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Research Article

Potential Growth Enhancement of Crops Using Pure Culture of Rhizobium Spp.

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ABSTRACT

Background: The quest for increased food production for ever-increasing population growth is a continuous venture. This has necessitated this research to use cheaply available resources, microorganisms in the production of biofertilizer.

Methods: A soil sample was obtained from a potato farm and a ten-fold dilution prepared up to 10-8. One milliliter from dilutions 10^{-4}, 10^{-5} and 10^{-6} was pipetted and transferred into 3 Petri dishes labeled accordingly. Molten Rhizobium agar medium was carefully poured into the plates, swirled gently to mix and incubated at 37°C for 24 hours. The discrete colonies were characterized. The broth culture of these colonies were prepared in nutrient agar. Seeds such as corn, beans, groundnut, and pumpkin were treated with broth cultures and planted in soil contained in plastic buckets. These were observed for 14 days at an interval of 2 days.

Results: A total of 5 strains of Rhizobium sp were identified but only 2 showed growth enhancement properties. These 2 were identified as Rhizobium phaseoli and Rhizobium meliloti but only Rhizobium phaseoli was used for the treatment of the seeds. After 2 days of planting, all the seeds treated with the Rhizobium phaseoli germinated except pumpkin. After 7 days of planting, all the seeds germinated and grew but the control for beans, soil treated and control for pumpkin did not germinate. After 14 days, the same pattern of growth as at 7 days except that the pumpkin germinated grew very fast and with impressive leaves. Conclusion: Based on these results it can be stated that the Rhizobium strain used for this research enhanced the growth of maize and groundnut, beans and pumpkin effectively and therefore can be used as biofertilizer for the cultivation of these crops. Rhizobium meliloti can be improved upon and also used to enhance crop growth.

Keywords: Biofertilizer; Rhizobium spp; Growth enhancement; Crops

INTRODUCTION

Demands for food in a growing human population is on the rise and so rises the pressure on orthodox farming [1]. Many farmers have taken to using of fertilizers (an organic or synthetic substance usually added to or spread onto soil to increase and enhance plant growth) to cushion the impact [2]. Based on the production process, fertilizers can be roughly categorized into three types: chemical, organic and biofertilizer. Chemical fertilizers are not without negative impact to the environment, in the context of nutrient supply, crop growth and environmental quality. The advantages need to be integrated in order to make optimum use of each type of fertilizer and achieve balanced nutrient management for crop growth [3]. One of the major concerns in today's world is the pollution and contamination of soil. The use of chemical fertilizers and pesticides has caused tremendous harm to the environment. An answer to this is the biofertilizer, an environmentally friendly fertilizer now used in most countries [4].

Biofertilizer is a substance that contains microoganisms, which when applied to seeds, plant surface or soil, colonizes the interior of the plant and promotes growth by increasing the availability or supply of primary nutrients to the host plant[5]. Biofertilizer facilitates nutrient uptake or by increasing nutrient availability in the rhizosphere [6]. The innovative view of farm production attracts the growing demand of biological based organic fertilizers exclusive of alternative to agro-chemicals. Farming with biofertilizer is an innovation, which guaranties food safety and adds to the biodiversity of soil [7]. Microbial based organic farming depends largely on the natural microflora of the soil mainly bacteria and fungi like mycorrhiza fungi/plant growth promoting rhizobacteria (PGPR) [8]. The upsides of biofertilizers outweighs its downsides. Top on the list is its ability to keep the soil environment rich in all kinds of micro- and macro-nutrients via nitrogen fixation, phosphate and potassium solubilisation or mineralization, release of plant growth regulating substances, production of antibiotics

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and biodegradation of organic matter in the soil [9]. In addition, when used as seed or soil inoculants, they multiply and participate in nutrient cycling and benefit crop productivity [10]. In sum, microbial inoculants have paramount significance in cohesive nutrient management systems to sustain agricultural productivity and healthy environment [11].

Microbes are often not as effective in natural environments as would be expected and therefore artificially multiplied in the laboratory. Cultures of keenly assembled organisms play an important role in appreciating the microbial processes in soil. Use of biofertilizer is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers. As we are experiencing tremendous increase in world population and environmental changes, increase in food production is also very urgently needed. It is therefore pertinent to source for plant nutrients that will increase crops yield but environmentally friendly.

MATERIALS AND METHODS

Sample collection and preparation

The soil sample was collected from a potato farm plantation (legume plant) in large containers and then transported to the laboratory. In the lab, 300 g of soil sample was weighed and transferred into an open mouth plastic container with a capacity of 500 g. A total of 12 of these containers were used.

Isolation of Rhizobium spp

Ten grams of soil sample was mixed with 90 ml sterile distilled water and shaken vigorously for 30 minutes to separate the microorganisms from the soil completely. The preparation was labelled "stock solution". From the stock, 10 ml was transfer to 90 ml sterile distilled water aseptically and shaken vigorously. The solution was labelled 10-1 dilution. This step was repeated till the eighth tube to get 10-2, 10-3, 10-4, 10-5, 10-6, 10-7 and 10-8 dilutions. Dilutions such as 10-4, 10-5 and 10-6 were chosen for plating.

One mililiter from 10-4dilution was taken and placed in a petri dish containing Rhizobium agar and evenly spread on the agar surface. The same procedure was repeated for 10-5 and 10-6 dilutions. The plates were incubated at 37°C for 24 hrs. After 24hours incubation, colonial characteristics of the growth on the plates were noted and recorded. A total of 5 strains, M1 – M5 were observed.

Purification and maintenance of isolates

The isolates were sub cultured and the pure cultures were then preserved on nutrient agar slants stored in the refrigerator at 4°C.

Biochemical characterization and identification of isolates

The cultural, morphological, biochemical and molecular characterization of the isolates were carried out. The isolates were gram stained and viewed under the microscope. Biochemical tests such as citrate utilization test, triple sugar iron test, glucose peptone agar utilization test, gelatine liquefaction test, starch utilization test and catalase test

Screening of the isolates (5 strains) of Rhizobium sp for growth enhancement of the crops

Preparation and planting of seeds: Four different types of crops were used for this research and they included beans, maize, groundnut and pumpkin. The beans, maize, groundnut were soaked in the Rhizobium broth cultures for 30 minutes while the pumpkin seeds were inoculated with the broth culture using a syringe. These were planted including control and soil treated for each crop type in separate containers and monitored at two days intervals. The growth characteristics of the crops were observed and recorded for 14 days at 2 days intervals.

Molecular characterization and identification of the Rhizobium strains showing enhancement of the crops growth: Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) (Thomas Scientific, USA) was spun at 14000 rpm for 3 min. The cells were re-suspended in 500 µL of normal saline and heated at 95°C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5 mL microcentrifuge tube and stored at -20°C for other downstream reactions. The extracted genomic DNA was quantified using the NanoDrop 1000 spectrophotometer[12]. The 16S rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix included: X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 min; denaturation, 95 °C for 30 s; annealing, 52°C for 30 s extension, 72°C for 30 s for 35 cycles and final extension, 72°C for 5 min. The product was resolved on a 1% agarose gel at 120 V for 15 min and visualized on a UV transilluminator.

The product of the PCR amplification was resolved on a 1% agarose gel at 120 V for 15 min and visualized on a UV transilluminator (Reyes-Escogido et al., 2010). Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa [13].

RESULTS

The morphological and biochemical characterization of the Rhizobium spp is presented in Table 1. All the selected isolates were confirmed to belong to the genera Rhizobium.

Table 2 presents the qualitative and quantitative analysis of plant growth after 2 days of planting using sp AC1. Plant morphological characteristics that were observed and recorded include seed germination, total height, number of leaves and branches. After 2 days of planting, it was observed that all the maize seed that were treated with Rhizobium spp AC1 grew with an average height of 8cm. However, only two out 4 seeds grew from the soil treated sample while in the control pot, only one seed grew with total heights of 5 and 2 cm respectively. For the groundnut and beans samples, growth was observed only in the seed treated pot while no growth was observed in the soil treated pot and control. There was no growth observed after 2 days for the pumpkin planted.

The qualitative and quantitative analysis of plant growth after 7 days of planting is presented in Table 3. Growth was observed in all the crops planted with the exception of control sample for beans and also, soil treated and control pot for pumpkin. All the seed treated with the Rhizobium spp (4) for maize and groundnut grew with total height of 15 and 11 cm. from the result, it was observed that for beans only the seed treated and soil treated pot grew while no growth was recorded for the control. For the pumpkin plant,

Table 1: Biochemical characterization and identification of the *Rhizobium* isolates.

Isolate code	Gram reaction	Cell shape	Colony morphology	Motility	Oxidase	Indole	Citrate	Catalase	MR	VP	Nitrate	Urease	Glucose	Lactose	Mannose	Sucrose	Mannitol	Probable organism
M_1	-	Rod	Mucoid, flat and entire	+	+	-	+	+	+	+	+	+	+	+	+	+	+	*Rhizobium* spp
M_2	-	Rod	Mucoid, smooth and entire	+	+	-	+	+	+	-	+	+	+	-	+	+	-	*Rhizobium* spp
M_3	-	Rod	Mucoid, flat and entire	+	+	-	+	+	+	+	+	+	+	+	+	+	+	*Rhizobium* spp
M_4	-	Rod	Mucoid, smooth and entire	+	+	-	+	+	+	+	+	+	+	+	+	+	+	*Rhizobium* spp
M_5	-	Rod	Mucoid, flat and entire	+	+	±	-	+	+	-	+	-	±	-	+	-	+	*Rhizobium* spp

Table 2: Qualitative and quantitative analysis of plant growth after 2 days of planting.

Crops	Treatment	Growth	Number of seeds planted	Number of seeds germinate d	Average height (cm)	Number of leaves	Number of branches
Maize	Seed treated	+	4	4	8	2	2
	Soil treated	+	4	2	5	2	1
	Control	+	4	1	3	1	1
Groundnut	Seed treated	+	4	2	4	2	2
	Soil treated	-	4	-	-	-	-
	Control	-	4	-	-	-	-
Beans	Seed treated	+	4	1	3	2	2
	Soil treated	-	4	-	-	-	-
	Control	-	4	-	-	-	-
Pumpkin	Seed treated	-	4	-	-	-	-
	Soil treated	-	4	po...	-	-	-
	Control	-	4	-	-	-	-

Key: - = Negative. + = Positive

Table 3: Qualitative and quantitative analysis of plant growth after 7 days of planting.

Crops	Treatment	Growth	Number of seed planted	Number of seed germinated	Total height (cm)	Number of leaves	Number of branches
Maize	Seed treated	+	4	4	15	8	4
	Soil treated	+	4	2	9	3	2
	Control	+	4	3	6	2	1
Groundnut	Seed treated	+	4	4	11	10	3
	Soil treated	+	4	3	6	4	2
	Control	+	4	2	4	3	2
Beans	Seed treated	+	4	1	7	3	2
	Soil treated	+	4	1	3	2	1
	Control	-	4	-	-	-	-
Pumpkin	Seed treated	+	4	1	4	4	3
	Soil treated	-	4	-	-	-	-
	Control	-	4	-	-	-	-

Key: + growth; - no growth

only the seed treated with the organism grew while no growth was observed for the soil treated sample and control pot.

The qualitative and quantitative analysis of plant growth after 14 days of planting is presented in Table 4. There was a significant improvement in the growth observed in all the crops planted with the exception of control sample for beans and also, soil treated and control pot for pumpkin. All the seeds treated with the Rhizobium spp for maize and groundnut grew with total heights of 15 and 11 cm. from the results, it was observed that for beans, only the seed treated and soil treated crops grew while no growth was recorded for the control. For the pumpkin plant, only the seed treated with the organism grew while no growth was observed for the soil treated sample and control crop.

DISCUSSION

The innovative view of farm produce production attracts the growing demand of biological based organic fertilizers exclusive of alternative to agro-chemicals [14]. In agriculture, the goal is to encourage alternate means of soil fertilization that relies on organic inputs to improve nutrient supply and conserve the field

Table 4: Qualitative and quantitative analysis of plant growth after 14 days of planting.

Crops	Treatment	Growth	Number of seed planted	Number of seed germinated	Total height (cm)	Number of leaves	Number of branches
Maize Seed	treated	+	4	4	20	14	6
	Soiltreated	+	4	2	14	8	4
	Control	+	4	3	10	4	4
Groundnut	Seedtreated	+	4	4	16	28	10
	Soil treated	+	4	3	10	14	6
	Control	+	4	2	8	15	5
Beans	Seedtreated	+	4	1	13	3	2
	Soil treated	+	4	1	7	6	1
	Control	-	4	-	-	-	-
Pumpkin	Seedtreated	+	4	1	11	6	3
	Soil treated	-	4	-	-	-	-
	Control	-	4	-	-	-	-

+ growth; - no growth

Table 5: Identification of the isolates by 16S rRNA genes amplification showing percentage similarities.

S/N	Gene Bank isolates	Accession number	Isolates code	% Similarity
1	*Rhizobium phaseoli* strain G16	CP021092.1	Ar_1	98.2
2	*Rhizobium meliloti*	KH131657.1	Ar_2	99.6

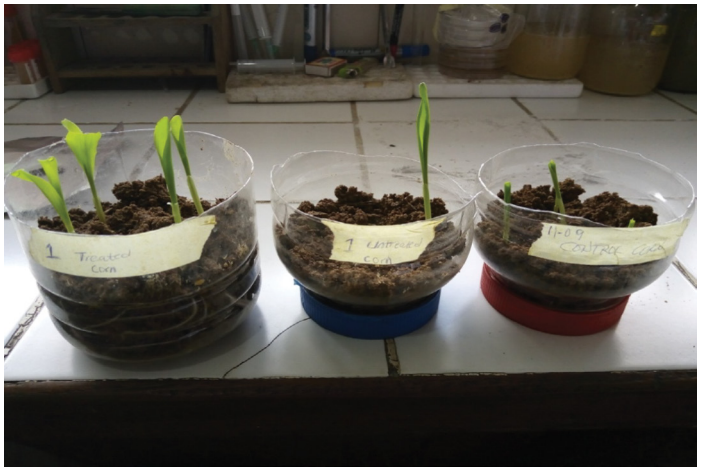

Figure 1a: Growth appearance of corn after 2 days of planting.

C B A

Key: A: Seed treated with *Rhizobium* spp; B: Soil treated with *Rhizobium* spp; C: Control

Figure 1b: Growth appearance of corn after 14 days of planting.

management [15]. Organic farming is one of such strategies that not only ensures food safety but also adds to the biodiversity of soil.

The results of the present study have established the effectiveness

A B C

Figure 2a: Growth appearance of groundnut after 2 days of planting.

of the Rhizobium strains for improving the growth of crops under natural conditions. The results showed that almost all the test strains of Rhizobium caused improvement in all the parameters of growth compared to control. The increment in the parameters in response to rhizobial inoculation endorsed the fact that the test strains were having one or more growth promoting mechanisms including mobilization and efficient uptake of nutrients [16,17], enhancement in stress resistance [18], solubilization of insoluble phosphates [19], induction of systemic disease resistance [20], inhibition of fungal growth [21], production of phytohormones [22], vitamins [23] and siderophores [24]. As shown from the results, the microbial inoculum enhanced the germination and growth of the crops as compared to the control. This gives an indication that microbial inoculum helped plant growth and has been able to provide the plant with nitrogen, which is one of the most important nutrients for plant growth, as it promoted rapid growth, increased leaf size and quality, hastened crop maturity, and promoted fruit and seed development. Nitrogen is an integral part of chlorophyll manufacture through photosynthesis [25] and

A B

Key:
A: Seed treated with Rhizobium spp
B: Soil treated with Rhizobium spp
C: Control

Figure 2b: Growth appearance of groundnut after 14 days of planting

A B C

Key:
A: Seed treated with *Rhizobium* spp
B: Soil treated with *Rhizobium* spp
C: Control

Figure 3: Growth appearance of beans after 7 days of germination.

A B

Key: A: Growth - Seed treated with *Rhizobium* spp 1
B: No growth – soil treated with *Rhizobium* sp 1

Figure 4: Growth appearance of pumpkin after 14 days of germination.

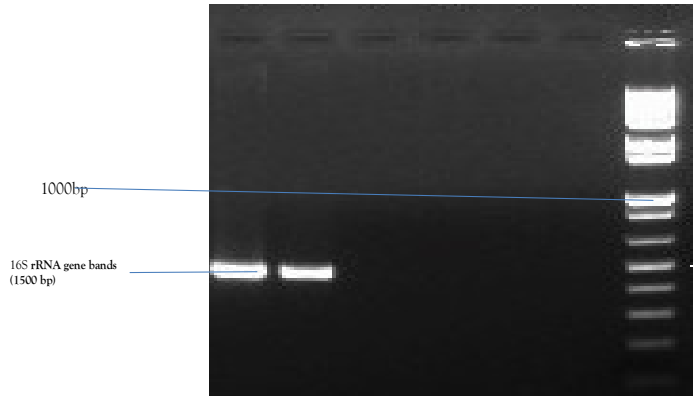

Figure 5: Agarose gel electrophoresis showing the 16S rRNA gene bands of the bacterial isolates. Lane M represents the 1000 bp DNA ladder.

therefore the greenish and healthy looking shoots of the crops is a manifestation of this.

These results are in line with [26] who have reported 16% increase in number of panicles per plant of rice and suggested that the improvement was due to increased availability of nutrients and phytohormones like indol acetic acid and ethylene. Similarly,[27] observed up to 23.63% increase in plant height of maize and rice over un-inoculated control and argued indol acetic acid and gibberellins production as the key mechanism for that improvement. Also, an increase in the number of leaves for pumpkin due to Rhizobium inoculation over uninoculated control was also recorded by [28] who have suggested efficient nutrient and water uptake as important mechanisms. In the same way, [29] showed significant increase in plant height up to 6.9% over un-inoculated control. Results from our study regarding seed germination showed up to 90% increase effectiveness in maize and groundnut respectively, over uninoculated control. These results are also similar with those of [30] who found significant increase in seed germination of 79.09% in rice and groundnut seeds by rhizobial inoculation over control and proposed phosphate solubilization and plant growth regulators production as the key mechanisms. Similarly, 42.55% increase in seed germination was observed due to auxin and gibberellin production by [31]. These findings are in conformity with who have already verified this kind of behavior from different rhizobium strains used as inoculant in maize. Furthermore, differential behavior of different PGPR strains of rhizobia against common host has also been reported by [31]. The results are also in concordance with most similar previous studies by, where crop yield were improved using Rhizobium spp (Figures 1-5).

CONCLUSION

From the results of this study it is clear that biofertilizer shows better results as compare to that of the control. The main advantage of biofertilizer is that it does not pollute the soil and also does not show any negative effect to environment and human health. Finally obtaining less amount of healthy products with less environmental disturbances is preferred over obtaining higher amount of non-healthy products with more environmental disturbances. Hence, the use of biofertilizer is highly beneficial. This isolated microorganism significantly enhanced the germination and growth of the planted seeds. These microbial inoculums can be mass produced and used to treat specified seeds for planting. These results indicate a significant increment in the productivity of plants treated with the newly developed biofertilizer.

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