In vitro Bioassay Of The Antagonistic Activity Of Some Bacteria Isolated From Compost Extracts

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ABSTRACT

In the present study, nine kinds of compost extracts were tested primarily for their activity against the causal agent of crown gall Agrobacterium tumefaciens (strain C58). The second step consisted on isolating bacteria from the most efficient extracts. For this, twenty-seven bacteria were isolated and investigated in vitro with the objective of selecting efficient antagonists against this disease. The bacterial activity is compared to the reference antagonist Agrobacterium rhizogenes K84 by the double layer method. In vitro analyzing the antagonistic activity revealed that, after incubation at 27°C with the pathogen, antagonists tested exhibited considerable inhibitory activity in vitro and reduced the development of the strain C58 of Agrobacterium tumefaciens with variable degrees. Statistical analyses revealed four groups of antagonist isolates. The first group contains the isolates that didn't induce inhibition zone; the second group is composed by isolates showing no significant activity compared to the control, whereas the third group is composed by the less efficient isolates and the final group is composed by the most efficient isolates. The highest level of inhibition zone diameter was observed with C58B2 (30.25 mm) against 19.37 mm in the control. Reduction of pathogen growth reached 38% compared to control. Compost extracts isolates tested in this study may be considered as potential sources of novel bioactive metabolites as well as promising candidates to develop new biocontrol agents for crown gall disease management.

Key Word: compost extracts, antagonists, Agrobacterium rhizogenes K84, Agrobacterium tumefaciens, inhibition, control,

Introduction

Crown gall caused by Agrobacterium tumefaciens is considered as an economically important bacterial disease. Over the world, it affects dicotyledonous plants from almost 100 different families (Belaskri, 2006; Matthysse, 2006), including stone fruits, grapevines, roses, some ornamental species, forest trees and tomato (Pulawska, 2001). Infected plants, especially those with tumors on the main roots and collar, are unfit for marketing and must be disposed of (Pulawska, 2010).

In Tunisia, the crown gall has frequently been observed on bitter almond (Rhonna et al., 2005). The disease has spread rapidly with the expansion of fruit tree cultivation and the establishment of new nurseries without adequate phytosanitary standards. Tunisian farmers are now facing problems in raising healthy stone fruit plants in nurseries, due to the lack of information about this disease and difficulties in identifying diseased stocks at an early stage. In spite of the preventive measures, that are being taken, crown gall continues to cause important damage in nurseries and in the field (Rhonna et al., 2008).

Biological control has been successfully applied using the non pathogenic strain Agrobacterium rhizogenes K84 (Ryder et al., 1991; Gupta et al., 2010) for almost three decades. It was the first example of biocontrol against pathogenic strains of Agrobacterium in different hosts and countries all over the world (Moore and Canfield, 1996; Rhonna et al., 2004). Nevertheless, the use of K84 has certain problems (Moore and Canfield, 1996). The failure of this strain is mainly due to transfer of genes controlling agrocin 84 production and so to the development of resistance to K84. Therefore, search for others antagonistic microorganisms with high activity for managing the crown gall, is necessary. Composts and compost extracts have been reported to control plant diseases caused by pathogens such as fungi (Hoitink and Fahy, 1986; Kerkeni et al., 2007a; KhanfirBenJenana et al., 2009), nematodes (Kerkeni et al., 2007b), bacteria (Al-Dahmani et al., 2003) and virus (Wahyuni et al., 2010). Inhibition induced by comports and extracts resulted from a combination of chemical and biological mechanisms. Biological factors included especially microflora (fungi and bacterial species) contained in these products (Nelson and Boehm, 2002). In fact, the effectiveness of microorganisms isolated from
composts and compost extracts against different pathogens was confirmed in several studies (Naidu et al., 2010). Bacteria of the genus Bacillus, Pseudomonas and Serratia and filamentous fungi of the genus Trichoderma were the most isolated and were known as biocontrol agents (Hoitink et al., 1991; Hoitink et al., 1997; Zhang et al., 1998; Quarles, 2001; Ingham, 2002; Camozzi, 2003).

Antifungal activity of microorganisms isolated from compost and compost extracts was widely investigated but studies about their antibacterial activity are fewer.

The aim of this study was to evaluate, in vitro, the antibacterial activity of some composts extracts and then the individually effect of some bacteria isolated from these compost extracts against Agrobacterium tumefaciens strain C58, and to compare their activity to the reference antagonist Agrobacterium rhizogenes K84.

Material And Methods

Compost extracts

Nine extracts prepared from different composts (C1, C2, C3, C4, C5, C6, C7, C8 and C9) and primarily composed of different animal manures (poultry, sheep, cattle and horse manures) were used (Table 1). Original composts were produced in the composting unit of the Technical Center of Organic Agriculture of Chott-Mariem (Tunisia), according to an aerobic process. Extract-production consists on suspending composts in tap water (1:5, v/v) in 20-liter plastic container and stirring the mixture daily for about 10 min during an extraction period of 5 days (Weltzien, 1992). After the incubation period, the mixtures were filtered through cheesecloth (250 µm) and the obtained extracts were stored at 4°C. They were taken out 30 min before use.

<table>
<thead>
<tr>
<th>Composts</th>
<th>Composition of composts used for extracts preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>50%CM+25%SM+25%PM</td>
</tr>
<tr>
<td>C2</td>
<td>60%CM+30%SM+10%ground straw</td>
</tr>
<tr>
<td>C3</td>
<td>50%CM+25%SM+25%HM</td>
</tr>
<tr>
<td>C4</td>
<td>50%CM+20%SM+20%PM+10%ground straw</td>
</tr>
<tr>
<td>C5</td>
<td>25%CM+25%SM+25%PM+25%HM</td>
</tr>
<tr>
<td>C6</td>
<td>30%CM+30%SM+30%PM+10% ground straw</td>
</tr>
<tr>
<td>C7</td>
<td>40%CM+40%SM+20% vegetable wastes</td>
</tr>
<tr>
<td>C8</td>
<td>25%CM+25%SM+25%PM+15%HM+10%ground straw</td>
</tr>
<tr>
<td>C9</td>
<td>25%CM+25%SM+25%PM+25%HM</td>
</tr>
</tbody>
</table>

C1-C9: compost1-compost9; CM: cattle manure; SM: sheep manure; PM: poultry manure; HM: horse manure

Isolation and growth conditions of tested bacteria

Compost extract bacteria

A serial dilution of compost extract up to 10^-3 was carried out, and then 10 µL aliquots of this dilution were spread onto Glutamate-Mannitol (MG) medium based on yeast agar (Oxoid) (0.5 g. L^-1), Glutamic acid (2 g. L^-1), Mannitol (5 g. L^-1), KH2PO4,3H2O (0.5 g. L^-1), NaCl (0.2 g. L^-1), MgSO4.7H2O (0.2 g. L^-1) and agar (Oxoid No.3) (20 g. L^-1). After 48 hours of incubation at 27°C, bacterial colonies formed in the seeded media were individually resuspended into MG medium. The same procedure was repeated until having a purified bacterial culture. A total of twenty seven bacterial isolates, showing different morphological characteristics were selected and designed by: C1A (ie : isolate A from compost C1), C1B1 (isolate B1 from compost C1), C1B2, C1C, C2A, C2B, C3A1, C3A2, C3B, C3C, C3D, C4A, C4B, C4C, C4D, C5A, C5D, C5B, C5C, C5E, C5B2, C7A1, C8A, C8B, C8C, C8D et C9A. They were sustained on King’s B medium at 27°C. For long storage, pure cultures were incubated at -20°C in eppendorf tubes (0.5 mL) containing 50% glycerol and 50% of sterile LB medium. Their identification was realized by means of the API system (Idris et al., 2007).

Agro bacterium rhizogenes K84 and Agrobacterium tumefaciens strain C58

The strain C58 of A. tumefaciens and the reference antagonist strain K84 were provided by the olive institute of Sfax (Tunisia). They were sustained on MG medium at 25°C.

In vitro Bioassay

Effect of compost extracts on Agrobacterium tumefaciens

The antibacterial activity of each extract against Agrobacterium tumefaciens strain C58 was tested via the double layer method (Rhouma et al., 2008). Test consist on suspending individually 10 µl of each extract on MG medium, in Petri plates, and incubating them for 24h and 48h at 27°C. In the same day of extract incubation, A. tumefaciens strain was streaked on the solidified surface of MG medium. After incubation, the plates were cleaned with alcohol (70%) then exposed to chloroform vapor for 30 min under laminar flow cabinet. After evaporation, One ml suspension of A. tumefaciens (10^6 CFU. mL^-1) was mixed with 3 mL of LBA (0.6% agar) at 45°C and was quickly overlaid to plates containing the extracts. Plates were incubated again at 27°C and checked after 24-48 h for the appearance of inhibition haloes surrounding the extracts’ spots.
Effect of compost extract isolated bacteria on Agrobacterium tumefaciens

Testing of in vitro sensitivity of A. tumefaciens strain C58 to the antagonists bacteria isolated was carried out according to the same method adopted for compost extracts (Rhouma et al., 2008). In this case, a bacterial suspension of antagonists (10^8 CFU mL^-1) was prepared in sterile distilled water, 20 μL aliquots were spot-inoculated on LBA medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 20 g agar in 1 liter of distilled water), and incubated at 25°C for 2 days. After two days incubation, the antagonistic bacteria were exposed to chloroform vapor and one ml suspension of A. tumefaciens (10^8 CFU, ml^-1) was mixed with 3 ml of LBA (0.6% agar) at 45°C and was overlaid to plates containing the bacterial isolates.

Control plates, were represented by the antagonistic bacteria K84. Plates were then incubated at 27°C and checked after 24 hours for the appearance of inhibition haloes surrounding the antagonist’s spots. The experiment was carried out with a completely randomized design with three replicates and was repeated twice.

Experimental design and statistical analysis

The experiment was carried out with a completely randomized design with three replicates and was repeated twice. Control plates, were represented by the antagonistic bacteria K84.

Data were subjected to analysis of variance (ANOVA) with the SPSS software (version 13). Significance of mean differences was determined using the Duncan’s test, and responses were judged significant at the 5% level (P=0.05).

Results

Effect of compost extracts on the development of A. tumefaciens

Results showed in figure 1, revealed that after 24 hours of incubation at 27°C, all the tested extracts were effective in reducing Agrobacterium tumefaciens strain C58 development. A significant difference were noted across the 9 extracts. The best antibacterial activity was recorded by the C7 extract, which showed an inhibition zone of 24.57 mm. The C1 was not effective compared to the control (19.5 mm). Whereas, C4 and C5 extracts were the least ones in reducing pathogen development, by respectively 6.09 and 18.96 mm.

Expanding the incubation period to 48 hours, showed that antibacterial activity of the extracts was improved. Inhibition zones were more important than those of 24 hours. The inhibition diameter ranged to 33 mm by the C6 extract. However, no significant difference was founded between extracts and the control K84.

![Figure 1 Diameters of inhibition zone measured by the different compost extracts after incubation at 27°C for respectively 24 and 48 hours. Each bar represents the mean of three replicates. Treatments affected by different letters were significantly different according to the Duncan test at the level of 5 %](image)

Antibacterial activity of compost extracts strains

Diameters of inhibition zone induced by the antagonists against the strain C58 are shown in Table 2. Results revealed that, after 24 hours of incubation at 27°C with the pathogen, compost extract bacteria decreased the development of A. tumefaciens by different degrees. In fact, statistic analyses revealed four groups of antagonistic bacteria, in comparison to the control K84. The first group contains the isolates without antibacterial activity; it regroups C1A, C1B2, C4A, C5B, C8A, C8B, C8C, C8D and C9A. The second group is composed by isolates that showed the same activity as the control (C4D, C1B1, C4C and C4B). The third group is composed by isolates with less effect than the control (C3A1, C3B C5C and C5E) and the final group contains isolates that had the best activity than the control (C5B2, C5D, C5A, 

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C7A1, C3D, C3C, C3A2, C2B, C2A and C1C). The highest level of inhibition zone diameter is observed with C5B2 strain (Figure 2).

Table 2. Inhibition zone diameter (mm) induced by compost extract bacteria against Agrobacterium tumefaciens strain C58 in double layer culture, after 24 hours of incubating. Data followed by different letters denote significant difference (p< 0.05), according to Duncan’s test.

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>A. tumefaciens strain C58</th>
<th>Antagonists</th>
<th>A. tumefaciens strain C58</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (K84)</td>
<td>19.38b</td>
<td>C1C</td>
<td>24.5a</td>
</tr>
<tr>
<td>C1B1</td>
<td>17 b</td>
<td>C2A</td>
<td>25.75a</td>
</tr>
<tr>
<td>C4C</td>
<td>17.75b</td>
<td>C2B</td>
<td>25.0a</td>
</tr>
<tr>
<td>C4B</td>
<td>19.75b</td>
<td>C3A2</td>
<td>23.5a</td>
</tr>
<tr>
<td>C4A</td>
<td>0 d</td>
<td>C3C</td>
<td>23.75a</td>
</tr>
<tr>
<td>C5B</td>
<td>0 d</td>
<td>C7A1</td>
<td>22.0a</td>
</tr>
<tr>
<td>C5A</td>
<td>0 d</td>
<td>C5A</td>
<td>27.75a</td>
</tr>
<tr>
<td>C5B2</td>
<td>0 d</td>
<td>C5B2</td>
<td>30.25a</td>
</tr>
<tr>
<td>C8C</td>
<td>0 d</td>
<td>C5D</td>
<td>28.5a</td>
</tr>
<tr>
<td>C1A</td>
<td>0 d</td>
<td>C5C</td>
<td>8.75c</td>
</tr>
<tr>
<td>C9A</td>
<td>0 d</td>
<td>C5E</td>
<td>6.25c</td>
</tr>
<tr>
<td>C8B</td>
<td>0 d</td>
<td>C3A1</td>
<td>11.0c</td>
</tr>
<tr>
<td>C8D</td>
<td>0 d</td>
<td>C3B</td>
<td>10.0c</td>
</tr>
</tbody>
</table>

Discussion

The crown gall caused by Agrobacterium tumefaciens constitute a serious disease causing important damage in nurseries and in the field (Rhouma et al., 2008). 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. However, some strains of A. tumefaciens were insensitive to the bacteriocin (agrocin 84) produced by strain 84 in vitro. This has encouraged workers to look for new alternatives to the strain 84-insensitive pathogens. Compost extracts have been reported to control different plant pathogens such as bacteria (Al-Dahmani et al., 2003). The present study showed that animal manure compost extracts inhibited the growth of Agrobacterium tumefaciens (strain C58). All the tested extracts were effective in reducing pathogen growth after 24 hours. However, the best antibacterial activity was recorded by the C7 extract (40%CM+40%SM+20% vegetable wastes), which showed an inhibition zone of 24.57 mm. This variability noted between compost extracts could be attributed to the nature of the microorganisms and substances liberated from those organic products.

After 48 hours of incubation, all the composts extracts were similar as the reference strain K84 in reducing disease development, but their activity was improved than after 24 hours. Reduction of disease reached 32% (C1 and C6). This improvement of extract activity is presumably du to the expression of all the microorganisms contained in this extracts, and then the production of higher amount of antibiotics and inhibitory substances in the culture media. Microorganisms and antibacterial substances of compost extracts may require more time to express their real biological potential.

According to Weltzien (1992), efficacy of compost extracts may vary considerably. This may in part be due to differences in procedures used for preparation of the extracts, the source, composition, quality, and maturity of the compost, length of storage, and possibly other factors. In addition and in previous studies, Penyalver et al. (2001), reported that Agrobacterium rhizogenes K84, produce iron-binding compounds (hydroxamate iron chelator) in large amounts compared to A. tumefaciens, when grown in iron-deficient medium (this is the case of the medium used in this study). This product may be identical to a previously described antimicrobial substance called ALS84. According to theses results we can attribute a part of the suppressive effect of compost extracts to their iron content. In fact, in a previous work, Kerkeni et al., (2009) showed that all compost extracts used for these in vitro tests contain more than 0.3 ppm of iron.
Concerning compost-isolated bacteria, in vitro tests showed that, after 24 hours of incubation, antagonistic isolates had inhibited the growth of A. tumefaciens strain C58 differently. Ten bacterial isolates, among the 27 tested, decreased the pathogen growth by more than 22% and showed better suppressive activity than the control K84. These results supported the findings of Ketterer (1990), Phae et al., (1990) and Weltzie (1992), who reported that compost extracts contain microorganisms, including bacteria with antagonistic potential. Contrarily to the results of Khanfir Ben Jenana (2009), suppressiveness of pure composts extracts was more accurate than this of isolated bacteria; this suggests the presence of interaction between all the components of the extract as microorganisms (Nelson and Hoitink, 1983). This is the case especially for C8 isolates (C8A, C8B, C8 C and C8D). In fact, when used individually, these four isolates had not showed suppressive activity, whereas the inhibition zone diameter noted with pure C8 extract was more than 32 mm. This result can be justified by the difference of the exposure period and by the fact that this extract has general suppressive potential.

Numerous studies have demonstrated that, the antagonistic activity of compost extracts bacteria is usually attributed to their high chitinolytic activity and the production of hydrolytic enzymes such as cellulases, glucanases or proteases (Cazorla et al., 2007). Others types of metabolites were detected such as volatiles, toxins, cyclic lipopeptides and antibiotics, which enable the genus to compete effectively (Haas and Keel, 2003).

In a previous study, Kerkeni et al. (2008) had identified by means of the API system (Idris et al., 2007), some bacteria from these extracts and revealed the presence of bacteria of the genus Pseudomonas, Serratia and Aeromonas, which were frequently found associated with plant roots and possess biological activity (Hoitink et al., 1997; Mekhel and Youssif, 2009).

The activity of these biological agents could be attributing to some others metabolites. In fact, in their study, Mekhela and Youssif (2009) had isolated a red pigment, called the red pigment prodigiosin (PG), from some species of genus Serratia, Pseudomonas and Aeromonas. They added that, this pigment appears only in the late stages of bacterial growth and it has been reported to have antifungal and antibacterial activity.

The main objectives of our study were the test of the antibacterial activity of several compost extracts and their bacterial strains. This work aimed also to found new alternatives that could be useful in the biological control of the crown gall disease caused by Agrobacterium tumefaciens. From this experiment, it is evident that compost extracts and their bacterial isolates have significant influence on A. tumefaciens strain C58 compared to the reference strain K84. Compost extracts and antagonistic bacteria tested in this study with a diverse range of antagonistic activities may be considered as potential alternative sources 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 to suppress the crown gall disease needs to be tested in vivo.

Acknowledgements

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