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In vitro suppression of the crown gall (*Agrobacterium tumefaciens*) by compost extracts bacteria

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ABSTRACT: Nine kinds of compost extracts were tested primarily for their efficiency, *in vitro*, against the causal agent of crown gall *Agrobacterium tumefaciens* (strain C58). The most efficient extracts were then selected and bacteria contained in these extracts were isolated. Twenty-seven isolates bacteria were obtained and investigated in vitro with the objective of selecting efficient antagonists against crown gall 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. In fact, statistical analyses revealed four groups of antagonistic isolates. The first group contains the isolates that didn't induce inhibition zone (C1A, C1B2, C4A, C5B, C8A, C8B, C8C, C8D et C9A); the second group is composed by isolates showing no significant activity compared to the control (C4D, C1B1, C4C et C4B), whereas the third group is composed by the less efficient isolates (C3A1, C3B C5C et C5E) and finally, the group composed by the most efficient isolates (C5B2, C5D, C5A, C7A1, C3D, C3C, C3A2, C2B, C2A and C1C). The highest level of inhibition zone diameter was observed with C5B2 (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.

KEYWORDS: compost extracts, antagonists, *Agrobacterium rhizogenes* K84, *Agrobacterium tumefaciens*, inhibition, control.

1 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 [2], [20], including stone fruits, grapevines, roses, some ornamental species, forest trees and tomato [28]. Infected plants, especially those with tumors on the main roots and collar, are unfit for marketing and must be disposed of [28].

In Tunisia, the crow gall has frequently been observed on bitter almond [31]. 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 [32].

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Biological control has been successfully applied using the non pathogenic strain *Agrobacterium rhizogenes* K84 [5], [33] 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 [22], [30]. Nevertheless, the use of K84 has certain problems. 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 [7], [12], [18], nematodes [13], bacteria [1] and virus [34]. Inhibition induced by composts and extracts resulted from a combination of chemical and biological mechanisms. Biological factors included especially microflora (fungi and bacterial species) contained in these products [25]. In fact, the effectiveness of microorganisms isolated from composts and compost extracts against different pathogens was confirmed in several studies [23]. 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 [3], [8], [9], [11], [29], [36].

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 the most efficient compost extracts, against *Agrobacterium tumefaciens* strain C58, and to compare their activity to the reference antagonist *Agrobacterium rhizogenes* K84.

2 MATERIAL AND METHODS

2.1 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 [35]. 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 1. Composition of composts used for extracts preparation

Composts	Composition
C1	50%CM+25%SM+25%PM
C2	60%CM+30%SM+10%ground straw
C3	50%CM+25%SM+25%HM
C4	50%CM+20%SM+20%PM+10%ground straw
C5	25%CM+25%SM+25%PM+25%HM
C6	30%CM+30%SM+30%PM+10% ground straw
C7	40%CM+40%SM+20% vegetable wastes
C8	25%CM+25%SM+25%PM+15%HM+10% ground straw
C9	25%CM+25%SM+25%PM+25%HM

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

2.2 ISOLATION AND GROWTH CONDITIONS OF COMPOST EXTRACT 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 [10].

Agrobacterium rhizogenes K84 and Agrobacterium tumefaciens strain C58: The strain C58 of *A. tumefaciens* and the reference antagonistic strain K84, used as control, were provided by the olive institute of Sfax (Tunisia). They were sustained on MG medium at 25°C.

2.3 IN VITRO BIOASSAY

Effect of compost extracts on A. tumefaciens

The antibacterial activity of each extract against *Agrobacterium tumefaciens* strain C58 was tested via the double layer method [32]. 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 over 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 (108 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 A. 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 [32]. In this case, a bacterial suspension of antagonists (108 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 (108 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.

2.4 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).

3 RESULTS

3.1 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 was 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.

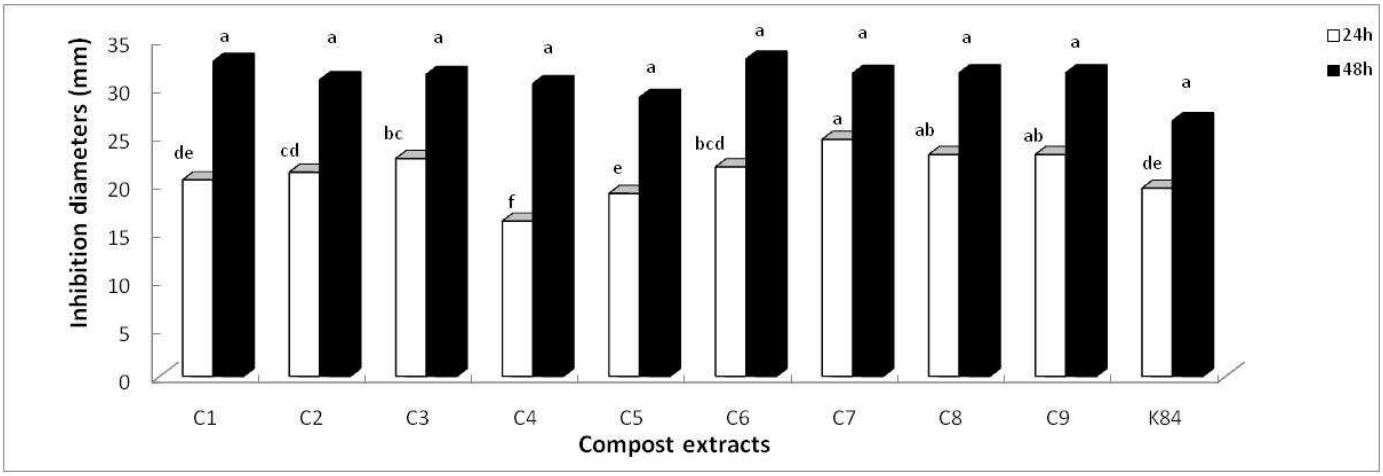

Fig. 1. Diameters of inhibition zone measured by the different compost extracts after incubation at 27°C for respectively 24 and 48 hours. Each bar represents the mean of three replicates. Treatments affected by different letters were significantly different according to the Duncan test at the level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

3.2 ANTIBACTERIAL ACTIVITY OF COMPOST EXTRACT STRAINS

Diameters of inhibition zone induced by the antagonists against the strain C58 of *A. tumefaciens* 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 the strain C58 of *A. tumefasiens* by different degrees. In fact, statistic analyses revealed four groups of antagonistic bacteria, in comparison to the control K84. The first group contains the no active isolates; it regroups C1A, C1B2, C4A, C5B, C8A, C8B, C8C, C8D and C9A. The second group is composed by isolates showing the same effect as the control (C4D, C1B1, C4C and C4B). The third group contains the least efficient bacteria compared to the control (C3A1, C3B C5C and C5E) and the final group includes the most efficient isolates (C5B2, C5D, C5A, 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.

Antagonists	Diameter of inhibition (mm)	Antagonists	Diameter of inhibition (mm)
Control (K84)	19.38b	C1C	24.5a
C4D	16.5 b	C2A	25.75a
C1B1	17 b	C2B	25.0a
C4C	17.75b	C3A2	23.5a
C4B	19.75b	C3C	23.75a
C4A	0 d	C3D	25.5a
C5B	0 d	C7A1	22.0a
C8A	0 d	C5A	27.75a
C8B	0 d	C5B2	30.25a
C8C	0 d	C5D	28.5a
C1A	0 d	C5C	8.75c
C8D	0 d	C5E	6.25c
C1B2	0 d	C3A1	11.0c
C9A	0 d	C3B	10.0c

Figures followed by different letters denote significant difference (p< 0.05), according to Duncan's test.

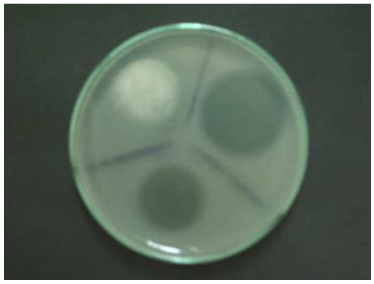

Fig. 2 inhibition zones induced by compost extract bacteria (C5B2) compared to the control K84

4 DISCUSSION

The crown gall caused by *Agrobacterium tumefaciens* constitute a serious disease causing important damage in nurseries and in the field [32]. [16] 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 [1].

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 different nature of 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 more improved. Reduction of disease had 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 in the culture media. Microorganisms and antibacterial substances of compost extracts may require more time to express their real biological potential. According to [35], efficacy of compost extracts may vary considerably. This may be, in part, 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, [26] 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, [15] 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 then 22% and showed better suppressive activity than the control K84. These results supported the findings of [17], [27],[35], reporting that compost extracts contain various microorganisms, including bacteria, with antagonistic potential. Contrarily to the results of [18], 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 [24]. 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 a 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 [4]. Others types of metabolites were detected such as volatiles, toxins, cyclic lipopeptides and antibiotics, which enable the genus to compete effectively [6].

In a previous study, [14] had identified by means of the API system [10] 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 [19], [21]. The activity of these biological agents could be attributing to some others metabolites. In fact, in their study, [21] had 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.

5 CONCLUSION

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*.

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