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The lipopeptide surfactin triggers induced systemic resistance and priming state responses in *Arachis hypogaea* L.

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Abstract Bioactive metabolites produced by multiple strains of *Bacillus* spp. stimulate plant defense responses. Among these, the cyclic lipopeptide surfactin was identified as an Induced Systemic Resistance (ISR) elicitor in different plant species. However, the underlying mechanisms involved in the ISR elicitation and the priming state costs in peanut plants (*Arachis hypogaea* L.) remain unknown. In this work, we demonstrated the ability of surfactin from *B. subtilis* to induce systemic resistance against *Sclerotium rolfsii* in peanut plants, and showed that this response involves key characteristics of priming-mediated resistance defense. Application of surfactin significantly reduced *S. rolfsii* disease incidence and severity on peanut plants, and an increased shoot and root dry weight was observed in surfactin pre-treated and pathogen challenged plants compared to non-treated challenged plants. In addition, peroxidase activity and phenolic compounds deposition underneath the fungal infection zone were significantly higher in surfactin pre-treated and challenged plants than in non-surfactin treated challenged plants. Collectively, results from this work indicate that ISR activity elicited by surfactin involves a priming defense state with low fitness-related costs, providing an enhanced protection against *S. rolfsii* in peanut plants.

Keywords ISR · Defense response · Priming · Peanut · Surfactin

Introduction

Peanut (*Arachis hypogaea* L.) is an economically important legume representing a key tool towards the development of an agro-alimentary complex that meets the growing global food demand. However, fungal diseases limit peanut production worldwide. The heavy reliance on chemical fungicides to control plant diseases has led to environmental pollution and increments in production costs (Perez-Garcia et al. 2011). Therefore, there is an increasing interest in finding biological alternatives to control pathogens, avoiding the environmental damage caused by the extensive use of chemical substances. As an alternative, combination of agrochemicals, beneficial microbes or biomolecules able to induce plant defense response could provide an excellent opportunity for reducing pesticide use in agriculture (Conrath et al. 2015).

Currently, much research are focused on the Induced Systemic Resistance (ISR) as a biological control mechanism. ISR is a long distance signaling mechanism that provides broad spectrum and long-lasting resistance to future infections throughout the plant. Some of the bacterial compounds involved in ISR elicitation include

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flagellin, lipopolysaccharides, and several secondary metabolites like siderophores, volatile, phenolic compounds (De Vleesschauwer and Höfte 2009; Lugtenberg and Kamilova 2009) and cyclic lipopeptides (surfactin, iturin and fengycin) (Ongena and Jacques 2008). ISR mechanism can be globally seen as a sequentially related three-step process. First, the phenomenon is initiated by plant perception of eliciting cells or molecules produced by elicitor agents. Second, signal transduction is activated (via ethylene and jasmonic acid signaling pathways) and the priming state is established. Finally, defense mechanisms that limit or inhibit the future entry of pathogens into plant tissues are expressed, including morphological adaptations such as lignification, accumulation of callose, phenolic compounds, among others (Mariutto and Ongena 2015).

A typical feature of ISR is the phenomenon known as priming. Primed plants show a faster and/or stronger activation of defense response when are challenged by a triggering stimulus. Historically, the lack of definition about the basic criteria for priming establishment has hindered the study of this phenomenon. Recently, Martinez-Medina et al. (2016) established the key features of priming. This defense state is characterized by the memory of the stimulus, a low metabolic cost for plants, a more robust defense response and a better performance of pathogen challenged plants. However, it can also entail some costs due to the allocation of resources or toxicity of defensive products (Heil 2002; Conrath et al. 2015; Hilker et al. 2016).

Surfactin is a lipopeptidic antibiotic produced by multiple strains of *Bacillus* able to induce ISR responses in diverse plant species (Ongena et al. 2007). This low specificity could be explained by the fact that its perception by plant cells may rely on a mechanism based on the direct interaction with the plasma membrane rather than the recognition by a receptor, as established for other elicitors (Cawoy et al. 2014). Studies about ISR activity and defense priming elicited by lipopeptides in peanut are very scarce. Considering that enhancing plant defenses is a more environmentally-friendly alternative than the application of pesticides, the objectives of this work were to determine the ability of surfactin from *B. subtilis* to induce systemic resistance effective against *S. rolfsii* in peanut and to assess if this response involves the key features of priming phenomenon.

Material and methods

Plant material and growth conditions

All experiments were conducted under controlled conditions with *Arachis hypogaea* (peanut) var. Runner cultivar Granoleico, susceptible to *S. rolfsii*.

Peanut seeds were surface sterilized as described by Vincent and IBP (1970). Briefly, seeds were soaked in 96% ethanol for 30 s followed by 20% H_2O_2 for 15 min, and then washed six times with sterile distilled water. The surface sterilized seeds were germinated at 28 °C in sterilized Petri dishes with one layer of Whatman No.1 filter paper and moist cotton, until the radicle reached approximately 2 cm.

Pathogen growth conditions and inoculum preparation

S. rolfsii was obtained from infected peanut plants and grown on Potato Dextrose Agar (PDA) (Kong et al. 2010) supplemented with streptomycin sulfate (100 g mL^{-1}) at room temperature for 7 days.

To obtain the pathogen inoculum, wet wheat seeds contained in a 50-mL Erlenmeyer flask were autoclaved and then infected with 5 mm diameter of *S. rolfsii* mycelial plugs. Erlenmeyer was maintained at room temperature until abundant mycelium growth was observed (7–10 days approximately) (Gupta et al. 2002).

Surfactin preparation

An aqueous solution of surfactin from *Bacillus subtilis,* ≥98.0% (HPLC) (Sigma–Aldrich Corporation, St. Louis, MO, USA) was used. Surfactin 5 and 10 μM were prepared using sterilized distilled water.

Induction of systemic resistance and defense priming evaluation

To guarantee physical separation between the lipopeptide and the phytopathogen, a system with two plastic cups was used (Figueredo et al. 2017) (Supplementary Fig. 1). Briefly, two plastic cups filled with sterilized vermiculite were placed one above the other and connected by a hole made in the bottom of the upper cup. A pre-germinated peanut seed was sown in the upper plastic cup so that the root reaches the bottom plastic cup through the hole that connected both cups. Radicles of peanut seedlings (contained in the bottom

cup) were previously treated by dipping in aqueous surfactin solution. At 24 h after lipopeptide treatment, the seedlings were challenged with the pathogen by adding on the plant crown (in the upper cup) one wheat seed infected with *S. rolfsii* mycelium (20 mg). Plants were covered with nylon bags for 72 h to favor disease development conditions. Non-pathogenized and non-surfactin treated control plants were also included. Plants were grown under controlled environment (light intensity of 200 μE m^{-2} s^{-1}, 16-h day/8-h night cycle, at a constant temperature of 28 °C and a relative humidity of 50%), watered regularly with sterilized tap water and once a week with Hoagland solution (Hoagland and Arnon 1950). At 14 days after phytopathogen inoculation, assessment of disease incidence (represented as the percentage of plants with the characteristic disease symptomatology) and disease severity (evaluated by shoot and root dry weights after dried at 60 °C for 10 days to a constant weight) were recorded. The experiment was repeated three times with 5 replicates per treatment.

Defense priming phenomena was analyzed by assessment of plant defenses and the associated cost–benefit balance, following the criteria proposed by Martinez-Medina et al. (2016).

Determination of peroxidase (PX) activity

PX activity was determined in a portion of peanut stems (0.1 g) cut about 1 cm above the crown. The term "crown" is used to designate the portion of the plant where the two lateral branches originate on the central stem. Plant tissues were homogenized with liquid nitrogen containing appropriate buffer solution (50 mM potassium phosphate and 1 mM EDTA, pH 7.8) and 1% PVP (polyvinylpyrrolidone). Tissue extract was centrifuged at 12,000 g for 20 min at 4 °C and the supernatant was stored at −20 °C until it was used for enzymatic activity determination. Protein concentration of the extracts was determined by the method described by Bradford, using bovine albumin (1 mg mL^{-1}) as standard.

PX activity was determined by measuring the absorbance at 470 nm according to Sosa Alderete et al. (2009). PX activity was expressed as units mg $proteins^{-1}$.

The experiment was repeated three times with five replicates per treatment.

Determination of total phenolic compounds accumulation

Phenolic compounds accumulation was determined in a portion of peanut stems (0.1 g) cut about 1 cm above the crown. Plant tissues were homogenized with liquid nitrogen in 1 mL of 95% methanol. The tissue extract was stored in the dark for 48 h, centrifuged at 13000×g for 5 min at room temperature and the supernatant was stored at −20 °C until it was used for total phenolic compounds determination.

Total phenolic content of the methanolic extracts were determined colorimetrically using Folin Ciocalteu reagent by measuring the absorbance at 765 nm according to Ainsworth and Gillespie (2007). Gallic acid (0.1 mg mL^{-1}) was used as a standard. Results were expressed as μM gallic acid equivalents g fresh $weight^{-1}$ of peanut plant material.

The experiment was repeated three times with 5 replicates per treatment.

Statistical analysis

Statistical analysis was performed by subjecting the data to Student *t*-test and analysis of variance (ANOVA), using Infostat software (1.0, FCA, UNC, Argentina) and GraphPad Prism for graphics. A $p \leq 0.05$ significance level was used throughout.

Results

Effectiveness of surfactin-induced defense response against *S. rolfsii* in peanut plants

In order to determine if surfactin induces a defense response against *S. rolfsii* in peanut, incidence of the disease was determined in challenged plants and surfactin treated and challenged plants. Two different surfactin concentrations were tested (5 and 10 μM). For both treatments, results showed that disease incidence was reduced compared with non-treated plants. However, the most effective surfactin concentration was 10 μM (Fig. 1).

According to these results, the surfactin concentration 10 μM was selected to continue with the analyses.

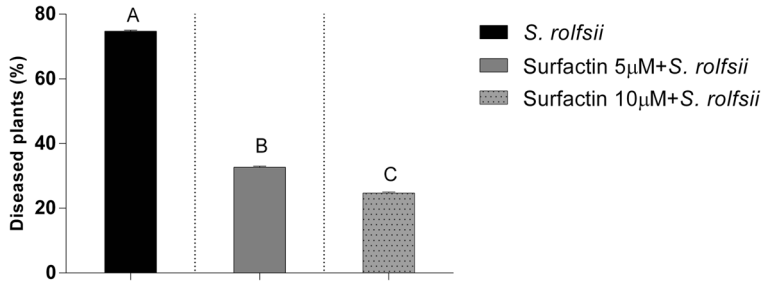

Fig. 1 Disease incidence of peanut plants challenged with *S. rolfsii*. Data represent the means ±SE of three independent experiments ($n = 15$ plants). Different letters indicate statistically significant differences according to the LSD Fisher test ($p < 0.05$)

Fitness-related responses and molecular analyses of plant defense modulation induced by surfactin in peanut plants

Biomass production

To evaluate if surfactin affects growth of both unchallenged and *S. rolfsii* challenged peanut plants, shoot and root dry weights were determined. Unchallenged peanut plants treated with surfactin did not show changes in biomass production, compared with control plants. However, in *S. rolfsii* challenged plants, the surfactin pre-treatment increased significantly their shoot dry weight (Fig. 2). Non-statistically significant differences were found in their root dry weight.

Accumulation of total phenolic compounds and PX activity

To evaluate the modulation of plant defense response mediated by surfactin treatment, content of total phenolic compounds and PX activity were measured. As expected, no differences were observed between unchallenged control and surfactin treated plants. However, surfactin treated plants showed an increase in the phenolic compounds content as well as in the PX activity when the plants were challenged with the pathogen (Figs. 3 and 4).

Discussion

The plants rhizosphere is colonized by a wide diversity of microorganisms including bacteria and fungi that can be involved in plant growth promotion (PGPB and PGPF) (Venturi and Keel 2016). The molecular mechanisms developed by plants and microbes to interact with one other provide a broad spectrum of benefits not only to the microbes but also to the plants (Babar et al. 2016; Wiesel et al. 2014). Based upon this complex molecular interplay, the use of microbes or microbe-derived products for inhibiting the growth of pathogenic agents are currently being employed for the agricultural, environmental, and health benefits (Wiesel et al. 2014).

Results obtained in this study demonstrated that *S. rolfsii* disease incidence was reduced in peanut plants pre-treated with surfactin, indicating that this lipopeptide activates the inducible defense responses in this legume.

Spatial and temporal separation between the lipopeptide (added to the roots) and the pathogen (in the crown) excludes the possibility of direct antagonism, leading to the conclusion that systemic resistance was

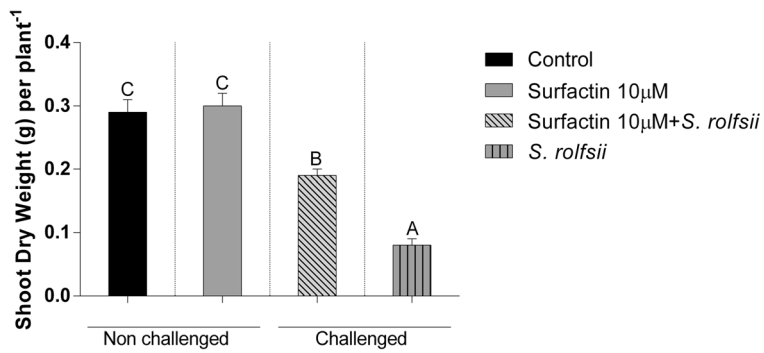

Fig. 2 Shoot dry weight of peanut plants after 10 dpt with surfactin 10 μM challenged and non-challenged with *S. rolfsii*. Data are the means ±SE of three independent experiments using 15 plants per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$)

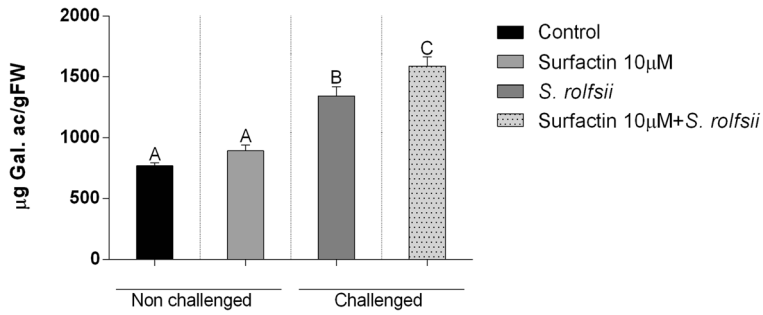

Fig. 3 Content of total phenolic compounds in peanut plants treated with surfactin. Data are the means ±SE of three independent experiments (n = 15). Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$)

induced. The fungal disease suppression ability of this lipopeptide in multiple plant patho-systems (including bean, tomato and tobacco cell suspension cultures) has been reported (Ongena et al. 2007).

In tomato plants, surfactin treatment significantly reduced the disease caused by *B. cinerea*. Moreover, disease levels were similar to those obtained with surfactin producing *Bacillus subtilis* S499 and 98S, indicating that the treatment with the biomolecule is as effective as inoculation with the bacteria (Cawoy et al. 2014).

ISR enhances the defensive capacity in plants, resulting in a faster and stronger defense reaction when they are exposed to biotic stress (Pieterse et al. 2014). Priming is an essential part of ISR phenomena. However, knowledge about the responses involved in defense priming is scarce, hindering the possibility to predict the cost and benefits of priming induction to enhance plant defense at field conditions. Therefore, it is necessary to redirect research focus towards the study of defense priming trough a methodological approach which integrates molecular analyses of plant defense responses and fitness-related trade-offs (Martinez-Medina et al. 2016). In this work we demonstrated that ISR elicited in peanut by surfactin involves the key characteristics of defense priming. Temporal separation between treatments (surfactin treatment and *S. rolfsii* challenge) and the reduction in the percentage of diseased plants are related with the storage of the first stimulus (surfactin treatment), indicating memory acquisition (a characteristic feature of priming defense). Furthermore, in absence of pathogen infection, surfactin treatment had no significant effect on plant growth. A positive effect on biomass production was evident in *S. rolfsii* challenged plants, with a significant increase in this parameter only in surfactin treated plants under pathogen pressure. These findings suggest that surfactin treatment incurs in low fitness cost for non-challenged peanut plants and, in the presence of *S. rolfsii* pressure these plants show a better performance than in non-treated plants. Surprisingly, few studies were directed to assess the fitness effects of defense priming (low fitness cost and better performance). In this study, we demonstrated that application of surfactin as ISR inductor does not represent a cost in plant fitness.

Plants possess a complex set of defense preformed structures and inducible responses that could be activated as a consequence of the perception of pathogenic microorganisms (Mikulic-Petkovsek et al. 2013). Peroxidases contribute to the formation of plant factors involved in disease resistance, such as lignin and hydrogen peroxide (Niranjan Raj et al. 2012). Moreover, reactive oxygen species like H_2O_2 are relevant to ISR because evidences support that they could act as long

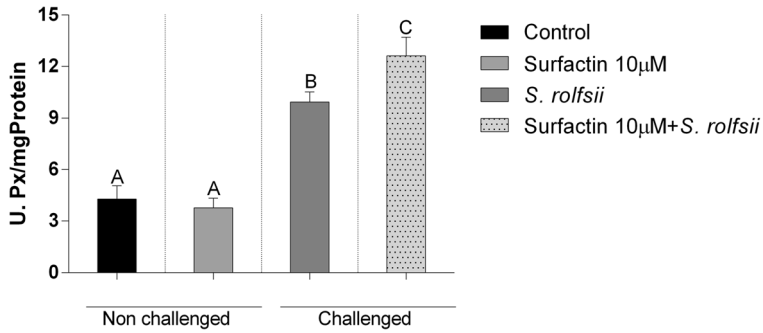

Fig. 4 PX enzyme activity in peanut plants treated with surfactin. Data are the means ±SE of three independent experiments (n = 15). Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$)

distance signaling molecules to systemic tissues (Mauch-Mani et al. 2017; Jourdan et al. 2009). Peanut plants previously treated with surfactin displayed an increased peroxidase activity 24 h after inoculation with *S. rolfsii*. This could be related with the expression of defense mechanisms, such as production of reactive oxygen species and cell wall reinforcement. In this sense, we speculate that surfactin stimulus before pathogen challenge primes the plant defense response and that this mechanism involves an increment in PX activity of peanut plants.

Phenolic compounds are accumulated in plant tissues exposed to pathogen attack, strengthening plant cell walls and limiting pathogen invasion (Cle et al. 2008). This response represents an important defense strategy towards necrotrophic pathogens such as *S. rolfsii* (Adandonon et al. 2017). As expected, the total content of phenolic compounds was higher in pathogen-challenged than in non-challenged peanut plants. Moreover, a higher accumulation of phenolic compounds in surfactin treated and challenged plants was observed, suggesting a fast onset of cell wall fortification enhanced by the previous surfactin stimulus. Other studies have reported significantly higher phenolic compounds in response to fungal, bacterial and viral infection (Schovánková and Opatová 2011; Koc and Ustun 2011). Considering that PX activity is involved in the enhance production and accumulation of phenolic compounds (Anand et al. 2009; Gogoi et al. 2001), we suggest that both the high activity of these enzymes and phenolic compounds accumulation in surfactin treated and challenged peanut plants are related with a more robust response induced by the lipopeptide, which acts as a priming stimulus preparing the plant defense mechanism to respond more efficiently upon pathogen challenge.

To our knowledge, this is the first report showing that surfactin is effective in activating defense responses against *S. rolfsii* in peanut. Taken together, results from this work indicate that the lipopeptide surfactin induces a systemic response in peanut plants, triggering a defense state characterized by the presence of key priming criteria such as a low fitness cost, a plant memory acquisition, a better performance and a more robust response.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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