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SHORT COMMUNICATION

Laboratory evaluation of botanical extracts, microbial culture filtrates and silver nanoparticles against *Botrytis cinerea*

Aly Soliman Derbalah · Gabr Abd Elweenes Elkot · Amany Mohamed Hamza

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Abstract In an attempt to find alternatives to fungicides, the efficacy of crude extracts of seven plant species (*Cassia senna*, *Caesalpinia gilliesii*, *Thespesia populnea* var. *acutiloba*, *Chrysanthemum frutescens*, *Euonymus japonicus*, *Bauhinia purpurea* and *Cassia fistula*), three microbial culture filtrates (*Epicoccum nigrum*, *Bacillus subtilis* and *Bacillus pumilus*) and silver nanoparticles were evaluated against *Botrytis cinerea*, the causative fungus of rot, under laboratory conditions. All tested materials were evaluated alone and combined with tolclofos-methyl, the recommended fungicide against *B. cinerea*. Gas chromatography–mass spectrometry analysis was performed to identify the possible biologically active components of the most effective plant extract and culture filtrate against *B. cinerea*. The results showed that *Euonymus japonicus* was the most effective plant extract and *Bacillus subtilis* was the most effective culture filtrate against *B. cinerea*. In addition, silver nanoparticles showed a high efficacy against *B. cinerea*. Combining each of the microbial culture filtrates, plant extracts and silver nanoparticles with the tolclofos-methyl improved the efficacy of the fungicide against *B. cinerea*. These non-traditional control methods can be regarded as providing effective control against *B. cinerea*, but their practical application and effect on human health need to be evaluated. If a combination of one or more of the tested materials and tolclofos-methyl were to reduce the amount of fungicide required to control *B. cinerea*, the adverse side effects of this fungicide on human health and the environment would also be reduced.

Keywords Nanosilver · Extract · Pathogen · Control

A. S. Derbalah (✉) · A. M. Hamza
Pesticides Department, Faculty of Agriculture,
Kafr-EL-Shiekh University,
Kafr EL-Sheikh 33516, Egypt
e-mail: aliderbalah@yahoo.com

G. A. E. Elkot
Agricultural Botany Department, Faculty of Agriculture,
Kafr El-Sheikh University,
Kafr EL-Sheikh 33516, Egypt

Introduction

Growth of fungal pathogens is the main cause of considerable economic loss during postharvest handling of fruits (Spadaro et al. 2004). *Botrytis cinerea* and *Penicillium expansum* can cause severe postharvest fruit diseases, including grey and blue mold even when the most advanced postharvest technologies are applied (Spadaro et al. 2004). *B. cinerea* is considered to cause one of the most important diseases of table grapes (Latorre et al. 1994). It is difficult to control fungal growth because fungi have developed resistance to many conventional fungicides, such as benzimidazoles and dicarboximides (Elad et al. 1998).

Moreover, as a result of a change in public attitudes towards the use of chemicals and the development of pathogen strains resistant to fungicides, new alternative control measures are required. Several alternative control agents, such as salts, plant extracts, culture filtrates of biocontrol agents (BCAs) and mineral oils, alone or in combination, have been tested against plant pathogens on different crops (Horst et al. 1992; Falk et al. 1995; Bélanger and Benyagoub 1997; Daayf et al. 1997). The practical use of culture filtrates of BCAs and plant extracts is gaining increasing acceptance as the cost of developing new active chemical ingredients is much higher than the cost of developing new adjuvants.

In recent years, nanoparticle (NP) materials have received increasing attention due to their unique physical

and chemical properties, which differ significantly from those of their conventional counterparts (Stoimenov et al. 2002). Recent studies have demonstrated that various NPs, including silver, have antimicrobial activities (Kim et al. 2008; Kumar et al. 2008). Silver is well known for its antimicrobial effects and has been used since ancient times. Developments in nanotechnology have enabled researchers to incorporate the use of silver ions and colloids into efficient antibacterial preventive measures (Kim et al. 2007; Tien et al. 2008). The large surface area to mass ratio that characterizes silver NPs is one of the properties that enables them to have such a strong antimicrobial effect (Morones et al. 2005).

The aim of this study was to evaluate the efficacy of newly used plant extracts, silver NPs and certain microbial culture filtrates, either alone or in combination with the fungicide tolclofos-methyl, against *B. cinerea* under laboratory conditions and to identify the biologically active compounds of the most effective plant extract and culture filtrate by gas chromatography-mass spectrometry (GC-MS) analysis.

Materials and methods

Preparation of plant crude extracts The leaves of seven medicinal plant species (*Cassia senna*, *Caesalpinia gilliesii*, *Thespesia populnea* var. *acutiloba*, *Chrysanthemum frutescens*, *Euonymus japonicus*, *Bauhinia purpurea* and *Cassia fistula*) were collected from a local nursery at Kafr El-Sheikh, Monofia, Gharbia and Alexandria Governorate, Egypt, respectively. The leaves were oven dried for 24 h at 70°C and then finely powdered using a blender. Each sample (25 g) was extracted twice with 300 ml of methanol at room temperature for 2 days. The extracts were filtered through filter paper (no.15; Whatman, Maidstone, Kent, UK), and the combined filtrate was concentrated to dryness by rotary evaporation at 40°C under vacuum.

Pathogen source and inoculation The *Botrytis cinerea* used in the study was originally isolated from grape plant. It was obtained as a culture slant from the Plant Pathology Research Institute, Giza, Egypt. Glass bottles (500 ml capacity) containing 100 g barley grain and 100 ml water were autoclaved for 30 min at 1.5 atm, inoculated with a plug cut of a 7-day-old fungal culture and incubated at 28± 1°C for 15 days.

The tested fungicide The fungicide tested in this study was tolclofos-methyl, which is sold under the trade name of Rizolex (Kafr-El-Zayat Co, Kafr-El-Zayat, Egypt).

Nanosilver The nanosilver (99.99%; diameter of particles 50 nm) used in this study was obtained from the Egypt Nanotech Co. Cairo, Egypt. Figure 1 shows the size and shape of the silver nanoparticles as observed by transmission electron microscopy.

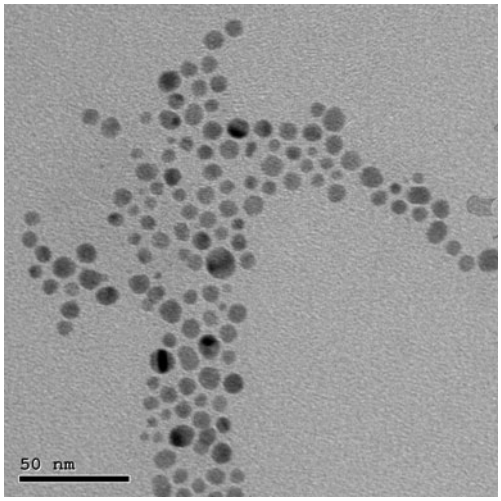

Fig. 1 Transmission electron microscopy image of silver nanoparticles

Preparation of bio-control agent's culture filtrates The microbial isolates used in this study were *Epicoccum nigrum*, *Bacillus subtilis* and *B. pumilus*. These biocontrol agents were previously isolated and identified by El-Kot and Hegazi (2008). The fungal isolate, *Epicoccum nigrum*, was cultured in potato dextrose broth (PDB) for 15 days at 20–25°C. The fungal biomass was then centrifuged at 10,000 rpm for 20 min and the culture medium put aside. The supernatant with culture broth was passed through a sterile membrane filter (pore size 0.2 μm) (El-Bogdady 1993). *B. subtilis* and *B. pumilus* isolates were cultured on Kings-B medium in 250-ml Erlenmeyer flasks on a rotary shaker at 150*g* and 28–30°C. After 24 h, the bacterial cell suspension was pelleted by centrifugation at 7,000 rpm for 10 min and the supernatant was filtered through a glass filter to obtain cell-free culture filtrate (El-Bogdady 1993).

Screening of the tested materials against B. cinerea in vitro test The seven plant extracts, the three microbial culture filtrates, and tolclofos-methyl were tested for their efficacy against *B. cinerea* under laboratory conditions using a completely randomized design. The efficacy of the tested materials was determined as the percentage of inhibition of the fungus radial growth relative to the control treatment. Four concentrations of each plant extract (50, 100, 200 and 300 ppm), culture filtrate (50, 100, 200 and 300 ppm), silver NPs (25, 50, 75 and 100 ppm) and fungicide (10, 20, 50 and 100 ppm) were used. The tested concentrations were obtained by adding the appropriate amount of stock solution to 60-ml portions of autoclaved PDA medium that had been cooled to about 45°C. Four petri dishes (diameter 9 cm) were used as a replicate for each concentration of each treatment, including the control. The control treat-

ments did not contain any of the microbial culture filtrates, nanosilver, plant extracts or tolclofos-methyl. The proportion of tested fungicide with the test substances depended on the concentrations fungicide and each tested material in the total used volume. Each dish was inoculated in the centre with an agar disk (diameter 5 mm) bearing mycelium growth from a 5-day-old *B. cinerea* culture. The inoculated dishes were sealed with parafilm to avoid the evaporation of volatile compounds and incubated at 28°C until the control plates were completely covered in mycelium. The inhibition percentage of radial growth of *B. cinerea* was calculated using the formula suggested by Vincent (1947). Each treatment (all concentrations for each treatment) was repeated three times. The inhibition percentage (I%) was calculated as:

$$I\% = A - B/A \times 100$$

where A=the radial growth of the tested fungus in the control treatment and B=the radial growth of the fungus in the specific test treatment

Chemical composition of the most effective plant extract and culture filtrate The GC/MS analysis was performed to identify the components of the most effective plant extract (*Euonymus japonicus*) and culture filtrate (*B. subtilis*) according to the methods described by Duarte-Almeida et al. (2004) and Mahboubi and Haghi (2008), respectively. Some of the detected compounds in the analyzed plant extract and microbial culture filtrate were identified first by comparison of their retention indices (RI) and mass spectra fragmentation with the available analytical standards (1,8-cineole, linalool, and tetradecanoic acid, octadecanoic acid and 9,12-octadecanoic acid). They were also identified by comparing their RI and mass spectra fragmentation with those stored in the Wiley registry and NIST reference library for GC-MS. Several compounds could only be identified by the second method. Analysis of the samples was performed by the Central Laboratory for Pesticides, Agriculture Research Centre, Cairo, Egypt.

Statistical analysis Data were subjected to the analysis of variance (ANOVA) test and Newman–Keuls' multiple range test using SAS ver. 6.12 (SAS Institute, Cary, NC).

Results

Efficacy of the tested materials alone and in combination with tolclofos-methyl against *B. cinerea* under laboratory conditions.

All of the test materials (plant extracts, microbial culture filtrates, silver NPs and fungicide) at their different concentrations significantly inhibited the radial growth of *B. cinerea* compared to the control. However tolclofos-methyl, the recommended fungicide against *B. cinerea*, was the most effective treatment relative to all tested treatments.

The extract of *Euonymus japonicus* was the most effective plant extract against *B. cinerea* followed by those of *Cassia senna*, *Chrysanthemum frutescens*, *Caesalpinia gilliesii*, *Bauhinia purpurea*, *Thespesia populnea* var. *acutiloba* and *Cassia fistula*, in descending order of effectiveness (Table 1). The combination of any one of the plant extracts with tolclofos-methyl improved the efficacy against *B. cinerea* compared to that of each plant extract and tolclofos-methyl alone (Table 2). The most effective combination against *B. cinerea* was *Caesalpinia gilliesii* plus tolclofos-methyl, while the lowest effective combination was *Bauhinia purpurea* plus tolclofos-methyl. The culture filtrate of *Bacillus subtilis* was the most effective microbial culture filtrate against *B. cinerea* followed by those of *Epicoccum nigrum* and *Bacillus pumilus*, in descending order of effectiveness (Table 3). The combination of any one culture filtrate with tolclofos-methyl improved the efficacy against *B. cinerea* compared to that of each culture filtrate and tolclofos-methyl alone (Table 3). The most effective mixture/combination against *B. cinerea* was *Epicoccum nigrum*+tolclofos-methyl, while the lowest effective one was *Botrytis cinerea*+tolclofos-methyl. The combination of silver NPs with tolclofos-methyl improved the efficacy against *B. cinerea* in comparison to that of either the silver NPs and fungicide alone (Table 4). The most effective mixture was silver NPs to tolclofos-methyl at a ratio of 100:10, respectively, while the lowest effective mixture was silver nanoparticles to tolclofos-methyl at 25:10, respectively.

Composition of the most effective plant extract and culture filtrate

The most effective plant extract (*Euonymus japonicus*) and microbial culture filtrate (*Bacillus subtilis*) against *B. cinerea* under laboratory conditions were analyzed by GC-MS to identify their active ingredients. Thirteen compounds were identified from the *Euonymus japonicus* plant extract, and eight compounds were identified from the *Bacillus subtilis* culture filtrate (Tables 5, 6). The identified compounds are eldyhydes, esters, alcohols and fatty acids.

Discussion

The efficacy of plant extracts and microbial cultural filtrates against *B. cinerea* under laboratory conditions has been reported previously (Dafereraa et al. 2003; Soylu et al. 2010; Elkot and Derbalah 2011). However, our study is the

Table 1 Efficacy of plant extracts against *B. cinerea* under laboratory conditions

Treatments	Concentrations (ppm)	Inhibition percentages[a]
Cassia senna	50	46.11 e
	100	56.56 g,f
	200	76.67 k,l
	300	85.44 q
Caesalpinia gilliesii	50	46.11 e
	100	66.33 h,i
	200	71.00 j
	300	81.56 n,p
*Thespesia populnea*var. *acutiloba*	50	38.33 d
	100	53.89 g,f
	200	68.56 i,h
	300	79.33 m,n
Chrysanthemum frutescens	50	54.11 g,f
	100	64.22 I
	200	76.67 k,l
	300	84.11 q
Euonymus japonicus	50	62.22 h
	100	68.78 i,h
	200	77.89 l,m
	300	90.78 r
Bauhinia purpurea	50	46.44 e
	100	54.33 g,f
	200	66.00 h,i
	300	80.89 n,p
Cassia fistula	50	16.00 b
	100	24.22 c
	200	51.56 f
	300	75.22 k
Control	0.00	0.00 a

Mean values followed by different lower-case letters within the column are sigficantly different at $p < 0.5$ according to the Student–Newman–Keuls' multiple range test

[a] Inhibition percentages, The reduction in radial growth diameter of the fungus relative to the control

first to evaluate and compare these seven plant extracts and three microbial culture filtrates, as well as the fungicide Tolclofos-methyl and silver NPs for their effectiveness against *B. cinerea*, both alone and in combination.

The antifungal activity of the *Euonymus japonicus* extract against *B. cinerea* may be due to the presence of high concentrations of different fatty acids and their derivatives (1,8-cineole, linalool, tetradecanoic acid, hexadecanoic acid methyl ester and 9,12 octadecadien-1-ol) (Wagh et al. 2007; Kelen and Tepe 2008; Chutia et al. 2009; Soković et al. 2009; Ahmadi et al. 2010).

The culture filtrate of the tested biocontrol agents showed a high efficacy against *B. cinerea* under laboratory conditions. The antifungal activity of microbial culture

Table 2 Efficacy of plant extracts combined with tolclofos-methyl against *B. cinerea* under laboratory conditions

Treatments	Inhibition percentages[a]
50 ppm *Cassia senna* +10 ppm tolclofos-methyl	78.95 b,c
50 ppm *Caesalpinia gilliesii* +10 ppm tolclofos-methyl	80.00 c,d
50 ppm *Thespesia populnea* var. *acutiloba* +10 ppm tolclofos-methyl	82.11 d,e
50 ppm *Chrysanthemum frutescens*+10 ppm tolclofos-methyl	77.89 b,c
50 ppm *Euonymus japonicus* +10 ppm tolclofos-methyl	96.84 f
50 ppm *Bauhinia purpurea* +10 ppm tolclofos-methyl	73.68 b
50 ppm *Cassia fistula* +10 ppm tolclofos-methyl	95.79 f
Control	0.00 a

Mean values followed by different lower-case letters within the column are sigficantly different at $p < 0.5$ according to the Student–Newman–Keuls' multiple range test

[a] See footnote of Table 1

filtrates against different plant pathogens has been reported previously (Fernando et al. 2005; Koitabashi 2005; Mercier and Manker 2005; Zou et al. 2007; Elkot and Derbalah 2011). Many researchers have also found that *Bacillus* sp. and their nonvolatile compounds can contribute considerably to the control of plant diseases (Hou and Forman 2000; Algam et al. 2004). The antifungal activity of the

Table 3 Efficacy of culture filtrate of certain biocontrol agents alone and mixed with tolclofos-methyl against *B. cinerea* under laboratory conditions

Treatments	Concentration (ppm)	Inhibition percentages[a]
Epicoccum nigrum	50	32.33 b
	100	49.89 c,d
	200	58.56 d,e
	300	70.20 f,g
Bacillus pumilus	50	42.22 c
	100	58.78 d,e
	200	67.89 f
	300	72.20 f,g
Bacillus subtilis	50	48.11 c,d
	100	56.56 d,e
	200	66.67 f
	300	75.6 h,i
Epicoccum nigrum+tolclofos-methyl	50+10	99.10 j
Bacillus pumilus+tolclofos-methyl	50+10	98.90 j
Bacillus subtilis+tolclofos-methyl	50+10	98.80 j
Control	0.00	0.00 a

Mean values followed by different lower-case letters within the column are sigficantly different at $p < 0.5$ according to the Student–Newman–Keuls' multiple range test

[a] See footnote of Table 1

Table 4 Efficacy of silver nanoparticles alone and combined with tolclofos-methyl against *B. cinerea* under laboratory conditions

Treatments	Concentration level (ppm)	Inhibition percentages[a]
Tolclofos-methyl	10	40.50 b,c
	20	73.25 e,f
	50	95.74 h,i
	100	100.00 j
Nanosilver	25	34.74 b
	50	63.16 d
	75	69.47 d,e
	100	77.89 f,g
Mixture	10+25	68.42 d,e
	10 + 50	89.15 h
	10 +75	90.42 h
	10 + 100	100.00 j
Control	0.00	0.00 a

Mean values followed by different lower-case letters within the column are sigficantly different at $p < 0.5$ according to the Student–Newman–Keuls' multiple range test

[a] See footnote of Table 1

Bacillus subtilis culture filtrate against *B. cinerea* may be due to the presence of different fatty acids and their derivatives (9, 12-octadecadienoic acid, 9-octadecanoic acid, 9,12-octadecanoic acid, 2-octenal and benothiazole) (Ultee et al. 2002**;** Goncalves et al. 2003; Dale et al. 2004; Fernando et al. 2005; Daniele et al. 2006; Neri et al. 2009).

Table 5 The main constituents of the *Euonymus japonicus* plant extract identified by gas chromatography-mass spectrometry analysis

No.	Identified compounds	Retention time (min)	Area[a] (%)
1	1,8-Cineole	7.94	10.30
2	1,6–Octadien-3-ol 3,7 dimethyl (Linalool)	8.75	7.20
3	3-Cyclohexene-1- methanol alpha alpha 4- methyl	9.78	3.22
4	1,6 Octadien-3-ol 3,7 dimethyl 2-aminobezoate	10.60	0.75
5	Cyclohexasiloxane dodecamethyl	10.78	1.88
6	2,6 Octadien-1-ol 3,7 dimethyl	11.55	1.50
7	Copaene	11.70	3.92
8	Caryphllene	11.94	5.20
9	1,6,10 Dodecantriene 7,11, dimethyl −3-methylene	12.30	5.72
10	Cycloheptasiloxane tetradecamethyl	12.45	13.0
11	Nerolidol	13.45	2.08
12	Tetradecanoic acid (Myristic acid)	14.61	8.67
13	Hexadecanoic acid methyl ester (methyl palmitate)	16.70	12.50

[a] Percentage of each component in the analyzed extract based on its peak area

Table 6 The main constituents of the *Bacillus subtilis* culture filtrate identified by gas chromatography-mass spectrometry analysis

No.	Identified compounds	Retention time (min)	Area[a] (%)
1	9, 12-Octadecadienoic acid	5.56	34.32
2	2-Amino 6-methyl benzoic acid	6.20	11.01
3	Hexadecanoic acid	9.70	23.99
4	Ethyl benzoic acid 2 pentyl ester	10.52	2.99
5	Carvacrol methyl ester	13.77	6.48
6	9, 12-Octadecanoic acid	17.70	4.70
7	2- Butyl benothiazole	19.72	7.49
8	2-Octenal	20.87	6.77

[a] Percentage of each component in the analyzed extract based on its peak area

Although the antimicrobial activity of the *Euonymus japonicus* plant extract and microbial culture filtrate of *Bacillus subtilis* is mainly attributed to its major identified compounds in the GC-MS analysis, the synergistic or antagonistic effect of the other compounds detected at only low levels also has to be considered (Ragas et al. 2005).

The results of our study confirm that silver NPs exhibit a high antifungal activity against *B. cinerea*. The antimicrobial activity of silver NPs against plant pathogens has been reported earlier (Kim et al. 2009; Kasprowicz et al. 2010), but our study is the first to evaluate the effectiveness of silver NPs as an antimicrobial agent against *B.cinerea*.

To date, several mechanisms have been postulated for the antimicrobial property of silver NPs. One proposal is that NPs may adhere to the cell surface, thereby altering membrane properties. In support of this mechanism, silver NPs have been reported to degrade lipopolysaccharide molecules, accumulate inside the membrane by forming "pits" and cause large increases in membrane permeability (Sondi and Salopek-Sondi 2004). Other proposed mechanisms include the penetration of silver NPs into the microbial cell, resulting in DNA damage, and the release of antimicrobial silver ions due to the dissolution of silver NPs (Morones et al. 2005).

The results of our study demonstrate the potential of using of microbial culture filtrates, silver NPs and plant extracts in combination with a fungicide to improve the efficiency of the antimicrobial activity (Fan and Tian 2001; Yoshida et al. 2001). However, our study has to be considered a first study, and one which used a selected plant pathogen and selected microbial culture filtrates and plant extracts. The results also reveal that the plant extracts and microbial culture filtrates behaved differently with the tested fungicide, indicating a major contribution of the different agents depending on their nature, with possible antagonism rather than synergism in some cases.

Tolclofos-methyl, a recommended fungicide against *B. cinerea*, was used to evaluate the efficacy of silver NPs,

botanical extracts and microbial culture filtrates against *B. cinerea* under laboratory conditions; all of these were found to be effective against *B. cinerea* under our laboratory conditions. Moreover, combining the fungicide with silver NPs and with any one of the plant extracts and microbial culture filtrates improved the efficacy against *B. cinerea*. Therefore, it can be concluded that silver NPs, botanical extracts and microbial culture filtrates alone or in combination with the fungicide can be used as effective agents against *B. cinerea*. However, exhaustive experimental trials under field conditions and with animals (safety evaluation) are needed before these non-traditional control methods can be used as potential antimicrobial agents.

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