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REGULAR ARTICLE

Enhanced chickpea growth-promotion ability of a *Mesorhizobium* strain expressing an exogenous ACC deaminase gene

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Abstract

Aims The main goal of the study reported herein was to assess the nodulation performance of a *Mesorhizobium* strain transformed with an exogenous ACC deaminase gene (*acdS*), and its subsequent ability to increase chickpea plant growth under normal and waterlogged conditions.

Methods The *Mesorhizobium ciceri* strain LMS-1 was transformed with the *acdS* gene of *Pseudomonas putida* UW4 by triparental conjugation using plasmid pRKACC. A plant growth assay was conducted to verify the plant growth promotion ability of the LMS-1 (pRKACC) transformed strain under normal and waterlogging conditions. Bacterial ACC deaminase and nitrogenase activity was measured.

Results By expressing the exogenous *acdS* gene, the transformed strain LMS-1 showed a 127% increased ability to nodulate chickpea and a 125% promotion of the growth of chickpea compared to the wild-type strain, under normal conditions. Plants inoculated with the LMS-1 wild-type strain showed a higher nodule number under waterlogging stress than under control conditions, suggesting that waterlogging increases nodulation in chickpea. No significant relationship was found between ACC deaminase and nitrogenase activity.

Conclusions The results obtained in this study show that the use of rhizobial strains with improved ACC deaminase activity might be very important for developing microbial inocula for agricultural purposes.

Keywords Rhizobia · Chickpea · ACC deaminase · Waterlogging · Nodulation

Introduction

Bacteria that express ACC (1-aminocycloproprane-1-carboxylate) deaminase can uptake and convert ACC into α-ketobutyrate and ammonia, thus, reducing plant ethylene levels (Glick et al. 1998). The use of bacteria that express high levels of ACC deaminase has been reported to help plants to overcome various stresses, including waterlogging (Belimov et al. 2001;

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Grichko and Glick 2001; Glick 2003; Mayak et al. 2004).

Rhizobial species producing ACC deaminase can promote nodulation of their host legumes (Ma et al. 2003a, 2004; Uchiumi et al. 2004), since they decrease ethylene levels that are known to inhibit nodule formation in many leguminous plant species (Guinel and Geil 2002; Gage 2004).

The chickpea (*Cicer arietinum* L.) microsymbiont *Mesorhizobium ciceri* has no ACC deaminase activity under free-living conditions (Ma et al. 2003b). In *Mesorhizobium loti* MAFF303099 ACC deaminase is expressed only inside the nodules under transcriptional regulation of the $NifA_2$ protein (Uchiumi et al. 2004; Nukui et al. 2006), suggesting that, in this case, the production of ACC deaminase is involved in symbiosis but not nodulation per se.

Conforte et al. (2010) showed that the expression of ACC deaminase in free-living conditions by a genetically engineered *M. loti* MAFF303099 strain improved nodulation efficiency and competitiveness of this strain in *Lotus* spp. This result demonstrates that using *Mesorhizobium* improved strains, with free-living ACC deaminase activity, can be a useful tool to promote nodulation and thus plant growth.

Chickpea is one of the most important leguminous plants being cultivated throughout the world. Its grain represents an important source of protein for both human and animal diets. Chickpea has also an important role in soil natural nitrogen fertilization, through its symbiotic relationship with rhizobia. In optimal conditions the chickpea-rhizobia symbiosis can lead to a natural nitrogen fixation up to 80–120 kg N ha^{-1} (Saxena and Singh 1987).

Under Mediterranean conditions chickpea is a traditionally spring/summer crop with water availability as the main limiting growth factor, with insufficient levels of water leading to reduced grain yields. However, the development of winter sown chickpea cultivars brought new possibilities in the agricultural use of this pulse crop, since it allows a longer vegetative period with a higher crop productivity potential (Duarte et al. 1992). Yet, new problems arise with the use of winter sown chickpea cultivars. Abiotic stresses, such as waterlogging due to the rainfall during the winter season in Mediterranean areas, becomes a stress factor of this crop, leading to plant death and reduced crop productivity (Schwinghamer 1994; Cowie et al. 1996a, b; Siddique et al. 2000).

Waterlogging consists of soil water saturation resulting from flooding or low soil drainage. This water saturation leads to the reduction of oxygen levels that can drop to critical levels, leaving the soil and the root systems in an anoxia or hypoxia state (Jackson 1985; Kozlowski 1984). Without sufficient oxygen levels, root nutrient and water uptake are disturbed resulting in lower photosynthetic rates by the plant (Vartapetian and Jackson 1997).

Under waterlogging conditions the symbiotic relationship between rhizobia and host plants is altered, since the O_2 levels in the root system that are necessary for optimal N_2 fixation are decreased (Sprent 1972; Minchin and Pate 1975). In addition, ethylene levels are increased in the shoots of waterlogged plants, and its accumulation is responsible for the development of the waterlogging symptoms including epinasty and chlorosis (Vartapetian and Jackson 1997). The high production and accumulation of ethylene in shoots results from the high ACC levels, synthesized in roots by ACC synthase, then transported from the root system to shoot where it is converted to ethylene by ACC oxidase (Bradford et al. 1982). Since oxygen concentrations of waterlogged root systems are too low or nonexistent, the conversion of ACC to ethylene by ACC oxidase cannot take place (Jackson and Campbell 1976; Bradford and Dilley 1978; Wang and Arteca 1992; Banga et al. 1997).

The aim of this study was to assess the nodule formation and plant growth promotion abilities of a mesorhizobia strain expressing an exogenous ACC deaminase under free-living conditions, and its impact on the capacity of chickpea plant to overcome waterlogging stress.

Material and methods

Bacterial strains, growth conditions and triparental conjugation

The plasmids and bacteria used in this work are presented in Table 1.

The Portuguese *Mesorhizobium ciceri* strain LMS-1 was transformed by triparental mating with plasmid pRKACC which contains the *acdS* gene of *Pseudomonas putida* UW4 cloned in pRK415 (Shah et al. 1998). The LMS-1 strain was chosen based on its symbiotic effectiveness and stress tolerance

Table 1 Bacterial strains and plasmids used in this work

Plasmids/Strains	Characteristics	Reference
pRK600	pRK2013 *npt*::Tn*9*, Cm^r	Finan et al. 1986
pRKACC	pRK415 containing *Pseudomonas putida* UW4 *acdS* gene and it's flanking regions	Shah et al. 1998
E. coli		
MT616	MT607 (pRK600), mobilizing strain	Finan et al. 1986
DH5α	SupE44 *Δ lacU169* (*φ80lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook and Russell 2001
Mesorhizobium		
LMS-1	*M.ciceri* species isolate with high symbiotic effectiveness and stress tolerance	Brígido, C., unpublished results
LMS-1 (pRKACC)	LMS-1 isolate carrying pRKACC	This work

characteristics, which makes it a high value candidate as a chickpea soil inoculant (Brígido, C., personal communication; Alexandre et al. 2009).

In the triparental conjugation method, *Escherichia coli* strains DH5α (pRKACC) and MT616 (pRK600) were used as donor and helper strains respectively (Shah et al. 1998; Ma et al. 2004). Strain LMS-1 was grown in the center of a TY plate for 2 days at 28°C. *E. coli* strains were then streaked onto this plate and the three cultures were mixed. After overnight growth at 28°C, the *Mesorhizobium* transformants were selected based on their ability to grow in modified minimal medium (Robertsen et al. 1981) containing sucrose as the only carbon source and 20 μg/ml tetracycline.

Mesorhizobium strains and transformants were grown in TY medium (Beringer 1974) and M9 minimal medium (Miller 1972), at 28°C, with 20 μg/ml tetracycline when necessary.

E.coli DH5α (pRKACC) was grown in LB medium (Sambrook and Russell 2001) containing 20 μg/ml tetracycline, at 37°C. *E.coli* MT616 was grown in LB medium containing 25 μg/ml chloramphenicol, at 37°C.

pRKACC plasmid extraction and visualization

Plasmid pRKACC was extracted from putatively transformed *Mesorhizobium* cells in order to confirm transformation success. Plasmid extraction was conducted using the GeneJET Plasmid Miniprep Kit (Fermentas Life Sciences) following the manufacturer's instructions. Plasmid pRKACC was cut using restriction enzymes *Hind*III and *Kpn*I and visualized by electrophoresis in agarose gel, as described by Shah et al. (1998).

ACC deaminase activity assay

The transformed *Mesorhizobium* LMS-1 (pRKACC) and wild-type strains were tested for ACC deaminase activity. *Mesorhizobium ciceri* UPM-Ca7 and *Mesorhizobium loti MAFF303099* were used as negative controls. *Rhizobium leguminosarum* bv.viciae 128C53K was used as a positive control (Ma et al. 2003a, b).

ACC deaminase induction in cells was performed as described by Duan et al. (2008). *Mesorhizobium* strains were grown in TY (supplemented with 20 μg/ml tetracycline when necessary) for 2–3 days at 28°C. Cells were washed twice with 0,1 M Tris–HCl (pH 7,5) and then resuspended in modified M9 minimal medium with a ACC final concentration of 5 mM. Cells were incubated with shaking for approximately 40 h at 28°C. After induction, ACC deaminase activity was measured based on the determination of α-ketobutyrate resulting from ACC cleavage by ACC deaminase, as described by Penrose and Glick (2003).

Total protein content of cells was quantified by the Bradford method (1976) using Bradford reagent (Sigma) according to the manufacturer protocol. Final ACC deaminase activity was expressed in μmol α-ketobutyrate/mg protein/h.

Plant growth conditions

Cicer arietinum winter cultivar CHK 3226 seeds were surface sterilized in a 14% calcium hypochlorite solution for 45 min. After sterilization, seeds were rinsed six times in sterilized distilled water and incubated for 2 h at 28°C. Seeds were placed in 0,75% agar plates and then incubated in the dark for 48 h at 28°C. After germination, one seed was distributed per pot, which contained 100 g of sterilized vermiculite and 100 ml of nitrogen-free nutrient solution (Broughton and Dilworth 1971), in a total volume of 600 ml.

The plants were grown in a growth chamber (Walk-in fitoclima, Aralab, Portugal) programmed for 65% humidity and a photoperiod of 16 h (Day Cycle: 22°C for 60 min, 24°C for 840 min and 20°C for 60 min; Night cycle: 20°C for 60 min, 18°C for 360 min and 22°C for 60 min). Plants were irrigated with 100 ml of nitrogen-free nutrient solution (Broughton and Dilworth 1971) whenever necessary.

Waterlogging stress conditions were imposed by immersing the pots in a container filled with nitrogen free nutrient solution until it reached about 1 cm above the soil (vermiculite) surface. Waterlogging was applied for 7 days (21 days after inoculation). Stress conditions were removed by allowing the pot to drain.

Plant growth assay

To evaluate the effect of an external ACC deaminase in plant and nodule development under normal and waterlogging conditions, a plant growth and nodulation assay was performed in a growth chamber. *Mesorhizobium* strains, LMS-1 wild-type and LMS-1 (pRKACC) were grown in TY medium (containing 20 μg/ml tetracycline when necessary), at 28°C for 72 h. After incubation, the cell suspension OD's were adjusted so that there were approximately 10^9 CFU ml^{-1}; 2 ml of bacterial suspension were used to inoculate each chickpea seed.

Four replicates were used per treatment; plants were harvested 31, 38 and 45 days after inoculation (3, 10 and 17 days respectively, after waterlogging conditions were removed), for evaluation of nodule number and weight, plant total biomass (shoot and root dry weight) and nitrogenase activity. After performing an acetylene reduction assay (see below), nodules as well as roots and shoots, were dried at 60°C for 48 h, and dry weights were determined.

Using the same conditions as described above, another assay was conducted with eight replicates per treatment. In this assay plants were harvested 45 days after inoculation. Nodule number and weight, plant total biomass and nitrogenase activity were evaluated. All assays were conducted as a randomized block design.

Recovery of pRKACC transformed strain from nodules

To assess the stability of plasmid pRKACC in nodules formed by the transformed strain LMS-1, the recovery of pRKACC-transformed bacteria from nodules was conducted. Nodules were surface sterilized by immersion in a 96% ethanol solution for 10 min, followed by 3 min in 3% H_2O_2, and rinsed six times with sterilized distilled water. After surface sterilization, nodules were crushed in a 1.5 ml tube containing 500 μl TY medium and 30 μl of crushed nodule suspension were incubated in a Congo red/YMA plate (Somasegaran and Hoben 1994) containing 20 μg/ml tetracycline. *Mesorhizobium* was identified by its growth characteristics on this medium (Somasegaran and Hoben 1994).

Acetylene reduction assay

Nitrogenase activity in nodules was evaluated by the acetylene reduction assay as described by Somasegaran and Hoben (1994). Acetylene and ethylene were quantified using a HP 5710A gas chromatograph (Hewlett-Packard, California) using N_2 as the carrier gas. A standard curve was based on known concentrations of ethylene.

Statistical analysis

The data obtained from the nodulation assay was characterized by analysis of variance, and means were compared with T-student test. Statistical analysis was carried out using SPSS statistics V.17 (SPSS Inc., IBM Company).

Results

Transformation, ACC deaminase activity and plasmid stability

The successful transformation of *Mesorhizobium* strain LMS-1 with plasmid pRKACC has been confirmed. After transformation, the plasmid pRKACC was extracted from transformed cells and linearized using the restriction enzymes *Hind*III and *Kpn*I, which resulted in two fragments of approximately 4 kb and 10 kb as expected (data not shown). This result shows that the plasmid introduced into *Mesorhizobium* cells has no obvious changes to its structure.

When ACC deaminase activity was assayed in wild-type LMS-1 and LMS-1 (pRKACC), the wild-type strain showed no ACC deaminase activity while LMS-1 (pRKACC) displayed a high level of ACC deaminase activity (2,035±0,210 μmol α-ketobutyrate/mg protein/h).

The stability of plasmid pRKACC in the plant nodules was demonstrated by recovering the pRKACC-transformed strain from root nodules of 45 day old chickpea plants subjected to control and waterlogging conditions and then growing the recovered bacterium on tetracycline (data not shown).

Plant growth under control conditions

The number of nodules formed on plants inoculated with LMS-1 (pRKACC) or LMS-1 wild type strain did not differ at 31 or 38 days after inoculation (DAI) (Table 2). However, at a later time point (45 DAI), plants inoculated with strain LMS-1 (pRKACC) had developed a significantly higher number of nodules than plants inoculated with the wild-type strain LMS-1 (Fig. 1a).

In addition, the nodule dry weight found on plants inoculated with LMS-1 (pRKACC), was significantly higher at 45 DAI, compared with plants inoculated with wild type strain (Table 2; Fig. 1b). Further, the rate of increase in nodule dry weight was significantly higher in plants inoculated with LMS-1 (pRKACC), as compared with those inoculated with wild-type strain (Table 2).

No significant differences were observed in the average weight per nodule at 45 DAI (Fig. 1c).

Statistically significant differences were observed at 31 DAI, when plants inoculated with LMS-1 (pRKACC) developed a higher average weight per nodule than plants treated with LMS-1 wild-type (Table 2). These results suggest that the expression of an exogenous ACC deaminase by *Mesorhizobium* can promote nodule development in chickpea plant.

Table 2 Results obtained from the nodulation assay of plants inoculated with LMS-1 wild- type or LMS-1 (pRKACC) under control and waterlogging conditions, at different times after inoculation. The data presented in the table represents the mean and standard deviation values of 4 plant replicates

Strain	Treatment	DAI	Nodule number per plant	Nodule dry weight (mg)	Average weight per nodule (mg)	Total biomass per plant (g)
LMS-1	Control	31	62±11	41±6	0,68±0,16*	0,529±0,067
	Control	38	43±13	52,1±10	1,29±0, 41	0,568±0,116
	Control	45	48±18 * #	109,2±43,9* (2,7x)	2,38±0,65	0,962±0,460*
LMS-1	Waterlogging	31	54±14	26±11	0,46±0,11	0,410±0,113
	Waterlogging	38	53±16	36,1±18,5	0,67±0,33#	0,366±0,142
	Waterlogging	45	75±10 #	62,9±16,3	0,84±0,18#	0,565±0,159
LMS-1 pRKACC	Control	31	67±10	63,6±9,4	0,96±0,14*	0,675±0,079
	Control	38	60±19	56,6±19,4	1,08±0,72	0,582±0,150
	Control	45	99±19 *	257, 8±47,8 * (4,1x)	2,65±0,56	2, 202±0,523*
LMS-1 pRKACC	Waterlogging	31	56±13	20,6±8,8	0,36±0,13	0,325±0,149
	Waterlogging	38	54±10	32,6±18,6	0,58±0,25	0,381±0,189
	Waterlogging	45	104±27	74,7±13	0,73±0, 06	0,689±0,158

Statistically significant differences ($P<0.05$) between the transformed and wild-type strains under control conditions are marked with *. Statistically significant differences ($P<0.05$) between the wild-type strain under control and waterlogging conditions are marked with #. DAI- Days after inoculation. In brackets is the rate of increase

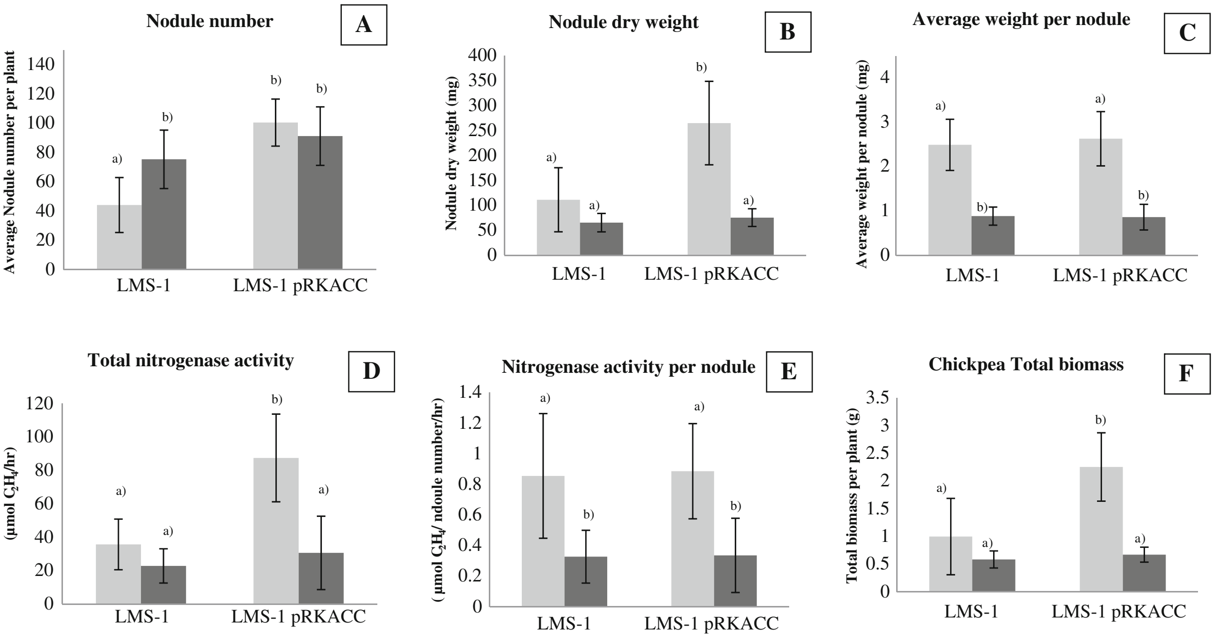

Fig. 1 Results obtained from a nodulation assay, 45 days post inoculation (17 days after removal of waterlogging conditions) with LMS-1 wild-type or LMS-1 (pRKACC) strain. Data correspond to the mean and standard deviation values of 8 plant replicates. *Light grey bars* correspond to results obtained under control conditions. *Dark grey bars* correspond to results obtained in plants subjected to waterlogging conditions. Different letters correspond to statistical significant differences ($P<0.05$)

At 31 DAI, nitrogenase activity was not detected in most replicates of LMS-1 wild-type inoculated plants. However, during the same time period, nitrogenase activity was detected in LMS-1 (pRKACC) nodules (data not shown). These results suggest that the timing of nodule nitrogenase activity may be accelerated by expression of ACC deaminase. At 45 DAI, statistically significant differences were found between total nitrogenase activity of plants inoculated with either LMS-1 or LMS-1 (pRKACC) (Fig. 1d). However, no statistical significance was found between the level of nitrogenase activity per nodule in plants inoculated with LMS-1 compared to LMS-1 (pRKACC) (Fig. 1e).

The expression of an exogenous ACC deaminase by the LMS-1 (pRKACC) strain resulted in a 125% increase of chickpea total biomass at 45 DAI, when compared to plants inoculated with the wild-type strain the LMS-1, under control conditions (Fig. 1f).

Plant growth under waterlogging conditions

No statistically significant differences were found in the number of nodules formed by either the wild-type, LMS-1, or the transformed, LMS-1 (pRKACC), strain, in plants subjected to waterlogging conditions (Fig. 1a) (Table 2).

Interestingly, the number of nodules formed by the LMS-1 wild-type strain in plants subjected to waterlogging conditions was higher when compared to the number of nodules formed by the same strain under control conditions (Fig. 1a) (Table 2).

Nodule dry weight, average weight per nodule, nitrogenase activity and chickpea total biomass were found to be similar in both LMS-1 wild-type and LMS-1 (pRKACC) inoculated plants, under waterlogging conditions (Fig. 1b–f).

Compared with control conditions, waterlogging conditions lead to a reduction in nodule dry weight, average weight per nodule, nitrogenase activity and chickpea total biomass in both LMS-1 wild-type and LMS-1 (pRKACC) inoculated plants (Fig. 1b–f).

Despite showing more nodules in waterlogging conditions, the plants inoculated with LMS-1 wild-type and LMS-1 (pRKACC) strains have reduced nitrogenase activity (Fig. 1d, e), suggesting that the nodules formed under waterlogging conditions are less effective.

Total plant biomass was also reduced in chickpea plants subjected to waterlogging, despite being inoculated with either the LMS-1 wild-type or the LMS-1 (pRKACC) strain (Fig. 1f). Chickpea plants subjected to waterlogging showed symptoms such as chlorosis and epinasty (data not shown) as expected and previously described by Cowie et al. (1996a).

Discussion

ACC deaminase has been shown to play an important role in the nodulation process conducted by different rhizobia genus in different plant hosts (Ma et al. 2003a, 2004; Uchiumi et al. 2004).

Conforte et al. (2010) showed that an engineered *Mesorhizobium loti* MAFF 303099 expressing its own ACC deaminase gene under free-living conditions can promote nodulation to a higher extent in *Lotus japonicus*. Ma et al. (2004) also showed that by expressing an exogenous *acdS* gene, *Sinorhizobium meliloti* increased its ability to nodulate alfafa by 35% to 40%.

Similar results were obtained in this work. By expressing the *P. putida* UW4 *acdS* gene, the LMS-1 (pRKACC) strain showed an increased ability to form nodules in chickpea plants (i.e. ~127%) compared to the wild type strain. Noticeably, chickpea plants inoculated with LMS-1 (pRKACC) showed an increase of 125% in its total biomass, compared to plants inoculated with the LMS-1 wild-type strain. These results indicate that ACC deaminase plays an important role in the chickpea-mesorhizobia symbiosis, suggesting that the use of chickpea *Mesorhizobium* with a higher level of ACC deaminase activity might be a useful tool to enhance the plant growth promotion abilities of these bacteria.

The *Mesorhizobium* transformed strain LMS-1 (pRKACC) showed relatively high ACC deaminase activity under free-living conditions when compared to results obtained in other studies. *P. putida* ATCC 17399 and *P. fluorescens* ATCC 17400 transformed with the plasmid pRKACC showed values for ACC deaminase activity of 0,507 and 0,490 μmol α-ketobutyrate/mg protein/h, respectively. These ACC deaminase activity levels were sufficient to promote the elongation of canola roots under gnotobiotic conditions (Shah et al. 1998). The natural ACC deaminase activity of *R. leguminosarum* bv.viciae 128Sm (1,56±0,23 μmol α-ketobutyrate/mg protein/h) is enough to promote nodulation of *P. sativum* L. cv. Sparkle (Ma et al. 2003a).

Although it has been previously demonstrated that expression of ACC deaminase promotes nodulation, no studies have been conducted to investigate the ACC deaminase role in the nodulation process per se. Ma et al. (2003a) suggested that ACC deaminase involvement on nodulation was restricted to nodule formation and not nodule function. Ma et al. (2004) suggested that rhizobia producing ACC deaminase could reduce ethylene levels in the root system, leading to a more successful rate of progression by the infections threads thus facilitating the formation of functional nodules. It was also proposed by the same authors that rhizobia producing ACC deaminase under free-living conditions can use ACC as nitrogen and carbon sources, improving the proliferation capacity of this strains thereby resulting in more efficient and competitive infections.

The results obtained in the present study indicate that ACC deaminase activity contributes to early nodule development but not nodule function, as proposed by Ma et al. (2003a, b). Under control conditions, at 31 DAI, nodules formed by the LMS-1 (pRKACC) strain were more developed than nodules formed by the wild-type strain (Table 2).

At 45 DAI, nitrogenase activity per nodule is similar between LMS-1 wild-type and LMS-1 (pRKACC) strains suggesting that, in this stage, the nitrogenase activity is not influenced by ACC deaminase. Similar results were reported by Ma et al. (2003a, 2004). Our results show that total nitrogenase activity was higher in plants inoculated with the LMS-1 (pRKACC) strain than with the wild-type, LMS-1. This result suggests that by forming more effective nodules the LMS-1 (pRKACC) strain can provide nitrogen fixation to a greater extent, even if the nitrogenase activity in the nodules occurs in the same extent as with the wild type strain.

Interestingly, no significant differences have been found in the nodule number formed by both strains at 31 DAI. At this time, both strains produce essentially the same nodule number. This result suggests that the role of ACC deaminase in nodulation occurs at the level of the nodule formation process and after root colonization and rhizobial entrance into plant cells. Conforte et al. (2010) showed that root colonization by *M. loti* MAFF 303099 SR strain (expressing ACC deaminase under free-living conditions), tended to be the same as the wild-type strain.

Under waterlogging conditions, no significant differences have been found between plants inoculated with either LMS-1 wild-type or LMS-1 (pRKACC) strains in any of the measured parameters. This suggests that expression of the exogenous ACC deaminase gene is unable to overcome the effects of waterlogging in chickpea plants under the conditions employed in these experiments.

When comparing the effect of the LMS-1 wild-type strain on plants in both control and waterlogging conditions, it seems that waterlogging induced nodule formation in chickpea. An increase in nodule formation resulting from waterlogging conditions was also reported by other authors in different plant species including *Vicia faba*, *Vigna unguiculata*, and *Glycine max* (Gallacher and Sprent 1978; Hong et al. 1977; Sánchez et al. 2011).

Sánchez et al (2011) found that *Bradyrhizobium japonicum* USDA110 formed more nodules in soybean plants subjected to waterlogging (for 14 days) when compared to control conditions. Even a *Bradyrhizobium japonicum* strain *norC* knockout mutant, known to have decreased nodulation abilities (Mesa et al. 2004) exhibited an increased nodule number to the same extent as the wild-type strain in plants subjected to waterlogging conditions. It seems that waterlogging can trigger changes in the host plant which result in promotion of nodulation.

It is known that waterlogging decreases O_2 in root systems to almost non-existent levels (Jackson 1985; Kozlowski 1984). Oxygen is necessary for many enzymatic processes such as the functioning of ACC oxidase (that is responsible for ACC oxidation to ethylene) and also for optimal N_2 fixation in the bacteroids (Abeles et al. 1992; Delgado et al. 1998). Since O_2 levels are decreased, it is likely that ethylene production in waterlogged roots is impaired. If the ethylene levels in roots are decreased, the negative effect of ethylene on nodulation should be decreased as well. Furthermore, if nitrogen fixation in the bacteroids is impaired by the exceedingly low O_2 levels, the plant which utilizes rhizobial nitrogen fixation as a unique source of nitrogen loses that nitrogen source. It is possible that the nitrate mechanism that is also known to regulate nodulation (Caba et al. 1998; Schmidt et al. 1999; Ferguson et al. 2010) might be deregulated under waterlogging conditions as well.

Thus, if fixed nitrogen and ethylene levels, both known to downregulate nodulation, are decreased under waterlogging conditions, it is possible that (IAA- synthesizing) bacteria can produce a higher number of nodules in the plant host. This could explain the high nodulation profile demonstrated by the LMS-1 wild-type strain under waterlogging conditions, compared to control conditions.

However, the nodules formed under waterlogging conditions are mostly ineffective. Nodule dry weight, average weight per nodule, nitrogenase activity and plant total biomass values are lower in plants subjected to waterlogging conditions than to control conditions. Similar results have been reported by Sanchez et al. (2011) in soybean plants inoculated with *Bradyrhizobium japonicum* USDA110 subjected to waterlogging for 7 days.

Although no significant plant promotion abilities by LMS-1 (pRKACC) strain have been found under waterlogging conditions, the plant growth-promoting effect of the LMS-1 (pRKACC) strain on chickpea plants under control conditions is noticeable. By expressing ACC deaminase under free-living conditions, the *Mesorhizobium* LMS-1 (pRKACC) increased its nodulation performance by 127% and increased chickpea plant total biomass by 125%, compared to LMS-1 wild type strain. These results show that the use of rhizobial strains with improved ACC deaminase activity might be very important for developing microbial inocula for agricultural purposes.

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