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# Effects of growth-promoting endophytic *Methylobacterium* on development of *Citrus* rootstocks

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**Endophytic *Methylobacterium* spp. were inoculated on citrus seed and evaluated for their ability to promote growth of *Citrus limonia* and *Citrus sunki* seedlings under commercial nursery conditions. The germination rate and seedlings growth differed according to the combination between *Methylobacterium* species and citrus rootstock, showing that the interaction depends on their compatibility. *Methylobacterium* had no effect on germination of both rootstocks, except AR 1.6/2 that reduced the germination of *C. limonia*. On the other hand, some strains from citrus significantly promoted biomass production and height of aerial part of both rootstocks. The pathway of Indole-3-acetic acid (IAA) biosynthesis was identified in *M. mesophilicum* SR1.6/6 genome and this ability was confirmed in culture medium, suggesting that this mechanism is probably involved in growth promotion observed in present study. Recovery of strains in culture medium and ARDRA analysis confirmed the endophytic colonization of rootstocks by *Methylobacterium*. Our analyses of *C. limonia* and *C. sunki* seeds revealed that *Methylobacterium* is not vertically transferred to citrus plants. These results suggest that *Methylobacterium* can endophytically colonize the plant and have a potential for plant growth promotion under commercial nursery conditions. However, this growth promoting effect depends on specific interactions between *Methylobacterium* and citrus species.**

**Key words:** *Methylobacterium*, *Citrus*, plant-bacteria interaction, growth promotion, Indole-3-acetic acid (IAA) pathway.

## INTRODUCTION

*Methylobacterium* spp. are pink-pigmented facultative methylotrophic (PPFM) bacteria able to metabolize one-carbon compounds (C1), such as methanol, as well as C2, C3 and C4 compounds (Toyama et al., 1998;

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Lidstrom, 2001). Member of this genus are ubiquitous in natural environments and have been isolated from soil, air, freshwater, sediments and plant surface (Gallego et al., 2005; Weon et al., 2008; Veyisoglu et al., 2013; Madhaiyan and Poonguzhali, 2014). In addition, *Methylobacterium* species have been found in symbiotic association with plants, colonizing the inner tissues (Bulgari et al., 2014; Madhaiyan et al., 2015).

The establishment of *Methylobacterium* in association with the host is thought to directly influence seed germination and plant growth by mechanisms, such as cytokinin and auxin synthesis (Omer et al., 2004; Tani et al., 2012; Eevers et al., 2015), nitrogen fixation (Sy et al., 2001, Madhaiyan et al., 2009; Madhaiyan et al., 2014) and plant protection (Ardanov et al., 2012; Yim et al., 2014). In this context, *Methylobacterium* seems to be an important bacterial group to be employed for improvement of crop productivity.

Nowadays, the availability of genetic tools such as the genome sequencing and studies of central metabolism have attracted attention to the genetic manipulation of *Methylobacterium* for the production of various bioproducts (Fitzgerald and Lidstrom, 2003; Choi et al., 2008, Sonntag et al., 2015) as well as for symbiotic control of phytopathogens (Gai et al., 2009; Ferreira Filho et al., 2012). The occurrence of new pests and diseases has affected the yield and production cost of several crops around the world. However, the economic importance of crops such as *Citrus* requires changes in the management and treatment of plants and use of new technologies to improve the productivity and quality of fruits (Duenhas et al., 2002; Donadio, 2011).

Therefore, bacteria associated with plants play an important role in the adaptation of their hosts, either in natural environments or in stressful conditions, and may be used as a strategy to promote better association between some crops and the environment (Hallmann et al., 1997; Sturz and Nowak, 2000; Bhattacharyya and Jha, 2012). In this way, the use of plant growth-promoting bacteria could be a possibility to improve the productivity and the quality of citrus orchards, reducing the use of chemical inputs, generating economic and ecological benefits, since these products might often affect the environment negatively.

Endophytic *Methylobacterium* has been isolated from citrus plants (Araújo et al., 2002) and previous studies have proposed an interaction between these bacteria and *Xylella fastidiosa* (Lacava et al., 2004; Araújo et al., 2002).

In citrus, one possibility to improve the productivity of the orchards could be through the inoculation of plant growth promotion endophyte (PGPE) into the rootstocks thereby, accelerating growth and consequently reducing the time that the plant remains in nursery. Therefore, in the present study we analyzed the effects of *Methylobacterium* species on seed germination and growth of *C. limonia* and *C. sunki* under commercial nursery conditions.

## MATERIALS AND METHODS

### Bacterial strains, culture conditions and plant material

In the present study, the effect of *Methylobacterium mesophilicum* (SR1.6/6, SR1.6/13, ER1/21, AR5/1), *M. extorquens* (AR1.6/2, AR1.6/11) and *M. radiotolerans* (AR1.6/4) isolated from *Citrus sinensis*; *M. mesophilicum* (PR1/3) and *M. zatmanii* (PR3/8) isolated from *Citrus reticulate*; *M. fujisawaense* (D5) isolated from *Saccharum officinarum* and *M. hispanicum* (TP4/2) isolated from *Capsicum annuum* were evaluated on the citrus growth promotion. For this, the bacteria were cultivated at 28°C in CHOI medium containing ($g\ L^{-1}$) $(NH_4)_2SO_4$, $KH_2PO_4$, $Na_2HPO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, and trace elements, supplemented with methanol 0.5% (v/v) as carbon source (Choi et al., 1989). The experiments were performed in greenhouse under commercial conditions for rootstocks (Rangpur lime - *Citrus limonia* and Sunki Tangerine - *Citrus sunki*) production (certified seeds, sterilized substrate, protected environment from insect vectors) at Horticitrus - Seedling Nursery, Cordeirópolis, SP, Brazil (22°28'59.9"S and 47°26'52.1"W).

### *In vivo* assays

#### *Evaluation of the effects of Methylobacterium spp. on seedlings growth of Citrus rootstocks*

For seedlings production, the bark seeds (stored for 4 months in cold chamber at 5/8°C) was removed and the seeds treated with bacteria ($10^8$ CFU $mL^{-1}$) or CHOI medium (control 1), for 5 h at 28°C with agitation by an orbital shaker at 80 rpm, and planted in dibble tubes (3 × 12cm; 50 $cm^3$) containing commercial substrate pH 6.0 (Golden Mix 11, Amafibra, Holambra, SP, Brazil). Each treatment was composed of 4 replicates, where each replicate consisted of 10 seedlings (40 seedlings per treatment). The germination rate was estimated as the number of seedling growth per replicate.

After germination, only one seedling was replanted per dibble tube containing substrate pH 6.0 (Golden Mix 11, Amafibra, Holambra, SP, Brasil) and maintained in greenhouse under controlled relative humidity (70%) and temperature ranging from 19 to 40°C.

The effects of the endophytic *Methylobacterium* spp. on seedling growth were compared to the effects of the controls (CHOI and without inoculation). The seed germination rate was evaluated 30 days after inoculation of bacterial strains. The plant height was evaluated after 30, 90 and 120 days after germination, while the seedlings biomass (shoot and root) were evaluated only after 120 days.

#### *Isolation of Methylobacterium from seeds and inoculated Citrus rootstocks*

The bacterial communities inside citrus seeds were evaluated from fresh seeds and those stored for 4 months in cold chamber at 5/8°C, the latter usually used for rootstocks production. For this, 100 seeds from each citrus species were surface disinfected (70% ethanol for 1 min, sodium hypochlorite solution (2% available $Cl^-$) for 3 min, 70% ethanol for 30 s, two rinses in sterilized distilled water for 1 min) and triturated in sterile PBS solution containing ($g\ L^{-1}$) $Na_2HPO_4$, 1.44; $KH_2PO_4$, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4. Serial dilution was plated on CHOI medium amended with 50 µg $mL^{-1}$ of the fungicide carbendazim and incubated at 28°C for 15

days. After growth, colonies were picked out, purified by streaking on CHOI agar medium and stored for further evaluation.

The citrus rootstocks are used for grafting 120 days after seed planting. Therefore, after this time we evaluated the presence of inoculated *Methylobacterium,* in order to assess the potential of these bacteria to be transmitted aerial plant from inoculated rootstock, increasing the plant growth promotion. For this 10 seedlings per treatment were sampled, roots and shoots were separated and surface disinfection was performed as following: 70% ethanol for 1 min, sodium hypochlorite solution (2% available $Cl^-$) for 3 min, 70% ethanol for 30 s, two rinses in sterilized distilled water for 1 min.

Both roots and shoots tissues were cut in small pieces, triturated in sterile phosphate buffered saline (PBS) solution and incubated at 28°C for 1 h under agitation (100 rpm). Appropriated dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$) were plated onto CHOI medium supplemented with 50 µg $mL^{-1}$ of the fungicide carbendazim and incubated at 28°C for 15 days. The disinfection process was checked by plating aliquots of the sterile distilled water, used in the last wash, on CHOI and incubated under the same conditions. Colonies were purified by streaking in CHOI medium and stored in 70% glycerol solution at -80°C for further analysis.

### *Amplified ribosomal DNA restriction analysis (ARDRA) and molecular identification of endophytic bacteria*

The bacterial community isolated from seeds was identified by 16S rRNA gene sequencing using colony-PCR. After growth on culture medium, the isolates were transferred to a tube containing 200 µL of sterilized ultra pure water. The bacterial suspension was used as the source of DNA in PCR reactions. Primers PO27F (5´-GAGAGTTTGATCCTGGCTCAG -3´) and 1387R (5´-CGGTGTGTACAAGGCCCGGGAACG - 3´) (Heuer and Smalla, 1997) were used in 50 µl PCR reaction containing 10 X buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3) (Fermentas Life Sciences, Brazil), 0.2 mM dNTP, 3.75 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Fermentas Life Sciences, Brazil), 0.2 µM of primers and 2 µl of the boiled cells (15 min at 90°C in 80 µl $H_2O$). Amplifications were performed in thermal cycler (PTC 200, MJ Research - USA) programmed for an initial denaturation (94°C for 4 min) followed by 35 cycles of 94°C for 30 s, 62.5°C for 1 min, 72°C for 1 min and a final extension of 10 min at 72°C. A negative control (PCR mixture without bacterial DNA) was included in all analysis. The PCR fragments were purified with polyethylene glycol (PEG) (20% PEG 8000; 2.5 mM NaCl) and sequenced at Human Genome Research Center (HGRC), (Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil). The nucleotide sequences were compared with sequences information available in the GenBank database, by BLASTn.

In order to compare with inoculated bacteria, the isolates obtained from seedlings rootstock shoot were identified by ARDRA technique and 16S rRNA gene sequencing. For this, the 16S rRNA gene was amplified by *Methylobacterium* colony-PCR using PO27F and 1387R primers as described above. The PCR products were digested with 2 units of *Alu*I restriction enzyme (Life Technologies, Brazil) according to the manufacture´s recommendations. The reaction mixture was incubated at 37°C for 1 h. The restriction patterns were examined using 2.5% (w/v) agarose gel and stained with ethidium bromide.

### *In vitro* assays for production of plant growth promoting substances

#### *Auxin production*

Auxin production (IAA) was evaluated by the colorimetric method as described by Gordon and Weber (1951) with modifications. For this, the bacteria were grown in 10% TSB medium (10 mL) amended with 5 mM of L-tryptophan and incubated in the dark at 28°C for 72 h. Cells were harvested by centrifugation (8 000 x g for 5 min), the supernatant (900 µl) was treated with 600 µl of Salkowski reagent (50 mL of perchloric acid (35%) and 1 mL of $FeCl_3$ solution (0.5 M) and incubated for 30 min at room temperature in the dark. IAA was quantified using a spectrophotometer (Pharmacia Biotech Ultroespec 3000) at 530 nm of absorbance. The readings were normalized by using the standard curve with different concentrations of IAA (µg $mL^{-1}$). All analysis was performed in triplicate.

#### *Biological nitrogen fixation*

The ability to fix $N_2$ in *Methylobacterium* strains was evaluated in strains able to improve plant growth. The strains were inoculated in nitrogen-free semi-solid NFb medium (Döbereiner et al., 1995) and, after growth the bacterial cells were re-inoculated in nitrogen-free NFb medium. This procedure was repeated three times and strains able to form a growth pellicle in this medium were considered able to fix nitrogen.

#### *Phosphorus solubilization*

The ability to solubilize phosphorus was assayed according to Verma et al. (2001) with modifications. Ten microliters containing about $10^8$ CFU $mL^{-1}$ of bacterial strains grown in CHOI medium were inoculated onto agar medium containing inorganic phosphate (agar, 15 g; glucose, 10 g; $NH_4Cl$, 5.0 g; NaCl, 1.0 g; $MgSO_4.7H_2O$, 1 g; $Ca_3(HPO_4)_2$, 0.8 g) in a final volume of 1 L filled with distilled water, pH 7.2. Bacteria were incubated at 28°C for 7 days. The ability to solubilize inorganic phosphate was characterized by a clear halo around bacterial colonies. All analysis was performed in triplicate using a *Burkholderia seminalis* (TC3.4.2R3) as a positive control.

### Screening of genes associated to nitrogen fixation, IAA biosynthesis and ACC deaminase in *Methylobacterium mesophilicum* SR1.6/6

*M. mesophilicum* SR1.6/6 genome was published as announcement (Almeida et al., 2013) under access ANPA01000000 in NCBI (www.ncbi.nlm.nih.gov/) thus some genomic re-annotation of genes that encode enzymes related to nitrogen fixation, IAA biosynthesis and ACC deaminase were made in order to confirm the physiological results approached in this present work. Artemis software (Rutherford et al., 2000) was used to overview the genome annotation as well as KEGG: Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and Biocyc (http://biocyc.org/) to study target pathways, such as IAA biosynthesis and ACC deaminase.

### Statistical analysis

The ESTAT (Barbosa et al., 1992) statistical package was used for ANOVA followed by 2×2 factorial design (plant height × month) and Tukey's test at 5% probability level for means comparison.

## RESULTS

### Checking the occurrence of *Methylobacterium* spp. in rootstocks seeds

No bacteria were recovered from fresh seeds indicating that in citrus cultivable endophytic bacteria are not

**Table 1.** Effect of *Methylobacterium* strains on seed germination and growth of *Citrus limonia* under commercial nursery conditions.

| Bacterial species | Strains | Seed Germination (%) | Plant height (cm) | | | Root dry biomass (g) | Root fresh biomass (g) | Shoot dry biomass (g) | Shoot fresh biomass (g) |
|---|---|---|---|---|---|---|---|---|---|
| | | | 30 days | 90 days | 120 days | | | | |
| Control | | 71.1$^{ab}$ | 7.62$^{a}$ | 19.01$^{abc}$ | 23.42$^{de}$ | 0.40$^{bc}$ | 1.62$^{c}$ | 1.23$^{abcd}$ | 3.66$^{ab}$ |
| CHOI control | | 73.5$^{a}$ | 8.81$^{a}$ | 18.74$^{bc}$ | 24.37$^{bcd}$ | 0.39$^{bc}$ | 1.56$^{c}$ | 1.16$^{bcd}$ | 3.55$^{ab}$ |
| *M. mesophilicum* | SR1.6/6 | 69.2$^{ab}$ | 8.19$^{a}$ | 20.48$^{ab}$ | 24.42$^{bcd}$ | 0.48$^{a}$* | 2.07$^{a}$* | 1.40$^{ab}$ | 4.16$^{a}$ |
| | AR5/1 | 68.6$^{ab}$ | 8.15$^{a}$ | 19.57$^{abc}$ | 25.70$^{abc}$ | 0.34$^{c}$ | 1.54$^{c}$ | 1.14$^{bcd}$ | 3.59$^{ab}$ |
| | SR1.6/13 | 62.8$^{b}$ | 8.27$^{a}$ | 18.75$^{bc}$ | 23.63$^{cd}$e | 0.37$^{bc}$ | 1.70$^{bc}$ | 1.09$^{cb}$ | 3.72$^{ab}$ |
| | ER1/21 | 70.8$^{ab}$ | 8.19$^{a}$ | 18.50$^{bc}$ | 24.02$^{cd}$ | 0.32$^{c}$ | 1.56$^{c}$ | 1.04$^{cd}$ | 3.74$^{ab}$ |
| | PR1/3 | 69.4$^{ab}$ | 8.69$^{a}$ | 19.67$^{abc}$ | 23.84$^{cd}$ | 0.40$^{bc}$ | 1.69$^{bc}$ | 1.27$^{abc}$ | 4.01$^{a}$ |
| *M. extorquens* | AR1.6/2 | 47.85$^{c}$* | 8.46$^{a}$ | 19.55$^{abc}$ | 23.18$^{de}$ | 0.41$^{bc}$ | 1.59$^{c}$ | 1.24$^{abcd}$ | 3.83$^{ab}$ |
| | AR1.6/11 | 63.4$^{b}$ | 7.83$^{a}$ | 21.02$^{a}$* | 26.56$^{a}$* | 0.45$^{ab}$ | 2.00$^{ab}$* | 1.40$^{ab}$ | 4.32$^{a}$ |
| *M. radiotolerans* | AR1.6/4 | 67.9$^{ab}$ | 8.10$^{a}$ | 20.29$^{ab}$ | 26.41$^{ab}$ | 0.43$^{abc}$ | 1.71$^{bc}$ | 1.27$^{abc}$ | 3.96$^{ab}$ |
| *M. zatmanii* | PR3/8 | 68.7$^{ab}$ | 9.13$^{a}$ | 19.24$^{abc}$ | 25.21$^{abcd}$ | 0.33$^{c}$ | 1.50$^{d}$ | 1.11$^{cd}$ | 3.41$^{ab}$ |
| *M. fujisawaense* | D5 | 70.1$^{ab}$ | 8.47$^{a}$ | 17.90$^{c}$ | 23.93$^{cd}$ | 0.39$^{bc}$ | 1.61$^{c}$ | 1.14$^{bcd}$ | 3.97$^{ab}$ |
| *M. hispanicum* | TP4/2 | 68.5$^{ab}$ | 8.60$^{a}$ | 18.57$^{bc}$ | 21.73$^{e}$ | 0.39$^{bc}$ | 1.63$^{c}$ | 0.99$^{d}$ | 3.06$^{b}$ |

*Means followed by the same letter show no significant differences by the Tukey's test at $p \leq 0.05$.

vertically transferred from plant to plant. However, bacteria were isolated from seeds stored for 4 months. This community ranged from $0.81.10^3$ to $0.76.10^1$ UFC $g^{-1}$ for *C. limonia* and *C. sunki*, respectively. Based on 16S rRNA gene sequencing, the microbial community from stored seeds of *C. limonia* and *C. sunki* was composed of *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Streptomyces* and *Mycobaterium*. Isolates close related (pink pigmented) to *Methylobacterium* spp. were not found, indicating that this group is not able to colonize the seeds under these conditions. No differences in bacterial communities were observed between *C. limonia* and *C. sunki.*

### Effects of *Methylobacterium* spp. on seed germination and seedlings growth of *C. limonia*

The seed germination ranged from 47.85 to 73.5%. The inoculation of *M. extorquens* AR1.6/2 reduced the seed germination, while the others strains have no effect on this plant growth parameter (Table 1). However, the presence of this *M. extorquens* AR1.6/2 strain has no negative effect on plant growth (Table 1) indicating that this bacterium could be inoculated only after seed germination.

*M. extorquens* AR1.6/11 and *M. mesophilicum* SR1.6/6 inoculation resulted in plant growth promotion. The strain AR1.6/11 was able to increase plant height after 90 and 120 and root fresh biomass at 120 days, while the strain SR1.6/6 increased root dry and fresh biomass at 120 days (Table 1). The TP4/2 strain promoted a negative effect on height plant 120 days after inoculation (Table 1). Based on these results, the strains AR1.6/11, AR1.6/2, SR1.6/6 and TP 4/2 were selected for further analysis in *C. sunki*. The colonization of rootstocks seedlings by AR1.6/11, AR1.6/2 and SR1.6/6 was evaluated 120 days after inoculation. Except for AR1.6/11 that was recovered only from *C. limonia* roots, all strains were re-isolated from shoots and roots tissues of both rootstocks at $10^2$ CFU $g^{-1}$ of plant tissue. The identity of the strains was successfully confirmed by ARDRA technique with *Alu*I restriction enzyme.

### Effects of *Methylobacterium* spp. on seed germination and seedlings growth of *C. sunki*

Unlike our finds in *C. limonia*, the strain AR1.6/2 has no negative effect on germination of *C. sunki* seeds (Table 2). All treatments induced seed germination when compared with the control, but this effect was due to the CHOI medium, since the germination rate between CHOI medium and bacterial inoculated seeds were not significantly different (Table 2). The strains SR1.6/6, AR1.6/2 and AR1.6/11 increased the plant height after 120 days. In addition, AR1.6/2 strain enhanced shoot fresh biomass (Table 2). *C. sunki* inoculated with

**Table 2.** Effect of *Methylobacterium* strains on seed germination and growth of *Citrus sunki* under commercial nursery conditions.

| Bacterial species | Strains | Germination (%) | Plant height (cm) | | | Root dry biomass (g) | Root fresh biomass (g) | Shoot dry biomass (g) | Shoot fresh biomass (g) | IAA production ($mg.ml^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 30 days | 90 days | 120 days | | | | | |
| Control | | 61.7[b] | 5.05[a] | 11.07[c] | 19.82[b] | 0.21[a] | 1.02[ab] | 1.20[ab] | 3.40[c] | |
| CHOI control | | 80.7[a] | 5.40[a] | 12.17[bc] | 19.71[b] | 0.15[b] | 0,93[ab]c | 1.13[b]c | 3.38[c] | |
| *M. mesophilicum* | SR 1.6/6 | 80.0[a] | 5.40[a] | 12.72[ab] | 21.37[a]* | 0.21[a] | 1.05[a] | 1.22[ab] | 3.80[ab] | 2.3 |
| *M. extorquens* | AR 1.6/11 | 81.0[a] | 5.25[a] | 12.87[a]* | 20.45[a]* | 0.19[ab] | 0.83[b]c | 1.21[ab] | 3.66[abc] | 1.8 |
| *M. extorquens* | AR 1.6/2 | 83.7[a] | 5.57[a] | 12.60[ab] | 21.22[a]* | 0.19[ab] | 1.00[ab] | 1.29[a] | 3.90[a]* | 2.1 |
| *M. hispanicum* | TP 4/2 | 79.2[a] | 5.57[a] | 12.32[bc] | 19.55[b] | 0.18[ab] | 0.92[ab]c | 1.05c | 3.36c | |

*Means followed by the same letter show no significant differences by the Tukey's test at $p \leq 0.05$.

TP4/2 strain no presented negative effect on height plant as observed to *C. limonia* (Table 2).

### *In vitro* plant growth promoting traits

Bacterial strains that exhibited positive effect on seedlings growth were evaluated *in vitro* for their ability to synthesize indole-3-acetic acid (IAA), phosphorus solubilization and $N_2$ fixation. The strains SR 1.6/6, AR 1.6/2, and AR 1.6/11 produced IAA at 2.3, 2.1 and 1.8 mg $ml^{-1}$ (Table 2), respectively. A clear zone halo around the colonies was not observed, indicating that these strains are not able to solubilize inorganic phosphorus. Although a growth had been observed in nitrogen-free medium after the first bacterial inoculation, it was not observed after successive inoculation in this medium, indicating that these strains were not able to fix nitrogen.

*M. mesophilicum* SR 1.6/6, which had the genome published (Almeida et al., 2013) was one of the most effective strain in promoting height plant and root biomass. We screened this genome and found at least 22 genes potentially related to IAA synthesis. According to these information, there are three possible pathways in *M. mesophilicum* SR 1.6/6 for the production of indole-acetic acid, (1) from idole-3-acetonitrile, (2) from indole-3-acetamide, (3) tryptamine (Figure 1). In the first route, the Indole-3-acetonitrile is catalyzed to Indole-acetic acid by Nitrilase/cyanide hydratase and N-acyltransferase. Through the second route, Indole-3-acetamide can be synthetized from Indole-3-acetonitrile by a nitrile-hydratase (subunit alpha and beta), and then to IAA by amidase. In the third route, IAA could be synthetized via tryptamine and indoleacetaldehyde. We did not find any Trp decaborxylase and tryptophan-2-monooxygenase enzymes to metabolize L-Tryptophan in *M. mesophilicum* SR 1.6/6 genome.

In addition, an evaluation of the SR1.6/6 genome showed that *nif* and *fix* gene clusters are not present in this strain, but a gene that encodes an ACC deaminase is present in the *M. mesophilicum* SR 1.6/6 genome, indicating that the suppression of stress response could be a mechanism present in these endophytic bacteria related to plant growth promotion.

## DISCUSSION

In sustainable agriculture the application of growth-promoting bacteria may increase productivity and quality of the crop, reducing the costs associated with the use of chemicals and the environmental impacts. Previously studies had shown that *Methylobacterium* species have the ability to induce shifts in physiological traits of their hosts, promoting positive, negative or any effect on seed germination and/or on plant development (Holland and Polacco, 1994, Abanda-Nkpwatt et al., 2006; Lee et al., 2011; Pohjanen et al., 2014). The specificity of plant-bacteria interaction has been considered an important factor for the generation of these different effects on plant host and the success of this approach for plant production.

Our results revealed that some *Methylobacterium* strains originally isolated from *Citrus sinensis* promoted significantly the height and biomass of *C. limonia* and *C. sunki*, as already described in other plant species with native *Methylobacterium* (Madhaiyan et al., 2005; Lee et al., 2006; Madhaiyan et al., 2015). In fact, some authors emphasize the need to employ native isolates or isolates adapted to their hosts, justifying the higher capacity of colonization and lower risk in the introduction of exogenous microorganisms in the plants (Enebak et al., 1998; Khalid et al., 2004). However, as we observed, bacterial strains isolated from *Citrus,* such as

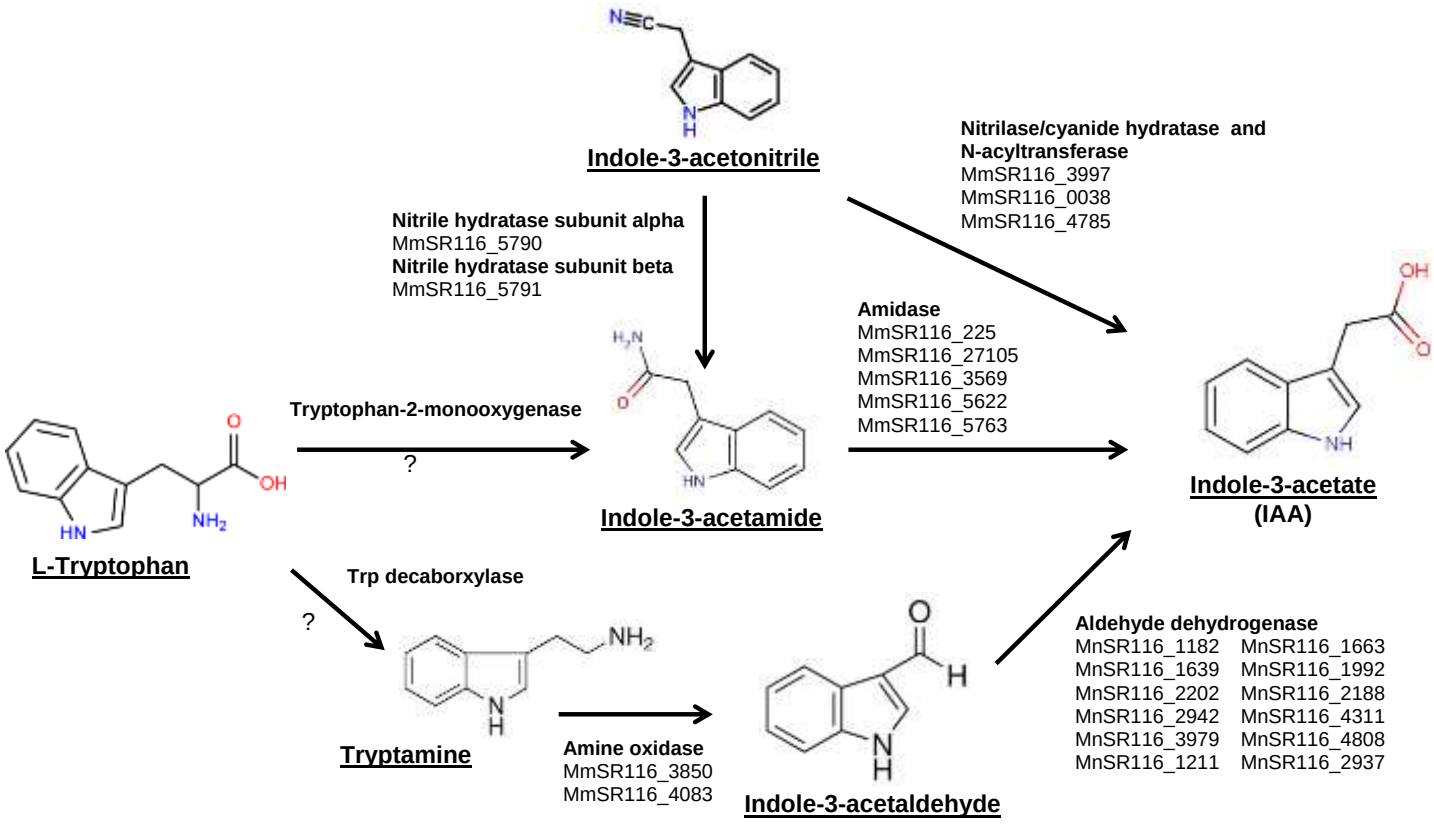


**Figure 1.** Predicted IAA biosynthesis pathways in *M. mesophilicum* SR 1.6/6.

AR1.6/2 induced negative effects on seed germination of *C. limonia,* but had no effect on plant growth of both *C. limonia* and *C. sunki.* In addition, this strain promoted the plant height and enhanced the shoot biomass.

During plant colonization, the cross-talking between bacteria and the host seems to has a key function (Rosenblueth and Martinez-Romero, 2006), which suggest that plants can communicate specifically to attract microorganisms for their own ecological and evolutionary benefits (Compant et al., 2005). Thus, endophytes may differentially express genes required to colonize the host and modulate plant genes stimulating plant growth (Rosenblueth and Martinez-Romero, 2006). In many cases, the plant responses to endophytes seem to be conditioned by the plant genotype (Nowak, 1998; Rosenblueth and Martinez-Romero, 2006). Besides, the environmental conditions and balance between other microorganisms can also be decisive for the final effect (Azevedo, 1998; Andrews and Harris, 2000; Montesinos et al., 2002).

Plant growth promotion by *Methylobacterium* isolates has been attributed to the nitrogen fixation and production of phytohormones such as auxins and gibberellins (Sy et al., 2001; Madhaiyan et al., 2014; Eevers et al., 2015).

In the present study, all *Methylobacterium* strains with potential to promote plant growth were able to synthetized IAA, but could not to solubilize phosphorus and growth in nitrogen-free medium. This result suggested that, although many factors, such as species-specific recognition may be related to the ability of bacteria to promote plant growth, the citrus growth promotion, based on height and biomass analysis seems to be only related to IAA production and ACC deaminase. These results were confirmed by the analysis of the genome of SR1.6/6 strain. Besides, a gene that encodes an ACC deaminase is present in the *M. mesophilicum* SR1.6/6 genome, indicating it could act in the suppression of the stress response resulting in plant growth promotion. The IAA plant hormone, under biotic and abiotic stress conditions, is able to activate the enzyme 1-aminocyclopropane 1-carboxylate (ACC) synthase, which synthetize ACC that is converted to ethylene by the enzyme ACC oxidase (Wang et al., 2002). The enzyme ACC deaminase, present in many endophytes, is known to compete with ACC oxidase, modulating the ethylene levels in plants, reducing the stress response triggered by ethylene and promoting plant growth under stress conditions (Hardoim et al., 2008).

The inoculated *Methylobacterium* strains were not present in seeds, but were able to colonize roots and

shoots endophytically after seed inoculation, as evident by their recovery from seedlings obtained from inoculated seeds. Generally, in experiments carried out in greenhouses the inoculated bacteria are subject to competition with other microorganisms present in the soil or inside the plant, as described previously (Omer et al., 2004), suggesting that the evaluated *Methylobacterium* strains present competitiveness to establish inside the plant even under this environment condition. The AR 1.6/11 was recovered only from root of inoculated plants, suggesting that the ability to colonize the plant shoot is not necessary for promoting the plant growth, since this strain was able to increase the plant height.

Our results obtained by *Methylobacterium* seed inoculation highlights the potential use of these bacteria to stimulate seed germination, plant height and biomass production of *C. limonia* and *C. sunki* under commercial nursery conditions. We observed that the non-specificity of bacteria-plant interaction could lead to an undesired effect on the plant. Thus, is very important to conduct a selection of strains in a breeding program and being careful to not extrapolate the results produced on one plant to another host. Future proposals may be the development of a bacterial consortium with specific strains that could be effective in citrus growth promotion and protection generating a low-cost and environmentally safe product.

## Conflict of Interests

The authors have not declared any conflict of interests.

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