



Nodule-associated microbiome diversity in wild populations of *Sulla coronaria* reveals clues on the relative importance of culturable rhizobial symbionts and co-infecting endophytes



Rosella Muresu^a, Andrea Porceddu^b, Leonardo Sulas^a, Andrea Squartini^{c,*}

^a Institute for the Animal Production System in Mediterranean Environment-National Research Council (ISPAAM CNR), Traversa La Crucca 3, 07100, Sassari, Italy

^b Dipartimento di Agraria, University of Sassari, Viale Italia 1, 07100, Sassari, Italy

^c Department of Agronomy, Food, Natural Resources, Animals and Environment, DAFNAE, University of Padova, Viale dell'Università 16, Legnaro, Padova, Italy

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ABSTRACT

The culturable bacteria from root nodules of *Sulla coronaria* growing in spontaneous conditions in Sardinia were characterized. This plant's peculiarity is to represent a legume still found in both wild and cropped statuses. We tested whether culturable bacteria would differ from those commonly isolated from its field-cropped varieties, to date exclusively represented by *Rhizobium sulae*. 63 isolates from 60 surface-sterilized nodules were analyzed by ARDRA and 16S rDNA sequencing. The official nitrogen-fixing symbiont *Rhizobium sulae* was found only in 25 nodules out of 60. The remaining nodules did not yield culturable rhizobia but a number of different endophytic genera including *Pseudomonas* sp. (17 nodules), *Microbacterium* sp. (15 nodules), *Pantoea agglomerans* (5 nodules). The situation appears therefore a hybrid between what is commonly observed in other Mediterranean legumes occurring only in wild status (featuring non-culturable rhizobia and arrays of culturable endophytes within nodules), as opposed to cropped legumes (endowed with fully culturable rhizobia and minimal endophytic occurrence). These findings, within a species bridging the ecology between native and cropped conditions, suggest insights on the relative importance of endophytic co-occupancy vs. true N-fixing symbiont culturability within nodules. The latter trait thus appears to accompany the domestication path of plants with a main trade-off of renouncing to interactions with a diversity of endophytic co-invaders; the relationships with those being critical in the non-cropped status. In fact, endophytes are known to promote plant growth in harsh conditions, which can be particularly stressful in the Mediterranean due to drought, highly calcareous soils, and pathogens outbreaks.

1. Introduction

Sulla (*Sulla coronaria* [L.] Medik.; syn. *Hedysarum coronarium* L.) is a short-duration perennial pasture legume expressing peculiar environmental tolerance, in particular, towards stressful conditions as soil alkalinity, salinity and drought. The qualities of its forage, featuring a high protein content, have made *sulla* widely cultivated throughout the Mediterranean basin where it is extensively used as forage crop for grazing, hay or silage production (Sulas et al., 2017). In a previous study (Squartini et al., 2002), we described the novel species, *Rhizobium sulae*, that specifically induces nodulation and symbiotic N fixation in *sulla* and it is regularly found in nodules of cropped plants throughout the Mediterranean irrespective of prior seed inoculation (Squartini et al., 2002; Muresu et al., 2005). Characterization of several bacterial strains nodulating this legume (Muresu et al., 2005; Struffi et al., 1988)

made us select *R. sulae* isolate IS123^T as type strain. The plant occurs as a cropped legume but it is still found as a spontaneous component of native flora in Mediterranean habitats (Fig. 1). The genome sequences of three strains of *R. sulae* have been published, including strain WSM1592 (Yates et al., 2015) isolated from cropped *sulla* on the island of Sardinia (Italy), the type strain IS123 (Sablok et al., 2017) isolated in southern Spain, and strain HCNT1 (de Diego-Diaz et al., 2018) isolated from root nodules of *sulla* in the highly calcareous pliocenic clays near Volterra (Tuscany, Italy). The microsymbionts of *sulla* are reported to express a high level of host specificity for nodulation (Kishinevsky et al., 2003). The introduction of *sulla* into new cultivation areas could require seed inoculation with *Rhizobium sulae* (Casella et al., 1894; Sulas et al., 2017). The use of rhizobial biofertilizers (Meng et al., 2015; Zahran, 2001) has relatively increased compared to the previous decades, with associated benefits of high N-fixation capabilities, enhanced

* Corresponding author.

E-mail address: squart@unipd.it (A. Squartini).

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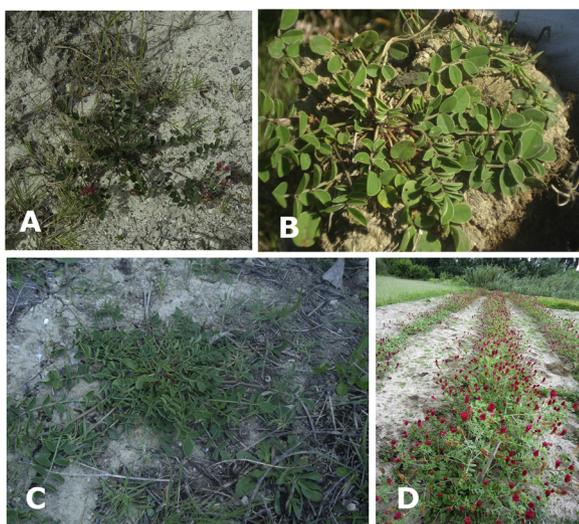


Fig. 1. *Sulla coronaria* occurring in spontaneous conditions (A,B,C) and as a field crop (D) in Sardinia.

productivity and improved soil fertility (Sulas et al., 2017).

Unlike the typical cropped varieties of Fabaceae, it has been reported that wild legume nodules may be colonized internally by several non-nodulating bacteria (Benhizia et al., 2004; de Lajudie et al., 1999; Muresu et al., 2008). It can be expected that cohabitation of nodules by rhizobia and other bacteria occurs frequently and mostly affects nodule physiology (Liu et al., 2017; Mrabet et al., 2006). It has been reported that *Enterobacter* spp., *Pseudomonas* spp., and *Bacillus* spp. are the most abundant endophytic bacteria in legumes (Peix et al., 2015). Other studies showed that such endophytes may co-exist in nodules with rhizobia (Ibanez et al., 2017; Lei et al., 2008; Muresu et al., 2008; Pandya et al., 2013). As the presence of rhizobia within those nodules could often be demonstrated only by PCR-amplification (Muresu et al.,

2008) we hypothesized that the limited culturability could be related to injury from oxidative stress caused by disruption of plant tissues during isolation (Muresu et al., 2013). Overall, these investigations showed that nodules induced by rhizobia may offer a habitat for a wide variety of bacteria that can accelerate seedling emergence, enhance plant growth under abiotic stress and protect plants from bacterial and fungal pathogen infections (Lata et al., 2018; de Almeida et al., 2018). Some of these endophytic bacteria have proven directly beneficial to their host legumes, being proficient in plant growth enhancement by producing plant hormones, solubilizing phosphate or being capable themselves of fixing atmospheric N₂ (Peix et al., 2015). Recently, a wide biodiversity among endophytic bacteria collected from nodules of other related species, namely *Sulla pallida* and *Sulla capitata*, was demonstrated in different Algerian and Tunisian soils. Members of the genera *Sinorhizobium*, *Neorhizobium*, *Rhizobium*, *Phyllobacterium*, *Arthrobacter*, *Variovorax* and *Pseudomonas* were isolated from nodules (Beghalem et al., 2017). As regards *Sulla coronaria*, a molecular analysis of strains isolated from nodules of a cropped variety, when grown in heavy metal-contaminated soil samples, showed a large diversity represented essentially by *Rhizobium* sp., *R. leguminosarum*, *Agrobacterium* sp., *Pseudomonas* sp., and *Rhizobium sullae* (Chiboub et al., 2016). In a different study on *Sulla capitata*, *S. carnososa*, *S. coronaria* growing in the wild in Tunisia, upon analyzing more than 50 nodules. Chriki-Adeeb and Chriki (2015) recovered only four culturable isolates, all of different nature (supposedly endophytic), which confirmed other studies and indicated that more than 92% of the nodules of these Mediterranean species collected in spontaneous conditions could not yield any colony.

The study of endophytic bacteria is also necessary for safety reasons in the food chain. As highlighted in a previous work (Muresu et al., 2010), bacteria not proficient in nitrogen fixing symbiosis belonging to taxa known as human pathogens were proven able to invade root nodules of wild legumes including the genus *Sulla*, and to multiply in place of the natural rhizobium symbionts. Strains isolated from those root nodules exhibited also effective phenotypic traits in the tests commonly used to assess virulence potential towards human cells.

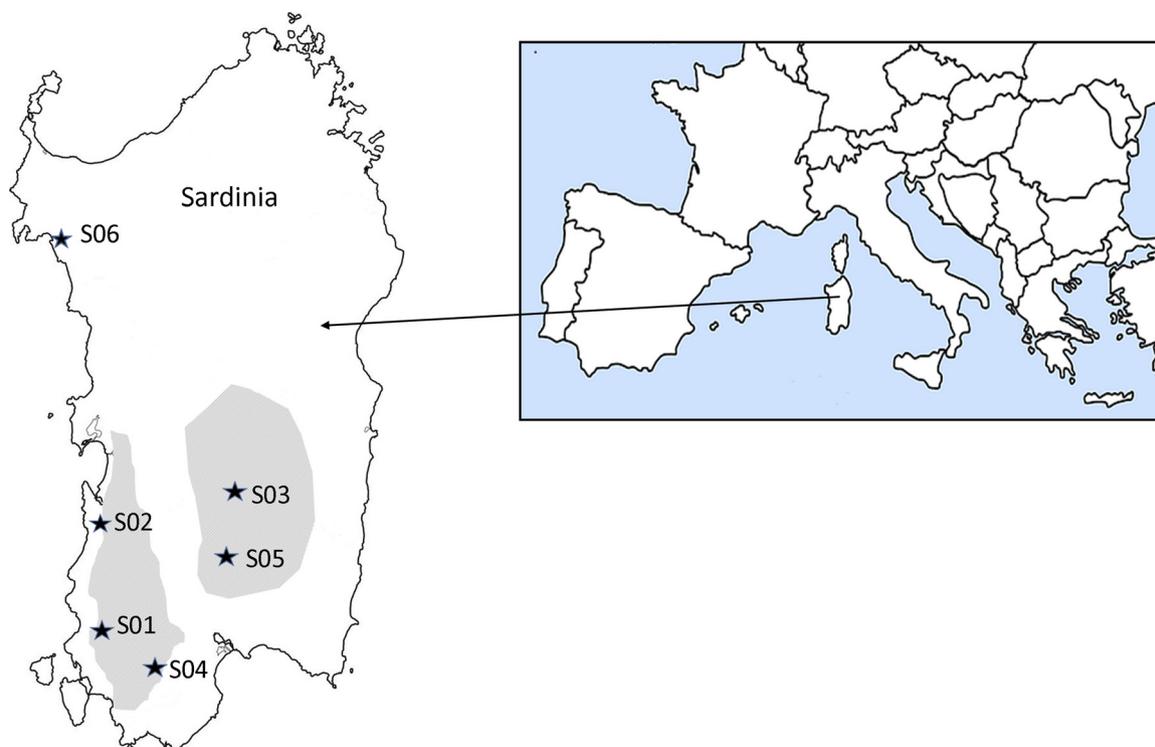


Fig. 2. Map of the Sardinia island showing the sampling sites of the spontaneous *Sulla coronaria* plants analyzed within this project. Shaded areas indicate the ranges in which botanical accounts from 1906 reported the distribution of this plant as wild occurrence (details cited in ref. 34).

Table 1
List of collection and surveying sites of *Sulla coronaria* germplasm in Sardinia.

Site	Code	FAO Soil Classification*	Altitude (m a.s.l.)	Latitude	Longitude
Bacu Abis	S01	Lithic leptosols	275	40° 13' 13"	8° 28' 03"
Torre dei Corsari	S02	Eutric and lithic leptosols	52	39° 40' 14"	8° 27' 47"
Ales	S03	Eutric, lithic leptosols	319	39° 48' 02"	8° 55' 08"
Nuxis	S04	Eutric, dystric and lithic leptosols	198	39° 13' 46"	8° 44' 55"
Furtei	S05	Eutric and lithic leptosols	22	39° 33' 27"	8° 59' 29"
La Speranza	S06	Eutric and lithic leptosols, Rock outcrop	9	40° 29' 50"	8° 22' 10"

* According to FAO (2006) Soil classification manual.

For all the above reasons, encompassing the evolutionary ecology aspect as well as the applied side, in this work we analyzed, through molecular genetics analysis, the microbial inhabitants collected from nodules of wild *sulla* populations throughout a transect spanning across various geographical areas of Sardinia. The choice was backed up by the notion that *sulla* had been widely present in the past as a spontaneous species in this island (Sulas, 2006).

2. Materials and methods

An extensive search was carried out to individuate plants of interest within Sardinia leading to the identification of six sites (Fig. 2, Table 1) in which wild specimens of *sulla* were found. The number of nodules collected from each site is reported in Table 2.

Isolation of bacteria from surface-sterilized nodules, followed by purity controls (streaking of surface-sterilized nodules on agarized nutrient media before nodule crushing), DNA extraction, amplified ribosomal DNA restriction analysis (ARDRA), 16S rDNA PCR amplification and sequencing were performed as previously described (Muresu et al., 2008). The 16S amplified ribosomal DNA restriction analysis was run, using as control the reference strain *Rhizobium sllae* IS123 in order to compare the restriction pattern and sort the isolates. *Rhizobium sllae* identification by the use of ISRh1 insertion element-specific internal primers was carried out as reported (Muresu et al., 2005).

3. Results

A total of 63 bacterial isolates from inside 60 surface-sterilized nodules were obtained. A number of culturable colonies appeared on yeast-extract mannitol agar (YMA) plates and the values ranged between fewer than ten colonies to an abundant growth along most of the streak length on culture plates. Colonies mostly displayed white-clear phenotypes and a minority of them were pigmented. The electrophoretic comparison of the restriction patterns of the 63 isolates revealed 5 different 16S rDNA types (patterns A–E). Table 2 shows the distribution of representative profiles observed. A total of 25 ARDRA profiles displayed the pattern of *Rhizobium sllae* IS123 reference strain (pattern A), 17 isolates belonged to pattern B, 10 had pattern C, 5

Table 2
Number of nodules and strains in which members of the ARDRA profile (A–E) were found in the various sampling site. The 16S sequencing results for each profile were the following: Profile A: *Rhizobium sllae* 100%; Profile B: *Pseudomonas* sp. 99%; Profile C: *Microbacterium* sp. 97%; Profile D: *Microbacterium* sp. 99%; Profile E: *Pantoea agglomerans* 99%.

Sampling site	Code	Number of nodules	Number of strain within each ARDRA profile
Ales	S03	13	2A, 3B, 2C, 3D, 3E
Bacu Abis	S01	5	3A, 1B, 1E
Torre dei Corsari	S02	20	9A, 7B, 1C, 2D, 1E
Nuxis	S04	18	11 A, 1B, 5C, 1E
Furtei	S05	2	4B
La Speranza	S06	2	1B, 2C

showed pattern D and 6 belonged to pattern E. Isolates showing different ARDRA types, and potentially belonging to different species, allowed the selection. Each representative 16S rDNA amplicon was sequenced from both strands. The sequences obtained were analyzed by BLAST at the NCBI website and the top homologies obtained are shown in Table 2. In order to further confirm the identity of *Rhizobium sllae* isolates, we also run a species-specific PCR using primers designed on an internal portion of a transposase gene within the insertion element ISRh1 (Muresu et al., 2005). The diagnostic amplicon was found in all the 25 samples displaying restriction profile A.

4. Discussion

This study provides the first comparative report on the full diversity of culturable bacteria in nodules of *Sulla coronaria* plants growing in spontaneous conditions. Regarding the nature and possible roles of the non-rhizobial root occupants recovered from 58% of these nodules, the following considerations can be done. Some species of the genus *Microbacterium* have already been reported to be endophytes of agronomic and prairie plants (Zinniel et al., 2002) and were isolated also from root nodules of spontaneous legumes growing in the arid zone of Tunisia where the presence of *nifH*-like gene sequences in them was also signaled (Zakhia et al., 2006). Endophytic *Microbacterium* isolated from common bean (*Phaseolus vulgaris*) showed antimicrobial activity and quorum sensing inhibition in Gram-negative bacteria; a mechanism that suggests the possible regulation of virulence factors production in pathogens (Lopes et al., 2015). That study also showed the potential of endophytic bacteria from common bean plants for the biological control of phyto-pathogens in this plant. Earlier, *Microbacterium* isolates from soil had been reported to exhibit plant-growth promoting traits such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, production of siderophores and indole-3-acetic acid (IAA) (Rashid et al., 2012). *Microbacterium* isolates from seeds of *Tylosema esculentum* showed the capability to produce proteases and *N*-acyl homoserine lactones (AHLs) besides IAA and siderophore synthesis, which makes these endophytes potential biofertilizers for plant growth promotion (Chimwamurombe et al., 2016). Other taxa that we found in wild *sulla* nodules were *Pseudomonas* sp. and *Pantoea agglomerans*, that had already been isolated from different Mediterranean wild legumes including *Hedysarum carnosum*, *Hedysarum spinosissimum* subsp. *capitatum* (Benhizia et al., 2004) and *Ornithopus compressus* (Muresu et al., 2008). The presence of endophytic *Pseudomonas* strains in legume nodules was subsequently reported by other authors (Deng et al., 2011; Hartman et al., 2017; Ibanez et al., 2009). Different studies have shown that non-rhizobial endophytic bacteria are not strictly passive hosts of nodules but may cooperate with rhizobia (Clúa et al., 2018) and could affect the growth performance of plants (Ibanez et al., 2017). They may control the host plant during their chronic infection of the nodules (Liu et al., 2010), in some cases they may help rhizobia to extend their nodulation host range (Liu et al., 2010), could increase number of nodules (Ibanez et al., 2009) or even improve the N₂ fixation of legume–rhizobia symbioses (Peix et al., 2015). Also *Pseudomonas* spp. have been found to synthesize 1-aminocyclopropane-1-carboxylate (ACC) deaminase,

which plays an active role in modulating ethylene levels in plants. This activity can lower the impact of different biotic and abiotic stresses on plant growth and development (Gamalero and Glick Bernard, 2015). Some *Pseudomonas* strains are ascertained plant growth-promoting rhizobacteria used as inoculants to enhance plant productivity and alleviate stress (Ahemad and Kibret, 2014), some have been reported as capable of inhibiting plant pathogens (Vijayabharathi et al., 2016). *Pseudomonas* sp., *Pantoea agglomerans* and *Microbacterium* sp. have all been found capable of synthesizing the major phytohormone IAA with the capability to control plant development in either beneficial or detrimental ways (Bal et al., 2013; Duca et al., 2014).

The fact that, in the present study, non-rhizobial endophytic bacteria were found in root nodules of spontaneous populations of *sulla* collected from different geographical sites, even within soils with different characteristics, suggests that nodule colonization by non-rhizobial bacteria does not appear a casual phenomenon but rather a common occurrence, at least in legumes growing in land not used for agricultural purposes. It could be then hypothesized that in agricultural fields the isolation of these non-rhizobial endophytic bacteria is less frequent, as legumes are usually cultivated as component of crop rotations and, sometime, in assisted and facilitated conditions of fertilization and irrigation. In plants growing spontaneously instead, endophytic bacteria could preferentially be selected as they can provide ecosystem-serviced benefits to plants, in contexts where it is more critical to receive assistance to counteract nutritional deficiencies, drought, herbivory and pathogenic attacks.

Atmospheric CO₂, global mean temperature and inter-annual variability in temperature and rainfall are expected to be significantly higher in Mediterranean regions by the end of the 21st century (Reverchon et al., 2012). Much of the *sulla* inoculation and fertilization practices has been carried out using the *Rhizobium sullae* that nodulates the cultivated crop, as the unique partner to improve its productivity (Sulas et al., 2017). While this can ensure agronomic-level exploitation, these practices may lead to an underestimation of the potential offered by the plant inner microbiome diversity exemplified by spontaneous legumes. Since climate change can also affect the growth of Mediterranean grassland, while warming, altered precipitation, and/or changes in soil physicochemical properties, can eventually modify microbial communities (Dumont et al., 2015), the interactions of legumes with their many microbial partners are important factors to be considered in order to select combinations that allow legumes to mitigate the impacts of such adverse and stressful environmental changes. All the endophytes recovered in this study belong to species in which active PGPR have been reported (Defez et al., 2017), and entail a potential to positively affect drought stress in nodulated legumes.

Another important aspect stemming from this research is that related to the evolutionary side of the plant-symbiont model. Out of the 60 nodules analyzed, only in 25 of them we detected culturable isolates of *Rhizobium sullae*. This situation is in part reminiscent of the findings of our prior work (Muresu et al., 2008) in which we analyzed eight other species of wild legumes native to the Mediterranean (but in that case none of them was featuring domesticated counterparts), from which no culturable rhizobia could be rescued at all from nodules, which yielded only colonies related to endophytes. Nevertheless, the direct 16S rRNA gene PCR analysis revealed that most of those nodules contained rhizobia as the predominant population.

In the present study, dealing for the first time with spontaneous specimens of a plant (*Sulla coronaria*) that at the same time exists also in cropped ecotypes, from more than half of the nodules we isolated bacteria different from rhizobia, while in 25 of them (41%) we still were able to find the ascertained true symbiont of *sulla*, *Rhizobium sullae* (Squartini et al., 2002) in culturable state. This scenario thus presents an interesting hybrid situation, reflecting, in the same legume species, both the wild and cropped kind of phenotypes hitherto reported only for one or the other plant status. One interpretive possibility could be that *sulla*, being still in transition from its spontaneous to

its cultivated status (or being able to revert and become naturalized), would be displaying a consequently intermediate condition in which its habitual N₂-fixing symbiont is turning culturable as it is in the cropped accessions of this legume and of all agricultural legumes in general. Nevertheless, in a majority of the nodules of wild stands of *sulla*, the situation appears still the one that is typical of wild Mediterranean legumes, in which the rhizobium is non-culturable and nodules are co-invaded by different endophytic taxa. Differences in the genomes of two strains of *Rhizobium sullae* one from a wild *sulla* and the other from a cropped one have been pointed out in our prior work (Sablok et al., 2017).

For a proper framing of this situation it is furthermore important to add another detail. As we previously pointed out (Sablok et al., 2017), there are countries of the Mediterranean in which *Sulla coronaria* has been recognized as “native” (<http://www.legumes-online.net/ildis/aweb/database.htm>). These are: Algeria, Morocco, Tunisia, Spain; in others (Corsica, Sardinia, Continental Italy, Sicily, Libya, Egypt) it is considered of “uncertain origin,” i.e., possibly introduced. The plant is also naturalized or cropped in other areas, where it has been intentionally introduced, including the Balearic Islands, Portugal, former Yugoslavia, Malta, Lebanon, and Syria.

From this scenario it follows that the spontaneous status of *sulla* in Sardinia can possibly be regarded even as a step closer to the cropped version if compared to the wild *sulla* occurring in its native territories. The *sulla* populations hereby sampled could actually be described as either wild or possibly naturalized after man-driven introduction. This happenstance could be in line with the observed ‘hybrid’ phenotypic situation of some nodules harbouring culturable *R. sullae*, as in the cropped *sulla*, and some yielding non-rhizobial endophytes, as in the fully wild legumes of the Mediterranean areas.

Additional evidence that adds to this interpretation can be drawn from a study by Chiboub et al. (2016) which, for different purposes (selecting strains for phytoremediation), addressed a complementary set up; those authors germinated seeds of an agronomical *sulla* accession in different soils contaminated with heavy metals including Cd, Zn and Cu, collected from different areas of Tunisia. Also in such case, featuring a reverse situation if compared to ours, from the nodules that were formed they were able to rescue either *R. sullae* or an array of other taxa.

This leads to hypothesize that the nodule-associated taxa would not be actually determined by the plant genotype but rather by the soil environment or with its contribution. It appears that also domesticated plant ecotypes, when grown in harsher, non-agricultural soils can be led to host a variety of endophytes in nodules supposedly induced by a non-recoverable rhizobium. The preferred selection of a single culturable rhizobium along with the absence of other endophytic co-occupants, would be instead typical of the agriculturally-cared situations which would not require extra bacteria to compensate plant needs. However at present these is essentially hypotheses and further evidences would be needed to corroborate this view or to find alternative interpretations

5. Conclusions

Results suggest that spontaneous populations of legumes could host bacteria with different identity and/or culturability when compared to their cropped counterparts. The fact could be possibly linked to the need to host a wider diversity of endophytes to cope with the harsher challenges of their wild status. Such aspect however needs to be proven by more extended analyses. In particular it would be necessary to perform the reciprocal experiments by planting cropped ecotypes of *sulla* in soils where the native one thrives and vice versa, collecting seeds from wild pods and planting them into the usual agronomical fields (with or without *R. sullae* inoculation), to inspect again in both cases the distribution of nodule occupants.

This study, provides the first report on the full diversity of bacteria in nodules of spontaneous populations of *sulla*. Further characterization

of the respective roles of nitrogen fixing symbionts and accompanying endophytes will allow better insights to exploit the potential to promote plant growth in the harsh conditions prevailing in increasingly arid climates. Such studies are of particular importance in a changing climate scenario where sustainable plant production must necessarily be obtained under environmentally friendly practices in order to preserve ecosystems balance and guarantee the conservation of soil resources biodiversity.

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Conflict of interest

The authors declare that they have no conflict of interest.

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