## Alternative rooting induction of semi-hardwood olive cuttings by several auxin-producing bacteria for organic agriculture systems

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#### Abstract

Southern Spain is the largest olive oil producer region in the world. In recent years organic agriculture systems have grown exponentially so that new alternative systems to produce organic olive cuttings are needed. Several bacterial isolates, namely *Pantoea* sp. AG9, *Chryseobacterium* sp. AG13, *Chryseobacterium* sp. CT348, *Pseudomonas* sp. CT364 and *Azospirillum brasilense* Cd (ATCC 29729), have been used to induce rooting in olive semi-hardwood cuttings of Arbequina, Hojiblanca and Picual cultivars of olive (*Olea europea* L). The first four strains were previously selected as auxin-producing bacteria and by their ability to promote rooting in model plants. They have been classified on the basis of their 16S rDNA gene sequence. The known auxin producer *A. brasilense* Cd strain has been used as a reference. The inoculation of olive cuttings was performed in two different ways: (i) by dipping cuttings in a liquid bacterial culture or (ii) by immersing them in a paste made of solid bacterial inoculant and sterile water. Under nursery conditions all of the tested bacterial strains were able to induce the rooting of olive cuttivars responded differently depending on the bacterial strain and the inoculation method. The strain that consistently gave the best results was *Pantoea* sp. AG9, the only one of the tested bacterial strains to express the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The results are also discussed in terms of potential commercial interest and nursery feasibility performance of these strains.

Additional key words: IBA; nursery; organic agriculture; plant growth promoting rhizobacteria (PGPR).

## Introduction

Synthetic chemical compounds used to prevent plant pest and disease symptoms or to fertilize plants can be detrimental to human health and they may also persist in natural ecosystems (Glick *et al.*, 2007; Pretty, 2008; Glick, 2010). In the last decade there has been an impetus to use biological agents such as microorganisms (bacteria and mycorrhizal fungi) to replace conventional chemical products. In fact, in organic agriculture systems the use of synthetic chemical products is forbidden [Commission Regulations (EC) 834/2007 (OJ, 2007) and 889/2008 (OJ, 2008)].

Free-living soil bacteria that provide some benefits to plants are often referred to as PGPR (plant growth

promoting rhizobacteria) (Kloepper & Schroth, 1978). They may stimulate plant growth and development indirectly through their ability to prevent or decrease the damage to plants (Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001) for example by synthesizing antibiotics that limit pathogen damage to plants or directly through biological activities as iron sequestration, phosphate solubilisation, the production of plant growth hormones such as indole-3-acetic acid (IAA), the most common auxin stimulating root elongation (Patten & Glick, 2002; Spaepen *et al.*, 2007; Glick, 2010) or by lowering the growth-inhibiting levels of ethylene in plant tissues by production of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase (Glick *et al.*, 1998; Penrose *et al.*, 2001; Toklikishvili *et al.*, 2010).

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Abbreviations used: ACC (1-aminocyclopropane-1-carboxylate); CFU (colony-forming unit); DAI (days after inoculation); DF (Dworkin and Foster); IAA (indole-3-acetic acid); IBA (indole-3-butyric acid); NB (nutrient broth); PGPR (plant growth promoting rhizobacteria).

*Azospirillum brasilense* has been widely used as a PGPR with different crops owing, not only to its ability to fix nitrogen, but also to its capacity to produce and release a broad spectrum of plant growth regulators (Holguin & Glick, 2003). For example *A. brasilense* strain Cd is a very efficient IAA producer (Li *et al.*, 2005).

Olive is the most important crop in the Andalusian region of southern Spain with about 1.5 million ha devoted to its cultivation, making this region the main olive oil producer in the world. 'Arbequina', 'Hojiblanca' and 'Picual' are the three main olive cultivars used in this area (Barranco et al., 2005). In recent years, organic agriculture systems in Spain, especially in Andalusia, have grown exponentially. Conventional plantations of olive trees are easily converted to a system of organic agriculture, so that, the area of organic olive groves in this region includes nearly 50,000 ha. Nowadays, the olive plant propagation system adopted by nursery growers mainly uses semiwoody olive cuttings. Although this system depends on several factors (the olive cultivar, the physical properties and temperature of the rooting media, the type of cutting material, the season, etc.), in general it provides a high level of efficiency. The induction of rooting with indole-3-butyric acid (IBA) is not authorized in organic agriculture. So that, it is necessary to find an alternative system to improve the percentage of natural rooting of the different cultivars. In this sense, some attempts have been made by using different organic products. Thus, Suarez et al. (2002) did not obtain higher rooting percentages with any of the products tested (commercial products included Auxym oligo, Roots and Myco+AA) compared to when no treatment was applied with 'Picual', 'Manzanilla' and 'Picudo'. Centeno & Gomez del Campo (2008) reported the use of some natural auxin sources such as germinating seeds, fungi and alga extract with 'Cornicabra'. However, they did not obtain higher rooting percentages than the IBA control with any of the products used with the exception of 'Terrabal Organico<sup>TM</sup>' which is obtained from a soluble fraction of an extract of macerated cereal seeds. Nevertheless this compound could produce a toxic effect on cuttings when treatment duration was increased and the subsequent influence on leaf growth has been not yet established.

Here we report a study of the rooting efficiency in olive cuttings of 'Arbequina', 'Hojiblanca' and 'Picual' by inoculation with different bacteria selected on the basis of their physiological traits as PGPR.

#### Material and methods

#### Bacterial strains used and culture conditions

Strains AG9 and AG13 were isolated from plant nursery well water (Montero-Calasanz *et al.*, 2006). Strains CT348 and CT364 were isolated from rhizosphere soil samples collected in an organic olive grove located at Mairena del Aljarafe (Sevilla). *A. brasilense* Cd (ATCC29729) was obtained from the American Type Culture Collection.

Bacterial isolates were grown in nutrient broth (NB) medium and *A. brasilense* Cd in modified NFb medium (Döbereiner, 1980). When needed, culture media were supplemented with 100 mg L<sup>-1</sup> L-tryptophan as an IAA biosynthesis precursor. All of the bacterial strains were incubated at 28°C on a shaker at 180 rpm for 48 h until they reached about 10° colony-forming unit (CFU) mL<sup>-1</sup>. IAA production was assessed in the bacterial cultures before they were used in the assays. Bacterial solid peat inoculants were prepared as previously described (Daza *et al.*, 2000). Viable PGPR cells from inoculants bags were estimated by plating 10-fold serial dilutions on NB agar and ranged from 0.5 to  $1 \times 10^9$  CFU g<sup>-1</sup>.

# *In vitro* analysis of putative PGPR characteristics

*IAA production.* IAA quantification was determined by the modified method described by Patten & Glick (2002). Briefly: 150 mL of  $H_2SO_4$  were mixed with 250 mL of distilled water and then 7.5 mL of cold 0.5 M FeCl<sub>3</sub> were added. This reagent was added to a culture supernatant (4:1 v/v reagent vs supernatant), mixed gently and, after 20 min in the dark, the absorbance was measured at 535 nm. The concentration of each sample was calculated from a standart plot ranging from 0.01 to 0.4 µg mL<sup>-1</sup> pure IAA (Sigma).

**Inorganic phosphorus solubilisation assay.** The ability of the bacterial strains to solubilise tricalcium orthophosphate was tested on PVK medium (Pikovskaya, 1948). For each isolate 10  $\mu$ L of a bacterial suspension (about 10<sup>9</sup> CFU mL<sup>-1</sup>) were dropped onto a Petri dish containing solid PVK medium. After incubation at 28°C for 3 days the clearing of the initially turbid medium indicated the presence of a phosphate solubilizing bacterial isolate.

Siderophore production assay. Semi-quantitative analysis of siderophore production by the bacterial strains was performed following the chrome azurol S (CAS) method of Alexander & Zuberer (1991). Ten microlitres of bacterial inoculum was spotted onto Petri plates and incubated at 28°C for three days; the discoloration of the medium (blue to yellow-orange) indicated siderophore-producing bacterial strains.

**Determination of ACC-deaminase activity.** ACCdeaminase activity was determined according to Penrose & Glick (2003). Bacterial cultures grown overnight (28°C, 180 rpm) in tryptic soybean broth (TSB) were centrifuged and pellets were washed twice with Dworkin and Foster (DF) salts minimal medium without nitrogen. Then, the cells were suspended in DF salts minimal medium with 3.0 mM ACC, which induces ACC deaminase activity, and incubated overnight under the same conditions. Cultures were then washed twice in 0.1 M Tris-HCl, pH 7.6. and finally suspended in 0.1 M Tris-HCl, pH 8.5. Then, 30 mL of toluene was added, to labilize the cell membranes, and the cell suspension was vortexed.

Using two tubes for each bacterial strain, 20 mL of 0.5 M ACC was added in one of the tubes. Both tubes were incubated at 30°C for 15 min. Following the addition of 1 mL of 0.56 M HCl, the mixture was vortexed and centrifuged. One mL of supernatant was mixed with 800 mL of 0.56 mM HCl, and 300 mL of 0.2% 2.4-dinitrophenyl-hydrazine in 2 M HCl was added. The mixture was vortexed and incubated at 30°C for 30 min. Two milliliters of 2 M NaOH were added for colour development which was measured at 540 nm. The absorbance of the tube without ACC was subtracted from the absorbance of the tube plus ACC and then compared to an a-ketobutyrate standard curve to calculate the amount of a-ketobutyrate generated.

#### **Gnobiotic assays**

**Rooting assay.** The effects of bacterial strains on the development of adventitious roots on mung bean cuttings (*Vigna radiata*) were assessed as described by Mayak *et al.* (1999), with minor modifications. Mung bean seeds (Wilczek) provided by Penelas Legumes, S.A. (León, Spain) were disinfected by 30 s treatment with 96% ethanol, 6 min with 6% sodium hypochlorite and six washes with sterile distilled water. Then seeds were placed on water agar plates (1% agar) at 28°C for 24-48 h in darkness to stimulate the seed germination. Seedlings were sowed individually in glass tubes  $(3 \times 25 \text{ cm})$  with cotton plugs containing quartz sand and nutrient solution (Rigaud & Puppo, 1975) under sterile conditions. Tubes were maintained in a growth chamber (Sanyo Electric Co., Japan) at 20-25°C, 80% RH and photoperiod of 12 h (4 LS). After 1 week, stems were cut, above the neck. Each cutting (8-10 cm in length) was placed in a glass tube  $(2.5 \times 15 \text{ cm})$  with cotton plug containing 6 mL sterile distilled water (negative control) or 6 mL bacterial suspensions (bacterial cultures centrifuged, washed twice with sterile distilled water and suspended in the same volume of water). Ten cuttings per treatment were incubated during 10 days in a growth chamber under the environmental conditions described previously. After this period, the number and length of adventitious roots were measured and analysed.

Canola root elongation assay. The effect of bacterial strains on root elongation from canola (Brassica napus) seeds was assessed by the method outlined by Patten & Glick (2002). Canola seeds (Thunder) provided by Department of Biology of the University of Waterloo (Ontario, Canada) were disinfected by 1 min treatment with 70% ethanol, 10 min with 1% sodium hypochlorite and six washes with sterile distilled water. Seeds were incubated with 4 mL of a suspension of each of the bacterial cells (final absorbance at 600 nm equals 0.15) in sterile 0.03 M MgSO<sub>4</sub> or in just sterile 0.03 M MgSO<sub>4</sub> (control) for one hour. Then, seeds were placed into sterile growth pouches (Mega International, Minneapolis, MN, USA) filled with 12 mL of distilled water and placed in a growth chamber under the same environmental conditions previously described. Each bacterial assay (nine growth pouches with eight treated seeds each) with its correspondient control, was performed independently. Two empty growth pouches (water only) were placed at the ends of each rack so that plants at the end of a rack were not subjected to extremes of light or air circulation. Primary root lengths were measured and analysed after 7 days. Seeds that had failed to germinate 3 days after they were sown were marked, and roots that subsequently developed from these seeds were not included in the final root length calculation.

*Olive nursery assays.* Three nursery assays were carried out in fall of 2006 (Assay 1), winter of 2006 (Assay 2) and summer of 2007 (Assay 3), using 'Arbequina', 'Hojiblanca' and 'Picual' olive semi-hardwood cuttings (12-15 cm long) with two pairs of

leaves at their tops. These were collected from vigorous one-year-old shoots of pathogen-free, 10-15 year old trees in Burguillos (Seville, Spain). The assays were performed in a commercial nursery taking advantage of its propagation squeme. Cuttings were inoculated in two different ways: in treatment 1, the cutting basal ends (1 cm) were submerged for 24 h in a bacterial culture whereas in Treatment 2, they were basally immersed for 4-5 s in a mixture of peat-based solid inoculant and sterile water just to form a film on the cutting basal end. In each assay, negative control by basal immersion in nursery water for 24 h and positive control by basal application of IBA solution (3000 mg  $L^{-1}$ ) for 5 s were used. Cuttings were planted at 2 cm depth in alveolar trays of 130 alveolus of 2 cm  $long \times 2$  cm wide  $\times 4$  cm depth containing mixed damp coconut fibre and perlite (3:1) (Assays 1 and 3). In Assay 2 only damp coconut fibre was used in order to simplify the transplant from alveolus to pots according to a nurseryman's request.

After planting, cuttings were placed in a propagation tunnel, built inside a climatised greenhouse, provided of electronic controll of air (20°C) and bed (22°C) temperatures. The cuttings were intermittently moistured by spraying water from 0.3 mm nozzles controlled by an electronic sensor. Two leaf treatments of aqueous solution of 0.3% Brotomax<sup>®</sup> (8% N, 1.8% Cu, 0.75% Mn, 0.5% Zn and natural plant extract, from Campbells Fertilisers, Australia) were applied on weeks 4<sup>th</sup> and 8<sup>th</sup> of the mist period.

All bacterial inoculants, plus the negative and positive controls had two replicates of 130 cuttings each, and were randomly distributed in the propagation tunnel. After 60 days ('Arbequina' and 'Picual') or 74 days ('Hojiblanca'), the porcentage of rooted cuttings per treatment was calculated.

#### Strain identification

Specific primers for 16S rDNA (forward primer b341: 5'-CCTACGGGAGGCAGCAG-3' and reverse primer b758: 5'-CTACCAGGGTATCTAATCC-3') (Juck *et al.*, 2000) were used for the identification of each bacterium. Isolation of genomic DNA was done using the 'Wizard Genomic DNA Purification Kit' (Promega, USA) in accordance with the manufacturer's specifications. PCR amplification was performed by using 'Go Taq Master Mix' from Promega and the PCR conditions were: one cycle at 94°C for 1 min, and 29 cycles at 92°C for 40 s, 55°C for 40 s, 75°C for 1.5 min, followed by a final 5 min at 75°C.

DNA sequencing was performed by the DNA sequencing facility at the Department of Biology of the University of Waterloo (Ontario, Canada).

The 16S rDNA gene sequence was edited and assembled by BioEdit v.7.0.9.0. software (Hall, 1999), carrying out an alignment with those of phylogenetically closely related organisms (as determined by EzTaxon server) (Chun *et al.*, 2007). The 16S rRNA gene sequences were deposited at DDBJ/EMBL/GenBank database.

#### Statistical analyses

Analysis of variance (ANOVA) was performed for the olive nursery assays and for the gnobiotic mung bean assay data, using the software program Statistix (version 8.0, NH Analytical Software, USA) for Windows. When the analysis of variance showed significant treatment effect, the least significant difference test (LSD, p < 0.05) was applied to make comparisons between the means. A Student's t-test was used to assess differences in root length of canola seeds between inoculated and non-inoculated growth pouches.

## Results

#### Strain characterization and identification

Bacterial isolates were characterised for a range of plant growth promoting traits including auxin and siderophore production, phosphorus solubilization, and ACC deaminase activity (Table 1). All of the strains tested were auxin producers, with the maximum amount of IAA being accumulated at the stationary growth phase. Strain *Pseudomonas* sp.CT364 was the only phosphorus solubiliser and the best siderophore producer. Strains *Pantoea* sp. AG9 and *Chryseobacterium* sp. CT348 produced siderophores but to a lesser extent. The capacity to produce ACC deaminase was only exhibited by strain AG9.

Comparing the 16S rDNA gene sequences of the isolates with the Eztaxon (http://www.Eztaxon.org) database, the highest similarities were obtained with members of well known plant associated bacterial

Bacterial strain <sup>1</sup>	IAA production <sup>2</sup> (mg L <sup>-1</sup> )	Siderophore production <sup>3</sup>	Phosphorus solubilization <sup>3</sup>	ACC <sup>4</sup> deaminase activity	Microbial group⁵
A.brasilense Cd ATCC 29729	$20 \pm 7.6$	_	_	_	
AG9 (EU336938)	$21 \pm 15$	+	_	+	Pantoea sp (99%)
AG13 (EU336941)	$7 \pm 2.3$	_	_	_	Chryseobacterium sp. (99%)
CT348 (EU336939)	$13 \pm 5$	++	_	_	Chryseobacterium sp. (99%)
CT364 (EU336940)	$45 \pm 13$	+++	+	_	Pseudomonas sp. (99%)

**Table 1.** Biochemical characterization of bacterial strains and phylogenetic identification by means of 16S rDNA polymerase chain reaction (PCR) amplification and sequencing

<sup>1</sup> Gen bank accession number in brackets. <sup>2</sup> N=6. Mean  $\pm$  SD. <sup>3</sup> Based on the hydrolysis halo produced onto the petri dish: +: 1-2.99 cm, ++: 3-5.99 cm, +++: > 6 cm). <sup>4</sup> ACC: 1-aminocyclopropane 1-carboxylate. <sup>5</sup> Percentage of similarity in brackets.

**Table 2.** Effects of bacterial strains on the number and total length of adventitious roots on mung bean cuttings under gnotobiotic conditions in  $2.5 \times 15$  cm glass tubes

Bacterial treatment	No. of roots/ cutting <sup>1</sup>	Total root length/cutting <sup>1</sup> (mm)	Root length (total root length/ no. roots)
Control	6.64 d	32.6 d	4.9
AG9	23.25 bc	206.4 a	8.9
AG13	33.93 a	142.4 b	4.2
CT348	19.60 c	98.6 c	5.0
CT364	26.37 b	164.8 ab	6.2

<sup>1</sup> Values are means of 10 replicates from three separate experiments. Plants were collected 10 days after inoculation (dai). Means followed by the same letter are not significantly different at p < 0.05 by LSD test.

families as *Pseudomonadaceae* and *Enterobacteriaceae* (Table 1).

#### **Gnobiotic assays**

**Rooting assay.** All of the bacterial treatments increased both the number of adventitious roots and the total root length from three to five fold when compared with the water control (Table 2). It is noteworthy that roots developed by cuttings placed in a bacterial suspension of strain *Pantoea* sp. AG9 were much longer (on average, 8.9 mm) than cuttings placed in any of the other bacterial strain suspensions (on average, from 4.2 to 6.2 mm) or in water (on average, 4.9 mm).

**Root elongation assay.** All of the tested strains were able to significantly stimulate the growth of canola seedling primary roots after seven days treatment (Table 3), with plants treated with strain *Pantoea* sp. AG9 having roots that attained the longest length.

#### **Olive nursery assays**

Assay 1. Bacterial strains A. brasilense Cd, Pantoea sp. AG9 and Chryseobacterium sp. AG13 were

**Table 3.** Effects of bacterial strains on the length of roots of canola seeds under gnotobiotic condition in  $17 \times 15$  cm growth pouches

Bacterial treatment	Root length <sup>1</sup> (mm)
AG9	80.7 a
Control	66.8 b
AG13	62.4 a
Control	53.0 b
CT348	60.3 a
Control	53.0 b
CT364	79.6 a
Control	69.7 b

<sup>1</sup> Values are means of 72 replicates from three separate experiments. Plants were collected 7 dai. Each bacterial treatment was compared with its own control . Means followed by different letter are significantly different at p < 0.05, based on Student's t-test.

Treatment —	<b>Arbequina</b> <sup>1</sup>		Picual <sup>1</sup>		Hojiblanca <sup>1</sup>	
	Method 1 <sup>2</sup>	Method 2 <sup>3</sup>	Method 1	Method 2	Method 1	Method 2
Cd	70 a	49 bc	35 cd	22 d	88 a	83 a
AG9	70 a	60 ab	87 a	85 a	68 b	70 b
AG13	58 ab	45 c	54 bc	80 a	70 b	64 b
Nursery water	17 d		40	) c	37	7 с
IBA	60	ab	60	) b	70	) b

**Table 4.** Effect of bacterial treatments on the percentages of rooted cuttings in 'Arbequina', 'Picual' and 'Hojiblanca' under two different inoculation methods in the fall assay (Assay 1)

<sup>1</sup> Values are means of two replicates, 130 cuttings each. <sup>2</sup> Cuttings were inoculated by submerging the cutting basal ends for 24 h in a bacterial culture. <sup>3</sup> Cuttings were inoculated by immersing them for 4-5 s in a mixture of peat-inoculant and sterile water. Cuttings were collected 60 dai, except cv. Hojiblanca that was harvested 74 dai. Means followed by the same letter are not significantly different at p < 0.05 by LSD test.

**Table 5.** Effect of bacterial treatments on the percentages of rooted cuttings in 'Arbequina', 'Picual' and 'Hojiblanca' under two different inoculation methods in the winter assay (Assay 2)

	<b>Arbequina</b> <sup>1</sup>		Picual <sup>1</sup>		Hojiblanca <sup>1</sup>	
Ireatment	Method 1 <sup>2</sup>	Method 2 <sup>3</sup>	Method 1	Method 2	Method 1	Method 2
Cd	63 ab	42 d	20 bcd	10 de	70 a	73 a
AG9	63 ab	60 bc	35 ab	38 a	61 ab	58 ab
AG13	60 bc	44 d	6 de	7 de	40 b	41 b
CT348	71 a	70 a	42 a	30 abc	52 ab	61 ab
CT364	56 c	39 d	17 cd	16 cd	70 a	67 a
Nursery water	28	e	2	l e	19	с
IBA	60	bc	40	) a	57	ab

<sup>1</sup> Values are means of two replicates, 130 cuttings each. <sup>2</sup> Cuttings were inoculated by submerging the cutting basal ends for 24 h in a bacterial culture. <sup>3</sup> Cuttings were inoculated by immersing them for 4-5 s in a mixture of peat-inoculant and sterile water. Cuttings were collected 60 dai, except cv. Hojiblanca that was harvested 74 dai. Means followed by the same letter are not significantly different at p < 0.05 by LSD test.

used to inoculate 'Arbequina', 'Hojiblanca' and 'Picual'. The data showed that, after 60 days, some of the bacterial strains produced a significantly higher rooting percentage than observed with IBA treatment for the three cultivars (Table 4).

In 'Arbequina' the best results were obtained when strains Cd and AG9 were applied by dipping the cuttings in the bacterial culture (Method 1). Strain AG9 provided similar results to the IBA control in Method 2. Strain Cd was a poor inducer of rooting in cv. Picual, while strains AG9 (both methods) and AG13 (Method 2) showed a high root induction efficiency. In 'Hojiblanca', strains AG9, AG13 and Cd produced similar or even higher rooting percentages than the IBA control. However, at 60 days after inoculation (dai) bacterial treated cuttings of this last cultivar displayed big callus and scarce emerging roots, requiring two additional weeks in the mist tunnel. This phenomenon was observed in all the performed assays.

Assay 2. All three strains used in Assay 1 and two new isolates obtained from an organic olive grove were included (Table 5). In 'Arbequina' all of the strains yielded good rooting in Method 1; however, only *Pantoea* sp. AG9 and *Chryseobacterium* sp. CT348 were as high as the IBA control in Method 2. In 'Picual', the strains AG9 and CT348 were similar to the IBA control in both methods while the other strains were less effective.

In 'Hojiblanca', all the five strains used induced rooting as the IBA control in both treatments.

*Assay 3.* In this assay only 'Arbequina' was used. In Method 1 strain *Pseudomonas* sp. CT364 was significantly more efficient than the IBA control while the other isolates did not differ from this positive control

**Table 6.** Effect of bacterial treatments on the percentages of rooted cuttings in 'Arbequina' under two different inoculation methods in the summer assay (Assay 3)

Treatment	Method 1 <sup>1,2</sup>	Method 2 <sup>1,3</sup>
Cd	58 cd	39 e
AG9	63 bc	77 ab
AG13	42 de	28 e
CT348	75 abc	41 e
CT364	85a	63bc
Nursery water	32	e
IBA	65	bc

<sup>1</sup> Values are means of two replicates, 130 cuttings each. <sup>2</sup> Cuttings were inoculated by submerging the cutting basal ends for 24 h in a bacterial culture. <sup>3</sup> Cuttings were inoculated by immersing them for 4-5 s in a mixture of peat-inoculant and sterile water. Cuttings were collected 60 dai. Means followed by the same letter are not significantly different at p < 0.05 by LSD test.

with the exception of strain *Chryseobacterium* sp. AG13 which was less efficient. Strains *Pantoea* sp. AG9 and *Pseudomonas* sp. CT364 both induced similar rooting percentages compared to the positive control in Method 2 (Table 6).

#### Discussion

The use of model plants provides methodological tools for comprehending the PGPR action mechanisms. Besides, they utilize a simple and idealised system where it is possible to check the biotechnological potential of the studied bacterium. The use of Vigna radiata and Brassica napus have been reported widely by others (Mayak et al., 1999; Penrose & Glick, 2003; Shaharoona et al., 2006; Rashid et al., 2012). They have been mostly used in studies on the involvement of phytohormones, more specifically auxin and ethylene, in the initiation and development of roots (Mayak et al., 1999). In this work the employ of these model plants has facilitated the pre-screening of a large number of PGPR and the subsequent selection of the more promising strains to be used for rooting in olive cuttings.

The effect of the inoculation method was studied by applying the inoculum in two different ways (Methods 1 and 2). The number of adhered bacteria was similar in both of them, and ranged between 10<sup>5</sup> and 10<sup>7</sup> cells/cutting (data not shown). Although both methods require similar and nursery-manageable amounts of microorganisms, probably Method 2, where cuttings are inmersed in a peat-based inoculant, is easier to commercialise and apply under nursery conditions owing to its similarity to the way in which peat based inoculants have been used since the end of the 19th century (Voelcker, 1896). Moreover, it has been described that some liquid inoculants (Method 1) quickly lose viability (Date, 2000).

İsfendiyaroğlu *et al.* (2009) found differences in 'Ayvalik' rooting when they tested twenty-five different types of rooting media. The substrates used in this study were a mixture of coconut fibre and perlite (Assay 1 and 3) or coconut fibre alone (Assay 2), substrates frequently used in olive nurseries. In assay 2, where perlite was removed from the rooting media, the high moisture level observed negatively affected the rooting of the olive cuttings, according to İsfendiyaroğlu *et al.* (2009), being cv. Picual the most affected.

Inoculation with Azospirillum induces the proliferation of lateral roots and root hairs (Glick, 1995; El-Khawas & Adachi, 1999). A. brasilense can also, effectively bind to a variety of surfaces including plant stems, as well as carnation cuttings (Li et al., 2005). In these previous studies, the inoculum was usually applied as a liquid culture. We have obtained good results with strain Cd using this method of inoculation (Method 1) but when it was applied as a solid inoculant (Method 2) rooting was significantly reduced in cultivars Arbequina and Picual. Other bacterial strains (AG13, CT348 and CT364) show a similar behaviour with cv. Arbequina. However, all the strains were as effective as the IBA control under both inoculation methods with cultivar Hojiblanca. This phenomenon could be explained in terms of different cultivar/strain interactions. In this sense, we also have observed a strong interaction between the effectiveness of the bacterial strain and the olive cultivar. Some strains that produced a high rooting percentage with one cultivar were nearly ineffective with other cultivars. Similar types of interactions have also been reported with other soil microorganisms (i.e. mycorrhizal fungi) and olive cultivars (Citernersi et al., 1998; Porras-Piedra et al., 2005; Soriano et al., 2006). Moreover, slight differences have been observed in the cultivar Hojiblanca with all of the bacterial strains, including strain Cd. Probably the bacteria-cultivar interaction mechanisms in this cultivar are different than in the others. In fact, our results showed that this cultivar needs more time in the mist tunnel before suitable rooting is obtained, independently of the season.

The strain AG9 is the only strain that always produced high rooting percentages independent of the mode of inoculation and in all of the tested cultivars. As it is not the best auxin producer, its effectiveness is likely explained by other mechanisms. In fact, it is the only strain tested that can synthesize the enzyme ACC deaminase. Bacteria possessing this enzyme have been described as highly effective PGPR owing to the fact that they can reduce the amount of ethylene produced by the plant and thereby promote root elongation (Penrose et al., 2001; Glick et al., 2007). In addition, our elongation results in mung bean cuttings agree with those obtained by Mayak et al., (1999) who observed that bacterial strains that either overproduced IAA or did not produce ACC deaminase yielded a large number of very small adventitious roots. On the other hand, also in agreement with Mayak et al. (1999), the use of bacteria with normal levels of IAA and ACC deaminase yielded plants with a moderate number of much longer roots. Strain AG9 was most similar to Pantoea sp (99%) or Erwinia sp (> 97%). In fact, some species of these genera have been renamed (e.g., Erwinia herbicola has been changed to Pantoea agglomerans) (Loiret et al., 2004) and its actual taxonomic position is not well established.

Our findings support the possible use of alternative methods for olive mist propagation under a context of 'organic agriculture system'. Although additional studies are needed, the results presented here strongly suggest that it is possible to obtain effective rooting induction of olive tree cuttings by the treatment of those cuttings with some plant growth-promoting bacteria that minimally synthesize and secrete the phytohormone IAA and actively produce the ethyleneregulating enzyme ACC deaminase. The method described here is a good starting point for a simple and straightforward approach that could easily be optimized by the olive nursery industry.

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#### References

- Alexander DB, Zuberer DA, 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol Fertil Soils 12: 39-45.
- Barranco D, Trujillo I, Rallo L, 2005. Libro I. Elaiografia hispanica. In: Variedades de olivo en España (Rallo L, Barranco D, Caballero JM, Del Rio C, Martin A, Tous J, Trujillo I, eds). Junta de Andalucía, MAPA and Ediciones Mundi-Prensa. Madrid.
- Centeno A, Gomez Del Campo M, 2008. Effect of rootpromoting products in the propagation of organic olive (*Olea europaea* L. cv. Cornicabra) nursery plants. Hortic Sci 43: 2066-2069.
- Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW, 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 57: 2259-2261.
- Citernesi AS, Vitagliano C, Giovanetti M, 1998. Plant growth and root system morphology of *Olea europaea* L. rooted cuttings as influenced by arbuscular mycorrhizas. S J Hort Sci Biotechnol 73: 647-654.
- Date RA, 2000. Inoculated legumes in cropping systems of the tropics. Field Crops Res 65: 123-136.
- Daza A, Santamaría C, Rodríguez-Navarro DN, Camacho M, Orive R, Temprano F, 2000. Perlite as a carrier for bacterial inoculants. Soil Biol Biochem 32: 567-572.
- Döbereiner J, 1980. Forage grasses and grain crops. In: Methods for evaluating biological nitrogen (Bergensen FJ, ed). John Wiley & Sons, NY. pp: 535-555.
- El-Khawas H, Adachi K, 1999. Identification and quantification of auxins in culture media of *Azospirillum* and *Klebsiella* and their effect on rice roots. Biol Fertil Soils 28: 377-381.
- Glick BR, 1995. The enhancement of plant growth by freeliving bacteria. Can J Microbiol 41: 109-117.
- Glick BR, 2010. Using soil bacteria to facilitate phytoremediation. Biotechnol Adv 28: 367-374.
- Glick BR, Penrose DM, Li J, 1998. A model for the lowering of plant ethylene concentrations by plant-growthpromoting bacteria. J Theor Biol 190: 63-68.
- Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B, 2007. Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26: 227-242.
- Hall, TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Sym Ser 41: 95-98.
- Holguin G, Glick BR, 2003. Transformation of *Azospirillum* brasilense Cd with ACC deaminase gene from *Enterobacter cloacae* UW4 fused to the Tet<sup>r</sup> gene promoter improves its fitness and plant growth promoting ability. Microbiol Ecol 46: 122-133.
- İsfendiyaroğlu M, Özeker E, Bafler S, 2009. Rooting of 'Ayvalik' olive cuttings in different media. Span J Agric Res 7: 165-172.
- Juck D, Charles T, Whyte LG, Greer CW, 2000. Polyphase microbial community analysis of petroleum hydrocarbon-

contaminated soils from two northen Canadian communities. FEMS Microbiol Eco 33: 241-249.

- Kloepper JW, Schroth MN, 1978. Plant growth-promoting rhizobacteria in radish. Proc 4th Int Conf of Plant Pathogenic Bacteria. Gilbert-Clarey, Tours, France. pp: 879-882.
- Li Q, Saleh-Lakha S, Glick BR, 2005. The effect of native and ACC deaminase-containing *Azospirillum brasilense* Cd1843 on the rooting of carnation cuttings. Can J Microbiol 51: 511-514.
- Loiret FG, Ortega E, Kleiner D, Ortega-Rodés P, Rodés R, Dong Z, 2004. A putative new endophytic nitrogen-fixing bacterium *Pantoea* sp. from sugarcane. J Appl Microbiol 97: 504-511.
- Mayak S, Tirosh T, Glick BR, 1999. Effect of wild-type and mutant plant growth promoting rhizobacteria on the rooting of mung bean cuttings. J Plant Growth Regul 18: 49-53.
- Montero-Calasanz MC, Gamane G, Albareda M, Santamaría C, Daza A, Camacho M, 2006. Estudio de sistemas alternativos a la utilización de hormonas en el estaquillado de olivo ecológico. In: Agricultura ecológica: gestión sostenible del agua y calidad agroalimentaria. SEAE, Zaragoza. Cap 116, pp: 1-10.
- OJ, 2007. Directive 834/2007/EC of the Council of June 28. Official Journal of the European Union. L 189 20/07/ 2007.
- OJ, 2008. Directive 889/2008/EC of the Council of September 5. Official Journal of the European Union. L 250 18/09/2008.
- Patten CL, Glick BR, 2002. Role of *Pseudomonas putida* indolacetic acid in development of the host plant root system. Appl Environ Microbiol 68: 3795-3801.
- Penrose DM, Glick BR, 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant 118: 10-15.
- Penrose DM, Moffat BA, Glick BR, 2001. Determination of 1-aminocycopropane-1-carboxylic acid (ACC) to asses the effects of ACC deaminase-containing bacteria on roots of canola seedlings. Can J Microbiol 47: 77-80.
- Pikovskaya RI, 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiologiya 17: 362-370.
- Porras-Piedra A, Soriano ML, Porras-Soriano A, Fernández-Izquierdo G, 2005. Influence of arbuscular micorrizas on

the growth rate of mist propagated olive plantlets. Span J Agric Res 3: 98-105.

- Pretty J, 2008. Agricultural sustainability: concepts, principles and evidence. Philos Trans R Soc Lond B Biol Sci 363: 447-465.
- Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R, 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Prot 20: 1-11.
- Rashid S, Charles TC, Glick BR, 2012. Isolation and characterization of new plant growth- promoting bacterial endophytes. App Soil Ecol 61: 217-224.
- Rigaud, J, Puppo, A, 1975. Indole-3-acetic-acid catalism by soybean bacteroids. J Gen Microbiol 88: 223-228.
- Shaharoona B, Arshad M, Zahir ZA, 2006. Effect of plant growth promoting rhizobacteria containing ACCdeaminase on maize (*Zea mays* L.) growth under axenic conditions and on nodulation in mung bean (*Vigna radiata* L.). Lett Appl Microbiol 42: 155-159.
- Soriano-Martínez ML, Azcón R, Barea JM, Porras-Soriano A, Marcilla Goldaracena I, Porras-Piedra A, 2006. Reduction of the juvenile period of new olive plantations through the early application of mycorrhizal fungi. Soil Sci 171: 52-58.
- Spaepen S, Vanderleyden J, Remans R, 2007. Indole-3-acetic acid in microbial and microorganism-plant signalling. FEMS Microbiol Rev 31: 425-448.
- Suarez MP, Lopez EP, Ordovas J, Perez I, Aguirre I, 2002. Utilización de distintos productos para mejorar el enraizamiento en estaquillado semileñosos de olivo. In: La olivicultura ecológica en España (Gonzalvez V, Muñoz R, eds). Mercadotecnia Grupo El Olivo, Ubeda, Spain. pp: 99-103.
- Toklikishvili N, Dandurishvili N, Vainstein A, Tediashvili M, Giorgobiani N, Lurie S, Szegedi E, Glick BR, Chernin L, 2010. Inhibitory effect of ACC deaminasa-producing bacteria on crown gall formation in tomato plants infected by *Agrobacterium tumefaciens* or *A. vitis*. Plant Pathol 59: 1023-1030.
- Van Loon LC, Bakker PAHM, Pieterse CMJ, 1998. Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36: 453-483.
- Voelcker JA, 1896. 'Nitragin' or the use of 'pure cultivation' bacteria for leguminous crops. J Royal Agron Soc 3rd Serv 7: 253-264.