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SYMBIOTIC NITROGEN-FIXATION AND YIELD OF SOYBEAN INOCULATED WITH ACID-TOLERANT RHIZOBIA STRAINS FOLLOWING SOIL AMENDMENTS UNDER LOW SOIL PH CONDITIONS OF RWANDA

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Abstract

Soil acidity is a limiting factor in legume system during plant growth and microsymbionts process. The aim of this work was to identify and document acid tolerant rhizobium strains and their response to soybean grain yield under low pH. Ninety-one rhizobia isolates were collected from two provinces (Eastern and southern) of Rwanda based on soil acidity in the two regions. Symbiotic nitrogen fixation and yield were determined for three soybean varieties (*PK6*, *SB24* and *Sc Squire*) inoculated with three acid-tolerant rhizobia strains (*BB18S*, *BB64S* and *SB88E*) across two sites with contrasting soil pH conditions. Interaction of soybean variety and soil amendments significantly revealed differences (p-value≤0.012), where organic manure increased the number of nodules for two promiscuous varieties (*SB24* and *Sc Squire*). Non-inoculated soybean control produced low N-fixed compared to all inoculated treatments, with high performance recorded for *SB88E* strain. The total dry matter biomass generated high grain yield, and this resulted from N-fixation due to the interaction between the acid tolerant rhizobia strains, soybean variety and application of N starter, P and K. Interaction of soybean variety and soil amendments revealed significant differences (p-value≤0.012), with organic manure application increasing the number of nodules for two the promiscuous varieties (*SB24* and *Sc Squire*). The biomass production generated high (*p*=000) grain yield by significantly (*p*=000) increasing the pod yields. The N-fixing efficiency of the strains was affected by N, P, and K application and legume isolates, and the grain yield increased when organic manure was applied on PK6 at the site with low pH and increased when lime and organic manure were applied on SB24 and Sc Squire. The study offers a technical solution for addressing soil acidity, nutrient availability, and enhancing soybean yield in Eastern and Southern Rwanda and similar agroecological regions.

Key words: N-fixation, Acid-tolerant rhizobia strains, Soybean varieties, Soil acidity, Soil amendments.

Introduction

Soybean is one of the major legumes grown in Rwanda and is being promoted by the Agriculture and Animal Resources Ministry (MINAGRI) as priority crop in the Crop Intensification Program (CIP), to enhance soil fertility and improve nutritional status of Rwandan households (Paul *et al.*, 2018). In Rwanda, soybean is a high nutritional crop and the most affordable source of protein compared to animal products. The nutritional value of soybean has prompted the government to start a program called 'one soybean milk cup/one egg per child' to reduce malnutrition among the vulnerable groups, especially women and children (Tukamuhabwa, 2016). Soybean also plays a significant role in increasing nitrogen in the soil through biological nitrogen fixation.

Nitrogen is an essential nutrient for plant growth and development and is the most limiting nutrient for crop production in Rwanda. To achieve maximum yields, crops may require high amounts of chemical fertilizers, which in turn may adversely affect the environment (Brusseau *et al.*, 2019). Use of legumes such as soybean could reduce the reliance on chemical fertilizers as they are N-fixing thus potentially providing sufficient amounts of N under favourable soil conditions (Kakraliya *et al.*, 2018). The amount of N-fixed by soybean promiscuous varieties in favourable conditions was estimated to be between 38 and 126 kg of nitrogen ha^{-1} (Tian *et al.*, 2001), but soil acidity has been one of the major problems during N-fixation process.

In Rwanda, almost 45% of the farmland is assumed to be acidic with a pH level of 5.2 and low in essential nutrients (N, P and K) (Nabahungu *et al.*, 2007). Soil acidity is one of the most critical barriers impeding soybean production in Rwanda. Research demonstrated that the application of lime and green manure would remedy soil acidity problem (Nabahungu *et al.*, 2007). Poor and ineffective nodulation coupled with low dinitrogen fixed in soybean may be attributed to soil acidity, absence of effective strains of rhizobia and crop genotype (Boivin *et al.*, 2020). Some studies have shown that the proportion of nodules from introduced strains decline where there are native rhizobia (Thilakarathna *et al.*, 2017). Inoculating soybean with compatible and efficient rhizobia was found to significantly increase plant growth and nitrogen fixation in acidic soils (Muleta *et al.*, 2017). Therefore, inoculation with a compatible and efficient native rhizobia strain improves the growth and yield of soybean in acid soils (Chibeba *et al.*, 2017).

However, plants use diverse mechanisms to survive under highly acidic conditions (Seifikalhor *et al.,* 2019) and microsymbionts growth rate mostly depend on the level of acid tolerance (Ngom *et al.,* 2016). Rwandan soils are a reservoir of untapped elite rhizobia strains and could be suitable to the local conditions, calling for their testing on legume crops for Biological Nitrogen Fixation under acid condition. The objective of this study were to (i) identify acid-tolerant elite rhizobia strains capable of nodulating soybean and (ii) determine the effect of inoculated seed symbiotic nitrogen-fixation, and yield of selected soybean varieties in low pH soils.

Material and Methods

Nodules collection: Ninety-one rhizobium isolates were collected from four legume crops (Soybean, Bush and climbing bean, cow pea and peas) grown in the eastern and southern province of Rwanda. The legume plants that formed nodules with rhizobia were carefully uprooted and nodules collected using sterile forceps. The nodules were then placed in a sterile container and transported to the laboratory for isolation and identification of the rhizobia strains. Based on soil pH, only eight rhizobia strains (three from soybeans, three from bush bean, one each from climbing bean and cowpea) responded well to soybean authentication. Soybean authentication involved growing soybean plants in a sterile acid growth medium without any nitrogen source and separately inoculating the roots with the eight rhizobia isolates and after few weeks of growth, the nodules were observed (Mburu *et al.,* 2020).

Strain morphological characteristics: Nodules were collected from different non inoculated legume crops (at flowering stage) and various places of Rwanda to get rhizobia strain isolates. To target indigenous rhizobia strains, only legume crops that did not receive inoculation were considered for nodules sampling. The collected nodules were first rinsed with ethanol to sterilize the nodule surface, then immersed in a bleach solution to kill any surface bacteria or fungi, and finally washed thoroughly with sterilized water to remove any remaining bleach residues. The nodules were then dried on sterile paper towels before being used for further analysis.

The nodules were cut open to examine the bacteroid zone, which contains the rhizobia. The size, color, and appearance of the bacteroid zone were examined to assess the effectiveness of rhizobia. The presence of dark or reddish pigmentation was a sign of healthy and active rhizobia, while a lack of pigmentation or small bacteroid zones indicated less effective symbiotic relationship (Agtuca *et al.,* 2020). The nodules were squashed to release the liquid that contains the rhizobium. The satirized inoculating loop was used to take the nodule fluid and streaked on Yeast Extract Mannitol Agar (YEMA) media (Howieson *et al.,* 2016). The plates were sealed and labelled with special codes for each individual isolates, and they were put in the incubator at 28ºC for a period between 3 to 10 days. Daily monitoring was done to score the date of growth for each isolate. By including Congo red in YEMA media, it was possible to determine the purity of the rhizobial cultures.

Pure cultures of rhizobia only marginally absorb the dye, whereas contaminants, considerably absorb it (Woomer *et al.,* 2011). In addition, observation of rhizobium was done using the gram-staining technique colour to identify bacteria as Gram negative or positive, and Rhizobium is a Gram-negative bacteria (Table 1).

Table 1. Rhizobium strains characteristics from collected field isolates.

Strains	Incubation period (days)	Bromothymol blue	Red Congo	Gram
BB18S	7	Green	Not absorbed	Negative
BB21S	7	Green	Not absorbed	Negative
CB23S	7	Green	Not absorbed	Negative
SB30S	3	Green	Not absorbed	Negative
BB64S	7	Yellow	Not absorbed	Negative
SB76S	3	Yellow	Not absorbed	Negative
CP84E	7	Green	Absorbed	Negative
SB88E	3	Yellow	Absorbed	Negative

Due to variations in chemical and physical properties, the Gram staining process prevents bacteria from maintaining the stain's color. Gram negative stains appear red or pink on microscope since the counterstain is safranin dye. Bromothymol blue is used to single out fast acid growing strains with yellow colour, from slow non-acid growing strains with green colour (Table 1). One loop of rhizobia from nodule suspension was streaked on YEMA plate with bromthymol blue for treatment (BTB), and each treatment was done on a separate plate. The petri dishes were covered with the aluminium foil before incubation to avoid light. After 3-5 days (for quick growers) and 5-7 days (for slow growers), plates were inspected for response (Somasegaran *et al.,* 1994).

Rhizobia strains authentication: The rhizobia isolates were authenticated using soybean variety SB24 to establish their nodule forming ability. Sterilized seeds were soaked in JIK (Sodium hypochlorite) and later rinsed in sterile water before inoculation. Plastic water bottles were cut (Woomer *et al.,* 2011) to open the lower larger part of the container (Riccillo *et al.,* 2000). The "cut" plastic bottles were suspended and connected to reservoirs containing Yeast Mannitol Broth (YMB) media (294.1 g/l of $Cacl_2.2H_2O$; 136 g/l of KH_2PO_4; 6.7 g of Fe-citrate; 123.3 g of $MgSO_4.7H_2O$; 87 g of K_2SO_4; 0.338 g of $MnSO_4.H_2O$; 0.247g of H_3BO_3; 0.288 g of $ZnSO_4.7H_2O$; 0.1 g $CuSO_4.5H_2O$; 0.056 g $CoSO_4.7H_2O$; 0.048 g $Na_2MoO_2.2H_2O$) (Broughton *et al.,* 1987).

The experiment was established in a greenhouse and all plants were watered at dawn and dusk. A nutrient control (without Rhizobia inoculation) of 1.0 g KNO_3 per liter (0.1%) was applied without rhizobia inoculation. The mineral form of nutrients solution concentration as source of N is preferred by soybean (Glycine max) in the absence of rhizobium, and plants only used N from KNO_3 (Alves *et al.,* 2000). Sixty-six rhizobia strains were tested with SB24 soybean variety in the Leonard bottle jar assemblies, and only eight strains produced nodules on roots of SB24 soybean variety.

Laboratory assessment of acid tolerance: The eight isolates identified from soybean authentication were subjected to acid Yeast Mannitol Broth media, to test the tolerance of strains to low pH. Autoclaving of agar was done separately and mixed when warm with acid solution to avoid hydrolysis; the media were poured into plate according to acid levels. The pH of the YEMA was adjusted to four levels (3.7, 4.4, 5.1 and 7.5) with filter sterilized HCl and NaOH solutions. The YEMA media with the four pH levels were inoculated with the rhizobia isolates and put in the incubator at 28ºC. After incubating for 3-7 days, colonies below 10^8 cells/g at pH 5.1 were considered as low pH tolerant (Vargas *et al.*, 1988), and only three with above threshold were evaluated in the field.

The nodules were collected across the two regions (Southern and Eastern provinces) of Rwanda (Fig. 1), with different agro-ecological conditions. In south the province (Fig. 2), soils are acidic with pH ranging between 5 and 5.5, whereas in the Eastern part, they are moderately acidic ranging between 5.7-7 (Table 2).

The three rhizobia isolates that performed well on pH 5.1 media were selected for further evaluation under field conditions, after laboratory assessment to determine their acid tolerance. To test the three performed rhizobium strains, trials were conducted at Huye and Bugesera research stations located in southern and eastern province, respectively.

MPN determination: The experiment employed two replications (n=2) and six dilution steps (S=6) to determine the Most Probable Number (MPN) of rhizobia by using Yeast Extract Mannitol Agar (YEMA) media. After counting the number of positive and negative records, the total number of nodulated units from all dilution steps were computed by adding the positive records from two replicates in all dilutions of the strain (m). The Most Probable Number (MPN) per gram of soil (X) was determined using a tenfold approach (Somasegaran *et al*., 1994).

m = N from the MPN table (Tenfold) determined by the total number of nodulated units
d = Low dilution (10^{-1})
v = Applied volume of aliquot during inoculation (1ml)

$$X = \frac{m \text{ X } d}{v}$$

Experimental design and treatments in the field experiment: In the treatment structure, a split-plot design was used with soil amendments and varieties as the main plot factor, and strains and fertilizer as the sub-plot factor. Soil amendments comprised 2 t/ha of manure, 2.5 t/ha of lime. The experiment consisted of 24 treatments, which were a combination of three varieties, and three strains, and two fertilizer inputs. The Urea and control treatments did not receive any inoculation to measure the effect of high inorganic N application, and the effectiveness of collected indigenous acid-tolerant rhizobia strains. The three soybean varieties were two promiscuous namely, *SB24* and *SC squire* and one non-promiscuous but acid tolerant (*PK6*) were subjected to soil amendment and rhizobia strains. Three types of starter input were applied (T1: Diammonium Phosphate **(**DAP); T2: Urea; T3: DAP + Potassium chloride (KCL) followed by the non-inoculated soybean as no-input control (T4) and maize as reference crops (Gwenambira *et al.*, 2021).

Agronomic practices: Field selection started with the background history check of cropping history and only fields previously under cereals crops were selected. Land preparation was done before planting soybean and the appropriate agronomic practices undertaken. Seeds were prepared one month before planting and the germination rate was 90%. Each plot measured 3 m by 3 m and the net plot gave room to have a 1 m stretch of legume to be sampled for nodulation assessment at full pod and 50% of grain filling (R4), dry matter yield, and N-tissue analysis. Soybean seeds were manually planted at 40 cm spacing between rows and 5 cm within row (spacing between seeds), with one seed planted in each hole. All fertilizers were applied during trial establishment and the second urea topdressing was done during the first weeding. Seeds were inoculated with 10^9 cells/g in solid form with peat as carrier and mixed using tap water to coat the seeds with inoculant, and the same strain code shared the same bucket during seed inoculation to avoid contamination. Germination dates was assessed two weeks after planting.

Soil sampling and analysis: The topsoils (0–20 cm) of 250 kg were collected for soil physico-chemical analyses during trial establishment from randomly selected points in the field using zig-zag technique. Soils in Bugesera district were characterized by near neutral pH and Huye district with high soil acidity. Both soils were sandy loam in texture with low nutrient contents.

Table 2. Soil physico-chemical characteristics of the experimental sites and description of rage categories.

Parameter	Unit	Area		Range			
		Bugesera district	Huye district	High	Moderate	Low	Very low
Ph		7.1	5.2	>7	6-6.5	5.5-5	<5
Tot. N.	(%)	0.07	0.11	0.25	0.12-0.25	0.05-0.12	<0.05
Org. C	(%)	1.66	1.78	3.00	1.5-3.0	0.5-1.5	<0.5
Av.P	mg kg^{-1}	14	22.2	>50	30-50	25	<25
Ca	mg kg^{-1}	620	116	1600-2400	1000-1600	500-1000	< 500
Mg	mg kg^{-1}	112.8	16.8	80-180	40-80	20-40	< 20
K	mg kg^{-1}	276.9	42.9	175-300	50-175	50-100	< 50
Sand	(%)	69	76	80-70	60-50	40-20	<20
Silt	(%)	14	10	90-80	60-70	40-60	<40
Clay	(%)	17	14	55-90	50-40	30-20	<25

Description of nodule collection and research trial sites

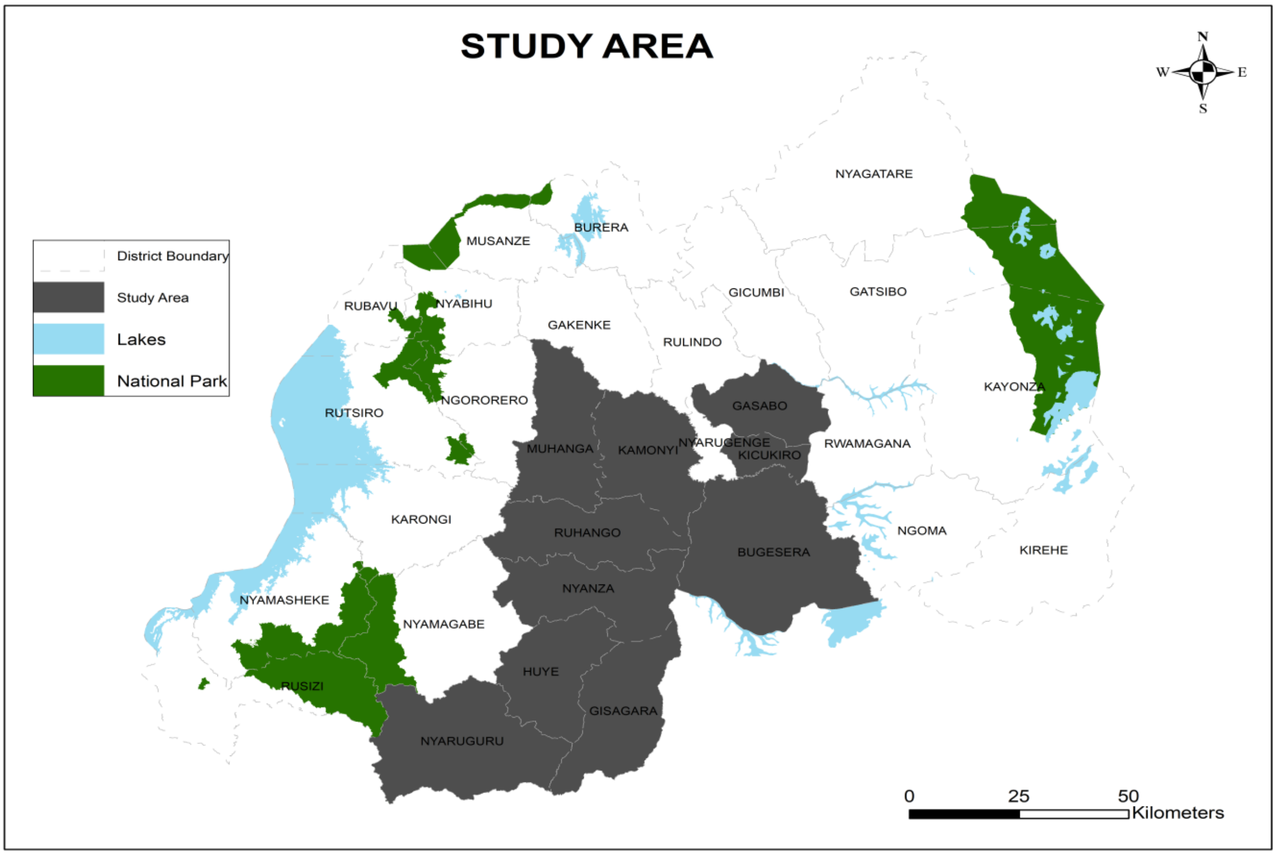

Fig. 1. Location of districts where nodules were collected (Adapted from Ministry of Local Government and Social Affairs (MINALOC).

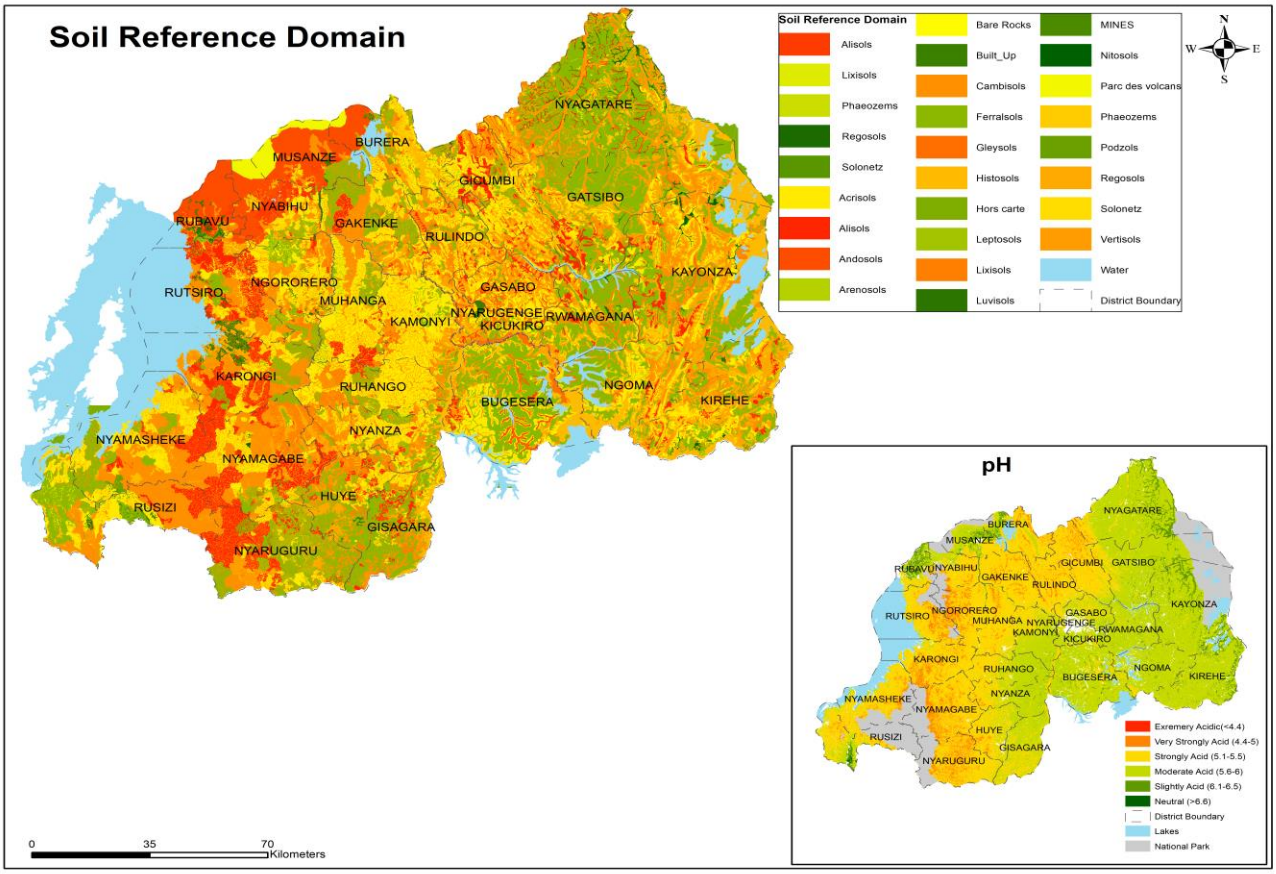

Fig. 2. Soil map of Rwanda showing soil types and pH.

Table 3. Soil nutrients composition across the sites following the nodule legume crops collection.

Strain	Area	Crop	pH	CEC mg kg^{-1}	Total N (%)	Org. C (%)	Av. P mg kg^{-1}	Ca mg kg^{-1}
BB18S	Nyaruguru	Bush bean	6.1	11.20	0.06	1.48	10.3	1.15
BB21S	Nyaruguru	Bush bean	5.9	13.96	0.08	1.85	16.8	1.24
CB23S	Nyaruguru	Climbing bean	6.0	13.05	0.08	1.80	13.4	1.27
SB30S	Nyaruguru	Soybean	5.2	12.88	0.09	1.56	12.2	0.70
BB64S	Save	Bush bean	6.0	19.19	0.07	2.10	7.7	2.82
SB76S	Huye	Soybean	5.1	13.39	0.09	2.15	13.0	1.98
CP84E	Bugesera	Cowpea	5.8	3.34	0.04	0.72	12.2	0.64
SB88E	Bugesera	Soybean	6.4	6.38	0.06	1.01	19.1	1.76

All the sites were very low in soil organic matter, with high nutrients depletion from soil layers (Table 3). Sub-samples of each collected samples were air dried, ground and passed through 2 mm mesh sieve for routine laboratory analysis of pH, organic C, available P, total N, exchangeable bases (Na, K, Ca and Mg) and texture following procedures described by (Okalebo *at al.,* 2002) for soil characterization. The analyses were conducted at the Soils laboratory, of the Rwanda Agriculture and Animal Resources Development Board (RAB), Rubona research station. Soil pH was determined using a pH-meter in soil: water ratio of 1:2.5 (w/v) after shaking for 30 minutes. Organic C. was determined by wet oxidation with potassium dichromate in an acidic medium and heated for 30 minutes at 150°C and then titrated with ferrous ammonium sulphate (Okalebo *et al.,* 2002). Total nitrogen was determined by steam distillation of soil digested with a mixture of sulphuric acid, hydrogen peroxide, salicylic acid and selenium catalyst (Okalebo *et al.,* 2002). Digestions were completed at 330°C on a heating block. Available P was determined calorimetrically on an Olsen solution extraction at a ratio of 1:20 (w/v) (Okalebo *et al.,* 2002). Olsen method is suitable for determining available P in a wide range of soil types and pH values (Okalebo *et al.,* 2002). Exchangeable Mg and Ca were determined on Atomic Absorption Spectrophotometer while Na and K were determined with a Flame Photometer after displacement with excess Ammonium acetate (Okalebo *et al.,* 2002). Soil texture was determined by particle size analysis using Hydrometer methods (Okalebo *et al.,* 2002). All the strains (Table 3) were found in soils with low pH although many areas in Bugesera district have slightly higher soil pH than the other districts.

Plant sampling and analysis

Nodulation and N-fixation: Assessment of nodulation, pod weight and amount of N-fixed were conducted at 45 days after planting (DAP). Ten plants were selected per plot and carefully uprooted from the soil after watering, ensuring that the root nodules were not severed. The roots were washed with water to have clean nodules and nodulation assessment and the other parameters were done following the methodology described by Unkovich *et al.,* (2008).

The procedure involved scoring each of the ten plants using predetermined criteria of classification (Fig. 3) by taking into consideration the number and position of nodules on plant roots.

The scale used to score case followed (Unkovich *et al.,* 2008):

Score 4-5 represents excellent potential for N2-fixation
Score 3-4 represents good potential for N2-fixation
Score 2-3 represents fair nodulation but N2 fixation, which may not be sufficient to supply N requirement of legume.
Score 0-2 represents poor nodulation, very low or no N2-fixation.

After scoring each plant, the total numbers of nodules per plant were counted and all the nodules were sliced into two to observe internal colouration, pinkish colour indicate effective nodulation.

Plant above ground biomass: Above ground plant biomass was determined at 50% of grain filling (R4) at 45 day after planting (DAP). The same plants selected in nodule assessment indicated the number and weight of pods. Plants were kept in clean pulp envelopes and immediately oven-dried, to a constant weight, at 70°C for 48 h and the dry weights were obtained. After weighing, the oven-dried plants were grounded and analysed for total N using procedures described by Okalebo *et al.,* (2002).

Determination of strain N-fixing /fixed N by difference method: The quantity of nitrogen fixed were determined by the N difference method. Plots planted with non-inoculated soybean and fertilizer input and maize was included alongside the experimental units as reference crop. Variation in the total shoot N-accumulated between inoculated legumes with non-inoculated legume control were regarded as a contribution of symbiotic nitrogen-fixation by the inoculated legume crop (Cerezini *et al.,* 2016). Tissue N data was used to calculate strain N_2-fixing (SNF) as described by (Herridge *et al.,* 2008) in Equation (1).

Equation 1: SNF for observed data = $\frac{N_{\text{uptake}}\text{ (with strain – with control)}}{N_{\text{uptake}}\text{ control}} \times 100$

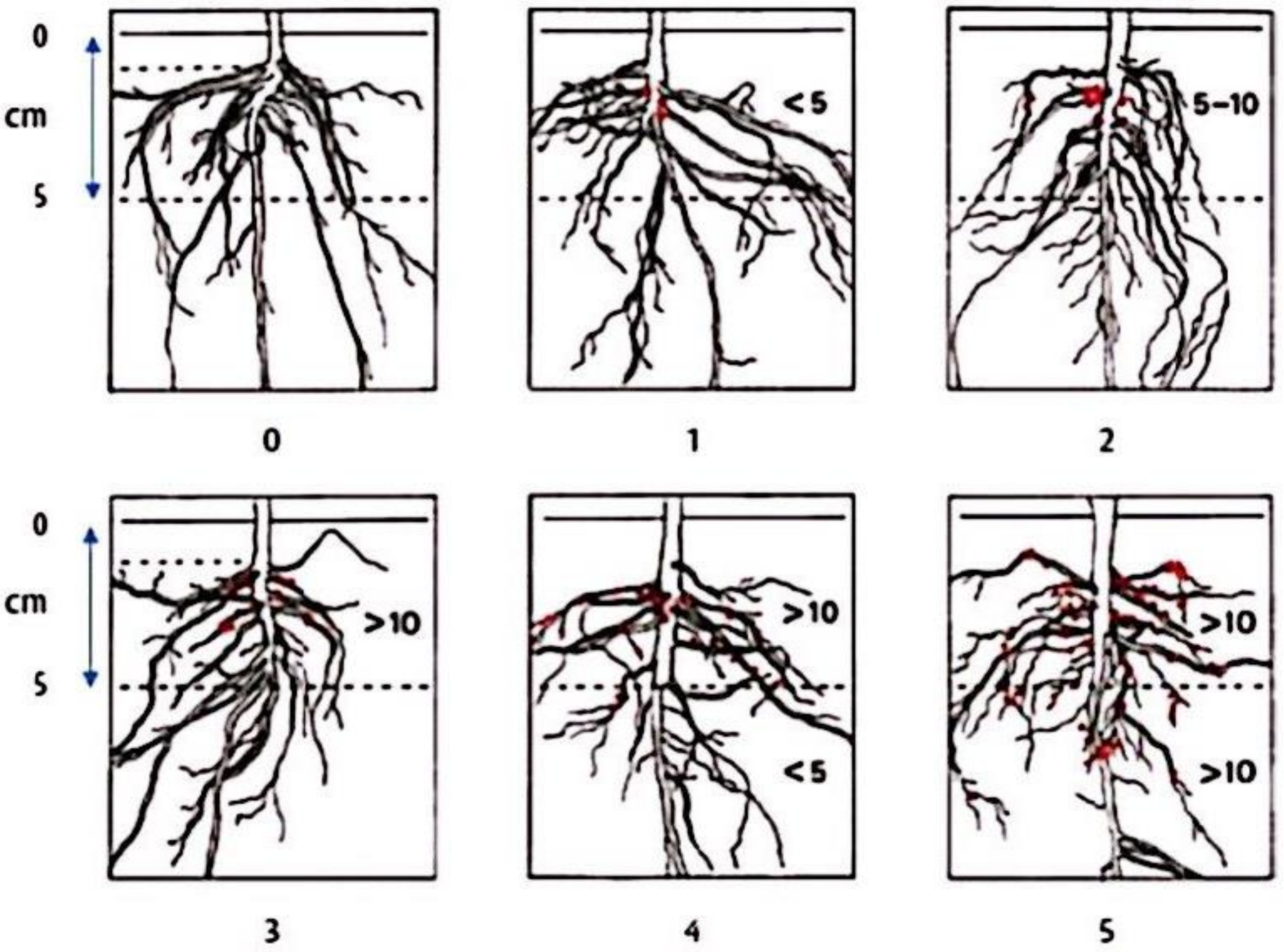

Fig. 3. Nodule scoring method from 0-5 ranks depending on the number and position of nodules.

Yield and yield components: All the plants within 1 m (0.4 m^2) radius were randomly selected to collect data on grain yield and yield components and the number of pods were counted and weighed. The grains were separated from the husks and their weights were recorded.

Statistical analysis

Number of colonies of four pH levels for the eight rhizobia isolates were transformed into log (x + 1) and subjected to the general analysis of variance with GenStat software 18th Edition. In the analyses, the pH levels represented the Y-variate with eight rhizobia isolates (*BB18S*, *BB21S*, *CB23S*, *SB30S*, *BB64S*, *SB76S*, *CP84E* and *SB88E*) as the treatment structure and this was calculated separately for each pH level and rhizobia isolates were compared by pH level. Means were compared to come up with its p-value and the Least Significant Difference (LSD) at 95% of confidence interval. Data collected on agronomic parameters were also subjected to the general analysis of variance and the differences among means were compared using LSD test at $p \leq 0.05$, and the treatment structure was constituted by soil amendments, fertilizer input, strains, and soybean variety. Multiple regression analysis of factors (Nodule formulation, N-fixed, biomass production) affecting pod yield and those affecting (Pods yield production and pods load) grain yields production were calculated using SPSS.

Results

At pHs 3.7 and 4.4, SB88E and SB76S, BB64S and CP84E isolates failed to grow except BB18S, BB21S, CB23S and SB30S (Table 4).

Surprisingly, SB76S, BB64S and SB88E grew at pH 5.1 and competed with only two high tolerant isolates (BB18S and CB23S) of pH 3.7. At pH 7, all the isolates grew well but significant differences (p=0.001) were observed.

Table 4. Number of strain's colonies of *R. leguminous* plant at four pH levels. Data is log (log (x+1)) transformed.

Strain	pH			
	3.7	4.4	5.1	7
BB18S	8.8^{a}	8.5^{a}	8.7^{ab}	8.8^{b}
BB21S	8.4^{a}	8.5^{a}	8.4^{c}	8.5^{de}
CB23S	8.7^{a}	8.6^{a}	8.5^{bc}	8.4^{e}
SB30S	8.4^{a}	8.5^{a}	8.3^{c}	8.7^{bc}
BB64S	NG	NG	8.7^{ab}	8.8^{b}
SB76S	NG	NG	8.7^{ab}	9.0^{a}
CP84E	NG	NG	NG	8.8^{b}
SB88E	NG	NG	8.9^{a}	8.6^{cd}
p-value	0.001	0.008	0.001	0.001
LSD	0.2	5.1	0.3	0.2

Within columns, values followed by different letters are significantly different at $p<0.05$, NG = No growth

Effect of soybean variety and soil amendment on nodulation across different sites: The interaction of soybean variety and soil amendments application was significant (p=0.012) due to low response of same variety without organic manure and lime application (Table 5).

Table 5. Effect of soybean variety and soil amendment on nodulation (nodule score) across different sites.

Variety	Amendments		
	Lime	Organic manure	Control
PK6	2.569^{a}	2.472^{a}	2.361^{ab}
SB24	2.582^{a}	2.375^{ab}	1.986^{bc}
Sc Squire	1.681^{c}	2.401^{ab}	1.736^{c}
p=0.012			
LSD=0.4406			
Site			
Bugesera	2.731^{a}	2.37^{a}	2.37^{a}
Huye	1.823^{b}	2.462^{a}	1.685^{b}
p=<.001			
LSD=0.3398			

Values with different letters across (columns and rows) are significantly different at p<0.05

Table 6 Effect of fertilizer inputs in the presence of strains inoculation and soil amendments on total dry matter yield (g/kg).

Amendments	Fertilizer application			
	DAP	DAP+KCL	Urea	Control
Lime	21.77^{ab}	16.82^{efj}	15.98^{j}	17.54^{defj}
Organic manure	19.61^{c}	18.26^{cde}	19.26^{cd}	17.35^{defj}
Control	19.86^{bc}	17.37^{defj}	23.54^{a}	16.29^{fj}
p=0.036				
LSD=1.954				
Strain				
BB18S	19.55^{b}	16.17^{f}	18.53^{bd}	16.96^{d}
BB64S	19.25^{bcd}	19.53^{b}	23.07^{a}	15.14^{f}
SB88E	22.44^{a}	16.76^{ef}	17.18^{cdf}	19.07^{bcde}
p=0.017				
LSD= 2.318				

Nodule score: score 4–5 represents excellent potential for n2-fixation, score 3–4 represents good potential for fixation, score 2–3 represents fair nodulation but n2 fixation, which may not be sufficient to supply n requirement of legume., score 0-2 represents poor nodulation

Organic manure application increased the number of nodules in all the three varieties (PK6, *SB24* and *Sc Squire*). The control PK6 nodulated well at the same level like where lime and organic manure were applied, but did not show any significant different with the control of SB24. PK6 and *SB24* responded well under lime application compared to the control. Nodulation was high in Bugesera district compared to Huye for all the strains (Fig. 4) (*p*<0.001), except *SB88E* strain inoculation which showed statistically similar performance.

Effect of soil amendments, inputs, strains and their interactions on dry matter yield: The combination of inorganic fertilizers and soil amendments significantly (*p*=0,036) increased the total dry matter yield compared to the control, except where fertilizer input was not applied (Table 6).

Lime application in combination with the starter nitrogen and phosphorus considerably increased the total dry matter (TDM) (Table 6). The application of nitrogen (Urea) without any amendment had the same results as obtained with treatment involving phosphorus and low nitrogen content (DAP) combined with lime. Most of strains performed well in the presence of all the essential nutrients, except BB18S and SB88E where DAP-KCL was applied (Table 6). *SB88E* strain showed good performance when N starter and P (DAP fertilizer) were applied, but not significantly different with the application of Urea alone.

Strain N-fixation of soybean varieties with microbial symbionts: Non-inoculated soybean legumes produced very low fixed N compared to all inoculated treatments (Fig. 5).

Analysis of variance revealed high response when PK6 was applied with N starter, phosphorus (DAP)+potassium (KCl) and Urea fertilizer compared to the control (Fig. 5). *SB24* highly responded well at Bugesera than the two varieties (*PK6* and *Sc Squire*) and then followed by *PK6* (Fig. 6). Under acidic condition at Huye, station, the SNF decreased and were similar in all varieties (Fig. 6).

All varieties had higher levels of N at Bugesera than at Huye with SB24 being the best followed by PK6. All legume isolates performed well under both soil conditions, with high SNF of more than 100 kg/ha of N-fixed.

The analysis of variance on strain performance indicated high nitrogen fixation at Bugesera district, compared to Huye (Fig. 7).

Multiple linear regression analysis: Multiple linear regression analysis revealed that the weight of pod was dependently significantly related to increase in biomass production, nodulation and the biological nitrogen fixation (Table 7).

Analysis of variance showed the association of all independent variables to have contributed significantly (*p*<0.000) to the pod formation (*p*=0.000 of the regression) with 40% (R^2) of the proportion of the variance for the dependent variable being explained by the three independent variables.

The results (Table 8) demonstrated the dependence of grain yield production on the number of pods and weight of pods, which were predicted by N-fixation and biomass production. However, both parameters (TDM and N-fixation) did not significantly affect the grain yield production (Table 8).

Effect of inoculation and NPK fertilizer on grain yield: Analysis of variance (Table 9) showed that the grain yield was significantly affected by rhizobia (*p*<0.011), soybean variety (*p*<.001), and interaction (*p*=0.029) between rhizobia inoculants, varieties and fertilizer inputs. It was noted that variety *Sc Squire* had a significantly higher grain yield than both *PK6* and *SB24*.

The inoculation of *Sc Squire* variety with *BB64S* combined with the application of DAP significantly increased the grain yield compared to the control. However, similar results were achieved with application of NPK and Urea) with no inoculation. Sc Squire responded well with all the strains compared to the other two varieties (*SB24* and *PK6*) followed by *SB24* variety.

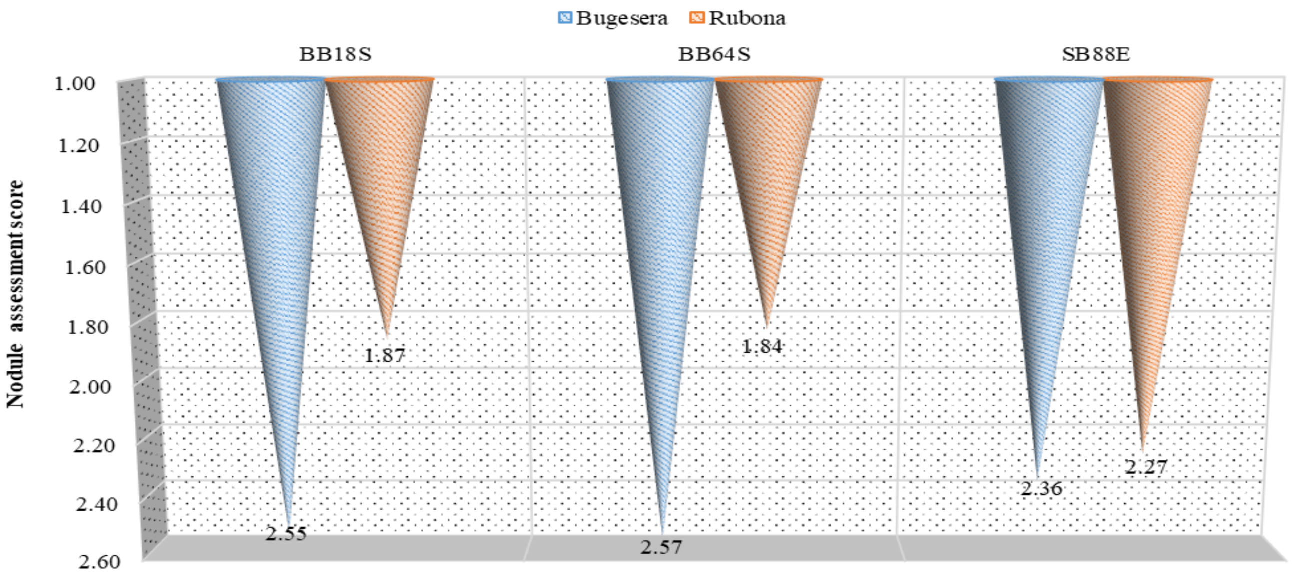

Fig. 4. Nodule assessment and response of rhizobia strains inoculants in two sites.

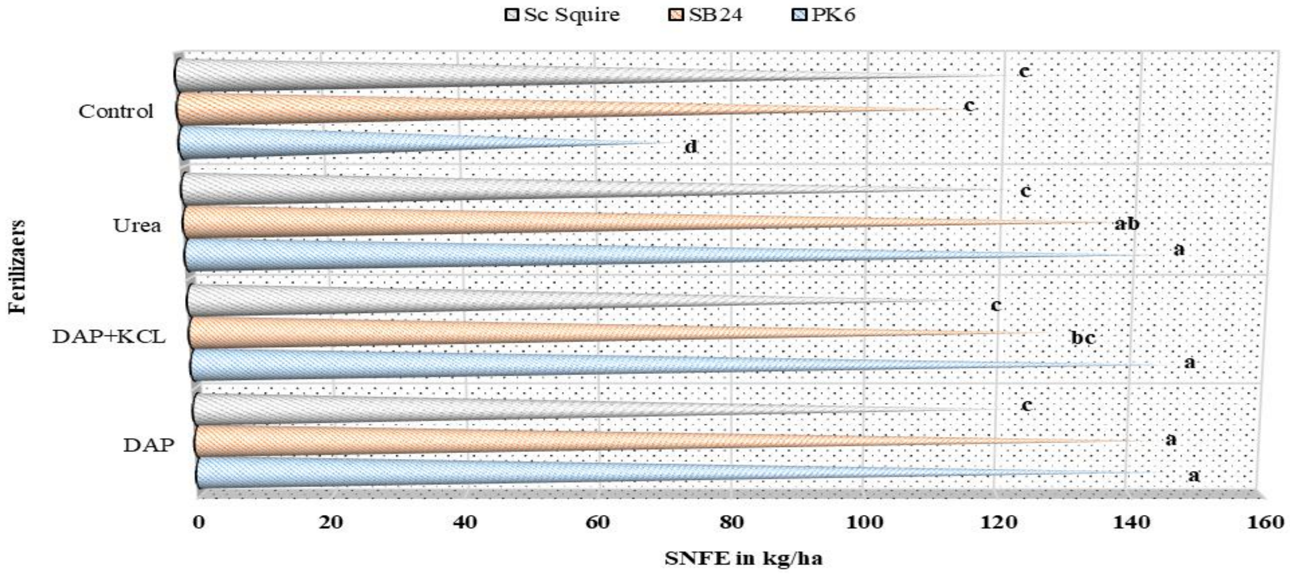

Fig. 5. Strain N-fixation response on varieties under fertility management.

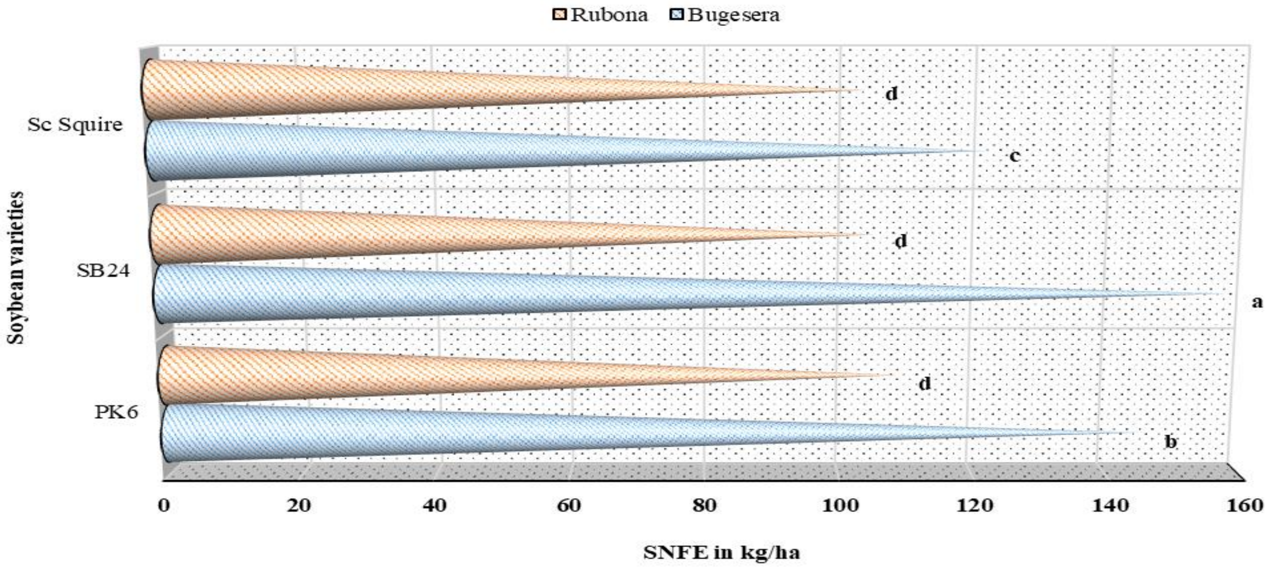

Fig. 6. Effect of site differences on SNF variety response.

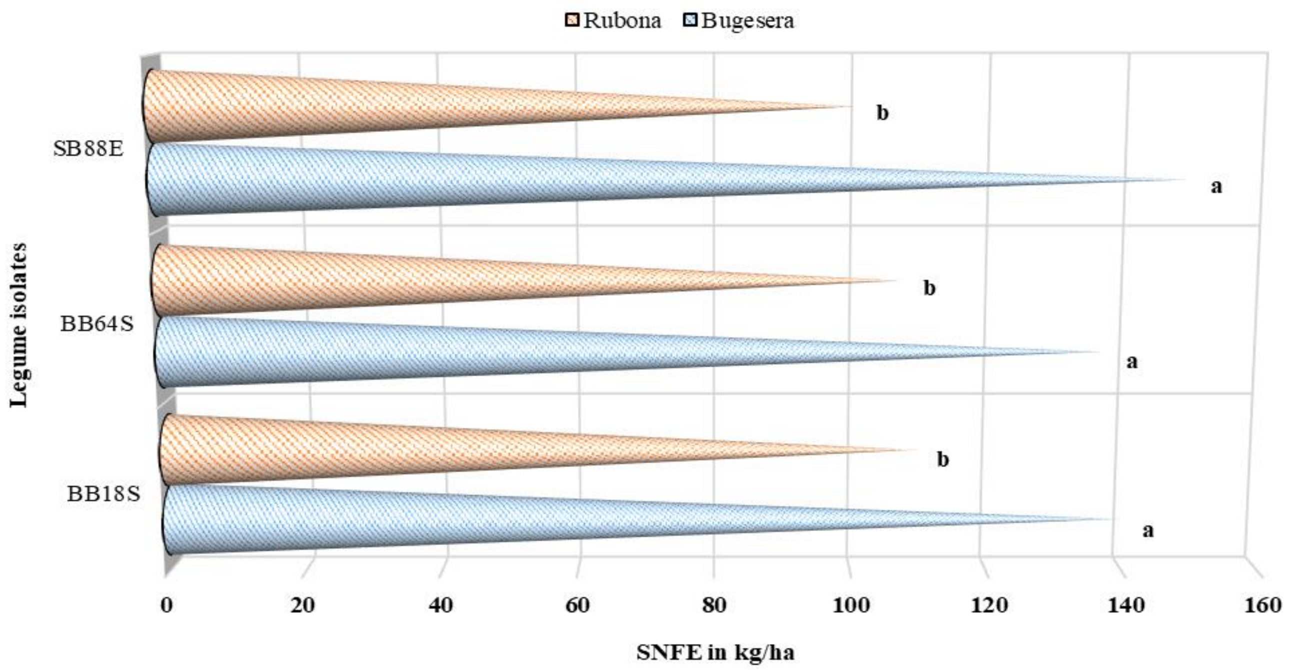

Fig. 7. Effect of site differences on strain N-fixation of three elite's legume isolates.

Table 7. Multiple regression analysis of the factors influencing pod yield production.

Model	**Unstandardized coefficients**		**Standardized coefficients**		
	B	**Std. Error**	**Beta**	**T**	**Sig.**
(Constant)	54.432	3.715		14.654	0.000
Nodule formulation	2.845	1.062	0.083	2.680	0.008
N-fixation	-0.410	0.102	-0.124	-4.020	0.000
Biomass production	0.089	0.004	0.625	20.101	000
Regression, p=0.000, R^2=0.404					

a. Dependent variable: Pod yield production

b. Independent variables: Nodule formulation, N-fixation, biomass production

Table 8 Multiple regression analysis of factors influencing the grain yield production.

Model	**Unstandardized coefficients**		**Standardized coefficients**		
	B	**Std. Error**	**Beta**	**T**	**Sig.**
(Constant)	-34,356	32,332		-1,063	,288
Pods yield production	14,022	,261	,888	53,636	,000
Total Dry Matter	-1,680	,975	-,027	-1,722	,086
N-fixation	-,914	,796	-,018	-1,148	,251
Pod load	,415	,076	,091	5,430	,000
Regression, p=0.000, R^2=0.863					

a. Dependent variable: Grain yield production

b. Predictors: Pods yield production and pods load

Table 9. Grain yield (kg/ha) of soybean varieties inoculated with rhizobia inoculants in combination with fertilizer inputs.

R. Strain	**Variety**	**Fertilizer inputs and control (T4)**			
		DAP	**DAP+KCL**	**Urea**	**Control**
BB18S	*PK6*	1295^{op}	1403^{nop}	1400^{nop}	1445^{lmno}
BB18S	*SB24*	1460^{lmn}	1549^{jklmn}	1537^{jklmn}	1421^{mnop}
BB18S	*Sc Squire*	1935^{bcd}	2050^{ab}	1800^{defg}	1912^{bcde}
BB64S	*PK6*	1522^{klmn}	1287^{p}	1549^{jklmn}	1467^{lmn}
BB64S	*SB24*	1731^{fghi}	1588^{ijkl}	1639^{hijk}	1567^{jklm}
BB64S	*Sc Squire*	2114^{a}	1974^{abc}	2084^{a}	1777^{efgh}
SB88E	*PK6*	1550^{jklmn}	1729^{fghi}	1439^{lmnop}	1563^{jklm}
SB88E	*SB24*	1687^{ghij}	1587^{ijkl}	1678^{ghij}	1521^{klm}
SB88E	*Sc Squire*	1860^{cdef}	1776^{efgh}	2122^{a}	2032^{ab}

p=0.029; *LSD*= 153.8

Table 10. Effect of soil amendments, varieties and sites on grain yield.

Amendments/Site	Varieties					
	PK6		SB24		Sc Squire	
	Bugesera	Huye	Bugesera	Huye	Bugesera	Huye
Lime	1629^{de}	1047^{hi}	1950^{c}	1270^{fgh}	2570^{ab}	1073^{hi}
Control	1901^{cd}	1168^{gh}	2039^{c}	850^{i}	2804^{a}	1249^{fgh}
Organic manure	1589^{de}	1492^{ef}	2019^{c}	1057^{hi}	2703^{a}	1320^{efgh}

p=<0.001, *LSD*=313.1

Effect of soil amendment and variety response on grain yield production: At Bugesera district, application of lime and organic manure increased the grain yield of soybean (Table 10). The application of organic manure on *PK6* and *Sc Squire* at Huye produced significantly higher grain yield compared to where lime was applied. Lime and organic manure effect was realized on the yield of SB24 soybean variety compared to the control.

Discussion

The soils in the study sites had neutral acidic conditions to strongly low pH, and overall, the regions had low nutrient levels. Most crops including soybean prefer a soil pH range of 5.5-6.5 for optimum growth (Mhango *et al.*, 2020). Acidic medium selectively reduces the diversity of soil microorganisms including rhizobia that symbiotically fix atmospheric N in leguminous host (Atieno *et al*., 2019). The low fertility potential of the soil, due to its deficiency in essential nutrients such as nitrogen, phosphorus, potassium, and calcium, as well as its low cation exchange capacity (CEC), was attributed to soil acidity, which creates complex interactions among physical, chemical, and biological properties of the soil, ultimately limiting plant growth (Agegnehu *et al*., 2017). The presence of rhizobia activities in the soil, which are responsible for the decomposition of organic carbon and availability of nutrients, were negatively impacted by soil acidity, resulting in reduced decomposition of soil organic carbon and decreased availability of nutrients (Chowdhury *et al*., 2021).

At very low pH, a number of strains could not grow due to low tolerance of high acidity condition, although a few grew in the same condition. In fact, more resistant rhizobium strains could survive under acidity conditions, and could be multiplied to get some pH tolerant effective *B. japonicum* strains (Ferreira *et al*., 2016). The abundant H^+ ion could be the most restrictive factor affecting the growth of rhizobia strains, although this will always be dependent on species sensitivity and resistance to acidity level (Paulo *et al.*, 2012). It is imperative that selection consider strains adapted to different conditions and focus be mainly on the knowledge of rhizobia strains diversity and most tolerant genetic resources for abiotic stresses (Freitas *et al*., 2013). The disruption of crop growth caused by acidic soil conditions serves as an indicator of plant disturbance (Shao *et al.*, 2009). It would not be easy for any introduced microorganism in partnership to survive under such conditions, and the stresses can lead to negative bio-physicochemical alterations of the soil (Gil-Sotres *et al.*, 2005). Selection of elite strains to improve the quality of nitrogen-fixation through the association of leguminous plants and indigenous microbial symbionts community would be a great biotechnology tool to address the acidity problem (Requena *et al.*, 2001). A large number of native rhizobia in competition against introduced strains leads to the weakening of the external symbiotic organism (Zhang *et al.*, 2007).

In most cases, slow growing strains of rhizobia are observed to be even less tolerant under high acidic condition than fast growing *Bradyrhizobia* strains (Boakye *et al.*, 2016), although in some research, the expectation proved otherwise indicating the ability of slow growing strains to withstand and produce much under such conditions depending on the soils from where they are coming from (Ndungu *et al.*, 2018). The study found that using acid tolerant isolates resulted in higher biomass production, which led to increased pod and grain yield. This approach is recommended as the most effective method for managing soil acidity and boosting productivity (Graham *et al*., 2000). In the field, the study results demonstrated the positive effects of lime and organic manure application on the nodule formulation (Table 5), dry matter (Table 6) and the grain yield production (Table 10). Soil acidity is among the most limiting factors affecting legume growth and microbial activities (Zahran, 2017). In most cases, farmers use resistant varieties to overcome acidity problem, but the result is still very poor (Gurmessa, 2021). At Huye where acidity was too high, the three varieties under lime application responded well compared to where organic manure was applied, though PK6 as low yielding and resistant variety demonstrated much yield than Sc Squire and SB24, respectively. Studies have shown that applying lime before manure under very acid conditions can significantly improve soil pH, increase nutrient availability, and ultimately lead to higher yields in soybean crops (Tadesse *et al.*, 2021).

Soil analyses showed that the two study sites had very low nutrient contents and varied soil pH. Soils of Huye district (Rubona) were acidic whereas Bugesera soils were near neutral. These soils are also nutrient-depleted and have low organic matter content. The type of soil pH and fertility status significantly affected soybean yield, with lower yields observed in soils with very low nutrient content and acidic soil pH (Makinde *et al.*, 2021). As such, soil organic residues enhance biological nitrogen fixation thus increasing nodule formation (Saha *et al.*, 2017). *Sc squire* soybean variety had low number of nodules in the control treatment compared to PK6 and SB24 probably due to low tolerance under acidic condition (Table 5). *PK6* variety on the nodules assessment score without any amendment gave the same results as where organic matter and lime were applied (table 2) but *SB24* responded well under lime application. This is principally attributed to genotypic expression where plant genetic factors influence nodulation potential and the amount of N-fixed (Goh *et al.,* 2016). Plant genotype also influences interaction with rhizobia strains, especially at varying soil pH (Zahran, 2017).

In legume-based cropping system, soil organic matter can restrain N-fixation due to competition by micro-organisms and N availability, but plant legumes in BNF formation play an elementary role to regulate the suppression effect of other micro-symbionts on N-fixation (Kuzmicheva *et al.,* 2017). The low number of nodules observed per plant ranging between 5 and 10 could be attributed to soil acidity. Whereas Sc Squire, a promiscuous variety responded poorly to inoculation under acid condition, other studies have reported nodule as high as 80 per plant on some soybean varieties (Mathenge *et al.,* 2019). Although soil acidity decreases N-fixation mechanisms, some rhizobia tolerant strains can adapt to low pH and nodulate under such condition due to their physiological mechanisms (Sena *et al.,* 2020). Under acid conditions in the presence of nitrogen and phosphorus, the three rhizobia strains (*BB18S*, *BB64S* and *SB88E*) produced high total dry biomass (Table 6), and the N-fixation was much realized at Bugesera district for all the strains than Huye district (Fig. 7). It is plausible to suggest that the three rhizobia strains performed well and increased N–fixation, which translated into increase plant growth as expressed by the aboveground biomass (Aserse *et al.,* 2020). Earlier studies have also demonstrated that under acidic conditions, inoculation of elite rhizobia strains increased nodulation and translated to higher aboveground biomass (Aserse *et al.,* 2020).

The study demonstrated that the application of lime and organic manure in the presence of inorganic nitrogen and phosphorus, increased the total dry matter of soybean crop. This integrated approach significantly increased the total dry matter of the soybean crop, emphasizing the benefits of a holistic nutrient management strategy that optimizes soil pH, enhances soil structure, and maximized yield potential in a sustainable manner (Maltais *et al.,* 2016). Soil amendments notably lime and soil organic manure have been translated into available essential nutrients thus increased total dry matter of soybean in legume-based cropping system (Da Costa *et al.,* 2016). Yield results (Table 9) showed that Sc Squire had significantly more grain yields than both *PK6* and *SB24*, especially when this variety was inoculated in presence of starter-N, phosphorus and potassium nutrients, and where also Urea was applied alone, but in many cases the controls had very low results. High-yielding soybean varieties have a greater capacity to take up and utilize nutrients such as N and P, resulting in higher yields (Tamagno *et al.,* 2017), and can be used in conjunction with appropriate nutrient management practices to meet the growing demand for soybean products.

In multiple regression analysis, an increase in biomass production positively influenced the pods yield, indeed biomass is a reliable indicator of yield in most crops (Kubar *et al.,* 2021). *PK6* responded well in the presence of organic manure under low pH, and furthermore, low and medium-yielding soybean varieties showed a better response to organic manure application than high-yielding varieties. This highlights the importance of considering varietal characteristics in nutrient management strategies, suggesting that a tailored approach with organic inputs may be more beneficial for enhancing the productivity of specific soybean varieties (Rurangwa *et al.,* 2018). At pH 7.1, the high yielding variety responded well on yield of soybean compared to the two varieties and SB24 came at the second place, and this explains how high yielding soybean varieties are more responsive to good soil pH levels than low yielding and resistant varieties (Ming *et al.,* 2017). The effect of lime on grain yield was realized with *Sc Squire* variety under acidic condition The results showed that the application of lime and manure had a greater impact on soybean yield in areas with soil pH of 7.1 (Bugesera district), compared to pH 5.2 (Huye district) where their effect was limited, indicating that soil acidity can hinder the effectiveness of soil amendment practices on improving soil fertility and soybean yield (He *et al.,* 2021).

Conclusion

Under low pH conditions, some of the strains were able to withstand the acidity, while others grew poorly. However, under neutral pH conditions, all of the strains grew well, and even the ones that had low performance under high acidity were able to produce a larger number of cells. While stresses such as acidity can suppress the growth of legumes and their microsymbionts, the selection of acid-tolerant rhizobia strains has the potential to minimize the impact of such abiotic conditions. By choosing rhizobia strains that are capable of withstanding acidic environments, the negative effects of acidity can be reduced, which may ultimately lead to improved growth and productivity of legumes. The findings revealed that all three rhizobia strains demonstrated good performance under both acidic soil conditions and in the field. Moreover, the use of these strains resulted in increased yield of selected soybean varieties, indicating their potential as effective inoculants for improving soybean productivity. Soil organic matter and lime played a significant role in nodules formation. All soybean varieties significantly nodulated when lime and organic manure were applied compared to control. However, amendments affected nodules formation and the strains responded under acid condition. Nutrient (N, P and K) content with application of soil amendments increased the total dry matter yield and nodulation. The SNF varied much under acid condition, and soybean varieties with their microbial symbionts responded differently. However, the dry matter yield as results of soil amendments, legume crop genotypes and acid-tolerant rhizobia strain inoculants were related to the grain yield production. N-fixation indirectly increased the grain yield by producing high biomass, weight and number of pods. The three acid tolerant rhizobia strains performed well under neutral and acid condition, therefore soil organic matter and lime could fix soil acidity problem and thereby increasing the biomass and productivity of legume crops with elite tolerant microsymbionts. This highlights the potential of using acid-tolerant rhizobia strains as effective inoculants for enhancing soybean productivity in acidic soil conditions.

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