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Fungicidal Activity of a Medium-chain Fatty Acids Mixture Comprising Caprylic, Pelargonic and Capric Acids

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Abstract: This study examines the fungicidal activity of a medium-chain fatty acids mixture comprising caprylic acid (C8:0), pelargonic acid (C9:0) and capric acid (C10:0), against *Rhizoctonia solani*, *Phytophthora infestans*, *Colletotrichum gloeosporioides*, *Botrytis cinerea*, *Fusarium oxysporum* and *Sphaerotheca cucurbitae*. The mixture of caprylic, pelargonic and capric acids (2/5/3, w/w/w) is prepared into a micro-emulsion concentrate and tested for its inhibitory effect on fungal growth using disc diffusion method except for *S. cucurbitae* using pot bioassay method. Results show that the fatty acids mixture is self-stabilized under either 4°C during a seven-day-storage or 54°C during fortnight. The doses of the mixed fatty acids completely inhibiting the mycelial growth are 100 ppm for *P. infestans* and 125 ppm *C. gloeosporioides* after three days and 200 ppm for *B. cinerea* after 4 days. A dose of 100 ppm reduces the mycelial growth in *R. solani* by 93.7% after 4 days and that in *F. oxysporum* by 92.9% after 3 days. For *S. cucurbitae*, a dose of 250 ppm results in a control effect of 81.0% in the pot bioassay. Our study provides so far the first report of the fungicidal activity of medium-chain saturated fatty acids mixture at relative low dosage rates.

Key words: Medium-chain fatty acids, micro-emulsion concentrates, fungicidal activity, inhibition rate, plant pathogenic fungi

INTRODUCTION

Fungi are commonly present in soil, air and on plant surfaces throughout the world. Plant pathogenic fungi may cause severe yield losses in crops by inducing various diseases. In soil-borne fungi, *Rhizoctonia solani* (teleomorph *Thanatephorus cucumeris*) causes diseases in many crop species (Lehtonen *et al.*, 2008). Late blight caused by the *Phytophthora infestans* (Mont.) de Bary is a highly destructive epidemic worldwide destroying the entire production of tomato (Mizubuti and Fry, 2006). *Colletotrichum gloeosporioides* (Penz.) Sacc. is the causative agent for anthracnose disease in tropical fruit trees such as citrus and mango (Peraza-Sanchez *et al.*, 2005). *Fusarium oxysporum* generates an illness called "Fusarium wilt", which is lethal to nearly all plants of agricultural importance, proves incredibly tough to eradicate since fungicides have only limited effects (Gordon and Martyn, 1997). The air-borne plant pathogen *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*) has been the reason for severe yield losses in more than 200 crop species (Elad *et al.*, 2004; Williamson *et al.*, 2007). The powdery mildew disease in melon

(*Cucumis melo* L.) is often caused by an air-borne fungus *Sphaerotheca cucurbitae* (McCreight, 2006). Although numerous fungicides have been developed, many of them failed due to fungicide resistance. Delaying fungicides resistance in pathogen populations is therefore a major goal in sustainable plant pathogen control (Brent and Hollomon, 2007), which requires integrated agents with superior fungicidal effectiveness as well as non-toxic and highly safe natures.

Fatty Acids (FAs) refer to a class of natural compounds which are of special interests in their fungicidal values against plant pathogenic fungi (Momin and Nair, 2002; Pohl *et al.*, 2011) for the nature of friendliness to environment (FRAC, 2012). Numerous FAs have been demonstrated capable of effectively controlling pathogenic fungi such as *R. solani* (Walters *et al.*, 2003), *P. infestans* (Avis and Belanger, 2001), *C. gloeosporioides* (Yenjit *et al.*, 2010), *B. cinerea* (Leyva *et al.*, 2008) and *F. oxysporum* (Liu *et al.*, 2008; Altieri *et al.*, 2009) that commonly occur worldwide. However, to the best of our knowledge, data are still largely missing with FAs for controlling *S. cucurbitae* which causes severe

powdery mildew disease in China. To investigate optimal FAs and preparations is therefore of crucial importance for fungal control.

Though, in general, the fungicidal efficiency of FAs progresses with an increase in chain length (Sado-Kamdem *et al.*, 2009), overlong chain length reduces their solubility in aqueous systems (Pohl *et al.*, 2011) while overabundant double bonds reduce their thermochemical stability (Wang, 1983). For instance, oleic acid (C18:1) and erucic acid (C22:1) had no inhibitory effect at any concentration examined on the mycelial growth of *R. solani* (Walters *et al.*, 2004) while the oleic acid up to 3200 $\mu\text{mol L}^{-1}$ had no apparent inhibitory effect on that of *F. oxysporum* (Liu *et al.*, 2008). Situation becomes even worse when considering the crystallization-induced phase separation during storage which greatly reduces the fungicidal activity of the final commodity under temperature lower than the melting points (Wang, 1983). The synergistic mixture of saturated medium-chain FAs in conjunction with hydroxyl carboxylic acid, phosphoric or phosphorus acid and anionic surfactants to form Micro-emulsion Concentrate (MEC) has been proven a feasible approach to solve these problems (Gibaud and Attivi, 2012). Our preliminary work showed that the mixture of caprylic, pelargonic and capric acids constituted effective fungicidal formulations and partly prevented the thermodynamical instability at low temperature or for long-period storage. It is also hypothesized that the multiple agents may provide a broad spectrum alternative comparative to single FA formulation. The aims of the present study are therefore (1) To optimize the formula of caprylic-pelargonic-capric acids mixture for low temperature and long-period storage, (2) To determine *in vitro* the fungicidal activity of various dilutions subject to the five pathogenic fungi strains and (3) To verify for the first time the effects of the FAs mixture on powdery mildew disease caused by the fungus *S. cucurbitae*.

MATERIALS AND METHODS

The caprylic acid, pelargonic acid and capric acid (purification: >98%), hydroxyl carboxylic acid, phosphoric and anionic surfactants were purchased from Cognis Chemicals, P. R. China. Individual of caprylic, pelargonic and capric acids alone and their combinations of a 2/3 caprylic/capric acids as well as a 6/5/9 and a 2/5/3 caprylic/pelargonic/capric acids mixtures on weight basis were homogenized and stored at 4°C for 7 days to observe crystallization by visual inspection. The optimized mixture was then prepared with n-octanesulfonate (NAS) surfactant solubilizer at pH 2-4 according to Wang (1983),

to gain a Micro-emulsion Concentrate (MEC) containing 10% FAs on weight basis. GC-MS analysis was performed as described by Schultz and Pugh (2001) to test the compositional change of this MEC that stored at 54°C for 14 days which is equivalent to 2 year storage at ambient temperature according to the “Guidelines on drafting specifications of pesticides, Chemical Industry Standard of P.R. China (HG/T 2467.1~2467.20-2003)”.

The fungi *B. cinerea*, *R. solani*, *C. gloeosporioides*, *P. infestans* and *F. oxysporum* were grown initially on potato dextrose agar (PDA, pH 6.50) in dark and the culture stocks were stored at 4°C. Solid sterile potato dextrose agar media (20 mL) was amended with MEC to obtain FAs concentrations ranging 25400 ppm and added aseptically to 90 mm plastic Petri dishes. Mycelial plugs of the five fungi were removed using a sterile 5 mm diameter cork borer from the peripheral growth zone of stock cultures, inverted and placed on the centre of each Petri dish and then incubated in darkness at 24°C. Mycelia growth was determined by measuring the diameter along two perpendicular lines passing through the centre of the dish after different periods of incubation within 2-7 days depending on preliminary results. The Inhibitory Rate (IR) was calculated according to Wang *et al.* (2005).

Sphaerotheca cucurbitae is an obligate parasite and was tested with cucumber seedlings in greenhouse. Cucumber seedlings with 2-3 leaves were transplanted individually in 250 cm³ plastic pots filled with 500 g artificial soil (dune sand/natural soil/organic fertilizer, 2/1/1, v/v). After 7 days of *S. cucurbitae* infection, cucumber seedlings were sprayed thoroughly with MEC dilutions at FAs concentrations of 400, 200, 100, 50 and 25 or 0 ppm as control at 27°C with six replications for each treatment. The percentage of leaf area covered with fungus before or after treatment was recorded after 7 days of inoculation. The Disease Index (DI) was calculated as described by Wheeler (1969).

All experiments were performed twice and exhibited similar trend. Data were analyzed by ANOVA-II (treatment and duration of stress as levels of classification) with SPSS (IBM® SPSS® software, version 16.0.0). Normality and variance homogeneity was achieved by arc-sin transformation for the percentage values. If the F-value indicated significant differences ($p < 0.05$), mean differences were compared according to the Student-Newman-Keuls test with a confidence limit of 95%.

RESULTS

As shown in Table 1, the 2/5/3 (w/w/w) mixed caprylic-pelargonic-capric acids formulation possesses

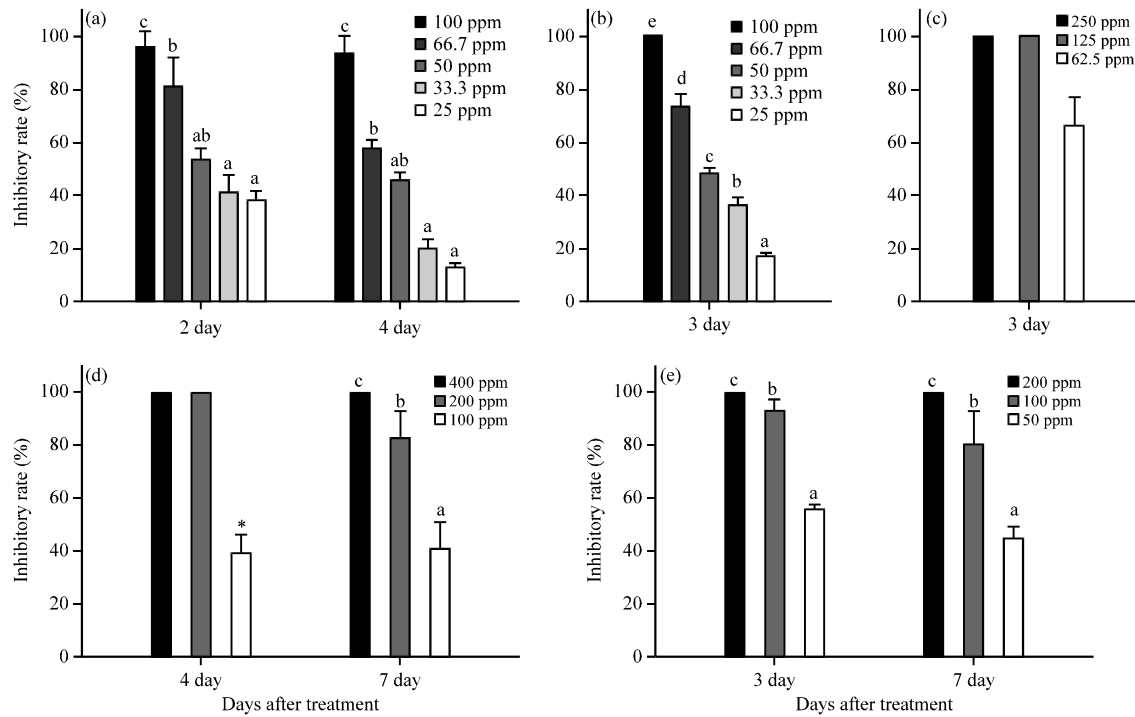


Fig. 1(a-e): The inhibitory rates of the micro-emulsion concentrate (MEC) against (a) *Rhizoctonia solani*, (b) *Phytophthora infestans*, (c) *Colletotrichum gloeosporioides*, (d) *Botrytis cinerea* and (e) *Fusarium oxysporum*. MEC-8910 is the micro-emulsion concentrate formulation of a 10% caprylic acid (C8:0), pelargonic acid (C9:0) and capric acid (C10:0) mixture (2/5/3, w/w/w) stabilized with surfactant. Concentrations refer to the final contents of FAs mixture after dilution rather to MEC. The set of MEC dilutions selected for different fungi strains is due to their variations in sensitivity based on preliminary experimental results. Values represent Mean±SE (n = 6) and different letters for a given duration of treatment or asterisks denote significant difference according to Student-Newman-Keuls test at the 5% level

Table 1: The cold solubility of individual or combinations of caprylic (C8:0), pelargonic (C9:0) and capric (C10:0) acids (in w/w) under the storage temperature of 4°C during 7 days

Parameters	C8	C9	C10	C8-C10(2/3, w/w)	C8-C9-C10	
					6/5/9	2/5/3
Titer point (°C)	16-17	11	31-32	1-6	-	-
1 day	Crystallized	Crystallized	Crystallized	Liquid	Liquid	Liquid
3 day	-	-	-	Crystallized	Liquid	Liquid
7 day	-	-	-	-	Crystallized	Liquid

the highest cold tolerance while the 6/5/9 mixture had crystal formed before the end of the period tested. Crystallization is also observed within 3 days for a 2/3 caprylic-capric acids mixture while the individual FAs alone form crystal within less than 1 day of cold treatment. For the long-term stability of this mixture, the compositional changes of the MEC are quantified with GC-MS method. Results show that the loss of the three FAs remains low after equivalent 2 year storage at ambient temperature (Table 2). For further analysis, 1000 or 2000 ppm of FAs mixture derived from the MEC with 4°C water of 500 ppm hardness also form

clear and stabilized solutions without the presence of crystallization after 24 h standing at 4°C.

The optimized FAs mixture manifests superior inhibitory effect to the mycelial growth of all five fungi (Fig. 1). At 100 ppm, the mycelial growth of *R. solani* is reduced by 95.85 and 93.83% after 2 and 4 days, while at 25 ppm, the relevant inhibition rates are 38.11 and 13.99%, respectively. After 3 days, at 100, 66.7, 50, 33.3 and 25 ppm, the mycelial growth of *P. infestans* is reduced by 100.00, 73.66, 48.86, 36.26 and 16.91% (Fig. 1). For *C. gloeosporioides*, a dose of 62.5 ppm mycelial growth by 62.98% after 3 days and higher doses inhibit it

Table 2: The compositional percentage of caprylic (C8:0), pelargonic (C9:0) and capric (C10:0) acids (in %) in the micro-emulsion concentrate (MEC) after 14 days storage at 54°C tested by GC-MS

Fatty acid	0 (day)	14 (day)	Loss (%)
Caprylic acid	1.98	1.90	4.04
Pelargonic acid	5.00	4.74	5.20
Capric acid	3.01	2.88	4.32
Σ	9.99	9.58	4.10

Table 3: The control effect of the gradient of fatty acids (FAs) mixture concentrations (ppm) derived from the dilution of a 2/5/3 (w/w/w) caprylic/pelargonic/capric acids mixture on *Sphaerotheca cucurbitae*-induced powdery mildew on cucumber leaves (in %) after 7 days of inoculation

Concentration (ppm)	Control effect
400	81.0
200	76.3
100	30.5
50	25.6
25	13.6

completely. In the cases of *B. cinerea*, the dose of 400 ppm also completely inhibits the mycelial growth after 4 or 7 days. At the lower dose of 200 ppm, the mycelial growth is reduced by 100 and 81.6% after 4 and 7 days, while at 100 ppm the inhibitory rates are 32.5 and 30.4%, respectively (Fig. 1). At 200 ppm, the mycelial growth of *F. oxysporum* is reduced by 100.00% after 3 or 7 days. At the dose of 100 ppm, the inhibition rates are 92.91 and 80.39% after 3 and 7 days, while at 50 ppm the rates are 55.67 and 44.58%, respectively (Fig. 1). For controlling the *S. cucurbitae*-induced powdery mildew, our assay in cucumber seedlings show that the 400, 200, 100, 50 and 25 ppm FAs mixture results in control effects of 81.0, 76.3, 30.5, 25.6 and 13.6% after 7 days of inoculation (Table 3).

DISCUSSION

Phase separation due to crystallization under temperatures below titer points induces significant decrease in the fungicidal activities of FAs-based fungicides (Wang, 1983). Titer points of FAs are a function of the degree of saturation and the chain length. Unsaturated FAs with shorter chain have lower titer points while the more saturated FAs with longer chain have higher titer points (Dorset *et al.*, 1991). The caprylic, pelargonic and capric acids have individual titer points of 15.41, 11.28 and 30.80°C (Knothe and Dunn, 2009), however, the mixture of FAs obtained a eutectic point lower than 4°C (Table 1). The particular nature of cold tolerance in this mixture gives the advantage to minimize the fungicidal efficacy loss under low temperature therefore avoiding the influence of seasonal parameters. Fungicides based on unsaturated FAs are also confronted with the problem of degradation resulting from the high activity of double bonds. Saturated FAs are more stable owing to the absence of double bonds and therefore

display much slower peroxidation and rancidity. GC-MS tests showed that the degradation-induced loss of the three FAs in MEC remains approximately 5% for 2 year storage under ambient temperature. We thus conclude that the 2/5/3 (w/w/w) mixture is the optimal formulation and the MEC is suitable for long-term storage and transportation in commercial practices.

The fungicidal FAs have been found to disrupt functions of the fungal cytoplasmic membrane by inducing the release of intracellular electrolytes and proteins due to increased membrane fluidity (Carballeira, 2008). According to Benyagoub *et al.* (1996), in artificial membranes constructed from sensitive and tolerant fungi strains, the elevation in fluidity of sensitive fungal membrane was indeed dose-dependently related with the content of phospholipid FAs while such a relation was not reliable in the tolerant strain. The membrane disorder induced by the elevated fluidity could thus modify membrane dynamics by affecting the activity of membrane-bound enzymes (Avis and Belanger, 2001). This interaction between fungicidal FAs and cellular enzymes in sensitive fungi has been proven to be indirect and nonspecific (Avis and Belanger, 2001). In our case, the ubiquitous fungicidal activities are apparently reliable against all the six fungi strains based on *in vitro* tests or pot bioassay suggesting a nonspecific Mode of Action (MOA) which is considered a prerequisite for broad-spectrum fungicides (Lyr, 1995). However, the variation in functional doses indicates that the inhibitory effects are not only dose-dependent but also fungus specific. It is therefore hypothesized that the MOA of MEC may relate to the physically penetration of lipophilic FAs across pathogen cell membranes and/or the hydrophobic interaction between the dissociated FAs (anionic form) and proteins, both of which could result in cell death in pathogen fungi as reported previously (Evtodienko *et al.*, 1996; Pohl *et al.*, 2008). The detailed mechanisms in the control of each fungus strain will need further investigation over the membrane integrity related parameters.

In conclusion, this study shows that the 2/5/3 (w/w/w) mixed caprylic-pelargonic-capric acids formulation has strong fungicidal activities against *B. cinerea*, *R. solani*, *C. gloeosporioides*, *P. infestans* and *F. oxysporum*. The MEC can be readily used in controlling phytopathological fungi and in fungicide resistance managements. Since the FAs ingredients are derived from coconut oils and have no Maximum Residue Limit (MRL) on food, vegetable and fruit surfaces (European Community, 2008), our formulation is suggested as a highly safe, bio-rational, environmentally-friendly and broad spectrum fungicidal agent. Moreover, this is the

first report so far on the control of *S. cucurbitae* that causes powdery mildew disease using Fas. More extensive study in the physiochemical characteristics and *in vivo* efficacy of this mixture remains to be performed.

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