxillus involutus under salt stress.

In loblolly pine, ECM fungi may also play an important role in salinity tolerance. Indeed, Dixon et al. (1993) observed an increased biomass in loblolly pine when inoculated with Suillus tomentosus or Pisolithus tinctorius under moderate salinity. However, reduced colonization was observed for P. tinctorius and Laccaria laccata at high salinity levels, and only salt-tolerant fungi were beneficial for the host in these conditions. The benefit of symbiosis for the host is also largely dependent on the fungal salt tolerance, which can vary greatly between species (Guerrero-Galán et al., 2019). Considerable salt tolerance has been observed in several ECM fungi, including P. involutus, which was observed to survive 500 mM of NaCl (Zhang et al., 2008). However, the salinity tolerance and host benefits of other ECM species in the Paxillaceae family have not been fully investigated.

The species Paxillus ammoniavirescens (Dessi & Contu, 1999) has been described as a generalist ECM fungus associating with diverse host trees and occurring in a wide range of environmental conditions (Jargeat et al., 2014). We recently observed that P. ammoniavirescens can associate with loblolly pine and enhance K\(^+\) nutrition while decreasing Na\(^+\) accumulation in pine shoot tissue under limited K\(^+\) conditions (Frank & Garcia, 2021), suggesting that this symbiosis may also help loblolly pine to mitigate Na\(^+\) uptake under saline conditions. However, the salt tolerance of P. ammoniavirescens, its response to K\(^+\) availability under saline conditions, its ability to colonize salt-stressed loblolly pine, and its impact on loblolly pine tolerance to salinity are still unknown. Also, while we reported multiple K\(^+\) transport proteins for another ECM fungus, Hebeloma cylindrosporum (Garcia et al., 2014, 2020; Guerrero-Galán, Delteil, et al., 2018; Guerrero-Galán, García, et al., 2018), none have been functionally characterized or even investigated so far for P. ammoniavirescens.

Therefore, we investigated here P. ammoniavirescens in axenic culture and in co-culture with loblolly pine under limited or sufficient K\(^+\) conditions with increasing exposure to salinity. By measuring growth parameters, root colonization rates, K\(^+\) and Na\(^+\) accumulations in fungal and plant tissues, and the expression of putative fungal K\(^+\) transporter-coding genes under these conditions, we aim to determine the impacts of K\(^+\) availability on (1) the salinity tolerance and cation uptake of P. ammoniavirescens, (2) the effect of salt exposure on loblolly pine root development and colonization by P. ammoniavirescens, and (3) the influence of the established ECM symbiosis on the growth and cation uptake of loblolly pine under increasingly saline conditions.

**EXPERIMENTAL PROCEDURES**

**Potassium and sodium treatments**

Fungi and plants were grown in controlled environments and supplied with N1 nutrient media described in Garcia et al. (2014) and modified for various K\(^+\) and Na\(^+\) conditions (Table S1). K\(^+\) treatment levels were chosen following Frank and Garcia (2021), containing either sufficient (SK, 1.0 mM K\(^+\)) or limited (LK, 0.05 mM K\(^+\)) K\(^+\) concentrations (Table S1; SK N1: 0.2 mM Ca(NO\(_3\))\(_2\), 0.6 mM KNO\(_3\), 0.2 mM KH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 0.2 mM KCl, 0.2 mM NaCl, 0.5 mM L\(^{-1}\) ferric citrate 1%, 0.2 mM L\(^{-1}\) micronutrient solution (Table S2), 10 μL L\(^{-1}\) thiamine; and LK N1: 0.4 mM Ca(NO\(_3\))\(_2\), 0.2 mM NaH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 0.05 mM KCl, 0.5 mM L\(^{-1}\) ferric citrate 1%, 0.2 mM L\(^{-1}\) micronutrient solution (Table S2), 10 μL L\(^{-1}\) thiamine). Five Na\(^+\) treatment levels were chosen, containing 0.2, 25, 50, 100, or 200 mM of Na\(^+\), which was supplied to all media as NaCl, except for 0.2 mM NaH\(_2\)PO\(_4\),
which was provided to LK solutions to compensate for the absence of KH₂PO₄ (Garcia et al., 2014). K⁺ and Na⁺ treatments were combined for a total of 10 unique treatment media, abbreviated below as LK+0.2/25/50/100/200Na and SK+0.2/25/50/100/200Na. Glucose (5 g L⁻¹) was added for fungi in axenic culture assays and subcultures, and agar (10 g L⁻¹) was added for fungal subcultures and radial growth assay only. All media were prepared using milli-Q water and autoclaved before use.

**Fungal and plant materials**

*P. ammoniavirescens* Pou9.2 (Dessi & Contu, 1999) was obtained from active cultures maintained at 26°C on solid modified Melin-Norkrans (MMN) medium (Marx, 1969). Subcultures for each experiment were prepared and maintained on LK+0.2Na medium (10 g L⁻¹ agar, 5 g L⁻¹ glucose) to minimize residual K⁺ or Na⁺ transferred to subsequent treatment conditions. All subcultures and axenic fungal experiments were incubated in the dark at 26°C for 1 month.

Loblolly pine seeds were obtained from Sheffield’s Seed Co. Inc. (Arkansas, USA; Lot no. 1830528). Seeds were surface sterilized in 35% H₂O₂ for 15 min, rinsed five times with sterile milli-Q water, submerged in the last rinse, and kept at 4°C for 72 h. Seeds were then germinated in sterile culture plates with solid medium containing 15 g L⁻¹ agar and 2 g L⁻¹ glucose. Seedlings were germinated in a Conviron® GEN1000 growth chamber (16 h day, 8 h night, 23°C, luminosity 210 μmol m⁻² s⁻¹, relative humidity 60%) for 4 weeks prior to seeding transplant.

**Mycelial growth rate measurement**

Axenic cultures of *P. ammoniavirescens* were grown on either LK or SK media, supplemented with each of the five Na⁺ treatment levels, and glucose (5 g L⁻¹). Culture plates containing 40 mL of solid media were each inoculated with one 1 cm agar plug taken from the advancing edge of subculture mycelia. Cultures were then incubated in the dark at 26°C and monitored daily until mycelial growth was observed. The diameter of visible mycelial growth on the agar medium was measured every 3 or 4 days until mycelia reached the edge of the plate for at least one replicate (25 days). The mycelial diameter (mm) was measured with a ruler along each of the three angles through the subculture origin (Figure S1). The radial growth was calculated as follows:

\[
\text{Radial growth} = \frac{1}{2} (\text{mycelium diameter} - \text{diameter of origin disc})
\]

The average of the three radial measurements for each replicate was calculated at each time point. A total of 10 replicates were prepared for each treatment.

**Axenic fungal culture assays**

Cultures of *P. ammoniavirescens* were grown at 26°C in 45 mL of liquid LK or SK media supplemented with each of the five Na⁺ treatment levels and 5 g L⁻¹ of glucose for 1 month to provide minimum tissue growth for accurate measurement in downstream elemental analyses. Radial growth curves generated from plate cultures described above were used to verify that growth was unlikely to reach a stable plateau phase at 1 month (Figure S2). Ten replicates per treatment were prepared. Each fungal thallus was then rinsed in 30 mL of milli-Q water to remove excess treatment media. Agar from the subculture plug was carefully removed with a scalpel, and the thallus was gently patted dry with a paper towel and weighed. Samples were then oven-dried at 70°C for 5 days, and dry biomass was recorded.

**Impact of NaCl exposure on pine root development and ECM mantle formation**

Germinated seedlings were randomly selected and transplanted into pots (Landmark Plastics, USA) containing 200 mL of triple-rinsed potting substrate (Safe T Sorb®, Ep Minerals®, Nevada, USA) and immediately watered with 30 mL per pot of either LK or SK media, supplemented with each of the five Na⁺ treatment levels. Pots were kept in the growth chamber (see above) in plastic trays (Yield Lab, USA) according to the assigned treatment. Thalli of *P. ammoniavirescens*, grown in 50 mL of liquid MMN for 1 month, were rinsed in 40 mL of LK+0.2Na or SK+0.2Na solutions, depending on the subsequent treatment. The rinsed thalli were ground in 80 mL of the same media using an immersion blender (Fisherbrand™ 150 Homogenizer). Each inoculant slurry was divided into two 40 mL aliquots, and one aliquot per seedling was used to inoculate roots 3 days after transplant. After inoculation, the nutrient solutions were added to the bottom of each tray at a rate of 30 mL per pot every 3 days. Plants were harvested 4 weeks after inoculation.

Roots were collected, water-rinsed, patted dry with a paper towel, and photographed. All short roots (<1 mm diameter, <3 mm length; Helmisaari et al., 2009; Raudaskoski & Salo, 2008) were counted along primary and lateral roots using a binocular scope, and the presence or absence of a developed ECM mantle was recorded. In this experiment, the presence of a developed mantle was used as a parameter to approximate the relative colonization rate, and the
percent colonization was calculated as the number of short roots with a developed mantle per hundred short roots for each plant. To quantify the number of lateral roots (defined here as all secondary or tertiary roots with length >3 mm), and the length of lateral and primary roots, ImageJ software (Schneider et al., 2012) was used to analyse root photographs.

Impact of established ectomycorrhizal symbiosis on salinity response in pine

Germinated seedlings were selected, transplanted, inoculated, and watered as described above. Non-ecorrhizal (NM) seedlings received 40 mL of fungus-free LK+0.2Na or SK+0.2Na solutions. Contrary to the previous experiment, to allow the establishment of ECM symbiosis prior to NaCl treatments, plants were first watered with LK+0.2Na or SK+0.2Na solutions only before starting the Na+ treatments. Three weeks later, NM and P. ammoniavirescens-inoculated (Pa) seedlings received the following treatment to prevent osmotic shock: the Na+ concentrations were increased every 3 days by 1/5 of the final Na+ concentration for each treatment (Table S3) until treatment media contained each of the five Na+ concentrations. This experiment was repeated twice. In the first experiment, root and shoot tissue were harvested as described above for determination of mantle development to assess ECM colonisation rate and biomass measurements, as well as nutrient concentrations. For the second experiment, harvested roots were immediately flash-frozen in liquid nitrogen and kept at −80°C before RNA extraction.

Nutrient quantification in fungal and plant tissue

Dry fungal tissues from axenic culture grown in liquid media and pine shoot tissues from co-culture experiments were digested in nitric acid and elemental concentrations of K+ and Na+ in the filtered digestate were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) at the NC State University Environmental Agricultural and Environmental Testing Services (NCSU EATS) laboratory.

RT-qPCR

Paxillus ammoniavirescens was first grown in 45 mL of either liquid LK+0.2Na or SK+0.2Na media for 2 weeks, then media was replaced with either LK or SK media containing each Na+ treatment level for three additional weeks. Each thallus was removed from media, the agar subculture plug was removed, and the thallus was immediately frozen in liquid nitrogen and stored at −80°C.

Fungal samples were homogenized using liquid nitrogen, and total RNAs were extracted using 1 mL of TRIZOL (Life Technologies, Carlsbad, CA, USA), treated with TURBO DNase (Thermo Fisher Scientific), and quantified by a NanoDrop One (Thermo Fisher Scientific).

RNA isolations were performed for pine root samples using a lithium chloride (LiCl) precipitation method (Liao et al., 2014). First, the frozen tissue was ground using mortar and pestle using liquid nitrogen and 1 mL of CTAB/chloroform extraction buffer (with 100 mM Tris-Cl, 25 mM EDTA, 2 M NaCl, 2% w/v CTAB, 2% w/v PVP, and 2% freshly added beta-mercaptoethanol) was immediately added to it. The samples were homogenized by vortexing, and incubated at 65°C for 10 min. Equal volumes of chloroform: isomyl alcohol mixture (24:1) were added to the lysates, mixed, and then centrifuged for 10 min at 10,000 rpm at 4°C. The upper aqueous phases containing RNAs were transferred carefully into other sterile 2 mL tubes. To precipitate the RNAs, 0.5 volume of 8 M LiCl was added to the supernatant and the tubes were kept at 4°C overnight. The tubes were then centrifuged at 10,000 rpm for 15 min at 4°C, the supernatants were discarded, and the pellets were washed with 70% ethanol. The pellets were dried and resuspended into 30 μL of RNase-free DEPC water.

One microgram of RNA was denatured for 5 min at 65°C with oligo(dT)18, and cDNAs were synthesized using the RevertAid Reverse Transcriptase Kit for 60 min at 42°C (Thermo Fisher Scientific). One microliter of cDNA (dilution 1:2) was used for RT-qPCR reactions using the Bio-Rad CF Connect Real-Time PCR system (Bio-Rad). Reactions were performed in 96-well plates using iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.5 μL of each primer (250 nM), and 1:20 (v/v) cDNA:water. The PCR conditions were as follows: 95°C for 3 min; 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s; dissociation up to 95°C, starting from 65°C with a gradual increment of 0.5°C. Expression levels of the putative transporters identified in the genome (https://mycocosm.jgi.doe.gov/Paxam1/Paxam1.home.html) PaTrk (protein ID 989605), PaACU1 (938511), and PaACU2 (147191) were determined relatively to the internal control α-tubulin (898703) on mycelium and colonized root samples using the corresponding primers (Table S4). NM roots were excluded from the analysis because no amplification was observed with these fungus-specific primers (Figure S3). The expression coefficients were calculated using the 2−ΔΔCt method. The results are presented as the average of two to four biological replicates and three technical replicates per biological replicates. The specificity and efficiency of
primer pairs were confirmed by analyses of dissociation curves (65°C–95°C) and serial dilutions, respectively.

**Statistical analyses**

All statistical analyses were performed in R v.4.0.3 (R Core Team, 2020), with a significance level of \( P \leq 0.05 \). Analysis of variance (ANOVA) and multiple comparison tests were performed in R using the agricolae package. All plots were created using ggplot2.

Two-sample \( t \)-tests were performed to compare radial growth and gene expression levels at each time point between LK and SK treatment groups within each \( \text{Na}^+ \) treatment level. All other significant differences among treatment groups were determined by 2-way or 3-way ANOVA, depending on the experiment, followed by Fisher’s post hoc LSD test. Correlations were also performed using R software.

**RESULTS**

*Paxillus ammoniavirescens* mycelial growth response to \( \text{NaCl} \) and \( K^+ \) supply

To determine the effects of \( K^+ \) availability and increasing \( \text{Na}^+ \) exposure on *P. ammoniavirescens* growth, two independent parameters were recorded: hyphal exploration over 4 weeks by regularly measuring thallus diameter on solid plates, and overall biomass production after 4 weeks of liquid culture. Active mycelial growth was observed in all \( \text{Na}^+ \) treatments, but growth rate and biomass were both decreased with increasing \( \text{Na}^+ \) exposure, regardless of \( K^+ \) treatment (Figures 1A,B and S4; Table S5). Regression analyses show that \( \text{Na}^+ \) treatment concentrations explained 37.1% and 57.2% of the variation in radial growth observed in LK and SK treatments, respectively (Figure S4a). \( \text{Na}^+ \) treatment concentration was also negatively correlated with the dry weight of cultures grown in LK media, explaining 68.8% of the variation in biomass, but only 13.4% of the biomass variation observed in SK treatments (Figure S4b). In LK and SK treatments, growth rate and biomass decreased significantly from 0.2 Na to +25Na (Figure 1A,B). At levels +25Na and higher, the growth rate decreased with increasing \( \text{Na}^+ \) levels in SK treatments, but this trend was not significant among LK treatments except between +50Na and +200Na (Figure 1A). A decrease in biomass was observed with increasing \( \text{Na}^+ \) levels among LK treatments from +25Na to +200Na, but biomass among SK treatment groups from +25Na to +200Na was not significantly different (Figure 1B). A significant effect of \( K^+ \) treatment was observed for mycelial growth, with higher radial growth rates in SK compared with LK treatments at all \( \text{Na}^+ \) levels (Figure 1A). Contrarily, biomass was not significantly different between LK and SK treatments at any \( \text{Na}^+ \) level (Figure 1B).

*Paxillus ammoniavirescens* nutrient uptake in response to \( K^+ \) and \( \text{Na}^+ \) treatments

To determine the impact of external \( K^+ \) availability and increasing \( \text{Na}^+ \) exposure on the concentrations of \( K^+ \) and \( \text{Na}^+ \) in 4-week-old fungal thalli, extracts of dried tissue were analysed by ICP-OES. Fungal \( K^+ \) concentrations and contents were significantly higher in SK compared with LK treatments for all \( \text{Na}^+ \) treatments. Additionally, they were significantly reduced in SK at +25Na and higher salt levels compared with the +0.2Na condition (Figures 2A and S5a; Table S5). However, \( \text{Na}^+ \) concentrations increased in all fungi with increasing \( \text{Na}^+ \) levels, but differences were spotted depending on \( K^+ \) availability (Figure 2B). This effect
Expression of PaTrk, PaACU1, and PaACU2 from Paxillus ammoniavirescens in response to K⁺ availability and Na⁺ exposure in axenic and symbiotic conditions

Coding sequences of the Na⁺–K⁺ transporter HcTrk1 from the ECM fungus H. cylindrosporum (Corratgé et al., 2007; Garcia et al., 2014) and the high-affinity K⁺ transporters MoACU1 and MoACU2 from Magnaporthe oryzae (Benito et al., 2011) were used to search by BlastP for the corresponding orthologues in the genome of P. ammoniavirescens. The identified gene sequences were named PaTrk, PaACU1, and PaACU2, respectively. Their expression level was determined by RT-qPCR in axenic liquid cultures grown in SK and LK conditions after 3 weeks of exposure to increasing Na⁺ treatments, as well as in symbiotic conditions with loblolly pine after 8 weeks of co-culture.

In axenic conditions, the PaTrk expression level was significantly higher in LK than in SK conditions, only at +200Na. Additionally, an oscillatory pattern can be seen, with a decrease at +25Na in comparison to the +0.2Na conditions, then an increase at +50Na to a maximum at +100Na, and a decrease again at +200Na, regardless of the K⁺ availability (Figure 3A). This oscillatory pattern was inverted in colonized pine roots, but only at SK, and no significant differences were detected between SK and LK conditions (Figure 3B). Concerning PaACU1 and PaACU2, different patterns were observed between their expression levels (Figure 3C–F). In axenic culture, PaACU1 showed a peak of expression in LK at +25Na (Figure 3C), while a similar peak was observed in symbiotic conditions, but in SK (Figure 3D). In axenic conditions and at SK, the expression levels of both transporters gradually increased up to +100Na in comparison to the +0.2Na conditions and then decreased at +200Na (Figure 3C,E). Also, at LK, their expression levels were the opposite of PaTrk, they increased at +25Na compared with the +0.2Na conditions, then decreased at +50Na and +100Na, and increased again at +200Na (Figure 3C,E). Additionally, the expression level of PaACU1 was significantly higher at LK+0.2Na, LK+25Na, and LK+200Na, and significantly lower at LK+100Na, compared with their respective SK conditions (Figure 3C). Similarly, PaACU2 expression levels were significantly higher at LK+0.2Na and LK+200Na, and significantly lower at LK+100Na, compared with their respective SK conditions (Figure 3E). In symbiotic conditions, the expression level of PaACU1 and PaACU2 increased from SK +0.2Na to SK+50Na, then decreased at SK+100Na and SK+200Na (Figure 3D,F). At LK, PaACU1 expression followed the same pattern, but was less pronounced (Figure 3D), while PaACU2 expression level gradually increased from +0.2Na to +200Na.
transporters was dependent on both Na\(^+\) and K\(^+\). Additionally, the major differences spotted in the expression level of these three genes between axenic and symbiotic conditions reveal an important influence of the host plant.

**Impact of NaCl exposure on Pinus taeda root formation and ECM colonization in response to K\(^+\) supply**

We evaluated the effect of increasing levels of Na\(^+\) exposure on root development and *P. ammoniavirescens* colonization in NaCl-exposed *P. taeda* seedlings after 4 weeks of co-culture in SK and LK conditions. The roots were collected and photographed for subsequent determination of primary and lateral root lengths (Figures 4A and S6a,b; Table S5). All short roots with or without a developed hyphal mantle were counted under a binocular scope, the mantle presence was used to assess the ECM colonization rate (Figure 4B,C; Table S5), and dry biomass was recorded (Figure 4C). The development of short roots decreased clearly with increasing salt concentrations (Figure 4A,B). For LK treatments, a significant decrease in the number of short roots was observed from LK +25Na to +50Na, and from LK +50Na to +200Na (Figure 4B). For SK treatments, significant decreases in short root counts occurred from SK +0.2Na to +25Na and from SK +50Na to +100Na (Figure 4B). Similar trends were observed for the length of primary and lateral roots (Figure S6a,b) and the density of short roots and lateral root counts relative to primary root length in response to Na\(^+\) treatment (Figure S7). However, external K\(^+\) availability at the two tested concentrations (0.05 mM and 1.0 mM) did not have a significant effect on the number of short roots (Figure 4B), but the length of primary and lateral roots was greater in SK than in LK conditions at +50Na (Figure S6a,b). Additionally, the primary root length was significantly higher in SK than in LK treatments at +0.2Na (Figure S6a). Root biomass also decreased significantly with increasing salinity, but no difference was observed between SK and LK conditions, except at +50Na (Figure S6c).

Short root colonization by *P. ammoniavirescens* was not significantly different among K\(^+\) or Na\(^+\) treatments, with an observed mean colonization rate above 70% for all groups (Figure 4C). However, it is worth noting that the significantly reduced number of short roots at higher salt exposure also meant a strongly reduced number of ectomycorrhizae per plant (Figure S8). No ectomycorrhizae were found on seedlings with limited or sufficient potassium availability at varying levels of Na\(^+\) supply.

**FIGURE 3** Relative expression of putative K\(^-\) transporters in *Paxillus ammoniavirescens* grown in axenic or symbiotic conditions with limited or sufficient potassium availability at varying levels of sodium exposure. Relative expressions of the putative K\(^-\) transporters *PaTrk* (A), *PaACU1* (C), and *PaACU2* (E) were determined by RT-qPCR in thalli grown in liquid media with 0.05 mM (LK) or 1.0 mM (SK) of potassium (K\(^+\)) and exposed for 3 weeks to either 0.2, 25, 50, 100, or 200 mM of sodium (Na\(^+\)). *PaTrk* (B), *PaACU1* (D), and *PaACU2* (F) expression levels were also determined by RT-qPCR in *P. taeda* roots colonized by *P. ammoniavirescens* (Pa) for eight weeds, in either LK or SK conditions, and exposed for 4 weeks to the same Na\(^+\) treatments. The results are based on two to four biological replicates and three technical replicates. Statistical differences between LK and SK conditions for each Na\(^+\) treatment point were determined using the Student's test. \(P<0.05\) (*) and \(P<0.01\) (**).
Impact of established ectomycorrhizal symbiosis and K⁺ supply on loblolly pine growth under increasing salinity

To determine the influence of ECM colonization and K⁺ treatment on the growth of loblolly pine in response to increasing Na⁺ exposure, seedlings exposed to these different conditions were harvested 8 weeks after inoculation, root colonization was quantified, and shoot and root dry biomass were measured (Figure 5; Table S5). Mean short-root colonization rates were above 80% for all plants inoculated with *Paxillus ammoniavirescens* regardless of K⁺ and Na⁺ levels (Figure 5A). All non-inoculated roots were confirmed to be NM.

The impact of inoculation on shoot biomass was only significant in LK+0.2Na conditions. Indeed, in LK+0.2Na treatments, shoot biomass was higher in...
ECM plants compared with NM ones, but the opposite was observed for seedlings growing at SK+0.2Na (Figure 5B). Surprisingly, shoots of ECM plants had lower biomass in SK than in LK conditions at +0.2Na (Figure 5B). Shoot biomass of ECM plants growing in LK conditions was significantly higher only at +0.2Na compared with the other Na\(^+\) treatments, while no difference was recorded at SK. However, the shoot biomass of NM seedlings decreased from +25Na to +100Na at LK, and from +50Na to +200Na at SK (Figure 5B). Root biomass increased in ECM plants compared with NM roots only in LK+0.2Na and SK+25Na conditions, though observed differences may be due to the presence of additional fungal biomass in the root samples of inoculated plants. Globally, root biomass decreased with increasing salt treatment but was not much affected by the two tested K\(^+\) conditions. Only significant differences were observed in ECM plants with higher root biomass in SK than LK conditions at +25Na, and in NM ones at +100Na.

**Influence of *Paxillus ammoniavirescens* colonization on loblolly pine nutrient uptake under various K\(^+\) and Na\(^+\) treatments**

We also assessed the impact of ECM colonization on nutrient acquisition in loblolly pine under SK and LK conditions and increasing Na\(^+\) levels. Therefore, K\(^+\) and Na\(^+\) concentrations and contents were determined in shoots of NM and ECM plants (Figure 6; Table S5). When plants were kept non-inoculated, shoot K\(^+\) concentrations were significantly higher in LK than SK conditions for all Na\(^+\) treatments, even if that was not significant at +50Na, but contents remained similar between all NM plants (Figures 6A and S9a). This indicates that low K\(^+\) availability enhanced K\(^+\) transfer by loblolly pine roots to shoots, potentially as a response to mitigate the stress effects and be able to survive. Therefore, it reflects a significant degree of tolerance of loblolly pine seedlings to low K\(^+\) conditions. Additionally, K\(^+\) concentrations significantly increased in shoots of NM plants at LK only when at least 100 mM of Na\(^+\) was supplied, compared with the +0.2Na condition. This increase was observed for NM seedlings in SK conditions only at +50Na and +200Na. Comparisons between ECM and NM plant status revealed that shoot K\(^+\) concentrations were higher in colonized seedlings than in NM ones among all SK treatments at all Na\(^+\) levels (Figure 6A). However, this observation was not true at LK, except under 100 mM of Na\(^+\). Interestingly, shoot K\(^+\) concentrations of ECM plants were not different between K\(^+\) conditions within each Na\(^+\) treatment, but significantly more K\(^+\) accumulated in shoots at LK in all Na\(^+\) treatments compared with the LK+0.2Na condition. This was also observed at SK, but only at +50Na and +100Na (Figure 6A). K\(^+\) contents were higher in ECM plants compared with NM plants at LK+0.2Na (Figure S9a).

At LK, NM plants accumulated more Na\(^+\) when exposed to +25Na, +100Na, and +200Na treatments, compared with those growing at SK and to the LK+0.2Na condition (Figures 6B and S9b). However, no differences were observed among Na\(^+\) treatments for

**FIGURE 6** Shoot potassium and sodium concentrations and ratios in *Pinus taeda* seedlings, inoculated or not with *Paxillus ammoniavirescens* and grown with limited or sufficient potassium availability at varying levels of sodium exposure. Potassium (K\(^+\), A) and sodium (Na\(^+\), B) concentrations were determined by ICP-OES, in the shoot of *P. taeda* seedlings 8 weeks after inoculation with *P. ammoniavirescens* (Pa) or not (NM) in either 0.05 mM (LK) or 1.0 mM (SK) of K\(^+\), and exposed for 4 weeks to either 0.2, 25, 50, 100, or 200 mM of Na\(^+\). (C) Ratios between shoot K\(^+\) and Na\(^+\) concentrations were calculated for each pine seedling. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from the first quartile to minimum and from the third quartile to maximum values, respectively. Different letters above boxplots indicate significant differences between treatments according to 3-way ANOVA followed by LSD post hoc test at \(P \leq 0.05\). \(n = 4–6\).
NM plants grown at SK, indicating that higher K⁺ availability prevented Na⁺ accumulation in loblolly pine. At these three Na⁺ treatments in LK, plants colonized by *P. ammoniavirescens* displayed lower Na⁺ concentrations than their NM counterparts, with significant differences only observed at +25Na and +200Na. However, this effect was reversed in SK at the highest Na⁺ levels when short root numbers were decreased, with significantly lower shoot Na⁺ concentrations and contents detected in NM plants than in ECM ones at SK+100Na (Figures 6B and S9b). When comparing ECM plants only, K⁺ availability seemed to have no effect on shoot Na⁺ concentrations for each Na⁺ treatment. Additionally, shoot Na⁺ concentrations in ECM plants were significantly higher at +100Na in LK, and at +100Na and +200Na in SK, compared with their respective +0.2Na condition (Figure 6B).

These observations on shoot K⁺ and Na⁺ concentrations resulted in the calculation of significantly higher K⁺:Na⁺ ratios in NM plants at +0.2Na compared with all other Na⁺ treatments as could be expected (Figure 6C). No other differences were found among any treatments for NM seedlings. When plants were colonized however, the K⁺:Na⁺ ratios were significantly higher than in NM seedlings in LK conditions at +0.2Na and +50Na, and also at SK+50Na, indicating an improved K⁺ nutrition or protection against Na⁺ increase (Figure 6C). Significantly higher K⁺:Na⁺ ratios were also observed in ECM plants grown at LK compared with SK only at +0.2Na. Interestingly, the K⁺:Na⁺ ratios significantly decreased at +25Na compared with the +0.2Na condition, then increased at +50Na before significantly decreasing again at +100Na and higher (Figure 6C).

**DISCUSSION**

**High salinity limits *Pinus taeda* root development and growth parameters, but K⁺ availability attenuates Na⁺ accumulation in non-mycorrhizal pines**

Increasing soil salinity is becoming a major issue in many coastal forests and tree plantations due to sea-level rise, saltwater intrusion, and storm surges (Antonellini & Mollema, 2010; Ury et al., 2020). Therefore, it is crucial to understand the ability of trees, including those from the *Pinaceae* family, to tolerate soil salinity. A negative impact of high salinity (≥ca. 100 mM of NaCl) on root growth has been observed previously in *P. taeda* (Domec et al., 2021; Poulier et al., 2008) in field or greenhouse experiments where tree roots are likely to be associated with naturally occurring ECM fungi in soil or nursery media. For other pine species, such as *Pinus banksiana* (Bois, Bigras, et al., 2006; Franklin & Zwiazek, 2004) and *Pinus thunbergii* (Wen et al., 2022), experiments conducted under laboratory-controlled conditions or greenhouse trials with non-mycorrhizal controls showed similar salinity response thresholds. However, some degree of tolerance has been described at moderate salinity (approximately 60–100 mM of NaCl) in non-mycorrhizal *P. banksiana* (Calvo Polanco et al., 2009), and *P. pinea* grown in hydroponic culture (Khalid et al., 2011). Here, we exposed loblolly pine seedlings to almost double the maximum NaCl concentrations tested by Poulier et al. (2008), who described a salinity tolerance threshold for this species at an equivalent of approximately 90 mM of NaCl. Similarly, our results indicate that increasing exposure to salinity inhibits root development and short-root formation of loblolly pine, particularly above 100 mM of NaCl (Figures 4 and 7). However, when sufficient K⁺ was supplied, biomass of seedlings exposed to 200 mM of Na⁺ was similar to plants growing under moderate salinity (Figure 5), suggesting that salt tolerance in loblolly pine may be enhanced by external K⁺ availability even in NM conditions. However, in natural and agro-ecosystems, trees from the *Pinaceae* family are always colonized by ECM fungi (Garcia et al., 2015; Hackman et al., 2022), making it critical to evaluate these responses to salinity in symbiotic conditions.

Since the excess of Na⁺ can be toxic for many trees, including loblolly pine (Pezeshki, 1992), the plant’s capacity to regulate Na⁺ concentrations in shoot tissue is crucial for preserving metabolic and cellular functions (Assaha et al., 2017; Kumari et al., 2021; Munns & Tester, 2008; Zhang et al., 2018). Particularly, the maintenance of an elevated cellular K⁺:Na⁺ ratio is an important feature in plants for tolerance to salinity (Assaha et al., 2017; Giri et al., 2007; Porcel et al., 2016; Zhang et al., 2018). In K⁺-limited conditions, we observed that more Na⁺ accumulated in loblolly pine shoots with increasing external salinity. However, with access to sufficient K⁺, shoot Na⁺ concentrations were not increased by high NaCl exposure in non-inoculated plants (Figure 7), suggesting that loblolly pine may utilize available soil K⁺ to attenuate Na⁺ accumulation and protect vital photosynthetic organs from toxicity (Maathuis, 2014). A similar decrease in shoot Na⁺ was observed with the addition of sufficient K⁺ in *Pinus pinaster* (Garcia et al., 2014), highlighting the importance of K⁺ availability for the mitigation of toxic Na⁺ accumulation in aboveground tissue (Benito et al., 2014; Wu et al., 2018). Additionally, a significantly higher K⁺:Na⁺ ratio was recorded in ECM plants under LK+0.2Na and in LK+50Na and SK+50Na compared with NM plants, but not in higher NaCl treatments (Figure 6C). This reveals the critical role of fungal colonization on loblolly pine tolerance to NaCl under moderate salinity only. Altogether, our data indicate that the external K⁺ availability positively influences loblolly pine tolerance to salt exposure (Figure 7), but the molecular mechanisms for this tolerance in *Pinaceae* species remain to be elucidated.
**P. ammoniavirescens** pure culture

- **[K⁺]**: SK > LK in SK
- **[Na⁺]**: SK < LK in SK
- **Radial growth**: SK > LK in SK
- **Biomass**: SK = LK in SK

**Symbiotic condition**

- **[K⁺]**: SK: ECM > NM
  - LK: ECM = NM
- **[Na⁺]**: SK: ECM > NM
  - LK: ECM = NM
- **Short roots**: SK = LK in SK
- **Colonization**: SK = LK in SK

**Figure 7** Summary of the impacts of NaCl exposure on *Paxillus ammoniavirescens* in pure culture and on the association with *Pinus taeda* under potassium-limited and -sufficient conditions. SK and LK: sufficient (1.0 mM) and limited (0.05 mM) potassium (K⁺) conditions, respectively. ECM and NM, ectomycorrhizal and non-ectomycorrhizal plants, respectively; [K⁺] and [Na⁺]: potassium and sodium concentrations, respectively; green arrow, significant increase; red arrow, significant decrease; grey equal sign, no change.

In pure culture, the fungus accumulated more K⁺ and less Na⁺ in SK compared with LK. The fungal radial growth was reduced in LK compared with SK, but its biomass remained similar. Additionally, the increase in NaCl exposure had a positive effect on [Na⁺], a negative effect on radial growth and biomass, and no effect on [K⁺], regardless of the external K⁺ availability. In symbiosis experiments, ECM plants generally displayed more [K⁺] and [Na⁺] in SK, less [Na⁺] in LK, and similar [K⁺] in LK compared with NM plants. Short root number and colonization were not affected by the external K⁺ availability, but the NaCl exposure had a negative effect on short root formation and no effect on colonization in both SK and LK conditions.

**Paxillus ammoniavirescens** can survive under high salinity and colonize salt-exposed loblolly pine

The ECM fungus *P. ammoniavirescens* has been described as a generalist species, able to form symbiosis with a diverse range of deciduous and coniferous trees (Jargeat et al., 2014), including loblolly pine (Frank & Garcia, 2021). In that recent study, we also reported that the biomass of this fungus was enhanced under mild salinity (1.0 mM). To understand how this fungal symbiont might influence the plant host response to salinity, it is important to investigate its ability to grow in higher saline conditions. Here, we report that *P. ammoniavirescens* can survive at high salinity, despite the negative impact of increasing NaCl on mycelial growth rate and biomass (Figure 7). Studies investigating salinity tolerance in the genus *Paxillus* have found that *P. involutus* is also tolerant to high saline conditions, in some cases even surviving at up to 500 mM of NaCl, despite toxic effects of salinity have also been observed at concentrations above 100 mM (Hutchison, 1990; Kernaghan et al., 2002; Langenfeld-Heyser et al., 2007; Zhang et al., 2008). Several other ECM species in the order Boletales were found to be salt-tolerant, though the degree of tolerance can vary among species in the same genus or even between isolates of the same species. Some species even exhibited enhanced growth with the addition of moderate amounts of NaCl (Bois, Bertrand, et al., 2006; Dixon et al., 1993; Matsuda et al., 2006). Similar observations were made in *P. ammoniavirescens* (Frank & Garcia, 2021), suggesting that this species may also be able to utilize moderate amounts of Na⁺ in metabolic processes. However, our observations indicate that exposure to Na⁺ levels at or above 25 mM can have a detrimental effect on fungal biomass production (Figure 7).

K⁺ availability appears to play an important role in the hyphal growth, nutrient status, and expression of putative K⁺ transporters of *P. ammoniavirescens* under saline conditions (Figure 7). At LK, we observed a reduction of the radial growth rate without a corresponding decrease in biomass, indicating that hyphal elongation may be suppressed, and mycelial density increased, in response to K⁺ deprivation. Soil exploration by extraradical hyphae may therefore be enhanced by sufficient K⁺, possibly increasing nutrient access as well as salt exposure in saline conditions. However, further research is necessary to understand these morphological responses of *P. ammoniavirescens* to K⁺ availability. Tissue K⁺ concentrations were only affected by K⁺ availability, and remained stable under high salinity even with increasing accumulations of Na⁺ in fungal tissue. Also, a significantly high fungal K⁺/Na⁺ ratio was observed only in SK conditions and without any NaCl added. Interestingly, although not significant, we can detect a trend of higher K⁺/Na⁺ ratio in all NaCl treatments at SK compared with LK (Figure 2C), indicating the importance of high external K⁺ availability for this fungus to prevent Na⁺ accumulation in the mycelium. Other research investigating the *P. involutus* isolate MAJ found a similar Na⁺ uptake response to...
increasing salt exposure, although K⁺ accumulation increased only under very high salinity (Zhang et al., 2008). In the present study, the fungal Na⁺ uptake was increased by K⁺ deprivation in all saline conditions up to 100 mM of NaCl, but at 200 mM, Na⁺ concentrations were decreased by K⁺ deprivation (Figure 2B). Finally, although their actual transported substrate and specificity remain to be demonstrated, the K⁺-dependent regulation of PaACU1 and PaACU2 in axenic and symbiotic conditions may suggest an ability to transport K⁺, as already described for other filamentous fungi including M. oryzae and Candida albicans (Benito et al., 2004, 2011; Ruiz-Castilla et al., 2021). Additionally, their change in expression levels under varying Na⁺ concentrations in both axenic and symbiotic conditions, particularly at 100 mM of NaCl, might suggest a regulation by salinity and an adaptive behaviour. Similarly, the oscillatory expression patterns observed for PaTrk expression level when NaCl treatments changed might indicate a better permeability for Na⁺ than K⁺, as already described for HcTrk1 in H. cylindrosorum (Corratgé et al., 2007). The numerous differences in the transcriptional pattern of these three genes when the fungus colonized the roots or not indicate an important influence of the host plant in their expression level. However, it is impossible to conclude further on their actual function in axenic vs. symbiotic conditions since no data on their substrate, specificity, as well as cellular, and sub-cellular localization have been collected so far. Further work toward that path will shed light on the role these putative transporters have in fungal and plant nutrition, particularly because no K⁺ transport systems from the HAK or TOK family were identified in P. ammoniavirescens genome (Garcia & Zimmermann, 2014; Houdinet et al., 2022), suggesting a more simplified ability for K⁺ movement in Paxillaceae.

Regardless of salinity and external K⁺ availability, P. ammoniavirescens was able to develop ECM mantles on nearly all available short roots of loblolly pine. This indicates that the capacity to form ECM partnership is equally independent of NaCl exposure, in contrast to the impact of salinity on root morphology (Figure 7). In an earlier study investigating loblolly pine under salinity in interaction with other ECM fungi, similar unaltered colonization rates were observed for Sulillus luteus and S. tomentosus, but a decrease was reported in plants inoculated with P. tinctorius (Dixon et al., 1993). P. ammoniavirescens is a known generalist that may associate with multiple tree species and in very diverse environments (Jargeat et al., 2014). Here, our observations indicate that the extent of ECM colonization of NaCl-exposed loblolly pine is not limited by the impacts of salinity on fungal behaviour, but by the reduced development of available short-roots in saline conditions. Therefore, the physiological responses of both symbionts are important for influencing their interaction in saline environments (Figure 7).

External K⁺ supply determines the influence of Paxillus ammoniavirescens on loblolly pine salinity response

Our previous observations using rubidium as a proxy demonstrated that P. ammoniavirescens was able to transport K⁺ to inoculated loblolly pine seedlings, thus improving shoot K⁺ concentrations (Frank & Garcia, 2021). Here we observed that P. ammoniavirescens-mediated enhancement of loblolly pine K⁺ uptake under saline conditions compared with NM plants was improved by sufficient K⁺ availability. This may be partly due to the increased mycelial growth rate observed under sufficient K⁺ conditions (Figure 1), suggesting that hyphal soil exploration and K⁺ transport ability may be enhanced by sufficient K⁺ availability. More research is therefore needed to understand how morphological responses in the fungus might influence nutrient uptake and transport in symbiosis with loblolly pine under saline conditions.

In K⁺-sufficient conditions, the shoot Na⁺ accumulation was not significantly decreased by fungal inoculation when seedlings were provided with sufficient K⁺ (Figure 7). This reinforces the idea that loblolly pine was independently capable of attenuating shoot Na⁺ uptake under salinity in these conditions, regardless of ECM symbiosis, probably by an avoidance strategy that limits root growth. Similarly, P. involutus inoculation did not decrease the Na⁺ content in poplar leaves compared with non-inoculated controls (Langenfeld-Heyser et al., 2007), and shoot Na⁺ concentrations were higher in P. thunbergii seedlings when inoculated with P. tinctorius compared with NM controls under high salinity, similar to our observations at SK+100Na⁺ (Figure 6B). However, in K⁺-limited conditions at high salinity, we observed lower shoot Na⁺ concentrations in inoculated plants than in NM plants. In one study with P. banksiana and S. tomentosus, shoot Na⁺ accumulations were similarly decreased by inoculation at 60 mM of NaCl (Calvo Polanco et al., 2009). However, a different experiment with the same species found that shoot Na⁺ concentrations were higher in inoculated plants than non-inoculated ones when exposed to 200 mM of NaCl (Bois, Bigras, et al., 2006), suggesting that concentration thresholds may exist for salinity levels. Moreover, for some ECM partnerships, the fungal influence on shoot Na⁺ regulation in the host plant may depend on the responses of both species to conditions in the soil environment (Guerrero-Galán et al., 2019).

In natural ecosystems, loblolly pine roots are often colonized simultaneously by multiple species of ECM fungi (Hackman et al., 2022), which can vary greatly in their response to salinity and benefits for host salt...
tolerance. Therefore, further research exploring interactions among ECM fungi with the same host would help to reveal the factors that shape the assembly of root-associated symbiotic fungi under increasing salinity.

**AUTHOR CONTRIBUTIONS**

Benjamin D. Rose: Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing -- original draft. Marissa A. Dellinger: Investigation, visualization, methodology. Clancy P. Larmour: Investigation, methodology. Mira I. Polishook: Investigation, visualization. Maria I. Higuita: Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing -- review & editing. Sabine D. Zimmermann: Supervision, writing -- review & editing. Kevin Garcia: Conceptualization, funding acquisition, methodology, project administration, supervision, writing -- original draft.

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**CONFLICT OF INTEREST STATEMENT**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.0p2ngf27k.

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**REFERENCES**


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