



THE EFFECT OF SUPPRESSION OF ENDOPHYTIC MANGROVE BACTERIA ON LEAF BLIGHT OF RICE CAUSED BY *Xanthomonas oryzae* pv. *Oryzae*

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Abstract

The bacteria isolated from mangrove plants are largely unexplored source for biocontrol agents with the potential to produce biologically active secondary metabolism. Therefore endophytic bacteria for biocontrol agent of bacterial blight (BB) caused by *Xanthomonas oryzae* isolated from *Avicenia alba*, *A. marina* and *Bruguiera gymnorhiza*, were explored. Fifty five bacteria strains were isolated from leaves, stems, flowers, fruits, and stalks of the mangrove plants in Bali and Manado, Indonesia. Seven out of the 55 strains, were antibiosis to the growth of *Rhizoctonia solani*, five strains were antibiosis to the growth of *Fusarium oxysporum*, while the two others were antibiosis to *X. oryzae*. None of them was antibiosis to *Pythium aphanidermatum* and *P. ultimum*. Eleven consortia of biocontrol agents (BCAs) which consist five of them were tested for suppression of rice BB. Sodium chloride 0.7% was added into the soil in order to know the ability of the BCAs consortia on suppression of BB in saline soil. The objective of this experiment was to screen the consortia of endophytic bacteria isolated from mangrove plants for biocontrol agents of bacterial blight caused by *X. oryzae* in saline soil. The results showed that BCAs03 reduced the highest BB by 67%, and the lowest was BCAs11 by 22%. FDA hydrolytic in soil on day 29 th, increased by the application of BCAs, except by BCAs11. All of BCAs tested (BCAs01 to BCAs11) were able to increase grain weight.

Key Words: biocontrol agent, *Xanthomonas oryzae*, bacterial endophytic mangrove, FDA hydrolytic, *Oryza sativa*, *Avicenia alba*, *A. marina*, *Bruguiera gymnorhiza*

Introduction

Mangroves are salt tolerance plants existing at the interface between land and sea in the tropical and subtropical latitudes. The mangrove forest is one among the world's most productive ecosystem with great ecological and economic significance. Mangroves, unique woody plant communities of intertidal coasts in tropical and subtropical coastal regions, are high productive ecosystems (Costanza *et al.*, 1997; Wang *et al.*, 2003), though surprisingly little is known about the microbial communities living there in (Hong and Yan 2008 ;Yan *et al.*, 2006).

Microbes that colonize living, internal tissue of plants without causing any immediate, over negative effects are termed as endophytes (Bacon and White, 2000). It is noteworthy that, of nearly 300,000 plants species that exist on the earth, each individual is the host to endophytes (Strobel and Daisy, 2003). It is estimated that there may be as many as 1 million different endophyte species; however, only handful of them are described (Guo *et al.*, 2008). Endophytic bacteria are important source for developing the novel drugs for effective treatment diseases in humans, plants and animals (Strobel *et al.*, 2004).

The United Nations General Assembly (UNGA) declared the year of 2004 as the International Year of Rice (IYR) with the slogan "Rice is Life". On global basis, UNGA declaration underlines the significance of rice (*Oryza sativa* L.) as staple crop for human consumption and accredited as primary food source for more than half of the world's population. In developing countries, rice accounts for 715 Kcal/capita/day, which involves 27% of dietary energy supply, 20% of dietary protein, and 3% of dietary fat (FAO, 2004). Bacterial blight (BB) caused by *X. oryzae* pv. *Oryzae* (Xoo), is one of the most important bacterial diseases of rice. The disease was first observed by the farmers of Japan in 1884 (Tagami and Mizukami, 1962). Subsequently, its incidence has been reported from different parts of Asia, northern Australia, Africa and USA. The disease is known to occur in epidemic proportions in many parts of the world, incurring severe crop loss of up to 50%. Crop loss assessment studies have revealed that this disease reduces grain yield to varying levels, depending on the stage of the crop, degree of the cultivar susceptibility and to a great extent, the conduciveness of the environment in which it occurs (Gnanamanickam *et al.*, 1999). Rice bacterial blight is vascular disease, the bacterium pathogen enter the epitheme, the tissue connecting the water pores with the xylem, where they multiply and move to the xylem vessels. Once in the vascular system, the pathogen continues to grow until the xylem vessels are clogged with the cell of bacterial pathogen and extracellular polysaccharide. Rice plants become wilted if infection occurs in latter stages, lessions of leaf blight enlarge in lenght and width and turn gradually from grayish green to chlorotic. During the infection the pathogen employs diverse tools to overcome the host innate defense system resulting in blight disease (Mew, 1993).

Leaf blight can be controlled by chemicals and resistant cultivar. Chemical pesticides are generally considered harmful to the environment, and sometimes the use of resistant cultivar caused problems such as host plant resistance which often based on a single gene, may not be durable in the field due to the frequent of the interference of resistancy. So the new methods to complement management strategy on bacterial leaf blight to achieve a better disease control are needed. Application of biocontrol agents is an important strategy in protection of plants from disease. Biocontrol of leaf blight has been tried by using bacteria such as *Lysobacter antibioticus* strain 13-1 (Ji *et al.*, 2008), *Bacillus licheniformis* BC98 (Tendulkar *et al.*, 2007), and *Pseudomonas fluorescens* strain pf7-14 (Krishnamurthy and Gnanamanickam, 1998).

Land area of rice field in Indonesia was 7,885,534 ha, and five percent was tidal rice field (BPS,2006). Rice production in tidal area were low and the rice field were saline. Salinity has got several problems for growth and development of plants by inducing physiological stresses (Munns, 2002; Saied *et al.*,2005). It also owns problems for microbial diversity and metabolic activities (Oren, 2002;Jiang *et al.*, 2007). For example, the abundance, composition, diversity, and metabolic functions of microbial communities are lower in saline and hypersaline terrestrial environments (Jiang *et al.*, 2007).

The purpose of this study was to obtain potentially consortia of endophytic bacteria as biocontrol agent of leaf blight of rice caused by *Xanthomonas oryzae* in saline soil.

Materials and Methods

Isolation of endophytic bacteria

Various of healthy mangrove plants in Bali and Manado, North Sulawesi, Indonesia, were collected, and kept in ice box and brought to laboratory. Various parts of them such as leaves, stalks, stems, flowers, and fruits were washed in running tap water and washing in 70% ethanol for 1-2 minutes and 5% sodium hypochlorite for 2 minutes, followed by three rinses with sterile distilled water. After removing extra water on sterile tissue paper, the parts of the plants were dried for 3-4 hours in a clean bench. After drying, cut them into pieces about 5-10 mm, and about six of plant pieces were put into ½ strength nutrient agar containing 1% NaCl, and supplemented with 100 µg mL⁻¹ of cyclohexamide. Plates were incubated at room temperature for about 10-14 days. Each colony that grew on the NA plates was purified by repeated transfers to new NA.

Antibiosis test

An antifungal test of the strains

Each of the fungal pathogen test (*F. oxysporum*, *P. aphanidermatum*, *P. ultimum*, and *R. solani*) was inoculated onto the center of PDA plate, three holes (diameter 9mm) were made using cork borer (the position of the holes were at the same distance from the center of the medium, where the fungal pathogens were placed). Hundred µl of a 7-day culture of bacterial strains in NO.3S medium (30 g polypepton S, 10 g glucose, 1 g KH₂PO₄, and 0.5 g MgSO₄·7H₂O, 1 L distilled water) was put into each hole. For the negative control distilled water was used. The plates were incubated for 3-5 days and growth inhibition were observed.

An antibacterial test of the strains

One loop of a bacterial pathogen (*X. oryzae*) colony was suspended into a sterile distilled water and hundred µl of the pathogen suspension was spread onto YDCA medium (Yeast Dextrose Carbonate Agar, 10 g yeast extract, 20 g dextrose, 20 g CaCO₃, 1L distilled water) plate by a sterile glass rod. Then endophytic bacterial strains were spotted on the plate and incubated at room temperature for 2-3 days. Antibacterial activity was judged by the clear zone formation around the colonies of the endophytic strains.

Germination of rice seeds (greenhouse experiment)

The sorting of pithy seed (varieties Ciherang) was performed by keeping the rice seeds into a bucket containing salt water. The seeds sink were collected, washed and soaked using with tap water for about 24-48 hours. Then the seed were dried and germinated in plastic tray covered with banana leaves in under side. The mixture of soil and compost (1:1) were put into the tray until they reached a height for about 4 cm. Three rice seeds pre-germinated for 10 days were transplanted into the pot containing 7 kg of soil mixed with 2 kg of compost, and NaCl 7 g. *X. oryzae* as pathogen was inoculated into the rice by dipping of the rice root, and biocontrol agent consortia which consist of five isolates of endophytic bacteria (BCAs 1- BCAs 11) were applied by pouring of 150 mL of their culture broth in No.3 medium (the same composition as No.3S, except for polypepton instead of polypepton S). Each the consortia consisting five isolates, control – (no inoculation of BCAs and pathogen) and control + (inoculation with pathogen only) were also prepared. Two weeks after planting, the rice plants were inoculated again with pathogen by injection of 0.5 mL of the pathogen. Watering was done in every two days, using a tap water. The experiments were performed in triplicate using a completely randomized design. After three months of planting, the rice was harvested; the percentage of the disease, weight of rice grain were observed and microbial activity was analyzed by measuring FDA hydrolysis, according to Schnurer and Rosswall (1982). Data were subjected to analysis of variance (ANOVA) with SPSS software, version 13.

Results and Discussion

Bacterial endophytic isolates

Fifty five bacterial endopytes were isolated from leaves, stems, stalks, flowers and fruits of healthy mangrove plants, *Avicenia alba*, *A. marina*, and *Bruguiera gymnorhiza*. Figure- 1 showed that the number of isolates obtained from leaves were 21 isolates (38% from the total number of isolates). Most of the isolates were obtained from leaf, it is probability due to the structure and the composition of leaves differs from the other part of the plant, such as the presence of stomata on leaves. Jose and Priscilla (2013) who isolated 26 bacterial strains from a mangrove plant of *Rhizophora mucronata*, also found the highest number bacterial isolates from leaf (38.5%) followed by root(34.5%) and stem (26.9%). Five of these bacterial strains showed broad-spectrum of antimicrobial activity against fungal and bacterial pathogen, and were identified as genus of *Serratia*, *Bacillus*, *Pseudomonas*, *Micrococcus* and *Enterobacter*. Hong *et al.*, (2009) isolated actinomycetes from *A.marina* and *B.gymnorhiza* that had bioactive compound activity to *Candida albican*, *Staphylococcus aureus*, and anti tumor-cell. *Actinomycetes* that isolated from *B.gymnorhiza* also inhibited to protein tyrosine phosphatase 1B (PTP1B), a protein related to diabetes. Hu *et al.*, 2010 isolated an

endophytic bacterium *Bacillus amyloliquefaciens* Bg-C31 from *B. Gymnorhiza* which antagonized some fungal and bacterial pathogens of plants and to be effective in the biocontrol of *Capsicum* bacterial wilt in pot and field experiment. Whereas Kathiresan *et al.*, (2013) isolated *Lysinibacillus* sp., from leaves of *A. marina* as producer of adenosine deaminase.

Ding *et al.*, 2010 who had isolated *Streptomyces* sp. GT2002/1503 from the stem of *Bruguiera gymnorhiza*. Found out crude extract from bacterial broth which indicated the presence of diverse secondary metabolites, one of them was xiamycin which has selective anti-HIV activity. Beside that Muduli *et al.*, (2013) isolated *Bacillus* spp. from pneupathopore of *A.alba* with biocontrol activity to *F.oxysporum*.

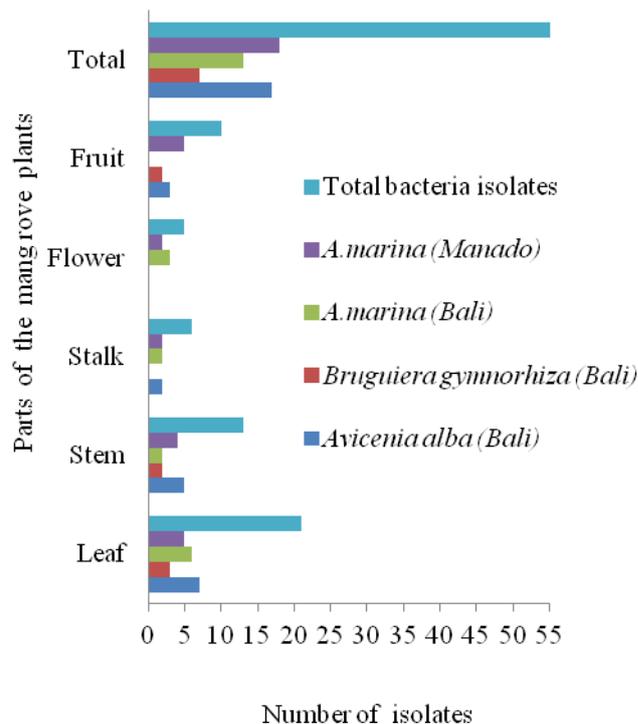


Fig. 1. Amount of endophytic bacteria isolated from *A. alba*, *A. marina* and *B. gymnorhiza*

Antagonistic activity

The antagonistic activity test on 55 bacterial strains showed that seven strains among them (MB06, MB16, MB20, MB31, MB32, MB41, and MB55) inhibited to the growth of *R.Solani*. Five strains of them (MB03, MB11, MB15, MB45, and MB52) were antagonistic to the growth of *F.oxysporum*, while the two others (MB04 and MB08) were antagonistic to the growth of *X. oryzae*. None of them that were tested could inhibit the growth of *P.aphanidermatum* and *P.ultimum* in PDA⁻¹ medium. The differences of the antagonistic activity of the tested bacterial strains may have related with the ability of a certain bacterial strains in producing bioactive compounds and its minimum concentration that was able to inhibit their growth of the antagonistic bacterial (MIC) test. It was reported by several researchers. *Streptomyces aureofaciens* CMUAc130 produced 5,7-dimethoxy-4-p-methoxyphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin compounds that inhibited to the growth of *Colletotrichum musae* and *F. oxysporum* in invitro test. MIC for *C. musae* was 120 µg mL⁻¹ and for *F. Oxysporum* was 150 µg mL⁻¹ (Taechowisan *et al.*, 2005). In case of antibiosis effect of the bacteria to the growth of *X. oryzae*, it has been revealed that *Pseudomonas fluorescens* produced antibiotics including compounds such as 2, 4-diacetylphloroglucinol (DAPG), phenazine, pyrrolnitrin, pyoluteorin and biosurfactant antibiotics. The antibiotics compound suppress the growth of *Xanthomonas oryzae*pv. *Oryzaea* causal agent of the bacterial blight of rice in vitro assay suppress up to 59%-64% (Velusamy *et al.*, 2006). Another genus bacteria, which is well known to produce antifungal was *Bacillus*, such as *Bacillus* sp. N produced 2,5-diketopiperazines (DKPs) against both medicinally and agriculturally important bacterium and fungi showed potent inhibitory values in the range of 1µg/mL. The cyclic dipeptides showed significantly higher activity than the commercial fungicide bavistin against agriculturally important fungi, viz., *Fusarium oxysporum*, *Rhizoctonia solani*, and *Pencillium expansum* (Kumar *et al.*, 2013). While another species of the bacteria antagonist, a *Pseudomonas jesseni* EC-S101 produced two metabolic compounds, 3-[(1R)-hydroxyoctyl]-5-methylene-2(5H)-furanone (4,5-didehydroacaterin) and 3-[(1R)-hydroxyhexyl]-5-methylene-2(5H)-furanone. In *invitro* test showed that the compounds inhibited to the radial growth and induced an abnormal growth, that was characterized by hypae swollen of *Pythium aphanidermatum* PA-5 (Deora *et al.*, 2010).

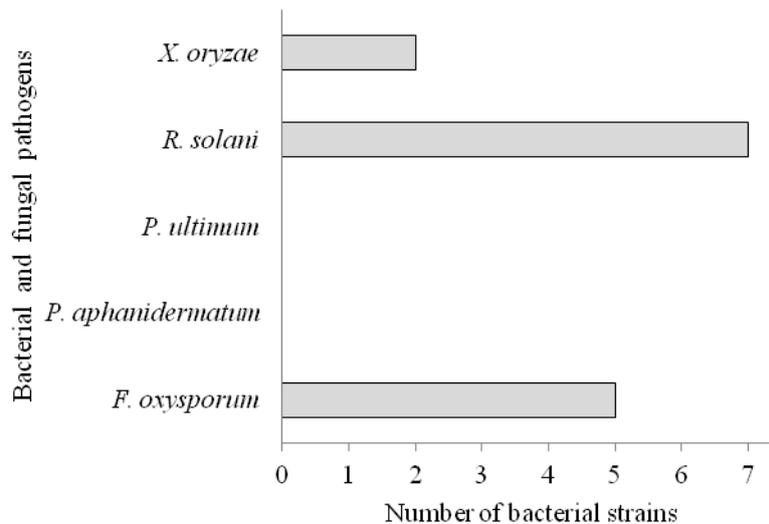


Fig. 2. Amount of endophytic bacterial strains showing antagonistic activity to plant pathogens

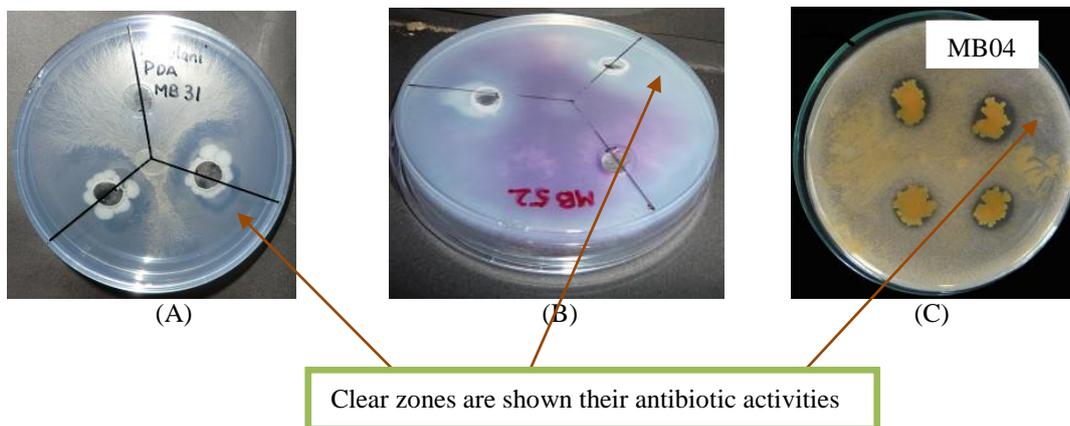


Fig. 3. Antibiosis of the culture broth of strain MB31 to *R. solani* (A), strain MB 52 to *F. oxysporum* (B) and strain MB04 to *X. oryzae* (C)

The Influence of the bacterial endophytic consortia on the suppression of bacterial blight

The results of screening for bacterial consortia biocontrol agents of leaf blight (Fig.4) showed that consortia BCA03 significantly reduced the disease about 67%, and BCAs 11 reduced the disease 22%. Ability of BCAs03 to suppress the disease was nearly the same as with the one reported by Ji *et al.*, (2008) using *Lysobacter antibioticus* strain 13-1, which was isolated from the rhizosphere of rice. It resulted the suppression efficiency of bacterial blight suppression efficiency up to 69.7%. Antibiotics and colonization of *L. antibioticus* on rice leaves may be involved on the suppression of the disease. Several mechanisms of disease suppression have been reported. Such as antibiotic metabolites production (Shoda, 2000; Alvarez *et al.*, 2011, Koberl *et al.*, 2013), inducing systemic resistance and siderophore production (De Boer *et al.*, 2003). Han *et al.*, (2005) revealed that the antagonistic bacteria *Delftia tsuruhatensis* strain HR4 that was isolated from rhizoplane of rice, suppressed bacterial blight in range 7-32% caused by a pathogenic bacterium *X. oryzae*, using foliar spraying and seed soaking inoculation methods. Another biocontrol agent of bacterial blight was reported by Velusamy *et al.*, (2013) who found that *Pseudomonas fluorescens* strain PDY7 that was able to suppress BB 58.83% and 51.88% under glass house and field condition respectively through antibacterial production of 2,4-diacetylphloroglucinol(DAPG).

Application of the BCAs consortia increased the weight of 1000 rice grains for about 200% more, by using the BCAs consortia the weight reached up to 17 g, and without BCAs the weight rice grain decreased to 6 g (Fig.5).The result was in agreement with the Velusamy *et al.*, (2013) who reported that the *P. fluorescens* PDY7 is the most effective in increasing grain weight of rice variety IR24 for about 2-3 folds. Nandakumar *et al.*,(2001) reported that the *P. fluorescens* strains PF1,FP7, and PB2 increased grain yield of rice 25.9% in combinations, and 17.7% in a single strain.

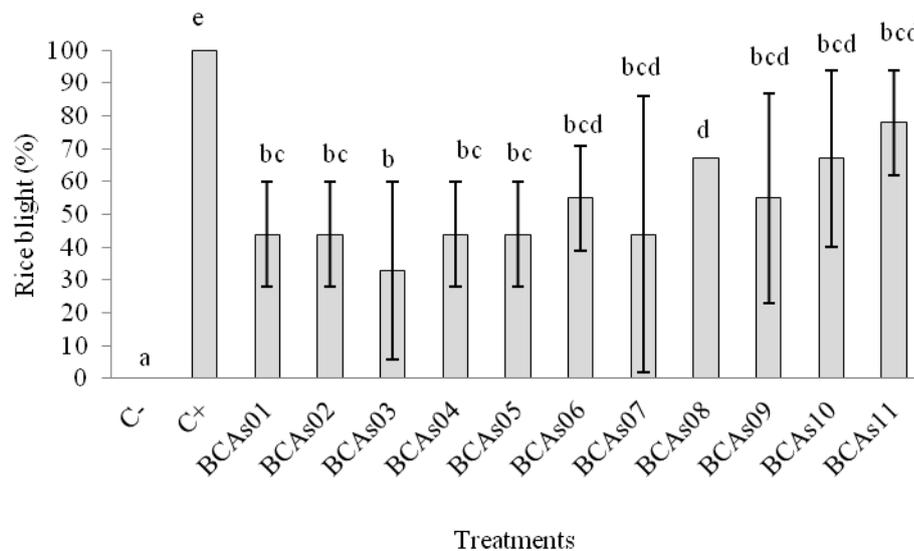


Fig. 4. Suppression of rice blight by biocontrol agents consortia

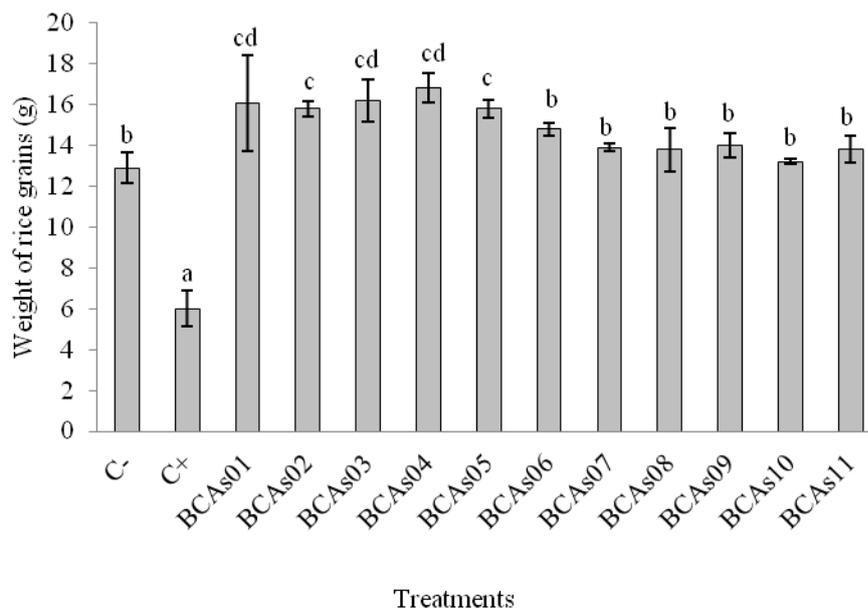
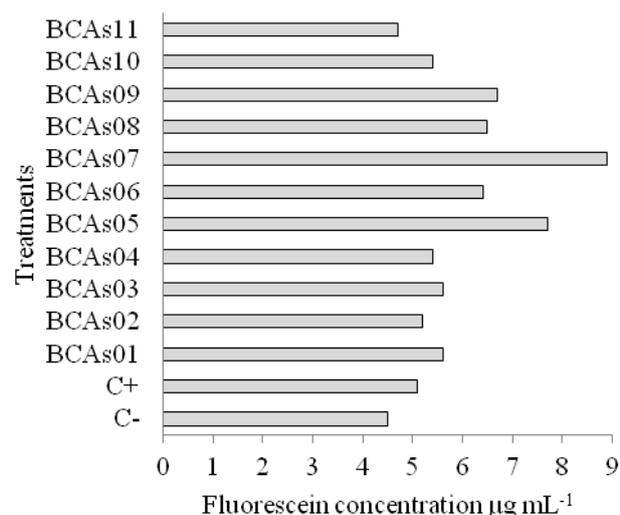
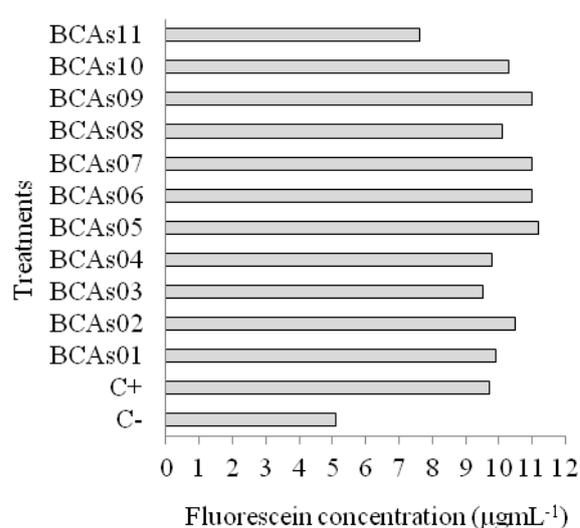


Fig.5. Influence of biocontrol agents consortia on weight of rice grains

Influence of the bacterial endophytic consortia on FDA hydrolytic activity

FDA hydrolytic activity increased after planting on day 49 with the application of all biocontrol agents consortia, except BCAs 11 consortia (Fig.6). It proved to be indicator suppression due to FDA hydrolysis. Several researcher reported about correlation between diseases suppression and FDA hydrolysis activity such as, the increasing of FDA hydrolysis was associated with lower disease incidence of *F.oxysporum* (Peng *et al.*, 1999) and of *Phytophthora lycopersici* on tomato in organic farms (Workhneh *et al.*, 1993). However Grunwald *et al.*,(2000a,b) found no relation between FDA hydrolysis and the presence of *Pythium aphanidermatum* in damping-off of tomato. Furthermore Pankhurst *et al.*, (2005) found no consistent association between FDA hydrolysis in amended soil and their suppressiveness towards sugarcane yield decline. FDA hydrolytic activities on day 56 th after planting tend to be increase in all treatments (Fig.7), so the activities are almost the same as the one of positive control with the application of BCAs01 to BCAs10 consortia, it is possible because the large number of pathogen population in soil which was indicated with 100% of the disease severity. Such condition lead to increase protease, lipase and esterase activity, automatically resulted the higher FDA activity.

Fig. 6. FDA hydrolisis in soil (Day 29th)Fig. 7. FDA hydrolisis in soil (Day 56th)

Conclusion

From the above result, it can be concluded that fifty five bacteria strains were successfully isolated from leaves, stems, flowers, fruits, and stalks of the mangrove plants *Avicenia alba*, *A.marina* and *Bruguiera gymnorhiza* in Bali and Manado, Indonesia.

Seven strains (MB06, MB16, MB20, MB31, MB32, MB41, and MB55) out of the 55 strains were antagonistic to the growth of *Rhizoctonia solani*, five strains (MB03, MB11, MB15, MB45, and MB52) were antagonistic to the growth of *F. oxysporum*, while the two others (MB04 and MB08) were antagonistic to the growth of *X. oryzae*. None of them that were tested could inhibit the growth of *P.aphanidermatum* and *P.ultimum* in PDA⁻¹ medium. The use of the bacterial consortia (BCAs01- BCAs 10) reduced bacterial blight diseases caused by *X. oryzae* significantly and increased the grain weight.

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