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Inoculation of Groundnut (*Arachis hypogaea* L.) with *Klebsiella* like Bacteria Isolated from Sudan using Charcoal and Groundnut Shells as Carriers

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ABSTRACT
The use of commercial bacterial inoculants and bacterial broth cultures in inoculation gave unacceptable results in most studies, this necessitateslooking for locally isolated and suitable inoculants carrier, therefore this study was designed to evaluate the efficiency of some locally fast growing *Klebsiella* like bacteria isolated from groundnut using charcoal and groundnut shells as carriers. In thisstudy groundnut was grown in pots and inoculated with different isolates using charcoal and groundnut shells as carriers and nodule numbers, plant height, branches number, shoot fresh weight and shoot dry weight were compared. The inoculation of groundnut with the isolates resulted in significant enhancement of its growth. When the groundnut shells were used as carrier the measured parameters were improved compared tocharcoal carrier. The study concluded that two of strains used were the best in groundnut growth promotion, the two strains isolated from groundnut grown in El-Obied with charcoal and El-Gezira with groundnut shells as carriers.

المستخلص:

إستخدام اللقاحات البكتيرية التجارية والمزارع البكتيرية السائلة في التلقيح أعطت نتائج غير مقبولة في معظم الدراسات، مما أدي إلي ضرورة البحث عن عزلات بكتيرية محلية وحوامل لقاحية مناسبة، لذلك هذه الدراسة صممت لتقييم كفاءة بعض العزلات المحلية السريعة النمو والتي تشبه البكتريا *Kelebsiella* عزلت من الفول السوداني وإستخدام الفحم النباتي وقشرة الفول السوداني كحوامل. في هذه الدراسة تمت زراعة الفول السوداني في أصص فخارية ولقحت بالعزلات المختلفة بإستخدام الفحم النباتي وقشرة الفول السوداني كحوامل وتمت مقارنة عدد العقد، طول النبات، عدد الأفرع، الوزن الرطب للمجموع الخضري والوزن الجاف للمجموع الخضري. تلقيح الفول السوداني بالعزلات أدي إلي زيادة واضحة في نموه. عند إستخدام قشرة الفول السوداني كحامل كل المقاييس كانت أفضل من إستخدام الفحم النباتي. خلصت الدراسة إلي أنالسلالات المعزولة من الفول السوداني المزروع في الأبيض بالفحم النباتي وسلالة الجزيرة بقشرة الفول السوداني كحوامل كانت الأفضل في زيادة نمو الفول السوداني.

***KEYWORDS*:**symbiotic nitrogen fixation, YEMA, inoculum

INTRODUCTION

Peanut is an important crop for small-scale farmers in semi-arid tropic regions, which are characterized by low soil fertility, and unpredictable rainfall and rain distribution. As soil nutrient are limited, peanut yield depends mainly on nitrogen derived from the atmosphere by symbiotic nitrogen fixation. Peanut kernel is an important source of inexpensive protein and oil essential for human diet. Peanut haulm, when incorporated into soils, provides nitrogen for the following crop; such as rice, cassava, maize and sugarcane. This helps to reduce the use of inorganic nitrogen fertilizer and provides sustainable means for agriculture. Improvement of nitrogen fixing ability in peanut can improve yield as well as soil fertility[(1)]. Cultivation of groundnut in Western Sudan is still lacking nitrogen fertilizers, hence three imported strains were compared to local strains. The results indicated that, the imported *Rhizobium* strain has no benefits for groundnut production in Western Sudan. It was recommended that nitrogen fixation by groundnut in this area should be directed to selection and identification of the most effective rhizobia strains from the adapted local population[(2)].

Efforts of research are directed to produce carriers characterized by good absorption and water holding capacity; easy in drying, milling, sterilization, mixing and packing; available and inexpensive[(3)]; non toxic, with good adhesion to seed, good pH buffering capacity, receptive for nutritive additions and suitable for all rhizobial strains[(4,5)].Therefore This work was conducted to evaluate the efficiency of some locally fast growing *Klebsiella* like bacteria isolated from groundnut using charcoal and groundnut shells as carriers.

MATERIALSAND METHODS

Nodules were collected in August 2011from three different regions in the Sudan, El-Obied with sandy soil (Arenosols) in western Sudan, El-Gezira (central Sudan) and El-Gadarif (eastern Sudan) with heavy clay soil (Vertisols). To collect the groundnut nodules the whole plants were pulled up carefully without detaching the nodules. Collected nodules were washed with sterile water and then surface sterilization was done using 70% ethanol and 0.1% $HgCl_2$ and repeatedly washed with sterile water. After surface sterilization, nodules were crushed and then the resulting suspension was streaked onto yeast extract mannitol agar (YEMA) at pH 6.8, the medium contains (g / l): mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.4; yeast extract, 1; agar, 20 with Congo Red or Bromothymole Blue. Purity was assured by routine plating on YEMA supplemented with Congo red and the uniform colonies were selected. The basal growth medium is that described by Vincent[(6)]. After repeated sub-culturing, pure culture was obtained from a single cell and preserved in 40% glycerol at -20℃. For experimental purposes growth was incubated at 28℃ for ten days. Growth was observed daily[(7)]. The carrier-based bacterialinoculant was prepared by modified method of Abdelrahim[(8)] by packing each 100g of Charcoal and groundnut shells separately in beakers and sealed with aluminum foil. Then it was autoclaved at 121℃, Ib/inch^2 for 4 hours continuous. Each beaker injected by 5 ml of inoculum broth aspetically. Sterile distilled water was added to each beaker to obtain a moisture content of 40%. Seeds of the groundnut local variety (Ghobish) were surface sterilized by 70% ethanol

and 0.2% $HgCl_2$ and inoculated by each of the four strains (three strains were locally isolated from groundnut nodules grown in El-Gadarif, El-Gezira and El-Obied and one reference strain, TAL169) by adding gum Arabic (40% solution) then charcoal and groundnut shells based inoculums was added separately to seeds till well coated, dried(15 minutes in the shade) then sown in pots containing sandy soil from El-Obied. The pots containing the soil were covered with aluminum foil and sterilized in oven at 180°C for 4 hours.

All pots were placed outside under natural conditions of light, temperature and humidity. Each treatment was replicated twice. The pots were watered as required with non sterile tap water. Plants were watered and harvested 8 weeks after sowing. The root systems of the individual plants were washed separately then the relative affectivity of the isolates was assessed by comparing the nodule number per plant[9].

Plant growth was determined by comparing branch number, plant height, shoot fresh weight and shoot dry weight of the shoot system. The dry weight was made when the shoot system was left to dry at 70°C for more than 72 hour in an air dry oven and then weighed as was described by Athar and Johnson[10].

All results obtained were compared with reference strain (TAL169) which was obtained kindly from Biofertilization Department, National Center for Research-Sudan.The experiments were carried in Food Technology, Microbiology Laboratory in Faculty of Natural Resources, University of Kordofan.

DATA ANALYSIS

The results were submitted to analysis of variance (ANOVA) by using sigma stat software program. When there were significant, means were compared with Multiple Range Test at $P < 0.05$.

RESULTS AND DISCUSSION

The results showed significant differences (P>0.05) for all parameters measured when charcoal and groundnut shells were used as carriers for inoculation of groundnut with the three isolates and the reference strains (Tables 1 and 2). For nodulation the majority of the nodules were found with small size but, the inoculation of El-Gezira, El-Obied and the reference strains resulted in one big nodule with other small size for each when charcoal used. The nodules were distributed in both the main and lateral roots. In contrast, the control showed no nodules. Inoculation of groundnut with El-Gadarif strain resulted in 14 nodules when groundnut shells were used compared to the charcoal (15). However the reference strain showed the best nodule number among all strains, the nodule number was 20 and 22.50 when the charcoal and the groundnut shells were used, respectively. The nodule formation by*Klebsiella* like bacteria in groundnut was reported before by Ibanez *et al*.[11] they stated that in spite of the fact that nodulation ability of bacteria was confirmed directly after isolation, several months later some strains failed to nodulate peanut, probably due to the loss of symbiotic genes. They also stated that the *16SrDNA* sequences of seven fast-growing

Table 1: Effect of the different isolates and the reference strain on groundnut (*Arachis hypogaea*) growth with charcoal as a carrier

Strains	Nodules number	Plant height (cm)	Branches number	Shoot fresh weight (g)	Shoot dry weight (g)
Control	0.00[d]	30.33[c]	10.75[c]	4.22[c]	1.07[b]
El-Gadarif	15.00[c]	29.25[d]	10.00[d]	4.00[d]	0.99[c]
El-Gezira	15.00[c]	29.00[e]	11.67[b]	3.57[e]	0.92[e]
El-Obied	16.00[b]	34.50[a]	14.00[a]	6.41[a]	1.77[a]
Reference	20.00[a]	31.63[b]	8.50[e]	4.62[b]	0.90[d]

*Each value is the mean of two replicates.
=*Values in column share same superscript letters show no significant differences as separate by Duncan Multiple Range Test.

Table 2: Effect of the different isolates and the reference strain on groundnut (*Arachis hypogaea*) growth with groundnut shells as a carrier

Strains	Nodules number	Plant height (cm)	Branches number	Shoot fresh weight (g)	Shoot dry weight (g)
Control	0.00[d]	29.67[e]	12.67[c]	3.91[e]	1.17[c]
El-Gadarif	14.00[b]	32.63[d]	15.50[a]	4.52[d]	1.27[b]
El-Gezira	13.00[c]	33.63[b]	13.00[b]	4.97[b]	1.33[a]
El-Obied	13.00[c]	35.38[a]	10.50[d]	4.72[c]	1.27[b]
Reference	22.50[a]	33.50[c]	10.25[e]	5.59[a]	1.17[c]

*Each value is the mean of two replicates.
*Values in column share same superscript letters show no significant differences asseparate by Duncan Multiple Range Test.

strains the phylogenetic analysis showed that these isolates belonged to the Phylum Proteobacteria, class Gammaproteo-bacteria, and included *Pseudomonas spp*., *Enterobacter spp*., and *Klebsiella spp*. After storage, these strains became unable to induce nodule formation in *Arachis hypogaea* plants, but they enhanced plant yield. For plant height charcoal inoculants showed significantly the least plant height than groundnut shells inoculants, El-Gezira and El-Gadarif strains have a negative effect on the plant height when charcoal used; they showed significantly less plant height than the control, this may be due to the nature of the soil (Vertisols)from which El-Gadarif and El-Gezira strains were isolated and the nature of the soil used in this study (Arenosols) as it was reported that differences in the nodulation of cowpea after inoculation with different *Bradyrhizobium* in decomposing rice as inoculants in the different soils, show that *Bradyrhizobium* inoculants should be matched to soil type [(12)]. However El-Obied strain showed significantly the best plant height (34 cm) followed by the reference strain which recorded (31.63cm). Plant height was the best when groundnut shells were used, all strains recorded best results than the control. As for the charcoal inoculants, El-Obied strain showed the best plant height (it was 35.38 cm) followed by El-Gezira strain (33.63 cm) then the reference strain (33.50 cm) and El-Gadarif strain (32.63 cm) compared to the control (29.67 cm). For branches number only two strains showed significantly best results than the control when charcoal was used, El-

Obied strain (14.00) and El-Gezira (11.67), all other strains including the reference strain showed significantly branches number less than the control. The branches number was significantly higher when groundnut shells used as a carrier. El-Gadarif strain and El-Gezira strain gave branches number more than the control while the other strains showed less branches numberthan the control. The fresh weight of groundnut inoculated with the different strains was only improved when El-Obied and the reference strains were used with charcoal. The other locally isolated strains gave fresh weight significantly less than the control. However, when groundnut shells used, all strains showed fresh weight better than the control, the best fresh weight was obtained by the reference followed by El-Gezira, El-Obied and El-Gadarif strains, respectively. The best dry weight was obtained by El-Obied strain when charcoal was used, the dry weight showed significant difference it was 1.77 g and it was (1.07 g) for the control, the other strains gave dry weight less than the control significantly. On the other hand all strains recorded significant difference in dry weights they were more than the control when groundnut shells used except the reference strain which showed dry weight the same as that of the control (1.17 g) i.e there is no significant difference. The dry weight was 1.33 g when the groundnut shells were inoculated with El-Gezira strain which was the best than the others. El-Gadarif and El-Obied strains gave same dry weight (1.27 g). From the above results using of groundnut shells as carrier was found better than charcoal. These results in agreement with others, they reported that among the sterilized carriers, groundnut shells was found to be the best in maintaining high number of rhizobia[8]. However the same author reported that charcoal is the best to be used as carrier for Rhizobia.Also it was stated that the use of carriers containing high organic matter could increase bacterial survival and led to efficiency improvement of biological inoculants[13]. The same authors also stated that the weakest carrier was activated charcoal.Since the shoot dry weights in this study were found significantly higher than the control when groundnut shells were used with all strains, this means that our isolates are affective but the affectivity depends on carrier type and increasing of shoot dry weights is indication of the ability of our isolated to fix nitrogen. Ibanez *et al*.[11] reported that although there was no direct evidence for the contribution of these peanut fast-growing strains to nitrogen fixation, at least the increase in the shoot dry weight of plants inoculated only with the *Klebsiella*-like isolates could be related to their ability to fix nitrogen. Other mechanisms, such as the secretion of phytohormones could also have contributed to the plant growth promotion observed[11].Somasegaran and Hoben[7] reported that increase in shoot dry weight of inoculated legume is occurring as a result of increasing rate of nitrogen fixation.Finally the application of selected carrier materials for the bacterial inoculants proves to be beneficial to protect the bacteria[14].

CONCLUSIONS

The promotion of the groundnut growth was significant when groundnut shells used as carrier. The strain isolated from El-Obied soil was the best in promoting groundnut growth when charcoal was used as carrier and El-Gezira strain was the best when groundnut shells were used. The affectivity of the isolates depends on the carrier type.

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