Journal of Agricultural Science; Vol. 7, No. 9; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Antimicrobial and Antifungal Activity of Volatile Extracts of 10 Herb Species against *Glomerella cingulata*

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Received: May 30, 2015 Accepted: June 24, 2015 Online Published: August 15, 2015

doi:10.5539/jas.v7n9p77 URL: http://dx.doi.org/10.5539/jas.v7n9p77

Abstract

We investigated the antimicrobial activity of volatile extracts from 10 herb species (coriander, oregano, chamomile, sage, thyme, chives, basil, fennel, lavender, and rosemary) against 4 plant pathogens (*Botryotinia fuckeliana*, *Glomerella cingulata*, *Fusarium oxysporum*, and *Pectobacterium carotovorum* subsp. *carotovorum*) to develop biological pesticides. We also determined the optimum quantity of each volatile extract for the highest activity against plant pathogens. Among these 10 herb extracts, the volatile extract of coriander leaves and stems exhibited high levels of antimicrobial activity against the 4 plant pathogens tested. The volatile extract prepared from chives also exhibited antifungal activity against *B. fuckeliana*, *G. cingulata*, and *F. oxysporum*, but exhibited no antimicrobial activity against *P. carotovorum* subsp. *carotovorum*. In particular, the antifungal activity of the volatile extract of coriander leaves and stems (equivalent to 0.25 g FW) against *G. cingulata* was higher than that of chemical fungicides. These results indicate that volatile extracts from coriander leaves and stems have high levels of antifungal and antimicrobial activity against plant pathogens.

Keywords: coriander, volatile, plant pathogen, antimicrobial, *Glomerella cingulata*

1. Introduction

In recent years, consumers have shown a growing interest in food safety. To date, agricultural crops have been supplied stably and effectively in part due to the use of chemical pesticides, which carry the risks of mammalian toxicity and environmental pollution. Such chemical products are a health hazard to farmers and consumers, and leave residues on or in the crops. Low-toxicity pesticides are desirable because the alternatives, such as organic farming or pesticide-free production, may be time-consuming or ineffective for reducing damage by plant pathogens and pests. It is worth noting that there is also great interest in developing biological, rather than synthetic chemical, agents for pest control (Kobayashi et al., 2012). Another serious problem regarding the use of certain chemical agents for crop protection is the development of resistance by fungal pathogens. Furthermore, the application of ever-greater concentrations of chemicals in an attempt to overcome this problem increases the risk of high levels of toxic residues in agricultural products (Daferera et al., 2003).

A variety of microorganisms, including pathogenic molds such as *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp., cause foodborne diseases or food spoilage (Betts et al., 1999). Therefore, health and economic considerations strongly motivate the search for antifungal and antimicrobial agents. Recently, there has been considerable interest in the use of plant volatile extracts and essential oils with antimicrobial properties to control such pathogens and toxin-producing microorganisms in foods (Daferera et al., 2003; Gilles et al., 2010; Okoh et al., 2010). The antimicrobial properties of these extracts are the result of multiple active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarins, and curcumines found in aromatic plants (Tepe et al., 2005). The extracts from such plants may provide an alternative method to protect foods, feed, and pharmaceuticals from fungal contamination.

Examples of natural antimicrobial phytochemicals in plant extracts include allyl isothiocyanate from mustard, which inhibits the growth of *P. expansum* (Mari et al., 2002), and phenolic compounds in thyme oil, which exhibit antimicrobial effects on foodborne bacteria (Cosentino et al., 1999). The Food and Drug Administration in the United States has defined these extracts a status of "Generally Recognized as Safe (GRAS)". Consequently, there are now many reports of the antimicrobial activity of herbal essential oils and extracts against foodborne pathogens (Kalemba & Kunicka, 2003). However, there have not yet been reports of herbs planted as companion plants for antimicrobial activity against plant pathogens.

Here, we investigated the antimicrobial activity of volatile extracts from 10 kinds of herb (coriander, oregano, chamomile, sage, thyme, chive, basil, fennel, lavender, and rosemary) of against 4 species of plant pathogens (*Botryotinia fuckeliana*, *Glomerella cingulata*, *Fusarium oxysporum*, and *Pectobacterium carotovorum* subsp. *carotovorum*). The objective of this research was to develop biological microbicides as alternatives to synthetic chemical microbicides for sustainable agriculture applications. To this end, the optimum amount of the herb volatile extract with the highest antimicrobial activity was determined.

2. Method

2.1 Materials

Coriander (*Coriandrum sativum* L.), oregano (*Origanum vulgare* L.), chamomile (*Matricaria recutit*a L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.), chive (*Allium schoenoprasum* L.), basil (*Ocimum basilicum* L.), fennel (*Foeniculum vulgare* L.), lavender (*Lavandula vera* L.), and rosemary (*Rosmarinus officinalis* L.) were cultivated in a greenhouse at Meiji University from April to September 2009. Seeds of each herb species (obtained from Takii & Co., Ltd., Kyoto, Japan) were planted in plastic pots containing propagation medium (Co-op Chemical Co., Ltd., Tokyo, Japan) and vermiculite (Showa vermiculite Co. Ltd., Kanagawa, Japan) (1:1). A hydroponic fertilizer (HYPONeX, HYPONeX JAPAN Corp. Ltd., Osaka, Japan) diluted 1000-fold in tap water (as a standard concentration) was added to the growth medium once per week. Aerial parts of plants were sampled at floral anthesis, frozen in liquid nitrogen, and stored at -40 °C until extraction of volatile compounds.

Fungal strains *B. fuckeliana* NBRC 9760, *G. cingulata* NBRC 5257, *F. oxysporum* NBRC 6385, and bacterial strain *P. carotovorum* NBRC 12380 were purchased from the National Institute of Technology and Evaluation (Kisarazu, Japan). Benomyl (Benlate wettable powder, 50% benomyl w/w, Sumitomo Chemical Co. Ltd., Osaka, Japan), which is effective against *B. fuckeliana*, *G. cingulata*, and *F. oxysporum*, and polycarbamate (bis-dithane wettable powder, 75% polycarbamate w/w, Sumitomo Chemical Co. Ltd., Osaka, Japan), which is effective against *P. carotovorum*, were used as positive fungicidal controls. Benomyl and polycarbamate treatments were prepared according to concentrations commonly used in agricultural applications.

2.2 Diethyl Ether Extraction Method

Volatile compounds were extracted using the modified method of Ikeura et al. (2012). Aerial parts (100 g FW) of each herb were added to liquid nitrogen and homogenized in a blender (Nissei Corp., Aichi, Japan), at 10,000 rpm for 2 min at 4 °C, then 400 ml of diethyl ether (special grade, Kanto Kagaku, Tokyo, Japan) was added. The mixture was stirred for 4 h at 4 °C, then filtered to remove the solid residue. The crude extract was dried overnight using anhydrous sodium sulfate (special grade, Kanto Kagaku, Tokyo, Japan), then concentrated to 20 ml under nitrogen gas flow.

2.3 Measurement of Antimicrobial Activity against Plant Pathogens by Herb Volatile Extracts

Suspensions (100 µL of 10^6 spore mL^{-1}) of *B. fuckeliana*, *G. cingulata*, and *F. oxysporum* were plated onto potato dextrose agar plates (Difco, BD, NJ). An aliquot of *P. carotovorum* suspension (100 µL of 10^4 CFU mL^{-1}) was plated onto standard plate count agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Paper disks containing ~1000 µL each of a single herb extract were placed onto the inside of the lid of each inoculated plate, which was then turned upside-down to dry the solvent from the extracts. Plates were incubated inverted at 25 °C for *B. fuckeliana*, *G. cingulata*, and *F. oxysporum*, or at 30 °C for *P. carotovorum*, for 7 d for inocula to grow and to allow extracts to evaporate onto the cultures, after which plates were sealed with Parafilm. Controls consisted of paper disks treated with the extract solvent, diethyl ether, alone. The antimicrobial activity of each extract is reported as the inhibition ratio calculated according to the following equation:

$$\text{Inhibition ratio (\%)} = [\text{diameter of inhibition circle (mm)}/\text{diameter of plate (85 mm)}] \times 100 \quad (1)$$

All experiments were performed in triplicate.

2.4 Statistical Analysis

Data are presented as mean with standard error values. Mean separation of antimicrobial activity was determined by Tukey-Kramer tests at $P < 0.05$.

3. Results and Discussion

3.1 Antimicrobial Activity of Volatile Extracts from 10 Herb Species against 4 Plant Pathogen Species

The antimicrobial activity of volatile extracts from 10 herb species tested on *B. fuckeliana, G. cingulata, F. oxysporum*, and *P. carotovorum* is shown in Table 1. Percent inhibition of the growth of *B. fuckeliana*, *G. cingulata, F. oxysporum*, and *P. carotovorum* by coriander volatile extract was 100%, 92.8%, 29.4%, and 40.9%, respectively. Coriander volatile extract had a particularly strong antifungal effect on *B. fuckeliana* and *G. cingulata.* Percent inhibition of the growth of *B. fuckeliana*, *G. cingulata*, and *F. oxysporum* by chive volatile extract was 16.1%, 34.4%, and 5.4%, respectively, which was lower than that by coriander volatile extract. Chive volatile extract had no antimicrobial effect on *P. carotovorum* at all. The volatile extracts of other herbs (oregano, chamomile, sage, thyme, basil, fennel, lavender, and rosemary) had no antimicrobial effects against the 4 plant pathogen species tested in these experiments. However, it is possible that higher doses of these extracts, or different extraction methods could increase the antimicrobial effects of these extracts.

There have been many reports of antifungal properties of plant volatile extracts and essential oils. Volatiles from thyme have been shown to have strong antifungal activity against *Aspergillus parasiticus* and *Cryptococcus neoformans*, although not against *Candida albicans* (Dorman & Deans, 2000; Gilles et al., 2010; Okoh et al., 2010).

The results of this study indicate that coriander and chive volatile extracts have antifungal activity against plant pathogens and that coriander volatile extract in particular has high levels of antifungal activity against *B. fuckeliana* and *G. cingulata.*

Table 1. Antifungal activity of volatile extracts from 10 herb species against *B. fuckeliana*, *G. cingulata*, *F. oxysporum*, and *P. carotovorum*

	Inhibition ratio (% ± SE)^z			
	B. fuckeliana	*G. cingulata*	*F. oxysporum*	*P. carotovorum*
Coriander	100.0 ± 0.00	92.81 ± 1.84	29.41 ± 1.70	40.94 ± 0.72
Chive	16.07 ± 0.49	34.37 ± 1.09	5.36 ± 1.12	-^{y}
Camomil	-	-	-	-
Oregano	-	-	-	-
Sage	-	-	-	-
Basil	-	-	-	-
Fennel	-	-	-	-
Rosemary	-	-	-	-
Lavender	-	-	-	-
Control(diethyl ether)	-	-	-	-

Note. ^{z} Inhibition ratio (%) = [diameter of inhibition circle (mm)/diameter of plate (85 mm)]×100; ^{y} Not inhibition.

3.2 Optimum Amounts of Coriander and Chive Volatile Extracts to Inhibit Growth of Plant Pathogens

The antimicrobial activity of coriander and chive volatile extracts, and benomyl or polycarbamate, against *G. cingulata*, *F. oxysporum*, *B. fuckeliana*, *and P. carotovorum* is illustrated in Figures 1-4. Antimicrobial activity against all plant pathogens tested increased with increased volumes of coriander and chive extracts. In particular, the growth of *B. fuckeliana* and *G. cingulata* was completely inhibited by equivalent to about 0.5 gFW and 0.25 gFW of coriander aerial parts, or by equivalent to about 1.5 gFW and 3.5 gFW of chive aerial parts.

Concentrations of benomyl and polycarbamate generally used in the field were 0.05 mg 100 μL^{-1} and 0.075 mg 100 μL^{-1}, respectively. At these concentrations, benomyl and polycarbamate had no antimicrobial activity against *B. fuckeliana*, *G. cingulata*, *F. oxysporum*, or *P. carotovorum* (data not shown).

In other studies, the antifungal activity of essential oils obtained from aerial parts of oregano, lavender, and rosemary, was tested against the fungal pathogen *Botrytis cinerea*, the causal agent of grey mold disease of tomato. A volatile vapor of oregano oil at 0.2 µg mL^{-1} was found to completely inhibit the growth of *B. cinerea*. However, complete inhibition of pathogen growth by essential oils of lavender and rosemary was observed at a concentration of 1.6 μg mL^{-1} (Soylu et al., 2010).

The antifungal activity of essential oils obtained from fennel, anise, peppermint, and cinnamon at concentrations 0-800 μg L^{-1} was investigated against *B. cinerea*, which causes strawberry fruit rotting and other negative quality effects. Growth of *B. cinerea in vitro* was completely inhibited by fennel, cinnamon, and anise essential oils at relatively low concentrations (400-800 μg L^{-1}) (Mohammadi et al., 2012).

Methanol, ethyl acetate, and chloroform extracts of *Terminalia chebula* seeds at concentrations of 1500 ppm $disc^{-1}$ revealed remarkable antifungal effects against *F. oxysporum*, *F. solani*, *Phytophthora capsici*, and *B. cinerea*, inhibiting growth in the range of 41.6-61.3%, with minimum inhibitory concentration (MIC) values ranging from 62.5 to 500 μg mL^{-1}. These extracts also had a strong detrimental effect on spore germination in all the plant pathogens tested, and inhibited *B. cinerea* spore germination in a concentration- and time-dependent manner (Bajpai et al., 2010). Thus, although many studies report antimicrobial activity in plant essential oil and volatile extracts, both the effective treatment concentrations and antimicrobial effects of these extracts differ depending on pathogens and plant host species.

Volatile compounds with a distinctive odor, similar to that of garlic, are released when chives are crushed or cut for culinary or medicinal purposes. Many organic sulfur compounds found in *Allium* species are known to have antimicrobial properties against various bacteria such as *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *Vibrio cholera*, and also against fungi (Rattanachaikunsopon & Phumkhachorn, 2008). Diallyl sulfides (diallyl monosulfide, diallyl disulfide, diallyl trisulfide, and diallyl tetrasulfide) in chive oil are believed to be responsible for its antimicrobial activity (Rattanachaikunsopon & Phumkhachorn, 2008). Thus, the chive volatile extract used in this study likely contained many sulfur compounds with antifungal activity against plant pathogens.

Furthermore, although there are many reports about coriander volatile analysis (Kohara et al., 2006; Ravi et al., 2007), antioxidant (Misharina & Samusenko, 2008) and antimicrobial activity against food-borne pathogens (Zivanovic et al., 2005), there have been no studies on the antimicrobial activity of coriander essential oil against plant pathogens. In this study, we show for the first time that the volatile extract of coriander leaves and stems has high levels of antibacterial activity against plant pathogens.

In addition to its use as seasoning, coriander has traditionally been used for its analgesic, aphrodisiac, antirheumatic, anti-inflammatory, diuretic, antispasmodic, circulatory stimulant, and antidiabetic effects (Gray & Flatt, 1999), and more recently for its possible cholesterol-lowering (hypolipidemic) effects (Momin et al., 2012). The essential oil extracted from coriander seeds has been reported to have antimicrobial activity against both gram-negative and gram-positive foodborne pathogenic bacteria, including *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *B. cereus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* (Alves-Silva et al., 2013), and *Candida* spp. (Begnami et al., 2010). Notably, coriander oil possessed the most powerful inhibitory bactericidal effect on *Campylobacter jejuni* (Rattanachaikunsopon & Phumkhachorn, 2010). Thus, the antimicrobial properties of coriander against foodborne pathogens, but not against plant pathogens have previously been described.

Components of plant extracts have several antimicrobial characteristics, depending on volatility and mechanistic properties. First, volatile extracts tend to show broader antibacterial, cytotoxic, and antiviral properties than do non-volatile ones (Sivropoulou et al., 1997). Secondly, the antifungal activity of many non-volatile plant extract components is very low (Farag et al., 1989), while the antifungal of volatile components tends to be high (Inouye et al., 2000). Thirdly, volatile components are effective against antibiotic-resistant bacteria because their antibacterial mechanism differs from those of antibiotic agents (Fanaki & EI-Nakeed, 1997). Because most antibacterial agents operate by interacting with the receptor proteins of target microbes, drug-resistant strains of bacteria emerge due to amino acid mutations in these receptors (Inouye et al., 2001). In contrast, volatile compounds act on microbial cell membranes, disturbing their fluidity or integrity (Pasqua et al., 2007). This kind of mechanism prevents emergence of drug-resistant strains of bacteria that arise due to amino acid mutations in membrane-localized receptor proteins. These antimicrobial essential oils exhibit two apparent modes of antifungal action: either weak and reversible activity, or potent and irreversible activity.

The reversible action of citron, lavender, and tea tree extracts might be associated with selective insertion of the non-polar components into the lipid-rich portion of the cell membrane, thereby disturbing membrane function, as do anesthetic agents (Teuscher et al., 1990). However, oils that have strong, irreversible effects often contain aldehydes as main constituents, and might irreversibly disrupt the cell membrane by crosslinking membrane proteins. For example, acetaldehyde causes electrolyte leakage and sugar and amino acid loss in *Rhizopus* *stolonifer* due to its crosslinking effects (Avissar et al., 1990). Thyme oil also contains a phenol component that is capable of denaturing membrane proteins. However, the relatively moderate action of cinnamon bark oil, which contains cinnamaldehyde, may be due at least partly to low absorption by fungal cells and agar upon short-term exposure.

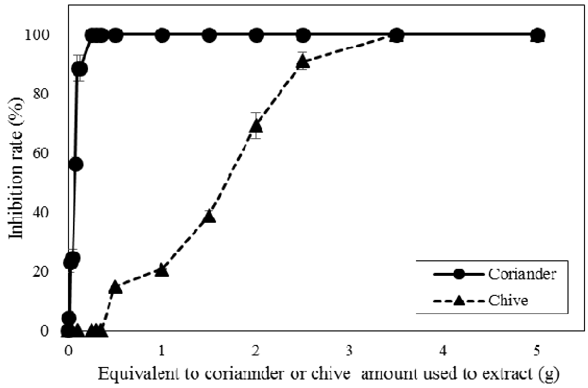

Figure 1. Figure 1 Antifungal activity of volatile extracts from coriander, chives on *G. cingulata*

Note. Vertical line indicates standard error (n = 3).

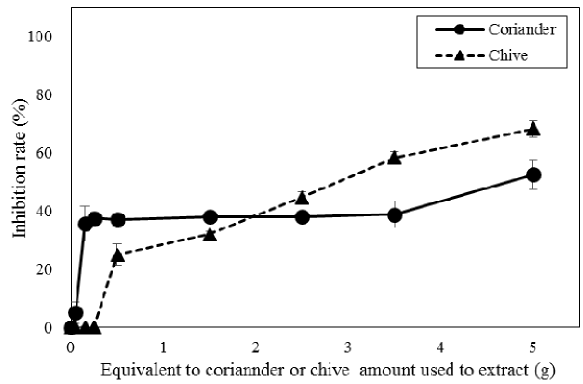

Figure 2. Antifungal activity of coriander and chive volatile extracts on *F. oxysporum*

Note. Vertical line indicates standard error (n = 3).

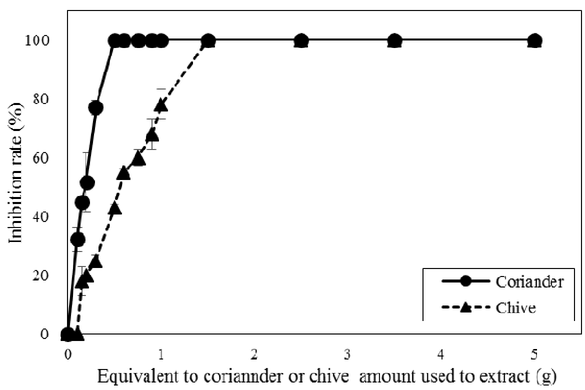

Figure 3. Antifungal activity of coriander and chive volatile extracts on *B. fuckeliana*

Note. Vertical line indicates standard error (n = 3).

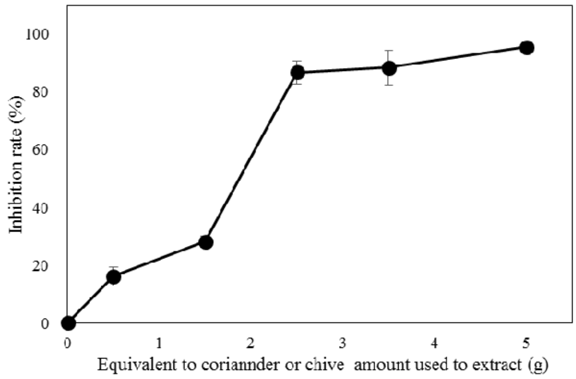

Figure 4. Antimicrobial activity of coriander and chive volatile extracts on *P. carotovorum*

Note. Vertical line indicates standard error (n = 3).

4. Conclusion

To develop biological microbicides as alternatives to synthetic chemical microbicides for sustainable agriculture applications, we investigated the antimicrobial activity of volatile extracts from 10 kinds of herb (coriander, oregano, chamomile, sage, thyme, chive, basil, fennel, lavender, and rosemary) of against 4 species of plant pathogens (*Botryotinia fuckeliana*, *Glomerella cingulata*, *Fusarium oxysporum*, and *Pectobacterium carotovorum* subsp. *carotovorum*). Antimicrobial activity against all plant pathogens tested increased with increased volumes of coriander and chive extracts. In particular, the growth of *B. fuckeliana* and *G. cingulata* was completely inhibited by equivalent to about 0.5 gFW and 0.25 gFW of coriander aerial parts, or by equivalent to about 1.5 gFW and 3.5 gFW of chive aerial parts. Experimental testing of coriander volatile extracts compared with common chemical microbicides in the field will be an important aspect of future research on development of these extracts as biological microbicides.

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