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Effect of Seed Inoculation of *Rhizobium leguminosarum on* Growth and Yield responses of Cowpea *Vigna unguiculata* (L.) Walp Under rain-fed Conditions

K. N. Kumara Sharma and A. G. B. Aruggoda

Department of Agricultural and Plantation Engineering, Faculty of Engineering Technology, The Open University of Sri Lanka, Nawala, Nugegoda, Sri Lanka

*Corresponding Author: email: agbar@ou.ac.lk, Tele: +94112881062

Abstract *– Legume is an important source of protein in the developed world and the main food in the developing world. Despite as a human diet legume can improv soil fertility through biological nitrogen fixation. .Thus, it reduces the nitrogen fertilizer requirement of legume and makes the legume-based cropping systems sustainable. Therefore, the impact of rhizobia inoculated seeds on overall growth and yield performances of Cowpea required to be evaluated to popularize the seed inoculations among farmers. A field experiment was conducted to investigate the response of cowpea to seed inoculated rhizobia, organic manure and recommended inorganic fertilizer. Waruni a local cowpea variety was used for this study as it is widely cultivated in Sri Lanka. Randomized complete block design with five replicates was used for the study. Seed inoculated rhizobia increased the number of nodules and nodules dry weight per plant, 100 seed weight, number of pods per plant, yield, hydration coefficient and cookability. Control treatment added with 100 kg/ha TSP only showed significant increase in total defects and non-soakers. Moisture content did not show a statistically significant difference among the treatments. The study indicated that the effect of seed inoculated rhizobia, organic manure and inorganic fertilizer on nodulation, yield and seed physical properties on cowpea. The study indicated that the Cowpea responded to inoculation in seeds containing Rhizobium showing positive interactions for most of the growth, yield, nodulation and seed physical properties and was more pronounced for yield and seed physical parameters.*

Keywords*: Rhizobia inoculated seeds, Cowpea, growth, yield*

1 INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] is one of the major grain legumes grown to acquire the protein requirement of humans. As per the researches done in the past, the protein content of cowpea leaves ranges from 27 to 43% and protein concentration of the dry grain range from 21 to 33% (Ahenkora et al, 1998; Ddamulira et al., 2015; Abudulai et al., 2016). In Sri Lanka generally Cowpea is supplied with the inorganic fertilizer mixture recommended by the Department of Agriculture. Commercial or synthetic fertilizers are tremendously increased in their price. Also, certain toxic compounds of synthetic fertilizers not only remain for a longer time in the soil but also enter into our food chain. Thus, the use of microbial fertilizers is a function in many ways to benefits the ecosystem. All the legumes are in symbiotic N_2 fixation which can compensate for missing soil nitrogen (N) by saving costly mineral N fertilizer (Guimarães et al, 2012). The farmers do not know or consider the ability of the root nodulation and fixing nitrogen by the

Cowpea. As per the Pule-Meulenberg et al, in 2010, Cowpea was shown to establish symbioses with several species and genera of the rhizobia. However, limited information is available on the performance of cowpea with inoculant strains in soils containing indigenous rhizobia population. It reduces environmental problems and enhances the productivity of the crop. Further, the benefits of microbial inoculation may be achieved when microbial cells are used along with compost (Gadalla et al. 2010). Therefore, enhancing the rhizobia nodulation in legumes would be beneficial to overcome the current problem of heavy use of fertilizers up to a certain level. Several studies are required to find the extent of inoculants used by the Rhizobia naturally present in soil and to access the requirement of inoculation to enhance the Nitrogen fixation.

Until recently, inoculation was not practiced for Cowpea assuming that the abundantly present indigenous *Bradyrhizobium* spp. in tropical soils effectively nodulate cowpea under natural environmental conditions (Singleton et al, 1992; Kimiti and Odee, 2010). However, recent studies have shown that cowpea responds to inoculation (Soares et al, 2006; Zilli et al, 2009; Almeida et al, 2010; Costa et al, 2011; Ferreira et al, 2013; Onduru et al, 2008; Nyoki and Ndakidemi, 2013, 2014). In these studies, the application of Bradyrhizobia inoculants improved nodulation, shoot dry matter as well as grain yield. Almeida et al, (2010), applied three inoculant strains separately which has increased cowpea grain yield by 29–50% compared with the non-inoculated control with no Nitrogen fertilization. In the trials by both Onduru et al, (2008) and Nyoki and Ndakidemi, (2013), inoculation increased nodulation, shoot dry weight, grain yields, and other growth variables.

Furthermore, as per some researchers' application of inoculants together with Phosphorus increased dry matter and grain yields more than applying inoculant or Phosphorus alone suggesting that cowpea growth and yield are limited by Phosphorus deficiency. The phosphorus is an essential nutrient for nodulation. The importance of Phosphorus in nodulation and grain yield of cowpea is well documented by several researchers (Bationo et al, 2002; Carsky and Toukourou, 2005; Jemo et al, 2006; Singh et al, 2011; Ayodele and Oso, 2014; Abaidoo et al, 2016). However, limited information is available on the performance of cowpea with inoculant strains and Phosphorus fertilization in soils containing indigenous rhizobia population. The present study was aimed to evaluate the overall performances of cowpea from germination to the consumption of seeds by establishing ***Rhizobium leguminosarum*** inoculated seeds under rainfed conditions. Organic manure as well as the application of recommended inorganic fertilizers at the recommended growth stages were also considered as treatments to conclude the outcomes with a comparative analysis.

2. METHODOLOGY

2.1 Site Description

A field study was conducted at the model farm of the Department of Agricultural and Plantation Engineering (DAPE), the Open University of Sri Lanka at Nawala, Nugegoda (6° 55' 37.4844'' N, 79° 51' 40.4784'' E, 3.1 m.a.s.l.) from July to December 2011. The selected field was abandoned for more than five years without any crop. As per the European Digital Archive of Soil Maps (EuDASM) the predominant soil type in the area is Red Yellow Podzolic soils with Soft and Hard Laterite (Panagos et al, 2011). The average annual temperature is 30 ^{0}C and the average annual precipitation is 2000 mm.

2.2 Analyzing the composition of soil and composted cattle manure

Composite soil samples from ten random locations in the field and composted cattle manure were analyzed for chemical composition and availability of microbes one week before start the experiment.

Composite soil samples were prepared by mixing ten random soil samples collected up to a depth of 20 cm using a soil auger. The pH was determined using a high impedance voltmeter on 1:2 soil–water suspension. Total organic carbon was determined by Walkley–Black method. Nitrogen was determined by Kjeldahl method (Black, 1965). Perchloric and nitric acid digestion was employed for Phosphorous and Potassium analysis (Jakson, 1958). As an initial study, 10 g of finely ground composted cattle manure dissolved in 100 ml of water was also analyzed for the pH, Phosphorous, Potassium, Nitrogen and total organic carbon by following the same methods above.

2.3 Experimental procedure and treatments

The experiment lied as a Randomized Complete Block Design with four treatments with four replications. The treatment combinations are given in table 02. To minimize the effect of phosphorous, 100 kg/ha phosphorous in the form of TSP was applied evenly to cover all replicates including control.

Table 01: Treatment combinations

Treatments	Fertilizer type and quantities
T1	Inorganic Fertilizer mixture (35 kg/ha of nitrogen, and 100 kg/ha TSP)
T2	Organic fertilizer (8 kg/ ha and 100 kg/ha TSP)
T3	Seed Inoculated Rhizobium (100 grams inoculants used for 10 kg seeds and 100 kg/ha TSP)
T4	Control (100 kg/ha TSP only)

2.4 Preparing inoculum for field application

Commercial pure culture of *Rhizobium leguminosarum (*Sri Bio-Tech Commercial Industries, Mawathagama, Padukka) was maintained at 32 ^{0}C by subculturing on Yeast Mannitol Agar. *Rhizobium leguminosarum* inoculum was prepared by adding a loop full of bacteria into the Yeast Mannitol Broth - YMB followed by employing ten steps, 10-fold (10^{-1} to 10^{-10}) serial dilution in the estimation of the total number of rhizobia in the soil samples, respectively, using a saline solution (0.89% NaCl) as the diluent. The concentration of the inoculating broth selected was 2 x 109 c.f.u/ml.

Seed inoculation was performed by weighing 20 g of seeds in plastic bags and adding 2 ml of water and 0.04 g sugar solution as a sticker and 200 ml of the prepared broth. The seeds and sugar solution were mixed thoroughly and 1 kg of Telcom powder was added for formulating (according to the manufacturers' recommendation), mixed thoroughly until all the seeds were completely covered with an inoculant. The inoculant was applied to supply approximately 106 rhizobia cells per seed. The seeds were treated in the field immediately before planting.

2.5 Land preparation, Field planting and crop management

Plots of 3.0 m × 5.0 m consisted of two rows with a spacing of 50 cm and 20 cm between and within rows respectively. Land preparation was accomplished by ploughing followed by two harrowing. Two to three seeds were placed in a hole to a 2 cm depth. To minimize contamination, the non-inoculated plots were planted first. Weed controlling was carried out whenever necessary. The plant was thinned to one plant per hill after emergence. The plants were grown under rainfed conditions.

2.6 Parameters Measured and Data Analysis

Seed germination percentage was analyzed on the third day after seeding. Shoot length was recorded and the number of leaves per plant counted at weekly intervals. At the end of the seventh week of field planting, three plants were selected randomly from each plot and washed the roots after removing the soil carefully. The nodules were picked from the roots and their numbers were recorded. Later, the dry weight of shoots as well as roots were determined by oven drying at 70 ^{0}C for 48 hours. The dry weight of the separated nodules was also determined by oven drying at the same temperature and time, practiced above. Number of flowers per plant was counted at four days intervals of each plant separately. Number of pods per plant were calculated by harvesting mature pods when they becoming yellow, nine weeks after planting from each plant. Number of seeds per pod was calculated by manually counting seeds of the cleaned pods from each plant separately. 100 seed weight was calculated by manually counting the number of pure seeds from randomly selected plants from each plot and by weighing to take the average value of replicates. Finally, the yield per plant was calculated in grams in each plant. Physical properties of cowpea seeds were determined. Seed moisture content (weight/weight) was determined using Low Constant Temperature Oven Method. Cookability test was conducted by weighing twenty grams of seeds followed by soaking in 200 ml of tap water for 16 hours then boiling at 110 ^{0}C for 30 minutes and by reweighing the cooked seeds. Non soakers were calculated by randomly selecting 100 seeds from each plant and by soaking in tap water by adding four times upper to the level of seeds for 16 hours followed by sorting and weighing the non-soaker seeds in each sample. Hydration coefficient percentage was calculated by dividing the weight of soaked seeds by initial weight. The total defects percentage was calculated by adding percentage of other defects to the non soaker seed percentage. Total other defects were calculated by dividing the weight of seeds having all other defects such as broken or physically damaged, failure to reach maturity and or being very small in size by the initial weight.

Data analysis was performed using SAS version 9.4 (SAS Institute, 2012). The Analysis of Variance (ANOVA) was carried out to test the significant ($p<0.05$) variation among treatments. Duncan's Multiple Range Test (DNMRT) was used to compare mean differences among treatments.

3.0 RESULTS AND DISCUSSION

The results of the composite soil sample and composted cattle manure analysis are given in table 01. The composted cattle manure has relatively high low total nitrogen and potassium content. Phosphorus and total organic carbon were low in both, soils of the experimented field as well as organic manure.

Table 02: Chemical properties and available *Rhizobium leguminosarum* population of the Composite Soil sample (0–20 cm depth) and composted cattle manure

Location	pH (1:2 H_20)	Total org. C (%)	Total N (%)	P (mg kg^{-1})	K (mg kg^{-1})	*Rhizobium leguminosarum* number (cells g^{-1} soil)
Soil	6.4	66	1.08	6.5	1.6	1.07 x 10^3
Composted cattle manure	7.2	12.6	10	3	6	-

Results of all treatments on seed germination and growth of cowpea are presented in table 03. Average seed germination was high in all treatments and the control had the lowest numerical value while showing significantly difference value with all other treatments (84) Inorganic fertilizer added treatment resulted with highest numerical mean value (98) while showing statistically significant. Control treatment was added with 100 kg/ha TSP only. Therefore, the seed germination was supported by the existing nutrients in the field only. These results are in line with the findings of (Kala et al, 2011), who reported that seed inoculated with rhizobium showed induced germination percentage than untreated seeds.

Table 03: Seed germination percentage, mean values of plant growth parameters, LSD and CV of Cowpea grown under different fertilizers

Treatment	Germination %	Plant Height (cm)	Number of Leaves /Plant	Shoot dry weight (g)	Root dry weight (g)
Inorganic fertilizer	98A	22.86BC	24.34A	61.80A	4.38A
Composted cattle manure	94A	23.30A	25.58A	70.22A	4.62A
Seed inoculated Rhizobium	96BA	23.10AB	26.21A	73.42A	5.12A
Control	84B	21.86C	24.83A	59.00A	3.84A
LSD	1.24	1.17	2.97	6.25	0.64
CV (%)	9.67	7.55	8.55	20.58	31.94

Note: Means with the same letters along the columns are not significantly different at p>0.05. Means are the average of the five replicates.

Composted cattle manure showed the significantly different plant height (23.30) when compared with other treatments which had lowest in control (21.86). However, number of leaves per plant, shoot as well as root dry weight did not show significant variation (P<0.05) among treatments. These results are in contrast to the findings of Patra and Bhattacharya in 1998, who reported that the cowpea plants produced from inoculated seeds exhibited significantly greater root and shoot length when compared to un-inoculated control cowpea plants. Cattle manure contains most of the important micro and macronutrients for the growth of Cowpea, however, essential nutrient supply is limited in other treatments.

Table 04: Linear correlation analysis between plant height, number of leaves per plant, germination percentage, shoot dry weight and root dry weight

	Plant Height	No. of leaves	Shoot dry weight	Root dry weight	Yield per ha
Plant Height	-	-0.18ns	-0.03ns	0.19ns	0.3ns
No. of leaves	-	-	0.51*	0.64**	0.25ns
Shoot dry weight	-	-	-	0.48*	0.22ns
Root dry weight	-	-	-	-	0.32ns
Yield per ha	-	-	-	-	-

*** Significant at P=0.0001 *Significant at P=0.05 ** Significant at P=0.01 ns non-significant at P=0.05

Correlation analysis was performed to evaluate the strength of relationship between the plant height, the number of leaves per plant, shoot dry weight, and root dry weight and yield per hectare (Table 4). Plant height showed a non-significant negative correlation with the number of leaves per plant and shoot dry weight while root dry weight and germination percentage showed a non-significant positive correlation with plant height. The number of leaves showed significant ($p<0.05$) positive correlation with shoot dry weight and moderately significant ($p<0.01$) positive correlation with root dry weight. Further, shoot dry weight showed a significant ($p<0.05$) positive correlation with root dry weight. Yield per hectare was non-significant with other parameters and however, it showed a positive correlation with other parameters.

Table 05: Mean valuses of nodule formation, LSD and CV of Cowpea grown under different fertilizers

Treatment	Nodule number	Nodule dry weight (mg)
Inorganic fertilizer	2.39BA	0.017A
Composted cattle manure	2.19B	0.033B
Seed inoculated Rhizobium	4.78A	0.056A
Control	1.79B	0.010c
LSD	2.57	0.023
CV (%)	66.79	57.52

Note: Means with the same letters along the columns are not significantly different at p>0.05. Means are the average of the five replicates.

Table 05 explaining the relationship between the mean nodule formation of Cowpea among different treatments. Seeds inoculated with Rhizobium showed significant difference on the number of nodule formation (4.78) and non significant in nodule dry weight (0.056). The similar records were resulted with the researches done by Hungria et al, 2000 and Mostasso et al, 2002 by having increased number of nodules due to seed inoculated Rhizobia in the field-grown common bean in Brazil. Further, composted cattle manure as well as inorganic fertilizer showed non significant difference in nodule number (2.19), (2.39) and nodule dry weight (0.033), (0.017) respectively when compared to the control. In both parameters control showed the lowest numerical value as well as non significant difference in both nodule number (1.79) and nodule dry weight (0.010). Nodule dry weight did not show significance difference among all the treatments. Moreover, the present experiment was recorded considerable higher response to the seeds inoculated with Rhizobium by resulting large number of nodules per plant in comparison to uninoculated treatments specially in a field with no cropping history of Cowpea.

Table 06: Mean yield components, LSD and CV of Cowpea grown under different fertilizers

	No. of flowers/plant	No. of pods/ plant	Seeds/pod	100 seed weight (g)
Inorganic fertilizer	10.94 BA	10.28A	10.42 BA	9.9B
Composted cattle manure	9.97B	8.79A	9.45B	9.3B
Seed inoculated Rhizobium	12.93A	10.11A	11.13A	12.2A
Control	8.93B	8.48A	9.67BA	8.9B
LSD	2.77	3.007	1.67	1.26
CV (%)	18.77	23.17	11.91	9.10

Note: Means with the same letters along the columns are not significantly different at p>0.05. Means are the average of the five replicates.

Table 06 explain the mean yield components of the all three treatments and the control. Treatments with seed inoculated Rhizobium showed statistically significant increase in total number of flowers (12.93), seeds per pod (11.13) and 100 seed weight (12.2) when compared to the other treatments whereas Composted cattle manure had non-significant difference among number of flowers (9.97), seeds per pod (9.45) and 100 seed weight (9.3) whereas the same in Inorganic fertilizer having non-significant difference in total number of flowers (10.94), seeds per pod (10.42) and 100 seed weight (9.9). Further, control which had the lowest numerical numbers and non significant difference in total number of flowers (8.93), seeds per pod (9.67) and 100 seed weight (8.9). High vegetative growth owing to the availability of nitrogen in soil so as the end of the vegetative stage nitrogen availability was reduced in the soil. The plants were used the nitrogen for their growth.

The effect of different group of fertilizer application on pods per plant was non-significant for all treatments including control. Inorganic fertilizer treated plant had higher mean (10.28) value than the other treatments. These results were similar with the findings of Otieno et al, (2007) who reported total numbers of pods were not significantly increased in the legume species with the added nitrogen. Further, the same results were experienced by Kyei-Boahen et al., in 2017 by having non-significant difference among the treated plants with Fertilizer treated plant had higher number of pods per plant compared to Rhizobia, manure and the control.

Rhizobia inoculated as well as other fertilizer applied treatments resulted significantly higher grain yield relative to the control (Figure 1). Highest weight, 1678.3 kg/ha was produced by seed inoculated treatment and lowest mean weight, 1243.4 kg/ha observed in control treatment. Control treatment and composted cattle manure added treatment showed statistically similar result.

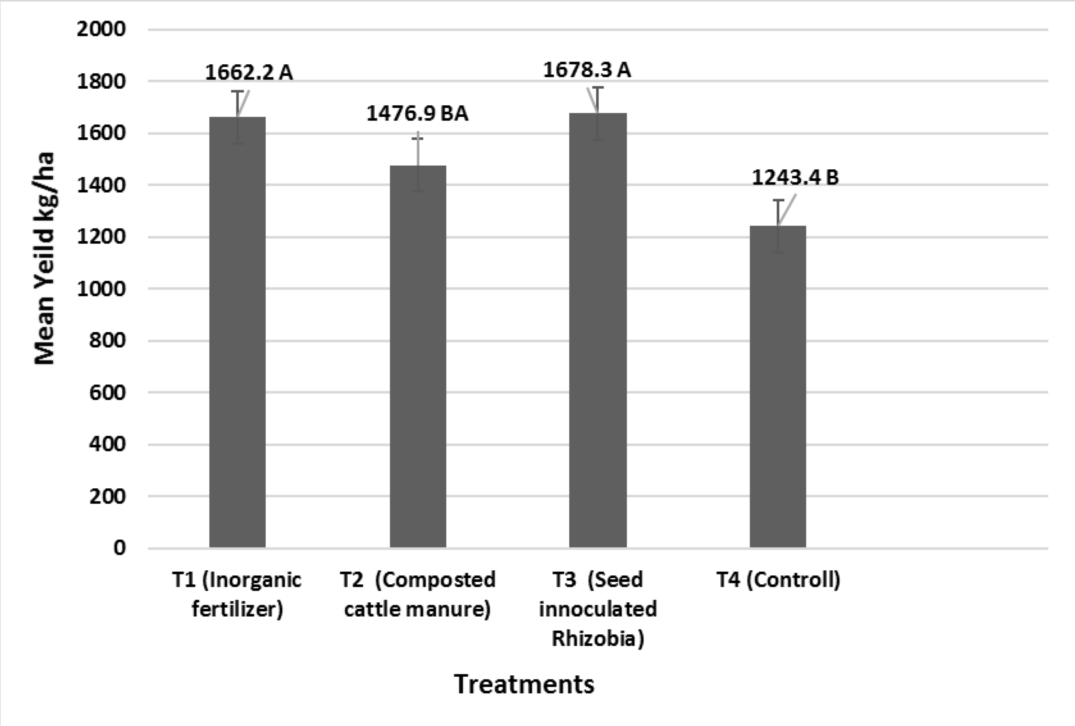

Figure 01: Mean yield of cowpea under different fertilizers

Table 07: Mean values of Seed Physical Properties, LSD and CV of Cowpea grown under different fertilizers

	Seed moisture %	**Cookability**	**Non soakers**	**Hydration coefficient**	**Total defects**
Inorganic fertilizer	0.05^{A}	127.7^{B}	5.1^{BA}	110.7^{BA}	9.2^{BA}
Composted cattle manure	0.03^{A}	123.3^{B}	3.8^{BC}	96.8^{B}	7.1^{B}
Seed inoculated Rhizobium	0.20^{A}	138.3^{A}	1.7^{C}	125.6^{A}	3.2^{B}
Control	0.23^{A}	121.3^{B}	6.5^{A}	100.2^{B}	11.2^{A}
LSD	0.42	8.46	2.41	15.43	3.84
CV (%)	230.53	4.81	40.94	10.34	36.30

Note: *Means with the same letters along the columns are not significantly different at p>0.05 Means are the average of the five replicates.*

Table 07 explaining the mean values of seed physical properties which is one of the most important parameter of consumable seeds. As per the results seeds inoculated with Rhizobium did not affect the moisture content of cowpea. Maximum mean value (0.21) was observed in control treatment and lowest mean value (0.03) was obsered in composted cattle manure treated experiments. Similar result were obtained in Faba bean by Elesheikh and Mohamedzein in 1998 and Fenugreek seeds by Abdelgani et. al. In 1999 as well as in ground nut seeds by Elsheikh and Ahamed, in 2000. However, few reports showed a significant increase in the moisture content of the seeds of inoculated plants (Elsheikh and Elzidany, 1997; Elsheikh and Ahamed, in 2000). Generally, the moisture content is affected by cultivar and the relative humidity of surrounding atmosphere at harvesting and storage. The cook ability of cowpea seed was significantly increased by inoculation with Rhizobia. Maximum cookability value (138.3) was in seed inoculated with Rhizobium. Lowest mean vales (121.3) was in control and the Inorganic Composted cattle manure as well as the control had statistically similar results. Similar results have been reported by Elsheikh and Elzidany in 1997 through a research done with groundnut in Sudan. Cookability is known to be affected by soaking time, type of water, time of cooking environmental factor, location and time of harvesting (Salih and Elmubarak, 1986, Elmubarark et al, 1988). The effect of different group of fertilizers on non-soaker percentage was significantly increased (6.5) in control teratment were are added with phosphorus only while lowest mean value (1.7) showed by the treastments innoculated with Rhizobium. The hydration coefficient whcih is a valuable attribute for both consumers and producers since it is a good indicator of seed quality because it plays a major role in defining the ability of the seed to absorb water and hence, become ready for the cooking process. Hydration coefficient of cowpea seeds were significantly diffrence in seed inoculated Rhizobium (126.6) relative to control (100.2). Low hydration coefficient indicates that's the seeds are not significantly capable of imbibing water when soaked (Elmubarak et al, 1998). Total defect range between 3.2 to 11.2% in different treatment shown in table number 7. The total defects followed a similar trend to that of the non-soakers' percentage. Control showed higher significant difference (11.2) than composted cattle manure (7.1) and seed inoculated Rhizobium (3.2).

Table 08: **Linear Correlation Coefficients of Seed Moisture Content, Cookability, Non-Soakers, Hydration coefficient and Total defects**

	Seed Moisture content %	Cookability %	Non-soakers %	Hydration coefficient %	Total defects %
Moisture content	-	0.31^{ns}	0.32^{ns}	0.10^{ns}	0.09^{ns}
Cookability	-	-	-0.36^{ns}	0.77^{***}	0.46^{*}
Non-soakers	-	-	-	-0.52^{*}	0.92^{***}
Hydration coefficient	-	-	-	-	-0.57^{**}
Total defects	-	-	-	-	-

**** Significant at P=0.0001 *Significant at P=0.05 ** Significant at P=0.01 ^{ns} non-significant at P=0.05*

Table 08 explain the correlation analysis among treatments applied for seed physical properties evaluation. Positive correlation was observed, for all treatments, between the non-soakers, hydration coefficient and cookability. The seed moisture content was non-significant with other seed physical properties. Cookability showed non-significant negative correlation with non-soaker seeds. The hydration coefficient showed a highly significant (p<0.0001) positive correlation with total defects. The hydration coefficient showed a significant positive correlation with cookability. Cookability showed a significant positive correlation with non-soaker seeds and nonsmoker seeds showed a highly significant (p<0.0001) positive correlation with total defects. The hydration coefficient showed a moderately significant (p<0.01) negative correlation with total defects.

4.0 CONCLUSION

Cowpea responded to inoculation in seeds containing Rhizobium showing positive interactions for most of the growth, yield, nodulation and seed physical properties and was more pronounced for yield and seed physical parameters. The study has demonstrated that using seed inoculant can enhance food security through increased grain yield and nutritional quality of many smallholder farmers in Sri Lanka. Furthermore, this management practice can contribute to the sustainability of the production system by replacing the inorganic fertilizer at least up to some level due to the reason of receiving higher yield from seed inoculated treatments. Farmers would benefit economically from using inoculant due to its low cost. Moreover, rhizobium inoculation is environmentally friendly application.

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