Isolation of Endophytic Microbes from Gunung Halimun National Park, West Java, Indonesia, and Bioassay their Potency for Eradicating Microbial Crops Pathogen

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Abstract

Gunung Halimun National Park (GHNP) –West Java, Indonesia is the largest preserved primary forest in West Java. Diversity of plants and animals of this park have been studied intensively during the last 15 years. Diversity of endophytic microorganisms, however, has never been reported. Endophytic microbes are those who reside in the interspatial tissues of plants, having a capacity to produce array of secondary metabolites. This paper illustrates the occurrence of endophyte microbes in diverse flowering plants of GHNP. Total of 160 bacteria and 337 fungi were isolated from 86 plants species in the area. Out of that, 159 bacterial isolates and 320 fungi isolates survived in our collection and tested against four major microbial crops pathogen namely *Xanthomonas campestris, Pseudomonas solanacearum, Colletotrichum glocosporoides* and *Fusarium oxysporum cubense*. Plate Agar Test Assay method reveals that 51 among 159 bacterial isolates and 62 among 320 endophytic fungal isolates have the ability to inhibit the growth of microbial crop pathogens. Endophytic bacteria can inhibit more microbial crops pathogen but the inhibition ability is less compared to that of endophytic fungi. Our preliminary study clearly shows that endophytic microbes of GHNP should have potential value in developing biological control agent to combat microbial crop pathogens and eventually reduce the use of synthetic chemicals.

Keyword: Gunung Halimun National Park, West Java, endophytic microbes, microbial crops pathogen

Introduction

Covering 40,000-hectare width, the Gunung Halimun National Park (GHNP) [106°2' to 106°38'E and 6°37' to 6°51'S], which was formally established in 1997, is the largest preserved primary forests in West Java, Indonesia. The park has a steep and much dissected topology, with the highest peaks, in the north part, reaches 1,929 m. The rainfall varies between 4,000 and 6,000 annually, with a distinct dry season occurring from May/June to September when the precipitation decreases to around 200 mm per month on average. The average daily temperature is 20–30°C. The dominant vegetation consists of lowland dipterocarp forest from 500–900(-1,000) m, the sub-montane transition forest from 1,000–1,500 m, and the montane lauraceous-ericaceous forest at attitudes above 1,500 m (Harada *et al*., 2003 and Tan *et al*., 2006).

Diversity of flora and fauna of GHNP has been intensively studied during the last 15 years (Tan *et al*., 2006; Ario, 2007; Mirmanto & Simbolon, 1998; Prawiradilaga & Marakarmah, 2004). The study has been intensified through cooperation between Research Center for Biology of the Indonesian Institute of Sciences (LIPI), Forest Research Agency of the Department of Forestry of the Republic of Indonesia and Japanese Scientist under the auspice of JICA Biodiversity Conservation Project. Study on microbial diversity of GHNP, however very limited. Sudiana & Rahmansyah (2002) briefly illustrates the species and functional diversity of soil microflora of GHNP. Distribution of soil fungi including arbuscular-mycorrhizal fungi and densities of soil bacteria including phosphate solubilizing and nitrogen fixing bacteria were noted by the authors. Meanwhile, endophytic microbe of GHNP remains unexplored and has never been reported.

Endophytic microbes appear to have capacity to produce an array of secondary metabolites exhibiting a variety of biological activity (Suryanarayanan *et al*., 2009). There are some evidence that endophytes

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may protect plants against diseases (Arnold *et al*., 2003), become ward of insect pests (Akello *et al*., 2007), and improve the fitness of plants. Plant becomes more tolerance to abiotic stress (Redman *et al*., 2002; Bae *et al*., 2008). Endophytes may be a source of natural products of pharmaceutical and agricultural importance (Tan & Zou, 2001; Gunatilaka, 2006).

Endophytes fungi are more innovative metabolically than soil fungi (Schluz *et al*., 2002). They produce unique bioactive metabolites (Mitchell *et al*., 2008; Stadler & Keller, 2008). Innovative and unique characters of bioactive metabolites produced by endophytes may be due to its constant interaction with the host milieu (Suryanarayanan *et al*., 2009). Endophytes-plant host interactions are different from pathogen-plant host interactions. The presence of endophytes does not create disease symptoms on the plant host, whereas the elimination of endophytes by the plant host does not occur (Pinto *et al*., 2000; Schulz *et a*l., 2002; Stone *et al*., 2004; Sieber, 2007; Saikkonen, 2007). Symbiotic interaction between endophytes and plant host sustained and prolonged reactions which could act as selection pressure for the developing of novel metabolites (Calhoun *et al*., 1992; Schulz *et al*., 1995; Lu *et al*., 2000; Wang *et al*., 2000; Tan & Zou, 2001; Weber *et al*., 2007; Suryanarayanan *et al*., 2009). Endophytes could therefore produce an array of metabolites of varied structural groups such as terpenoids, steroids, xanthones, chinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasines and enniatines (Schulz *et al*., 2002). The metabolites of endophytes include antibacterial, antiviral, and antifungal (Gunatilaka, 2006).

The target in our study would be to search endophytes having the ability to produce metabolites for preventing the growth of *Xanthomonas campestris*, *Pseudomonas solanacearum* and *Fusarium oxysporium-cubense* growth. Those three species of microbes create a huge damage in crops industry in Indonesia. The research is also dedicated to find endophytes producing active metabolite to halt the growth of *Colletotrichum glocosporoides*. *C. glocosporoides* creating problem on fruit industry (Hamdayanty *et al*., 2012; Bolkan *et al*., 1976; Dickman *et al*., 1982; Dickman & Alvarez, 1983; Hunter & Buddenhagen, 1972; Trujillo & Obrero, 1969). This microbe attacks fruit crops during storage and handling. Loss in export of fruit commodities is tremendous. Wastie (1972) reports several factors affecting the production, germination, and viability of spores of *C. gloeosporioides* related to the secondary leaf fall of *Hevea brasiliensis*.

Materials and Methods

Isolation of endophytes. A portion of plant sample from GHNP was washed using clean water. A branch of the sample plant, or leaf, flower, fruit was cut to the size of 1 cm. It was then sterilized through the step as following: submerge it onto 75% (v/v) ethanol for 1 minute and soak it into sodium hypochlorite 5.3 % (w/v) for 5 minutes. Sample again was transferred into 75% (v/v) ethanol for 0.5 minute. Sample was longitudinally cut so that the inner part plant sample was exposed. Each portion of plant sample was aseptically put on Nutrient Agar (NA) and Potato Dextrose Agar (PDA), and then incubated at 30°C for 2 to 5 days until the colonies could be visually observed (Tomita, 2003). Those colonies (bacteria or fungi) are transferred into fresh sterile medium. Purification of those microbial isolates were to be continued to get pure isolate for further study.

Purification and maintenance of endophytes. Purification of bacterial or fungal isolates was carried out by transferring those isolates into fresh sterile medium. NA was used for purifying bacterial isolates and PDA for purifying fungal isolates. For bacterial isolates, microscopic observation and Gram stained was applied. Purified culture was transferred into slant NA/PDA, incubated for 2 to 3 days and stored at 4°C for maintenance. The pure culture was then stored in a 10% (v/v) of glycerol at -20°C. The culture was also stored using lyophilization techniques at 4°C.

Plate agar assay. Plate agar assay techniques were adopted from Son & Kqueen (2002) to determine the ability of endophytes in preventing the growth of microbial crop pathogens (*X. campestris*, *P. solanacearum*, *F oxysporium-cubense*, and *C. glocosporoides*). Microbial crop pathogens were cultivated on their growth medium and suspended on sterile aquadest. The amount of 1 mL of cell suspension was poured onto melted NA/PDA medium with the temperature of 35-40°C on petri dish. The culture was gently shaken to evenly distribute the microbial cells. On the top of the culture plate, a sterile punch of filter paper disk was put. The amount of 10 µL of endophyte suspension was dropped on filter paper disk. The culture plate was then incubated at 30°C for 1 to 3 days. Inhibition zones outside the paper filter disk were observed daily and measured.

Results and Discussion

This study that was conducted between 2003 and 2005 showed that diverse plant species of GHNP are good sources of isolation of endophytic microbes. During the course of our study, the total number of 497 isolates (160 bacteria and 337 fungi) was successfully isolated from 86 plant species. Based on visual observation of colony form on agar plate, the diversity of endophytic bacteria and fungi isolated from 86 different species of flowering plants of GNHP was obvious. The occurrence of the endophytic fungi was more dominant (68%) compared to that of endophytic bacteria which was only 32%.

The occurrence of bacteria and fungi are varied depending on plant species. As depicted in Figure 1, we successfully isolated fungi from all plant species studied, except two plant species namely *Lophatherium gracile* and *Calamus javanensis*. The number of fungus isolated from each species of plant varied greatly between 1 and 7. The most common number of fungi isolated is between 3 and 5 isolates. There are 7 species of plants namely *Gironniera subaeqalis*, *Lasianthus laevigatus*, *Prunus arborea*, *Sarcanda glabra*, *Melanochyla caesia*, *Paphniphyllum glaucescens* and *Thea lanceolata* from which we could isolate 7 different fungal isolates.

On the contrary, there are more plant species of GNHP from which we could not be able to isolate bacterial endophytes. Those plant species are *Ficus hispida*, *Zyzigium* sp., *L. laevigatus*, *Syzygium lineatum*, *Engelhardia spicata*, *Saurauia pendula*, *M. caesia*, *Polyosma ilicifolra*, *Glochidion arborescens*, *Melastoma speciosa*, *Rhodamnia cinerea*, *T. lanceolata*, *Garcinia rostrata*, *Pinanga coronate*, and *Weinmannia* sp. The reason for this not clear. More works need to be done. Sample size and isolation procedure may need to be considered. The number of bacteria isolated from each species of plant is slightly low compared to that of fungi. It varied between 0 and 6. The most common number of bacteria isolated is between 2 and 3 isolates. There are only 3 species of plants namely *Alpinia scraba*, *Forrestia mollissima* and *Calamus javanensis* from which we could isolate 6 different bacterial isolates.

Out of 337 fungal endophytes, 320 isolates survived during maintenance in the laboratory, while out of 160 bacterial endophytes, 159 isolates survived during maintenance in the laboratory. All survival microbes were tested against 4 microbial plant pathogens, *X. campestris*, *P. solanacearum*, *C. glocosporoides* and *F. oxysporum cubense*. For this purpose, agar plate assay was used. The total of 258 fungal isolates (81%) could not inhibit and only 62 (19%) could inhibit the growth of microbial plant pathogen. Meanwhile, among 159 bacterial isolates, 108 isolates (68%) could not inhibit and 51 (32%) could inhibit the growth of microbial plant pathogen.

As shown in Figure 2, the inhibition ability (indicated by clearing zones in mm) of the 51 endophytic bacteria of GHNP on 4 microbial crops pathogen was weak. The inhibition zone commonly was not more than 1 mm. Only 3 isolates (HL38B.83, HL39B.88, and HL68B.140) showed better inhibition ability with the inhibition zone to 2 mm on *X. campestris* or *C. glocosporoides*. Three isolates (HL11B.16, HL12B.19, and HL39B.88) had wide spectrum, their inbition zone could be detected on *X. campestris*, *P. solanacearum*, and *C. glocosporoides*. Although the inhibition zone was weak, HL108B.14, HL31B.65, HL32.B.72, HL42.B.95, HL.50B.106, and HL64B.131 could inbhibit *F. oxysporum-cubense*.

In contrast, as depicted in Figure 3, the inhibition ability of fungal endophytes were stronger compared to that of shown by bacterial endophytes. Clearing zone of some fungal isolates could be until 4 mm. Strains of HL11F.44 and HL23F.107 showed their superiority against *C. glocosporoides*, while HL85F.408 and HL110F.485 were superior against *X. campestris* and HL102F.462 was potential for developing bioactive agent against *P. solanacearum*. Four isolates (HL4F.15, HL9F.36, HL11F.41, and HL45F.207) could inhibit *F. oxysporum cubense*, but the inhibition ability was weak.

HL11F.44 and HL23F.106 which showed their superiority against *C. glocosporoides*, should had great potential value in fruit industry. One of the most important issues in fruit industry is that fruit is naturally perishable. The fruit can easily be attacked by *C. gloeosporioides* which eventually is the major handicaps in fruit industry especially in papaya industry if it is marketed overseas (Harianingsih. 2010). *C. gloeosporioides* is a fungus pathogen to a wide variety of hosts, particularly papaya. This fungus is widely distributed or cosmopolitan. The disease caused by this fungus is regularly seen in the field on ripe or overripe fruits. It is not a serious problem for unrefrigerated fruit sold in the local markets, but it can cause disadvantage on fruits refrigerated and exported to overseas markets. *C. gloeosporioides* is the major cause on papaya anthracnose. The first symptoms of papaya anthracnose are round, waters-oaked, sunken spots on the body of the ripening fruit. Lesions may become as large as 5 cm in diameter (Dickman *et al*, 1982). The pathogen initially infects intact, non-wounded immature green fruit in the field. Spores germinate and form appressoria on the fruit surface. The fungus, using its appressorium, enzymatically penetrates the cuticle and then remains as sub-cuticular hyphae until the post climacteric stage of fruit growth is attained. At this point, for reasons that are not understood, the fungus resumes growth and causes the characteristic symptoms. Thus, papaya anthracnose has a latent stage in its development that is similar to many other anthracnose diseases of tropical fruits (Dickman *et al*, 1982). *C. gloeosporioides* is an agent disease to fruits and papaya industry in Indonesia. Papaya is an important tropical fruit commodity with high economic value and highly nutritious. Production of papaya between 2008 and 2010 had been greatly fluctuated due to *C. gloeosporioides* infection. Anthracnosa cause by *C. gloeosporioides* could reach to 70% and reported to be the most significant after harvest loss of papaya. Finding metabolites which is effective to prevent the growth of *C. gloeosporioides* should be of alternative advantages replacing fungicides to effectively reducing anthracnose. This could eventually be a common practice especially for fruits shipped to overseas markets.

Bacterial spot caused by *X. campestris* is one of the most serious diseases in many part of the world (El-Hendawi *et al*., 2005). The disease affects stems, leaves and fruits (Agrios, 1997) and causes significant losses when environmental conditions are suitable for the pathogen (Pohronezny & Volin, 1983). Different strategies have been employed for controlling the disease, including sanitation, the use of pathogen-free seed and other cultural practices (Goode & Sasser, 1980; Sherf & MacNab, 1986; Jones *et al*., 1991), not to mention the use of cultivars resistant to *X. campestris*. Chemical control by using copper and streptomycin sprays have also been used (Jones and Jones, 1985). Disadvantages of chemical applications such as potential chemical residues on fruit as well as cost and development of resistant bacterial strains have been reported (Stall & Thayer, 1962; Marco & Stall, 1983; Jones & Jones, 1985; Stall *et al*., 1986; Ritchie & Dittapongitch, 1991). Endophytic fungi namely HL85F.408 and HL110F.485 which showed superiority against *X. campestris* (Figure 4) would be good candidates for the development of metabolites against this disease.

P. solanacearum is an important soil-borne bacterial plant pathogen which causes bacterial wilt, a widely distributed plant disease in tropical, subtropical and warm temperate regions of the world (Hayward, 1991). This pathogen has an unusually wide host range of 200 plant species belonging to more than 50 families (Hayward, 2000). Some of its economically important hosts include tomato, pepper, potato, tobacco, banana, eggplant, cowpea, peanut, cashew, papaya and olive (Guo *et al*., 2004). Bacterial wilt is reported to be among the top five diseases (Elphinstone, 2005). Management of bacterial wilt in eggplant and in other crops has been difficult due to the diversity in *P. solanacearum* strains, their ability to survive in adverse soil conditions, worldwide distribution, varied hosts including asymptomatic hosts and efficient mechanism of invading host. Various strategies, including resistant varieties (Dalal *et al*., 1999), soil amendments (Islam & Toyota, 2004), soil solarization (Kumar & Sood, 2001), the use of bio-fumigants (Pradhanang *et al*., 2003), transgenic resistant plant (Jia *et al*., 1999), plant growth promoting rhizobacteria (Guo *et al*., 2004), and the use of SAR inducers (Anith *et al*., 2004) had been developed with limited success in the bacterial wilt management. Biological control has been accepted and emerged as one of the important methods in the management of soil-borne plant pathogens. Laboratory bioassay to detect the antagonistic activity of 48 endophytic bacteria against *P. solanacearum* has been carried out by Ramesh & Phadke (2012). Their study indicated that most of the potential antagonists from endophytic tissue are *Pseudomonas* sp. In recent years, endophytic bacteria are reported as potential biological control agents in plant disease management. Endophytic *Bacillus* sp. (Algam *et al*., 2004; Li *et al*., 2006) and endophytic *Pseudomonas* sp. (Rahman & Khan, 2002; Long *et al*., 2004) are major bacterial group inhibitory to *P. solanacearum* in tomato and other crops. In our study, that antagonistic ability of bacterial endophytes was weak. We found that endophytic fungi, HL102F.462 did have remarkable antagonistic ability and should be potential for developing bioactive agent against *P. solanacearum*.

Banana (*Musa* sp.) is considered as the fourth most widely consumed food crop in the world after rice, wheat and corn. Main obstacle affecting banana cultivation is *Fusarium* wilt caused by *F. oxysporum* f. sp. *cubense*. This disease is considered one of the most important threats in Asia, Africa, Australia and tropical America (Hwang & Ko, 2004). Various control measures have been practiced to manage this disease, including destruction of diseased plants, sanitary measures, the use of disease-free tissue culture planting material, the use of tolerant variety and other integrated management methods (Akila *et al*., 2011). Chemicals are also widely utilized for the management of this disease. However, indiscriminate use of chemicals is known to cause health hazards to human beings besides warranting repeated application (Akila *et al*., 2011). As an alternative approach, biocontrol agents are being used for the management of various diseases (Kavino *et al*., 2008; Harish *et al*., 2009). Botanicals with antifungal compounds have been identified and these can be exploited for the management of diseases (Kagale *et al*., 2004). It is the need of the day to find an alternative approach for the management of Fusarium wilt. Our study showed that 4 endophytic fungi namely HL4F.15, HL9F.36, HL11F.41, and HL45F.207 have demonstrated their ability to inhibit the growth of *F. oxysporum-cubense*. The inhibition ability, however was relatively weak. Search for new endophytic microbes with better inhibition ability against *F. oxysporum cubense* therefore is challenging.

Conclusions

This study indicated that the number of endophytes, both bacteria and fungi isolated from diverse flowering plant species of GHNP are a good source of active metabolites to inhibit the growth of plant pathogenic diseases such as *X. campestris*, *P. solanacearum*, *F. oxysporium-cubense* and *C. glocosporoides*. Endophytic bacteria have an ability to inhibit more crops pathogenic microbes compared to that of endopytic fungi. But, the inhibition ability against plant pathogenic diseases generally lower compared to that of produced by endophytic fungi. Endophytic bacteria strain HL38B.83 and HL68B.140 have inhibition ability against *X. campestris*, while HL39B.88 could inhibit *C. glocosporoides* with the inhibition zone to 2 mm. Meanwhile, endophytic fungi strain HL85F.408 and HL110F.485 have inhibition ability against *X. campestris*, whereas HL11F.44 and HL23F.107 inhibit *C. glocosporoides* with the inhibition zone to 4 mm. In addition, strain of HL102F.462 could inhibit *P. solanacearum* with the same inhibition zone (4 mm). Microbes isolated from flowering plant species of GHNP do have potential for developing active metabolites as biocontrol agents to be used in the future agricultural practices.

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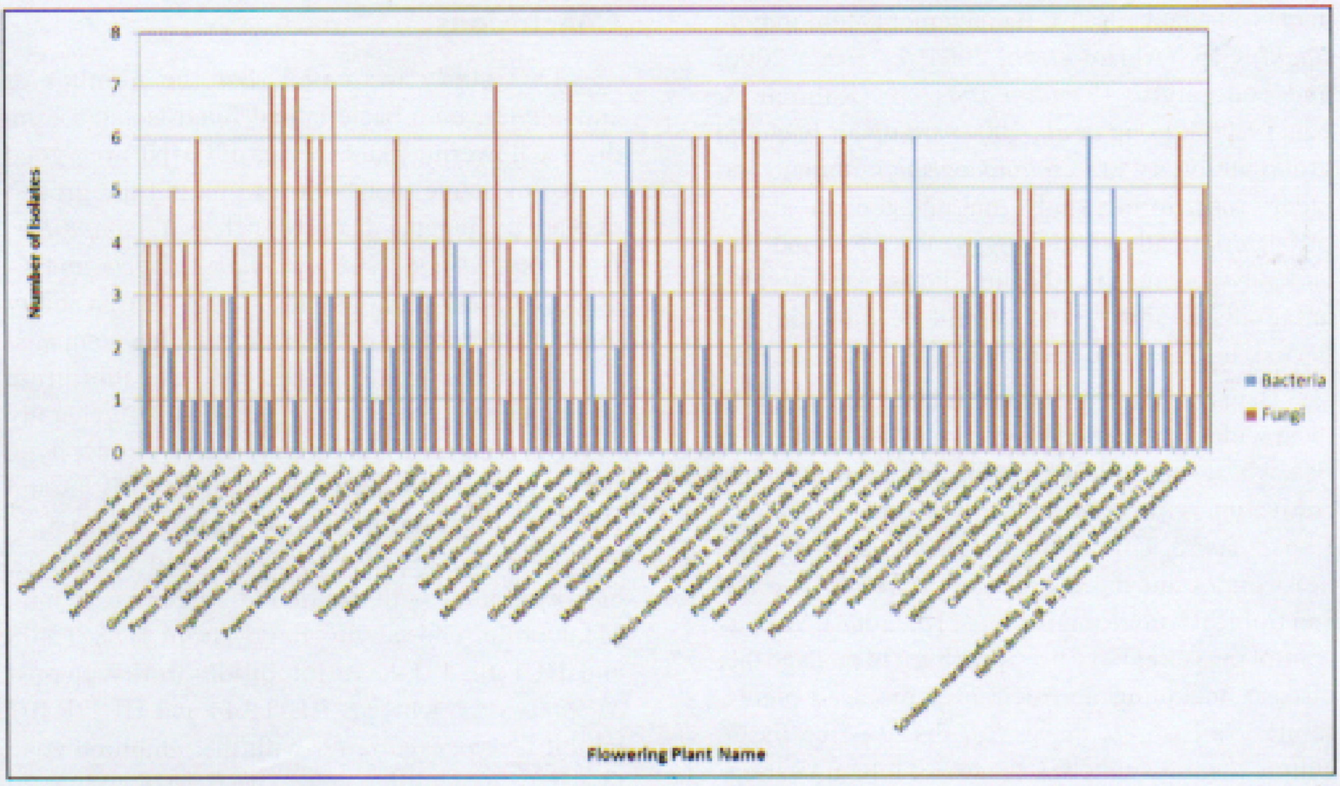

Figure 1. The occurrence of endophytic bacteria and fungi in 86 different flowering plants species of Gunung Halimun National Park (GHNP), West Java – Indonesia.

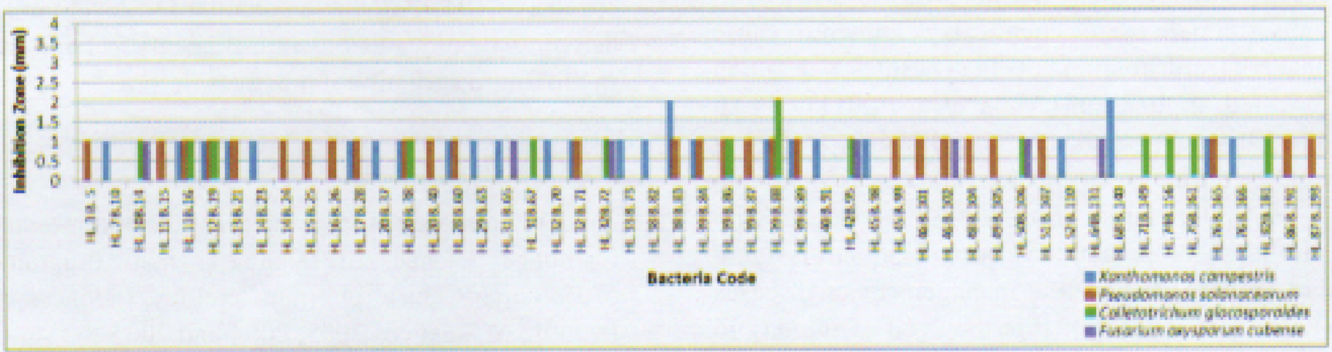

Figure 2. Inhibition profile (indicated by clearing zones on agar plate assay in mm) of 51 endophytic bacteria of Gunung Halimun National Park (GHNP), West Java - Indonesia on 4 microbial crops pathogen namely *X. campestris*, *P. solanacearum*, *C. glocosporoides* and *F. oxysporum cubense.*

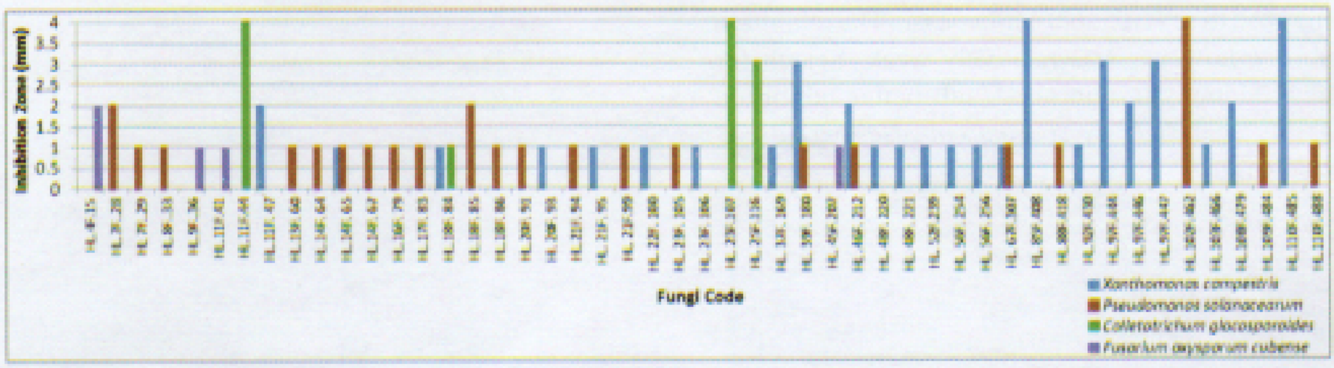

Figure 3. Inhibition profile (indicated by clearing zones on agar plate assay in mm) of 49 endophytic fungi of Gunung Halimun National Park (GHNP), West Java - Indonesia against 4 microbial crops pathogen namely *X. campestris*, *P. solanacearum*, *C. glocosporoides*, and *F. oxysporum cubense.*

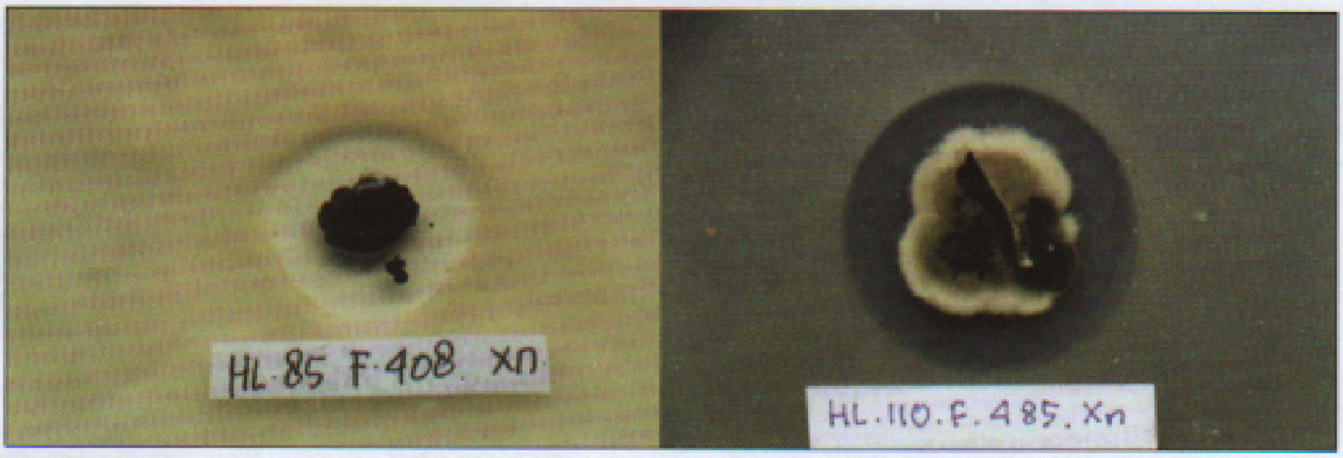

Figure 4. Agar plate assay of two fungal endophytic strain, HL85F.408 and HL110F.485, showing their superioriority against *X. campestris*.

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