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Applied Microbiology
Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Mesorhizobium ciceri LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints

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Keywords
1-aminocyclopropane-1-carboxylate deaminase, *acdS*, chickpea, *Mesorhizobium*, root rot, soil.

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2012/0247: received 8 February 2012, revised 27 March 2012 and accepted 29 March 2012

doi:10.1111/j.1472-765X.2012.03251.x

Abstract

Aims: Our goal was to understand the symbiotic behaviour of a *Mesorhizobium* strain expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which was used as an inoculant of chickpea (*Cicer arietinum*) plants growing in soil.
Methods and Results: *Mesorhizobium ciceri* LMS-1 (pRKACC) was tested for its plant growth promotion abilities on two chickpea cultivars (ELMO and CHK3226) growing in nonsterilized soil that displayed biotic and abiotic constraints to plant growth. When compared to its wild-type form, the *M. ciceri* LMS-1 (pRKACC) strain showed an increased nodulation performance of *c.* 125 and 180% and increased nodule weight of *c.* 45 and 147% in chickpea cultivars ELMO and CHK3226, respectively. *Mesorhizobium ciceri* LMS-1 (pRKACC) was also able to augment the total biomass of both chickpea plant cultivars by *c.* 45% and to reduce chickpea root rot disease susceptibility.
Conclusions: The results obtained indicate that the production of ACC deaminase under free living conditions by *Mesorhizobium* strains increases the nodulation, plant growth abilities and biocontrol potential of these strains.
Significance and Impact of the Study: This is the first study regarding the use of a transformed rhizobial strain expressing an exogenous ACC deaminase in different plant cultivars growing in soil. Hence, obtaining *Mesorhizobium* strains with high ACC deaminase activity is a matter of extreme importance for the development of inoculants for field applications.

Introduction

Plants cultivated in soil are subjected to different abiotic and biotic stress that ultimately limit and reduce plant growth and development (Glick *et al.* 2007). From an agricultural perspective, this may be reflected in a loss in crop productivity leading to major economic problems. Chickpea (*Cicer arietinum*, L.) is a legume crop with great agricultural and economic importance (Yadav *et al.* 2007). Chickpea is also responsible for atmospheric nitrogen fixation and soil fertility maintenance through its symbiotic association with rhizobia (Saxena and Singh 1987). Abiotic stresses like drought, waterlogging, extreme temperatures and salinity are major problems affecting chickpea plant growth, resulting in yield losses from about 6·4 million tons per year (Ryan 1997). Biotic stresses like fungal, viral and insect borne diseases also limit and reduce chickpea plant growth. For example, diseases like *Ascochyta* blight, *Fusarium* wilt and root rot have been reported to severely affect chickpea crop productivity (Nene 1982; Trapero-Casas and Jimenez-Diaz 1985; Kaiser 1997; Sharma and Muehlbauer 2007) and together with other biotic stresses may account for losses of up to 4·8 million tons per year (Ryan 1997).

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Stress conditions induce ethylene production and accumulation in plant tissues (Hyodo 1991). When present in high concentrations, ethylene can trigger the initiation of senescence, chlorosis and abscission processes and ultimately lead to plant death (Abeles *et al.* 1992). Ethylene is also known to be an inhibitor of nodule formation in various leguminous plants (Penmetsa and Cook 1997; Oldroyd *et al.* 2001; Gage 2004).

The use of bacteria that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase under free living conditions has been shown to be a useful tool for promoting plant growth under various stress conditions, including drought, waterlogging, heavy metal contamination and pathogen attack (Wang *et al.* 2000; Grichko and Glick 2001; Mayak *et al.* 2004; Reed and Glick 2005). ACC deaminase converts ACC into ammonia and α-ketobutyrate (Honma and Shimomura 1978), leading to a reduction in plant ACC content and therefore lowering the ethylene concentration in plant tissues and its deleterious effects on plant development and growth (Glick *et al.* 2007).

Rhizobial strains expressing exogenous ACC deaminase genes show a higher nodulation profile and increased plant growth promotion abilities when compared to their wild-type forms. In the study conducted by Ma *et al.* (2004), a *Sinorhizobium meliloti* strain expressing an exogenous ACC deaminase gene increased its nodulation abilities by 40%. More recently, Conforte *et al.* (2010) demonstrated that *Mesorhizobium loti* MAFF303099 expressing ACC deaminase under free living conditions induced the formation of a higher number of nodules in *Lotus* plants. When *Mesorhizobium ciceri* LMS-1 was transformed to express the *Pseudomonas putida* UW4 ACC deaminase gene (*acdS*), its nodulation performance was increased resulting in an increased chickpea plant total biomass (Nascimento *et al.* 2012).

All the reported studies regarding the effect of ACC deaminase in rhizobia-leguminous plant symbiosis have been conducted under laboratory conditions. However, in order to be considered as potential field inoculants, more information is needed about the symbiotic performance of engineered rhizobial strains in soil, where they face potentially adverse conditions. Therefore, a plant growth assay was conducted under greenhouse conditions using nonsterilized soil to evaluate the nodulation performance and plant growth promotion abilities of the *M. ciceri* LMS-1 strain expressing an exogenous ACC deaminase.

Material and methods

Bacterial growth conditions

In this study, the *M. ciceri* LMS-1 and *M. ciceri* LMS-1 (pRKACC) expressing *acdS* from *Ps. putida* UW4 strains were used. No ACC deaminase activity was found in the wild-type strain. On the other hand, the (pRKACC) transformed strain is able to produce ACC deaminase (2·035 ± 0·210 μmol α-ketobutyrate per mg protein per h) (Nascimento *et al.* 2012).

The strains were maintained in Tryptone Yeast (TY) medium (Beringer 1974) and supplemented with tetracycline (20 $\mu g\ ml^{-1}$) when necessary. For the plant growth assay, strains were grown in 100 ml Erlenmeyer flasks with TY medium at 28°C for 3 days. When the growth reached the late exponential phase, the cells were collected and the optical density was measured at 540 nm. The OD's were adjusted so that the cell concentration was *c.* 10^9 CFU ml^{-1}, and 4 ml of the bacterial suspension was used to inoculate each chickpea seed.

Soil characterization

To evaluate *M. ciceri* LMS-1 (pRKACC) nodulation performance and its ability to promote chickpea plant growth, a pot experiment was performed under greenhouse conditions using a Cambisoil derived from granites collected from the Ap horizon of a field located at the 'Herdade da Mitra' (+38·52, −8·02), University of Évora, Portugal. This soil is known to induce manganese toxicity in wheat crops (Goss and Carvalho 1992), and, as observed in Table 1, it presents other constraints to plant growth, namely low phosphorus, zinc and boron availability.

Chickpea plant growth assay

To investigate the symbiotic performance of the two *Mesorhizobium* strains (i.e. wild-type *vs* pRKACC transformed), two independent assays were performed using two chickpea plant types.

Table 1 Analitycal characteristics of the soil used in the experiment

O.M.	pH	MgO	P_2O_5	K_2O	Na_2O	Zn	Mn	Cu	Fe	B
%	H_2O	ppm								
1·1	6·0	200	7	68	25	0·4	22·6	0·9	65·8	0·1

O.M., organic matter content.

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Cicer arietinum cultivars CHK 3226 (Kabuli type) and ELMO (Desi type) seeds were surface-sterilized by incubation in a 2·5% sodium hypochlorite solution for 20 min. After sterilization, seeds were rinsed six times in sterilized distilled water and incubated for 2 h at 28°C. Seeds were placed in sterilized vermiculite and then incubated in the dark for 48 h at 25°C. After germination, two seeds were distributed per pot (containing 2 kg of soil under field capacity) and inoculated with the specific *Mesorhizobium* culture. No bacteria suspension was added to the negative control. Four pots were used per treatment. Plants were irrigated with distilled water whenever necessary. The assay was conducted as a randomized block design.

Forty-five days after inoculation, plants were harvested for evaluation of nodule number and dry weight and plant total biomass (shoot and root dry weight). Plant material was dried at 60°C for 48 h, and dry weights were determined.

Identification of fungal pathogens

To identify a possible causal agent for the disease found in all studied chickpea plants, necrotic roots were washed five times with sterilized distilled water and cut into small samples that were then incubated in potato dextrose agar for 1 week. After growth, fungal material was stained with a lactophenol blue solution and visualized using light microscopy. Also, small necrotic root sections were cut and stained as described by Phillips and Hayman (1970), and later visualized under light microscopy. The fungal agent was identified based on morphological characteristics (Leslie and Summerell 2006).

Statistical analysis

The data obtained from the chickpea plant growth assay were characterized by analysis of variance, and means were compared by one-way ANOVA. Statistical analysis was carried out using SPSS statistics V.17 (SPSS Inc; IBM New York, USA).

Results

When inoculated in two different chickpea plant cultivars grown in nonsterilized soil, the *M. ciceri* LMS-1 (pRKACC) strain showed a higher nodulation performance and plant growth promotion abilities than its wild-type form.

When inoculated in the chickpea 'Desi' cultivar ELMO, the *M. ciceri* LMS-1 transformed strain showed an increase in the nodulation performance of *c.* 125% (18 nodules formed by *M. ciceri* LMS-1 (pRKACC) *vs* eight nodules formed by *M. ciceri* LMS-1) (Fig. 1a). *Mesorhizobium ciceri* LMS-1 (pRKACC)-induced nodules also showed an increased weight (*c.* 45%) compared to the nodules formed by wild-type strain (0·158 mg/nodule *vs* 0·109 mg/nodule) (Fig. 1b). Moreover, ELMO chickpea plant's total biomass was augmented by *c.* 48% in plants inoculated with *M. ciceri* LMS-1 (pRKACC) compared to the wild-type strain (0·747 g *vs* 0·506 g) (Fig. 1c).

In the chickpea 'Kabuli' type cultivar CHK3226, the *M. ciceri* LMS-1 (pRKACC) transformant again demonstrated an increase in its nodulation abilities (*c.* 180%) compared to the wild-type strain (28 nodules formed by LMS-1 (pRKACC) *vs* 10 nodules formed by LMS-1 wild type) (Fig. 2a). The average weight per nodule was also increased (*c.* 147%) with the transformed strain (0·200 mg/nodule *vs* 0·081 mg/nodule) (Fig. 2b). In addition, the chickpea total biomass was also increased (*c.* 42%) in the plants inoculated with the *M. ciceri* LMS-1 (pRKACC) strain compared to the wild-type (0·930 g *vs* 0·656 g) (Fig. 2c).

No nodules were found in noninoculated plants, suggesting that rhizobial populations able to nodulate both chickpea cultivars ELMO and CHK3226 do not exist in the soil samples used in this study.

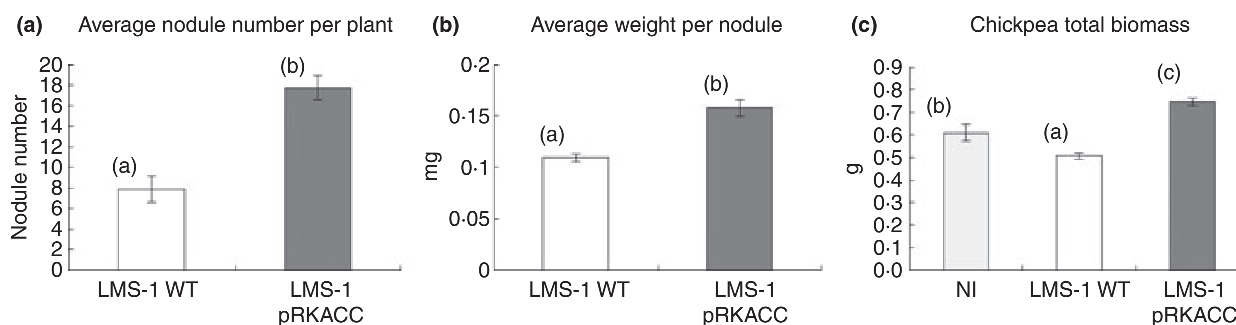

Figure 1 Results obtained from the plant growth assay using chickpea cultivar ELMO (Desi type), 45 days postinoculation with LMS-1 wild-type or LMS-1 (pRKACC) strain. Data correspond to the mean and standard error values of eight plant replicates (four pots). Different letters (a, b, c) correspond to statistical significant differences ($P < 0·05$). NI – noninoculated. No nodules were found in noninoculated plants.

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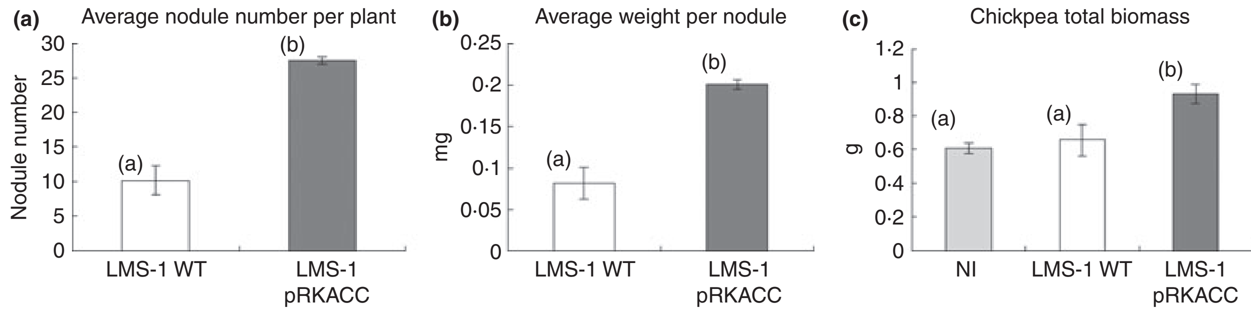

Figure 2 Results obtained from the plant growth assay using chickpea cultivar CHK3226 (Kabuli type), 45 days postinoculation with LMS-1 wild-type or LMS-1 (pRKACC) strain. Data correspond to the mean and standard error values of eight plant replicates (four pots). Different letters (a, b) correspond to statistical significant differences ($P < 0{\cdot}05$). NI – noninoculated. No nodules were found in noninoculated plants.

Interestingly, following their planting in soil, all cultivated chickpea plants (including noninoculated plants) demonstrated stress symptoms to some extent. Leaf yellowing and reduced aerial plant growth were observed. Also, chickpea roots demonstrated the typical disease symptoms of the root rot disease complex (Fig. 3). Fungal growth was observed in all of the roots sampled, with the fungus being morphologically identified as *Fusarium* spp. (Fig. 4).

Root tissue necrosis was observed upon examination of plant tissue following growth in soil, and it was observed that plants inoculated with the *M. ciceri* LMS-1 (pRKACC) had more developed roots and were less affected by the root rot disease when compared chickpea plants inoculated with the *M. ciceri* LMS-1 wild-type strain (Table 2; Fig. 3). Moreover, the protective role of *M. ciceri* LMS-1 (pRKACC) was more evident in the chickpea cultivar ELMO as the chickpea cultivar CHK3236 was naturally less affected by the fungal disease when compared to the ELMO cultivar (Table 2, Fig. 3).

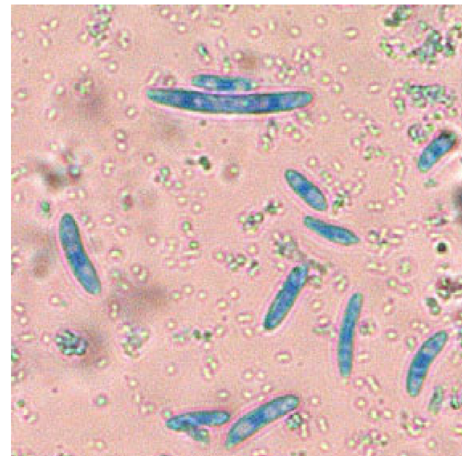

Figure 4 *Fusarium* spp. isolated from necrotic chickpea roots obtained in this study. Magnification 400×.

Discussion

The use of genetically transformed *Mesorhizobium* strains with improved ACC deaminase activity has been shown to be a useful tool in augmenting the nodulation and plant growth promotion abilities of these strains under laboratory conditions (Conforte *et al.* 2010; Nascimento *et al.* 2012)). Despite the already known high nodulation performance and plant growth promotion abilities demonstrated by *Mesorhizobium* strains expressing ACC

Figure 3 Chickpea plant roots demonstrating the typical aspect of the root and collar rot disease complex. (a) Roots of chickpea plants (ELMO) inoculated with *Mesorhizobium ciceri* LMS-1 wild-type strain and (b) *M. ciceri* LMS-1 pRKACC. (c) Roots of chickpea plants (CHK3226) inoculated with *M. ciceri* LMS-1 wild-type strain and (d) *M. ciceri* LMS-1 pRKACC.

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Table 2 Root development and root rot disease severity in chickpea plants obtained in this study

Chickpea cultivar	Strain	Root development – root rot disease severity				
		Pot 1	Pot 2	Pot 3	Pot 4	Average
ELMO	Noninoculated	1–2	2–3	2–3	1–3	2–3
	Mesorhizobium ciceri LMS-1	2–3	1–2	2–3	1–3	2–3
	M. ciceri LMS-1 (pRKACC)	3–3	3–2	3–2	3–2	3–2
CHK3226	Noninoculated	2–1	1–1	1–1	3–1	2–1
	M. ciceri LMS-1	1–1	3–1	2–1	1–1	2–1
	M. ciceri LMS-1 (pRKACC)	3–1	3–1	3–1	3–1	3–1

Root development level: 1– Low; 2– Medium; 3– High.
Root rot disease severity: 0 – no *Fusarium* root rot symptoms, no roots coloured black; 1 – Low, <30% of the roots coloured black; 2 – Medium, 60% of the roots coloured black; 3 – High, more than 60% of the roots coloured black.

deaminase under free living conditions, not much is known about its behaviour in soil where many factors can affect symbiosis and plant growth.

In this work, the previously studied *M. ciceri* LMS-1 (pRKACC) was used to inoculate two different chickpea plant cultivars grown in nonsterilized soil under greenhouse conditions. When compared to the *M. ciceri* LMS-1 wild-type strain, the pRKACC transformed strain showed an increased nodulation performance. The average weight per nodule and the chickpea plant total biomass was also increased in plants inoculated with the *M. ciceri* LMS-1 (pRKACC) strain. By producing ACC deaminase, the pRKACC transformed strain is able to increase its nodulation abilities and promote chickpea plant growth. Similar results were obtained in the previous study conducted by Nascimento *et al.* (2012), where chickpea plants (CHK3226) growing in sterilized vermiculite under growth chamber conditions and inoculated with the *M. ciceri* LMS-1 (pRKACC) demonstrated an increased nodule number (127%) and plant total biomass (125%) in comparison with plants inoculated with the wild-type strain (no ACC deaminase activity).

However, it is observed that under greenhouse conditions, and using nonsterilized soil, the nodulation performance and plant growth promotion abilities of the *M. ciceri* LMS-1 (pRKACC) strain were affected. Under growth chamber conditions and using sterilized vermiculite, the *M. ciceri* LMS-1 (pRKACC) was able to form an average of 100 nodules per plant, 45 days after inoculation in CHK3226 chickpea plants (Nascimento *et al.* 2012). When inoculated in the same chickpea plant cultivar (CHK3226) growing in soil under greenhouse conditions, the *M. ciceri* LMS-1 (pRKACC) was only able to form an average of 28 nodules per plant in the same time period. *Mesorhizobium ciceri* LMS-1 (pRKACC) plant growth promotion abilities were also decreased by 80% when comparing the two situations. This result may reflect the fact that all chickpea plants used in this study demonstrated stress symptoms, and the roots were found to present the typical aspect of the root rot disease. Necrosis of root tissues was observed, and *Fusarium* spp. was found associated with the diseased chickpea roots. Fungal agents like *Fusarium* and *Rhizoctonia* are known to be major participants in the chickpea root rot disease complex that affects crops worldwide and particularly in Mediterranean regions (Westerlund *et al.* 1974; Trapero-Casas and Jimenez-Diaz 1985; Hwang *et al.* 2003; Chang *et al.* 2004; Dubey *et al.* 2011). Plants suffering from root rot disease also show a decreased nodulation profile (Jones and Curnow 1986; Muthomi *et al.* 2007).

Here, it is shown that, despite the fungal disease pressure, different chickpea plant cultivars inoculated with the *M. ciceri* LMS-1 (pRKACC) produced more nodules and showed more developed and healthier root systems than the plants inoculated with the *M. ciceri* LMS-1 wild-type strain. Despite forming fewer and lighter nodules in the cultivar ELMO, the *M. ciceri* LMS-1 (pRKACC) was able to promote plant growth to the same extent as in cultivar CHK3226 (*c.* 45%).

The results obtained in this study suggest that, by expressing the *Ps. putida* UW4 *acdS* gene, *M. ciceri* LMS-1 (pRKACC) not only increased its nodulation abilities, but also enhanced its potential as a biocontrol agent. Similar results were obtained by Wang *et al.* (2000), where *Ps. fluorescens* CHA0 expressing an exogenous *Ps. putida* UW4 *acdS* gene showed an improved ability to protect cucumber against *Pythium* damping-off, and potato tubers against *Erwinia* soft rot. Hao *et al.* (2007) and Toklikishvili *et al.* (2010) demonstrated that ACC deaminase-producing bacteria, including *Ps. putida* UW4, can inhibit crown gall formation in tomato and castor bean plants infected by *Agrobacterium* strains. When the *Ps. putida* UW4 *acdS* gene was absent, this strain lost the ability to inhibit gall formation.

It is possible that the reduction in the amount of stress ethylene by ACC deaminase can lead to a reduction in

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the pathogen-induced disease symptoms (Robison *et al.* 2001a,b). In addition, by degrading ACC, bacteria producing ACC deaminase can gain an extra nitrogen and carbon source, thus becoming more competitive and better able to colonize roots (Ma *et al.* 2004). If so, these bacteria may be more capable of competing with the pathogen and thereby reducing its deleterious effects. Low levels of ethylene are known to be a participant in the plant's defence system against pathogen attack (Dong 1998), while high ethylene levels may contribute to the damage caused by a pathogen (Glick *et al.* 2007). By decreasing plant ethylene levels and concomitantly promoting plant growth, ACC deaminase-producing bacteria may also indirectly increase host plant defence systems.

Previous reports regarding the use of rhizobial ACC deaminase transformed strains show that the production of ACC deaminase does not affect the nitrogen fixation abilities of these strains (Ma *et al.* 2004; Nascimento *et al.* 2012)). These studies also indicate that the plant growth promotion abilities of the ACC deaminase transformed strains are because of the increased nodule formation and also by decreasing plant stress ethylene levels, as proposed by Glick *et al.* (2007).

While additional studies may be needed to understand the precise role of ACC deaminase in plant protection against pathogen attack, these results indicate that ACC deaminase production by *Mesorhizobium* may provide their cognate legumes with a variety of benefits. This is evident when examining the results obtained here, where the symbiotic performance of the *M. ciceri* LMS-1 (pRKACC) strain is significantly higher than the wild-type strain despite the presence of biotic and abiotic constraints to plant growth. Therefore, obtaining rhizobial strains with enhanced ACC deaminase activity is an important strategy for the development of inocula with agronomic importance.

Acknowledgements

This work has received funding from FCT (Fundação para a Ciência e a Tecnologia), co-financed by EU-FEDER (PTDC/BIO/80932/2006) and from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 247669. C. Brígido acknowledges a PhD fellowship (SFRH/BD/30680/2006) from FCT. We thank G. Mariano for technical assistance. We thank Prof. Isabel Brito (Universidade de Évora), Prof. Maria do Rosário Félix (Universidade de Évora) and M. Mário (Universidade de Évora) in the identification of the fungal pathogens. Chickpea seeds were generously provided by the Instituto Nacional de Recursos Biológicos, Elvas, Portugal.

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