Plant growth promoting traits of microorganisms isolated from saltmarsh plants rhizosphere

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Resumo

Os estuários e as suas zonas de sapal são ecossistemas de elevado valor ecológico e desempenham um papel importante na mitigação da poluição. As zonas costeiras, historicamente atrativas para a fixação humana, têm enfrentado uma degradação ambiental significativa devido às atividades humanas. As zonas de sapal sapais têm sido fortemente afetadas, devido à poluição por metais e compostos orgânicos, levando à redução da sua resiliência. Os métodos tradicionais são frequentemente dispendiosos e não são ideais para aplicações em ambientes naturais, como é o caso dos estuários. Desta forma, é crucial o desenvolvimento de tecnologias sustentáveis e adequadas, como a bioremediação e a fitoremediação, para a remoção destes poluentes, utilizando microrganismos ou plantas ou a combinação de ambos para remediar e recuperar ambientes contaminados.

Alguns microrganismos da rizosfera de plantas de sapal são capazes de produzir compostos ou solubilizar nutrientes essenciais (conhecidos como caraterísticas promotoras de crescimento de plantas (PGP)), que são cruciais para as plantas e para promover uma remoção eficiente de poluentes. Assim, este estudo teve como objetivo avaliar as caraterísticas PGP de 41 estirpes bacterianas, previamente isoladas de culturas enriquecidas com cobre (Cu) e venlafaxina (VFX), utilizando rizosedimentos de *Phragmites australis* como inóculo. Foram realizados diferentes ensaios para avaliar os diferentes PGP de cada estirpe bacteriana fixação de azoto, solubilização de fosfato, atividade de ACC deaminase, produção de sideróforos e produção de biofilme. Depois, as estirpes com as melhores características PGP foram selecionadas paraavaliar a sua influência na germinação de sementes de *P. australis*, em cultura axénica ou em consórcio Para tal, foi necessário optimizar as melhores condições de germinação para as sementes de *P. australis*.

Os resultados demonstraram que a maioria das estirpes bacterianas foram capazes de formar biofilme (85%). Além disso, metade dos isolados testados apresentaram potencial para fixação de azoto (51%), a atividade de ACC deaminase (51%) e a produção de sideróforos (59%). Contudo, só 4 estirpes bacterianas apresentaram capacidade solubilizar fosfato. As estirpes PCV3 (*Pseudomonas* sp.) e PCV7 (*Pseudomonas* sp.) foram as duas estirpes que demonstraram as caraterísticas mais promissoras de promoção do crescimento das plantas, apresentando resultados positivos em todos os cinco testes. No entanto, outras estirpes bacterianas tais como PC6 (*Hydrogenophaga* sp.), PCV1 (*Alcaligenes* sp.), PCV2 (*Brucella* sp.), PCV8

(*Alcaligenes* sp.), PCV11 (*Alcaligenes* sp.) e PCV12 (*Microbacterium* sp.), apresentaram resultados positivos em quatro dos cinco testes avaliados.

Relativamente aos resultados de germinação das sementes de *P. australis* sem a presença das estirpes, os meios MS e 0,5% NaCl foram os que apresentaram as percentagens de germinação mais elevadas. A manutenção da humidade e o fotoperíodo de 16h/8h de luz/escuro exerceram um efeito positivo na germinação. Nos ensaios com as estirpes bacterianas selecionadas (PCV3 E PCV7), o consórcio com ambas as estirpes teve uma influência positiva na germinação das sementes de *P. australis* em dois dos meios analisados (MS e NaCl 0,5% + PCB).

Este estudo demonstrou o potencial de diferentes estirpes bacterianas para produzir diferentes PGPs. Além disso, ensaios preliminares demostraram que estas estirpes podem ter um efeito positivo na germinação de sementes de *P. australis*. Estes resultados devem ser considerados no futuro, para o desenvolvimento de ferramentas de Biorremediação-Fitorremediação.

Palavras-chave: [Plantas de sapais, caraterísticas promotoras do crescimento das plantas, estirpes bacterianas, biorremediação, fitorremediação]

Abstract

Estuaries and their saltmarsh areas are ecosystems with high ecological value and can play an important role in pollution mitigation. Coastal areas, historically attractive to human settlement, have faced significant environmental degradation due to human activities. Salt marshes have been heavily impacted by metal and organic pollution, reducing the ecosystems resilience and functions. Traditional methods are often expensive and are not suitable to be applied in natural environments, such as estuarine areas. Thus, is crucial the development of sustainable technologies like bioremediation and phytoremediation, by using microorganisms or plants or the combination of both, to remediate and recover polluted environments. Some microorganisms from saltmarsh plant's rhizosphere are able to produce compounds or solubilize essential nutrients (known as plant growth promoting traits (PGP)), that are crucial for plants endurance and survival and to promote an efficient removal of pollutants.

Thus, this study aimed to evaluate the PGP traits of 41 bacterial strains, previously isolated from enriched cultures with copper (Cu) and venlafaxine (VFX), using rhizosediments from *Phragmites australis* as inoculum Different PGP assays were conducted: nitrogen fixation, phosphate solubilization, ACC deaminase activity and siderophore and biofilm production. The bacterial strains with the best PGP traits were selected and the influence of this consortium was tested on the germination percentages of *P. australis* seeds. For that, optimization of germination conditions for P. australis seeds were performed.

Results showed that most bacterial strains exhibited biofilm formation (85%). In addition, 50% of the tested bacterial strains showed positive results for nitrogen fixation (51%), ACC deaminase activity (51%), and siderophore production (59%). On the contrary, only 4 bacterial strains were capable of solubilize phosphate. The bacterial strains PCV3 (*Pseudomonas* sp.) and PCV7 (*Pseudomonas* sp.) were the two strains that demonstrated the most promising plant growth-promoting) traits, exhibiting positive results in all five tests. However, bacterial strains PC6 (*Hydrogenophaga* sp.), PCV1 (*Alcaligenes* sp.), PCV2 (*Brucella* sp.), PCV8 (*Alcaligenes* sp.), PCV11 (*Alcaligenes* sp.) and PCV12 (*Microbacterium* sp.) showed positive results for 4PGP assays, and should be considered in the future.

Regarding the *P. australis* germination assays, the MS and 0.5% NaCl media were the ones with the highest germination percentages. In addition, humidity and 16h/8h light/dark photoperiod proved to be the best conditions for the germination of P. australis seeds. When the bacterial strains with PGP traits were tested, the consortium (composed

by PCV3 and PCV7) proved to have a positive influence on the germination of *P. australis* seeds in two treatments (MS and 0.5% NaCl + PCB).

This study highlighted the potential of different bacterial strains to produce different PGPs. Furthermore, preliminary trials have demonstrated that these bacterial strains can have a positive effect on the germination of *P. australis* seeds. These results should be considered in the future, for the development of bioremediation-phytoremediation tools, for the recovery of contaminated environments.

Keywords: Salt marsh plants, plant growth-promoting traits, bacterial strains, bioremediation, phytoremediation.

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List of Abbreviations

mg	MILLIGRAMS
C	CARBON
ha	HECTARE
G	GRAMS
m^2	SQUARE METER
Yr	YEAR
h	HOUR
ng	NANOGRAM
L	LITER
Kg	KILOGRAM
CEC	CONTAMINANTS OF EMERGING CONCERN
POP	PERSISTENT ORGANIC POLLUTANTS
Cu	COPPER
Cd	CADMIUM
Co	COBALT
Cr	CHROMIUM
Fe	IRON
Mn	MANGANESE
Ni	NICKEL
Pb	LEAD
Sb	ANTIMONY
Sn	TIN
V	VANADIUM
Zn	ZINC
Al	ALUMINUM
Hg	MERCURY
As	ARSENIC
WWTP	WASTEWATER TREATMENT PLANT
VFX	VENLAFAXINE
PGPB	PLANT GROWTH-PROMOTING BACTERIA
PGPT	PLANT GROWTH-PROMOTING TRAITS
N	NITROGEN
P	PHOSPHORUS
IAA	INDOLE-3-ACETIC ACID

ECM	EXTRACELLULAR MATRIX
ACC	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID
PCA	PLATE COUNT AGAR
MM	MINERAL SALTS MEDIA
NFGMM	NITROGEN FREE GLUCOSE MINERAL MEDIA
PVK	PIKOVSKAYA PHOSPHATE GROWTH MEDIUM
CAS	CHROME AZUROL S
HDTMA	HEXADECYLTRIMETHYLAMMONIUM BROMIDE
PIPES	PIPERAZINE-1,4-BIS (2-ETHANESULFONIC ACID)
DFO	DESFERRIOXAMINE
DF	DWORKIN AND FOSTER
MS	MURASHIGE AND SKOOG
NACL	SODIUM CHLORIDE
PCB	PLATE COUNT BROTH
NACLO	SODIUM HYPOCHLORITE
OD	OPTICAL DENSITY

1. Literature Review

1.1. Salt marshes

Salt marshes are dynamic coastal ecosystems characterized by their high salinity levels and fluctuating conditions. They are typically found in the upper intertidal zone, favouring shallow slopes shorelines with low-energy waves (Balke et al., 2016). These coastal hotspots are widespread at mid and high latitudes, but in tropical and subtropical areas, mangrove ecosystems take over, although not completely (Adam, 2002). Salt marshes often experience periodic tidal or non-tidal flooding due to fluctuations in water levels (Bakker, 1993).

Beyond their historical significance as a source of food and materials (Gedan et al., 2009; Knottnerus, 2015), salt marshes, known for their ecological resilience, offer a range of critical ecosystem services that are of highly valuable (Barbier, 2011; Costanza et al., 2014). The unique flora and fauna of these habitats have adapted to survive and thrive in the face of constant salt stress. Notably, these ecosystems are highly productive, making them ecologically important (Morris, Sundberg, & Hopkinson, 2013; Curcó et al., 2002). Salt marshes significantly contributes to overall biodiversity conservation, (Lefeuvre et al., 2003). Moreover, their complex and densely plant structures provides a nursery for juvenile fish, crustaceans, and shellfish and plays a crucial role in protecting shorelines. (Boesch & Turner, 1984). In fact, the upper portion of the vegetation attenuates water velocity, diminishes turbulence, and enhances sediment deposition (Christiansen et al., 2000). The vegetation within these coastal ecosystems plays a crucial role in protecting the shorelines by acting as natural barriers that dissipate more hydrodynamic energy, thus mitigating coastal erosion (Gedan et al., 2011; Temmerman et al., 2013). When water moves across marshes, the upstanding grasses provide resistance, which slows the water down. This induces the deposition of suspended sediments, facilitating their absorption by the plants in the salt marsh (Morgan et al., 2009). The subterranean decomposition of roots contributes to the enrichment of soil organic matter, and soils with a fine, organic-rich composition generally experience slower erosion compared to mineral soils in wetland environments (Feagin et al., 2009).

Salt marshes have a high carbon accumulation rate (Duarte et al., 2013; Mcleod et al., 2011) and are recognized as essential blue-carbon ecosystems (Lovelock & Duarte, 2019; Miller et al., 2023). They engage in a two-fold process of blue carbon sequestration: short-term storage (decennial) in biomass and long-term accumulation (millennial) in sediments (Duarte et al., 2005; Mcleod et al., 2011). In fact, it is estimated

that the soils from salt marshes have the capability to sequester an average of 400 Mg C ha^{-1} (Temmink et al., 2022), in which the average carbon accumulation rate in salt marsh sediment worldwide stands at 244.7 ± 26.1 g C m^{-2} yr^{-1} (Ouyang & Lee, 2014).

1.2. Salt marshes – Anthropogenic Influences and Pollution

Throughout history, coastal areas have consistently drawn human populations for settlement and cultural development (Reimann et al., 2023; Small & Nicholls, 2003). Over one-third of the Earth's population is concentrated in coastal areas and small islands (Assessment, 2005), and by 2060, it is predicted that coastal populations will increase by 50% to 120% relative to the year 2000 (Neumann et al., 2015). The scale of population growth in coastal areas is evident in the latest research, with a figure of 2.15 billion inhabitants by 2020 (Reimann et al., 2023).

In the last century, humanity has witnessed the depletion of more than 50% of the world's original salt marshes (Adams et al., 2021; Barbier et al., 2008). The alarming trend of salt marsh loss persists, occurring at an annual rate ranging from 1% to 2% (Duarte et al., 2008; Lotze et al., 2006). Globally, salt marshes experienced a net loss of approximately 1,452.84 km^2 between 2000 and 2019. While some areas (1,390.40 km^2) were gained or recovered, the overall loss amounted to an average of 2,733.33 km^2 h^{-1} (Campbell et al., 2022).

Human activities have exerted deep alterations on salt marshes, challenging their resilience to environmental changes (Lowe & Peterson, 2014). The conversion of salt marshes for urban development or industrial purposes, known as land claiming, poses a significant danger (Allen, 2000; Du et al., 2022; X. Huang et al., 2014; Wolff et al., 2020). This process not only leads to habitat destruction but also disrupts crucial hydrological patterns and the rapid decrease in the sediment flux, which is estimated to be declining at a rate of 1.4 billion tonnes per year due to anthropogenic influence (Du et al., 2022; Neumann et al., 2015; Syvitski et al., 2005).

Sediments in these areas are alarmingly contaminated with metals and other organic and inorganic pollutants (Adrees et al., 2015). These contaminants can enter the food web, disrupting a range of vital physiological and biochemical processes within the marsh ecosystem and, through a process called biomagnification, accumulate in progressively higher concentrations as they move up the food chain. This means that humans, who often occupy the top of the food chain, can be exposed to significantly higher levels of these contaminants, posing a significant threat to human health (Clemens, 2006).

As the global human population continues to prosper, pollution emerges as an unavoidable repercussion. Whether stemming from inadvertent mishaps or deliberate choices endorsing a certain threshold of pollution for economic sustenance, the consequences are being felt across different ecosystems (Adam, 2019). Salt marshes, in particular, find themselves entwined in this intricate web of environmental challenges, as the pollution seeping into their habitats disrupts the delicate balance of these coastal landscapes (Adam, 2002). Many industrial facilities were built close to salt marshes before stricter regulations on pollution control were put in place. As a result, the sediments and waters of these urbanized estuaries often contain significant amounts of metals and organic pollutants (Beeftink & Nieuwenhuize, 1986; Negrin et al., 2021).

Solid waste contamination, including anthropogenic litter, has become a significant global issue (Galgani et al., 2015; Mazarrasa et al., 2019). Numerous studies have reported the staggering amount of litter entering the world's oceans each year (Jambeck et al., 2015). Plastic, often originating from food packaging, fishing activities, and personal use, is the predominant component of marine litter. For instance, a study carried out by Almeida et al., (2023) shows that microplastic levels in sediment samples from the Lima estuary marsh ranged from 250 to 2,500 particles per kilogram of dry sediment. Most of these particles were between 0.201 and 5 millimetres in size. However, other materials such as metal, glass, paper, and wood are also commonly found in marine and coastal ecosystems (Anastacio et al., 2023). Point source pollution comes from identifiable and localized sources, such as industrial discharge pipes or sewage treatment plants, as well as vehicle exhaust and metals from smelting and mining, which are significant contributors (Adam, 2019) . Nonpoint source pollution from diffuse sources are harder to trace back to a single origin point, including insecticides, pesticides and runoff from agricultural fields or urban areas (Wu & Chen, 2013).

Salt marshes are natural filters that can effectively remove excess nitrogen and phosphorus from water. (Celis-hernandez et al., 2021; Crain, 2007; Wimp et al., 2019). However, the intensified use of fertilizers, urban runoff, and agricultural practices has led to an elevated nutrient load and subsequent eutrophication in adjacent water bodies (Galloway et al., 2008). This nutrient enrichment, when reaching salt marshes, sparks a cascade of ecological changes, reshaping patterns of productivity, carbon sequestration, and species distribution (Canfield et al., 2010; Morris et al., 2013; Wang et al., 2018). Belowground biomass, such as roots and rhizomes, decreases as a consequence of eutrophication, (Deegan et al., 2012; Wasson et al., 2017), reducing the stability of marsh

edges and impeding vegetation expansion (Herbert et al., 2020), resulting in increased lateral erosion (Deegan et al., 2012).

In recent decades, the emergence of contaminants of emerging concern (CECs) has become a major cause for concern due to their potential harm to human health and ecosystems (Stefano et al., 2022). CECs encompass a diverse range of substances, including persistent organic pollutants (POPs), pharmaceutical compounds, personal care products, veterinary antibiotics, and nanomaterials (Taheran et al., 2018; Teng et al., 2015). These contaminants have been identified in several environmental matrices, with soil serving as a significant sink for many of these chemicals (Yadav et al., 2021). The inefficient treatment processes of wastewater treatment plants were considered as the primary sources of CECs in the aquatic environment (Liu et al., 2018).

1.2.1 Metals

Metals are a group of naturally occurring elements with high atomic weight and density, often exhibiting metallic properties (Tchounwou et al., 2014). Some of these elements are essential for enzyme function, while others do not serve any vital function in living organisms and can exhibit high toxicity even at minimal exposure levels (Theron et al., 2012). Metals are non-degradable and cannot be destroyed biologically, transitioning only between different oxidation states or organic complexes (Suman et al., 2018). On a global scale, different reports indicated that over 10 million areas are affected by soil pollution, with more than half of these locations showing contamination with metals and/or metalloids (He et al., 2015; C. Li et al., 2019). The presence of metals in different environmental matrices, such as soil and water, have been reported in different studies. In fact, several studies have reported the presence of several metal(loid)s, such as Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni), Lead (Pb), Antimony (Sb), Tin (Sn), Vanadium (V), and Zinc (Zn), in surface soil samples, at concentrations ranging between 0.03 and 701 mg Kg^{-1} (Adamcová et al., 2016; Adelopo et al., 2018; Hiller et al., 2024; Hussein et al., 2020). Additionally, heavy metal ions such as chromium (VI), cadmium (II), lead (II), arsenic (V and III), mercury (II), nickel (II), and copper (II) have been detected in wastewater sludge (Aziz et al., 2023; Vardhan et al., 2019; Zhang et al., 2023).

Natural processes like rock weathering and volcanic eruptions contribute to the presence of metals in the environment (Bradl, 2005). However, contamination with toxic metals has increased markedly in recent years, largely due to the significant exacerbation caused by human activities associated with global industrialization (Manousaki et al., 2008). Improper mining practices, excessive use of pesticides and fertilizers, and the

discharge of untreated wastewater all lead to a dramatic increase in metal contamination (Geetha et al., 2023).

In a study carried out by Jezycki et al., (2024), it was reported the presence of metals in soil samples from salt marshes, in which Cd, Cr, Cu, Ni, Pb, and Zn were detected. Moreover, the depth-weighted concentration of Cr and Zn exceeded the threshold limits concentrations for these metals (43.4 and 121 μg g^{-1} respectively). In another study carried out by Zhou et al., (2020) it was evaluated total metal concentrations in river and lake water bodies worldwide from 1972 to 2017. The results showed that metal concentrations in the water bodies tested were higher in Africa, Asia, and South America, and lower in Europe and North America. Among the 12 metals identified, 6 (Cd, Zn, Ni, Al, Mn, and As) exceeded the standard thresholds in Europe in the 2010s. with concentrations of around 6, 1339, 137, 569, 258 and 19 μg L^{-1} respectively.

The presence of metals in the environment can cause deleterious effects in the organism that inhabits these ecosystems. For instance, research conducted by Kumar et al. (2023) revealed that acute exposure to a low concentration (0.112 mg L^{-1} and 0.224 mg L^{-1}) of mercuric chloride caused a significant tissue damage in gills, liver, kidneys, and muscles of *Channa punctatus*. Another study conducted by Tang et al., (2023) the presence of As, Cu, Pb, Hg and Zn, in water samples from the estuary studied, was found, at concentrations of 0.65-9.25 μg L^{-1}, 0.07-11.57 μg L^{-1}, 0.05-9.09 μg L^{-1}, (< 0.40) μg $L^{-1}$and (0.67-86.12) μg L^{-1}, respectively. In this study, it was also observed that, at the concentrations observed, Zn had lethal effects on the crustaceans *Temora Stylifera*.

Moreover, The persistent presence of metals in the environment is alarming due to their potential carcinogenic effects on humans (Lone et al., 2008). These contaminants pose significant health risks, with exposure occurring through ingestion, inhalation, and dermal contact (El-Kady et al., 2018). Dermal exposure, though less common, can occur through contact with contaminated soil or water (Chaparro et al., 2018). Once in the body, metals can accumulate and cause various health problems, including cancer, developmental retardation, kidney damage, immunological impairment, and neurological disorders (Ismael et al., 2022; Vardhan et al., 2019).

1.2.1.1 Copper

In nature, Cu can be found as native Cu, Cu sulphides, Cu sulfosalts, Cu carbonates, Cu(I) and Cu (II) oxides (Hammond, 2004). Copper's industrial significance is undeniable, having diverse applications across numerous industrial processes, being the second most used non-ferrous metal. Due to its unique properties, copper is extensively

used in power generation, electronics, energy, petrochemicals, machinery, metallurgy, transportation, light industry and emerging technologies (Beylot & Villeneuve, 2015).

The presence of this metal in the environment can be due to natural processes such as volcanic eruptions, windblown dust carrying eroded minerals, sea spray, forest fires, and even the decomposition of vegetation (Rehman et al., 2019). However, human activities significantly amplify this problem (Panagos et al., 2018). Factories producing or using copper and related compounds, copper mining (Wojtkowska et al., 2015), the burning of fossil fuels and waste materials, domestic wastewater discharged without proper treatment, and copper-based pesticides (Neaman et al., 2024), are the main anthropogenic inputs of Cu into the environment.

Small concentrations of Cu (usually 5-30 mg kg^{-1}), are crucial for the plant's health, assisting in numerous physiological processes essential for survival and growth (Ballabio et al., 2018), like respiration, photosynthesis, and the metabolism of carbohydrates, proteins, and cell walls (Quartacci et al., 2000; Printz et al., 2016). While Cu deficiency can hinder plant growth, excessive Cu can cause alterations in DNA, cell membrane integrity, respiration, photosynthesis, and enzyme activity (Chaffai et al., 2007; Zvezdanović et al., 2007; Chen et al., 2022), and lead to stunted growth and even threaten plant survival (Printz et al., 2016). Higher concentrations of Cu can also cause negative effects on non-target organisms. In fact, Bat et al., (2017) showed a decrease of the population of *Crangon crangon* and *Syngnathus acus* in the Black Sea, due to the increased of Cu concentrations. In humans, studies have shown that concentrations of 1-5 mg L^{-1} of Cu can cause dizziness, lethargy, and headache (Pizarro et al., 1999; Royer & Sharman, 2023) and in more severe cases, can result in epigastric pain, gastrointestinal bleeding, diarrhea, vomiting, tachycardia, hematuria, respiratory difficulties, hemolytic anemia, liver damage, kidney dysfunction, and even death (Rahimzadeh et al., 2024).

Several studies reported the presence of copper in high concentrations in salt marshes. For example, in the study conducted by Chadwick & Bury, (2023) copper concentrations between 3.8 and 4420 µg Cu L^{-1} were observed in the estuarine water. Moreover, Cunha and co-authors reported the seasonal presence of different metals in non-vegetated sediments from the Lima river estuary in which concentrations of Cu ranging between 5 – 21 µg g^{-1} were found. Moreover, this study also reported the presence of Zn, Mn, Pb, Cr, and Ni (concentration between 2 - 85 µg g^{-1}) Fe (concentrations between 11 and 20 mg g^{-1}) (Cunha et al., 2024).

1.2.2. Pharmaceuticals

Pharmaceuticals, a well-kwon CECs, have been essential for the maintenance of our society (Fernandes et al., 2021). Different classes of pharmaceuticals, such as antibiotics, antidepressants, anti-inflammatory drugs, among others, are widely used in human and veterinary medicine, to treat different diseases (Fernandes et al., 2021; Larsson & Flack, 2022) However, their extensive consumption has led to their presence in different environments, at different concentrations, which can cause negative effects on the ecosystems and non-target organisms (Osuoha et al., 2023).

Wastewater treatment plants (WWTPs) are one of the major sources of pharmaceuticals in the environment (Pereira et al., 2017). In fact, WWTPs were not designed to completely remove pharmaceuticals, and they end up to be discharged into the environment (Aukidy et al., 2012; Bueno et al., 2012; Kümmerer, 2010). Pharmaceuticals can enter the environment through various pathways. Direct emissions from drug manufacturing facilities, as well as the use of sewage sludge and animal-derived fertilizers in agriculture, are significant contributors (Yang et al., 2021). Improper disposal of unused or expired pharmaceuticals, accidental spills, and leachates from solid waste landfills can also lead to environmental contamination. Pharmaceuticals contamination can leach into groundwater, ultimately contaminating drinking water (Nikolaou et al., 2007; Fernandes et al., 2021).

The diverse nature of pharmaceuticals can make their removal more difficult since these pollutants are characterized by a high degree of heterogeneity, encompassing a wide range of substances with different physicochemical properties, environmental persistence and mobility, and biological activities (Bottoni et al., 2010; Caracciolo et al., 2014). This variation makes it challenging to develop effective strategies for mitigating their presence and impact on aquatic ecosystems.

The presence of pharmaceuticals and their byproducts in different environments have been reported, including surface waters, groundwaters, coastal marine waters, drinking water, soils, and sediments (Caracciolo et al., 2015; Paíga et al., 2015). In fact, Gros & Barceló, (2012) reported the presence of pharmaceuticals. such as Clarithromycin (17 ng L^{-1}) Ibuprofen (16 ng L^{-1}) and Gemfibrozil (23 ng L^{-1}) in seawater samples from Spain. Another study by López-Serna et al., (2013) identified 95 different pharmaceuticals and their byproducts in Barcelona's urban groundwater. While most concentrations were low, for some analgesics, antibiotics, and diuretics, the concentrations exceeded 100 ng L^{-1}. Moreover, azithromycin and spiramycin were detected at concentration over 1 mg L^{-1} in

certain cases, and ibuprofen and diclofenac were also detected, with maximum concentrations of f 988 and 380 ng L^{-1}, respectively.

While these contaminants are often found in very low concentrations (ranging from ug L^{-1} to ng L^{-1}), their impact in the ecosystems can be significant due to their biological effects (Pereira et al., 2017). Moreover, pharmaceuticals can pose a significant environmental risk due to their potential to harm a variety of non-target aquatic organisms with similar biological functions and receptors (Arnold et al., 2014). For instance Hejna & Kapu, (2022) reported growth inhibition for *Lemna minor* after exposure to 22.0 mg L^{-1} of Ibuprofen . In addition, a study conducted by Rocco et al., (2010) reported DNA fragmentation, apoptosis and genomic alterations in *Danio rerio*, after exposure to 0.000092 mg L^{-1} of Ibuprofen. Nowakowska and co-authors (2020) further demonstrated that exposure to *Danio rerio* larvae to antidepressants (paroxetine, sertraline, fluoxetine, and mianserin) led to an increased rate of abnormal development, accelerated hatching time, and influenced the total hatching rate. Furthermore, the widespread of antibiotics in the environment can contribute to the development of antibiotic resistance among microbial communities, posing a significant challenge to the management of infectious diseases (Larsson & Flach, 2022).

1.2.2.1 Venlafaxine

Venlafaxine (VFX) is anorepinephrine reuptake inhibitors (SNRIs) antidepressant (Mitchell, 2020), that is prescribed to treat major depressive disorders, generalized anxiety disorder, panic disorder, and social phobia (Llorca & Castellet-rovira, 2019). VFX can enter the environment via wastewater treatment plants and through human waste and flushed medications (Schultz et al., 2010).

The low biodegradation efficiency of VFX and the ineffectiveness of existing municipal wastewater treatment processes in removing VFX can pose a challenge, leading to its persistence in effluents and raising concerns about potential impacts on aquatic ecosystems (Huang et al., 2021). While VFX's environmental presence is concerning, the combined action of venlafaxine and its metabolite (O-desmethylvenlafaxine), can be stronger than the parental compound alone (European Commission, 2022). VFX can be found in WWTP effluents at concentrations ranging between 68 ng L^{-1} to 268 ng L^{-1} (Santos et al., 2013), however, VFX exhibits the lowest removal efficiency among similar antidepressants, with conventional treatment processes only managing to eliminate up to 6.5% of VFX (Cao et al., 2020; Lajeunesse et al., 2012).

Due to the lack of removal efficiency of this pharmaceutical, VFX has been found in different environmental matrices such as surface water, groundwater, sediments and even in water for human consumption (Bueno et al., 2014; Lehmphul, 2016). Some studies point to the presence of VFX in surface water samples at concentrations ranging from 5 to 575 ng L^{-1} (Gracia-lor et al., 2011; Petrovic et al., 2014). In the study carried out by Gros & Barceló, (2012), the presence of this drug was confirmed in seawater samples at concentrations of 52 ng L^{-1}. Furthermore, Osorio et al., (2016) detected VFX in sediment and soils samples at concentrations between 0.05 and 1.94 ng g^{-1}. Due to VFX widespread presence, the European Union has included this pharmaceutical on the 3rd and 4th EU Watch List to assess its distribution and potential risks to aquatic ecosystems (European Commission 2020; European Commission 2022).

The potential deleterious effects of VFX in different organisms have been investigated. According to Bastos et al. (2017), toxic effect on *Pseudokirchneriella subcapitata* were observed due to exposure to VFX (EC50 of 11 mg L^{-1}). Complete locomotion inhibition of freshwater snails, *Lymnaea stagnalis*, was reported by Raman and co-authors, for concentration of 50 µg L^{-1} of VFX (Raman et al., 2024).

Bearing this in mind, it is essential to prevent the introduction of organic and inorganic pollutants into environment, to protect the ecosystems and the living organisms. The presence of organic and inorganic pollutants in natural environments and their potential impacts and/or toxicity on non-target organisms emphasize the urgent need for the development of sustainable remediation techniques for the recovery of contaminated environments.

1.3. Bioremediation and Phytoremediation as sustainable technologies to recover contaminated environments

Traditional methods available for the removal of metals and pharmaceuticals from polluted environments often rely on physical or chemical processes. Since pharmaceuticals have a more diffuse environmental distribution, technologies like ozonation, nanofiltration, advanced oxidation, and adsorption (Sivaprakash et al., 2022) are available but they have mostly been developed for artificial reservoirs (WWTP). On the other hand, technologies such as soil isolation, soil substitution, vitrification, thermal desorption, immobilisation, chemical washing, encapsulation, chemical fixation are used to remove metal pollutants from the environment (Azhar et al., 2022).

Although these chemical approaches have demonstrated high efficiency in removing these pollutants from the environment, most of this technologies are not suitable to be

applied in natural environments, (Dhangar and Kumar 2020), can be expensive and lacks public support (Khalid et al., 2017) Thus, the development of sustainable and suitable technologies to remediate and recover polluted environments is crucial for the preservation of environmental health and promotion of sustainable conservation practices (Gerhardt et al., 2017; Hasan et al., 2019).

Bioremediation and phytoremediation are sustainable and cost-effective technologies suitable for the removal of pollutants, promoting the recovery of the affected ecosystems. Bioremediation is a technology that relies on the capacity of microorganisms to reduce, eliminate, contain, or transform pollutants, taking advantage of natural detoxification processes (Kensa, 2011). Bioremediation techniques offer effective and minimally disruptive methods for treating contaminated soil, water, wastewater and groundwater. Bioremediation can be applied using different approaches, being the most common biostimulation and bioaugmentation. Biostimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation (Khan et al., 2017). By altering the physical and chemical properties of the contaminated site (addition of various forms of limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon), biostimulation can stimulate the bioremediation process (Adams et al., 2015). For example, bioventing, involves injecting air into the soil to stimulate indigenous bacteria. On the other hand, bioaugmentation is the addition of specialized microorganisms exhibiting specific functional machineries to degrade the target pollutant that cannot be readily biodegraded by microorganisms that are present in that environment (Fernandes et al 2022).

Bioremediation processes are greatly influenced by environmental factors (Adams et al., 2015). Even if microorganisms possess the necessary genetic traits for degradation, they cannot effectively break down pollutants unless the environmental conditions align with their metabolic requirements (Gaylarde et al., 2005). Key factors affecting bioremediation include the physical nature of the contaminated site, temperature, light, chemical composition, pH, humidity, oxygen levels, redox potential, and the structure of the pollutant (Nazir et al., 2021). Additionally, the presence of other contaminants can also impact bioremediation as they can be toxic for the microorganisms (Gaylarde et al., 2005; Nazir et al., 2021).

In recent years, significant advancements have been made in bioremediation field, with the discovery of microorganims capable of degrading considered non-degradable compounds. For instance, Fortunato et al., (2016) reported that bacterial species *Comamonas aquatica* and *Bacillus* sp., showed up to 92% degradation capacity of 100

mg L^{-1} of ibuprofen. Moreover, a group of endophytic bacteria, belonging to the *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* phyla, isolated from the plant *Phragmites australis*, demonstrated high efficiency (90%) in removing the pharmaceutical compound carbamazepine (Sauvêtre and Schröder, 2015).

Phytoremediation is a cost-effective and environmentally friendly technology, that harnesses the ability of plants to remediate contaminated sites by immobilizing, sequestering, detoxifying, stabilizing, or degrading pollutants originating from various sources (Kafle et al., 2022). Plants have the ability to absorb and accumulate metals in their tissues and degrade organic pollutants, thereby reducing the toxicity of these pollutants in soil and water resources (Tan et al., 2023).

Phytoremediation strategies can be developed using different strategies: . (Ali et al., 2013; Arthur et al., 2007; Kafle et al., 2022; Salt et al., 1998):

- Phytoextraction is a process that involves the uptake of metals or organic contaminants by plants that concentrate these pollutants in their harvestable tissues, effectively removing them from the soil.
- Phytostabilization is a process that involves the inactivation or immobilization of pollutants within the roots or in the rhizosphere.
- Rhizofiltration employs plant roots to absorb or adsorb contaminants, particularly metals, from water and liquid waste.
- Phytovolatilization involves the transformation of contaminants into volatile forms that are then released into the atmosphere by plants.
- Phytodegradation leverages the combined action of plants and their associated microorganisms to break down organic pollutants.

The plant species selected for phytoremediation should exhibit hardiness, high biomass production, tolerance to the toxic effects of metals and contaminants, ease of cultivation and a high capacity for absorbing pollutants (Kafle et al., 2022).

The combination of bioremediation and phytoremediation technologies can improve the removal of the target pollutants (Agnello et al., 2016; Glick, 2003). This synergistic approach, particularly beneficial for mixed pollution contaminated soils, enhances the interaction between plants and microorganisms, leading to increased uptake of metal and the reduction, elimination or transformation of organic contaminants (Huguenot et al., 2015; Lebeau et al., 2008). Microorganisms can improve root absorption and increase the bioavailability of metals in the rhizosphere, facilitating their uptake by plants (Lin et al., 2008; Sessitsch et al., 2013).

For instance, Fahid et al., (2019), reported that the combination of *P. australis* and microorganisms can significantly enhance hydrocarbon degradation in wetland ecosystems, resulting in a 28.8% higher reduction compared to the use of plants alone. Teixeira et al., (2014) evaluated the influence of a bacterial consortium to improve phytoremediation of *Phragmites australis* and *Juncus maritimus* from salt marsh sediments contaminated with cadmium. They concluded that the addition of the consortia increased *Juncus maritimus* metal phytostabilisation capacity and promoted metal phytoextraction potential in *Phragmites australis.*

1.3.1. *Phragmites australis*

The perennial macrophyte, *Phragmites australis* (Cav.) Trin. Ex Steud (also known as the common reed) exhibits remarkable resilience due to its robust rhizome system. *P. australis* is characterized by high propagation ability, extended growth periods, and exceptional adaptability across diverse environments (Fraser et al., 2004; X. Liu et al., 2012). The adaptability of *Phragmites sp*. is evident in their geographic distribution, colonizing wetlands from cold temperate to hot tropical zones across most continents (Havens, 1997; Vymazal, 2013). This species thrives in Portuguese wetlands across the country, except for those at high altitudes, exhibiting a widespread presence in numerous coastal salt marshes (Pacheco et al., 2012).

Phragmites australis is a salt marsh plant with high potential for phytoremediation process. Its extensive root system, high biomass production, and well-documented tolerance to pollutants are backed by numerous studies demonstrating its effectiveness in cleaning up contaminated sites (Kalu et al., 2021; Milke, 2020; Rezania et al., 2019). *P. australis* can play a role in the removal of high concentration nutrients, such as nitrogen and phosphorus (Milke, 2020). This nutrient uptake helps to prevent eutrophication, a process that can lead to algal blooms and oxygen depletion in aquatic ecosystems (Eid et al., 2020; Geurts et al., 2020; Meuleman et al., 2002).

Additionally, this plant demonstrates an impressive ability to accumulate high concentrations of metals from contaminated soils and sediments, effectively sequestering them within its tissues and preventing their release back into the environment, thereby mitigating ecological risks (Cicero-fernández et al., 2015; Fawzy et al., 2011). A study conducted by Bonanno (2013) showed that *P. australis* has the ability to uptake Al, Mn, Zn, Cu, Pb, Ni, Cr, Hg. Moreover, a study conducted by (Rzymski (2014) demonstrated the ability of *P. australis* to accumulate a variety of metals (Cr, Cd, Cu, Co, Fe, Pb, Mn, Ni and Zn) in its roots, with Cd and Pb being translocated to the leaves.

This plant plays a crucial role in the biodegradation of organic pollutants, such as hydrocarbons, pesticides, and pharmaceuticals. Its associated rhizosphere microbiome facilitates the breakdown of these complex molecules into more harmless byproducts (Toyama et al., 2013; Zhang et al., 2023). For example, the study carried out by Fernandes et al., (2015) shows that in an experiment in constructed wetland microcosms with *P. australis* at least 94% of tetracycline and 98% of enrofloxacin (two veterinary antibiotics) were removed. Moreover, *P. australis* was associated to the high removal efficiency of diclofenac and atenolol in a constructed wetland system (Francini et al., 2018). *P. australis* is one of the most used species in constructed wetland treatments, to treat various types of wastewaters, effluent (Bhatia & Goyal, 2013, Hardej & Ozimek (2002).

1.4. Plant Growth Promoting bacteria

The microbiome around plant roots is a complex and ever-changing ecosystem. The composition of the plant's microbiome is shaped by a confluence of factors, such as the interaction between microbes and the plant itself, environmental conditions, and the root exudates (Huang et al., 2014). Plant root exudates, which contain amino acids, organic acids, and specific sugars, can attract microorganisms to the root zone. These compounds not only attract bacteria but also provide them with a readily available carbon source to fuel their activity and competition (Feng et al., 2021).

The term "plant growth-promoting bacteria" (PGPB) was first introduced by Kloepper & Schroth (1981) to describe these beneficial bacteria. PGPB are a fundamental element in the intricate network of soil-plant interactions (Hoque et al., 2023). PGPB not only ensure the availability of essential nutrients to plants but also enhance their use. In addition, these microorganisms produce phytohormones that stimulate plant growth (Andrade et al., 2023). Furthermore, PGPB can help plants withstand abiotic stresses like salinity and drought (Khalid et al., 2009).

In the rhizosphere, the zone of influence around plant roots (Hartmann et al., 2008), PGPB establish a complex web of interactions with plants that significantly influence their development and growth through a variety of mechanisms (Glick, 1995; Hoque et al., 2023). These mechanisms can be broadly categorized into two main strategies: direct stimulation and indirect promotion (Ortíz-Castro et al., 2009; Penrose & Glick, 2003a). Direct stimulation by PGPB involves providing plants with essential nutrients in forms readily available for uptake. This includes fixing atmospheric nitrogen, solubilizing minerals (like phosphates), and offering sequestered iron through the production of

siderophores (Glick, 2012). Indirect promotion, on the other hand, focuses on protecting plants from detrimental pathogens and reduce disease incidence. PGPB employ various strategies to achieve this biological control (Khalid et al., 2009). Some bacteria produce antibiotics, enzymes, or fungicidal compounds that directly target and inhibit the growth of pathogenic organisms. Some microorganisms produce or used siderophores to compete with pathogens for essential iron, limiting their ability to establish themselves within the rhizosphere (Paredes-páliz et al., 2016). PGPB can improve plant stress by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that helps regulate stress hormone levels in plants (Ahemad & Kibret, 2014; Glick, 2012; Paredes-páliz et al., 2016).

Research has consistently demonstrated that individual PGPB strains frequently exhibit a diverse range of beneficial mechanisms (Kloepper, 2003; Saleem et al., 2007; Vessey, 2003). PGPB can be classified based on their functional activities (Antoun & Prévost, 2006):

- Biofertilizers: Enhance the availability of nutrients for plants.
- Phytostimulators: Promote plant growth, often through the production of beneficial phytohormones.
- Rhizoremediators: Degrade organic pollutants in the soil.
- Biopesticides: Control plant diseases, primarily through the production of antibiotics and antifungal metabolites.

1.4.1. Nitrogen Fixation

Nitrogen (N), a crucial element for plant growth and development, serves as foundation for many essential biomolecules (Rascio & Rocca, 2013). However, despite its high atmospheric concentration (around 78%), most nitrogen is unavailable for direct uptake by plant roots (Vocciante et al., 2022). The strong triple bond between nitrogen atoms (N≡N) can difficult its utilization by the plants (Rascio & Rocca, 2013).

PGPB play a crucial role in nitrogen fixation, making nitrogen available for plants. PGPB can overcome this limitation through two primary nitrogen fixation mechanisms: symbiotic and free-living (Aasfar et al., 2021; Goswami et al., 2016).In symbiotic nitrogen fixation, some plants form a mutually beneficial association with specific bacteria. These bacteria live within specialized root nodules, where they convert atmospheric nitrogen into ammonia for the plant's consumption (Bhattacharjee et al., 2008). This symbiotic partnership enables plants to fulfil their nitrogen requirements without relying solely on external sources (Andrade et al., 2023). Conversely, free-living nitrogen-fixing PGPB

exists independently within the rhizosphere without forming a physical symbiosis (Bhattacharjee et al., 2008). These bacteria fix atmospheric nitrogen and release it into the surrounding soil, making it accessible for plant uptake through their root systems (Aasfar et al., 2021). This indirect contribution enhances the overall nitrogen budget of the associated plant. A study conducted by García-Fraile et al., (2012) reported that the using of nitrogen-fixing PGPB increased the growth in both plants tested (*Solanum lycopersicum* and pepper plants), where the dry weight of inoculated seedlings (shoots and roots) was more than double that of uninoculated seedlings. In another study, free-living nitrogen-fixing bacteria *Azotobacter* and *Azospirillum* were used in an experiment to evaluate the influence of non-pathogenic nitrogen-fixing plant growth-promoting rhizobacteria on vegetable production. The authors observed that the presence of these bacterial strains increased growth of the plants tested (Zaidi et al., 2017). Ferreira and co-authors characterized the halotolerant bacteria from the roots of the saltmarsh plant *Salicornia ramosissima*, and they reported the presence of halotolerant nitrogen-fixing bacteria belonging to the genera *Halomonas*, *Kushneria*, *Psychrobacter*, *Stenotrophomonas* and *Brevibacterium* (Ferreira et al., 2021).

1.4.2. Phosphate Solubilization

Phosphorus (P) is an essential macronutrient for several plant functions, such as cell division, energy transfer, DNA synthesis, and the formation of phospholipids the building blocks of cell membranes (Isidra-arellano et al., 2021). Moreover, plant health hinges on the adequate availability of phosphorus, since phosphorus is important for the synthesis and activation of enzymes responsible for the acquisition and transport of other vital nutrients within the plant (Lambers, 2022; Khan et al., 2023). In addition, phosphorus levels are crucial for plants to effectively regulate their response to environmental stresses like heat, drought, salinity, and metal toxicity (Hawkesford et al., 2012).

Even though phosphorus is widely available in the earth's crust (Paz-ares et al., 2021), the primary form readily utilized by plants, inorganic orthophosphate (Pi), exists predominantly in an insoluble state, exhibiting limited mobility within the soil profile, making it mostly unavailable for plants (Etesami, 2020). Due to the limited bioavailability of phosphorus in soil and its crucial role in promoting plant development, plant growth is consequently restricted (Rebi et al., 2022).

Plant access to phosphorus is highly influenced by the dynamic interactions between the soil, rhizosphere, and plants (Gopalakrishnan et al., 2015). A critical factor within this intricate relationship is the presence of phosphate-solubilizing bacteria within the rhizosphere microbiome (Pantigoso et al., 2023). They actively promote the dissolution

of mineral forms of phosphorus by using phosphatase enzymes, secreting organic acids (including gluconic acid), releasing protons, and producing chelating substances (Chung et al., 2005; Etesami et al., 2015; Rodríguez & Fraga, 1999), making this essential nutrient more readily available for plant uptake and utilization (Richardson & Simpson, 2011; G. Wang et al., 2022).

Several studies reported the potential of some bacterial strains to solubilize phosphate. In fact, a study conducted by Ferreira et al., (2021) studied the root−associated microbial community of *Salicornia ramosissima* and they reported that bacteria from the genera *Bacillus, Halomonas, Pseudomonas, Micrococcus, Kushneria, Psychrobacter, Staphylococcus* had potential to solubilize P. Moreover, Tagele and co-authors showed that *Burkholderia contaminans* also had P solubilization potential (Tagele et al., 2018).

1.4.3. Siderophore Production

Iron is essential for plants, as it underpins several crucial functions for instance electron transport, reactions involving oxidation and reduction, detoxification of harmful oxygen radicals, and the synthesis of precursors for DNA (Hider & Kong, 2010). Although it is the fourth most abundant and common element present in the soil, it is rarely found in free form (Sayyed et al., 2013), and like phosphorus, a great amount of insoluble iron in the soil is not available for plants (Peng et al., 2023).

Under iron scarcity conditions, different microorganisms, that inhabits the plant rhizosphere, can produce siderophores (Neilands, 1981; Gomes et al., 2024), small, highly-affinity iron-chelating molecules, that are crucial for iron uptake. Microorganisms can produce different siderophores, with a wide range of chemical structures and different capacity to bind to different metals besides iron. Siderophores can be classified as hydroxamates, catecholates or carboxylate (Ferreira et al., 2019). Environmental pH influence siderophore prevalence, with hydroxamates being more common in neutral to acidic conditions and catecholates in neutral to alkaline environments (Winkelmann & Drechsel, 2018; Ferreira et al., 2019). Several bacteria have been identified as siderophore producers such as bacteria from the genera *Pseudomonas* (Sharma & Johri, 2003) , *Bacillus* (Sultana et al., 2021), *Streptomyces* (Sadeghi et al., 2012), *Azotobacter*, *Azospirillum*, *Nocardia*, *Paenibacillus* and *Serratia* (Ferreira et al., 2019).

1.4.4. IAA Production

Indole-3-acetic acid (IAA), a well-characterized auxin phytohormone is crucial for plant growth and development (Etesami & Glick, 2024). IAA has an influence in several physiological processes including intercellular signal transduction, regulation of plant growth, and induction of plant defence system (Tian et al., 2018). Additionally, it can also

influence photosynthesis, metabolite production, and even mediate plant resistance to stress conditions (Li et al., 2021). Studies have shown that IAA triggers a range of plant responses, including rapid cell elongation for faster growth and long-term changes like cell division and differentiation (Cleland, 1990; Hagen, 1990; Mohite, 2013).

Different bacteria can produce IAA, being determinant in plant-bacteria interactions, particularly affecting plant growth and nodule formation (Etesami & Glick, 2024; Lana et al., 2023). The study conducted by Duca et al.(2014) demonstrated that several bacterial strain, belonging to the genera *Pseudomonas*, *Azospirillum*, *Bacillus*, *Klebsiella*, *Serratia*, and *Enterobacter*, were able to produce IAA, directly affecting growth processes of the plant *Vigna radiata*.

Overall, these bacteria enhances plant nutrient acquisition by promoting the development of a more extensive root system with a larger surface area for nutrient absorption (Khandelval et al., 2022). Additionally, IAA bacterial producers can pass through the plant cell walls, increasing the availability the root exudates, providing additional source of nourishment for the bacteria (Peng et al., 2023).

Different studies suggests that 80% of bacteria isolated from plant rhizosphere possess the ability to synthesize IAA (Etesami & Glick, 2024; Patten & Glick, 1996). In fact, different bacterial strains isolated from plants rhizosphere showed potential to produce IAA, such as bacteria belonging to the genera *Acinetobacter*, *Acetobacter*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Herbaspirillum*, *Klebsiella*, *Mesorhizobium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Rouxiella*, *Serratia*, *Stenotrophomonas*, *Streptomyces*. (Morales-Cedeno et al., 2021; Orozco-mosqueda et al., 2019).

1.4.5. Biofilm Formation

Biofilms are structured communities of microorganisms, embedded in a self-produced extracellular matrix (ECM), composed of a diverse array of exopolysaccharides, extracellular nucleic acids, and proteins, that adhere to biotic or abiotic surfaces (Niu et al., 2020). This ECM provides both mechanical stability and a platform for cell-cell communication, ultimately leading to the formation of synergistic micro-consortia (Flemming et al., 2016). Moreover, it also serves as a protective shield, enhancing bacterial resistance to harsh environmental conditions and safeguarding them from pathogens (Singh et al., 2021).

These biofilms facilitate crucial interactions between the bacteria and the plant. For instance, they can act as nutrient exchange channels, providing the plant with essential

minerals while the bacteria benefit from root exudates (Niu et al., 2020). This symbiotic relationship can significantly enhance the growth and health of both organisms. The ability of PGPB to rapidly colonize root surfaces and compete for space and nutrients plays a key role in protecting plants from a variety of potential threats, including pathogenic bacteria, fungi, and even viruses (Ahemad & Kibret, 2014).

Biofilms can serve as a protective shield for microorganisms against metal exposure, changes in pH, osmolarity, lack of nutrients, and various external mechanical forces, but it can also provide crucial protection for plants, as demonstrated by Chien et al.(2013). In addition, the use of biofilm-forming plant growth-promoting rhizobacteria in agriculture and phytoremediation has gained traction in recent years as a strategy to mitigate various environmental stresses (Li et al., 2024). Research shows that the biofilm produced by PGPB in plant rhizosphere protects plants from drought stress by keeping a high-water potential in the rhizosphere, reducing osmotic stress (Fetsiukh et al., 2021). In another study, under salt stress, plants inoculated with biofilm-forming PGPB were better able to withstand salt stress and maintain ionic balance and ion homeostasis (Haque et al., 2022).

1.4.6. ACC deaminase activity

Endogenous production of the gaseous plant growth hormone ethylene (C_2H_4) is a ubiquitous phenomenon observed in nearly all plant species (Saleem et al., 2007). This gas triggers a range of physiological responses that influence the plant functions. Ethylene sees its production significantly accelerate in plants under stressful conditions (salinity, drought, waterlogging, metals and pathogen exposure), leading to disruptions on roots growth, ultimately hindering overall plant development (Siddikee et al., 2015). Moreover, investigations suggest that ACC deaminase offers additional benefits beyond simply reducing the inhibitory effects of ethylene (Li et al., 2021). This enzyme can enhance functionality of other plant growth hormones like IAA, ultimately promoting optimal plant growth under both stress and non-stress conditions (Khandelval et al., 2022).

Certain PGPB strains harbour a beneficial enzyme known as ACC (1-aminocyclopropane-1-carboxylic acid) deaminase, that hydrolyses the ethylene precursor into α-ketobutyric acid and ammonia (Khandelvalet al., 2022), thereby mitigating the negative effects of stress-induced ethylene production on plants (Glick, 2005; Glick et al., 1998; Saleem et al., 2007). A study carried out by Siddikee et al., (2015) shows that the ACC deaminase activity of a *Bacillus* strain plays an important role in reducing ethylene levels in plants in the presence of salt stress.

Notably, the presence of ACC deaminase has been identified in a diverse range of microorganisms encompassing both gram-negative (Gontia-Mishra et al., 2017) and gram-positive bacteria (Babalola et al., 2003) and even some fungal species (Rauf et al., 2021).

ACC deaminase activity is frequently found in the plant microbiome, particularly in environments with challenging conditions (Timmusk et al., 2011). Several authors have been reported the presence ACC deaminase activity in different bacterial genera, for instance in *Aneurinibacillus*, *Arthrobacter*, *Achromobacter*, *Bacillus*, *Brevibacterium*, *Burkholderia*, *Citrobacter*, *Enterobacter*, Leclercia, *Micrococcus*, *Ochrobactrum*, *Parastrephia*, *Pseudomonas*, *Ralstonia* and *Serratia* genera (Orozco-Mosqueda et al., 2020; Ferreira et al., 2019; Kamran et al., 2019).

1.5. Objectives

Phytoremediation and bioremediation (through bioaugmentation) can be presented as a promising solution to remove different pollutants, including metals. However, more studies are needed to further address whether combining plants and bacteria with PGPB traits can enhance plants endurance and improve of the remediation process.

The main objective of this study was to evaluate the plant growth promotion traits of different microorganisms isolated from *P. australis* rhizosediments. To achieve this major goal, this work was divided in different sub-objectives:

- Unveil the PGP potential of 41 bacterial strains, through different screening assays: nitrogen fixation, phosphate solubilization, ACC activity, siderophores and biofilm production. After that, select the bacterial strains with the best PGP traits for the experiments with *P. australis* seeds.
- In parallel optimise the best conditions for the germination of *P. australis* seeds.
- Evaluate the effects of the selected bacterial strains on the germination of *P. australis* seeds, either alone or in a consortium.

2. Materials and Methods

2.1. Material Sterilization

To avoid contamination during the experiments, all materials and solutions were sterilized. Biological sterilization was performed by autoclaving the material and solutions (VWR VAPOUR-Line lite) in cycles of 20 minutes at 121°C. All assays were made in aseptic conditions in a flow chamber (LABOGENE SCANLAF MARS) after a UV cycle of 20 minutes.

Moreover, chemical decontamination was achieved by submerging the material in a chloride acid bath (10% (v/v)) for 24 h, being hereafter washed with deionized water.

2.2. Sampling, bacterial isolation and identification of the bacterial strains used in the current study

In a previous study conducted by Pereira et al. (in prep), an enrichment process was performed by exposing rhizosediments from *Phragmites australis,* collected in the Lima River Estuary, to 1mg L^{-1} of target contaminants: copper (Cu), venlafaxine (VFX) and a mixture of both (Cu + VFX).

Briefly, after twelve cycles of 15 days, the enriched cultures were spread in several tenfold dilutions (up to 10^{-5}) onto Plate Count Agar (PCA) (Liofilchem S.r.l.) and in Mineral Salts Medium (MM) (Alexandrino et al., 2018) doped with 1 mg L^{-1} of Cu and VFX. After that, individual colonies with different morphologies were visually identified. Each colony was purified by repetitive streaking in the same agar plates where the colonies were found. Considering the different enriched cultures and different media, the bacterial strains were named as follows: PC or PCV, bacterial strains isolated in PCA media, from the enriched culture with Cu and Cu and VFX, respectively; and MC and MCV, bacterial strains isolated in the MM medium, from the enriched cultures doped with Cu and Cu and VFX, respectively. Afterwards, the bacterial strains were identified through 16S rRNA gene sequence analysis (regions V1–V9 of the 16S rRNA gene) and consensus sequences were submitted to GenBank for taxonomic identification. To ensure and compare the results, EzBioCloud database (Yoon et al., 2017) was also used. In the end, 41 bacterial strains were isolated and identified. All 41 bacterial strains were considered for this study (Table 1).

Table 1 - Taxonomic identification of bacterial isolates, their ID used throughout the work, the enriched culture medium from which they were isolated and the respective contaminants used for enrichment.

ID	Taxonomic identification	Culture Media	Pollutant
MC1	*Albirhodobacter* sp.	MM w/Cu	Cu
MC2	*Albirhodobacter* sp.	MM w/Cu	Cu
MC3	*Pseudomonas* sp.	MM w/Cu	Cu
MC4	*Pseudomonas* sp.	MM w/Cu	Cu
MC5	*Hydrogenophaga* sp.	MM w/Cu	Cu
MC6	*Pseudomonas* sp.	MM w/Cu	Cu
MCV1	*Alcaligenes* sp.	MM w/ Cu and VFX	Cu and VFX
MCV2	*Oceanisphaera* sp.	MM w/ Cu and VFX	Cu and VFX
MCV3	*Oceanisphaera* sp.	MM w/ Cu and VFX	Cu and VFX
MCV4	*Alcaligenes* sp.	MM w/ Cu and VFX	Cu and VFX
MCV5	*Oceanisphaera* sp.	MM w/ Cu and VFX	Cu and VFX
MCV6	*Alcaligenes* sp.	MM w/ Cu and VFX	Cu and VFX
MCV7	*Albirhodobacter* sp.	MM w/ Cu and VFX	Cu and VFX
MCV8	*Pseudomonas* sp.	MM w/ Cu and VFX	Cu and VFX
PC1	*Albirhodobacter* sp.	PCA	Cu
PC2	*Pseudogemmobacter* sp.	PCA	Cu
PC3	*Alcaligenes* sp.	PCA	Cu
PC4	*Chryseobacterium* sp.	PCA	Cu
PC5	*Albirhodobacter* sp.	PCA	Cu
PC6	*Hydrogenophaga* sp.	PCA	Cu
PC7	*Pseudogemmobacter* sp.	PCA	Cu
PC8	*Pseudomonas* sp.	PCA	Cu
PC9	*Pseudomonas* sp.	PCA	Cu
PC10	*Hydrogenophaga* sp.	PCA	Cu
PC11	Not identified	PCA	Cu
PC12	*Paenibacillus* sp.	PCA	Cu
PC13	*Microbacterium* sp.	PCA	Cu
PC14	*Brevundimonas* sp.	PCA	Cu
PC15	*Niallia* sp.	PCA	Cu
PCV1	*Alcaligenes* sp.	PCA	Cu and VFX
PCV2	*Brucella* sp.	PCA	Cu and VFX
PCV3	*Pseudomonas* sp.	PCA	Cu and VFX
PCV4	*Albirhodobacter* sp.	PCA	Cu and VFX
PCV5	*Alcaligenes* sp.	PCA	Cu and VFX
PCV6	*Chryseobacterium* sp.	PCA	Cu and VFX
PCV7	*Pseudomonas* sp.	PCA	Cu and VFX
PCV8	*Alcaligenes* sp.	PCA	Cu and VFX
PCV9	*Pseudomonas* sp.	PCA	Cu and VFX
PCV10	*Hydrogenophaga* sp.	PCA	Cu and VFX
PCV11	*Alcaligenes* sp.	PCA	Cu and VFX
PCV12	*Microbacterium* sp.	PCA	Cu and VFX

2.3. Screening for Plant-Growth Promoting Traits

Plant-growth promoting traits were explored in all 41 bacterial strains, previously isolated as described in section 2.2. For that, different screening assays were performed, namely nitrogen fixation, phosphate solubilization, siderophores production, biofilm production and ACC deaminase activity. All the methodologies were optimised in the current work. All tests were preceded by a bacterial subculturing on PCA (PC and PCV bacterial strains) and on MM doped with Cu (MC bacterial strains) or Cu + VFX (MCV bacterial strains), for 48 hours, at 28 ºC.

2.3.1. Nitrogen Fixation

Bacterial strains described in section 2.2 were tested in screening assays to evaluate their ability for N fixation. The assays were performed on Nitrogen Free Glucose Mineral Media (NFGMM), without the addition of any nitrogen source, containing per 1 L of deionized water: glucose (10 g), malic acid (10 g), K_2HPO_4 (1 g), $CaCl_2$ (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), $MnSO_4 \cdot 5H_2O$ (0.01 g), NaCl (25 g), agar (15 g) and $C_27H_{28}Br_2O_5S$ (bromothymol blue, 0.0025 g) (Ferreira et al., 2021). In the end, pH was adjusted to 7.0, and the media was further autoclaved.

Each bacterial strain was cultivated in triplicate in NFGMM mediaand incubated for a week at 28 °C (VWR INCU-Line). During this time, two evaluations were carried out, the first after 72 hours and the second after one week of incubation. The result was considered positive when in the medium colour shifted from green to blue (Figure 1).

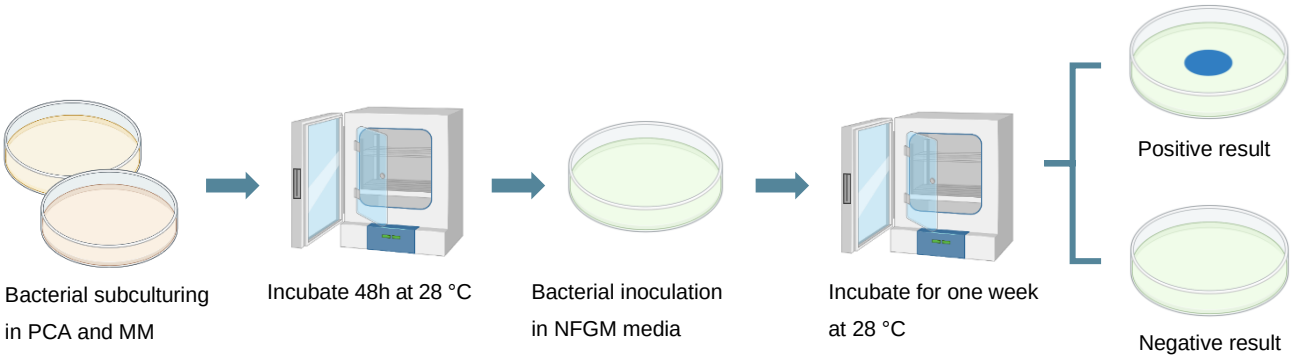


Figure 1 - Schematic of the nitrogen fixation screening procedure.

2.3.2. Phosphate Solubilization

Phosphate solubilization ability by each bacterial strain was evaluated using the Pikovskaya (PVK) (Pikovskaya, 1948), further adapted by Onyia & Anyanwu (2013), containing per litter of deionized water: glucose (10 g), $Ca_3(PO_4)_2$ (5 g), $MgCl_2.6H_2O$ (5 g), $MgSO_4.7H_2O$ (0.25 g), KCl (0.2 g), $(NH_4)_2SO_4$ (0.1 g) and Agar (15 g). Two additional media were prepared by replacing glucose with tryptone (PVK-T) or yeast extract (PVK-Y) in the same quantities, since some bacterial strains may not use glucose as main carbon source. The medium was adjusted to pH 7.0 and subsequently autoclaved. To prevent sedimentation and ensure uniform distribution, the medium was gently agitated during the pouring procedure into the petri dishes.

Afterwards, each bacterial strain was cultivated in triplicate in PVK medium and incubated at 28 °C for one week (Figure 2). Bacterial growth was assessed after 72 hours and after one week of incubation. The results were considered positive (meaning phosphate solubilization) when a clear zone surrounding the bacterial colonies was observed. In addition, the bacterial isolates that presented growth but with non-appearance of a transparent halo were considered “Negative results with growth" (Figure 2).

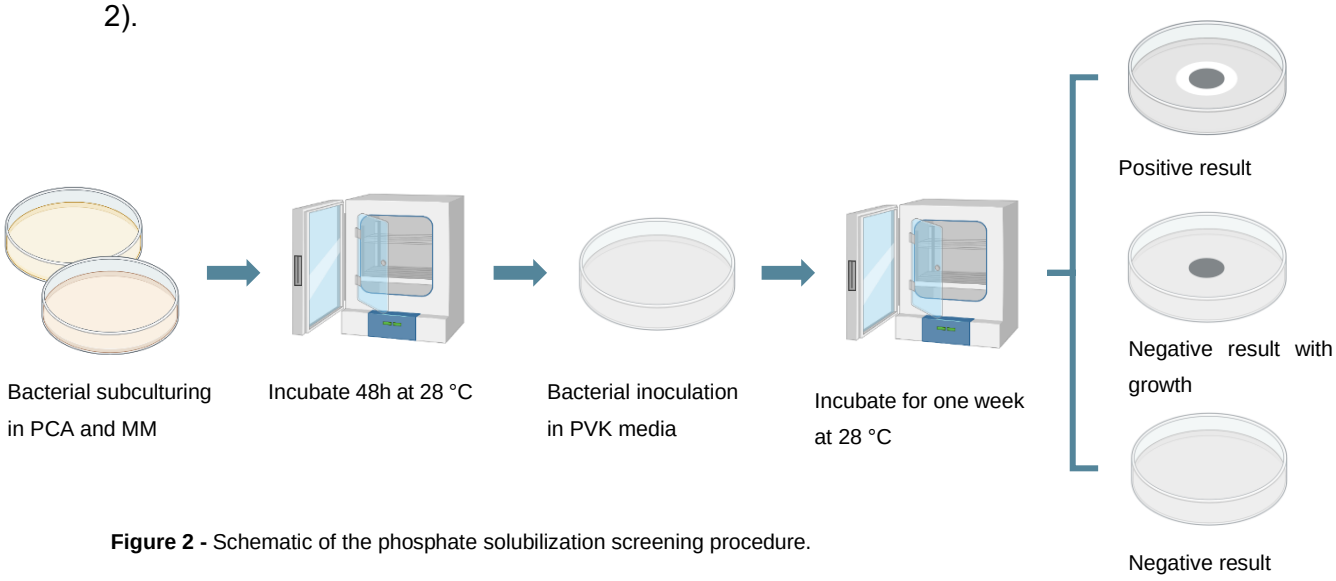


Figure 2 - Schematic of the phosphate solubilization screening procedure.

2.3.3. Siderophore Production

Siderophore production assay was performed in PCA and MM media which were deferrated by adding 50 µM 2,2'-dipyridil (Balado et al., 2015). All the glassware used to prepare the culture media was previously washed in an acid bath (hydrochloric acid, 10% v/v) to avoid iron residues. Each bacterial strain was cultured in the respective deferrated media and incubated for 48 hours at 28 °C. Following incubation, plates showing

bacterial growth were overlaid with pre-made circular plugs of Chrome Azurol S agar (Lankford, 1994) and incubated for 48 hours (Figure 3). For each plate, 2 circular plugs were placed in the culture media with bacterial growth and one was placed in the culture media that had no bacterial growth. The change in colour of the overlay from blue to orange indicated siderophore production. To prepare Chrome Azurol S agar, per litter of deionized water were measured 60.5 mg of Chrome azurol S (CAS) and 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA), Then, 10 mL of a solution containing 2.7 mg of FeCl3 · 6H2O in 10 mM HCl were added. While stirring, the pH was adjusted to 6 and 30.24 g Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) was slowly added, since PIPES does not dissolve in pH below 5. After that,15 g of agar was added.

Given the sensitivity of this medium to $FeCl_3$ and pH levels (Louden et al., 2014) and to validate the methodology, a test was carried out with the commercial siderophore desferrioxamine (DFO) on a plate containing iron-free PCA as a positive control. Circular plugs of CAS medium were then placed in the iron-free PCA medium with DFO and the colour change was assessed.

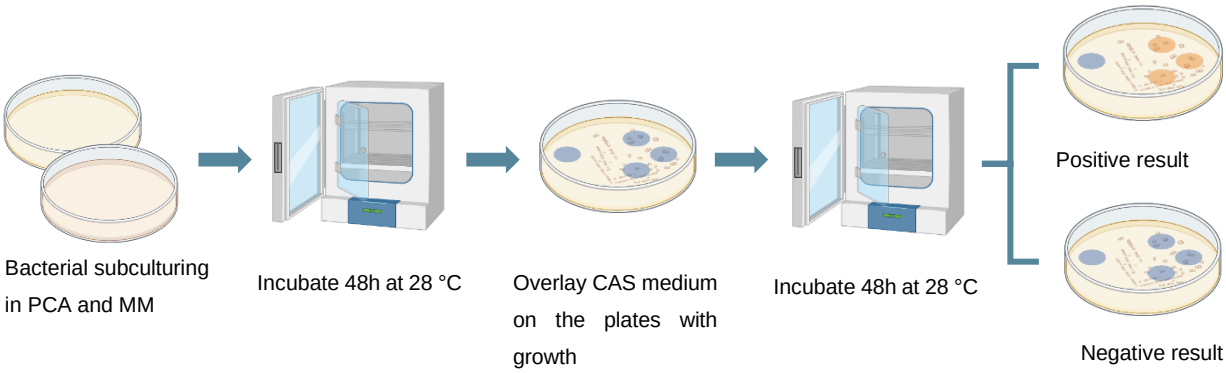


Figure 3 - Schematic of the siderophore production screening procedure.

2.3.4. Biofilm Formation

To evaluate biofilm production, the PC and PCV bacterial strains were cultivated in Plate Count Broth (PCB, with the same composition of PCA, 5 g of tryptone, 1 g of glucose, and 2.5 g of yeast extract per litter of deionized water) and MC and MCV bacterial strains were cultivated in MM liquid medium, and incubated for 48 hours at 28°C, under orbital agitation at 100 rpm (Optic Ivymen System/Comecta SA). The culture optical density (OD) was measured at λ = 600 nm (OD600) and adjusted to 1.0 using sterile PCB or MM medium. After that, the normalized bacterial cultures were inoculated in triplicate (60 µL per each well) into 96-well microtiter plates (Kartell LABWARE). A control row of wells

containing 60 µL of sterile PCB or MM medium was also used, as shown in Figure 4. The 96-well microtiter plates were incubated for 4 days at 28 °C, under static conditions (Paredes-páliz et al., 2016).

After incubation, the cultures were removed by inverting the plates, followed by washing the wells 5 times with deionised water to eliminate non-adherent cells. Biofilms were stained with a 0.01% crystal violet solution and incubated for 20 minutes. After that, excess crystal violet was rinsed off by washing the wells 3 times with deionised water. The remaining dye was solubilized using a solution of 96% ethanol and glacial acetic acid (65% 35% v/v respectively). The absorbance at λ = 570 nm was measured using a microplate spectrophotometer reader (BioTek SYNERGY HT, Gen5 V2.04), to evaluate biofilm formation (Figure 5).

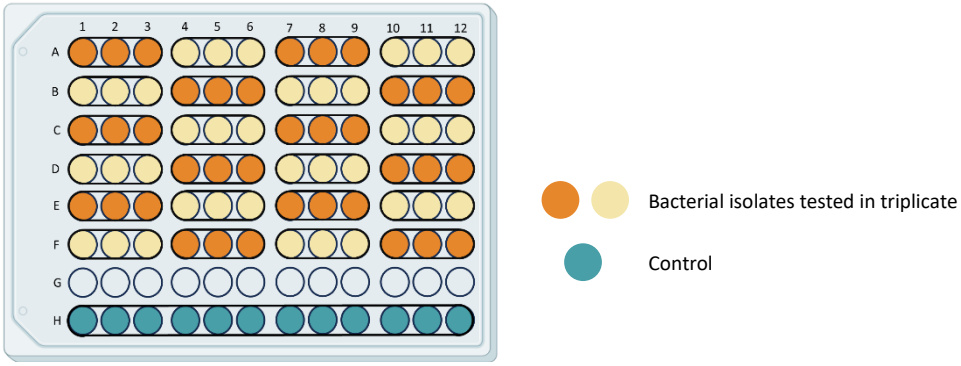


Figure 4 - Distribution of triplicates of the bacterial strains as well as the control into 96-well microtiter plates.

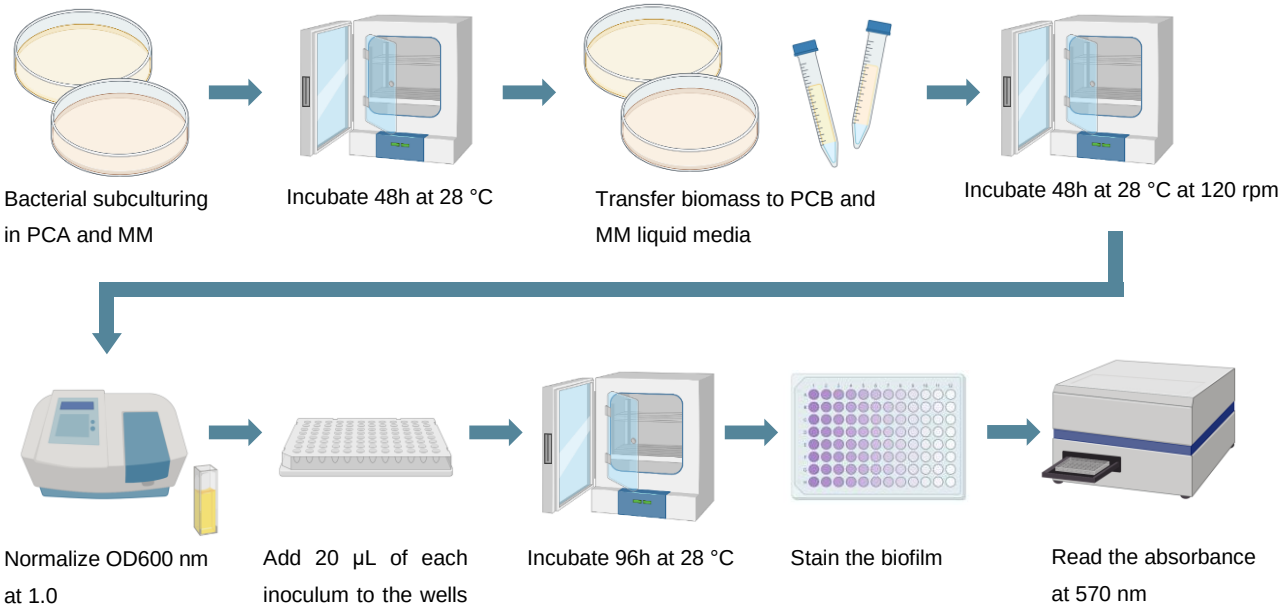


Figure 5 - Schematic of the biofilm production screening procedure.

2.3.5. ACC Deaminase Activity

Bacterial strains were tested for ACC deaminase activity (Gupta & Pandey, 2019) on sterile minimal Dworkin and Foster (DF) salts media. The culture media was modified by replacing $(NH_4)2SO_4$ with 3 mM ACC as sole nitrogen source (Dworkin & Foster, 1958; Penrose & Glick, 2003b)), with the following composition per litter of deionized water: KH_2PO_4 (4 g L), Na_2HPO_4 (6.0 g), MgSO4.7H2O (0.2 g), glucose (2.0 g), gluconic acid (2.0 g), citric acid (2.0 g) and agar (15 g), along with trace elements: $FeSO_4.7H_2O$ (1 mg), H_3BO_3 (10 mg), $MnSO_4.H_2O$ (11.19 mg), $ZnSO_4.7H_2O$ (124.6 mg), $CuSO_4.5H_2O$ (78.22 mg), MoO_3 (10 mg). The pH was adjusted to pH 7.2 and further autoclaved.

Just prior to the inoculation, 30 µmol plate^{-1} of 3.0 mM ACC solution was spread on sterile DF media and allowed to dry fully. After that, each bacterial strain was cultivated in the prepared media, in triplicate, and incubated at 28 °C for one week. Bacterial growth was evaluated after 72 hours and after one week of incubation (Figure 6). Colonies exhibiting growth on the plates were identified as ACC deaminase producers.

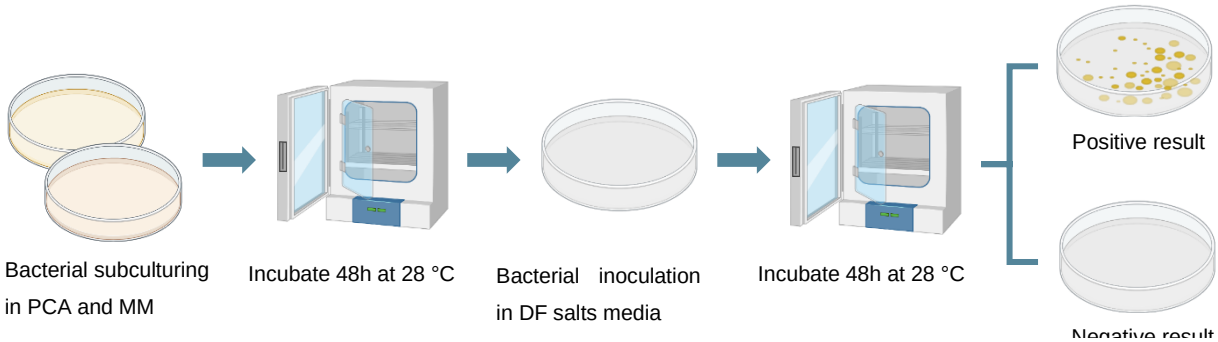


Figure 6 - Schematic of the ACC deaminase activity screening procedure.

2.4. *P. australis* Germination Assay

P. australis seeds were selected for this germination assays since these bacterial strains were isolated from enriched cultures, using rhizosediments from this plant as inoculum.

2.4.1. Media Preparation

Murashige and Skoog (MS) culture medium (Murashige & Skoog, 1962) was prepared by dissolving 4.4 g of Murashige and Skoog Basal Salt Mixture (Duchefa Biochemie) and 20 g of sucrose in one liter of deionized water (Csaba et al., 2001). Plate Count Broth was prepared as previously described in section 2.3.4. Saline solutions at 0.5% and 1% were prepared by dissolving 5 g and 10 g of sodium chloride (NaCl), per liter of deionised water, respectively. The saline solution (0,5% NaCl) + PCB medium was created by combining equal volumes of each media. All the culture media and the saline solutions were autoclaved.

2.4.2. Sterilisation of Seed Surface

To ensure a sterile environment, all sterilization steps were performed near a flame. The surface of the seeds was sterilized (Figure 7) by immersing them in 70% ethanol for 1 minute with agitation, followed by a single rinse in sterile deionized water. Subsequently, seeds were disinfected in 2% sodium hypochlorite solution (NaClO) for 5 minutes with agitation. In the end, all seeds were thoroughly rinsed with sterile deionized water five times.

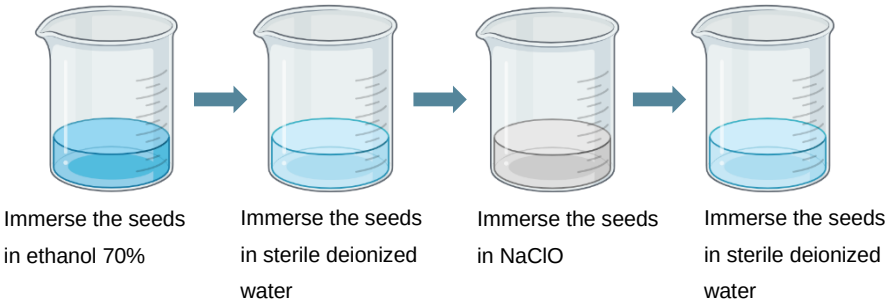


Figure 7 - Schematic of the seed surface sterilisation procedure.

2.4.3. Optimisation of Germination Conditions for *P. australis*

To optimize the germination of *P. australis* seed, four treatments were conducted in triplicates: 1) germination with 0.5% NaCl; 2) germination only with sterile deionized water; 3) germination with 1% NaCl and 4) germination on MS medium. For the germination assay, 1 mL of saline solution or Ms medium or deionized water was added to the respective sterile filter paper placed in a petri dish, and then, 10 sterile seeds were placed on top of the paper filter. All the petri dishes were sealed with the lid, but without the addition of parafilm. The petri dishes were placed in a phytoclimatic chamber (Matrix by ColdKit) (23-26 °C) under a 16h/8h light/dark photoperiod. Germination was assessed after one week of incubation, using a stereo microscope (Leica EZ 4W). The images were obtained with Leica Application Suite LAS EZ v3.4.0.

In a second experiment, the same treatments were tested, but after adding the seeds, the Petri dishes were sealed with parafilm. In this experiment, 6 replicates were performed for each medium, in which 3 plated were placed in the same conditions as in the previous test (under a photoperiod of 16h/8h light/dark) while the other 3 replicates were subjected to 0h/24h light/dark in a phytoclimatic chamber (Matrix by ColdKit) (23-26 °C). Germination was assessed after one week of incubation, using a stereo microscope (Leica EZ 4W). The images were obtained with Leica Application Suite LAS EZ v3.4.0.

2.4.4. Bioaugmentation assisted Germination of *P. australis* seeds with the bacterial strains with the best PGP traits

Considering the results from each PGP screening assays, the bacterial strains with the best PGP traits were selected for the germination assays: PCV3 (*Pseudomonas* sp.) and PCV7 (*Pseudomonas* sp.). To assess their potential, each bacterial strain was tested alone and in a consortium (mixture of both). Moreover, treatments with Ms medium and 0.5% NaCl displayed the best germination percentages, thus, they were selected for this experiment.

For each bacterial strain and the bacterial consortium, 4 treatments were selected: 1) germination with 0.5% NaCl; 2) germination on MS medium; 3) germination with 0.5% NaCl + PCB medium and 4) germination with PCB medium. The treatment 3 was selected as PCB was the bacterial medium with the suitable nutritional composition to sustain the growth of each bacterial strain. The treatment 4 was conducted as control for the treatment 3, to assess if this media could influence the germination of *P. australis* seeds.

For the assay, both bacterial strains were cultivated in PCA, for 48h, at 28 ºC. Biomass of the selected strains (PCV 3 and PCV 7) was inoculated in falcon tubes containing each base medium (MS, 0.5% NaCl, 0.5% NaCl + PCB). The culture optical density (OD) was measured at λ = 600 nm (OD600) and adjusted to 0.1 (Figure 8). To prepare the bacterial consortium, equal proportions of each strain was inoculated in each medium, with a final OD of 0.1. Under strictly aseptic conditions, and to ensure sterility, all steps were carried out in a flame-sterilized environment.

A sterile filter paper disk was placed in each petri dish. After that, 1 ml of each inoculum (PCV 3, PCV 7, MIX) was added to the filter paper to ensure complete saturation. As a control,1 ml of each non-inoculated medium was added to the paper filters. Then, 10 sterile *P. australis* seeds were carefully transferred onto the moistened disk, as outlined in Figure 8. All tests were carried out in triplicate. Afterwards, each Petri dish was sealed with parafilm, to prevent evaporation. All plates were placed in a phytoclimate chamber (Matrix by ColdKit) (23-26 °C) under a 16h/8h light/dark photoperiod. Germination was evaluated after one week of incubation, using a stereo microscope (Leica EZ 4W). The images were obtained with Leica Application Suite LAS EZ v3.4.0.

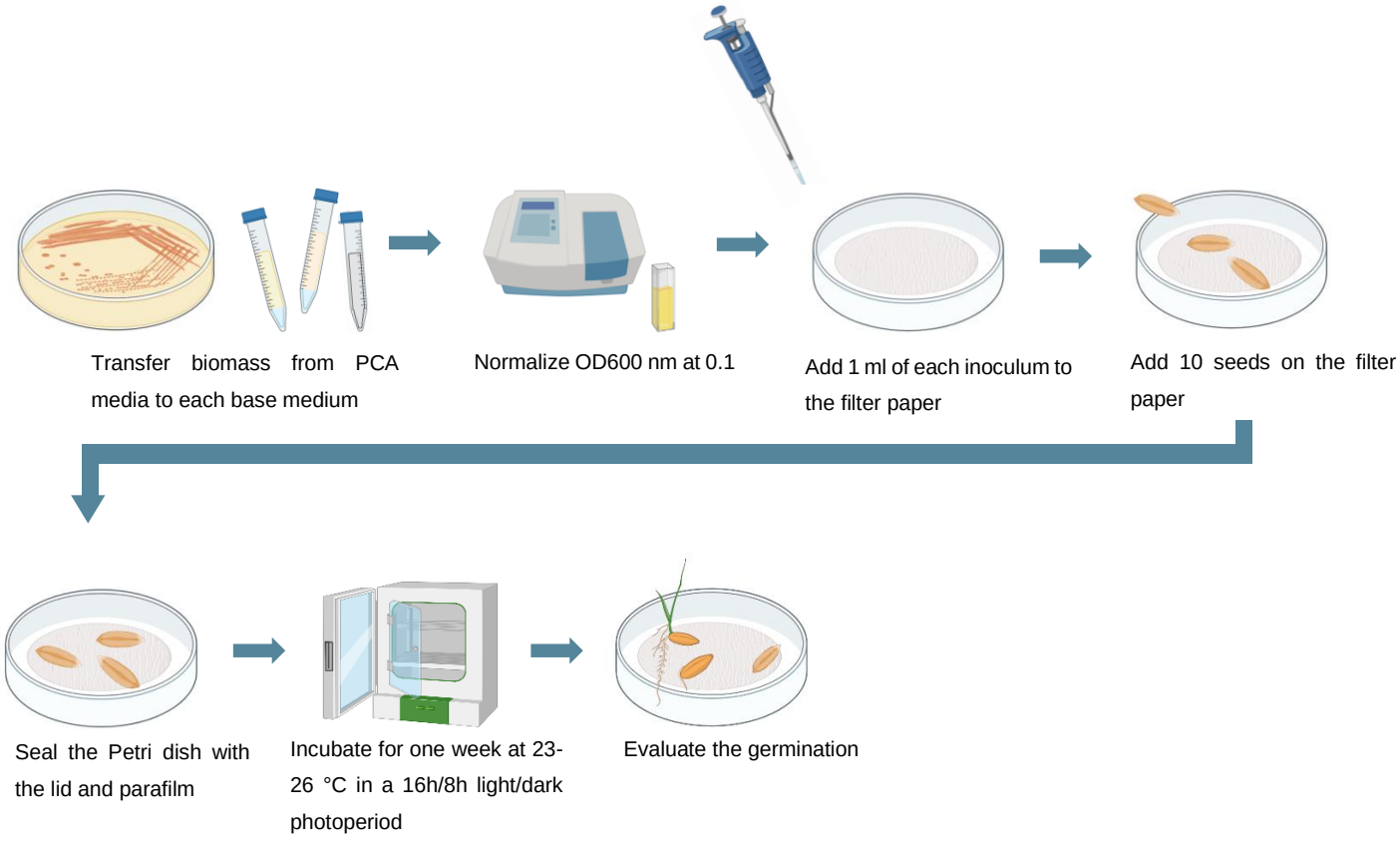


Figure 8 - Schematic of the procedure germination of *P. australis* with the bacterial consortium.

2.5. Statistical Analysis

For the biofilm formation and germination assays, significant differences among samples were evaluated through Kruskal-Wallis test, using mean values and corresponding standard deviations of the replicates (n=3). Significant differences were assumed for p-value below or equal to 0.05 (confidence level of 95 %).

3. Results and Discussion

3.1. Plant-Growth Promoting Traits

3.1.1. Nitrogen Fixation

Nitrogen-fixing bacteria typically assimilate atmospheric nitrogen and release ammonia as a byproduct into their environment (in this case, the culture medium). This rise in ammonium concentration caused a change in the pH of the medium, which, in turn, resulted in a visible colour change.

Nitrogen fixation was detected in 21 out of 41 bacterial strains, showing growth and a color change in the medium, visible in the Figure 9a. The remaining 20 bacterial strains exhibited no growth and no change in the colour of the medium (Figure 9b).

Among the tested bacterial strains, *Alcaligenes* (7 bacterial strains), *Pseudomonas* (5 bacterial strains), *Albirhodobacter* (4 bacterial strains), *Oceanisphaera* (3 bacterial strains), *Brucella* (1 bacterial strain) and *Hydrogenophaga* (1 bacterial strain) were identified as positive for nitrogen fixation. Moreover, the bacterial strain MCV8 (*Pseudomonas* sp.) was the only strain isolated from MCV media that did not show potential for nitrogen fixation.

Nitrogen fixation is a demanding process for bacteria (Hrynkiewicz et al., 2019; Rosenblueth et al., 2018). In this study, the bacterial strains isolated from enriched cultures with Cu and VFX (MCV and PCV) showed a higher potential for nitrogen fixation than the bacterial strains isolated from MC and PC, where the VFX was not added (Figure 10).

Previous studies had established nitrogen fixation as a trait absent from *Pseudomonas* species (Setten et al., 2013).This notion was even proposed as a defining characteristic of the genus (Anzai et al., 2000; Solanki et al., 2016). However, recent research has challenged this assumption, demonstrating that certain strains within the *Pseudomonas* genus have the ability to fix nitrogen (Li et al., 2017; Setten et al., 2013; Solanki et al., 2016). In fact, a study conducted by Li et al., 2017 showed that *Pseudomonas entomophila* isolated from sugarcane rhizosphere, displayed N fixation potential. Moreover, Pinkwart et al. (1983) proved the nitrogen-fixing capacity of *Alcaligenes latus*.

In this study, none of the bacterial strains belonging to the *Brevundimonas, Chryseobacterium, Microbacterium, Niallia, Paenibacillus* and *Pseudogemmobacter* genera showed the ability to fix nitrogen. However, in other studiesspecies from these genera have been associated to this trait. Naqqash et al. (2020) stated that

Brevundimonas spp. isolated from rhizospheric soil of potato had the potential to fix nitrogen. Dhole et al., (2017) showed that *Chryseobacterium indologenes* isolated from *Vigna radiata* also showed this characteristic. In the study carried out by Zheng et al, (2024) *Microbacterium dauci* sp. nov. isolated from carrot rhizosphere soil was capable of fixing nitrogen. Rashad et al., (2024) affirmed the ability of *Niallia circulans* to fix atmospheric nitrogen. Moreover, in a study by Liu et al. (2019), *Paenibacillus* strains showed ability to fix nitrogen. For the *Pseudogemmobacter genus*, it was not found studies reporting nitrogen fixation potential for this strain.

To our best knowledge, this study is the first report describing the nitrogen fixation potential of a bacterial strain belonging to the genera *Albirhodobacter*, *Brucella* and *Oceanisphaera.*

In the future, further tests such as nitrogenase activity detected by the acetylene reduction method (Rice et al., 1994, Puri et al., 2018), micro-Kjeldahl method or even molecular identification are needed to prove the ability of these strains to fix atmospheric nitrogen (Zhang et al., 2022).

a) b)

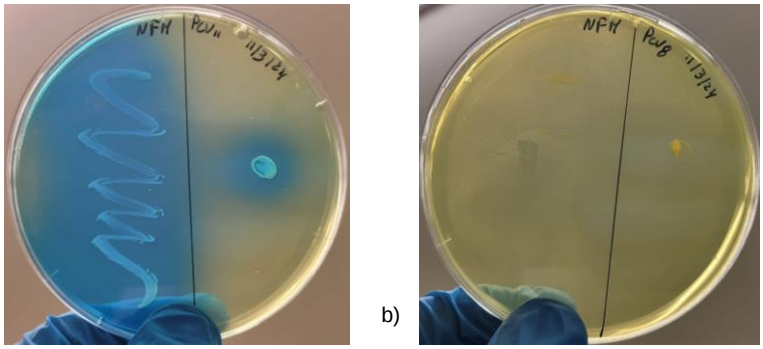


Figure 9 - Nitrogen fixation assay using NFGMM medium (Ferreira et al., 2021). a) Positive result in the nitrogen fixation test; b) Negative result in the nitrogen fixation teste (b).

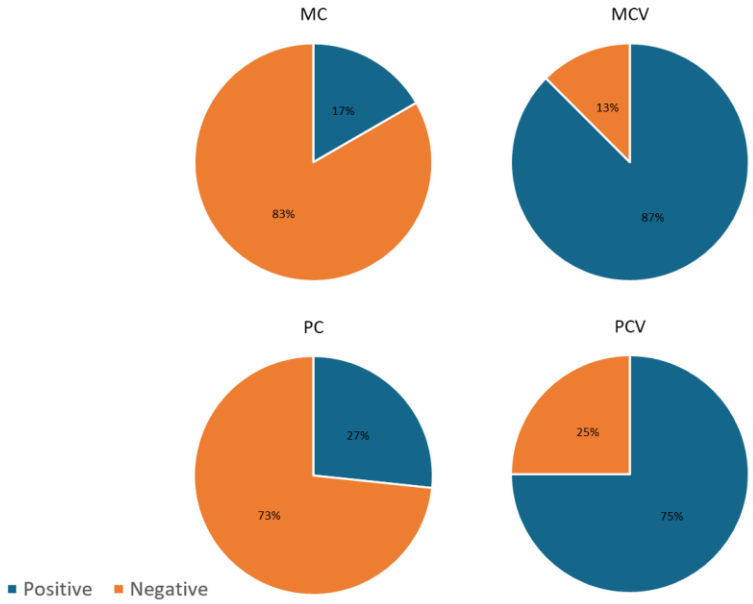


Figure 10 - Nitrogen fixation percentages of the bacterial strains isolated from different enriched cultures, using different culture media.

3.1.2. Phosphate Solubilization

P solubilization was tested for all 41 bacterial isolates. Out of the 41 bacterial strains tested, only 4 bacterial strains were capable of P solubilisation (formation of a transparent halo around the inoculum, as shown in Figure 11a). The bacterial strains with positive P solubilization belong to the genera *Pseudomonas* and *Microbacterium*. The 4 bacterial stains with P solubilization potential were isolated from PCA media, in which no MC and MCV bacterial strains phosphate solubilisation potential (Figure12).

Moreover, 24 bacterial strains did not grow in the culture medium, as shown in the Figure 11b. In addition, 13 bacterial strains were characterized as 'negative with growth' (colony grew without P-solubilisation, non-appearance of a transparent halo, figure 11c), being unable to solubilize phosphate (considered negative). Moreover, 13 bacterial strains were tested on two additional media PVK-T and PVK-T, replacing glucose with tryptone and yeast extract respectively, however for both media the ‘negative with growth’ result remained for all strains (Figure 11d and Figure 11e).

Bacterial strains from the genus *Pseudomonas* are among the most powerful phosphate solubilizers (Blanco-Vargas et al., 2020; Rodríguez & Fraga, 1999). The study by Zeng et al. (2016) showed the phosphate solubilisation capacity of *Pseudomonas frederiksbergensis,* isolated from the rhizosphere of *Populus euramericana*. Additionally, *Microbacterium* species have also been reported as phosphate solubilizers (Lunggani & Suprihadi, 2019; Rivas et al., 2004; B. Zhang et al.,

2016). For example, Rivas et al. (2004) characterised *Microbacterium ulmi* sp. nov. isolated from *Ulmus nigra* sawdust as capable of solubilising phosphate.

On the other hand, and contrary to what was found in this study, other studies point to some of the genera as being able to solubilise phosphates. For example, Behera et al. (2017) showed the potential of *Alcaligenes faecalis*, isolated from mangrove soil, to solubilise phosphates. Shazmin et al. (2023) confirmed the phosphate solubilising capacity of *Brucella intermedia* Msd2 isolated from cotton plant. Singh and co-authors (2018) stated that this trait was also present in *Chryseobacterium* sp.

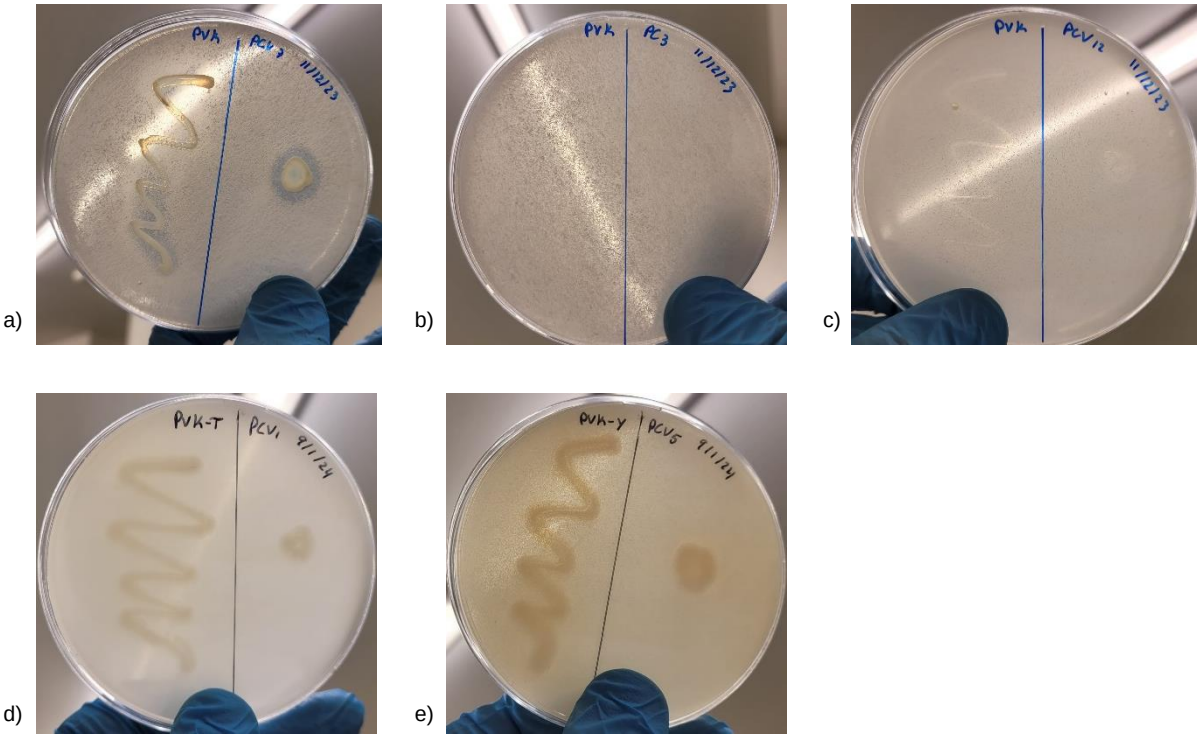


Figure 11 - Phosphate solubilization assay using PVK medium (Pikovskaya, 1948). a) Positive result in PVK media; b) Negative result in PVK media; c) Negative result with in PVK media; d) Negative result with PVK-T media; e) Negative result with growth PVK-Y media.

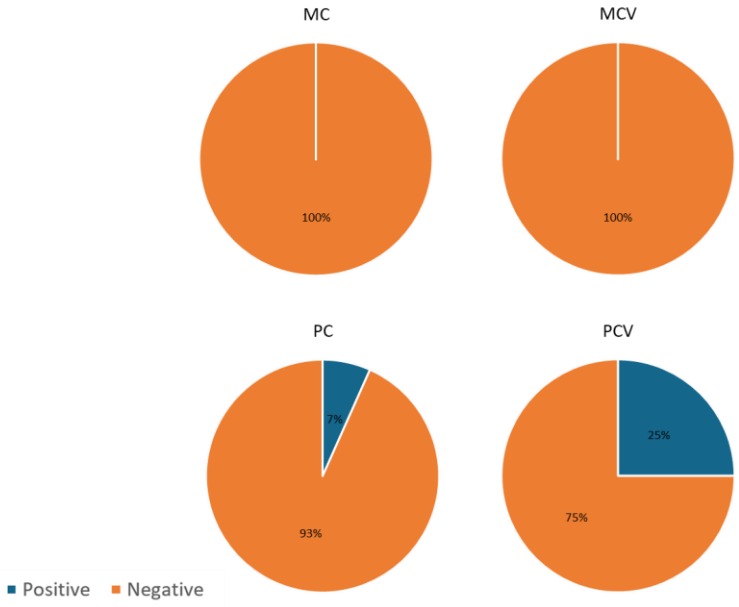


Figure 12 - P. solubilization percentages of the bacterial strains isolated from different enriched cultures, using different culture media.

3.1.3. Siderophore Production

Bacterial isolates capable of producing siderophores are common in the rhizosphere of salt marsh plants. All 41 bacterial strains were tested for their potential to produce siderophores.

Results showed that 24 bacterial strains displayed a positive result, indicated by a colour change from blue to orange of the CAS agar disc placed on the bacterial colony (a result of iron removal from the dye complex by these strong iron chelators) (Figure 13a). All bacterial strains isolated from MCV medium did not produce siderophores when subjected to this screening assay (Figure 14). On the other hand, strain PCV5 (*Alcaligenes sp.)* was the only one with a negative result out of all the strains isolated from PCV medium

All the bacterial strains from the to the genera *Brevundimonas* (PC14), *Brucella* (PCV2), *Chryseobacterium* (PC4 and PCV6), *Hydrogenophaga* (MC5, PC6, PC10, PCV10), *Paenibacillus* (PC12), and *Pseudogemmobacter* (PC2, PC7) presented positive results for siderophores production, which are in agreement with the literature (Singh et al., 2016; Carreró et al., 2002; Kutsuna et al., 2021; Zhang et al., 2020). However, all the strains belonging to the *Hydrogenophaga* (MC5, PC6, PC10, PCV10) and *Pseudogemmobacter* (PC2, PC7) genera have also shown the ability to produce

siderophores, and as far as we know, no studies were reported for these bacterial genera. To our best knowledge this is the first report describing siderophore production potential for these genera.

In addition, some bacterial strains from the genera *Albirhodobacter* (1), *Alcaligenes* (3), *Microbacterium* (1) and *Pseudomonas* (7) also showed positive results in the screening test. On the other hand, all strains of the genera *Niallia* and *Oceanisphaera* were unable to produce siderophores, since the disc remained blue after the incubation time (Figure 13b).

a) b)

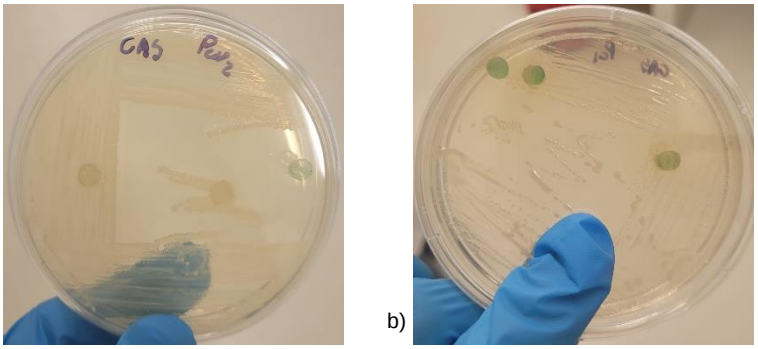


Figure 13 - Siderophore production assay with CAS agar overlay (Lankford, 1994). Positive result in the siderophores production teste (a); Negative result in the siderophores production teste (b).

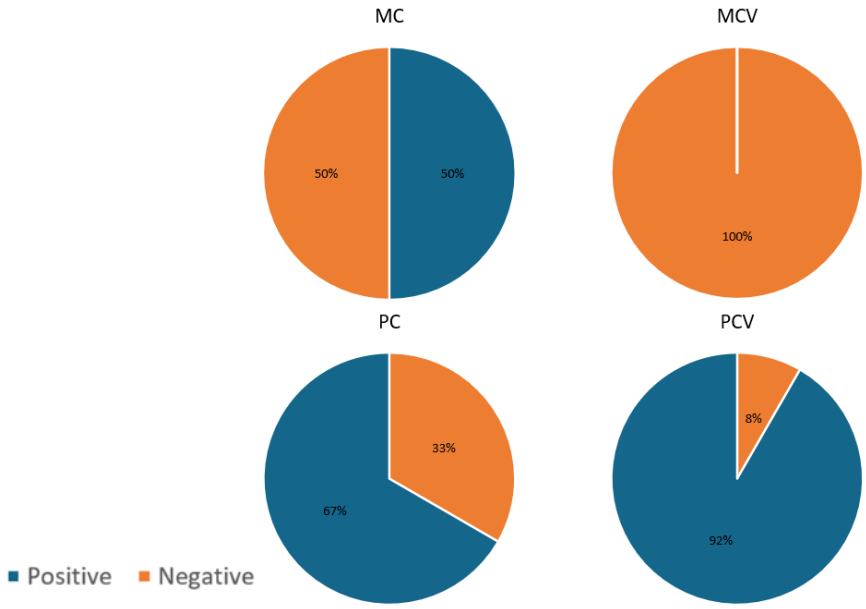


Figure 14 – Siderophore production percentages of the bacterial strains isolated from different enriched cultures, using different culture media

3.1.4. Biofilm Formation

The capacity of different bacterial strains to form biofilm was assessed through the adhesion to the surface of wells of 96-well microtiter plates (Figure 15).

Results showed that 35 out of 41 bacterial strains were able to form biofilm: bacterial strains from the genus *Pseudomonas*, *Alcaligenes*, *Albirhodobacter*, *Hydrogenophaga*, *Oceanisphaera*, *Chryseobacterium*, *Microbacterium*, *Pseudogemmobacter*, *Brevundimonas* and *Brucella.*

Moreover, the bacterial strains from *Niallia* and *Paenibacillus genera* were not capable to form biofilm. These results are not in line with previous studies, where strains belonging to the genera *Niallia* and *Paenibacillus* have been categorized as 'notable biofilm formers (Gil et al., 2023; B. Li et al., 2011; Majhi et al., 2023). In the other hand, the bacterial strains MC2 (*Albirhodobacter* sp.), MC5 (*Hydrogenophaga* sp.), MCV8 (*Pseudomonas* sp.) and PC3 (*Alcaligenes* sp.) were the only representatives of their respective genera with negative biofilm formation results.

In general, all bacterial strains retrieved from PCV medium tested positive for biofilm formation (Figure16). Among the bacterial strains from the MCV medium, only MCV8 (*Pseudomonas sp.*) failed to produce biofilm.

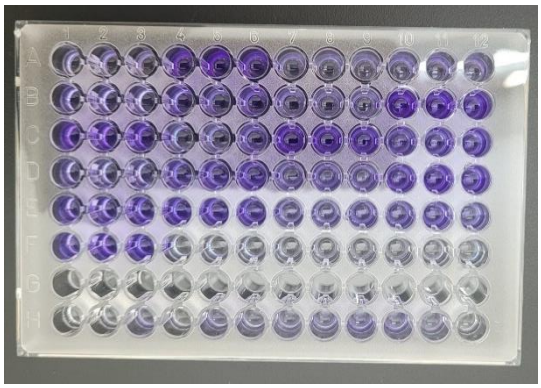


Figure 15 - Illustrative example of a 96-well microtiter plate with isolates stained with 0.01% crystal violet solution for the assessment of biofilm production.

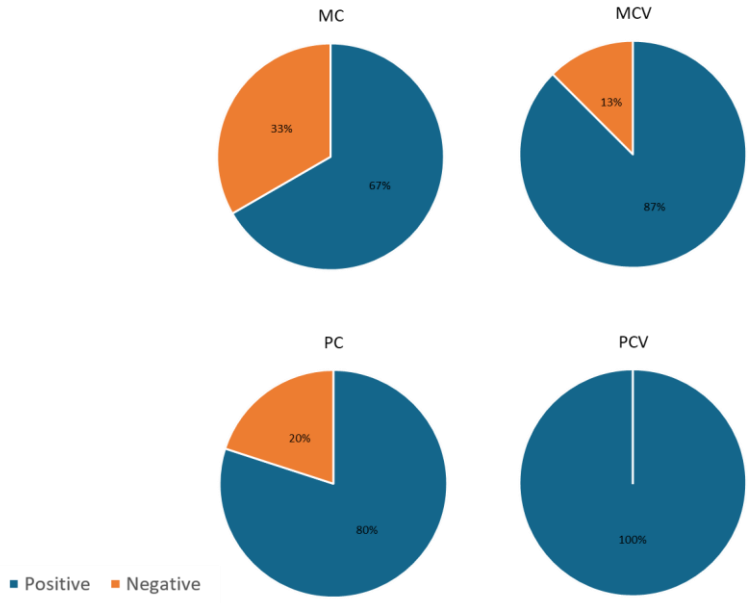


Figure 16 Biofilm formation percentages of the bacterial strains isolated from different enriched cultures, using different culture media.

3.1.5. ACC Deaminase Activity

ACC deaminase activity assay was performed by culturing each bacteria strain in DF salts media where 3 mM ACC was used as the sole source of nitrogen. From the 41 bacterial strains tested, 21 belonging to the genera *Albirhodobacter*, *Alcaligenes, Brucella, Hydrogenophaga, Microbacterium, Niallia, Oceanisphaera, Pseudomonas* presented ACC deaminase activity, presenting bacterial growth, as shown in Figure 17a.

The results obtained in this study are, as far as we know, the first positive results for ACC deaminase activity from the *Albirhodobacter*, *Alcaligenes* and *Oceanisphaera*. Zhang et al. (2023) reported to the possibility of *Hydrogenophaga* being capable of ACC degradation. Moreover, Shaharoona et al. (2006) also confirmed the ability of ACC deaminase in *Pseudomonas* spp. to improve growth and yield of maize (*Zea mays* L.). The study carried out by Shrivastava & Kumar (2013) showed the capacity for ACC deaminase in bacterial strains belonging to the *Microbacterium* genus, which is in line with the results presented in this work.

In this study, *Brucella sp*. PCV2 presented ACC deaminase activity. In a study conducted by Shazmin et al. (2023) *Brucella intermedia* Msd2 did not show ACC deaminase activity. Moreover, none of the bacterial strains from *Brevundimonas, (Chryseobacterium*, *Paenibacillus*, or *Pseudogermmobacter* genera exhibited ACC-deaminase activity (Figure 17b).

In the future, more studies are needed to validate these results, by using, for example, quantitative methodologies by analysing released ammonia and α–ketobutyrate, as described in (Honma & Shimomura, 1978).

a)

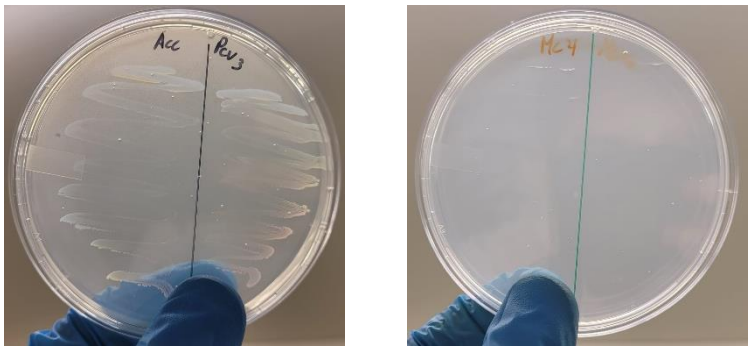


Figure 17 - ACC deaminase activity in DF salts media Positive result in the ACC deaminase activity teste (a); Negative result in the ACC deaminase activity teste (b).

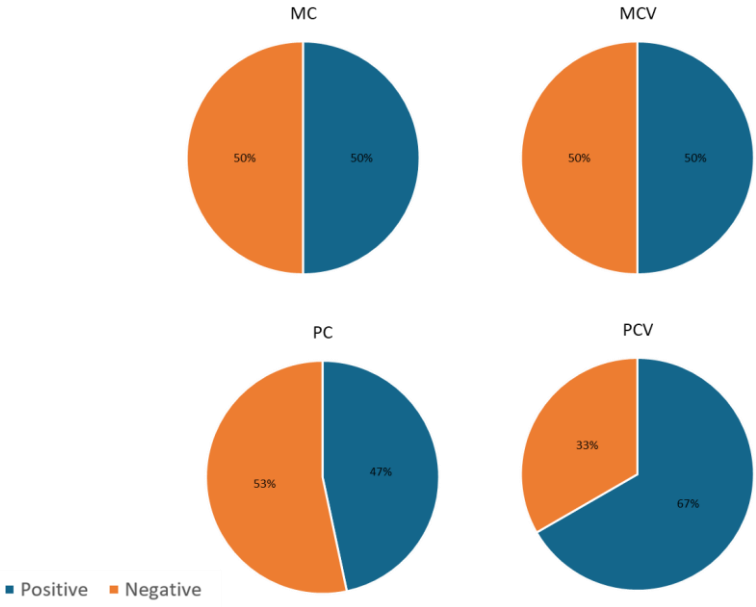


Figure 18 – ACC deaminase percentages of the bacterial strains isolated from different enriched cultures, using different culture media

3.1.6. PGP traits across all bacterial strains – which are the best PGPB?

All PGP results (nitrogen fixation (N FIX), phosphate solubilization (P SOL), siderophore production (S PROD), 1-aminocyclopropane-1-carboxylic acid deaminase activity (ACC DEA) and the biofilm production (BIOFILM)) for the 41 bacterial isolates are generally described in figure 19 and summarized in Table 2.

Overall, biofilm formation was the most prevalent trait, observed in 85% of the bacterial strains, as also stated by Ferreira et al., (2021) when they analysed the root microbiome of *Salicornia ramosissima*. Nitrogen fixation, ACC deaminase activity and siderophore production were also common, detected in 51 – 59 % of the bacterial strains. In the other hand, phosphate solubilization was the less frequent PGP trait, observed in only 10% of the bacterial strains (Figure 19).

With the exception of phosphate solubilization, which was not detected in bacteria isolated from MC and MCV enriched media, all the other PGP traits are represented in all enriched media. As observed in Table 2, the bacterial strains isolated from enriched culture exposed to VFX and Cu (MCV and PCV) presented, in general, positive results for nitrogen fixation. In the other hand, MC strains were the ones that showed the least positive results for the all PGP traits. Independently from the enriched culture they were isolated from, the bacterial strains isolated in PCA medium were more capable of producing siderophores comparing with those isolated in MM medium.

Overall, the bacterial strains PCV3 (*Pseudomonas* sp.) and PCV7 (Pseudomonas sp.), presented the best PGP traits, testing positive in all screening assays (Table 2, Figure 20). These results are in line with many other studies that point to *Pseudomonas* as excellent PGPBs (Lugtenberg and Kamilova 2009; Pathma et al. 2011; Jain and Pandey 2016). In fact, Kumar et al., (2017) states that this genus is the most widely used for plant and soil inoculants to enhance the plant growth and disease management. Besides, *Pseudomonas* strains are versatile in their ability to degrade a wide range of water pollutants, from organic compounds to heavy metals and pesticides (Wasi et al., 2011; Singh et al., 2011). Both bacterial strains were isolated from the PCA medium from the enriched culture with Cu and VFX, showing that they can survive in their presence. Bacterial strains PC6 (*Hydrogenophaga* sp.), PCV1 (*Alcaligenes* sp.), PCV2 (*Brucella* sp.), PCV8 (*Alcaligenes* sp.), PCV11 (*Alcaligenes* sp.) and PCV12 (*Microbacterium* sp.) had four positive results thus proving to be good bacterial isolates to apply in future PGPB consortia. As far as we know, no studies have shown bacterial strains from the *Hydrogenophaga* and *Brucella* genera to be excellent PGPBs. On the other hand, various studies have shown that bacterial strains belonging to the *Alcaligenes* genus have proved to be excellent PGPBs involved in salt tolerance and improving plant growth under salinity stress (Dixit et al., 2020; Fatima et al., 2020). Tsavkelova et al., (2024) reported that *Microbacterium sp.,* have a good auxin biosynthesis and tolerant of NaCl concentration up to 10%, thus favouring the idea that this genus can be applied as a PGPB in environments where plants are under salt stress.

In the other hand, the bacterial strains MC2 (*Albirhodobacter* sp.), MC3 (*Pseudomonas* sp.), PC3 (*Alcaligenes* sp.), PC12 (*Paenibacillus* sp.) and PC15 (*Niallia* sp.) only had one positive result for all evaluated traits. Furthermore, the bacterial strains PC13 (*Microbacterium* sp.), PCV3 (*Pseudomonas* sp.), PCV7 (*Pseudomonas* sp.) and PCV12 (*Microbacterium* sp.)) were the only bacterial strains isolated with P solubilization potential (Table 2 e Figure 19).

The results for PGP traits were different among different media and different enriched cultures. In fact, the use of different culture media for the isolation of the bacterial strains of each enriched culture allowed the isolation of different bacterial strains. The MM media was used as it replicates the conditions used in the enrichment process, but is a poor media, with only sodium acetate and VFX as carbon source. PCA media was selected as a richer and non-selective culture media, which can favour the isolation of other groups of bacteria. Still, a fraction of cultivated bacteria may have been lost, as the isolation conditions may not be adequate for their isolation. Moreover, a part of the prokaryotic community present in the enriched cultures can be recalcitrant or not culturable, since culturable bacteria are estimated to represent only 0.01–1% of the total microbial species present in a given environment (Garza & Dutilh, 2015; Shi et al., 2015). Among the bacterial strains retrieved from each culture, *Alcaligenes* and *Pseudomonas* genera were the most abundant. In fact, *Pseudomonas* genus was isolated in all cultures, independently of the media that was used. In the other hand, the genus *Alcaligenes* was predominantly isolated in MC medium but was isolated in both enriched cultures (exposed to Cu or Cu + VFX, Notably, *Oceanisphaera* was only isolated in the MCV medium). In addition, genera like *Brevundimonas*, *Brucella*, *Niallia* and *Paenibacillus* were isolated only in PCA media (from both enriched cultures).

For all the results obtained in this study, we have to keep in mind that within the same genera, different species can have different traits. The taxonomic identification of the 41 bacterial strains did not allow to identify the correspond specie and the discussion was performed considering strains from the same genera. Still, these results gave an insight of PGP traits among different species, from different genera, which contributes to the knowledge regarding this PGP traits in different bacterial species.

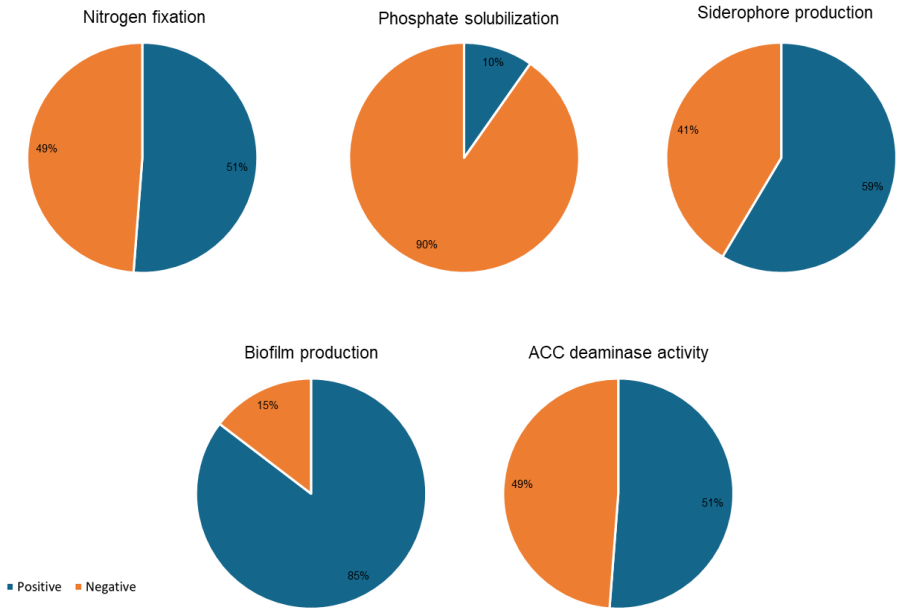


Figure 19 - Percentage of positive and negative results obtained in each PGP screening.

Table 2 - PGP trait results for the 41 bacterial strains, isolated from rhizosediments from *Phragmites australis* and their taxonomic identification. MC stands for MM medium doped with Cu; MCV stands for MM medium doped with Cu and VFX; PC stands for PCA medium doped with Cu; PCV stands for PCA medium doped with Cu and VFX.

ID	Bacterial genera	N Fix	P Sol	Biofilm	ACC Dea	S Prod
MC1	*Albirhodobacter* sp.	-	-	+	+	-
MC2	*Albirhodobacter* sp.	-	-	-	+	-
MC3	*Pseudomonas* sp.	-	-	+	-	-
MC4	*Pseudomonas* sp.	+	-	+	-	+
MC5	*Hydrogenophaga sp.*	-	-	-	+	+
MC6	*Pseudomonas* sp.	-	-	+	-	+
MCV1	*Alcaligenes* sp.	+	-	+	-	-
MCV2	*Oceanisphaera* sp.	+	-	+	+	-
MCV3	*Oceanisphaera* sp.	+	-	+	-	-
MCV4	*Alcaligenes* sp.	+	-	+	-	-
MCV5	*Oceanisphaera* sp.	+	-	+	+	-
MCV6	*Alcaligenes* sp.	+	-	+	-	-
MCV7	*Albirhodobacter* sp.	+	-	+	+	-
MCV8	*Pseudomonas* sp.	-	-	-	+	-
PC1	*Albirhodobacter* sp.	+	-	+	-	-
PC2	*Pseudogemmobacter* sp.	-	-	+	-	+
PC3	*Alcaligenes* sp.	-	-	-	+	-
PC4	*Chryseobacterium* sp.	-	-	+	-	+
PC5	*Albirhodobacter* sp.	+	-	+	+	-
PC6	*Hydrogenophaga* sp.	+	-	+	+	+
PC7	*Pseudogemmobacter* sp.	-	-	+	-	+
PC8	*Pseudomonas* sp.	-	-	+	-	+
PC9	*Pseudomonas* sp.	+	-	+	-	+
PC10	*Hydrogenophaga* sp.	-	-	+	+	+
PC11	**Not identified**	-	-	+	+	+
PC12	*Paenibacillus* sp.	-	-	-	-	+
PC13	*Microbacterium* sp.	-	+	+	+	-
PC14	*Brevundimonas* sp.	-	-	+	-	+
PC15	*Niallia* sp.	-	-	-	+	-
PCV1	*Alcaligenes* sp.	+	-	+	+	+
PCV2	*Brucella* sp.	+	-	+	+	+
PCV3	*Pseudomonas* sp.	+	+	+	+	+
PCV4	*Albirhodobacter* sp.	+	-	+	-	+
PCV5	*Alcaligenes* sp.	+	-	+	+	-
PCV6	*Chryseobacterium* sp.	-	-	+	-	+
PCV7	*Pseudomonas* sp.	+	+	+	+	+
PCV8	*Alcaligenes* sp.	+	-	+	+	+
PCV9	*Pseudomonas* sp.	+	-	+	-	+
PCV10	*Hydrogenophaga* sp.	-	-	+	-	+
PCV11	*Alcaligenes* sp.	+	-	+	+	+
PCV12	*Microbacterium* sp.	-	+	+	+	+

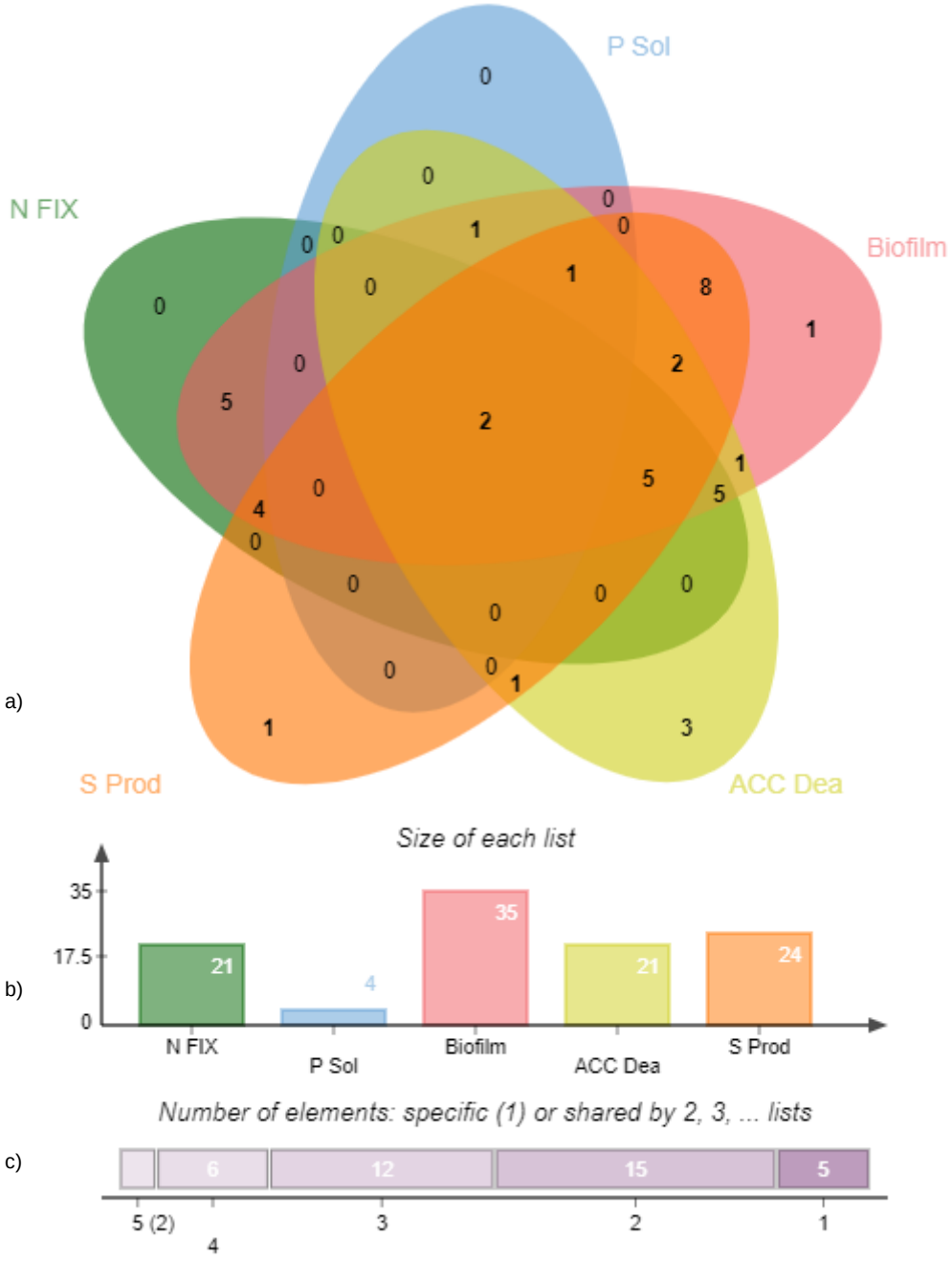


Figure 20 - Venn diagram representing the unique and shared PGP among the 41 bacterial strains (a); number of isolates displaying each plant-growth promoting trait (b); number of isolates combining the expression of 1, 2, 3, 4 or 5 plant-growth promoting traits.

3.2. *P. australis* Germination Assay

3.2.1. Optimisation of Germination Conditions for *P. australis*

Different germination assays were conducted to optimize the best conditions for the germination of *P. australis* seeds. Germination was deemed to have occurred when the radicle protruded beyond the seed coat by at least 1 mm (Figure 21a and b). In the first germination assay, the treatment with MS medium displayed higher germination percentages, reaching 50% of germination (Figure 22). In addition, the treatment with 0.5% NaCl salt solution medium showed 30% germination and the 1% NaCl medium showed 20% germination (Figure 22). These results are in agreement with previous studies, in which germination percentage is negatively affected by increased salinity (Gorai et al., 2006; Greenwood & MacFarlane, 2006). Finally, no seeds germinated was observed on the filter paper moistened with sterile H_2O (Figure 21c).

In first germination assay, it was observed that germination percentage was relatively low. A possible explanation can be related with the fact that Petri dishes were only sealed with the lid, which allowed the culture media to evaporate over the week (after one week, all the filter papers were completely dry). The lack of humidity could have an influence in the germination percentage. In fact, previous studies have shown that the germination of *P. australis* seeds is favoured by constant humidity (Lissner & Schierup 1996; Kettenring & Whigham 2009).

a) 

b) 

c) 

Figure 21 - Germinated *P. australis* seed (a, b); Non-germinated *P. australis* seed (c).

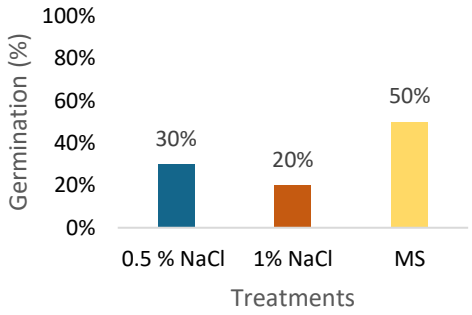


Figure 22 - Germination percentages in each treatment in a 16h/8h light/dark photoperiod). * No germination was observed in sterileH_2O treatment

Regarding the second germination assay, in general, the germination percentages were higher than in the previous evaluation. In this test, all Petri dishes were sealed with parafilm to prevent evaporation (at the end of a week the filter papers were still damp). For the treatments subjected to 16h/8h light/dark photoperiod (Figure23a), 70% of seeds germination was observed for the MS medium, followed by 60% germination for the 0.5% NaCl medium, 30% germination for 1% NaCl medium and 0% for sterile H_2O treatment. Regarding the germination percentages on the plates subjected to total darkness (Figure23b), 30% seeds germination was observed for 0.5% NaCl and MS treatments, followed by 20% germination for 1% NaCl treatment and 0% for sterile H_2O treatment. The 16h/8h light/dark photoperiod presented higher germination percentages comparing with 0h/24h light/dark photoperiod. These results are in agreement with research conducted by Wei et al., (2023) and (Haslam, 1973) who stated that light is essential for optimal seed germination. For instance, Hayasaka et al., (2020) indicated that light levels had positive impact on germination rates of *Spartina alternifora*. In another study conducted by Ekstam et al. (1999), although variations were minimal, seeds germination was higher with the presence of the light comparing with dark conditions. The discrepancy in the impact of light on germination can be attributed to variations between different plant populations, and their light sensitivity can be different (Ekstam et al. 1999).

A comparison of these two preliminary tests shows that for the studied seeds, germination was favoured by the 16h/8h light/dark photoperiod. Germination also increased when the humidity in the filter papers was maintained throughout germination. In both trials, the highest germination percentages were obtained when the seeds were germinated on MS medium. Seeds germinated in 0.5% NaCl saline solution had a higher germination percentage comparing with the seeds germinated in 1% NaCl saline solution, showing that the increase in salinity negatively affected germination (Gorai et

al., 2006). None of the seeds germinated on the filter papers moistened with sterile H_2O (autoclaved deionised water) in any of the tests.

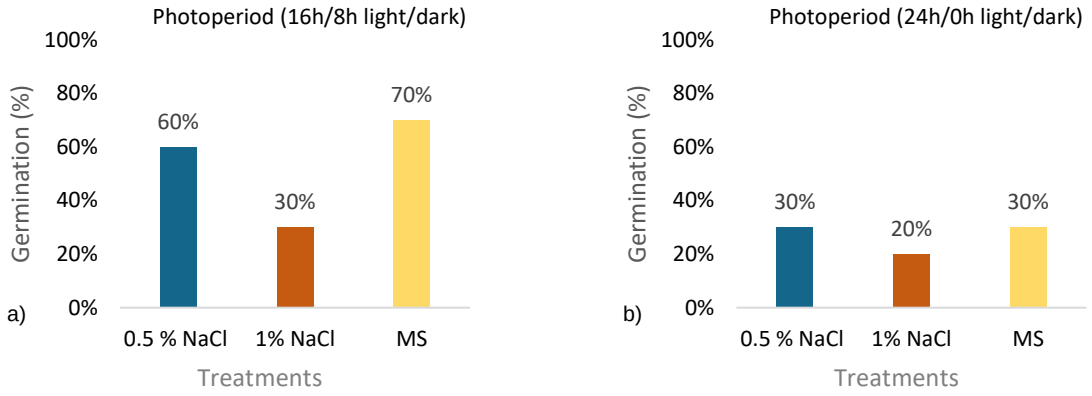


Figure 23 - Germination percentages in each treatment: a) 16h/8h light/dark photoperiod; b) 0h/24h light/dark photoperiod (b). *No germination was observed in sterileH_2O treatment

3.2.2. Bioaugmentation assisted Germination of *P. australis* seeds with the bacterial strains with the best PGP traits

The bacterial strains with the best PGP traits (*Pseudomonas* sp. PCV3 and *Pseudomonas* sp. PCV7) were selected for the bioaugmentation assisted germination of *P. australis* seeds. The bacterial strains were tested either alone or in a consortium, using the best conditions, optimized in the previous germination assays.

Results showed that in all treatments, the germination percentage was higher when the seeds were inoculated bacterial consortium composed by PCV3 and PCV7 (MIX) (Figure 24), in the treatments containing MS medium and 0.5% NaCl + PCB. However, comparing with the control, only the 0.5% NaCl + PCB treatment was significant ($p<0.05$). On the contrary, in the 0.5% NaCl medium, the seeds when inoculated with the MIX, did show a lower germination percentage comparing with the ones that were inoculated only with the bacterial strains.

Germination percentage was slightly higher when the seeds were inoculated with the strain PCV7, but no significant differences were observed ($p>0.05$). However, the seeds germination was higher when the mix was used. No seed germinated in the PCB medium was found, showing that PCB does not contribute for the germination of *P. australis* seeds.

The 0.5% NaCl salt solution medium was the one that showed the lowest germination percentages, comparing with the control. These results may have been influenced by the inexistence of a carbon source (no culture medium used in this treatment).

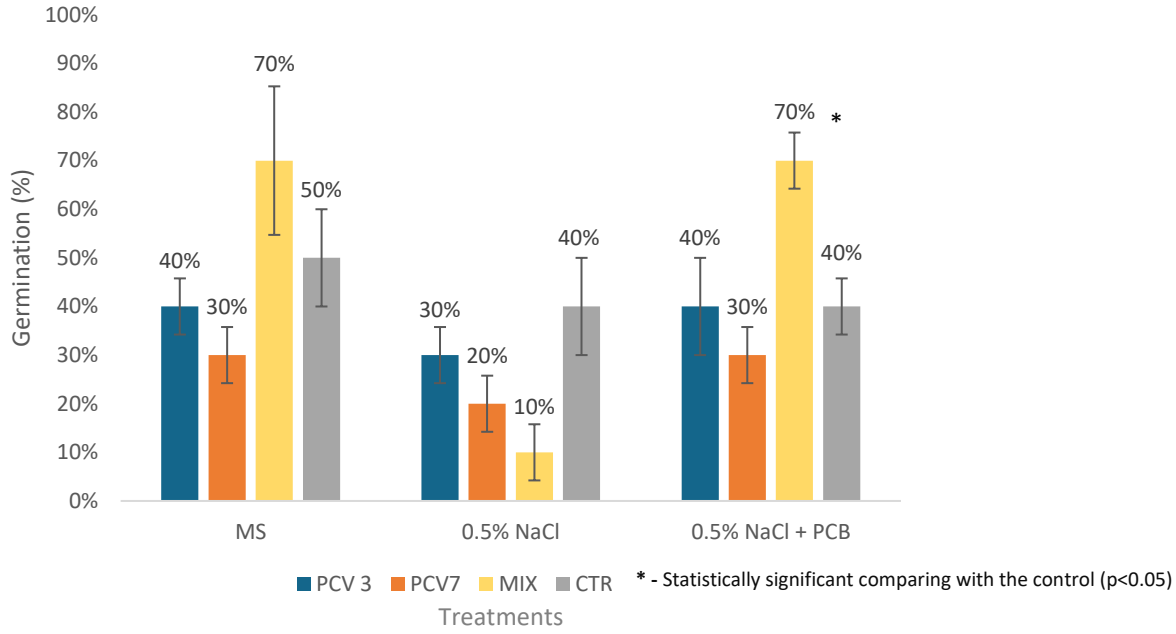


Figure 24 - Germination percentages of *P. australis* seeds Germination percentages of P. australis seeds for each treatment, in the presence of *Pseudomonas* sp. PCV3, *Pseudomonas* sp. PCV7 and the consortium composed by PCV3 AND PCV7 bacterial strains

Different studies showed the potential influence of bacterial strains with PGP traits on plant's germination (Hashmi et al., 2019; Ibarra-villarreal et al., 2023). For example, Raza et al., (2024) evaluated the influence of seed coating with the synthetic consortium on the germination of *Pisum sativum* L. They reported that seed coating with the consortium had a significant positive effect on seed germination, seedling height and biomass accumulation.

The findings of this study provide a foundation for future research regarding the potential of tested bacterial consortium to promote plant germination. Further studies are needed to clarify the specific mechanisms underlying the observed effects of the bacterial consortium on *P. australis* seed germination and how the PGP traits displayed by each bacterial strain can contribute for the results observed in the germination assays. Increasing the number of bacterial isolates within the consortium would allow for a more comprehensive assessment of the individual and combined effects of different bacterial species. Additionally, evaluating the viability and representation of each bacterial strain at the end of the experiment would provide valuable insights into the dynamics of the bacterial community over time.

Furthermore, plant-bacteria system in microcosm experiments would enable us to explore the potential of this microorganisms in a more complex environment, in the

presence of pollutants, to access if they can improve the remediation process. These results are the first insight into the PGP potential of different bacteria, isolated from plants rhizosphere, and how they can influence the germination of P. australis. These results should be considered in the future in the development of bioaugmentation assisted phytoremediation technologies for the removal of mix pollutants.

Conclusions

The rhizosphere of *P. australis* proved to be a hotspot for PGPB, several of which displayed multiple PGP traits. Halophyte microbiomes remain a valuable model for studying how bacteria influence host adaptation. Additionally, these microbiomes offer potential applications as sources of plant growth-promoting bacteria (PGPB).

In agreement with other research, in this study, the genera *Pseudomonas*, *Alcaligenes* and *Microbacterium* were found to be good PGPBs. However, as far as we know, this was the first study to identify PGP traits in bacterial strains from the *Hydrogenophaga* and *Brucella* genera.

PCV3 (*Pseudomonas* sp.) and PCV7 (*Pseudomonas* sp.) were the two strains that demonstrated the most promising plant growth-promoting (PGP) traits, exhibiting positive results in all five screening assays. However, other promising bacterial strains were observed, such as PC6 (*Hydrogenophaga* sp.), PCV1 *(Alcaligenes* sp.), PCV2 (*Brucella* sp.), PCV8 (*Alcaligenes* sp.), PCV11 (*Alcaligenes* sp.) and PCV12 (*Microbacterium* sp.), which showed 4 out of 5 PGP traits.

The germination assays with *P. australis* seeds showed that germination benefited from maintaining humidity during germination, as well as exposure to light (16h/8h light/dark photoperiod). On the contrast, increased salinity proved to be a limiting factor in the germination rates of *P. australis* seeds. The MIX of bacterial strains PCV3 and PCV7 have shown to have a positive influence on *P. australis* seed germination however, more studies are needed to understand the role of each bacterial strain in the germination of *P. australis* seeds.

Since these bacterial strains were isolated from enriched cultures capable of degrade VFX and remove Cu, planted microcosms should be assembled in the future to assess the VFX degradation and Cu removal in the present of the PGPB strains characterized in this study, and how they improve the remediation process. Moreover, a consortium can be built with bacterial strains with PGP traits and degradation potential, to be applied in Bioaugmentation-Phytoremediation processes.

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