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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.

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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