In vitro antifungal activity screening of crude extracts of soil fungi against plant pathogenic fungi

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ABSTRACT

The ethyl acetate extracts of the culture of *Eupenicillium parvum, Gelasinospora brevispora, Neosartoryap seudofischeri, N. quadricincta* and *N. multiplicata* were assessed for their antifungal activity against ten economically important plant pathogenic fungi: *Pythiumaphanidermatum, Phytophthorapalmivora, Alternaria* sp., *Fusariumoxysporum, Colletotrichumgloeosporioides, Lasiodiplodiatheobromae, Helminthosporiummaydis, Sclerotiumrolfsii* and *Rhizoctoniasolani*,which arecausative agents of fruit and vegetable diseases. The bioassay for the antifungal activity of the fungal crude extracts was based on the dilution plate method. Although all the extracts exhibited a complete inhibition of the mycelial growth of some plant pathogenic fungi at the highest concentration tested (10,000 ppm), an interesting antifungal effect was observed for the crude extract of *N. pseudofischeri* KUFA 0060 against *Ph. palmivora* and *C. capsici*at 100 ppm, as well as for *N. quadricincta* KUFA 0064 against *Ph. palmivora* and *Alternaria* sp. at 10 ppm. The results obtained from this screening allow us to identify new potential sources for the development of alternative fungicides.

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Key words: Antagonistic fungi,*Neosartorya*,*Eupenicillium*, *Gelasinospora*, phytopathogenic fungi.

INTRODUCTION

It is interesting to note that considerable efforts have recently been made for the development of biocontrol agents and biofungicide from medicinal plants as environmentally safe alternatives (Gurjar*et al*., 2012). Even though fungi are still not widely exploited to control plant pathogens, some fungal strains such as *Trichoderma harzianum* (Binab T®, Trichodex®), *Pythium oligandrum* (Polygandron®, Polyversum®), *Fusarium oxysporum* (Fusaclean®, Biofox C®), *Gliocladium virens*(SoilGard®) and *Gliocladium catenulatum* (Primastop®) are currently commercialized for this purpose (Kaewchai *et al*., 2009). This fact clearly reflects the potential of antagonistic fungi for the development of alternative antifungal agents as well as for several other agricultural applications (Frisvad *et al*., 1998; Butt and Copping, 2000; Dethoup *et al*., 2007). Although the fungal antagonistic effect depends on several mechanisms such as mycoparasitism (Whipps, 2001; Irtwange, 2006; Viterbo *et al*., 2007), competition (Irtwange, 2006; Viterbo *et al*., 2007), induced resistance (Harman *et al*., 2004; Pal and Gardener, 2006; Viterbo *et al*., 2007) and antimicrobial effect (Kanokmedhakul *et al*., 2006; Woo and Lorito, 2007; Vinale *et al*., 2008), they are supposedly dependent on the products of fungal secondary metabolism that are toxic to a variety of plant pathogenic fungi (Nakadate *et al*., 2007; Wang *et al*., 2012).

The potential of fungi as producers of valuable bioactive metabolites is clearly demonstrated by several commercial fungicides developed from fungal metabolites such as strobilurin fungicides (Bartlett *et al*., 2002), however the focus on soil fungi for the purpose of

developing new environmentally safe and easily biodegradable antimicrobial agents for use in agriculture is still scarce. Nevertheless, there are a few reports dealing with the antifungal activity of soil fungi collected in Thailand against some plant pathogenic fungi (Kaewchai *et al*., 2009; Talubnak and Soytong, 2010; Sibounnavong *et al*., 2012). On the other hand, the soil fungi of the genera *Neosartorya*, *Eupenicillium* and *Gelasinospora* have been reported to produce a variety of secondary metabolites with interesting biological activities (Nakadate *et al*., 2008; Eamvijarn *et al*., 2012, 2013). The fact that many of these fungal metabolites exhibited antimicrobial activity against human pathogens can be indicative that they can also have some potential for developing biofungicide against plant pathogens which can be potential substitutes for the synthetic fungicides in current use. Consequently, we have selected ten strains of important plant pathogenic fungi which cause severe diseases to economic plants in Thailand such as mangoes, chilies, durians, as targets for antifungal activity evaluation of the crude extracts of these five soil fungal species.

It is important to point out also that although there are previous reports on the antagonistic activity of soil fungi, to the best of our knowledge, this is the first report on the *in vitro* antifungal activity of the crude extracts of*E. Parvum* (Raper & Fennell) Stolk & D.B. Scott, *G. brevispora* R.S. Khan & J.C. Krug, *N. pseudofischeri* S.W. Peterson, *N. quadricincta* (J.L. Yuill) Malloch & Cain and *N. multiplicata* Yaguchi, Someya & Udagawa against plant pathogenic fungi.

MATERIALS AND METHODS

Isolation and Identification of Fungi

Soil samples were collected from agricultural and forest fields in Chiang Mai (*E. parvum* KUFA 0056), Chonburi (*G. brevispora* KUFA 0072), Chanthaburi (*N. pseudofischeri* KUFA 0060 and *N. multiplicata* KUFA 0081) and Phang Nga (*N. quadricincta* KUFA 0064) provinces. For fungal isolation, 1 gram of soil sample was placed in a sterile test tube and kept in a water bath at 65ºC for 15 minutes. After eliminating the excess of water, the soil particles were transferred into Petri dishes and immediately poured with warm glucose ammonium nitrate agar mixed with streptomycin, and incubated at 25ºC in the dark for 3 days. Hyphal tips from pure fungal strains were transferred onto potato dextrose agar (PDA) slants and stored in the Culture Collection at the Department of Plant Pathology, Kasetsart University for subsequent fungal identification.

Based on macro- and microscopic morphological features observed under stereo, light, and scanning electron microscopes, and also supported by sequence analysis of the β-tubulin gene, the selected fungal cultures were identified as *Eupenicillium parvum*, *Gelasinospora brevispora*, *Neosartoryap seudofischeri*, *N. quadricincta* and *N. multiplicata*. Fungal strains gene sequences were deposited in GeneBank with accession numbers as follows: KM095493 (*E. parvum* KUFA 0056), KM980630 (*G. brevispora* KUFA 0072), KM095495 (*N. pseudofischeri* KUFA 0060), KM095498 (*N. quadricincta* KUFA 0064) and KM095491 (*N. multiplicata* KUFA 0071).

Extraction Procedure

Twenty-five 1,000 mL Erlenmeyer flasks, each containing 200 g of rice and 100 mL of water, were autoclaved at 121ºC for 15 minutes, inoculated with 5 mycelial plugs from each of the selected fungi, and incubated at 25ºC for 30 days, after which the moldy rice was macerated with ethyl acetate for 7 days. After filtration with the filter paper, the ethyl acetate solutions were combined and the solvent was evaporated by rotary evaporator to furnish the crude ethyl acetate extracts.

Antifungal Activity Assay

Plant pathogenic fungal isolates

Ten plant pathogenic fungi were obtained from several host plants as described in Table 1. Stock cultures were maintained on PDA plates and stored at -20 ºC.

Table 1. Plant pathogenic fungi from various fruits and vegetables diseases used for antifungal activity test.

Plant pathogenic fungi	Host plant	Disease	Class
Pythiumaphanidermatum (Edson Fitzp.)	*Cucumissativus* (cucumber)	Pythium root and stem rot	Oomycetes
Phytophthora palmivora (E.J. Butler)	*Duriozibethinus* (durian)	Durian root rot	Oomycetes
Colletotrichumcapsici (E.J. Butler &Bisby)	*Capsicum annuum* (chilli)	Chili anthracnose	Coelomycetes
Colletotrichumgloeosporioides (Penz. &Sacc.)	*Pyruspyrifolia* (pear)	Anthractnose	Coelomycetes
Lasiodiplodiatheobromae ((Pat.) Griffon &Maubl.)	*Garciniamangostana* (mangosteen)	Fruit rot	Coelomycetes
Alternaria sp.	*Pyruspyrifolia* (pear)	Fruit rot	Hyphomycetes
Fusariumoxysporum (E.F. Sm. &Swingle)	*Lycopersiconesculentum* (tomato)	Fusarium wilt	Hyphomycetes
Helminthosporium maydis (Y. Nisik. & C. Miyake)	*Zea mays* (corn)	Southern corn leaf blight	Hyphomycetes
Sclerotiumrolfsii (Sacc)	*Vigna radiate* (mungbean)	Basal stem rot	Agonomycetes
Rhizoctonia solani (J.G. Kühn)	*Oryza sativa* (rice)	Sheath rot	Agonomycetes

Dilution Plate Method

The dilution plate method was used for the evaluation of the *in vitro* antimycelial growth of the plant pathogenic fungi.

Briefly, 1 g of each crude extract was dissolved in 10 ml of methanol to prepare a stock solution of 100,000 ppm, which was serially diluted to prepare four different concentrations (10,000; 1,000; 100 and 10 ppm). Then, 1 mL from each solution was added into 9 ml of warm PDA, mixed, and poured into the middle of Petri dishes. After solidification, the plates were inoculated with young mycelia of the plant pathogenic fungi, and incubated at 25ºC for 7-14 days. The PDA Petri dish void of the fungal crude extract was used as a control. The inhibition levels were calculated using the formula: G1-G2/G1 × 100, where G1 = colony radius of the plant pathogenic fungi in the control, and G2 = colony radius of plant pathogenic fungi in the presence of the tested crude extract. Each treatment was performed in triplicate with complete randomized design.

RESULTS AND DISCUSSIONS

Antifungal activity screening of the crude extract of *N. multiplicata* KUFA 0071 revealed a complete inhibition of the mycelial growth in almost all of the plant pathogenic fungi at 10,000 ppm, except for the

Coelomycetous plant pathogenic fungus, *C. gloesporioides* (Fig. 1). Despite its antifungal activity at the highest concentration tested, the extract was still effective at 1,000 ppm against six plant pathogenic fungi, displaying a strong activity against *Ph. palmivora*, *C. capsici*, *C. gloeosporioides*, *F. oxysporum*, *R. solani*, and causing a complete inhibition of themycelial growth in *S. rolfsii*.

Similarly, the ethyl acetate crude extract of *E. parvum* KUFA 0056 showed antifungal activity in most of the tested isolates, causing a complete mycelial growth inhibition at 10,000 ppm in eight plant pathogenic fungi, except for *L. theobromae* and *F. oxysporum* (Fig. 2). Additionally, a strong antifungal activity (more than 50 % inhibition of mycelial growth) was also observed at the concentration of 1,000 ppm against the two Coelomycetous plant pathogenic fungi *C. capsici* and *C. gloeosporioides*, causing 64.81 and 70.00 % of a mycelial growth inhibition, respectively.

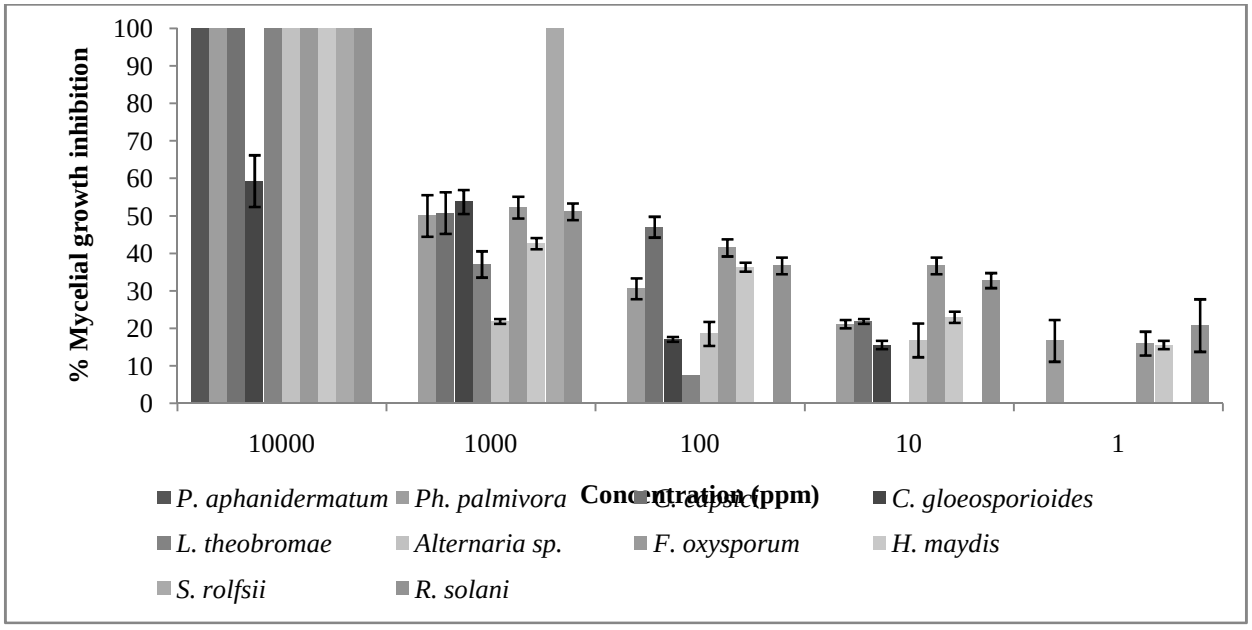

Fig 1. Inhibitory effect of the ethyl acetate crude extract of *N. multiplicata* KUFA 0071 on the mycelial growth of 10 selected plant pathogenic fungi. Each value is mean of three replicates and bars represent the standard deviations of the mean.

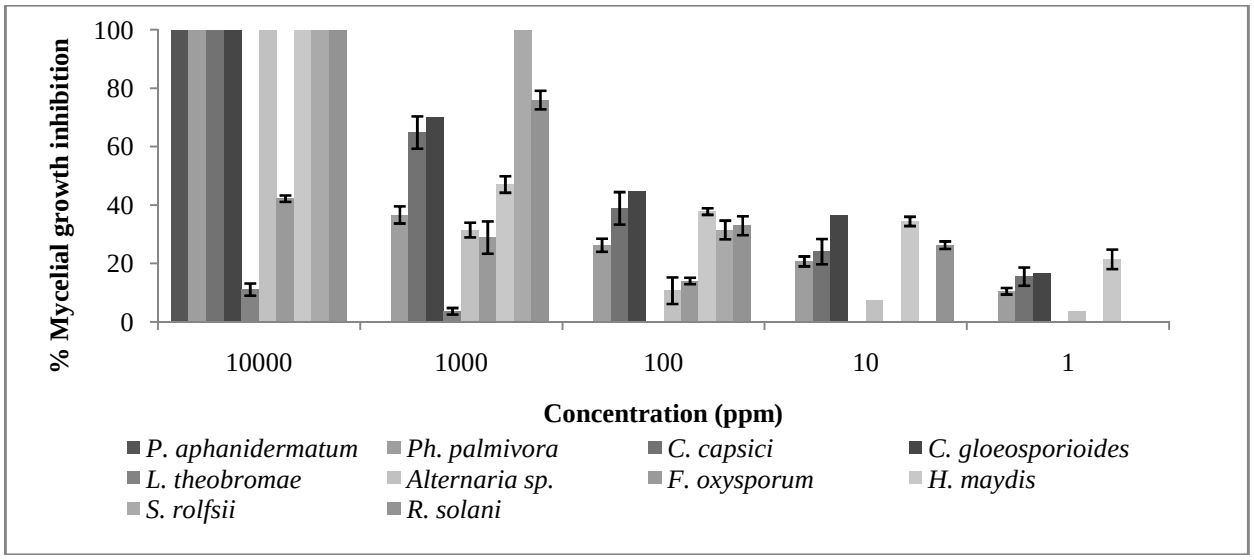

Fig 2. Inhibitory effect of the ethyl acetate crude extract of *E. parvum* KUFA 0056 on the mycelial growth of 10 selected plant pathogenic fungi. Each value is mean of three replicates and bars represent the standard deviations of the mean.

It also inhibited the mycelial growth of the Agronomycetous plant pathogenic fungus*R. solani* (75.93 %) and a complete inhibition of mycelial growth of *S. rolfsii* (Fig. 2). On the other hand, the extracts of the culture of *Neosartorya* species showed some interesting aspects of their antifungal activity. When compared to the extract of *N.multiplicata* KUFA 0072, the extract of *N. quadricincta* KUFA 0064 exhibited a complete inhibition of the mycelial growth in less plant pathogenic fungal species at the highest concentration tested (10,000 ppm), however it revealed the most promising results at lower concentrations (Fig. 3).

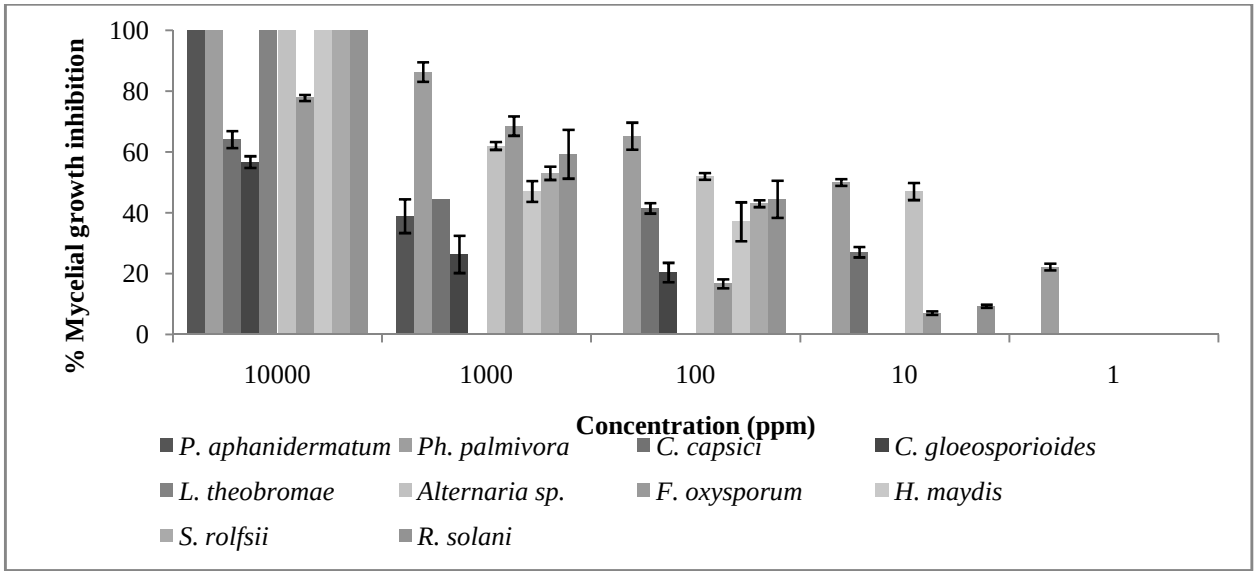

Fig 3. Inhibitory effect of the ethyl acetate crude extract of *N. quadricincta* KUFA 0064 on the mycelial growth of 10 selected plant pathogenic fungi. Each value is mean of three replicates and bars represent the standard deviations of the mean.

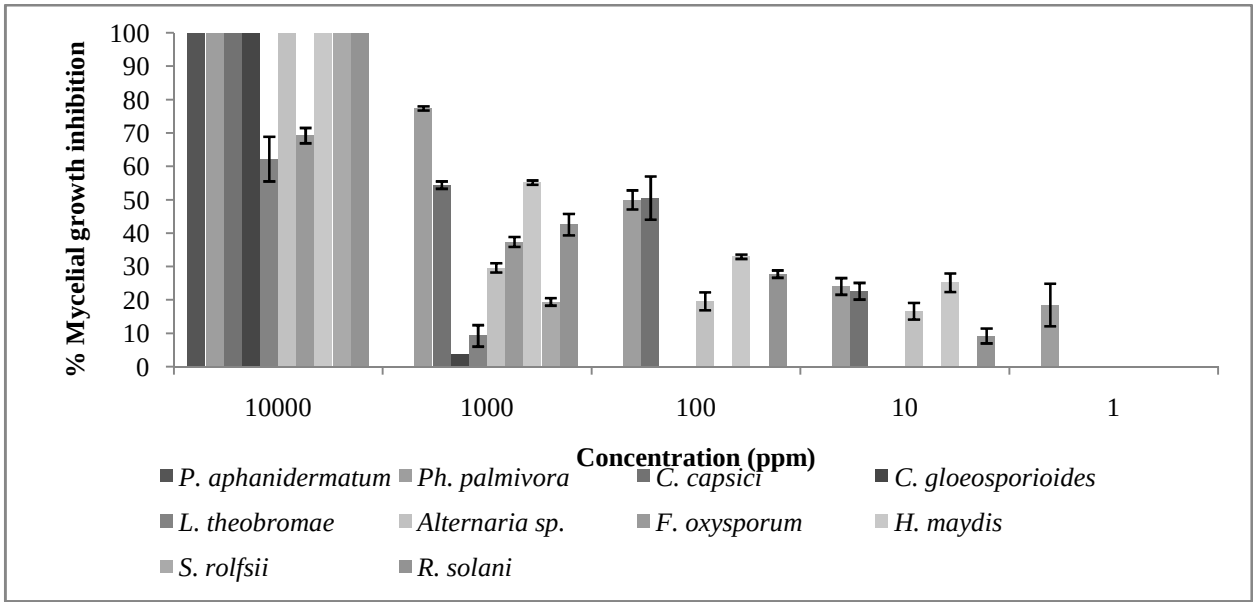

Fig 4. Inhibitory effect of the ethyl acetate crude extract of *N. pseudofischeri* KUFA 0060 on the mycelial growth of 10 selected plant pathogenic fungi. Each value is mean of three replicates and bars represent the standard deviations of the mean.

Although, at a concentration of 1,000 ppm, the extract of *N. quadricincta* KUFA 0064 exhibited lower inhibitory activity on the mycelial growth of the ten plant pathogenic fungi, it remained highly effective against five species causing more than 50 % inhibition, namely *Ph. palmivora*, *Alternaria* sp., *F. oxysporum* and both Agonomycetous plant pathogenic fungi (*S. rolfsii* and *R. solani*).

Furthermore, it was observed that *Ph. palmivora* and *Alternaria* sp. exhibited significant sensitivity towardthe extract of *N. quadricincta* KUFA 0064 at lower concentrations (100 and 10 ppm). At 100 ppm, the extract of *N. quadricincta* KUFA 0064 was found to suppress a mycelial growth of *Ph. palmivora*and *Alternaria* sp. at 65.19 and 52.00 % respectively, while at the lowest concentration tested, i.e. 10 ppm, its

antifungal activity remained strong against plant pathogenic fungi, causing respectively, 50.00 and 47.00 % of a mycelial growth inhibition.

Analogous to the extracts of *E. parvum* KUFA 0056 and *N. multiplicata* KUFA 0071, the extract of *N. pseudofischeri* KUFA 0060 also displayed a complete mycelial growth inhibition for the majority of the plant pathogenic fungi at the highest concentration tested (10,000 ppm) (Fig. 4). Moreover, the results clearly revealed a strong antifungal activity against *Ph. palmivora*, *C. capsici* and *H. maydis* at a concentration of 1,000 ppm (Figure 4). Interestingly, even at 100 ppm concentration, it was able to prevent the mycelial growth of *Ph. palmivora* and *C. capsici* at 50.00 and 50.56 %, respectively.

Finally, the crude ethyl acetate extract of *G. brevispora* KUFA 0072 was found to have less promising results on the inhibition of themycelial growth, showing only weak inhibition in all plant pathogenic fungi tested at concentrations lower than 1,000 ppm, and with no inhibition observed on the growth of *P. aphanidermatum*, *L. theobromae*, *H. maydis* and *S. rolfsii* (Fig. 5). Even at the highest concentration tested (10,000), it was found to exhibit a complete mycelial growth inhibition of only two (*P. aphanidermatum* and *R. solani*) of the ten plant pathogenic fungi tested.

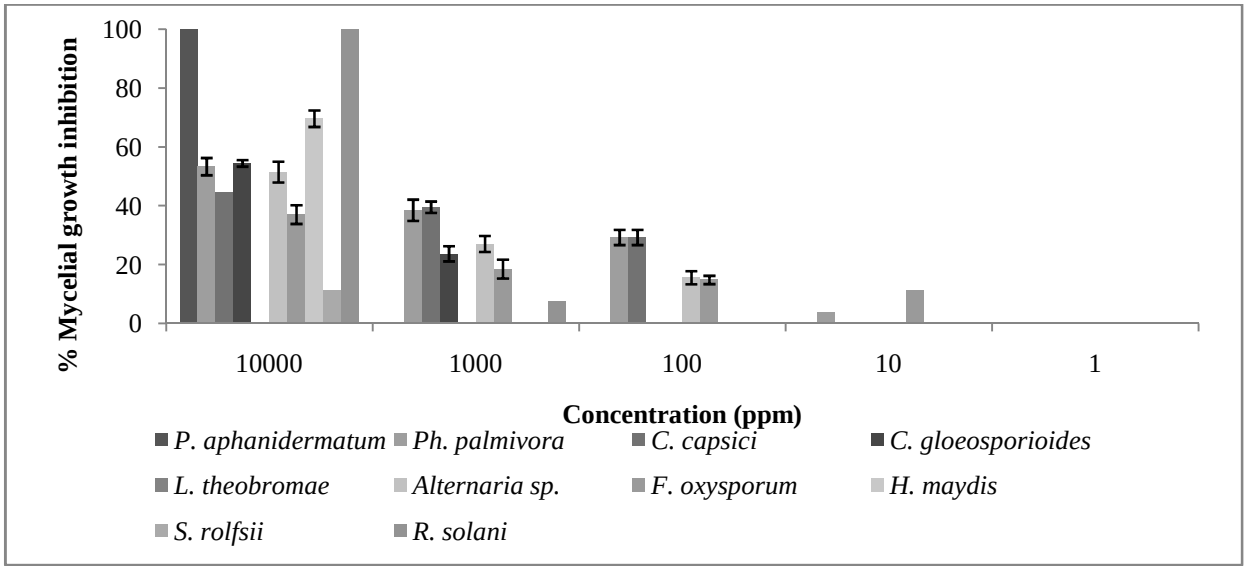

Fig 5. Inhibitory effect of the ethyl acetate crude extract of *G. brevispora* KUFA 0072 on the mycelial growth of 10 selected plant pathogenic fungi. Each value is mean of three replicates and bars represent the standard deviations of the mean.

Five soil fungi were selected for the screening on the antifungal activity against ten plant pathogenic fungi belonging to four different classes. The results obtained in this study revealed that these fungal species, with the exception of *G. brevispora* KUFA 0072, were able to completely inhibit the mycelial growth of these ten plant pathogenic fungi, at the highest concentration tested (10,000 ppm).

For the mycelial growth inhibition of both species belonging to Oomycetes class, the ethyl acetatecrude extracts of *N. pseudofischeri* KUFA 0060 and *N. quadricincta* KUFA 0064 were found to exhibit strong inhibitory activity (50%) against *Ph. palmivora*, at 100 ppm concentration. Besides the first report on the antifungal activity of *Chaetomium cochliodes* against *P. aphanidermatum* by Pornsuriya *et al*. (2010), this is the only study on the antagonistic activity of the soil fungi against the plant pathogenic fungi *P. aphanidermatum*

and *Ph. palmivora*. Interestingly, we have found that the crude extracts of *N. pseudofischeri* KUFA 0060 and *N. quadricincta* KUFA 0064 exhibited higher antifungal activity than that of the plant extracts (Suleiman and Emua, 2009; Ghasemi *et al*., 2012; Bahraminejad *et al*., 2013; Salehan *et al*., 2013).

The antifungal activity screening of the crude extracts against both plant pathogenic *Colletotrichum* species revealed a promising antifungal activity for the extracts of *E. parvum* KUFA 0056 and *N. multiplicata* KUFA 0071 at 1,000 ppm, when compared to the previous reports (Changkasiri and Wongroung, 2009; Johnny *et al*., 2010; Talubnak and Saytong, 2010; Mukherjee *et al*.,2011; Rahman *et al*., 2011; Bussaman *et al*., 2012; Chen *et al*., 2013). Interestingly, although the extract of *N. pseudofischeri* KUFA 0060, at 100 ppm, showed strong mycelial growth inhibitory activity against *C. gloeosporioides*, it was found to havesignificantly lower activity than that of *Piper bettle* L. leaves (Johnny *et al*., 2011).
The inhibitory effects of the extracts of the *Neosartorya* species on the mycelial growth of plant pathogenic fungi of the Hyphomycetes class showed some interesting features. While *F. oxysporum*was very sensitive toward the extract of *N. multiplicata* KUFA 0071, *H. maydis* was more sensitive to the extract of *N. pseudofischeri* KUFA 0060. On the other hand, the extract of *N. quadricincta* KUFA 0064 was found to inhibit the mycelial growthof*Alternaria* sp., even at the concentrations low as 10 ppm. Despite a wide variety of plant extracts used to control the mycelial growth of *Alternaria* sp., *F. oxysporum* and *H. maydis* (Shrestha and Tiwari, 2009; Thobunluepop *et al*., 2009; Abdolmaleki *et al*., 2011; Dellavalle *et al*., 2011; Dissanayake,2014; Shen *et al*., 2014), our results demonstrated that the extracts of soil fungi can be a potential source for the control of these plant pathogens, especially the extract of *N. quadricincta* KUFA 0064 since it showed a significant inhibition of the mycelial growth of *Alternaria* sp. at low concentration.

Amin *et al*. (2013) have previously reported the antimycelial growth activity of the tobacco leaf and turmeric rhizome extracts against the plant pathogenic fungus *S. rolfsii*, however these two extracts were many folds less active than that of the crude ethyl acetate extracts of *E. parvum* KUFA 0056 and *N. multiplicata* KUFA 0071, which displayed a complete mycelial growth inhibition at 1,000 ppm. It was also found that the crude ethyl acetate extracts of *E. parvum* KUFA 0056, *N. multiplicata* KUFA 0071 and *N. quadricincta* KUFA 0064 exhibited a significant antimycelial activity against *R. solani*, even though they were less active than the extracts of *Pelargonium graveolens* and *Polyalthia longifolia*, at the same concentration (Seema *et al*., 2011).

Although there are some emerging reports on the isolation and biological activity evaluation of the secondary metabolites from the fungi of the genus *Neosartorya*, to date there is no study of the effects of these fungi for the control of plant diseases. Recently, we have reported some interesting bioactive secondary metabolites from the cultures of the Thai collections of *Neosartorya* spp., isolated from soil (Kijjoa *et al*., 2011; Buttachon *et al*., 2012; Eamvijarn *et al*., 2012, 2013). These include the cytostatic alkaloid eurochevalierine and cadinenesesquiterpene, as well as brasiliaminde B, pyripyropene A, 1,4-diacetyl-2,5-dibenzylpiperazine 3,7-oxide, a quinazolinone-containing indole derivative and a phenyl ester of 2,4-dihydroxy-6-methylbenzoic acid from the culture of *N. pseudofischeri* (Eamvijarn *et al*., 2012). Later on, Masi *et al*., (2013) reported the isolation of a new pyrroloindoleterpenoid, fischerindoline, and other compounds from another strain of *N. pseudofischeri*. Interestingly, a derivative of dihydroisocoumarin which exhibited a potent insecticide property acting on insect GABA receptor, was isolated from the culture of *N. quadricincta* (Ozoe *et al*., 2004).
The fungi of the genus *Eupenicilium* also produce interesting secondary metabolites.

Example of these is *E. javanicum* whose cultures yielded the derivatives of decalin, eujavanicols A-C, eujavanoic acids A, B, and a cyclic depsipeptide, eujavanicin A (Okamoto *et al*., 2004; Nakadate*et al*., 2007, 2008). Interestingly, eujavanicin A was found to exhibit antifungal activity against the human pathogenic filamentous fungus, *Aspergillus fumigates* (Nakadate*et al*., 2008) . The fungi of the genus *Gelasinospora* have also been widely investigated for their secondary metabolites. While multiforisins A–E were isolated from *G. multiforis*, kobiin and kobifuranones A, B, and C were produced from the culture of *G. kobi* (Fujimoto *et al*., 1995, 1998). Later, the same author reported the isolation of immunomodulatory constituents including 2-pyrone derivatives named multiforisins G, H, I and hexa ketidesordarial from *G. heterospora*, *G. multiforis* and *G. longispora* (Fujimoto *et al*., 1999).To the best of our knowledge, there is no report on the secondary metabolites of *G. brevispora*.

Although there are considerable efforts to search for plant extracts for the control of plant pathogenic fungi, with several studies focused on their potential for the development of new antifungal agents, there were only a few studies on soil fungi for this purpose. Thus, this study constitutes the first report on the antagonistic activity of the ethyl acetate extracts of the culture of *E. parvum*, *G. brevispora*, *N. multiplicata*, *N. pseudofischeri* and *N. quadricincta*, against plant pathogenic fungi that cause diseases on economically important plants of Thailand. The results obtained from this study identify *N. quadricincta* KUFA 0064 as a promising source for the development of antifungal agents against the plant pathogenic fungi *Ph. palmivora* and *Alternaria* sp. However, despite the demonstrated potential of soil fungi as sources of extracts for the development of new natural fungicides, further studies on the chemical constituents of the extracts and the mechanism underlying their antifungal effect are necessary to shed light on the activity of these fungal extracts.

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