RESISTANCE OF TWO *B. japonicum* STRAINS TO ANTIBIOTICS AND THEIR EFFECT WITH OR WITHOUT SOLID CARRIERS ON GROWTH AND BIOMASS YIELD OF COWPEA.

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ABSTRACT

RESISTANCE OF TWO *B. japonicum* STRAINS TO ANTIBIOTICS AND THEIR EFFECT WITH OR WITHOUT SOLID CARRIERS ON GROWTH AND BIOMASS YIELD OF COWPEA.

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Biological Nitrogen Fixation (BNF) is one of the major source of nitrogen to inherently low tropical soils (Bohool et al.,1992). The level of resistances of microorganisms to diferent concentrations of antibiotics can be a factor for determining the degree of tolerance of microbes to stressfull environment, especially microbes which take part in BNF. Carriers are also required for effective inoculant preparation because they facilitate the survival and multiplication of rhizobium strains(microbes). Parent rhizobium strains USDA 3384 and USDA 3451 were exposed to antibiotics at different concentrations to develop (to determine their intrinsic resistance level) mutant strains which were further seeded into three different solid carriers (Peat, composted maize cob and cowdung). Mutant strains inoculated with the solid carriers and mutant strains inoculated without the solid carriers were evaluated in the laboratory and pot experiments and their effect with or without solid carriers on growth and dry matter yield of cowpea (Ife bimpe) were assayed. Data on plant growth and dry matter yield of cowpea were analysed using ANOVA and means were separated using Duncan Multiple Range Test (DMRT) at 5 % probability level.

USDA 3384 and USDA 3451 were resistant to Ampicillin trihydrate pensy at 500 mg/l and Streptomycin sulphate at 1000 mg/l respectively. Use of solid carriers significantly influenced ($p<0.05$) the various parameters investigated positively compared to mutant strains inoculated without solid carriers. The strains investigated responded to antibiotics differently which shows the extent at which each of the stains can survive environmental stress. Use of solid carriers for inoculant preparation is better compared to inoculation without solid carrier this will always influence positive plant growth and optimal yield.

Key word: Antibiotics, Bradyrhizobium, Cowpea, , Growth, Solid carrier

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INTRODUCTION

Several environmental conditions facilitate limitations to the growth and activity of the N_2-fixing plants. Crop production level can not be more than that allowed by the maximum limiting factor"[1]. In a N_2-fixing system,especially in the *Rhizobium*-legume symbiosis the process of N_2 fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors such as mineral toxicity, salinity, unfavorable soil pH, temperature extremes, nutrient deficiency, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing are imposing limitations on the vigor of the host legume [1,2,3]. When studying any living organism, it is important to know how each species grows and responds to certain conditions that can be found in their natural environment. Ascertaining how bacteria respond to environmental signals, or stressful conditions, is a vital part to understanding how those microbes live, thrive and survive. Every bacterium has optimum conditions that make this process easier. However, in order to survive in a changing environment (or any other form of stress), the bacteria must be able to adapt.

Antibiotics have been used increasingly in agriculture to create selective pressure in the environment which leads to the mutation in organism thus allowing the microbes to survive better and hence multiply. Presence of Genes on a plasmid, are important survival make up of organisms in an environment with multiple stresses, which are most likely transferred together in the event of conjugation. Thus, it would be more ecologically favorable in terms of survival for a bacterium to acquire resistance to any environmental stresses. Studies have shown that the level of resistances of microorganisms to antibiotics and their diferent concentrations can be a factor for determining the degree of tolerance of microbes to stressfull environment[4,5,6].

Cropping history of a given soil determines the population of rhizobia present in such soil. Studies have shown that population of indigenous rhizobia may be low or absent in a particular soil. This may occur due to unfavourable conditions especially when legume crops such as cowpea is planted in such soil for the first time [7].

Inoculation of legume with high population of specific and efficient strains of rhizobia is important for optimal and improve crop productivity which can be enhanced by appropriate carriers.

Carriers are required for effective inoculant preparation because they facilitate the survival and multiplication of rhizobium strains and also determines the transfer of the desired rhizobia strain in sufficient number to the targeted legume [8]. Peat, the conventional solid carrier has been used for more than 9 decade due to its ability to foster multiplication of rhizobia. However, peat is not readily available in most countries, hence the evaluation of locally available solid materials is important for use as substitute for inoculant preparation. Kibunja [9] reported that some locally available materials can be used as substitutes for peat in legume inoculant production. Among the materials investigated, filtermud was significantly better with comparable survival rate of *Rhizobium phaseoli* to that in peat, The objective of this investigation was to assay two strains of *B.japonicum* strains on their intrinsic resistance to different concentrations of selected antibiotics and also to evaluate their effect with or without solid carriers on growth and biomass of cowpea.

MATERIALS AND METHODS

Development of Antibiotic Resistant Strains

Two parent Bradyrhizobium strains (obtained from Soil Microbiology Laboratory, International Institute of Tropical Agriculture, Ibadan (IITA) which are of most infective (ability to nodulate) and effective (ability to fix nitrogen) in nodulation were exposed to antibiotics: Streptomycin sulphate and Ampicillin trihydrate pensy of the following concentrations 250, 500, 1000 and 1500 mg/l from a stock solution of 10,000 mg/l antibiotic which were used to amend 500 mls of prepared Mannitol Yeast Extract Agar (MYEA) respectively. Antibiotics were sterilized using membrane filtration (0.2 µm size). Melted antibiotic amended agar was poured into sterile Petri dish in duplicate (15 ml each) and was allowed to set. Following the procedure of [10] the parent strains were streaked aseptically on the set agar and incubated at 28 ^{o}C for 7 days after which Resistances (+) or susceptibilities (-) of the strains to the antibiotic concentrations (250 – 1500 mg/l) were scored.

Characteristics of experimental soil

Low – N soil, used for the pot experiment was collected at a depth of 0 -15 cm from the Teaching and Research Farm, Department of Agronomy and Landscape Design, Babcock University, Ilishan Remo, Ogun State. The selected soil physical, chemical and biological characteristics were as follows: 720 g/kg Sand, 114 g/kg Silt, 166 g/kg Clay; pH in (H_20) 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 Cmol/kg respectively) while the Indigenous rhizobium population was 10 viable cells/g soil. The physical and chemical analysis of soil were carry out according to the method [11] while the biological assay was done according to [12].

Solid Carriers

Selected solid carrier assayed were cowdung, composted maize cob and no carrier while peat was used as a reference carrier. They were analysed for water holding capacity, pH reaction, organic carbon, percent nitrogen and for other macro and micro nutrients.

Effect of Antibiotics Resistant strains with or without solid carriers on growth and biomass yield of Cowpea

Two hundred and fourty grammes of each of the three sterilized solid carriers were aseptically mixed with hundred and sixty millilitres (160 mls) suspension of 7 day old broth culture of the infective antibiotics resistant strains (isolate) for the two parent strains (3:2 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 mixed with bulk (640 kg) air dried soil passed through 2 mm screen. Five kilogrammes (5 kg) portion of the soil was introduced into pots perforated basally to facilitate drainage of excess water.

Surface sterilized cowpea seeds of the Ife Bimpe variety was used for this experiment which was moistened with 30 % gum arabic. They were coated with each of the three solid carrier / antibiotics resistant strains. The seeds were spread aseptically inside the laminar flow to dry over night. Four seeds were sown at approximately 2 cm depth the 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 antibiotics resistant strains were dispensed per seedling in each pot.

Experimental Design

The experiment was a 2 × 4 factorial using completely randomised design with four replicates. The factors were two strains of *Bradyrhizobium* and four levels of carriers. USDA 3384 strain was resistant to ampicillin at 500mg/l while USDA 3451 strain was resistant to streptomycin at 1000mg/l. The carriers were peat (reference carrier) cowdung, composted maize cob, and no carrier.

Measurement and Harvest

Plant height and number of leaves were determined at 2 weeks interval till the 8th week after sowing (WAS), while shoot and root dry matter yield were assessed at 6 and 8 WAS. Percent (%) tissue N of cowpea used was also determined at 6 and 8 WAS. Shoot and root dry weight were taken after oven drying the samples at 70 ^oC for 48 hours. Percent (%) tissue N of cowpea was determined after digestion of the samples with concentrated H_2SO_4 in the presence of catalyst [13].

Statistical Analyses

Data collected were subjected to ANOVA while treatment means were separated using Duncan Multiple Range Test (DMRT) at 5 % probability level.

Result and Discussion

Using moderate growth as standard, (USDA 3384) was resistant to Ampicillin trihydrate pensy at 500 mg/l while (USDA 3451) was resistant to Steptomycin sulphate at 1000 mg/l (Table 1) and they are similar to the finding of [14]. The resistance of rhizobial strains to antibiotics is being widely used in investigation of their survival in soil.

At two weeks after sowing, the lowest height of cowpea was observed when USDA 3384 was inoculated without solid carrier (Table 2). It was significantly lower compared to other treatments except when USDA 3451 was inoculated without solid carrier and USDA 3384 with cowdung as solid carrier. Inoculation of USDA 3384 with peat and composted maize cob significantly increased height of cowpea although they were not significantly different from USDA 3384 inoculated with cowdung as solid carrier. Inoculation of USDA 3451 with or without solid carrier did not significantly affect height of cowpea although inoculation of USDA 3451 with solid carriers resulted in increase in height of cowpea compared to when it was inoculated without solid carriers.

Inoculation of USDA 3384 and USDA 3451 without solid carrier resulted in significantly lower height of cowpea at four weeks after sowing compared to other treatments (Table 2). Height of cowpea was significantly increased when USDA 3451 was inoculated with peat as solid carrier compared to inoculation of USDA 3451 with cowdung as solid carrier. Inoculation of USDA 3384 with solid carriers within the treatments at four weeks after sowing significantly increased height of cowpea compared to inoculation of USDA 3384 without solid carriers.

At six weeks after sowing, inoculation of USDA 3451 with peat as solid carrier significantly increased height of cowpea compared to other treatments but was similar to when USDA 3384 was inoculated with composted maize cob and peat respectively (Table 2). Height of cowpea was significantly lower when USDA 3384 was inoculated without solid carrier compared to when it was inoculated with solid carriers except cowdung. Peat and composted maize cob used in inoculation of USDA 3384 showed similar trend with respect to increase in height of cowpea compared to USDA 3384 with cowdung as a solid carrier. When USDA 3451 was inoculated with peat as solid carrier height of cowpea significantly increased compared to other treatments, however USDA 3451 inoculated with cowdung increased height of cowpea compared to that of composted maize cob and USDA 3451 inoculated without solid carrier.

At eight weeks after sowing, there was no significant difference in the least height of plants under USDA 3384 having cowdung as well as that of USDA 3451 with composted maize cob except when both strains were without solid carriers. In addition, height of cowpea at 8 WAS under 3451 strain when peat was used as solid carrier was not significantly different from that of composted maize cob as carrrier (Table 2). Inoculation of USDA 3451 with or without solid carriers had no significant effect on height of cowpea, however, when USDA 3451 was inoculated with peat and cowdung respectively they resulted in higher plant height compared to USDA 3451 with composted maize cob and USDA 3451 without solid carrier. Generally, mutant strains inoculated with any of the solid carriers resulted to higher performance compared to when mutant strains (USDA 3384 and USDA 3451) were inoculated without solid carrier with respect to plant height at different weeks after sowing (Table 2). This indicates mutant strains preference for solid carrier (Peat, composted maize cob and cowdung) with respect to their pattern of influence on plant height compared to mutant strains without solid carrier. Inoculation of both strains with peat supported high performance with significant ($p<0.05$) increase in plant height which comfirms the findings of [1] who reported that Peat is the best solid carrier with respect to it characterics and functions, however the consistent positive performance of cowdung in both strains indicates its ability to stand as a close substitute for peat compared to composted maize cob. Usually, organisms occur where ever organic matter (solid carriers) are present, microorganisms are concentrated in litter, on humus, on the surface of soil aggregates and in spaces between aggregates, around root zones [15].

The lowest number of leaves were observed at two and four weeks after sowing when USDA 3384 was inoculated without solid carrier and was not significantly different from that of USDA 3451 without solid carrier (Table 3). USDA 3384 inoculated with any of the solid carriers significantly increased number of leaves compared to when USDA 3384 was inoculated without any of the solid carriers. Similar trend was observed when USDA 3451 was used for inoculation. Highest significant number of leaves were recorded when USDA 3451 was inoculated with cowdung compared to other treatments at six weeks after sowing. However, Inoculation of both USDA 3384 and USDA 3451 with peat resulted to an increase in number of leaves although they not significantly different from other treatments (Table 3).

At eight weeks after sowing, USDA 3384 inoculated with composted maize cob significantly increased root dry weight compared to other treatments except when USDA 3451 was inoculated with peat (Table 4). The lowest root dry weight was observed when USDA 3384 was inoculated without solid carrier. On the other hand, USDA 3451 resulted to increased root dry weight when cowdung was used for inoculation compared to composted maize cob (Table 4). Increase in root dry weight in composted maize cob and cowdung could be due to their high nitrogen content. Similar work has been reported by [16]. 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 [17].

Shoot dry weight was significantly lower at eight weeks after sowing when USDA 3384 was inoculated without solid carrier and also when USDA 3451 was inoculated with composted maize cob. USDA 3384 inoculated with composted maize cob increased shoot dry weight compared to when it was inoculated with other solid carrier although it was not significantly different from them. USDA 3451 inoculated with peat increased shoot dry weight although it was not significantly different from shoot dry weight obtained when inoculated with cowdung.

Mutant strains inoculated without solid carrier resulted in significantly reduced shoot dry weight unlike when solid carrier base inoculants were used. This could be due to the fact that strains require solid carrier which determines their rate of survival and transfer to legume for effective shoot dry weight productivity. This finding is consistent with the work of [18] who reported that solid carriers facilitates multiplication and survival of rhizobium. USDA 3451 also maintained its preference for cowdung in comparison with composted maize cob with respect to shoot dry weight yield. The more positive result observed with the use of cowdung might be due to its higher potassium content compared to composted maize cob, hence its ability to maintain increase in plant vigour resulting in increase in shoot dry weight by the two strains [19].

At eight weeks after sowing, USDA 3384 inoculated without solid carrier significantly reduced shoot tissue N compared to other treatments with exception of shoot tissue N obtained when USDA 3384 was inoculated with cowdung and when USDA 3451 was inoculated without solid carrier (Table 4). USDA 3384 inoculated with composted maize cob resulted to significantly increased shoot tissue N compared to cowdung and USDA 3384 without solid carrier but was not significantly different from shoot tissue N observed when USDA 3384 was inoculated with peat. USDA 3451 with cowdung as solid carrier significantly increased shoot tissue N which was not significantly different from that obtained from peat. Significant increase in shoot tissue N observed at eight weeks after sowing from USDA 3384 with composted maize cob as solid carrier and USDA 3451 with cowdung as solid carrier respectively could be as a result of preference of mutant strains for solid carriers. This corresponds to similar work by [20] who reported that *Rhizobium* strains differed in their ability to metabolize carriers, hence preference for different solid carriers.

CONCLUSION

The strains investigated responded to antibiotics differently. Response of the strains to solid carriers also vary. USDA3381 was more responsive to composed maize cob while USDA 3451 was more responsive to cowdung.

Inoculation with the use of solid carrier performed better with respect to all parameters which includes plant height, number of leaves, dry matter yield (shoot and root), shoot tissue N compared to when no carrier was used.

Results from this study shows that *the strains investigated responded to antibiotics differently which shows the strength of each of the strains and the level of their ability to adapt and survive environmental stress.* Apart from peat which is the conventional solid carrier, some locally available agricultural materials can also be used as substitutes for peat in legume inoculant production instead of preparing inoculant without solid carriers. This will help in production of higher inoculant during legume inoculation which can increase nitrogen in low N soil. This is required to increase crop prodductivity.

Table 1: Growth Pattern of Parent Bradyrhizobium Strains in Antibiotics Amended Agar

Rhizobium Strains	MYEA/Amp concentration/mg/l				MYEA/ Streptomycin concentrationIn mg/l				Plain Agar	
	250	500	1000	1500	250	500	1000	1500		
USDA 3384	+	++	+	-	+	+	+	+	+++	+++
USDA 3451	+	+	-	-	+	+	++	-	+++	+++

LEGEND

+ Slight growth

++ moderate growth

+++ Luxuriant growth

- No growth (Susceptible)

MYEA = mannitol yeast extract Agar

Table 2: Effect of Bradyrhizobium Mutant Strains with or without Solid Carriers on Height (cm) of Cowpea at Different Weeks after Sowing

Bradyrhizobium Strains	Carriers	Weeks After Sowing			
		2	4	6	8
USDA 3384	None	14.1b	23.0c	30.7c	36.5c
USDA 3384	Peat	18.6a	33.5ab	56.5ab	104.1a
USDA 3384	Composted Maize cob	19.4a	34.2ab	57.1ab	98.0a
USDA 3384	Cowdung	17.1ab	34.1ab	45.3bc	61.8bc
USDA 3451	None	16.3ab	22.8c	41.3bc	60.1bc
USDA 3451	Peat	19.7a	35.5ab	66.2a	73.0ab
USDA 3451	Composted Maize cob	19.0a	31.5ab	41.7bc	50.0bc
USDA 3451	Cowdung	18.6a	30.6b	44.2bc	73.0ab
ANOVA					
Solid carrier		*	**	**	**
Bradyrhizobium		NS	**NS**	*	NS
BS*C		**NS**	**	NS	***

Means with same letter(s) in a column are not significantly different at 5 % level of probability by Duncan Multiple Range Test (DMRT)

NS = Not significant, ***** = p> 0.05, ****** = p> 0.01, ******* = p> 0.001

Table 3: Effect of Bradyrhizobium Mutant Strains With or Without Solid Carriers on Number of Leaves of Cowpea at Different Weeks after Sowing

Bradyrhizobium Strain	Carriers	Weeks After Sowing		
		2	4	6
USDA 3384	None	5.0b	13.0b	25.0b
USDA 3384	Peat	8.0a	21.3a	37.6b
USDA 3384	Composted maize cob	8.0a	22.5a	42.0b
USDA 3384	Cowdung	8.0a	21.4a	34.4b
USDA 3451	None	5.0b	13.0b	28.1b
USDA3451	Peat	8.0a	21.6a	41.9a
USDA 3451	Composted maize cob	8.0a	21.5a	40.0b
USDA 3451	Cowdung	8.0a	21.3a	66.9a
ANOVA				
Solid carrier		**NS**	***	***
Bradyrhizobium		**NS**	**NS**	*
BS *C		**NS**	**NS**	***

Means with same letter(s) in a column are not significantly different at 5 % level of probability by Duncan Multiple Range Test (DMRT)

NS = Not significant, * = p> 0.05, ** = p> 0.001, *** = p>0.001

Table 4 : Effect of Bradyrhizobium Mutant Strains with or without Solid Carriers on Root Dry Weight, Shoot Dry Weight (g) and shoot tissue N (%) of Cowpea at eight Weeks after Sowing

Bradyrhizobium Strain	Carriers	Root dry wt/plt	Shoot dry wt/plt	Shoot tissue N
USDA 3384	None	1.7c	4.7b	2.0d
USDA 3384	Peat	2.1bc	7.3ab	4.4ab
USDA 3384	Composted maize cob	3.0a	7.7a	4.9a
USDA 3384	Cowdung	2.0bc	6.8ab	3.2bcd
USDA 3451	None	1.9bc	5.2ab	2.7cd
USDA 3451	Peat	2.8ab	7.8a	4.7a
USDA 3451	Composted maize cob	1.8bc	4.7b	3.9abc
USDA 3451	Cowdung	2.1bc	7.5a	4.8a
ANOVA				
Solid carrier		**NS**	**	***
Bradyrhizobium		**NS**	**NS**	**NS**
BS * S		*	**	*

Means with same letter(s) in a column are not significantly different at 5 % level of probability by Duncan Multiple Range Test (DMRT)

NS = Not significant, ***** = p> 0.05, ****** = p> 0.01, ******* = p>0.001

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