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Identification of plant growth promoting rhizobacteria for Ballona Wetlands Restoration

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ABSTRACT

The Ballona Wetlands Ecological Reserve (BWER) is home to many California native plant species which contribute to ecosystem services of flood and erosion mitigation, water quality improvement, carbon sequestration, and habitat provisions for threatened and endangered species. Plant growth promoting rhizobacteria (PGPR) improve plant tolerance to abiotic stressors and suppress pathogens through the alteration of nutrient absorption, water uptake, and enzymatic activity, and have potential in conservation strategies by aiding revegetation methods. Eight bacterial strains were isolated from native Frankenia salina and Baccharis pilularis in the BWER to determine whether these microbes act as PGPR. The strains were characterized using PGPR biochemical assays such as phosphate solubilization, cellulase activity, and salt tolerance. Results have shown that all strains are negative for phosphate solubilization while four strains demonstrate cellulase activity, seven strains are tolerant of 5% NaCl and five are tolerant of 10% NaCl. The high salt tolerance of these bacteria may be contributing to the salt tolerance of native species in the BWER. Given these findings, in vitro germination and growth assays using Camissoniopsis cheiranthifolia seeds were carried out to determine the role of these microbes in facilitating the restoration of native coastal species in the BWER.

INTRODUCTION

Coastal wetlands are habitats located between the land and ocean, most prominently referring to mangroves and marshes, but broadly including various water bodies such as seagrass meadows, coral reefs, estuaries, and coastal water bodies within -6m depth (Li et al. 2018). Wetland ecosystems may be defined as salt, brackish, or freshwater tidal marshes, forested wetlands such as riparian and floodplain, or inland freshwater and saline systems including sedge meadows, wet prairies, emergent, or seasonal wetlands such as mudflats (Rodrigo 2021). Despite vast variation, wetland ecosystems share the common characteristics of small surface areas (in comparison to other terrestrial ecosystems) yet extremely high productivity. For example, the average net primary productivity of temperate salt marshes (1.7 $kgm^{-2}year^{-1}$) is higher than that of temperate forests (1.2-1-3 $kgm^{-2}year^{-1}$) and provide a vast array of ecosystem services including food and resources, wildlife habitat, carbon sequestration, storm surge protection, sediment accumulation, and water purification (Li et al. 2018). Historically, anthropogenic impacts such as development, subsurface mining, and pollution have resulted in a loss of more than half of global wetland habitats and climate change impacts including sea level rise and drought continue to threaten these habitats (Li et al. 2018, Rodrigo 2021) One particularly problematic issue is the introduction of invasive species, which further degrade wetland habitats and contribute to the overall loss of biodiversity. This causes concern due to the role of biodiversity in promoting ecosystem resilience against the negative impacts of environmental fluctuations (Elliot, Igl, & Johnson 2020).

In Southern California, the Ballona Wetlands Ecological Reserve (BWER) represents the last remaining major coastal wetlands in Los Angeles County and its watershed represents one of the most developed in the United States, with urbanized areas making up approximately 80

percent of the 130 square miles. Once encompassing over 2000 acres of land, development and channelization has resulted in less than 100 acres of the wetland habitats continuing to receive estuarine waters. Hydrological impacts include fill deposits from construction, resulting land surface elevation changes and the alteration of soil permeability, conversion of marsh to agricultural fields, tidal exchange reduction, and rerouting of natural freshwater and tidal flows (Bergquist et al. 2012). In Area A of the BWER, much of the habitat is degraded as a result of said fill deposits from the construction of Marina Del Rey. Native plants including *Baccharis pilularis* (coyote brush) and *Frankenia salina* (alkali heath) can be found, however the area lacks much of the plant diversity which can be found in adjacent areas, for example the disappearance of species such as *Camissoniopsis cheiranthifolia* (beach evening primrose). These species are representatives of 407 taxa which comprise the flora of BWER, 40% of which are not native to California. This percentage is alarmingly high in comparison to the 17% of non-native taxa in California as a whole (Read 2015).

B. pilularis is a shrub in the family Asteraceae which grows up to 3 meters tall with leaves 8 to 55 millimeters long. In California, it ranges from the northwestern coast south and eastward to the Sierra Nevada foothills and is found on coastal bluffs and oak woodlands from sea levels up to 1500 meters. Its seed dispersal begins in November and germination occurs in winter and early spring. Historically, it has been used by Native Americans medicinally in a tea to treat poison oak rash (Francis 2004). *F. salina* is a rhizome-producing, woody perennial subshrub inhabiting coastal and inland salt marshes. These plants can form extensive mats up to several meters across and may form adventitious roots at the nodes. Additionally holding cultural significance, this plant was used by indigenous people for salt extraction due to its glandular excretion mechanisms which function to remove excess salt, aiding in ionic homeostasis in salt

marsh habitats (Whalen 1987). *F. salina* is a member of the Frankeniaceae family and was selected for the West Steve Marsh Revegetation to be established in the middle marsh zone based on its salt tolerance, tidal inundation tolerance, and competition ability (Aquatic Outreach Institute 2004). Additionally, *F. salina* was reported as the third most abundant tidal marsh species at Tijuana Estuary and the third most resistant to invasion, owing to its ecological importance in estuarine habitats (Bonin & Zedler 2008).

Plant growth promoting bacteria (PGPB) and rhizobacteria (PGPR) have been used in the agricultural realm based on features such as nitrogen fixation, indole acetic acid (IAA) production, siderophore secretion, and phosphate solubilization (Montanez et al. 2012). PGPR are root-colonizing non-pathogenic bacteria which have been found to increase plant tolerance against phytopathogens through antibiotic synthesis, antifungal metabolites, direct competition, and induction of systemic resistance in plants. In addition to increasing plant tolerance to biotic stressors, PGPR can increase tolerance to abiotic stressors such as flooding, drought, salinity, heavy metal contaminants, and high and low temperatures through 1-aminocyclopropane-1-carboxylate (ACC) deaminase production, which is a precursor of the plant hormone ethylene and is theorized to decrease ethylene levels in plants (Maheshwari & Dubey 2008). Inoculation with PGPR has promoted plant growth with and without stress applications for important crops such as tomatoes, maize, rice, raspberries, wheat, and many more (Almaghrabi, Massoud, & Abdelmoneim 2013, Pereira et al. 2020, Lavakush et al. 2014, Orhan et al. 2006, Kumar, Maurya, & Raghuwanshi 2014). PGPR have widely established applications in agriculture, however recently have been applied in the realm of restoration ecology. *Bacillus tequilensis* SH1RP8, isolated from indigenous coastal sand dune plants improved the shoot growth and dry weight of

Peucedanum japonicum (coastal hog fennel) and *Arundo donax* (giant reed), and is being used in an effort to restore vegetation and prevent coastal sand dune erosion (Hong & Lee 2014).

PGPR have been studied in the context of wetland restoration to determine the role of beneficial microbes in promoting plant growth and stress tolerance of dominant wetland vegetation (Belding 2018, Bledsoe & Boopathy 2016). Furthermore, PGPR have been investigated in the BWER specifically in the effort to improve native plant restoration (Lyford et al. 2023, Alverson & Johnston 2021). In this study, bacterial strains isolated from *B. pilularis* and *F. salina* were used to promote germination and growth of *C. cheiranthifolia* in the attempt to improve its establishment during restoration activities in the BWER. This study focuses on addressing current gaps in the research of PGPR which specifically benefit wetland vegetation and promote stress tolerance mechanisms and sustainable restoration planning for the BWER.

METHODS

Bacterial Isolation

To determine the role of bacteria isolated from native plant species, bacteria were isolated from native seeds. Seeds of *Frankenia salina* were collected from BWER in September 2023 and seeds of *Baccharis pilularis* were collected from BWER in October 2023. Seeds were subsequently surface sterilized in 70% EtOH for one minute, rinsed with sterile water, soaked in a 50% NaClO solution for 10 minutes, and rinsed five times with sterile water. Seeds were then vortexed in a suspension of 1 mL 10 mM $MgSO_4$ and 250 µL of the supernatant was spread plated on tryptone yeast extract (TY) agar and yeast mannitol agar (YMA) plates to serve as controls. Seeds were transferred to sterile mortar and pestles and ground with 1 mL 10 mM $MgSO_4$. Solution was transferred into a sterile microcentrifuge tube and 100 µL was spread

plated onto TY and YMA plates. Three replicates were plated for each type of media and for *F. salina* and *B. pilularis* seeds. Plates were incubated at 30°C and growth was observed after five days, upon which selected colonies were restreaked and isolated. Strains were suspended in TY supplemented with 20% glycerol for permanent storage in a -80°C freezer.

Bacterial Characterization and Identification

To characterize the isolated bacteria, salt tolerance of the strains was determined by growth on TY agar media supplemented with 0%, 5%, and 10% NaCl. Eight strains were evaluated on a single plate and three replicates were performed for each salt concentration. Cellulase activity was determined by the formation of a halo on carboxymethyl cellulose (CMC) plates flooded with Gram's iodine. Phosphate solubilization was determined by the formation of a halo on phosphate media. Plates were incubated at 30°C and growth or halos were observed after three days. Motility was determined by movement throughout TY + 0.25% agar and was observed after 24 hours.

To identify isolated bacteria, DNA extraction was performed using Chelex 100 Resin (BioRad). Bacterial colonies were suspended in 250 µL of 10% Chelex, vortexed for 10-15 seconds, centrifuged for 10-15 seconds on maximum speed, and incubated in a 100°C heating block for 20 minutes. Following the heating, samples were again vortexed for 10-15 seconds and then centrifuged for one minute at maximum speed. Forty microliters of the supernatant were transferred to new microcentrifuge tubes and PCR reactions were performed by using 5 µL of the DNA for a 30 µL PCR reaction to amplify the 16S rRNA gene using the 8F and 1492R primer pair. PCR samples underwent the PHU16S cycle in the thermal cycler. To verify the presence of a PCR product, a 1% agarose gel was made using 0.5 g agarose in 50 mL 1X TAE and 2.5 µL

SafeView. Gel electrophoresis was performed using 7 µL 1kb DNA ladder, and 5 µL of the PCR samples combined with 2 µL loading dye. The gel ran at 100V for 30 minutes, upon which the gel was illuminated and visualized. DNA samples were then sent for sequencing by Laragen and were identified using the National Institute of Health (NIH) Basic Local Alignment Search Tool (BLAST). A nucleotide BLAST search was performed using the rRNA/ITLS databases and the search was limited to sequences from the type material.

Germination and Growth Assays

To determine the salt concentration that significantly inhibits *Camissoniopsis cheiranthifolia* germination, a preliminary pipette tip assay was performed using sand-filled 1000 µL pipette tips placed in deep 96-well racks in black boxes. *C. cheiranthifolia* seeds were surface sterilized in 70% EtOH for one minute followed by a sterile water rinse, 50% NaClO (bleach) for 10 minutes followed by six rinses with sterile water, and soaked in deionized water for 30 minutes. Seeds were dried for two days under sterile conditions. Per tip, three seeds were planted and 2 mL of 1/8X Murashige and Skoog (MS) basal medium + 0 mM, 100 mM, 150 mM, 200 mM, or 300 mM NaCl were aliquoted with eight replicates per treatment. Pipette tips in the racks were covered with plastic wrap to prevent drying out and covered with the lid of the box for germination in the dark. The setup was placed in the Percival growth chamber. Growth media was refilled every two days by adding 500 µL of 1/8X MS treatments to each tip. Germination was measured at three and five days after planting.

To assess the effects of the isolated bacterial strains on *C. cheiranthifolia* seeds, the same preliminary pipette tip assay was performed with treatments of 1/8X MS + 0 mM NaCl, 1/8X MS + 150 mM NaCl, and 1/8X MS + 150 mM NaCl + 23CE1, 23CE2, 23CE4, 23CE7, and

23CE8. The strains listed were chosen out of the eight isolated strains due to their tolerance to 10% NaCl. For preparation of the bacterial inoculum, individual colonies were suspended in 1.5 mL $MgSO_4$ and absorbances of 1:10 dilutions were measured using the spectrophotometer. Suspensions of 1.5 mL bacteria in $MgSO_4$ were made with bacterial concentrations of OD600 = 0.1, and 100 µL of these suspensions or $MgSO_4$ were added to the tips. Germination was measured at three, five, and seven days after planting.

To evaluate the effect of the isolated bacterial strains on *C. cheiranthifolia* seeds on a larger scale, a sand-filled pot assay was performed under salt stress using the most effective strains from the preliminary pipette tip assay (23CE1, 23CE2, 23CE4, and 23CE7) in addition to a co-inoculated treatment with equal amounts of the four strains. A layer of cheesecloth was used on the bottom of each pot to prevent sand loss. Fifty seeds were planted per pot and 200 mL of 1/8X MS or 1/8X MS + 150 mM NaCl were added to black boxes. One milliliter of bacterial inoculum or $MgSO_4$ was added to the boxes. Germination was assessed after seven days and after 10 days, bacteria was isolated from the roots of co-inoculated *C. cheiranthifolia* roots by suspending root segments in 1 mL 10 mM $MgSO_4$ and spread plating three serial dilutions of the suspension. After five days, bacteria were visually assessed and quantified, and representatives of each bacterium present were re-isolated for comparison with original cultures.

To more accurately simulate restoration activities, the above assay was repeated using an alternate method of bacterial inoculation. Surface sterilized *C. cheiranthifolia* seeds were coated with a 1:10 dilution of bacterial inocula in ultraviolet light sterilized 0.2% xantham gum for 30 minutes, upon which liquid was removed and seeds were allowed to dry. During planting, 200 mL of Hoagland's complete media was added to pots and after three days of germination, stress

was induced using 1/8X MS + 150 mM NaCl or 1/8X MS for non-stressed pots. Seedling shoot and root length was measured after three weeks.

RESULTS

Bacterial Isolation, Characterization, and Identification

From the seeds of *Frankenia salina* and *Baccharis pilularis*, eight bacterial strains with distinct colony morphologies on TY agar were isolated (Fig. 1, Table 1). Six of those strains (23CE1, 2, 3, 4, 6, and 8) were tolerant to 5% NaCl and two (23CE4 and 6) were tolerant to 10% NaCl on TY agar (Fig. 2). None of the strains demonstrated phosphate solubilization, but four strains (23CE2, 4, 6, 8) demonstrated cellulase production with one strain's (23CE5) cellulase activity not determined (Fig. 3). Four strains that demonstrated initial PGPR properties in the preliminary pipette tip assay were tested for motility (Fig. 4), with two strains, 23CE1 and 23CE2, showing no motility, 23CE4 showing high motility, and 23CE7 showing motility after 24 h of incubation. Biochemical test results are summarized in Table 2.

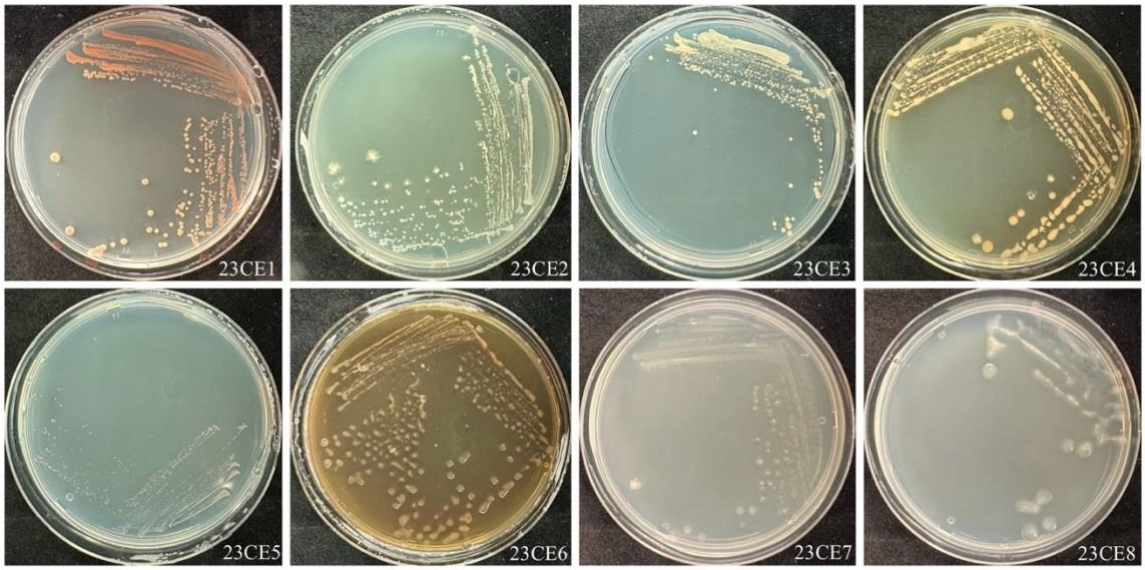

Figure 1. Bacterial strains isolated from coyote brush and alkali heath on TY agar.

Table 1. Bacterial colony morphologies on TY agar. Coyote brush (CB) and alkali heath (AH).

Strain ID	Description
23CE1	Pink medium smooth round colonies, isolated from CB
23CE2	White medium rhizoid colonies, isolated from CB
23CE3	Pale yellow small smooth round colonies, isolated from CB
23CE4	Yellow large smooth round colonies, isolated from AH
23CE5	Pink tiny smooth round colonies, isolated from AH
23CE6	Yellow large smooth round colonies w/ EPS, isolated from AH
23CE7	Cloudy medium umbonate colonies, isolated from AH
23CE8	Cloudy large umbonate colonies, high EPS, isolated from AH

Figure 2. Strains 23CE1–23CE8 on TY supplemented with 0%, 5%, or 10% NaCl after three days.

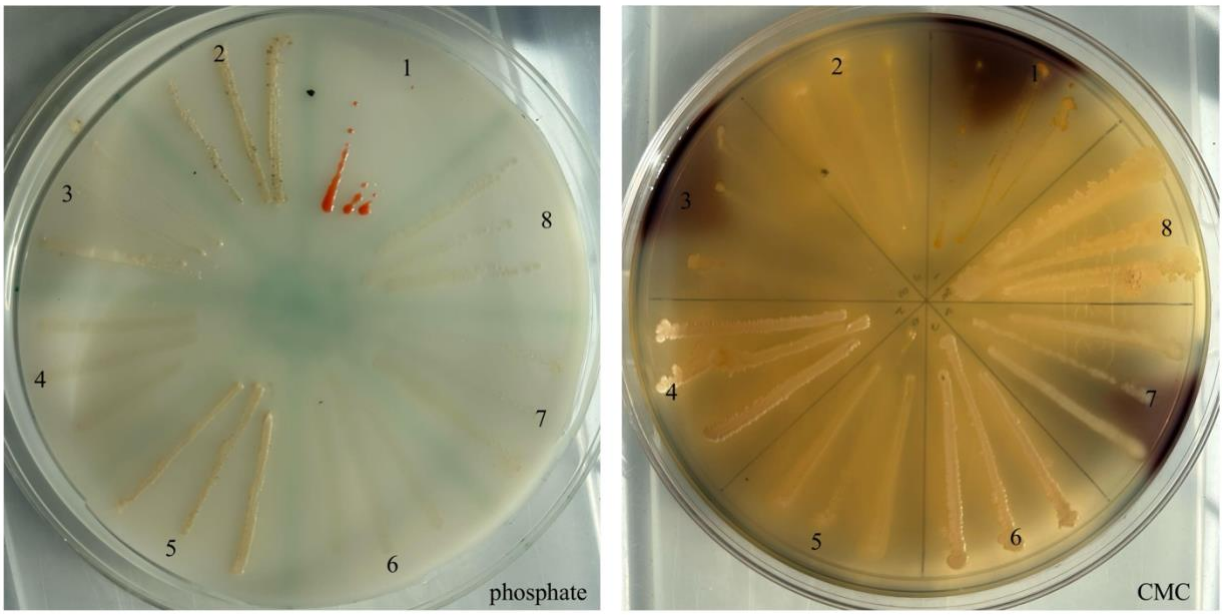

Figure 3. Strains 23CE1–8 on phosphate media and carboxymethyl cellulose (CMC) media after three days.

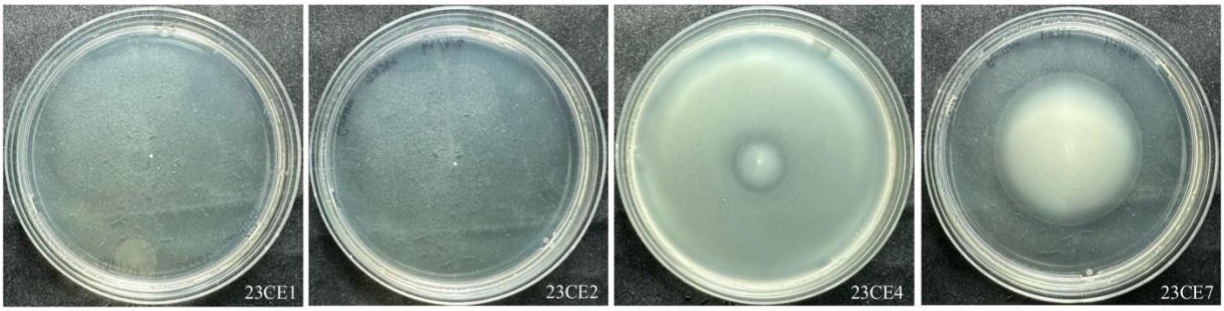

Figure 4. Strains 23CE1, 2, 4, and 7 on TY + 0.25% agar after 24 hours.

Table 2. Biochemical characterization of 23CE1–8.

Strain ID	Growth on 5% NaCl	Growth on 10% NaCl	Phosphate solubilization	Cellulase activity	Motility
23CE1	+	-	-	-	-
23CE2	+	-	-	+	-
23CE3	+	-	-	-	N/A
23CE4	+	+	-	+	++
23CE5	-	-	-	ND	N/A
23CE6	+	+	-	+	N/A
23CE7	-	-	-	-	+
23CE8	+	-	-	+	N/A

The strains chosen for motility testing based on preliminary PGPR behavior in the preliminary pipette tip assay (23CE1, 2, 4, and 7) were additionally sequenced to assess any known PGPR benefits of the strains (Table 3), however only 23CE4 and 7 were identified due to complications in the sequences of 23CE1 and 2. 23CE4 was identified as *Bacillus atrophaeus* strain NBRC 15539 at 100% identity. 23CE7 was identified as *Bacillus safensis* FO-36b at 99.59% identity.

Table 3. Top results from nucleotide BLAST search for strain sequences using the rRNA/ITLS databases, limiting to sequences from type material, and megablast

Strain ID	Accession	Description	Max Score	Query Cover	E Value	% Identity
23CE4	NR_112723.1	Bacillus atrophaeus strain NBRC 15539	1247	100%	0.0	100%
23CE7	NR_041794.1	Bacillus safensis FO-36b	889	100%	0.0	99.59%

Germination and growth assays

To determine the NaCl concentration at which primrose germination is significantly inhibited, a preliminary pipette tip assay was performed using NaCl concentrations ranging from 0 mM to 300 mM (Fig. 5), and a negative correlation between NaCl stress and percent germination was observed. 100 mM NaCl reduced both germination and growth of primrose seedlings, though not significant statistically based on the Tukey HSD test from a One-Way ANOVA. All other tested salt concentrations (150, 200, and 300 mM) demonstrated significant reduction of germination ($p = 4.351e-12$). Therefore, 150 mM NaCl was used in subsequent assays for significant inhibition of primrose germination.

The next assay preliminarily assessed PGPR potential of the 5% NaCl tolerant strains using a pipette tip setup. One week after planting, the uninoculated and unstressed control had high rates of germination (75%) while the uninoculated and stressed control had no germination (Fig. 6, 7). 23CE1, 2, 4, and 7 all demonstrated capability of rescuing primrose germination under 150 mM NaCl stress, with 23CE1 bringing back the highest amount of germination. While the unstressed and uninoculated control was significantly different from the treatments ($p = 5.79243e-9$), none of the treatments different from the stressed and uninoculated control. Of the five strains tested, only 23CE8 failed to rescue germination. Based on this, 23CE1, 2, 4, and 7 were analyzed in subsequent assays while 23CE8 was eliminated for further testing.

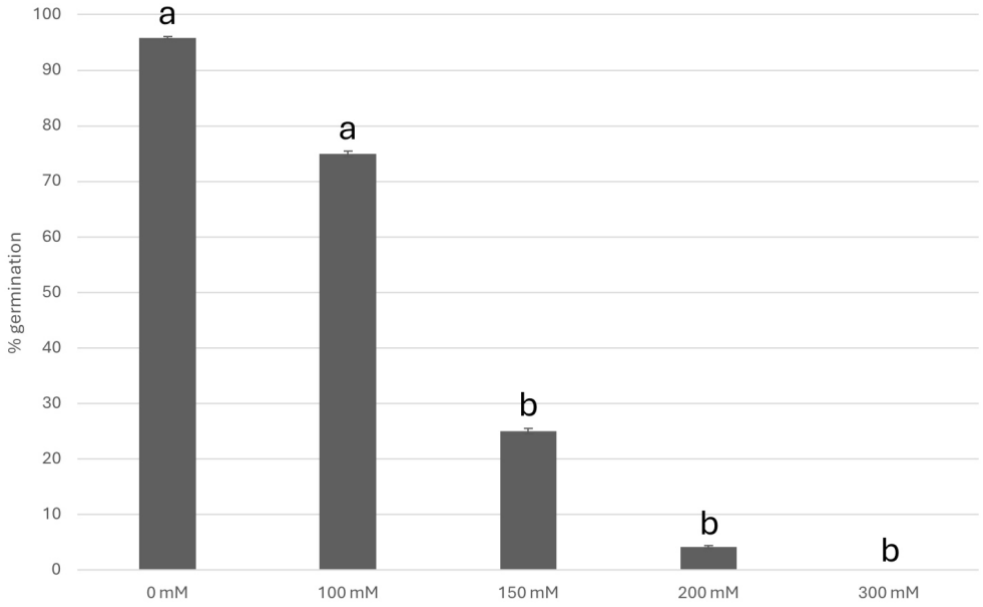

Figure 5. Percent germination of primrose seeds five days after planting in pipette tips. Three seeds were planted per tip with eight replicates per treatment. Treatments include 0 mM, 100 mM, 150 mM, 200 mM, and 300 mM NaCl stress. Error bars indicate standard error and lowercase letters indicate statistical difference.

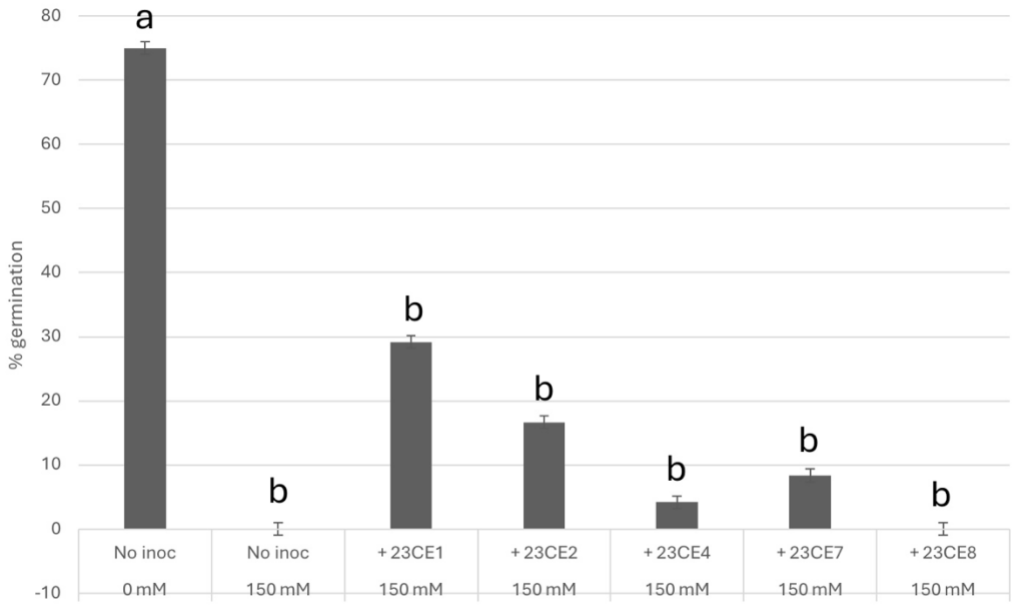

Figure 6. Percent germination of primrose seeds seven days after planting in pipette tips. Three seeds were planted per tip with eight replicates per treatment. Treatments include 0 mM NaCl + no inoculation, 150 mM NaCl + no inoculation, or 150 mM NaCl + inoculation with 23CE1, 2, 4, 7, and 8. Error bars indicate standard error and lowercase letters indicate statistical difference.

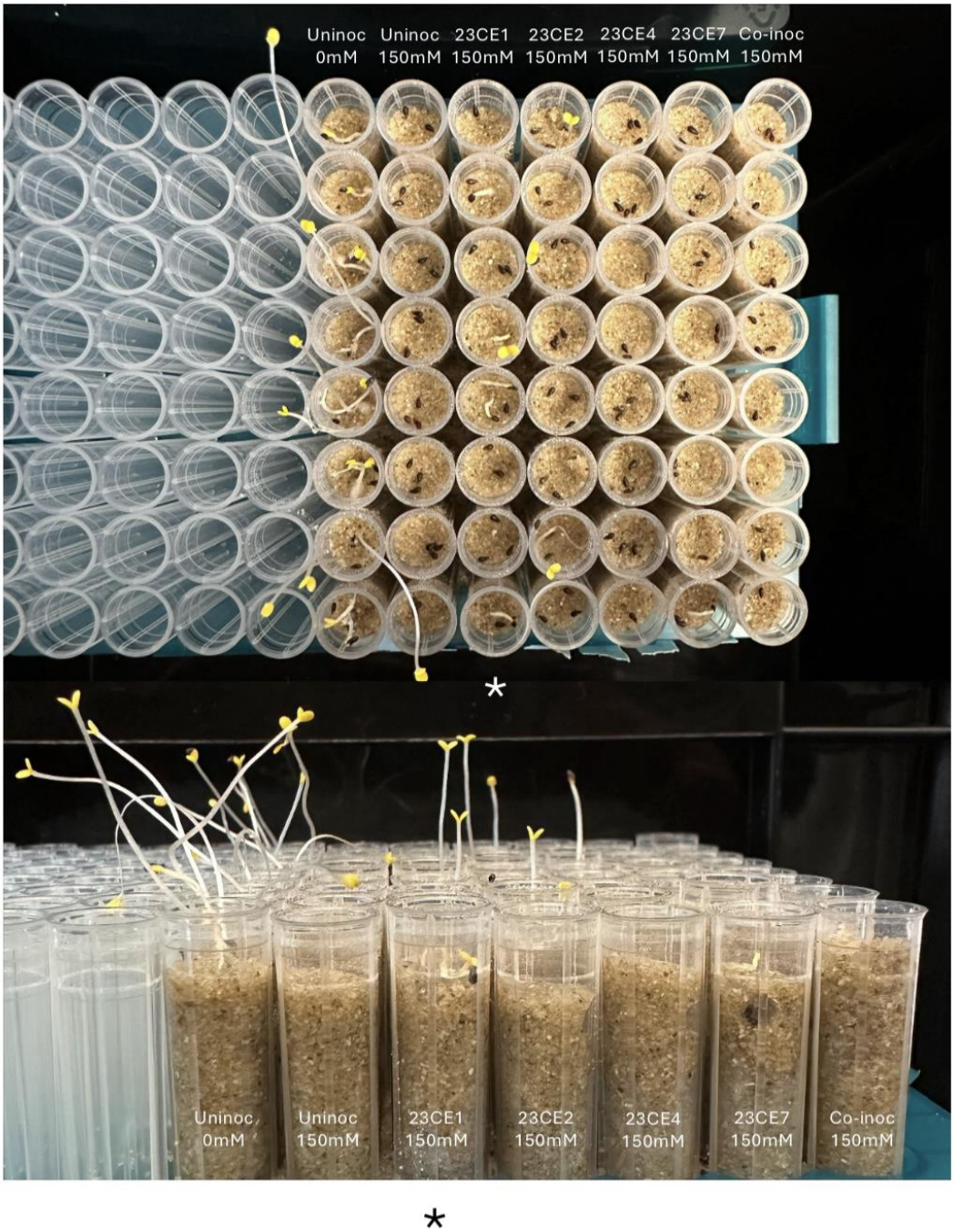

Figure 7. Germinated primrose seeds seven days after planting in pipette tips. Three seeds were planted per tip with eight replicates per treatment. Treatments include 0 mM NaCl + no inoculation, 150 mM NaCl + no inoculation, or 150 mM NaCl + inoculation with 23CE1, 2, 4, 7, and 8. Asterisks indicate the most effective treatment.

For larger-scale experimentation, the four strains found to bring back primrose germination in the preliminary pipette tip assay (23CE1, 2, 4, and 7) were used in a pot assay along with a treatment of a co-inoculation using all four strains. The co-inoculated treatment was included to determine whether such a method, which would be more efficient for restoration, could adequately function to improve native germination. Again, germination was high in the uninoculated and unstressed control (62%) and low in the uninoculated and stressed control (2.67%). 23CE1, 7, and the co-inoculated treatment improved primrose germination with only 23CE1 yielding significant improvement ($p = 1.656 \times 10^{-13}$).

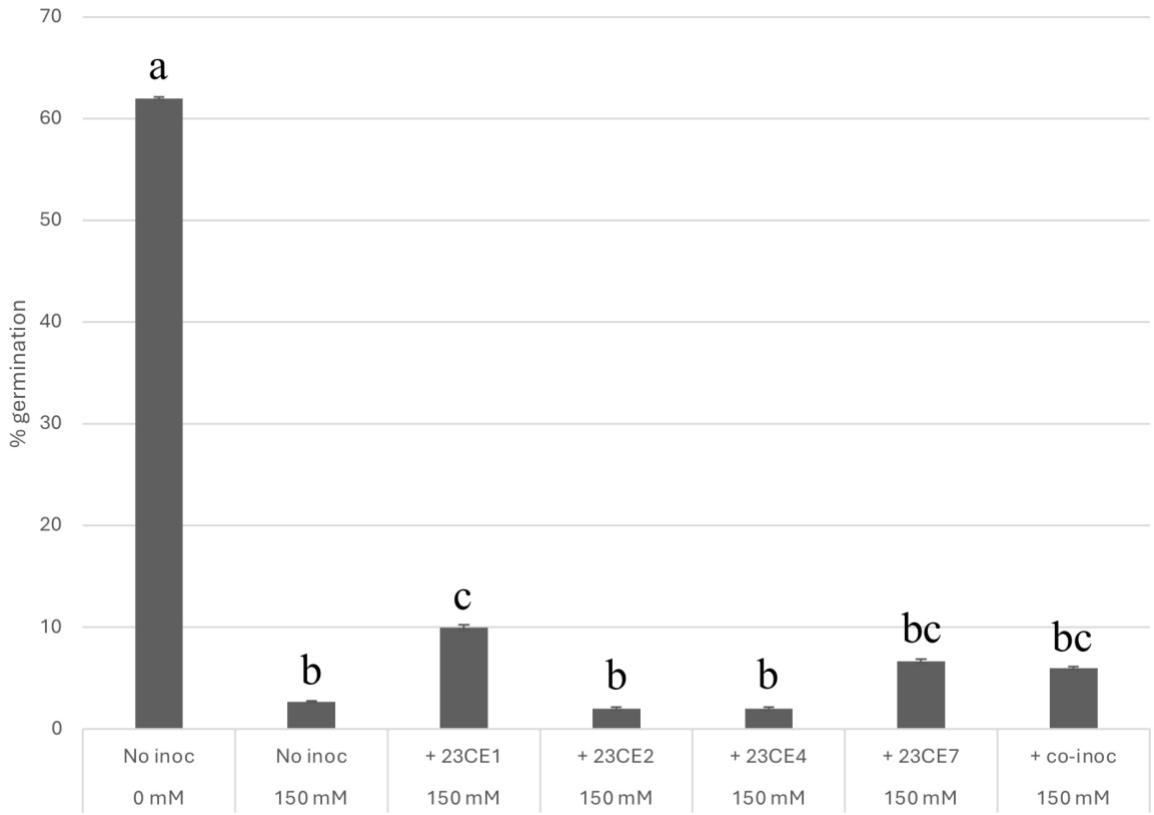

Figure 8. Germinated primrose seeds seven days after planting in pots. Fifty seeds were planted per pot with three replicates. Treatments include 0 mM NaCl + no inoculation, 150 mM NaCl + no inoculation, or 150 mM NaCl + inoculation with 23CE1, 2, 4, 7, and a co-inoculation of 23CE1, 2, 4, and 7. Error bars represent standard error. Lowercase letters indicate statistical difference.

Following the pot assay, root bacteria from germinated co-inoculated seedlings was re-isolated to determine the bacteria established in symbiosis with the plant which may be conferring PGPR properties. Of the four strains co-inoculated (23CE1, 2, 4, and 7), 23CE7 was most prevalent through colony morphology observation and comparison alone. Additionally abundant was a possible contaminant (marked by the asterisk in Table 4) which did not morphologically resemble any of the four strains initially inoculated.

Table 4. Colony morphologies and percent abundance of re-isolated root bacteria from co-inoculated treatment.

Strain name	Strain ID	Colony morphology on YMA	# colonies in 10^{-3}	% in 10^{-3}
Unidentified	23CE1	Pink smooth and round	0	0
Unidentified	23CE2	White filamentous	0	0
Bacillus atrophaeus	23CE4	White undulate	0	0
Bacillus safensis	23CE7	White smooth and round	44	80
*	*	Yellow spindle	11	20

Finally, a pot assay was completed using seeds coated with bacterial inoculum using xantham gum as an adhesive. Salt stress was applied three days after germination and after three weeks, seedling root length was measured (Fig. 9). Unstressed and uninoculated seedlings demonstrated the highest amount of growth and the salt impact decreased root length for most of the treatments compared to the unstressed and uninoculated control. Only the co-inoculated treatment significantly brought back germination compared to the uninoculated and stressed control ($p = 1.003e\text{-}9$), rescuing growth to levels of the uninoculated and unstressed control.

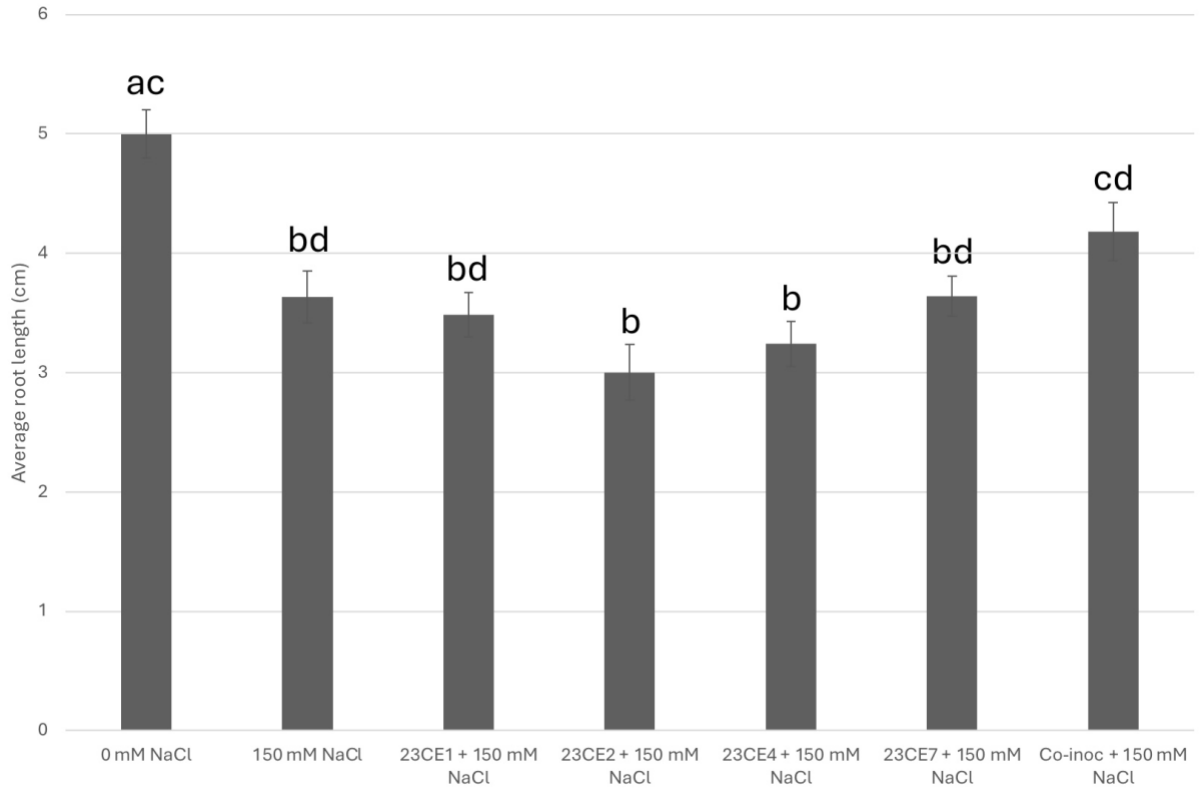

Figure 9. Seedling root length three weeks after salt stress application. Fifty seeds were planted per pot with three replicates. Treatments include 0 mM NaCl + no inoculation, 150 mM NaCl + no inoculation, or 150 mM NaCl + inoculation with 23CE1, 2, 4, 7, and a co-inoculation of 23CE1, 2, 4, and 7. Error bars represent standard error. Lowercase letters indicate statistical difference.

DISCUSSION

The initial isolation of bacteria from *F. salina* and *B. pilularis* seeds yielded strains lacking common PGPR properties of phosphate solubilization and cellulase production, however the tolerance of the strains to 5% and 10% NaCl provided evidence of PGPR potential given the high salinity of wetlands hydrology. Given the adaptation of these bacteria to survive under salt stress, there could be a mechanistic symbiosis conferring salt tolerance to associated plants. In addition, high degrees of motility in 23CE4 and 23CE7 provides evidence of their PGPR

potential given their ability to spread throughout soil or water, impacting more seeds in a restoration setting than those initially coated. Despite the direct application of PGPR to seeds via an adhesive material such as xantham gum, bacteria must be able to reach plant roots using motility to colonize the root surface (Amaya-Gómez et al. 2020).

The bacterial identification from DNA sequencing allows for the analysis of PGPR potential of the isolated bacteria in the literature. *Bacillus atrophaeus* has been identified as a potential biocontrol agent for anthracnose disease caused by *Colletotrichum* fungal species through its antagonism against micelial growth and spore germination of two virulent *C. gloeosporioides* strains isolated from soursop and avocado. *B. atrophaeus* produces antibiotics such as surfactin, bacillomycin, and iturin, owing to its PGPR potential of inducing systemic resistance against plant pathogens (Guardado-Valdivia et al. 2018). *B. atrophaeus* strain HAB-5 additionally promotes the growth of tobacco plants and serves as a biocontrol agent for tobacco mosaic virus (Rajaofera 2019).

Bacillus safensis FO-36b has been found to be genetically similar to *Bacillus pumilus* (Tirumalai et al. 2018). *B. pumilus* is a known PGPR capable of offsetting high saline impact on wild barley growth (Mahmoud et al. 2024) and exhibiting biocontrol properties against fungal phytopathogens (Dobrzyński et al. 2023). Furthermore, *B. safensis* PM22 has been found to improve maize growth under salinity stress with PGPR properties of producing extrapolysaccharide (EPS), indole-3-acetic acid (IAA), siderophores, and 1-aminocyclopropane-1-carboxylic acid deaminase (ACC-deaminase) under saline conditions, in addition to stimulating antioxidant production (Azeem et al. 2022). Another *B. safensis* strain VRKK2 demonstrating PGPR properties of IAA production, phosphate solubilization, HCN production, ammonia production, nitrogen fixation and siderophore production was found to improve

cowpea seed germination (Kannan et al. 2019). This is contradictory to our finding that *B. safensis* FO-36b was negative for phosphate solubilization capabilities, however as further biochemical tests were not completed, it is difficult to establish a full comparison between the strains. Further work should undergo the biochemical tests found for other *B. safensis* strains in previous studies in addition to generating accurate sequences of the remaining strains to yield identification and analysis of those strains.

In the preliminary primrose germination assay to determine the statistically significant inhibitory concentration of NaCl, an inverse trend between salt concentration and primrose germination was observed. This is not a surprising result given the ability of NaCl to induce stress in plants, notably through the generation of low osmotic potential around the seed, preventing water uptake (Guo et al. 2020). The tolerance of primrose to 100 mM NaCl is additionally unsurprising given its distribution being Pacific coastal dunes in North America, which is a saline environment (López-Villalobos & Eckert 2019). Such salt tolerance may be supplemented by halophytic PGPR such as *B. safensis*.

In the preliminary pipette tip assay involving inoculation, 23CE6 was not tested due to its morphologic and biochemical similarity to 23CE4, which was chosen to represent the two suspected identical strains. While *B. safensis* demonstrated a rescue in germination compared to the salt stressed and uninoculated control, it demonstrated less of an effect that 23CE1 or 23CE2. In addition to its lesser effect on germination, it demonstrated a lesser effect on plant growth in comparison to 23CE1 and 23CE2. Furthermore, while some strains rescued germination compared to the uninoculated and stressed control, none significantly rescued germination to the level of the uninoculated and unstressed control. This suggests that the isolated strains may not be ideal inocula for a wide scale restoration event during which germination should be

maximized in a laboratory setting to mitigate the unpredictability and high mortality of seeds in wetland restoration (Kettenring & Tarsa 2020).

In the pot assay, however, 23CE1 exhibited a significant effect on germination while *B. safensis* and the co-inoculated treatment affected germination, but to a lesser extent. Therefore, 23CE1 represents a potential PGPR strain for inoculation in seed-based restoration, however it would be beneficial to test more strains in order to identify an improved co-inoculation treatment. Again, while the 23CE1 strain rescued germination compared to the uninoculated and stressed control, it did not rescue germination to the extent of the uninoculated and unstressed control. Therefore, while this strain demonstrates potential to improve primrose germination in the lab, the extent may not be great enough to affect field-based restoration approaches in wetlands.

In the root isolation of co-inoculated seedlings, it was surprising that *B. safensis* was dominant with a lack of *B. atrophaeus* given the much higher motility of *B. atrophaeus.* However, the lack of 23CE1 in the roots may be consistent with the significant effect of 23CE1 on primrose germination if the strain is acting at the seed level to promote germination but its non-motile nature in fact inhibits its colonization of the roots. Because strain re-isolation and identification from co-inoculated seeds was based on colony morphology observations alone, it is possible that strains were incorrectly identified. Future work could investigate this further through sequencing the re-isolated strains or by tagging inoculated strains with a plasmid-borne growth fluorescent protein (GFP) gene (Timmusk, Grantcharova, & Wagner 2005).

In the seedling growth assay with inoculum coated seeds and salt stress application post-germination, uninoculated and unstressed seeds showed the highest amount of growth, followed by decreases in all stressed treatments, with the smallest decrease being the co-inoculated

treatment. The co-inoculated treatment significantly rescued seedling growth back to levels of the unstressed and uninoculated control, indicating that such an inoculum may be beneficial for use in seed-based wetlands restoration. In addition, the efficacy of this inoculum could be improved using an integrated approach combining inoculation with the addition of chemical stimulants or osmo-protectants (Enebe & Babaloba 2018). The potential of improving plant germination and growth in this way certainly provides a basis for future work in the field of restoration.

In conclusion, bacteria isolated from native plants demonstrates potential as PGPR for improving beach evening primrose germination and growth despite lacking common PGPR properties. Therefore, a more comprehensive initial isolation and biochemical screening may result in better candidates for bacterial inoculum for wetlands restoration. Following identification of potentially improved candidates, investigation into root colonization could be visualized using GFP or sequencing. Finally, an integrated approach using both bacterial inoculation and osmo-protectants may be investigated to maximize the germination and plant survival of ideal seedlings used in restoration efforts. Once a maximized approach has been identified using primrose seeds as a model, next steps would include experimentation on native seeds of alkali heath, coyote brush, and any wetland vegetation desirable for restoration.

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Appendix A

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Developing Growth Promotion Strategies for *Cressa truxillensis* to Improve Success of Restoration Activities

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Abstract.—*Cressa truxillensis*, commonly known as alkali weed, is native to western North America and is used in revegetation projects in saline or alkaline soils at locations such as the Ballona Wetlands Ecological Reserve. This research aimed to (i) determine methods to improve *C. truxillensis* seed germination, (ii) characterize the impact salt has on seed germination and growth, and (iii) identify and characterize bacterial seed endophytes and their potential as plant growth promoting bacteria (PGPB). Results showed that seed scarification, either through mechanical or chemical methods, substantially improved seed germination rates. The presence of salt at 300 mM NaCl delayed germination, and both 150 mM and 300 mM NaCl decreased seedling size. Two different strains of *Paenibacillus peoriae* were found to reside within *C. truxillensis* seeds collected from the Ballona Wetlands. Although neither strain alleviated the salt sensitivity displayed by *C. truxillensis,* both strains showed tolerance to heavy metals, salt, and showed additional properties suggestive that they may function as PGPB. Methods used in this study can serve as guidelines for preparation of seed of *C. truxillensis* prior to seeding in appropriate habitats throughout the species' range.

Wetlands provide important ecosystem services such as supporting biodiversity, filtering water, sequestering carbon, and protecting against storm surge (Zedler and Kercher 2005; Duarte et al. 2013; Benson et al. 2019). Anthropogenic impacts have resulted in a global loss of approximately half of the world's wetlands (Zedler and Kercher 2005). Wetland losses in California have exceeded 90% in the last 200 years, thus creating a continued need for wetland research, conservation, and restoration or enhancement to prevent and slow further loss (Allen and Feddema 1996). Wetlands have increasingly faced threats by invasive species, anthropogenic impacts, and changes in the climate; however, restoration by revegetation with native plants provides one possible solution to returning wetland ecosystem services. Revegetation approaches include transplantation of plants or rhizomes, planting plugs, and direct seeding (Godefroid et al. 2011; Kettenring and Tarsa 2020). Understanding the optimal conditions to promote seed germination and plant establishment of target species is of substantial importance to wetland restoration and management.

A seed-based approach to vegetative restoration has the advantage of being less expensive and logistically less challenging to implement (Kettenring and Tarsa 2020). However, there is a range of soil conditions and hydrology that are likely to affect *in situ* field germination (e.g., nutrient availability, soil grain size and porosity, invasive species presence, etc.), and the downside is that many plant species exhibit extremely low seed germination

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rates, especially due to abiotic stresses such as salt or the presence of a hard seed coat that results in dormancy and prevents imbibition (Almansouri et al. 2001; Baskin and Baskin 2014, 2020). To break dormancy, scarification can break the seed coat and allow a seed to imbibe and eventually germinate (Baskin and Baskin 2014). A better understanding of effective scarification techniques for individual species that are targets of restoration projects is essential for the success of a seed-based approach.

A plant's tolerance to abiotic stress can also be manipulated, with numerous examples of plant growth promoting bacteria (PGPB) forming beneficial relationships with host plants (Cassán et al. 2009; Enebe and Babalola 2018). PGPB can reside within the seed, as well as on and inside plant roots, promoting plant growth and resilience via biochemical properties, resistance to disease, and acting as a buffer to abiotic stressors (Eida et al. 2018). Determining optimal seed germination to account for these inhibitory factors can be important for seeding plants during restoration, leading to a higher percentage of germinated seeds. With a growing need for restoration and improving seeding success of suitable plants for revegetation, this study aimed to inform effective growth strategies of the California native wetland plant, alkali weed, *Cressa truxillensis*.

Cressa truxillensis, commonly known as akali weed, is a facultative wetland plant native to the western United States and commonly found in many southern California wetlands (Lichvar 2012). *Cressa truxillensis* is known to have a late spring bloom period with hallmark characteristics including a high salinity tolerance and high calcium carbonate tolerance (Jepson and Hickman 1993). Studies on *C. cretica,* another species in the genus, found that the plant could grow in soils with an upper range of 22% calcium carbonate and saline concentrations up to 400 mM (Kabir et al. 2010; Sivasankaramoorthy et al. 2011). Although often found in wetlands, the plant can also thrive as a native in many arid and alkaline habitats, making it an important focus for the conservation or enhancement of multiple habitat types. The *Cressa* genus has very low germination rates in its naturally occurring habitats, which has been attributed to a hard seed coat and suboptimal environmental conditions (Etemadi et al. 2020). This research project aimed to inform the current state of knowledge regarding the use of this species in revegetation projects by investigating *C. truxillensis* scarification techniques and through characterization of its microbial endophytes.

Materials and Methods

Different scarification methods were applied to seeds of *C. truxillensis* to determine optimal conditions to increase the number of seeds that imbibe and germinate. Seeds for the scarification experiment were obtained from S&S Seeds (www.ssseeds.com). *C. truxillensis* seeds are ovular, brown, and 2-4 mm in length (Austin 1998). Four approaches were taken to scarify *C. truxillensis* seeds, including three mechanical and one chemical approach (Table 1). Each treatment had five replicate batches of 20 seeds. Chemical scarification was performed by immersing seeds in 0.5 mL of concentrated sulfuric acid in a centrifuge tube for 45 min. The seeds were then rinsed with sterile distilled water five times to remove all traces of the acid. The three methods of mechanical scarification were scratching individual seeds against sandpaper, rubbing seeds in a batch between sandpaper, and nicking the seed coat with a razor blade. For scarification of individual seeds by sandpaper, each seed was scratched across 220-grit sandpaper for 2.5 cm. For the batch treatment, seeds were randomly dispersed between two pieces of 220-grit sandpaper, a book placed on top to apply even pressure, and a circular motion applied to rub the seeds between the sheets

Table 1. Methods for scarification of *C. truxillensis* seeds

	Chemical	Mechanical		
Scarification Type	Acid	Individual	Batch	Razor Blade
Description	Seeds immersed for 45 min in sulfuric acid (Etemadi et al. 2020).	Seeds individually scratched on sandpaper (Hassen et al. 2005).	Seeds sandwiched between sandpaper, scratched (Hassen et al. 2005).	Seed coat nicked (Mackay et al. 1996).

of sandpaper for 10 passes. To scarify seeds with a razor blade, each seed was nicked once to break the surrounding seed coat. Batches of untreated seeds were placed in petri dishes to serve as controls. For all treatments and the controls, seeds were placed in autoclaved petri dishes containing filter paper saturated with 7 mL of sterile deionized water. The petri dishes with seeds were wrapped in parafilm to prevent evaporation and then placed in the dark at 24°C. The number of germinated seeds within each dish was counted after seven days.

Bacteria associated with *C. truxillensis* were identified, as many plant-associated bacteria have plant-growth promoting properties. *C. truxillensis* seeds were collected from the ground, within *C. truxillensis* and *Salicornia pacifica* plant communities in the non-tidal high salt marsh and transition habitats of the western area B marsh at the Ballona Wetlands Ecological Reserve (Los Angeles, CA, 33.963025°, -118.447015°). No more than 10% of available seed was collected from within individual polygons that were identified throughout the appropriate habitat areas, ensuring that no seed banks were impacted during the collection. A total of 150 seeds were collected from the Ballona Reserve. Seeds were scarified and surface sterilized with 95% ethanol for five minutes, followed by 10% bleach for 10 min, and then rinsed with five washes of sterile deionized water. The water from each wash step was plated onto Tryptone Yeast extract agar (TY; 5 g/L tryptone, 3 g/L yeast extract, 0.0662 g/L $CaCl_2{\cdot}2H_2O$, 15 g/L agar) and incubated at 30°C to rule out contamination by a lack of microbial growth on the media. Following sterilization, seeds were crushed individually in 5 mL of sterile saline (0.9% NaCl) using a sterile mortar and pestle. Dilutions of the bacterial suspensions were plated onto TY agar and incubated at 30°C (Siddikee et al. 2010). Dilutions were made so that individual colonies would be easily identified and isolated when grown on TY agar. Two individual bacterial strains, C15 and C3G, were selected to represent the different colony morphologies seen on Yeast extract Mannitol agar (YM; 0.5 g/L yeast extract, 10 g/L mannitol, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.2g/L NaCl, 15 g/L agar). A simple stain with safranin and a spore stain were performed on each strain and observed by brightfield microscopy (Zeiss Axioscope 5).

The bacterial strains were characterized for biochemical properties associated with plant growth promotion and their ability to tolerate salt and heavy metal stress. Strains were tested for nitrogen-fixation by growth on nitrogen-free Jensen's media (Jensen 1942) after 7 d. Cellulase activity was tested by growing the strains on Carboxymethyl Cellulose (CMC) agar (Kasana et al. 2008), flooding the plates with Gram's iodine, and checking for the presence of clear halos around the streaked bacteria. The production of exopolysaccharide was determined by growing bacteria on YM agar and visually identifying the presence of a mucoid colony morphology. Phosphate solubilization was tested by visually

identifying the presence of clear halos around bacteria on Pikovskaya's agar (Pikovskaya et al. 1948) after 7 d of growth. Auxin production was determined by culturing bacteria in TY supplemented with 1 mg/ml tryptophan and adding Salkowski reagent (Ehmann 1977; Gordon and Weber 1951) after growth. The presence of red coloration indicated the production of indole acetic acid (IAA). For analysis of bacterial tolerance to abiotic stressors, bacteria were streaked on TY agar that had been supplemented with zinc (50, 100, 200, 400, 750, or 1000 μM $ZnSO_4$), cadmium (50, 100, or 200 μM $CdCl_2$) or salt (1%, 2%, 3%, 4%, or 5% NaCl), incubated for seven days at 30°C, and growth compared to that seen on TY. All assays were done in triplicate.

To identify bacterial strains C3G and C15, DNA was obtained from each strain using a Chelex extraction (Walsh et al. 2013). The DNA was used to amplify the 16S rRNA gene by the polymerase chain reaction using universal primers 27F and 1492R (Weisburg et al. 1991). The PCR products were sequenced, and the sequences were compared to that of bacterial type strains using tools of the Ribosomal Database Project (Cole et al. 2014) and NCBI BLAST (Altschul et al. 1990). Closely related sequences were retrieved from the GenBank database. A phylogenetic tree of the 16S rRNA gene was generated using MEGAX (Kumar et al. 2018; Stecher et al. 2020) and the maximum-likelihood algorithm. Bootstrap analysis was done for statistical support using 1000 re-samplings with gamma distribution (G) and the Tamura 3-parameter (T92) model (Tamura 1992). The following new sequences were deposited in the GenBank database: 16S rDNA of *Paenibacillus* sp. strain C15 (OK178930) and *Paenibacillus* sp. strain C3G (OK178931).

For the seed germination assays including salt stress and bacterial inoculation, *C. truxillensis* seeds were scarified and surface sterilized as described above. Bacterial strains C3G and C15 were grown overnight in TY media, centrifuged and the bacterial pellet washed once with 10 mM $MgSO_4$ to remove any residual TY, and then centrifuged again and the bacteria resuspended in 10 mM $MgSO_4$ to an absorbance at 600 nm of 0.1 (Montañez et al. 2012). *C. truxillensis* seeds were incubated for 1 hr in the bacterial suspension or 10 mM $MgSO_4$ as a control. The 35 seeds were transferred in triplicate to a petri-dishes containing 0.8% agar (Sigma A1296) with either 0, 150, or 300 mM NaCl. The number of seeds germinated were counted after 7, 14, and 21 days. Wet weight of germinated seedlings was taken at 21 d.

Results

Cressa truxillensis control seeds exhibited a low average percentage of germination when seeds were not scarified (5.8% ± 3.4%) (Fig. 1). Chemical scarification in concentrated sulfuric acid for 45 min resulted in the greatest average germination (54% ± 6.1%), substantially higher than the unscarified control, although mechanical scarification of individual seeds via sandpaper (34% ± 9.8%) and nicking with a razor blade (29% ± 3.4%) also improved average germination (Fig. 1). However, batch scarification did not show substantial improvement in the percentage of germination (11% ± 6.1%) as compared to the control treatments.

Results indicated that the low salinity treatment (150 mM NaCl) had no impact on percent seed germination of *C. truxillensis* at 7 days (61% ± 1.2%) as it was comparable to the control (59% ± 5.1%) (Fig. 2). However, the high salinity treatment (300 mM NaCl) caused a reduction in germination (8% ± 0.7%) (Fig. 2). By 21 d the percentage of germinated seeds did not vary between saline treatments (Fig. 2). Although the final germination percentages were similar, seedlings exposed for 21 d to 150 mM or 300 mM NaCl showed

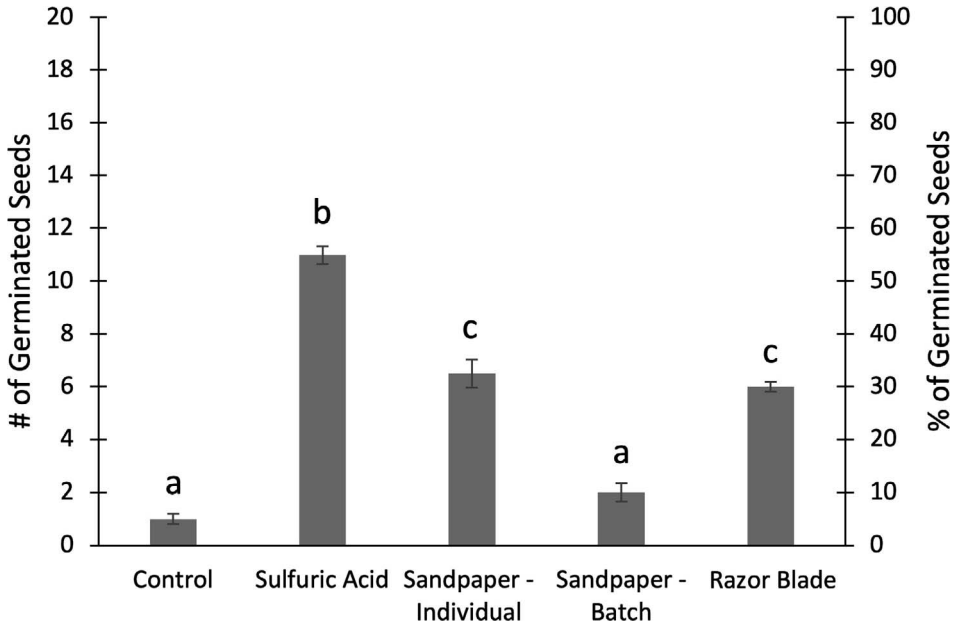

Fig. 1. Effect of four seed scarification treatments on the number of germinated *C. truxillensis* seeds (left Y-axis) and the percentage of germinated *C. truxillensis* seeds (right Y-axis). Values are the mean of five replicates (20 seeds each replicate) and vertical bars indicate standard error. Different letters indicate a statistical difference based on One-Way ANOVA with post-hoc Tukey ($p < 0.05$).

a reduction in growth compared to unexposed seedlings (Fig. 3A and 3B). In addition, the higher the concentration of salt, the greater the reduction in seedling size and mass (Fig. 3A and 3B).

Bacteria were isolated from within *C. truxillensis* seeds that had been collected from the Ballona Wetlands. Although bacterial colonies looked similar on TY, when transferred to YM agar they showed two distinct colony morphologies. Strains C15 and C3G were chosen as representatives of each colony type (Fig. 4A and 4B). Most notably, the texture was a differentiating characteristic between strains C3G and C15; on YM agar, strain C15 had a mucoid appearance indicating the presence of exopolysaccharide while strain C3G did not (Fig. 4B). Microscopy of stained bacteria showed that both strains looked similar and were bacillus in shape (Fig. 4C and 4D). In addition, both strains were found to produce endospores (Table 2). The 16S rRNA gene sequences of strains C3G and C15 were 100% identical to each other and identified them as species of *Paenibacillus*, with 99.71% (1393/1397 n.t.) identity to and phylogenetically grouping with *P. peoriae* DSM8320 (Fig. 5).

Table 2. Biochemical characteristics of endophytic bacteria of *C. truxillensis* seeds.

	Tolerance to abiotic stress			PGPB properties				
Strain	$ZnSO_4$	$CdCl_2$	NaCl (%)	N- fixation	Cellulase	IAA	Phosphate	Endospore
C3G	400 μM	-	4%	+	+	+	+	+
C15	1 mM	50 μM	4%	+	+	+	+	+

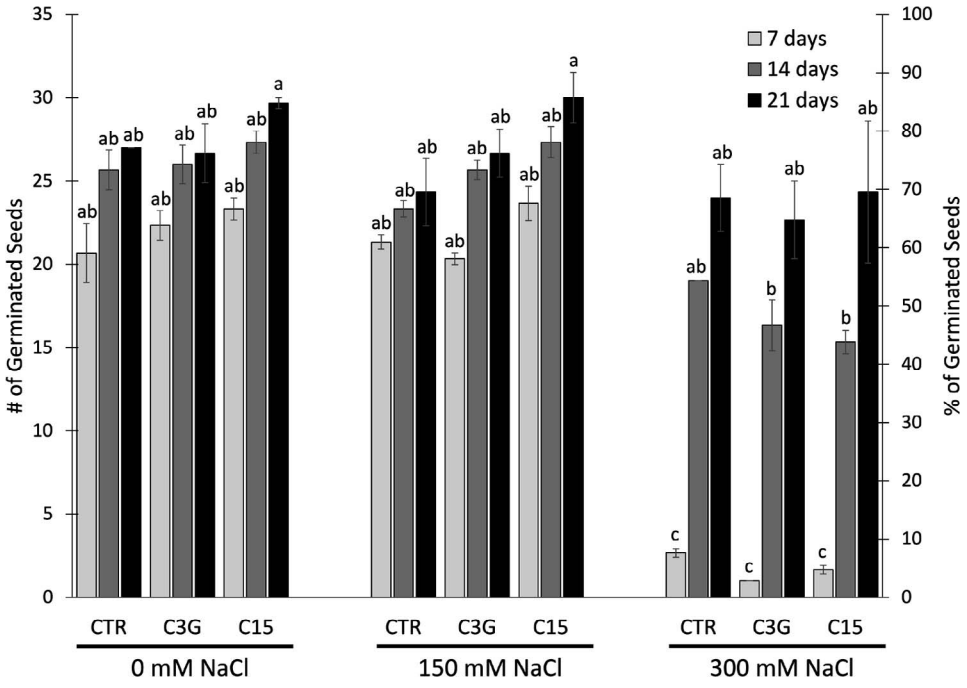

Fig. 2. Effect of NaCl and bacterial inoculation on the number of germinated *C. truxillensis* seeds (left Y-axis) and the percentage of germinated *C. truxillensis* seeds (right Y-axis) over time. Values are the mean of three replicates (35 seeds each replicate) and vertical bars indicate standard error. Different letters indicate a statistical difference based on One-Way ANOVA with post-hoc Tukey ($p < 0.05$). CTR indicates uninoculated control treatments.

Strains C15 and C3G were tested for biochemical properties associated with plant growth-promoting bacteria. Both strains grew on nitrogen-free media (Table 2), indicating that they were able to fix nitrogen, similar to many previously characterized *Paenibacillus* strains (von der Weid et al. 2002). In addition, both strains had cellulase activity, produced auxin, and solubilized phosphate (Table 2). Strains C15 and C3G were tested for growth at varying concentrations of zinc, cadmium, and sodium chloride in order to look at the impact of various abiotic stressors. C3G grew with up to 400 μM $ZnSO_4$, however C15 grew even in the presence of 1 mM $ZnSO_4$ (Table 2). The strains also showed varying tolerance to cadmium. C3G was sensitive to cadmium, but C15 grew in up to 50 μM $CdCl_2$ (Table 2), highlighting the higher tolerance C15 has to heavy metals. Both strains behaved similarly to salt stress and could grow in as much as 4% NaCl but were inhibited at 5% NaCl (Table 2). When *C. truxillensis* seeds were inoculated with either strain of bacterium, no difference was seen in the percentage of seeds that germinated, whether or not salt was present (150 and 300 mM) (Fig. 2). However, in the absence of salt, inoculation with C3G resulted in a slight increase in seedling wet weight (Fig. 3B).

Discussion

The seed coat can be a major barrier to germination for many plants. How to overcome this seed coat-imposed dormancy, or "hardseededness", and thus increase the seed's permeability to water, has been the basis of numerous studies (Tadros et al. 2011; Mousavi

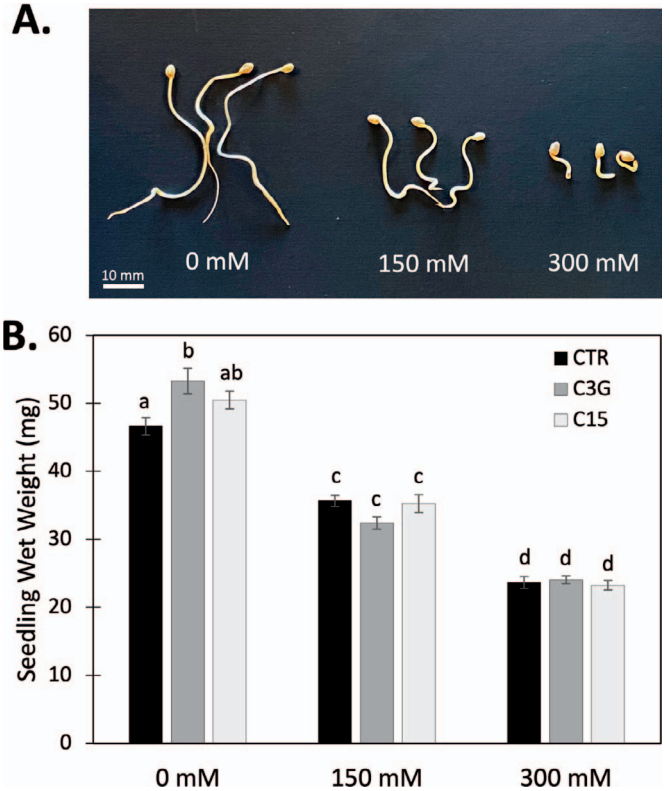

Fig. 3. Effect of NaCl and bacterial inoculation on *C. truxillensis* seedlings. A) Seedlings after 21 d of growth with 0, 150, or 300 mM NaCl. B) Average wet weight of seedlings after 21 d of growth on different concentrations of NaCl. Data represents the mean seedling wet weight from 30 seedlings from three independent replicates, and vertical bars indicate standard error. Different letters indicate a statistical difference based on One-Way ANOVA with post-hoc Tukey ($p < 0.05$).

et al. 2011). Typical scarification strategies include hot water treatment, chemical scarification with acid, or mechanical scarification to nick the seed coat (Rusdy 2017). Though differing in method, chemical and mechanical scarification work to obtain similar results of breaking the seed coat. Mechanical scarification may require more labor on fewer seeds via physical removal or permeation of the barrier, while chemical scarification is effective for larger batches and uses concentrated acid to dissolve part of the seed coat (Majd et al. 2013). The use of sulphuric acid to overcome germination difficulties in seeds has been well-established in the literature as a highly effective scarification technique (Kheloufi et al. 2017). Congruent with studies of *C. cretica*, which found that naturally occurring seeds have very low germination rates (Etemadi et al. 2020), the non-scarified control *C. truxillensis* seeds in this study showed a low percentage of germination. This suggests that restoration or revegetation projects seeding with non-scarified seeds may experience low initial germination rates due to seed coat-imposed dormancy, which would delay bringing back native plant cover to an area. An experimental study at the University of Wisconsin comparing hand seeding and transplantation as restoration techniques found hand seeding to be more successful in producing greater diversity and growth of

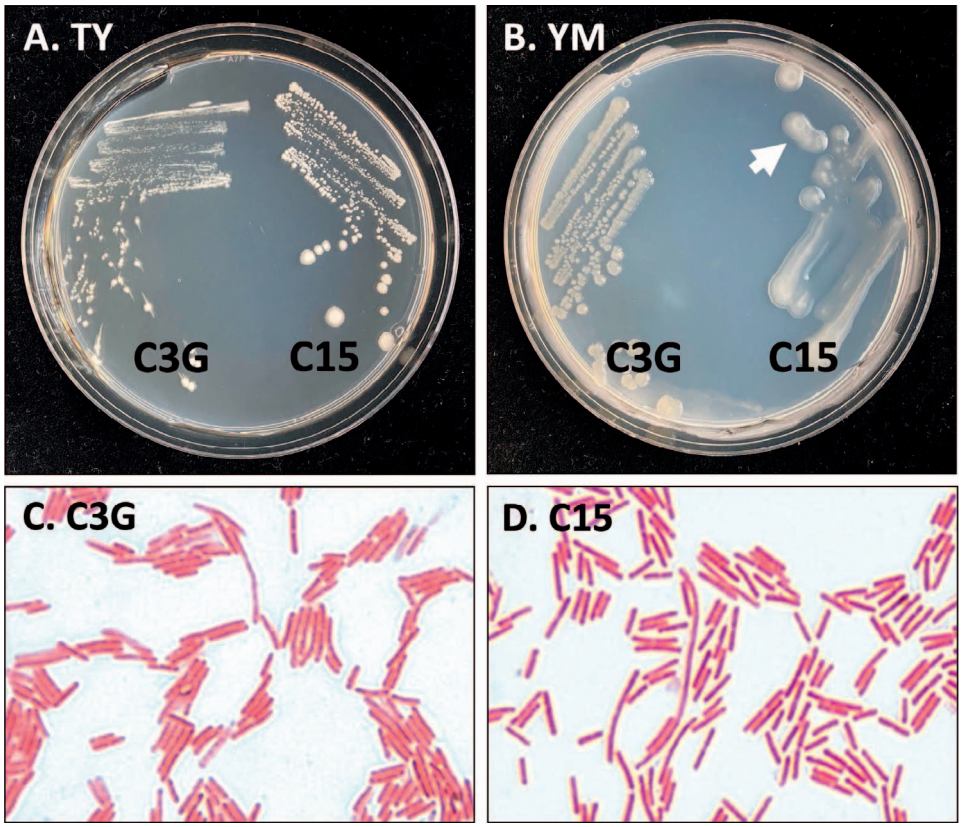

Fig. 4. Colony and cellular morphology of bacterial isolates C3G and C15. Bacterial growth on (A) TY and (B) YM agar. Cellular morphology after staining with safranin of (C) C3G and (D) C15. Arrow points to mucoid colonies of C15.

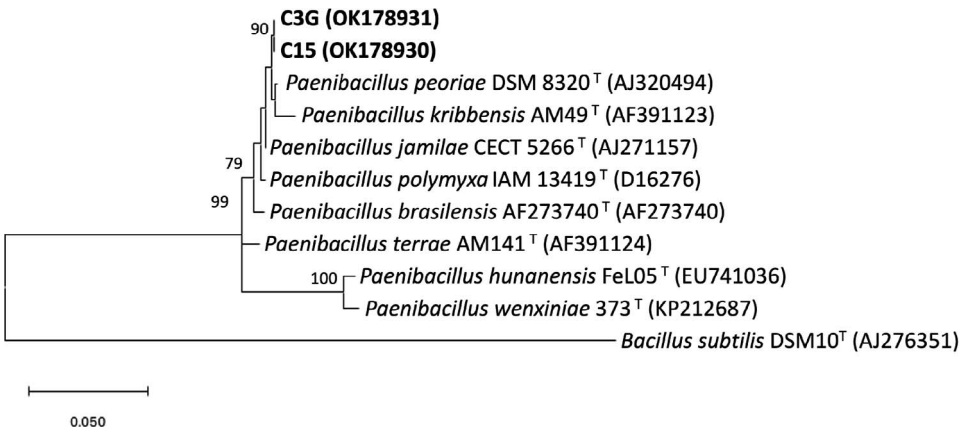

Fig. 5. Maximum-likelihood phylogenetic tree of the 16S rRNA gene sequences from *C. truxillensis* endophytic strains, C15 and C3G, and closely related *Paenibacillus* type strains. Genbank accession numbers are in parentheses. Bootstrap values greater than 70% are shown in the nodes. *Bacillus subtilis* was used as the outgroup.

native plants in addition to greater cost and time effectiveness; however, they noted that an extended dormant period for unscarified seeds may present problems with the regrowth of non-native plants (Weiher et al. 2003). Results from this study indicated both mechanical and chemical scarification methods improved *C. truxillensis* seed germination, showing that scarification is a useful strategy to overcome dormancy. The sulfuric acid scarification technique, which was also effective in scarifying *C. cretica* (Etemadi et al. 2020), substantially improved germination rates of *C. truxillensis* compared to other treatments. Additionally, the sulfuric acid scarification method allowed for many seeds to be scarified at the same time in a batch, meaning this technique could be a feasible option for preparing large quantities of seeds prior to seeding for restoration or revegetation activities, particularly because of the reduced time and effort. If the percentage of seeds that are germinating at each restoration site can be increased using pre-scarified seeds, restoration projects may be able to see more rapid success in reaching native plant cover objectives.

Bacterial endophytes reside intracellularly or intercellularly within the plant, often acting as beneficial PGPB and helping the plant survive abiotic stress (Rashid et al. 2012; Egamberdieva et al. 2017). An increasing number of studies have shown bacteria residing as endophytes in seeds as well (Li et al. 2019; Khalaf and Raizada 2016), with these bacteria likely derived from the parent plant and contributing to increased fitness and survival of the seed in its environment (Li et al. 2019; Mastretta et al. 2009; Zhang et al. 2010). *Paenibacillus* sp. are known to be PGPB for numerous plant species and have been found residing as seed and root endophytes (Eida et al. 2018; Khalaf and Raizada 2016). *Paenibacillus* has been studied in *Arabidopsis thaliana* and found to protect the plant from abiotic stressors and plant pathogens (Hong et al. 2016). Further studies have tagged the bacteria with green fluorescent protein and found that the bacteria form biofilms and colonize root tips of plants (Timmusk et al. 2005). The *C. truxillensis* bacterial strains isolated in this study were identical to each other in 16S rRNA gene sequence and were most similar to *Paenibacillus peoriae*. Although the strains showed variation in exopolysaccharide production and tolerance to cadmium, they both showed plant growth promoting traits such as nitrogen fixation, cellulase production, phosphate solubilization, and auxin production. Consistent with this, after inoculation of *C. truxillensis* seeds, one of the strains resulted in an enhancement of seedling mass. In addition, *Paenibacillus* produce endospores. Bacterial endospores are a dormant form of bacteria that can withstand harsh chemical and thermal environments and allow the bacteria to survive stressful conditions, thus making them ideal for inoculant formulations. If these *Paenibacillus* strains were to be used for plant growth promotion in the field, their endospores could be explored as a form of inoculant as this could increase inoculant shelf life due to the ability of endospores to survive variable conditions (Kim et al. 2010).

Studies have shown that plant associated bacteria, including strains of *Paenibacillus*, can help plants better cope with heavy metal stress (Kumari and Thakur 2018; Sukweenadhi et al. 2018; Eida et al. 2020). The *Paenibacillus* strains from this study showed tolerance to zinc and cadmium. Previous studies in the Ballona Reserve showed that zinc and cadmium concentrations are relatively high, presumably due to urban runoff and other anthropogenic impacts (Johnston et al. 2012). Thus, it is not surprising that bacterial strains isolated from the Ballona Wetlands have increased tolerance to these heavy metals. A study of *C. truxillensis* at a salt marsh in Patagonia, Argentina, found that *C. truxillensis* was sensitive to heavy metals, with morphology of the leaves altered depending on concentrations of pollutants such as zinc (Pollicelli et al. 2018).

Halophytes, which include *C. truxillensis*, are plants that can tolerate NaCl levels greater than 200 mM (1.17%). This is consistent with the study findings for *C. truxillensis*, which could germinate with at least 300 mM NaCl. This finding, as well as the decreased seedling growth seen with increasing salt concentrations, is analogous to what has been reported for *C. cretica* (Etemadi et al. 2020). Recent studies suggest that plant-associated microbes can play a key role in the ability of halophytes to grow in high salinity (Etesami and Beattie 2018) but may also help non-halophytes tolerate salt stress. *Paenibacillus* sp. JZ16, isolated from inside the roots of the halophyte *Zygophyllum simplex*, was found to promote salinity tolerance of *Arabidopsis* (Eida et al. 2018). The *Paenibacillus* strains from this study grew in both the absence of salt as well as in up to 4% NaCl. Both strains also produced auxin, a trait frequently associated with salt tolerant PGPB (Dodd et al. 2010). It is thought that the altered hormonal signaling, which can influence lateral root development (Yang et al. 2009), contributes to the ability of these bacteria to increase the plant's fitness in highly saline environments (Siddikee et al. 2010; Tiwari et al. 2011). Other studies have found that exopolysaccharide may allow bacteria to promote plant growth under saline stress (Abbas et al. 2019). Since *C. truxillensis* is known to grow in saline soils, inoculation with an exopolysaccharide producing strain may allow for optimized plant growth in high saline conditions. However, although one of the *Paenibacillus* strains in this study showed some growth promotion of seedlings in the absence of salt, neither strain, whether or not it produced exopolysaccharide, alleviated the negative impact salt had on seed germination or growth under the laboratory conditions tested. Future studies might look at whether these bacterial strains have a beneficial impact on plant growth with salinity stress using greenhouse and field conditions.

Many studies have focused on the use of PGPB in agriculture; however, few studies have explored applications of the microbial community on restoration and native plant revegetation projects (Ahn et al. 2007). The application and results of this study could inform habitat restoration projects throughout the range of *C. truxillensis*, including at the Ballona Wetlands. Wetland restoration projects that have focused on returning native cover to an area have quickly discovered a vast lack of available peer reviewed literature when it comes to best practices for species-specific cleaning, storage, and breaking dormancy, despite breaking seed dormancy being a widely known requirement for revegetation (Kettenring and Tarsa 2020; Barton et al. 2016). Scarification techniques recommended by this study could be used by practitioners working on habitat restoration projects with the goal of improving native plant cover. Past restoration efforts within the Ballona Wetlands have focused on reducing anthropogenic uses and removal of invasive plants within the area (Johnston et al. 2021); however, the findings from this study could allow for a unique opportunity to combine knowledge of revegetation techniques with the microbial community for successful revegetation with *C. truxillensis*. Application of these findings at a restoration project would allow for a greater knowledge base around the use of PGPB for revegetation, as well as development of best practices for germinating wild seeds.

Utilizing the findings from this study, a few key recommendations can be made for future revegetation and restoration projects. Regarding improving germination, sulfuric acid scarification should be used on seeds prior to deployment: this method resulted in the greatest average percent germination and would also allow for large batches of seeds to be scarified together, reducing energy and time. Seeds can be inoculated with PGPB's prior to seeding, such as the ones identified in this study, to increase plants' protections against heavy metals and provide biochemical advantages. Finally, considerations should be taken to deploy seeds in habitats that include some freshwater hydrology, especially in salt marsh

soils. Findings suggested that high salinity had a delaying effect on germination of *C. truxillensis* seeds, and freshwater sources may ameliorate this delay from salt stress to provide maximum germination.

Future directions with these findings will aim to test scarification methods and microbial applications *in situ* for applications within restoration projects. Scarification techniques from this study should also be explored for other native plants commonly seeded in restoration and revegetation projects, including rare species. To further contribute to the available knowledge informing habitat restorations, pre-scarified seeds could be dispersed within an area to measure the success and feasibility of scarification methods in varying field conditions. Based on findings in the Pollicelli et al. (2018) study that *C. truxillensis* has sensitivity to heavy metals, future studies might look at whether the *Paenibacillus* strains from this study are able to increase the tolerance of *C. truxillensis* to certain heavy metals. The endophytic nature and presence of plant growth promoting traits of the two strains isolated in this study suggest they are PGPB, although the strains did not alleviate the impact of salt stress under the conditions tested in this study. Future studies can further assess the conditions in which these strains might promote plant growth and assist *C. truxillensis* withstand abiotic stress, and if this would be helpful when planting seeds collected from other locations that might not carry the same microbes. Formulating inoculants for the plants and measuring growth could give definitive answers on how well each strain is able to promote plant growth and other characteristics that may be advantageous for future restoration projects.

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