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ORIGINAL ARTICLE

Efficacy of native antagonistic bacterial isolates in biological control of crown gall disease in Egypt

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Abstract *In vitro* analyzing the antagonistic activity of seventy native bacterial isolates towards plant tumorigenic *Agrobacterium tumefaciens* resulted in a selection of eight potential biocontrol agents. These isolates were screened for their antagonistic effect *in vitro* as well as their efficacy in reducing gall formation *in planta*. They were identified using Biolog microplates system as *Bacillus megaterium*, *Paenibacillus polymyxa*, *Pseudomonas fragi* (two isolates), *Pseudomonas viridilivd*, *Pseudomonas asplenii*, *Curtobacterium flaccumfaciens* and *Curtobacterum* sp.

All antagonists tested exhibited considerable inhibitory activity *in vitro* and significantly reduced incidence and size of galls in rose shoots, kalanchoe leaves and squash fruits with variable degrees on the tested hosts. *C. flaccumfaciens* reduced the incidence of crown gall up to 100% in the case of rose shoots and kalanchoe leaves whereas the same antagonist reduced galling of squash fruits to 75%. Likewise, *P. asplenii*, *P. viridilivd* and *P. polymyxa* reduced the incidence of crown gall up to 100% in the case of kalanchoe leaves and squash fruits, whereas they reduced galling of rose shoots to 66.7%, 55.6% and 44.5% respectively. In the same manner, the two isolates of *P. fragi* reduced galling up to 100% in squash fruits, while it was 88.9% in rose shoots and kalanchoe leaves. Interestingly, *B. megaterium* isolate completely suppressed the gall development in rose shoots, whereas the gall incidence was 100% in kalanchoe leaves and 25% in squash fruits. Bacterial isolates characterized in this study may be considered as potential sources of novel bioactive metabolites as well as promising candidates to develop new biocontrol agents for controlling crown gall disease.

Introduction

Plant bacterial diseases are very difficult to control owing to the lack of effective chemicals. Antibiotics could be used, but they are expensive and, in any case, the compounds that are valuable for human therapy are not allowed to be used in agriculture. Many strategies are used to management of crown gall disease including chemicals, pre-plant application of soil

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sterilents, soil solarization, herbicides, soil amendments (Gupta and Kamal, 2006; Moore and Canfield, 1996).

Bacteria are numerically the most abundant organisms in soil, and some of them have shown great potential for the biological control of soil-borne and other plant diseases. Extensive investigations have been conducted over the last decades to assess their potential to control crown gall disease (Lopez et al., 1987, 1989; Farrand, 1990; Moore and Canfield, 1996; Rhouma et al., 2004). These research efforts have found that antagonistic bacteria are distributed broadly, possess diverse modes of action, and have broad host ranges.

Kerr (1972) discovered and developed the first biocontrol system by isolating non-pathogenic strains of *Agrobacterium radiobacter*, from disease sites, and testing their ability to compete with pathogenic strains in mixed inoculations. He found several non-pathogenic strains helped to reduced infection, but one strain in particular, *A. radiobacter* strain designated as K84 completely prevented disease when added to wound sites at a 1:1 ratio with cells of *Agrobacterium tumefaciens*. This strain is the one that is successfully used against pathogenic strains of *Agrobacterium* on different hosts (Farrand, 1990; Lopez et al., 1987). It is used until now and marketed globally by several companies under a range of trade names. However, some strains of *A. tumefaciens* were insensitive to the bacteriocin (agrocin 84) produced by strain 84 *in vitro* (Kerr and Htay, 1974; Kerr and Panagopoulas, 1977; Moore, 1979; Schroth and Moller, 1976) and in some instances strain 84 did not prevent tumor production by these pathogens on susceptible hosts (Kerr and Panagopoulas, 1977; Moore, 1979). The success of strain 84 has encouraged workers to look for new antagonists for the strain 84-insensitive pathogens, but other *A. radiobacter* strains that inhibit pathogenic *Agrobacterium* species *in vitro* have been ineffective as control agents on plants (Garrett, 1979; Kerr and Panagopoulas, 1977; Moore, 1977).

The purpose of the present study was to explore the potential of native biocontrol agents outside the genus *Rhizobium* for their efficacy to control gall formation *in planta*.

Materials and methods

Microorganisms and culture conditions

Antagonistic bacteria were selected among a collection of 70 isolates which were isolated from plant tumors or the rhizosphere of galled plants which collected from different locations in Egypt. The selection was based on the ability of bacterial isolates to inhibit growth of *A. tumefaciens* on potato dextrose agar medium (Bechard et al., 1998). The strain of *A. tumefaciens* used as the crown gall pathogen in this study was the isolate designated 5A. This strain was originally isolated from rose plant with typical symptoms of crown gall disease.

Inocula of bacteria were prepared according to Eastwell et al. (2006). The isolates were grown in nutrient broth for 48 h at 28 °C. Cultures were chilled on ice for 30 min, concentrated by centrifugation and washed two times in saline solution 0.85% NaCl to remove media and any extra-cellular components released by the bacteria. They were then diluted in saline solution to a final concentration to obtain a density equal to 0.5 × McFarland (absorbance at wavelength of 600 nm). This gave final cell density approximately equal to 1.5×10^8 CFU/ml.

Pure cultures of bacterial isolates were preserved under sterile tap water at 5 °C. The retention of antimicrobial activity of antagonistic bacteria was periodically confirmed by the development of a zone of inhibition in the growth of the same isolate of *A. tumefaciens* comparable to that produced in the preliminary test.

Antagonistic activity in vitro

Cell suspension of *A. tumefaciens* was swabbed on the surfaces of PDA medium in 90 mm plates using sterile cotton swab. Subsequently, uniform size filter paper disks (6 mm in diameter) were impregnated by 10 µl of the specific antagonistic isolate suspension and left to dry in laminar flow cabinet. placed on surface of each inoculated plate. The plates were incubated in the upright position at 28 °C for 3 days. Simultaneously, addition of the saline solution instead of antagonistic isolates was served as control. Three replicates were carried out for each isolate. After incubation, the diameters of the growth inhibition zones formed around the disk were measured with transparent ruler in millimeter, averaged and the mean values were tabulated. Isolates of antagonistic bacteria that yielded considerable inhibition zones were selected for further analysis in this study.

Biocontrol activity in planta

Eight isolates that yielded the greatest inhibition zones for growth of *A. tumefaciens in vitro* were selected to demonstrate its biocontrol activity *in planta* against gall development. These isolates were examined for their ability to suppress gall formation by *A. tumefaciens* in rose shoots (*Rosa gallica*), kalanchoe (*Kalanchoe daigremontiana*) leaves and summer squash (*Cucurbita pepo* cv. Eskandarany) fruits. Each test plant was inoculated with one of the biocontrol agents or sterile saline.

In the case of rose, an 18-gauge needle (no bezel) was used to produce five holes in tender stem at 5 cm intervals starting 15 cm from the growing tip. Into each wound, 5 µl of saline solution or specific antagonistic suspension was pipetted. After the liquid was absorbed by the plant tissue, the wounded sites were wrapped in Parafilms. After 24 h, each site was re-wounded and 5 µl of *A. tumefaciens* suspension was introduced in each site. After the suspension was absorbed into the wound, the stem was again wrapped in Parafilms and loosely wrapped in polyvinyl chloride laboratory wrap to increase humidity at the inoculation site (Eastwell et al., 2006). This latter step was required for reliable gall formation. The plants were maintained in the greenhouse until, galls measuring were assessed. Number and size of formed galls were recorded after 40 days.

In the case of kalanchoe, the midrib of young detached leaves was stabbed by toothpick to make holes at three sites in each leaf. Into each hole, 2 µl of bacterial biocontrol suspension or saline solution was pipetted. After the liquid was absorbed by the plant tissue, the leaves were maintained for 24 h in sterile Petri dishes (15 cm) with wetted cheesecloth. After 24 h, sites were re-wounded and 2 µl of *A. tumefaciens* cell suspension was pipetted in each site. After the suspension was absorbed into the wound, the leaves were again backed to the dishes and kept wetted in growth chamber at 27 ± 2 until

galls measuring were assessed. Number and size of formed galls were recorded after 15 days.

In the case of squash fruits, uniform fruits were stabbed by toothpick to make holes at 12 sites distributed over three rows (four sites/row) per fruit. Inoculation was performed in the same manner as in kalanchoe leaves. The treated fruits were maintained in plastic containers with transparent plastic covers and kept at 27 ± 2 in growth chamber until galls measuring were assessed. Number and size of formed galls were recorded after 10 days.

Characterization of antagonistic bacteria

The ability of the antagonists to produce agrocin was tested using the agar well diffusion assay as reported by Tagg and Given (1971). Potential pathogenicity of each biocontrol agent was measured by testing its ability to evoke a hypersensitive reaction on tobacco leaves using leaf infiltration assay (Klement et al., 1964). Potential tumorigenicity of each biocontrol agent was assayed by stem inoculation of tobacco (*Nicotiana tabacum* cv. White Burley), tomato (*Lycopresicon esculentum* cv. Riogrande), Datura (*Datura stramonium*) and kalanchoe (*K. daigremontiana*).

For biochemical identification, bacteria were first determined to be either Gram-positive or Gram-negative using potassium hydroxide (New and Kerr, 1972). Carbon source utilization was determined using Biolog microplates comparing outputs to the MicroLog System 2 database, release 4.01B (Biolog, Inc., Hayward, Calif.). Gram-positive isolates were identified using GP2 MicroPlates (Biolog), whereas Gram-negative isolates were identified using GN2 MicroPlates (Biolog), according to the instructions of the manufacturer. Identification was based on the similarity index of carbon source utilization by each isolate relative to that of identified reference strains in the Biolog GP and GN databases.

Statistical analysis

Quantitative data of inhibition zone and number of wounds inoculated, wounds showing galls and size of galls per replication were subjected to analysis of variance (ANOVA) with SPSS software (version 16). The $P < 0.05$ was considered significant.

Results

*Antagonistic activity **in vitro***

Among 70 screened isolates, many isolates showed antagonistic activity toward *A. tumefaciens* isolate in variable degrees. Among these isolates, only eight isolates (designated: 2B, 4B, 7C, 26C, 4C, 11A, 72M and 2M) exhibited considerable inhibitory activity. The mean values of zone diameter resulted from these isolates fluctuated between 18 and 45 mm (Table 1 and Fig. 1). The greatest inhibition zone resulted from isolate 72M flowed by isolate 2M which reached 45 and 40 mm, respectively. Isolates 2B, 4B, 7C, 26C and 4C showed moderately antagonistic reaction indicated by the inhibition zone values (30, 29, 28, 28, and 25 mm, respectively). Isolate 11A showed relatively low antagonistic activity (18 mm).

Table 1 Inhibitory activity of eight bacterial isolates against *Agrobacterium tumefaciens in vitro*.

Isolate code	Mean of zone diameter (nearest whole mm)	Isolation origin
2B	30	Rose tumor
4B	29	Rose tumor
7C	28	Rose tumor
26C	28	Galled rose rhizosphere
4C	25	Galled rose rhizosphere
11A	18	Rose tumor
72M	45	Grapevine tumor
2M	40	Galled rose rhizosphere
LSD	4.2	

Biocontrol activity in planta

Inoculation of wounded sites on rose shoots, kalanchoe leaves and squash fruits with antagonistic bacterial isolates provide significant reduction in incidence and size of galls formed in response to subsequent inoculation with *A. tumefaciens* comparatively with 100% gall incidence resulted with saline solution alone (control treatment). There was significant difference between the tested isolates in its prophylactically effect on the different tested plants (Table 2 and Fig. 2A–C). Generally, the effect of tested isolates on gall formation or gall size in rose shoots were significantly low than in other tested plants.

Isolate 26C (*Curtobacterium flaccumfaciens*) reduced the incidence of crown gall up to 100% in the case of rose shoots and kalanchoe leaves whereas it reduced galling of squash fruits to 75%. The mean size of formed gall was relatively low in comparable to control treatment (3 mm vs. 10 mm). Isolate 7C (*Curtobacterium* sp.) reduced the incidence of crown gall up to 100% in the case of kalanchoe leaves only whereas it reduced galling to 33.3% in rose shoots and to 25% in squash fruits. The mean of gall size was relatively high in comparable to control treatment (8 mm vs. 18 mm in rose and 4 mm vs. 10 mm in squash fruits).

Isolates, 72M (*Pseudomonas asplenii*), 4C (*Pseudomonas viridilivd*) and 11A (*Paenibacillus polymyxa*) reduced the incidence of crown gall up to 100% in the case of kalanchoe leaves and squash fruits whereas the same antagonists reduced galling in rose shoots to 66.7%, 55.6% and 44.5%, respectively (gall incidence = 33.3%, 44.4% and 55.5%, respectively). In the same manner, the two isolates 2B and 4B (*Pseudomonas fragi*) reduced galling up to 100% in the case of squash fruits, while they reduced galling of rose shoots and kalanchoe leaves to 88.9% (11.1% gall incidence).

Characterization of antagonists

None of the tested isolates were able to produce agrocin. These results indicated that, none of the tested isolates are belongs to *A. radiobacter* type.

No hypersensitive reaction resulted from infiltration of tobacco leaves by antagonists suspensions.

No evidence of tumorigenicity was associated with stem inoculation of any tested plants (tomato, datura and kalanchoe). These results indicated that the used biocontrol

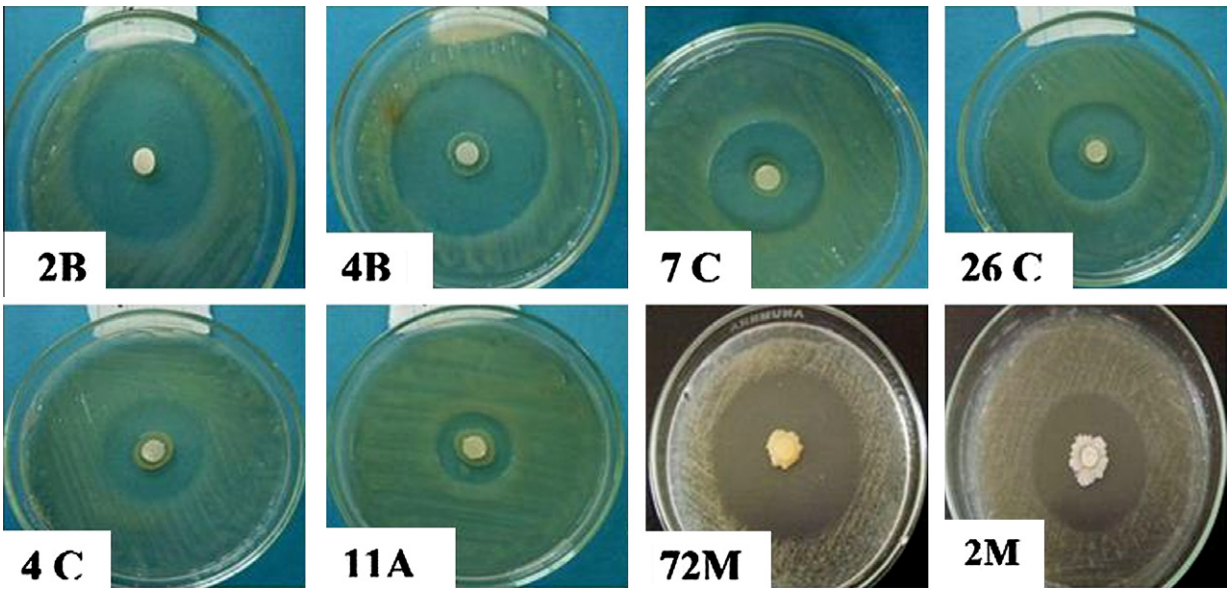

Fig. 1 Inhibition zones resulted from challenge of eight antagonistic bacterial isolates toward *Agrobacterium tumefaciens*.

Table 2 Effect of eight antagonistic bacterial isolates on incidence and size of galls induced by *Agrobacterium tumefaciens* on rose shoots, kalanchoe leaves and squash fruits, under artificial conditions.

Isolate	Identified	Gall on rose shoots			Gall on kalanchoe leaves			Gall on squash fruits		
		Size[a] (mm)	Incidence (%)	Reduction (%)	Size[a] (mm)	Incidence (%)	Reduction (%)	Size[a] (mm)	Incidence (%)	Reduction (%)
2B	*Pseudomonas fragi*	2	11.1	88.9	5	11.1	88.9	–	0.0	100
4B	*Pseudomonas fragi*	1	11.1	88.9	3	11.1	88.9	–	0.0	100
7C	*Curtobacterum* sp.	8	66.6	33.3	–	0.0	100	4	75	25
26C	*Curtobacterium flaccumfaciens*	–	0.0	100	–	0.0	100	3	25	75
4C	*Pseudomonas viridilivd*	6	44.4	55.6	–	0.0	100	–	0.0	100
11A	*Paenibacillus polymyxa*	6	55.5	44.5	–	0.0	100	–	0.0	100
72M	*Pseudomonas asplenii*	5	33.3	66.7	–	0.0	100	–	0.0	100
2M	*Bacillus megaterium*	–	0.0	100	6	100	0.0	3	25	75
Control		18	100	0.0	6	100	0.0	10	100	0.0
LSD		2.1	22.3	20.7	2.2	22.9	21.2	2.1	23. 3	20.4

[a] Mean, nearest whole mm.

agents is non-plant pathogenic and consequently could be used as biocontrol agents.

Identification of antagonistic bacteria was carried out using Biolog microplates system. Resulted identities (Table 3) included, *P. polymyxa, P. fragi* (two isolates), *P. viridilivd, P. asplenii, C. flaccumfaciens*, *Curtobacterum* sp. (unidentified speacis) and *Bacillus megaterium*.

Table 3 Biolog system for identification of eight bacterial isolates with strong inhibitory activity toward *Agrobacterium tumefaciens*.

Isolate	Gram staining	Identity	Similarity
11A	+	*Paenibacillus polymyxa*	0.762
2B	–	*Pseudomonas fragi*	0.658
4B	–	*Pseudomonas fragi*	0.587
7C	+	*Curtobacterum*	0.445
26C	+	*Curtobacterium flaccumfaciens*	0.669
4C	–	*Pseudomonas viridilivd*	0.524
72M	–	*Pseudomonas asplenii*	0.637
2M	+	*Bacillus megaterium*	0.894

Discussion

To maximize the chance for isolating bacteria suitable as rich sources of bioactive metabolites as well as for potential development of biological control agents for use against *A. tumefaciens*, we screened 70 bacterial isolates isolated from the rhizosphere of galled plants and plant tumors collected from Egyptian environment. The idea provided an opportunity to select effective biocontrol strains capable of antagonizing *A. tumefaciens* in the same environment. Keeping in view that, the production of an extremely wide array of bioactive compounds by bacteria and their potential for use in biocontrol programs is completely dependent on parameters such as taxonomical position, physiological characters, geographic condi-

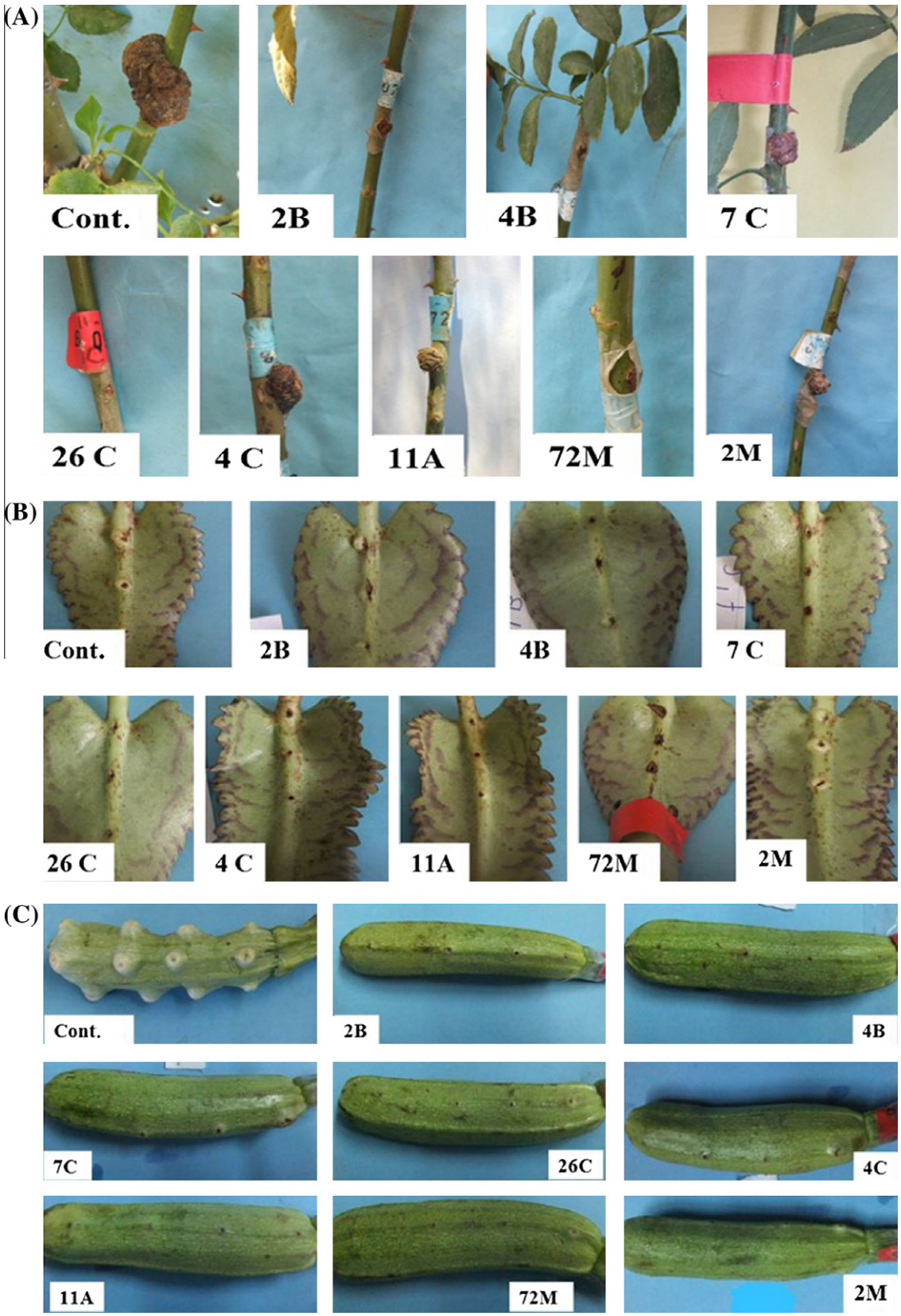

Fig. 2 Effect of eight antagonistic bacterial isolates on incidence and size of galls induced by *Agrobacterium tumefaciens* in rose shoots (A), kalanchoe leaves (B) and squash fruits (C).

tion, and soil composition. Thus, screening of a large number of bacteria from different geographic locations may increase the chance of finding novel bioactive compounds with broad spectrum antagonistic activity.

Among the screened isolates, eight antagonistic isolates with strong inhibitory activity against *A. tumefaciens* were selected and subsequently identified in the genera *Bacillus* (*B. megaterium*) *Paenibacillus* (*P. polymyxa*), *Pseudomonas* (*P. fragi*, *P. viridilivd* and *P. asplenii*) and *Curtobacterum* (*C. flaccumfaciens* and *Curtobacterum* sp.).

Since *in vitro* experiments have certain limitations in that biocontrol effectiveness may not be expressed under *in planta* conditions (Inam-ul-Haq et al., 2003), the antagonistic isolates under study were tested for their efficacy against *A. tumefaciens in planta*. In general, the selected antagonistic isolates proved to be efficient *in vitro* and significantly reduced the percentage of galled plants. This correlation between *in vitro* and *in planta* results have been documented in the study of Gupta et al. (2010). On the other hand, lack of this correlation has been documented in other studies with other antagonists (Ran et al., 2005 and Rajkumar et al., 2005).

The level of *in planta* control achieved with most of our antagonists was comparable to that reported initially by Kerr and Htay (1974) using K84. They reported that, galling was reduced from 79% to 31% when peach seeds were treated with strain K84. Our results showing that, all antagonists exhibited considerable inhibitory activity toward gall formation in variable degrees on different tested hosts. Proof of this, *C. flaccumfaciens* reduced the incidence of crown gall up to 100% in rose shoots and kalanchoe leaves and 75% in squash fruits. Likewise, *P. asplenii*, *P. viridilivd* and *P. polymyxa* reduced the incidence of crown gall up to 100% in the case of kalanchoe leaves and squash fruits. In the same manner, the two isolates (2B and 4B) of *P. fragi* reduced galling up to 100% in squash fruits, while they reduced galling of rose shoots and kalanchoe leaves to 88.9%. Interestingly, there is quite in consistent results with *B. megaterium* isolate. Where this isolate gave a level of resistance equivalent to 100% in rose hoots sits, gave a level equal to 0% in kalanchoe leaves. Much of the inconsistency in the performance of antagonistic bacteria has been attributed to variability in the physical and chemical properties within the niches occupied by biocontrol agents, and by the host, which affect both colonization and expression of biocontrol mechanisms (Ryan et al., 2004).

It is important to make sure that the biocontrol agents do not have the ability to cause a plant disease. Therefore, potential pathogenicity of each biocontrol agent was measured by testing its ability to produce a hypersensitive reaction using a tobacco leaf infiltration assay (Klement et al., 1964) and potential tumorigenicity was assayed by stem inoculation of tomato plants, *D. stramonium* and kalanchoe leaves. There was no evidence on the ability of any of the tested isolates to evoke a hypersensitive reaction on tobacco leaf in addition to the inability to produce tumors on any of the tested plants. These results indicated that the used biocontrol agents were not plant pathogens and consequently could be used as biocontrol agents.

A variety of antagonistic organisms have been isolated from soil and host-plant tissues and they affect plant pathogen by a variety of modes: for example parasitizing; producing toxins, antibiotics, or enzymes; interfering with pathogen–plant-host recognition; competing for nutrients; inducing systemic resistance of plants; and promoting plant health (Siddiqui and Mahmood, 1999).

Bacteria have a wide range of suppressive activities on different plant pathogen species. The strain K-84 harbors pAgK84 plasmid (Hayman and Farrand, 1988; Kerr and Htay, 1974) which codes of production of an inhibitor agrocin (bacteriocin). In spite of the success of K-84, some potential problems could be associated with its application (Lopez et al., 1989). The principal cause of failure of efficacy of strain K-84 is related to the pAgK84 transfer because the genes controlling agrocin 84 production and resistance can be transferred from strain K-84 to a pathogenic Agrobacterium (Ryder and Jones, 1990; Stockwell et al., 1996) becoming resistant to agrocin 84. In order to avoid this transfer and safeguard the biocontrol of crown gall, the genetically modified strain K1026 was successfully developed in the frame of an Australian and USA cooperation (Jones et al., 1988). The plasmid pAgK1026 is incapable of conjugal transfer at a detectable frequency in the laboratory (Jones et al., 1988). However, due to restrictions of the use of genetically modified organisms, K1026 is currently not used in many countries. Besides, crown gall biocontrol using K1026 could also breakdown via the transfer of Ti plasmid from a pathogenic Agrobacterium donor to K1026, which thus become pathogenic.

Members of the genus *Bacillus* were reported to be effective in controlling a wide range of fungal and bacterial diseases (Commare et al., 2002 and Kim et al., 2003). *Bacillus* spp. produce a wide range of secondary metabolites such as antibiotics, non-volatile and volatile compounds (Parke and Gurian-Sherman, 2001), and lytic enzymes (Frandberg and Schnurer, 1994).

Pseudomonads have a protective effect on the roots of plants infected by plant pathogenic bacteria by producing metabolites that include lytic enzymes (Berg, 1996), auxins (Ramamoorthy and Samiyappan, 2001), siderophores (Dwivedi and Johri, 2003), and antibiotics (Ran et al., 2005).

Bacteria of the genus *Curtobacterium* have been isolated as endophytes from many crops, including red clover (Sturz et al., 1998), rice (Elbeltagy et al., 2000), potato (Sturz and Matheson, 1996), yam (Tor et al., 1992), prairie plants (Zinnier et al., 2002), and citrus (Araújo et al., 2001). Several reports have indicated that *C. flaccumfaciens* can function as a biological control agent against many pathogens, and may function either by the triggering of induced systemic resistance (Raupach and Kloepper, 1998) or by antibiosis (Sturz and Matheson, 1996).

Bacterial isolates identified in this study with a diverse range of antagonistic activities may be considered as potential sources of novel bioactive metabolites as well as promising candidates to develop new biocontrol agents for controlling crown gall disease. Future studies to identify the bioactive metabolites of antagonistic bacteria isolated here, to determine their mechanisms of action as effective biocontrol agents are recommended and their ability for root colonization and survival in the rhizosphere needs still to be tested.

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