Keywords: Oryza sativa L., Azospirillum sp., Pseudomonas fluorescens, Arbuscular mycorrhizal fungi, Pelleted biofertilizer

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most important staple food for more than two billion people in Asia and hundreds of millions in Africa and Latin America. It is a chief source of carbohydrates and protein in Asia and provides minerals and fibers also [1]. Due the prominent and valuable role of rice, different fertilizers are being made to increase rice productivity to ensure food security throughout the globe [2]. Environmental degradation has become the major threat confronting the world therefore there is a need for the replacement of synthetic fertilizers with biofertilizers [3, 4]. Therefore, ending the indiscriminate use of chemical fertilizers, biofertilizers can be used for sustainable rice farming systems. Biofertilizers have their more advantages over chemical fertilizers and are economically and environmentally friendly [5].

Biofertilizer are the products containing carrier based (solid or liquid) living microorganisms which are useful in agriculture [6]. The core function of carrier material is providing the suitable micro-environment for introduced microbes to enhance the shelf life and efficacy of inoculum as biofertilizers [7]. The co-inoculation of different types of beneficial bacterial strains and AMF create positive effects on growth and yield of plants and soil microbial communities comparing with single microbial inoculant [5, 6,7]. Providing suitable nutrient source to microbes by incorporating with carrier materials is a good option to further improve the effectiveness of biofertilizers [7]. Natural element compounds such as rock phosphate and potassium feldspar can be used as nutrient supplements for microorganisms [8]. Among different physical types of biofertilizers, pelleted biofertilizers should be a quality product with several desirable qualities [9,10,11].

This study was focused on evaluating three compatible substances for development of effective pelleted biofertilizer for rice (*Oryza sativa* L.) using co-inoculated bacteria and arbuscular mycorrhizal fungi. Aquatic weed *Salvinia* sp., biochar and compost were tested for their effectiveness as carrier materials in pelleted biofertilizer applied rice plants. Findings of this study is helpful to farmers to get high rice yield through an environmentally friendly, easily applicable fertilization.

2. MATERIALS AND METHODS

2.1 Preparation of Bioinoculum

Azospirillum sp. and *Pseudomonas fluorescens* two bacterial inoculants and mycorrhizal inoculum were used as the co-inoculum which used in biofertilizer production.

2.1.1 Preparation of bacterial inoculants

Two required bacterial species were isolated by serial dilution method from the randomly selected soil samples which were collected from undisturbed water logged area in Mihintale Sri Lanka. *Azospirillum* sp. and *Pseudomonas fluorescens* were cultured respectively by using standard spread plate technique on *Azospirillum* [12] and King's B agar media (KB) [13] respectively in triplicates and incubated at 30 °C for 2 days.

2.1.2 Characterization of Isolates

Colonies of fluorescent *Pseudomonas* strains were identified under UV illuminator at 366 nm and sub cultured on KB agar plates and pure cultures were made. *Azospirillum* pure cultures were also prepared after characterized by colony morphology, Gram's staining and biochemical methods described by Bergey's Manual of Determinative Bacteriology [13]. They were Gram positive, oxidase positive, indole test negative, methyl red positive, catalase positive, motility test positive and the identified *Azospirillum* colonies were sub cultured on *Azospirullum* medium [14].

2.1.3 Preparation of arbuscular mycorrhizal fungal (AMF) inoculum

In order to prepare the inoculums with sufficient indigenous AMF population, trap cultures were established. Composite soil samples with fine root fragments were collected from the upper layer (0-15 cm) of the organically grown rice field at Ranpathwela, Anuradapura district and used as an indigenous AMF inoculum. Such soil with root fragments was thoroughly homogenized with sand (grain size 0.7–1.2 mm) in a ratio of 1:4 (v/v) and added into 1000 ml plastic pots. Before sowing, seeds of maize (*Zea mays* L.) were surface disinfected by immersing them in a 0.5% sodium hypochlorite solution for 15 minutes. These seeds were washed with distilled water and they were sown at 2 cm depth in each pot and covered with autoclaved sand. Approximately 50-60 seeds of maize were sown per pot and were kept in the planthouse at Faculty of Applied Sciences, Rajarata University of Sri Lanka for one month. Root fragments of maize together with rhizosphere soil is considered as an AMF inoculum.

2.2 Preparation of carrier material

Aquatic fern *Salvinia* sp. were collected from the Mihintale tank, Anuradapura, Sri Lanka. Compost was collected from a home garden at Mihintale and wood chip biochar was prepared by double barrel method. They were air dried and ground to powder and sieved through 2 mm sieve. The prepared carrier materials were packed in autoclavable polythene covers, sealed using an electric sealer and prepared 250 g weight of bags. They were sterilized at 121 ^{0}C for 20 minutes to destroy contaminated microbes.

2.3 Development of biofertilizers as pellets

The pure cultures of isolated bacteria were used to prepare 10^8 bacterial inoculants of relevant cultures by using sterile water according to McFarland method. Each pre-sterilized, 1 kg weight of ground carrier material was inoculated with 50 g of AMF propagules and 20 ml of 1.5 x 10^8 (CFU/ml) of each bacterial inoculant. Then it was mixed by hand or by shaker until the microbial inoculum has been uniformly spread in to the carrier substances. Then microbial inoculants and carrier substance were packed in the polythene bag and was immediately sealed. Prepared biofertilizers were used to make pellets under applied pressure. There were six biofertilizer types and 10 g of rock phosphate (RP) and 10 g of potassium feldspar were used as nutrient supplement mixture in three biofertilizer types among them. The different types of pellets were aquatic weed and bioinoculum (P_1), aquatic weed, bioinoculum and nutrient supplement mixture (P_2), biochar and bioinoculum (P_3), biochar, bioinoculum and nutrient supplement mixture (P_4), compost and bioinoculum (P_5), compost, bioinoculum and nutrient supplement mixture (P_6). After the preparation, pelleted biofertilizers were packed properly. The packages were placed under two temperature conditions (0 ^{0}C and 30 ^{0}C) for appropriate period (7 days). After 7th day interval, biofertilizer pellets were tested for the microbial survivability with the time by determining viable cell count of bacteria at two storage temperatures of 0 ^{0}C and 30 ^{0}C. All the experiments were performed in triplicates.

2.4 Pot experiment

The pot experiment was conducted from February 2019 to May 2019 inside a planthouse under natural light conditions at the Faculty of Applied Sciences, Rajarata University of Sri Lanka in Mihintale Sri Lanka. The day temperature in the planthouse during the trial was 31 ^{0}C -35 ^{0}C and night temperature was 30 ^{0}C with the 68% relative humidity. Annual precipitation in the area is between 1000-1500 mm. The treatments were field soil only (T_0), field soil and biofertilizers type P_1 (T_1), field soil and biofertilizers type P_2 (T_2), field soil and biofertilizers type P_3 (T_3), field soil and biofertilizers type P_4 (T_4), field soil and biofertilizers type P_5 (T_5) and field soil and biofertilizers type P_6 (T_6). The treatments were arranged in complete randomized block design (CRBD) with five replicates. Pots were filled with sieved soil and rock phosphate and potassium feldspar was added to the all treatments as to provide phosphate and potassium respectively. Control (without biofertilizers) and pelleted biofertilizers were added to soil in pots (Table 01). Seeds of variety Bg 360 were grown in a tray and one plant was transplanted to each pot. Fifteen pellets of biofertilizers (15 x 2g) were added to one pot initially and repeated the application after one half month period.

Table 01- Treatment combinations of the pot experiment

	Treatments	Amount of soil (g)	No. of pellets (1 pellet=2g)	Amount of rock phosphate (g)	Amount of feldspar (g)
T0	Field soil	2800	-	100	100
T1	Field soil + biofertilizer type P_1	2740	30	100	100
T2	Field soil + biofertilizer type P_2	2740	30	100	100
T3	Field soil + biofertilizer type P_3	2740	30	100	100
T4	Field soil + biofertilizer type P_4	2740	30	100	100
T5	Field soil + biofertilizer type P_5	2740	30	100	100
T6	Field soil + biofertilizer type P_6	2740	30	100	100

2.5 Data collection

2.5.1 Viable cell counts of the applied bacterial inoculants in tested biofertilizer pellets stored at 0 ^{0}C and 30 ^{0}C

The survival of *Azospirillum* sp. and *Pseudomonas fluorescens* in formed pellets of different carriers at two temperature storage conditions (0 ^{0}C and 30 ^{0}C) were determined by standard viable cell counting. Randomly selected three pellets of each biofertilizer types was taken separately for estimating viable cells at 7days after stored at 0 ^{0}C and 30 ^{0}C, using standard dilution plate count method on *Azospirillum* media and King's B medium respectively. Serial dilution was prepared by transfer of 2 g each of pellet into 18 ml sterile water blanks to get 10^{-1} dilution. Similar dilutions were made serially up to 10^{-9} from 10^{-1} dilution. One ml the diluted bacterial suspensions was pipetted out into sterile glass Petri plates and collected *Azospirillum* medium or King's B medium for respective cultures. The plates were rotated clockwise and anticlockwise direction for uniform spread of the dilution mixture and the plates were incubated at 30 ^{0}C for 2 days. After incubation, *Azospirillum* sp. and *Pseudomonas fluorescens* colonies were counted using colony counter and recorded as CFU/ml. The plate count was carried out in duplicates and the mean value was accounted for the analysis.

2.5.2 Agronomic data

Final plant height (cm), relative growth rate, fresh weight of plant (g) and dry weight of plant (g) as growth parameters and number of panicles per plant, number of grans per panicle and weight of 100 grains (g) as yield parameters were measured. Shoot height (cm) was measured as the length from the base of the plant to the tip of the shoot at harvesting stage of rice plants and relative growth rate was calculated. Initial shoot height of plants was obtained on the date of transplanting and final shoot height was obtained at harvesting stage. After harvest total weight of fully-grown plant was recorded as fresh weight of each plant. After recording the fresh weight of plants at harvest, they were air dried naturally and then oven dried at 60 °C temperature overnight and dry weight was determined.

2.5.3 Soil pH and electrical conductivity (mS/m)

Ten grams of soil from each treatment type were weighed and 40 ml of distilled water was added to it. The samples were stirred for one hour at 15 rpm in shaker to get uniform mixing of carrier with the distilled water and was allowed to settle for 30 minutes. Then Hanna Multiparameter Water Quality Meter was calibrated and used to measure final pH and electrical conductivity.

2.5.4 Final microbial population count in biofertilizer treated soil (CFU/ml)

The microbial population in the soils of each treatment pots were estimated by serial dilution plate count technique. Soil samples were collected from the rhizosphere of each treated pots after harvesting to observe the final microbial population size. One gram of experimental soil of each treatment was taken to prepare dilution series. Final *Azospirullum* sp. and *Pseudomonas fluorescens* colony counts were estimated by using pour culture technique on *Azospirullum* medium and King's B medium and were kept for incubation at 30 °C for 48 hours. *Azospirillum* sp. and *Pseudomonas fluorescens* colonies were counted using colony counter and recorded as CFU/ml.

2.5.5 Observation of AMF colonization in biofertilizer treated rice roots

Biofertilizer applied rice plant roots were screened for potential of AMF colonization following the standard staining procedures [15]. Root sub-samples were rinsed with distilled water and fixed in a formaldehyde-acetic acid-ethanol solution (90:5:5 by volume). After cutting fine roots of a sample into 1 cm long segments, they were washed thoroughly in distilled water and then placed in 10% KOH and heated to 90 ^{o}C for 15-30 minutes in a water bath and washed in distilled water. The heavily pigmented root samples were bleached by immersing them in alkaline 3% H_2O_2 solution for 60 minutes at room temperature. The roots were thereafter acidified with 1% HCl for 1 minute before staining. The root segments were stained with preheated 0.05% trypan blue in lactoglycerol for 5 minutes at 75 ^{o}C. The roots were first rinsed in deionized water and destained in a lactic acid: glycerol: deionized water solution [1: 2: 2 (v: v: v)]. Stained roots were stored in glycerine. Approximately 25-30 segments of 1 cm long root segments were randomly selected from each stained sample and mounted in glycerine on microscopic slide gently squashed under a cover glass and viewed under a compound microscope (Olympus SZH10, China) at x 400 magnification and percentage AMF colonization was determined using modified grid transection method [15].

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2.6 Data analysis

One-way ANOVA was used for normally distributed data Analysis of Variance (ANOVA) for all treatments in Randomized Block Design. Means were compared by using the Tukey's Studentized Range Test at a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Viable cell count of microbes (CFU/ml) in tested biofertilizer pellets after 7 days of storage at 0 ^{0}C and 30 ^{0}C

Viable cell count (CFU/ml) of microbes in tested biofertilizer pellets stored in sealed packets at 0 ^{0}C and 30 ^{0}C temperatures after 7 days were presented in figure 01 and 02. Initially 10^8 CFU/ml of each *Azospirillum* sp. and *Pseudomonas fluorescens* were used in carrier inoculation and pellet formation. The results revealed that there was no any significant difference ($p \leq 0.05$) in *Azospirillum* and *Pseudomonas fluorescens* colony counts among different types of pellets as P_1 to P_6 and two storage temperatures of 0 ^{0}C and 30 ^{0}C after 7 days respectively.

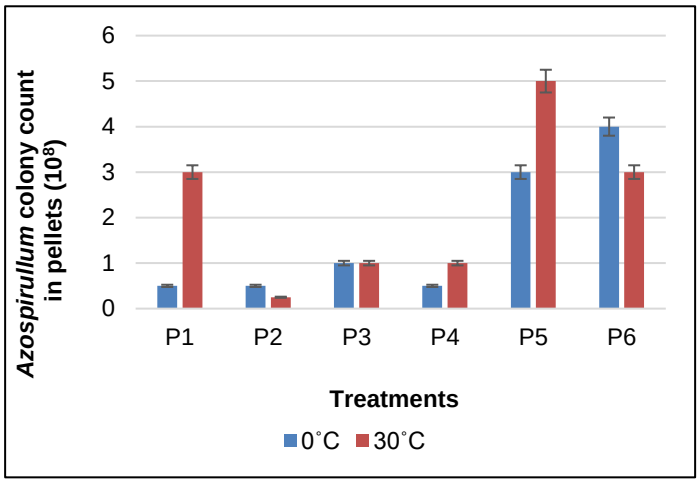

Figure 01- Changes of *Azospirillum* sp. colony count (×10^8 CFU/ml) in biofertilizer pellets formed with different carriers after 7 days at 0 ^{0}C and 30 ^{0}C

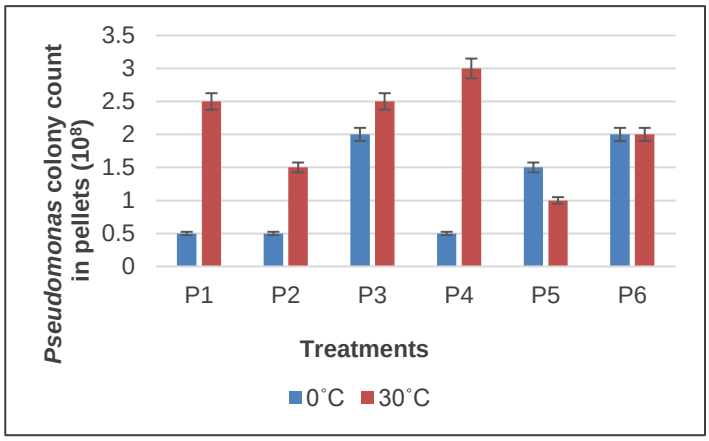

Figure 02- -Changes of *Pseudomonas fluorescens* colony count (×10^8 CFU/ml) in biofertilizer pellets formed with different carriers after 7 days of storage at 0 ^{0}C and 30 ^{0}C

P_1: Aquatic weeds + bioinoculum, P_2: Aquatic weeds + bioinoculum + nutrient supplement mixture, P_3: Biochar+ bioinoculum, P_4: Biochar+ bioinoculum + nutrient supplement mixture, P_5: Compost+ bioinoculum, P6: Compost+ bioinoculum + nutrient supplement mixture

However, *Azospirullum* sp. and *Pseudomonas fluorescens* colony counts in biofertilizer pellets were comparatively higher at 30˚C temperature than 0˚C. Therefore, 30˚C can be considered as suitable temperature for biofertilizer storage than 0˚C.

3.2 Agronomic parameters of the pot experiment in with added different biofertilizer pellets

Final plant height (cm), relative growth rate, fresh weight of plant (g) and dry weight of plant (g) as growth parameters and number of panicles per plant, number of grains per panicle and weight of 100 grains (g) as yield parameters were measured. There were significant differences ($p \leq 0.05$) for shoot height, number of grains per panicle and 100 grains weight were observed in the treatments of different pellet types Figure 03, 04 and 05). However, there was no significant difference ($p \geq 0.05$) observed for relative growth rate, plant dry and fresh weights, soil pH and electrical conductivity (Table 2). Height of shoots was measured once a month after the transplanting of rice seedlings. Final shoot height of plants was obtained after 75 days of transplanting. Changes of shoot height of plants with different treatments were shown in figure 03.

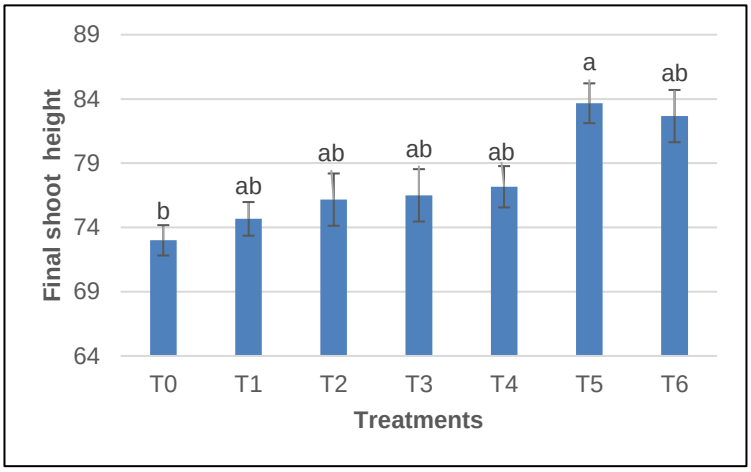

Figure 03- Changes of shoot height of plants with different treatments. Means denoted with different letters are significantly different at p<0.05

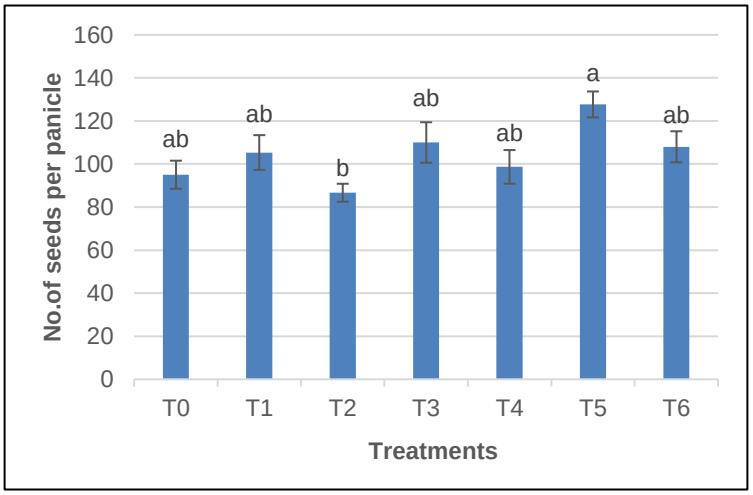

Figure 04- Changes of Number of seeds per panicle with different treatments. Means denoted with different letters are significantly different at p<0.05

Total number grains per panicle was counted in fully matured panicles after harvesting. Changes of Number of seeds per panicle with different treatments were given in figure 04. The 100 grains obtained from each treated rice plants were weighed and the test weight of grains per plant was calculated (figure 05).

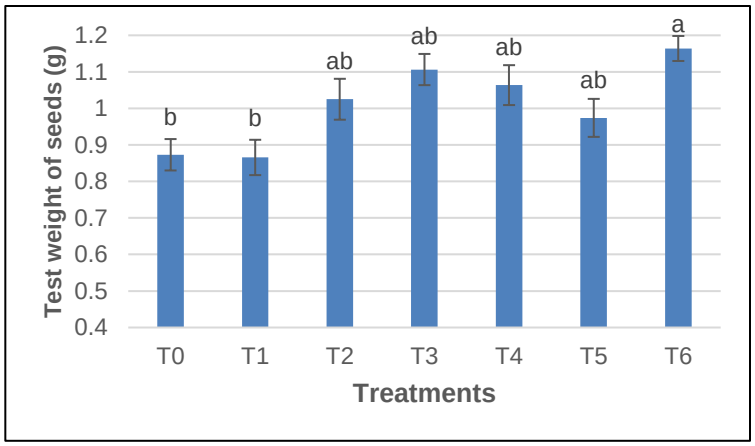

Figure 05- Changes of test seed weight with different treatments. Means denoted with different letters are significantly different at p<0.05.

Table 02 - Mean values of growth parameters, yield parameters and soil parameters for different treatments

Treatment No	Treatment type	Growth Parameters			Yield parameters	Soil parameters	
		Relative growth rate	Fresh weight of plant (g)	Dry weight of plant (g)	No. of panicles per plant	Electrical Conductivity (mS/m)	Soil pH
T_0	Field soil without biofertilizers	0.998±0.03	7.83±3.21	6.79±2.25	2.67±0.70	102.33	6.58
T_1	Field soil + biofertilizer type 1	1.091±0.05	5.55±1.98	5.55±1.98	3.00±1.41	154.17	6.41
T_2	Field soil + biofertilizer type 2	0.993±0.04	9.06±3.65	7.36±3.07	3.33±0	189.33	6.05
T_3	Field soil + biofertilizer type 3	1.100±0.12	5.47±2.29	5.47±2.29	2.33±0.70	194.50	6.49
T_4	Field soil + biofertilizer type 4	1.091±0.13	6.83±2.37	6.83±2.37	3.33±0	231.83	6.01
T_5	Field soil + biofertilizer type 5	0.993±0.07	11.78±2.55	8.45±6.02	4.33±0.70	146.10	6.46
T_6	Field soil + biofertilizer type 6	1.078±0.03	7.64±1.18	7.24±1.86	3.33±0	105.13	6.22

3.3 Final microbial population count in biofertilizer treated soil (CFU/ml)

After 75 days of growth rice, colony counts of *Azospirullum* sp. and *Pseudomonas fluorescens* were estimated and it was observed that the counts were lower than the initial application in pellets (figure 06 and 07).

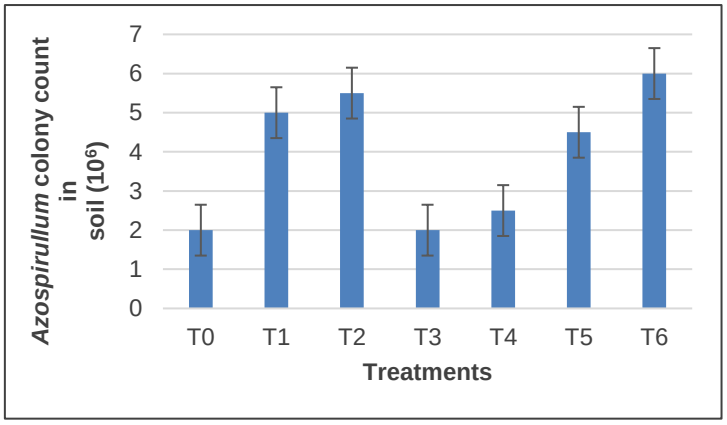

Figure 06- Changes of *Azospirullum* sp. colony count (CFU/g) in soil with different treatments

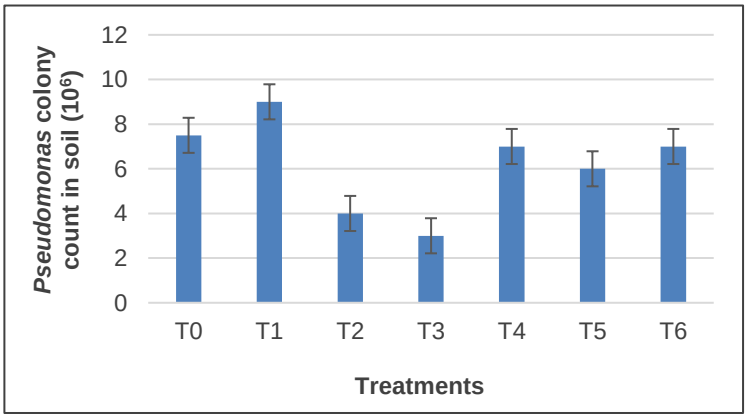

Figure 07- Changes of *Pseudomonas fluorescens* colony count (CFU/g) in soil with different treatments

3.4 Arbuscular mycorrhizal fungi colonization

Arbuscular mycorrhizal fungi colonization was observed the rice roots after harvesting. However, percentage AMF colonization was very low in the rice roots. Rice was grown in the flooded conditions that may be the reason of very low percentage AMF colonization. Although, the percentage AMF colonization was approximately in between 15% - 20 % in the application of different bioinoculum, this was the first reported evidence of AMF colonization of rice roots in Sri Lanka.

The key challenge in the development of effective biofertilizers is supporting regular survival rates of the inoculum. Carrier materials can influence inoculum efficacy and viability by altering the soil structure for making it more beneficial for microbial colonization [16,17]. Most of the carrier materials contain a high organic matter content to increase bacterial survival and enhance the efficacy of bacterial inoculum [18]. In the present study, compost with mixed bioinoculant pellets application exceedingly enhanced the rice growth and yield among different pelleted biofertilizers. Compost, bioinoculum and nutrient supplement mixture (P_6) added pellets were shown highest bacterial survivability at 30 ^{0}C for seven days. Compost is an organic soil amendment and served as a good carrier material for the production of efficient biofertilizer [19].

4. CONCLUSION

Among tested carriers compost is the most suitable carrier substance in production of pelleted biofertilizers for rice. Although AMF colonization of rice roots were low this was the first report of citing the presence of AMF in lowland flooded rice roots in Sri Lanka. These pelleted biofertilizers have the potential to be used for improved productivity of rice variety Bg 360.

Therefore, developing such bioinoculants as a biofertilizer could be the solution to the many problems associated with the use of chemical fertilizers in rice cultivation in Sri Lanka and worldwide.

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