Introduction

Paraseriathes falcataria (L.) Nielsen is one species of Nitrogen-fixing leguminous trees (NFLTs) planted worldwide and used in a reforestation program in Indonesia. It is a valuable multipurpose tree for the humid tropics. One of the fastest growing of all tree species, it is used for pulp and other wood products, fuel-wood, ornamental plantings and shade for coffee, tea, cacao and cattle and has good effect in the protection for soil erosion.

Despite the increasing importance of *Paraseriathes* plants in tropical agroforestry systems, little information is available on the synergistic effect of plant growth-promoting rhizobacteria (PGPR) on improving growth of legume trees. The use of PGPRs on tree species is gaining interest since unusual pathogens in agricultural crops also appear in tree nurseries (Enebak et al., 1997). Inoculation of PGPRs in forest-tree nurseries has proved to be crucial in enhancing survival of young seedlings when transplanted to the field. Inoculated seedlings with a more developed root system achieve more sufficient nutrition and survival after transplanting (Probanza et al., 2001).

However, many factors influence the competitive ability of a PGPR strain and affect legume plant growth. Soil acidity is one factor that causes yield loss due to low nutrient availability particularly in tropical soils. The synergistic effect of PGPRs and *Rhizobium* is well documented (Requena et al, 1996, Tilak et al., 2006). However, the beneficial traits of PGPR and *Rhizobium* spp. have been mainly studied separately. Only recently the effects of PGPR and *Rhizobium* spp. have been studied with respect to their combined beneficial impacts on legume plants and their interactions with other beneficial microorganism in the soil. Very few studies have examined the possible interactions between plant growth-promoting rhizobacteria in terms of improved growth of legume plants and mycorrhiza development. The aim of this study was to investigate the synergistic effects of two plant growth-promoting rhizobacteria and *Rhizobium* on the seedling growth of *Paraseriathes falcataria* (L.) Nielsen and indigenous mycorrhiza development in two soils with contrasting level of pH.

Materials and methods

Plant and microbial inoculums

Seeds of *Paraseriathes falcataria* (L.) Nielsen were treated by dipping in sterile de-ionized hot water (100°C) for 30 seconds and then in cold water (25°C) for 24 hours. Thereafter, the seeds were treated with or without PGPRs by seed bed application with *Pseudomonas sp.* "Proradix®" (DSMZ 13134, Proradix®, Sourcon-Padena GmbH & Co. KG, Tübingen, Germany, 1.5 x 10^{10} cfu l^{-1} sterile distilled water) or seed coating with *Bacillus amyloliquefaciens* FZB42 (RhizoVital® 42 TB, ABiTEP, Berlin, Germany, 5 – 15 g kg^{-1} seed). As a control, the seeds were treated with water only. In the following, each pot containing 1.5 kg substrate were prepared. A loamy sandy soil was collected from a tsunami affected field site at Banda Aceh, (Nanggroe Aceh Darussalam, Indonesia) with the following characteristics: pH ($CaCl_2$) 6, P 13 mg, K 22 mg and Mg 35 mg 100 g^{-1} soil, Mn 137 mg, Zn 5 mg, B 0.45 and Fe 236 mg kg^{-1} soil (soil pH and plant available nutrients analyzed according to VDLUVA, 2007). A clay loam soil was collected from a field site at Giessen (Germany) with the following characteristics: pH ($CaCl_2$) 5, P 1 mg, K 1.9 mg and Mg 18 mg 100 g^{-1} soil, Mn 71 mg, Zn 0.6 mg, B 0.13 mg and Fe 115 mg kg^{-1} soil (VDLUVA, 2007). Each soil was mixed with sand (3:1) before using it as planting substrate. Additional N, P, K, Mg and Fe was added at 50, 50, 100, 50 and 0.06 mg kg^{-1} substrate. The *Rhizobium leguminosarum* strain YS1 was grown in Yeast Extract Mannitol (YEM) agar medium (Vincent, 1970) in a rotary shaker for 7 days at 28°C. *Rhizobium* cultures

were then centrifuged and the resulting pellets were re-suspended in saline solution (NaCl 0.85%). Root systems of three weeks-old seedlings received 10 ml *Rhizobium* inoculum or not.

The pots were arranged in a completely randomized design in the greenhouse and cultivated for fifteen weeks with 75% air humidity. Additional light was supplied during phases with low light intensity and plants were irrigated as required according to 15% soil moisture based on dry weight and were harvested 15 weeks after planting.

Plant harvest, nutrient concentration analysis, and mycorrhizal root colonization.

At harvest, roots were thoroughly washed and blotted and a weighed subsample was taken for assessment of mycorrhiza formation. Shoots and roots were dried at 65°C for 72 hours for dry weights were determined. Mineral elements were determined by atomic absorption spectrophotometry (Zn and Cu) and photo-spectrophotometry (P) after wet digestion. Assessment of mycorrhizal root colonization was based on Koske and Gemma (1989) and Kormanik and McGraw (1984).

Statistical analysis

The experimental design was a completely randomized design pattern consisting of eight treatments, i.e. CONTROL (No microbial inoculation), *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PF), *Bacillus amyloliquefaciens* FZB42 (BA), PF+BA, *Rhizobium leguminosarum* (RH), PF+RH, BA+RH and PF+BA+RH. Tukey tests ($P<0.05$) were conducted on transformed data after one-way ANOVA to identify significant differences among the treatments. Results in the figures are given as means. Statistical analyses were performed using the Sigma Stat version 2.03 statistical software (SPSS Inc., Chicago, IL, USA).

Results

In general, root and shoot dry weights, and shoot P and Cu concentrations of *Paraseriathes* plants were significantly bigger in the soil with pH 6 than in the soil with pH 5. Single and combined PGPR inoculation and single *Rhizobium* inoculation significantly increased mycorrhization, root and shoot dry weight, and shoot nutrient concentrations of *Paraseriathes* plants (Figs. 1 to 6). In the soil with pH 5, *P. sp.* "Proradix®" increased shoot nutrient concentrations more than did *B. amyloliquefaciens* FZB42. Combining *P. sp.* "Proradix®" and *B. amyloliquefaciens* FZB42 did not induce stronger effects than using the single PGPRs alone. However, in the soil with pH 5, co-inoculation with *P. sp.* "Proradix®" and *Rhizobium* inoculation led to a further increase of the measured variables compared to *Rhizobium* inoculation alone. In all other treatments, co-inoculation of PGPRs and *Rhizobium* did not lead to a significant increase of any of the measured variables as compared to the individual inoculants. In contrast, combined inoculation with both PGPRs in addition to *Rhizobium* inoculation decreased the shoot Cu contents compared to *Rhizobium* inoculation alone.

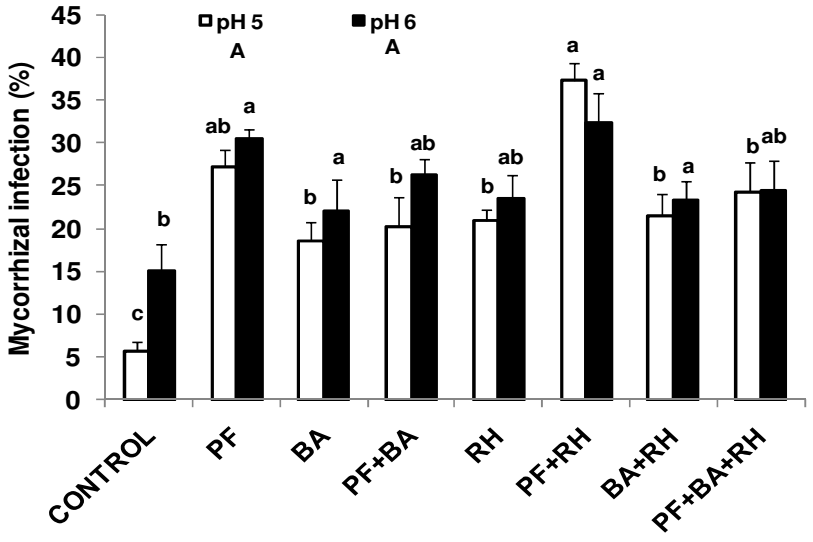

Figure 1. Effect of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and *Rhizobium leguminosarum* (RH) on mycorrhizal root infection (%) 15 weeks after planting in two types of soil differing in soil pH. Different capital letters indicate significant differences between the pH-values. Different small letters indicate significant differences between the treatments within one soil pH.

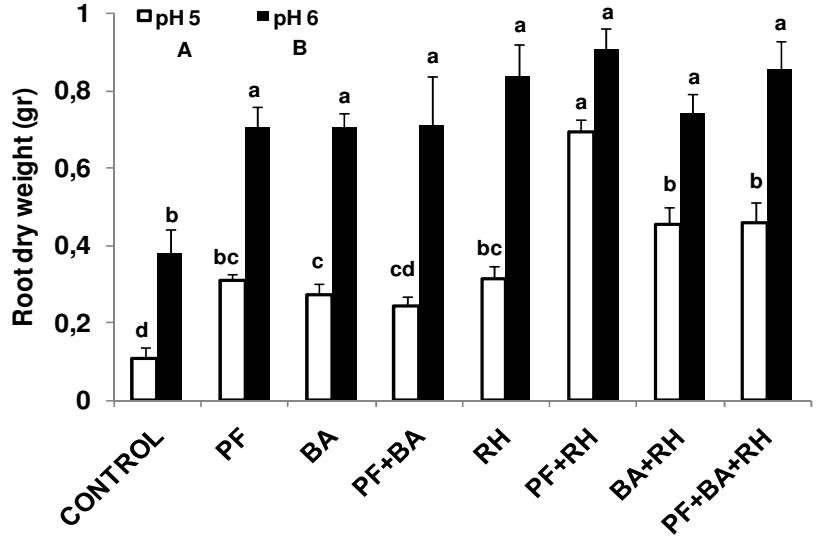

Figure 2. Effect of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and *Rhizobium leguminosarum* (RH) on the root dry weight 15 weeks after planting in two types of soil differing in soil pH. Different capital letters indicate significant differences between the pH-values. Different small letters indicate significant differences between the treatments within one soil pH.

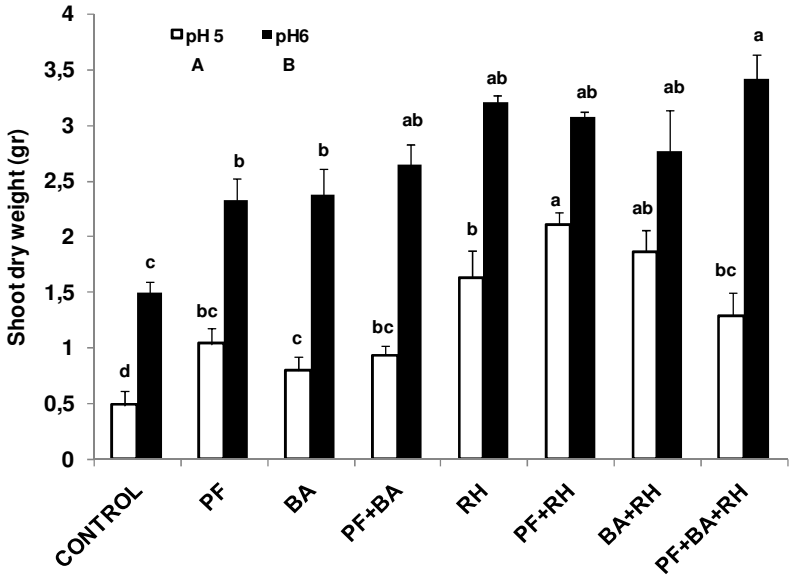

Figure 3. Effect of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and *Rhizobium leguminosarum* (RH) on the shoot dry weight per plant 15 weeks after planting in two types of soil differing in soil pH. Different capital letters indicate significant differences between the pH-values. Different small letters indicate significant differences between the treatments within one soil pH.

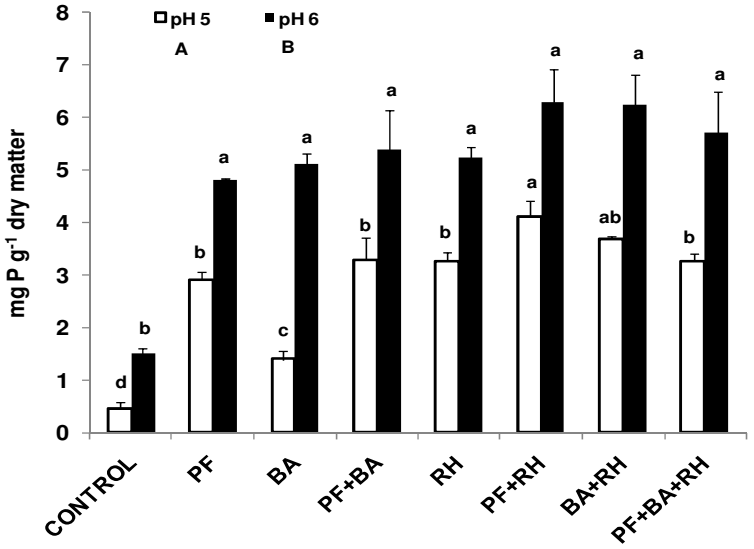

Figure 4: Effect of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and *Rhizobium leguminosarum* (RH) on the P concentration in the shoot of *Paraseriathes* 15 weeks after planting in two types of soil differing in soil pH. Different capital letters indicate significant differences between the pH-values. Different small letters indicate significant differences between the treatments within one soil pH.

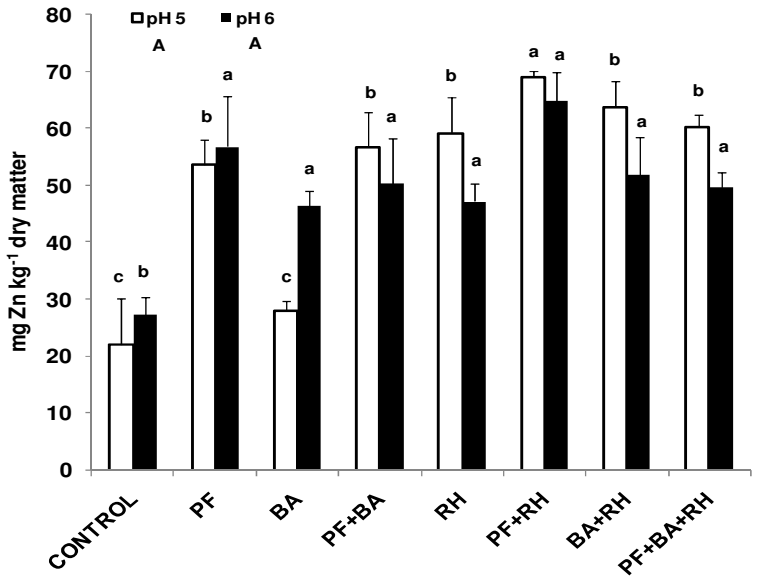

Figure 5: Effect of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and *Rhizobium leguminosarum* (RH) on the Zn concentration in the shoot of *Paraseriathes* 15 weeks after planting in two types of soil differing in soil pH. Different capital letters indicate significant differences between the pH-values. Different small letters indicate significant differences between the treatments within one soil pH.

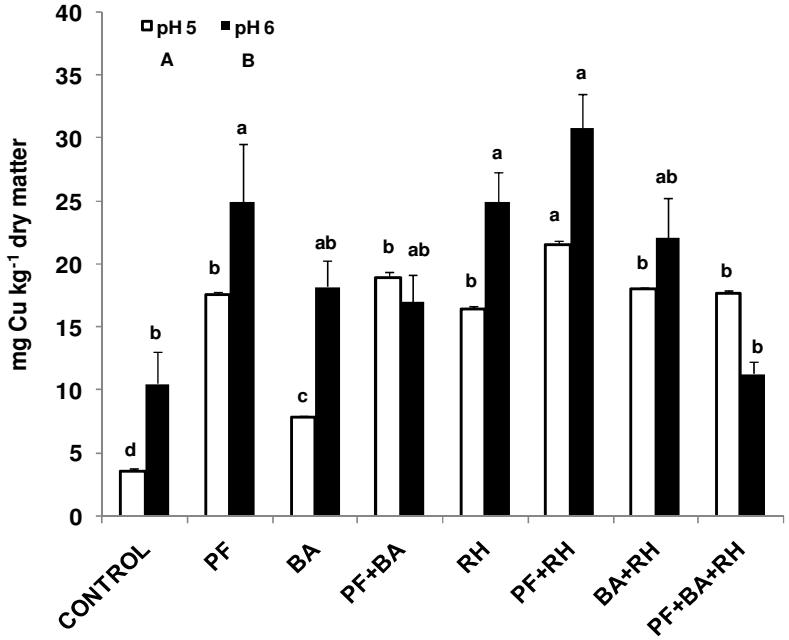

Figure 6: Effect of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and *Rhizobium leguminosarum* (RH) on the Cu concentration in the shoot of *Paraseriathes* 15 weeks after planting in two types of soil differing in soil pH. Different capital letters indicate significant differences between the pH-values. Different small letters indicate significant differences between the treatments within one soil pH.

Discussion and conclusion

The results demonstrate that PGPRs have the potential to promote indigenous arbuscular mycorrhizal fungi (AMF) establishment and the growth of *Paraseriathes* seedlings. The investigated PGPRs, thus, act as mycorrhiza helper bacteria (Garbaye, 1994).

The effect of PGPRs on *Paraseriathes* seedling growth was comparable with the effect of *Rhizobia* inoculation. Consequently, an inocculation with PGPRs in combination or in addition to *Rhizobia* inocculation did not lead to additional effects in general. Slightly synergistic effects could only be observed for the combination of *Rhizobia* and *P.* sp. "Proradix®" inoculation in the soil with pH 5. This indicates, that N-deficiency was not an important factor in the treatments without Rhizobium inoculation and that the effects PGPRs and *Rhizobia* were related to other growth factors than N availability. In conclusion, general synergistic effects as reported in the literature could not be confirmed for the system investigated here.

The differences in nutritional status and growth between *Paraserianthes* seedlings cultivated in the two types of soil, may be the result of overlapping effects which are difficult to distinguish from each other: (1) differences in general nutrient status of the soil, (2) nutrient mobilising/immobilising effects of low soil pH, (3) nutrient dilutional effect of higher plant biomass production in the Indonesian soil, (4) soil specific differences in mycorrhization, (5) nutrient mobilization/immobilization by rhizosphere acidification due to N_2-fixation and finally (6) soil specific effects of PGPRs on mycorrhization, N_2-fixation and nutrient mobilization. The latter was indicated by soils specific effects of the two different PGPRs on mycorrhization, plant growth and shoot nutrient concentrations. Egamberdiyeva (2007) reported that three different bacteria strains *P. alcaligenes* PsA15, *B. polymyxa* BcP26 and *Mycobacterium* phlei MbP18 had a stronger stimulatory effect on plant growth and nutrient uptake of maize in nutrient deficient calcisol soil. Our results indicate the importance of PGPR selection for specific soil conditions.

Soil acidity is one factor that causes yield loss due to low nutrient availability particularly in tropical soils. Our results indicate that improvement of mycorrhization by *P.* sp. "Proradix®" and *B. amyloliquefaciens* FZB42 in the soil with and without *Rhizobium* inoculation can improve P, Zn and Cu acquisition measured as the concentration in *Paraseriathes* shoot. Many previous studies demonstrated that AMF contributes to plant growth via enhancement of mineral nutrient uptake, especially of immobile soil nutrients such as P, Cu, and Zn (Al-Karaki and Clark, 1998, George et al., 1994, Marschner and Dell, 1994, Bethlenfalvay et al., 1988, Yusran et al., 2009). Several authors reported that dual inoculation of AMF and rhizobia can improve legume nutrient status and productivity (Pacovsky et al., 1986; Kucey and Bonetti, 1988; Lynch, 1999: Saxena and Tilak, 1994; Latta et al., 2000; Azcon-Aguilar and Barea, 1981; Grimes and Mount, 1984; Yusran and Rukmi, 2008). An inoculants-induced increase in nodule numbers (data not presented) might be caused by a direct preferential enhancement of nodule functions by AMF mediated through P uptake (Fitter and Garbaye, 1994). This is similar to the results obtained by Manjunath et al. (1984) who reported that inoculation with *Glomus fasciculatum* and a *Rhizobium* sp. significantly improved mycorrhizal colonization, root nodulation, dry weight, N and P content compared to the single inoculation and un-inoculated control plants of *Leucaena leucocephala*. Our study demonstrated clear effects of two PGPR strains and *Rhizobium* on seedling growth of *Paraseriathes* and indigenous arbuscular mycorrhizal development particularly at low soil pH where indigenous rhizobia availability might be very low. This is highly relevant for reforestation programs in humid tropic countries where soils may lack adequate plant available nutrients.

Field experiments will be required in order to investigate improved nutrient acquisition and

survival of seedlings by application of beneficial microorganisms, such as PGPRs at different sites and under natural environmental conditions.

Acknowledgements
Yusran thanks the Asia link – European Commission project ASIA LINK/008/110-005 and the Directorate General for Higher Education, The Ministry of National Education, Republic of Indonesia for postgraduate scholarships, and Tadulako University for study leave. We also express our thanks to Sourcon Padena GmbH and Co. KG. Tübingen (Germany) for providing *Pseudomonas* sp. "Proradix®" inoculums and ABiTEP, Berlin (Germany) for providing *Bacillus amyloliquefaciens* FZB42 inoculums.

References

Al-Karaki GN, Clark RB. Growth, mineral acquisition and water use by mycorrhizal wheat grown under water stress. J. Plant Nutr. 1998; 21: 263–276.

Azcon-Aguilar C, Barea JM. Field inoculation of *Medicago* with VA mycorrhiza and *Rhizobium* in phosphate-fixing agricultural soil. Soil Biol. Biochem. 1981;13:19 –22.

Bentlenfalvy GJ, Brown MS, Ames RN, Thomas RS. Effect of drought on host and endophyte development in mycorrhizal soybeans in relation to water use and phosphate uptake. Physiol. Plant. 1988; 72:565-571.

Egamberdiyeva D. The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Appl. Soil Eco. 2007;36:184-189.

Enebak SA, Wei G, Kloepper JW. Effects of plant-growth-promoting rhizobacteria on loblolly and slash pine seedlings. Forest Sci. 1997;44:139-144.

Fitter AH, Garbaye J. Interaction between mycorrhizal fungi and other soil microorganisms. In Management of mycorrhizas in agriculture, horticulture and forestry. Development in plant and soil sciencies. A.D. Robson, L.K. Abbott and N. Malajezuk (Eds.). Kluwer Academic Publishers, Netherlands; 1994:56:123–13.

Garbaye J. Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol. 1994;128:197-210.

George E, Kothari SK, Li X-L, Weber E, Marschner H. VA mycorrhiza: benefits to crop plant growth and costs. In: Expanding the production and use of cool season food legumes (FJ. Muehlbauer and WJ. Kaiser, eds.), 1994:822–846.

Grimes HD, Mount MS. Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. Soil Biol. Biochem. 1984;16:27–30.

Kormanik PP, McGraw AC. 1984. Quantification of Vesicular-arbuscular mycorrhizae in plant roots. In Methods and Principles of Mycorrhizal Research. Ed. NC Schenck. The American Phytopathological Society. St. Paul Minn. 1984:37-45.

Koske RE, Gemma JN. A modified procedure for staining roots to detect VA mycorrhizas. Mycol. Res. 1989;92:486–505.

Kucey RMN, Bonetti R. Effect of vesicular-arbuscular mycorrhizal fungi and captan on growth and N2 fixation by *Rhizobium*-inoculated field beans. Can. J. of Soil Science. 1988;68:143–149.

Lata Saxena AK, Tilak KVBR. Biofertilizers to augment soil fertility and crop production. In: Soil Fertility and crop production (ed. KR. Krishna). Science Publishers, Enfield, NH, USA. 2000:279-312.

Lynch JM. Beneficial interactions between microorganisms and roots. Biotechnology Advances. 1990:8:335–346.

Manjunath A, Bagyaraj DJ, Gowda HS. Dual inoculation with VA mycorrhiza and *Rhizobium* is beneficial to *Leucaena*. Plant and Soil. 1984;78:445–448.

Marschner H, Dell B. Nutrient uptake in mycorrhizal symbiosis. Plant Soil. 1994;159: 89 - 102.

Pacovsky RS, Fuller G, Stafford AE. Nutrient and growth interactions in soybeans colonized with *Glomus fasciculatum* and *Rhizobium japonicum*. Plant and Soil. 1986;92:37–45.

Probanza A, Mateos JL, Lucas JA, Ramos B, de Felipe MR, Gutierrez Mańero FJ. Effects of inoculation with PGPR *Bacillus* and *Pisolitus tinctorius* on *Pinus pinea* L, growth, bacterial rhizosphere colonization and mycorrhizal infection. Microb. Ecol. 2001;41:140-148.

Requena N, Jimenez I, Toro M, Barea JM. Interaction between Plant growth promoting Rhizobacteria, arbuscular mycorrhizal fungi and *Rhizobium* spp in the rhizosphere of *Anthillys cytisoides*, a model legume for revegetation in mediteranian semi aird ecosystems. New Phytol. 1997;136:667–677.

Saxena AK, Tilak KVBR. Interactions among beneficial soil microorganisms. Indian J. of. Microb. 1994;34:91–106.

Tilak KVBR, Ranganayaki N, Manoharachari. Synergistic effects of plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*). Europ. J. Soil scie. 2006;57:67-71

VDLUFA. Methodenbuch Bd. 1: Die Untersuchung von Böden. VDLUFA-Verlag, Darmstadt. 2007.

Vincent JM. A manual for the practical study of root-nodule bacteria. IBP Handbook No. 15. Blackwell Scientific Publications Ltd, Oxford. 1970:164

Yusran Y, Rukmi R. Synergistic effects of *Rhizobium* sp, *Thelepora* sp. and *Arbuscular* mycorrhiza inoculation in improving seedling growth of *Paraseriathes falcataria* (L.)

Nielsen. In: Eric Tielkes, Competition for resources in a changing world: new drive for rural development. Tropentag 2008. Cuvillier Verlag Göttingen. 2008:440.

Yusran Y, Weinmann M, Roemheld V, Mueller T. Suppression of soilborne pathogens in onion (*Allium ascalonicum* L.) by rhizobacteria; related mechanism. In: Perner H, George A, Zaitun, Syahabuddin, International Symposium Proceeding, Land use after the tsunami, supporting education, research and development in the Aceh region. Banda Aceh, Indonesia. 2009:122-130.