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Effect of alternative strategies for the disinfection of tomato seed infected with bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*)

A.-M. Kasselaki[a,*], D. Goumas[a], L. Tamm[b], J. Fuchs[b], J. Cooper[c], C. Leifert[c]

[a] *School of Agricultural Technology, Technological Educational Institute of Crete, Heraklion, Crete, Greece*
[b] *Research Institute of Organic Agriculture (FiBL), Frick, Switzerland*
[c] *Nafferton Ecological Farming group, Newcastle University, Stocsfield, UK*

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ABSTRACT

Currently there is a lack of effective seed treatments for bacterial pathogens, with Cu-based compounds (the only chemical treatments permitted under organic farming standards) only providing partial control. The aim of this study was to quantify the effect of alternative treatments for the control of bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*), a major seed-borne bacterial disease in tomato. Treatments assessed were acidified nitrite (a treatment previously shown to control the seed-borne fungal disease *Didymella lycopersici*), antagonistic strains of *Bacillus* spp. and compost extracts, which were not previously evaluated as treatments for seed-borne diseases. Efficacy of treatments was determined in a seed disinfection assay. Ten-minute immersion of seed in 300 mmol l^{-1} acidified nitrite resulted in 98% being pathogen free. Copper hydroxide, certain strains of *Bacillus* spp. and all compost extracts resulted in 100% pathogen free seed.

1. Introduction

Seed-borne fungal and bacterial diseases are a serious concern in conventional as well as organic/low input crop production systems. The use of untreated seed infected with seed-borne diseases can have a direct effect on yield, on the build-up of pathogen inocula in the soil, the introduction of diseases into previously unaffected areas, and the re-emergence of diseases that were effectively controlled by pesticide seed treatments [1,2].

Effective seed treatments for fungal diseases, based on fungicides are available for conventional production systems [2–4]. Also for organic production systems a range of alternative treatments has recently been described [3]. However, currently available treatments for seed-borne bacterial pathogens (e.g., copper fungicides) are only partially effective, and the control of bacterial seed diseases remains a challenge in both organic and conventional production [2,4]. As a result there is an urgent need for the development of new alternative treatments for seed-borne bacterial pathogens [2].

The aim of this study was to test seed treatments based on acidified nitrite, previously shown to control the seed-borne fungal disease *Didymella lycopersici* [3], compost extracts and different *Bacillus* spp. strains against seed-borne inocula of *Clavibacter michiganensis* subsp. *michiganensis* (coded Cmm) on tomato seed using a standard seed disinfection assay.

2. Materials and methods

2.1. *Clavibacter michiganensis subsp. michiganensis strains*

Different strains of Cmm were tested on tomato plants at the fourth true leaf stage for their pathogenicity using the method of the hypersensitive reaction on *Mirabilis jalapa* [5] and for their virulence by inoculation. A mixture of the two most virulent strains, Cmm 4019 and Cmm 4089, originating from diseased tomato plants of the region of Crete, was used for the inoculation of the tomato seeds.

2.2. *Production of infected tomato seeds*

The Cmm strains 4019 and 4089 were cultured on the semi-selective medium m-SCM [6]. After autoclaving, the following chemicals were added to the medium through a membrane filter of 0.2 μm (Pall, Michigan, USA): 200 mg cycloheximide (in 1 ml methanol), 30 mg nalidixic acid (in 1 ml of 0.1 mol l^{-1} NaOH) and 100 mg nicotinic acid (in 20 ml sterilized distilled water). Bacteria were collected after 72 h at 27 °C, and suspended in 0.1 mol l^{-1} phosphate buffered saline (PBS), pH 7.2.

A 10^5 colony forming units (cfu) ml^{-1} Cmm (mixture of 4019 and 4089 strains) suspension was prepared for inoculation of the

* Corresponding author. Tel.: +30 2810 379 459; fax: +30 2810 318 204.
E-mail address: kaselaki@staff.teicrete.gr (A.-M. Kasselaki).

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tomato seeds by adjusting the optical density of the initial suspension (10^8 cfu ml^{-1}) to 0.1 at 600 nm. Seeds (50 g) of the tomato cultivar Packmore (Geoponiko Spiti, Athens, Greece) were packed in a cheesecloth bag and placed in a 1-l flask (Millipore, Schwalbach, Germany) containing 400 ml of the bacterial suspension. A vacuum was created by applying negative pressure (−40 kPa) for 5 min, after which the seeds were left to dry completely on sterile blotting paper, at room temperature, in a laminar flow cabinet.

The seeds were checked for the presence of the pathogen, using the immunofluorescent assay (IF) method, adapted for the combination 'tomato-Cmm' [7]. The antiserum used was the 'anti-Cmm 25' 1/800 diluted in PBS. The conjugate used was the Alexa Fluor (Molecular Probes Inc., Portland, USA) 1/100 diluted in PBS [7]. Inoculated seeds were stored at 5 ± 1 °C until further use.

2.3. Assessment of seed treatment efficacy

In preliminary trials, all treatments included in the study were applied to healthy tomato seeds to test for potential phytotoxicity effects. One hundred seeds were used per treatment. After application of treatments by soaking, seeds were placed on Petri dishes between deionized-sterilized water-moist blotting paper and incubated in the dark, at 25 °C for 10 days. None of the treatments appeared to be phytotoxic to the tomato seeds, since their germination rates (75–80%) were similar to the untreated control. Subsequently the following treatments were applied to Cmm-infected tomato seeds: (1) acidified nitrate, (2) *Bacillus* spp. strains, (3) compost extracts, and (4) a control.

- Acidified nitrite solution (300 mmol l^{-1}, pH 2) was freshly prepared immediately prior to seed treatment by adding 20.7 g sodium nitrite in 1 l of citric acid (57.63 g) buffer.
- *Bacillus* spp. strains (coded d1, d2, d3, d5, d9, f3, f4, f5, f9, f11 and f12) were cultured on Tryptone Soy Agar at 22 °C for 1 day. Bacterial cell suspensions were prepared in sterilized de-ionized water and the optical density at 600 nm was measured and adjusted to 0.1 for all strains.

  For the liquid *Bacillus* spp. cultures, 100 μl of each strain suspension was added to 100 ml Tryptone Soy Broth (TSB), in 200-ml flasks. The flasks were placed in an orbital incubator at 27 ± 1 °C. Liquid *Bacillus* spp. cultures were used for inoculation of the tomato seed 3 days later. Plain TSB was used as control (TSB control).
- Compost extracts. The seven different composts tested were provided by the Research Institute of Organic Agriculture (FiBL, Frick, Switzerland). The compost samples were selected in 2005 at seven commercial compost plants [8,9].

All compost samples fulfilled the Swiss quality guidelines (e.g., source separated organic material) [10]. The seven composts extracted were: (1) 074 (green waste for horticultural use), (2) 075 (digestate from co-fermentation of 2/3 cow manure and 1/3 green waste and mesophilic fermentation), (3) 076 (green waste for covered cultures), (4) 077 (green waste mixed with 10–15% soil at the start/horse manure), (5) 078 (green waste with a high percentage of lignin material), (6) 079 (green waste with a high percentage of tree leaves collected in autumn) and (7) 080 (digestate from biowaste). The numbers given to the composts are the FiBL sample numbers.

Each of the composts had been previously characterized in terms of origin and type of raw material, processing history and system, chemical analyses for plant nutrients, contaminants, and biological activity. The compost samples were stored in the dark, at 4 °C before further processing for the seed experiments.

A 100-ml sample of each of the composts was mixed with 200 ml tap water and incubated for 3 days at room temperature. The crude extracts were then filtered through four layers of cheesecloth and immediately used in the experiments.

Kocide 101 (copper hydroxide 50% WP, Griffin LLC, Valdosta, USA) at the rate of 3 g l^{-1} and inoculated untreated seeds were used as control treatments in all experiments.

Treatments were applied by immersion of the seed into prepared solutions for 10 min except for the compost extracts, where the seeds were soaked overnight. Seeds were left to dry completely at room temperature and were then placed on Petri dishes containing m-SCM, and incubated at 27 ± 1 °C.

The experiment was carried out twice, with four replicate Petri dishes (25 seeds per dish) per treatment in each experiment. Percentage of infected seeds (% Cmm incidence) was recorded per Petri dish, 21 days after application of the treatments. Assessment was based on (1) the visual examination of the bacterial colonies formed (typical Cmm colony phenotype) and (2) the application of the IF assay on randomly selected colonies.

Table 1
Effect of seed treatment with acidified nitrite and copper hydroxide on the incidence of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) on inoculated tomato seeds. Results of a disinfection assay.

Treatment	Mean % Cmm incidence	SEM[a]
Copper hydroxide (3 g l^{-1})	0.0	0.0a[b]
Acidified nitrite (300 mmol l^{-1})	2.0	0.9a
Inoculated-untreated control	48.0	4.9b

[a] SEM, standard error of the mean.
[b] Means followed by the same letter are not statistically different ($p < 0.05$).

2.4. Statistical analysis

Data (% Cmm incidence) from the two experiments were pooled and analysed using one-way analysis of variance (ANOVA). Treatment means were compared using Tukey's HSD test for homogenous subsets ($p \leq 0.05$). The statistical package SPSS 11 for Windows was used.

3. Results

Seed treatment with acidified nitrite was highly effective at reducing Cmm incidence in the disinfection assay (Table 1).

Of all *Bacillus* spp. strains tested, d1, d3 and d9 completely inhibited Cmm incidence. In contrast, Cmm incidence was high on seed treated with the TSB (control), as was expected for the bacteriological culture medium (Table 2).

Table 2
Effect of tomato seed treatment with *Bacillus* spp. strains on the incidence of seed-borne *Clavibacter michiganensis* subsp. *michiganensis*. Results of a disinfection assay.

Treatment	Mean % Cmm incidence	SEM[a]
Copper hydroxide	0.0[b]	0.0a
Bacillus strains		
d1	0.0a	0.0
d2	3.0ab	1.2
d3	0.0a	0.0
d5	4.5ab	0.8
d9	0.0a	0.0
f3	30.6d	2.2
f4	10.1b	0.6
f5	29.0d	1.9
f9	9.5b	1.5
f11	5.7ab	1.5
f12	17.7c	1.7
TSB control	46.9e	3.6
Inoculated-untreated control	23.6cd	0.8

[a] SEM, standard error of the mean.
[b] Means followed by the same letter are not statistically different ($p < 0.05$).

Table 3
Effect of seed treatment with compost extracts on the incidence of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) on inoculated tomato seeds. Results of a disinfection assay.

Treatment	Mean % Cmm incidence	SEM[a]
Inoculated-untreated control	49.0	6.1
Copper hydroxide	0.0	0.0
Compost extracts		
074	0.0	0.0
075	0.0	0.0
076	0.0	0.0
077	0.0	0.0
078	0.0	0.0
079	0.0	0.0
080	0.0	0.0

[a] SEM, standard error of the mean.

There were no statistically significant differences in reducing disease incidence amongst the different compost extracts: all extracts were 100% effective against Cmm (Table 3).

The above alternative seed treatments were as effective as copper hydroxide in all disinfection assays.

4. Discussion and conclusions

Acidified nitrite is a highly reactive substance that upon reaction with acids releases gaseous compounds with antimicrobial activity such as nitric oxide [11]. It was previously shown to be highly effective against the fungal seed-borne pathogen *D. lycopersici* without being toxic to the tomato seeds [1]. In the present study it was also found to have disinfection activity against Cmm. Nitrite is known to have significant bactericidal activity against a range of food pathogens [12] and is currently permitted as a food additive (e.g., for curing bacon) under organic processing standards [13]. However, whether it will be permitted as a seed disinfection treatment under organic farming standards is currently unclear.

The inhibition of Cmm incidence on tomato seeds by antagonistic *Bacillus* spp. strains was high. This was most probably due to their ability of producing antibiotics and/or the ability to elicit resistance in plants against diseases, especially when used as seed treatments [14]. Future studies should therefore quantify the effect of *Bacillus* spp. strains against Cmm *in vivo*, since inoculation with *Bacillus* spp. strains appears to be a promising approach for the control of bacterial diseases that currently cannot be controlled effectively by chemical seed treatments.

On infected tomato seeds treated with any of the compost extracts, despite their great variety in waste materials and processes, Cmm incidence was 0%. Compost extract mode of action is poorly understood. Their efficacy may be due to competition between micro-organisms that are antagonistic to the pathogen and present in the compost, and/or antimicrobial compounds produced by some of these micro-organisms; but this should be further investigated. The use of compost extracts is permitted under EU organic farming standards and they are used by organic farmers for the control of foliar pathogens [13]. Since compost extracts can be produced on-site, they could reduce the dependence of growers on commercial crop protection products.

Results of the study presented here indicate that acidified nitrite, certain *Bacillus* spp. strains and all compost extracts tested, are promising alternative treatments for seed-borne Cmm in tomato.

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