



Evaluating the effectiveness of rhizobacteria producing 1-aminocyclopropane-1-carboxylic acid deaminase in inhibiting tumor formation by *Agrobacterium tumefaciens*

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Abstract

Crown gall is one of the most dangerous bacterial diseases affecting the production of fruit tree nurseries in Egypt and many countries of the world. In the present study, ten isolates of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing rhizobacteria were isolated from the rhizosphere of apricot (*Prunus armeniaca* L.) and plum (*Prunus domestica* L.) trees to evaluate their ability to decrease tumor formation by *Agrobacterium tumefaciens* (synonym *Rhizobium radiobacter*). The ten isolates were identified as *Pseudomonas* strains based on 16S rRNA gene sequence analysis and deduced protein sequences obtained from a partial ACC deaminase structural gene (*acdS*) sequence. Co-inoculating castor bean (*Ricinus communis* L.) and kalanchoe (*Kalanchoe* sp.) plants with *A. tumefaciens* and four ACC deaminase-producing *Pseudomonas* isolates decreased tumor formation. However, six ACC deaminase-producing *Pseudomonas* isolates produced varying results in these two plant species. The results showed that isolates of *Pseudomonas vancouverensis* reduced tumor formation when co-inoculated with *A. tumefaciens* in castor bean and kalanchoe plants. However, the isolate *P. putida* inhibited tumor formation in castor bean plants but did not achieve the same effect in kalanchoe plants. Additionally, isolates of *P. frederiksbergensis* and *P. kilonensis* decreased tumor formation in kalanchoe plants while increasing tumor formation in castor bean plants. The results showed that ACC deaminase-producing *P. vancouverensis* is a promising biocontrol agent against *A. tumefaciens*.

Keywords *Pseudomonas* spp. · *acdS* gene · Biocontrol · Crown gall

Introduction

Crown gall, caused by *Agrobacterium tumefaciens* (synonym *Rhizobium radiobacter*), is one of the most dangerous diseases affecting the production of fruit tree nurseries.

Crown gall disease affects more than 750 species of plants, including ornamentals, vegetables, and fruit trees (Pulawska 2010; Horuz et al. 2018). Biological control of crown gall is more successful in controlling the disease than chemical treatments (Abd-El-Aziz et al. 2021). Some chemical compounds were used to control crown gall, but they are not enough to completely control the disease. *Agrobacterium radiobacter* strain K84 (synonym *Rhizobium rhizogenes* strain K84) was successfully used to protect against crown gall disease (Trong et al. 2022). Besides successful examples of biological control of crown gall, such as using *Agrobacterium radiobacter* strain K84, several studies are being conducted to develop biological control methods for crown gall using rhizobacteria with certain characteristics, such as bacteria that produce hydrogen cyanide (HCN) or the ACC deaminase enzyme (Toklikishvili et al. 2010; Abd El-Rahman et al. 2019).

ACC is the direct precursor of the phytohormone ethylene. Ethylene regulates many physiological processes in plants. The role of ethylene in plant growth cannot be

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separated from its role in plant development. By interacting with other phytohormones, ethylene triggers the network of signaling pathways and influences the regulation of several processes. The transition from vegetative to reproductive phases and senescence in plants is controlled by ethylene and its interactions with other plant hormones. These interactions affect not only the ethylene content but also the sensitivity of the tissues. In addition, crop adaptability and performance under various stress conditions are influenced by the balance between ethylene perception and biosynthesis (Iqbal et al. 2017). However, ethylene production in plants causes significant reductions in plant growth and development and can result in plant death. The increase in ethylene synthesis from its immediate precursor ACC has been observed in nearly all plants growing under stress conditions (Gupta and Pandey 2019).

When the plant is infected with *A. tumefaciens*, a segment of the Ti-plasmid carrying oncogenes (*onc*) is transferred and inserted into the host cell genome for disease induction. Oncogenes are expressed in transformed plants and are involved in plant hormone synthesis. This causes a hormonal imbalance in infected plants, and tumor growth occurs due to the change in the rate of plant cell division. The high ethylene concentration in tumors indicates that ethylene is a regulating factor in tumor formation, in addition to the known roles of auxin and cytokinin (Tzfira and Citovsky 2008; Toklikishvili et al. 2010).

The plant enzymes ACC synthase and ACC oxidase catalyze the main steps in the biosynthesis of the plant hormone ethylene. ACC is synthesized from S-adenosyl-L-methionine by ACC synthases. The ACC subsequently oxidized to ethylene by ACC oxidases. The microbial enzyme ACC deaminase catalyzes the hydrolytic cleavage of ACC, the immediate precursor of ethylene, and thus acts as an inhibitor of ethylene biosynthesis (Penrose and Glick 1997; Polko and Kieber 2019). The ACC deaminase enzyme, which is encoded by the *acdS* gene in some species of plant growth-promoting rhizobacteria (PGPR), is responsible for the breakdown of ACC into ammonia and ketobutyrate, which bacteria use as nitrogen and carbon sources. The ACC deaminase activity of PGPR promotes plant growth and protects plants from environmental stresses. Additionally, this activity can also protect plants from bacterial and fungal pathogens. The synergistic interaction between ACC deaminase and both plant and bacterial auxin is critical for these PGPRs to operate effectively (Glick 2014; Gupta and Pandey 2019; Bianco et al. 2021). Rhizobacteria with ACC deaminase activity reduce the detrimental effects of salt stress on plants by lowering ACC levels, which reduces excessive ethylene production and is one of the most effective ways to induce salt stress tolerance in plants (Gupta and Pandey 2019; Orozco-Mosqueda et al. 2020). Drought stress causes poor plant growth and yield. Nevertheless, rhizobacteria

that produce ACC deaminase are expected to enhance crop growth and production in drought-affected lands by protecting plants from oxidative damage caused by drought stress and improving plant growth (Danish et al. 2020; Gowtham et al. 2020). Crown gall formation can be inhibited by ACC deaminase, which can protect plants from this disease to a certain degree. Pathogenic strains of *Agrobacterium* can be controlled by PGPR which produces the ACC deaminase enzyme. There are hypotheses that ethylene plays an essential role in *Agrobacterium* tumor formation, and that reducing ethylene levels by ACC-producing rhizobacteria may protect plants infected with pathogenic strains of *Agrobacterium* from crown gall (Toklikishvili et al. 2010). Avirulent strain *A. tumefaciens* D3 was additionally demonstrated to have a putative *acdS* and a regulatory gene (*acdR* = *lrpL*). The crown gall formation was significantly decreased when wounded castor bean plants were co-inoculated with the virulent strain “*Agrobacterium fabrum*” C58 and the wild-type *A. tumefaciens* D3 or the *acdS* and *lrpL* double mutant strain *A. tumefaciens* D3-1 (with no ACC deaminase activity). These results indicate that ACC is not the main factor in reducing crown gall formation, and other factors in bacteria may play the same role (Hao et al. 2011).

This study aims to isolate and identify ACC deaminase-producing rhizobacteria from the rhizosphere of some fruit trees. Evaluate the ability of ACC deaminase-producing rhizobacteria to inhibit tumor formation caused by *A. tumefaciens* on castor bean (*Ricinus communis* L.) and kalanchoe (*Kalanchoe* sp.) plants to determine the possibility of their use in biocontrol of *A. tumefaciens*.

Material and methods

Isolation of *A. tumefaciens*

Seedlings of peach (*Prunus persica* L.) and apricot (*Prunus armeniaca* L.) with crown gall symptoms were collected from nurseries of stone fruit in Nubaria at Beheira Governorate. The samples were packed in plastic bags and transferred to the laboratory. Tumors were separated from seedlings and washed with sterile distilled water several times to remove adherent soil particles. Tumors were cut into small pieces, placed in a mortar, and crushed with a few sterilized water by a pestle. The resulting suspension was left at room temperature for an hour before streaking onto the surface of plates of D1 Agar medium (D1 Agar medium: 15 g mannitol, 5 g NaNO₃, 6 g LiCl, 20 mg Ca (NO₃)₂·4H₂O, 2 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 bromothymol blue, 20 g agar, distilled water to 1L, and pH 7.2) plates. The inoculated plates were incubated at 28 °C for 5 days, and the growth of bacterial colonies was observed.

Two single colonies similar to *Agrobacterium* were selected from the colonies that developed from each plant species and re-streaked on the same medium to obtain a pure culture. All selected isolates were kept at 4 °C in a refrigerator on the King's B agar medium (KBAM: 20 g peptone, 10 mL glycerol, 1.5 g K₂HPO₄, 1.5 g MgSO₄·7H₂O, 20 g agar, distilled water to 1L, and pH 7.2) slants for later use (Kado and Heskett 1970; Gupta et al. 2013).

Preparation of DNA templates

Isolates were cultured on the Luria–Bertani agar medium (LBAM: 10 g peptone, 5 g yeast extract, 10 g NaCl, 20 g agar, distilled water to 1L, and pH 7) and incubated for 48 h at 28 °C before DNA extraction. Two colonies of the tested isolate were suspended in 200 µL molecular grade water in a 2-mL microcentrifuge tube. The tube was tightly closed with parafilm and microwaved using a JAC Microwave 25L – Model NGM-25D2 (Nafea Group, Egypt) for 2 min at maximum power before centrifuging for 2 min at 12,000 rpm. Two microliters of supernatant were used as a DNA template for the polymerase chain reaction (PCR) for each 25 µL amplification reaction (Dashti et al. 2009; Li et al. 2015).

Detection of *Agrobacterium* Ti plasmid using PCR and biovar determination

The tmr236F (5'-TTATTGGAGTGCGGATTTTCGTT-3') and tmr236R (5'-CGGATGTGATCTGGTTCTGGCTA-3') primers were used to amplify the sequence of tmr locus of *Agrobacterium* Ti plasmid in isolates with an expected amplicon size of 236 bp (Yang et al. 2011). Twenty-five microliters were used in amplification reactions consisting of 12.5 µL PCR reaction mixture (amaR OnePCR, GeneDireX, Taiwan), 1 µL of each 10 pmol/µL dilution of reverse and forward primer (Eurofins Genomics Germany GmbH, Ebersberg, Germany), 2 µL DNA template of tested isolate, and 8.5 µL molecular grade water. As a negative control, the amplification reaction was used with 2 µL of molecular grade water instead of the 2 µL template DNA of the tested isolate. The amplification reaction with 2 µL of *A. tumefaciens* 27AS_Pp4 (accession number in GenBank of the National Center for Biotechnology Information is OK559740) instead of the 2 µL template DNA of the tested isolate was used as a positive control. The PCR conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR was carried out using an applied biosystems 2720 thermal cycler (Life Technologies Holdings Pte Ltd, Singapore). The PCR products were

detected by electrophoresis in 1.5% agarose gel containing ethidium bromide in 1 × TAE (Tris–acetate–EDTA) buffer.

Biovar determination of isolates was made based on the ability of isolates to produce 3-ketolactose, growth at 35 °C, growth at 2% NaCl, and acid production from erythritol according to Schaad et al. (2001).

Tumorigenicity test

The pathogenicity of isolates was confirmed on 4-week-old castor bean plants. Plants were inoculated by wounding the crown region with a sterile scalpel contaminated with a two-loopful of 24-h-old bacterial culture. Three castor bean plants were used for each isolate. Three castor bean plants wounded with a sterile scalpel without inoculum were used as a control. All castor bean plants were placed in a lath house under natural conditions. Wounds were examined for the presence or absence of tumors during the four weeks following the castor bean plant inoculation (Hao et al. 2007; Gupta et al. 2013; Abd El-Rahman et al. 2020). The diameters (mm) and weights (g) of the tumors formed on the plants were measured at the end of the four weeks (Rhouma et al. 2005).

Collection of rhizosphere samples

In 2021 and 2022, root samples of healthy peach, apricot, and plum (*Prunus domestica* L.) plants were collected from different regions. Peach root samples were collected from Wadi El Natrun, Beheira Governorate (30°11'37.9"N 30°30'35.0"E), apricot root samples were collected from Sadat City, Menofia Governorate (30°22'16.2"N 30°42'22.8"E), and plum root samples were collected from Atfih, Giza Governorate (29°22'45.6"N 31°14'17.5"E). The soil adhering to the plant roots was separated for each plant species separately.

Isolation of rhizobacteria

A serial dilution technique was followed to isolate rhizobacteria from rhizosphere soil samples on KBAM and LBAM. Ten grams of the soil adhered to plant roots were transferred to a 250-mL Erlenmeyer flask containing 90 mL sterile distilled water and shaken in an incubator shaker at 150 rpm for 30 min under 28 °C. The sample was serially diluted up to 10⁻⁶ in sterilized water. A hundred microliter of sample dilution (10⁻⁵) was plated on each plate of KBAM and LBAM and incubated for 48 h at 28 °C. Individual bacterial colonies were randomly selected and streaked on KBAM plates to be purified. Pure isolates were stored in slants of KBAM at 4 °C until further testing (Kifle and Laing 2016; Gupta and Pandey 2019).

Screening rhizobacteria for the presence of ACC deaminase activity

The presence of ACC deaminase activity in rhizobacteria isolates was determined by using specific amplification of the partial *acdS* gene sequences with consensus-degenerate hybrid oligonucleotide (CODEHOP) primers and the ability of the isolates to grow on plates of Dworkin and Foster minimal salts agar medium (DFMSAM) supplemented with ACC.

Detection and sequencing of *acdS* gene

DNA template was prepared using the same method as mentioned above. The *acdSf3* (5'-ATCGGCGGCATCCAGWS-NAAYCANAC-3') and *acdSr3* (5'-GTGCATCGACTTGCCCTCRTANACNGGRT-3') primers were used to amplify partial *acdS* gene sequences with an expected amplicon size of ~680 bp (Li et al. 2015; Alemneh et al. 2021). Twenty-five microliters were used in amplification reactions consisting of 12.5 µL PCR reaction mixture (amaR OnePCR, GeneDireX, Taiwan), 1 µL of each 10 pmol/µl dilution of reverse and forward primer (Eurofins Genomics Germany GmbH, Ebersberg, Germany), 2 µL DNA template of tested isolate, and 8.5 µL molecular grade water were used. As negative controls, the amplification reaction with 2 µL of molecular grade water and the amplification reaction with a 2 µL DNA template from *Escherichia coli* O157:H7 (obtained from the Microbiology Department, Faculty of Agriculture, Cairo University, Cairo, Egypt) instead of the 2 µL template DNA the tested isolate was used. The PCR conditions were 95 °C for 4 min, followed by 35 cycles of 95 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR was carried out using an applied biosystems 2720 thermal cycler (Life Technologies Holdings Pte Ltd, Singapore). PCR products were detected by agarose gel electrophoresis, purified with QIAquick Gel Extraction Kit and the QIAquick PCR and Gel Cleanup Kit (Qiagen, Hilden, Germany), and sequenced with the *acdSr3* primer using the Sanger sequencing method at Colors Medical Labs, Cairo, Egypt. The deduced protein sequences were obtained from the partial *acdS* gene sequences using the ORFFinder (Blaha et al. 2006) of the National Centre for Biotechnology Information (NCBI) website.

Qualitative determination of ACC deaminase activity of the rhizobacteria isolates

The activity of ACC deaminase in the selected rhizobacteria isolates was assessed qualitatively on DFMSAM containing 3 mM ACC as the sole nitrogen source. Rhizobacteria isolate was streaked on the surface of sterile DFMSAM [4.0 g KH_2PO_4 , 6.0 g Na_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g glucose,

2.0 g gluconic acid, 2.0 g citric acid, 0.1 mL $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution (100 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ is dissolved in 10 mL sterile distilled water), 0.1 mL trace elements solution (10 mg H_3BO_3 , 11.19 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 124.6 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 78.22 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 10 mg MoO_3 are dissolved in 100 mL sterile distilled water), 20 g agar, distilled water to 1.0 L, and pH 7.2] amended with 3 mM ACC (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) instead of 2.0 g $(\text{NH}_4)_2\text{SO}_4$ as sole nitrogen source. The DFMSAM was sterilized for 20 min at 121 °C, whereas the ACC substrate was sterilized separately using a 0.2 µm membrane filter. The negative control plates contained only DFMSAM without ACC and $(\text{NH}_4)_2\text{SO}_4$, while the positive control plates contained DFMSAM with $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source. The inoculated plates were incubated at 28 °C for 3 days, with daily growth monitoring. The growth of isolates on ACC-supplemented plates was compared to that of negative and positive controls. The experiment was repeated three times. The ability of the isolates to grow on ACC-supplemented plates compared to negative control proves that they are ACC deaminase producers (Penrose and Glick 2003; Zafar-Ul-Hye et al. 2007; Ali et al. 2014; Palaniyandi et al. 2014; Gupta and Pandey 2019).

Antagonistic activity assay

Bacterial isolates (selected isolates of rhizobacteria and *Agrobacterium* isolate BAAg4) were grown in King's B agar medium. The *Agrobacterium* isolate BAAg4 culture was diluted in sterile water until the OD600 ranged between 0.07 and 0.1, equivalent to 108 CFU/ml. One milliliter of *Agrobacterium* isolate BAAg4 suspension was used as inoculum per 250 ml of sterile nutrient glucose agar medium (NGA medium: 5 g peptone, 3 g beef extract, 10 g glucose, 20 g of agar; distilled water to 1.0 L, pH 7.2) after cooling to 50 °C in a water bath. The inoculated NGA medium (250 mL) was poured into 14 plates (90 mm diameter). A loopful of the rhizobacterial isolate (24-h-old culture) was placed on the surface of the plate inoculated with *Agrobacterium* isolate BAAg4. Three replicates were used for each isolate of rhizobacteria. The zones of inhibition formed around the growth of the rhizobacteria after incubating the plates for 48 h at 28 °C were measured to the nearest millimeter.

The 16S rRNA gene sequences

The 16S rRNA genes of *Agrobacterium* isolate BAAg4 and selected rhizobacterial isolates were partially amplified with 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWTGTACAAGGC-3') primers (Marchesi et al. 1998) at Sigma Scientific Services Co, Head Scientific Office, 6 of October, Cairo, Egypt. PCR amplification was performed in a total volume of 50 µL containing 4 µL

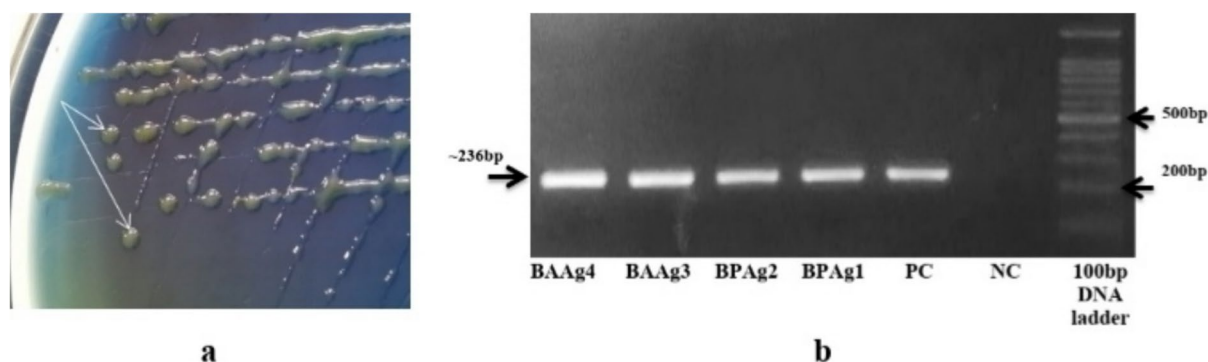


Fig. 1 Colony shapes of *Agrobacterium* on the D1 medium and detection of Ti plasmid of *Agrobacterium* by PCR using tmr236F and tmr236R primers. Circular convex colonies with olive green color and entire margins were recovered on D1 medium (A). PCR products of 236 bp were detected on agarose gel electrophoresis for DNA of four isolates from tumors of peach (BPAg1 and BPAg2) and apricot

(BAAg3 and BAAg4) using tmr236F and tmr236R primers run at an annealing temperature of 60 °C (B). With molecular grade water (NC), no PCR products were detected. With DNA of the reference isolate *A. tumefaciens* 27AS_Pp4 (PC), a PCR product of 236 bp was detected

of the template (DNA extract), 1 µL of 20 pmol/µL dilution of 63f primer, 1 µL of 20 pmol/µL dilution of 1387r primer, 25 µL of COSMO PCR RED Master Mix (Willowfort), and 19 µL of molecular grade water. The PCR conditions were 94 °C for 6 min, 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The DNA Clean & Concentrator-25 (Zymo Research) was used to purify the PCR products. The primers 63f / 1387r were used to sequence the purified DNA using the Sanger method at GATC Biotech, Germany.

Perform similarity and phylogenetic analyses of the sequences

The Nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) and Protein-protein Basic Local Alignment Search Tool (BLASTp) of the NCBI GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) were used to obtain DNA and protein sequences comparable to partial 16S rRNA gene and deduced protein sequences obtained in this study, respectively. DNA and deduced protein sequences were aligned with the MUSCLE program. The phylogenetic trees were generated using the neighbor-joining method with 1000 bootstrap (The Kimura-2-parameter model was used for DNA sequences and the Poisson correction method for deduced protein sequences) using molecular evolutionary genetics analysis MEGA11 software (Tamura et al. 2021).

Sequence accession numbers

Partial sequences of the 16S rRNA and *acdS* genes were submitted to the NCBI GenBank database. Accession

number PP506592 was obtained for partial 16S rRNA gene sequence of *Agrobacterium* isolate BAAg4. Accession numbers OR197536, OR197537, OR197538, OR197539, OR197540, OR197541, OR197542, OR197543, OR197544, and OR197545 for partial 16S rRNA gene sequences and OR227281, OR227282, OR227283, OR227284, OR227285, OR227286, OR227287, OR227288, OR227289, and OR227290 for partial *acdS* gene sequences were obtained for the isolates BApK9, BApL11, BApL12, BApL18, GPIK17, GPIK18, GPIK19, GPIK20, GPIK23 and GPIL23, respectively.

Evaluation of the ability to inhibit tumor formation

To evaluate the effects of rhizobacteria with ACC deaminase activity on the tumor induced by *A. tumefaciens*, the bacterial isolates of *A. tumefaciens* BAAg4 and rhizobacteria were grown in the LBBM in an incubator shaker at 150 rpm for 48 h under 28 °C. The cultures were then diluted with LBBM to an OD600 of 2.0 (approximately 5×10^9 cfu/ mL). Castor bean plants were grown in pots from seeds of similar size and color, and a V-shaped wound was made in the middle of the stems of 4-week-old castor bean plants with a sterile scalpel. The plant wound was then treated with 10 µL of *A. tumefaciens* BAAg4 suspension mixed with 10 µL of the culture suspension of the tested rhizobacteria isolate. Ten microliters of the *A. tumefaciens* BAAg4 suspension were mixed with 10 µL of uninoculated LBBM in the positive control. Plants were wounded and inoculated with 20 µL uninoculated LBBM and were used as a negative control. The same experiment was conducted with the same conditions on kalanchoe plants. The kalanchoe plants were inoculated into

Fig. 2 Crown gall disease. Natural infection on the apricot seedling (A). A tumor (s) appears on the crown region of the castor bean plant after wounding the crown region with a sterile scalpel contaminated with a two-loopful of the isolates recovered on D1 medium from tumors of peach and apricot seedlings and placing castor bean plants in a lath house (B). No tumor (s) appeared on the crown region of the castor bean plant after being wounded with a sterile scalpel without bacterial growth and placed under the same conditions (C). All recovered isolates were able to produce tumor (s) on castor bean plants (D)



Table 1 Tumor formation on castor bean plants by four selected isolates recovered on D1 medium from tumors of peach and apricot seedlings

Isolate	Gall diameter (mm)	Gall weight (g)
BPAg1	9.02 ab	1.12 a
BPAg2	7.73 b	0.90 ab
BAAg3	5.42 c	0.75 b
BAAg4	9.75 a	1.22 a
Negative control	0.00 d	0.00 c

Means of three replicates; Means in the same column that do not share a letter are significantly different using Duncan post hoc test ($p \leq 0.05$)

the leaf axils. The inoculated plants were placed in a lath house under natural conditions for 6 to 8 weeks before the size and weight of the formed tumors were measured. The experiments were repeated twice, with 3 replicates for each treatment. The total number of replicates for each treatment was 6 (Hao et al. 2007, 2011).

Statistical analysis

A completely randomized block design was used in tumorigenicity tests and the ability to inhibit tumor formation experiments. The collected data were subjected to analysis of variance (ANOVA) with P value < 0.05 . Duncan's post

Table 2 Districts of isolated rhizobacteria and detection of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity

Districts and sampling date	Plant	Total number of isolates			Number of isolates containing <i>acdS</i> gene	Number of isolates grow on DFMSAM supplemented with ACC
		Total number	King's B agar medium	Luria–Bertani agar medium		
Wadi El Natrun (August 20, 2021)	Peach	17	8 (from BPeK1 to BPeK8)	9 (from BPeL1 to BPeL9)	None	None
Sadat City (December 19, 2021)	Apricot	19	8 (from BApK9 to BApK16)	11 (from BApL10 to BApL20)	4	4
Atfih (March 28, 2022)	Plum	19	12 (from GPIK17 to GPIK28)	7 (from GPIL21 to GPIL27)	6	6

DFMSAM Dworkin and Foster minimal salts agar medium

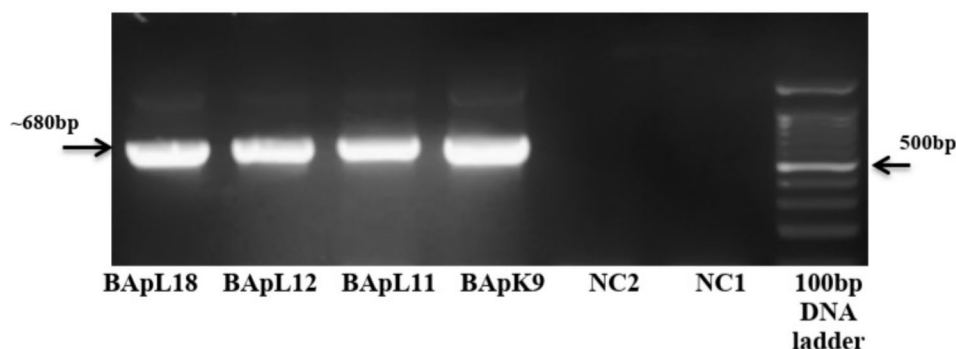


Fig. 3 Detection of *acdS* gene in rhizobacteria by PCR using *acdSf3/acdSr3* primers. PCR products of around 680 bp were detected on agarose gel electrophoresis for DNA of four isolates (BApK9, BApL11, BApL12, and BApL18) from the rhizosphere of apri-

cot using *acdSf3/acdSr3* primers run at an annealing temperature of 53 °C. With molecular grade water (NC1) and DNA of *E. coli* O157:H7 (NC2), no PCR products were detected

hoc test was used to compare the treatment means using IBM SPSS (SPSS Inc., IBM Corporation, NY) Statistics version 25.0 (IBM Corporation 2017). For the ability to inhibit tumor formation experiments, the homogeneity of error terms was tested before the combined analysis as described by the Levene test (Levene 1960). The homogeneity of individual error variances for all studied measures was satisfied and allowed to run a combined analysis across two experiments.

Results

Agrobacterium tumefaciens isolates

Four isolates with authentic descriptions of *Agrobacterium* (Circular convex colonies with olive green color and entire margins) were recovered on D1 medium (Fig. 1a) from tumors of peach and apricot (Fig. 2a) seedlings, two from peach (BPAg1 and BPAg2) and two from apricot (BAAg3 and BAAg4). The four isolates carry Ti plasmid

and produced positive results with primers *tmr236F* and *tmr236R*. PCR detected products of 236 bp on agarose gel electrophoresis for the DNA of the four isolates (Fig. 1b). Castor bean crown regions were used to test the pathogenicity of these isolates (Fig. 2b, c). All recovered isolates were able to produce tumor (s) on castor bean plants. There were no differences in tumor shapes formed by the four isolates. Relatively larger tumors were formed on the castor bean plant with BAAg4 isolate compared to the other isolates (Table 1 and Fig. 2d). Also, all isolates gave positive reactions with 3-ketolactose production, growth at 35 °C, and growth at 2% NaCl. These isolates gave negative reactions with acid production from erythritol. The results showed that all recovered isolates belonged to biovar 1.

Rhizobacteria isolation and screening for ACC deaminase activity

Fifty-five rhizobacteria were isolated from the rhizosphere of peach, apricot, and plum plants on KBAM and LBAM (Table 2). After purification on KBAM, all isolates were tested for ACC deaminase activity using specific

Table 3 The deduced protein sequences obtained from partial *acdS* gene sequences of the 10 apricot and plum rhizobacteria isolates using ORFfinder of the NCBI website

Isolate	Deduced protein sequence (obtained from partial <i>acdS</i> gene sequence)
BApK9	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVREERGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIRC
BApL11	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVREERGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIRC
BApL12	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVREERGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIDC
BApL18	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVREERGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIDC
GPIK17	MKCRLVQESWVPPDAVYDRVGNILMSRVLGAEIEFVDEGFDIGIRESWERALEDVKAKGGKPYAIPAGASVHKY- GLGYVGFAAEEVRQETRMGIRFDYIIVCTVTGSTHAGMLVGFAKDGRARNVVGIDASATPEQTRAQVLAIAQQTAK- LIGLQEIADDVVLKEEYAYPAYGIPSEETNAA
GPIK18	MSRIMGADVRLDAAGFDIGIRPSWEKAMSDVVEQGGKPFPIAGCSEHPYGGLFVGFAAEEVRQQEKELGFKFDYIVVCS- VTGSTQAGMVVGFAADGRSRNVIGVDASAKPEQTKAQILRIARHTAELVELGREITEEDVVLDTFRFAYPEYGLPNEG- T
GPIK19	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVVEQGGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSRNVIGIDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIRC
GPIK20	MGCHLVQESWVPLPDAGYDRVGNILMSRVLGAEIEFVEVGLDIGIRESWERALEDLKAKGGKPYAIPAGASVHKY- GLGYVEFAEEVRQETRMGIRFDYIIVCTVTGSTHAGMLVGFAKDGRARNVIGIDASATPDQTRAQVFSIAHHTAKLVG- VVQEITAEDLIHKEEYAYPAVWHPDQK
GPIK23	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVVEQGGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSRNVIGIDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIRC
GPIK23	MKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPSWEKAMSDVVEQGGKPFPIAGCSEHPYGG- LFVGFAAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVEL- GREITEEDVVLDTFRFAYPEYGLPNEGTLAIRC

amplification of the partial *acdS* gene (Table 2). The results showed that 10 out of 55 isolates contained the *acdS* gene, 4 isolates (BApK9, BApL11, BApL12, and BApL18) from the rhizosphere of apricot trees (Fig. 3), and 6 isolates (GPIK17, GPIK18, GPIK19, GPIK20, GPIK23, and GPIL23) from the rhizosphere of plum trees. PCR detected products of around 680 bp on agarose gel electrophoresis for the DNA of the 10 isolates. Deduced protein sequences obtained from the *acdS* gene sequences of the 10 isolates using ORFfinder of the NCBI website are shown in Table 3. No isolates from the rhizosphere of peach trees contained the *acdS* gene. These 10 isolates grew well on the DFMSAM amended with ACC compared to the negative control [DFMSAM without ACC and (NH₄)₂SO₄], indicating that they can use ACC as a nitrogen source (Fig. 4).

Antagonistic activity against *Agrobacterium*

The antagonistic activity of the 10 isolates of ACC deaminase-producing rhizobacteria against *Agrobacterium* isolate BAAg4 on nutrient glucose agar medium showed that 9 (BApK9, BApL11, BApL12, BApL18, GPIK17, GPIK18, GPIK19, GPIK20, and GPIK23) out of the 10 isolates gave unclear inhibition zones ranged from 9.7 ± 2.08 to 35 ± 2.65 mm. The other isolate (GPIL23) showed a clear inhibition zone of 8.3 ± 0.58 mm (Table 4 and Fig. 5).

The similarity between sequences and phylogenetic analysis

The partial 16S rRNA gene sequence of *Agrobacterium* isolate BAAg4 was compared with the 16S rRNA gene sequences of other strains in the NCBI GenBank database using the BLASTn tool. *Agrobacterium* isolate BAAg4 showed the highest 16S rRNA gene sequence identity of 95.98, 95.72, and 95.55% with 16S rRNA gene sequences

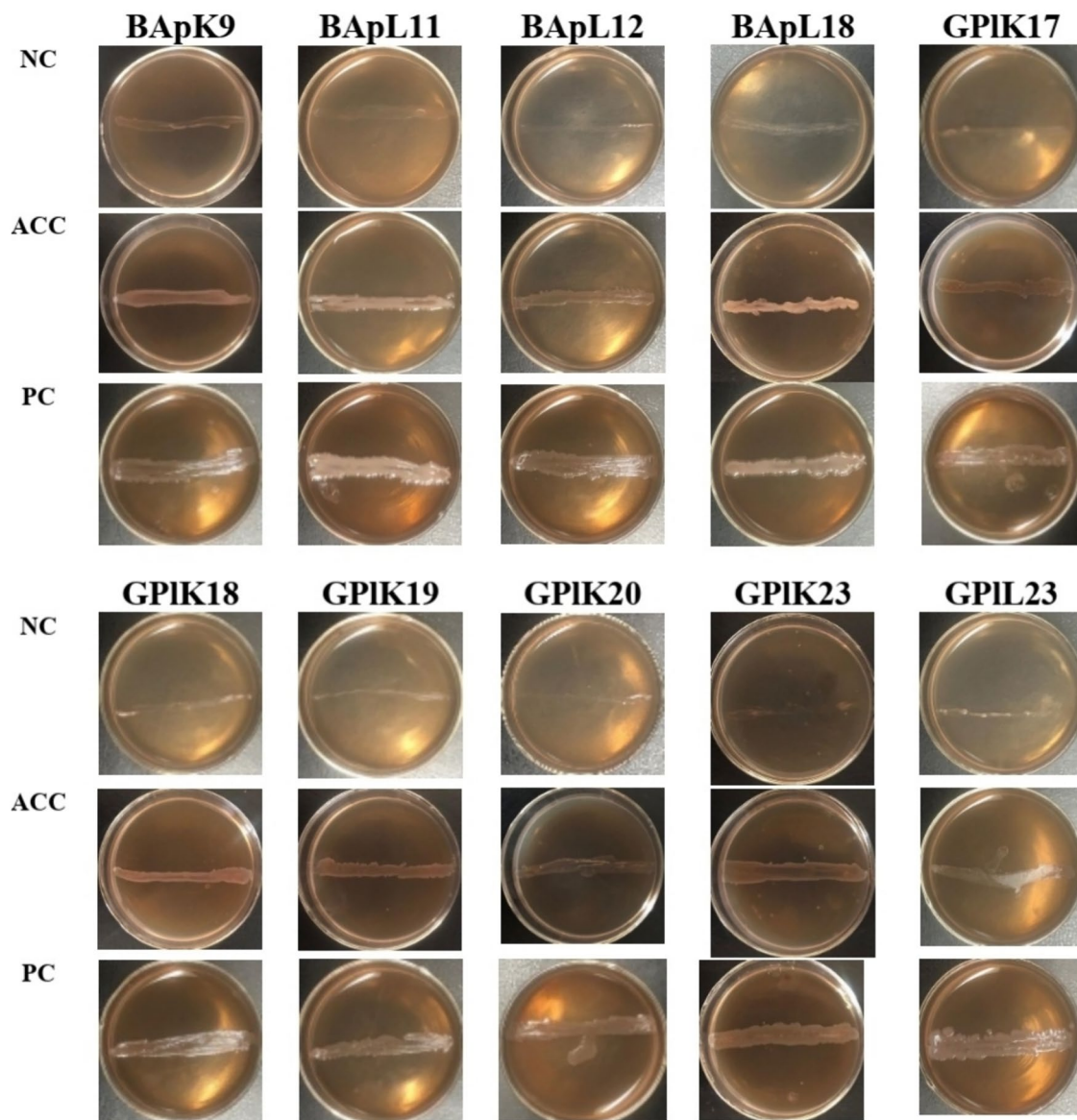


Fig. 4 Growth of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing rhizobacteria on Dworkin and Foster minimal salts agar medium (DFMSAM) containing ACC. Apricot and plum rhizosphere isolates containing the *acdS* gene grew well on

DFMSAM amended with ACC as a nitrogen source compared to the negative control. Negative control, DFMSAM without ACC and $(\text{NH}_4)_2\text{SO}_4$ (NC). DFMSAM amended with ACC (ACC). Positive control, DFMSAM containing $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source (PC)

from *A. radiobacter* strain UQM 1685 (NR_116306.1), *A. radiobacter* strain IAM 12048 (NR_041396.1), and *A. tumefaciens* strain NCPPB2437 (NR_115516.1), respectively. The phylogenetic tree of *Agrobacterium* isolate BAAg4 and closely related isolates based on partial 16S rRNA gene sequences using the neighbor-joining method with 1000 bootstraps is shown in Fig. 6.

The partial 16S rRNA gene sequences of 10 apricot and plum rhizosphere isolates were compared with the 16S rRNA gene sequences of other strains in the NCBI GenBank database using the BLASTn tool. All ten isolates showed

the highest identity of the 16S rRNA sequences with the *Pseudomonas* strains. Isolates BApK9, BApL11, BApL12, and BApL18 exhibited the highest identities of 16S rRNA gene sequences ranging from 99.80% to 99.93% with 16S rRNA gene sequence from *P. vancouverensis* strain DhA-51 (NR_041953.1). Also, isolates GPIK17 and GPIK20 showed the highest identities of 16S rRNA gene sequences of 99.33% and 99.46% with 16S rRNA gene sequence from *P. frederiksbergensis* strain DSM 13022 (NR_117177.1), respectively. While isolates GPIK18, GPIK19, and GPIK23 showed the highest identities of 16S rRNA gene sequences

Table 4 The antibacterial activity of 1-aminocyclopropane-1-carboxylic acid deaminase-producing rhizobacteria against *A. tumefaciens* BAAg4 on nutrient glucose agar medium

Isolate	<i>A. tumefaciens</i> inhibition zone (mm)*
BApK9	10.7 ± 1.15**
BApL11	14.3 ± 0.58**
BApL12	17.3 ± 1.53**
BApL18	9.7 ± 2.08**
GPIK17	33.7 ± 2.51**
GPIK18	21 ± 1**
GPIK19	25.3 ± 2.08**
GPIK20	35 ± 2.65**
GPIK23	17.3 ± 0.58**
GPIL23	8.3 ± 0.58

*Mean of three replicates; values expressed as mean ± Standard Deviation (SD)

**unclear inhibition zone

of 99.93% with 16S rRNA gene sequence from *P. kilonensis* strain 520-20 (NR_028929.1). Whereas isolate GPIL23 showed the highest 16S rRNA gene sequence identity of 99.52% with 16S rRNA gene sequence from *P. putida* strain NBRC 14164 (NR_113651.1). The phylogenetic tree of 10 apricot and plum rhizosphere isolates and closely related isolates based on partial 16S rRNA gene sequences using

the neighbor-joining method with 1000 bootstraps is shown in Fig. 7.

The deduced protein sequences obtained from the partial *acdS* gene sequences of 10 apricot and plum rhizosphere isolates were compared with the protein sequences of the *acdS* gene sequences of other strains in the NCBI GenBank database using the BLASTp tool. All ten isolates showed the highest protein sequences identity with the *Pseudomonas* strains. Isolates BApK9, BApL11, BApL12, and BApL18 exhibited the highest identities of protein sequences ranging from 99.47 to 100% with protein sequences from *Pseudomonas* sp. SaCS17 (AFQ62710.1), *P. fluorescens* (ATN40279.1), unclassified *Pseudomonas* (WP_095195256.1), and *Pseudomonas* sp. LAMO17WK12:I5 (WP_256581065.1). Also, isolate GPIK17 showed the highest protein sequence identity of 98.39% with protein sequences from *Pseudomonas* sp. GM55 (WP_008016613.1), *P. fluorescens* (WP_057398053.1), and *P. frederiksbergensis* (WP_205890920.1). Isolates GPIK18, GPIK19, and GPIK23 also showed highest identities of protein sequences ranging from 99.37 to 100% with protein sequences from *Paraburkholderia caledonica* (ABE66287.1), *P. ogorae* (WP_079302828.1), and *P. ogorae* (WP_014338921.1), while isolate GPIK20 showed the highest protein sequence identity of 85.16% with a protein sequence from *P. frederiksbergensis* (WP_205890920.1).

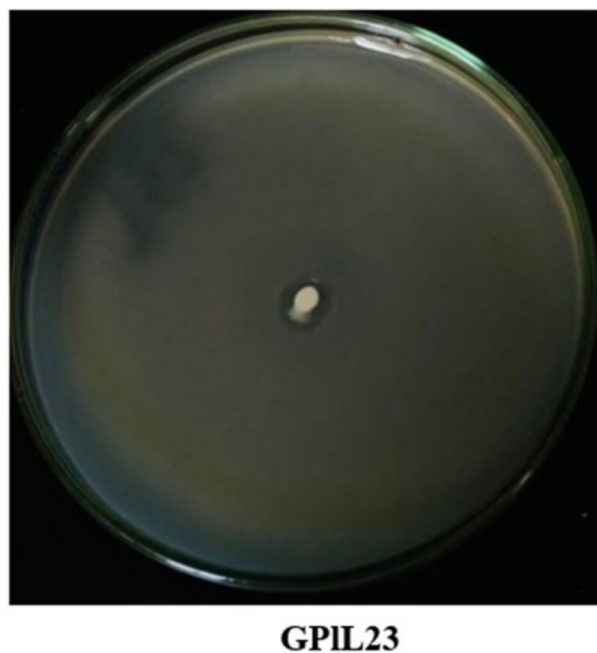
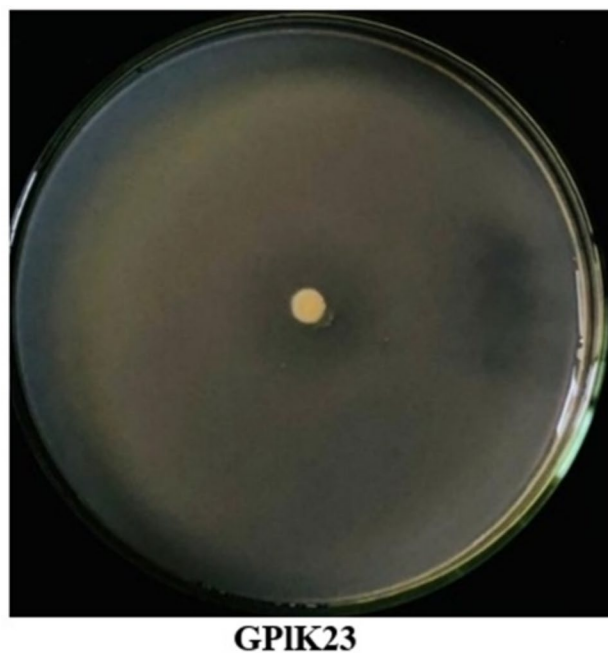


Fig. 5 The antagonistic activity (Inhibition zone) of ACC deaminase-producing rhizobacteria against *Agrobacterium* isolate BAAg4. One milliliter of a suspension (10^8 CFU/ml) of *Agrobacterium* isolate BAAg4 was used as an inoculum per 250 ml sterilized NGA medium. A loopful of a 24-h-old culture of the isolate of rhizobacteria (e.g.,

GPIK23 and GPIL23) was placed on the surface of the plate seeded with *Agrobacterium* isolate BAAg4; then, the plates were incubated for 48 h at 28 °C. Isolate GPIK23 showed an unclear zone of inhibition, while GPIL23 showed a clear zone of inhibition

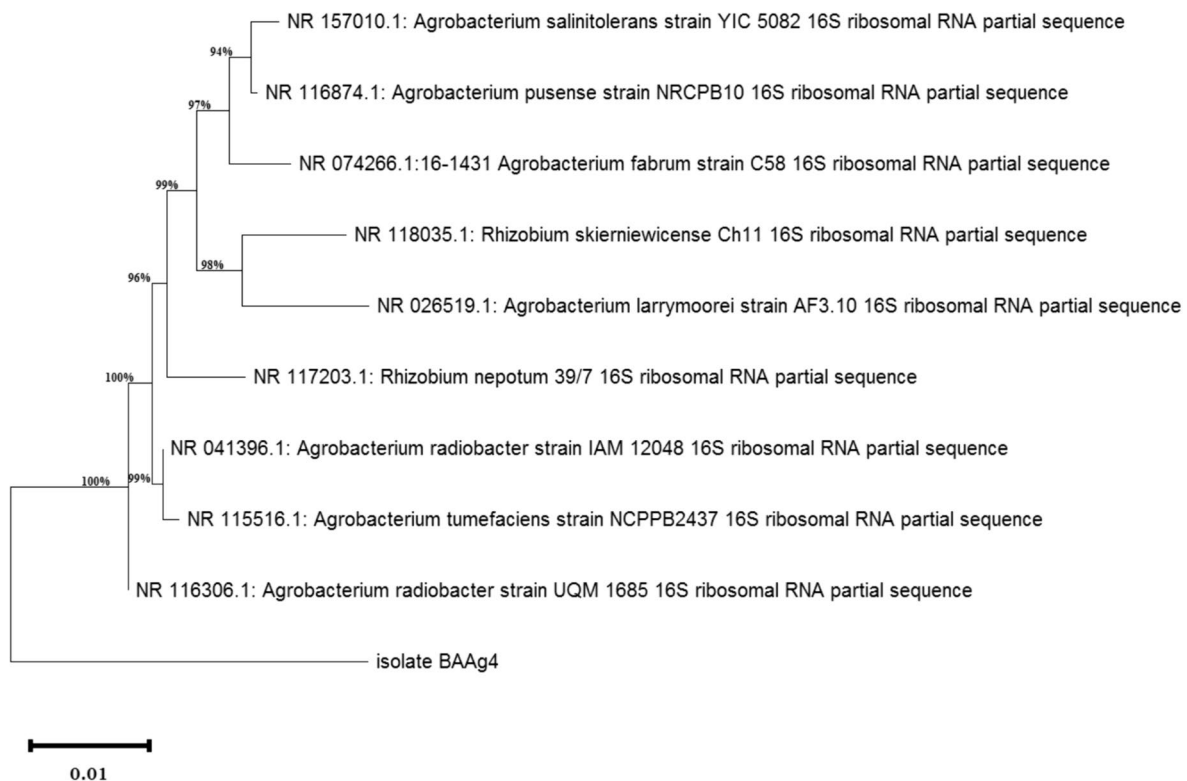


Fig. 6 Phylogenetic relationships between *Agrobacterium* isolate BAAg4 and closely related isolates based on partial 16S rRNA gene sequences. The evolutionary history was inferred using the neighbor-joining method with 1000 bootstraps. The percentages of replicate

trees in which associated taxa clustered together according to the bootstrap test are shown above the branches. The Kimura-2-parameter model was used to calculate the evolutionary distances

Whereas isolate GPIL23 exhibited the highest protein sequence identity of 100% with protein sequences from *P. klonensis* (WP_024616701.1), *Pseudomonas* (WP_058542122.1), and *Pseudomonas* (WP_053178893.1). The phylogenetic tree of 10 apricot and plum rhizosphere isolates and the closely related isolates based on protein sequences obtained from partial *acdS* gene sequences using the neighbor-joining method with 1000 bootstraps is shown in Fig. 8.

The extent of tumor inhibition

Co-inoculation (Mixed inoculum) of castor bean and kalanchoe plants with *A. tumefaciens* BAAg4 and ACC-producing isolates resulted in different effects on tumor formation. Isolates of *P. Vancouverensis* BApK9, BApL11, BApL12, and BApL18 significantly ($p \leq 0.05$) decreased the diameter and weight of tumors in castor bean (Except for tumor weight with BApL11) to 3.48 and 0.19, 3.72 and 0.31, 2.61 and 0.17, and 3.60 and 0.21 compared to 4.83 mm and 0.46 g in the positive control (*A. tumefaciens* BAAg4 only), respectively. Also, these isolates decreased the diameter and weight of tumors in kalanchoe to 4.56 and 0.22, 8.23 and 0.67, 6.78

and 0.31, and 6.79 and 0.41 compared to 15.4 mm and 1.67 g in the positive control, respectively. Although the isolate *P. putida* GPIL23 completely inhibited tumor formation in the castor bean, this effect was not achieved in the kalanchoe plants. Co-inoculation of castor bean and kalanchoe with *A. tumefaciens* BAAg4 and some other ACC deaminase-producing isolates, *P. frederiksbergensis* GPIK17, *P. frederiksbergensis* GPIK20, *P. klonensis* GPIK18, *P. klonensis* GPIK19, *P. klonensis* GPIK23 decreased the diameter and weight of tumors in kalanchoe and increased the diameter and weight of tumors in castor bean compared to the positive control (Table 5 and Fig. 9).

Discussion

In this study, four isolates displaying distinctive characteristics of *Agrobacterium* were successfully obtained from tumors found in peach and apricot seedlings on D1 medium. Furthermore, PCR analysis of the DNA from these isolates revealed these isolates carry Ti plasmid. All recovered isolates were able to produce tumor (s) on castor bean plants and gave positive reactions with 3-ketolactose production

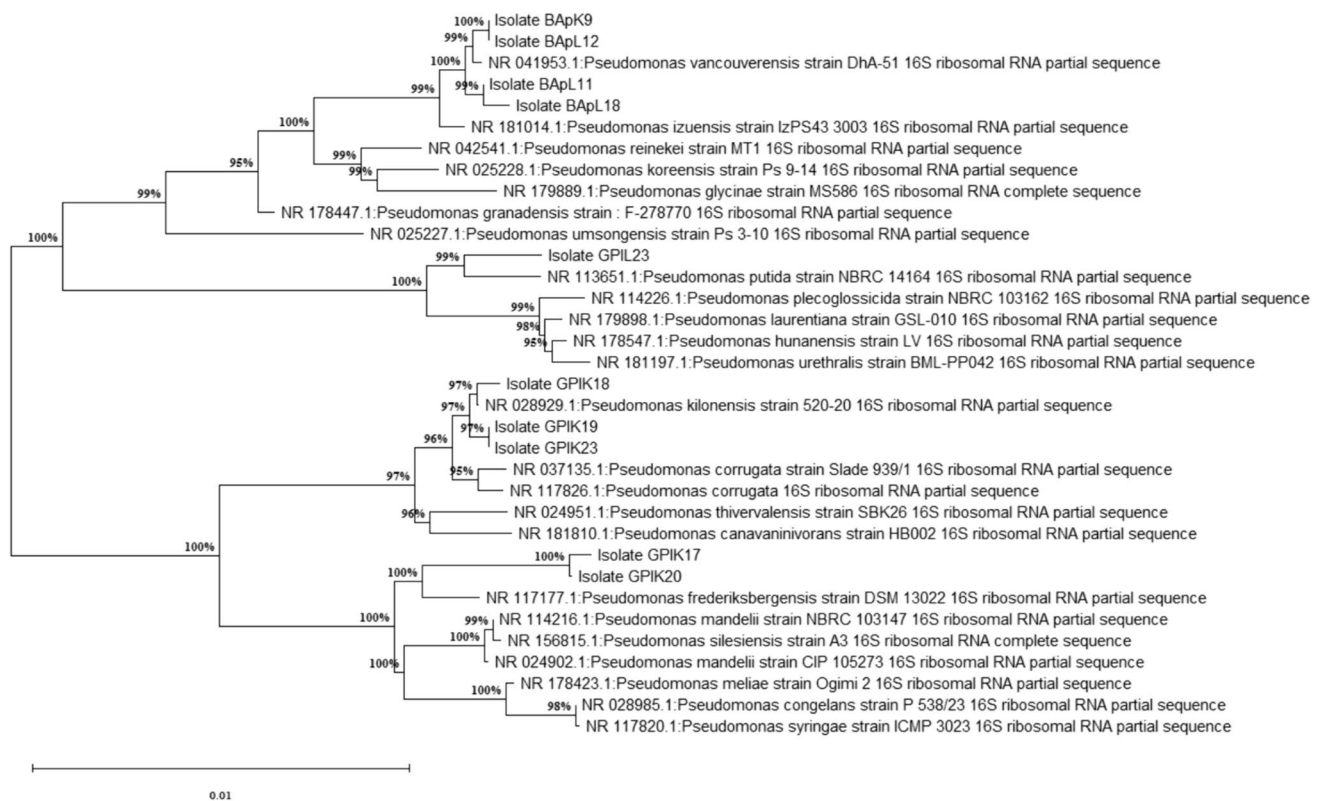


Fig. 7 The phylogenetic relationship between ACC deaminase-producing isolates and closely related isolates based on partial 16S rRNA gene sequences. The phylogenetic relationships between the ten apricot and plum rhizosphere isolates (BApK9, BApL11, BApL12, BApL18, GPIK17, GPIK18, GPIK19, GPIK20, GPIK23, and GPIL23) and closely related isolates in the NCBI GenBank were

conducted using the neighbor-joining method with 1000 bootstraps. The percentages of replicate trees in which associated taxa clustered together according to the bootstrap test are shown above the branches. The Kimura-2-parameter model was used to calculate the evolutionary distances

test. One of these isolates (BAAg4) was identified as *A. tumefaciens* using 16S rRNA gene sequence analysis. On the D1 medium, *Agrobacterium* species grow well while other bacterial genera are inhibited. *Agrobacterium* colonies appear circular and convex, initially light blue and turning to dark olive green (Kado and Heskett 1970). The primers tmr236F and tmr236R were designed based on the *tmr* locus sequence for the sensitive and specific detection of Ti plasmid. Only the *Agrobacterium* strains that carry Ti plasmid can produce the 236 bp target fragment. (Yang et al. 2011).

The 3-ketolactose production test is considered very reliable for assigning isolates to biovar 1 or biovar 2; biovar 1 gives positive results with the test, while biovar 2 gives a negative result (Ridé et al. 2000; Peluso et al. 2003). Infection of castor bean plants by *A. tumefaciens* resulted in the formation of large tumors, making it a suitable plant for testing the pathogenicity of *A. tumefaciens* isolates (Hao et al. 2007).

The misidentification of ACC deaminase and the *acdS* gene increases the estimate of the number and function of ACC deaminase-containing bacteria in the ecosystems. The nonspecific amplification of *acdS* homologs resulted in an overestimation of *acdS* gene horizontal transfer. CODEHOP primers (*acdS*f3/*acdS*r3) were designed based on distinguishing key residues in ACC deaminases from those of homologs for specific amplification of partial *acdS* genes (Li et al. 2015). This work showed that 10 out of 55 isolates from peach, apricot, and plum rhizosphere contained the *acdS* gene. These 10 isolates grew well on the DFM-SAM amended with ACC and can use ACC as a nitrogen source. In vitro, bacterial strains are tested for ACC activity on DFMSAM supplemented with 3 mM ACC (Gupta and Pandey 2019). Bacterial isolates that cannot grow in this medium are considered negative isolates for the production (Alemneh et al. 2021).

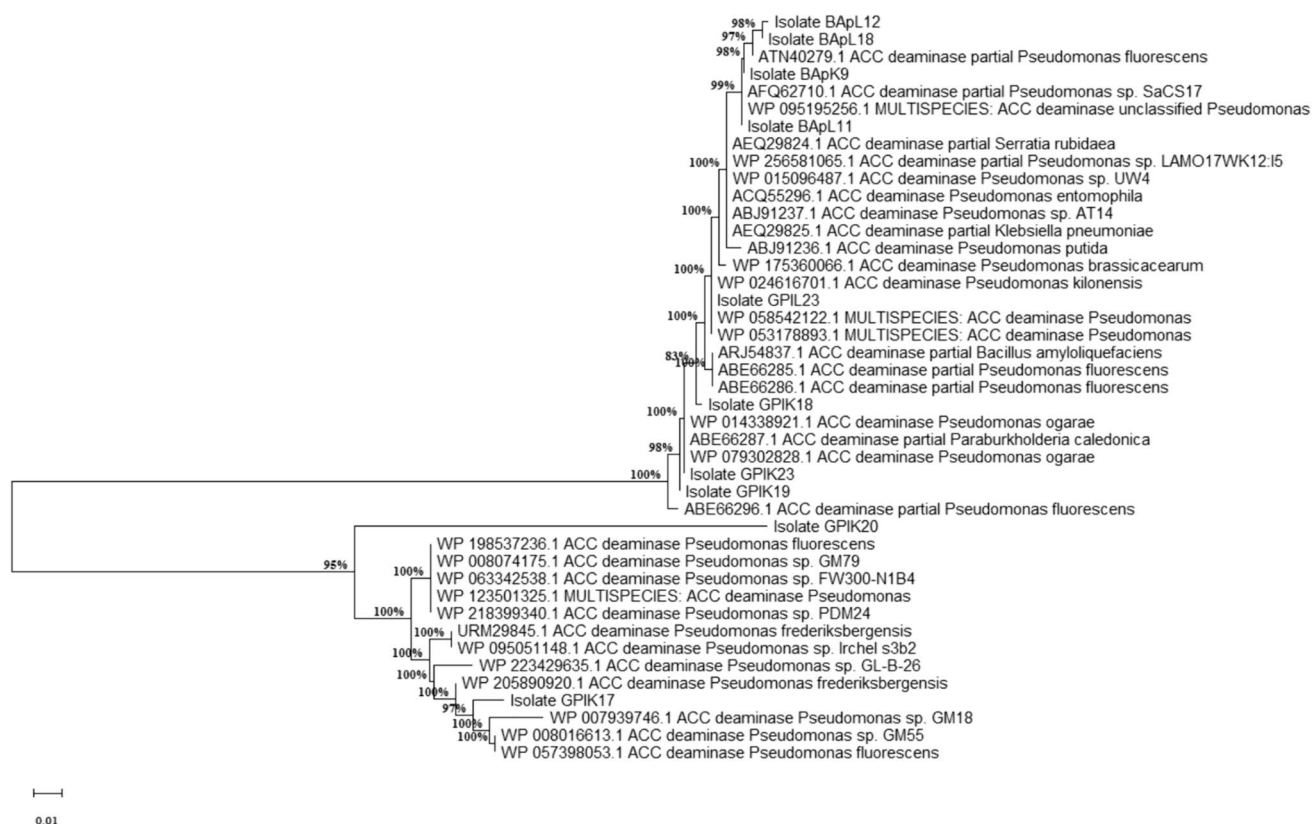


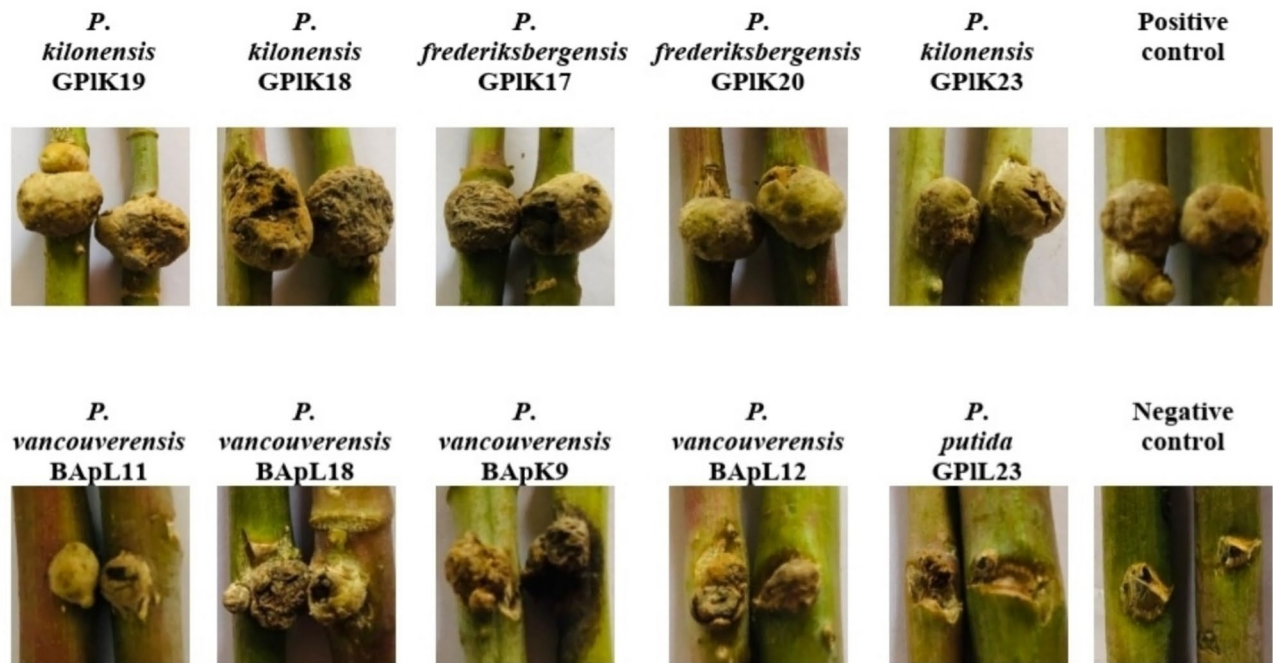
Fig. 8 The phylogenetic relationship between ACC deaminase-producing isolates and closely related isolates based on deduced protein sequences obtained from the partial *acdS* gene sequence. The phylogenetic relationships between the ten apricot and plum rhizosphere isolates (BApK9, BApL11, BApL12, BApL18, GPIK17, GPIK18, GPIK19, GPIK20, GPIK23, and GPIL23) and closely related isolates

in the NCBI GenBank were conducted using the neighbor-joining method with 1000 bootstraps. The percentages of replicate trees in which associated taxa clustered together according to the bootstrap test are shown above the branches. The Poisson correction technique was used to calculate the evolutionary distances

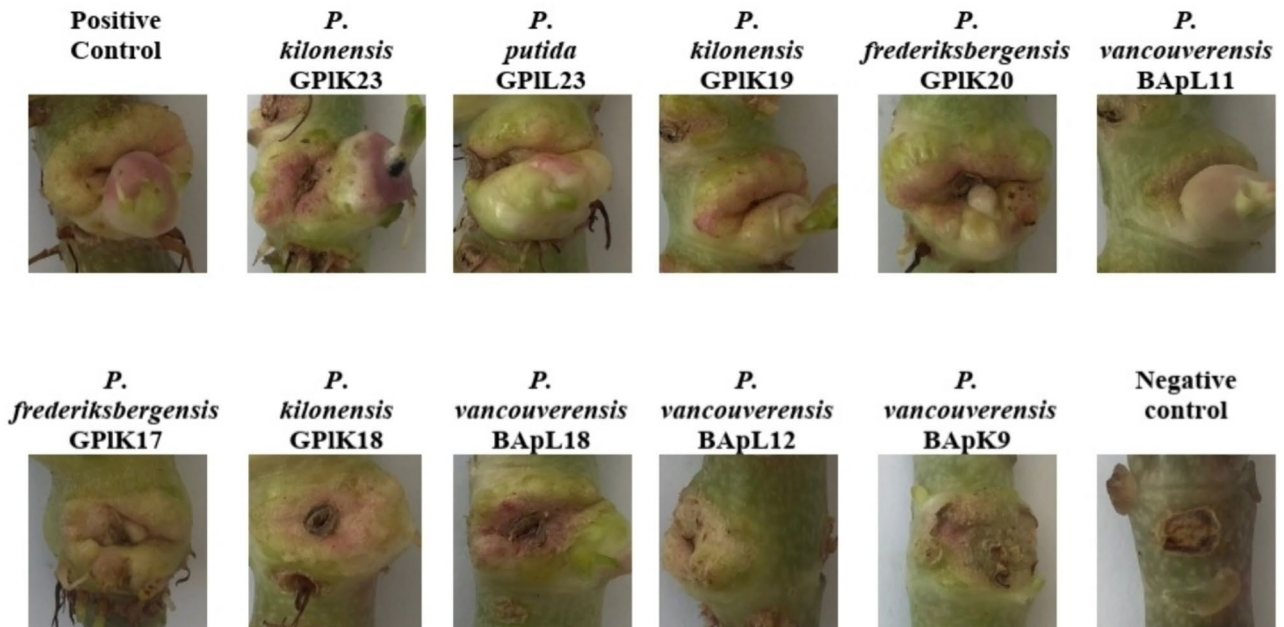
Table 5 Effect of 1-aminocyclopropane-1-carboxylic acid deaminase-producing *Pseudomonas* in inhibiting tumor formation by *A. tumefaciens* BAAg4 on castor bean and kalanchoe plants

Isolate	Tumor diameter (mm)		Tumor weight (g)	
	Castor bean	Kalanchoe	Castor bean	Kalanchoe
<i>P. vancouverensis</i> BApK9	3.48 e	4.56 g	0.19 e	0.22 e
<i>P. vancouverensis</i> BApL11	3.72 e	8.23 e	0.31 de	0.67 cd
<i>P. vancouverensis</i> BApL12	2.61 e	6.78 f	0.17 e	0.31 e
<i>P. vancouverensis</i> BApL18	3.60 e	6.79 f	0.21 e	0.41 de
<i>P. frederiksbergensis</i> GPIK17	7.11 c	6.92 ef	0.83 b	0.45 de
<i>P. kilonensis</i> GPIK18	8.36 b	6.80 f	1.08 a	0.43 de
<i>P. kilonensis</i> GPIK19	10.46 a	10.23 cd	1.18 a	1.11 b
<i>P. frederiksbergensis</i> GPIK20	5.53 d	9.91 d	0.63 bc	0.94 bc
<i>P. kilonensis</i> GPIK23	4.92 d	12.67 b	0.47 cd	1.56 a
<i>P. putida</i> GPIL23	0.00 f	11.45 bc	0.00 f	1.22 b
Positive control	4.83 d	15.4 a	0.46 cd	1.67 a
Negative control	0.00 f	0.00 h	0.00 f	0.00 f

Means of six replicates; Means in the same column that do not share a letter are significantly different using Duncan post hoc test ($p \leq 0.05$). Positive control=inoculated by *A. tumefaciens* BAAg4 only. Negative control=inoculated by sterilized Luria–Bertani broth medium



Castor bean



kalanchoe

Fig. 9 Results of co-inoculation of castor bean and kalanchoe plants with *A. tumefaciens* BAAg4 and ACC deaminase-producing *Pseudomonas* on tumor formation. On castor bean: isolate *P. putida* GPIL23 completely inhibited tumor formation. Isolates of *P. van-*

couverensis (BApK9, BApL11, BApL12, and BApL18) decreased tumor formation contrary to other isolates. On kalanchoe: all isolates decrease tumor formation and results ranging between significant and non-significant ($p \leq 0.05$) results

Many *Pseudomonas* strains, particularly those with ACC deaminase activity, are of tremendous interest for the development of innovative sustainable agricultural and biotechnological solutions. The ACC deaminase gene was found in 2591 *Pseudomonas* genomes (Glick and Nascimento 2021). The partial 16S rRNA gene sequences and the deduced protein sequences obtained from the partial *acdS* gene sequences of the ten *acdS* gene-containing isolates reported herein compared to the strains in the NCBI GenBank database showed the highest identity with *Pseudomonas* strains.

The findings in this study indicated that when *A. tumefaciens* BAAg4 was co-inoculated with four isolates of ACC deaminase-producing *Pseudomonas*, tumor formation decreased in castor bean and kalanchoe plants. However, contrasting outcomes were observed when six isolates of ACC deaminase-producing *Pseudomonas* were used, with varying effects on the two plant species. This could be due to the difference between plants used in the evaluation experiments or that ACC deaminase production by bacteria had a role in reducing tumorigenesis with other factors in bacteria. The synergistic interaction between ACC deaminase and both plant and bacterial auxin is necessary for ACC-producing bacteria to function at their best to protect plants against bacterial and fungal pathogens (Glick 2014). Hao et al. (2011) indicated that ACC deaminase is not the main factor in the avirulent strain *A. tumefaciens* D3 that reduces the pathogenicity of *A. tumefaciens* C58 and one explanation is that *A. tumefaciens* D3 may produce some other compounds that inhibit the growth of *A. tumefaciens* C58. The results here show that the ten ACC deaminase-producing *Pseudomonas* isolates have an effect on the growth of *A. tumefaciens* on NGA medium in vitro. The results showed that isolates of *P. vancoverensis* reported herein (BAPK9, BAPL11, BAPL12, and BAPL18) had a significant ($p \leq 0.05$) effect in decreasing tumors (diameter and weight) resulting from co-inoculation with *A. tumefaciens* BAAg4 in castor bean and kalanchoe plants. Ten isolates with better plant growth promoting (PGP) characteristics were chosen out of 252 isolates to evaluate their influence on seed germination and early development of eelgrass (*Vallisneria spiralis*) under various stress conditions (Li et al. 2023). It was shown that IAA concentration and ACC deaminase activity were negatively associated, and strains with higher ACC deaminase activity produced higher siderophores. Under the stress of low light intensity, *P. vancoverensis* with higher ACC deaminase activity demonstrated the most significant growth-promoting effect (Li et al. 2023). Another study showed that *P. vancoverensis* inhibited the growth of *Erwinia amylovora* on three media (NAS, KB, and NAG) and did not inhibit *E. amylovora* on two (LB and R2A) of the six media used (Mikiciński et al. 2020). In contrast, *P. vancoverensis* grown on the sixth medium (925) activated

the growth of the pathogen, which showed no growth in the absence of *P. vancoverensis*. However, experiments on apple trees and detached apple branches revealed that *P. vancoverensis* protects blossoms from fire blight (Mikiciński et al. 2020). The isolates of *P. frederiksbergensis* (GPIK17 and GPIK20) and *P. kilonensis* (GPIK18, GPIK19, and GPIK23) reported herein had decreased the diameter and weight of tumors in kalanchoe and increased the diameter and weight of tumors in castor bean plants compared to the positive control treatment. According to several studies, *P. frederiksbergensis* can control fungal plant pathogens. *P. frederiksbergensis* inhibited the growth of *Botrytis cinerea*, the causal agent of gray mold disease on strawberries (Melo et al. 2016). Also, *P. frederiksbergensis* significantly reduced the severity of *Fusarium* head blight disease caused by *Fusarium culmorum* on wheat and barley (Khan and Doohan 2009). Beneficial species that belong to the *P. corrugata* subgroup (*P. brassicacearum*, *P. kilonensis*, and *P. thivervalensis*) are closely related. It has been suggested that *P. kilonensis* be considered a junior synonym of *P. brassicacearum*. *P. brassicacearum*-like organisms are common in soils and have good biocontrol capabilities (Höfte 2021). Although the isolate of *P. putida* GPIL23 reported herein completely inhibited tumor formation in the castor bean, this effect was not achieved in the kalanchoe plants. Inoculation of maritime pine (*Pinus pinaster*) seedlings with ACC deaminase-producing *Pseudomonas putida* UW4 reduced symptoms of pine wilt disease caused by the nematode *Bursaphelenchus xylophilus* (Nascimento et al. 2013).

In conclusion, ACC deaminase production by *Pseudomonas* strains can contribute to reduced tumor formation. This role can be assisted by other compounds, such as antibacterial compounds produced by *Pseudomonas* strains. It is necessary to perform confirmatory experiments on the target host because the results were affected by the host or the *Pseudomonas* strain used as a biocontrol agent. The ACC deaminase-producing *P. vancoverensis* can be used as a biocontrol agent against *A. tumefaciens*.

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Author contributions All authors contributed to the development and design of the research plan. The IAAA prepared the samples and conducted the experiments under the supervision of the other authors. AFA and IAAA analyzed and interpreted the results and wrote the manuscript. MSM and MMY reviewed the final version of the manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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