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Effectiveness of solid carriers on multiplication of bradyrhizobium mutant strain and nodulation of cowpea (*Vigna unguiculata* L)

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Effective nodulation of legume is usually solid carriers dependent. Peat, the most effective solid carrier is not readily available in Nigeria, hence, alternative solid carriers were evaluated with Bradyrhizobium strains using cowpea as a test crop. Mutant strains (USDA 3384 and USDA 3451) were evaluated in the laboratory using solid carriers and pot experiments. The survival (multiplication), infectivity and nodulating ability of the Bradyrhizobium mutant strains using cowpea as a test crop were assayed in peat, cowdung and composted maize cob. Mutant strains inoculated in the three solid carriers got to their peak of growth at 16th day of incubation. Peat significantly ($p<0.05$) increased the population of both USDA 3384 and USDA 3451 compared to cowdung and composted maize cob. USDA 3451 inoculated in cowdung significantly ($p<0.05$) increased multiplication of *Bradyrhizobium* mutant strain and nodulation of cowpea. Investigation shows it can be used as peat substitute when preparing inoculant. USDA 3384 inoculated in both composted maize cob and cowdung, respectively had consistent and significant positive effect on nodulation of cowpea and nodule dry weight. These results reveal their ability to subtitute peat. Hence, blind use of alternative solid carriers for inoculant preparations may hinder high infectivity and optimal nodulation which could facilitate positive effects of nitrogen fixation in low N tropical soil.

Key words: Solid carriers, Bradyrhizobium,survival, infectivity, nodulation.

INTRODUCTION

Tropical soils are inherently low in fertility. Constant exposure of soil of this region to forces of degradation which causes rapid deterioration in their physical, chemical and biological properties is due to the intensification of agricultural production (Obi and Ebo, 1995). Organic matter content and nutrient status especially related to nitrogen, which is largely important in crop production, are generally low in tropical soil (Lal

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and Kang, 1982; Henao and Baanante, 2006). The growing world population is presenting a challenge of adequate and balanced nutrition, thus, the need for sustainable crop production that will preserve the environment. The nitrogen reserve of agricultural soils must be replenished regularly in order to maintain crop production. Replacement of soil nitrogen can be accomplished by the addition of inorganic fertilizers and by biological nitrogen fixation (Giller et al., 2009).

More than 50% of inorganic N – fertilizer applied to remedy the problem of low soil nitrogen in the tropics polluted the environment (Ladha et al., 1998) through acidification of soil (Kennedy and Tchan, 1992) and nitrate pollution of ground water through leaching (Shrestha and Ladha, 1998). Under intensive agriculture, use of inorganic fertilizers alone has not been helpful because it aggravates soil degradation (Sharma and Mittra, 1991) due to loss of organic matter and resulting in soil acidity, nutrient imbalance and low crop yields (Ayoola and Makinde, 2007).

Biological nitrogen fixation (BNF) can be a major source of nitrogen to tropical soils especially when symbiotic N_2 – fixing systems are used. The contribution to soil nitrogen economy through this system is as high as 360 kg/ha (Bohlool et al., 1992). Biofertilizers, which are preparations containing living cells of efficient strain of microorganisms, such as *Rhizobium*, help crop plants to uptake nutrients (Jeyabal and Kuppswamy, 2001).

Problems asssociated with N_2-fixation in *rhizobium* - legumes symbiosis include inappropriate, specificity or insufficiency of strains of bradyrhizobium which can be corrected by inoculation of appropriate strains (Lindemann and Glover, 2003). Therefore the need to inoculate legumes with respect to specificity and sufficiency for effectiveness and infectivity have been observed and researchers on use of Rhizobium inoculants for production of grain legumes also reported its effectiveness in agronomic practice for ensuring adequate N nutrition of legumes, compared with the application of N fertilizer (Chianu et al., 2008).

Roy et al. (2010) discovered that population of *Bradyrhizobium* strains declined progressively shortly after introduction to the soil. This may be as a result of heterogenous and unpredictable environment of soil. For effective survival and multiplication of *rhizobium* strains, carriers are needed for inoculant preparation. This also helps to transfer sufficient number of desired *rhizobia* strain to target legumes (Brockwell and Bottomley, 1995). High water capacity, ability to foster multiplication in rhizobium, non-toxicity, easy to sterilize by autoclave or gamma irradiation, readily available and not expensive, pH buffering capacity and high adhesiveness for effective application on to seeds are characteristics of a good carrier, observed by Keyser et al. (1992). Research has discovered that peat has these qualities but has been on use for long, hence needs a substitute due to its unavailability in most countries.

Freire and Vernetti (1999), confirmed that liquid carrier inoculants are good for mechanical sowing. Some locally available agricultural materials like filtermud, bagasse, sawdust, coffee husks, coir dust, charcoal dust and forest soil were investigated in Kenya to select those which had similar characteristics to peat (Kibunja, 1991). Filtermud was observed to increase the survival rate of *Rhizobium phaseoli* as determined by plate count method.

Rhizobium strains differ in their ability to utilize whatever carbon-compound and other nutrient solid carriers, due to their different rate of metabolizing these carriers. A particular *Bradyrhizobium* strain can perform well in a particular solid carrier but not in another over a period of time. Apparently, there should be strict investigation on the preference of a particular *rhizobium* strain for different alternative solid carriers as substitute for peat. This research covered both laboratory and pot investigations of *Bradyrhizobia* strains survival, multiplication and their infectivity and nodulating ability in solid carriers using cowpea as a test crop.

MATERIALS AND METHODS

Soil sample

Low – N soil, used for the pot experiment (so as to relate some of the variables assessed) were taken at a depth of 0 -15 cm and were obtained from the Teaching and Research Farm, Department of Agronomy and Landscape Design, Babcock University, Ilishan Remo, Ogun State. Low – N soil's physical, chemical and biological characteristics were as follows: 720 g/kg Sand, 114 g/kg silt, 166 g/kg clay; pH in (H_2O) 5.7; 14 g/kg organic carbon; 8 mg/kg extractable P (Bray 1) soils; 1.0 g/kg total N and Exchangeable Ca, Mg, K were 14.6, 1.1, 3.0 Cmol/kg respectively while the indigenous *rhizobium* population was 10 viable cells/g soil (1×10^1 cfu/g).

Experimental design

The experiment was a completely randomised design replicated four times with a 3 × 2 factorial combination comprising of two *bradyrhizobium* strains (USDA 3384 and USDA 3451) and three solid carrier (peat, composted maize cob and cowdung).

The solid rhizobium carriers

The solid *Rhizobium* carrier namely peat (imported conventional carrier) was obtained from International Institute of Tropical Agriculture (IITA). Other two locally sourced possible alternative carriers, cowdung and composted maize cob were obtained from the Teaching and Research Farm of the Department of Agronomy and Landscape Design, Babcock University. They were autoclaved at 121°C for 30 min and were analysed for water holding capacity and other chemical properties as shown in Table 1

Determination of growth of bradyrhizobium mutant strains in solid carriers

Two parent *Bradyrhizobium* strains (obtained from Soil Microbiology Laboratory, IITA) which are the most infective (ability to nodulate) and effective (ability to fix nitrogen) in nodulation were developed

Table 1. Water holding capacity and other chemical properties of the three solid carriers.

Properties	Peat	Composted maize cob	Cowdung
Water holding capacity (%)	53	75	75
pH in H_2O	6.20	6.72	9.24
Total nitrogen (%)	2.35	4.51	5.03
Organic carbon (%)	61.78	37.52	23.78
Total P (%)	0.28	0.95	0.31
Ca (%)	0.01	3.59	0.71
Mg (%)	0.31	1.56	1.11
K (%)	0.62	0.52	3.13
Na (mg/g)	555.88	967.30	762.96

into mutant strains by screening them using Mannitol Yeast Extract Agar (A selective medium for isolation and quantification of bradyrhizobium spp) with various antibiotics concentrations (Tas et al., 1996). Three ratio two (3:2) w/v of each of the six treatments, which was the mixture of 240 g solid *bradyrhizobium* carrier and 160 ml of seven day old broth culture of the two mutant strains, were introduced into sterilized flasks and incubated at a temperature of 24 ± 2°C in a lab laminar flow. At 8, 16 and 24 days after incubation, 1.0 g of the mixtures was aseptically taken to make serial dilution (10^{-2} to 10^{-8}) according to the procedure of Vincent (1970). For the determination of the mutant (isolate) population in the carriers, 1.0 ml of each of 10^{-7} and 10^{-8} dilution were pipetted out in triplicates into sterile Petri dishes and 15 ml of melted MYEA was added (Packialakshmi and Riswana, 2014). The diluent and the agar were thoroughly mixed before the plates were incubated invertedly (two to five days incubation) at 28°C.

Effect of solid carriers/mutant strain mixture on nodulation of cowpea

Treatment combination was formulated from the solid carriers and mutant factor and they were assessed in terms of root nodulation (number and dry weight), and %strain nodule occupancy of *Vigna unguiculata* species.

One hundred and sixty millilitres (160 ml) of a seven days old broth culture of the infective mutant (isolate) for the two different stains USDA 3384 (3.0×10^{10} cell/ml) and USDA 3451 (3.3×10^{10} cell/ml) were aseptically dispensed into 240 g of each of the three sterilized solid carriers (2:1 w/v), respectively. These mixtures were incubated at room temperature for 16 days before use. Basal rate of urea (20 kg N/ha), muriate of potash (30 kg K/ha) and single super phosphate (40 kg P/ha) were thoroughly mixed with bulk (640 kg) air dried soil passed through 2 mm sieve. Five kilogrammes (5 kg) portion of the soil was introduced into pots perforated basally to facilitate drainage of any excess water.

Surface sterilized cowpea seeds of the Ife Bimpe variety were used for this experiment which was moistened with 30% gum arabic. The seeds were coated with each of the three solid carrier / mutant treatment and were spread aseptically inside the laminar flow to dry overnight. After this time, four seeds were sown 1 to 1.5 cm deep into the soil in each pot. The pots were watered when necessary after seed sowing. Ten days after sowing, the seedlings were thinned to two per pot. To ensure high *bradyrhizobial* population in the rhizosphere, 4 ml of *rhizobial* broth culture of each mutant was dispensed per seedling in each pot.

Measurement and harvest

Number of plant nodules were assessed two weeks after sowing (WAS) while the plant nodule dry weight and root dry matter yield were assessed at 6 and 8 WAS. Eight nodules were chosen randomly and detached from each of the treatment at 8 WAS, they were surface sterilized and used for typing back nodule strain. Each of the nodules chosen was crushed in 1 ml of sterile saline water (0.85%) and a loopful of the suspension was cultured using the spread plate method on the antibiotic amended agar. *Bradyrhizobial* growth was examined after incubation for five days at 28°C. The number of nodules that formed colonies per treatment in the plates were counted and data collected was used for the computation of percent strain nodule occupancy. This was equated to the proportion of nodules formed by the mutant strain on the cowpea species used (Typing back).

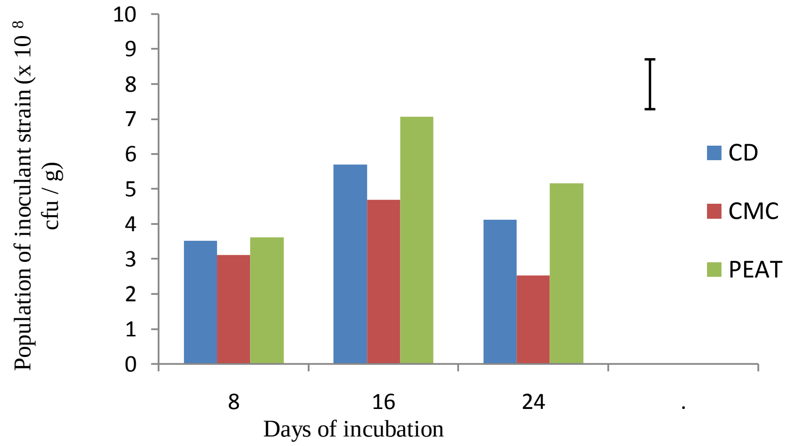
Statistical analyses

Data collected were subjected to analysis of variance (ANOVA) and descriptive statistics while treatment means were separated using Duncan multiple range test (DMRT) at 5% probability level.

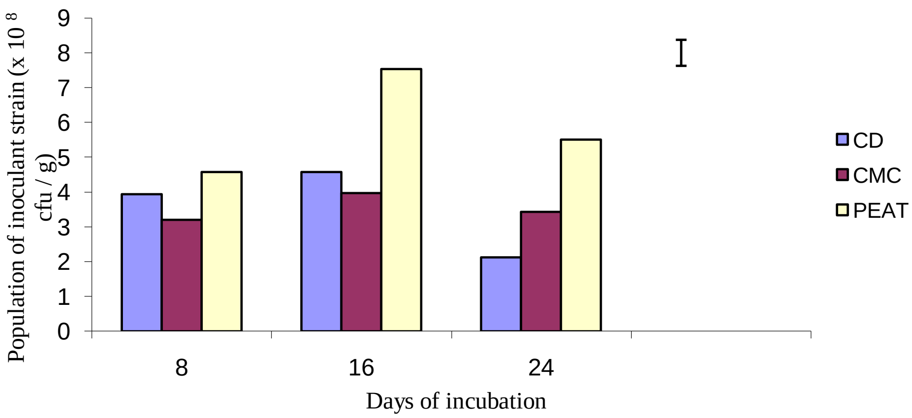
RESULTS AND DISCUSSION

Water holding capacity of peat was the lowest of the three carriers. Composted maize cob and cowdung were similar in their water holding capacity (Table 1). pH showed that cowdung was alkaline, composted maize cob was near neutral while peat was slightly acidic. Total nitrogen was lowest in peat and highest in cowdung while composted maize cob was in between but almost twice that of peat. Organic carbon was highest in peat, lowest in cowdung and intermediate in composted maize cob. Total P, Mg and Na were highest in composted maize cob, lowest in peat and intermediate in cowdung. Highest Ca was obtained in composted maize cob followed by cowdung with the observed in lowest in peat. K was highest in cowdung followed by peat, the lowest was observed in composted maize cob.

At 8 and 16 days of post - inoculation, the trend of population of the two isolates were similar in all the three solid carriers, but at 24 days post - inoculation, composted maize cob enhanced the population of USDA 3541 more than cowdung, whereas, the trend of peat > cowdung > composted maize cob was observed with USDA 3384. In both strains, the highest population of



(a) USDA 3384



(b) USDA 3451

Figure 1. Effect of incubation period of Bradyrhizobium strain in peat, composted maize cob and cowdung solid carriers. LSD (0.05). CD = cowdung; CMC = composted maize cob; USDA = United State Department of Agriculture; Bar represent Least Significant Different (LSD).

Bradyrhizobium was obtained at 16th day after incubation (Figure 1). Hence, the strains differ in their ability to grow in any given medium which is facilitated by their environmental and nutritional needs (Richardson, 2001). Figueiredo et al. (1992) and Khavazi et al. (2007) also reported that the chemical and physical characteristics of alternative carrier materials determines the maintenance of initial concentrations of *Bradyrhizobium* cells in inoculants. It is evident that the ability of the mutant strains to grow in a medium depending on their specific need significantly resulted in their differential growth. None of the solid carriers used was significantly higher compared to peat in terms of promoting mutant survival and growth in carriers. This is in line with the findings of Brockwell and Bottomley (1995), who observed that peat was the most suitable solid carrier for inoculant production.

At two weeks after sowing, the highest nodule number was obtained in cowpea when composted maize cob was used as solid carrier for strain USDA 3384 while the lowest was also obtained using composted maize cob as solid carrier for strain USDA 3451 (Table 2). Number of nodules obtained with peat and cowdung were not significantly different. At 4 weeks after sowing, the highest nodule number was obtained when cowdung was used as carrier with mutant strain USDA 3384 which was not significantly different from other treatments except composted maize cob and cowdung with strain USDA 3451. At six weeks after sowing, nodule numbers reduced compared to those obtained at 4 weeks after sowing. Despite the highest number of nodules obtained with cowdung using strain USDA 3451, there was no significant difference among the treatments. At eight weeks after sowing, the highest number of nodules was obained on cowdung with strain USDA 3384, but this was not signifcantly different from composted maize cob with the same strain as well as cowdung with strain USDA 3451. Peat as solid carrier at the early stage of cowpea

Table 2. Effect of Bradyrhizobium mutant strains with three solid carriers on number of nodules/plant of cowpea at different weeks after sowing.

Treatment	Weeks afer sowing			
	2	4	6	8
C1M1	54.3[b]	92.8[a]	71.5[ab]	76.8[b]
C2M1	64.3[a]	94.1[a]	72.6[ab]	92.6[ab]
C3M1	52.8[b]	96.0[a]	65.1[abc]	106.3[a]
C1M2	54.8[b]	94.0[a]	58.8[abc]	72.4[b]
C2M2	22.9[c]	50.9[bc]	58.6[abc]	40.6[c]
C3M2	52.3[b]	60.0[b]	76.0[a]	97.0[ab]

Means with same letter(s) in a column are not significantly different at 5% level of probability according to Duncan multiple range test (DMRT). C1M1 = peat/mutant strain 1; C2M1 = composted maize cob/mutant strain 1; C3M1 = cowdung/mutant strain 1; mutant strain 1 = USDA 3384; C1M2 = peat/mutant strain 2; C2M2 = composted maize cob/mutant strain 2; C3M2 = cowdung/ mutant strain 2; Mutant strain 2 = USDA 3451.

Table 3. Effect of Bradyrhizobium mutant strains with three solid carriers on dry weight of nodules (g) of cowpea at different weeks after sowing.

Treatment	Weeks after sowing		
	4	6	8
C1M1	2.2[a]	1.1[a]	2.0[ab]
C2M1	2.1[ab]	1.2[a]	2.1[a]
C3M1	1.6[c]	1.1[a]	1.8[ab]
C1M2	1.6[bc]	1.3[a]	2.1[a]
C2M2	1.0d	1.1[a]	1.2[bc]
C2M3	1.6[c]	1.1[a]	1.8[ab]

Means with same letter (s) in a column are not significantly different at 5% level of probability according to Duncan multiple range test (DMRT). C1M1 = peat/mutant strain 1; C2M1 = composted maize; cob/mutant strain 1; C3M1 = cowdung/mutant strain 1; mutant strain 1 = USDA 3384; C1M2 = peat/mutant strain 2; C2M2 = composted maize cob/mutant strain 2; C3M2 = cowdung/mutant strain 2; mutant strain 2 = USDA 3451.

growth did not provide a significantly higher number of nodules compared to other treatments. The increase in performance observed in cowdung was similar to the findings of Dharma (1996) who reported that farmyard manure (cowdung) has the ability to stimulate the activities of microorganisms which can help make nutrients readily available to plant, thus increasing cowpea nodulation. This result also corresponds with the earlier work where it was discovered that rhizobum inoculation can improve nodulation, N_2 fixation and yield (Delić et al., 2009)

At six weeks after sowing, all treatments were significantly different with respect to nodule dry weight (Table 3). At four weeks after sowing, the highest nodule dry weight was obtained under the peat as carrier with mutant strain USDA 3384, while the lowest was observed on composted maize cob with mutant strain USDA 3451. The lowest nodule dry weight was significantly lower compared to other nodule dry weights, while the highest nodule dry weight was also significantly higher compared to other treatments except composted maize cob with mutant strain USDA 3384. At eight weeks after sowing, only composted maize cob with mutant strain USDA 3451 was significantly lower compared to treatments with the highest nodule dry weight.

The root dry weight of cowpea under peat as solid carrier with mutant strain USDA 3451 was the highest at six weeks after sowing but it was only significantly higher than the root dry weight of plants under composted maize cob with mutant strain 3451. Other treatments were not significantly different (Table 4). At eight weeks after sowing, the composted maize cob with USDA 3384 inoculated plant had the highest root dry weight which was significantly higher than other treatments except peat with USDA 3451. Other treatments were not significantly different. Increase in root dry weight in composted maize cob and cowdung could be due to their high nitrogen content (Table 1). Similar work was reported by Shaukat (1994) on nitrogen effect on root weight. Growth and development of crops depend largely on development of root system hence the need for major macro and micro nutrients which solid carriers can provide is high

Table 4. Effect of Bradyrhizobium mutant strains with three solid carriers on root dry weight/plant of cowpea at different weeks after sowing.

Treatments	Root dry weight /plant	
	6 Weeks after sowing	8 Weeks after sowing
C1M1	1.9[a]	2.1[bc]
C2M1	1.6[ab]	3.0[a]
C3M1	1.8[a]	2.0[bc]
C1M2	2.0[a]	2.8[ab]
C2M2	1.2[b]	1.8[bc]
C2M	1.7[ab]	2.1[bc]

Means with same letter(s) in a column are not significantly different at 5% level of probability according to Duncan Multiple Range Test (DMRT). C1M1 = peat/mutant strain 1; C2M1 = composted maize cob/ mutant strain 1; C3M1 = cowdung/ mutant strain 1; mutant strain 1 = USDA 3384; C1M2 = peat/mutant strain 2; C2M2 = composted maize cob/ mutant strain 2; C3M2 = cowdung/ mutant strain 2; mutant strain 2 = USDA 3451.

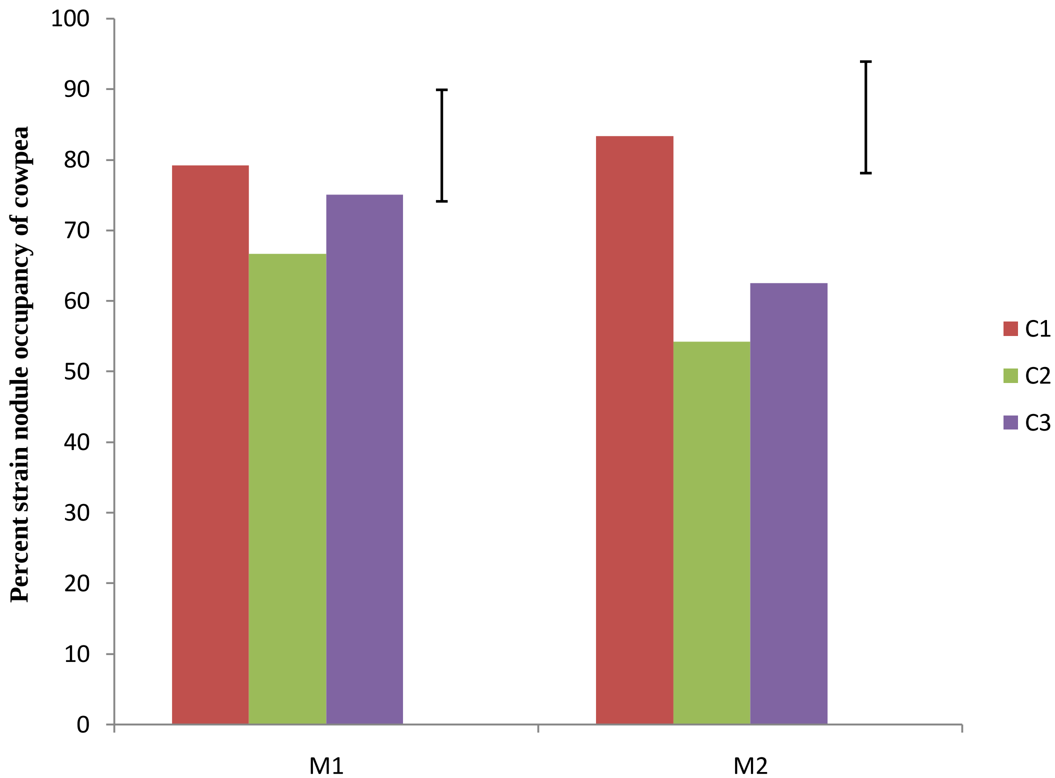


Figure 2. Effect of Bradyrhizobium mutant strains with three solid carriers on nodule occupancy of cowpea. LSD (0.05); C1 = peat; C2 = composted maize cob; C3 = cowdung; Bar represent Least Significant Difference; M1 = Mutant strain USDA3384; M2= Mutant strain USDA3451.

(Veeramani and Subrahmaniyan, 2011).

The relative competitiveness or contribution of the introduced strain to legume nodulation in the presence of soil native *Rhizobium* can be marked by an index known as percent strain nodule occupancy. USDA 3384 inoculated with peat was responsible for 79.17% strain nodule occupancy compared to USDA 3384 inoculated with cowdung and composted maize cob, which resulted to 75.0 and 66.67% strain nodule occupancy, respectively (Figure 2). This might be due to the fact that rhizobium is required in sufficient number for optimal nodulation and efficient nitrogen fixation, especially when a legume is being cultivated on a particular soil or land for the first time (Catroux et al., 2001). Strain nodule occupancy significantly increased up to 83.33% when USDA 3451 was inoculated with peat compared to USDA 3451 inoculated with cowdung, which was responsible for 62.50% strain nodule occupancy and USDA 3451 inoculated with composted maize cob, which resulted in 54.17% strain nodule occupancy. Peat maintained its superiority as the most suitable carrier (Smith, 1992), resulting in best performance in respect to its percentage strain nodule occupancy. However, cowdung also showed that it can be a suitable peat replacement when USDA 3451 is used, compared to composted maize cob (Stephens and Rask, 2000).

Most of the percent strain nodule occupancy obtained in this experiment fall within 47 to 80% as reported by Jensen and Sorensen (1987). The high percent strain nodule occupancy obtained indicates that the mutant strains (USDA 3384 and USDA 3451) used for inoculant preparation in this experiment possess the ability to compete favourably with indigenous rhizobium present in the soil and are capable of forming nodules on a plant host (Martensson, 1989), hence have high ability of fixing nitrogen with their target host legume (Howieson et al., 2000). An obvious explanation for this could be because the introduced *Bradyrhizobium* strains used for this experiment which was higher in population (compared to native rhizobium which was as low as 1×10^{1} cfu/g in population) most probably enhanced by use of solid carriers resulted to high percent strain nodule occupancy, since use of carrier based inoculant determines the transfer of the desired *rhizobium* strain with sufficient numbers to targeted legume (Brockwell and Bottomley, 1995).

Conclusion

The use of the three solid carriers for *Bradyrhizobium* inoculation showed a promising outcome with respect to infectivity and nodulation, dry nodule weight, root dry weight and percent strain nodule occupancy in cowpea. Both composted maize cob and cowdung showed qualities as peat (suitable carrier according to Brockwell and Bottomley (1995) substitutes when USDA 3384 strain was used for inoculation with respect to all the parameters, while cow dung was preferable as a peat substitute when USDA 3451 was used for inoculant preparation with respect to the majority of estimated parameters. *Bradyrhizobium* strains' compatibility with solid carrier should be ascertained before using such solid carrier for inoculant preparation. This will help to avoid blind use of solid carrier which can cause inefficiency of introduced strains when used for inoculation of legumes.

Conflict of Interests

The authors have not declared any conflict of interests.

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