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MANNOSYLERYTHRITOL LIPIDS AS GREEN BIOSTIMULANT FOR SEEDS GERMINATION OF MONICA LETTUCE SF 31 (*LACTUCA sativa* L.)

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Florianópolis
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O que realmente se fixa na memória é o que se vive,
e o que se vive precisa de emoção (Byington, 2004).

ABSTRACT

There is a rapid growth of the global population, consequently the increasing for the food demands, in particular sustainable and economically viable alternatives. Pesticides are commonly applied in the agricultural sector in order to reach high productivity, nevertheless they are also responsible for several environmental problems and damage to human health. In recent years, biosurfactants have aroused the interest of the scientific community and the industrial sector due to their properties similar to pesticides (for example, antibacterial and antifungal properties) and the possibility of production from natural sources. Among the biosurfactants, mannosylerythritol lipids (MELs) stand out due to their physicochemical properties such as biodegradability, antiphytopathogenic, and antimicrobial activities, in addition to their lower toxicity compared to conventional surfactants. This work presents a critical discussion of the current state of the art and future trends on the use of MELs as a biostimulant. Additionally, an empirical investigation was carried out on the application of MEL-B (one out of for homologues of MELs) in the germination of Monica SF 31 lettuce seeds. From the correlation between the properties of MELs and other glycolipids with potential applications in agriculture, a screening of MEL-B concentration was carried out (3.16; 31.6; 158; 316; 632 mg of MEL-B/L) where the potential biostimulant property was investigated. In other words, the germinated seeds were evaluated according to the morphological behavior, physiological characteristic, and physical-chemical and biochemical analyses performed after the germination stage. It was verified that the seeds treated with MEL-B at 158 mg/L presented the best germination and root development performance compared to the control and the subsequent concentrations. MEL-B at 316 and 632 mg/L showed inhibition of seed germination and had a negative influence on root development. The biostimulant property of MEL-B was noticed in the roots germinated in the cultivation condition containing MEL-B at 158 mg/L.

Keywords: Biosurfactants; Germination; Biostimulant.

RESUMO

O rápido crescimento da população global e o aumento da demanda por alimentos exige do setor agroindustrial alternativas sustentáveis e economicamente viáveis. Os pesticidas comumente utilizados no setor agrícola garantem um bom desempenho de produtividade, porém são responsáveis por diversos problemas ambientais e danos à saúde humana. Nos últimos anos, os biossurfactantes têm despertado o interesse da comunidade científica e do setor industrial devido suas propriedades similares aos pesticidas (por exemplo: propriedades antibacterianas e antifúngicas) e a possibilidade de produção a partir de fontes naturais. Dentre as diferentes classes de biossurfactantes, os manosileritritol lipídios (MELs) destacam-se devido as suas propriedades físico-químicas como biodegradabilidade, atividades antifitopatogênica e antimicrobiana, além de toxicidade inferior comparada aos surfactantes convencionais. Neste trabalho foi apresentada uma discussão crítica do estado da arte atual e as tendências futuras do MELs como bioestimulante agrícola. Adicionalmente, foi realizada uma investigação empírica sobre a aplicação do MEL-B (um dos homólogos dos MELs) na germinação de sementes de alface Mônica SF 31. A partir da correlação feita entre as propriedades do MELs e outros glicolipídios com potenciais aplicações na agricultura relatados na literatura, foi realizado um *screening* de concentrações (3,16; 31,6; 158; 316; 632 mg/L) de MEL-B onde a potencial propriedade bioestimulante foi investigada. Ou seja, as sementes germinadas foram avaliadas conforme o comportamento morfológico, característica fisiológica, assim como as análises físico-químicas e bioquímicas foram feitas após a etapa de germinação. Verificou-se que as sementes tratadas com MEL-B a 158 mg/L apresentaram o melhor desempenho de germinação e desenvolvimento das raízes quando comparados ao controle e as concentrações subsequentes. MEL-B a 316 e 632 mg/L apresentou inibição na germinação das sementes e influenciou negativamente no desenvolvimento das raízes. A propriedade bioestimulante do MEL-B foi notada nas raízes germinadas na condição de cultivo contendo MEL-B a 158 mg/L.

Palavras-chave: Biossurfactante. Germinação. Bioestimulante.

Resumo expandido

MANOSILERITRITOL LIPÍDIOS COMO BIOESTIMULANTE PARA GERMINAÇÃO DE SEMENTES DE ALFACE MÔNICA SF 31 (*LACTUCA sativa* L.)

Introdução

A busca por uma produção agroindustrial mais sustentável cresce proporcionalmente com o crescimento da população. Os biossurfactantes são biocompostos com propriedades atrativas para a comunidade científica e o setor industrial, pois os mesmos apresentam vantagens em relação aos surfactantes químicos, entre as quais baixa toxicidade, biodegradabilidade, redução da tensão superficial e produção a partir de fontes naturais. Em particular, os biossurfactantes apresentam-se como uma alternativa promissora aos pesticidas na agricultura. A propriedade bioestimulante desses biocompostos pode contribuir com práticas agrícolas sustentáveis, beneficiando a absorção de nutrientes, eficiência de germinação, tolerância ao estresse abiótico, rendimento e qualidade das colheitas. Todavia, ainda que os biossurfactantes sejam amplamente aplicados nos setores alimentício e farmacêutico, existe uma lacuna sobre a aplicação destes compostos no setor agrícola.

MELs são biossurfactantes produzidos por pseudo-leveduras e pertencentes à classe dos glicolipídios. Sua estrutura química é composta por uma parte hidrofóbica e outra hidrofílica - que é diretamente influenciada pelo microrganismo produtor e disponibilidade de oxigênio e nitrogênio no meio de cultivo. Neste sentido, este biocomposto pode ser classificado como monoacetilado (MEL-A), di-acetilado (MEL-B e MEL-C) e não acetilado (MEL-D). MELs se constituem em uma alternativa promissora aos pesticidas químicos, devido suas características antimicrobianas, anfifílicas, de biodegradabilidade e potencial propriedade bioestimulante.

A possibilidade da aplicação do MELs como bioestimulante na agricultura é baseada em dados de outros biossurfactantes glicolipídicos (ramnolipídios e soforolipídios), que apresentaram resultados promissores na bioestimulância de plantas e inibidores fitopatogênicos, simultaneamente. Embora a literatura científica contemple diversos estudos recentes sobre a necessidade de alternativas ambientalmente seguras para o setor agrícola, faltam pesquisas que explorem a aplicação dos MELs como bioestimulante e investiguem, a partir de abordagens mais profundas, a interação biossurfactante-planta. Logo, compreender

essas lacunas possibilita avançar com estudos que corroborem para reduzir os problemas ambientais e sociais causados no setor agrícola.

Objetivo

O principal objetivo deste trabalho foi investigar a propriedade bioestimulante do MEL-B em sementes de alface Mônica SF 31 (*Lactuca sativa* L.), considerando as modificações morfológicas e fisiológicas na etapa de germinação.

Metodologia

A primeira etapa deste trabalho envolveu uma discussão crítica baseada no estado da arte e tendências futuras da aplicação de MELs no setor agrícola. A discussão foi baseada em três pilares, sendo eles: (i) as propriedades físico-químicas do biossurfactante, (ii) a atividade bioestimulante; e (iii) a atividade antimicrobiana contra fitopatógenos. Uma abordagem sobre as propriedades bioestimulantes e antimicrobianas, simultaneamente, também foi relatada com o intuito de corroborar com a viabilidade de aplicação deste biocomposto na agricultura.

A segunda etapa deste trabalho contemplou a aplicação do MEL-B em sementes de alface Mônica SF 31 (*Lactuca sativa* L.). Desta forma, foi possível avaliar experimentalmente o efeito bioestimulante do biossurfactante nas sementes germinadas. Inicialmente, foi feito um *screening* de concentrações (0; 3,16; 31,6; 158; 316; 632 mg de MEL-B/L) para suplementar o meio de cultivo onde as sementes foram germinadas. Os testes de germinação foram avaliados a partir do índice de velocidade de germinação, tempo médio de germinação e incidência de raízes germinadas por dia. Em paralelo, a influência das diferentes concentrações de MEL-B na medida do ângulo de contato e tensão superficial foram realizadas com o auxílio de um goniômetro. As análises morfológicas das raízes foram conduzidas a partir do cultivo das sementes sob diferentes condições de tratamento com MEL-B. Assim, o comportamento das raízes laterais, raízes estressadas, comprimento e massa foram observados diariamente durante o período de experimento. Em relação às caracterizações físico-químicas, foi realizado um estudo da influência do MEL-B na germinação e na indução de condições de estresse em cultivo. Nesta etapa, as observações foram realizadas a partir da quantificação de proteínas totais e análise da atividade das enzimas peroxidase (POD) e polifenoloxidase (PPO).

Resultados e Discussão

Na primeira etapa do trabalho foi possível identificar a potencial ação bioestimulante agrícola dos glicolipídios. Em relação aos MELs, eles podem promover melhorias na qualidade

biológica do solo e das sementes, além de melhorar a nutrição das raízes e estimular o crescimento das plantas. Porém, ainda é necessário compreender as lacunas existentes na literatura sobre a interação MELs-planta em diferentes formas de cultivo. Além disso, a análise da compilação de dados sobre os MELs indica que esses biossurfactantes podem, potencialmente, agir na agricultura simultaneamente como bioestimulante e antifitopatogênico.

Em relação à segunda etapa do trabalho, notou-se que algumas concentrações de tratamento promoveram comportamento adverso nas sementes de alface. Na etapa de germinação, o cultivo com 316 e 632 mg/L de MEL-B influenciou na redução de raízes germinadas e velocidade de germinação. Este comportamento foi observado em comparação com o controle e as demais concentrações (3,16; 31,6 e 158 mg/L de MEL-B) utilizadas do trabalho. A redução na germinação e no desenvolvimento das raízes pode ser relacionado à diminuição das divisões celulares devido às alterações morfológicas e fisiológicas após tratamento utilizado na germinação das sementes. Como esperado, as respostas obtidas com as análises morfológicas e caracterizações físico-químicas corroboram para a compreensão do efeito fitotóxico causado com o tratamento feito a 316 e 632 mg/L de MEL-B. Contudo, foi possível identificar que o tratamento com MEL-B a 158 mg/L correspondeu à maior concentração de tratamento que agiu como bioestimulante do crescimento das raízes e não inibiu o processo de germinação das sementes.

Conclusão

Biossurfactantes, incluindo MELs, são uma alternativa promissora aos pesticidas químicos. MEL-B a 158 mg/L no meio de cultivo de sementes de alface promoveu desempenho satisfatório de germinação e crescimento das raízes. Além disso, não influenciou o estresse oxidativo das sementes, conforme notado nas análises bioquímicas. Porém, a aplicação do MEL-B na germinação de sementes de alface indicou dose-dependência. Portanto, em concentrações superiores a 158 mg/L de MEL-B foi observada a inibição na germinação e estresse das sementes no meio de cultivo.

Palavras-chave: Biossurfactantes; Agricultura; Germinação; Bioestimulância.

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LIST OF ABBREVIATIONS

ATP Adenosine triphosphate

CMC Minimum micellar concentration

HPLC High performance liquid chromatography

MBC Minimum bactericidal concentration

MELs Mannosylerythritol lipids

SEM Scanning electron microscopy

MIC Minimum inhibitory concentration

POD Peroxidase

PPO Polyphenol oxidase

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CHAPTER 1

In this chapter, it is presented a brief introduction to the research developed, the problem and motivation, hypotheses, research question, objectives, and a conceptual diagram.

1 INTRODUCTION

The use of pesticides has become essential to agricultural practices, since they guarantee good production conditions and high levels of quality, in addition to promoting actions to control biotic and abiotic stress (HALPERN et al., 2015; MÜLLER et al., 2002). However, the indiscriminate and continuous use of these products is linked to environmental impacts, including contamination of water, plants, soil and increased pathogenic resistance (SHENG et al., 2001). In addition, pesticides are related to multiple adverse effects on human health, ranging from nausea and skin irritation to chronic problems such as diabetes and cancer (LOPES; ALBUQUERQUE, 2018). Some alternatives to the use of chemicals in agriculture may be promising, such as the use of biosurfactants, which have properties similar to conventional chemical pesticides.

Biosurfactants are compounds excreted from microorganisms, such as bacteria, filamentous fungi and yeasts, enzymes, rhizobacteria and algae extracts, in diversified carbon sources (BITTERLICH et al., 2018; ROUPHAEL et al., 2020). These biomolecules have an amphipathic nature, lower toxicity compared to chemical surfactants, biodegradability, variety of chemical structures and reduce the surface tension of the medium where they are produced. In addition, they have a biostimulant property for seed germination (SIEVERDING, Ewald, 2017) and biocontrol of infection and biotic stress by phytopathogenic fungi in plants (YAN et al., 2014). These characteristics make biosurfactants a highly advantageous alternative to chemical surfactants (DAS; KUMAR, 2018; GEETHA; BANAT; JOSHI, 2018).

In this context, MELs are an abundant group of biosurfactants belonging to the class of glycolipids (FUKUOKA; MORITA; KONISHI; IMURA; SAKAI; et al., 2007; KIM, H.-S. et al., 2006). This biomolecule is commonly excreted from *Pseudozyma* ssp. and fungi of the genus *Ustilago* ssp. The chemical structure of MELs varies according to the producing microorganism and nitrogen and carbon sources available in the culture medium. Furthermore, they are surface active molecules and have two domains, one hydrophobic and one hydrophilic (MORITA et al., 2008). MELs can be classified as diacetylated (MEL-A), monoacetylated (MEL-B and MEL-C) and non-acetylated (MEL-D), according to the position and number of acetyl radical present in their structure (DE ANDRADE; PASTORE, 2017; GÜNTHER et al., 2015).

According to Yoshida *et al.* (2014), MELs stimulated the defense mechanism of plants and controlled the development of the fungus *Pseudomonas antarctica* on the surface of rice

leaves. In another study, Yoshida *et al.* (2015) observed antimicrobial activity of MELs against phytopathogenic fungal conidia infection on wheat and rice leaves. This biosurfactant has biological properties of high interest for several industrial sectors, including agricultural applications (PAULINO et al., 2016).

The antimicrobial activity together with the potential biostimulant property of MELs expands the prospects of sustainable agriculture with high productivity, production quality, and less environmental impact.

1.1 HYPOTHESIS

Population growth reflects on increased agro-industrial demand. Although pesticides are associated with production processing, these products are also directly related to environmental problems, as well as human health. Therefore, sustainable alternatives are needed to minimize the damage caused by the excessive use of pesticides and maintain production quality. Based on this challenge, the work presents the following hypotheses:

(i) Taking into account the characteristics of glycolipids, MELs may have biostimulant properties;
(ii) The metabolism of fatty acids, erythritol and mannose, which make up the chemical structure of the MELs, are closely related to energy generation (ATP);
(iii) MELs, in addition to act as biostimulant for seeds, may have a phytotoxic effect at certain concentrations;
(iv) MELs can partially or totally replace chemical pesticides.

1.2 AIM OF THE WORK

1.2.1 General aim

The general objective of this work was to investigate the biostimulant property of MEL-B from the direct treatment of Monica SF 31 lettuce seeds (*Lactuca sativa* L.), considering the morphological and physiological changes in the germination stage.

1.2.2 Specific aims

(i) Verify the influence of MEL-B on the germination of Monica SF 31 lettuce seeds (*Lactuca sativa* L.), from a concentration screening (3.16, 31.6, 158, 316 and 362 mg/L) in agar-agar culture media;

(ii) Evaluate root development from morphological (lateral roots, stressed roots, length and weight) and physiological (total proteins and enzymatic activity) analyzes for each treatment concentration;
(iii) Quantify total proteins and the activity of POD and evaluate PPO enzymes through physicochemical methods in order to identify the biostimulant or phytotoxic effect of MEL-B on seed germination;
(iv) Determine, under the conditions of the study, the best concentration of treatment with MEL-B for the cultivation of lettuce seeds and the concentration that inhibits the process of germination and root development.

1.3 STRUCTURE OF DISSERTATION

This dissertation is divided into four chapters, that the main points are presented below:

In the current chapter, a general contextualization and objectives are presented.

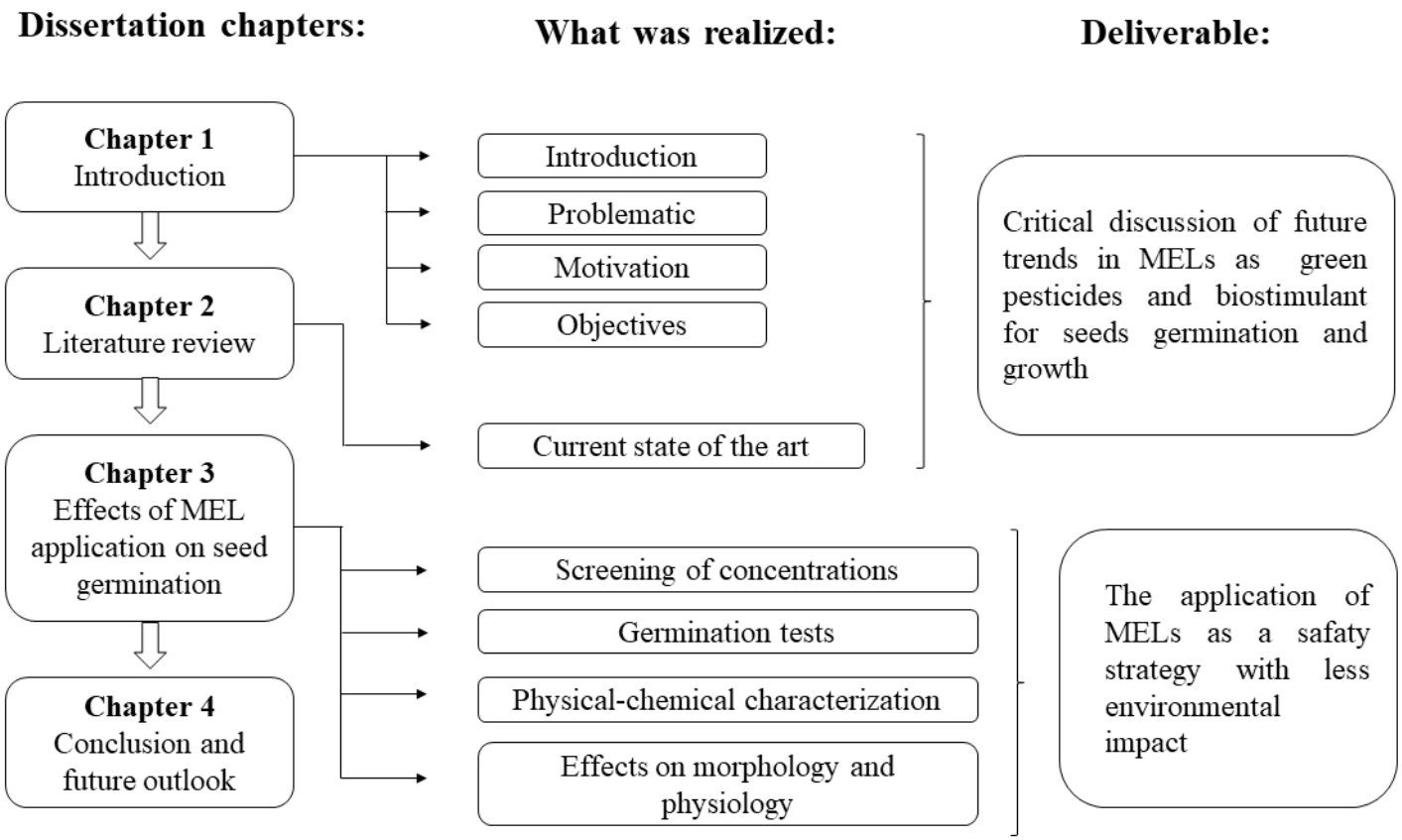
Chapter 2 is related to the literature review article (Accepted for publication in Journal of the Science and Food Agriculture) on MELs as biostimulant in seed germination. In this review, it was carried out a critical assessment of the current state of the art and future trends on the application of MELs as a green pesticide and biostimulant for seed germination and growth. The discussion is based on the physicochemical properties of this biosurfactant, the potential biostimulant activity and the antimicrobial activity against phytopathogens.

Chapter 3 shows the experimental results achieved to confirm the main hypotheses proposed in this master thesis. The research paper was recently submitted to Journal of Agricultural and Food Chemistry. In this chapter, the MEL-B biosurfactant was applied to the germination of SF 31 lettuce seeds through treatments of the culture media. That is, an application strategy was defined and then, from a screening of concentrations, the MEL-B was inserted into the culture medium where the seeds were germinated. Chapter 3 presents the first experimental work results on this subject, in which the preparation steps, cultivation method and morphological, physiological and biochemical evaluations are described, with direct application of MEL-B to seeds. Furthermore, this chapter proved the biostimulant property of this biosurfactant.

Chapter 4 presents a summary of the main conclusions, including the future perspectives on the subject of this work.

1.3.1 CONCEPTUAL DIAGRAM

Figure 1 - Conceptual diagram of the master thesis showing the problem and motivation, master thesis structure, what was evaluated in experimental chapter, and deliverables.



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CHAPTER 2

In this section, the review article “Mannosylerythritol Lipids as Green Pesticides and Plant Biostimulants” is presented. This chapter aims to give the reader an overview of the MELs and their potential seed biostimulant property. The current state of the art and a discussion of future trends based on the physicochemical, antimicrobial, and antiphytopathogenic properties of MELs are reported. This review article is linked to the jornal “Journal of the Science of Food and Agriculture” published in July 2022 (DOI: 10.1002/jsfa.12100).

2 MANNOSYLERYTHRITOL LIPIDS AS GREEN PESTICIDES AND PLANT BIOSTIMULANTS

Abstract

Biosurfactants can be applied to the formulation of personal care products, as food additives, and as biocontrol agents in the agricultural sector. Glycolipids and lipopeptides represent an important group of microbial-based biosurfactants with biostimulating properties. Among them, the MELs also presented antimicrobial activity, mostly against Gram-positive bacteria and phytopathogenic fungi. In this sense, MELs are a potential safer green alternative for partially replacing synthetic pesticides. This review aimed to critically discuss the current state-of-the-art and future trends of MELs as green pesticides and biostimulants for seed germination and plant growth. Due to the chemical structure, MELs are likely related to the energy pathways such as glycolysis, Krebs cycle, and others; that is, a direct cellular biostimulant potential. In this case, experimental evidence from other glycolipids indicated that structural and chemical changes as a potential drug vehicle due to morphological changes caused by the biosurfactant-membrane interaction. In addition, like other biosurfactants, MELs can trigger self-defense mechanisms, leading to a lower frequency of phytopathogen infections. Therefore, MELs have the potential for biostimulation and antiphytopathogenic action, despite, to the date, no data is available on MELs as biostimulant and green pesticide, simultaneously. Based on the current state-of-the-art, on MELs have a great potential for a biotechnological advance towards more sustainable agriculture.

2.1 INTRODUCTION

MELs are biosurfactants belonging to the glycolipid class. The chemical structure of these molecules varies in the position and number of the acetyl radical. In this sense, MELs can be classified according to 4 different structures (MEL-A, MEL-B, MEL-C and MEL-D). The main producers of MELs are the yeast-like *Pseudozyma* ssp. and fungi of the genus *Ustilago* ssp. (FUKUOKA *et al.*, 2007; SAIKA *et al.*, 2017). MELs have remarkable biological properties that have been drawn attention to the cosmetic and pharmaceutical industries. Notably, antimicrobial activity, anti-aging and moisturizing characteristics, induction of apoptosis and stopping the cell cycle of cancer cells, and increased transfection of genetic

material trapped in liposome systems (FEUSER *et al.*, 2021; KITAMOTO *et al.* 1990; MANIGLIA, *et al.* 2019; PAULINO *et al.* 2016; COELHO *et al.* 2020ab).

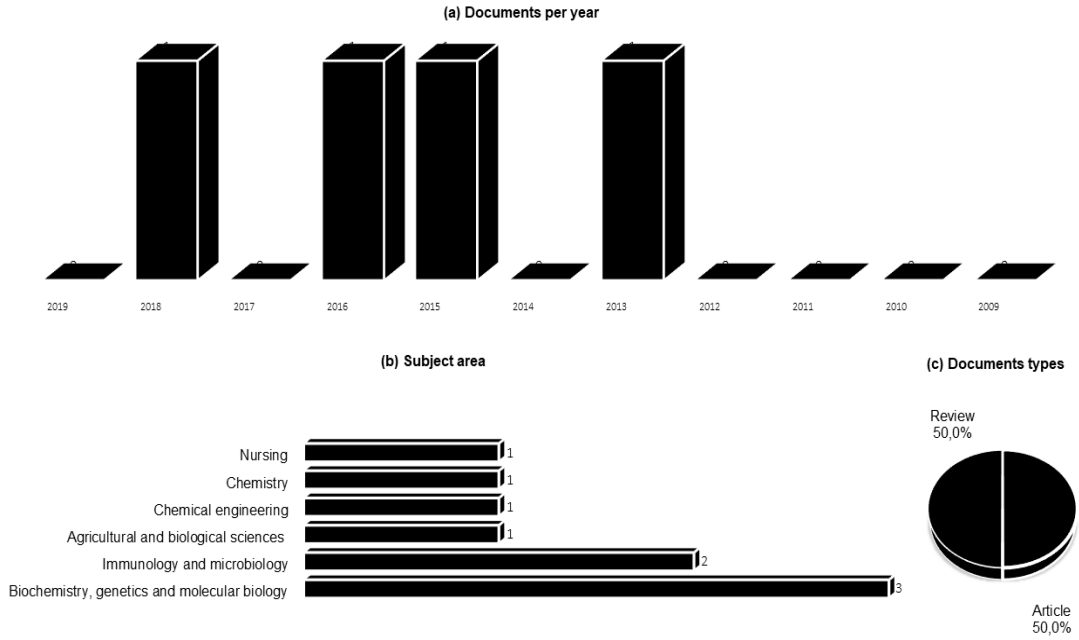
Chemical pesticides are essential for modern agriculture. However, they are directly related to acute and chronic diseases. In addition, the excessive use of pesticides can compromise the quality and safety of drinking water system, i.e., they are massive anthropogenic pollutants.

Worldwide, the indiscriminate use of pesticides aims at high productivity. However, it contributes to ecological imbalances such as the stability of agricultural systems, the conservation of natural resources (water, soil and air), and food quality. Although 184 pesticides are regulated around the world, water quality standards vary by country. In this sense, the increase in the different regulations of the countries leads to controversial situations. For example, the herbicide glyphosate, detected in fresh waters around the world, has only 6 (Australia, New Zealand and United Kingdom) water quality standards. In Brazil, one of the main agricultural producers in the world, the use of Azinphos-methyl and Carbaryl is not legal. However, countries like the United States and regions of high agricultural production like the US still use this pesticide. Persistent organic pollutants are regulated 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane, 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene, toxaphene and pentachlorophenol are regulated in Brazil. However, they are semi-volatile, persistence, bioaccumulatiors and, toxics at -2.4 x 10^{-8} mg/L, 1.8 x 10^{-8} mg/L, 2.8 x 10^{-7} mg/L mg/L and 9 x 10^{-6} mg/L, respectively, as the lowest standard water quality values. According to the Brazilian regulation about pesticides presence in water (Brazilian Regulatory Standard, 2005), potable water is defined as suitable for human consumption after different treatments such as simplified, conventional and, advanced treatments. In this sense, it is necessary to identify the levels of pesticide residues in water from agricultural practices, i.e. technological needs (yields) must line up with environmental regulations.

Pseudozyma ssp. (for example, *P. aphidis, P. antarctica* and *P. churashimaensis*) have been used as a biocontrol agent (CALDERÓN et al., 2019; LEE et al., 2017; LIU et al., 2018). The potential of MELs to stimulate the spread of *Pseudozyma* ssp. on the surface of plant leaves was identified and inhibited the growth of the opportunistic phytopathogen. However, it is worth noting that the direct agriculture application of MELs as an active agent is still incipient (YOSHIDA, Shigenobu et al., 2014).

A survey conducted on June 2021 in the Scopus and Web of Science databases relating the keywords "pesticides" AND "phytopathogenic" AND "biostimulant" AND "mannosylerythritol lipids". No document was found using this combination of words. When the research was carried out using only the keyword "mannosylerythritol lipid", it was found that the studies are concentrated in different areas, such as: biochemistry, genetics and molecular biology, nursing, chemistry, chemical engineering, agricultural and biological sciences and, immunology and microbiology. Taking into account specific area as agricultural and biological sciences, 23 documents were found - however, none correlating the biostimulant and antimicrobial potentials of these compounds for agriculture. From 2009 to 2021, when the term “mannosylerythritol lipid” was associated with the keyword “agriculture”, only 4 documents were found - two empirical articles and two review articles, all developed in Asia (Japan 50%, India 25% and Tunisia 25%), as shown in Figure 1, despite the potential of MELs acting as biostimulants and antimicrobial in agriculture, there is a clear lack of available information.

Figure 2 - Documents per year (a), subject area (b) and documents types (c), in the Scopus and Web of Science databases, from 2009 to the present.



Source: From the author.

Therefore, the present manuscript aimed to critically evaluate the current state-of-the-art and discuss future trends on the application of MELs as green pesticides and biostimulants

for seed germination and growth based on (i) the physicochemical properties of MELs; (ii) the potential biostimulant activity; and (iii) the antimicrobial activity against phytopathogens.

2.1.1 Mannosylerythritol lipids: Chemical structures and microbial producers

MELs are surfactants synthetized by microbial metabolism with antimicrobial and self-assembly properties. These compounds can reduce the surface tension of water from 72 to around 30 mN/m (KIM, H.-S. et al., 2006; ROSENBERG; RON, 1999; VAN HAMME; SINGH; WARD, 2006). MELs are versatile molecules since they can reduce environmental toxicity - due to their ability to interact with heavy metals (Pb^{2+}, Cd^{2+}, Hg^{2+} and Zn^{2+}) - and increase the solubility of agrochemicals, acting as dispersing agents for pathogenic microorganisms on the surfaces of leaves and animals (ARUTCHELVI et al., 2008). Among the various applications, an emulsifying activity of MELs has potential industrial application, such as bioremediation. Since an interaction of this bioemulsifier with aliphatic, aromatic hydrocarbons and hydrocarbon mixtures form stable emulsions. In this sense, they efficiently contribute to the biodegradation process of pollutants - such as diesel oil (GUDIÑA et al., 2015). Regarding healthcare, MELs can enhance the transfection efficiency of genes delivered by liposomes (RODRIGUES, 2015). In cosmetology, MELs have remarkable skin hydrating activity when added in skincare formulations (KIM, M. K. et al., 2014).

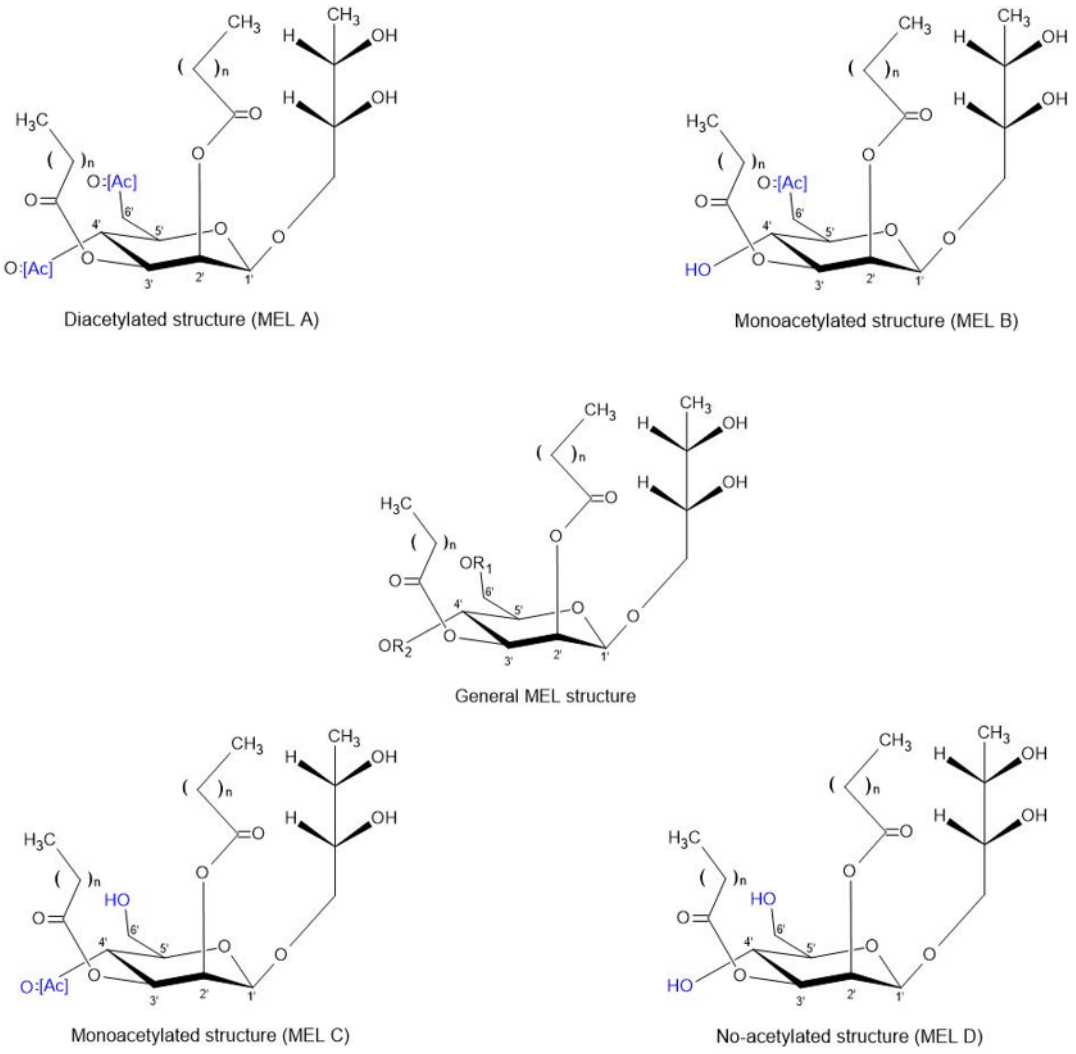
The interfacial and biological properties of MELs are related to their chemical structure. MELs are composed of a hydrophobic portion (acetyl and/or fatty acid group) and a hydrophilic fraction (4-*O*-β-D-mannopyranosyl-D-erythritol). In this sense, MEL-A, MEL-B, MEL-C and MEL-D can be classified according to the position and number of the acetyl group in carbon atoms C-4 and C-6, as shown in Figure 2 (ANDRADE et al., 2017; COELHO et al., 2020b; GÜNTHER et al., 2015). The length and unsaturation degree of the fatty acid chains, and erythritol configuration (conventional or diastereomers) affect MELs properties. Moreover, the iso-MEL-B, a diastereomer produced from *P. tsukubaensis*, has similar NMR spectrum patterns when compared with MEL-B. However, there is a chirality difference between MEL-B and iso-MEL-B, particularly erythritol-mannose binding positions (FUKUOKA; MORITA; KONISHI; IMURA; KITAMOTO, 2007).

It is worth noting that the MELs structure is consequence of the producing microorganism, sources of carbon and nitrogen in the culture medium, bioreactor configuration

and mode of operation. Furthermore, a biosynthesis pathway was identified through genomic sequencing. However, genetic engineering techniques can improve the understanding of the MELs biosynthesis pathway directly related to the increased production of MELs (ARUTCHELVI et al., 2008; COELHO et al., 2020b; PAULINO et al., 2016; SAIKA et al., 2017).

As shown in Table 1, the production of biosurfactants by microorganisms is theoretically related to solubilization of hydrophobic compounds. According to Andrade and Pastore (2017), more hydrophobic homologues are synthetized when a culture medium containing hydrophobic molecules are used; for instance a culture medium composed of olive or soybean oil. In this sense, Fukuoka *et al.* (2007a) produced MEL-A by *P. antarctica* T-34 in a high soybean oil concentration (from 80 to 120 g/L) and described sort of MEL-A, with a third fatty acid linked to erythritol (tri-acylated MEL-A), that makes the molecule even more hydrophobic. This study contributed to correlate the production of MELs by microorganisms with the hydrophobicity of the culture medium. Morita *et al.* (2015) showed that species with a genetic relation tend to obtain similar MELs products. This fact was reinforced by Wang *et al.* (2015) in their study that classifies MELs-producing species. For example, the secretion of MEL-A is the product of preference for several producing species, such as *P. antarctica* T-34*, P. aphidis* DSM 70725 and *P. rugulosa* NBRC 10877.

Figure 3 - MELs chemical structures



Source: (COELHO et al., 2020a)

In addition to product specifications for *Pseudozymas* (they tend to secret MEL-A), Kakugawa *et al.* (2002) investigated *Kurtzmanomyces* sp. I-11. In this study, the new yeast strain was isolated under similar cultivation conditions as other MELs homologues and the results demonstrate a high level of production of glycolipids recognized as MEL-B with the fatty acid components C8:0 (36.4%), C12:0 (11.9%) and C14:2 (25.9%).

Also, the taxonomic analyzes of *P. siamensis* CBS 9960 based on rRNA gene sequencing demonstrated effective production of MEL-C, as elucidated by Morita *et al.* (2008).

In this case, the strains were cultivated in a basal medium containing 4.0% (w/v) of safflower oil. The results observed by HPLC indicated that the highest peak of glycolipids produced corresponds to MEL-C (84.6%), represented by the singular hydrophobic structure, potential surface activity and self-assembly property. In addition, it was found that the CMC increased as the carbon source increased, in this sense, the authors observed that the best yield of MELs produced was 12 g/L obtained by adding 1% (w/w) of glucose. In other words, glucose was an efficient supplement for the production of MELs from safflower oil. Thus, carbon sources, specification of producing yeasts, as well as production yield were carefully elucidated. In conclusion, the authors report for the first time in the literature that *P. siamensis* CBS 9960 is a new MEL-C producer.

Table 1 - MELs producers and surface activity properties. Adapted from Arutchelvi *et al.* (2008)

Microorganism	MELs homologues (major component)	Sugar	Fatty acid profile	Primary Carbon source	Inducer	CMC	Surface tension (mN/m)	Reference
Pseudozyma aphidis DSM 70725	MEL-A	4-*O*-*β*-D-mannopyranosyl (1→4)-D-meso-erythritol	$C_{10:0}$, $C_{10:1}$ and $C_{8:0}$	Yeast extract and glucose	Soybean oil	2.7 x 10^{-6}	28.4	(Nakahara *et al.* 1990; Rau *et al.* 2005)
Pseudozyma rugulosa NBRC 10877	MEL-A	4-*O*-*β*-D-mannopyranosyl (1→4)-D-meso-erythritol	$C_{8:0}$ (28.09%), $C_{10:0}$ (21.68%), $C_{10:1}$ (22.94%)	Yeast extract	Soybean oil	2.7 x 10^{-6}	28.4	(Morita *et al.* 2006)
Pseudozyma antarctica T-34	MEL-A	4-*O*-*β*-D-mannopyranosyl-erythritol	$C_{8:0}$ (27.26%), $C_{10:0}$ (21.28%), $C_{10:1}$ (27.22%)	Malt extract, beef extract, polypetonrice bran, corn steep liquor and yeast extract.	Soybean oil, safflower oil, coconut oil, cottonseed oil, corn oil and palm oil.	2.7 x 10^{-6}	28.4	(Nakahara *et al.* 1990; Kitamoto *et al.* 2001)
Pseudozyma antarctica T-34	MEL-B	4-*O*-*β*-D-mannopyranosyl-erythritol	$C_{8:0}$ (27.26%), $C_{10:0}$ (21.28%), $C_{10:1}$ (27.22%)	Malt extract, beef extract, polypetonrice bran, corn steep liquor and yeast extract.	Soybean oil, safflower oil, coconut oil, cottonseed oil, corn oil and palm oil.	4.5 x 10^{-6}	28.2	(Nakahara *et al.* 1990; Kitamoto *et al.* 2001)
Kurtzmanomyces sp. I-11	MEL-B	6-*O*-*β*-D-mannopyranosyl (1→4) -D-meso-erythritol	$C_{8:0}$ (36.4%), $C_{12:0}$ (11.9%), $C_{14:2}$ (25.9%)	Soybean oil	Not specified	4.5 x 10^{-6}	28.2	(Kakugawa *et al.* 2002)
Candida (Pseudozyma) sp. B7	MEL-B	4-*O*-*β*-D-mannopyranosyl-D-erythritol	C_7–C_{14}	Yeast extract and glucose	Soybean oil	4.5 x 10^{-6}	28.2	(Hideki Kawashima *et al.* 1983)
Pseudozyma siaensis CBS 9960	MEL-C mixture of monoacetylation and deacetylation	4-*O*-*β*-D-mannopyranosyl-(1→4)-D-meso-erythritol	$C_{14:2}$, $C_{16:0}$, $C_{16:1}$, $C_{16:2}$ (C2 or C4) at the C-2' position and (C_{16}) at the C-3' position of the mannose moiety	Yeast extract and glucose	Safflower oil	4.5 x 10^{-6}	30.7	(Rodrigues *et al.* 2006; Morita *et al.* 2008)

2.1.2 Antimicrobial activity of MELs against phytopathogenic microorganisms

As previously discussed, biosurfactants can be applied as vegetable biostimulants. They also have antimicrobial activity against phytopathogenic microorganisms, including MELs (VARNIER et al., 2009; VATSA et al., 2010). In this sense, Yoshida *et al.* (2014) evaluated the effect of MELs against *P. antarctica* on the surface of plants (rice and wheat), focusing on morphological changes. The results showed single cells in the form of hyphae that play a role in the development of fungal colonization in the studied surfaces. The authors also noted that after 3 days MELs reduced the phytopathogenic areas on leaf surfaces. However, deeper research should be carried out on the specific mechanisms - direct or indirect implications on morphological changes**.**

Yoshida *et al.* (2015) studied the feasibility of MELs - MEL-A, MEL-B, MEL-C - and (iso-MEL-B) to reduce hydrophobicity of plastic surfaces. The authors also evaluated suppressive activity against the early infection behaviors of several phytopathogenic fungal conidia on the aforementioned material, as well as on the host leaves two *Gramineae* plants (surface with high hydrophobic nature) and two non-*Gramineae* plants (surface with more hydrophilic features than *Gramineae*): wheat, rice, strawberry and mulberry, respectively. The step which follows the previously mentioned study was to assess the efficacy of bio- and chemical surfactants in inhibiting germination of conidial fungi, on the treated and untreated Gelbond surfaces. The most promising results were obtained for *Blumeria graminis f.* sp. *tritici* strain T-10 (Bg), where germination was strongly suppressing (<0.7%) by MEL-A and iso-MEL-B, when compared to control condition (31%). *Colletotrichum dematium* strain S9733 (Cd) showed a germination percentage of 89% on the untreated surface, a value reduced to 57 and 68% when the films were treated with MEL-B and MEL-C, respectively.

For the *Glomerella cingulata* strain S0709 (Gc), MELs did not show any suppressive effect. Regarding *M. grisea* strain Kyu89–246 (Mg), it was verified that MEL-A, MEL-C and iso-MEL-B significantly reduced their germination. Finally, disease suppression trials were carried out with wheat, blackberry, strawberry and rice leaves. For this, the leaves underwent a treatment similar to that reported for the Gelbond film. In this case, however, the experimental protocol was different for each leaf, due to the peculiarities in physical and growth characteristics. The strains were grown on the surface of wheat, blackberry, strawberry and rice leaves, respectively. Bg showed more significant results with MEL-A being applied. The inhibition of MEL-B and MEL-C stood out from the others when applied to Cd conidia's

germination. Germination of Gc conidia showed no inhibition by any treatment and Mg showed significant inhibition by iso MEL-B (YOSHIDA, S. et al., 2015).

The MELs produced from *Pseudozyma* sp. were isolated from the organic layer of the culture. The authors followed the separation and then evaporation process; in this sense, the concentrated glycolipid solution chloroform was added later purified by chromatography. Compared to other types of MELs (MEL-B, MEL-C and (iso-MEL-B)), treatment with MEL-A was the most effective among its counterparts in suppressing approximately <1/3 of the necrotic symptoms caused by *Blumeria graminis*. Regarding the treatment of strawberry leaves, the data were the opposite, as the application of MELs contributed to the increased severity of the results caused by *Glomerella cingulata*. Probably, glycolipids can be act as a nutrient, contributing to the fungus growth. The increase in the severity of the disease was also observed in the treatments performed with Tween 20 and Brij 35. Additionally, appressorium formation in vegetable pathogens (*Blumeria graminis f.* sp. *tritici, Colletotrichum dematium, Glomerella cingulata* and *Magnaporthe grisea*) was not observed in experiments with films, however it was found in those made with leaves. Due to such behavior in biotic materials is linked with several physiological factors of both organisms, plant and fungus, which in the specific case of MELs still require extensive investigation (YOSHIDA, S. et al., 2015).

Interestingly, tests conducted by Yoshida *et al.* (2015) related that MEL-A, MEL-B and MEL-C, separately, do not have inhibitory activity on the growth of vegetable pathogen *Magnaporthe grisea,* also known to be a cause of disease in the rice plant, which was only evidenced when glycolipids were used simultaneously in the following proportion of 58 (MEL-A): 25 (MEL-B): 10 (MEL-C). Thus, the results indicated that the ability of MELs to act as pesticides of plant depends on the plant and on the target pathogen; since in the leaves of rice no effect was observed and in leaves of strawberry the symptoms of the disease were significantly increased.

Although the aforementioned results are promising, it can be driven when the effects of MELs on the fungal conidia at molecular level become clear. (LEE et al., 2017), for example, reported that antibiotic glycolipid flocculosin, produced by *Pseudozyma flocculosa*, well known for efficiently controlling powdery mildew fungi, modified the expression of cyp 1 gene in the powdery mildew cells, which is related to the synthesis of the monooxygenase enzyme that modifies the physiology of fungus, promoting a fast plasmolysis of cells, in addition to limiting/inhibiting its growth.

Shu *et al.* (2020) explored the bioactive activity of MELs to assess the antibacterial and antibiofilm capacity of this biosurfactant against gram-positive *Staphylococcus aureus*. MELs were produced by *Pseudozyma aphidis* DSM 70725 and purified before being applied to the study. The MIC and MBC of MELs were determined to be 0.625 mg/mL and 2.50 mg/mL, respectively, and these values indicate that MELs have great antibacterial activity against *S. aureus* planktonic cells. Another interesting fact is that as the concentration of MELs was increased, the survival rate of bacteria declined. In conclusion, the authors indicated that MELs are advantageous over chemical surfactants and are a safe alternative to food preservatives.

2.1.2.1 Association of MELs with nanoparticulate systems

The application of biosurfactants to replace chemicals harmful to the environment has attracted attention, mainly due to the special bioactivity as stabilizing agents to synthesize nanoparticles. Biosurfactants applied in nanoparticulate systems are known as the green method - advantageous over synthetics due to better biodegradability, having more stable production conditions, environmental compatibility and low toxicity of by-products. Previous studies have shown that nanoparticles permeated by biosurfactants can improve antibacterial activities for most bacterial species (BAKUR; ELSHAARANI; et al., 2019; COELHO et al., 2020b)

Bakur *et al.* (2019) used MELs produced from *U. maydis* CGMCC 5203 like green-reducing and green-stabilizing agents in the synthesis of gold nanoparticles (AuNPs). The authors used three concentrations for the study and realized that synthesized materials showed a considerable antibacterial effect against *E. coli* and *S. aureus* strains in the minimum concentration of 25 μg/mL. Whereas (BAKUR; NIU; et al., 2019) used MELs as a reducing and stabilizing agent. The objective was to investigate the prevention of diseases caused by microorganisms (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* and *Clostridium perfringens*) in food products. Through ecological nanocomposites, the study investigated the antimicrobial effects of MEL@AgNPs, MEL@ZnONPs and Ag-ZnO/MEL/GA and the results indicated that the nanoparticles do not present a statistically significant difference ($p>0.05$) among them, with exception for *E. coli*, which showed a higher level of inhibition by MEL@ZnONPs.

So, MELs can be considered a promising component for new green technologies. They are biosurfactants produced from safe microbial sources and renewable materials - which enhances their structural variety and contributes to the reduced impact on the environment.

Studies such as those carried out by Bakur *et al.* (2019b) reported the good performance of MELs within particles designed with antimicrobial characteristics. In addition to this, remarkably inhibition against Gram-positive bacteria through its antibiofilm property was also observed. The bioactivity and excellent physicochemical properties of MELs against phytopathogenic microorganisms have vast potential for new applications in agriculture.

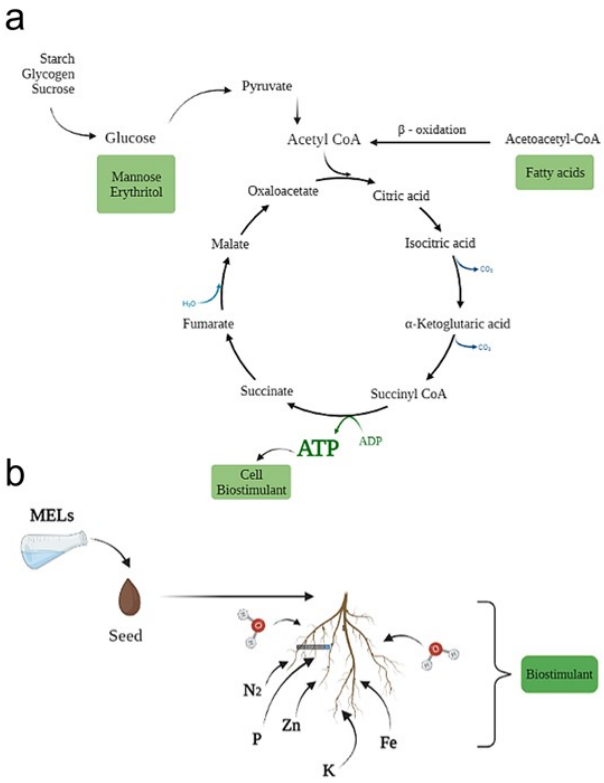
2.1.3 Glycolipids

Glycolipids are the most relevant biosurfactants in industrial terms. Their chemical structures are composed of carbohydrate(s) (hydrophilic moiety) bounded to lipid(s) (hydrophobic moiety). This class of biosurfactants includes MELs, rhamnolipids, and sophorolipids (DRAKONTIS; AMIN, 2020; MARIA et al., 2020).

2.1.3.1 Mannosylerythritol lipids

MELs are chemically composed of two fatty acids (hydrophobic moiety) and one mannose and one erythritol (hydrophilic moiety) (ANDRADE et al., 2017; COELHO et al., 2020b; GÜNTHER et al., 2015). The metabolisms related to mannose and erythritol, very likely, is intimately related to energetic metabolic pathways MELs-derived compounds can be completely oxidized, that is, increasing the production of ATP (glycolysis, Krebs cycle, among others). In addition, both fatty acids can also generate ATP from β-oxidation – Figure 3a. Thus, the MELs metabolization can be related to their biostimulating potential, or even cellular membrane changes, since they are surface-active agents (increase nutrient absorption rate) – Figure 3b.

Figure 4 - Presumable metabolism related to MELs - biostimulant effects



Source: From the author.

2.1.3.2 Rhamnolipids

Rhamnolipids are biosurfactants that have great antimicrobial activities. The applicability as agricultural additives and their potential function against bacteria contribute to their act as direct antimicrobial agents and stimulants to increase plant immunity, controlling the growth and spread of various phytopathogenic fungi (ABDEL-MAWGOUD; LÉPINE; DÉZIEL, 2010; MARIA et al., 2020; VATSA et al., 2010). Chong and Li (2017) showed that rhamnolipids could induce the synthesis of hormones responsible for signaling important pathways for plant immunity. Other researchers showed that rhamnolipids can induce genes involved in the defense systems of plants, being them grapevine (*Vitis vinifera* L.), thale-cress (*Arabidopsis thaliana*) and cherry tomato (*Lycopersicon esculentum*). This study also identified the effective defense of foliar tissues caused by applied rhamnolipids. In the first case, after 96 hours of inoculation with conidia of *B. cinerea*, the leaves that were previously immersed in a solution of rhamnolipids at 0.1 mg/mL did not present any change in color, corresponding to the maceration symptoms caused by the fungus. The observed defense responses may be related

to increased resistance to the fungus. In the second study, the bacterial resistance observed was directly related to the activation of the plant defense responses.

Vasconcelos *et al.* (2020) compiled data on rhamnolipids as plant immunity stimulants against phytopathogenic microorganisms, as presented in Table 2. According to the data reported in this table, rhamnolipids have a high potential to promote sustainability in agricultural production and replace conventional agrochemicals. However, studies that investigate the ability to biocontrol diseases, grow seedlings and activate defense genes against pathogens should be further investigated.

Table 2 - Rhamnolipids to stimulate plant defense mechanisms. Adapted from Vasconcelos *et al.* (2020)

Pathogen	**Plant**	**Application responses**	**Defense mechanisms activate**	**References**
Botrytis cinerea	Grapevine Thale-cress Rapeseed	The applications demonstrated resistance against the phytopathogenic fungus, when comparing treated and untreated tissues, as well as antimicrobial properties and induction of plant defense.	Expression of defense genes; Production of reactive oxygen species.	(Varnier *et al.* 2009; Vatsa *et al.* 2010; Monnier *et al.* 2018)
Alternaria alternata	Cherry tomato	Under laboratory conditions, low concentrations of *R. glutinis* and rhamnolipids, presented an expressive and safe alternative for the control of infection caused by *A. alternata.*	Peroxidase, polyphenoloxidase and phenylalanine ammonialyase activities of cherry tomato fruit.	(Yan *et al.* 2014)
Pseudomonas syringae pv tomato	Thale-cress	Immune responses, activation of the defense gene and, consequently, protection of the plant was induced by synthetic elicitor. This generates amphiphilic compounds with wide application in the agricultural market and helps to understand the molecular mechanisms of plants.	Interaction with plant plasma membrane; Early signaling activation.	(Luzuriaga-Loaiza *et al.* 2018)

2.1.3.3 Sophorolipids

Sophorolipids are a class of biosurfactant that present few number studies related to improve plant immunity. However, experiments with different sophorolipids samples in different plant growth stages were projected. The results show that these biosurfactants can defend plants from insect infestation and illness at a primary stage of growth (MARIA et al., 2020; SIEVERDING, Ewald, 2017).

According Gross and Schofield (2014), rhamnolipids are effective biopesticides, their production reach ≈ 45 g/L which is exponentially lower than sophorolipids production ≈ 700 g/L. The sophorolipids application prior to germination promoted a significant seed stimulation of seeds (KRAWCZYŃSKA et al., 2012). Thus, sophorolipids can be applied in agriculture formulation since they simultaneously act against phytopathogens (fungi and bacteria) and plant biostimulants.

2.1.4 The potential of mannosylerythritol lipids as plant biostimulants: a parallel with rhamnolipids, sophorolipids, and lipopeptides

The plant biostimulants are associated with their vital and structural processes, from root to flowers and fruits, from seeds to bushes (DJOULLAH; SAUREL, 2021). In this sense, they can be applied to seeds, plants, and soil, reducing the need of using fertilizers and promoting positive performance even at low concentrations - as they increase nutritional efficiency and tolerance to abiotic stress (DU JARDIN, 2015; YARONSKAYA et al., 2006). Thus, the biostimulants present a new sustainable approach for modern agriculture.

In this sense, tomato seeds were subjected to normal conditions and treated with a biostimulant Vivema Twin. As a result, the authors observed that even at low concentrations (75 μM), there was a high incidence of lateral roots in the germinated roots (CAMPOBENEDETTO et al., 2021). Similarly, Kim *et al.* (2014) applied a germination stimulant isolated from the black oat plant named as Avenaol at only 10 nM. As a result, they evaluated that it significantly induced (≈ 50%) the germination index of *Phelipanche ramosa* when compared to *Orobanche minor* and *Striga hermonthica* (0%). It was necessary to apply Avenaol at 100 nM for germination (< 20%) of *O. minor* and *S. hermonthica*.

However, biosurfactants play an essential role - in particular rhamnolipids, sophorolipids and lipopeptides (DU JARDIN, 2012; YARONSKAYA et al., 2006). Regarding the application of MELs in this area, Yoshida *et al.* (2015) demonstrated that MELs and other biosurfactants can be applied as antimicrobial agents against Gram-positive bacteria and phytopathogenic fungi - as a promising alternative to synthetic pesticides. Similarly, Buxdorf *et al.* (2013) identified inhibitory phytopathogenic fungi (*in vitro*) effects of metabolites from *P. aphidis*.

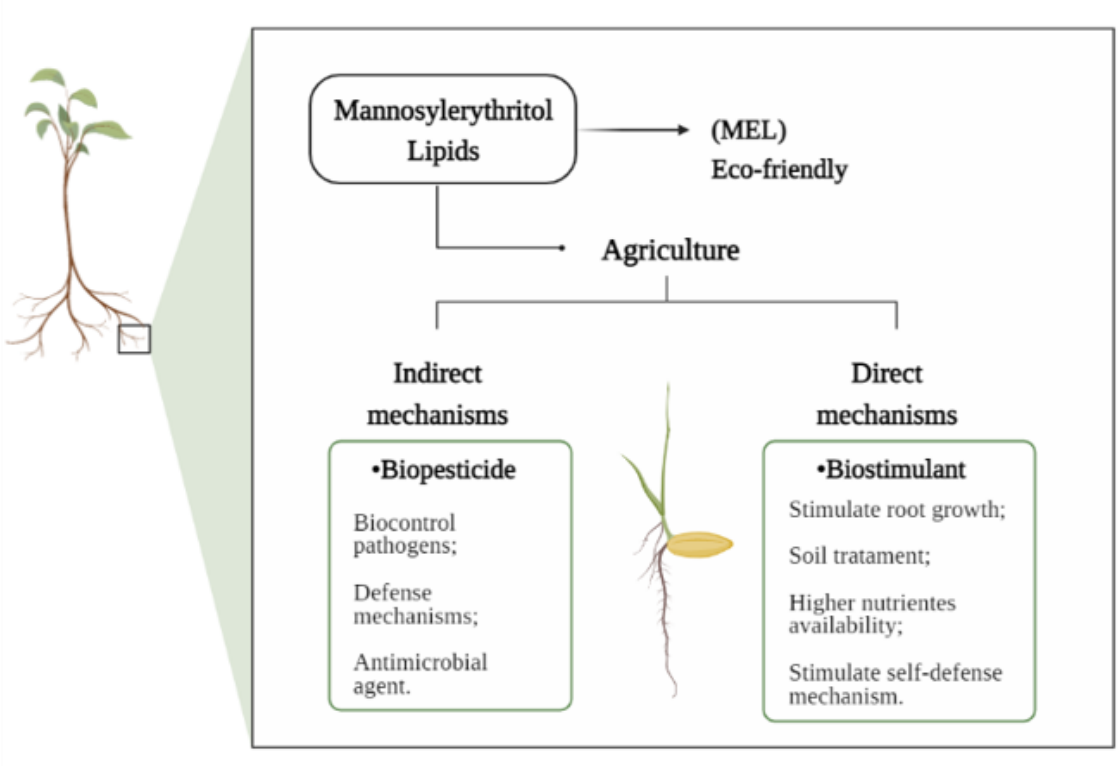
Although, to the best of our knowledge, the plant biostimulating effects of MELs were never investigated. MELs can also enhance the plant self-defensive mechanisms against phytopathogens. Thus, due to the lack of information on MELs biostimulating properties, the following biosurfactants rhamnolipids, sophorolipids, and lipopeptides were used to provide scientific evidence on the potential of MELs biostimulating. In addition, it was carried out a detailed discussion on the antimicrobial properties of MELs, in particular against phytopathogenics.

2.1.5 A perspective on mannosylerythritol lipids as green pesticides and biostimulants, simultaneously

It is fundamental to develop sustainable alternatives for modern agriculture. Biosurfactants are an interesting alternative to pesticides, at least for a partial replacement. In addition, it is critical to determine the biosurfactant glycolipid-based metabolism in germination, which will replace, partially or fully, the application of pesticides.

MELs are a promising seed biostimulating agents, since their molecules may be involved in the vital processes of plants, stimulating cell reproduction (growth) in seeds. Thus, as MELs induce in seed cultivation, they can promote beneficial plant morphology and physiology changes. The seed modifications can be noticed in the germination stage - where a root epidermis will be stimulated to the absorption of nutrients from the culture medium (piliferous region), as well as the vascular tissues will be stimulated to the growth of secondary structures. MELs-producing yeast-like species (*Pseudozyma ustilaginal*, *Pseudozyma ustilaginales* and, *P. aphidis*) may be involved in improving plant resistance mechanisms against phytopathogens, in addition to simultaneously presenting antimicrobial properties – Figure 4.

Figure 5 - MELs application in the agriculture



Source: From the author.

Therefore, the effects of MELs as a biostimulant and biopesticide simultaneously must be further investigated, to expand the understanding of the influence of MELs metabolic pathways on plant development, as well as their anti-phytopathogenic ability in environments of biotic stress.

2.1.6 Conclusion

It was possible to verify the biostimulating action of glycolipids in different forms of application. Under laboratory conditions (500 µg/mL), rhamnolipids expressed promising responses to control the fungus *A. alternata* in cherry tomatoes, where it inhibited 48% of spore germination. In these hands, the application of sophorolipids at 0.05g/L in the biopreparation of Avena sativa seeds culture medium stimulated 6% more the germination index than the control. An application of MELs on the leaf surface of a plant can present microbial inhibition. The mode of action of the fungus *P. aphidis* (MELs-producer) and the ability to control the

disease caused by *B. cinerea* on the surface of plants were found, that is, the protective effect and the resistance induced by *P. aphidis* was confirmed. The glycolipid properties of MELs can improve the biological quality of soil and seeds, contribute to the nutrition of germinated roots and stimulate plant growth. Still, there are more questions than answers regarding its application as a biostimulant. In this sense, it is recognized that it is necessary to investigate the biosurfactant-plant interactions in the different stages of seed cultivation and understand the biostimulant and antimicrobial potentials, simultaneously - a new perspective of the biotechnological and environmental application. The characteristics observed in biosurfactants indicate that these surfactants can be simultaneously biostimulant and antiphytopathogenic. Although MELs belong to the glycolipid group and are a safe strategy with less environmental impact, there is a lack of research in the literature with deeper approaches to this mechanism. Understanding these gaps along with ecological demands creates a relevant and promising approach to more sustainable agriculture.

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CHAPTER 3

In this section, the experimental article “The Biosurfactant Mannosylerythritol Lipids (MELs) as Stimulant on the Germination of *Lactuca sativa* L.” is presented. This chapter aims to give the reader an insight into MEL-B as a biostimulant in lettuce seeds. This chapter comprises the data obtained in the experimental part of this work from the direct application of MEL-B in the seed germination stage is reported and discussed according to the observations made in the morphological, physical-chemical and biochemical tests. The article was submitted for publication in Journal of Pesticide Biochemistry and Physiology.

3 THE BIOSURFACTANT MANNOSYLERYTHRITOL LIPIDS AS STIMULANT ON THE GERMINATION OF *LACTUCA SATIVA* L.

Abstract

Currently, the application of pesticides in the agricultural is fundamental. However, the continuous use of these products is related to environmental impacts. In this sense, the MELs - a promising alternative to modern agriculture - is a biosurfactant with antimicrobial, amphiphilic characteristics, and reduced toxicity compared to chemical surfactants. The aim of this study was to evaluate the biostimulant effect of MEL-B on the germination of SF 31 monic lettuce (*Lactuca sativa* L.) seeds and the influence on plant growth and root development. The seeds were germinated for seven days in Petri dishes under a culture medium containing different concentrations of MEL-B (0, 3.16, 31.6, 158, 316, and 632 mg/L). The incidence of germinated seeds, the index, and the average germination time were evaluated. Regarding root morphology, the length of the seedlings, gross mass, development of lateral roots, and roots under biotic stress were evaluated. The influence of MEL-B on the physiological behavior of the roots was also assessed based on the enzyme activity (peroxidase and polyphenol oxidase). The MEL-B at 158 mg/L stimulated seed germination, growth, and seedling development parameters. It was also noticed the appearance of lateral roots and a lower incidence of stressed roots. In addition, MEL-B at 158 mg/L was the highest concentration in which there was no phytotoxic effect of MEL-B on the seeds, whereas MEL-B from 316 mg/L showed an inhibitory effect on seed germination and contributed to the oxidative stress of the medium. The increase on the enzymatic activities corroborates the phytotoxic effect and consequent stress of seeds at 316 and 632 mg/L concentrations. In an unprecedented way, this study proved that MEL-B has a biostimulant and phytotoxic effect related to its concentration. Therefore, MEL-B has potential replacement, even partially, to conventional pesticides.

3.1 INTRODUCTION

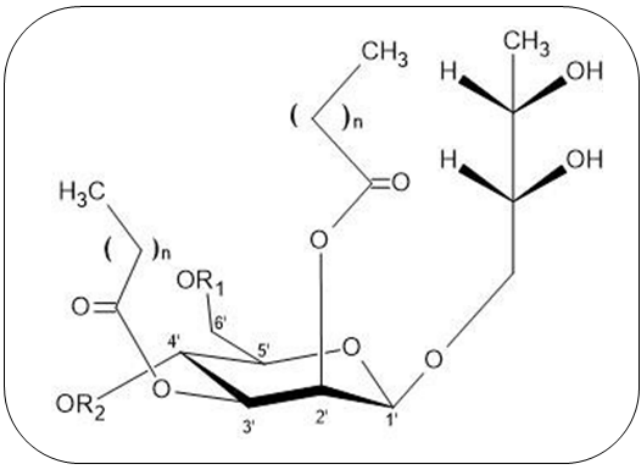
The application of pesticides in agricultural leads to higher productivity. However, pesticides are easily diffused in soil, air, and water – resulting in large environmental impact (MÜLLER et al., 2002; SHENG et al., 2001). In addition, they are vectors for simple and chronic human health problems, such as nausea and headaches, diabetes, and cancer (LOPES; ALBUQUERQUE, 2018). The commercialization of active ingredients used in the production of pesticides exceeds 4 million tons annually (MORAES, 2019). In Brazil, since 1990 the use of pesticides has been increasing over time. In 2019, approximately 13.300 chemicals were registered (AENDA, 2019). Pesticides are essential to high crop yields and high level of quality. On the other side, the modern agriculture should be efficient and also environmentally friendly.

Biostimulants are eco-friendly compounds that can stimulate plant metabolism and improve the absorption of nutrients in the soil (EUROPEAN UNION REGULATIONS, 2019). Biostimulants can be classified into four main groups, based on amino acids and protein hydrolysates; on humic substances; on microorganisms and; on inoculum and algae extracts (EMBRAPA, 2016).

To date, the biostimulant potential of biosurfactants has been subtly investigated. For example, the inhibitory effects (*in vitro*) of *Pseudozyma aphidis* metabolites on phytopathogenic fungi was studied. The results indicate that *P. aphidis* has potential application as a biocontrol agent for fungal pathogens (BUXDORF et al., 2013). In another study, the authors applied *Rhodotorula glutinis* and rhamnolipids on cherry tomatoes infected with *Alternaria alternata*. They concluded that, even at low concentrations, the mixture of *R. glutinis* and rhamnolipids is a safe alternative for controlling *A. alternata* infection (YAN et al., 2014). The screening of cultivation conditions with sophorolipids and application of them at different stages of plant growth was investigated. In response, it was observed that sophorolipids present efficient biocontrol activity for biotic and abiotic stress in the primary stage of plant germination (SIEVERDING, Ewald, 2017). The production of the biosurfactant with an anionic characteristic from *Candida sphaerica* UCP 0995 was investigated and then, the germination index was used to evaluate the toxicity of the biosurfactant in the germination of *Lactuca sativa* L., indicating that the solutions of 0.125, 0.25, and 0.5 g/L did not inhibit germination seeds or elongation of roots (LUNA et al., 2013). The biosurfactant production from *Candida lipolytica* UCP 0988 at 0.15, 0.3, and 0.6 g/L did not inhibit the germination of seeds *Lactuca sativa* L (RUFINO et al., 2014).

MELs are glycolipid biosurfactants (FUKUOKA; MORITA; KONISHI; IMURA; KITAMOTO, 2007; KIM, H.-S. et al., 2006). The acetylation-based classification of MELs includes MEL-A, MEL-B, MEL-C, and MEL-D, as demonstrated in Figure 5 (ANDRADE et al., 2017; COELHO et al., 2020b; GÜNTHER et al., 2015). This biosurfactant are amphiphilic molecules composed of a hydrophobic moiety (an acetyl group and/or a fatty acid) and a hydrophilic moiety (4-O-β-D-mannopyranose-erythritol) (ARUTCHELVI, J., DOBLE, 2010).

Figure 6 - General chemical structure of MELs. MEL-A, R1 and R2 = acetyl group; MEL-B, R1 = acetyl group and R2 = H; MEL-C, R1 = H and R2 = acetyl group; MEL-D, R1and R2 = H



Source: COELHO *et al.* (2020a)

Therefore, the aim of this study was to evaluate, unprecedentedly, the biostimulant potential of the MEL-B on Monica SF 31 lettuce (*Lactuca sativa* L.) seeds, in particular seed germination plant growth, root development and stressed roots.

3.1.1 Material and methods

3.1.1.1 Material

Monica SF 31 Lettuce (*Lactuca sativa* L.) seeds were purchased from the company Feltrin Sementes Ltda., registered in the National Registry of Seeds and Seedlings (RENASEM) of the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA). MEL-B at 95% purity was kindly provided by Toyobo JP Ltda., Osaka, Japan. Guaiacol, sodium phosphate, phosphoric acid, hydrogen peroxide, PBS and BSA reagents are analytical grades obtained from Sigma and Merck.

3.1.1.2 Growing medium containing MEL-B for lettuce seed germination

The germination tests were carried out in a Petri dish containing purified agar (% agar) at different concentrations of MEL-B (0, 3.16, 31.6, 158, 316, and 632 mg/L). Simultaneously, the MEL-B was weighed on an analytical balance (AD-500, Marte) and subsequently solubilized into agar using a vortex mixer (K45-2820, Kasvi). After homogenizing, the media were transferred to Petri dishes inside a flow chamber. The plates were sealed and stored in the refrigerator until use (SIEVERDING, Ewald, 2017).

3.1.1.2.1 Contact angle and surface tension

The influence of different concentrations of MEL-B on the measurement of contact angle and surface tension was performed in a goniometer where drops of soybean oil or diiodomethane were placed on the surface of agar containing MEL-B by using a micropipette of 100 µL. The procedure was performed in triplicate at 25 ºC. The drops were photographed by a digital camera (Ramè-Hart, 250-F1). The photo was subjected to digital processing to obtain the width and height (LIMA; FERNANDES, 2019). The contact angle and surface tension values were provided by the Drop Image.

3.1.1.3 Germination test

Lettuce seeds were sterilized with aqueous alcohol solution (95%) alcohol for 5 minutes and then abundantly washed with distilled water. Then, the 100 seeds were distributed in 10 plates and incubated in a BOD chamber (New Lab, NL-41-02) with controlled relative humidity (60%) for seven days, with day and night simulation at 25 and 20 °C, respectively. The number of lettuce germinated seeds was monitored for each concentration of treatment with MEL-B (0, 3.16, 31.6, 158, 316, and 632 mg/L). The germination speed index (GSI) and mean germination time (MGT) were calculated using Equations 1 and 2 (BRASIL, 2009).

3.1.1.3.1 Germination speed index (GSI)

During the germination period, the germination speed index of emerged seedlings was performed daily and calculated according to Maguire (1962):

$$Germination\ speed\ index\ (GSI) = {}^{N1}/_{D1} + {}^{N2}/_{D2} + \cdots + {}^{Nn}/_{Dn} \qquad \text{Equation 1}$$

Where: GSI = germination speed index; N1, N2, Ni = number of seeds germinated in the first count, second count, i-th count, respectively; D1, D2, Di= number of days in the first count, second count, i-th count, respectively. Unit: dimensionless.

3.1.1.3.2 Mean germination time (MGT)

The plates were monitored daily, and the average germination time was calculated as proposed by Labouriau (1983):

$$Mean\ germination\ time\ (MGT) = {}^{\sum ni\ .\ ti}/_{\sum ni} \qquad \text{Equation 2}$$

Where: MGT = mean germination time; ni = number of seeds germinated in time ti (not the accumulated number, but the one referred to the i-th observation); ti= time between the beginning of the experiment and the i-th observation. Unit: days.

3.1.1.4 Morphological parameters in lettuce cultivation

The seeds were cultivated with different concentrations of MEL-B. The behavior of lateral roots, stressed roots, length, and mass were evaluated. Regarding the appearance of lateral roots, the emergence of lateral roots was evaluated from the 3th day of cultivation. One hundred Petri dishes containing 10 seeds per dish were monitored. All samples were observed visually and under a magnifying microscope (Technical, stereoscopic) (ZHANG; HASENSTEIN, 1999).

Stressed roots were counted as they did not germinate and/or showed low performance. In addition, the seeds that showed delay in the germination process during the observation of the 7 days of the experiment were taken into account. The accumulated records were represented in percentage at the end of the experiment.

The length of the roots (cm) was measured from the third day of germination to the seventh day. The procedure was performed in triplicate (LUIZ et al., 2015).

The mass of the samples used to prepare the crude enzymatic extract was determined using an analytical balance (Marte, AD-500). Seedlings (leaves and roots) were collected from Petri dishes daily and weighed. For each concentration, three runs were performed.

3.1.1.5 Morphological analysis by Scanning Electron Microscopy (SEM)

The lettuce root samples were fixed with glutaraldehyde (2.5 %) for 30 minutes. Then, they were dehydrated with an alcohol series (10, 30, 50, 70, 80, 90, and 100 %) and dried at room temperature. For the analysis, the lettuce was distributed on carbon tapes on the surface of stubs and then coated with a layer of gold. After recovering, the samples were analyzed in SEM (JEOL JSM (6390LV)), with a tungsten electron source secondary electron detector at 10 kv (AL SHEHADAT S., 2018).

3.1.1.6 Physicochemical characterizations of total proteins and activity of peroxidase and polyphenol oxidase enzymes

The study of the influence of MEL-B on germination and the induction of stress conditions in cultivation was carried out by quantifying total proteins and analyzing the activity of peroxidase and polyphenol oxidase enzymes.

3.1.1.6.1 Crude enzyme extract

Crude enzyme extraction was performed daily from the 3th day of cultivation. After this period, the seeds that visibly started the germination process were selected. To extract the enzymes, the selected roots were weighed and macerated in a crucible under an ice bath. The addition of 1 mL of 50 mM sodium phosphate buffer was added until a homogeneous mass was obtained. Then, the plant material was transferred to microcentrifuge tubes and then centrifuged at 15952 G-force for 10 minutes. The supernatant was used to determine enzymatic activity and protein quantification (BRADFORD, 1976).

3.1.1.6.2 Protein content

For protein quantification, the traditional Lowry method was used, proposed by Pomory (2008), with modifications. The test was performed in triplicate. 100 µL of crude extract and 2 mL of solution C (Na_2CO_3 (2%) in 1M NaOH + $CuSO_4$ (0.5%)) were added to test tubes. The mixtures stand for 10 minutes. Subsequently, 200 µL of Folin reagent was added, shaken and left to rest for another 30 minutes. The reading was performed under absorbance at a wavelength of 750 nm and calculated concerning the mass (g) of the sample used to prepare the crude extract. An average of the absorbances obtained was made to determine the protein concentration in each analyzed sample. The calibration factor of the calibration curve was determined and, finally, the calculation of the protein concentration for each sample was performed, according to the equation:

$$C = {Abs\ of\ sample\ x\ F}/{mass\ of\ \ the\ sample\ (g)} \qquad \text{Equation 3}$$

Where: C = concentration of protein in each sample; F = calibration curve factor.

This determination was performed in triplicate.

3.1.1.6.3 Peroxidase activity

A 140 µL aliquot of sodium phosphate buffer (50 mM, pH 6.4) containing 0.3 % (v/v) guaiacol was used. An aliquot of 100 µL of crude enzyme extract and 60 µL of H_2O_2 (0.3 %) was added. Enzyme activity was determined by spectrophotometer by observing the variation in absorbance at 470 nm and 25 °C for 5 minutes (GUO et al., 2014).

3.1.1.6.4 Polyphenoloxidase activity

Polyphenoloxidase (PPO) activity was performed according to the methodology presented by Matsuno e Uritani. This analysis was determined using catechol (0.02 mol/L) as a substrate for the enzyme. The reading was determined in proportions of 0.30 mL of sample and 1.85 mL of 0.10 M solution of phosphate buffer pH 6.0 with catechol. The absorbance was read in a UV-Vis spectrophotometer (Spectra Max, 384 plus) at 395 nm. The reading was

performed every 1 min for 10 min, and water was used as a blank (MATSUNO; URITANI, 1972).

3.1.1.7 Statistical analysis

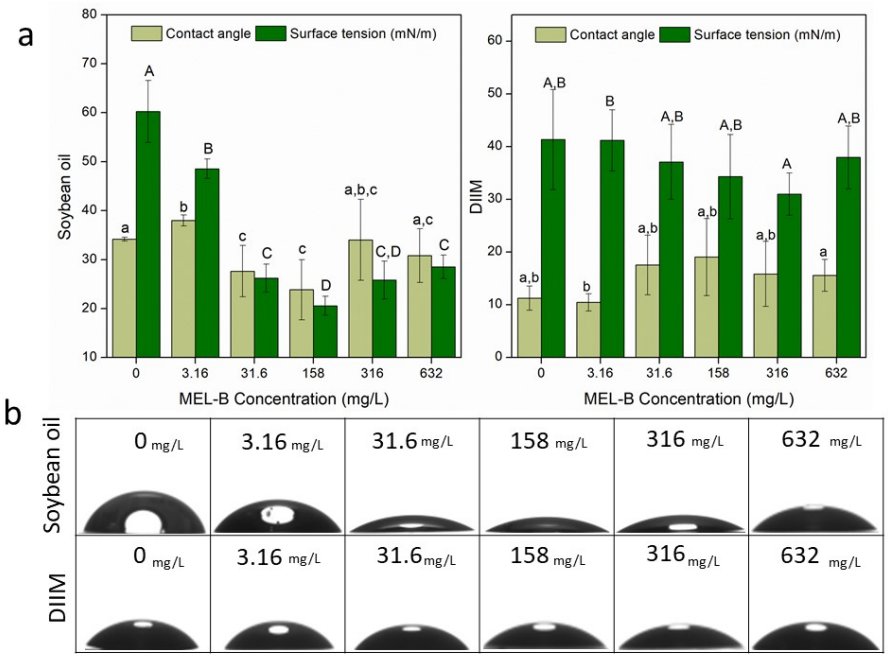
The experimental plots consisted of 100 seeds at each concentration of treatment with MEL-B for daily monitoring of germination and 1.600 destructive samples to evaluate the behavior of germinated seeds and physical-chemical analyses, totaling 2.200 analyzed seeds in the period of 7 days of cultivation. Data were submitted to a one-way analysis of variance (ANOVA) significance test, and the difference was compared using Tukey's test ($p \leq 0.05$).

3.1.2 Results and discussion

3.1.2.1 Contact angle and surface tension

Hydration of the plant cell is essential for biochemical reactions and seed metabolism (ÖRDÖG; ZOLTÁN, 2011). In this sense, MEL-B is an surface active agent - it reduces the surface tension (COELHO et al., 2020b). Thus, the Figure 6 shows the influence of different concentrations of MEL-B on the interaction of drops of diiodomethane (DIIM) and soybean oil with the treated surfaces.

Figure 7 - (a) Contact angle and surface tension using oil and diiodomethane, (b) drops in contact with the surface of the medium containing different concentrations of MEL-B. Means followed by the same letter do not differ from each other by the Tukey Test (P<0.05)



Source: From the author.

DIIM is a highly non-polar molecule, truly little water-soluble (VENTUROSO, 2009). Figure 6a shows that this molecule did not undergo significant variation in contact angle and surface tension, after contact with agar surfaces treated with different concentrations of MEL-B. This behavior was expected, since naturally the DIIM molecule has low surface tension (ADAMSON; GAST, 1997). Therefore, despite having a contact angle lower than 90° and showing a wetting aspect, MEL-B did not promote the interaction of DIIM at the surface. Furthermore, the variation of MEL-B concentrations did not imply the reduction of the contact angle, as shown in Figure 6b.

On the other hand, a different response was observed after adding soybean oil. The surface tension reduced as the MEL-B concentration increased up to 31.6 mg/L, followed by a surface tension (quasi)plateau for higher MEL-B concentrations. MEL-B at 158 mg/L showed a significant difference in surface tension reduction about the control (Fig. 6a). The behavior of the contact angle corroborates with this speculation since from the treatment carried out with 31.6 mg/L of MEL-B, wettability tended to increase. Due to the glycolipidic characteristics of MEL-B, the culture media supplemented with the biosurfactant suffered a weakening of the binding of water molecules. Thus, the surface tension reduced as the concentration of MEL-B

increased. In the case of the soybean oil drop, the observed behavior may be related to the lipophilic character of the biosurfactant (SCHULTZ; ROSADO, 2020).

In general, the behavior observed with soybean oil under different cultivation media can predict the behavior of the lettuce seeds in the present study. In this sense, the lower surface tension - due to the increase in MEL-B concentration in the culture medium - may reflect higher levels of seed wettability in contact with the treated surfaces. Therefore, greater seed hydration is expected and, consequently, a more significant influence of the bioactive properties of MEL-B on seed germination as biosurfactant concentrations increase.

3.1.2.2 Germination properties

Figure 2 shows the influence of the MEL-B concentration added to the media on the number of germinated lettuce seeds (Figure 7a), germination speed index - GSI and mean germination time - MGT (Figure 7b), and the appreciation of secondary roots appearance (Figure 7d).

Figure 8 - (a) Cumulative germination of seeds, (b) GSI represented by vertical bars and MGT represented by points, and (c) representative image of Lettuce seeds germinated after 24 hours of cultivation (d) representative image of Lettuce seeds germinated after 4 and 7 days of cultivation. Means followed by the same letter do not differ from each other by Tukey's test (P<0.05)

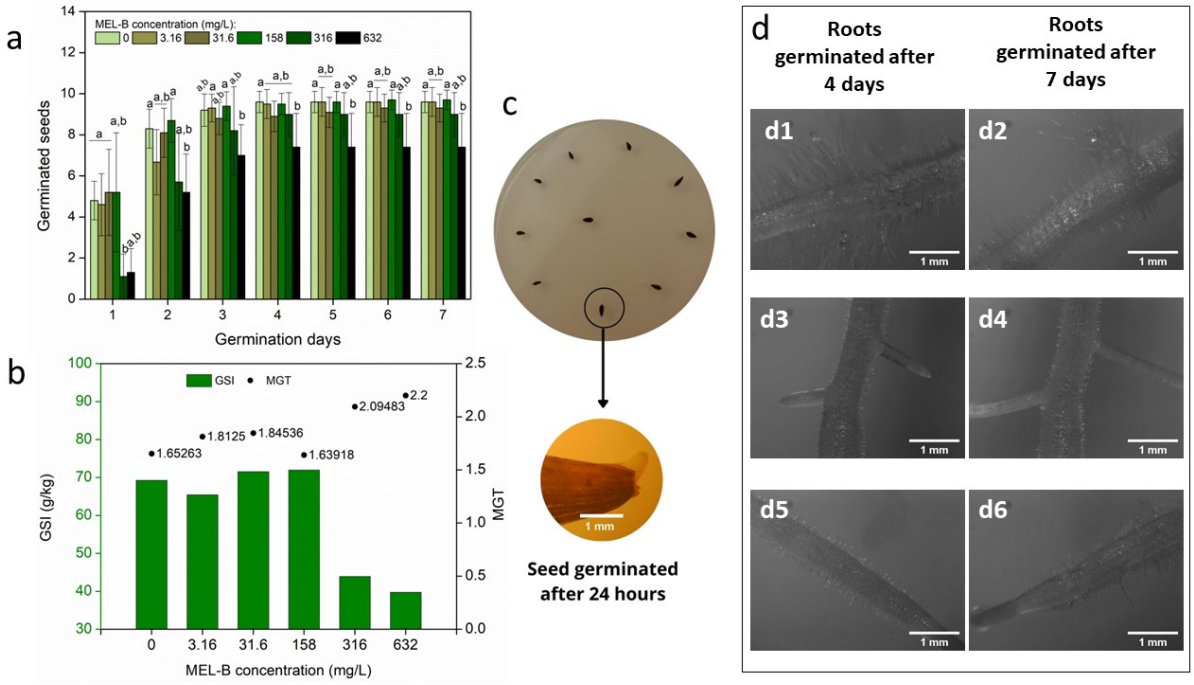


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Figure 7c reports the characteristic behavior of seeds that germinated after 24 hours of cultivation. The observation was realized using an optical microscopical at 10x enlargement. The concentrations of MEL-B added the culture medium influenced the incidence of germination (Figure 7a), however all the seeds germinated on the 1th day showed the same morphological characteristic, independently of the MEL-B content present.

The incidence of germination was observed cumulatively during the germination lettuce seeds (Fig. 7a). On the 1th day of the experiment, it was observed that MEL-B at 316 and 632 mg/L affected seed germination, where only about 15 % of the seeds germinated out of 100 %. In contrast, at lower concentrations (0, 3.16, 31.6 and, 158 mg/L) of MEL-B, more seeds (greater than 40 %) germinated under each treatment condition. The differences in relation to the control were significant ($p \leq 0.05$) only in the concentration of 316 mg/L of MEL-B; it is noticed that the seeds cultivated with 316 and 632 mg/L of MEL-B germinated less in comparison with the other conditions. Even though, germination was higher than 80 % in all growing conditions, except for the concentration of 632 mg/L of MEL-B, which was the highest used for seed germination and had lower levels since the first day of germination (Fig. 7a). The same level of incidence in soybean seed germination (less than 80%) after treatment with rhamnolipids was observed. However, the concentrations of rhamnolipids used for seed treatment were higher than the concentrations used in this study (SANCHETI; JU, 2020). In other study, evaluated the effect of rhamnolipid on lettuce germination and growth. They noted that the concentration of 750 mg/L stimulated lettuce seed germination but impaired radicle development compared to control (LUIZ et al., 2015).

One of the indicators of seed vigor is the GSI, which is directly proportional to each other. That is, the higher the GSI, the more vigorous the seed (FERREIRA, A. G.; BORGHETTI, 2004). About GSI, MEL-B promoted similar responses for control and intermediate conditions (3.16, 31.6, and 158 mg/L), indicating values above 65% for GSI. Differing significantly from concentrations of 316 and 632 mg/L, GSI showed an inhibitory effect by MEL-B with values of 43.9 and 39.7%, respectively (Fig. 7b).

The results obtained for the GSI were corroborated by the MGT, where the time required for germination was greater for concentrations of 316 and 632 mg/L of MEL-B. For the culture containing 158 mg/L of MEL-B, there was a decrease in MGT compared to subsequent concentrations, returning to an increase in following treatments. This observation demonstrates that the average germination time of lettuce seeds is progressively increased under biotic stress.

Treatment with MEL-B reduced GSI, increasing MGT at 316 and 632 mg/L. At the concentration of 158 mg/L, the results for the same parameters were the opposite, confirming a less pronounced inhibitory effect than the other cultivation conditions (Fig. 7b). MEL-B showed more significant interaction with the external tissue surrounding the seed due to its chemical structure. MEL-B increased the permeability of the seeds and contributed to a better performance in the germination of the seeds in some concentrations (NORMAN, 1965).

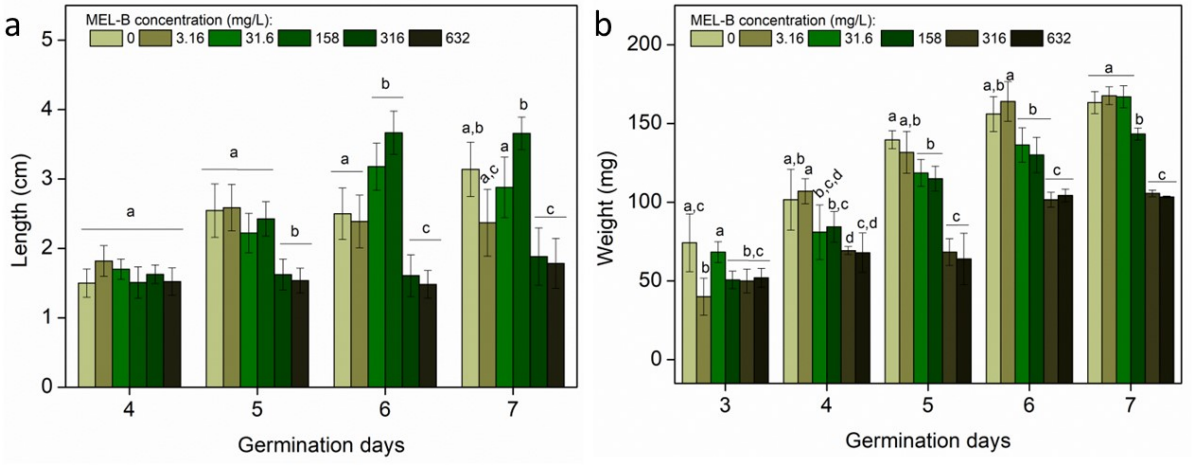
Figure 7c shows the behavior of all roots after 24 h of cultivation. In all conditions, the same behavior of the germinated seeds was observed. In Figure 7d, morphological observations of the germinated roots after 4 and 7 days of cultivation. Figure 7d1 and d2 demonstrates the predominant behavior of seeds grown in the medium without MEL-B treatment (control) in the medium containing MEL-B at 3.16 and 31.6 mg/L. In the records represented on 7d3 and d4, the evolutionary behavior of the germinated seeds in the culture medium containing MEL-B at 158 mg/L was compiled. The treatment performed with MEL-B at 316 and 632 mg/L is represented in Figure 7d5 and d6. Thus, observations recorded with the aid of a microscope indicate that the different treatments with MEL-B caused morphological changes in the roots.

3.1.2.2.1 Morphology of the roots

Root growth was monitored from the 4th day of seed germination (Fig 8). It is noted that on this 1th day of observation, all cultivation conditions showed similar behavior. That is, all verified roots showed similar size in the measurements (~1.8 cm).

However, from the 5th day of monitoring, the concentrations of 316 and 632 mg/L of MEL-B showed a significant difference in relation to the size of roots grown under control conditions. On the 6th day of cultivation, the roots cultivated at a concentration of 158 mg/L of MEL-B reached the largest size (less than 3.5 cm in length) compared to the other treatment conditions. In addition to presenting the largest size among the concentrations, it was the largest size observed among all the experiment days. In general, the seeds grown without MEL-B and the low concentrations (3.16, 31.6, and 158 mg/L) of MEL-B tend to grow with the days of cultivation. However, seeds germinated with 316 and 632 mg/L of MEL-B were less than 2 cm in length and did not show significant development since the 1th day of monitoring.

Figure 9 - Monitoring (a) length and (b) weight of roots in each treatment performed with MEL-B. Means followed by the same letter do not differ from each other by Tukey's test (P<0.05)



Source: From the author.

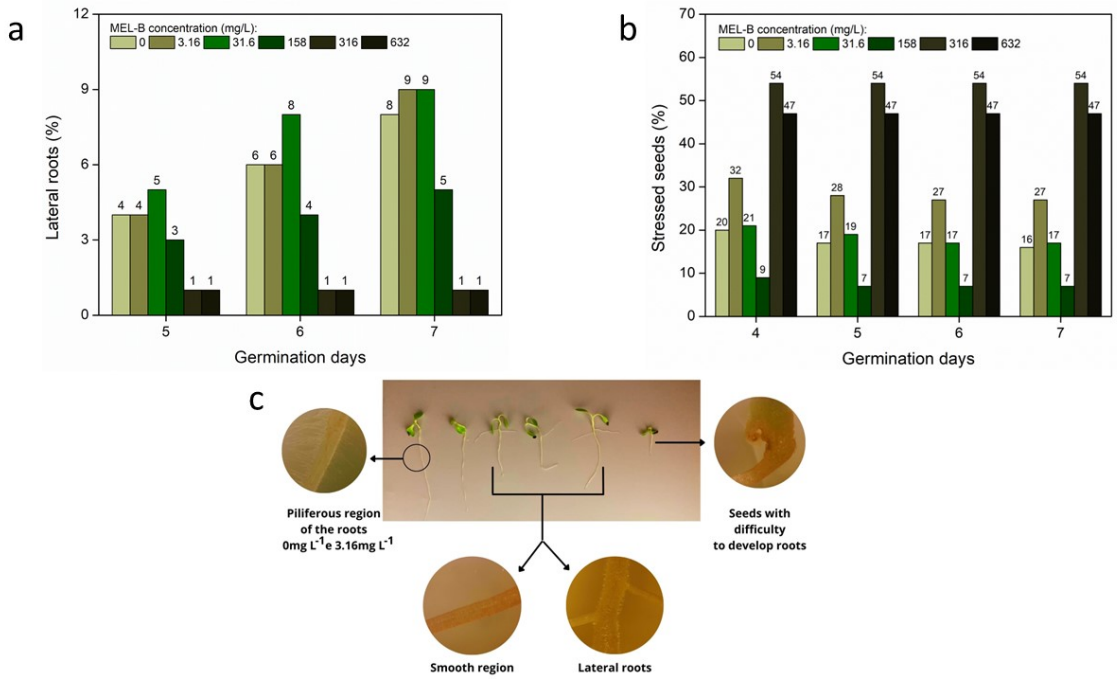
Another parameter monitored was the weight of the germinated roots (Fig. 8b). On the 3th day of the experiment, the roots germinated in the control conditions showed an average difference of 34.33 mg in relation to the roots germinated with medium containing 3.16 mg/L of MEL-B. On the 4th day of germination, the weight of roots grown in the control and 3.16 mg/L of MEL-B were >100 mg and showed a significant difference in treatments made with 316 and 632 mg/L of MEL-B, which were lower than only 70 mg. From the 5th day, the behavior was similar for all concentrations. The tendency to increase the weight as the roots developed was observed. However, at MEL-B concentrations of 316 and 632 mg/L, the same pattern observed in Figure 8a was observed for the same treatment conditions. The roots developed less when compared to the control and other concentrations. On the last day of monitoring, the roots that developed the most (in this parameter) were without treatment and with 3.16 and 31.6 mg/L of MEL-B, obtaining an average weight >160 mg. The opposite was observed for roots treated with 632 mg/L of MEL-B. The lowest average weight (<105 mg) was observed in this condition compared to the control and the other concentrations on 7th day.

Similarly, was noted that seeds subjected to rhamnolipid treatment showed a decrease in root length as the concentration increased. This observation may suggest a phytotoxic effect of this by-product against seedlings at high concentrations. In relation to root mass, the highest treatment concentration with rhamnolipid (1 g/L) showed a lower mass than control and other treatments (LUIZ et al., 2015). This behavior was also observed in the treatment with MEL-B at 316 and 632 mg/L in this work. In another study, using the biostimulant Coveron in lettuce

germination the observed effects were positive about the control, where the length of the lettuce roots grew up to 2.1 cm at the end of the experiment (POŠTIĆ et al., 2021). Compared to this study, MEL-B showed superior results, with roots up to 3 cm in length under treatment at a concentration of 158 mg/L.

Factors that cause adverse reactions in the development of lettuce seeds were noted in the experiment (Fig. 9). The treatment performed with MEL-B on seed germination promoted the development of lateral roots (Fig. 9a) at intermediate concentrations and created a stress (Fig. 9b) medium at higher concentrations. In addition to the morphological observations, as illustrated in Figure 9c.

Figure 10 - Monitoring (a) stressed roots, (b) secondary roots and (c) illustrative image of root regions on the 7th day of cultivation under different concentrations of MEL-B



Source: From the author.

As observed on the 4th day of germination (Fig. 9b), there was an increase of 34 and 27% for treatments made with 316 and 632 mg/L of MEL-B compared to the control. For the treatment with MEL-B at 158 mg/L, the stressed roots reduced by 11% when compared to the control. From the 5th day of cultivation, intermediate concentrations (3.16, 31.6, and 158 mg/L) and the control showed reduced stressed seeds. This behavior may reflect seed dormancy, a phenomenon that causes an intrinsic temporal block that provides additional time for

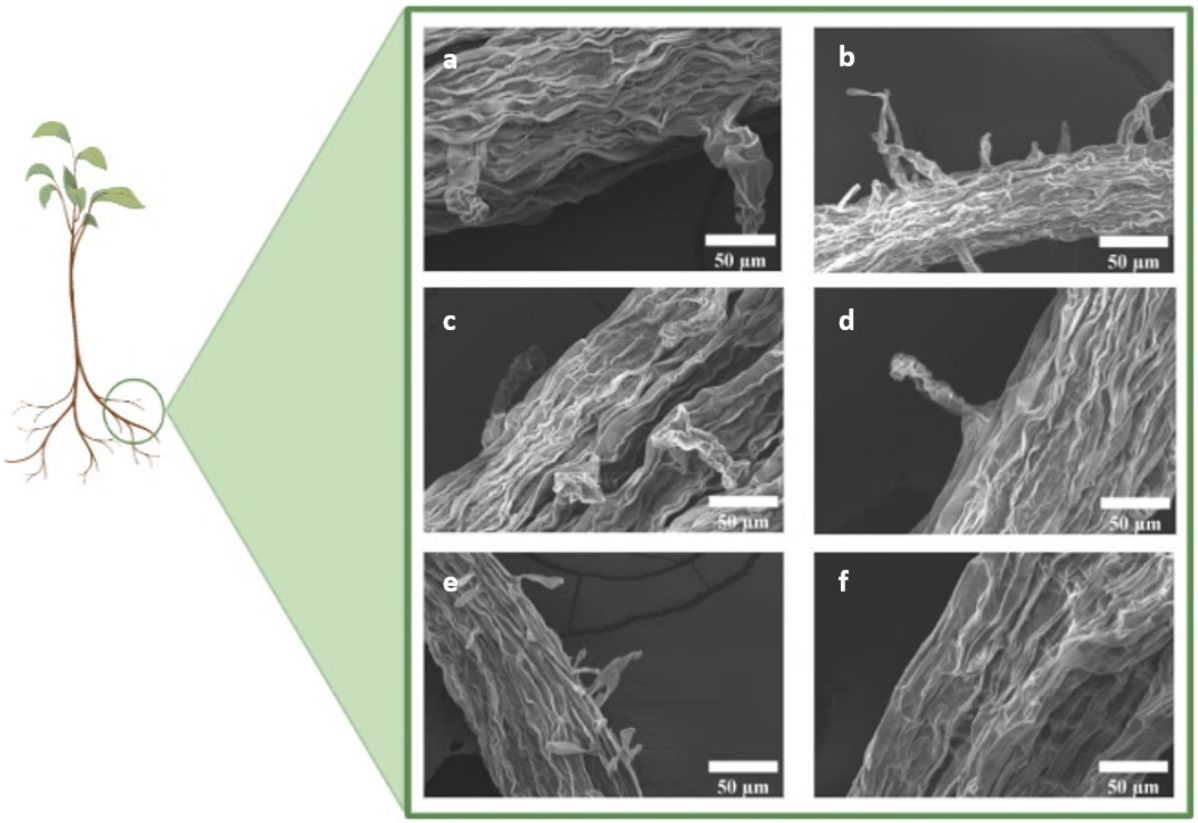
germination. On the other hand, seeds grown under treatment of 316 and 632 mg/L of MEL-B remained with 54 and 47% of the stressed samples (Fig. 9b). At the end of the experiment, it was noticed that the treatment performed with 158 mg/L of MEL-B presented only 7% of the roots stressed. That was the only concentration that showed a reduction of stressed roots among the treatments made with MEL-B compared to the control.

Sophorolipids were applied to the germination of barley seeds. In 10 days of germination, 195 mg/L of this biosurfactant stimulated the development of 9 lateral roots. In comparison to the control, the application of sophorolipid was superior in 2% in the stimulation of the lateral roots (SIEVERDING, E., 2017). However, the treatment performed with MEL-B at 31.6 mg/L stimulated the same number of lateral roots in seven days of cultivation (Fig. 9a). Regarding stressed roots, noted that the development of germinated roots after treatment with rhamnolipid at 1 g/L was lower compared to control and intermediate concentrations (LUIZ et al., 2015). This behavior was also observed when seeds were germinated with MEL-B at 316 and 632 mg/L (Fig 9b). In this sense, at high concentrations these bioproducts have an inhibitory effect on seed germination. However, the biostimulant effect of MEL-B can be noticed in lower amounts when compared to rhamnolipid and sophorolipid.

The influence of different treatments with MEL-B is also recorded in Figure 9c. The behavior of the germinated roots after 7 days of cultivation show that the intermediate treatments (3.16, 31.6, and 158 mg/L of MEL-B) did not inhibit the development of the roots. The opposite is registered when the seeds were germinated in the culture medium treated with MEL-B at 316 and 632 mg/L. Under these conditions, the roots showed adverse behavior in relation to the control and other treatments.

The microstructural analysis of the root surface was performed after 7^{th} days (Fig. 10). SEM is another alternative that makes it possible to interpret the surface of the roots, evaluate the microstructure and correlate with the potential influences of treatments with MEL-B in the cultivation. The evaluated roots presented plant tissue with an irregular shape and contracted cellular aspect in all treatments performed. In this way, the cell walls characterize the appearance of withered plant cells.

Figure 11 - Scanning electron microscopy (SEM) of radicle and primary root of Monica lettuce after seven days of cultivation. (a) Root grown without treatment, and Radicle treated with MEL-B (b) 3.16 mg/L, (c) 31.6 mg/L, (d) 158 mg/L, (e) 316 mg/L and (f) 632 mg/L



Source: From the author.

Under control conditions and treated with 3.16 mg/L of MEL-B, the plant tissue of the primary roots showed a well-developed hairy region (Fig. 10a and 10b). Although apparently in smaller amounts, the development of the piliferous region was observed in seeds cultivated under treatment of 31.6 and 158 mg/L of MEL-B (Fig. 10c and 10d). For 7 days of cultivation, the seeds treated with up to 158 mg/L of MEL-B presented root growth superior to those treated with 316 and 632 mg/L of MEL-B. The observations made with SEM corroborate the behavior illustrated in Figure 9c, where a visual comparison of root development under different treatment concentrations was performed.

Furthermore, it was noted that the plant tissue of the primary roots of seeds treated with 316 mg/L of MEL-B has a shape and structure similar to those observed in the root tissue of seeds germinated with 632 mg/L (Fig. 10f). However, a considerable reduction in the development of the hairy region with these treatments was seen. In addition, the integrity of the roots was compromised. However, with a low rate of seeds germinating under these conditions,

root growth and morphological development were significantly lower than the control and other treatment concentrations.

Thus, it can be said that the mechanisms of action of MEL-B in lettuce seeds contribute to the oxidative stress of roots when treated with MEL-B at 316 and 632 mg/L. Furthermore, this imbalance in plant tissue and root development indicates a lack of control in primary plant metabolism in which ATP synthesis may be compromised.

3.1.2.3 Quantification of protein and enzyme activity

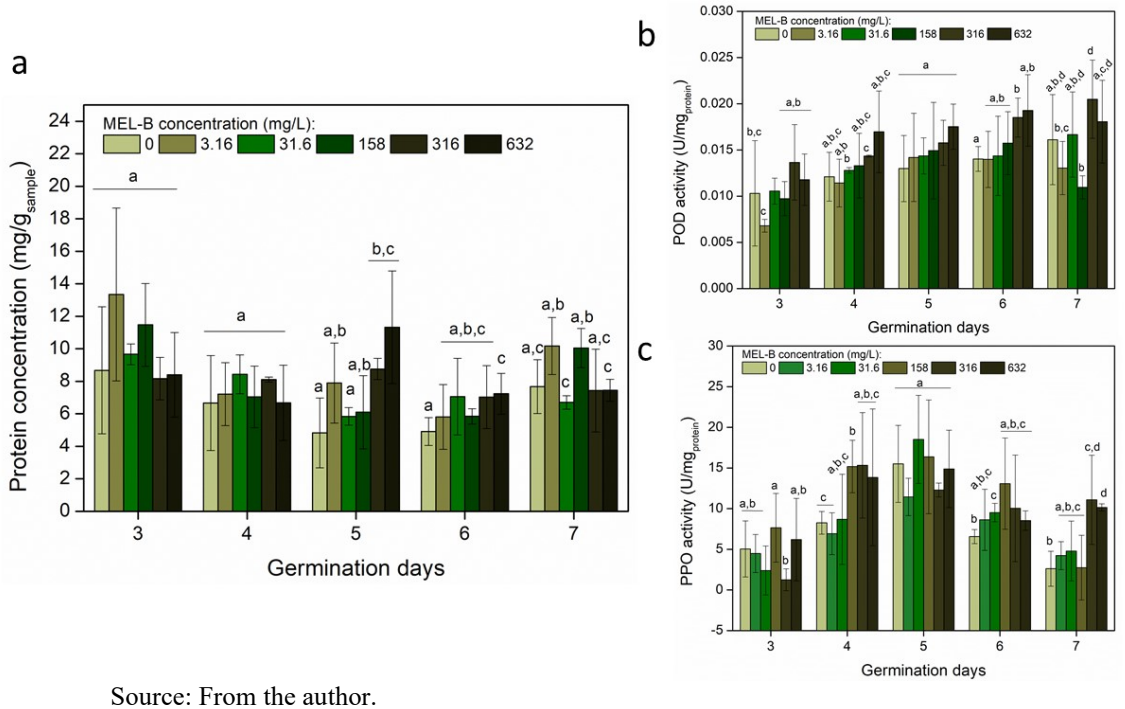
The reduction in germination and root length can be attributed to the decrease in cell divisions due to morphological and physiological changes caused by the treatment used in seed germination. Figure 11 illustrates the biochemical responses in relation to enzymatic activities and the quantification of total proteins after seed cultivation with MEL-B.

The protein quantified in each treatment condition was evaluated from the crude extract of the sprouted roots. The crude extract obtained by the roots on the 3th day of germination was produced with roots of lower size and mass than the roots that germinated for 4 days and successively until the 7th day of germination. In this sense, it was observed that even though the root mass decreased with increasing concentrations of MEL-B in the culture medium (Fig. 8b), the protein quantification remained at similar values and without a statistical difference ($p \leq 0.05$) - for the roots germinated until the 4th day of the experiment (Fig. 11a). In contrast, subsequent quantifications showed that the protein did not change significantly even when the root masses decreased. Studies indicate that proteins are covalently linked to the lignin molecule (DIEHL, 2014). The protein molecule is associated with cellulose in the cell wall and can confer rigidity, impermeability and resistance against biological attacks to plant tissues. In addition, lignification of root tissues can promote anatomical changes and influence water absorption, affecting root cell elongation. This physiological alteration can corroborate with the interpretation of the behavior of the germinated roots under treatment of 316 and 632 mg/L of MEL-B and validate the stress that these concentrations cause in the germination of the seeds. From the 4th day of the experiment, it is noted that all concentrations present a higher or equal amount of protein than the control. In addition, the highest amount of protein obtained in this study was 11.33 mg/g on the 5th day of germination, with the extract of germinated roots under treatment performed with 632 mg/L of MEL-B.

Peroxidase and polyphenol oxidase enzymes are pathogenesis-related enzymes involved in the cell wall lignification process and plant defense development processes in response to biotic and abiotic stresses.

When analyzing the peroxidase activity (Fig. 11b), MEL-B treatments caused changes in the peroxidase enzyme activity. On the 3th day of germination, only 316 and 632 mg/L of MEL-B concentrations showed higher enzyme activity than the control. In the evaluations carried out from the fourth day onwards, it was noted that there was no difference in enzyme activity between the treatments and the control. On the 7th day of the experiment, the highest enzyme activity was 0.0205 U/mg$_{protein}$ in the treatment with 316 mg/L of MEL-B. On the other hand, the concentration of 158 mg/L showed the lowest enzyme activity for the same day of culture. In general, about enzymatic activities, the results showed that the levels of the peroxidase enzyme are low. However, plants may have suffered oxidative stress after treatment with MEL-B, which resulted in higher levels of peroxidase enzyme activity at higher treatment concentrations.

Figure 12 - (a) Total protein in cultivated lettuce seeds, (b) Peroxidase enzymatic activity, and (c) Polyphenoloxidase. Means followed by the same letter do not differ from each other by Tukey's test (P<0.05)



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The polyphenol oxidase enzyme (Fig. 11c) had its activity increased until the 5th day of germination. On the 6th and 7th days, lower levels of activity were observed. The treatment performed with 31.6 mg/L of MEL-B showed 18.5 U/mg$_{protein}$ on the 5th day of germination,

which was the highest level of enzyme activity among all the days of cultivation. On the 7th day of germination, treatments made with 316 and 632 mg/L of MEL-B showed the greatest differences in enzyme activity in control, 8.48 and 7.54 U/mg$_{protein}$, respectively. In addition, treatments performed with 316 and 632 mg/L of MEL-B influenced lower root development when compared to control and intermediate concentrations. However, it is noted that the enzyme activity levels remained above 10 U/mg$_{protein}$. The increase in polyphenol oxidase activity occurred without increasing the protein concentration indicating that the roots were subjected to water stress.

For both enzymes, the size and mass of the roots decreased as MEL-B concentrations were increased. The enzymatic activity was evaluated using the crude extract of these roots. The responses obtained in this step corroborate the observations made in the previous steps, indicating that the highest treatment concentrations (MEL-B at 316 and 632 mg/L) caused stress in seed cultivation.

3.1.3 Conclusion

Unprecedentedly, it is the first report on the influence of MEL-B on seed germination. MEL-B at 158 mg/L showed promising results in the biostimulation of cultivated seeds. On the other hand, the responses observed in the physiological and biochemical behavior indicate that MEL-B at 316 and 632 mg/L influenced oxidative stress and inhibited the germination and development of the seeds. However, it is fundamental to identify the mechanisms of biosurfactant-plant interaction. Nevertheless, the analysis of obtained results indicated that MEL-B has great potential to replace, even if partially, the chemical components present in conventional pesticides.

3.1.4 References

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CHAPTER 4

In this chapter, there are presented the conclusion and the suggestion to suggestions for future studies.

4 CONCLUSION AND SUGGESTIONS FOR FUTURE STUDIES

4.1 CONCLUSION

The critical discussions carried out in the compilation of information on MELs made it possible to understand the diverse biochemical, biocompatible functions and a wide range of applications for MELs. In addition to the substantial contribution, mainly for researchers in the environmental and agro-industrial areas, the review article (section 2.1) demonstrates that the glycolipid properties of MELs can promote benefits in the biological quality of the soil, contribute to the nutrition of germinated roots and stimulate the growth of plants. The application of MEL-B promotes influences the seeds germination and the lettuce roots development. The responses observed in the physiological and biochemical behavior indicate that MEL-B at 316 and 632 mg/L influenced oxidative stress and inhibited the germination and development of lettuce seeds. In addition, the activity of peroxidase and polyphenol oxidase enzymes was expressive at these concentrations. This observation corroborates the fact that these concentrations are responsible for oxidative stress. Although MEL-B is an environmentally better product than pesticides, it can present undesired behavior at high concentrations. Thus, concentrations higher than 316 mg/L of MEL-B can promote negative changes in plant metabolism and prevent root development. On the other hand, MEL-B at 158 mg/L showed favorable behavior for seed germination. Under the conditions used in this study, MEL-B at 158 mg/L was the highest concentration at which there was no phytotoxic effect on the seeds. However, the work validated the hypothesis of the biostimulant property of this biosurfactant from the direct application of MEL-B in the germination of lettuce seeds.

4.2 SUGGESTIONS FOR FUTURE STUDIES

- Evaluate the efficiency of MEL-B associated with other conventional surfactants in the seed germination stage;
- Evaluate the synergistic effect of MEL-B associated with other biosurfactants for seed biostimulation;
- Investigate the antimicrobial activity of MEL-B in phytopathogenic fungi (*in vitro*);
- Monitor the development of plants and evaluate better conditions for treatment with biosurfactant;
- Prospect new formulation of a biopesticide using MEL-B.