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Research article

Restrictive water condition modifies the root exudates composition during peanut-PGPR interaction and conditions early events, reversing the negative effects on plant growth

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ABSTRACT

Water deficit is one of the most serious environmental factors that affect the productivity of crops in the world. *Arachis hypogaea* is a legume with a high nutritional value and 70% is cultivated in semi-arid regions. This research aimed to study the effect of water deficit on peanut root exudates composition, analyzing the importance of exudates on peanut-PGPR interaction under restrictive water condition.

Peanut seedlings were subjected to six treatments: 0 and 15 mM PEG, in combination with non-inoculated, *Bradyrhizobium* sp. and *Bradyrhizobium-Azospirillum brasilense* inoculated treatments. We analyzed the 7-day peanut root exudate in response to a water restrictive condition and the presence of bacterial inocula. Molecular analysis was performed by HPLC, UPLC and GC. Bacteria motility, chemotaxis, bacterial adhesion to peanut roots and peanut growth parameters were analyzed.

Restrictive water condition modified the pattern of molecules exuded by roots, increasing the exudation of Naringenin, oleic FA, citric and lactic acid, and stimulation the release of terpenes of known antioxidant and antimicrobial activity. The presence of microorganisms modified the composition of root exudates. Water deficit affected the first events of peanut-PGPR interaction and the root exudates favored bacterial mobility, the chemotaxis and attachment of bacteria to peanut roots.

Changes in the profile of molecules exuded by roots allowed *A. hypogaea-Bradyrhizobium* and *A.hypogaea–Bradyrhizobium-Azospirillum* interaction thus reversing the negative effects of restrictive water condition on peanut growth. These findings have a future potential application to improve plant-PGPR interactions under water deficit by formulating inoculants containing key molecules exuded during stress.

1. Introduction

Arachis hypogaea is a legume with high nutritional value and is the sixth most important source of oil and the third most important source of vegetable protein in the world (Raval et al., 2018). In Argentina, about 85% of peanut production takes place in Córdoba province and the peanut obtained is of a very high quality and almost all the production is exported to European Union, Indonesia, Canada, among others (INTA, 2017). Taking into account the agronomic importance of peanut crop, used for food (raw, roasted or boiled, cooking oil), animal feed (pressings, seeds, green material, and straw) and industrial raw material, it is important to develop strategies that increase its production. But peanut production process, from planting to storage, is affected by different types of biotic and abiotic agent. An increase in the periods of water deficit is expected in many regions of the world (Dai, 2011), including the province of Cordoba, the focus area of this study. Inoculation with plant promoting bacteria (PGPB) is a widespread practice since it help to maintain adequate nutrition of plants and reduce the negative effects of abiotic stress (Sandhya et al., 2009; Glick, 2010). Thus, the knowledge of the impacts of water stress on plants, including root exudation, rhizospheric microorganism and their interactions, is consequently vital for agricultural development.

Root exudates (RE) encompass a wide array of chemical constituents including primary and secondary metabolites, ions, mucilage, amino

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acids, sugars, nucleotides, organic acids, fatty acids, phenolic compounds as flavonoids, and few other miscellaneous chemicals (Bais et al., 2006). These exudates are known to build a network of interactions with plant roots and their surrounding rhizospheric microbes through various physical, chemical, or biological interactions (Haichar et al., 2014). The quantity of RE depends mainly on plant species, age, cultivar type, plant root metabolic attributes, root system architecture, and environmental conditions that come across during plant growth (Haichar et al., 2008; Compant et al., 2010). Flavonoids, organic acids or sugars in RE play specific roles as carbon sources and molecular signals in plant–microbe interactions (Kloss et al., 1984). Flavonoids are a large subgroup of secondary metabolites categorized as phenolic compounds and their functions include auxin transport regulation, modulation of reactive oxygen species, protection against UV light and the induction of several *nod* genes in the *Rhizobium* spp. to produce nod factors (Fox et al., 2011; Amalesh et al., 2011; Falcone Ferreyra et al., 2012). Organic acids present in the RE are involved in metabolic processes including the assimilation of carbon and nitrogen, the regulation of cytosolic pH and osmotic potential, the balancing of charges during excess cation uptake. These compounds can also stimulate microbial activity in the rhizosphere, which is likely to influence the availability of other minerals and nutrients (Ryan et al., 2001).

Root exudates modulate positive plant-microbe interactions and thereby regulate the plant growth, development, and yield. *Arachis hypogaea* L. cultivar Granoleico (Criadero El Carmen) is widely used in Argentina, since it presents a high yield of grain per hectare. However there are few reports on the molecular composition of peanut root exudate, the profile of flavonoids exuded by *Arachis hypogaea* cv. Tegua has been described in the literature (Taurian et al., 2008). A better understanding of root exudation should contribute to improve the crop adaptation to stressful environments, such as water deficit, and to more sustainable and profitable farming.

The symbiosis between rhizobia and its legume host plants is an important example for plant growth-promoting rhizobacteria (PGPR). Bacteria of the genus *Bradyrhizobium* are able to establish a symbiotic relationship with peanut (*Arachis hypogaea*) and metabolize root exudates and in turn provide nitrogen to the plant for amino acid synthesis (Nievas et al., 2012). The infection occurs when rhizobia directly colonize the subepidermal root tissue (the root cortex) by crack entry at the lateral root base in an intercellular manner, without the formation of infection thread (Sprent, 2007). The ability to fix nitrogen also occurs in free-living bacteria like *Azospirillum*. This genus is able to colonize hundreds of plant species and improve their growth, development and productivity by several mechanisms such as indol acetic acid (IAA) production, and improve general plant performance under normal and/or stressing growth conditions (Bashan & de-Bashan, 2010). Previous results of our working group show that the simple inoculation with SEMIA6144 reversed the negative effects of a RWC on peanut plants of 30 days of growth, with better results compared to double inoculation (Cesari et al., 2019). However, it is unknown whether this response is conditioned by a modification in the early events of the plant-microorganisms interaction, given by signal molecules.

Effective colonization of the root system by PGPR depends on molecular signals and early events such as bacterial motility, chemotaxis and the attachment of soil bacteria to plant root cells. All this is crucial for the exercise of afore mentioned beneficial effects. Thus, the aim of this study was to evaluate whether a restrictive water condition impact on the root exudation pattern of *Arachis hypogaea* cv. Granoleico and the early step required in plant-microbe with *Bradyrhizobium* SEMIA6144 and *Azospirillum brasilense* Az39.

2. Material and methods

2.1. Plant material and bacterial strains

Arachis hypogaea L. (peanut) cv. Granoleico (provided by El Carmen S.A, General Cabrera, Córdoba, Argentina) seeds were surface-sterilized as described by Vincent (1970) and germinated at 28 °C in sterile water-agar in petri dishes.

The bacterial strains used in this work were *Bradyrhizobium*. sp strain SEMIA6144 (MIRCEN/FEPAGRO, Brazil) and *Azospirillum brasilense* strain Az39 (Rodriguez Cáceres, 1982). *Bradyrhizobium* sp. SEMIA6144 (SEMIA6144 in the rest of the text) was grown in B-medium (van Brussel et al., 1977; Medeot et al., 2010). *Azospirillum brasilense* (Az39 in the rest of the text) was grown in NFb (Döbereiner and Day, 1976). Both cultures were incubated at 28 °C with shaking at 150 rpm (Allied Fisher Scientific) until the stationary phase (24 h for Az39 and 110 h for SEMIA6144) for use in subsequent tests. To simulate a growth restrictive water condition (RWC), the bacterial media were supplemented with 15 mM of non-permeating solute polyethylene glycol (PEG, average MW 5489 Da, Sigma Chemical Co., St. Louis, MO, USA) (Dardanelli et al., 2008; Cesari et al., 2016, 2018).

2.2. A. hypogaea root exudate collection and experimental design

To collect the peanut RE, each germinated seed was aseptically transferred to a hydroponic system consisting of a glass tube containing 30 ml of Hoagland (pH 6.5) nutrient solution (Hoagland and Arnon, 1938). The plants were incubated aseptically for 7 days in a growth chamber subjected to a photoperiod of 16 h of light at 24 °C alternating with 8 h of darkness at 20 °C, preserving the roots of light (Dardanelli et al., 2008b). Polyethylene glycol (PEG; MW 6000) was used for induction of restrictive water condition.

The experiment had a factorial structure with a completely randomized 2X3 design:

1. Availability of water with two (2) levels: a. Non-restrictive water conditions (NRWC): Hoagland solution (−0.07 MPa); b. Restrictive water conditions (RWC): Hoagland solutions supplemented with PEG6000, 15 mM (−0.28 MPa). 2. Inoculation with three (3) levels: a. Plants un-inoculated; b. Single inoculation: 1 ml (10^8 cells) of *Bradyrhizobium* SEMIA6144 (SEMIA6144 in the rest of the text) per tube; c. Double inoculation: 1 ml (10^8 cells) of SEMIA6144and 1 ml (10^6 cells) of *Azospirillum brasilense* Az39 (Az39 in the rest of the text) per tube.

On the seventh day, plants were removed from the tubes. To check sterilization, a sample of exudate (100 μL) was inoculated in TY medium and growth was assessed after overnight incubation at 28 °C. Sterile samples were kept at 4 °C. Exudates were collected and centrifuged at 10,000 rpm for 20 min to remove root debris and microorganisms, and 150 ml of exudate were concentrated by lyophilization and stored at −20 °C. Exudates concentrated by lyophilization, were dissolved in water and analyzed by chromatography.

2.3. Molecular characterization of the peanut RE

All peanut RE samples collected were analyzed to characterize the presence of molecules of interest.

Flavonoids, auxins and tryptophan: high performance liquid chromatography coupled to a mass detector (HPLC-MS) was used. The lyophilized material was dissolved in 1 ml of deionized water and 20 μl aliquots were injected in an electrospray HPLC electrospray ionization tandem. Chromatographic separation was performed using a PerkinElmer 200 Series HPLC system (Wellesley, U.S.A.) coupled to an Applied Biosystems QTRAP LC/MS/MS (Foster City, USA). The reverse phase ODS2 C18 column with a particle size of 5 mm was used (Teknokroma, Barcelona, Spain). The flow rate was 0.3 ml min^{-1}. Commercial controls were used to identify the various flavonoids. The separation and detection procedures are described by Dardanelli et al. (2009).

Fatty acids (AG): lipids were extracted as described by Bligh and Dyer (1959) and dried under nitrogen. Generation of methyl esters of fatty acids (FAMEs) was performed with boron trifluoride in methanol

(F_3BMeOH), as indicated by Morrison and Smith (1964). For the identification of AG, the obtained FAMEs were dried under nitrogen stream and resuspended in hexane for analysis by Gas Chromatography (GC, Hewlett Packard 5890 Series II) equipped with a highly polar (HP 88) column. Also, the molecules found were confirmed by GC-MS analysis. The following GC-MS conditions were used: injector temperature, 240 °C; column temperature, 180 °C, maintained for 30 min; increase of 5 °C.min^{-1} to 240 °C, maintained for 10 min. Run time: 46 min. MS: full SCAN, 40–500. Injection volume: 1 µl. Split: 1:10.

Organic acids: were identified using the method described by Cawthray (2003) with modifications, using ultra-liquid chromatography coupled to a mass detector (UPLC-MS), WATERS model ACQUITY H CLASS (Center of scientific instrumentation of the University of Granada, Spain). For the identification commercial witnesses were used. As mobile phase the methanol, buffer KH_2PO_4 and HPLC-grade acetonitrile (HiPerSolv) from Merck (Darmstadt, Germany) were used. In order to adjust the pH of the mobile phase H_2PO_3, H_2SO_4 and analytical grade NaOH were used. All aqueous solutions were prepared with Milli-Q water and vacuum stripped and filtered using 0.2 µm membrane filters.

Terpenes: lyophilized material was diluted in 1 ml of methanol: distilled water: formic acid (85: 14: 1, v/v/v) and 2 ml dichloromethanol and left overnight at 4 °C. The samples were eluted in micro-columns oasis and C18. A 100 µl aliquot was placed in inserts with 1 ng of 1-nhexadecane as an internal standard, and 2 µl were injected into GC-EIMS. The oven temperature program was: initial temperature at 45 °C.min^{-1}, followed by an increase from 2 °C min to 130 °C, then from 130 °C to 250 °C at a rate of 20 °C.min^{-1} and held for 10 min at 250 °C. Terpenes were identified by comparison with commercial controls. Separation and detection procedures are described by Salomón et al. (2013) and Gil et al. (2012).

2.4. Bacteria motility assays

Cells of SEMIA6144 and Az39 were grown under NRWC, RWC (15 mM of PEG). 5 µl of a bacterial suspension (OD: 1) of each strain was inoculated in the middle of a petri plate, with 20 ml of 0.5% water-agar for swarming assays. For swimming assays, petri plates with 20 ml of 0.3% water-agar were inoculated with the strains by puncturing in the center. Motility diameter was measured 7 days after this inoculation.

The effect of RE on motility diameter was evaluated by adding 8 µl of 10x concentrated exudates to culture medium (Vicario et al., 2015).

2.5. Chemotaxis

A capillary assay was performed with slight modification to Yuan et al. (2015) using a multichannel pipette. Peanut root exudates collected at 7 days were previously examined by TSA medium, to verify that they were free of microbial contamination. Then the selected exudates were filtered with sterilizing filters (Sartorius Minisart® Filter Sterilization 0.1–0.2 µm). One hundred µL of RE from NRWC (RE-NRWC) and RE from RWC (RE-RWC) were pipetted and sterile water, Hoagland and Hoagland with 15 mM of PEG served as the control. The set-up was placed by just touching the tips into a 96-well plate containing 200 µL of SEMIA6144 and Az39 exponential phase culture to an OD of 0.4 in physiological solution (10^8 CFU ml^{-1}). After 30 min of incubation, under sterile conditions in a laminar flow, the number of bacteria attracted to test solutions was counting by the microdroplet technique (Somasegaran and Hoben, 1994).

2.6. Bacterial adhesion assay

Stationary cultures of SEMIA6144 and Az39 were centrifuged at 10000*g* for 5 min. The pellets were washed three times with 25 mM PO_4Na buffer (pH 7.5) and suspended in the same solution to an OD 0.4 to give a bacterial concentration of 1.10^8 CFU ml^{-1} for both strains. To study the effects of root exudates on adherence, bacterial cultures were centrifuged and diluted in RE (from plants grown under NRWC and RWC) to an OD of 0.4. Five lateral roots of 2 cm (0.1 gr) of 7-day old peanut plants growing under NRWC and RWC treatment were immersed in 1 ml of bacterial suspension for 2 h, with agitation at room temperature. Then, roots were washed 10 times in phosphate buffer to remove free and weakly attached bacteria. For the quantification of the number of bacteria adhered, the roots were crushed with 500 µl of PO_4Na buffer. The counting was carried out by the microdroplet technique (Somasegaran and Hoben, 1994). The assay was performed in triplicate for each condition and the results were expressed as CFU.mg^{-1} RDW).

2.7. Plant growth parameters and lipid peroxidation

Shoot length (SL) and root length (RL) were determinate and shoot and root dry weights (SDW and RDW) were measured after 24 h of drying at 80 °C until constant weight.

Lipid peroxidation was measured by the level of malondialdehyde (MDA), a product of lipid peroxidation, using a reaction with thiobarbituric acid (TBA) as described by Hodges et al. (1999). Fresh samples (100 mg) were ground in a mixture of 1 ml trichloroacetic acid (TCA) (20% w/v) and 0.2 ml of 4% (w/v) butylatedhidroxytoluene in ethanol, at 4 °C. After centrifugation (10,000 × *g* for 15 min), 0.25 ml aliquots of the supernatant were mixed with 0.75 ml of 0.5% (w/v) thiobarbituric acid in 20% TCA and the mixture was incubated at 94 °C for 30 min. The reaction was stopped by cooling in an ice bath for 15 min. Reaction tubes were centrifuged at 10,000 × *g* for 15 min and supernatants were used to determine the absorbance at 532 nm. The value for non-specific absorption at 600 nm was subtracted.

2.8. Plant experiment design and statistical analysis

Plant experiment had a factorial structure with a totally randomized design with two (2) factors: 1. Availability of water with two (2) levels: NRWC and RWC. 2. Inoculation with three (3) levels: Plants not inoculated; plants inoculated with SEMIA6144, plants doubly inoculated with SEMIA6144 and with Az39; with three (3) repetitions for each combination of treatment levels, totaling 30 plants. The analysis of variance (ANOVA) and the means compared to Fisher's minimal difference test (LSD) were performed on the data of the treatments and their interactions ($p < 0.05$). The software program used was Infostat 1.0 (Di Rienzo et al., 2016).

The bacteria data were subjected to analysis of variance (ANOVA) with multiple comparison variables by Fisher's least significant difference (LSD) test. Differences between means were considered to be significant at $p \leq 0.05$. The software program used was Infostat 1.0 (Di Rienzo et al., 2016).

3. Results

3.1. *A. hypogaea* RE profile change in response to RWC and rizobacteria presence

Flavonoids, IAA and Trp identified in peanut RE are shown in Table 1. The interaction between inoculation treatments and water conditions was $p < 0.05$. Under NRWC, Rutin, Naringin and Naringenin were the main flavonoids presented in REs of non-inoculated plants. Flavone family was found in lower concentration compared to the rest of the families. In the RE of plants inoculated with SEMIA6144, Luteolin and Naringenin increased by 66% and 23% respectively in relation to the non-inoculated plants, while Chrysin and Genistein were not detected. In contrast to these results, in RE of double inoculated plants, Chrysin and Genistein levels increased 5.8 and 2.4 times compared to the values found in the non-inoculated plants, while

Table 1
Chemical composition of root exudates of seven days old plants: flavonoids (μg.L^{-1}), indole-3-acetic acid and tryptophan (μg.L^{-1}).

Treatment	Flavone			Flavonone		Flavonol		IAA	Trp
	Apigenin	Chrysin	Genistein	Luteolin	Naringenin	Naringin	Rutin		
NRWC									
UI	0.4 a	0.6 a	0.4 a	0.9 a	1.3 a	2 a	2.4 c	2.6 b	172 a
SEMIA6144	0.4 a	ND	ND	4.3 b	1.2 a	4 b	1.1 b	1.8 a	195 b
SEMIA6144 + Az39	ND	3.5 b	0.9 b	1.5 a	1.6 b	1.8 a	0.6 a	34.2c	390 c
RWC									
UI	0.3	ND	0.2	2.3 b	3.7 b	1.7 a	1.4 a	1.8 a	156a
SEMIA6144	ND	ND	ND	3.5 c	0.7 a	2.1 b	2 b	1.6 a	422b
SEMIA6144 + Az39	ND	ND	ND	1 a	0.85 a	2.05 b	ND	19 b	755c

Data represent mean values of three replicates. All variables had significant interaction (P < 0.05) between the factors. The analysis of variance (ANOVA) and the means compared to Fisher's minimal difference test (LSD) were performed on the single effect of treatments of their interactions (p < 0.05). Different letters indicate a significant difference between the treatments in each column for each growth condition (NRWC and RWC), (*p* < 0.05).ND: not detected. UI: Uninoculated plants.

glycosylated flavonoids such as Naringin and Rutin decreased (7.5% and 75.8% respectively) (Table 1).

The RE profile changed in response to RWC. Under this condition an inhibition of the flavone was observed. Respect to flavonove family, Naringenin (flavonoid precursor) was 2.8 times higher than the value found under NRWC. For Luteolin, the increase was 150% respect to the NRWC. After single or double inoculation, Flavone was not detected in the RE. Interestingly, in plants inoculated with SEMIA6144 an increase of Luteolin (52%) and of glycosylated flavonoids such as Naringin and Rutin (15% and 30% respectively) were observed respect to non-inoculated plants under RWC.

IAA and Trp were also found in RE under NRWC (Table 1). Interestingly, we detected that in the RE of double-inoculated plants, AIA and Trp exudation increased 13 times and 2.3 times respectively in relation to the RE of non-inoculated plants. Under RWC a reduction by 30% in the levels of AIA in the RE was observed. Single inoculated plants released Trp by 2.7 times more than the non-inoculated plants. Double inoculated plants released 4.8 times more Trp and 10.5 times more AIA than no inoculated plants under RWC.

Under NRWC the main fatty acid (FAs) detected in RE were saturated long chain FA such as 16:0 and 18:0 and lesser amounts of unsaturated FA and FA short chain (Table 2).

In the RE of plants inoculated with SEMIA6144, 12:0, 14:0 and 16:1 were not detected. Interestingly, an increase of 476% in the amount of FA 18:1 was observed, compared to the RE of non-inoculated plants. Similar, in the RE of double inoculated plants, the increase of 18:1 was 355% compared to non-inoculated.

Under RWC, 18:1Δ9 FA was 2 times the concentration value observed in RE under NRWC (Table 2) and this value increases in the presence of microorganisms, being 4 times higher compared to non-inoculated plants.

Under NRWC malic, citric, succinic, lactic and acetic acids were detected in the peanut RE. The presence of SEMIA6144 increased the exudation of lactic and acetic acid being 3.8 and 1.2 times higher than the values detected in the RE of non-inoculated plants. Double inoculation increased the exudation of lactic (716%), malic (550%) and citric acid (220%) (Table 2).

Under RWC the amount of the organic acids detected in the RE were increased except for malic and acetic acid (Table 2). The presence of microorganisms caused a reduction in the levels of organic acids exuded by the plant, except for lactic acid which increased 209% in the RE of simple inoculated plants, and acetic acid wich increased 620% in RE of doubly inoculated plants (Table 2).

Under NRWC, peanuts RE four types of non-volatile terpenes, ocimene, carene, menthatriene and farnesene. A RWC increased the exudation of terpenes, the value of carene and menthatriene found was twice higher than that found under NRWC. Also the RWC induced the exudation of terpinolene, hymachelene, nerodiol and farnesol, which had not been detected in the NRWC (data not shown).

Table 2
Chemical composition of root exudates from seven days old plants grown under NRWC and RWC, uninoculated or inoculated with *B.* sp SEMIA6144 y *A. brasilense* Az39. Fatty acid (%), Organic acid (μg.L^{-1}).

Chemical Family		NRWC			RWC		
		UI	SEMIA6144	SEMIA6144 + Az39	UI	SEMIA6144	SEMIA6144 + Az39
Fatty Acid	Lauric acid (12:0)	2.1	ND	ND	ND	ND	ND
	Myristic acid (14:0)	7.8 a	ND	9.1 b	7.9 a	ND	10.2 c
	Palmitic acid (16:0)	40 b	37a	35.3 ba	37.4 c	35 b	21 a
	Palmitoleic acid (16:1Δ9)	2.2 a	ND	7.7 b	ND	ND	8.5 b
	Stearic acid (18:0)	42 c	32.5 b	29.5 a	36.2 c	24.5 b	13.5 a
	Oleic acid (18:1Δ9)	5.1 a	24.3 c	18.1 b	9.8 a	40 b	41 b
Organic Acid	Malic acid	0.21 a	0.38 b	1.17 c	0.19 b	0.11 a	0.08 a
	Citric acid	0.05 a	0.09 b	0.11 b	0.29 b	0.21 a	0.19 a
	Succinic acid	0.20 a	0.21a	0.20 a	0.39 c	0.30 b	0.04 a
	Lactic acid	0.06 a	0.23b	0.43 c	0.31b	0.65 c	0.18 a
	Acetic acid	0.18 a	0.22 b	0.25 b	0.05 a	0.05 a	0.31b

Data represent mean values of three replicates. All variables had significant interaction (P < 0.05) between the factors. The analysis of variance (ANOVA) and the means compared to Fisher's minimal difference test (LSD) were performed on the single effect of treatments of their interactions (p < 0.05). Different letters indicate a significant difference between the treatments in each column for each growth condition (NRWC and RWC), (*p* < 0.05).ND: not detected. UI: Uninoculated plants.

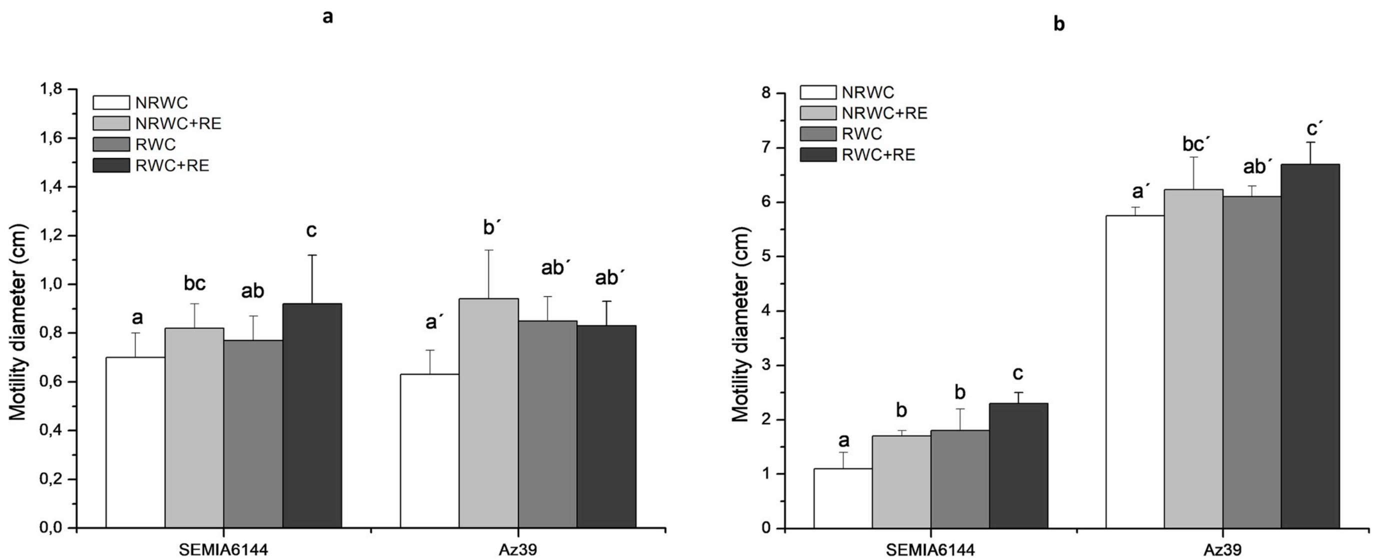

Fig. 1. Swarming (a) and Swimming (b) motility diameters of rhizobacteria grown under NRWC and RWC with or without RE in 0.3% (a) or 0.5% (b) water-agar medium. The values shown are mean ± SD of three independent pairs of triplicate experiments. Differing letters above the bars indicate statistically significant (ANOVA, Fisher's LSD test, $P < 0.05$) differences between means.

3.2. Bacteria motility depends on the growth conditions and RE presence

The effect of previous bacterial growth under RWC and the effect of the addition of RE to the medium on the swarming (Fig. 1a) and swimming motility (Fig. 1b) was studied.

Fig. 1 a shows the swarming motility (swa) diameter of SEMIA6144 and Az39. Both microorganisms grown under NRWC had a diameter of 0.7 cm. Regarding SEMIA6144, the presence of RE increased the swa by 17% as well as under RWC increased 8.6% respect to NRWC. The combined effect of the RWC + RE resulted in an increase of 31.5% with respect to the control (NRWC).

About Az39, the presence of RE in the motility agar increased the swa motility by 57% compared to the NRWC. The previous growth of Az39 under RWC as well as the simultaneous effect of RWC + RE did not affect the swa significantly (Fig. 1a).

Fig. 1 b shows the swimming (swi) motility diameter of SEMIA6144 and Az39. Regarding SEMIA6144, the presence of RE in the motility plate as well as the previous growth of the bacterium under RWC increased by 54% and 63% respectively with respect to NRWC. The combined effect of the RWC + RE resulted in a 109% increase over the NREWC (Fig. 1b). Az39 has swi motility 5.6 times greater than that of SEMIA6144. The presence of RE in the motility agar caused an increase in swi by 8% compared to NRWC. The greatest effect on the motility diameter was observed when the bacteria previously grown under RWC and RE were added on the motility agar. In this case, the increase was 16.5% with respect to the NRWC (Fig. 1b).

On the other hand, we characterize the size of mobile cells. The growth under RWC did not modify the length of SEMIA6144 vegetative cell, while Az39 cell length increased from 1.6 to 2.15 μm (data not shown). Both microorganisms showed a significant increase in cell length of Swa cells with respect to the vegetative cells, from 1.5 μm to 1.8 μm for SEMIA6144, and from 1.6 μm to 2.3 μm for Az39. The differentiation of vegetative cell to swi cell was also highlighted by an increase from 1.5 μm to 2.5 μm for SEMIA6144 and from 1.6 μm to 2.4 μm for Az39.

3.3. Bacteria chemotaxis is favored by RE from peanut grown under RWC

Fig. 2 show the chemotactic response of SEMIA6144 and Az39 towards the peanut RE of plants growing under NRWC and RWC. SEMIA6144 presented chemotactic response to the Hoagland solution,

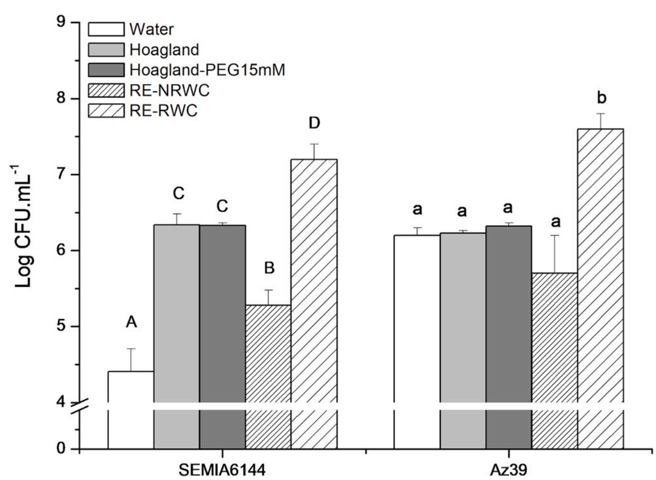

Fig. 2. Effect of root exudates from peanut plants grown under NRWC and RWC, on. chemotaxis fo *B.* sp SEMIA6144 and *A. brasilense* Az39. The values shown are mean ± SD of three independent pairs of triplicate experiments. Differing letters above the bars indicate statistically significant (ANOVA, Fisher's LSD test, $P < 0.05$) differences between means.

30% higher than water. Our results confirm the absence of a chemotactic effect of the PEG molecule, since the chemotactic response was the same for both Hoagland and Hoagland with the addition of 15 mM PEG. When chemotaxis was evaluated against RE, it was observed that SEMIA6144 showed a high chemotaxis towards RE from plants of RWC, being 27% higher than chemotaxis against RE of plants grown in NRWC.

Regarding Az39, the number of cells observed in experimental chemotaxis was 1. 10^6 CFU ml^{-1} for water and the solutions of Hoagland tested. Similar to SEMIA6144, when chemotaxis was evaluated against peanut RE, Az39 showed higher chemotaxis to RE from plants grown under RWC (25% higher than RE-NRWC). Compared with SEMIA6144, the chemotactic response of Az39 to RE was greater, being 7.3% higher for RE-NRWC and 5.3% higher for RE-RWC.

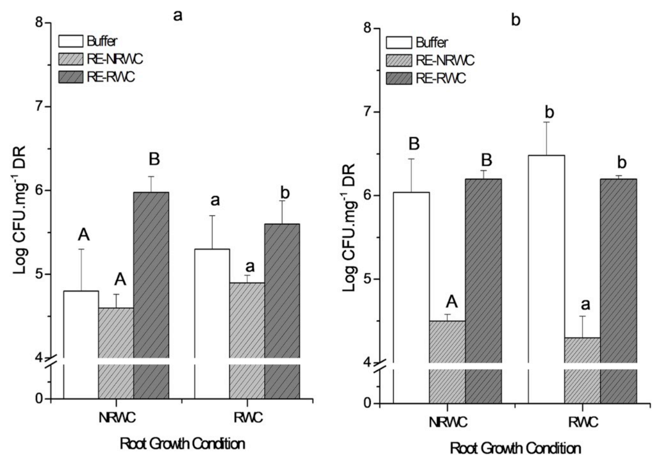

Fig. 3. Adhesion of *B.* sp SEMIA6144 (a) and *A. brasilense* Az39 (b) to lateral roots of 7-days old peanut plants grown under NRWC and RWC.

3.4. The adhesion of rhizobacteria to the roots of A. hypogaea is promoted by RE-RWC

Fig. 3 shows the CFU.mg^{-1} RD (dry root) of SEMIA6144 (a) and Az39 (b) adhered to the 7 days-lateral roots of peanut from NRWC and RWC.

Statistical analysis of the data indicated interaction between the NRWC and RWC factors, which would indicate that the adhesion of the rhizobacteria to the peanut roots depends on the previous condition of plant growth. Fig. 3 a shows the adhesion of SEMIA6144 to peanut roots. Regarding the plants grown under NRWC 6.8.10^4 CFU mg^{-1} RD was the number of cells adhered when the adhesion test was performed in buffer (pH 7). In order to know if the molecules present in the peanut root exudate grown under NRWC and RWC can modify the adhesion, the adhesion test was performed replacing the buffer by RE (pH 5). In the presence of RE-NRWC, the number of cells adhered to root was similar to that obtained in the presence of buffer. Interestingly, in the presence of RE-RWC, adhesion was 23% higher than in RE-NRWC. Regarding the plants cultivated under RWC 2.4. 10^5 CFU mg^{-1} RD was the number of cells adhered when the adhesion test was performed in buffer. In the presence of RE-NRWC, the number of cells adhered to the root was similar to that obtained in the presence of buffer. Interestingly, under the presence of RE-RWC, adhesion was 12.5% higher than in RE-NRWC.

Fig. 3 b. shows the adhesion values of Az39 to peanut roots. Regarding the plants grown under NRWC, when the adhesion test was performed in buffer, the adhesion was 3.10 ^{6} CFU mg^{-1} RD. Surprisingly, when the adhesion was evaluated in the presence of RE-NRWC, the number of cells adhered to the root was 25.5% less than the adhesion in buffer. Similar to that observed with SEMIA6144, in the presence of RE-RWC the adhesion was 38% higher than in RE-NRWC.

Regarding the plants cultivated under RWC 2.2.10^7 CFU mg^{-1} RD was the number of cells adhered when the adhesion test was performed in buffer. In the presence of RE-NRWC the number of cells adhered to the root was 33% less than the adhesion in buffer. Interestingly, under the presence of RE-RWC adhesion was 44% higher than in RE-NRWC.

3.5. SEMIA6144 and Az39 improves peanut growth under RWC

After the statistical analysis we determined that all growth variables studied had significant interaction ($p < 0.05$) between the factors. In an early event defined to 7 days, the growth of un-inoculated *A. hypogaea* plants were negatively affected by RWC (−0.28 MPa), as demonstrated by a reduction of the relative growth rate (RGR) of 44% with respect to growth under NRWC (−0.07 MPa) (Table 3). Shoot length decreased by 32%, while the RL decreased by 12% under RWC with respect to NRWC. Both root and shoot dry biomass was reduced by 43% compared to the control (Table 3).

Under NRWC, the greatest effect of inoculation was observed at the level of root growth, which increased 8% with single inoculation (SEMIA6144) while double inoculation (SEMIA6144 + Az39) increased 46% compared to non-inoculated plants.

Under RWC, both single and double inoculation mitigates the negative effects during the first 7 days of *A. hypogaea* growth. The RGR of the plants inoculated with SEMIA6144 increased by 25%, while with double inoculation the increase was 50% respect to non-inoculated plants. SEMIA6144 inoculation favored to a greater extent the aerial growth, being the SDW 41% higher than that in the non-inoculated plants. The double inoculation favored mainly root growth, being RDW 93% higher than in non-inoculated plants.

The lipid peroxidation, estimated as the MDA content, increased in leaves and roots of peanut plants exposed to a RWC, reaching 3.2 for leaves and 2.5 for root respect to NRWC (Table 3). Double inoculation increased MDA levels in NRWC, while under RWC the presence of rizobacteria reduced the MDA level in leaves by 14% for simple inoculation and 7% for double inoculation.

4. Discussion

The aim of this study was to evaluate whether a restrictive water condition impact on the root exudation pattern of *Arachis hypogaea* cv. Granoleic and on the very early step required in plant-microbe interaction with *Bradyrhizobium* SEMIA6144 and *Azospirillum brasilence* Az39.

After the chemical analysis of the RE, we detected the presence of three flavonoids families, flavonol (Rutin), flavonone (Luteolin, Naringenin and Naringin) and in lower concentration the flavone (Apigenin, Chrysin, Genistein). Unlike our results, Taurian et al. (2008) showed a high concentration of the flavonoids Daidzein, Genistein and Chrysin in the RE of *A. hypogaea* L. cv. Tegua. These data taken together show that the variety of metabolites presents in the RE is dependent on the cultivar. Our work is the first demostrating that a restrictive water condition on plant growth modified the profile of flavonoids present in the RE of peanut. RWC caused a reduction in the exudation of the flavones, and the presence of bacterial inoculums did not reverse this effect. Under this growth condition, SEMIA6144 inoculation induced Luteolin exudation while the amount of Naringenin decreased. Luteolin is the main nodulation gene induced by rhizobia and acts as a chemoattractant (Peters et al., 1986). Some authors have reported that Naringenin has antagonistic activity toward the expression of nodulation genes and abolishes chemotaxis of Luteolin (Peters et al., 1986; Caetano-Anollés, 1997). Thus, our results suggest that the reduction of Naringenin level might reinforce the positive effect of Luteolin during the rizobio-plant interaction as demonstrated by Morel et al. (2015). Although the *Bradyrhizobium-Arachis hypogaea* symbiosis occurs through "crack entry", Boogerd and Rossum (1997) showed that nod factors induced by the plant flavonoids play an important role in the establishment of the symbiosis. Interestingly, in our work we observe that the double inoculation had an opposite effect on the exudation of some flavonoids with respect to single inoculation, the concentration of Luteolin decreased while Naringenin increased. This change in the root exudates composition could modify the early capacity of SEMIA6144 to interact with peanut roots in co-presence of Az39 and thus explain why double inoculation does not effectively reverse the negative effects of water deficit on 30-day-old plants (Cesari et al., 2019).

In our work, the main FA detected in the *A. hypogaea* RE were palmitic (16: 0), stearic (18: 0) and oleic FA (18: 1Δ9) among others. Similar to our results, others authors report that peanut roots released a higher proportion of 16:0, 18:0 and 18:l and a lower proportion of 18:2 and 18:3 FA (Thompson and Hale, 1983). In our study, the simple and double inoculation of *A. hypogaea* modified the exudation profile of FA, finding an important increase in the concentration of the oleic FA. This

Table 3
Effect of RWC and inoculation with *B.* sp SEMIA6144 *A. brasilense* Az39 on growth parameters of seven days old *A. hypogaea* plants.

Treatments		RGR (g.g.day^{-1})	SL (cm)	SDW (mg)	RL (cm)	RDW (mg)	SMDA (nmol.g^{-1})	RMDA (nmol.g^{-1})
NRWC	UI	3.6	11 ± 2 ab	137 ± 20 a	11.5 ± 2 b	74 ± 16 a	13 a	2 a
	SEMIA6144	3.4	10 ± 1 a	135 ± 21 a	11.7 ± 2 b	80 ± 19 b	21 b	3 a
	SEMIA6144 + Az39	4.2	11 ± 1.5 b	147 ± 18 b	8.9 ± 1 a	108 ± 2 c	24 c	4 b
RWC	UI	2	7.5 ± 2 a	78 ± 21 a	10.1 ± 2. a	43 ± 15 a	42 b	5 a
	SEMIA6144	2.5	8.8 ± 1 b	110 ± 15 c	11.5 ± 1.a	67 ± 15 b	36 a	5 a
	SEMIA6144 + Az39	3	8.7 ± 2 a	103 ± 11 b	11.1 ± 2 a	83 ± 16 b	39 c	17 b

Data represent mean values of three replicates. All variables had significant interaction ($P < 0.05$) between the factors. The analysis of variance (ANOVA) and the means compared to Fisher's minimal difference test (LSD) were performed on the single effect of treatments of their interactions ($p < 0.05$). Significant differences ($p < 0.05$) between values within a column, for independents treatments NRWC and RWC are indicted by different letters. NRWC: non-restrictive water condition; RWC: restrictive water condition. UI: Uninoculated plants. RGR: root growth relative (g.g.day^{-1}), SL: shoot length (cm), SDW: shoot dry weight (mg.plant^{-1}), RL: root length (cm), RDW: root dry weight (mg.plant^{-1}). shoot and root MDA (nmol.g^{-1}).

FA is involved in the regulation of the membrane fluidity, and the elevation of oleic FA may simply be a reflection of an up-regulation of metabolic pathways necessary for the synthesis of new membranes required during the infection process, important for colonization of roots by microorganisms, and the invasion of cortical cells during the "crack entry" process (Brechenmacher et al., 2010; Muñoz et al., 2014). Here, when peanut grew under RWC, we observed a significant increase in oleic FA in the RE and that increase was even greater when the plant was inoculated. Similar to our results, Svenningsson et al. (1990), reported that *Brassica napus* exposed to water deficit (−0.4 MPa) induced by the addition of PEG, increase the release of 18:1 FA. Increases of free FA might have been caused by increased synthesis, liberation from triglycerides or phospholipids, or both (Thompson and Hale, 1983).

Organic acids found in peanut RE grown under NRWC were malic, succinic, acetic, citric and lactic acid, while under RWC the exudation of lactic, citric and succinic acid had a significant increase. Citric, malic and acetic acids have been reported as important constituents of the exudates of other legume as *Lupinus albus* and are considered to be related to phosphorus absorption (Neumann and Romheld, 2007; Kamh et al., 1999). Similar to our results, Song et al. (2012) showed an increase in the exudation of malonic, lactic, acetic and succinic organic acids by corn roots grown under water deficit induced by PEG. Also, an increase in lactic acid exudation has been reported in *Quercus ilex* and in *Zea Mays* ground under water deficit (Song et al., 2012; Gargallo-Garriga et al., 2018). Xia and Roberts (1994) reported that plants escape the toxic effects of accumulated ethanol and lactic acid that can accumulate under abiotic stress conditions, by secreting these metabolites from their roots. Interestingly, the values of the lactic acid found in peanut RE were high when the plants were inoculated with SEMIA6144, both under NRWC and RWC, in comparison with the non-inoculated plants. Brechenmacher et al. (2010) showed that lactic acid accumulated specifically in root hairs, after inoculation with *B. japonicum*. In double-inoculated plants, an increment in the acetic acid exudation was observed, mainly under RWC. Acetic acid has been reported as an efficient mobiliser of phosphorus and iron in soils for pigeonpea, rice, soybean and sorghum, among others (Strom et al., 1994). High levels of acetic acid have been found in root exudates of wheat and other monocotyledonous species grown in hydroponic cultures but there are no reports in peanut RE (Rovira, 1969; Kloss et al., 1984; Kraffczyk et al., 1984).

Although antimicrobial compounds such as terpenoids have been reported in *Arabidopsis*, soybean, corn and alfalfa RE (Bais et al., 2004, 2006; Gargallo-Garriga et al., 2018), our work is the first to describe the presence of non-volatile terpenes in peanut RE. RWC caused an increase in the exudation of terpenes, mainly monoterpenes with known antioxidant activity and sesquiterpene oxygenated species, such as farnesol and nerodiol, both with known antimicrobial properties.

Plant roots initiate interaction with soil microbes by producing signals that are recognized by microbes inducing motility, chemotaxis and root colonization (Bais et al., 2006). Both rhizobacteria used in this study are mobile, SEMIA6144 and Az39 showed swarming and swimming motility. Here we show that the previous growth of bacteria with PEG simulating RWC favored both types of motility. This could be related to changes in the composition of the cytoplasmic membrane of bacteria when they grow under a water deficit, demonstrated by the increase of 51% and 21% of phosphatidylcholine in the membrane of Az39 and SEMIA6144 respectively (Cesari et al., 2016, 2018). The relationship between bacterial motility and phosphatidylcholine levels have been previously demonstrated by our working group, showing that a SEMIA6144 mutant in phosphatidylcholine biosynthesis presented reduced motility (Medeot et al., 2010). In our work, the addition of peanuts RE in the culture medium, favored the motility of both microorganisms. In addition, some authors have shown that some chemoattractants such as malic acid and aromatic compounds increase swi speed of various *Azospirillum* strains (Zhulin and Armitage, 1993; López de Vittoria and Lovell, 1993; Borisov et al., 2007).We also demonstrate that SEMIA6144 and Az39 presented a positive chemotactic response towards peanut RE. Interestingly for both bacteria the chemotaxis was greater when RE-RWC were used, suggesting that this could be related with the molecules exuded by the root under RWC, as Luteolin, Naringenin, citric, succinic and lactic acid, and oleic FA. Also, we observed that the chemotaxis towards RE is greater for Az39 than for SEMIA6144. Barak et al. (1983) demoted that *A. brasilense* show positive chemotaxis towards the organic acids malate, citrate and succinate, compounds that we detected in high concentration in the peanut RE-RWC. Two types of chemotactic response have been reported for Rhizobia: a general non-inducible chemotactic response and a specific inducible response to plant phenolic compounds (Dowling and Broughton, 1986). In our work, we observed an increase in the chemotaxis of SEMIA6144 towards RE-RWC, which could be related to high concentrations of Luteolin present in this exudate.

In this work, we show that the PGPR adhesion to the roots depended on the plant growth condition and varies in response to the root exudate composition. The previous plant growth condition was determinant in the adhesion to the roots, finding a greater adhesion to the roots that previously grew under RWC. Under this condition the roots have more radical hairs (Cesari et al., 2019) and, therefore, greater adhesion surfaces. Similar to our results, Albareda et al. (2006) reported a greater number of *A. brasilense* Sp7 and *Rhizobium etli* adhered to roots of *P. vulgaris* under salt stress. The adhesion of SEMIA6144 and Az39 to roots grown under NRWC and RWC increased when RE-RWC was present with respect to RE-NRWC.

RWC show a negative effect on growth parameters of the 7-day peanut plants, mainly with a significant reduction in the RGR and a decrease in the shoot biomass. Simple and double inoculation reversed the effect of RWC on the RGR of peanut. Inoculation with SEMIA6144 favored the shoot biomass, while inoculation with SEMIA6144-Az39 favored mainly the root biomass.

Oxidative stress is a collateral effect of water deficit which led us to determined malonic dialdehyde (MDA) used as a marker for lipid peroxidation. Similar to that reported by Celikol et al. (2010), we observed a 2.5-fold increase in MDA levels in leaves of peanut plants exposed to a RWC with respect to NRWC. Interestingly, we observed that simple and double inoculation increased MDA levels in NRWC, while under RWC the presence of rhizobacteria reduced the MDA level in the leaves.

Taking into account the results obtained in this study, we demonstrate for the first time that a RWC affects the profile of molecules exuded by *A. hypogaea* during its first days of growth, increasing the exudation of precursor flavonoids (Naringenin), oleic FA and organic acids principally citric and lactic acid, and stimulation in the exudation of terpenes of known antioxidant and antimicrobial activity. The first events during the interaction between peanut-SEMIA6144-Az39 were also affected by the RWC. In addition our results indicate that the molecules exuded by the roots of peanut growing under RWC exert a chemoattractant effect and favor the adhesion of the bacterial to the roots. These results not only deepened our understanding of the PGPR-root interaction, but also provided useful information to improve the mobility, chemotaxis and the future colonization of the roots by the PGPR.

Author contributions

AC, NP and MD conceived and designed the experiments. MLG, JHC and CL provided equipment to perform the molecular determinations in the RE. AC, MLG, JHC performed the measurements in the RE and the MDA content. AC and NP performed chemotaxis and adhesion experiments. AC performed the statistical analysis. AC, NP and MD wrote the manuscript, with contributions from all the authors. All authors read and approve.

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