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Effect of Phosphate Solubilizing Bacteria (PSB) on Growth and P Uptake of Wallapatta (*Gyrinops walla* Gaertn.) Seedlings

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ABSTRACT

Gyrinops walla Gaertn (wallapatta) is a highly valuable agarwood producing plant species endemic to Sri Lanka. Due to over-exploitation of the species, the natural habitats are tremendously under pressure, thus commercial-scale cultivation is needed to meet the increasing demand. Propagation through seeds is found to be hampered by inadequate availability of seeds, poor viability and germination and delayed rooting of seedlings. As the use of Phosphate Solubilizing Bacteria (PSB) is recognized to be a sound technology that could enhance the growth of different crops, the present research was carried out to investigate the effect of isolated five PSB strains on growth and P uptake of wallapatta seedlings. Significant *($P \leqslant 0.05$)* increase in plant height, stem diameter, shoot and root dry weight of wallapatta plants were recorded from the seedlings raised with the PSB inoculated seeds compared to non-inoculated seeds. The best growth performances (52.67 cm, 7.52 mm, 7.62 g/plant, and 3.89 g/plant for plant height, stem diameter, shoot and root dry weight, respectively) were recorded from the seedlings inoculated with the strain PSB5 followed by PSB3. Significant *($P \leqslant 0.05$)* decrease in soil pH and increased available P contents were recorded in rhizosphere soil samples taken from PSB inoculated seedlings than that of the non-inoculated seedlings. The lowest soil pH and the highest available soil P contents (6.05 and 18.24 mg/Kg soil respectively) were recorded from the rhizosphere soils inoculated with the strain PSB5 followed by PSB3. The strain PSB5 which enhanced the highest growth performances and P uptake of wallapatta seedlings was identified as *Enterobacter cancerogenous*. Therefore, present results provide much needed baseline information for future studies on the use of PSB as bioinoculants to enhance the early growth of wallapatta seedlings.

1. Introduction

Agarwood also referred to as gaharu is a highly demanded aromatic resin used in perfume industry, local medicines, food industry and for some religious purposes [1]. It has been used over the years as an attractive incense in religious events by Buddhists, Hindus and Islamic people in many different parts of the world [2]. The active ingredients found in agarwood make it very useful in treating some diseases and health-related disorders as it shows antimalarial, aphrodisiac, antirheumatic, analgesic, deobstruent, tonic, and diuretic properties [3]. Agarwood receives steady and increasing demand at the world market where Middle East region and East Asian countries are reported to be the major consumers [4].

Aquilaria and *Gyrinops* species of the family Thymelaeaceae are found to have the ability to produce agarwood [5]. Species of the *Aquilaria* genus has not been naturally found in Sri Lanka where genus *Gyrinops* (wallapatta) is distributed scattered in some parts of the country. The agarwood formation is known to be linked with some form of physical damages to the heartwood of the stem in combination with a fungal infection [2, 6]. The plant then responds to the damages, by secreting a resin which deposits around the wounds

and forms agarwood with the accumulation of the volatile compounds [7]. The infected area becomes dark brown or black with time [8] though distinguishing from outside is difficult as agarwood formation takes place under the bark. The uncontrolled wild harvest is continued to meet the demand which threatens the existence of natural habitats [8]. The intensified wild harvest hampers the natural regeneration as well thus future prospects are bleak. Under this background, International Conservation Initiatives have identified nine *Aquilaria* sp. which should come under the Red List of Threatened Species of International Union for Conservation of Nature (IUCN). At present all *Aquilaria* and *Gyrinops* spp. have been placed under Appendix II of the Convention on International Trade in Endangered Species (CITES) [9]. *Gyrinops walla* (wallapatta), also known as Sri Lankan agarwood is an indigenous tree species in Sri Lanka [10]. Wallapatta plant often shows slow growth at the early stages thus special care is needed at the nursery stage. Seed propagation is identified as the most reliable method of propagation of the species. However, due to lack of uniform matured seeds, poor seed viability, poor germination and, delayed rooting of seedlings, hamper the propagation and commercial-scale cultivation. Seedlings have to be maintained at the nursery for about 12 months before field planting. Thus, attention has been paid on enhancing the early growth of seedlings in order to reduce nursery period.

Many tropical soils are deficient in nutrients especially phosphorous resulting in poor plant growth. Soluble forms of P quickly convert to insoluble forms (Ca/Al/Fe – bound P) and become unavailable to plants. Consequently about 95- 99% of P in soil is in the form of insoluble phosphates, which cannot be absorbed by plants [11]. There are several rhizospheric microorganisms called Phosphate Solubilizing Microorganisms (PSMs) which possess the ability to convert the insoluble phosphatic compounds into soluble forms enabling them available to plant uptake [12]. In addition to phosphate solubilization, PSMs could solubilize potassium, enhance nitrogen fixation, and produce plant growth promoting substances like auxins, cytokinins, and gibberellins while protecting plants from pathogens. However, no information is available regarding the effect of microbial seed inoculation on the early growth of *Gyrinops* species. Under this background present research was carried out to investigate the effect of isolated PSB strains on seedling growth of wallapatta.

2. Material and Methods

2.1 Isolation of bacterial strains and inoculum preparation

Soils employed in isolating bacterial strains were collected from different agricultural lands in Matara District, Southern Sri Lanka. Based on the performance of quantitative phosphate solubilization (published data), five bacterial strains (PSB-1, PSB-2, PSB-3, PSB-4 and PSB-5) were selected for the inoculum preparation and they were grown on nutrient agar plates initially. A single colony of each strain was transferred to 500 ml flasks containing nutrient broth, and grown aerobically in flasks on a rotating shaker (150 rpm) for 48 h at 30 ℃ and then the broth was centrifuged at 10,000 rpm for 10 min separately. The supernatant was discarded, and the pellet was re-suspended and washed with sterilized distilled water three times. The bacterial suspensions were then diluted in sterile distilled water to a final concentration of 10^8 CFU/ml, and the resulting suspensions were used to treat wallapatta (*Gyrinops walla*) seeds.

2.2 Seeds

Wallapatta seeds were collected from a well matured mother plant grown in a secondary forest in Matara district, Sri Lanka. The seeds were soaked in water for about 2 hours and then surface-sterilized by shaking in 5% NaClO for 5 min. They were thoroughly rinsed twice in sterile distilled water. Surface sterilized seeds were soaked in separate bacterial suspensions approximately 30 min prior to planting. Seeds soaked in diluted nutrient broth without bacteria were used as the control. PSB treated and untreated seeds were sown separately in a plastic tray containing autoclave-sterilized sand media and kept under shady conditions (55%).

2.3 Soil preparation and planting

The experiment was carried out in a greenhouse located at the Faculty of Agriculture, University of Ruhuna, Sri Lanka. The experimental soil used as potting media was collected from 0-15 cm soil depth from a previously cultivated land at the faculty research farm. As listed in the USDA soil taxonomy, the soil of this area comes under Red Yellow Podzolic great soil group. The soil was air dried, crushed, sieved (2 mm mesh), and analyzed for its physico-chemical characteristics (Table 01). Then the soil was autoclaved at 121 ℃ for 20 minutes to eliminate native PSB and exactly 3 kg of the soil was weighed and placed in each sterilized pots (25 cm diameter, 35 cm height). The pots were arranged in a completely randomized block design with three replications per treatment.

Bacteria inoculated and non-inoculated 21-day old seedlings were transplanted to plastic pots (two plants/plot). Immediately after planting, an inorganic fertilizer mixture (N:P:K at the ratio of 16:16:16) was added at the rate of 5 g/pot. After 4 weeks, the weak seedling of each plot was removed allowing the

remaining plant to grow. Plants were protected from rain and grown under natural light (mean temperature 30 ℃ day and 25 ℃ night, relative humidity 16–89%, day length approximately 12 h, maximum light intensity 196 W/m^2). They were watered to a level approximately equal to the field capacity twice a week with tap water. Weed and pest controls were carried out manually.

Table 01: Some important physico-chemical properties of soil used in the study. Values given here are the means (n = 4) ± standard deviation.

Soil properties	Value
Sand (%)	84 ± 2.46
Silt (%)	12 ± 1.01
Clay (%)	4 ± 0.73
Soil texture	Loamy sand
Bulk density (g/cm^3)	1.28 ± 0.06
pH	6.73 ± 0.41
Organic carbon (%)	0.85 ± 0.07
Total N (%)	0.15 ± 0.01
NH_4^+ - N (mg/kg soil)	82 ± 3.24
NO_3^- - N (mg/kg soil)	31 ± 2.25
Available P (mg/kg soil)	4.8 ± 0.82
Available K (mg/kg soil)	118 ± 4.78
CEC (cmol $^{(+)}$/kg soil	12.1 ± 0.85

Growth promoting effects of bacterial treatments were assessed by measuring the plant height, stem diameter, dry weights of shoot and root, and P uptake of wallapatta plants after six months of planting.

2.4 Plant height and stem diameter

Seedling height was measured from the collar region to the tip of the main stem using a meter scale in centimeters (cm). The stem diameter was measured at 3.8 cm above the collar region using a Vernier caliper in millimeters (mm).

2.5 Dry matter content of plants and phosphorous uptake

At the end of the experiment, all plants were harvested, root and shoot portions were separated and air dried for two days. Roots of wallapatta plants were washed gently over a 2-mm sieve under running tap water to separate them from soil particles. They were then oven dried at 70 ℃ to a constant weight. The shoot and root dry weights were recorded separately and the average dry weight of plants was expressed in g/plant. Plant samples were finely ground after drying and used to determine phosphorous content following Vandomolybdate phosphoric yellow color method as described by Jackson [13].

2.6 Soil analysis

The samples of rhizosphere soil were aseptically separated from roots to measure soil pH, phosphorous content and population densities of PSB. Soil pH was measured in 1:2.5 soil:water suspension with a pH meter. Available phosphorous was extracted by the bicarbonate method and determined following the molybdate blue color method. PSB population density was assessed using pour plate method. For that rhizosphere soil was collected by uprooting the plants. The soil adhering to the roots was serially diluted and aliquots of 0.1 ml of the sample from each of these dilutions were spread on to a Petri dish containing NBRIP medium. The plates were incubated for three days in an incubator at 30 ℃. The colonies with clear halos were counted at the end of the incubation.

2.7 Statistical analyses

The data were subjected to analysis of variance (ANOVA) using SAS package. The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $P \leqslant 0.05$.

3. Results

3.1 Isolation of bacterial strains

A total of fifteen bacterial strains (PSB-1 to PSB-15) that exhibited large clear zones on the agar plates were isolated from soil samples collected from rhizosphere soils and they were identified as phosphate solubilizing strains. It was evident that a high proportion of phosphate solubilizing microorganisms are concentrated in the rhizosphere region of plants compared to non-rhizosphere region as observed by Ramachandran et al. [14] also. Isolated bacterial strains were capable of solubilizing tricalcium phosphate [$Ca_3(PO_4)_3$] present in NBRIP medium and they formed large halos with varied intensity indicating their P solubilizing capacities. Based on the phosphate solubilizing capacities, five contrasting bacterial isolates were selected as the most efficient phosphate solubilizing organisms for the present study (low "PSB1", moderate "PSB2 and PSB4" and high "PSB3 and PSB5" P-solubilizing capacity "PSC").

3.2 Growth and P uptake in wallapatta plants

There were differences in plant growth among wallapatta seedlings inoculated with five PSB strains. Significant ($P \leqslant 0.05$) increased in plant height, stem diameter, shoot and root dry weight of

wallapatta plants were recorded from the seedlings raised with the PSB inoculated seeds compared to that of non-inoculated seeds (Table 2). The best growth performances (52.67 cm, 7.52 mm, 7.62, and 3.89 g/plant for plant height, stem diameter, shoot and root dry weight, respectively) were recorded from the seedlings inoculated with the strain PSB5 followed by PSB3 (46.67 cm, 5.65 mm, 5.26 and 3.35 g/plant for plant height, stem diameter, shoot and root dry weight, respectively). Moreover, they were significantly ($P \leqslant 0.05$) higher than the corresponding figures of the control and the other strains. Although seeds inoculation with other PSB strains (PSB1-PSB4) resulted in better growth performances, no significant ($P \leqslant 0.05$) differences in shoot length, root length, shoot and root dry weight were observed among them.

Table 02: Effect of PSB inoculation on the growth of wallapatta seedlings

Treatments	Plant height (cm)	Stem diameter (mm)	Shoot dry Weight (g)	Root dry weight (g)
Seeds without PSB	31.67±0.58^{d}	3.50±0.44^{c}	4.39±0.27^{c}	2.22±0.02^{d}
Seeds with PSB1	43.67±2.08^{bc}	5.38±0.34^{b}	5.10±0.12^{b}	3.28±0.18^{bc}
Seeds with PSB2	44.67±0.58^{bc}	5.59±0.43^{b}	5.18±0.09^{b}	3.11±0.13^{bc}
Seeds with PSB3	46.67 ±0.53^{b}	5.65±0.22^{b}	5.26±0.12^{b}	3.35±0.06^{b}
Seeds with PSB4	43.00±1.00^{c}	5.44±0.10^{b}	4.97±0.28^{b}	3.06±0.08^{c}
Seeds with PSB5	52.67±0.58^{a}	7.52±0.40 ^{a}	7.62±0.45^{a}	3.89±0.09

Table 03: Effect of PSB inoculation on soil pH, soil available P content and plant P uptake

Treatments	Soil pH	Soil available P (mg/Kg soil)	Shoot P (mg/plant)	Root P (mg/plant)
Seeds without PSB	6.75±0.06^{a}	14.2±0.87^{d}	81.20±2.48^{c}	15.28±1.19^{c}
Seeds with PSB1	6.57±0.02^{b}	15.85±0.38^{bc}	90.71±2.48^{ab}	18.43±1.40^{bc}
Seeds with PSB2	6.33±0.08^{c}	15.98±0.22^{bc}	90.04±2.01^{b}	18.95±0.13^{b}
Seeds with PSB3	6.14±0.03^{de}	16.48±0.87^{ab}	92.73±4.93^{ab}	20.97± 2.25^{b}
Seeds with PSB4	6.21±0.07^{cd}	15.72±0.44^{bc}	91.76±1.04^{ab}	19.85±0.79^{b}
Seeds with PSB5	6.05±0.03^{e}	18.24±0.95^{a}	98.58±3.25^{a}	29.29±1.28^{a}

The P contents in shoot and root of wallapatta seedlings were higher in seedlings raised with the PSB inoculated seeds than that of in control seedlings (Table 3) and there were differences in shoot and root P contents of wallapatta seedlings among PSB inoculated seedlings.

The P contents in shoots and roots of wallapatta seedlings inoculated with PSB strains were significantly ($P \leqslant 0.05$) different from those of control seedlings. The highest shoot and root P contents (98.58 and 29.29 mg/plant for shoot and root P, respectively) were recorded from the seedlings inoculated with the strain PSB5 followed by PSB3 (92.73, 20.97 g/plant for shoot and root P, respectively). Similar to the other parameters, seedlings inoculated with other PSB strains resulted in higher shoot and root P contents, though no significant ($P \leqslant 0.05$) differences were observed among them (PSB1-PSB4).

3.3 Changes in soil pH and available soil P contents

The effect of PSB inoculation with wallapatta seedlings on soil pH and soil available P contents are shown in Table 3. A significant ($P \leqslant 0.05$) decrease in soil pH and increased available soil P contents in soil were recorded in rhizosphere soil samples taken from PSB inoculated seedlings than non-inoculated seedlings (control). The lowest soil pH and highest available soil P contents (6.05 and 18.24 mg/Kg soil for soil pH and available soil P content) were recorded from the seedlings inoculated with the strain PSB5 followed by PSB3 (6.14 and 16.48 mg/Kg soil for soil pH and available soil P content).

4. Discussion

Use of phosphate solubilizing microorganisms (PSMs) as microbial inoculants has been identified

as a technology which could minimize possible ecological and environmental hazards associated with high dependency on chemical fertilizers. The technique is regarded as non-toxic, economically viable, and environmentally friendly approach for sustainable agriculture [15]. Therefore, in connection with this, different aspects of the technique have been extensively studied for the last decades and tested with varying degrees of success under a variety of growing environments.

Enhanced seed germination, seedling growth and P uptake of several crop plants due to PSB inoculation have been reported in a number of studies conducted under both growth chamber and greenhouse conditions [16]. As seen in the present results, the increase in plant height, stem diameter, shoot and root weight of wallapatta seedlings inoculated with PSB strains could be attributed to a greater absorption of nutrients, especially P as evident in higher P contents in shoot, root and soil samples taken from PSB inoculated seedlings compared to the non-inoculated seedlings.

The growth promoting ability of PSB is not only attributed to greater phosphate solubilization, but to the stimulation of the efficiency of plant hormone production such as auxins, cytokinins, gibberellins, and production of some volatile compounds also [17]. The combine treatment of gibberellic acid (GA3) and cytokinin (6-Benzylaminopurine/6-BAP) at 50 ppm and 100 ppm respectively resulted in significantly ($p<0.05$) higher stem height, stem diameter, leaf area, fresh and dry weight of shoots and roots in wallapatta seedlings compared to those of the control (personal communication). Enhanced seedling growth after inoculation of PSB strains can be attributed to the ability of the strains to make P available and to simultaneously produce plant growth-promoting substances [18]. All the PSB strains used in this study exhibited the capacity to produce indoleacetic acid (IAA) in the order of PSB5 > PSB4 > PSB3 > PSB2 > PSB1 (published data). Therefore, IAA might also have contributed to enhance plant height, stem diameter, shoot and root dry weights through cell elongation and multiplication induced by greater absorption of nutrients, particularly phosphorous.

Further to this, Dharmasena and Arunakumara [19] also investigated the influence of GA3, IAA and indol-3-butyric acid (IBA) at 800 ppm, 1000 ppm, and 1200 ppm concentrations on seed germination of the *G. walla* and reported the highest seed germination (65%) with the presence of GA3 at 1200 ppm followed by IAA at 1000 ppm (53.33%) and 800 ppm (50%).

Significant ($P \leqslant 0.05$) decreased in soil pH and increased available P content in soil after inoculation of PSB strains is in agreement with Elhaissoufi et al. [20] who observed similar results after soil inoculation with five contrasting PSB (*Pseudomonas* spp.) isolates. In accordance with our results, they also observed the lowest pH and the highest P content in soil when PSB inoculation. Phosphate solubilization potential has been attributed to the strains' ability to reduce pH of the surroundings, either by releasing organic acids or protons. This leads to increased P availability, which ultimately increases plant P uptake [21]. The detectable reductions of soil pH are slightly lower in the present study which might be due to high buffering capacity of the soil.

Although, no information is available regarding the effect of microbial inoculation on the early growth of *G. walla*, many studies have been conducted on arbuscular mycorrhizal (AM) fungal inoculation on early growth of *Aquilaria* species [22, 23]. According to research findings of Tujaman et al. [23], seed inoculation with AM fungi increased N and P content, plant growth and survival rates of *Aquilaria malaccensis* and *Aquilaria crasna* seedlings at 6 months after transplantation under greenhouse conditions. This is an agreement with Yuwono et al. [22] who observed the highest percentage of root infection by AM in 4 months old *A. malaccensis* with combination of ameliorants of phosphate rock (PR) and AMF. They categorized the relative mycorrhizal dependence (RMD) as highly dependent. A high RMD value is an indicator of increased plant growth and biomass of *A. malaccensis*. RMD value is changed with the soil types, AMF types, and host plants implying that the inoculated AMF is very effective in seedling growth of *A. malaccensis*. Mycorrhizal association improves soil structure, increase nutrient solubility, water and nutrient uptake, and protects plants from root pathogens and toxic elements [24].

As reported by several researchers for *Aquilaria* sp. and other tropical tree species [23], inoculation with AM fungi can reduce fertilizer requirement in plant production during seedling growth at the nursery stage. Although cost benefit analysis was not performed in this study, the results undoubtedly indicate that microbial inoculations can substantially reduce chemical fertilizer requirement in *Gyrinops* sp. during the seedling stage.

Among the bacterial strains, the strain PSB5 showed the highest growth performances and P uptake in wallapatta seedlings. According to 16S rRNA sequence analysis, the strain was identified as *Enterobacter cancerogenous* and the sequences of the strain were deposited in the GenBank nucleotide sequence data library under KX815170 accession number.

5. Conclusion

Present study demonstrated that inoculation of wallapatta seeds with indigenously isolated PSB strains could significantly increase the seedling growth. The growth enhancement is mainly attributed to the P solubilization by bacterial strains in the soil and thereby increased P uptake. Though other plant growth promoting abilities of PSB such as IAA, cytokinin, and gibberellins production could have also positively contributed, they were not tested in the present study. Based on the results, it could be concluded that PSB have the potential to use as a promising approach in improving seedling growth and P absorption in wallapatta seedlings. The economic feasibility and the performance under field conditions should be studied before making recommendations for large-scale applications.

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