Journal of Medical and Biological Science Research
Vol. 1 (8), pp. 100-106, October, 2015
ISSN: 2449-1810
Research Paper
http://pearlresearchjournals.org/journals/jmbsr/index.html

Rhamnolipids: A New Application in Seeds Development

Vinícius Luiz da Silva[1], Roberta Barros Lovaglio[1], Henrique Hespanhol Tozzi[2], Massanori Takaki[2] and Jonas Contiero[1*]

Accepted 24 September, 2015

[1]UNESP, Universidade Estadual Paulista, Department of Biochemistry and Microbiology, Institute of Biological Sciences, Rio Claro, SP, Brasil.
[2]UNESP, Universidade Estadual Paulista, Departament of Botany, Institute of Biological Sciences, Rio Claro, SP, Brasil.

ABSTRACT

Growing environmental concerns have contributed to the search for, and development of, the use of environmentally friendly substances. Such innovative thinking will lead to the replacement of chemicals by biological surfactants, culminating in an increased use of biosurfactants in several key areas, including agriculture. Hence the crucial importance of evaluating the effect of biosurfactants on seed germination and development. *In vitro* tests of seed germination were conducted on different species of cultivars: lettuce, corn, soybean and sunflower. Evaluated biosurfactant concentrations ranged from 0.25 to 1.00 g·L^{-1}; distilled water was used as the control. After treatment of seeds, lettuce showed an increase of up to 75.50% in its rate of germination, while for corn, seed stimulation occurred at 0.25 g·L^{-1} of rhamnolipids. Regarding to soybeans, biosurfactants did not influence germination, however favored seedling development. The tensioative increased both the germination and development of sunflower seeds.

Key words: Biosurfactants, *Pseudomonas aeruginosa* LBI 2A1 and Seeds Germination.

*Corresponding author.E-mail: jconti@rc.unesp.br.

INTRODUCTION

Rhamnolipids are organic surfactants with low toxicity and high biodegradability and, as such, are environmentally friendly. Their environmental compatibility characteristics, including the use of renewable carbon sources and their production by microorganisms, make these compounds excellent substitutes for surfactants of chemical origin, since both have the same physicochemical properties (Desai and Banat, 1997; Banat et al., 2000). Due to their ability to reduce surface and interfacial tensions, these molecules can be used in many areas of human activity, among them: the cosmetics, pharmaceutical and agricultural industries; food; oil recovery; and bioremediation (Sandrin et al., 2000; Rahman et al., 2003).

The main attraction of biosurfactants for the agro-industrial sector is their antimicrobial activity against phytopathogens (Varnier et al., 2009; Vatsa et al., 2010). According to Sanchez et al. (2012) rhamnolipids may activate the immunological system of thale cress (*Arabidopsis thaliana),* which may help it combat pests and diseases. According to Sachdev and Cameotra (2013), in 2004, 0.2 million tons of harsh chemical surfactants were used in crop protection and pesticide formulation. Due to the increasing demand for products with a low environmental impact, it is probable that environmentally damaging chemical surfactants can be replaced by biosurfactants, if their efficiency can be shown within the agro-industry. With this in mind, the

present study evaluated the influence of these metabolites on the germination of seeds from cultivated plants, such as lettuce (*Lactuca sativa*), corn (*Zea mays*), soybean (*Glycine max*) and sunflower (*Helianthus annuus*).

MATERIALS AND METHODS

Rhamnolipid Production

The *Pseudomonas aeruginosa* strain, LBI 2A1, was used for the production of rhamnolipids. Culture media and growth conditions used for rhamnolipid production were previously described by Müller et al. (2010). Fermentation, using sunflower oil as carbon source, was performed in a 13L bioreactor (Infors HT – Suiça) whit 4 L of culture media. Ramnolipids isolated from sunflower oil were used in the experiments.

Rhamnolipid Extraction

The rhamnolipids extraction carried out were previously described by Lovaglio et al. (2014). The fermented broth was centrifuged at 4000 rpm for 30 min, and equal volumes of cell-free supernatant and n-hexane were thoroughly mixed in a volumetric flask. The mixture was then allowed to settle until the organic and aqueous phases separated. The organic phase was removed and 85% H_3PO_4 1:100 (v/v) was added to the aqueous phase to precipitate rhamnolipids. Biosurfactants were extracted with ethyl acetate 1:1.25 (v/v). The mixture was then shaken for 10 min, allowed to settle, and the upper phase was removed. This extraction process was then repeated using the lower phase. The extracted rhamnolipids were concentrated using a rotary evaporator, and the viscous yellowish product was dissolved in methanol and concentrated again by evaporation of residual solvent at 45°C.

External Seed Sterilization

The external sterilization of lettuce, sunflower, soybean and corn seeds was carried out using alcohol 70% (v/v) for 1 min, after which a 2% (v/v) hypochlorite solution was added for one more min and, then seeds were washed with sterile distilled water.

Seed Germination Tests

Thirty seeds were placed on two filter papers in a sterile plate, and 7.5 mL of water control or rhamnolipid solution were added. Soybean seeds were sandwiched between two filter papers to increase the contact surface of the seeds with the solution. Experiments were carried out under constant luminosity provided by two 20 W daylight-type fluorescent lights (GE; Hungary). The temperature was maintained at 25ºC. The evaluated solutions were: control (distilled water); or 0.25, 0.50, 0.75 or 1.00 $g \cdot L^{-1}$ of rhamnolipids (the rhamnolipids were dissolved in distilled water); with the pH adjusted to 6.5. Throughout the experiment, seeds showing 2 mm roots were considered germinated. Four replicates of each treatment were tested.

Measurement of Root Growth and Dry Biomass

At the end of the experiments, seedling roots were measured, and dry biomass was quantified after drying in an oven at 95 ± 3°C.

Statistical Analysis

Data were compared by one-way analysis of variance followed by Tukey test when significant differences were found at $P = 0.05$ (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

Almost all rhamnolipids concentrations tested stimulated lettuce seed germination, with the greatest stimulation seen at concentrations of 0.75 and 1.00 $g \cdot L^{-1}$, and the average number of seeds that developed were 18.50 and 21.50, respectively (Figure 1a). In contrast, lettuce seedlings subjected to rhamnolipid treatment showed slightly smaller root development, but the accumulation of biomass was favored when compared to control (Figure 1b). The relative frequency of germination indicates the germination distribution over time. When *L. sativa* seeds were subjected to a 1.00 $g \cdot L^{-1}$ rhamnolipid solution, germination reached a relative frequency of 100% in 24 h, indicating that the rhamnolipid solution increased seed germination speed and synchronicity relative to control group seeds (Figure 2).

It was observed partial inhibition of corn seed germination and root growth at 1.00 $g \cdot L^{-1}$ of rhamnolipids, (Figure. 3). However, at lower concentrations 0.50 and 0.75 there are not significantly difference compared to control ($P = 0.075$). The rate of germination increased at 0.25 $g \cdot L^{-1}$, reaching 19.75 of germinated seeds (Figure 3a); the development of root length and the seedling dry weight, was also favored at that concentration of biosurfactant, (Figure 3b). Although root growth was impaired at concentrations of 0.75 and 1.00 $g \cdot L^{-1}$, the dry mass were close to the control, due to the increased number of lateral roots (Figure 3b). The germination of soybean seeds was not significantly affected by rhamnolipid application ($P = 0.3$); after 48 h, 98% of the seeds in all

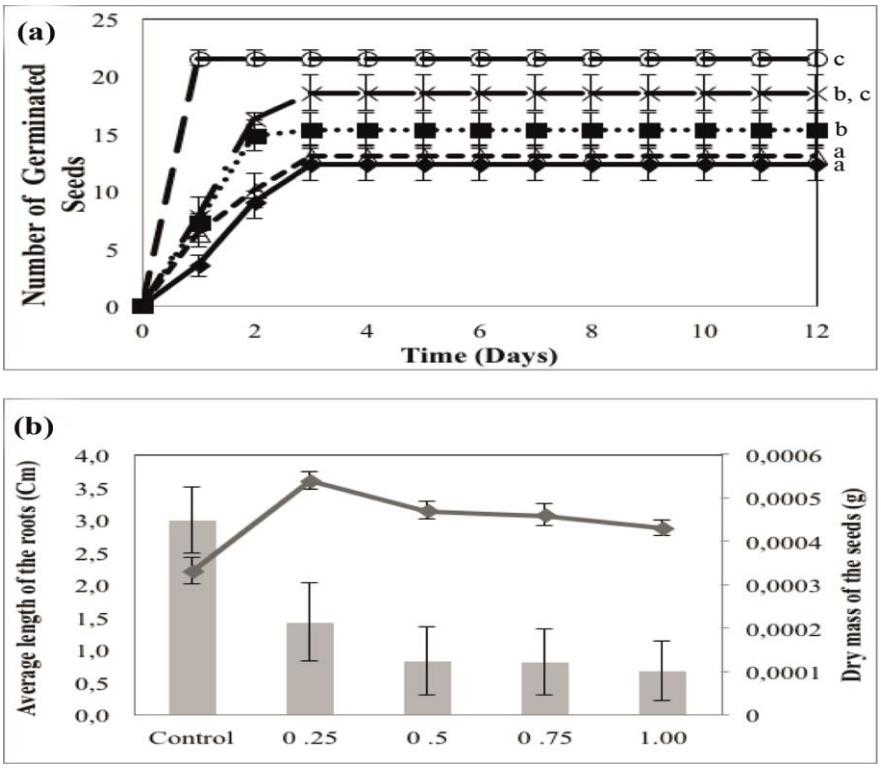

Figure 1. Cumulative germination of *L. sativa* seeds in different rhamnolipid solutions. Thirty seeds were exposed to: control (—◆—) (distilled water), or 0.25 (—■—), 0.50 (—▲—), 0.75(—✕—) and 1.00 (—⊖—) $g \cdot L^{-1}$ of rhamnolipid solution. **(a)** Germinated seeds were counted every day and **(b)** root length (▬) and dry biomass weight (—◆—) of lettuce seedlings measured after 12 days.

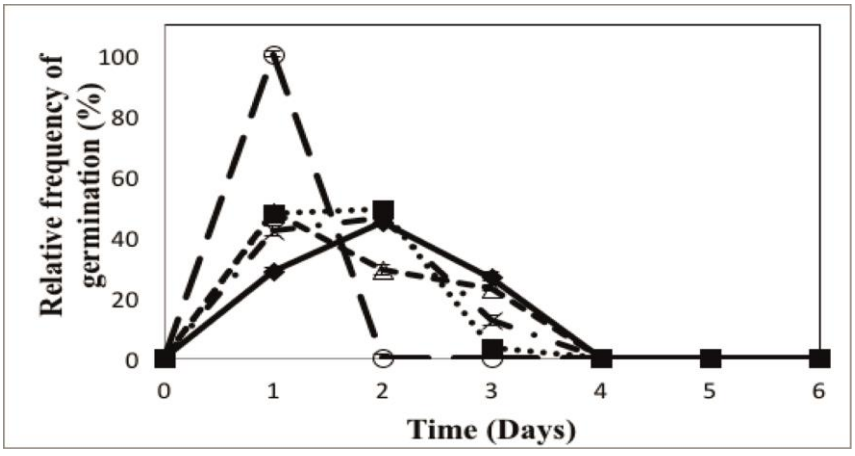

Figure 2. Relative frequency of *L. sativa* seeds germinating over time in different rhamnolipid solutions. Thirty seeds were exposed to: control (—◆—) (distilled water), or 0.25 (—■—), 0.50 (—▲—), 0.75(—✕—) and 1.00 (—⊖—) $g \cdot L^{-1}$ of rhamnolipid solution, and the relative frequency of germinated seeds counted every day.

treatments had germinated (Figure 4a). When analyzing root development and biomass accumulation, a positive effect for all rhamnolipids treatments was noted (Figure 4b). Rhamnolipids favored sunflower seed germination (Figure 5a).

The best germination rate occurred with 1.00 $g \cdot L^{-1}$ of

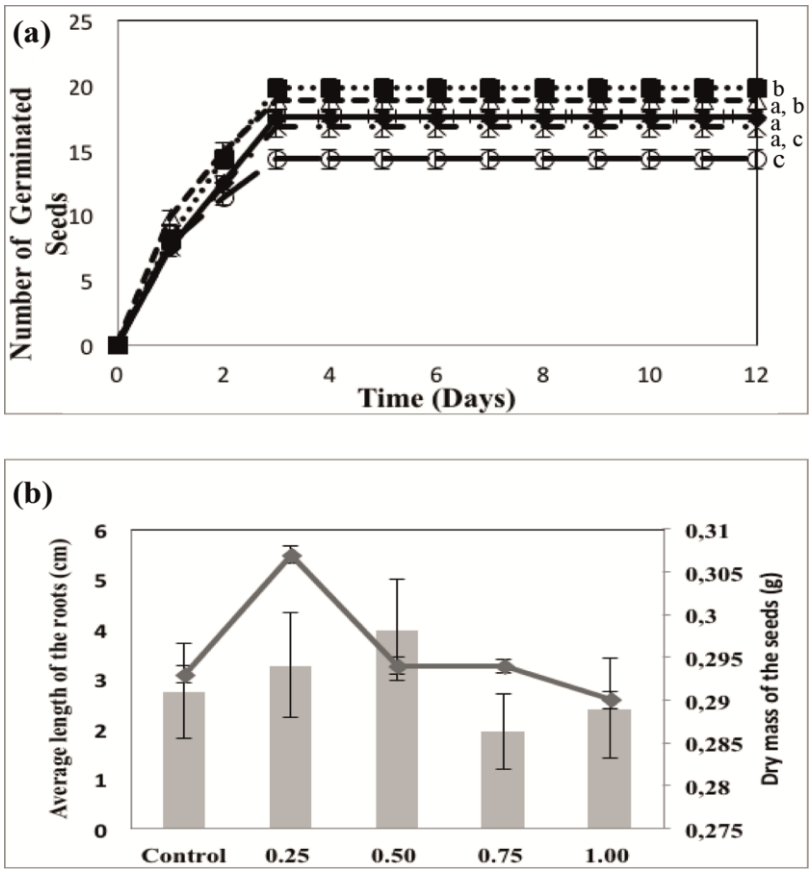

Figure 3. Cumulative germination of *Zea mays* seeds in different rhamnolipid solutions. Thirty seeds were exposed to: control (—◆—) (distilled water), or 0.25 (—■—), 0.50 (—▲—), 0.75 (—✕—) and 1.00 (—●—) g·L^{-1} of rhamnolipid solution and (a) the number of germinated seeds counted every day. (b) Root length ( ) and dry biomass weight (—◆—) of corn seedlings were measured after 12 days.

rhamnolipids, being 30% greater when compared to control. Furthermore, the application of 0.25 and 0.50 g·L^{-1} of this product has made the seeds begin to germinate faster, when compared to other treatments. The use of rhamnolipids also increased sunflower seedling development, as seen in higher biomass accumulation and root growth (Figure 5b). All biosurfactant concentrations evaluated were significantly different, compared to control (P = 0.0009), however there were no statistic difference among them. These results demonstrate that, the application of rhamnolipids can be positive for the tested species. This therefore suggests it is possible to use this microbial metabolite when growing such species, in order to replace synthetic surfactants or in new applications. According to Kerbauy (2008), the process of germination begins when the seed coat allow the entrance of water in the seed, which activates metabolism and leads to growth of the embryonic axis. For this process to take place, it is necessary that wrapping embryonic tissues be permeable to water (Kerbauy, 2008). Similarly, rhamnolipids, due to their amphipathic structure, probably acted on external wrapping tissue, increasing the permeability of seeds and thereby facilitating germination, as reported by Parr and Norman (1965) for surfactants. Soybean cultivars display fast wicking due to the presence of small cracks in the cuticle of the palisade layer, which is the layer responsible for the permeability of water in this species (Fengshan et al., 2004). However, rhamnolipids did not affect soybean germination, maybe because this species has its own evolutionary strategies that increase the permeability of tissues.

In relation to root development and biomass, rhamnolipids may have penetrated soybean and sunflower seeds, helping to mobilize oleaginous reserve tissue from these species, thus supporting seedling development. Kerbauy (2008) reported that the main limiting factor for the start of root axis extension is seed coating resistance; thus the action of rhamnolipids in decreasing the strength of wrapping tissue will favor

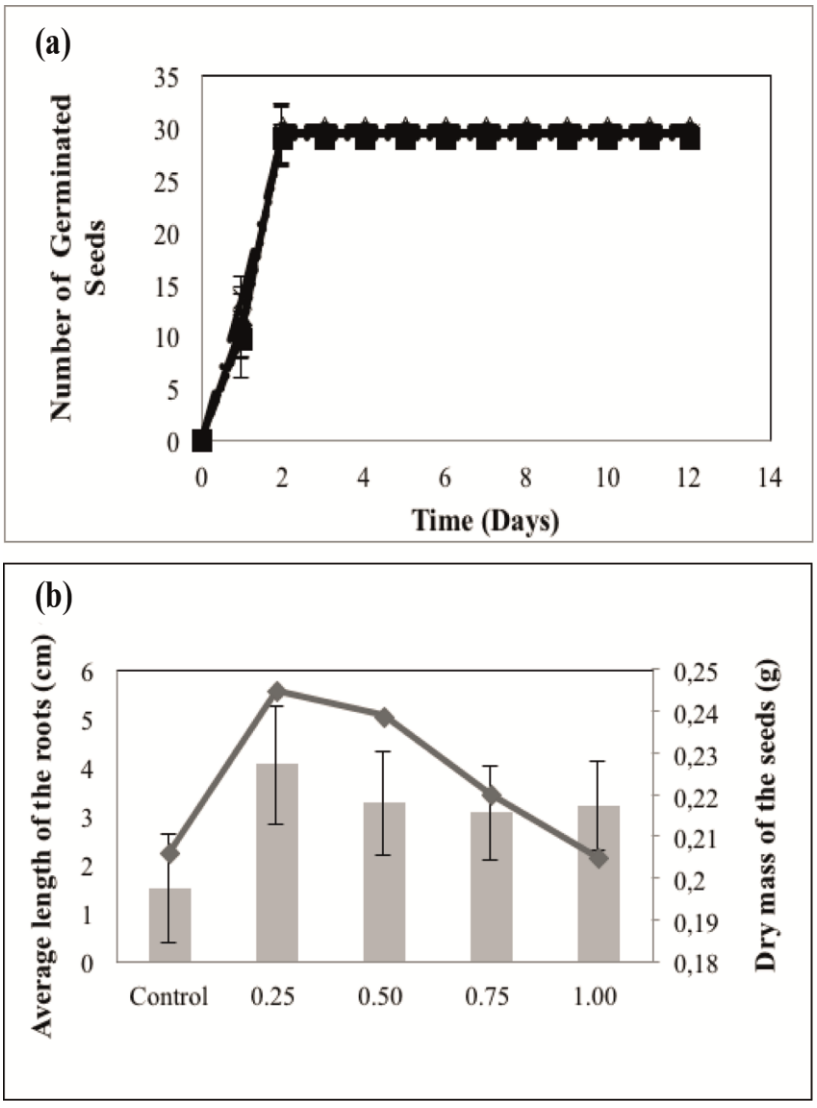

Figure 4. Cumulative germination of *Glycine max* seeds in different rhamnolipid solutions. Thirty seeds were exposed to: control (—◆—) (distilled water), or 0.25 (—■—), 0.50 (—▲—), 0.75(—✕—) and 1.00 (—○—) g·L^{-1} of rhamnolipid solution and (a) the number of germinated seeds counted every day. (b)Root length (▬) and dry biomass weight (—◆—) of soybean seedlings were measured after 12 days.

radicle protrusion, which characterizes germination. In experiments in which lettuce seeds were treated with rhamnolipid solution, it was observed that the leaf portion emerged before the root region; therefore the roots in treatments using this biosurfactant showed less growth. This atypical emergence of the leaf before the root may be due to a large decrease in the resistance of the seed coat in the leaf axis region.

An increase in the synchrony of germination was observed in lettuce seeds that were subjected to rhamnolipid treatments. According to Heydecker and Coolbear (1977), this behaviour is important for farming, because plants present at the same stage of development at harvest time, reducing costs and optimizing the work of the producer. Hurtt and Hodgson (1987) reported that the use of Tween 20 and 80 at 0.5 to 4 g·L^{-1} favored *Echinochloa crus-galli* germination. However, germination inhibition can also occur, and Spurrier and Jackobs (1955) found that Tween 80 at 10 g·L^{-1} inhibited the germination of *Triticum aestivum*. For some species, there is a limit on the amount of surfactant that can be employed in order to promote development, as observed in these studies with corn seeds. In this study, the action of rhamnolipids varied depending on the

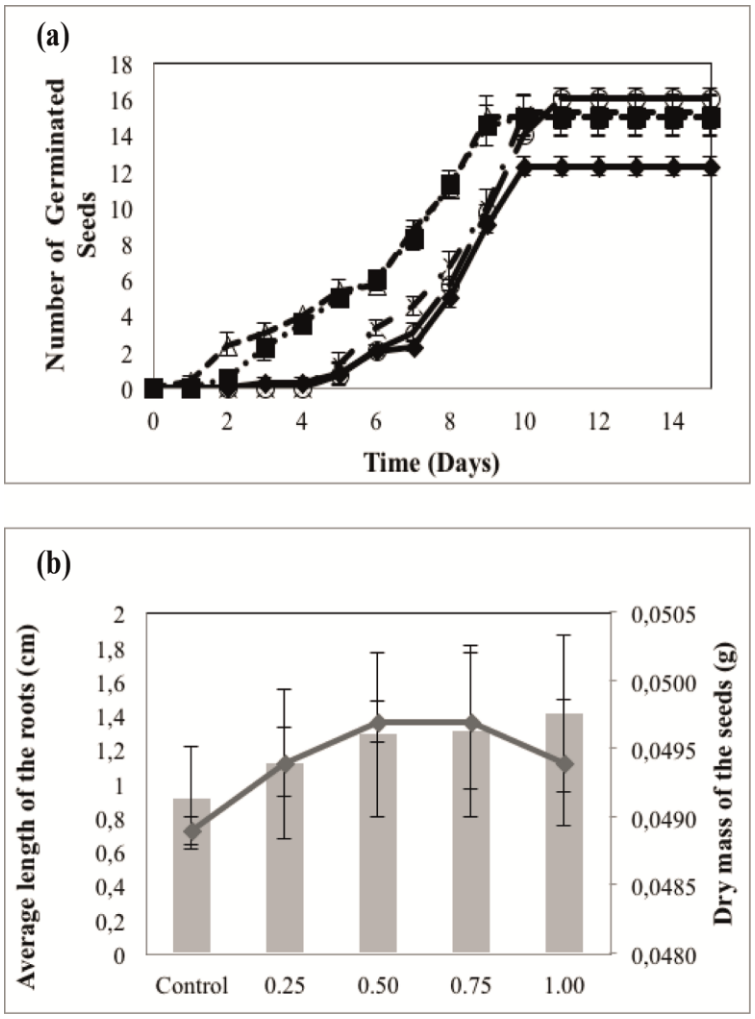

Figure 5. Cumulative germination of *Helianthus annuus* seeds in different rhamnolipid solutions. Thirty seeds were exposed to: control (◆) (distilled water), or 0.25 (■), 0.50 (▲), 0.75(✕) and 1.00 (⊖) $g{\cdot}L^{-1}$ of rhamnolipid solution and (a) the number of germinated seeds counted every day. (b) Root length (▬) and dry biomass weight (◆) of soybean seedlings were measured after 15 days.

concentration employed, and also according to the type of cultivar used. As well as their use in farming, the positive effect of rhamnolipids on the germination and development of these cultivars may also allow the successful planting of these cultivars at sites undergoing bioremediation processes and containing rhamnolipid traces.

CONCLUSION

Rhamnolipids application is favorable for the germination and/or growth of lettuce, corn, soybean and sunflower cultivars, this combined with the high environmental compatibility of these biological molecules can lead to a new application of rhamnolipids in agriculture

ACKNOWLEDGEMENTS

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), for fellowships and financial supports (Process 2011/ 15345-8).

REFERENCES

Banat IM, Makkar RS, Cameotra, SS (2000). Potential commercial application of microbial surfactants. Appl. Microbiol. Biotechnol.

53:495-508.
Desai JD, Banat IM (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev.61:47-64.
Fengshan MA, Cholewa EWA, Mohamed T, Peterson CA, Gijzen M (2004). Cracks in the palisade cuticle of soybean seed coat with their permeability to water. Ann. Bot. 94:213-228.
Heydecker W, Coolbear P (1977). Seed treatments for improved performance survey and attempted prognosis. Seed Sci. Technol. 5:353-42.
Hurtt WW, Hodgson HR (1987). Effects of Nonionic Surfactants, Temperature, and Light on Germination of weed seeds. Weed Sci. 35:52-57.
Kerbauy GB ,2008. *Fisiologia vegetal* 2nd ed. Rio de Janeiro: Guanabara Koogan Ltd.pp. 389-406.
Lovaglio R B, Silva VL, Capelini TL, Eberlin MN, Hausmann R, Henkel M, Contiero J (2014). Rhamnolipids Production by a *Pseudomonas aeruginosa* LBI Mutant: Solutions and Homologs Characterization. Tenside. Surf. Det. 51:397-405.
Müller MM, Hörmann B, Syldatk C, Hausmann R (2010). Pseudomonas aeruginosa PAO1 as a model for rhamnolipid production in bioreactor systems. Appl. Microbiol. Biotechnol. 87:167-174.
Parr JF, Norman AG (1965). Considerations in the use of surfactants in plant systems: a review. Botan. Gaz. 126:86-96.
Rahman KSM, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, Banat IM (2003). Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol. 90:159–168.
Sachdev DP, Cameotra SS (2013). Biosurfactants in agriculture. Appl. Microbiol. Biotechnol. 97:1005-1016.
Sanchez L, Courteaux B, Hubert J, Kauffmann S, Renault J, Clément C, Baillieul F, Dorey S (2012). Rhamnolipids elicit defense responses and induce disease resistance against biotrophic, hemibiotrophic, and necrotrophic pathogens that require different signaling pathways in arabidopsis and highlight a central role for salicylic acid. Plant Physiol. 160:1630–1641.
Sandrin TR, Chech AM, Maier RM (2000). A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Appl. Environ. Microbiol. 66:4585-4588.
Sokal RR, Rohlf FJ, 1995. Biometry: The Principles and Pratices of Statistics in Biological Research. New York.pp. 200-230.
Spurrier EC, Jackobs J A (1955). Some effects of an anionic sodium sulfonate type surfactant upon plant growth. Agron. J. 47:462-465.
Varnier AL, Sanchez L, Vatsa P, Boudesocque L, Garcia-Brugger A, Rabenoelina F, Sorokin A, Renault JH, Kauffmann S, Pugin A, Clement C, Baillieul F, Dorey S (2009). Bacterial rhamnolipids are novel MAMPs conferring resistance to Botrytis cinerea in grapevine. Plant Cell Environ. 32:178-193.
Vatsa P, Sanchez L, Clement C, Baillieul F, Dorey S (2010). Rhamnolipid Biosurfactants as New Players in Animal and Plant Defense against Microbes. Int. J. Mol. Sci. 11:5095-5108.