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Antimicrobial efficacy of some plant extracts on bacterial ring rot pathogen, *clavibacter michiganensis ssp. sepedonicus*

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Abstract

Control of plant bacterial diseases remains difficult due to the limited availability of efficient plant protection products with reduced negative effects either in the environment or with human and animal health. In order to reduce the usage of chemical pesticides alternative strategies for controlling plant pathogens and improve plant disease resistance are promoted. The aim of the study was to investigate the antibacterial activity of some natural compounds (plant extracts of *Tamarix ramosissima, Rosmarinus officinalis, Chelidonium majus, Silybum marianum*, *Satureja hortensis* essential oil and propolis) against bacterial ring rot pathogen, *Clavibacter michiganensis ssp. sepedonicus* (*Cms*). An agar diffusion method was used for the screening of the inhibitory effect of natural compounds on bacterial strains' growth. Minimum inhibition concentrations (MICs) were determined by a twofold serial dilution method. The anti-pathogenic activity was investigated by the study of anti-biofilm activity of natural substances. The analyzed natural substances showed a good microbicidal activity and anti-biofilm activity. The results obtained from this study may contribute to the development of new bio-control agents as alternative strategies for prevention and control of ring rot pathogen.

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Introduction

Clavibacter michiganensis subsp. *sepedonicus* (*Cms*), the causal agent of bacterial ring rot (BRR) of potato (*Solanum tuberosum*), is a globally recognized quarantine pathogen that is managed in the European Union through zero tolerance regulation within the certified seed industry (1, 2). Unfortunately, zero tolerance for BRR alone has been insufficient for long-term management of the disease since ring rot infections can remain symptomless or latent (3). By laboratory testing for latent infections, infected lots can be detected early and eliminated from seed programmes before further spread of the pathogen occurs. Implementation of crop rotation, disinfection and other sanitation practices is most important whenever the disease has occurred to prevent its recurrence and spread of the pathogen (4, 5).

At the moment, there is no method for direct chemical or biological control available for *Cms*. Breeding for resistance has produced some (mainly) tolerant cultivars that are not much used (6, 7). Control is mainly based on seed treatments and on hygiene and cultivation measures reducing the risks of introductions and dissemination.

The significant use of phytosanitary products it is correlated with crop production enhancement and with control of economically important plant diseases. However, the extensive use of pesticides in agriculture can entail risks for human health, environment and non-target organisms. Significant studies on the development of biological control methods have been initiated as legislation and government policy have demanded less reliance on chemical pesticides and greater adoption of integrated pest management. Therefore, there is a growing interest in research concerning the alternative pesticides and antimicrobial active compounds, including plant extracts and essential oils of aromatic plants (8, 9). The objective of the present study was to investigate the antibacterial activity of some natural compounds (plant extracts, essential oils, propolis) against *Cms* strains.

Materials and Methods

Natural compounds

Natural compounds tested for antibacterial activity against *Cms* strains were represented by: methanolic plant extracts of *Tamarix ramosissima, Rosmarinus officinalis, Chelidonium majus* and *Silybum marianum* as well as the essential oil of *Satureja hortensis* and an aqueous solution of propolis.

Antimicrobial activity

The antimicrobial activity was tested on ten *Cms* strains isolated from infected potatoes designated: *Cms*01-*Cms*10, and one reference strain, PD406. The strain identification was performed using an indirect immunofluorescence cell staining PCR test and bioassay on eggplant in accordance with the EPPO standard diagnostic protocol (10). The strains were routinely cultivated on solid YGM medium, at 21°C.

The qualitative screening of the antimicrobial activity

The qualitative antimicrobial activity screening was performed by an adapted disk diffusion technique (11, 12). Petri dishes containing the solid YGM medium were inoculated with microbial suspensions of density corresponding to $OD{\sim}0.2_{600nm}$, obtained from cultures grown for 3 days at 21°C in YGM medium. The inoculated plates were spotted with 5 µL of each plant extract. After a period of 20-30 minutes at room temperature, the plates were incubated for 72 h at 21°C. The occurrence of a growth inhibition area, surrounding the spot, was interpreted as an antibacterial effect. The diameters of the inhibition zones were measured and expressed in mm.

Quantitative assay of the antimicrobial activity

Quantitative inhibitory tests were performed in 96-well, flat-bottom plates using a serial binary micro-dilution method (13, 14). Serial binary dilutions of the tested compounds, ranging between 500 µL/mL and 0.1 µL/mL were performed in a total volume of 200 µL YGM liquid medium. After completion of the twofold serial dilutions for each plant extract, 100 µl of a suspension of the culture of *Cms* with optical density at 650 nm, 0.2 was added to each well in the microtiter plate. All plant extracts and each serial dilution were tested in duplicate. Each plate also contained eight wells of uninoculated medium serving as the sterility control; eight inoculated wells of *Cms*, devoid of any plant extract, were used as the growth controls. The plates were incubated at 21°C for three days and then subjected to macroscopic evaluation. The concentration of the tested plant extract from the last well that did not show the signs of culture growth (the culture medium remained clear) was considered the Minimum Inhibitory Concentration (MIC) for that plant extract.

The study of the influence of the tested plant extracts against microbial biofilm development and inert substratum colonization

The anti- biofilm properties were measured by the biofilm microtiter method as previously described (15, 16). The microbial cells were incubated for seven days at 21°C in the presence of two-fold dilutions of the tested plant extracts performed in YGM medium. The liquid medium was removed by inverting the assay plate and washed 3 times with dH_2O and then fixed with 95% ethanol. In order to stain the cells, a 0,1% crystal violet solution was added to every well and allowed to act for 30 minutes. After completion of the staining reaction, excess stain was removed by repeated washing (3–4 washes) with dH_2O. The crystal violet was eluted from stained biofilms by adding 150µl 30% acetic acid to each well, and measuring the OD490nm. The smallest concentration of the plant extract that inhibited biofilm development was considered the minimum biofilm eradicating concentration (MBEC), expressed in mg/mL.

Results

The qualitative screening of the antimicrobial activity

The qualitative screening can be considered a preliminary analysis regarding the antimicrobial activity of a compound. The results of the tests are presented in **Table 1**. All the natural compounds analyzed prove to be active. They exhibited good antimicrobial activities against all the tested *Cms* strains with diameters (expressed in mm) of the growth inhibition zones ranging between 18 mm and 4 mm. Two of the natural compounds analyzed gave similar results to the disinfectant control (sodium hypochlorite 10%).

Quantitative assay of the antimicrobial activity

The results of the minimum inhibitory concentrations (MIC) assay are presented in **Table 2**. They show a very good antibacterial activity for the tested natural compounds with the MIC ranging between 500 and 0.1 µL/mL. Significant antimicrobial activity was detected for essential oils of *S. hortensis*, and *T. ramosissima* plant extracts and propolis tincture 30%.

The influence of the tested molecules against microbial biofilm development and inert substrate colonization

Microorganisms present the ability to adhere to different substrata and form biofilms. This dynamic process is considered a virulence factor. The resistance to disinfectants of plant pathogenic bacteria in the biofilm state has important implications for disease control strategies. The natural substances tested exhibited inhibitory effects on artificially grown *Cms* biofilms. The minimum biofilm eradicating concentrations (MBEC) values were similar to the obtained MICs values. The results obtained showed that the inhibitory effect on microbial adhesins synthesis was expressed at concentrations as low as 0.1 µL/mL. The most efficient antibiofilm compound proved to be *T. ramosissima* plant extract and essential oils of *S. hortensis* (1:1 DMSO) with a dilution as low as 0.1 µL/mL, indicating that it could represent a potential alternative to chemical disinfectants used for ring rot control on surfaces of agricultural machines and other equipment.

Table 1. Results of antibacterial sensitivity assay using agar diffusion

Natural compouds tested against Cms strains	Cms bacterial strains										
	Diameters of the growth inhibition zones (mm)										
	Cms01	Cms02	Cms03	Cms04	Cms05	Cms06	Cms07	Cms08	Cms09	Cms10	Cms PD406
T. ramosissima plant extract	16	16	15	16	14	16	15	15	15	15	15
R. officinalis plant extract	7	7	7	7	7	7	6	7	7	7	7
C. majus plant extract	10	10	11	11	11	10	10	10	10	10	10
Propolis tincture 30%	12	10	10	10	10	10	11	11	11	11	11
Propolis aqueous solution	4	4	4	5	4	4	4	4	4	5	4
Essential oils of *S. hortensis* (1:1 DMSO)	17	18	18	18	18	18	18	18	17	17	17
S. marianum plant extract (10 mg/ml aqueous solution)	10	9	10	10	10	10	10	10	10	10	10
Sodium hypochlorite 10%	20	20	21	22	20	20	21	22	20	20	20

Discussion

T. ramosissima commonly known as tamarisk or salt cedar belongs to the family *Tamaricaceae*. Saltcedars are used as occasional ornamental shrubs or small trees, especially in the southwestern states of the USA, because of their attractive flowers and foliage, and tolerance of drought, heat and salinity.

Tamarix species are employed in traditional medicine as astringent, aperitif, stimulus of perspiration and diuretic. It is used as an anthelmintic, antihaemorrhoid haemostat and for diarrhea and gingivitis (17). Several researches have demonstrated antioxidant and antimicrobial activities of *Tamarix* species such as *T. ramosissima*. *In vitro* studies have shown that an antibacterial activity of ethyl acetate and water-acetone extracts of *T. ramosissima* is associated with the presence of polyphenolic substances. An ethyl acetate and tannin fraction exhibited antibacterial activity against *Clostridium diphtheriae* (25 mg/ml) and *Proteus mirabilis* (100 mg/ml). A butanolic fraction also showed activity against *Salmonella typhi* (100 mg/ml) and *Clostridium diphtheriae* (100 mg/ml) (18). Leaves of *T. ramosissima* have been used for the traditional treatment of rheumatism and jaundice. The plant is found to be rich in polyphenolic compounds such as flavonoids, sulphur-containing flavonoids, phenolic acids, hydrolyzable tannins and coumarins (18). Flavonoids are known to be synthesized by plants in response to microbial infection (19), and so it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against *Cms* strains. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. Moreover, lipophilic flavonoids may also disrupt microbial membranes (19).

Table 2. The minimum inhibitory concentrations (µL /mL) obtained from the quantitative antimicrobial assay

Natural compounds analyzed	Mean values of the minimum inhibitory concentrations (µL/mL)
T. ramosissima plant extract	0.1
R. officinalis plant extract	62.5
C. majus plant extract	15.62
Propolis tincture 30%	3.9
Propolis aqueous solution	125
S. marianum plant extract (10 mg/ml aqueous solution)	500
Essential oils of *S. hortensis* (1:1 DMSO)	0.1
Sodium hypochlorite	0.5 %

Among the aromatic plant species, genus *Satureja* L. occupies a special position because of the fact that essential oils and extracts of these species showed very significant antimicrobial activity against various species of bacteria and fungi. Recent studies are suggesting the potential use of hexane–methanol extract mixture of *S. hortensis* as a seed disinfectant and as a botanical pesticide for management of plant bacterial disease. *S. hortensis* L. essential oil proved to have a strong antibacterial activity against bacteria on Petri plates against plant pathogenic bacteria: *Clavibacter michiganensis* ssp. *michiganensis*, *Xanthomonas axanopodis* pv. *vesicatoria* and/or *Xanthomonas axanopodis* pv. *vitians* (20). Our experimental results have shown that *S. hortensis* oil exhibited a high inhibitory effect on *Cms* strains' growth indicating the potential inclusion of this natu-

ral compound in the management control strategies of ring rot disease. Research on the chemical composition of the natural compounds (plant extracts, bee-related products and essential oils) analyzed for their antibacterial activities against different *Cms* strains are currently under investigation. Additional basic research into active antimicrobial substances is urgently needed for the development of commercially competitive natural based disinfectants. Thus, the immediate problem to be addressed is how to extract active ingredients from plants, develop new disinfectants, and improve plant disease control.

Conflict of interest statement

The author declare no conflict of interest.

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