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Frequency of endophytic fungi isolated from *Dendrobium crumenatum* Sw. (Pigeon orchid) and antimicrobial activity

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ABSTRACT

Mangunwardoyo W, Suciatmih, Gandjar I. 2012. Frequency of endophytic fungi isolated from Dendrobium crumenatum *Sw. (Pigeon orchid) and antimicrobial activity. Biodiversitas 13: 34-39.* Endophytic microorganisms were found in orchid such as *Dendrobium crumenatum* Sw. (pigeon orchid), an orchid that sometimes used in traditional medicine. The fungi were isolated from roots, bulbouses, stems, and leaves of *D. crumenatum* collected from Tanah Baru Housing area, Botanical Garden Bogor, and Herbarium Bogoriense, respectively. Twelve species were identified from 60 samples obtained. *Guignardia endophyllicola* Okane, Nakagiri, and Ito (anamorph: *Phyllosticta capitalensis* P. Herm.) were the dominant endophytic mould. Screening of the anti-microorganism activity of the samples revealed that *Fusarium nivale* (Fr.) Ces. inhibited *Candida albicans* ATCC 2091 and *Candida tropicalis* LIPIMC 203. All specimens did not inhibit the growth of *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25022, and *Staphylococcus aureus* ATCC 25023.

Key words: anti-yeast, anti-bacterial, *Dendrobium crumenatum,* endophytic.

INTRODUCTION

Endophytic fungi are fungi associated with plants. Those fungi can be found in the root, stem, leaf, flower, fruit and seed, without any disease or impairness showed by the host. Zhang et al. (2006) reported that endophytic fungi refer to fungus colonization in the healthy tissue and it is able to produce secondary metabolites such as mycotoxin, enzyme, antibiotic and anti-cancer.

Petrini (1986) classified endophytic fungi into Ascomycotina, Basidiomycotina, Deuteromycotina, and Oomycotina, includes genera *Cladosporium* (Mahesh et al. 2005; Rubini et al. 2005), *Colletotrichum* (Cannon and Simmons 2002), *Curvularia* (Nakagiri et al. 2005), *Diaporthe* (Agusta et al. 2005, 2006; Shibuya et al. 2005), *Fusarium* (Bacon et al. 2001), *Gibberella* (Rubini et al. 2005), *Guignardia* (Okane et al. 1998), *Nectria* and *Pleurotus* (Rubini et al. 2005), *Phyllosticta* (Okane et al. 2001), and *Xylaria* (Rubini et al. 2005).

Petrini (1986) informed that the plants are able to be the host of endophytic fungus, including orchid. Endophytic fungi *Rhizoctonia* sp. and *Xylaria* spp. were isolated from the leaf and root of orchid *Lepanthes* (Bayman et al. 1997); *Rhizoctonia* spp. from root of *Anoectochilus formosanus* Hayata and *Haemaria discolor* var. *dawsoniana* (Chou and Chang 2004); *Phyllostictina pyriformis* Cash and Watson (syn. *Phyllosticta capitalensis* P. Hrm.) from the orchid of *Cypripedium* sp., *Arundina graminifolia* (Don) Hochr. and *Dendrobium moniliforme* (L.) Sw. (Okane et al. 2003).

Orchid is not only valuable from the aesthetic aspect but also from the medical aspect. People in China, Mongolia and Japan, use bulbous of *Bletilla striata* Reichg. Fil. to cure tuberculosis, bleeding and to relieve scar on hand and foot. Moreover, they also use these plants to cure some diseases in appendix. They used the stem of *Dendrobium nobile* Lindley for mouth disease, the rhizome and stem of *Gastrodia elata* Blume for headache, epilepsy, rheumatic and sickness (Ming et al. 2003). Chou and Chang (2004) informed that *A. formosanus* and *H. discolor* var. *dawsoniana* protect the heart, against cancer, cardiovascular, and relieve diabetes. Orchid contains alkaloid and steroid. Alkaloid dendrobine and nobilonine have been isolated from *D. nobile* and *D. findlayanum* Parr; alkaloid crepidine and stigmastane steroid glycoside have been isolated from *D. crepidatum* Lindley (Arditti 1992).

The objective of this research is to isolate and study the frequency of endophytic fungi from roots, bulbous, stems, and leaves of pigeon orchid, also to assess for antimicrobial against *Candida albicans* ATCC 2091, *Candida tropicalis* LIPIMC 203, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923.

MATERIALS AND METHODS

Plants materials

Roots, bulbouses, stems, and leaves from pigeon orchid were collected in Tanah Baru Housing area, Herbarium Bogoriense, and Bogor Botanical Garden, Indonesia. The samples were grown for two years old plants. The experiment was carried out at the period of April to November (in dry seasons).

Microorganisms

Microorganisms used for production of antimicroorganism were isolated from pigeon orchid. *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 2091 were gained from National Quality Control Laboratory Drug and Food, National Agency of Drug and Food control. *Candida tropicalis LIPIMC 203* was acquired from Research Center for Microbiology, Indonesian Institute of Science, Cibinong-Bogor, West Java, Indonesia.

Procedures

Fungi isolation

The five materials in the size of 10 mm x 5 mm from top, middle and bottom of healthy roots, bulbouses, stems, and leaves pigeon orchid washed with distilled water that were used for fungi isolation. Then, the material were cut into 10 subsamples in the size of 2 mm x 2.5 mm, so there were 50 subsamples for each part of plan. A total of 200 subsamples were examined (Cannon and Simmon 2002). The samples were sterilized using 70% alcohol for 1 minute and 5,3% sodium hypochlorite for 2 minutes and then rinsed 3 times using distilled water, followed by blotting for 3-4 hours. Direct isolation was used for 8 subsamples on PDA 50% added with 200 mg/l of chloramphenicol and incubated at room temperature (26-28^o C) (Nakagiri et al. 2005).

Fungi purification

Purification was done using single spore isolation method (Gandjar et al. 1992). Selected specimen inoculated at PDA slant and incubated for 5 days. Spore suspension was prepared by adding 5 mL distilled water, scraped and diluted at 10^{-3}. An amount 0.1 mL of spore suspension was spreaded on PDA medium incubated in room temperature (26-28^o C).

Fungi identification

Single specimen from endophytic fungi was identified using the books of Domsch et al. (1980), Ellis (1993) and Nakagiri et al. (2005).

Inoculum preparation and fungi endophytic enumeration

An amount of 2 mL distilled water was added in the slant of 7 days olds culture, scraped it and next, rotated it. The amounts of spore were calculated using Colony Forming Unit (CFU) (Gandjar et al. 1992).

Inoculum preparation and bacterial or yeast enumeration

The bacteria of *B. subtilis*, *E. coli*, and *S. aureus* were subcultured using NA medium and were incubated at 37^o C for 24 hours. The yeast *C. albicans* and *C. tropicalis* were subcultured in PDA medium and were incubated at 30^o C for 48 hours. A total of 5 mL NB and PDB was added, scraped and rotated. The next step was pouring in the 15 mL NB and PDB, and then be incubated in waterbath shaker in 90 rpm at 37^o and 30^{o}C for 24 hours (Agusta et al. 2005). The amount of cell was calculated using Colony Forming Unit (CFU) (Gandjar et al. 1992).

Screening endophytic fungi that produce antimicrobial

An amount of 2 mL spore suspension of endophytic fungi (2.4-3.0) x 10^4 cfu/mL was added to 100 mL Erlenmeyer that consists of 20 mL medium PDY, and then incubated in shaker incubator in room temperature (26^o-28^o C). Next, the suspension was agitated in 90 rpm for 5 days (Syarmalina et al. 2003; Agusta et al. 2005; Kumala 2005). Harvesting the antimicrobial by centrifugation was done by rotating the suspension in 6000 rpm for 10 minutes (Prihatiningtias et al. 2005). Supernatant was used as crude antimicrobial agent for bioassay. The tube with 17 mL of MH medium was inoculated with 0,2 mL bacterial suspension *B. subtilis*, *E. coli* and, *S. aureus* (7.1-93) x 10^8 cfu/mL; *C. albicans* and *C. tropicalis* (4.4-6.5) x 10^7 cfu/mL, was being swiveled for homogenous. The tube was poured on Petri dishes and was allowed to solidify. Kirby-Bauer disc were used to assess the activity of endophytic fungi (Harmita and Radji 2004). An amount of 50 µl supernatant was dropped onto Kirby-Bauer disk. Each Petri dish with the MH medium consists of 5 paper discs. Three of the discs have the supernatant; one of them has distilled water (negative control). Incubation environment for bacteria and yeast were 37^o and 30^o C for 24 hours. The experiment was done in triplicate.

Data analysis

The percentage of colonization species endophytic samples from roots, bulbouses, stems, and leaves was calculated using Cannon and Simmons' formula (2002):

$$FK = \frac{\sum \text{organ plant colonized by fungi} \times 100\%}{\sum \text{organ plant examined}}$$

FK = Frequency of fungi colonization

Index of antimicroorganism was used in the parameter of screening. The clear zone (halo) around the paper disc was divided by the diameter of the disc. As the result is the index of antimicroorganism (Sudiana et al. 2001).

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

A total of 60 endophytic fungi specimens that consist of 12 species belongs to 9 genera were isolated from pigeon orchid from Tanah Baru Housing area, Bogor Botanical Garden, and Herbarium Bogoriense. Two genera belong to Ascomycotina and 10 species of 7 genera belongs to *mitosporic fungi* (Deuteromycotina). The endophytic fungi were identified as *Cladosporium cladosporioides* (Fres.) de Vries, 1952, *Cladosporium sphaerospermum* Penzig, 1882, *Colletotrichum gloeosporioides* (Penzig) Sacc., *Colletotrichum* sp., *Curvularia brachyspora* Boedijn, *Fusarium nivale* (Fr.) Ces., 1895, *Fusarium solani* (Mart.) Sacc., 1881, *Guignardia endophyllicola* Okane, Nakagiri, and Ito, 2001 (anamorf: *Phyllosticta capitalensis*), *Pestalotiopsis* sp., *Scolecobasidium* sp., *Westerdikella* sp., and *Xylohypha* sp.. *Guignardia and Westerdikella* as a Ascomycotina. *Cladosporium, Colletotrichum, Curvularia, Fusarium, Pestalotiopsis, Scolecobasidium* and *Xylohypha* as a *mitosporic fungi*. *Cladosporium*, *Colletotrichum*, and *Fusarium* have two species (Figure 1).

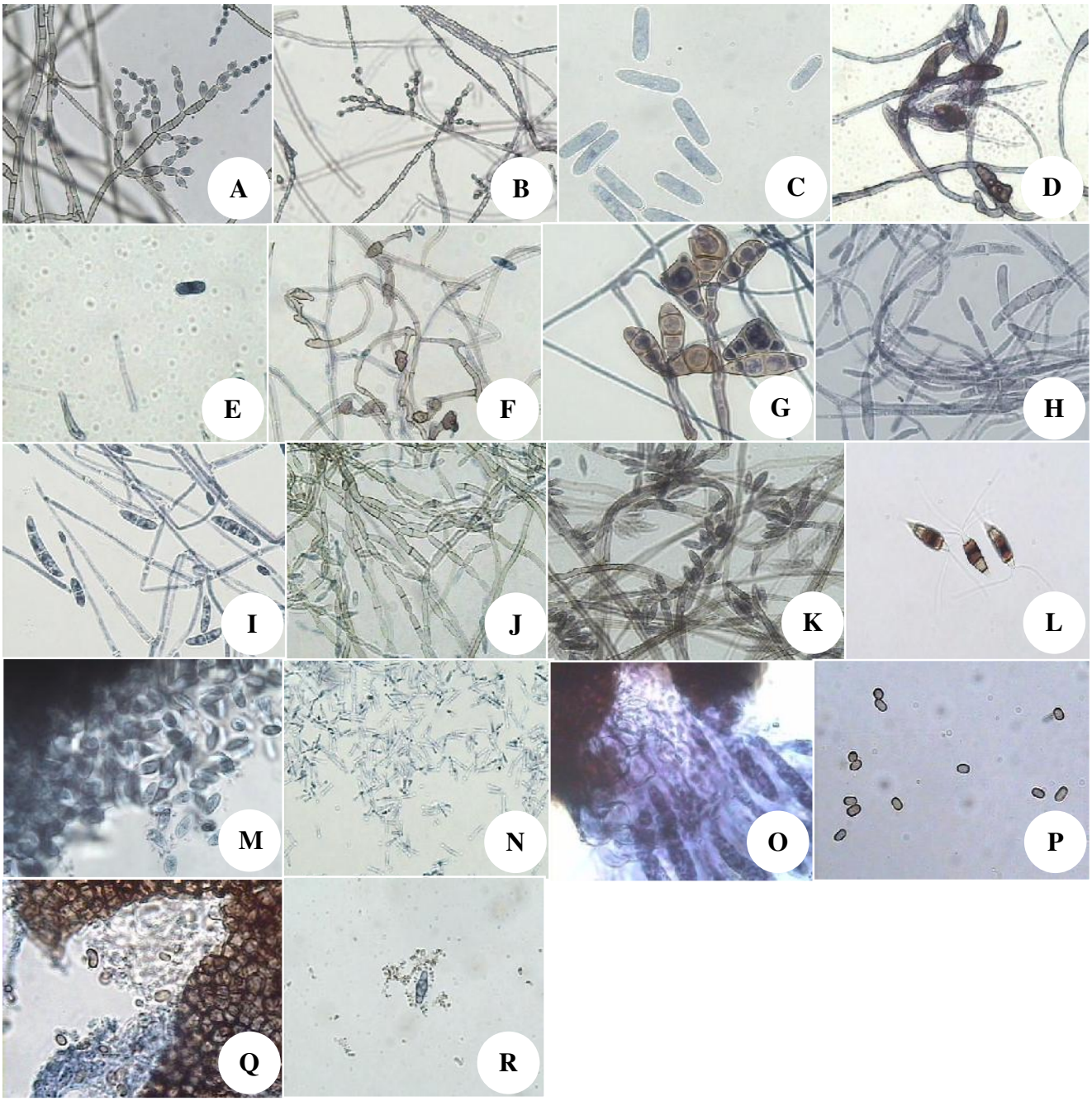

Figure 1. Microscopic character of endophytic molds isolated from *D. crumenatum.* A. conidia *Cladosporium cladosporioides*; B. conidia *Cladosporium sphaerospermum*; C & D. conidia & apresoria *Colletotrichum gloeosporioides*; E & F. conidia & apresoria *Colletotrichum* sp. G. conidia *Curvularia brachyspora*; H. conidia *Fusarium nivale*; I. conidia *Fusarium solani*; J. conidia *Pestalotiopsis* sp.; K. conidia *Scolecobasidium* sp.; L. conidia *Xylohypha* sp.; M & N. conidia & spermatia *Phyllosticta capitalensis*; O & P. ascomata, ascus & ascospora *Guignardia endophyllicola*; Q & R. ascomata & ascospora *Westerdikella* sp. (1000 X)

The fungi endophytic that was isolated from pigeon orchid belong to Ascomycotina and *mitosporic fungi*. Basidiomycotina and Oomycotina did not isolate during the research. This is because the medium used for isolation might not suitable for slow growing fungi and required some specific growth factors. With the exception of the genera *Scolecobasidium*, *Westerdikella*, and *Xylohypha*, the endophytic fungi like *Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Guignardia* (anamorf: *Phyllosticta*), and *Pestalotiopsis* are commonly found and isolated (Lodge et al. 1996; Nakagiri et al. 2005; Zhang et al. 2006). *Scolecobasidium* sp., *Westerdikella* sp., and *Xylohypha* sp. were new information that has been isolated from the pigeon orchid.

Cladosporium, *Colletotrichum*, *Curvularia*, *Fusarium*, *Guignardia* and *Pestalotiopsis* are not specific host (Petrini 1986). The fungi had been collected from various host with different plant and environment. *Cladosporium cladosporioides* isolated from *Juncus* spp. (Cabral et al. 1993), from *Mitracarpus hirtus* (L.) D.C. (Pereira and Barreto 2004), and from *Azadirachta indica* A. Juss (Neem) (Verma et al. 2005); *C. sphaerospermum* isolated

from *Livistona chinensis* Rebr. (Guo et al. 2000) and *Chromolaena odorata* (L.) King and Robinson (Prashanthi and Kulkarni 2005); *C. gloeosporioides* isolated from *Rhododendron* spp. (Okane et al. 1998), from 11 species of plants from Nusakambangan, Cilacap and 2 species of plants from Muara Angke, Jakarta (Nakagiri et al. 2005); *C. brachyspor*a isolated from *Aloe* sp., *Saccharum*, and *Triticum* (Ellis 1993); *F. solani* isolated from *Glycine max* L. and *Zea mays* L. (Domsch et al. 1980); *F. nivale* isolated from *Agrostis stolonifera* L. (Warnke 2003), *Festuca arundinacea* Schreb, *G. max*, and *Triticum aestivum* L. (Petit et al. 2003); *Pestalotiopsis* spp. Isolated from *Rhododendron* spp., and *Pieris japonica* D. Don ex G. Don (Okane et al. 1998), from *A. indica* (Mahesh et al. 2005), and *Theobroma cacao* L. (Rubini et al. 2005).

With the exception of *Guignardia*, the composition of endophytic fungi that were isolated from pigeon orchid (*Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Pestalotiopsis*, *Scolecobasidium*, *Westerdikella*, and *Xylohypha*) was different from the isolation from orchid *Dendrobium* spp. and *Lepanthes*. Genera *Aspergillus*, *Penicillium*, *Pestalotia*, *Rhizoctonia*, and *Xylaria* that were isolated from the root of orchid *Lepanthes* (Bayman et al. 1997); *Physalospora* from *Dendrobium* sp.; *Phomopsis orchidophila* Cash and Watson from the root, stem and carpel of *Dendrobium atroviolaceum* Rolfe; *Septoria selenophomoides* Cash and Watson from *D. nobile*, and *D. phalaenopsis* (Cash and Watson 1955).

Frequency of colonization of endophytic fungi

The species and the frequency of colonization of endophytic fungi isolated from various sources of pigeon orchid showing various results. The specific and dynamic environment of the habitat causing various composition and frequency of colonization on roots, bulbuos, stems and leaves of pigeon orchid. Okane et al. (1998) reported that the composition and frequency of a colonization related with the place and the host condition. Araujo et al. (2002) recorded that community of endophytic fungi depends on the interaction of microbial endophytic or any other pathogen. The existence of endophytic fungi is influenced by the variation of the season (Halmschlager et al. 1993), the environmental factors (Clay 1986) and the type of its host tissue (Rodrigues 1994).

Figure 2 showed the frequency of colonization of endophytic fungi isolated from pigeon orchid. The high colonization of *G. endophyllicola* 28 specimens (4.7%), *C. gloeosporioides* 13 specimens (2.17%) and *C. cladosporioides* 7 specimens (1.2%). The other colonization were lower (0.2-0.3%). *Cladosporium sphaerospermum* (2 specimens), *F. solani* (2 specimens), and *Xylohypha* sp. (2 specimens) (0.3%), *Colletotrichum* sp. (1 specimen), *C. brachyspora* (1 specimen), *F. nivale* (1 specimen), *Pestalotiopsis* sp. (1 specimen), *Scolecobasidium* sp. (1 specimen) and *Westerdikella* sp. (1 specimen) (0.2%).

Figure 3 illustrated the result of the frequency of endophytic fungi colonization that were isolated from the root of pigeon orchid. Six species of endophytic fungi were isolated as *C. cladosporioides* (4 specimens), *C. sphaerospermum* (1 specimen), *C. gloeosporioides* (1 specimen), *F. solani* (1 specimen), *Pestalotiopsis* sp. (1 specimen), and *Scolecobasidium* sp. (1 specimen). Frequency of colonization range between 0.7-2.7%. The highest frequency was *C. cladosporioides* (2.7%).

Five endophytic fungi were isolated as *C. cladosporioides* (1 specimen), *C. sphaerospermum* (1 specimen), *C. gloeosporioides* (10 specimens), *Colletotrichum* sp. (1 specimen), and *Xylohypha* sp. (2 specimens). They were isolated from pseudobulbus of pigeon orchid. The frequency of colonization is varying with the range between 0.7-6.7%. The highest frequency was *C. cladosporioides* (6.7%) (Figure 4).

Five endophytic fungi were isolated as *Cladosporium cladosporioides* (1 specimen), *C. gloeosporioides* (2 specimens), and *G. endophyllicola* (2 specimens), from the stem of pigeon orchid. The frequency of colonization was varying between 0.7-1.3%. The highest frequency of colonization was for *C. gloeosporioides* and *G. endophyllicola* with 1.3% for each (Figure 5).

Six of endophytic fungi were isolated as *C. cladosporioides* (1 specimen), *C. brachyspora* (1 specimen), *F. nivale* (1 specimen), *F. solani* (1 specimen), *G. endophyllicola* (26 specimens) and *Westerdikella* sp. (1 specimen). They were taken from the leaf of pigeon orchid. The frequency of colonization range was between 0.7-17.3% with the highest colonization was for *G. endophyllicola* (17.3%) (Figure 6).

As a compare, the frequency of colonization of (4.7%) *G. endophyllicola* (anamorf: *P. capitalensis*) has the highest value with the specimen that was taken from the leaves and stems of the pigeon orchid. A similar result was reported that *Phyllostictina pyriformis* (syn: *P. capitalensis*) isolated from the same part of the plant of *Dendrobium canaliculatum* Rebr., *D. phalaenopsis* Griff. ex Lindley, and *D. undulatum* Pers. (Cash and Watson 1955). The similar specimens were obtained from the orchid of *Cypripedium* sp., *A. graminifolia*, and *D. moniliforme* (Okane et al. 2003), from 64 species and 3 varieties from Kyoto Herbal Garden (Okane et al. 2003), *Rhododendron* spp., from 7 species in Muara Angke, Jakarta, and 12 species in Nusakambangan, Cilacap (Nakagiri et al. 2005).

Screening of endophytic fungi that produce antimicrobial activity

Only one specimen, which was *F. nivale* (1.7%) out from 60 specimens of endophytic fungi were showing the inhibiting growth of *C. albicans* and *C. tropicalis*. Index of antimicroorganism was *C. albicans* (1.7 ± 0.02) and *C. tropicalis* (1.3 ± 0.06).

Filtration of *F. nivale* showed potential antibiosis activity for human pathogen. The microbial effect of the fungus *F. nivale* during the attachment with the host plant or during saprophytic produced mycotoxin of nivalenol and fusarenon-x (Ueno et al. 1973), and deoxynivalenol (DON, vomitoxin) (Logrieco et al. 1991). Strobel and Daisy (2003) informed that *Fusarium* sp. that was isolated from *Selaginella pallescens* (Presl.) Spring produced pentaketide that gave antiyeast effect against *C. albicans*.

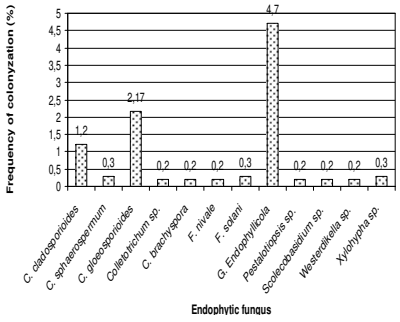

Figure 2. Frequency colonization of endophytic fungi of *D. crumenatum*.

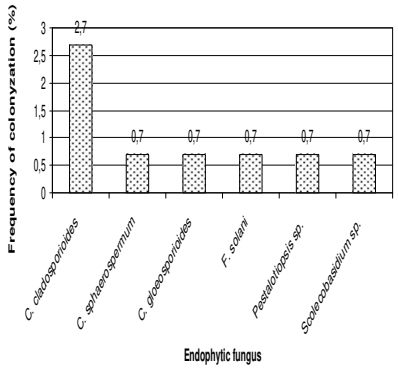

Figure 3. Frequency colonization of fungi endophytic from root *D. crumenatum*.

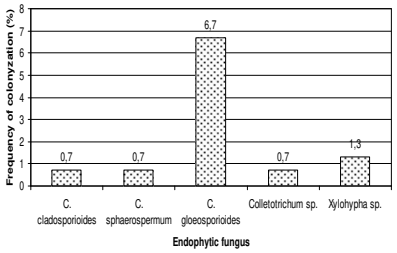

Figure 4. Frequency of colonization of endophytic fungi from pseudobulbus of *D. crumenatum*.

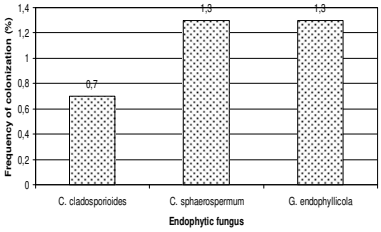

Figure 5. Frequency colonization of fungi endophytic from stem *D. crumenatum*.

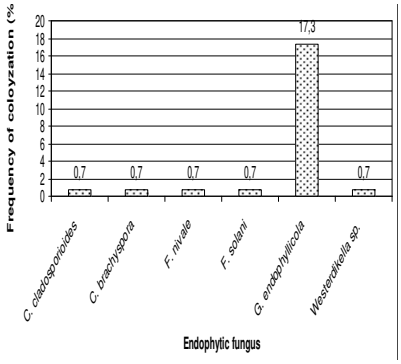

Figure 6. Frequency colonization of fungi endophytic from leaf *D. crumenatum*

Inability of the other fungi to inhibit the growth of *B. subtilis*, *C. albicans*, *C. tropicalis*, *E. coli*, and *S. aureus*, might be caused by the fact that the fungi did not produce antimicrobial secondary metabolites against bacteria or yeast. And also, it might be caused by the very low concentrations of the secondary metabolites (50µl/disc). The other possibility is that the endophytic fungi have secondary metabolites that give different function such as, anticancer, antimalaria, antioxidant, and precursor. *Pestalotiopsis* spp. isolated from *Taxus wallichiana* Zucc., produced taxol that has the ability as antitumor (Mahesh et al. 2005), *Pestalotiopsis* sp. associated with *Torreya taxifolia* Arnot, produced ambuic acid as antifungi (Zhang et al. 2006). *Pestalotiopsis microspora* (Speg.) Batista produced pestacine and isopestacine as antifungi and antioxidant (Zhang et al. 2006). *Colletotrichum* sp. was isolated from plant *Artemisia annua* L., that produced secondary metabolite artemisine which has a potential for antimalaria (Strobel and Daisy 2003), antibacteria to Gram-positive *B. subtilis*, *S. aureus*, and *Sarcina lutea*, Gram-negative *Pseudomonas* sp., and antifungi to *Phytophthora capsici* Lionian, *Rhizoctonia cerealis* Van Der Hoeven, *Gaeumannomyces graminis* var. *tritici*, and *Helminthosporium sativum* Pammel, King and Bakke (Tan and Zou 2001). *Phyllosticta* sp. (teleomorf: *Guignardia*) isolated from plant *Abies balsamea* Miller, produced heptelidic acid and hydroheptelidic acid that has a toxic effect for larvae *Choristoneura fumiferana* (Tan and Zou 2001). *Guignardia* sp. associated with *Spondias mombin* L., produced precursor guignardic acid (Zhang et al. 2006). Alkaloid asperfumoid, aspernigrine A, and aspernigerine isolated from *Cladosporium herbarum* (Pers.) Link ex S.O. Gray interaction with *Cynodon dactylon* K. Nov. which are able to inhibit *C. albicans* and cancer cell (Zhang et al. 2006).

CONCLUSION

An amount 60 specimens consist of 12 species endophytic fungi have been isolated from root, bulbous, stems, and leaves of pigeon orchid. Endophytic fungi isolated from roots were 6 species, bulbous 5 species, stems 3 species, and leaves 6 species. The genus isolated from endophytic fungi were *Cladosporium cladosporioides*, *Cladosporium sphaerospermum*, *Colletotrichum gloeosporioides*, *Colletotrichum* sp., *Curvularia brachyspora*, *Fusarium nivale*, *Fusarium solani*, *Guignardia endophyllicola* (anamorf: *Phyllosticta capitalensis*), *Pestalotiopsis* sp., *Scolecobasidium* sp., *Westerdikella* sp., and *Xylohypha* sp. The dominant endophytic fungi were *Guignardia endophyllicola*. *Fusarium nivale* was able to inhibit the growth of *Candida albicans* and *Candida tropicalis* with antimicrobial index of 1.7 ± 0.02 and 1.3 ± 0.06. However, others specimens did not inhibit the tested bacterial, which are Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*.

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