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Review

Is quantitative genetics still necessary in this age of genomics?

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Quantitative genetics and genomics are two different disciplines that have separate evolutions. The quantitative genetics has enormous applications and has contributed a lot in four main distinct fields of plant breeding, animal breeding, evolutionary genetics and human genetics. This field is based on study of inheritance patterns and their underlying mechanisms using biometrical or statistical methods. The analysis of genome aims to identify genes of interest and understand gene expression profile and gene function. This analysis exploits different molecular biology approaches. This review discusses the quantitative genetics and molecular approaches in studies of quantitative traits. It also tries to find out the connection and complementation between approaches of quantitative genetics and molecular biology in the studies of quantitative traits. The information gathered in this review will assist breeders and geneticists in their regular research works.

Key words: Gene, genome, genotype, phenotype, trait.

INTRODUCTION

Quantitative genetics and genomics are two different disciplines that have separate evolutions. Quantitative genetics provides the means to estimate heritability, genetic correlations and predicted responses to various selection schemes (Keurentjes et al., 2008). Genomics offers powerful tools for mass screening of desired traits (Holland and Cardinal, 2008). Both disciplines are currently applied as tools for crop and animal improvement and for human and evolution genetics (Ellgren and Galtier, 2016). This paper discusses the quantitative genetics and molecular approaches in studies of quantitative traits and also tries to find out the connection and complementation between approaches of quantitative genetics and molecular biology in the studies of quantitative traits. Then, it gave a general view.

QUANTITATIVE GENETICS

Genetic traits can be qualitative or quantitative and each category has its own specificity. Qualitative traits are controlled by single genes and characterized by clear phenotypic classes. Inversely, quantitative traits are controlled by many genes and they present continuous variations in phenotypes. Moreover, these traits are extremely affected by non-genetic effects and their

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complexity is enhanced by interactions between genes and environment (Holland, 2007; Keurentjes et al., 2008; Kroymann and Mitchell-Olds, 2005). The study of inheritance patterns of quantitative traits and their underlying mechanisms by using biometrical or statistical methods is named quantitative genetics (Falconer et al., 1996).

The quantitative genetics has enormous applications and has contributed a lot in four main distinct fields of plant breeding, animal breeding, evolutionary genetics and human genetics. The general objective of studies related to these fields is to determine the contribution of genetic and non-genetic factors to the phenotype. However, the specific objectives of each field differ from one another. Plant and animal geneticists focus on development of new lines and identify among these lines, individuals which present desirable and stable traits. The human geneticists focus on identification of genotype associated with diseases and contribution of non-genetic factors for the disease development (Wray and Visscher, 2015). On the side of evolutionary genetics, geneticists concentrate on pining out the genetic makeup of specific phenotype and try to understand its past and its probable future evolutions (Kearsey et al., 2003; Walsh, 2001).

Even though the quantitative genetics has contributed to solve different problems in agriculture and animal breeding, human genetics and evolution genetics, it presents some drawbacks. Quantitative genetics does not provide facility to study effects of isolate genes on variation of a specific variation (Kearsey et al., 2003). In addition, with quantitative genetics, it is not easy to understand the genetic basis of quantitative traits and their mechanisms of maintenance during evolution and to understand the relationship between genetic variation and phenotypic variation (Mackay et al., 2009). This is a particularity of molecular approaches which facilitate following and localizing the transmission of small pieces of chromosomal region from parents to offspring (Kearsey et al., 2003). Therefore, the progress in molecular approaches including genomics could have a positive effect on evolving the quantitative genetics.

Molecular approaches

Currently, many studies in molecular biology aim to understand the gene function and gene expression profile. To achieve this goal, different molecular approaches such as analysis of genome, transcriptome, metablome and proteome were developed (Carpentier, 2007; Lappalainen, 2015).

The analysis of an organism's genome is a complex study and this discipline is known as genomics. The origin of genomics is genetics on which, there is aim to understand the structure, function and the evolution of genomes. Genomics is based on a complete genome analysis and involves DNA sequencing, assembly of sequences, annotation and mapping of genes (Arabidopsis, 2000).

The study of gene expression and its regulation is another approach to understanding the gene function. This approach is known as transcriptome. The most efficient tools to carry out the transcriptome analysis include microarray analysis, cDNA fragment fingerprinting and serial analysis of gene expression (SAGE) (Brown and Botstein, 1999; Schena et al., 1998).

Metabolome represents the collection of all metabolites in a biological organism at a specific time point and under specific conditions. These metabolites are the end products of the biological organism genes expression. The study of metablome (metabolomics) is the comprehensive, qualitative and quantitative study of all small molecules (less than or equal to 1500 daltons) participating in important metabolic functions and fulfilling critical roles such as signalling molecules or secondary metabolites in an organism (Oliver et al., 1998). The main methods for metablome analysis are metabolite profiling and metabolite fingerprinting (Hall, 2006).

The last approach towards understanding the gene function and gene expression profile is proteomics. Proteomics focuses on the characterization of the cellular proteome which is defined as a set of protein species present in a biological unit at a specific developmental stage and under determined external biotic and abiotic conditions (Jorrín et al., 2006; Klug et al., 2000; Prescott et al., 2005). Proteomics involves protein biochemistry and bioinformatics to determine the spatial and temporal expression of proteins in cells and tissues of a living organism (Karr, 2007). Expression measurements of mRNA levels show the dynamics of gene expression and show what might occur in the cell, whereas, proteomics discovers what is actually happening (Prescott et al., 2005; Ghatak et al., 2017). The main tool of proteome analysis is a two dimensional gel electrophoresis (2-DE).

All these approaches (genomics, transcriptomics, metabolomics and proteomics) are powerful tools for massive screening of several genes and aim to reveal the changes of what might be occurring in a cell (Rute et al., 2016). However, each approach has its own strength and weakness.

The comparisons of mRNA expression and protein expression revealed a poor correlation between RNA transcription and protein abundance (Greenbaum et al., 2003). This observation was attributed to the fact that there are many complicated and varied regulation mechanisms of gene expression and post-transcriptional mechanisms. Therefore, the expressed proteins of the same gene may differ significantly in their abundance and structures (Giavalisco et al., 2005).

A genome project provides information on the number and kinds of genes present in an organism (Klug et al., 2000). Sequencing has revealed that the link between gene and gene product is often complex and one gene can produce several types of transcripts as a result of an

alternative splicing (Celotto and Graveley, 2001). It is estimated that 40 to 60% of human genes produce more than one protein because of the alternative splicing and post-translational modification (Klug et al., 2000; Kwon et al., 2006). These variations of end products of the same genes have effects on variation of phenotypes.

The transcriptomics and the proteomics studies are based on the available information of genome sequence. Therefore, transcriptomics studies are still hindered by the lack of full sequence of genome of many livings things (Greenbaum et al., 2003). The sequences of genes are infrequently identical between species. On the contrary, functional protein domains are well conserved. Therefore, it is possible to identify the function of new gene product by its comparison with well-known homologous proteins (Carpentier et al., 2008).

These molecular approaches are powerful tools to identify candidates with desired traits but the manifestation of these traits depends on non-genetic factors. This requires the investigation of appearance of those traits in different environments before taking a final conclusion on identified candidates. From this observation, it is also evident that the quantitative genetics assists the molecular approaches to reconfirm their findings. Therefore, there is a close link between quantitative genetics and molecular approaches.

LINK BETWEEN QUANTITATIVE GENETICS AND GENOMICS

In the quantitative genetics, a trait is controlled by many genes. In the past, there was a gap of knowledge on a theoretical work of individual genes determining the quantitative trait. Currently, a method to study these genes is available and these genes are known as quantitative trait loci (QTLs). The identification of individual genes leads to several applications. It can facilitate the selection process for traits with low heritability and allow their applications in a genetic engineering of quantitative traits. In the medical field, the identification of individual genes responsible for hereditary diseases can assist to improve the prevention methods. This discovery has also a positive effect in the understanding of evolution process (Falconer et al., 1996).

The main methods of quantitative genetics to identify the genes underlying quantitative traits are multimodal distribution, backcrossing with selection, non-normal distribution, heterogeneity of variance, offspring parent resemblance and complex segregation analysis. However, these methods do not give any information on how the individual genes contribute to the traits. Therefore, the new approach to study these individual genes is to identify all individual genes that have effect on the trait, try to set up their linkage map and finally use molecular cloning of relevant sequences of DNA (Falconer et al., 1996).

The difference between individuals is mainly due to variation at a genomic level and this variation affects the quantitative traits. The variation observed in these traits are derived specifically from the variation in DNA sequences and this polymorphism at DNA level is the most excellent marker of variation between individuals (Keurentjes et al., 2008). Nevertheless, this polymorphism needs a careful analysis because in some cases, they are meaningless. On one hand, the polymorphisms in coding DNA sequences and in regulatory sequences can result in variations in protein expression, function and stability. Consequently, these variations affect strongly the phenotypes. On the other hand, effects of polymorphism on non-coding DNA sequence are extremely low when affecting the phenotype. The study of these polymorphisms could assist to predict quantitative traits in breeding programs (Borevitz and Nordborg 2003; Keurentjes et al., 2008).

Quantitative genetics uses mainly the variance to evaluate different traits in the population, whereas, genomics uses precise markers. In quantitative genetics, there are challenges because genotypes are generally unknown and their appearance in population is a random process. On the side of genomics, there are tools for quantitative genetics to overcome this challenge. These tools include molecular markers mainly established from single nucleotide polymorphisms (SNPs) and or microsatellites. These genomic tools for quantitative genetics assist to identify a QTL mapping and to estimate the degree of relatedness between individuals (Walsh, 2001). These tools are the results of progress of molecular biology.

The progress in molecular biology techniques has changed the focus of quantitative genetics from one characteristic to a broad analysis. These techniques permit geneticists to identify the relationship between gene, its product and its biological function. The combination of these molecular techniques and progress in the statistics through quantitative genetics permitted the establishment of regulatory network that put together diverse stages of biological information from gene to function (Keurentjes et al., 2008).

The study on connection between genetics and genomics was first carried out on yeast in 2002 and this work opened the window for other similar studies (Brem et al., 2002). The progress in genome sequence offers the possibilities to compare genomic positions of genes with the map positions of QTLs that affect the expression of these genes. This comparison gives information on cis- and trans- regulation of gene expression (duplication, transcription and translation). In this process of gene expression, transcription is the initial stage of connection of sequences of genotype to phenotype. The variation in quality and quantity of successive products (proteins and metabolites) resulting from this expression process are responsible for variation in phenotype. These were also

confirmed by high analysis of proteome and metablome of physiologically stressed individuals and between individuals with different genetic makeup (Chevalier et al., 2004; Fiehn et al., 2000; Keurentjes et al., 2008).

Even though proteomics and metablomics are good candidates to study the functional consequences of natural genetic variation, they present some limitations. The complete analysis of proteome or metablome which is equivalent to full genomics analysis is impossible. This impossibility is due to the complexity and diversity of proteins and metabolites in a living organism and their analysis requires different and many protocols. Moreover, even for full sequenced genome, it is not possible to precisely predict proteins or metabolites that a living organism can express. This is because of variation in gene expression where one gene can be expressed in products varying in quality and quantity (Fiehn, 2002; Jansen, 2003).

The progress of findings in genomics has positive effects on quantitative genetics. After having a complete genome sequence, it is possible to scan the potential variations among individuals. These variations can be used to choose microsatellite makers and to construct different DNA chip microarrays for identified DNA sequences. In addition, other techniques such as DNA probing, *in situ* hybridization and others are based on the availability of full genome sequence. With full genome sequence, it is possible to propose candidates presenting genes for the traits of interest (Walsh, 2001). All these improvements in genomics have positive effects by shortening the screening process in the specific studies of quantitative traits. Therefore, it seems that in addition to the link between quantitative genetics and genomics, these two fields complement one another.

COMPLEMENT BETWEEN QUANTITATIVE GENETICS AND GENOMICS

Quantitative genetics and genomics have different levels of screening process but both levels contribute to the availability of good results. In the quantitative genetics, the selection of complex traits in the animal and plant breeding is totally based on phenotypes. Currently, genomics allows a direct selection to genotype level. This facilitates and shortens the selection process (Walsh, 2001). However, this selection at genome level has some shortcomings. In breeding process, specifically for horizontal resistance, the frequencies of genes controlling quantitative traits increase with time under selection pressure. The probability that the frequencies of these genes will increase in population with selection to genotype level is extremely low. Moreover, the expression of gene depends on many factors. Therefore, the presence of a gene does not mean the presence of a phenotype.

The ability to screen plant cells in tissue culture and then grow the identified and surviving individuals to develop whole and fertile plants greatly increases the efficiency of selection process for certain characters. However, results from the controlled artificial environment need to be confirmed in natural environment because many studies revealed divergent results in these two different environments (Walsh, 2001). This shows the need for the phenotype evaluation to be part of the screening process. Therefore, genomics can be used to check the presence of the genes and then the quantitative genetics intervenes to explore the end products of genes expression.

The French breeding program of daily sheep is a good practical case that combines the genomics and quantitative genetics. This program was able to develop very good French daily sheep breeds using conventional phenotypic selection for milk production and other valuable traits. To emphasize the disease resistance in this program, genomic tools were incorporated in the breeding program for the management of the PrP gene associated with spongiform encephalopathies. These new tools were used for PrP genotyping of one year old rams and allowed to identify the status of PrP gene in young ram before sending them into pipeline of breeding program (Barillet, 2010).

Currently, some developed molecular makers are available and applied in selection. The study of fatty acid biosynthesis pathway in plants and sequencing of genes in that pathway make DNA markers to assist in the selection for specific change in fatty acid traits in soybean (Holland and Cardinal, 2008). The molecular makers associated with diseases and pest resistance, drought and frost tolerance and others have been developed and are under use in the breeding program, but all these markers are used at the initial stages of the screening process (Mohan et al., 1997; Staub et al., 1996; Tanksley, 1983). The identified individuals undergo other studies with quantitative genetic approaches. This process of current breeding program shows the manner in which both genomics and quantitative genetics are important in the breeding works.

CONCLUSION

Quantitative genetics provide the methods to measure heritability and genetic correlation, and to predict the responses in selection process and assist the breeders to improve crops and livestock. This selection is mainly based on phenotypic variation which is determined by the combination of genetic makeup of individuals and environments. The main challenge of quantitative genetics is to understand the connection between genetic makeup (variation at DNA sequence) and variation in phenotype (quantitative traits), the mechanisms of maintenance and evolution of quantitative traits in population. At this point, quantitative genetics is effectively supported by genomics due to the availability of DNA sequencing, abundant markers, fingerprinting,

reverse genetic methods, studies on gene expression, development of statistical method for analyzing quantitative trait locus mapping and others. In combination with other molecular approaches (transcriptome, metabolite and proteome analysis) based on the availability of full genome sequence, genomics evolved the quantitative genetics. Moreover, information on quality and quantity of variation in proteins and metabolites, understanding the cis- and trans-regulation in the process of gene expression assist in understanding and obtaining a complete picture of genetic and phenotypic variation within the same and between different populations. However, in some cases, there is a contradiction between results from molecular approaches and those from quantitative genetics approaches.

In many molecular works, sometimes, cells or small tissues are used as a living organism mode. Results from this living organism mode are useful specifically in the screening process of breeding program. However, unexpected results are frequent when identified and selected individuals at cell level are tested in natural environment. This recalls the power of quantitative genetics on which the final conclusion is based on phenotypes. Therefore, both quantitative genetics and genomics approaches could complement each other to generate conclusive results.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Efficiency of pre-inoculation of soybeans with *Bradyrhizobium* up to 60 days before sowing

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The inoculation of nitrogen-fixing bacteria in soybean crops allows the achievement of high crop yield by reducing or eliminating the application of nitrogen fertilizers. Pre-inoculation of seeds can reduce costs and increase the efficacy of this agricultural practice. The objective of this study was to assess the efficacy of pre-inoculation of soybean seeds with a commercial inoculant of *Bradyrhizobium* (RIZOLIQ LLI® manufactured by Rizobacter do Brasil) at 60, 45, and 30 days before sowing using a cell protector and different chemical treatments. The study was conducted in four municipalities in the state of Paraná (Londrina, Pato Branco, Ponta Grossa, and Santa Tereza do Oeste) and included 14 treatments, a negative control, standard inoculation of the recommended bacterial strains on the day of sowing, and pre-inoculation at 30, 45, and 60 days before sowing using the commercial inoculant RIZOLIQ LLI® together with the cell protector and chemical treatment of seeds with Imidacloprid (Rocks®), Fipronil+Thiophanate-methyl+Pyraclostrobin+ (Standack®Top), Metalaxyl-M+Fludioxonil (Maxim®XL), and Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®). The nodulation, plant biomass, nitrogen concentration in shoot and grain, and grain yield were evaluated. The cell protector was efficient in maintaining the bacterial inoculant viable in the seed for up to 60 days. All treatments of pre-inoculation of soybean chemically-treated seeds up to 60 days before sowing could be performed without impairment of nodulation, plant biomass, nitrogen concentration in shoot and grain, and grain yield. Therefore, pre-inoculation of soybean seeds up to 60 days before sowing is an efficient and practical inoculation strategy for sowing soybean crops.

Key words: Inocula, pre-sowing inoculation, rhizobia, symbiosis.

INTRODUCTION

Glycine max (L.) Merr. (soybean) is actually the most cultivated oilseed plant worldwide. Soybean production in Brazil was the second largest worldwide and reached 113.9 million tons of grains in the 2016/2017 harvest (CONAB, 2017).

Nitrogen (N_2) is the most required nutrient in soybean crops and biological nitrogen fixation (BNF) under the growth conditions of Brazil can provide all the nitrogen needed by the crop (Mourtzinis et al., 2018), making this process indispensable for the economic and

Table 1. Chemical characteristics of the soil of the experimental areas located in Londrina, Santa Tereza, Pato Branco, and Ponta Grossa, state of Paraná, Brazil.

Study site	P	C	pH	Al	H+Al	Ca	Mg	K
	mg/dm^3	g/dm^3		cmol$_c$ dm^3 de solo^{-1}				
Londrina	12.1	15.42	5.2	0.00	4.60	3.60	2.09	0.50
Santa Tereza do Oeste	15.4	29.96	4.7	0.15	8.35	4.52	2.59	0.44
Pato Branco	6.2	21.23	6.6	0.00	2.94	7.35	5.59	0.25
Ponta grossa	29.7	16.20	5.3	0.00	4.27	2.87	1.52	0.38

*P and K: Melich I; Ca and Mg (KCl) and Al: SMP; pH: $CaCl_2$ 0.01 mol L^{-1}; C: Walkley Black.

environmental viability of soybean cultivation in Brazil. This biological process is performed by bacteria of the genus *Bradyrhizobium*, which can fix the atmospheric N_2 naturally and in synchrony with crop requirements and cycle, supplying more nitrogen to grains than chemical fertilizers (Kaschuk et al*.,* 2010).

The efficiency of BNF in leguminous plants, especially in tropical regions, has been affected by several edaphoclimatic factors, management practices and seed treatment with fungicides, and these factors could compromise the viability of bacterial cells and symbiosis (Hungria et al., 2007). For this reason, there is a growing interest in research on innovative strategies that can help maximize BNF and increase crop productivity.

The practice of the inoculation of *Bradyrhizobium* species has been recognized for its advantages and economic and environmental gains. However, some factors limit the application of this technology, including inoculation on the day of sowing, which generates extra work for the producer and increase labor and time to prepare the seeds (Aguiar et al., 2014). These difficulties have stimulated the non-utilization of this inoculation practice in the soybean crop by producers (Zilli et al., 2010).

Pre-inoculation or early inoculation of *Bradyrhizobium* strains recommended for soybeans might be the solution as long as the bacterial cells are viable on the day of sowing and the chemical treatment of the seeds does not reduce the viability of the inoculant. Therefore, pre-inoculation allows amplifying the effects of BNF in soybeans by the increased inoculation of soybeans with *Bradyrhizobium* by farmers because the seeds could be commercialized in the pre-inoculated homogenized form and seed quality is guaranteed (Anghinoni et al., 2017; Araújo et al., 2017).

The objective of this study was to assess the agronomic efficiency of soybean pre-inoculation with the inoculant Rizoliq LLI + Premax cell protector at 30, 45, and 60 days before sowing seeds chemically treated with different products.

MATERIALS AND METHODS

Treatments and experimental areas

In the agricultural harvest of 2015/2016, four field experiments were conducted in experimental areas of the Agronomic Institute of Paraná (Instituto Agronômico do Paraná–IAPAR) located in the municipalities of Londrina (23° 21’ 26” S and 51° 10’ 08” W, altitude of 565 m), Santa Tereza do Oeste (25° 05’ 19” S and 53° 35’ 19”’ W, altitude of 750 m), Pato Branco (26° 07’ 32” S and 52° 38’ 56” W, altitute of 720 m), and Ponta Grossa (25° 09’ 04” S and 50° 09’ 14” W, altitude of 865 m), with different edaphoclimatic conditions but with adequate conditions for soybean cultivation. The chemical characterization of the soil of each region was performed at the Soil and Tissue Laboratory of IAPAR in Londrina following the methodology of Pavan et al. (1992) (Table 1). Soil correction and fertilization was performed according to the EMBRAPA soybean fertilization recommendation bulletin (2008).

The study included four replicates arranged in a completely randomized block design and was conducted in plots of 4.05 × 6.00 m (24.3 m^2) with nine crop rows each with 6 m in length, spaced at 0.45 m, with approximately 12 plants per linear meter. The soybean varieties cultivated in the state of Paraná were BMX Potência RR in the municipalities of Londrina and Pato Branco and BMX Apolo RR in Santa Tereza do Oeste and Ponta Grossa.

The inoculants *Bradyrhizobium japonicum* strains Semia 5079 and Semia 5080 used in this study were manufactured and marketed by Rizobacter do Brasil (Paraná, Brazil). The treatments consisted of pre-inoculation with the inoculant Rizoliq LLI® (*B. japonicum* - strains Semia 5079 and Semia 5080 at the concentration of 7 × 10^9 CFU mL^{-1}) + Premax® Cell Protector at 30, 45, and 60 days before sowing, combined with chemical treatments of seeds with Imidacloprid (Rocks® manufactured by FMC), Fipronil+Thiophanate-methyl+Pyraclostrobin+ (Standack®Top manufactured by Basf), Metalaxyl-M+Fludioxonil (Maxim®XL manufactured by Syngenta) and Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced + Cruiser® manufactured by Syngenta) at the dosages of 3.5, 2.0, 1.0, and 0.5 mL Kg^{-1} of seed, respectively, as recommended by the manufacturers. A standard treatment using the inoculant RIZOLIQ® (*B. japonicum* strains Semia 5079 and Semia 5080 at 5 × 10^9 CFU mL^{-1}) on the day of sowing and a

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Table 2. Pre-inoculation treatments of soybean seeds.

Treatment	Inocula	Inoculation	Seeds treatment
Control	Control	Without inoculation	-
Standard	RIZOLIQ®[1]	Sowing	-
LLI30S	RIZOLIQ-LLI®[2] + Premax®	30 days pre-sowing	Standak®Top
LLI30MC			Maxim®Advanced + Cruiser®
LLI30M			Maxim®XL
LLI30R			Rocks®
LLI45S		45 days pre-sowing	Standak®Top
LLI45MC			Maxim®Advanced + Cruiser®
LLI45M			Maxim®XL
LLI45R			Rocks®
LLI60S		60 days pre-sowing	Standak®Top
LLI60MC			Maxim®Advanced + Cruiser®
LLI60M			Maxim®XL
LLI60R			Rocks®

[1]RIZOLIQ®: *Bradyrhizobium japonicum* (strains Semia 5079 and Semia 5080). [2]RIZOLIQ-LLI®: *Bradyrhizobium japonicum* (strains Semia 5079 and Semia 5080) + Premax®.

treatment without addition of inoculant or mineral nitrogen were used (Table 2). The inoculated and treated seeds were stored in paper bags and kept at ambient temperature (25 ± 3°C) protected from the sun and humidity until the time of sowing (MAPA, 2010).

Bacterial survival in inoculated seeds

After storage of pre-inoculated seeds, viable cells of *Bradyrhizobium* spp. were recovered and quantified on the surface of the seeds according to Brazilian regulations (MAPA, 2010). Standard inoculation with RIZOLIQ® was also subjected to analysis. For this procedure, samples of 100 seeds of each treatment were aseptically transferred to an Erlenmeyer containing 100 mL of a solution of 0.85% NaCl (w/v) and 0.01% Tween 80 (w/v) and shaken in an orbital shaker for 15 min at 150 rpm. The obtained suspension was serially diluted ten-fold in a 0.85% NaCl solution (w/v). After that, 0.1-mL aliquots of the dilutions 10^{-1} to 10^{-9} were transferred to Petri dishes containing Ikuta semi-selective culture medium (IKUTA, 1995), Congo red (0.25 g/100 mL), and the antimicrobials nalidixic acid (20 mg L^{-1}), neomycin (20 mg L^{-1}), chloramphenicol (20 mg L^{-1}), actidione (10 mg L^{-1}), and triazole (2.5%). The aliquots were spread over the culture medium using a drigalski loop with three replicates on distinct plates. The plates were kept in an oven at 28 ± 2°C for 10 days and then the colonies were counted. Only the dilutions whose average of the three plates were 30 to 300 CFU were considered in the count. The number of *B. japonicum* recovered from the seeds was transformed into $\log_{10}$ (MAPA, 2010).

Experimental procedures and analyses

The execution of the experiments followed the agroclimatic zoning of each region (MAPA, 2015) and sowing were performed in October. The cultivation practices adopted for managing weeds, pests, and diseases complied with the recommendations for soybean cultivation. During flowering (phenological stage R1), five plants of each treatment were collected and the following parameters were evaluated: number of nodules per plant, dry mass of nodules per plant, dry mass of root and shoot, and concentration of nitrogen (N) of the shoot following the methodology described by Miyazawa et al. (1992). Grain yield and nitrogen concentration in grains (Miyazawa et al., 1992) were evaluated after 50% of the crop reached the R8 phenological stage.

Data analysis

The results were subjected to analysis of variance (ANOVA) with a level of significance of $p<0.1$. The mean number of viable bacterial cells on the seeds were compared using Duncan's test ($p<0.1$). The mean values of the pre-inoculation treatments for the variables nodulation (number and dry mass of nodules), biomass (root and shoot dry mass), nitrogen concentration in shoot and grain, and grain yield were compared by pairs with the standard treatment (inoculation at sowing using RIZOLIQ) using the bilateral Dunnett test ($p<0.1$) (Dunnet, 1964).

RESULTS

Bacterial survival on inoculated seeds

The recovery and quantification of viable *Bradyrhizobium* cells on the seed surface are shown in Table 3. The mean number of viable bacterial cells on seeds inoculated with RIZOLIQ® on the day of sowing but not treated chemically (standard inoculation) was 3.3 $\log_{10}$ CFU of $seed^{-1}$.

Pre-inoculation with RIZOLIQ LLI® + Premax® cell protector at 30 days before sowing seeds chemically treated with different products ensured the survival of

Table 3. Viability (colony forming units–CFU) of *Bradyrhizobium japonicum* in soybean seeds inoculated with RIZOLIQ® on the day of sowing or pre-inoculated with RIZOLIQ-LLI® + Premax® cell protector.

Treatment	Viable cells of *Bradyrhizobium*
	Log_{10} CFU of seeds^{-1}
Standard	3.30[b]
LLI30S	2.89[b]
LLI30MC	3.79[b]
LLI30M	4.03[a]
LLI30R	4.08[a]
LLI45S	2.46[c]
LLI45MC	2.66[c]
LLI45M	2.30[c]
LLI45R	2.76[b]
LLI60S	2.79[b]
LLI60MC	2.50[c]
LLI60M	2.62[c]
LLI60R	2.86[b]
CV%	6.9

The means followed by the same letter were not significantly different using the Duncan test (p<0.05). Data transformed to log_{10} (CFU + 1). CV=Coefficient of variation.
Standard inoculation with RIZOLIQ® (*B. japonicum* strains Semia 5079 and Semia 5080) on the day of sowing in seeds not chemically treated; LLI30S, LLI30MC, LLI30M, and LLI30R, inoculation with RIZOLIQ LLI® + Premax® cell protector at 30 days before sowing of seeds chemically treated with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standack®Top manufactured by Basf), Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser® manufactured by Syngenta), Metalaxyl-M+Fludioxonil (Maxim®XL manufactured by Syngenta) and Imidacloprid (Rocks® manufactured by FMC), respectively; LLI45S, LLI45MC, LLI45M, and LLI45R, inoculation with RIZOLIQ LLI® + Premax® cell protector at 45 days before sowing seeds chemically treated loc.cit.; LLI60S, LLI60MC, LLI60M, and LLI60R, pre-inoculation with RIZOLIQ LLI® + Premax® cell protector at 60 days before sowing seeds chemically treated loc. cit.

bacterial cells. The mean number of bacterial cells on seeds pre-inoculated at 30 days before sowing and treated with Metalaxyl-M+Fludioxonil and Imidacloprid (Maxim®XL and Rocks®, respectively) (LLI30M and LLI45R) was significantly higher than that in the standard inoculation. The mean number of bacterial cells on seeds pre-inoculated at 30 days before sowing chemically treated with other treatments (LLI30S and LLI30MC) was similar to that of seeds subjected to the standard inoculation.

In addition, the mean bacterial population on seeds pre-inoculated at 45 days before sowing and treated with Imidacloprid (Rocks®) (LLI45R) and seeds pre-inoculated at 60 days before sowing and treated with Imidacloprid and Fipronil+Thiophanate-methyl+Pyraclostrobin (Rocks® and Standak®Top) (LLI60R and LLI60S) was not significantly different from the population on seeds subjected to standard inoculation. The bacterial population in seeds of other treatments pre-inoculated at 45 and 60 days before sowing (LLI45S, LLI45MC, LLI45M, LLI60MC, and LLI60M) was smaller than that on seeds inoculated with RIZOLIQ® on the day of sowing.

Symbiotic efficiency and grain yield

The number of nodules, dry mass of nodules, dry mass of roots and shoot, nitrogen concentration in shoot and grain, and grain yield in Londrina, Santa Tereza do Oeste, Pato Branco and Ponta Grossa are shown in Tables 4, 5, 6 and 7, respectively.

Nodulation in the flowering period was high in all tested locations and all treatments. In most cases, pre-inoculation with RIZOLIQ LLI® + Premax® cell protector at 30, 45 and 60 days before sowing did not reduce nodule number and dry mass compared with inoculation with RIZOLIQ® on the day of sowing. In Londrina, pre-inoculation at 45 days before sowing and chemical treatment with Imidacloprid (Rocks®) (LLI45R) and pre-inoculation at 30 days before sowing and treatment with Fipronil + Thiophanate-methyl + Pyraclostrobin (Standak®Top) (LLI30S) caused a signifcant increase in

Table 4. Number of nodules, dry mass (DM) of the nodules, dry mass of roots and shoot, nitrogen concentration in shoot and grains, and yield of soybean cultivar BMX Potência RR treated with different pesticides and pre-inoculated in Londrina, Paraná, Brazil, in the 2015/2016 harvest.

Treatment	Nodulation		Dry mass		Nitrogen		Grain yield
	Nodules	DM	Root	Shoot	Shoot	Grains	kg ha^{-1}
	nº plant^{-1}	g plant^{-1}	g plant^{-1}	g plant^{-1}	g kg^{-1}	g kg^{-1}	
Control	457.5	1.81	5.48	42.86	50.58	62.74	4044.11
Standard	362	1.62	4.4	39.35	48.6	64.69	4150.88
LLI60S	327	1.27	5.11	40.77	44.69	60.96	4291.98
LLI60MC	392	1.48	5.61	33.52	39.98	60.72	4223.92
LLI60M	425	1.52	5.68	40.94	39.37*	58.71*	4218.31
LLI60R	385	1.47	3.88	33.54	41.71	59.32*	4170.23
LLI45S	441	2.02*	5.2	40.83	43.83	62.78	3924.85
LLI45MC	416	1.42	5.97	38.2	51.03	59.42*	4236.41
LLI45M	457	1.34	4.65	38.08	39.46*	59.62*	4106.54
LLI45R	577*	1.7	6.64*	39.33	38.37*	59.88*	4190.21
LLI30S	423	1.79	4.78	30.72	50.65	63.94	3844.15
LLI30MC	305	1.43	4.71	33.67	50.72	63.74	4270.13
LLI30M	534*	1.82	4.65	39.29	50.73	62.39	4048.5
LLI30R	439	1.85	4.05	38.93	51.98	60.74	4208.32
p-value	<0.0001	0.0001	0.0533	<0.0001	<0.0001	0.0109	0.1878
CV %	15.6	13.1	21.6	7.1	10.5	3.9	5.3
HSD	122.77	0.3923	2.0312	4.9571	8.922	4.501	407.3

The means followed by the same letter were not significantly different using the Duncan test (p<0.05). CV=Coefficient of variation. HSD: Honestly significant difference.
Standard. inoculation with RIZOLIQ® (*B. japonicum* strains Semia 5079 and Semia 5080) on the day of sowing in seeds not chemically treated; LLI30S, LLI30MC, LLI30M, and LLI30R, inoculation with RIZOLIQ LLI® + Premax® cell protector at 30 days before sowing of seeds chemically treated with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standack®Top manufactured by Basf), Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser® manufactured by Syngenta), Metalaxyl-M+Fludioxonil (Maxim®XL manufactured by Syngenta) and Imidacloprid (Rocks® manufactured by FMC), respectively; LLI45S, LLI45MC, LLI45M, and LLI45R, inoculation with RIZOLIQ LLI® + Premax® cell protector at 45 days before sowing seeds chemically treated loc.cit.; LLI60S, LLI60MC, LLI60M, and LLI60R, pre-inoculation with RIZOLIQ LLI® + Premax® cell protector at 60 days before sowing seeds chemically treated loc. cit.

the number of nodules compared with standard inoculation. In addition, pre-inoculation at 45 days before sowing and treatment with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standak®Top) (LLI45S) caused a significant increase in nodule dry matter compared with standard inoculation (Table 4). In Santa Tereza do Oeste, pre-inoculation at 30 days before sowing and chemical treatment with Imidacloprid (Rocks®) (LLI30R) caused a significant increase in nodule dry mass (Table 5). In Pato Branco, pre-inoculation at 30 and 60 days before sowing and treatment with Imidacloprid (Rocks®) (LLI30R and LLI60R) increased nodule number and dry mass, respectively, compared to standard inoculation (Table 6). In Ponta Grossa, only pre-inoculation at 30 days before sowing and treatment with Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®) (LLI30MC) increased the number of nodules. However, pre-inoculation at 60 days before sowing and treatment with Imidacloprid (Rocks®) (LLI60R) and pre-inoculation at 30 days before sowing and treatment with Metalaxyl-M+Fludioxonil (Maxim®XL) (LLI30M) decreased the number of nodules when compared with standard inoculation. Moreover, pre-inoculation at 60 days before sowing and combined treatment with Fipronil+Thiophanate-methyl+Pyraclostrobin and Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Standak®Top and Maxim®Advanced+Cruiser®, respectively) (LLI60S and LLI60MC), preinoculation at 45 days before sowing and treatment with Metalaxyl-M+Fludioxonil (Maxim®XL) (LLI45M), and pre-inoculation at 30 days before sowing and treatment with Imidacloprid (Rocks®) (LLI30R) yielded a lower nodule dry mass when compared with the standard inoculation (Table 7). The results of the other treatments did not differ from the standard inoculation.

Plant biomass also showed a good response to pre-inoculation with RIZOLIQ LLI® + Premax® cell protector. Pre-inoculation at 45 days before sowing and treatment with Imidacloprid (Rocks®) (LLI45R) in Londrina, and pre-inoculation at 45 days before sowing and treatment with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standak®Top)

Table 5. Number of nodules. dry mass (DM) of the nodules. dry mass of roots and shoot. nitrogen concentration in shoot and grains. and yield of soybean cultivar BMX Apolo RR treated with different pesticides and pre-inoculated in Santa Tereza do Oeste, Paraná, Brazil. in the 2015/2016 harvest.

Treatment	Nodulation		Dry mass		Nitrogen		Grain yield
	Nodules	DM	Root	Shoot	Shoot	Grains	kg ha^{-1}
	nº plant^{-1}	g plant^{-1}	g plant^{-1}	g plant^{-1}	g kg^{-1}	g kg^{-1}	
Control	235	1.6	5.15	29.64	45.88	62.73	4005.8
Standard	299	1.39	3.86	24.7	45.21	65.87	4195.59
LLI60S	306	1.39	4.08	31.38	37.03*	65.09	4280.35
LLI60MC	266	1.74	4.95	29.26	37.80*	65.06	4135.45
LLI60MS	298	1.49	4	30.17	46.69	64.17	3931.81
LLI60R	278	1.46	4.61	23.34	43.39	67.39	4315.1
LLI45S	239	1.33	5.34*	29.85	41.08	62.61	4310.28
LLI45MC	280	1.25	4.31	27.49	41.55	64.89	4253.75
LLI45MS	255	1.45	4.93	30.18	35.42*	62.59	4094.79
LLI45R	330	1.82	4.36	30.95	38.39	64.6	4222.5
LLI30S	270	1.42	3.49	29.2	45.4	64.03	4377.74
LLI30MC	280	1.54	5.26*	28.04	40.31	64.57	4130.84
LLI30MS	261	1.2	4.13	30.22	45.16	63.3	4057.15
LLI30R	354	2.19*	4.24	31.51	45.7	62.55	4283.09
p-value	0.0079	0.0182	0.0173	0.1853	0.0007	0.0812	0.0791
CV %	13.5	21.6	16.41	13.79	9.31	3.3	4.65
HSD	72.51	0.61	1.36	7.42	7.27	3.94	361.08

The means followed by the same letter were not significantly different using the Duncan test (p<0.05). CV: Coefficient of variation; HSD: honestly significant difference.

Table 6. Number of nodules. dry mass (DM) of the nodules. dry mass of roots and shoot. nitrogen concentration in shoot and grains. and yield of soybean cultivar BMX Potência RR treated with different pesticides and pre-inoculated in Pato Branco, Paraná, Brazil. in the 2015/2016 harvest.

Treatment	Nodulation		Dry mass		Nitrogen		Grain yield
	Nodules	DM	Root	Shoot	Shoot	Grains	kg ha^{-1}
	nº plant^{-1}	g plant^{-1}	g plant^{-1}	g plant^{-1}	g kg^{-1}	g kg^{-1}	
Control	274	1.01	4.62	19.46	38.59	59.99	2543.65*
Standard	234	0.92	4.79	16.78	37.8	60.2	2951.62
LLI60S	289	1.24	4.39	17.35	41.89	63.2	2902.79
LLI60MC	268	1	4.93	23.22*	43.84	62.59	2780.16
LLI60MS	272	1.04	5.13	22.28*	39.09	60.6	2860.93
LLI60R	290*	1.17	5.81	24.39*	41.25	62.27	2604.7
LLI45S	272	1.2	5.2	19.79	44.54*	58.53	3117.03
LLI45MC	219	1.02	4.52	17.88	40.34	54.03	2855.79
LLI45MS	247	1.03	5.3	19.07	37.49	56.96	2523.55*
LLI45R	238	1.23	4.5	20.29	43.94*	59.42	2624.13
LLI30S	257	1.19	3.59*	17.81	40.51	59.29	2782.65
LLI30MC	217	1.06	4.27	18.28	43.05	58.52	2866.73
LLI30MS	248	1.16	5.07	22.02*	43.58	60.13	2569.00*
LLI30R	274	1.33*	4.69	20.48*	43.8	60.38	2539.34*
p-value	0.0140	0.0898	0.0006	<0.0001	0.0261	0.0876	0.0014
CV %	11.7	16	11.6	9.9	8	5.9	7.3
HSD	56.033	0.3279	1.0277	3.6726	6.1222	6.5815	370.34

The means followed by the same letter were not significantly different using the Duncan test (p<0.05). CV=Coefficient of variation. HSD: Honestly significant difference.

Table 7. Number of nodules. dry mass (DM) of the nodules. dry mass of roots and shoot. nitrogen concentration in shoot and grains. and yield of soybean cultivar BMX Apolo RR treated with different pesticides and pre-inoculated in Ponta Grossa, Paraná, Brazil. in the 2015/2016 harvest.

Treatment	Nodulation		Dry mass		Nitrogen		Grain yield
	Nodules	DM	Root	Shoot	Shoot	Grains	kg ha^{-1}
	nº plant^{-1}	g plant^{-1}	g plant^{-1}	g plant^{-1}	g kg^{-1}	g kg^{-1}	
Control	387	1.67*	9.1	60	39.2	61.52	5518.2
Standard	456	2.29	10.39	63	41.93	61.25	5130.65
LLI60S	363	1.50*	7.77	43.5	39.46	60.33	5565.15
LLI60MC	376	1.66*	8.24	49	39.73	59.9	4821.94
LLI60MS	366	2.08	11.17	82	39.8	59.74	4844.22
LLI60R	335*	1.46*	10.24	59.5	38.72	61.49	4993.23
LLI45S	418	1.93	7.7	50.5	41.89	60.36	5122.91
LLI45MC	466	2.06	10.77	57	42.42	60.16	5195.27
LLI45MS	337	1.40*	6.45*	44.5	42.85	57.99	5202.32
LLI45R	337	1.82	9.39	61	42.3	55.36*	4885.43
LLI30S	456	2.2	12.67	63	40.91	60.21	4992.11
LLI30MC	627*	2.12	11.99	56	40.34	59.25	4973.47
LLI30MS	250*	1.42*	7.98	31.0*	40.59	61.24	4721.05
LLI30R	342	1.51*	6.55*	48	40.67	60.75	4951.51
p-value	<0.0001	<0.0001	0.0009	<0.0001	0.1390	0.3409	0.0015
CV %	16.4	15	22.1	19.9	5.2	5	5.3
HSD	119.61	0.4993	3.8286	20.242	3.9213	5.6202	494.99

The means followed by the same letter were not significantly different using the Duncan test (p<0.05). CV: Coefficient of variation; HSD: honestly significant difference.

(LLI45S) and pre-inoculation at 30 days before sowing and treatment with Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®) (LLI30MC) in Santa Tereza do Oeste promoted an increase in root dry mass (Tables 4 and 5). However, shoot dry mass was similar to that of the standard inoculation in these locations. In Pato Branco, pre-inoculation at 30 days before sowing and treatment with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standak®Top) (LLI30S) decreased root dry mass. However, pre-inoculation at 60 days before sowing and treatment with Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®), Metalaxyl-M+Fludioxonil (Maxim®XL) and Fipronil+Thiophanate-methyl+Pyraclostrobin (Standak®Top) (LLI60MC, LLI60M, and LLI60S) and pre-inoculation at 30 days before sowing and treatment with Metalaxyl-M+Fludioxonil and Imidacloprid (Maxim®XL and Rocks®, respectively) (LLI30M and LLI30R) promoted an increase in shoot dry mass when compared with standard inoculation (Table 6). In Ponta Grossa, pre-inoculation at 45 days before sowing and treatment with Metalaxyl-M+Fludioxonil (Maxim®XL) (LLI45M), and pre-inoculation at 30 days before sowing and treatment with Imidacloprid (Rocks®) (LLI30R) promoted a decrease in root dry matter. Furthermore, pre-inoculation at 30 days before sowing and treatment Metalaxyl-M+Fludioxonil (Maxim®XL) decreased shoot dry mass. The other pre-inoculation treatments did not differ from the standard inoculation (Table 7).

The amount of nitrogen provided to the shoot and grain by pre-inoculation at 30 days before sowing with RIZOLIQ LLI® + Premax® cell protector was similar that of the standard inoculation in all analyzed areas. In Londrina, pre-inoculation at 45 and 60 days before sowing and treatment with Metalaxyl-M+Fludioxonil (Maxim®XL) (LLI60M and LLI45M) and pre-inoculation at 45 days before sowing and treatment with Imidacloprid (Rocks®) (LLI45R) caused a decrease in nitrogen concentration in the shoot. Pre-inoculation at 60 days before sowing and treatment with Imidacloprid (Rocks®) (LLI60R) and pre-inoculation at 45 days before sowing and treatment with Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®) (LLI45MC) promoted a decrease in the concentration of nitrogen in the grains (Table 4). In Santa Tereza do Oeste, pre-inoculation at 60 days before sowing and treatment with Fipronil+Thiophanate-methyl+Pyraclostrobin and Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Standak®Top and Maxim®Advanced+Cruiser®, respectively) (LLI60S and LLI60MC) and pre-inoculation at 45 days before sowing

and treatment with Metalaxyl-M+Fludioxonil (Maxim®XL) (LLI45 M) decreased the nitrogen concentration in the shoot. However, these pre-inoculation treatments did not decrease nitrogen concentration in the grains. In Pato Branco, pre-inoculation at 45 days before sowing and treatment with Fipronil+Thiophanate-methyl+Pyraclostrobin and Imidacloprid (Standak®Top and Rocks®, respectively) (LLI45S and LLI45R) caused an increase in nitrogen concentration in the grains, and the concentration was similar to that of the standard inoculation (Table 6). However, pre-inoculation at 45 days before sowing and treatment with Imidacloprid (Rocks®) (LLI45R) decreased the nitrogen concentration in the grains (Table 7).

Although pre-inoculation treatments showed differences in nodulation, plant biomass, and nitrogen concentration in Londrina, Santa Tereza do Oeste, and Ponta Grossa, grain yield by pre-inoculation with different chemical products at 30, 45, and 60 days before sowing was statistically similar to that of the standard inoculation. In Pato Branco, because of the occurrence of pests and diseases in the study period, the mean grain yield in the region was lower than that in the other regions and presented variability between the evaluated treatments. In Pato Branco, only pre-inoculation at 30 and 45 days before sowing and treatment with Imidacloprid (Rocks®) (LLI45R and LLI30R) and pre-inoculation at 30 days before sowing and treatment with Metalaxyl-M+Fludioxonil (Maxim®XL) (LLI30M) caused lower yields compared with the standard inoculation, whereas grain yield in nine of the twelve pre-inoculation treatments tested was not significantly different from that in the standard inoculation, suggesting the potential use of this strategy even under unfavorable conditions.

DISCUSSION

Soybean crops require high amounts of nitrogen, and it is estimated that 80 kg of nitrogen is needed for producing 1000 kg of grains (Hungria et al., 2007). The supply of nitrogen to soybean culture in Brazil is provided primarily by inoculation of *Bradyrhizobium* spp., and the major factor involved in the successful supply of nitrogen by these bacteria to crops is the guarantee of bacterial survival on the seeds (Dall'Agnol, 2016).

In this study, the pre-inoculation of soybean seeds was tested with RIZOLIQ-LLI® + Premax® cell protector before sowing. Pre-inoculation at 30 days before sowing and treatment with Metalaxyl-M+Fludioxonil and Imidacloprid (Maxim®XL and Rocks®, respectively) promoted an increase in the population of *Bradyrhizobium* spp. on the seeds. The increase in the bacterial population may have been stimulated by the integrated action of Premax® cell protector combined with a positive effect of the products used in seed treatment (Maxim®XL and Rocks®). However, this hypothesis was not investigated. Although in the pre-inoculation treatments LLI45S, LLI45MC, LLI45M, LLI60MC, and LLI60M, bacterial survival on the seeds was lower than that in the standard inoculation in all the studied areas (Table 3), in all treatments were recovered at least 10^5 viable cells $seed^{-1}$ as recommended for soybean crop (Hungria et al., 2007) and this difference did not significantly decrease nodulation or grain yield, evidencing that the survival of viable cells is enough to ensure the nitrogen supply required by the crop (Tables 4 to 7) .

Although some authors report the toxicity of plant protection products used in seed treatment to the bacteria present in the inoculants (Araujo and Araújo, 2006; Hartley et al., 2012), the seeds inoculated with RIZOLIQ LLI® + Premax® cell protector and chemically treated with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standack®Top), Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®), Metalaxyl-M+Fludioxonil (Maxim®XL) and Imidacloprid (Rocks®) had a large amount of viable cells even after storage for 30, 45, and 60 days at room temperature. Silva et al. (2018) found a drastic reduction in the number of viable cells in seeds pre-inoculated 10 days before sowing and chemically treated with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standack®Top) and Metalaxyl-M+Fludioxonil (Maxim®XL). Nevertheless, in the present study, the use of the cell protector ensured the survival of bacteria even with chemical treatments and these results are in accordance with Araújo et al. (2017), who hypothesize that for establishing pre-inoculation technologies, especially in seeds treated chemically with crop protection products, the use of chemicals that improve bacterial survival is necessary.

Pre-inoculation of soybean seeds before sowing may stimulate the use of inoculants in these crops and optimize sowing. Some studies have shown the efficacy of seed pre-inoculation on bacterial survival (Gemell et al., 2005) and crop yield (Zilli et al., 2010; Anghinoni et al., 2017) even with a cell protector (Marks et al., 2013). However, to date, few inoculation technologies used before sowing ensured bacterial survival on seeds treated chemically with crop protection products under long storage periods and ensured good agronomic performance in the field. Therefore, in this study, the use of the Premax® cell protector promoted the survival of the bacteria present in the RIZOLIQ LLI® inoculant, even on seeds treated chemically with crop protection agents during storage.

The crop yield was the parameter that best reflected the efficacy of pre-inoculation. The grain yield achieved by pre-inoculation at 30, 45, and 60 days before sowing in the three study areas was similar to that of the standard inoculation, demonstrating the effectiveness of pre-inoculation with these two producs. Positive results in soybean grain yield with pre-inoculation technology have already been found in other studies. Silva et al. (2018) also observed that even with a drastic reduction in numbers of cells with chemical treatmens of the seeds,

pre-inoculation 10 days before sowing did not reduce nodulation nor grain yield. Zilli et al. (2016) verified that the pre-inoculation five days before sowing had a similar performance to the standard inoculation.

Although grain yield in three treatments (LLI45M, LLI30M, and LLI30R) in Pato Branco was lower than that in the standard inoculation, grain yield in nine of the twelve pre-inoculation treatments tested was not significantly different from that of the standard inoculation. Araujo et al. (2017) observed that pre-inoculation of soybeans with cell protector was effective for up to 30 days before sowing, and grain yields were similar to that of the standard inoculation even under adverse environmental conditions, indicating the potential of using this technology even under unfavorable conditions.

Several studies had reported the effectiveness of the pre-inoculation technology in soybean crop for five days before sowing (Zilli et al., 2016), up to 10 days (Anghinoni et al., 2017; Silva et al., 2018) and up to 30 days before sowing (Araujo et al., 2017). However, this study is the first to report the efficacy of pre-inoculation of soybean seeds for up to 60 days before sowing. Therefore, we showed that the pre-inoculation of soybeans with RIZOLIQ LLI®+Premax® cell protector can be used in seeds chemically treated with Fipronil+Thiophanate-methyl+Pyraclostrobin (Standack®Top), Metalaxyl-M+Fludioxonil+Thiabendazole with Thiamethoxam (Maxim®Advanced+Cruiser®), Metalaxyl-M+Fludioxonil (Maxim®XL) and Imidacloprid (Rocks®) and stored for up to 60 days at room temperature without loss in nodulation, plant biomass, nitrogen concentration in the plant shoot and grains, and grain yield (Tables 4 to 7).

Conclusions

The inoculation of soybean seeds with RIZOLIQ LLI®+Premax® cell protector allowed the survival of bacterial cells on seeds chemically treated with crop protection agents and stored for up to 60 days before sowing.

The pre-inoculation of soybean seeds with RIZOLIQ LLI®+Premax® cell protector up to 60 days before sowing can be performed on seeds treated chemically without impairment of nodulation, plant biomass, nitrogen concentration in shoot and grain, and crop yield and is an efficient and practical inoculation strategy for sowing soybean crops.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of seed rate on upland cotton (*Gossypium hirsutum*) seedling emergence

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The most important factor to achieving profitable cotton yields is obtaining a uniform stand of healthy and vigorously growing seedlings. Cotton seedling emergence highly depends on the number of seeds that are planted on the same planting station. The cotton seedling stalk is weak and may fail to push up and crack the soil in order to emerge. Therefore several seedlings put together may use the power of numbers to push out of the soil. Cotton seedling emergence percentage and stand are closely related to seed rate. In Zimbabwe the question of which seed rate is optimal took centre stage in input negotiations between contractors and farmers. A research project was therefore conducted at Cotton Research Institute, Tokwane, Mahuwe, and Muzarabani communal areas during two seasons of 2014 to 2015 in order to determine the effect of seed rate on cotton seedling emergence. The experiment was laid in a randomized complete block design with eight treatments of varying seed rates that ranged from two to nine seeds per planting station and with four replications. Results showed significant differences on stand counts among seed rates. At C.R.I and Mzarabani communal area three seeds per station achieved better stand counts while at Tokwane, five seeds per station resulted in better stand counts. In Mahuwe communal area, six seeds per station performed better. However, six seeds per station was the median seed rate that produced the highest stand counts across sites and across seasons. It is therefore recommended that farmers can plant three up to six seeds per station depending on environmental conditions

Key words: Cotton, seed rate, seed, stand counts, emergence, seedling.

INTRODUCTION

Cotton germination begins as the seed absorbs water and oxygen through its seed coat after planting. The hypocotyl elongates from the radicle and forms an arch or crook that begins to push up through the soil, a brief period often referred to as the “crook stage” (Cotton Germination and Seedling Development, 2012). At this stage, the cotton seedling may fail to push out the soil on its own. Rapid seed germination and emergence is an

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important factor in crop successful establishment, (Somayeh et al., 2015). Achieving even establishment of a cotton crop is critical in getting the crop off to a good start, as it can influence how the crop is to be managed. The aim of every cotton grower should be to plant the crop once and achieve the desired plant stand and evenness and get the crop off to a great start. The emergence of cotton seedlings is influenced by several factors, these being mainly the seed, the environment, and various mechanical factors. The environment influences seedling emergence through biotic and abiotic factors, (Gitz et al., 2015). Biotic factors such as presence of soil pathogens, nematodes, bacteria, or fungi affect the survival of seedlings during or shortly after emergence. The effects of biotic factors on seedling emergence vary greatly from site to site. The environment provides the basic requirements of light, heat, oxygen and moisture as the abiotic factors affecting cotton seedling emergence. The mechanical factors provide such aspects of the planting configuration as row spacing, seed placement distance, depth of sowing, seed rate, and degree of seed-soil contact.

The seed is one of the most important inputs in cotton production, (Nazir et al., 2014).The use of delinted cotton seeds in cotton planting instead of fuzzy cotton seeds has spread recently, (Zeybek et al., 2010). Planting of cotton seed when soil temperature and conditions are favorable at the proper depth and seeding rate is very important to give the crop the best chance of emerging properly and getting off to a good start. About 3.33 kg of seed of the cotton variety SZ9314 would be sufficient for a hectare if one seed was planted per planting station. The cotton seedling stalk is weak and may fail to push up and crack the soil in order to emerge. Therefore several seedlings put together may use the power of numbers to push out of the soil. That is the reason why several cotton seeds are planted at the same position and at as shallow a depth as 20 mm. The general and traditional seed rate recommendation of 25 kg/hectare, (Cotton Agronomy Manual, 2012), was viewed as too luxurious by some key players of Zimbabwe's cotton industry with claims that 15 kg/hectare would suffice. This study therefore sought to determine the optimum seed quantity required per unit area for optimum seedling emergence under rain fed cotton production in different agro ecological conditions of the cotton growing regions in Zimbabwe.

MATERIALS AND METHODS

The experiment was carried out for two seasons, 2014 and 2015 at four sites namely Cotton Research Institute in Mashonaland West Province, Tokwane communal area in Masvingo Province, Mahuwe and Mzarabani communal areas in Mashonaland central Province (Table 1). Treatments consisted of eight different seed rates of the cotton variety SZ9314 which has an average cotton seed weight of is 0.1g. The treatments used are shown in Table 2. Six seeds per planting station was used as the standard treatment. The treatments were laid out in a Randomised Complete Block Design with four replications. The recommended plant spacing of 1m between rows and 30cm within rows was used. The gross plot was 6 rows of 8 metres length and the net plot was 4 rows of 6 metres length. Basal fertilizer application was applied using soil analysis results. Stand counts were recorded one week after crop emergence. Analysis of variance on data collected of stand counts was performed using GenStat 14th edition for Windows, (Payne et al., 2011). The differences among treatment means were compared by Fisher's Protected Least Significant Differences test (LSD) at 0.05 level of probability

RESULTS AND DISCUSSION

Stand counts

Results in Table 3 indicated significant interactions at 5% level on stand counts among seed rate, site, and season. Thus, the relationship between seed rate and stand counts varied from site to site and from season to season, hence the results of the interactions on the effects of seed rate on seedling counts are presented by site and by season. Table 3 indicates the combined performance of the treatments over the two seasons. C.R.I represents Cotton Research Institute. At C.R.I three and five up to nine seeds per station had comparably the highest stand counts while two seeds per station had the lowest stand counts. At Tokwane communal area, five, six, eighty and nine seeds per station produced the highest stand counts. Results also indicated that six up to nine seeds per station had comparably the highest stand counts at Mahuwe communal area and at Mzarabani, three up to nine seeds per station produced the better stand counts. C.R.I represents Cotton Research Institute.

In 2014 season, results indicated significant differences on stand counts at C.R.I, Tokwane and Mahuwe and no significant differences at Mzarabani. At C.R.I, two seeds per station gave the lowest stand counts while three to nine seeds per station had comparably the highest stand counts. At Tokwane communal area, two seeds per planting station gave the lowest stand counts, while 3, 4, 5, 6, 7 and 9 seeds were comparable. Eighty seeds per station gave the highest stand counts which was comparable to 4, 5, 6, and 9 seeds per station. At Mahuwe, two seeds per station had the lowest stand while four to nine seeds resulted in comparably the highest stand counts (Table 4).

In 2015 season, results showed significant differences on cotton stand counts at C.R.I, Dande and Mzarabani and no significant differences on stand counts at Tokwane. At C.R.I two seeds per station had the lowest stand counts which were comparable to 3, 4, 5, 7 and 8 seeds per station. Stand counts for three seeds per station were comparable to that of six and seven seeds per station (Table 5).

Nine seeds per station had the highest stand counts which were comparable to that of six and three seeds per station. At Mahuwe communal area, two seeds had the lowest stand counts. Three to five seeds per station had

Table 1. Characteristics of experimental locations.

Location	Longitude and latitude	Altitude (asl) (m)	Soil type	Average rainfall (mm)
C.R.I	18° 20′ S and 29° 54′ E	1156	red clay loamy soils	666
Mahuhwe	16° 23′ S and 30° 44′ E	455	upland loamy sandy soils	754
Mzarabani	15° 45′ S and 29° 19′ E	600	clayey alluvial soils	909
Tokwane	19° 49′ S and 30° 20′ E	547	clay loamy soils	521

asl represents above sea level.

Table 2. Description of treatments used in this project.

Treatments	Number of seeds per station	Seed rate per hectare (kg)
1	2	9
2	3	12
3	4	15
4	5	18
5	6	20
6	7	24
7	8	27
8	9	30

Table 3. Effect of cotton seed rate on stand counts across season.

Treatments	Sites			
Seeds/station	**C.R.I**	**Tokwane**	**Mahuwe**	**Mzarabani**
2	26 719^{a}	15 364^{a}	16 771^{a}	24 688^{a}
3	30 781^{bc}	21 823^{b}	23 177^{b}	27 969^{b}
4	30 312^{b}	21 771^{b}	24 844^{bc}	28 698^{b}
5	31 198^{bc}	22 031^{bc}	26 719^{cd}	30 104^{b}
6	31 667^{bc}	22 188^{bc}	28 438de	29 869^{b}
7	31 250^{bc}	21 667^{b}	28 594de	29 427^{b}
8	31 354^{bc}	24 010^{c}	29 474^{e}	29 167^{b}
9	32 292^{c}	22 552^{bc}	30 156^{e}	29 636^{b}
Grand mean	**30 697**	**21 426**	**26 022**	**28 698**
P	*<0.001*	*<0.001*	*<0.001*	*0.017*
L.S.D	1807.30	2177.7	2564.5	3005.3
CV (%)	5.8	10.1	9.8	10.4

Means followed by the same letter are not significantly different at p = 0.05 and means were separated by the Fishers' LSD. C.R.I represents Cotton Research Institute.

comparable stand counts while five to eighty seeds per planting station had comparable stand counts. Nine seeds per station gave the highest stand counts, comparable to six, seven and eighty seeds per station. At Mzarabani, two seeds per station had the lowest stand counts while the other seed rates resulted in comparably the highest stand counts.

Though the performance of the seed rates varied from season to season and site by site, however six seeds per station was the median seed rate across sites and across seasons that resulted in the highest cotton stand counts. The targeted stand count 0f 33 333 per hectare was not achieved at any site for the two seasons. The failure to achieve the targeted stand count could have been due these factors that influence the seed germination and seedling emergence apart from the seed rate. Wanjura (undated) has also noted these factors to be the reasons for causing poor emergence even if seeds were planted properly, as some of these factors are largely uncontrollable.

Table 4. Effect of cotton seed rate on stand counts per hectare in 2014 season**.**

Treatments	Sites			
Seeds/station	**C.R.I**	**Tokwane**	**Mahuwe**	**Mzarabani**
2	24271[a]	13958[a]	20729[a]	23646
3	31 146[b]	24 792[b]	27 396[b]	26250
4	30 417[b]	26 979[bc]	31 250[c]	27500
5	32 187[b]	26 771[bc]	30 729[c]	30104
6	31 458[b]	26 146[bc]	32 708[c]	28750
7	32 187[b]	26042[b]	32 083[c]	28854
8	32 604[b]	30000[c]	32 812[c]	27604
9	32 604[b]	28021[bc]	32 708[c]	28229
Grand mean	**30 859**	**25 339**	**30 052**	**27 617**
P	*<0.001*	*<0.001*	*<0.001*	*0.176*
L.S.D	3265.2	3885.2	3100.5	4499.8
CV (%)	7.2	10.4	7.0	11.10

Means followed by the same letter are not significantly different at p = 0.05 and means were separated by the Fishers' LSD. C.R.I represents Cotton Research Institute.

Table 5. Effect of cotton seed rate on stand counts per hectare in 2015 season.

Treatments	Sites			
Seeds/station	**C.R.I**	**Tokwane**	**Mahuwe**	**Mzarabani**
2	29167a	16771	12812a	25729a
3	30417abc	18854	18958b	29688b
4	30208a	16563	18438b	29896b
5	30208a	17292	22708bc	30104b
6	31875bc	18229	24167cd	31042b
7	30313ab	17292	25104cd	30000b
8	30104a	18021	26146cd	30729b
9	31979c	17083	27604d	31042b
Grand mean	**30534**	**17513**	**21992**	**29779**
P	0.030	0.089	<0.001	0.002
L.S.D	1641.1	1592.7	4333.4	2317.4
CV (%)	3.7	6.2	13.4	5.3

Means followed by the same letter are not significantly different at p = 0.05 and means were separated by the Fishers' LSD. C.R.I represents Cotton Research Institute.

Conclusions

At C.R.I and Mzarabani communal area three seeds per station achieved better stand counts while at Tokwane, five seeds per station resulted in better stand counts. In Mahuwe communal area, six seeds per station performed better. However, six seeds per station was the median seed rate that produced the highest stand counts across sites and across seasons.

Recommendation

It is therefore recommended that farmers can plant three up to six seeds per station depending on environmental conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Soil organic carbon stock under different land use types in Kersa Sub Watershed, Eastern Ethiopia

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Understanding and assessing soil organic carbon stock (SOCS) within the framework of greenhouse gas emissions and land degradation is so crucial in combating climate change and enhancing ecological restoration. The goal of this study was to quantify the current SOCS in major land use types in Kersa sub watershed, eastern Ethiopia. Replicated soil samples from 0 to 20, 20 to 40, and 40 to 60 cm depth were collected from three major land uses types: grazing, cultivated, and fallow lands. Analysis of variance (ANOVA) was used to compare means and Pearson correlation analysis was used to see relationships between selected soil parameters. The results of the study revealed significant difference in soil organic carbon stock under the different land use types (P≤ 0.05). Soil under grazing land use type had significantly higher values of SOCS (42.9 t/ha and 32.9 t/ha) than cultivated land use type (32.6 t/ha and 26.3 t/ha) and fallow land use type (23 t/ha and 12.5 t/ha) in surface and sub surface layers, respectively. Similarly, SOCS decreased with soil depth in all the land use types and showed positive and significant correlation (P≤ 0.05) with clay content while negatively and significantly correlated with bulk density. The results show potential contribution of vegetation cover in land use to enhance soil organic carbon sequestration and environmental protection.

Key words: Land use, organic carbon, soil organic carbon stock, carbon sequestration.

INTRODUCTION

In the presence of climate change, land degradation and biodiversity loss, soils have become one of the most vulnerable resources in the world (FAO, 2017). Soil plays crucial role in combating climate change and ecological restoration through controlling the global carbon cycle.

Managing soil organic carbon (SOC) through sustainable agricultural practices has become a widely recognized strategy for restoring vulnerable soil resources. This is because soils are a major carbon reservoir in terrestrial ecosystems. The SOC pool stores an estimated amount of 1500 petagram of carbon (Pg C) in the first meter of soil which is more carbon than what is

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contained in the atmosphere (roughly 800 Pg C) and terrestrial vegetation (500 Pg C) combined (Batjes,1996). Therefore, a relatively small change in the soil C pool can considerably mitigate or enhance CO_2 concentrations in the atmosphere.

The distribution of SOC varies spatially and temporally. This is because SOC can be influenced by various factors such as soil type, land use types, land use change, climate, landscape, and soil management practices. As a result, soils in different geographic areas have different potential as carbon sources and sinks. However, vegetation is the major source of soil organic matter hence land uses are known to play a major role in SOC stock build up through organic matter input. That is the reason after the burning of fossil fuels land use and land cover change is the largest anthropogenic source of carbon into the atmosphere (Houghton et al., 2012; IPCC, 2014).

Many studies have suggested that land use type is the main factor determining SOC content by directly altering soil properties and supply of soil nutrients (Woldeamlak and Stroonsnijder, 2003; Lemenih and Itanna, 2004; Li et al., 2012; Yan et al., 2012). The impact of land use change varies according to the land use types. Land use change from natural forest to agricultural land and plantation result in lowering of SOC through intensive soil disturbance of soil structure and oxidation of soil organic matter. For example, conversion of forest to crop land invariably results in a loss of 20 to 50% of soil carbon (Post and Mann, 1990).

Similarly, 59% carbon lose through the conversion of pasture to cropland has been reported (Guo and Gifford, 2002; Murty et al., 2002). However, the conversion of forest to pasture did not results in significant loss of soil carbon (Murty et al., 2002). Similarly, when cropland is converted into natural vegetation, SOC will accumulate (Kwon, 2000; Zhang et al., 2010). Moreover, in the surface soil degradation in the form of deforestation and erosion results in significant loss of soil organic carbon in top soil (Sombroek et al., 1993; Lal, 2002). As a result, SOC is expected to vary along with soil depths in addition to land use types. Ingram and Fernandes (2001) reported that apart from land use, the level of SOC is determined by soil attributes including soil depth, texture and climate factors.

Currently, sequestering carbon in agricultural soils is seen as one way of decreasing atmospheric carbon dioxide (CO_2) concentrations and mitigating climate change. The potential increases in soil organic carbon associated with land use could be achieved through improved retention of plant/animal residues and greater inputs (Hoyle, 2013). As a result, identifying land uses that increase net plant/animal organic carbon inputs to the soil and then understanding how these changes will impact soil function is so indispensable (Murphy et al., 2011).

Land use change is the main primary net C release in Africa, much of it released through burning of forests (Williams et al., 2007). In sub-Saharan Africa, the increasing demand for food can encourage farmers to reduce the length of fallow periods, cultivate continuously, overgraze fields, or remove much of the above ground biomass through fuel collection or for building materials. Such practices can result in the reduction of SOC, water holding capacity, nutrients, as well as enhance soil erosion (Lal, 2004).

In Ethiopia, rural population is currently growing rapidly, resulting in massive conversions of land use and land cover with negative impacts (Woldeamlak and Stroosnijder, 2003). Similarly, the highlands of Eastern Ethiopia, due to continuous intensive cultivation for many years, are highly degraded, being degraded, and prone to degradation (Kibebew, 2014). The Kersa sub watershed which is part of Eastern Hararghe highland is facing a similar problem. It is clearly observed that the study area is characterized by high population pressure and intensive cultivation for many years. The increase in population has resulted in encroachment of crop production to the marginal and steeper slopes. This conversion of land use is likely to result in loss of CO_2 through vegetation removal and rapid oxidation of SOC following intensive cultivation.

Therefore, understanding the influence of land use types on soil organic carbon is an important step in line with the United Nations Framework Convention on Climate Change (UNFCCC) plan to reduce the effect of climate change and greenhouse gases (GHG) emission and developing potential future CO_2 mitigation strategies. However, little is known about the impact of different land use types on SOC stocks in Eastern Ethiopia. Therefore obtaining information on the SOC stocks of adjacent land use types is essential and imperative. Hence, this study was aimed at generating data to build scientific evidence that could be available to land managers and policy makers based on hypothesis that different land use types affect the soil organic carbon stock in Kersa sub watershed. Therefore, the objective of this study was to quantify the soil organic carbon stock and assess the relationship between SOC and land use types.

MATERIALS AND METHODS

Description of the study area

Geographically, the study area, Kersa sub watershed, is located in Kersa District, Eastern Harerghe zone of Oromia National Regional State between 9° 26' 28" N to 9°27' 50"N, and 41°52' 0 "E to 41°53'50"E (Figure 1). The total area of the watershed is 622 ha.

Climate

Based on 19 years (1995 to 2014) data obtained from Ethiopian National Meteorology Authority, the study area receives a mean annual rainfall of 732 mm. The rainfall pattern in the area is bi-modal with high amount of rainfall occurring during the main rainy

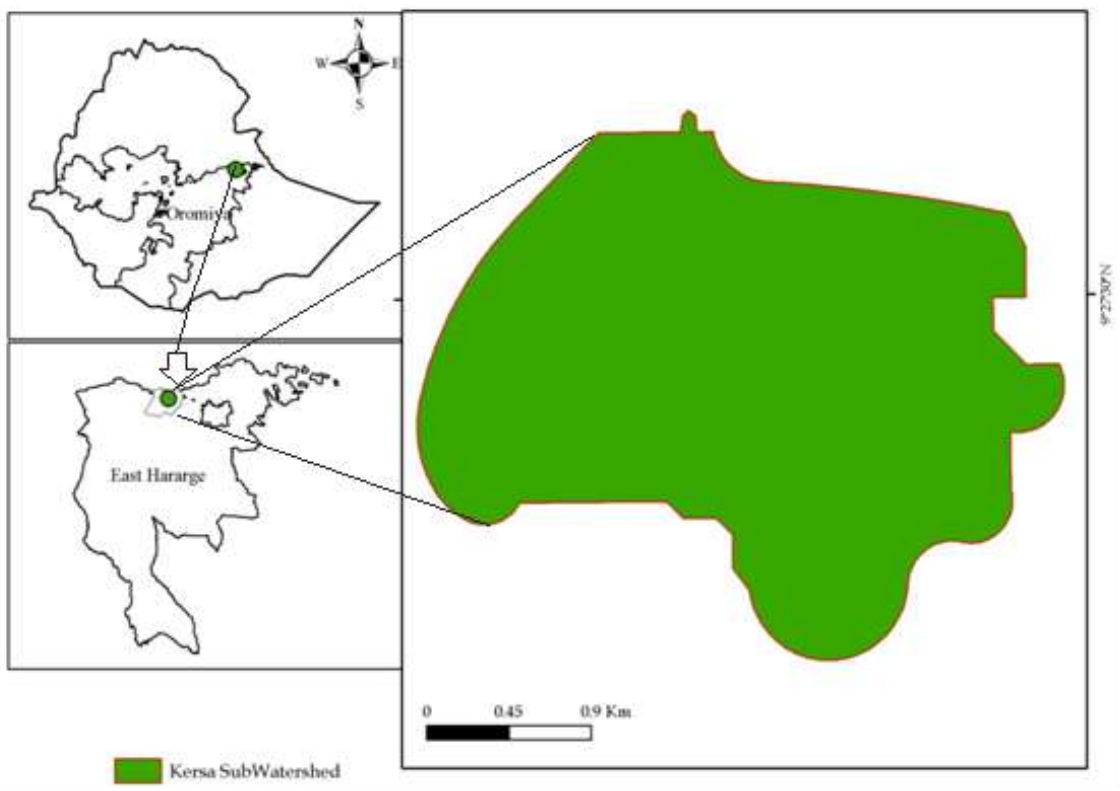

Figure 1. Map of the Kersa sub watershed.

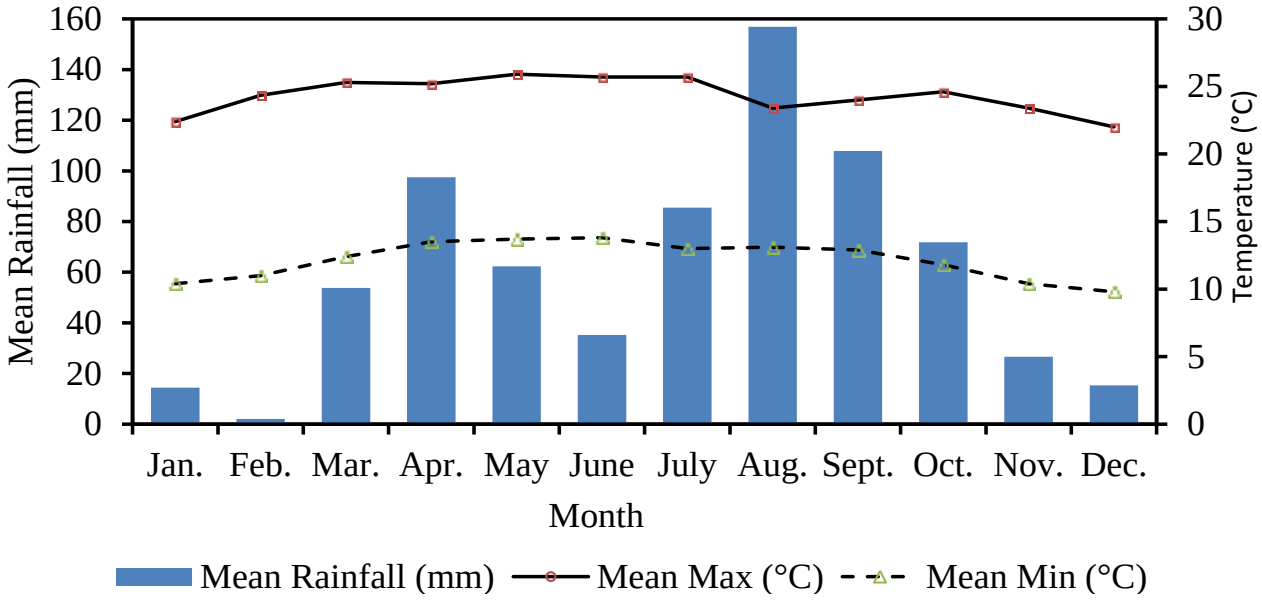

Figure 2. Mean monthly rainfall and mean monthly minimum and maximum temperatures in Kersa sub watershed.

season between July to September and the short rainy season stretching from March to June (Figure 2). The highest mean rainfall is received in August. Based on 17 years climate data (1997 to 2014), the mean minimum and maximum annual air temperatures of the area are 12 and 24°C, respectively, with mean annual air temperature of 18°C.

Table 1. Description of land use within the Kersa sub watershed in Eastern Ethiopia.

Land use	Description
Grazing	Land used as communal grazing land for cattle and it is managed through controlled system whereby livestock is confined in a stall and fed with cut and carry system
Cultivated	This includes land used for cultivation of crops under rainfed conditions. The main cropping system is mixed cropping where khat (*Catha edulis*) is intercropped with sorghum. Small amount of organic matter is returned to the soil because no crop residue is returned to the soil due to its use for other purposes, such as animal feed, fuel wood, source of cash, and construction material
Fallowing	This includes land that has been once under intensive cultivation and is now relieved from use for crop production since 2012

Geology and soil

According to the geological map of Ethiopia, first published in 1973 at a scale of 1:2000, 000, the geology of the Kersa district is covered by Adigrat formation constituted by sandstones and shell. Hamanlei series formation that contains Oxfordian limestone covers the lower part of the landscape and lower complex undifferentiated pre Camberian rock cover the upper part of the landscape. Moreover, Mohr (1964) indicated that Hararghe highlands lie over the crystalline bed rock composed mainly of granitic rock and gneiss material. According to FAO/WRB (2014) classification, the soils of the study area consist of Luvisols, Cambisols, Vertisols, Leptosols, and Regosols. Altitude of the watershed ranges from 1968 to 2127 meters above sea level.

Land use and farming systems

The study area encompasses different land use types and the dominant land uses are grazing, cultivated, and fallow land (Table 1). The farming system of the area is predominantly subsistence farming based on mixed crop-livestock production. Livestock are integral part to the farming system, supplying draught power for cultivation, food and income to households. The major rainfed field crops grown are sorghum and maize intercropped with common bean and Khat. Besides these, around homesteads, the vegetation is dominated by *Eucalyptus globules and Eucalyptus camaldulensis* trees.

Land use selection and soil sampling

Before soil sample collection, field observation and a reconnaissance soil survey was carried out, and informal group discussions with agricultural experts was made to identify representative land use types. Accordingly, three adjacent land use types: fallow, cultivated, and grazing lands were selected. Purposive sampling method was employed and selection for sampling considered adjacent land use types in order to minimize differences in climate, slope and soil type. Soil samples were taken from cultivated, fallow and grazing land use types with three replications based on a sampling plot size of 10 m by 10 m. In each plot, an auger was used to collect soil samples from the corners and in the centre of the square plots at three depths, at 0 to 20, 20 to 40, and 40 to 60 cm , and mixed to form a composite sample.

Analysis of soil physical and chemical properties

Determination of particle size distribution was carried out by the Bouyoucos hydrometer method (Bouyoucos, 1962) using sodium hexametaphosphate as dispersing agent as described in Sahlemedhin and Taye (2000). Bulk density was determined from undisturbed (core) soil samples collected using core sampling method (Black and Hartge, 1986). The bulk density was then calculated by dividing the mass of oven dry soil by volume as it exists naturally under field conditions. Measurement of soil pH was conducted using pH meter in the supernatant suspension of a 1:2.5 soil to water ratio as described by Van Reeuwijk (1993). Organic carbon of the soils was determined using the Walkley and Black wet oxidation method (Walkley and Black, 1934). Coarse fraction was determined during sample preparation after crushing of clods by hand and mechanical grinding and sieving until the sample was passed through a 2 mm sieve. Subsequently, coarse fraction was weighted and its proportion was determined using the following formula as described in Zhang et al. (2008):

$$\text{Coarse fraction (\%)} = \left(\frac{\text{Total weight} - \text{weight of fraction} < 2\text{mm}}{\text{Total weight}}\right) \times 100 \quad (1)$$

Soil carbon stock calculation

Soil carbon density (kg C m^{-2}) for each sample and depth was computed using the following equation (Zhang et al., 2008):

$$\text{SOCD}_i = \frac{L_i \times \text{SOC}_i \times \rho b_i \left(1 - \frac{F_i}{100}\right)}{100} \quad (2)$$

where, $SOCD_i$ is the total amount of soil organic carbon between the soil surface and depth of i^{th} layer per unit area (kg C m^{-2}); i is the i^{th} layer and L_i, SOC_i, ρbi and F_i are thickness (cm),SOC concentration (g kg^{-1}), bulk density (g cm^{-3}), and the proportion (%) of coarse (> 2mm) fragments in the i^{th} layer, respectively and 100 conversion factor. Carbon stock for each layer of the dominant land use was calculated by multiplying the C stock obtained by Equation 1 by the total area covered by a particular land use. Subsequently, C stock in each soil layer thickness was summed up to determine total C stock contained up to 60 cm depth for each land use type.

Data analysis

Measured data were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure in which land use types and depth were considered as independent variables (factors) and the selected soil properties as dependent variables. Mean separation was done using LSD at $P < 0.05$ level. Furthermore, correlation analysis was used to check the degree and magnitude of relationships between soil organic carbon and selected soil properties using SAS 9.2 software.

Table 2. Selected soils physical and chemical properties Kersa sub watershed, Eastern Ethiopia.

Variable	Depth cm	Land use			LSD	CV (%)
		Grazing	Cultivated	Fallow		
Sand %	0-20	54[a]	54[a]	51[ab]	5.13	6.1
	20-40	52[a]	50a[b]	46[bc]		
	40-60	53.3[a]	40.6[d]	42[cd]		
Silt%	0-20	17[ab]	16.7[ab]	16.66[ab]	2.6	9.33
	20-40	17.7[a]	14.67[bc]	13.66[c]		
	40-60	17.33[a]	18[a]	13.66[c]		
Clay%	0-20	29[b]	29.3[b]	28[b]	5.33	9.22
	20-40	30[b]	32[b]	40.33[a]		
	40-60	29.33[b]	41.33[a]	44[a]		
pH(H_20)	0-20	7.17[ab]	7.29[a]	6.72[d]	0.2	2.18
	20-40	6.95[cd]	6.98[bc]	6.96[cd]		
	40-60	6.89[cd]	6.81[cd]	7.0[bc]		
BD g/c^3	0-20	1.18[d]	1.30[cb]	1.33[ab]	0.09	4.22
	20-40	1.21d[c]	1.32[b]	1.38[ab]		
	40-60	1.32[b]	1.38[ab]	1.42[a]		
Textural class	0-20	Sandy clay loam	Sandy clay loam	Sandy clay loam		
	20-40	Sandy clay loam	Sandy clay loam	Sandy clay		
	40-60	Sandy clay loam	clay	clay		

Means followed by the same letter(s) across columns and rows are not significantly different ($p > 0.05$) with respect to land uses and depth.

RESULTS AND DISCUSSION

Physical and chemical properties of the soils

Particle size distribution

Across the land uses and depth sandy clay loam is the dominant textural class in all the land use types (Table 2). However, cultivated and fallow land use types revealed sandy clay and clay textural class in their sub surface soils. This could be related to intensive cultivation in cultivated and fallow land uses.

ANOVA (Table 2) showed that both land use and soil depth ($p<0.05$) had a significant effect on sand and clay content but the interaction effect between the two factors was not significant ($p > 0.05$). Across the land use types, mean particle size distributions of clay (44%) and sand (54%) were the highest in fallow and grazing lands respectively. The silt fraction was also highest in cultivated soils. The more clay content recorded under continuous cropping might be due to cultivation which might have enhances physical and chemical weathering in addition to mixing soils from the subsurface layers. Similar results were reported by Awdenegest (2013) in which higher clay fraction was recorded in soils under farm land use types

However, the clay percentage increased while the sand percentage decreased from the surface to the subsurface layers in almost all land use types (Table 2). The relatively higher clay content in sub surface layer might be due to movement of clay from upper to lower layer. These results are in conformity with the findings of Atofarati et al. (2012) and Desalegn et al. (2015) who found vertical clay migration content with depth and high sand content in topsoil. In addition, the highest ($r=-0.94$, $p<0.01$) negative and significant correlation between clay and sand in Table 5 indicates that removal of clay results in a relative increase of sand. Moreover, according to particle size distribution rating proposed by Hazelton and Murphy (2007), the soils of the sub watershed are

characterized by moderate to high clay, low level of silt and high to very high level of sand.

Soil bulk density

Bulk density (BD) varied significantly with land use types and depth ($p < 0.05$), but the interaction effect between the two factors was not significant ($p > 0.05$). The bulk density of soils under different land uses ranged between 1.18 g cm^{-3} in grazing land (0-20) to1.42 g cm^{-3} in fallow land (40-60) (Table 2). On average in the surface soil, cultivated and fallow land had 2.25 and 11.3% higher BD than grazing land. The smallest value of bulk density recorded in grazing land use type could be related to less soil disturbance and relatively higher organic matter (OM) content.

By contrast, study by Woldeamlak and Stroosnijder (2003) reported that bulk density was the highest in grazing land. On the other hand, the higher bulk density value in fallow land could be related with less aggregation of soil as result of organic matter degradation which in turn affects pore space and water holding capacity. This result is in consonance with Teshome (2016) and Zhang et al. (2009) who stated that the highest BD in the fallow land is due to organic matter degradation, animal tracking and human activity.

In all the land uses, the lowest bulk density values were found at the surface layers. This could be ascribed to high organic matter, better aggregation, particle size distribution, and root penetration in the surface layers. The results are in agreement with the findings of Ahmed (2002) and Bessah et al. (2016) who reported that bulk density values revealed increasing trend with depth in all land uses. Moreover, the critical values of bulk density for plant growth at which root penetration is likely to be severely restricted in clay loam soil is 1.6 g/cm^3 (Jones, 1983). In reference to this critical value of bulk density, all the surface and sub surface bulk density values of the soils were below the critical values. This implies that no excessive compaction and restriction of root development occurred in the soils of the study area.

Furthermore, the correlation analysis revealed SOC (%) and SOCS (t/h) had a significant negative relationship with bulk density (Table 5). These result evidenced that the lower bulk density the higher will be the SOC and SOCS in the study area. Similar finding (Lal and Kimble, 2001; Murty et al., 2002; Don et al., 2011) indicated the inverse relationship between SOC and SOCS with bulk density (Table 2).

Soil pH

ANOVA results indicated that there was no significant difference ($p > 0.05$) in soil pH value among land uses and depth but their interaction effect showed significant ($p < 0.05$) difference. Continuous cultivation practices, leaching of bases from the bare surfaces and application of inorganic fertilizers could be some of the factors which are responsible for the variation in pH in the soil. These results are in consonance with Aweke et al. (2013) who explained that intensive farming over a number of years with nitrogen fertilizers resulted in decline of soil pH more rapidly. The result, however, contradicts with previous study by Kaleem (2005) who found the lowest pH under grass because of presence of high OM. In addition, the soil pH of different land use types ranged between 6.72 to 7.29 and 6.82 to 7.00 in surface and sub surface soils respectively. It revealed decreasing trend with depth. This was supported by a negative correlation between OC and pH for the 20-40 and 40-60 cm depth (Table 5). According to soil pH (H_2O) ratings of Tekalign (1991), the overall pH (H_2O) range of the studied soils fall under the neutral (6.72-7.29) soil reaction range, which is favourable range for availability of most nutrients and activities of microorganisms.

Effect of land use on soil organic carbon content and soil organic carbon stock

The data pertaining to soil organic carbon (SOC) content (g kg^{-1}) and soil carbon stock (SOCS) (t/ha) in Tables 3 and 4 showed significant variations with respect to both land use type and depth ($p < 0.05$). However, the interaction effect of land use type and depth was not significant ($p > 0.05$). In the surface soils, the mean SOC and SOCS in the grazing land (18.5 g kg^{-1}, 42.9 t/h) was significantly higher than cultivated (13 g kg^{-1}, 32.6 t/h) and was the lowest in fallow land (9.7 g kg^{-1}, 23.0 t/ha) respectively. SOC and SOCS consistently declined with depth in all the land uses.

The highest SOC and SOCS in the grazing land use could be related to the high amount roots of grass and high grass root biomass turnover rate, which is important as protection from erosion and lack of tillage. In addition, the controlled grazing management practice where livestock is confined in a stall and fed with cut and carried fodder might have contributed to the high SOC and SOCS in Kersa sub watershed. In relation to this high total organic carbon stock under grazing land due to high grass root biomass turnover rate and also lack of tillage was reported (Guo and Gifford, 2002; Urioste et al., 2006; Qi et al., 2012; Yoseph et al., 2017). Similarly, Girmay and Singh (2012) reported higher mean SOCS in northern Ethiopia due to animal excrement. Our result clearly shows that in the study area controlled grazing land use types accrue significantly higher soil organic carbon stock than cultivated and fallow land use types.

Conversely, SOC and SOCS were significantly lower in the cultivated land compared to the grazing land use type, in which the cultivated lands accumulated 28 and 27% less SOC and SOCS than the grazing land, respectively. These could be due to fast decomposition and mineralization as continuous cultivation affect the soil

Table 3. Effect of land use types and depth on soil organic carbon content in Kersa sub watershed.

Land use types	SOC (g kg^{-1})		
	0-20cm	20-40 cm	40-60cm
Grazing	18.5^{a}	13.9^{b}	11.6^{b}
Cultivated	13.0^{b}	10.6^{cd}	07.8^{e}
fallow	09.7^{d}	05.2^{f}	03.8^{f}
Overall mean	10.5	-	-
LSD (0.05)	0.15	-	-
CV (%)	8.40	-	-

Means followed by the same letter(s) across columns and rows are not significantly different ($p > 0.05$) with respect to land uses and depth.

Table 4. Effect of land use types on soil organic carbon stock (t/ha) in Kersa watershed.

Land use types	SOCS (t/h)		
	0-20cm	20-40 cm	40-60cm
Grazing	42.9^{a}	32.9 ^{b}	32.6^{bc}
Cultivated	32.6^{bc}	26.3^{dc}	20.3^{f}
fallow	23.0^{ef}	12.5^{g}	09.5^{g}
Overall mean	25.5	-	-
LSD(0.05)	0.039	-	-
CV (%)	8.85	-	-

Means followed by the same letter(s) across columns and rows are not significantly different ($p > 0.05$) with respect to land uses and depth.

moisture and aeration which in turn results in oxidation of soil organic matter and less accumulation of organic matter through harvesting plant as well as plant residues. The continuously removed plant residues from fields for various purposes like source of fuel wood and livestock feed ultimately result in low organic carbon stock besides increasing surface runoff and removal of other essential nutrients from the soil. The results indicate that cultivation causes SOC loss, which is in conformity with other studies (Post and Kwon, 2000; Yimer et al., 2006; Wang et al., 2008; Don et al., 2011; Itanna et al., 2011) who reported that cultivation reduced soil organic carbon through high decomposition and minimum protection of SOC.

Similarly, the lowest SOC and SOCS found in fallow land could be related to the fact that the fallow land is situated on a relatively steep slope area which is prone to high soil erosion and soil degradation as well as the process of succession was slow and the fallow time was short. In line with this, Yoseph et al. (2017) indicated that soil organic matter plays significant roles in soil aggregate stability and nutrient availability which subsequently contributes to enhanced soil quality. As a result, low soil organic carbon in fallow land can lead to severity of soil degradation in terms of nutrient availability and water holding capacity.

SOC and SOCS also showed variability with depth. SOC and SOCS decreased consistently with depth for each land use types. The highest SOC and SOCS were observed in the top layer (0 to 20 cm) than in the middle layer (20 to 40 cm) and in the bottom layer (40 to 60 cm).The difference in SOC and SOCS between different land use types narrowed with soil depth. In the study area about 42.3 and 43% of SOCS and SOC was found in the top 0-20 cm layer, while 30.8 and 31.56% was in the 20 to 40 cm layer and 26.8 and 24.4% in the 40 to 60 cm layer respectively. The finding revealed that not only land use but soil depth also significantly affected the level of SOC and SOCS in the study area. The high SOC and SOCS in surface soil could be due to incessant addition of undecayed and partially decomposed plant and animal remains in the surface soils. This finding is in consonance with the results of studies (Yimer et al., 2007; Awdenegest et al., 2013; Nega and Heluf, 2013).

In addition, the correlation analysis in the surface layer showed significant ($p < 0.05$) and negative relationship between soil organic carbon stock (SOCS) and sand, while positive and significant relation with clay was recorded (Table 5). This indicates that the amount of SOC in soil increased with the amount of clay. This could be related to SOC coating around clay minerals and protected against weathering and microbial degradation which helps the SOC to stay in the soil for long periods.

In consistent with Plante et al. (2006) and Hoyle et al. (2011) report clay content increase the amount of SOC through physical protection from microbial breakdown.

Despite a significant difference in SOC and SOCS content among the major land use types in Kersa sub watershed, according to the OC content rating criteria established by Tekalign (1991), the overall OC content of the soils was in the range of very low to low. The low amount of organic carbon in the study area could be related with inadequate application of organic input and intensive cultivation. Similarly, Tegbaru et al. (2014) and Okubay et al. (2015) reported intensive cultivation and low application of organic inputs were the factors which reduced soil OC in Ethiopian soils respectively.

Conclusion

Based on the result of the study grazing, cultivated and fallow land use showed significant differences in their soil organic carbon and soil organic carbon stock content. SOC stock in grazing land was significantly higher than those of cultivated and fallow land use types. Similarly, the surface layer (0 to 20 cm) stored significantly higher SOC in all land use types. However, the soil organic carbon status in all land use types was found to be low. This indicates the presence of a good potential to

Table 5. Correlation matrix for selected soil parameters in Kersa sub watershed.

Variable	Depth (cm)	Sand	Silt	clay	pH	BD	OC	SOCS
	0-20	1.00	-	-	-	-	-	-
Sand	20-40	1.00	-	-	-	-	-	-
	40-60	1.00	-	-	-	-	-	-
	0-20	0.368	1.00	-	-	-	-	-
Silt	20-40	0.571	1.00	-	-	-	-	-
	40-60	0.292	1.00	-	-	-	-	-
	0-20	-0.86***	-0.50	1.00	-	-	-	-
Clay	20-40	-0.93**	-0.72**	1.00	-	-	-	-
	40-60	-0.94**	-0.582	1.00	-	-	-	-
	0-20	0.548	0.276	-0.392	1.00	-	-	-
pH	20-40	0.410	-0.218	-0.246	1.00	-	-	-
	40-60	0.006	-0.605	0.196	1.00	-	-	-
	0-20	0.187	0.31	-0.453	-0.184	1.00	-	-
BD	20-40	-0.033	-0.67**	0.242	0.406	1.00	-	-
	40-60	- 0.69**	-0.79**	0.85**	0.369	1.00	-	-
	0-20	0.21	0.1	0.08	0.48	-0.76**	1.00	-
OC	20-40	0.35	0.56	-0.52	-0.11	-0.80**	1.00	-
	40-60	0.76**	0.74**	-.89**	-0.47	-0.86**	1.00	-
	0-20	-0.27**	-0.24	0.6*	0.23	-0.80**	0.82**	1.00
SOCS	20-40	-0.25	-0.15	0.11	-0.14	-0.60*	0.54 **	1.00
	40-60	0.15	0.58	-0.33	-0.63	-0.39	0.69**	1.00

sequester carbon in soils of the study area. Therefore, appropriate farming and management practice which increase inputs and reduces losses of the soil organic carbon should be designed and implemented in the study area. These improve the soil potential to sequester more SOC and minimize the effect of climate change.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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