From the sea to the field, marine bacteria as potential plant bioherbicide

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“You may chain my hands, you may shackle my feet; you may even throw me into a dark prison; but you shall not enslave my thinking, because it is free!”

— Kahlil Gibran

We did it little one….

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Resumo

O controlo de ervas daninhas na agricultura moderna é uma prioridade. A utilização de herbicidas sintéticos para mitigar este problema gerou outros desafios: estas ervas estão a tornar-se resistentes aos herbicidas utilizados mundialmente, e estes compostos representam riscos para a saúde e o meio ambiente. Portanto, métodos alternativos devem ser considerados e investigados. Uma alternativa aos herbicidas sintéticos é a utilização de bioherbicidas, compostos de fontes naturais mais ecológicos, menos prejudiciais para a saúde pública e mais seletivos no seu modo de ação. Uma possível fonte destes compostos podem ser os microrganismos encontrados no oceano. Dado que os microrganismos marinhos são largamente inexplorados, têm um grande potencial para a descoberta de novos compostos. Como tal, o presente estudo tem como objetivo determinar o potencial herbicida de extratos obtidos a partir de bactérias marinhas. Neste estudo foi avaliado o potencial herbicida de extratos brutos produzidos a partir de três bactérias marinhas, *Mycrobacterium oxydans* estirpe Berg02_79, *Kocuria polaris* estirpe PMIC_1H7A e *Rhodopirellula rubra* estirpe LF2^{T}, pertencentes aos filos *Actinomycetota* e *Planctomycetota*. As três bactérias foram cultivadas em vários meios com diferentes concentrações de carbono e azoto. Os extratos brutos foram obtidos destas bactérias e utilizados em ensaios de crescimento de plantas em placas de Petri, onde foram expostas três espécies modelo, *Arabidopsis thaliana*, *Lactuca sativa* e *Lolium spp*, aos mesmos. Os efeitos foram avaliados pela medição de parâmetros biométricos. Foram também realizados ensaios de crescimento em vasos em que o material vegetal foi utilizado para determinar os níveis de stress oxidativo induzidos pelos extratos nas plantas. Além disso, a produção de ácido índole-acético (IAA) pelas bactérias foi também analisada na tentativa de encontrar uma correlação entre os efeitos observados nas plantas e os níveis desta fitohormona nos extratos, aumentando o nosso conhecimento sobre o modo de ação destes extratos. Este estudo revelou que a *Rhodopirellua rubra* estirpe LF2 demonstrou o maior potencial para a produção de compostos herbicidas. Os extratos de LF2 não só inibiram significativamente todos os parâmetros biométricos analisados, como também exibiram seletividade contra *A. thaliana*, enquanto as restantes plantas modelo permaneceram inalteradas. Os ensaios de produção de IAA confirmaram a capacidade destas bactérias em sintetizar a auxina. No entanto, isto por si só não foi suficiente para explicar os efeitos inibitórios observados. Por fim, as experiências em vasos confirmaram a seletividade em relação a *A. thaliana* e forneceram evidências de stress oxidativo induzido pela exposição aos extratos, culminado na morte da planta.

Concluindo, foram observados efeitos inibitórios significativos dos extratos, e determinou-se que a espécie *Rhodopirellula rubra* LF2 possui potencial para a produção de novos compostos com atividade bioherbicida seletiva para a planta daninha modelo *A. thaliana*. Este estudo fornece evidências não apenas do potencial das bactérias marinhas como fontes de bioativos, mas também destaca especificamente o potencial bioherbicida dos extratos de *Rhodopirellula rubra*. Tanto quanto sabemos, este é o primeiro estudo a relatar atividade bioherbicida de bactérias marinhas.

Palavras-chave: Bioherbicidas, bactérias marinhas, extratos bacterianos, efeitos inibitórios em plantas, IAA, stress oxidativo

Abstract

Weed management in modern agriculture is a top priority. The use of synthetic herbicides to mitigate this problem has generated other challenges, weeds are getting resistant to the herbicides used worldwide, and these compounds are proven to pose health risks and environmental concerns. Therefore, alternative methods should be considered and investigated. One alternative to synthetic herbicides is the use of bioherbicides, compounds from natural sources that are more eco-friendly, less harmful to public health and are more selective in their mode of action. One possible source of these compounds could be microorganisms found in the ocean. Given that marine microorganisms are widely unexplored, they have great potential for the discovery of novel compounds. As such, this study had the objective to determine the herbicidal potential of extracts obtained from three strains of marine bacteria. In this study, the herbicidal potential of crude extracts produced from three marine bacteria, *Mycrobacterium oxydans* strain Berg02_79, *Kocuria polaris* strain PMIC_1H7A and *Rhodopirellula rubra* strain LF2^T, belonging to the *Planctomycetota* and *Actinomycetota* phyla was evaluated. The three bacteria were grown in various media with different concentrations of carbon and nitrogen. Crude extracts were obtained and tested in Petri dish growth assays, in which three model plants, *Arabidopsis thaliana*, *Lactuca sativa* and *Lolium spp.*, were exposed to them. Subsequently, biometric parameters were measured, and the inhibitory effects of the extracts were evaluated. Plants were also grown in pots and treated with bacterial extracts to assess oxidative stress levels induced by these treatments. In addition, the production of indole-3-acetic acid (IAA) by the bacteria was analysed to identify a correlation between auxin levels and the observed effects, aiming to better understand the possible mode of action of the extracts. This study revealed that *Rhodopirellula rubra* strain LF2 demonstrated the highest potential for producing herbicidal compounds. LF2 extracts not only significantly inhibited all the biometric parameters analysed but also exhibited selectivity against *A. thaliana*, while the other model plants remained unaffected. IAA production assays confirmed the ability of this bacterium to synthesize auxins. However, this alone could not account for the inhibitory effects observed. Finally, pot experiments further confirmed the selectivity toward *A. thaliana* and provided evidence of oxidative stress induced by exposure to the extracts, ultimately leading to plant death. In conclusion, significant inhibitory effects were observed, and it was determined that the species *Rhodopirellula rubra* LF2 has the potential to produce novel compounds with selective bioherbicidal activity against the model weed *Arabidopsis thaliana*. This study provides evidence not only of the potential of marine bacteria as

sources of bioactive compounds, but also specifically highlights the bioherbicidal potential of *Rhodopirellula rubra* extracts. As far as we know, this is the first study reporting a bioherbicide activity of marine bacteria.

Keywords: Herbicides, synthetic. Bio-herbicides, marine bacteria, bacterial extracts, inhibitory effects, IAA, oxidative stress

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2,4-D	2,4-DICHLOROPHENOXYACETIC ACID
2-4-5-T	2,4,5-TRICHLOROPHENOXYACETIC ACID
ALS	ACETOLACTATE SYNTHASE
DMSO	DYMETHYL SULFOXIDE
DTNB	5, 5'-DITHIOBIS-(2-NITROBENZOIC ACID)
EDTA	ETHYLENEDIAMINE TETRA ACETIC ACID
F.W.	FRESH WEIGTH
GM	GENETICALLY MODIFIED
GSH	REDUCED GLUTATHIONE
IAA	INDOLE–3–ACETIC ACID
MDA	MALONDIALDEHYDE
MOA	MODE OF ACTION
OD	OPTICAL DENSITY
PGPB	PLANT GROWTH PROMOTING BACTERIA
PPFD	PHOTOSYNTHETIC PHOTON FLUX DENSITY
TBA	THIOBARBITURIC ACID
TCA	TRICHLOROACETIC ACID
TRP	TRYPTOPHAN
ROS	REACTIVE OXYGEN SPECIES

1. Introduction

Agriculture is the backbone of human civilization, ensuring food security and supporting livelihoods around the world. It also contributes significantly to economic growth and sustainable development. One of the major challenges in agriculture is effective weed management. Weeds are unwanted plants that compete with crops light, water, macro-nutrients such as nitrogen, phosphorus and potassium as well as micro-nutrients, implying lower quality and yield during the harvest (Oerke, 2006; Kumar et al., 2024; Saito, 2010; Lindquist et al., 2010). This competition, combined with the seed dormancy exhibited by many weeds, allowing them to remain inactive until field preparation or the onset of the crop growing season, makes them particularly difficult to control and eliminate from agricultural fields. (Acker et al., 1993; Clay et al., 2005).

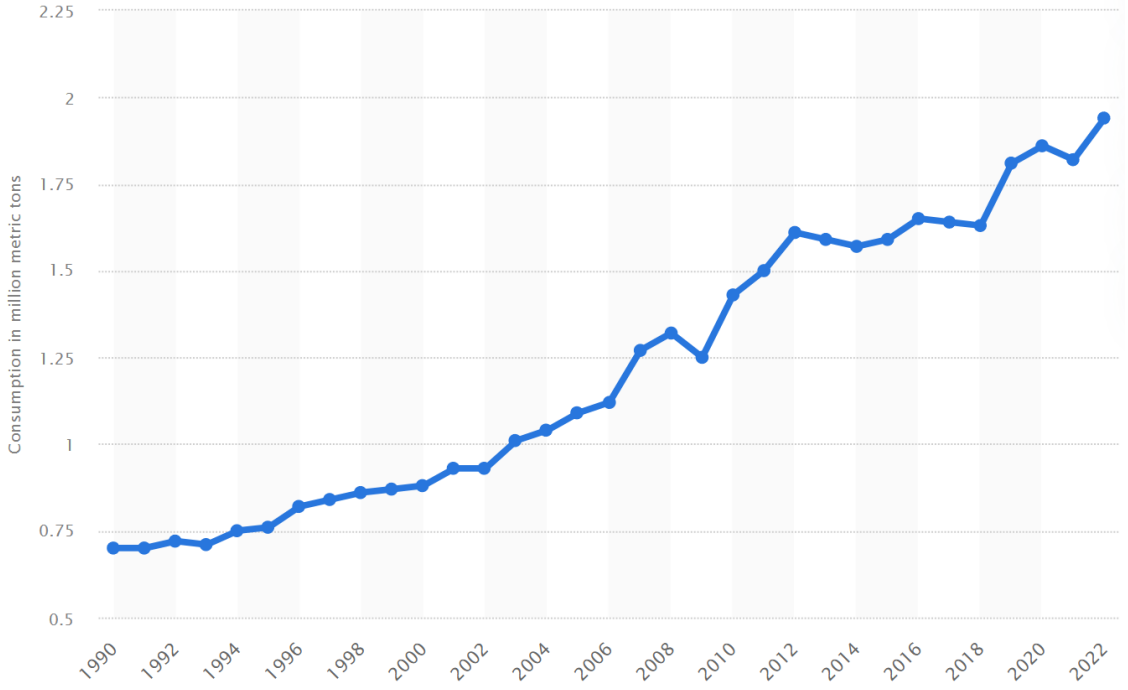

Figure 1 - Agricultural consumption of herbicides worldwide from 1990 to 2022 in million metric tons. Image source: Statista Research Department, 2023. Accessed via https://www.statista.com/statistics/1312103/global-herbicide-agricultural-use/

The most common weed control practices include manual removal, mowing, and, most widely used, the application of herbicides (Melander et al., 2005), which are substances used to control undesired plants. Among these practices, herbicide use is considered the most cost-effective method for weed management and is the preferred choice for large-scale agricultural producers (Holt, 2001). Given this, it is not surprising that the use of herbicides has been increasing over the years, which can be seen in

Figure 1. Herbicide usage was approximately 0.71 million metric tons in 1993 and almost tripled in 2022 with 1.94 million metric tons of herbicide consumed.

The increased use of herbicides is closely linked to the rise of genetically modified (GM) crops engineered to tolerate potent herbicides such as glyphosate (Bonny, 2016). Initially, this combination led to a reduction in overall herbicide usage, as the resistant crops allowed for more targeted and efficient applications. However, over time, herbicide-resistant weed species began to emerge due to the selective pressure imposed by repeated herbicide use. As a result, herbicide application rates have started to rise again to combat these resistant weed populations (Benbrook, 2016; Raman, 2017 Bonny, 2016).

Glyphosate, a chemical developed and introduced to the market by Monsanto in 1974, has been one of the main drivers behind the global spread of herbicide resistance. However, it is not the sole contributor to the emergence of herbicide-resistant weeds in recent years. Other herbicides, such as atrazine, imazamox, and 2,4-dichlorophenoxyacetic acid (2,4-D), have also played significant roles (Holt, 2001; Ofosu et al., 2023). These herbicides, along with their respective chemical families, will be discussed in more detail later.

The rise in herbicide resistance poses a significant threat, not only because resistant weeds will increasingly reduce crop yields over time, but also because existing herbicides may no longer be sufficient to meet the demands of modern agriculture (Holt & Lebaron, 1990; Owen & Zelaya, 2005). This growing challenge, alongside with health and environmental concerns, highlights the urgent need for new solutions, whether through the development of novel herbicides or alternative strategies to combat herbicide-resistant weeds.

Unfortunately, the discovery of new herbicides has proven to be a significant challenge, due to a range of issues spanning from industry and field application constraints to research and development hurdles (Owen & Zelaya, 2005; K. N. Reddy & Nandula, 2012). The efficiency of glyphosate, especially following the introduction of Roundup Ready® cultures by Monsanto in 1996, ushered in an era of convenience for farmers. The widespread adoption of both glyphosate and glyphosate-resistant crops allowed for extensive herbicide use without damaging the crop (Powles, 2022). However, the emergence of herbicide-resistant weeds also means resistance to specific herbicide modes of action (MoA), making the search for novel herbicides with new mechanisms of action an increasingly difficult but essential task for researchers (Torra et al., 2022).

1.1. Differences between bio-herbicides and synthetic herbicides

Herbicides fall into two main categories, biological or synthetic, depending on the source. Synthetic herbicides are compounds that are chemically synthesized in laboratory and are not commonly found naturally. These compounds are typically cheaper and easier to produce when compared to their biological counterparts. Not only that but synthetic herbicides are harder to degrade in nature and have long half times (Oaya et al., 2019).

On the other hand, bioherbicides are substances of biological origin that exhibit phytopathogenic activity, are not so harmful to human health, and can be used in sustainable agriculture. These include, but are not limited to, metabolites produced by microorganisms, essential oils from plants, crude extracts of fungi, bacteria or plants, the microorganisms themselves, among others. These herbicides are generally less harmful to the environment and human health, are safer to use and have a shorter half-life when compared to their synthetic counterparts. But at the same time, they are usually harder to produce and are more expensive (Algandaby & El-Darier, 2018; Cordeau et al., 2016; Islam et al., 2024).

1.2. Synthetic herbicide and their mode of action (MoA)

The first synthetic selective herbicides, 2,4-D and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), were formulated and introduced to the market in the 1940s, shortly after World War II (Newton & Young, 2004; Peterson et al., 2016). These two herbicides are widely used selective herbicides that primarily targets broadleaf weeds while leaving grasses largely unaffected. They are analogous to one group of phytohormones, the auxins. This family of hormones, of which indole-3-acetic acid (IAA) is the most common, plays a key role in regulating plant growth, particularly in the development of leaves and roots. Auxins control growth by varying their concentration throughout different parts of the plant (Newton & Young, 2004). These herbicides (2,4-D and 2,4,5-T), due to their structural similarity to natural auxins, disrupt normal plant growth by inducing abnormal and uncontrolled cell division and elongation, particularly in the leaves. This overstimulation leads to premature senescence and ultimately plant death (Naqvi, S.," 2001). Both herbicides became subjects of controversy, with later studies revealing significant public health risks, especially in the case of 2,4,5-T, which ultimately led to their ban under the Rotterdam Convention (Rotterdam Convention-Operation of the Prior Informed Consent Procedure for Banned or Severely Restricted Chemicals Decision Guidance Documents 2,4,5-T and Its Salts and Esters Secretariat for the Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade; Song, 2014).

Atrazine is an herbicide that belongs to the triazine organic compound family and was the most used herbicide in the world before the commercialization of glyphosate. This herbicide is highly effective and relatively inexpensive to produce and distribute, which has contributed to its widespread global use (Frumkin, 2003). This herbicide acts by entering the chloroplasts and disrupting the electron transport chain at photosystem II, thereby inhibiting photosynthesis. This disruption leads to cell death and, ultimately, the death of the entire plant (Jablonowski et al., 2011). Several studies have identified this herbicide as an endocrine disruptor, causing gonadal abnormalities in frogs by promoting the conversion of testosterone to oestrogen (Bai et al., 2015; Frumkin, 2003; Hayes et al., 2002). Additionally, this herbicide has been at the centre of many controversies, making it a lasting example of the challenges and drawbacks associated with synthetic herbicides in agriculture (He et al., 2019).

Imazamox is an herbicide from the imidazolinone class, which also includes compounds such as imazapic and imazethapyr (Lee, 2014.; Rohr, 2021). These herbicides act by inhibiting acetolactate synthase (ALS), a key enzyme in plants responsible for the biosynthesis of the essential amino acids valine, leucine, and isoleucine. Blocking ALS not only deprives the plant of these amino acids but also disrupts vital metabolic processes (including photosynthesis and respiration) by depleting pathway intermediates and inducing the production of alternative oxidase proteins (Aubert et al., 1997; Bernasconi et al., 1995). Ultimately, this cascade of effects leads to plant death. In addition to its herbicidal activity, imazamox has been reported to exert toxic effects in mammals, including the induction of apoptosis in the liver and pancreas of rats (Duke & Powles, 2008).

Glyphosate's MoA is unique among widely used herbicides because it exclusively targets the shikimate pathway, ultimately leading to plant death (Zhou et al., 2007). This pathway is an effective herbicide target since it is present in plants and microorganisms but absent in animals. The shikimate pathway regulates shikimate levels and produces essential aromatic amino acids (phenylalanine, tyrosine, and tryptophan) as well as other critical cellular metabolites. Inhibition of this pathway by glyphosate causes shikimate to accumulate within cells, disrupting metabolism and leading to cell death, and consequently, the death of the plant (Duke & Powles, 2008; Schönbrunn et al., 2001). However, glyphosate is non-selective in its activity and affects a broad range of plants. To mitigate crop losses, genetically modified crops resistant to glyphosate were developed and commercialized, a practice that has generated considerable controversy in agriculture (Becerril et al., 1989). Beyond its herbicidal action, glyphosate has been

reported to exert toxic effects across diverse organisms, including invertebrates, fish, reptiles, mammals, and even humans, raising significant health concerns (Gill, Sethi, & Mohan, 2017; McHenry, 2018). Notably, glyphosate exposure has also been associated with an increased risk of non-Hodgkin lymphoma (Gill, Sethi, Mohan, et al., 2017).

Synthetic herbicides with selective activity have been widely used since the introduction of 2,4-D to the market (Torra et al., 2022). However, as noted earlier, many of these herbicides have raised significant health concerns and, in some cases (such as atrazine) posed serious risks to public health. Their extensive use has also contributed to the emergence of herbicide-resistant weeds, which in turn has driven both the increased application of herbicides worldwide and the expansion of genetically modified herbicide-resistant crops, most notably Monsanto's Roundup Ready® plants (Raman, 2017). These challenges are difficult to resolve, highlighting the need for continued research into alternative weed management strategies. One promising avenue is the development of bioherbicides, which may provide more sustainable solutions with reduced risks of resistance and toxicity (Zhang et al., 2019).

1.3. Bioherbicides, the next generation

Compounds with herbicidal properties can be found in plants, fungi, bacteria and algae. These compounds may arise as by-products of the metabolism or be intentionally produced by the organism to suppress competitors or inhibit plant growth. Most of the literature available focuses on metabolites produced by fungi and other phytopathogenic microorganisms. Common examples include fungal genera such as *Irpex*, *Cladosporium*, *Penicillium*, *Mucor*, and *Talaromyces*, as well as bacterial genera such as *Staphylococcus*, *Micrococcus*, *Escherichia*, and *Pseudomonas* (Adetunji et al., 2018; Islam et al., 2024; Kong et al., 2025; W. Wang et al., 2022; Wu et al., 2023). However, plants can also serve as important sources of herbicidal compounds, particularly through essential oils (e.g., eucalyptus oil) and aqueous extracts (Algandaby & El-Darier, 2018; Cordeau et al., 2016).

Bioherbicides offer significant advantages over synthetic herbicides, as they are generally more environmentally friendly and pose lower risks to human and ecosystem health (Benchaa et al., 2018). In some cases, the bioherbicide consists of the live organism itself, which slows the development of resistance in weeds due to the natural interactions between plants and microorganisms (Islam et al., 2024). These herbicides typically act through the spread of a phytopathogenic agent that targets a specific weed species, or through a combination of microorganisms that broaden the spectrum of activity to affect multiple weed types (Mortensen, 1988).

Bioherbicides have also been reported to exhibit a shorter half-life compared to synthetic herbicides. While this can be a disadvantage, since it requires more frequent applications, it also means that bioherbicides do not persist in the field for long periods, reducing the risk of environmental contamination associated with synthetic herbicides (Ortiz-Ribbing et al., 2011). As reported by Vischetti & Esposito (1999) the methyl ester of fusaric acid a toxin produced by the fungus *Fusarium nygamai* with herbicidal properties, presented a half-life of less than 10 days degrading into butylpyridine, in three different soils. On the other hand, glyphosate despite having a half-life of about 6 days in the soil, its toxic degradation product, aminomethylphosphonic acid, has a half-life of about 32 days (Simonsen et al., 2008).

Another advantage of bioherbicides over synthetic herbicides lies in their MoA. With the rise of resistance to many of the herbicides currently available, the conventional MoAs have become less effective and less desirable as targets for new herbicide development (Duke et al., 2000). Bioherbicides, however, are derived from natural sources and often act through MoAs that differ from those of synthetic herbicides, giving them a broader range of targets for inhibiting weed growth. This variability in MoAs is a key strength: it helps slow the development of resistance, enhances selectivity toward specific weed families or species, and improves safety (Duke et al., 2014; Owen & Zelaya, 2005). Moreover, the diversity of MoAs among bioherbicides allows for greater flexibility in product design, enabling combinations of different compounds to cover a wide spectrum of weeds while maintaining high specificity (Bordin et al., 2021).

1.4. Bacteria as herbicide factories

Bacteria have proven to be a reliable source of novel compounds of interest. Many antibacterial compounds have been isolated from bacteria, as well as pigments, antioxidants, and anticancer compounds, amongst others (Raaijmakers et al., 2002; Sajjad et al., 2020; Y. Wang et al., 2017).

Many compounds with herbicidal activity have also been isolated from bacteria. As reported by Kennedy et al. (1991), a strain of *Pseudomonas* spp. isolated from the rhizosphere of *Triticum aestivum* showed strong inhibitory effects on *Bromus tectorum*. Similarly, a strain of *Pseudomonas fluorescens* was reported to produce compounds that block the germination of *Poa annua* seeds (Banowetz et al., 2008). In addition, it was also reported by Adetunji et al. (2018), that extracts from a strain of *Pseudomonas aeruginosa* demonstrated potent herbicidal activity against several plant species across different concentrations.

As reported by Sindhu et al. (2018) many microorganisms present in the rhizosphere are an important source of compounds with herbicidal potential. This is consistent with the literature, which shows that most reported compounds and microbial species with herbicidal activity belong to fungal and bacterial groups. This is not surprising, as it is well established that the rhizosphere microbiome engages in complex interactions with plant root systems. Such interactions can either stimulate or inhibit root growth and significantly influence nutrient uptake (Berendsen et al., 2012; Jones & Hinsinger, 2008). Consequently, research into bioherbicidal activity often focuses on these interactions, aiming to identify compounds with potential biotechnological applications.

However, the rhizosphere is not the only source of compounds with biotechnological potential. One largely unexplored environment with immense promise for the discovery of novel compounds is the ocean.

1.5. Unleashing the potential of the ocean

The ocean remains largely unexplored, holding vast untapped potential. Over the years, there has been a growing effort to investigate and explore this environment, leading to the discovery of novel compounds with significant biotechnological potential each year (Debbab et al., 2010; Soliev et al., 2011).

As reported by Pagels et al. (2021) cyanobacteria can produce carotenoids with potential biotechnological applications. These compounds have been shown to exhibit antioxidant, anti-inflammatory, and antitumoral activities. However, cyanobacteria are not the only sources of bioactive compounds; several bacterial phyla are of great interest and hold significant potential for novel compounds. Two of these phyla are the *Actinomycetota* and the *Planctomycetota*.

Actinomycetota have proven to be a reliable source of bioactive compounds, particularly antibiotics (Soliev et al., 2011). This phylum is even described by some authors as a “never-ending source of bioactive compounds” (De Simeis & Serra, 2021), a fitting characterization for a group of bacteria that continues to yield numerous novel bioactive molecules. As reported by Santos et al. (2020), several extracts obtained from bacteria isolated from marine sponges exhibited bioactivity against the fungus *Aspergillus fumigatus*, the bacteria *Staphylococcus aureus* and *Escherichia coli* (including methicillin-resistant strains), the parasite *Trypanosoma cruzi*, and the human liver cancer cell line HepG2. Furthermore, extracts from Actinomycetota isolated from the Tagus River estuary demonstrated bioactivity against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus fumigatus*, *Candida albicans*, and *Trichophyton rubrum (Santos et al., 2024)*.

Planctomycetota have also proven to be a promising source of bioactive compounds. Although this phylum is not as extensively studied as Actinomycetota, it has shown significant potential (Graça et al., 2016). As reported by Calisto et al. (2019), extracts from Planctomycetota bacteria exhibited anticancer activity against the human prostate cancer cell line PC3 and the human acute myeloid leukaemia cell line MOLM-13. Additionally, a genomic study of a bacterium belonging to the genus *Stieleria* within the phylum *Planctomycetota* revealed potential bioactivity against *Staphylococcus aureus*. In another study involving Planctomycetota, including *Rhodopirellula rubra* used in this research, bacteria from this phylum demonstrated potent inhibition of *Pseudomonas* strains (Vitorino et al., 2022).

Given their potential for novel compound discovery and bioactivity, these two phyla represent excellent candidates to be explored in this study, which aims to identify new potential bioherbicides.

1.6. Bacteria

For this study three strains of bacteria were used, belonging to two phyla, *Planctomycetota* and *Actinomycetota*.

1.6.1. Planctomycetota

LF2^T is the type of strain of *Rhodopirellula rubra*, a species of bacteria typically found in oceanic or marine related environments (Bondoso et al., 2014). This species was first isolated from the biofilm surface of the alga *Laminaria* sp. collected in Praia da Luz, Foz, Porto. As seen in Figure 2, this species has been reported all over the world and mainly in aquatic samples (Rhodopirellula Rubra LF2 | Type Strain | DSM 25459, CECT 8075 | BacDiveID:130536).

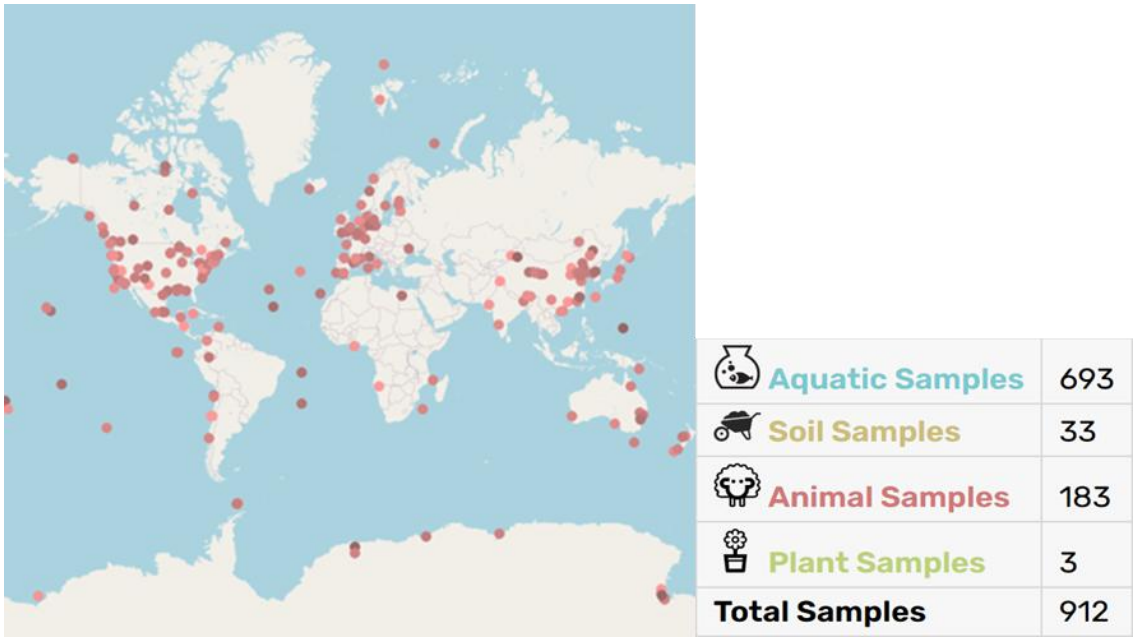

Figure 2 – *Rhodopirellula rubra* strain LF2 isolates global distribution based on 16S rRNA gene sequence identity (>99%), along with number of samples from aquatic, soil, animal and plant sources. Image source: Microbe Atlas Project, 2025. Accessed via https://bacdive.dsmz.de/strain/130536

Research has been done to evaluate the potential of this species as a supplement and/or nutritional food source for *Daphnia magna* (Marinho et al., 2019). In this study, it was concluded that nutritionally supplementing *Daphnia magna* with *R. rubra* resulted in a significant increase in daphnids size and in the production of offspring. Therefore, this bacterium demonstrates significant potential for development as a food supplement.

1.6.2. Actinomycetota

Berg02_79, a strain of *Microbacterium oxydans* was isolated from a marine sponge belonging to the genus *Erylus* found at the continental shelf in Berlengas (Portugal) (Santos et al., 2019). This species presents a wide distribution around the world (Fig. 3) given its tolerance to salt concentrations up to 6% (Microbacterium oxydans X98 | Type Strain | DSM 20578, CIP 6612, NCIB 9944, NRRL B-24236, CCUG 58056, JCM 12414, CIP 66.12, KCTC 3256, BCRC 12117, CGMCC 1.8870, IAM 15197, IFO 15586, NBRC 15586, NCIMB 9944, VKM Ac-2116, IFO (Now NBRC) 15586, NCIB (Now NCIMB) 9944 | BacDiveID:7369).

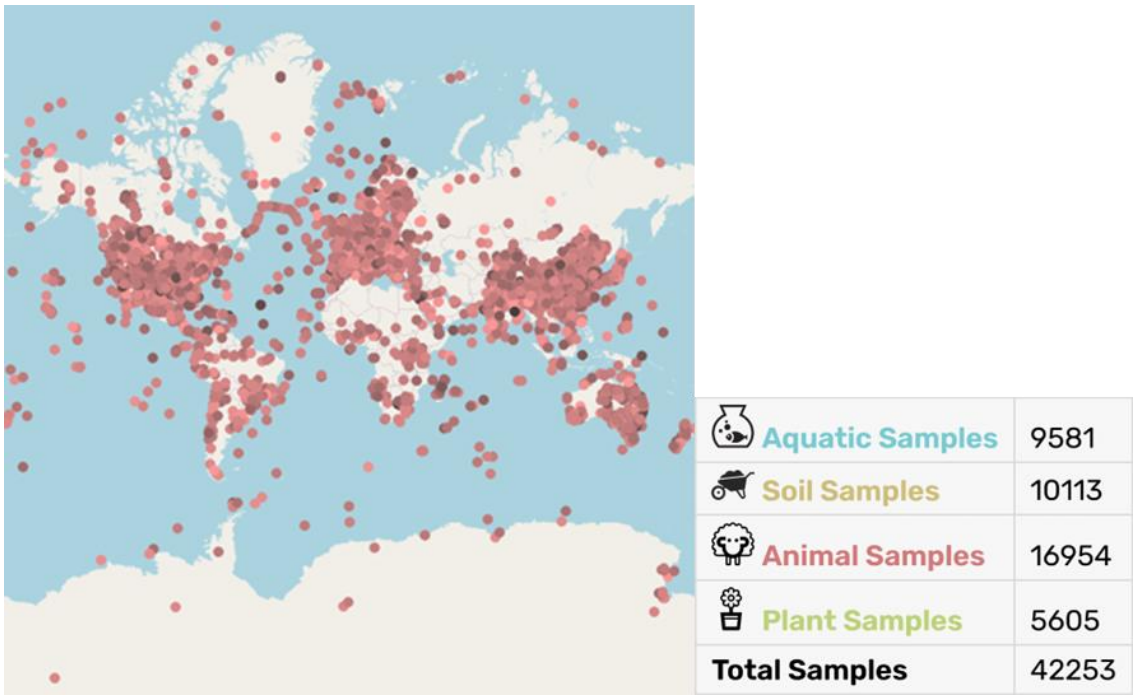

Figure 3 - *Microbacterium oxydans* strain Berg02_79 global distribution based on 16S rRNA gene sequence identity (>99%), along with number of samples from aquatic, soil, animal and plant sources. Image source: Microbe Atlas Project, 2025. Image source: Microbe Atlas Project, 2025. Accessed via https://bacdive.dsmz.de/strain/7369#ref121487

This species has been reported to produce compounds with antimicrobial properties against methicillin-resistant *S. aureus* (Santos et al., 2019), indicating a potential for novel molecules discovery. It has also been reported to have potential as an effective bioremediatory, removing metal ions from liquid cultures at different temperatures and pH (Heidari et al., 2020). Furthermore, this species was reported to promote the growth of *Solanum lycopersicum* under drought stress and to produce IAA (Gan et al., 2025; Siraj et al., 2022) making it a species with great PGPB potential.

PMIC_1H7, a strain of *Kocuria polaris,* isolated from Memoria beach in Perafita, Matosinhos (Santos et al., 2022). This species is a psychrophilic bacterium, first isolated from a cyanobacterial mat sample in a pond located in McMurdo Dry Valley, Antarctica (Reddy et al., 2003). Members of this species have a wide distribution around the world, being present in all sorts of environments (Fig. 4).

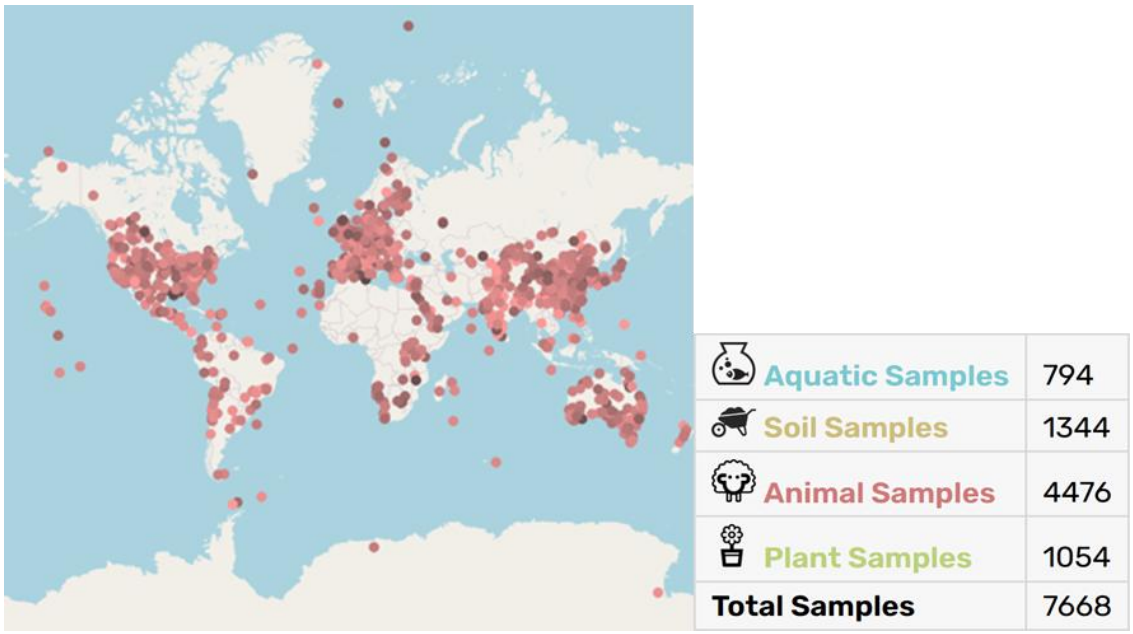

Figure 4 - *Kocuria polaris* strain PMIC_1H7A global distribution based on 16S rRNA gene sequence identity (>99%), along with number of samples from aquatic, soil, animal and plant sources. Image source: Microbe Atlas Project, 2025. Image source: Microbe Atlas Project, 2025. Accessed via https://bacdive.dsmz.de/strain/7650

Bacteria from *Kocuria* genus were reported to have the capability to produce IAA, as well as genomic traits for adaptation to desert environments (Li et al., 2025). Furthermore, they have also been reported to produce UV-protective pigments, with the carotenoid's family being the most prominent pigments (Paredes Contreras et al., 2025).

1.7. Plants

For this study, three model plants were used, two dicotyledonous and one monocotyledonous. *Arabidopsis thaliana* is a dicot plant widely used as a biological model (Woodward & Bartel, 2018). This plant was used as a representative of a weed for which a negative impact of the extracts would represent an herbicidal effect. *Lactuca sativa* is a dicot plant with great agroeconomic value and will serve as a model plant for which no negative effect of the extracts should be obtained. Similarly, *Lolium spp.* were used as an example of a crop monocot.

1.8. Aims

This study focuses on the herbicidal potential of crude extracts from three marine bacteria belonging to two phyla: *Actinomycetota* and *Planctomycetota*. The selected bacteria were grown in different media, and their crude extracts were tested on three model plants. The study evaluated the effects of these extracts on plant germination, morphology, and biochemical stress markers when plants were grown in a solid medium substrate supplemented with bacterial extracts or in pots sprayed with bacterial extracts.

Additionally, the ability of these bacteria to produce IAA was assessed as it could potentially be responsible for the observed effects. By analysing these results, the study aimed to determine the potential of these bacteria to produce herbicidal compounds, acting as bioherbicides.

2. Material and Methods

2.1. Culture media

For this study, solid and liquid bacterial cultures were obtained using three different culture media adapted from Lage & Bondoso, (2011). These media differ in the concentrations of carbon and nitrogen, whereas M600 medium has 4x more concentration of C and N than M607 medium and 1:10 M607 has one tenth of the C and N concentrations from M607 medium (Tables 4 -7 from Annex). Following the One Strain Many Compounds (OSMAC) approach, varying the conditions where the bacteria were grown, different compounds could be produced and thus increasing the potentiality of herbicidal effects by the extracts produced from the bacteria, being observed. This OSMAC approach on marine microorganisms has been described in the literature either by Romano et al. (2018) and Santos et al. (2020).

The plants in the Petri dish trials were grown in an adaptation of Hoagland's agarized solution (Río et al., 2014). The plants in the pot trials, on the other hand, were watered using Hoagland's non-agarized solution (Table 8 from Annex)

2.2. Extract preparation

2.2.1. Solid cultures

For the screenings of the effects of the bacteria on Petri dish plant growth assays, extracts were prepared by growing bacteria in 10 Petri dishes with 20 mL of culture for each medium, at 28ºC, for 1 or 2 weeks, for *Actinomycetota* and *Planctomycetota*, respectively. After, the cultures were sliced, submerged in an organic solvent (ethyl acetate), and the mixture was sonicated for 5 min and incubated overnight. These procedures aimed to enable the lysis of the bacteria and the subsequent release of intracellular content with potentially relevant compounds. The extract mixture (from here on designated by organic phase) was then evaporated in a rotary-evaporator, operated at 40º C for the water bath, -2º C for the chiller and 120 mbar of atmospheric pressure in the system. The resulting powder was then solubilized in 2% DMSO.

The bacterial extracts will be referred to in this study based on the name of the bacterium and the name of the respective medium where it was cultivated (*e.g.* LF2 M600).

2.2.2. Liquid cultures

New extracts were prepared from the three strains grown in M607 liquid media at 200 rpm, 27ºC, for 7 days. Afterwards, the bacterial cultures were centrifuged at 5000 g for 20min, and both the pellet and the supernatant were recovered separately and further processed. The pellet was submerged in about 5 mL of ethyl acetate, sonicated for 5min, ethyl-acetate was then added to a total volume of 100 mL and bacterial compounds were extracted overnight at room temperature. Then, the ethyl acetate phase was collected with a pipette, and the pellet was washed once more with 50 mL of solvent. The ethyl acetate phase was again collected, and the pellet was discarded. The supernatant was subjected to liquid–liquid extraction with ethyl acetate. Given that ethyl acetate and the supernatant are not miscible, and the latter is denser than the ethyl acetate, both were poured into a separatory funnel in a proportion of 1:1, shaken vigorously and waited until separation of the two phases occurred, the aqueous phase (the supernatant) and the organic phase. The aqueous phase was then washed with ethyl acetate in a 2:1 proportion, and the organic phase was also collected. Both the ethyl acetate phase of the pellet extraction and the organic phases of the liquid-liquid extraction were dried out in a rotary evaporator as previously described, and the resulting powder was dissolved in 10 mL of 99.8% DMSO.

These extracts were also labelled with the name of the bacterium and the name of the culture medium from which the extracts were obtained. In addition, the extracts from liquid cultures were also labelled with the phase from which they were obtained (pellet or supernatant).

2.3. Petri Dish plant growth assays

Petri dish assays followed a design, with sets of three conditions (three plates for M600 extracts, three plates for M607 extracts and three plates for 1:10 M607 extracts) plus the control (three plates) tested simultaneously.

To assess if DMSO induced toxicity on our test plants, increasing concentrations of DMSO (0%, 1%, 2%, 5%) were tested on *L. sativa*, *Lolium spp.* and *A. thaliana*.

Petri dish assays were performed by adding 2.5 mL of bacterial extracts obtained from the extraction process or DMSO at the three concentrations to melted Hoagland solution (plant medium) and stirring to ensure a good incorporation of the extract or the DMSO solution (control). After media solidification, seeds previously disinfected with a solution of 50% bleach for 3 minutes and thoroughly washed with sterile water, were placed in line on the medium surface. The plates were then sealed with parafilm and placed

vertically in a greenhouse at 25ºC under a 16h:8h light:dark cycle and a Photosynthetic Photon Flux Density (PPFD) of 190 µmol m^{-2} s^{-1}. For *A. thaliana,* 20 seeds were sown per plate, and grown for three weeks, whereas for *L. sativa* and *L. spp.*, 12 seeds were sown per plate, and grown for two weeks.

At the end of each assay, the plants were removed from the medium, placed side by side and photographed for root length measurements using the ImageJ software. Additionally, total plant fresh weight and the number of leaves were also recorded. For *Lolium spp.*, leaf length was measured instead of leaf number.

2.4. Plant growth pot assays

For the pot assays, the seeds of each species were placed in a mixture of perlite and vermiculite at a ratio of 1:1, watered with Hoagland solution every two days and grown under a controlled light cycle. For *A. thaliana,* the light cycle was 8h:16h light:dark, whereas for *L. sativa* and *Lolium perenne* were submitted to a cycle of 16h:8h light:dark, both light cycles were performed under a PPFD of 190 µmol m^{-2} s^{-1}. A shorter light cycle allowed a slower and more controlled development of the flower stem in *A. thaliana,* preventing the appearance of flowers.

After an initial growth of around 10 cm in *L. sativa* and *L. perenne*, and the formation of the third pair of leaves in *A. thaliana*, the pots were sorted randomly and the plants treated with the bacterial extracts. For that, the liquid culture extracts were diluted to a final DMSO concentration of 8% and 0.05% Triton X-100 was added. This DMSO concentration was used to test all plant species. The plants were sprayed twice a week with either the extracts or the control solution (8% DMSO and 0.05% Triton X-100) for two weeks. The aerial parts of the plants were ground with liquid nitrogen and divided into ~ 200 mg aliquots and stored at -80ºC for further biochemical analysis.

2.5. Biochemical analyses

2.5.1. Proline and reduced glutathione quantifications

Plant biomass (~200 mg) was homogenized in 2 mL of 3% sulfosalicylic acid with the help of quartz sand and incubated on ice for 10min before centrifuged at 12,000 *g* for 10 minutes. The supernatant was retrieved and used to carry out proline and reduced glutathione (GSH) quantification separately.

The proline quantification was performed by following the procedure described by Bates et al. (1973). Two hundred µL of supernatant were mixed with 200 µL of ≥99% glacial acetic acid and 200 µL of acid ninhydrin (2.5% ninhydrin dissolved in glacial acetic acid

and 2.4 M phosphoric acid). The mixture was incubated at 95ºC for 1 hour and cooled down in ice for 10 minutes. After the cooling, 1 mL of toluene was added, and the mixture was vortexed for 15 seconds and then kept undisturbed for toluene phase separation. From this organic phase, 340 µL were added to a 96-well plate to determine the optical density (OD) in a spectrophotometer, at 520 nm, using toluene as a blank. The proline content was expressed in µg mg^{-1} f. w. (fresh weight), based on a standard curve of known proline concentrations.

The reduced glutathione quantification was performed by following the procedure described by Rahman et al. (2006), adapted for microplate spectrophotometry. Twenty µL of supernatant were mixed with 70 µL of dH_2O and 250 µL of reaction mix (100 mM of potassium phosphate buffer, 1 mM of ethylenediamine tetra acetic acid (EDTA) and 0.1 M 5,5′-dithiobis-(2-nitrobenzoic acid)-DTNB) in a 96-well plate and incubated in darkness for 10 minutes. For the blank, 20 µL of sulfosalicylic acid was used instead of the supernatant. The OD was measured at 412 nm, and the reduced glutathione content was expressed in µmol g^{-1} f. w. using a standard curve of known GSH concentrations.

2.5.2. Lipid peroxidation and H_2O_2 quantification

The plant biomass (200 mg) was homogenized in 1 mL of 0.1% trichloroacetic acid (TCA) with the help of quartz sand. The resultant mixture was centrifuged at 12,000 *g* for 10 minutes, and the supernatant was retrieved for lipid peroxidation and H_2O_2 quantification.

The quantification of lipid peroxidation was made through the quantification of the malondialdehyde (MDA) levels, using the procedure developed by Heath & Packer (1968) adapted for microplate spectrophotometry. For that, 50 µL of the supernatant were mixed with 200 µL of 0.5% thiobarbituric acid (TBA) in TCA, incubated at 95ºC for 30 minutes and then cooled down for 10 minutes in ice. The blank was made using 50 µL of TCA instead of the supernatant. The mixture was then centrifuged at 12,000 *g* for 10 minutes. After, 250 µL of the supernatant were transferred to a 96-well plate and the ODs were measured at both 532 nm and 600 nm in a spectrophotometer. Then the OD values obtained at 600 nm were subtracted from the ODs obtained at 532 nm. MDA concentration was then calculated considering $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ and the results expressed in nmol g^{-1} f. w.

As for the H_2O_2 quantification, an adaptation of the procedure described by Alexieva et al. (2001) was performed. First, 50 µL of the supernatant were mixed with 200 µL of potassium iodide and 50 µL of 100 mM potassium phosphate buffer, pH 7, in a 96 well plate and incubated for 1 hour in darkness. For the blank, 50 µL of TCA were used in place of the supernatant. After, the OD was measured at 390 nm and the H_2O_2 content

was calculated considering ε = 0.28 μM^{-1} cm^{-1} and the H_2O_2 content expressed in nmol g^{-1} f. w.

2.5.3. Indole-3-acetic acid quantification

Using an adaptation of the quantification method developed by Gordon & Weber (1951) IAA production was evaluated by growing bacteria in each of the three different liquid culture media, both in the presence and the absence of tryptophan, a precursor of IAA.

Initially, the pre-inocula of the three bacteria were prepared in M607 medium, until the culture reached an OD ~ 0.3 by incubating at 27ºC, with agitation (200 rpm). Then, 1 mL of each pre-inoculum was transferred to previously prepared Falcon tubes with 19 mL of each culture medium (M600, M607 and 1:10 M607), in triplicate. These cultures grew at 27ºC, with agitation (200 rpm), for different periods of time, depending on the bacterial phylum and presence/absence of tryptophan (200 mg L^{-1}). For *Actinomycetota* bacteria in the presence of tryptophan (Trp), the cultures grew for 24h, while for cultures in the absence of Trp, incubation was carried out for 24h and 48h. As for *Planctomycetota* bacteria, in the presence of Trp the cultures grew for 48h, while for cultures in the absence of tryptophan, incubation was carried out for 48h and 72h.

After the growth period, the cultures were centrifuged at 3,200 *g* for 20 minutes, and the supernatant was discarded. Two mL of the respective culture medium used for each culture were added to the pellet and then sonicated for 1 min and 30 s. This mixture was then centrifuged at 3,200 *g* for 20 minutes. In a 96-well plate, 100 µL of the supernatant was mixed with 200 µL of Salkowski reagent (0.5 M ferric chloride diluted in 35% perchloric acid). For the blank, 100 µL of the respective medium were used instead of the supernatant. After, the OD was measured at 530 nm, and the IAA content was expressed in µg mL^{-1} using a standard deviation curve of known IAA concentrations.

2.6. Statistical analysis

The statistical analysis was performed using the SPSS (version 30.0.0.0). The quantitative variables analysed were the root and leaf length, number of leaves and fresh weight of the plants used in the plate assays. Approximately 60 seeds of *A. thaliana* and 20 seeds of *L. sativa* and *L. spp.* were used in each experiment. The data was expressed as average ± standard deviation. The biochemical analyses were carried out on a set of 3-5 plants of each treatment, and each analysis was performed at least in triplicate, and the results were expressed as mean ± standard deviation (SD).

To test the difference between the experimental groups and the control group, a multivariate ANOVA was used. When significant differences were observed, a Dunnett

post hoc test was used to compare each individual experimental group to the control group.

For this study a significant level of 5% ($p < 0.05$) was adopted in all analyses.

Graphs and outlier removal were performed using the software GraphPad Prism (version 8.4.3). To remove outliers the ROUT method was used and a Q value of 5% was adopted.

3. Results and Discussion

3.1. Petri Dish plant growth assays

3.1.1. Effect of dimethyl sulfoxide on plant growth

To test the bacterial extracts, the bacterial material had to be solubilized in the universally used solvent, dimethyl sulfoxide (DMSO), an organosulfur polar compound ($(CH_3)2SO$) with low toxicity (Kaczor-Kamińska et al., 2024). This organic solvent is regularly used to solubilize poorly soluble polar and non-polar compounds. DMSO toxicity was assessed in plant studies, namely in *Solanum lycopersicum* and *L. sativa* where 0.5% to 2% through nebulization on young plant leaves were well tolerated (Gholami et al., 2025). Despite the protective effect on plant regeneration (Sharafat et al., 2023) and protection of fungi infections it can present some degree of toxicity as reported in rice seedlings, leading to oxidative stress of the plants and accumulation of H_2O_2 in roots (Sankpal et al., 2012).

To solve this potential problem and minimize the possible effects of DMSO on the plants during the tests, preliminary toxicity tests were conducted with four DMSO concentrations (0%, 1%, 2% and 5%) on three model plants. For that, the seeds of *A. thaliana*, *L. sativa* and *L. spp* were germinated in Petri dishes containing Hoagland agar medium supplemented with DMSO at different concentrations, the seedlings were grown for 2-3 weeks, and the germination rate, the root length, the number of leaves and the fresh weight were assessed.

The DMSO concentration that induced fewer effects on the model plants was chosen to solubilize the bacterial material and obtain the extracts used in further tests.

3.1.1.1. Effects on *Arabidopsis thaliana*

A. thaliana plants exposed for 3 weeks to concentrations of 1%, 2% and 5% of DMSO (Figs. 5 B, 5C and 5D), have very similar morphological aspects compared to the control plants (Fig. 5A). However, the plants exposed to 5% DMSO (Fig. 5 D) appear to be

smaller (Fig. 5A) and more yellowish. This change in colour is indicative of leaf senescence due to lability of chlorophyll induced by DMSO (Bhandari et al., 2021).

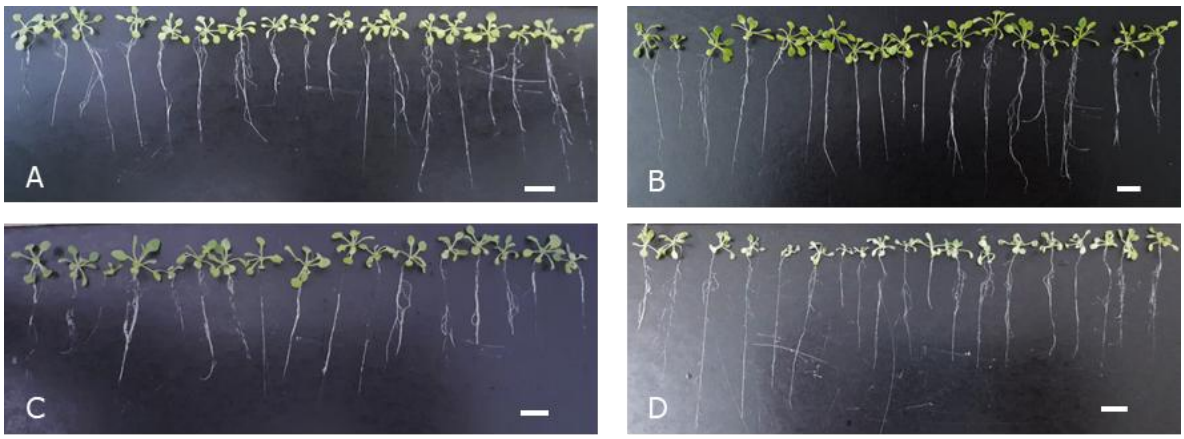

Figure 5 - *A. thaliana* after 3 weeks of growth, under various concentrations of DMSO. A - control plants (0% DMSO). B - plants tested with 1% DMSO. C - plants tested with 2% DMSO. D - plants tested with 5% DMSO. The scale represents 1 cm.

The root length, number of leaves, plants' fresh weight and germination rate were assessed (Fig. 6). Regarding root length, no significant differences were observed between the plants treated with DMSO and the control, except at the 1% concentration, where a significant increase was observed (Fig. 6A). Concerning the number of leaves, differences were only observed in plants exposed to 5% DMSO, where a significant decrease compared to the control was detected (Fig. 6B). As for the fresh weight, plants exposed to both 1% and 2% concentrations of DMSO experienced a significant increase compared to control plants. (Fig. 6C) Lastly, a slight decrease in germination rate was observed in plants exposed to all three DMSO concentrations (1%, 2% and 5%) (Fig. 6D).

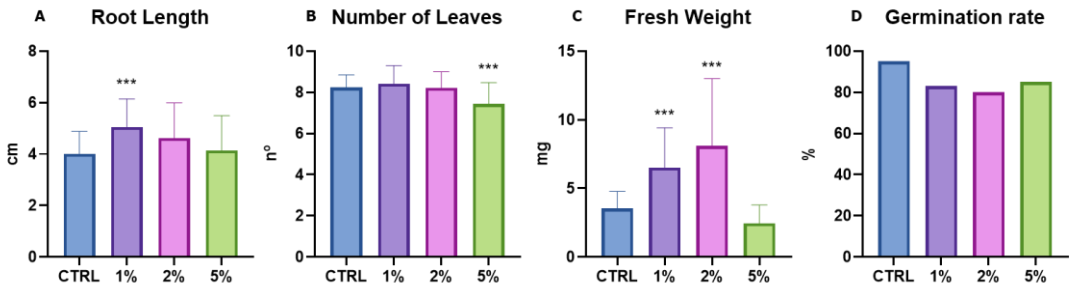

Figure 6 - Effect of DMSO on *A. thaliana* plant growth. Plants were grown for 3 weeks in Petri dishes with Hoagland agar medium supplemented with 0% (CTRL), 1%, 2% and 5% of DMSO and root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with *** ($p<0.001$)

Given this, contrary to what was observed in *A. thaliana* plants exposed to 5% DMSO, where negatively affects were observed (development of yellow colour), plants exposed to 1% and 2% DMSO revealed a beneficial effect, increasing some biometric parameters.

3.1.1.2. Effects on *Lactuca sativa*

L. sativa plants exposed for 2 weeks to concentrations of 1%, 2% and 5% of DMSO (Fig. 7B, 7C and 7D) showed very similar morphological aspects compared to the control plants (Fig. 7A).

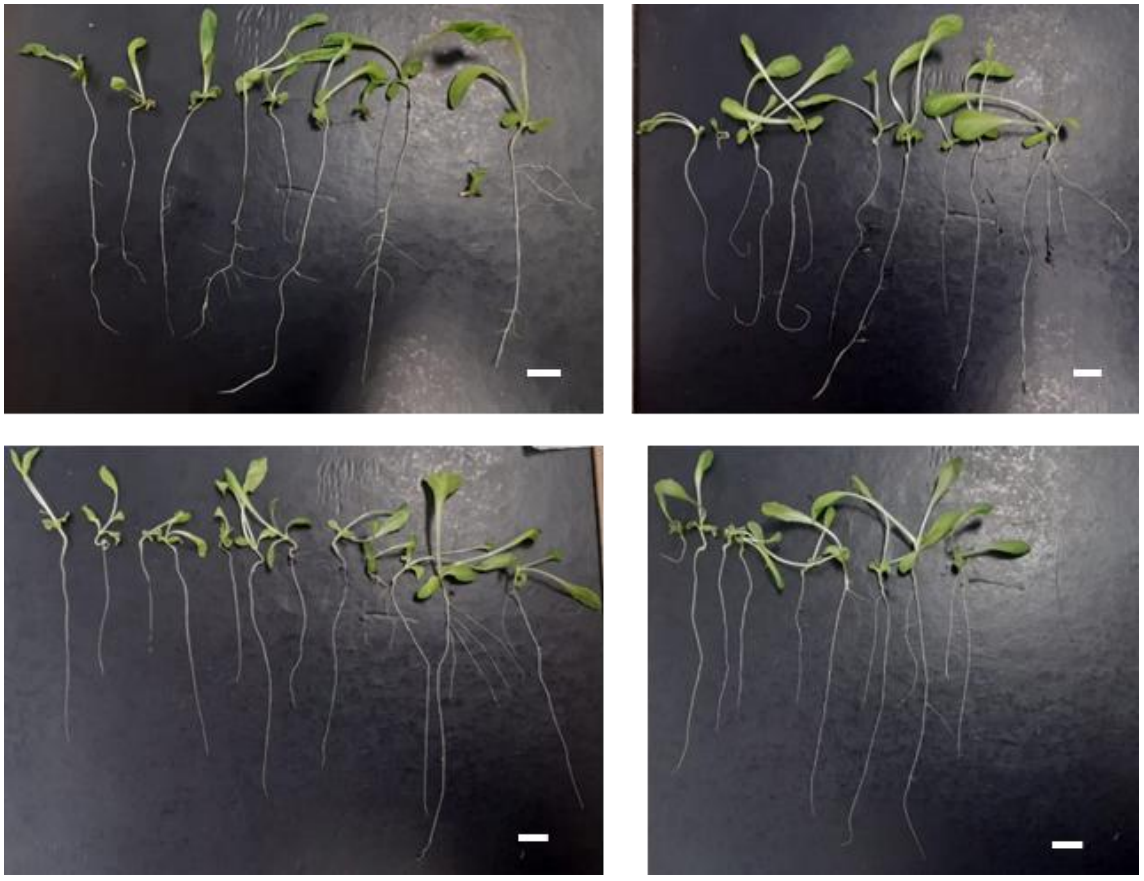

Figure 7 – *Lactuca sativa* after 2 weeks of growth, under various concentrations of DMSO. A - control plants (0% DMSO). B - plants tested with 1% DMSO. C - plants tested with 2% DMSO. D - plants tested with 5% DMSO. The scale represents 1 cm.

The root length and the fresh weight were not significantly affected by any of the concentrations of DMSO tested (Figs. 8A and 8C). Concerning the number of leaves, a significant decrease was observed in plants exposed to 1% DMSO (Fig. 8B). No changes were detected in the plant fresh weight (Fig. 8C). The germination rate decreased only in plants that were exposed to 5% DMSO (Fig. 8D).

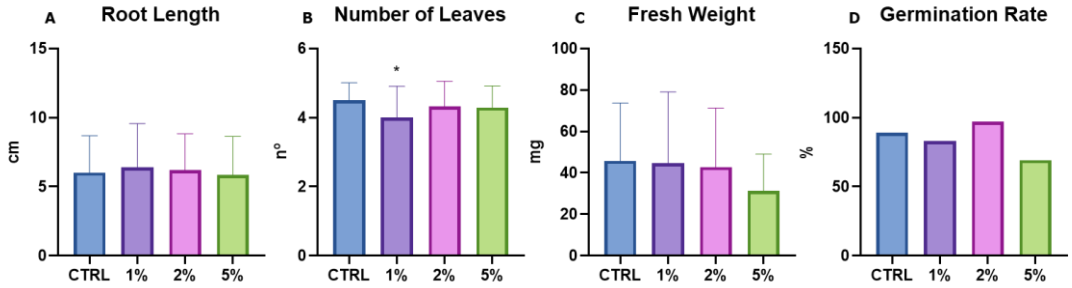

Figure 8 - Effect of DMSO on *L. sativa* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented with 0 (CTRL), 1%, 2% and 5% of DMSO and root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with * ($p<0.05$).

In summary, *L. sativa* plants exposed to the different concentrations of DMSO were, in general, not affected by this solvent.

3.1.1.3. Effects on *Lolium perenne*

Lolium perenne plants grown for 2 weeks exposed to 1%, 2% and 5% of DMSO can be observed in Figs. 9B, 9C and 9D, respectively. By observing the plants, it was evident that plants exposed to 1% and 2% DMSO are similar to control plants seen in Fig 9A. Contrary to that, plants exposed to 5% DMSO present smaller roots.

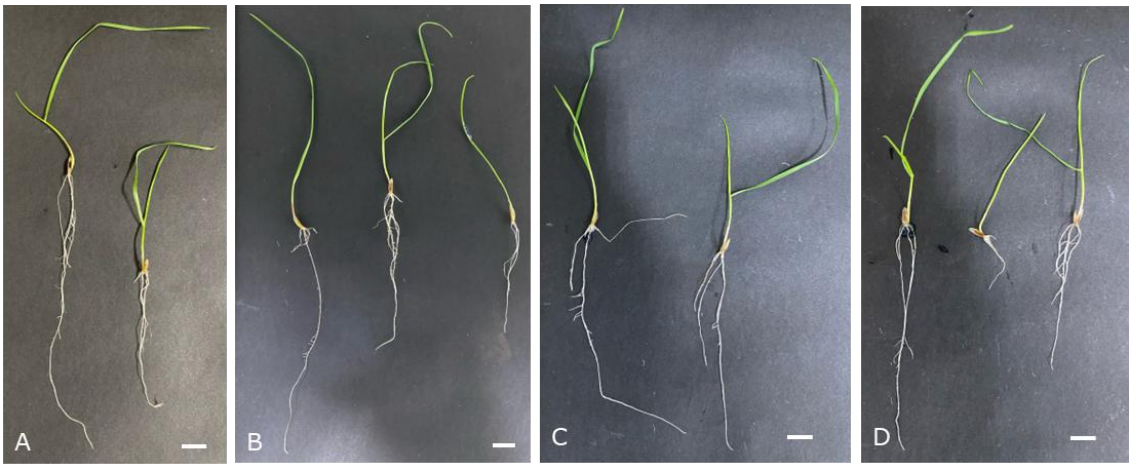

Figure 9 – *Lolium perenne* after 2 weeks of growth, under various concentrations of DMSO. A - control plants (0% DMSO). B - plants tested with 1% DMSO. C - plants tested with 2% DMSO. D - plants tested with 5% DMSO. The scale represents 1 cm.

Confirming what was observed previously, plants exposed to 5% DMSO presented significantly shorter roots than control plants, while plants exposed to 1% and 2% DMSO did not present any significant differences (Fig. 10A). Regarding length of leaves (Fig. 10B), no significant differences were observed for any of the treatments. On the other hand, fresh weight of plants exposed to 2% and 5% DMSO experienced a significant

increase compared to control plants (Fig. 10C). Lastly, germination rate was inhibited below 80% in plants exposed to 1% and 5% DMSO, while plants exposed to 2% DMSO stayed above 80%, closer to the control germination rate (Fig. 10D).

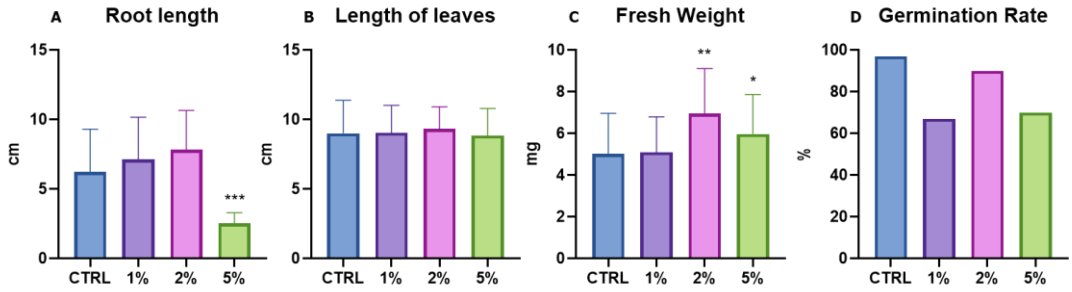

Figure 10 - Effect of DMSO on L. sativa plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented with 0 (CTRL), 1%, 2% and 5% of DMSO and root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with * ($p<0.05$), ** ($p<0.005$) and *** ($p<0.001$).

In conclusion and based on the results obtained for the three plants, we consider that a DMSO concentration of 2% is the most appropriate for solubilizing the bacterial material to obtain the extracts, which will be applied to the plants. This concentration of DMSO presented a great compromise between the minimal effects this organic solvent had on plants and the feasibility of the solubilization of the extracts.

3.1.2. Effect of the bacterial extracts on plant growth

Managing weeds has been a necessary battle for a good yield on a farm. Weed plants compete for space, resources such as light, water and nutrients, or even inhibit the growth of the desired plants leading to economic losses (Baktash & Naes, 2016; Dangwal et al., 2010). To combat this, the use of herbicides has been increasing all over the world, and with that bioherbicides alternatives have also been increasing to be sustainably used in modern agriculture (Fang et al., 2022; Hasan et al., 2021).

In this study, extracts obtained from the bacteria *Microbacterium oxydans* strain Berg02_79, *Kocuria polaris* strain PMIC_1H7A and *Rhodopirellula rubra* strain LF2, grown in three culture media (M600, M607 and 1:10 M607 - with different carbon and nitrogen concentrations) were tested in three model plants, *A. thaliana, L. sativa* and *Lolium spp*., to determine the potential of these bacteria to produce compounds of agronomical interest. For that, seeds of the 3 species were germinated in Petri dishes containing Hoagland agar medium supplemented with the bacterial extracts and after 3 weeks (*A. thaliana*) or 2 weeks (*A. sativa* and L. *perenne*) of growth, several biometric parameters were evaluated.

3.1.3. Effects of *Microbacterium oxydans* strain Berg02_79

3.1.3.1. *Arabidopsis thaliana*, a weed model plant

The extracts from strain Berg02_79 tested on *A. thaliana* produced notable effects on treated plants when compared to the control plants (2%DMSO). Plants treated with M600, M607 and 1:10 M607 extracts (Figs. 11B, 11C and 11D), when compared to those of the control (Fig. 11A), had visually shorter roots. This effect was confirmed by measurement of root length, and detection of a statistically significant decrease in root length induced by all three extracts (Fig. 11A). Furthermore, a pronounced increase in lateral root production, indicating a loss of apical dominance, was also observed in plants grown in the presence of all the extracts. The plants still presented a central root with secondary roots shooting from it; however, the central root growth was halted and secondary root growth stimulated in such a way that it was difficult to discern the central root from the secondary ones (Figs. 11B, 11C and 11D). This effect resulted in *A. thaliana*, a plant with a tap root system, growing roots in a way resembling a fibrous root system, typically found in a monocot plant (Chen et al., 2018).

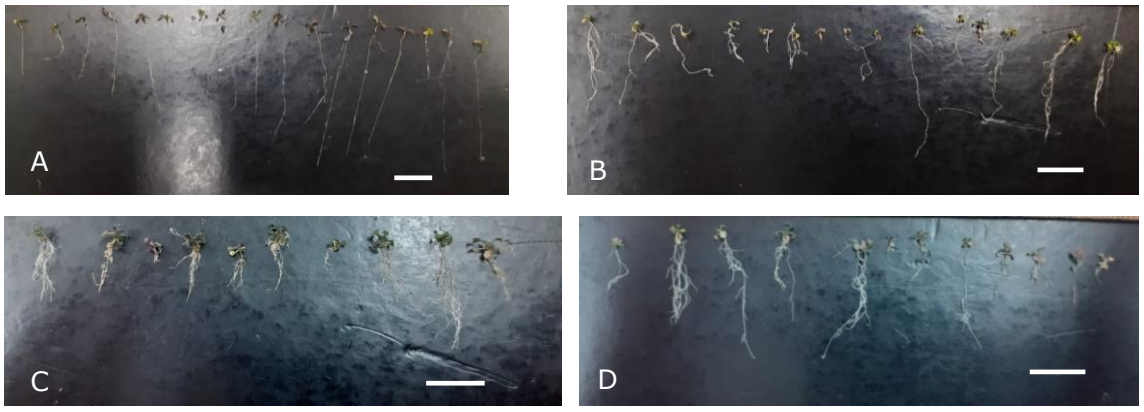

Figure 11 – Exposure of *A. thaliana* to Berg02_79 extracts after 3 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 1 cm.

Additionally, the number of leaves, fresh weight and germination rate were also recorded. Except for the M607 extract, where a significant increase was observed for both number of leaves and plant fresh weight biometric parameters (Figs. 12B and 12C), no significant differences were observed. Despite the significant inhibition in root length, the significant increase in fresh weight of plants exposed to M607 extract could be related to the significant increase in the number of leaves and number of lateral roots observed.

Seeds' germination rate decreased relevantly in the presence of the three bacterial extracts as seen in Fig. 12D.

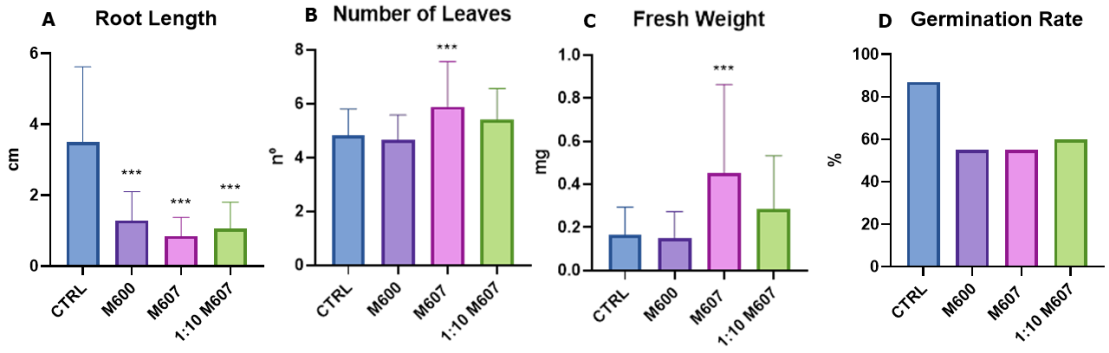

Figure 12 - Effect of Berg02_79 extracts on *A. thaliana* plant growth. Plants were grown for 3 weeks in Petri dishes with Hoagland agar medium supplemented with 2% DMSO (CTRL), M600, M607 or 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with *** ($p<0.001$).

Based on these results, we consider that Berg02_79 extracts altered *A. thaliana* root growth, inhibiting its elongation but stimulating the formation of roots, without negatively affecting the number of leaves and fresh weight. In fact, plants exposed to M607 extract had these parameters increased, revealing a significant biostimulator effect.

3.1.3.2. *Lactuca sativa*, an important dicot crop

As seen in Figure 13, after two weeks of growth, *Lactuca* plants exposed to Berg02_79 M607 and 1:10 M607 extracts (Figs. 13C and 13D) presented morphological differences when compared to the control (Fig. 13A). These plants had shorter roots and an under-developed aerial part, particularly the plants exposed to the M607 extract. On the other hand, plants exposed to the M600 extract (Fig. 13B) presented development of aerial part and of the root length quite similar to the control plants (Fig. 13A).

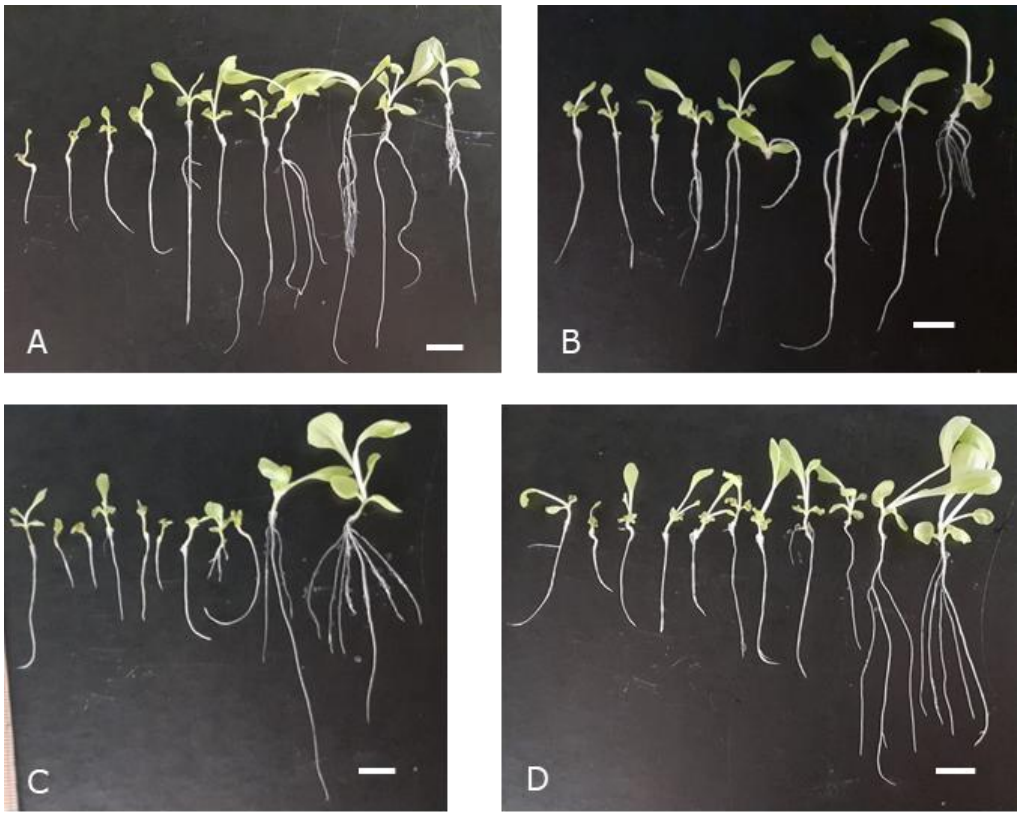

Figure 13 - Exposure of *L. sativa* to Berg02_79 extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 1 cm.

The quantification of the biometric parameters detected statistically significant differences in plants exposed to the M607 extract. As seen in Figure 14, root length (A) and number of leaves (B) were significantly decreased in the treated plants. Regarding fresh weight, a decrease was also detected in plants exposed to M607 extract, although not significant (Fig. 14C). The germination rate was not affected by the exposure of the plants to either of the 3 extracts (Fig. 14D).

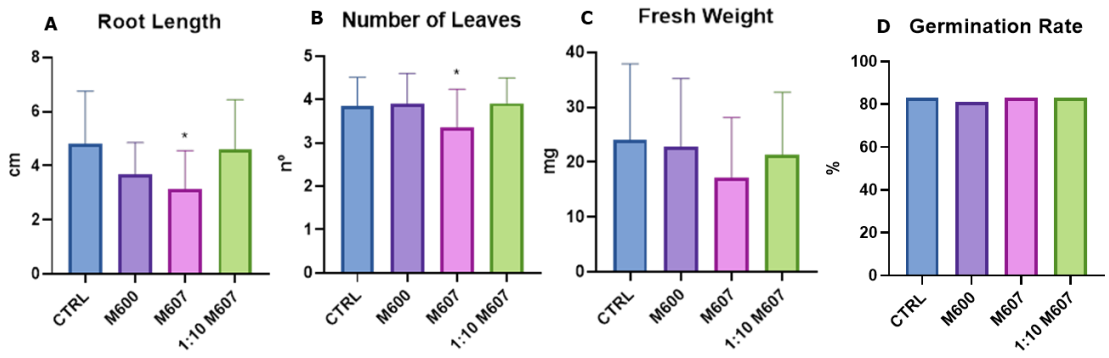

Figure 14 - Effect of Berg02_79 extracts on *L. sativa* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with * ($p<0.05$).

In summary, apart from plants exposed to M607 extracts, *L. sativa* plants were not significantly affected by Berg02_79 extracts when compared to the control. However, a slight inhibitory effect was visible in plants treated with the M607 medium, both at root and aerial part growth, an effect distinct from what was observed in *A. thaliana*.

3.1.3.3. *Lolium perenne,* a monocot crop

As seen in Figure 15, after two weeks of growth, *L. perenne* plants exposed to M600 (Fig. 15B) and M607 (Fig. 15C) extracts exhibited morphological similarities with plants from the control (Fig. 15A), showing similar roots and aerial parts. On the other hand, plants exposed to 1:10 M607 (Fig.15D) extract clearly presented longer roots, but the aerial parts seem similar when compared to control plants (Fig. 15A).

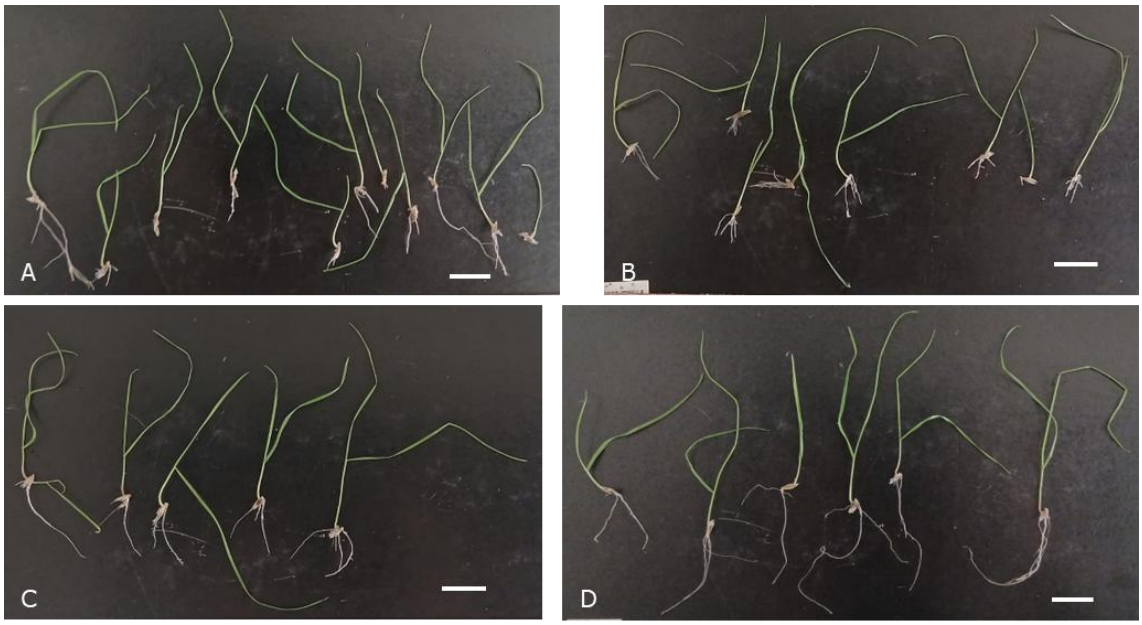

Figure 15 - Exposure of *L. perenne* to Berg02_79 extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 2 cm.

Biometric parameters measurements can be seen in Figure 16. Regarding root length (Fig. 16A), plants exposed to M600 extract exhibited significantly shorter roots than the control and plants exposed to 1:10 M607 extract exhibited significantly longer roots than those of the control plants.

Length of leaves did not differ significantly between plants exposed to the extracts and the control plants, except for plants exposed 1:10 M607 extract, where longer leaves were also observed (Fig. 16B). Fresh weight of plants exposed to M600 and 1:10 M607 extracts increased significantly when compared to the control plants (Fig. 16C). Lastly, germination rate was steadily the same throughout the conditions tested (Fig. 16D).

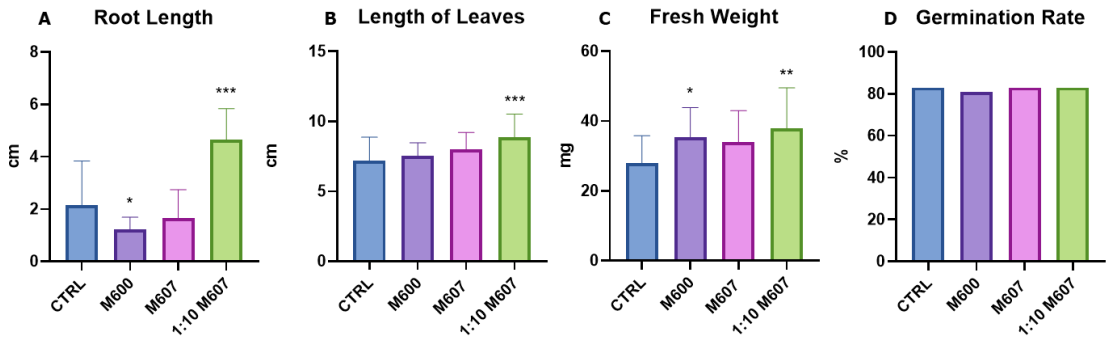

Figure 16 - Effect of Berg02_79 extracts on L. perenne plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with * ($p<0.05$), ** ($p<0.005$) and *** ($p<0.001$).

With these results we determined that no consistent inhibitory effects were observed. Although when *L. perenne* was exposed to M600 extract, root length was inhibited, there were no other significant inhibitions. In fact, 1:10 M607 extract favoured the growth of *L. perenne*.

In conclusion, no consistent inhibitory effects were observed when *A. thaliana, L. sativa* and *L. perenne* plants were exposed to Berg02_79 extracts. This is a clear indication that Berg02_79 does not have a bioherbicidal capacity against the model weed.

3.1.4. Effects of *Kocuria polaris* strain PMIC_1H7A

3.1.4.1. *Arabidopsis thaliana*, a weed model

Extracts produced from strain PMIC_1H7A, grown in M607 and 1:10 M607 culture media, were shown to greatly affect *A. thaliana* development. After three weeks of growth, exposure to these extracts resulted in inhibited root growth (Figs.17C and 17D) compared to the control plants (Fig. 17A). This inhibition led to plants having extremely short roots. In a similar way to Berg02_79 extracts, plants exposed to PMIC_1H7A M607 and 1:10 M607 extracts presented inhibition of root apical dominance and promotion of lateral root growth when compared to control plants, leading to plants with root systems resembling a fibrous root system. In contrast, M600 extracts did not induce similar effects, plants exposed to this extract present long and single roots, similar to the control plants (Figs. 17A and 17B, respectively).

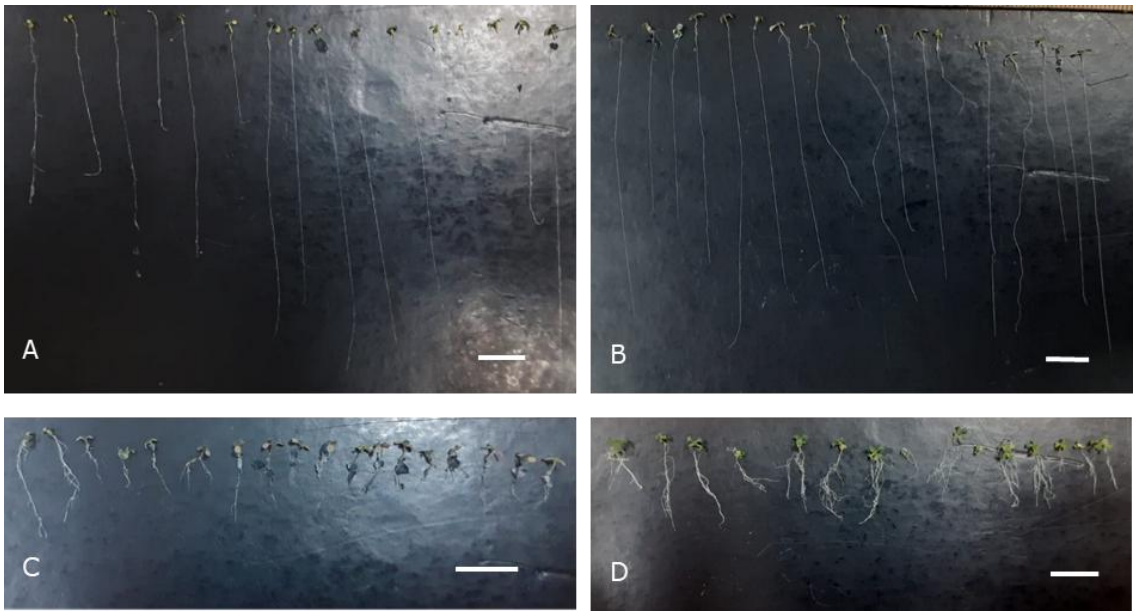

Figure 17 - Exposure of *A. thaliana* to PMIC_1H7A extracts after 3 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 1 cm.

The assessment of several biometric parameters confirmed the visual effects. Root length (Fig. 18A) was significantly decreased as a result of exposure to M607 and 1:10 M607 extracts, but it was significantly increased by M600 extracts. Concerning the fresh weight (Fig. 18B), no significant effect was observed, except for the extract 1:10 M607 which induced a significant increase. The number of leaves (Fig. 18C) was significantly affected by all the extracts: negatively by M600 and M607 extracts, but positively by 1:10 M607. Lastly, germination rate (Fig. 18D) was affected, decreasing greatly in 1:10 M607 and having a small decrease in M607.

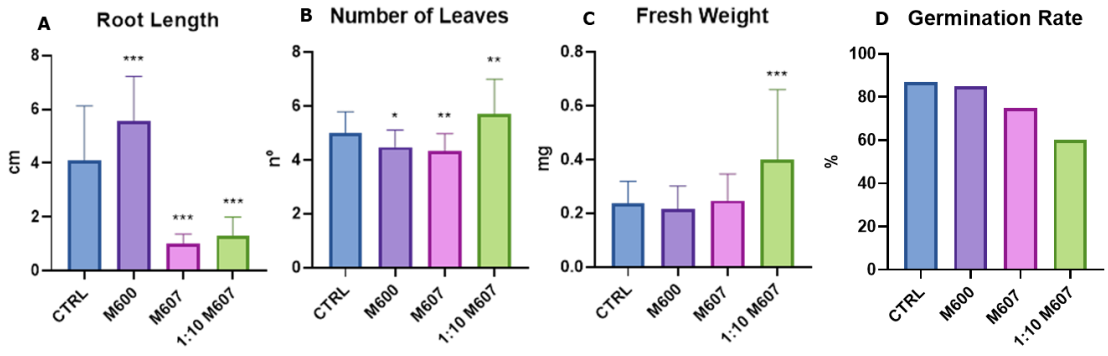

Figure 18 - Effect of PMIC_1H7A extracts on *A. thaliana* plant growth. Plants were grown for 3 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with * ($p<0.05$), ** ($p<0.005$) and *** ($p<0.001$).

These results indicate that PMIC_1H7A extracts do not exhibit a clear and consistent inhibitory effect on *A. thaliana*. While some parameters are negatively affected (i.e. root length and germination rate in M607 and 1:10 M607 extracts), others are stimulated (i.e. root length in M600 and number of leaves and fresh weight both in 1:10 M607). A similar effect of the M607 media extracts on root growth, inhibition of elongation and stimulation of root formation, growth of aerial part and total plant fresh weight, was observed in both Berg02_79 and PMIC_1H7A extracts in *A. thaliana*, particularly M600 in the first bacterium and 1:10 M607 in the second one.

3.1.4.2. *Lactuca sativa*, an important dicot crop

As seen in Figures 19C and D, plants exposed to M600 and 1:10 M607 extracts, presented longer roots and a more robust aerial part when compared to control and to M600 exposed plants, Figures 19A and 19B.

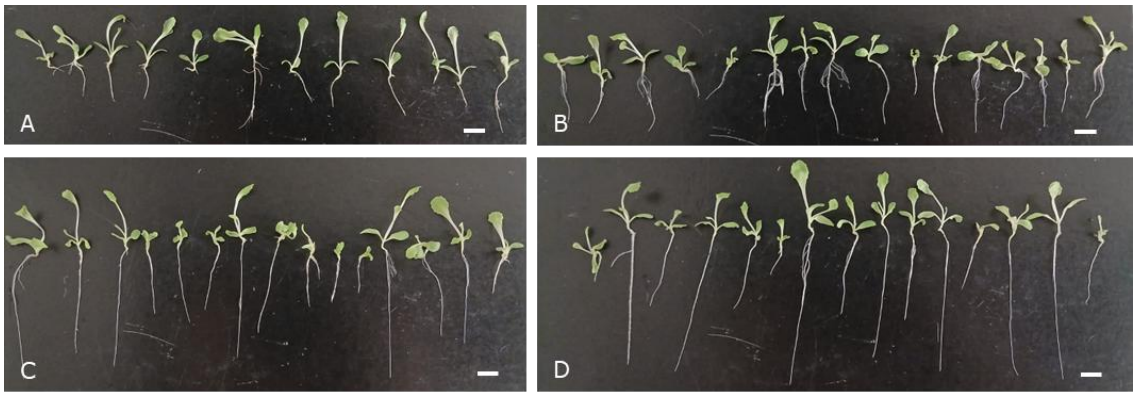

Figure 19 - Exposure of *L. sativa* to PMIC_1H7A extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 1 cm.

Confirming the visual observation, the quantification of biometric parameters in Figure 20, reveals root lengths (Fig. 20A) of plants exposed to the 3 extracts significantly longer when compared to the control plants, with the most significant difference being in plants exposed to M607 and 1:10 M607 extracts. The number of leaves (Fig. 20B) was only affected negatively in plants exposed to M600 extract. Regarding fresh weight (Fig. 20 C) compared to the control plants, statistically significant differences were only observed for plants exposed to M607 extract. Germination rate (Fig. 20D) increased in plants exposed to the extracts when compared to the control plants. Germination rate between M607 and 1:10 M607 extracts was relatively similar between them, whereas M600 extract presented the highest germination rate.

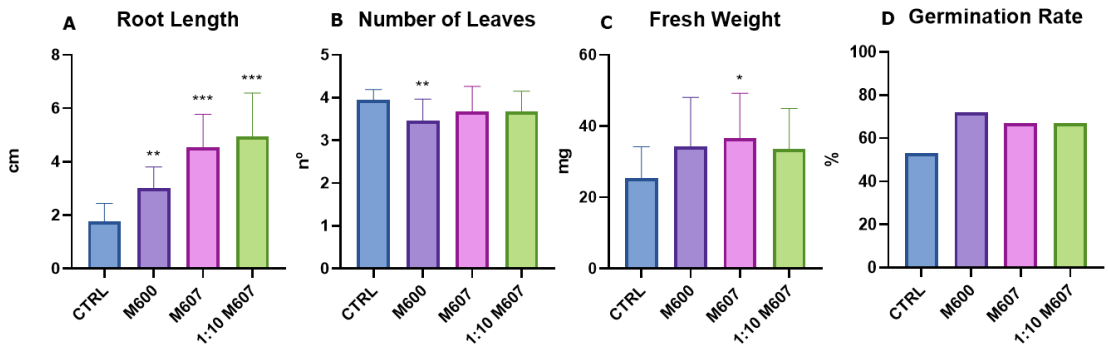

Figure 20 - Effect of PMIC_1H7A extracts on *L. sativa* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated with * ($p<0.05$), ** ($p<0.005$) and *** ($p<0.001$).

In summary, significant inhibition of *L. sativa* plants by exposure to PMIC_1H7A extracts was only observed in the number of leaves of plants exposed to M600 extract. Given

these results, we determined that PMIC_1H7A extracts do not present clear inhibition effects, but stimulate, in general, the root growth in *L. sativa*.

3.1.4.3. *Lolium perenne*, a monoct crop

After two weeks of growth, *L. perenne* plants exposed to PMIC_1H7A extracts presented notable morphological differences when compared to the control plants. As seen in Figure 21, plants exposed to M600, M607 and 1:10 M607 (Figs. 21B, 21C and 21D, respectively) had longer roots then control plants (Fig. 21 A).

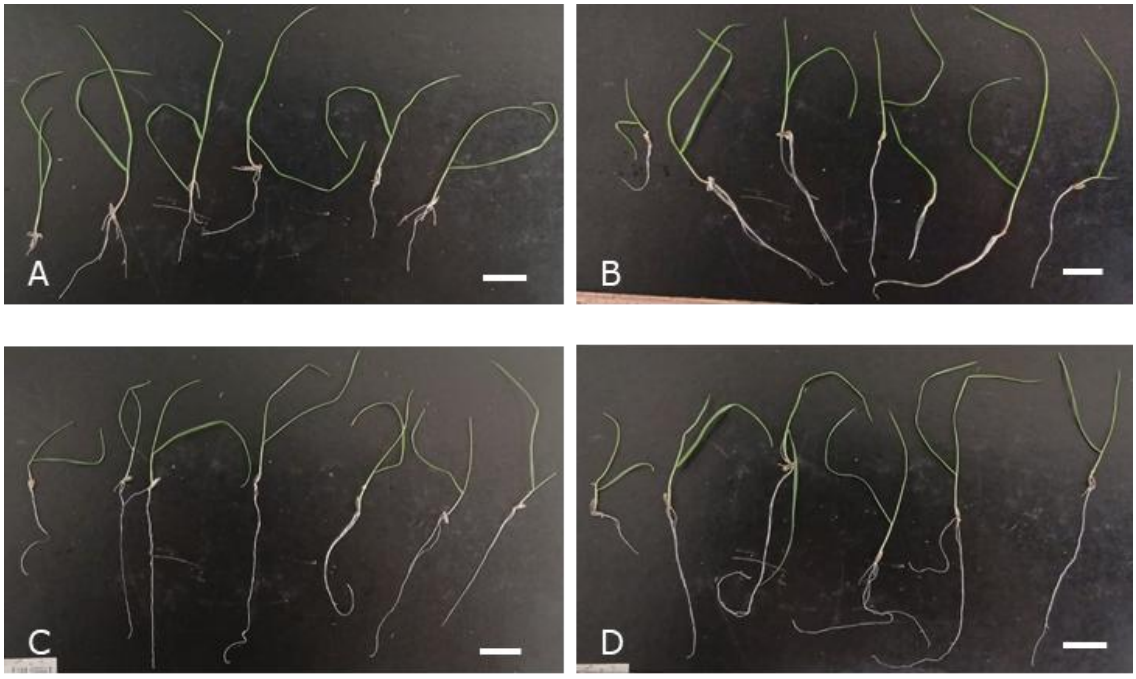

Figure 21 - Exposure of *L. perenne* to PMIC_1H7A extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 2 cm.

Regarding root length (Fig. 22A), all PMIC_1H7A extracts significantly increased root growth. Moreover, plants exposed to M:607 extracts presented the longest roots. On the other hand, the length of the plant's leaves (Fig. 22B) did not differ significantly between any of the tested conditions. As for the fresh weight (Fig. 22C), significant differences were not observed apart from plants exposed to M600 extract, which induced a significantly higher fresh weight. Lastly, germination rate (Fig. 22D) did not differ between plants exposed to the extracts.

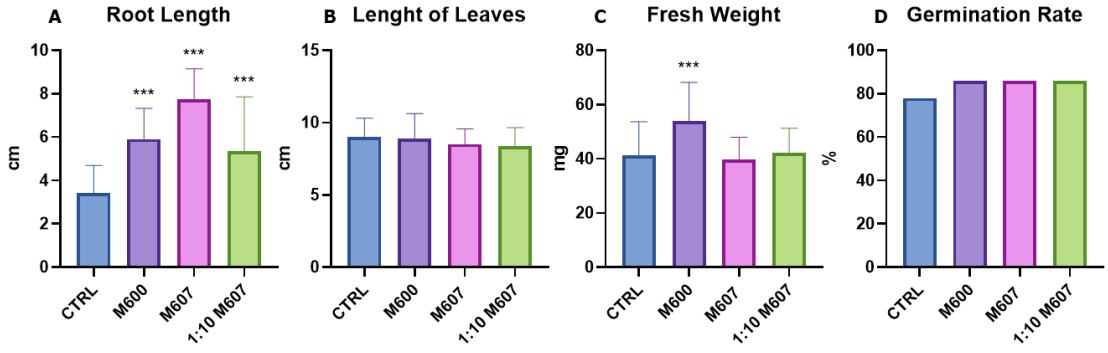

Figure 22 - Effect of PMIC_1H7A extracts on *L. perenne* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated *** ($p<0.001$).

Exposure of *L. perenne* to PMIC_1H7A did not show any inhibitory effects, on the contrary, a stimulatory effect was observed at the root level, similar to what was observed in *Lactuca*. An increase in fresh weight in plants exposed to M600 extract was also observed.

In conclusion, no inhibitory effect of the PMIC_1H7A extracts was observed in any of the model plants. Although *A. thaliana* plants when exposed to PMIC_1H7A presented a significant inhibition of root length and loss of apical dominance at root level, inhibitory effects were not consistent across all extracts tested and parameters measured.

3.1.5. Effects of *Rhodopirellula rubra* strain LF2

3.1.5.1. *Arabidopsis thaliana,* a model plant

Extracts from strain LF2 were tested on two batches of *A. thaliana* seeds. The repetition was carried out to evaluate the reproducibility of the initial results and to confirm the observations obtained in the first test with LF2 extracts.

Regarding the first *A. thaliana* assay, all extracts produced by LF2 resulted in a strong inhibition of plant development. This inhibition was significantly stronger on plants exposed to M607, and in 1:10 M607, with germination being completely inhibited. Extract M600 inhibited root length and increased the production of lateral roots (Figs. 23A and 23B).

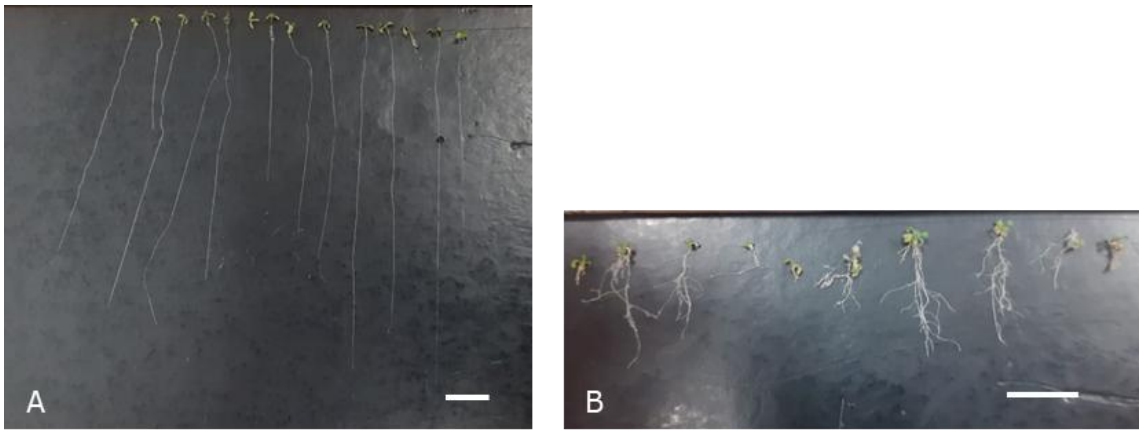

Figure 23- Exposure of the first batch of *A. thaliana* to LF2 extracts after 3 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 1 cm.

Since plants exposed to M607 and 1:10 M607 extracts did not germinate, the quantification of the biometric parameters was only shown for control plants and plants exposed to M600 (Fig. 24). Confirming the visual observation, root length (Fig. 24A) was significantly inhibited by the M600 extract. However, the number of leaves (Fig. 24B) significantly increased. Fresh weight (Fig. 24C) was not affected by the extract. Germination rate (Fig. 24D) was strongly affected by the M600 extract; as such, control plants had a germination rate above 60%, and plants exposed to M600 extract had a germination rate below 20%.

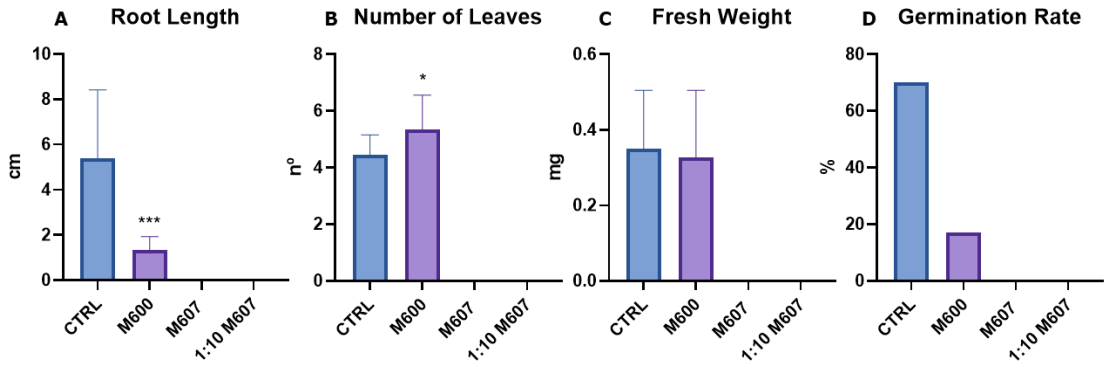

Figure 24 - Effect of LF2 extracts on the first batch of *A. thaliana* plant growth. Plants were grown for 3 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated * ($p<0.05$).

In the second assay, as shown in Figures 25B, 25C, and 25D, the roots of *A. thaliana* plants exposed to LF2 M600, M607, and 1:10 M607 extracts, respectively, are noticeably shorter than those of the control plants depicted in Figure 25A.

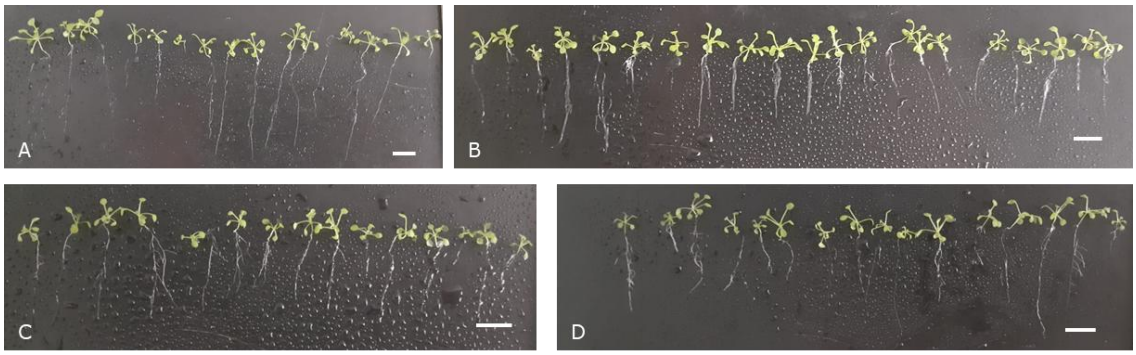

Figure 25 - Exposure of the second batch of *A. thaliana* to LF2 extracts after 3 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607 extract. The scale represents 1 cm.

As seen in Figure 26A, confirming the visual results, root length of the plants exposed to the three LF2 extracts was significantly decreased compared to control plants. These differences were particularly prevalent in plants exposed to 1:10 M607 extract, which had the shortest root length. The number of leaves (Fig. 26B) and the fresh weight (Fig. 26C) of plants exposed to the LF2 extracts significantly decreased when compared to the control plants. Also, the germination rate (Fig. 26D) decreased for plants exposed to M607 and 1:10 M607 confirming the result previously observed with the first test. However, germination rate was 100% for plants exposed to M600 extract, an increase not in line with the result obtained in the first batch. This discrepancy should be further investigated with confirmation tests.

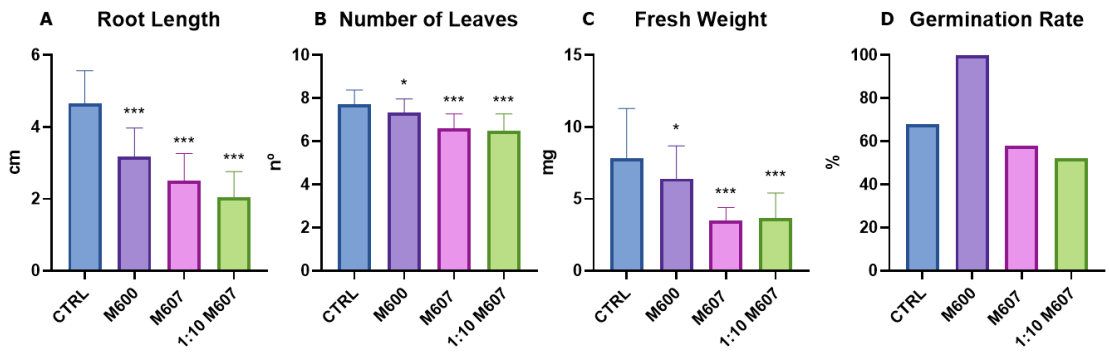

Figure 26 - Effect of LF2 extracts on the second batch of *A. thaliana* plant growth. Plants were grown for 3 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of > X biological replicates. Significant differences compared to the control (CTRL) were indicated * ($p<0.05$) and *** ($p<0.001$).

Although the inhibitory effects of the M607 and 1:10 M607 extracts were not as strong as in the first batch, a clear and negative effect was also observed for all the biometric parameters under study. These results are indicative of a potential herbicidal effect of *R. rubra* strain LF2 extracts on *A. thaliana* plants.

3.1.5.2. *Lactuca sativa*, an important crop

Plants of *L. sativa* exposed to LF2 extracts did not show apparent differences relative to the control plants. As seen in Figure 27, plants of all treatments (Figs. 27B, 25C and 25D) are morphologically similar to the control plants (Fig. 27A). However, it was observed that plants exposed to M607 and 1:10 M607 extracts have more root ramifications compared to the control plants, indicating a stimulation of lateral root growth by the extracts.

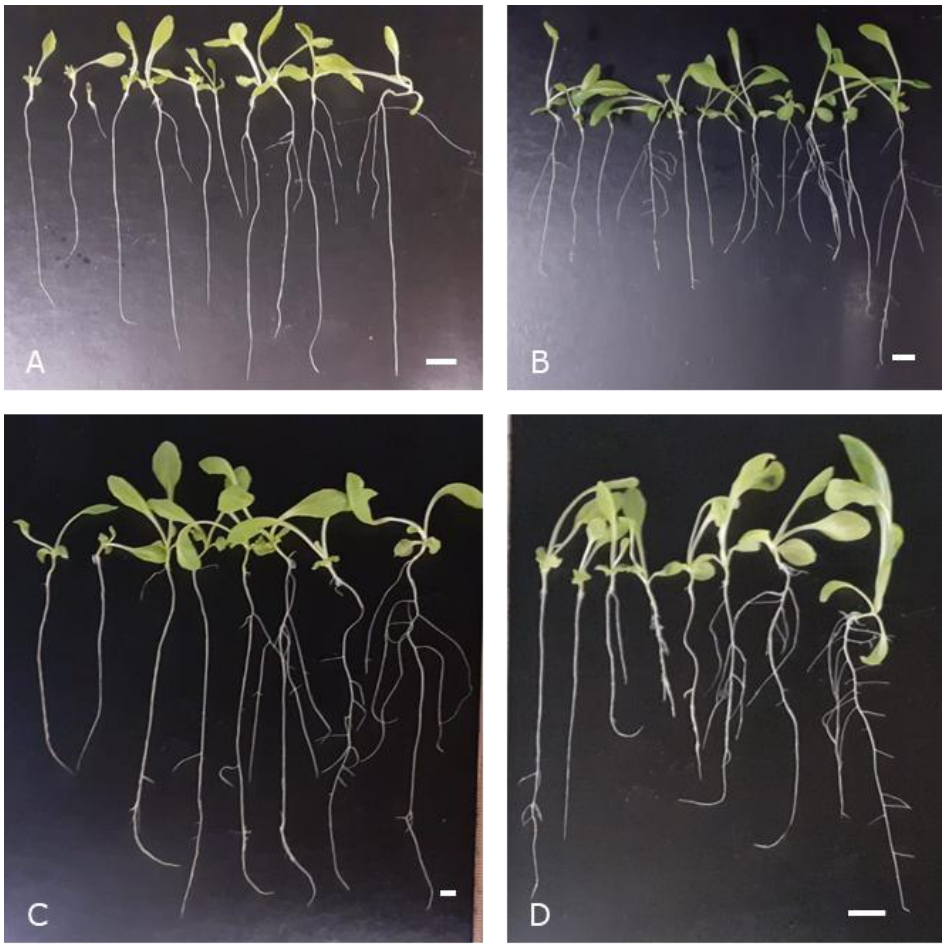

Figure 27 - Exposure of the *L. sativa* to LF2 extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607 extract. The scale represents 1 cm.

The morphological similarities between the exposed plants and the control plants were confirmed by the statistical analysis of the biometrical parameters as seen in Figure 28.

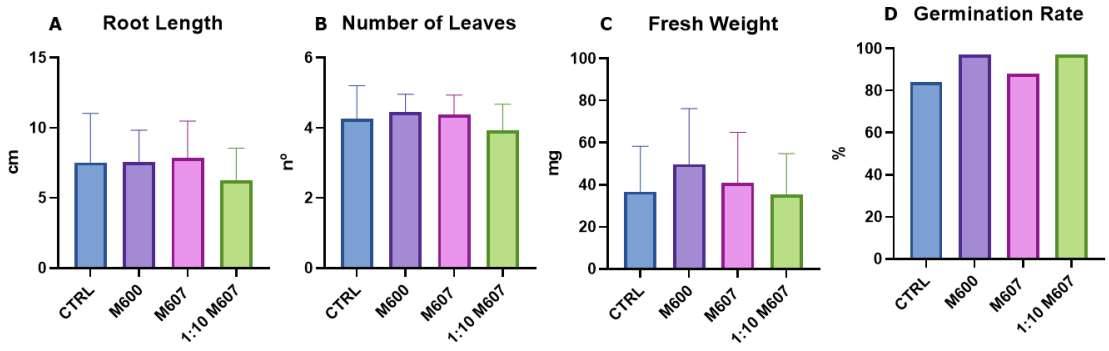

Figure 28 - Effect of LF2 extracts on *L. sativa* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), number of leaves(B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were not observed.

Given the results of the tests in *L. sativa,* we concluded that none of the LF2 extracts significantly affects growth of the plant, contrary to what was observed for Arabidopsis.

3.1.5.3. *Lolium perenne,* an important monocot crop

Plants of *L. perenne* exposed to LF2 extracts are shown in Figure 29. As can be seen, plants exposed to all the extracts (Figs. 29B, 29C and 29D) are morphologically similar to the control plants (Fig. 29A).

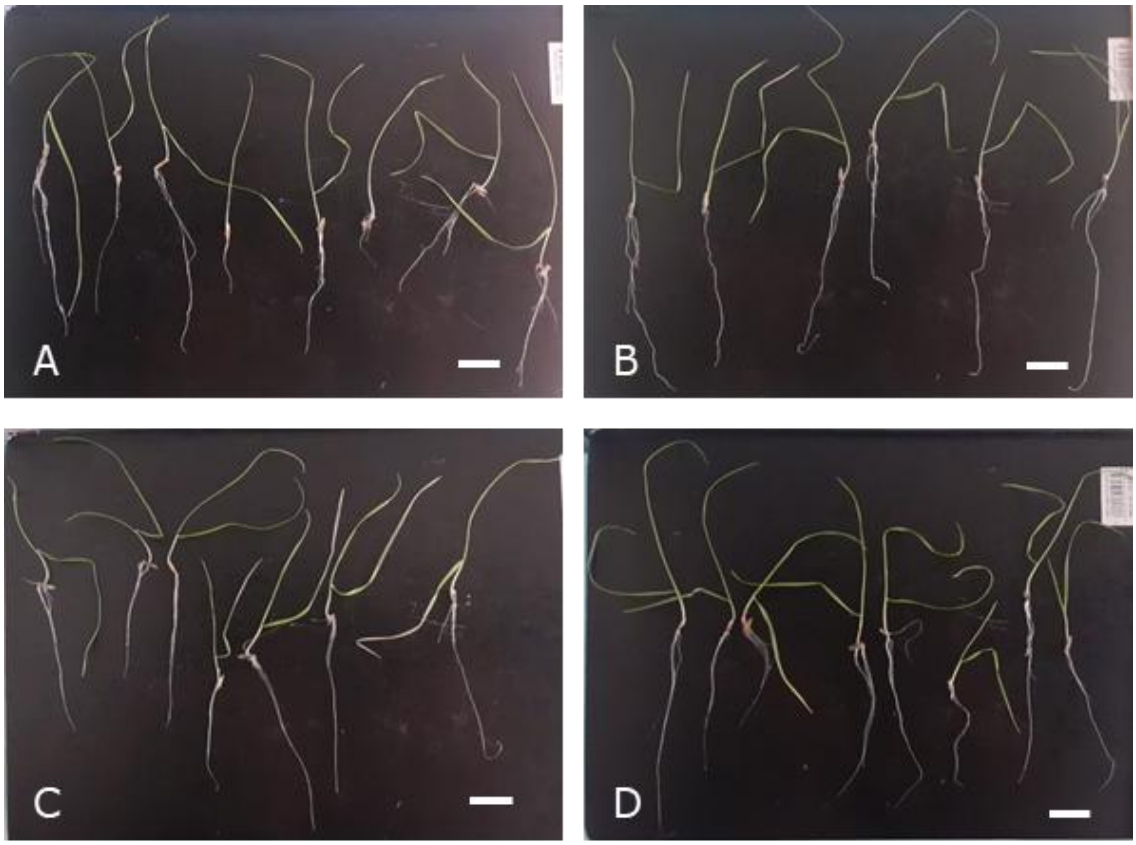

Figure 29 - Exposure of the L. perenne to LF2 extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 2 cm.

By analyzing the root length, length of leaves and germination rate (Figs. 30A, 30B and 30D, respectively) no significant differences between plants exposed to the extracts and the control plants were observed. However, significant differences were observed in the fresh weight of plants exposed to the 1:10 M607 extract, as seen in Figure 30D.

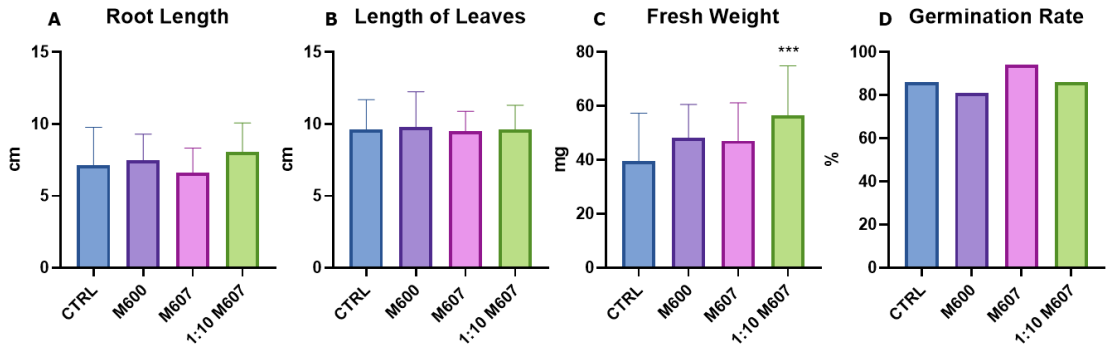

Figure 30 - Effect of LF2 extracts on *L. perenne* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented with 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), length of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were indicated *** ($p<0.001$).

Similar to what was observed in *L. sativa*, no significant inhibitory effects were observed with *L. perenne* exposed to LF2 extracts.

3.1.5.4. *Lolium multiflorum*, a monocot crop

Plants of *L. multiflorum* tested with LF2 extracts are shown in Figure 31. No relevant morphological differences were observed between plants exposed to the extracts (Figs. 31B, 31C and 31D) and the control plants (Fig. 31A).

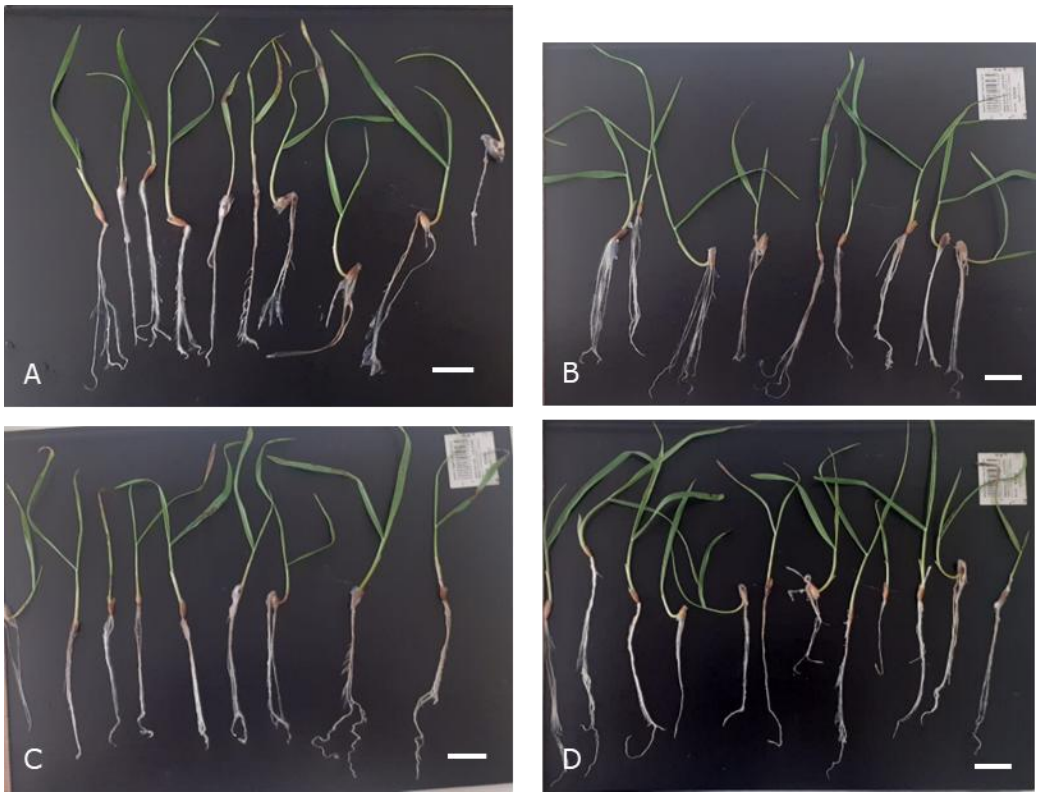

Figure 31 - Exposure of the *L. multiflorum* to LF2 extracts after 2 weeks of plant growth. A - control plants (2% DMSO). B - plants tested with M600 extract. C - plants tested with M607 extract. D - plants tested with 1:10 M607. The scale represents 2 cm.

Confirming the visual results, no significant differences were observed in three of the biometrical parameters measured (root length, length of leaves and fresh weight) for any of the extracts tested (Figs. 32A, 32B and 32C, respectively), with the exception of the germination rate that decreased manly in plants exposed to M600 and M607 extracts (Fig. 32D).

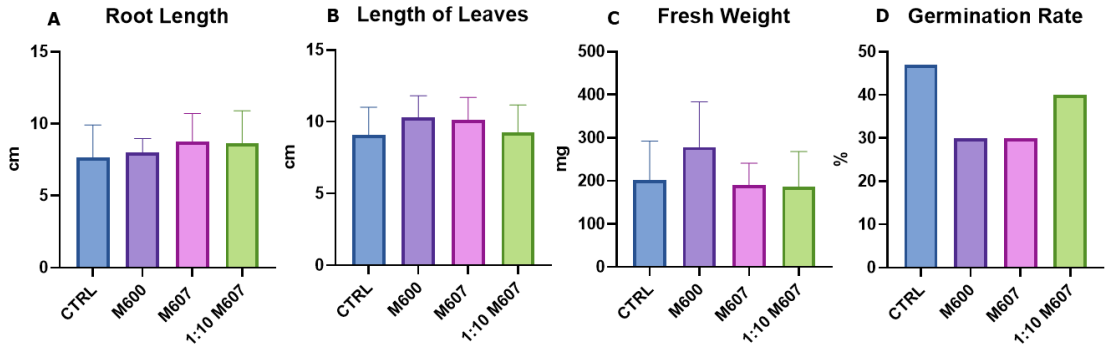

Figure 32 - Effect of LF2 extracts on *L. multiflorum* plant growth. Plants were grown for 2 weeks in Petri dishes with Hoagland agar medium supplemented exposed to 2% DMSO (CTRL), M600, M607 and 1:10 M607 extracts. Root length (A), lenght of leaves (B), fresh weight (C) and germination rate (D) were assessed. Results are expressed as mean ± SD of 20-30 biological replicates. Significant differences compared to the control (CTRL) were not observed.

Results observed in the test with *L. multiflorum,* as for *L. perenne,* show that morphological parameters of the monocots tested are not affected by any extracts.

In summary, *R. rubra* LF2 extracts showed a selective inhibitory activity against *A. thaliana*. This inhibitory activity is consistent throughout the exposure of the plants to the different extracts. On the other hand, no significant inhibitory activity against the other plants tested (*L. sativa* and *Lolium* spp.). Given these results, LF2 extracts show potential for selective herbicide activity.

In the following Tables (Tables 1, 2 and 3) all the results obtained from the assays performed with the extracts of the three bacteria, *Microbacterium oxydans* Berg02_79, *Kocuria polaris* PMIC_1H7A and *Rhodopirellula rubra* LF2 are summarized.

Table 1 - Summary of the results obtained for the tests of Microbacterium oxydans Berg02_79 in all three model plants. These results are presented by the significant increase (↑), significant decrease (↓) or no significant difference (=) in each of the parameters of each plant exposed to each extract, when compared to the respective control conditions.

Berg02_79 *Microbacterium oxydans*		Root Length	Leaf Growth	Fresh Weight	Germination Rate
A. thaliana	M600	↓	=	=	↓
	M607	↓	↑	↑	↓
	1:10 M607	↓	=	=	↓
L. sativa	M600	=	=	=	=
	M607	↓	↓	=	=
	1:10 M607	=	=	=	=
L. perenne	M600	↓	=	↑	=
	M607	=	=	=	=
	1:10 M607	↑	↑	↑	=

Table 2 - Summary of the results obtained for the tests of *Kocuria polaris* PMIC_1H7A in all three model plants. These results are presented by the significant increase (↑), significant decrease (↓) or no significant difference (=) in each of the parameters of each plant exposed to each extract, when compared to the respective control conditions.

PMIC_1H7A *Kocuria polaris*		Root Length	Leaf Growth	Fresh Weight	Germination Rate
A. thaliana	M600	↑	↓	=	=
	M607	↓	↓	↑	↓
	1:10 M607	↓	↑	↑	↓
L. sativa	M600	↑	↓	=	↑
	M607	↑	=	↑	↑
	1:10 M607	↑	=	=	↑
L. perenne	M600	↑	↑	=	=
	M607	↑	=	=	=
	1:10 M607	↑	=	=	=

Table 3 - Summary of the results obtained for the tests of *Rhodopirellula rubra* LF2 in all three model plants. These results are presented by the significant increase (↑), significant decrease (↓) or no significant difference (=) in each of the parameters of each plant exposed to each extract, when compared to the respective control conditions. In addition, complete seed germination inhibition was indicated with (-).

LF2 *Rhodopirellula rubra*		Root Length	Leaf Growth	Fresh Weight	Germination Rate
A. thaliana 1	M600	↓	↑	=	↓
	M607	-	-	-	↓
	1:10 M607	-	-	-	↓
A. thaliana 2	M600	↓	↓	↓	↑
	M607	↓	↓	↓	↓
	1:10 M607	↓	↓	↓	↓
L. sativa	M600	=	=	=	↑
	M607	=	=	=	=
	1:10 M607	=	=	=	↑
L. perenne	M600	=	=	=	=
	M607	=	=	=	=
	1:10 M607	=	=	↑	=
L. multiflorum	M600	=	=	=	↓
	M607	=	=	=	↓
	1:10 M607	=	=	=	↓

Considering all these results, the only bacterium from which extracts were produced that presented a consistent inhibitory and selective effect was *R. rubra* LF2. As evidenced in Table 3, extracts from this bacterium consistently inhibited the growth of *A. thaliana* and did not affect the growth of the other model species. In contrast, the extracts of Berg02_79 and PMIC_1HA7 (Tables 1 and 2) did not consistently inhibit the various biometric parameters in *A. thaliana*, not demonstrating a clear herbicidal effect.

Based on the results obtained, M607 extracts were the ones inducing most of the significant differences observed. Therefore, this culture medium was chosen to grow the bacteria to obtain the extracts that were later used to spray the plants in the Plant Growth Pot assays.

3.2. Plant Growth Pot Assays

Seeds of the three model plants were germinated in pots with perlite and vermiculite, and the seedlings were sprayed 2 times a week with the respective M607 extract of each bacterium until some plant death started to be observed. After which the tests were terminated and the aerial parts processed for biochemical analysis. Visual results observed for *L. sativa*, *L perenne* and *A. thaliana* plants can be seen in Fig. 33, Fig. 34 and Fig. 35, respectively. Plant death was only observed in *A. thaliana* plants after the 4^{th} spray. Plants presented yellow leaves (Fig. 35B, C and D), indicative of leaf senescence, with the most intense effects observed when exposed to Ber02_79 M607 extract. (Pyung et al., 2007). *L. sativa* (Fig. 34b and C) and *L. perenne*, and (Fig 34B, C and D) treated with the extracts did not show any notable visual difference when compared to the respective control plants.

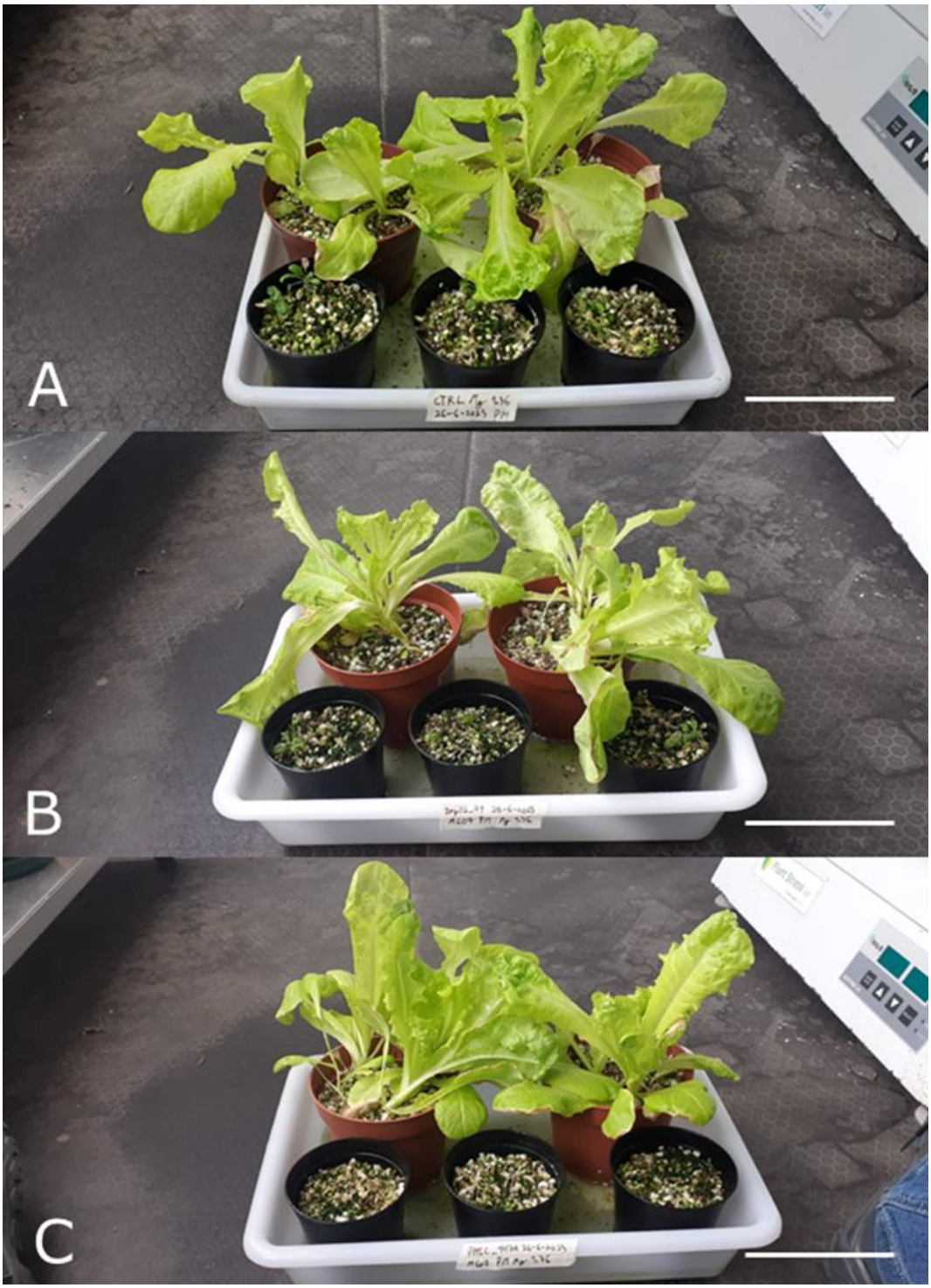

Figure 33 - *L. sativa* plants after 2 weeks of assay where no noticeable cues of stress can be observed in plants treated with the extracts. A) CTRL plants treated with 8% DMSO. B) plants treated with Berg02_79 M607 extract. C) plants treated with PMIC_1H7A M607 extract. The scale represents 10 cm.

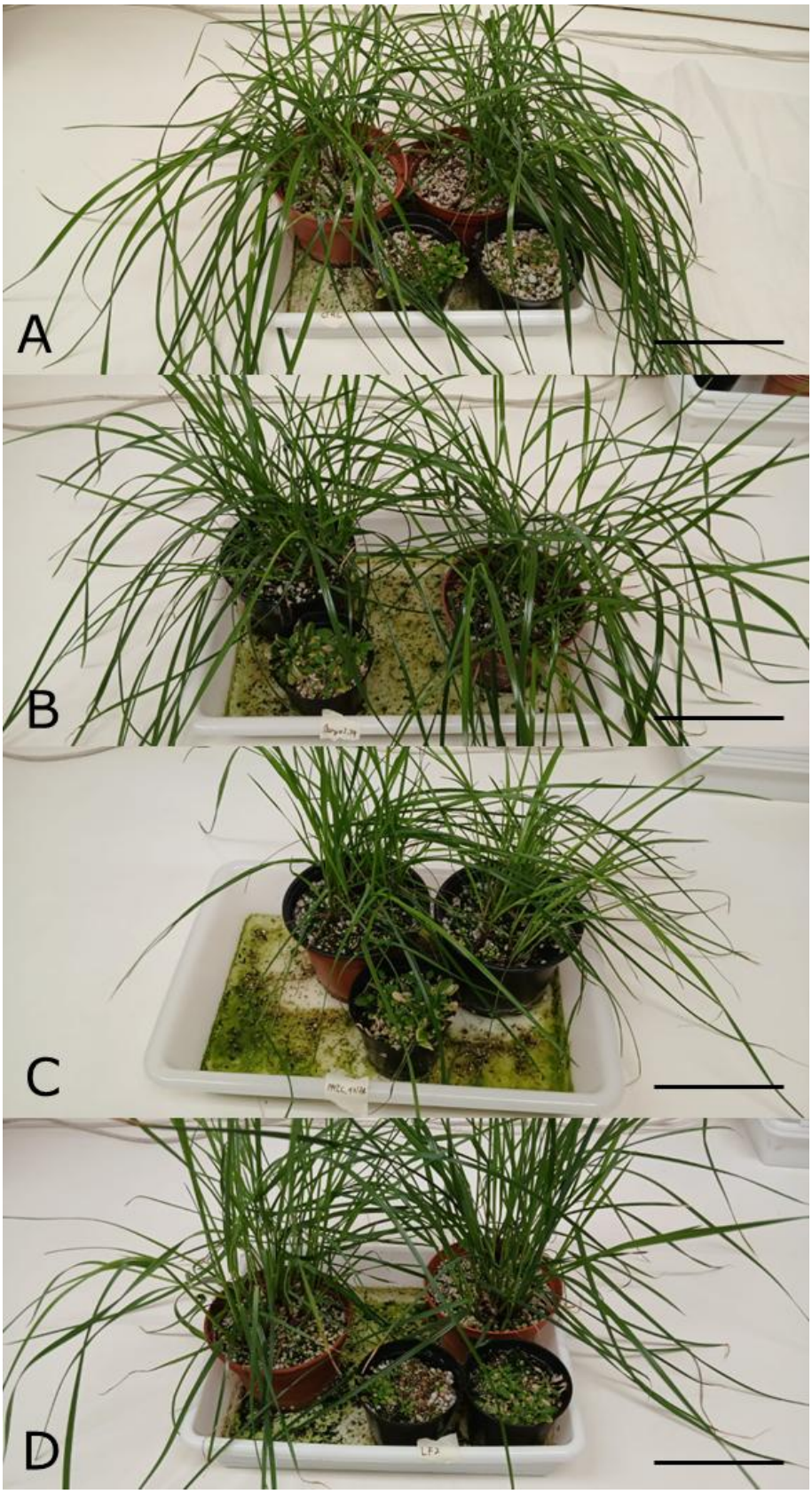

Figure 34 - *L. perenne* plants after 2 weeks of assay where no noticeable cues of stress can be observed in plants treated with the extracts. A) CTRL plants treated with 8% DMSO. B) plants treated with Berg02_79 M607 extract. C) plants treated with PMIC_1H7A M607 extract. D) plants treated with LF2 M607 extract. The scale represents 10 cm.

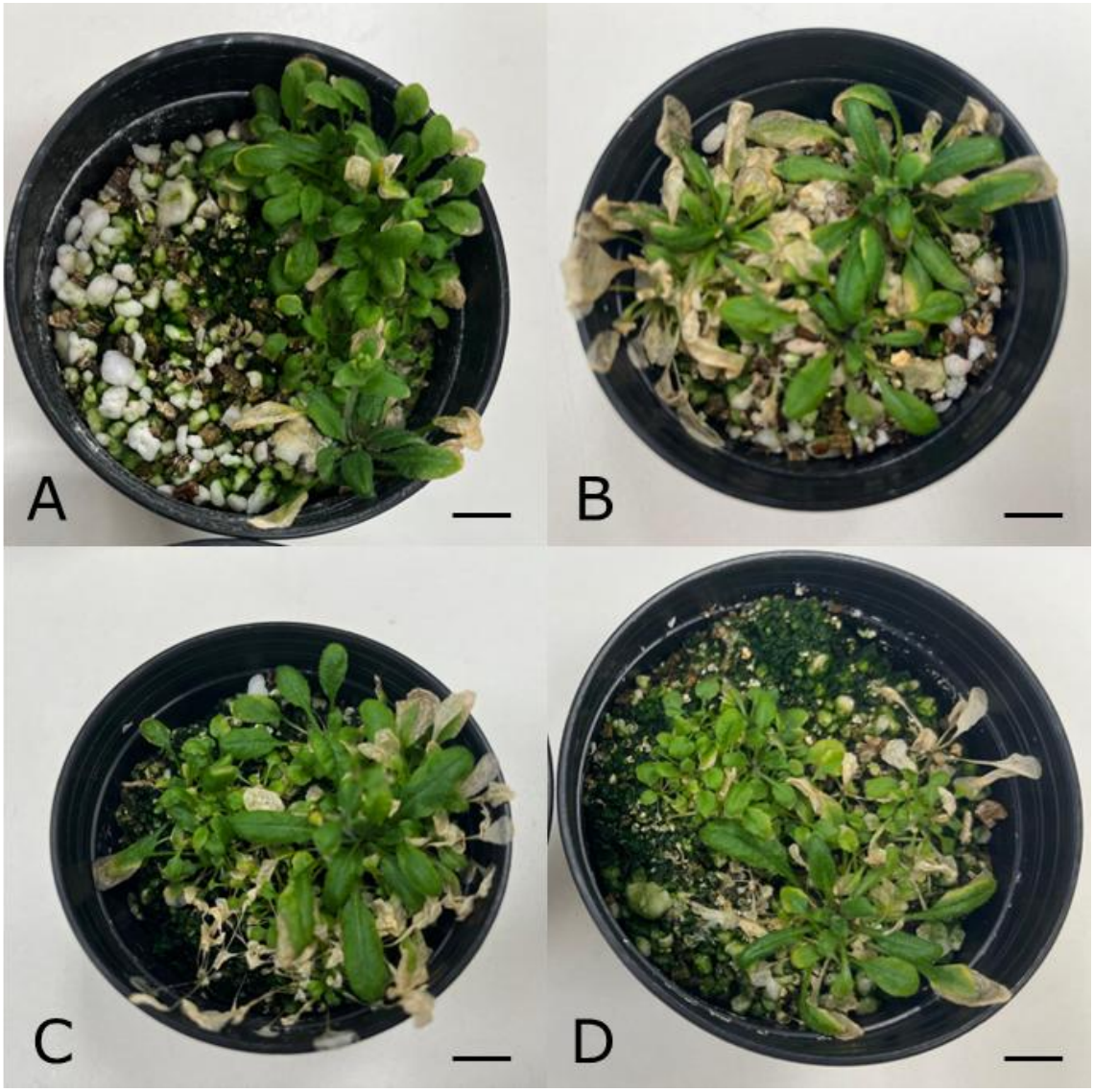

Figure 35- *A. thaliana* plants after 2 weeks of assay where noticeable cues of stress can be observed in plants treated with the extracts. A) CTRL plants treated with 8% DMSO. B) plants treated with Berg02_79 M607 extract. C) plants treated with PMIC_1H7A M607 extract. D) plants treated with LF2 M607 extract. The scale represents 1 cm.

3.2.1. Proline

Proline is an amino acid present in plants and is commonly quantified as an indicator of biotic and abiotic stresses such as drought, salinity, pathogen infection and temperature stress, just to name a few (Verbruggen & Hermans, 2008).

No significant differences were observed between plants exposed to the extracts and their respective controls. However, a slight decrease in proline levels was detected in *A. thaliana* and *L. sativa* treated with the PMIC_1H7A extract (Figs. 36A and B), as well as in *L. perenne* exposed to the Berg02_79 extract (Fig. 36C). Conversely, a slight increase

in proline content was observed in *L. sativa* (Fig. 36B) and *L. perenne* (Fig. 36C) treated with the Berg02_79 and LF2 extracts, respectively.

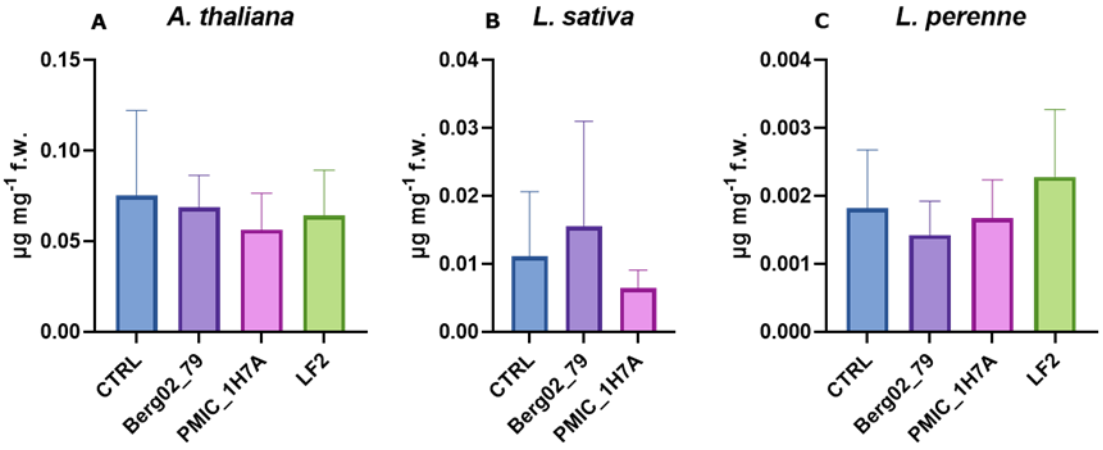

Figure 36 - Proline contents in plants exposed to the bacterial extracts. A – *A. thaliana* plants, B-*L. sativa* - C - *L. perenne* Results are expressed as mean ± SD of 3 biological replicates.

However, looking at the result only with proline quantifications is not enough to determine the oxidative stress of the plant, as such GSH and H_2O_2 contents were quantified.

3.2.2. Glutathione

Reduced glutathione is a major molecule that alleviates oxidative stress from the plant, thus its quantification and in combination with proline and H_2O_2 helps understand the levels of oxidative stress felt by the plant (Couto & Zipfel, 2016; Khan et al., 2020; Liedschulte et al., 2010).

A significant increase in GSH levels was observed in *L. perenne* exposed to Berg02_79 and PMIC_1H7A (Fig. 37A). This increase indicates activation of the antioxidant defense system to mitigate oxidative stress. However, no visual symptoms of stress were observed in the plants, suggesting that the oxidative stress was efficiently controlled, preventing visible damage. In contrast, no significant differences in GSH levels were detected in *L. sativa* or *L. perenne* for any of the extracts (Figs. 37B and C), although a slight decrease in GSH content was noted in plants of both species exposed to Berg02_79 extracts. Finally, in *A. thaliana*, an increase, although not significant, was

detected after treatment with LF2, probably representing an attempt to resist to the bioherbicide effect of this extract.

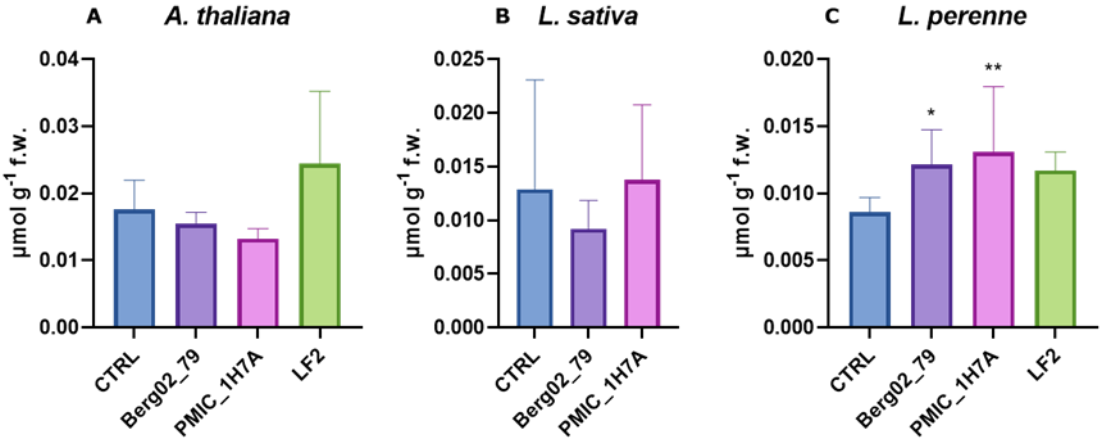

Figure 37 - GSH contents in plants exposed to the bacterial extracts. A – *L. sativa* plants, B - *A. thaliana*. C - *L. perenne* Results are expressed as mean ± SD of 3 biological replicates.

3.2.3. Lipid peroxidation

Lipid peroxidation is an indicator of the cellular damage caused by reactive oxygen species (ROS). This indicator is measured by the quantification of MDA content and is important to determine the damage in the cellular membrane caused by ROS (Pospíšil & Yamamoto, 2017).

In contrast to the patterns observed for proline and GSH, MDA levels in *A. thaliana* significantly increased in response to all extracts (Fig. 38A). This increase is a clear indicator of lipid peroxidation, reflecting membrane damage in the cells, which can ultimately lead to cell death and tissue damage. These results are consistent with the visual observations of plant yellowing and death, particularly in plants treated with LF2 extracts. In *L. sativa*, a significant increase in MDA content was observed following exposure to PMIC_1H7A (Fig. 38B), suggesting membrane damage. However, this response was not accompanied by visible symptoms in the plants. Finally, and in contrast to the other species, MDA levels in *L. perenne* significantly decreased (Fig. 38C). Based on the previous results, we hypothesize that the observed decrease in MDA reflects an oxidative stress response in which the plant activated defence mechanisms, leading to increased GSH levels and subsequent repair of cellular damage. This repair would

explain the reduced MDA content, indicate restored membrane stability and confirm the absence of visible oxidative stress symptoms such as leaf yellowing or plant death.

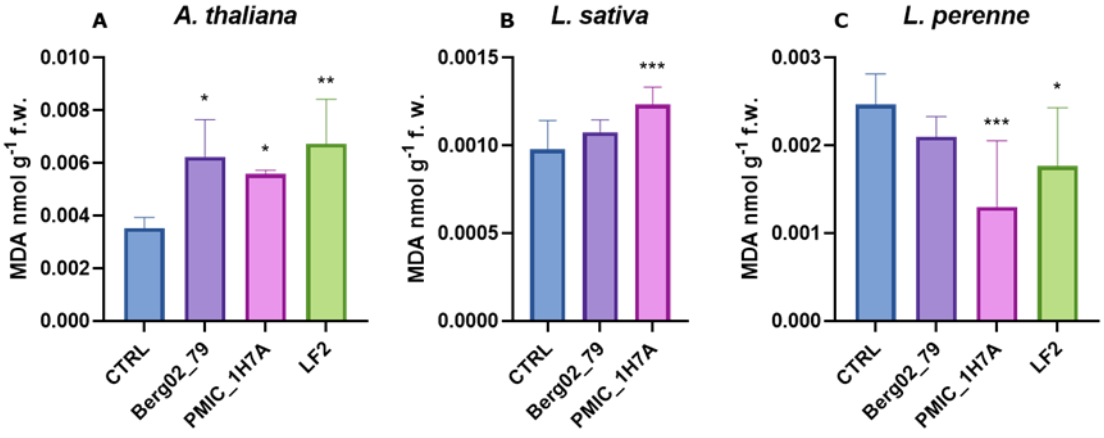

Figure 38 - MDA contents in plants exposed to the bacterial extracts. A – *L. sativa* plants, B - *A. thaliana*. C - *L. perenne* Results are expressed as mean ± SD of 3 biological replicates.

3.2.4. H_2O_2

The quantification of H_2O_2 provides information not only on oxidative stress, since it is a reactive oxygen species (ROS), but also on its role as a signalling molecule in plant cellular metabolism, where controlled levels of H_2O_2 are typically associated with the activation of defense and adaptation mechanisms (Černý et al., 2018).

In *A. thaliana*, H_2O_2 levels significantly increased in response to all extracts (Fig. 39A). This rise in H_2O_2 content strongly supports the results observed for MDA levels and the visual symptoms, confirming that *A. thaliana* experienced severe oxidative damage, which led to cellular injury and ultimately plant death. Since plant death occurred only in *A. thaliana*, these biochemical results provide insight into the plate tests, where this species was selectively targeted by some of the extracts.

In *L. sativa*, no significant differences in H_2O_2 levels were detected, which is consistent with the lack of visible stress symptoms. In *L. perenne*, exposure to PMIC_1H7A extracts caused a significant increase in H_2O_2 levels, while no other significant changes were observed.

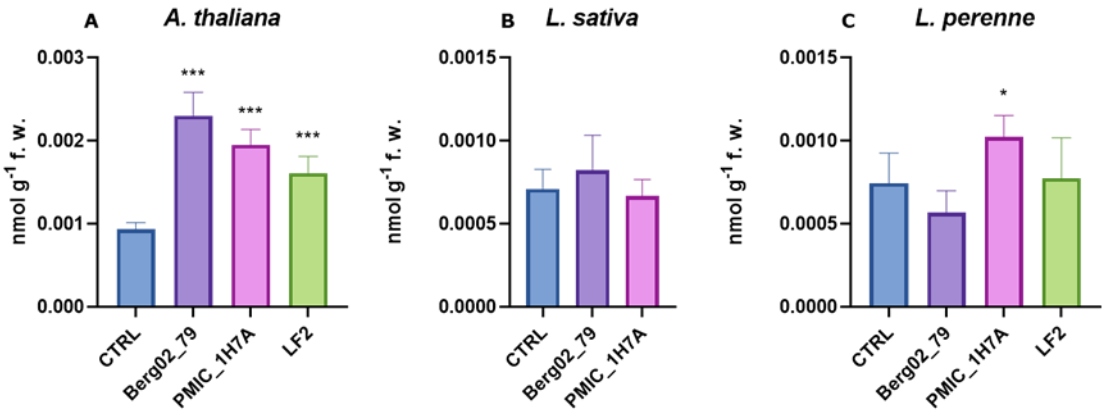

Figure 39 – H_2O_2 contents in plants exposed to the bacterial extracts. A – *L. sativa* plants, B - *A. thaliana*. C - *L. perenne* Results are expressed as mean ± SD of 3 biological replicates.

In summary, the biochemical analyses provided valuable insight into the stress experienced by plants exposed to the bacterial extracts. Considering the combined results from the pot and plate assays, it is evident that *A. thaliana* was specifically targeted by the compounds produced, particularly by *R. rubra* LF2^T extracts, where the consistent inhibitory effects were confirmed through biochemical tests. This selectivity became even clearer when examining the results as a whole: *A. thaliana* was not only the most affected species but also displayed a rapid onset of inhibition following extract application in the pot assays. Unlike *L. perenne*, which appeared able to mount a stress response, *A. thaliana* was unable to do so effectively, ultimately leading to plant death.

3.3. Indole-3-Acetic Acid

Root growth is mainly controlled by IAA and gibberellins. Leaf growth is mainly controlled by IAA, gibberellins and abscisic acid (“Plant Growth Hormones: Growth Promoters and Inhibitors,” 2001; Tanimoto, 2005). And given that some of the most used herbicides in the world (i.e. 2,4-D) are analogous to IAA, production of this plant hormone by the bacteria used in this study was evaluated. This production tests also served to possibly find a correlation between the results obtained in the plant exposure tests and the possible IAA production.

However, the method used for IAA quantification has its limitations, as described by (Guardado-Fierros et al., 2024) using Salkowski reagent to quantify IAA does not provide an exact quantity of the amount produced, it provides an inflated measurement due to other compounds also being detected. Given this, it is important to note that the values of IAA quantities obtained in this study do not reflect the real quantity produced by the

bacteria but rather provide a way to compare qualitatively the production between culture media used, time periods and presence or absence of Trp.

3.3.1. *Microbacterium oxydans* strain Berg02_79

Strain Berg02_79 was evaluated relatively to the production of IAA in the presence and absence of a precursor of the vegetal hormone, the amino acid tryptophan (Trp). As seen in Figure 37, this bacterium could produce IAA in both the presence and absence of the precursor. Production was higher when the bacterium grew in M600 culture medium and in the presence of Trp (Figure 40A), however, in the absence of Trp the production was higher after 24h of growth (Figure 40B).

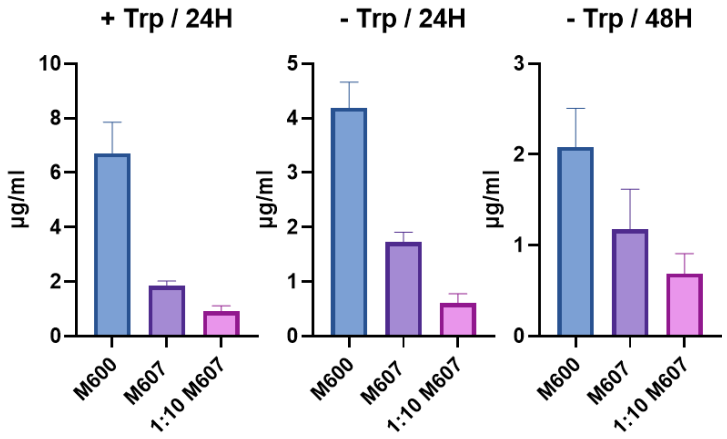

Figure 40 - Production of IAA by the strain Berg02_79 grown in M600, M607 and 1:10 M607 culture media in both presence and absence of tryptophan (Trp). A - IAA production in presence of Trp at 24h, B - IAA production in absence of Trp at 24h and C - IAA production in absence of Trp at 48h. IAA content is expressed in µg/ml.

When comparing IAA production by the bacterium with the results from the plant assays, no correlation could be established. Growth promotion and inhibition did not follow the same progression as IAA production but instead appeared more dispersed across the tested extracts. Considering the differences between the patterns observed in the plant tests and the IAA production profile of the bacterium, along with the apparent selectivity toward *A. thaliana*, it became evident that these effects could not be explained by IAA alone and are likely due to the presence of other compounds.

3.3.2. *Kocuria polaris* strain PMIC_1H7A

Strain PMIC_1H7A was evaluated relatively to the production of IAA in the presence and absence of Trp. The strain grew for 24H and for 48H in the absence of Trp. As seen in Figure 41, production of IAA was higher in the presence of the precursor (Figure 41A). Not only that but, production was higher when the bacterium grew in M607 culture medium in the present of Trp. On the other hand, production was similar in both times

with the highest production in both being when the bacteria were grown in M600 medium (Figures 41B and 41C).

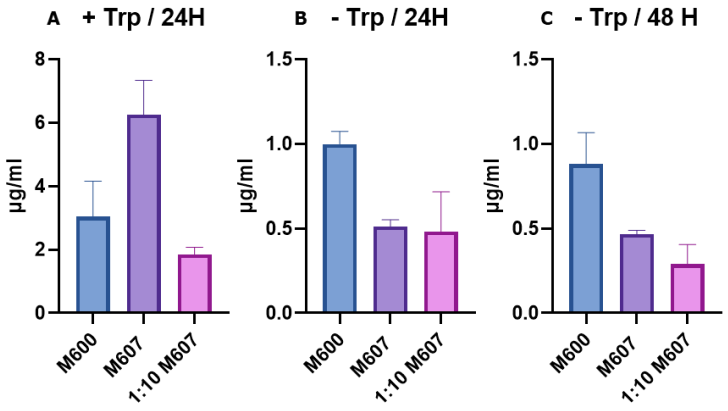

Figure 41 - Production of IAA by the strain PMIC_AH7A grown in M600, M607 and 1:10 M607 culture media in both presence and absence of tryptophan (Trp). A - IAA production in presence of Trp at 24h, B - IAA production in absence of Trp at 24h and C - IAA productio in absence of Trp at 48h. IAA content is expressed in µg/ml.

Based on the pattern of IAA production by this bacterial strain in the absence of tryptophan and the results observed in the plant assays, no clear correlation could be established between the two. This indicates that the inhibitory or stimulatory effects observed in the tested plants cannot be explained by IAA production from PMIC_1H7A alone, suggesting that other compounds may be responsible for the observed effects.

3.3.3. *Rhodopirellula rubra* strain LF2

LF2 grew 48 hours in the presence of tryptophan, a precursor of IAA, and 48 and 72 hours in the absence of tryptophan in M600, M607, and 1:10 M607 culture media. IAA production by LF2 in the presence of tryptophan followed a pattern similar to that observed for PMIC_1H7A, with the highest levels detected in cultures grown in M607 medium. Unfortunately, during this quantification experiment, LF2 cultures grown in M600 medium without tryptophan became contaminated, and no results could be obtained for that condition.

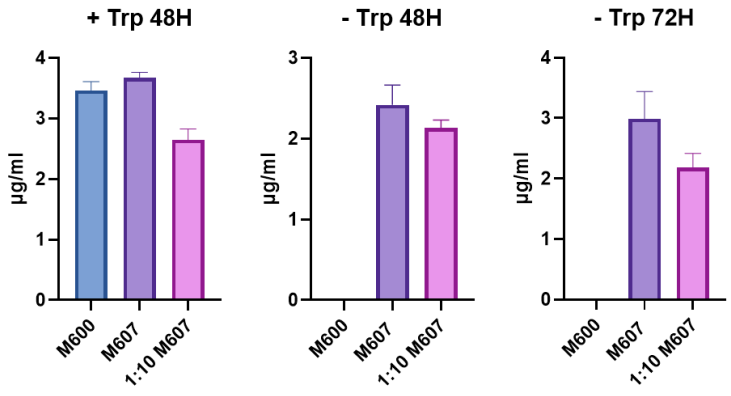

Figure 42 - Production of IAA by the strain LF2 grown in M600, M607 and 1:10 M607 culture media in both presence and absence of tryptophan (Trp). A - IAA production in presence of Trp at 24h, B - IAA production in absence of Trp at 24h and C - IAA productio in absence of Trp at 48h. IAA content is expressed in µg/ml.

Comparing the IAA production data with the plant exposure experiments, no clear correlation was found between IAA levels and the observed effects of the extracts. This suggests that IAA alone is not responsible for the effects, and that other compounds present in the extracts may contribute to the herbicidal activity.

In summary, with none of the IAA production tests performed on the bacteria we were able to find any correlation with the results obtained in the plate tests. Therefore, we hypothesized that the compounds present in the tested extracts that presented inhibitory results, could not have the same MoA of herbicides such as 2,4-D which are analogous to IAA. This is specifically relevant to LF2 extracts given that we considered that this was the only bacterium of the three used in this study as a potential producer of bio-herbicidal compounds.

In line with what was discussed previously, and considering the many problems associated with the widespread use of synthetic herbicides, there is a strong interest in identifying novel compounds as alternatives to those currently available. As reported by (Ocán-Torres et al., 2024) research on bioherbicides has been steadily increasing, particularly those derived from microorganisms such as *Pseudomonas* and *Xanthomonas* bacteria, as well as fungi from the genera *Alternaria*, *Colletotrichum*, and *Trichoderma*. However, the same authors noted that the use of microorganisms as bioherbicides presents several challenges and limitations. To overcome these issues, they proposed alternative strategies, such as utilizing microbial extracts instead of live cultures. This approach not only mitigates the practical difficulties associated with handling and applying live microorganisms but also enables the combination of these extracts with other bioherbicides or even synthetic herbicides, potentially broadening their spectrum of action in the field. Moreover, the extraction and isolation of active

compounds allow for their integration into advanced delivery systems, such as encapsulation technologies, which can improve the efficiency, stability, and specificity of bioherbicidal formulations (La Iacona et al., 2025).

Although the exploration of microbial secondary metabolites is not entirely new, recent years have seen a growing interest in the bioactivity of compounds derived from marine bacteria. As highlighted by Orfali et al. (2021), these metabolites possess significant potential for biotechnological applications due to their complex chemical structures and diverse biological properties (Karthikeyan et al., 2022). Further supporting this, (Santos et al., 2020) emphasized the relevance of marine bacterial secondary metabolites in the medical field, underscoring their promise in pharmaceutical development. This potential is further illustrated by Calisto et al. (2019) who reported notable anticancer activity in secondary metabolites produced by *Planctomycetota* bacteria, reinforcing the value of marine microorganisms as prolific sources of novel bioactive compounds.

Taken together, the present work clearly highlights the potential of marine microorganisms as valuable sources of novel bioactive compounds. However, to the best of our knowledge, except for a previous master's dissertation performed in our research group (Santos et al., 2017), there are no published reports specifically addressing herbicidal activity in marine bacteria. The present study, therefore, offers a novel perspective in the field of bioherbicides by demonstrating that marine bacteria can produce compounds with herbicidal properties. Further research is needed to explore the full potential of these microorganisms in weed management. Additionally, this study contributes to the growing body of knowledge on the biotechnological applications of *Rhodopirellula rubra*. This species has already demonstrated its versatility, as described by Marinho et al. (2022) who explored its potential as a supplementary food source for *Daphnia magna*.

4. Conclusion

This study revealed not only the potential of marine bacteria to produce bioactive compounds with agroeconomic interest but also demonstrated the specificity of compounds with possible bioherbicidal properties. The extracts demonstrated a preference to target *A. thaliana* plants instead of any of the other two plants with agronomic interest used in this study (*L. sativa* and *Lolium* spp.). This selectivity was observed with extracts obtained from *Rhodopirellula rubra* strain LF2^{T} demonstrating the remarkable potential of its extracts. The referred selectivity was evident by the Petri Dish assays where LF2 extracts affected consistently the germination rate, fresh weight, root

length and number of leaves of *A. thaliana*. This bacterium also demonstrated to induce oxidative stress in *A. thaliana*, proving once again its potential for novel bio-herbicidal compounds. The effect induced by this bacterium could not be due to the IAA production, as such, we hypothesize that this bacterium may be producing selective compounds that act differently than the herbicides homologous to IAA.

Given the novelty of this study in extracting compounds from marine bacteria and testing them in model plants, it remarks a great milestone in research of bioherbicides and the biotechnological applications of marine bacteria and their extracts. However, we recognize that this study presented some limitations, such as the number of biological replicates and species tested, as well as the application in field plants. However, as stated above, this study is pioneer in the research of marine bacterial extracts as novel bioherbicides, as such we encourage that, in the future, marine bacteria are more explored and investigated for their agronomical use, as well as for the isolation and identification of the compounds produced by the bacterium and its mode of action.

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Annex

Table 4 - Composition of each culture media, standardized for 1 L of culture, used to grow the bacteria used in this study.

Components / Media	1L		
	M600	M607	M607 1/10
Peptone	1 g	0,25 g	0,025 g
Yeast extract	1 g	0,25 g	0,025 g
Glucose (2,5%)	40 ml	10 ml	1 ml
Vitamins *	10 ml	10 ml	10 ml
Hutner's Solution **	20 ml	20 ml	20 ml
Tris-HCl	50 ml	50ml	50 ml
Sea Water	880 ml	900 ml	900 ml
Distiled Water	-	10 ml	19 ml
Agar	16 g	16 g	16 g

Table 5 - Composition of stock Vitamin solution needed to make the bacterial culture media.

Vitamin *	concentration on media (mg/L)
Biotin	0,02
Folic acid	0,02
Riboflavin	0,05
Thiamine-HCl	0,05
Nicotinamide	0,1
D calcium Pantothenate	0,05
B12	0,001

Table 6 - Composition of stock Hurner's solution needed to make the bacterial culture media.

Hutner's Solution	
Compound	Amount/L
NTA	10 g
$MgSO_4.7H2O$	29,7 g
$CaCl_2.2H2O$	3,34 g
$NaMoO_4.2H2O$	12,67 mg
$FeSO_4.7H2O$	99 mg
Metals "44"	50 ml
Distilled Water	950 ml

Table 7 - Composition of stock Metals "44" solution needed to make the bacterial culture media.

Metals "44"	
Compound	Amount/ 100mL
Na2-EDTA	250 mg
ZnSO4.7H2O	1,095 g
FeSO4.7H2O	500 mg
MnSO4.H2O	154 mg
CuSO4.5H2O	39,2 mg
CoCl2.6H20	20,3 mg
H3BO3	11,47 mg

Table 8 - Composition of modified Hoagland solution standardized to 1L used to grow the plants for the plate assays.

Compound	Molecular weight	Concentration of stock solution		Volume of stock solution per litre of final solution	Element	Final concentration of element	
	g mol-1	mM	g l-1	ml		µm	ppm
Macronutrients							
KNO3	101.10	1000	101.10	6	N	16000	224
Ca(NO3)2.4H2O	236.15	1000	236.16	4	K	6000	235
NH4H2PO4	115.03	1000	115.08	2	Ca	4000	160
MgSO4.7H2O	246.48	1000	246.48	1	P	2000	62
					S	1000	32
					Mg	1000	24
Micronutrients							
KCl	74.55	25	1.864		Cl	50	1.77
H3BO3	61.83	12.5	0.773		B	25	0.27
MnSO4.H2O	169.02	1	0.169	2	Mn	2	0.11
ZnSO4.7H2O	287.54	1	0.288		Zn	2	0.13
CuSO4.5H2O	249.68	0.25	0.062		Cu	0.5	0.03
Na2MoO4.2H2O	241.95	0.25	0.040		Mo	0.5	0.05
FeEDTA	367.10	50	18.36	1	Fe	50	2.8