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Application of Selected Plant Extracts to Inhibit Growth of *Penicillium expansum* on Apple Fruits

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Abstract: *Penicillium expansum* is an important postharvest pathogen that not only causes decay on apple and pear fruit but also produces the carcinogenic mycotoxin patulin in spoiled fruit and processed fruit. Although synthetic fungicides are effective to protect against fruit decay, their potential effects on human health and the environment are a concern. Plant extracts are one of several non-chemical control alternatives that inspiring great interest due to their availability, non-toxicity and friendliness to the environment. In this study, screening of antifungal activity against *P. expansum* from sixteen plants (garlic, clove, dokudami, kumasasa, dandelion, kusagi, yomogi, ginkgo, marigold, lavender, thyme, hot pepper, ginger and lemon basil) by means of solvent extraction with either dichloromethane or diethyl ether was conducted. By the solution contact method and the vapor contact method, plant extraction of 16 plants was treated on PDA and the diameter of a clear inhibition zone was recorded daily for 5 d. Next, 100 μL of conidia suspension was added to each wound on apple fruits. Lesion diameter of the treated fruits was observed daily for 6 d. The antifungal activity against *P. expansum* of garlic, thyme, lavender, ginkgo and dandelion which directly contacted the fungal spore, was distinguishingly affective with a clear inhibition zone diameter higher than 12 mm over 5 days of incubation. Apple fruits were treated with garlic extracts by the solution contact method. Growth inhibition activity of *P. expansum* in an amount of 50 μL was higher than that in 20 μL which was equally effective control. Apples exposed to vapor of garlic extract at 1, 2 and 3 ml L^{-1} for 24, 48 and 72 h show different antifungal effects on *P. expansum*. Vapor contact of *Allium sativum* at a concentration of 1 ml L^{-1} with 72 h of exposure time demonstrated the most optimal performance in terms of fruit appearance. In these results, dichloromethane is an appropriate solvent for use in extracting active compounds from plants presenting antifungal activity against *P. expansum*. Crude extract of garlic was the most effective, both in the form of solution and vapor contact, for inhibiting mycelium growth of *P. expansum*. In addition, garlic extract is applicable at relatively low concentration to reduce blue mold rot on apple fruits.

Key words: *Penicillium expansum*, antifungal activity, *Allium sativum*, solvent extraction, plant extract

INTRODUCTION

Apple is the most important fruits species in the world and is produced in many countries, almost on all continents (Mehmood *et al*., 2008). The world is produced 64,255,520 t of apple in 2007. The biggest planetary producers are China, USA, Iran and Turkey. Italy is European leader in apple production (Lana and Marko, 2009). *Penicillium expansum* causes blue mould, a serious postharvest disease of apples and is the main producer of the mycotoxin patulin. Since control by synthetic fungicides is less accepted by consumers, the demand for alternative means is pressing (Ryu and Holt, 1993; Sanzani *et al*., 2010). Synthetic fungicides are effective to protect against fruit decay. However, their potential effects on human health and the environment are a concern. In addition, prohibition of commercial application of some chemical fungicides has recently been enacted (U.S. Environmental Protection Agency, 2003). Many trials control plant diseases with ignorance of using

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chemical. Plant extracts are one of several non-chemical control alternatives that inspiring great interest due to their availability, non-toxicity and friendliness to the environment (Aqil *et al*., 2010).

Many plant extracts have potential as natural antimicrobial agents that can be applied to agricultural produces, foods and pharmaceutics (Horburg, 1998; Maoz and Neeman, 1998) because they contain a phytochemical that exhibits antimicrobial and cytotoxic effects on microorganisms (Feldberg *et al*., 1988). Examples include allyl isothiocyanate from mustard which is able to inhibit the growth of *P. expansum* (Mari *et al*., 2002), phenolic compounds in thyme oil which exhibited antimicrobial effects on food-borne bacteria (Cosentino *et al*., 1999). These extracts are of Generally Recognized As Safe (GRAS) status.

This study was aimed to examine the antifungal properties of 16 plant extracts against *P. expansum* Link. The comparative study of their activities by the method of solution contact and vapor contact was undertaken both *in vitro* and by application on apple fruits.

MATERIALS AND METHODS

This study was conducted at the laboratory Prefectural University of Hiroshima from June 1st to December 20th 2005.

Plant materials: The nomenclature of the plant materials, shown in Table 1 was as follows: garlic: clove (*Allium sativum*); dokudami: leaves and stem (*Houttuynia cordata*); kumasasa: leaves (*Sasa veitchii*); tampopo: leaves (*Taraxacum* spp.); kusagi: leaves (*Clerodendron trichotomum*); yomogi: leaves and stem (*Artemisia vulgaris*); ginkgo: leaves (*Ginkgo biloba* L.); pot marigold: flower (*Calendula officinalis*); lavender: flower (*Lavendula angustifolia*); thyme: leaves and stem (*Thymus vulgaris*); hot pepper: fruit (*Capsicum chinense*); ginger: root (*Zingiber officinale*) and lemon basil: leaves (*Ocimum citriodorum*). Plants were collected in the area of Shobara city, Hiroshima prefecture, Japan. The coriander seed (*Coraindrum sativum*) and lesser galangal root (*Kaempferia pandurata*) were from Thailand and neem leaves (*Azadirachta indica*) were from India. Raw garlic clove, thyme, lemon basil and marigold were washed and crushed by a porcelain mortar. Other plant samples were cut into small pieces, dried in a ventilated chamber and ground using a Waring blender (Waring, New Hartford, Connecticut, USA).

Plant extraction: Ten grams of respective plant samples were put in an Erlenmeyer flask to which 100 mL of either dichloromethane (Kanto Chemical Co., Inc., Tokyo, Japan) or diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added. The flask was placed on a magnetic stirrer at a temperature 20°C for 3 h. The mixture was centrifuged at 12000 g, 4°C, for 20 min. The supernatant was separated by filter paper (Whatman 5B). The solvent was evaporated from the filtrate in vacuo by means of a rotary evaporator, utilizing a water bath at 35°C. The extract was refused with 5 mL of dichloromethane or diethyl ether. The extract was sterilized by passing through a sterilized Millipore filter (45 μm).

Cultures: *Penicillium expansum* (ATCC 42710) was obtained from Microbiological Laboratory, Prefectural University of Hiroshima and then grown at 28±2°C for

Table 1: The volatile extracts were tested on the probability to be fungicide against *Penicillium expansum* at 5 days of incubation

	Dichloromethane		Diethyl ether	
Plants	Solution[a]	Vapor[b]	Solution	Vapor
Control	-	0	-	0
Allium sativum	45.0±0.00	+++	35.0 + 0.00	+++
Artemisia vulgaris	-	+	-	+
Azadirachta indica	-	++	-	+
Calendula officinalis	9.5±1.73	++	-	+
Capsicum chinense	-	+	-	+
Clerodendron trichotomum	-	++	-	+
Coraindrum sativum	-	+	-	+
Ginkgo biloba L.	13.0±0.00	+	-	+
Houttuynia cordata	-	0	-	0
Kaempferia pandurata	-	+	-	+
Lavendula angustifolia	13.2±0.5	+	-	+
Ocimum citriodorum	-	+	-	+
Sasa veitchii	-	+	-	+
Taraxacum spp.	12.0±2.45	++	-	+
Thymus vulgaris	18.8±2.63	+	-	+
Zingiber officinale	-	+	-	+

[a]Solution : clear inhibition zone diameter examined by solution contact method, included 8 mm in diameter of a paper disc (mm+SD); $p<0.05$ (-) Mean the absence of clear inhibition zone. [b]Vapor: growth inhibition activity examined by vapor contact method when 0, +, ++, +++ stand for uninhibited, slightly inhibited (growth area 30-50%), distinguishingly inhibited (growth area <30%) and definitely inhibited (no mycelium growth), respectively

7-14 days on Potato Dextrose Agar (PDA). Water suspension of conidia from this culture was prepared by washing conidia from the culture surface with 5 mL of sterile distilled water containing 0.05% v/v Tween 80. Then 1 mL of water solution was pipetted into test tubes filled with 9 mL of distilled water. The concentration of spore suspension was adjusted to 10^5-10^6 spores mL^{-1} as determined by a Haemacytometer (Erma, Tokyo, Japan).

Screening of extracts on pathogen growth *in vitro*: One hundred microliters of spore suspension was added and spread over the surface of PDA plates (9 cm diameter). For the solution contact method, 50 μL of plant extract was impregnated on an individual paper disc of 8 mm diameter (Advantec, Japan). The solvent was left to vaporize for a minute, then 2 discs were placed directly on the plate. By the vapor contact method, a drop of the extract was treated in the same manner as that used for the solution contact method, whereas 2 discs were placed on a lid of a reversed plate. All treatments included incubation at 28°C and the diameter of a clear inhibition zone was recorded daily for 5 day. Three replications of each treatment were performed.

Disease control of selected plant extract on apple fruits: Apple fruits cv. 'Tsugaru' were harvested from a demonstration orchard of Prefectural University of Hiroshima, Shobara, Hiroshima, Japan in August 2004. They were free from chemical treatment and showed no damage from pathogenic infection. Sampling fruits were soaked in 100 ppm of sodium hypochlorite and washed twice with distilled water then kept dry under ambient condition. Each fruit was wounded with a sterilized cork borer of 6 mm diameter and 5 mm depth, for a total of 3 wounds per fruit. Then 100 μL of conidia suspension was added to each wound. An hour after that, three replications were subjected to all treatments, as follows (1) Control: 20 μL of dichloromethane was added to each wound of control fruit. (2) Solution contact: 20 and 50 μL of plant extracts were directly applied to infected fruits. (3) Vapor contact: each inoculated fruit was exposed to vapor of 1, 2 and 3 ml L^{-1} of plant extract concentration for 24-72 h in the chamber. The treated fruits were kept under the ambient condition at a temperature of 20±5°C and lesion diameter was observed daily for 6 day.

Statistical analysis: Mean separation of contents of antifungal activity was determined by Turkey-Kramer test at $p = 0.05$ and standard division of the mean (SD) using Excel statics 2008 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

The antifungal activity against *Penicillium expansum* of plant extracted with dichloromethane shows a difference between the two methods, solution contact and vapor exposure (Table 1). Among 16 species of candidate plants, the antifungal activity of *Allium sativum*, *Thymus vulgaris*, *Lavendular angustifolia*, *Ginkgo biloba* and *Taraxacum* spp. which directly contacted the fungal spore, were distinguishingly affective with a clear inhibition zone diameter higher than 12 mm over 5 day of incubation, whereas crude extracts from other plants did not show this phenomenon. By the vapor exposure method, the extracts of *Allium sativum*, *Clerodendron trichotomum*, *Calendula officinalis*, *Azadirachta indica* and *Taraxacum* spp. showed a strong growth inhibiting effect on *P. expansum* within 5 day of incubation compared to the control. *Allium sativum* extract exhibited the most powerful inhibiting effect on *P. expansum* through 14 day of incubation, without any growth of fungi (data not shown). This confirms the fungicidal effect of *Allium sativum* extract on *P. expansum* by both solution contact and vapor contact methods.

Dichloromethane and diethyl ether control have no inhibiting effect in either solution or vapor contact method (Table 1). Dichloromethane has greater potential to extract antifungal compounds from candidate plants by both solution and vapor contact methods. Of the diethyl ether extracts, only *Allium sativum* exhibited an inhibiting effect of a clear inhibition zone with a diameter of 35 mm by means of solution contact method and clearly inhibited fungal growth by the vapor contact method. No other crude extracts showed any inhibition effect.

Results in Table 1 showed that the *Allium sativum* extract was the most effective fungicide. Apple fruits were treated with garlic extracts by the solution contact method (Fig. 1). In 2 days of incubation, lesion diameter of 50 μL of garlic extract was suppressed stronger than 20 μL but lesion diameter of 20 and 50 μL of garlic extracts were increased the same ratio with day. In 6 days of incubation, growth inhibition activity of *P. expansum* by 50 μL of garlic extracts was higher than that by 20 μL which was equally effective control. Apple fruits exposed to vapor of *Allium sativum* extract at 1, 2 and 3 ml L^{-1} for 24, 48 and 72 h show different antifungal effects on *P. expansum* (Fig. 2). Vapor of *Allium sativum* extract at a concentration of 1-3 ml L^{-1} and long exposure period (72 h) was more effective at inhibiting fungal growth than the same concentration for a shorter period. Although the antifungal activity of *Allium sativum* extract at a concentration more than 1 mL L^{-1} was appreciable, the

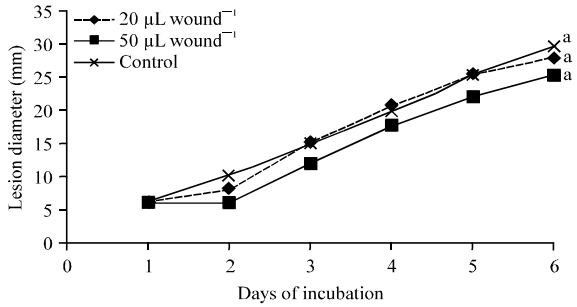

Fig. 1: Apple fruits infected by *P. expansum* after directly contacted with garlic extracts solution and incubated for 6 day under the ambient condition. Vertical bars represent the standard division of the mean (n = 3)

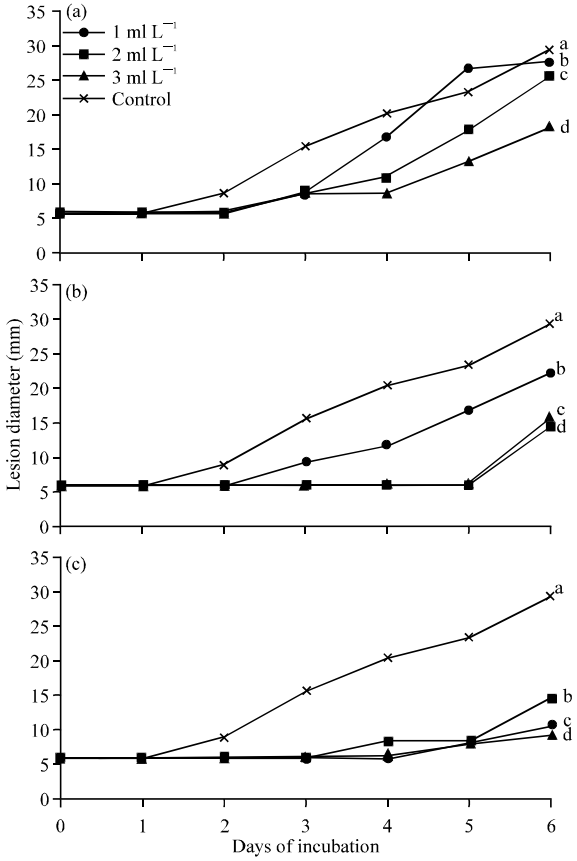

Fig. 2 (a-c): Apple fruits infected by *P. expansum* after fumigation with vapor of garlic extract concentration of 1, 2 and 3 ml L^{-1} for (a) 24 h, (b) 48 h and (c) 72 h; then incubated for 6 day under the ambient condition. Vertical bars represent the standard division of the mean (n = 3). Different letters indicate a difference significant at the 5% level by Turkey-Kramer test between treatments

Fig. 3: Apples infected with *P. expansum*, then fumigated with garlic extract concentration of 1, 2 and 3 ml L^{-1} for (a) 24 h (b) 48 h and (c) 72 h, on 6 day of incubation

appearance of fruits was not preferable due to the presence of discoloration on the peel of the apple fruits (Fig. 3).

DISCUSSION

The results demonstrated that active components of each plant are stable at different states. To our knowledge, the antimicrobial activity of plant extracts has been studied by both the solution contact and the vapor contact method (Suhr and Nieken, 2003). The reports concluded that the activity depended on the state (liquid or gas) in which the active compounds were stable. The antimicrobial effects of the essential oils depended on the application method which related to the molecular size of the active compounds in each plant. Solution contact is the most widespread technique of antimicrobial activity assessment (Zafar *et al*., 2002; Ezeifeka *et al*., 2004; Somda *et al*., 2007; Ajibesin *et al*., 2008). The essential oil components were allowed to diffuse in the agar. The

active compounds are able to directly inhibit the metabolism of microbial cells (Brogden, 2005). However, some volatile components that do not diffuse well in the agar broth may evaporate with the dispersing solvent during incubation which resulted in the presence of uninhibited activity in many extracts. The vapor contact method is suitable for estimation of the effect of volatile compounds. The vapor of volatile compounds not only circulates within the headspace above the agar medium but also adsorbed in the agar medium (Moleyar and Narasimham, 1986). The findings in this study are in agreement with those in other reports showing that essential oil components are efficient in preventing fungal growth by gaseous contact species (Inouye *et al*., 2000). Inouye *et al*. (2001) reported that vapor of lavender oils and thyme show better antimicrobial activity than solution contact. Nonetheless, the bioactivity on hamster cells revealed that thyme oil in the aqueous state had a higher activity than that in gaseous contact. This result is supported by Suhr and Nieken (2003) reported that a large phenolic compound such as thymol in thyme in direct contact with the medium was very effective to control rye bread fungi. In this experiment, the inhibiting activity of vapor contact for *Lavendula angustifolia* and *Ginkgo biloba* was less than that of the solution contact method for these extracts. This would be due probably to insufficient vapor concentration which is related to the evaporation or decomposition in the vapor state of some of the components during long incubation periods. This was suggested by Kalemba and Kunicka (2003). Dorman and Deans (2000) supported this result, finding that some essential oil components such as limonene and α-pinene were unstable in the vapor state, causing a rapid gas phase reaction with the atmospheric oxidants to yield oxygenated products, though these oil components were stable in an aqueous medium.

The efficacy of solvents for extracting volatile compounds from plants is different. This contributes to polarity of the solvent and the functional components. Volatile compounds are largely lipophilic products (Dudareva *et al*., 2004). In present experiment, we found that dichloromethane has less polarity than diethyl ether and could be expected to perform better in an extract active aroma compound designed to have high antifungal activity.

In practical application on fruits, garlic extracts in the liquid state were less effective than those in the vapor state. This phenomenon was in contrast to that in the *in vitro* test. Storage in an open container contributes to loss of activity of compounds in solvent. Dichloromethane has a low boiling point which promoted the vapor pressure of the mixture and accelerated the volatilized rate of the key compounds.

A long fumigation period even at a low concentration can well control *P. expansum* growth. This is advantageous for use of garlic extract on fruits subjected to long-term storage. Volatile compounds in garlic were able to sterilize the container and surface of fruits. Residual components in the vapor phase will be easily disintegrated under the normal air condition. However, discoloration of the peel might be a side effect from the toxicity of dichloromethane to fruit. The most appropriate eluted solvent would be optimal in terms of preserving stability of components and being non-toxic to fruit. Obagwu and Korsten (2003) recommended adding garlic extract to vegetable oil which mixture was effective as a fungicide for controlling both blue and green mold in Valencia orange.

CONCLUSION

In summary, dichloromethane is an appropriate solvent for use in extracting active compounds from plants presenting antifungal activity against *P. expansum*. Crude extract of *Allium sativum* was the most effective plant extract investigated here, both in the form of solution and vapor contact, for inhibiting mycelium growth of *P. expansum*. Other extracts, of *Thymus vulgaris*, *Lavendular angustifolia* and *Ginkgo biloba*, showed higher antifungal activity by solution contact method than by vapor contact method. In addition, *Allium sativum* extract is applicable at relatively low concentration to reduce blue mold rot on apple fruits. However, the extract should be added to an appropriate eluted solvent that is non-toxic to the host fruit and capable of maintaining stability of the active compounds during storage.

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