Isolation, diversity, and the evaluation of multi-trait plant growth promoting rhizobacteria associated to roots of saffron (Crocus sativus L.)

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Research Article

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Abstract

The cultivation of *Crocus sativus* L. (saffron) is of particular interest in view of the sale of this spice on the world market. It is a very popular product throughout the world, especially for its culinary and medicinal virtues. In order to maintain the quality of the product, the organic production method without chemical inputs should be maintained and improved by the application of organic fertilizers and biofertilizers based on beneficial micro-organisms such as plant growth-promoting rhizobacteria (PGPR), which are capable of boosting the productivity. In this context, a better knowledge of the microbial populations of the rhizosphere of saffron is needed. The isolation of rhizobacterial strains was carried out from saffron soil collected from Taliouine-Talakhat region (Morocco). Plant growth promoting traits (PGP) activities of these isolates were evaluated by measuring, auxin production, siderophore production, phosphate solubilization, biological nitrogen fixation, aminocyclopropane -1-carboxylate (ACC) deaminase activity, cellulose activity, and antagonistic activity. Moreover, molecular identification of the isolates was determined by using 16S rDNA gene sequencing. Results showed that a collection of 88 bacteria strains were isolated from rhizospheric soils. The evaluation of PGP of these isolates showed that most of them possessed one or more PGP activity studied. The molecular identification of the isolates by 16S rDNA gene sequencing revealed eight genera: *Pseudomonas*, *Rahnella*, *Variovorax*, *Delftia*, *Bacillus*, *Rhizobium*, *Luteibacter,* and *Pantoea*, while, *Pseudomonas* was the most abundant genus. The findings of this study suggest that the isolates with higher levels of PGP activities can be used for the conception of bacterial biofertilizers that will be applied in saffron field inoculation trials.

1. Introduction

The saffron is one of the rare and most expensive spices in the world, it consists in dehydrated stigmas of flowers of a bulbous plant named *Crocus sativus* L. (Crozet et al., 2012). Almost 180000 flowers are needed for 1kg of dry saffron (Mir et al., 2011). Saffron is recognized since antiquity for its beneficial medical effects. Different studies showed the strong effect of the saffron against depression (Hausenblas et al., 2013), cancer (Samarghandian et al., 2013), in addition to immunological, antioxidizing, anti-tumoral activities (Bolhassani et al., 2014) and a potential therapy for Alzheimer's disease (Finley and Gao, 2017). It contains a large number primary metabolites such as carbohydrates, minerals, fats, and vitamins (Sampathu et al., 1984), and large number of components belonging to different classes of secondary metabolites such as carotenoids, monoterpenoids, flavonoids, and anthocyanins (Dhar et al., 2017). The molecules of saffron related to the color (crocin), the flavor (picrocrocin), and the aroma (safranal) makes saffron usable in cosmetics, perfume and textile manufacturing industries. These metabolites are the essential parameters used in the determination of the category of the saffron according to the International Organization for Standardization ISO 3632.

C. sativus belongs to the *Iridaceae* family, it's a sterile geophyte plant, which can reproduce only by the vegetative multiplication of corms (Mir et al., 2010). It requires a particular combination of climatic, edaphic, and hydric factors for flowering (Gresta et al., 2009). Saffron is a perennial crop culture adapted to the semi-arid and arid areas, in temperate climates, and subtropical climates. However, the

Mediterranean area is recognized all over the world as being the best region for the production of good quality saffron (Lage and Cantrell, 2009). Traditional producers are mainly Spain, Iran, India, Pakistan, Turkey, Italy, Switzerland, Greece, Central Asia and Morocco (Gresta et al., 2008). The world production of saffron is estimated at approximately 300 tons a year and is dominated by Spain and Iran, with the later producing approximately 76 % of the total (Jalali-Heravi et al., 2010; Samarghandian et al., 2013). However, an increase of saffron productivity is desirable, but must be sustainable and environmentally friendly. The use of chemical fertilizers at high rates is responsible of massive pollution of soils and reduces saffron productivity (Moghadam et al., 2013). Compared to chemical fertilizers, the biological fertilization can have positive effects on saffron plants. The fertilization with natural manure or composted cattle manure, increases significantly yield parameters of saffron (flower fresh weight, stigma length, stigma fresh and dry weights) (Amiri, 2008; Koocheki and Seyyedi, 2015), and can also affect microbial flora of soil as *arbuscular mycorrhizal fungi* (Chamkhi et al., 2019).

Use of microbial biofertilizers is another interesting alternative to chemical fertilizers. Indeed, the study of Sharaf-Eldin et al. (2007) proved that incubation of *C. sativus* with PGPR *Bacillus subtilis* FZB24 strain improved importantly the quality of saffron metabolites, boost the length of the leaves, the number of flowers, the biomass of stigmas, and decreased significantly the time required for the seeding of corms.

In fact, PGPR strains are known to colonize the rhizosphere, where they ensure several functions that improve and stimulate plants growth and increase their yield by direct and indirect mechanisms (Chamkhi et al., 2022). Indeed, direct mechanics of PGPR include biological nitrogen fixation, phosphate solubilization, and phytohormone production such as *indole-3-acetic acid* (IAA), gibberellins, cytokinins, and abscissic *acid. Moreover, the* indirect mechanics of PGPR as siderophore production, biocontrol mechanisms (secretion of chitinase, glucanase, cellulase, protease…), production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, antibiotic production, inducing systematic resistance have been proved as indirect mechanics of PGPR (Goswami et al., 2016). On the other hand, the rhizobacteria release elicitors that bind to plant cell receptors and mediate signaling pathways involved in secondary metabolites synthesis (terpenoids, phenolic acids, and flavonoids) in medicinal plants (Chamkhi et al., 2021). There are only few studies concerning PGPRs isolated from the rhizosphere of saffron and no one in Morocco, where high quality saffron is produced specially in the area of Taliouine-Taznakht.

The objectives of the present work were the isolation, molecular identification and evaluation of the plant growth promoting activities of the rhizobacteria associated with saffron. The main PGP traits studied were inorganic phosphate solubilization, biosynthesis of auxin, siderophore production, biological nitrogen fixation, ACC deaminase, cellulase activity and antagonistic activity. The molecular identification was carried out by using 16S rDNA gene sequencing.

The most performing isolates will be used later for field applications as inoculums for saffron biofertilization.

2. Materials And Methods

2.1. Sample collection and soil physicochemical analysis

Saffron soil was collected from a field of saffron in “1.2.3 SAFRAN” farm in Taliouine-Talakhat area, located in the South of the province of Taroudant, Souss-Massa region Morocco (N30°28’12.997”/W7°46’22.479”). Soil samples were sent for analyses to «Laboratoire des Moyens Analytiques de l’Institut de Recherche pour le Développement» (LAMA, IRD, Dakar, Senegal) for the physico-chemical analysis like pH, total organic carbon, total nitrogen, etc. Samples of saffron rhizospheric soil were kept at +4°C until they were used for isolation of rhizobacteria.

2.2. Isolation of rhizobacterial strains

In order to increase the probability of obtaining rhizobacteria possessing the most important PGP traits we used three specific solid media for bacteria isolation. Phosphate solubilizing bacteria were obtained on modified *Pikovskaya medium (PVK)* (Pikovskaya, 1948) (1glucose, 0.02 g KCl, 0.01 g $MgSO_4.7H_2O$, 0.002 g $FeSO_4.7H_2O$, 0.002 g $MnSO_4.7H_2O$, 4 mL bromophenol blue, 0.5 g natural phosphate, 0.01 g yeast extract, 15 g agar, with pH adjusted to 6.7). Siderophore producing bacteria, were isolated on Modi medium (Berraho et al., 1997). While auxin producing isolates, were isolated on Yeast Extract Mannitol (YEM) medium, (Vincent, 1970) supplemented with tryptophan (YEM-Try) (5 g/L Mannitol, 1 g/L yeast extract, 0.46 g/L KH_2PO_4, $3H_2O$, 0.12 g/L KH_2PO_4, 0.2 g/L $MgSO_4$,$7H_2O$, 0.1 g/L NaCl, 1 L distilled water and 0.5 mg/mL of tryptophan). Four grams of *rhizospheric soil samples* were suspended in 16 mL sterile of in sodium Chloride solution (0.9 %) and shaken for 30 min, then the suspensions were diluted serially (Becerra-Castro et al., 2012). Petri dishes of each medium, were spiked with 0.1 mL of each soil dilution, and incubated at 28°C for 48 hours. Colonies obtained were purified and stored at -80 °C in 40 % glycerol.

2.3. Evaluation of PGP activities

2.3.1. Auxin production

Salkowski colorimetric method is the most used to quantify *in vitro* indolic acid biosynthetic capacity of the isolates. Liquid pre-cultures of isolates are inoculated to liquid YEM-tryptophan medium, and then incubated at 28 °C for 24 hours. Two mL of each culture were centrifuged at 12000 rpm for 10 min, the supernatants were recuperated and filtered through a sterile nylon membrane of 0.2 µm (Millipore) following the method of Glickmann and Dessaux, (1995).

2.3.2. Siderophore production

Schwyn and Neilands, (1987) method was used to highlight siderophore production by saffron isolates. Fifty mL of liquid Modi medium was inoculated with a fresh culture of initial optical density equal to 0.1. Inoculated medium was incubated at 28 °C in the Shaker-Incubator at 180 rpm/min. After 7 days of incubation, 10 mL of culture were centrifuged at 10000 rpm for 20 min. The supernatants were filtered on a nylon membrane of 0.45 µm (Millipore) (Khan et al. 2006). Three ml of supernatant were added to 3mL

of reagent CAS then were incubated in the dark between a minute until 24 h (Berraho et al., 1997). Siderophore production was indicated by the time when CAS reagent color changed from blue to orange. A semi-estimation of siderophores produced by the best isolates was estimated spectrophotometrically at 630 nm by calculating the ratio A/Ar (He et al. 2013).

2.3.3. Phosphate solubilization

Qualitative test of phosphate solubilizing activity was carried out on modified Pikovaskaya agar medium. Ten µL of each bacterial suspension were dispensed into the center of Petri dishes containing the medium, incubated for 3 days at 28 °C (Xie et al., 2009). The presence of a clearing zone around bacterial colonies (halo zone) is an indicator for positive phosphate solubilization.

A quantitative test of phosphate solubilization was studied by the vanadate-molybdate method (Tandon et al. 1968). In 250 mL Erlenmeyer flasks containing 100 mL of liquid PVK medium was inoculated with fresh pre-cultures of each strain. The incubation was done for 48h at 28°C in the Shaker-Incubator at 185 rpm, then 2 mL of the culture was centrifuged and the pH of the culture determined. Soluble phosphorus concentrations were measured using a spectrophotometer at 400 nm.

2.3.4. Biological nitrogen fixation

Atmospheric nitrogen fixation by saffron isolates was studied by inoculating plates containing Nitrogen fixing bacteria (Nfb) medium with or without the addition of NH_4Cl. Plates were incubated at 28 °C for 7 days (Zhou et al., 2013).

2.3.5. Aminocyclopropane -1-carboxylate (ACC) deaminase activity

The ACC deaminase qualitative test was determined according to the method described by Jacobson et al., (1994). The tested strains were spotted in triplicate on minimal media plates containing ACC (only nitrogen source), ammonium sulphate (positive control), and no nitrogen source (negative control). All the inoculated plates were incubated at 28 °C. The growth of test strains was measured at 600 nm at 0h and after 24h.

2.3.6. Cellulose activity

Cellulose-degrading ability of bacterial isolates was detected by spotting the culture on the cellulose Congo-Red agar media (Yang et al., 2014). After spot inoculation of bacterial culture, the plates were incubated at 28 ◦C for 5–7 days, and observed for clearing zone around the colonies.

2.3.7. Antagonistic activity

The antifungal activity of all the isolates were tested against *Fusarium oxysporum* on potato dextrose agar (PDA). Fungal disc was placed in the middle of agar plate from 2 cm away from the inoculated isolates on the surface of the agar plate. Antagonist activity was observed after incubation at 28 °C up to

7 days. The percentage of inhibition was measured using the following equation: I %= (C−T/C) x100; T: treatment; C: control (Petri plate without bacterial inoculation served as control).

2.4. Molecular Identification

2.4.1. DNA extraction and PCR amplification

After the extraction of the genomic DNA (Moulin et al., 2004), the quality and quantity of DNA in the samples were checked on a 0.8% agarose gel and using Nanodrop spectrophotometer. PCR amplification of partial nucleotide sequences of 16S rRNA gene was performed by using the universal primers 1488 (CGGTTACCTTGTTACGACTTCACC)/41F (GCTCAGATTGAACGCTGG CG) and MyTaq TM HSMix (Bioline, London, UK) in 25 µL final volume. The PCR program used was as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 64 °C for 45 s and extension at 72 °C for 1 min 30 s. Purified PCR products were then sequenced in both senses by using 1488 and 41F primers at Genoscreen compagny, Lille, France.

2.4.2. Construction of phylogenetic trees

The sequences of sense 1488 were converted in inverse complement (http:// www. Bioinformatics .org /sms /revcomp.html), then aligned with the sequences of sense 41F by the online software ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/). Sequence identities and similarities were determined using the BLAST program and the GenBank database on the NCBI. All sequences were deposited in GenBank under accession numbers. The phylogenetic tree was constructed from the ClustalX results and the Maximum Like-hood test with Kimura 2-parameter model by using a MEGA5 program. The bootstrap method was used as the phylogeny test. Reference sequences were added to optimize the comparison.

2.5. Statistical analysis

Statistical analyses were performed by XLSTAT program (version 2014.5.03). With a one-way ANOVA and compared by Fisher's Least Significant Difference (LSD) with a 5 % probability threshold to determine differences between means. The correspondence analysis (CA) as an exploratory data analysis technique was performed by XLSTAT software.

3. Results

3.1. Physico-chemical soil characteristics

The analyses revealed that the saffron soil studied is a sandy clay loam according to the USDA texture classification and a Luvisol according to the WRB soil classification. It’s an alkaline soil with a low percentage of nitrogen (0.04%) and total carbon (0.89%), a high C/N ratio (22), and a very low amount of available phosphorus (18 mg/kg). All these parameters are indictors of low soil fertility (Chamkhi et al., 2019).

3.2. Isolation of bacterial strains

A collection of 88 isolates was obtained from rhizospheric soil of saffron by using 3 selective media: PVK, Modi, and YEM-try. Among them, 34 isolates were obtained on PVK medium, 22 isolates on Modi medium, and 32 isolates on YEM-try medium.

3.3. Evaluation for PGP activities

3.3.1. Auxin production

The colorimetric method of Salkowsky demonstrated that saffron isolates tested produced different amounts of IAA, classified at 7 different levels from Group1: Interval [125.3 µg/mL; 123.83µg/mL] to Group7: Interval [14.9µg/mL; 1.1µg/mL] (Fig. 1). The best auxin producing strains were S11S2 (125.3 µg/mL), S11A1a (124. 4 µg/mL) and S11P12 (123.8 µg/mL) respectively group in interval 1 (Grp1).

3.3.2. Siderophore production

The qualitative test by using the chrome-Azul S reagent (CAS) detects the siderophores produced by the isolates, which can be classified the isolates into 4 categories according to the time and the color change of CAS reagent from blue to orange color. Percentage of 48.31 % of isolates produce highly the siderophores (+++), 23.60 % produce siderophores moderately (++), 11.24 % (+) weakly produce siderophores and only 16.85 % (-) of isolates were unable to produce these chelators (Fig.2).

The ratio A/A_{ref} (semi-quantitative test) was calculated to the best strains screening on the first qualitative test of siderophores production. The results of siderophores production in decreasing order as follows: S12S4, S11P12, S11A11b, S13P5, S11A10a, S12S1, S13P9 represented in table 1, were the best siderophore producing isolates according to the ratio A/ A_{ref} (Table 1).

3.3.3. Phosphate solubilization

The qualitative test of phosphate solubilization on solid Pikovskaya medium revealed that almost all the bacteria isolated on selective PVK medium were surrounded by transparent halos, what indicates the capacity of these bacteria to solubilize rock phosphate *in vitro*. The halo of phosphate solubilization is caused by the production of organic acids in the medium which turns the color of the bromophenol blue from mauve towards white/yellow. As a result, the strains S13P4 (19 mm), S12P9 (17 mm) and S13P11 (13 mm) have the biggest halo diameter size on solid Pikovskaya medium (Fig.3).

The quantification of soluble phosphate released confirmed that all of the tested bacteria possessed the ability to solubilize inorganic phosphate (Fig. 4). The measured concentrations were very close to each other for the majority of isolates, with the high amount of soluble phosphate released by strains S12A7 (65.13 mg/L), S11A8 (63.60 mg/L) and S11A5 (63.30 mg/L) (group 1).

3.3.4. Biological nitrogen fixation

Obtained results of atmospheric nitrogen fixation showed that only 9 % of the isolates had a high capacity of nitrogen fixation, 29% showed a medium capacity of nitrogen fixation, while 27% were low nitrogen fixers and 14% were unable to fix nitrogen (Fig. 5).

3.3.5. Aminocyclopropane -1-carboxylate (ACC) deaminase activity

Among the isolates screened only 55% of isolates growth in the minimal medium by measuring absorbance at 600 nm, where they have the ACC as a sole source of nitrogen. While, 9 % produce ACC deaminase highly, 22% produced ACC deaminase moderately, 24% isolates produced ACC deaminase lowly. Nevertheless, 45 % isolates not able to produce ACC deaminase (Fig. 6).

3.3.6. Cellulose activity

From all the collection of saffron, 11.36 % formed a clear halo around bacterial spots on plates containing carboxymethylcellulose (CMC-Na) degradation selective medium, which is indicative of cellulose activity. The isolates S12S3 (7mm), S13A4 (5mm) show the biggest halo which indicates high cellulose activity, then the isolates S13A6b, S13A2, S11P13, S11P10, S11A1b, S13P2, S11P5, S13S1 respectively go after Fig. 7.

3.3.7. Antagonistic activity against pathogenic fungus

The screening of antagonistic activity demonstrates that the percentage of 20.45 of the isolates shows an antagonistic effect against *F. oxysporum*. The maximum inhibition percentage in radial growth of *F. oxysporum* caused by strains was S11A3 (79 %), S12P3 (70%), S12P4 (67.4%), S11A2a and S12S4 (66.7%), S12A4b (65.2%), S12P1 (63%) and the minimum inhibition percentage was 35.6% caused by strain S13P9 (Fig. 8).

3.4. Molecular identification

The 16S rRNA gene sequences of 88 isolates obtained were analyzed using bioinformatic tools and deposit under NCBI gene bank accession number, from **KU569610** to **KU569698**. Bacterial isolates belonged to 13 different bacterial species namely *Pseudomona putida*, *Pseudomona fluorescens, Pseudomonas sp., Rahnella aquatilis, Variovorax paradoxus, Bacillus simplex*, *Bacillus subtilis, Rhizobium radiobacter (Agrobacterium tumefaciens), Rhizobium rhizogenes, Pantoea sp., Luteibacter sp., Delftia sp.,* and *Rhizobium sp*. Among the 8 genera identified, *Pseudomonas* is the predominant genus with 34.83%, followed by the genus *Rahnella* with 24.71%, *Variovorax* with 15.73% and *Delftia* with 11.23%. The other genera are weakly represented in the saffron rhizosphere (Fig. 9).

3.5. Correspondence analyses

The analyses showed a relationship between the biological activities and the genera of the isolated bacteria. At this respect genera *Luteibacter*, *Pantoea,* and *Rahnella* have presented the ability of solubilizing inorganic phosphate (Fig.10A), while *Rahnella* and *Pantoea* were the best producers of auxin

(Fig.10B). Five genera, *Variovorax*, *Pseudomonas*, *Rahnella*, *Luteibacter*, and *Pantoea* are siderophore producers (Fig.10C), while biological nitrogen fixers were found in two genera, *Rhizobium* and *Rahnella* (Fig.10D). While *Pseudomonas* was the only genus showed cellulose activity (Fig.10E), however, the ACC deaminase activity includes *Pseudomonas*, *Rahnella* and *Variovorax* genera (Fig.10F) and finally, the antagonistic activity *Bacillus* was the only genus manifested this activity (Fig.10G).

4. Discussion

The physicochemical analysis of soil showed that saffron soil of Taliouine is very poor in organic matter and mineral elements including nitrogen and available phosphorus. At the opposite, the C/N ratio is high, which indicate low mineralization that can be a result of a reduced biological activity caused by a limited microbial population. Additionally, soil pH is alkaline, which can affect the availability of nutrient elements for plants, such as phosphorus and iron, especially regarding the high calcium content of the soil. These results indicate that this soil needs diverse amendments to improve the microbial activity and consequently its fertility and requires phosphate fertilization to ensure good growth and yields of plants.

In this context, it is important to identify, for the first time in Morocco, the rhizobacteria colonizing saffron rhizosphere in the main production area in Morocco and evaluate their potential beneficial activities for plant growth.

A collection of 88 isolates associated with saffron plants in Taliouine were isolated and identified by using 16S rDNA sequencing. Eight genera were identified: *Pseudomonas*, *Rahnella*, *Variovorax*, *Delftia*, *Bacillus*, *Rhizobium*, *Luteibacter* and *Pantoea*, with *Pseudomonas* as the most abundant genus. There are very restricted number of studies dealing with this aspect in countries producing saffron, especially India (Parray et al., 2013; Ambardar and Vakhlu, 2013; Ambardar et al., 2014 ; Kour et al., 2018) and Iran (Al-Ahmadi et al., 2017), and this is the first one in Morocco. Like found in India, the rhizosphere of Moroccan saffron is colonized predominantly by Gram-negative bacteria belonging to genera *Pseudomonas* and *Pantoea*. Moreover, *Pseudomonads* are the most dominant genera in saffron rhizosphere. Rouatt and Katznelson, (1961) suggested that the preponderance of *Pseudomonads* in the root zone is due to at least three factors which may operate singly or together depending on environmental conditions: their rapid growth, their ability to produce acidic substances, and to elaborate fluorescent pigments that are giving a competitive advantage to this genera over the other rhizospheric microorganisms. Thus, due to their PGP properties these bacteria become the most important component of the microbial population surrounding the roots of young healthy plants. Additionally, the interesting bacteria for the plants are chemically attracted to the root exudates and are selected over other microbes (Saharan and Nehra, 2011).

Apparently, all the rhizobacteria isolated from the saffron demonstrated at least one plant growth property. Biosynthesis of auxin is one of the most interesting properties of the PGPR, which influences the growth of plants at different levels (Vanneste and Friml 2009). All saffron isolates of the collection were able to produce auxin, while, the highest concentration was found was 125.3 µg/mL on liquid medium

YEM added to tryptophan a precursor of auxin (tryptophan-dependent pathway). Though it, is widely known that the tryptophan-dependent auxin biosynthesis pathway is the most important among the six metabolic routes known in bacteria (Cassán et al., 2014). While, in natural conditions, tryptophan is an exometabolite supplied richly by root exudates of host plants stimulated by rhizobacteria (Kravchenko et al., 2004). Our results are in accordance with those of Parray et al. (2013), who found that almost 83.33% of strains isolated from the rhizosphere of saffron growing in India were able to synthesize auxin. Based on 16S rRNA gene sequence analysis, the genera *Pantoea* and *Rahnella* were demonstrated very high *in vitro* auxin production, has also been reported by Sergeeva et al., (2007; Vyas et al., (2010).

On the other hand, saffron isolates selected on PVK medium are the best bacteria solubilizing natural phosphate and, also produced important amounts of auxin. However, the highest concentration found was 65.13 mg/L. Similar results were reported by Leinhos and Vacek (1994) who found that approximately 80% of the phosphate-solubilizing bacteria strains were able to synthesize auxin in a liquid culture medium, contrary to the isolates producing IAA were not able to solubilize phosphate strongly (Leinhos and Vacek, 1994).

As found by many authors, the concentration of free phosphate measured increased proportionally with the decrease of the pH in the culture medium. This decrease is thought to be responsible for the dissolution of phosphate in the culture medium. It may be caused by the biosynthesis of different organic acids that have a relevant role in P solubilization, like oxalic, citric, gluconic acids and other organic acids that were commonly found in the media where phosphate solubilizing bacteria were grown (Rashid et al. 2004). In this activity, the genera *Pantoea*, *Rahnella* and *luteibacter* were the best solubilizes of inorganic phosphate *in vitro,* in accordance with the study of Costa et al., (2014).

The majority of tested saffron isolates possess the ability to fix freely atmospheric nitrogen and part of them may be good nitrogen fixers (38%). Regarding the rarity of nitrogen in the soils of Taliouine, diazotrophs may have an important role during cultivation of saffron plants because of their beneficial effects on the yield on flowers and quality of the saffron (Ünal and Çavuşoğlu, 2005). In this activity, *Rhizobium* genus was the potent biological nitrogen fixator in the saffron collection which includes the species *Rhizobium* sp., *Rhizobium rhizogenes,* and *Rhizobium radiobacter* (*Agrobacterium tumefaciens)* as confirmed by Kanvinde and Sastry (1990). Indeed, *Rhizobium radiobacter* is a *diazotrophic* bacterium that can fix nitrogen in free-living conditions. These species belong to the *Rhizobiaceae* family, which contains genus bacteria able to induce nodules in legumes named *Rhizobium* as well as genus bacteria able to produce hairy roots or plant tumors named *Agrobacterium* (Velázquez et al., 2005). The classification of theses bacteria is changing constantly. Indeed, Young et al. (2001) proposed that *Agrobacterium tumefaciens* become *Rhizobium radiobacter* and *Agrobacterium rhizogenes* become *Rhizobium rhizogenes.*

The majority of saffron isolates tested, 48.31 %, produce the siderophores *in vitro*. Among the whole rhizobacterial collection, strains isolated on the selective strict minimal medium Modi were the most productive of siderophores. In fact, in addition to their role in the plants iron nutrition, the rhizobacteria

producing siderophores may play an important role in the biocontrol of rhizospheric pathogens that can affect plants in general and saffron in particular (Kour et al., 2018). The 16S rRNA gene sequence analysis allowed the identification of *variovorax*, *Pantoea,* and *luteibacter* as the best genera producing siderophores in this study. Indeed, these strains have been reported also as the most producing of siderophores by Costa et al., (2014).

A percentage of 55 % of saffron rhizobacteria demonstrated ACC deaminase activity for converting ACC into ammoniac. The ACC deaminase activity represents an important role under stress conditions by decreasing ethylene levels (Glick et al., 2007). The genera *variovorax*, *Rahnella* and *pseudomonas* were produced the most ACC deaminase in this study. In other hand, the cellulolytic bacteria are important in the decomposition of cellulosic remains to assure carbon source to improve the soil fertility (Yang et al., 2014), however, 11.36 % of saffron isolate produced enzymatic hydrolysis as cellulose, which *Pseudomonas* was the most cellulolytic genus in the saffron collection.

Many saffron isolates inhibited fungal growth of *Fusarium oxysporum*, a fungus which known to induces corm rot of saffron, is the most destructive disease of saffron, causing severe yield losses were observed in Italy and in Spain (Di Primo et al., 2002). Moreover, in this study, the genus *Bacillus* was the most antagonistic to these fungal pathogens. Indeed, literature has also reported antagonist properties this genus (Gupta and Vakhlu, 2015).

5. Conclusion

In conclusion, this explorative study of the rhizosphere of Moroccan saffron has revealed the diversity of the rhizospheric bacteria associated with this plant at the genetic and functional levels. The promising results found allow us to consider the use of selected strains from this collection as biofertilizers in the field to improve saffron growth, yield and quality. They can be also used for other plants, which can lead to a sustainable agriculture that provides a way to replace, at least partially, the use of chemical fertilizers and pesticides known for their adverse effects on the environment and health.

Abbreviations

PGPR**:** plant growth-promoting rhizobacteria; PGP: plant growth promoting; IAA: *indole-3-acetic acid;* ACC: 1-aminocyclopropane-1-carboxylic acid; ISO: the International Organization for Standardization; PVK: Pikovskaya; YEM: Yeast Extract Mannitol; NFb: Nitrogen fixing bacteria, PDA: potato dextrose agar; LSD: *Least Significant Difference;* ANOVA: analysis of variance; CA: Correspondence analysis.

Declarations

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This study has not received funding.

Data availability

All used data in this study were cited in the manuscript.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1. The semi-estimation of siderophore production by saffron isolates.

Strains	Evaluation test on liquid Modi medium	Estimation of siderophores production (A/Aref)
S12S4	+++	0.020
S11P12	+++	0.028
S11A11b	+++	0.05
S13P5	+++	0.058
S11A10a	+++	0.071
S12S1	+++	0.083
S13P9	+++	0.088
S12S6	+++	0.108
S13S1	+++	0.108
S12S5	+++	0.117
S12A4a	+++	0.13
S12P2	+++	0.144
S13P2	+++	0.152
S11P5	+++	0.166
S13A6a	+++	0.174
S11P3	+++	0.176
S12A3	+++	0.19
S12P1	+++	0.193
S11A6	+++	0.198
S13P1	+++	0.204
S12P8	+++	0.208
S13A6b	+++	0.216
S12A7	+++	0.232
S13P6	+++	0.239
S11A5	+++	0.251
S11P13	+++	0.255
S13P4	+++	0.278
S12S2	+++	0.279

S12A1b	+++	0.312
S12S3	+++	0.323
S12P6	+++	0.324
S13S4	+++	0.338
S12P4	+++	0.358
S11A8	+++	0.377
S12P9	+++	0.397
S12P5	+++	0.398
S13A5	+++	0.401
S11A4	+++	0.413
S11P7	+++	0.439
S12A5	+++	0.562
S13S3	+++	0.577
S13S2	+++	0.610

A: absorbance at 630 nm**; A ref:** absorbance at 630 nm of the reference.

Figures

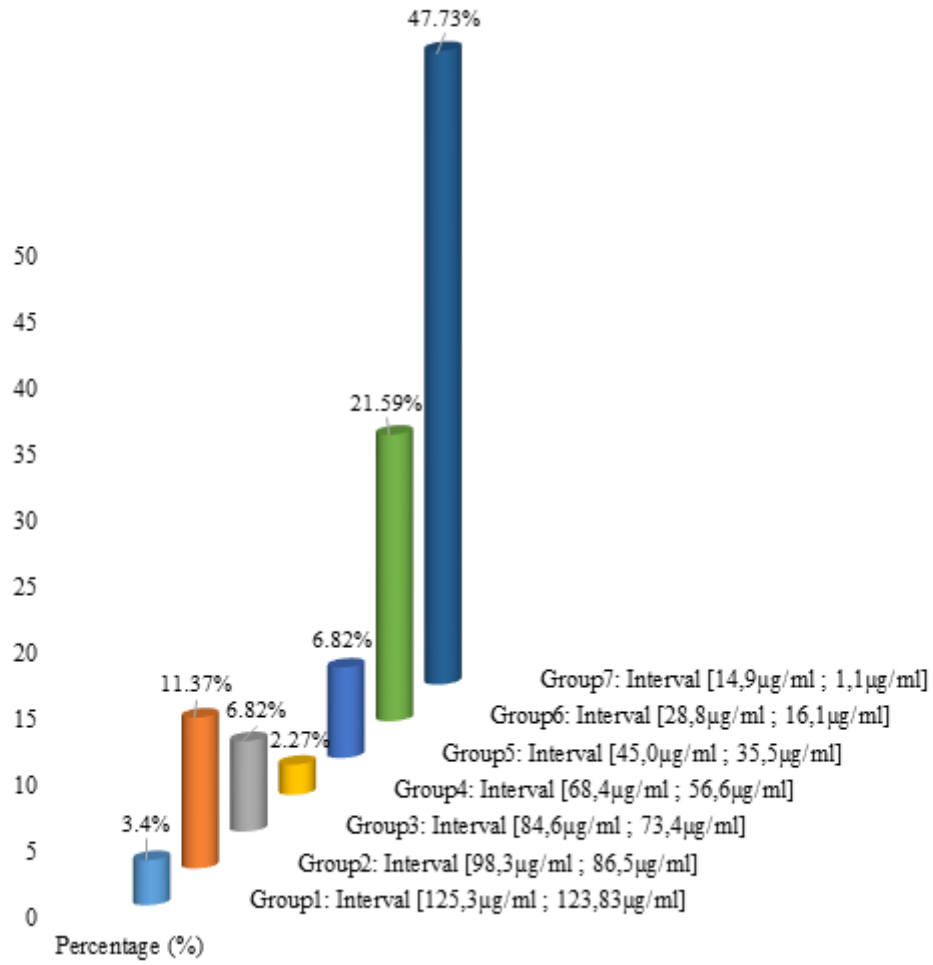

Figure 1

Auxin (IAA) concentration produced by saffron isolates

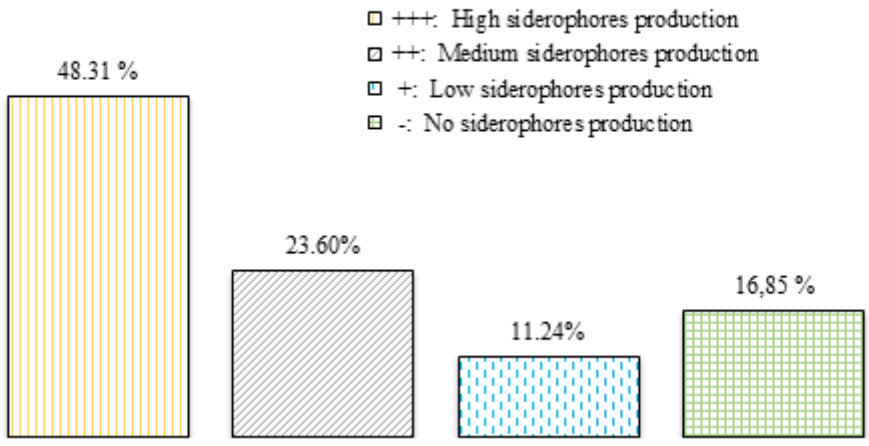

Figure 2

Qualitative test of siderophores production

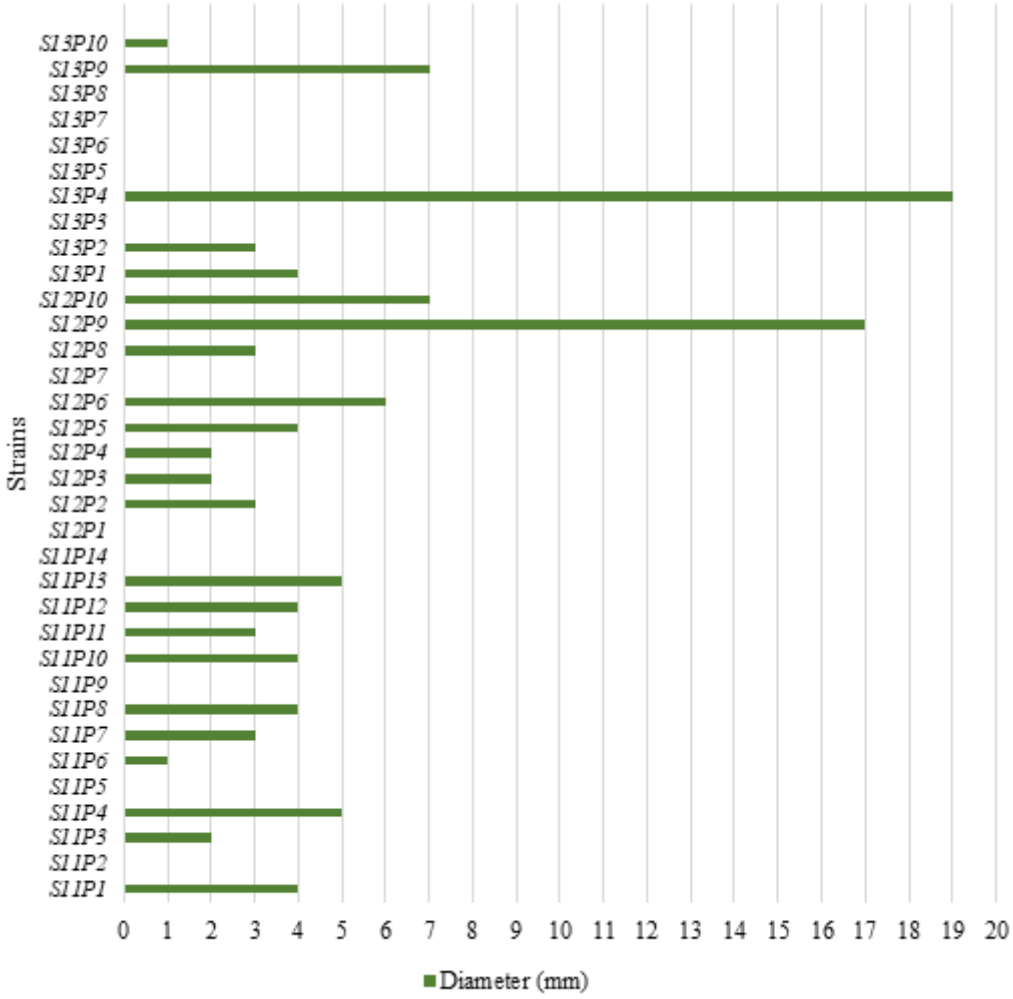

Figure 3

Qualitative test of phosphate solubilization

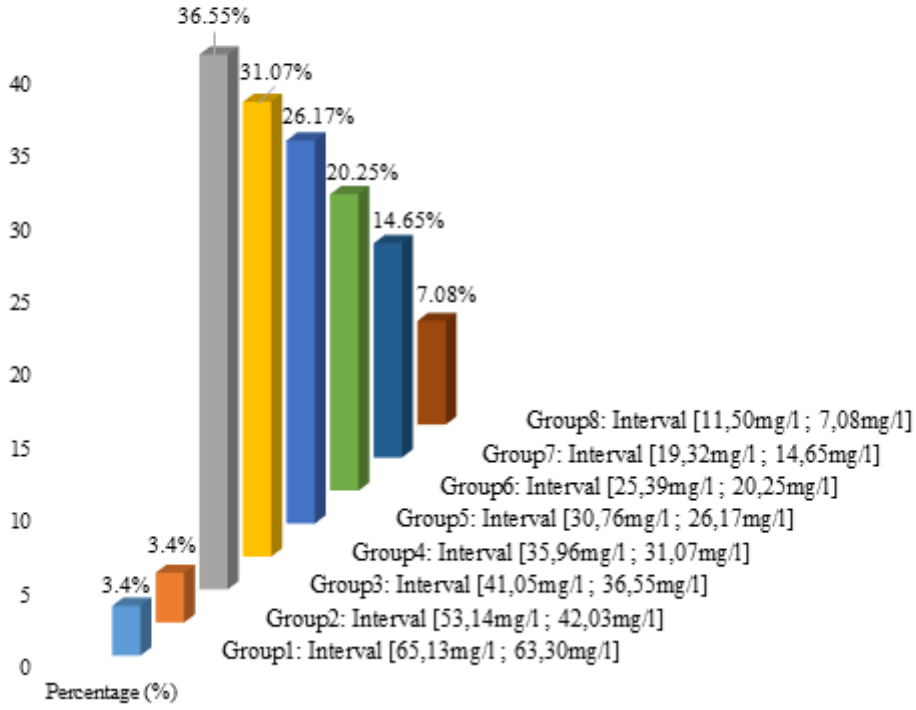

Figure 4

Phosphate released concentration by saffron isolates.

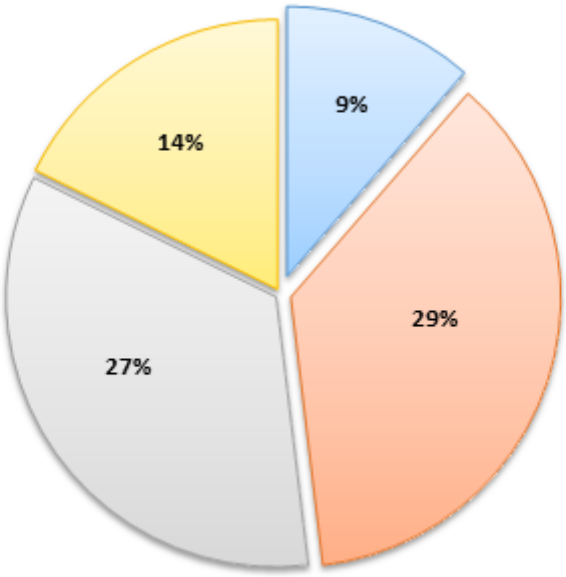

Figure 5

Biological nitrogen fixation activity.

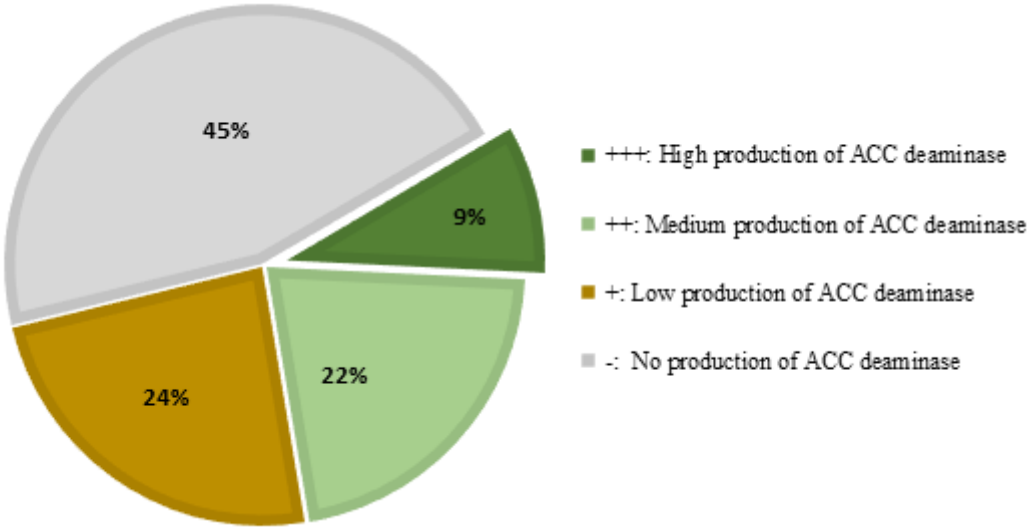

Figure 6

Aminocyclopropane -1-carboxylate (ACC) deaminase activity

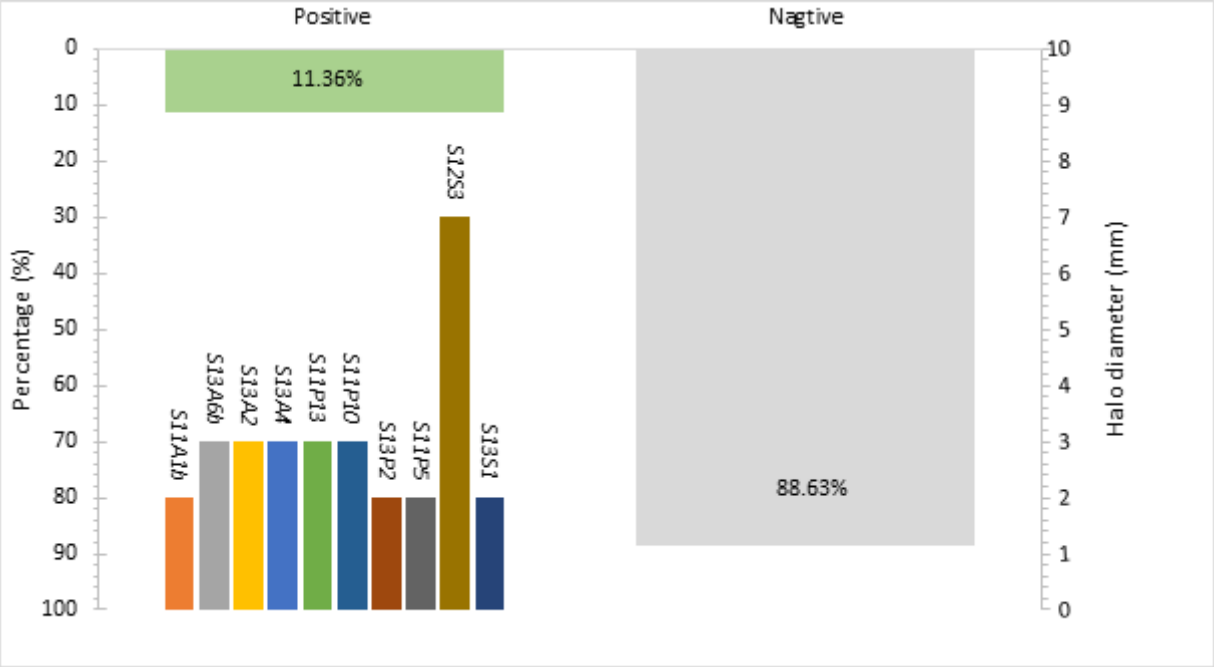

Figure 7

Cellulose activity

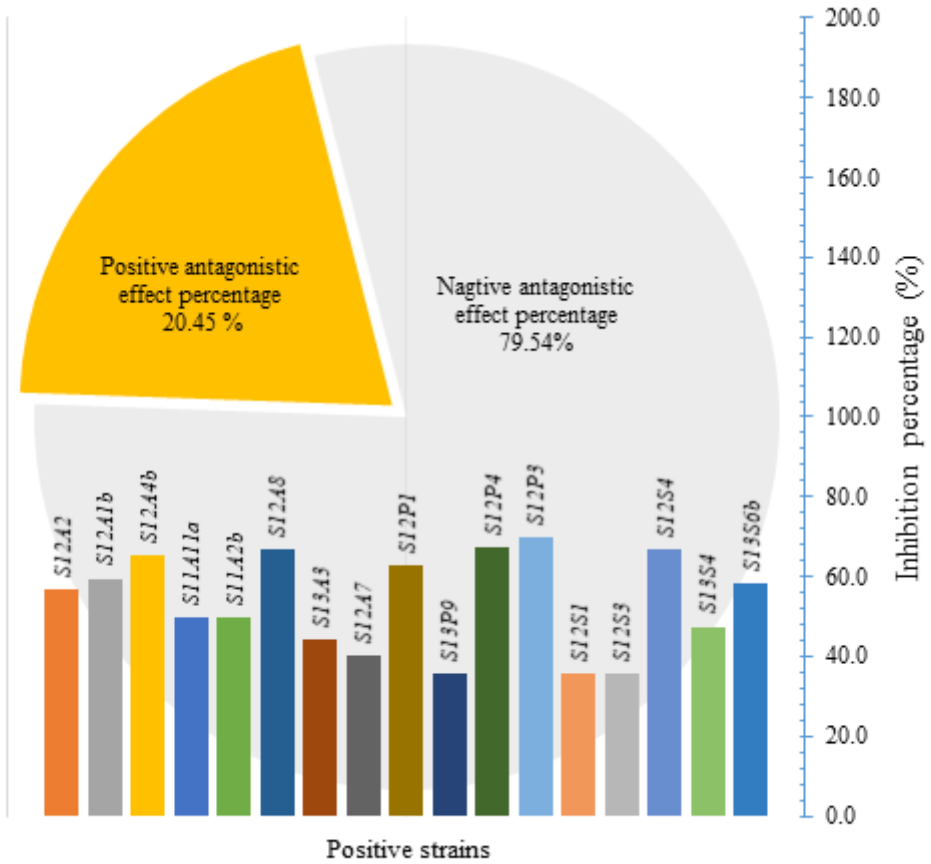

Figure 8

Antagonistic activity

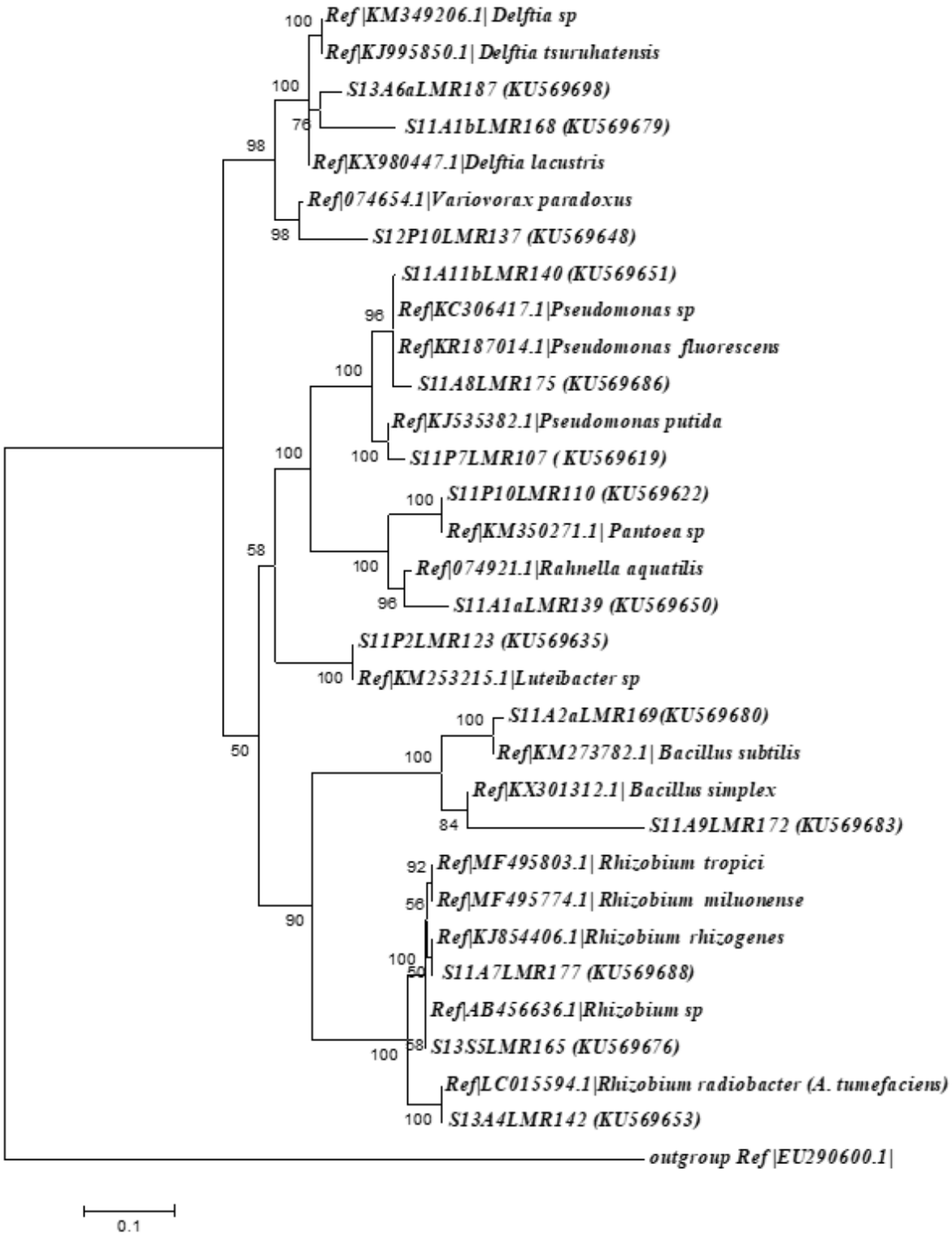

Figure 9

Phylogenetic tree based on the 16S rRNA gene sequences on the both sense corresponding to saffron PGPR. The reference sequences are shown by species name followed by the GenBank accession number. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model with 1000 bootstrap replications. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 14 representative sequences. Evolutionary analyses were conducted in MEGA6.

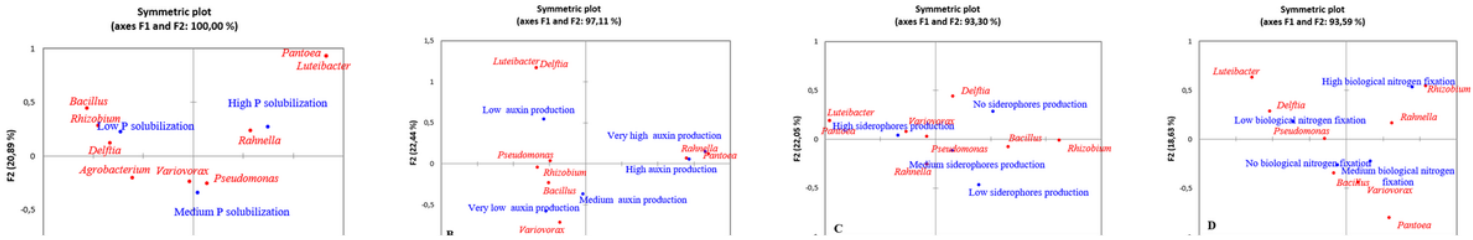

Figure 10

Correspondence analyses (CA) of the relationship between the isolate's genera and the biological activities tested. (A): Phosphate solubilization, (B): Production of auxin, (C): Siderophores production, (D): Biological nitrogen fixation, (E): Cellulose activity, (F): ACC deaminase activity and (G): Antagonistic activity.