Auxin-Producing Bacteria Isolated from the Roots of Illinois Spiranthes Orchids

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ABSTRACT

Orchid conservation efforts often focus on utilizing fungal symbionts to increase germination rates. Less well known is the role specific endophytic root bacterial associates play in promoting orchid growth. Notably, auxin-producing endophytic bacteria in orchid roots have been shown to induce orchid germination in the absence of a fungal symbiont. To determine if bacteria that produce auxin were present in *Spiranthes* orchids, bacteria were isolated from three native Illinois species and colorimetric analysis was used to quantify auxin production. Using 16s rRNA sequencing, a total of 15 isolates were found to produce measurable concentrations of auxin. *Spiranthes* seeds plated with media supernatant, which contained auxin from four isolates, did not significantly increase germination percent compared to seeds plated with a known fungal symbiont. To quickly determine if these isolates impacted root development in plants, the model plant *Arabidopsis* was utilized. *Arabidopsis* seeds did show decreased primary root formation

when plated on supernatant media containing auxin from the four isolates, suggesting the auxin has biological activity in plants. Two of the isolates were labelled with green fluorescent protein and found to colonize root structures of *Spiranthes magnicamporum* Sheviak. Overall, these findings suggest that root-associated bacteria that produce auxin should be examined more thoroughly for their impact on orchid growth for conservation purposes.

Keywords: *Spiranthes* orchids**,** Endophytic bacteria**,** Auxin production**,** Root-associated bacteria**,** Orchid conservation, Fungal symbionts

INTRODUCTION

The genus *Spiranthes* Rich. (Orchidaceae) contains terrestrial orchids typically found throughout temperate zones in North America and are commonly known as Ladies' Tresses (NatureServe 2022). These orchids have white flowers (occasionally pink) that are arranged helically along a terminal inflorescence (Dueck et al. 2014). Currently, there are 35 species recognized within the genus *Spiranthes* with nine being native to Illinois (Pace and Cameran 2017). Three of these Illinois native species, *Spiranthes cernua* (L.) L.C. Rich, *Spiranthes magnicamporum* Sheviak, and *Spiranthes vernalis* Engelm. & Gray, are in danger of population loss (NatureServe 2022) (Fig. 1). Notably, *S. magnicamporum* is listed as globally vulnerable, while *S. cernua* and *S. vernalis* are globally secure but listed, respectively, as vulnerable and critically imperiled in Illinois (NatureServe 2022). Both *S. cernua* and *S. magnicamporum* are autumn-flowering species with a widespread range that extends further north into Canada and are agamospermic depending on geographical location (Catling 1981; Pace and Cameran 2017; NatureServe 2022). Conversely, *S. vernalis* is a summer-flowering species in Illinois and is not known to be agamospermic, possibly contributing to its critically imperiled status (Catling 1981). To combat further population decline due to overcollection from wild populations and habitat

loss from urbanization, current conservation efforts for *Spiranthes* orchids involve the use of mycorrhizal fungi to improve germination and promote later stages of development such as flowering (Zettler and McInnis 1993).

Mycorrhizal fungi are required for orchid development because nearly all species start off as tiny, dust-like seeds that lack resource-containing endosperm (Barthlott et al. 2014). Because orchid seeds lack endosperm, they must obtain energy from another source to fuel growth and development prior to establishing photosynthesis. This energy requirement is fulfilled by mycorrhizal fungi which forms a beneficial relationship with the seeds to provide carbohydrates and nitrogen necessary for development (McCormick et al. 2006; Mohammadi et al. 2011). This union results in development of a protocorm which houses the fungal symbiont and produces a shoot apical meristem (Yeung et al. 2017). Thus, most orchid conservation efforts employ *in vitro* symbiotic germination (Fig. 2) with the seeds and mycorrhizal fungi collected from natural orchid populations (McCormick et al. 2006).

Because low germination rates and mycorrhizal fungi incompatibility significantly limit current conservation efforts, the development of new techniques should be explored (Zettler and McInnis 1993). Notably, bacteria have been discovered on the rhizoplane and endophytically within several terrestrial orchid roots (Li et al. 2017; Alibrandi et al. 2020; Kaur and Sharma 2021). Promising research has shown that endophytic Root Associated Bacteria (RAB) can protect against pathogens by priming plants for systematic resistance through ethylene, jasmonic acid, and salicylic acid defense signaling pathways (Brock et al. 2013; Glaeser et al. 2016). Root associated bacteria can also aid in plant nutrient uptake by solubilizing phosphorous, fixing atmospheric nitrogen, and processing insoluble iron present in soil (Rajkumar et al. 2009; Gupta et al. 2013; Afzal et al. 2019). More pertinent to orchid conservation is the ability of RAB to

promote seed germination possibly through the production of plant hormones (Tsavkelova et al. 2007b; Kaur and Sharma 2021).

One of the most common plant hormones produced by RAB in orchids is auxin in the form of Indole-3-acetic acid (IAA) (Júnior et al. 2011; Shah et al. 2021; Tsavkelova et al. 2016; Yang et al. 2014). Auxin is involved in almost every aspect of plant growth and development including root formation, germination, post embryonic development, vascular tissue development, and tropisms (Evans et al. 1994; Liu et al. 2013; Teale et al. 2006). Past research has indicated that exogenous application of auxins such as IAA and 1-Naphthaleneacetic acid (NAA) positively promote orchid germination by increasing protocorm diameters, trichomes, and DNA contents through endoreduplication (Lim and Loh 2003; Novak and Whitehouse 2013).

One conservation-focused advantage of RAB that produce auxin may be their ability to promote vegetative growth as well as germination in multiple orchid species (Tsavkelova et al. 2007b; Tsavkelova et al. 2016; Kaur and Sharma 2021). Germinated seeds from epiphytic orchid species outperformed asymbiotic germination controls, while established plantlets displayed increased root numbers and foliar area when inoculated with auxin producing RAB such as *Bacillus*, *Sphingomonas*, and *Enterobacter* (Tsavkelova et al. 2007b; Júnior et al. 2011).

Auxin is naturally transported cell-to-cell in a unidirectional manner known as polar auxin transport (PAT). Polar auxin transport inhibitors, such as monensin, obstruct the unidirectional flow of auxin and impede the allocation of auxin within certain plant regions thereby impacting organ development. When orchid seedlings were placed on media containing such PAT inhibitors, first leaf formation was diminished (Novak and Whitehouse 2013). In *Arabidopsis*, PAT occurs from an area known as the auxin organizing center to the site of

cotyledon initiation. This could suggest that protocorms have an auxin organizing center and that auxin plays an integral role in orchid germination by facilitating shoot development through PAT post-embryogenesis (Novak et al. 2014). Overall, the role of auxins produced by RAB and their potential to induce growth benefits are still unclear.

The purpose of this study is to: 1) isolate and compare the endophytic auxin-producing bacteria in three different *Spiranthes* species, 2) determine the genus of auxin-positive bacteria using 16s rRNA sequencing, 3) track bacterial localization within root structures using fluorescently labelled cells, and 4) perform *in vitro* symbiotic germination experiments with *Spiranthes* and *Arabidopsis* seeds to uncover potential benefits for growing orchids *in vitro* with auxin-producing bacteria.

METHODS

Root collection

Roots from three *S. cernua, S. magnicamporum,* and *S. vernalis* orchids were collected from populations in Illinois while the plants were flowering. Each species was collected from populations within 30 miles of each other (Table 1). Single roots were collected in sterile falcon tubes, stored at 4°C and processed within 48 hours of collection.

Root Surface Sterilization for Bacterial Collection

Roots were initially rinsed with DI water to remove residual soil. Roots were then surface sterilized with a 5:5:90 sterilization solution of alcohol (95% ethanol), bleach (7.5% sodium hypochlorite), and DI autoclaved water for two minutes with shaking. After sterilization, the roots were washed three consecutive times with DI water for one minute with shaking.

Isolation of Root Associated Bacteria

Surface-sterilized root samples were crushed using a sterile mortar and pestle with 5 mL of sterile DI water. A series of tenfold dilutions were carried out six times with each root sample. After this, 50 µL of each dilution was plated on BD™ Bacto™ Tryptic Soy Agar (TSA) plates and left to incubate at ambient temperature (21°C-23°C). Newly formed bacterial colonies were streaked on new TSA plates every 24 hours. Isolated colonies were grown in BD™ Bacto™ Tryptic Soy Broth (TSB) for 12 hours at 115 rpm at ambient temperature (21°C-23°C). Colonies cultured in TSB were mixed with glycerin and frozen at -80°C for storage.

Auxin Quantification

Bacteria were screened for auxin production using a colorimetric analysis assay. Colorimetric analysis is utilized to quantify an unknown concentration of a particular compound in a sample with the aid of a color reagent. In this case, colorimetric analysis is being used to determine the concentration of auxin in RAB supernatant samples and Salkowski reagent is the color reagent. Salkowski reagent reacts with auxin to produce a pink color change which can be measured and compared to a set of standards with known auxin concentrations to determine the amount of auxin in a sample.

Individual bacterial colonies were grown in TSB for 12 hours at 30°C with shaking at 115 rpm. Samples were diluted to an OD_{600} of 1.0 with sterile TSB. Following this, 100 µL of each diluted sample was used to inoculate 100 mL of TSB cultures in triplicate. These were incubated at 30°C at 115 rpm for 48 hours. One mL was centrifuged at 1000 xg for 5 minutes to remove cell bodies.100 µL of supernatant were transferred to a 96 well plate. A standard curve was made by adding 3-Indoleacetic acid (IAA) (Sigma Life Science, St. Louis, MO) to TSB to

create the following concentrations: 3.75, 7.5, 15, 30, 60 µg/mL Salkowski reagent (50 ml of 35% $HClO_4$ and 1 ml of 0.5 M $FeCl_3$) was added to each well in a 2:1 (v/v) ratio. The plate was incubated at ambient temperature for 60 minutes, followed by Abs 530 nm readings. The linear trendline constructed from the colorimetric curve was used to quantify the amount of auxin concentration using absorbance values.

Sequencing of Root Associated Bacteria

DNA was extracted from auxin-positive colonies using the DNeasy® PowerSoil Pro® Kit (Qiagen). After extraction, the Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich) was used to prep samples for PCR Amplification. The following primers were used to amplify the 16s rRNA gene: 16F27 Forward= 5'- AGAGTTTGATCMTGGCTCAG -3' and 16R1492 Reverse=5'- TACGGYTACCTTGTTACGACTT -3' (Heuer et al., 1997). The parameters of the thermocycler were set to run one cycle at 94 °C for 5 minutes, 35 cycles at 90 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 1 minute, and one cycle at 72 °C for 4 minutes. The PCR products were cleaned with the Zymo Research DNA Clean & Concentrator™ kit and sent to the Core DNA Sequencing Facility at the University of Illinois, Urbana-Champaign for sequencing. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used to identify the bacterial isolates that produced auxin (Altschul et al., 1990).

Creating Green Fluorescent Protein Labelled Bacteria using the Triparental Mating Method

Green Fluorescent Protein (GFP) is a protein that was originally isolated from jellyfish and is often used to label microorganisms as it produces a detectable phenotype (green fluorescence) when the protein is exposed to light in the blue to ultraviolet range. Therefore,

bacteria were created using a triparental conjugation system to insert a mini-Tn7 transposon containing P_{lac}:GFP into auxin producing RAB. Donor strains of *Escherichia coli* WM3064 containing the plasmid pURR25 (mini-Tn7-KSGFP; Teal et al., 2006) or pUX-BF13 (Tn7 transposase; Bao et al. 1991) were grown in TSB supplemented with 100 µg/mL Ampicillin (Amp) and 300 µg/mL 2,6-Diaminopimelic acid (DAP). The recipient auxin producing bacteria were cultured in regular TSB with no supplements. All samples were grown for 12 hours at 30°C. *E. coli* pURR25 and *E. coli* pUX-BF13 were subsequently diluted to 0.1 A in TSB supplemented with 300 µg/mL DAP and recipient bacteria were diluted to 0.1 A in TSB. Samples were incubated at 30°C for three hours with shaking at 115 rpm to reach log phase. 1 mL of each culture was harvested and mixed as follows: Recipient only (negative control); *E. coli* pURR25 only (negative control); *E. coli* pUX-BF13 only (negative control); Recipient + *E. coli* pURR25 + *E. coli* pUX-BF13 (positive control) and centrifuged at 10,000 rpm for 1 min to pellet. The supernatant was removed, leaving about 20 µL of residual media which was used to resuspend and spot the pellet onto TSA plates supplemented with 200 µg/mL DAP for a 12-hour incubation at ambient temperature (21°C-23°C). Bacterial spots were then collected in 1 mL of TSB and 200 µL of each were spread onto TSA plates supplemented with 20 µg/mL kanamycin (Kan). Plates were incubated at ambient temperature (21°C-23°C) for three days until colonies developed. GFP positive colonies were identified using a transilluminator, restreaked on new TSA plates, cultured in TSB and frozen with 20% glycerol at -80°C for storage. To confirm the recipient identity of GFP positive colonies, sequencing was performed as described above.

Inoculation of Orchids with GFP Labelled Bacteria

Roots of soil-established two-year-old *S. magnicamporum* orchids were sterilized as described above. Only *S. magnicamporum* orchids were used in this particular experiment due to

their availability. GFP-labelled *Enterobacter* spp. were grown in TSB and incubated at 30°C at 115 rpm for 48 hours. Each bacterial culture was placed in a centrifuge tube and pelleted at 1000xg for five minutes. The supernatant was decanted, and the bacterial pellet was resuspended in fresh TSB and sterile water in a 1:1 (v/v) ratio. The *S. magnicamporum* roots were then submerged in the diluted TSB media for 10 minutes at 115 rpm. Plants were transplanted into closed plastic containers with sterilized soil. After the initial transplant, 1 mL of bacterial broth was pipetted into the soil near the rhizosphere of each transplant. Root cross sections were prepared after 48-96 hours and fluorescently imaged using a Leica DM50000 B microscope. Brightfield and fluorescent images were merged using ImageJ.

Seed Collection and Processing

Spiranthes magnicamporum and *S. cernua* inflorescences were collected from the same root collection sites described above when flowers were wilted and seed capsules had begun to turn slightly brown. The inflorescences were placed in a 15 mL falcon tube without a lid. Tubes were placed in a mason jar containing approximately 6 cm of WiseSorbent Technology Indicating Silica Gel beads. Inflorescences were stored in a closed jar for a minimum of 10 days to remove moisture. Under a dissecting microscope, 2-3 capsules were placed on a piece of paper. Sterile forceps and scalpels were used to open capsules followed by manual shaking to dispense all seeds. Seeds were placed in a glass tube with a lid and parafilmed. Individual jars were then placed in a flacon tube filled a fourth of the way with silica beads. Tubes were stored in -20 °C freezer.

Preparation of OMA Plates Supplemented with Bacterial Auxin

Tryptic soy broth was inoculated with frozen bacterial isolates 3,4,5, and 8 and grown at 30°C with shaking at 115 rpm for 12 hours. Isolate concentration was then measured at 600 nm

and normalized to 1 A by diluting in sterile TSB. Following this, 100 mL of diluted bacterial sample was added to 100 mL of Oatmeal Broth (OMB) media with 200 μg/mL L(-)-Tryptophan, 99% (Acros Organics). Isolate cultures were collected after 48 hours of growth with shaking at 115 rpm and 30°C. Cultures were then placed in 50 mL tubes centrifuged at 1000 G for 5 minutes to collect supernatant. The pH of the supernatants was adjusted to 6.0 using HCl and NaOH and Bacto™ Agar was added to a final concentration of 1%. Supernatants were then autoclaved for 20 minutes and poured into petri dishes to make plates for germination studies. These plates were wrapped in aluminum foil and stored at 4°C to avoid deterioration of auxin by light and temperature.

Colorimetric Analysis of Bacterial Auxin Before and After Autoclaving

To determine if autoclaving would degrade bacterial auxin, each isolate was grown as described above. Supernatant from the culture was then tested for auxin concentration before and after a 20-minute autoclave exposure. To make blanks and standards for colorimetric analysis, OMB (pH 6) media supplemented with 200 μg/mL L(-)-Tryptophan, 99% (Acros Organics) was used.

Orchid Seed Sterilization and Sowing

About 200 *S. magnicamporum* seeds collected from Edwardsville, Illinois and *S. cernua* seeds collected from Pocahontas, Illinois were placed on filter paper that was folded and stapled. Packets were then placed in a clean container with 1 drop Tween/100 mL DI water. Seeds were agitated at 112 rpm using an orbital shaker for 10 minutes while soaking to eliminate surface tension. Packets were then removed and placed in a vessel with 0.5% NADCC (Sodium Dichloroisocyanurate) and 1 drop of Tween/100 mL. The seeds were agitated for 30 minutes in the bleach solution and subsequent processing was carried out in a biological safety cabinet to

ensure sterility. Packets were removed and put into sterile DI water with manual shaking for three minutes to dilute remaining NADCC. Packets were then cut open with sterile scalpels using forceps to stabilize them. Seeds were sowed on Oatmeal agar (OMA) plates supplemented with bacterial supernatant, OMA plates with 1 cm. fungus (positive control), and OMA plates without fungi. The fungus used in the positive control plates was *Ceratobasidium* collected from Stoddard County, MO from *Spiranthes praecox* in 2018 by Caleb Dvorak. Plates were then wrapped in aluminum foil and stored at ambient temperature (21°C-23°C). Germination scores were assigned after 90 days using a dissection microscope to examine seeds.

Arabidopsis Seed Sterilization, Sowing, and Root Analysis

Wildtype (Col-0) *Arabidopsis* seeds were submerged in 1 mL of a 30% bleach (7.5% sodium hypochlorite) and 0.1% Tween20 solution and mixed for 15 minutes horizontally on a vortex. Seeds were then pelleted by centrifuging at 6,000 G for 10 seconds and the supernatant was replaced with sterile dd H_2O water. This wash procedure was repeated three times for a total of four rinses. For the last step, the water was not decanted and kept in the tube. The tube was covered with aluminum foil and placed at 4 °C for 72 hours before sowing on ½ Murashige and Skoog (MS) plates (Caisson Labs) (0.8% Bacto™ Agar, pH=5.8, and 1% sucrose), OMA plates (pH=6), and OMA plates containing supernatant from isolates 3, 4, 5, and 8. For each plate, 10 seeds were sowed in a single row 0.7 cm apart. Plates were incubated at ambient temperature (21°C-23°C) for 12 hours in the dark, uncovered, and then placed vertically in a growth chamber with a 18:6 L/D cycle. Photos of the plates were captured every three days using a Canon EOS Rebel T3i Digital SLR Camera with EF-S 18-55mm f/3.5-5.6 IS lens. Photos were then analyzed using ImageJ to measure primary root length.

RESULTS

Isolation of RAB and Quantification of Auxin Production in *Spiranthes* Orchids

To determine if any *Spiranthes*-associated endophytic bacteria were capable of auxin production, RAB were isolated from three species of *Spiranthes* orchids and tested with Salkowski reagent to identify auxin production. A total of 15 isolates were found to produce auxin. Of these, six belonged to the *Enterobacter* genus, six belonged to the *Agrobacteria* genus, two belonged to the *Pantoea* genus, and one belonged to the *Bacillus* genus as determined from 16s rRNA sequence analysis (Table 2). *Enterobacter* isolated from *S. cernua* produced the most auxin with an average concentration of 12.92 µg/mL. In comparison, the lowest auxin-producing RAB belonged to the *Bacillus* genus isolated from *S. vernalis*. Supernatent from this strain showed an average auxin concentration of 0.24 µg/mL (Fig. 3). Notably in *S. ceruna*, all auxin-producing isolates belonged to the *Enterobacter* genus, while *Agrobacteria* were commonly found in *S. vernalis* specimens. *Spiranthes magnicamporum* orchids housed a mixture of *Enterobacter* and *Pantoea* bacteria (Table 2)

Localization of Auxin Producing Root-Associated Bacteria in Roots

To determine the location of auxin-producing isolates in *S. magnicamporum* roots, isolates two and four were transformed with a constitutively expressed GFP gene. Both isolates were *Enterobacter* sp. with a 99.85% 16S rRNA sequence identity. After 48 hours, fluorescent microscopy revealed that these bacteria colonized root hairs, epidermal layers, the cortex, and steles (Fig. 4). Notably, a high concentration of bacteria was observed colonizing root hairs, while a low concentration of bacteria was observed colonizing root steles.

Impact of Auxin by *Spiranthes* RAB on Orchid Germination

To determine if the auxin produced by the bacterial isolates was biologically active and could impact germination or root growth, *S. magnicamporum* and *S. cernua* were grown on agar plates supplemented with bacterial culture supernatant. The auxin concentrations of supernatants from individual isolates grown in oatmeal broth was determined using colorimetric analysis before and after being autoclaved. These analyses indicated that the supernatant from all isolates still appeared to contain comparable levels of auxin before and after sterilization, suggesting that the auxin is stable during this process. Specifically, supernatant from isolate 4 contained the most auxin with an average of around 33 μg/mL (Fig. 5a). Slightly higher concentrations post-autoclaving may be due to evaporation. Increased concentrations of auxin in autoclaved supernatant compared to initial quantification of auxin in samples is most likely contributed to the addition of tryptophan in culture broth.

To examine if auxin produced by the isolates could improve orchid germination in the absence of a fungal symbiont, *S. magnicamporum* and *S. cernua* seeds were sown on supernatant-supplemented plates. For *S. magnicamporum* germination, the OMA bacterial supernatant plates containing auxin were comparable to the negative control which had seeds placed on plain OMA plates with no fungal symbiont (Fig. 5b). The positive control plates containing a known fungal symbiont had the highest rate of germinated seeds with 21.7% of seeds reaching the germination stage. Seeds grown on plates made with supernatant from isolate 8 contained the most germinated seeds (6.7%) among isolate plates that germinated; however, rhizoid production was not observed. *Spiranthes cernua* germination plates performed similarly with isolate 8 plates showing the most seeds in stage three (2.7%). However, *S. cernua* seeds grown on plates with a fungal symbiont only resulted in 2 out of 197 seeds successfully germinating, suggesting that this overall germination approach may not be suitable for this

species. All isolate plates were also comparable to the negative control that did not contain a fungal symbiont, suggesting that in the absence of mycorrhizal fungi, the auxin produced by these bacteria do not impact germination (Fig. 5c).

Impact of RAB Auxin on *Arabidopsis* Germination and Root Growth

To determine if the auxin produced by the RAB was biologically active, *Arabidopsis* germination and root growth were quantified on agar plates supplemented with RAB supernatant. *Arabidopsis* was utilized because of its high germination reliability, quick root development compared to *Spiranthes* orchids, and the ample amount of auxin-related literature on its growth. *Arabidopsis* seeds plated on OMA bacterial supernatant plates containing RAB supernatant had comparable germination results to the control plates (OMA and 1/2MS) (Fig. 5d). Notably, ½ MS plates displayed 100% seed germination while OMA and all other isolate plates displayed a 90% germination rate. Seeds grown on isolate 8 plates displayed the lowest germination rates at 84.2%. Nine days after sowing seeds on auxin plates, the primary root lengths of *Arabidopsis* seedlings were an average of 2.5 cm when grown on 0.5x MS media. Plants grown on OMA media resulted in root lengths that averaged 0.5 cm. All plants grown on isolate plates (containing bacterial supernatant grown in OMA media) resulted in root lengths that were less than 0.2 cm in length and were significantly different than the root lengths of plants grown on OMA media as determined by a one-way ANOVA test ($p < 0.05$) (Fig. 5e), suggesting that the auxin produced by the RAB is active and able to influence *Arabidopsis* growth.

<div align="center">**DISCUSSION**</div>

Colorimetric Analysis of RAB for Auxin Quantification

Auxin assays involving colorimetric quantification revealed endophytic microbes that were auxin-positive in *Spiranthes* roots. *Pantoea agglomerans* and *Enterobacter cloacae* were high-auxin producers while *Agrobacterium tumefaciens* and *Bacillus manliponensis* were low-auxin producers. It should be noted that while auxin compounds were produced by isolates from *Spiranthes* orchids, the specific types of auxins were not deduced. Various research shows that common auxin compounds produced by RAB from orchids are indole-3-lactic acid (ILA), indole-3-acetaldehyde (IAAld), indole-3-propionic acid (IPA), and IAA (Tsavkelova et al. 2007b; Júnior et al. 2011). Previous research using height performance liquid chromatography (HPLC) suggests that *Enterobacter* isolates are primarily producing IAA and IPA, while *Bacillus* isolates are primarily producing ILA (Júnior et al. 2011). A limitation of using colorimetric analysis with Salkowski reagent to quantify auxin production is that IAA concentrations have been observed to be as much as 19 times greater than HPLC detection methods. This discrepancy could be attributed to the addition of tryptophan, an IAA precursor, often added in other studies to increase IAA production by RAB or by the accumulation of other indole compounds which are sensitive to Salkowski reagent (Tsavkelova et al. 2007b). Therefore, IAA levels determined using Salkowski reagent are likely inflated, with the functional auxin produced at levels lower than reported. In future studies, HPLC quantification could improve IAA concentration accuracy. (Sachdev et al., 2009; Júnior et al., 2011).

Characterization of RAB that Produce Auxin in *Spiranthes* Orchids

The breadth of auxin-producing RAB reported here is consistent with orchids collected from Vietnam and Brazil (Tsavkelova 2007a; Júnior et al. 2011). However, these results are not directly comparable as many of these RAB were found in orchid species that were epiphytic, while our research identified *Enterobacter*, *Agrobacterium*, and *Pantoea* in terrestrial species.

Thus, this could suggest that various orchid species, despite type and location, share a common auxin-producing endophytic profile. These findings further implicate the need for RAB to be characterized for conservation purposes as their use could be implemented across several orchid species. This would offer great advantages compared to conservation efforts that use fungal symbionts, which can be species dependent.

Location of Auxin-Producing RAB in *S. magnicamporum* Plants

Isolated auxin-producing RAB, specifically *Enterobacter* sp., was confirmed to be present endophytically within *Spiranthes* roots. Large concentrations of bacteria colonizing root hairs suggest this route as a common source of entry. *Enterobacter* was also discovered to colonize cortical cells but appeared to be in greater abundance in cortical cell walls. These results support previous research in which GFP labelled *Pseudomonas*, another RAB commonly found in terrestrial orchids, greatly colonized root hairs and cortical intercellular spaces of olive roots (Prieto et al. 2011; Alibrandi et al. 2020). Our results also found that *Enterobacter* was present in small abundances within the root stele supporting prior research detailing *Enterobacter* sp. colonization in *Brassica oleracea* (Tanaka et al. 2006).

Impact of Auxin produced by RAB on Plant Growth

Plants are known to produce a wide variety of auxins, not all of which show the same levels of activity and modes of regulation (Zhao et al. 2010). While the likely function of bacterial-produced auxin is to somehow regulate plant growth and development in a way that benefits the bacteria, compounds reacting with the Salkowski reagent are not necessarily biologically active in plants. As a quick test to determine if this auxin is biologically active, we chose an *Arabidopsis* root-growth assay because of the well-established root-growth-inhibition phenotype caused by excess auxin in growth media (Evans et al. 1994). Indeed, addition of the

RAB supernatant to growth media suppressed almost all root growth, suggesting that these compounds are active in plants. In spite of this, the RAB supernatant failed to have an impact on germination in either *Spiranthes* or *Arabidopsis*. This was not surprising for *Arabidopsis*, as auxin has not been shown to have much impact on its germination (Liu et al. 2013). While previous work in other orchid species has suggested that auxin-producing RAB can have a positive impact on germination, our results did not support this (Tsavkelova et al. 2007b; Tsavkelova et al. 2016). Conversely, our results were loosely supported by conflicting research which has shown that exogenous application of auxin upregulates production of Abscisic Acid (ABA), a plant hormone that delays germination (Shuai et al. 2017; Liu et al. 2013). The concentration of IAA present in the supernatant plates may have also played a role in deterring germination in *Spiranthes* orchids. Evident by past research on orchids, IAA concentrations that are too high or too low can have a negative impact on protocorm diameter size and first leaf emergence (Novak and Whitehouse 2013). Past studies on orchids with increased germination rates due to RAB utilized living bacteria, while in this study bacteria were killed by autoclaving and only the supernatant was collected and used for germination experiments. These bacteria could therefore be producing other metabolites that could benefit germination other than auxin (Tsavkelova et al. 2007b). When considering that complicated germination requirements present a barrier to efficient orchid conservation efforts, improvements promoted by auxin-producing RAB would be welcomed in the field. Unfortunately, the isolates reported here do not appear to have a beneficial impact on *Spiranthes* germination, although this does not preclude eventual discovery of beneficial impacts of this microbe-plant association in other species of orchids.

CONCLUSIONS

Root associated bacteria that produced auxin were found endophytically within the roots of *Spiranthes* orchids. GFP-labelled RAB confirmed that bacteria could colonize root structures and suggested root hairs as a common source of entry. While culture supernatant from these bacteria did not appear to promote germination in *S. magnicamporum*, *S. cernua*, or *Arabidopsis*, they did significantly decrease the length of primary roots in *Arabidopsis,* suggesting biological activity in plants. While more tests would need to be conducted to confirm that the auxin produced by the RAB was directly impacting root growth, these results are consistent with prior research on exogenous application of auxin in plants. Overall, these results outline the need to examine root microbes in concurrence with mycorrhizal fungi for conservation purposes.

ACKNOWLEDGEMENTS

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Table 1: Sample collection information. Information regarding the location identity and collection dates for root samples.

Orchid Species	Location	Collection Dates
S. cernua	Pocahontas, IL	September 24, 2022
S. magnicamporum	Edwardsville, IL	September 20, 2022
S. vernalis	Edwardsville, IL	July 14, 2022

Table 2: Auxin-producing bacteria isolated from *Spiranthes* roots. Top NCBI nucleotide BLAST scores for each bacterial isolate that produced auxin. Isolate 1 served as a negative control and did not produce auxin.

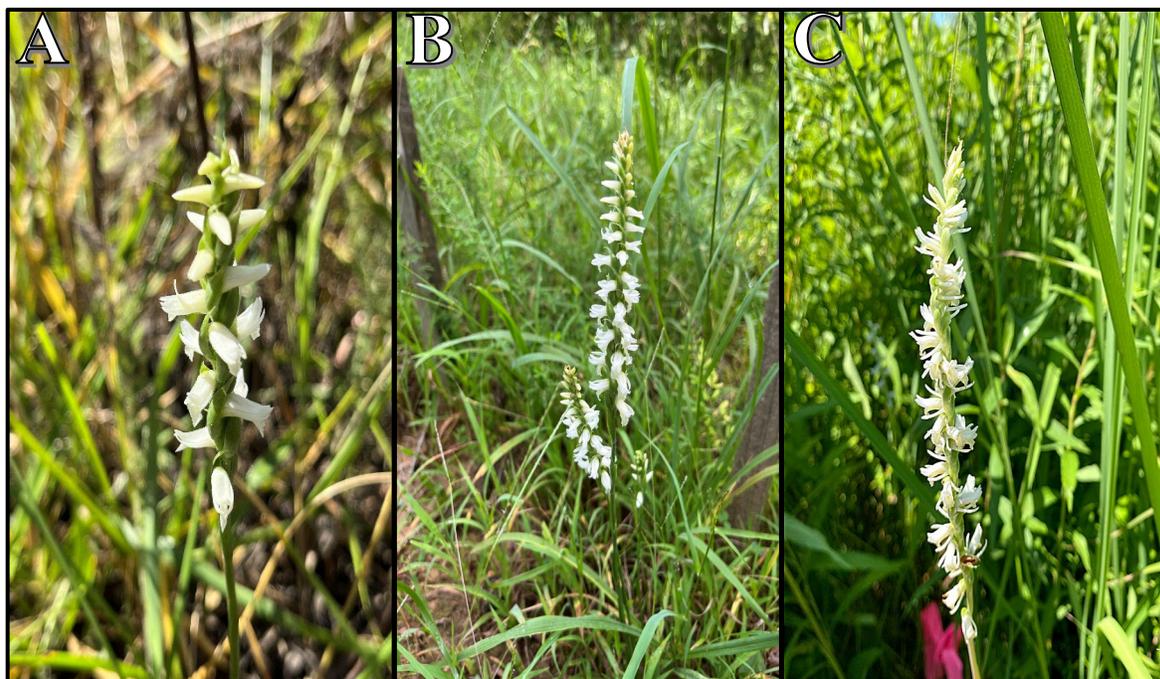| Isolate Number	Species Isolated From	BLAST Description	Bit Score	E Value	Percent Identity	Query Cover	Accession
1	*S. magnicamporum*	*Bacillus cereus* strain yasmun8	1,443.35	0	96.9	100	OK632087
2	*S. magnicamporum*	*Enterobacter huaxiensis* strain WCHEHu090008	1,238.38	0	100.0	100	OP818075
3	*S. magnicamporum*	*Pantoea agglomerans* strain DAPP-PG734	1,264.23	0	100.0	100	OW970315
4	*S. magnicamporum*	*Enterobacter huaxiensis* strain WCHEHu090008	1,284.54	0	99.9	100	OP818075
5	*S. magnicamporum*	*Pantoea agglomerans* strain DBM 3797	1,275.31	0	99.3	100	CP086133
6	*S. magnicamporum*	*Enterobacter huaxiensis* stain WCHEHu090008	1,415.65	0	99.6	100	OP818075
7	*S. cernua*	*Enterobacter Cloacae* isolate 96	1,389.80	0	100.0	100	OW969784
8	*S. cernua*	*Enterobacter Cloacae* isolate 732	1,308.55	0	100.0	100	OW969624
9	*S. cernua*	*Enterobacter Cloacae* isolate 96	1,424.89	0	100.0	100	OW969784
10	*S. vernalis*	*Agrobacterium tumefaciens* P8-19	1,788.68	0	100.0	100	MN181152
11	*S. vernalis*	*Agrobacterium tumefaciens* strain RB`-076	1,781.29	0	100.0	100	MT453929
12	*S. vernalis*	*Agrobacterium tumefaciens* strain GU-RP21	1,714.81	0	100.0	100	OQ421733
13	*S. vernalis*	*Agrobacterium tumefaciens* strain GU-RP21	1,554.15	0	100.0	100	OQ421733
14	*S. vernalis*	*Agrobacterium tumefaciens* strain P8-19	1,899.48	0	100.0	100	MN181152
15	*S. vernalis*	*Bacillus manliponensis* strain 12L-2	1,790.52	0	100.0	100	KT720103
16	*S. vernalis*	*Agrobacterium tumefaciens* strain GU-RP21	1,779.44	0	99.9	100	OQ421733

Figure 1. Photos of *Spiranthes* orchids: A) *Spiranthes cernua* collected in Pocahontas, IL**;** B) *Spiranthes magnicamporum* collected in Edwardsville, IL; C) *Spiranthes vernalis* collected in Edwardsville, IL.
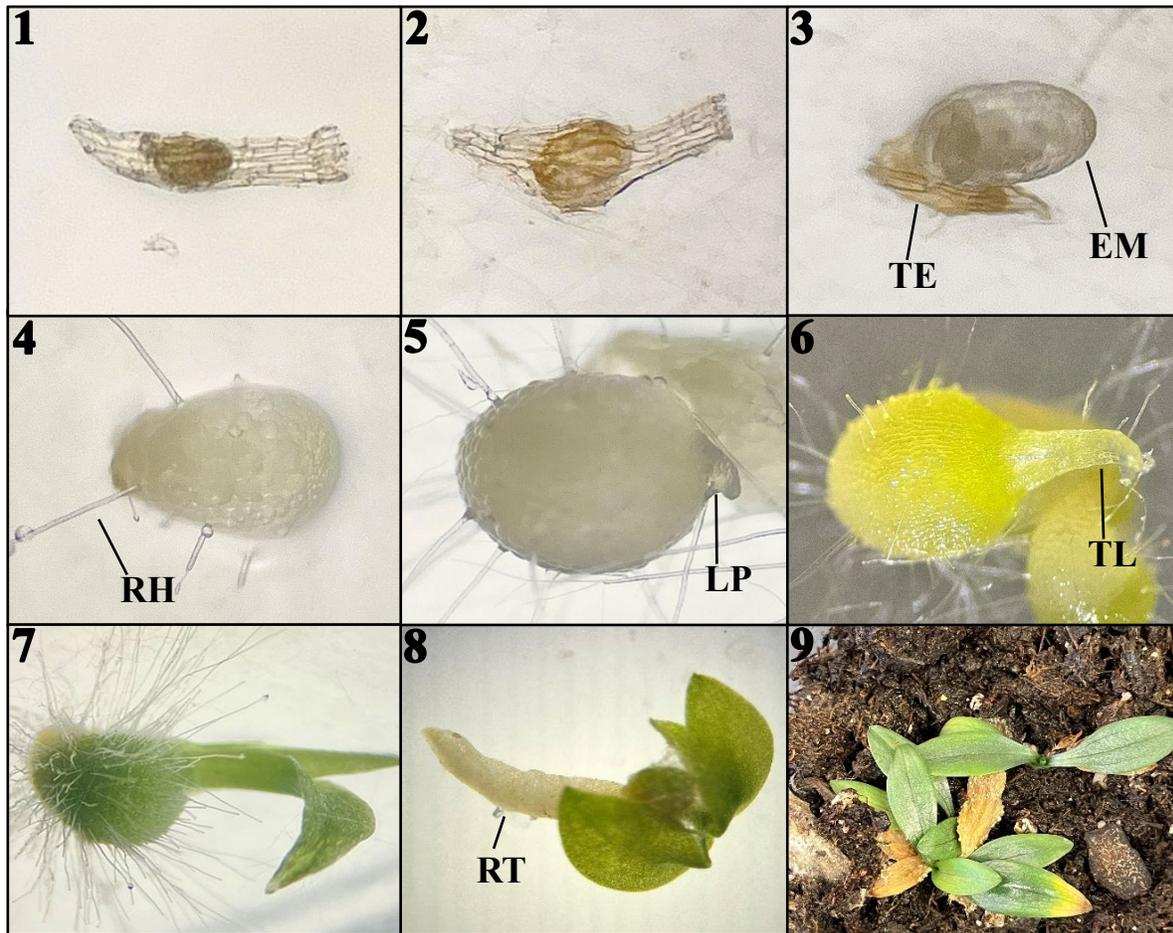
Figure 2. *Spiranthes* germination stages: 1. No germination; 2. Embryo swells; 3. Germination-swollen embryo (EM) ruptures testa (TE); 4. Protocorm produces rhizoids (RH); 5. Emergence of leaf primordium (LP); 6. Development of first true leaf (TL); 7. Elongation of leaves; 8. Emergence of roots (RT); 9. Orchids established in soil.
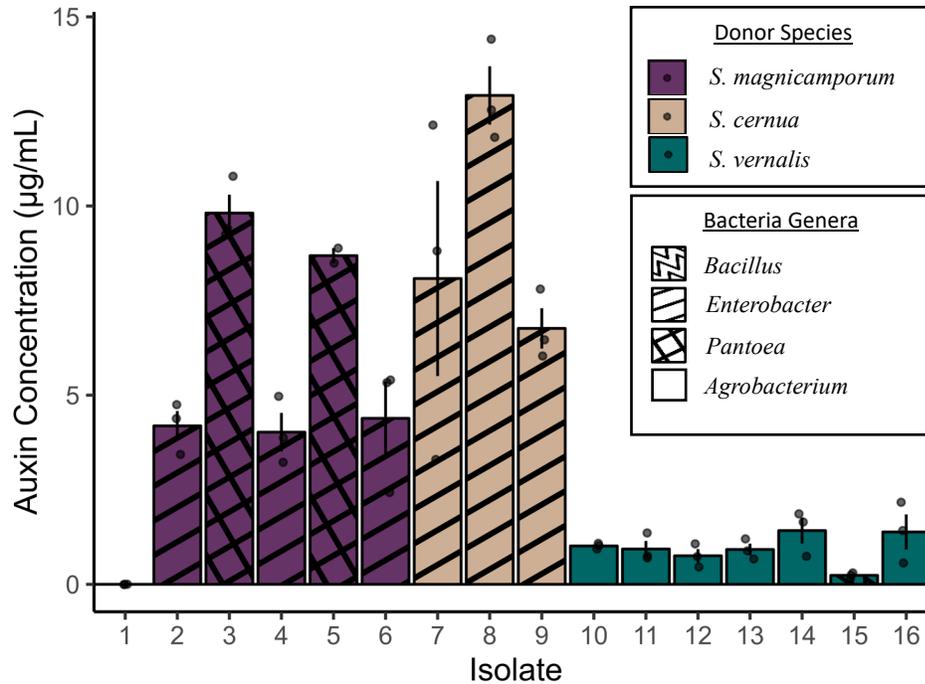
Figure 3. Auxin quantification in RAB supernatent. Amount of auxin produced by each bacterial isolate grown in tryptic soy broth for 48 hours at 30°C. Black dots represent the average of three technical replicates. Each bar represents an average of three biological replicates. Bar color corresponds to *Spiranthes* species from which the isolates were identified. Isolate 1 served as a negative control and did not produce auxin. Bars represent +/- standard error of the three biological replicates.
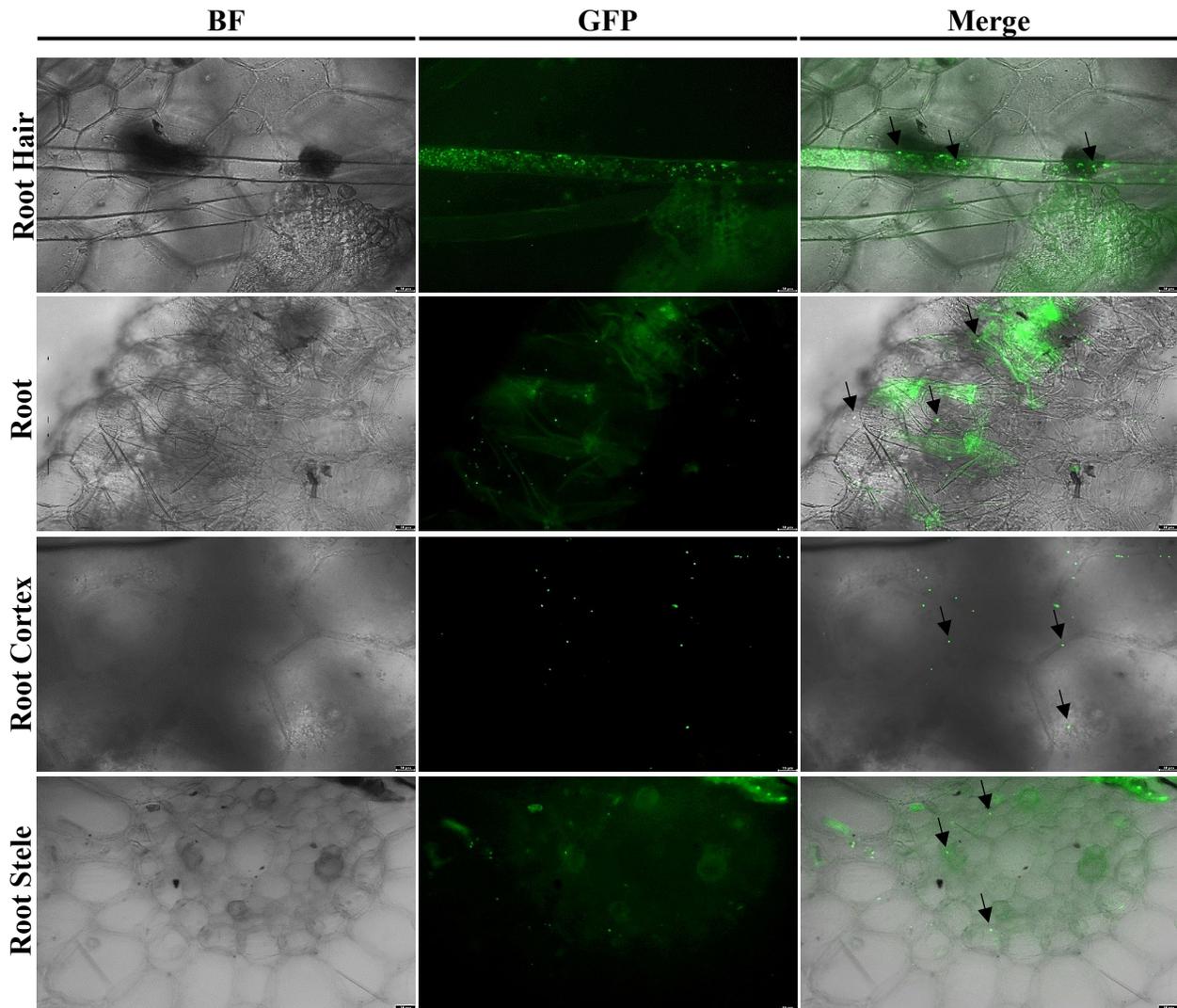
Figure 4. Infection of *S. magnicamporum* plants with GFP-labelled auxin producing RAB. *Enterobacter* sp. expressing GFP was introduced into *S. magnicamporum* plants and analyzed using fluorescent microscopy. Black arrows represent *Enterobacter* sp. that successfully infiltrated the roots. Scale bars present in each image are 15 μm. The image columns are labelled BF (images generated using bright-field microscopy), GFP (images generated using fluorescent microscopy which detects GFP labelled bacteria), and Merge (BF and GFP images are combined).
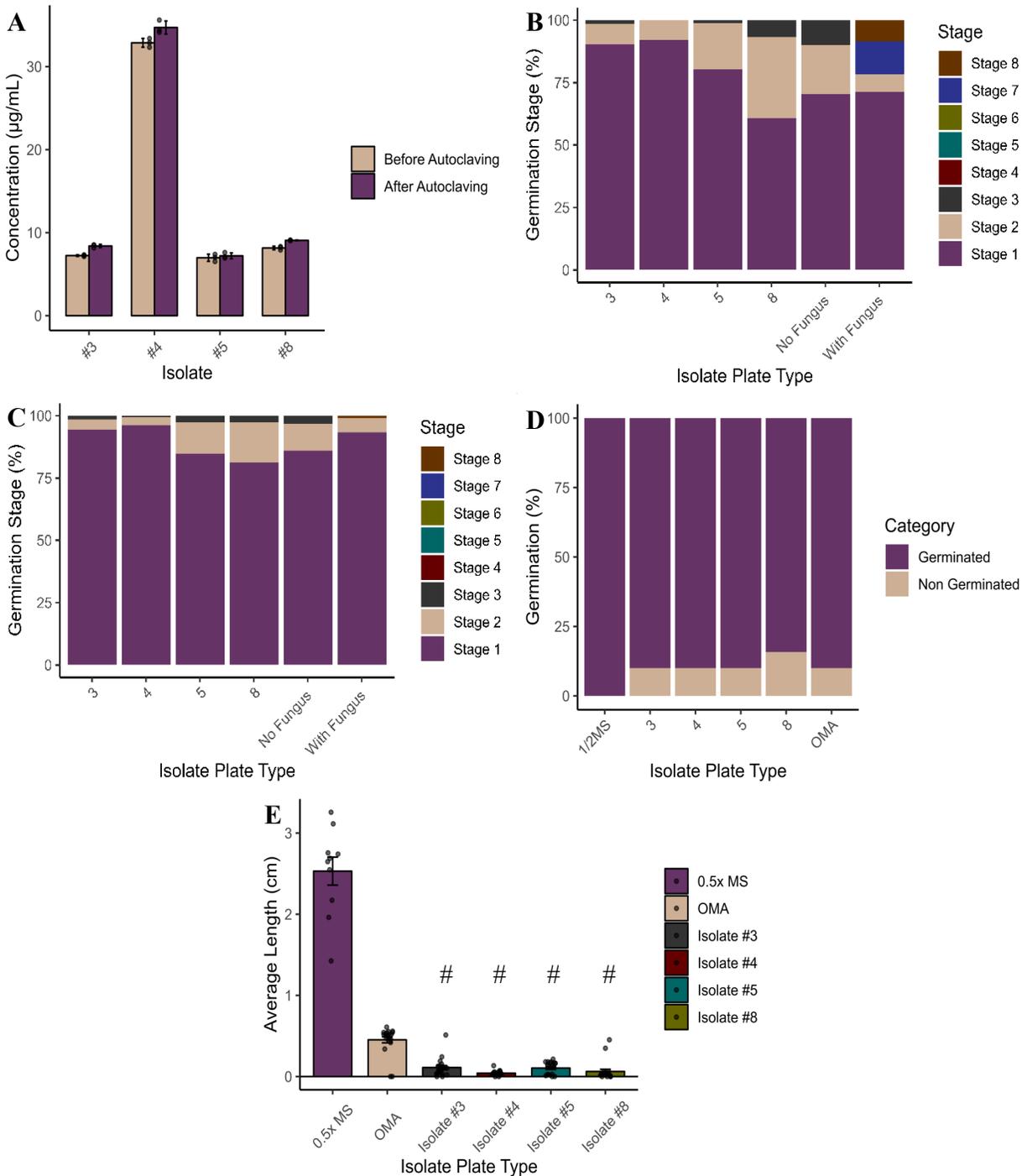
Figure 5. Impact of auxin-producing RAB on *Spiranthes* and *Arabidopsis* germination and growth: A) Concentration of auxin present before and after supernatant from isolates was autoclaved for 20 minutes. For each isolate tested, n=3; B) Percent germination of *S. mangicamporum* seeds grown on plates supplemented with RAB culture supernatent. Stages correspond to the stages represented in Figure 2. Therefore, true seed germination occurs at stage

three. For isolate plate type 3 (n=135), 4 (n=102), 5 (n=258), 8 (n=253), No Fungus (n=91), With Fungus (n=129); C) Percent germination of *S. cernua* seeds grown on plates supplemented with RAB culture supernatent. For isolate plate type 3(n=125), 4(n=153), 5(n=197), 8 (n=75), No Fungus (n=122), With Fungus (n=197); D) Percent germination of *Arabidopsis* seeds grown on plates supplemented with the supernatant of auxin producing bacteria. For each treatment, n=20, except for plants grown on ½ MS media (n=10); E) Average primary root lengths of *Arabidopsis* plants grown on plates containing the supernatant of auxin producing bacteria. For each treatment, n=20, except for plants grown on ½ MS media (n=10). Error bars represent+/- standard error. The # represents a significant decrease in primary root length compared to the OMA control as determined by one-way ANOVA test ($p < 0.05$).