Symbiosis, 36 (2004) 257–268
Balaban, Philadelphia/Rehovot

Occurrence and Role of Vanillin in Root Exudates of Peanut (*Arachis hypogaea*)

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Received December 5, 2003; Accepted March 1, 2004

Abstract

The purpose of this work was to identify phenolic compounds released by young peanut roots growing hydroponically under low nitrogen supply and to investigate if these substances may be implicated in the establishment and functioning of an effective *Bradyrhizobium*-peanut symbiosis. Vanillin was found among the phenolics exuded by young peanut roots growing under these conditions. The effect of this compound on the growth rate of a *Bradyrhizobium* strain infective for peanut was further investigated. At concentrations ranging from 0.2 to 20 μg ml^{-1} vanillin had detrimental effects on the multiplication rate of *Bradyrhizobium* sp. (Arachis) strain C145. Bacterial cultures exposed to 20 μg ml^{-1} of this compound showed a two-fold increase in their lag phase and after 5 days of incubation optical densities were 48% decreased in respect to control cultures. In an inoculation pot experiment using this strain significant decreases in total dry weight, nodule biomass, acetylene reduction activity and nitrogen content were observed when 10 μg of vanillin were added to each germinating peanut seed prior to inoculation. However, no adverse effects were detected at a lower vanillin dose (2.5 μg ml^{-1}) or when peanut seeds were inoculated using rhizobia cells previously exposed to 1 μg ml^{-1} of vanillin.

Keywords: *Arachis hypogaea*, Leguminosae, peanut, root exudates, vanillin, *Bradyrhizobium*

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0334-5114/2004/$05.50

1. Introduction

Secondary metabolites play a significant role in the interactions occurring between plant roots and soil microflora. There are numerous studies showing phenolic compounds to be of major importance in plant response to plant pathogens (Matern and Kneusel, 1988; Dixon et al., 1994; Dixon and Paiva, 1995) or as positive effectors in the symbiotic interactions between leguminous plants and rhizobia (Fisher and Long, 1992; Phillips, 1993). During the early stages of the symbiotic process, plant defense-like reactions are induced by the invading rhizobia (Djordjevic et al., 1987; Schmidt et al., 1992). The coordination of these processes involves an intensive exchange of signal molecules between both symbiotic partners. The signal molecules produced by the bacterial partner, usually termed as *Nod* factors, are responsible for the root morphological changes (nodule meristem formation and root hair curling) leading to nodule development. Synthesis of *Nod* factors is triggered by the transcription of certain rhizobial genes known as *nod* genes (Schultze et al., 1994; Dénarié et al., 1996).

As symbiotic plant signals, phenolic compounds such as flavonoids and other phenylpropanoids are considered of special significance (Shirley, 1996). It was reported that they act as chemoattractants (Aguilar et al., 1988; Caetano-Anollés et al., 1988) and influence the expression of rhizobial *nod* genes in legume seeds and legume root exudates (Long, 1996; Zuanazzi et al., 1998). Although many of these compounds have already been extracted and purified from different legume species (Peters et al., 1986; Kosslak et al., 1987; Maxwell et al., 1989; Hungria et al., 1991), little is known about the signal molecules involved in the nodulation process of peanut plants. Flavonoid excretion by groundnut has not been demonstrated yet (Boogerd and van Rossum, 1997), although coumarin may be among the exuded compounds (van Rossum, 1994).

The aim of this work was to identify phenolics released by the roots of young peanut plants growing under limited nitrogen supply and to investigate if they may be implicated in the establishment and functioning of an effective *Bradyrhizobium*-peanut symbiosis.

2. Materials and Methods

Biological material

Seeds of *Arachis hypogaea* subsp. *fastigiata*, corresponding to the Valencia-type cultivar Colorado Irradiado INTA, were obtained from INTA-Manfredi, Argentina. *Bradyrhizobium* sp. (Arachis) strain C145, isolated from *Arachis hypogaea* nodules found under field conditions, was provided by IMYZA, INTA-Castelar, Argentina.

Chemicals and culture media

Analytical grade vanillin (3-methoxy-4-hydroxybenzaldehide) was purchased from Merck (No. 818718). The *Bradyrhizobium* strain used was routinely maintained on yeast extract mannitol (YEM) agar slants. Purity was periodically checked by plating on YEM agar supplemented with Congo red.

Collection of root exudates

Collection of root exudates was performed by duplicate as following. Fifteen peanut plants were pregerminated and then hydroponically grown for 3 weeks in 3-l containers using Hoagland solution (Hoagland and Arnon, 1950) with 10% of the standard nitrogen content. This solution was weekly replaced (the old solution was filtered and frozen). Root exudates were analyzed at 7, 14 and 21 days. At these times, the entire plants were carefully removed from the containers. After being air-dried for 1 hour, each root system was sprayed using 20 ml of methanol 80% and the liquid collected. The nutrient solution where the plants had been growing was filtered, lyophilized and resuspended in 20 ml of methanol 80%. All methanolic fractions thus obtained were combined and the solvent evaporated under reduced pressure. The remaining aqueous fraction was twice extracted with ethyl acetate. This solvent was evaporated under reduced pressure and the residue obtained was finally resuspended in 3 ml of methanol 100%.

Detection of vanillin and other phenolic substances

Aliquots of 100 µl of the extracts obtained as described above were subjected to two-dimensional TLC analysis on cellulose plates using butanol:acetic acid:water (4:1:2) as the first mobile phase and acetic acid (15%) as the second one. Under UV_{254} light, several spots were detected, scrapped off and scanned between 220 and 380 nm in a Gilford UV-VIS spectrophotometer.

Purification and identification procedures

Purification was achieved by means of HPLC. A LKB Bromma equipment supplied with a ODS column (Microsorb MV 100, 250 × 4.6 mm i.d.; Varian Ltd.) was used. A mixture of acetonitrile and water containing 0.01% of *o*-phosphoric acid was used as mobile phase. Chromatographic conditions applied were those described by Ehlers (1999), with a flow rate of 1 ml min^{-1} and UV detector set at 278 nm. Main peak collected was further identified by means of GC-MS of electronic impact, using a Shimadzu GP-5000 equipment.

Effect of vanillin on bacterial growth

The effect of vanillin on the growth rate of *Bradyrhizobium* sp. (Arachis) strain C145 was assessed by analyzing the growth curves patterns of vanillin-amended liquid cultures at three different concentrations: 0.2, 2 and 20 μg ml^{-1}. Primary cultures started from agar slants and incubated at 28°C in a rotary shaker (150 rpm) until the early logarithmic phase (OD=0.15) was reached provided inocula for these experiments. Flasks containing 50 ml of fresh YEM were inoculated with 0.5 ml of primary cultures and grown at the conditions cited above for 5 days. Optical density was regularly monitored. Three independent experiments with three replicates each were performed.

The effect of vanillin on *Bradyrhizobium* sp. (Arachis) strain C145 was also checked by placing in the center of Petri dishes containing YEM agar previously inoculated with a suspension of this bacterium, a sterilized paper disc soaked up with 50 μl of a vanillin solution. Three different concentrations were tested: 1, 10 and 100 μg ml^{-1}. Controls were achieved by placing paper discs previously soaked in distilled water.

Effect of vanillin on growth, nodulation and nitrogen fixation of peanut plants

Arachis hypogaea subsp. *fastigiata* seeds were surface sterilized, pregerminated at 20°C for 48 h and sown in 1-l plastic pots filled with vermiculite (6 per pot). To obtain treated plants 1 ml of a vanillin solution containing 2.5 or 10 μg ml^{-1} was added on each germinating seed just after transferring them to the pots. Two days later, the young seedlings were inoculated using 1 ml of a *Bradyrhizobium* sp. C145 cell suspension with an OD_{600} of 0.8, equivalent to 1.2×10^8 CFU ml^{-1}. Ten replicated pots were prepared for each treatment. Plants were kept in a growth chamber at 30–23°C under 16/8 h light/dark photoperiod, and watered daily with N-free Hoagland solution. After 7 weeks five of the pots were used to determine nitrogen fixation by measuring acetylene reduction activity of whole root systems; the remaining pots (containing 3 to 6 plants each) were destined to measure fresh weight of shoots and roots, total dry weight, nodule number and weight and total nitrogen content. Plant survival was also recorded.

Nitrogen fixation rate was measured as acetylene reduction activity (ARA), according to Hardy et al. (1968). Peanut whole root systems contained in each pot were enclosed in 1-l bottles sealed with rubber stoppers. After 10% of the atmosphere had been replaced by acetylene, the bottles were kept at 28°C. Gas samples (0.5 ml) were taken after 2 h of incubation and analyzed for ethylene production in a Shimadzu gas chromatograph supplied with a H_2-FID detector and a Porapak T column. Three replicated measurements were performed for

each bottle. Total nitrogen content of oven-dried (55°C) peanut plants was estimated using a semi-micro Kjeldahl procedure (Bremner, 1965).

In a second experiment, the same experimental design was followed, but instead of being added to each individual seed, vanillin was included in the culture medium where the *Bradyrhizobium* strain was grown, at a concentration of 1 μg ml^{-1}. Four days old cultures (OD_{600}=0.6) were centrifuged at 5,000 g for 20 min and the pellets obtained were washed 3 times with sterile buffer phosphate (0.05 M, pH 6.8) in order to remove vanillin completely. Pellets were finally resuspended in the same buffer giving an optical density of 0.8. Once emerged, seedlings were inoculated using 1 ml of these *Bradyrhizobium* sp. C145 cell suspensions. Control plants were obtained by inoculating peanut seeds with a vanillin-untreated cell suspension having the same optical density. After 7 weeks, the entire plants were harvested. Nodule biomass and total dry weight were determined.

Statistical analysis

Data shown in text and tables indicate mean values ± SE. Differences among treatments were analyzed by one-way ANOVA (P=0.05), followed by Tukey's multiple range test.

3. Results

Vanillin detection

Under UV_{254}-light, several spots were detected on cellulose TLC plates. Only three spots appeared repeatedly and with similar intensity at the different sampling times tested. These spots were scrapped off and spectrophotometrically scanned between 220 and 380 nm. Since only one of these spots (R_f=0.55) showed two well defined peaks at 279 and 309 nm, this spot was selected for further purification and identification. Under the chromatographic conditions used this compound showed a retention time of 6.5 minutes. This retention time was identical to those obtained using commercial vanillin as a standard. The peak was collected and further analyzed. To confirm the identity of this compound, GC-MS for the unknown compound and a vanillin standard were performed. The presence of the characteristic ions, $[M]^+$ (m/z: 152), $[M–Me]^+$ (m/z: 137), $[M–CHO]^+$ (m/z: 123), $[M–Me–CHO]^+$ (m/z: 109) and ring cleavage products (m/z: 81 and 53), were observed in both spectra. Data obtained from both HPLC and mass spectrometry analysis clearly demonstrated that the unknown compound was vanillin.

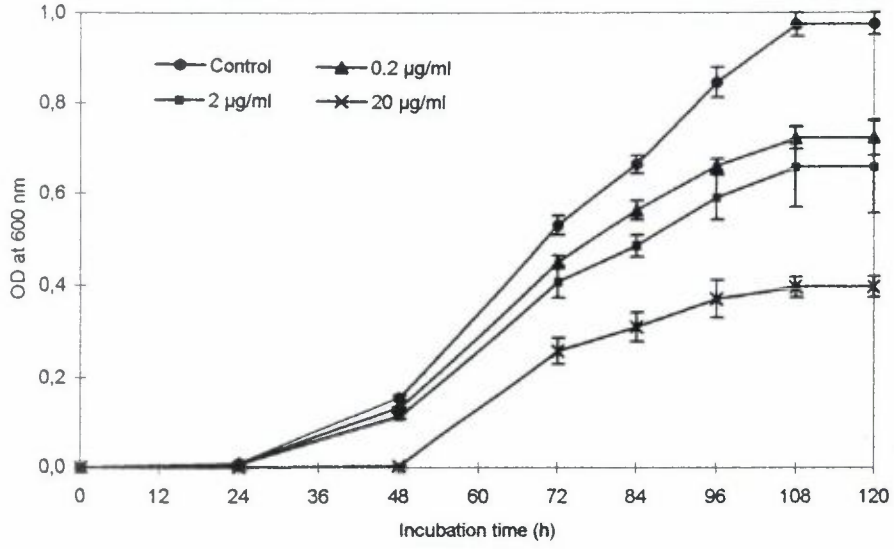

Figure 1. Effect of vanillin concentrations in culture media on the *in vitro* growth of *Bradyrhizobium sp.* (Arachis) strain C145. Data are the mean of three independent experiments with three replicates each, and bars indicate SE. *Significant differences (P<0.05) according to Tukey's multiple range test.

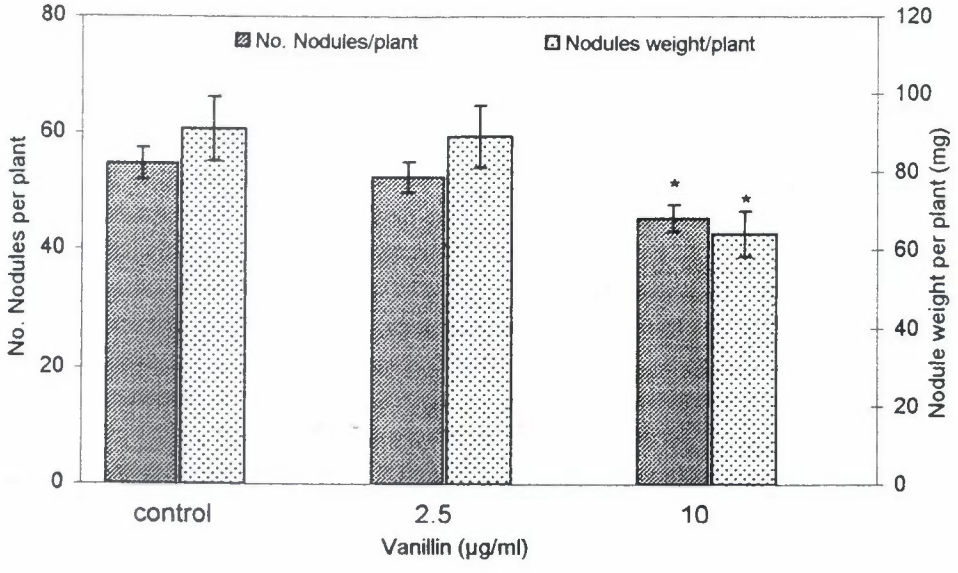

Figure 2. Effect of vanillin on nodule number and nodule biomass of inoculated peanut plants. Data are the mean of 18-26 plants, and bars indicate SE. *Significant differences (P<0.05) according to Tukey's multiple range test.

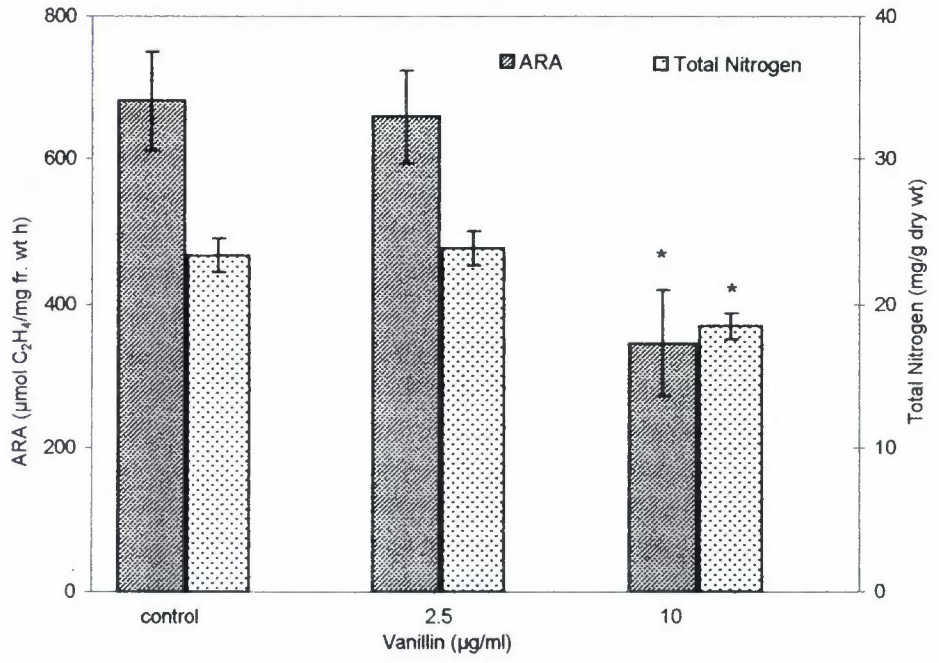

Figure 3. Effect of vanillin on acetylene reduction activity and total nitrogen content of inoculated peanut plants. Data of acetylene reduction activity are the mean of five replicated pots with three replicated measurements each, and bars indicate SE. Data of nitrogen content are the mean of 18-26 plants, and bars indicate SE. *Significant differences ($P<0.05$) according to Tukey's multiple range test.

Table 1. Effect of vanillin addition before inoculation on growth parameters of peanut plants.

	Vanillin concentration (µg ml^{-1})		
	Control	2.5	10
Number of plants/pot	5.2 ± 0.7 a	4.4 ± 0.5 ab	3.6 ± 0.5 b
Fresh weight/nodule (mg)	1.7 ± 0.3 a	1.7 ± 0.3 a	1.4 ± 0.4 a
Shoot fresh weight/plant (g)	6.4 ± 0.6 a	6.8 ± 0.5 a	5.2 ± 0.6 a
Root fresh weight/plant (mg)	671 ± 52 a	530 ± 59 b	453 ± 62 b
Total dry weight/plant (mg)	881 ± 75 a	686 ± 65 b	701 ± 60 b

Vanillin solution was added to each seed just after transferring them to the pots. Data are the mean ± SE of five pots, containing 3 to 6 plants each at harvest (18–26 plants per treatment). Different letters within rows indicate significant differences ($P<0.05$) according to Tukey's multiple range test.

Effect of vanillin on bacterial growth

Fig. 1 shows the effect of different concentrations of vanillin on the growth rate of *Bradyrhizobium* sp. (Arachis) strain C145. It is evident that this compound has adversely affected bacterial multiplication. After 5 days of incubation, optical densities of rhizobial cultures exposed to 0.2, 2 and 20 μg ml^{-1} of vanillin were 19, 23 and 48% decreased respect to control units. At the highest vanillin concentration tested, not only the growth rate was significantly affected but the lag phase too, showing this treatment no turbidity before 48 h of incubation. In concordance with these results, inhibition zones of 12 and 3 mm were measured around the paper discs moistened with vanillin solutions of 100 and 10 μg ml^{-1}, respectively, in the Petri dishes assay. No inhibition zones could be detected around the paper discs treated with 1 μg ml^{-1} of vanillin (data not shown).

Effect of vanillin on growth, nodulation and nitrogen fixation of peanut plants

Vanillin was added to the plant before inoculation with *Bradyrhizobium*. Its effects on nodulation and growth parameters of inoculated peanut plants are summarized in Fig. 2 and Table 1. Fig. 2 shows that both nodule number and biomass were significantly affected when 1 ml of vanillin solution at a concentration of 10 μ ml^{-1} was added to each peanut seed. It may be noticed that in this case the number of nodules per plant was 17% decreased in respect to controls, while nodule biomass was diminished in 30%. No adverse effects were found when the vanillin dose applied was 2.5 μg $plant^{-1}$. Nodule fresh weight and shoot fresh weight per plant remained unaltered at both vanillin doses tested (Table 1). However, significant decreases in root fresh weight (21–32%) and dry matter production (ca. 20%) were detected for vanillin treated plants, as compared to controls. When peanut plants were inoculated with rhizobial cells previously exposed to 1 μg ml^{-1} of vanillin, neither nodule biomass nor plant dry weight were affected (data not shown). Acetylene reduction activity was drastically diminished (50%) at the highest vanillin concentration assayed (10 μg $plant^{-1}$), being in this case total nitrogen content of the plants significantly affected with a 21% decrease, respect to control values (Fig. 3).

4. Discussion

As far as we know, the occurrence of vanillin had not been described previously in *A. hypogaea*. Devi and Reedy (2002) have demonstrated the presence of several phenolic compounds in peanut, but they did not report the occurrence of vanillin. In the present study, vanillin was found as the major

phenolic compound extracted with methanol from the root exudates of peanut plants grown on vermiculite. Le Strange et al. (1990) reported the first case of two simple phenolic compounds, vanillin and isovanillin, contributing to transcriptional activation of the nodD-dependent *nod* genes of strain NGR234 of *Rhizobium* sp., a broad range host rizobia able to nodulate dozens of legume genera including groundnut (Stanley and Cervantes, 1991). They identified these hydroxybenzaldehyde-derived molecules in extracts of wheat seedlings and demonstrated that they are active inducers of *Rhizobium nod* genes expression, although less potent than other compounds such as daidzein, genistein and apigenin. However, they also reported inhibition of bacterial cell growth at concentrations of 10^{-3} to 10^{-4} M, being the latest comparable to the highest vanillin concentration tested in this study (20 μg ml^{-1}). We therefore conclude that lower concentrations of vanillin than those reported by Le Strange et al. may be harmful to peanut-specific rhizobia populations. The dose tested when vanillin was added to *Bradyrhizobium* sp. (Arachis) strain C145 in the culture medium (1 μg ml^{-1}), before using these cultures to inoculate peanut plants, was in the range of those reported as nodD inducing concentrations (10^{-6}–10^{-7} M); however no changes in the nodulation parameters assessed could be observed. Sène et al. (2000) reported allelopathic effects of *Sorghum bicolor* on subsequent peanut crops, and demonstrated that several phenolic acids and their associated aldehydes found in the soils where this species had been cultivated displayed adverse effects on peanut: reduction of germination, emergence and seedling growth. Vanillin was one of the phenolics found by these authors, at concentrations ranging from 25 to 75 ng per gram of dry soil. Assuming that each peanut plant in our system was theoretically affected by 1/5 of the mass filling the pot (200 g); the vanillin dose applied to each seed in this pot experiment (2.5 or 10 μg) are in the range of those possibly found in cultivated soils.

Although at the lowest vanillin concentration tested only root fresh weight and total dry matter production were adversely affected, at a higher dose assayed vanillin had also detrimental effects on symbiotic parameters such as nodule number, nodule mass and acetylene reduction activity. Under these conditions, total dry weight and nitrogen content were significantly diminished too.

Data presented here show categorically the antagonistic effect of vanillin on the growth of *Bradyrhizobium* sp. (Arachis) strain C145. Peanut is considered a highly promiscuous species because it forms nodules with rhizobia, which are able to nodulate a diverse group of legumes (Alwi et al., 1989). However, not all peanut microsymbionts are equally effective for nitrogen fixation in symbiosis with legumes (Castro et al., 1999). Under field conditions, well-adapted native strains with high nodulation ability but sometimes with low nitrogen-fixing ability may have a competitive advantage (Martensson and Gustafsson, 1985).

Therefore, it is important to identify not only the mechanisms and substances involved in nodulation process but also in rhizobial proliferation and survival in the rhizosphere.

Because of its antimicrobial properties, vanillin has successfully been used as a natural food preserver (Cerrutti et al., 1997). Antifungal activity of vanillin is well documented (López-Malo et al., 1997). On the other hand, Weiss et al. (1997, 1999) found a tissue-specific and development-dependent accumulation of vanillin and other phenylpropanoids during the early stages of mycorhization of larch (*Larix decidua* Mill.) and other members of the Pinaceae family. These authors hypothesized that fungus-induced increased levels of phenylpropanoids may restrict the extent of fungal colonization and pointed out the existence of a careful balance between both partners in regard to the activities of the cell wall-degrading enzymes. Taking this association as a model of mutualistic symbiosis interaction, it is possible to speculate that in peanut-rhizobia symbiosis vanillin and perhaps other phenylpropanoids may be restricting proliferation of less effective strains in the rhizosphere or even inside the roots. The effect of interstrain rhizobial competition in the soil or rhizosphere is not clearly understood, and this sometimes results in the selection of competitive but ineffective rhizobial strains (Athar and Johnson, 1996).

According to the results obtained we conclude that vanillin affects peanut plant growth and *Bradyrhizobium* sp. (Arachis) multiplication rather than rhizobial infectivity, although at relatively high concentrations (10 µg per plant) symbiotic parameters may be also compromised. This study may be a contribution to the understanding of the role of simple phenolic compounds in the establishment and functioning of peanut and perhaps other legume-rhizobia symbiosis.

Acknowledgments

This work was supported by grants of Universidad de Buenos Aires, Argentina, and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. M.A. del P.M. and M.L.T. are career investigators from CONICET.

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