



## Research article

Shedding light on the presymbiotic phase of *C. arietinum*

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## ABSTRACT

A complex network of symbiotic events between plants and bacteria allows the biosphere to exploit the atmospheric reservoir of molecular nitrogen. In seeds, a series of presymbiotic steps are already identified during imbibition, while interactions between the host and its symbiont begin in the early stages of germination. In the present study, a detailed analysis of the substances' complex delivered by *Cicer arietinum* seeds during imbibition showed a relevant presence of proteins and amino acids, which, except for cysteine, occurred with the whole proteinogenic pool. The imbibing solution was found to provide essential probiotic properties able to sustain the growth of the specific chickpea symbiont *Mesorhizobium ciceri*. Moreover, the imbibing solution, behaving as a complete medium, was found to be critically important for the symbiont's attraction, a fact this that is strictly related to the presence of the amino acids glycine, serine, and threonine. Here, the presence of these amino acids is constantly supported by the presence of the enzymes serine hydroxymethyltransferase and formyltetrahydrofolate deformylase, which are both involved in their biosynthesis. The reported findings are discussed in the light of the pivotal role played by the imbibing solution in attracting and sustaining symbiosis between the host and its symbiont.

## 1. Introduction

Nitrogen is the most abundant element in the atmosphere where is mainly present in its molecular form (N<sub>2</sub>) (Moore, 1977). However, its presence in the biosphere is strictly dependent on the conversion of molecular nitrogen (N<sub>2</sub>) into ammonia, nitrites and nitrates that are the most common forms in living organisms (Mylona et al., 1995; Lodwig et al., 2003).

This conversion takes place in the rhizosphere, and, in most cases, it is mediated by bacteria (Bond, 1941; Lodwig et al., 2003; Di et al., 2009). Nitrogen is a limiting factor for plant growth (Kolber et al., 1988; Kant et al., 2010) due to its very dynamic nature and being an important element of amino acids, nucleic acids, and photosynthesis-

related cofactors. Given the extreme stability of the N<sub>2</sub> triple-covalent bond and the lack of non-bacterial nitrogenases (Raymond et al., 2004), plants had to evolve a complex symbiotic system to break these molecules, relying on nitrogen-fixing bacteria (e.g. *Rhizobium* and *Mesorhizobium* spp.) to sustain their needs (Bond, 1941; Mylona et al., 1995; Poole and Allaway, 2000; Hichri et al., 2015). To establish the symbiosis, complex signaling pathways on the host induce important physiological and morphological changes mediated by the activation of early-nodulation genes in both the plant (e.g. ENOD genes – Geurts and Bisseling, 2002) and microbes (NOD genes - Debelle et al., 2001). Although late stages of pre-nodulation and molecular bases of symbiotic interactions have been extensively described (Caetanoanollés et al., 1988; Hartwig et al., 1990; Prell et al., 2009; Kamboj et al., 2010;

**Abbreviations:** FDR, False Discovery Rate; FTHFD, formyltetrahydrofolate deformylase; MS, Mass Spectrometry; MW, Molecular Weight; N<sub>2</sub>, molecular nitrogen; NCR, nodule-specific cysteine-rich peptides; PITC, phenylisothiocyanate; SDS-PAGE, denaturing Polyacrylamide Gel Electrophoresis; SHMT, serine hydroxymethyltransferase; TY, Tryptone, Yeast Extract; TLC, Thin Layer Chromatography; UHPLC, Ultra-High Performance Liquid Chromatography

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Hassan and Mathesius, 2012; Liu and Murray, 2016), little is known about the very early stages of this process. Initial events leading to symbiosis begin during seeds' imbibition and subsequent germination (Johansson and Bergman, 1992; Rasmussen et al., 1994; Kato et al., 1997; Ndakidemi and Dakota, 2003), implying that mechanisms of chemoattraction may already take place during the heterotrophic phase of the host's life cycle by a costly release of molecules in the soil. During the imbibition osmotic fluctuations induce a consistent uptake of water, resulting in a large exchange of small molecules with the environment. This event triggers the chemoattraction and the subsequent symbiotic interactions (Mandimba et al., 1986; Knight and Adams, 1996; Liu and Murray, 2016; Webb et al., 2016). Considering that most seeds have been found to release amino acids (Vančura and Hanzlíková, 1972; Lanfermeijer et al., 1989; Webb et al., 2016) and that nitrogen-fixing bacteria have to find nutrients in the soil (Mora et al., 2014), we have investigated the early trophic relationship between *Cicer arietinum* L. and its symbiont *Mesorhizobium ciceri* (Romdhane et al., 2007; Kim et al., 2014). The composition and the chemotactic ability of the imbibing solution were found to comply with an amino acids-mediated mechanism able to promote and establish symbiosis.

Most of what has been reported so far suggests that the attraction of bacteria during germination is actively controlled by roots through the production of flavonoids and other plants secondary metabolites (Segev et al., 2010; Liu and Murray, 2016), while only few reports describe the attraction mediated by amino acids (Webb et al. 2014, 2016). In particular, the production of amino acids by the host is reported to have an important role in modulating growth and migration of symbionts (Kato et al., 1997; Webb et al., 2016). In this work, we report a role played by amino acids and discuss its implications in symbionts attraction and symbiosis establishment.

## 2. Materials and methods

### 2.1. Isolation of the seeds' hydrophilic extract

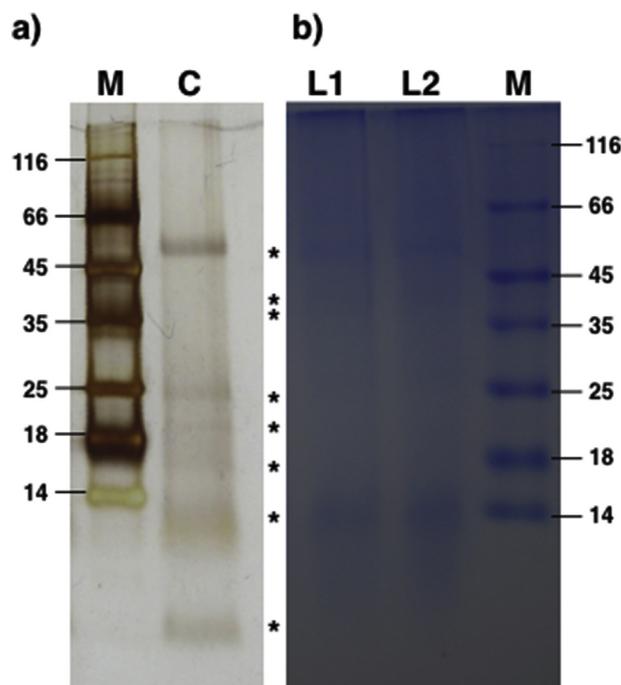
The soaking solution was obtained by incubating 200 g of dry chickpea seeds (*C. arietinum* L.), in 500 mL of spring water for 8 h at 4 °C in the dark. During incubation the imbibing solution color gradually turned into deep yellow, indicating the release of soluble compounds. After incubation, seeds were removed and the imbibing solution was filtered with a 0.22 µm cutoff nitrocellulose filter. The filtered solution was then either concentrated by using Vivaspin 20 ultrafiltration membranes with 5 kDa MW cutoff, or lyophilized. The concentrated solution and the powder were both analyzed by denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Mass Spectrometry (MS) analyses.

### 2.2. Polyacrylamide Gel Electrophoresis

The protein composition of the imbibing solution from *C. arietinum* seeds was analyzed with 10% SDS-PAGE. Electrophoresis was performed as described by Collu et al. (2017). Before loading, samples were diluted in a 3:1 ratio with loading buffer (Rotiload, Roth) and denatured at 100 °C for 5'. After electrophoretic separation, performed at 75 V for 3 h, gels from ultra-filtrated samples were silver-stained according to Piano et al. (2010), while gels from lyophilized samples were incubated overnight in a Coomassie staining solution (0.2% Coomassie Brilliant Blue G250, 10% acetic acid, 50% ethanol). After Coomassie staining, gels were destained for 1 h in a solution of 10% ethanol and 7% acetic acid in water. A commercial molecular marker (SM0431, Fermentas) was used for mass estimation.

### 2.3. Mass spectrometry

Mass spectrometry analysis (MS) was performed on lyophilized samples and the two main bands resolved by SDS-PAGE (Fig. 1, first and



**Fig. 1.** Analysis of the imbibing solution by gel electrophoresis. (a) Silver stained SDS-PAGE of the concentrated imbibing solution by ultrafiltration (C); (b) Coomassie Blue Stained SDS-PAGE of the lyophilized imbibing solution in a loading buffer 3:1 (L1) or 1:1 (L2). Lane M indicates the molecular marker in both gels; asterisks (\*) indicate the resolved bands. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fourth asterisks). These bands were excised and digested with trypsin before being processed as reported by Farci et al. (2016). Raw data were processed in Proteome Discoverer (v2.0, Thermo) using Mascot (version 2.5.1, Matrix Science) as the search engine against several plant species including *C. arietinum* from various Data Banks (Ensembl Plants DB, NCBI nr green plants DB and UniProt DB) with a list of common contaminants appended. For the search were allowed 1 missed cleavage, a Peptide Mass Tolerance of  $\pm 10$  ppm and a Fragment Mass Tolerance of  $\pm 0.5$  Da. Oxidation (methionine) and acetylation (N-term) were considered as variable modifications, while carbamidomethyl (cysteine) was fixed. A 1% False Discovery Rate (FDR) cut-off was applied in percolator. Protein's hits from Mascot were first filtered for a minimum peptide score of 20 and the top 10 'hits' were further selected. Both top 10 "hits" derived from each of the two analyzed bands were pooled and ranked according to their emPAI. As shown in Table 1, from the ranking all proteins with emPAI lower than 0.2 were excluded. The relative protein abundance was also ranked by defining the ratio ( $i$ ) between the emPAI and the mass (kDa) of each protein. Finally, as shown in Table 1, each  $i$  value obtained was clustered according to three relative groups defined as follows:  $+ = i \leq 0.01$ ;  $++ = 0.01 \leq i \leq 0.1$ ;  $+++ = i > 0.1$ .

### 2.4. Thin Layer Chromatography

Thin Layer Chromatography (TLC) plates (200 µm tick on aluminum foil, Sigma) were spotted with 10 µL of sample by gradually loading 1 µL at a time and drying it with a hairdryer. After spotting, TLC was run for 20 min with a mobile phase composed of 60% ethanol, 20% acetic acid, and 20% distilled water. The plate was then dried and sprayed with a solution of acetone containing 0.1% ninhydrin (w/v) and 0.5% acetic acid (v/v). Finally, the plate was warmed until ninhydrin reaction occurred (Niederwieser, 1972).

Table 1

**Mass spectrometry analysis.** The imbibing solution characterized by MS analysis revealed a typical pattern of proteins. Besides SHMT and FTHFD, a set of other enzymes involved in seeds' protection, imbibition, germination, symbiosis, and chemotaxis was found.

Identified Protein	Gene	Function	Mass (kDa)	emPAI	Protein presence <sup>a</sup>
Formyltetrahydrofolate deformylase	KZV07010	one carbon metabolism	15.5	3.52	+++
Serine hydroxymethyltransferase	KZV06781	one carbon metabolism	25.4	2.66	+++
DING protein	AAU95561	protection against bacteria	16.0	1.40	++
Heme-binding protein 2-like	XP_004511057	chemotaxis and/or prenodulation mechanisms	24.9	1.13	++
Pectinesterase 2-like	XP_004514850	imbibition, expansion	56.5	1.12	++
Alpha-galactosidase-like	XP_004495485	imbibition, expansion	44.1	1.11	++
Putative PTS protein	Q9M3Z3_CICAR	phosphotransferase	45.1	0.87	++
Chitinase	A0A076KWH5_CICAR	protection against fungi and arthropods	39.4	0.82	++
Heparanase-like protein 3	XP_004488517	imbibition, expansion	59.7	0.48	+
Uncharacterized protein LOC1011500557	XP_004504132	unknown	73.0	0.47	+
PI-PLC x domain-containing protein At5g67130 like	XP_004511919	protection and/or prenodulation mechanisms	47.2	0.35	+
Polygalacturonase-like	XP_004513766	imbibition, expansion	50.1	0.33	+
Alpha-galactosidase 1	XP_004502389	imbibition, expansion	73.4	0.29	+
Acidic mammalian chitinase-like	XP_004501097	protection against fungi and arthropods	39.3	0.27	+
CO <sub>2</sub> -response secreted protease-like	XP_004500924	germination/root stomata formation	83.3	0.25	+
Alpha-galactosidase-like	XP_004495485	imbibition, expansion	44.1	0.24	+
Polygalacturonase-like	XP_004513766	imbibition, expansion	50.1	0.21	+

<sup>a</sup> The relative protein abundance was ranked by the ratio (i) between the emPAI and the mass (kDa) of each protein. Finally, the value for each protein was clustered according to three relative groups defined as follows: + =  $i \leq 0.01$ ; ++ =  $0.01 \leq i \leq 0.1$ ; +++ =  $i > 0.1$ .

## 2.5. Ultra-high performance liquid chromatography

UHPLC was performed on an Acquity UPLC system (Waters) with a BEH C18 column (2.1 mm × 10 cm) at a temperature of 48 °C and a flow rate of 325 µL/min. After equilibration, injection (5 µL) and baseline stabilization in 100% Buffer A (140 mM Na Acetate pH 6.05, 6% Acetonitrile and 0.05% trimethylamine), the mobile phase was switched to 100% Buffer B (60% Acetonitrile). The whole run was monitored at a wavelength of 254 nm. The presence of amino acids and their respective amount in the imbibing solution was determined after a step of filtration with 0.22 µm nitrocellulose filters. Analyses were performed through the Waters Empower 2 Chromatography Software, using as internal standard the Amino Acid Standard H (Pierce) derivatized with phenylisothiocyanate (PITC, Sigma) (Szkudzińska et al., 2017).

## 2.6. Growth of *Mesorhizobium ciceri*

Tests of growth and chemotaxis were performed on *M. ciceri* strains LMS-1 and CC1192 (Nascimento et al., 2012; Haskett et al., 2016). The growth of these two strains, repeated in triplicate, was performed in 1.5% agar plates of both sterilized imbibing solution and TY (Tryptone 0.5%, Yeast Extract 0.3%, 6 mM CaCl<sub>2</sub>). A 10 µL volume of cultures grown overnight on TY (O.D.<sub>590 nm</sub> = 0.5) was spotted according to a series of four dilutions equivalent to 10, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> times, respectively.

Chemotaxis assays were performed by spotting 10 µL of cultures grown overnight on TY (O.D.<sub>590 nm</sub> = 0.5) on either semisolid-soaking solution (0.2% Agar) as a control or semisolid-soaking solution supplemented with 1 or 5 mM of a specific amino acid (either serine, glycine, or threonine). Each growth condition, including the control, was repeated in triplicates at 7 different time points (0, 24, 72, 96, 120, 240, 288 h). The quality of the data was estimated by calculating mean and standard deviation from a set of three independent measurements (experiments) at each time point. The circular expansion of the culture versus time was used as an indicator of the effect in absence (control) or presence of a specific amino acid.

## 3. Results

### 3.1. The imbibing solution consists of a typical pattern of proteins

During water soaking, *C. arietinum* seeds release specific

components that turn the soaking solution into a yellow color. This turning in colour suggests the presence of yellow metabolites, such as flavones, folates, luteolin, cyclic β-1,2-glucan, and others, which are already known to be characteristic components of chickpea seeds (Segev et al., 2010; Liu and Murray, 2016) and promoters of symbiotic interactions during late stages of pre-nodulation (Halverson and Stacey, 1986; Hassan and Mathesius, 2012).

The soaking solution, hereinafter referred to imbibing solution, was clarified and subjected to a step of ultrafiltration to concentrate possible proteinaceous components. This procedure washed, separated, and, finally, concentrated any possible macromolecular fraction heavier than 5 kDa. Alternatively, clarified samples were subjected to lyophilization allowing to reach higher concentrations. To identify the presence of proteins, samples obtained in both ways were subjected to SDS-PAGE analysis (Fig. 1). The lyophilization not only allowed to work with higher amounts of proteins, but, alternatively to ultrafiltration, also avoided the loss of small peptides. When resuspended in a small volume of denaturing solution, lyophilized samples showed a pattern of bands similar to the one of the samples concentrated by ultrafiltration (Fig. 1a), but this time visible under the less sensitive and MS-compatible Coomassie staining (Fig. 1b). When analyzed by MS, either on the specific bands separated by SDS-PAGE or directly on the lyophilized powder, the identity of a specific pattern of proteins was defined. A summary of the main proteome associated with these samples is reported in Table 1. As expected, identified proteins were enzymes whose activity could be ascribed to seeds' protection, imbibition, germination, symbiosis, and chemotaxis.

### 3.2. The imbibing solution consists of an almost complete set of proteinogenic amino acids where only cysteine is missing

Two of the main proteins identified by the MS analysis, the serine hydroxymethyltransferase (SHMT) and the formyltetrahydrofolate deformylase (FTHFD), are directly involved in the metabolisms of amino acids and one-carbon units. Both these enzymes are required during the pre-nodulation and nodulation phases (Caetanoanollés et al., 1988; Hartwig et al., 1990; Kamboj et al., 2010; Hassan and Mathesius, 2012; Liu and Murray, 2016). Their presence suggests a possible role of amino acids in promoting the interaction between *C. arietinum* and its specific endosymbionts. Considering the pivotal role that SHMT plays in amino acids' metabolism and, specifically, in the metabolism of serine, threonine, and glycine (Dunn, 2014), we have investigated the possible presence and relative amounts of these amino acids in the imbibing

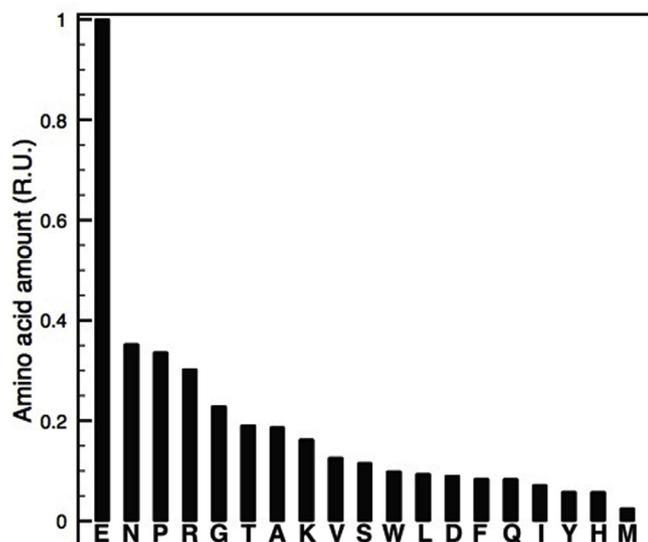


Fig. 2. Amino Acid composition of imbibing solution analyzed by UHPLC. Amounts of amino acids in the imbibing solution are expressed in relative units (R.U.). Except for cysteine, which was not identified in the analyses, the whole set of proteinogenic amino acids is present.

solution. A preliminary TLC of the imbibing fluid confirmed the presence of amino acids in the sample (Supplementary Fig. 1), and a more detailed analysis by UHPLC revealed the presence of the whole proteinogenic amino acid set, except for cysteine (Fig. 2). The most abundant amino acid was glutamic acid, and among others, except for serine present in lower amounts, consistent amounts of potential SHMT substrates, such as threonine and glycine were also found (Fig. 2). These amino acids are reported to be essential in the phases of pre-nodulation and nodulation (Roszbach and Hennecke, 1991; Barnett et al., 2001; Brencic and Winans, 2005; Dunn, 2014).

### 3.3. The imbibing solution is a complete medium for *M. ciceri* strains

Considering the completeness in amino acids composition, we checked whether the imbibing solution could be used to grow two different strains of *M. ciceri*, LMS-1 and CC1192, known to be the specific symbionts of *C. arietinum* (Nascimento et al., 2012; Haskett et al., 2016). The growth in this medium was then compared with the one in the standard TY medium, which is the election media for this bacterium. As shown in Fig. 3, the growth of both strains in the solidified imbibing solution at four different dilutions takes place with comparable levels (if not superior) to the one in TY.

### 3.4. The imbibing solution of *C. arietinum* induces chemotaxis in *M. ciceri*

Considering the role of amino acids in rhizobiology and, in particular, glycine, threonine, and serine as SHMT substrates and products (Lodwig et al., 2003; Calderón-Flores et al., 2005; Tegeder, 2014), we have tested the possible involvement of these amino acids as chemoattractants. Standard chemotaxis experiments were performed on plates containing a semi-solid imbibing solution supplemented with either glycine, threonine or serine, by spotting in the center of the plate 10  $\mu$ L of overnight grown cultures. After incubation, cultures resulted in a concentric growth with visible fronts of expansion. Taking into account that media tend to dry over time, thus affecting the migration rate, swarms of the two strains grown under different conditions were completed in 5 days and monitored for the following 7 days. Chemotaxis was estimated from the degree of cultures' expansion and fronts' formation (Fig. 4). This analysis showed the qualitative influence of a specific amino acid supplementation on the growth of the cultures. In

particular, both strains appeared significantly inhibited by glycine with a more pronounced effect on the CC1192 strain (Fig. 4). This strain resulted also more sensitive to the stimulant effect of serine or threonine than the LMS-1, which, on the contrary, has a growth comparable to the control (Fig. 4).

The effect of glycine on the LMS-1 strain could be estimated by fitting into a curve the time needed by each expansion front to reach its position at a given glycine concentration (Fig. 5a). Furthermore, the first derivative of the fitting curves in the interval of time required for cultures swarms, allowed to estimate the migration speed under different conditions (Fig. 5b). Similar analyses were performed also on the CC1292 strain for which a stronger concentration-dependent and stimulant effect of serine (Fig. 6) and threonine (Fig. 7) was observed.

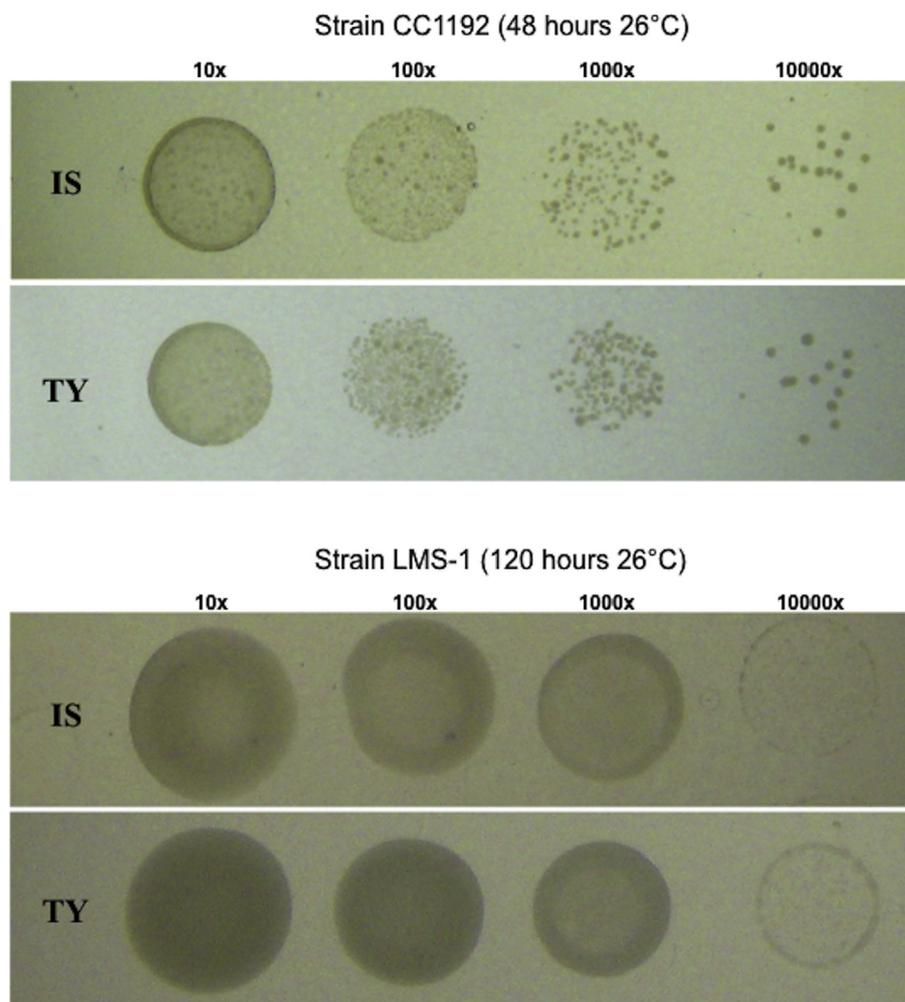
## 4. Discussion

The fixation of atmospheric molecular nitrogen ( $N_2$ ) by bacteria represents the only access of nitrogen into the biosphere (Newton, 2000; Lodwig et al., 2003). This process and the subsequent incorporation of nitrogen into organic molecules are essential for organisms, and for these reasons plants rely on symbiotic relationships with nitrogen-fixing bacteria to maximize their nitrogen uptake (Vance, 2000; Lodwig et al., 2003; Okumoto and Pilot, 2011). Although the general dynamics of this symbiosis are well characterized (Caetanoanollés et al., 1988; Hartwig et al., 1990; Kamboj et al., 2010; Hassan and Mathesius, 2012; Liu and Murray, 2016), a detailed description of early chemotactic events triggering symbiosis in seeds is still missing.

In this study, the composition and the biological activity of the imbibing solution from *C. arietinum* seeds were characterized to understand whether it may lead the early symbiotic events between *C. arietinum* and its symbiont *M. ciceri*. In previous works it was suggested that early steps of symbiosis may already start during imbibition and germination of seeds (Johansson and Bergman, 1992; Rasmussen et al., 1994; Kato et al., 1997; Ndakidemi and Dakota, 2003; Webb et al., 2016). Accordingly, in the present study the imbibing solution was found to contain a significant amount of proteinogenic amino acids (Fig. 2) delivered by seeds after 6–8 h of imbibition and shown to play a potential role in sustaining symbiosis by feeding and attracting specific symbionts (Figs. 3–7).

The role of amino acids in inducing nodulation and their consequent role in nitrogen fixation was already reported for several species (Vančura and Hanzlíková, 1972; Johansson and Bergman, 1992), including the *Leguminosae* family (Kato et al., 1997; Lanfermeijer et al., 1989; Lodwig et al., 2003; Prell et al., 2009; Okumoto and Pilot, 2011; Moe, 2013) and, in particular, chickpea (Esfahani et al., 2014). Moreover, the secretion of branched chained amino acids is known to regulate the symbiosis between *Leguminosae* and rhizobia (Lodwig et al., 2003), while in the same group of plants glutamine and asparagine are critically important for regulating nodulation events (Mohd-Radzman et al., 2013). Our analysis revealed that the imbibing solution, which was studied through several techniques (TLC, UHPLC, SDS-PAGE, and MS), consists of a complete set of proteinogenic amino acids where only cysteine is missing (Fig. 2). Moreover, the protein pool of the imbibing solution (Table 1) was found to be highly represented by SHMT and FTHFD. Both these enzymes are strictly involved in the metabolism of several amino acids and, in particular, of glycine, serine, and threonine (Dunn, 2014), which are also well represented in the imbibing solution (Fig. 2).

Among the whole proteinogenic pool, glycine, threonine, and serine were studied because of their significance as components of the imbibing solution and because of their role as SHMT substrates. The effect of glycine on the growth was found to be inhibitory with a degree dependent on the symbiont strain tested. In the CC1192 strain even at low concentrations, the glycine exerted its bacteriostatic properties, while at the same concentrations the LMS-1 strain growth was subject to a



**Fig. 3. Bacterial growth assays.** Growth tests on the two strains of *M. ciceri*, CC1192 and LMS-1, were performed in agar plates of sterilized imbibing solution (IS) and TY medium (TY). A series of four dilutions of the starting culture, equivalent to 10,  $10^2$ ,  $10^3$ , and  $10^4$  times, was used. The growth in IS takes place with levels comparable to the one in TY for both strains.

concentration-dependent effect (Figs. 4 and 5). According to these observations, glycine was previously reported to play an important role in chemotactic mechanisms of *Rhizobia*, especially with the inhibition of motility caused by its excess (Sherwood, 1972; Vanderlinde et al., 2011).

Contrary to glycine, the amino acids serine and threonine, also SHMT substrates, were found to enhance growth and migration. As in the case of glycine, this effect was much more pronounced in the cc1192 strain (Figs. 4, 6 and 7).

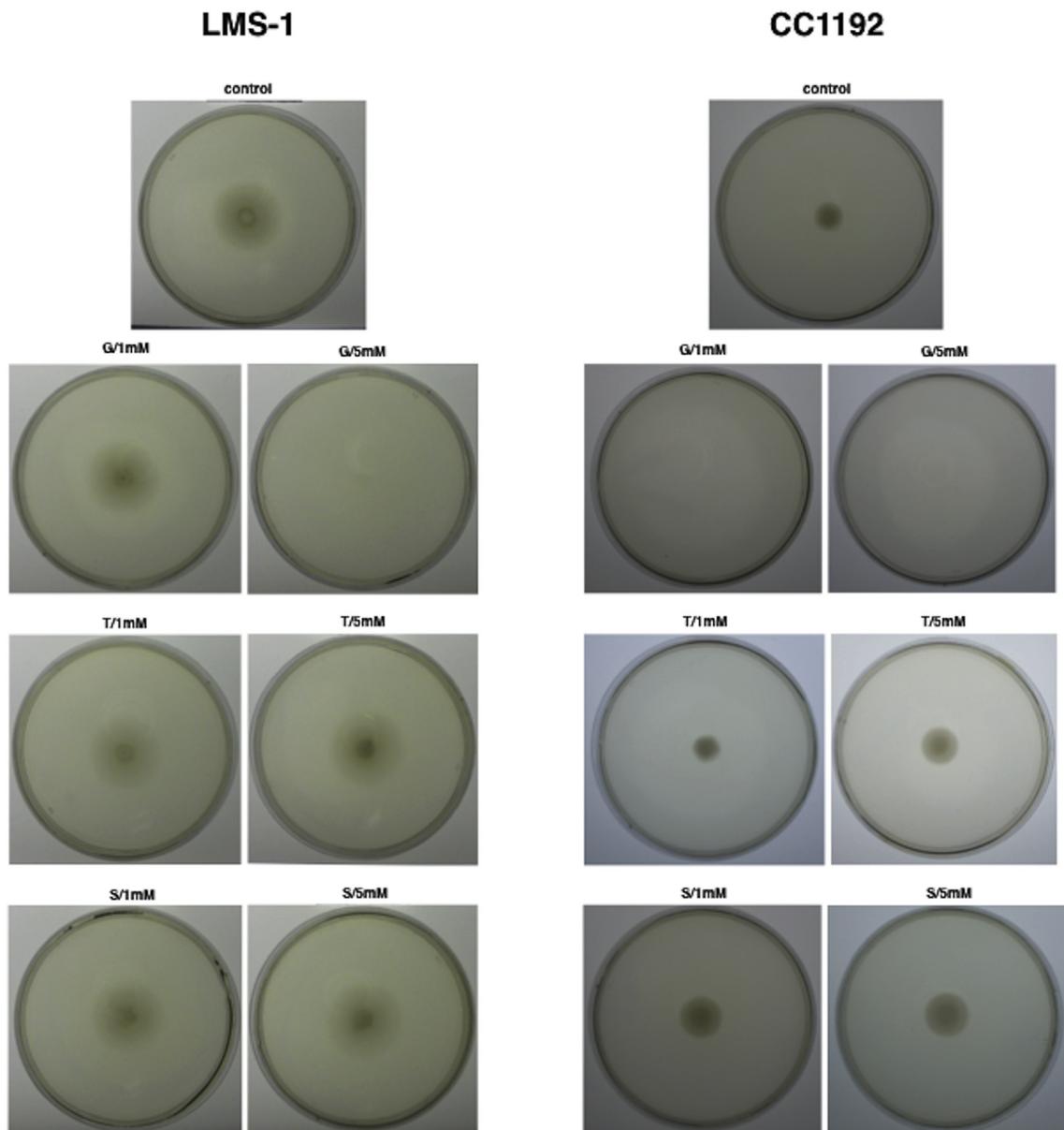
The shown results support a model where amino acids produced by the host can modulate growth and migration of symbionts, as already hinted in other reports (Kato et al., 1997; Webb et al., 2016).

The complementary role of glycine, serine, and threonine become particularly important when taking into account their relevant presence in the imbibing solution together with the enzyme SHMT, of which they are typical substrates and modulators. The co-presence of these factors suggests an extracellular mechanism driven by diffusion where SHMT and its substrates are released to the environment. In this mechanism amino acids and SHMT would create a dynamic system able to drive the symbiont in proximity of the seed, allowing the short distance mechanisms of symbiosis to take place. In particular, starting from the seed, a diffusion gradient of these amino acids may well be essential in defining a critical distance on which symbionts are attracted. This effect would take place driven by the bacteriostatic properties of glycine and the proliferative properties of serine and threonine, creating a system

able to modulate migration, speed, and growth. Into these dynamics, the role of SHMT would be essential in balancing the interconversion between the three amino acids only on the base of the substrates equilibrium and availability. To regulate the levels of tetrahydrofolate, this mechanism would need the enzyme FTHFD, also found in our MS analysis (Table 1). On its side, the FTHFD would require the availability of folates also typical for chickpea seeds and its imbibing solution (Segev et al., 2010; Liu and Murray, 2016).

Among other amino acids, glutamic acid was identified as the most abundant in the imbibing solution, while cysteine was not detected (Fig. 2). These observations are consistent with glutamic acid playing a primary role in promoting the growth of nitrogen-fixing bacteria (Hosie et al., 2002) and inhibiting primary roots' development (Kim et al., 2010). On the other hand, the absence of cysteine might be aimed at avoiding a conflict between its strictly bacteriostatic properties (Tiricz et al., 2013; Mikuláss et al., 2016) and the modulable effect of glycine on proliferation (Fig. 4), as well as to cysteine's involvement in the synthesis of nodule-specific cysteine-rich peptides (NCR). These peptides are used by the seed as antimicrobial compounds (Tiricz et al., 2013; Mikuláss et al., 2016), but are also essential during bacteroid and symbiosome development to induce membrane permeabilization (Wang et al., 2010; Haag et al., 2011; Marshall et al., 2011).

The present study provides a direct evidence on the pivotal role played by amino acids in the preparatory events preceding nodulation. Amino acids are shown to sustain symbiont attraction and growth,



**Fig. 4.** Chemotaxis assay on the two *M. ciceri* strains CC1192 and LMS-1. Chemotaxis experiments were performed on semi-solid imbibing solution (0.2% agar) either in absence of additional amino acids (control), or in presence of glycine (either, 1 or 5 mM), or in presence of threonine (either, 1 or 5 mM), or in presence of serine (either, 1 or 5 mM). The specific effect on the growth induced by the excess of a supplemented amino acid in the medium is shown. Both strains appeared significantly inhibited by glycine.

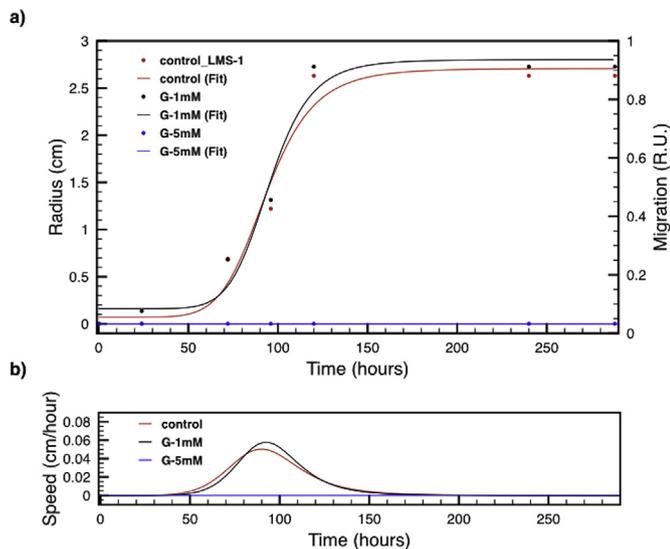
allowing, through the subsequent nodulation processes, a long-lasting symbiotic relationship that is fundamental for the whole biosphere. A detailed understanding of composition and mechanisms through which the imbibing fluid mediates the early events of symbiosis and nodulation are essential for implementing the current agricultural practices. The use of inoculants in agriculture became an essential requirement on crops, especially in poor soils and/or environments where water availability is limited. Knowing the mechanisms underlying the composition of the imbibing fluids and their roles will not only be essential for a rational sustaining of symbiosis, but will also help in enhancing symbionts survival. This will result in fine optimizations of future inoculants and will promote a more rational preparation of the seeds to the post-sowing events. Future perspectives are aiming at a detailed study of the germination proteome which, by complementing the present findings, might have a huge potential in crop production under a more sustainable agriculture.

## 5. Conflicts of interest

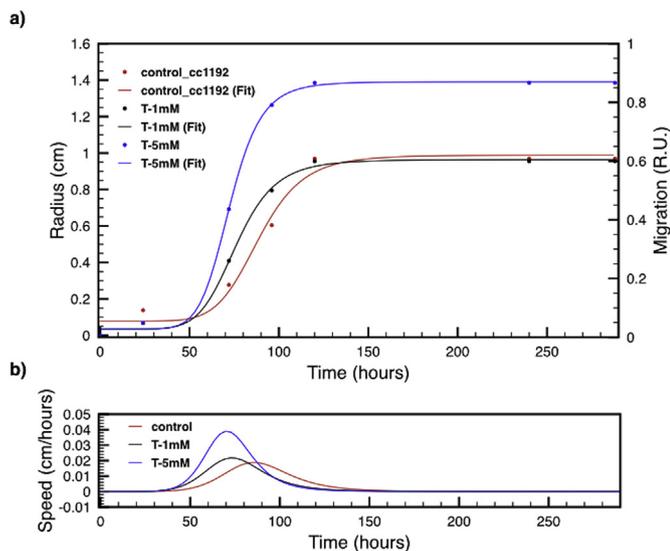
The authors declare no competing financial interest.

## Authors contributions

DP conceived of the study, participated in its design and coordination, carried out the chickpea and bacterial preparation, performed the biochemical and bioinformatic studies and drafted the manuscript. CS conceived of the study, participated in its design and coordination and drafted the manuscript. JK performed the mass spectrometry analysis and helped in drafting the manuscript. DF participated in the chickpea and bacterial preparation, performed the biochemical and bioinformatic studies and drafted the manuscript. RM and FP helped in the biochemical studies and in drafting the manuscript. HMK helped in preparation the final version of the manuscript.



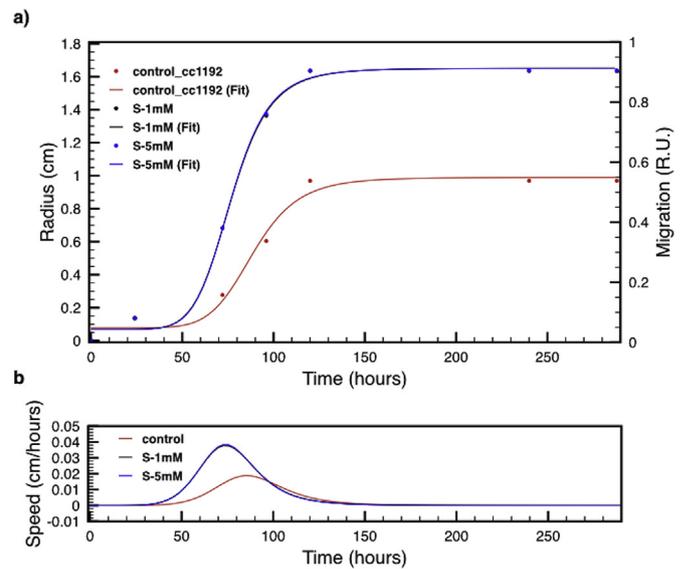
**Fig. 5.** Glycine effect estimated on the LMS-1 strain. (a) Curve fitting for the position/time of each expansion pattern either in absence (control) or in presence of 1 mM and 5 mM of glycine (G-1mM and G-5mM respectively); (b) First derivative of the fitting curve for each condition (control, G-1mM, G-5mM) in the interval of time needed for cultures swarms (see details in the picture legend). Growth condition and quality of the data were estimated by calculating mean and standard deviation from a set of three independent measurements (experiments) from each time point.



**Fig. 6.** Threonine effect estimated on the CC1192 strain. (a) Curve fitting for the position/time of each expansion pattern either in absence (control) or in presence of 1 mM and 5 mM threonine (T-1mM or T-5mM); (b) First derivative of the curve for each condition (control, T-1mM, T-5mM) in the interval of time needed for cultures swarms (see details in the picture legend). Growth condition and quality of the data were estimated by calculating mean and standard deviation from a set of three independent measurements (experiments) from each time point.

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**Fig. 7.** Serine effect estimated on the CC1192 strain. (a) Curve fitting for the position/time of each expansion pattern either in absence (control) or in presence of 1 mM and 5 mM of serine (S-1mM or S-5mM); (b) First derivative of the curve for each condition (control, S-1mM, S-5mM) in the interval of time needed for cultures swarms (see details in the picture legend). Growth condition and quality of the data were estimated by calculating mean and standard deviation from a set of three independent measurements (experiments) from each time point.

the *M. ciceri* strain LMS-1, and to Dr J. Terpolilli at the Centre for Rhizobium Studies, Murdoch University, Perth (Australia) for providing the *M. ciceri* strain CC1192. This work was done in the frame of: Projekt nr POIR.01.01.01-20-1911/15 „Triffid – produkt przyszłości Klastra Obróbki Metali (KKK). White Hill synergia kooperacji w obszarze B + R” realizowany w ramach Programu Operacyjnego Inteligentny Rozwój; “Centrum Badawczo-Rozwojowe – Misja White Hill – Podlaska Strategia Sukcesu B + R + I” Projekt współfinansowany jest ze środków Unii Europejskiej z Europejskiego Funduszu Rozwoju Regionalnego.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.09.014>.

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