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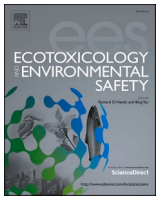

Phytotoxic response of ryegrass (*Lolium multiflorum* L.) to extreme exposure to two anionic surfactants

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ABSTRACT

Bioremediation is an effective and environment-friendly treatment used to clean up hydrocarbon-contaminated soil. However, the effectiveness of this treatment is often limited by the low bioavailability of the target contaminants. Surfactants addition thus appears as a way to increase solubility of these hydrophobic molecules and consequently improve their bioavailability. The use of biological surfactants is often favoured over synthetic ones because they are claimed to be non-toxic to the environment though few studies have addressed this issue. The present work evaluated the effects of a synthetic surfactant, sodium dodecyl sulphate (SDS) and a biosurfactant (rhamnolipids) on germination and growth of ryegrass over a wide range of concentrations, between one up to ten times their respective critical micellar concentration (CMC). Experimental results showed that SDS inhibited seed germination of *Lolium multiflorum* at high concentrations (10 × CMC), unlike rhamnolipids, which did not induce any toxicity symptom at germination stage. At the growth stage, high rhamnolipid concentrations induced chronic phytotoxicity by significantly reducing root length, decreasing biomass production and disrupting the enzymatic defence system. Thus, biosurfactants are less toxic than synthetic ones but their application at high doses in bioremediation treatments might still induce phytotoxicity symptoms and thus negatively affect the environment.

1. Introduction

In France, just over 10,045 polluted sites and soils have been identified as of mid-2022 (Antoni et al., 2023). Of these polluted sites and soils, 80% are affected by organic contamination, the majority of which is petroleum hydrocarbon pollution (Colombano, 2022). Petroleum hydrocarbons are persistent organic contaminants in the environment and are toxic to living organisms (Ehis-Eriakha et al., 2024). It is therefore essential to rehabilitate soils contaminated by these organic compounds.

Petroleum hydrocarbons can be removed from contaminated soil by various treatments. Among these treatments, bioremediation is a technique that involves using living organisms such as bacteria, fungi and/or plants that can contain, degrade or eliminate soil contaminants (Khan et al., 2004). Certain microorganisms, known as hydrocarbonoclastic, are used in bioremediation for their ability to use hydrocarbons as a source of carbon and energy (Dashti et al., 2015; Ehis-Eriakha et al., 2024) and thus biodegrade them. However, the effectiveness of this biodegradation process is often limited by the water solubility and the lack of bioavailability of hydrocarbon compounds (Kebede et al., 2021). The addition of surfactants can be used to enhance the solubilization and the bioavailability of hydrophobic pollutants such as hydrocarbons due to their amphiphilic nature when their concentration is equal to or exceeds the critical micellar concentration (CMC), corresponding to the concentration of micelles formation (Nagtode et al., 2023).

Biosurfactants are often favoured over synthetic surfactants because they are claimed to be less toxic, more easily biodegradable and more compatible with the environment (Zargar and Srivastava, 2024). However, few ecotoxicological studies have been carried out to confirm these claims and many authors cite their biological origin as a guarantee of their reduced impact on the environment (Akbari et al., 2018; Shao et al., 2017), although there is very little data available to confirm this (Parus et al., 2024). In addition, it is often necessary to use relatively high concentrations of biosurfactants in soil systems to overcome the problems of surfactant adsorption to the soil and to be able to reach the CMC in this type of system and thus solubilise more hydrocarbons later on. Herman et al. (1995) have demonstrated the strong adsorption of biosurfactants such as rhamnolipids (between 78% and 100%) for

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relatively low added concentrations (between 12.5 mM and 25 mM). It is then necessary to add much higher concentrations of rhamnolipids in hopes of reaching the CMC_{soil}, which corresponds to the critical micellar concentration for the surfactant solution-soil system. In some cases, the CMC_{soil} is 70–140 times higher than the CMC_{water} applied in a standard aqueous solution-air system, according to the review of Parus et al. (2023).

The need to use relatively high concentrations of biosurfactants in bioremediation processes raises the issue of their environmental impact, and more specifically toxicological effect on plants in a potential biosurfactant-assisted phytoremediation treatment strategy. A recent review by Parus et al. (2023) concluded that among the previous studies carried out on the phytotoxicity of rhamnolipids, 75% concerned studies on phytoremediation of heavy metals and/or hydrocarbon contaminations. Only 25% of these studies aimed at investigating the toxicity of rhamnolipids independently from any contamination. In these studies, experiments were carried out under controlled conditions in Petri dishes or soil-filled pots, but very rarely using hydroponic systems. The plant species tested included mainly model crops and vegetables (maize, rice, sunflower, white mustard, lettuce) or perennials (ryegrass, alfalfa and cuckoo flower). Few of these studies directly exposed plants to rhamnolipids and they did not look into cellular functions consequences to rhamnolipid exposure. Toxicity was therefore mainly assessed on measurements of germination indexes, root and leaf length or wet and dry biomass produced.

This work proposes to study in greater depth the phytotoxic effects of biosurfactants in terms of acute and chronic toxicity, before considering their *in situ* application in bioremediation and also to provide concrete answers justifying their preferential use compared to synthetic surfactants. In this context, the aims of this study were (i) to evaluate the acute toxicity of two anionic surfactants, one biological or one synthetic, at germination stage, (ii) to assess the chronic toxicity of the biological surfactant composed of rhamnolipids, through their impact on plant growth parameters and cell functions and thus (iii) to identify the conditions that make it possible to apply surfactants in the presence of a plant. To this end, ecotoxicological tests on the germination and growth of ryegrass (*Lolium multiflorum*), were carried out in the presence of a synthetic surfactant (sodium dodecyl sulphate) or a commercial bacterial surfactant, composed of a mixture of mono-rhamnolipids produced by *Pseudomonas aeruginosa*.

2. Materials and methods

2.1. Reagents and ryegrass seeds

For the germination test, inert quartz sand (Sand Fontainebleau, TECHNICAL) was used as a substrate and purchased from VWR Chemicals (Belgium). Its grain size was between 0.18 mm and 0.5 mm and SiO_2 purity above 95.0%.

Chloroform was from Carlo Erba Reagents (France). Molecular biology-grade water was from Merck Millipore (France). KH_2PO_4 and ethanol were from VWR Chemicals (Belgium). K_2HPO_4 was from Acros Organics (Belgium). Coomassie Brilliant Blue was from BIO-RAD (USA). Phosphoric acid was from Thermo Fisher Scientific (USA). Trichloroacetic acid, thiobarbituric acid, ascorbic acid, hydrochloric acid, sodium tetraborate and methylene blue were from Sigma-Aldrich (USA). All these chemicals were of analytical purity.

Seeds of ryegrass (*Lolium multiflorum*), a perennial plant often used in the hydrocarbon rhizodegradation processes (Hussain et al., 2022, 2018; Kaimi et al., 2007, 2006; Olson et al., 2007), were used in germination and hydroponic growth tests (Magellan-Bio, France). Hydrogen peroxide (30 % purity) used to sterilize the seeds was from VWR Chemicals (Belgium).

2.2. Surfactants

In this study, the anionic synthetic surfactant sodium dodecyl sulphate (SDS, Sigma-Aldrich, USA) and a commercial mixture of mono-rhamnolipids produced by *Pseudomonas aeruginosa* (R90, AGAE Technologies, batch number: AGA011519b, USA), an anionic bacterial surfactant, were used. These surfactants were of analytical purity. SDS is a model surfactant molecule with a known critical micellar concentration (CMC) value of 8.2 mM. The CMC of rhamnolipid was determined at about 20 °C using the oil-spreading test method (D'Incau et al., 2024). The CMC value of rhamnolipids used in this study was established at 0.030 g L^{-1} ± 0.005 g L^{-1} (data not shown), which is consistent with previous studies (Clifford et al., 2007; Kłosowska-Chomiczewska et al., 2017; Sabturani et al., 2016).

2.3. Germination test

Before germination, seeds were surface sterilized in a 10% (v/v) hydrogen peroxide solution for 5 min and then carefully washed three times with demineralised water. The germination test was adapted from the standard method ISO/DIS 17126:2023. Seeds were germinated in the dark at 20 °C ± 2 °C and 70% atmospheric humidity was maintained in a phytotronic chamber (Aralab FITOCLIMA 10000 EHHF). The concentrations tested for each surfactant (SDS and rhamnolipid) were as follows: 1 × CMC_{water}, 5 × CMC_{water} and 10 ×CMC_{water}. These concentrations corresponded respectively to 2.36 g L^{-1}, 11.82 g L^{-1}, 23.65 g L^{-1} for SDS treatments and to 0.03 g L^{-1}, 0.15 g L^{-1}, 0.3 g L^{-1} for rhamnolipid treatments. A positive control containing boric acid at 10 g L^{-1} and a negative control containing only demineralised water were also carried out. For each treatment, four replicates were performed and 15 seeds were placed per replicate on 50 g of inert quartz sand in a 90 mm diameter Petri dish. Each replicate was moistened with 11.1 mL (corresponding to 80% of the water holding capacity for quartz sand) of surfactant solution, demineralised water or acid boric solution depending on the conditions tested. Substrate moistening was kept constant (± 10%) by watering every two days with demineralised water on a weight basis. Germinated seeds were scored daily for 14 days. Germination was considered complete when the radicle reached approximately 2 mm in length. After counting, each germinated seed was removed so as not to physically constrain the germination of the other seeds. At the end of the experiment, the final germination percentage (FGP) and the mean germination time (MGT) were calculated. The mean germination time was calculated for each condition using the equation of Ranal et al. (2009), as follows:

$$MGT = \frac{\sum_{i=1}^{k} n_i \times t_i}{\sum_{i=1}^{k} n_i}$$

where MGT is the mean germination time (day) calculated for each condition; t_i the time from the start of experiment to the ith observation (day); n_i the number of seeds germinated in the ith time (not the accumulated number, but the number correspondent to the ith observation) and k the last time of germination.

2.4. Hydroponic growth test

After surface sterilization of the seeds as with the germination test, seed priming for the hydroponic growth test was carried out in hydroponics. One ryegrass seed was placed in a 1.5 mL Eppendorf tube filled with cotton to act as a support, with the base cut out to allow the roots to develop in the nutrient medium (Fig. S. 1). Hydroponic seeds were primed for 14 days in a phytotronic chamber (Aralab FITOCLIMA 10000 EHHF) maintained at 70 % humidity, 16 h/8 h photoperiod (350 µmol m^{-2} s^{-1}) and a temperature of 20 °C/15 °C (day/night). The Hoagland

nutrient solution used for seed priming (Table S. 1) was prepared according to the Hoagland and Arnon (1950) study.

Fourteen days after priming, 30 uniform seedlings (equivalent length) were selected for the rest of the experiment. Seedlings were then introduced into an Erlenmeyer flask 100 mL using a device adapted from Chaîneau et al. (2000). Each Erlenmeyer flask received one seedling. The Eppendorf tube used as a support during the seed priming phase has been preserved. The Erlenmeyer flasks were also covered with aluminum foil to limit the development of photosynthetic organisms during incubation (Fig. 1). Based on the germination results, the effects of surfactants on the growth plant were studied only for rhamnolipid treatments at 1 × CMC_{water} (*i.e.* 0.03 g L^{-1}) and 10 × CMC_{water} (*i.e.* 0.3 g L^{-1}). Negative control was also conducted in parallel without surfactant in the assay sample. All conditions were prepared in Hoagland nutrient solution. Five replicates were performed per treatment. This exposure phase lasted 14 days and was carried out in the same phytotronic chamber as the priming period, with the same temperature, lighting and humidity conditions. The mass of each Erlenmeyer flask was checked every two days and adjusted when necessary, with demineralized water to account for water loss through evapotranspiration. Total water loss (TWL ± 0.1 g) was thus calculated at the end of the cultivation (after 14 days of growth). At the end of the culture, plants were removed from the Erlenmeyer flasks avoiding root damage and various analyses were realized on each plant.

2.5. Analysis of morphological plant growth parameters

At the end of the hydroponic growth test, plant roots were carefully washed with demineralized water, dried with filter paper and the total fresh weight (TFW, ± 0.01 mg) was measured. Root and shoot lengths were measured using a ruler. Root lengths (RL, ± 0.01 cm) were measured on the main root of each plant. For shoot length (SL, ± 0.01 cm), the length of the largest shoot was taken into account for each plant. Then, roots and shoots were separated using a pair of scissors disinfected with a 70 % ethanol solution. Root dry weight (RDW, ± 0.01 mg) and shoot dry weight (SDW, ± 0.01 mg) were measured after drying each part at 70 °C for 48 h and the total dry weight (TDW) was calculated (TDW = RDW + SDW). The plant water content (PWC) was then evaluated as PWC= [(TFW – TDW)/TDW]. Water use efficiency for each plant at the end of the culture was analysed as WUE = TDW/TWL.

2.6. Analysis of plant cell functions

2.6.1. Chlorophyll content

The chlorophyll content in shoots of ryegrass was measured using a SPAD chlorophyll meter (SPAD-502Plus, Konica Minolta). The measurement was conducted on the largest shoot of each plant. Each measurement was repeated three times at the same point on the shoot to limit the variability of the measurement. Chlorophyll contents using this method are semi-quantitative and expressed in U (arbitrary unit).

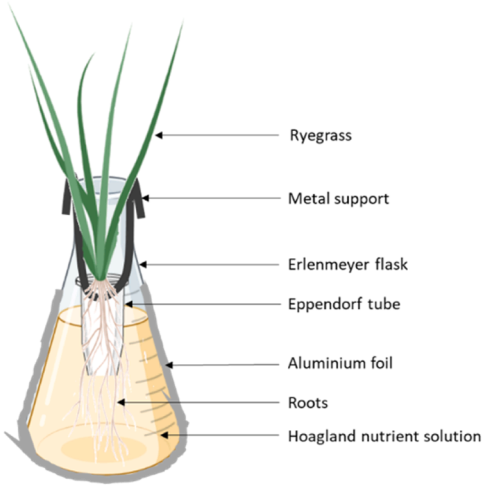

Fig. 1. Experimental device for hydroponic growth test.

2.6.2. Soluble protein, antioxidant enzymes and lipid peroxidation

The assays were carried out on a plant extract obtained using two different protocols depending on the analysis performed. For the assays of soluble protein content, catalase and ascorbate peroxidase activities, 200 mg of fresh shoot biomass was harvested and stored at − 80 °C in liquid nitrogen. The shoot biomass was then ground to powder in liquid nitrogen with a cold pestle. The powder was then homogenised in 1.2 mL of potassium phosphate buffer (0.2 M, pH 7.8) and centrifuged at 15,000 × *g* for 20 min at 4 °C. The 0.2 M potassium phosphate buffer was made with 14.894 g of K_2HPO_4 and 1.972 g of KH_2PO_4 dissolved in 500 mL of molecular biology-grade water. The supernatant was then recovered and set aside, while the pellet was resuspended in 0.8 mL potassium phosphate buffer and centrifuged (15,000 × *g*, 15 min, 15 °C). This second supernatant was mixed with the first supernatant recovered upstream to form the plant extract.

The soluble protein content was determined according to the method of Bradford (1976). Firstly, Bradford's solution was prepared with 100 mg Coomassie blue dissolved in 50 mL of 95% ethanol and 100 mL of 85% (v/v) phosphoric acid. Once the dye was completely dissolved, the volume was made up to 1 L q.s. with demineralised water. Before each use, the Bradford reagent was filtered through Whatman paper. The protein concentration was determined for each plant extract by spectrophotometry by measuring the absorbance at 595 nm of 30 µL of undiluted plant extract homogenised in 1.4 mL of Bradford's solution. This absorbance was then compared to a standard range performed with protein concentrations (bovine serum albumin medium) ranging from 0 mg mL^{-1} to 4 mg mL^{-1}.

The catalase activity was determined spectrophotometrically using the protocol of Aebi (1984). First, 667 µL of plant extract was diluted 200-fold in potassium phosphate buffer (50 mM, pH 7) made with 7.447 g of K_2HPO_4 and 0.986 g of KH_2PO_4 dissolved in 500 mL of molecular biology-grade water. The diluted extract was then mixed with 333 µL of 30 mM hydrogen peroxide in a quartz spectrophotometric cell. The absorbance at 240 nm was then determined for one minute against a blank containing 667 µL of 200-fold diluted plant extract and 333 µL of potassium phosphate buffer (50 mM, pH 7). Catalase activity was calculated as a function of the extinction coefficient of 40 mM cm^{-1}, which corresponded to the difference in absorbance observed during the dismutation of one micromole of hydrogen peroxide (H_2O_2).

The ascorbate peroxidase activity was assayed using the method of Nakano and Asada (1981). First, 10 µL of undiluted plant extract was homogenised with 980 µL of potassium phosphate buffer (50 mM, pH 7), 5 µL of 100 mM ascorbic acid and 5 µL of 100 mM hydrogen peroxide. The absorbance at 290 nm was then determined for one minute against a blank containing 10 µL of undiluted plant extract, 5 µL of 100 mM hydrogen peroxide and 980 µL of potassium phosphate buffer (50 mM, pH 7). Ascorbate peroxidase activity was calculated as a function of extinction coefficient of 2.8 mM cm^{-1}.

The specific enzyme activity for catalase and ascorbate peroxidase was expressed on a protein content basis as unit mg^{-1} protein.

For the assays of malondialdehyde (MDA) content, 200 mg of fresh shoot biomass was harvested and stored at − 80 °C in liquid nitrogen. The shoot biomass was then ground to powder in liquid nitrogen with a cold pestle. The powder was then homogenised in 1.5 mL of 1 % (v/v) trichloroacetic acid and centrifuged at 15,000 × *g* for 15 min at 4 °C.

Malondialdehyde (MDA) content, a product of lipid peroxidation, was determined by the thiobarbituric acid (TBA) reaction using the method described by Velikova et al. (2000). Half a millilitre of trichloroacetic extract was mixed with 3 mL of reaction solution containing 0.5 % (v/v) thiobarbituric acid and 20 % (v/v) trichloroacetic acid.

The mixture was heated in a water bath at 90 °C for 30 min, then cooled in ice to stop the reaction and centrifuged at 5590 × *g*, 4 °C, for 15 min. Absorbance of the supernatant was measured at 532 and 600 nm. The content of MDA was calculated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm and calibrated by using the extinction coefficient of 155 mM cm^{-1}.

2.7. Statistical analyses

Statistical analyses were carried out using RStudio software (version 4.2.1) and statistical significance was set at p-value < 0.05. One-way ANOVAs (analysis of variance) tests were performed and the average values were compared using the Tukey HSD test for multiple *post hoc* comparisons between treatments. Before the ANOVA analysis, the absence of autocorrelation in the residuals was checked using a Durbin-Watson test, the normality of residuals was evaluated using a Shapiro-Wilk test and the homogeneity of the variation in the data was investigated with a Levene test. When necessary, data were log-transformed to meet ANOVA validity criteria. Otherwise, a non-parametric one-way ANOVA (Kruskal-Wallis test) was applied.

3. Results

3.1. Germination

The test's validity criteria, *i.e.* a minimum final germination rate of 80.0% for the negative control (no surfactant) and significant inhibition of the germination for the positive control (10 g L^{-1} boric acid), were fully respected. Indeed, the final germination rates for the negative and positive controls were 81.4% ± 6.2% and 0.0% ± 0.0% respectively.

The germination kinetics curves presented a sigmoidal shape with three phases: an initial lag phase, followed by an exponential phase analogous to an acceleration of germination and ending with a plateau at maximum germination (Fig. 2). The ryegrass started to germinate 3 days after sowing for all the treatments, except for the positive control with a zero-germination rate until the end of incubation. In the negative control (no surfactant), the lag phase lasted only 3 days and the exponential phase 8 days to reach the stationary phase before reaching maximum germination rate. This germination kinetics of ryegrass was similar for all the conditions with rhamnolipids and lowest concentrations of SDS (p-value > 0.05). In the presence of the highest SDS concentration, 23.65 g L^{-1}, germination rates significantly differed from the control 7 days after sowing with germination rates lower by 41% (p-value < 0.05). Seed germination kinetics were therefore not significantly affected by rhamnolipids, unlike by SDS which limited ryegrass germination.

Mean seed germination indexes were calculated after 14 days of incubation (Table 1). The rhamnolipids, surfactants of bacterial origin, had no significant impact on the germination of *L. multiflorum* (p-value > 0.05), and even tended to improve the final germination percentage (FGP) for supplied rhamnolipid concentrations of 1 × CMC_{water} and 5 × CMC_{water} with respective FGP values normalized on the negative control of 102.0% ± 4.4% and 103.8% ± 12.4%. Contrary to rhamnolipids, the synthetic surfactant SDS significantly inhibited the ryegrass germination at a concentration of 10 × CMC_{water} (p-value < 0.05), with a final germination rate of only 62.6%. Mean germination time (MGT) of the different conditions was between 3.7 d ± 0.4 d and 4.9 d ± 0.9 d and was not statistically different (p-value > 0.05) from the negative control (4.4 d ± 0.8 d).

3.2. Growth conditions and ryegrass responses to biosurfactants

3.2.1. Effect of rhamnolipids on growth parameters

Since SDS induced acute toxicity symptoms, its effect was not further tested in the following hydroponic growth tests carried out to check

Table 1
Mean germination time (MGT) and normalized final germination percentage (FGP) of *L. multiflorum*. (Different letters indicate statistically significant differences at the 5 % level among treatments using Tukey HSD with p-values < 0.05 and n = 4).

Parameters	Treatment	Surfactant concentration in CMC_{water}			
		0	1	5	10
FGP (%)	Rhamnolipids	100.0 a	102.0 ± 4.4 a	103.8 ± 12.4 a	89.1 ± 9.4 a
	SDS		94.7 ± 4.2 a	91.8 ± 10.4 a	62.6 ± 6.4 b
MGT (day)	Rhamnolipids	4.4 ± 0.8 a	3.9 ± 0.6 a	4.2 ± 0.7 a	3.7 ± 0.4 a
	SDS		4.4 ± 0.5 a	4.6 ± 0.3 a	4.9 ± 0.9 a

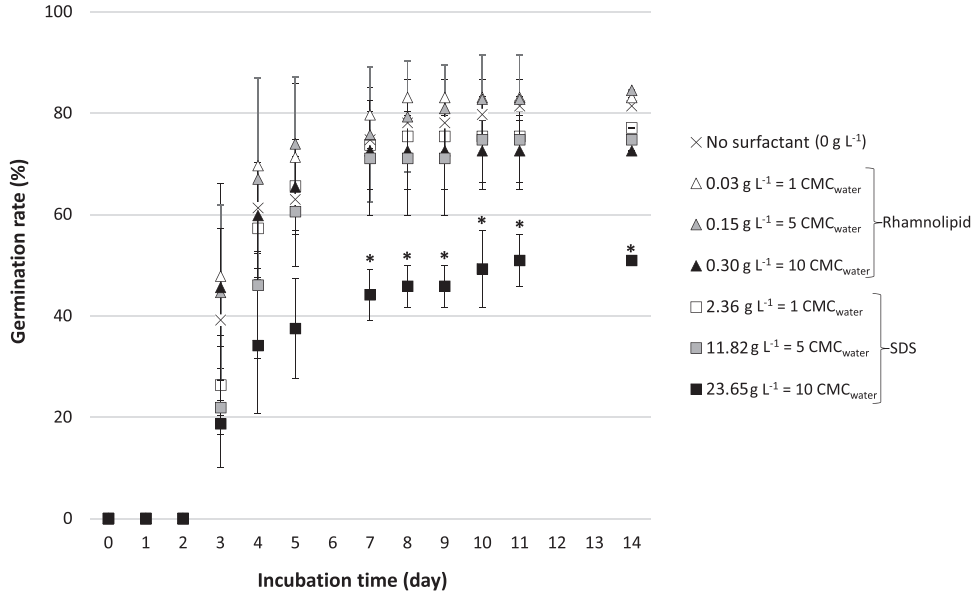

Fig. 2. Effect of surfactants (rhamnolipid or SDS) on the germination of *L. multiflorum* seeds. (Asterisks indicate a significant difference at the 5 % level compared to the no surfactant treatment using Kruskal-Wallis test, p-value < 0.05, n = 4).

whether rhamnolipids could induce chronic phytotoxicity.

For all parameters, the exposure to the lowest rhamnolipid concentration of 1 × CMC did not induce any effect (p-value > 0.05). However, except for shoot length, total fresh weight and water use efficiency, the ryegrass exposure to high concentrations of rhamnolipids of 10 × CMC significantly reduced (p-value < 0.05) plant biomass indicators (Table 2). This toxic impact of rhamnolipids at 10 × CMC is reflected in a reduction in shoot and root dry weights with respective masses of 0.68 g ± 0.15 g and 0.10 g ± 0.03 g, which are significantly lower (p-value < 0.05) than the control condition (without surfactants, concentration of 0 × CMC) with respective shoot and root dry weights values of 1.06 g ± 0.23 g and 0.19 g ± 0.04 g. Root length was also affected by this relatively high concentration of rhamnolipids, with a root length of 13.28 cm ± 1.38 cm which was significantly shorter than the control root length of 18.48 cm ± 2.58 cm (p-value < 0.05). Regarding plant evapotranspiration, total water loss (TWL) reached 121.00 mL ± 30.22 mL and 121.12 mL ± 31.06 mL respectively for rhamnolipid concentrations of 0 × CMC and 1 × CMC. These water losses by evapotranspiration were significantly greater (p-value < 0.05) than the 10 × CMC rhamnolipid concentration condition with a TWL value of only 74.60 mL ± 17.73 mL.

3.2.2. Effect of rhamnolipids on chlorophyll content

The chlorophyll content of ryegrass shoots at the end of incubation for initial rhamnolipid concentrations of 1 × CMC and 10 × CMC were 40.6 U ± 1.7 U and 41.6 U ± 2.4 U respectively. These data were not statistically different from the chlorophyll content of the control condition (0 × CMC) of 40.9 U ± 3.0 U (p-value > 0.05).

3.2.3. Effects of rhamnolipids on antioxidant enzyme activities and lipid peroxidation

When the ryegrass plants were exposed to 10 × CMC of rhamnolipids, the shoot catalase activity increased significantly by 52.0% (p-value < 0.05) compared to the control (Fig. 3a). However, no significant difference in catalase activity was observed between 1 CMC and the control (p-value > 0.05).

Shoot ascorbate peroxidase activities were 872.5 U mg^{-1} protein ± 194.4 U mg^{-1} protein, 792.9 U mg^{-1} protein ± 256.9 U mg^{-1} protein and 730.4 U mg^{-1} protein ± 166.4 U mg^{-1} protein respectively for rhamnolipid concentrations of 0 × CMC, 1 × CMC and 10 × CMC (Fig. 3b). There was no significant difference (p-value > 0.05) in shoot ascorbate peroxidase activity between any of these treatment plants.

In addition, no difference in MDA content in ryegrass shoot tissues (p-value > 0.05) was observed between the control and other two rhamnolipid regimes (1 × CMC and 10 × CMC) with respective MDA contents of 383.7 nmol g^{-1} dry weight ± 50.3 nmol g^{-1} dry weight, 295.5 nmol g^{-1} dry weight ± 79.6 nmol g^{-1} dry weight and 300.8 nmol g^{-1} dry weight ± 70.1 nmol g^{-1} dry weight.

Table 2
Effect of rhamnolipid concentration on the growth of *L. multiflorum*. (Asterisks indicate a significant difference at the 5 % level compared to the no surfactant treatment using Tukey HSD, p-value < 0.05, n= 4).

	Rhamnolipid concentration in CMC		
Growth parameters	**0**	**1**	**10**
Shoot length (cm)	36.94 ± 2.91	40.78 ± 8.69	33.58 ± 5.02
Shoot dry weight (g)	1.06 ± 0.23	0.99 ± 0.23	0.68 ± 0.15 *
Root length (cm)	18.48 ± 2.58	15.98 ± 1.75	13.28 ± 1.38 *
Root dry weight (g)	0.19 ± 0.04	0.16 ± 0.02	0.10 ± 0.03 *
Total fresh weight (g)	5.78 ± 0.77	6.29 ± 0.99	4.62 ± 0.95 *
Total dry weight (g)	1.25 ± 0.26	1.16 ± 0.24	0.77 ± 0.17 *
Plant water content (mL)	3.69 ± 0.50	4.49 ± 0.41 *	5.00 ± 0.43 *
Total water loss (mL)	121.00 ± 30.22	121.12 ± 31.06	74.60 ± 17.73 *
Water use efficiency (g L^{-1})	10.48 ± 0.95	9.72 ± 1.22	10.49 ± 1.15

4. Discussion

4.1. A synthetic surfactant is more toxic than a biological one

Our results indicate that rhamnolipids are non-toxic for concentrations ranging from 0 g L^{-1} up to 0.3 g L^{-1} for ryegrass germination. SDS however, a synthetic surfactant, significantly inhibits ryegrass germination for high concentrations exposure, namely 23.65 g L^{-1} corresponding to 10 × CMC_{water} (p-value < 0.05). This contrasted response to both surfactant types is in agreement with results of Adetunji et al. (2017) who studied the germination inhibition of five different plant species (*Sorghum bicolor*, *Solanum lycopersicum*, *Triticum aestivum*, *Vigna unguiculata* and *Capsium annuum*) in the presence of synthetic surfactants (Tween 20) or biosurfactants (rhamnolipids). That study also showed that the germination of the five tested plant species was inhibited only in the presence of the synthetic surfactant. No inhibitory effect of rhamnolipids was observed on the germination rates of these different plant species, which were all higher than 80 % for an applied concentration of rhamnolipids of 0.5 g L^{-1}, close to the one used in our study. Synthetic surfactants (SDS and Tween 20) therefore appear to be toxic to certain plant species at the germination stage, unlike biosurfactants (rhamnolipids). In contrast, Gidudu and Chirwa (2022) reported that a concentration of rhamnolipids 3 times lower than what was tested in this study (0.1 g L^{-1}) reduced the germination to 54.29%, 26.16% and 25.22% for the plant species *Brassica napus*, *Pisum sativum* and *Phaseolus vulgaris* respectively. They also found that for the same applied SDS concentration of 0.1 g L^{-1}, a much lower concentration than the maximum SDS concentration of 23.65 g L^{-1} tested in our study, the negative effects on seed germination were even greater with the extremely low germination index values of 1.19%, 3.28% and 8.93% respectively for *Brassica napus*, *Pisum sativum* and *Phaseolus vulgaris* species. Here again, though the tested plant species seemed more sensitive than ryegrass, the higher toxicological effect of the synthetic surfactant compared to the biological one was highlighted.

Toxic effects of synthetic surfactants have also been observed in the study of Gálvez et al. (2019) testing the effect of non-ionic surfactants (Triton X-100, Brij35, Tween 80 and Glucopone 600) and anionic surfactants (SDS and Aerosol 22) on seeds of onion (*Allium cepa*) and lettuce (*Lactuca sativa*). In this study, SDS significantly inhibited seed germination compared to the control at surfactant concentrations of 6.5 g L^{-1} and 0.65 g L^{-1} for onion and lettuce respectively (p-value < 0.05). The highest surfactant concentration of 32.5 g L^{-1} (close to the maximum concentration of 23.65 g L^{-1} tested in our study) significantly reduced germination to 40 % for onion and completely inhibited lettuce germination (p-values < 0.05). Chang et al. (2015) noticed also the acute toxicity of SDS on wheat (*Triticum aestivum*) germination from a low surfactant concentration of 0.48 g L^{-1}, *i.e.* about 50 times lower than the maximum SDS concentration in our study. This toxicity was found to be greater as the surfactant concentration increased, with significantly lower germination rates (p-values < 0.05) compared with the control of 70.6%, 41.7% and 0.0% for SDS concentrations of 0.48 g L^{-1}, 0.72 g L^{-1} and 0.8 g L^{-1} respectively. All these results show that synthetic surfactants are acutely toxic and inhibit the germination of terrestrial plant species when exposed to SDS. However, this inhibitory effect appears to be species-dependent. Based on the information listed above, terrestrial plants could be classified from the most to the least sensitive to SDS exposure in the order lettuce > wheat > onion > ryegrass.

Regarding the phytotoxicity of biosurfactants, Marecik et al. (2012) observed an inhibitory effect of rhamnolipids on the germination of alfalfa (*Medicago sativa*), mustard (*Sinapis alba*) and sorghum (*Sorghum saccharatum*) but for concentrations greater than the one used in our study (*i.e.* above 75 mg kg^{-1}). Millioli et al. (2009) observed as well that increasing rhamnolipid concentration decreased the germination index of lettuce (*Lactuca sativa*) and estimated the EC_{50} value between 4000 mg kg^{-1} and 6000 mg kg^{-1} rhamnolipids. For the highest rhamnolipid concentrations tested (10,000 mg kg^{-1} and 15,000 mg kg^{-1}),

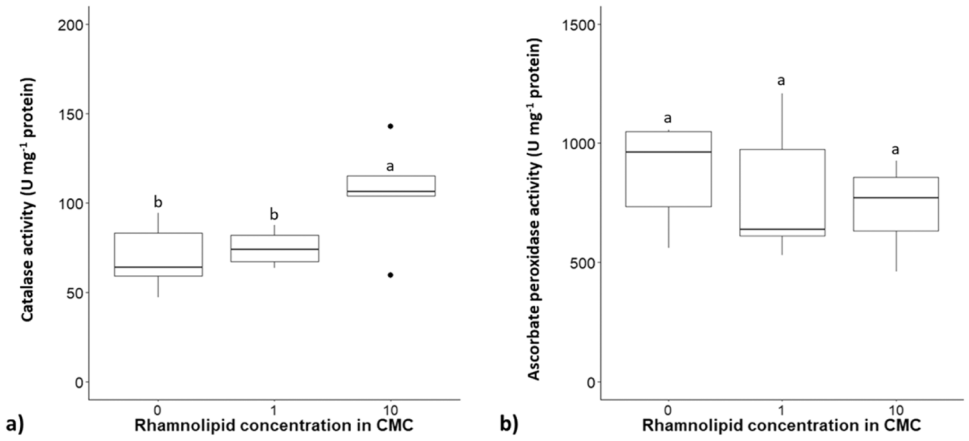

Fig. 3. Effect of rhamnolipid concentration on antioxidant enzyme activities of shoot tissues of *Lolium multiflorum* **a)** Catalase activity and **b)** ascorbate peroxidase activity. (This figure corresponds to an analysis of 5 replicates, but due to outliers and important variability, statistical analyses have been done on 3 replicates without the highest and the lowest values. Different letters indicate statistically significant differences at the 5% level among treatments using Tukey HSD with p-values < 0.05 and $n = 3$).

germination inhibition reached up to 80%. These concentrations are much higher than the maximum value tested in our study of 66.6 mg kg^{-1}, but suggest that acute toxicity response may still occur for extremely high biosurfactant concentrations. Other studies involving possibly more sensitive species, namely mung bean and rice, observed that rhamnolipid concentrations just above the CMC value could induce a reduction in germination index (Bharali et al., 2018). Oppositely, several authors have shown that low concentrations of rhamnolipids can promote the germination of some plant species. This is the case of Millioli et al. (2009) who observed an increase in the germination index of lettuce (*Lactuca sativa*) of around 26% for a rhamnolipid concentration of 1000 mg kg^{-1} compared with the non-contaminated control soil. They suggested that low rhamnolipids concentrations may increase the availability of some nutrients improving soil fertility. Silva et al. (2010) also showed the positive impact of rhamnolipids on cabbage (*Brassica oleracea*) germination for concentrations of 0.175 g L^{-1}, 0.350 g L^{-1} and 0.525 g L^{-1} with an increase in germination indexes of 145%, 87% and 32% respectively compared to the negative control. These results are in agreement with those obtained in our study, since a trend towards an increase in the final germination percentage (FGP) was observed for rhamnolipid concentrations of 0.03 g L^{-1} and 0.15 g L^{-1}, with FGP values of 102.05% and 103.80% respectively.

SDS and rhamnolipids are both anionic surfactants with similar properties. Anionic surfactants are known for their ability to interact with phospholipid or protein compounds in cell membranes and thus modify the chemical structure of the cell surface. This alteration of the cell surface integrity can sometimes lead to membrane rupture (Bjerk et al., 2021) and therefore inhibit the germination process. Many studies have shown that rhamnolipids could modify the cell surface hydrophobicity (CSH) *via* their adsorption on the cell surface (Guzmán et al., 2024) or *via* induction of lipopolysaccharides release from the cell surface by adsorbed rhamnolipids (Vara Prasad et al., 2021). Furthermore, anionic surfactants are highly polar compounds and are likely to interact with the seed's covering layer and form a barrier around the seed. This degree of hydrophilicity is reflected in the Hydrophilic-Lipophilic Balance (HLB) value of the surfactant, which is higher the greater the solubility of the surfactant in water. The HLB value greater than 10 indicates that the surfactant is more hydrophilic, leading to the formation of direct emulsions (O/W, oil in water emulsifier). SDS has a very high HLB value of 40, which is 4 times higher than the HLB value of rhamnolipids estimated at 10.9 by Long et al. (2013). This much higher HLB value for SDS could explain its higher degree of phytotoxicity. The micelles formed around the seed at SDS concentrations higher than the CMC value are likely to create a more resistant barrier around the seed than rhamnolipids and inhibit the initial imbibition phase of the germination cycle. This inhibited soaking phase could block any water absorption into the seed tissue, preventing it from swelling. In our study, the plant water content was statistically higher in the seedling exposed to rhamnolipids than in the control seedling. These results therefore highlight the interaction of these biosurfactants with the cell surface and prove that biosurfactants do not have the same waterproofing effect as SDS and other synthetic surfactants. Rhamnolipids are also more readily biodegradable than synthetic surfactants such as SDS (Lavanya, 2024). Zeng et al. (2007) demonstrated that rhamnolipid had no toxicity and could be degraded by *Bacillus subtilis* and compost microorganisms in liquid culture media contrary to CTAB, Triton X-100 and SDS synthetic surfactants studied. In another study, the intensity of SDS degradation was approximately 10 times lower than that of biosurfactant degradation by *Pseudomonas sp.* (Lima et al., 2011). Zhu et al. (2024) also reported the highly toxic effect of SDS on the species, quantity and genetic functions of microorganism communities in natural water at a very low SDS concentration ($\leq$ 0.006 g L^{-1}), well below CMC. We can therefore assume that the impact of rhamnolipids on the cell surface of seeds could also have been attenuated by their biodegradation during germination. Conversely, SDS, which is known to be difficult to biodegrade, was not degraded during seed germination and therefore had more time to modify the cell structure and impact the membrane integrity of the seeds.

4.2. High rhamnolipid concentrations have a moderate impact on plant growth but still disrupt plant cell functions

4.2.1. Ryegrass biomass is affected by relatively high rhamnolipid concentrations

The addition of moderate amounts of rhamnolipids, concentration close to CMC value (0.03 g L^{-1}), did not affect plant growth. Rhamnolipids significantly reduced the root and shoot dry biomass of ryegrass (p-value < 0.05) only when present at an extremely high concentration equivalent to ten times the CMC value (0.3 g L^{-1}). These results are in line with those of Marecik et al. (2012) who also observed a significant decrease in dry matter values for shoots and roots (p-values < 0.05) for high rhamnolipids concentration (600 mg kg^{-1}) when a lower rhamnolipid concentration of 150 mg kg^{-1} did not induce any significant growth response (p-value > 0.05). In this case, the toxic effect of rhamnolipids was more important for sorghum (*Sorghum saccharatum*) than alfalfa (*Medicago sativa*), mustard (*Sinapis alba*) and cuckooflower (*Cardamine pratensis*). Sancheti and Ju (2020) demonstrated a severely reduced root and shoot weights of soybeans with visible browning and

damages of roots at high rhamnolipid concentrations between 5 g L^{-1} and 20 g L^{-1} after 7 days' exposure of seeds to rhamnolipid solutions. However, it is noteworthy noting that these rhamnolipids concentrations corresponded to extreme concentrations, ranging from 166 to 666 times the CMC value, and much higher than the concentrations used in our study. Johnson et al. (2009) also found that high concentrations of rhamnolipids (*i.e.* 0.2 g L^{-1} equivalent to about 6.7 × CMC) caused some toxicity to plant shoot growth resulting in wilting of Indian mustard (*Brassica juncea*) stems but with no inhibitory effect on perennial ryegrass (*Lolium perenne*) growth. Lower rhamnolipid concentrations (*i.e.* 0.025 g L^{-1} equivalent to about 0.8 × CMC) had no impact on the growth of Indian mustard and ryegrass, unlike Triton X-100 (a synthetic surfactant also tested in this study), which significantly reduced the shoot mass of both plant species (p-values < 0.05) for low and high synthetic surfactant levels tested of 0.2 g L^{-1} (equivalent to 0.5 × CMC) and 1.6 g L^{-1} (equivalent to 4 × CMC). These rhamnolipids concentrations are similar to those tested in our study and therefore confirm that rhamnolipids do not affect ryegrass growth at concentrations below 10 × CMC, *i.e.* 0.3 g L^{-1}, unlike synthetic surfactants. The use of bacterial surfactants to improve the bioremediation of organic pollutants should therefore be favoured over synthetic ones, but here again plant sensitivity to rhamnolipids is species dependant.

In addition, a significant reduction (p-value < 0.05) in the root length of ryegrass by 28% compared to the control was observed for the highest rhamnolipids concentration (10 × CMC equivalent). In the case of shoots, shoot lengths were similar and no significant toxic effect of rhamnolipids was demonstrated on this growth parameter (p-value $>$ 0.05). These results are consistent with those of Szczepaniak et al. (2015), where the root lengths of white mustard (*Sinapis alba*) were significantly reduced (p-value < 0.05) by 36% (for 0.15 g L^{-1} of rhamnolipids), 64% (for 0.3 g L^{-1} of rhamnolipids) and 69% (for 0.6 g L^{-1} of rhamnolipids) compared to the control sample. They also observed that the toxic effect of rhamnolipids was not significant (p-value > 0.05) on the shoot length for rhamnolipids concentrations between 0.15 g L^{-1} and 0.6 g L^{-1}. Sydow et al. (2018) observed an inhibition of root and shoot growth after four days of germination and concluded that this inhibition increased as a function of the concentration of rhamnolipids added for concentrations between 1 × CMC (*i.e.* 0.15 g L^{-1}) and 10 × CMC (*i.e.* 1.5 g L^{-1}). The level of phytotoxicity observed was also dependent on the plant species used. For cress (*Lepidium sativum*) the inhibition reached $>$ 40%, for white mustard (*Sinapis alba*) $>$ 10% and in the case of sorghum (*Sorghum saccharatum*), the inhibition was $<$ 3%. This impact on root morphology is related to the total water loss data in our study, which were significantly lower (p-value < 0.05) for the highest rhamnolipids concentration. Disruption to root growth caused by water stress due to the presence of a toxic agent can result in reduced plant transpiration as highlighted in the study by Wen et al. (2010) for a rhamnolipid concentration of 10 × CMC. This can be explained by an alteration in membrane processes, such as water and nutrient uptake, in the presence of surfactants, which ultimately affects plant growth as indicated in the study by Salvatori et al. (2021). Surfactants can also modify the physicochemical properties of the growth medium, for example by increasing its osmotic potential. The process of water uptake by the plant's roots could then be further reduced as observed in the study by Wiel-Shafran et al. (2006). However, this effect was not observed in our study, since the water use efficiency of 10 × CMC rhamnolipid was equivalent to that of the control (p-value > 0.05). This result can be explained by the difference in the nature of the anionic surfactants used in the study by Wiel-Shafran et al. (2006). Indeed, in this study an Ariel Ultra laundry detergent solution was used with a synthetic anionic surfactant concentration in the form of MBAS of 0.25 g L^{-1} (this anionic surfactant concentration was relatively close to the maximum concentration of 10 X CMC tested in our study).

4.2.2. *Rhamnolipids affect plant cell functions of ryegrass*

The influence of rhamnolipids was also studied on the cellular functions of ryegrass by measuring chlorophyll content, antioxidant enzyme activities (catalase and ascorbate peroxidase activities) and the presence of lipid peroxidation products (malondialdehyde).

The chlorophyll content of ryegrass was not affected (p-value > 0.05) by the addition of rhamnolipids for all tested concentrations (up to 10 × CMC). The rhamnolipids did not affect the chlorophyll production of the ryegrass and did not therefore interfere with its photosynthetic activity. However, other authors have noted a negative impact of rhamnolipids on the photosynthetic performance of maize (Liao et al., 2016; Parus et al., 2024). A trend towards a decrease in chlorophyll content of *Zea mays* in the presence of rhamnolipids was observed in the study by Liao et al. (2016) but for very extreme rhamnolipids concentrations of 10 g L^{-1}, approximately 33 times higher than the maximum concentration tested in our study, applied every half a month for an experimental period of 3 months. It is therefore important to take a step back when comparing our results with those of the Liao et al. (2016), given the significant methodological differences. Indeed, the rhamnolipid concentration was much higher, a different plant species was used, and the experimentation time was 6 times longer than in our study (3 months *vs* only 14 days in our study). Chlorophyll content was also measured differently (chlorophyll fluorescence was measured with a Junior-PAM chlorophyll fluorometer to estimate the quantum efficiency of photosynthesis). If the ryegrass had been grown under the same conditions for 3 months, alterations in chlorophyll content could therefore have been observed. Parus et al. (2024) have also highlighted a negative impact of rhamnolipids on the chlorophyll fluorescence parameters for maize (*Zea mays*) and the amount of chlorophyll produced but for lower rhamnolipid concentrations of 1 × CMC_{soil} (*i.e.* 0.5 g L^{-1}). This effect increased with increasing rhamnolipids content, resulting in a reduction in chlorophyll fluorescence parameters of 20–40% for rhamnolipids contents of 1.5 × CMC_{soil} (*i.e.* 0.75 g L^{-1}) and 2 × CMC_{soil} (*i.e.* 1.0 g L^{-1}) respectively. These results initially showed that concentrations up to 3 times higher in rhamnolipids than those tested in our study could reduce the plant's production of chlorophyll. Secondly, this highlighted the need to use high concentrations of rhamnolipids in bioremediation processes of contaminated soils that exceed their adsorption to the soil as shown in the study by Li et al. (2014) with CMC values of TX100 and B35 surfactants 2.75 and 6.31 times their corresponding CMC values in aqueous solutions, respectively. A concentration of 1 × CMC_{soil} (*i.e.* 0.5 g L^{-1}) in the study by Parus et al. (2024) was approximately 17 times higher than 1 × CMC_{water} (*i.e.* 0.03 g L^{-1}) in our hydroponic experiment. Consequently, it is not surprising that no toxic effect of rhamnolipids was found on the ryegrass chlorophyll content since the maximum concentration tested of 10 × CMC corresponded to 0.3 g L^{-1} and was therefore much lower than the biosurfactant concentrations tested in previous studies. This difference in CMC values between our study and those of previous studies can also be explained by the purity level and properties of rhamnolipids, with less pure variants with higher CMCs (Kłosowska-Chomiczewska et al., 2017). Thus, in the study by Parus et al. (2024), the source of rhamnolipids used were a commercial mixture of type JBR 425, whereas in our study 90 % pure rhamnolipids (R90) were used. According to the rhamnolipid CMC prediction model and data synthesized in the article by Kłosowska-Chomiczewska et al. (2017), the CMC_{water} of commercial JBR 425 rhamnolipids is around 0.07 g L^{-1}, while that of pure rhamnolipids (90–95%) is around 0.026 g L^{-1}, close to the CMC_{water} determined in our study of 0.03 g L^{-1}.

Catalase activity in shoots of ryegrass increased significantly (p-value < 0.05) by 52% after exposure to the highest rhamnolipid concentration of 10 × CMC. This symptom results from an oxidative stress caused by high concentration of rhamnolipids. Indeed, catalase is one of the most important intracellular enzymes in the detoxification of the oxidant hydrogen peroxide which turns H_2O_2 into H_2O and O_2 (Gebicka and Krych-Madej, 2019). The increase in catalase activity observed in our study may have occurred due to H_2O_2 increase in shoot cells, which triggered a warning signal and increased the catalase production to detoxify the hydrogen peroxide in the cellular compartments. Yan et al.

(2016) also observed an increase in antioxidant enzyme activities, including superoxide dismutase and catalase, in rhamnolipids-treated cherry tomato (*Lycopersicon esculentum* Qianxi) inoculated with *Alternaria alternata* within 12 h, while contents of reactive oxygen species (H_2O_2 and glutathione) decreased. The concentration of rhamnolipids applied to cherry tomatoes treated with this biosurfactant was 0.5 g L^{-1} and close to that of the 10 × CMC rhamnolipid-treatment in our study. Relatively high rhamnolipid concentrations (between 0.3 g L^{-1} and 0.5 g L^{-1}) can cause oxidative stress in certain plant species such as ryegrass or cherry tomatoes, increasing catalase activity. This change in enzyme activity could be explained by the increase in the permeability of the cell membrane and/or the alteration of the enzymatic structure under the effects of rhamnolipids, as mentioned in the review of Shao et al. (2017). Previous studies showed that rhamnolipids enhance enzymatic production because they increase the permeability of the cell membrane which results in increasing the secretion of enzymes out of the cells (Guzmán et al., 2024; Shao et al., 2017; Zeng et al., 2018). However, all these studies have demonstrated the effect of biosurfactants on microbial cell membranes and not on plant cell membranes at root-solution interaction, as is the case in our study. It would therefore be interesting to study in-depth the uptake of rhamnolipids by plant roots and their transfer to the shoot system to understand the toxic response induced through the antioxidant enzymatic activities measured. The presence of rhamnolipids can also cause an alteration in the enzymatic structure of catalase. Indeed, electrostatics interactions between the rhamnose head group and the charged amino residues of the catalase and/or hydrophobic interactions between the alkyl chains of the surfactant tail group and the hydrophobic amino acid residues of the enzyme could occur (Karbassi et al., 2003).

Concerning the ascorbate peroxidase activity of ryegrass shoots, the production of this enzyme was not affected by the addition of rhamnolipids in our study. Yan et al. (2016) obtained similar results, observing equivalent ascorbate peroxidase activities between rhamnolipid-treated and untreated cherry tomatoes. This enzymatic activity did not seem to be affected by the presence of rhamnolipids at relatively high concentrations. Adetunji et al. (2018) found that 2% (w/v) rhamnolipids coating induced an increase in superoxide dismutase, catalase and peroxidase activities of orange fruit compared with the control. Moreover, they observed that rhamnolipids significantly delayed the accumulation of malondialdehyde in rhamnolipids-coated fruits compared to the control. Malondialdehyde is a marker of lipid peroxidation and is often quantified to measure the level of oxidative damage caused by oxidative stress. In their study, they concluded that this rhamnolipid application lowered malondialdehyde content and maintained the membrane integrity in coated fruits. These results once again confirm the impact of rhamnolipids on catalase activity, although a reduction in the resulting malondialdehyde content was not observed in our study. However, antioxidant enzymatic activities may be more affected in the case of application of biosurfactants in the context of assisted-phytoremediation treatments. In the context of biosurfactant application for phytoremediation of cadmium (Cd) in polluted paddy field, Wang et al. (2021) found higher superoxide dismutase, peroxidase and catalase activities in roots and leaves of *Iris sibirica*. These were attributed to the abundancy of heavy metals in the apoplast for the roots and in the symplast and apoplast for the leaves. The phytotoxicity of rhamnolipids analysed here through an increase in antioxidant enzymatic activities can be explained by an indirect impact of biosurfactants on the plant, since their contribution led to an increase in the bioavailability of xenobiotics and thus an increase in the concentration of toxic compounds in the plant in synergetic way (Parus et al., 2023).

Regarding the future application of biosurfactants for phytoremediation applications, our results clearly showed that rhamnolipids are likely to harm plant growth when applied at relatively high concentrations (*i.e.* 10 × CMC_{water}), although many authors had previously put forward their biological origin as a guarantee for their environmental safety (Akbari et al., 2018; Shao et al., 2017). This phytotoxicity could be explained by the amphiphilic nature of surfactants, which then interact with the phospholipids in cell membranes and consequently disrupt the selective barrier function of the membranes (Crouzet et al., 2020; Herzog et al., 2020). However, the phytotoxicity of biosurfactants depends on the plant species, and it is therefore essential to assess the impact of biosurfactants on the plant species selected before carrying out biosurfactant-assisted phytoremediation treatment as already pointed by Parus et al. (2023). It is then necessary first to characterize the properties of the rhamnolipids used prior to their application, and more specifically to determine their CMC, which is bound to vary greatly depending on the purity level of the rhamnolipids used, as highlighted by the rhamnolipid CMC prediction models of Kłosowska-Chomiczewska et al. (2017). In addition, since rhamnolipids are anionic compounds, they interact with the soil and often tend to adsorb on soil particles. It is therefore often necessary to add larger quantities of anionic surfactants to the soil in bioremediation processes for hydrocarbon-contaminated soils to exceed their sorption to the soil and achieve CMC in the aqueous phase (Parus et al., 2023). Nevertheless, these higher concentrations are likely to interact directly with plant tissues and therefore cause greater phytotoxic effects.

5. Conclusions

The results obtained in this study show that rhamnolipids are not acutely toxic to ryegrass for concentrations up to 10 × CMC (*i.e.* 0.3 g L^{-1}). Conversely, sodium dodecyl sulphate (synthetic surfactant) inhibits ryegrass germination at a high concentration of 23.65 g L^{-1}, corresponding to 10 × CMC. Relatively high concentrations of 10 × CMC rhamnolipids still moderately impacts ryegrass growth (dry biomass, root length, total water loss) and affects cellular functions (catalase activity) of the ryegrass. These observations can be explained by a change in the permeability of ryegrass root cells in the presence of high rhamnolipid concentrations. The application of biosurfactants in bioremediation processes appears to be more environmentally friendly than the use of synthetic surfactants but to overcome the adsorption of surfactants to the soil the use of very high concentrations of biosurfactants is often necessary in bioremediation processes of contaminated soils. Our results thus suggest that the future application of rhamnolipids in phytoremediation could prove limited due to their phytotoxic potential. Given the different sensitivity of plant species, this parameter should be taken into account when selecting plant species used for phytoremediation applications. In addition, it is to be expected that in real phytoremediation applications additional cross-response of contaminants and biosurfactants are to be expected and should be further assessed.

CRediT authorship contribution statement

Stéphanie Ouvrard: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Sonia Henry:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Antoine Spaudo:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Emmeline D'Incau:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117320.

Data availability

Data will be made available on request.

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