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Full Length Research Paper

Diversity and Symbiotic Effectiveness of *Rhizobium* Isolates Collected from Different Faba bean (*Vicia faba*) Growing Areas of North and South Gondar, Ethiopia

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Fifty seven *Rhizobium* isolates were retrieved based on infection method from different faba bean growing areas of North and South Gondar, Ethiopia. In sand culture, only 35.5% of isolates showed nodulation. Analyses of variance indicated that inoculation of isolates improved tested traits significantly ($p< 0.05$) at all measured investigated parameters such as shoot length, shoot dry weight, and plant total nitrogen as 11, 28 and 31.3%, respectively, over nitrogen treated plants, 2, 10 and 29.4% respectively over standard *Rhizobium* and 55, 82.3 and 85.7% respectively over negative control treatments. Shoot dry weight was found to be strongly positively correlated with symbiotic effectiveness ($r = 0.994$, $P <0.01$). Based on symbiotic effectiveness, 80% of the tested isolates were found to be highly effective, 13% effective and only one isolate less effective. Therefore, there is a need for detail study of effective isolates tested under field conditions and molecular characterization for better classification.

Key words: Authentication, Legumes, Nitrogen fixation, *Rhizobium*, total nitrogen.

INTRODUCTION

Ethiopia is home to about a dozen species of tropical grain legumes that are extensively grown in many parts of the country contributing the major diet both in rural and urban population. Grain legumes have a nitrogen fixing symbiosis with soil root nodule bacteria. Among these, Faba bean (*Vicia faba* L.) is an annual grain legume widely cultivated, which serves as foods for human and animal nutrition in many countries, since it is rich in protein, minerals and vitamins (Tekle et al., 2016). Cultivation of faba bean plays important roles in maintaining sustainable agriculture system in many marginal areas; due to its high nutritional value, multiple uses and ability to grow over a wide range of climatic and soil conditions.

Faba bean is a dominant pulse crop in Ethiopia in terms of area coverage and amount of production (CSA, 2014), however its average yield under small holder farmers is not more than 1.6 t ha^{-1} (CSA, 2013) due to lack of improved varieties, insects/pests and diseases. Historically, Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean. The

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world's foremost producing countries for faba bean are China, Ethiopia, Egypt and the United Kingdom. Ethiopia is the leading producer of faba bean in Africa accounting for 56% of the production (FAOSTAT, 2014). Faba bean popularity has increased recently as its high yield makes it attractive to producers while its high protein content and low-priced makes it attractive to consumers (Stagnari et al., 2017).

The productivity of faba bean in Ethiopia is low. Most farmers in Ethiopia cultivate local faba bean varieties which are susceptible to biotic and abiotic factors (Link et al., 2010). The Central Statistical Agency (CSA, 2013) reported that faba bean is planted in to 4.34% of the grain crop area with an annual production of about 9.917 million quintals, 3.94% of the total grain production and yield of 18.42 q/ha in Ethiopia.

Legumes are able to establish nitrogen-fixing symbioses with bacterial microsymbionts (rhizobia), thus reducing the need for chemical fertilizers. This reduction may help to minimize greenhouse- gas emissions and to avoid contamination of ecosystems. This association further provides a nitrogen supplement for the subsequent crops. Faba bean like other legumes crops has the ability to form symbiotic association with root nodulating bacteria (rhizobia) group called *Rhizobium leguminosarum bv. Viciae*.

Polyphasic characterization approach and selection of potential *R. leguminosarum bv. viciae* that show better nitrogen fixing capacity are still insufficient. Hence, the present study aimed to identify efficient *Rhizobium* isolates and their effect on nodulation from soil samples collected from major faba bean producing areas of North and South Gondar, Ethiopia.

MATERIALS AND METHODS

Collection of soil samples

Three representative faba bean growing woredas were selected from North Gondar and South Gondar zone (Lay Gayint and Farta woreda from South Gondar; Chilga from North Gondar) based on their productivity of faba bean in the last few years. From each woreda, the productive areas were selected based on their productivity of faba bean. From each woreda of productive area, representative farmer fields were selected based on the previous history of cultivating faba bean crop. Thus, 20 farmer fields from each of Chilga and Farta woredas and 17 farmer fields from Lay Gayint were selected. About 3 Kg of soil sample was collected from each selected farmer fields at a depth of 20 cm using sterile (fresh) plastic bags.

The presumptive test, *Rhizobium* isolation, identifications and pot experiments were carried out at the Department of Biology Laboratory, University of Gondar. Plant total nitrogen and Soil chemical analysis were done at Adet Agriculture Research Center, Ethiopia.

Colony morphological and biochemical characterization

Colony morphology of rhizobial isolates was studied on Yeast Extract Mannitol Agar (YEMA) according to Jordan (1984). Gram staining and acid/base reactions were evaluated on YEMA containing 25 µg ml^{-1} bromothymol blue (BTB) (Lupwayi and Haque, 1994).

Utilization of carbon source

Carbon source utilization of isolates was determined following the method of Somasegaran and Hoben (1994) on 15 carbohydrates prepared as 10% (w/v) in sterile distilled water. Each 10 ml of the carbohydrate stock solutions was added to 90 ml of the carbohydrate free basal medium and their growth was observed after 2-3 days of incubation and YEMA medium plates incubated at 28°C were used as controls.

Acidity, alkalinity, salinity and temperature tolerance

All experiments on tolerance to acidity, alkalinity, temperature and salinity were performed according to McVicar et al, (2005). Tolerance to acidity and alkalinity of each isolate was evaluated on YEMA with pH adjusted between 4.0 to 9.0. For salt tolerance, the isolates were transferred to YEMA plates supplemented with NaCl at concentrations of 0.1, 0.5, 1, 2, 3, 4, 5% (w/v). The ability of bacterial strains to grow at high and low temperatures was monitored at incubation temperatures of 5, 10, 15, 35, 40 and 45°C and YEMA medium plates incubated at 28°C were used as controls. Strains were considered salt tolerant, resistant to acidity and temperature resistant when growth was similar to the growth in the control plates.

Intrinsic antibiotic resistance

The intrinsic resistance of isolates was determined by inoculating 10 µl of each culture (10^9 cells/ml)) on solid YEMA medium containing four filter sterilized with Millipore filters antibiotics at different concentrations of water and ethanol according to Taye (2010).

Authentication of the strains (Symbiotic effectiveness)

In order to determine the isolates performance (effectiveness) in nitrogen fixation, authentication test was done as described by Vincent (1970). Out of 57 isolates, 40 best isolates were selected from the presumptive test based on nodule color, nodule number, nodule fresh weight and shoot height. For each isolate three surface sterilized pots were filled with approximately 3 kg acid washed and heat sterilized river sand. In each pot four to five healthy surface sterilized Adet faba bean variety seeds obtained from Adet Agricultural Research Centre were germinated and planted according to the methods of Somasegarian and Hoben (1994). A total of 120 pots were sown for this experiment. As a starter, 20 ppm of nitrogen was included in each pot before planting. Each seedling was inoculated with 1 ml of each isolate with an inoculum size of 10^9 cells/ml. After a week, the seedlings were reduced into three per pot. Two treatments were used as control: one without nitrogen supply and an uninoculated (*i.e*. negative control) and the other an uninoculated but with provision of 0.05% (W/V) KNO_3 per week (*i.e.* positive control). This experiment was conducted in triplicates and the plants were grown under glasshouse condition. The pots were arranged in Completely Randomized Design (CRD) and plants were also fertilized with the quarter strength Broughton and Dilworth nitrogen-free nutrient solution once a week and received water every three days. After 45 days of planting, the plants were carefully uprooted and nodule

color, nodule number, nodule fresh weight, shoot length were counted and measured, nodule dry weight, root biomass and shoot dry weight were also estimated after drying at 70°C for 24 h in under dry oven, and total nitrogen was analyzed by modified Kjeldahl method after Sahelemedihin and Taye (2000). The Relative symbiotic effectiveness of the isolates was calculated according to the equation proposed by Date et al, (1993) cited in Purchino et al, (2000) [100 X inoculated plant dry matter (DM)/ N-fertilized plant DM) with Nitrogen fixing effectiveness classified as ineffective <35%; poorly-effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%.

Nodulation status survey

The general survey was conducted during the identification and collection of soil samples. In most cases, attempts were made to meet the owner of the field to establish cropping history.

Data analysis

Statistical data analysis was done by using SAS software version 9.2. Analysis of variance (ANOVA) was done for the comparison of means for all treatments and Duncan's multiple range test was used to detect the significant difference among treatment means at $p \leq 0.05$. Correlation coefficients were calculated to study the association among the measurement traits using Pearson correlations. Data from all physiological studies was also used for cluster analysis and similarities between isolates and a dendrogram was constructed based on average linkage hierarchical cluster analysis between groups using SPSS version 20.0 software statistical program.

RESULTS AND DISCUSSION

Morphological and Biochemical characteristics

All the 57 isolates recovered from the root nodule fulfilled the characteristics of rhizobia (Lupwayi and Haque, 1994): as gram-negative, rod-shaped, the color of colonies was milky-white opaque with a circular shape, regular borders and raised, showing intermediate to high production of mucus after 2 to 3 days of growth on YEMA medium at 28°C. Furthermore, the colony diameter of all isolates of *Rhizobium leguminosarum* bv. *viciae* was within the range of 1.5-4.5 mm after 2 to 3 days of incubation at 28°C (data not shown). According to the report of Jordan (1984), *R. leguminosarum* isolates usually show colony size between 2-4mm in diameter and 95 % of our isolates fall to this group (data not shown).

All the isolates changed the YEMA-BTB medium to yellow color and did not absorb Congo red on YEMA-CR medium, indicating that all the isolates are acid producer and fast growing rhizobia. Similar classification has been done by Ondieki et al. (2017). The color formation is due to the utilization of the sugar component of the medium for their growth. This finding is similar with the previous work of Mulisa and Fassil (2011) indicating that many *Rhizobium* strains isolated from each sampling field of Northern Ethiopia were fast growing and acid producing. All the strains were gram negative and rod shaped as revealed by Gram's staining technique. These results confirmed the finding obtained by Tagelsir and Mohamed (2015).

Hierarchal cluster analysis

Hierarchal cluster analysis for 57 isolates of *Rhizobium* based on their physiological characteristics including carbon source utilization, pH tolerance, salinity tolerance, temperature tolerance and different antibiotic tolerance at different concentrations (data not shown) were used to construct a dendrogram using average linkage clustering method between groups. Soil temperature, physical and chemical composition, moisture content in soil varies within small areas and these variations affect the populations of the soil inhabitants. Therefore, differences in response towards salinity, pH and temperature are expected. Based on the above parameters, *Rhizobium* isolates from different North and South Gondar locations essentially grouped in to six main clusters and the individual main cluster contains sub clusters to analyze their similarity using Pearson's coefficient on the dendrogram (Figure 1).

The highest similarity was computed between isolate (KD-4) from Kimir Dingay and (LG-3) from Lay Gayint in cluster 4 and isolate (KD-12) from Kimir Dingay and (LG-9) from Lay Gayint in cluster 5 which were nearly 100% similar. This 100% similarity indicates that the isolates were retrieved from the same soil pH, temperature, salinity, and showed resistance of different antibiotics at different concentrations and carbon source utilization (data not shown). On the other hand, CHI-6 and CHI-19 isolates from Chilga in cluster 2, CHI-14 and CHI-20 isolates from Chilga in cluster 1 and KD-6 and KD-10 isolates from Kimir Dingay in cluster 6 were revealed 88% similarity on the dendrogram. Whereas, the lowest similarity was computed between isolate CHI-15 from Chilga and isolate KD-6 from Kimir Dingay which presented in to two different clusters (that is CHI-15 in cluster 1 and KD-6 in cluster 6). This lowest similarity shows that the isolates were retrieved from different soil pH (CHI-15 ranging 5-9 and KD-6 between 5.5-8), temperature (CHI-15 between 15-40^{0}Cand KD-6 ranging from 5-35^{0}C, salt concentration (CHI-15 at 0.5 % w/v and KD-6 at 1% w/v), carbon utilization (CHI-15 uses 87% and KD-6 uses 100% of tested carbon sources) and different antibiotics at different concentrations (data not shown). Similarity increases as we read from right to left on the dendrogram, based on Pearson's correlation coefficient value (Figure 1).

Analysis of variance

Forty isolates obtained from presumptive test of faba

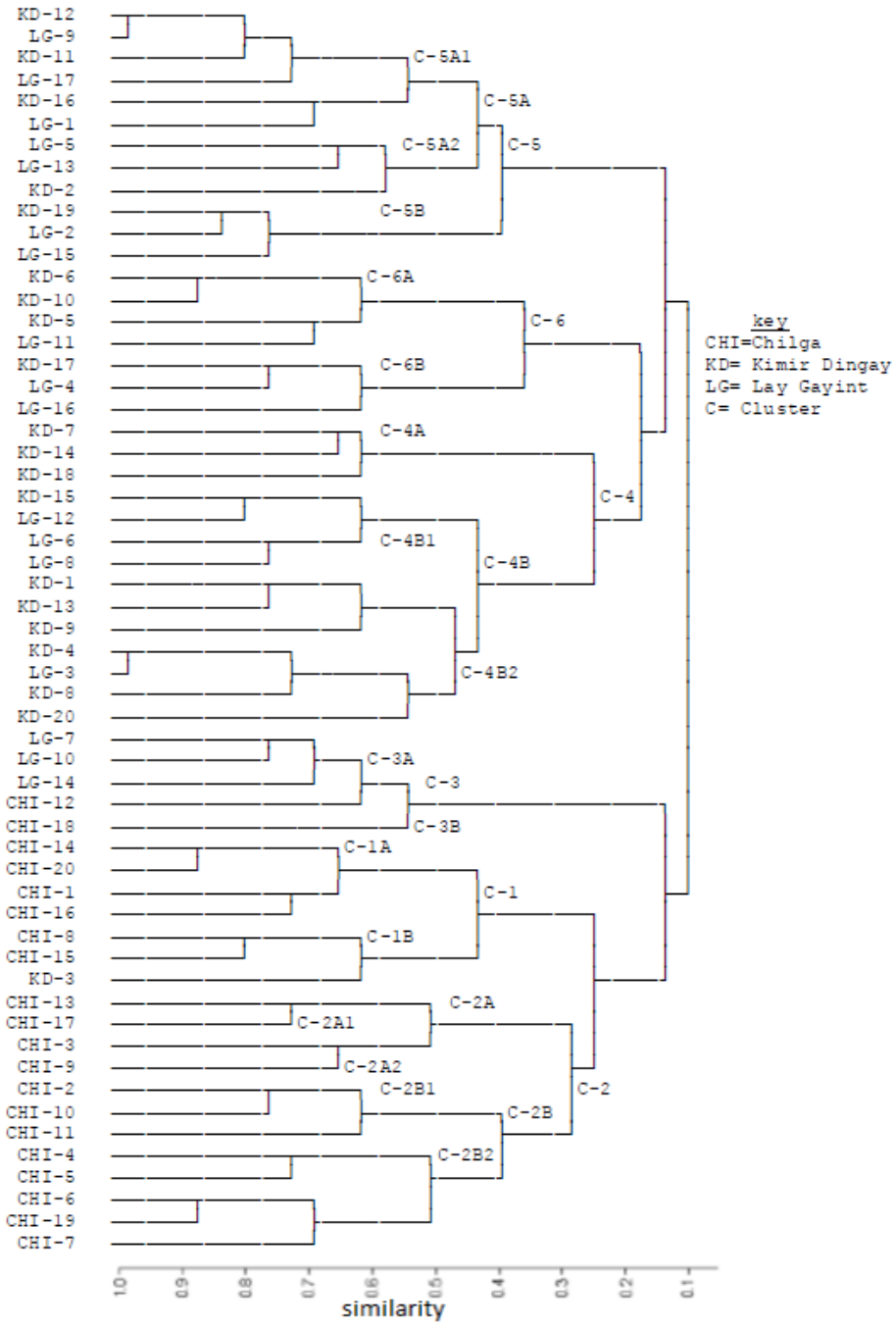

Figure 1. Dendrogram Showing Similarity Using Pearson's Coefficient.

bean nodules were assessed for their infectiveness and effectiveness of nitrogen fixation on Adet faba bean on sterile and acid treated sand in a pot experiment conducted under Greenhouse. Out of these, only fifteen isolates formed nodules on the test faba bean root authenticating as *Rhizobium* (Table 1). The ability to form nodules along with the subsequent nitrogen fixing capacity is widely used as means of assessing the association between rhizobia and respective hosts (Anteneh, 2012a).

The results of analyses of variance showed that *Rhizobium* inoculation significantly ($P<0.05$) increased at all investigated parameters such as, number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments (Table 1). This finding is similar with Dereje et al. (2015) showing symbiotic effectiveness of faba bean nodulating on sand culture.

The nodulating isolates showed difference with nodule

Table 1. Nodulation and relative effectiveness of nitrogen fixation of *R. leguminosarum bv. viciae* isolates of North Gondar (Chilga) and South Gondar (Farta and Lay Gayint) tested on Adet faba bean variety on sand using pot experiment under greenhouse condition.

Isolates	NC	NN p^{-1}	NDM (mg)p^{-1}	NFW (mg) p^{-1}	SDW (g) p^{-1}	SL (cm)p^{-1}	RB (g)p^{-1}	PTN (%)	SE (%)
CHI-10	P	82.00cdefg	100.00bcdef	173.7bcde	4.693c	43.67bc	1.695cdefgh	3.639j	93.33c
CHI-2	P	70.33bcde	91.67bcde	149.0bcd	2.257ab	46.67cde	0.73 ab	3.051f	45.00ab
CHI-3	P	89.67efgh	94.00bcde	150.0bcd	6.389efg	46.00cde	3.400i	3.364h	127.00def
CHI-7	P	94.00fgh	110.67ef	231.0ef	5.158cde	43.67bc	1.652bcdefg	3.671k	102.67cd
CHI-8	P	69.00bcd	83.33b	162.0bcd	4.453c	43.67bc	1.197bcdef	2.969e	87.67c
CHI-9	P	95.67gh	105.67def	141.0bc	6.970g	47.67de	2.190gh	4.160m	138.33f
KD-1	P	90.00fgh	117.33f	265.3f	5.427cde	42.00b	1.853efgh	3.419i	110.33cdef
KD-13	P	61.33b	83.67b	116.0b	4.644c	43.33bc	1.902efgh	2.848c	92.33c
KD-19	P	63.67bc	88.33bcd	140.3bc	2.735 b	46.00cde	0.817abcd	4.111 l	54.33b
KD-4	P	88.00defgh	102.00bcdef	149.0bcd	6.808fg	48.67e	1.753defgh	4.292n	135.67ef
KD-9	P	64.67bc	85.67bc	149.0bcd	2.871b	43.67bc	0.983abcde	3.359h	56.67b
LG-1	P	103.00h	104.33cdef	153.0bcd	5.747cdefg	48.67e	2.437gh	2.760b	114.33cdef
LG-16	P	86.33defgh	101.00bcdef	186.0cde	6.481efg	43.00bc	2.647hi	3.200g	129.00def
LG-17	P	69.33bcd	99.00bcdef	186.7cde	5.496cdef	44.33bcd	0.758abc	3.677k	109.00cde
LG-9	P	76.00bcdef	105.67def	208.3def	5.535cdef	46.00cde	1.983fgh	2.925d	110.33cdef
Standard	P	81.33cdefg	90.67bcd	200.0cdef	6.290defg	47.67de	2.020fgh	3.029f	124.67def
N+		0.00a	0.00a	0.00a	5.040cd	43.33bc	1.070bcdef	2.947de	100.00cd
N-		0.00a	0.00a	0.00a	1.230a	22.00a	0.080a	0.612a	24.33a
Grand Mean		71.4	86.8	153.4	4.901	43.89	1.621	3.2241	97.5
CV (%)		16.4	13.7	26.1	14.4	4.6	30.7	0.5	15.5
LSD (0.05)		19.37	19.70	66.28	1.1693	3.312	0.8238	0.0277	25.05

P= pink; NC= nodule color; NN= nodule number; NDW= nodule dry weight; NFW= nodule fresh weight; SDW= shoot dry weight; SL=shoot length; RB= root biomass; PTN=plant total nitrogen; SE=symbiotic effectiveness; p^{-1}=per plant. N- = without chemical and inoculation; N+ =with optimum amount of N fertilizer; CV= Coefficient of variation; LSD= least significant difference. 0 = not found. Means within a column followed by the same letters are not significant at $p < 0.05$.

number of 61.33 per plant for (KD-13) and 103 for (LG-1) p^{-1} (Table 1) which were less than 67 and 168 nodule number p^{-1} of faba bean on sand culture reported by Zerihun and Fassil (2011), but higher than 17 p^{-1} for isolate AUFR-11 (Semema) to 91 p^{-1} for isolate AUFR-5 (Koyetsa) of degaga variety of faba bean on sand culture reported by Solomon and Fassil (2014). The mean nodule number p^{-1} recorded in this study was 71.4 and which was less than both 88 nodule number p^{-1} for degage and 92 nodule number p^{-1} for dosha faba beans on sand culture studied by Dereje et al, (2015) and 98 nodule number p^{-1} of faba bean on acidic soil reported by Girmaye et al. (2014). Fifty three percent of isolates showed higher number of nodules p^{-1} as compared to standard *Rhizobium* isolate from national soil laboratory, Addis Ababa.

The highest plant shoot length 48.67cm was recorded for both isolate (KD-4) and (LG-1) inoculated with Adet faba bean variety (Table 1). These improvements in shoot length were equivalent to 54.8% over the negative control (without inoculation and nitrogen supplement), 11% over the positive control (nitrogen treated plants) and over the standard *Rhizobium* 2.1% with Adet faba bean. This result was the best compared with the results of Dereje et al.(2015) study on faba bean inoculation with Degaga variety which was measured shoot height of 43.3 cm of isolate HUGAVf1 collected from acidic soils of Ethiopia, which was 32.94% over the negative control and 16.9% over the positive controls. Our study found better result than previously reported by Anteneh (2012a). Their study on Degaga variety displayed shoot length of of 49.7cm for isolate NSFBR-48 and 51cm over negative control. This improvement of shoot length could be regard as; the rhizobia may increase plant growth by providing products through nitrogen fixation (Kumar et al, 2014).

Plant total nitrogen ranges from 2.76 % for isolate (LG-1) to 4.29 % for isolate (KD-4) and the mean plant total nitrogen of isolates was 3.22% (Table 1). In general, inoculation of *Rhizobium* isolates resulted in a significant difference at ($p<0.05$) in plant total nitrogen over negative treatments. Analysis of variance shows that inoculation increased the plant total nitrogen at 31.3, 29.4 and 85.7% over nitrogen treated plants, standard *Rhizobium* and negative treatment (plants without nitrogen sources and inoculation) respectively. The application of nitrogen had

negatively affected nodule color (could not be pink), and nodule number, nodule dry weight and nodule fresh weight (0.00 mg plant-1) (Table 1) and this indicates the negative effect of nitrogen fertilizer application on nodulation of the legume plants. In this experiment, application of mineral nitrogen fertilizer did not improve growth and development of plants; instead it delayed and inhibited nodulation and effectiveness of nitrogen fixation potential of *Rhizobium* isolates. Similarly, Ouslim et al. (2015) reported that addition of nitrogen fertilizer had a negative effect on the nodulation and nitrogen fixation of *Rhizobium* isolates.

Based on the percentage differences of shoot dry weight of inoculated and nitrogen-fertilized plants as a measure of effectiveness, more than 80% of the isolates were found to be highly effective, 13 % were effective and 7 % were poorly effective nitrogen fixers (Table 1). The highest scores of 87.67 % to 138.33 % effectiveness were displayed by isolates CHI-3, CHI-7, CHI-8, CHI-9 and CHI-10 from Chilga, KD-4 and KD-13 from Kimir Dingay and LG-1, LG-9, LG-16 and LG-17 from Lay Gayint areas (Table 1). The data showed that, more than 93% of the rhizobial isolates from North and South Gondar were highly effective and effective *Rhizobium* on the sand culture. This result reveals that, the effectiveness of our isolates was relatively high compared with the finding of Zerihun and Fassil (2011) where 80% of the isolates from North Gondar were effective indicating variation in effectiveness of isolates was found to be widespread in Ethiopia.

The first parameter for a *Rhizobium* strain used as inoculant or biofertilizer is it must be superior and highly effective in nitrogen fixing ability forming symbiotic association with the host legume. Nine isolates identified with Adet faba bean variety showed effectiveness ranging from 102.7% to 138.3% as compared to the nitrogen treated plants (Table 1). Mainly the best four isolates (CHI-3, CHI-9, KD-4, and LG-16) showed effectiveness of 127, 138.33, 135.67 and 129% respectively as compared to inoculants of standard *Rhizobium* which showed effectiveness of 124.67%. The standard *Rhizobium* inoculated plants also showed effectiveness over nitrogen treated plants (Table 1).

In this study, more highly effective isolates were obtained compared to other investigator reports. Dereje et al*,* (2015) observed that 56% of the isolates were highly effective in both Degaga and Dosha varieties collected from acidic soils of Ethiopia. Girmaye et al*.* (2014) reported that 16% of the isolates of faba bean were highly effective collected from acidic soils of Wollega, Western Ethiopia and Anteneh (2012b) result showed that 20.9% of isolates were very effective collected from major lentil growing areas of Ethiopia. Generally, the results of this study indicates that, screening of local *Rhizobium* isolates gives paramount importance for enhancement of dinitrogen fixation in faba bean.

Correlation analysis

Nodule number was found to be positively correlated with nodule fresh weight (r = 0.469 P <0.01) and strongly positive correlated with nodule dry mass (r =0.677, P <0.01), shoot dry weight (r = 0.591, P <0.01), root biomass (r = 0.561, P <0.01) and symbiotic effectiveness (r =0.586, P <0.01) (Table 2). Shoot dry weight was found to be positively correlated with nodule dry mass (r = 0.393, P <0.01), strongly positive correlated with root biomass(r =0.614, P <0.01) and symbiotic effectiveness(r = 0.994, P <0.01) (Table 2). Shoot dry weight and nodule dry weights are usually positively correlated revealing that shoot dry weight can be used as an indicator of relative symbiotic effectiveness.

Shoot length was found to be positively correlated with nodule dry mass (r = 0.396, P <0.01) and with nodule fresh weight (r = 0.391, P <0.01) (Table 2). Nodule fresh weight was negatively correlated with soil nitrogen content (r =-0.318, P <0.05) and there was also strongly positive correlation among nodule fresh weight (r =0.775, P <0.01), root biomass (r =0.610, P <0.01) (Table 2). A similar result was reported on lentil, using growth pot experiment by Anteneh (2012b). Many research findings reveal that nodulation status positively correlated with plant tissue nitrogen, symbiotic effectiveness and shoot biomass or dry weight (Ashenafi and Mekuria, 2015; Tekle et al, 2016).

Conclusion and recommendation

In the present study, most of our isolates displayed abundant diversity with respect to their response to morphological and physiological characteristics. Inoculation of isolates significantly increased at all investigated parameters such as, number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments. About 80% of the isolates collected from major faba bean growing areas of North and South Gondar, Ethiopia were found to be highly effective, 13 % were effective, only one isolate (7 %) from Chilga area categorized as poorly effective and none of isolates grouped as ineffective.

The best nine effective isolates were selected over nitrogen treated plants with Adet faba bean variety from Chilga (3), Kimir Dingay (2) and Lay Gayint (4) study areas. The best four effective isolates, two of them from Chilga, one from Kimir Dingay and one from Lay Gayint were also selected as compared to the standard *Rhizobium* isolate from National Soil Laboratory, Addis Ababa.

Inoculation of selected *Rhizobium* isolates revealed shoot dry weight enhancement over nitrogen treated plants of Adet faba bean on sand culture using pot

Table 2. Correlation coefficients among investigated parameters in faba bean.

Parameter	NN p^{-1}	NDM (mg) p^{-1}	NFW (mg) p^{-1}	SDW(g) p^{-1}	SL (g) p^{-1}	RB (g) p^{-1}	PTN (%)	SE (%)	Soil pH	Soil N (%)
NN p^{-1}	1	0.677**	0.469**	0.591**	0.136ns	0.561**	0.115ns	0.586**	0.106ns	-0.285ns
NDM (g)p^{-1}	0.677**	1	0.775**	0.393**	0.396**	0.300*	0.158ns	0.416**	-0.055ns	-0.270ns
NFW (g) p^{-1}	0.469**	0.775**	1	0.217ns	0.391**	0.099ns	0.018ns	0.239ns	-0.060ns	-0.318*
SDW (g) p^{-1}	0.591**	0.393**	0.217ns	1	0.098ns	0.614**	0.181ns	0.994**	-0.088ns	-0.205ns
SL (cm) p^{-1}	0.136ns	0.396**	0.391**	0.098ns	1	0.124ns	0.190ns	0.082ns	0.070ns	-0.081ns
RB (g) p^{-1}	0.561**	0.300*	0.099ns	0.614**	0.124ns	1	0.144ns	0.610**	-0.053ns	-0.236ns
PTN (%)	0.115ns	0.158ns	0.018ns	0.181ns	0.190ns	0.144ns	1	0.178ns	0.284ns	0.202ns
SE (%)	0.586**	0.416**	0.239ns	0.994**	0.082ns	0.610**	0.178ns	1	-0.095ns	-0.202n
Soil pH	0.106ns	-0.055ns	-0.060ns	-0.088ns	0.070ns	-0.053ns	0.284ns	-0.095ns	1	-0.410**
Soil N (%)	-0.285ns	-0.270ns	-0.318*	-0.205ns	-0.081ns	-0.236ns	0.202ns	-0.202ns	-0.410**	1

** = significant at P < 0.01, * = significant at P < 0.05 and ns = not significant at p <0.05. NN=nodule number; NDM (g)=nodule dry mass; NFW (g)=nodule fresh weight; SDW (g)=shoot dry weight; SL (cm)=shoot length; RB (g)=root biomass; PTN (ppm)= plant total nitrogen; SE (%)= symbiotic effectiveness; soil N(ppm)= soil nitrogen; p^{-1}=per plant.

experiment under controlled greenhouse condition. Finally, further investigations of very effective isolates need to be tested under greenhouse and field condition on soil culture to assess their competitiveness ability, adaptability to the wide edaphic condition and survival and colonization within soil. Further research work with various molecular approaches should be conducted to investigate the protein and DNA pattern for better classification of the *Rhizobium* strains.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Agronomic performance of soybean treated with *Bacillus amyloliquefaciens*

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The development of practices that maintain or increases soybean yield can increase the profitability of this crop. In this context, the use of microorganism-based products in crops has been extensively studied. Among the species, *Bacillus amyloliquefaciens* has shown significant potential for agronomic use due to its ability to control phytoparasitic microorganisms and its effects in promoting plant growth. The aim of this study is to evaluate the effects of *B. amyloliquefaciens* application on soybean. Fields experiments were conducted at four sites. The experimental design used was randomized block design, with six treatments and four replications. The treatments consisted of seed treatment with *B. amyloliquefaciens* strain MBI600 (Integral II SC)-based product at 2.5, 5, 10, 15 and 20 mL c.p. 100 kg^{-1} of seeds, plus a control without treatment. The variables evaluated were plant stand, phytotoxicity, plant height, root and shoot dry mass, number of nodules and crop yield. Increasing doses of the *B. amyloliquefaciens*-based product promote an increase in all variables related to crop development. All doses of the *B. amyloliquefaciens*-based product provided an increase in soybean yield compared to the control. The dose that produces the maximum agronomic efficiency was 15 mL c.p. 100 kg^{-1} of seeds.

Key words: Biological control, *Bacillus subtilis*, *Glycine max*, growth promoters, seed treatment.

INTRODUCTION

In recent years Brazil has been facing severe economic crisis (Filho, 2017). However, the agricultural sector has contributed to a far less gloomy situation than might otherwise be occurring. Soybean is the main crop produced in Brazilian agriculture, and forecasts show Brazil becoming the world's largest producer of this oilseed in upcoming seasons (USP, 2018).

The mean domestic soybean yield is 3,185 kg ha^{-1}

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according to provisional data from the 2017/2018 season (CONAB, 2018). When analyzing data among seasons or within the same season in different Brazilian states, it is common to observe large fluctuations in mean yield. This is due to several factors, including those that cannot be controlled by man, such as climate, to those that can be managed, such as adequate plant nutrition and health control.

In this context, it is important to develop technologies and management practices that maintain productivity levels of soybean or that can even promote higher yields, as these will enable farmers to obtain higher profits when growing this crop. One of the primary lines of research that has grown considerably in the agricultural scenario in recent years is related to the use of biological control products. The factors that have contributed to this growth include the potential for reducing the use of pesticides, the provision of a more specific spectrum of control of phytoparasitic pests and microorganisms, and greater selectivity for natural enemies (Dreistadt, 2014).

In addition to the benefits related to microorganism control provided by biological products (Lobna and Zawam, 2010; Alfonzo et al., 2012), recent research has shown that several of these agents act as promoters of plant growth, favoring the development of plants to which they are applied (Wang et al., 2012; Szilagyi-Zecchin et al., 2015). In the scientific literature, beneficial effects on plant development have already been reported for bacteria, which are called plant-growth-promoting rhizobacteria (PGPR) (Araújo, 2008). Among the species most studied for this purpose are those of the genus *Bacillus* sp, which are characterized by cosmopolitan habits—their natural habitat is the soil, and they are commonly found associated with the rhizosphere and endophytic environment of plants (Meng et al., 2012).

The species *Bacillus amyloliquefaciens* has been extensively studied and has phylogenetic affinity with *B. subtilis*; for a long time it was even considered its subspecies. Studies developed with *B. amyloliquefaciens* have associated the use of this bacterium with increased plant defense, showing positive results in the control of nematodes and other microorganisms. In addition, there are reports that, when used in different crop species, the benefit is not only restricted to the control of diseases but also promotes plant growth, as observed in such diverse crops as rice, eucalyptus, wheat and tomato, among others (Ng et al., 2012; Paz et al., 2012; Kasim et al., 2013; Szilagyi-Zecchin et al., 2015).

Based on the results available in the literature, we hypothesized that *B. amyloliquefaciens* may positively influence soybean development. However, it is necessary to determine how the agronomic performance of this important crop is affected by different doses of the bacterium in order to determine the concentration that can generate the greatest benefits in soybean. Therefore, this work sought to evaluate the effects of the application of different doses of a *B. amyloliquefaciens*-based product on soybean development and yield.

MATERIALS AND METHODS

The study was conducted at four sites to evaluate the potential use of a *B. amyloliquefaciens*-based product on the treatment of soybean seeds and its potential effects as a growth promoter. The experiments were conducted in the municipalities of Francisco Alves (Paraná State-PR), latitude 25º08'9.65"S, longitude 53º52'36.72"W and 321 m altitude; Terra Roxa (PR), 24º17'15.87"S, 54º02'31.11"W and 332 m altitude; Maringá (PR), 23º23'48.33"S, 51º56'58.20"W and 501 m altitude; and Ourizona (PR), 23.27,3.7,S, 52.15,28.99,W and 367 m altitude. All experiments were carried out from January to June 2015.

For all experimental sites, Cfa - humid mesothermal – was the predominant climate according to the Köppen classification, which is characterized by warm summers and infrequent frosts with a tendency towards rainfall accumulation in the summer months and with no well-defined dry season. Mean air temperatures in the warmer months are higher than 22°C (below 13°C in the colder months) and the mean annual precipitation is between 1,600 and 1,800 mm (IAPAR, 2014). Figure 1 shows precipitation data for the time period during the experiments.

Prior to the initiation of the experiments, soil samples (0 to 20 cm layer) were collected in the experimental areas, and the soil physical and chemical characteristics, together with the ultimate crop data, are presented in Table 1. To ensure there were no negative effects on soybean development caused by pests and diseases, or losses due to weed interference, the phytosanitary treatments recommended by EMBRAPA (2010) were performed in all experimental areas.

In all experiments, the experimental design adopted was a randomized block design (RBD), with six treatments and four replicates. The treatments consisted of application of five doses of a *B. amyloliquefaciens*-based product, plus one control without product, to seeds (Table 2). The formulation used was isolated from *B. amyloliquefaciens* strain MBI600 (II Integral SC) and provided by BASF (BASF Corporation Agricultural Products Research Triangle Park, USA; http://agro.basf.com).

In all experiments, soybean seeds were treated with the *B. amyloliquefaciens*-based product following the method described below, using the dose established for each treatment. Initially, four samples of soybean seeds were weighed to obtain 3 kg samples, sufficient to sow the four replicates corresponding to each treatment. Subsequently, these samples were placed in plastic bags and then the dose of the biological product corresponding to each treatment was added directly into the bags containing the seeds. After this procedure, the seeds were homogenized by vigorously shaking the bag for 3 min.

The experimental units measured 5.5 m wide by 5 m long, totaling a gross area of 27.5 m^2. All the response variables were measured in the useful area of the experimental units, which measured 22.5 m^2 after excluding the borders.

In order to evaluate the effect of the treatments on soybean development, the response variables plant stand, phytotoxicity, plant height, root and shoot dry weight, number of nodules and crop yield were evaluated. The plant stand was measured at 7 and 14 days after emergence (DAE), counting the number of seedlings in 2 linear m using the two central rows of the experimental unit for evaluation.

The toxicity of soybean plants was evaluated at 7 and 14 DAE using a percentage scale as a reference to assign the scores, in which 0% corresponded to the absence of symptoms and 100% corresponded to death of the plants. Two plant height evaluations were performed, one at 14 and one at 35 DAE. To calculate this variable, the distance from the soil level to the apical meristem of

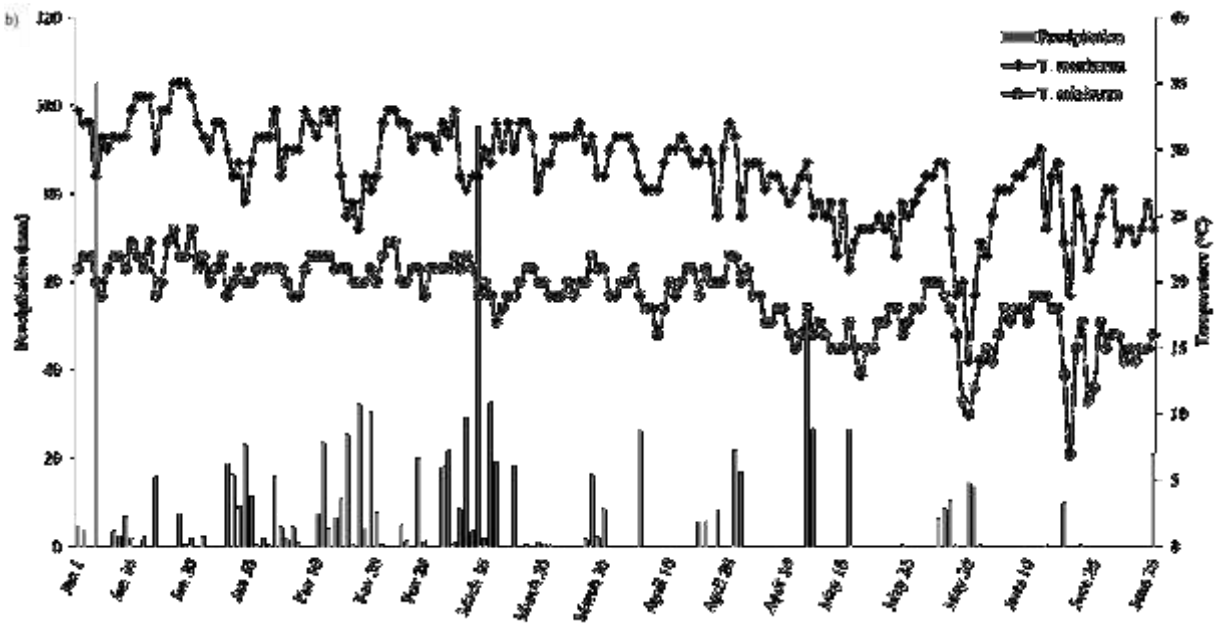

Figure 1. Temperature (maximum and minimum) and precipitation (mm) during the experimental period for the regions of Francisco Alves and Terra Roxa (a) and Maringá and Ourizona (b), Paraná, Brazil (2015 season).
Source: INMET - National Institute of Meteorology.

Table 1. Physical and chemical characteristics of the soils of the experimental areas and crop data in the experiments with the *B. amyloliquefaciens*-based product applied to soybean seeds. 2015 season.

Parameter	Francisco Alves	Ourizona	Terra Roxa	Maringá
Physical and Chemical Characteristics of the Soil				
pH (H_2O)	4.8	7.1	6.1	6.0
CEC ($cmol_c\ dm^{-3}$)	4.55	13.20	12.22	9.43
$H^+ + Al^{3+}$ ($cmol_c\ dm^{-3}$)	2.64	1.50	2.10	2.60
OM ($mg\ dm^{-3}$)	10.54	2.80	2.90	2.80
Clay ($g\ kg^{-1)}$	180	718	684	730
Silt ($g\ kg^{-1)}$	20	225	213	218
Sand ($g\ kg^{-1)}$	800	57	103	52
Textural class	Loamy-sandy	Very clayey	Very clayey	Very clayey
Crop data				
Sowing date	01/06/2015	01/29/2015	02/26/2015	03/03/2015
Variety	V-Max RR®	Monsoy 6210 IPRO®	Monsoy 6410 IPRO®	V-Max RR®
Spacing (m)	0.45	0.40	0.45	0.45
Density ($seeds\ m^{-1}$)	18	16	17	14
Fertilization ($kg\ ha^{-1}$)	250 (00-20-20)	280 (00-20-20)	300 (00-20-20)	250 (00-20-20)

OM, Organic matter.

the plant was measured, averaging across ten plants per experimental unit.

At 14 and 35 DAE, the dry weights of the roots and shoot were measured. For this evaluation, the soybean plants were carefully removed from the experimental units, separated into roots and shoots and placed in a forced-air oven for 72 h at an average temperature of 65°C to obtain the dry weight of each material. Ten plants were used per experimental unit. The number of nodules was quantified at 14 and 35 DAE, counting the nodules present in the roots of the soybean plants. For this variable, ten plants were used per experimental unit, and the mean number of nodules for all plants sampled was used (Costa et al., 2013).

Table 2. Treatments and respective doses of a *B. amyloliquefaciens*-based product applied to soybean seeds. 2015 season.

Treatments		Dose	
		mL c.p. 100 kg^{-1} of seeds	g a.i. 100 kg^{-1} of seeds
1	Control	-	-
2	*B. amyloliquefaciens* strain MBI600 *	2.5	0.0045
3	*B. amyloliquefaciens* strain MBI600	5	0.009
4	*B. amyloliquefaciens* strain MBI600	10	0.018
5	*B. amyloliquefaciens* strain MBI600	15	0.027
6	*B. amyloliquefaciens* strain MBI600	20	0.036

*Integral II SC (MBI 600). Concentration: 2 x 10^{10} viable spores mL^{-1}.

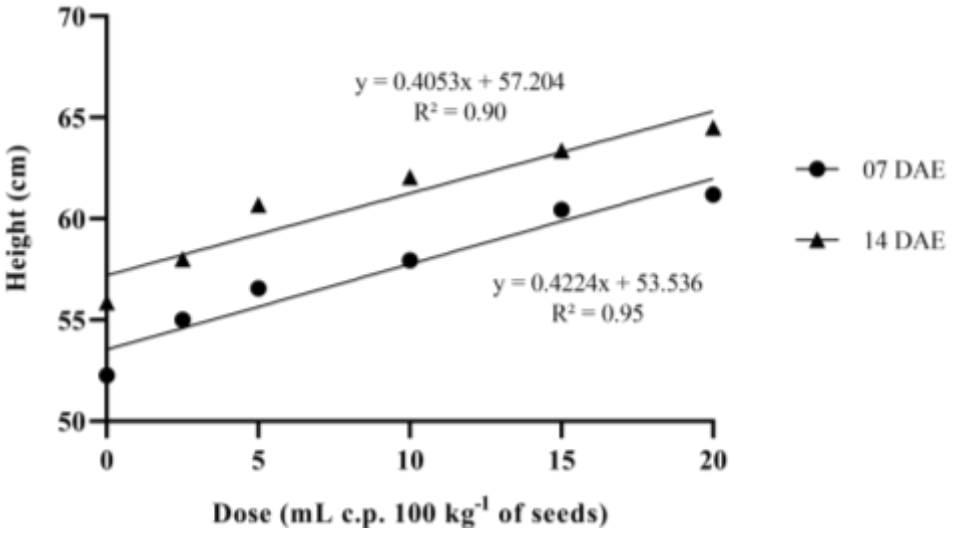

Figure 2. Density of soybean plants at 7 and 14 DAE as a function of the application of different doses of a *B. amyloliquefaciens*-based product to soybean seeds (2015 season).

Regarding the yield, the soybean plants present in the useful area were harvested and threshed, and the moisture corrected to 13%. The production value obtained per experimental unit was converted to hectares, and the yield results were expressed in kg ha^{-1}.

Statistical analysis was performed using the Sisvar software (Ferreira, 2011). After the end of the study period, the data were subjected to analysis of variance ($p \leq 0.05$). In addition, a joint analysis of the experiments was carried out to assess whether there was any effect of the specific experimental site. When a significant effect of the doses evaluated was observed, the data were subjected to regression analysis ($p \leq 0.05$).

RESULTS AND DISCUSSION

Joint analysis of the experiments showed similar behaviors for all response variables, with no effect of the experimental site observed. Therefore, the data from the different evaluation sites performed are presented together, and the means of the four experiments are shown in the graphs.

The plant population per hectare is directly related to the productive potential of the crop, as crop stand losses can lead to lower yields (Scheeren et al., 2010). In this sense, the use of products that benefit the initial development of soybean seedlings, ensuring adequate stand, is an intriguing tool for potential use in crop management. At 7 DAE of soybean, there was a positive linear effect of increasing doses of the *B. amyloliquefaciens*-based product on the plant stand variable (Figure 2). Comparing the increase in the plant stand generated by the highest dose of the *B. amyloliquefaciens*-based product to the control, in which the product was not applied to the seeds, there was a 15% increase in the overall plant population.

The behavior displayed in the first evaluation of the stand persisted until 14 DAE, with increasing dose of the *B. amyloliquefaciens*-based product again promoting an increase in the number of emerged seedlings. Based on the fitted equation, a dose of approximately 2.5 mL c.p. 100 kg^{-1} of soybean seeds would be required to promote

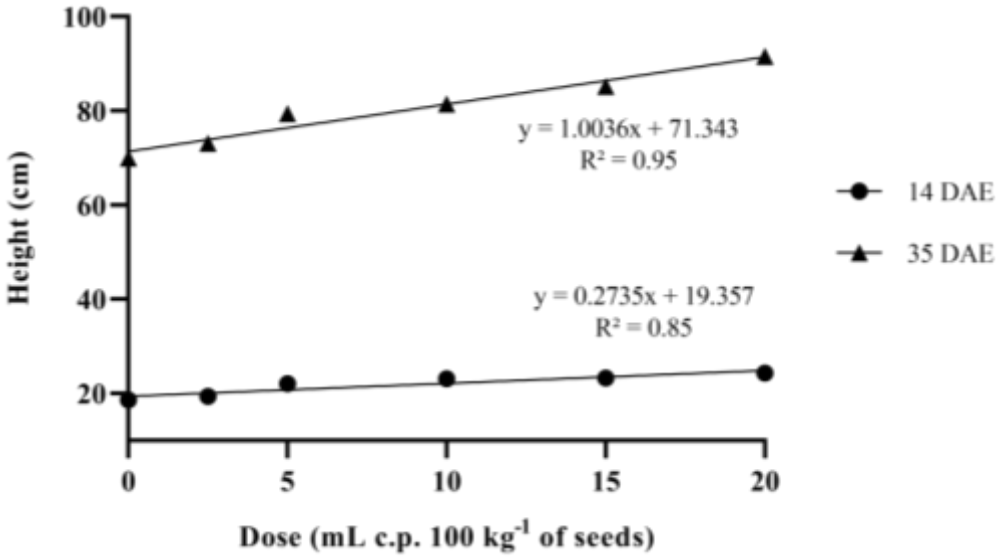

Figure 3. Height of soybean plants at 14 and 35 DAE as a function of the application of different doses of a *B. amyloliquefaciens*-based product to soybean seeds (2015 season).

an increase of approximately one plant every 2 linear m.

The application of the *B. amyloliquefaciens*-based product to seeds did not produce toxic effects to soybean plants in any of the evaluations performed (data not shown). The evaluation of the potential for this biological product to have negative effects on the crop when applied to soybean seeds is essential; in the event of damage to plants, it is necessary to carry out new studies to evaluate if the injuries are caused by the formulation of the commercial product developed or the phytotoxic action of the bacteria.

Regardless whether the evaluation was performed at 14 and 35 DAE, increasing doses of the *B. amyloliquefaciens*-based product showed a linear relationship with the height of the soybean plants, with this variable increasing with increasing doses applied to seeds (Figure 3). The effect of the product on soybean plant height was so dramatically evident at 35 DAE that for each 1 mL of c.p. used 100 kg^{-1} of seeds, there was an increase of 1 cm in this variable.

Plants with more aggressive root systems have a better capacity to explore the edaphic environment, mainly in the search for water and nutrients, in addition to being more tolerant to drought periods (Pitol and Broch, 2012). At 14 DAE, the increasing doses of the *B. amyloliquefaciens*-based product in the treatment of soybean seeds showed a linear relationship with root dry weight (Figure 4). In the evaluation at 35 DAE, a similar trend was observed, with higher doses producing higher soybean root weights.

The increases promoted by the *B. amyloliquefaciens*-based biological product were not only restricted to the soybean root system; an increase was also observed in the shoots of the plants (Figure 5). The increase in shoot dry weight was observed at both 14 and 35 DAE, with a mean increment of 5% observed relative to the control (without the product) for each mL of c.p. used in the treatment of soybean seeds.

Another response variable positively influenced by the use of the *B. amyloliquefaciens*-based product (MBI 600) was the number of nodules (Figure 6). At 14 DAE, a linear relationship was observed, with an increase in the number of nodules as a function of the use of increasing doses of the biological product. This demonstrates that the use of *B. amyloliquefaciens*-based products can aid the soybean nodulation process, increasing the number of nodules and, consequently, making the biological nitrogen fixation (BNF) process more efficient (Costa et al., 2013).

At 35 DAE, there was a quadratic effect of increasing doses of the biological product on the number of nodules, with all doses within the range studied associated with increases in this variable relative to the control (Figure 6). This result indicates that there is potentially no competition among bacteria in the colonization of the rhizosphere environment of soybean plants. On the other hand, given the increase in the number of nodules of the root system of soybean plants, there is likely a positive interaction between *Bradyrhizobium japonicum* (responsible for BNF) and *B. amyloliquefaciens*. This behavior has been previously reported in soybean and common bean crops (Souza et al., 2008; Araújo et al., 2009).

All doses of the *B. amyloliquefaciens*-based biological product applied to the seeds provided an increase in soybean yield relative to the control (Figure 7). According to the fitted equation, the dose that provided maximum soybean yield was approximately 15 mL c.p. 100 kg^{-1}

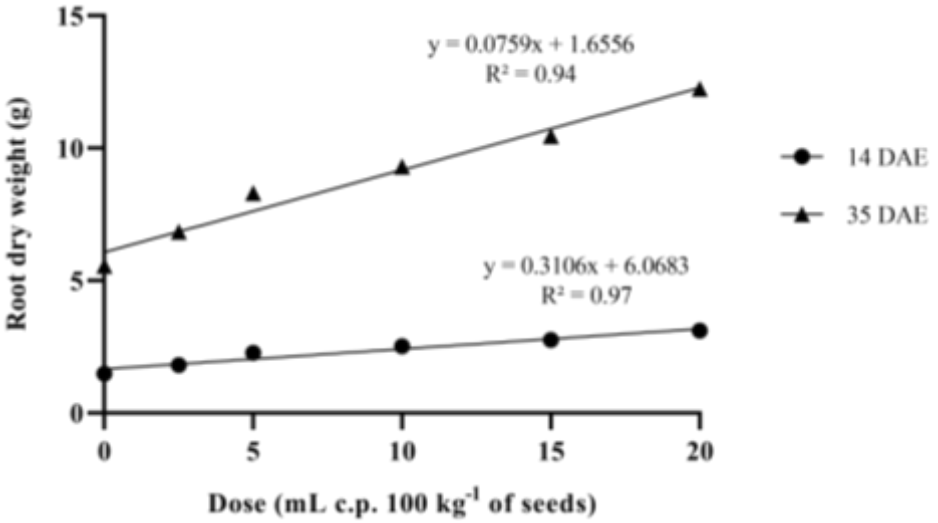

Figure 4. Root dry weight of soybean plants at 14 and 35 DAE as a function of the application of different doses of the *B. amyloliquefaciens*-based product to soybean seeds (2015 season).

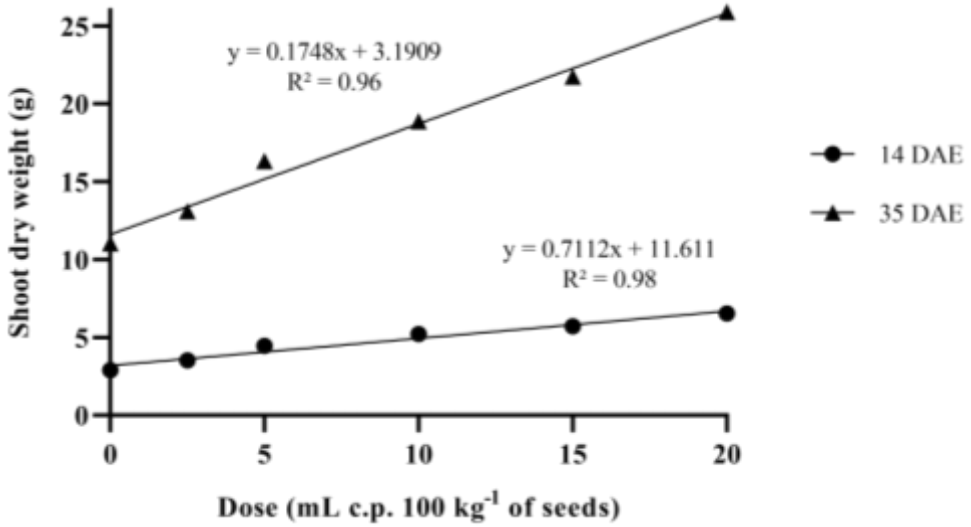

Figure 5. Shoot dry weights of soybean plants at 14 and 35 DAE as a function of the application of different doses of the *B. amyloliquefaciens*-based product to soybean seeds (2015 season).

seed. At this dose, the yield was close to 3,749.7 kg ha^{-1}, an increase of approximately 16% above the control.

The increase in soybean yield promoted by the use of the *B. amyloliquefaciens*-based product on seeds can be explained by several factors, such as the increase in plant emergence, the increase in the number of nodules and greater development of the roots and shoots of the plants. A previous study also reported higher grain yields due to the use of rhizobacteria in soybean crops (Araújo and Hungria, 1999). Additionally, Kim et al. (2017) found that the use of *B. amyloliquefaciens* in soybean crops provided tolerance to salt stress, demonstrating that the use of this bacterium can bring additional advantages in regions with salinized soils.

The results of the present study demonstrate that the advantages associated with the use of *B. amyloliquefaciens* (MBI 600) are not limited to those related to the biological control of other phytoparasitic microorganisms (Araújo et al., 2002), which in and of itself already represents a substantial benefit to soybean

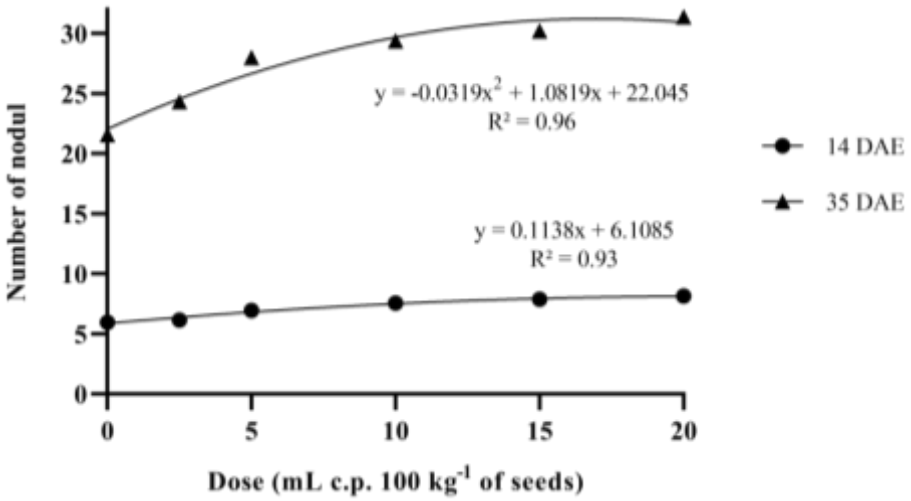

Figure 6. Number of nodules in soybean plants at 14 and 35 DAE as a function of the application of different doses of the *B. amyloliquefaciens*-based product to soybean seeds (2015 season).

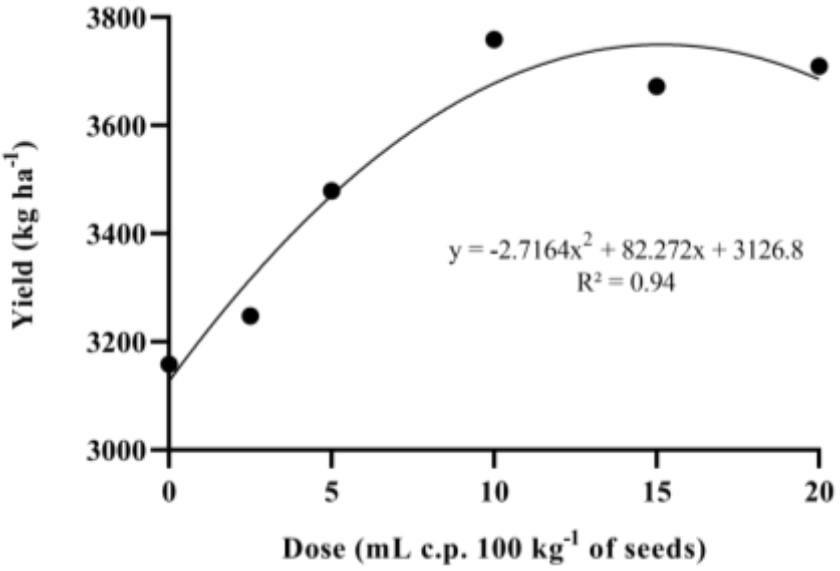

Figure 7. Soybean grain yield as a function of the application of different doses of the *B. amyloliquefaciens*-based product to soybean seeds. 2015 season.

management, but further includes the potential to generate higher crop yields. However, to ensure that the use of *B. amyloliquefaciens* (MBI 600) in soybean is successful, it is necessary to carry out additional studies to evaluate the selectivity of the phytosanitary products used in seed treatment procedures for this rhizobacteria.

Conclusions

Under the conditions in which this study was conducted, the following can be concluded:

i) The use of increasing doses of a *B. amyloliquefaciens* (MBI 600)-based product, through seed treatmen, promotes increases in plant stand, height, root and shoot dry weight and number of nodules per plant;
ii) The benefits observed due to the use of the *B. amyloliquefaciens* (MBI 600)-based product resulted in an increase in soybean yield; and
iii) All doses of the *B. amyloliquefaciens*-based product provided greater soybean yields compared to the control.

The dose that generated the maximum agronomic efficacy was approximately 15 mL c.p. 100 kg^{-1} seed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Seroprevalence of Chikungunya during outbreak in Dhaka, Bangladesh in 2017

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Chikungunya (CHIK) infection is re-emergence public health problem globally including Bangladesh. It is an arthropod-borne disease, which is transmitted by mosquitoes bite. The virus was first isolated in Newala district of Tanzania in 1953. In 2017, an outbreak of Chikungunya, has struck Bangladesh's capital, Dhaka. This study was conducted to know the seroprevalence, clinical presentations and seasonal trends of CHIK infection. This study was conducted in the Ibn Sina Diagnostic & Consultation Center, Uttara from January to November, 2017. Serum samples from about 1060 Chikungunya suspected cases were tested for immunoglobin M (IgM) and IgG antibodies by Immuno-Chromatographic test (ICT) method. Out of total tested cases, 524 (49.43%) were seropositive for Chikungunya, among the seropositive 379 (72.32%) were IgM positive, 98 (18.70%) were IgG positive and 47 (8.96%) were both IgM and IgG positive. The most affected age group was 11 to 40 years. Females were more affected than males. A high percentage of Chikungunya seropositive cases were found among suspected patients.

Key words: Chikungunya, IgM and IgG antibodies, seroprevalence, outbreak in Dhaka.

INTRODUCTION

The Chikungunya virus infection as an important mosquito-borne disease of an alpha genus belongs to the Togaviridae family (Ang et al., 2017). The virus consists of single-stranded RNA genome, a 60 to 70 nm diameter capsid and phospholipids envelop. Chikungunya fever is predominantly transmitted by bites of mosquitoes of *Aedes* genus (*Aedes aegypti* and *Aedes albopictus*). Probably, Chikungunya virus originated in East Africa (Dash et al., 2011). Chikungunya virus was first isolated from the serum of a febrile human during an epidemic outbreak by Ross in Newala district of Tanzania in 1953 (Khatun et al., 2015). Since then, Chikungunya virus has become a more global concern (Kabir et al., 2017). In Asia, *Ae. aegypti* is believed to be the principal vector for transmission during the human outbreak. Only female mosquitoes are infective and bite human in daytime

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Table 1. Distribution of suspected Chikungunya case patients.

Parameter	No. of cases	Percent
Positive	524	49.43
Negative	536	50.57
Total	1060	100

Table 2. Distribution of serologically positive Chikungunya case.

Antibody	No. of cases	Percent
Anti-Chikungunya IgM positive	379	72.32
Anti-Chikungunya IgG positive	98	18.70
Both IgM and IgG Positive	47	8.96

(especially early morning and late evening). A high vector density in the post-monsoon season accentuates virus transmission (World Health Organization, Special Programme for Research, Training in Tropical Diseases, 2009). In 2008, the first Chikungunya outbreak occurred in the northern area at Rajshahi and Chapainawabganj districts of Bangladesh. Outbreak was investigated by Institute of Epidemiology, Disease Control and Research (IEDCR) and International Centre for Diarrhoeal Disease Research Bangladesh (ICDDR, B). An outbreak of fever with prolonged joint pain was investigated at Dohar of Dhaka District in 2011; suspected cases were identified by house-to-house surveys. Approximately, 29% of the village residents have symptoms consistent with Chikungunya fever during the outbreak (Khatun et al., 2011). After that six confirmed cases of Chikungunya were reported in 2014. In 2017, Chikungunya outbreak occurred at Dhaka of Bangladesh. Clinical confirmed cases had 2,314; reported in different hospitals and clinics of Dhaka from May to September 2017 and more than 1 million people were affected in the capital city of Bangladesh (Kabir et al., 2017). Chikungunya viral fever occurs in the victim of all ages in both sexes. Acute Chikungunya virus infection usually has the onset of high fever, severe joint pain, myalgia, erythematous, and maculopapular rash, which can range in severity from a mild, localized rash to an extensive rash involving more than 90% of the skin (Miner et al., 2015). The joint pain begins to improve after the first week, although some patients have persistent joint pain, swelling and morning stiffness. These symptoms can last for up to 3 years (Burt et al., 2017). There are different ways for diagnosis of Chikungunya fever; however, blood specimen is collected from an infected patient within 7 days for the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to detect the viral RNA. Both anti-Chikungunya antibodies immunoglobin M (IgM) and IgG can be detected in either the acute or the convalescent-phase samples (Wahid et al., 2017). ELISA and ICT test were performed for detection of IgM and IgG (Dash et al., 2011).

METHODOLOGY

The study was conducted in Ibn Sina Diagnostic & Consultation Center Uttara from January to November 2017. Inclusion criteria include the patient who reported to the clinic with high fever, joint pain, headache, skin rash, and received a doctor's referral to the diagnostic centre for Chikungunya test. Exclusion criteria include patients who were unwilling to participate. Respondents were selected and inform consent taken at the time of blood collection and also written permission was taken from branch Manager. About 2 to 3 ml of whole blood samples were collected from each patient using sterile aseptic precautions, and serum was separated by standard methods. Collected serum samples were tested for IgM and IgG anti-Chikungunya antibodies by immune chromatographic method (ICT) according to the SD STANDARD DIAGNOSTICS, PHA instruction. In this method, IgM and IgG were detected by using an antibody capture method and gold-labeled anti-Chikungunya virus monoclonal SD Chikungunya antibody. The patient serum (10 µl) and 3 drops (90 µl) of buffer solution are added for dilution of serum. The result was taken within 15 to 20 min after the appearance of the color of control line and test line. Results of all tests were written in the laboratory resister and data collection sheet. Data obtained were statistically analyzed by SPSS software version 23. P-value <0.05 was considered as statistically significant.

RESULTS

A total of 1060 Chikungunya suspected cases were studied to detect anti-Chikungunya IgG and IgM antibodies in serum samples. Out of these, 524 (49.43%) samples were positive for Chikungunya infection and 536 (50.57%) were negative (Table 1).

Out of total 524 Chikungunya positive cases, IgM anti-Chikungunya antibody was found positive in 524 (72.32%) samples, IgG anti-Chikungunya antibody was positive in 153 (18.70%) and both IgM and IgG were positive in 47 (8.96%) cases (Table 2).

Table 3 shows that respondents in the age group 31 to 40 years were more infected than other age groups. There was no statistical significance (P>0.05) between age and anti-Chikungunya antibody. Female respondents were more infected than male and statistical (P<0.05) differences were found between sex and anti-Chikungunya antibody.

Figure 2 shows the clinical symptom of the respondents which includes high fever and joint pain was the most common symptom of most cases, joint swelling (48.03%), rash (69.33%), headache (73.59%), and body pain (83.09%) of seropositive cases.

DISCUSSION

Chikungunya virus is an important re-emerging disease of the tropical and sub-tropical regions in last decade.

Table 3. Age-sex distribution of Chikungunya suspected cases.

Variable	CHI-IgM (ICT)	
	Negative	Positive
Age of the respondents		
0-10 years	21	36
11-20 years	146	98
21-30 years	129	87
31-40 years	121	123
41-50 years	71	77
51-60 years	34	71
Above 60 years	14	32
Total	536	524
Significance	χ^2=7.45, df=6, p=0.281	
Sex of the respondents		
Female	359	331
Male	177	193
Total	536	524
Significance	χ^2=19.27, df=1, p=0.01	

Chikungunya has been occurring regularly with periodic surges in a number of cases (Singh, 2007). The differential diagnosis associated with Chikungunya fever includes a wide variety of viral which includes Dengue, bacterial and parasitic infections that produce a similar syndrome. A definitive diagnosis is confirmed by virus isolation and/or serological test. This study describes the seroprevalence of Chikungunya virus in Dhaka resident population. A total of 1060 serum samples from suspected cases of Chikungunya infection were received during the study period, out of which 524 (49.43%) samples were positive for Chikungunya infection. It was found that 379 (72.32%) anti-Chikungunya IgM positive. Khatun et al. (2015) reported 29% Chikungunya infection in Dhaka Dohar, Chopra et al. (2014) reported 49.0%, Divya and Krishna (2016) reported 21.8% and Wadekar et al. (2017) reported 8.17% and Cunha et al. (2017) reported 35%. The study also showed that most (58.77%) affected age group was 11 to 40 years; these results are comparable with Wadekar et al. (2017) and Cunha et al. (2017). Less than 10 years age group was 4.2% least affected. According to gender distribution, female were more infected than male. These findings are comparable with the study done by Kawle et al. (2017), Divya and Krishna (2016) and Mohanty et al. (2013). The highest percentage of morbidity was found in female and females were more frequently affected than males (Ang et al., 2017). Clinical presentation of Chikungunya seropositive cases showed that fever, joint pain, joint swelling, headache, and the rash were the most common symptom in all the cases. Headache was seen in 73.59% and body pain in 83.09% of seropositive cases. Joint swelling and rashes were observed in 48.03 and 69.33% seropositive cases, respectively. Similar findings correlated with other studies conducted by Mohanty et al. (2013) and Balasubramaniam et al. (2011) showed that fever and joint pain were the most common symptom (Figure 1).

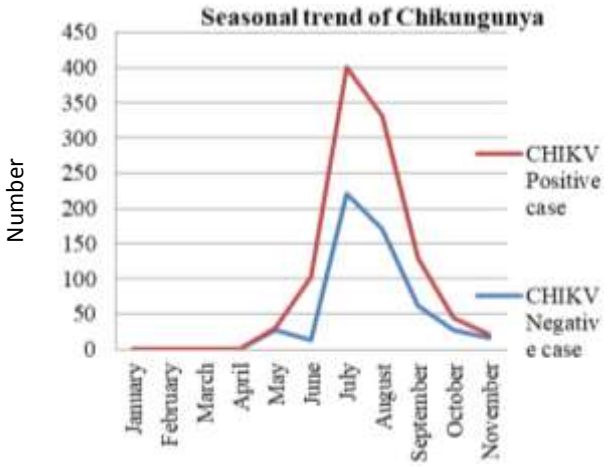

Figure 1. A seasonal peak was seen in the month of June to September.

This study shows seroprevalence of 49.43%. The geographical distribution had a significant influence on the prevalence of antibodies to the virus. The Chikungunya infected number of cases was more in the months of May to September and less during the months of January to April. Most of the studies represent seasonal variation, because of the increase in vector density during the rainy season (Dwibedi et al., 2011).

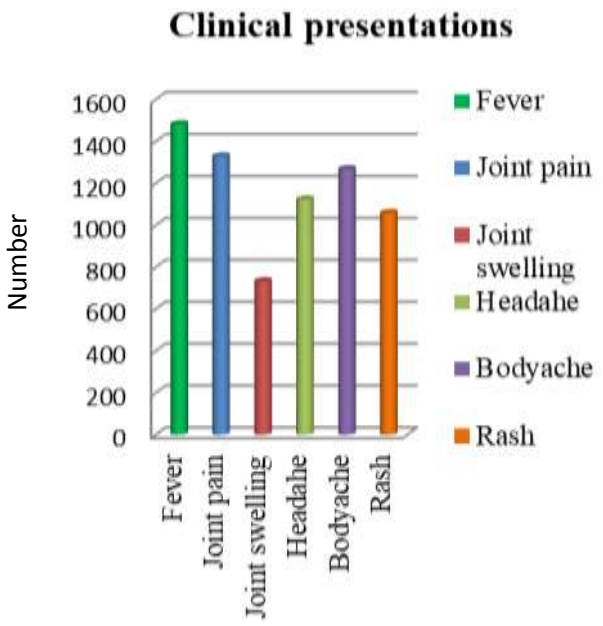

Figure 2. Clinical symptom of the respondents.

This might be explained by the possible impact of ecological characteristics of the areas on the natural cycles of the arthropod-borne viruses under consideration (Shrihari et al., 2012).

Conclusion

Chikungunya affects the humans of all age in both sex groups worldwide. In this study, there was no mortality but morbidity rate was high in affected cases, most affected age groups belonged to 11 to 40 years. The seroprevalence of Chikungunya in the study was 49.43%. The finding suggests it continuance as a major health threat in the present scenario. The *Aedes* mosquito is present in varying density in the different season. The virological surveillance of CHIKV and other vector-borne diseases should, therefore, be given utmost attention that will in turn help in the prediction, prevention, and control of impending and sporadic outbreaks in developing countries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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